SURVIVAL IN *PARVOCALANUS CRASSIROSTRIS* CULTURES TREATED WITH COMMERCIAL PROBIOTICS

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ABSTRACT

The production of copepod nauplii is essential for the first feeding of many marine fish species. Nauplii production remains a bottleneck to production of marine ornamental and food fish species. To the best of our knowledge, survival from nauplii to adult for *Parvocalanus crassirostris* has not been documented in the literature. Researchers at the Oceanic Institute in Oahu, Hawai'i have been rearing *Parvocalanus crassirostris* for approximately a decade. These researchers report that nauplii survival to adult is generally less than 50%, and the mechanisms contributing to variability in copepod production remain unclear. Pathogenic bacteria can negatively affect survival across species, raising the question of whether reducing bacterial loads can improve survival. In this study, we compared the survival of *Parvocalanus crassirostris* treated with two commercial probiotic preparations (INVE Sanolife ™ MIC and MIC-F) that were reported as being beneficial in shrimp culture and a control to determine if probiotic treatments affect nauplii survival to adult. Following the doses recommended by the manufacturers, these probiotics had no significant differences on nauplii survival to adult compared to the control group (n = 3). The causes of high mortality and variable copepod survival remain unknown and future studies should focus on understanding these causes.

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1. INTRODUCTION

1.1 Culturing live feeds for aquaculture

Aquaculture is a diverse sector that encompasses the farming of hundreds of species of animals and plants in various aquatic environments (Lucas 2015). Within this broad category, marine ornamental aquaculture is the sector which includes the rearing of both freshwater and seawater species for home and public aquaria. Research to develop technologies and methodologies for closing the life cycle of marine ornamental species is a requisite to achieving commercial successes in the marine ornamental industry. One of the challenges to achieving commercial success is rearing live prey items (also known as live feeds) that are required by most pelagic marine ornamental fish larvae due to behavioral traits and physiological needs.

Live feeds offer a multitude of benefits to larval fish, such as: 1) the ability to elicit a feeding response; 2) the ability to be size sorted to provide an ideal consumable size; and 3) providing fish larvae with the digestive enzymes and essential fatty acids required for growth and survival (Buskey 2005; Conceição et al. 2010; Olivotto et al. 2011; Garcia-Ortega et al. 2013). There are many types of live feeds that have been utilized in aquaculture including microalgae such as *Isochrysis galbana* and *Nannochloropsis occulata*, and many types of zooplankton such as *Artemia* spp. (brine shrimp), rotifers, and copepods (Conceição et al. 2010; Olivotto et al. 2017). Fish hatcheries, especially those specialized in rearing freshwater species, have typically raised live feeds such as *Artemia* and rotifers. However, *Artemia* range in length from 100-340 μm, and rotifers range in width from 50–200 μm, these are too large for the majority of newly hatched pelagic marine ornamental larvae (Lavens & Sorgeloos 1996; Conceição et al. 2010). Marine ornamental larvae body and mouth gape are highly variable among species, and thus far, the most widely accepted prey items that meet the criteria for first feeding are copepod nauplii, which can be as small as 50 μm in width (Lavens & Sorgeloos 1996; Lee et al. 2005). This is primarily because *Artemia* and rotifers are generally too large to be utilized as a first feed; however, they can be introduced to fish larvae at later stages (DiMaggio et al. 2017; Callan et al. 2018). Therefore, a typical feeding regime for marine ornamental larvae starts with copepod nauplii as a first feed, then progresses to rotifers, and finally, *Artemia* before transitioning to

inert feeds such as weaning diets/pellets and frozen food items (Figure 1; Modified from Callan et al. 2018).

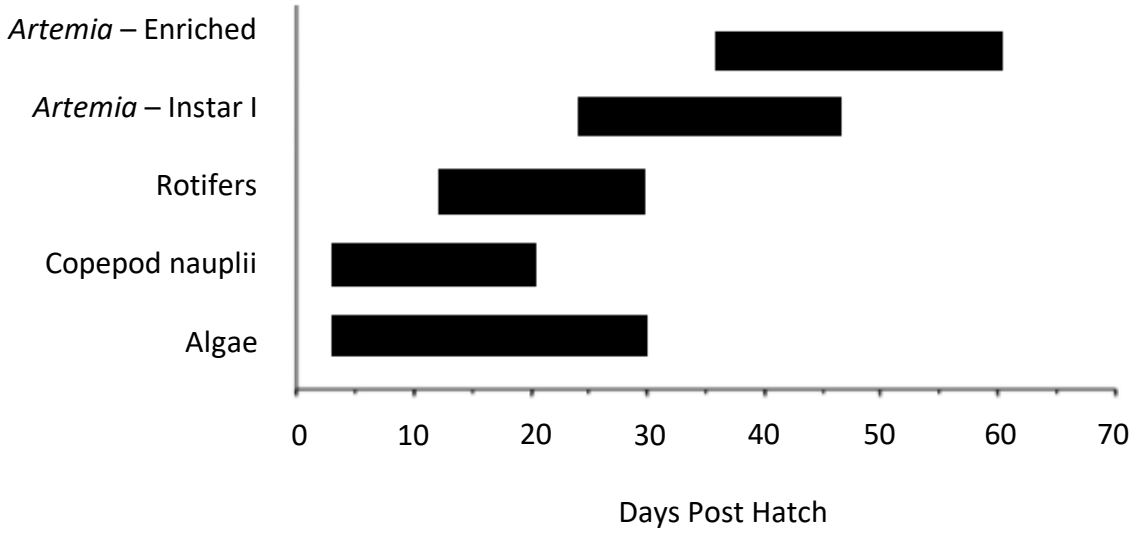


Figure 1. A typical feeding regime for marine ornamental larvae starts with copepod nauplii as a first feed along with microalgae, then progresses to rotifers, and finally *Artemia* before transitioning to inert feeds (Modified from Callan et al. 2018).

Copepods and their nauplii offer many benefits that render them ideal as a feed item for larval fish. These include swimming in a pattern that stimulates a feeding response (Buskey 2005), being appropriately sized for small-mouthed larvae (Schipp 2006; Laidley et al. 2008; Gopakumar et al. 2009; DiMaggio et al. 2017; Callan et al. 2018; Zeng et al. 2018; Anzeer et al. 2019), and naturally providing essential nutrients such as the essential fatty acids DHA, EPA, and ARA (McKinnon et al. 2003; Schipp 2006).

Many marine ornamental aquaculturists raise *Parvocalanus crassirostris*, a calanoid copepod found in the subtropics and tropics (Kline 2011; Alajmi 2015). Identification of *Parvocalanus crassirostris* as a first feed has resulted in successful captive rearing of flame angelfish, *Centropyge loriculus* (Laidley et al. 2008), blue tang, *Paracanthurus hepatus* (DiMaggio et al. 2017), Marcia's anthias, *Pseudanthias marcia* (Anil et al. 2018) and yellow tang, *Zebrasoma flavescens* (Callan et al. 2018). The utilization of *P. crassirostris* in conjunction with

other live feeds has also been shown to increase survival in species such as the green mandarinfish, *Synchiropus splendidus* (Zeng et al. 2018).

Understanding the life cycle of copepods is an important part of aquaculture. Eggs hatch into the first of six naupliar stages, often referred to as nauplii 1 (N1). Like all crustaceans, copepods molt, marking the transitions through different life-history stages. After completing the development through the six naupliar stages, there are five copepodite stages, followed by the transition into an adult copepod (Figure 2; Anzeer et al. 2018). *P. crassirostris* becomes reproductively mature in approximately 8 days at which point females can lay up to 20 eggs per day (McKinnon et al. 2003; Kline 2011; Alajmi 2015). Culture conditions such as temperature, salinity, and density dictate the adult life span of *P. crassirostris,* which averages 20 days (Anzeer et al. 2018). Peak egg production in adult *P. crassirostris* is 2-5 days after becoming an adult (Alajmi et al. 2014). Overcoming challenges to production within the complex life cycle of *P. crassirostris* will be discussed in more detail below.

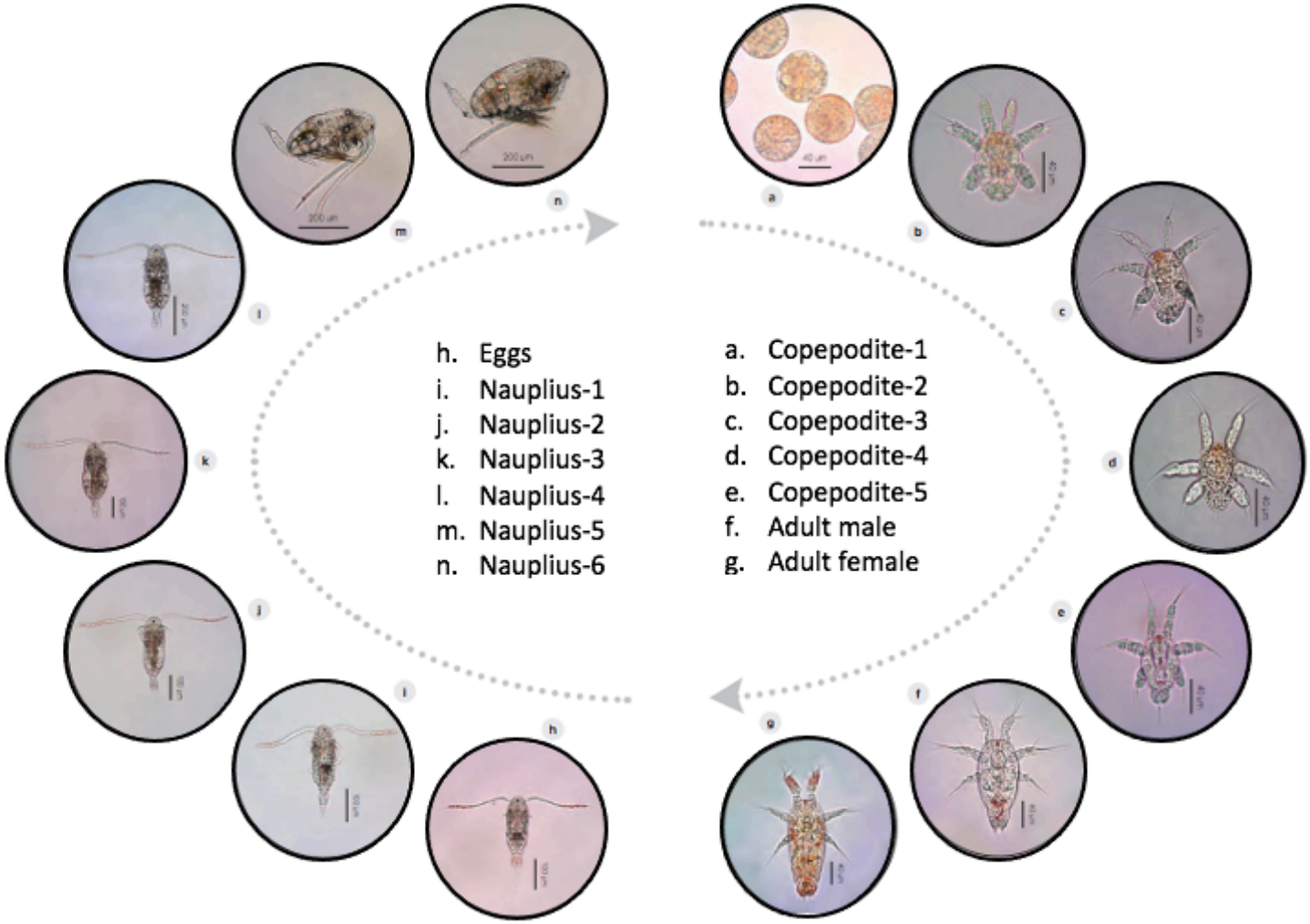


Figure 2. Developmental stages of the calanoid copepod, *Parvocalanus crassirostris* (Modified from Anzeer et al. 2018).

Reliable technologies for mass culturing copepods have not yet been developed, mainly because of biological and technical challenges such as the requirement for live microalgae, long generation times, low culture densities, low survival from nauplii to adult, and sensitives to water quality (Payne & Rippingale 2001; Schipp 2006; Støttrup 2006; Kline 2011; Olivotto et al. 2011; Alajmi 2015; Santosh et al. 2018). In comparison to other live feeds, copepods such as *Parvocalanus crassirostris* have long generation times. This is problematic because it takes more than a week to reach sexual maturity and produce subsequent generations/nauplii for aquaculture use (Kline 2011; Alajmi 2015). Planning for production of nauplii requires additional foresight due to extensive maturation times. Additionally, some species of copepods exhibit decreased fecundity (Kline 2011), and increased mortality (Alajmi & Zeng 2014) with increasing densities; due to these constraints the density of adults needs to be kept low, which requires more tank space for producing similar densities of live feeds. Another challenge is that production tanks must be replenished with new adult copepods regularly to maintain consistent levels of egg production, because adult copepods are only reproductive for a short amount of time. Furthermore, small increases in female mortality can cause negative population growth, and peak production of eggs lasts only a few days (Kiørboe 1998). Several facilities have adopted a production method developed at the Oceanic Institute in Hawai‘i, where maturation tanks are used to rear nauplii to adults; these adults are then stocked daily into production tanks (Figure 3). The primary problem is that survival from nauplii to maturation is highly variable and relatively low. Thus, these factors which lead to highly variable production, poses one of the most significant challenges for producing larval fish.

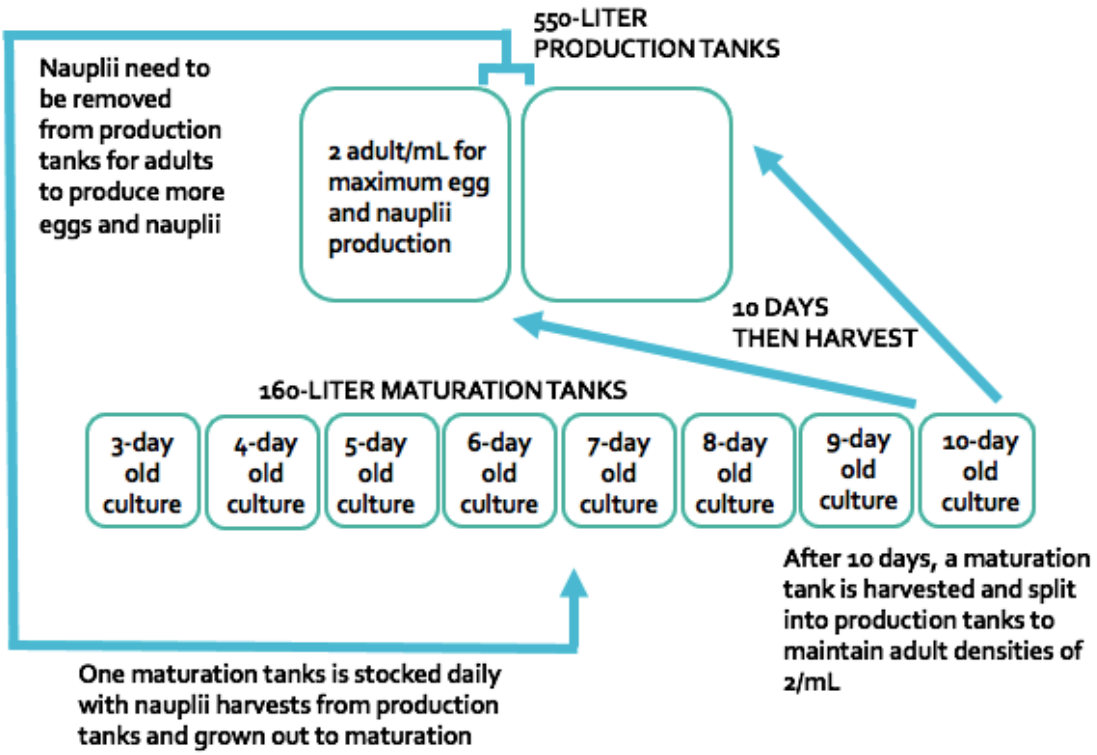


Figure 3. Schematic of copepod production at the Pacific Aquaculture and Coastal Resources Center. These production methods were adopted from those developed at the Oceanic Institute.

Copepod mortality has been attributed to many factors including but not limited to variations in microalgae diets (Camus & Zeng 2012), photoperiod (Camus & Zeng 2008), poor algae quality (Koski & Breteler 2003; Ismar et al. 2008), handling stress (Nilsson et al. 2018), and suboptimal rearing conditions such as high densities (Alajmi & Zeng 2014). Although these stressors contribute to mortality, mortality in copepod production systems is highly variable and the causes of low and variable survival are unknown (C. Callan, personal communication, 2021). Mortality rates vary with developmental stage in copepods. The highest mortality rates occur at the copepodite I and copepodite 5 stages and this may be due to stress induced by physiological changes required for the transition from nauplii to copepodite and copepodite to copepod (Ohman & Wood 1995). If suitable rearing conditions are not provided at various developmental stages, the stress caused to copepods could exacerbate mortality rates during these critical transitions. For that reason, it is essential to understand the causes of mortality and how they can be alleviated with changes to aquaculture methodologies.

Handling stress is a contributing factor to mortality in copepod production although tolerance to handling stress varies with species and developmental stages. Adult *Acartia tonsa* survival decreased by 44 ±7% (mean ± SD) after 10 minutes of handling stress, but nauplii exposed to the same handling stress did not seem affected (Nilsson et al. 2018). In contrast, another study reported nauplii mortality in *Acartia* sp. from handling individuals by sieving (Marshall 2002). Although handling stress likely contributes to mortality, designing a study around this factor can be difficult due to variations in sieving methods and challenges in standardizing the process.

Another challenge with rearing copepods is that almost all copepod species must be fed live microalgae, which is labor-intensive, requires specialized infrastructure, and technical skills (Conceição et al. 2010; Ohs et al. 2012; Alajmi 2015). *Apocyclops* sp., *Nitokra lacustris*, and *Robertsonia* sp. have successfully been reared on algae paste (Lee et al. 2005). However, these copepods are larger than calanoid copepods such as *Parvocalanus crassirostris* which are suitably sized for marine ornamental larvae. Beyond requiring specialized infrastructure and skills to grow microalgae, it can be difficult to produce consistent cultures of algae and these inconsistencies may have effects on copepod cultures. Copepod mortality is affected by feeding but varies with copepod species, the concentration of feed offered, and feed quality (Koski & Breteler 2003; Ismar et al. 2008; Camus & Zeng 2012). In a study on calanoid copepods, mortality was measured in response to high quality versus nutritionally-deficient microalgae at low and high concentrations. High concentrations of high-quality *Rhodomonas* sp. and *Dunaliella* sp. resulted in the lowest mortality rates (Koski & Breteler 2003). Thus, utilization of high-quality algal feeds for copepods should be prioritized. This may be easier to achieve indoors with a controlled production environment, however, a cost-analysis would need to be conducted in order to determine feasibility. The density of microalgae is equally important, and suboptimal densities can result in lower survival even when high-quality food is offered. A study by Ismar et al. 2008 showed that *Rhodomonas* sp. offered to the copepod *Acartia tonsa* resulted in high survival rates and faster naupliar and copepodite development at concentrations of 600 μm C^{-1} but at lower concentrations of 75-150 μm C^{-1} survival and

development time decreased. Fortunately, densities of algal cultures can easily be managed within copepod production tanks.

Moreover, copepods have demonstrated sensitivities to water quality changes which further exacerbates the difficulties with intensive rearing (Payne & Rippingale 2001; Hoff & Snell 2014; Jepsen et al. 2015). A study on the copepod *Parvocalanus crassirostris* revealed that concentrations of $N\text{-}NH_3 \leq 0.4$ mg/L did not affect copepod survival or fecundity (Kline 2011). However, ammonia concentrations as low as 0.03 mg/L negatively affected egg production and survival from nauplii to adult in other copepod species (Payne & Rippingale 2001; Jepsen et al. 2015). Given the range of toxicity levels of ammonia in copepods, measures should be taken to mitigate levels of ammonia within copepod production systems. Nitrate toxicity has not been studied in copepods and is generally understudied in aquatic invertebrates. However, nitrate toxicity has been examined in the amphipod *Gammarus pseudolimnaeus*, a marine crustacean. The study showed no significant differences in mortality when exposed to nitrate levels up to 128 mg/L suggesting that nitrate toxicity at low levels does not produce sub-lethal or lethal effects to aquatic invertebrates (Stelzer & Joachim 2010).

Although small naupliar sizes are the primary reason that *P. crassirostris* has been selected as an ideal first feed for many marine ornamental larvae there are other reasons that have made it a preferred live feed. *Parvocalanus crassirostris* broadcast their eggs, making it possible for eggs to be collected on a sieve for transfer into larval rearing tanks (Anzeer et al. 2018). Copepod eggs can be stocked in larval rearing tanks where they hatch making the first naupliar stage immediately available to larval fish. This developmental stage can be readily consumed by even very small fish larvae (Schipp 2006). Additionally, *P. crassirostris* are herbivorous and compared to parasitic or cannibalistic copepods, they do not cause harm to copepod cultures by ingesting nauplii or causing harm to the fish larvae (Alajmi & Zeng 2015).

Parvocalanus crassirostris egg production, naupliar, copepodite survival, and population growth are primarily affected by diet. To obtain high production efficiencies, *P. crassirostris* must be fed a live microalgae diet rather than alternative algal pastes (Alajmi & Zeng 2015). The demand for live algae by *P. crassirostris* requires that copepod production facilities have adequate microalgae production which adds significantly to production, labor costs and increases the number of variables that may affect copepod production. Various feeding densities for successful rearing of *P. crassirostris* have been reported, with the optimal ranges between 150,000 - 400,000 microalgae cells/mL (Kline 2011; Santosh et al. 2018). Therefore, optimal growth of *P. crassirostris* requires high-density cultures or large-scale microalgae production to meet production needs.

Although considerable advancements have been made to develop intensive culture methods for copepod culture, research should continue to address challenges to production (Schipp 2006; Kline 2011; Alajmi & Zeng 2014, 2015; Alajmi et al. 2014). Studies on *Parvocalanus crassirostris* have primarily focused on egg and nauplii production at different stocking densities (Kline 2011; Alajmi & Zeng 2014), improvements with selective breeding (Alajmi et al. 2014), and egg production under different diet treatments (Alajmi & Zeng 2015). One aspect of production that needs to be addressed is the survival from nauplii to adult. Survival is highly variable in intensive copepod cultures, but historically, the mean survival of *Parvocalanus crassirostris* is around 50% at the Oceanic Institute (OI), Waimānalo, O'ahu.

However, survival can be as low as 10% following the same methodologies (C. Callan, personal communication, 2020).

Although studies documenting copepod mortality in intensive production systems are sparse, it is probable that infections by bacteria and viruses could be a contributing factor. It is well documented that bacteria and viruses account for the majority of mortalities in other cultured crustaceans, such as shrimp (Flegel 2019). A study by Ninawe & Selvin, 2009 also showed that bacteria and viruses can impede fish and shellfish production. Increases in mortality can be linked to intensive aquaculture practices. This includes raising organisms at higher densities, higher temperatures, and constant food additions to increase growth and maximize production efficiencies. However, these conditions support the proliferation of opportunistic bacteria, viruses, and other pathogens and could make the organisms more susceptible to disease due to higher pathogen pressure, disease transmission, or stress that leads to reduced immune responses (Olafsen 2001; Bondad-Reantaso et al. 2005; Prado et al. 2005; Balcázar et al. 2006a; Ninawe & Selvin 2009; Walker & Mohan 2009; Walker & Winton 2010). For example, bacterial infections are a significant problem in shellfish aquaculture, causing mass mortalities, particularly in the larval and juvenile stages (Karim et al. 2013).

Several types of bacteria, including *Vibrio* spp., *Aeromonas* spp. and *Pseudomonas* spp., which include human- and animal-pathogenic species, have been associated with laboratory-reared and wild-collected copepods (Sochard et al. 1979; Gugliandolo et al. 2008; De Corte et al. 2014; Zidour et al. 2017; Rasmussen et al. 2018). Although associations of bacteria with copepods have been documented, few studies have examined the nature of the interactions between bacteria and copepods and whether or not there is a positive or negative correlation with survival. Such investigations could close a crucial gap to gain much needed insight into the variability of copepod survival. Additionally, the transfer of pathogenic bacteria from copepods to fish larvae could be a concern if infected copepods are used as a live feed in larviculture (Olafsen 2001). One study revealed that the fish pathogen *Vibrio anguillarum* colonized the exterior and the intestines of the copepod *Acartia tonsa* without causing mortality to the copepod (Rasmussen et al. 2018). This study highlighted the importance of using disease-free live feeds for feeding to larval fish.

Antibiotics have traditionally been used in aquaculture to prevent and treat diseases and promote growth (Irianto & Austin 2002; Kesarcodi-Watson et al. 2008). Although antibiotics can treat certain bacterial diseases effectively, misuse can result in environmental impacts and lead to antibiotic resistances (Verschuere et al. 2000; Balcázar et al. 2006a; Vine et al. 2006; Kesarcodi-Watson et al. 2008). For example, the overuse of antibiotics in shrimp farms throughout Asia resulted in the emergence of antibiotic-resistant bacteria and led to large-scale mortality due to disease problems (Moriarty 1999). As a more sustainable alternative practice to antibiotics, probiotic bacteria are becoming more widely used in aquaculture (Verschuere et al. 2000; Irianto & Austin 2002; Kesarcodi-Watson et al. 2008). Probiotics have reduced antibiotic use in disease treatment and prevention, provided water quality amelioration, and increased organism growth, survival, and health (Verschuere et al. 2000; Irianto & Austin 2002; Kesarcodi-Watson et al. 2008; Newaj-Fyzul et al. 2014).

1.2 Probiotics

Probiotics applications have been used extensively in aquaculture to mitigate harmful effects from pathogenic bacteria (Gomez-Gil et al. 2000; Prado et al. 2005; Walker & Winton 2010; Newaj-Fyzul et al. 2014). The Food and Agricultural Organization (FAO 2006) defines probiotics as live microorganisms administered in appropriate dosages that benefit the host (FAO 2006). However, this definition applies more to terrestrial organisms versus aquatic organisms because in aquatic environments, there is a constant interaction between the bacteria in the environment and the bacteria in the organisms' gut (Verschuere et al. 2000). An aquaculture-appropriate definition of a probiotic is a live microbial additive that benefits the host or host's close environment (Verschuere et al. 2000). Microbial additives refer to bacteria which are unicellular microorganisms with cell walls that lack organelles and an organized nucleus. Probiotics can come in various application forms and are administered via feed supplements, water additives, and enriched live feeds (Gomez-Gil et al. 2000; Daniels et al. 2010, 2013; Sharifuzzaman & Austin 2017). Probiotic products can be applied as single strains, multiple strain cocktails, in conjunction with prebiotics (as synbiotics), and as live or dead forms (Hai 2015; Sharifuzzaman & Austin 2017). Prebiotics are non-digestible food additives that

enhance the survival and efficacy of beneficial gastrointestinal microbes (Akhter et al. 2015). A range of probiotic bacteria used for aquaculture has been reviewed by Newaj-Fyzul et al. (2014).

Although probiotic use in aquaculture has become more widespread, limited research has involved applications to copepods (Divya et al. 2012; Sun et al. 2013; Forbes 2016; Zidour et al. 2017). One study observed increased female size, egg production, and hatching success in the copepod *Acartia tonsa* when *Rhodomonas salina* feed was supplemented with a commercial probiotic additive (Drillet et al. 2011). In addition, Forbes (2016) examined the application of probiotics to the copepod *Parvocalanus crassirostris,* its effects on the bacterial community, and on yellow tang, *Zebrasoma flavescens* larval growth and survival. Although no significant differences in growth and survival of yellow tang larvae were reported, the bacterial community of the copepod cultures was found to be significantly different, with the probiotic group having greater species diversity and reduced levels of *Vibrio* spp. (Forbes 2016).

Studies generally focus on the effects of probiotic-enriched copepods to the targeted larval fish (Divya et al. 2012; Sun et al. 2013). Sun et al. 2013 showed that probiotic-enriched copepods fed to Orange-Spotted Grouper, *Epinephelus coioides*, resulted in a microbial shift in the gut from potential pathogenic bacteria to *Bacillus clausii* DE5 and *Bacillus pumilus* SE5. Divya et al. 2012 showed that probiotic enriched copepods fed to Rosy Barb, *Puntius conchonius,* caused a reduction of bacterial fish pathogens in the intestines. In another study, the bacterial strain *Bacillus pumilus* was isolated from *Acartia tonsa* eggs and inhibited the growth of several pathogens *in vitro*, including *Vibrio alginolyticus*, *Vibrio anguillarum*, *Listeria monocytogenes*, and *Staphylococcus aureus* (Zidour et al. 2017). The aforementioned studies suggested that use of probiotics in copepod cultures used as live feeds for larval fish could have a beneficial effect.

The control of host-microbe interactions in intensive systems can help stabilize production (Gomez-Gil et al. 2000; Olafsen 2001; Kesarcodi-Watson et al. 2008). Therefore, it is reasonable to assume that controlling pathogens in intensive copepod cultures could decrease the variability in copepod survival from nauplii to adult, but research on this topic was limited when this research was initiated. The study detailed in this thesis evaluated INVE probiotics'

efficacy on survival of copepod stages from nauplii to adult. Although INVE offers many probiotic products, INVE Sanolife ™ MIC and INVE Sanolife ™ MIC-F (Belgium), hereinafter referred to as MIC and MIC-F, were selected for this study and will be discussed in more detail below. MIC contains *Bacillus subtilis, Bacillus licheniformis,* and *Bacillus pumilus* and was chosen because it is a commercially available, shelf-stable product widely used in aquaculture. MIC-F also consists of *Bacillus subtilis, Bacillus licheniformis,* and *Bacillus pumilus* but has a higher concentration of *Bacillus pumilus,* which are putatively more effective against pathogens. Silva et al. (2012) found positive effects on growth and survival of certain stages in Pacific white shrimp, *Litopenaeus vannamei,* with INVE Sanolife. Another study that evaluated field data from a Thai and a Brazilian shrimp aquaculture facility showed that the application of MIC increased survival in the naupliar stages at both hatcheries (Decamp et al. 2008).

Bacillus spp. are rod-shaped, gram-positive, facultative anaerobic bacteria that have been widely utilized as probiotics in aquaculture (Irianto & Austin 2002; Soltani et al. 2019). Species of this genus are spore-forming, a desirable trait for creating a shelf-stable commercial product (Soltani et al. 2019). *Bacillus* spp. have yielded many benefits in aquaculture, including but not limited to: improved water quality, promoting species' growth, enhanced immune status, enzyme or bacteriocin production, antibacterial properties, and pathogen inhibition (Soltani et al. 2019). Additionally, *Bacillus* spp. can produce digestive enzymes such as lipase, protease, and carbohydrase, which can be beneficial as a feed additive (Leonel Ochoa-Solano & Olmos-Soto 2006). *Bacillus subtilis*, also known as grass bacillus, are naturally found in the soil and GI tract of ruminants and humans. Newaj-Fyzul et al. (2014) reviewed the extensive use of *Lactobacillus* spp. and *Bacillus subtilis* in aquaculture, detailing various successes, including pathogen control, infection resistance, feed conversion ratio improvements, and improved growth and enzyme activity. *Bacillus licheniformis* are found in aquatic environments, specifically in soil and vegetation, and are responsible for the cycling of nutrients. The strain *Bacillus pumilus* used in MIC-F produces antimicrobial peptides like bacteriocin for inhibiting pathogens (SANOLIFE ™ - INVE Aquaculture, Belgium). Among the gram-positive bacteria, *Bacillus* spp. have been the most widely assessed genus in aquaculture, followed by

Lactobacillus spp. (Sharifuzzaman & Austin 2017). For this reason, many of the probiotics listed below belong to these genera (Table 1).

Table 1. A selection of probiotics used in aquaculture, their host species, and method of administration.

Species Targeted	Probiotic	Source of Probiotic	Administration	Results	Source
Copepod *Acartia tonsa* and Microalgae *Rhodomonas salina*	*Phaeobacter inhibens*	Unknown	Co-culture	Significantly inhibited fish pathogen *Vibrio anguillarum* .	Rasmussen et al. 2018
Copepod *Acartia tonsa*	*Lactobacillus farciminis* , *Lactobacillus rhamnosus*	Sorbial A/S (DANISCO, Allonnes, France)	Feed additive	Increased individual size of the adult females and their egg production and increased the hatching success.	Drillet et al. 2011
White shrimp juveniles Litopenaeus vannamei	*Bacillus* sp., *Saccharomyces cerevisiae (yeast), Nitrosomonas sp., Nitrobacter sp.*	Commercial Probiotic (Unspecified)	Feed additive	Significantly higher survival rate, feed conversion rate, and final production. Reduction in concentrations of nitrogen and phosphorous and increased dissolved oxygen. Shift from pathogenic species such as *Vibrio* sp. to beneficial bacteria.	Wang et al. 2005
White shrimp larvae *Litopenaeus vannamei*	*Bacillus coagulans*	Pond sediment	Water additive	Significantly increased survival over the control across all treatments. Increased digestive activities at some of the ontogenetic stages.	Zhou et al. 2009
White shrimp larvae and post-larvae Litopenaeus vannamei	*Bacillus* spp.	INVE MIC ™ - INVE Aquaculture, Belgium	Water additive and feed additive	Increase in survival in growth of zoea and mysis phases, especially when probiotic administration via water additive. Reduction in presumptive *Vibrio* for water and shrimp.	Silva et al. 2012
White shrimp *Litopenaeus vannamei*	*Bacillus licheniformis*	Microbial biofloc	Water and feed additive	Significantly decreased the concentration of *Vibrio* spp. Increased the response of hemato-immunological parameters in marine shrimp.	Ferreira et al. 2015

Black tiger shrimp juveniles *Litopenaeus monodon*	*Bacillus* sp.	Intestines of *Litopenaeus monodon* , pond sediment	Live-sprayed and freeze-dried probiotic feeds	Lengths and survival rates of shrimp were significantly higher than the control. Hepatopancreas and intestinal bacteria shifted from *Vibrio* spp. to *Bacillus* spp.	Boonthai et al. 2011
Black tiger shrimp *Litopenaeus monodon* and White shrimp *Litopenaeus vannamei*	*Bacillus foraminis* , *Bacillus cereus biovar toyoi* , *Bacillus fusiformis*	Hydrogen-producing fermented solution	Water additive –	*B. cereus biovar toyoi* resulted in significantly deleterious effects on survival of *Litopenaeus monodon* . *B. fusiformis* showed highest survival rate but no statistically significant difference from control in *Litopenaeus monodon* . *B. fusiformis* significantly increased survival in *Litopenaeus vannamei* .	Guo et al. 2009
Giant freshwater prawn *Macrobrachium rosenbergii*	*Bacillus* NL110, *Vibrio* NE17	*Macrobrachium rosenbergii* larvae and eggs	Feed and water additive	Significant improvement in specific growth rate and weight gain. Significant improvements in water quality parameters such as nitrate and ammonia. Improvements in immune parameters including total hemocyte count, phenol oxidase activity and respiratory burst.	Mujeeb Rahiman et al. 2010
Rainbow trout *Oncorhynchus mykiss*	*Bacillus subtilis*, *Bacillus licheniformis*	BioPlus2B (USA)	Feed additive	Improved resistance to pathogen *Yersinia ruckeri* .	Raida et al. 2003

Cardinal tetra *Paracheirodon axelrodi*	*Bacillus subtilis*, *Bacillus licheniformis*, *Lactobacillus acidophilus*, *Saccharomyces cerevisiae*	Efinol®L (Bentoli Agrinutrition, Austin TX, USA)	Water additive	Survival during transport at 24 hours was higher and cortisol levels were significantly lower. Ammonia was significantly lower. No significant difference in dissolved oxygen, temperature, conductivity, pH, and alkalinity between treatments.	Gomes et al. 2009
Rohu *Labeo rohita*	*Bacillus subtilis*	Gastrointestinal tract of Mrigal Carp *Cirrhinus mrigala*	Feed additive	Colonization of gut by *Bacillus subtilis* suppressed the pathogen *Aeromonas hydrophila*. Survival and weight gain were significantly higher. Increased immune responses indicated by increased total leucocyte counts, increased total serum protein and globulin.	Kumar et al. 2006
Rohu *Labeo rohita*	*Bacillus licheniformis*, *Bacillus pumilus*	Gut of *Labeo rohita*	Immunized intraperitoneally and feed additive	Significantly lower mortality when challenged with pathogen *Aeromonas hydrophila*.	Ramesh et al. 2015

1.21 Selection and administration of probiotics

There are several criteria for selecting a successful probiotic. First, to be considered a probiotic, it should benefit the host. Additionally, the probiotic administration should not harm humans, the targeted host, other trophic levels involved in the culture of the host, or induce adverse effects to the rearing environment (Soltani et al. 2019). To be effective, the probiotic must be able to survive at the host's rearing conditions and be shelf-stable (Balcázar et al. 2006a). Furthermore, the selected probiotic should not contain virulence resistance genes or antibiotic resistance genes (Verschuere et al. 2000; Vine et al. 2006).

To select a specific probiotic against a pathogen, inhibitory activity against the pathogen can be tested *in vitro* or *in vivo* (Irianto & Austin 2002; Balcázar et al. 2006a; Hai et al. 2007; Kesarcodi-Watson et al. 2008). For *in vitro* testing, the efficacy of a probiotic can be tested with the application of a known pathogen on a petri dish or well plate. If the probiotic is effective against the pathogen, a zone of inhibition will show that the pathogen cannot exist in the same area as the probiotic. Alternatively, probiotic treatments can be tested *in vivo* against a control to determine what consequences the probiotic treatment has on the culture water or cultured organism (Verschuere et al. 2000).

Once a probiotic has been selected, the method of administration must be decided for the targeted species. The administration of probiotics depends on the targeted species and the desired outcome of the application. Probiotics are most commonly administrated as an additive to the feed or the culture water (Hai 2015). However, probiotic administration via enrichment or encapsulation of live feeds is becoming more common (Daniels et al. 2010, 2013; Avella et al. 2011; Divya et al. 2012; Sun et al. 2013; Akbarali et al. 2014; Lobo et al. 2014; Dey et al. 2015; Forbes 2016; Tarnecki et al. 2019; Table 2). Bioencapsulation is the process by which the nutritional or therapeutic value of live feeds is enhanced (Dey et al. 2015). The utilization of bioencapsulated live feeds by fish larvae has been documented for *Artemia* (Makridis et al. 2009; Daniels et al. 2010, 2013; Avella et al. 2011; Akbarali et al. 2014; Lobo et al. 2014), rotifers (Avella et al. 2011), and copepods (Sun et al. 2013; Forbes 2016). When probiotic bioencapsulated live feeds are fed to fish larvae, they are delivered directly to the gut of the larvae where competition from the probiotic can prevent the colonization of pathogenic

bacteria (Ringø & Gatesoupe 1998). This is important because many pathogens harm fish larvae by entering via gut mucosa during ingestion. Besides preventing pathogen colonization, probiotics produce other beneficial effects in the guts, such as competition for nutrients with potential pathogens, and the production of antimicrobial compounds that kill the pathogen (Balcázar et al. 2006b; Kesarcodi-Watson et al. 2008; Bermudez-Brito et al. 2012). Additional modes of action will be discussed further in Section 2.54.

Although probiotics are commonly bioencapsulated in live feeds, the effects of probiotics are generally studied on the fish or aquatic organism ingesting the live feed rather than the live feed itself (Irianto & Austin 2002). Drillet et al. (2011) observed increased female size, egg production, and hatching success in the copepod *Acartia tonsa* when *Rhodomonas salina* feed was supplemented with a commercial probiotic additive. In another study, the bacteria *Alteromonas* sp. enhanced rotifer growth rates (Douillet 2000). The results of these studies show promise for applications of probiotics having a direct positive effect on the live feeds.

Table 2. A selection of probiotics administered in aquaculture using bioencapsulated live feeds.

Species Targeted	Probiotic	Sources of Probiotic	Administration	Results	Source
European lobster zoea I *Homarus gammarus*	*Bacillus* spp., mannan oligosaccharides (MOS)	SANOLIFE ™ - INVE Aquaculture, Belgium, MOS (BIO- MOS® - Alltech Inc., KY USA)	Bioencapsulated *Artemia*	Significantly improved weight, carapace length and weight gain. Significantly increased survival with highest survival in synbiotic treatment. Enhanced tolerance to salinity stress. Colonization of larvae guts by probiotic. Reduction in *Vibrio* levels.	Daniels et al. 2013
Yellow tang larvae *Zebrasoma flavenscens*	*Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis*	INVE MIC-F ™ - INVE Aquaculture, Belgium	Water additive and/or bioencapsulated *Parvocalanus crassirostris*	Greater species diversity and reduced levels of *Vibrio* spp. in copepod cultures. No significant differences to growth or survival of Yellow Tang.	Forbes 2016
Orange-spotted grouper larvae *Epinephelus coioides*	*Bacillus clausii* DE5, *Bacillus pumilus* SE5	Gut of *Epinephelus coioides*	Bioencapsulated *Pseudodiaptomus annandalei* copepods	Increased total length and body weight. Improved survival. Shifts from pathogenic bacteria to *Bacillus clausii* and *Bacillus pumilus* .	Sun et al. 2013
Common sole *Solea solea*	*Enterococcus faecium*	Gut of *Solea solea* broodstock	*Brachionus plicatilis* , *Brachionus rotundiformis* , *Artemia salina* depending on larval stage	*Vibrio* populations of the intestines were significantly reduced. Significantly reduced the stress marker Heat Shock Protein 70. Higher body weight and total length over control by 50 DPH. Survival was not affected.	Avella et al. 2011
Senegalese sole *Solea senegalensis*	*Shewanella putrefaciens* Pdp11	Skin mucus of healthy cultured gilthead seabream *Sparus aurata*	Bioencapsulated *Artemia*	Significantly modulated larval and fry microbiota. Growth of larvae was significantly higher.	Lobo et al. 2014

Common snook larvae *Centropomus undecimalis*	*Bacillus licheniformis* , *Bacillus amyloliquefaciens*	Proprietary probiotic strains	Water additive and bioencapsulated rotifers	Significantly higher survival and resistance to transport stress in probiotic-treated larvae as compared to controls.	Tarnecki et al. 2019
Zebrafish post-larvae *Brachydanio rerio*	*Lactobacillus ramnosus* , *Bacillus coagulans* , *Bacillus mesentericus* , *Bifidobacterium infantis* , *Bifidobacterium longum*	Gut of *Brachydanio rerio*	Bioencapsulated *Artemia parthenogenetica* nauplii	Probiotic established in the gut of *B. rerio* . Significantly decreased levels of pathogenic bacteria in the gut microflora.	Akbarali et al. 2014
Rosy barb post-larvae *Puntius conchonius*	*Bacillus coagulans* , *Bacillus mesentericus* , *Bifidobacterium infantis*	Unknown	Bioencapsulated *Thermocyclops decipiens* copepod	Significantly decreased levels of pathogenic bacteria in the gut microflora.	Divya et al. 2012

1.22 Potential modes of action

The interactions between probiotics and their hosts are complex, and generally, their mode of action is poorly understood (Irianto & Austin 2002; Balcázar et al. 2006a; Vine et al. 2006; Newaj-Fyzul et al. 2014; Hai 2015; Sharifuzzaman & Austin 2017). There are many potential modes of action by which probiotics and prebiotics can produce beneficial effects on a host (Balcázar et al. 2006b; Vine et al. 2006). Possible modes of action include but are not limited to: 1) immune stimulation; 2) sources of nutrients and enzymes; 3) improvement of gut microbiota; 4) antagonistic compound production; 5) competition for adhesion sites; and 6) competition for nutrients or chemicals (Figure 4, Sharifuzzaman & Austin 2017).

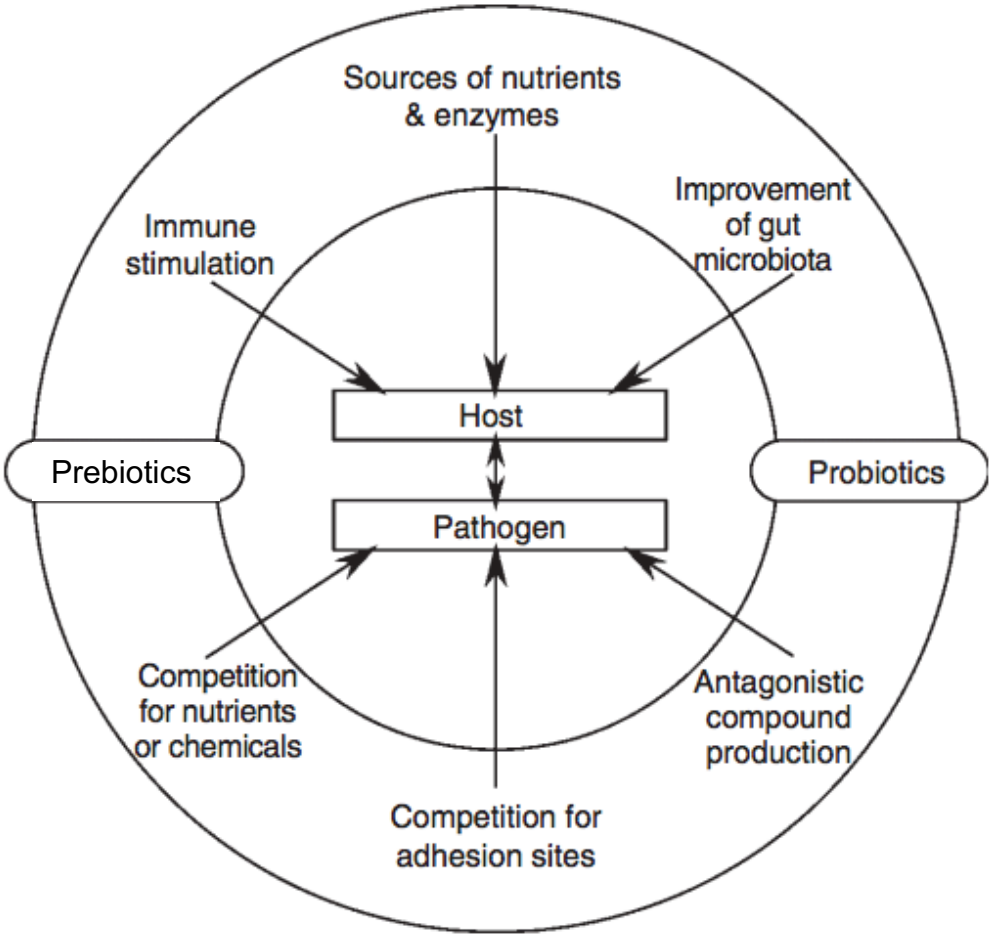


Figure 4. Potential modes of action include but are not limited to: 1) immune stimulation; 2) sources of nutrients and enzymes; 3) improvement of gut microbiota; 4) antagonistic compound production; 5) competition for adhesion sites; and 6) competition for nutrients or chemicals (Modified from Sharifuzzaman & Austin 2017).

Direct antagonism and competitive exclusion are two methods by which probiotics inhibit the growth of potential pathogens (Irianto & Austin 2002). Direct antagonism describes the processes in which a probiotic kills or inhibits a pathogen by secreting a molecule or by attaching to it and injecting toxins into the pathogen. Direct antagonism is the easiest method to test, as it is easily testable *in vitro* by growth curves or cell counts (Kesarcodi-Watson et al. 2008). For example, *Lactobacillus* spp. JK-8 and JK-11 produced organic acids, namely, lactic acid and acetic acid, which caused a drop in pH, killing or, at least, inhibiting the pathogens *Vibrio parahemolyticus*, *Vibrio harveyi*, and *Edwardsiella tarda* (Ma et al. 2009). Competitive exclusion describes how probiotics compete with pathogens for nutrients, space, and adhesion receptors (Moriarty 1999). It is very challenging to test competitive exclusion, so this mode of action is often poorly understood (Kesarcodi-Watson et al. 2008).

Probiotics can benefit the physiological or immunological responses of cultured organisms by improving growth and survival, increasing food utilization, and enhancing the digestibility of feeds (Newaj-Fyzul et al. 2014). *Bacillus* spp. and other probiotics have been shown to increase survival and growth in many crustaceans, including freshwater prawn *Macrobrachium rosenbergii* (Mujeeb Rahiman et al. 2010), black tiger shrimp, *Litopenaeus monodon* (Boonthai et al. 2011), and Pacific white shrimp, *Litopenaeus vannamei* larvae (Guo et al. 2009; Zhou et al. 2009; Silva et al. 2012). For example, applications of *Bacillus coagulans* to the culture water of *Litopenaeus vannamei* larvae resulted in significantly increased survival over the control across all treatments (Zhou et al. 2009). In addition to benefitting survival, probiotics can contribute to increases in growth by producing digestive enzymes such as protease, amylase, and lipase (Balcázar et al. 2006a; Zhou et al. 2009; Newaj-Fyzul et al. 2014).

Although not described by Sharifuzzaman & Austin (2017), another mode of action by which probiotics can have beneficial effects on a host or its environment is water quality improvement (Soltani et al. 2019). There are various mechanisms by which this occurs, including increases to dissolved oxygen (Wang et al. 2005), reduction in organic matter (Verschuere et al. 2000; Wang et al. 2005), stabilizing effects on pH (Wang et al. 2005), reduction of nitrogen concentrations (Wang et al. 2005; Mujeeb Rahiman et al. 2010), reduction of metabolic wastes like ammonia (Gomes et al. 2009; Mujeeb Rahiman et al. 2010)

and reduction of pathogenic bacteria (Avella et al. 2011; Boonthai et al. 2011; Divya et al. 2012; Daniels et al. 2013; Sun et al. 2013; Akbarali et al. 2014; Rasmussen et al. 2018). Improvements to water quality are most commonly associated with *Bacillus* spp., which can convert organic matter into carbon dioxide thereby minimizing sludge accumulation (Balcázar et al. 2006a; Soltani et al. 2019). In summary, probiotics may positively affect the host or environment by improving the water quality through the reduction of potentially harmful waste, organic matter, and bacteria.

This study aimed to determine whether survival in *Parvocalanus crassirostris* from nauplii to adult (Day 8) is affected by probiotics that have been shown to improve survival in crustacea. In the first round of experiments, two commercial probiotics, containing *Bacillus subtilis, Bacillus licheniformis,* and *Bacillus pumilus* (SANOLIFE ™ - INVE Aquaculture, Belgium), were administered to determine the effects on survival of *Parvocalanus crassirostris*. The administration of probiotics to copepod cultures could result in health benefits leading to increased survival and ease of culture.

2. MATERIALS AND METHODS

Parvocalanus crassirostris stocks were initially collected from Kaneohe Bay (Oahu, Hawai'i) in 2004 and were maintained at Pacific Planktonics in Kona (Hawai'i Island). Copepod stocks for this experiment were obtained from Pacific Planktonics and gradually scaled up at the Pacific Aquaculture and Coastal Resources Center in Keaukaha (Big Island, Hawai'i). Cultures were maintained in 540-liter semi-square black tanks (Pentair Aquatic Eco-Systems, Apopka, FL). These production tanks were maintained during the course of the experiments to provide nauplii for stocking into experimental tanks. All water used in these experiments was supplied via a seawater well at 20°C ± 1°C and a pH of 7.8 ± 1. Culture water was mechanically filtered to 1 μm and UV treated at a dosage rate of 30 MJ/cm^2 prior to filling culture tanks, where water was heated and maintained at 26.5°C ± 1°C. Light aeration was provided via two air diffusers spaced evenly around a central standpipe. Overhead fluorescent lights (24L:0D) provided indirect lighting to tanks, and no supplemental lighting was provided directly above tanks.

The algal species *Chaetoceros calcitrans*, *Tisochrysis lutea,* and *Rhodomonas salina* were obtained from NOAA and NEFSC's Milford Laboratory (Connecticut, United States). Microalgae stocks were maintained in a temperature-controlled laboratory. Erlenmeyer flasks for stock cultures were filled with treated natural seawater filtered to 1-μm and UV sterilized at a dose of 30 MJ/cm^2. F medium was added to the stock cultures, and flasks were autoclaved prior to being inoculated with microalgae under a transfer hood. Stock cultures were transferred every four days to 1-L flasks using the same protocol. After four days, 1-L flasks were transferred to 15-L carboys. Carboys were filled with treated seawater and chlorinated with sodium hypochlorite and dechlorinated using sodium thiosulfate prior to being inoculated with microalgae. All lab cultures >1-L were vigorously aerated through 0.2-μm disc filters. Cool white fluorescent lights provided 24-hour lighting to all laboratory microalgae cultures. Large-scale microalgae culture was conducted outdoors in a continuous algae production system (Seacaps Ltd., UK) in 500-L bags. In this system, new medium is constantly added to provide space and nutrients for microalgae and to facilitate overflow harvesting into a reservoir. Seawater for this system was supplied via a well and filtered through 5-μm, 1-μm, and 0.02-μm mechanical filtration before UV sterilization at a dose of 180 MJ/cm^2. The temperature of the cultured

microalgae varied depending on the ambient temperature. Individual algae culture bags were filled with approximately 100-L of filtered seawater and were inoculated with a 15-L carboy of microalgae. Algae bags were filled to about 500 liters in about one week, at which point the microalgae reached a harvest volume and flowed into a reservoir for daily pumping of algae to *P. crassirostris* cultures. About 10% of the volume of the bag was harvested daily. A pH controller linked to a solenoid valve dosed CO_2 into all of the bags when the pH was > 8.2.

To stock maturation tanks for the trials, copepod production tanks were drained, and the copepods were caught on a submerged 30-μm sieve. A secondary screening on a 55-μm screen was conducted to separate copepodites and copepods. Eggs and early-stage nauplii of *Parvocalanus crassirostris* are similar in size and when sieved, both stages are collected. The remaining eggs and early-stage nauplii were rinsed into a 3-L pitcher for enumeration. After enumeration, the eggs and nauplii were split into three beakers at equal densities. Three 1-mL samples were taken from each beaker to determine that densities across the beakers were similar. Beakers were stocked into randomly assigned 100-L semi-square experiment tanks. In designing this study, we chose to test this hypothesis in 100-L tanks instead of in flasks or beakers that are typically used at the experimental scale. There were only four experiment tanks available at the time of this study, so each treatment was tested once per trial and replicates were conducted over several months. Although this was a constraint, it was determined that conducting experiments on the same scale that would be used to stock production tanks would yield the most accurate results as tank size and shape can affect survival in some aquacultured species (Wittenrich et al. 2012). Standard protocols were followed, and environmental conditions were matched as much as possible. All experimental tanks were set up with the same treated natural seawater as the copepod production tanks and maintained under the same conditions. Tanks and trials were used as independent variables in a model to account for tank effects or trial effects.

MIC, MIC-F, and a control (no probiotic treatment) were tested over eight days in the maturation tanks at which point *Parvocalanus crassirostris* becomes reproductive. Tanks were inoculated with probiotics according to the dosages recommended by the manufacturer (MIC 0.1 grams/100 L tank and MIC-F 0.5 grams/100 L tank). Each probiotic was weighed, suspended

in 1 L of seawater, and dissolved using a magnetic stir bar (for 5 minutes) before being added as a solution. The initial dose was allowed to sit for 24 hours before each trial (according to the manufacturer's recommendation). *Parvocalanus crassirostris* cultures were incubated in the probiotic treatment tanks for the eight-day duration to accumulate the probiotic strains.

Background microalgae levels were counted daily in *P. crassirostris* treatment tanks to account for algae that had been consumed or had fallen out of suspension. Cell densities were determined with a hemocytometer. Background microalgae levels were increased to 400,000 cells/mL once daily with a ratio of 1:1:1 *Chaetoceros calcitrans*, *Tisochrysis lutea*, and *Rhodomonas salina*. To replace probiotics that had been flushed from the tank during daily microalgae feeding, the amount of probiotic that needed to be added was calculated based on the volume lost, the type of INVE probiotic, and the appropriate dose. After the initial inoculant of probiotics was added, probiotics were added daily as a solution after the morning feeding. All stages of copepods were retained in the tank by a 30-μm sieve.

Survival and water quality were recorded for the probiotic trials. On day eight, tanks were drained through a 30-μm sieve retaining the copepods which were rinsed into 2-L of filtered SW to assess survival. The number of adult copepods was enumerated by taking a 1-mL aliquot from the 2-L sample and euthanizing the copepods with sodium hypochlorite (12%) prior to counting on a Sedgewick Rafter Cell. Three counts were averaged. Percentage survival was calculated at the end of each trial as $\%S = (S_t/S_0)*100$, where S_t is the number of copepods that survived by the end of the trial, and S_0 is the number of nauplii that were initially stocked.

Water quality parameters, including temperature, pH, salinity, dissolved oxygen, ammonia, alkalinity, hardness, nitrate, nitrite, pH, and phosphate, were measured daily to determine if treatments could be having an effect on water quality. Salinity was measured with a refractometer. Alkalinity, ammonia, hardness, nitrate, nitrite, pH, and phosphate were measured with a WaterLink Spin Touch FF (LaMotte, Chestertown, Maryland, United States), and temperature and dissolved oxygen were measured with a YSI ODO meter. Optimum ranges for temperature (25-28°C), pH (8.0-8.5), dissolved oxygen (>2 mg/L), and ammonia (<1ppm) have been identified for *P. crassirostris* (Anzeer et al. 2018; Santosh et al. 2018). These parameters were measured daily to ensure that optimal conditions were met.

In an effort to elucidate whether other factors might have existed that would have confounded the experimental results, a *post-hoc* trial was conducted simultaneously using three 100-L maturation tanks in which the "control" conditions (i.e. without probiotics) were used. Survival and water quality were recorded for the control tanks.

All statistical analyses were performed using program R version 4.0.5 (R Core Team, Boston, MA). The response of copepod survival to the fixed effects of tank, treatment and trial was analyzed using an ANOVA. The influence of tank, treatment, and trial on water quality parameters was analyzed using a MANOVA. *A priori* significance levels were set at $p < 0.05$. A t-test was used to compare survival of control groups from the first set of experiments to *post-hoc* control tanks run simultaneously.

3. RESULTS

The survival of nauplii to adults did not differ among probiotic treatment levels ($F_{df1,\ df2} = {}_{2,\ 6} = 0.06$, $p = 0.94$; Figure 5). Regardless of tank, trial, or treatment, copepods exhibited a mean survival rate 24.66% ± 10.75, but was highly variable ranging from 12%-41%. A *post-hoc* power analysis indicated that the β for the experiment was 0.09 and >4% difference in group means would be needed in order to detect an effect of treatment under this experimental design. Mean survival from initial control groups (26.6. ± 14.5) did not differ from mean survival in *post-hoc* control groups (19.9 ± 6.88, $df = 1$, $t = -0.72$, $p = 0.5$).

Water quality parameters remained consistent among treatment groups for dissolved oxygen (DO), alkalinity, pH, phosphate, calcium, magnesium, ammonia (NH_3), and nitrate ($F_{df1,df2} = {}_{2,\ 69} = 1.59$, $p = 0.06$, Table 3). However, mean DO, pH, magnesium, and ammonia (NH_3) concentrations were statistically different among trials ($F_{df1,df2} = {}_{2,\ 69} = 5.74$, $p < 0.001$, Table 3). Mean DO (6.23 mg/L), mean pH (7.93), and mean magnesium were highest during Trial 1 (1331 ppm, Appendix 1), and mean ammonia was lowest during Trial 3 (0.009 ppm, Appendix 1). Mean nitrite concentrations were consistently higher in Tanks 3 and 4 than Tanks 1 and 2 regardless of the treatment ($F_{df1,df2} = {}_{3,\ 68} = 2.01$, $p << 0.01$; Table 3).

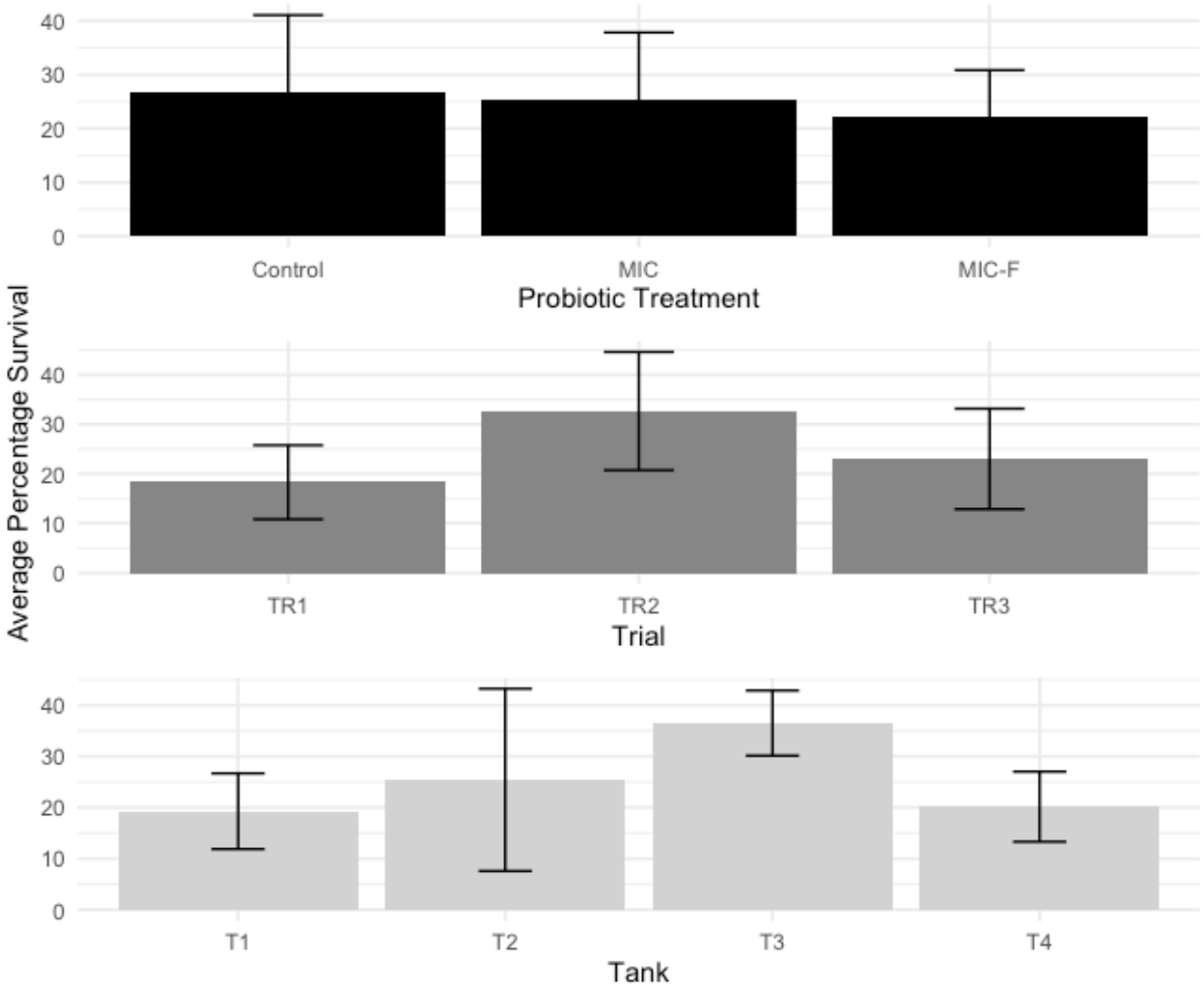


Figure 5. Mean percent survival (± SD) of *Parvocalanus crassirostris* nauplii to adults in experimental rearing tanks receiving INVE Sanolife ™ MIC (MIC, *n* = 3), INVE Sanolife ™ MIC-F (MIC-F, *n* = 3), or no probiotics (Control, *n* = 3) during 8-day trials conducted during November 2020-January 2021. No significant differences were detected across treatments, tanks, or trials ($p = 0.94$, $p = 0.78$, $p = 0.66$).

Table 3. Mean (± SD) temperature, dissolved oxygen (DO), salinity, alkalinity, pH, phosphate, calcium, magnesium, total available nitrogen (TAN), ammonia, nitrite and nitrate concentrations in experimental rearing tanks containing *Parvocalanus crassirostris* receiving INVE Sanolife ™ MIC (MIC; *n* = 3), INVE Sanolife ™ MIC-F (MIC-F, *n* = 3), or no probiotics (Control; *n* = 3) during 8-day trials conducted during November 2020-January 2021.

Treatment	Temperature (℃)	DO (ppm)	Salinity (ppt)	Alkalinity (ppm)	pH	Phosphate (ppm)
Control	26.59 ± 0.45	5.79 ± 0.80	31.5 ± 0.00	125.13 ± 7.62	7.84 ± 0.18	2.95 ± 0.71
MIC	26.49 ± 0.32	5.79 ± 0.53	31.5 ± 0.00	125.33 ± 7.06	7.78 ± 0.18	2.94 ± 0.71
MIC-F	26.76 ± 0.53	5.95 ± 0.49	31.5 ± 0.00	126.04 ± 7.17	7.85 ± 0.16	2.9 ± 0.76
Treatment	**Calcium (ppm)**	**Magnesium (ppm)**	**TAN (ppm)**	**Ammonia (ppm)**	**Nitrite (ppm)**	**Nitrate (ppm)**
Control	401.46 ± 17.00	1308.75 ± 61.56	0.61 ± 0.50	0.02 ± 0.02	0.43 ± 0.35	46.29 ± 18.76
MIC	403.75 ± 13.31	1294.75 ± 58.92	0.62 ± 0.52	0.01 ± 0.01	0.52 ± 0.51	41.79 ± 20.68
MIC-F	395.92 ± 17.84	1292.13 ± 49.24	0.65 ± 0.51	0.02 ± 0.01	0.3 ± 0.17	43.83 ± 19.65

Table 4. *p*-values from a MANOVA for temperature, dissolved oxygen (DO), alkalinity, pH, phosphate, calcium, magnesium, ammonia, nitrite and nitrate concentrations in experimental rearing tanks containing *Parvocalanus crassirostris* receiving INVE Sanolife ™ MIC (MIC, $n = 3$), INVE Sanolife ™ MIC-F (MIC-F, $n = 3$), or no probiotics (Control, $n = 3$) during 8-day trials conducted during November 2020-January 2021. Treatments were assigned to one of four tanks and randomized between runs. Significant differences were detected for temperature among treatments (df = 2, f = 4.06, $p = 0.02$). Significant differences were detected for temperature (df = 2, f = 21.06, $p << 0.001$), DO (df = 2, f = 17.15, $p << 0.001$), pH (df = 2, f = 20.57, $p << 0.001$), magnesium (df = 2, f = 7.50, $p < 0.01$), NH_3 (df = 2, f = 5.08, $p < 0.01$) and nitrate (df = 2, f = 4.95, $p = 0.01$) among trials. Significant differences for temperature (df = 3, f = 5.49, $p < 0.01$) and nitrites (df = 3, f = 8.66, $p << 0.001$) were detected among tanks.

Water Quality Parameter	p-value Treatment	p-value Trial	p-value Tank
Temperature	0.02	<<0.001	<0.01
DO	0.5	<<0.001	0.7
Alkalinity	0.91	0.62	0.76
pH	0.23	<<0.001	0.35
Phosphate	0.97	0.12	0.98
Calcium	0.23	0.09	0.95
Magnesium	0.51	<0.01	0.78
NH_3	0.59	<0.01	0.93
Nitrite	0.07	0.73	<<0.001
Nitrate	0.71	0.01	0.48

4. DISCUSSION

In an attempt to reduce high mortality rates during maturation in *Parvocalanus crassirostris*, INVE Sanolife ™ MIC (MIC, *n* = 3), INVE Sanolife ™ MIC-F (MIC-F, *n* = 3), or no probiotics (Control, *n* = 3) were applied to experimental rearing tanks during 8-day trials. There were no significant differences on mean nauplii survival to adult stages compared to the control group, suggesting that these probiotics were not effective at reducing mortality at the doses and duration tested (Figure 5). A *post-hoc* power analysis indicated that a >4% difference in group means would be needed to detect an effect of treatment; differences between treatments were <4% for these experimental trials. Given this outcome, additional trials were not justified.

There are several possible reasons that INVE Sanolife ™ MIC and MIC-F were ineffective as a probiotic for copepods under the experimental conditions. The ability of *Bacillus* spp. to affect a host is dose-dependent and applications of alternative doses could be effective (Hai 2015; Kuebutornye et al. 2019). The dose-effect relationship is a principal consideration as underdosing can be detrimental to the host (Sharifuzzaman & Austin 2017). INVE claims that there is no toxicity towards shrimp and fish even at doses >100 times the recommended dose. However, the effects on copepods have not been studied, and the increase in bacteria at higher doses could lead to low DO levels caused by oxygen consumption of bacteria. *Bacillus* spp. found in both of the INVE probiotics tested were isolated from terrestrial and aquatic sources. The use of terrestrial probiotics has had limited success in aquaculture (Ninawe & Selvin 2009). Alternatively, bacteria that can be isolated from the aquatic rearing environment such as the water, tank surfaces, biofilm, and the gastrointestinal tract would be more suitable for use as a probiotic in aquaculture (Vine et al. 2006; Ninawe & Selvin 2009). Lastly, it is plausible that the probiotics tested may not have worked due to the presence of bacteriophages. Bacteriophages are viruses that kill bacteria and if present in the water, could have inactivated the *Bacillus* spp. present in the probiotic (Mateus et al. 2014).

Considering that survival from nauplii to adult for *Parvocalanus crassirostris* has not been documented in the literature, it is difficult to evaluate whether the variation observed for survival in this experiment are typical or not. At the Oceanic Institute, nauplii survival to adult is

generally less than 50%, and is highly variable with numbers as low as 10% (C. Callan, personal communication, 2021). To better understand variability in copepod survival within this system without probiotic treatments, a *post-hoc* trial was run with three control tanks (no probiotic treatments). Variability in mean survival among *post-hoc* control groups was similarly high, ranging from 14%-27%. The mean survival from initial control groups and *post-hoc* control groups did not differ (19.9 ± 6.88, $df = 1$, $t = -0.72$, $p = 0.5$). The research conducted at PACRC corroborated the low and variable survival associated with copepods at Oceanic Institute and given this degree of variability, pursuing further trials with these brands of probiotics is probably not merited.

The most critical problem in copepod culture remains the low and variable survival to maturation. Gaining a better understanding the causes of variability in mortality is necessary step in efficient production. Future studies should aim to identify the causes of the high and variable mortality rates. There are several factors that could be examined such as handling stress, microalgae quality, and suboptimal rearing conditions particularly having to do with water quality. Although water quality parameters were examined in this study, it would be beneficial to conduct a time series of the variables over the course of the entire experiment instead of a single daily reading because water quality parameters are dynamic and could negatively affect survival. It is important to note that although significant differences were detected among treatments, trials and tanks for various water quality parameters (Table 4), none of the differences corresponded to lower survival. Further, microalgae quality and handling stress can cause differences in copepod survival, but these can be hard to quantify. The effect of microalgae quality on copepod survival has been examined in the context of nutrient concentrations and feed densities; however, it would be beneficial to examine algal health indicators such as motility, flocculation, bacteria levels, and ciliate levels to help understand if variations in algal quality are affecting copepods. Harvesting copepods on a sieve contributes to handling stress but because harvest methods are usually done by different individuals, it is difficult to standardize this process under standard aquaculture production conditions.

In conclusion, although no significant results for survival were observed with probiotics administration, the current study represents an area of novel research that could be further explored to improve commercial production. The commercial probiotics chosen for this study were selected based on their efficacy with certain species of crustacea, but this didn't translate to a beneficial effect in copepods. These results suggest caution in assuming that probiotics will have similar effects on species within the same subphylum, although it is common in aquaculture to rely on commercial products that may not be well-tested as prophylactics since so few products are available for some types of aquaculture. Rather than rely on the currently available commercial probiotics designed for shrimp culture, future work should focus on isolating bacteria from thriving copepod cultures to be evaluated as putative probiotic candidates to increase production yields. This method has shown to be an effective strategy to isolate effective probiotic cocktails for use in other aquaculture production systems, such as shellfish (C. Schubiger, personal communication, 2021). Since copepod production continues to be a crucial bottleneck to marine larval fish production, future studies should aim to identify sources of variability in survival during maturation and explore the use of probiotics as a means to control this variability where appropriate.

6. APPENDICES

Figure A.1 Mean temperature (°C) by treatment, trial, and tank in experimental rearing tanks containing *Parvocalanus crassirostris* receiving INVE Sanolife ™ MIC (MIC; *n* = 3), INVE Sanolife ™ MIC-F (MIC-F, *n* = 3), or no probiotics (control; *n* = 3) during 8-day trials conducted during November 2020-January 2021.

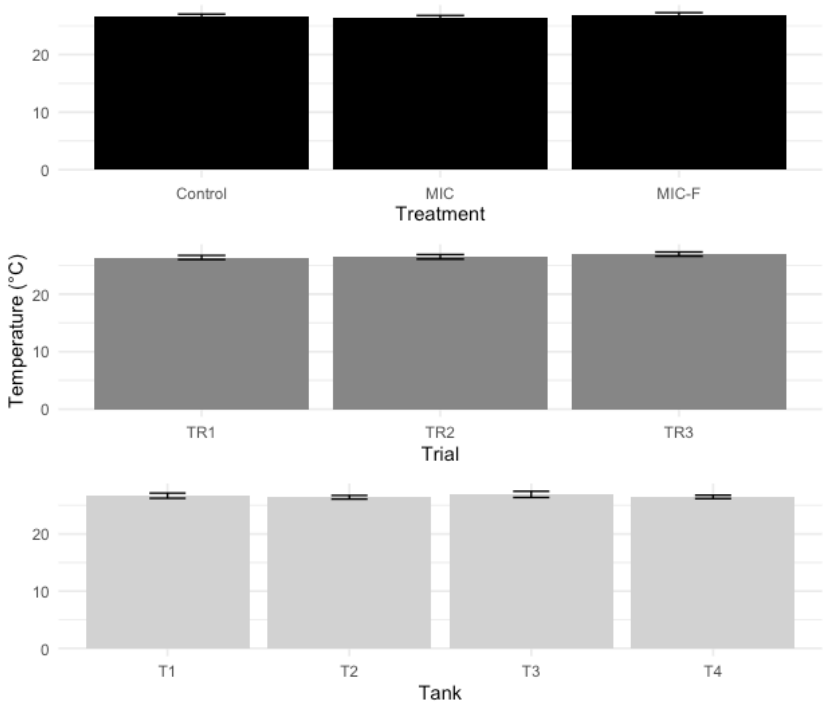


Figure A.2 Mean dissolved oxygen ± SD (mg/L) by treatment, trial, and tank in experimental rearing tanks containing *Parvocalanus crassirostris* receiving INVE Sanolife ™ MIC (MIC; *n* = 3), INVE Sanolife ™ MIC-F (MIC-F, *n* = 3), or no probiotics (Control; *n* = 3) during 8-day trials conducted during November 2020-January 2021.

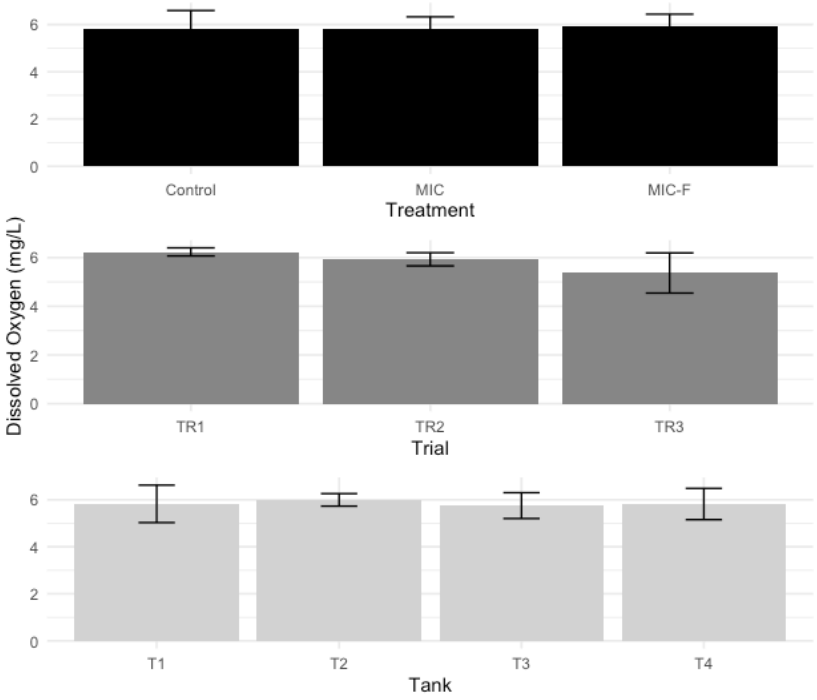


Figure A.3 Mean alkalinity ± SD (ppm) by treatment, trial, and tank in experimental rearing tanks containing *Parvocalanus crassirostris* receiving INVE Sanolife ™ MIC (MIC; *n* = 3), INVE Sanolife ™ MIC-F (MIC-F, *n* = 3), or no probiotics (Control; *n* = 3) during 8-day trials conducted during November 2020-January 2021.

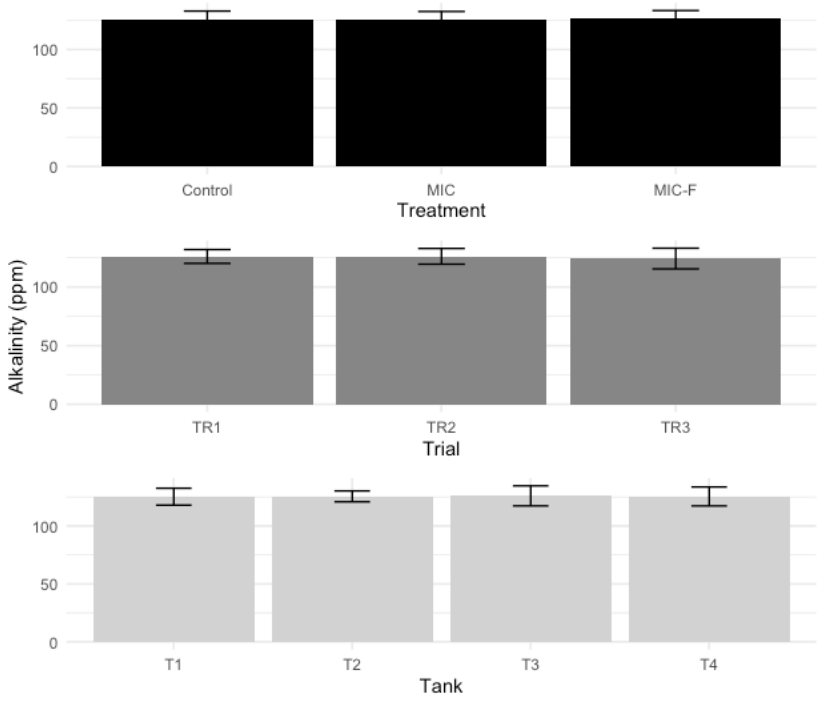


Figure A.4 Mean pH ± SD by treatment, trial, and tank in experimental rearing tanks containing *Parvocalanus crassirostris* receiving INVE Sanolife ™ MIC (MIC; *n* = 3), INVE Sanolife ™ MIC-F (MIC-F, *n* = 3), or no probiotics (Control; *n* = 3) during 8-day trials conducted during November 2020-January 2021.

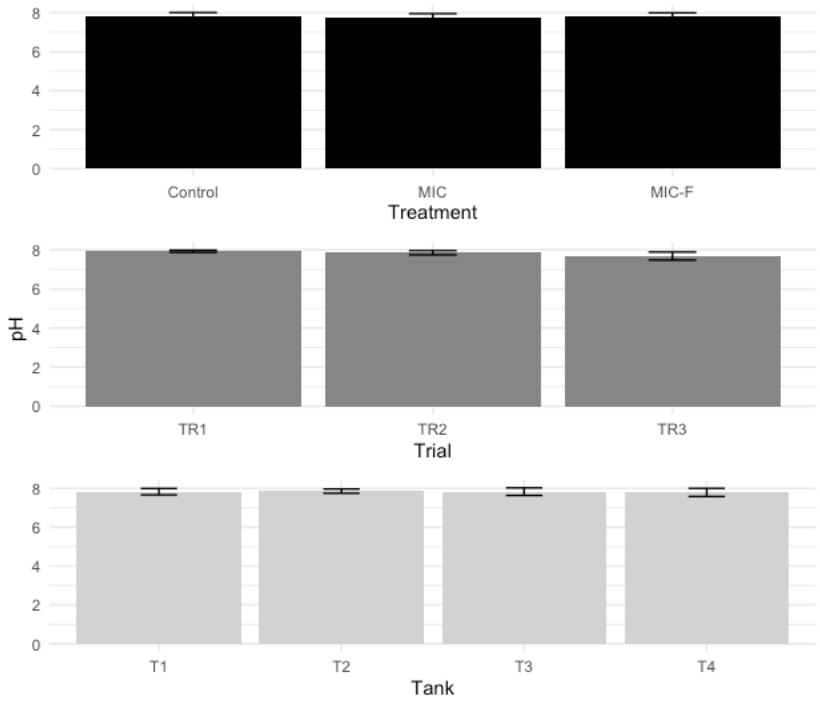


Figure A.5 Mean phosphate ± SD (ppm) by treatment, trial, and tank in experimental rearing tanks containing *Parvocalanus crassirostris* receiving INVE Sanolife ™ MIC (MIC; *n* = 3), INVE Sanolife ™ MIC-F (MIC-F, *n* = 3), or no probiotics (Control; *n* = 3) during 8-day trials conducted during November 2020-January 2021.

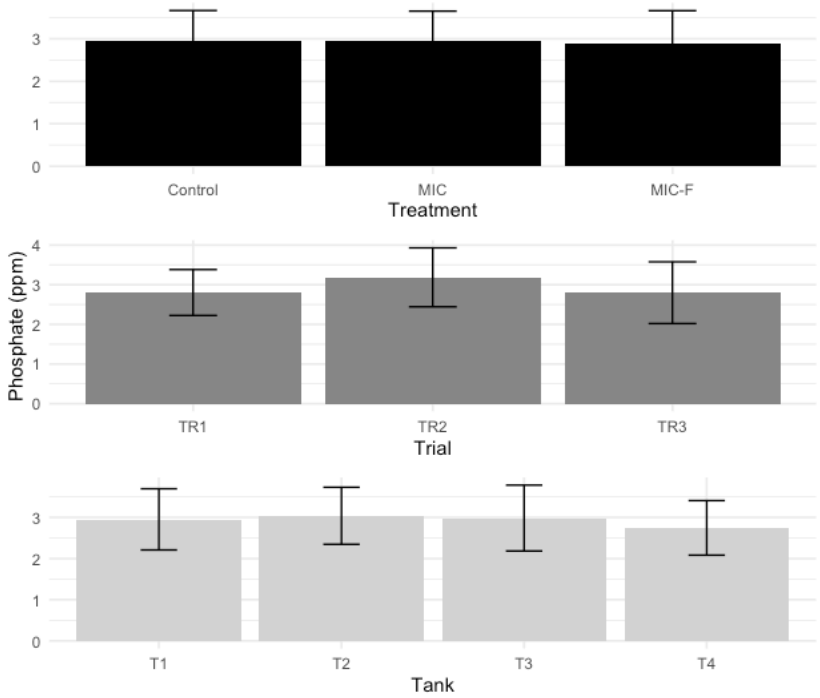


Figure A.6 Mean calcium ± SD (ppm) by treatment, trial, and tank in experimental rearing tanks containing *Parvocalanus crassirostris* receiving INVE Sanolife ™ MIC (MIC; *n* = 3), INVE Sanolife ™ MIC-F (MIC-F, *n* = 3), or no probiotics (Control; *n* = 3) during 8-day trials conducted during November 2020-January 2021.

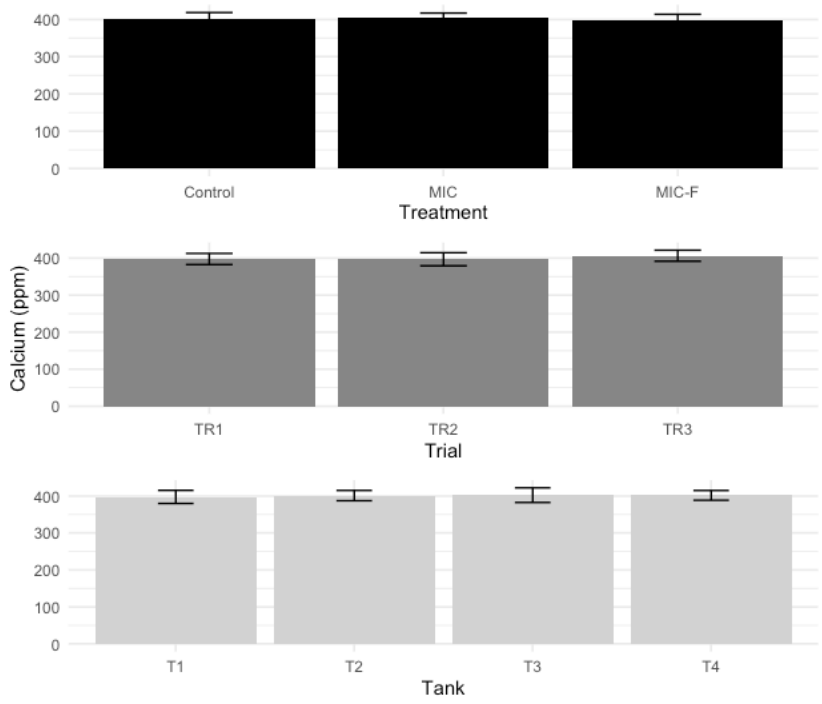


Figure A.7 Mean magnesium ± SD (ppm) by treatment, trial, and tank in experimental rearing tanks containing *Parvocalanus crassirostris* receiving INVE Sanolife ™ MIC (MIC; *n* = 3), INVE Sanolife ™ MIC-F (MIC-F, *n* = 3), or no probiotics (Control; *n* = 3) during 8-day trials conducted during November 2020-January 2021.

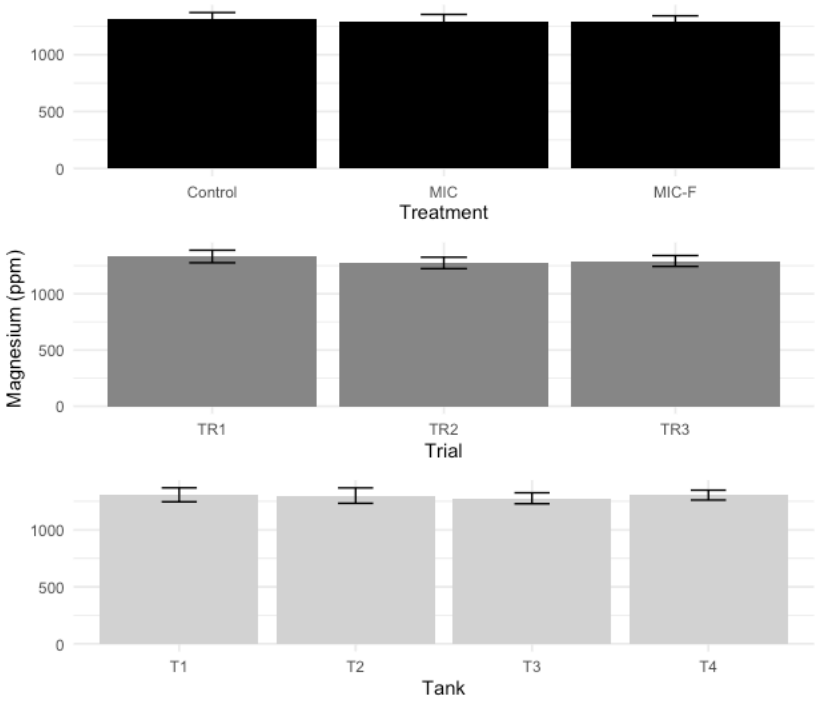


Figure A.8 Mean ammonia ± SD (ppm) by treatment, trial, and tank in experimental rearing tanks containing *Parvocalanus crassirostris* receiving INVE Sanolife ™ MIC (MIC; *n* = 3), INVE Sanolife ™ MIC-F (MIC-F, *n* = 3), or no probiotics (Control; *n* = 3) during 8-day trials conducted during November 2020-January 2021.

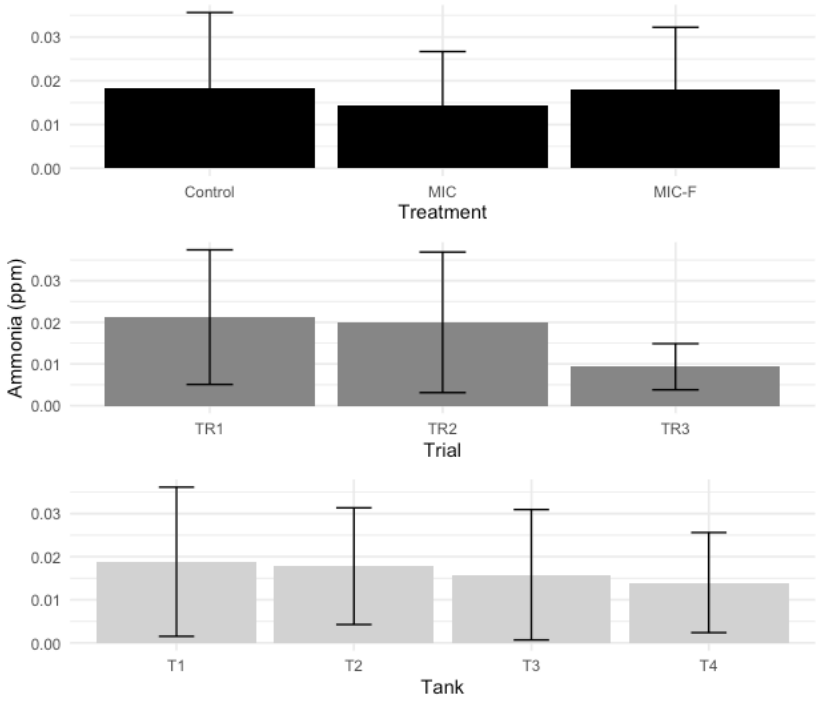


Figure A.9 Mean nitrite ± SD (ppm) by treatment, trial, and tank in experimental rearing tanks containing *Parvocalanus crassirostris* receiving INVE Sanolife ™ MIC (MIC; *n* = 3), INVE Sanolife ™ MIC-F (MIC-F, *n* = 3), or no probiotics (Control; *n* = 3) during 8-day trials conducted during November 2020-January 2021.

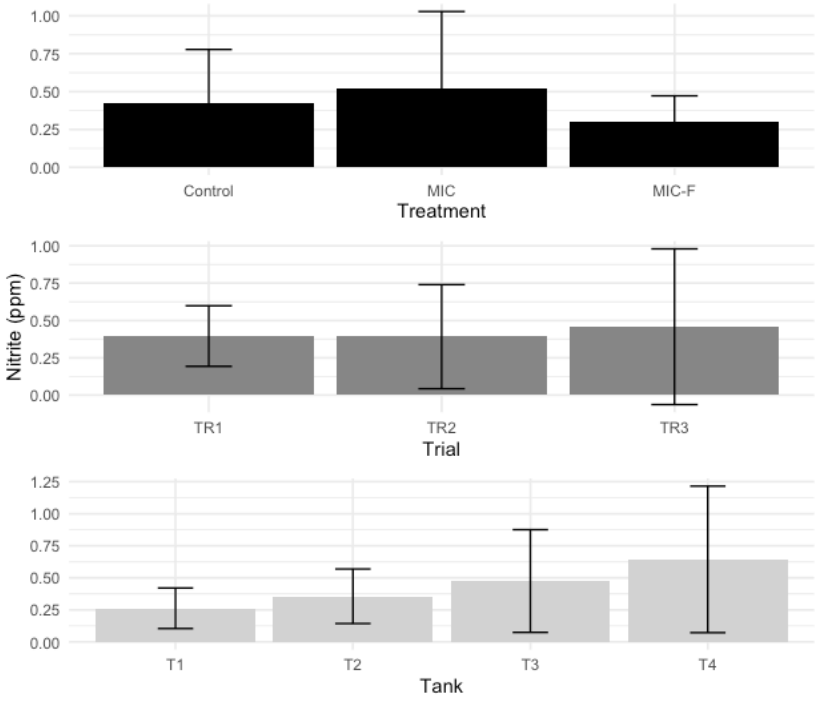
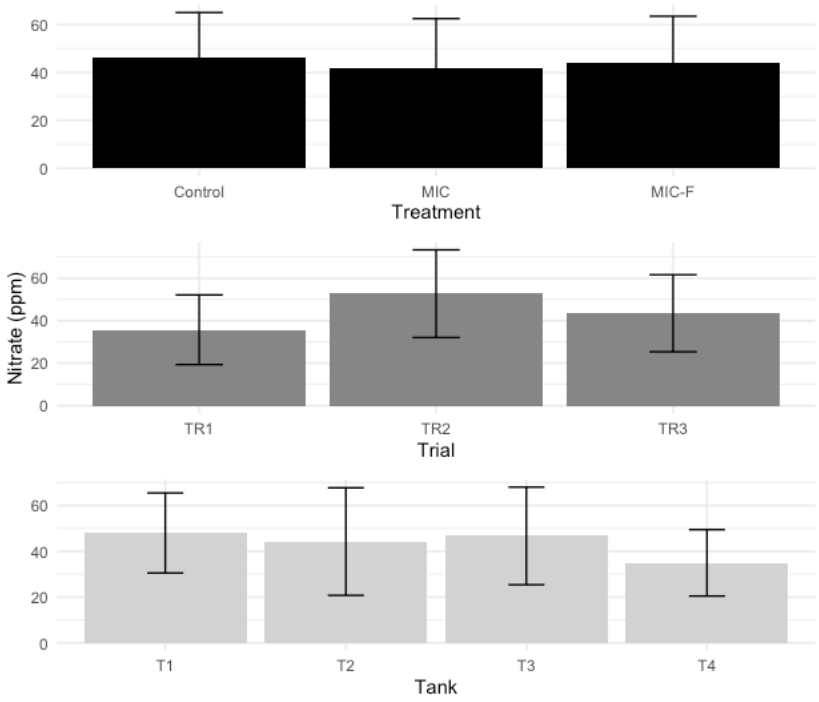


Figure A.10 Mean nitrate ± SD (ppm) by treatment, trial, and tank in experimental rearing tanks containing *Parvocalanus crassirostris* receiving INVE Sanolife ™ MIC (MIC; *n* = 3), INVE Sanolife ™ MIC-F (MIC-F, *n* = 3), or no probiotics (Control; *n* = 3) during 8-day trials conducted during November 2020-January 2021.



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