# EVALUATING THE EFFECT OF PREBIOTICS AND PROBIOTICS ON ROTIFER AND JUVENILE RED DRUM (*SCIAENOPS OCELLATUS*) PRODUCTION

A Thesis

by

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# ABSTRACT

Commercially produced probiotics and prebiotics show potential to enhance growth performance and disease resistance of various animals but have not been evaluated as enrichments in live foods for larval fish. Therefore, four separate trials were conducted to evaluate changes in the production and microbial composition of rotifers (*Branchionus plicatilis*) exposed to the probiotics Bactocell™ and Aquablend™ and prebiotics Grobiotic®-A, and SILOhealth 108P added to the water during 4-day cultures compared to the control with no supplement. Grobiotic®-A showed potential to increase rotifer production while Bactocell™ decreased production. Denaturing gel gradient electrophoresis (DGGE) analysis demonstrated rotifers cultured with aforementioned additives except SILOhealth 108P altered microbiota composition compared to the control.

Bactocell™, a commercial probiotic consisting of *Pediococcus acidilactici*, has shown benefits to immunomodulation, nutrient digestion, and feed utilization in shrimp but has not been evaluated in fish. Therefore, a feeding trial was conducted with juvenile red drum (*Sciaenops ocellatus*) fed diets with graded concentrations of Bactocell™ (0, 0.05, 0.1, 0.2, 0.4, and 0.8% of dry weight) for 8 weeks. The diets were formulated primarily from soybean protein sources with low amounts of menhaden fishmeal to contain 44% crude protein and 10% lipid. Each diet was fed to fish in quadruplicate 38-L aquaria for 8 weeks. Digesta from red drum was obtained at the end of weeks 4 and 8 to characterize microbiota by DGGE analysis. Red drum fed experimental diets grew rapidly and achieved a 1,300% increase in initial body weight after 8 weeks; however, no significant ($P \leq 0.05$) differences in weight gain, feed efficiency, or survival could be attributed to different concentrations of Bactocell™. After 8 weeks of feeding, DGGE analysis

showed that Bactocell™ altered the microbiota of red drum digesta compared to fish fed the basal diet.

The prebiotics and probiotics tested except SILOhealth 108P altered the microbiota of rotifers after a 4-day cycle. Bactocell™ altered the digesta microbiota of red drum but did not improve fish growth or survival. Additional investigations under harsh environmental conditions in which immunological responses and/or disease resistance are assessed may be required to demonstrate potential benefits of dietary Bactocell™ supplementation.

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# CONTRIBUTORS AND FUNDING SOURCES

## Contributors

The experiments preformed to complete this thesis were directed by my advisor Dr. Gatlin (Ecology and Conservation Biology Department), Dr. Hume (USDA Agricultural Research Service), and Dr. Todd Sink (Range, Wildlife and Fisheries Management Department).

Brian Ray provided guidance and a helping hand while conducting the research detailed in this thesis. All other work detailed in this report was conducted independently by the student.

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# CHAPTER I

# INTRODUCTION

Aquaculture is an efficient method for producing seafood and is practiced all around the world. According the Food and Agriculture Organization (FAO), 80 million metric tonnes of seafood were produced by aquaculture in 2016, which has drastically increased from the 0.6 million metric tonnes produced in 1950 (FAO, 2018). These numbers show the increasing potential of aquaculture to help support a growing global population that has caused wild-caught fisheries around the world to be pushed to the brink of unsustainability. While the demand for seafood continues to increase with human population increases, the supply of seafood from capture fisheries has remained stagnant since the mid-1990s (Chassot et al., 2010). Despite improvements in fishing methods, fishermen are spending more time at sea while traveling further distances to catch similar quantities of seafood due to the decreasing availability of wild fish. Aquaculture provides an efficient option for meeting the increasing global demand for seafood. In addition to increasing seafood production, aquaculture is also practiced in various parts of the world for enhancing stocks of native fish species for recreational purposes as well as for commercial harvest.

Stock enhancement has been defined as, "a set of management approaches involving the release of cultured organisms to enhance, conserve or restore fisheries," (Lorenzen, 2008). Red drum (*Sciaenops ocellatus*) is a species commonly cultured in Texas for stock enhancement as well as seafood production due to its popularity as a recreational and food fish, with a history of being overfished (Murphy and Crabtree, 2011).

Due to its popularity as a food fish, red drum stocks were severely depleted before conservation measures were put into place in the 1980s to help preserve their population. To

help combat their declining populations, commercial fisheries in state and federal waters were banned, sport harvest regulations were put in place, and production for stock enhancement was implemented (McEachron et al., 1998).

Texas is the leader in stock enhancement in the United States, with the Texas Parks and Wildlife Department reporting to have released 20-30 million red drum fingerlings (25-30 mm in length) each year in repopulation efforts. Due to these extremely effective measures, by the 1990s, populations of subadult red drum (1-5 years old) had doubled, populations of fish greater than 711 mm in length increased by 750%, and recreational catches also increased compared to data from the 1980s (McEachron et al., 1998).

While red drum is produced for stock enhancement, it is also produced in commercial aquaculture for seafood in various forms including fillets, steaks and sometimes as whole fish (FAO, 2020). Red drum first became widely produced in 2003 as worldwide production increased from about 2,000 metric tonnes in 2002 to 40,000 metric tonnes of fish in 2003 (FAO, 2020). As of 2016, about 75,000 tons of red drum are produced annually for human consumption with China being the leader in food fish production, producing 94% of global output (FAO, 2020). Out of the 75,000 tons of red drum produced, the state of Texas produces each year between 1,000 and 1,150 tons of fish (Treece and Sink, 2017).

The red drum is a prime subject to be raised in aquaculture systems due to their ability to breed predictably in a hatchery setting. Photoperiod can be manipulated along with water temperature, enabling the brood stock of red drum to be predictably induced to volitionally spawn on a regular basis. This type of photothermal cycling is practiced in red drum aquaculture for both stock enhancement and commercial seafood production so fish can produce fertilized eggs multiple times per year. Once fertilized eggs have been collected from the broodfish tanks

and allowed to hatch, they are typically transferred to fertilized ponds in which phytoplankton and zooplankton, such as copepods and rotifers, provide natural foods for the larval fish to grow to juvenile size in approximately 30 days (Davis, 1990). An issue with raising juvenile red drum in fertilized ponds is that there is limited control over what the fish may be eating, and potential changes in environmental conditions can cause high mortality and limited recovery of juvenile fish from the ponds. Red drum larvae also can be cultured indoors to achieve greater control over production conditions. However, indoor larviculture requires culturing or natural harvesting of live foods such as rotifers and brine shrimp (*Artemia salina*) nauplii to feed the larval red drum. Providing a constant supply of live foods in a hatchery setting is much more labor intensive and cost prohibitive than allowing larval red drum to feed on zooplankton in fertilized ponds. However, improvements in live foods production or nutritional quality may allow more controlled and enhanced production of red drum juveniles under indoor, environmentally controlled conditions.

One potential area for improving live foods for hatchery rearing of larval fish is to supplement live food organisms with additives to allow them to confer nutritional and health benefits to the larvae. Rotifers (*Brachionus plicatilis)*, due to their relatively small size, limited swimming speed, and relative ease of cultivation, are one of the most commonly used live food organisms (Wang et al., 2019). In general, compared to proximate composition of other live foods such as copepods, rotifers have been shown to have similar protein and higher carbohydrate content while having a slightly lower lipid content (Rajkumar and Kumaraguru, 2006). It has been shown that the nutritional value of the larval fish can be beneficially altered depending on the food resources that rotifers consume (Wang et al., 2019). For example, in a study involving turbot (*Scophthalmus maximus*) larvae consuming rotifers differing in dietary

protein content affected the amount of protein transferred to the larval fish after consumption (Oie et. al, 1997). In another study, rotifers supplemented with increasing taurine concentrations (up to 400 mg/g) were shown to increase the survival of yellowtail (*Seriola dumerili*) larvae (Matsunari et al., 2013). These are two examples of numerous studies indicating there are opportunities to transfer potential health benefits to larval fish by supplementing rotifers with various nutrients. In addition, other diet additives such as prebiotics and probiotics, which have been shown to confer health benefits to various animals including juvenile fish, may have potential for supplementation to live foods.

Prebiotics and probiotics are diet additives which have been used for numerous years in human and terrestrial animal nutrition, and more recently are being utilized in juvenile fish to help with immunomodulation by stimulating the innate, cellular, and humoral immune responses (Akhter et al., 2015). Prebiotics are defined as "…non-digestible food ingredients that selectively stimulate growth and/or the metabolism of health-promoting bacteria in the intestinal tract, thus improving the organism's intestinal balance," (Gibson and Roberfriod, 1995). Probiotics, on the other hand, are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host," (Sanders, 2008). These definitions help to better understand the differences between these two different additives, with probiotics being the live beneficial bacteria, while prebiotics are ingredients which selectively target the growth of beneficial bacteria in the gastrointestinal tract (GIT).

These types of feed additives have potential to improve growth, act as antimicrobial compounds, as well as enhance the immune system of fish consuming them (Gatlin and Peredo, 2012). Probiotics and prebiotics in some instances have been shown to increase feed utilization and digestion of nutrients by cultured fish (Kesarcodi-Watson et al., 2008; Buentello et al.,

2010). In several previous studies, probiotics and prebiotics added to the diet of juvenile fish could alter the microbiota present in the GIT, thus improving the survival and growth of various fish species including red drum (Buentello at al., 2010). These additives have many potential beneficial roles in the body ranging from assisting with digestion to preventing establishment of disease-causing organisms.

To combat bacterial diseases in a hatchery setting, probiotics and prebiotics are being explored as possible solutions for improving production and disease resistance of larval organisms (Gomez-Gil et al., 2000). Traditionally, antibiotics were the main way to help control bacterial diseases in aquaculture**;** however, the overuse of these antimicrobials has led to antibiotic-resistant strains of bacteria such as *Vibrio harveyi* (Defoirdt et al., 2011). These bacterial pathogens may have an even greater effect on larval fish whose immune systems are not fully developed. Therefore, one component of this project was to evaluate the application of commercially available prebiotics and probiotics on rotifers to be fed to larval red drum. The second part of this research involved adding Bactocell™, one of the probiotics selected for trial on rotifers, to the diet of juvenile red drum in a feeding trial to determine if it has an effect on survival and growth parameters of these fish.

# CHAPTER II
# ROTIFER PRODUCTION WITH PREBIOTIC AND PROBIOTIC SUPPLEMENTATION

## 2.1 INTRODUCTION

Rotifers are one of the main food sources for larval fish and show potential to be manipulated by beneficial nutrient supplementation (Oie et. al, 1997; Matsunari et al., 2013). Because larval fish production in aquaculture is fundamental to sustained industry growth, it is critical to improve growth and survival during this production stage. No studies have been conducted to evaluate the effect of prebiotics and probiotics on the production and composition of rotifers to improve larval fish production. The effects of these dietary additives have not been extensively tested in larval fish; however, some probiotics have been shown to have beneficial effects when supplemented in culture water and the diet of larval shrimp (Castex et al., 2008). Such findings have prompted interest in evaluating the effects of prebiotic and probiotic supplements to live foods such as rotifers used in the culture of larval fish. Because prebiotic and probiotic supplementation is on the verge of becoming more readily integrated into aquaculture production, it is important to determine their effects on rotifers before feeding them to larval fish.

## 2.2 METHODS

### *2.2.1 Prebiotic and probiotic supplements*

During four separate trials, selected commercially available prebiotics and probiotics were directly added to rotifer cultures to determine whether they affected growth and population densities of rotifers. The different probiotic and prebiotic treatments that were evaluated

included Bactocell™ (Lallemand Animal Nutrition, Montreal, Canada), Grobiotic®-A (International Ingredient Corporation, St. Louis, MO), Aquablend™ (BIO-CAT Microbials, Troy, VA) and SILOhealth 108P (BASF, Ludwigshafen, Germany). Bactocell™ is a feed additive composed of viable *Pediococcus acidilactici* and is an approved additive for use in aquaculture (EFSA, 2012). This probiotic has been used mainly in larval shrimp culture, where it has been shown to improve survival of shrimp larvae when exposed to the pathogen *Vibrio nigripulchritudo* (Castex et al., 2008). Bactocell™ also has demonstrated an antioxidant effect by lowering the oxidative stress levels of shrimp consuming the probiotic prior to exposure to *Vibrio nigripulchritudo* (Castex et al., 2009).

Aquablend™ is a commercial probiotic which contains non-pathogenic bacteria of the genus *Bacillus* (Gonzalez-Felix et al., 2018). This probiotic is considered optimal as a feed supplement due to the spore forming characteristics of *Bacillus*, making bacteria of this genus resistant to adverse environmental conditions during feed manufacture and storage. This dietary supplement has shown no positive or health effects or altered gut microbiota of fish evaluated thus far; however, little evaluation has been conducted using this particular supplement in aquaculture (Ju, 2019). In animals such as chickens, *Bacillus* spp. have been shown to produce beneficial immunological responses, enhancing antibody response after vaccine administration (Talazadeh et al., 2016). Because of this finding as well as being marketed as an aquaculture supplement, Aquablend™ was evaluated to determine if there was any alterations in production from adding *Bacillus* spores to rotifer cultures.

Grobiotic®-A is a prebiotic mixture of partially autolyzed brewer's yeast, dairy ingredient components and dried fermentation products (Li and Gatlin, 2005). In previous research, juvenile red drum fed diets supplemented with Grobiotic®-A demonstrated increased weight

gain, feed efficiency and non-specific immunity, as well as enhanced survival following *Amyloodinium* challenge (Buentello et al., 2010). These immensely positive results with juvenile red drum, as well as other fish species (Peredo and Gatlin, 2012), prompted the evaluation of GroBiotic®-A during live foods production to determine if it was possible to alter rotifer microbiota before feeding them to larval fish.

The feed additive SILOhealth 108P is a blend of short- and medium-chain fatty acids with a prebiotic-like effect intended to enhance growth of fish as well as prevent harmful bacteria such as *Vibrio parahaemolyticus* and *Aeromonas hydrophila* from establishing within the fish and affecting their health. SILOhealth 108P has been reported to show great promise in the aquaculture industry; however, this product requires more comprehensive examination to determine its effectiveness in larval fish.

*2.2.2 Rotifer cultures*

A series of rotifer culture experiments were conducted in which the prebiotics and probiotics mentioned above were individually added to rotifer cultures in isolated culture systems. Rotifers were cultured using 15-L bucket systems from Reed Mariculture (Campbell, CA). This culture system utilizes the circulation of air bubbles to create a bell-shaped current within the bucket, ensuring that the organisms obtain adequate aeration and access to feed while removing waste from the system. Prior to initiation of the rotifer culture experiments, ten million rotifers (*Branchionus plicatilis)* were purchased from Reed Mariculture and cultured for 1 week to ensure there were adequate numbers for the various trials.

Rotifers in each bucket were counted and adjusted to 1 million organisms at the initiation of each trial. Rotifers were enumerated by taking a 100-mL sample after the culture was stirred with a probe to ensure it was homogenous throughout. Then a 1-mL aliquot of the culture was

removed and a 10x dilution was made for counting the rotifers in each sample. Lugol's solution (Sigma-Aldrich, St. Louis, MO) was then added at a rate of 5 drops per 10-mL to euthanize and stain the rotifers in the sample. A Sedgewick Rafter counting cell (M415, Pentair, Minneapolis, MN) was used to plate 1 mL of the diluted and stained rotifer sample to be viewed under 40x magnification. Female rotifers with eggs were counted separately from rotifers which did not have eggs. Counting females with eggs helps to determine the health of the population as higher numbers of female rotifers with eggs lead to a higher overall population of rotifers the next day. The total number of rotifers counted on the slide with eggs and without eggs were multiplied by 10 to compensate for the dilution. Once the numbers of rotifers for each bucket were quantified, they were added to each of four buckets to initiate each experiment. The buckets were filled with approximately 15 L of artificial seawater (20 g salt per L) at 20 ppt, and an aliquot of water calculated to contain 1 million rotifers was added to each bucket. Rotifer cultures were maintained at a constant 28 C throughout the duration of their growth.

Each of the experiments began on a Monday morning and ended on a Friday morning of the same week to replicate a standard 4-day production cycle of rotifers. Two buckets of control rotifers without the addition of any prebiotic or probiotic and two buckets in which the experimental rotifers were treated individually with each of the selected prebiotics or probiotics in the water were grown during each 4-day trial. This process was replicated four times until all four experimental treatments (GroBiotic®-A, Aquablend™, Bactocell™ and SILOhealth 108P) had been evaluated.

To feed the rotifers in each experiment, RotiGrow Complete One Step™, (Reed Mariculture, Campbell, CA, US), was used throughout the experiment to feed rotifer cultures. During each experiment, 11 mL of RotiGrow Complete One Step ™ was added daily to each

bucket per million rotifers, with population density of rotifers in each bucket determined daily as described above. The feed was divided into two portions, and the rotifers were fed once in the morning at approximately 10 AM and once in the evening at approximately 5 PM. After the morning feeding, all rotifer buckets received 0.5 g of Cloram-X™ (Reed Mariculture, Campbell, CA, US) to help ensure good water quality.

The individual prebiotic and probiotic supplements were added to the experimental buckets at a rate of 0.07 g per bucket (4.5 mg/L) each day following the manufacturer's recommendation for Bactocell™. The other three treatments did not have a manufacturer recommendation for dosage rates, so to keep the experimental comparisons uniform, the same amount of each probiotic and prebiotic were used in equal amounts as recommended for Bactocell™.

After the 4-day production cycle with each experimental treatment and control, the rotifers from each bucket were harvested, enumerated and placed in a freezer for preservation to determine the quantity of rotifers produced during the trial and for denaturing gradient gel electrophoresis (DGGE) analysis to characterize their microbial composition. To determine the total production of rotifers in each treatment, a 100-mL sample was removed from each culture and enumerated to quantify how many rotifers were produced during the 4-day cycle. Harvesting was done by sieving the contents of each bucket with gradually decreasing sieve size to remove all non-rotifer contents from the bucket water. The first sieve had a mesh size of 200 μm and the final sieve used to ensure containment of the rotifers had a mesh size of 55 μm. Once the solution with rotifers had been filtered through the 55-μm sieve, rotifers contained with the sieve were vigorously rinsed with water to ensure no leftover feed or other organisms remained within the sieve besides rotifers. Rotifers were then placed in 2-mL Eppendorf tubes

and stored at -20°C until denaturing gradient gel electrophoresis (DGGE) analysis to determine sample microbial diversity.

After the rotifer samples were collected from the experimental trials, DGGE analysis was performed according to the protocol of Dr. Michael Hume, USDA Southern Plains Agricultural Research Center, as summarized by Buentello et al. (2010). The DGGE is a polymerase chain reaction process in which bacterial 16S rDNA contained in the rotifers was extracted and compared to determine microbial diversity within the rotifers. This analysis makes it possible to determine whether the prebiotics and probiotics had any effect on production and altering the microbiota of the rotifers.

*2.2.3 Statistical analysis*

To interpret the results of the dendrograms from DGGE analysis, Dice Percentage Similarity Coefficient (DPSC) values were computed as a method for characterizing the similarity of the different samples being analyzed with DGGE (Hume et al., 2003). Samples which were considered not similar to each other were based on DPSC values below 79%, samples with DPSC values between 80 and 84% were considered somewhat similar, samples with DPSC values between 85 and 89% were considered similar, samples with DPSC values between 90 and 94% were considered very similar to each other and samples that were the same or identical were based on DPSC values of 95% or higher.

## 2.3 RESULTS

During the 4-day trial in which rotifers were cultured while being exposed to the probiotic Aquablend™, the experimental treatment produced an average of 6.45 million rotifers while the control produced an average of 7.1 million rotifers (Table 1).

**TABLE 1** Production data from rotifers exposed to control and Aquablend™ treatments during a 4-day trial.

| Aquablend Trial | | | | | | |
|---|---|---|---|---|---|---|
| Day | 1 | 2 | 3 | 4 | 5 | |
| Treatment | Rotifer Count | | | | | Final Avg. |
| Control 1 | 1,000,000 | 1,300,000 | 2,500,000 | 3,600,000 | 6,600,000 | |
| Control 2 | 1,000,000 | 1,500,000 | 2,800,000 | 3,600,000 | 7,600,000 | 7,100,000 |
| Aquablend 1 | 1,000,000 | 1,300,000 | 2,800,000 | 3,500,000 | 4,900,000 | |
| Aquablend 2 | 1,000,000 | 1,700,000 | 2,700,000 | 4,900,000 | 8,000,000 | 6,450,000 |

In the second trial in which rotifers were cultured while being exposed to the probiotic Bactocell™, the experimental cultures with the probiotic produced an average of 6.9 million rotifers while the control produced 10.3 million rotifers (Table 2).

**TABLE 2** Production data from rotifers exposed to control and Bactocell™ treatments during a 4-day trial.

| Bactocell Trial | | | | | | |
|---|---|---|---|---|---|---|
| Day | 1 | 2 | 3 | 4 | 5 | |
| Treatment | Rotifer Count | | | | | Final Avg. |
| Control 1 | 1,000,000 | 1,400,000 | 2,900,000 | 4,700,000 | 11,100,000 | |
| Control 2 | 1,000,000 | 1,400,000 | 2,700,000 | 6,100,000 | 9,500,000 | 10,300,000 |
| Bactocell 1 | 1,000,000 | 1,500,000 | 1,700,000 | 5,400,000 | 6,300,000 | |
| Bactocell 2 | 1,000,000 | 1,400,000 | 1,900,000 | 5,300,000 | 7,500,000 | 6,900,000 |

During the third trial in which rotifers were cultured while being exposed to the prebiotic GroBiotic®-A, treatment groups produced an average of 13.05 million rotifers while the control produced an average of 8.65 million rotifers (Table 3).

**TABLE 3** Production data from rotifers exposed to control and GroBiotic®-A treatments during a 4-day trial.

| GroBiotic®-A Trial | | | | | | |
|---|---|---|---|---|---|---|
| Day | 1 | 2 | 3 | 4 | 5 | |
| Treatment | Rotifer Count | | | | | Final Avg. |
| Control 1 | 1,000,000 | 1,900,000 | 2,600,000 | 4,100,000 | 8,900,000 | |
| Control 2 | 1,000,000 | 1,600,000 | 5,000,000 | 6,700,000 | 8,400,000 | 8,650,000 |
| GroBiotic®-A | 1,000,000 | 1,600,000 | 2,900,000 | 4,700,000 | 11,700,000 | |
| GroBiotic®-A | 1,000,000 | 2,100,000 | 3,900,000 | 4,700,000 | 14,400,000 | 13,050,000 |

In the last 4-day trial in which rotifers were cultured while being exposed to the prebiotic-like additive SILOhealth 108P, the experimental treatment produced 11.15 million rotifers while the control produced 13.15 million rotifers (Table 4).

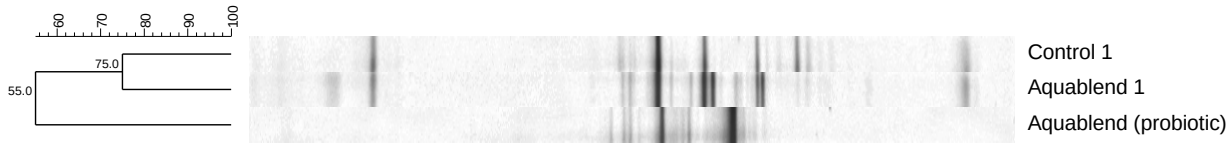
**TABLE 4** Production data from rotifers exposed to control and SILOhealth 108P treatments during a 4-day trial.

| SILOhealth 108P Trial | | | | | | |
|---|---|---|---|---|---|---|
| Day | 1 | 2 | 3 | 4 | 5 | |
| Treatment | Rotifer Count | | | | | Final Avg. |
| Control 1 | 1,000,000 | 1,500,000 | 3,800,000 | 6,000,000 | 15,700,000 | |
| Control 2 | 1,000,000 | 1,500,000 | 2,900,000 | 5,300,000 | 10,600,000 | 13,150,000 |
| SiloHealth 1 | 1,000,000 | 2,400,000 | 3,900,000 | 9,900,000 | 10,200,000 | |
| SiloHealth 2 | 1,000,000 | 1,800,000 | 4,200,000 | 7,700,000 | 12,100,000 | 11,150,000 |

Samples of rotifers from the various production trials were then subjected to DDGE analysis to characterize potential changes in microbial composition. The DGGE results from the Aquablend™ trial indicated the treatment rotifers were different compared to the control with a similarity coefficient below 79% (Figure 1). Only one of the images for the replicate buckets of

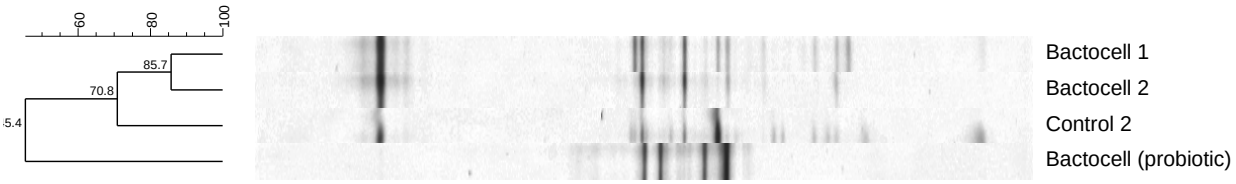
rotifers could be used as the image of rotifers from the second Aquablend™ treatment bucket had bands that were too faint for analysis.

**FIGURE 1** Dendrogram analysis following bacterial 16S rDNA denaturing gradient gel electrophoresis of rotifer samples after 4-days of Aquablend supplementation in their culture water. The treatment labels indicate the comparison of gut microbiota of rotifers treated with Aquablend™, control rotifers and the probiotic Aquablend by itself. Numbers to the left of the dendrogram are percentage similarity coefficients.




The results from the 4-day study comparing rotifers exposed to the probiotic Bactocell™ were interpreted as having no similarity to control rotifers grown at the same time as the similarity coefficient was 70.8% (Figure 2). When comparing the replicates of rotifers which were exposed to Bactocell™, the similarity coefficient was 85.7% meaning that the first Bactocell™ sample and second Bactocell™ sample were similar to each other (Figure 2). Compared to treated rotifers from the experiment, the Bactocell™ additive showed no similarity with a similarity coefficient of 5.4% (Figure 2).

**FIGURE 2** Dendrogram analysis following bacterial 16S rDNA denaturing gradient gel electrophoresis of rotifer samples after 4-days of Bactocell™ supplementation in their culture water. The treatment labels indicate the comparison of gut microbiota of rotifers treated with Bactocell™, control rotifers and the probiotic Bactocell™ by itself. Numbers to the left of the dendrogram are percentage similarity coefficients.

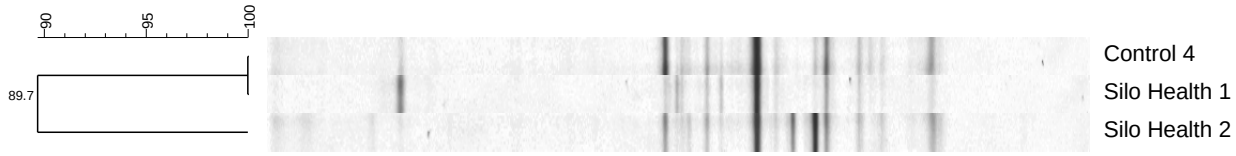


The results from the 4-day study comparing rotifers exposed to Grobiotic®-A and control rotifers showed there was no similarity between the two treatment groups with a similarity coefficient of 40% (Figure 3). Only one of the images for replicates of the experimental rotifers could be used as the image of one of the samples of Grobiotic®-A had bands that were too faint for analysis.

**FIGURE 3** Dendrogram analysis following bacterial 16S rDNA denaturing gradient gel electrophoresis of rotifer samples after 4-days of including Grobiotic®-A in culture water. The treatment labels indicate the comparison of gut microbiota of rotifers treated with Grobiotic®-A and the control rotifers. Numbers to the left of the dendrogram are percentage similarity coefficients.

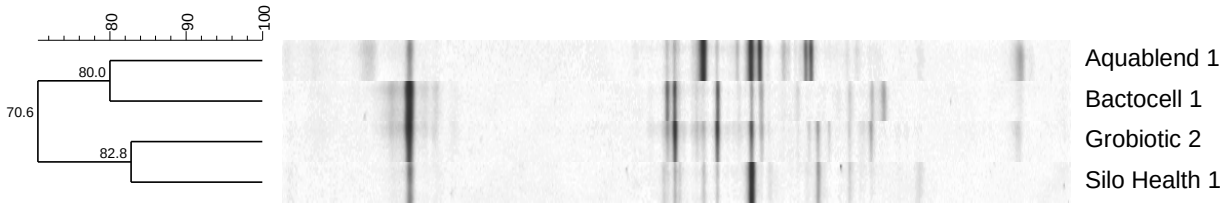

Results from the 4-day study comparing rotifers exposed to the prebiotic-like additive SILOhealth 108P and control rotifers showed that all treatments were very similar to each other with a similarity coefficient of 89.7% between all treatments (Figure 4).

**FIGURE 4** Dendrogram analysis following bacterial 16S rDNA denaturing gradient gel electrophoresis of rotifer samples after 4-days of treatment with SILOhealth 108P in culture water. The treatment labels indicate the comparison of gut microbiota of rotifers immersed in SiloHealth 108P and the control rotifers. Numbers to the left of the dendrogram are percentage similarity coefficients.



Rotifers exposed to each prebiotic and probiotic additive also had their DGGE patterns compared to each other. It was shown that the rotifers exposed to the probiotic additives (Aquablend™ and Bactocell™) were somewhat similar to each other with a similarity coefficient of 80% (Figure 5). Those exposed to the prebiotic additives (Grobiotic®-A and SiloHealth 108P) were also somewhat similar to each other with a similarity coefficient of 82.8% (Figure 5). When comparing the rotifers exposed to prebiotics and probiotics to each other, there was no similarity as the similarity coefficient reported was 70.6% (Figure 5).

**FIGURE 5** Dendrogram analysis following bacterial 16S rDNA 3denaturing gradient gel electrophoresis comparing rotifer samples from each treatment after 4-day of treatment with prebiotic or probiotics in culture water. Numbers to the left of the dendrogram are percentage similarity coefficients.




## 2.4 DISCUSSION

During the 4-day production trials involving the prebiotics GroBiotic®-A and SILOhealth 108P as well as the probiotic Aquablend™, the rotifer cultures showed similar production results when compared to the control. Rotifers cultured with Aquablend and SILOhealth 108P resulted in similar production numbers when compared to rotifers which had been cultured without the addition of prebiotic and probiotic supplements. When cultured with GroBiotic®-A, rotifers showed a higher rate of growth when compared to the control, producing about 66% more organisms than those which had no added supplements. On the other hand, rotifers cultured with

the addition of Bactocell™ showed about a 67% decrease in production when compared to those without the probiotic in the culture system.

After reviewing the results from the trial where rotifers were grown while exposed to prebiotics and probiotics, there were many notable results which were gained after DGGE analysis was performed. The first 4-day trial involving the addition of Aquablend™ demonstrated that there was an ability to alter the microbiota of rotifers. Treatment rotifers were shown to have a different microbial composition than the control rotifers, and they also were determined to have a different microbial composition than the probiotic when it was isolated by itself. This result shows that although the microbiota of the rotifers had been altered by Aquablend™, the composition of the microbiota was not able to be completely altered to the same composition of Aquablend™.

It was determined that the two replicates of rotifers grown with the addition of Bactocell™ had similar microbial composition while showing a difference in microbiota when compared to the control rotifers. The interesting part of this result is that the probiotic Bactocell™ (*Pediococcus acidilactici*) was also analyzed to determine how the gut microbiota of treatment rotifers compared to the probiotic itself. It was seen that the treatment rotifers, though different from the control, were not similar to the probiotic itself. This means that though Bactocell™ did alter the microbiota of the treated rotifers, the composition of microorganisms in the rotifer was still much different from the Bactocell™ probiotic.

GroBiotic®-A was the next additive to be included in rotifer cultures to determine if it could alter the bacterial composition of the rotifer upon addition to the system. This additive contributed to a general increase in rotifer production compared to the control. In a previous experiment by James et al. (1987) where rotifers were grown by feeding various species of yeast,

which is a component of GroBiotic®-A, it was shown that rotifers could be cultured using yeast as the main food source. The yeast in GroBiotic®-A could potentially have led to the increased production numbers from rotifers in this treatment.

DGGE analysis of prebiotic-like additive SILOhealth 108P showed that there was a very strong similarity between the bacterial composition in the control rotifers and those which had been exposed to SILOhealth 108P. This strong similarity was interpreted as SILOhealth 108P having almost no effect on the microbiome of rotifers. Because SILOhealth 108P is not a true prebiotic and is a prebiotic-like additive, this makes sense that there would be no alteration of rotifer microbiota.

Once the different prebiotics and probiotics had been compared to their respective controls separately, they were then compared to each other with no control present in the analysis to see if there was any correlation between treatments. Interestingly, rotifers exposed to prebiotics were determined to be similar to each other while those exposed to the probiotics were also shown to be similar to each other overall. Though there was similarity within prebiotic and probiotic treatments, when comparing the different types of additives to each other, they were shown to have different microbial compositions. This means that although most of the treatments had an effect on altering the bacterial composition of rotifers, prebiotics and probiotics appeared to produce different results in terms of the bacterial composition of the rotifer samples. DGGE analysis demonstrated the ability of the different prebiotic and probiotic supplements to selectively culture different species of microbial organisms within the rotifers leading to varying microbial profiles.

Though the DGGE results from samples of rotifers enriched with probiotics did not show that the microbiota of rotifers was the same as the microbial composition of the probiotics

themselves after 4 days of exposure, this exposure time was not sufficient to completely alter the microbiome of rotifers to reflect the composition of the probiotics. It is possible that given a longer time for growth of rotifers enriched with the probiotic supplements that their microbial composition could more closely reflect the composition of the probiotics themselves, but this would require further study.

# CHAPTER III
# JUVENILE RED DRUM BACTOCELL™ FEEDING TRIAL

## 3.1 INTRODUCTION

There has not been ample success in growing juvenile fish of various species for food and recreational purposes; however, further improvements may be achieved with the dietary supplementation of nutritional additives to potentially enhance the immune system and increase survival of fish grown in a hatchery setting. Improvements of larval nutrition food additives have the potential to lead to increased food fish production in commercial aquaculture or more fish being restored to their native habitats through stock enhancement. Aquaculture of red drum is practiced in Texas for both food fish production and stock enhancement.

Prebiotics are a group of diet additives which have received considerable attention in recent years. Some prebiotics which have previously been studied with juvenile red drum include fructooligosaccharides, galactooligosaccharides, Bio-MOS® (containing yeast-derived mannan oligosaccharides), and Previda™ (containing oligosaccharides with over 50% mannan oligosaccharides) among several other products (Zhou et al., 2010). It has been shown that prebiotics such as Previda™ had beneficial health impacts in red drum such as improved growth performance and immune responses (Zhou et al., 2010). Other prebiotics such as mannan oligosaccharide, transgalactooligosaccharide, and GroBiotic®-A (partially autolyzed brewer's yeast, dairy ingredient components and dried fermentation products) also have been shown to increase survival and feed efficiency of juvenile red drum (Buentello et al., 2010). Supplementation of GroBiotic®-A also increased survival of juvenile red drum when exposed to the disease-causing parasitic dinoflagellate *Amyloodinium ocellatum* (Buentello et al., 2010).

However, no studies have been conducted to evaluate probiotics in the diet of juvenile red drum. Therefore, a feeding trial was conducted to evaluate the effects of Bactocell™, a probiotic consisting of *Pediococcus acidilactici,* on juvenile red drum.

## 3.2 METHODS

### *3.2.1 Experimental diets*

Bactocell™ was added to a formulated diet at different inclusion concentrations of 0 (basal), 0.05, 0.1, 0.2, 0.4, 0.8% of dry weight. The basal diet was formulated from soybean meal, soy protein concentrate and a limited amount of menhaden fishmeal to contain 40% crude protein (Table 5). Only 17% of the protein in the diet was contributed by fishmeal while the other 83% came from soybean products. Menhaden oil was supplemented to all diets to achieve a total of 10% lipid, and along with 10% dextrinized starch achieved an estimated digestible energy level of 3.4 kcal/g. Vitamin and mineral premixes were included in the diets to ensure they were nutritionally complete for juvenile red drum.

Diets for this trial were formulated and ingredients were mixed at the Texas A&M Aquacultural Research and Teaching Facility using a V- mixer (Blendmaster Lab Blender; Patterson-Kelly, Stroudsburg, PA) to mix the dry ingredients including varying concentrations of Bactocell™, which was utilized in a powdered form. Once the dry ingredients were adequately mixed, they were combined with oil and water using an industrial mixer (Model A-200; Hobart, Troy, OH). Once the ingredients were homogenous throughout, they were pressure-pelleted using a commercial meat grinder attachment with a 3-mm die plate on the same industrial mixer. After the diets were pelleted, they were air-dried overnight and broken down into adequately sized pellets for the experimental fish to consume.

**TABLE 5** Formulations and proximate composition (g/100 g dry weight) of diets containing the Bactocell™ additive.

| **Dietary Treatments** | | | | | | |
|---|---|---|---|---|---|---|
| **Ingredients** | **Basal** | **0.05% BC** | **0.1% BC** | **0.2% BC** | **0.4% BC** | **0.8% BC** |
| Menhaden Fishmeal[1] | 10 | 10 | 10 | 10 | 10 | 10 |
| Soybean Meal[2] | 45 | 45 | 45 | 45 | 45 | 45 |
| Soy Protein Conc.[3] | 13.8 | 13.8 | 13.8 | 13.8 | 13.8 | 13.8 |
| Menhaden Oil[4] | 7.7 | 7.7 | 7.7 | 7.7 | 7.7 | 7.7 |
| Vitamin Premix[5] | 3 | 3 | 3 | 3 | 3 | 3 |
| Mineral Premix[5] | 4 | 4 | 4 | 4 | 4 | 4 |
| Dex. Starch[5] | 10 | 10 | 10 | 10 | 10 | 10 |
| Carboxymethyl Cellulose[6] | 2 | 2 | 2 | 2 | 2 | 2 |
| Glycine[6] | 1 | 1 | 1 | 1 | 1 | 1 |
| L-Lysine[6] | 1 | 1 | 1 | 1 | 1 | 1 |
| Taurine[6] | 1 | 1 | 1 | 1 | 1 | 1 |
| DL-Methionine[6] | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| Celufil[6] | 1.1 | 1.05 | 1 | 0.9 | 0.7 | 0.3 |
| Bactocell™[7] | 0 | 0.05 | 0.1 | 0.2 | 0.4 | 0.8 |
| **Analyzed proximate composition,[8] g/100g dry wt** | | | | | | |
| Crude Protein | 44.4 | 44.0 | 44.0 | 43.8 | 43.9 | 44.0 |
| Crude Lipid | 8.6 | 8.8 | 9.1 | 9.3 | 9.6 | 9.1 |
| Ash | 8.1 | 7.8 | 8.0 | 8.0 | 8.3 | 8.4 |

[1]Special Select™ Omega Protein Inc., Abbeville, LA, USA
[2]Rangen Inc., Angleton, TX, USA
[3]The Solae Company, St. Louis, MO, USA
[4]Omega Protein, Reedville, VA, USA
[5]MP Biomedicals, Solon, OH, USA
[6]USB, Cleveland, OH, USA
[7]Lallemand Animal Nutrition
[8]Represented as a mean from samples analyzed in duplicate

*3.2.2 Feeding trial design and culture system*

Juvenile red drum for the comparative feeding trial were obtained from the Texas Parks and Wildlife Department Perry R. Bass Hatchery and conditioned for 1 week using the basal diet to prepare them for the experiment. The juvenile red drum were cultured in an indoor

recirculating aquaculture system (RAS) consisting of 38-L aquaria equipped with a settling chamber, biofilter, sand filter, and UV light to maintain water under optimal conditions. The system was maintained with brackish (7 ppt) water prepared from well water and synthetic sea salts (Red Sea U.S.A., Houston, TX). Water temperature was maintained at 28ºC by conditioning ambient air. To ensure optimal conditions for the fish, temperature, water quality, salinity, and pH were measured throughout the experiment and corrections were made when necessary. Specific water quality parameters which were measured throughout the trial were concentrations of ammonia, nitrate, nitrite, pH, dissolved oxygen, as well as temperature.

After conditioning, 12 juvenile red drum were stocked into each of 24, 38-L aquaria. These fish were selected and weighed with each aquarium in the experiment averaging 2 grams per fish to ensure there was no difference in total biomass of fish in each aquarium at the beginning the trial. Each diet was fed to fish in three randomly selected aquaria as treatment replicates. All fish in each aquarium were group-weighed once a week to determine total biomass and to adjust feed quantities based on a fixed percentage of body weight beginning at 6% and reducing to 4% by the end of the trial. This adjustment ensured the fish were fed at a rate approaching apparent satiation without overfeeding. The feeding trial was continued for a total of 8 weeks after which weight gain (percentage of initial weight), and feed efficiency (FE, g fish weight gain/g dry feed offered) were computed for each aquarium.

*3.2.3 Sample collection and analysis*

At the end of week 4 of the feeding trial, two fish per aquarium were euthanized using tricane methanesulfonate (MS-222, Sigma-Aldrich) at a concentration of 150 mg/L. Fish digesta was aseptically removed under a sterile hood from their gastrointestinal tract (GIT), placed into 2-mL Eppendorf tubes, and frozen in liquid nitrogen (-200 C). The samples were stored at -80ºC

prior to DGGE analysis. After 8 weeks of feeding the experimental diets, three more fish from each aquaria were euthanized using MS-222, after which they were weighed, total length was determined, bled from the caudal vasculature using heparinized needles, and then their digesta was aseptically removed, frozen in liquid nitrogen, and stored for later DGGE analysis. Three additional fish per aquarium were euthanized and dissected to remove the liver, intraperitoneal fat (IPF), and muscle to compute body condition indices including hepatosomatic index (HSI) ratio, IPF ratio, and muscle ratio according to the formulas: liver weight x 100/fish body weight, IPF weight x 100/fish body weight, and muscle fillet weight x 100/fish body weight, respectively.

Denaturing gradient gel electrophoresis was used to characterize microorganisms in the digesta of the red drum (Muyzer et al., 1993). The methods for preparation of red drum digesta samples and DDGE analysis were as previously described by Hume et al. (2003).

Neutrophil oxidative radical production of the whole blood of three red drum per aquarium was determined by the Nitroblue-Tetrazolium (NBT) assay (Sigma Aldrich, St. Louis, MO, US). This provided one measure of the non-specific immune response of fish fed the different concentrations of Bactocell™ by assessing oxidative radical production by blood leukocytes. This assay was performed as described by Siwicki et al. (1994).

Proximate composition of composite whole-body samples of three red drum per aquarium also was determined at the end of the 8-week feeding period. The moisture content of the whole-body samples was determined by homogenizing the samples in a blender (Grindomix GM 200, Retsch, Haan, Germany), then placing aliquots of the sample into pre-weighed aluminum sample dishes before placing them into an oven to dry at 125ºC for 3 hours. The changes in weights of the dried samples were recorded to calculate the amount of moisture

within the samples before drying (AOAC, 1990). Next, ash was determined by placing a crucible with a measured amount of sample into a furnace at 650ºC for 3 hours and dividing the mass of remaining ash by the initial sample weight (AOAC, 1990). To determine crude protein content, a LECO protein analyzer (FP-528; LECO, St. Joseph, MI) was used to measure total nitrogen which was multiplied by 6.25 for estimating crude protein (AOAC, 2005). Lipid content within the whole-body samples was determined using the Folch extraction method (Folch et al., 1957).

*3.2.4 Statistical analysis*

Data collected from the feeding trial were subjected to analysis of variance (ANOVA) and orthogonal polynomial contrasts using JMP® Pro version 14.1.0 (SAS Institute Inc., Cary, NC). Significance was set at $P < 0.05$. To interpret the results of the DGGE analysis, the resulting dendrograms were analyzed by computing Dice Percentage Similarity Coefficients (DPSCs). Samples which were considered not similar to each other were based on DPSC values below 79%, samples with DPSC values between 80 and 84% are considered somewhat similar, samples with DPSC values between 85 and 89% are considered similar, samples with DPSC values between 90 and 94% are considered very similar to each other and samples that were the same or identical were based on DPSC values of 95% or higher.

## 3.3 RESULTS

Overall, the growth performance of juvenile red drum fed the various Bactocell™ concentrations was quite high with fish in each dietary treatment obtaining over 1,300% increase in initial body weight, but values did not vary significantly with different concentrations of Bactocell™ (Table 6). In the analysis of the growth parameters of red drum fed diets with

different concentrations of Bactocell™, no significant differences were found among treatments for weight gain, FE, protein efficiency or survival (Table 6).

**TABLE 6** Growth performance, feed utilization, and survival of red drum fed differing concentrations of the Bactocell™ probiotic diets for 8 weeks[1].

| Bactocell™ (%) | Weight Gain | Feed Efficiency | Protein Efficiency | Survival |
|---|---|---|---|---|
| | % | | | % |
| Basal | 1366.4 | 0.660 | 1.49 | 91.7 |
| 0.05 | 1398.1 | 0.682 | 1.55 | 91.7 |
| 0.10 | 1399.0 | 0.665 | 1.51 | 89.7 |
| 0.20 | 1328.5 | 0.689 | 1.57 | 95.9 |
| 0.40 | 1455.4 | 0.697 | 1.59 | 89.6 |
| 0.80 | 1365.9 | 0.652 | 1.48 | 83.3 |
| PSE[2] | 83.132 | 0.020 | 0.046 | 4.665 |
| ANOVA (Pr>F) | 0.9252 | 0.5875 | 0.4780 | 0.5800 |
| Linear Trend (Pr>F) | 0.3901 | 0.6484 | 0.7430 | 0.0926 |
| Quadratic Trend (Pr>F) | 0.6189 | 0.1990 | 0.1407 | 0.1934 |

[1]Values of means of 4 replicate groups (n=4)
[2]PSE = Pooled Standard Error

Red drum fed the diets with various Bactocell™ concentrations did not demonstrate any significant ($P > 0.05$) differences in body condition indices (Table 7). Also, no statistical differences due to diet were observed in the NBT values (Table 7).

**TABLE 7** Condition indices of red drum fed differing concentrations of the Bactocell™ probiotic diets for 8 weeks[1].

| Bactocell™ (% dietary inclusion) | Hepatosomatic index[2] % | Intraperitoneal Fat Ratio[3] % | Muscle Yield[4] % | Nitroblue Tetrazolium Test[5] mg/mL |
|---|---|---|---|---|
| Basal | 2.3 | 0.514 | 31.4 | 5.7 |
| 0.05 | 2.2 | 0.674 | 31.6 | 5.1 |
| 0.10 | 1.9 | 0.620 | 32.8 | 5.4 |
| 0.20 | 2.0 | 0.603 | 32.1 | 5.5 |
| 0.40 | 2.3 | 0.694 | 33.1 | 5.9 |
| 0.80 | 2.4 | 0.758 | 32.2 | 6.1 |
| PSE[6] | 0.129 | 0.108 | 0.639 | 0.371 |
| ANOVA (Pr>F) | 0.1382 | 0.6933 | 0.6661 | 0.4743 |
| Linear Trend (Pr>F) | 0.1702 | 0.1538 | 0.7810 | 0.0941 |
| Quadratic Trend (Pr>F) | 0.1825 | 0.3565 | 0.6226 | 0.2479 |

[1]Values of means of 4 replicate groups (n=4).
[2]Calculated as follows: liver weight x 100/fish body weight.
[3]Calculated as follows: intraperitoneal fat weight x 100/fish body weight.
[4]Calculated as follows: skeletal muscle weight x 100/fish body weight.
[5]Calculated as follows: 80 x (absorbance – 0.0245)/5.8564
[6]PSE = Pooled Standard Error

When analyzing whole-body proximate composition, differences ($P < 0.05$) were observed for lipid retention and lipid content among fish fed the various diets while differences in protein retention, crude protein, moisture, and ash content were not significantly different (Table 8). Lipid retention and whole-body lipid content did not show linear or quadratic trends with increasing concentrations of Bactocell™; however, ash content showed a negative ($P < 0.05$) linear trend.

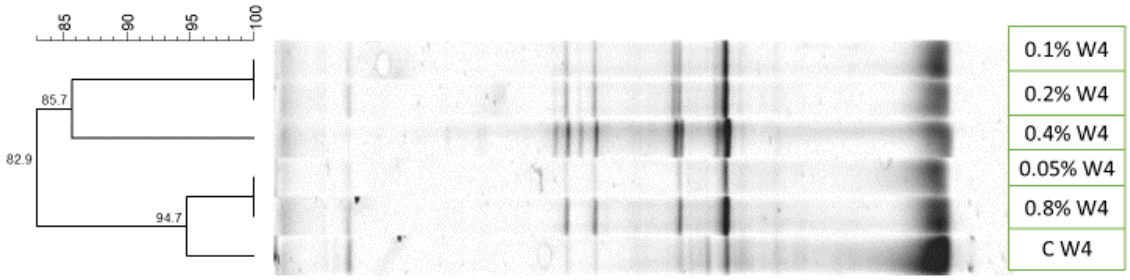
**TABLE 8** Whole-body proximate composition of red drum fed differing concentrations of the Bactocell™ probiotic diets for 8 weeks[1].

| Bactocell™ (% dietary inclusion) | Lipid Retention[2] % | Protein Retention[3] % | Moisture % | Crude Protein % | Lipid % | Ash % |
|---|---|---|---|---|---|---|
| Basal | 26.4 | 23.3 | 76.9 | 15.9 | 3.5 | 15.0 |
| 0.05 | 26.1 | 24.9 | 76.4 | 15.9 | 3.7 | 14.1 |
| 0.10 | 24.8 | 24.7 | 76.5 | 16.1 | 3.4 | 14.1 |
| 0.20 | 32.8 | 25.3 | 76.2 | 19.9 | 4.3 | 14.4 |
| 0.40 | 27.3 | 25.3 | 76.4 | 15.9 | 3.8 | 13.1 |
| 0.80 | 25.5 | 23.8 | 77.2 | 15.9 | 3.6 | 13.1 |
| PSE[4] | 1.632 | 0.798 | 0.374 | 0.168 | 0.133 | 0.481 |
| ANOVA (Pr>F) | 0.0283 | 0.4052 | 0.3851 | 0.9482 | 0.0004 | 0.0692 |
| Linear Trend (Pr>F) | 0.4574 | 0.8498 | 0.2119 | 0.6410 | 0.9156 | 0.0059 |
| Quadratic Trend (Pr>F) | 0.2793 | 0.1231 | 0.1015 | 0.8951 | 0.0448 | 0.0129 |

[1]Values were obtained from composite samples of three fish per replicate group (n = 4).
[2]Calculated as follows: (final body lipid – initial body lipid) x (100/total lipid fed).
[3]Calculated as follows: (final body protein – initial body protein) x (100/total protein fed).
[4]PSE = Pooled Standard Error

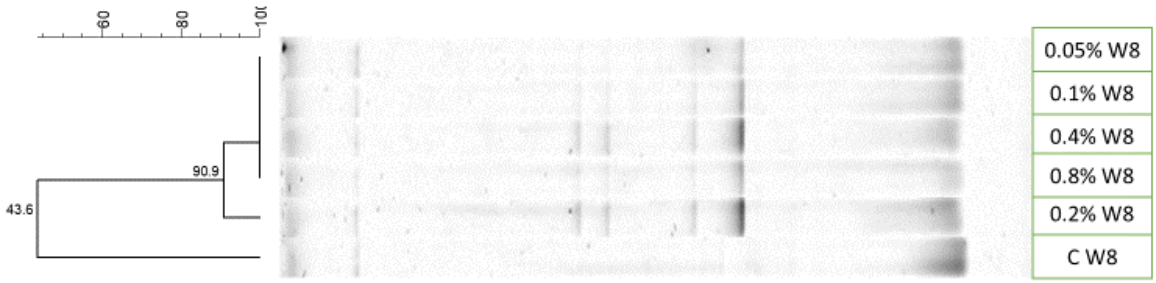
Bacterial diversity of the digesta samples obtained at week 4 was somewhat similar with similarity coefficient values between 80 and 84% for fish fed the various dietary treatments (Fig. 6). The digesta samples from fish fed 0.1, 0.2, and 0.4% Bactocell™ concentrations were determined to be similar (85.7%), and the digesta samples from fish fed the diets with 0.05, 0.8, and 0% Bactocell™ (basal) were determined to be very similar (94.7%).

**FIGURE 6** Dendrogram analysis following bacterial 16S rDNA denaturing gradient gel electrophoresis of digesta samples from juvenile red drum after 4 weeks of feeding the experimental diets. Numbers in the treatment labels indicate the percent of Bactocell™ inclusion except C which was the control or basal diet. Numbers to the left of the dendrogram are percentage similarity coefficients.




In contrast to the week 4 results, the DGGE analysis of digesta samples after week 8 indicated the microbiota of the digesta from red drum fed the basal diet was different when compared to fish fed experimental diets containing Bactocell™ having no similarity (43.6%) to each other (Fig. 7). These results show that dietary Bactocell™ feeding for 8 weeks altered the microbiota of digesta from the GIT of juvenile red drum.

**FIGURE 7** Dendrogram analysis following bacterial 16S rDNA denaturing gradient gel electrophoresis of digesta samples from juvenile red drum after 8 weeks of feeding the experimental diets. Numbers in the treatment labels indicate the percent of Bactocell™ inclusion except C which was the control or basal diet. Numbers to the left of the dendrogram are percentage similarity coefficients.




## 3.4 DISCUSSION

Over the 8-week feeding trial, red drum gained over 1,300% of their initial body weight, which is considered an exceptional growth rate. In a similar feeding trial involving prebiotics, red drum of similar initial weight had weight gain values averaging around 1,100% over a 9-week period (Rossi et al., 2017). Feed efficiency values in the present study were not particularly high, ranging from 0.65 to 0.7 compared to other studies with red drum in which feed efficiency values ranged between 0.8 and 1.0 (Rossi et al., 2017; Castillo and Gatlin, 2018; Castillo et al., 2015). The lower feed efficiency values obtained in the current experiment are undesirable, but there was no obvious reason why they were lower. The fish in the study were fed at a fixed percentage of body weight which was adjusted each week to ensure a level close to apparent satiation without overfeeding (Rossi et al., 2017). Though low feed efficiency values

are undesirable, there are other studies involving red drum which have reported lower feed efficiency values ranging from 0.5 to 0.6 (Zhou et al., 2010, Buentello et al., 2010). In experiments involving other species grown in similar conditions such as hybrid striped sass (*Morone chrysops* x *M. saxatilis*), feed efficiency values have been reported to range from 0.5 to 0.96 (Gaylord and Rawles, 2005 and Li and Gatlin, 2003) which at the lower end of the spectrum are similar to the results obtained in the present trial.

Survival of red drum in the present study also was on par with previous red drum experiments with survival values of approximately 90% or higher (Rossi et al., 2017). However, other studies with red drum (e.g., Buentello et al., 2010) have reported lower values than in the present trial. Mortalities throughout the present experiment were infrequent and fish which died during the experiment showed signs of predation or scavenging by other fish as there were living fish which had eyes missing before they had died, and dead fish commonly had open wounds on their tails and midsection.

Protein retention is another common metric of diet utilization which estimates the amount of dietary protein retained by the fish; values between 33 and 40% commonly have been reported for juvenile red drum (Rossi et al., 2017; Castillo and Gatlin, 2018). The protein retention values obtained in the current feeding trial were lower than values commonly seen in other experiments with results ranging from 23 to 25%. However, other studies involving red drum have reported similar and even lower protein retention values (Castillo et al., 2015; Castillo and Gatlin, 2018), making these results not completely uncommon. In feeding trials with other fish such as the hybrid striped bass grown in similar recirculating systems, protein retention values have been reported to range anywhere from 18 to 30% (Rawles et al., 2006; Li and Gatlin, 2003).

Reduced levels of fishmeal protein in the diet of red drum could have contributed to some of the reduced growth values which were collected from this experiment. Substituting fishmeal-based protein feedstuffs with plant-protein feedstuffs demonstrates potential drawbacks, as it has been seen that carnivorous fish fed plant-based diets tend to have reduced growth when compared to those fed fishmeal-based diets (Boucher et al., 2011). However, the potential benefits of probiotic or other health-related supplements may be accentuated in plant-based diet formulations.

In the present trial a complete assessment of disease resistance or immunological responses of red drum fed various concentrations of Bactocell™ was not performed. However, the Nitroblue-Tetrazolium test, conducted on blood drawn from the experimental fish, was used as a measure of innate immunity of fish fed the various diets. The observed values ranged from 5.1 to 6.1 mg/mL. When comparing these values to other prebiotic experiments involving red drum, these values were higher than typically reported, with other feeding trials reporting NBT values ranging from 3.8 to 5.2 mg/mL (Buentello et al., 2010; Rossi et al., 2017; Zhou et al, 2010). Studies which have experimented with other potential probiotic dietary supplements on fish species, such as Nile Tilapia (*Oreochromis niloticus*), reported NBT values ranging from 1.7 to 7.1 mg/mL (Aly et al., 2008; Peredo et al., 2015). According to Choi et al. (2006), higher NBT values as those observed in the present study may have indicated the presence of a bacterial pathogen as the fish's immune system was possibly upregulated to fight the infection. However, this could not be determined because no tests were conducted to determine if there were bacteria present which were detrimental to the health of the fish.

After 4 weeks of feeding diets with incremental concentrations of the probiotic Bactocell™ to the juvenile red drum, there was no noted alteration of the microbiota of the

digesta. However, after 8 weeks there was a difference between fish fed the basal diet and the diets with different inclusion concentrations of Bactocell™. This result is consistent with those taken from previous studies where prebiotics such as Grobiotic®-A (Burr et al., 2007) and inulin (Burr et al., 2009) were shown to alter the gut microbiota of juvenile red drum after an 8-week period. However, in the present trial no improvements in fish performance or immune status were evident.

# CHAPTER IV
# SUMMARY AND CONCLUSIONS

Rotifers were cultured in 4-day production trials to evaluate the addition of different prebiotic and probiotic supplements to the culture water and their influences on overall productivity as well as microbial composition of resulting rotifers. It was determined that Aquablend, ™ Bactocell™, and Grobiotic®-A influenced the composition of microbiota within the rotifers when compared to the control culture without any supplements. These results show that there is potential for altering the microbiota of live foods in an attempt to use prebiotic or probiotic supplements as nutritional amendments for larval fish. Results of the DGGE analysis of rotifers fed Grobiotic®-A were similar to results from rotifers enriched with both probiotic supplements. Prebiotic and probiotic supplements in live foods should now be evaluated with larval fish with the goal of increasing their survival and growth during hatchery production.

In the second experiment as part of this thesis, juvenile red drum were fed graded concentrations of the probiotic Bactocell™ to evaluate its effect on growth performance and microbial composition of their digesta. It was determined that using the probiotic Bactocell™ as a dietary supplement was not extremely successful in contributing to improved growth of these fish. There also was no effect of Bactocell™ inclusion on neutrophil oxidative radical production as a measure of non-specific immunostimulation. However, Bactocell™ was able to alter the microbiota of the digesta based on DGGE analysis after 8 weeks of feeding. Before implementing the use of Bactocell™ in the diet of juvenile red drum, further experimentation needs to be performed to further explore the immunological responses of fish fed ddiets supplemented with the probiotic. Possible immunological evaluations to be performed are measuring head kidney reactive oxygen species production, bactericidal assay, and disease

challenges. Upon this further work, Bactocell™ may be incorporated into the diet of red drum during production for stock enhancement or as a food product to decrease the amount of mortalities and potentially increase growth during the juvenile stage.

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