Effects of Selenium-enriched Prebiotic on the Growth Performance, Innate Immune Response, Oxidative Enzyme Activity and Microbiome of Rainbow Trout *(Oncorhynchus mykiss)*

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Abstract:

Using prebiotics as functional ingredients in aquafeed is an emerging approach to prevent disease and improve fish production. This study explored the effect of supplementing aquafeed with selenium-enriched prebiotic on the growth performance, plasma lysozyme activity, and oxidative enzymes activities of various tissues of the rainbow trout, *Oncorhynchus mykiss.* The fish scale and intestinal microbiomes were also characterized. A genetically- modified, whole-cell inactivated *Schizosaccharomyces pombe* that produces a novel form of selenium called selenoneine was used as a prebiotic supplement in this study. Selenoneine is a novel selenium-containing molecule with an antioxidant activity, suggesting it has potential to improve the production of fish when used as a functional ingredient in aquafeeds. To investigate if this selenium-enriched *S. pombe* can strengthen the production metrics, modulate the surface and internal microbiome, and improve the health status of fish, an 8-week fish feeding trial was conducted. A commercial feed was supplemented with no addition (control), 5 g Kg^{-1} (0.5%), or 10 g Kg^{-1} (1.0%) of selenium-enriched *S. pombe*. Fish were harvested at the middle (week 4) and end of the trial (week 8). There were no significant differences ($p>0.05$) in food conversion ratio, condition factor, or survival of fish during the trial. The microbiome also remained stable during the experiment. No effect ($p>0.05$) was observed in the growth performance of the fish at week 4; however, at week 8 fish that were fed 0.5% yeast had a significantly higher weight gain ($p<0.05$), hepatosomatic index, and viscerosomatic index than the fish fed control and 1.0% diets. At week 4, the concentration of plasma lysozyme in fish fed 0.5% yeast was significantly higher ($p<0.05$) than the control fish and fish fed the 1.0% diet. No significant differences ($p>0.05$) were observed in glutathione peroxidase activity in the liver, kidney, or plasma of fish fed experimental diets at the two different sampling times. However, the superoxide dismutase

activity of plasma was significantly higher with the 0.5% diet than the control and 1.0% fed fish at week 4. At week 8, both the 0.5% and 1.0% diets led to significant increases ($p<0.05$) in superoxide dismutase activity in the plasma of fish when compared to the control diet. This study demonstrates that supplementation of aquafeed with selenoneine-producing *S. pombe* can improve the growth, immune response, and oxidative status of fish. Thus, this ingredient holds a promising potential to enhance sustainability within the aquaculture industry.

Keywords: Microbiome; Oxidative enzymes; Prebiotic; Salmonid; Selenoneine

1. Introduction:

Salmonids have become a highly valuable, globally-traded food commodity. They account for about 19% of the total value of the internationally traded fish in 2018 (FAO, 2020), and contribute tremendously to the aquaculture industry. The success and sustainability of salmonid aquaculture depends on the control of disease. The health status of fish is one of the main factors affecting the level of economic return for the salmonid aquaculture industry.

Application of functional ingredients as immunostimulants is an emerging alternative to antibiotics that is increasingly being used to improve resistance against pathogens and enhance fish production. A prebiotic is a dietary supplement that can be used by the aquaculture industry to prevent disease. Prebiotics are defined as food ingredients that are not digestible by the host, but that can be fermented by natural microbiota within the intestines, which in return will change the composition and/or activity of the gastrointestinal tract (GIT) microbiota. Prebiotics can selectively stimulate the proliferation and/or activity of beneficial bacteria, resulting in the improved health and well-being of the host (Burr et al., 2008, 2010; Gibson et al., 2004). Prebiotics have also been shown to enhance the growth and immunological response in fish (Buentello et al., 2010; Zhou et al., 2010), with an increased area of intestinal absorption (Zhou et al., 2010), and improved survival after challenges against pathogens like bacteria or parasites (Buentello et al., 2010).

Selenium (Se), an essential trace element, has a fundamental role in maintaining the cellular oxidative homeostasis of fish (Watanabe et al., 1997). It has been proposed as a natural prophylactic that can support aquaculture sustainability by promoting fish health. This element is co-translationally incorporated into an amino acid, resulting in selenocysteine (Sec) (Shetty et al., 2014), which is a building block of multiple physiologically-essential proteins called

73 selenoproteins. These macromolecules are necessary for protecting DNA, other proteins, and
74 lipids against an attack from reactive oxygen species (ROS) that are produced during the normal
75 metabolism of humans and animals (Rayman, 2012; Labunsky et al., 2014).

76 Selenium maintains cellular oxidative status primarily via a selenoprotein called
77 glutathione peroxidase (GPX, EC 1.11.1.9) (Rotruck et al., 1973). In fish, four isoenzymes of
78 GPX have been identified (Kryukov & Gladyshev, 2000; Tujebajeva et al., 2000). Selenium
79 deficiency in fish can compromise their immunity and overall health. Salmonids, in particular,
80 require higher Se content in their diet due to the elevated levels of polyunsaturated fatty acids
81 (PUFAs) in the fillets that are prone to lipid peroxidation (Winston & Di Giulio, 1991). Thus,
82 selenium deficiency in salmonids has been shown to cause lipid peroxidation, ataxia, muscular
83 dystrophy, and decreased plasma and hepatic GPX activity (Bell et al., 1986; Bell et al., 1987;
84 Poston et al., 1976; Hodson et al.,1983).

85 A previous study has shown that fish fed a commercial diet require additional Se for the
86 maintenance of optimal oxidative status (Kucukbay et al., 2009). The Se content in tissues of
87 farm-raised rainbow trout has been shown to be significantly lower than their wild counterparts
88 (Poppe et al., 1985; Felton et al., 1990). This might be due to the lower bioavailability of Se in
89 the commercial diet and the nature of intensive fish production systems that can cause chronic
90 stresses (Telli et al., 2014), leading to increased demand for oxidative enzymes and Se
91 utilization. Therefore, Se supplementation is necessary for maintaining the optimal health and
92 growth of farm-raised fish.

93 Selenium exists in multiple forms that can affect its absorption, bioaccumulation, and
94 bioavailability. Selenoneine is a novel selenium-containing imidazole compound (2-selenyl-
95 Nα, Nα, Nα-trimethyl-L-histidine) that has been found in the blood and muscle of tuna

(Yamashita et al., 2010). Strong antioxidant activity by selenoneine has been observed during *in vivo* and *in vitro* studies making it an interesting molecule for use as a dietary supplement (Yamashita et al., 2013; Yamashita et al., 2015). There is a wide body of knowledge in the literature reporting the effects of selenium-producing yeast, containing the selenomethionine form of Se, on fish physiology. However, the work described here is a novel study in which selenoneine-producing *Schizosaccharomyces pombe* is used as a dietary supplement in aquafeed. A Se-enriched prebiotic combines the virtues of a prebiotic with those of organic Se, and can thereby exert dual effects simultaneously. Therefore, a genetically modified *S. pombe* that synthesizes selenoneine when the culture medium is supplemented with sodium selenate (Pluskal et al., 2014, Achouba et al., 2019) was used as a selenium-enriched, whole-cell inactivated yeast prebiotic supplement.

The effect of selenoneine-producing *S. pombe* on fish was evaluated focusing on the production and health status of rainbow trout, which was used as a model species for salmonid farming. The effect on fish growth was evaluated by comparing the feed conversion ratio (FCR), weight gain, survival, hepatosomatic index (HSI) and viscerosamatic index (VSI), conditioning factors (CF), and microbiome of fish fed the different experimental diets. Additionally, the study sought to assess the effect of the prebiotic on the oxidative status and the innate immune response of fish in the different experimental groups.

2. Materials and Methods:

2.1. Culture of selenoneine-producing *S. pombe*

Genetically-modified *S. pombe* was purchased from the Yeast Resource Center of Osaka City University (Yeast strain FY25320, NPRP/YGRC, Osaka, Japan) and grown in Edinburgh

minimal medium (EMM2, Fisher Scientific, Hampton, NH, USA) supplemented with 10 μM sodium selenate (Sigma Aldrich, St. Louis, USA). Culture conditions were 26°C, pH = 5.8, and 300 rpm for 48 hours, using a 2 L BioFlo® 120 Bioprocess control station fermenter (Eppendorf, Hauppauge, NY, USA). After 48 hours, the cells were harvested by centrifugation (10,000 g for 15 min) and then washed with deionized (dH_2O) water and stored at –80°C. Proximate composition of the lyophilized yeast powder was analyzed using a commercial service (Midwest lab, Omaha, NE, USA). Inductively coupled mass spectroscopy (ICP-MS) (Agilent 7900) was used to measure the Se in the yeast cell powder.

2.2. Diet preparation

Lyophilized yeast powder was mixed with 100 mL of deionized water for 1 minute. The mixture was spray coated on the commercial feed (3 mm Finfish Gold, Zeigler, Gardner, PA, USA). The prepared feed was dried for 48 hours at room temperature. Diets were stored in a commercial refrigerator at a temperature that ranged between 0 and 3.5 ºC until they were used. All diets were analyzed to confirm the proximate nutritional value and minerals using a commercial service (Midwest lab, Omaha, NE, USA) (Table 1).

2.3. Feeding trial experimental design

All animal procedures were approved by Virginia Tech's Institute of Animal Care and Use Committee (VT-IACUC-#16–099). An 8-week growth trial was conducted to evaluate the effect of selenoneine-enriched *S. pombe* on rainbow trout production and health. One hundred fifty fish were obtained from a commercial fish farm (Goshen, VA, USA) and were acclimated to laboratory conditions for 6 weeks. Fish with an average weight of 52.4 ± 0.28 g, (mean ±

142 standard error [SEM]) were randomly stocked into 15 polyethylene tanks (170 L each) in a
143 single recirculating aquaculture system (RAS) at 10 fish per tank. The RAS was equipped with
144 bead and sand filters for mechanical filtration, a bioreactor for nitrification, UV disinfection
145 units, a commercial chiller, and distributed diffuse aeration.

146 During the trial, fish were weighed weekly on a per tank basis. The amount of feed was
147 adjusted based on the weight gain, and feed rates were kept consistent between the three
148 treatment groups. The feeding rate was on a percent body weight per day basis. Fish were hand-
149 fed twice daily with a minimum of 4 hours between each feeding. Water quality parameters were
150 analyzed using methods adapted from HACH (AOCS, 2010) and the American Public Health
151 Association (APHA) (2012). Dissolved oxygen (DO) and temperature were measured using a
152 meter with a DO probe (YSI ProDO, Cole-Parmer, IL, USA) daily. Alkalinity was also measured
153 daily using a titration method. Nitrite, nitrate, total ammonia nitrogen, and pH were measured
154 three times a week.

155

156 **2.4. Sampling**

157 Fish were sampled midtrial (week 4) and at the end of the trial (week 8). On both
158 sampling days, 15 fish from each diet were arbitrarily sampled. Individual fish were humanely
159 euthanized using 250 mg L^{-1} tricaine methanesulfonate solution (MS 222, Western Chemical,
160 Ferndale, WI, USA) buffered with sodium bicarbonate. The weight and length of each fish were
161 measured and recorded before necropsy. On each harvest day, nine total fish from each diet were
162 used for microbiome sampling. Immediately after capturing each fish, 50 mL of sterile dH_2O
163 was flooded over the left side, posterior end of each fish to remove transient microorganisms. A
164 sterile swab (Fisher brand, Pittsburgh, PA) was then used to harvest scale-associated bacteria

over a 4 cm^2 area between the caudal and dorsal fins, after which, the fish were humanely euthanized in a buffered MS-222 water bath. Each intestinal tissue sample was harvested by removal of a ~10 cm segment of the intestinal tract (mainly midgut tissue) using flame-sterilized dissecting tools. All tissue samples were flash-frozen in a dry ice bath and subsequently stored at -20°C.

2.4.1. Plasma collection

Blood was immediately collected postmortem from the caudal vessels of each fish using a 1 mL syringe and 23-gauge needle. Blood samples were placed in ethylenediaminetetraacetic acid (EDTA)-coated tubes and centrifuged for 15 minutes at 10,000 x *g*. After centrifugation, plasma samples were collected and stored at –80°C until analysis. Lysozyme, superoxide dismutase (SOD), and GPX activities were measured in each individual plasma sample.

2.4.2. Liver and kidney collection

Liver and kidney samples were collected for analysis of GPX activity within the tissue. Liver and total viscera weights (g) were measured individually. Tissues were flash-frozen in sample tubes/containers in an isopropanol-dry ice bath and stored at –80ºC immediately after collection.

2.5. Growth performance of fish

Production variables, survival rates (%), FCR, and weight gain were used to compare fish production between the three treatment groups. Survival was assessed daily. Weight gain and FCR were measured weekly. The HSI, VSI, and CF were assessed for each treatment group at

188 the middle and end of the feeding trial. Growth and nutritional indices were calculated as
189 follows:
190
191 CF = 100 × body weight (g)/length^3 (cm^3); FCR = feed intake (g)/fish weight gain (g); HSI = 100
192 × liver weight (g)/body weight (g); VSI = 100 × viscera weight (g)/body weight (g).
193
194 **2.6. Lysozyme concentration**
195 A commercial kit (EnzChek™ Lysozyme Assay Kit, Thermofisher Scientific, Waltham,
196 MA, USA) was used for measuring lysozyme activity. This kit measures lysozyme activity based
197 on a novel method, which uses the fluorophore fluorescein incorporated in *Micrococcus*
198 *lysodeikticus* cell walls, which quenches the fluorescent signal. Active lysozyme hydrolyzes the
199 ß-(1-4)-glycosidic linkages between the N-acetylmuramic acid and N-acetyl-D-glucosamine
200 residues in the mucopolysaccharide cell wall, relieving the cell wall-mediated signal quenching
201 and yielding a dramatic increase in fluorescence that is proportional to lysozyme activity.
202
203 **2.7. Superoxide dismutase activity**
204 Superoxide dismutase (SOD) activity was measured using a commercial kit (Sigma
205 Aldrich, St. Louis, USA), which uses nitro blue tetrazolium (NBT). Absorption at 440 nm is
206 proportional to the amount of superoxide anion, and the SOD activity can be quantified by
207 measuring the decreases in color development at 440 nm. The rate of change in A_{440} was
208 directly proportional to the SOD activity of the sample.
209
210 **2.8. Total GPX activity measurement**

211 Liver and plasma GPX activity were measured using a commercially available kit
212 (Cayman Chemical, Ann Arbor, MI, USA). This assay measured the decrease in absorbance at
213 340 nm (A_{340}) as the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)
214 was oxidized to $NADP^+$. The rate of change in A_{340} was directly proportional to the GPX activity
215 of the sample. Before analysis, liver samples were homogenized in 10 mL of cold buffer (50 mM
216 Tris HCl, pH 7.5, 5 mM EDTA and 1 mM dithiothreitol (DTT)) per gram of tissue.
217 Homogenized samples were centrifuged for 15 minutes at 10,000 x *g*. The supernatant was then
218 collected and protein concentrations of samples were measured using a bicinchoninic acid (BCA)
219 assay kit (Fisher Scientific, UK). Plasma samples were diluted 1:2 with cold buffer (50 mM Tris-
220 HCl, pH 7.6, containing 5 mM EDTA and 1 mg ml^{-1} of bovine serum albumin (BSA)) prior to
221 analysis.
222
223 **2.9. Microwave digestion**
224 Yeast powder and feed samples were digested using a modified method adopted from
225 Olmedo et al. (2013). Briefly, 0.5 g of each sample was weighed in MARS 5 microwave Teflon
226 vessels (CEM Corporation, Matthews, NC, USA) in duplicate, that had been subjected to an anti-
227 static gun. Samples were digested using 8 mL of 35 % (w/w) nitric acid and 2 mL of 30 %
228 hydrogen peroxide (w/w). Before digestion, the vessels were briefly swirled to mix and allowed
229 to sit for approximately 10 minutes. The microwave program performed a ramp to 180°C over
230 20 minutes and then held that temperature for 10 minutes before cooling to 50°C. After
231 digestion, the samples were transferred to polypropylene (PP) tubes and the vessels were
232 quantitatively rinsed using 2% (w/w) nitric acid.
233

234 **2.10. Inductively Coupled Plasma Mass Spectroscopy (ICP-MS)**

235 The freeze-dried digested samples were separately diluted twice with 2% (w/w) nitric
236 acid prior to analysis. The dilutions were necessary to reduce contamination as well as the
237 concentrations of acids going onto the instrument and to allow the analyte concentrations to fall
238 within the calibration range. Sample acquisition was performed on an Agilent 7900 ICP-MS
239 (Agilent, Santa Clara, CA, USA) using a custom method (Ed McCurdy & Potter, 2006) that
240 utilized the helium mode octopole reaction system (ORS) to reduce and/or remove potential
241 matrix interferences, while simultaneously maintaining the lower detection limits needed for the
242 analysis. A certified reference material (DORM-4, National Research Council of Canada) was
243 digested and analyzed along with the samples to ensure proper digestion and analysis.
244

245 **2.11. DNA Extraction, Sequencing and Bioinformatics Analysis**

246 Total genomic DNA (gDNA) was extracted from fish tissue samples using the Qiagen
247 PowerSoil kit (Germantown, MD) with the following procedural alterations. Frozen intestinal
248 samples were thawed on ice and homogenized with an OmniTip homogenizer (Omni
249 International, Kennesaw, GA). Addition of intestinal tissue samples involved addition of 10–20
250 mg of intestinal homogenate or the cotton swab tips, containing scale samples, directly to the
251 PowerBead tubes. Samples were incubated with pre-warmed C1 buffer at 70°C for 10 minutes
252 prior to vortex mixing. gDNA was eluted in 50 μL sterile dH_2O following a 5-minute 70°C
253 incubation.

254 PCR reactions consisting of gDNA as a template with universal primers were used to
255 amplify the V4 region of the bacterial 16S rRNA gene for comparative bioinformatics analysis
256 (Caporaso et al., 2011). Samples were amplified in triplicate reactions using the following

reaction conditions: 10 μL of 2.5X 5 Prime HotStart master mix (Quantabio, Beverly, MA), 200 nM (final concentration) each of forward primer (515f;AATGATACGGCGACCACCGAGATCTACACGCTxxxxxxxxxxxxTATGGTAATTGT GTGYCAGCMGCCGCGGTAA, with the x region representing the bar code) and reverse primer (806r;CAAGCAGAAGACGGCATACGAGATAGTCAGCCAGCCGGACTACNVGGGTWTC TAAT), 13 ng μl^{-1} (final concentration) gDNA was used as the template, and sterile dH_2O used to bring the total reaction volume to 25 μL. The following program settings for the thermocycler (Thermo Scientific, Waltham, MA) were used: initial denaturation for 2 min at 94°C; 35 cycles with denaturation for 45 sec at 94°C, annealing for 1 min at 50°C, and elongation for 30 sec at 68°C; final elongation for 10 min at 68°C. Triplicate reactions were then pooled together and analyzed on a 1% agarose gel. The band encompassing the V4 region amplicons was extracted from the gel and purified using the Qiagen gel extraction kit. Gel purified samples were sent to the Virginia Tech Biocomplexity Institute (BI) for Qubit fluorometry quality analysis. Samples that returned with DNA concentrations higher than 0.8 nM (n=6 for each treatment group) were pooled together, diluted to 9.5 pM (based on qPCR with 20% phiX spiking), and 250 bp paired-end Illumina MiSeq sequencing was done. Sequences were uploaded to the NCBI Sequence Read Archive under the accession number PRJNA577276.

The Quantitative Insights Into Microbial Ecology (QIIME 2) (Bolyen et al., 2018) pipeline was used to analyze microbial communities from the sequencing data. Briefly, the default parameters of DADA2 were used to remove low-quality reads and artifactual sequences. Resulting sequences were clustered together at 97% identity using the VSEARCH program (Rognes et al., 2016) to create operational taxonomic units (OTU). Taxonomy of the OTUs was

assigned by OTU alignment to the SILVA database (Yilmaz et al., 2014) and further filtered to remove contaminating host sequences (i.e., from mitochondria). Filtered sequences and taxonomic information were rarefied to 8560 reads per sample to enable sample comparisons. Rarefied sequences were used as inputs to construct the family-level relative abundance plot and analyze diversity within the R program (Bisanz, 2018; McMurdie and Holmes, 2013; Oksanen et al., 2019; R Core Team, 2019; Wickham, 2016; Wickham, 2017; Wickham et al., 2019). Alpha diversities were calculated in QIIME 2 using the Shannon diversity metric. Principal Coordinates of Analysis (PCoA) beta diversity plots were created in R using weighted UniFrac dissimilarity calculated in QIIME 2.

2.12. Data analysis

Statistical analysis was performed using GraphPad prism and JMP Pro 13 for Apple (Cary, NC, USA). One-way ANOVA was utilized when more than two means were compared to determine dietary effects on dependent variables (fish performance, biometrics, and activities of lysozyme and oxidative enzymes). When appropriate, Tukey's post-hoc test was applied to determine where the significant ($p<0.05$) differences occurred amongst the means. Data from each sampling point were compared to each other (i.e., week 4 data were compared to week 4 data, week 8 data were compared to week 8).

3. Results

3.1. Feed and *S. pombe* proximate and mineral composition:

The proximate and mineral compositions of the prebiotic yeast and experimental diets are summarized in Table 1 and 2, respectively.

303

304 **3.2. Water quality**

305 Water quality parameters that were monitored during the trial are presented in Table 3.

306

307 **3.3. Growth parameter and biometrics**

308 Data for growth performance and biometrics are shown in Table 4. There were no
309 significant differences for FCR, weight gain, HSI, VSI, survival, or CF between fish fed the
310 different diets at four weeks. At week eight, fish fed 0.5% diet had significantly ($p<0.05$) higher
311 weight gain, HSI and, VSI in comparison to fish fed the control and 1.0% diet. There were no
312 significant differences in the survival, FCR, or CF of fish fed different experimental diets during
313 any observation of the trial.

314

315 **3.4. Plasma lysozyme activity**

316 Fish fed the control diet had significantly higher ($p<0.05$*)* lysozyme activity than fish fed
317 the 1.0% diet at week 4. However, at week 8, lysozyme activity in the plasma of fish from all
318 treatments was lower than week 4. Furthermore, no significant differences in plasma lysozyme
319 activity of fish from any treatment group were observed at week 8 (Figure 1).

320

321 **3.5. Superoxide dismutase activity**

322 The SOD activity in the plasma of fish fed the 0.5% diet was significantly higher than
323 fish fed control and 1.0% diets at week 4. Fish fed the 1% diet had intermediate SOD activity
324 levels similar to those of both the control fish and the fish fed the 0.5% diet. At week 8, both
325 diets enriched with *S. pombe* had significantly higher ($p<0.05$) SOD levels in the plasma than the

326 control diet. Meanwhile, no significant differences were observed at either timepoint regarding
327 plasma SOD activity between fish fed the 0.5% or 1.0% diets at week 8 (Figure 2).
328
329 **3.6. Glutathione peroxidase activity**
330 There were no significant differences in the GPX activity of the plasma, liver or kidney
331 from fish fed the experimental diets at week 4 or week 8.
332
333 **3.7. Microbiome analysis**
334 Analysis of the scale-associated microbiomes revealed similar taxa between the treatment
335 groups. The treatment groups shared a similar pattern of dominance of the top 10 families
336 (Figure 4) at both harvest time points as well as similar alpha and beta diversity values (data not
337 shown).
338 The dietary supplementation with selenium also had minimal effect on the intestinal
339 epithelial-associated microbiomes. Unlike the scale-associated microbiome, the microbiome
340 associated with the intestinal epithelium had near-complete dominance of *Mycoplasmataceae*
341 (Figure 5) with similar alpha and beta diversity values between different groups (data not
342 shown).
343
344 **4. Discussion**
345 Optimal supplementation of aquafeed with micronutrients and functional ingredients such
346 as Se and prebiotics is necessary to support the health and growth of fish, and hence sustainable
347 production of seafood. The present study investigated the effect of different levels of
348 selenoneine-producing, whole-cell inactivated *S. pombe* as a prebiotic supplement on the growth,

349 enzymatic activities of lysozyme and two of the most important oxidative status markers (SOD
350 and GPX), and composition of the external and internal microbiomes of rainbow trout. While Se
351 deficiency can result in growth depression and increased mortality, high levels of Se can also
352 have toxic effects resulting in reduced growth and FCR (Bell et al., 1987; Kim et al., 2003).
353 Thus, there is a fine line between deficiency and toxicity, requiring that the optimal
354 concentration of supplement per fish species must be established.

355 The selenium requirement of fish, based on species and life stage of the fish, is between
356 0.1 and 4 mg Kg^{-1} (dry mass). In this study, diets were supplemented with 5 (0.5%) or 10 (1.0%)
357 g Kg^{-1} *S. pombe* yeast to achieve dietary Se concentrations ranging from approximately 1 to 4
358 mg Kg^{-1} (Table 2). This range of Se concentration has been shown to be beneficial for rainbow
359 trout without any toxic effects (Pacitti et al., 2016). The United States Food and Drug
360 Administration (FDA) allows the inclusion of organic Se as high as 3 to 5 mg Kg^{-1} in animal
361 feed. Therefore, Se-enriched *S. pombe* was included in the diet at prebiotic levels of 0.5% and
362 1.0% to keep the Se concentrations of the diet within the FDA limit and within the range that has
363 been shown to be beneficial for rainbow trout. However, the Se concentration in the control diet
364 was higher than the European food legislation upper limit set at 0.5 mg Kg^{-1} (88% dry matter) of
365 organic Se in fish feed (Aquilina et al., 2016).

366 Supplementation of aquafeed with selenoneine-producing *S. pombe* is novel in salmonid
367 feeds. Results of previous studies investigating the impact of aquafeed supplementation with
368 various species of Se on fish growth are contradictory. Pacitti et al. (2016) demonstrated no
369 effect on the growth of rainbow trout after the supplementation of feed with increasing
370 concentrations of selenomethionine-rich yeast. Contrary to this, the supplementation of a
371 rainbow trout diet with 3 mg Se kg^{-1} in the form of selenomethionine was shown to increase

372 rainbow trout growth significantly. Similarly, Se supplementation of feed for channel catfish,
373 *Ictalurus punctatus* (Gatlin and Wilson, 1984), *Cyprinus carpio* (Gaber, 2008), and *Carassius*
374 *auratus gibelio* (Zhou et al., 2009) was associated with significant increases in fish weight gains.
375 The discrepancy in the results of studies focused on the effect of Se supplementation on fish
376 growth might stem from the variety of factors that affect Se metabolism: digestibility,
377 bioavailability, and the fine threshold between toxic and beneficial doses of selenium.
378 Bioavailability of Se depends on many factors such as the form and concentration of Se, and the
379 species and life stage of fish. In this study, supplementation of feed with 0.5 % *S. pombe*, used to
380 supply selenium-enriched prebiotic, improved the weight gain of fish, whereas supplementation
381 with 1.0% *S. pombe* did not have any effect on weight gain.

382 In addition, a significant increase ($p<0.05$) was observed in the HSI of the fish fed 0.5%
383 selenium-enriched prebiotic in comparison to the control and 1.0% diets. The HSI is a very good
384 indicator of total energy reserves and fish condition (Lambert and Dutil, 2000). Increased HSI in
385 this study presumably has some relation to the fish condition and feeding activity, playing a role
386 in nutrient transfer between the different organs. In particular, lipids of dietary origin that are not
387 used immediately could be transferred from the liver to the muscle and viscera and may later be
388 used in reproduction. Interestingly, an increase in the HSI has been observed when the fish were
389 exposed to contaminants such as high heavy metal concentrations (Lambert and Dutil 2000). In
390 this study, a significant increase in the HSI was observed in the fish that were supplemented with
391 the lowest level of S. *pombe*. Therefore, increased HSI in this study is probably an indicator of
392 good energy reserve and nutrient transfer. A significant increase in weight gain in the same
393 group of fish was consistent with this conclusion as well.

Lysozyme is constitutively expressed, synthesized and secreted by neutrophils, monocytes, and macrophages and is considered as one of the most important bactericidal enzymes (Klüter et al., 2014). Lysozyme is an indispensable tool for fish to fight infectious agents. Fish fed a diet with 0.5% *S. pombe* had significantly ($p<0.05$) higher lysozyme activity in the plasma than fish fed the control and 1.0% *S. pombe* diets 4 weeks after the beginning of the trial, indicating this dietary supplement can potentially improve this aspect of animal health.

Two critical enzymes, SOD and GPX, are important aspects of the innate immune system in fish and are responsible for detoxification of harmful reactive oxygen species that are produced during normal metabolism. Superoxide dismutase reduces the superoxide anion (O_2^-) to hydrogen peroxide (Dörr et al., 2008). Glutathione peroxidase is one of the most studied Se-containing enzymes. It serves to protect the cell membranes against oxidative damage by catalyzing the conversion of hydrogen peroxide and fatty acid hydroperoxides to water and fatty acid (Rotruck et al., 1973). Glutathione peroxidase is also measured to evaluate the bioactive status of Se in humans and animal tissues. There were no significant differences in hepatic, plasma, or kidney GPX activities of fish fed the different experimental diets, indicating all three diets contained sufficient Se to meet the cellular needs for maximizing GPX activity. However, there was an increasing trend in activity over time in the liver and plasma of fish fed the 1.0% *S. pombe* diets compared to the control diet. Supplementation of the diet with 0.5% *S. pombe* led to a significant increase in SOD activity in the plasma of fish at 4 weeks. At week 8, both diets supplemented with *S. pombe* showed significantly higher SOD activity in the plasma. These results indicate the beneficial effect of selenoneine-producing *S. pombe* dietary supplement for sustaining and improving the oxidative status of rainbow trout.

Dietary supplementation with selenoneine-producing *S. pombe* does not appear to affect the external microbiome of trout (Figure 4). The 4-week period between samples also appears to have little to no effect on the microbiomes. Instead, alpha and beta diversity analyses indicated highly similar, diverse microbiomes (data not shown). Dominant taxa within these microbiomes are known environmental- and host-associated microorganisms (Pohlner et al., 2019; Teixeira, 2014; Santos, 2014). The microbiome associated with the intestinal tract also appears to remain largely unaltered with supplementation of selenoneine-producing *S. pombe* and over time between harvests (Figure 5). The intestinal microbiomes are very similar as indicated by the alpha and beta diversity analyses (data not shown); however, the intestinal microbiomes were not as diverse as the scale-associated microbiomes. Further investigation into the intestinal microbial community reveals the near-complete dominance of organisms from the *Mycoplasma* genus. Though this organism does not appear to be pathogenic in this system or other fish microbiome studies (Bano et al., 2007; Llewellyn et al., 2016), its function in fish intestines is not known. Apart from the *Mycoplasma* dominance, other taxa present within the top 10 families appear to vary little between timepoints in relative abundance between the fish fed the 0.5% and 1.0% diets.

5. Conclusions

There is a growing interest in improving the sustainable production of aquaculture industry products using natural dietary supplements. In this study, we used a yeast engineered to produce selenoneine, a novel Se-containing molecule, to evaluate its potential as a functional dietary additive in aquafeed. Supplementation of rainbow trout diet with 5 g Kg^{-1} selenoneine-enriched yeast improved the weight gain and oxidative enzyme status of the fish. Increases were

439 also observed in the plasma lysozyme activity. The microbiomes associated with the scales and
440 intestinal tract remained largely unaltered with supplementation of selenoneine over the two time
441 points examined. These results suggest value in using selenium-enriched *S. pombe* as a
442 promising dietary prebiotic for the aquaculture industry.
443

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630

631 **Figures Legend:**

632

633 **Figure 1**. Lysozyme activity of plasma of rainbow trout (*Oncorhynchus mykiss*) fed different
634 experimental diets. Week 4 results (left) and week 8 results (right) are depicted. Bars denoted
635 with different letters are significantly different ($p < 0.05$). Data is presented as the mean ±
636 standard error. Six samples per diet were evaluated in duplicate.

637

638 **Figure 2**. Superoxide dismutase activity levels (%) of plasma of rainbow trout (*Oncorhynchus*
639 *mykiss*). Week 4 results (left) and week 8 results (right) are depicted. Bars denoted with different
640 letters are significantly different ($p<0.05$). Data is presented as the mean ± standard error. Six
641 samples per diet were evaluated in duplicate.

642

643 **Figure 3**. Glutathione peroxidase activity of rainbow trout (*Oncorhynchus mykiss*) in (a) liver,
644 (b) kidney and (c) plasma of fish at week 4 (left) and 8 (right) after the trial began. Unit (U) is
645 nano moles of NADPH oxidized per milligram of total protein per minute. There were no
646 significant differences ($p>0.05$) in the liver, hepatic, or plasma GPX activity. Data are
647 represented as the mean ± standard error. Six samples per diet were evaluated in duplicate.

648

649 **Figure 4.** Scale-associated microbiome. The reads produced from sequencing the 16S rRNA
650 gene V4 region extracted from six fish per diet were averaged for the two harvests (Week 4 and
651 Week 8). The relative abundance of the top 10 OTUs as represented by the assigned bacterial
652 families are plotted. Dietary treatments include commercial feed control (Control), feed with

653 0.5% *S. pombe* (0.5%), and feed with 1.0% *S. pombe* (1.0%). Yeast was top coated onto the
654 commercial feed as a percentage of the total feed weight.
655
656 **Figure 5.** Intestinal epithelial-associated microbiome. The reads produced from sequencing the
657 16S rRNA gene V4 region extracted from six fish per diet were averaged for the two harvests
658 (Week 4 and Week 8). The relative abundance of the top 10 OTUs as represented by the assigned
659 bacterial families are plotted. Dietary treatments include commercial feed control (Control), feed
660 0.5% *S. pombe*, and feed with 1.0% *S. pombe*. Yeast was top coated onto the commercial feed as
661 a percentage of the total feed weight.

662 **Table 1.** Proximate and mineral composition (as-is) of selenoneine producing *S. pombe*.

Proximate level (%)

Crude Protein	34.0
Carbohydrate*	50. 9
Ash	5.56
Total Fat	3.45
Crude Fiber	n.d.
Moisture	6.01
Trace mineral level (mg kg-1)	
Iron	34.1
Copper	5.90
Zinc	44.8
Manganese	2.10
Selenium	264

663 n.d.: not detected

664 *Calculated value (Merrill and Watt, 1973): Carbohydrate = total - (ash + crude protein +
665 moisture + total fat)

Table 2. Proximate and mineral composition of experimental diets (as-is) for rainbow trout *(Oncorhynchus mykiss)*.

Proximate level (%)	**Control/commercial**	**0.5%**	**1.0%**
Crude Protein	43.1	41.8	42.4
Carbohydrate*	23.5	24.9	22.4
Ash	7.68	7.3	7.2
Total Fat	16.5	16.4	17.8
Crude Fiber	1.73	0.7	1.2
Moisture	7.51	8.9	9.04
Trace mineral level (mg kg^{-1})			
Iron	273	282	310
Copper	50.8	55.4	57.4
Zinc	108	114	112
Manganese	75.8	81.1	82.1
Selenium	1.10	2.50	3.91

*Calculated value (Merrill and Watt, 1973): Carbohydrate = total - (ash + crude protein + moisture + total fat)

673 **Table 3**. Water quality results for the RAS system during the 8-week trial.

Water quality parameters	
Dissolved Oxygen (mg L^{-1}), n=56	7.8 ± 0.30
Alkalinity (mg L^{-1} as $CaCO_3$), n=56	124 ± 12.11
Temperature (°C), n=56	17.1 ± 0.78
Nitrate-N(mg L^{-1}), n=8	7.30 ± 3.49
Nitrite-N (mg L^{-1}), n=33	0.02 ± 0.03
pH, n=33	7.61 ±0.18
Total ammonia-N(mg L^{-1}), n=11	0.05 ± 0.03

674

675 Number of sampling events is denoted by n.

676 Data are mean ± SEM

Table 4. Growth performance and biometrics of rainbow trout *(Oncorhynchus mykiss)* at week 4 and week 8 of the trial.

	Control	**0.5%**	**1.0%**
Initial Weight (g)	53.5 ± 0.30	52.9 ± 0.46	52.2 ± 1.02
Weight (Wt.) Week 4 (g)	74.5 ± 2.01	75.8 ± 0.58	73.4 ± 2.50
Wt. Week 8 (g)	115.4 ± 6.61[a]	122.9 ± 4.62[b]	115.8 ± 2.23[a]
Wt. Gain Week 1-4 (g)	23.9 ± 2.63	21.7 ± 1.38	21.2 ± 1.58
Wt. Gain Week 4-8 (g)	47.0 ± 2.94[a]	57.0 ± 3.68 [b]	48.8 ± 3.29 [a]
HSI Week 4	1.7 ± 0.12	1.8 ± 0.11	1.9 ± 0.13
HSI Week 8	1.7 ± 0.10 [a]	3.1± 0.07[b]	1.4 ± 0.07 [a]
VSI Week 4	9.3 ± 0.38	8.9 ± 0.30	9.0 ± 0.33
VSI Week 8	9.5 ± 0.40[a]	11.1 ± 0.29[b]	9.5 ± 0.61 [a]
CF Week 4	1.7 ± 0.02	1.7 ± 0.03	1.7 ± 0.04
CF Week 8	1.6 ± 0.13	1.4 ± 0.02	1.3 ± 0.03
FCR Week 1-4	1.4 ± 0.13	1.5 ± 0.15	1.5 ± 0.13
FCR Week 4-8	1.0 ± 0.27	0.9 ± 0.08	0.7 ± 0.10
Overall FCR	1.2 ± 0.4	1.3 ± 0.1	1.2 ± 0.11
Survival (%)	98	92	98

Data are mean ± SEM

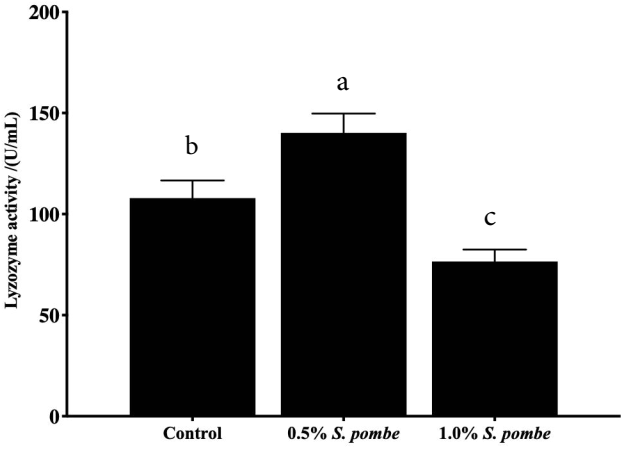
Means with different letters were significantly different ($p<0.05$)

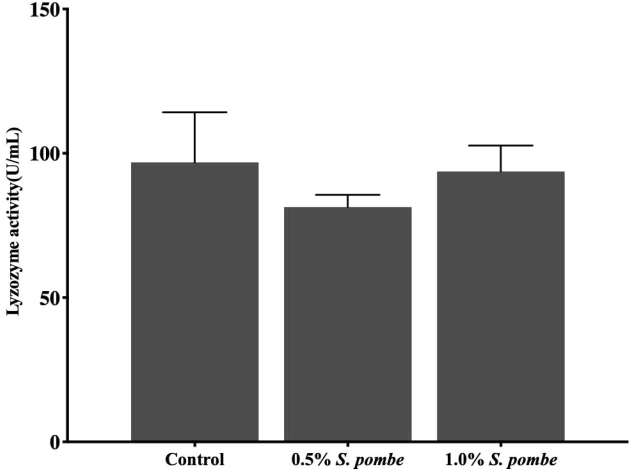
HSI: hepatosomatic index

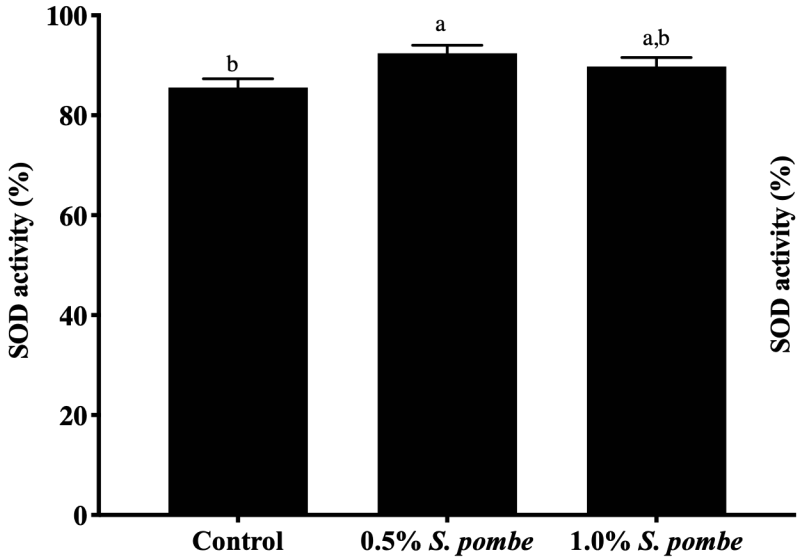
VSI: viscerosomatic Index

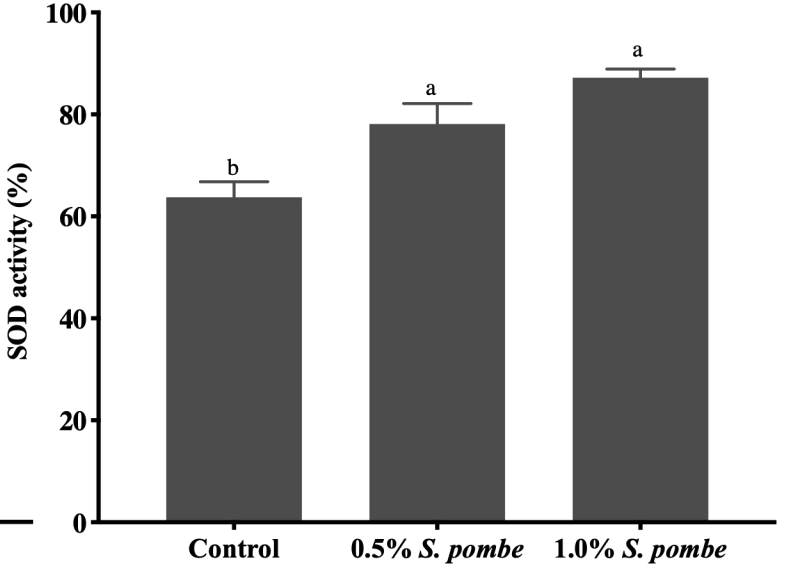
CF: conditioning factor

FCR: food conversion ratio

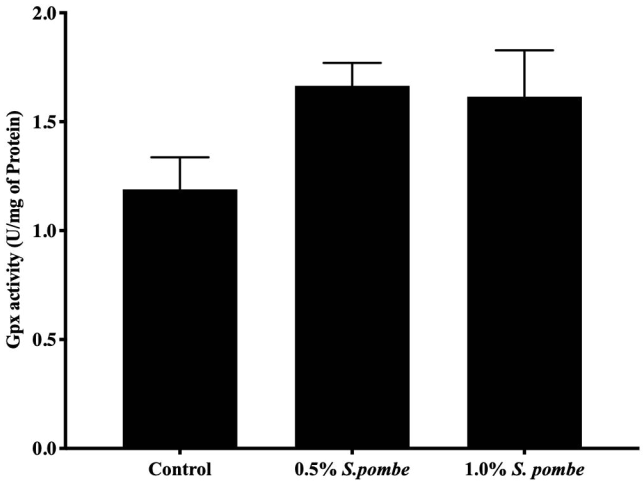

200
150
100
50
0
Lyzozyme activity /(U/mL)
a
b
c
Control
0.5% S. pombe
1.0% S. pombe

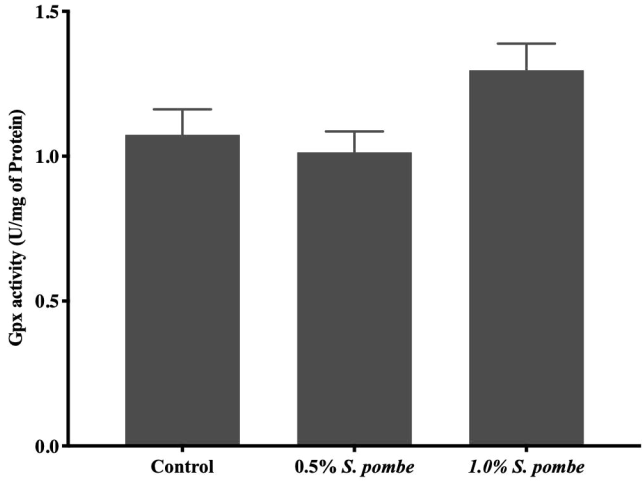

150
100
50
0
Lyzozyme activity(U/mL)
Control
0.5% S. pombe
1.0% S. pombe


100
80
60
40
20
0
SOD activity (%)
b
a
a,b
Control
0.5% S. pombe
1.0% S. pombe

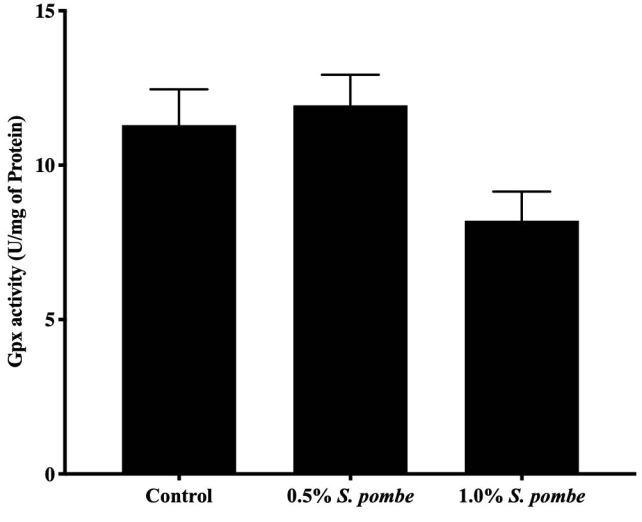

100
80
60
40
20
0
SOD activity (%)
b
a
a
Control
0.5% S. pombe
1.0% S. pombe

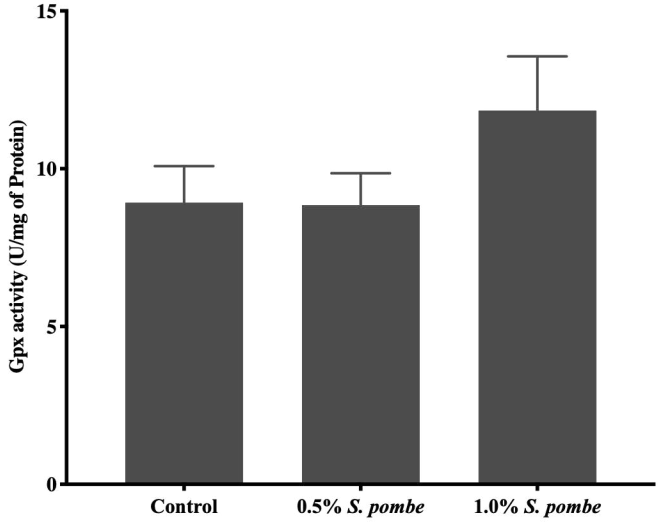
a. Liver



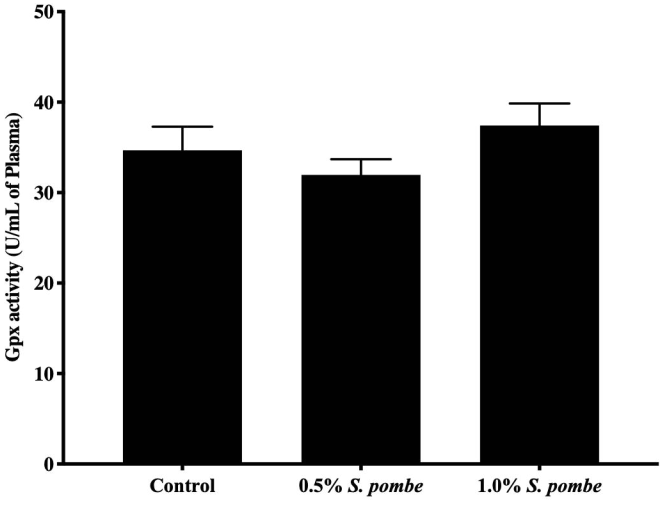


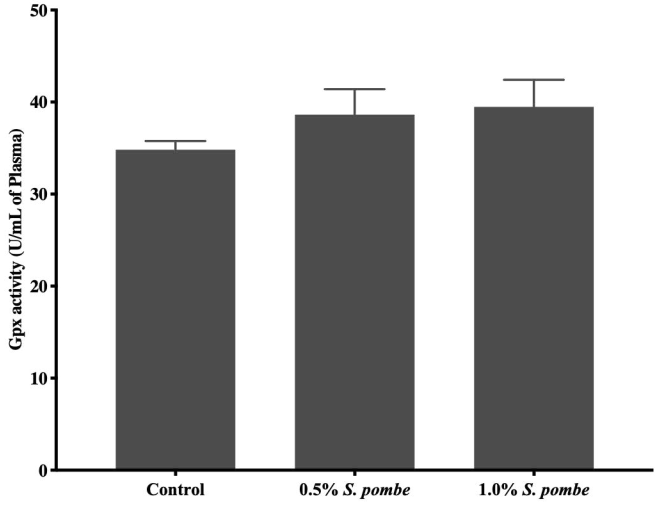
b. Kidney

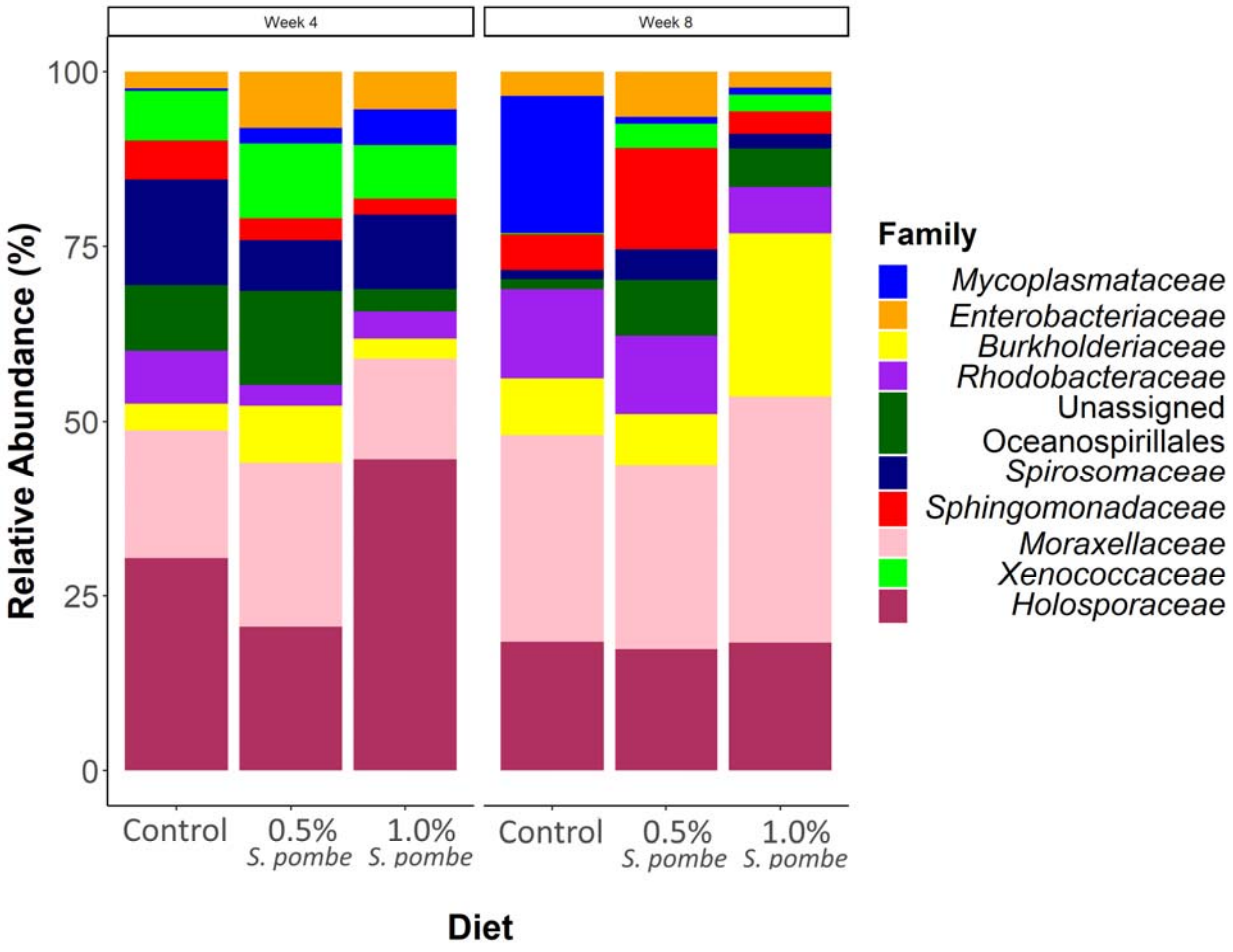


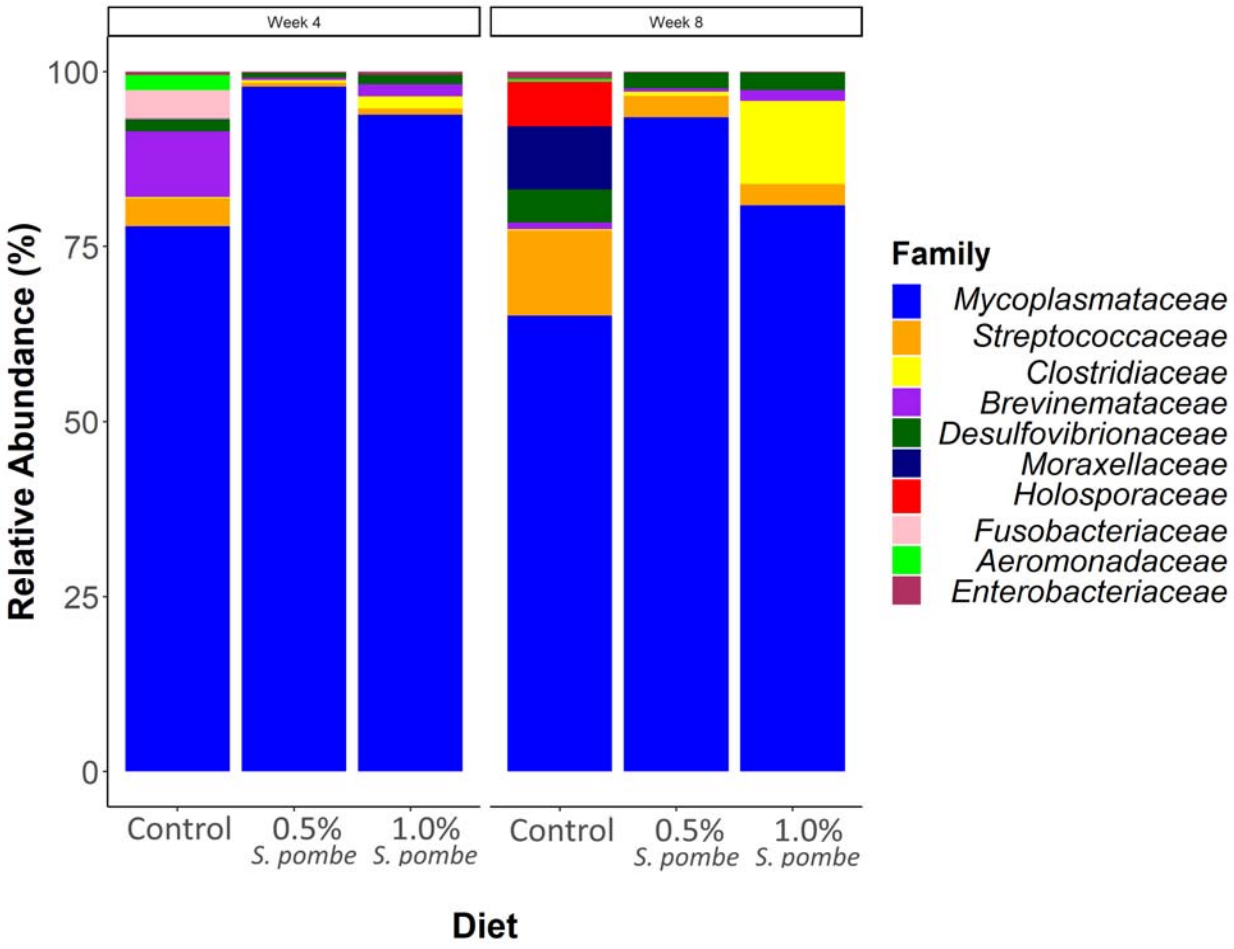


c. Plasma






Week 4
Week 8
100
75
50
25
0
Relative Abundance (%)
Control
0.5%
S. pombe
1.0%
S. pombe
Control
0.5%
S. pombe
1.0%
S. pombe
Diet
Family
Mycoplasmataceae
Enterobacteriaceae
Burkholderiaceae
Rhodobacteraceae
Unassigned
Oceanospirillales
Spirosomaceae
Sphingomonadaceae
Moraxellaceae
Xenococcaceae
Holosporaceae


Week 4
Week 8
100
75
50
25
0
Relative Abundance (%)
Control
0.5%
S. pombe
1.0%
S. pombe
Control
0.5%
S. pombe
1.0%
S. pombe
Diet
Family
Mycoplasmataceae
Streptococcaceae
Clostridiaceae
Brevinemataceae
Desulfovibrionaceae
Moraxellaceae
Holosporaceae
Fusobacteriaceae
Aeromonadaceae
Enterobacteriaceae