

1 Excessive *Aurantiochytrium acetophilum* docosahexaenoic acid supplementation decreases
2 growth performance and breast muscle mass of broiler chickens

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12 **Abstract:**

13 Docosahexaenoic acid (DHA) is an n-3 polyunsaturated fatty acid with health-promoting
14 potential. This study was to investigate effects of supplemental DHA from *Aurantiochytrium*
15 *acetophilum* on growth performance, health status, meat quality, and protein synthesis signaling
16 of broiler chickens. Day-old male chicks were housed in an environmental control room (6
17 cages/treatment, 8 chicks/cage), and fed a corn-soybean meal basal diet supplemented with the
18 DHA-rich *A. acetophilum* biomass (Heliae, Gilbert, AZ) at 0, 1, 2, and 4% (0, 1.7, 3.4 and 6.8 g
19 DHA/kg diet) for 6 weeks. Growth performance were measured weekly. Blood samples were
20 collected at weeks 3 and 6 (2 chicks/cage). Four tissues were sampled (2 chicks/cage) for
21 biochemical and meat quality analyses. Data were analyzed by one-way ANOVA and regression.
22 Compared with the control, the 4% *A. acetophilum* diet decreased ($P < 0.05$) body weight gain
23 (19%) and gain to feed ratio (19%) during weeks 4-6. The *A. acetophilum* supplementation dose-
24 dependently decreased ($P < 0.05$, $R^2 = 0.21-0.54$) plasma alanine amino transferase activity and
25 glucose concentrations, but had little effect on plasma activity of alkaline phosphatase or
26 concentrations of inorganic phosphorus and uric acid at weeks 3 and 6. Compared with the
27 control, the 4% *A. acetophilum* diet decreased ($P < 0.05$) breast muscle weight by 21%, and
28 down-regulated ($P < 0.05$) mRNA levels of mammalian target of rapamycin and ribosomal s6
29 protein (S6), and protein levels of phosphorylated S6 to S6 and phosphorylated S6 kinase beta 1
30 to S6 kinase beta 1. The *A. acetophilum* supplementation linearly increased ($P < 0.01$) lipid
31 peroxidation ($R^2 = 0.62-0.90$) and hardness and chewiness ($R^2 = 0.34-0.44$) of breast and thigh
32 muscles. In conclusion, supplemental 4% (6.8 g DHA/kg), but not 1 or 2%, of *A. acetophilum*
33 impaired growth performance, breast muscle mass accumulation, and(or) protein synthesis
34 signaling of broilers.

35 *Keywords:* Broiler, DHA, Meat quality, *Aurantiochytrium*, Protein synthesis

36

37 *Abbreviation:* AKP, plasma alkaline phosphatase; ALT, plasma alanine aminotransferase;

38 AMPK, AMP-activated protein kinase; DHA, Docosahexaenoic acid; EPA, eicosapentaenoic

39 acid; MAFbx, muscle atrophy F-box; MDA, malondialdehyde; mTOR, mammalian target of

40 rapamycin; MURF1, muscle RING-finger protein-1; PIP, plasma inorganic phosphorus; P-P70,

41 phosphorylated P70; P-S6, phosphorylated S6; P70, ribosomal protein S6 kinase B1; S6,

42 ribosomal protein S6; S6K1, ribosomal protein S6 kinase B1; TPA, texture profile analysis;

43 TRAP, tartrate-resistant acid phosphatase; WHC, water holding capacity; 4E-BP1, eukaryotic

44 translation initiation factor 4E-binding protein 1.

45

46 **Highlights:**

- 47 • Supplemental *A. acetophilum* DHA at 6.8 g/kg diet impaired growth of chickens.
- 48 • Supplemental *A. acetophilum* DHA at 6.8 g/kg diet decreased breast muscle weight.
- 49 • Supplemental *A. acetophilum* DHA decreased plasma glucose concentrations of chickens.
- 50 • Supplemental *A. acetophilum* DHA elevated meat chewiness, hardness, and peroxides.
- 51 • *A. acetophilum* DHA at 6.8 g/kg diet modulated muscle protein synthesis signaling.

52 1. Introduction

53 Docosahexaenoic acid (DHA) is an omega-3 polyunsaturated fatty acid (n-3 PUFA), and
54 an important component of human neural and retina tissues [1]. Due to their anti-inflammatory
55 and hypolipidemic effects, elevating intakes of DHA and eicosapentaenoic acid (EPA, another n-
56 3 PUFA), may lower risks of cardiovascular disease [2], non-alcoholic fatty liver [3],
57 hypertriglycerolemia [4], and Alzheimer disease [5]. Therefore, the World Health
58 Organization recommended daily intakes of DHA and EPA at 1 to 2% of total energy [6], and
59 the European Food Safety Authority recommended daily intakes at 250 mg of DHA and EPA [7].
60 Because fish is an excellent source of n-3PUFA and fish consumption is rather low in many parts
61 of the world, many populations are unable to ingest those recommended amounts of DHA and
62 EPA [8]. Consequently, alternative food sources of DHA and EPA have been actively sought to
63 overcome their inadequacies.

64 One of the most economical and feasible approaches for generating such food source is to
65 feed broiler chickens with DHA and EPA rich ingredients. These ingredients include linseed oil
66 [9], fishmeal [10], fish oil [11–13], flaxseed oil [12,14], and microalgae or protists [15–20].
67 Among the available and tested single cell biomass, *Aurantiochytrium*, a heterotopic marine
68 microorganism in the *Thraustochytrids* family, has high cell density and DHA content [21].
69 Taxonomically, *Aurantiochytrium* is a protist, but is often called microalgae and compared with
70 DHA-producing microalgae species. Supplementing moderate amounts of *Aurantiochytrium* (1%
71 to 2% or 2.9 to 5.8 g DHA/kg of diet) to diets effectively enriched DHA in poultry meat, without
72 impairing growth performance or meat quality [16,18,19]. In fact, our laboratory fed hatchling
73 Cornish male broilers a corn–soybean meal basal diet supplemented with the DHA-rich
74 *Aurantiochytrium acetophilum* at 0%, 1%, 2%, and 4% for 6 wk and demonstrated dose-

75 dependent enrichments of DHA and decreases of n-6/n-3 fatty acids (FAs) in plasma, liver,
76 muscle, and adipose tissue [22]. However, that study did not determine effects of the graded
77 levels of the *Aurantiochytrium* supplementation, in particular the highest dose of *A. acetophilum*
78 DHA (6.8 g/kg diet), on growth performance, health status, or muscle protein synthesis.

79 Several groups have explored supplemental dietary DHA and(or) microalgae on muscle
80 protein synthesis in different species. In healthy young and middle-aged adults, an increased
81 dietary n-3 PUFA/DHA intake stimulated muscle protein synthesis and activated the elements of
82 the mammalian target of rapamycin (mTOR)/ribosomal protein S6 kinase (S6K1) signaling
83 pathway [23]. Feeding pigs a DHA-enriched diet promoted muscle growth, along with
84 upregulated mTOR and downregulated eukaryotic translation initiation factor 4E-binding protein
85 1 (4E-BP1) [24]. Likewise, feeding broiler chickens 4 to 8% defatted *Nannochloropsis oceanica*
86 microalgae enhanced the protein production of mTOR, phosphorylated ribosomal protein S6 (P-
87 S6), 4E-BP1, eukaryotic translation initiation factor 4E (eIF4E) in the breast muscle without
88 affecting growth performance [25]. In another study, feeding broilers 15% defatted
89 *Desmodesmus sp.* elevated mTOR protein expression in the breast and liver, while decreased
90 protein level of PS6 [26]. Apparently, supplemental dietary DHA and microalgae could affect
91 muscle protein synthesis and(or) signaling, but that type of effect and its correlation with growth
92 performance and the DHA enrichment remain inconclusive.

93 Therefore, the objective of this study was to extent our above-described research [22] and
94 to reveal effects of the graded levels of DHA-rich *A. acetophilum* supplementation on growth
95 performance, health status, muscle mass, meat quality, and protein synthesis signaling of broiler
96 chickens.

97

98 **2. Materials and methods**

99 *2.1 Animal, diets, and management*

100 Detailed animal experimental design, diet preparation, and daily care were reported
101 previously [22]. Briefly, a total of 192 Cornish Broiler chicks was purchased from local hatchery
102 (Moyer's Hatchery, PA) and housed in an environment-controlled room for 6 weeks. Broiler
103 chicks were allotted to 4 treatments (6 cages/treatment, 8 chicks/cage) to keep similar body
104 weights between cages at day 0. The DHA-rich biomass (*A. acetophilum*, Heliae, Gilbert, AZ,
105 USA) was supplemented as 0, 1, 2, 4% (to provide 0, 1.7, 3.4 and 6.8 g DHA/kg diet) in a corn-
106 soybean meal basal diet. All experimental diets were isocaloric and isoproteinic with balanced
107 amino acid profile and formulated to meet the requirement of all nutrients by chickens. Our
108 experimental protocols were approved by the Institutional Animal Care and Use Committee of
109 Cornell University.

110

111 *2.2 Sample collection and biochemical analysis*

112 At the end of weeks 3 and 6, two chicks from each cage were euthanized by carbon
113 dioxide after 8 h fasting and blood was drawn from heart by heparinized needle. Then, blood
114 plasma was separated at 3000 x g centrifugation for 15 min for further analysis. Activities of
115 plasma alanine aminotransferase (ALT) was determined by Infinity ALT liquid stable reagent kit
116 (Thermo Scientific, Waltham, MA, USA). Plasma alkaline phosphatase (AKP) and tartrate-
117 resistant acid phosphatase (TRAP) was analyzed using the methods of Bowers et al. [27] and Lau
118 et al. [28], respectively. Plasma inorganic phosphorus (PIP) concentrations were determined as
119 described previously [29]. Plasma glucose and uric acid concentrations were determined using

120 assay kits of Sigma Aldrich (St. Louis, Missouri, USA) and of Thermo Scientific, Inc. (Waltham,
121 MA, USA), respectively.

122

123 *2.3 Carcass yield, meat quality, and malondialdehyde (MDA) levels*

124 Texture profile analysis (TPA) was performed by measuring the compression force of
125 meat samples using the texture analyzer (TA.XT*plus*, Stable Micro Systems, Hamilton, MA,
126 USA). Frozen breast and thigh muscle were thawed and cut into 0.5-inch diameter cubes, cooked
127 in oven at 175°C for 30 min, then subjected to analysis of chewiness, springiness, and hardness
128 using the method of Huidobro et al [30]. The meat pH was determined using a previous method
129 [31] with modifications: 250 mg of meat was homogenized for 30 sec in 2.5 mL of solution
130 composed of 5 mM sodium iodoacetate and 150 mM potassium chloride at pH 7. The mixture
131 pH was measured using Accumet pH probe (AB150, Fisher Scientific, Waltham, MA, USA).
132 The water holding capacity (WHC) was determined using a centrifugal method [32,33].
133 Approximately 1 g of meat was weighed into a centrifuge tube with three pieces of Whatman
134 filter paper folded into a thimble. The tube and its contents were subjected to 7,710 g force for 30
135 min and the meat residue were separated from the filter papers and weighed to determine the
136 WHC. Malondialdehyde (MDA) was measured using a method modified from Jo et al. and
137 1,1,3,3-tetraethoxypropane as the standard [34].

138

139 *2.4 Real-time PCR and immunoblotting*

140 Abundances of mRNA of genes involved in the protein anabolic or catabolic pathway,
141 including AMP-activated protein kinase (AMPK), mTOR, S6K1, 4E-BP1, muscle RING-finger
142 protein-1 (MURF1), muscle atrophy F-box (MAFbx), in the breast and liver were quantified. The

143 primer sequences were shown in **Supplemental Table 1**. TRIzol Reagent (Life Technologies,
144 Carlsbad, CA) was used to isolate total mRNA from tissue homogenates, then the resultant RNA
145 was reverse transcribed to cDNA by High-Capacity cDNA Reverse Transcription Kit (Applied
146 Biosystems, Grand Island, NY, USA) and detected by Real-time qPCR (7900 HT; Applied
147 Biosystems). The relative quantification of gene expression for each sample was adjusted with
148 the control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using the 2-delta delta
149 Ct ($\Delta\Delta Ct$) equation [35] and normalized to the control group (as ‘‘1’’). Western blot analyses of
150 proteins in mTOR/ S6K1/S6 pathway such as mTOR, ribosomal protein S6 (S6), P-S6,
151 ribosomal protein S6 kinase B1 (P70), phosphorylated P70 (P-P70) were quantified in the breast
152 and liver as previously described [36]. Information on the primary antibodies used for Western
153 blot analysis is presented in **Supplemental Table 2**. The relative density of the protein bands
154 was quantified by using ImageJ software (NIH) and normalized to GAPDH as the loading
155 control [37].

156

157 **3. Results**

158 *3.1 Growth performance*

159 Growth performance including body weight gain (BWG), feed intake (FI) and gain: feed
160 ratio (G: F ratio) are summarized during grower (weeks 0-3), finisher (weeks 4-6) and overall
161 (weeks 0-6) period in **Table 1**. Compared with the control group, birds fed 1% or 2% DHA-rich
162 *A. acetophilum* had similar BWG, FI and G: F ratio, whereas those fed 4% DHA-rich *A.*
163 *acetophilum* had lower ($p < 0.05$) BWG (13 to 19%) and G: F (15 to 19%) during finisher and
164 overall periods. Regression analyses indicated that supplemental DHA-rich *A. acetophilum*

165 resulted in linear decreases of BWG and G: F ratio ($p < 0.01$, $R^2 = 0.32$ to 0.60) during finisher
166 and overall periods.

167

168 3.2 Plasma health parameters

169 Supplemental DHA-rich *A. acetophilum* caused dose-dependent linear decreases ($p <$
170 0.01 , $R^2 = 0.34$) of plasma ALT activity at week 6 and plasma glucose concentrations ($p < 0.05$,
171 $R^2 = 0.22$ to 0.54) at weeks 3 and 6, without effects on plasma AKP activities or PIP
172 concentrations at either week (**Table 2**). Plasma uric acid concentration was 1.1-fold greater ($p <$
173 0.05) in chickens fed 2% DHA-rich *A. acetophilum* than the control at week 3.

174

175 3.3 Meat yield and quality

176 Supplemental DHA-rich *A. acetophilum* produced no significant differences in carcass
177 weights or dressing percentage at week 6 (**Table 3**). However, the supplementation caused dose-
178 dependent linear decreases ($p < 0.05$, $R^2 = 0.29$ to 0.4) in the breast muscle weight and its
179 relative percentage of the muscle. Moreover, supplementing 4% DHA-rich *A. acetophilum*
180 lowered ($p < 0.05$) the breast muscle weight and the relative percentage of muscle by 21% and
181 12%, respectively, compared with the controls. The supplementation did not affect thigh muscle
182 weight, but produced a weak linear decrease in the relative percentage of muscle ($p < 0.05$, $R^2 =$
183 0.18). The supplementation caused a linear increase trend ($p < 0.05$, $R^2 = 0.18$ to 0.22) in the
184 relative heart weight to the body weight and the liver weight.

185 The supplementation of d DHA-rich *A. acetophilum* did not affect WHC, pH, or
186 springiness of the breast or thigh muscles. In contrast, the supplementation produced linear
187 increases ($p < 0.01$, $R^2 = 0.34$ to 0.44) in chewiness and hardness of the breast and thigh muscles.

188 Birds fed supplemental DHA-rich *A. acetophilum* had 58% to 118% higher ($p < 0.05$) of
189 chewiness and hardness in the breast and thigh muscles than the birds fed the control diet. In
190 addition, the supplementation caused linear increases ($p < 0.05$, $R^2 = 0.62$ to 0.91) in the MDA
191 concentrations in the breast, thigh, liver and adipose tissue of chickens at week 6 (**Table 4**).

192

193 3.4 Expression of genes and proteins related to protein synthesis signaling

194 The 4% DHA-rich *A. acetophilum* diet decreased ($p < 0.05$) mRNA levels of AMPK,
195 mTOR, S6K1, 4E-BP1, and MAFbx in the breast at week 3, while the 2% DHA-rich *A.*
196 *acetophilum* diet increased mRNA levels of MURF1 ($P < 0.05$) and MAFbx ($P = 0.05$),
197 compared with the control diet (**Figure 1**). Supplemental DHA-rich *A. acetophilum* showed no
198 effect on mRNA level of these genes in the breast at week 6 except for that the 4% diet increased
199 ($p < 0.05$) 4E-BP1 mRNA level over the control. Meanwhile, the 4% DHA-rich *A. acetophilum*
200 diet decreased ($p < 0.05$) mRNA levels of mTOR, S6K1, 4E-BP1, and MURF1 at week 3 and
201 mTOR and 4E-BP1 at week 6 in the liver, compared with the control (**Figure 2**).

202 The DHA-rich *A. acetophilum* supplementation showed little or no consistent effect on
203 protein levels of mTOR, P70 and PP70: P70 ratio in the breast of chickens at week 3, compared
204 with the control (**Figure 3**). In contrast, the 2% DHA-rich *A. acetophilum* diet elevated ($p <$
205 0.05) PP70 protein levels in the breast by 41% compared with the control. In addition, the breast
206 S6 protein levels were enhanced ($p < 0.05$) in the birds fed the DHA-rich *A. acetophilum*
207 supplemented diets by 64 to 97% whereas the breast PS6 levels were enhanced ($p < 0.05$) in the
208 birds fed the 2% DHA-rich *A. acetophilum* diet (41%, $p < 0.05$) over the control. The breast PS6:
209 S6 protein ratios were decreased (26% to 40%, $p < 0.05$) by the DHA-rich *A. acetophilum*
210 supplemented diets compared with those of the control diet. The supplementations decreased (p

211 < 0.05) the protein levels of mTOR in the liver of chickens at week 3 (33% to 46%) compared
212 with the control (**Figure 4**). The 4% DHA-rich *A. acetophilum* decreased ($p < 0.05$) the hepatic
213 PP70 protein level by 46% over the control.

214

215 **4. Discussion**

216 Our previous research demonstrated dose-dependent DHA enrichments in the plasma,
217 breast, thigh, and liver [22] by feeding broiler chickens the DHA-rich *A. acetophilum* biomass at
218 1, 2, and 4% of diets. However, the exact same supplementations (to provide 0, 1.7, 3.4 and 6.8 g
219 DHA/kg diet, respectively) in the present study led to linear decreases in the body weight gain
220 and feed use efficiency. In particular, the actual differences in these two measures between the
221 highest inclusion (4%, 6.8 g DHA/kg of diet) and the control reached statistical significance (13-
222 19%). Comparatively, the two lower supplementations ((1% to 2% or 1.7 to 3.4 g DHA/kg of
223 diet) in the present study did not result in significant decreases in the growth performance. The
224 outcomes were largely similar to previous reports with moderate inclusion levels of
225 *Aurantiochytrium* (1% to 2% or 2.9 to 5.8 g DHA/kg of diet) [16,18,19]. In contrast, the negative
226 results associated with our highest supplementation (4%, 6.8 g DHA/kg) were in disagreement
227 with those of Moran *et al.* who fed broilers up to 5% *A. limacinum* biomass (9 g DHA/kg of diet)
228 and observed no adverse effect on growth performance [20]. It is difficult for us to assess if the
229 discrepancy was due to the species used between the two experiments. Our interpretation is
230 somewhat limited by the lack of lipid-extracted (DHA removed) *A. acetophilum* control in our
231 dietary treatments and of the toxicological information of the strain used in our experiment.
232 However, another *Aurantiochytrium* species, *A. limacinum*, caused no mortality or signs of acute
233 toxicity via oral administration of 276 mg/kg body weight and 2000 mg/kg body weight as food

234 additive to humans [38]. Because *A. acetophilum* is closer to *A. limacinum* than other species
235 from the phylogenomic tree including 219 informative orthogroups of *Thraustochytrids* and
236 other *Stramenopiles* [39], there is only 5% difference in their proteomes [40]. Thus, we might
237 infer that it was safe to supplement 4% DHA-rich *A. acetophilum* in the broiler diets. Most likely,
238 the negative effects of the highest supplementation of *A. acetophilum* were caused by the high
239 levels of DHA.

240 In fact, birds fed up to 15% redfish meal or 4.2% redfish oil (rich with n-3 PUFA)
241 showed decreased body weight, feed intake, and feed use efficiency [10]. Likewise, feeding
242 broilers 8% fish oil, in comparison with lard, for 23 days decreased body weights [12]. However,
243 broiler chickens fed 7.4% DHA Gold (around 1.5 g DHA/kg), a product extracted from
244 *Schizochytrium* biomass, the similar genus to *Aurantiochytrium*, had improved body weight gain,
245 feed intake, and feed conversion ratio [15]. Supplementing another DHA-rich *Schizochytrium*
246 *limacinum* up to 2% (5.8g DHA/kg of diet) also improved body weight and feed conversion ratio
247 [16]. These discrepancies underscore the complexity and challenge in establishing the maximal
248 and optimal inclusion levels of marine biomass DHA for the highest DHA enrichment efficiency
249 without adverse effects on their growth and health of these animals. Practically, our present study
250 suggested that supplemental *A. acetophilum* DHA at 3.4 g/kg diet was safe, but at 6.8 g/kg diet
251 was excessive or harmful to the chickens.

252 Another undesired outcome was the 21% decrease in the breast muscle weight in the
253 birds fed the 6.8 g DHA/kg (4% *A. acetophilum*) compared with the control. In fact, this loss was
254 associated with down-regulation of AMPK, mTOR, S6K1 and 4E-BP1 mRNA levels at week 3.
255 Intriguingly, mRNA levels of protein catabolic genes such as MURF1 and MAFbx were
256 upregulated in the breast muscle by 3.4 g *A. acetophilum* DHA/kg. Similarly, that level of dietary

257 DHA upregulated PP70 and PS6 protein levels in the breast muscle. Seemingly, 3.4 g *A.*
258 *acetophilum* DHA/kg could stimulate muscle protein synthesis via differential regulations of the
259 expression of S6K1/S6 biosynthesis pathway vs the MURF1 and MAFbx catabolic pathway.
260 Indeed, a number of studies have shown the potential of appropriate amounts of DHA in
261 stimulating muscle growth [23]. Dietary DHA supplementation promoted muscle growth in pigs
262 at fed state by upregulating mTOR and downregulating 4E-BP1 [24]. Kamolrat et al. found that
263 DHA enhanced protein synthesis by increasing P70S6K phosphorylation but no effect on protein
264 breakdown in C2C12 myotube [41]. Previously, our group observed up-regulations of protein
265 synthesis signaling in broiler chickens fed 10% defatted *Nannochloropsis oceanica* [25] and 15%
266 defatted *Desmodium sp.* [26]. Based on the little or inconsistent effect of the lower
267 supplementations of DHA-rich *A. acetophilum* on the gene expression and protein production
268 involved in the protein synthesis and catabolism signaling and the above-assumed biosafety of
269 4% DHA-rich *A. acetophilum* biomass, we postulate that the decreased breast muscle weight and
270 the down-regulated protein synthesis pathway in the birds fed the 4% DHA-rich *A. acetophilum*
271 were induced by the relatively high level of DHA (6.8 g/kg).

272 The DHA-rich *A. acetophilum*-mediated elevation of liver weight and the relative heart
273 weight percentage to the body weight in the present study may be interpreted in two ways. The
274 negative view is that the supplementation induced metabolic burden requiring larger tissue to
275 cope with. The positive view is that an increased liver weight was associated with elevated
276 metabolic capacity to improve the general health of animals [16]. Besides, the *A. acetophilum*
277 dose-dependent decreases in plasma activities of ALT might be an indirect indicator of liver
278 health [42], whereas the *A. acetophilum* dose-dependent linear decreases in the fasting plasma

279 glucose concentrations could be mediated through an improved insulin sensitivity by the elevated
280 DHA [43].

281 Similar to the results of Long et al. [16], supplemental the DHA-rich *A. acetophilum*
282 showed no effect on WHC or pH of the breast and thigh muscle. However, the supplementation
283 caused dose-dependent increases in chewiness and hardness of both muscles. Because no such
284 responses were caused by 10% defatted *Nannochloropsis* [44] or 2% astaxanthin-rich
285 *Haematococcus* [45], the observed effects on chewiness and hardness in the present study were
286 likely due to the DHA concentrations. In fact, shear forces of meat before cooking were
287 enhanced by feeding flaxseed oil rich in n-PUFA [46]. Whereas supplemental the DHA-rich *A.*
288 *acetophilum* enriched DHA in the breast and thigh muscles [22], the supplementation caused
289 dose-dependent increases in MDA concentrations in these tissues. As the secondary products
290 from lipid peroxidation and a practical indicator of food lipid peroxidation, MDA is related to
291 off-flavor of meat products [47] and shortened shelf life. Because DHA contains 6 double bonds
292 prone to oxidation or peroxidation, appropriate rather than maximal enrichments of DHA in the
293 breast and thigh muscles should be considered to avoid off flavor and instability [48].

294 In conclusion, the present study reveals a new panel of metabolic outcomes from feeding
295 hatchling Cornish male broilers the DHA-rich *A. acetophilum* at 0%, 1%, 2%, and 4% for 6 wk,
296 contrary to the desired dose-dependent enrichments of DHA and decreases of n-6/n-3 PUFA in
297 tissues of broilers observed in our previous study [22]. Specifically, supplemental 4% *A.*
298 *acetophilum* (6.8 g DHA/kg diet), but not lower levels, produced adverse effects on body weight
299 gain, feed use efficiency, and breast muscle weight. The decrease was associated with
300 upregulation of protein catabolic pathway and downregulation of protein anabolic pathway.
301 Although the excessive DHA level was likely the primary cause of the adverse responses, future

302 research is needed to evaluate the relative contribution and mechanism of other factors in this
303 DHA-rich protist biomass.

304

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309 **Declaration of author contributions**

310 XGL designed the experiment and edited the manuscript. TS performed the experiment and
311 wrote the manuscript. ST, AM and GL assisted with the animal care and (or) laboratory analysis,
312 ST performed the enzyme activity assays, mRNA quantification and Western blot. All authors
313 have read the manuscript and approved this submission.

314 **Statement of informed consent, human/animal rights**

315 No conflicts, informed consent, or human rights are applicable. All protocols of this experiment
316 were approved by the Institutional Animal Care and Use Committee of Cornell University.

317 **Competing financial interests**

318 The authors declare no competing financial interest.

319

320 **Reference:**

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Table 1. Effects of different concentrations of supplemental DHA-rich *A. acetophilum* on overall growth performance of broiler chickens*

Treatment	Week	<i>A. acetophilum</i> biomass				SEM	P value		
		0%	1%	2%	4%		Anova	Linear	Quad
BWG** g/chick/d	0-3	41.3	40.0	41.4	42.1	1.02	0.531	0.345	0.511
	4-6	97.0 ^a	94.7 ^a	91.4 ^a	78.9 ^b	2.40	<0.01	<0.01	<0.01
	0-6	71.6 ^a	69.7 ^a	68.7 ^a	62.5 ^b	1.55	<0.01	<0.01	<0.01
FI** g/chick/d	0-3	53.5 ^{ab}	52.1 ^b	55.8 ^{ab}	57.4 ^a	1.48	0.091	0.028	0.09
	4-6	164	180	166	165	5.59	0.192	0.606	0.51
	0-6	109	116	111	111	3.04	0.419	0.955	0.66
G: F ratio**	0-3	0.773	0.767	0.772	0.736	0.018	0.423	0.028	0.06
	4-6	0.594 ^a	0.534 ^{ab}	0.551 ^a	0.479 ^b	0.022	0.014	<0.01	0.01
	0-6	0.660 ^a	0.606 ^{ab}	0.619 ^{ab}	0.563 ^b	0.020	0.021	<0.01	0.02

*Data are expressed as mean (n = 6) and analyzed by one-way ANOVA and Duncan's multiple-range test.

^{a,b}Means in the same row without a common letter differ ($p < 0.05$).

**BWG: body weight gain, FI: feed intake, G: F ratio: gain: feed ratio.

Table 2. Effects of different concentrations of supplemental DHA-rich *A. acetophilum* on plasma indicators of broiler chickens*

Treatment	<i>A. acetophilum</i> biomass				SEM	P value		R ²
	0%	1%	2%	4%		Anova	Linear	
ALT, U/L**								
Week 3	2.40	2.77	2.98	2.61	0.198	0.409	0.653	0.015
Week 6	1.29 ^a	1.02 ^{ab}	0.947 ^{ab}	0.422 ^b	0.194	0.060	<0.01	0.335
AKP, U/mL**								
Week 3	298	305	208	252	45.3	0.573	0.406	0.041
Week 6	157	161	131	172	15.9	0.492	0.741	0.006
PIP, mg/dL**								
Week 3	78.2	85.4	91.4	91.3	11.3	0.854	0.428	0.035
Week 6	66.2 ^a	60.8 ^{ab}	57.6 ^b	63.3 ^{ab}	2.43	0.114	0.501	0.022
Glucose, g/L								
Week 3	2.79 ^a	2.28 ^b	1.94 ^{bc}	1.72 ^c	0.144	<0.01	<0.01	0.536
Week 6	3.02 ^a	2.93 ^{ab}	2.89 ^{ab}	2.72 ^b	0.091	0.200	0.028	0.219
Uric acid, mmol/L								
Week 3	0.125 ^b	0.189 ^{ab}	0.262 ^a	0.218 ^{ab}	0.027	0.072	0.095	0.175
Week 6	0.195	0.218	0.281	0.205	0.034	0.453	0.714	0.009

*Data are expressed as mean (n = 6) and analyzed by one-way ANOVA and Duncan's multiple-range test.

^{a,b,c}Means in the same row without a common letter differ ($p < 0.05$).

**ALT: Alanine aminotransferase; AKP: Alkaline phosphatase; PIP: plasma inorganic phosphorus; TC: total cholesterol; TG: triglycerides; NEFA: non-esterified fatty acid.

Table 3. Effects of different concentrations of supplemental DHA-rich *A. acetophilum* on carcass traits of broiler chickens at week 6*

Treatment	<i>A. acetophilum</i> biomass				SEM	P value		R ²
	0%	1%	2%	4%		Anova	Linear	
Breast muscle								
Weight, g	656 ^a	581 ^{ab}	622 ^a	520 ^b	28.5	0.029	0.011	0.285
Muscle yield, %	27.4 ^a	25.8 ^{ab}	26.1 ^a	24.1 ^b	0.56	0.010	<0.01	0.400
pH	6.31 ^{ab}	6.11 ^{ab}	6.00 ^b	6.53 ^a	0.144	0.078	0.233	0.064
WHC, %**	40.3	39.3	41.1	39.2	1.44	0.783	0.731	0.005
Chewiness	1373 ^c	2168 ^b	2291 ^{ab}	2993 ^a	250	<0.01	<0.01	0.442
Springiness	0.765 ^{ab}	0.756 ^b	0.758 ^b	0.797 ^a	0.001	0.035	0.021	0.237
Hardness	3656 ^b	6368 ^a	6335 ^a	7189 ^a	563	<0.01	<0.01	0.362
Thigh muscle								
Weight, g	583	564	588	574	21.5	0.871	0.927	<0.01
Muscle yield, %	24.3	25.0	24.7	25.8	0.49	0.201	0.046	0.177
pH	6.30	6.39	6.32	6.43	0.131	0.901	0.558	0.017
WHC, %	39.1	37.9	39.9	39.2	0.876	0.472	0.651	<0.01
Chewiness	1964 ^b	3008 ^{ab}	3163 ^a	3855 ^a	361	0.020	<0.01	0.369
Springiness	0.880	0.994	0.875	0.870	0.006	0.467	0.162	0.100
Hardness	3607 ^b	5426 ^a	5292 ^a	6129 ^a	455	0.012	<0.01	0.340
Other organs								
Heart, g	14.8	15.6	16.4	16.2	0.827	0.577	0.256	0.061
Heart, %	0.620 ^b	0.690 ^{ab}	0.692 ^{ab}	0.729 ^a	0.029	0.098	0.023	0.224
Liver, g	64.4 ^{ab}	59.3 ^b	67.2 ^{ab}	71.7 ^a	3.23	0.106	0.050	0.179
Liver, %	2.67	2.75	2.96	2.95	0.203	0.711	0.292	0.053
Intestine, g	114	126	118	117	8.01	0.767	0.989	<0.01
Intestine, %	4.74	5.60	4.96	5.23	0.259	0.144	0.541	0.018
Carcass, g	2397	2248	2379	2229	86.9	0.431	0.299	0.051
Dressing, %	76.5	75.6	76.1	74.6	0.58	0.181	0.514	0.022

*Data are expressed as mean (n = 6) and analyzed by one-way ANOVA and Duncan's multiple-range test.

^{a,b}Means in the same row without a common letter differ ($p < 0.05$).

**WHC: water holding capacity.

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Table 4. Effects of different concentrations of supplemental DHA-rich *A. acetophilum* on lipid peroxidation (malondialdehyde) in various tissues of broiler chickens*

Treatment	<i>A. acetophilum</i> biomass				SEM	P value		R ²
	0%	1%	2%	4%		Anova	Linear	
WK3, $\mu\text{mol/g}$ protein								
Breast	0.204	0.140	0.285	0.247	0.052	0.272	0.312	0.046
Thigh	0.511	0.408	0.492	0.529	0.067	0.599	0.571	0.015
Liver	1.49	1.44	1.76	1.72	0.224	0.686	0.338	0.044
Adipose	0.912	0.663	1.19	1.25	0.282	0.266	0.166	0.099
WK6, $\mu\text{mol/g}$ protein								
Breast	0.108 ^c	0.233 ^b	0.330 ^a	0.380 ^a	0.024	<0.01	<0.01	0.688
Thigh	0.124 ^d	0.231 ^c	0.315 ^b	0.454 ^a	0.017	<0.01	<0.01	0.905
Liver	1.49 ^b	2.29 ^a	2.66 ^a	2.79 ^a	0.181	<0.01	<0.01	0.622
Adipose	0.313 ^c	0.851 ^{bc}	1.148 ^b	2.311 ^a	0.162	<0.01	<0.01	0.766

*Data are expressed as mean (n = 6) and analyzed by one-way ANOVA and Duncan's multiple-range test.

^{a,b} Means in the same row without a common letter differ ($p < 0.05$)

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500 **Figure legends:**

501

502 **Figure 1. Effects of different concentrations of supplemental DHA-rich *A. acetophilum* on**

503 **relative mRNA levels of genes involved in protein synthesis and degradation in the breast**

504 **muscle of broiler chickens at weeks 3 (A) and 6 (B).** Values are means \pm SEs, n = 6. Asterisk

505 symbol indicates significance at $p < 0.05$. AMPK: AMP-activated protein kinase; mTOR:

506 mammalian target of rapamycin; S6K1: ribosomal protein S6 kinase-1; 4EBP1: eukaryotic

507 translation initiation factor 4E-binding protein 1; MURF1: muscle RING finger 1. MAFbx:

508 muscle atrophy F-box.

509

510 **Figure 2. Effects of different concentrations of supplemental DHA-rich *A. acetophilum* on**

511 **relative mRNA levels of genes involved in protein synthesis and degradation in the liver of**

512 **broiler chickens at weeks 3 (A) and 6 (B).** Values are means \pm SEs, n = 6. Asterisk symbol

513 indicates significance at $p < 0.05$. AMPK: AMP-activated protein kinase; mTOR: mammalian

514 target of rapamycin; S6K1: ribosomal protein S6 kinase beta-1; 4EBP1: eukaryotic translation

515 initiation factor 4E-binding protein 1; MURF1: muscle RING finger 1. MAFbx: muscle atrophy

516 F-box.

517

518 **Figure 3. Effects of different concentrations of supplemental DHA-rich *A. acetophilum* on**

519 **the protein production of factors involving in protein synthesis signaling in the breast**

520 **muscle of broiler chickens at weeks 3 (A) and 6 (B).** Values below the protein bands were

521 relative densities and are expressed as means \pm SEs, n = 3. Means without a common letter

522 differ, $p < 0.05$. mTOR: mammalian target of rapamycin; GADPH: Glyceraldehyde 3-phosphate

523 dehydrogenase; P70: ribosomal protein S6 kinase; PP70: phosphorylated ribosomal protein S6
524 kinase; S6: ribosomal protein S6; PS6: phosphorylated ribosomal protein S6.

525

526 **Figure 4. Effects of different concentrations of supplemental DHA-rich *A. acetophilum* on**

527 **the protein production of factors involved in protein synthesis signaling in the liver of**

528 **broiler chickens at weeks 3 (A) and 6 (B).** Values below the protein bands were relative

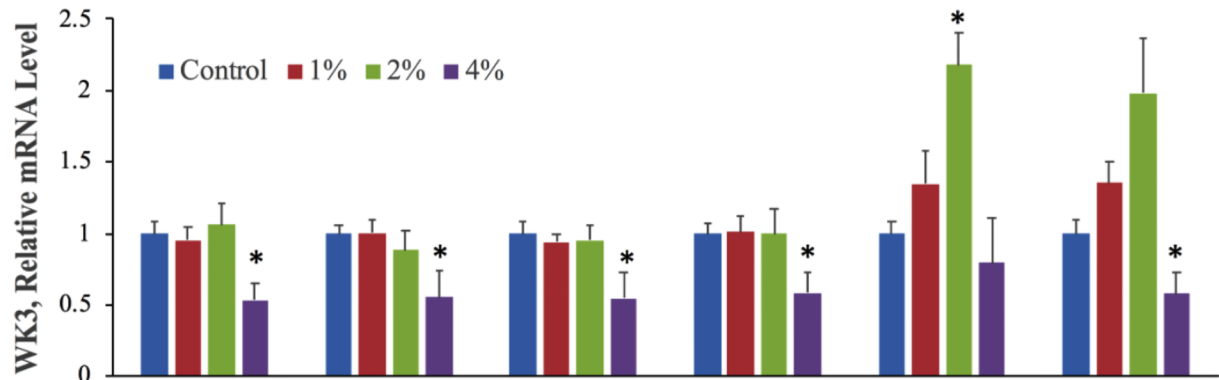
529 densities and are expressed as means \pm SEs, n = 3. Means without a common letter differ, $p <$

530 0.05. mTOR: mammalian target of rapamycin; GADPH: Glyceraldehyde 3-phosphate

531 dehydrogenase; P70: ribosomal protein S6 kinase; PP70: phosphorylated ribosomal protein S6

532 kinase; S6: ribosomal protein S6; PS6: phosphorylated ribosomal protein S6.

533

A**Breast****B**