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Article

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A Comprehensive Approach to Assess Feathermeal as an Alternative Protein Source in Aquafeed

Mohammad Sedigh Jasour [1#], Liane Wagner [1#], Ulrik K. Sundekilde [1*], Bodil K. Larsen [2], Ines Greco [3], Vibeke Orlien [3], Karsten Olsen [3], Hanne T. Rasmussen [4], Niels H. Hjermitslev [4], Marianne Hammershøj [1], Anne J. T. Dalsgaard [2], Trine K. Dalsgaard [1*]

1: Aarhus University, Science and Technology, Department of Food Science, Blichers Allé 20, Tjele/ Kirstinebjergvej, 5792 Årslev, Denmark

2: Technical University of Denmark, DTU Aqua, Section for Aquaculture, The North Sea Research Centre, P.O. Box 101, DK-9850 Hirtshals, Denmark

3: University of Copenhagen, Department of Food Science, Rolighedsvej 26, 1958 Frederiksberg, Denmark

4: Biomar A/S, R&D Process Technology, Mylius Erichsensvej 35, 7330 Brande, Denmark

# Authors contributed equally

Corresponding author:

Trine Kastrup Dalsgaard

Phone +45 87157998

trine.kastrup.dalsgaard@food.au.dk

and

Ulrik K. Sundekilde

Phone: +45 87154882

uksundekilde@food.au.dk

1 **ABSTRACT**

2 The effect of partially replacing fishmeal in aquafeed with feathermeal (FTH) at three
3 levels (0%: FTH0, 8%: FTH8, 24%: FTH24) and two extrusion temperatures (100 and
4 130 °C) were evaluated in rainbow trout (*Oncorhynchus mykiss*) with respect to growth
5 performance, metabolism response, and oxidative status of the feed proteins. Multivariate
6 data analyses revealed that FTH24 correlated positively with high levels of: oxidation
7 products, amino acids (AA) racemization, glucogenic AAs level in liver, feed intake (FI),
8 specific growth rate (SGR), and feed conversion ratio (FCR); and low AAs digestibility.
9 Both FI and SGR were significantly increased when 8 and 24% feathermeal was included
10 in the feed extruded at 100 °C, while there was a negative effect on FCR in fish fed
11 FTH24. In conclusion, higher oxidation levels in FTH24 may give rise to metabolic
12 alterations while lower levels of FTH may be considered as fishmeal substitute in
13 aquafeed for rainbow trout.

14 ***Keywords***: fishfeed; fishmeal; feathermeal; extrusion; oxidation; metabolite; growth
15 performance; rainbow trout; *Oncorhynchus mykiss*

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19

20 **INTRODUCTION**

21 In recent years, the soaring global demand for protein has boosted commercial fish
22 farming dramatically. Hence, global production of aquafeeds is increasing and expected to
23 reach 71 million metric tonnes by 2020, corresponding to an increase at an average rate of
24 11 percent per year [1-2]. Feed costs are a significant part of the total production costs,
25 mostly due to high cost of fishmeal [2-3]. However, there are several ways to reduce the
26 fishmeal proportion in aquafeed. Increasing attention has been paid to utilization of more
27 economically and environmentally sustainable alternative protein sources to reduce
28 production costs [4-6]. Feathermeal (FTH) is becoming attractive due to high supply options,
29 low costs, its high content of protein and essential amino acids (AAs), and the lack of anti-
30 nutritional factors [7]. Recently, the potential of utilizing FTH in extruded fish feed was
31 investigated and showed promising results [8]. Overall, it was found that the formation of
32 oxidation products and heat-induced cross-links increased with a high inclusion level of
33 feathermeal (24%). However, it was also found that an inclusion of 8% FTH in the feed
34 resulted in the highest *in vitro* digestibility [8]. These preliminary findings underlined that
35 the relationship between chemical and physicochemical changes of proteins and
36 digestibility is more or less straightforward; hence, the biochemical and biological effects,
37 especially the bioavailability, must be taken into account upon evaluating protein
38 replacement. In previous studies on replacement of fishmeal with feathermeal [9-11] there
39 has, to the best of our knowledge, been no focus on the relationship between feed protein
40 chemical changes and fish biological performance.

41 During extrusion, feed ingredients undergo extensive heat treatments at high-pressure
42 conditions. Therefore, heat sensitive AAs such as methionine, lysine, and tryptophan may
43 suffer from oxidative damages, which may reduce the digestion or absorption of nutrients
44 and consequently affect growth performance and even induce toxicity [12-13]. Knowledge

45 about the effect of extruded proteins on the chemical and biological characteristics in
46 relation to aquafeed is, however, scarce. The current study was therefore conducted to
47 provide more knowledge about the interactions between extrusion and fishmeal
48 replacement with FTH and the chemical effects in feed and biological effects in rainbow
49 trout (*Oncorhynchus mykiss*). Based on recent results (own unpublished data), feed with
50 inclusion levels of 8% (the best candidate) and 24% (worst-case scenario) were produced
51 in industrial settings at two different extrusion temperatures (100 and 130 °C). Extrusion
52 processing effect on proteins was monitored as protein oxidation products, amino acid
53 digestibility, and amino acid racemization (AAR). Furthermore, the effects of protein
54 changes on fish growth performance and liver and plasma metabolites were monitored.

55

56 **MATERIALS AND METHODS**

57 **Extrudate and Feed Production**

58 Feed was produced and extruded by Biomar A/S (Biomar A/S, Tech Center, Brande,
59 Denmark). A feed production experiment was designed according to a 3 × 2 factorial
60 model with three feathermeal inclusion levels (0, 8, and 24%) and two extrusion
61 temperatures (100 and 130 °C). The feeds were formulated to have similar level of
62 macronutrients, to be iso-nitrogeneous, iso-energetic by balancing with wheat flour, and to
63 meet rainbow trout requirements [14]. The content of lysine, histidine, methionine, and
64 tryptophan was maintained constant by adding L-lysine HCl, L-histidine, DL-methionine,
65 and L-tryptophan respectively, while phosphorus was optimized by adding mono-calcium
66 phosphate. Yttrium oxide was added as internal marker. Recipes and the chemical
67 composition of fishmeal and feathermeal, the meal mixes, the extrudates, and
68 experimental feed after oil coating are given in Table 1.

69 Feed ingredients were milled with a hammer mill to pass through a 0.75 mm screen. The
70 formulation mixtures were subsequently extruded in a five-section twin-screw extruder
71 (Clextral BC 45 extruder, Clextral, France) equipped with a 2.4 mm die. Moisture content
72 of the dough during extrusion was set at 25%. Following extrusion processing, the
73 extrudates were dried and coated by fish- and rapeseed oil using a vacuum oil pump.
74 Samples labelled 'extrudate' were sampled immediately after the extrusion process and
75 stored in closed plastic containers at 4 °C until analysis for oxidation and heat-induced
76 products. Samples labelled 'feed' (6 codes: FTH0/T100, FTH0/T130, FTH8/T100,
77 FTH8/T130, FTH24/T100, and FTH24/T130) refer to the extrudates after drying and oil
78 coating and were stored in bags at 4 °C until used in the fish trials.

79

80 **Protein Extraction and Determination of Solubilized Proteins**

81 Samples (50 mg) of meal mixes and extrudates were shaken for 4 h in 10 mL of 6 M
82 guanidine hydrochloride (GuHCl). Samples were then centrifuged for 1 min at 1000 rpm,
83 and the supernatants (protein solutions) were collected for analysis of solubilized protein
84 content and oxidation products. The solubilized protein content of the raw meal mixes and
85 extrudates was determined by a bicinchoninic acid (BCA) assay kit (Pierce, Bonn,
86 Germany) according to the manufacturer instruction using the microplate procedure (25
87 μL sample/200 μL BCA reagent; 37 °C/30 min). Bovine Serum Albumin (BSA from
88 Sigma, Munich, Germany) was used as protein standard. The absorbance of the solution
89 was measured using a spectrophotometer at a wavelength of 562 nm. Each sample was
90 assayed in triplicate.

91

92 **Oxidation and Heat-induced Changes**

93 All analyses of heat-induced changes of proteins were performed on the extrudates. The
94 following measurements of oxidation products were all based on soluble proteins (ca. 0.6
95 mg/mL) obtained from protein extraction in 6 M GuHCl as mentioned above and selected
96 wavelengths from following references;

97 *Protein hydroperoxides (PHP)*: the content of PHP was obtained by mixing an aliquot of
98 the protein solution with a xylenol orange and ammonium ferrous reagent following
99 incubation and measurement of absorbance at 560 nm [15] using a spectrofluorometer
100 (Synergy 2 spectrofluorometer (BioTek, Winooski, VT, USA)). The standard curve for
101 quantification (μmol PHP/mg protein) was based on hydrogen peroxide (0 to 30 μM).

102 *Total carbonyl*: the content of total carbonyl (arbitrary intensity units (AU)/mg protein) of
103 an aliquot of the protein solution was obtained by fluorescence detection with excitation at
104 350 nm [16] and emission intensity at 447 nm in non-transparent microtiter plates (96-
105 Corning-Costar (Lowell, MA, USA)) on a Perkin Elmer LS 50B spectrofluorometer
106 (PerkinElmer, Massachusetts, United States).

107 *N-formylkynurenine (NFK)*: the content of NFK (arbitrary intensity units (AU)/mg
108 protein) of an aliquot of the protein solution was obtained by fluorescence detection with
109 excitation at 330 nm [15, 17] and emission intensity at 449 nm in non-transparent microtiter
110 plates (96-Corning-Costar (Lowell, MA, USA)) using a Perkin Elmer LS 50B
111 spectrofluorometer (PerkinElmer, Massachusetts, United States).

112 *Schiff base products*: the content of Schiff base products (arbitrary intensity units
113 (AU)/mg protein) of an aliquot of the protein solution was obtained by fluorescence
114 detection with excitation at 345 nm and emission intensity at 449 nm [18-19] in non-
115 transparent microtiter plates (96-Corning-Costar (Lowell, MA, USA)) using a Perkin
116 Elmer LS 50B spectrofluorometer (PerkinElmer, Massachusetts, United States).

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117 *Lanthionine and furosine*: Quantification of lanthionine and furosine was carried out
118 following acidic hydrolysis of the extrudates. Briefly, an amount of sample corresponding
119 to 10 mg protein was suspended in 1 mL 6M hydrochloric acid (HCl) and incubated for 24
120 hours at 100 °C. HCl was subsequently removed by purging with nitrogen gas, and the
121 dried sample was resuspended in 1 mL MilliQ water and sonicated for 5 min in water bath
122 (Marshall Scientific Branson 1210 Ultrasonic Cleaner) at room temperature. The samples
123 were centrifuged (10 min, 19000 g, 4 °C) and supernatants were diluted 1:50 in MilliQ
124 water containing 1 μg/mL internal standard deuterated lysine (L-Lysine-4,4,5,5-d4
125 hydrochloride, Sigma Aldrich). Diluted samples were centrifuged (10 min, 19000 g, 4 °C)
126 and 10 μL of the supernatants were injected into a LC-MS/MS system. Quantification was
127 performed via an RP-UPLC (Thermo-Scientific) featuring a C18 column (Phenomenex
128 Aeris XB-C18. 1.7 μm particle size, 150 x 2.1 mm) coupled with mass spectrometer (Q-
129 Exactive Orbitrap) using electrospray ionization in positive mode. The analytes were
130 eluted from the LC column using a 27-min method with aqueous (A) and organic buffers
131 (100% acetonitrile, B) both containing 5 mM perfluoropentanoic acid. The method was
132 designed as follows: 100% A (0 to 5 minutes), 100 to 50% A (5 to 15 min), 50 to 100% B
133 (15 to 17 min), 100% B (17 to 22 min), 100% B to 100% A (22 to 24 min) and 100% A
134 (24 to 27 min) at a constant flow rate and oven temperature of 0.25 mL/min and 40 °C,
135 respectively. Direct injection of standard solutions in the mass spectrometer was used to
136 determine ionization source parameters (auto-tuning).

137 Peaks were identified and quantified by monitoring the specific m/z ratios for each
138 analytes (Table 2). Processing and quantification was performed using the
139 ThermoScientific Xcalibur software. A standard curve (5 to 10000 ng/mL) was derived
140 for every standard prior to sample analysis, using for every point the analyte/internal
141 standard peak area ratio (PAR). The internal standard was present in each point of the

142 curve and for each analyte at the same concentration (1 μg/mL). The calculated furosine
143 and lanthionine concentrations in the samples were then compared to the known protein
144 concentration of each material and expressed in μg/mg protein.

145

146 **Amino Acid Racemization**

147 The method for measuring amino acid racemization (AAR) was based on Tojo et al.
148 (2012) [20], which combines derivatization using Marfey´s reagent (2,4-Dinitro-5-
149 fluorophenyl; FDAA, Sigma Aldrich 71478) with separation and quantification of D- and
150 L-amino acids by HPLC. D-AAs derivatized with Marfey's reagent exhibit strong
151 intramolecular bonding, which reduces their polarity relative to the corresponding L-
152 amino acid derivates. Consequently, the D-derivates are selectively retained on reverse
153 phase columns and elute later than the corresponding L-derivates.

154 Approximately 10 mg of grinded feed were added to hydrolysis tubes (Thermo Scientific
155 29571) in addition to 1.7 mL 6 *N* HCl containing 0.2% phenol (w/v). Air/oxygen was
156 removed by flushing the tubes with nitrogen gas followed by application of vacuum
157 (alternating 3 times, 30 sec each) and tightening the lid under vacuum. The samples were
158 hydrolysed for 24 hours at 110 °C. Nor-Leucine (Sigma Aldrich N8513) was added as an
159 internal standard for estimation of recovery. The hydrolysed samples were transferred to
160 glass tubes and the HCl evaporated in a vacuum-concentrator (CentriVap, VWR 531-
161 0224). The samples were re-suspended in 33% acetonitrile and filtered by 0.2 μm
162 centrifugation filters (VWR 516-0234) and could hereafter be derivatized with 1% FDAA
163 in acetone, according to Thermo Scientific online protocol (MAN0016377), using 50 μL
164 for standards or feed samples. As derivatized samples are rather unstable, they were
165 analyzed immediately after derivatization. Identification and quantification was performed

166 by means of an uHPLC system (Flexar FX-10, PerkinElmer Inc., Waltham, MA, USA)
167 using gradients of 50 mM trimethylamine-phosphate buffer, pH 3.5, containing either 10
168 or 40% acetonitrile (mobile phases A and B, respectively). Standard curves were prepared
169 using a standard mix of L- amino acids (Sigma Aldrich A9781) added the D-isoforms
170 (Sigma Aldrich) of methionine (M9375), lysine (L8021), threonine (T8250),
171 phenylalanine (P1751) and valine (855987). The chromatographs for the different samples
172 were analyzed using the CHROMORA FLEXAR v3.2.0 4847 software (PerkinElmer
173 Inc.). The amount of D-AA per kg feed were subsequently calculated, taking recovery of
174 nor-leucine into account. The degree of AAR of each individual AA was calculated as:

175 Degree of AAR= D/(D+L); where D and L refer to the two isoforms of the amino acid.

176 **Fish trial**

177 An 8-weeks fish performance trial was carried out in a recirculating freshwater
178 aquaculture system at the Biomar Research Center in Hirtshals, Denmark. The trial was
179 carried out in accordance with EU legislation and Danish Animal Welfare Regulations.
180 All six feed codes (FTH0/T100, FTH0/T130, FTH8/T100, FTH8/T130, FTH24/T100, and
181 FTH24/T130) were fed to triplicate tanks containing 90 rainbow trout each with a start
182 weight of 111.2 ± 2.60 g. Fish were fed ad libitum every 6h each day, and uneaten pellets
183 were collected and weighted. Water temperature (12 °C), oxygen (>92%), and a light:dark
184 ratio (16:8 h) were kept constant for the duration of the trial. Upon finalizing the feeding
185 period, the fish were anaesthetized, gently cleaned with soft tissue and weighed, stripped
186 for feces, and plasma and liver samples were obtained. Growth performance parameters
187 including the specific growth rate in % day^{-1} (SGR; 100*(ln final weight – ln initial
188 weight)/feeding days), feed conversion ratio (FCR, dry feed intake/wet weight gain), and

189 daily feed intake in % day^{-1} (FI; 100*((daily feed load - daily feed loss)/feeding days)
190 were calculated for each replicate at the end of the study.

191 ***In vivo* Amino Acid Digestibility**

192 The amino acid composition of the feed and stripped feces from the rainbow trout was
193 analyzed according to ISO 13903 [21], and the apparent digestibility (ADC) of the amino
194 acids [22] was calculated as follows: $ADC_i = (1-(F_i/D_i \times D_y/F_y)) \times 100$; where F_i and D_i refer
195 to the percentages of the amino acid (i) in the feces and diet, respectively, and F_y and D_y
196 refer to the percentage of yttrium (y) in the feces and diet, respectively.

197

198 **^{1}H NMR-based Metabolomics Analyses**

199 ***Sample Preparation for Metabolomics Study of Plasma and Liver***

200 Preparing fish plasma samples for ^{1}H NMR analyses were carried out as described
201 previously [23], with slight modification. Briefly, Nanosep centrifugal filters with 3 kDa cut-
202 off (Pall Life Science, Port Washington, NY, USA) were washed three times with MilliQ
203 water (2000 g, 12 min, 30 ºC) to remove glycerol from the filter membrane. For plasma
204 analyses, pooled samples from 5 fish per tank were used. This included mixing, 100 µL of
205 each plasma sample into one tube (total= 500 µL) and centrifuging at 13000 g at 4 ºC. 200
206 µL of the pooled plasma samples were subsequently mixed with 350 µL of deuterium
207 oxide (D_2O) and 50 µL D_2O containing 0.05 wt% of sodium-3-(trimethylsilyl)-2,2,3,3-
208 tetradeuteriopropionate (TSP) as internal standard. The pooled plasma samples were
209 prepared in duplicates.

210 Fish liver samples were extracted and prepared for ^{1}H NMR analyses following the
211 method described previously [24], with few modifications. Hence, 20 mg of lyophilized,
212 grinded homorganic liver powder was whirl-mixed in 3 steps of 1 min duration each: first

213 in 300 µL ice-cold methanol, then in 300 µL ice-cold chloroform and third in 300 µL ice-
214 cold water. The samples were placed on ice for 10 min between each step and finally
215 stored at 4 °C overnight for separation. The following day the samples were centrifuged
216 (30 min, 1400 g, 4 ºC) (Eppendorf centrifuge 5417, USA), and following phase separation
217 the aqueous and chloroform supernatant was collected in separate tubes. The collected
218 aqueous phase samples were dried using an evacuated centrifuge (Eppendorf Concentrator
219 Plus, Germany) for approximately 3 hours and re-dissolved with 550 µL D_2O, 25µL
220 MilliQ water and 25 µL D_2O containing 0.05 wt% TSP. The chloroform phase samples
221 were dried for approximately 1 hour and re-dissolved in 575 µL $CDCl_3$ (99.96 atom% D)
222 and 25 µL $CDCl_3$ containing 0.05 wt% TSP. The liver samples were prepared in
223 quintuplicate (5 individual fish per tank and diet). The plasma and liver samples were
224 subsequently analyzed with ^{1}H NMR in 5 mm NMR tubes (Bruker Spectrospin Ltd,
225 BioSpin, Karlsruhe, Germany).

226

227 ***^{1}H NMR Spectroscopy, data processing and identification of the signals***

228 The plasma and liver samples were analyzed with a Bruker 600 MHz spectrometer
229 (Bruker Biospin GmbH, Rheinstetten, Germany) using zgpr pulse sequence at 25 ºC with
230 64 scans, a spectral width of 7,288 Hz collected into 32,768 data points, an acquisition
231 time of 2.24 sec, and an interscan relaxation delay of 5 sec. The ^{1}H NMR spectra for the
232 chloroform liver phase was obtained using zg30 pulse sequence (Bruker) at 20 ºC with 64
233 scans and 65,536 data points over a spectral width of 12,335 Hz. Acquisition time was
234 2.65 sec and relaxation delay 1sec.

235 All data were processed using the Bruker Topspin 3.0 software (Bruker) and Fourier-
236 transformed after multiplication by line broadening of 0.3 Hz. The spectra were

237 referenced to standard peak TSP (chemical shift 0 ppm), phased and baseline corrected.
238 Each NMR spectrum was integrated using Matlab R2011b (Mathworks, USA) into 0.01
239 ppm integral region (buckets) between 0.5-9.5 ppm and 0.8-9.0 ppm for aqueous liver
240 phase and plasma extracts, respectively, in which area between 4.7 and 5.0 ppm (4.7 and
241 5.15 ppm for plasma) corresponding to water signal was excluded and for chloroform
242 liver samples between 0.6 and 5.5ppm. For the aqueous and chloroform liver phase each
243 spectral region was normalized to the intensity of internal standard (TSP) for quantitative
244 measurements and for plasma samples was normalized to the sum of total area. The
245 chloroform samples were not analyzed further. For the plasma and aqueous liver samples
246 the ChenomX NMR Suite version 8.1 profiler (ChenomX Inc, Edmonton, AB, Canada)
247 was used to identify and quantify compounds. A total of 55 metabolites in the plasma and
248 aqueous liver phase were identified by overlapping with standard spectra, and their
249 concentrations were expressed in µmol/mg for liver and µmol/L for plasma. Assignments
250 of the ^{1}H NMR signals were carried out using ChenomX NMR Suite 8.1 library
251 (ChenomX Inc), the Human Metabolome Database (www.hmdb.ca) and previous
252 literature [24-26], and confirmed with 2D-NMR in case of multiplicity.

253 **Data Analyses**

254 The Simca-P software (version 14.0; Umetrics, Umeå, Sweden) was applied for
255 multivariate data analyses of the absolute concentrations of the metabolites. All variables
256 were "unit variance" (UV)-scaled. Principal component analyses (PCA) was used to get a
257 first overview of the data and search for outliers. Outliers were observed using PCA-
258 Hotelling T^2 Ellipse (95% confidential interval (CI)). Data on protein oxidation
259 compounds, fish growth performance parameters, and metabolites were subjected to one-
260 way and two-way analysis of variance (ANOVA) and Duncan's multiple range tests to
261 compare the effects of different experimental conditions examined and their main effects

262 and interactions. An independent Student's t-Test analysis was performed to find out
263 whether significantly different liver and plasma metabolites existed between fish fed the
264 low (FTH0) and high (FTH24) level of feathermeal feeds. Statistical analyses were carried
265 out using the IBM SPSS STATISTICS statistical program, (version 22.0, IBM
266 Corporation, New York, USA). Differences were considered significant when $P < 0.05$
267 unless otherwise indicated.

268

269 **RESULTS**

270 In the present study, we investigated the effects of two extrusion temperatures (100 and
271 130 °C) and three feathermeal inclusion levels (0, 8, and 24%) on the chemical properties
272 of proteins in the extrudates (i.e., protein oxidation products and AAR) and on fish growth
273 performance (FCR, FI, and SGR) and *in vivo* AAs digestibility. In order to explain the
274 underlying mechanism of how the feed parameters affected the growth performance, liver
275 and plasma metabolites in individual rainbow trout were also examined following a 8
276 weeks feeding study. Hence, all results were subjected to a multivariate data analysis to
277 assess the overall relationships. In addition, the most common oxidation products and
278 growth performance results are presented and discussed in details, while other results are
279 presented in the supplementary material.

280

281 **Growth performance**

282 In general, all feeds were well accepted by the fish and the average body weight increased
283 from 111.2 ± 2.6 g to 212.2 ± 10.9 g during the 8 weeks of feeding. There was a
284 significant main effect of the level of FTH on the growth parameters (FI, SGR, and FCR),

285 while no main effect of the extrusion temperature was found (Table 3). Meanwhile, there
286 was a significant interaction between the extrusion temperature and FTH level on FI and
287 SGR (Table 3). A significantly higher FI was seen in fish fed feathermeal diets (FTH8 and
288 FTH24) extruded at 100 °C compared to the control group (FTH0), while no similar effect
289 was observed for diets processed at 130 °C (Fig. 1A). The feed intake in the control group
290 (FTH0) was significantly higher when fed the diet extruded at 130 °C compared to 100 °C
291 (Fig. 1A). The SGR of fish fed the FTH0 diet extruded at 100 °C was significantly lower
292 than that of fish fed any of the other experimental feed (Fig. 1B). At the same time,
293 replacing fishmeal with a high level of feathermeal (24%) significantly increased the FCR
294 compared to the other groups independently of the extrusion temperature (Fig. 1C).

295

296 **Protein Oxidation and Amino Acid Racemization**

297 The PCA analysis (Fig. 2B) showed that the protein oxidation products, e.g. total
298 carbonyl, NFK, and Schiff base grouped together, indicating a similar variance of the data.
299 Hence, the oxidation and heat-induced products including PHP, carbonylation, and
300 lanthionine together with methionine racemization (Fig. 3) serve as representative markers
301 of changes in the primary protein structure following extrusion processing. For a detailed
302 overview of the other protein degradation products (NFK, Schiff base, furosine, and
303 specific AA racemization) the reader is referred to supplementary material (Fig. S1). The
304 primary oxidation product, PHP, did not change significantly due to increased extrusion
305 temperature to 130 °C irrespectively of the FTH inclusion levels (Fig. 3A). Only FTH24
306 showed a significant increase in PHP as an effect of increasing the extrusion temperature
307 from 100 to 130 °C. Carbonylation is a measure of the protein oxidation propagation and,

308 as seen in Fig. 3B, increasing both the extrusion temperature and the feathermeal inclusion
309 level resulted in a significant increase in the level of total carbonyls.

310 Regarding changes in the physical characteristics of the proteins, the amount of FTH had a
311 significant effect on the formation of cross-links. The content of lanthionine was
312 significantly higher in the extrudates with the highest level of feathermeal (FTH24)
313 compared to that without feathermeal (FTH0), whereas no effect of processing
314 temperature was found (Fig. 3C). Amino acid AAR can have a great impact on protein
315 bioavailability and the degree of methionine racemization represents the physical changes
316 due to AAR. Hence, increasing the level of feathermeal increased the degree of
317 methionine racemization whereas no effect of processing temperature was found (Fig.
318 3D). The same pattern was observed for racemization of phenylalanine (Fig S1F) while
319 not similar effect was observed for the other tested amino acids (i.e., lysine, threonine, and
320 valine; Fig S1B, D, and G, respectively)

321 **Correlation of Feed Variables with Growth Responses Variables**

322 In order to compare the results presented in Fig. 1 and 3 (and S1), PCA modelling was
323 carried out. The resulting PCA plot of the feed variables and growth performance data
324 show that the model was principally able to separate the different feeds by the first two
325 components with the first principal component (PC1) explaining 72% of the variance in
326 the data matrix and the second PC (PC2) explaining 14% (Fig. 2A). The extrudates, feed
327 characteristics and fish growth performance data clearly grouped by the feathermeal level
328 (Fig. 2), while samples were not separated according to temperature (Fig. S2). In general,
329 an increase in feathermeal correlated with an increase in FCR, SGR, and FI (Fig. 2B).
330 Moreover, the highest level of feathermeal correlated positively with an accumulation of
331 oxidation and heat-induced products in the extrudates (e.g. PHP, carbonyls, NFK, Schiff

332 base, lanthionine, and furosine) compared to extrudates with no or low inclusion of
333 feathermeal (Fig. 2A, B). The lack of a similar dependency of FTH level and extrusion
334 temperature on the specific AAR (Fig. 3D, Fig. S1 B, D, F, G) is seen from the scattering
335 of these data in the PCA plot, though a high accumulation level of AAR was correlated
336 with samples with feathermeal included, especially with FTH24 (Fig. 2B). Furthermore,
337 high *in vivo* digestibility of amino acids correlated largely with a lack of feathermeal in
338 the feed (Fig. 2B).

339

340 **Correlation of Fish Metabolites (Liver and Plasma) with Growth Response Variables**

341 A PCA was also used to examine the covariance between fish growth performance,
342 metabolites (liver and plasma), extrusion temperatures, and feathermeal inclusion levels
343 (Fig. 4 and S3). The PCA scores plot of the liver and plasma metabolites and growth
344 performance data displayed group separation according to the feathermeal inclusion levels
345 along PC1, explaining 21.6 % of variation, whereas PC2 explained 12.6% of variation
346 (Fig. 4A). Hence, separation was not as confined as the protein changes (Fig. 2), but
347 similar to the protein changes no separation was observed with respect to extrusion
348 temperature (Fig. S3).

349 The variables important for the observed grouping included mainly AAs and organic acids
350 (Fig. 4B, Table 4 & 5). Among the plasma AAs, phenylalanine, proline, valine, serine,
351 tyrosine, leucine, and methionine correlated positively with the inclusion level of
352 feathermeal. In contrast, plasma lysine and arginine were negatively correlated to the level
353 of feathermeal (Fig. 4B). Only tyrosine, valine and phenylalanine were significantly
354 different between dietary treatments following univariate statistics (Table 4). For the liver
355 AAs, phenylalanine, arginine, methionine, valine, isoleucine, tyrosine, alanine, and

356 leucine were positively associated with fish fed FTH24, while liver lysine correlated
357 positively with the control diet (FTH0). Detailed comparisons of liver metabolites are
358 presented in Table 5. Furthermore, pyruvate level in the liver, plasma levels of lactate and
359 glucose, FCR, SGR, and FI were positively correlated with FTH24 (Table 4 and Fig. 4B).
360 Creatinine, creatine, acetate, NAD+, ATP, ADP, in liver were positively correlated with
361 the control group (FTH0).

362 **Significantly Different Plasma and Liver Metabolites between FTH0 and FTH24**

363 The largest effects on plasma and liver metabolites (Fig. 4A) were seen between fish fed
364 the high feathermeal diet and fish fed the control diet. A high inclusion of feathermeal
365 resulted in a decrease in plasma creatinine, dimethylamine, trimethylamine,
366 trimethylamine-n-oxide, n-methylhydantoin, and an increase in plasma phenylalanine,
367 valine, methionine, tyrosine independently of the extrusion temperature (Table 4). In
368 contrast, liver metabolites were with a few exceptions more affected by the extrusion
369 temperature. Hence, a high inclusion of feathermeal in the diet extruded at a high
370 temperature resulted in a decrease of creatine, creatinine, NADP+, taurine and threonine,
371 whereas the low extrusion temperature of the same diet resulted in an increase in
372 isoleucine, phenylalanine, valine and beta-alanine (Table 5).

373 **DISCUSSION**

374 The global shortage of fishmeal as a primary source of protein forces the aquafeed
375 industry to use unconventional protein ingredients in formulated aquafeed [27]. A large
376 number of poultry-industry waste materials such as feathermeal can potentially be used.
377 However, high concentrations of sulfur-containing AAs that are more susceptible to
378 oxidation than fishmeal makes feathermeal questionable with respect to digestibility.

379 Hence, reduced fish growth [28-30] and altered immune response [31] have been reported when
380 oxidized feeds have been used in aquaculture.

381 In the present study, the extent of protein oxidation in the extruded feed was found to be a
382 function of both extrusion cooking temperature and feathermeal inclusion level. Hence,
383 mildly treated samples (100 °C) had fewer protein oxidation products compared to the
384 more harshly treated samples (130 °C). Furthermore, the degree of racemization of the
385 examined AAs correlated positively with the FTH inclusion, being highest in FTH24. The
386 higher levels of AAR and protein oxidation products in feed with feathermeal proteins
387 might be attributed to the transformation of free radicals formed from sulfur-containing
388 AAs oxidized during extrusion cooking and turned into other AAs [32].

389 The accumulation of lanthionine in feed with high FTH inclusion did not seem to be
390 affected by the extrusion temperature. In comparison, previous studies have shown that
391 heat treatment has a significant effect on the formation of unnatural AAs, particularly
392 lanthionine, leading to a reduction in protein digestibility [33-34]. The results in the current
393 study are consistent with the observation that oxidative cross-linking occurring in
394 feathermeal can reduce AAs digestibility *in vivo*. The digestibility of proteins typically
395 decreases when the ratio of AAR increases due to the stereospecificity of proteinases and
396 peptidases [32]. Furthermore, the positive correlation between FCR and oxidation products
397 found (Fig. 2) indicates that protein, being one of the most valuable components of the
398 feed from a nutritional standpoint, can be made partly unobtainable to the fish due to heat-
399 induced damages. Hence, the levels of oxidation products and AAR in the feed combined
400 with reduction in *in vivo* AAs digestibility and higher FI in fish fed FTH24 all suggest that
401 the heat-induced damages on proteins plays a significant role in energy demanding
402 process.

403 In aquafeed, an optimal proportion of all essential AAs is required for efficient protein
404 utilization and growth of the fish [35]. Feathermeal is deficient in several AAs including
405 methionine, lysine, histidine, and tryptophan [36]. In the present study, these essential AAs
406 were therefore supplemented as free AAs to diets containing FTH (Table 1). It has
407 previously been reported that lysine, sulfur-containing amino acids, and the indole ring of
408 tryptophan are the AAs most susceptible sensitive to oxidation [37-38]. Consistent with this,
409 high level of protein oxidation was observed in the feed containing FTH supplemented
410 with free essential AAs. Similar to the current study, a previous study has also shown that
411 FCR increases when diets are supplemented with free AAs [39]. The higher FI in fish fed
412 high amounts of FTH may thus be a reflection of an increased energy demand deriving
413 from de novo protein synthesis from AAs damaged during extrusion cooking or catabolic
414 expenses associated with deaminating and excreting the damaged AAs. Furthermore,
415 increased energy demand due to consumption of oxidized proteins might also be the
416 reason for higher FI in the control group fed the diet extruded at 130 °C, in which higher
417 protein oxidation were found compared to the diet extruded at 100 °C.

418 A high inclusion level of feathermeal resulted in a higher hepatic pyruvate level. Pyruvate
419 can be produced from glucose via glycolysis in the cytosol. It usually penetrates the
420 mitochondria and is converted to acetyl Co-A which enters the tricarboxylic acid (TCA)
421 cycle generating energy in form of Adenosine triphosphate (ATP) [40]. An increase of
422 pyruvate in the liver may thus indicate that its use in the TCA cycle was somehow
423 affected. Consistent with this, lower levels of ATP correlated with a high dietary inclusion
424 level of feathermeal, indicating that the liver cells were energy limited. Furthermore, the
425 high correlation between FTH24 and the levels of lactate in both liver and plasma samples
426 corroborate that pyruvate did not efficiently enter the TCA cycle, explaining that there
427 was no need to deplete lactate from blood. Consistent with these results, a higher glucose

428 level in the plasma correlated with a high inclusion level of feathermeal. A high glucose
429 content in the plasma of fish fed feed containing high levels of feathermeal may be related
430 to an impaired TCA cycle leading to a reduction in the glycolytic activity and
431 consequently an insufficient transfer of glucose from the blood stream into the body cells.
432 The low levels of NAD^+ in the liver of fish fed feed containing high levels of feathermeal
433 are consistent with this hypothesis. Under normal conditions, NAD^+ promotes the release
434 of energy from pyruvate via the TCA cycle [40]. Furthermore, a lack of pyruvate in the
435 mitochondria would activate glutamine metabolism to ensure a persistent TCA cycle
436 function [41]. Consistent with this, the low concentrations of glutamine in the liver in the
437 present study correlating with a high inclusion level of feathermeal might indicate
438 glutamine depletion due to a lack of pyruvate.

439 It is well known that dietary ingredients can be reflected in fish tissues or biofluids. In the
440 present study, the hepatic levels of leucine, isoleucine, tyrosine, valine, methionine,
441 arginine, and phenylalanine correlated positively with fish fed a high inclusion level of
442 feathermeal. All of the above mentioned AAs are involved in energy metabolism [24, 40].
443 The increase in the concentration of these AAs in the liver thus indicates that they were
444 inhibited from entering the TCA metabolic pathway and thereby hindered from generating
445 energy, potentially explaining the higher FI in fish fed FTH24 compared to the control
446 group. Furthermore, tyrosine synthesized from the essential AA phenylalanine is a
447 precursor for thyroid hormones and neurotransmitters [42]. Thyroid hormones play an
448 important role next to energy metabolism and protein synthesis, and indirectly affect the
449 feed intake, feed conversation efficiency and growth performance. Thus, higher tyrosine
450 (plasma) and phenylalanine (liver and plasma) levels in fish fed FTH24 may indicate a
451 thyroid promoting effect of the feathermeal diet due to higher energy demands,
452 subsequently leading to the observed increased feed intake.

453 Trimethylamine-n-oxide (TMAO) has protein-stabilizing capabilities and prevents
454 oxidative damages [43-44]. High TMAO levels can be obtained either from the diet or by
455 endogenous biosynthesis from the trimethylamine moiety of choline [45]. In the current
456 study, we observed a lower level of TMAO with higher inclusion of feathermeal
457 independently of the extrusion temperature in plasma and for high extrusion temperature
458 in the liver. The observation might be explained with the lower levels of TMAO found in
459 the diets (i.e., FTH0 vs. FTH24 for mild extrusion: 0.56 ± 0.06 µM/mg vs. 0.44 ± 0.02
460 µM/mg, respectively; for harsh extrusion: 0.60 ± 0.00 µM/mg vs. 0.50 ± 0.00 µM/mg,
461 respectively).

462 In summary, the results in the current study showed that protein oxidation increases as a
463 function of the extrusion temperature and dietary feathermeal inclusion level. Although,
464 harshly treated feed samples with a higher level of feathermeal were more prone to
465 oxidation and led to lower amino acid digestibility, the liver and plasma metabolites of the
466 fish appeared to be affected mainly by the feathermeal inclusion level. Furthermore, the
467 observed increase in certain AAs in the liver was presumably associated with energy
468 metabolism, suggesting a metabolic disturbance at the hepatic level that may explain the
469 higher FI and FCR in fish fed the highest FTH inclusion level. In comparison, a lower
470 level of feathermeal (FTH8) resulted in an increased SGR without any adverse effect on
471 FCR. To our knowledge, this is the first study correlating dietary protein oxidation effects,
472 amino acid digestibility, and liver and plasma metabolomics with growth performance of
473 fish as a means to explore the effects of replacing fishmeal with feathermeal in the diet.
474 The study demonstrated that the studied variables were useful as indexes for monitoring
475 fishmeal replacement with a new protein source in extruded fish feed. However, further
476 in-depth research is needed to determine the fate of oxidation products, metabolic

477 pathways, and pyruvate carrier inhibitors potentially affected by feathermeal included in
478 extruded aquafeed.

479 **ABBREVIATIONS USED**

480 (FTH: feathermeal, AA: amino acids; FI: feed intake; SGR: specific growth rate; FCR:
481 feed conversion ratio; AAR: amino acid racemization; PHP: Protein hydroperoxides; AU:
482 arbitrary intensity units; FDAA: 2,4-Dinitro-5-fluorophenyl; ADC: apparent digestibility;
483 TSP: sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate; PCA: Principal
484 component analyses; CI: confidential interval; ANOVA: analysis of variance; NFK: N-
485 formylkynurenine; ATP: Adenosine triphosphate; TCA: tricarboxylic acid; TMAO:
486 Trimethylamine-n-oxide
487

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492

493 **DECLARATION OF INTEREST**

494 There is no conflict of interest.
495

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499

500 **SUPPORTING INFORMATION**

501 Following supporting information are available free of charge on the ACS Publications
502 website at: DOI:

503 Metabolite differences in liver and plasma of fish fed the control diet (FTH0) and the diet
504 with high inclusion of feathermeal (Table S1); Effect of different inclusion levels of
505 feathermeal (0, 8, and 24%) and extrusion temperatures (100 and 130°C) on Schiff Base, *N*-
506 Formylkynurenine, furosine, and racemization of amino acids (lysine, threonine, phenylalanine,
507 and valine) in the different extrudates (Fig. S1); Effect of different extrusion temperatures
508 (100 and 130°C) on differentiation of the studied observations based on protein and feed
509 functional characteristics, and growth performance of the fish fed extruded feed
510 containing different levels of feathermeal (Fig. S2); Effect of different extrusion
511 temperatures (100 and 130°C) on differentiation of the studied observations based on
512 growth response variables and metabolites from liver and plasma of the fish fed extruded
513 feed containing different levels of feathermeal (Fig. S3)

514

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FIGURE CAPTIONS

Fig. 1: Effect of different inclusion levels of feathermeal (0, 8, and 24%) and extrusion temperatures (100 and 130 °C) on growth performance: feed intake (A), specific growth rate (B), feed conversion ratio (C) of rainbow trout after 8 weeks feeding trial with the different feeds, FTH0: 0% feathermeal, FTH8: 8% feathermeal, FTH24: 24% feathermeal. Results are expressed as mean ± SD and statistically significant differences between individual treatments assessed by Duncan test at $P < 0.05$ are indicated with different letters in superscript.

Fig. 2: Scores (A) and loading (B) plots based on extrudates and feed protein characteristics and fish growth performance for different levels of feathermeal, FTH0: 0% feathermeal, FTH8: 8% feathermeal; FTH24: 24% feathermeal. FCR: feed conversion ratio, SGR: specific growth rate, FI: feed intake. ◯: Amino acids digestibility; ☆: Oxidation (lanthionine (1), total carbonyls (2), Schiff base (3), N-formyl kynurenine (NFK) (4), protein hydroperoxides (PHP) (5), and furosine (6); ◇: Growth performance (FCR, FI, and SGR).

Fig. 3: Effect of different inclusion levels of feathermeal (0, 8, and 24%) and extrusion temperatures (100 and 130 °C) on protein hydroperoxides (A), carbonylation (B), lanthionine (C), and racemization of methionine (D) in the different extrudates, FTH0: 0% feathermeal, FTH8: 8% feathermeal, FTH24: 24% feathermeal. Results are expressed as mean ± SD, except for lanthionine in FTH0 and racemization of methionine in FTH8 extruded at 100 °C, which was measured once. Statistically significant differences between individual treatments assessed by Duncan test at $P < 0.05$ are indicated with different letters in superscript, except for those stated before.

Fig. 4: Scores (A) and loadings (B) plot based on growth response variables and metabolites from liver and plasma of the fish fed extruded feed containing different levels of feathermeal. FTH0: control fish fed feed with 0% feathermeal, FTH8: fish fed feed with 8% feathermeal; FTH24: fish fed feed with 24% feathermeal. ■: Plasma metabolites; ●: Liver metabolites; ◇: Growth Performance.

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Table 1: Recipes and Nutrient Composition of Meal Mix, Extrudates, and Feed Including Three Inclusion Levels of Feathermeal (0, 8, and 24%)

Nutrient composition	FTH0 (g/100 g DM)	FTH8 (g/100 g DM)	FTH24 (g/100 g DM)
Recipe			
Fishmeal	52.57	42.84	20.67
Feathermeal		8.00	24.00
Wheat flour	19.70	21.00	24.07
L-Lysine HCI			1.08
DL-Methionine		0.01	0.37
L-Histidine	0.16	0.31	0.66
L-Tryptophan		0.01	
Mono-calcium phosphate			0.7
Yttrium	0.05	0.05	0.05
Fish oil*	6.40	6.40	6.50
Rapeseed oil*	19.10	19.30	19.50
Fishmeal			
Protein	71		
Lipid	10.30		
FTH			
Protein	86.40		
Lipid	6.90		
Meal mixes[a]			
Protein	51.20	53.60	52.60
Lipid	9.10	8.60	7.50
Extrudates[b]			
Protein	53.3±1.00	53.0±0.30	54.7±1.40
Lipid	9.50±0.20	8.90±0.10	7.60±0.10
Feed[c]			
Protein	39.9±0.50	39.7±1.50	40.2±0.70
Lipid	34.7±0.20	33.7±0.10	32.5±1.40
Total ash	8.95±0.07	7.55±0.07	5.40±0.00

DM: dry matter. FTH: feathermeal. *: oils used for coating the final feed. [a]sampled after meal mixer and before pre-conditioning. [b]sampled at the end of the extruder. [c]sampled after oil coating. The values for extrudates and feed are given as the mean±SD.

Table 2: m/z Values and Fragments Used for LC-MS/MS Analysis

Compound	**[M+H]** ***m/z***	**Fragments** ***m/z***
Deuterated lysine	151	88.1 and 134.1
Lanthionine	209	120.0
Lysinoalanine	234	130.1 and 84.1

Table 3: Effects of Extrusion Temperatures (100 and 130 °C) and Feathermeal Levels (0, 8, and 24%), and Their Interaction on Protein Oxidation and Growth Parameters of Fish Fed with the Experimental Feeds

	Temperature	**FTH level**	**Temperature x FTH level**
FCR	NS	*	NS
SGR	NS	*	*
FI	NS	*	*
PHP	*	*	*
Carbonyls	*	*	*
NFK	*	*	*
Schiff base	*	*	*
Lanthionine	NS	*	NS
Furosine	NS	NS	NS

FTH: Feathermeal, FCR: feed conversion ratio; SGR: specific growth rate; FI: feed intake; PHP: protein hydroperoxides; NFK: N-formylkynurenine. *: Significant at *P*-value <0.05, NS: not significant

Table 4: Significantly Different Absolute Concentrations of Metabolites (μmol/L) in Plasma of Rainbow Trout Fed the Experimental Diets with Different Inclusion Levels of Feathermeal (0, 8 and 24%) at Different Extrusion Temperatures (100 and 130 °C)

Metabolites	FTH0		FTH8		FTH24	
	100 °C	130 °C	100 °C	130 °C	100 °C	130 °C
Aromatic amino acid						
Phenylalanine	131 ± 14.7 ^{a}	119 ± 1.35 ^{a}	150 ± 13.7 ^{abc}	126 ± 6.06 ^{a}	152 ± 10.2 ^{b}	173 ± 14.5 ^{b}
Tyrosine	50.6 ± 2.87 ^{a}	51.2 ± 6.50 ^{a}	58.2 ± 19.1 ^{abc}	46.5 ± 10.4 ^{a}	68.8 ± 6.91 ^{b}	77.2 ± 4.79 ^{b}
Branched-chain amino acid						
Valine	472 ± 60.0 ^{a}	479 ± 5.84 ^{a}	575 ± 146 ^{abc}	528 ± 47.7 ^{ab}	712 ± 120 ^{c}	664 ± 19.0 ^{bc}
Other amino acid						
Aspartate	19.0 ± 3.48 ^{b}	16.2 ± 0.18 ^{ab}	13.5 ± 2.95 ^{ab}	20.0 ± 6.46 ^{b}	13.4 ± 3.23 ^{ab}	11.7 ± 2.21 ^{a}
Methionine	148.5 ± 21.7 ^{a}	145.2 ± 23.0 ^{a}	164.4 ± 42.1 ^{a}	142.5 ± 33.6 ^{a}	295.7 ± 55.6 ^{b}	354.3 ± 22.5 ^{b}
Glutamate	67.0 ± 31.3 ^{ab}	59.3 ± 25.5 ^{a}	126 ± 27.2 ^{b}	72.3 ± 22.9 ^{ab}	84.6 ± 52.8 ^{ab}	74.2 ± 10.2 ^{ab}
Organic acid and derivates						
Betaine	42.8 ± 1.96 ^{a}	60.3 ± 3.77 ^{ab}	58.9 ± 5.33 ^{ab}	61.1 ± 9.83 ^{ab}	79.1 ± 27.02 ^{b}	71.3 ± 9.62 ^{b}
Organic heterocyclic compounds						
Creatinine	128 ± 24.8 ^{cd}	180 ± 29.2 ^{d}	107 ± 36.7 ^{bc}	114 ± 53.1 ^{bc}	47.1 ± 1.37 ^{a}	58.7 ± 12.9 ^{ab}
N-Methylhydantoin	177 ± 23.0 ^{c}	173 ± 16.5 ^{c}	164 ± 25.5 ^{bc}	138 ± 6.54 ^{b}	83.7 ± 12.0 ^{a}	87.5 ± 10.6 ^{a}
Organic nitrogen compounds						
Dimethylamine	10.2 ± 1.51 ^{b}	11.8 ± 4.75 ^{b}	9.95 ± 2.31 ^{ab}	9.45 ± 5.07 ^{ab}	4.60 ± 0.69 ^{a}	5.05 ± 0.48 ^{a}
Trimethylamine	3.90 ± 0.60 ^{bc}	4.75 ± 0.09 ^{c}	3.20 ± 1.53 ^{abc}	3.45 ± 1.04 ^{abc}	2.00 ± 0.46 ^{a}	3.00 ± 0.54 ^{ab}
Trimethylamine N-oxide	24.9 ± 0.79 ^{c}	21.9 ± 1.66 ^{bc}	19.9 ± 5.68 ^{ab}	16.0 ± 4.61 ^{ab}	14.0 ± 2.80 ^{a}	17.2 ± 0.71 ^{ab}
Organic oxygen compounds						
Acetone	9.60 ± 1.19 ^{ab}	11.4 ± 2.5 ^{b}	8.75 ± 0.74 ^{a}	7.80 ± 0.84 ^{a}	7.85 ± 0.57 ^{a}	7.40 ± 0.48 ^{a}
Glucose	9719 ± 1138 ^{ab}	9198 ± 643 ^{a}	12367 ± 2103 ^{b}	10772 ± 1599 ^{ab}	13212 ± 2679 ^{b}	11432 ± 2170 ^{ab}
myo-Inositol	78.4 ± 29.2 ^{ab}	101 ± 34.9 ^{b}	55.6 ± 8.68 ^{a}	80.0 ± 13.6 ^{ab}	63.6 ± 21.7 ^{ab}	61.2 ± 14.3 ^{ab}
trans-4-Hydroxy-L-proline	88.0 ± 16.3 ^{ab}	93.3 ± 8.17 ^{ab}	106 ± 22.4 ^{b}	81.7 ± 15.1 ^{ab}	69.1 ± 10.9 ^{a}	86.7 ± 15.2 ^{ab}

Experimental diets: FTH0: fish fed control feed without feathermeal, FTH8: fish fed the 8% feathermeal diet; FTH24: fish fed the 24% feathermeal diet. Absolute concentration values are expressed as mean ± SD (n=21 per diet). ^{abcd}mean values across rows with different superscripts assessed by Duncan test are significantly different ($P < 0.05$)

Table 5: Significantly Different Absolute Concentrations of Metabolites (µmol/mg) in Liver of Rainbow Trout Fed the Experimental Diets with Different Inclusion Levels of Feathermeal (0, 8 and 24%) at Different Extrusion Temperatures (100 and 130 °C)

Metabolites	FTH0		FTH8		FTH24	
	100 °C	130 °C	100 °C	130 °C	100 °C	130 °C
Aromatic amino acid						
Phenylalanine	0.68 ± 0.04 ^{ab}	0.70 ± 0.09 ^{ab}	0.81 ± 0.18 ^{ab}	0.66 ± 0.05 ^{a}	0.86 ± 0.03 ^{b}	0.82 ± 0.09 ^{ab}
Branched-chain amino acid						
Isoleucine	0.78 ± 0.10 ^{a}	0.91 ± 0.14 ^{ab}	1.00 ± 0.11 ^{b}	0.80 ± 0.02 ^{a}	1.01 ± 0.06 ^{b}	0.86 ± 0.06 ^{b}
Leucine	1.88 ± 0.20 ^{ab}	2.17 ± 0.24 ^{ab}	1.98 ± 0.22 ^{ab}	1.79 ± 0.15 ^{a}	2.00 ± 0.26 ^{ab}	2.25 ± 0.28 ^{b}
Valine	1.28 ± 0.25 ^{a}	1.64 ± 0.18 ^{bc}	2.14 ± 0.28 ^{e}	1.46 ± 0.03 ^{ab}	2.07 ± 0.11 ^{dc}	1.80 ± 0.08 ^{cd}
Other amino acids						
Alanine	23.3 ± 2.44 ^{ab}	21.2 ± 2.69 ^{a}	22.3 ± 2.60 ^{ab}	24.7 ± 1.91 ^{ab}	27.2 ± 3.88 ^{b}	24.3 ± 3.03 ^{ab}
Creatine	1.57 ± 0.14 ^{ab}	1.68 ± 0.19 ^{b}	1.49 ± 0.09 ^{ab}	1.38 ± 0.18 ^{a}	1.42 ± 0.18 ^{ab}	1.32 ± 0.05 ^{a}
Glutamate	23.8 ± 3.72 ^{ab}	29.4 ± 4.78 ^{b}	23.6 ± 0.59 ^{ab}	23.5 ± 2.51 ^{a}	24.3 ± 3.11 ^{ab}	26.4 ± 0.57 ^{ab}
Lysine	2.11 ± 0.39 ^{ab}	2.65 ± 0.15 ^{ab}	1.81 ± 0.34 ^{a}	1.97 ± 0.23 ^{a}	1.66 ± 0.37 ^{a}	1.67 ± 0.31 ^{b}
Methionine	0.24 ± 0.03 ^{a}	0.49 ± 0.14 ^{b}	0.62 ± 0.18 ^{cd}	0.34 ± 0.08 ^{ab}	0.58 ± 0.11 ^{cd}	0.70 ± 0.02 ^{a}
Threonine	5.11 ± 0.81 ^{ab}	5.22 ± 0.42 ^{b}	4.57 ± 0.21 ^{ab}	5.05 ± 0.37 ^{ab}	4.65 ± 0.38 ^{ab}	4.25 ± 0.20 ^{a}
Nucleosides, nucleotides and analogues						
NADP+	0.19 ± 0.04 ^{b}	0.18 ± 0.02 ^{cd}	0.16 ± 0.02 ^{ab}	0.15 ± 0.03 ^{ab}	0.17 ± 0.01 ^{ab}	0.14 ± 0.01 ^{a}
UDP-glucose	1.55 ± 0.02 ^{d}	1.44 ± 0.12 ^{cd}	1.24 ± 0.17 ^{ab}	1.33 ± 0.07 ^{bc}	1.1 ± 0.05 ^{a}	1.31 ± 0.03 ^{bc}
UDP-glucoronate	1.74 ± 0.17 ^{b}	1.82 ± 0.04 ^{b}	1.55 ± 0.03 ^{a}	1.55 ± 0.05 ^{a}	1.58 ± 0.03 ^{a}	1.45 ± 0.07 ^{a}
Organic acid and derivates						
2-Aminobutyrate	0.21 ± 0.01 ^{ab}	0.29 ± 0.09 ^{b}	0.24 ± 0.03 ^{ab}	0.19 ± 0.04 ^{a}	0.18 ± 0.04 ^{a}	0.20 ± 0.01 ^{ab}
3-Hydroxyisobutyrate	0.15 ± 0.03 ^{ab}	0.17 ± 0.03 ^{ab}	0.17 ± 0.03 ^{ab}	0.14 ± 0.00 ^{a}	0.17 ± 0.01 ^{ab}	0.19 ± 0.04 ^{b}
5-Aminopentanoate	3.14 ± 1.00 ^{c}	2.56 ± 0.46 ^{abc}	1.87 ± 0.21 ^{ab}	2.68 ± 0.51 ^{bc}	2.20 ± 0.25 ^{abc}	1.63 ± 0.25 ^{a}
Formate	0.36 ± 0.09 ^{ab}	0.31 ± 0.05 ^{ab}	0.41 ± 0.07 ^{b}	0.29 ± 0.02 ^{a}	0.38 ± 0.03 ^{ab}	0.33 ± 0.06 ^{ab}
N,N-Dimethylglycine	0.03 ± 0.00 ^{abc}	0.04 ± 0.01 ^{c}	0.03 ± 0.01 ^{bc}	0.02 ± 0.00 ^{ab}	0.02 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}
Sarcosine	0.21 ± 0.09 ^{a}	0.06 ± 0.01 ^{ab}	0.04 ± 0.01 ^{a}	0.05 ± 0.01 ^{a}	0.03 ± 0.00 ^{a}	0.06 ± 0.02 ^{a}
Taurine	141 ± 7.94 ^{b}	138 ± 3.13 ^{b}	136 ± 1.51 ^{b}	133 ± 5.33 ^{b}	134 ± 5.72 ^{b}	115± 9.34 ^{a}
β-Alanine	2.63 ± 0.45 ^{ab}	2.22 ± 0.37 ^{a}	2.95 ± 0.38 ^{bc}	2.49 ± 0.09 ^{ab}	3.55 ± 0.38 ^{c}	2.49 ± 0.33 ^{ab}
Organic heterocyclic compounds						
Creatinine	0.82 ± 0.24 ^{b}	0.85 ± 0.27 ^{b}	0.44 ± 0.05 ^{a}	0.60 ± 0.12 ^{ab}	0.52 ± 0.18 ^{ab}	0.39 ± 0.05 ^{a}
N-Methylhydantoin	0.59 ± 0.04 ^{d}	0.51 ± 0.10 ^{c}	0.42 ± 0.07 ^{b} ^{c}	0.45 ± 0.02 ^{bc}	0.39 ± 0.03 ^{b}	0.26 ± 0.04 ^{a}
Organic nitrogen compounds						
O-Phosphocholine	4.22 ± 0.72 ^{b}	4.09 ± 0.13 ^{ab}	3.04 ± 0.52 ^{ab}	4.28 ± 1.84 ^{b}	2.47 ± 0.70 ^{a}	2.67 ± 0.21 ^{ab}
Trimethylamine N-oxide	0.81 ± 0.22 ^{c}	0.62 ± 0.10 ^{abc}	0.43 ± 0.04 ^{a}	0.68 ± 0.15 ^{bc}	0.48 ± 0.04 ^{ab}	0.44 ± 0.04 ^{a}
Organic oxygen compounds						
Glucose	54.7 ± 5.07	69.7 ± 24.0 ^{b}	71.6 ± 13.0	41.0 ± 11.8 ^{a}	63.5 ± 9.83	51.2 ± 5.84

ab b ab ab

Experimental diets: FTH0: fish fed control feed without feathermeal, FTH8: fish fed the 8% feathermeal diet; FTH24: fish fed the 24% feathermeal diet. Absolute concentration values are expressed as mean ± SD (n=21 per diet). [abcd]mean values across rows with different superscripts assessed by Duncan test are significantly different (P<0.05)

Fig. 1:

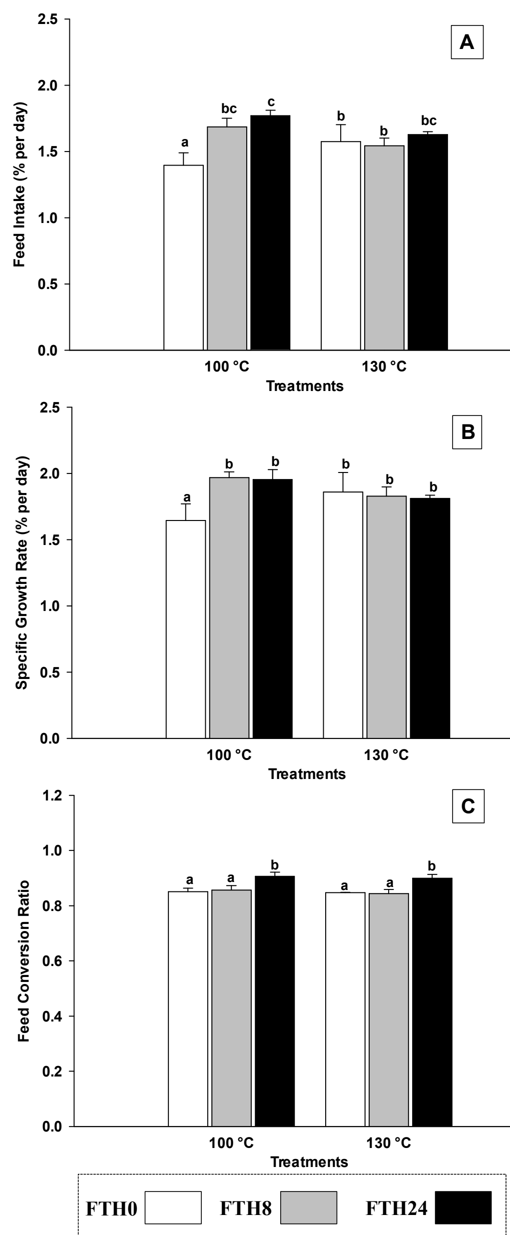

Fig. 2:

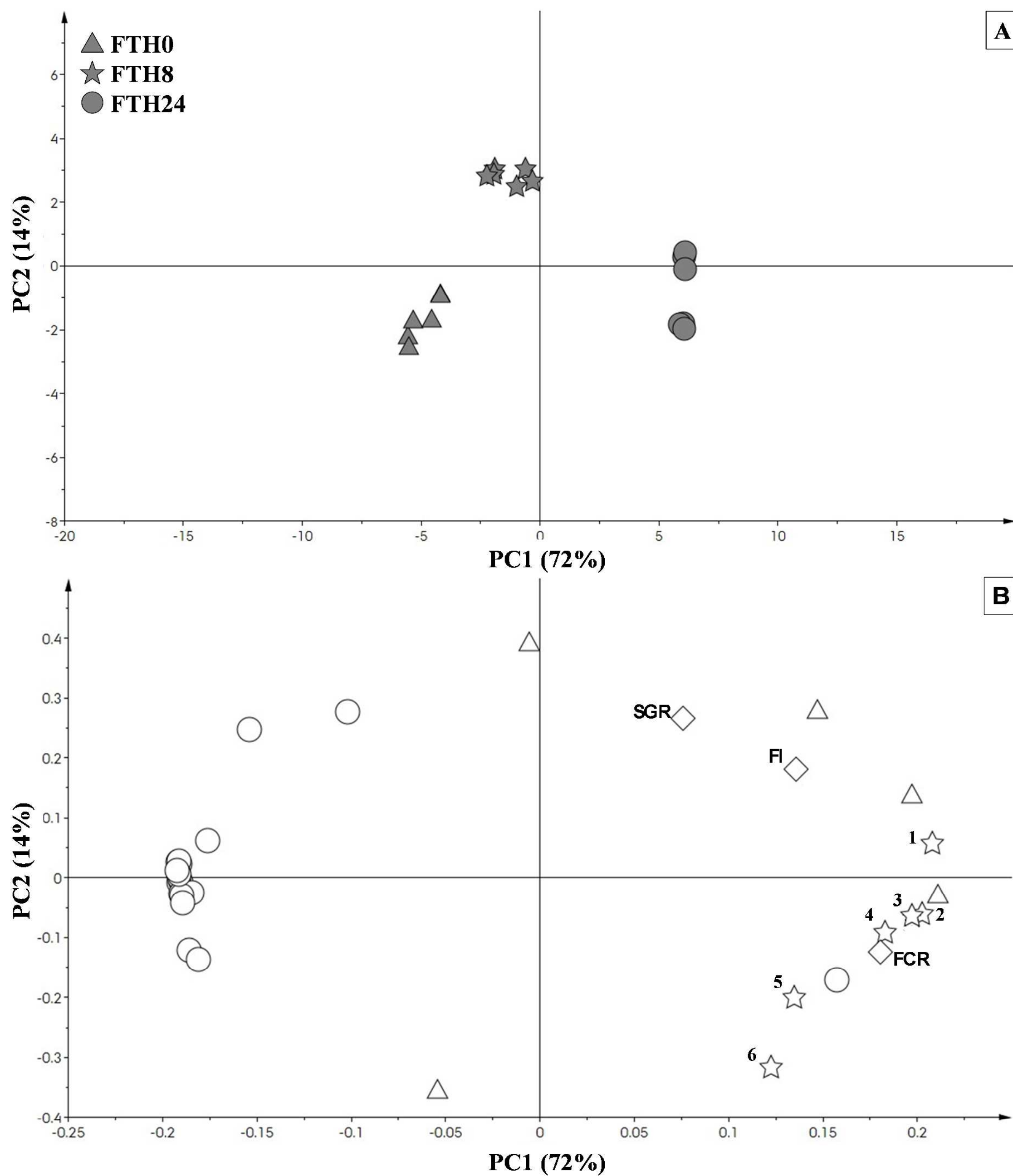

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Fig. 3:

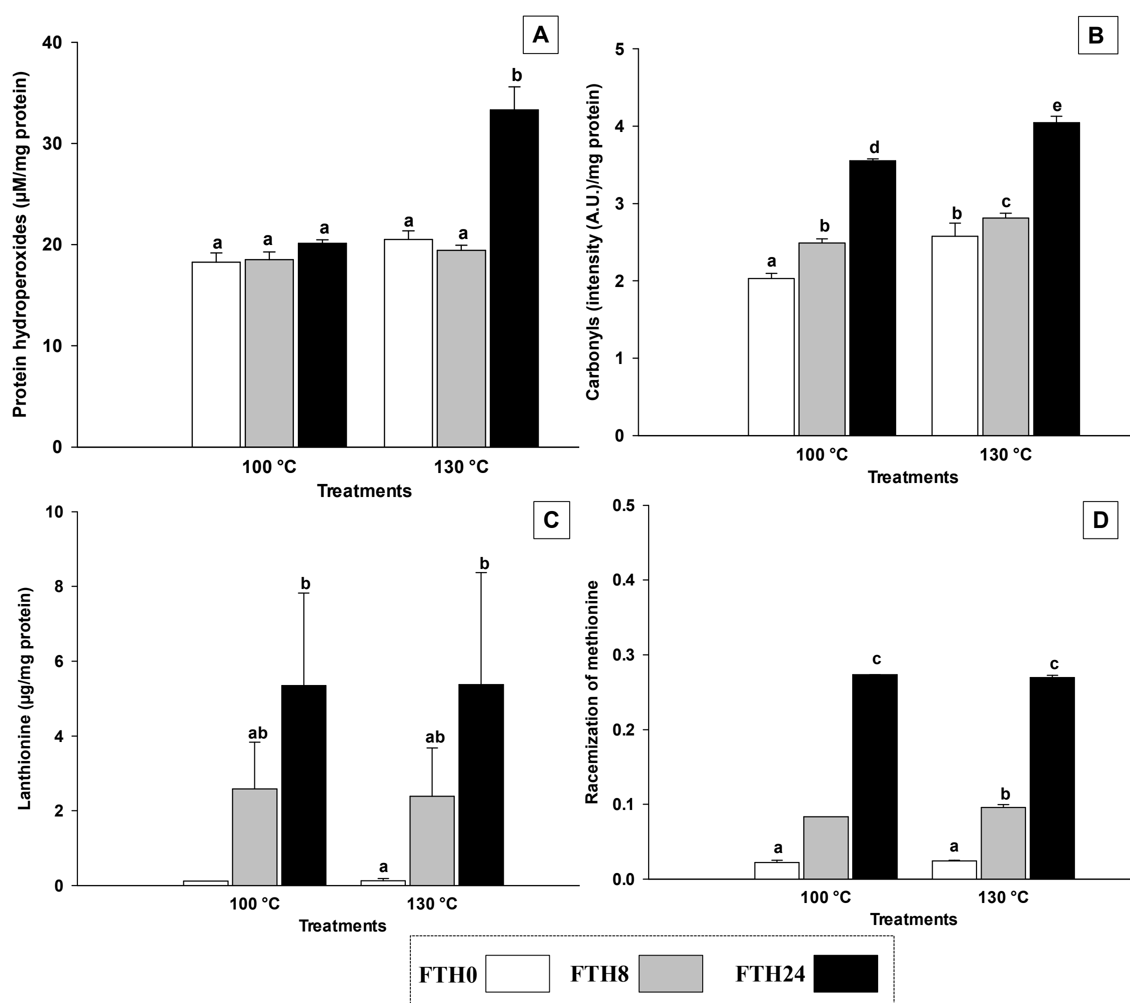

Fig. 4:

TOC Graphic