SUSTAINABLE PRODUCTION OF BIODEGRADABLE BIOPOLYMERS AND THEIR APPLICATIONS IN SUPPORT OF ORGANIC AQUACULTURE

A Dissertation

by

FAHAD M A R ASIRI

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Chair of Committee,	Kung-Hui Chu
Committee Members,	Delbert M Gatlin III
	Xingmao Ma
	Yongheng Huang
Head of Department,	Zachary Grasley

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ABSTRACT

Polyhydroxyalkanoates (PHA) are a class of microbially-produced biodegradable biopolymers obtained from renewable feedstocks. Due to variations in the properties of PHA, they have received growing attention to develop several practical applications, particularly bioplastics. Recently, a common PHA type, poly(3-hydroxybutyrate) (PHB), has been identified as an effective biocontrol agent to replace antibiotics and improve growth and disease resistance in aquaculture. However, the production and application of PHB are associated with several challenges, such as expensive feedstocks, costly sterilization, high-energy input harvesting techniques, and toxic extraction and purification processes. The overall goal of this three-manuscript dissertation was to establish a sustainable and economical process for PHB production, which in turn, can be applied as an effective biocontrol agent and aquafeed. The implications of this process can overcome the traditional PHB challenges, as well as challenges associated with commercial aquaculture, such as waste management, high feed cost, and the use of antibiotics to control pathogens. In paper I, a novel PHB production and supplementation system, called recirculating aquaculture system for PHB-rich microorganisms (RAS-PHB), was developed. This system integrates the treatment of agro-industrial wastes, including aquaculture wastewater/wastes, with the production and harvest of PHB-rich *Zobellella denitrificans* ZD1 (designated as ZD1 hereafter) using chitosan as a biocoagulant. In paper II, chitosan-harvested PHB-rich ZD1 demonstrated multifunctional effects, such as improving growth, survival, immune response, and

altering gut microbiome in an aquaculture animal model, brine shrimp *Artemia*. In paper III, results showed the effects of providing different agro-industrial wastes/wastewaters as substrates on PHA polymer composition in ZD1. Furthermore, bacterial cells that accumulated fractions of longer PHA monomers, along with PHB, magnified the biocontrol efficacy by enhancing antipathogenic properties, providing additional energy to *Artemia*, and improving survival against pathogens. Overall, the engineering approach of PHB production and application, proposed in this dissertation, yields promising potentials toward sustainable PHB production and organic aquaculture practices to enhance commercial production.

DEDICATION

I dedicate my dissertation to God, the most gracious and most merciful, my source of inspiration, knowledge, and blessings; to my mother who sacrificed her life to raise and educate me for the future; to my father's soul who always believed in me and wished to see me in this position; and to my wife and kids who supported me and surrounded my life with love.

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This work was supervised by a dissertation committee consisting of Dr. Kung-Hui Chu (advisor) and Dr. Xingmao Ma from the Department of Civil and Environmental Engineering, Dr. Delbert Gatlin III from the Department of Ecology and Conservation Biology, and Dr. Yongheng Huang from the Department of Biological and Agricultural Engineering.

All other work conducted for the dissertation was completed by the student independently.

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NOMENCLATURE

PHA	Polyhydroxyalkanoates
SCL-PHA	Short-chain-length polyhydroxyalkanoates
MCL-PHA	Medium-chain-length polyhydroxyalkanoates
LCL-PHA	Long-chain-length polyhydroxyalkanoates
PHB	Poly(3-hydroxybutyrate)
PHV	Polyhydroxyvalerate
PHH	Polyhydroxyhexanoate
PHV	Polyhydroxyoctanoate
SCFAs	Short-chain fatty acids
MCFAs	Medium-chain fatty acids
3-HB	3-hydroxybutyrate
3-HV	3-hydroxyvalerate
3-HH	3-hydroxyhexanoate
3-HO	3-hydroxyoctanoate
PUFAs	Polyunsaturated fatty acids
SCP	Single-cell protein
ZD1	*Zobellella denitrificans* ZD1
RAS	Recirculating Aquaculture System
RAS-PHB	Recirculating Aquaculture System for PHB-rich microorganisms
AW	Aquaculture wastewater

COD	Chemical oxygen demand
TN	Total nitrogen
TP	Total phosphorus
OD_{600}	Optical density at 600 nm
CDW	Cell dry weight
M_w	Molecular weight
LB	Luria−Bertani medium
R2A	Reasoner's 2A medium
TSB	Tryptic soy broth
MSM	Mineral salt medium
CDW	Cell dry weight
P-ZD1	Poly(3-hydroxybutyrate)-rich *Zobellella denitrificans* ZD1
CP-ZD1	Chitosan-harvested PHB-rich *Zobellella denitrificans* ZD1
RHA1	*Rhodococcus jostii* RHA1
G–	Gram-negative bacteria
G+	Gram-positive bacteria
COS	Chitosan oligosaccharides
MIC	Minimum inhibitory concentration
IC_{50}	Median inhibitory concentration
LD	Lethal dose
Hsp70	Heat shock protein 70
ftn	Ferritin

pxn	Peroxinectin
RT-qPCR	Quantitative real time-polymerase chain reaction
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
ASVs	Amplicon sequence variants
PCA	Principal component analysis
GC-FID	Gas chromatography-flame ionization detector
SWS	Sugary waste slurry
CWW	Cheese whey wastewater
SCG	Synthetic crude glycerol
HSSW	High-strength synthetic wastewater
FWFL	Food waste fermentation liquid
BP	Banana peels
OP	Orange peels
AFWW	Anchovy fishmeal wastewater

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1. CHAPTER I INTRODUCTION

1.1. Introduction and Background

Polyhydroxyalkanoates (PHA) are a broad class of intracellular biodegradable biopolymers[1] that are synthesizable by various microorganisms growing on different renewable feedstocks.[2, 3] The chemical structure of PHA consists of repeating units (i.e., monomers) of hydroxy-fatty acids and can be classified into three groups, short-chain-length PHAs (SCL-PHAs) with 3–5 carbon (C) atoms, medium-chain-length PHA (MCL-PHA) with C6–14 (C-x represents a chain length of x C atoms), and long-chain-length PHA (LCL-PHA) with C >14. Due to the variations in the structure and properties, PHA have attracted attention to develop many applications such as bioplastics for packaging purposes, biocompatible implants,[4] bacterial substrate in self-healing concrete,[5] paper coating,[6] bio-based glue,[7] and slow-release fertilizer/herbicide.[8, 9]

Poly(3-hydroxybutyrate) (PHB) is the most common SCL-PHA consists of repeating units of the C-4 short-chain fatty acid (SCFA) (3-hydroxybutyrate; 3-HB).[2] Owing to the similar physical and chemical characteristics of PHB to those of petroleum-based polymers (i.e., polypropylene and polyethylene), PHB has been highly regarded as a promising bioplastic in several practical applications.[10] However, this kind of application is restricted by several factors such as expensive downstream steps to extract and purify PHB, high quality requirements to meet consumer needs, and a fierce competition with the traditional petroleum-based plastics.[3] Accordingly, PHA/PHB application has been extended as a potential biocontrol agent in aquaculture, where minor variations in polymer properties are not regarded as problematic.

Aquaculture is the fastest-growing food production sector with an average growth rate of 8%.[11, 12] It consists of intensive farming systems of all forms of aquatic animals (i.e., fish,

crustaceans, mollusks, etc.) and plants (seaweeds) in all aquatic environments (fresh, brackish, and marine). Due to the limited resources and supplies of wild-capture fisheries and the growing seafood demand, the global aquaculture production is estimated around 82 million tonnes of aquatic animals in 2018 (527% increase since 1990), valued at 250 billion US dollars.[13] Despite the advantages of aquaculture, current commercial aquaculture practices are not entirely sustainable due to the challenges on aquaculture wastewater (AW)/sludge management, high feed cost, and most importantly, disease outbreaks due to the proliferation of various pathogens.[11, 12] To control bacterial pathogens, antibiotics have been traditionally used in aquafeeds. However, the long-term use of antibiotics pose a significant risk to food safety and public health, as antibiotics might accumulate in the tissues of aquatic species[14] and promote antibiotic-resistant microbes in aquaculture and human beings.[15, 16] Therefore, various alternative biocontrol agents have been suggested to overcome this problem.[16, 17]

SCFAs and pure crystalline PHB are promising alternatives to antibiotics.[1] SCFAs are known for combating bacterial diseases in aquaculture animals[18] and enterobacteria such as *Salmonella typhimurium*, *Escherichia coli*, and *Shigella flexneri*.[17, 19-24] Once penetrating the cell membrane of the pathogens, SCFAs release protons (H^+) from their undissociated acid forms that reduces the pH of cytoplasm to an acidic level. In response to the rapid pH change, pathogens redirect their energy to pump out the excess protons, leading to growth limitation and eventually cell death.[25] Among many different tested SCFAs (formate, acetate, propionate, and valerate), butyrate showed promising results in decreasing the pathogenic viability and invasion.[24] However, prophylactic SCFAs such as butyrate are highly soluble, leading to inefficient uptake by filter-feeding aquatic animals.[1, 26]

PHB is a biopolymer of SCFA (3-HB) categorized under the SCL-PHA family of microbial polymers.[10] Uptake of PHB is more efficient than soluble SCFAs for filter-feeding animals due to its insolubility and biodegradability to the desired intermediates/SCFAs (3-HB and butyrate) in the animal gut.[1, 27] Supplementing aquafeeds with PHB in crystalline form (i.e., extracted from microbial biomass) or amorphous form (still inside the cells) has been shown to improve growth and disease resistance of many fish species[28-30] and crustaceans.[31-34] Different mechanisms have been suggested for PHB and its intermediate 3-HB to promote the survival and health of aquatic animals, such as inhibiting the pathogenic growth and virulence factors, enhancing the immune system, serving as an energy source to aquatic animals, and improving the beneficial microbes in the gut microbiota. Nevertheless, the cost of PHB production and supplementation in aquafeed is remarkably high due to expensive substrates for cultivating PHB-accumulating microorganisms,[35] costly sterilization processes to avoid microbial contamination,[36, 37] and the high-energy input to harvest and dry PHA-filled microorganisms before the application of toxic solvents for PHA extraction and purification.[25, 38]

Although previous studies have shown PHB to be an effective biocontrol agent, several literature gaps still need to be filled. Previous studies were not considering the bioprocess with which PHB is produced; rather focusing on the outcome itself (i.e., supplementing PHB to aquatic animals). As a result, shortcomings related to high PHB production/recovery costs continue to persist. Apparently, the application of waste-derived amorphous PHB, produced without sterilization and harvested with an energy-efficient method, can provide a more practical and economical PHB production process. Interestingly, a PHB-hyperaccumulating salt-tolerant bacterium, *Zobellella denitrificans* ZD1 (designated as ZD1 hereinafter), tested in our previous study,[37] has shown ability to adapt and utilize various salty organic wastes to produce PHB without

sterilization. Yet, the potential of integrating aquaculture wastewater/wastes (containing high organics, nutrients, and salt contents)[39, 40] with the production of cheap PHB-rich ZD1 has not been examined. Additionally, the impact of various wastes on PHA polymer composition in ZD1 has not been investigated. By extension, the focus of previous literature on the outcomes solely results in ignoring the optimal harvesting method of amorphous PHB. The economic feasibility and performance of PHB as a biocontrol agent is highly dependent on PHB recovery, particle sizes, and delivery methods. Thus, this dissertation evaluates organic chitosan as a harvesting method of PHB-rich biomass to assess its impact on PHB delivery to an aquaculture animal model, brine shrimp *Artemia*. *Artemia* is a filter-feeding aquatic species that has been used as an important live food in aquaculture[41] and tested for PHB application.[1] Furthermore, a third gap in the literature is pertaining to limiting the investigation of defensive mechanisms to PHB, a SCL-PHA type. Hence, this dissertation aims to expand the investigation further to include MCL-PHA.

Accordingly, the **overarching goal** of this three-manuscript dissertation is to establish a sustainable and economical process for PHB production, which in turn, can be applied as an effective biocontrol agent and aquafeed to maintain organic aquaculture. The implications of this process can overcome the traditional PHB challenges, such as costly sterilization, high-energy input harvesting, and toxic extraction and purification, as well as challenges associated with aquaculture industry, such as waste management, high aquafeed cost, and the use of antibiotics to control pathogens.

1.2. Dissertation Specific Objectives and Tasks

To accomplish the overall goal of this dissertation, three objectives and hypotheses are proposed here. The supporting experimental designs are articulated in different tasks.

1.2.1. Objective 1: Develop a novel Recirculating Aquaculture System (RAS) that can concurrently treat aquaculture wastewater/waste while producing PHB-rich biomass.

Hypothesis: Aquaculture wastewater/sludge can be used for production of PHB-accumulating strain ZD1, which could lead to the development of a sustainable and economical aquaculture system.

- Task 1a: Examine the ability of zeolite to adsorb nitrogen (N) from aquaculture wastewater and indirectly release them for PHB-producing ZD1.
- Task 1b: Investigate the potential for nonsterile ZD1 cultivation in aquaculture wastewater with the supplementation of agro-industrial wastes as additional substrates.
- Task 1c: Characterize and compare the quality of PHB-rich ZD1 biomass against conventional aquafeeds.
- Task 1d: Determine and optimize a harvesting method for ZD1 biomass using chitosan as a coagulant in comparison with common $FeCl_3$.
- Task 1e: Perform an economic analysis to assess the advantages of implementing the proposed RAS over a conventional RAS.

1.2.2. Objective 2: Assess the effects of chitosan-harvested PHB-rich ZD1 as a feed on *Artemia's* health.

Hypothesis: Pathogenic stress is the most common threat in aquaculture. PHB-rich ZD1 (P-ZD1) can be used as an effective feed against pathogens. Particularly, chitosan-harvested PHB-rich ZD1 (CP-ZD1) can improve the antimicrobial efficacy and disease resistance of aquatic animals.

- Task 2a: Assess the antimicrobial efficacy of PHB and chitosan biodegradation intermediates against several Gram-negative (G-) and Gram-positive (G+) bacteria and predominant aquaculture pathogens.
- Task 2b: Examine whether CP-ZD1 is an effective energy source for *Artemia.*
- Task 2c: Determine the survival and immune response of *Artemia* challenged with aquaculture pathogens and supplemented with CP-ZD1.
- Task 2d: Investigate the effects of CP-ZD1 exerted on the gut microbiome of *Artemia*.

1.2.3. Objective 3: Investigate the effects of supplementing different forms of SCL- and MCL-PHA on the growth and disease resistance of *Artemia.*

Hypothesis: MCL-PHA are more effective than SCL-PHA in inactivating aquaculture pathogens. PHA/PHB-rich ZD1 can be used as an effective aquaculture feed supplement.

- Task 3a: Assess the antimicrobial efficacy of SCL- and MCL-PHA intermediates against G- and G+ aquaculture pathogens.
- Task 3b: Examine the potential of utilizing different agro-industrial wastes/wastewaters by ZD1 and their implications on PHA composition.
- Task 3c: Investigate whether SCL- and MCL-PHA are used as energy/food sources by *Artemia.*
- Task 3d: Determine the survival of *Artemia* against aquaculture pathogens, when *Artemia* was supplemented with SCL- and MCL-PHA.

1.3. Dissertation Overview

This dissertation consists of six chapters. **Chapter I** lays out the background, objectives and tasks, and overview of the dissertation. **Chapter II** presents a literature review of the major topics related to this dissertation. These topics include the types of the PHA biopolymers, PHB properties and applications, PHA-producing microorganisms, properties of ZD1 as a promising PHA/PHB producer, substrates used for PHA production, aquaculture industry, and PHB application in aquaculture.

In **Chapter III**, a novel PHB production and supplementation system in aquaculture, called recirculating aquaculture system for PHB-rich microorganisms (designated as RAS-PHB hereafter) was proposed. It is an upgraded version of the conventional recirculating aquaculture system (RAS). The developed RAS-PHB integrates the treatment of agro-industrial wastes, such as aquaculture wastewater/wastes with the production of PHB-rich ZD1, which is effectively harvested using chitosan biocoagulant derived from crustacean wastes.

In **Chapter IV**, the potential functions of supplementing the chitosan-harvested PHB-rich ZD1 (CP-ZD1) on an aquaculture animal model, brine shrimp *Artemia*, were investigated. Therefore, the study examined the impacts of CP-ZD1 exerted on aquaculture pathogens, growth, disease resistance, immune response, and gut microbiome of *Artemia*.

In **Chapter V**, the effect of providing different agro-industrial wastes/wastewaters as substrates on PHA polymer composition in ZD1 (i.e., SCL- and MCL-PHAs) was investigated. The study further evaluated the potential of supplementing those SCL- *vs.* MCL-PHAs on the growth and disease resistance of *Artemia.* Finally, in **Chapter VI**, conclusions, implications, and directions for future research are discussed.

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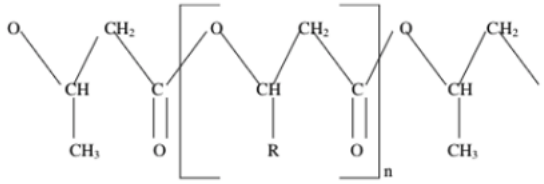
2. CHAPTER II LITERATURE REVIEW

2.1. The Polyhydroxyalkanoates (PHA) Family

Polyhydroxyalkanoates (PHA) are a broad class of biodegradable biopolymers (200–500 nm diameter size)[1] that are synthesizable by various microorganisms growing on different renewable feedstocks.[2, 3] They are accumulated inside the bacterial cells as energy reserve polymers, particularly under stressful conditions of nutrient limitation (e.g., nitrogen (N), phosphorous (P), and oxygen (O)). The chemical structure of PHA consists of repeating units (i.e., monomers) of hydroxy-fatty acids and can be classified into three groups, short-chain-length PHA (SCL-PHA) with 3–5 carbon (C) atoms, medium-chain-length PHA (MCL-PHA) with C6–14 (C-x represents a chain length of x C atoms), and long-chain-length PHA (LCL-PHA) with C >14. Figure 2.1 illustrates a general molecular structure of PHA and some representative members.



n varies from 600 to 3500

R = hydrogen	Poly(3-hydroxypropionate)
R = methyl	Poly(3-hydroxybutyrate)
R = ethyl	Poly(3-hydroxyvalerate)
R = propyl	Poly(3-hydroxyhexanoate)
R = pentyl	Poly(3-hydroxyoctanoate)
R = nonyl	Poly(3-hydroxydodecanoate)
R = hydrogen	Poly(4-hydroxybutyrate)
R = hydrogen	Poly(5-hydroxyvalerate)

Figure 2.1. General polyhydroxyalkanoates (PHA) structure and some representative members.[4, 5]

The application of PHA biopolymers depends on the physicochemical properties of PHA, which are affected by several factors, such as the type of PHA-accumulating microorganisms,

growth conditions, fermentation mode (i.e., batch, fed-batch, and continuous), and type of feedstocks (i.e., substrates) used as C-sources.[3] Due to the variations in the structure and properties, PHA have attracted attention to develop many applications (Figure 2.2) such as bioplastics for packaging purposes (e.g., consumer products, automotive components, and plastic gift cards), biocompatible implants,[6] bacterial substrate in self-healing concrete,[7] paper coating,[8] bio-based glue,[9] and slow-release fertilizer/herbicide.[10, 11] Only recently, it has been also demonstrated that PHA, particularly the short-chain type poly(3-hydroxybutyrate) (PHB), can be used as an affective feed additive and biocontrol agent in aquaculture to improve growth and disease resistance.[1, 12, 13]

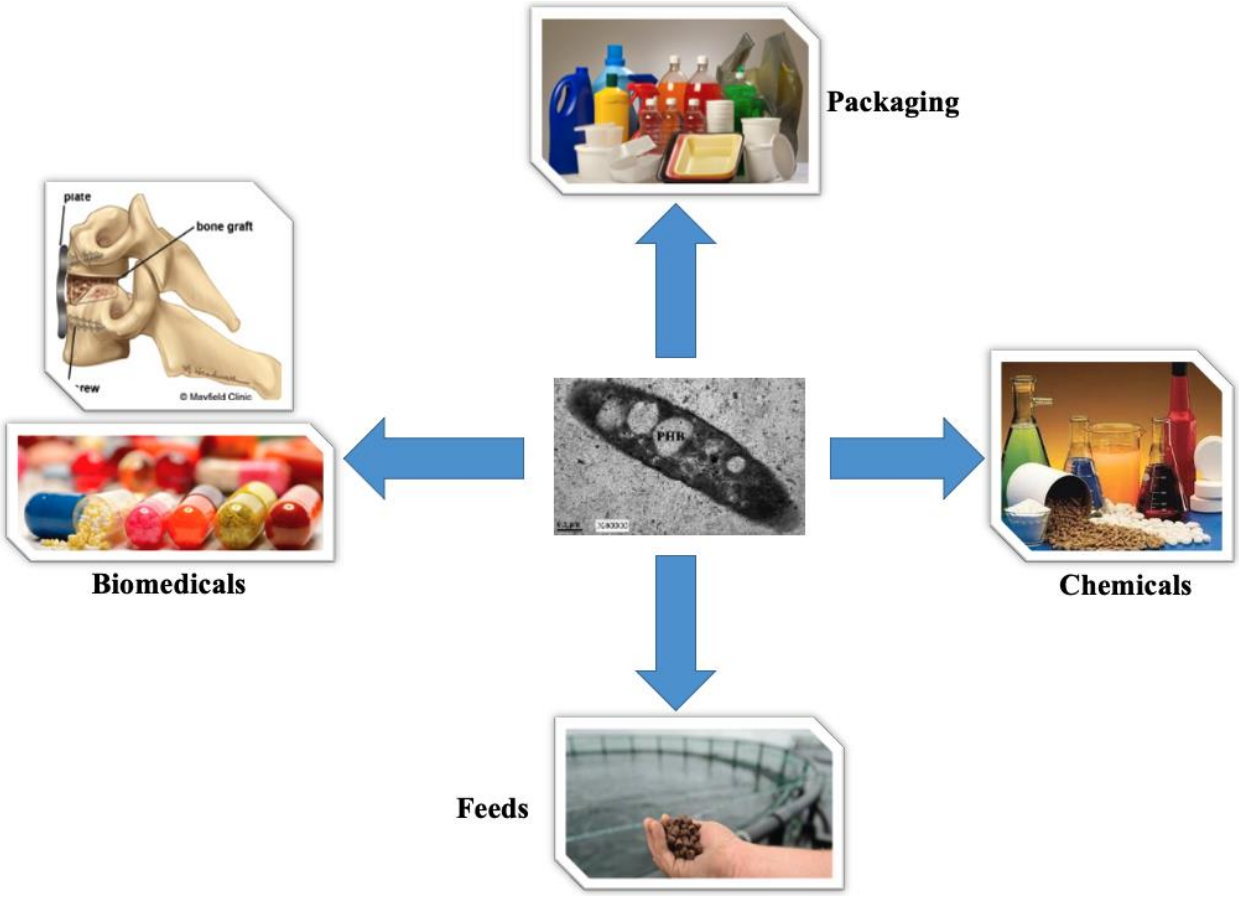


Figure 2.2. Some of polyhydroxyalkanoates (PHA) applications. Inset image shows transmission electron micrograph of a *Bacillus* sp. JL47 strain containing intracellular poly(3-hydroxybutyrate) (PHB).[1]

2.1.1. Poly(3-hydroxybutyrate) (PHB) Properties and Applications

PHB is a SCL type of PHA consists of repeating units (monomers) of C-4 short-chain fatty acid (SCFA) (3-hydroxybutyrate; 3-HB).[2] It is the most commonly used and widely studied PHA that was first discovered by Lemoigne[14] in 1926, when a significant pH drop in the cultivation medium was observed due to the production of 3-HB acid during the autolyzation of *Bacillus subtilis* in distilled water (DI). Owing to the similar physical and chemical characteristics of PHB to those of petroleum-based polymers (i.e., polypropylene and polyethylene), PHB has been highly regarded as a promising bioplastic in several practical applications.[15] It has been also found that the mechanical properties of the bioplastic improves by incorporating higher molecular weight (M_w), i.e., longer PHA polymers in the PHB structure.[15] For example, incorporating hydroxyvalerate (C-5) monomer in PHB (i.e., poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV)) enhances the thermomechanical properties of the polymer, allowing it to be used for wider applications as medical materials (sutures and bone nails/pins), disposable items (pens and tableware), and film products (covering films, shopping bags, and compost bags).[16]

Only recently, the PHB application has been extended to the aquaculture industry. Because PHB consists of repeating units of SCFA (3-HB), it has been suggested that PHB could be used as a promising biocontrol agent to replace antibiotics by inhibiting pathogens and improving growth and immune system functions of aquatic animals.[13] Briefly, the main mechanism for PHB is that it is biodegraded in the gastrointestinal tract into its monomers/SCFAs (i.e., 3-HB and butyrate). Many SCFAs, particularly butyrate,[1, 17] are known to inhibit the growth of various enterobacteria and decrease pathogen invasion by diffusing into the pathogenic cell membrane and acidifying the cytoplasm.[1, 17-20] As a consequence, pathogens consume high cellular energy to maintain homeostasis by pumping out excess protons (H^+), leading eventually to cell death (Figure 2.3).[21]

Therefore, PHB can be used as an effective biocontrol agent[22, 23] and an immunostimulant[24-28] for aquatic species, thus replacing the widespread use of unsafe antibiotics.[29, 30]

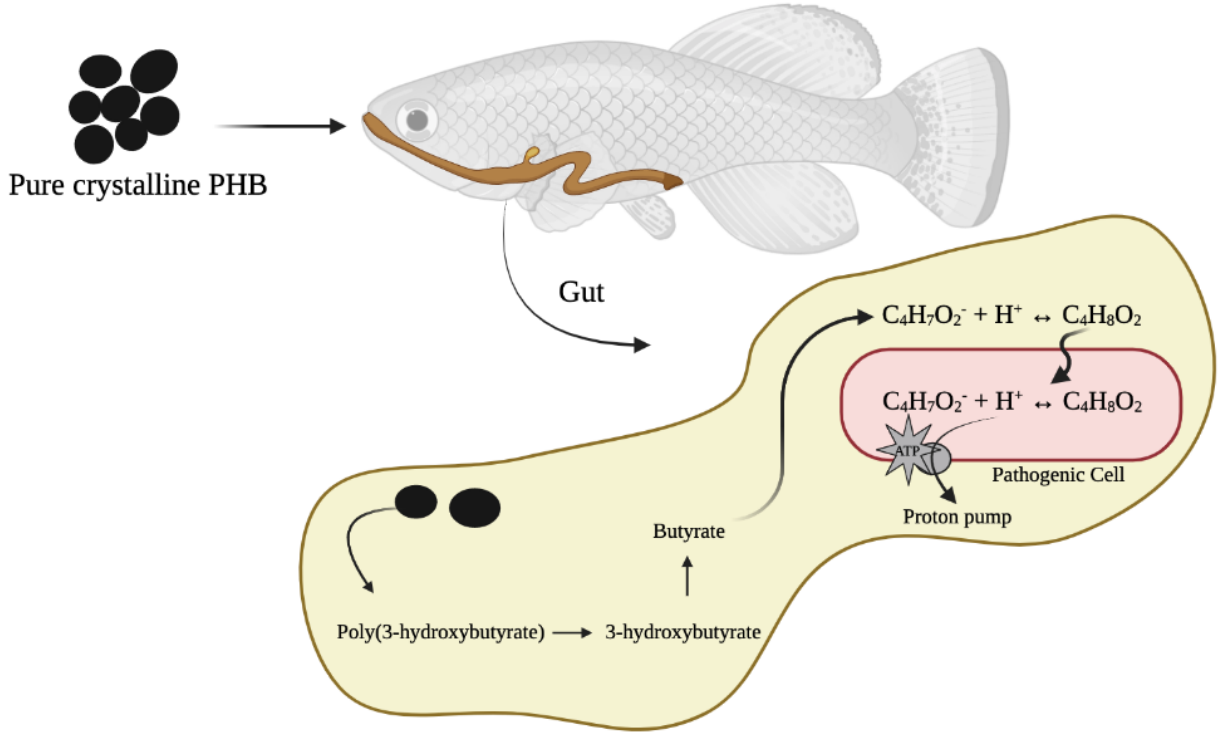


Figure 2.3. The main PHB mechanism in inhibiting pathogens in aquaculture.

2.1.2. PHA-Producing Microorganisms

There are a variety of microorganisms, such as Gram-negative (G–) and Gram-positive (G+) heterotopic bacteria, photosynthetic bacteria (cyanobacteria and purple non-sulfur bacteria), and archaea, which can accumulate different PHA co-/polymers for energy storage.[31, 32] Those microorganisms originate from diverse habitats and ecosystems, such as estuarine sediments, rhizosphere, mangrove sediments, groundwater sediments, and wastewater treatment plants.[31] Besides being used as an energy reserve by microorganisms, PHA acts as a multifunctional agent by reducing the harmful effect of osmotic and other stress factors, maintaining anoxic photosynthesis and sulfur (S) cycle, promoting sporulation, and enhancing N fixation.[31] Among various PHA-accumulating microorganisms, PHA production from bacterial strains has great

potential due to their high accumulation capacity and their ability in utilizing a wide range of substrates. The PHA production from various bacterial strains has been intensively studied and recently reviewed.[3, 16, 31-33] In general, PHA-accumulating bacteria can be classified into two groups based on stress conditions required for PHA accumulation. For the first group, the accumulation of PHA does not occur during bacterial growth. The limitation of essential nutrients (e.g., N, P, O, and S) with an excess of C is required for bacteria to accumulate PHA. Those type of bacteria are called non-growth-associated PHA producers. Most common types are *Alcaligenes eutrophus*, *Protomonas extorquens*, and *Protomonas oleovorans*.[34] In contrast, the second group is called growth-associated PHA producers, which are unaffected by the limitation of nutrients; therefore, bacteria are able to store PHA during growth.[31, 35] Most common growth-associated bacteria include *Alcaligenes latus*, a mutant strain of *Azotobacter vinelandii*, and recombinant *Escherichia coli*.[32]

2.1.2.1. *Zobellella denitrificans* ZD1 as a Promising PHA/PHB Producer

Zobellella denitrificans ZD1 (designated as ZD1 hereinafter) is a G– heterotopic, salt-tolerant bacteria isolated from sediment samples collected from various mangrove ecosystems in Taiwan.[36] It is a growth-associated PHA producer with encoding enzymes (phaB, phaA, PFP, and phaC) involving PHB production[37, 38] and 98.5% 16S rRNA similarity to a known PHB-accumulating strain *Zobellella denitrificans* MW1.[39] Unlike non-growth-associated PHB producers that can only accumulate PHB under nutrient-limited conditions, requiring a complex two-stage fermentation bioprocess,[35] ZD1 can grow fast with a cultivation period of 24 h and accumulate high levels of PHB (up to 84% in cell dry weight (CDW) in a growth-associated manner.[40, 41] Therefore, PHB production from ZD1 can be implemented in a simple and

continuous single-stage bioprocess. The high PHB accumulation capacity can place strain ZD1 as one of the highest PHB-accumulating strains among the previously reported strains. Furthermore, a three-gene cluster (*ectA, ectB,* and *ectC*) responsible for the synthesis of ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid), which is a common osmolyte in many halophiles that protects them against high salinity, has been previously identified in the ZD1 genome.[38] Therefore, the advantage of using salt-tolerant ZD1 that it eliminates the need for costly sterilization by growing the strain in salty mediums (30 g/L NaCl); hence, inhibiting the growth of non-salt-tolerant microorganisms.[40] Interestingly, ZD1 has shown ability to adapt and utilize various saline and non-saline agro-industrial wastes/wastewaters, such as activated sludge, high-strength synthetic wastewater, synthetic crude glycerol, and aquacultural wastewater to produce PHB without sterilization.[40, 42] Yet, the effects of different agro-industrial wastes as C-sources on the composition of PHA co-/polymer in ZD1 and its application in aquaculture has not been investigated.

2.1.3. Substrates used for PHA Production

Selecting a substrate for bacterial growth can represent 28–50% of the total PHA production cost.[31, 43] Therefore, a diverse range of cheap substrates utilization by various PHA-accumulating bacterial strains have been intensively studied and reviewed.[3, 16, 31-33] Those substrates vary from simple pure organics to complex waste streams, including industrial byproducts, fats and oils, lignocellulosic biomass, agricultural and household waste materials, glycerol, sugars, alcohols, and wastewater. Using agro-industrial wastes/wastewaters that are rich in C and other nutrients has been described as promising alternatives to traditional pure substrates like glucose. Moreover, studies have shown that the choice of substrates could impact the PHA

composition in the cells. For instance, when hexanoate was used as a C-source for *Pseudomonas oleovorans*, PHA composition consisted of a major polyhydroxyhexanoate (PHH) with 18% of polyhydroxyoctanoate (PHO), while the PHO content reached 86% when octanoate was used as a C-source. The study further indicated that using an even-chain-length fatty acid as the sole C-source will promote even-chain-length PHA and vice versa.[44] It is important to note that the selection of renewable substrates in the form of waste streams and/or byproducts should meet some basic requirements, such as high availability and constant quality throughout the year, storing suitability, no conflict with other feedstock applications, and easy collection and transportation. Optimally, PHA production should be integrated into existing industrial production lines.[31]

Despite the great potential of using waste streams for reducing PHA production cost, this strategy comes with few drawbacks. For example, agro-industrial wastes contain a large fraction of complex organics and inhibitors that limit their utilization by bacteria and decrease PHA yield.[31, 33] This leads to the necessity of applying some toxic or energy-intensive pretreatments, such as solvents or thermal processes to purify the substrates and enhance the availability of fermentable sugars.[33] Another major drawback is the difficulty to control the composition PHA accumulated in the cells due to the complexity of applied substrates.[31] Finally, the migration of pollutants (heavy metals) from organic wastes to PHA is also another concern that should be taken into consideration. It was reported that total content of heavy metals in PHA produced from fruit wastes or crops were lower than PHA samples produced from municipal wastewater and sludge.[45] However, the results indicated that heavy metals were below the migration limits specified by the Commission Regulation (EU) October 2011 on plastic materials and articles for contact with food under frozen and refrigerated conditions.[45] Overall, further studies to discover new utilization

techniques, carbon substrates, and hyper PHA-accumulating strains are needed to overcome those obstacles and achieve high and sustainable PHA production.

2.1.4. Biosynthesis Pathways of PHA/PHB

There are three well-known pathways for PHA biosynthesis. Those pathways depend on the provided substrates (C-sources) that shape the monomer composition, metabolic pathways, and specificity of PHA synthase.[33] A general scheme of the PHA biosynthesis pathways from different C-sources, including main intermediates, metabolites, enzymes, and cofactors is illustrated in Figure 2.4. Pathways I (tricarboxylic acid (TCA) cycle), which is responsible for SCL-PHA biosynthesis from sugars and was well studied for *Cupriavidus nectar*, starts with the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA by 3-ketothiolase (PhaA), which is then reduced to 3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (PhaB) using NADPH as the electron donor. Finally, 3-hydroxybutyryl-CoA is polymerized to PHB by PHA synthase (PhaC class I). Also, 3-hydroxyvalerate (3-HV) monomer units could be synthesized by the addition of propionate or valerate as precursors to the cultivation medium causing the condensation of propionyl–CoA and acetyl-CoA by the action of 3-ketothiolase to 3-ketovaleryl-CoA.[33]

On the other hand, the biosynthesis of MCL-PHA has been reported through Pathway II and III (Figure 2.4), particularly in *Pseudomonas putida* and *P. oleovorans*. Pathway II (β-oxidation) involves the degradation of fatty acids (C-sources) to generate substrates/intermediates (e.g., trans-2-enoyl-CoA) that can be converted into (R)-hydroxyacyl-CoA by (R)-specific enoyl-CoA hydratase, yielding MCL-PHA monomers.[33] Pathway III (*de novo* fatty acid synthesis) converts the fatty acids to (R)-3-hydroxyacyl intermediates that are then converted from their acyl carrier protein form to the CoA form by acyl-ACP-CoA transacylase (encoded by phaG). This *de*

novo fatty acid pathway is of particular interest because it uses simple, inexpensive, and related C-sources such as glucose, sucrose, and fructose for the biosynthesis of PHA monomers.[3, 33]

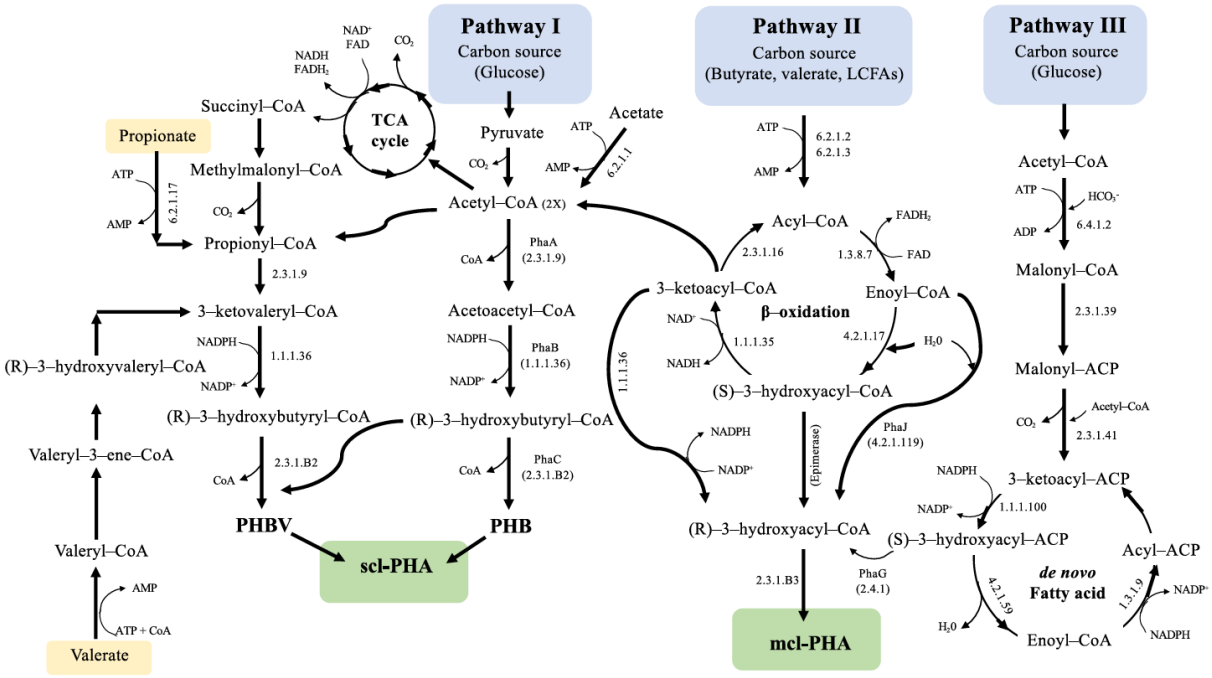


Figure 2.4. PHA biosynthesis pathways from different C-sources, including main intermediates, metabolites, enzymes, and cofactors.[33]

2.1.5. Challenges of PHA Production and Potential Solutions

Despite the undisputed advantages of the applications of PHA biopolymers, PHA production cost is hampered with some challenges, limiting their use in niche applications like biomedical products (sutures, bone nails/pins, implants, biodegradable carriers, etc.).[16] Some of the major challenges are (i) the use of expensive substrates for cultivating PHA-accumulating microorganisms[46] and producing specific PHA co-/polymers,[6] (ii) the need of costly sterilization process to avoid microbial contamination in the cultivation reactor,[40, 47] (iii) and the high-energy input to harvest and dry PHA-filled microorganisms before (iv) the application of toxic solvents for PHA extraction and purification.[48, 49]

To overcome the above mentioned PHA production challenges, new strategies for bacterial cultivation and downstream processing have been explored. For instance, a nonsterile production of PHA have already been sought, including the use of halophilic archaeal and bacterial strains (requiring salts for cell growth) to outcompete non-halophiles, thereby successfully producing PHB from glucose, acetate, or synthetic seawater without sterilization.[50-53] In parallel, extensive studies have been conducted to test different waste streams as substrates.[3, 16, 31-33] Therefore, using inexpensive substrates for cultivating PHA-accumulating strains in saline nonsterile growth medium is a promising solution for the viability of PHA production.[40] Furthermore, various cost-effective harvesting approaches of microorganisms, such as the use of organic coagulation, palletization, co-cultivation of bacteria and microalgae, have been explored to substitute traditional high-energy input centrifugation, flotation, filtration, or toxic metal coagulation.[2, 54, 55] Finally, studies have investigated various downstream extraction techniques of biolipids and PHA/PHB to overcome inefficient and toxic physical and chemical extractions. Examples of those efficient and safe extractions are enzymatic cell lysis,[56] bacteriophage-based extraction (i.e., viruses that lyse bacteria),[57] and cell autolysis.[58]

The purpose of PHA application is a crucial factor that determines the cultivation methods, downstream processes, and polymer characteristics, all of which affect the production cost of PHA. Taking the use of PHA as a bioplastic for example. Although PHA has attracted a widespread attention as an effective alternative to conventional plastics, this kind of application is restricted by several factors, such as expensive chemical-based downstream processes to extract and purify PHA, high polymer quality requirements to meet consumer needs, and a fierce competition with the traditional petroleum-based plastics. As a result, the market price of PHA (2.25–2.75 US$/lb) is approximately 3–4 times higher than synthetic plastics (e.g., polypropylene and polyethylene).[3]

Accordingly, PHA could have higher chance of success if used in different applications, where minor variations in polymer properties are not regarded as problematic.

As discussed before, PHB, the most common SCL-PHA, has recently been revealed as a promising alternative to antibiotics in aquaculture by serving as a biocontrol agent and immunostimulant to improve growth and disease resistance. Such a novel application eliminates the cost associated with PHB extraction and purification by feeding whole PHB-rich microorganisms (i.e., amorphous PHB) as microbial proteins to aquatic animals. Furthermore, the presence of other PHA monomers would not interfere with the application of PHB. In fact, longer-chain PHA (i.e., MCL-PHA), after their biodegradation into their intermediates (medium-chain fatty acids (MCFAs)) could have greater inhibitory activities against aquaculture pathogens, thereby reducing the required dosage and associated costs.[59] Moreover, the cultivation of PHA-accumulating microorganisms could be integrated with agro-industrial wastes, including aquaculture wastewater/wastes as substrates under saline mediums. This strategy will facilitate the implementation of waste-derived PHA and lift the energy-intensive sterilization by cultivating salt-tolerant PHB-accumulating strains; hence, inhibiting the growth of non-salt-tolerant microorganisms. Overall, this new application will facilitate PHA feasibility, while maintaining sustainable and economical aquaculture.

2.2. The Aquaculture Industry

2.2.1. Background and Common Practices

Aquaculture is the fastest-growing food production sector with an average growth rate of 8%, exceeding the growth rates of grain (1.4%), livestock (2.2%), and poultry (4.6%).[13, 60] It consists of intensive farming of all forms of aquatic animals (i.e., fish, crustaceans, mollusks, etc.) and plants (seaweeds) in all aquatic environments (fresh, brackish, and marine). As a result, aquaculture plays a vital role in food security with fishery protein contributing more than 33% of the total animal protein supply for human uptake.[61] Furthermore, due to the limited resources and supplies of wild-capture fisheries and the growing seafood demand, the global aquaculture production is estimated around 82 million tonnes of aquatic animals in 2018 (527% increase since 1990), valued at 250 billion US dollars (Figure 2.5).[62] This global aquaculture production was increased to approximately 87 million tons in 2019, accounting for more than half of the total fish consumed by humans.[1] Therefore, aquaculture essentially contributes to the "sustainable intensification" envisioned by Godfray regarding the challenge of feeding 9 billion people in the upcoming decades.[63]

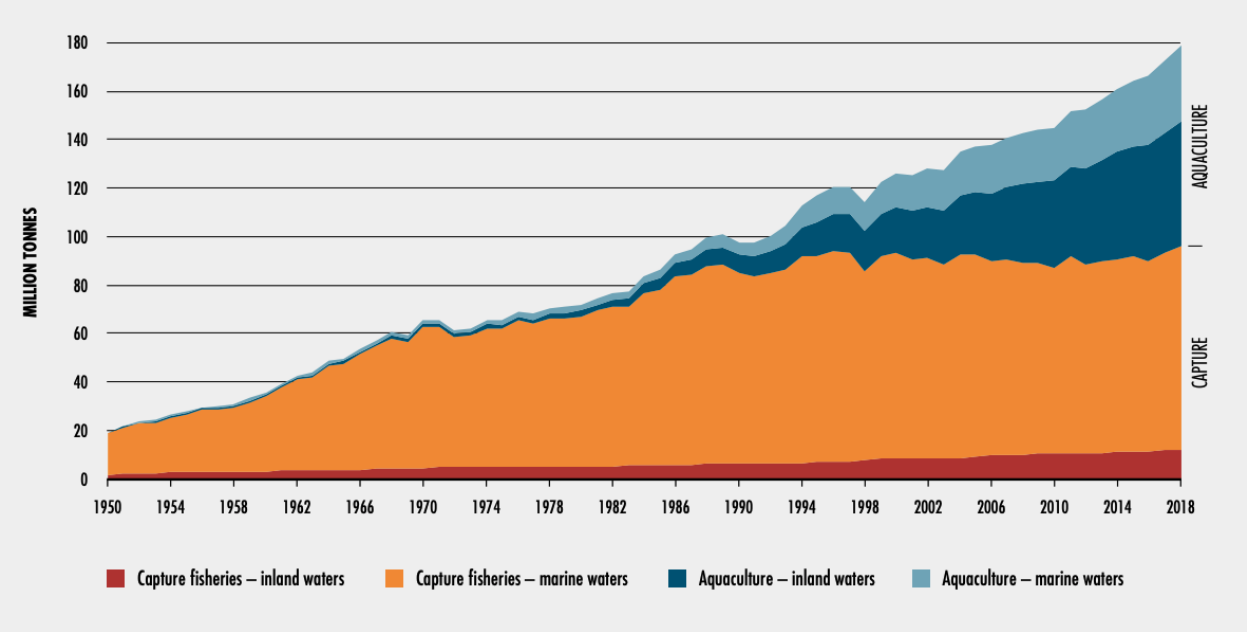


Figure 2.5. World capture fisheries and aquaculture production from 1950–2018.

There are various farming sytems for culturing aquatic animals in the aquaculture industry. Those systems are catogerized into water-based systems (inshore/offshore), land-based systems (ponds, flow-through systems, and raceways), and most importantly, recycling systems (high-control enclosed systems). A recirculating aquaculture system (RAS) is an indoor farming system commonly used to overcome the shortcomings of traditional outdoor systems/practices. It prevents the escape of wastes and animals into the environment and eliminates the adverse impacts of climate on farming. Additionally, RAS plays a vital role in supporting the sustainability and commercial viability of aquaculture by reducing water consumption and footprint and allowing year-round intensive production under controlled conditions.[64] Conventional RAS consist of aquatic animals' tanks, solid settlers, biofilters, sand filters, and UV disinfection units (Figure 2.6). Briefly, aquaculture wastewater (AW) from the aquatic animals' tanks first enters solid settlers to precipitate suspended particles (feces, sand, silts, food debris, etc.). Next, the supernatant from AW moves to biofilters to remove toxic ammonia by the nitrification process through nitrifiers

grown in the biofilter. Then, the water passes sand filters as a polishing step to remove small particles. Finally, the water goes through UV disinfection units to destroy harmful bacteria, viruses, and microbes by damaging their DNA, before being recirculated to the fish tanks.

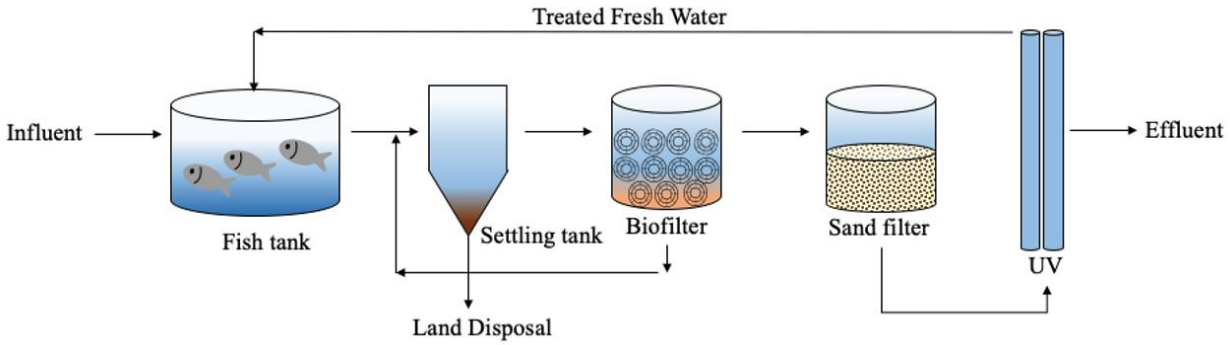


Figure 2.6. Conventional Recirculating Aquaculture System (RAS).[42]

2.2.2. Aquaculture Challenges

Despite the advantages of aquaculture and improvements in farming systems like RAS, current aquaculture systems/practices are outengineered and faces challenges. The major challenges in aquaculture are (i) waste management, (ii) high feed costs, and most importantly, (iii) disease outbreaks due to the proliferation of various infectious diseases (Figure 2.7).[13, 60] Those challenges are considered limiting constraints for the sustainable development of safe and economical aquaculture.

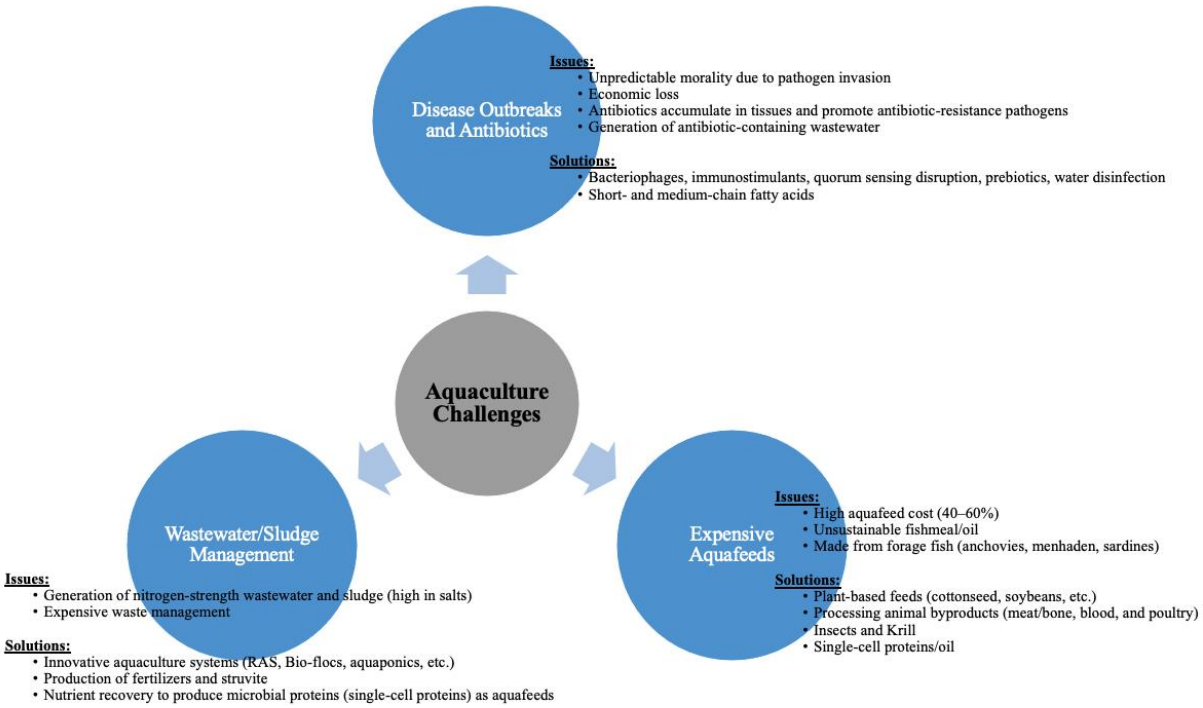


Figure 2.7. Aquaculture challenges and potential solutions.

2.2.2.1. Management of Aquaculture Wastewater and Solid Waste

Aquaculture generates wastewater and solid wastes (sludge) that contain high N along with high salt content (in case of farming marine species), which need be to be treated before discharge and/or utilized for resource recovery to prevent any environmental issues, such as eutrophication, salinization, and sodification.[65, 66] Although the components in AW are simpler than those of agro-industrial and municipal wastewaters, AW includes concentrated streams with suspended solids (SS), total nitrogen (TN), and total phosphorus (TP) that need to be treated. Generally, AW contains approximately 5–50 mg/L of SS, 0.12–14.7 mg/L of NH_4^+-N, 0.02–1.5 mg/L of NO_2-N, 0.01–5.3 mg/L of NO_3-N, and 3.1–17.7 mg/L of PO_4^{3-}-P.[66] Among those constituents, ammonia is of particular interest because it is the major component, contributed by unconsumed aquafeed (around 70–80% of the ammonia in AW)[67, 68] and aquatic animals' wastes in farming tanks,[69] and considered toxic to most aquatic species. In terms of aquaculture sludge, this waste mainly consists

of animal feces and organics, mostly in particulate form.[65] As grain and plant materials are common ingredients in aquafeed, undigested fibers contribute to a large fraction of nonbiodegradable complex organics in the aquatic animal feces.

AW is typically treated for reuse in the aquaculture system,[66, 70] while sludge most often applied as a fertilizer or disposed into sewage systems.[65] However, the cost of sludge transfer to fields along with odor can hamper its application as a fertilizer. There is also a concern about the discharge of AW and sludge application due to its high salinity, which might potentially cause salinization and/or sodification of soil, groundwater, and local surface water.[65] While AW and sludge have been considered wastes for removal, previous studies have reported that AW can be used to produce microbial proteins (i.e., single-cell proteins) as aquafeeds.[66, 71] Therefore, the high organics, nutrients, and salts in aquacultural wastes could become a great asset to produce microbial proteins, including salt-tolerant PHA-accumulating microorganisms, and prevent sterilization by inhibiting the growth of non-salt-tolerant microorganisms. Therefore, an optimum growth medium for PHA-accumulating microorganisms can be provided by replacing precious freshwater that is conventionally used for this purpose with AW.

2.2.2.2. Expensive and Unsustainable Aquafeeds

The use of unsustainable costly aquafeeds is considered another major bottleneck in the aquaculture industry. Feeding aquaculture highly depends on the capture of forage/trash fish (e.g., anchovies, menhaden, and sardines) to produce fishmeal and fish oil to be used in the diet. Forage fish play an essential role in the marine ecosystem by primary production from plankton to larger fish, mammals, and birds.[13] For several decades, 20–30 million tonnes of fish (~1/4–1/3 of the global fish catch) have been removed from the marine food web each year to produce fishmeal/oil

for animal feeds and other industrial purposes.[72] Despite the improvements in food conversion ratio (FCR) in aquaculture, which is the amount of feed (in kilograms) needed to increase the aquatic animal's bodyweight by one kilogram, the expansion in aquaculture has continued the pressure on forage fish capture. Similarly, the ratio of wild fisheries inputs to farmed fish output (i.e., fish-in to fish-out ratio) has significantly decreased to 0.63 for the overall aquaculture sector but remains as high as 5.0 for Atlantic salmon.[72]

To overcome the aquafeed issue, various feed alternatives have been suggested to lower the aggregate level of fishmeal/oil inputs in aquafeeds and alleviate the pressure on forage fish. Examples of those substitutes are plant-based feeds (e.g., barley, canola, corn, cottonseed, soybeans, and wheat), rendered terrestrial animal products (e.g., meat and bone meal, feather meal, blood meal, and poultry by-product meal), processing seafood by-products, different species of insects and krill, and single-cell proteins.[72] However, these alternatives are not without shortcomings. For instance, plant-based alternatives may contain antinutritional factors (e.g., protease inhibitors, phytates, glucosinolates, saponins tannins, lectins, gossypols, cyanogens, mimosine, canavanine, antivitamins, etc.),[73] and high level of fibers and nonsoluble carbohydrates, limiting their digestibility by aquatic animals.[72] On the other hand, the use of animal by-/products in aquafeeds is limited by consumer acceptance and potential risk of disease transmission to aquatic animals.[72]

Among different aquafeed alternatives, single-cell proteins/oil (SCP/O) have high potential to reduce aggregate levels of forage fish in aquafeeds. SCP/O are microbial biomass that are produced by various microorganisms (bacteria, algae, yeast, and fungi) growing on pure organics and/or agro-industrial wastes. Such microorganisms can be used effectively as animal feed because they are rich in proteins and contain readily digestible nutrients (e.g., lipids and minerals)

for animal consumption.[13, 72, 74-77] Table 2.1 illustrates the comparison between common microbial biomasses (SCPs) composition with other protein sources, such as fishmeal and soybean meal. As the protein and energy content are comparable with conventional feeds, SCPs can address a significant sustainability metric that is the reduction in the "fish-in to fish-out" ratio, which have been continuously endorsed by many scientists and professionals in the aquaculture industry.[72] The development and production of SCP is tied to food sacristy and high costs of food previously experienced during wars.[13] In the early 20th century, scientists from Germany and England during World War I evaluated SCP production by yeast grown on molasses and examined their potential as a feed component for pigs. After World War II, the focus has shifted from yeast to bacteria, allowing the investigation of broader range of feedstocks as C-sources and cultivation conditions for large-scale production. In the late 20th century, extensive research has been conducted with producing SCP from different microorganisms (yeast, bacteria, and algae) and feedstocks due to the concerns related to rapid human population growth. It is not until the 21st century when renewed interest in SCP production has been reinforced due to the rise of global environmental issues and a boom in the aquaculture industry.[13]

Table 2.1. Comparison of microbial biomass (SCPs) composition with other protein sources.

Protein Source		Energy Source/Substrate	Protein[a] (%)	Lipid (%)	Ash (%)	Energy (MJ/kg)	References
Microbial biomass (SCPs*)	*Bacillus licheniformis*	Potato processing waste	38	-	11	-	[78]
	Purple phototrophic bacteria	Light/Poultry WW[a]	~75[b]	~20	-	22	[79]
		Light/Dairy WW	~61	~29	-		
		Light/Sugar WW	~42	~20	-		
	Methane-oxidizing bacteria	Biogas methane	60	8-11	6-9	-	[80-82]
	Hydrogen-oxidizing bacteria	Hydrogen	75	-	-	-	[80]
	Microalgae: *Chlorella vulgaris* and *Scenedesmus* species	Light/Poultry WW	~65	~27	-	-	[79]
		Light/Dairy WW	~37	~59	-	-	
		Light/Sugar WW	~14	~15	-		
	Yeast	Organic carbon	45-55	1-6	5-10	19.9	[83, 84]
Fishmeal		-	63	11	16	20.1	[85]
Soybean meal		-	44	2.2	5	21.3	[85]

[a] WW refers to wastewater. [b] The values were estimated from Figure 3 in Hülsen et al.[79]

Conventional SCP/O are phototrophic microorganisms such as algae and purple phototrophic bacteria that are cultivated with energy-intensive illumination to low cell densities.[77, 79] Furthermore, microorganisms known for producing polyunsaturated fatty acids (PUFAs) such as microalgae and fungi[86-88] have been used for SCP/O production to serve as a feed supplement. PUFAs, particularly eicosapentaenoic and docosahexaenoic acids, are known to be essential supplements in the aquafeed as they can improve fish health and the quality of seafood produced (e.g., increased omega-3 content in the seafood).[72, 89, 90] Interestingly, previous studies have also reported successful cultivations of different PUFA-rich microalgal strains using fish farm effluents.[90, 91] Other microalgae, such as *Chlorella sp.* and marine green algae *Platymonas subcordiformis*, have been also successfully cultivated in aquaculture wastewater.[66, 71] However,

such an approach was limited by the low lipid content (25%) and low biomass yields (usually <400–850 mg/L) in the cultivated microorganisms.[86, 90]

On the contrary, the production of bacterial heterotrophs as SCPs is more energy-efficient than phototrophic microorganisms. It only requires approximately 230-MJ C/electron donor to produce 1 kg of biomass.[74, 92, 93] Most importantly, some of heterotrophic microorganisms have also shown ability to accumulate PHB biopolymer in their cells, which is a SCP coproduct that has a great potential to replace antibiotics and improve growth and disease resistance in aquaculture.[1, 13] Therefore, PHB-rich SCP from heterotopic bacteria can simultaneously tackle the major aquaculture challenges represented in the reduction of expensive aquafeeds and combating disease outbreaks. Besides the excellent accumulation of the healthy feed additive (PHB) as a replacement for antibiotics, the produced PHB-rich SCP has many other advantages: higher biomass yield than methane or hydrogen-oxidizing bacteria[13] and lower land/water requirement and anti-nutritional factors compared with soybean meal.[13, 75] It is also imperative to recognize that heterotrophic bacteria lifts the energy-intensive illumination required for traditional SCPs (such as purple phototropic bacteria or algae).[79] Such an advantage agrees with the "dark food chain" envisioned previously,[13] wherein chemoheterotrophy substitutes photosynthesis of SCPs as animal feed or human food. Accordingly, a novel strategy is to design aquaculture systems/configurations that can concurrently produce healthy PHB-rich heterotopic bacterial biomass as SCP while treating aquaculture wastewater/wastes for reuse.

2.2.2.3. Disease Outbreaks and the Use of Antibiotics

Disease outbreaks and unpredictable high mortality due to pathogen infection is a significant constraint on the economic viability of aquaculture. Compared to terrestrial environment, the aquatic environment is more vulnerable to pathogens, which can be ingested with aquafeed or reach high densities in the water.[94] The infected animals commonly result in weak growth and thus lead to a substantial economic loss. For example, the global economic loss due to acute hepatopancreatic necrosis caused by *Vibrio* has been estimated at over $ 1 billion US dollars per year in shrimp farming alone.[95] To prevent such damage, antibiotics have been widely used to control pathogens in aquaculture. However, the long-term use of antibiotics pose a significant risk to food safety and public health, as antibiotics might accumulate in the tissues of aquatic species[29] and promote antibiotic-resistant microbes in aquaculture and human beings.[30, 94] This risk associated with resistance transmission to human pathogens led to a controversial issue in the U.S. and Europe in which a ban was initiated in Europe in 2006 in regards to applying antibiotics in either aquaculture or terrestrial animal production.[21, 96] However, antibiotics remain in use in many other countries in aquaculture industry. Furthermore, conventional aquaculture practices (e.g., RAS and ponds) have been shown to generate wastewater/wastes containing toxic antibiotics if administered to animals in those culture systems, and are also considered contaminants of emerging concern in the wastewater treatment industry.[29, 97-99] Therefore, there is an urgent need to develop safe and effective antibiotic alternatives to overcome this issue and improve the aquaculture industry.

To control disease outbreaks and limit/prevent the use of antibiotics, different protective strategies, such as bacteriophages, immunostimulants, SCFAs, quorum sensing disruption, prebiotics, water disinfection, etc., have been tested.[20, 94] Developing an effective antibiotic

alternative depends on targeting the strong and complex interactions between the cultured animal (host), pathogen, and environment depicted in Figure 2.8. In fact, a successful alternative should have several modes of action targeting each member to maximize the effectivity of the antibiotic alternative. Among different alternatives, SCFAs are known for combating bacterial diseases in aquaculture animals.[21] SCFAs can also inhibit the growth of enterobacteria such as *Salmonella typhimurium*, *Escherichia coli* and *Shigella flexneri*.[17-20, 100-102] Once penetrating the cell membrane of the pathogens, SCFAs acidifies the cytoplasm. In turn, the pathogens must redirect their cellular energy to maintain homeostasis, suppressing their growth, and causing cell death.[49, 103] Among many different SCFAs (formate, acetate, propionate, and valerate) tested, butyrate is the most effective SCFA in decreasing the invasion of the pathogens.[104] It was described that butyrate-supplemented media dramatically reduced pathogenic invasion in comparison to acetate-supplemented media. Only recently, it was also reported that medium-chain fatty acids (MCFAs) such as hexanoate and 3-hydroxyhexanoate are effective in inhibiting the growth of shrimp-pathogenic bacterium *Vibrio penaeicida*.[59] Nevertheless, the prophylactic use of fatty acids to aquatic animals is limited by their high solubility in water due to their polar nature. This high solubility of fatty acids, even with effective butyrate, weakens their uptake efficiency by aquatic animals as they are mostly considered as filter-feeding animals, which separate food particles from water.[105] Therefore, it is advantageous to use insoluble compounds of butyrate, such as the use of PHB for filter-feeders, as PHB is insoluble making the uptake of PHB more efficient, which can be later biodegraded to the desired intermediates (3-HB and butyrate) in the animals' guts.[1, 95]

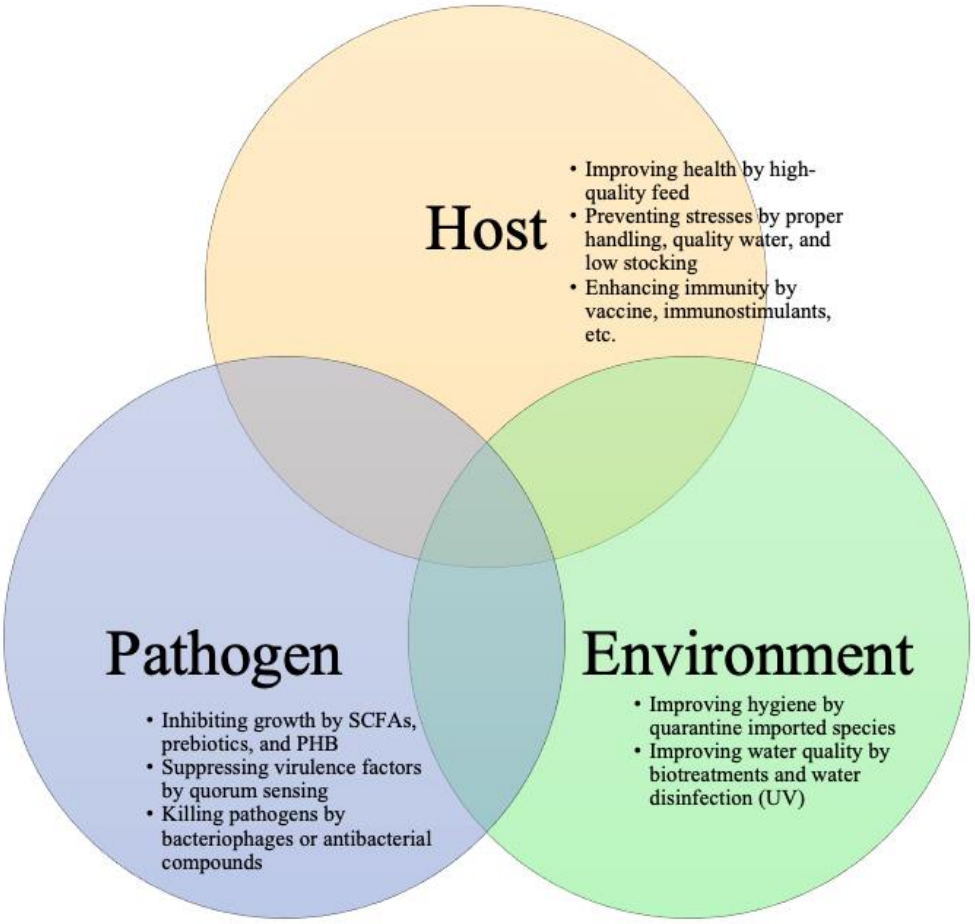


Figure 2.8. Host-pathogen-environment interaction in aquaculture and strategies to prevent and control diseases without using antibiotics (redrawn from Defoirdt et al.).[20, 94]

2.3. PHB Application in Aquaculture

2.3.1. PHB as a Promising Biocontrol Agent in Aquaculture

PHB is a promising alternative to antibiotics and inefficient soluble SCFAs. It is a nontoxic biodegradable biopolymer that can be produced by various microorganisms.[2] Uptake of PHB is more efficient than the soluble SCFAs for filter-feeding animals because PHB is insoluble and can be converted into soluble 3-HB in the animal gut.[20] Recent studies have reported that PHB can act as a biocontrol agent[22, 23] and an immunostimulant[24-28] and that PHB-supplemented tilapia achieved an 85% survival rate,[105] a similar survival rate when using a common oxytetracycline

antibiotics.[106] As previously discussed, PHB consists of repeating units of a SCFA (3-HB). PHB can be biodegraded in the animal gastrointestinal tract into its intermediates/SCFAs (3-HB and then to butyrate), which are known for combating the growth of pathogens.[1, 17]

Supplementing aquafeed with PHB in crystalline form (i.e., extracted from microbial biomass) or amorphous form (i.e., the PHB inside the microbial cells) has been shown to improve the growth of aquatic animals[1, 13] and the disease resistance of many fish species[107-109] and crustaceans (Table 2.2).[12, 25, 28, 110] The major advantage of supplementing amorphus PHB (i.e., PHB-accumulating microorganisms) that it avoids the application of toxic solvents for PHB extraction and purification.[48, 49] Amorphus PHB is also accompanied with other cell nutrients (e.g., proteins, lipids, and minerals)[42] that are likely to elicit additional energy, lipid deposition, and immune response in aquatic animals.[105, 111, 112] Furthermore, amorphous PHB particles have smaller size and lower crystallinity compared to crystalline PHB particles, making it more susceptible to biodegradation.[1] Examples of PHB-accumulating microorganisms that were applied to aquafeeds are *Alcaligenes eutrophus* H16,[49, 113-115] *Halomonas* spp.,[116] *Brevibacterium casei* MSI04,[117] *Bacillus* sp. JL47,[12] *Comamonas testosteroni* CNB-1,[110] and *Brachymonas denitrificans* AS-P1.[110] Apparently, the application of amorphous PHB can be more practical and economical when compared to the high price of pure crystalline PHB.

Table 2.2. Overview of PHB application and effects on different aquatic animals.

Animal name	Species name	Life stage	PHA form	Effects observed	References
Brine shrimp	*Artemia franciscana*	Nauplii	Crystalline PHB	- Prolonged survival of starved *Artemia* - Increased survival against pathogen *Vibrio campbelli*	Defoirdt 2007[22]
	A. franciscana	Nauplii	Amorphous PHB-containing *Brachymonas denitrificans*	- Enhanced survival against pathogen *V. campbelli*	Halet 2007[110]
	A. franciscana	Nauplii	Amorphous PHB-containing cultures	- Higher survival against pathogen *Vibrio harveyi*	Van Cam 2009[24]
	A. franciscana	Nauplii	Crystalline PHB	- Increased survival against pathogen *Vibrio campbelli* - Induced the expression of defensive genes (heat shock protein, prophenoloxidase, and transglutaminase immune)	Baruah 2015[26]
	A. franciscana	Nauplii	Crystalline PHB	- Enhanced survival against pathogen *Vibrio* PUGSK8 - Degradation intermediates inhibited expression of virulence factors and biofilm formation	Kiran 2016[117]
	A. franciscana	Nauplii	Amorphous PHB-containing *Bacillus* sp. JL47	- Higher survival against pathogen *V. campbelli*	Laranja 2018[12]
	A. franciscana	Nauplii	Crystalline PHB	- Increased 3-HB concentration in the intestinal tract	Defoirdt 2018[95]
	A. franciscana	Nauplii	Crystalline PHB or amorphous PHB-containing *Ralstonia eutropha* DSM545	- Increased the whole-body lipid contents - PHB was rapidly assimilated in *Artemia* tissues	Ludevese-Pascual et al. 2020[112]
Freshwater prawn	*Macrobrachium rosenbergii*	Larvae	PHB-enriched *Artemia* (bioencapsulation)	- Increased survival and development of the larvae - Reduced total bacterial counts and *Vibrio* spp. counts	Nhan et al. 2010[118]
	Macrobrachium rosenbergii	Larvae	Amorphous PHB-containing *Alcaligenes eutrophus* H16	- Increased the survival against *V. harveyi* and larval development - Better efficiency in feeding PHB-containing *A. eutrophus*	Thai et al. 2010[49]

Table 2.2. Continued.

Animal name	Species name	Life stage	PHA form	Effects observed	References
Kuruma shrimp	*Marsupenaeus japonicus*	Adult 1.25 g	PHB-hydroxyhexanoate extracted from a recombinant strain of *Cupriavidus necator*	- Increased survival against *Vibrio penaeicida* - No significant effect on body weight, daily feeding rate, and feed conversion ratio	Fukami et al. 2021[59]
Pacific white shrimp	*Litopenaeus vannamei*		Crystalline PHB	- Increased protease, trypsin, chymotrypsin, and amylase - Enhanced digestibility of polysaccharides and lipids - Increased length, width, and perimeter of intestinal villi and integrity of mucus membrane - Increased the growth performance - Lowered the feed conversion ratio	Silva et al. 2016[119]
	Litopenaeus vannamei	Juvenile	Crystalline PHB	- Increased intestinal amylase, lipase, and trypsin activity - Induced total antioxidant capacity, nitric oxide synthase, and lysozyme - Induced heat shock protein 70, Toll and immune deficiency genes	Duan et al. 2017a[28]
	L. vannamei	Juvenile	Crystalline PHB	- Increased the intestinal SCFA and body composition (protein and lipid content) - Altered composition and diversity of intestine microbiota - Increased mammalian target of the rapamycin signaling-related genes	Duan et al. 2017b[27]
	L. vannamei	Postlarvae	PHB-based biodegradable plastic as artificial substratum	- Enhanced the survival and weight of larvae compared to using conventional PVC substratum - Increased trend of visit of the shrimp in the PHB-based substratum - Improved water quality	Ludevese-Pascual et al. 2019[120]
	L. vannamei	Postlarvae	PHB-enriched *Artemia* with amorphous PHB-containing *Halomonas* strain or crystalline PHB	- Enhanced survival, growth, and robustness against *Vibrio anguillarum* and salinity stress - Suppressed *Vibrio* in gut microbiome and promoted beneficial bacteria	Gao et al. 2019[116]

Table 2.2. Continued.

Animal name	Species name	Life stage	PHA form	Effects observed	References
Pacific white shrimp	*L. vannamei*	Juvenile	Gelatinized PHB extracted from *Brevibacterium casei* MSI04	- Increased weight gain and - Enhanced prophenol oxidase, superoxide dismutase, and total antioxidant activity - Resulted in 100% survival against *Vibrio parahaemolyticus* and showed no pathological changes in the muscle fibers and gills.	Kiran et al. 2020[121]
	L. vannamei	Postlarvae	Crystalline PHB	- Increased survival and growth with or without *V. harveyi* - Increased final body weight, total biomass, SGR, and weight gain - No effect on total abundance of heterotrophic bacteria and *Vibrio* spp. - Decreased tubular epithelial cell lesions due to *V. harveyi*	Situmorang et al. 2020[122]
	L. vannamei	Postlarvae	Amorphous PHB-containing *Ralstonia eutropha* DSM545	- Enriched pentadecanoic and palmitic fatty acids in shrimp - PHB was assimilated in tissues and could act as an energy source	Ludevese-Pascual et al. 2021[111]
Asian tiger shrimp	*Penaeus monodon*	Postlarvae	Different amorphous PHB-containing *Bacillus* strains	- Increased the survival before challenge - Enhanced survival against *V. campbelli*	Laranja et al. 2014[23]
	P. monodon	Postlarvae	Amorphous PHB-containing *Bacillus* sp. JL47	- Stimulated the innate immune-related genes, particularly prophenoloxidase and transglutaminase	Laranja et al. 2017[25]
	P. monodon	10–15 g	Crystalline PHB	- Enhanced survival and increased the level of haemocytes and prophenoloxidase when challenged with White Spot Syndrome Virus	Monica et al. 2017[123]
	P. monodon	Postlarvae	Crystalline PHB-enriched *Artemia*	- Enhanced survival against *V. campbelli* and ammonia stress	Ludevese-Pascual et al. 2017[124]

Table 2.2. Continued.

Animal name	Species name	Life stage	PHA form	Effects observed	References
Chinese mitten crab	*Erioceir sinensis*	Zoea 3 larvae	PHB-enriched rotifer or *Artemia*	- Enhanced the survival and growth of unchallenged larvae - Enhanced survival and development stage against *V. anguillarum*	Sui et al. 2012[125]
	E. sinensis	Zoea 2 to megalopa	PHB-enriched rotifer or *Artemia*	- Enhanced the survival, development stage, and tolerance to osmotic stress. - Improved weight gain, moulting frequency, and survival	Sui et al. 2014[126]
	E. sinensis	Juvenile	Crystalline PHB	- Increased hepatopancreatic pepsin, trypsin, lipase, total superoxide dismutase activities - Reduced alkaline and acid phosphatase - Increased richness, diversity, and evenness of gut community	Sui et al. 2016[127]
Siberian sturgeon	*Acipenser baerii*	Fingerling	Crystalline PHB	- Improved weight gain, specific growth rate, and survival - Increased diversity and richness in the gastrointestinal tract - Stimulated beneficial belonging to *Bacillus* and *Ruminococcaceae* - Decreased growth performance	Najdegerami et al. 2012[108]
	A. baerii	Larvae	PHB-enriched *Artemia*	- Increased whole body lipid content and decreased total saturated, monoenoic, n3, n6, and DHA in the larvae - Increased pepsin activity and suppressed amylase - Decreased survival when challenged with salinity and ammonia stresses	Najdegerami et al. 2015[128]
Persian sturgeon	*Acipenser persicus*	Hatchlings	PHB-enriched *Artemia*	- Decreased growth performance - Increased the total saturated fatty acids and n6, but decreased the total MUFAs, C18:3n3, n3 and n3/n6. - Decreased the total protease, amylase, and lipase	Najdegerami et al. 2015[129]

Table 2.2. Continued.

Animal name	Species name	Life stage	PHA form	Effects observed	References
	Dicentrarchus labrax	Juvenile	Crystalline PHB	- Decreased gut pH from 7.7 to 7.2 - Increased survival, weight gain, and microbial richness in the intestine	De Schryver et al. 2010[103]
European sea bass	*D. labrax*	Postlarvae	PHB-enriched *Artemia* with amorphous PHB-containing *A. eutrophus*	- No effect on the survival and growth performance - Upregulated insulin-like growth factor1 expression - Elevated the expression of dicentracin, hepcidin, mhc class lla and class llb	Franke et al. 2017[113]
	D. labrax	Yok-sac larvae	Amorphous PHB-containing *A. eutrophus*	- Affected the expression of ferritin and dicentracin expression - Improved survival against *V. anguillarum* - No effect on growth or microbial community	Franke et al. 2017[115]
Red drum channel bass	*Sciaenops ocellatus*	Juvenile	Crystalline PHB	- Reduced weight gain and feed efficiency - Enhanced phagocytic activity	Rodriguez et al. 2017[48]
Rainbow trout	*Oncorhynchus mykiss*	fry	Crystalline PHB	- Decreased weight in the first 2 weeks and then increased the final weight after 6 weeks - Improved protease, pepsin, and pancreatic enzyme secretion - Increased concentrations of Na and K in the body - Enhanced survival against *Yersinia ruckeri*	Najdegerami et al. 2015/7[109]
	O. mykiss	Fingerling	Crystalline PHB	- No effect on growth performance or digestive enzymes, except for pepsin and amylase - Reduced gut pH - Enhanced survival against *Y. ruckeri*	Najdegerami et al. 2017[130]
	O. mykiss	Fingerling	Crystalline PHB	- No effect on mean weight gain, specific growth rate, and feed conversion ratio - No effect on chemical composition, PCR-DGGE profile of the intestinal bacterial community, total bacterial count, lactic acid bacteria, psychrophilic bacteria, and liver histopathology - Reduced total coliform counts and Enterobacteriaceae counts - Increased intestinal villus length and width	Sahin et al. 2021[131]

Table 2.2. Continued.

Animal name	Species name	Life stage	PHA form	Effects observed	References
Nile tilapia	*Oreochromis niloticus*	Juvenile	Crystalline PHB	- Increased weight gain but no effect on final weight - Increased lipase and total body lipid content	Situmorang et al. 2016[105]
		Larvae	PHB-enriched *Artemia*	- Enhanced survival against *Edwardsiella ictaluri*	
Mozambique tilapia	*Oreochromis mossambicus*	Adult	PHB-hydroxyvalerate extracted from *Bacillus thuringiensis B.t.*A102	- Stimulated specific and nonspecific immune mechanisms - Increased survival against *Aeromonas hydrophila*	Suguna et al. 2014[132]
Soiny mullet	*Liza haematocheila*	Adult	Crystalline PHB	- Increased total antioxidant capacity, catalase, and superoxide dismutase - Enhanced transcriptome-based signaling	Qiao et al. 2019[133]
Gibel carp	*Carassius auratus gibelio*	Adult	PHB extracted from bioflocs	- Upregulated eight immune-related genes - Decreased cumulative mortality and early Cyprinid herpesvirus 2 replication in spleen - Changed the microbial structure but not diversity, and increased beneficial bacteria such as *Bacillus* sp.	Qiao et al. 2020[134]
Large yellow croaker	*Larimichthys crocea*	Adult	PHB extracted from *Halomonas bluephagenesis* TD01	- Enhanced specific growth rate, final body weight gain, and survival rates - Increased the serum activities of glutamic-pyruvic transaminase and glutamic-oxaloacetic transaminase	Wang et al. 2019[135]
Half-smooth tongue sole	*Cynoglossus semilaevis*	Juvenile	Crystalline PHB or Amorphous PHB-containing *Halomonas* strain	- Crystalline and amorphous PHB improved the growth and survival against *V. anguillarum* - Increased intestine fold, epithelium height and surface - PHB modified and stabilized the intestine microbial community	Gao et al. 2020[136]
Sea cucumber	*Apostichopus japonicus*	Juvenile	Gut PHB producers	- Increased abundance of microbiome retaining PHB metabolism genes in the largest individual - Provided a link between microbial PHB producers and potential growth promotion	Yamazaki et al. 2016[137]

Table 2.2. Continued.

Animal name	Species name	Life stage	PHA form	Effects observed	References
Blue mussel	*Mytilus edulis*	Larvae	Crystalline PHB and amorphous PHB-containing *A. eutrophus*	- No effect on growth or metamorphosis - Amorphous PHB enhanced the survival - No relationship between changes in the microbiota composition and the improved survival	Van Hung et al. 2015[114]
	M. edulis	Larvae	Amorphous PHB-containing *R. eutropha*	- Enhanced survival against *Vibrio splendidus* or *Vibrio coralliilyticus*	Van Hung et al. 2019[138]
	M. edulis	Larvae	Amorphous PHB-containing *R. eutropha*	- Enhanced survival against *V. coralliilyticus* - Upregulated the expression of antimicrobial peptides - Increased phenoloxidase activity	Van Hung et al. 2019[139]
	M. edulis	Larvae	Crystalline PHB microplastics	- Decreased activity levels of catalase and glutathione S-transferase in gills, superoxide dismutase in digestive glands and SeGPx in both tissues.	Magara et al. 2019[140]

2.3.2. Main PHB Mechanisms in Aquaculture

Previous studies have suggested that PHB presence and uptake may elicit different protective mechanisms in the aquatic species. In general, it has been proposed that PHB can be biodegraded into its intermediates (3-HB and butyrate) in the gastrointestinal tract of animals, and these SCFAs exert beneficial effects such as inhibiting the growth of pathogenic bacteria, reducing virulence factors, and delivering energy to aquatic animals.[1, 26] Recently, studies have also indicated that PHB can stimulate the expression of stress- and immune-related genes in the supplemented animals.[25-27, 113, 115, 132] Overall, it has been suggested that PHB can improve the survival and disease resistance in aquatic animals by three main mechanisms:[1] (i) inhibiting pathogenic growth[22, 95, 141] and/or suppressing virulence factors,[117] (ii) enhancing immune system in aquatic animals,[25-27, 113, 115, 132] and (iii) serving as an energy source for aquatic animals.[22, 111]

2.3.2.1. Antipathogenic Impacts of PHB

The antipathogenic effects of PHB could be achieved once PHB is ingested and partially converted to its intermediates/SCFAs (butyrate and 3-HB monomers and oligomers) by digestive enzymes[22] or PHB degraders[137, 142] in the gut. The release of SCFAs in the gut may hinder the pathogenic growth directly by the diffusion of those intermediates through the pathogen cell membrane, leading to the release of protons (H+) from their undissociated acid forms to effectively lower the cytoplasm pH in the pathogens. To resist the rapid pH change, the pathogens must redirect their energy to pump out the excess H+, causing exhaustion and growth inhibition, and eventually cell death (Figure 2.3).[1, 21] This has been hypothesized to be the major mechanism for pathogenic inhibition caused by SCFAs. It is important to note that studies have indeed documented the release of 3-HB in the intestinal tract of brine shrimp *Artemia* fed with varying

PHB levels.[95] Also, higher intestinal SCFAs content (e.g., acetic, propionic, and butyric) has been reported in Whiteleg shrimp *Litopenaeus vannamei* fed with PHB-supplemented diet.[28]

Another proposed antipathogenic impact of PHB is the ability of released SCFAs to reduce the phenotypic expression of virulence factors in pathogens, such as biofilm formation, luminescence, motility behavior, hemolysin production, N-acyl-homoserine lactone (AHL)-mediated quorum sensing, phospholipase, and protease activities.[95, 117] This antipathogenic mechanism could be attributed to PHB intermediates reducing motility mediated by flagella and pili adhesion factors, thus disrupting biofilm formation and inhibiting phenotypic expression of bioluminescence, hemolysin, and quorum-sensing.[117] PHB intermediate/degradation product (3-HB) has shown ability to inhibit the virulence activities of the pathogen *Vibrio campbellii*[95] and luminescent *Vibrio* sp. PUGSK8.[117] Further studies are needed to investigate the efficacy of PHB intermediates against other predominant G– and G+ aquaculture pathogens.

2.3.2.2. PHB Enhances the Immune System in Aquatic Animals

Recently, studies have indicated that PHB enhances the immune system by triggering the expression of specific (adaptive) and nonspecific (innate) responses.[25-27, 113, 115, 132] For example, higher immune effect on specific (i.e., increased antibody response) and nonspecific (i.e., increased lysozyme, total peroxidases and antiprotease activity) immunity have been reported in Mozambique tilapia *Oreochromis mossambicus* fed with PHB-hydroxyvalerate (PHB-HV) extracted from *Bacillus thuringiensis*.[132] Amorphous PHB (contained in *A. eutrophous*) has also shown ability to promote insulin-like growth factor 1, antimicrobial peptides dicentracin, hepcidin genes (innate immunity), and histocompatibility complex (mhc) class IIa and mhc class IIb (adaptive immunity) in European seabass larvae.[113] The immune-enhancing effect of PHB was

also investigated in crustaceans. Crustaceans lack adaptive immunity and depend on their innate immune system, i.e., expressing stress- and immune-related genes when faced with pathogens. For instance, higher expression of prophenoloxidase (*proPO*) and transglutaminase (*Tgase*) genes were observed with *Vibrio*-challenged giant tiger prawn *Penaeus monodon* supplemented with PHB.[25] Previous studies have also reported the positive impact of PHB to protect *Vibrio*-challenged brine shrimp *Artemia* by stimulating the expression of stress-response gene, heat-shock protein 70 (*hsp70*), which in turn regulated the expression of other immune-related genes (e.g., *proPO*, *tgase*, and ferritin (*ftn*)).[26] PHB supplementation has also increased the expression of *hsp70* as well as the activity of lysozyme, total antioxidant, inducible nitric oxide synthase, nitric oxide content, and activated mammalian target of the rapamycin signaling pathway of *L. vannamei*.[27, 28] The main mode of action for PHB to trigger the immune response is associated with cellular acidification induced by 3-HB release in the animal gut, leading to the expression of defensive genes.[1] However, the exact underlying mechanisms and the impact of PHB on immune response are complex; thus, more research is needed to analyze other defensive genes, focusing on the immune response at different challenging times and life stages.

2.3.2.3. PHB Serves as a Food/Energy Source

Fatty acids are known to be considered as typical sources of energy.[143] Previous studies have indicated that fatty acids (SCFAs and MCFAs) are important substrates for the energy metabolism and anabolic processes,[144] which could be further used as blood fuel for energy purposes or lipid synthesis by aquatic animals.[145] It is thus hypothesized that PHB, as a biopolymer of SCFAs (3-HB and butyrate), can serve as an energy source for aquatic animals. Indeed, feeding crystalline PHB has shown ability to prolong the survival of starved *Artemia*, proving the energy

source hypothesis.[22] Furthermore, the lipid-saving effect of PHB, which is an indicator for lipid deposition and growth of aquatic animals, have been previously confirmed with *Artemia*[112] and Nile tilapia,[105, 111] reporting higher lipid and whole-body contents. Furthermore, replacing 0.1–5% (w/w) of standard diet with PHB incorporated with a small fraction of MCFA (hydroxyhexanoate) (i.e., PHB-HH) improved the survival of Kuruma shrimp *Marsupenaeus japonicus* without any adverse effects on body weight, feeding rate, and FCR.[59] This MCFA incorporation into PHA signals the potential of other longer PHA monomers to provide additional energy to aquatic animals. Overall, the ability of PHB and its intermediates to serve as energy source could not only increase the growth but also improve the robustness of farmed animals against pathogens and stressful environmental conditions.

It is notworthy to mention that PHB has also shown ability to influence the microbial community in the gatsrointentsital tract of different aquatic animals (Table 2.3). For example, PHB induced bacterial richness and diversity as well as stimulated the abundance of probiotic (beneficial bacteria), such as *Bacillus* spp., *Lactobacillus*, *Lactococcus*, *Clostridium*, *Bdellovibrio*, and *Paenibacillus* in aquatic animals (Table 2.3), enhancing survival and disease resistance. PHB supplementation could have served as energy source, favoring the growth of beneficial bacterial while suppressing the growth of pathogens like Vibrionales-associated populations, the most common pathogen.[95] Nevertheless, most previous PHB studies did not consider the potential effects on G– and G+ non-pathogens in the gut microbiomes of the aquatic species. Therefore, PHB effects on G– and G+ bacteria (including aquaculture pathogens), immune response, and gut microbiome are imperative and should be examined to better understand the mechanisms and reinforce antipathogenic characteristics in aquatic animals.

Table 2.3. Overview of published studies on the impact of PHB on the microbial community in different aquaculture animals.

Aquatic Animal	Technique	Main Findings	Phylum	Class	Order	Genus	Species	References
Siberian sturgeon, European sea bass, and giant river prawn	- PCR targeting 16S rRNA - DNA Sequencing and BLAST	- Isolated six PHB degrading bacteria from a gastrointestinal environment					- Closely related to *Acidovorax* spp., *Acinetobacter* spp., and *Ochrobactrum* spp.	Liu et al. 2010[142]
European sea bass	- PCR-DGGE	- PHB induced bacterial richness and larger changes in the bacterial community						De Schryver et al. 2010[103]
Siberian sturgeon	- PCR-DGGE - CLPPS	- PHB improved the intestinal microbial species richness and diversity. - PHB increased aerobic metabolism of culturable bacteria in the GI tract				- PHB stimulated *Bacillus* and *Ruminococcaceae*		Najdegerami et al. 2012[108]

Table 2.3. Continued.

Aquatic Animal	Technique	Main Findings	Phylum	Class	Order	Genus	Species	References
Chinese mitten crab	- PCR-DGGE - DNA Sequencing and BLAST	- PHB enhanced richness, diversity, and evenness of gut microbiome - A total of 165 bands (presumed species) were observed					- Similarities with uncultured bacterium from Chinese mitten crab, black tiger shrimp, and white-leg shrimp	Sui et al. 2016[127]
European sea bass	- Illumina-NGS	- High PHB from mouth had the highest proportion of bacteria belonging to *Firmicutes*. - *Firmicutes* includes a variety of probiotic bacteria such as *Bacillus* spp. - PHB did not affect OTUs, bacterial species richness, evenness, and diversity	- *Proteobacteria* and *Bacteroidetes* were > 92.4% except for low PHB from mouth (74.5%) - *Firmicutes* were increased to 1.3-2.5% in high/low PHB from mouth					Franke et al. 2017[115]

Table 2.3. Continued.

Aquatic Animal	Technique	Main Findings	Phylum	Class	Order	Genus	Species	References
Pacific white shrimp	- Illumina-NGS	- PHB altered the composition and diversity of intestine microbiota - Increasing PHB doses decreased the diversity	- PHB increased *Proteobacteria*, *Tenericutes*, and *Bacteroidetes* - PHB decreased *Planctomycetes*, *Actinobacteria* and *Verrucomicrobia*	- PHB increased *Gammaproteobacteria* and decreased *Alphaproteobacteria*		- PHB increased beneficial bacteria *Bacillus*, *Lactobacillus*, *Lactococcus*, *Paenibacillus*, and *Bdellovibrio* - PHB (3-5%) increased *Ruminococcus*, *Brevibacterium*, and *Clostridium*		Duan et al. 2016[27]
Pacific white shrimp	- Illumina-NGS	PHB-rich *Halomonas* suppressed *Vibrio* and increased biodiversity and probiotic *Bacillus* and *Lactobacillus*	- Dominated by Proteobacteria, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*			- In unchallenged groups, *Halomonas* was mainly existed in PHB-rich/free HM groups. - In challenged, *Bacillus* and *Lactobacillus* in amorphous PHB groups >> Control.		Gao et al. 2019[116]

Table 2.3. Continued.

Aquatic Animal	Technique	Main Findings	Phylum	Class	Order	Genus	Species	References
Sea Cucumber	- High-throughput 16S rRNA sequencing - Shotgun metagenome	- Rhodobacterales retaining PHB metabolism genes might contribute to the production of larger individuals - Abundance of microbiome retaining PHB metabolism genes in the largest individuals - 67% of total prokaryotic reads annotated to *pha*ABC genes were affiliated to *Rhodobacterales*, in accordance with sequencing data, where higher abundance of *Rhodobacterales* in the larger individuals	- Insignificant difference in abundance of *Proteobacteria* and *Bacteroidetes* between larger and smaller individuals. - Minor phyla (*Actinobacteria*, *Firmicutes*, *Fusobacteria*, *Spirochaetes*) were significantly different.	- *Alphaproteobacteria* and *Deltaproteobacteria* were significantly different between larger and smaller individuals	- *Rhodobacterales*, *Desulfobacterales*, and *Oceanospirillales* were significantly abundant in larger individuals - *Marinicellales* and *Acidimicrobiales* were more abundant in smaller individuals			Yamazaki et al. 2016[137]

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3. CHAPTER III A NOVEL RECIRCULATING AQUACULTURE SYSTEM FOR SUSTAINABLE AQUACULTURE: ENABLING WASTEWATER REUSE AND CONVERSION OF WASTE-TO-IMMUNE-STIMULATING FISH FEED*

Recirculation aquaculture system (RAS) is a unique indoor fish farming method. Yet, the RAS system needs to address challenges such as effective wastewater/waste management, reduction of feed cost, and usage of antibiotics during farming to become a sustainable aquaculture system. Here, we report a novel RAS-polyhydroxybutyrate (PHB) system to overcome these challenges simultaneously. The RAS-PHB enables effective aquaculture wastewater (AW) treatment for reuse while producing and harvesting PHB-rich biomass as a protein-rich, immune-stimulating fish feed without the need for antibiotics. The feasibility of nitrogen recovery from AW for non-sterile cultivation of a salt-tolerant PHB-producing strain (*Zobellella denitrificans* ZD1) with or without supplementing agro-industrial wastes/wastewaters (e.g., glycerol, cheese whey wastewater, and aquaculture solid waste) were examined. Glycerol-grown *Z. denitrificans* ZD1 showed high contents of PHB (48%) and proteins (45.5%). High harvest efficiency of PHB-rich *Z. denitrificans* ZD1 (97%) was achieved by using small amounts of medium-molecular-weight chitosan. A simple economic analysis showed that the production costs were 0.6–0.7 $/kg of fish produced for the RAS-PHB and 1.2–1.6 $/kg for the conventional RAS, suggesting a reduction of 56% of the total cost by using a RAS-PHB. Overall, this proof-of-concept study showed that the RAS-PHB system is promising for future sustainable aquaculture practice.

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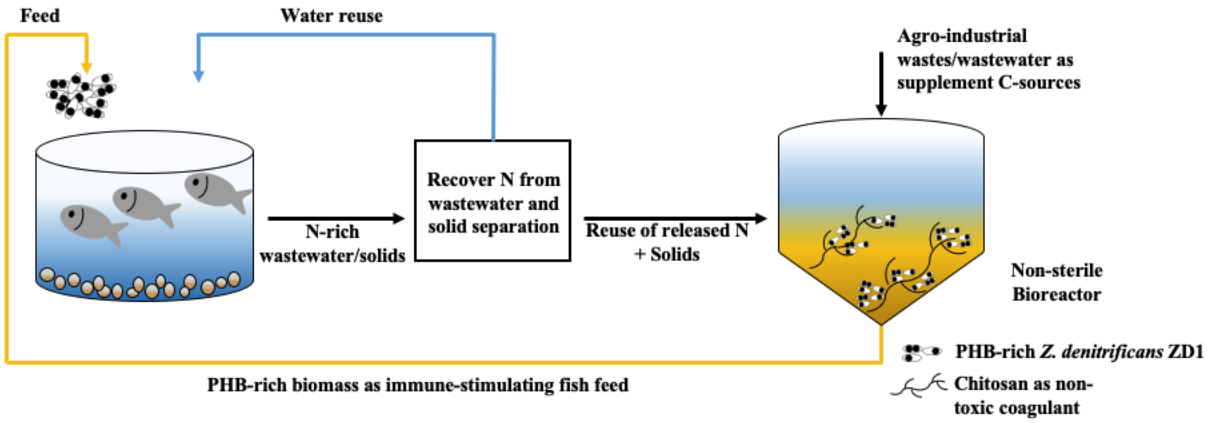

Synopsis

A novel recirculating aquaculture system for treating high-nitrogen-strength agro-industrial wastewaters while producing PHB-rich biomass as immune-stimulating fish feed.

3.1. Introduction

Aquaculture has become a rapidly growing industry with a 4% annual growth rate due to the limited resources and supplies of wild fishery and the growing seafood demand. The global aquaculture fish production was estimated to be 87 million tons in 2019, which accounts for more than half of the total fish consumed by humans.[1] However, current aquaculture practice is unsustainable due to the challenges on aquaculture wastewater (AW)/waste management, high feed cost, and unrestricted usage of antibiotics during farming. The widespread use of antibiotics also poses public health concerns, as antibiotics are bioaccumulative[2] and they might exert the emergence of antimicrobial-resistant microbes in aquaculture species and human beings.[3] A promising alternative to antibiotics is polyhydroxybutyrate (PHB), a non-toxic and biodegradable biopolymer that can be produced by microorganisms.[4, 5] Recent studies have reported that PHB can act as a biocontrol agent[6, 7] and an immunostimulant[8-10] for aquatic species and that PHB-supplemented tilapia achieved an 85% survival rate,[11] a similar survival rate when using oxytetracycline antibiotics.[12] Nevertheless, the application of PHB as a fish diet supplement is limited by its high price. The high PHB production cost is due to the use of sterilization processes, expensive cultivation medium for cultivating PHB-accumulating microorganisms,[13] and the costly PHB extraction and purification processes from the PHB-filled microorganisms.[14, 15]

Recirculating aquaculture system (RAS) is an indoor fish farming system developed to overcome shortcomings of traditional outdoor fish farming.[16] The RAS requires less water and footprint and allows year-around fish production under controlled conditions.[17] AW is low in chemical oxygen demand (COD) but high in ammonia, contributed by unconsumed fish feed (around 70–80% of the ammonia in AW)[18, 19] and fish wastes in fish tanks.[20] As high ammonia levels are toxic to most fish species, the challenges associated with RAS are to effectively remove

nitrogen (N) from the AW for recirculation and manage the sludge produced in the fish tanks. While AW and sludge have been considered wastes for removal, previous studies have reported that AW can be used for biomass production.[21, 22] Accordingly, one can use the untapped resources of N and carbon (C) in the AW and sludge for producing microorganisms as fish feed.

Such microorganisms, used as animal feed, are also called single-cell proteins (SCPs) because they are rich in proteins and contain readily digestible nutrients (e.g., lipids and minerals) for animal consumption.[23-28] Conventional SCPs are phototrophic microorganisms such as algae and purple phototrophic bacteria that are cultivated with energy-intensive illumination to low cell densities.[28, 29] On the contrary, the production of heterotrophs as SCPs is more energy-efficient; for example, it only requires ~230-MJ C/electron donor to produce 1 kg of biomass.[23, 30, 31] *Zobellella denitrificans* ZD1 is a heterotrophic hyper-PHB-accumulating (up to 84%) bacterium, capable of growing on different saline and non-saline organic wastes.[32, 33] Accordingly, a novel strategy is to concurrently produce PHB-rich *Z. denitrificans* ZD1 biomass as SCP from AW and wastes and high-quality treated wastewater for reuse. Most importantly, the PHB-rich *Z. denitrificans* ZD1 is a valuable fish feed, as it can not only reduce the feed cost but also provide a biocontrol benefit similar to antibiotics.

To this end, a novel system called RAS-PHB is proposed (as described below). This proof-of-concept study reported the production of PHB-producing *Z. denitrificans* ZD1 from AW, aquaculture solid wastes, and various cheap agro-industrial wastes/wastewater such as crude glycerol and cheese whey wastewater (CWW). These wastewaters are rich in N and high in COD,[29, 34-36] thus suitable for *Z. denitrificans* ZD1 production.

Proposed RAS-PHB. The schematics of a conventional RAS and the proposed RAS-PHB are illustrated in Figure 3.1A and B, respectively. The conventional RAS consists of fish tanks,

solid settlers, biofilters, sand filters, and UV disinfection units (Figure 3.1A). In the RAS-PHB (Figure 3.1B), the sand filter (in Figure 3.1A) is replaced with a sorbent unit with a regeneration step to recover the N in the effluent of the solid settler, and the biofilter in the RAS (Figure 3.1A) is replaced with a non-sterile bioreactor for cultivating PHB-accumulating SCP. Then, coagulants are added to the PHB-rich SCP to form coagulant-PHB-rich SCP aggregates as fish feed.

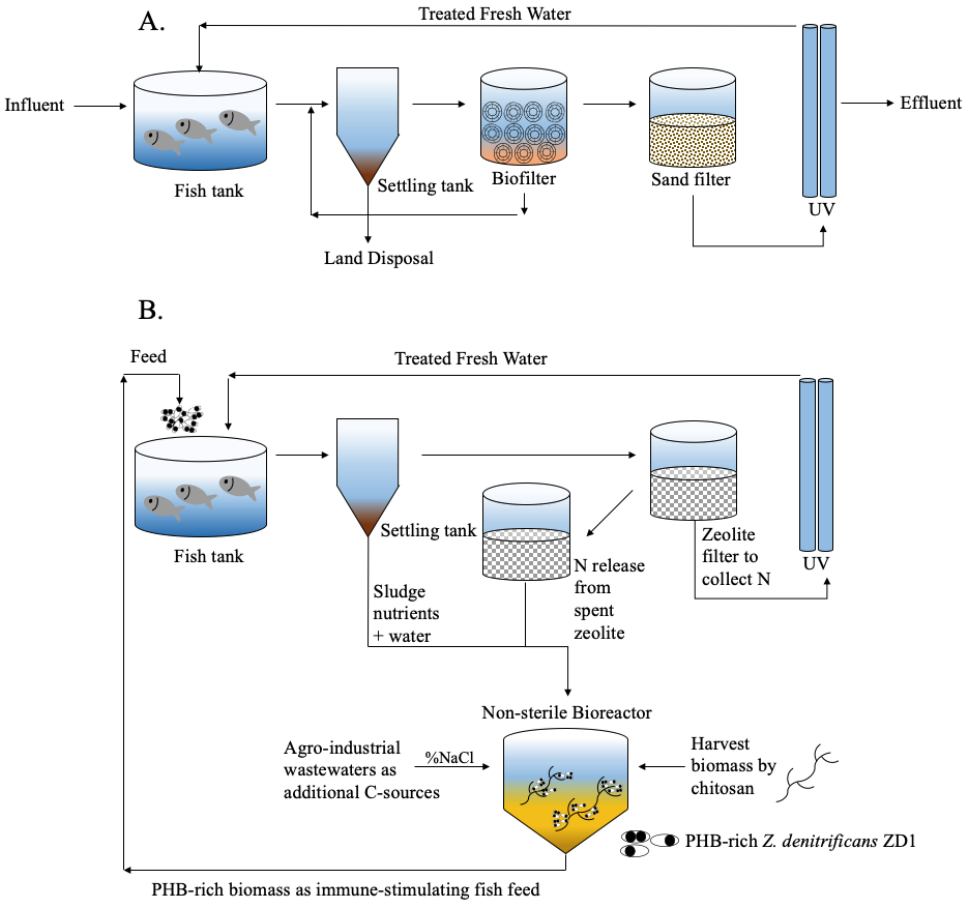

Figure 3.1. Conventional Recirculating Aquaculture System (RAS) (A), and proposed RAS-PHB system allowing treatment of aquaculture wastewater and production of PHB-rich biomass for fish feed (B). Reprinted with permission from Fahad Asiri and Kung-Hui Chu. *ACS Sustainable Chemistry & Engineering* 2020, 8 (49), 18094-18105.

To demonstrate the feasibility of the proposed RAS-PHB, natural zeolites, agro-industrial wastes/wastewaters, PHB-accumulating *Z. denitrificans* ZD1, and coagulants were used. Natural

zeolites were selected for N adsorption–desorption experiments because they are inexpensive, reusable, and can adsorb ammonia nitrogen (in particular, the protonated form, NH_4^+) from water, wastewater, and synthetic wastewater.[37, 38] Different desorption solutions (DI water and acidic, basic, and saline water) were investigated to determine the optimal condition for ammonia desorption from spent zeolites. Then, the desorbed N and/or non-sterile agro-industrial wastes/wastewaters (e.g., glycerol, CWW, and aquaculture solid waste) were used to cultivate PHB-accumulating *Z. denitrificans* ZD1. As successful demonstration of non-sterile cultivation of *Z. denitrificans* ZD1 from activated sludge, synthetic high-strength wastewater, and synthetic crude glycerol under saline conditions has been previously reported,[33] it is feasible to produce PHB-rich *Z. denitrificans* ZD1 biomass from non-sterile agro-industrial wastes/wastewaters in this study. The non-sterilized production approach is expected to reduce the cost associated with the sterilization process.

To determine a cost-effective harvesting method for the PHB-rich *Z. denitrificans* ZD1 biomass, we proposed using organic, non-toxic chitosan, as opposed to conventional inorganic coagulants such as iron (III) chloride ($FeCl_3$).[39, 40] Besides its safe properties, chitosan has been reported to be beneficial to the health of aquaculture.[41, 42] For comparison, both chitosan and $FeCl_3$ were tested over a range of different pH and dosages to determine optimal operating conditions for cell harvesting. Finally, the experimental results were used to assess the economic advantages of implementing the proposed RAS-PHB over a conventional RAS for tilapia and red drum farming.

3.2. Materials and Methods

3.2.1. Chemicals, Strain, and AW Collection.

Natural clinoptilolite-type zeolites, with a particle size of 4–7 mm, were procured from Marineland, Blacksburg, VA. Chitosan with low and medium molecular weight (M_w) (75%–85% deacetylation degree) and $FeCl_3$ as a traditional coagulant) were purchased from Sigma-Aldrich, USA. Glycerol (≥99%) and commercial PHB were obtained from Sigma-Aldrich, USA. Strain *Z. denitrificans* ZD1 (JCM 13380) was obtained from Riken BRC Microbe Division, Japan.

AW was collected from the Texas A&M Aquacultural Research and Teaching Facility in College Station, Texas. The AW contained 3-g/L NaCl and was centrifuged to separate the liquid and solids. The supernatant and solid fraction were stored at 4 °C before use.

3.2.2. Nitrogen Adsorption and Desorption Experiments

The natural zeolite was rinsed with DI water and oven-dried at 105 °C for 6 h before use. Ammonium was used as the model N form in the adsorption/desorption experiments as it is the major N content in AW (constitutes ~90%,[43] range = 0.12–345 mg NH_4^+-N/L),[22, 36] depending on the type of aquatic species and the culture system. For the adsorption experiments, a series of flasks was prepared by adding 1.25 g zeolite to 25 mL DI water containing ammonium-nitrogen concentrations ranging from 10–500 mg NH_4^+-N/L. The flasks were incubated at room temperature while shaking at 150 rpm for 24 h. Liquid samples were collected at different regular intervals and analyzed for NH_4^+-N concentration. Data collected from the 24-h samples were used to estimate the ammonium adsorption capacity of zeolite (q in mg NH_4^+-N/g) using Eq. (1):

$$q = (C_0 - C_f) \times \left(\frac{V}{m}\right) \quad (1)$$

where C_0 and C_f are concentrations of NH_4^+-N before and after adsorption, respectively. V represents the liquid sample volume (L), and m represents the zeolite mass (g).

The desorption experiments were conducted in a series of flasks as described below. Briefly, spent zeolites were produced by incubating zeolites with 500 mg NH_4^+-N/L for 4 h similar to those described in the adsorption experiments. Then, a known amount of spent zeolites (50 g/L of N-laden zeolite) was added to a flask containing 25 mL of one of the following extraction solutions: 1 M HCl, 1 M NaOH, 3–10% NaCl, or DI water. The flasks were then incubated at 25 °C for 24 h before collection for NH_4^+-N analysis. The N desorption efficiency (%) was determined by dividing the total mass of NH_4^+-N in the solution over the total mass of NH_4^+-N retained in the spent zeolites. For the set that was extracted with 3% NaCl, the desorption process was repeated two more cycles to assess the maximum amount of ammonium that can be desorbed.

Another set of adsorption–desorption experiments using the supernatant fraction of AW were conducted similarly. All the experiments were conducted in duplicate.

3.2.3. *Z. denitrificans* ZD1 Cultivation Experiments

Two sets of *Z. denitrificans* ZD1 cultivation experiments were conducted using (i) N released in the 3% NaCl extraction solutions obtained from the spent zeolites as described above in N adsorption–desorption experiments, and (ii) different non-sterile agro-industrial wastes/wastewater. All the growth experiments were conducted in 250-mL flasks, and Luria-Bertani medium (LB)-grown *Z. denitrificans* ZD1 was used as an inoculum (4% v/v), which was prepared by growing *Z. denitrificans* ZD1 in LB medium at 30 °C and shaking at 150 rpm for 24 h. After incubation, the culture was pelleted at 4,500 × g for 10 min at 4 °C, and the pellet was re-

suspended in N-free mineral salt medium (MSM)[44] to an optical density (OD_{600}) of 0.9–1.0. Experimental details are described below.

3.2.3.1. *Z. denitrificans* ZD1 Cultivation using N released from the Spent Zeolites

As described in the desorption experiments, the spent zeolites were subjected to three cycles of desorption using 3% NaCl extraction solution, resulting in three extracts containing different levels of NH_4^+-N (hereinafter designated extract-1, extract-2, and extract-3). The 3% NaCl in these solutions provided an ideal condition for the non-sterile cultivation of PHB-rich *Z. denitrificans* ZD1, and thus there will be no need for sterilization.[33]

The growth experiments were conducted in a series of 250-mL flasks containing 5 g/L glycerol, one of the three extracts, and LB-grown *Z. denitrificans* ZD1 (4% v/v) in 50 mL of N-free MSM. The N-free MSM medium was modified from the MSM by removing the ammonium component. The flasks were incubated at 30 °C and 150 rpm, and samples were intermittently collected to monitor the growth of *Z. denitrificans* ZD1. Another parallel set of growth experiment was conducted similarly, except that the 3% NaCl extraction solution derived from the supernatant of AW adsorption–desorption experiments (hereinafter extract-AW) was used. *Z. denitrificans* ZD1 cultivation in the supernatant of AW before and after adsorption were used as positive and negative controls, respectively.

3.2.3.2. *Z. denitrificans* ZD1 Cultivation using Different Non-sterile Agro-industrial Wastes/Wastewater

Non-sterile agro-industrial wastes/wastewaters (glycerol, CWW, and aquaculture solid waste) were used as additional C-sources to increase the biomass production of PHB-rich *Z.*

denitrificans ZD1. Glycerol (10 g/L) was prepared in MSM as reported previously.[33] CWW, containing the last remnant from ricotta cheese production, was prepared as described previously,[45, 46] with some modifications. Briefly, 4 L of whole milk was heated up to 82 °C before acidification with citric acid (7% v/v), followed by gentle mixing to form cheese. After the curd was firmed, CWW was flocculated by adding 750 mg/L medium M_w chitosan (dissolved in 1% v/v acetic acid). The mixture was settled at room temperature, and a clear supernatant (or so-called CWW) was collected for experimental use. Aquaculture solid waste previously prepared by centrifugation of AW was used and pretreated with three solubilization methods: (i) using one cycle of heat and pressure by sterilization at 120 °C, (ii) increasing the pH to 10 by adding 2 M NaOH,[33] or (iii) adding different amounts of peracetic acid (1–5% v/v).[47] Then, the samples were incubated for 24 h and centrifuged (10,000 × g, 4 °C), and then the supernatant was collected and neutralized with 2 M HCl to bring the pH to 7.5. The physicochemical properties (such as COD, total nitrogen (TN), salinity, and pH) of these agro-industrial wastes/wastewaters are listed in Table S3.1 in the Supporting Information.

Similarly, the growth experiments were conducted in 250-mL flasks containing LB-grown *Z. denitrificans* ZD1 (4% v/v) in 50 mL of one of the wastewater/wastes (glycerol, CWW, and aquaculture solid wastes). Liquid samples were collected at the stationary growth phase to determine cell dry weight (CDW), PHB content, TN, COD, and biomass composition. Removal efficiencies of COD and TN of the tested wastes/wastewaters were also determined. The entire experimental process did not involve sterilization.

3.2.4. *Z. denitrificans* ZD1 Biomass Harvesting Experiments

Batch experiments were performed to investigate the efficacy of two coagulants, $FeCl_3$ and chitosan, on harvesting the stationary culture of *Z. denitrificans* ZD1 (OD_{600} ~2.0) grown on glycerol in the supernatant of AW. Briefly, the cell suspension (20 mL) was supplied with 5–500 mg/L chitosan or 10–500 mg/L $FeCl_3$ under different pH (5, 7, and 9) conditions. The mixtures were then placed on a rotary shaker at 150 rpm for 2 min, followed by a slow mixing at 40 rpm for 15 min, before settling for 20 min without disturbance. The supernatant was then collected for OD_{600} measurement. The effects of high salinity (3% NaCl) on cell harvesting were also examined using the optimum coagulation condition determined above. All the harvesting experiments were conducted in triplicate. The harvesting efficiency was determined using Eq. (2):

$$\text{Harvesting Efficiency (\%)} = \left(1 - \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}}\right) \times 100 \qquad (2)$$

where OD_{sample} and $OD_{control}$ refers to the optical densities of the supernatant of samples and controls, respectively. The controls refer to those with *Z. denitrificans* ZD1 cell suspension only. The samples refer to the supernatant of treatments (i.e., *Z. denitrificans* ZD1 cell suspensions received different dosages of coagulant).

3.2.5. Analytical Methods

Physicochemical parameters such as cell growth, absorbance, CDW, COD, NH_4^+-N, and TN-N were determined according to standard methods and can be found in the Supporting Information. The PHB content in CDW (glycerol- and CWW-grown *Z. denitrificans* ZD1 biomasses) was spectrophotometrically determined via conversion of PHB to crotonic acid.[33, 48] The biomass composition (i.e., crude protein, lipid, and ash contents) was determined as described previously.[49]

3.2.6. Economic Analysis

An economic analysis was conducted on farm fish by using commercial fish feed and antibiotic in conventional RAS as a benchmark against the scenario of farming fish with PHB-rich *Z. denitrificans* ZD1 and reusing the treated AW in the proposed RAS-PHB. The key elements of the analysis include the fish species (tilapia and red drum), annual production rate (500 ton/year), RAS volume (1000 m^3), and expected stock density (50 kg fish/m^3). A detailed description of the assumptions and calculations is provided in the Supporting Information.

3.3. Results and Discussion

3.3.1. Effectiveness of Natural Zeolite for Ammonium Recovery.

As shown in Figure 3.2, zeolites were able to adsorb a wide range of NH_4^+-N concentrations (10 to 500 mg NH_4^+-N/L) in DI water within the first 4 h and reached greater than 88% of ammonium removal efficiency. By using the last data points of the tests, the adsorption capacity was estimated to be 7.4 mg NH_4^+-N/g zeolite, which falls in a range previously reported for clinoptilolite-type zeolites (6–9 mg NH_4^+-N/g).[37, 50]

After 24 h of incubation, ammonium was desorbed from the NH_4^+-laden zeolites into the tested extraction solutions with different desorbing efficiencies and quantities (shown in parenthesis) from low to high: DI (13%; 0.61 mg NH_4^+-N) < 1 M NaOH (17%; 1.64 mg NH_4^+-N) < 3% NaCl (28%; 2.54 mg NH_4^+-N) = 5% NaCl (28%) < 10% NaCl (30%; 2.75 mg NH_4^+-N) < 1 M HCl (39%, 3.56 mg NH_4^+-N) (Figure 3.2 and Figure S3.2). Additional two desorption cycles with 3% NaCl (Figure S3.3) released a total of 3.9 mg NH_4^+-N from the zeolites, which corresponded to 42% of the amount sorbed into the zeolites. This desorption efficiency was similar

to the observation reported by Tarpeh et al. that 43% of ammonium was released from the zeolites.[51]

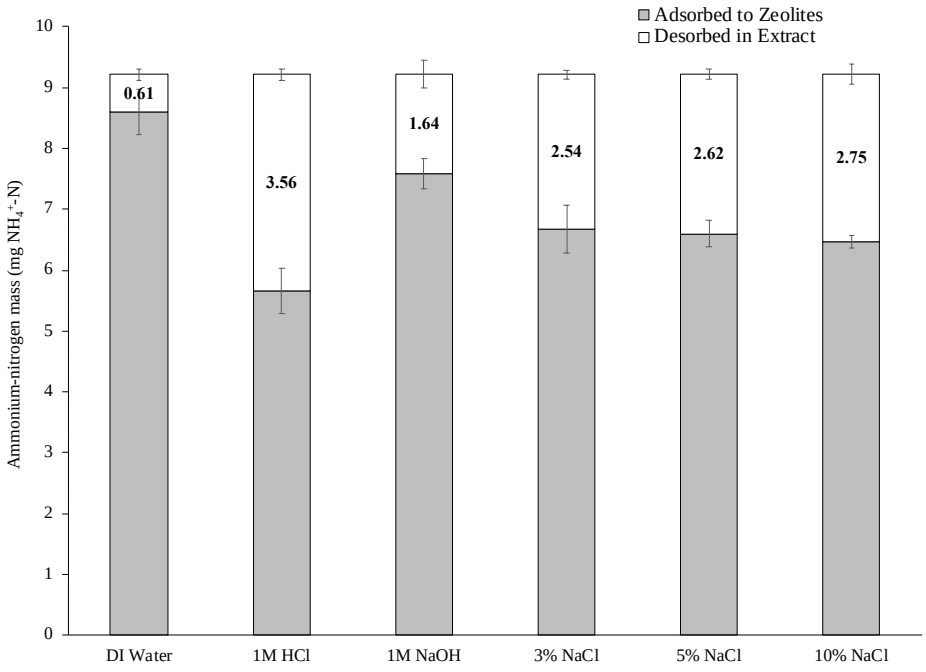

Figure 3.2. Different amounts of NH_4^+-N were desorbed from the spent zeolites, based on 1.25 of zeolites, in different extraction solutions. The total mass of NH_4^+-N adsorbed in spent zeolites was 9.25 mg. Reprinted with permission from Fahad Asiri and Kung-Hui Chu. *ACS Sustainable Chemistry & Engineering* 2020, 8 (49), 18094-18105.

The different ammonium desorption efficiencies in the extraction solutions might be explained by the ammonium adsorption mechanisms in zeolites. Previous studies have suggested that NH_4^+ removal by zeolites can be attributed to the high ion-exchange with Ca^{2+}, Na^+, K^+, and Mg^{2+} in zeolites.[50-53] Moreover, based on the pK_a of ammonium (9.25), the pH of the extraction solution will impact the predominant ammoniacal species attached to zeolites (i.e., NH_4^+ in acidic medium and NH_3 in alkaline medium),[51] resulting in the highest and lowest levels of desorption in the HCl and NaOH extraction solutions, respectively (Figure 3.2). In saline solution, the Na^+ competes with NH_4^+ for the adsorption sites in zeolites. As salinity increased, more NH_4^+ on the

zeolites were displaced and released into the extraction solution. This explanation was supported by the desorption results observed with using DI water only, as there was no Na^+ for ion exchange.[54] Furthermore, the incomplete ammonium recovery (i.e., 42% of adsorption capacity) after three cycles of 3% NaCl desorption can be attributed to the hydrated ionic radius (i.e., Na^+ radius > NH_4^+), which might have blocked Na^+ from percolating and reaching the adsorption sites.[51] Overall, our adsorption results at various initial ammonium concentrations represent most scenarios in which zeolite has been applied for treating several ammonium-strength wastewaters, such as dairy processing wastewaters,[54] landfill leachate,[55] and sewage sludge leachate.[56] Based on the desorption results, a high salinity solution (3–5% NaCl) would be the best method for regenerating NH_4^+-laden zeolites, as using the acidic extraction method would render an unsuitable medium for the subsequent *Z. denitrificans* ZD1 cultivation. The total amount of released NH_4^+-N was considered sufficient to secure microbial biomass production needs.

3.3.2. Feasibility of using N in the Extracts of Spent Zeolites for *Z. denitrificans* ZD1 Cultivation

3.3.2.1. *Z. denitrificans* ZD1 Cultivation by using the Ammonium in the Extracts of Spent Zeolites. Z. denitrificans

ZD1 was able to grow on glycerol and N in the three saline extracts (3% NaCl) of the spent zeolites in N-free MSM (Figure S3.4). The growth of *Z. denitrificans* ZD1 was the highest in extract-1 (OD_{600} = 3.5), followed by those in extract-2 (OD_{600} = 1.2) and extract-3 (OD_{600} = 0.7). The observed cell concentrations were directly proportional to the amounts of released NH_4^+-N in the extracts (Figure S3.3), demonstrating that it is feasible to cultivate *Z. denitrificans* ZD1 with the desorbed ammonium from the spent zeolites.

3.3.2.2. *Z. denitrificans* ZD1 Cultivation using N Recovered from AW

After 4 h of adsorption, zeolites were able to remove 100% TN-N from real AW with a TN concentration of 25 mg TN-N/L (Table S3.1). This zeolite's ammonium adsorption performance for AW was similar to those for 30 mg NH_4^+-N/L in DI water (Figure S3.2). These results were also similar to those reported by Zhou and Boyd[57] of which zeolite removed ~90% of TN in AW with TN <10 mg/L.

However, after 24 h of incubating the N-laden zeolites with 3% NaCl extraction solution, only 28% of the adsorbed TN (i.e., 8 mg TN-N/L in the extract-AW) was released. The desorption efficiency of the spent zeolites used for adsorbing ammonium in AW was much lower when compared with those used for adsorbing ammonium in DI water. One possible explanation might be due to the presence of organics (proteins and organic acids) in AW (COD = 205 mg/L in Table S3.1) that might have also been attached to zeolites during the adsorption process.[58] The adsorbed organics on the zeolites might compete with the adsorbed ammonium for Na^+ in the NaCl extraction solution, limiting the release of adsorbed ammonium. Previous studies have reported that the presence of organics, which originated from common ingredients in aquaculture feed,[49] influences the adsorption–desorption mechanism by reducing the surface tension of water.[58] However, future studies would be needed to determine any definitive link between organics and the degree of desorption.

The extract-AW from the desorption process was used for *Z. denitrificans* ZD1 cultivation. As shown in Figure 3.3A, *Z. denitrificans* ZD1 was able to grow with glycerol as an additional C-source and the extract-AW in N-free MSM. The growth of *Z. denitrificans* ZD1 reached an OD_{600} of 0.65, while no growth was observed in samples supplied with glycerol and zeolite-treated AW. However, using AW supernatant (without using zeolites for N removal) and glycerol, *Z.*

denitrificans ZD1 was able to grow to a high OD_{600} of 2. The low OD observed in the samples with extract-AW was probably attributed to the low N supply (8 mg TN-N/L). Similarly, Markou et al. reported a higher *Arthrospira platensis* biomass in poultry wastewater than in zeolite-treated wastewater.[37] Overall, our results demonstrated that it is feasible to treat and recover N from AW for *Z. denitrificans* ZD1 cultivation.

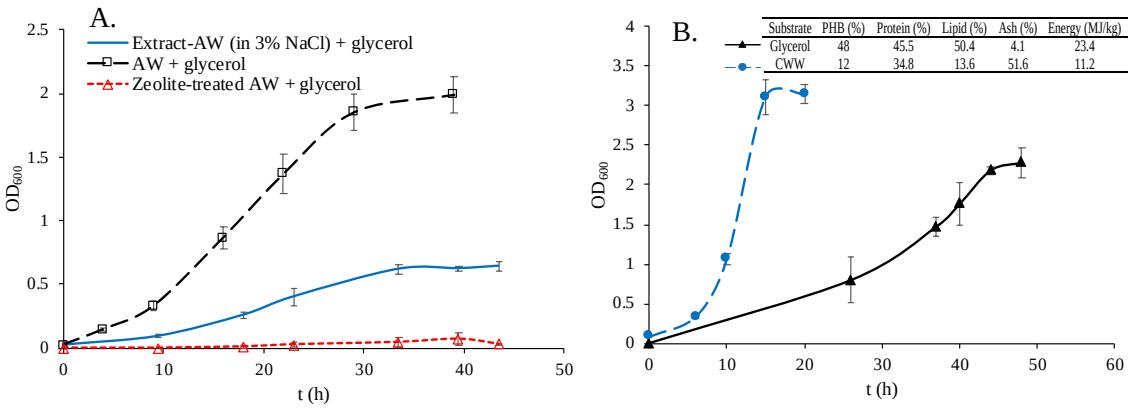

Figure 3.3. (A) Growth curves of *Z. denitrificans* ZD1 in N-free MSM with 3% NaCl-desorption solution (Extract-AW) from spent zeolite obtained from aquaculture wastewater (AW), AW before adsorption (positive control), and zeolite-treated AW (negative control). Glycerol was supplied in all cultivations as a carbon source. (B) Growth curves and biomass characterization of *Z. denitrificans* ZD1 grown in glycerol and CWW. Reprinted with permission from Fahad Asiri and Kung-Hui Chu. *ACS Sustainable Chemistry & Engineering* 2020, 8 (49), 18094-18105.

3.3.3. Non-sterile Cultivation of PHB-rich *Z. denitrificans* ZD1 using Agro-industrial Wastes/Wastewaters

3.3.3.1. Cultivation of *Z. denitrificans* ZD1 using Aquaculture Solid Waste, Glycerol, and CWW.

Z. denitrificans ZD1 was unable to use aquaculture solid waste as a C-source (data not shown). Solid waste mainly consists of fish feces and organics, and they are mostly in particulate form.[59] As grain and plant materials are common ingredients in fish feed, undigested fibers

contribute to a large fraction of non-biodegradable complex organics in the fish feces. It is possible that *Z. denitrificans* ZD1 was unable to use the complex organics for growth. Future studies are required to confirm this assumption.

Unlike aquaculture solid wastes, *Z. denitrificans* ZD1 was able to grow in glycerol and CWW as supplementary C-sources (Figure 3.3B). The CWW-supplied *Z. denitrificans* ZD1 grew faster and reached a higher OD (OD_{600} = 3.3) in 20 h compared with glycerol-supplied *Z. denitrificans* ZD1 (OD_{600} = 2.3 in 44 h). The maximum CDW of CWW-grown *Z. denitrificans* ZD1 was 3.24 g/L, which was 1.65-fold higher than that of glycerol-grown *Z. denitrificans* ZD1 (Table S3.1). Surprisingly, a high COD removal (80%) was observed in glycerol-amended samples, whereas a low COD removal (33%) was observed in CWW-supplied samples. The differences in COD removals might be attributed to the rapid decrease in pH in the growth medium (Figure S3.5), which was associated with the growth of *Z. denitrificans* ZD1. The pH profile depressed with *Z. denitrificans* ZD1 growth until the stationary phase, reaching the lowest pH value of 6.35 for CWW at the end of cultivation. This low pH compared with glycerol might have hindered *Z. denitrificans* ZD1 to further grow and utilize CWW. Additionally, the initial COD in CCW was 50 g/L compared with 12.2 g/L in glycerol (Table S3.1), which explains the final measured COD. These results suggest that *Z. denitrificans* ZD1 can effectively utilize glycerol and CWW for growth while treating these wastes.

3.3.3.2. SCP Characterization

The inset table in Figure 3.3B describes the key elements in the SCP biomass of glycerol- and CWW-grown *Z. denitrificans* ZD1. Despite the lower CDW achieved by glycerol-grown *Z. denitrificans* ZD1, their biomass contained a higher PHB content (48% in CDW) than that of

CWW-grown *Z. denitrificans* ZD1 biomass (12%). By taking the total biomass of glycerol- and CWW-grown *Z. denitrificans* ZD1 into consideration, the total mass of PHB from glycerol (47.5 mg) was significantly higher than that from CWW (20 g).

The accumulation of PHB by *Z. denitrificans* ZD1 is growth-associated, i.e., it is stored during growth and unaffected by the limitation of nutrients.[33] In our previous study, when *Z. denitrificans* ZD1 was cultivated in crude glycerol, high-strength wastewater, and activated sludge, comparable PHB production (0.38–3.44 g/L) was yielded.[33] Therefore, the PHB production in this study further confirmed the importance of using *Z. denitrificans* ZD1 as it allows the production of PHB from organic wastes in a continuous single-stage bioprocess without the need to operate under nutrient-limitation conditions. Nevertheless, the high PHB observation in glycerol compared with CWW can be attributed to the lower N supply in the media, which intensifies PHB accumulation.[4] CWW contained higher COD and initial TN than glycerol (Table S3.1); this explains the faster growth rate and higher biomass production in CWW. Lower N supply in glycerol may have had influenced *Z. denitrificans* ZD1 to prioritize PHB synthesis over biomass production.[60] Overall, the results demonstrated that both CWW and glycerol as C-sources successfully produced PHB-rich biomass. Thus, both waste options could be considered in future RAS processes upon their availability.

Compared with CWW-grown *Z. denitrificans* ZD1, glycerol-grown *Z. denitrificans* ZD1 contains more than 10% higher protein content, representing a higher protein quality by 45.5%. The lipid content of glycerol-grown *Z. denitrificans* ZD1 biomass was 50.4% (3.7 times more than in CWW). The energy content of glycerol-grown cells (23.4 MJ/kg) was greater than that of strains in CWW (11.2 MJ/kg). Finally, the CWW-grown *Z. denitrificans* ZD1 assimilated remarkably higher mineral content by 51.6%, whereas *Z. denitrificans* ZD1 on glycerol only assimilated 4.1%.

The nutritional analysis highlighted an imperative strategy that could be implemented for our proposed RAS-PHB; that is, different C-sources could be used based on the aquatic species being cultured (i.e., carnivorous and omnivorous) since each requires various fish feed quality. For example, to increase the protein content, glycerol may be considered as a C-source. Similarly, to obtain SCP with high mineral content to overcome mineral deficiency in fish feed, cultivation of *Z. denitrificans* ZD1 with CWW could be an optimal option. Other agro-industrial wastewaters (poultry, red meat, dairy, and sugar) could also be employed in our proposed RAS-PHB as these wastewater/wastes have been previously shown to yield different biomass quality.[26, 29] Most importantly, the high protein content in the glycerol-grown *Z. denitrificans* ZD1 meets the theoretical demand of protein in the fish feed for omnivorous or carnivorous fish, where their dietary protein demand is 40–55%.[25, 26] *Z. denitrificans* ZD1 biomass has the potential to be an ideal replacement of feed for omnivorous fish species, such as tilapia, channel catfish, and common carp.[11, 61-63] It can even supplement some types of carnivorous fish such as red drum *Sciaenops ocellatus*, which needs lower protein content (40%).[14, 49] Nonetheless, in vivo fish trials should be conducted to determine the fish species and optimum inclusion levels of the prescribed diet. From nutrimental viewpoints, glycerol-grown *Z. denitrificans* ZD1 is considered a more favorable SCP source than other microbial protein sources (microalgae and yeast) (Table S3.2). Notably, the energy content of glycerol-grown biomass (23.4 MJ/kg) is higher than that of commercially available fishmeal and soybean (20.1–21.3 MJ/kg) (Table S3.2) or in meat and bone meals (9.4–13.9 MJ/kg).[64]

3.3.4. Harvesting *Z. denitrificans* ZD1 using Coagulants

3.3.4.1. Effects of pH and Coagulant Dosage on Cell Harvesting

Medium M_w chitosan showed the highest harvesting efficiencies with broad ranges of pH and coagulant dosage (Figure 3.4A). Under the ideal pH range between 7 and 9, a maximum harvesting efficiency of 97% for both medium and low M_w chitosan were obtained (Figure 3.4A and B) compared with those using $FeCl_3$ (77%) (Figure 3.4C) and those of gravitational settling (i.e., controls) (39%). At pH 7 (i.e., also a typical pH value of AW),[17] the harvesting efficiency of both types of chitosan (87%) (Figure 3.4A and B) was still higher than that of $FeCl_3$ (52%) (Figure 3.4C), indicating the advantage of chitosan application without any pH adjustment. To enhance *Z. denitrificans* ZD1 biomass recovery, the performance of all coagulants was also examined in terms of their applied dosages. Figures 3.4A-C show that harvesting efficiency increased as coagulant dosage increased, except for $FeCl_3$ and low M_w chitosan at pH 5. Noted that $FeCl_3$ could not reach 80% efficiency at all dosages or pH values (Figure 3.4C). In contrast, only 10 mg/L of the medium M_w chitosan was required to achieve 70% harvesting efficiency, which was 20× lower than that of the $FeCl_3$ dosage (Figure 3.4C).

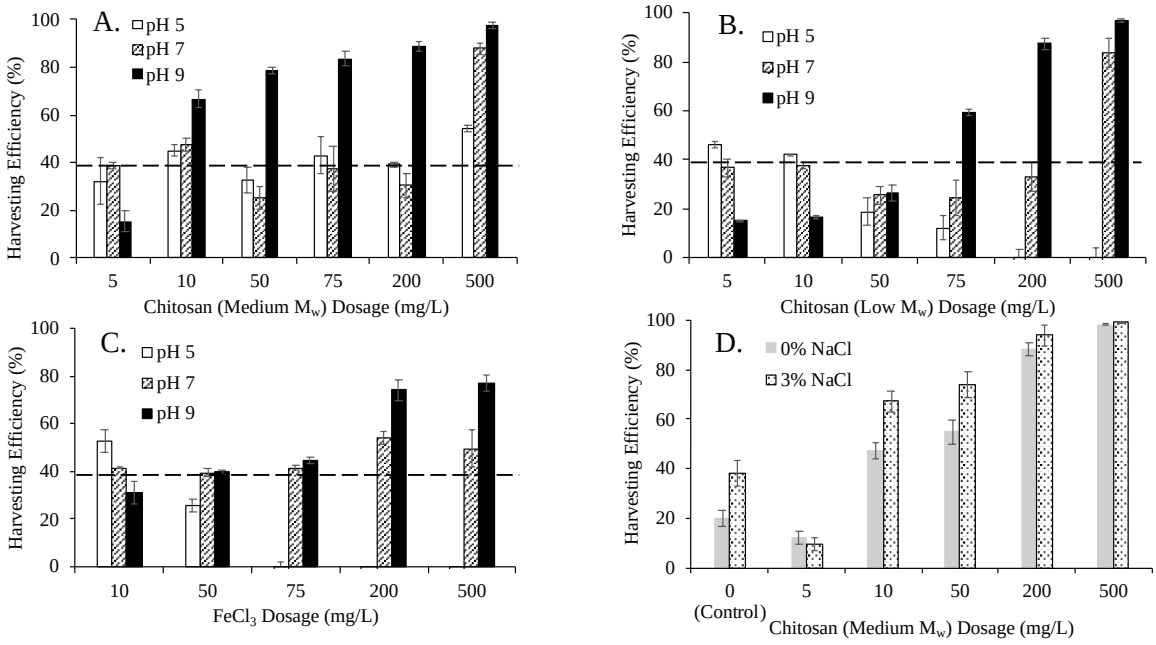

Figure 3.4. Harvesting efficiency (%) of *Z. denitrificans* ZD1 biomass under various pH values and coagulant dosages of (A) medium M_w chitosan, (B) low M_w chitosan, and (C) $FeCl_3$. The dashed lines represent the harvesting efficiency of the control (gravitational settling of cells without adding coagulants). (D) Effects of salinity on *Z. denitrificans* ZD1 biomass harvesting efficiency using various dosages of medium M_w chitosan at pH of 9. Reprinted with permission from Fahad Asiri and Kung-Hui Chu. *ACS Sustainable Chemistry & Engineering* 2020, 8 (49), 18094-18105.

The harvesting efficiency of medium M_w chitosan significantly surpassed that of $FeCl_3$ due to charge neutralization and/or bridging, whereby the suspended particles aggregated[40] over a broader pH range. Under low pH conditions, $FeCl_3$ lacks charge neutralization because of lower amounts of positively charged species such as $FeOH^{2+}$ and $Fe(OH)_2^+$, decreasing coagulation with the negatively charged cells.[40, 65] The higher the M_w of chitosan, the higher is the polymerization and cationic charge density. It was not surprising that medium M_w chitosan showed a higher efficiency than low M_w chitosan. Our findings are consistent with previous studies, which substantiated the supremacy of bridging over charge neutralization in higher M_w chitosan.[40, 60, 66, 67] To conclude, based on harvesting efficiency and food safety concerns, organic chitosan

coagulant is a better choice over the conventional inorganic $FeCl_3$ to harvest Z. denitrificans ZD1 biomass for fish feed.

3.3.4.2. Effects of Salinity

As the non-sterile production of *Z. denitrificans* ZD1 is possible under saline conditions (i.e., high salinity to prevent sterilization), the effects of salt levels on harvesting efficiency were investigated using medium M_w chitosan. As shown in Figure 3.4D, regardless of dosages, salinity improved the coagulation performance. At low chitosan dosages of 10–50 mg/L, high salinity (3%) enhanced the harvesting efficiency by a 1.4-fold increase compared with the medium without salts. However, the addition of low chitosan dosages of 5 mg/L caused more turbidity in the medium, leading to a lower harvesting efficiency (10%). This might be attributed to the cationic charge density, which was not enough to destabilize the cells and reach the coagulation/flocculation threshold; thus, *Z. denitrificans* ZD1 cells remained suspended. Furthermore, the salt effects decreased at a high chitosan dosage (200–500 mg/L), and a maximum efficiency of 98% was observed at 500 mg/L of chitosan despite of salinity. Moreover, without adding chitosan (control), 3% NaCl showed 39% harvesting efficiency (Figure 3.4D).

The efficient harvesting performance of low dosages of medium M_w chitosan under higher NaCl is attributed to the higher medium's ionic strength.[67] The Z. denitrificans ZD1 cells, as other bacteria, were stabilized in the suspension due to the electrostatic repulsion within the long double layers.[67] Therefore, as ionic strength increases, the double layer becomes compressed, bringing cells closer via van der Waals forces.[67] Therefore, the high NaCl content acted as an aid, thus increasing the harvesting efficiency and lowering the required chitosan dosage. In contrast, insignificant difference in efficiencies at high chitosan dosages is attributed to the bulky chemical structure of chitosan, which contains an acetyl group exhibiting a high degree of hydrophobicity.[67]

Overall, lower chitosan dosages can be applied under high salinity to achieve cost-effective biomass recovery. The optimal Z. denitrificans ZD1 biomass recovery (80%) can be obtained using medium M_w chitosan (50 mg/L) at pH < 9 and 3% NaCl.

3.3.5. Economic Assessment, Significance, and Limitations of the Proposed RAS-PHB

Three main processes were considered in the feasibility of using the proposed RAS-PHB for sustainable aquaculture. These processes are (i) implementation of zeolite-based adsorption and desorption mechanisms to recover and reuse ammonium from AW, (ii) supplementing agro-industrial wastes/wastewater along with ammonium recovered from AW to produce large quantities of PHB-rich SCP in saline medium without sterilization, and (iii) application of chitosan to effectively harvest PHB-rich *Z. denitrificans* ZD1 for fish feed.

3.3.5.1. Economic Assessment

A comparative economic analysis of RAS-PHB with RAS is summarized in Table 3.1, where only the monetary costs influencing the final price were considered. Farming with a conventional RAS, the total annual tilapia (omnivorous fish species) production costs were estimated to be 1.2 $/kg for using antibiotic supplement (Scenario TA) and 1.6 $/kg for using pure commercial PHB as a feed supplement (Scenario TB) (Table 3.1). While farming with the proposed RAS-PHB, total production costs were 0.7 or 0.6 $/kg for glycerol-grown PHB-rich *Z. denitrificans* ZD1 (Scenario TC) or CWW-grown *Z. denitrificans* ZD1 (Scenario TD), respectively, when PHB-rich *Z. denitrificans* ZD1 was used as an alternative to biocontrol agents and as a protein and energy source in fish feed. Both Scenarios TC and TD suggest that the employment of the proposed RAS-PHB will result in a 41–56% profit margin. The feed cost was

lowered 2.5-fold in RAS-PHB by replacing 60% of the regular feed with PHB-rich biomass (Table 3.1). As shown in the supplementary materials, the glycerol-grown *Z. denitrificans* ZD1 containing high PHB content (48%) is an ideal replacement for commercial fish feed, as the PHB-rich *Z. denitrificans* ZD1 biomass can not only eliminate the use of antibiotics but also reduce the high cost of pure commercial PHB. In fact, by using the glycerol-grown *Z. denitrificans* ZD1, the amount of PHB to be supplemented in the feed will be 0.37 kg/kg, which is about 3× higher than what is required to promote the growth and survival of Nile tilapia against *Edwardsiella ictaluri*.[11] A second economic analysis conducted on red drum, which is a carnivorous fish species and has been intensively cultured worldwide over the past decades,[14] yielded similar results (Table 3.1 and Supporting Information).

Table 3.1. Annual Costs of the Conventional and Proposed RAS-PHB Based on Normalized Fish Production Capacity. Reprinted with permission from Fahad Asiri and Kung-Hui Chu. *ACS Sustainable Chemistry & Engineering* 2020, 8 (49), 18094-18105.

Item	Normalized Production Costs ($/kg)							
	Conventional RAS				Proposed RAS-PHB			
	Tilapia		Red drum		Tilapia		Red drum	
	Scenario TA	Scenario TB	Scenario RA	Scenario RB	Scenario TC	Scenario TD	Scenario RC	Scenario RD
• Regular fish feed	0.9	0.9	0.8	0.8	0.36	0.36	0.32	0.32
• Water replacement	0.024	0.024	0.024	0.024	-	-	-	-
• Solid waste disposal	0.15	0.15	0.2	0.2	-	-	-	-
• Antibiotics	0.08	NS[a]	0.08	NS	NS	NS	NS	NS
• Pure commercial PHB	NS	0.48	NS	0.48	NS	NS	NS	NS
• PHB-rich ZD1	NS	NS	NS	NS	0.17	0.08	0.21	0.09
• Chitosan coagulant	-	-	-	-	0.19	0.14	0.24	0.21
Total ($/kg)	1.2	1.6	1.1	1.5	0.7	0.6	0.8	0.6

Note: RAS was assumedly used to culture tilapia (T) or red drum (R) with a production of 500 ton/year, a volume of 1000 m^3, and a stock density of 50 kg fish/m^3. Scenarios TA and TB and Scenarios RA and RB represent the conventional RAS with the supplementation of antibiotics or pure commercial PHB, respectively, to achieve the same overall tilapia (T) and red drum (R) production. Scenarios TC and TD and Scenarios RC and RD represent the proposed RAS-PHB with the supplementation of glycerol- or CWW-grown PHB-rich *Z. denitrificans* ZD1, respectively, as alternatives. [a]NS = Not Supplemented. See supporting information for assumptions and calculation details of each item.

3.3.5.2. Significance

In the proposed RAS-PHB system, using zeolites to adsorb–desorb ammonium from AW for sustainable SCP production is advantageous. This approach can address the first main

challenge in aquaculture industry – treatment and management of ammonia-strength wastewater and solid waste. In addition, it allows a better control of the N content in the cultivation of SCP to minimize potential growth inhibition, which may arise through the direct application of wastewaters as substrates because of the high suspended solids.[37] Mostly, zeolite exhibits a lower material cost than the conventional biological N removal technologies, such as biofilters in RAS.[22]

In the second process, the different *Z. denitrificans* ZD1 performance in the agro-industrial wastes indicates the high potential of this strain to adapt to varying nutrient dynamics to produce SCP as fish feed. This produced SCP can tackle another major aquaculture challenge represented in the reduction of expensive aquafeeds. Furthermore, the produced SCPs can address a significant sustainability metric that is the reduction in the ratio of wild fisheries inputs (i.e., forage fish: anchovies, menhaden, and sardines) to farmed fish outputs or the "fish-in to fish-out" ratio, which have been continuously endorsed by many scientists and professionals in the aquaculture industry.[25] Besides the excellent accumulation of the healthy feed additive (PHB) as a replacement for antibiotics, which is a major aquaculture challenge, the produced PHB-rich SCP has many advantages: higher biomass yield than methane or hydrogen-oxidizing bacteria[24] and lower land/water requirement and anti-nutritional factors compared with soybean.[24, 26] It is also imperative to recognize that our proposed system lifts the energy-intensive illumination required for traditional SCPs (such as purple phototropic bacteria or algae).[29] Such an advantage agrees with the "dark food chain" envisioned previously,[24] wherein chemoheterotrophy substitutes photosynthesis of SCPs as animal feed or human food.

The digestibility of aquaculture feed is commonly assessed as apparent digestibility coefficients. In general, microbial biomass has shown high apparent digestibility. Some bacterial biomass has higher digestibility than algae or yeast because it has a more digestible cell wall.

Algae and yeasts' cell walls are characterized to be rough (i.e., comprises 25–30% and 10% of the yeast and algal dry matter, respectively),[23, 68] and composed of complex heteropolysaccharides, mannoprotein, and glucan.[69] It is thus reasonable to assume that *Z. denitrificans* ZD1, as a fish feed, would be highly digestible. This issue, however, warrants in vivo fish tests to assess the digestibility of *Z. denitrificans* ZD1 and the potential outcomes of the accumulated PHB. In this regard, the application of PHB as an aquaculture feed supplement in some studies reported significant improvement in growth, survival, and immune system, whereas insignificant improvement has been reported in other studies.[24, 70] The findings suggest that PHB application might be species- or life stage-specific.[24] Therefore, other microorganisms known for producing polyunsaturated fatty acids (PUFAs) such as microalgae and fungi[71-73] could be employed in RAS-PHB and serve as a feed supplement. PUFAs, particularly eicosapentaenoic and docosahexaenoic acids, are known to be essential supplements in the aquaculture feed as they can improve fish health and the quality of seafood produced (e.g., increased omega-3 content in the seafood).[25, 74, 75] In this context, our proposed RAS-PHB system is still an optimal technology as the system can be further expanded and generalized for cultivating other microbial biomasses such as PUFAs-producing microalgae under mixotrophic conditions (i.e., autotrophic and heterotrophic).[71, 76, 77] Previous studies have reported successful cultivations of different PUFA-rich microalgal strains using fish farm effluents under non-sterile conditions.[75, 76] However, such an approach was limited by the low lipid content (25%) and low biomass yields (usually < 400–850 mg/L) in the cultivated PUFA-producing microorganisms.[71, 75]

Finally, the use of chitosan in SCP harvesting can be considered remedial and safe for fish consumption, as it is obtained from crab and shrimp wastes.[24] Studies have attributed some positive effects of chitosan as a growth-promoting compound and as an antimicrobial and

immunostimulant in aquatic animal feed.[41, 42] Additionally, previous literature has documented improved re-cultivation of microalgae as SCPs in the chitosan spent medium compared with the (toxic) alum-harvested medium due to the adaptation of the unharvested cells to the environment and substrate.[66] Thus, re-cultivation of *Z. denitrificans* ZD1 could be an effective technique in reducing associated SCP production costs.

3.3.5.3. Limitations

It is important to note that this study mainly intended to validate RAS-PHB as a proof-of-concept. The effects of environmental and operational changes due to the differences in farmed aquatic animals, carbon feedstocks, ammonia-nitrogen concentrations, and pH in the wastewater were not investigated. Nevertheless, the results of this study demonstrated that RAS-PHB is feasible and economical; the proposed system operated efficiently with real aquaculture wastewater and successfully produced and harvested biomass under typical aquaculture conditions. Thus, future research is needed to examine the effects of those environmental and operational changes on the long-term RAS-PHB performance to provide the required knowledge for future development of a full-scale RAS-PHB system.

Fish feed ingredient has been recently suggested as a potential source of off-favors in aquaculture.[78] Geosmin and 2-methylisoborneol (MIB) are the two most recognized off-flavor compounds in fish. Accordingly, to consider *Z. denitrificans* ZD1 as a viable fish feed, it is important to examine if *Z. denitrificans* ZD1 can produce and/or accumulate these off-flavor compounds. By examining the genome of *Z. denitrificans* ZD1 (https://www.ncbi.nlm.nih.gov/nuccore/NMUO00000000), no genes encoding enzymes involved in the synthesis of geosmin (i.e., *geoA, cyc2, spterp13,* and *tpc*)[78] and MIB (i.e., *mtf, mic, sco7700,*

and *sco7701*)[79] were identified, suggesting that *Z. denitrificans* ZD1 is unable to produce these compounds. Furthermore, zeolites have been shown to remove or decrease geosmin and MIB in water.[80, 81] Thus, off-flavors compounds in AW might be controlled by the natural zeolite filter unit of the PHB-RAS system.

Other salt-tolerant non-PHB-producing microorganisms might be present in 3% saline AW, resulting in co-cultivation of non-PHB-producing microorganisms and PHB-producing *Z. denitrificans* ZD1. One measure to minimize and suppress the growth of these salt-tolerant non-PHB-producing microbes is to increase salinity (higher than 3%) in the medium, as *Z. denitrificans* ZD1 was able to survive salt concentration as high as 5% (50 g/L),[33] and up to 12% (120 g/L),[82] the conditions that many microorganisms cannot tolerate. Alternatively, increasing *Z. denitrificans* ZD1 inoculation population might be able to outcompete other salt-tolerant microorganisms during non-sterile cultivation.

3.4. Conclusions

We developed and demonstrated a new RAS-PHB system that integrates the treatment of high-N-strength wastewaters with the production of PHB-rich SCPs. An economic analysis based on experimental data obtained in this study strongly indicated that our proposed RAS-PHB system is more efficient and economical than the conventional RAS. The advantages of this approach include (i) valorizing organic wastes/wastewater before treatment or discharge, (ii) producing PHB-rich SCP as a potential replacement to traditional fish feed and antibiotics, and (iii) efficiently harvesting the generated biomass by chitosan.

3.5. Supporting Information.

The Supporting Information for Chapter III can be found in Appendix A.

3.6. References

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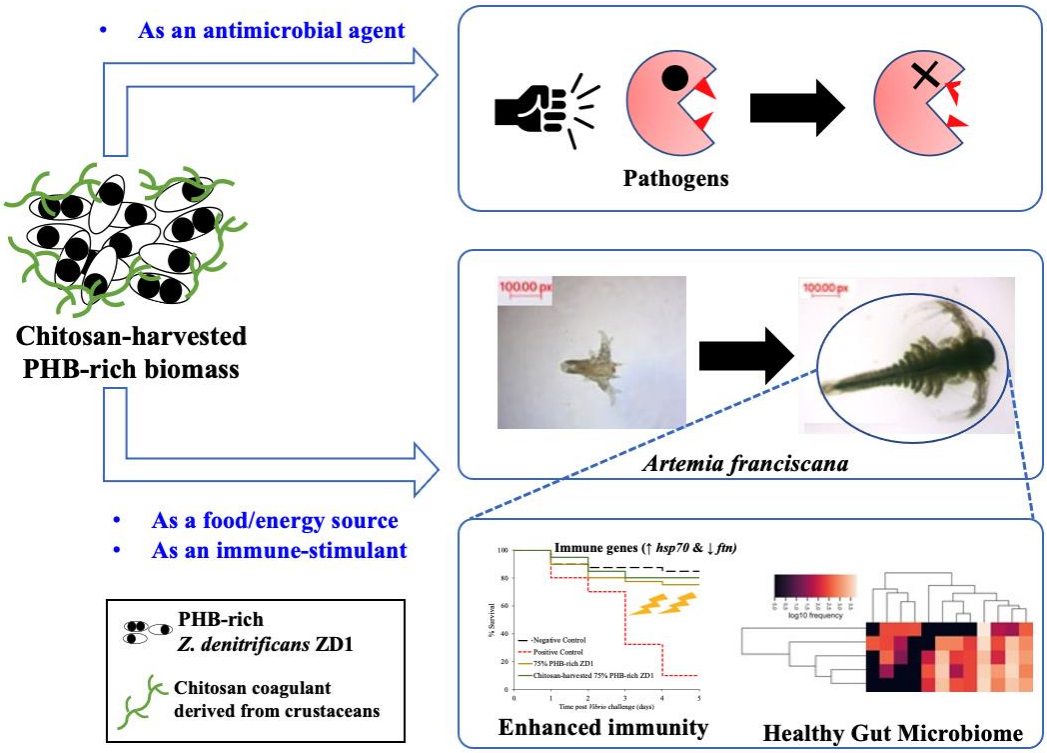
4. CHAPTER IV CHITOSAN-HARVESTED POLYHYDROXYBUTYRATE-RICH *ZOBELLELLA DENITRIFICANS* ZD1 AS A MULTIFUNCTION FEED FOR SUSTAINABLE AQUACULTURE

Aquaculture industry is essential to the security of our sustainable seafood supply but faces challenges of antibiotics overuse for pathogen control and increasing demand for aquaculture feeds, which remain heavily derived from capture fisheries. Biopolymer poly(3-hydroxybutyrate) (PHB) and microorganisms are promising replacements for antibiotics and aquaculture feed, respectively. To simultaneously address these challenges, we investigated the potential of using chitosan-harvested PHB-rich *Zobellella denitrificans* ZD1 (designated as CP-ZD1 hereafter) as a multifunction feed for brine shrimp *Artemia* as a model species. Both PHB and chitosan and their intermediates (3-hydroxybutyrate and chitosan oligosaccharides) showed antimicrobial properties toward three known aquaculture pathogens (*Vibrio campbellii*, *Aeromonas hydrophila*, and *Streptococcus agalactiae*) and non-pathogens (*Escherichia coli*, *Bacillus megaterium*, and *Rhodococcus jostii* RHA1). Low doses of 3-hydroxybutyrate and chitosan oligosaccharides mixtures effectively suppressed the growth of the pathogens. When supplied with CP-ZD1, the starved *Artemia* showed prolonged survival and a healthy gut microbiome, suggesting that CP-ZD1 can serve as a food/energy source for *Artemia*. Enhanced survival (up to 80%) and immune response were observed in CP-ZD1-fed *Artemia* when challenged with pathogenic *V. campbellii*. Overall results showed that CP-ZD1 can serve as an effective biocontrol agent, a food/energy source, and an immunostimulant for aquatic animals for sustainable aquaculture.



Synopsis

Chitosan-harvested PHB-rich biomass serves as an antibiotic-free multifunctional aquafeed by suppressing pathogens, stimulating immunity, and enhancing growth and gut microbiome.

4.1. Introduction

Aquaculture is the fastest-growing food production sector with 8% annual growth, far exceeding the growth rates of grain (1.4%), livestock (2.2%), and poultry (4.6%).[1, 2] By providing around 82 million tonnes of aquatic animals for seafood in 2018 (527% increase since 1990), valued at 250 billion US dollars,[3] aquaculture plays an essential role in securing the global food demand. Due to the rapid growth, aquaculture industry encounters several challenges such as waste management, high feed costs, and most importantly, widespread use of antibiotics to avoid economic loss due to disease outbreaks.[1, 2] For example, the global economic loss due to acute hepatopancreatic necrosis caused by *Vibrio* has been estimated at over $ 1 billion US dollars per year in shrimp farming alone.[4] However, the long-term use of antibiotics in aquaculture feed reduces effectiveness against pathogens. Mostly, antibiotics pose a risk to food safety and public health, as antibiotics might be accumulated in the aquaculture species[5] and promote antibiotic-resistant microbes in aquaculture species and human beings.[6]

Poly(3-hydroxybutyrate) (PHB) has been suggested as a promising alternative to antibiotics.[1, 4, 7, 8] Various microorganisms[9] can produce PHB as an intercellular biopolymer, which consists of repeating units of 3-hydroxybutyrate (3-HB). PHB can also be degraded into its intermediates 3-HB and then to butyrate, a short-chain fatty acid (SCFA). Several SCFAs, particularly butyrate,[10, 11] inhibit the growth of various enterobacteria and decrease pathogen invasion. It was suggested that SCFAs could diffuse into the cell membrane, which in turn acidifies the cytoplasm and leads to high cellular energy consumption to maintain homeostasis, causing cell death.[7, 10-13] It is advantageous to use PHB rather than soluble butyrate for filter-feeders, as PHB is insoluble, making the uptake of PHB more efficient, and the PHB can be later biodegraded to the desired intermediates, such as 3-HB and/or butyrate in the animals' guts.[4, 11]

Supplementing feed with PHB in crystalline form (i.e., extracted from microbial biomass) or amorphous form (i.e., the PHB inside the microbial cells) has been shown to improve the growth of aquatic animals[1, 11] and the disease resistance of many fish species[14-16] and crustaceans.[17-20] Yet, the PHB application in aquaculture is hindered by the high cost of the crystalline form of PHB. Crystalline PHB is conventionally produced by first using expensive growth substrates to cultivate PHB-accumulating microorganisms under sterile conditions to avoid microbial contamination,[21] followed by high-energy input to harvest and dry PHB-filled microorganisms before the application of not environmentally friendly solvent extraction and purification for PHB.[22, 23] To overcome the issues described above, we recently developed a sterile-free cultivation strategy and low-energy input harvesting method, called recirculating aquaculture system for PHB-rich microorganisms (designated as RAS-PHB hereafter), to enable the production of PHB-accumulating *Zobellella denitrificans* ZD1 (designated as ZD1 hereafter) from agro-industrial wastes such as aquaculture wastewater/wastes.[24] ZD1 is a salt-tolerant PHB-hyperaccumulating bacterium that can accumulate high PHB (up to 84% of its cell dry weight) and utilize various organic wastes without sterilization.[25] In the RAS-PHB process, the PHB-filled ZD1 is effectively harvested using chitosan (i.e., a nontoxic biocoagulant derived from crab and shrimp shell wastes[26]). While our previous study has successfully addressed the PHB production challenges, the effectiveness of the chitosan-harvested PHB-rich ZD1 (designated as CP-ZD1 hereafter) as an aquafeed to enable sustainable aquaculture has not been investigated.

The presence and uptake of PHB and chitosan has been suggested to elicit different protective mechanisms in aquatic species. It has been suggested that PHB can inhibit pathogenic growth[4, 8, 27] and/or suppress virulence factors,[4, 28] enhance the immune system,[19, 29-32] and serve as an energy source for supplemented aquatic animals.[8, 33] Some studies proposed that PHB was

biodegraded into its intermediates (3-HB and butyrate) in the gastrointestinal tract of animals, and these SCFAs exert beneficial effects, such as inhibiting the growth of pathogenic bacteria, reducing virulence factors, and delivering energy to aquatic animals.[11, 32] Recently, studies have also indicated that PHB stimulates the expression of stress- and immune-related genes in the supplemented animals.[19, 29-32]

Most previous PHB studies derived from the findings of *in vitro* challenge tests using selective aquatic pathogens only, without considering the potential effects on non-pathogens in the gut microbiomes of the aquatic species. Mostly, the impacts of PHB and/or chitosan on other non-pathogenic Gram-negative (G–) and Gram-positive (G+) bacteria in the animal gut have not been investigated. PHB and chitosan effects on G– and G+ bacteria (including aquaculture pathogens), immune response, and gut microbiome are imperative and should be examined to better understand the mechanisms and impacts of such a combination in reinforcing antipathogenic characteristics in aquatic animals. To date, no studies have investigated the synergetic effects of PHB and chitosan on aquaculture animals' health.

Likewise, chitosan has been shown to have antimicrobial activities[26, 34] and can improve the immune response and hematological parameters;[34, 35] inhibiting pathogens and promoting the growth of aquatic animals.[36] Chitosan's beneficial effects stem from its ingestion and degradation to water-soluble, shorter products (intermediates) like chitosan oligosaccharides (COS),[34, 37-42] and providing positive effects to aquatic animals by disrupting pathogenic cells through the electrostatic interaction between the positively charged chitosan and negatively charged bacterial surface[43-45] and acting as an immunostimulant[35, 46-48] for aquatic animals.

To this end, this study investigated the potential functions of CP-ZD1 elicited in an aquaculture animal model, brine shrimp *Artemia*. *Artemia* is a filter-feeding zooplankton species

used as an essential live food in aquaculture[49] and tested for PHB application.[11] The specific objectives of this study were to (i) assess antimicrobial efficacy of 3-HB, COS, and 3-HB + COS against several G– and G+ bacteria and predominant aquaculture pathogens (*Vibrio campbellii*, *Aeromonas hydrophila*, and *Streptococcus agalactiae*), (ii) examine whether CP-ZD1 is an effective energy source for *Artemia*, (iii) determine the survival and immune response of *Artemia* challenged with aquaculture pathogens and supplemented with CP-ZD1, and (iv) investigate the effects of CP-ZD1 exerted on the gut microbiome of *Artemia*.

4.2. Materials and Methods

4.2.1. Bacterial Strains, Chemicals, and *Artemia*

The PHB-accumulating strain, *Zobellella denitrificans* ZD1 (JCM 13380), was obtained from the Japan Collection of Microorganisms, Japan. *Vibrio campbellii* (DSM 19270), a G– aquaculture pathogen, was obtained from DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Germany. *Aeromonas hydrophila* (G– aquaculture pathogen) and *Streptococcus agalactiae* (G+ aquaculture pathogen), isolated from diseased fish during an outbreak, were kindly provided by Dr. Delbert Gatlin, Texas A&M University, USA. Non-pathogenic strains used in this study included *Bacillus megaterium* (ATCC 14581) (G+ strain) and *Escherichia coli* (ATCC 10798) (G– strain) that were obtained from the American Type Culture Collection, and *Rhodococcus jostii* RHA1 (G+ strain, designated as RHA1 hereafter) that was kindly provided by Dr. Bill Mohn, University of British Columbia, Canada.

Medium molecular weight (M_w) chitosan (190–310 kDa; 75–85% deacetylation degree), chitosan oligosaccharide lactate (COS) (M_w = 4–6 kDa), 3-hydroxybutyrate (3-HB) (≥99.0% pure), butyrate (98% pure), crystalline PHB, and glycerol (≥ 99.5%pure) were purchased from

Sigma-Aldrich, USA. All other chemicals used in this study were obtained from Sigma-Aldrich or Fisher Scientific, USA. High-quality hatching cysts of brine shrimp (*Artemia franciscana*, EG® Type) for the challenge tests were obtained from INVE Aquaculture, Great Salt Lake, Utah, USA.

4.2.2. Growth Inhibition Tests: Antimicrobial Properties of 3-HB, butyrate, COS, and 3-HB + COS

Growth inhibition tests were conducted in a series of 55-mL culture tubes containing 10-mL Luria-Bertani (LB) medium with one microbial strain type and one of these compounds (3-HB, butyrate, COS, or 3-HB + COS). The PHB intermediates (3-HB and butyrate) and chitosan intermediate (COS) are water-soluble and expected to be the degradation products present in the gastrointestinal tract of aquatic animals, allowing to assess the antimicrobial efficacy of PHB and chitosan under the best scenario. Various concentrations of PHB intermediates (5–125 mM), COS (0.2–3 mM), or Mixtures of 3-HB + COS (i.e., Mixture 1 (4 mM 3-HB + 0.1 mM COS), Mixture 2 (12 mM 3-HB + 0.3 mM COS), Mixture 3 (24 mM 3-HB + 0.6 mM COS), and Mixture 4 (60 mM 3-HB + 1.5 mM COS) were used based on the previously tested PHB[4, 8, 27, 50] and chitosan[43, 44, 51-53] *in vitro* antimicrobial concentrations. Pre-grown bacterial strains, *S. agalactiae*, RHA1, *A. hydrophila*, *V. campbellii*, *B. megaterium*, and *E. coli*, were used for inoculation. For *S. agalactiae* and RHA1, tryptic soy and Reasoner's 2A media were used, respectively. The medium pH was adjusted to 6.0 based on the typical pH value found in aquatic animals' gut[54, 55] and the optimized antimicrobial activity of PHB reported previously.[4, 8, 54] The culture tubes were inoculated with 2% v/v pre-grown strains (optical density (OD_{600}) of 1.0) and incubated at 30°C under 150 rpm. Liquid samples were collected to monitor the bacterial growth as determined by absorbance at OD_{600}. The growth of tested strains in the absence of PHB intermediates or chitosan were used as controls. The inhibition efficiency (%) was calculated using Eq. (1):

$$\text{Inhibition Efficiency (\%)} = \left(\frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}}\right) \times 100 \qquad \text{Eq. (1)}$$

where $OD_{control}$ and OD_{sample} refer to the highest optical densities of the growth curves for the controls and samples, respectively. The antimicrobial activity was calculated as the minimum or median inhibitory concentrations (MIC and IC_{50}), representing the concentrations of the tested compounds that inhibited 100% or 50% bacterial growth, respectively. MIC is determined by taking the regression through the highest OD_{600} measured at different compound concentrations. IC_{50} is estimated by taking the regression through percentage inhibition efficiencies to fit the 4-parameter logistic model.[56]

4.2.3. Production and Preparation of CP-ZD1 Biomass.

CP-ZD1 biomass was produced and prepared as previously described in the RAS-PHB system.[24] Glycerol was used as a carbon (C)-source to produce PHB-rich ZD1 biomasses (P-ZD1) under nonsterile conditions. The CP-ZD1 biomasses were prepared by harvesting the P-ZD1 at the stationary growth phase with a chitosan biocoagulant agent according to the optimized conditions and operations described in the RAS-PHB.[24] A detailed description of the ZD1 cultivation procedures and harvesting operations is provided in the Supporting Information (SI).

4.2.4. *Artemia* Starvation and Pathogen Challenge Tests

Gnotobiotic *Artemia* nauplii were hatched and prepared as previously described,[4, 57] with a minor modification. The procedures of the axenic hatching of *Artemia* are provided in the SI. Hatched *Artemia* nauplii (one day old) were used in (i) starvation and (ii) pathogen challenge tests. These tests were designed to elucidate the effects of different supplementation strategies, from

individual chemicals such as 3-HB, PHB, and chitosan to CP-ZD1, on the growth, the survival and immune response, and the gut microbiome of the *Artemia*.

The starvation challenge tests were conducted to examine whether starved *Artemia* can grow and obtain energy from the individual solid form of supplemental feeds (PHB, chitosan, PHB + chitosan, P-ZD1, and CP-ZD1) in terms of modifying the *Artemia*'s gut microbiome and affecting *Artemia*'s survival. The ability of the starved *Artemia* to survive after receiving the individual supplemental feed would indicate that the supplemented feed can be used by the starved *Artemia* as an energy source. The pathogen challenge tests were conducted to demonstrate the effects of different supplementation strategies on the survival and immune response of *Artemia* challenged with *Vibrio*.

4.2.4.1. Starvation challenge tests

The starvation challenge tests were conducted by transferring hatched *Artemia* nauplii (one day old) to new sterilized 55-mL glass tubes containing 20 mL of FASW with a stock density of 1 nauplii/mL, followed by one-time feeding of one of the supplements. Briefly, the *Artemia* in the glass tubes were fed with 1 g/L of one of the following supplements: crystalline PHB, chitosan, PHB + chitosan (1:1 w/w), and ZD1 biomass containing 60% and 75% of PHB (hereafter P60-ZD1 and P75-ZD1), and chitosan-harvested 60% and 75% PHB-rich ZD1 (hereafter CP60-ZD1 and CP75-ZD1). Starved (unfed) *Artemia* and yeast-fed *Artemia* were used as negative and positive controls, respectively. The culture tubes were placed on a rotor (4 cycles/min) at room temperature with continuous illumination. The survival of *Artemia* was monitored daily for five days, and then the swimming *Artemia* nauplii were collected for microscopic imaging and microbial community analyses.

4.2.4.2. Pathogen challenge tests

Pathogen challenge tests were conducted as described in the starvation tests, except that *Artemia* cultures (one day old) with each of the abovementioned supplements (1 g/L) were exposed to a lethal dose (10^8 cells/ml) of live *V. campbellii*. In all treatments, 250 mg/L of yeast was added initially as a feed. The lethal dose was determined by performing preliminary experiments challenging *Artemia* with different concentrations of *V. campbellii* (10^6–10^8 cells/ml) and recording the survival of *Artemia* (Figure S4.1). A dose of 10^8 cells/ml, delivering 90–95% mortality, was selected as the LD_{90} challenge dose. Unchallenged and *Vibrio*-challenged *Artemia* were used as negative and positive controls, respectively. *Artemia* survival was monitored daily for four days, and then the swimming *Artemia* nauplii were collected to analyze their immune response.

4.2.5. Analysis of Immune-Related Genes Expression and Gut Microbiome in *Artemia*

The expression of immune-related genes (heat shock protein (*hsp70*), ferritin (*ftn*), and peroxinectin (*pxn*)) and housekeeping gene (*β-actin*) in *Artemia* samples (~60 mg) from the pathogen challenge tests was assessed using quantitative real time-polymerase chain reaction (RT-qPCR) as previously described, [32, 58] with some modifications. Details regarding primer sets (Table S4.1) and methods of RT-qPCR are provided in the SI.

The *Artemia* samples collected from the starvation challenge tests were analyzed for their gut microbiome using Illumina MiSeq sequencing (Texas A&M Institute for Genome Sciences and Society, USA). Details regarding DNA extraction, sequencing, and data processing are provided in the SI. The raw sequences are available in NCBI Sequence Read Archive under BioProject number PRJNA765685. The abundance of PHB or chitosan degradation genes from

the retrieved amplicon sequence variants (ASVs) in *Artemia*'s gut microbiome were identified and predicted using Tax4Fun2 version 1.1.5.[59] Furthermore, the abundance of *Vibrio spp.* in *Artemia* in relevant to total bacteria was determined using qPCR as previously described.[60] The primer sets used for qPCR is available in Table S4.1.

4.2.6. Physicochemical and Statistical Analyses

Physicochemical analyses, such as bacterial growth, cell dry weight (CDW), PHB content in ZD1 biomass, and *Artemia* microscopic imaging, were determined and described in the Supporting Information.

Statistical analyses were conducted using independent Student's t-test between two groups and one-way analysis of variance for multiple groups followed by Tukey−Kramer post-test for identifying significant difference ($p < 0.05$) in JMP Pro 14 Statistical software. Additionally, principal component analysis (PCA) was conducted using PAST4.06b software.

4.3. Results and Discussion

4.3.1. Antimicrobial Properties of 3-HB, COS, and 3-HB + COS

4.3.1.1. Growth Inhibition Toward G– and G+ Bacteria and three Aquaculture Pathogens

Growth inhibition of non-pathogens and three known aquaculture pathogens were observed in the presence of PHB intermediates (3-HB and butyrate), chitosan intermediate (COS), and a mixture of 3-HB + COS (Figure S4.2), where the extents of the inhibition were concentration-dependent. Generally, the PHB intermediates (3-HB and butyrate) showed more inhibitory effects on aquaculture pathogens (*V. campbellii, A. hydrophila,* and *S. agalactiae*) than non-pathogens (*E. coli, B. megaterium,* and RHA1), whereas COS showed more inhibitory effects on non-pathogens

than pathogens (Figure S4.2). Mostly, low concentrations of 3-HB and COS in mixtures effectively inhibited the growth of the tested strains, suggesting synergistic antimicrobial effects of 3-HB and COS.

PHB intermediates showed a significant inhibitory effect on G– strains than on G+ strains (Figure S4.2). *V. campbellii* and *A. hydrophila* were highly susceptible to PHB intermediates where the growth was significantly inhibited at 50–125 mM of 3-HB, with an increasing lag phase as the concentrations of 3-HB increased. For example, the growth of *V. campbellii* and *A. hydrophila* was significantly reduced from an OD_{600} of 3.5–4 in the control to an OD_{600} of 2 in 50-mM 3-HB with a longer lag phase (30 h) (Figure S4.2A and E). Compared to 3-HB, the butyrate inhibitory effect was more pronounced with lower concentrations (25–50 mM). In contrast, *S. agalactiae* was slightly more resistant to PHB intermediates, requiring higher concentrations (>50 mM), particularly with 3-HB (Figure S4.2M and N). Additionally, no significant inhibition effects on *E. coli*, *B. megaterium*, and RHA1 were observed; the latter two strains used PHB intermediates as additional C-sources (Figure S4.2).

Compared to PHB intermediates, COS showed significant antimicrobial property toward G+ strains, i.e., suppressing the strains growth or extending the lag phase at low concentrations (<1.2 mM) (Figure S4.2). A concentration of 0.6 mM was sufficient to inhibit the growth of *S. agalactiae* (Figure S4.2O). The inhibitory effects on *B. megaterium* and RHA1 at even lower concentration (0.2 mM) were observed (Figure S4.2S and W). On the contrary, much higher COS concentrations (>1.2 mM) were required to inhibit the growth of G– strains, *V. campbellii* and *E. coli* (Figure S4.2C and G), and *A. hydrophila* was not inhibited even at higher COS concentrations (>3 mM COS (Figure S4.2G)). This observation might be explained by the ability of *A. hydrophila* to secrete chitinolytic enzymes that can effectively degrade chitin and chitosan for their growth.[61]

Interestingly, mixtures of 3-HB + COS showed inhibitory effects on pathogens and non-pathogens, regardless of G+ or G– strains (Figure S4.2). Compared to the less inhibitory effects observed in the individual compound of 25-mM 3-HB or 0.6-mM COS, the mixture 2 (i.e., 12-mM 3-HB + 0.3-mM COS) exhibited effective inhibition effects toward all tested strains, except for *A. hydrophila*. Mixture 1 (4-mM 3-HB + 0.1-mM COS) and Mixture 3 (24-mM 3-HB + 0.6-mM COS) effectively inhibited G– strain *V. campbellii* and G+ strain *S. agalactiae*, respectively (Figure S4.2D and P). As *A. hydrophila* grew in Mixture 4 (60-mM 3-HB + 1.5-mM COS), higher concentrations of both compounds are needed to inhibit the growth (Figure S4.2H).

Table 4.1 presents the MIC and IC_{50} of 3-HB, butyrate, COS, and 3-HB + COS determined by taking the regression through the highest OD_{600} in Figure S4.2. The percentage inhibition efficiencies shown in Table S4.2 was used to fit a 4-parameter logistic model as shown in Figure S4.3.[56] For *V. campbellii* and *A. hydrophila,* similar MIC and IC_{50} of 111.5–133 and 53–58 mM of 3-HB, respectively, were observed. Yet, much lower values of MIC and IC_{50} of butyrate (25.7–118 and 5.8–39.7 mM) were observed for these two strains (Table 4.1). In contrast, *S. agalactiae* was slightly more resistant to PHB intermediates, requiring higher MICs and IC_{50} of 3-HB and butyrate (Table 4.1). As indicated by the lower MICs and IC_{50} values, COS had stronger antimicrobial effects on G+ strains than G– strains (Table 4.1). For example, a MIC of 1.08 mM COS was required for *S. agalactiae*, but 2.36 mM COS for *V. campbellii*, and higher (> 3 mM) for *A. hydrophila* (Table 4.1). Much lower MIC and IC_{50} were observed for all strains when using mixtures of 3-HB + COS compared to those receiving individual treatment of 3-HB and COS (Table 4.1).

Table 4.1. MICs and IC_{50} (mM) of 3-HB, butyrate, COS, and 3-HB + COS against various Gram-negative and Gram-positive bacterial strains.

		Gram-negative strains			Gram-positive strains		
Compound	Parameter	*V. campbellii*	*A. hydrophila*	*E. coli*	*S. agalactiae*	*B. megaterium*	*R. jostii* RHA1
3-HB	MIC	111.5	133	510	945	N.I.	N.I.
	IC_{50}	53	57.7	223.5	493.4	N.I.	N.I.
Butyrate	MIC[a]	25.7	118	179	135	53	N.I.
	IC_{50}[b]	5.82	39.7	61.2	83.4	26.2	N.I.
COS	MIC	2.36	N.I.	1.88	1.08	<0.2[c]	<0.2
	IC_{50}	0.07	0.74	0.08	0.16	N.A.	N.A.
3-HB + COS[d]	MIC	< 4 + 0.1	N.I.	72 + 1.8	22 + 0.55	< 4 + 0.1	< 4 + 0.1
	IC_{50}	0.20 + 0.01	3056 + 76.1	9.30 + 0.23	2.69 + 0.07	N.A.	N.A.

[a]MIC was determined by taking regression through the highest optical densities measured at different compound concentrations. [b]IC_{50} is estimated by taking regression through % inhibition efficiencies calculated in Table S2 to fit the 4-parameter logistic model.[56] [c]< symbol was provided when total inhibition was reached within low tested concentrations. [d]Concentrations are determined based on tested Mixtures 1–4 of 3-HB + COS. 3-HB = 3-hydroxybutyrate; COS = chitosan oligosaccharides; N.I. = no inhibition; N.A. = not applicable (strains have already exhibited full inhibition at the lowest compound concentration).

Consistent with the aforementioned results, the biplot based on IC_{50} values (Figure 4.1) revealed that G+ bacteria were more sensitive to COS. Specifically, pathogenic *S. agalactiae* was more susceptible to COS and mixtures, whereas *A. hydrophila* was far less suspectable. Particularly, butyrate and 3-HB showed better inhibitory effects toward G– pathogens (*V. campbellii* and *A. hydrophila*) but not as effective toward G+ pathogen *S. agalactiae*.

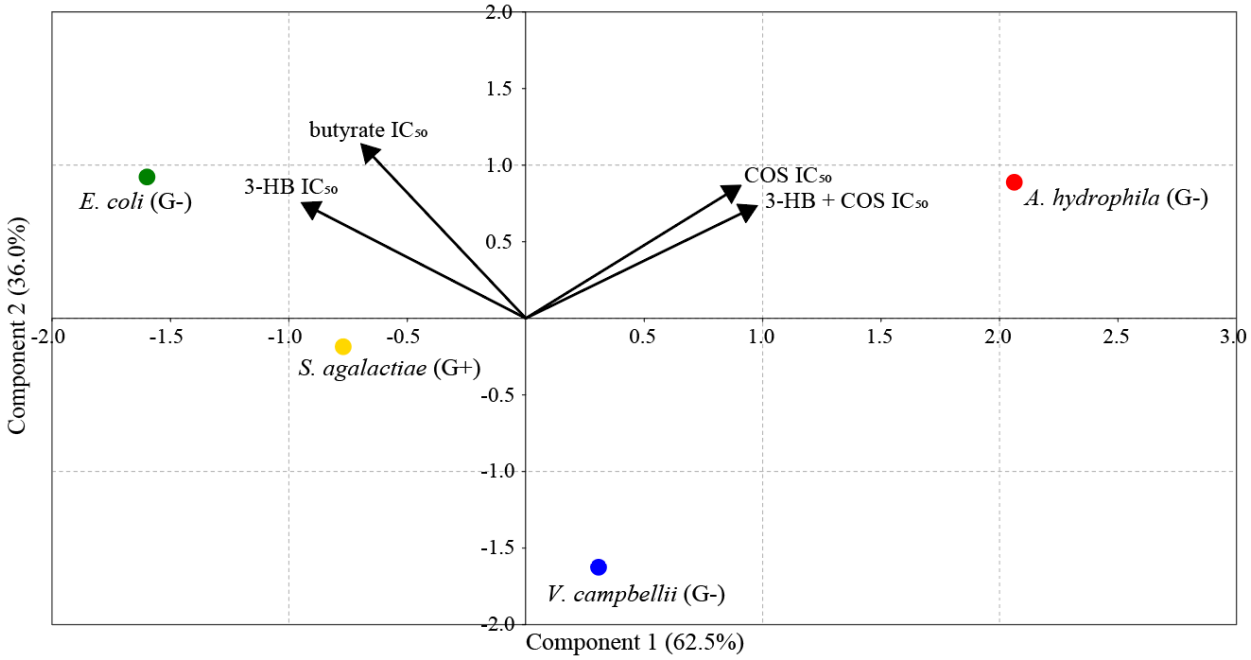


Figure 4.1. Biplot derived from principal component analysis (PCA) of median inhibitory concentrations (IC_{50}) of 3-hydroxybutyrate (3-HB), butyrate, chitosan oligosaccharides (COS), and 3-HB + COS against Gram-negative and Gram-positive bacterial strains. The plot reveals that G– aquaculture pathogens (*V. campbellii* and *A. hydrophila*) were highly sensitive, i.e., located far from butyrate and 3-HB but not as effective toward G+ pathogen *S. agalactiae*. Also, *S. agalactiae* and *V. campbellii* was more susceptible to the mixture of 3-HB + COS than *A. hydrophila*, which was located closer to COS or 3-HB + COS.

4.3.1.2. New Perspectives of Antimicrobial Properties of 3-HB, Butyrate, and COS

Consistent with previous studies,[4, 8, 50] this study observed that PHB intermediates (3-HB and butyrate) inhibited growth of aquaculture pathogens and further demonstrated the potential of PHB as a biocontrol agent in aquaculture. Most of all, for the first time, this study demonstrated that PHB intermediates inhibited not only aquaculture pathogens but also non-pathogens, such as *E. coli*, *B. megaterium*, and RHA1. A higher antimicrobial efficacy against pathogens could be attributed from the ability of PHB intermediates to inhibit phenotypic expression of pathogens' virulence factors.[4, 28, 62] For example, PHB intermediate (3-HB) reduced *Vibrio*'s motility which was mediated by flagella and pili adhesion factors, and inhibited *Vibrio*'s phenotypic expression of bioluminescence, hemolysin, and quorum-sensing compounds that led to disruption of biofilm

formation.[28] Furthermore, butyrate is an effective SCFA against enterobacteria and is commonly used as a reference due to having approximately the same pK_a (4.82) as 3-HB.[8, 10, 27, 63] Diffusion of the intermediates (monomers) through the cell membrane to acidify the cytoplasm of the pathogens has been suggested as the underlying mechanism of PHB against pathogens. Consequently, the pathogens must redirect their cellular energy to maintain homeostasis, suppressing their growth, and finally causing cell death.[11, 64] The stronger inhibition activity of PHB intermediates against G– strains is likely attributed to the higher diffusion of intermediates through the thin peptidoglycan layer of G– cell wall, while hindered by the thick peptidoglycan layer of G+ strains.[63] This explained our observation that PHB intermediates had better antipathogenic performance against G– strains (*V. campbellii* and *A. hydrophila*) than G+ *S. agalactiae* (Figure S4.2).

In this study, chitosan intermediate (COS) showed antimicrobial property toward bacteria and common aquaculture pathogens. Our observations are consistent with previous studies reporting that chitosan and COS effectively inhibited common warm-water finfish pathogens (*A. hydrophila, Edwardsiella ictaluri, Flavobacterium columnare,* and *S. agalactiae*)[43, 44, 51-53] and the cold-water fish pathogen (*Aliivibrio salmonicida*).[65] Chitosan has also been shown to be effective against common *Vibrio* species that infect crustaceans.[66, 67] It has been suggested that the positively charged amino group of chitosan and COS could adsorb onto the negatively charged bacterial cell wall due to the ionic interaction, leading to cell disruption, leakage of intercellular components, and/or blockage of DNA synthesis.[36, 43, 44] Depending on the strain type (i.e., G+ vs. G–), chitosan and COS have to first penetrate the cell membrane by binding with teichoic acid in the peptidoglycan layer in case of G+ strains, or with metal cations (chelation effect) and anions of the outer lipopolysaccharide of G– strains.[36] Notably, our study observed low difference to

slightly higher inhibition effect of COS on G+ than G– strains. This finding, however, warrants further investigations. Previous studies have reported inconsistent findings on chitosan efficacy against strain type (i.e., G+ vs. G–). Chitosan has been shown to have a broad-spectrum against various microorganisms and strain type (i.e., G+ vs. G–).[43, 68] This discrepancy stems from varying chitosan properties, such as molecular weight and degree of deacetylation and polymerization.[68] It is thus reasonable to speculate that the low difference in inhibitory observations between G– and G+ strains in this study could be attributed to chitosan's strong polycationic chemical structure, overshadowing the type of cell wall and/or surface components.

The mixture of 3-HB + COS at low doses resulted in strong inhibition against aquaculture pathogens, particularly against *V. campbellii* and *S. agalactiae*. This enhanced inhibitory effect is likely attributed to the combined inhibitory mechanisms elicited by PHB and chitosan intermediates as described above. Furthermore, the low pH (average pH 5.9–6.7 in *Artemia*) in aquatic animals' guts, particularly after feeding PHB,[54, 55] can increase the concentration of undissociated fatty acids in the cells and thus improve the antimicrobial efficacy of PHB intermediates.[7] The low pH environment can also boost higher positive amino charges in chitosan,[43] magnifying the antimicrobial properties, and cutting down the MICs. Thus, the mixture of 3-HB + COS combats bacteria and pathogens and vitalizes other beneficial microbes, leading to improvement of microbiome in aquatic animals (see microbial community analysis section). Our results suggested that the co-application of chitosan with PHB represents as an even better biocontrol agent than PHB or chitosan alone to promote survival and disease resistance of aquaculture.

4.3.2. Chitosan-Harvested PHB-Rich ZD1 (CP-ZD1) as an Effective Food/Energy Source for *Artemia*

Starvation challenge tests were performed to examine whether the supplemented feeds can be used as food/energy sources by the starved *Artemia* and affected *Artemia*'s gut microbiome. After five days, only 5% survival was observed in the negative controls (i.e., starved unfed *Artemia*) and 48% in the positive controls (i.e., yeast-fed *Artemia*) (Figure 4.2A). A higher survival of the supplement-fed *Artemia* compared to the starved unfed *Artemia* (i.e., 5%) would indicate that the supplemented feed can be used by the starved *Artemia*'s gut as a food/energy source. A survival of 16% for crystalline PHB-fed *Artemia* and a survival of 20% for chitosan-fed *Artemia* were observed. Interestingly, co-feeding crystalline PHB + chitosan (1:1 w/w) significantly enhanced the survival to 35%.

Feeding P-ZD1 yielded a survival rate of 63–75% compared to 85% survival rate for CP-ZD1, about 1.7-fold higher than that of the yeast-fed *Artemia* (Figure 4.2B). Interestingly, a positive link between the PHB content in ZD1 (i.e., 75% PHB in CDW compared to 60% PHB) and the survival rate of the P-ZD1-fed *Artemia* was observed, regardless the presence of chitosan (i.e., use of chitosan for ZD1 harvesting). Mostly, microscopic analysis revealed that *Artemia* fed with amorphous PHB, particularly CP-ZD1, had an advanced nauplii life stage developing rudimentary thoracopods, primordial bilateral compound eyes, and a longer body length (Figure 4.2C). Furthermore, in contrast to all other feeds, *Artemia* fed with CP-ZD1 adopted a darker color because of higher tissue development, indicating greater thickness and density.

The improvement in survival of CP-ZD1- and P-ZD1-fed *Artemia* compared to traditional single-cell protein (SCP) (yeast) could be attributed to ZD1's simple and more digestible cell wall. Yeasts' cell walls are characterized to be rough (i.e., comprises 25−30% of dry matter)[69, 70] and

are composed of complex heteropolysaccharides, mannoprotein, and glucan,[71] all of which complicate yeast's digestibility. Additionally, ZD1 biomass is a PHB-accumulating SCP that contains essential nutrients (e.g., proteins, lipids, and minerals)[24] that contribute as energy content for *Artemia* and lead to lipid deposition in aquatic animals' tissues,[33, 72] including *Artemia*.[73]

The proportional relationship between the PHB content in ZD1 (i.e., CP60-ZD1 and CP75-ZD1) and *Artemia*'s survival might be explained by the property of PHB itself. PHB is a biopolymer of fatty acid (3-HB and butyrate); hence, they act as additional energy source.[4, 8, 74] Taken together, feeding pure crystalline PHB drove prolonged survival of *Artemia* (Figure 4.2A). The latter finding is in agreement with previous studies.[8]

The starved *Artemia* obtained the most survival benefit when supplied with P-ZD1 and CP-ZD1, suggesting that ZD1 biomass an easily assimilated feed and an effective energy source for *Artemia*. The combination of distinctive contributions of ZD1 biomass, PHB, and chitosan might have promoted the beneficial effects of CP-ZD1 on the starved *Artemia*. Aside from the contributions of ZD1 biomass and PHB content described above, positive impacts of chitosan and COS in aquacultural diet to improve disease resistance, growth, and nutrient digestion have been reported frequently .[34, 37-42] As derived from the crab and shrimp shell wastes, chitosan may contribute to the growth and biosynthesis of crustaceans.[34, 75] This hypothesis was confirmed by a higher survival rate and size of *Artemia* fed with pure chitosan or with pure PHB + chitosan (Figure 4.2A). Furthermore, chitosan contains 5−8% N (depending on the degree of deacetylation) that is considered a major part of amino acids, which are the building blocks of proteins.[34] Chitosan degradation, mainly by lysozyme (a nonspecific protease), occurs by randomly splitting β-1,4-glycosidic bonds (depolymerization) followed by N-acetyl linkage (deacetylation). This process produces oligosaccharides and functional groups (amino, carbonyl, amido, and hydroxyl),

which can then be incorporated into glycosaminoglycans and glycoproteins.[40] Therefore, chitosan may contribute to protein content and/or serve as an energy source for *Artemia*. Finally, chitosan significantly improved the culture water quality by reducing turbidity by 94% and 98% compared to ZD1- and yeast-supplemented media, respectively (data not shown). Coagulating ZD1 with chitosan creates a protective barrier around the biomass that enhances the integrity of pellets[35] and improves water quality by lowering the turbidity, total ammonia, chemical oxygen demand, and total bacterial count.[35, 48]

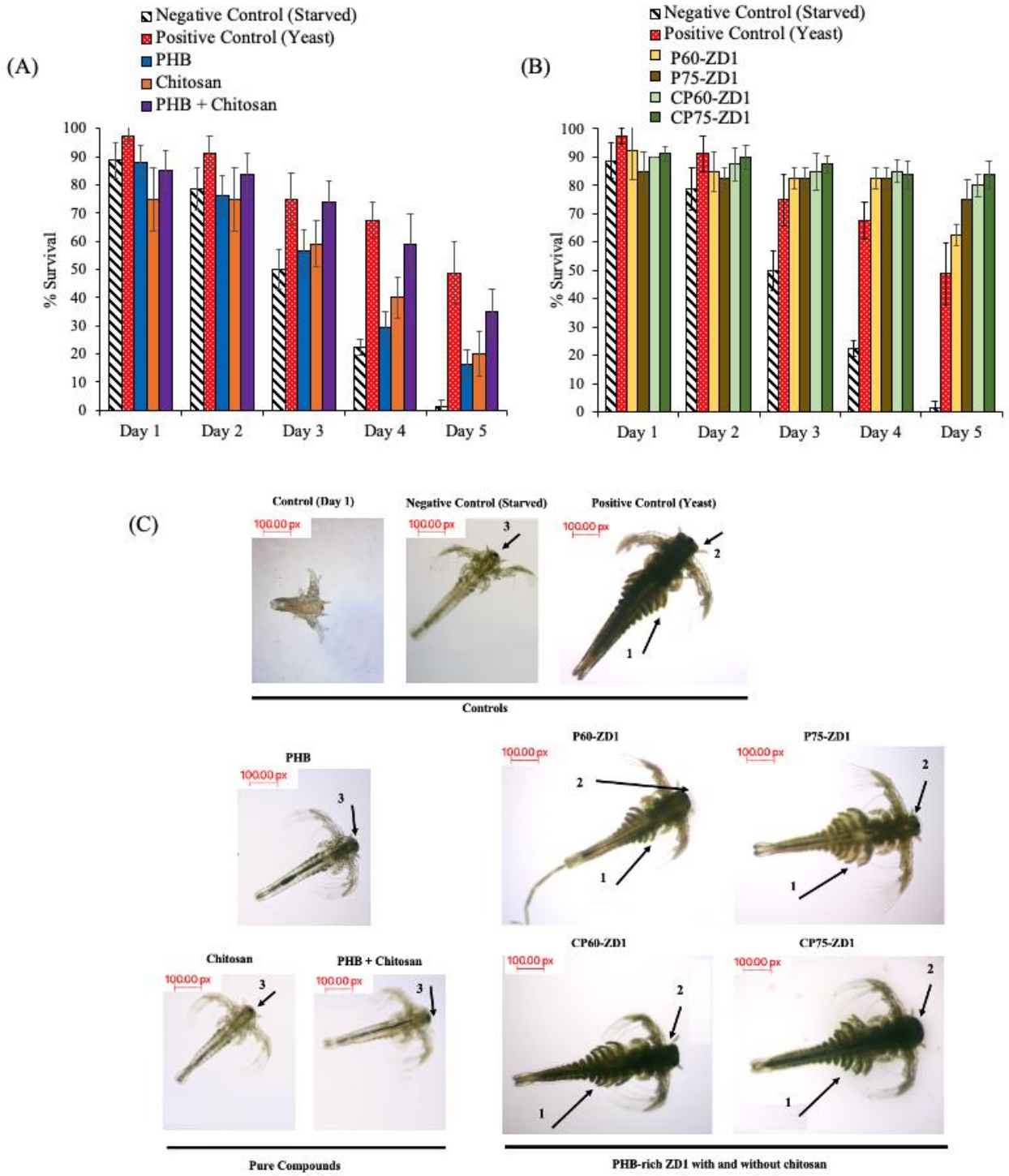


Figure 4.2. The survival of starved *Artemia* fed with: (A) Poly(3-hydroxybutyrate) (PHB), chitosan, and PHB + chitosan. (B) Chitosan- and non-chitosan-harvested PHB-rich ZD1 biomass (CP-ZD1 and P-ZD1). (C) Representative microscopic images of *Artemia* after five days of different feeds. Starved unfed *Artemia* and yeast-fed *Artemia* were used as negative and positive controls, respectively. *Artemia* after one day was used as a representation. 1, thoracopods; 2, bilateral compound eyes; 3, median/naupliar eye.

4.3.3. Chitosan-Harvested PHB-Rich ZD1 (CP-ZD1) as an Effective Immune-Stimulating Feed

4.3.3.1. Enhance Pathogen Resistance in *Artemia*

The effects of different supplements on the survival of *Vibrio*-challenged *Artemia* that were pre-grown with yeast were shown in Figure 4.3. The survival of unchallenged *Artemia* on day 5 was 85%. Pure crystalline PHB improved *Artemia*'s survival (60%) compared to 10% for the positive control (i.e., unsupplemented *Vibrio*-challenged *Artemia*) (Figure 4.3A). Supplementing with chitosan yielded a survival rate of 40%. Interestingly, when supplementing with pure crystalline PHB + chitosan (1:1 w/w), the survival was boosted considerably to 73%. Mostly, supplementing P-ZD1 yielded a 60–70% survival rate compared to 60–80% survival rate for supplementing CP-ZD1 (Figure 4.3B), indicating the superiority of CP-ZD1 biomass in protecting *Artemia* against *Vibrio* infection.

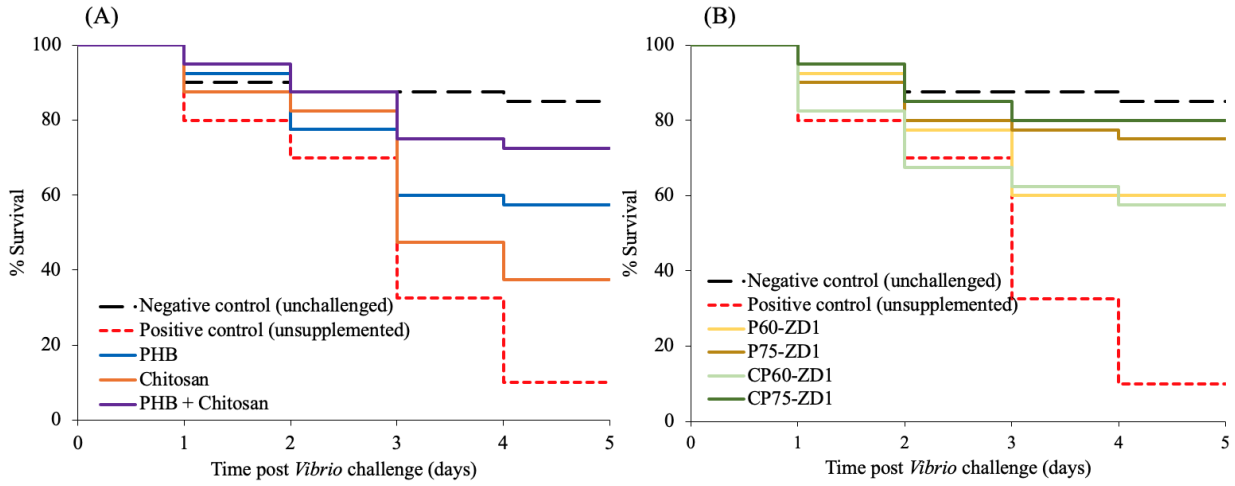


Figure 4.3. The survival rates of *Vibrio*-challenged *Artemia* supplemented with: (A) Poly(3-hydroxybutyrate) (PHB), chitosan, and PHB + chitosan. (B) Chitosan- and non-chitosan-harvested PHB-rich ZD1 biomass (CP-ZD1 and P-ZD1). Unchallenged and *Vibrio*-challenged *Artemia* (unsupplemented) were used as negative and positive controls, respectively.

The higher survival of CP-ZD1-fed *Artemia* following *Vibrio* challenge might be contributed by a combination of the antimicrobial and immune-stimulating effects triggered by the presence of PHB and chitosan in *Artemia*. This observation is supported by the low MIC and IC_{50} of 3-HB, butyrate, COS, and 3-HB + COS mixtures listed in Table 4.1. As discussed previously, *V. campbelli* was the most susceptible strain to PHB and chitosan intermediates, particularly after combining 3-HB + COS. Therefore, PHB and chitosan in CP-ZD1 could have been biodegraded in *Artemia*'s gut into their intermediates (i.e., 3-HB, butyrate, COS), leading to higher resistance to pathogen invasion. In fact, it has been reported that approximately 24 mM (2.5 g/L) of 3-HB was released in *Artemia*'s gut after feeding 1 g/L of PHB.[4] Considering CP75-ZD1 treatment in this study, the biodegradation would theoretically lead to 1.88 g/L (i.e., 18 mM) of 3-HB. Therefore, feeding CP75-ZD1 (with ~0.75 g/L PHB and ~0.05 g/L chitosan) is about 17 and 300 times more efficient if compared with 3-HB and COS concentrations that have shown to inhibit *V. campbelli* (Table 4.1). Furthermore, previous studies have confirmed that PHB promotes the survival and disease resistance of aquacultured animals,[1, 11] including *Artemia* challenged with *Vibrio*.[4, 17, 18, 28] Chitosan has also been shown to be effective against common *Vibrio* species that infect crustaceans.[66, 67] The survival rates of *Vibrio*-challenged *Artemia* in this study were consistent with previous literature.[8, 23, 28, 32] However, previous studies reported survival rates after two days of challenge; whereas, it took four to five days to reach the same survival rate in this study. Slower infection and mortality rate in this study might have resulted from a lower culturing temperature at 20°C compared to the 30°C as previously tested.[4, 17, 18, 28] This condition was nonideal for *Vibrio* to grow, leading to a slower growth rate (Figure S4.5).

4.3.3.2. Effects on the Immune Response in *Artemia*

Figure 4.4 illustrates the expression of stress- and immune-related genes (*hsp70*, *ftn*, and *pxn*) in *Vibrio*-challenged *Artemia* supplemented with different treatments. As shown in Figure 4.4A, the relative expression of *hsp70* in pure PHB-supplemented *Artemia* after 12 h was significantly higher than the negative controls (i.e., unchallenged *Artemia*). While *Artemia* supplemented with P-ZD1 and CP-ZD1 had relatively higher *hsp70* expression (1.2–1.6 fold), the *hsp70* expression after 24 h decreased to a similar level as that of the controls and showed insignificant differences among treatments. An opposite trend was observed for the expression of *ftn* and *pxn*. For *ftn*, the relative expression was downregulated in all treatments after 12 h with a significant decrease in the PHB + chitosan treatment, and then increased to the control levels after 24 h (Figure 4.4B). Interestingly, *ftn* expression in *Artemia* supplemented with CP-ZD1 remained unaltered (downregulated) even after 24 h. Similarly, the relative expression of *pxn* was declined (0.4-fold) in all treatments after 12 h. However, no significant difference between the treatments was observed, and the trend remained at the same condition even after 24 h (Figure 4.4C).

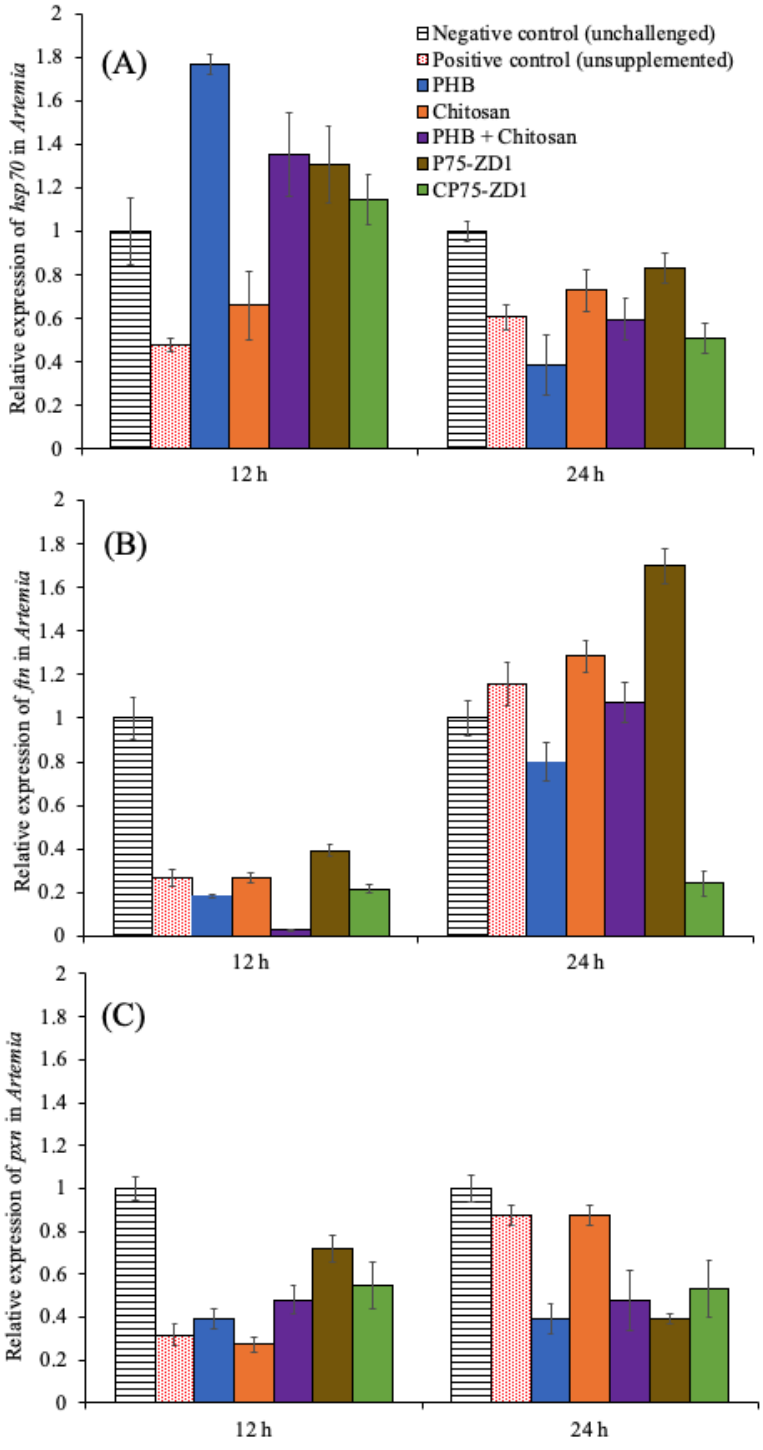


Figure 4.4 Relative expression of immune-related genes (A) *hsp70*, (B) *ftn*, and (C) *pxn* in *Vibrio*-challenged *Artemia* supplemented with different treatments. Unchallenged and *Vibrio*-challenged *Artemia* (unsupplemented) were used as negative and positive controls, respectively. The expression of target genes in the negative control was regarded as 1.0.

Invertebrates such as *Artemia* lack adaptive immunity and depend on their innate immune system, i.e., expressing stress- and immune-related genes when faced with pathogens. Furthermore, studies have suggested that chitosan can act as an immunostimulant,[35, 46-48] triggering immune responses and improving hematological parameters.[34] For instance, supplementing chitosan in fish and crustacean diets enhanced phagocytic and lysozyme activities.[75-77] It also regulated antioxidant enzyme activities and reduce lipid oxidation.[78] PHB has also been shown to stimulate an immune response in *Artemia*,[19, 29-31] particularly innate (nonspecific) genes due to cellular acidification induced by 3-HB.[32] Among various innate responses, *hsp70*, *ftn*, and *pxn* have been suggested as the most important defensive genes in many invertebrates.[30, 32, 42, 58, 79] Previous studies have demonstrated that PHB can elicit protective effects against pathogens by stimulating the expression of stress-response gene (*hsp70*), which in turn regulates the expression of immune-related genes in aquatic animals.[19, 32] Consistent with previous reports, we observed higher expressions of *hsp70* in *Vibrio*-challenged *Artemia* supplemented with P-ZD1 and CP-ZD1, and specifically in pure PHB-supplemented *Artemia*. This observation also further confirmed that the PHB might play a key role in increasing disease resistance and improving *Artemia*'s survival.

We also observed *ftn* gene downregulation, which was in agreement with previous studies.[32, 58] The *ftn* gene encoding the protein ferritin participates in the defensive mechanism by withholding iron as an essential element required for the growth of pathogens.[58] Therefore, *ftn* downregulation observed in this study indicated that a defensive strategy of the host to deprive iron from pathogenic bacteria.[32, 80] The prolonged *ftn* downregulation in CP-ZD1-supplied *Artemia* might explain for a higher survival observed in the challenged *Artemia*. The *pxn* gene encoding the protein peroxinectin is a multifunctional immune component involved in various biological processes.[58] The observation of no significant change in *pxn* expression/trend

suggested there was no association between the *pxn* gene and the supplement feed or between the *pxn* gene and the *Vibrio* challenge. Overall, the results suggest that supplementing amorphous PHB, particularly CP-ZD1, induced the expression of defensive genes in *Artemia* to resist pathogen *Vibrio.* As the exact underlying mechanisms and the impact of supplements on immune response are complicated, more research is needed to analyze other defensive genes, focusing on the immune response at different times and life stages of *Artemia*.

4.3.4. Chitosan-Harvested PHB-Rich ZD1 (CP-ZD1) Shaped a Healthier *Artemia* gut Microbiome

4.3.4.1. Microbial Diversity *Artemia*

The microbial community analysis indicated that the CP-ZD1-fed *Artemia* had a higher diversity (Simpson and Shannon = 0.94–0.95 and 4.61–4.78, respectively) than of the *Artemia* fed with pure PHB + chitosan (Simpson and Shannon = 0.79 and 3.31, respectively) (Table S4.3). High values of diversity indices were also observed in *Artemia* fed with P-ZD1. Similarly, Chao1 index was the highest (72.05–73.37) in the *Artemia* fed with CP-ZD1. Furthermore, the high phylogenetic diversity (i.e., Faith PD index) provided a separation between different treatments, i.e., feeding pure PHB + chitosan *vs.* P-ZD1 and CP-ZD1. *Artemia* fed with PHB + chitosan exhibited a high Faith PD (7.08) followed by that fed with CP-ZD1 (Faith PD of 4.66–4.97) and that fed with P-ZD1 (Faith PD of 3.08–3.96). Also, no significant difference in diversity indices was observed in those fed ZD1 biomass with different PHB contents (i.e., P60-ZD1, P70-ZD1, CP60-ZD1, and CP70-ZD1).

4.3.4.2. Overview of Microbial Community Compositions in *Artemia* Gut

The microbial community compositions revealed that G+ bacteria were dominant in *Artemia* received all feeding treatments, i.e., 188 amplicon sequence variants (ASVs) belong to G+ bacteria among 251 of total retrieved ASVs. However, *Artemia* fed with P-ZD1 had higher G+ bacteria (99.5–99.8%) than *Artemia* fed with CP-ZD1 or PHB + chitosan (86.1%) (Table S4.4 and Figure 4.5A). Higher relative abundance of G+ bacteria in CP-ZD1- and P-ZD-fed *Artemia* was observed compared to those fed with PHB + chitosan. The microbiome in *Artemia* fed with PHB + chitosan had high populations of *Psychrobacillus* and *Solicibacillus* and a low population of *Bacillus* (Figure 4.5A). Three *Bacillus*-associated ASVs (i.e., ASV4, 10, and 14) decreased from 36.1, 26.2, and 9.1% to 1.7, 1.4, and 4.7%, respectively; while the other seven *Bacillus*-associated ASVs (i.e., ASV7, 12, 16, 17, 31, 15, and 18) increased compared to PHB + chitosan treatment (Figure S4.6). Among them, ASV15 and ASV18 were identified to be closely related to *B. infantis* and *B. solimangrovi* (Figure S4.7). Interestingly, *Bacillus*-associated ASV21, closely related to *B. horikoshii*, significantly increased in the chitosan-included treatments, i.e., CP-ZD1 and PHB + chitosan (Figure S4.6).

In an opposite trend, the abundances of G– bacteria were the highest in *Artemia* fed with PHB + chitosan (7.64%), followed by that fed with CP-ZD1 (0.96–1.3%) and that fed with P-ZD1 (0.21–0.51%) (Table S4.4 and Figure 4.5B). For G– bacteria-associated ASVs, a total of nine different order-level populations were detected (Figure 4.5B). The relative abundances of G– bacteria were significantly lower than G+ bacteria. However, a substantial decrease in Aeromonadales was observed for all treatments when compared to those supplied with pure PHB + chitosan. Aeromonadales and Burkholderiales decreased significantly in the P-ZD1 treatments (i.e., from 5.8% to 0.13%), but not as rapid in the CP-ZD1 treatments (i.e., from 5.8% to 1.2%).

Vibrionales were not detected in all treatments, except in the pure PHB + chitosan treatment. Four ASVs were identified to be Gram-variable bacteria, *Paenibacillus uliginis*.[81] However, these four ASVs were only present in chitosan-included treatments such as pure PHB + chitosan (6.15%) and CP-ZD1 (0.17%).

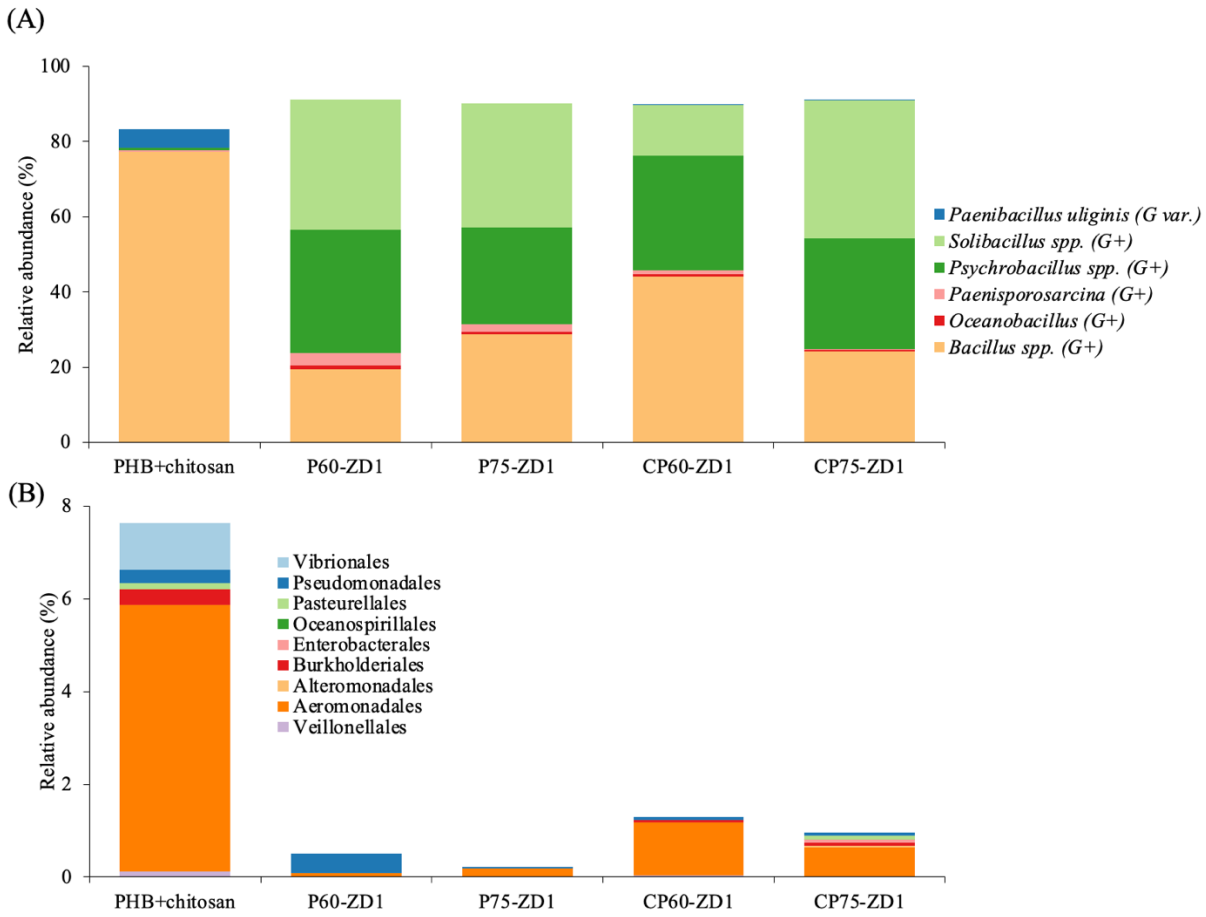


Figure 4.5 Relative abundance of (A) Genus level bacterial populations (>1% within at least one sample of the five samples) and (B) all G– associated order-level bacterial populations.

4.3.4.3. New Insights into Possible Links between Gut Microbiome, Microbial Inhibition, and Survival of *Artemia*

The higher microbial diversity observed in *Artemia* fed with CP-ZD1 or P-ZD1 is due to the increased richness of *Bacillus* spp. and the appearance of closely related *Psychrobacillus* and *Solibacillus* spp. This higher diversity could have been facilitated due to the effectiveness of

amorphous PHB being more readily biodegradable and concurrently supplied with enriched nutrients in ZD1 biomass.[24] Amorphous form of PHB is known to have smaller PHB particles, lower crystallinity, and surrounded by a layer of phospholipids and proteins, making it more susceptible to biodegradation compared to crystalline PHB.[11] In contrast, the low microbial diversity in *Artemia* fed with pure crystalline PHB + chitosan is due to the significant dominance of a few *Bacillus* spp. (ASV4, ASV10, and ASV14), which could have been propagated due to their possible presence as direct crystalline PHB degraders.[15, 82] Notably, this higher microbial diversity observed with feeding CP-ZD1 and P-ZD1 supports previous studies that have indicated crystalline and amorphous PHB induce bacterial richness and diversity as well as stimulates probiotic (i.e., beneficial bacteria), such as *Bacillus* spp., *Lactobacillus*, *Lactococcus*, and *Paenibacillus* (Table S4.5).[15, 31, 83, 84] Additionally, the presence of chitosan in CP-ZD1 could have enhanced the diversity and composition of microbial community.[85] Interestingly, *P. uliginis* and *B. horikoshii* were only observed in chitosan-included treatments, suggesting its possible participation in chitosan degradation like other *Bacillus* spp. that harbors chitosanase enzymes[86-88] and could take a role in the beneficial effects of chitosan in the diet.[26, 34] Therefore, these beneficial effects of feeding CP-ZD1 (i.e., higher diversity and beneficial bacteria) could have led to the higher growth and disease resistance of *Vibrio*-challenged *Artemia* observed in our previous results.

The lower abundance of G+ bacteria in *Artemia* fed with chitosan-included treatments (i.e., PHB + chitosan or CP-ZD1) was supported by our previous microbial growth inhibition results, which indicated that chitosan intermediate (i.e., COS) has stronger antimicrobial efficacy against G+ bacteria (Figure S4.2). This inhibitory observation was more apparent when pure chitosan was fed (i.e., PHB + chitosan treatment), resulting in a significantly lower abundance of G+ bacteria.

In contrast, the lower abundance of G– bacteria in CP-ZD1 compared to PHB + chitosan is associated with the decrease in Aeromonadales and Burkholderiales as well as the absence of Vibrionales-associated populations, such as *Vibrio*, the most common aquaculture pathogen (Figure 4.5B).[4] This observation was further supported with qPCR analysis, which have shown a lower abundance of *Vibrio* spp. in *Artemia* fed with CP-ZD1 and P-ZD1 than other treatments (Figure S4.8). Therefore, CP-ZD1 could have served as a strong biocontrol agent, inhibiting *Vibriosis* and promoting the survival and disease resistance in *Vibrio*-challenged *Artemia*.

4.3.4.4. Prediction of PHB or Chitosan Degradation in *Artemia*'s Gut Microbiome

As PHB and chitosan are biodegradable, the abundance of PHB or chitosan degradation genes in *Artemia*'s gut microbiome were identified and predicted using Tax4Fun2 (Figure 4.6). For the genes involved in the PHB degradation, PHB depolymerase (KO:K05973; EC 3.1.1.75), poly(3-hydroxyoctanoate; 3-HO) (PHO) depolymerase (KO:K00019; EC 3.1.1.76), and 3-HB dehydrogenase (KO:K00019; EC 1.1.1.30) were identified (Figure 4.6A). Polyhydroxyalkanoates (PHA) depolymerases (EC 3.1.1.75, EC 3.1.1.76) catalyze the hydrolysis of the polymer to mono- and/or oligomeric hydroxy-alkanoic acids (3-HB and 3-HO), which can be subsequently utilized as a source of carbon and energy by microorganisms (Figure S4.9A).[89] 3-HB dehydrogenase (EC 1.1.1.30) catalyzes the reversible oxidation of 3-HB to acetoacetate, which is then yield two molecules of acetyl-CoA that are metabolized via the tricarboxylic acid cycle, providing energy (Figure S4.9A).[90] Overall, the prediction showed that the PHA depolymerization enzymes (PHB and PHO depolymerase) be relatively dominant in pure PHB + chitosan treatment.[91] 3-HB dehydrogenase was relatively evenly distributed for CP-ZD1 and P-ZD1 treatments as its requirements might be rather significant with readily degraded, less crystalline PHB.

For chitosan degradation, chitin- and chitosan-degradation genes associated with chitosanase (KO:K05973; EC 3.2.1.132), glucosamine kinase (KO:K18676; EC 2.7.1.8), and glucosamine 6-phostphate deaminase (KO:K02564; EC 3.5.99.6) were identified (Figure 4.6B). Chitosanases (EC 3.2.1.132) are glycosyl hydrolases that catalyze the endohydrolytic cleavage of β-1,4-glycosidicbonds between monomers in order to release COS (Figure S4.9B).[92] Glucosamine kinase (EC 2.7.1.8) catalyzes the conversion of glucosamine to glucosamine 6-phosphate (Figure S4.9B).[93, 94] Glucosamine-6-phosphate deaminase (EC 3.5.99.6) catalyzes the reversible conversion of glucosamine-6-phosphate into fructose-6-phosphate and ammonia (Figure S4.9B).[95] Chitosanase and glucosamine kinase were dominant in pure PHB + chitosan treatment, which could be attributed to their higher activities through direct exposure to pure chitosan.[96] For CP-ZD1, chitosanase had higher Z-scores (this score signifies the abundance of PHB and chitosan degradation genes) (-0.43–0.02) than P-ZD1 (-0.66), suggesting the relative consistency of chitosanase requirements in treatments with chitosan inclusion. Furthermore, *P. uliginis* str. N3/975^{T} and *B. horikoshii* str. a20 were identified to possess chitinase (accession#: WP_208919439 and WP_208914389) and chitin disaccharide deacetylase (accession#: WP_088017227), respectively, which could explain the higher trend of genes relevant for chitin or partially acetylated chitosan degradation (i.e., KO:01183 and KO:K03478) on chitosan-included treatments.

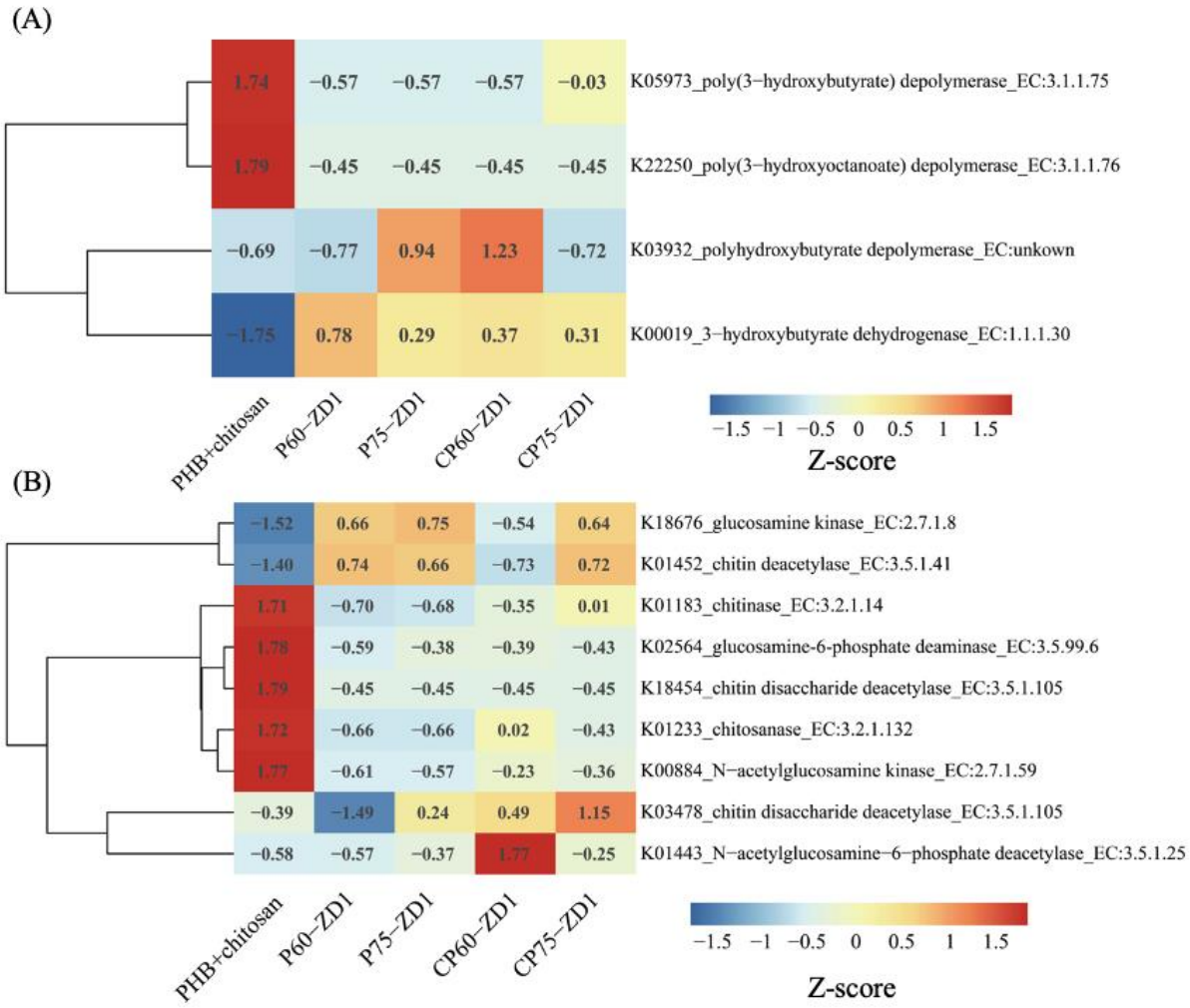


Figure 4.6 Heatmap of predicted (A) PHB and (B) chitosan degradation relevant genes by Tax4fun2. Color coding indicates row scaled Z-scores for increased resolution purposes. This Z-score signifies the abundance of PHB and chitosan degradation genes. Dendrogram generated based on hierarchical clustering from Euclidean distances.

Overall, the functional prediction analysis indicated the higher abundance of PHB and chitosan degradation genes in *Artemia*'s gut microbiome, particularly in the pure PHB + chitosan treatment. The presence of pure PHB and chitosan could have promoted microbiome with relevant PHB depolymerases and chitosanase. However, the trend of those genes was less observable in treatments with P-ZD1 and CP-ZD1 nor with ZD1 containing different PHB contents.

4.3.5. Significance and Implications for Safe and Sustainable Aquaculture

This study demonstrated the efficacy of chitosan-harvested PHB-rich ZD1 (CP-ZD1) as an antibiotic-free multifunction feed to improve health and disease resistance in aquaculture. The combination of chitosan with PHB-rich ZD1 (i.e., CP-ZD1) have shown to suppress common pathogens through the mixture of PHB and chitosan intermediates (i.e., 3-HB, butyrate, and COS), induce high survival rates and immune responses in aquatic animals, and provide them with the energy needed for growth and a healthy microbiome.

These findings showed CP-ZD1 as an efficient biocontrol agent to replace antibiotics or other inefficient and uneconomical alternatives (i.e., soluble SCFAs and expensive crystalline PHB) in aquaculture. Antibiotics are used extensively worldwide but with few countries monitoring the quantities and regulations of their use in aquaculture, leading to scarce and/or variation in the data on the amounts and types of applied antibiotics.[97] For example, a large variation in the usage of antibiotics was reported between different countries, such as 1 g of antibiotics was used per tonne of aquaculture production in Norway compared to 700 g per tonne in Vietnam.[97, 98] Unfortunately, the widespread and unrestricted usage of antibiotics has led to many environmental and public health concerns. Up to 75% of antibiotics used in aquaculture maybe lost into the surrounding environment[99] through diffusion into farm sediments[6] and discharge of antibiotic-containing aquaculture wastewater/waste.[100-102] Consequently, the released antibiotics negatively impact the beneficial bacterial flora in sediments and water,[6] and promote the emergence of antibiotic-resistance bacteria and the spread of antibiotic resistance genes,[103] which is considered a major public health problem of the 21st century.[99] There are a list of common antibiotics used in aquaculture and resistant pathogenic bacteria previously reported.[97] Some of those antibiotics (e.g., oxolinic acid, flumequine, and sulfadiazine) were detected in surface waters

from fish farm effluents,[104] which in turn could lead to the development of antibiotic-resistance pathogenic bacteria such as *Aeromonas* spp. and *Pseudomonas* spp.[97] Another health concerns are the transfer of antibiotics' resistance to human pathogens and their bioaccumulation in the aquaculture species, which could lead to allergy and toxicity after human consumption.[5, 6]

Moreover, CP-ZD1 as an aquafeed can reduce the dependency on wild-capture forage fish (e.g., anchovies, menhaden, and sardines) in fishmeal. This reduction will contribute significantly to an important sustainability metric that is the ratio of wild fisheries inputs to farmed fish outputs or the "fish-in to fish-out" ratio, which has been continuously endorsed by many scientists and professionals in the aquaculture industry.[105] The fish-in to fish-out ratio has significantly decreased to 0.63 for aquaculture sector but remains as high as 5.0 for Atlantic salmon due to the expansion in aquaculture and continuous pressure on forage fish capture.[105] Forage fish play an essential role in the marine ecosystem by primary production from plankton to larger fish, mammals, and birds.[1] For several decades, 20–30 million tonnes of fish (1/4–1/3 of the global fish catch) have been removed from the marine food web each year to produce fishmeal/oil for animal feeds and other industrial purposes.[105] In 2018, the world fishmeal production reached approximately 6 million tonnes with an expensive market price of 2000–2400 US dollars per tonne.[3] Among various fishmeal alternatives (e.g., plant-based products, rendered terrestrial animal products, processing seafood by-products, and krill),[105] single-cell proteins (SCPs) are promising substitutes that could alleviate the pressure on forage fish. The advantages of feeding SCPs such as CP-ZD1 that they contain essential nutrients (e.g., proteins, lipids, and minerals) comparable to conventional aquafeeds[24] and prevent issues associated with other fishmeal alternatives, such as the high presence of nonsoluble carbohydrates[105] and antinutritional factors in plant-based products,[106] and the potential risk of disease transmission to aquatic animals with

using animal by-/products in aquafeeds.[105] Furthermore, the use of CP-ZD1 as a SCP is advantageous since it also contains amorphous form of PHB, which is known to have smaller PHB particles, lower crystallinity, and surrounded by a layer of phospholipids and proteins, making it more susceptible to biodegradation compared to crystalline PHB.[11]

Finally, the production of CP-ZD1 as an aquafeed could be effectively integrated with the treatment of aquaculture wastewater (AW)/waste in typical aquaculture practices (e.g., RAS and ponds). AW is typically treated for reuse in the aquaculture system or discharged into the environment,[107, 108] while sludge most often applied as a fertilizer or disposed into sewage systems.[109] However, the cost of sludge transfer to fields along with odor can hamper its application as a fertilizer. There is also a concern about the discharge of AW and sludge application due to its high salinity, which might potentially cause salinization and/or sodification of soil, groundwater, and local surface water.[109] While AW and sludge have been considered wastes for removal, previous studies have reported that AW can be used to produce SCPs as aquafeeds.[108, 110] Therefore, the high organics, nutrients, and salts in aquacultural wastes[108] could become a great asset to produce SCPs such as CP-ZD1. Remarkably, we recently developed a novel RAS-PHB system that integrates the treatment of AW/wastes with the production of CP-ZD1 as a healthy aquafeed. A simple economic assessment was also provided, which demonstrated the economic advantage of using CP-ZD1 in the RAS-PHB.[24]

The study has shown CP-ZD1 to have high digestibility in an aquaculture model species, brine shrimp *Artemia*. This digestibility, however, warrants future fish trials to further validate the findings reported in this study. Furthermore, future studies should consider the application of chitosan with other PHB-accumulating microorganisms or even expanded to other SCPs such as microalgae and fungi known for accumulating important supplements, such as polyunsaturated

fatty acids that increase omega-3 content in seafood.[111] Other medium-chain-length PHA-accumulating microorganisms could also be investigated, which could have a stronger biocontrol potential due to the presence of longer-chain PHA. Overall results indicated that CP-ZD1 serves as an effective biocontrol agent, a food/energy source, and an immunostimulant for aquatic animals to support sustainable aquaculture. The aquaculture industry would benefit from developing economical, sustainable, and safe biocontrol agents.

4.4. Supporting Information.

The Supporting Information for Chapter IV can be found in Appendix B.

4.5. References

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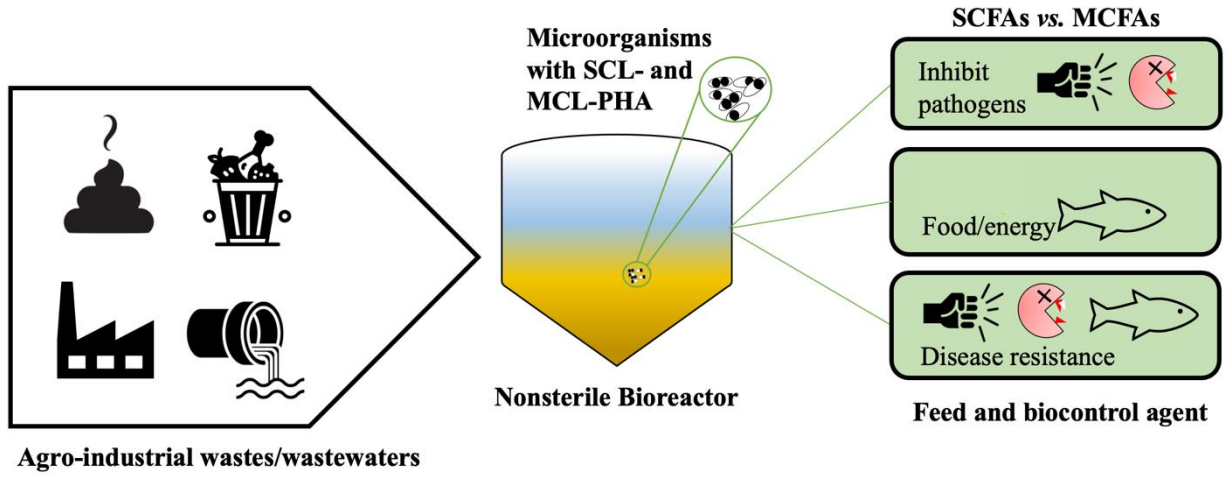
5. CHAPTER V FROM AGRO-INDUSTRIAL WASTES TO HEALTHY AQUAFEED: EFFECTIVENESS OF SHORT- AND MEDIUM-CHAIN-LENGTH POLYHYDROXYALKANOATES ON THE GROWTH AND DISEASE RESISTANCE IN AQUACULTURE

Polyhydroxyalkanoates (PHA) are a class of microbially-produced biopolymers that have several practical applications. Recently, a common PHA type, polyhydroxybutyrate (PHB), has been identified as an effective biocontrol agent to replace antibiotics in aquaculture by its degradation in the animal gut into short-chain fatty acids (SCFAs) with antimicrobial properties. However, the biocontrol investigation has been limited to only short-chain-length (SCL) PHA produced from pure substrates as carbon sources, leading to persistent challenges such as high dosages of SCL-PHA to effectively control pathogens and costly PHA production due to the expensive cultivation feedstocks and the need to sterilize the cultivation medium. This study investigated the effects of supplementing various forms of short- and medium-chain-length PHA (SCL- and MCL-PHA), produced from cheap and renewable agro-industrial wastes/wastewaters, on the growth and disease resistance of an aquaculture animal model, brine shrimp *Artemia*. *In vitro* assay demonstrated that low dosages of PHA intermediates (i.e., SCFAs, and particularly MCFAs) efficiently suppressed the growth of Gram-negative and Gram-positive aquaculture pathogens. Moreover, successful PHA co-/polymers production from *Zobellella denitrificans* ZD1 and *Pseudomonas oleovorans* on different pure substrates and agro-industrial wastes/wastewaters was demonstrated. Furthermore, *Artemia* starvation and pathogen challenge tests indicated that PHA-rich ZD1 and *P. oleovorans* biomasses (particularly cells contain MCL-PHA) can be used as food/energy sources in aquafeeds and protect *Artemia* against the pathogen infection. Overall, SCL- and MCL-PHA, produced from various agro-industrial wastes, demonstrated its

effectiveness to reduce PHA dosage and production cost while improving growth and disease resistance in aquaculture.



Synopsis

- SCL- and MCL-PHA-accumulating bacteria, produced from agro-industrial wastes, are effective in promoting growth and disease resistance in aquaculture.

5.1. Introduction

Polyhydroxyalkanoates (PHA) are a broad class of intracellular biodegradable biopolymers that are synthesizable by different microorganisms growing on renewable feedstocks.[1, 2] PHA consist of repeating units of hydroxy-fatty acids and classified mainly into two groups, short-chain-length PHA (SCL-PHA) with 3–5 carbon (C) atoms and medium-chain-length PHA (MCL-PHA) with C6–14 (C-x represents a chain length of x carbon atoms). The variations in the structure of PHA have attracted attention to develop various applications such as bioplastics for packaging purposes, biocompatible implants,[3] bacterial substrate in self-healing concrete,[4] paper coating,[5] bio-based glue,[6] and slow-release fertilizer/herbicide.[7, 8] Only recently, it has been demonstrated that PHA, particularly the short-chain type poly(3-hydroxybutyrate) (PHB), can be used as an affective feed additive and biocontrol agent in aquaculture to improve growth and disease resistance.[9-11]

Aquaculture is the fastest-growing food production sector with 8% annual growth rate.[11, 12] It essentially contributes to the global food security by supplementing around 82 million tonnes of aquatic animals, valued at 250 billion US dollars.[13] However, commercial aquaculture production encounters challenges such as waste management, high feed cost, and most importantly, disease outbreaks due to the proliferation of various pathogens.[11, 12] Recent studies have reported that PHB, a polymer of short-fatty fatty acid (SCFA) (3-hydroxybutyrate; 3-HB), can act as a biocontrol agent[6, 7] and an immunostimulant[8-10] for aquatic animals, thus replacing the widespread use of unsafe antibiotics in aquaculture.[14, 15] PHB gets biodegraded in the gastrointestinal tract into its intermediates/SCFAs (i.e., 3-HB and butyrate), which are known along with medium-chain fatty acids (MCFAs) to inhibit the growth of various enterobacteria and pathogens.[9, 16-19] The fatty acids inhibit pathogens by diffusing through the cell membrane and

acidifying the cytoplasm, leading to high cellular energy consumption to maintain homeostasis and eventually cell death. It is more advantageous to use PHB than soluble SCFAs because it is insoluble making the uptake of PHB more efficient for aquatic filter-feeders.[9, 20]

Supplementing aquafeeds with PHB in crystalline form (i.e., extracted from microbial biomass) or amorphous form (i.e., the PHB still inside the microbial cells) has been shown to improve the growth of aquatic animals[9, 11] and disease resistance of many fish species[21-23] and crustaceans.[10, 24-26] Yet, most of the research, if not all, has limited their investigation on supplementing the SCL-PHA type (i.e., PHB), while ignoring the biocontrol potential of MCL-PHA co-/polymers that consist of repeating units of MCFAs. As a result, shortcomings related to high polymer dosages to effectively control pathogens continue to persist. Besides, PHA production and application is hampered by different challenges, including expensive feedstocks (substrates) used for cultivating PHA-accumulating microorganisms[27] and producing different types of PHA,[3] energy-intensive sterilization to avoid microbial contamination, and high-energy toxic solvents to extract and purify PHA from PHA-filled microorganisms.[28, 29] Therefore, it is expected that longer-chain PHA, produced from inexpensive substrates, could have greater inhibitory activities against aquaculture pathogens after their biodegradation into intermediates/fatty acids in the animal gut, thereby reducing the required dosage and associated costs.

To overcome the PHA production and supplementation challenges described above, different types of agro-industrial wastes/wastewaters as substrates and PHA-accumulating strains have been explored.[2, 30, 31] Agro-industrial wastes/wastewaters such as crude glycerol, food wastes, fermentation leachates, fishmeal processing wastewater, cheese whey wastewater, and waste derived from the sugar industry are rich in carbon (C) and nutrients (nitrogen (N), phosphorus, and

trace elements) that could serve as excellent substrates for PHA-accumulating microorganisms. Furthermore, among different PHA-accumulating microorganisms, *Zobellella denitrificans* ZD1 (designated as ZD1 hereafter) is a salt-tolerant PHB-hyperaccumulating (up to 84%) bacterium, has shown great potential of utilizing various organic wastes without sterilization.[32, 33] In our previous study, we developed a novel recirculating aquaculture system (called RAS-PHB) that integrates the treatment of agro-industrial wastes, including aquaculture wastewater/wastes, with the production of PHB-rich ZD1 as an aquafeed.[32] Yet, the effects of different agro-industrial wastes/wastewaters on PHA composition in ZD1 (i.e., SCL- and MCL-PHA) have not been investigated. Moreover, the viability of using other PHA-accumulating strains seems interesting in order to exploit the full potential of RAS-PHB. *Pseudomonas oleovorans* is another bacterium known for producing MCL-PHA, such as polyhydroxyhexanoate (PHH) and polyhydroxyoctanoate (PHO).[2, 33] However, no studies have been conducted on using *P. oleovorans* containing MCL-PHA on the health and disease resistance in aquaculture.

Therefore, this study investigated the potential of supplementing different forms of SCL- and MCL-PHA (i.e., intermediates, crystalline, and amorphous), produced from various agro-industrial wastes/wastewaters, on the growth and disease resistance of an aquaculture animal model, brine shrimp *Artemia*. *Artemia* is a filter-feeding aquatic species that has been used as an important live food in aquaculture[34] and tested for PHB application.[9] The specific objectives of this study were to (i) assess the antimicrobial efficacy of SCFAs (butyrate and valerate) and MCFAs (hexanoate and octanoate) against several Gram-negative (G-) and Gram-positive (G+) predominant aquaculture pathogens (*Vibrio campbellii, Aeromonas hydrophila,* and *Streptococcus agalactiae*), (ii) examine the potential of utilizing different agro-industrial wastes/wastewaters by ZD1 and their implications on PHA composition, (iii) investigate whether SCL- and MCL-PHA

are used as food/energy sources for *Artemia*, and (iv) determine the survival of *Artemia* against aquaculture pathogens, when *Artemia* was supplemented with SCL- and MCL-PHA.

5.2. Materials and Methods

5.2.1. Bacterial Strains, Chemicals, and *Artemia*

The PHA-accumulating strains, *Z. denitrificans* ZD1 (JCM 13380) and *P. oleovorans* (ATCC 29347), were obtained from the Japan Collection of Microorganisms and the American Type Culture Collection, respectively. *Vibrio campbellii* (DSM 19270), a G- aquaculture pathogen, was obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures GmbH. *Aeromonas hydrophila* (G- aquaculture pathogen) and *Streptococcus agalactiae* (G+ aquaculture pathogen), isolated from diseased fish during an outbreak, were kindly provided by Dr. Delbert Gatlin, Texas A&M University, USA.

SCFAs (C-4 butyrate and C-5 valerate), MCFAs (C-6 hexanoate and C-8 octanoate), crystalline PHB, poly(3-hydroxybutyrate-co-3-hydroxyvalerate; PHB:9% HV), and poly(3-HB-co-3-HV-co-3-hydroxyhexanoate; PHB:2.3% HV:4.1% HH) were purchased from Sigma-Aldrich, USA. All other chemicals used in this study were obtained from Sigma-Aldrich or Fisher Scientific, USA. High-quality hatching cysts of brine shrimp (*Artemia franciscana*, EG® Type) for the challenge tests were obtained from INVE Aquaculture, Great Salt Lake, Utah, USA.

5.2.2. *In Vitro* Antipathogenic Properties of SCFAs and MCFAs

In vitro antipathogenic assays were conducted in a series of 55-mL culture tubes containing 10 mL of Luria-Bertani (LB) medium with one pathogenic strain type and one of these SCL-PHA intermediates (SCFAs: butyrate and valerate) and MCL-PHAs intermediates (MCFAs: hexanoate,

and octanoate). The PHA intermediates (SCFAs and MCFAs) are water-soluble and expected to be the degradation products present in the gastrointestinal tract of aquatic animals, allowing for the assessment of the antipathogenic efficacy of PHA under the best scenario. Various concentrations of PHA intermediates (5–125 mM) were used based on previously tested *in vitro* antimicrobial concentrations.[20, 35-38] Pre-grown bacterial strains, *V. campbellii, A. hydrophila, S. agalactiae* were used for inoculation. For *S. agalactiae,* tryptic soy media was used. All mediums' pH was adjusted to 6.0 based on the typical pH value found in the gut of aquatic animals[38, 39] and optimized antimicrobial activity of PHB reported previously.[20, 36, 38] The culture tubes were inoculated with 2% v/v pre-grown strains (optical density (OD_{600}) of 1.0) and incubated at 30 °C under 150 rpm. Liquid samples were collected to monitor the bacterial growth as determined by absorbance at OD_{600}. The growth of tested strains in the absence of PHA intermediates were used as controls. The inhibition efficiency (%) was calculated using Eq. (1):

$$\text{Inhibition Efficiency (\%)} = \left(\frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}}\right) \times 100 \quad (1)$$

where $OD_{control}$ and OD_{sample} refer to the highest optical densities of the growth curves for the controls and samples, respectively. The antibacterial activity was calculated as the minimum or median inhibitory concentrations (MIC and IC_{50}), representing the concentrations of the tested compounds inhibited 100% or 50% bacterial growth, respectively. MIC is determined by taking the regression through the highest OD_{600} measured at different compound concentrations. IC_{50} is estimated by taking the regression through % inhibition efficiencies to fit the 4-parameter logistic model.[40]

5.2.3. Sources and Pretreatment of Agro-Industrial Wastes/Wastewaters

Various agro-industrial wastes/wastewaters such as sugary waste slurry (SWS), cheese whey wastewater (CWW), synthetic crude glycerol (SCG), high-strength wastewater (HSSW),

food waste fermentation liquid (FWFL), banana peels (BP), orange peels (OP), and anchovy fishmeal wastewater (AFWW) were used as substrates (C-sources) for the cultivation of *Z. denitrificans* ZD1 and to determine their potential influences on PHA composition. SWS was collected from a local sweat factory (preferred to stay anonymous) in College Station, Texas. SWS was initially dissolved in DI water and filtered (0.45-μm) before adding to the cultivation medium described in the following section. CWW, containing the last remnant from ricotta cheese production, was prepared as described previously,[41, 42] with some modifications. Briefly, 4 L of whole milk was heated up to 82 °C before acidification with citric acid (7% v/v), followed by gentle mixing to form cheese. After the curd was firmed, CWW was flocculated by adding 750 mg/L medium-molecular-weight chitosan (dissolved in 1% v/v acetic acid). The mixture was settled at room temperature, and a clear supernatant (or so-called CWW) was collected for the cultivation experiments. SCG was prepared by adding 15 g/L glycerol, 10% methanol (weight of methanol/weight of glycerol), 1% fatty acids (i.e., 0.33% of each of stearate, oleate, and linoleate), and 30 g/L NaCl (3%) to the cultivation medium.[43] HSSW consisted of sodium acetate (8.2 g/L), glucose (10.1 g/L), $(NH_4)_2SO_4$ (2 g/L), Na_2HPO_4 (0.137 g/L), $MgSO_4{\cdot}7H_2O$ (37 mg/L), $CaSO_4{\cdot}2H_2O$ (12.1 mg/L), $FeSO_4{\cdot}7H_2O$ (22.2 mg/L), and K_2SO_4 (171 mg/L).[44] FWFL was prepared by mixing four different acids (acetic, propionic, butyric, and valeric acid) with ethanol at a fixed chemical oxygen demand (COD)-based ratios (16:12:57:11:4).[45] BP and OP were prepared as described previously,[46] with some modifications. Briefly, fresh peels were shredded, dried at 60 °C for 24 h, and boiled with water (100 g/L) for 45 min. The mixture was then squeezed and filtered using a cloth, and the filtrate was centrifuged at 3000 rpm for 15 min. pH of the supernatant was adjusted to 7 before adding to the cultivation medium. AFWW was prepared by boiling dried anchovies (Fisher Queen, Korea) in water (250 g/L) for 1 h. The mixture was then

squeezed using a cloth and the filtrate was centrifuged at 3000 rpm for 15 min. The supernatant pH was adjusted to 7 before adding to the cultivation medium.[47] The physicochemical properties (such as COD, total nitrogen (TN), salinity, and pH) of these agro-industrial wastes/wastewaters are listed in Table S5.1 in the Supporting Information.

5.2.4. Cultivation Experiments of PHA-Accumulating Strains

Two sets of cultivation experiments were conducted using (i) ZD1 in pure organic compounds and agro-industrial wastes/wastewaters as C-sources to examine potential PHA accumulation and (ii) *P. oleovorans* in pure hexanoate and octanoate as C-sources to produce PHH- and PHO-rich *P. oleovorans* biomasses, respectively, for *Artemia* challenge tests. All of the cultivation experiments were conducted using modified mineral salt medium (MSM)[48] containing NH_4Cl (1 g/L), Na_2HPO_4 (9 g/L), KH_2PO_4 (1.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), $CaCl_2.2H_2O$ (0.02 g/L), Fe(III)NH_4-citrate (0.0012 g/L), and 0.1% (vol/vol) trace mineral solution. The trace mineral solution contained EDTA (50 g/L), $FeCl_3$ (8.3 g/L), $ZnCl_2$ (0.84 g/L), $CuCl_2.2H_2O$ (0.13 g/L), $CoCl_2.6H_2O$ (0.1 g/L), $MnCl_2.6H_2O$ (0.016 g/L), and H_3BO_3 (0.1 g/L). Experimental details are described below.

5.2.4.1. ZD1 Cultivation in Pure Organic Compounds and Agro-Industrial Wastes/Wastewaters

The growth experiments were conducted in a series of 50 mL MSM supplemented with different pure organic compounds as C-sources such as sugars (glucose, fructose, sucrose, xylose, and lactose), SCFAs (acetate, propionate, butyrate, and valerate), MCFAs (hexanoate and octanoate), and other pure organics (glycerol and citric acid). In other cultivations sets, agro-

industrial wastes/wastewaters (i.e., SWS, CWW, SCG, HSSW, FWFL, BP, OP, and AFWW) were supplemented as C-sources. The initial COD in the cultivation medium after supplementing the abovementioned C-sources was set at 18.3 g/L, except for lactose (8.4 g/L), BP (2.8 g/L), OP (6.6 g/L), and AFWW (8.8 g/L), based on preliminary cultivation experiments to test optimum growth conditions (Figure S5.1). LB-grown ZD1 (OD_{600} = 1.0) after resuspending pellet in MSM was added to the cultivation medium as an inoculum (4% v/v). High salinity (30 g/L NaCl) was used in the cultivation experiments to provide an ideal condition for the nonsterile cultivation of ZD1.[43] The flasks were incubated at 30 °C under 150 rpm. Liquid samples were periodically collected to monitor ZD1 growth, and samples collected at the stationary growth phase were used to quantify cell dry weight (CDW), COD, TN, pH, and PHA content and composition. Removal efficiencies of COD and TN of wastes/wastewaters were also determined.

5.2.4.2. *P. oleovorans* Cultivation to Produce PHH- and PHO-Rich Biomasses

Another set of growth experiments in MSM were conducted as described above. However, MCFAs (hexanoate and octanoate) were used as C-sources to produce PHH- and PHO-rich *P. oleovorans*, respectively, as described previously.[33] Briefly, Reasoner's 2A (R2A)-grown *P. oleovorans* culture (OD_{600} = 1) was inoculated (4% v/v) in MSM containing 20 mM of hexanoate or octanoate and 0.5 g/L NaCl. After sterilization, the flasks were incubated at 30 °C under 150 rpm. Liquid samples were periodically collected to monitor the growth, and samples collected at the stationary growth phase were used to quantify CDW and PHA content and composition.

For the *Artemia* challenge tests, PHA-rich biomasses (i.e., PHB/V-rich ZD1 and PHH/O-rich *P. oleovorans*) were prepared from the cultivation experiments by collecting liquid samples

at the stationary phase, centrifuging the samples (4,500 × *g* for 10 min at 4°C), drying the pellets for 24 h at 105°C, and finally grounding the pellets with pestle and mortar before supplementation.

5.2.5. Axenic Hatching of *Artemia*

Gnotobiotic *Artemia* nauplii was prepared as described previously,[20, 49] with a minor modification. Briefly, 50 mg of cysts was hydrated in 18 mL of sterilized DI water for 30 min with moderate 0.2 μm-filtered aeration. Then, sterile decapsulation of hydrated cysts was achieved by adding 660 μL of NaOH (32% w/v) and 10 mL of NaOCl (13% active chlorine). After 3 min, decapsulation was stopped by adding 14 mL of $Na_2S_2O_3$ (10 g/L) and then immediately washed in filtered autoclaved artificial seawater (FASW) containing 35 g/L of sea salt (Instant Ocean, USA). The cysts were re-suspended in 50-mL tube containing 30 mL FASW (pH = 7.5) and allowed to hatch in a rotary (4 cycles/min) at room temperature for 30 h with constant illumination. After 30-h incubation, *Artemia* instar II nauplii were harvested, washed with FASW, and used for all *Artemia* challenge tests.

5.2.6. *Artemia* Starvation and Pathogen Challenge Tests

Artemia was used in (i) starvation and (ii) pathogen challenge tests. These tests were designed to elucidate the effects of different supplementation strategies, from individual chemicals such as SCFAs, MCFAs, to crystalline SCL- and MCL-PHA and amorphous SCL-PHA-rich ZD1, and MCL-PHA-rich *P. oleovorans*, on the growth, survival, and disease resistance of *Artemia*. Starvation challenge tests were performed to examine whether starved *Artemia* can grow and obtain energy from the individual supplemental feeds. The ability of the starved *Artemia* to survive after receiving the individual supplemental feed would indicate that the supplemented feed can be

used by the starved *Artemia* as an energy source. Pathogen challenge tests was conducted to demonstrate the effects of different supplementation strategies on the survival of *Artemia* challenged with *Vibrio*.

5.2.6.1. Starvation challenge tests

The starvation challenge tests were conducted by transferring hatched *Artemia* nauplii (1-day old) to new sterilized 55-mL glass tubes containing 20 mL of FASW with a stock density of 1 nauplii/mL, followed by one-time feeding of one of the supplements. Briefly, the *Artemia* in the glass tubes were fed with 1 g/L of one of the following supplements: SCFAs (butyrate and valerate), MCFAs (hexanoate and octanoate), crystalline PHB, crystalline PHB:9% HV, crystalline PHB:2.3% HV:4.1% HH, amorphous SCL-PHA (PHB-rich ZD1 and PHV-rich ZD1), and amorphous MCL-PHA (PHH-rich *P. oleovorans* and PHO-rich *P. oleovorans*). Starved (unfed) *Artemia* and yeast-fed *Artemia* were used as negative and positive controls, respectively. The culture tubes were placed on a rotor (4 cycles/min) at room temperature with continuous illumination. The survival of *Artemia* was monitored daily for four days.

5.2.6.2. Pathogen challenge tests

Pathogen challenge tests were conducted as described in the starvation tests, except that *Artemia* cultures (1-day old) with each of the abovementioned supplements (1 g/L) were exposed to a lethal dose of live *V. campbellii* (10^8 cells/ml) as determined previously.[50] In all treatments, 250 mg/L of dried and autoclaved yeast was added initially as a feed. *Artemia* was supplemented with 1 g/L of the abovementioned treatments in the starvation challenge tests. Unchallenged and

Vibrio-challenged *Artemia* were used as negative and positive controls, respectively. The survival of *Artemia* was monitored daily for three days.

5.2.7. PHA Quantification and Characterization

To determine the PHA content and composition in ZD1 and *P. oleovorans* grown on different substrates, a gas chromatography-flame ionization detector, GC-FID (Model 6890N, Agilent, USA) equipped with a DuraGuard J&W DB-5ms column (30 m, 0.25 mm, 0.25 μm) was used as described previously.[33, 51] Briefly, 30 mg of dried bacterial biomass was subjected to methanolysis in a screw-cap glass tube by reacting with 2 mL chloroform and 2 mL methanol (containing 15% v/v H_2SO_4) in a heating block at 100°C for 4 h. After cooling, 1 mL of DI water was added, and the mixture was vigorously shaken for 5 min to separate and collect the bottom organic phase after settling for 5 min. Then, 1 μl of the 0.2-μm filtered organic phase (hydroxyalkanoic acid methyl esters) was automatically injected (splitless) with helium used as the carrier gas (1.2 mL/min) in the GC-FID (80°C for 4 min; temperature ramp of 8°C/min; 160°C for 6 min; and temperature ramp of 25°C/min; 200°C for 1 min). The temperatures of the injector and detector were 250 and 280°C, respectively. Under these conditions, the retention times of the different hydroxyalkanoic acid methyl ester standards were as follows (min): C-4, 5.91; C-5, 7.49; C-6, 9.33; C-8, 10.55. The weight percentage of PHA of the total biomass was calculated by comparing peak areas to the standard of known concentrations.

5.2.8. Physicochemical Analyses

All bacterial growth was determined by OD_{600} using a UV–visible scanning spectrophotometer (VWR, 3100 PC). The CDW of ZD1 and *P. oleovorans* was determined after

centrifuging the collected liquid samples at the stationary phase (4,500 × g for 10 min at 4°C). Subsequently, the pellets were washed with DI water, recentrifuged, and desiccated at 105°C. The cell concentration was determined by dividing the weight of the dried pellet by the sample volume. The COD in the growth media after filtering (0.45-μm) was determined according to standard methods (with potassium dichromate in sulfuric acid) using a CHEMetrics Inc. mercury-free COD Test Kit, 0–1,500 ppm (HR). TN was determined in the filtered samples by wet-digested using peroxo-disulphate, before being analyzed according to standard methods (HACH assay kits, TNT 826).

5.2.9. Statistical Analysis

Statistical analyses were conducted using independent Student's t-test between two groups and one-way analysis of variance (ANOVA) for multiple groups followed by Tukey−Kramer post-test for identifying significant difference ($p < 0.05$) in JMP Pro 14 Statistical software. Principal component analysis (PCA) was performed using PAST4.06b software.

5.3. Results and Discussion

5.3.1. Antipathogenic Properties of SCL- and MCL-PHA Intermediates

5.3.1.1. Effects of SCFAs and MCFAs on Growth, Inhibition Efficiency, and Sensitivity of G- and G+ Pathogens

In vitro assays of the presence of PHA intermediates such as SCFAs (C-4 butyrate and C-5 valerate) and MCFAs (C-6 hexanoate and C-8 octanoate) were conducted in liquid media to assess their efficacy against common G- and G+ aquaculture pathogens. All SCFAs and MCFAs affected the pathogenic growth (V. campbellii, A. hydrophila, and S. agalactiae) in a concentration-dependent manner compared to the control (i.e., without PHAs intermediates) (Figure S5.2). However, despite the strain type, the inhibitory effects were more proportional with the carbon chain-length of the amended fatty acids, i.e., more pronounced with MCFAs (hexanoate and octanoate) than SCFAs (butyrate and valerate). Table 5.1 presents the MIC and IC50 of butyrate, valerate, hexanoate, and octanoate determined by taking the regression through the highest OD600 in Figure S5.2 and % inhibition efficiencies (Table S5.2) to fit the 4-parameter logistic model (Figure S5.3).[40]

Table 5.1. MICs and IC_{50} of PHA intermediates/fatty acids against common aquaculture pathogens.

Compound	Parameter	*V. campbellii*	*A. hydrophila*	*S. agalactiae*
Butyrate	MIC[a]	70.1	124.6	127.9
	IC_{50}[b]	8.97	30.9	57.5
Valerate	MIC	62.4	77.9	120.2
	IC_{50}	0.84	11.1	30.2
Hexanoate	MIC	22.9	37.6	72.2
	IC_{50}	3.73	5.27	13.4
Octanoate	MIC	< 5[c]	< 5	< 5
	IC_{50}	N.A.[d]	N.A.	N.A.

[a]MIC was determined by taking regression through highest optical densities measured at different compound concentrations. [b]IC_{50} is estimated by taking regression through % inhibition efficiencies calculated in Table S5.2 to fit the 4-parameter logistic model.[40] [c]< symbol was provided when total inhibition was reached within low tested concentrations. [d]N.A. = not applicable (strains have already exhibited full inhibition at the lowest compound concentration).

SCFAs were effective (valerate impact > butyrate) against the aquaculture pathogens, particularly against G- strains (V. campbellii and A. hydrophila) compared to G+ S. agalactiae (Figure S5.2). For example, MICs of 70.1–124.6 mM butyrate and 62.4–77.9 mM valerate were required to inhibit V. campbellii and A. hydrophila, while 127.9 mM butyrate and 120.2 mM valerate were required for S. agalactiae (Table 5.1). Furthermore, the IC50 occurred at 8.97–30.9 mM butyrate and 0.84–11.1 mM valerate for V. campbellii and A. hydrophila (Table 5.1). S. agalactiae was slightly more tolerant, requiring higher IC50 (57.5 mM butyrate and 30.2 mM valerate).

Similarly, MCFAs had strong antipathogenic performance against G- strains (V. campbellii and A. hydrophila), with octanoate effect > hexanoate (Figure S5.2). However, the inhibition efficiencies were significantly stronger compared to SCFAs. For instance, the growth of pathogens was clearly suppressed with low concentrations of hexanoate and octanoate (5–25 mM) (Figure S5.2). MICs of only 22.9–37.6 mM hexanoate and < 5 mM of octanoate were sufficient to terminate the growth of both V. campbellii and A. hydrophila (Table 5.1). This strong inhibitory effects of hexanoate and octanoate were also observed on G+ S. agalactiae, but with slightly higher MIC (72.2 mM of hexanoate). Octanoate was the most effective fatty acid against all pathogenic growth (Figure S5.2) with a MIC of < 5 mM (Table 5.1).

Biplot of the MIC values based on the highest optical densities is shown in Figure 5.1. Consistent with the aforementioned results, the plot revealed that aquaculture pathogens were highly susceptible to MCFAs (octanoate > hexanoate) followed by SCFA (valerate > butyrate). Furthermore, G- strains were more sensitive to the PHA intermediates. In specific, both V. campbellii and A. hydrophila were more sensitive to the SCFAs and MCFAs than G+ S. agalactiae, which was susceptible to octanoate.

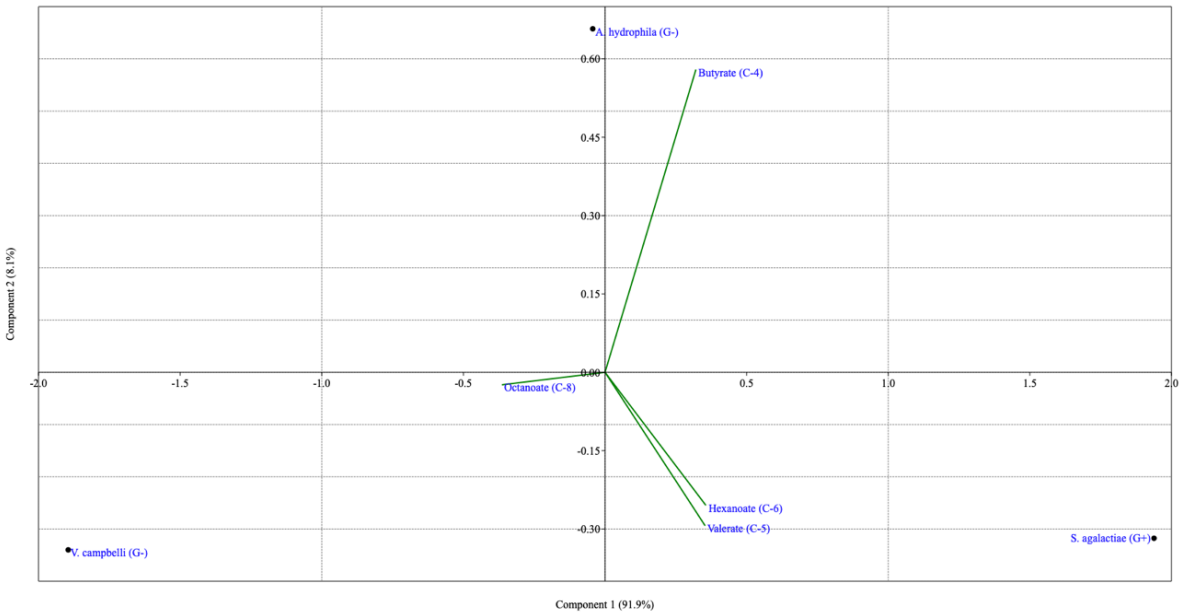


Figure 5.1. Biplot derived from PCA of MIC of PHA intermediates (butyrate, valerate, hexanoate, and octanoate) against common aquaculture pathogens.

5.3.1.2. Understanding the Antipathogenic Properties of SCFAs and MCFAs

The *in vitro* antipathogenic results demonstrated the efficacy of SCL- and MCL-PHA intermediates/fatty acids against common G- and G+ aquaculture pathogens. The observed inhibitory impacts of SCFAs were consistent with the literature.[20, 35-37] SCFAs such as formic, acetic, propionic, valeric acids, and particularly butyric,[9, 18] have shown to be effective against various enterobacteria,[18, 35, 36, 52] confirming the high potential of applying PHA as biocontrol agents in aquaculture. Furthermore, the stronger antipathogenic efficacy of MCFAs (hexanoate and octanoate) compared to SCFAs were in harmony with previous studies, which have demonstrated greater inhibitory activities in proportional to the carbon chain-length of fatty acids.[35, 38] The increase in carbon atoms in the compound elevates complexity and molecular weight, which could interfere with cell metabolism and eventually lead to cell inactivation. To our knowledge, this is the first study to examine the efficacy of SCFAs and MCFAs against G- and G+ aquaculture pathogens.

Although the exact underlying mechanism of PHA intermediates against pathogens is not known, one hypothesis maintains that fatty acids diffuse through the cell membrane and release protons (H^+) from their undissociated forms to lower the cytoplasm's pH. Consequently, the pathogens have to redirect their cellular energy to maintain homeostasis, suppressing their growth and causing cell death.[9, 53] Nevertheless, higher antipathogenic activity against G- strains could be attributed due to higher diffusion of the PHA intermediates thorough the thin peptidoglycan layer of G- cell wall compared to the thick peptidoglycan layer of G+ strains.[52] This finding, however, warrants further investigations. Overall, PHA intermediates, particularly MCFAs, are shown to be effective against pathogens and seems to serve as promising biocontrol agents in aquaculture to promote survival and disease resistance. Therefore, an effective strategy to combat pathogens in aquaculture could be by delivering PHA intermediates efficiently through PHA-accumulating microorganism to aquatic animals.

5.3.2. ZD1 Growth and PHA Accumulation in Different Pure Organic Compounds

The ability of ZD1 to utilize various substrates and accumulate different PHA was first assessed by cultivating the strain in various pure organic compounds as C-sources (e.g., sugars, SCFAs, MCFAs, and other organics). Apart from xylose, ZD1 was able to grow on all tested sugars (glucose, fructose, sucrose, lactose) and other pure organics such as glycerol and citric acid (Figure S5.4A). However, ZD1 growth was the fastest and highest (OD_{600} = 12–14) in sugars, followed by glycerol (OD_{600} = 6) and citric acid (OD_{600} = 3.5). Similarly, the highest cell concentration (i.e., CDW) achieved by ZD1 was in sugars (3–3.4 g/L), followed by glycerol (2 g/L) and then citric acid (1.2 g/L) (panel A in Table 5.2). In addition, ZD1 was able to grow on SCFAs (OD_{600} of 8–10), such as C-2 acetate, C-4 butyrate, and C-5 valerate, but not C-3 propionate

(Figure S5.4B). However, no growth was observed on MCFAs such as C-6 hexanoate and C-8 octanoate (Figure S5.4B). The cell concentration in SCFAs reached 1.8–2.5 g/L (panel A in Table 5.2), but the growth lag phase was significantly longer than other tested organics and elongated in proportional to the carbon chain length (e.g., 290 h for valerate > 120 h for butyrate > 37 h for acetate 37 h) (Figure S5.4B).

Table 5.2. Growth of ZD1 and PHA accumulation from different pure organic substrates and agro-industrial wastes/wastewaters.

Panel A: ZD1 growth and PHA accumulation in pure organic compounds as carbon sources

Carbon Source	Incubation time (h)	CDW (g/L)	%PHA[a]	%HB	%HV	PHA (g/L)	PHA productivity (g/L/d)	Final pH	%COD Removal	Yield[b]
Glucose	57	3.40	63.32	63.07	0.24	2.16	0.91	5.8	59.2	0.20
Fructose	48	3.08	66.89	66.61	0.28	2.06	1.03	5.9	60.9	0.19
Sucrose	48	3.16	54.74	54.51	0.23	1.73	0.87	5.8	54.8	0.17
Xylose	-	-	-	-	-	-	-	-	-	-
Lactose	247	0.72	0.81	-	0.81	0.01	0.00	6.8	50.3	0.00
Glycerol	48	1.98	32.29	31.88	0.41	0.64	0.32	6.4	26.2	0.13
Citric	50	1.22	25.79	25.15	0.64	0.32	0.15	9	78	0.02
Acetate	120	2.48	59.38	59.10	0.29	1.47	0.29	9	18.2	0.44
Propionate	-	-	-	-	-	-	-	-	-	-
Butyrate	222	1.84	60.40	60.07	0.33	1.11	0.12	8.7	15.9	0.38
Valerate	380	2.34	52.04	1.41	50.63	1.22	0.08	8.6	20.7	0.32
Hexanoate	-	-	-	-	-	-	-	-	-	-
Octanoate	-	-	-	-	-	-	-	-	-	-

Panel B: ZD1 growth and PHA accumulation in agro-industrial wastes/wastewaters as carbon sources

Carbon Source	Incubation time (h)	CDW (g/L)	%PHA[a]	%HB	%HV	PHA (g/L)	PHA productivity (g/L/d)	Final pH	%COD Removal	Yield[b]
SWS	69	2.81	65.60	65.18	0.42	1.85	0.64	5.9	30.1	0.34
CWW	20	1.55	13.29	12.90	0.39	0.21	0.25	6.5	31.5	0.00
SCG	43	1.72	29.81	29.26	0.55	0.51	0.29	6.6	18.3	0.14
HSSW	43	0.15	49.41	49.41	0.00	0.07	0.04	6.1	12.7	0.03
FWFL	65	1.11	17.50	14.78	2.72	0.19	0.07	8.6	24.8	0.04
BP	16	0.70	0.75	-	0.75	0.01	0.01	6.9	25.7	0.00
OP	22	1.75	25.59	25.00	0.59	0.45	0.49	6.6	28.2	0.01
AFWW	16	0.66	0.89	-	0.89	0.01	0.01	7.3	15.5	0.00

[a]The percentage of the PHA content in the cell dry weight (CDW) after reaching the stationary growth phase. [b]PHA yields were calculated based on grams of PHAs produced per gram of COD consumed. SWS = sugary waste slurry; CWW = cheese whey wastewater; SCG = synthetic crude glycerol; HSSW = high-strength wastewater; FWFL = food waste fermentation liquid; BP = banana peels; OP = orange peels; AFWW = anchovy fishmeal wastewater

The effective growth and high cell concentrations achieved by ZD1 in sugars could be attributed to the simple chemical structure and zero oxidation number of carbon atoms in sugars, making them readily consumable C-sources for many bacteria, compared with reduced fatty acids.[54] However, ZD1 inability to grow on xylose could be due to the lack of xylose enzymatic activities in ZD1.[55] In contrast, successful growth of ZD1 on SCFAs (i.e., acetate, butyrate, and valerate) indicated that the strain contains genes encoding enzymes for the utilization of those fatty acids. However, as the carbon chain-length increases (> 5C), the fatty acid becomes more difficult to assimilate. This hypothesis was confirmed by ZD1 incapability to grow on MCFAs and/or the elongated lag phase observed with longer SCFAs. However, the inability of ZD1 to grow on propionate could be related to its cell damaging effects due to odd and short n-alkyl-carbon-chain fatty acids (i.e., C-3).[30] Previous studies have reported toxic effects of propionate on the growth and PHA accumulation when used as a (co)-substrate for *Z. denitrificans* MW1[48] and *Ralstonia eutropha*.[56] Furthermore, a cultivation concentration of < 7 g/L was recommended.[56]

The highest PHA accumulations in ZD1 were observed with sugars as C-sources, particularly in glucose and fructose (63–67% gPHA/gCDW), followed by 52–60% in SCFAs (acetate, butyrate, and valerate), and 25–32% in glycerol and citric acid (panel A in Table 5.2). Most importantly, the main polymer composition of the produced PHA from all C-sources was HB monomer unit with a small fraction of HV unit (< 1%). Notably, valerate-grown ZD1 biomass contained 52% HV along with < 2% HB (panel A in Table 5.2). Furthermore, the initial pH in sugars-supplied mediums dropped from 7.5 to 5.8 as ZD1 grew, while it raised to 8.6–9 in mediums containing acids (panel A in Table 5.2). Moreover, a high COD removal efficiency (78%) was observed in citric acid, followed by sugars (55–60%), and fatty acids (15–20%) (panel A in Table 5.2).

The high PHA accumulation in ZD1 grown on sugars and fatty acids compared with other organics (glycerol and citric acid) could be related to the determined PHA biosynthesis pathways from sugars and fatty acids that are described previously.[30] However, the major accumulation of SCL-PHA (i.e., mainly PHB with a small fraction of HV), regardless of the C-source, could indicate the absence of genes encoding for MCL-PHA in ZD1 and that the strain possesses genes for SCL-PHA biosynthesis, which have been previously identified.[57, 58] The biosynthesis of SCL-PHA can take two pathways. Pathways I (tricarboxylic acid (TCA) cycle), which is related to sugars, starts with the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA by 3-ketothiolase (PhaA), which is then reduced to 3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (PhaB) using NADPH as the electron donor. Finally, 3-hydroxybutyryl-CoA is polymerized to PHB by PHA synthase (PhaC class I). 3HV monomer units could be synthesized by the addition of propionate or valerate to the cultivation medium causing the condensation of propionyl–CoA and acetyl-CoA by the action of 3-ketothiolase to 3-ketovaleryl-CoA. 3HV units could also be synthesized by pathway II (β-oxidation), which involves the degradation of fatty acids to generate substrates that can be polymerized by PHA synthase yielding SCL-PHA copolymer, mainly PHBV.[30] Nevertheless, there are some microorganisms that are able to generate the key precursor of 3HV-CoA for PHBV biosynthesis from unrelated C-sources.[30] Interestingly, the high HV accumulation in valerate-grown ZD1 suggests that pathway II could be the major PHA biosynthesis pathway for fatty acids, and that HB and HV accumulation could be related to the strain's capability to grow on butyrate and valerate, respectively.

The drop in pH value in sugars-supplied cultivation mediums is due to the secretion of acids and release of protons (H^+) during the oxidation of the energy source (sugars) and vice versa (i.e., protons consumption) for acids-supplied mediums.[54] However, based on the remaining COD

in all mediums, it is possible that high ZD1 biomass and PHA production could be achieved by controlling pH and supplying additional N. Overall, the broad spectrum of substrates exploitation and high PHA accumulation demonstrated the remarkable potential of ZD1 to treat various agri-industrial wastes/wastewaters, particularly sugars-containing wastes, while producing relatively inexpensive and tailor-made PHA (co-/polymers).

5.3.3. Feasibility of Using Different Agro-Industrial Wastes/Wastewaters for ZD1 Growth and PHA Accumulation

As the chemical composition of real substrates like agro-industrial wastes/wastewaters are source-dependent and may potentially contain some inhibitory compounds, further investigations using more diverse substrates were essential to test the robustness of ZD1 to grow and accumulate PHA. Figure S5.5 and Table 5.2 (panel B) illustrates cell growth and PHA accumulation of ZD1 on various real and synthetic agro-industrial wastes/wastewaters. ZD1 grew effectively in all tested organic wastes, reaching OD_{600} and CDW (shown in parenthesis) from high to low: SWS (10; 2.81 g/L) > OP = SCG (5.2; 1.75 g/L) > CWW (3.3; 1.55 g/L) > FWFL (3.1; 1.11 g/L) > BP = AFWW (2; 0.7 g/L) > HSSW (1; 0.15 g/L). Interestingly, PHA contents in ZD1 biomass were comparable to those accumulated in ZD1 grown in simple pure substrates with a maximum PHA accumulation (65%) observed in SWS (panel B in Table 5.2). However, no significant PHA accumulation (<1%) was observed in ZD1 grown on BP and AFWW. As noted with pure substrates, the PHA polymer composition in ZD1 grown in organic wastes was mainly HB along with <1% HV. Notably, ZD1 grown on FWFL contained a larger fraction of HV unit (3%) along with 14.8% HB. Likewise, the pH after ZD1 growth dropped to 5.9–6.5 in all cultivation mediums, while it increased to 8.6 in FWFL (panel B in Table 5.2).

The effective growth of ZD1 and high PHA accumulation in real agro-industrial wastes/wastewaters (without the need for sterilization and nutrient-limitation condition) are promising, placing strain ZD1 as a favorable candidate among the previously tested PHA-accumulating strains.[2, 30, 31] The fast growth and short lag phase could be attributed to the presence of various organics in the wastes, which have provided the energy for growth and biomass production. Furthermore, PHA accumulation in ZD1 grown in wastes were comparable to those accumulated using pure substrates. However, poor PHA accumulation in BP- or AFWW-grown ZD1 could be attributed to the presence of more complex organics, which may have prioritized biomass production over PHA accumulation by ZD1. Additionally, the major HB content with low HV fraction observed also with agro-industrial wastes further suggests that the ZD1 contains genes encoding for SCL-PHA. Nevertheless, a higher fraction of HV in FWFL-grown ZD1 is due to the high presence of valeric acid (i.e., 11% COD-based) in FWFL, which could have facilitated the accumulation of HV unit as discussed earlier. Although low HV fraction is obtained in the accumulated PHA, the presence of low HV confers to the polymer properties (i.e., better thermal and mechanical properties than PHB)[31] and possibly stronger antimicrobial activities due to the presence of longer SCFAs, thereby offering improved biocontrol efficacy against aquaculture pathogens. Overall, the efficient ZD1 growth and PHA accumulation observed with different agro-industrial wastes/wastewaters demonstrate the potential of using ZD1 to exploit various wastes while producing PHA for different applications, including biocontrol agents in aquaculture.

5.3.4. Ability of SCL- and MCL-PHA to Serve as Food/Energy Sources for *Artemia*

Starvation challenge tests were performed to examine whether starved *Artemia* can obtain energy from the administrated feeds (i.e., SCFAs, MCFAs, crystalline PHA (co)-polymers, and

amorphous PHA-rich biomasses). Prolonged survival of *Artemia* would indicate that the feed has been used as a food/energy source and degraded in the gut. Feeding *Artemia* with individual fatty acids, particularly MCFAs (hexanoate and octanoate), sustained the survival of *Artemia* (22.5–25%) after four days of starvation compared to 2.5% in the negative control (i.e., starved unfed *Artemia*) (Figure 5.2A). Interestingly, a proportional relationship between the carbon chain group and the survival rate was observed (i.e., C-8 octanoate and C-6 hexanoate higher survival than C-5 valerate and C-4 butyrate). However, insignificant difference in survival was observed within the same chain group. Furthermore, feeding crystalline PHA (e.g., PHB and PHB:9%HV) did not significantly improve the survival of starved *Artemia* (Figure 5.2B). However, feeding crystalline PHB:2.3%HV:4.1%HH prolonged the survival to 25% after 4 days. Most importantly, feeding PHA-rich biomasses (i.e., PHB/V-rich ZD1 and PHH/O-rich *P. oleovorans*) significantly improved the survival of starved *Artemia* (77.5–87.5%) at even a higher level compared to the positive control, i.e., yeast-fed *Artemia* (67.5%) (Figure 5.2C). However, insignificant difference in survival was observed between feeding different types of PHA-rich biomasses. Notably, by visualizing *Artemia* with a 30x glass magnifier, *Artemia* fed with PHA-rich biomasses clearly had an advanced nauplii life stage (e.g., grew larger, moved faster, and developed rudimentary thoracopods and bilateral compound eyes).

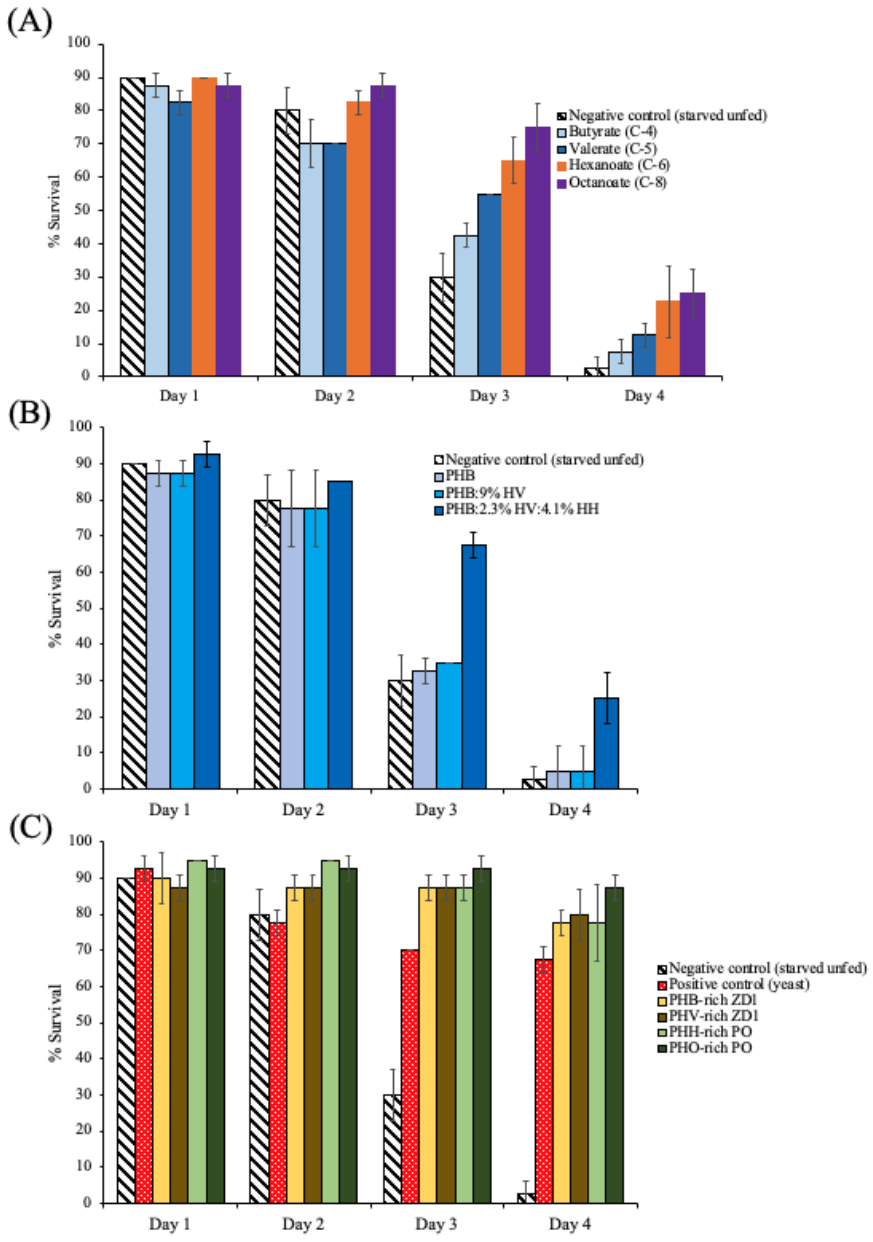


Figure 5.2. Survival of starved *Artemia* fed with: (A) SCFAs (butyrate and valerate) and MCFAs (hexanoate and octanoate). (B) Crystalline PHA (PHB, PHB:9% HV, and PHB:2.3% HV:4.1% HH). (C) Amorphous SCL-PHA (PHB-rich ZD1 and PHV-rich ZD1) and amorphous MCL-PHA (PHH-rich *P. oleovorans* and PHO-rich *P. oleovorans*). Starved (unfed) *Artemia* and yeast-fed *Artemia* were used as negative and positive controls, respectively.

The *Artemia* starvation results indicated that fatty acids, particularly MCFAs, could serve as additional energy sources for *Artemia*. Previous studies have showed that SCFAs (3-HB and

butyrate) could provide energy for *Artemia*.[50, 59, 60] Nevertheless, the enhanced *Artemia* survival with feeding MCFAs (hexanoate and octanoate) could be attributed to their longer carbon chain-length (>5C), thus sustaining the survival by providing extra energy. Previous studies have indicated that SCFAs and MCFAs are important substrates for the energy metabolism and anabolic processes,[61] which could be further used as blood fuel for energy purposes or lipid synthesis by aquatic animals.[62]

In terms of feeding different pure crystalline PHA co-/polymers, the results indicated no adverse effects on *Artemia*. In fact, PHA with longer chain-length monomers (i.e., PHB with 2.3%HV and 4.1%HH) significantly prolonged the survival of *Artemia*. This observation is in harmony with feeding pure MCFAs described above and consistent with previous studies. For instance, feeding crystalline PHB has shown to serve as an additional energy source for starved *Artemia*.[36] Furthermore, replacing 0.1–5% (w/w) of standard diet with PHB:11%HH improved the survival of Kuruma shrimp and was shown to have no negative effects on body weight, feeding rate, and feed conversion ratio.[38] Those findings further confirmed the potential of PHA, particularly with longer monomers, to provide energy (i.e., lipid deposition) to aquatic animals. The lipid-saving effects of PHA (e.g., PHB) have been previously confirmed with *Artemia*[63] and Nile tilapia,[64, 65] reporting higher lipid and whole-body contents.

Aside from the distinctive contributions of pure PHA polymers and monomers, the improvement in survival of *Artemia* fed with amorphous PHA-rich biomasses compared to unfed or yeast-fed *Artemia* were promising. However, the insignificant difference in survival between feeding different chain-length PHA-accumulating biomasses could be attributed to the biomass (SCP) content. ZD1 and *P. oleovorans* are PHA-accumulating SCP that contain essential nutrients (e.g., proteins, lipids, and minerals),[32] which can contribute energy for *Artemia*. Therefore, the

presence of other cell components could have overshadowed the distinctive impacts of different accumulated chain-length PHA. Nevertheless, higher survival compared to traditional SCP (yeast) could be attributed to more simple and digestible cell wall of bacteria compared to yeasts' cell walls that are characterized to be rough (i.e., comprises 25−30% of dry matter)[66, 67] and composed of complex heteropolysaccharides, mannoprotein, and glucan,[68] all of which complicate yeast's digestibility. Overall, the *Artemia* starvation results indicated that amorphous PHA-rich biomasses are easily assimilated (degraded in the gut) and used as effective energy sources by aquatic animals.

5.3.5. Effectiveness of SCL- and MCL-PHA on the Survival of *Vibrio*-Challenged *Artemia*

Figure 5.3 illustrates the effects of various supplementation strategies (i.e., SCFAs, MCFAs, crystalline PHA co-/polymers, and amorphous PHA-rich biomasses) on the survival of *Artemia* challenged with *Vibrio*. Pure MCFAs significantly enhanced the survival of *Artemia* (30% with C-6 hexanoate and 80% with C-8 octanoate) after three days of challenge compared to SCFAs (10% for C-4 butyrate and C-5 valerate). The survival with SCFAs was insignificant compared to the positive control, i.e., unsupplemented *Vibrio*-challenged *Artemia* (5%). Among the supplemented fatty acids, octanoate showed the highest potential to improve *Artemia*'s survival, even greater than the negative control, i.e., unchallenged *Artemia* (47.5%) (Figure 5.3). Additionally, feeding crystalline PHA co-/polymers enhanced the survival of *Artemia* compared to the positive control; however, there was insignificant difference between the presence of longer monomers (i.e., 9%HV and 2.3%HV:4.1%HH). Most importantly, supplementing amorphous PHA-rich biomasses (i.e., PHB/V-rich ZD1 and PHH/O-rich *P. oleovorans*) yielded significantly higher survival (42.5–55%) compared to the positive control, and even higher than the negative

control (Figure 5.3). Notably, as observed with pure fatty acids supplementation, the survival trend was slightly proportional with the accumulated chain-length PHA (i.e., longer PHH/O-rich PO had greater impact than shorter PHB/V-rich ZD1). However, insignificant difference in survival was observed within the same PHA chain group.

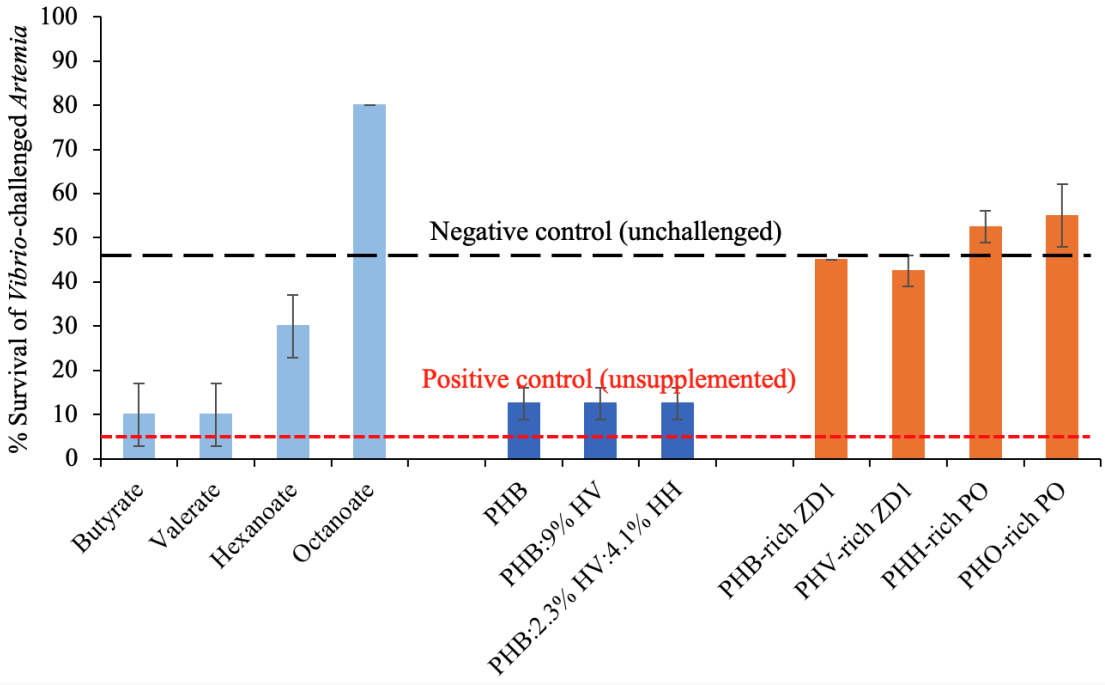


Figure 5.3. Survival rates of *Vibrio*-challenged *Artemia* supplemented with SCFAs (butyrate and valerate), MCFAs (hexanoate and octanoate), crystalline PHA (PHB, PHB:9% HV, and PHB:2.3% HV:4.1% HH), amorphous SCL-PHA (PHB-rich ZD1 and PHV-rich ZD1), and amorphous MCL-PHA (PHH-rich *P. oleovorans* and PHO-rich *P. oleovorans*). Unchallenged and *Vibrio*-challenged *Artemia* (unsupplemented) were used as negative and positive controls, respectively.

The *Artemia* pathogen challenge results demonstrated the high potential of PHA-rich biomasses as effective biocontrol agents, protecting *Artemia* against the *Vibrio* infection. Particularly, the presence of longer monomers in the accumulated PHA could have intensified the protection. Previous studies have confirmed that SCL-PHA (e.g., PHB and PHBV) promotes the survival and disease resistance of aquaculture animals,[9, 11, 69] including *Artemia*,[10, 20, 24, 70] with several key mechanisms. This could be achieved once PHB gets ingested and partially converted

to SCFAs (3-HB and butyrate monomers and oligomers) by digestive enzymes[36] or PHB degraders[71, 72] in the animal gut. Studies have indeed documented the release of 3-HB in the gastrointestinal tract of *Artemia* fed with varying levels of PHB.[20] The release of SCFAs may hinder the pathogenic growth and/or reduce the expression of virulence factors such as biofilm formation, luminescence, motility, hemolysin production, and the N-acyl-homoserine lactone-mediated quorum sensing.[70] Another protective mechanism of PHB is stimulating stress- and immune-response in *Artemia*,[25, 69, 73, 74] particularly the innate (non-specific) genes.[75] This mechanism is associated with the cellular acidification induced by 3-HB release in the animal gut, leading to higher expression of defensive genes.[9]

Interestingly, the enhanced survival of *Artemia* in response to supplementing longer MCL-PHA (i.e., PHH/O-rich *P. oleovorans*) could be attributed to their higher number of carbons (> 5C), providing greater protection against *Vibrio* infection. This hypothesis is supported by the greater survival observed with supplementing pure MCFAs to *Artemia* compared to SCFAs. Therefore, the presence of MCFAs, in water or *Artemia*, could have inhibited the growth of *Vibrio* (as noted in our *in vitro* results with hexanoate and octanoate), allowing *Artemia* to survive. Furthermore, the release of MCFAs intermediates after the digestion of MCL-PHA in the gut could have served as additional energy sources as described in the starvation challenge test. Notably, the higher disease resistance observed with supplementing pure octanoate and/or PHO-rich *P. oleovorans* further signifies the antipathogenic activity of longer MCFAs. In consistence with the present study, caprylic acid (also known as octanoic acid) was shown to promote the survival of challenged *Artemia* and to inhibit shrimp pathogens (*V. harveyi* and *V. parahaemolyticus*)[76] and even fish parasites.[77-79] Octanoic acid (C-8) is longer than SCFAs and less hydrophobic (i.e., more soluble) than long-chain fatty acids, thereby more effective in killing bacteria.[76]

Notwithstanding the benefits of PHA, *Artemia* survival rate when using crystalline PHA was lower than amorphous PHA-rich biomasses. This observation could be attributed to smaller particle size and lower crystallinity in amorphous PHA, making it more susceptible to enzymatic and microbial degradation.[9] Moreover, crystalline PHA lack other cell nutrients (e.g., proteins, lipids, and minerals)[32] found in amorphous PHA, which might have elicited additional immune responses. Few studies have applied amorphous PHA-accumulating microorganisms to aquafeeds such as *Alcaligenes eutrophus*,[29, 73, 80] *Halomonas* spp.,[81] *Brevibacterium casei*,[70] *Bacillus* sp.,[10] *Comamonas testosteroni*,[24] and *Brachymonas denitrificans*.[24] The advantages of using ZD1 is that it can accumulate PHB/V in a growth-associated manner (i.e., without nutrient limitation),[32, 43] allowing a simple and continuous single-stage production bioprocess. Furthermore, ZD1 with a common osmolyte (ectoine)[58] eliminates the need for energy-intensive sterilization by growing in high salt concentration (30 g/L); thus, inhibiting the growth of non-salt-tolerant microorganisms.[43] *P. oleovorans* is another important strain traditionally used to accumulate MCL-PHA such as PHH and PHO.[2, 33] Therefore, the application of *P. oleovorans* with MCL-PHA provided additional biocontrol benefits to aquatic animals.

PHA-rich ZD1 and *P. oleovorans* evinced encouraging results in protecting *Artemia* against the *Vibrio* infection. To our knowledge, this is the first study to investigate the application of PHA-rich ZD1 and *P. oleovorans* in aquaculture, and most importantly, to compare the biocontrol potential of various forms of SCL- and MCL-PHA (i.e., intermediates, crystalline, amorphous). Nevertheless, it was reported that supplementing PHB:11%HH extracted from recombinant *Cupriavidus necator* increased the survival of Kuruma shrimp challenged with *V. penaeicida*, and that the *in vitro* supplementation of 3-HH had greater antibacterial effect than 3-HB.[38] In another study, it was reported that supplementing PHBV extracted from *Bacillus*

thuringiensis enhanced disease resistance of Mozambique tilapia challenged with *A. hydrophila*.[69] Overall, the *Artemia* pathogen challenge results suggest that amorphous PHA, particularly MCL-PHA-rich biomasses, can provide strong protection for aquatic animals against pathogens, thereby serving as effective biocontrol agents.

5.4. Conclusions, Scaling-Up Limitations, and Future Directions

This study demonstrated, for the first time, the efficacy of supplementing different types and forms of SCL- and MCL-PHA (i.e., intermediates, crystalline, and amorphous) on the growth and disease resistance of an aquaculture model species, brine shrimp *Artemia*. Particularly, the study evaluated the efficacy of supplementing different amorphous PHA-accumulating microorganisms, produced from various inexpensive agro-industrial wastes/wastewaters. Low doses of PHA intermediates/fatty acids, specifically MCFAs, were shown to suppress the growth of common G− and G+ aquaculture pathogens. Strain ZD1 demonstrated a remarkable potential to accumulate high levels of SCL-PHA (PHBV copolymer) by treating different pure organic compounds and agro-industrial wastes/wastewaters. Finally, *Artemia* starvation and pathogen challenge results indicated that ZD1 and *P. oleovorans* rich with SCL- and MCL-PHA, respectively, served as additional food/energy sources in aquafeeds and protected *Artemia* against pathogen infection, particularly in the presence of longer MCL-PHA.

Despite the great potential of producing waste-derived PHA-rich biomasses as single-cell proteins (SCP) for aquafeeds, cultivating SCP and scaling-up the entire production bioprocess come with few limitations. Generally, SCP production encounters challenges such as high C/electron donor requirement, energy-intensive illumination, large footprint, and poor mass transfer of sparingly gases (i.e., carbon dioxide, hydrogen, and methane) in the bioreactor during

cultivation. For example, approximately 361−5000 MJ of C/electron donor is required to produce 1 kg of biomass by traditional SCPs such as phototrophic (algae and purple phototrophic bacteria), lithotrophic (hydrogen-oxidizing bacteria), and methylotrophic (methane-oxidizing bacteria).[66] Comparatively, the use of organotrophic (heterotrophs) like ZD1 and *P. oleovorans* as SCPs is more energy-efficient as ~230-MJ C/electron donor is only required to produce 1 kg of biomass.[66, 82, 83] Furthermore, the use of heterotrophs lifts the energy-intensive illumination and large footprint required for the production of traditional phototropic SCPs.[66, 84] Such an advantage agrees with the "dark food chain" envisioned previously,[11] wherein chemoheterotrophy substitutes photosynthesis of SCPs as animal feed or human food. Moreover, the supplementation of agro-industrial wastes as C/electron donor for heterotrophs avoids the poor mass transfer associated with the supplementation of carbon dioxide, hydrogen, and methane for phototrophic, lithotrophic, and methylotrophic microorganisms, respectively.[11] The solubility of those sparingly gases are orders of magnitude less than other soluble organic feedstocks such as glucose;[11] therefore, the use of heterotrophs can lead to higher production of SCP biomass.

Furthermore, this study proposed the integration of PHA-rich SCP production as aquafeed with the treatment of inexpensive and renewable waste streams. This novel combination would successfully lower large-scale PHA production costs and extend the sustainability and commercial viability of aquaculture. However, this strategy is accompanied with few drawbacks. For example, agro-industrial wastes/wastewaters contain a large fraction of complex organics and inhibitors that may limit their utilization by bacteria and decrease biomass and PHA yield.[30, 85] This leads to the necessity of applying some toxic or high-energy input pretreatments, such as solvents or thermal processes to purify the substrates and enhance the availability of fermentable sugars.[30] Another major drawback is the difficulty to control the composition of PHA in the cells

due to the complexity of applied substrates.[85] Finally, the migration of pollutants such as heavy metals from organic wastes to PHA is also another concern that should be taken into consideration. However, the careful selection of agro-industrial wastes/wastewaters could overcome the previous issues. For example, high-strength wastewater from food and agricultural industries or livestock effluents are the type of organic wastes that can be used for PHA-rich SCP production. These waste streams are typically high in COD and nitrogen and low in pollutants.[86-89] It was reported that the total content of heavy metals in PHA produced from fruit wastes or crops were significantly lower than PHA samples produced from municipal wastewater and sludge.[90] The heavy metals were also below the migration limits specified by the Commission Regulation (EU) October 2011 on articles for contact with food under frozen and refrigerated conditions.[90] Therefore, it is essential to choose agro-industrial wastes that meet some basic requirements, such as high and constant quality and availability throughout the year, storing suitability, absence of conflict with other feedstock applications, and easy collection and transportation. Optimally, PHA-rich biomass production should be integrated into existing industrial production lines.[85]

Remarkably, we recently developed a novel RAS-PHB system as a proof-of-concept, which integrates the treatment of aquacultural wastes with the production and harvest of PHB-rich SCP as a healthy aquafeed. A simple economic assessment was also demonstrated the economic advantage of the RAS-PHB.[32] Nevertheless, future research is needed to examine the effects of environmental and operational changes (e.g., different farmed aquatic animals, carbon feedstocks, and other PHA-producing strains) on the long-term RAS-PHB performance to provide the required knowledge for future development of a full-scale RAS-PHB system.

Future studies to explore new PHA-rich SCP production techniques and safe and inexpensive carbon substrates are warranted to overcome the above-mentioned limitations and

achieve large-scale, sustainable PHA production and application. Future innovations could aim for dark heterotopic production of PHA-rich SCP using renewable substrates, such as acetate generated by microbial bioelectrosynthesis or ethanol, fermentation of syngas (CO and H_2), or by degradation of cellulosic waste streams.[11] Furthermore, to understand the difference in underlying mechanisms between the produced SCL- and MCL-PHA in fighting pathogens in aquaculture, further studies that focus on analyzing the immune response and change in the gut microbiome are needed. Finally, this study provided an overview of the application of different types of PHA (e.g., SCL- and MCL-PHA) in their various forms (intermediates, crystalline, and amorphous). Future studies could use this overview to predict the effects of supplementing different PHA-accumulating microorganisms to aquaculture.

5.5. Supporting Information.

The Supporting Information for Chapter V can be found in Appendix C.

5.6. References

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6. CHAPTER VI CONCLUSIONS, IMPLICATIONS, AND FUTURE DIRECTIONS

6.1. Conclusions

This three-manuscript dissertation proposed a sustainable and economical engineering approach for poly(3-hydroxybutyrate) (PHB) production, which in turn, can be applied as an effective biocontrol agent and aquafeed to support organic aquaculture. The implications of this process can overcome the traditional PHB challenges, such as costly sterilization, high-energy input harvesting, and toxic extraction and purification, as well as challenges associated with the aquaculture industry such as waste management, high aquafeed cost, and the use of antibiotics to control pathogens.

In paper I, a novel PHB production and supplementation system in aquaculture, called recirculating aquaculture system for PHB-rich microorganisms (RAS-PHB) that integrates the treatment of agro-industrial wastes, including aquaculture wastewater, with the production of PHB-rich single-cell proteins (SCPs) was successfully developed. An economic analysis based on experimental data strongly indicated that the proposed RAS-PHB system is more efficient and economical than the conventional RAS in terms of (i) valorizing organic wastes/wastewater before treatment or discharge, (ii) producing PHB-rich SCPs as a potential replacement to traditional aquafeed and antibiotics, and (iii) efficiently harvesting the generated biomass by chitosan biocoagulant. In paper II, chitosan-harvested PHB-rich ZD1 (CP-ZD1) produced from RAS-PHB demonstrated multifunctional effects as an antibiotic-free feed in improving the growth and disease resistance in aquaculture. The combination of chitosan with amorphous PHB (i.e., CP-ZD1) was shown to suppress common pathogens, induce high survival rates and immune response among aquatic animals, and provide them with the energy needed for growth and a healthy microbiome. Finally, paper III further confirmed that low doses of polyhydroxyalkanoates (PHA) intermediates,

particularly medium-chain-length PHA (MCL-PHA), suppress the growth of common aquaculture pathogens. Furthermore, bacterial cells that accumulated fractions of longer PHA monomers, along with PHB, magnified the biocontrol efficacy of PHB. Overall, the engineering approach of PHB production and application, proposed in this dissertation, yields promising potentials toward large-scale PHB production and sustainable, organic aquaculture industry.

6.2. Implications

The findings of this dissertation have several implications that relate to the production of PHA/PHB, the applications of CP-ZD1 in the aquaculture industry, and recirculating aquaculture systems (RAS). In terms of the production of PHA/PHB, nonsterile production of PHA/PHB from PHA-accumulating microorganisms, such as ZD1, growing on renewable agro-industrial wastes/wastewaters significantly lower the production cost of PHA and ensure its sustainability. Furthermore, using chitosan, the most widespread polysaccharide in nature after cellulose,[1] as a biocoagulant to harvest the PHA/PHB-accumulating microorganisms, as opposed to toxic metal coagulants or energy-intensive centrifugation, allows the recultivation of these microorganisms and reduces the PHA/PHB production cost.

In terms of the application of CP-ZD1 in the aquaculture industry, CP-ZD1 has the potential to replace traditional antibiotics in aquafeed. CP-ZD1 improves the growth and disease resistance of aquatic animals, while maintaining antibiotic-free wastewater/wastes, an issue of emerging concern in the industry.[2-4] Moreover, feeding CP-ZD1 as SCPs can reduce the dependency on wild-captured forage fish (e.g., anchovies, menhaden, and sardines) in fishmeal. This reduction will contribute significantly to an important sustainability metric, the “fish-in to fish-out” ratio, which have been continuously endorsed by many scientists and professionals in the

aquaculture industry.[5] Finally, CP-ZD1 is harvested by chitosan; thus, it is deemed remedial and safe for fish consumption, as it is obtained from crab and shrimp wastes.[6, 7] Altogether, the use of CP-ZD1 may promote safe and sustainable aquacultural production.

Finally, the findings reported in paper I have direct implications on recirculating aquaculture systems (RAS). The developed RAS-PHB must be seen as an upgraded version of conventional RAS. This RAS-PHB encompasses enhancements that further improve the effectiveness and sustainability of conventional RAS. First and foremost, RAS-PHB successfully produces PHA/PHB-accumulating microorganisms as SCPs, a function that is lacked in conventional RAS. The internal production of SCPs within RAS-PHB lifts the energy-intensive illumination required for traditional SCPs (such as purple phototropic bacteria or algae),[8] which in turn lower the aquafeed/SCP production cost. Secondly, the use of zeolite as a nitrogen-recovery adsorbent allows a better control of nitrogen in the cultivation of SCP to minimize potential growth inhibition, which may arise through the direct application of wastewaters, with high suspended solids, as substrates.[9]

6.3. Future Directions

The developed RAS-PHB warrants further investigations. This dissertation mainly intended to validate RAS-PHB as a proof-of-concept. The effects of environmental and operational changes due to the differences in farmed aquatic animals, carbon feedstocks, ammonia-nitrogen concentrations, and pH in the wastewater were not investigated. Thus, future research is needed to examine the effects of those environmental and operational changes on the long-term RAS-PHB performance to provide the required knowledge for future development of a full-scale RAS-PHB system. Furthermore, this dissertation used RAS-PHB to produce PHA-accumulating

microorganisms solely. Future research should expand the use of RAS-PHB for cultivating other microbial biomasses, such as polyunsaturated fatty acids (PUFAs)-producing microalgae and fungi.[10-12] PUFA-producing microorganisms seems particularly suitable to be tested in RAS-PHB because they are known to grow on aquaculture wastewater without sterilization,[11, 13] and their ability to improve fish health and quality of seafood (e.g., higher omega-3 content) is documented.[5, 11, 14]

For the applications of PHA/PHB and chitosan-harvested SCPs in the aquaculture industry, several research topics seems of particular interest. For example, this dissertation showed microbial biomasses (SCPs), represented in the PHA-accumulating microorganisms with or without chitosan, to have high digestibility in an aquaculture animal model, brine shrimp *Artemia*. This digestibility, however, warrants *in vivo* fish trials to further validate the findings reported in this dissertation. Moreover, the application of PHA-accumulating microorganisms showed remarkable potential to improve growth and disease resistance in aquaculture, particularly with longer-chain PHA. However, further studies that focus on analyzing immune response, hematological parameters, and change in the gut microbiome are warranted to understand the difference in underlying mechanisms between SCL- and MCL-PHA. Finally, this dissertation provided an overview of the application of different types of PHAs (e.g., SCL- and MCL-PHA) in their various forms (intermediates, crystalline, and amorphous). Future studies could use this overview to predict the effects of supplementing different PHA-accumulating microorganisms to aquaculture.

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APPENDIX A.

SUPORTING INFORAMATION FOR CHAPTER III

Supporting Information: 16 pages total including text describing physicochemical analysis, 2 tables, 5 figures, and economic analysis

Table of Content

Physicochemical Analysis. The COD of AW or growth media was determined according to standard methods (with potassium dichromate in sulfuric acid) using a CHEMetrics Inc. mercury-free COD Test Kit, 0–1,500 ppm (HR). To determine total nitrogen-nitrogen (TN-N) in AW and NH_4^+-N in media, the samples were first filtered (0.45-μm) and wet-digested using peroxo-disulphate, before being analyzed according to standard methods (HACH assay kits, TNT 826 and 828). The *Z. denitrificans* ZD1 growth and supernatant absorbance were monitored based on absorbance (OD_{600}) using a UV–visible scanning spectrophotometer (VWR, 3100 PC). The CDW was measured after centrifuging the collected sample at the stationary phase using a Sorvall

Legend XTR centrifuge (4,500 × g for 10 min at 4°C). Subsequently, the pellets were washed with DI water and desiccated at 105°C in pre-weighed glass tubes. The cell concentration was determined by dividing the weight difference (before and after drying) by the sample volume. To determine the PHB content, the dried biomass was digested with 1 mL of concentrated H_2SO_4 at 70°C for 4 h and then neutralized by adding 4 M NaOH. The digested solution was centrifuged, and crotonic acid was determined at 235-nm against that of pure PHB standards treated alike.

Table S3.1. Characteristics of agro-industrial wastewaters used for the non-sterile production of PHB-rich *Z. denitrificans* ZD1 biomass

Organic Waste	COD (g/L)	TN (g-N/L)	Salinity (g/L)	pH
AW	0.205	0.025	3	8.2
Glycerol	12.2	-	30	7.4
CWW	50.4	1.47	0	6.5

Note: AW = Aquaculture wastewater (i.e., fish tank effluent)
CWW = Cheese production wastewater

Table S3.2. Comparison of Biomass Composition in this Study with other Protein Sources.

Protein Source		Energy Source/Substrate	PHB (%)	Protein[a] (%)	Lipid (%)	Ash (%)	Energy (MJ/kg)	References
Microbial biomass (SCPs)	*Z. denitrificans* ZD1	Glycerol	48	45.5	50.4	4.1	23.4	This study
		CWW	12	34.8	13.6	51.6	11.2	
	Bacillus licheniformis	Potato processing waste		38		11		[1]
	Purple phototrophic bacteria	Light/Poultry WW[b]		~75[c]	~20		22	[2]
		Light/Dairy WW		~61	~29			
		Light/Sugar WW		~42	~20			
	Methane-oxidizing bacteria	Biogas methane	43-73	60	8-11	6-9		[3-5]
	Hydrogen-oxidizing bacteria	Hydrogen	57	75	-			[3]
	Microalgae: *Chlorella vulgaris* and *Scenedesmus* species	Light/Poultry WW		~65	~27			[2]
		Light/Dairy WW		~37	~59			
		Light/Sugar WW		~14	~15			
	Yeast	Organic carbon		45-55	1-6	5-10	19.9	[6, 7]
Fishmeal		-		63	11	16	20.1	[8]
Soybean meal		-		44	2.2	5	21.3	[8]

Notes: Results are expressed as dry-weight basis and presented as mean values from triplicate experiments. [a] The protein content in this study was adjusted (1–5%) to reduce the overestimation caused by the interference with the non-protein nitrogen compounds using the Dumas method. [b] WW refers to wastewater. [c] The values were estimated from Figure 3 in Hülsen et al.[2]

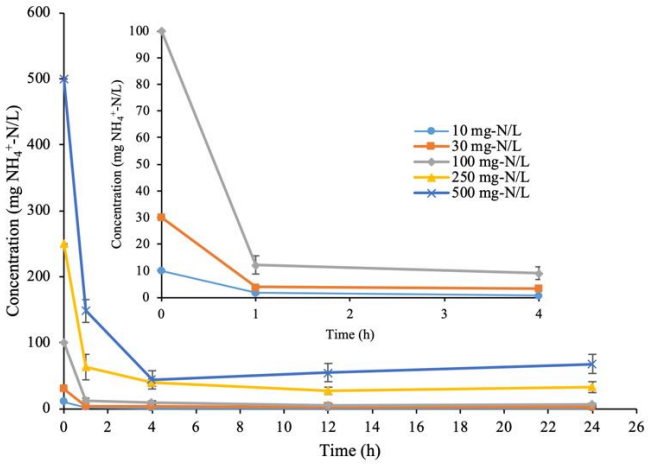

Figure S3.1. Change in NH_4^+-N concentration with time after adsorption by natural zeolite under different initial NH_4^+-N concentrations.

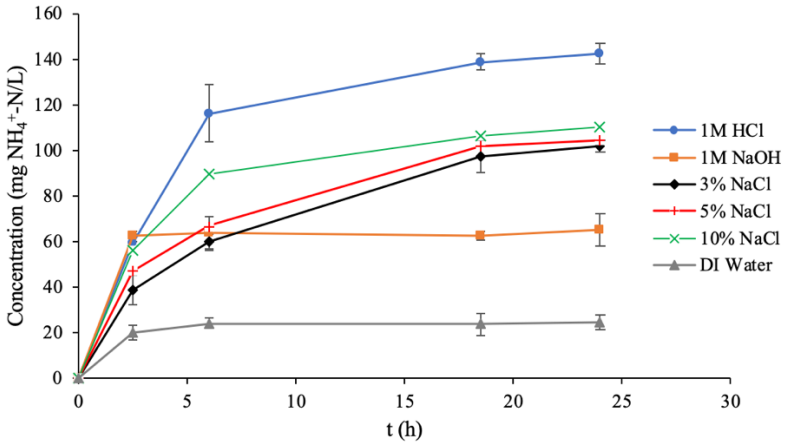

Figure S3.2. Concentration of released NH_4^+-N from ammonium-laden zeolite using various desorption solutions.

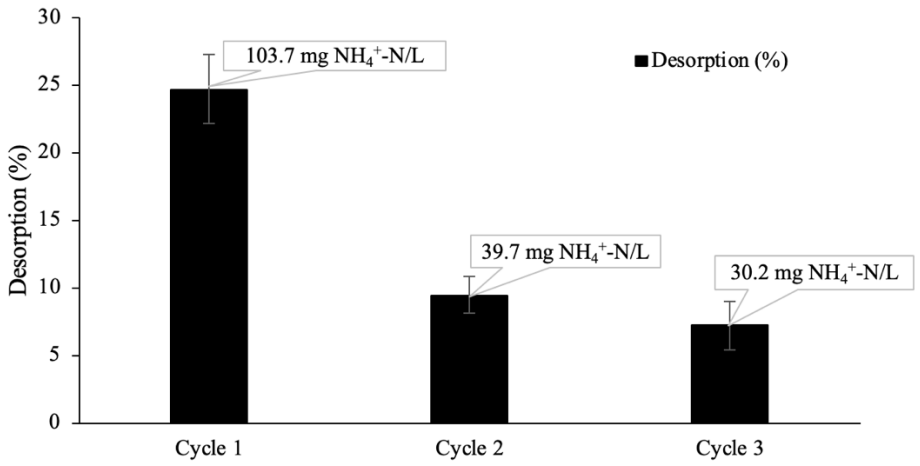

Figure S3.3. Desorption (%) from each cycle after extraction of ammonium-laden zeolite with 3% NaCl. NH_4^+-N concentration detected in the extract after each cycle was shown on the top of the bar.

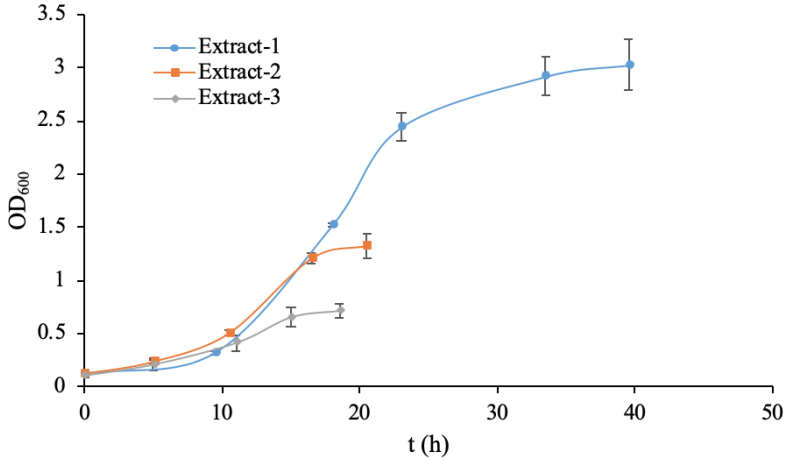

Figure S3.4. *Z. denitrificans* ZD1 cultivation in N-free MSM, glycerol (5 g/L), and one of the three extracts obtained from spent zeolite after three cycles of 3% NaCl desorption.

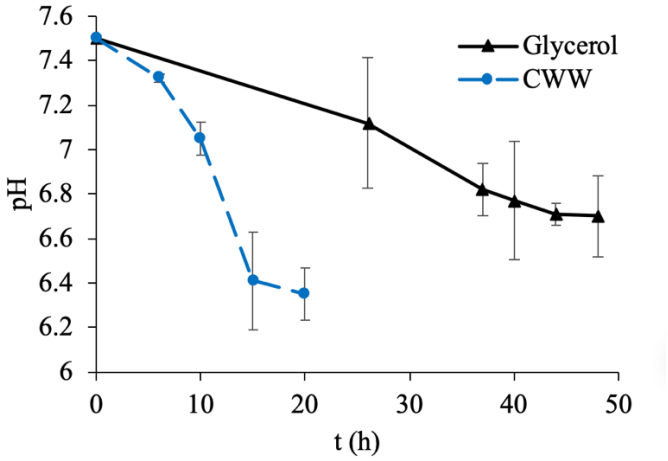

Figure S3.5. Changes in pH during the growth of *Z. denitrificans* ZD1 in agro-industrial wastes/wastewaters, glycerol and CWW.

Economic Analysis

The assumptions and calculations for computing the annual production cost of farmed fish using the conventional RAS or the proposed RAS-PHB is provided in this section. The economic analysis was separated into eight scenarios; four scenarios for each RAS based on the farmed fish species (tilapia (T) and red drum (R)). Scenarios TA and TB and Scenarios RA and RB represent the conventional RAS with the supplementation of antibiotics or pure commercial PHB, respectively, to achieve the same overall tilapia and red drum production. Scenarios TC and TD and Scenarios RC and RD represent RAS-PHB with the supplementation of glycerol- or CWW-grown PHB-rich *Z. denitrificans* ZD1 biomass, respectively, as alternatives.

The following assumptions were made for computing production cost for all scenarios:

Assumptions applied to all scenarios:

- An annual production of 500 ton of farmed tilapia or red drum using RAS.
- Volume of aquaculture system is 1000 m^3
- Stock density is 50 kg fish/m^3
- The aeration energy required for *Z. denitrificans* ZD1 growth in the bioreactor is similar to that required for the conventional biofilter in RAS.
- The cost of the zeolite filter is similar to the sand filter in RAS.

- Feed cost is \$0.18/lb = \$0.40/kg feed.[9]
- The average food conversion ratio for tilapia and red drum is 2.2[10, 11] and 2.0 kg feed/kg fish produced,[12] respectively.
- The protein content of a typical tilapia and red drum feed is 30%[13] and 40%,[14, 15] respectively.
- The application of antibiotics, commercial pure PHB, or PHB-rich *Z. denitrificans* ZD1 exhibits similar fish survival rates and the final kg of fish produced.

Assumptions applied to Scenarios TA and TB and Scenarios RA and RB:

- Average daily water replacement in the conventional RAS is 10% of the water volume.[9]
- Water replacement cost is ~\$0.32/m^3.[11]
- Average solid waste produced in tilapia culture is 230 kg/ton fish produced = 0.25 kg/kg fish produced.[16]
- Average solid waste produced in red drum culture is ~ 0.31 kg/kg fish produced.[17]
- Solid waste disposal cost is ~\$0.62/kg of waste. Such estimate is based on \$8726 annual operating cost for 127 mt of solid waste.[18]
- **For Scenario TA and RA only:**
    - Antibiotics use in the conventional RAS is 0.53 kg/ton.[19]
    - Average cost of antibiotics is \$150/kg.[20]
    - The survival rate of fish receiving 75 mg dose/kg bodyweight/day of oxytetracycline antibiotic is 85%.[21]
- **For Scenario TB and RB only:**
    - Pure commercial PHB use in the feed (5% w/w).[22-24]
    - Commercial PHB price is \$0.48/kg.[25]
    - The survival rate of fish receiving 5% (w/w) PHB in the feed is 85%.[24]

Assumptions applied to Scenarios TC and TD and Scenarios RC and RD:

- The PHB-rich *Z. denitrificans* ZD1 biomass to be used in the proposed RAS-PHB is 60% of the feed.
- Chitosan dosage of 50 mg/L is used to harvest 80% of biomass.
- Price of chitosan is \$7/kg.[26]
- **For Scenario TC and RC only:**
    - Glycerol is the organic waste to cultivate *Z. denitrificans* ZD1.
    - Price of glycerol is \$0.04/kg.[27]
- **For Scenario TD and RD only:**
    - Cheese whey wastewater (CWW) is the organic waste to cultivate *Z. denitrificans* ZD1.
    - Although CWW is considered a dairy waste that can be obtained at no cost, its price assumed to be €25/ton = \$0.028/kg.[28]

Production Cost of Tilapia Farming using Conventional RAS (Scenarios TA and TB)

(i) Annual feed cost for producing 1 kg of fish:

➔ 2.2 kg feed/kg fish produced × \$0.40/kg feed = <u>\$0.9/kg fish produced</u>

▪

(ii) Annual water replacement cost for producing 1 kg fish:

➔ 0.1 m^3 water replaced/m^3 water volume/d × 1000 m^3 water volume × 365 d/yr × \$0.32/m^3 = \$11680/yr

➔ \$11680/yr /(500 ton/yr) = \$24/ton = <u>\$0.024/kg fish produced</u>

▪

(iii) Annual solid waste disposal cost for producing 1 kg of fish:

➔ 0.25 kg solid waste/kg fish produced × \$0.62/kg solid waste = <u>\$0.15/kg fish produced</u>

▪

(iv) Annual antibiotics/ pure commercial PHB cost for producing 1 kg fish:

➔ **Scenario TA – Use of antibiotics:** 0.53 kg antibiotics/ton fish produced × 1 ton fish/1000 kg fish × \$150/kg antibiotics = <u>\$0.08/kg fish produced</u>

▪

➔ **Scenario TB – Use of pure commercial PHB:** 2.2 kg feed/kg fish produced × 0.05 kg PHB/kg feed × \$4.4/kg PHB = <u>\$0.48/kg fish produced</u>

Scenario TA: Total cost = \$0.9 + \$0.024 + \$0.15 + \$0.08 = \$1.2/kg fish produced/yr
Scenario TB: Total cost = \$0.9 + \$0.024 + \$0.15 + \$0.48 = \$1.6/kg fish produced/yr

<u>Production Cost of Red Drum Farming using Conventional RAS (Scenarios RA and RB)</u>

(v) Annual feed cost for producing 1 kg of fish:

➔ 2.0 kg feed/kg fish produced × \$0.40/kg feed = <u>\$0.8/kg fish produced</u>

▪

(vi) Annual water replacement cost for producing 1 kg fish:

➔ 0.1 m^3 water replaced/m^3 water volume/d × 1000 m^3 water volume × 365 d/yr × \$0.32/m^3 = \$11680/yr

➔ \$11680/yr /(500 ton/yr) = \$24/ton = <u>\$0.024/kg fish produced</u>

▪

(vii) Annual solid waste disposal cost for producing 1 kg of fish:

➔ 0.31 kg solid waste/kg fish produced × \$0.62/kg solid waste = <u>\$0.20/kg fish produced</u>

▪

(viii) Annual antibiotics/ pure commercial PHB cost for producing 1 kg fish:

➔ **Scenario RA – Use of antibiotics:** 0.53 kg antibiotics/ton fish produced × 1 ton fish/1000 kg fish × \$150/kg antibiotics = <u>\$0.08/kg fish produced</u>

▪

➔ **Scenario RB – Use of pure commercial PHB:** 2.2 kg feed/kg fish produced × 0.05 kg PHB/kg feed × $4.4/kg PHB = $0.48/kg fish produced

Scenario RA: Total cost = $0.8 + $0.024 + $0.20 + $0.08 = $1.1/kg fish produced/yr Scenario RB: Total cost = $0.8 + $0.024 + $0.20 + $0.48 = $1.5/kg fish produced/yr

Production Cost of Tilapia Farming using Proposed RAS-PHB (Scenarios TC and TD)

According to De Schryver et al., in a conventional RAS, tilapia can be produced with an average food conversion ratio of 2.2 kg feed/kg fish and 30% protein content in the regular feed.[11] In the proposed RAS-PHB, 60% of the regular fish feed is assumed to be replaced by PHB-rich *Z. denitrificans* ZD1 biomass that was produced from glycerol (Scenario TC) or CWW (Scenario TD). The protein contents of glycerol- and CWW-grown *Z. denitrificans* ZD1 were 45.5% and 34.8%, respectively. Therefore, the amount of *Z. denitrificans* ZD1 biomass needs to be added to the feed to produce 1 kg of fish is:

➔ 0.3 kg protein/kg feed × 2.2 kg feed/kg fish produced = 0.66 kg protein/kg fish produced
➔ **Scenario TC – Use of glycerol:** (0.66 kg protein/kg fish produced × 0.60) /(0.455 kg protein/kg ZD1 biomass) = 0.87 kg ZD1 biomass/kg fish produced
➔ **Scenario TD – Use of CWW:** (0.66 kg protein/kg fish produced × 0.60) /(0.348 kg protein/kg ZD1 biomass) = 1.13 kg ZD1 biomass/kg fish produced

▪

(i) Total feed cost for producing 1 kg fish in RAS-PHB = Cost of 40% regular feed + Cost to produce kg ZD1 biomass/kg fish produced based on the organic waste type

➔ Cost of 40% regular feed: 0.40 × (2.2 kg feed/kg fish produced × $0.40/kg feed) = $0.36/kg fish produced
➔ Cost to produce kg ZD1 biomass/kg fish produced based on the organic waste type
  ⇒ **Scenario TC – Use of glycerol:** Cost to produce 0.87 kg ZD1 biomass/kg fish produced.
    - ZD1 biomass yield = (1.97 g ZD1 biomass/L)/(10 g glycerol/L) = 0.20 g ZD1 biomass/g glycerol = 5 kg glycerol/kg ZD1 biomass
    - 0.87 kg ZD1 biomass/kg fish produced × 5 kg glycerol/kg ZD1 biomass = 4.35 kg glycerol/kg fish produced
    - 4.35 kg glycerol/kg fish produced × $0.04/kg glycerol = $0.17/kg fish produced

- The total feed cost = $0.36 + $0.17 = $0.53/kg fish produced

▪

⇒ **Scenario TD – Use of CWW:** Cost to produce 1.0 kg ZD1 biomass/kg fish produced.

- ZD1 biomass yield = (3.24 g ZD1 biomass/L)/(8 g CWW/L) = 0.4 g ZD1 biomass/g CWW = 2.4 kg CWW/kg ZD1 biomass
- 1.13 kg ZD1 biomass/kg fish produced × 2.4 kg CWW/kg ZD1 biomass = 2.7 kg CWW/kg fish produced
- 2.7 kg CWW/kg fish produced × $0.028/kg CWW = $0.08/kg fish produced
- The total feed cost = $0.36 + $0.08 = $0.44/kg fish produced

Note: In the conventional RAS, the amount of pure PHB supplied in the feed is 0.05 kg PHB/kg feed. In the proposed RAS-PHB, the amount of PHB supplemented through PHB-rich ZD1 biomass is:

➔ **Scenario TC – Use of glycerol:**

⇒ 0.87 kg ZD1 biomass/kg fish produced × 0.48 kg PHB/kg ZD1 biomass = 0.42 kg PHB/kg fish produced

⇒ 0.42 kg PHB/kg fish produced × 1 kg fish produced/2.2 kg feed = 0.19 kg PHB/kg feed

⇒ (0.19 kg PHB/kg feed)/(0.05 kg PHB/kg feed) = 3.8 times higher than the needed pure PHB in the conventional RAS, which is also the amount required to promote growth and survival of Nile tilapia.[24]

➔ **Scenario TD – Use of CWW:**

⇒ 1.13 kg ZD1 biomass/kg fish produced × 0.12 kg PHB/kg ZD1 biomass = 0.14 kg PHB/kg fish produced

⇒ 0.14 kg PHB/kg fish produced × 1 kg fish produced/2.2 kg feed = 0.06 kg PHB/kg feed

⇒ (0.06 kg PHB/kg feed)/(0.05 kg PHB/kg feed) = 1.2 times higher than the needed pure PHB in the conventional RAS.

▪

(ii) The chitosan coagulant cost:

➔ **Scenario TC – Use of glycerol:**

⇒ Required chitosan amount = 0.80 kg ZD1 biomass harvested/kg ZD1 biomass × (1.97 g ZD1 biomass/L) × 1 L/0.05 g chitosan = 31.5 kg ZD1 biomass/kg chitosan = 0.032 kg chitosan/kg ZD1 biomass

⇒ 0.032 kg chitosan/kg ZD1 biomass × 0.87 kg ZD1 biomass/kg fish produced = 0.028 kg chitosan/kg fish produced

⇒ 0.028 kg chitosan/kg fish produced × $7/kg chitosan = $0.19/kg fish produced

➔ **Scenario TD – Use of CWW:**

⇒ Required chitosan amount = 0.80 kg ZD1 biomass harvested/kg ZD1 biomass × (3.24 g ZD1 biomass/L) × 1 L/0.05 g chitosan = 51.8 kg ZD1 biomass/kg chitosan = 0.02 kg chitosan/kg ZD1 biomass

⇒ 0.02 kg chitosan/kg ZD1 biomass × 1.13 kg ZD1 biomass/kg fish produced = 0.02 kg chitosan/kg fish produced

⇒ 0.02 kg chitosan/kg fish produced × $7/kg chitosan = $0.14/kg fish produced

▪

- Scenario TC: Total cost = $0.36 + $0.17 + $0.19 = $0.7/kg fish produced
- Scenario TD: Total cost = $0.36 + $0.08 + $0.14 = $0.6/kg fish produced

Production Cost of Red Drum Farming using Proposed RAS-PHB (Scenarios RC and RD)

Red drum can be produced with an average food conversion ratio of 2.0 kg feed/kg fish[12] and 40% protein content in the regular feed.[14, 15] Therefore, the amount of *Z. denitrificans* ZD1 biomass needs to be added to the feed to produce 1 kg of fish is:

➔ 0.4 kg protein/kg feed × 2.0 kg feed/kg fish produced = 0.8 kg protein/kg fish produced

➔ **Scenario RC – Use of glycerol:** (0.8 kg protein/kg fish produced × 0.60) /(0.455 kg protein/kg ZD1 biomass) = 1.05 kg ZD1 biomass/kg fish produced

➔ **Scenario RD – Use of CWW:** (0.8 kg protein/kg fish produced × 0.60) /(0.348 kg protein/kg ZD1 biomass) = 1.38 kg ZD1 biomass/kg fish produced

(i) Total feed cost for producing 1 kg fish in RAS-PHB = Cost of 40% regular feed + Cost to produce kg ZD1 biomass/kg fish produced based on the organic waste type

➔ Cost of 40% regular feed: 0.40 × (2.0 kg feed/kg fish produced × $0.40/kg feed) = $0.32 kg fish produced

➔ Cost to produce kg ZD1 biomass/kg fish produced based on the organic waste type

⇒ **Scenario RC – Use of glycerol:** Cost to produce 1.05 kg ZD1 biomass/kg fish produced.

- ZD1 biomass yield = (1.97 g ZD1 biomass/L)/(10 g glycerol/L) = 0.20 g ZD1 biomass/g glycerol = 5 kg glycerol/kg ZD1 biomass
- 1.05 kg ZD1 biomass/kg fish produced × 5 kg glycerol/kg ZD1 biomass = 5.3 kg glycerol/kg fish produced
- 5.3 kg glycerol/kg fish produced × $0.04/kg glycerol = $0.21/kg fish produced
- The total feed cost = $0.32 + $0.21 = $0.53/kg fish produced

⇒ **Scenario RD – Use of CWW:** Cost to produce 1.38 kg ZD1 biomass/kg fish produced.

- ZD1 biomass yield = (3.24 g ZD1 biomass/L)/(8 g CWW/L) = 0.4 g ZD1 biomass/g CWW = 2.4 kg CWW/kg ZD1 biomass
- 1.38 kg ZD1 biomass/kg fish produced × 2.4 kg CWW/kg ZD1 biomass = 3.31 kg CWW/kg fish produced
- 3.31 kg CWW/kg fish produced × \$0.028/kg CWW = \$0.093/kg fish produced
- The total feed cost = \$0.32 + \$0.093 = \$0.41/kg fish produced

(ii) The chitosan coagulant cost:

➔ **Scenario RC – Use of glycerol:**

⇒ Required chitosan amount = 0.80 kg ZD1 biomass harvested/kg ZD1 biomass × (1.97 g ZD1 biomass/L) × 1 L/0.05 g chitosan = 31.5 kg ZD1 biomass/kg chitosan = 0.032 kg chitosan/kg ZD1 biomass

⇒ 0.032 kg chitosan/kg ZD1 biomass × 1.05 kg ZD1 biomass/kg fish produced = 0.033 kg chitosan/kg fish produced

⇒ 0.033 kg chitosan/kg fish produced × \$7/kg chitosan = \$0.24/kg fish produced

➔ **Scenario RD – Use of CWW:**

⇒ Required chitosan amount = 0.80 kg ZD1 biomass harvested/kg ZD1 biomass × (3.24 g ZD1 biomass/L) × 1 L/0.05 g chitosan = 51.8 kg ZD1 biomass/kg chitosan = 0.02 kg chitosan/kg ZD1 biomass

⇒ 0.02 kg chitosan/kg ZD1 biomass × 1.38 kg ZD1 biomass/kg fish produced = 0.03 kg chitosan/kg fish produced

⇒ 0.03 kg chitosan/kg fish produced × \$7/kg chitosan = \$0.21/kg fish produced

- Scenario RC: Total cost = \$0.32 + \$0.21 + \$0.24 = \$0.8/kg fish produced
- Scenario RD: Total cost = \$0.32 + \$0.093 + \$0.21 = \$0.6/kg fish produced

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APPENDIX B.

SUPORTING INFORAMATION FOR CHAPTER IV

Supporting Information: 24 pages total including methods, 9 figures, and 5 tables

Table of Content

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Production of PHB-Rich ZD1 Biomass

The growth of the salt-tolerant strain, Z. denitrificans ZD1, took place under controlled condition of using glycerol as a carbon (C)-source to produce PHB-rich ZD1 biomass (P-ZD1).1 Briefly, LB-pregrown ZD1 culture (OD600 of 1) was inoculated (4% v/v) in 50 mL of mineral salt medium (MSM) containing glycerol (10 g/L), KNO3, K2HPO4 (2.85 g/L), Na2SO4 (0.5 g/ L), MgSO4·7H2O (0.4 g/L), NaCl (30 g/L), and 0.1% (vol/vol) trace mineral solution. The trace mineral solution contained FeSO4·7H2O (2.78 g/L), MnCl2·4H2O (1.98 g/L), CoSO4·7H2O (2.81 g/L), CaCl2·2H2O (1.47 g/L), CuCl2·2H2O (0.17 g/L), and ZnSO4·7H2O (0.29 g/L). High salinity (30 g/L NaCl) resembles the non-sterile PHB-rich ZD1 production, eliminating the sterilization process.1 To obtain two accumulation levels of PHB in the ZD1 biomass (i.e., P60-ZD1 and P75-ZD1), the concentration of KNO3 in MSM was adjusted to create either a nitrogen (N)-balanced condition (C/N = 4; 60% PHB in DCW) or a N-limited condition to enhance PHB accumulation (C/N = 21.5; 75% PHB in DCW).1 The pH value of the medium was originally adjusted to 7.5. The flasks were incubated at 30 °C under 150 rpm, and samples were periodically collected to monitor the ZD1 growth. Liquid samples were collected at the stationary growth phase to quantify cell dry weight (CDW) and PHB content.

Preparation of Chitosan-Harvested PHB-Rich ZD1

The harvest of PHB-rich ZD1 at the stationary phase was carried out by adding chitosan as a coagulant agent to produce chitosan-harvested PHB-rich ZD1 (CP-ZD1) based on optimized conditions and operations previously conducted.[2] Briefly, 50 mg/L of medium M_w chitosan (dissolved in 1% v/v acetic acid) was added to the cell suspension and pH was adjusted to 9. The mixtures were then placed on a rotary shaker at 150 rpm for 2 min, followed by a slow mixing at 40 rpm for 15 min, before settling for 20 min without disturbance. The precipitates (i.e., chitosan-harvested PHB-rich ZD1 biomass) were collected and desiccated at 105°C. The process is

illustrated in Figure S4.4 in the Supporting Information. Non-chitosan-harvested PHB-rich ZD1 biomass was also prepared by collecting the biomass through a centrifuge (4,500 × g for 10 min at 4°C) and desiccated at 105°C.

Axenic Hatching of *Artemia*

Gnotobiotic *Artemia* nauplii were prepared as previously described,[3, 4] with a minor modification. Briefly, 50 mg of cysts were hydrated in 18-mL sterilized DI water for 30 min with moderate 0.2 μm-filtered aeration. Then, sterile decapsulation of hydrated cysts was achieved by adding 660-μL NaOH (32% w/v) and 10-mL NaOCl (13% active chlorine). After 3 min, decapsulation was stopped by adding 14-mL $Na_2S_2O_3$ (10 g/L) and then immediately washed in filtered autoclaved artificial seawater (FASW) containing 35-g/L sea salt (Instant Ocean, USA). The cysts were then resuspended in a 50-mL tube containing 30-mL FASW (pH = 7.5) and placed on a rotary (4 cycles/min) at room temperature for 30 h with constant illumination to hatch. After 30 h of incubation, *Artemia* instar II nauplii were harvested, washed with FASW, and used for all *Artemia* challenge tests.

Analysis of Immune-Related Genes Expression in *Artemia*

The expression of immune-related genes (heat shock protein (*hsp70*), ferritin (*ftn*), and peroxinectin (*pxn*)) and housekeeping gene (*β-actin*) in *Artemia* receiving different treatments was assessed using quantitative real time-polymerase chain reaction (RT-qPCR) as described previously.[5, 6] Table S4.1 lists the primer sets used for RT-qPCR. Briefly, *Artemia* samples (~60 mg) from pathogen challenge tests were homogenized in RNA lysis buffer using a pestle for 1.5-mL microcentrifuge tubes and stored at -80°C before RNA extraction. Total RNA was extracted

using the SV Total RNA Isolation Kit (Promega, USA). The total RNA concentration was determined using a NanoDrop Lite spectrophotometer (Thermo Fisher, USA), and the quality was checked by PCR/gel electrophoresis for any DNA contamination. Then, cDNA was synthesized using OneStep Ahead RT-PCR Kit (QIAGEN, Germany). PCR amplification was conducted in a 20-μL reaction mixture containing 1-μL cDNA template (diluted ×100), 0.5 μL each of forward and reverse primer, 10-μL Power SYBR Green Master Mix (Thermo Fisher, USA), and 8-μL nuclease-free water. RT-qPCR was conducted using IQ5 multicolor real-time PCR detection system (Bio-Rad, USA) with the following amplification conditions: 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. All reactions were performed in triplicate. The relative expression ratio of the target genes versus *β-actin* was calculated using the $2^{-\Delta\Delta CT}$ method. The expressions of target genes in the negative control (i.e., unchallenged *Artemia*) were used as references and regarded as 1, and all other data were normalized accordingly.

Analysis of *Artemia* Gut Microbiome

Artemia samples collected from the starvation challenge tests were transferred into ethanol and stored at 4°C before DNA extraction. Because the *Artemia* nauplii were too small to remove the intestinal tract, whole *Artemia* samples were homogenized using a pestle for 1.5-mL microcentrifuge tubes and then DNA extracted by DNeasy Blood and Tissue Kit (QIAGEN, Germany), according to the manufacturer's instructions. Triplicates were pooled together to prepare one corresponding DNA extract sample. NanoDrop Lite spectrophotometer was used to measure the concentration and quality of the extracted DNA. The DNA extracts were stored at -20°C before further processing. The extracts were used as templates for sequencing the V3–4 region with universal primers (341F/785R) on the Illumina MiSeq platform (Texas A&M Institute for Genome Sciences and Society, USA) using the bulk 2 × 300 bp paired-end sequencing strategy.

Furthermore, the abundance of *Vibrio spp.* in *Artemia* in relation to total bacteria was determined with specific primer sets (Table S4.1) by qPCR as previously described.[7]

The obtained sequences were processed using QIIME2 version 2021.4.[8] Briefly, the paired-end sequences were merged with VSEARCH and denoised through DADA2, which generated high-resolution amplicon sequence variants (ASV).[9, 10] Low sequence depths (i.e., < 1,000 reads per sample) were excluded from downstream analysis. Taxa was assigned from the SILVA 138 database, generated with a 99% identity criterion, using a trained naive Bayes classifier with the aforementioned primers.[11, 12] Phylogenetic trees were constructed using ARB with the same database used for taxonomy assignment through QIIME2.[13] Sequences were added with parsimony, and the neighbor-joining method was used for tree construction. The abundance of PHB or chitosan degradation genes from the retrieved amplicon sequence variants (ASVs) in *Artemia*'s gut microbiome were identified and predicted using Tax4Fun2 version 1.1.5.[14] The NCBI RefSeq 99% similarity threshold genome dataset generated with UCLUST[15] was used as the reference. The raw sequences were deposited in NCBI Sequence Read Archive under BioProject number PRJNA765685.

Physicochemical Analyses

All bacterial growths were determined by OD_{600} using a UV–visible scanning spectrophotometer (VWR, 3100 PC). The cell dry weight (CDW) of ZD1 was determined after centrifuging the collected sample at the stationary phase (4,500 × g for 10 min at 4°C). Subsequently, the pellets were washed with DI water, centrifuged, and desiccated at 105°C. The cell concentration was determined by dividing the weight of the dried pellet by the sample volume. The PHB content in CDW was spectrophotometrically determined via conversion of PHB to crotonic acid.[1, 16] Briefly, the dried biomass was digested with 1 mL of H_2SO_4 at 70°C for 4 h and

then neutralized by adding 7 mL of 4 M NaOH. The digested solution was 0.45-μm filtered, and crotonic acid was determined at 235-nm against pure PHB standards treated alike. In parallel, a gas chromatography-flame ionization detector, GC-FID (Model 6890N, Agilent, USA) equipped with a DuraGuard J&W DB-5ms column (30 m, 0.25 mm, 0.25 μm) was used to identify and quantify PHB following a previously developed method.[17] Briefly, 30 mg of CDW was subjected to methanolysis by reacting with 2 mL chloroform and 2 mL methanol (containing 15% v/v H_2SO_4) in a heating block at 100°C for 4 h. After cooling, 1 mL of DI water was added, and the mixture was vigorously shaken to separate and collect the bottom organic phase. 1 μl of the 0.2-μm filtered organic phase was automatically injected in the GC-FID (80°C for 4 min; temperature ramp of 8°C/min; 160°C for 6 min; and temperature ramp of 25°C/min; 200°C for 1 min). The temperatures of the injector and detector were 250 and 280°C, respectively. The concentration of PHB was calculated by comparing peak areas to the standard of known concentrations. For microscopic images of *Artemia*, samples were collected and examined with an Amscope T720 microscope equipped with an Amscope MU130 camera.

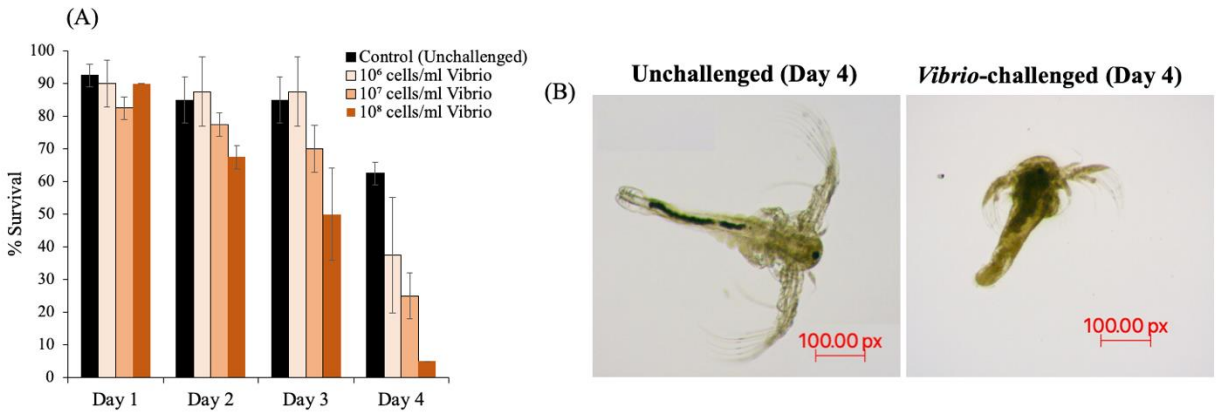

Figure S4.1. (A) Survival of *Artemia* challenged with various concentrations of live *V. campbellii* (10^6–10^8 cells/ml). (B) Representative microscopic images of *Artemia* after four days of challenge. The bars represent ranges of duplicate samples.

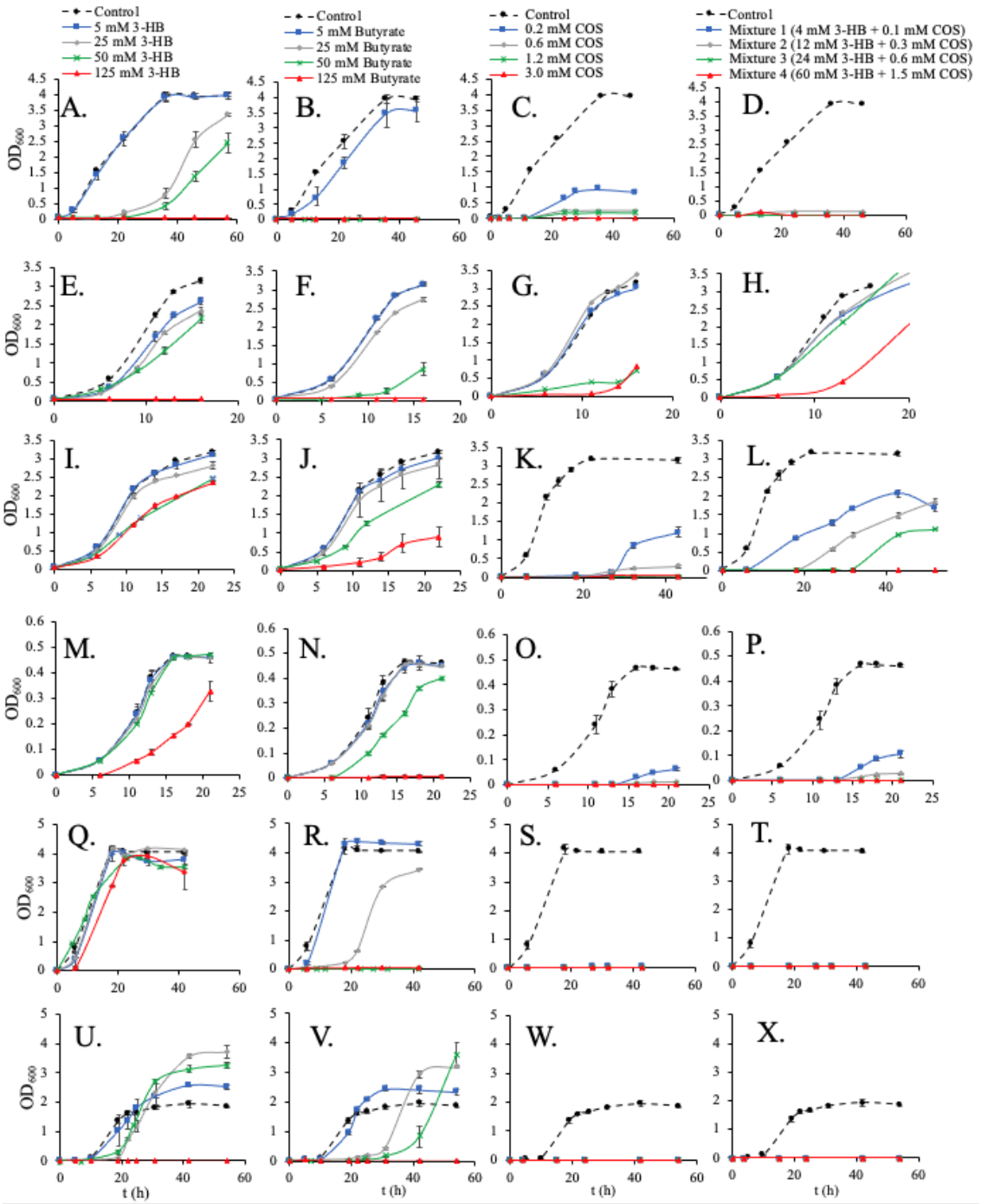

Figure S4.2. The growth curves of Gram-negative strains: *V. campbellii* (A–D), *A. hydrophila* (E–H), and *E. coli* (I-L) and Gram-positive strains: *S. agalactiae* (M–P), *B. megaterium* (Q–T), and *R. jostii* RHA1 (U-X) incubated in liquid media with various concentrations of 3-hydroxybutyrate (3-HB), butyrate, chitosan oligosaccharides (COS), and Mixtures 1–4 of 3-HB + COS. Strains cultivated without PHB or chitosan intermediates were used as controls. The bars represent ranges of duplicate samples.

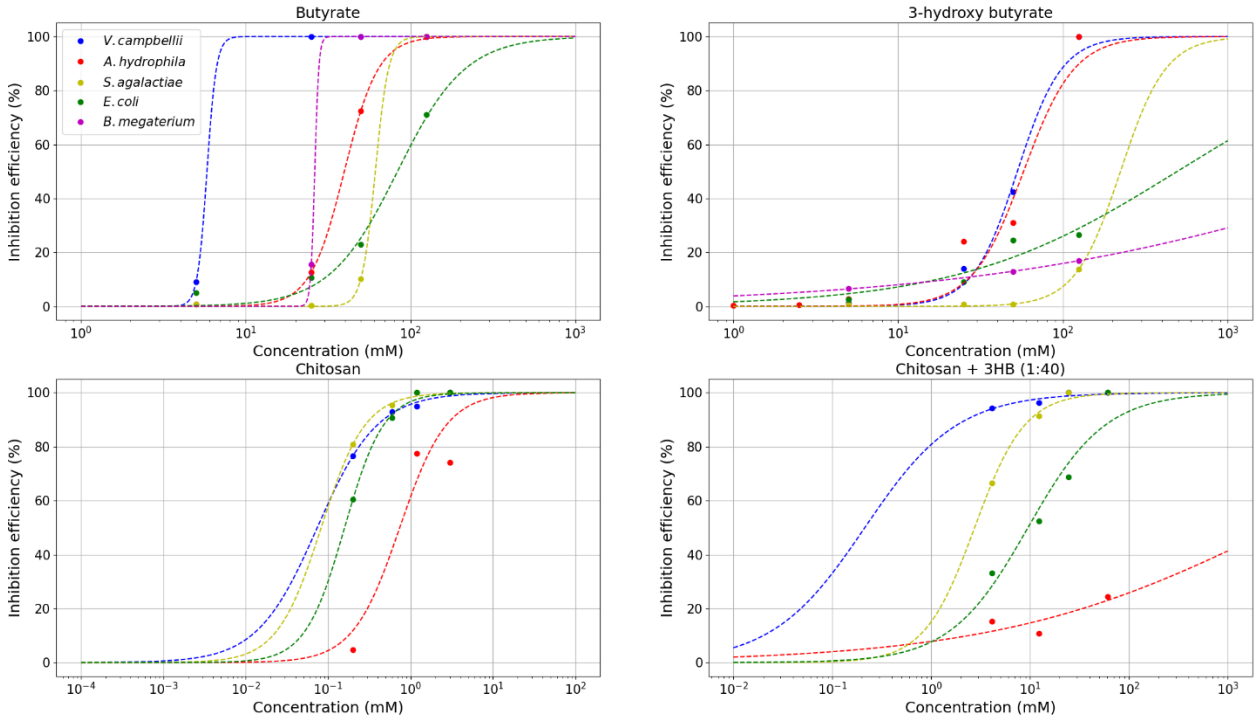

Figure S4.3. Inhibition efficiency curves (fitted through 4-parameter logistic model) of 3-hydroxybutyrate (3-HB), butyrate, chitosan oligosaccharides (COS), and Mixtures 1–4 of 3-HB + COS against Gram-negative and Gram-positive bacterial strains. Each treatment was conducted in duplicate. Average values are shown with ranges of duplicate samples.

Figure S4.4. Chitosan-harvested PHB-rich ZD1 biomass by adding 50 mg/L of medium M_w chitosan to the cultivation medium.

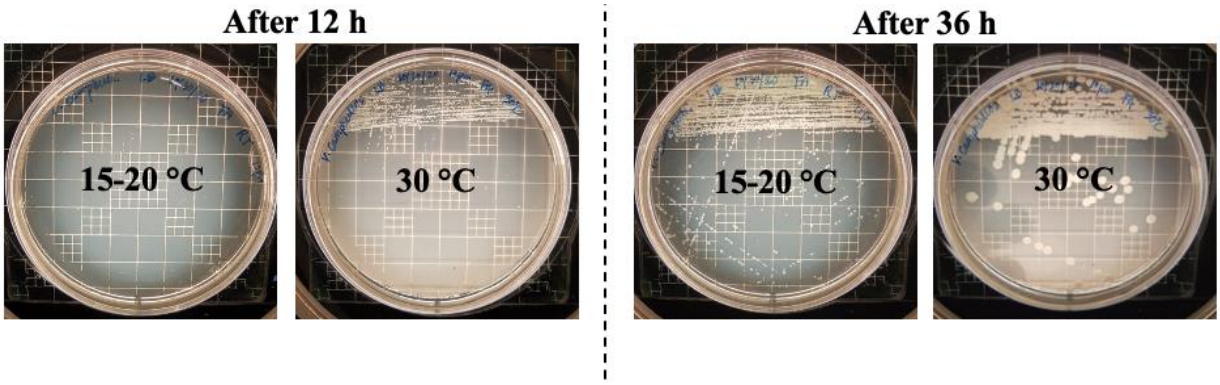

Figure S4.5. Colony formation of *V. campbellii* in LB agar after 12 and 24 h of incubation at room temperature (15–20°C) or 30°C. Pictures shown were representatives of three replicates.

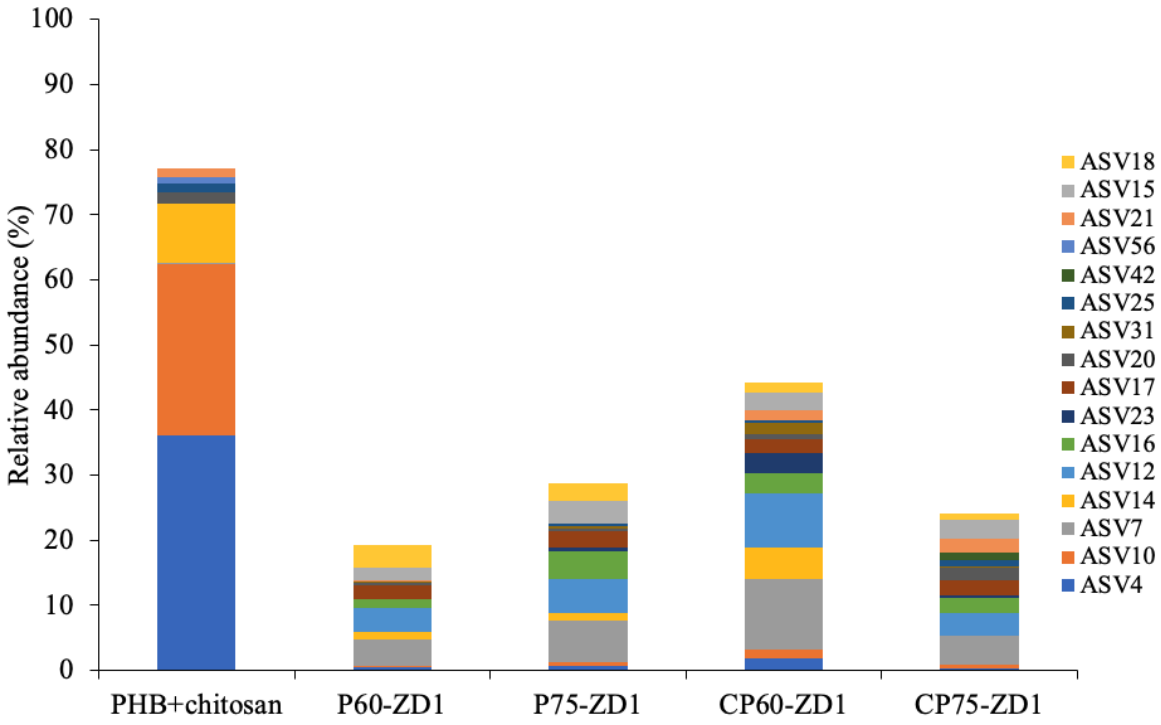

Figure S4.6. Relative abundance of 16 *Bacillus* spp. (>1% within at least one sample of the five samples) associated ASV level sequences.

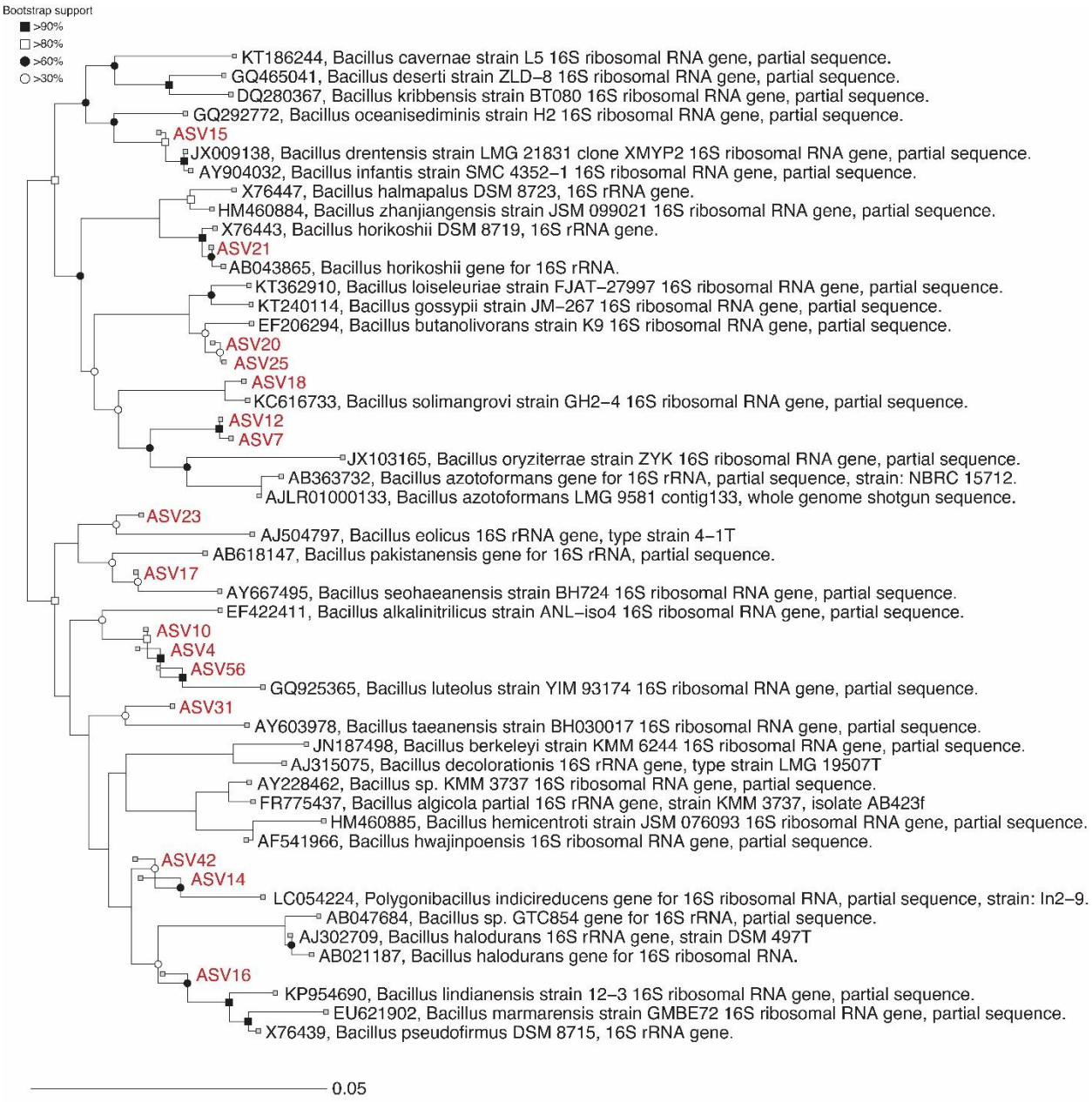

Figure S4.7. Phylogenetic tree of 16 *Bacillus* spp. relevant ASVs and closely associated known *Bacillus* spp.

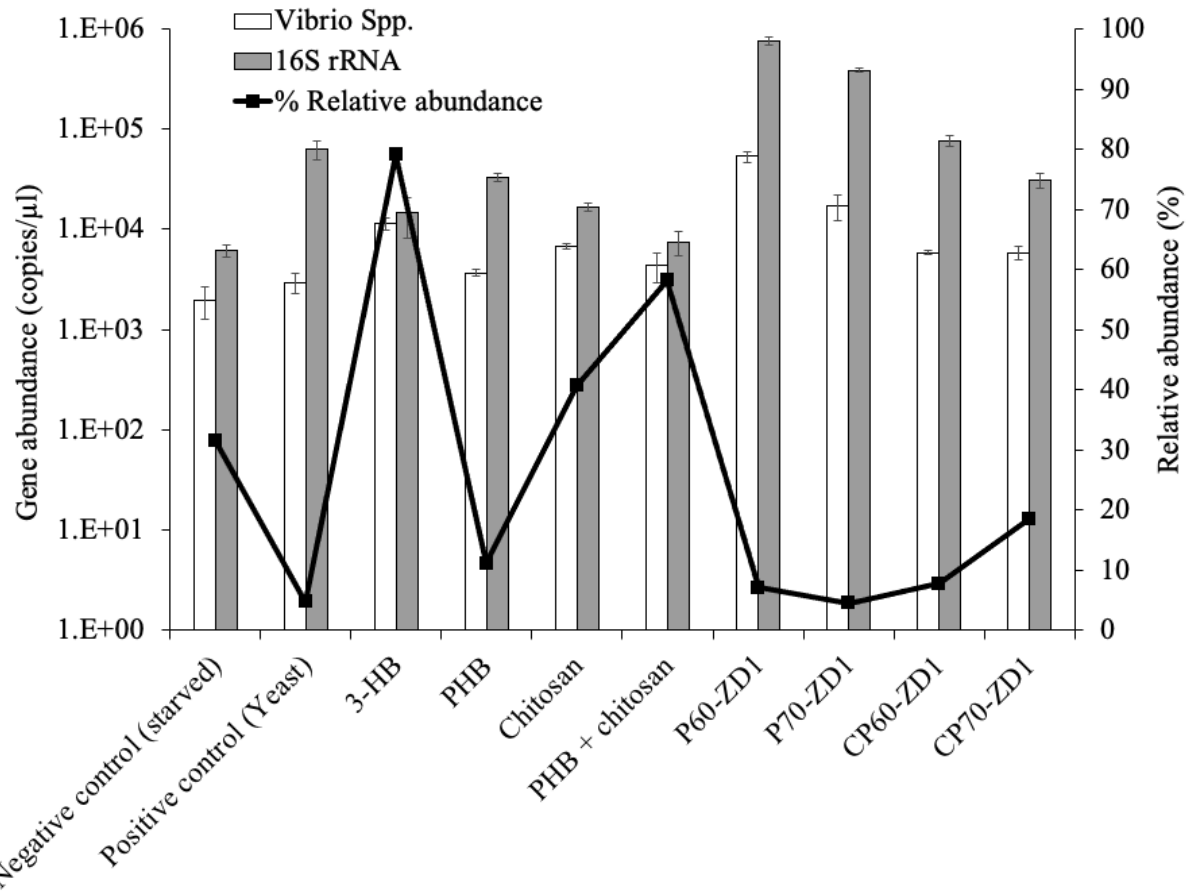

Figure S4.8. Concentrations and relative abundance determined by qPCR of *Vibrio* spp. in *Artemia* samples fed with different treatments in relevant to total bacteria based on 16S rRNA. The bars represent ranges of triplicate samples.

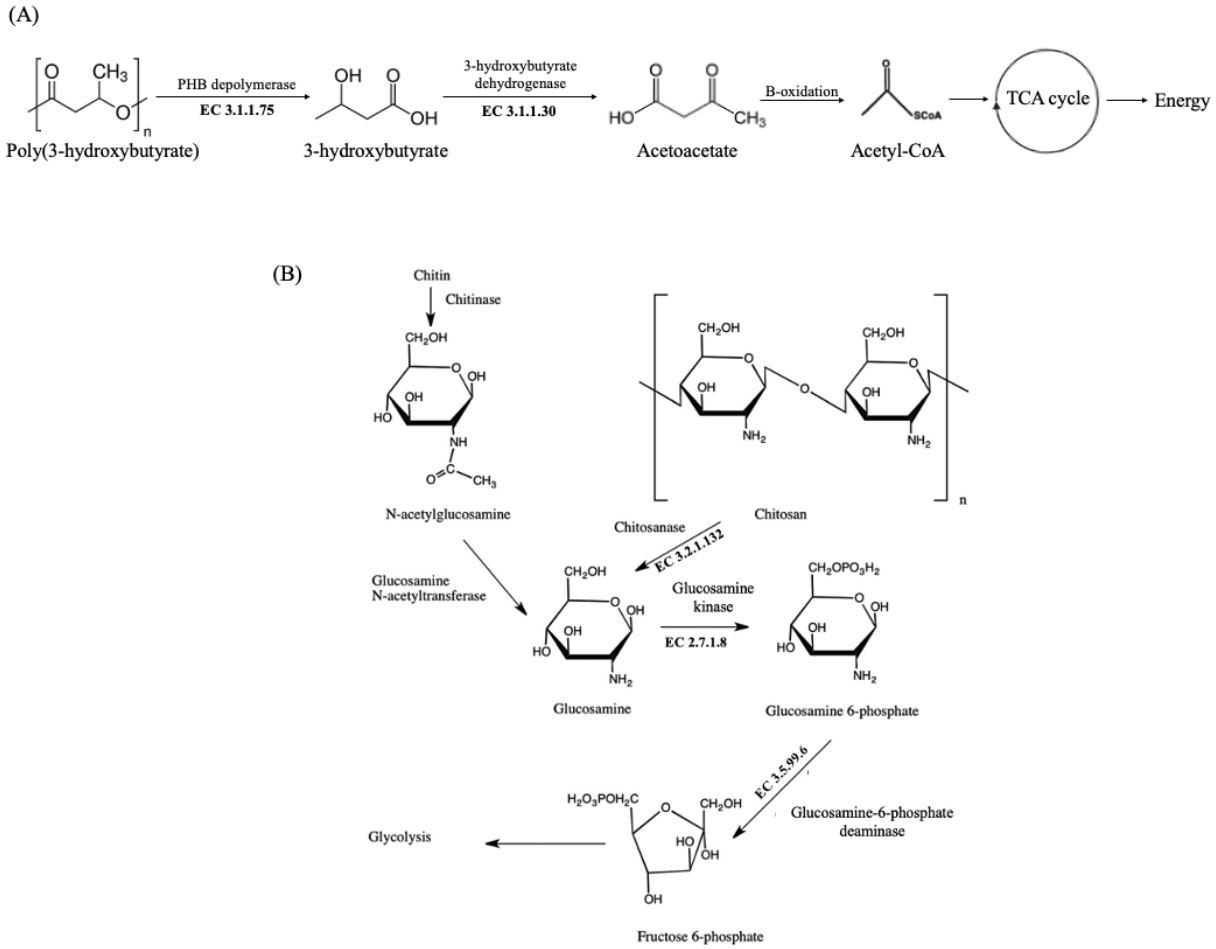

Figure S4.9. Degradation pathways with specific enzymes of (A) Poly(3-hydroxybutyrate) (PHB) (adapted from Hankermeyer and Tjeerdema 1999,[18] Singh et al. 2017,[19] and Roohi et al. 2018[20]). (B) Chitin and chitosan (modified from Yan and Fong 2015).[21]

Table S4.1. Primer sets used for immune response analysis in *Artemia* by RT-qPCR.[6] Heat shock protein 70 (*hsp70*), ferritin (*ftn*), peroxinectin (*pxn*), and *β-actin*. Primer sets used for relative abundance of *Vibrio spp*. and total bacteria by qPCR.[7]

Gene	Primer	Sequence (5'-3')
hsp70	Forward	cgataaaggccgtctctcca
	Reverse	cagcttcaggtaacttgtccttg
ftn	Forward	tccaaggcttatccgatgaaca
	Reverse	atgaccaagtgagtgcttctct
pxn	Forward	gagctaccgatgaagatccag
	Reverse	cgtttcctgaacagcgaataaa
β-actin	Forward	agcggttgccatttcttgtt
	Reverse	ggtcgtgacttgacggactatct
Vibrio spp. 16S rRNA	Forward	cggtgaaatgcgtagaga
	Reverse	ttactagcgattccgagttc
Total bacteria 16S rRNA	Forward	atggctgtcgtcagct
	Reverse	acgggcggtgtgtac

Table S4.2. Inhibition efficiencies of 3-HB, butyrate, COS, and 3-HB + COS against various Gram-negative and Gram-positive bacterial strains.

		Inhibition Efficiency (%)[a]					
		Gram-negative strains			Gram-positive strains		
Compound	Concentration (mM)	*V. campbellii*	*A. hydrophila*	*E. coli*	*S. agalactiae*	*B. megaterium*	*R. jostii* RHA1
3-HB	0 (Control)	0	0	0	0	0	0
	5	1.27 ± 1.07	2.7 ± 1.26	2.68 ± 0.67	0.75 ± 0.01	6.52 ± 3.48	-32.3 ± 2.20
	25	14.0 ± 1.78	24.1 ± 2.70	9.15 ± 3.12	0.75 ± 0.03	-1.35 ± 1.04	-87.3 ± 4.00
	50	42.4 ± 5.88	31.1 ± 3.59	24.5 ± 2.23	0.75 ± 0.01	12.9 ± 0.94	-61.7 ± 6.58
	125	100	100	26.5 ± 1.34	13.8 ± 0.15	17.0 ± 14.4	100
Butyrate	0 (Control)	0	0	0	0	0	0
	5	9.14 ± 8.90	0.80 ± 0.22	5.0 ± 0.89	0.75 ± 0.01	-5.80 ± 1.74[b]	-25.2 ± 4.93
	25	100	12.7 ± 1.80	10.7 ± 12.0	0.43 ± 0.15	15.5 ± 1.56	-70.8 ± 2.65
	50	100	72.4 ± 5.40	23.0 ± 6.24	10.2 ± 1.83	100	-92.2 ± 21.6
	125	100	100	71.1 ± 8.25	100	100	100
COS	0 (Control)	0	0	0	0	0	0
	0.2	76.5 ± 1.13	4.81 ± 0.11	60.6 ± 4.56	80.9 ± 3.20	100	100
	0.6	93.0 ± 1.10	-7.30 ± 0.40	90.6 ± 0.91	95.4 ± 0.01	100	100
	1.2	95.0 ± 0.23	77.6 ± 0.28	100	100	100	100
	3	100	74.2 ± 0.61	100	100	100	100
3-HB + COS	0 (Control)	0	0	0	0	0	0
Mixture 1	0.1 + 4	94.2 ± 0.18	15.2 ± 1.39	33.2 ± 3.65	66.5 ± 5.60	100	100
Mixture 2	0.3 + 12	96.4 ± 0.17	10.7 ± 4.84	52.4 ± 2.50	91.4 ± 0.03	100	100
Mixture 3	0.6 + 24	100	-10.5 ± 0.70	68.7 ± 0.91	100	100	100
Mixture 4	1.5 + 60	100	24.5 ± 3.14	100	100	100	100

[a]% Inhibition efficiencies were determined using Eq. (1) that considers the highest optical densities in relevant to the control (i.e., strains cultivated without PHB or chitosan intermediates). Data were presented (average ± SD) from duplicate measurements. [b]Negative efficiency means that the strain grew more than the control. 3-HB = 3-hydroxybutyrate; COS = chitosan oligosaccharides.

Table S4.3. Diversity indices of gut microbiome of *Artemia* fed with different treatments at 20,000 sequence depth.

Feeding type	Simpson	Shannon	Chao1	Faith PD
PHB + chitosan	0.79	3.31	69.00	7.08
P60-ZD1	0.94	4.50	56.90	3.08
P75-ZD1	0.95	4.70	74.15	3.96
CP60-ZD1	0.95	4.78	72.05	4.97
CP75-ZD1	0.94	4.61	73.37	4.66

Table S4.4. Relative abundance (%) of total G+, G-, Gram-variable, and unknown bacterial populations.

Feeding type	Gram-positive bacteria	Gram-negative bacteria	Gram-variable bacteria[a]	Unknown
PHB + chitosan	86.1	7.64	6.15	0.11
P60-ZD1	99.5	0.51	0	0
P75-ZD1	99.8	0.21	0	0
CP60-ZD1	98.6	1.30	0.09	0.01
CP75-ZD1	98.9	0.96	0.17	0.01

[a]Four ASVs associated with Gram-variable bacteria belong to *Paenibacillus uliginis.*

Table S4.5. Overview of published studies on the impact of PHB on the microbial community in different aquaculture animals.

Aquatic Animal	Technique	Main Findings	Phylum	Class	Order	Genus	Species	References
Siberian sturgeon, European sea bass, and giant river prawn	- PCR targeting 16S rRNA - DNA Sequencing and BLAST	- Isolated six PHB degrading bacteria from a gastrointestinal environment					- Closely related to *Acidovorax* spp., *Acinetobacter* spp., and *Ochrobactrum* spp.	[22]Liu et al. 2010
European sea bass	- PCR-DGGE	- PHB induced bacterial richness and larger changes in the bacterial community						[23]De Schryver et al. 2010
Siberian sturgeon	- PCR-DGGE - CLPPS	- PHB improved the intestinal microbial species richness and diversity. - PHB increased aerobic metabolism of culturable bacteria in the GI tract				- PHB stimulated *Bacillus* and *Ruminococcaceae*		[24]Najdegerami et al. 2012

Table S4.5. Continued.

Aquatic Animal	Technique	Main Findings	Phylum	Class	Order	Genus	Species	References
Chinese mitten crab	- PCR-DGGE - DNA Sequencing and BLAST	- PHB enhanced richness, diversity, and evenness of gut microbiome - A total of 165 bands (presumed species) were observed					- Similarities with uncultured bacterium from Chinese mitten crab, black tiger shrimp, and white-leg shrimp	[25]Sui et al. 2016
European sea bass	- Illumina-NGS	- High PHB from mouth had the highest proportion of *Firmicutes*. - *Firmicutes* includes a variety of probiotic bacteria such as *Bacillus* spp. - PHB did not affect OTUs, richness, evenness, and diversity	- *Proteobacteria* and *Bacteroidetes* were > 92.4% except for low PHB from mouth (74.5%) - *Firmicutes* were increased to 1.3-2.5% in high/low PHB from mouth					[26]Franke et al. 2017

Table S4.5. Continued.

Aquatic Animal	Technique	Main Findings	Phylum	Class	Order	Genus	Species	References
Pacific white shrimp	- Illumina-NGS	- PHB altered the composition and diversity of intestine microbiota - Increasing PHB doses decreased the diversity	- PHB increased *Proteobacteria*, *Tenericutes*, and *Bacteroidetes* - PHB decreased *Planctomycetes*, *Actinobacteria* and *Verrucomicrobia*	- PHB increased *Gammaproteobacteria* and decreased *Alphaproteobacteria*		- PHB increased beneficial bacteria *Bacillus*, *Lactobacillus*, *Lactococcus*, *Paenibacillus*, and *Bdellovibrio* - PHB (3-5%) increased *Ruminococcus*, *Brevibacterium*, and *Clostridium*		[27]Duan et al. 2017
Pacific white shrimp	- Illumina-NGS	PHB-rich *Halomonas* suppressed *Vibrio* and increased biodiversity and probiotic *Bacillus* and *Lactobacillus*	- Dominated by Proteobacteria, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*			- *Halomonas* was existed in PHB-rich/free HM unchallenged groups. - In challenged, *Bacillus* and *Lactobacillus* in amorphous PHB >> Control.		[28]Gao et al. 2019

Table S4.5. Continued.

Aquatic Animal	Technique	Main Findings	Phylum	Class	Order	Genus	Species	References
Sea Cucumber	- High-throughput 16S rRNA sequencing - Shotgun metagenome	- Rhodobacterales retaining PHB metabolism genes might contribute to the production of larger individuals - Abundance of microbiome retaining PHB metabolism genes in the largest individuals - 67% of total prokaryotic reads annotated to *pha*ABC genes were affiliated to *Rhodobacterales*, in accordance with sequencing data, where higher abundance of *Rhodobacterales* in the larger individuals	- Insignificant difference in abundance of *Proteobacteria* and *Bacteroidetes* between larger and smaller individuals. - Minor phyla (*Actinobacteria*, *Firmicutes*, *Fusobacteria*, *Spirochaetes*) were significantly different.	- *Alphaproteobacteria* and *Deltaproteobacteria* were significantly different between larger and smaller individuals	- *Rhodobacterales*, *Desulfobacterales*, and *Oceanospirillales* were significantly abundant in larger individuals - *Marinicellales* and *Acidimicrobiales* were more abundant in smaller individuals			[29]Yamazaki et al. 2016

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APPENDIX C.

SUPORTING INFORAMATION FOR CHAPTER V

Supporting Information: 9 pages total including 5 figures and 2 tables

Table of Content

List of Figures

List of Tables

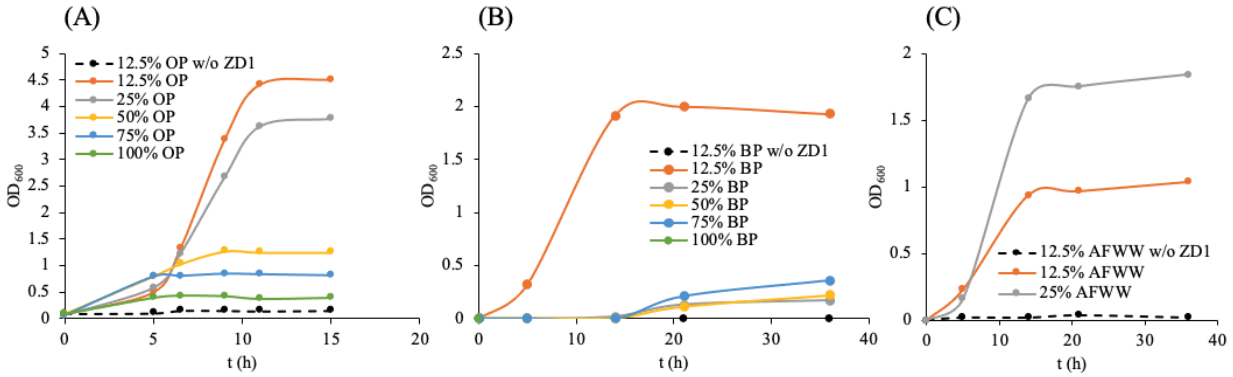

Figure S5.1. Growth of ZD1 in different concentrations of (A) banana peels (BP), (B) orange peels (OP), and (C) anchovy fishmeal wastewater (AFWW).

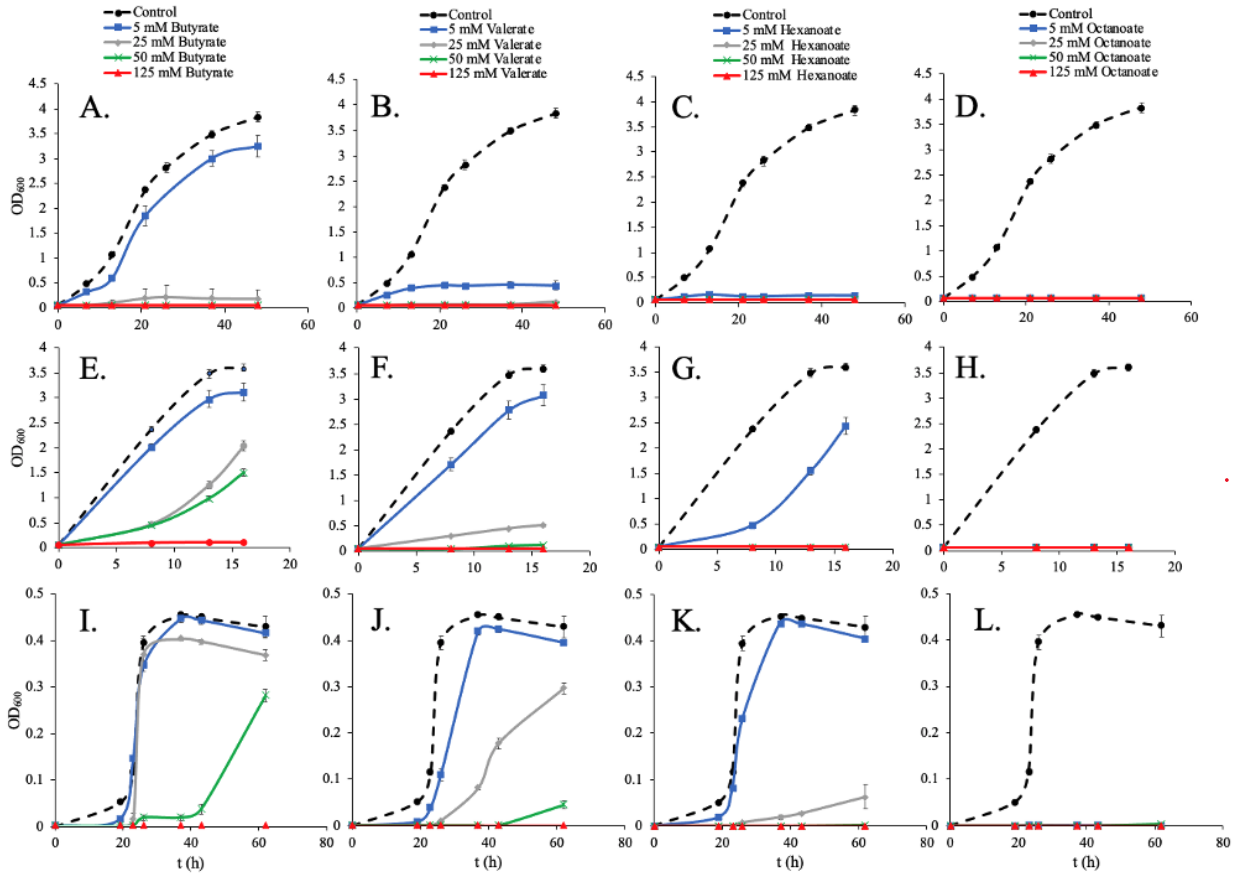

Figure S5.2. The growth curves of aquaculture pathogens: gram-negative *V. campbellii* (A–D), *A. hydrophila* (E–H), and gram-positive *S. agalactiae* (I–L) incubated in liquid media with 5–125 mM of SCFAs (C-4 butyrate and C-5 valerate) and MCFAs (C-6 hexanoate and C-8 octanoate). Strains cultivated without PHA intermediates were used as controls.

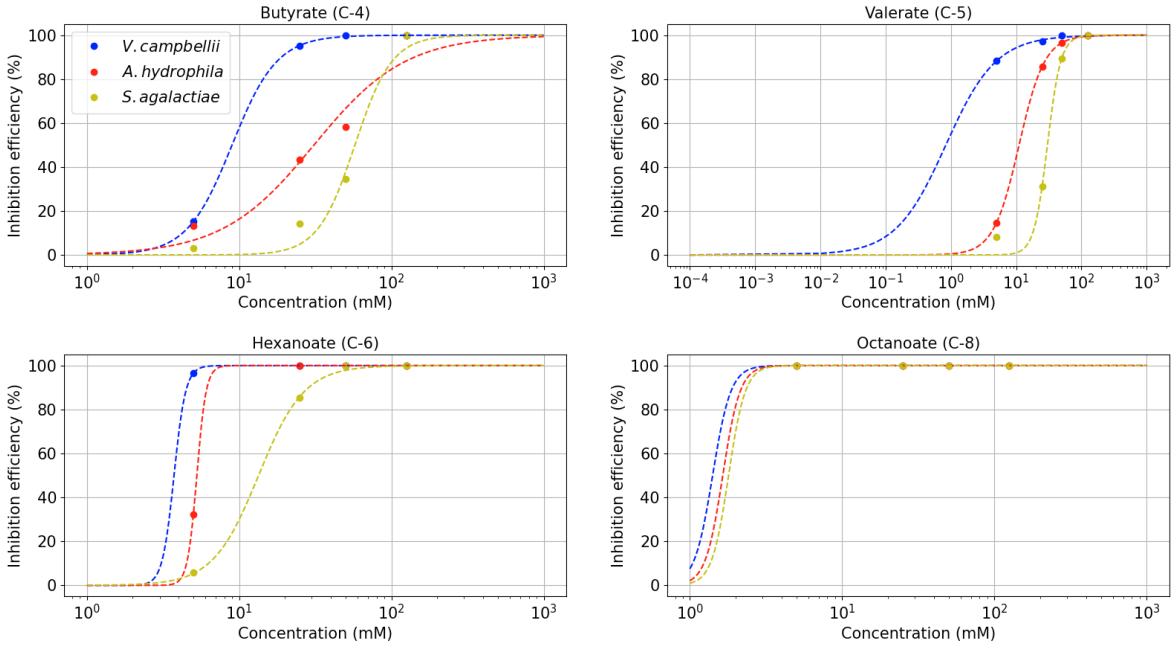

Figure S5.3. Inhibition efficiency curves (fitted through 4-parameter logistic model) of various SCL- and MCL-PHA intermediates (C-4 butyrate, C-5 valerate, C-6 hexanoate, and C-8 octanoate) against common aquaculture pathogens. Note: The inhibition efficiencies for octanoate against pathogens were assumed close to 100% to be able to visualize the inhibition curves since the strains have already exhibited full inhibition at the lowest compounds concentration.

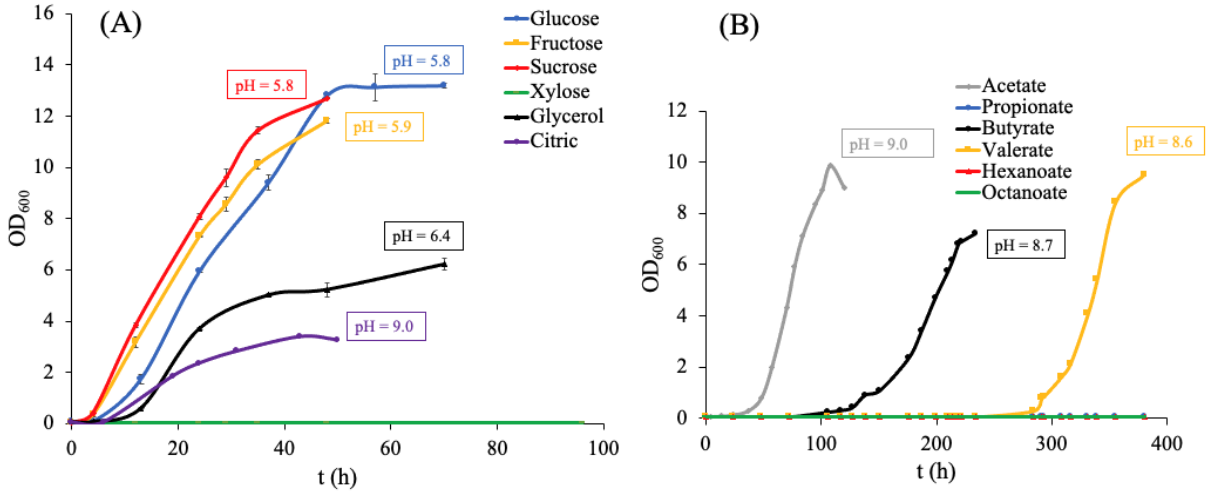

Figure S5.4. Growth curves and final pH values of ZD1 grown on different pure organic compounds such as (A) sugars (glucose, fructose, sucrose, and xylose), glycerol, and citric acid and (B) SCFAs (acetate, propionate, butyrate, and valerate) and MCFAs (hexanoate and octanoate).

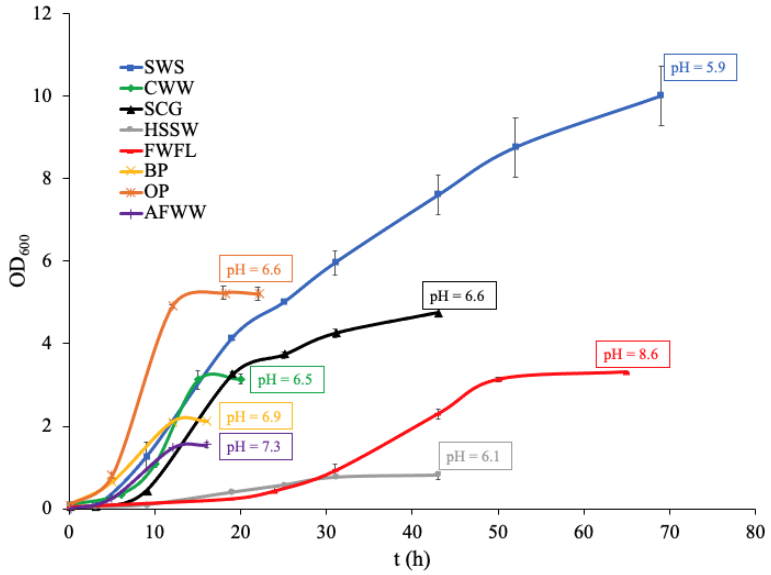

Figure S5.5. Growth curves and final pH values of ZD1 grown on different agro-industrial wastes/wastewaters such as sugary waste slurry (SWS), cheese whey wastewater (CWW), synthetic crude glycerol (SCG), high-strength wastewater (HSSW), food waste fermentation liquid (FWFL), banana peels (BP), orange peels (OP), and anchovy fishmeal wastewater (AFWW).

Table S5.1. Characteristics of agro-industrial wastes/wastewaters used for the cultivation of Z. denitrificans ZD1 for PHA accumulation.

Organic Waste	COD (g/L)	TN (g-N/L)	Salinity (g/L)	pH
SWS	N.A.[a]	-	-	-
CWW	50.4	1.47	0	6.5
SCG	18.3	0.26	30	7.5
HSSW	18.3	0.42	30	7.5
FWFL	18.3	0.26	30	7.5
BP	22.5		-	4.7
OP	53.2		-	5.4
AFWW	35.4		-	5.9

[a]N.A. = not applicable. SWS is a solid waste with 1 g of SWS was equivalent to 1 g of COD. SWS = sugary waste slurry; CWW = cheese whey wastewater; SCG = synthetic crude glycerol; HSSW = high-strength wastewater; FWFL = food waste fermentation liquid; BP = banana peels; OP = orange peels; AFWW = anchovy fishmeal wastewater

Table S5.2. Inhibition efficiencies of SCL- and MCL-PHA intermediates/fatty acids against common aquaculture pathogens.

Compound	Concentration (mM)	Inhibition Efficiency (%)[a]		
		V. campbellii	*A. hydrophila*	*S. agalactiae*
Butyrate	0 (Control)	0	0	0
	5	15.2 ± 5.6	13.3 ± 5.1	3.14 ± 1.9
	25	95.2 ± 4.7	43.4 ± 2.8	14.3 ± 2.6
	50	100	58.3 ± 1.9	34.5 ± 3.3
	125	100	100	100
Valerate	0 (Control)	0	0	0
	5	88.3 ± 2.2	14.7 ± 5.8	8.0 ± 1.0
	25	97.1 ± 0.4	85.7 ± 0.6	31.1 ± 2.6
	50	100	96.5 ± 0.2	89.5 ± 1.9
	125	100	100	100
Hexanoate	0 (Control)	0	0	0
	5	96.5 ± 0.2	32.3 ± 4.8	5.8 ± 1.5
	25	100	100	85.4 ± 6.1
	50	100	100	100
	125	100	100	100
Octanoate	0 (Control)	0	0	0
	5	100	100	100
	25	100	100	100
	50	100	100	100
	125	100	100	100

[a]% Inhibition efficiencies were determined using Eq. (1) that considers the highest optical densities in relevant to the control (i.e., growth medium without PHA intermediates). Data were presented (average ± SD) from duplicate measurements.