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2 Title

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4 Full:

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6 Gluten-free Fish? Marine Carnivores Cobia (*Rachycentron canadum*) and European Sea Bass

7 (*Dicentrarchus labrax*) Have Different Tolerances to Dietary Wheat Gluten

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9 Short:

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11 Gluten-free Fish? Marine Carnivores Have Different Tolerances to Dietary Wheat Gluten

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15 aquaculture feed

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37      **Abstract**

38      In developing more sustainable fishmeal-free diets for a broad range of fish species, a

39      "one-size-fits-all" approach should not be presumed. The production of more ecologically

40      sustainable aquaculture diets has increased the incorporation of plant-based protein sources

41      such as wheat gluten. Here we show that wheat gluten at even less than 4% inclusion in a

42      compound feed has a negative impact on growth and survivorship in juvenile cobia (*Rachycentron*

43      *canadum*). In addition, plasma factors capable of binding wheat gluten were detected in the

44      plasma of cobia fed diets containing this ingredient but not in wild cobia with no exposure to

45      dietary wheat gluten. Furthermore, there is evidence that supplementary taurine partially

46      mitigates the deleterious effects provoked by wheat gluten. Based on these results, we propose

47      that wheat gluten should be added with caution to aquaculture diets intended for juvenile cobia

48      and potentially other marine carnivores. After observing that dietary wheat gluten can cause

49      deleterious effects in cobia, we sought to evaluate a possible effect in European sea bass

50      (*Dicentrarchus labrax*), another large, carnivorous, marine species. There were no major effects

51      in terms of growth rate, plasma biochemical parameters, or detectable induction of plasma IgM,

52      IgT, or factors capable of binding gliadin in response to 4% dietary wheat gluten. However,

53      plasma levels of taurine doubled and there were considerable changes to the intestinal

54      microbiome. There was increased diversity of predominant taxonomic orders in the pyloric caeca,

55      anterior, middle, and posterior intestinal sections of fish consuming wheat gluten. Despite these

56      measurable changes, the data suggest that dietary inclusion of 4% wheat gluten is well tolerated

57      by European sea bass in feed formulations. Together these findings underscore the need to

58      evaluate tolerance to ingredients in aquaculture formulations on a species by species basis.

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59

60    Introduction

61    Reducing fishmeal inclusion in favor of more sustainable and cost-effective protein

62    sources is a high priority for the aquaculture industry (1). Aquaculture diets that incorporate low-

63    fat, high-protein concentrates derived from plants such as soy or wheat (including processed

64    wheat gluten) have been described for many species (2)(3). Feed formulations often incorporate

65    wheat gluten as a protein source and for binding and adding a desirable chewy texture to pellets.

66    Wheat flour is processed to remove soluble fibers and starches, and the vital wheat gluten

67    product that remains contains two fractions: soluble gliadins and insoluble glutenins (4)(5). Vital

68    wheat gluten, marketed as a dry powder, regains its elastic properties when rehydrated (6). In

69    addition to being a low-cost protein source, it is useful for the binding together of feed

70    ingredients into granules and pellets. Gliadins are known to trigger an immune response in

71    susceptible people, such as those with celiac disease (7–9).

72    When plant proteins replace fishmeal, essential amino acids (e.g. lysine, methionine,

73    threonine), as well as various vitamins and minerals, often need to be supplemented to

74    counteract deficiencies in the protein source (10–12). One such necessary nutritional constituent

75    found in fishmeal but is absent in plants is taurine. Some fish species are inadequate synthesizers

76    of taurine and require dietary intake (13). Previous work by our laboratory showed that cobia is

77    one such species, and it is essential to add taurine to a plant-based feed formulation intended

78    for their consumption (14). Findings presented in this work suggest that taurine may help to

79    mitigate some of the deleterious effects prompted by dietary wheat gluten in cobia.

80    The original intent of the cobia study described here was to test various plant-based diets

81  formulated from available and cost-effective ingredients that had been previously found to be

82  highly digestible by cobia and to be effective fishmeal replacements in rainbow trout

83  (*Oncorhyncus mykiss*) and other species (14). Commercial feed formulations tend to vary based

84  on the availability and cost of ingredients, with different batches potentially containing

85  significantly different proportions and quality of ingredients, or even different ingredients

86  altogether. The assessment of multiple components and combinations is necessary in the

87  development of optimized fishmeal replacement diets. It is also necessary to identify which

88  components might need to be supplemented for particular species when plant proteins replace

89  fishmeal in a formulation.

90       In a previous study, when a plant protein-based diet lacking taurine was fed to cobia,

91  poor growth and palatability were observed. However, when taurine was added to a similar plant

92  protein-based diet, growth performance was as good if not better than a fishmeal-based

93  commercial diet (14). It is likely that taurine supplementation greatly contributed to the

94  improved performance of the diet. However, two of the plant protein sources were also replaced:

95  barley meal (10.4%) and wheat gluten (8.2%) in exchange for solvent-extracted soybean meal

96  (12.1%) and wheat flour (22.7%). The barley meal was replaced because of poor digestibility (<

97  60%), and the wheat gluten was replaced with wheat flour as part of a slight reformulation

98  unrelated to specific concerns regarding wheat gluten. In the current study, which was not

99  originally designed to evaluate wheat gluten as an ingredient, we prepared plant-based diets for

100  cobia with varying concentrations of taurine (0 to 5%) at a fixed vital wheat gluten level of 2.2%.

101  Wheat flour in the formulations also contributed a small amount of wheat gluten. Dietary wheat

102  gluten at less than 4% inclusion appeared to be poorly tolerated, causing poor growth and even

5

103 mortalities in juvenile cobia. We also detected the presence of a plasma factor(s) capable of

104 binding gliadin that was present only in cobia exposed to dietary wheat gluten. Our laboratory

105 wished to expand on this study to further delineate the impacts of wheat gluten and taurine but

106 were unable to procure another cohort of cobia. We decided to investigate the impact of dietary

107 wheat gluten in European sea bass (*Dicentrarchus labrax*), another marine carnivore.

108 European sea bass are a globally important aquaculture fish, with approximately 60,000

109 tons of fish produced each year as part of a greater than 300 million dollar market. They can be

110 found on restaurant menus as branzino or branzini (15). A study in European sea bass suggests

111 that they perform well on a diet containing wheat gluten (~25% of formulation) when fishmeal is

112 also included in the formulation (16). Another study included 20% gluten in an entirely plant-

113 based formulation and noted differences in growth rates between the fish consuming that diet

114 as compared to a fishmeal-based diet (17). It is important to note than in the dietary formulations

115 for the study, the added wheat flour contributes a small amount of gluten. However, results from

116 cobia suggest that wheat flour does not elicit the same negative effects as the processed vital

117 wheat gluten. Being uncertain as to the taurine requirements of European sea bass, we

118 supplemented the diets in the study with 1.5% taurine to ensure that taurine deficiency would

119 not be a confounding factor in evaluating the effects of wheat gluten in European sea bass.

120 In the current study, we sought to determine if the inclusion of 4% wheat gluten into the

121 diet of European sea bass impacted overall growth, health and immune status, and the intestinal

122 microbiome. This entailed tracking growth rates, assaying plasma parameters and tissue weights,

123 detecting adaptive immune factors IgM and IgT, and evaluating differences in the intestinal

124 microbiome. European sea bass, like other teleost fish, have both innate and adaptive immunity.

6

125    European sea bass and other teleosts have three types of immunoglobulins: IgM, IgT, and IgD

126    (18,19). The functions of the first two types have been well described. The role of IgM in fish is

127    similar to its role in other organisms. It is the principal immunoglobulin found in teleost plasma

128    and though its primary role is in systemic immunity, a role in mucosal immunity has also been

129    described. IgT is an integral part of mucosal immunity and functions similarly to IgA in mammals.

130    Like IgM, it is detectable in plasma, though levels tend to be lower (20).

131        Regarding the gut microbiome, it is known that predominant intestinal microbes in fish

132    by diet, including with the addition of plant-based protein sources such as soybean meal (21–23).

133    A study of European sea bass fed combined fish- and plant-based protein sources reported the

134    main gut genera to be *Lactobacillus*, *Pseudomonas*, *Vibrio*, and *Burkholderia* (24). In this study,

135    we wished to characterize both the microbiome of European sea bass fed a plant-based diet

136    and the contribution of wheat gluten to the microbial landscape.

137        European sea bass fed the 0 and 4% wheat gluten diets had similar growth rates and levels

138    of common plasma parameter, and neither group had detectable induction of plasma IgM, IgT,

139    or factors capable of binding gliadin. Notable differences between the groups included twice the

140    plasma levels of taurine in the fish fed 4% wheat gluten and considerable differences in the

141    predominant taxonomic orders of the intestinal microbiome. Despite these measurable changes,

142    the data suggest that dietary inclusion of 4% wheat gluten is well tolerated by European sea bass

143    in an aquaculture feed formulation.

144

145    Materials and Methods

146    *Fish System Maintenance and Care*

7

147     *Cobia*

148          This study was carried out in accordance with the guidelines of the International Animal

149     Care and Use Committee of the University of Maryland Medical School (IACUC protocol

150     #0610015). Approximately 500 juvenile (~2 g) cobia were obtained from the Virginia Agricultural

151     Experiment Station, Virginia Tech, Hampton, VA, USA, for the first and second trials and

152     approximately 500 juveniles (~2 g) were obtained from the University of Miami in Miami, FL, USA,

153     for the third trial. Juveniles were housed at the Institute of Marine and Environmental

154     Technology's Aquaculture Research Center in Baltimore, MD. Fish for the first and second trials

155     (PP1-PP4) were maintained on a fishmeal diet until they reached an average weight of ~10 g (Trial

156     1) or ~120 g (Trial 2), at which point 18 fish were stocked into each of 12 identical tanks and

157     randomly assigned one of the four experimental diets using three replicate tanks per dietary

158     treatment. Six 340-liter tanks connected to bubble-bead and biological filtration as well as

159     protein skimmers constituted the recirculating systems. Four replicate systems were occupied

160     simultaneously during trials with the photoperiod maintained at 14 hours light, 10 hours dark

161     throughout the trials. The first trial was conducted for 8 weeks, with tank weights recorded and

162     feeding rates adjusted weekly to 5% bw day$^{-1}$. The second trial commencing with ~120 g fish was

163     conducted for 8 weeks, with tank weights recorded weekly and feeding rates adjusted from 3.5%

164     bw day$^{-1}$ to 2.5% bw day$^{-1}$, with a bi-weekly 0.25% bw day$^{-1}$ reduction throughout the trial.

165          The third trial (EPP3 diet) was initiated with ~18 g average weight individuals, with 12 fish

166     stocked per tank. The third trial was conducted for 12 weeks, with tank weights recorded and

167     feeding rates adjusted weekly to 5% bw day$^{-1}$ for the first 6 weeks, reduced to 3.5 % from 6 weeks

168     through 10 weeks, and 3.0% for the final 2 weeks of the trial as feed conversion ratio gradually

169     increased. Fish received the daily ration by hand over the course of 4 feedings.

170

171     *European sea bass*

172     This study was carried out in accordance with the guidelines of the International Animal

173     Care and Use Committee of the University of Maryland Medical School (IACUC protocol

174     #0616014). European sea bass were obtained from the laboratory of Yonathan Zohar, PhD, and

175     maintained in the Aquaculture Research Center at the Institute of Marine and Environmental

176     Technology. Starting at ~25 g of body weight, fish were fed diets containing either 0 or 4% wheat

177     gluten (Zeigler Bros., Gardners, PA, USA), starting with 75 fish per diet. Fish were maintained on

178     these diets for 6 months, at which time the average weight was 250 g. Fish were fed 3.5% of their

179     body weight per day over 3-4 feedings.

180     Temperature was maintained at 27 degrees C and salinity was 25 ppt. Fish were divided

181     by diet and housed in one of 2 eight-foot diameter, four cubic meter, recirculating systems

182     sharing mechanical and bio-filtration as well as life support systems. The recirculating system has

183     a filtration system which including protein skimming, ozonation, mechanical filtration in the form

184     of bubble-bead filters, and biological filtration. Water samples were tested 2-3 times per week

185     and analyzed by the National Aquarium water quality lab at IMET. Water quality was not

186     significantly different between systems utilized (ANOVA, p>0.05) during the study and overall

187     parameters were: dissolved oxygen, 5.69 $\pm$ 1.62 mg/L ; temperature, 26.85 $\pm$ 1.77 degrees C; pH

188     (4,500-H$^+$), 7.61 $\pm$ 0.27; total ammonia nitrogen (4,500-NH$_3$ ), 0.06 $\pm$ 0.06 mg/L ; nitrite (4,500-

189     NO$_2^-$ ), 0.12 $\pm$ 0.08 mg/L ; nitrate (4,500-NO$_3^-$ ), 49.28 $\pm$ 8.87 mg/L, alkalinity (2,320), 95.77 $\pm$ 23.11

190     meq/L ; and salinity (2,510) 24.91 $\pm$ 1.65 ppt.

9

191

192    *Diet preparation*

193    *Cobia*

194        Formulations of the five plant protein (PP) diets used in the feed trials are shown in S1

195    Table: Feed formulations for the cobia dietary study. For all diets, ingredients were ground using

196    an air-swept pulverizer (Model 18H, Jacobsen, Minneapolis, MN) to a particle size of < 200 μm.

197    All ingredients for PP1, PP2, PP3, and PP4 were mixed prior to extrusion, while EPP3 was top-

198    coated with the oil ingredient after extrusion. Pellets were prepared with a twin-screw cooking

199    extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) with an 18-second exposure to 127 °C in the

200    extruder barrel. Pressure at the diet head was approximately 26 bar, and a die head temperature

201    of 71 °C was used. The pellets were dried for approximately 15 minutes to a final exit air

202    temperature of 102 °C using a pulse bed drier (Buhler AG, Uzwil, Switzerland) followed by a 30

203    min cooling period to product temperature less than 25 °C. Final moisture levels were less than

204    10% for each diet. Diets were stored in plastic lined paper bags at room temperature and were

205    fed within six months of manufacture. Portions of each diet were analyzed by New Jersey Feed

206    Labs, Inc. (Trenton, NJ, USA) for proximate composition (S2 Table: Proximate composition and

207    measured taurine values of the cobia diets). Calculations of feed gluten content are based on

208    wheat flour containing 8% gluten plus any added vital wheat gluten (25)

209

210    *European sea bass*

211        The formulations for the 0 and 4% wheat gluten diets are shown in S3 Table: Feed

212    formulations for the wheat gluten European sea bass dietary study (Zeigler Bros., Gardners, PA,

1

213    USA).  The proximate composition of the feeds in shown in S4 Table: Proximate composition and

214    measure taurine values of the European sea bass diets. The analysis was performed by New

215    Jersey Feed Laboratory, Inc. (Ewing Township, NJ, USA).

216

217    *Blood and Tissue Sampling and Analysis*

218    *Cobia*

219       At the conclusion of the first trial, two individuals from each tank were sacrificed for

220    intestinal analysis. Portions of the anterior intestine were preserved in 4% paraformaldehyde and

221    dehydrated from 70% to 90% EtOH in 10% increments over eight hours. Dehydrated samples

222    were sent to AML Laboratories (Baltimore, MD) for sectioning, mounting, and H&E staining.

223    Slides were analyzed for pathologies and abnormalities with the aid of the acknowledged

224    pathologist, Dr. Renate Reimschuessel, VMD, PhD, (FDA in Laurel, MD). Gall bladders were

225    removed and bile was extracted and stored at -20 °C prior to bile salt analysis. Total bile salts

226    were assayed with 3 α-hydroxysteroid dehydrogenase  (26). Blood samples were taken from the

227    caudal vein with heparinized needles, plasma was separated by centrifugation (16,000 x g for 20

228    min), and total plasma protein was quantified after a 1:600 dilution utilizing a Micro BCA™ Protein

229    Assay Kit (ThermoFisher Scientific, Waltham, MA, USA).

230       At the conclusion of the second trial, two fish from each tank (total of six per dietary

231    treatment), were randomly selected for sampling. Fish were anesthetized with Tricaine

232    methanosulfonate (MS-222, 70 mg L$^{-1}$, Finquel, Redmond, WA, USA), blood samples were taken

233    from the caudal vein with heparinized needles, after which fish were euthanized with MS-222

234    (150 mg L$^{-1}$) and gall bladders were removed for bile analysis as in Trial 1. Liver and fillet samples

1

235 were also taken for histology. Blood plasma was separated by centrifugation (16,000 x g for 20

236 min at 4 °C) and plasma osmolality measured in triplicate (10 µl) on a Vapro™ Model 5520 vapor

237 pressure osmometer (Wescor, Logan, UT, USA). Plasma samples from three fish per dietary

238 treatment were sent to the Pathology and Laboratory Medicine Services department at the

239 University of California at Los Angeles for constituent analysis. Remaining plasma, fillet, and liver

240 samples were frozen and stored at -80 °C and portions of each were lyophilized to constant

241 weight for water and taurine content analysis. Triplicate samples of each liver (~10 mg), fillet (~50

242 mg), plasma (~10 µL), and diet (~50 mg) sample were used for taurine extractions based on

243 Chaimbault et al., with samples homogenized in cold 70% EtOH, sonicated for 20 min, dried, and

244 resuspended in 1 ml $H_2O$ prior to injection into the LC-MS (27).

245

246 *European sea bass*

247 At the conclusion of the 6-month trial, food was withheld for 24 hours and 10-12 fish from

248 each diet were sedated with 25 mg/L MS-222 (Syndel, Ferndale, WA, USA) buffered with 50 mg/L

249 sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA) and exsanguinated via the caudal vein to

250 collect blood for plasma analysis. Following blood collection, the spinal cord was severed, and

251 tissues were harvested for analysis.

252 Approximately 1 ml blood from the caudal vein was put into a tube containing 20 µL of

253 1000 units/ml heparin and gently inverted to mix. Samples were centrifuged at 2000 x g for 15

254 minutes at 4 degrees C and the plasma fraction retained. This processing was sufficient for plasma

255 chemistry and immunoblotting. In preparation for taurine analysis by HPLC, 10 µL plasma was

256 mixed with 90 µL (1:10) 70% ethanol containing 0.154 mM D-norleucine. The solution was

1

257     vortexed and centrifuged at 2000 x g for 5 min. 50 µL supernatant was retained and dried down

258     at 70 degrees C overnight.

259

260     Plasma Analysis

261         Plasma analysis was performed by Jill Arnold of the National Aquarium in Baltimore, MD.

262     Samples were processed using standard procedures for biochemistry analytes (S5 Table:

263     Common plasma analytes measured) using the ChemWell-T analyzer (CataChem, Oxford, CT,

264     USA). Calibration and quality control materials were used per manufacturer's instructions

265     (Catacal and Catatrol control level 1 and 2, CataChem, Oxford, CT, USA).  Osmolality was

266     measured using the Wescor Osmometer (Wescor, Inc., Logan, UT, USA) after calibration with two

267     levels of standards (290 and 1000 mmol/kg, OPTIMOLE, ELITechGroup Biomedical Systems,

268     Logan, UT, USA).

269

270     Plasma taurine analysis by HPLC

271         Dry samples were resuspended in 300 µL 0.1 N HCl and filtered through 0.45 micron filters

272     (EMD Millipore, Billerica, MA, USA). 5 µL of the filtered extracts was derivatized according to the

273     AccQTag$^{TM}$ Ultra Derivitization Kit protocol (Waters Corporation, Milford, MA, USA). Amino acids

274     were analyzed using an Agilent 1260 Infinity High Performance Liquid Chromatography System

275     equipped with ChemStation (Agilent Technologies, Santa Clara, CA, USA) by injecting 5 µL of the

276     derivatization mix onto an AccQTag™ Amino Acid Analysis C18 (Waters, Milford, MA, USA)

277     4.0 um, 3.9 x 150 mm column heated to 37 °C. Amino acids were eluted at 1.0 mL min$^{-1}$ flow

278     with a mix of 10-fold diluted AccQTag$^{TM}$ Ultra Eluent (C) (Waters Corporation, Milford, MA, USA),

1

279    ultra-pure water (A) and acetonitrile (B) according to the following gradient: Initial, 98.0% C/2.0%

280    B; 2.0 min, 97.5% C/2.5% B; 25.0 min, 95.0% C/5.0% B; 30.5 min, 94.9% C/5.1% B; 33.0 min, 91.0%

281    C/9.0% B; 38 min, 40.0% A/60.0% B; 43 min, 98.0% C/2.0% B. Derivatized amino acids were

282    detected at 260 nm using a photo diode array detector. Signals were referenced to AABA (alpha-

283    Aminobutyric acid), D-norleucine, and standard hydrolysate amino acids.

284

285    *SDS-PAGE and western blotting*

286        A solution of gliadin (Sigma, St. Louis, MO, USA) was made to an original concentration of

287    2 mg/ml and subsequently diluted 2-fold to 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.125 mg/ml,

288    all in Laemmli sample buffer.  All samples were heated to 95 °C for 3 min and centrifuged at

289    10,000 x g for 1 min prior to electrophoresis. Recombinant *Amphidinium carterae* eIF4A-1A

290    (obtained from Grant Jones at IMET, 7/28/14) was diluted into Laemmli sample buffer to a

291    concentration of 57.5 ng/μl. 15 μl of a 1:1 volume ratio of gliadin and eIF4A was loaded into each

292    lane of a Novex NuPAGE 4-12% Bis-Tris gel and electrophoresed in a Bolt® Mini Gel Tank at 165 V

293    for 1 h with MOPS SDS running buffer (Life Technologies, Frederick, MD, USA).

294        PVDF membrane was activated by a brief dip in 100% methanol and equilibrated for 5

295    min in Novex™ NuPAGE® transfer buffer. The gel was electroblotted onto a prepared PVDF in a

296    Bolt Mini Blot Module at 30 V for 1 h in Novex™ NuPAGE® transfer buffer (Life Technologies,

297    Frederick, MD, USA). Following transfer, the membrane was washed in ddH$_2$0 for 5 min. Duplicate

298    transferred lanes were divided and designated as "No Plasma Block" or "With Plasma Block" for

299    subsequent incubation procedures.

300        For the "No Plasma Block" sections, the membrane was incubated at room T for 1 h

301    followed by overnight at 4 °C with 5 ml 5% nonfat milk in TBS-T. For cobia, the "With Plasma

302    Block" membrane was incubated at room T for 1 h followed by overnight at 4 °C with either mixed

303    plasma from a total of 7 fish from Trial 2 fed diets containing 3.2-3.6% gluten (PP1-PP4) or mixed

304    plasma from two wild-caught cobia diluted 1:10 into TBS-T containing 5% nonfat milk. For

305    European sea bass, the "0 Wheat Gluten Plasma" and "4% Wheat Gluten Plasma" membranes

306    were incubated at room T for 1 h followed by overnight at 4 °C with mixed plasma from a total

307    of 3 fish fed diets containing either 0 wheat gluten or 4% wheat gluten, respectively, diluted 1:12

308    into TBS-T containing 5% nonfat milk. The following day, blots were washed 4 times for 10 min

309    each time with TBS-T. Both blots were incubated with polyclonal anti-gliadin antibody (Biorbyt,

310    Cambridge, UK) and rabbit anti-*A. carterae* eIF4E-1A (GenScript, Piscataway, NJ, USA) diluted

311    1:500 and 1:2000, respectively, into TBS-T containing 5% nonfat milk at room temperature for 1

312    h. The blots were again washed 4 times for 10 min each time with TBS-T. Both blots were

313    incubated with goat anti-rabbit IgG-HRP conjugate (Bio-Rad, Hercules, CA, USA) diluted 1:2500

314    into TBS-T containing 5% nonfat milk at room temperature for 1 h followed by four 10-min

315    washes with TBS-T. The HRP signal from bound antibody was visualized using Clarity™ Western

316    ECL Substrate (Bio-Rad, Hercules, CA, USA). Imaging was performed in a Flourchem™, and the

317    AlphaView program was used to analyze densitometry (ProteinSimple, San Jose, CA, USA).

318

319    *IgM and IgT immunoblotting in European sea bass*

320    Plasma was diluted 1:40 in 1x SDS-PAGE sample buffer. Samples were heated for 3

321    minutes at 95 degrees C and centrifuged for one minute at 10,000 x g. 13 µL of each sample was

322    electrophoresed on a 4%–12% Bis-Tris protein gel (NuPAGE Novex, (ThermoFisher Scientific,

1

323     Waltham, MA, USA) for 35 minutes at 200 V using MOPS buffer in a PowerPac™ HC Power Supply

324     (Bio-Rad, Hercules, CA, USA. Proteins were transferred to a PVDF membrane for 14 minutes on

325     the high molecular weight setting (25 V) in the Trans-Blot® Turbo™ Transfer System (Bio-Rad,

326     Hercules, CA, USA). Immunoblotting was performed in the iBind™ Western System

327     (ThermoFisher Scientific, Waltham, MA, USA).

328     For IgT detection, an anti-European sea bass IgT polyclonal antibody (rabbit IgG "RAIgT1,"

329     kindly provided by Giuseppe Scapigliati, Tuscia University, Italy) was used at a dilution of 1:1000

330     as the primary antibody, and goat anti-rabbit IgG H&L HRP conjugate at a dilution of 1:2000 (Bio-

331     Rad, Hercules, CA, USA) was used as the secondary antibody. For IgM detection, the Magic™ anti-

332     European sea bass IgM monoclonal antibody (mouse IgG) (Creative Diagnostics, Shirley, NY, USA)

333     was used at a dilution of 1:1000 as the primary antibody, and goat anti-mouse IgG H&L HRP

334     conjugate (Bio-Rad, Hercules, CA, USA) was used as the secondary antibody at a dilution of

335     1:2000. A chemiluminescent signal was generated with addition of Clarity™ Western ECL

336     substrate and imaged in a ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA). Image

337     Lab software (Version 5.2.1, Bio-Rad, Hercules, CA, USA) was used to visualize immunoblots and

338     analyze protein molecular weights.

339

340     *Microbiome analysis in European sea bass*

341     DNA Extraction

342     For sampling of tank water, 1 L of water was filtered through a pore size of 0.2 microns.

343     For the feed, 0.127 g (3 pellets) of each diet was used. For intestinal samples (pyloric caeca,

344     anterior intestine, mid-intestine, and posterior intestine), ~0.25 g tissue was collected. DNA

1

345    extraction was performed using the Qiagen DNeasy® Powerlyzer® Powersoil® kit (Qiagen,

346    Germantown, MD, USA). Samples were manually processed through the beat beating step 6 m/s

347    for 30 sec, repeated once (FastPrep®-24, MP Biomedicals, Santa Ana, CA, USA) and centrifugation

348    procedure for two minutes at 10,000 x g (Eppendorf 5415 D, Sigma-Aldrich, St. Louis, MO, USA).

349    In the case of the water analysis, the filter was treated as the sample for processing. Following

350    Step 5 in the manufacturer's protocol, DNA extracted was completed in a Qiacube® (Qiagen,

351    Germantown, MD, USA) according to manufacturer's instructions for DNA extraction including

352    the optional PCR inhibitor removal. DNA was stored at -20 degrees C.

353

354    PCR and Gel Electrophoresis

355        PCR was performed to confirm the presence of amplifiable DNA for 16s rRNA sequencing.

356    One μL of extracted DNA was used in a 25 μL reaction volume with Promega™ PCR Master Mix

357    (Thermo Fisher Scientific, Waltham, MA) and amplified in a DNA Engine Dyad (MJ Research,

358    Quebec, Canada) using primers 16S_27F 5' AGAGTTTGATCMTGGCTCAG 3'

359    and 16S_1492R 5' TACGGYTACCTTGTTACGACTT 3' with the following reaction conditions:

360    95 degrees C for 5 min, 92 degrees C for 30 sec, 50 degrees C for 2 min, 72 degrees C for 1 min,

361    30 sec, cycle to step 2 for 39 more times, incubate at 72 degrees C for 5 minutes, hold at 4 degrees

362    C. Agarose gel electrophoresis was performed on each PCR product to detect the presence of a

363    1465 bp DNA band spanning bacterial rRNA variable regions 1-9. Samples were electrophoresed

364    in a 1% agarose gel containing 0.5 μg/mL ethidium bromide at 150V for 40 minutes and imaged

365    in a ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA).

366

1

367    Sequencing

368        Sequencing was performed at the BioAnalytical Services Laboratory (BAS Lab) at IMET on

369    an Illumina MiSeq™ (San Diego, CA, USA) using 5 ng DNA from each sample. Primers

370    complementary to the V3-V4 hypervariable region of the bacterial 16s rRNA gene were designed

371    based on those characterized by Klindworth et al.: S-D-Bact-0341-b-S-17 5'-

372    CCTACGGGNGGCWGCAG-3' and S-D-Bact-0785-a-A-21 5'-GACTACHVGGGTATCTAATCC-3' (28).

373    Including the Illumina adaptor sequences, the full-length primers were F 5'

374    TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and R 5'

375    GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. CLC Genomics

376    Workbench 8 (Version 8.5.1, Qiagen, Germantown, MD, USA) was used to trim, pair, and merge

377    sequences (default parameters used for all functions). They were exported as a merged FASTA

378    file and imported into the Quantitative Insights into Microbial Ecology (QIIME) program (Version

379    1.9.1) (29) for open reference operational taxonomic unit (OTU) and taxonomic classification

380    using the Silva 128 reference database (30). Rarefaction curves were generated based on

381    observed OTUs. Identify threshold was set to 97%. Representative sequence alignments for each

382    OTU were generated using Python Nearest Alignment Space Termination (PyNAST) (31), and R

383    (Version 3.4.3) (32) was used to generate a bar graph of the bacterial orders, as well as a PCA

384    plot. QIIME was used to generation the rarefaction plot.

385

386    *Statistics*

387        Statistical significance was evaluated using analysis of variance (ANOVA) and Student's t-

388    test (2-tailed) with a 95% confidence interval.

389

## Results

*Cobia Trials*

392     Diets 1-4 for both PP (plant protein) and FM (fishmeal) were formulated to contain graded

393     levels of taurine of approximately 0, 0.5%, 1.5% and 5%. PP1-4 contain approximately 3.6%, 3.6%,

394     3.5%, and 3.2% total wheat gluten, respectively. This calculation includes any wheat gluten

395     present in the wheat flour, estimated to be ~8% of the total weight. The FM1-4 diets contain an

396     average of 1.8% total wheat gluten. These diets are part of a previously published study

397     performed by our laboratory (33). The EPP3 diet contains approximately 1.2% wheat gluten and

398     1.5% taurine.

399

Growth Data

401     Growth data for Trial 1 of diets PP1-PP4 are displayed in Figure 1 (A). For this trial, the

402     average initial fish weight was ~10 g, and fish were maintained on the diets for 8 weeks. Data

403     from Trial 3 with fish of average initial weight ~18 g fed the EPP3 diet are also included on this

404     graph, as well as averaged growth data for fish fed various fishmeal diets (FM1-FM4). Growth

405     data from Trial 2 (~120 g initial weight) are shown in Figure 1 (B). The trial continued for 8 weeks

406     and as with the graph for the first trial, data are included for comparison from the EPP3 and FM1-

407     FM4 studies. For both trials, the fishmeal and EPP3 diets trend higher in terms of growth. PP1

408     appears to be the least favorable diet in terms of growth. It contains the greatest amount of

409     wheat gluten but also contains no taurine.

410

1

411 **Figure 1: Growth data from 8-week dietary trials in juvenile cobia.**
412 Plot A presents data from the first growth trial starting with ~10 g average weight juveniles for
413 four plant protein based diets (PP1, PP2, PP3, and PP4) with graded levels of supplemental
414 taurine and the average of four fishmeal-based diets (FM, data from Watson et al. 2014) (33).
415 Plot B presents data from the second growth trial starting with ~120 g average weight juveniles.
416 Included are the growth data for the trial with diet EPP3 starting with ~18 g average weight
417 juveniles. Plotted are the average tank weights from 3 replicates ± standard deviation.
418
419 *Survival and performance characteristics*

420 Performance characteristics for fish fed PP1-PP4 are shown in Table 1. Differences in

421 survival are substantial, with PP4 containing the lowest amount of wheat gluten and highest

422 amount of taurine being the best performer. There were no significant differences among

423 surviving individuals in percent weight gain, feed conversion ratio (FCR), specific growth rate

424 (SGR), total plasma protein concentration, or total bile salt concentration (ANOVA, P>0.05). Due

425 to low survival in several replicates of PP1 and PP2 resulting in 0 individuals in some tanks, these

426 diets were not included in statistical analyses other than survival for the first trial.

427

428 **Table 1: Performance characteristics from Trial 1 with cobia (~10 g initial weight).**
429 Within a row, values that share common superscripts are not significantly different from one
430 another (P>0.05).
431

| Characteristic | PP1 (0.02% T) | PP2 (0.39% T) | PP3 (1.35% T) | PP4 (4.08% T) |
|---|---|---|---|---|
| Survival (%) | 1.96 ± 1.96[a] | 9.80 ± 5.18[a,b] | 9.83 ± 1.97[a,b] | 19.6 ± 7.06[b] |
| Weight Gain (%) | 315.55 [3] | 1194.73 ± 21.22 [3] | 1155.92 ± 410.70 | 1200.68 ± 214.22 |
| FCR[1] | 1.93 [3] | 0.96 ± 0.02 [3] | 1.17 ± 0.44 | 0.95 ± 0.11 |
| SGR[2] | 2.54 [3] | 4.57 ± 0.03 [3] | 4.26 ± 0.72 | 4.53 ± 0.31 |
| Total Plasma Protein (g dL$^{-1}$) | 3.49[3] | 3.40 ± 0.17[3] | 3.35 ± 0.17 | 3.48 ± 0.23 |
| Total Bile Salts (mM) | 40.84[3] | 42.17 ± 0.46[3] | 41.37 ± 1.99 | 41.50 ± 1.36 |

432
433
434 T=Taurine
435 [1] FCR =feed conversion ratio = (g fed/g gained)

2

436    [2] SGR=specific growth rate = $((\ln BW_f - \ln BW_i)*(\text{days of growth trial}^{-1}))*100$

437    [3] Not included in statistical analyses due to lack of replicates (survivors)

438

439      Performance characteristics from the second trial (~120 g initial weight) are shown in

440    Table 2. Percent weight gain was significantly lower in diets PP1 and PP2 compared to the other

441    four fishmeal-based diets (p<0.05), but not significantly different from diets PP3 and PP4

442    (p>0.05). Diet PP1 resulted in significantly lower FCR and SGR than the other diets (p<0.05). Diets

443    PP2, PP3, and PP4 did not result in significantly different SGRs than one another (p>0.05). The

444    compared outcomes from diets PP1 and PP2 containing roughly the same amount of wheat

445    gluten with and without taurine supplementation reinforce the importance of the amino acid for

446    promoting growth and suggest that it may partially alleviate adverse effects resulting from wheat

447    gluten. Total bile salt concentration was not significantly different between any of the 4 dietary

448    treatments (p>0.05).

449

450    **Table 2: Performance characteristics from Trial 2 with cobia (~120 g initial weight).**
451    Within a row, values that share common superscripts are not significantly different from one
452    another (P>0.05).
453
454

| Characteristic | PP1 (0.02% T) | PP2 (0.39% T) | PP3 (1.35% T) | PP4 (4.08% T) |
|---|---|---|---|---|
| Weight Gain (%) | 23.35 ± 22.82[a] | 80.57 ± 60.24[a] | 130.87 ± 25.20[b] | 133.82 ± 10.83[b] |
| FCR | 6.38 ± 1.49[a] | 2.97 ± 1.17[b] | 1.98 ± 0.19[b] | 2.12 ± 0.23[b] |
| SGR | 0.57 ± 0.12[a] | 1.31 ± 0.26[b] | 1.47 ± 0.19[b] | 1.51 ± 0.09[b] |
| Total Bile Salts (mM) | 38.76 ± 7.40 | 28.37 ± 1.94 | 36.30 ± 5.69 | 28.75 ± 3.22 |

455
456

457    T=Taurine

458    [1] FCR =feed conversion ratio = (g fed/g gained)

459    [2] SGR=specific growth rate = $((\ln BW_f - \ln BW_i)*(\text{days of growth trial}^{-1}))*100$

460

2

461   For the EPP3 diet in the third trial (initial weight ~18 g), weight gain, FCR, and SGR were

462 much improved from the first two trials, and were an improvement over all previous plant protein

463 diets tested in our laboratory. Performance characteristics included weight gain of 1673.52% ±

464 192.75, FCR of 1.22 ± 0.04, and SGR of 3.42 ± 0.13. The EPP3 diet contains the lowest gluten

465 content of all diets tested in this study (1.2%).

466

467 Wheat gluten: Survival and pathology

468   Figure 2 presents survival curves based on percent wheat gluten for fish fed diets PP1-

469 PP4 (Trial 1), EPP3 (Trial 3), or fishmeal-based diets FM1-FM4 (averaged) from a previous study

470 (33). These data suggest that wheat gluten inclusion may be deleterious to cobia at this early

471 stage of development, though it may be tolerated at later stages. Histological analysis of the

472 proximal intestinal epithelium showed no distinct differences for fish fed the PP1-PP4 and FM1-

473 FM4 diets. However, pathological effects in the distal intestinal region cannot be ruled out. No

474 histological analysis was performed on the EPP3 fish. Survival was approximately 100% for fish in

475 Trial 2 fed the PP1-PP4 diets and in Trial 3 with the EPP3 diet. Cannibalism was not observed to

476 be a contributing factor to the low survival in Trial 1, and dead individuals were promptly

477 removed from the tanks so as to not be a nutritional/taurine source for remaining fish.

478

479 **Figure 2: Dietary wheat gluten level affects survival of young cobia.**
480 For Trial 1 fish (growth data shown in Figure 1(A)), probability of survival is graphed as a function
481 of dietary gluten. For fish fed PP1-PP4, (> 3% gluten) or FM1-FM4 (< 3% gluten) (33), survival data
482 from the diet subtypes (1,2,3, and 4) are averaged. Also shown is survival data from fish fed EPP3
483 (1.2% gluten).
484
485 Muscle and liver characteristics

486     Fillet and liver characteristics from the second and third trials are shown in S6 Table: Fillet

487     and liver characteristics from the second and third cobia growth trials. Fillet water content was

488     highest in fish fed the PP1 diet with a gradual reduction in fillet water content as dietary taurine

489     level increased. Hepatosomatic index show showed a similar trend of reduction as dietary taurine

490     level increased. Fillet yield, liver water, fillet taurine, and liver taurine contents all showed

491     increasing trends with increasing dietary taurine levels.

492

493     Plasma analysis

494     Results of the plasma analysis are shown in Table 3. Plasma water content significantly

495     decreased as dietary taurine level increased (p<0.05). Concentrations of glucose, calcium,

496     phosphorus, and magnesium ions, as well as overall osmolality, are indicators of health status.

497     Total protein levels also often correlate with nutritional status and general health (34)·(35).

498     Triglycerides are an energy substrate, and a fasting study in European sea bass demonstrated

499     their potential as a marker for nutritional condition (36). AST (aspartate aminotransferase) and

500     ALP (alkaline phosphatase) are indicators of liver health and function (37).

501     Plasma taurine levels were significantly lower in diets PP1 and PP2 than the other diets

502     (p<0.05). Plasma cholesterol, phosphorous, and albumin all showed similar trends of significantly

503     increasing with dietary taurine level (p<0.05). Lower creatine kinase levels can be indicative of

504     muscle wasting (38). Though biochemical markers of liver failure are not well characterized in

505     fish, elevated bilirubin is a classic sign (39). Stress on the liver may be a result of insufficient

506     taurine required for bile acid conjugation or a manifestation of gluten-induced enteritis (40). The

507     substantial difference in plasma glucose levels between PP1-PP4 and EPP3 is likely an artifact of

2

508    MS-222 and not a barometer of health status (41).

509

510    **Table 3: Plasma measures from fish from the second and third cobia growth trials.**

511    Values represent the mean ± standard error for three fish per dietary treatment. Within a row,

512    values that share common superscripts are not significantly different from one another (p

513    >0.05).

514

515

| Plasma Component | PP1 (0.02% T)[1] | PP2 (0.39% T)[1] | PP3 (1.35% T)[1] | PP4 (4.08% T)[1] | EPP3 (1.05% T)[2] |
|---|---|---|---|---|---|
| Water Content (%) | 96.75 ± 0.19[a] | 94.75 ± 0.21[b] | 95.19 ± 0.29[a,b] | 94.73 ± 0.13[b] | 94.94 ± 1.21[a,b] |
| Osmolality (Osm L$^{-1}$) | 329.17 ± 5.57 | 343.00 ± 5.57 | 325.17 ± 18.94 | 336.33 ± 4.60 | 332.84 ± 9.65 |
| Taurine (nmol ml$^{-1}$) | 421.25 ± 41.03[a] | 565.79 ± 17.91[a] | 658.47 ± 50.92[b] | 723.86 ± 89.62[b] | 601. 29 ± 47.97[a,b] |
| Albumin (g dL$^{-1}$) | 0.53 ± 0.12[a] | 0.70 ± 0.06[a,b] | 0.80 ± 0.00[a,b] | 0.87 ± 0.03[b] | 0.52 ± 0.07[a] |
| Total Bilirubin (mg dL$^{-1}$) | 0.40 ± 0.10[a] | 0.30 ± 0.00[a] | 0.23 ± 0.03[a] | 0.23 ± 0.03[a] | 0.12 ± 0.00[b] |
| Calcium (mg dL$^{-1}$) | 9.70 ± 0.88 | 10.90 ± 0.61 | 11.10 ± 0.10 | 11.37 ± 0.27 | 11.69 ± 0.30 |
| Cholesterol (mg dL$^{-1}$) | 49.33 ± 12.17[a] | 68.33 ± 1.20[a,b] | 80.33 ± 3.18[b] | 79.33 ± 4.26[a,b] | 46.59 ± 11.43[a] |
| Creatine Kinase (U L$^{-1}$) | 96.67 ± 46.77 | 386.67 ± 276.01 | 301.33 ± 81.63 | 552.00 ± 113.15 | nd |
| Creatinine (mg dL$^{-1}$) | 0.17 ± 0.03 | 0.20 ± 0.00 | 0.17 ± 0.03 | 0.17 ± 0.03 | nd |
| Glucose (mg dL$^{-1}$) | 48.00 ± 4.36[a] | 51.33 ± 3.48[a] | 48.33 ± 3.92[a] | 41.67 ± 3.93[a] | 109.13 ± 19.13[b] |
| Phosphorous (mg dL$^{-1}$) | 7.13 ± 1.34[a] | 8.37 ± 0.69[a] | 8.87 ± 0.38[a] | 9.33 ± 0.23[a] | 13.82 ± 1.78[b] |
| Magnesium (mg dL$^{-1}$) | 2.37 ± 0.22 | 1.97 ± 0.07 | 2.13 ± 0.03 | 2.23 ± 0.08 | 2.77 ± 0.18 |
| Triglycerides (mg dL$^{-1}$) | 66.67 ± 27.43 | 112.00 ± 19.67 | 91.00 ± 36.12 | 109.67 ± 20.96 | 75.73 ± 17.35 |
| Sodium (mmol L$^{-1}$) | 169.10 ± 3.56 | 178.67 ± 2.89 | 177.33 ± 2.32 | 174.83 ± 3.89 | nd |
| Potassium (mmol L$^{-1}$) | 8.78 ± 1.09 | 8.07 ± 0.36 | 8.92 ± 0.53 | 9.35 ± 0.23 | nd |
| Chloride (mmol L$^{-1}$) | 169.97 ± 1.93 | 173.97 ± 2.08 | 168.83 ± 0.88 | 169.43 ± 3.85 | nd |

2

516

517

518     T=Taurine

519     [1] UCLA DLAM analysis

520     [2] NOAA-NWFSC analysis by A.W. (excluding water content, osmolality, and taurine)

521

522     Liver histology

523         Figure 3 shows H&E-stained liver sections from Trial 2 fish fed PP1 (Panel A) or PP4 (Panel

524     B). Supplementation with taurine prevents pervasive steatosis. This effect was also seen in fish

525     fed FM1 vs. FM4 (data not shown). Taurine supplementation may have therapeutic potential in

526     the treatment of nonalcoholic fatty liver disease, suggesting that supplementation may be of

527     value even in cases where there is not an underlying deficiency (42).

528

529     **Figure 3: Taurine supplementation mediates hepatic steatosis.**
530     Histological sections from fish were stained with H&E and microscopically examined. Panel A
531     presents a representative section from fish fed PP1 (0.02% taurine). Panel B presents a
532     representative section from fish fed PP4 (4.08% taurine). Pervasive lipidosis is apparent in Panel
533     A.
534

535     Detection of immune factors capable of binding to wheat gluten

536         Suspecting that wheat gluten might be a contributing factor to poor growth performance,

537     we assayed in Trial 2 fish plasma an immune response to gliadin, a potentially immunogenic

538     component of wheat gluten. Standard methods for detecting specific antibody responses to

539     wheat gluten in human celiac patients cannot be utilized as they are designed to detect IgA. Cobia

540     and other teleosts have only three classes of immunoglobulins: IgM, IgT, and IgD, and there are

541     currently no reagents to detect these immunoglobulins in cobia (43). It is not known if antibodies

542     designed to detect these adaptive factors have cross-reactivity to these immune components in

543     cobia.

544        To detect plasma factors capable of binding to gliadin, varying concentrations of the

545    protein as well as a fixed amount of eIF4E-1A from the dinoflagellate *A. carterae* were subjected

546    to immunoblotting. eIF4E-1A, a translation protein, was used as a control for loading, transfer

547    efficiency, and specificity of binding. After electroblotting, membranes were pre-incubated in

548    blocking buffer only or blocking buffer containing plasma. The 30-40 kDa bands visible on the

549    immunoblots correspond to a range of α/β and γ gliadins (44). These gliadin bands are less visible

550    over the course of 2-fold dilutions (Figures 4 and 5).

551        Pre-incubation with plasma from cobia fed diets containing 3.2-3.6% wheat gluten

552    diminished binding of an anti-gliadin polyclonal antibody, even at the highest concentration of

553    gliadin (Figure 4). A comparison of slopes from linear regressions based on densitometry data for

554    "No Plasma Block" vs. "With Plasma Block" relatively quantifies this difference in signal as

555    approximately 10-fold. A similar dot blot experiment with gliadin but no eIF4E-1A confirmed the

556    results of the western blot (not shown). Similar results were obtained using plasma from fish fed

557    diets containing 1.5-1.9% wheat gluten, suggesting that a small amount is sufficient to mobilize

558    a response (not shown).

559

560    **Figure 4: Factor(s) that specifically bind gliadin are present in plasma of fish fed plant-based**
561    **diets containing 3.2-3.6% wheat gluten.**
562    Immunoblotting of gliadin without pre-incubation of plasma (A) or with pre-incubation with
563    plasma from fish fed diets containing 3.2-3.6% wheat gluten from Trial 2 (B) demonstrates the
564    ability of factor(s) in plasma to bind to gliadin (30-40 kDa) and inhibit binding of anti-gliadin
565    polyclonal antibody. Each lane also contains 431 ng recombinant A. *carterae* protein eIF4E-1A (50
566    kDa).
567

568        Pre-incubation with plasma from wild-caught cobia with no dietary exposure to wheat

569    gluten does not diminish binding of an anti-gliadin polyclonal antibody, even at the lowest

570 concentrations of gliadin (Figure 5). For all experiments, there was no reduction in eIF4E-1A signal

571 as a result of pre-incubation with plasma, demonstrating the specificity of plasma factors for

572 gliadin. There was also no detection of a component capable of binding gliadin found in the

573 plasma of fish fed the FM1-4 diets (data not shown).

574

575 **Figure 5: Factor(s) that bind gliadin are absent in plasma from wild cobia with no dietary gluten**
576 **exposure.**
577 Immunoblotting of gliadin without pre-incubation of plasma (A) or with pre-incubation with
578 plasma (B). No factors capable of binding to gliadin (30-40 kDa) and inhibiting binding of anti-
579 gliadin polyclonal antibody are detectable. Each lane also contains 431 ng recombinant *A.*
580 *carterae* protein eIF4E-1A (50 kDa).

581
582 *European sea bass*

583 Growth data

584 Growth rates were equivalent (p>0.05) for the fish fed plant-based diets with or without

585 added wheat gluten (4%). The study commenced with fish of an average weight of ~25 g and

586 continued for 6 months. The growth curve is shown in Figure 6.

587

588 **Figure 6: Dietary inclusion of 4% wheat gluten does not affect growth of European sea bass.**
589 European sea bass were fed plant-based diets containing 0 or 4% wheat gluten starting at an
590 average weight of ~25 g.  The study continued for 6 months, and growth rates were similar
591 throughout the study (p>0.05).

592

593 Plasma analysis

594 As shown in Table 4, analysis of common plasma analytes using a bioanalyzer revealed no

595 differences in levels between the 0 or 4% wheat gluten dietary groups with the exception of

596 calcium and AST (aspartate aminotransferase) (p<0.05). Plasma calcium is higher in the group fed

597 4% wheat gluten, whereas plasma AST levels are lower.

2

598

**Table 4: Levels of common plasma parameters for European sea bass fed diets with 0 or 4% wheat gluten are similar in value except for levels of calcium and AST.**

| Plasma Component | 0 Wheat Gluten | 4% Wheat Gluten |
|---|---|---|
| Glucose (mg/dL) | 120.727 ± 27.335 | 113.874 ± 32.011 |
| **Calcium (mg/dL)** | ***11.045 ± 0.465** | ***11.75 ± 0.567** |
| Magnesium (mg/dL) | 3.545 ± 0.398 | 3.7 ± 0.25 |
| Phosphorus (mg/dL) | 7.909 ± 0.76 | 8.125 ± 0.784 |
| Triglycerides (mg/dL) | 410.364 ± 77.747 | 388 ± 129.836 |
| Cholesterol (mg/dL) | 199.545 ± 33.074 | 206.75 ± 43.702 |
| ALP (U/L) | 24.091 ± 1.486 | 25.125 ± 1.511 |
| **AST (U/L)** | ***47.8 ± 28.558** | ***35.25 ± 17.484** |
| Osmolality (mOsmol/kg) | 363 ± 5.773 | 368.917 ± 6.851 |
| Total Protein (g/dL) | 4.291 ± 0.241 | 4 ± 0.525 |

603

*Statistically different between diets
ALP=Alkaline phosphatase
AST=Aspartate aminotransferase

604

Body and tissue weights

Whole body and tissue weights are similar between fish fed either 0 or 4% wheat gluten with the exception of the mid-intestine ($p < 0.05$). This is not a typical place for major changes to the intestine to manifest, so this is an interesting result. These data are summarized in S7 Table: Body and tissue weights for fish fed 0 or 4% wheat gluten are similar with differences manifesting in the mid-intestine. Hepatosomatic indices were an average of 0.01 for both dietary groups.

Plasma taurine analysis

Though there were minimal differences between the diets for levels of most common plasma analytes, a separate analysis of plasma taurine levels by HPLC showed that levels were

2

615    approximately twice as high in the fish fed the diet containing wheat gluten (Figure 7). One

616    possible explanation is that the fish are producing or sequestering more taurine to counter some

617    effect induced by the wheat gluten.

618

619    **Figure 7: 4% dietary wheat gluten substantially raises plasma taurine levels in European sea**
620    **bass.**
621    Taurine levels in plasma were measured using HPLC. Average concentrations of plasma taurine
622    for fish fed the 0 or 4% wheat gluten diets are 33.82 ± 7.133 or 62.515 ± 15.719 nmol/mL,
623    respectively.
624
625

626    Gliadin immunoblotting

627        To detect plasma factors capable of binding to gliadin, varying concentrations of the

628    protein as well as a fixed amount of eIF4E-1A from the dinoflagellate *A. carterae* were subjected

629    to immunoblotting. eIF4E-1A, a translation protein, was used as a control for loading, transfer

630    efficiency, and specificity of binding. After immunoblotting, membranes were pre-incubated in

631    blocking buffer only or blocking buffer containing plasma. In the immunoblot, bands are visible

632    in the 30-40 kDa range corresponding to various α/β and γ gliadins (Figure 8) (44). These gliadin

633    bands are less visible over the course of 2-fold dilutions. There does not appear to be a

634    component in European sea bass plasma produced in response to dietary wheat gluten that is

635    capable of binding gliadin. This would be evidenced by decreased binding of the primary

636    antibody, and subsequently the secondary signal antibody. This is in contrast to data presented

637    in Chapter 2 showing that juvenile cobia produce a plasma factor capable of binding gliadin when

638    they are fed a diet containing 3.2-3.6% wheat gluten.

639

2

640 **Figure 8: Plasma factors capable of binding gliadin are not detectable in European sea bass fed**
641 **4% wheat gluten.**
642 Western blot analysis of gliadin without pre-incubation of plasma (a) or with pre-incubation with
643 plasma (b). No factors capable of binding to gliadin (30-40 kDa) and inhibiting binding of anti-
644 gliadin polyclonal antibody are detectable. Each lane also contains 431 ng recombinant *A.*
645 *carterae* protein eIF4E-1A (50 kDa).
646

647 Ig and IgM

648 We used immunoblotting to assay for IgT and IgM to see if dietary inclusion of wheat

649 gluten alters their levels in plasma. The results shown in Figure 9 suggest that levels do not

650 change in response to dietary wheat gluten. Any immune response mounted against gliadin or

651 some other component of wheat gluten is not likely due to an adaptive response, in this case.

652 The size of ~73 kDa for the heavy chains of the antibodies is similar to the size of ~78 kDa

653 detected by Picchietti et al. (45).

654

655 **Figure 9: Dietary wheat gluten does not induce changes to levels of plasma IgT or IgM.**
656 IgT and IgM were measured using immunoblotting, and their levels do not appear to be altered
657 by 4% dietary wheat gluten.
658

659 Intestinal microbiome analysis

660 To characterize the microbiome of the water, feed, and intestinal sections of the

661 European sea bass, 16S rRNA gene analysis was performed using the MiSeq platform. Figure 10

662 shows a bar graph of order-level taxonomic abundance. Proteobacteria dominate in the tank

663 water. The "cyanobacteria" in feed are most likely chloroplasts from plant ingredients. This is

664 also true for "cyanobacteria" in the pyloric caeca, which likely corresponds to undigested feed

665 despite the fact that food was withheld from these fish for 24 hours before tissue sampling. For

666 fish consuming no dietary gluten (Tank 6-11), the predominant phylum for all intestinal sections

667    analyzed (pyloric caeca, anterior intestine, mid-intestine, and posterior intestine) is

668    Proteobacteria. The same is true for the intestinal sections of the fish fed 4% gluten (Tank 6-12),

669    but there is a greater diversity of predominant orders of Proteobacteria, and Bacteroidetes

670    presents in the mid- and posterior intestines.

671

672    **Figure 10: European sea bass fed 4% wheat gluten as part of a plant-based diet exhibit a**
673    **greater diversity of predominant taxonomic orders across the intestine.**
674    DNA extracted from water, feed, and various sections of the intestine for the two diet groups
675    underwent *16S* rRNA gene analysis using the MiSeq platform to characterize the microbial
676    landscape. The addition of 4% wheat gluten to a plant-based diet dramatically shifts the
677    intestinal microbiome of European sea bass.
678
679    Figure 11 shows an alpha-diversity rarefaction curve based on OTU data. The PCA

680    (principal component analysis) plot in Figure 12 shows that samples cluster by absence or

681    presence of dietary wheat gluten. The ellipse shows the 95% confidence interval.

682

683    **Figure 11: Species richness for each microbiome sample.**
684    Species richness of the microbiome samples is shown in a rarefaction curve of observed OTUs vs.
685    sequences per sample.
686

687    **Figure 12: Samples cluster by absence or presence of dietary wheat gluten in a PCA plot.**
688    Samples cluster by absence or presence of dietary wheat gluten. The ellipse shows the 95%
689    confidence interval.
690

691    **Discussion**

692    Overall, in the cobia study, dietary wheat gluten negatively impacted growth and survival

693    in juvenile cobia, and taurine supplementation appeared to partially ameliorate those effects.

694    Negative effects observed in the first trial are not likely due to a poor cohort as other fish from

695    this brood used for separate feeding trials grew well on both fishmeal-based formulations and

3

696    commercial feeds in the IMET ARC facility. Palatability issues with plant protein diets have been

697    well described for many species, and it is known that taurine has the potential to serve as a feed

698    attractant due to its small nitrogenous structure (46,47). Increased feed palatability rather than

699    solely physiological benefits from taurine supplementation may have contributed to the

700    differences in growth and survival during the first trial. Concerns over palatability and the overall

701    formulation prompted the second trial, which was initiated with a size that readily accepted and

702    performed well on plant protein formulations. Although growth and feed conversion in the

703    second trial were lower than for some of the other plant protein formulations such as EPP3,

704    survival was 100% in all treatments, a significant improvement over the first trial. As with the first

705    trial, there was a significant increase in performance with increasing dietary taurine, indicating

706    that taurine is promoting growth and is possibly remediating the negative impacts of this

707    formulation.

708        The fact that levels of taurine and wheat flour are similar between the high-performing

709    EPP3 diet and the poorer performing PP3 diet suggests that 2.2% added wheat gluten is

710    contributing to a reduction in performance characteristics. There is the possibility that the soy

711    protein concentrate HP300 in EPP3 is greatly enhancing growth characteristics, but trials with

712    another diet, EPP2, point to the gluten as a critical component. EPP2 has an identical formulation

713    to PP3, except EPP3 contains no added wheat gluten with the exception of what is introduced

714    from the wheat flour itself (which is in slightly greater proportion). EPP2 contains roughly 1.8%

715    wheat gluten, and as was the case with EPP3, fish consuming this diet outperformed fish fed the

716    other plant protein-based diets.

717        The identity of the plasma factor(s) capable of binding gliadin cannot be determined

718    without the necessary reagents to isolate non-specific and specific immune factors in cobia. It is

719    probable that constituents of the cobia innate immune system recognize gliadin, the potentially

720    pathogenic component of wheat. Enzyme-solubilized wheat gluten extracts have been shown to

721    initiate the alternative complement pathway, and it has been suggested that similar to some

722    pathogens, gliadin may be capable of binding to toll-like receptors and initiating an innate

723    immune response (48,49).

724        Wheat gluten has been shown to have negative impacts on humans, often but not always

725    affiliated with celiac disease, and other mammalian models (50,51),(52,53). It is possible that IgT,

726    the primary mucosal antibody in teleosts, might play a similar role to human IgA. In gluten-

727    sensitive humans, IgA mediates the transport of gliadin across the gastrointestinal epithelium

728    and induces an adaptive immune response (54,55). Without purified antibodies against cobia IgM

729    or IgT, the role of adaptive immunity cannot be assessed. However, it is clear that ingestion of

730    wheat gluten triggers the mobilization or production of plasma factors capable of binding gliadin,

731    be they innate or adaptive.

732        The glutamine to glutamate deamidation reaction in gliadin catalyzed by tTG (tissue

733    transglutaminase) is instrumental in gluten-induced pathology. During transamidation, another

734    reaction catalyzed by tTG, the reaction of glutamine with lysine or lysine methyl ester abrogates

735    the T-cell mediated immunotoxic effects of gliadin in the intestine (56). It is possible that the

736    success of wheat gluten incorporation into many fish feeds is due in part to the addition of lysine

737    favoring the transamidation rather than deamidation of gluten catalyzed by tTG. Zebrafish fed a

738    diet containing 60% wheat gluten without adequate lysine supplementation had compromised

739    growth (57). A low pH environment favors deamidation over transamidation, which offers a

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740   possible explanation for why gluten may be less tolerated in the gastrointestinal tract of

741   carnivorous fish compared to omnivorous fish (58,59).

742         Taurine is present in several tissues and possesses powerful anti-inflammatory

743   properties.  It mitigates inflammation by scavenging free radicals, ameliorating oxidative injury,

744   and modulating the immune response (60). Intestinal enteritis has been observed in several

745   species with specific plant protein inclusion such as soy ingredients in salmon and carp  (61,62).

746   Wheat gluten may have triggered inflammation in the cobia intestinal tract, leading to the

747   proliferation of circulating plasma factor(s) capable of binding gliadin.

748         Previous studies have demonstrated the ability of taurine to mitigate degeneration of the

749   intestinal mucosa and inflammatory bowel disease. It is possible that taurine supplementation of

750   the cobia diet may partially counteract intestinal inflammation caused by dietary ingredients such

751   as wheat gluten (60,63). Taurine may also offer some protection in the gut against deamidated

752   gluten (64). Unfortunately, samples of the distal intestine were not preserved from fish in this

753   study. This would have been useful for histological analysis of any gluten-induced enteritis.

754         Genetic predisposition is the primary risk factor for CD in humans, but there is evidence

755   that early and abrupt introduction of gluten-containing foods into the infant diet alters the

756   microbiome and increases likelihood of developing the disease (65). Several microorganisms are

757   known to uptake taurine via Tau transporters for utilization as sulfur or carbon sources (66).

758   Though no characterization has been performed of the cobia microbiome, such analysis has been

759   performed in cats, another strict carnivore. Dietary studies have revealed that cats, like cobia,

760   require dietary intake of taurine due to insufficient synthesis. By virtue of the fact that they have

761   similar dietary needs (high protein: carbohydrate ratio) and metabolism (can only conjugate bile

762    acids to taurine), there may also be common commensal bacteria. Similar to cats, alterations to

763    populations of intestinal bacteria in cobia caused by intake of fermentable feed ingredients might

764    increase deconjugation of bile salts and contribute to taurine depletion (67). Rapid shifts in the

765    microbiome induced by diet are not isolated to carnivores, as it has also been well documented

766    in humans along with celiac disease-associated dysbiosis (68,69). In the case of juvenile cobia in

767    Trial 1, it is possible that gut immaturity and incomplete establishment of commensal microbiota

768    may have increased injurious effects from gluten. However, alterations to the normal carnivore

769    microbial landscape from ingredients such as gluten could have the potential to affect the overall

770    health of cobia at any stage of development.

771    The most significant physiological impacts of 4% dietary wheat gluten in European sea

772    bass are the substantial increase in plasma taurine and induction of greater diversity of

773    predominant taxonomic orders of intestine microbiota. These changes in no way suggest a

774    poorer fitness outcome, and there were no significant differences in growth between the two

775    groups. Therefore, there is no reason to contraindicate the addition of 4% wheat gluten addition

776    to a completely plant-based diet for European sea bass.

777    The common plasma measures of overall health that differed between the 0 and 4%

778    groups were calcium and AST. Plasma calcium was higher in the group fed 4% wheat gluten,

779    whereas plasma AST levels were lower than they were in the fish fed the diet without wheat

780    gluten. European sea bass stressed by hydrogen peroxide exposure exhibited higher levels of

781    plasma calcium, so it could be indicative of a health effect (70). AST is an indicator of liver health

782    and function, along with ALP (alkaline phosphatase) (71). In a fasting study in European sea bass

783    performed by Peres et al., AST levels increased during the fasting period (36).

784     Our values for all plasma markers measured are similar to those reported by Peres et al.

785     in European sea bass maintained on a fishmeal diet with the exception of cholesterol, ALP, and

786     AST. For ALP and AST, our values were substantially lower, almost half of the values obtained by

787     their group. This may be a function of a plant-based vs. fishmeal-based diet.  Changes to

788     cholesterol were not apparent in our study, but they have been in other studies with wheat

789     gluten as a feed component. Plasma cholesterol decreased in European sea bass fed diets with

790     graded levels of wheat gluten partially replacing up to 70% of the fishmeal as a protein source,

791     but that may be due to the greater proportion of plant protein incorporation rather than an effect

792     unique to wheat gluten (72). Interestingly, a partial replacement of fishmeal with corn as a

793     protein source and no added wheat gluten was shown to raise plasma cholesterol and

794     phospholipid levels. The authors attributed this effect to the higher carbohydrate content in the

795     diet containing corn (73). Different plant sources of protein may have a variety of influences on

796     plasma parameters.

797     The only change in measured tissue weights between diets was for the mid-intestine.

798     There have been no reports of major histological changes to only the mid-intestine prompted by

799     dietary ingredients. However, a gene expression study performed in European sea bass

800     suggested that there is functional specialization across the length of the intestinal tract (47).

801     Another study found that mid-intestine lactic acid bacteria (order *Lactobacillales*) are highly

802     modulated by diets in a recirculating aquaculture system (74).

803     There are no studies correlating wheat gluten to higher levels of taurine in plasma.

804     Recently there have been reports of diets for pets containing legumes marketed as "grain-free"

805     causing cardiomyopathies related to taurine deficiency (75). It is possible that a feed ingredient

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806    such as wheat gluten might be influencing sequestration of taurine in plasma. Alternatively,

807    endogenous levels of taurine may be increased as a result of dietary wheat gluten, perhaps to

808    counter some pro-inflammatory effect of the wheat gluten.

809         The lack of detectable induction of higher levels of plasma IgM, and IgT suggest a lack of

810    adaptive immune response. We also attempted to detect TNF-α in the plasma samples but

811    obtained a high standard error suggesting that our antibody was non-specific. There was no

812    detectable plasma factor capable of binding to gliadin. This is in contrast to our data presented

813    in Chapter 2 for cobia consuming a diet containing less than 4% wheat gluten in which a gliadin-

814    binding plasma factor(s) was produced.

815         The dietary inclusion of wheat gluten induced marked changes to the intestinal

816    microbiome. In the intestines of fish fed 4% wheat gluten, there were increased predominant

817    diversities of Proteobacteria as compared to the 0 wheat gluten group as well as the presence of

818    Bacteroidetes in the mid- and posterior intestines.  Several studies have linked dietary changes

819    to alterations in the intestinal microbiome, and a small number have probed for this effect

820    specifically with wheat gluten. Human studies in which participants shifted to a gluten-free diet

821    showed variations to the microbiome, though the most significant variation was inter-patient.

822    One of these studies found a significant decrease in the family *Veillonellaceae* of the *class*

823    *Clostridia* (76)⁻(77). One study in zebrafish in demonstrated that fish fed diets containing wheat

824    gluten (~50% of formulation) had heightened abundances of *Legionellales, Rhizobiaceae,* and

825    *Rhodobacter* over fishmeal-fed fish (78). In another study in zebrafish, fish fed wheat gluten had

826    decreased *Bifidobacterium* relative to fish fed brine shrimp  (79). Only an abstract could be

827    located for this study, so the actual percentage of wheat gluten is unknown. In a study of Atlantic

828     salmon, wheat gluten (~14-20% of formulation) mixed with a legume protein (soybean meal or

829     guar meal) increased abundance of lactic acid bacteria in the gut as compared to the reference

830     fishmeal diet (80). Our data do not appear to correlate with these other studies, but many of the

831     differences are likely attributable to the difference between species and the overall constitution

832     of the diet: Plant-based, fishmeal-based, or a combination. Overall, the study results do not seem

833     to indicate some type of disease state induced by the addition of 4% wheat gluten, as the fish

834     have overall health comparable to that of the fish consuming a dietary containing no wheat

835     gluten.

836         Taken together, these studies suggest that different species and life stages, even among

837     the marine carnivores, may have different reactions to dietary ingredients such as wheat gluten.

838     It is possible that the ability to synthesize taurine is significant in determining tolerance.

839     Preliminary data from another study performed by our laboratory in European sea bass using

840     diets containing 0 or 5% taurine suggest that unlike cobia, European sea bass are adequate

841     endogenous synthesizers of taurine and do not require supplementation. Future studies of the

842     effects of dietary wheat gluten in other marine carnivorous species with various taurine

843     requirements will clarify this connection. Additionally, the observed partial amelioration of the

844     negative effects induced by dietary wheat gluten in cobia should spur future research into

845     taurine's ability to mitigate effects elicited by other general stress factors.

846

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852

853     1.      Olsen RL, Hasan MR. A limited supply of fishmeal: Impact on future increases in global
854             aquaculture production. Trends in Food Science and Technology. 2012.
855     2.      Rust MB, Barrows FT, Hardy RW, Lazur A, Naughten K, Silverstein J. The Future of
856             Aquafeeds. NOAA Tech Memo NMFS F/SPO-124. 2011.
857     3.      Hardy RW. New developments in aquatic feed ingredients , and potential of enzyme
858             supplements. Av en Nutr Acuicola V Memorias del V Simp Int Nutr Acuicola. 2000.
859     4.      Apper-Bossard E, Feneuil A, Wagner A, Respondek F. Use of vital wheat gluten in
860             aquaculture feeds. Aquatic Biosystems. 2013.
861     5.      Wieser H. Chemistry of gluten proteins. Food Microbiol. 2007.
862     6.      Day L, Augustin MA, Batey IL, Wrigley CW. Wheat-gluten uses and industry needs. Trends
863             in Food Science and Technology. 2006.
864     7.      Gujral N, Freeman HJ, Thomson ABR. Celiac disease: Prevalence, diagnosis, pathogenesis
865             and treatment. World J Gastroenterol. 2012.
866     8.      Sollid LM. Coeliac disease: Dissecting a complex inflammatory disorder. Nature Reviews
867             Immunology. 2002.
868     9.      Di Sabatino A, Corazza GR. Coeliac disease. The Lancet. 2009.
869     10.     Barrows FT, Gaylord TG, Sealey WM, Porter L, Smith CE. The effect of vitamin premix in
870             extruded plant-based and fish meal based diets on growth efficiency and health of
871             rainbow trout, *Oncorhynchus mykiss*. Aquaculture. 2008.
872     11.     Gibson Gaylord T, Barrows FT, Teague AM, Johansen KA, Overturf KE, Shepherd B.
873             Supplementation of taurine and methionine to all-plant protein diets for rainbow trout
874             (*Oncorhynchus mykiss*). Aquaculture. 2007.
875     12.     Zhou QC, Wu ZH, Chi SY, Yang QH. Dietary lysine requirement of juvenile cobia
876             (*Rachycentron canadum*). Aquaculture. 2007.
877     13.     Salze GP, Davis DA. Taurine: A critical nutrient for future fish feeds. Aquaculture. 2015.
878     14.     Watson AM, Kissil GWM, Barrows FT, Place A. Developing a plant-based diet for cobia
879             (*Rachycentron canadum*). Int Aquafeed. 2012;15:34–8.
880     15.     Bagni M. Cultured Aquatic Species Information Programme. *Dicentrarchus labrax*.
881             [Internet]. FAO Fisheries and Aquaculture Department. 2005. Available from:
882             http://www.fao.org/fishery/culturedspecies/Dicentrarchus_labrax/en
883     16.     Kaushik SJ, Covès D, Dutto G, Blanc D. Almost total replacement of fish meal by plant
884             protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus
885             labrax*. Aquaculture. 2004.
886     17.     Geay F, Ferraresso S, Zambonino-Infante JL, Bargelloni L, Quentel C, Vandeputte M, et al.
887             Effects of the total replacement of fish-based diet with plant-based diet on the hepatic
888             transcriptome of two European sea bass (*Dicentrarchus labrax*) half-sibfamilies showing
889             different growth rates with the plant-based diet. BMC Genomics. 2011.
890     18.     Danilova N, Bussmann J, Jekosch K, Steiner LA. The immunoglobulin heavy-chain locus in

891        zebrafish: Identification and expression of a previously unknown isotype,
892        immunoglobulin Z. Nat Immunol. 2005.

893  19.  Hansen JD, Landis ED, Phillips RB. Discovery of a unique Ig heavy-chain isotype (IgT) in
894        rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish.
895        Proc Natl Acad Sci. 2005.

896  20.  Xu Z, Parra D, Gomez D, Salinas I, Zhang Y-A, von Gersdorff Jorgensen L, et al. Teleost
897        skin, an ancient mucosal surface that elicits gut-like immune responses. Proc Natl Acad
898        Sci. 2013.

899  21.  Egerton S, Culloty S, Whooley J, Stanton C, Ross RP. The gut microbiota of marine fish.
900        Frontiers in Microbiology. 2018.

901  22.  Ringø E, Sperstad S, Myklebust R, Refstie S, Krogdahl Å. Characterisation of the
902        microbiota associated with intestine of Atlantic cod (*Gadus morhua*). Aquaculture. 2006;

903  23.  Green TJ, Smullen R, Barnes AC. Dietary soybean protein concentrate-induced intestinal
904        disorder in marine farmed Atlantic salmon, *Salmo salar* is associated with alterations in
905        gut microbiota. Vet Microbiol. 2013.

906  24.  Guerreiro I, Serra CR, Oliva-Teles A, Enes P. Short communication: gut microbiota of
907        European sea bass (*Dicentrarchus labrax*) is modulated by short-chain
908        fructooligosaccharides and xylooligosaccharides. Aquac Int. 2018.

909  25.  Huebner FR, Wall JS, Regional N. Fractionation and Quantitative Differences of Glutenin
910        from Wheat Varieties Varying in Baking Quality. Cereal Chem. 1974.

911  26.  Coleman BR, Iqbal S, Godfrey PP, Billington D, Box PO, Birmingham B. Membranes and
912        Bile Formation: Composition of Several Mammalian Biles and their Membrane-Damaging
913        Properties. J Biochem. 1979.

914  27.  Chaimbault P, Alberic P, Elfakir C, Lafosse M. Development of an LC-MS-MS method for
915        the quantification of taurine derivatives in marine invertebrates. Anal Biochem. 2004.

916  28.  Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of
917        general 16S ribosomal RNA gene PCR primers for classical and next-generation
918        sequencing-based diversity studies. Nucleic Acids Res. 2013.

919  29.  Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME
920        allows analysis of high-throughput community sequencing data. Nature Methods. 2010.

921  30.  Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal
922        RNA gene database project: Improved data processing and web-based tools. Nucleic
923        Acids Res. 2013.

924  31.  Caporaso JG, Bittinger K, Bushman FD, Desantis TZ, Andersen GL, Knight R. PyNAST: A
925        flexible tool for aligning sequences to a template alignment. Bioinformatics. 2010.

926  32.  R Development Core Team R. R: A Language and Environment for Statistical Computing.
927        R Foundation for Statistical Computing. 2011.

928  33.  Watson AM, Barrows FT, Place AR. Effects of Graded Taurine Levels on Juvenile Cobia. N
929        Am J Aquac. 2014;

930  34.  Rehulka J, Minarik B, Adamec V, Rehulkova E. Investigations of physiological and
931        pathological levels of total plasma protein in rainbow trout, *Oncorhynchus mykiss*
932        (Walbaum). Aquac Res. 2005.

933  35.  Maita M. Dietary Supplements for the Health and Quality of Cultured Fish. Fish Heal
934        Assessment, Intl. 2007;10–34.

4

935   36.   Peres H, Santos S, Oliva-Teles A. Blood chemistry profile as indicator of nutritional status
936         in European seabass (*Dicentrarchus labrax*). Fish Physiol Biochem. 2014.
937   37.   Gowda S, Desai PB, Hull V V, Math AAK, Vernekar SN, Kulkarni SS. A review on laboratory
938         liver function tests. Pan Afr Med J. 2009.
939   38.   Rosalki SB. Low serum creatine kinase activity. Clinical chemistry. 1998.
940   39.   P Sharma O. Clinical Biochemistry of Hepatotoxicity. J Clin Toxicol. 2014.
941   40.   Wyllie R, Hyams JS, Kay M. Pediatric Gastrointestinal and Liver Disease. Pediatric
942         Gastrointestinal and Liver Disease. 2006.
943   41.   Eames SC, Philipson LH, Prince VE, Kinkel MD. Blood Sugar Measurement in Zebrafish
944         Reveals Dynamics of Glucose Homeostasis. Zebrafish. 2010.
945   42.   Gentile CL, Nivala AM, Gonzales JC, Pfaffenbach KT, Wang D, Wei Y  et al. Experimental
946         evidence for therapeutic potential of taurine in the treatment of nonalcoholic fatty liver
947         disease. Am J Physiol Regul Integr Comp Physiol. 2011;301(6):1710–22.
948   43.   Fillatreau S, Six A, Magadan S, Castro R, Sunyer JO, Boudinot P. The astonishing diversity
949         of Ig classes and B cell repertoires in teleost fish. Frontiers in Immunology. 2013.
950   44.   Urade R, Sato N, Sugiyama M. Gliadins from wheat grain: an overview, from primary
951         structure to nanostructures of aggregates. Biophysical Reviews. 2018.
952   45.   Picchietti S, Nuñez-Ortiz N, Stocchi V, Randelli E, Buonocore F, Guerra L, et al. Evolution
953         of lymphocytes. Immunoglobulin T of the teleost sea bass (*Dicentrarchus labrax*):
954         Quantitation of gene expressing and immunoreactive cells. Fish Shellfish Immunol. 2017.
955   46.   Glencross BD, Booth M, Allan GL. A feed is only as good as its ingredients - A review of
956         ingredient evaluation strategies for aquaculture feeds. Aquaculture Nutrition. 2007.
957   47.   Gómez-Requeni P, Mingarro M, Calduch-Giner JA, Médale F, Martin SAM, Houlihan DF, et
958         al. Protein growth performance, amino acid utilisation and somatotropic axis
959         responsiveness to fish meal replacement by plant protein sources in gilthead sea bream
960         (*Sparus aurata*). Aquaculture. 2004.
961   48.   Unsworth DJ, Würzner R, Brown DL, Lachmann PJ. Extracts of wheat gluten activate
962         complement via the alternative pathway. Clin Exp Immunol. 1993.
963   49.   Moossavi S. Gliadin is an uncatalogued Toll-like receptor ligand. J Med Hypotheses Ideas.
964         2014.
965   50.   Briani C, Samaroo D, Alaedini A. Celiac disease: From gluten to autoimmunity.
966         Autoimmunity Reviews. 2008.
967   51.   Kuiper HA KG. The scientific basis for risk assessment and regulation of genetically
968         modified foods. Trends Food Sci Technol. 2003;14(5):277–93.
969   52.   Penttila IA, Devery JM, Gibson CE, LaBrooy JT, Skerritt JH. Cellular and humoral responses
970         in coeliac disease. 1. Wheat protein fractions. Clin Chim Acta. 1991.
971   53.   Rivabene R, Mancini E, De Vincenzi M. In vitro cytotoxic effect of wheat gliadin-derived
972         peptides on the Caco-2 intestinal cell line is associated with intracellular oxidative
973         imbalance: Implications for coeliac disease. Biochim Biophys Acta - Mol Basis Dis. 1999.
974   54.   Heyman M, Menard S. Pathways of gliadin transport in celiac disease. Ann N Y Acad Sci.
975         2009.
976   55.   Matysiak-Budnik T, Moura IC, Arcos-Fajardo M, Lebreton C, Ménard S, Candalh C, et al.
977         Secretory IgA mediates retrotranscytosis of intact gliadin peptides via the transferrin
978         receptor in celiac disease. J Exp Med. 2008.

4

979  56.  Gianfrani C, Siciliano RA, Facchiano AM, Camarca A, Mazzeo MF, Costantini S, et al.
980       Transamidation of Wheat Flour Inhibits the Response to Gliadin of Intestinal T Cells in
981       Celiac Disease. Gastroenterology. 2007.
982  57.  Smith DL, Barry RJ, Powell ML, Nagy TR, D'Abramo LR, Watts SA. Dietary Protein Source
983       Influence on Body Size and Composition in Growing Zebrafish. Zebrafish. 2013.
984  58.  Day RD, Tibbetts IR, Secor SM. Physiological responses to short-term fasting among
985       herbivorous, omnivorous, and carnivorous fishes. J Comp Physiol B Biochem Syst Environ
986       Physiol. 2014.
987  59.  Fleckenstein B, Molberg Ø, Qiao SW, Schmid DG, Von Mülbe F Der, Elgstøen K, et al.
988       Gliadin T cell epitope selection by tissue transglutaminase in celiac disease. Role of
989       enzyme specificity and pH influence on the transamidation versus deamidation reactions.
990       J Biol Chem. 2002.
991  60.  Çetiner M, Şener G, Şehirli AÖ, Ekşioğlu-Demiralp E, Ercan F, Şirvanci S, et al. Taurine
992       protects against methotrexate-induced toxicity and inhibits leukocyte death. Toxicol Appl
993       Pharmacol. 2005.
994  61.  Romarheim OH, Overland M, Mydland LT, Skrede A, Landsverk T. Bacteria Grown on
995       Natural Gas Prevent Soybean Meal-Induced Enteritis in Atlantic Salmon. J Nutr. 2011.
996  62.  Urán PA, Gonçalves AA, Taverne-Thiele JJ, Schrama JW, Verreth JAJ, Rombout JHWM.
997       Soybean meal induces intestinal inflammation in common carp (*Cyprinus carpio L*.). Fish
998       Shellfish Immunol. 2008.
999  63.  Son M, Kim HK, Kim WB, Yang J, Kim BK. Protective effect of taurine on indomethacin-
1000      induced gastric mucosal injury. Arch Pharm Res. 1996.
1001 64.  Ripps H, Shen W. Review: Taurine: A "very essential" amino zcid. Mol Vis. 2012;
1002 65.  Myléus A, Ivarsson A, Webb C, Danielsson L, Hernell O, Högberg L, et al. Celiac disease
1003      revealed in 3% of Swedish 12-year-olds born during an epidemic. J Pediatr Gastroenterol
1004      Nutr. 2009.
1005 66.  Cook AM, Denger K. Metabolism of taurine in microorganisms: A primer in molecular
1006      biodiversity? In: Advances in Experimental Medicine and Biology. 2006.
1007 67.  Backus RC, Rogers QR, Morris JG. Microbial degradation of taurine in fecal cultures from
1008      cats given commercial and purified diets. J Nutr. 1994.
1009 68.  David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet
1010      rapidly and reproducibly alters the human gut microbiome. Nature. 2014.
1011 69.  Sjöberg V, Sandström O, Hedberg M, Hammarström S, Hernell O, Hammarström ML.
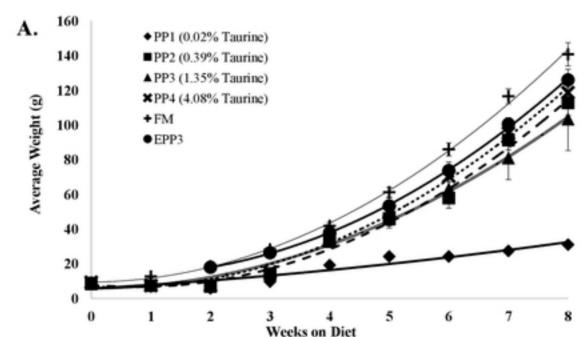1012      Intestinal T-cell Responses in Celiac Disease - Impact of Celiac Disease Associated
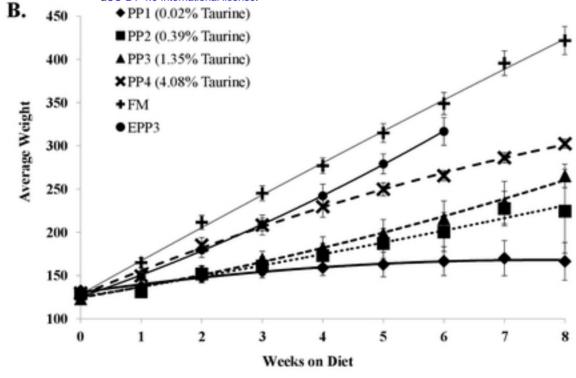1013      Bacteria. PLoS One. 2013.
1014 70.  Roque A, Yildiz HY, Carazo I, Duncan N. Physiological stress responses of sea bass
1015      (*Dicentrarchus labrax*) to hydrogen peroxide (H2O2) exposure. Aquaculture. 2010.
1016 71.  Al-Khashali MS, Al-Shawi SAS. Effect of salt stress on ALT and AST enzymes activity and
1017      cortisol level in adults of *Carassius auratus*. Pakistan J Nutr. 2013.
1018 72.  Messina M, Piccolo G, Tulli F, Messina CM, Cardinaletti G, Tibaldi E. Lipid composition
1019      and metabolism of European sea bass (*Dicentrarchus labrax L*.) fed diets containing
1020      wheat gluten and legume meals as substitutes for fish meal. Aquaculture. 2013.
1021 73.  Castro, C, Corraze G, Perez-Jimenez A, Larroquet L, Cluzeaud M, Panserat S O-TA. Dietary
1022      carbohydrate and lipid source affect cholesterol metabolism of Eu.opean sea bass
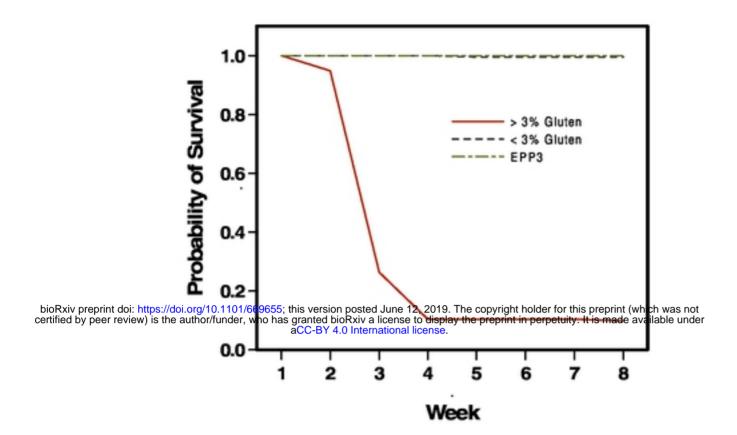
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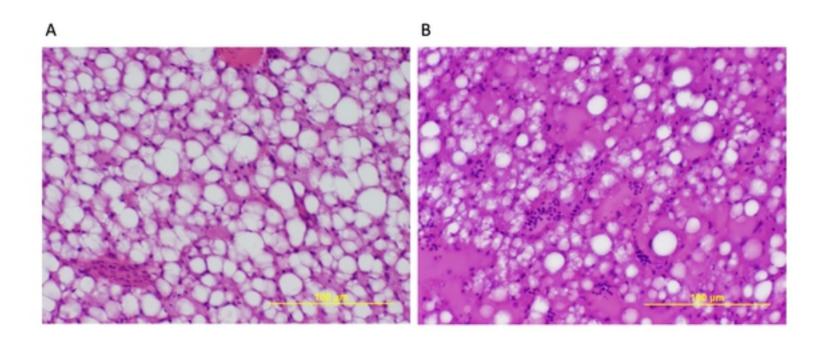1023         (*Dicentrarchus labrax*) juveniles. Br J Nutr. 2015;114(8):1143–56.
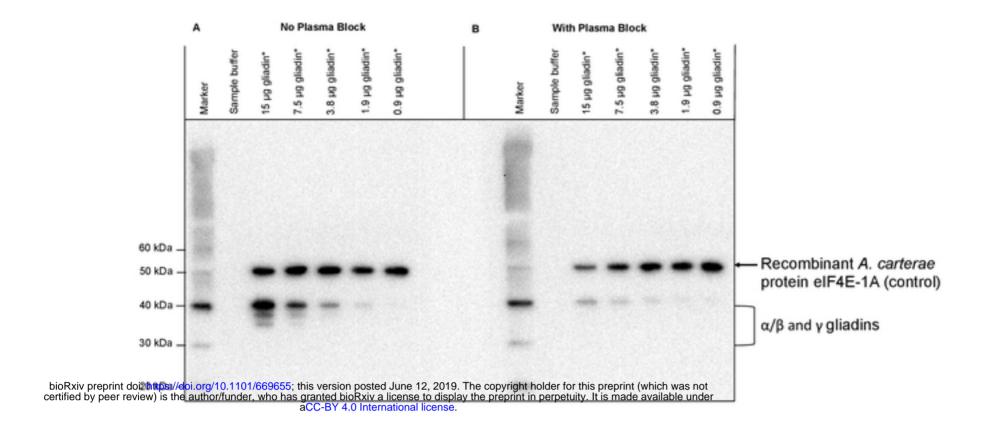
1024 74. Schmidt V, Amaral-Zettler L, Davidson J, Summerfelt S, Good C. Influence of fishmeal-free
1025      diets on microbial communities in Atlantic Salmon (*Salmo Sala*r) recirculation
1026      aquaculture systems. Appl Environ Microbiol. 2016.

1027 75. Freeman LM. A broken heart: Risk of heart disease in boutique or grain-free diets and
1028      exotic ingredients [Internet]. 2018. Available from:
1029      http://vetnutrition.tufts.edu/2018/06/a-broken-heart-risk-of-heart-disease-in-boutique-
1030      or-grain-free-diets-and-exotic-ingredients/

1031 76. Bonder MJ, Tigchelaar EF, Cai X, Trynka G, Cenit MC, Hrdlickova B, et al. The influence of
1032      a short-term gluten-free diet on the human gut microbiome. Genome Med. 2016.

1033 77. Nylund L, Kaukinen K, Lindfors K. The microbiota as a component of the celiac disease
1034      and non-celiac gluten sensitivity. Clin Nutr Exp. 2016.

1035 78. Koo H, Hakim JA, Powell ML, Kumar R, Eipers PG, Morrow CD, et al. Metagenomics
1036      approach to the study of the gut microbiome structure and function in zebrafish *Danio*
1037      *rerio* fed with gluten formulated diet. J Microbiol Methods. 2017.

1038 79. Savarese K, Davis Tousignant K, Uno J. The effect of wheat products on the colonization
1039      of the microbiota in the intestines of zebrafish (903.2). FASEB. 2014;28(1).

1040 80. Gajardo K, Jaramillo-Torres A, Kortner TM, Merrifield DL, Tinsley J, Bakke AM, et al.
1041      Alternative protein sources in the diet modulate microbiota and functionality in the distal
1042      intestine of Atlantic salmon (*Salmo salar*). Appl Environ Microbiol. 2017.

1043

Figure 1

Figure 2

Figure 3

Figure 4

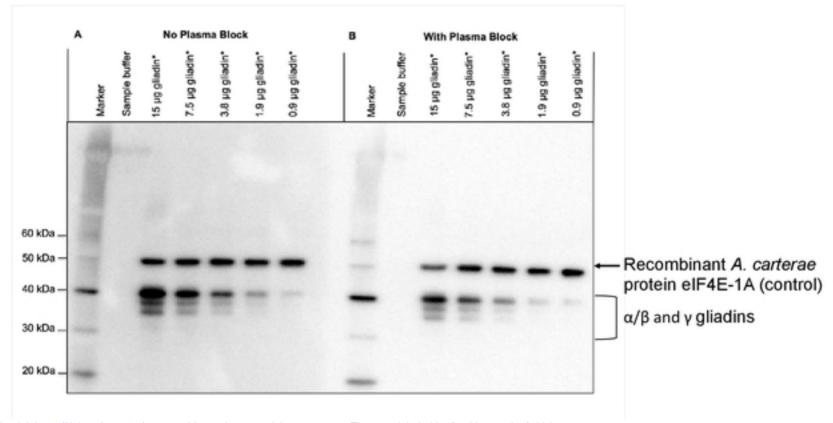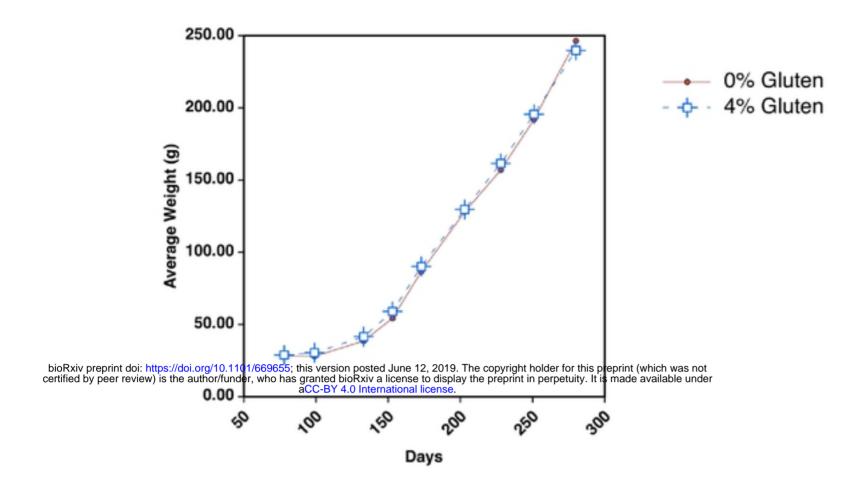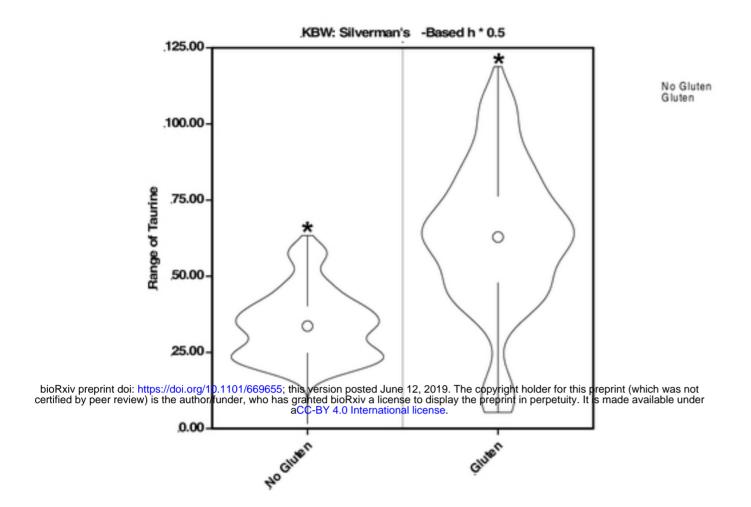Figure 5

Figure 6

KBW: Silverman's -Based h * 0.5

Range of Taurine

No Gluten
Gluten

*Statistically different between diets

Figure 7

Figure 8

73 kDa ——

| 0 Gluten-1 | 0 Gluten-2 | 0 Gluten-3 | 4% Gluten-1 | 4% Gluten-2 | 4% Gluten-3 | 0 Gluten-1 | 0 Gluten-2 | 0 Gluten-3 | 4% Gluten-1 | 4% Gluten-2 | 4% Gluten-3 |

———— IgT ———— ———— IgM ————

# Figure 9

Order Level Abundance

Figure 10

observed_otus: Name

| | |
|---|---|
| ■ 6-7 anterior intestine fish 4 | ■ 6-8 anterior intestine fish 8 |
| ■ 6-7 mid intestine fish 4 | ■ 6-8 anterior intestine fish 9 |
| ■ 6-7 posterior intestine fish 2 | ■ 6-8 anterior intestine fish 10 |
| ■ 6-7 posterior intestine fish 3 | ■ 6-8 mid intestine fish 8 |
| ■ 6-7 posterior intestine fish 4 | ■ 6-8 mid intestine fish 9 |
| ■ 6-7 pyloric caeca fish 2 | ■ 6-8 mid intestine fish 10 |
| ■ 6-7 pyloric caeca fish 4 | ■ 6-8 mid intestine fish 8 |
| | ■ 6-8 mid intestine fish 9 |
| | ■ 6-8 posterior intestine fish 10 |
| | ■ 6-8 pyloric caeca fish 8 |
| | ■ 6-8 pyloric caeca fish 9 |

| | |
|---|---|
| ■ Feed from 6-7 |
| ■ Feed from 6-8 |
| ■ Water from 6-7 |
| ■ Water from 6-8 |

Figure 11

Figure 12