

# ***Candida utilis* yeast as a functional protein source for Atlantic salmon (*Salmo salar* L.): Local intestinal tissue and plasma proteome responses**

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16 **Abstract**

17 Microbial ingredients such as *Candida utilis* yeast are known to be functional protein sources with  
18 immunomodulating effects whereas soybean meal causes soybean meal-induced enteritis in the distal  
19 intestine of Atlantic salmon (*Salmo salar* L.). Inflammatory or immunomodulatory stimuli at the local  
20 level in the intestine may alter the plasma proteome profile of Atlantic salmon. These deviations can  
21 be helpful indicators for fish health and therefore potential tools in diagnosis of fish diseases. The  
22 present work aimed to identify local intestinal tissue responses and changes in plasma protein profiles  
23 of Atlantic salmon fed inactive dry *Candida utilis* yeast biomass, soybean meal, or combination of  
24 soybean meal based diet with various inclusion levels of *Candida utilis*. A fishmeal based diet was  
25 used as control diet. Inclusion of *Candida utilis* yeast to a fishmeal based diet did not alter the  
26 morphology, immune cell population or gene expression of the distal intestine, but gave a plasma  
27 proteome profile different from the fishmeal based control. Lower levels of *Candida utilis* combined  
28 with soybean meal modulated immune cell populations in the distal intestine and reduced the severity  
29 of soybean meal-induced enteritis, while higher inclusion levels of *Candida utilis* were less effective.  
30 The results suggest that *Candida utilis* could induce systemic responses without altering intestinal  
31 morphology, and thus could be a high-quality alternative protein source with potential functional  
32 properties in diets for Atlantic salmon.

## 33 **Introduction**

34 The composition of feeds used in salmon aquaculture has undergone significant changes over the last  
35 decades. The rapid growth in the aquaculture industry, but stable production of the major protein  
36 resource, fishmeal (FM), has led to increasing use of alternatives. Alternative protein sources are  
37 required to contribute to a well-balanced diet and to support optimal fish growth performance, health  
38 and disease resistance. Currently, proteins derived from insects [1], terrestrial animal co-products [2,

39 3] and microbial ingredients [4] are considered to be valuable alternatives. In commercial diets, plant  
40 derived proteins have already replaced two third of the proteins of marine origin [5].

41 Plant ingredients are the most attractive protein sources due to their low cost of production, high protein  
42 content and availability [6]. Inclusion of plant ingredients in salmonid feeds can, however, result in  
43 reduced growth performance and feed utilization, and health issues due to anti-nutritional factors  
44 (ANF) [6, 7]. ANFs in plant-based diets have been associated with detrimental effects on the intestine  
45 of several salmonid species [7], and soybean meal-induced enteritis (SBMIE) is a well described  
46 condition in Atlantic salmon (*Salmo salar* L.) [8, 9]. Currently, in commercial salmon diets, the refined  
47 soy product with reduced level of ANF, soy protein concentrate, is the major protein source of plant  
48 origin [5] and has not shown to cause pathological changes in the distal intestine (DI) of salmonids  
49 after short-term dietary exposure [9]. However, certain degree of inflammation and ectopic epithelial  
50 cells have been observed in the DI of salmonids when fed diets based on FM and soy protein  
51 concentrate over a longer period of time [10].

52 Microbial ingredients have proven to be high quality protein sources with the ability to mitigate the  
53 negative effects of plant-derived proteins [11]. Microbial ingredients such as yeast and bacteria contain  
54 bioactive compounds with immunomodulating properties that improve the changes caused by SBM  
55 [12-14]. Moreover, bacterial meal has been shown to prevent the development of SBMIE in a dose-  
56 dependent manner [15]. Mannan oligosaccharides, compounds found in yeast cell walls, have been  
57 used as a prebiotic and shown to protect the intestinal mucosa and reduce inflammation and leukocyte  
58 infiltration [16]. However, the degree of bioactivity of these compounds depends on the microbial  
59 origin as well as the fermentation conditions and downstream processing of the microbial product  
60 before incorporation into the salmon diet [11, 17].

61 Assessing the bioactivity of novel dietary microbial ingredients has traditionally involved the  
62 evaluation of local intestinal tissue responses, such as changes in morphology, gene expression or  
63 microbiome. Local intestinal responses can induce changes in the whole organism, inducing systemic  
64 responses that could contain new biomarkers for health and disease [18]. Innate immune system may  
65 respond to local inflammatory or immunomodulatory stimuli in the intestine of fish and in turn elicit  
66 changes systemically. Release of cytokines into the circulation stimulates hepatocytes to produce  
67 proteins and release them into the circulation to regain homeostasis [19]. Plasma proteomic analysis is  
68 a post-genomic tool that allows investigation of complex biological systems involved in physiology  
69 and pathology. Plasma proteome profiles in response to certain inflammatory or immunomodulatory  
70 stimuli can be useful diagnostic tools for fish diseases and indicators of fish health.

71 In this study, an inactive dry yeast strain *Candida utilis* (*C. utilis*) was used as an alternative protein  
72 source with functional properties in FM and SBM based diets. SBM was used as a dietary challenge to  
73 induce SBMIE. Increasing levels of *C. utilis* were included in the diets to evaluate the  
74 immunomodulating properties of the yeast, in particular the ability to counteract SBMIE. We combine  
75 histomorphological evaluation, immunohistochemistry, morphometry and gene expression of the DI  
76 with plasma proteome analysis. By combining these methods, we aim to identify local intestinal tissue  
77 responses and changes in plasma protein profiles in Atlantic salmon resulting from dietary treatments.  
78 Our results show that inclusion of *C. utilis* as an alternative protein source could induce a systemic  
79 response in plasma proteins without altering the local morphology and immune cell population in the  
80 DI of Atlantic salmon.

## 81 **Materials and methods**

### 82 **Experimental ingredient and diet preparation**

83 The inactive dry *C. utilis* yeast corresponded to a commercial product called Lakes States® Type B  
 84 produced by LALLEMAND SAS (Blagnac, France). Supplementary Table 1 (S1 Table) shows the  
 85 proximate composition of the test inactive dry yeast. The crude protein content was determined by

Ingredient (g/kg)	Experimental diets						
	FM	SBM	SBM25CU	SBM50CU	SBM100CU	SBM200CU	FM200CU
Fishmeal <sup>a</sup>	425.4	193	193	190	190	190	269
Soybean meal <sup>b</sup>		200	200	200	200	200	
<i>Candida utilis</i> <sup>c</sup>			25	50	100	200	200
Wheat gluten	150.6	150	139.4	115.2	93.5	72.9	118.8
Corn gluten meal		60	60	60	60		36.8
Wheat flower	168.6	181	167.4	169.6	142.9	132.3	166
Fish oil <sup>d</sup>	240	186	186	186	186	179.2	186
Choline chloride <sup>e</sup>	1.5	1.5	1.5	1.5	1.5	1.5	1.5
MCP <sup>f</sup>	6.2	10.8	10.8	10.9	10.9	10.8	9.6
L-Threonine <sup>g</sup>	0.9	2.4	2.2	2.2	1.7	1.2	1.0
Premix Fish <sup>h</sup>	6.3	6.3	6.3	6.3	6.3	6.3	6.3
Rhodimet NP99 <sup>i</sup>		2	2	2.2	2.2	2.6	1.5
L-Lysine monohydrochloride <sup>j</sup>	0.5	7	6.4	6.1	5	3.2	3.5

86 multiplying nitrogen content by a conversion factor of 6.25. All diets used in this study were formulated  
 87 to meet or exceeded the nutrient requirements of Atlantic salmon [20] (Table 1), produced by extrusion  
 88 technology at the Center for Feed Technology (FôrTek) at the Norwegian University of Life Sciences  
 89 (Aas, Norway), and stored at -20°C prior to feeding. The extruded pellets were dried to ~ 6 % moisture  
 90 content before vacuum coating with fish oil. The diets consisted of a FM-based control diet (FM group)  
 91 and the following six experimental diets; a diet containing 200 g/kg *C. utilis* (FM200CU group), and  
 92 five diets containing 200 g/kg SBM together with 0 (SBM group), 25, 50, 100 or 200 g/kg *C. utilis*  
 93 (SBM25CU, SBM50CU, SBM100CU and SBM200CU groups, respectively).

94 **Table 1. Ingredient and proximate chemical composition (g/kg) of control (FM) and**  
 95 **experimental diets.** FM = Fishmeal; SBM = soybean meal; SBM25CU = soybean meal + 25 g/kg *C.*  
 96 *utilis*; SBM50CU = soybean meal + 50 g/kg *C. utilis*; SBM100CU = soybean meal + 100 g/kg *C.*  
 97 *utilis*; SBM200CU = soybean meal + 200 g/kg *C. utilis*; FM200CU = fishmeal + 200 g/kg *C. utilis*.

Proximate chemical composition (g/kg)

Dry matter	936	942	936	943	956	937	932
Crude protein	445	409.1	401.9	397.5	400.3	383	394.4
Crude lipid	24	22.4	22.4	22.4	22.7	22.2	22.4
Starch	253.2	182.0	181.7	175.6	186.5	177.5	187.1
Gross energy MJ/kg	90.5	63.4	64.7	67.2	69.3	72.3	73.1
Total ash	14.0	8.9	9.3	9.2	8.8	9.0	10.2
Phosphorus	113.2	143.8	140.3	144.3	130.6	116.2	138.1

98 <sup>a</sup>LT fishmeal, Norsildmel, Egersund, Norway. <sup>b</sup>Soybean meal, Non-GMO, Denofa AS, Fredrikstad, Norway. <sup>c</sup>Lake  
99 States<sup>®</sup> Torula, Lallemand, USA. <sup>d</sup>NorSalmOil, Norsildmel, Egersund, Norway. <sup>e</sup>Choline chloride, 70 % Vegetable,  
100 Indukern s.a., Spain. <sup>f</sup>Monocalcium phosphate (MCP), Bolifor<sup>®</sup> MCP-F, Oslo, Norway Yara, <sup>g</sup>L-Threonine, CJ Biotech  
101 CO., Shenyang, China. <sup>h</sup>Premix fish, Norsk Mineralnæring AS, Hønefoss, Norway. Per kg feed; Retinol 3150.0 IU,  
102 Cholecalciferol 1890.0 IU,  $\alpha$ -tocopherol SD 250 mg, Menadione 12.6 mg, Thiamin 18.9 mg, Riboflavin 31.5 mg, d-Ca-  
103 Pantothenate 37.8 mg, Niacin 94.5 mg, Biotin 0.315 mg, Cyanocobalamin 0.025 mg, Folic acid 6.3 mg, Pyridoxine 37.8  
104 mg, Ascorbate monophosphate 157.5 g, Cu: CuSulfate 5H<sub>2</sub>O 6.3 mg, Zn: ZnSulfate 151.2 mg, Mn: Mn(II)Sulfate 18.9  
105 mg, I: K-Iodide 3.78 mg, Ca 1.4 g. <sup>i</sup>Rhodimet NP99, Adisseo ASA, Antony, France. <sup>j</sup>L-Lysine CJ Biotech CO.,  
106 Shenyang, China.

## 107 **Fish husbandry and feeding trial**

108 Vaccinated salmon were acquired from Sørsmolt AS (Sannidal, Norway) and maintained according to  
109 the guidelines established by the Norwegian Animal Research Authority at the Research Station  
110 Solbergstrand of Norwegian Institute of Water Research (Drøbak, Norway). Fish were acclimated to  
111 seawater, housed in 300 L tanks supplied with ultraviolet light treated seawater (8 °C; 34.5 g/L NaCl)  
112 in a 7–8 L per min flow-through system, and fed with a commercial marine-based compound feed not  
113 containing soybean-derived products (3-mm pellet; Polarfeed AS, Europharma, Leknes, Norway)  
114 under continuous light during a 4-month period prior to conducting the feeding trial. The water  
115 temperature, dissolved oxygen and pH level were measured and recorded daily. At the beginning of  
116 the experiment, 360 fish (average initial body weight of 526 g) were randomly assigned to 18 tanks  
117 (20 fish/tank) and acclimated to the FM based control diet for two weeks prior to feeding experimental

118 diets. Feeding was approximately 20% in excess twice daily using automatic belt feeders based on a  
119 daily estimate of fish biomass and uneaten feed per tank, which was collected from the tank outlet after  
120 each feeding period. Following the acclimation period, each experimental diet was randomly allocated  
121 to the fish tanks (two tanks/diet) and fed for 30 days as described above. During the experiment, fish  
122 groups were batch weighed at 0, 7, 30 and 37 days and individual weight and length of sampled fish  
123 were recorded. After 30 days, the feeding strategy were changed and fed for 7 days as illustrated in Fig  
124 1.

125 **Fig 1. Experimental design.** The adaptation period of 14 days was followed by experimental period  
126 1 that lasted for 30 days. In experimental period 2, there was a shift in diets and this period lasted for  
127 7 days. Sampling points are day 0, 7, 30 and 37. FM = Fishmeal; SBM = soybean meal; SBM25CU =  
128 soybean meal + 25 g/kg *C. utilis*; SBM50CU = soybean meal + 50 g/kg *C. utilis*; SBM100CU =  
129 soybean meal + 100 g/kg *C. utilis*; SBM200CU = soybean meal + 200 g/kg *C. utilis*; FM200CU =  
130 fishmeal + 200 g/kg *C. utilis*.

## 131 **Sample collection**

132 At each sampling point, 8 fish per diet (4 fish per tank) were randomly selected and anaesthetized by  
133 immersion in 60 mg/l of tricaine methanesulfonate (MS-222, Sigma-Aldrich, MO, USA) and  
134 subsequently euthanized by a sharp blow to the head prior to dissection at the following sampling  
135 points: 0, 7, 30 and 37 days after starting feeding experimental diets. Before dissecting fish, blood  
136 samples were taken from the *vena caudalis* (tail vein) using heparinized syringe and centrifuged (1300-  
137 2000 x g for 10 min) to isolate blood plasma, which was aliquoted and stored at -80°C, until proteomic  
138 analysis was performed. DI tissue was sampled and kept in RNAlater® (Merck KGaA, Darmstadt,  
139 Germany) at 4°C for 24 h, and then at -80°C, until RNA extraction. DI tissue samples were also  
140 collected and preserved for subsequent histology, morphometry and immunohistochemistry.

## 141 **Histology, immunohistochemistry and morphometric measurements**

### 142 **Histology**

143 Approximately 1 cm segment of the DI was open longitudinally and the intestinal content were  
144 carefully removed. The tissue was fixed in 10% formalin for 48 h at room temperature and further  
145 processed according to routine histological procedures. Briefly, tissue was embedded in paraffin with  
146 an orientation to ensure longitudinal sectioning. Sections (2  $\mu\text{m}$ ) of paraffin-embedded DI tissue were  
147 mounted on glass slides (Menzel Gläser, Thermo Fisher scientific, Braunschweig, Germany) and  
148 processed for staining with hematoxylin and eosin. A blinded, semi-quantitative histological scoring  
149 of the DI was performed using the criteria described in detail by Baeverfjord and Krogdahl [8]. Briefly,  
150 the criteria were: 1) shortening of both the simple and complex intestinal mucosal folds, 2) appearance  
151 of the enterocytes including supranuclear vacuolization, cellular heights and presence of intraepithelial  
152 lymphocytes (IELs), 3) widening of the central lamina propria of the simple and complex folds by  
153 connective tissue and 4) infiltration of leucocytes in the lamina propria. Each criterion was given a  
154 score ranging from 0 to 2, and half scores were included (i.e. 0, 0.5, 1, 1.5, 2) [13]. Score 0 indicated  
155 normal morphology and score 2 represented marked changes. Score 0.5 was regarded as changes within  
156 normal range.

### 157 **Immunohistochemistry**

158 Fish subjected for immunohistochemical analysis were from sampling at day 30, and the following diet  
159 groups were included: FM, FM200CU, SBM, SBM25CU and SBM200CU. CD3 and CD8 positive T  
160 lymphocytes were identified in DI tissue sections by immunohistochemistry using a monoclonal anti-  
161 CD3 $\epsilon$  antibody (dilution 1:600) [21] and a monoclonal anti-CD8 $\alpha$  antibody (kindly supplied by Karsten  
162 Skjødt, dilution 1:50) [22], respectively. This analysis was performed as follows: formalin-fixed,



163 paraffin embedded DI sections (4  $\mu\text{m}$ ) were mounted on poly-L-lysine-coated glass slides (Superfrost  
164 Plus, Thermo Fisher scientific, Braunschweig, Germany) and left to dry at 37°C. The slides were  
165 incubated at 58°C for 30 min before deparaffinised in xylene and rehydrated in graded alcohols to  
166 distilled water. Antigen retrieval was done by using hydrated autoclaving at 121°C for 15 min in 0.01  
167 M citrate buffer, pH6. Endogenous peroxidase was inhibited with 0.05 % phenylhydrazine (0.05%;  
168 Sigma-Aldrich, MO, USA) in phosphate buffered saline, preheated to 37°C, for 40 min. The sections  
169 were stored in PBS overnight at 4°C and then incubated with normal goat serum (dilution 1:50; Sigma-  
170 Aldrich) in 5 % bovine serum albumin /0.05 M tris-buffered saline to avoid non-specific binding for  
171 20 min. The blocking solution was tapped off without washing, and the sections were incubated with  
172 primary antibody diluted in 1% bovine serum albumin/Tris-buffered saline for 1 h. Control sections  
173 were incubated with only 1% bovine serum albumin. The sections were incubated in the kit polymer-  
174 HRP anti-mouse (Dako En Vision+ System-HRP, Dako, Glostrup, Denmark), as secondary antibody,  
175 for 30 min. Peroxidase activity was detected with 3,3'-diaminobenzidine following the kit procedure.  
176 The sections were counterstained with haematoxylin for 30 s followed by washing in distilled water  
177 before mounting with Aquatex (Novoglas Labortechnik Langenbrinck, Bern, Germany). Unless  
178 otherwise stated, the sections were washed three times with phosphate buffered saline for 5 min  
179 between each step. All incubations took place in a humid chamber at room temperature.

## 180 **Morphometric measurements and calculation of immune cell density**

181 Morphometric measurements and calculation of the density of immune cells were performed from  
182 immunohistochemically labelled sections mentioned above. ImageJ software, version v1.51r [23], was  
183 used to perform the measurements and calculations. Images were captured with a Zeiss AxioCam 506  
184 color camera connected to a light microscope (Zeiss Axio Imager M2, Carl Zeiss, Germany) at a 10 X  
185 magnification. The measurement scale was set to 2.26 pixels/ $\mu\text{m}$  in ImageJ and the measurements were  
186 converted from  $\mu\text{m}$  to mm. Counting of immunohistochemically labelled cells was performed using

187 the multi point tool. The freehand selection and segmented line selection were used to measure fold  
188 area and height, respectively. The fold height was measured from stratum compactum to the tip of the  
189 epithelium lining the fold (S1 Fig.). The fold area was measured from stratum compactum, including  
190 the middle of the fold base on each side, and the whole simple fold (S1 Fig.). The  
191 immunohistochemically labelled cells were counted within the measured area of the simple fold. The  
192 density of CD3 $\epsilon$ - or CD8 $\alpha$ -labelled cells was calculated as follow: Cell density = (no. of labelled  
193 cells)/area. Simple folds were subjected to all the measurements mentioned above and the folds were  
194 selected as the first appropriate simple fold located to the left of a complex fold. An appropriate fold  
195 was defined as a fold that appeared long, not bent and had an intact epithelium that was attached to the  
196 basement membrane all the way to the tip of the fold. Between 2 - 6 measurements were collected from  
197 each individual with a total of at least 30 measurements from each group for each measurement. A  
198 mean for each individual was calculated based on the measurements.

## 199 **RNA isolation, cDNA synthesis, quantitative PCR**

200 DI from FM, FM200CU, SBM and SBM200CU diet groups (8 fish/diet) at day 30 were subject to gene  
201 expression analysis. Total RNA was extracted and purified using RNeasy® 96 kit (Qiagen, Valencia,  
202 USA) and QIAcube® HT system (Qiagen), according to the manufacturer's protocol. After the first  
203 washing step, on-column DNase treatment was performed using PureLink™ DNase kit (Thermo  
204 Fisher Scientific, Waltham, Massachusetts). RNA concentration and quality were measured using  
205 NanoDrop™ 8000 spectrophotometer (Thermo Fisher Scientific). Purified total RNA was stored at  
206  $-80^{\circ}\text{C}$  until further analysis.

207 Prior to cDNA synthesis, all samples were normalized to 400 ng/ $\mu\text{L}$ . cDNA synthesis was performed  
208 using AffinityScript QPCR cDNA Synthesis kit following the manufacturer's guidelines (Agilent

209 Technologies, Santa Clara, CA, USA). The total reaction volume was 10  $\mu$ L using 5  $\mu$ L of Mastermix,  
210 1.5  $\mu$ L random hexamer primers, 0.5  $\mu$ L AffinityScript RT/ RNase Block enzyme mixture and 3  $\mu$ L  
211 DNase treated RNA. The resulting cDNA was stored at  $-80^{\circ}\text{C}$  before use.

212 All quantitative PCR (qPCR) reactions were performed in duplicates and conducted in 96 well plates  
213 on LightCycler® 480 system (Roche Diagnostics, Mannheim, Germany). Each reaction consisted of a  
214 total amount of 12  $\mu$ L divided into 6  $\mu$ L LightCycler 480 SYBR Green I Master (Roche Diagnostics),  
215 2  $\mu$ L primers, and 4  $\mu$ L cDNA. The qPCR conditions were  $95^{\circ}\text{C}$  for 5 min before a total of 45 cycles  
216 of  $95^{\circ}\text{C}$  for 5 seconds,  $60^{\circ}\text{C}$  for 15 seconds, and  $72^{\circ}\text{C}$  for 15 seconds. To confirm amplification  
217 specificity, each PCR product was subject to melting curve analysis ( $95^{\circ}\text{C}$  5 s;  $65^{\circ}\text{C}$  60 s;  $97^{\circ}\text{C}$   
218 continuously). Primers tested are listed in Supplementary Table 2 (S2 Table). Glyceraldehyde-3-  
219 phosphate dehydrogenase (GDPH) and hypoxanthine phosphoribosyltransferase I (HPRTI) were  
220 chosen as reference for normalization since these genes did not show significant differential expression  
221 between the diets and have previously been described as suitable reference genes in the DI of salmon  
222 [24]. The crossing point (Cp) values were determined using the maximum second derivative method  
223 on the basis of the LightCycler® 480 software release 1.5.1.62 (Roche Diagnostics). The geometric  
224 mean of the CP-values for GDPH and HPRTI was used as an index. The qPCR relative expression of  
225 mRNA was calculated using the  $\Delta\Delta\text{Ct}$  method [25].

## 226 **In-solution digestion and protein sequence analysis by LC-MS/MS**

227 Proteomic analysis was performed, according to methods described by Lagos *et al.* 2017 [26], using  
228 four biological replicates per treatment of plasma taken at the end of the first feeding period (30 days)  
229 (FM, SBM, SBM200CU and FM200CU). In brief, frozen plasma ( $-80^{\circ}\text{C}$ ) was thawed and diluted to  
230 40  $\mu$ g of total protein in PBS, and the pH was adjusted to 8 by adding ammonium bicarbonate (Sigma-  
231 Aldrich, Darmstadt, Germany). The samples were then subjected to overnight incubation at  $37^{\circ}\text{C}$ . The

232 tryptic peptides were dissolved in 10  $\mu$ L 0.1% formic acid/2% acetonitrile and 5  $\mu$ L analyzed using an  
233 Ultimate 3000 RSLCnano-UHPLC system connected to a Q Exactive mass spectrometer (Thermo  
234 Fisher Scientific, Bremen, Germany) equipped with a nano electrospray ion source. The proteomic  
235 analysis was performed by the Proteomic core facility of University of Oslo. The mass spectrometry  
236 proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [27] partner  
237 repository with the dataset identifier PXD012051.

## 238 **Data analysis**

### 239 **Histology, morphometric measurements, T-cell density and gene expression**

240 Non-parametric data from the histological evaluation were analyzed by Kruskal-Wallis followed by  
241 post hoc Dunn's test with comparison of mean rank using GraphPad Prism, version 7.00 (GraphPad  
242 Software Inc., San Diego, CA, USA). Morphometric analyses and T-cell density were analyzed by  
243 one-way ANOVA followed by Tukey-Kramer HSD by using JMP statistical software (JMP®Pro  
244 13.0.0, SAS Institute Inc, NC, USA). Morphometric analyses and T-cell density analyses were  
245 performed at the individual level using the mean of measurements of between 2-6 simple folds per fish.  
246 Results of qPCR (means  $\pm$  standard deviations) were analyzed using One-way ANOVA with Dunnett's  
247 multiple comparison test ( $\alpha < 0.0001$ ) on GraphPad Prism 8.0. 1.

### 248 **Proteomic data analysis**

249 The resulting proteomic data, MS raw files, were analyzed using MaxQuant and identifications were  
250 filtered to achieve a protein false discovery rate (FDR) of 1%. Raw data files were processed using  
251 Scaffold4 (Proteome Software, Portland, OR, USA), and a non-redundant output file was generated  
252 for protein identifications with log (e) values less than -1. Peptide identification was determined using  
253 a 0.8 Da fragment ion tolerance. MS/MS spectra were searched against the salmon proteome, and

254 reverse database searches were used in estimation of false discovery rates. The analysis was restricted  
255 to proteins reproducibly identified in at least two of the four replicates per diet, making the minimum  
256 number of peptides used to identify each protein an average value of 2. Row-wise normalization was  
257 applied to provide Gaussian-like distributions [28] for adjusting the differences among protein data.  
258 Protein raw data transferred to log normalization and then performed on autoscaled data (mean-  
259 centered and divided by the standard deviation of each variable) [29]. A diagnostic plot was utilized to  
260 represent normalization procedures for normal distribution assessments [30]. Spearman's correlation  
261 analysis, multivariate statistical analysis and data modeling were performed in R, using R package  
262 MetaboAnalystR [28]. In hierarchical cluster analysis, each individual begins as a separate cluster and  
263 the algorithm proceeds to combine them until all individuals belong to one cluster. The clustering  
264 results are also presented in the form of a heatmap, with levels of protein expression across the dietary  
265 groups. Hierarchical clustering was performed with the hclust function in R package stat. UniprotKB  
266 database was used for functional annotation of the proteins. A gene ontology (GO) analysis on the  
267 proteomic data was performed to provide insights into the complex structure and biological processes  
268 of the plasma proteome across the diets.

269 A series of principal component analysis (PCA) was performed using the prcomp package in R. PCA  
270 was used as an unsupervised method to find the directions of maximum covariance among FM, SBM,  
271 SBM200CU and FM200CU. In addition, a series of Partial least squares discriminant analysis (PLS-  
272 DA) regression was performed using the pls function provided by R pls package. The classification  
273 and cross-validation were performed using the corresponding wrapper function by the caret package  
274 in R. Briefly, to define the optimal number of PCs (principal components), “7-fold cross-validation”  
275 (CV) was applied[31]. Using CV, the predictive power of the model was verified. Two parameters  
276 were calculated for evaluating the models: R<sup>2</sup> (goodness of fit) and Q<sup>2</sup> (goodness of prediction). A  
277 model with Q<sup>2</sup>>0.5 is considered good, Q<sup>2</sup>>0.9 excellent [32]. As cross-validation only assesses the

278 predictive power without a statistical validation, the performance of PLS-DA models was also  
279 validated by a permutation test (200 times).

280 In order to help interpretation of results from PLS-DA, we considered the variable importance in the  
281 projection (VIP) scores. This allowed visualization of protein influence (including prediction  
282 performance) on the model and identified the best descriptors of the differences among the four  
283 conditions. The VIP score is a weighted sum of squares of the PLS loading weights taking into account  
284 the amount of explained Y-variation in each dimension [33]. VIP scores “greater than 2” rule is  
285 generally used as a criterion to identify the most significant variables [33].

## 286 **Results**

### 287 **Growth and general health**

288 All groups of fish accepted their allocated diets and no significant differences were found in feed intake  
289 among dietary treatment. The average initial weight was 526 g and average final weight was 667 g at  
290 day 37, indicating that all fish gained weight during the experimental period. The general health of the  
291 fish in this experiment was good, but two fish died during the experimental period, one from  
292 SBM50CU group and one from SBM100CU group, for unknown reasons.

### 293 **Histology**

294 All fish sampled at day 0 showed normal DI morphology. Briefly, simple folds were long and slender  
295 with a thin lamina propria, whereas the complex folds were tall with a narrow lamina propria and a  
296 partial central core of smooth muscle. Intestinal epithelial cells were tall with the nucleus located  
297 basally, large vacuoles located apically and many IELs. Goblet cells were scattered among the

298 epithelial cells towards the apex, and there was a higher presence of goblet cells at the apex of the  
299 complex folds. The lamina propria adjacent to the stratum compactum was thin and numerous  
300 eosinophilic granule cells were present (data not shown).

301 At day 7 (Fig 2A), FM and FM200CU groups showed normal DI morphology as described above. In  
302 general, all fish groups fed diets containing SBM, including SBM diets with *C. utilis* inclusion,  
303 displayed changes in the DI morphology consistent with SBMIE (described in detailed below).  
304 Nevertheless, in SBM25CU and SBM50CU, there was variation within the groups ranging from  
305 individuals showing no changes to other individuals with moderate changes in DI morphology.

306 At day 30 (Fig 2B), no morphological changes were seen in the DI of FM and FM200CU groups.  
307 However, a moderate SBMIE was present in the DI of SBM, SBM100CU and SBM200CU groups.  
308 Both simple and complex folds were shorter with a widening of the lamina propria within the folds and  
309 adjacent to the stratum compactum. Fusion of the simple folds was frequently observed. There was an  
310 increased presence of connective tissue in the lamina propria and the increased infiltration of leucocytes  
311 consisted mainly of eosinophilic granule cells and to a lesser extent of lymphocytes, some macrophages  
312 and neutrophils were present. The intestinal epithelial cells shown a reduction in height with nucleus  
313 displaced in a more apical position and small supranuclear vacuoles. There was an increased presence  
314 of IELs. In SBM25CU and SBM50CU groups, there were still a variation within the groups as seen at  
315 day 7.

316 At day 37 (Fig 2C), fish previously fed SBM, either alone or in combination with *C. utilis*, had normal  
317 DI morphology after being fed FM for 7 days. Similarly, most of the fish that had diets changed from  
318 SBM to FM200CU had DI morphology regarded as normal, but there were some fish in these groups  
319 that had a mild enteritis. Changing diet from FM200CU to SBM induced in general a mild SBMIE,

320 whereas a shift from SBM to SBM200CU maintained a moderate SBMIE. The FM control group was  
321 normal, as described above.

322 No tank effects were observed at any of the sampling points. In Fig 2A and 2B, only fish from the tanks  
323 that were subjected to immunohistochemical analysis at day 30 are presented, i.e., two SBM-groups  
324 are omitted from the figure. Fish with diet shift from SBM combined with *C. utilis* to FM at day 37  
325 had normal DI morphology but are excluded from Fig 2C as this group did not differ from the SBM  
326 group.

327 **Fig 2. Histological evaluation.** Histological evaluation of the distal intestine of Atlantic salmon based  
328 on atrophy, lamina propria, epithelium and edema at 7 (A), 30 (B) and 37 (C) days. Changes are scored  
329 from 0 to 2 where 0 indicates no changes and 2 indicates severe changes. Data are expressed as mean  
330 and standard deviation, n = 8 for all groups. Significant difference from the control fish fed FM based  
331 diet is denoted by asterisk (\*) ( $p < 0.05$ ; Dunn's test). FM = fishmeal; FM200CU = fishmeal combined  
332 with 200 g/kg *C. utilis* (CU); SBM = soybean meal; SBM25CU = soybean meal with 25 g/kg CU;  
333 SBM50CU = soybean meal with 50 g/kg CU; SBM100CU = soybean meal with 100 g/kg CU;  
334 SBM200CU = soybean meal with 200 g/kg CU.

### 335 **Immunohistochemistry, morphometry, density of immune cells**

336 At day 30, CD3 $\epsilon$  positive cells showed an abundant presence at the base of the epithelium and  
337 extending along the entire length of simple folds of FM and FM200CU (Fig 3A and 3B) groups. Only  
338 a few CD3 $\epsilon$  positive cells were observed in the lamina propria adjacent to the stratum compactum, and  
339 were rarely present in the lamina propria of the simple folds. A weak diffuse labelling was observed in  
340 the smooth muscle, which was interpreted as background labelling. The negative controls were blank.



341 The density of CD3 $\epsilon$  positive cells in the simple folds of groups fed diets containing SBM was  
342 increased compared with the density in FM group. IEL's that showed labelling for CD3 $\epsilon$  were located  
343 as individual cells at the base of the epithelium but occasionally clusters of CD3 $\epsilon$ -labelled IEL's were  
344 observed. CD3 $\epsilon$  positive cells were more frequent in the lamina propria adjacent to the stratum  
345 compactum of fish fed SBM compared with fish fed FM (Fig 3D), but there were only a few CD3 $\epsilon$ -  
346 positive cells present in the lamina propria of the simple folds (Fig 3C).

347 CD8 $\alpha$ -labelled IEL's showed the same distribution as the CD3 $\epsilon$ -labelled IEL's being located basally  
348 between the intestinal epithelial cells in all diets (Fig 3E-G). In general, the presence of CD8 $\alpha$ -labelled  
349 IEL's was lower than the presence of CD3 $\epsilon$ -labelled IEL's.

350 **Fig 3. Immunohistochemical staining for CD3 $\epsilon$  and CD8 $\alpha$  positive cells at day 30.**

351 Immunohistochemical labelling (brown) showed an abundant presence of CD3 $\epsilon$  positive cells at the  
352 base of the epithelium along the entire length of simple folds in all diet groups (A: FM200CU; D:  
353 SBM). At higher magnification, CD3 $\epsilon$  positive cells were rarely present in the lamina propria of the  
354 simple folds in any of the diets (B: FM200CU; C: SBM). However, there was a higher number of CD3 $\epsilon$   
355 positive cells in the lamina propria adjacent to the stratum compactum in groups fed diets with SBM  
356 (D: SBM). CD8 $\alpha$  positive cells were mainly found between the epithelial cells of all individuals of all  
357 diet groups (E: FM200CU; F: SBM200CU; G: SBM). Image A and D captured at 10x magnification,  
358 Image B, C, E, F and G captured at 40x magnification.

359 Morphometric measurements of simple folds, both length and area, revealed no significant differences  
360 between FM and FM200CU groups. The simple folds in the DI of fish fed SBM, SBM25CU and  
361 SBM200CU were significantly shorter and had a significantly smaller area than the simple folds in fish  
362 fed FM200CU and FM. There were no statistically significant differences between the simple folds of  
363 fish fed diets containing SBM either alone or combined with *C. utilis* (Fig 4A-B).

364 The density of CD3 $\epsilon$  and CD8 $\alpha$ -labelled cells in fish of FM and FM200CU groups was significantly  
365 lower compared with the density in fish from groups fed diets containing SBM. There was a statistically  
366 significant difference between the density of CD8 $\alpha$ -labelled cells in fish of SBM group and the density  
367 CD8 $\alpha$ -labelled cells in fish from the SBM25CU ( $p = 0.0465$ ) (Fig 4D).

368 **Fig 4. Morphometry of simple folds in distal intestine at day 30.** Morphometric measurements of  
369 fold length (**A**) and fold area (**B**) of the simple folds of the distal intestine, and the density of CD3- and  
370 CD8-positive T-cells in simple folds including the lamina propria adjacent to the stratum compactum  
371 (**C** and **D**). Data are expressed as mean  $\pm$  standard deviation (SD) for each individual,  $n = 7$  for the  
372 SBM diet and  $n = 8$  individuals per diet for the remaining groups. Groups with different letters on the  
373 upper x-axis are significantly different ( $p < 0.05$ ; Dunn's test)

## 374 Gene expression

375 Among the tested genes, only mRNA expression of Aquaporin 8 (*aqp8*) was significantly down  
376 regulated in the SBM and SBM200CU groups compared with the FM control group (Fig 5E). There  
377 was no significant difference between FM200CU and the FM control group. The transcription levels  
378 of superoxide dismutase 1 (*sod1*), glutathione S-transferase alpha 3 (*gsta3*), annexin A1 (*anxa*), and  
379 catalase (*cat*) were not different among dietary groups ( $p > 0.05$ ) (Fig 5A-D).

380 **Fig 5. Gene expression.** Quantitative PCR analyses of (**A**) superoxide dismutase 1 (*sod1*), (**B**)  
381 glutathione S-transferase alpha 3 (*gsta3*), (**C**) annexin (*anxa*), (**D**) catalase (*cat*) and (**E**) aquaporin 8  
382 (*aqp8*) genes in the DI of Atlantic salmon fed a control fishmeal-based diet (FM), a diet containing 200  
383 g/kg *Candida utilis* (FM200CU), and a diet containing 200 g/kg soybean meal (SBM) and one diet  
384 with 200 g/kg SBM in combination with 200 g/kg of *C. utilis* (SBM200CU) for 30 days. Data are mean  
385  $-\Delta\Delta CT \pm SE$  ( $n = 7$  for FM diet,  $n = 8$  for the other groups).

## 386 **Plasma proteome**

387 We performed proteomic analysis on plasma sampled at day 30 from four individual fish from  
388 treatment FM, SBM, SBM200CU and FM200CU. In order to simplify the reading of the data, the  
389 treatment FM is named **D1**, SBM **D2**, SBM200CU **D6** and FM200CU **D7**. In total, 367 salmon proteins  
390 were identified across the four dietary groups. A Venn diagram shows the overlap between plasma  
391 protein sets detected across the four dietary treatments (Fig 6A). There were 304 plasma proteins shared  
392 between the four groups. Moreover, each dietary treatment presented unique proteins in at least half of  
393 the replicates (Fig 6B). It is important to mention that due to the variability within the groups; just two  
394 proteins were expressed in all the four replicates in FM fed group (highlighted in Fig 6B).

395 After filtering for plasma proteins present in at least two of the four individuals per diet, 256 proteins  
396 were selected for further analyses (S3 Table). This criterion was used due to the variability among fish.  
397 To determine whether certain proteins share particular expression patterns, we produced a hierarchical  
398 clustering heatmap showing 50 significantly different proteins with positive and negative correlations  
399 based on Spearman non-parametric correlation coefficients (Spearman's R) (Fig 6C).

400 **Fig 6. Common and unique proteins expressed in plasma of salmon fed different diets.** (A) Venn-  
401 diagram showing the overlap between plasma protein sets detected across the four diet groups FM  
402 (D1), SBM (D2), SBM200CU (D6) and FM200CU (D7). (B) Unique proteins expressed in each dietary  
403 group. (C) Hierarchical clustering heatmap shows Spearman's correlation between the proteins.  
404 Positive correlation values are in red, and negative correlation values are in blue.

405 The GO analysis revealed that plasma has mainly proteins involved in metabolic and cellular processes,  
406 and biological regulation (S2F Fig). GO analysis also showed that many of the plasma proteins have  
407 roles in response to stimulus and immune system processes. Further, most of the proteins belong to the  
408 signal protein domain, followed by repeat and transmembrane helix domains (S2E Fig).

409 PCA and PLS-DA multivariate analyses were carried out first with all selected proteins across dietary  
410 groups (Fig 7A). It is apparent that all dietary groups tend to cluster together, with the exception of the  
411 FM control group in PCA, whereas a better separation of the groups is reached by using PLS-DA (Fig  
412 7B). The results also indicate that the resulting two principal components explain a small proportion  
413 of the total variability observed in the data, and the PLS-DA analysis offers poor prediction properties  
414 ( $Q^2 < 0.5$ ) (S2A-D Fig). This means that the resulting PCA and PLS-DA models are highly over  
415 parameterized as they used all variables. Furthermore, it also confirms that high goodness of fit (i.e.  
416  $R^2$ ) can be expected in over parameterized models. The proteins (variables) that fulfilled VIP criterion  
417 ( $VIP > 2$ ) were determined, followed by re-running PCA and PLS-DA with the resulting 10 VIP proteins  
418 (Fig 7C).

419 **Fig 7. Multivariate analyses and identification of VIP proteins.** (A) Principal components analysis  
420 (PCA) and (B) Partial least squares discriminant analysis (PLS-DA)'s scores plots based on selected  
421 proteins from FM (D1), SBM(D2), SBM200CU (D6) and FM200CU (D7) dietary groups. (C)  
422 Variable importance in the projection (VIP) scores to identify the proteins that discriminate FM (D1),  
423 SBM (D2), SBM200CU (D6) and FM200CU (D7) among the proteins selected by ANOVA plot with  
424 p value threshold 0.05.

## 425 Discussion

426 Research on the effects of nutrition on fish health and disease has mainly focused on intestinal local  
427 immune responses rather than evaluating overall health impact. Therefore, the present study used an  
428 integrated approach to achieve a better understanding of the effect of feeding inactive dry *C. utilis*  
429 yeast, SBM and increasing levels of *C. utilis* yeast to Atlantic salmon in presence of SBMIE. Herein,  
430 we discuss how changes in the DI morphology and immune cell profile could reflect challenges posed

431 by dietary treatments, while plasma protein profiling provided an association between systemic  
432 responses and outcomes of nutritional challenges.

433 A previous study has shown that there were no significant negative effects on feed intake, specific  
434 growth rate or feed conversion ratio when up to 30 % *C. utilis* was included in the diet for salmon [4],  
435 but DI morphology was not assessed. The present study is the first to demonstrate that FM200CU diet  
436 maintains a DI morphology similar to the FM based control diet in sea-water adapted farmed salmon.  
437 As a dietary challenge, fish were fed diets with high levels of SBM and were observed to have  
438 morphological changes in the DI that are consistent with SBMIE development in salmon [9]. Adding  
439 lower levels of *C. utilis* to SBM diets (i.e. SBM25CU and SBM50CU) resulted in a large variation  
440 within the groups, ranging from normal morphology to moderate SBMIE. The severity of SBMIE in  
441 fish fed the highest inclusion levels of *C. utilis* (i.e. SBM100CU and SBM200CU) in combination with  
442 SBM was similar to that of fish fed the SBM diet. Thus, partial prevention of SBMIE occurred only  
443 with lower inclusion levels of *C. utilis*. This finding does not agree with previous work, where feeding  
444 200 g/kg *C. utilis* together with SBM (200 g/ kg) prevented SBMIE development in salmon [13]. This  
445 inconsistency could be due to differences in the degree of bioactivity of the *C. utilis* yeast, or in the  
446 ANF content of the SBM, and/or the experimental conditions. For example, Øverland and Skrede [11]  
447 have speculated that inconsistent effect of yeast on host immunity can be attributed to yeast strain,  
448 fermentation conditions and downstream processing when manufactured. The shift in diet at d 30 of  
449 the experiment showed that the developed enteritis observed in the DI of fish fed either SBM alone or  
450 in combination with *C. utilis* was resolved after feeding FM diet for 7 d. It is also important to point  
451 out that the degree of SBMIE were reduced from moderate to mild after 7 d feeding FM200CU diet to  
452 those fish fed SBM diet for 30 d. It can be suggested that all fish would have returned to normal state  
453 if the experiment had lasted longer.

454 Local DI response to SBM has been described as a T-cell mediated inflammatory response [34]. At d  
455 30, the SBM, SBM25CU and SBM200CU groups had increased CD3 $\epsilon$  and CD8 $\alpha$  cell populations in  
456 the DI, which support this description. The CD3 $\epsilon$ - and CD8 $\alpha$ -lymphocytes were mainly confined to  
457 the basal part of the DI epithelium with only a few CD3 $\epsilon$ -labelled cells scattered in the lamina propria  
458 adjacent to stratum compactum. However, Bakke McKellep *et al.* [34] reported that lamina propria  
459 adjacent to stratum compactum and stroma of complex folds were rich in CD3 $\epsilon$ -labelled cells in DI  
460 presenting with SBMIE. It is relevant to indicate that there was a decreased presence of CD8 $\alpha$ -cell  
461 population in the SBM25CU group compared with the SBM group. The CD3 $\epsilon$ - and CD8 $\alpha$  related  
462 observations might imply that the SBM used in these studies differed in immunostimulatory properties,  
463 and that *C. utilis* has an immunomodulating effect locally in the DI when included at a low level in the  
464 SBM diet. On the other hand, FM200CU presented a similar T-cell population profile in the DI  
465 compared with control FM group, indicating no stimulation of the T-cell population when SBMIE was  
466 not present.

467 The mRNA expression profile of *aqp8* gene in the DI in the diet groups (FM, FM200CU, SBM,  
468 SBM200CU) indicates an association of *aqp8* with the resulting DI morphological and immune cell  
469 responses to nutritional challenges observed in this study. Our results confirm previous findings  
470 showing suppression of *aqp8* gene expression in intestinal inflammatory processes in salmon such as  
471 SBMIE [13, 35]. The relation between *aqp8* expression and inflammation is consistent with cell-based  
472 studies showing that reduced *aqp8* expression is linked to increased oxidative cell stress damage [36]  
473 and implying that *aqp8* is a key player in the maintenance of redox cellular status. DI. In contrast, the  
474 similar mRNA *aqp8* expression levels observed in the DI from the FM200CU and FM groups can  
475 support the notion that feeding *C. utilis* to salmon promotes intestinal homeostasis. Together, *aqp8* can

476 be considering as a good indicator to differentiate between inflamed and non-inflamed intestines in  
477 salmon.

478 The systemic nature of the response of salmon towards dietary treatments was pursued by the  
479 proteomic profiling of blood plasma from four diet groups at day 30 of the experiment, namely the FM,  
480 SBM, SBM200CU and FM200CU groups. By applying advanced proteomic methodologies, 10 VIP  
481 proteins and top 50 significant proteins, presented in a hierarchial clustering heatmap, visualize the  
482 changes in plasma protein levels across the dietary groups. Differences between the plasma protein  
483 profiles of fish receiving SBM diets (SBM and SBM200CU) and FM diet were expected, as systemic  
484 changes may accompany a local tissue inflammation. However, the difference in plasma protein profile  
485 between FM control group and FM200CU experimental group was noteworthy as these two dietary  
486 groups responded similarly at a local tissue level. *C. utilis* containing diets are known to have  
487 immunomodulatory effects, therefore, the proteins that are related to an immune response/function are  
488 of particular interest.

489 VIP protein HA-S (i.e. H-2 class I histocompatibility antigen, Q10 alpha chain) has been involved in  
490 the modulation of natural killer cells in the mouse [37], implying a role in immune tolerance [38].  
491 Interestingly, the strongest reduction in HA-S expression was detected in the FM200CU group, which  
492 at the same time presented with DI morphology similar to the FM group. Complement factors are part  
493 of the innate immune system that enhance the ability of antibodies and phagocytic cells to clear  
494 microbes and damaged cells and promote inflammation. Previous study in Atlantic salmon have shown  
495 that intraperitoneal injection of glucans from *Saccharomyces cerevisiae* resulted in increased activities  
496 of complement in plasma 2-4 weeks after injection [39]. In our study, the FM200CU group had an  
497 increased expression of COME.19 and C4L (i.e. complement 4 like), and COME.16 (complement C4-  
498 B) as shown in the hierarchial clustering heatmap. On the other hand, the VIP protein VEFA (i.e.  
499 venom factor like) that also has a suggested role in complement activation [40] has a lower expression

500 when compared to FM group. These findings are relevant as they highlight differences between the  
501 FM and FM200CU groups, indicating a systemic effect on the complement system by *C. utilis* without  
502 causing DI inflammation [41].

503 HIIN, LYME and HMP are among the significant proteins that are highly expressed in SBM, and  
504 separate the SBM group from the other groups, especially the SBM200CU group. Lysozyme (LYME)  
505 is primarily associated with innate immune defense against bacteria but is also known to activate the  
506 complement system [42]. Our results show that DI inflammation is associated with high plasma levels  
507 of LYME (i.e. Lysozyme g) in blood as seen in human with active Chron's disease [43]. This  
508 observation also suggests that LYME expression during SBMIE in Atlantic salmon might vary  
509 depending on the tissue as mRNA down-regulation of LYME has been observed in DI with SBMIE  
510 [13]. Thus, further proteomic analysis in inflamed DI is needed to elucidate the differences between  
511 protein and gene expression analyses. Hemopexin (HMP) is regarded as an acute phase protein in  
512 mammals that possesses a high affinity for heme released from hemoglobin [44]. The sequestering of  
513 circulating heme iron is an important dual defense mechanism as bacterial growth relies on the  
514 acquisition of iron from their host [45] and free heme can cause oxidative damage [46]. Therefore,  
515 increased expression of HMP can be expected in inflammation and in infectious diseases to promote  
516 tissue healing by reducing tissue oxidative damage. This is supported by increased HMP expression  
517 during bacterial infections in Atlantic salmon [47] and rainbow trout [48]. These observations are  
518 further supported by our study showing a distinct strong HMP plasma protein expression in salmon  
519 presenting with DI inflammation induced by nutrition challenge. Fish with a high degree of DI  
520 inflammation (SBM group) also showed high HIIN (i.e. histidine-rich glycoprotein-like) levels  
521 compared with the other groups. HIIN has been shown to modulate tumor progression and anti-tumor  
522 immune responses in humans, and its high expression made it useful as a potential glycoprotein



523 biomarker in some cancers [49]. These three proteins separate SBM from SBM200CU, which is of  
524 interest as both of these groups present with enteritis in the DI and decreased gene expression of *aqp8*.

525 This study utilized plasma proteomic analysis to achieve a better understanding of the effects of  
526 nutrition on fish health and disease and to identify systemic protein profiles in response to the dietary  
527 treatments. The inclusion of 200 g/kg of *C. utilis* yeast to a FM based diet (i.e. FM200CU) as a novel  
528 protein source with potential functional properties produced similar DI morphology, immune cell  
529 population and gene expression profiles, compared with a FM based control. Feeding the SBM diet  
530 induced SBMIE while feeding lower inclusion levels of *C. utilis* in combination with SBM (i.e.  
531 SBM25CU and SBM50CU) reduced the severity of SBMIE. Interestingly, higher inclusion levels of  
532 *C. utilis* yeast (i.e. SBM100CU and SBM200CU) did not show any significant protection against  
533 SBMIE, as observed in previous studies. A key finding from this study is that dietary *C. utilis* could  
534 influence the expression of plasma proteins related to immune function without altering DI  
535 morphology. Further research is needed to evaluate the impact of yeast strain and the fermentation  
536 and/or down-stream processing conditions of the yeast on functional properties in relation to gastro-  
537 intestinal health and systemic responses.

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683

## 684 **Supplementary material**

685 **S1 Table. Chemical composition (g kg<sup>-1</sup>) of dry *Candida utilis* biomass.**

686 **S2 Table. Primers used in qPCR analysis.**

687 **S3 Table. List of 256 proteins identified**

688 **S1 Fig. Morphometric measurement.** Red line indicates measurement of fold height from the tip of  
689 the simple fold to the stratum compactum. The yellow line indicates the fold area including the  
690 simple fold and the lamina propria adjacent to the stratum compactum.

691 **S2 Fig. Multivariate analyses and Gene ontology analyses of plasma proteins.** Plots **(A)** and **(B)**  
692 shown R<sup>2</sup> (goodness of fit) and Q<sup>2</sup> (goodness of prediction) for the PLS-DA models before and after  
693 discrimination of VIP variables. The red star indicates the best classifier. The explained variances are  
694 shown in brackets. The explained variances are shown in brackets. Score plots **(C)** and **(D)** shown PCA  
695 and PLS-DA based on VIP variables. **(E)** Protein domain analysis reveals that plasma is enriched in  
696 proteins with signal domain, repeat and coiled domain. **(G)** Gene ontology analysis reveals that plasma  
697 is enriched in proteins with functions in metabolic and cellular processes and biological regulation.  
698 The numbers of proteins in each Gene Ontology class are shown.

699



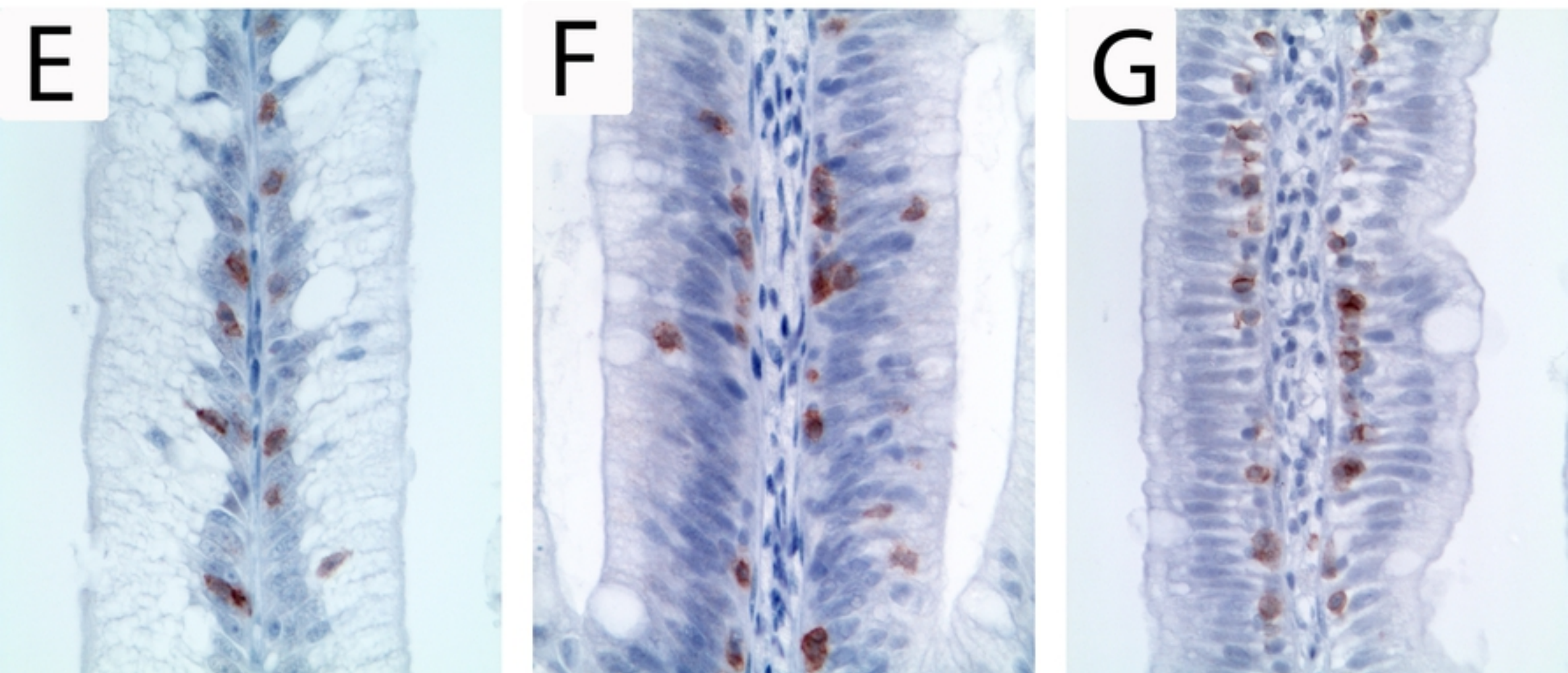
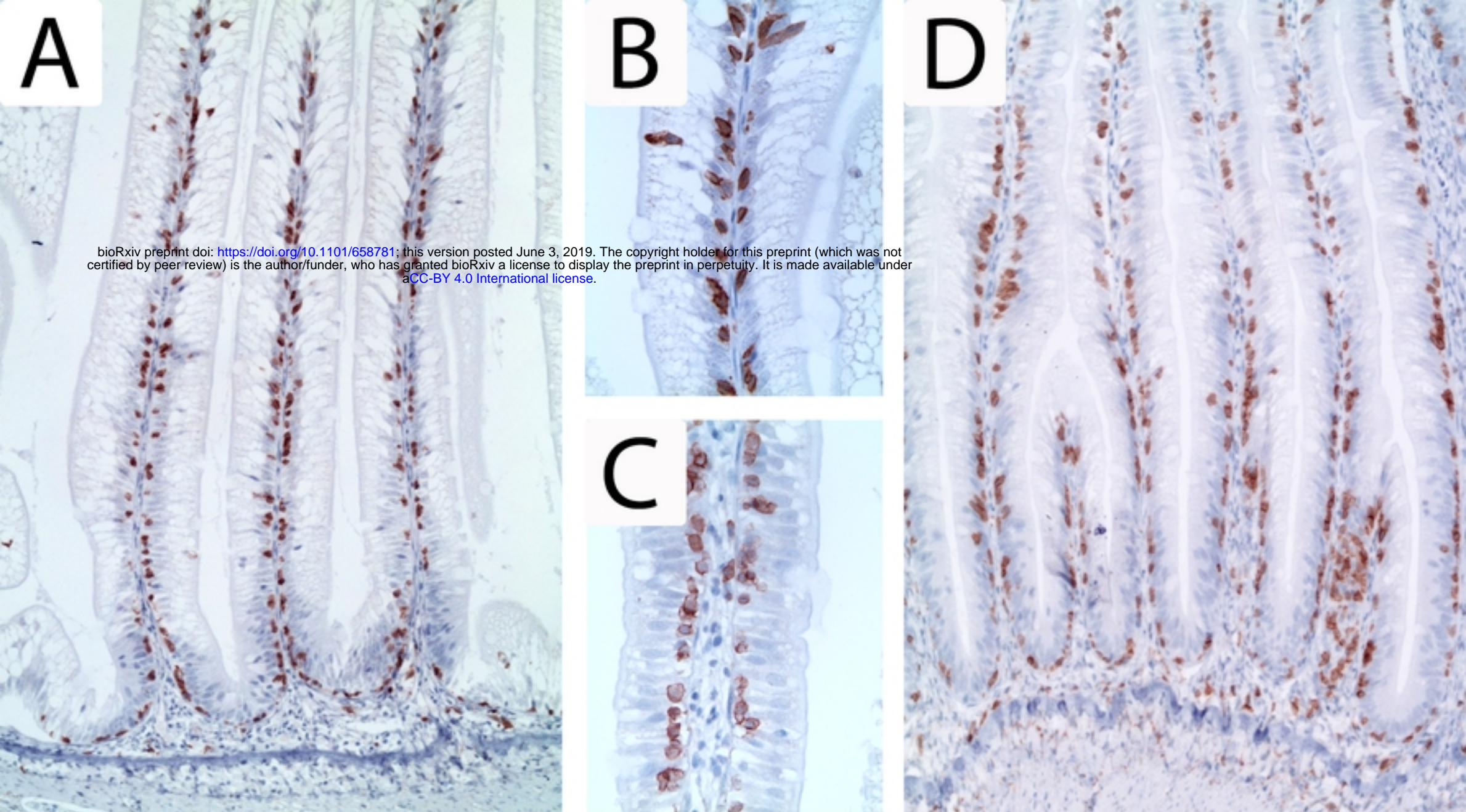


Figure 3



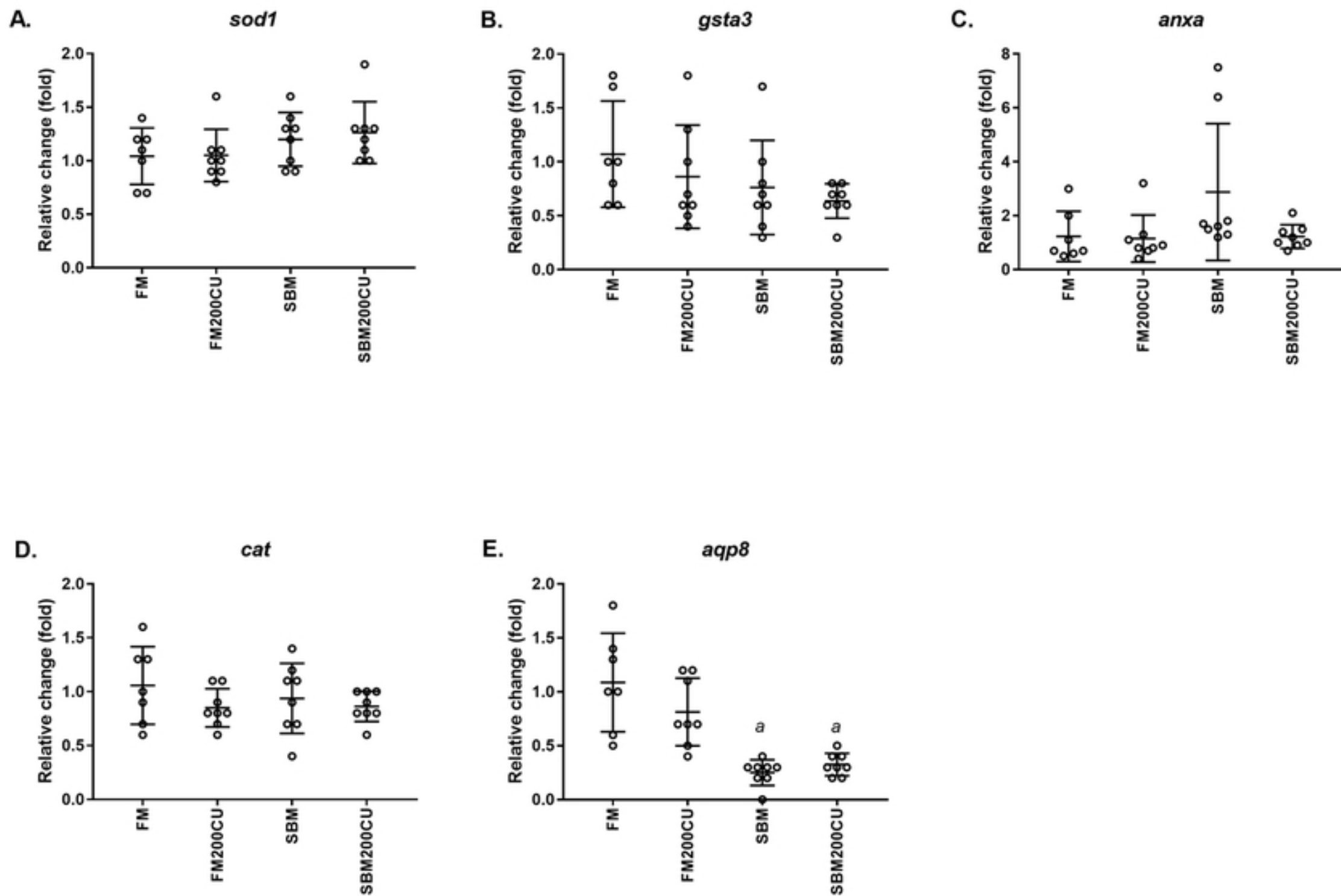
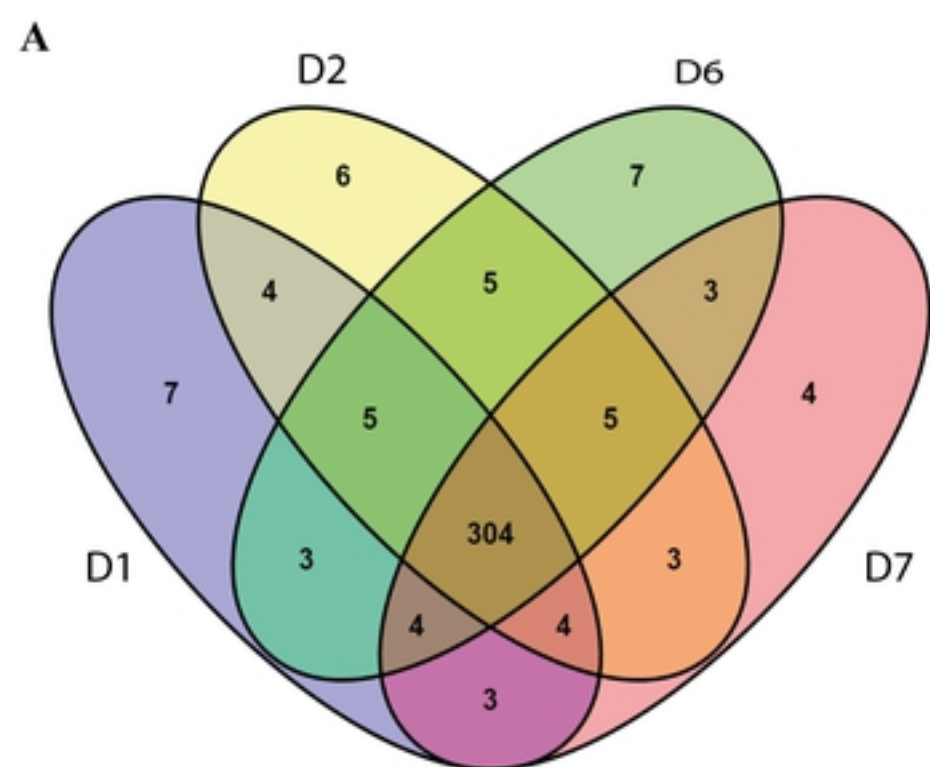


Figure 5



**B**

Diet	Accession numbers	Protein Name
D1	C0H808	Tubulin beta chain
	A0A1S3LLQ8	Tubulin alpha chain
	A0A1S3N4T8	Elongation factor 1-alpha
	Q9W6K6	Heat shock protein 90-beta-1
	A0A1S3LGM9	Complement C1q
	A0A1S3KR13	neutrophil gelatinase-associated lipocalin
D2	B5X2X5	Spondin-2 precursor
	A0A1S3QHK3	Cartilage acidic protein 1-like
	B5X8D4	Trypsin-1
	A0A1S3KRP3	Contactin-1a-like
	A0A1S3QZH9	Apolipoprotein D-like
	B5X4D7	SPARC-like
D6	A0A1S3PL33	Collagen alpha-3(VI) chain-like
	A0A1S3LER5	Inhibin beta chain-like
	A0A1S3R9N1	Hemoglobin subunit beta-1-like
	A0A1S3LK21	Myosin light polypeptide 6-like
	B5DFX8	Phosphoglycerate kinase
	A0A1S3NEN6	Snaclec 1-like
D7	B5X3K2	Glyceraldehyde-3-phosphate dehydrogenase
	B5X1B5	Alpha-enolase
	A0A1S3MALS	Meprin A subunit
	A0A1S3MA64	Metalloendopeptidase
	B5X4D9	Digestive cysteine proteinase 2

**C**

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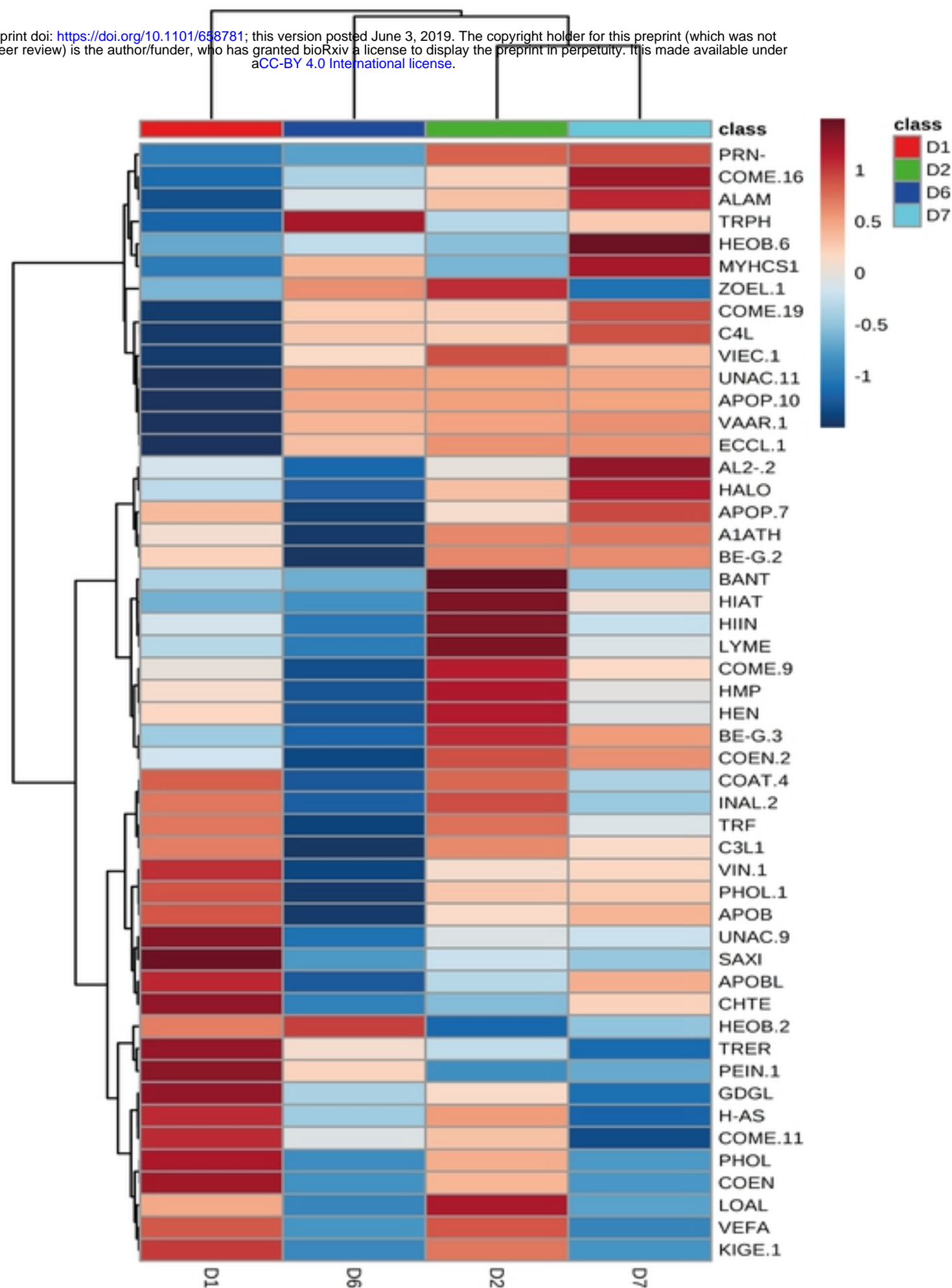


Figure 6

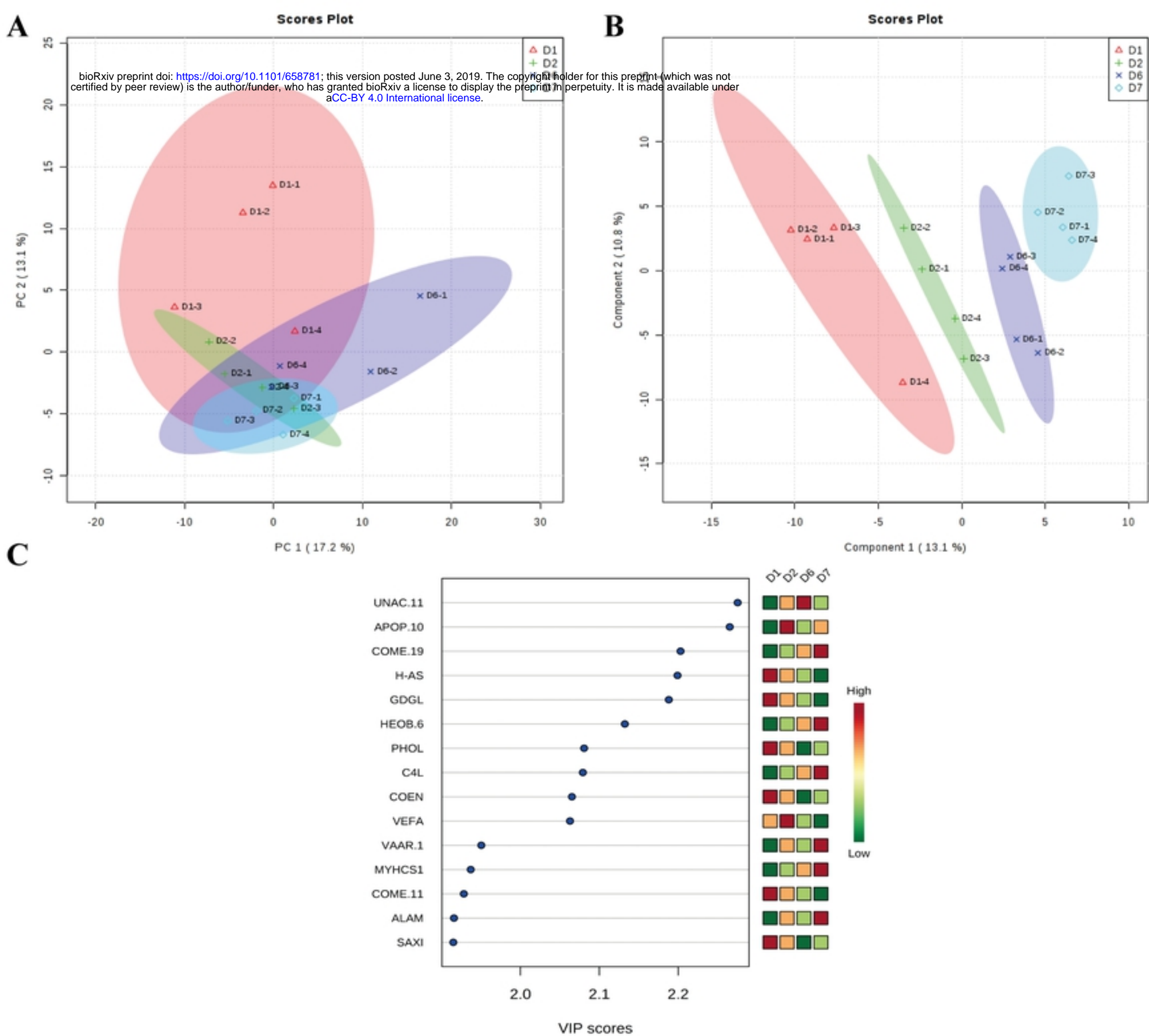


Figure 7

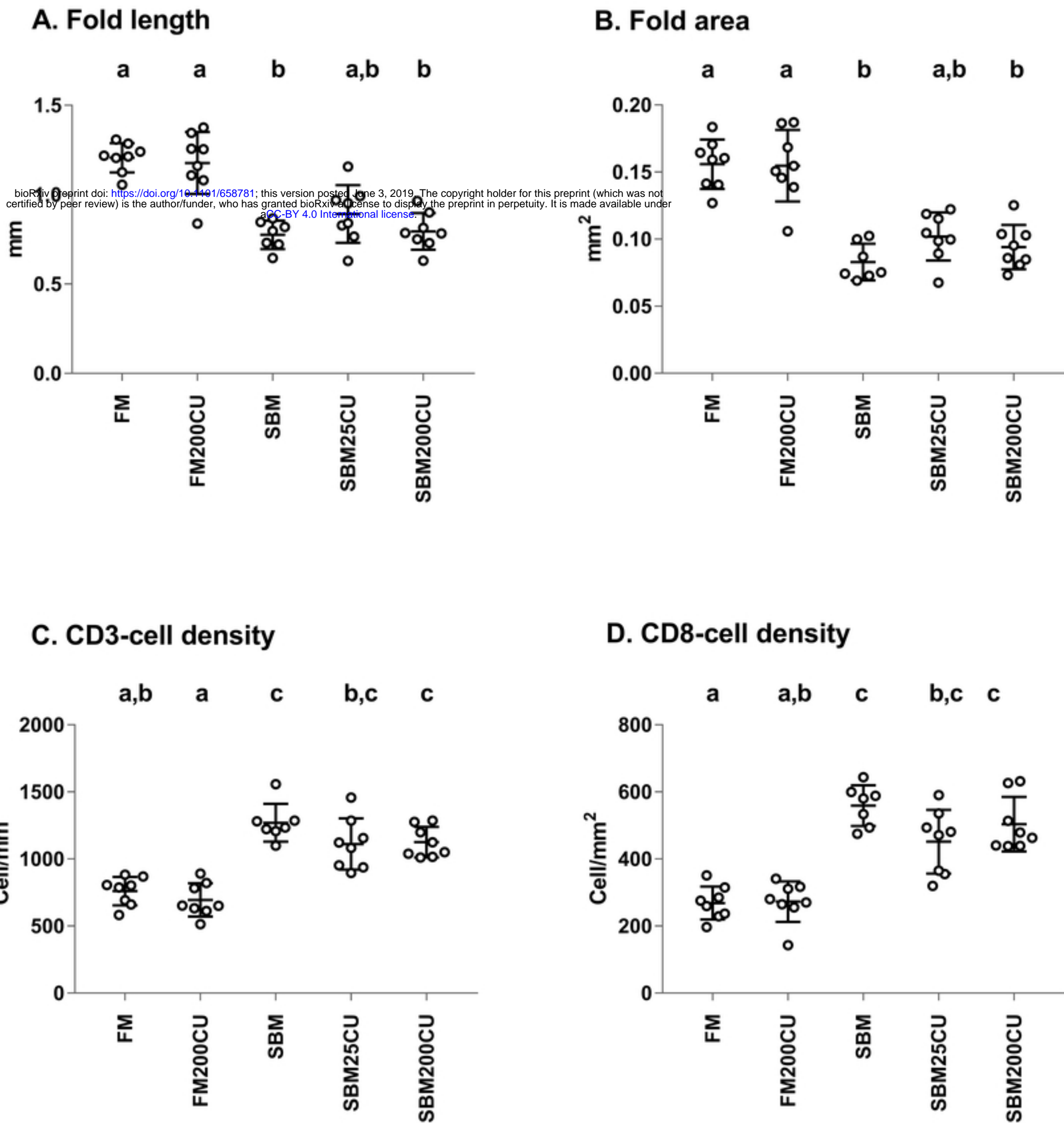
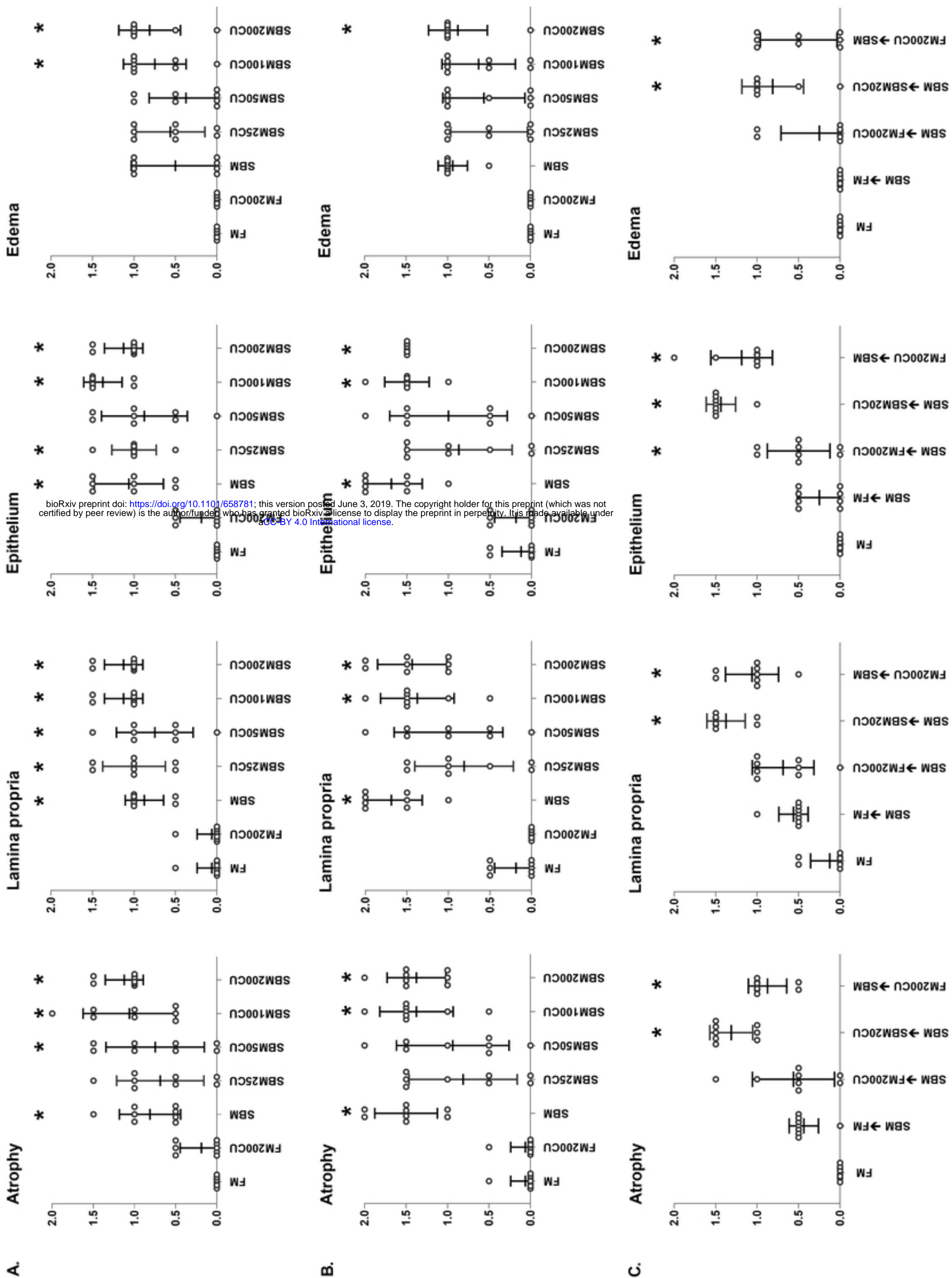


Figure 4





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Figure 2

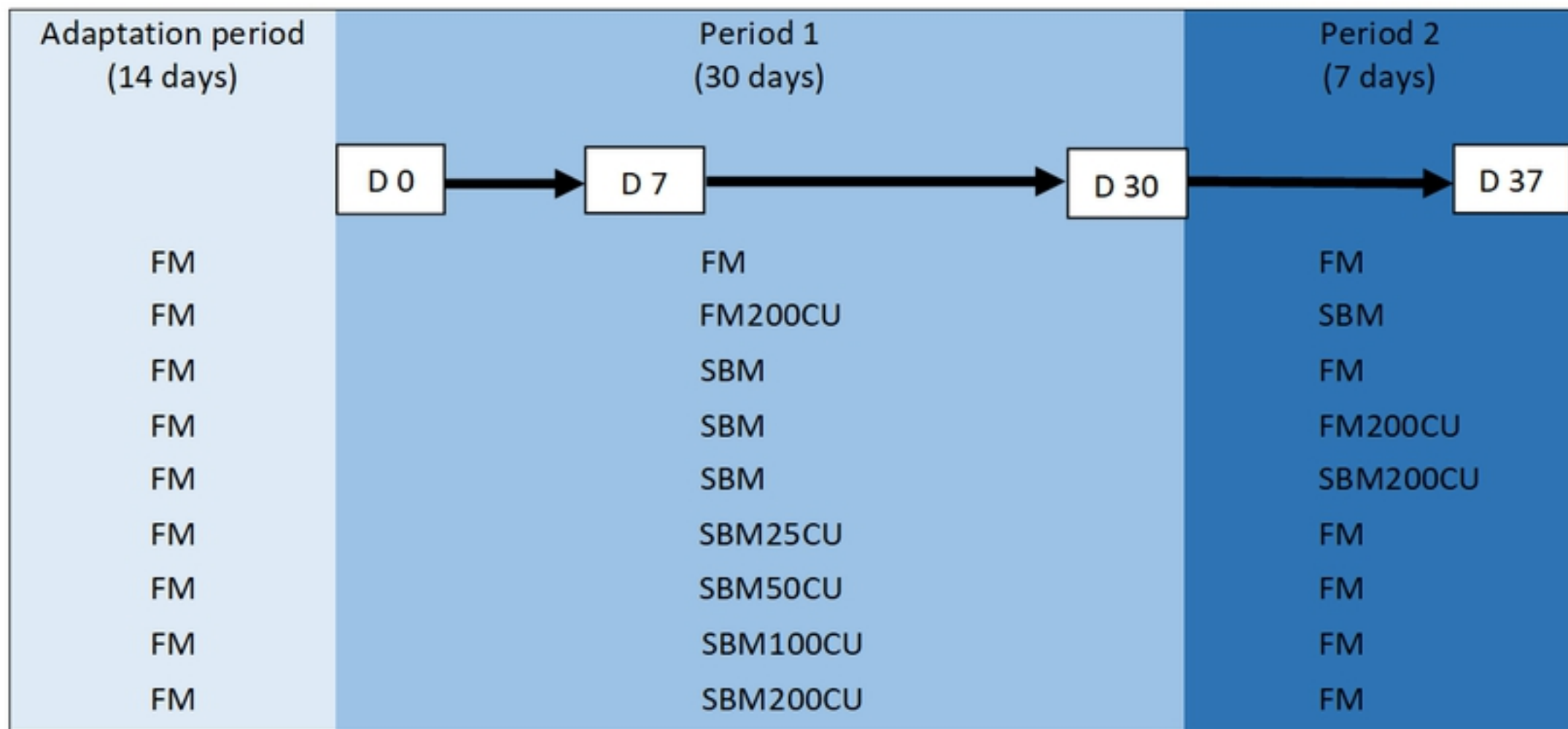


Figure 1