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White Abalone (Haliotis sorenseni) Restoration Aquaculture: An Assessment of Formulated Diets and Probiotics

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WHITE ABALONE (*HALIOTIS SORENSENI*) RESTORATION AQUACULTURE: AN ASSESSMENT OF FORMULATED DIETS AND PROBIOTICS

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To my friends and family, who helped and encouraged me in my graduate studies and thesis work.

ABSTRACT

White Abalone (*Haliotis sorenseni*) Restoration Aquaculture: An Assessment of Formulated Diets and Probiotics
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White abalone (*Haliotis sorenseni*) are an endangered species found along the California coast. They are at historically low densities, nearing extinction. Aquaculture facilities throughout California are currently involved in the captive breeding of the species and grow out of juveniles for outplanting to wild habitat. White abalone have historically suffered from an infectious bacterial disease known as withering syndrome or Ca.Xc. This disease can be treated with an antibiotic, but antibiotic treatments can lower immune functions and create antibiotic resistance genes. A probiotic treatment could replace this need for antibiotics and increase overall health and growth rates in white abalone. White abalone restoration activity aims to enhance the species recovery by developing self-sustaining populations, which is costly due to the species' slow growth, high early mortality rate, and reliance on seasonal macroalgae feed. These limiting factors warrant an assessment of alternative diets and probiotic treatments to shorten the culture time and lower costs before outplanting. Diet administered probiotics have previously shown improved growth rates, feed digestibility, and survivorship in abalone species, while formulated feeds can provide adequate nutrition and reduce costs for several cultured species.

The probiotic, *Bacillus licheniformis* was added to macroalgae feed for white abalone exposed to and unexposed to Ca.Xc. No Ca.Xc was detected in any of the white abalone at the end of the study. This indicated that white abalone may be able to combat Ca.Xc with a bacteriophage (p*C*Xc). Shell loss during the first probiotic experiment hindered data collection on feeding and growth rates. In the first probiotic study 42% of the white abalone and 53% of the red abalone lost their shells. Two subsequent studies were conducted to understand the cause of the shell loss. These studies were inconclusive, but one experiment showed that low stocking density (588 abalone/m^2) increased feeding and growth rates compared to high densities (1,176 abalone/m^2).

The second probiotic study investigated the effects of *B. licheniformis* on a formulated abalone feed, ABKelp®. Three diet treatments were assessed: 1) formulated, 2) formulated + probiotic, 3) standard (*Devaleraea mollis* and *Macrocystis pyrifera*). The standard treatment resulted in the fastest growth and feed intake. The formulated + probiotic treatment had the lowest growth and feeding rates, which may be due to reduced palatability from the probiotic. Despite comparatively inferior growth metrics, the formulated treatment resulted in adequate growth and survivorship in white abalone. The use of a formulated diet is feasible for white abalone restoration aquaculture when considering additional costs associated with fresh macroalgae feeding, including permits, diving, and boat operations for *M. pyrifera* collection and culture facilities devoted to macroalgae culture and storage. The growth rates, feeding rates, and proximate analysis suggest that formulated feed is not a viable alternative diet for white abalone, but could be used as a supplemental feed for conservation aquaculture facilities with limited access to fresh macroalgae or during times of the year when fresh feed is unavailable.

TABLE OF CONTENTS

List of Tables

Page

List of Figures

Background and Rationale

Threats to Abalone

Abalone populations throughout the world are in decline (Rogers-Bennet et al., 2002a). For generations, abalone have been utilized as a resource by humans. They were used for food, jewelry, decorations, ceremonial purposes, spiritual practices, and alternative medicine. During the 1900s, abalone were commercially harvested and prized for their meat and shells. This led to overfishing and a subsequent decline in their populations. Currently, there is significant concern for wild abalone populations. Discussions on rebuilding these populations are taking place in Australia, Canada, Japan, South Africa, and the United States (Campbell, 2000). Along the West Coast of the U.S. there are now six abalone species considered species of concern or endangered: flat abalone (*Haliotis walallensis*), pinto abalone (*Haliotis kamtschatkana*), black abalone (*Haliotis cracherodii*), green abalone (*Haliotis fulgens*), pink abalone (*Haliotis corrugata*) and white abalone (*Haliotis sorenseni*) (Vileisis, 2020).

Along the California coast red, green, pink, black, and white abalone were heavily fished in California up until 1969, when these landings decreased because of significantly reduced abalone populations (Karpov et al., 2000). There was a total take of 2,000 tons of red and pink abalone from 1950-1970 (Karpov et al., 2000). Red and pink abalone were the shallower and thus most popular abalone species taken, but when these populations declined to unsustainable numbers deeper species were targeted including white abalone (Karpov et al., 2000). Beyond the well documented fishing pressure on abalone populations, environmental stressors have further contributed to the decline in abalone numbers (Wessel et al., 2018). Warming waters associated with climate change have been shown to impact shell formation and development in larval stages, followed by later shell dissolution of juvenile and adult abalone due to ocean acidification

(Wessel et al., 2018). These environmental factors and disease have severely depleted the population sizes of many abalone species, but have particularly led to the near extinction of white abalone, the focus of this study.

White Abalone

White abalone (*Haliotis sorenseni*) are mollusks in the class Gastropoda, order Vetigastropoda, and family Haliotidae. They are found along the West Coast of North America from Southern California to Baja California (Stierhoff et al., 2012). White abalone occupy rocky substrates at depths from 15-50 m, with most found between 25-30 m (Stierhoff et al., 2012). The species can live up to 35 years (Stierhoff et al., 2012) and grows to a maximum of 770 g and 250 mm in shell length (NOAA Fisheries). Abalone in the North Pacific typically graze on drifting brown kelp species and have been shown to influence algal and invertebrate communities through their feeding activities and space use (Hamer et al., 2010). The loss of abalone may lead to other invertebrate species colonizing these environments, with effects on algal and invertebrate community structure (Hamer et al., 2010).

Threats to White Abalone Survival

White abalone have experienced drastic population declines due to overharvesting. During 1969-1978, an estimated 263 tons or 362,759 adult white abalone were harvested along the California coast (Stierhoff et al., 2012). They were commonly targeted following the serial depletion of other abalone species between 1970-1997, at which point a moratorium on fishing occurred for both the commercial and recreational abalone sectors (Karpov et al., 2000; Burton & McCormick, 2008). White abalone are one of the most at-risk abalone species in the world with only about 1% remaining of their previous estimated historical population size of 360,000 individuals in the United States (Rogers-Bennet et al., 2002b). As their populations continued to

decline, white abalone became the first marine invertebrate to be recognized under the Endangered Species Act of 2001 (Burton & McCormick, 2008). Despite these protections, white abalone populations in California further declined, with a standing population of around 15,000 declining to below 5,000 individuals at Tanner Banks near Catalina Island by 2014 (Catton et al., 2016).

Overfishing was the main reason for the near extinction of white abalone, but certain life history characteristics of white abalone also contribute to their unsuccessful population recovery to date. The species has slow growth and maturation rates, usually reaching sexual maturity between 4-6 years or 88-143 mm in shell length (Tutschulte & Connell, 1981). As broadcast spawners, their current low population densities and limited mobility reduces their rate of successful gamete fertilization during spawning events (Rogers-Bennet et al., 2002a). Males and females are required to be within a few meters of each other for successful fertilization. Scientists estimate that only 11% of remaining individuals in the wild are within relative proximity to each other to result in reproductive success (Hobday et al., 2001). The current large distances between males and females in the wild reduces the probability of the suspended gametes coming into contact and fertilizing within the water column. White abalone also spawn seasonally from February to April with one gametic cycle a year (Tutschulte & Connell, 1981).

Research suggests that there has not been significant recruitment of white abalone since the 1960s (Stierhoff et al., 2012). Small population sizes from limited successful recruitment events reduce gene flow, which increases inbreeding (Anders, 1998). This likely increases the species susceptibility to succumb to future unforeseen threats like environmental change or disease (Anders, 1998). The white abalone is more sedentary compared to other species, like the green and pink abalone; thus, the possibility for immigration or aggregation to increase encounters of

individuals is very unlikely (Stierhoff et al., 2012). Taken together, the reproductive biology, short larval dispersal, and sedentary nature compounds the external threats to the species.

Additional threats to white abalone health and survival include environmental changes such as ocean acidification, warming temperatures, and bacterial infections (Wessel et al., 2018). Ocean acidification increases mortality and developmental deformities. Shell mineralization in abalone utilizes amorphous calcium carbonate and aragonite deposition. Ocean acidification significantly alters the solubility of these compounds, limiting their availability for abalone to build their shells and decreasing survivorship (Wessel et al., 2018). Along with the increased acidic conditions in the ocean, warming ocean temperatures also hinder abalone survival. Warmer oceans exacerbate bacterial infections in white abalone and increase death rates compared to abalone with the same infection under lower water temperatures (Wessel et al., 2018).

While there are many threats to white abalone and intrinsic characteristics of the species that threaten its survival, restoration aquaculture could aid in its recovery. The small populations of wild white abalone could be supplemented through restoration aquaculture practices until their population reaches a self-sustaining critical mass. Restoration aquaculture has been successfully employed for other endangered species in the past with a notable example being the white sturgeon (*Acipenser transmontanus*). The white sturgeon suffered from drastic population declines and the threat of extinction before aquaculture methods were used to supplement and increase their wild populations (Anders, 1998).

White Abalone Restoration

The drastic decline in white abalone populations has led to the implementation of the White Abalone Project at the University of California Davis, Bodega Marine Laboratory. The White

Abalone Project is working to raise white abalone in captivity and outplant them into the wild. The recovery plan includes monitoring current wild populations, breeding captive white abalone, and outplanting cultured white abalone. The White Abalone Project partners with facilities along California coast including Moss Landing Marine Laboratories (MLML), Aquarium of the Pacific, Cultured Abalone Farm, Cabrillo Marine Aquarium, NOAA's Southwestern Fisheries Science Center, The Bay Foundation, California Department of Fish and Wildlife, and the National Marine Fisheries Service. These organizations receive white abalone embryos spawned at Bodega Marine Labs. The embryos are transported to the partner facilities where they are reared to 20 mm in shell length before being outplanted into the wild in San Diego.

To overcome the current bottlenecks to successful natural recruitment, the White Abalone Project takes adult white abalone isolated from other white abalone of reproductive size from the wild and spawns them in captivity. Despite the application of aquaculture technology, the growth rates of white abalone complicate this restoration aquaculture work because white abalone grow at slow and varying rates compared to other abalone species. It takes multiple years for the abalone to reach outplanting size. Individual growth rates vary, meaning that some abalone may take twice as long as others to reach outplating size. During the many years it takes to grow-out white abalone, they are susceptible to disease, stress, and mortality. They also require increasing amounts of food, which is a limiting factor in white abalone restoration aquaculture.

White abalone in culture are typically fed fresh giant kelp (*Macrocystis pyrifera*) and the red algae, dulse (*Devaleraea mollis*), which are either collected from the wild or grown in aquaculture. Wild stocks of *M. pyrifera* are seasonally depleted by winter storms and wave action in the wild, while *D. mollis* is typically cultured via tank-based tumble culture, and as such is limited in production depending on culture capacity. Urchin barrens have depleted wild

M. pyrifera, further reducing its availability for white abalone feed (Wernberg, 2018). The low light conditions in the winter months limit the growth and supply of both wild and cultured *M. pyrifera* and *D. mollis* (Stephens & Hepburn, 2014). During the winter, White Abalone Project partners struggle to supply their white abalone with enough feed. Additionally, there are many costs associated with the collection of *M. pyrifera* including permitting and boat operations. Cultured *M. pyrifera* and *D. mollis* require culture space, tanks, and culture care, increasing the abalone feeding cost. Costs also occur when the fresh *M. pyrifera* must be shipped to partner organizations to supply feed for the abalone in each facility. A formulated feed could provide an alternative feed during winter months when fresh seaweed is limited. A formulated feed could reduce the cost of feeding abalone and increase operational resilience if used as a replacement or supplement for fresh seaweed feed.

Beyond the limited feed supply, the health of white abalone reared in aquaculture is a concern. White abalone are a fragile species that suffer high mortality rates (Vater et al., 2018). Specifically, juvenile white abalone in their young developmental stages pre- and post-settlement are susceptible to high rates of disease and mortality. Research into the disease and overall health of the cultured white abalone is vital to the success of this aquaculture restoration plan and survival of this species. The small population size of white abalone limits their reproductive success, necessitating captive rearing of the species to enhance wild populations. The goal of this restoration work is to ultimately increase the population size so that it is self-reliant. One of the most significant health conditions ailing captive white abalone is withering syndrome.

Withering syndrome lowers digestion rates, leading to muscle atrophy, a withering appearance, and eventual death of the abalone. Some abalone species have been less affected by withering syndrome through genetic diversity and a bacteriophage that targets the bacterial infection

(Brokordt et al., 2017; Vater, 2017; Vater et al., 2018). White abalone are believed to not have this same genetic diversity or the bacteriophage (Brokordt et al., 2017; Vater, 2017; Vater et al., 2018). The current antibiotic treatment, Oxytetracycline (OTC), has been shown to significantly reduce the impacts of withering syndrome in white abalone (Parker-Graham et al., 2020). The antibiotic also reduces the health of the animal and the environment through runoff (Jang et al., 2018). Probiotics have been used to treat and prevent bacterial infection in livestock and seafood (Patterson & Burkholder, 2003). They have been used to improve the growth rates and health of other abalone species, but have not been assessed in white abalone (Gao et al., 2018; 2020). Probiotics could reduce antibiotic use to and provide other health and growth benefits.

The first probiotic study in Chapter 1 of this thesis used *Bacillus licheniformis* to assess its impact on white abalone survivorship, feeding rates, growth rates and ability to combat Ca.Xc infections in white abalone. This first probiotic study was interrupted by shell loss syndrome, which spread thought the study species (white abalone) and Ca.Xc infecting agent (red abalone), hindering data collection. Two shell loss challenge experiments were conducted to investigate the cause of the shell loss. One shell loss challenge experiment assessed shell loss in white abalone. The other experiment assessed shell loss in red abalone and the impact of tank density on abalone shell loss, growth rates, and feeding rates. These shell loss challenge experiments are discussed in Chapter 2. Chapter 3 covers the second probiotic study and alternative feed experiment, which assessed *B. licheniformis* impact on feeding and growth rates without the impacts of shell loss. This study also assessed an alternative feed to replace or supplement seasonally limited seaweed currently fed in white abalone restoration. This study evaluated the effects of feeds and probiotics on endangered white abalone, which may contribute to future restoration efforts for their populations

Chapter 1: First Probiotic Study

Introduction

Withering Syndrome: Causes and Susceptibility in Abalone Species

Withering syndrome in abalone is caused by an infection of *Candidatus Xenohaliotis californiensis* bacterium (Ca.Xc). The first instance of withering syndrome was detected in black abalone (*Haliotis cracherodii*) along the Channel Islands in 1985, but the bacteria causing the disease was not described for several years (Crossen et al., 2014). Ca.Xc infections are characterized in abalone by the presence of cellular inclusions within the digestive epithelia of the gut and post-esophagus (Friedman et al., 2000; Friedman & Crosson, 2012). Infections of Ca.Xc result in a shrunken foot, reduced digestive gland, and lowered feeding rates (Friedman et al., 2000). This infection hinders the ability of abalone to secrete digestive enzymes and absorb nutrients. Since infected abalone are unable to digest food effectively, the organism begins to catabolize its own tissue for sustenance (Friedman et al., 2000). This causes a withering effect in the abalone foot, mantle, and digestive tract, which is the source of the disease's initial name, withering syndrome. The epithelial infections can be detected in the diverticula of the intestine and post-esophagus (Crossen et al., 2014). Atrophy occurs in the abalone foot due to catabolism of the pedal muscle and if left untreated this infection will lead to mortality of the abalone. The bacterium occurs within numerous abalone species and is also free-living in the water column along the California coast (Crossen et al., 2014). The infection is transmitted fecal-orally among individuals and thus can be spread though the water column (Crossen et al., 2014).

Infection of Ca.Xc is more extensive and deadly within white abalone populations compared to other abalone species, like red and black abalone (Brokordt et al., 2017; Vater, 2017; Vater et al., 2018). Vater et al. (2018) reported that white abalone infected with Ca.Xc in warm water

(18.5°C) had mortality rates 10.9 times higher than infected red abalone held at the same temperature. The combination of the Ca.Xc infection and increased water temperature resulted in 100% mortality in the white abalone at 250 days, while 91% of the infected red abalone reared in an elevated water temperature survived at 350 days (Vater et al., 2018). This higher resistance to the Ca.Xc infection in the red abalone could be due to the presence of bacteriophage in this species that targets Ca.Xc.

A bacteriophage, known as *Xenohaliotis* phage (p*C*Xc), has been detected within Ca.Xc bacteria (Cruz-Flores et al., 2018). p*C*Xc is a virus that inhibits the bacteria's ability to reproduce, ultimately aiding in the reduction and potential eradication of the infecting Ca.Xc bacteria. Black and red abalone containing both Ca.Xc and the phage have shown a reduction in the Ca.Xc bacterial loads shed from the abalone (Brokordt et al., 2017; Vater et al., 2018). p*C*Xc has been documented to occur in white abalone, but the phage does not appear to protect the white abalone from the Ca.Xc infection in the same way as in other abalone species (Vater, 2017, Vater et al., 2018). Instead white abalone are still susceptible to high Ca.Xc infection rates and death even with p*C*Xc present (Vater et al., 2018).

Genetic variation between abalone species is hypothesized to play an important role in resistance to the Ca.Xc bacteria. The genetics of the infecting bacteria and the host play roles in the infection rates of the bacteria (Brokordt et al., 2017). Some species are highly resistant to the bacterial infection including pink abalone (*Haliotis corrugata*), green abalone (*Haliotis fulgens*) and variously coloured abalone (*Haliotis diversicolor*) due to specific genes in these species, which resist the bacteria (Moore et al., 2009; Wetchateng et al., 2010; Brokordt et al., 2017). Disk abalone (*Haliotis diversicolor supertexta*) appear not to be susceptible to the Ca.Xc bacterial infection at all, which is attributed to their genetic composition (Wetchateng et al.,

2010; Brokordt et al., 2017). Genetic variability and heritability in resistance to Ca.Xc has been detected in red abalone (*Haliotis rufescens*) (Brokordt et al., 2017). Similar genes or a nonspecific genetic resistance to Ca.Xc have not been found in white abalone (Wetchateng et al., 2010).

No specific mortality rates of wild white abalone have been attributed to Ca.Xc, but a high infection and mortality rate in captive white abalone has been reported (Vater et al., 2018). The prevalence of Ca.Xc bacteria within abalone increases in relation to warm water conditions. Significant increases in Ca.Xc loads in abalone are found at 18°C in many different abalone species (McCormick et al., 2016). In white abalone, Ca.Xc loads have been observed to increase at temperatures of 15°C or higher (Burton & McCormick, 2008). Warming water temperature and natural phenomena like El Niño events, which also raise water temperatures, are correlated with increased bacterial loads of Ca.Xc within abalone (Tissot, 1995; Altstatt et al., 1996; Raimondi et al., 2002). The warmer water temperatures and lack of resistance to the Ca.Xc bacteria are theorized to cause higher mortality rates in white abalone compared with other abalone species (Vater, 2017).

Antibiotic Treatment for Ca.Xc

The only reported effective treatment for the Ca.Xc bacterial infection to date is an administration of antibiotic Oxytetracycline (OTC). OTC has been shown to significantly reduce Ca.Xc within white abalone (Parker-Graham et al., 2020). OTC can be administered to abalone through baths and infused feed. OTC is one of only three antibiotics approved for aquaculture use in the U.S. (Benbrook, 2002). This limited antibiotic selection is partly due to concerns for non-targeted and prophylactic effects of antibiotic treatments in open environments (Benbrook, 2002). Antibiotics can kill beneficial bacteria within the organism as well as the infecting

bacteria. Reduced gut flora can diminish digestion and growth efficiency, similar to the effects of the Ca.Xc bacteria. Additionally, the overuse of antibiotics can lead to bacterial resistance in the form of Antibiotic Resistance Genes (ARGs). ARGs can be transferred between marine bacterial species (Jang et al., 2018) and to the gut flora that consume the bacteria in the human intestine (Centner, 2016). This transmission reduces antibiotic effectiveness to treat pathogens in animals and humans over time and is recognized nationally and globally as a health crisis. Antibiotic resistance led to an estimated thousand human deaths worldwide in 2013 (Centner, 2016).

Antibiotics can create harmful effluent if they are released into the environment. Jang et al. (2018) reported a significantly high number of ARGs in wild flatfish offshore of aquaculture facilities in South Korea. These ARGs were attributed to the runoff of antibiotic treated waters and animals in the offshore aquaculture facilities (Jang et al., 2018). An estimated 75% of antibiotics used in aquaculture, including OTC, make their way into the natural environment from fish farms (Bôto, 2016). Effluent antibiotics can create antibiotic resistance and health issues in other wild species. The presence of the effluent antibiotics in the wild alters the bacterial communities found in the water column and sediments (Bôto, 2016).

Detrimental health and environmental issues related to antibiotic use have shifted the public perception of this once revolutionary treatment. Antibiotics are no longer seen as the solution to disease, but as a last resort for treating bacterial infections. This viewpoint of conservative antibiotic use is prevalent among the general public as well as legislative bodies around the world. Strict regulations with monetary fines for the release of antibiotic effluent are present in Portugal, China, the European Union, and the United States (Broughton & Walker, 2010; Bôto, 2016; Liu, 2017).

Probiotics for Growth, Overall Health, and Ca.Xc in Abalone

Antibiotics are no longer accepted as an effective prophylaxis measure because of the multiple issues associated with antibiotic treatments. In contrast, probiotics have been shown to be an effective alternative to antibiotic use for both preventative and therapeutic measures, by creating competitive exclusion against foreign pathogens (Macey & Coyne, 2006). Additionally, probiotics are able to provide nutrients, increase digestive enzyme activity, promote growth, improve feeding rates, and support immune health within the organism (Macey & Coyne, 2006). *Lactobacillus* sp., *Bacillus* sp., *Enterococcus* sp., and *Saccharomyces* yeasts have been administered to livestock for many years to successfully treat and prevent bacterial infections and improve overall health (Patterson & Burkholder, 2003). Several studies have found health benefits from *Lactobacillus* sp., *Bacillus* sp., *Bacillus subtilis, and Bacillus licheniformis* when used to treat livestock and seafood species (Table 1). These probiotics along with others have been successful in improving growth rates, increasing immune response, and reducing bacterial infections in various abalone species (Table 2).

Table 1. Probiotic use in livestock and seafood production. Literature review of organisms treated with a probiotic and the impact metrics of the probiotic assessed in the study.

Probiotics	Treated Organism	Impact	Study
Lactobacillus sp.	Poultry	Improved growth and feeding rates	Jin et al., 1997
Bacillus sp.	Poultry	Reduced *Salmonella* and *Clostridium* in poultry	Tellez et al., 2012
Bacillus subtilis F6 and *Enterococcus* sp. S2	Black tiger shrimp *(Penaeus monodon)*	Increased enzyme production and activity as well as increased growth	Nimrat et al., 2013
Bacillus subtilis	Rainbow trout *(Oncorhynchus mykiss)*	Improved respiratory burst and intestinal lysozyme activity (immune response) and reduced mortality	Newaj-Fyzul et al., 2014
Bacillus subtilis	Black tiger shrimp (*Penaeus monodon*)	Reduced the infections *vibrio* pathogens	Vaseeharan & Ramasamy, 2003
Bacillus subtilis	Cattle	Increased antibody production and immune response	Chen et al., 2013
Bacillus subtilis	Silverfish (*Trachinotus ovatus*)	Increased growth rates and feed conversion efficiency	Zhang et al., 2018
Bacillus licheniformis	Dairy cows	Increased growth rates in calves, increased milk production, elevated milk protein content, and increased nutrient digestion within the rumen	Chen et al., 2013

Table 2. Examples of probiotic use in abalone. Literature review of studies on probiotic use in abalone based on abalone species and the measurable impacts of the probiotic.

Probiotic	Abalone Species	Impact	Study
Vibrio midae SY9	South African abalone (*Haliotis midae*).	Increased growth and digestive enzyme production	Huddy & Coyne, 2015
Cryptococcus sp. SS1, *Debaryomyces* sp AY1 and *Vibrio midae* SY9	South African Abalone (*Haliotis midae)*	Increased immune response in elevated phagocytic activity and hemocyte count	Macey & Coyne, 2006
Vibrio JH1, *Exiguobacterium* JHEb1 and *Enterococcus* JHLD	New Zealand Abalone (*Haliotis iris*)	Increased shell length and wet weight, reduced mortality	Hadi et al., 2014
Shewanella colwelliana WA64 and *Shewanella olleyana* WA65	Disk Abalone (*Haliotis diversicolor supertexta*)	Improved growth and immune response	Jiang et al., 2013
Bacillus stratosphericus A3440 *and Phaeobacter daeponensis* AP1220	Variously Coloured Abalone (*Haliotis diversicolor*)	Increased shell length and wet weight, reduced bacterial infection of *Vibrio harveyi*	Zhao et al., 2018
Bacillus licheniformis	Disk Abalone (*Haliotis diversicolor supertexta*)	Increased growth, reduced bacterial infection of *Vibrio alginolyticus*	Gao et al., 2020

B. licheniformis was selected for examination in this study due to a number of attributes including localization within the abalone, probiotic availability, and prior demonstrated growth and health benefits to abalone. Specifically, *B. licheniformis* is a gram-positive bacterium that populates the digestive tract of the abalone (Gao et al., 2018). *B. licheniformis* occupies regions that are most affected by Ca.Xc, the post-esophagus and the intestine (Gao et al., 2018). The

probiotic is readily available commercially, while other potential probiotic candidates are restricted to specific non-commercial laboratories. This probiotic has been shown to significantly increase feeding rate, survivorship, and growth in the abalone species, *Haliotis discus hannai Ino* (Gao et al., 2018; 2020).

The mechanism by which *B. licheniformis* affords such attributes in abalone is via an increase in starch and cellulose decomposition in abalone digestive systems and increasing feed digestibility (Gao et al., 2018). The probiotic, *Bacillus licheniformis* is able to increase enzyme secretions like alginate lyase, amylase, and protease, which increase feeding rates (Gao et al., 2018). Food intake in abalone treated with *B. licheniformis* can be 0.08-1.41 g/day greater than those not treated with the probiotic (Gao et al., 2018). Survival rates of abalone treated with *B. Licheniformis* increased by 5-10% along with growth rates of 5-9% (Gao et al., 2018). The availability, localization, increased growth, survival, and feeding rates in *Haliotis discus hannai Ino* as well as the probiotics historical response to bacterial infections make *B. licheniformis* an ideal candidate to test the effects of a probiotic treatment on white abalone exposed to Ca.Xc (Gao et al., 2018; 2020).

Probiotics are increasingly being used in aquaculture to increase productivity and as an alternative to antibiotics for disease therapeutics and prophylaxis. The *B. licheniformis* probiotic had not yet been administered to captive reared white abalone. This study assessed if *B. licheniformis* is able to reduce Ca.Xc loads, optimize feeding rates, and increase survivorship in treated white abalone. This experiment also compared growth rates in shell length, wet weight, and Condition Factor in abalone treated with and untreated with *B. licheniformis.*

Methodology

Experimental Design

White abalone were supplied by the White Abalone Project at the UC Davis Bodega Marine Laboratory. Juvenile abalone with a mean shell length of 10 mm were used because of their availability and because abalone at 10 mm are the ideal size in terms of disease susceptibility and the potential to observe treatment success. The abalone were held in a 9.5 L glass aquarium on water tables in the aquarium room at MLML.

To infect white abalone with Ca.Xc, a tank of previously infected red abalone from the Monterey Abalone Company (MAC) were held in the same tanks as the uninfected white abalone. The red and white abalone in the experiment were separated by a screen in the tanks. The infected red abalone were then exposed to passively infect all of the white abalone in the infection treatment as they shed bacterial particles into the water through their feces. Uninfected white abalone were obtained from the Bodega Marine Laboratory, where they were cultured in sterilized water, free of Ca.Xc. As a control for the presence of the infected red abalone, uninfected red abalone were sourced from Bodega Marine Labs and housed on the opposite side of the screen from the uninfected white abalone. Quantitative Polymerase Chain Reaction (qPCR) was used on pooled abalone feces at the end of the experiment to confirm the infection in white abalone exposed to Ca.Xc and lack of infection in unexposed white abalone. The methods for this qPCR work is described below in the "Presence and Abundance of Ca.Xc" section.

The aquaria holding abalone had aeration stones to provide dissolved oxygen. Flow rates were maintained at 200 mL min^{-1} (Wassing et al., 2010). Water was filtered through a 10 μm and 25 μm particle filter and a 100 mW sec^{-1} cm^{-2} UV sterilization system to prevent the spread of bacteria and pathogens. The water tables of infected and uninfected treatments were separated on

either side of the experiment room to limit the spread of the Ca.Xc infection to uninfected abalone treatments. Separate tools were used for the uninfected abalone compared to those that were exposed to the Ca.Xc infection. Iodine was used to sanitize the tools and human hands used in the uninfected tanks. A plastic tent was set up around the uninfected white and red abalone to ensure unfiltered water carrying the Ca.Xc pathogen would not spread from other tanks into the uninfected tanks. The temperature of the water was maintained between 13-15°C for all abalone with aquarium heaters to increase Ca.Xc infections rates in the infected treatments.

Abalone were assigned to four separate treatment groups including the control group C (–Ca.Xc, -*B. licheniformis*), Treatment 1 (T1) (-Ca.Xc, +*B. licheniformis*), Treatment 2 (T2) (+Ca.Xc, +*B. licheniformis*), and Treatment 3 (T3) (+Ca.Xc, -*B. licheniformis*). Treatment 1 was used to determine the probiotic's impact on growth, survival, and feeding rates on uninfected white abalone. Treatment 2 was used to test the impact of the probiotic on the Ca.Xc infection and growth, survival, and feeding rates in infected abalone. Treatment 3 tested the difference in growth, survival, and feeding rates between abalone with and without the probiotic as well as with or without the infection. There were a total of 24 tanks with six tanks for each treatment. Each tank contained 44 white abalone. The entire study lasted for six months from June 1, 2021 to December 1, 2021.

Feeding Rates and Probiotic Administration

The abalone in each treatment group were fed an *ad libitum* base diet of *M. pyrifera* and *D. mollis* every three days of the study. The *M. pyrifera* and *D. mollis* were soaked in freshwater for 10 minutes before being fed to the abalone in the uninfected treatments to remove any bacteria that could infect the abalone. The initial feeding rate was 125 g of *M. pyrifera* and 64 g of *D. mollis* for all abalone in each tank every three days. The feeding rate of the abalone was

calculated by removing excess feed from the tanks and weighing it before each new feeding period and subtracting the remaining feed weight from the initial feed weight. As the abalone grew in size, the amount of *M. pyrifera* and *D. mollis* added to tanks increased to compensate for increased consumption.

The probiotic was acquired from the commercial provider, Bacillus Genetic Stock Center in Columbus, Ohio and cultured to scale at MLML. The probiotic, *Bacillus licheniformis* was grown with Luria-Bertani (LB) broth for 18 hours before being added to the feed. The probiotic was aerated in a liquid media and was cultured in an Erlenmeyer flask in a shaker and water bath at 37°C (Brody & Price, 1998). The probiotic, *B. licheniformis* cells, were diluted to 10^8 cfu/ml and placed in a spray bottle. In T1 and T2 the *B. licheniformis* probiotic was sprayed on the *M. pyrifera* and *D. mollis* feed at 10^8 cfu/ml (Gao et al., 2018). The *M. pyrifera* was then allowed to air dry for about one hour before it was fed to the abalone. The feed treated with the probiotic was prepared twice a week.

Presence and Abundance of Ca.Xc

To determine the presence and abundance of the Ca.Xc bacteria, qPCR and histology were employed. qPCR was used on 250 mg of fecal matter per treatment, at the end of the study. The qPCR tests detected the presence and abundance of the Ca.Xc bacteria. The feces collected from each tank were pooled by treatment, weighed, and DNA was extracted using a QIAamp Fast DNA Stool Mini Kit from QIAGEN (Vater et al., 2018). The solution was kept at 20°C for analysis (Vater et al., 2018). The qPCR assays were conducted using methods from Friedman et al. (2014a, 2014b), including primers for the 16S rRNA for Ca.Xc.

Histology was used to detect inclusions from the Ca.Xc within the post-esophagus and digestive tract. Histology was conducted on a subsample of six abalone per tank at the beginning of the study (day 0), the middle of the study (day 90), and end of the study (day 180). The abalone were sedated with MS-222 at a concentration of 20 ppm during the three collection periods. After sedation, the abalone shell was removed from the abalone with a scalpel and the entire abalone was cross sectioned for the histology work. A cross section was used instead of dissecting out the organs due to the small size of the abalone used in this experiment. The cross section of the whole abalone was made through the pericardium, digestive gland, and post-esophagus. This histology cross section was used to observe the progression of inclusions in the post-esophagus and digestive tract between the treatment groups.

Tissue from cross sections of the six subsampled and dissected abalone was fixed in Davidson's Fixative for 24 hours at room temperature during the three sampling periods throughout the experiment (day 0, day 90, and day 180) (Moore et al., 2011). Samples were then rinsed in a series of ethanol concentrations from 25% to 50% and rinsed three times with 70% ethanol. The samples were shipped in ethanol soaked paper towels for histology work at Histology Consulting Services Inc. in Everson, Washington. The sections were embedded, sectioned, and stained with Hematoxylin and Eosin (H & E). The resulting slides were blind coded before they were evaluated to eliminate biased slide interpretation (Huddy & Coyne, 2014). Finally, the histological tissue sections were viewed with a Leica (model number $MZ12_5$) compound light microscope at 100x magnification.

Presence of B. licheniformis in Treated White Abalone

The presence of the probiotic in the abalone was checked at the end of the study by plating the gut of the white abalone. HiCrome^{TM} Bacillus agar plates also known as the Bacillus

ChromoSelect agar (Alippi & Abrahamovich, 2019) were used to culture the gut bacteria. The yellow to green coloration of the *Bacillus* species specific HiCrome^{TM} plates and rod-shaped bacteria, and colonies with lichen characteristics were used to identify the *B. licheniformis* bacteria. The HiCrome^{TM} Bacillus agar served as a selective medium that allowed the *B. licheniformis* to grow, while prohibiting the growth of other bacteria species. The probiotic, *Bacillus licheniformis* is a gram-positive bacterium, which can thrive in external conditions that are considered unfavorable for other bacteria species (Němečková et al., 2011). Only the presence of *B. licheniformis* was assessed and not the quantity of the probiotic in the abalone gut. A subsample of six abalone from each treatment at the start and end of the study were sacrificed for this plating process. The gut was removed from the abalone with a scalpel. It was mixed in a 1.5 ml centrifuge tube with a rotor to grind up the tissue for plating. The gut tissue was then spread on the plate using a sterilized glass pipette tip and plated in triplicate. The plates were labeled by treatment, sealed with parafilm, and placed inverted in an incubator at 37°C. The plates were checked for bacteria growth and the change in plate color after 24 hours and again after 48 hours. Contamination in the *B. licheniformis* cultures was assessed by plating the sterilized LB broth HiCrome^{TM} plates prior to adding the probiotic.

Growth and Feeding Measurements

Individual abalone growth was recorded at the end of each month of the study. MS-222 was used to safely remove the abalone from the aquaria. Shell length measurements were taken with calipers to the nearest 0.1 mm. The abalone were weighed wet on to the nearest 0.1 g. Condition Factor (CF) was calculated as weight (g)/shell length $(mm)^3$ * 100 to assess the relationship between abalone weight and length growth.

Overall Health Measurements

To assess the overall potential health benefits of the *B. licheniformis* treatment feeding rates and survival rates of the abalone were assessed throughout the study. The feeding rates of the abalone were assessed as wet weight feed (g)/wet weight abalone (g)/day to account for the difference in abalone growth rates and to standardize abalone biomass in each tank. The tanks were fed and cleaned twice a week to assess the feeding rate. Biweekly tank cleaning was used to reduce the contribution of tank biofilms to the abalone diets. Abalone survival was assessed monthly.

Data Analysis

Absolute quantification was used for the qPCR analysis to determine the bacterial load measurements of the Ca.Xc via copy number of the 16S rRNA gene. Methods for qPCR analysis were conducted based on data interpretation methods from Friedman et al. (2014a, b). The C_T values for the test samples were compared to a standard curve. The standards for the curve were supplied by the Bodega Marine Laboratory. Growth measurements for abalone including wet weight and shell length were converted into means for each treatment over time. Repeated Measures ANOVAs were used on mean tank growth metrics and feeding rates to assess significant changes in growth over time. Shell loss in the study was assessed over time with a Kaplan-Meier Survival Analysis.

Results

Growth Measurements

There were no significant differences in growth rates between all four of the treatment groups and there was no evidence that the growth rates diverged over time (treatment x date interaction effect): control group C (–Ca.Xc, - *B. licheniformis*), Treatment 1 (T1) (- Ca.Xc, +*B. licheniformis*), Treatment 2 (T2) (+ Ca.Xc, +*B. licheniformis*), and Treatment 3 (T3) (+ Ca.Xc, -

B. licheniformis) (RM-ANOVA, shell length: treatment $F_{3,72}$=1.71, p=0.17, date $F_{5,72}$=3.58, p=0.17, treatment x date $F_{15,72}$=0.88, p=0.59; wet weight: treatment $F_{3,72}$= 1.70, p=0.18, date F_{5720}=7.89, p<0.0001, treatment x date $F_{15,72}$=1.07, p=0.41). When the treatment groups were collapsed to probiotic exposure only, there was also no significant difference in mean growth or the growth trajectories between abalone that were treated with the probiotic compared to those that were not treated with the probiotic (RM-ANOVA shell length: treatment $F_{1,72}$= 0.52, p=0.47, date $F_{5,72}$=3.43, p=0.08, treatment x date $F_{5,72}$=0.52, p=0.76; wet weight: treatment $F_{1.72}$ = 0.03, p =0.87, date $F_{5.72}$=7.08, p<0.0001, treatment x date $F_{5,72}$=0.16, p=0.98). There was no significant difference in mean growth rates or growth trajectories between abalone that were exposed to the Ca.Xc infection compared to those that were in the uninfected treatments (RM-ANOVA shell length: exposed $F_{1,72}$ =2.91, p=0.09, date $F_{5,72}$ =3.57, p=0.006, exposed x date $F_{5,72}$ =0.63, p=0.68; wet weight: exposed $F_{1,72}$=2.05, p=0.16, date $F_{5,72}$=7.53, p<0.0001, exposed x date $F_{5,72}$=0.67, p=0.65). There was no significant difference in CF between all four treatments (ANOVA, $F_{2,86}$=1.02, p=0.36).

Feeding Rates

The mean feeding rate of abalone untreated with the probiotic was 0.06 dry feed (g)/abalone (g)/ day while abalone treated with the probiotic consumed 0.07 dry feed (g)/abalone (g)/day. The feeding rate of abalone exposed to Ca.Xc was 0.05 dry feed(g)/abalone(g)/day, while the feeding rate of abalone unexposed to Ca.Xc was 0.08 dry feed(g)/abalone(g)/day. There was a difference in feeding rates between treatments, but the difference in feeding rates remained constant in all four treatments over time and there was no change in the trajectory of any treatment feeding rate (RM-ANOVA, treatment $F_{3,95}$=9.54, p<0.0001, date $F_{5,95}$=9.86, p<0.0001, treatment x date $F_{15,95}$=0.82, p= 0.62). There was a difference in feeding rates between abalone that were treated

with the probiotic and abalone that were untreated with the probiotic. Abalone treated with the probiotic had higher feeding rates than abalone untreated with the probiotic, but the difference in feeding rate remained constant over time (RM-ANOVA, treatment $F_{5,95}$=5.42, p=0.02, date $F_{5,95}$=2.89, p=0.014, treatment x date $F_{5,95}$=1.03, p=0.40). There was a difference in feeding rates between abalone that were exposed to Ca.Xc and abalone unexposed to Ca.Xc. Abalone unexposed to Ca.Xc had higher feeding rates than abalone exposed to Ca.Xc, but the difference in feeding rate remained constant over time and there was no change in the trajectory of the treatments during the study (RM-ANOVA, exposed $F_{1,95}$=159.97, p<0.0001, date $F_{5,95}$=4.53, p<0.0001, exposed x date $F_{5,95}$=2.30, p=0.43).

Survivorship

Over the six-month study, treatment C had 82% survivorship, T1 had 93% survivorship, T2 had 65% survivorship, and T3 had 81% survivorship. During the second month of the study an abalone in the T1 treatment was found with its shell hanging from its body connected via residual foot tissue. Over the next several days, this shell loss syndrome was observed in abalone in each of the four treatments (Figure 1). Shell loss occurred from July 22, 2021 through the end of the study on December 1, 2021. Shell loss was seen in both the white abalone and the red abalone. The shell loss syndrome observed in this first probiotic experiment hindered the shell length, wet weight, feeding rate, and survivorship data collection. The abalone that lost their shells, but were still alive, were left in their tanks for the first two weeks of the shell loss outbreak. After this period, all abalone with shell loss syndrome were removed from the experiment in case the syndrome was caused by an infectious agent. These results of this study were skewed since almost half of the white abalone in this original study suffered from shell loss and had to be removed from the experiment.

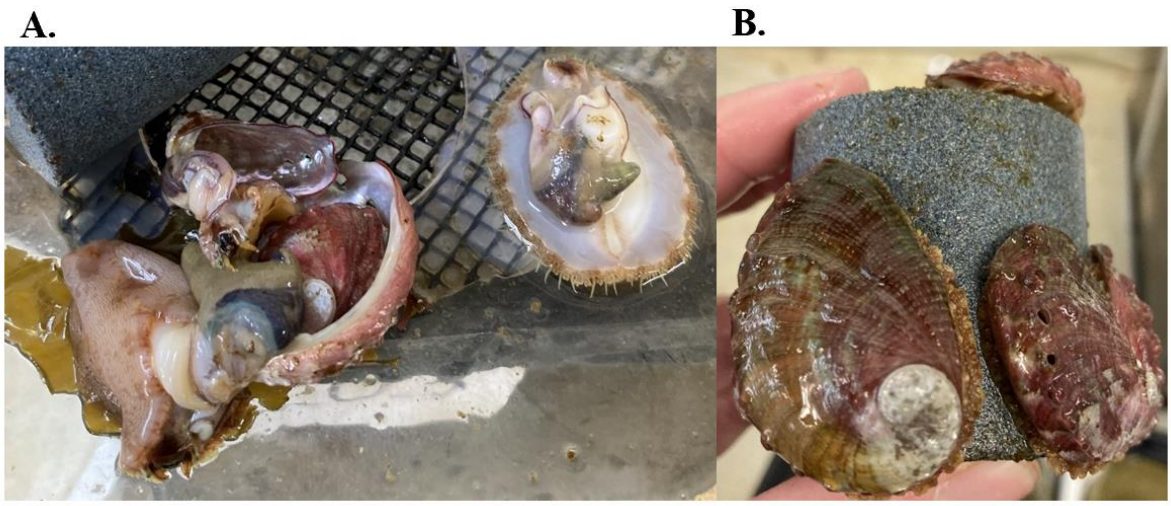

Figure 1. (A) White abalone with shell loss from the first proboitc study. **(B)** White abalone with shell from the first probiotic study.

Shell Loss in the First Probiotic Study

Shell loss was observed two months after the start of the first probiotic experiment. The treatments in the first probiotic study had no impact on shell loss. Similar shell loss rates were seen in each of the four treatments. There were a total of 110 cases of shell loss or 46% of the abalone in C suffered from shell loss syndrome. There was a total of 43% or 103 symptomatic abalone in T1. There were 47% or 112 cases of symptomatic abalone in T3 and 49% or 118 symptomatic abalone in T3. The red abalone used in this study to infect the white abalone with Ca.Xc, also experienced shell loss during the study. There were 454 symptomatic white abalone out of 1,056 white abalone or 43% of the white abalone in the first probiotic experiment lost their shells. Out of 200 red abalone, 104 were symptomatic or 52% of the red abalone in the first probiotic experiment lost their shells (Figure 2). There was a significant variance in shell loss rates between the red and white abalone with higher percent shell loss in the red abalone population over the study (Kolmogorov-Smirnov Test, $D=0.43$, $p=0.0012$). The weekly percentage of shell loss showed a similar trend of more red abalone with shell loss week by week (Figure 3). The rate, impacts, and possible causes of shell loss are discussed further in Chapter 2.

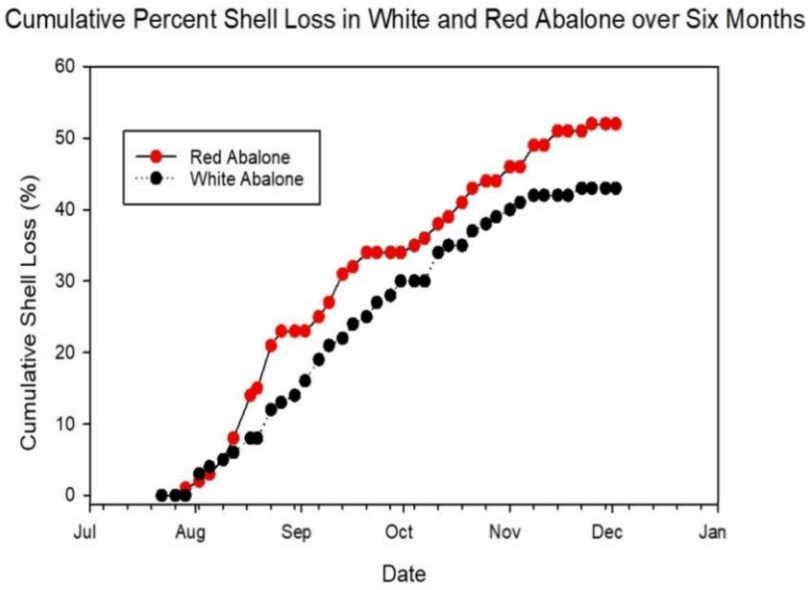

Figure 2. Cumulative percent shell loss in red and white abalone over the last four months of the first probiotic study. The total shell loss in red abalone was 52% and in white abalone the shell loss was 43%.

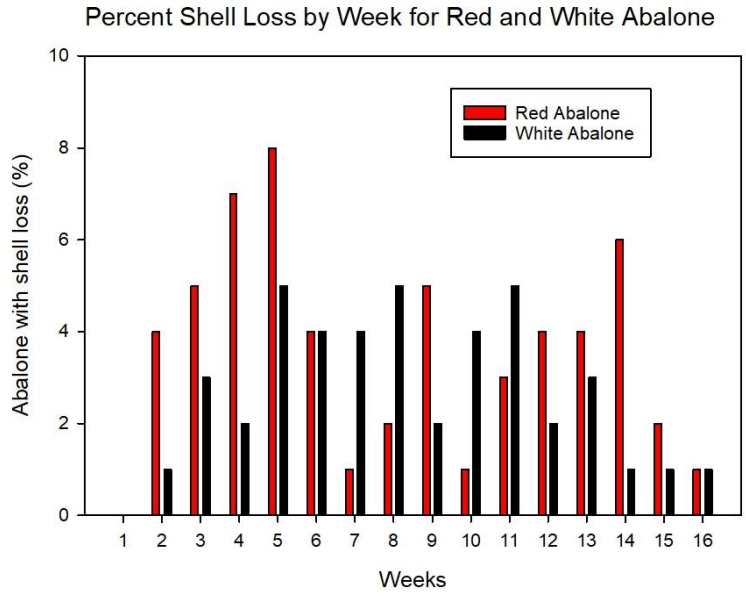

Figure 3. Percent of symptomatic abalone by week for red and white abalone. The percentage of shell loss for each species was based on the total number of red and abalone used in the study. There were a total of 200 red abalone and 1,056 white abalone used at the start of the study.

Ca.Xc Infection Status and Presence of the Probiotic

There was no indication of Ca.Xc infections detected within the exposed abalone treatments.

According to qPCR assessments from month 6, there was no Ca.Xc detected in any of the

treatment groups (mean Ct=33.93 ± 1.02 SE). Histology slides of the abalone intestine tract were examined under a microscope and no Ca.Xc inclusions were found in any of the sampled abalone from the start of the study, month 3 or month 6 of the study (Figure 4). The plated gut samples on HiCrome^{TM} plates did not show the presence of the probiotic in any of the treatments.

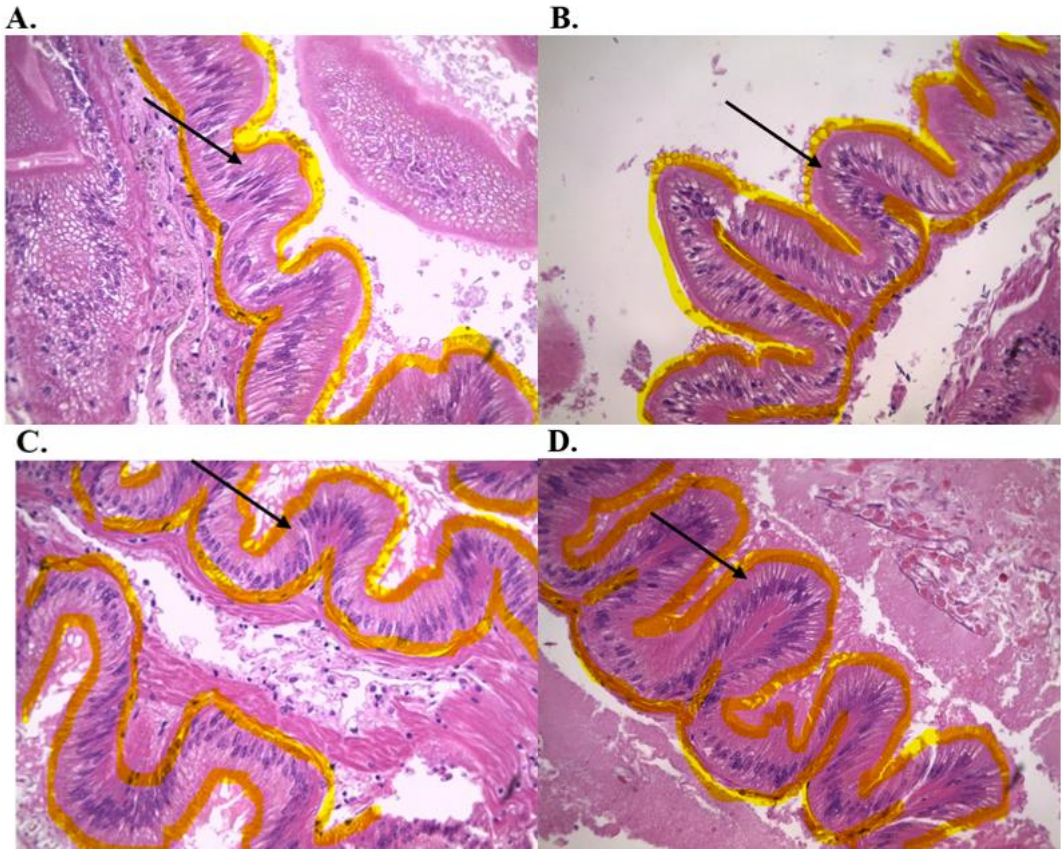

Figure 4. Diverticula are the folds of the post-esophagus tissue used in digestion and are highlighted in yellow. **(A)** Abalone post-esophagus diverticula from C (–Ca.Xc, - B. licheniformis) sampled at month 6 of the study. **(B)** Abalone post-esophagus diverticula from (T1) (- Ca.Xc, +B. licheniformis) sampled at month 6 of the study. **(C)** Abalone post-esophagus diverticula from T2 (+ Ca.Xc, +B. licheniformis) sampled at month 6 of the study. **(D)** Abalone post-esophagus diverticula from T3 (+ Ca.Xc, -B. licheniformis) sampled at month 6 of the study. No inclusions from the Ca.Xc infection were observed, and the post-esophagus looked healthy in both infected treatments. If inclusions were present they would appear as large purple circles at the end of the arrow. The purple ovals in the diverticula are the cell nuclei. All images magnified to 100x viewed with a Leica (model number MZ125).

Discussion

This study aimed to assess whether probiotics could mitigate the effects of Ca.Xc infection and enhance growth of captive-reared white abalone. However, there was no evidence of Ca.Xc infection at the end of the experiment, even in the individuals that were exposed to Ca.Xc-infected individuals. Furthermore, this experiment was influenced by an unexplained, high occurrence of shell loss, which may have affected the experiment results. These results are discussed in the following sections in the context of what is known about the Ca.Xc infection and the potential future use of probiotics in restoration aquaculture.

Ca.Xc Infection Resistance in White Abalone

There was no Ca.Xc infection detected in any of the treatment groups, which could be due to the water temperature or presence of the bacteriophage p*C*Xc (Vater, 2017; Vater et al., 2018). The experiment temperature was maintained between 13-15°C, but the abalone may have needed higher water temperatures to prompt the Ca.Xc infection. Vater et al. (2018) increased their water temperature to 18.5°C to increase the Ca.Xc. infection. Alternatively, the white abalone may now be more protected from the Ca.Xc. bacteria because of the pCXc bacteriophage.

The existing literature for Ca.Xc exposure of white abalone indicated that the presence of p*C*Xc in white abalone did not provide any of the therapeutic effects as observed recently in red abalone (Vater et al., 2018). Vater et al. (2018) reported p*C*Xc in 2 of 14 abalone tissue samples infected with Ca.Xc in the post-esophagus at ambient water temperature of 13.6℃. This sample size of 14 white abalone is very low and does not provide an adequate assessment of p*C*Xc presence in the white abalone population. Vater et al. (2018) reported 100% mortality of white abalone in the elevated treatment of 18.5℃, but there were no data on the presence of Ca.Xc or p*C*Xc in the white abalone elevated temperature treatment (Vater et al., 2018). This paper stated

that the bacteriophage is present in white abalone, but ineffective (Vater et al., 2018). However, the small sample size and lack of data on the infection or bacteriophage in white abalone at elevated temperatures do not support this claim, and further research is needed. The temperature used in this experiment could have hindered infection rates in white abalone. Red abalone were once highly susceptible to Ca.Xc, but the presence of the p*CX*c in red abalone has protected them from the deadly impacts of this infection. The p*CX*c could have mutated to become more prevalent and effective in white abalone. The p*CX*c could have been transferred to white abalone due to their increased time in aquaculture facilities and in tanks with red abalone carrying the p*CX*c. Based on the lack of infection rates in the white abalone within the first probiotic study, it is possible that p*CX*c could be more prevalent in the white abalone than previously seen. This recent discovery for white abalone populations negates the assessment of probiotic therapeutic and prophylactic effects on white abalone exposed to Ca.Xc, and as such Ca.Xc was not considered for the second probiotic experiment discussed in Chapter 3.

Probiotic Presence in White Abalone

The lack of significant differences between the treatments expressed as shell length, wet weight, and survivorship could be due to the reduced sample size of abalone used in the study since abalone with shell loss had to be removed from the experiment. Alternatively, it could also be possible that the probiotic treatments were not conveyed to the gut of the white abalone in an appropriate manner and condition. The probiotic was not detected in the gut of abalone from any treatment group. The probiotic application on the seaweed may have been inadequate and potentially washed off the *D. mollis* and *M. pyrifera* feeds once introduced to the experimental tanks. To better understand the impacts of *B. licheniformis* on white abalone, a second probiotic study was conducted (described in Chapter 3). This second study used a new pellet feed that

would allow the probiotic to fully impregnate into the feed before being fed to the white abalone to ensure that the probiotic makes it to the abalone gut (Martinez et al., 2012; Jiang et al., 2013; Iehata et al., 2014; Hadi et al., 2014; Zhao et al., 2018; Grandiosa et al., 2018; Gao et al., 2018; Xialong et al., 2020).

Shell Loss in Other Farms and at MLML

The shell loss was an unexpected phenomenon in this study. Although some other abalone farmers have witnessed cases of shell loss, this study had a significantly higher rate of shell loss than previously seen by experts in the field. The cause of the shell loss was not determined. There were several factors or conditions in the system during that time, which could have caused or contributed to the shell loss syndrome. Observations of shell loss began approximately around the period that a large algal bloom in Monterey Bay was detected (July 22, 2021). One diatom species detected off the Monterey Municipal Wharf II at this time was *Pseudo-nitzschia* (Anderson, 2021). This species is responsible for harmful algal blooms or HABs. Other diatoms including *Dinophysis*, *Prorocentrum*, and *Ceratium* were also detected in high quantities at the time, indicating a HAB (Bower, 2023). This could have negatively impacted aspects of water quality leading to the shell loss. There are however some factors that discredit the theory that the shell loss was caused by the algal bloom. The white abalone housed at the nearby MLML Aquaculture Center, which received very similar flow through seawater from Monterey Bay, did not experience any discernible shell loss or other significant health issues at this time. Water quality parameters were subsequently measured including pH, salinity, alkalinity, temperature, and DO. Values measured for each of these parameters were all considered within acceptable ranges. The shell loss also continued after the HAB had passed.

The pipe transferring water from the MLML Aquaculture Center to the MLML Aquarium Room was cleaned when the shell loss was first observed. Bacteria from the built-up sludge in this pipe could have caused an infection in the abalone leading to the shell loss. There could also be other water quality factors in the pipe leading to the shell loss syndrome. The water lines were clogged by sludge during the pipe cleaning period, which reduced the water clarity and increased the particles in the water. This dirty water and the lack of continuous water flow could have increased abalone stress, leading to, or contributing to the shell loss. Experimental tank specific effects were considered as possible causes for the syndrome at the time and as such alterations to the system and husbandry were implemented. Airlift pumps were added to the experimental tanks to improve dissolved oxygen concentrations and water movement in the hopes of halting or reducing the shell loss syndrome.

Although the cause of the shell loss was not determined during this study, the longevity of and course of infection in shell less abalone was recorded. The symptomatic live abalone were left in the tanks for 14 days and after August 2, 2021 these abalone were removed from the experimental tanks for fear of possibly transmitting an unknown pathogen to the remaining abalone in the tanks. Shell-less abalone were held in separate holding tanks. The shell-less abalone continued to survive without their shell for up to three months. Over this time their digestive glands degraded. Strands of the abalone intestines unraveled from their digestive system. The abalone died between a day and three months after the initial signs of shell loss. To further investigate the cause of this shell loss syndrome two subsequent shell loss experiments were conducted (Chapter 3).

Chapter 2: Shell Loss Syndrome

Preface

Shell Loss Study Rationale

The cause of the shell loss in the probiotic study was undetermined during the course of the study. Considering the relatively novel nature of the shell loss observations in abalone, two subsequent studies were conducted. The goal of these two studies were to determine if the shell loss syndrome could be caused by an infectious agent. These infection chain challenge studies were designed and conducted to discover if the shell loss was transmitted from shell-less or symptomatic abalone to healthy shelled abalone. The studies in this chapter investigate if the density of both shell-less and healthy abalone contributed to the shell loss.

Introduction

Shell Loss in the Wild and in Aquaculture Facilities around the World

Abalone shells are an important structure in abalone anatomy. The shell protects the abalone from predators and protects the digestive gland of the abalone, which is housed under the shell. The detachment of the shell from the abalone tissue is termed shell loss. Shell loss has been observed in wild shellfish species like whelks (Koy, 2007). Abalone farms around the world have also experienced shell loss on a smaller scale compared to this thesis study (Huchette et al., 2006). In the wild, the shell-less abalone are more prone to predation, but abalone in the laboratory are able to survive for up to three months without shells. Their shell is slowly shed from their body over time. First, the attachment muscle detaches from the shell. Then the surrounding mantle tissue is disconnected from the edge of the shell. Lastly, the shell hangs from the connective tissue at the base of the digestive glands before it is completely separated from the abalone (Figure 5).

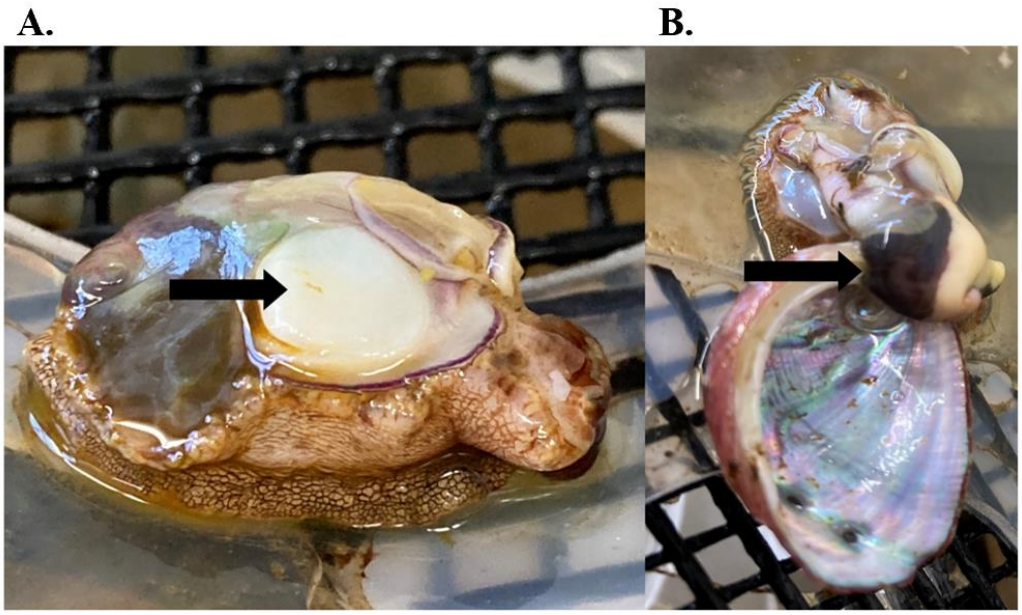

Figure 5. (A) The attachment muscle, which is the first part of the abalone to disconnect from the shell, indicated by the arrow. **(B)** The final attachment point for the shell is the digestive gland (the dark brown color wrapped in white gonad) indicated by the arrow.

The cause of this shell loss has not been confirmed, but there are several possible factors that could cause the shell loss including: water quality, notably Ca^{+} and nitrate concentration, physical disturbance from aeration, irritants lodged between the shell and the abalone tissue, parasites like boring polychaetas, and bacterial and fungal infections. Abalone aquaculture practitioners have anecdotally observed shell loss in abalone species periodically. Shell loss has been seen in red abalone within a recirculating system, which could be caused by poor water quality (*pers comm* Nancy Caruso, Get Inspired). Bodega Marine Laboratory observed red abalone using their foot to push smaller white abalone, resulting in the detachment of the white abalone connective tissue from their shell (*pers comm* Kristin Aquilino). Boring polychaetas observed in abalone have been linked to shell loss (Sato-Okoshi & Abe, 2012; Grindley et al., 1998). Qualitative evidence suggests that shell loss could be caused by an irritant found between the abalone foot and shell leading to an infection and disconnection between the shell and foot

(*pers comm* Doug Bush, The Culture Abalone Company). Bacterial and fungal infections have also been pointed to as the cause of the shell loss (Huchette et al., 2006).

Due to the relative novelty and large-scale observations of shell loss during the previous probiotic study, it was both opportunistic and prudent to investigate the incident to determine potential causal relationships. The shell loss progressed slowly in the abalone population in the first probiotic study. At first only a few abalone were found to have shell loss, but the rate of shell loss increased to 43% of the white abalone population and 52% of the red abalone population over four months. It was believed that the shell loss syndrome could have been caused by an infectious agent that spread through both the white and red abalone populations. Population densities can impact the spread of an infectious disease and the infection can be amplified by high densities (Lafferty & Kushner, 2000; Behringer et al., 2020). Higher urchin population densities have been associated with increased disease outbreaks compared to healthy, lower urchin population sizes (Lafferty & Kushner, 2000). Increased fish densities in aquaculture can increase infection rates within the population (Behringer et al., 2020). The stocking density of abalone could impact the transmission of any infectious agent that may cause shell loss.

Two separate shell loss experiments were conducted to determine if the shell loss was caused by an infectious agent like a bacterial, fungal, or viral pathogen. The first experiment on shell loss exposed healthy white abalone to possibly infectious shell-less white abalone. The expectation of this study is that if a pathogen was responsible for the shell loss, these shell-less animals would pass the infection through the infection chain to healthy host animals, resulting in similar shell loss outcomes. Low- and high-density treatments of symptomatic white abalone were used to assess the transmission model of the potential causative infectious agent. This experiment was set up on the basis that all symptomatic abalone are equally infectious and all healthy abalone

that are exposed to the symptomatic abalone are susceptible to the infection (Velthuis, 2007). Therefore, potentially infections abalone with shell loss were housed with healthy abalone, susceptible to the infection chain. The second study utilized healthy red abalone isolated in the same tanks as symptomatic red abalone. It used low versus high tank stocking densities of healthy shelled red abalone to discover if extra stressors like tank density could increase the transmission of shell loss between shell-less and healthy red abalone. This second shell loss challenge experiment was used to determine if stocking density stressors increase shell loss or impact other health metrics like growth and feeding rates. The two shell loss challenge experiments assessed if the shell loss was caused by an infectious agent and if stocking densities of both symptomatic and healthy, shelled abalone influence the rate of shell loss.

Methodology

White Abalone Shell Loss Infection Chain Challenge

The first shell loss study used white abalone and investigated the possible infection transmission chain. This study was conducted over six months from October 18, 2021 to April 18, 2022. Nine 9.5 L tanks replicated three times each with 10 healthy white abalone per tank were used for the study. Two treatment conditions were assessed for the study including infection chain transmission and density impact on transmission rates. The experiment aimed to understand if the shell loss could be spread through exposure of symptomatic abalone to healthy abalone. The abalone density was assessed to better understand stocking density impacts on transmission rates of any causative agent. The low transmission treatment had 1 symptomatic white abalone and 10 healthy abalone separated by a tank screen. The high transmission treatment had 10 symptomatic white abalone and 10 healthy abalone. The no transmission treatment served as a control with 10 healthy abalone on each side of the tank screen (Figure 6).

All experimental tanks were cleaned once a week and fed fresh *D. mollis* or *M. pyrifera*. The abalone in each tank were fed *ad libitum*. Tanks were checked weekly for mortalities and shell loss. As symptomatic abalone died in the experiment, they were replaced with symptomatic abalone for the first three months of the study. After three months, additional symptomatic animals were no longer available, at which point the symptomatic mortalities were then removed from the tanks and not replaced.

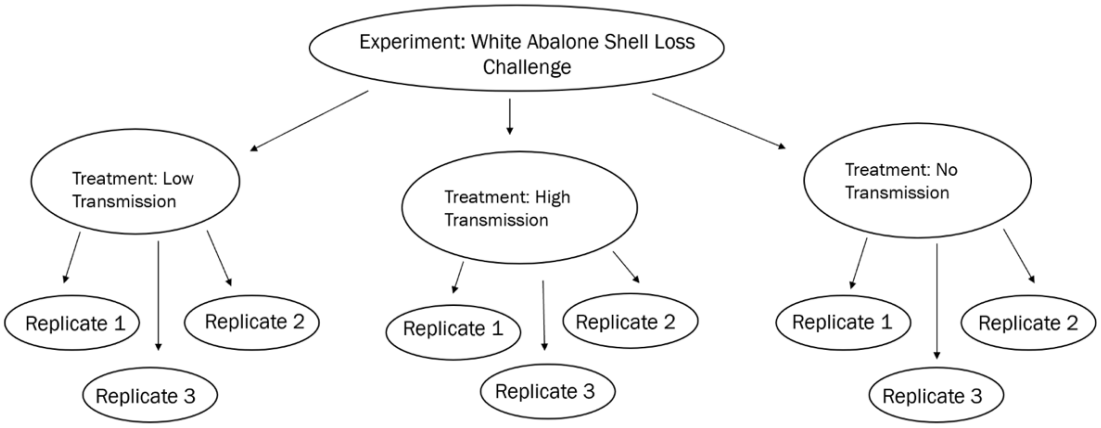

Figure 6. White abalone shell loss infection chain challenge experiment. There were 3 treatments each done in triplicate.

Red Abalone Shell Loss Infection Chain Challenge

The second shell loss study used red abalone to understand the infection chain and if it was influenced by the density of healthy and symptomatic abalone. The experiment was used to better understand the stressors of density on abalone feeding rates and growth rates. The study was conducted from January 31, 2022 through May 2, 2022. Twelve 9.5 L tanks were used. There were four treatments with three replicates per treatment. The treatments consisted of low and high stocking density with and without the presence of symptomatic abalone. The low-density tanks were stocked at 588 abalone m^{-2}. The high-density tanks were stocked at 1,176 abalone m^{-2}. The low density + symptomatic treatment had 5 healthy abalone on one side of the

tank screen and 5 symptomatic abalone on the other side of the screen. The high density + symptomatic treatment had 25 healthy abalone on one side of the screen and 5 symptomatic abalone on the other side of the tank screen. The low-density treatment had 5 healthy abalone on each side of the tank screen. The high-density treatment had 25 healthy abalone on one side of the screen and 5 healthy abalone on the other side of the screen (Figure 7). As the symptomatic red abalone died, they were removed from the tanks, but were not replaced due to the unavailability of symptomatic abalone. Tanks were cleaned twice a week and feed intake measured. Growth rates including shell length, wet weight, and tank biomass were collected at the end of every month. Differences in mean tank growth rates and feeding rates between treatments were assessed with a Repeated Measures ANOVA.

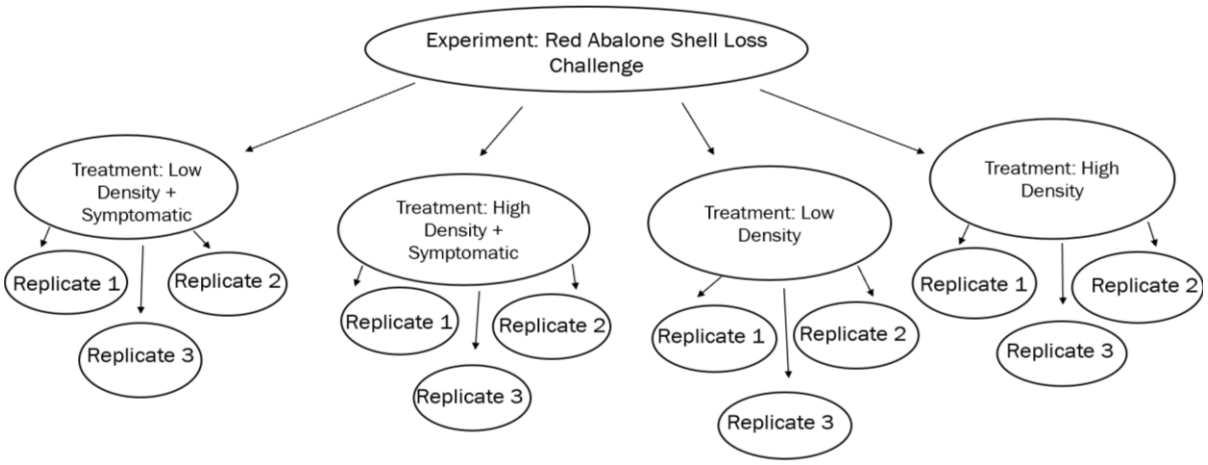

Figure 7. Red abalone shell loss infection chain challenge experiment. There were four treatments used and each treatment was done in triplicate.

Histology and Shell Observations

Abalone tissue samples from the symptomatic and healthy white and red abalone from this experiment were sent to Histology Consulting Inc. for histology slide preparation. The gut and post-esophagus of 20 shell-less red abalone were assessed with histology at the UC Davis, Bodega Marine Laboratory by a shellfish pathologist. The thickness and health of the diverticula

and space in the vacuoles were assessed. The symptomatic white abalone histology slides were compared to gut and post-esophagus from the healthy white abalone from the MLML Aquaculture Center (Figure 8). All of these slides were blind coded before evaluation to ensure there was no bias in slide interpretation. The shells from white and red shell-less abalone were assessed for conchiolin deposits and boring polychaetas to determine if boring polychaetas or other irritants causing conchiolin to accumulate within the shell led to shell loss. The area where the foot attached to the shell was viewed with a Leica (model number $MZ12_5$) compound light microscope at 100x magnification. Ten shell-less red abalone were assessed under the microscope to determine if there were any issues with the attachment muscle that may have led to the shell loss. The shell observations and histology were qualitative. Any boring polychaetas, irritants, or conchiolin observed was recoded and reported.

Results

White and Red Abalone Shell Loss Infection Chain Challenge

Throughout the white abalone shell loss infection chain challenge experiment, one abalone experienced shell loss. The shell loss was in one of the low transmission treatment tanks. There was no shell loss observed in the red abalone shell loss infection chain challenge. Since no shell loss was seen in the red abalone experiment, the treatments were combined into low versus high density treatments. There was a 16% increase in cumulative shell length in the low-density treatments (low density + symptomatic and low density) and 10% cumulative growth in shell length in the high-density treatments (high density + symptomatic and high density) at the end of the study. The growth in shell length in the low-density treatments was significantly higher than the high-density treatments throughout the study, but the difference in shell length did not diverge or increase over time (RM-ANOVA, treatment $F_{1,40}=13.91$, $p<0.0001$, date $F_{3,40}=22.16$,

p<0.0001, treatment x date: $F_{3,40}$=0.92, p=0.43; Figure 8A). The low-density treatments had a mean cumulative wet weight growth rate of 57%. The high-density treatments had a mean cumulative wet weight growth rate of 25%. The growth in wet weight between treatments diverged over time and increased more in the low-density treatments compared to the high-density treatments throughout the study (treatment x date interaction effect) (RM-ANOVA, treatment $F_{1,40}$= 2.47, p=0.12, date $F_{3,40}$=29.50, p<0.0001, treatment x date $F_{3,40}$=7.06, p<0.0001; Figure 8B). The mean feeding rate in the low-density treatment was 0.04 g feed /wet weight abalone g/d. The mean feeding rate in the high-density treatment was 0.03 g feed/wet weight abalone g/d. The red abalone in the low-density treatments had significantly higher feeding rates compared to the high-density treatments throughout the study. The feeding rates diverged over time and the feeding rate in the low-density treatments increased at a higher rate over time compared to the high-density treatments (RM-ANOVA, treatment $F_{1,318}$=2706.70, p<0.0001, date: $F_{2,318}$=314.70, p<0.0001, treatment x date: $F_{2,318}$=121.70, p<0.0001).

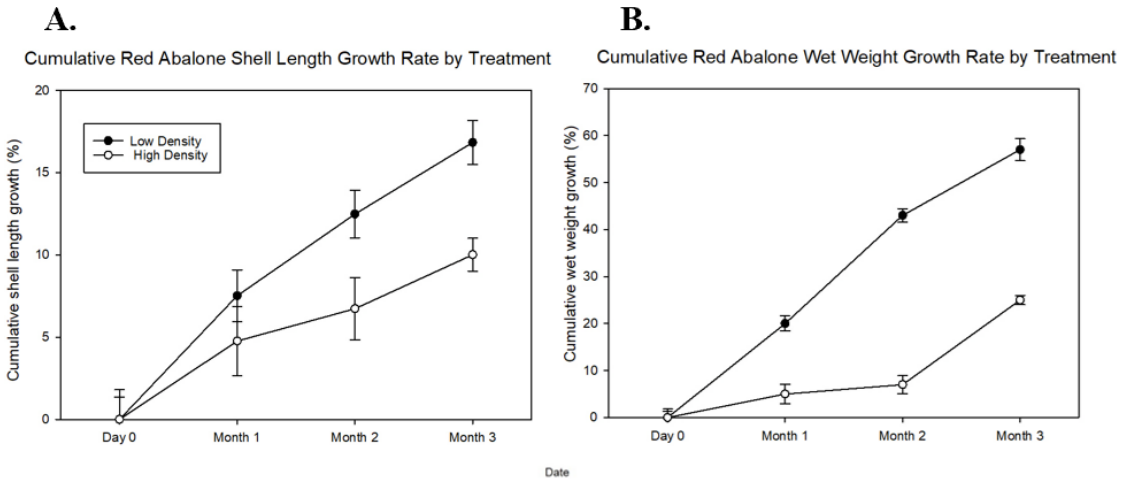

Figure 8. (A) Red abalone cumulative percent change in shell length from day zero to month three ± SE for low and high density treatments. **(B)** Red abalone cumulative percent change in wet weight from day to month three ± SE for low and high density treatments.

Shell and Tissue Assessments

Histological examination of tissues from symptomatic white abalone showed more degraded, undigested food in the digestive gland than in healthy abalone indicating poor digestive capabilities and overall health in the abalone. Histological examination of the foot and shell attachment site showed no degradation or necrosis. A morphological assessment of the shells from both symptomatic and healthy abalone showed no discernable differences. Conchiolin deposits were present in both healthy and symptomatic abalone shells. There were no holes or markings in the shells of healthy or symptomatic red and white abalone showing that boring polychaetas disease was not the source of the shell loss.

Discussion

The shell loss syndrome in the first probiotic study began in July, 2021 and continued for a year. The cause of this shell loss in both red and white abalone was particularly perplexing based on the high rates of shell detachment. A total of 43% of white abalone and 52% of red abalone suffered from shell loss in this first study. In an attempt to better understand the cause and spread of the shell loss syndrome, symptomatic and healthy abalone were housed together, to determine if the shell loss was due to contagious factors or if tank density stressors were responsible for the shell loss syndrome.

Shell Loss Infection Status

To determine if a specific microbe is responsible for a certain disease in other organisms a four-step process was created. These steps are called “Koch’s postulates.” These postulates state: *1) The organism must be present in characteristic form and arrangement in the diseased tissue. 2) The organism, which from its relationship to the diseased tissue appears to be responsible for the disease, must be isolated and grown in pure culture. 3) The pure culture must be shown to*

induce the disease experimentally. 4) The organism should be re-isolated from the experimentally infected subject. The final postulate in this series was later added to the protocol for determining if certain microorganisms are responsible for a specific disease. (Walker et. al., 2021).

The tissue of symptomatic abalone was dissected for histology and assessed by the California Department of Fish and Wildlife, Shellfish Health Lab, but no specific bacterium was detected. Since there were limited resources and time for the shell loss infection chain challenges no further PCR, sequencing, or plating work was done to detect the presence of a microorganism in the symptomatic abalone tissue as stated in the first of Koch's postulates. Without a microorganism detected in the symptomatic abalone the rest of Koch's postulates could not be satisfied. Instead, the shell loss infection chain challenges focused on the spread of shell loss from symptomatic abalone to healthy abalone to understand if the shell loss syndrome was caused by a contagion.

The white abalone shell loss infection chain challenge experiment did not indicate that an infectious agent was responsible for the shell loss. The white abalone shell loss challenge experiment tested the transmission and spread of the shell loss syndrome. Infectious agents can be spread through exposure and higher densities of symptomatic abalone housed with healthy abalone would likely transmit the infection at a higher rate. Increased fish densities in aquaculture can increase infection rates within the population (Behringer et al., 2020). The stocking density of abalone could impact the transmission of an infectious agent that may cause shell loss syndrome. With only a single instance of shell loss being observed in the six-month study, there was no robust indication that shell loss syndrome is transmitted in an infectious manner. The abalone individual that experienced shell loss in this experiment was housed with

only one symptomatic abalone. No shell loss was observed in any of the other replicate tanks for that treatment or the high transmission treatment which exposed healthy abalone to ten symptomatic abalone. The lack of infection in abalone exposed to a larger number of symptomatic abalone and therefore higher loads of an infectious agent suggest that the shell loss syndrome was not caused by an infection.

Abiotic Conditions

The initial observations of shell loss syndrome prior to the infection chain challenge studies coincided with a poor water quality event at the research facility. Water quality parameters were not measured during this period, but the event was consistent with exposure to anaerobic materials due to the discharge from a seawater supply pipe. This pipe had previously been inactive and contained stagnant seawater for several months. During pipe cleaning, some proportion of the stagnant water was inadvertently discharged into the culture tanks with a detectable odor indicating the presence of hydrogen sulfide. A similar scenario being the presence of contaminants including ammonia and hydrogen sulfide from stormwater runoff has been observed as a stressor in red abalone embryos (Bay & Greenstein, 1996). Sulfide has also been detected as a toxic by-product from *Ulva* decomposition that resulted in Japanese abalone mortalities (Chao et. al., 2011). Exposure of the abalone to these unusual hydrogen sulfide and other contaminant conditions may have manifested as a specific stressor resulting in abalone shell loss syndrome. The pipe cleaning led to unreliable water flow rates, which was not experienced at the MLML Aquaculture Center. The debris built up in the pipe transporting water to the aquarium room clogged the water lines that delivered water to the abalone experiment tanks. This debris reduced and temporarily stopped the water flow into the experiment tanks for hours at a time until they were able to be flushed out.

What is incongruent with this event and shell loss syndrome is the relatively slow and latent response seen by the abalone. The abalone showed consistent shell loss months after the initial shell loss was observed. The shell loss was a continuous process that began during a period of degraded water quality, but even after the water quality and inconsistent flow rates improved shell loss syndrome continued for several months. This inconsistent and sludge filled water from the cleaned-out pipes could have negatively impacted the abalone health, but the continued shell loss after the water cleared indicated that poor water quality was not likely the cause of shell loss. The water quality could have made the abalone more susceptible to infection or other cause of the shell loss syndrome.

Biotic Conditions

Abalone farms around the world have experienced shell loss on a smaller scale in comparison to this study. Previously observed shell loss has been linked to biotic conditions including infection (Huchette et al., 2006). Brown protein deposits called conchiolin have been seen on the inside of abalone and clam shells, which could lead to shell loss in the organism. Boring polychaetas observed in abalone have also been linked to shell loss (Grindley et al., 1998; Huchette et al., 2006; Sato-Okoshi & Abe, 2012). The abalone tissue of symptomatic abalone in the first probiotic study and shell loss infection chain challenge experiments were assessed and showed no signs of degradation, necrosis, or parasites that have been seen in other abalone species (Muznebin et al., 2021). The shells were assessed for boring polychaetae holes and the polychaetas themselves, but neither were found. The conchiolin deposits were assessed in abalone shells, but these protein deposits were found in both healthy and symptomatic abalone showing that these deposits were not likely responsible for the shell loss syndrome.

Harmful Algal Blooms (HABs) have been known to cause mortality in farmed and wild abalone species in South Africa (Botes, 2003). Toxins from these blooms pose a threat to the health and survivorship of abalone species. It was theorized that these toxins may have contributed to the shell loss observed in the first probiotic study. Shell loss observations in the abalone in the first probiotic study coincide approximately one week after the algal blooms of *Pseudo-nitzschia, Dinophysis, Prorocentrum,* and *Ceratium* in the Monterey Bay; however, the water used in the first probiotic study was also used for white abalone at the MLML Aquaculture Center where no shell loss was observed. Considering that the algal bloom subsided after a three-week period and abalone in the first probiotic study continued to experience this shell loss syndrome months after the algal bloom had cleared, it is highly unlikely that the HAB is responsible for the shell loss syndrome.

Shell Loss, Stress, and Density

A combination of stressors over time can weaken an organism's immune system making them more susceptible to illness. Combined stressors can make animals more susceptible to infection, irritants, or other poor health metrics. Stress from altered water quality, flow rates, or tank density could have induced and allowed for the continued shell loss. The red abalone infection chain challenge experiment however, showed it is unlikely that the tank density is responsible for the shell loss since no shell loss was seen in this experiment. The red abalone shell loss challenge experiment compared the growth, feeding rates, and spread of shell loss syndrome between abalone in high density tanks (1,176 abalone/m^2) versus low density tanks (588 abalone/m^2). Other studies have used high densities at 909 abalone/m^2 and low densities at 227 abalone/m^2 and medium densities at 455 abalone/m^2 (Lloyd & Bates, 2008). The low-density treatment in this experiment was closer to the medium stocking density of other studies, but since it was

lower than the high-density treatment it was labeled the low-density treatment. Tank density impacted feeding rates in red abalone even though all abalone in the experiment were fed *ad libitum*. Higher feeding rates are likely responsible for the higher growth rates in red abalone within the low-density treatments compared to the high-density treatments. The stress of the tank density did not result in any shell loss. As the shell loss continued throughout the first probiotic study the symptomatic abalone were removed from the tanks further reducing tank density and indicating that tank density is not a likely contributor to the shell loss.

Possible Shell Loss Factors

The initial observations of shell loss syndrome and the results of the subsequent infection chain challenge studies were somewhat incongruent considering the relatively long and consistent nature of the initial shell loss observations. The initial shell loss in the first probiotic study corresponded with the algal bloom and water line pipe cleaning that reduced water quality and reliable water in the experiment tanks. The initial theory was that shell loss was caused by poor water quality. However, after the algae bloom and sludge from the pipe cleaning cleared resulting in optimal water quality for the abalone, the shell loss syndrome continued.

Thus, water quality was not considered to be the main or only cause of the shell loss. Therefore, the two shell loss challenges were conducted. The outcome of these studies showed that shell loss syndrome was unlikely transmissible from symptomatic abalone to healthy abalone when cultured in the same water and in close proximity to each other. It should be noted that the symptomatic abalone used in the challenge studies, having experienced shell loss already, may no longer have been infectious or that physical contact between abalone is required for an infection to transmit. More research is required to discern the true cause of the shell loss and how to treat the syndrome, but this syndrome could not be replicated in subsequent challenge studies.

Chapter 3: Second Probiotic and Alternative Feed Study

Preface

The first probiotic study was broadly interrupted by the shell loss syndrome and subsequent shell loss infection chain challenge experiments. Therefore, the potential health, growth, and feeding rate benefits of the probiotic *B. licheniformis* could not be assessed well in white abalone. A secondary probiotic study was conducted to better understand how the use of this probiotic could improve white abalone restoration aquaculture efforts. Similarly, during this first probiotic study, qPCR assays at the end of the trial suggested that infecting white abalone with Ca.Xc proved unsuccessful or not long-lasting. The initial study did not show an effect of the bacterium on body condition for white abalone either, which I hypothesize is due to the recently reported presence of an antagonistic bacteriophage, p*CX*c, found in other species like red and black abalone (Cruz-Flores & Cáceres-Martínez, 2020; Cáceres-Martínez et al., 2021). As such, the second probiotic study omitted the Ca.Xc infection component. Instead, the second experiment focused on the utility of formulated feeds with and without probiotics for white abalone aquaculture, since fresh macroalgae feed is seasonally and spatially limited. Furthermore, inland aquaculture sites can struggle to grow adequate feed for their abalone and winter months particularly hinder macroalgae growth due to winter storm action and light availability.

Introduction

Alternative abalone feed has been produced and utilized as a nutritional alternative and additive in abalone aquaculture (Martinez et al., 2012; Jiang et al., 2013; Iehata et al., 2014; Zhao et al., 2018). Alternative formulated pellet feeds have a longer shelf life than fresh macroalgae. Formulated feed can last for six months when stored properly in a sealed container to prevent

moisture degradation of the feed (Gouveia & Empis, 2003). The feed is easily stored in a resealable container in laboratory or aquaculture facility spaces. The alternative feed could be cheaper than cultured macroalgae when comparing the required culture space and hired help needed for macroalgae cultivation to the price per kilogram of alternative feed. For example, the use of formulated feed has been shown to lower tilapia feeding costs by 8% (Borski et al., 2011).

Other benefits of alternative formulated feeds compared to fresh feeds are the ability to ship and maintain feed quality over time with little to no care. Formulated feed can easily be shipped to facilities without the same concerns in packaging compared to the fresh feeds, which need temperature control and a moist environment while shipping. The shipping of fresh seaweed can require permitting and material must be assessed for disease when transported across state or national borders. However, these regulations are not a concern when shipping formulated feed. Disease and feed degradation are not issues in alternative feed, but are a matter of great importance in feeding fresh macroalgae (Egan et al., 2014).

In white abalone aquaculture restoration work, there is a lack of fresh feed for multiple partner facilities. Fresh macroalgae has to be driven for several hours to transfer the feed from one facility to another. This requires White Abalone Project team members to drive, pick up, and transport the feed since it cannot be mailed in its fresh form. This transfer requires an ice chest and ice or ice packs to keep the feed fresh. The formulated feed could easily be shipped to partner facilities to support white abalone growth and restoration work.

The caloric density and nutrient composition in alternative feed could aid in white abalone restoration work. Formulated abalone feed is higher in calorie and macronutrient density than fresh macroalgae feeds (O’Mahoney et al., 2014). The protein and lipid content in abalone feed

is of particular concern in abalone nutrition. A protein content of about 27% and lipid content of 3.6% is considered optimal for tropical abalone (*Haliotis asinina*) (Bautista-Teruel et al., 2016). Macroalgae sources do not contain these protein and lipid ratios on their own and therefore abalone aquaculture requires a combination of algae species to suit the dietary requirements of abalone (O'Mahoney et al., 2014; Bautista-Teruel et al., 2016). The use of the probiotic in tandem with the formulated feed could provide benefits in protein utilization. Proximate analysis on abalone feed showed higher protein digestibility in the probiotic treated feed compared to the formulated feed alone (Hadi et al., 2014).

Although the macronutrient makeup of the formulated diet suits the nutrient needs of abalone, the palatability of formulated feeds reduces their effectiveness (O'Mahoney et al., 2014; Bautista-Teruel et al., 2016). The formulated feed requires careful formulation with highly palatable ingredients (O'Mahoney et al., 2014; Bautista-Teruel et al., 2016). The incorporation of algae species like *Laminaria digitata* has been used in a milled formulated feed form to increase abalone feed palatability (O'Mahoney et al., 2014; Bautista-Teruel et al., 2016). The stability of the formulated feed in seawater also reduces the suitability of the feed to abalone. Abalone are naturally grazers. They need feed that can hold together in the seawater conditions for multiple days without degradation (O'Mahoney et al., 2014). The stability of formulated feeds and their palatability for abalone are of concern when considering the replacement of fresh macroalgae feeds with formulated feeds.

Probiotic Administration

Other probiotic studies have used pellets to feed abalone in aquaculture (Martinez et al., 2012; Jiang et al., 2013; Iehata et al., 2014; Zhao et al., 2018). These pellet feeds have been utilized in administering probiotics to abalone because the probiotic can adhere to the pellet and remain

viable on the pellets when properly preserved at 4°C prior to feeding (Martinez et al., 2012; Jiang et al., 2013; Iehata et al., 2014; Hadi et al., 2014; Zhao et al., 2018; Grandiosa et al., 2018; Gao et al., 2018; Xialong et al., 2020). When the formulated feed is used in combination with a probiotic, the microbiome health, growth, and feeding rates of abalone can improve (Sahu et al., 2008). Hadi et al. (2014) showed a significant increase in growth rates when feeding a formulated feed with living probiotics over a two-month period. These improvements in abalone digestion, growth rates, and gut health indicate that the combination of a probiotic and formulated feed may benefit white abalone restoration aquaculture work.

Bacillus licheniformis was unable to improve growth rates, feeding rates or survivorship in the first study (Chapter 1), which could be because the probiotic was inefficient in improving these metrics in white abalone. Alternatively, the lack of health improvements could be because the probiotic was unable to be delivered to the abalone gut as a live, viable culture. The probiotic's inability to populate the white abalone gut could be due to the probiotic administration protocol. The probiotic may have washed off the *M. pyrifera* and *D. mollis* before it made it to the abalone's gut. Thus, the second probiotic study, discussed in this chapter, was conducted utilizing a commercial pellet feed called ABKelp®.

Feed Experiment

There is a need to identify and test alternative feeds beyond the currently used standard feeds for white abalone, which include whole fresh seaweed. Fresh macroalgae feeds have been traditionally used by commercial abalone farms since it is the natural abalone diet in the wild (Bautista-Teruel, 2016). These fresh macroalgae feed sources have proven nutritionally adequate for commercial abalone production purposes. Although macroalgae is nutritionally acceptable for white abalone, the economics of sourcing fresh seaweed for smaller scale restoration partners

is less feasible. These smaller partner facilities lack suitable equipment and staff associated with regular seaweed harvesting or culture. In addition, due to the current *M. pyrifera* crisis affecting much of California's coastline the ability to acquire fresh *M. pyrifera* consistently year-round is now also in question. Urchin barrens, warming ocean temperatures, and large storm action threaten *M. pyrifera* stock and production (Wernberg, 2018). Pellet feeds in contrast are typically available as needed from commercial suppliers, are nutritionally balanced, have prolonged shelf lives, and do not usually require special storage conditions (Gouveia & Empis, 2003). The utility of pellet feeds on white abalone performance is unknown, making such an investigation valuable for restoration efforts for the endangered species. Beyond the utility of repeating the first study to better deliver probiotics, there is another benefit to this second probiotic study, which is understanding the performance of formulated feeds in white abalone.

Instead of focusing on a probiotic treatment for Ca.Xc, this second probiotic study concentrated on the application of the probiotic using an alternative feed. It investigated if the probiotic could be successfully administered to the white abalone to reach and populate their gut. This study assessed the probiotic's impact on white abalone growth, feeding, and survivorship rates to better understand if *B. licheniformis* is a worthwhile additive in white abalone restoration aquaculture work. The alternative feed was assessed in this experiment as a tool for administering the probiotic and as an alternative or additive feed in white abalone aquaculture. The use of an alternative feed would be vastly beneficial for the white abalone restoration work since it could allow for more accessible and reliable feed. It could also improve the feeding, growth, and survivorship in white abalone. This secondary probiotic and feed study utilized the *B. licheniformis* probiotic to better understand its potential for improving growth in shell length, wet weight, and Condition Factor. It assessed survivorship and feeding rates in white abalone

treated with versus untreated with *B. licheniformis*. This experiment also investigated an alternative pellet feed to determine if this formulated feed could improve growth and feeding rates in white abalone, while replacing the seasonally limited traditional fresh macroalgae feed for white abalone.

Methodology

Tank Set up

This study consisted of three treatment groups. One treatment received the ABKelp® pellet feed untreated with the probiotic and was termed the formulated treatment. The second treatment group was fed the ABKelp® pellet feed treated with the probiotic and was referred to as the formulated + probiotic treatment. The last treatment group received the commercial standard diet of fresh *D. mollis* and *M. pyrifera,* termed the standard treatment. Each treatment was replicated with three tanks, each containing 10 white abalone. This experiment had nine 9.5 L tanks and 90 white abalone in total. Seawater was supplied to each replicate tank on a flow through basis at 200 mL min^{-1} and filtered to 20 μm via sand filters. The temperature fluctuated between 11℃ and 13℃ based on the ambient flow through water temperature.

Pellet Feed Preparation and Feeding

The probiotic was cultured weekly in 250 ml of liquid LB broth. The glassware and LB broth were autoclaved for 20 minutes prior to use. Once cooled, the frozen probiotic sample was thawed and added to the culture media. The probiotic was cultured for 18 hours at a density of 10^8 cfu/ml. The resulting probiotic culture was sprayed onto the pellet feed before being allowed to dry at room temperature for 24 hours. After this drying period, the probiotic treated ABKelp® was stored in the fridge at 4℃ for one week and used for daily feedings.

The average dry ABKelp® weight and average probiotic treated ABKelp® pellets were taken before the start of the study. Both pellet forms had an average weight of 0.3 g. The feeding rates were collected by subtracting the final count of pellets from the initial number of pellets added to the tanks each day. There were ten pellets added to each tank every day to accommodate the ten abalone in each tank. This totaled 3 g of ABKelp® per tank for the formulated and formulated + probiotic treatments. The difference in pellet count from the initial to final feed was converted into grams and the mean feeding rates for the formulated and formulated + probiotic treatments were calculated as dry feed mg/wet weight g abalone/d.

The standardized feed treatment utilized *D. Mollis* or *M. pyrifera* depending on availability throughout the experiment. There was a total of 20-30 g of feed added to the standardized feed treatments. The final feed was weighed out once a week, subtracted from the initial feed, and divided by seven for mean daily feeding rates. The *M. pyrifera* and *D. mollis* were weighed at the start and end of feeding. Feeding rate of white abalone (dry feed mg/wet weight g abalone/d) in each feed treatment, while accounting for 88% moisture in *M. pyrifera*, 91% moisture in *D. mollis*, 9% moisture in the formulated feed, and 22% moisture in the formulated + probiotic feed. These moisture values were taken from the proximate analysis results discussed below in the "Proximate Analysis" section.

Growth Rate and Survivorship Data Collection

There were a limited number of abalone available for this study, therefore two treatments (the standard and formulated + probiotic) had abalone with a starting mean shell length of 29 mm. There were not enough smaller abalone to make a third treatment of the same mean size, so abalone in the formulated treatment had a starting mean shell length of 32 mm. The abalone were not randomized by size and animals of a similar size class were used to reduce noise in the data.

All abalone were permanently labeled with an identification tag affixed to their shells using Corafix adhesive to track individual growth rates. Abalone were measured for shell length in millimeters and wet weight in grams individually on day zero of the study and at the end of each subsequent month of the six-month study. Abalone survivorship in each tank and treatment was assessed at the beginning of each week. Mortalities were also checked during the daily feeding. All mortalities per tank were recorded and removed from the tanks. The mortalities were not replaced by new abalone. If an abalone was removed from the tank their growth rates were removed from the entire study.

Proximate Analysis

At the end of the study 50 ml samples of feed, abalone tissue, and abalone feces from each treatment was sent to NP Analytical Laboratories in St. Louis, Missouri for proximate analysis. The samples were assessed for total ash, crude fiber, lipid, carbohydrates, calories, moisture, and protein. Ash was calculated by igniting the sample in a muffle furnace at 600°C to remove the organic portion. The residue was then calculated as percent ash in wet products. The crude fiber was found by drying the sample, washing it in dilute sulfuric acid, filtering it, and then washing it in dilute sodium hydroxide. The sample was then filtered again, washed, dried, weighted, ignited, and re-weighted. The remaining residue was then converted into percent fiber in wet products. Lipid was calculated by acid hydrolysis and converted into percent lipid in wet products. Carbohydrates and calories were assessed by calculation. Calories were calculated as kcal/100 g and carbohydrates were measured as percent carbohydrates in wet products. Moisture was found by drying the sample at 133°C and calculated as a percent moisture per sample. Protein was found using combustion and recorded as percentage in wet products. All of these

results were converted into percent in dry products except for the calories, which were reassessed as kcal/100 g in dry products.

Probiotic Detection

To determine if the probiotic was successfully delivered to the abalone, the feed and abalone tissue were plated using HiCrome™ plates used in the first probiotic study in Chapter 1. The pellets were crushed and added to a *Bacillus sp*. selecting agar plate to test that the *B. licheniformis* was still viable on the dried pellets before being fed to the abalone. The treated and untreated ABKelp® feeds were plated 24 hours after the probiotic was added to the feed and at the end of the week to ensure that the probiotic is still alive on the ABKelp® after a week of using it to feed the abalone. At the end of the six-month study 27 abalone from each treatment were sacrificed and their gut tissue was ground up using a rotor and plated on HiCrome™ plates to determine the presence of the probiotic in the abalone gut.

Data Analysis

Growth rates in terms of shell length (mm) and wet weight (g) were calculated as percent growth rate to normalize all treatments. This standardization allowed all three treatments to have the same starting point of 0% on day zero of the study. The difference in individual growth rates, Condition Factor (CF), feeding rates, Feed Conversion Ratio (FCR), Weight Specific Growth Rate (WSGR), Length Specific Growth Rate (LSGR), and Protein Efficiency Ratio (PER) between all three treatments were assessed with Repeated Measures ANOVA analyses in RStudio. Since individual abalone growth rates were assessed, each tank was used as a random factor in the Repeated Measures ANOVA analysis to account for potential non-independent growth rates in each tank. Significant differences in these factors were further assessed with a pairwise analysis to determine where the significant differences were between each treatment.

These analyses were done in RStudio version 4.2.2. The CF, feeding rate, FCR, WSGR, LSGR, and PER calculations used are as follows:

Condition Factor (CF)=($\frac{W}{L^3} * 100$)

Feeding Rate=($\frac{Fe(mg)}{W}$)/D

Feed Conversion Ratio (FCR)=$\frac{Fe}{Wg}$

Weight Specific Growth Rate (WSGR)=100*($Ln\ Wf - Ln\ Wo$)/190

Length Specific Growth Rate (LSGR)=100*($Ln\ Lf - Ln\ Lo$)/190

Protein Efficiency Ratio (PER)=$\frac{Wg}{P}$

where W is wet weight in grams, Wo is initial wet weight in grams, Wf is final wet weight in grams, and Wg is wet weight gained in grams. L is shell length in millimeters, Lo is initial shell length in millimeters, Lf is final shell length in millimeters, and Lg is shell length gained in millimeters. Fe is dry feed consumed in grams, except in the feeding rate where Fe is measured in milligrams. P is for dry protein consumed in grams. D stands for days since the last sample was taken and 190 is used for the total number of days in the experiment. The total Fe and total Wg are used for the total feed consumed in grams and total weight gained in grams in abalone throughout the study.

Results

Growth Metrics

The white abalone in the standard treatment exhibited the fastest growth rates in shell length and wet weight (Table 3). The mean starting shell length of the abalone in the formulated feed treatment was 3 mm higher than the other two treatments. The mean weight of the formulated feed treatment was also about 1 g heavier than the other two treatments at the start of the

experiment. After six months the abalone in the standard treatment had higher average growth rates in shell length and wet weight compared to the formulated and formulated + probiotic treatments (Table 3, Figure 9). There was a significant difference in cumulative percent change in shell length over six months between the standard treatment and both formulated treatments. (RM-ANOVA, diet $F_{2,90}$=69.23, p<0.0001, date $F_{5,90}$=74.54, p<0.0001, diet x date $F_{10,90}$=3.72, p<0.0001) and the trajectory of cumulative shell length diverged over time with the highest growth rate in the standard treatment (Figure 9A, C). There was no difference between the formulated and formulated + probiotic treatments over time and these treatments did not diverge during the study, as shown by the pairwise analysis.

There was a significant difference in cumulative percent change in wet weight between the standard verses the formulated and the formulated + probiotic treatments at all time points (diet effect). The trajectory of cumulative wet weight diverged over time with the highest growth rate in the standard treatment (RM-ANOVA, diet $F_{2,90}$=38.71, p<0.0001, date $F_{5,90}$=67.85, p<0.0001, diet x date $F_{10,90}$=6.85, p<0.0001; Figure 9B, D). There was no difference between the formulated and formulated + probiotic treatments over time and these treatments did not diverge during the study.

The condition factor (CF) compared the weight to the shell length of each abalone, with a heavier abalone for a given size indicating an abalone in better condition. There were no significant differences in the CF of each treatment and the CF values did not diverge over time between treatments (RM-ANOVA treatment: $F_{2,90}$=0.97, p-value=0.38, date: $F_{6,90}$=5.78, p<0.0001, treatment x date: $F_{12,90}$=1.23, p=0.26; Table 3, Figure 10). Instead, the CF remained similar and there was no change in trajectory over time between all treatments. The standard treatment had a significantly higher WSGR than the other two formulated treatments, which

were not significantly different from each other (treatment effect). The difference in WSGR between the standard treatment and the formulated treatments increased over time (treatment x date interaction effect) (RM-ANOVA, treatment $F_{2,54}$ =14.67, p <0.0001, date $F_{5,54}$=21.84, p<0.0001, treatment x date $F_{10,54}$=3.10, p<0.001; Figure 11A). The LSGR was significantly higher at all time points in the standard treatment compared to both of the formulated treatments, which were not significantly different from each other. The difference in LSGR between the standard treatment and the formulated treatments increased over time (RM-ANOVA, treatment F_{254} =23.70, p<0.0001, date $F_{5,54}$=8.41, p<0.001, treatment x date $F_{5,54}$=1.58, p=0.029; Figure 11B).

Table 3. Growth metrics for feed, cumulative growth rates, specific growth rates, and CF.

Treatment	Initial Wet Weight (g)	Final Wet Weight (g)	Initial Shell Length (mm)	Final Shell Length (mm)	Shell Length Growth Rate (%)	Wet Weight Growth Rate (%)	Condition Factor	Specific Length Growth Rate	Specific Weight Growth Rate
Standard	3.95±1.23	8.72±0.49	29.4±0.97	38.3±1.44	31.38±0.84	132.6±4.40	1.40E-02±1.07E-04	0.14±0.01	0.42±0.03
Formulated	4.63±1.19	8.1±0.46	32.28±0.78	37.79±1.44	16.95±0.31	76.36±1.46	1.39E-02±7.93E-04	0.08±0.041	0.29±0.02
Formulated + probiotic	3.61±0.93	5.75±0.36	29.57±0.52	34.47±1.07	16.89±0.34	65.28±0.87	1.37E-02±7.12E-04	0.06±0.01	0.25±0.03

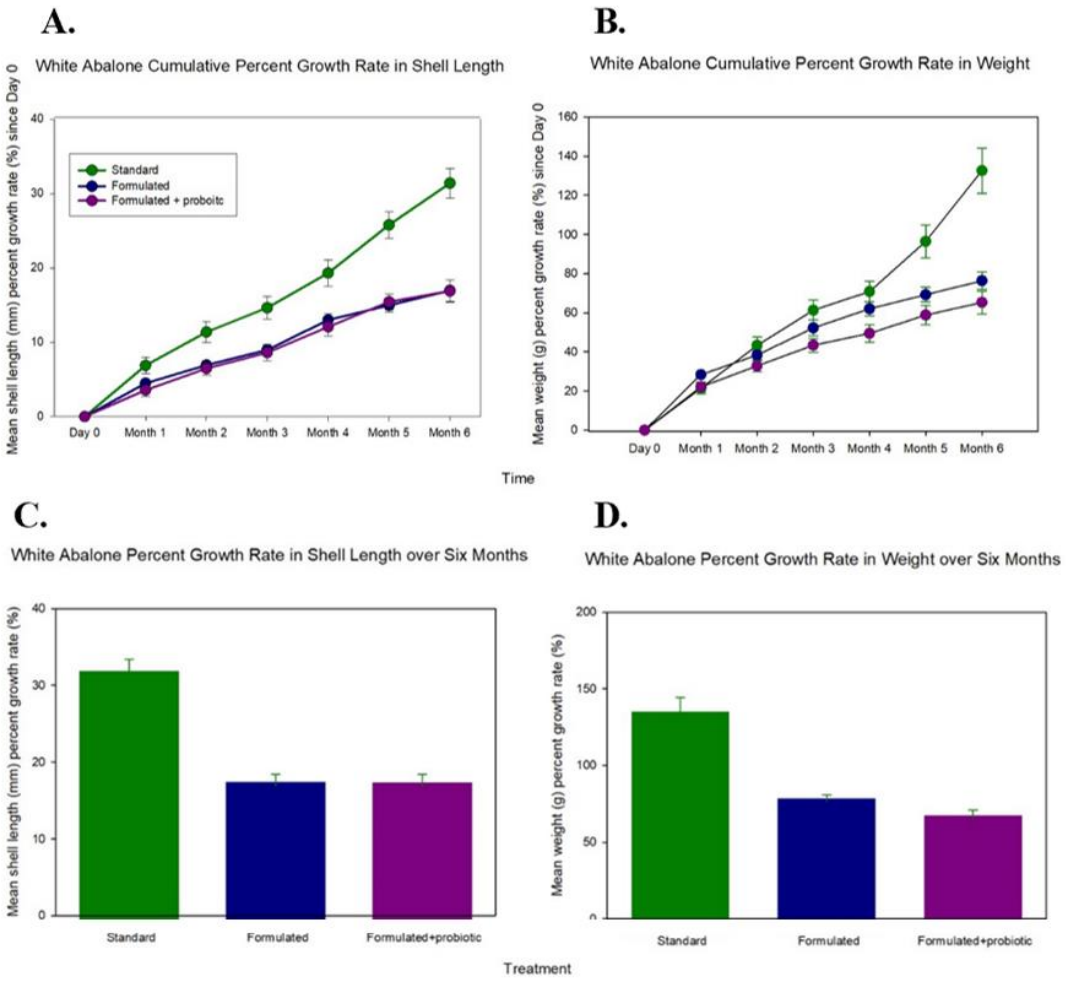

Figure 9. (A) Cumulative percent growth in shell length over 6 months by treatment. **(B)** Mean cumulative abalone wet weight growth rate over 6 months by treatment. **(C)** Cumulative percent growth rate in shell length from the start to the end of the study. **(D)** Mean cumulative wet weight at month 6 by treatment. Shown are mean values of each diet treatment ± SE.

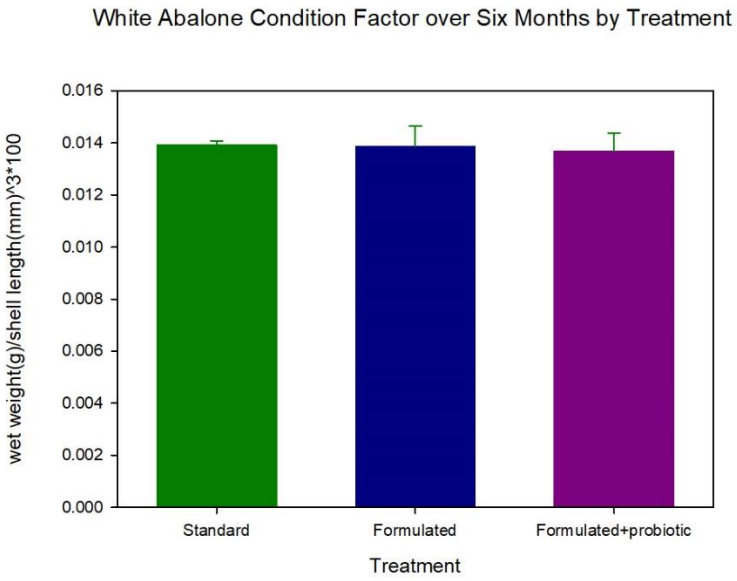

Figure 10. Condition factor of white abalone fed the three diet treatments. Shown are the mean values per treatment ± SE.

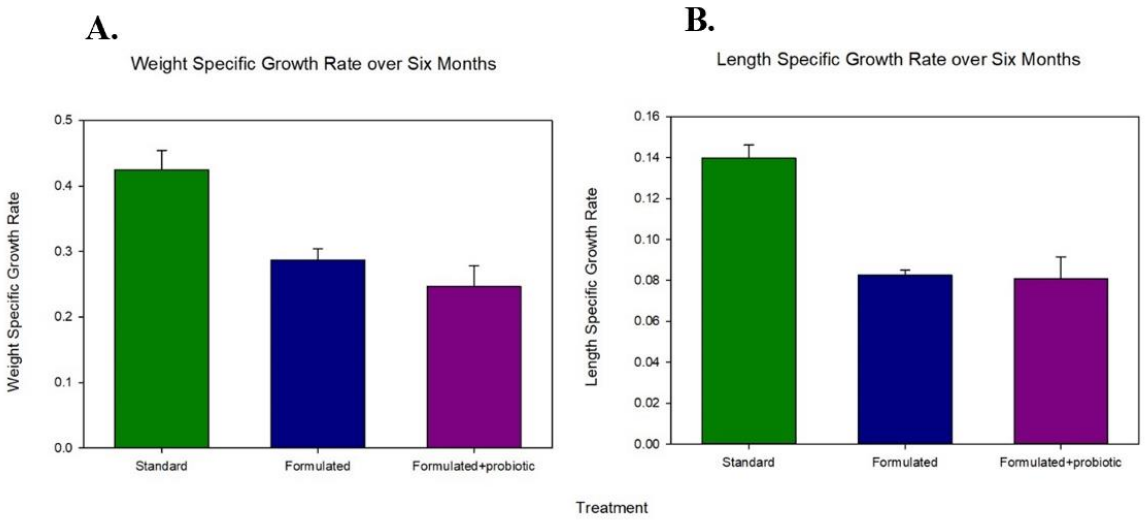

Figure 11. (A) Mean specific growth rate by weight (WSGR) at month 6 of the study by treatment. **(B)** Mean specific growth rate by shell length LSGR at month 6 by treatment. Shown are mean values of each diet treatment ± SE.

Feeding Rates

There was a significant difference in feeding rates among the three diet treatments. The highest feeding rates were in the standard treatment, followed by the formulated treatment, and the formulated + probiotic had the lowest feeding rate. All treatments were significantly different from each other shown in a Tukey HSD test and Figure 12A (ANOVA $F_{2,54}=21.93$, $p<0.0001$).

The standard treatment had a significantly higher FCR compared to the formulated feeds, indicating that the abalone required more of the standard feed to attain the same growth rate as abalone fed the formulated diet. The lowest FCR and thus the best feed conversion into new abalone biomass occurred in the formulated + probiotic diet. The standard treatment FCR was significantly higher than the formulated treatments throughout the study and the FCR diverged over time with the highest FCR rates remaining in the standard treatment over time compared to the formulated treatments, which remained constant and did not diverge from each other over the study (RM-ANOVA treatment: $F_{2,54}$=188.36, p<0.0001, date: $F_{5,54}$=20.87, p<0.0001, treatment x date: $F_{5,54}$ =11.89, p<0.0001; Figure 12B). The mean daily calorie intake was highest in the formulated treatment and lowest in the formulated + probiotic treatment. The standard treatment mean daily calories consumed was 0.57 kcal. The formulated mean daily calories consumed was 0.92 kcal and the formulated + probiotic was 0.37 kcal.

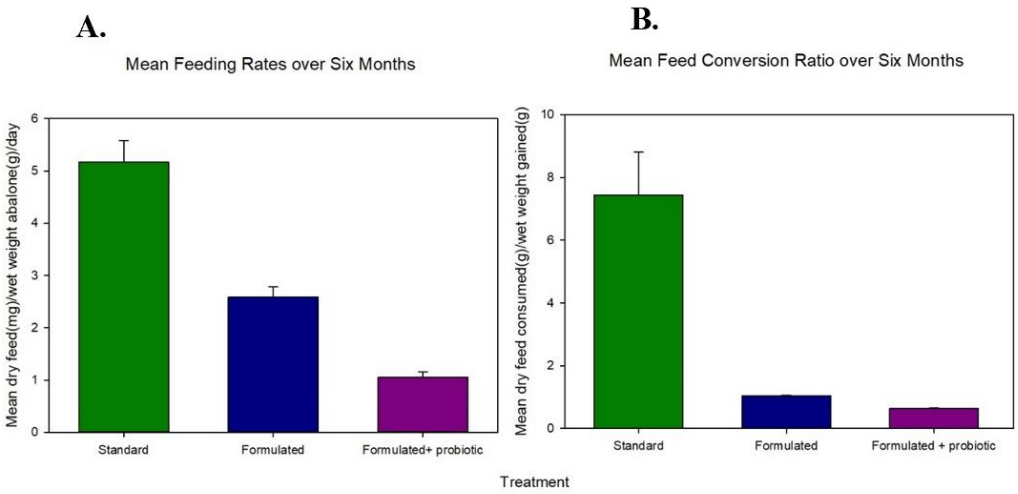

Figure 12. (A) Mean feeding rate over the 6 months of the study for each diet treatment ± SE. **(B)** Mean feed conversion ratio (FCR) for each diet treatment. Shown are mean values of each diet treatment ± SE.

Digestive Analysis

The mean Protein Efficiency Ratio (PER) is wet weight gained based on the amount of protein consumed in grams. PER was significantly higher in the formulated and the formulated +

probiotic feeds compared to the standard treatment across all sample time points. The formulated PERs were not significantly different from one another. The formulated and formulated + probiotic treatments diverged from the standard treatment over time (treatment x date interaction effect) (RM-ANOVA, treatment $F_{2,54}$=17.38, p<0.0001, date: $F_{5,54}$=2.74, p=0.034, treatment x date: $F_{5,54}$=6.29, p <0.0001; Figure 13).

The proximate feed analysis showed that the formulated and formulated + probiotic treatment feeds had higher calorie, protein, and carbohydrate content compared to the macroalgae species in the standard treatment. The formulated feeds had lower moisture content than the standard feed species (Table 4). The fecal proximate analysis indicated higher protein content in the formulated treatments compared to the standard treatment. The carbohydrate content was similar across all three treatments, but the lipid content was lower in the formulated treatments versus the stranded treatment. The fecal calorie content was highest in the formulated treatment and similar between the standard and formulated + probiotic treatments (Table 5). The tissue proximate analysis showed the highest protein and lipid content in the standard treatment. The lowest lipid content was in the formulated + probiotic treatment. The highest calorie density was in the standard treatment, followed by the formulated and then the formulated + probiotic treatment. The standard treatment had the lowest carbohydrate content of all three treatments, while the formulated treatments had similar carbohydrate concentrations (Table 6).

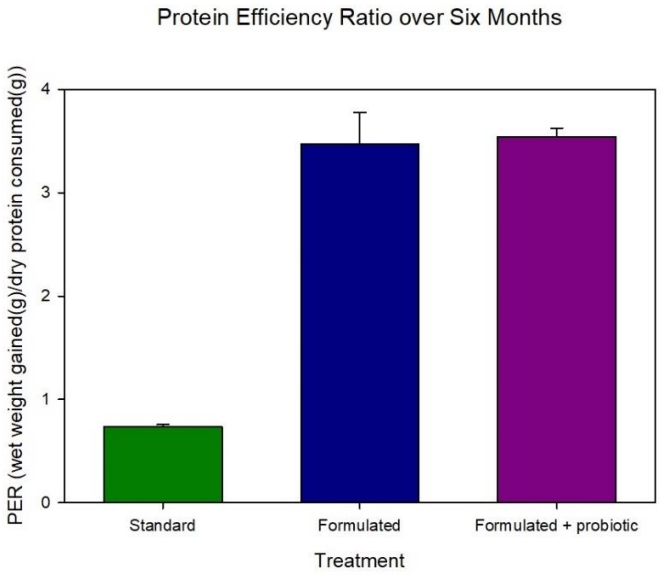

Figure 13. Protein Efficiency Ratio (PER) for abalone fed the three diet treatments. Shown are mean PER for each treatment ± SE.

Table 4. Analytical analysis of three treatment feeds dry matter (%). Analysis by NP Laboratories as wet matter (%) and converted into dry matter (%) except for calories, which was given as wet product in kcal/100 g converted into dry product in kcal/100 g.

Analysis	*M. pyrifera* Feed	*D. mollis* Feed	Formulated Feed	Formulated + probiotic Feed
Ash	44.78%	48.62%	13.85%	13.97%
Crude Fiber	5.42%	2.30%	3.25%	2.33%
Lipid (Acid Hydrolysis)	0.88%	1.91%	2.46%	1.91%
Calories (by calc) as kcal/100 g	225.7	216.1	356.7	353.8
Carbohydrates (by calc)	43.81%	24.71%	54.28%	55.00%
Moisture	88.70%	91.30%	9.72%	22.00%
Protein (combustion)	10.71%	24.94%	29.35%	29.10%

Table 5. Analytical analysis of fecal dry matter (%). Analysis by NP Laboratories as wet matter (%) and converted into dry matter (%) except for calories, which was given as wet product in kcal/100g converted into dry product in kcal/100g.

Analysis	Standard Feces	Formulated Feces	Formulated + probiotic Feces
Ash	71.02%	59.85%	68.46%
Crude Fiber	2.54%	3.94%	3.46%
Lipid (Acid Hydrolysis)	1.61%	<0.100%	<0.100%
Calories (by calc) as kcal/100 g	124.6	163.5	129.2
Carbohydrates (by calc)	16.10%	17.74%	16.13%
Moisture	88.20%	86.30%	87.00%
Protein (combustion)	11.27%	21.53%	13.54%

Table 6. Analytical analysis of abalone tissue dry matter (%). Analysis by NP Laboratories as wet matter (%) and converted into dry matter (%) except for calories, which was given as wet product in kcal/100g converted into dry product in kcal/100g.

Analysis	Standard Tissue	Formulated Tissue	Formulated + probiotic Tissue
Ash	9.33%	8.96%	9.00%
Crude Fiber	1.03%	0.95%	0.95%
Lipid (Acid Hydrolysis)	3.69%	2.25%	1.14%
Calories (by calc) as kcal/100g	382.1	375.4	368.9
Carbohydrates (by calc)	11.79%	34.79%	31.29%
Moisture	80.50%	78.90%	79.10%
Protein (combustion)	75.38%	54.03%	58.37%

Survivorship

There were no mortalities in any of the treatments. One abalone in tank six in the formulated treatment was removed from the entire study. During month two data collection, this abalone's shell was accidentally, manually detached in the process of removing the abalone from the edge

of tank six. Thus, the formulated feed treatment had a total of 29 abalone, while the other two treatments had 30 abalone per treatment.

Probiotic Detection

The HiCrome™ plates determined the presence of the probiotic on the abalone feed. They showed the presence of the probiotic on all ABKelp® treated with the probiotic and absence of the probiotic on the ABKelp® untreated with the probiotic. The plated tissue samples were less conclusive. The HiCrome™ plates were not able to clearly show the presence or absence of the probiotic. The probiotic was detected in abalone from all treatments. There were also numerous inconclusive plates where the plate color and presence of *B. licheniformis* could not be determined (Table 7).

Table 7. The presence of the probiotic in sampled abalone gut tissue by treatment, determined by HiCrome™ plates. There were 3 abalone samples per tank and each sample was plated/replicated three times for a total of 27 samples per treatment.

Treatment	Probiotic Present	Probiotic Absent	Inconclusive
Standard	6	19	2
Formulated	9	13	5
Formulated + probiotic	10	15	2

Discussion

Investigating the utility of a formulated diet for white abalone aquaculture is valuable because of the need to provide feed year-round despite seasonal limitations and ease of supplying a feed to abalone culture facilities. The formulated feed must also be assessed for its ability to increase growth rates in white abalone, since white abalone growth rates are inherently slow, which presents an additional impediment to the restoration species. This study assessed whether use of

the probiotic *B. licheniformis* as an additive to improve growth rates, feeding rates, and survivorship in white abalone aquaculture. In other species, probiotics provide nutrients and increase digestive enzyme activity within the organism; they also promote growth, feeding rates, and immune health (Macey & Coyne, 2006). Specifically for abalone, *Vibrio* JH1, *Exiguobacterium* JHEb1 *and Enterococcus* JHLD have been shown to enhance shell length and wet weight growth in New Zealand abalone (*Haliotis iris*) (Hadi et al. 2014). *Shewanella colwelliana* WA64 *and S. olleyana* WA65 have been reported to improve growth and immune response in Disk abalone (*Haliotis diversicolor supertexta*) (Jiang et al. 2013) (Table 2). This study examined both the suitability of a formulated diet and the effectiveness of probiotic *B. licheniformis* for white abalone growth. The results of this thesis work showed that fresh macroalgae feed produced the greatest feeding rates and growth rates in white abalone compared to formulated feeds and probiotic treatments, while the probiotic treated feed had the lowest feeding and growth rates. However, abalone fed the formulated feed and probiotic treatments had significantly better FCRs and PERs compared to those fed the fresh standard seaweed feed.

Probiotic Detection

Previous studies have shown that the presence of different probiotics in formulated abalone feed have resulted in increased growth rates, improved immune response, and reduced infection in abalone (Jiang et al., 2013; Hadi et al., 2014; Zhao et al., 2018; Gao et al., 2020) (Table 2). It is important to consider the delivery method of the probiotics to the abalone to be confident that the probiotic is reaching the target locations of the animals in a viable state. This confirmation of the probiotic presence in the abalone is especially important if no significant effect on the animals are observed (as in Chapter 1).

The presence of the probiotic should be accurately assessed to ensure low growth rates in the probiotic treatment was due to the ineffectiveness of the treatment as opposed to inadequately delivering the probiotic to the animal. Previous studies that have shown a significant effect in abalone used experimental treatment designs to determine that the results were related to the probiotic and no other confounding factors. These studies used methodologies like growth rates, feeding rates, FCR, PER, specific growth rates, mortality rates, bacterial infection status, phagocytic activity, hemocyte count, and bacterial culture techniques to determine if their delivery systems were performing as expected. This study assessed growth rates, feeding rates, FCR, PER mortality rates, specific growth rates, and proximate analysis to test the probiotic delivery method. The experimental design also included plating the fed and abalone gut tissue to test the presence and viability of the probiotic.

The presence of the probiotic was confirmed on the probiotic treated ABKelp® feed by the use of diagnostic HiCrome™ culture plates. The probiotic was able to survive on the ABKelp® feed for one week following application to the feed. This result is in line with previous studies that also checked for probiotic viability on feed using bacterial culture techniques (Macey & Coyne, 2006; Jiang et al., 2013). This method has been used in past studies to confirm probiotic delivery methods, but the protocol does not specifically account for whether the probiotic successfully colonized the abalone gut tissue as is intended with digestive system probiotics. Macerated gut tissue samples were cultured on the HiCrome™ plates to confirm if the probiotic was able to survive in the abalone intestine. The outcome of this effort was largely inconclusive, with plated gut tissues showing varied presence of a *Bacillus sp.* in treated and untreated fed treatments (Table 7). This could be due to other experimental factors in the abalone gut confounding the results of these plates. Factors that may produce negative identification include gut enzymatic

inhibition, other inhibiting bacteria, or a lack of sufficient amounts of *B. licheniformis* for detection (Steinhaus, 1960; Walker et al., 1999). Unexpected positive identification of the probiotic in gut tissue from untreated fed treatments may be the HiChrome^{TM} plates detecting other *Bacillus sp.* bacteria closely related to *B. licheniformis.* The presence of the probiotic in the gut or ability for the probiotic to survive in the abalone gut was unclear in this study. Nevertheless, the positive probiotic detection on the feed and the lower abalone growth and feeding rates in the probiotic treatment indicate that this probiotic may not be beneficial for white abalone. The lower feeding rates and in turn lower growth rates in the probiotic treated white abalone may be due to a combination of factors. The probiotic may not be able to colonize the abalone gut. Merrifield et al. (2010) found that when enterococci, *B. subtilis*, and *B. licheniformis* were fed to rainbow trout (*Oncorhynchus mykiss*) these three probiotics together accounted for less than 10% of the viable bacteria population in digestive tract. Thus, the *B. licheniformis* may not be able to populate enough of the abalone gut to provide growth and feeding rate benefits. The probiotic might inference with digestion and the taste and texture of the probiotic treated feed may hinder feeding and growth rates (Figure 9 and 12A). The chemical composition, texture, and taste of feed could be altered by the addition of the probiotic, which can impact the abalone tissue, digestion, growth, and feeding (Kemp et al., 2015). This probiotic would not be a recommended additive in white abalone aquaculture based on its poor growth and feeding performance in the abalone.

Growth Rates

The higher cumulative percent abalone growth rate in wet weight compared to shell length across all treatments in this study were consistent with other abalone formulated feed studies (Bautista-Teruel & Millamena, 1999; Naidoo et al., 2006). Naidoo et al. (2006) compared various kelp and

algae diets including the formulated feed ABKelp® from Sea Plant Productions Ltd., South Africa. They found that although the shell length growth rates remained relatively constant over the nine-month study, there was a significant increase in wet weight growth rates for abalone fed the standard feed (Naidoo et al., 2006). These results indicate that more energy is put into weight gain in juvenile abalone compared to shell growth. Furthermore, shell growth may be less linked to metabolism and more a function of time and water chemistry. Abalone shells are formed with about 95% $CaCO_3$ and approximately 5% organic compounds (Sarikaya et al., 1989). These are added to the shell through biomineralization where these molecules are taken up from the surrounding saltwater environment (Lin & Meyers et al., 2005). Ajili et al. (2022) found that carbonate used in shell formation is primarily sourced from seawater. Thus, the abalone shell growth could be more associated with water chemistry than diet.

In the first two months of the study, abalone in all three treatments had similar wet weights. The separation in growth rates between the standard treatment compared to the other formulated treatments began in month three of the experiment with noticeable increase in standard growth rates (based on wet weight) in month five of the study. The similar growth rates between all three treatments at the start of the study and the separation in growth rates over time could indicate that the probiotic colonized the abalone gut, but had a negative effect on growth rates. Alternatively, the probiotic may have altered the chemical composition of the feed and reduced palatability in the treated feeds resulting in lower feeding and growth rates compared to the untreated formulated feed and standard feed treatments despite higher caloric content of the formulated feeds.

Specific growth rates in this study were similar to those of other abalone feed studies ranging from 0.118-0.101 mm in length and 0.223-0.399 g in weight (Nelson et al., 2002) (Figure 11).

Formulated and formulated + probiotic feeds resulted in slower feeding and growth rates compared to standard feed, at the scale of the whole abalone and at a specific rate (per-gram-wet-weight). This difference was only statistically significant at the scale of the whole abalone, but LSGR and WSGR could have continued to separate and differ over time because of the higher feeding rate in the standard treatment compared to the formulated treatments.

The FCR was higher in the standard treatment using two macroalgal species compared to experiments that used a single macroalgae species. Studies that used one macroalgae species had FCRs of 2.8-3.4 compared to the 7.6 FCR in the standard treatment of this study (Table 3 and Figure 12B) (Britz, 1996; Sales & Britz, 2008). The higher FCR in the study compared to other studies could be due to difference in the macroalgae used. These studies used *Gracilis verrucosa* and *Ecklonia maxima*, which could have contributed to the higher FCR values in those found in this study (Britz, 1996; Sales & Britz, 2008). The formulated feed FCR in other studies was 0.7-1.0, which is similar to the 1.1 FCR in the formulated treatment in this experiment (Figure 12B) (Britz, 1996; Sales & Britz, 2008). The formulated + probiotic FCR was 0.65, which was lower than the formulated feed FCR values in other studies. This lower FCR in the formulated + probiotic could be due to the presence of the probiotic. Thus, the probiotic may have improved the FCR, but the lower feeding rate in the formulated + probiotic treatment also could have contributed to the lowest FCR of all three treatments. The FCR could have varied between different feeds treatments based on nutritional values, feeding rates, or proximate analysis.

The lower PER in the standard treatment compared to the formulated and formulated + probiotic feed treatments relates to the protein content in each feed treatment. The formulated and the formulated + probiotic treatments had higher protein content compared to the *M. pyrifera* and *D. mollis* in the standard feed treatment. The PER could also be associated with protein sparing.

Protein sparing is the conservation of protein for purposes other than energy such as tissue repair and muscle production (Faturrahman et al., 2014). Beamish & Medland, (1986) found that increased lipid and protein content in rainbow trout feed can cause protein sparing. The higher protein content found in the tissue of abalone from the standard treatment compared to the formulated treatments corroborates this protein sparing theory.

The lipid content in each treatment feed, feces, and tissue could have contributed to the difference in growth rates between treatments. The mean lipid content in the standard treatment feed was lower than the formulated treatment feeds, but higher in the standard treatment feces and abalone tissue. The reduced feeding rate in the formulated and formulated + probiotic treatments could have reduced digestibility of the feed lipid content, resulting in the lower length and wet weight growth rates in the formulated treatments compared to the standard treatment.

Feed and Probiotic Palatability

Differing feeding rates among the treatments in this study are likely related to the familiarity and palatability of the feeds. The standard feed treatment had the highest feeding rate, which is consistent with previous studies (Reyes & Fermin, 2003; Bansemer et al., 2014; O'Mahoney et al., 2014) and is likely related to feed familiarity. All abalone used in this study were fed *M. pyrifera* and *D. mollis* conventionally used in California for aquaculture prior to the beginning of the experiment. It is possible but unlikely that conditioning abalone alone caused the lower feeding and growth rates because this study was long enough that the first month could have been viewed as a conditioning period. Over the five months that followed growth rates in the standard feed treatment continued to be faster than the growth rate in the two formulated treatments, suggesting that acclimation alone was not the cause of the lower feeding and growth rates observed (Figure 9 and 12A).

It is unclear from these results what components in standard fresh seaweed result in superior feed consumption rates compared to formulated feeds. One consideration is the basic presence of natural organoleptic feed attractants in seaweed that appeal to the abalone's taste and smell physiology (Mau & Jha, 2018). Other algae species such as *Laminaria digitata, Palmaria palmata,* and *Ulva lactuca* have been used in a milled formulated form to increase abalone feed palatability (O'Mahoney et al., 2014; Bautista-Teruel et al., 2016). The formulated feed treatments in this study consisted of ABKelp®, a commercial feed whose full list and exact proportions of ingredients is not publicly available, but which is composed of *M. pyrifera,* vegetable protein, and other proprietary ingredients. Despite the inclusion of dried *M. pyrifera* in ABKelp®, the feed differed in many aspects to the standard treatment of fresh *M. pyrifera* and *D. mollis*. Based on this experiment it is reasonable to surmise that the addition of natural seaweed attractants in dried, milled *M. pyrifera* alone are unable to match the palatability of the fresh feed. The addition of multiple milled seaweed species like *Laminaria digitata, Palmaria palmata,* and *Ulva lactuca* could add more natural attractants to the formulated feed and increase ABKelp® feeding rates.

Other studies have not investigated the palatability of probiotic additives, but instead blame palatability solely on the composition of formulated feeds (Lee et al., 2013; Mau & Jha, 2018 Bullon et al., 2021; Dexfooli et al., 2021; Yu et al., 2022; Dexfooli et al., 2023). Although feed palatability is considered an issue in formulated feed production, the palatability of the added probiotics may also require more investigation. It is still unclear from this study if *B. licheniformis* was present in a viable state in the abalone gut, as would be required to confer any probiotic benefits to the abalone. Nonetheless, the potentially poor palatability of the probiotic

coupled with reduced growth metrics, indicate that this particular probiotic and its delivery method is not a viable treatment to enhance white abalone growth in aquaculture.

Proximate Feed, Tissue, and Fecal Analysis

A protein content of about 27% and lipid content of 3.6% is considered optimal for tropical abalone (*Haliotis asinina*) (Bautista-Teruel et al., 2016). The ABKelp® feed had lipid and protein content close to those optimal for abalone species, but the *M. pyrifera* and *D. mollis* individually had below optimal protein and lipid content (Table 4). Macroalgae sources typically do not contain enough protein and lipid ratios individually; therefore, abalone aquaculture requires a combination of algae species to suit the dietary requirements of abalone (O'Mahoney et al., 2014; Bautista-Teruel et al., 2016). This feed proximate analysis is only qualitative and only one sample from each treatment was analyzed. Proximate analysis in the feed showed that the formulated and formulated + probiotic feed treatments had higher caloric densities and macronutrient compositions compared to the standard feed. These higher values may be responsible for the superior FCRs and PERs in both formulated treatments versus the standard treatment (Figure 12B and 13).

The conversion of the feed into fecal matter showed higher conversion rates of calories, protein, carbohydrates, and lipids in the standard treatment versus the formulated treatment and formulated + probiotic treatment (Table 5). The lipid content in the formulated feeds was higher than the standard feed, but the feces of the standard treatment had higher lipid content that the formulated feeds. This could be related to the higher feeding rates in the standard treatment compared to the formulated treatments. The assimilation of lipids in the standard treatment could also be more efficient compared to the formulated treatments. Apparent digestibility of the feed could not be calculated because an inert digestibility marker could not be included in the feed

treatments (Montaño-Vargas et al., 2002). Similarly, total feces from a known feeding volume and acid-insoluble ash could not be collected due to experimental logistics (Montaño-Vargas et al., 2002). This fecal analysis is only qualitative and only one sample from each treatment was analyzed. The feces from the formulated treatments showed lower lipid values, but similar calories, protein and carbohydrates compared to the standard treatment. This could be based on the higher feeding rates in the standard feed compared to the formulated feeds. The standard feed had lower lipid, calorie, protein, and carbohydrate content compared to the formulated feed, but the significantly higher feeding rates could have increased the quantities of calories and macronutrients in the standard treatment feces. Lower lipid content in the formulated treatments feces could have contributed to lower digestion and growth rates in these treatments compared to the standard treatment.

Proximate analysis of the abalone tissue showed lower macronutrient compositions and caloric densities in the animals fed the formulated feed treatments compared to the standard treatment (Table 6). Despite greater lipid content in the formulated abalone tissue fed formulated feed had lower lipid content than those fed standard treatment. This could be related to the higher feeding rates in the standard treatment compared to the formulated treatments, a result found in other studies where it was attributed to differing macronutrient composition of the feeds and the higher productive energy value in the macroalgae feed (Mulvaney et al., 2013). Although the formulated diet had greater protein content than the standard diet, the amino acid composition of those proteins may play a larger role in building muscle and storing energy than total protein content alone (Gómez-Montes et al., 2003; Sales & Britz, 2008; Dunstan, 2010; Mulvaney et al., 2013). Regardless of the higher protein content in the formulated feed, the make-up of essential amino acids in the macroalgae could be attributed to the higher protein content found in the

tissue of abalone from the standard treatment (Gómez-Montes et al., 2003; Sales & Britz, 2008; Dunstan, 2010; Mulvaney et al., 2013).

Formulated ABKelp® Feed use in White Abalone Restoration Aquaculture

There are tradeoffs in formulated feed and fresh macroalgae feeds. The standard feed had higher feeding rates and growth rates in shell length, wet weight, LSGR, and WSGR compared to the formulated treatment and formulated + probiotic treatment. These results are likely due to the increased palatability of the standard feed compared to the formulated feeds. The natural attractants, flavors, and texture of the standard feed could have contributed to the increased feeding rates and in turn higher growth rates in the standard treatment over the formulated feeds. The formulated treatments had significantly better FCRs and PERs compared to the standard treatment. These results are likely based on the composition of the formulated feed, which had higher macronutrient and calorie density compared to the standard treatment. Thus, less of the formulated feed is required for the equivalent growth rates on the standard feed, but due to the significantly higher feeding rates in the standard feed compared to the formulated feed the standard feed was still able to out-perform the formulated feed growth rates. Additionally, while the feed stability of the formulated feeds was anecdotally shorter than the standard seaweed feed, leaving the formulated feed in the tank for more than 24 hours could result in abalone eating more food as the pellet softens in the water over time. This in turn could increase feeding and growth rates in the formulated treatments.

Although the formulated feed treatments had lower growth and feeding rates compared to the standard feed treatment, the formulated treatment still showed positive growth rates in both shell length and wet weight. This feed could be used in abalone aquaculture during periods of limited access to fresh macroalgae. Strong storms and short days during the winter result in reduced *M.*

pyrifera and *D. mollis* growth and availability. Formulated feed could be used to supplement seaweed during the winter. An economic analysis would be required to better understand how formulated feed could lower the cost of abalone aquaculture. Based on the growth rates in this study it would take abalone fed the standard diet approximately 13.5 months to reach outplanting size. Abalone on the formulated feed alone could reach outplanting size at 24 months.

Formulated feed could reduce ancillary and direct costs associated with harvesting *M. pyrifera* and *D. mollis* from the wild. The use of alternative pellet feed could also improve kelp forest health. The macroalgae needed for formulated feed production could be grown in aquaculture at production facilities to limit kelp deforestation. The formulated feed could then be easily shipped to White Abalone Project partners to ensure adequate feed supply during the winter.

There is a trade off in the formulated feed compared to the standard fresh feed. The standard fresh feed has higher palatability leading to higher feeding and in turn growth rates as seen in this and other studies (Bansemer et al., 2014). The formulated feed has higher protein, carbohydrate, lipid, and calorie content. This creates better FCRs and PERs compared to the standard fresh feed. The use of formulated feed alone may not be optimal for white abalone production. Other studies, however, have seen success in supplementing fresh seaweed feeds with a formulated feed (Naidoo et al., 2006; Dlaza et al., 2008). The formulated diet requires improved palatability to completely replace seaweed with a formulated diet.

Conclusion

The first study reported in this thesis focused on a probiotic treatment for Ca.Xc infection. This bacterial infection in abalone typically results in impaired digestion, degradation of gut tissue, atrophy of the foot and mantle, and if left untreated, eventual mortality. The only current

treatment for Ca.Xc is the administration of the antibiotic OTC, but antibiotics come with numerous caveats including reduced feeding and growth rates, the reduction of beneficial gut bacteria, and the increase of ARGs in the organism. An alternative mitigation measure to prevent and treat intestinal ailments in abalone and other species is the use of probiotics which may provide improvements in gut flora health, feeding rates, and growth rates.

The initial study in this thesis investigated the utility of probiotics to prevent or mitigate the effects of Ca.Xc on white abalone. However, during the investigation it was discovered that Ca.Xc may not pose the same threat to the species' health as it once did. None of the abalone in this experiment contracted the Ca.Xc infection, despite their prolonged exposure to red abalone from which Ca.Xc is found endemically (Crossen et al., 2014). Beyond this experimental issue, the study was further confounded during the experimental period by unexpected observations of substantial and sustained shell loss in white and red abalone. The shell loss severely hindered the feeding and growth rate data collection in this experiment making it impractical to accurately determine the effects of probiotics on feeding and growth rates.

The shell loss observed in the first probiotic study was on a larger scale than previously observed in other studies or abalone aquaculture facilities. In Chapter 2, the infection chain challenge experiments were conducted for red and white abalone to determine if an infectious agent was responsible for the shell loss syndrome. Only one white abalone lost its shell over the course of the experiment. This abalone was housed in the low transmission treatment, which does not support the assumption that an infectious agent would spread more rapidly win a denser setting. No red abalone lost their shells during their shell loss challenge experiments, but it was noted that lower stocking densities can significantly improve feeding and growth rates in red abalone. With only one abalone losing its shell across the two shell loss infection chain challenge

experiments, this study could not determine whether an infectious agent was the root cause of the shell loss syndrome.

A pathological health assessment done by the California Fish and Wildlife Shellfish Health Lab indicated that the shell-less abalone were generally in poor health with reduced gut functions and undigested feed in the gut. The shell-less abalone were able to persist in captivity for up to three months without their shell. The cause of the shell loss is still unknown and requires further investigation for the health of captive and wild abalone.

The use of probiotics to mitigate Ca.Xc infection in white abalone proved inconclusive and potentially irrelevant due to the inability to detect Ca.Xc in white abalone tissues (Chapter 1); however, the use of probiotics to improve culture methods is established in the literature for other abalone species. Chapter 3 assessed the feeding and growth rates of white abalone treated with *B. licheniformis*. The application of the *B. licheniformis* probiotic to the ABKelp® resulted in the lowest feed intake and wet weight growth rates of all three treatments. Abalone treated with the probiotic had the lowest (and thus best) FCR, which could be due to the presence of the probiotic or may be associated with the lowest feeding rates in the probiotic treated abalone compared to the other treatments due to the palatability of the probiotic. Based on these results, *B. licheniformis* is not considered to improve white abalone aquaculture and could even decrease growth rates. Other more palatable probiotics may prove to be more beneficial for improving the growth rates and digestibility in white abalone for future work in white abalone restoration aquaculture.

Despite the poorer performance of formulated feeds, they may still have an important role as part of a holistic feeding program that includes traditional fresh macroalgae feed as well. Fresh

macroalgae is seasonally limited in winter by storms and reduced light availability. Many partners of the White Abalone Project are challenged to adequately feed their white abalone during winter months. A formulated dry feed that supplements fresh seaweed would address these issues. This comparative feed study showed that white abalone fed the standard fresh seaweed feed produced the highest growth, which is likely related to a superior palatability compared to that of the formulated and formulated + probiotic feeds. Despite the formulated feed being calorically denser than the standard seaweed feed, abalone did not consume adequate amounts of the formulated feed, as evidenced by significantly lower growth rates. Enhancing feed intake, by improving feed palatability of the formulated feed, would be necessary for this feed to be considered a viable alternative to the standard fresh seaweed feeds. Additionally, a mix of formulated feed with standard seaweed feed could improve growth and feeding metrics. Overall, while the formulated ABKelp® feed proved comparatively inferior to standard fresh seaweed for growth metrics in this study, formulated feed could be a viable feed source for white abalone when the standard fresh seaweeds are limited, or the cost of seaweed collection or cultivation is too great. Taken together, results from this thesis lay the foundation for enhancing future white abalone restoration aquaculture through an understanding of tank stocking density, feed composition, Ca.Xc, shell loss syndrome, and probiotics that could affect restoration aquaculture of white abalone.

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