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1 # The effect of dietary chitin on Atlantic salmon (*Salmo salar*) chitinase activity, gene expression,
2 # and microbial composition

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10 **Running head:** Dietary chitin and salmon gene expression

11 **Keywords:** chitin; chitinase; chitin synthase; chitin metabolism; shrimp shell; black soldier fly

12

13 ## ABSTRACT

14 **Background:** Chitin is a common component in the natural diet of many fish, and a range of chitinases
15 with the potential to down chitin have been identified. Yet whether chitin is metabolized in fish is still
16 unclear. Here we used a combination of chitinase activity assay, transcriptomics, and 16S rRNA
17 bacterial analysis to assess the effect of chitin supplementation on Atlantic salmon gene expression and
18 microbial community.

19 **Results:** Atlantic salmon express multiple genes associated with chitin metabolism, and we show that
20 the expression and activity of Atlantic salmon chitinases are not affected by the addition of dietary
21 chitin. We do, however, demonstrate an association between gut microbial composition, chitinase
22 activity in the gut, and host chitinase expression.

23 **Conclusion:** The findings presented here support the idea that chitin metabolism genes are linked to
24 the maintenance of a chitin-based barrier in the teleost gut. These results contribute to a greater
25 understanding of chitin metabolism in fish.

26

27 ## INTRODUCTION

28 Chitin is a tough and insoluble polymer consisting of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc)
29 residues that serves as a protective and structural component in arthropod exoskeletons such as insects
30 and crustaceans (Kramer *et al.* 1995; Kurita 2006). This complex carbohydrate is commonly found in
31 the natural diet of many fish, including Atlantic salmon (*Salmo salar*), and chitinous organisms like

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32 Antarctic krill (*Euphausia superba*) and black soldier fly (*Hermetia illucens*) are considered two
33 sustainable feed sources for Atlantic salmon farming (Hansen *et al.* 2010; Belghit *et al.* 2019).
34 Supplementation of chitin in Atlantic salmon feed has been shown to promote the growth of potentially
35 beneficial gut microbes that *in vitro* inhibit the growth of common fish pathogens (Askarian *et al.* 2012).
36 However, some studies have reported a reduced growth rate in salmon fed chitin-rich diets and it has
37 been hypothesized that chitin acts as an energy sink when fish are not able to digest and utilize this
38 polysaccharide properly (Karlsen *et al.* 2017; Renna *et al.* 2017).

39 Chitinase and chitobiase are the enzymes responsible for the hydrolysis of chitin and the production of
40 GlcNAc monomers. Their activity has been detected in the digestive tract of a variety of fish species
41 (Jeuniaux 1961; Fänge *et al.* 1979; Lindsay *et al.* 1984; Lindsay 1984; Kono *et al.* 1987; Gutowska *et*
42 *al.* 2004; Krogdahl *et al.* 2005; Abro *et al.* 2014), including salmonids such as rainbow trout
43 (*Oncorhynchus mykiss*) (Lindsay *et al.* 1984), and a range of fish chitinases have been identified and
44 biochemically characterized (Ikeda *et al.* 2009, 2012, 2013, 2017; Zhang *et al.* 2012; Koch *et al.* 2014;
45 Teng *et al.* 2014; Kakizaki *et al.* 2015; Kawashima *et al.* 2016; Pohls *et al.* 2016; Gao *et al.* 2017). Most
46 of these enzymes are detected in the gastrointestinal tract and show acid-resistant activities toward
47 insoluble chitin substrates, indicating that they could have the ability to digest chitin (Ikeda *et al.* 2017).
48 As far as we know, no one has characterized chitinase proteins in Atlantic salmon.

49 Another family of enzymes involved in chitin metabolism is chitin synthases (CHS), which synthesize
50 chitin from GlcNAc. Since chitin is present in their scales (Tang *et al.* 2015), it is hypothesized that
51 salmon express CHS and that these are active, however very little is known about the role of these
52 enzymes in metabolic processes. In this study we investigated the effect of chitin supplementation on
53 the expression of host chitinases, chitobiase, and CHS as well as on the microbiome of Atlantic salmon.

54

55 MATERIALS AND METHODS

56 Feeding trial

57 The fish used in this experiment were Atlantic salmon post-smolts obtained as fry from AquaGen
58 Breeding Centre, Kyrksæterøra, and reared in fresh water at the Centre for Fish Research, NMBU. The
59 fish (n = 32) were acclimatized to experimental conditions for 41 days before the trial and were fed a
60 standard commercial diet. Groups of six fish were placed in each tank with an average weight of 812 ±
61 113 g and diets were tested in two replicate tanks. The fish were fed one of three different diets (Table
62 1) over 29 days through an automatic feeding system. Starting with a standard core feed composition,
63 Diet 1 – "control" was supplemented with 6 % cellulose bought from FôrTek, NMBU, Diet 2 – "shrimp
64 shell" was supplemented with 6 % shrimp (*Pandalus borealis*) shell chitin bought from Primex
65 (ChitoClear Chitin, Iceland) and in Diet 3, a portion of the fish meal was substituted with insect meal
66 from defatted black soldier fly larvae (*Hermetica illucens*) obtained from Protix Biosystems BV

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67 (Dongen, Netherlands) to a final concentration of approximately 6% chitin. After 29 days fish were
68 euthanized by a blow to the head in accordance with the national regulations of animal
69 (Dyrevelferdsloven 2015). Tissue samples and the contents from the stomach and pyloric caeca were
70 collected separately, flash-frozen in liquid nitrogen, and stored at -80 °C until analysis of gene
71 expression and chitinase activity. Contents from the distal intestine (DI), the most distal compartment
72 of the gut, were sampled for 16S rRNA sequencing as previously described (Rudi *et al.* 2018).
73

74 **Table 1**. Ingredients and proximal composition of the experimental diets.

	Experimental diets		
	Control	*Shrimp shell*	*Fly larvae*
Ingredients (g kg^{-1})			
Cellulose	**4.7**	0.0	0.0
Fish meal	32.2	32.2	9.1
Wheat gluten	9.4	9.4	9.4
Pregel potato starch	8.6	8.6	8.6
Defatted black soldier fly larvae meal	0.0	0.0	**32.4**
Shrimp shell chitin	0.0	**4.7**	0.0
Gelatine	5.8	5.8	5.8
Choline chloride	0.1	0.1	0.1
Fish oil	10.8	10.8	6.1
Vitamin/mineral mixture	0.5	0.5	0.5
Proximate composition (%)			
Ash	9.17	8.79	6.38
Moisture	6.91	6.58	4.71
Lipid	19.64	19.64	19.25
Crude protein (N · 6.25)	46.9	46.9	47.36
Chitin	**0**	**6.11**	**6.35***
Cellulose	6.11	0	0

75 * This is a proximate measure using the following formula to calculate chitin in insect meal: chitin (%)
76 = ash-free ADF (%) - ADIP (%) as previously done by Marono S. *et al* (2015) (Marono *et al.* 2015)
77

78 **RNA-sequencing**

79 Total RNA was extracted from the stomach and pyloric caeca tissue samples stored at -80 °C (n = 4 for
80 control and fly larvae, n = 5 for shrimp shell) using the RNeasy Plus Universal Kit (QIAGEN). RNA
81 quality was assessed using a 2100 Bioanalyzer with the RNA 6000 nano kit (Agilent) and the
82 concentration was measured using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific).
83 Extracted RNA with RNA integrity number (RIN) ≥7 was used as input for the TruSeq Stranded mRNA
84 HT Sample Prep Kit (Illumina) according to the manufacturer's recommendations. Mean library length
85 was measured on 2100 Bioanalyzer using the DNA 1000 kit (Agilent) and the library concentration was
86 quantified with the Qbit Broad Range kit (Thermo Fisher Scientific). Single-end sequencing (100bp
87 reads) was performed at the Norwegian Sequencing Center (Oslo, Norway) using a HiSeq4000
88 instrument (Illumina).
89

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90 **Gene expression**

91 Trimming, mapping, and counting of reads were done using the bcbio-nextgen pipeline
92 (https://github.com/bcbio/bcbio-nextgen). The sequencing reads were aligned to the Atlantic salmon
93 genome (ICSASG_v2) (Lien *et al.* 2016) using the STAR aligner with default settings (Dobin *et al.*
94 2013) after adapter trimming. FeatureCounts was then used to count reads aligned to genes (Liao *et al.*
95 2014). Raw gene counts were transformed to transcripts per million (TPM) values to normalize for
96 gene length before comparison of chitinase gene expression levels (Welch's t-test, $\alpha = 0.05$). Gene
97 expression values were normalized by library size (see TMM normalization in EdgeR user guide
98 (Robinson and Oshlack 2010)) before differential expression analysis. Based on PCA analysis, two
99 samples (X8.CFE.7.F4.PC and X9.CFE.13.F1.PC) showed unusual gene expression patterns and were
100 removed from further analysis of pyloric caeca for fish fed control and fly larvae. All statistical analysis
101 was done in R (v.3.6.0).

102

103 **Differential expression analysis**

104 Lowly expressed genes ($\log_2$(TPM + 1) values < 1) were filtered out prior to differential expression
105 analysis. The analysis was carried out using the standard EdgeR protocol (Robinson *et al.* 2010) where
106 an exact test of expression values between the experimental diet (shrimp shell or fly larvae) and control
107 diet gave a log2-fold change, p-value, and false discovery rate (FDR) for each gene. Genes with an FDR
108 < 0.05 and absolute log2 fold change (logFC) > 0.5 were defined as differentially expressed.

109

110 **Gene enrichment analysis**

111 Gene enrichment analysis of the differential expressed genes was performed using KEGG (Kyoto
112 Encyclopedia of Genes and Genomes) and GO (Gene Ontology). KEGG analysis was carried out using
113 the "kegga" function in the limma-package (Ritchie *et al.* 2015) with the argument "species.KEGG =
114 "sasa"" and p-values < 0.05. GO analysis was carried out using the package GOstats (Falcon and
115 Gentleman 2007), following the steps outlined here
116 (https://www.bioconductor.org/packages/release/bioc/vignettes/GOstats/inst/doc/GOstatsForUnsuppo
117 rtedOrganisms.pdf), using the argument "ontology = "BP"" (biological processes) and Bonferroni
118 adjusted p-values (q) < 0.05.

119

120 **Chitinase activity test**

121 We measured the chitinase activity of fish analyzed for gene expression that contained stomach and
122 pyloric caeca content (n = 3) as described previously (Ohno *et al.* 2013). Approximately 200 mg of
123 contents were homogenized using TissueLyser II (QIAGEN) in 900 uL of 50 mM sodium acetate (pH
124 = 5.5) containing 1X Halt protease inhibitor cocktail (Thermo Fisher Scientific). The samples were

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125 centrifuged at 14.000 g for 20 min at 4°C to pellet particulates, and the supernatant was collected for
126 protein quantification and chitinase activity. Protein concentration was measured using Bradford protein
127 assay (Quick Start^{TM} Bradford Assay, BioRad) with BSA (Bovine Serum Albumin) used as standard,
128 according to manufacturer's instructions. Chitinase activity was measured by monitoring the hydrolysis
129 of 4-Methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside (Sigma-Aldrich, M5639), a fluorogenic
130 chitin substrate suitable for measuring endochitinase activity, according to the Fluorimetric Chitinase
131 Assay Kit (Sigma-Aldrich, CS1030) with the following modifications. Briefly, 10 µg of pyloric caeca
132 proteins and 1 µg of stomach proteins were incubated with 200 µM 4-Methylumbelliferyl β-D-N,N′,N″-
133 triacetylchitotrioside (4-MU-GlcNAc$_3$) in McIlvaine's buffer (0.1 M citric acid and 0.2 M Na_2HPO_4,
134 pH 6) in a volume of 100 µL at 28 °C. The pH and temperature were determined from pilot experiments
135 on a recombinant Atlantic salmon chitinase (rChia.8, unpublished) where pH 6 and 28 °C were optimal
136 conditions for activity of this enzyme which is expressed in both stomach and pyloric caeca of Atlantic
137 salmon. The reaction was terminated after 30 min by adding 400 mM sodium carbonate and the
138 fluorescence of the released 4-Methylumbelliferone (4-MU) was measured at an excitation wavelength
139 of 360 nm and an emission wavelength of 450 nm using a SpectraMax M2 plate reader (Molecular
140 Devices) no later than 30 minutes after stopping the reaction. The assay was performed in triplicates
141 and a 4-MU standard curve was used to quantify 4-MU resulting from the hydrolytic reaction. The
142 measured fluorescence was corrected for hydrolysis of the substrate without enzyme added. The
143 chitinase activity was expressed as unit/mg of total protein in the sample where 1 unit is defined as 1
144 umol of 4-MU formed per minute. Statistical comparisons were done using Student's t-test ($\alpha = 0.05$
145 and $\alpha = 0.1$).
146
147 **Illumina sequencing of the 16S rRNA gene**

148 DNA was extracted from ground samples of Diet 1-3 fish feed (n=2 per feed) and the contents collected
149 from the distal intestine (DI) from all fish (n=12 per feed) using the Mag midi kit (LGC Genomics, UK)
150 following the manufactures instructions. Preparation of amplicon library using the primer pair
151 341F/806R (Yu *et al.* 2005) and sequencing was done as previously described (Rudi *et al.* 2018). The
152 resulting reads were processed as previously described (Angell *et al.* 2020) using a sequence depth of
153 10,000 sequences per sample.
154
155 **RESULTS**

156 **Impact of dietary chitin on chitinase activity and expression**

157 We performed an *in vitro* quantification of chitinase activity from crude extracts of total soluble material
158 collected from the stomach and pyloric caeca of fish fed one of three diets differing in chitin content.
159 Chitinase activity relative to the amount of protein was consistently much higher (ranging from 5 to11-

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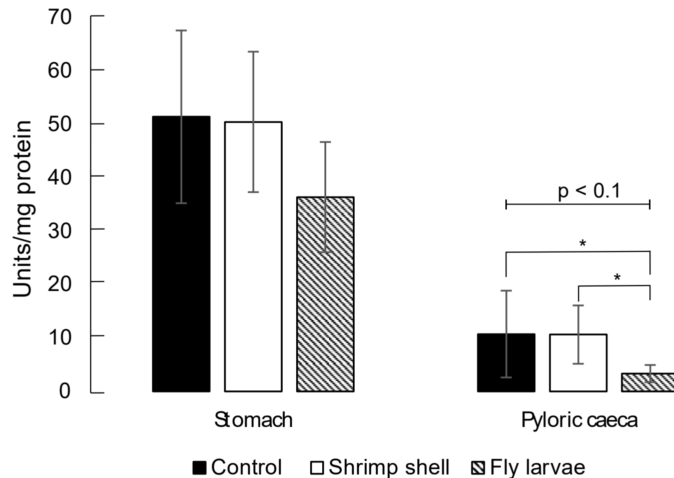
160 fold difference) in the stomach than in pyloric caeca irrespective of diets. Chitinase activity was
161 unaffected by the inclusion of dietary chitin when compared to control (Student's t-test, $p > 0.05$; Figure
162 1). However, a trend towards lower activity in fish fed fly larvae compared to the fish fed control and
163 shrimp shell was observed (Student's t-test, $p < 0.1$).



164

165 **Figure 1.** Chitinase activity (units/mg protein) in the stomach- and pyloric caeca contents of fish (n=3)
166 fed control, shrimp shell, and fly larvae using 4-MU-GlcNAc$_3$. Crude soluble protein solutions from
167 stomach (1 µg per reaction) and pyloric caeca (10 µg per reaction) was incubated with 200 µM 4-MU-
168 GlcNAc$_3$ for 30 minutes at 28 °C in McIlvaine's buffer, pH 6. 1 unit is defined as 1 µmol of 4-MU
169 formed per minute. The values shown are means of triplicates ± SD.

170

171 According to the NCBI Salmo Salar RefSeq annotation (GCF_000233375.1; release 100), Atlantic
172 salmon codes for 10 chitinase-like genes from the glycosyl hydrolase family 18 (hereafter named
173 *chia.1-10*; see Supplementary Table 1 for the corresponding gene IDs), a single chitobiase-like gene
174 from the glycosyl hydrolase family 18 (hereafter named *ctbs*), and four genes with putative chitin-
175 synthase domains (hereafter named *chs1a*, *chs1b*, *chs2,* and *chs3*). Eight chitinase genes and two CHS
176 genes showed tissue-specific expression (stomach; *chia.3*, *4, 5* and *7*, pyloric caeca; *chia.1*, *2*, *6*, *9* and
177 *10, chs1a* and *chs1b*) with *chia.8* and *ctbs* being ubiquitously expressed in both tissues (Figure 2A).
178 Notably, three of the chitinase genes expressed in the stomach (*chia.3*, *4,* and *7*) were among the most
179 abundant transcripts present in the tissue, whereas *chia.1*, *chia.2,* and *chia.6* were among the most
180 abundant transcripts in pyloric caeca. Two CHS genes, *chs2* and *chs3* were not expressed in any of the
181 two tissues. To assess the response of chitinase-, chitobiase- and CHS genes to the inclusion of dietary
182 chitin, we compared their expression levels in the stomach (Figure 2B) and pyloric caeca (Figure 2C).
183 One chitinase, *chia.5*, was lowly expressed ($\log_2(\text{TPM}) < 2$) and is therefore not shown in Figure 2B.
184 The inclusion of dietary chitin had no significant effect on chitinase, chitobiase, and CHS gene
185 expression (Welch's t-test, $p > 0.05$), although the expression of *chia.1, chia.2,* and *chia.6* in pyloric
186 caeca appeared to be slightly lower in fish fed fly larvae ($p < 0.13$).

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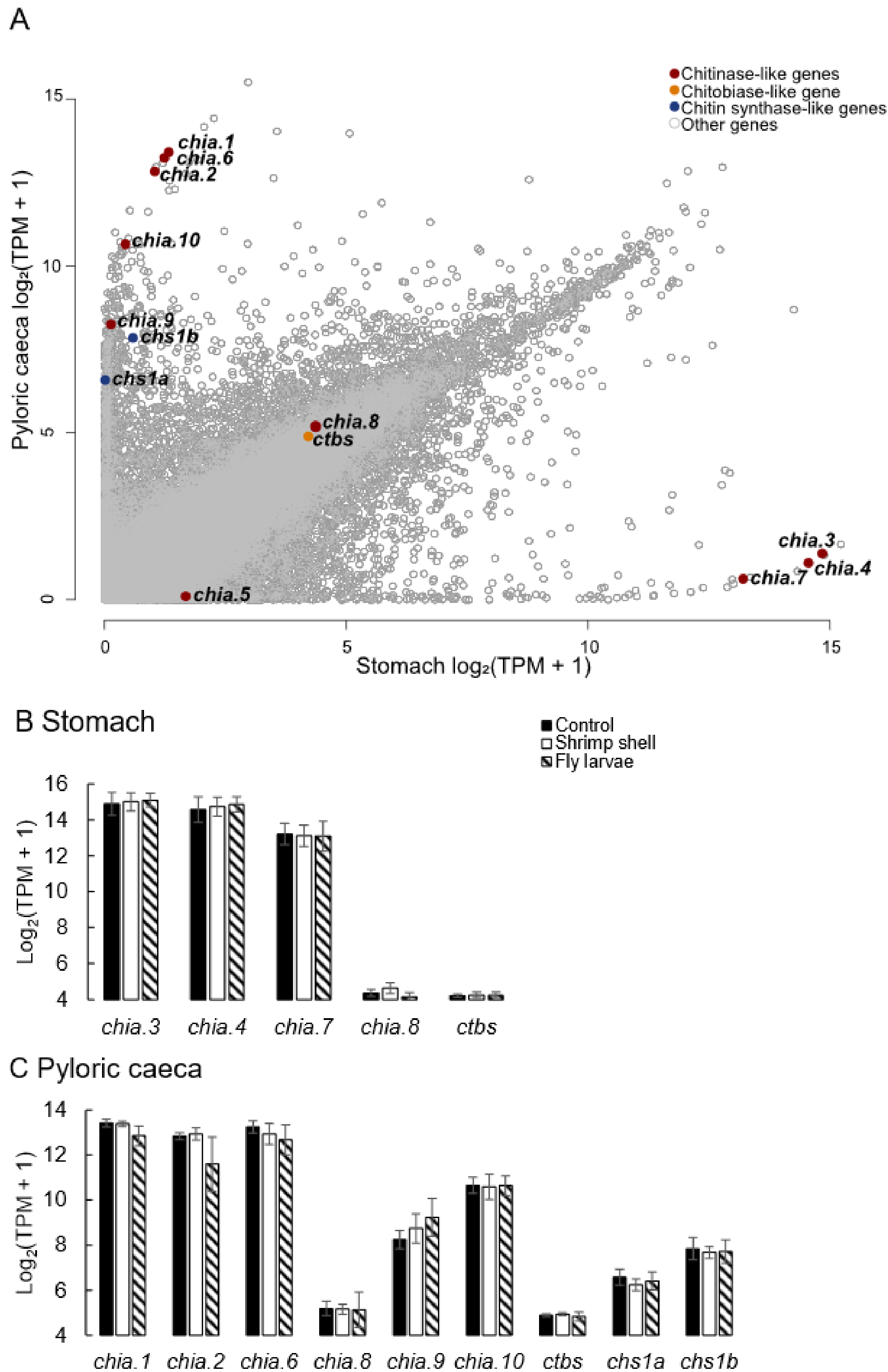

187

188 **Figure 2.** A) Gene expression levels ($\log_2$(TPM + 1) of chitinase-, chitobiase- and CHS genes compared
189 to the expression levels of all genes in the stomach and pyloric caeca for fish fed the control diet (n=4
190 for stomach and n=3 for pyloric caeca). B) Gene expression levels ($\log_2$(TPM + 1) of chitinase and
191 chitobiase genes in the stomach (n=4 for control and fly larvae, n=5 for shrimp shell). C) Gene
192 expression levels ($\log_2$(TPM + 1) of chitinase and CHS genes in pyloric caeca (n=3 for control and fly
193 larvae, n=5 for shrimp shell). Please note that the y-axis does not extend to 0.
194

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195 **Gene enrichment analysis**

196 In addition to focusing on genes related to chitin metabolism, we examined how the transcriptome more
197 generally responded to the inclusion of dietary chitin as a replacement to cellulose. Beginning with the
198 stomach, feeding fish a diet including fly larvae had very little effect on the transcriptome compared to
199 the control diet with only one differentially expressed gene (DEG), namely dual-specificity protein
200 phosphatase 1-like (*dusp1*). In contrast, a shrimp shell containing diet provoked a larger effect with 889
201 upregulated and 570 downregulated genes. The most enriched GO (biological process) and KEGG
202 terms among the upregulated genes were processes and pathways involved in cell organization
203 including muscle structure development, focal adhesion, and extracellular matrix (ECM) receptor
204 interactions (Table 2).

205

206 **Table 2.** Top GO terms and KEGG pathways of upregulated (up) and downregulated (down) DEGs in
207 the stomach of fish fed shrimp shells. The rich factor is the ratio of DEG number and the total number
208 of genes annotated to this pathway.

Enriched term	DEGs	Rich factor	P-value	Method	Mode
Muscle structure development	184	0.108	7.69E-35	GO_BP	Up
Anatomical structure morphogenesis	396	0.063	3.18E-27	GO_BP	Up
Cell adhesion	189	0.087	5.58E-25	GO_BP	Up
Focal adhesion	41	0.064	1.25E-12	KEGG	Up
ECM-receptor interaction	19	0.067	8.75E-07	KEGG	Up
Regulation of actin cytoskeleton	30	0.048	9.88E-07	KEGG	Up
Synaptic vesicle recycling	19	0.116	2.91E-05	GO_BP	Down
Glycoprotein biosynthetic process	38	0.066	3.29E-05	GO_BP	Down
Protein glycosylation	30	0.071	5.77E-05	GO_BP	Down
UDP-N-acetylglucosamine biosynthetic process	5	0.42	3.45E-04	GO_BP	Down
Metabolic pathways	80	0.025	4.59E-10	KEGG	Down
Amino sugar and nucleotide sugar metabolism	11	0.085	1.20E-06	KEGG	Down
Glutathione metabolism	9	0.084	1.20E-05	KEGG	Down
Mucin type O-glycan biosynthesis	8	0.09	2.28E-05	KEGG	Down

209

210 Among the downregulated genes in the stomach, the most enriched processes and pathways were those
211 where glycosylation plays a central role, including synaptic vesicle recycling, glycoprotein biosynthetic
212 processes, and amino sugar and nucleotide sugar metabolism. Notably, several of the downregulated
213 genes are central in the pathway whereby fructose-6-phosphate is converted to uridine diphosphate N-
214 acetylglucosamine (UDP-N-acetylglucosamine biosynthetic process), the substrate molecule used by
215 CHS to synthesize chitin (Figure 3). This includes glutamine-fructose-6-phosphate aminotransferase 2-

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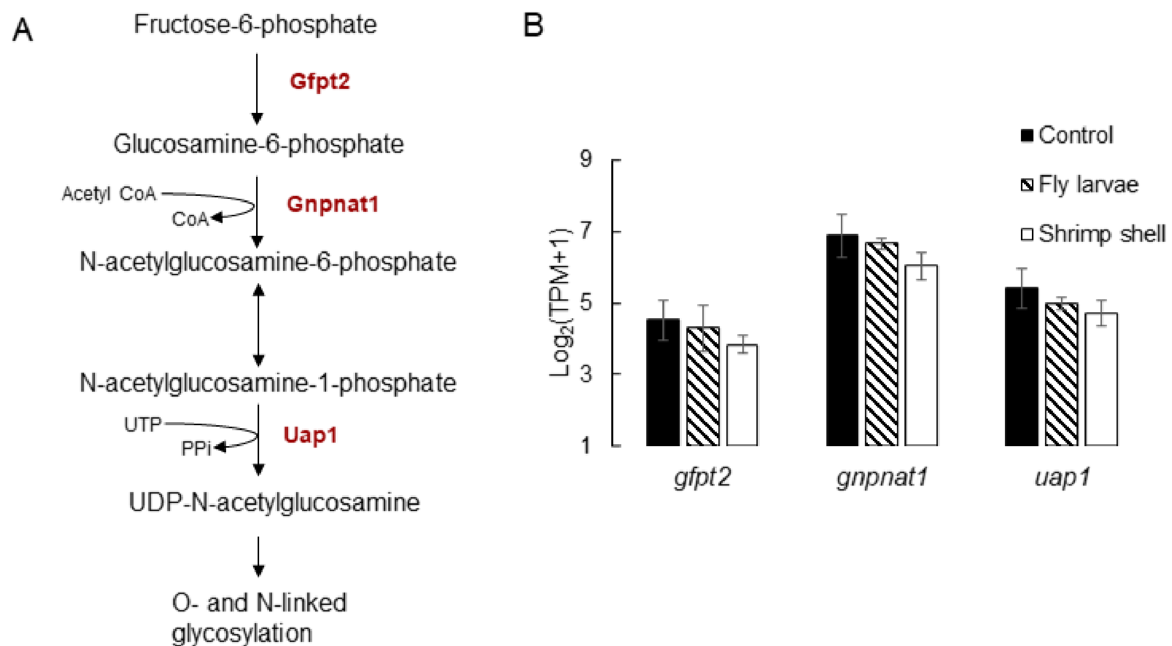
216 like (*gfpt2*), glucosamine-phosphate N-acetyltransferase 1 (*gnpnat1*) and UDP-N-acetylhexosamine
217 pyrophosphorylase-like (*uap1*).
218



219

220 **Figure 3.** A) The hexosamine biosynthetic pathway leading to the production of UDP-N-
221 acetylglucosamine (UDP-GlcNAc) where central enzymes discussed in the text are marked in red. B)
222 Gene expression levels (log_2(TPM + 1) of differentially expressed hexosamine biosynthetic pathway
223 genes in the stomach (n=4 for control and fly larvae, n=5 for shrimp shell). Please note that the y-axis
224 does not extend to 0.

225

226 When analyzing data from the pyloric caeca only two genes (endonuclease domain-containing 1
227 protein-like; *endd1*, and ribonuclease P protein subunit p30-like; *rpp30*) were differentially expressed
228 when replacing cellulose with shrimp shell chitin, whereas 53 genes were upregulated and eight
229 downregulated in response to feeding fly larvae. GO enrichment of the 53 upregulated genes revealed
230 that many of these genes (n=22) are involved in the biosynthesis of cholesterol including HMG-CoA
231 reductase (*hmgr*) which is responsible for the rate-limiting step in the cholesterol biosynthesis pathway.
232 No common enriched term was found for the downregulated genes, but the 8 genes detected included
233 acid-sensing (proton gated) ion channel 1 (*asic1*), collagen alpha-1(XXIV) chain-like (*col24a1*), solute
234 carrier family 25 member 48-like (*slc25a48*), peptidase inhibitor R3HDML-like (*r3hdml*), lecithin
235 retinol acyltransferase-like (*lrat*) and three uncharacterized genes.

236

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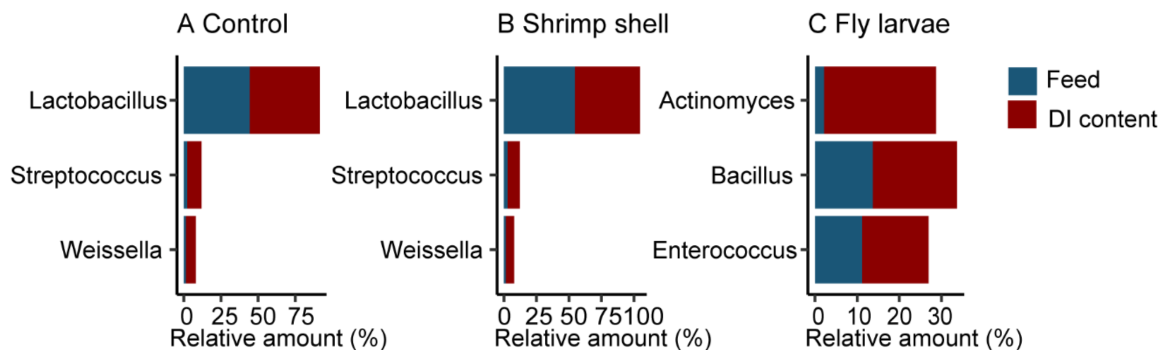
237 **The bacterial composition of distal intestine contents**

238 To investigate the impact of dietary chitin on the microbial community, we analyzed the bacterial
239 metapopulation of feed and DI using 16S rRNA gene sequencing. We observed a clear relationship
240 whereby bacteria present in the raw feedstuff were also present in the DI (Figure 4). The microbial
241 profiles of DI contents from fish fed control and shrimp shell diets were almost identical, at the genus
242 level the taxa dominating the distal intestinal contents of fish fed control and shrimp shell were
243 *Lactobacillus* (45.7% and 50.7%), *Streptococcus* (9.4% and 9.3%) and *Weissella* (6.4% and 6.5%). For
244 fish fed fly larvae, the dominant taxa were *Actinomyces* (28.2%), *Bacillus* (21.6%), and *Enterococcus*
245 (16.9%). All prevalent taxa in distal intestinal contents were also present in the feed samples, although
246 the relative amount of *Actinomyces* in fish fed fly larvae was substantially higher than in the feed (28.2%
247 vs 2.18%).

248



249

250 **Figure 4.** The three most abundant bacteria found in distal intestine (DI content) and fish feed as
251 assessed by 16S rRNA gene sequencing of fish fed A. Control diet (mean value of n=11 for DI
252 content and n=2 for feed), B. Shrimp shell diet (mean value of n=9 for DI content and n=2 for feed),
253 and C. Fly larvae diet (mean value of n=10 for DI content and n=2 for feed).

254

255 **DISCUSSION**

256 Transcriptome analyses revealed that the stomach and pyloric caeca react differently to the diets given
257 in this trial. The largest changes in terms of DEGs were observed in the stomach when fish were fed a
258 diet supplemented with chitin from shrimp shells. GO and KEGG analysis lead us to conclude that these
259 changes involve the upregulation of genes involved in tissue organization and extracellular matrix-cell
260 interactions and are linked to structural changes in the gastric tissue. The mucosal barrier that covers
261 the gastrointestinal tract of Atlantic salmon consists of highly glycosylated mucins (Jin *et al.* 2015) and
262 the downregulation of genes involved in glycosylation of these proteins further implies a structural
263 change or a response to the extracellular environment. We hypothesize that this change is partly caused
264 by chitin degradation, with the release of products such as GlcNAc as the result of the activity of highly
265 expressed chitinases in the stomach (Figure 2A-B). In line with what we observe, higher GlcNAc
266 concentrations are associated with the downregulation of genes involved in the biosynthesis of UDP-
267 GlcNAc (Figure 3). UDP-GlcNAc is a substrate required for O – and N-glycosylation of proteins,

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268 including mucins that are heavily glycosylated. In mice, orally administered GlcNAc was shown to
269 enter the hexosamine biosynthetic pathway and increase the abundance of UDP-GlcNAc (Ryczko *et al.*
270 2016). UDP-GlcNAc has shown to act as an end-product inhibitor of this pathway (Chiaradonna *et al.*
271 2018) and regulates the gene expression of *gfpt2*, which converts fructose-6-phosphate and glutamine
272 to glucosamine-6-phosphate and glutamate, eventually modulating glycosylation homeostasis.

273 There is evidence for the presence of chitinous structures surrounding the mucosal barrier of ray-finned
274 fish (Tang *et al.* 2015; Nakashima *et al.* 2018) and the results presented here are consistent with the
275 evolutionary conservation of host chitinases and chitobiase to participate in the remodeling of these
276 structures. Previous studies show that Atlantic salmon is not able to utilize chitin to a significant extent
277 (Olsen *et al.* 2006). In line with our results, increasing the dietary chitin content has previously been
278 shown not to correlate with increased chitinase activity in fish (Lindsay *et al.* 1984; Kono *et al.* 1987;
279 Abro *et al.* 2014), and it seems as if the chitinase activity is always present independent of dietary chitin.
280 There could be two possible reasons for this: 1) gut chitinase activity is not regulated by the addition of
281 chitin because Atlantic salmon is exposed to a relatively constant supply of chitin during its life cycle,
282 and/or 2) the chitinase and chitobiase genes are constitutively expressed because of their role as chitin
283 remodelers in the intestinal mucosa. Relatively high CHS gene expression levels in the same intestinal
284 segments of Atlantic salmon as chitinases and chitobiase genes are expressed favor the second
285 hypothesis.

286 Compared to stomach, few DEGs were detected in pyloric caeca with the greatest changes observed in
287 fish fed fly larvae; this included the significant upregulation of cholesterol biosynthetic genes. Such an
288 upregulation could be expected as a response to lower cholesterol levels in the feed, as insect lipids
289 usually contain low cholesterol levels, but a substantial amount of phytol sterol (Secci *et al.* 2018). This
290 has previously been shown to induce the cholesterol biosynthetic pathway in the pyloric caeca of
291 Atlantic salmon (Jin *et al.* 2018).

292 Chitinase activity in the stomach and pyloric caeca contents of Atlantic salmon did not seem to be
293 significantly affected by the addition of dietary chitin, but fish fed fly larvae had slightly lower chitinase
294 activity in pyloric caeca contents than fish fed control and shrimp shell diets (Figure 1). This is in line
295 with the slight decrease in gene expression levels of three chitinase genes of fish fed fly larvae; *chia.1*,
296 *chia.2,* and *chia.6,* all being exclusively expressed in pyloric caeca (Figure 2C). In general, the
297 transcriptome of Atlantic salmon did not seem to respond strongly to changing the standard commercial
298 diet with fly larvae diet, but the bacterial composition of fish fed fly larvae was different from the
299 composition of fish fed control and shrimp shell diets (Figure 4). In accord with our findings, an increase
300 in the relative abundance of *Actinomyces*, *Bacillus,* and *Enterococcus* when Atlantic salmon is fed fly
301 larvae have previously been reported (Li *et al.* 2021). Since *Actinomyces* and *Bacillus* are potential
302 chitin degraders (Askarian *et al.* 2012; Beier and Bertilsson 2013; Wang *et al.* 2018), we hypothesize

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303 that the observed decrease in Atlantic salmon chitinase gene expression levels and activity under fly
304 larvae diet is an effect of the activity of bacterial chitinases, reducing the level of dietary and host-
305 derived chitin available as a substrate to endogenous chitinases in the gastrointestinal tract.

306

307 CONCLUSION

308 We show that the stomach and pyloric caeca transcriptome of Atlantic salmon did not respond to a great
309 extent to the presence of dietary chitin, in support of the idea that evolutionary conservation of host
310 chitinases is mostly linked to remodeling of chitin as a structural element in the gut lining (Nakashima
311 *et al.* 2018). Furthermore, we demonstrate an association between gut microbial composition, chitin
312 activity in the gut, and host chitinase gene expression, and hypothesize functional interconnection
313 between chitinase-secreting gut bacteria (e.g. *Actinomyces*) and chitinase gene regulation in the host.
314 These results contribute to a greater understanding of chitin metabolism in fish in general.

315

316 DATA AVAILABILITY STATEMENT

317 Raw RNA-seq data are available on ArrayExpress with the accession number E-MTAB-10594. The
318 raw 16S sequence data are available at NCBI's Sequence Read Archive (SRA) with accession number
319 PRJNA820557.

320

321 WELFARE STATEMENT

322 The experiment was conducted in accordance with Norwegian and European regulations related to
323 animal research. Formal approval of the experiment by the Norwegian Animal Research Authority
324 (NARA) was not required as the experimental conditions were following routine practices at the Centre
325 for Fish Research at NMBU and no compromised welfare was expected.

326

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331

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