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Effects of Selected Commercial Diets and Yeast Substitution on the Growth and Associated Microbiota of Rotifer (*Brachionus plicatilis*)

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UNIVERSITY OF MIAMI

EFFECTS OF SELECTED COMMERCIAL DIETS AND YEAST SUBSTITUTION
ON THE GROWTH AND ASSOCIATED MICROBIOTA OF ROTIFER
(Brachionus plicatilis)

By

Ryan D. Lind

A THESIS

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Master of Science

Coral Gables, Florida

December 2014

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(*Brachionus plicatilis*)

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The rotifer (*Brachionus plicatilis*) is an essential feed organism for successful marine larviculture. Cost effective feeding regimes and the control of harmful bacteria are critical components of rotifer production. Supplementation of commercial diets with yeast can reduce costs, but its effects on bacteria such as *Vibrio spp.* are not well documented. Two experiments were conducted to identify a practical culture diet. The first experiment evaluates different commercial diets on rotifer growth and total *Vibrio*. The second experiment determines a practical level of yeast substitution, with regard to rotifer growth, culture *Vibrio* loads, and cost. The diets evaluated in the first trial include INVE S.parkle, Reed RotiGrow *Nannochloropsis*, Reed *Tetraselmis*, and Aquafauna Bio-marine Algamac Protein Plus. The second trial substituted yeast for 50%, 75%, and 85% of the top performing commercial diet from the first trial. Total rotifer growth (%) and *Vibrio* (CFU rotifer⁻¹) were measure in both trials, and results were evaluated with one-way ANOVAs and Tukey post-hoc tests. Rotifer growth was highest for INVE S.parkle and Reed RotiGrow *Nannochloropsis*. Reed *Tetraselmis* contained the lowest *Vibrio* (CFU rotifer⁻¹) on day 2 and was significantly different than all other treatments. In the second experiment, no significant differences were found in growth between levels of

yeast substitution for *S. parkle*. There was, however, a significantly greater concentration of *Vibrio* within the 75% substitution group on day 3.

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CHAPTER 1: INTRODUCTION

Despite attempts to develop artificial micro-diets, live feed organisms are still essential for culturing marine larval fish (Lee 2003). The rotifer *Brachionus plicatilis* (Müller) is commonly used as a first feed organism due to its small size and ease of culture (Watanabe et al. 1983; Lubzens et al. 1989; Lubzens & Zmora 2003). Rotifers are selective feeders (Chotiyaputta and Hirayama, 1987), but may be grown on a number of diets. Rotifers lack dietary value, but can be fed a diet designed to match the nutritional profile required by larval fish. Some diets are formulated to enrich rotifers with maximum nutrition (enrichment diet), while other diets are formulated for rotifer growth (culture diet).

Typically rotifers are fed microalgae such as *Chlorella spp.*, *Isochrysis spp.*, *Nannochloropsis spp.*, and *Tetraselmis spp.* (Korstad et al. 1989). Microalgae are administered fresh, frozen, or dried. The high cost of microalgae, due to labor intensity of cultivation and harvest, may limit rotifer production and success in marine fish hatcheries. A single fish larvae may require more than 40,000 – 100,000 rotifers before it is weaned to another diet (Okauchi et al. 1980). Therefore, the production demand of rotifers is extremely high in order to support marine fish culture.

Nagata & Whyte (1992) have identified baker's yeast as a cost effective substitute for algal-based diets for rotifers. Yeast is ten times less expensive than microalgae diets, and rotifers can be successfully grown on yeast alone. Rotifers fed only yeast are subject to potential stagnation in rotifer growth; however, high growth can be achieved by feeding rotifers a combination of yeast and algae (Tamaru 1993).

Compared to microalgae, yeast is deficient in omega-3 amino acids and other essential nutrients. Striped mullet and milkfish fed rotifers grown on yeast have lower survival than fish fed rotifers that are grown on microalgae or a combination of microalgae and yeast (Clyde et al. 1993). However, most hatcheries today employ a two-step process for producing rotifers that eliminates the nutritional effect of yeast on fish larvae. In this two-step process, rotifers are mass cultured on a standard diet and part of the culture is set aside to enrich with a more complete diet in order to reduce total feed costs. The enrichment diet includes all of the nutrients required by the fish larva. Rotifers fed yeast initially and later enriched are nutritionally similar to rotifers fed only enrichment, especially during periods of enrichment greater than 6 hours (Rodriguez et al, 1996). Longer enrichments up to 24 hours offer many advantages including a stable fatty acid profile, a lipid content comparable to wild zooplankton, decreased loss in rotifers and lower labor costs (Dhert et al. 2001). Therefore, given long enrichment periods, hatcheries may reduce costs in the culture stage without adversely effecting nutrition in the enrichment stage. Still, a combination of yeast and algae is preferred over yeast alone for mass rotifer production as mentioned above (Tamaru 1993).

Recent studies on rotifers have focused on optimizing an enrichment diet (Ludwig 2008; Haché and Plante, 2011; Ma 2014). Few studies, in contrast, have explored optimizing a diet for mass culture of rotifers prior to enrichment. While it is important to understand the effect of enrichment on larval success, it is also critical to optimize a diet for mass production of rotifers. Feed is the largest cost of marine fish farms (Urban et al. 1991), and practices that reduce feed costs without compromising nutrition are critical for developing a viable industry. Cost savings can be achieved in the mass production of

rotifers by combining baker's yeast with an algal-based diet. Baker's yeast is cheap and readily available. In contrast, microalgae is expensive and has a shorter shelf life. Some hatcheries culture algae on site, while others purchase commercial microalgae products.

While it is important to consider rotifer growth and cost of the diet, it is equally important to understand the diet's effect on the culture's microbial community. Various commercial microalgae diets are available on the market but differ in rotifer growth and associated microbial communities (Qi et al. 2009). Some diets may promote harmful bacteria that are later transferred to the fish larvae. Thus, it is critical to understand the diet's effect on bacteria when selecting a rotifer diet.

The most common method of rotifer production is batch culture. Batch cultures promote highly variable microbial communities, as pioneer bacteria bloom with new cultures (Rombaut 2001). Batch cultures are static systems without continuous water exchange or recirculation. After several days batch cultures need to be rinsed and reset, as the water becomes polluted. The process of resetting a culture leads to opportunistic bacteria. Continuous or recirculating systems utilize constant exchange of water. Since this method of culturing rotifers typically maintains relatively undisturbed cultures for weeks or months, microbial communities become more stable as opportunistic bacteria are outcompeted by slower-growing, potentially less harmful bacteria.

High-density rotifer production is associated with large feed inputs of organic matter, which increases the susceptibility to harmful heterotrophic bacteria. Not all bacteria are detrimental to rotifer cultivation; however, pioneer bacteria associated with rotifer batch cultures have negative impacts on rotifer production and subsequently effect larval growth and survival (Skjermo and Vadstein, 1993).

Marine larvae are susceptible to a host of harmful bacteria. *Vibrio spp.* are the dominant harmful species associated with rotifer cultures (Verdonck et al. 1997; Gomez-Gil et al. 2003). Bacteria thrive in all parts of the rotifer culture system, attaching to tank walls, or residing within the gut of the rotifer. Thus rotifers act as a vector, which transmit opportunistic bacteria to fish cultures. Successful marine larviculture depends on sound microbial management in live feeds production. Attempts to control bacteria in rotifer cultures include the use of antibiotics and probiotics (Benetti et al. 2008; Rotman et al. 2011; Zink et al. 2013) as well as continuous culture systems (Rombaut 2001).

A third method for controlling bacteria in rotifer cultures is selecting the optimal diet that minimizes harmful bacteria. Qi et al. (2009) has shown that commercial rotifer diets have different effects on bacteria in low-density rotifer cultures. Commercial diets vary in digestibility and consequent different levels of organic waste. Heterotrophic bacteria feed on organic waste supplied by the rotifer diet, so it is important to choose a diet that minimizes waste, and harmful bacteria that feed on organic matter. Some diets may have anti-microbial or anti-*Vibrio* properties that may bestow benefits to rotifer cultures (Olsen et al. 2000; Regunathan and Wesley 2004).

The present study is composed of two experiments. The first experiment seeks to identify a commercial diet with the greatest performance in terms of rotifer growth and *Vibrio* reduction. The second experiment identifies a practical diet by evaluating incremental yeast substitution for the commercial diet from experiment I. A practical diet will be evaluated based on three parameters: rotifer growth, cost of the diet, and concentrations of total *Vibrio*.

CHAPTER 2: EXPERIMENT I – COMPARISON OF SELECTED COMMERCIAL DIETS FOR MASS ROTIFER CULTIVATION

2.1 Background

Many studies on rotifer diets have focused on enrichment because of its influence on larval growth and survival. Fewer studies focus on improving feed strategies for mass rotifer cultivation before enrichment. Commercial diets may not be optimal for application of increasing rotifer biomass. Greater efficiencies are achieved by selecting a diet that maximizes growth with respect to cost. Yeast, in combination with commercial diets, can reduce costs of rotifer production (Nagata and Whyte, 1992). These savings are achieved without sacrificing larval production in most hatcheries for which secondary rotifer enrichment is inevitable. While it is important to reduce costs of mass rotifer production, it may be unwise to do so at the expense of elevated opportunistic bacterial levels that may later impact fish larvae.

Qi et al. (2009) has evaluated select commercial rotifer diets for the effects on rotifer growth and changes in microbial communities during low-density rotifer trials (initial density ~100 individuals per mL). Qi et al. found increased growth of rotifer cultures fed *Nannochloropsis* algae than yeast-based Culture Selco.¹ Authors found a different dominant bacteria species between the two diets, indicating an effect on bacteria. In an earlier study, Reitan and Olsen (1994) found a yeast-based diet to promote more bacteria than algal diets. Reitan and Olson also showed better rotifer growth performance on algal-based diets than yeast and capelin oil.

Recent improvements have been made to create a better yeast-based diet. INVE's Selco S.parkle is a product that is formulated of mostly yeast, but also includes dried

¹ Culture Selco was a common rotifer diet manufactured by INVE. INVE discontinued producing Culture Selco and now produces a new yeast-based diet, S.parkle, which is evaluated in this experiment.

algae. Limited research has been done comparing recent formulations of rotifer culture diets, such as Selco S.parkle, (Qi et al. 2009). Our study sought to answer two questions regarding culture diets: i) how recently formulated diets effect rotifer growth and levels of opportunistic bacteria in commercial-density rotifer production; and ii) whether yeast substitution yields greater per-dollar growth in commercial-density rotifer production without effecting levels of opportunistic bacteria. Experiment I answers the first question, and experiment II answers the second.

The first experiment analyzed and compared rotifer growth and total *Vibrio* concentrations between the treatment groups of four commercially available rotifer diets i) INVE Selco S.parkle, a combination of dry yeast and algae, ii) Algamac Protein Plus (P.plus) from Aquafauna Bio-marine, a dry algae diet, iii) Reed RotiGrow *Nannochloropsis* (Nanno) algae paste, and iv) Reed *Tetraselmis* (Tetra) algae paste. Two hypotheses were tested during this experiment: 1), whether significant differences exist in growth between the diet treatments; 2), whether significant differences exist in *Vibrio* loads between treatments. By examining these parameters this study may help determine if selected commercial diets exhibit higher growth in rotifer cultures and lower growth in opportunistic bacteria.

In the second experiment, the best performing diet from the first experiment was substituted with yeast in different amounts to answer the second question: whether yeast substitution in rotifer diets results in production cost savings, without negative effects on the levels of opportunistic bacteria.

2.2 Materials and Methods

2.2.1 Experimental Setup

This study was conducted at the University of Miami Experimental Hatchery (UMEH). Stock cultures of *Brachionus plicatilis* were obtained from UMEH. Culture tanks consisted of static batch-culture 5-gallon buckets, maintained within a continuously flowing, ambient temperature bath to control fluctuations between night and day. Culture systems and aeration apparatus were disinfected in a 60-min immersion in a 10-ppm sodium hyperchlorite solution, followed by a sodium thiosulfate solution rinse. Water was filtered to 1 μm and Ultraviolet (UV) sterilized. The stock culture water was disinfected by the addition of sodium hypochlorite at 10 mg L^{-1} , which was then neutralized with sodium thiosulfate after 30 min. Sodium hypochlorite neutralization was verified using a colorimetric chlorine presence test kit. Final culture volumes of 15 L were achieved by distribution of proper predetermined volume of concentrated stock rotifers and stock culture water to obtain a target initial stocking density of 500 rotifers mL^{-1} .

Cultures were aerated with 0.35 μm filtered air using a diffuser airstone suspended 1 inch above the bottom at the center of the bucket. Supplemental oxygen was supplied by diffusers and dissolved oxygen levels were maintained between 7-12 mg L^{-1} . Ambient light was reduced by 70% shade cloth fitted over the UMEH roof. Natural daylight cycles were maintained. Rotifers were acclimated to treatments by being fed treatment diets for 3 days prior to stocking in culture systems. The trial duration was 3 days.

2.2.2 Feeding Regime

Treatment groups were fed per manufacturers recommendations. The Nanno treatment ($n = 3$) was fed every 6 hrs at 1.8 ml of diet per million rotifers per day. The S.parkle treatment ($n = 3$) was fed every 6 hrs, according to a variable feed schedule: 0.5g per million rotifers per day (for rotifer densities of 500-750 rotifers/mL), then 0.45g per million rotifers per day (750-1,000 rotifers/mL), and 0.4g per million rotifers per day (1,000-1,500 rotifers/mL). The Algamac Protein Plus treatment group ($n = 3$) was fed 0.5g per million rotifers twice a day. Control treatment replicates ($n = 3$) were fed our UMEH diet every six hours at 0.32 g per million rotifers per day. Care was taken to avoid cross contamination between cultures by disinfecting feeding equipment with ethanol before preparing the feed for the next treatment group. Feed amounts were prepared based on morning density counts of rotifer cultures. When required, feeds were mixed with municipal water using a Cuisinart hand blender. Rations were stored in a refrigerator at 8 °C to be fed throughout the day.

2.2.3 Daily Population and water quality sampling

Temperature (°C), dissolved oxygen (DO, mg L⁻¹), and pH were recorded daily in the evening. Care was taken to prevent bacterial cross contamination by rinsing water quality probes with 70% ethanol followed by 1 µm, UV sterilized seawater between sampling individual tanks. Population estimates were obtained for each culture by taking ~300 mL samples from individual cultures and subsequently analyzing four 1 mL subsamples to determine rotifer densities.

2.2.4 Bacteriology

Water samples were obtained in sterile 1.5 ml microcentrifuge tubes from culture tanks on day 0 and day 2 to determine changes in total *Vibrio* communities. Each sample was plated in triplicate on TCBS (thiosulfate citrate bile sucrose, BD Diagnostics) using a membrane filter technique and incubated at 40 °C for 24 hrs following a protocol outlined by Hernández-López et al. (1995). Total *Vibrio* colony forming units (CFU ml⁻¹) were counted 24 hrs after plating. Standardization of CFU's was achieved by calculating (CFU rotifer⁻¹) to allow for comparison across treatments with different growth.

2.2.5 Data Analysis

One-way ANOVA tests were run to determine whether significant differences exist in rotifer growth and total *Vibrio* concentrations between treatments. Differences between treatments were assessed by Tukey post-hoc analysis. All results are reported as means ± SE. All statistical analyses were conducted using IBM® SPSS® Statistics, Version 22 (International Business Machines Corp., Armonk, NY, 10504-1722).

2.3 Results

No statistically significant differences were seen in temperature 27.4 ± 0.1 ($P = 0.929$) or DO 9.3 ± 0.4 ($P = 0.668$). Salinity remained constant at 35 (with no observed variation), but pH was significantly different between treatment groups $F(4,10) = 5.547$, ($P = .013$). Tukey post-hoc analysis revealed that pH of S.parkle was significantly different from Nanno ($P = .047$) and Tetra ($P = .008$), but no other group differences in pH were statistically significant.

Table 1. Rotifers (millions), total growth (%), ciliates (1=presence, 0=absence), and Vibrio (CFU/rotifer) with means \pm SE.

	Day	S.parkle	P.plus	UMEH	Nanno	Tetra	Level of Significance
Rotifers (millions)	1	6.34 \pm 0.24 ^{ab}	1.77 \pm 0.37 ^c	6.44 \pm 0.19 ^{ab}	7.26 \pm 0.30 ^a	5.64 \pm 0.23 ^b	***
	2	8.38 \pm 0.25 ^a	3.03 \pm 0.83 ^b	6.59 \pm 0.27 ^a	7.83 \pm 0.39 ^a	4.48 \pm 0.18 ^b	**
	3	9.22 \pm 0.29 ^a	3.42 \pm 0.72 ^b	4.97 \pm 0.31 ^b	8.74 \pm 0.70 ^a	4.02 \pm 0.35 ^b	***
Total growth (%)	-	41.85 \pm 4.40 ^a	-47.38 \pm 11.14 ^b	-23.49 \pm 4.76 ^b	34.45 \pm 10.76 ^a	-38.17 \pm 5.36 ^b	***
Ciliates (1=presence, 0=absence)	3	0	1	1	0	1	
Total <i>Vibrio</i> (CFU rotifer ⁻¹)	0	0.05 \pm 0.05	0.10 \pm 0.05	0.10 \pm 0.05	0.15 \pm 0.15	0.20 \pm 0.13	NS
	2	6.48 \pm 1.77 ^a	113.67 \pm 27.17 ^a	1.39 \pm 0.34 ^a	3.56 \pm 1.77 ^a	0.65 \pm 0.15 ^b	**

Means in the same row having different superscripts are significantly different determined by ANOVA and Welch ANOVA ($P < 0.05$)
 Significance levels are denoted by: NS (No Significance), * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$)
 Total growth calculation assumes the rotifer stock of 6.50 million on Day 0

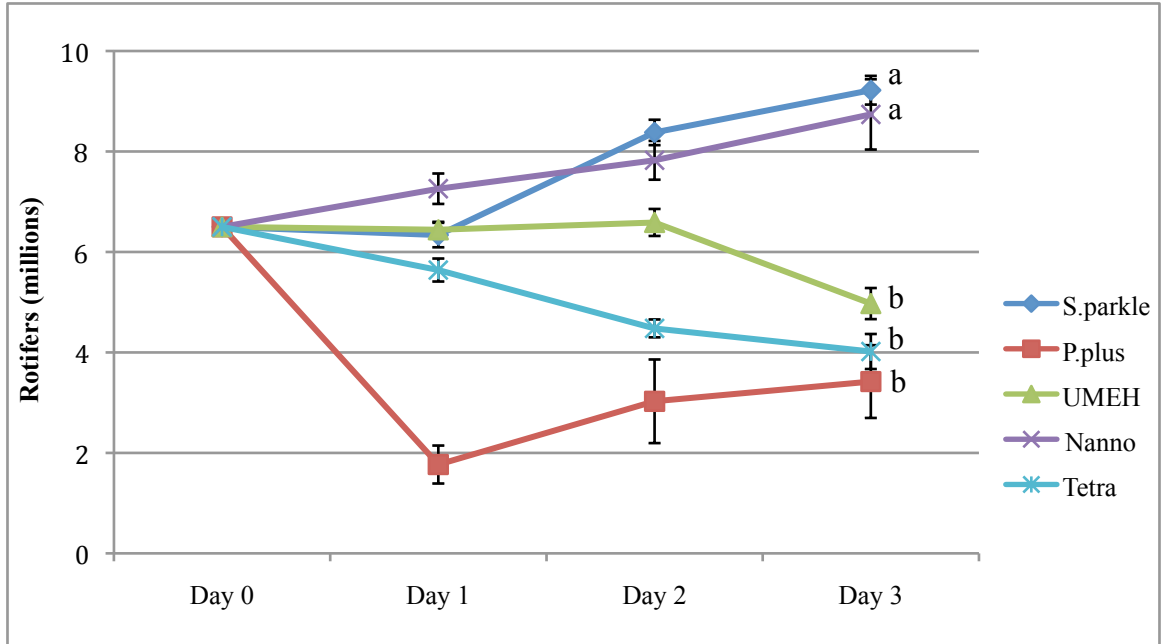


Figure 1. Population sizes (means \pm SE) for *B. plicatilis* ($n = 3$) by treatment for days 0, 1, 2, and 3. Values with different letters are significantly different determined by Tukey HSD post-hoc analysis ($P < 0.05$).

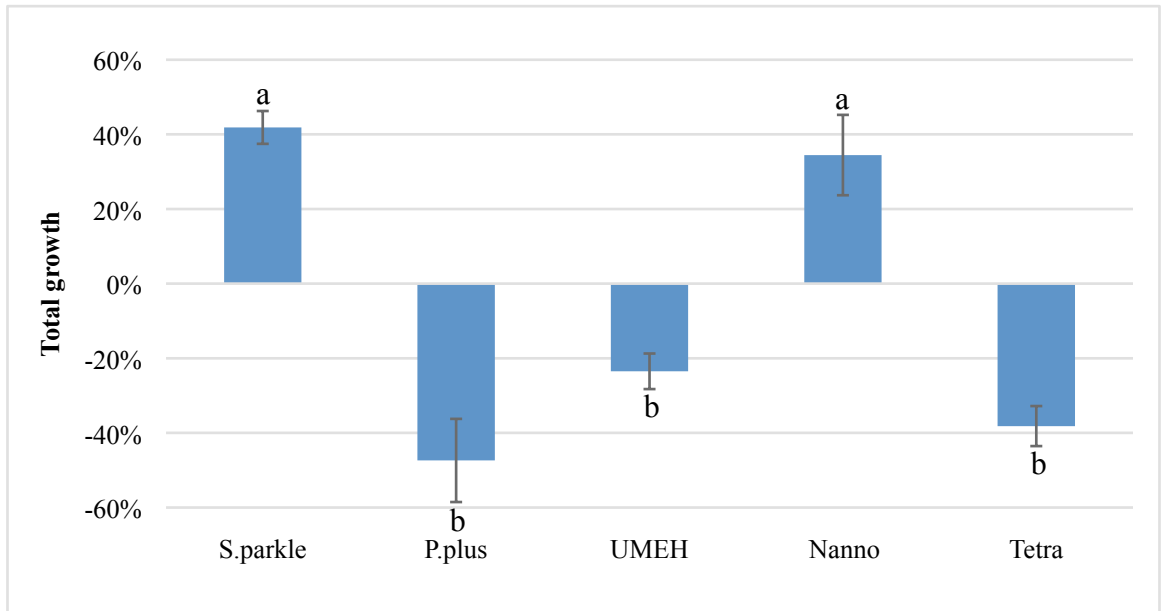


Figure 2. Total growth (means \pm SE) of *B. plicatilis* ($n = 3$) by treatment for days 0, 1, 2, and 3. Values with different letters are significantly different determined by Tukey HSD post-hoc analysis ($P < 0.05$).

Table 1 lists the main parameters of the first experiment. S.parkle and Nanno diets showed greater numbers in total rotifers (Figure 1). Total growth (%) was significantly different between groups, $F(4,10) = 28.059$, ($P < .0005$) (Figure 2). Tukey post-hoc analysis revealed that S.parkle ($41.85\% \pm 4.40\%$) and Nanno ($34.45\% \pm 10.76\%$) had significantly higher growth than other diets: P.plus ($-47.38\% \pm 11.14\%$), UMEH ($-23.49\% \pm 4.76\%$), and Tetra ($-38.17\% \pm 5.36\%$).

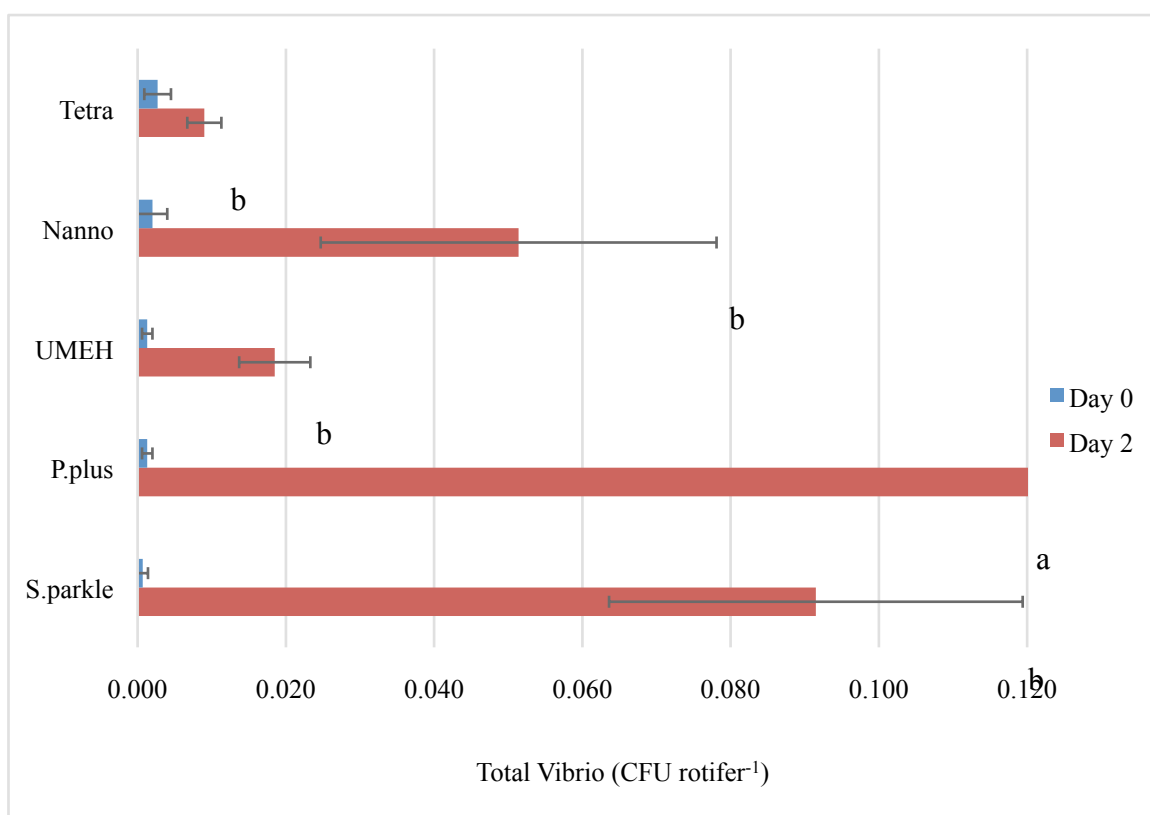


Figure 3. Total *Vibrio* (means \pm SE) for *B. plicatilis* ($n = 3$) by treatment for days 0, 2. Values with different letters are significantly different determined by Tukey HSD post-hoc analysis ($P < 0.05$). P.plus was graphically cut to allow for visible scale of other treatments.

On day 3, Ciliates were absent from all cultures of S.parkle ($n = 3$) and Nanno ($n = 3$), but ciliates were observed in all cultures of P.plus ($n = 3$), UMEH ($n = 3$), and Tetra ($n = 3$). No statistically significant differences were seen in Total *Vibrio* (CFU rotifer⁻¹) on day 0 (Figure 3). However, day-2 *Vibrio* (CFU rotifer⁻¹) was statistically significantly

different between treatments, $F(4,10) = 12.394$, ($P = .001$). Tukey post-hoc analysis revealed that *Vibrio* (day 2) in the Tetra group was significantly lower than all other treatments: S.parkle ($P = .002$), P.plus ($P = .001$), UMEH ($P = .045$), and Nanno ($P = .006$), but no other group differences in *Vibrio* were statistically significant.

2.4 Discussion

Significant differences in growth were found in *B. plicatilis* fed different commercial diets. Yeast-based S.parkle exhibited the highest growth and least variation in growth, over a 3-day period. Nanno showed slightly lower growth, but the difference was not statistically significantly from S.parkle. Past studies have shown algal-based diets to out-perform other yeast-based diets such as Culture Selco (Qi et al. 2009) and yeast with capelin oil (Reitan and Olsen 1994). The present experiment shows improved formulations of yeast-based diets, such as S.parkle, may outperform algae paste as a feed for rotifer cultures.

P.plus, UMEH, and Tetra showed negative growth, which was likely due to sub-par culture conditions across treatments. In fact, all diets showed lower growth than expected, likely caused by settling of food and rotifers in the flat-bottom culture systems. During previous studies, rotifers at lower densities (100 individuals per milliliter) performed better in similar culture systems (Zink, et al., 2013). But, higher stocking densities in this experiment combined with flat-bottom culture systems may require increased aeration to suspend higher feed inputs associated with commercial-density rotifer production. This experiment should be repeated with increased aeration to determine whether top-performing diets would be the same under ideal culture conditions.

No significant difference was found in total *Vibrio* (CFU rotifer⁻¹) between groups upon stocking (day 0). Significant differences were found in day-2 total *Vibrio*. The CFU per rotifer was significantly lower in Tetra than all other treatments. Reduced *Vibrio* may have resulted from anti-*Vibrio* properties of Tetra. The use of Tetra as a supplemental feed in aquaculture has been shown to inhibit growth of *Vibrio* (Olsen et al. 2000; Regunathan and Wesley 2004), even as inert, spray-dried Tetra (Austin and Day 1990). Future studies should evaluate Tetra as an anti-*Vibrio* agent in rotifer cultures.

No ciliates were seen in any treatment on days 0, 1, and 2. On day 3, Ciliates were observed in all cultures, except the two diet groups showing the best growth: S.parkle and Nanno. Presence of ciliates may be influenced by poor rotifer growth in P.plus, UMEH, and Tetra groups. Indeed, suppressed rotifer growth is associated with an increase in protozoa such as ciliates (Reguera, 1984). Although ciliates may not directly harm rotifer cultures, some ciliates compete by consuming the same food, thereby reducing the production efficiency of rotifer cultures.

Overall, S.parkle appeared to perform best compared to other rotifer diets in this study. Sparkle showed the highest mean total growth, no ciliates, and lower concentrations of total *Vibrio*. Thus, yeast-based S.parkle is a viable alternative to algal-based rotifer culture diets. Hatcheries should consider using S.parkle as part of their strategy to produce quality and quantity rotifers to be enriched and fed to marine fish.

In post hoc analysis, S.parkle was greater than 20% more cost-effective at producing rotifers than Nanno. While S.parkle offers distinct advantages as a stand-alone rotifer culture diet, it is hypothesized that further cost efficiencies be achieved by adding baker's yeast. The goal of the next experiment is to identify a practical diet by finding the

level of yeast substitution for S.parkle that optimizes growth, reduces costs, and maintains low *Vibrio* concentrations.

CHAPTER 3: EXPERIMENT II – YEAST SUBSTITUTION IN A COMMERCIAL ROTIFER DIET

3.1 Background

The success of marine larviculture depends on large amounts of high quality rotifers. Hatcheries spend vast amounts of time and money producing rotifers. Cost savings by yeast substitution into rotifer diets are likely achievable in the mass production of rotifers, where a secondary enrichment is conducted. Especially for those hatcheries using long enrichment periods, substituting yeast (*Saccharomyces cerevisiae*) for algae may have a marginal effect on the nutritional composition of the rotifer after enrichment.

Limited research has been conducted on the effect of different levels of yeast substitution for rotifer culture diets. Nagata and Whyte (1992) found that yeast alone supports 30% daily rotifer growth of compared to 40% for algae (*Chlorella saccharophila*). Tamaru et al (1993) showed no significant difference in rotifer growth between different diets of yeast, Nanno, and a combination of yeast and Nanno. No reports have yet to be found on the effect of substituting yeast for *S.parkle* on rotifer growth and bacteria. If partial substitution is attainable for this product, considerable cost savings can be achieved.

The primary assumption of this experiment is that partial yeast substitution of *S.parkle* has no effect on rotifer growth or levels of opportunistic bacteria. Consequently, there are two hypothesis tested in the second experiment. The first hypothesis is that there are no significant differences in growth between treatment groups of different level yeast substitution. The second hypothesis is that there are no differences in total *Vibrio* concentrations between treatments. Treatment groups for trial 2 include different levels of

yeast substitution for S.parkle. The control diet ($n = 3$) is S.parkle only. Treatment one ($n = 3$) is S.parkle with 50% substitution of yeast by weight; the second treatment ($n = 3$) is S.parkle with 75% yeast substitution (YS); and the third treatment is S.parkle with 85% YS.

3.2 Materials and Methods

3.2.1 Experimental Setup

This study was conducted at the University of Miami Experimental Hatchery (UMEH). Stock cultures of *Brachionus plicatilis* were obtained from UMEH. Culture tanks consisted of static batch-culture 5-gallon buckets, maintained within a continuously flowing, ambient temperature bath to control fluctuations between night and day. Culture systems and aeration apparatus were disinfected in a 60-min immersion in a 10-ppm sodium hyperchlorite solution, followed by a sodium thiosulfate solution rinse. Water was filtered to 1 μm and Ultraviolet (UV) sterilized. The stock culture water was disinfected by the addition of sodium hypochlorite at 10 mg L^{-1} , which was then neutralized with sodium thiosulfate after 30 min. Sodium hypochlorite neutralization was verified using a colorimetric chlorine presence test kit. Final culture volumes of 15 L were achieved by distribution of proper predetermined volume of concentrated stock rotifers and stock culture water to obtain a target initial stocking density of 500 rotifers mL^{-1} .

Cultures were aerated with 0.35 μm filtered air using a diffuser airstone suspended 1 inch above the bottom at the center of the bucket. Supplemental oxygen was supplied by diffusers and dissolved oxygen levels were maintained between 7-12 mg L^{-1} .

Ambient light was reduced by 70% shade cloth fitted over the UMEH roof. Natural daylight cycles were maintained. Rotifers were acclimated to treatments by being fed treatment diets for 3 days prior to stocking in culture systems. The trial duration was 3 days.

3.2.2 Feeding Regime

Rotifer cultures were fed at 0500, 1100, 1700, and 2300 hrs. The control group was fed *S. parkle* the recommended daily feed rate. Treatment groups received 0.4 grams of *S. parkle* per million rotifers per day, except a partial substitution of yeast as outlined above. Feeds were mixed with municipal water using a Cuisinart hand blender. Daily feed rations were stored in a refrigerator at 8 °C.

3.2.3 Daily Population and water quality sampling

Temperature (°C), dissolved oxygen (DO, mg L⁻¹), and pH were recorded daily in the evening. Care was taken to prevent bacterial cross contamination by rinsing water quality probes with 70% ethanol followed by 1 µm, UV sterilized seawater between sampling individual tanks. Population estimates were obtained for each culture by taking ~300 mL samples from individual cultures and subsequently analyzing four 1 mL subsamples to determine rotifer densities.

3.2.4 Bacteriology

Water samples were obtained in sterile 1.5 ml microcentrifuge tubes from culture tanks on day 0 and day 2 to determine changes in total *Vibrio* communities. Each sample was plated in triplicate on TCBS (thiosulfate citrate bile sucrose, BD Diagnostics) using a membrane filter technique and incubated at 40 °C for 24 hrs following a protocol outlined by Hernández-López et al. (1995). Total *Vibrio* colony forming units (CFU ml⁻¹)

were counted 24 hrs after plating. Standardization of CFU's was achieved by calculating (CFU rotifer⁻¹) to allow for comparison across treatments with different growth.

3.2.5 Data Analysis

One-way ANOVA tests were run to determine whether significant differences exist in rotifer growth and total *Vibrio* concentrations between treatments. Differences between treatments were assessed by Tukey post-hoc analysis. All results are reported as means \pm SE. All statistical analyses were conducted using IBM[®] SPSS[®] Statistics, Version 22 (International Business Machines Corp., Armonk, NY, 10504-1722).

3.3 Results

Table 2. Summary of Yeast Substitution parameters assessed by treatment and days post stocking during the course of the trial.

	Day	Control	50% YS	75% YS	85% YS	Level of Significance
Rotifers (millions)	1	6.63 ± 0.24 ^b	6.90 ± 0.08 ^{ab}	7.57 ± 0.30 ^{ab}	7.61 ± 0.14 ^a	*
	2	10.35 ± 0.32	9.13 ± 0.59	8.93 ± 0.16	9.19 ± 0.13	NS
	3	12.79 ± 0.84	12.17 ± 0.25	11.83 ± 0.20	10.70 ± 0.36	NS
Final Growth (%)	-	155 ± 17	143 ± 5	136 ± 4	115 ± 7	NS
Final Ciliates (1=presence/0=absence)	-	1	1	1	1	
Total Vibrio (CFU rotifer ⁻¹)	2	0.07 ± 0.02	0.13 ± 0.07	0.11 ± 0.08	0.02 ± 0.01	NS
	3	0.05 ± 0.01 ^b	0.05 ± 0.03 ^b	0.68 ± 0.12 ^a	0.18 ± 0.09 ^b	**

Means in the same row having different superscripts are significantly different determined by ANOVA and Welch ANOVA ($P < 0.05$)
Significance levels are denoted by: NS (No Significance), * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$)

Total growth calculation assumes the rotifer stock of 5.00 million on Day 0

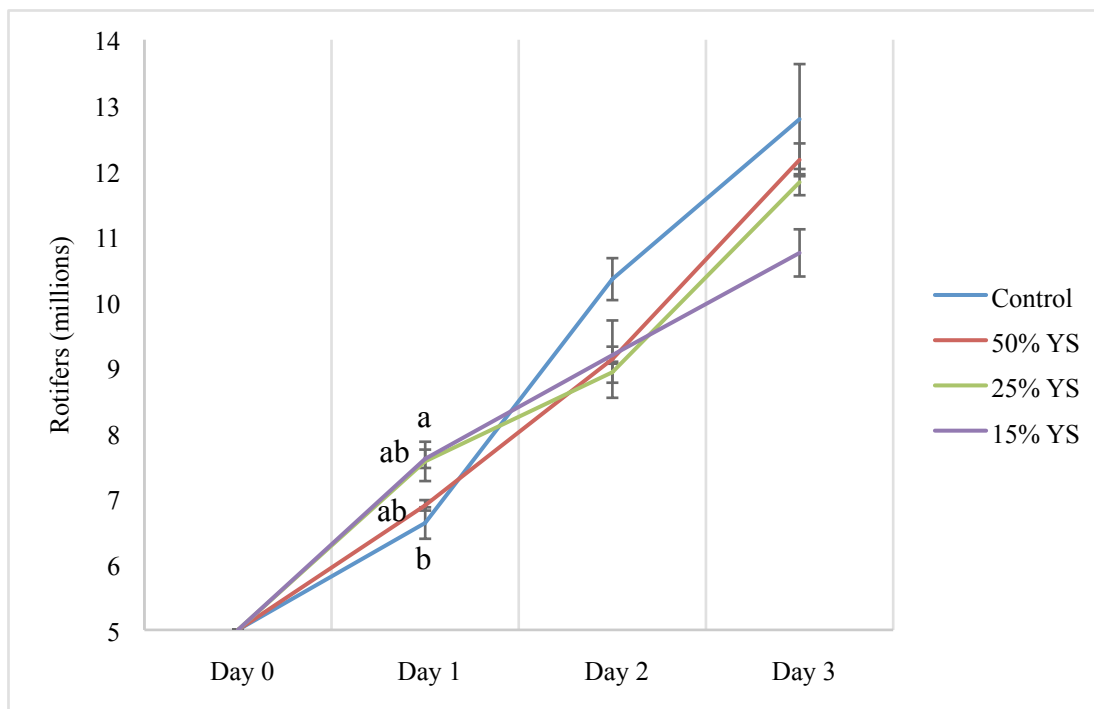


Figure 4. Population sizes (means \pm SE) for yeast substitution groups ($n = 3$) for days 0, 1, 2, and 3. Values with different letters are significantly different determined by Tukey HSD post-hoc analysis ($P < 0.05$).

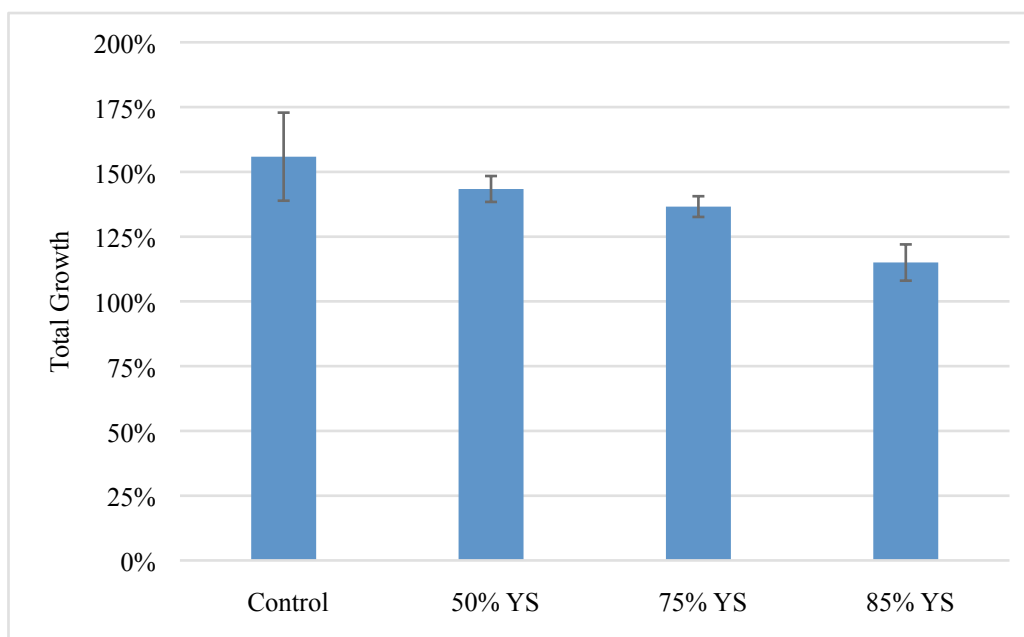


Figure 5. Total growth (means \pm SE) for yeast substitution groups ($n = 3$). Values with different letters are significantly different determined by Tukey HSD post-hoc analysis ($P < 0.05$).

On day 1, rotifer population size was significantly different between treatments, $F(3,8) = 5.498$, ($P = 0.024$). Post-hoc analysis revealed that the control (6.63 ± 0.24) was significantly different than the 85% yeast substitution diet (7.61 ± 0.14). On days 2 and 3, population size was not significantly different. Total growth was not statistically significantly different between the groups, $F(3,8) = 3.085$, ($P < .090$).

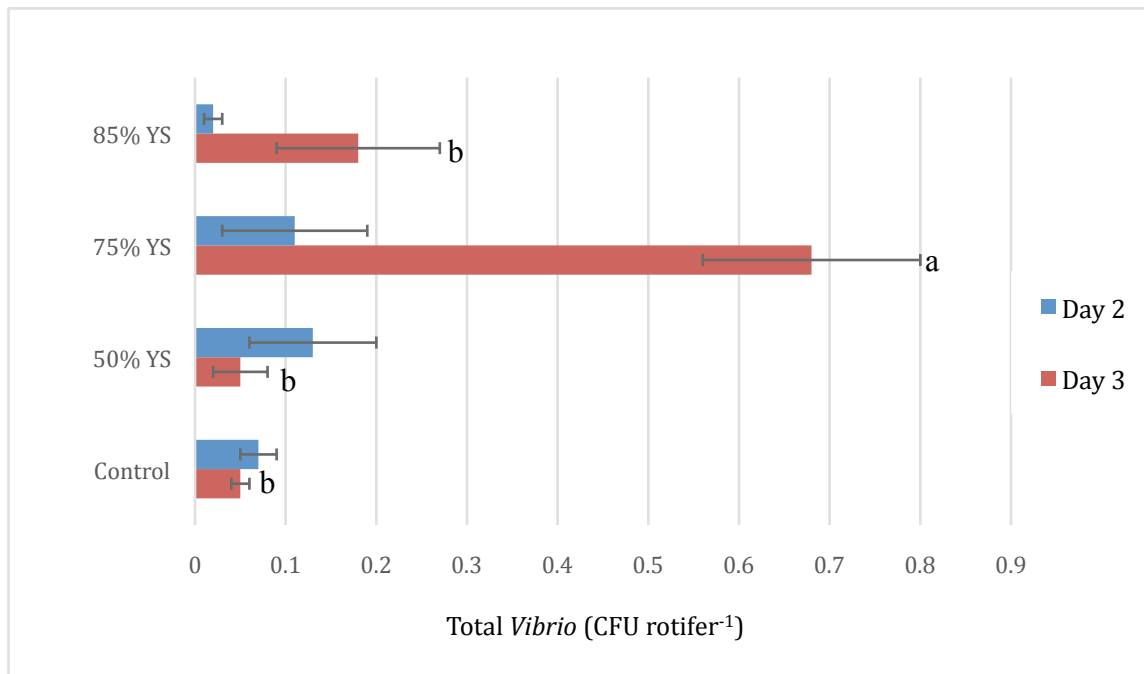


Figure 6. Population sizes (means \pm SE) for yeast substitution groups ($n = 3$) for days 0, 1, 2, and 3. Values with different letters are significantly different determined by Tukey HSD post-hoc analysis ($P < 0.05$).

No differences were found in day-2 *Vibrio* (CFU rotifer⁻¹) between treatments, $F(3,8) = 0.857$, ($P = 0.501$). Significant differences were seen in day-3 *Vibrio*, $F(3,8) = 11.631$, ($P = 0.003$). Tukey post-hoc analysis revealed that the 75% YS (0.68 ± 0.12) was significantly different than 85% YS (0.18 ± 0.09), 50% YS (0.05 ± 0.03), and the control (0.05 ± 0.01).

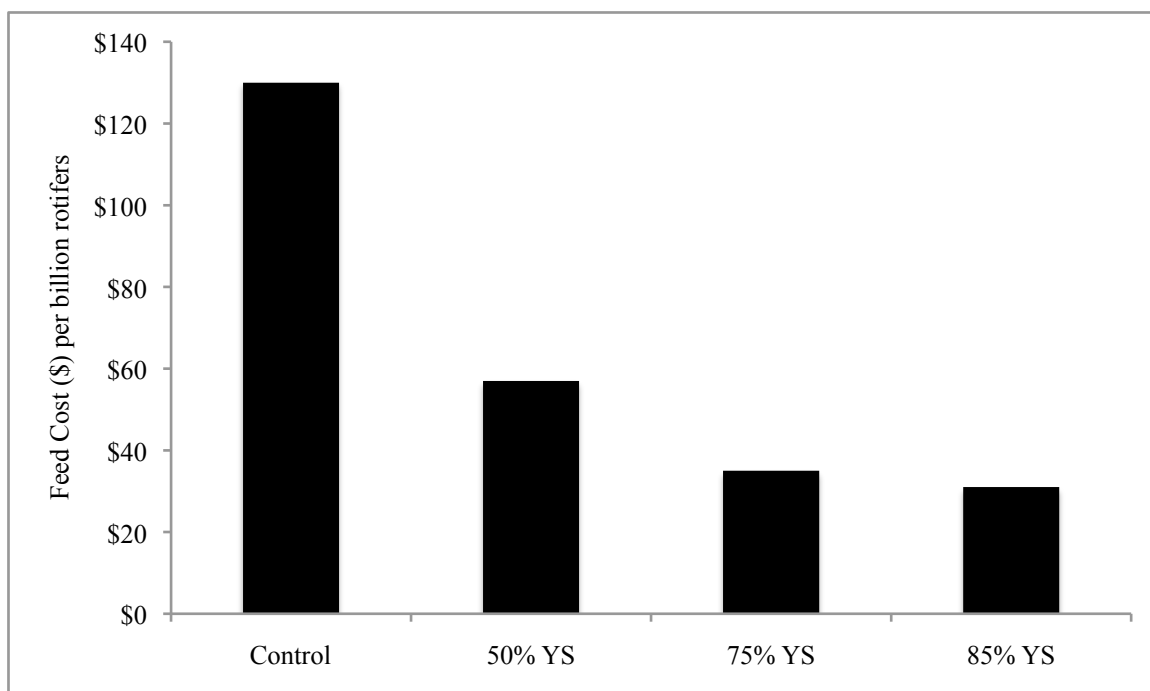


Figure 7. Feed cost (\$) per billion rotifers produced between different levels of yeast substitution.

Figure 7 illustrates the financial cost of substituting different levels of yeast for *S.parkle*. The cost of producing a billion rotifers is \$131 using *S.parkle* alone. The cost drastically decreases when yeast is substituted for *S.parkle*. At 50% yeast substitution the cost is \$57, at 75% yeast substitution the cost is \$38 and at 85% YS the cost is reduced to \$34.

3.4 Discussion

Differences in total growth were not significant between different levels of yeast substitution within high-density rotifer cultures. Total growth was observed to decrease slightly with increasing yeast, corroborating a previous study on yeast substitution at low-density rotifer cultures (Tamaru et al. 1993). On day 1, a significant difference in rotifer population sizes between treatments was shown. The 85% YS group had more rotifers than all other groups. Growth in this treatment declined in relation to all other treatments,

however, and no other statistically significant differences in growth between treatments was found after day 1. Differences in day-1 growth may be explained by the effect of *S.parkle* on recently rinsed rotifers. For example, egg percentages were observed to be higher in the *S.parkle* group on day 1 compared to the yeast substitution groups. A delayed egg release on day 2 could have been the cause of decreased relative growth on day 1 but no difference in days 2 or 3.

Ciliates were present in all cultures on days 0, 1, 2, and 3. Since some ciliates are known to feed on bacteria, presence of ciliates on all days may explain relatively stable levels of *Vibrio* compared to the previous experiment. No significant difference was found in total *Vibrio* on day 2. Similar levels of *Vibrio* were seen in Day 3, except for the 75%-yeast group, which had significantly more colony forming units per rotifer.

Overall, this study suggests that yeast can be partially substituted for *S.parkle* up to 50%, with no adverse effect on growth of *Vibrio*. No statistical differences were seen in growth or bacteria between the 50% YS group and the control, yet costs were significantly reduced. It is recommended for hatcheries producing rotifers at commercial-scale densities to consider substituting yeast for *S.parkle* up to 50%.

This yeast-substitution study should be repeated for other commercial diets, such as the Reed Rotigrow Nanno, which showed similar performance to *S.parkle* in experiment I. Future research on yeast substitution should focus on multi-generational grow-out trials to explore any long-term effects, and studies should be repeated using continuous culture systems to see if the same benefits apply to other rotifer production systems.

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