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1 **Title:** Lipidome profiles of postnatal day 2 vaginal swabs reflect fat composition of gilt's
2 postnatal diet.
3
4 **Running Title.** Effect of diet on lipidome of perinatal vagina
5
6 **Summary sentence.** Vaginal swab lipidome profiles at 48 h reflect the fat composition of
7 neonatal diet during first two days postnatal.
8
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27 **Key words.** lipidome, vagina, developmental origins of health and disease, colostrum,

28 biomarker

29

30 **Abbreviations.** Precursor (Prec) and neutral loss (NL) scan modes. phosphatidylethanolamine

31 (PE), acylcarnitines (AC), cholesteryl esters (CE), glycosylinositolphosphoceramides (GIPC),

32 phosphatidic acids (PA), phosphatidylcholines (PC), alkenyl-acyl PCs (ePC), sphingomyelin

33 (SM), phosphatidylinositols (PI), phosphatidylglycerols (PG), phosphatidylserines (PS), fatty

34 acids (FA), triacylglycerol (TAG), bottle fed milk replacer (B); suckled colostrum (S); bottle fed

35 milk replacer and supplemented with fat (BF); suckled and supplemented with fat (SF),

36 receiver operating characteristic (ROC); area-under-the-curve (AUC); multiple reaction

37 monitoring (MRM); postnatal day (PND)

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38 **Abstract.**

39 We hypothesized that postnatal development of the vagina is impacted by early nutritional
40 environment. Our objective was to determine if lipid profiles of vaginal swabs were different
41 between gilts suckled by sow or fed milk replacer the first 48 h postpartum, with and without a
42 lard-based fat supplement. Gilts (>1.3 kg) were selected at birth across 8 litters and assigned to
43 treatments: colostrum suckled (S, n=8); S plus fat supplement (SF, n=5); bottle-fed milk replacer
44 (B, n=8); or B plus fat supplement (BF, n=7). At 48 h postnatal, vaginal swabs were taken with
45 a cytology brush, immersed in ultrapure water to burst cells, and lipids extracted for analysis
46 using multiple reaction monitoring (MRM)-profiling. Lipids extracted from serum collected at
47 48 h from gilts and milk collected from sows at 24 h were also analyzed with MRM-profiling.
48 Receiver operating characteristic curve analysis found 18 lipids highly distinguished [area-under-
49 the-curve (AUC) > 0.9] between S and B gilts, including phosphatidylethanolamine with 34
50 carbon and four unsaturations in the fatty acyl residues [PE(34:4)]. Twelve lipids from vaginal
51 swabs highly correlated ($r > 0.6$; $p < 0.01$) with nutrition source. Lipids more abundant in milk
52 replacer drove association. For example, mean intensity of PE (34:4) was 149-fold higher in
53 milk replacer than colostrum, with 1.6- and 2.12-fold higher levels in serum and vaginal swab
54 samples ($p < 0.001$), respectively, of B versus S gilts. Findings support that vaginal swabs can be
55 used to noninvasively study effects of perinatal nutrition on tissue composition.

56

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57 ## Introduction

58 Early nutritional environment affects long term health and fertility. In swine, colostrum ingestion
59 is essential for postnatal piglet survival, growth, and development because it provides immunity,
60 nutrients, energy, and bioactive factors [1, 2]. The window of opportunity for milk-borne bioactive
61 factors to influence neonate development is limited, and primarily occurs prior to closure of tight
62 junctions between cells lining the piglet's gut. Closure of the gut occurs by 48 h postnatal [3].
63 During the first 48 h postnatal, piglets ingest up to 30% of their body weight in milk [4]. This time-
64 period is a critical developmental period for the gilt reproductive system, including the formation
65 of uterine glands, otherwise known as adenogenesis [5, 6]. Colostrum ingestion significantly
66 affected the developmental trajectory of uterine tissue [7-10]. Replacement gilts with less
67 colostrum consumption than littermates as indicated by blood immunocrit values had reduced litter
68 sizes relative to other sows [11, 12]. Colostrum-deprivation also resulted in significantly different
69 patterns of uterine gene and protein expression [13].

70

71 The link between early nutritional environment, uterine development, and subsequent
72 reproductive potential led to the hypothesis that early nutritional environment affects
73 reproductive tract development and subsequently predicts long-term reproductive performance of
74 gilts. However, in order to evaluate uterine development, the animal must be euthanized. We
75 previously proposed that since the vagina is embryologically related to the uterus [14], its
76 postnatal developmental trajectory may also be responsive to early nutritional environment.
77 Moreover, we proposed that using vaginal swabs to non-invasively sample lower reproductive
78 tract may serve as a means to evaluate differences in nutritional exposures on gilt development.
79 Using a biomarker-discovery technique known as multiple reaction monitoring (MRM) profiling,

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80 we found that lipid profiles of vaginal swabs taken on postnatal day 14 differed between gilts
81 that were fed milk replacer during the first 48 h postpartum before return to litter versus gilts that
82 suckled sow's milk continuously from birth [15].
83
84 While our previous studies supported the potential of using biological material obtained from
85 vaginal swabs to distinguish between gilts exposed to different nutritional environments the first
86 2 days postnatal [16], the lapse of time from colostrum exposure and relatively small sample size
87 limited interpretation. In this study, we further investigated the efficacy of using MRM-profiling
88 of vaginal lipids to differentiate PND 2 vaginal swabs between gilts suckled by sow or fed milk
89 replacer. Secondly, we tested the effect of a lard based supplement on vaginal lipid profiles of
90 gilts [17-20].
91

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92 **Materials and Methods**

93 ***Animals and study design***

94 Prior to beginning studies involving animals the protocol was reviewed and approved by Purdue
95 University's Institutional Animal Care and Use Committee (Protocol #1605001416). Standard
96 farrowing protocols for the Purdue University Animal Sciences Research and Education Swine
97 facility were followed. All lipidomics sample preparation and analysis was completed at the
98 Proteomics and Metabolomics Core Facilities in the Bindley Bioscience Center at Purdue
99 University.

100

101 Three to four gilts were selected per litter from eight different sows which were monitored
102 during parturition (Supplemental Figure S1). Immediately after delivery, all gilts were towel-
103 dried, weighed, and placed in a holding cart until at least three gilts above 1.3 kg were delivered.
104 Within litter, each gilt was randomly assigned to one of four treatment groups and ear tagged for
105 identification. The four treatment groups were: suckled (S; n=8); suckled plus fat-supplement
106 (SF; n=5); bottle-fed with milk-replacer (B; n=8), and; bottle-fed milk replacer plus fat-
107 supplement (BF; n=7). Body weights were recorded at birth and 48 h. All gilts were administered
108 a 2 ml dose of Camas experimental antibody product (Camas Incorporated; Le Center, MN)
109 using Pump It™ Automatic Delivery System (Genesis Industries, Inc.) at birth, and at 3 h and 9
110 h after the first dose.

111

112 Gilts in B and BF treatment groups were taken to a separate temperature-controlled nursery,
113 where they were placed in holding cages in groups of 2-3 piglets. Birthright™ milk replacer
114 (Ralco Nutrition, Inc.) was mixed using the recommended concentration for orphaned piglets,

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115 which was 1:2 replacer to tap water. Reconstituted milk replacer was stored in a refrigerator and
116 warmed to room temperature prior to feedings. Piglets were fed using Evenflo Classic slow-flow
117 0-3-month infant bottles (Evenflo Feeding, Inc.) approximately every 2 h, with a minimum of 5
118 ml fed per time point. In the event piglets did not consume at least 30 ml after two consecutive
119 feedings, 15 ml of milk replacer was oral gavage-fed using a 30 ml syringe and Kendall™ 4.7
120 mm x 41 cm feeding tube (Medtronic, Minneapolis, MN). The fat supplement was administered
121 at the back of the throat in 3 ml room-temperature doses using a 5 ml syringe at 6 h, 12 h, 24 h,
122 and 36 h after birth. Swallowing was observed to ensure the supplement was delivered.
123
124 Blood samples were collected from gilts at 48 h postnatal. A 22 g x 1.5-inch needle and a 3 ml
125 serum Vacutainer® (BD Life Sciences) tube was used to collect blood by jugular venipuncture.
126 Blood samples were refrigerated and allowed to clot overnight. Serum was collected following
127 centrifugation (Horizon Model 614B Centrifuge; Fisher Scientific) for 25 minutes at 2,500 g and
128 stored at -20°C until analysis.
129
130 Gilts were euthanized approximately 48 h after birth using CO_2 inhalation. Following
131 euthanasia, skin of the abdominal and genital regions was cleaned thoroughly using 70% ethanol,
132 and vaginal swabs were taken using a cytology brush (Puritan 2196 Removable Stiff Bristle Tip
133 Brush; QuickMedical; Issaquah, WA) by inserting the tip of the brush into the vulva angled
134 dorsally at 45˚. Once inserted to the base of the bristles, the brush was rotated 360˚ against the
135 vaginal surface. Two consecutive swabs were collected from each animal, and swabs were
136 placed in separate 15 ml sterile polypropylene conical tubes (Falcon™, Fisher Scientific, San

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137 Jose, California) and immediately placed on dry ice for transport. Samples were stored in a -

138 80°C freezer until lipid extraction and analysis.

139

140

141 *Fat supplement preparation*

142 The fat supplement was created by emulsifying pork lard (Berkshire pork lard, EPIC; Austin,

143 TX). Pork lard was used as the fat source because the fatty acid composition of lard is similar to

144 sow colostrum [21]; Tween 80 was used as an emulsifying agent. A 30% volume/volume

145 solution of lard/2% aqueous solution of Tween 80 was prepared using a homogenizer to

146 microscopically distribute the lipid aggregates with low heat. The emulsion was stored at 4˚C.

147

148 *Milk collection*

149 Sows were milked during farrowing, and at 24 h after delivery of first piglet. For milk collection

150 piglets were removed from the sow for approximately an hour and then 1 ml oxytocin (VetOne;

151 Boise, ID; 20 USP/ml) was administered IM using a 20g x 1.5-inch needle into the vulva to

152 stimulate milk letdown. Colostrum samples were collected manually from all teats and

153 combined to create a uniform sample. Samples were stored until further analysis at -20°C.

154

155 **Lipidomics Analysis**

156 *Lipid extraction*

157 The Bligh & Dyer [22] lipid extraction technique was slightly modified to extract lipids from the

158 vaginal swab samples. A volume of 500 µl distilled water was added to the conical tube

159 containing the swab brushes and vortexed to remove biological material from the brush. The

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160 brushes were removed, the sample homogenate was transferred to a new tube, and phase
161 separation was performed by mixing with chloroform/methanol/distilled water (1:2:0.8).
162 Samples were centrifuged, the organic phase (bottom phase) was separated from aqueous phase,
163 divided into four aliquots, and dried in a centrifugal evaporator (Savant SpeedVac AES2010,
164 ThermoFisher Scientific, San Jose, CA). Dried lipid extracts were stored at -20˚C until mass
165 spectrometry analysis.
166
167 The Bligh & Dyer [22] lipid extraction technique was also used to extract lipids from 48 h serum
168 samples from suckled and bottle-fed piglets; colostrum samples taken from all eight sows
169 (during parturition, 6 h after first piglet born, 12 h after, and 24 h after); and milk replacer used
170 for bottle-feeding. The procedure for these samples began at the phase separation step (i.e. no
171 water was added to the samples).
172
173 *Multiple reaction monitoring (MRM)-profiling*
174 For the discovery phase of MRM-profiling, vaginal lipid extracts were pooled by treatment to
175 determine the functional groups present in samples. Pooled samples were prepared by combining
176 equal volumes of dried lipid extracts diluted in 200 µl of the flow injection solvent
177 [acetonitrile/methanol/ammonium acetate 300 mM at 3:6.65:0.35 (*v*/v)]. Pooled sample
178 injections (8 µl) were delivered to the micro-autosampler (G1377A) in a QQQ6410 triple
179 quadrupole mass spectrometer (Agilent Technologies, San Jose, CA) equipped with an ESI ion
180 source. The solvent pumped between injections (CapPump G1376A, Agilent Technologies, San
181 Jose, CA) was acetonitrile with 1% formic acid at 10µL/min. Between sample injections,
182 methanol/chloroform was injected to remove any remaining lipids before the next sample

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183 injection. Samples were examined for fragmentation features of specified chemical classes using
184 Precursor (Prec) and neutral loss (NL) scan modes as previously reported [15]. Scans were used
185 to profile chemical classes, which included: phosphatidylethanolamine (PE), acylcarnitines (AC),
186 cholesteryl esters (CE), glycosylinositolphosphoceramides (GIPC), phosphatidic acids (PA),
187 phosphatidylcholines (PC), alkenyl-acyl PCs (ePC), sphingomyelin (SM), phosphatidylinositols
188 (PI), phosphatidylglycerols (PG), phosphatidylserines (PS), fatty acids (FA), ceramides, sugar
189 lipids, and glycerolipids (see Supplemental Table S1 for classes scanned). Initial data processing
190 of the profiles obtained was carried out by converting each set of MRM-profiling method data
191 into mzML format using MSConvert20. Next, the signal intensity for the ions present in NL and
192 Prec mass spectra was obtained using an in-house script. Ions with values of counts >1000 were
193 selected as parent ions and the product ion or neutral loss information was used for selecting ion
194 pairs (1486) for the screening phase.
195
196 For Screening Phase I, individual sample extracts were analyzed for the MRMs identified in the
197 discovery phase (1486), as well as triacylglycerol (TAG) MRMs (Supplemental Table S2). TAG
198 have no polar head or diagnostic functional group fragment, precluding them from the discovery
199 step of analysis. During screening phase, TAG were profiled using parent ions and a product ion
200 related to the presence of specific fatty acyl residues (16:0, 16:1, 18:0, 18:1, 18:2, and 20:4).
201 MRMs identified in the discovery step and TAG MRMs were divided into 8 groups, or 8
202 screening *methods*. The methods were organized by factors such as lipid class to optimize the
203 efficiency of the machine and to limit the number of precursor ion/product ion pairs screened per
204 sample injection. Thus, for each of the 8 methods, intensities of approximately 250 MRMs were
205 measured per 8µl sample injection. Since cellular content in each sample may vary when using

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206 swabs, prior to screening lipid extract amount was recorded across samples by scanning for the
207 precursor ion of *m/z* 184, which profiles the most abundant lipids in cells, namely PC and SM
208 lipids. In this way, intensities for PC lipids were measured, and samples were diluted using the
209 same flow injection solvent to achieve a similar concentration for comparative relative analysis.
210
211 Data from Screening Phase I were uploaded into MetaboAnalyst 4.0 (www.metaboanalyst.ca) to
212 identify MRMs that best discriminated between suckled and bottle-fed gilts. A second set of
213 MRMs were selected based on discrimination between gilts with and without fat
214 supplementation. There were 146 MRMs identified that best discriminated between suckled and
215 bottle-fed vaginal lipids (S and SF piglets versus B and BF piglets, respectively), and 197 MRMs
216 that discriminated not-supplemented and fat supplemented vaginal lipids (S and B piglets versus
217 SF and BF piglets, respectively). The 146 MRMs (Supplemental Table S3) and 197 MRMs
218 (Supplemental Table S4) were organized into two distinct methods (Method 1 and Method 2,
219 respectively) for Screening Phase II analysis.
220
221 Method 1 and Method 2 were used to evaluate the vaginal lipids from all piglets in Screening
222 Phase II. The resulting ion pair intensities were normalized to reflect percent intensity for
223 subsequent comparative analysis. These data were used to verify the effectiveness of each
224 method to differentiate between piglets that were: A.) suckled or bottle-fed, or; B.) fat
225 supplemented or not. Data were uploaded in MetaboAnalyst 4.0 by method for statistical
226 analysis to evaluate the potential effectiveness of selected MRMs as biomarkers for
227 distinguishing treatment groups.
228

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229 *MRM-profiling data analysis in MetaboAnalyst*

230 Ion intensities of MRMs from the screening phase were used to calculate the relative ion
231 intensity of ion pairs in each sample and these data was uploaded into MetaboAnalyst 4.0 [23]
232 and normalized using autoscaling. The Biomarker Analysis tool was used to identify MRMs that
233 discriminated between suckled versus bottle-fed gilts and fat supplemented versus non-fat
234 supplemented gilts. Biomarker analysis tools applied included principal component analysis,
235 partial least square-discriminant analysis, and classical univariate receiver operating
236 characteristic (ROC) curve analysis. Using data from Screening Phase I, a cut-off area-under-the-
237 curve (AUC) value of 0.70 and 0.80 was used to select MRMs to discriminate between vaginal
238 lipids of piglets suckled versus bottle-fed (Method 1), and those with and without fat
239 supplementation (Method 2).

240

241 *Lipid identification*

242 Precursor ion/product ion pairs were tentatively assigned identities using attributions in Metlin
243 (https://metlin.scripps.edu) and Lipid Maps (http://www.lipidmaps.org/). Potential identities
244 were assigned using the associated functional group and biological information. Metlin MS/MS
245 Spectrum Search was used with the following settings: precursor ion mass from the mass
246 spectrometer reading for *m/z* value, maximum tolerance at 100, collision energy at 10 eV or 20
247 eV, MS/MS tolerance at 0.5, peaks included the product ion mass *m/z* with an intensity of 100,
248 positive mode, and M+H was selected for the adduct. If the suspected ion pair was a TAG,
249 $M+NH_4$ was also selected as an adduct to account for ammonium acetate.

250

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251 Phospholipids were assigned identity by their class (PS, PI, PE, or PC), the number of carbon
252 atoms in the esterified fatty acid, and the number of carbon-carbon double bonds present in the
253 molecule, eg. PE(34:4). TAGs were similarly assigned tentative attributions. First, the number of
254 carbon atoms in each fatty acyl chain was summed, and the number of carbon-carbon double
255 bonds was assigned. Since searches in Lipid Maps can only be performed using neutral masses,
256 and ammonium acetate was present in the injection solvent, TAGs were detected as ammonium
257 adducts. For this reason, 18 mass units (NH_4) was subtracted from the parent mass observed in
258 the MS analysis to obtain the neutral mass for each TAG. Other lipids were searched by
259 accounting for a single mass unit loss to account for a hydrogen ion only. Results were narrowed
260 using the associated functional group and biological information, and attributions were assigned
261 where possible.
262
263 *Swab, serum, and colostrum correlation analysis*
264 Method 1 was also used to analyze serum lipids collected from the S and B animals, milk
265 replacer, and colostrum at 0 h, 6 h, 12 h, and 24 h post-parturition. These resulting intensities
266 were converted to percent intensity for comparative analysis. Pearson's correlation coefficient
267 analysis of percent intensities between milk and serum samples, milk and vaginal swab samples,
268 and serum and swab samples was performed for each of the 38 most discriminating MRMs from
269 Method 1 using the CORR procedure of SAS 9.4 (Cary, NC). Variables compared were percent
270 intensities of: 48 h swab samples and 48 h serum samples; 48 h swab samples and respective
271 source of nutrition at 24 h; and 48 h serum samples and respective source of nutrition at 24 h. Six
272 MRMs overlapped as highly correlating between source of nutrition and swab lipids, and source
273 of nutrition and serum lipids. Percent ion intensities were compared between milk replacer and

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274 colostrum for those six MRMs. Statistics were not performed on colostrum versus milk replacer
275 since ion intensity results were from only one sample of milk replacer. In addition, MIXED
276 procedure of SAS 9.4 (Cary, NC) was used to analyze the statistical difference in ion intensities
277 of the six most highly correlated MRMs between suckled versus bottle-fed gilt serum, as well as
278 suckled versus bottle-fed gilt swab samples to compare.
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288 **Results**

289 *Effect of postnatal diet on vaginal lipid profiles.*

290 The discovery phase identified a total of 1486 unique MRMs in pooled isolates from vaginal
291 swabs. Glycerolipids with saturated bonds encompassed 37% of identified lipids (Figure 1A;
292 Supplemental Table S2). Glycerolipids with unsaturated bonds were next in abundance with 25%
293 of identified lipids, and acylcarnitines were third most abundant (15.81%). Intensities of the 1486
294 MRMs identified in the discovery phase, plus approximately 400 triacylglycerols (TAGs), were
295 measured in individual samples in Screening Phase I (Supplemental Table S2). Screening Phase I
296 analysis found 146 MRMs (Supplemental Table S3) that best discriminated between suckled and
297 bottle-fed vaginal lipids, and 197 MRMs (Supplemental Table S4) that best discriminated
298 between vaginal lipids of gilts with and without fat supplementation.

299

300 For Screening Phase II, the 146 MRMs that distinguished between suckled and bottle-fed
301 treatments were organized into Method 1 analysis, which consisted primarily of TAGs,
302 acylcarnitines, and glycerolipids (Figure 1B). Tentative attributions were assigned to 91/146
303 lipids in Method 1 (Supplemental Table S5). Principal component and hierarchical cluster
304 analysis showed distinct clusters by treatment groups of suckled versus bottle-fed milk replacer
305 (Figure 2A and 2B). ROC curve analysis identified 18 MRMs with AUC values greater than
306 0.90. Of the 18, 15 were assigned tentative attributions, which included hydroxy octadecanoyl
307 carnitine, two TAG(46:1), TAG(48:2), TAG(48:1), PG(36:2)/PI(30:4), PC(36:7), TAG(44:1),
308 PG(34:6), PE(34:4), PE(36:5), PE(38:5), two TAG(44:0), and TAG(48:3). The proportion of
309 TAGs identified in this group (44%) was greater than proportion of TAGs present in the entire

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310 list of Method 1 ion pairs (27%). The 20 most discriminating lipids recovered from the vaginal
311 swabs showed a greater relative abundance in most bottle-fed versus suckled gilts (Figure 2B).
312
313 Hierarchical cluster and principal component analysis of the effect of fat supplementation on
314 lipidome profiles showed several groupings, with some distinction between plus and minus fat
315 supplemented gilts (Figure 2C and 2D). The 197 MRMs that most distinguished between fat
316 supplemented piglets and non-supplemented piglets and selected for Method 2 Screening Phase
317 II analysis were primarily glycerolipids (Figure 1C). ROC curve analysis identified 15 MRMs
318 with AUC values ranging from 0.65 to 0.78. Mean ion intensity of these MRMs was compared
319 between treatments, and overall treatment effects ($p < 0.05$) were found for a cholesteryl ester
320 and a ceramide, with numerical differences between treatments in two glycerol lipids with EPA
321 residues (Figure 3).
322
323 *Correlation of lipids isolated from Swabs, Blood Serum, and Colostrum-Milk Replacer*
324 To investigate whether there was a relationship between piglet nutrition and either serum or
325 vaginal lipidome profiles, lipids from piglet serum, sow colostrum, and milk replacer were
326 analyzed using Method 1 MRMs. Linear regression analysis was used to determine if there was a
327 relationship between intensities of the top 38 MRMs (AUC > 0.8) that discriminated most
328 between vaginal swab samples of S and B groups with intensity in piglet serum and nutrition
329 source (sow colostrum or milk replacer; Table 1). The correlation coefficient between twelve
330 MRMs in swab and milk samples was highly significant ($r > 0.60$ and $P < 0.01$), and included:
331 three PE lipids; a PG lipid; a PC lipid; five TAGs; stearoylcarnitine, hexadecanedioic acid mono-
332 L-carnitine ester; and PG (36:2)/PI(30:4). Analysis of relationship of lipid intensities by nutrition

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333 source and serum found seven MRMs were significant including three PE lipids, one PG lipid,
334 and three TAGs. One MRM, related to TAG (48:3), had a negative correlation between nutrition
335 source and serum (r = -0.62 and P = 0.01).

336

337 Six MRMs overlapped amongst the strongest correlations between source of nutrition and serum
338 and between source of nutrition and swab [tentative lipid attributions: PE (34:4), PE(36:5),
339 PG(34:6), TAG(46:1), PE(38:5), and TAG(44:0)]. Levels of all six lipids were 159-fold to 2.41-
340 fold (Figure 4 and Supplemental Figure S2) higher in milk replacer than colostrum, which
341 correlated to significantly higher levels across all serum and swab samples of botte-fed versus
342 suckled animals (Figure 4).

343

344

345

346

347

348

349 **Discussion**

350 Screening phase II Method 1 analysis aimed at identifying lipids that discriminated between gilts
351 which suckled sow colostrum versus gilts fed milk replacer the first 48 h postnatal. ROC curve
352 analysis found 18 lipids with AUC $\geq$ 0.9. The AUC value is a measure of the usefulness of a
353 test, in terms of sensitivity and specificity [24]. Biomarkers with AUC values from 0.9-1.0 are
354 excellent, 0.8-0.9 are good, 0.7-0.8 are fair, and 0.6-0.7 are poor [25]. The 18 lipids are thus
355 excellent candidate markers to detect differences between suckled gilts compared to those that

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356 consumed only milk replacer. The most discriminatory lipids identified in Method 1 exhibited
357 higher intensities in bottle-fed animals when compared to suckled gilts. We found a positive
358 correlation of ion intensities of MRMs between nutrition source and swabs of suckled versus
359 bottle-fed gilts, and nutrition source and serum of suckled versus bottle-fed gilts. Comparative
360 analysis of mean ion intensities of the most highly correlated lipids found much higher intensities
361 of lipid signals in milk replacer than in 24 h colostrum samples, and correspondingly higher
362 intensities in serum and vaginal swabs of bottle-fed animals relative to suckled gilts.
363
364 The high correlation of MRM intensities between nutrition source and swab were primarily driven
365 by lipids that were significantly more abundant in milk replacer than colostrum samples. These
366 findings demonstrate that lipid content in milk replacer formulas changes the composition of
367 neonate tissues, and component fats may become part of the tissues. Lipids are bioactive
368 molecules, and play a central role in metabolism and influence and modulate cell function. For
369 example, lipids from the diet function in cytokine and (steroid) hormone synthesis, cell
370 differentiation and growth, cell membrane structure, myelination, signal transmission, and are
371 substrates used for prostaglandin synthesis. Fatty acids and their metabolites, such as eicosanoids,
372 have a major signaling function and regulate gene transcription by serving as transcriptional co-
373 activators, with polyunsaturated fatty acids activating the transcription factor peroxisome
374 proliferator–activated receptors [26, 27]. Thus, fatty acid composition of diet has the potential to
375 affect significant biological response in the neonate, which in turn can affect the course of neonatal
376 development and health. In humans, there is evidence that the quality of dietary lipids provided
377 to infants has a marked impact on health outcomes [28], with our study supporting that dietary
378 lipids became components of tissues. Although infant formula has been amended some, currently

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379 available products continue to be markedly different from breast milk in their lipid composition;
380 these differences might be of importance for infant health, development and long-term fertility
381 [28]. Correlation of lipids in nutrition source (milk replacer and colostrum) with serum and swab,
382 suggest some lipids may be directly absorbed without modification in the gastrointestinal tract,
383 potentially prior to intestinal barrier closure, which is within 36-48 h after birth [3, 29-31]. Thus
384 this period of time may be viewed as either a window of opportunity or vulnerability for
385 influencing neonate's developmental program.
386
387 Functional groups of Screening Phase II Method 2 analysis were primarily glycerol lipids with
388 both saturated and unsaturated FA residues. Of the most discriminatory lipids, four MRMs had
389 AUC values between 0.7 and 0.8, thus achieving a biomarker utility rating of fair [25]. Two of
390 these were glycerol lipids with eicosapentaenoic acid (EPA) residues, one was a ceramide, and the
391 final was a cholesteryl ester. Mean intensity of the cholesteryl ester was significantly higher in
392 suckled plus fat supplemented gilts versus non-supplemented gilts. Unfortunately, MRM-profiling
393 was not completed with the fat supplement. Thus we can only speculate that the higher cholesteryl
394 ester content in swabs was a response to the higher fat diet. Two glycerol lipids with EPA residues
395 and ceramide were numerically lower in vaginal swabs of fat supplemented gilts. EPA is either
396 absorbed from the diet or synthesized from the essential fatty acids linoleic and alpha linoleic acid.
397 A potential explanation of these findings is that direct supplementation of piglets with animal fats
398 lowered the bioavailability of EPA (or linoleic and alpha linoleic acids). In the mature gut,
399 triglycerides and phospholipids are hydrolyzed by pancreatic lipase, but the ethyl ester of EPA
400 requires additional digestion with bile salt–dependent lipase [32]. Digestion and absorption of
401 EPA is thus considerably lower than triglycerides and phospholipids, and dependent on high-fat

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402 content in meals, which stimulates bile-salt dependent lipase and enhanced absorption [32]. In
403 porcine and human neonates the levels of pancreatic lipase and bile acids are much lower than at
404 maturity [33]. Thus, despite the higher lipid content of fat supplement treated gilts, the low
405 availability of bile acids levels may have limited EPA absorption. Further research into how lipids
406 ingested by early postnatal gilts are metabolized and used in the reproductive tract is needed.
407

408 **Conclusion**

409 Vaginal swab lipidomes were significantly affected by composition of early postnatal diet.
410 Multiple lipid molecules were differentially abundant between gilts exposed to colostrum versus
411 gilts fed milk replacer and gilts fed with and without fat supplement the first 48 h of life. Several
412 lipids collected from vaginal swabs were highly correlated with the nutrition source a piglet
413 ingested. Lipids more abundant in milk replacer appeared to be driving the association in the
414 current study. These findings demonstrated that lipid composition of neonatal diet affects tissue
415 composition and thus may affect development, function and overall health and fertility.
416 Moreover, while data support the efficacy of using MRM-lipid profiling of vaginal swabs to
417 detect differences in early postnatal nutritional exposures, findings also highlight a need to
418 modify our approach to determine if biomarkers of colostrum intake can be specifically
419 identified in vaginal swab samples.
420
421

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523 **Figure Legends.**

524 Figure 1. Lipid class distribution of MRMs: (A) detected in initial discovery phase; (B) Selected
525 for method 1; and (C) Selected for method 2.

526

527 Figure 2. (A) Principal component analysis and (B) heat map of lipids selected for Method 1;
528 within the hierarchical analysis, red signifies bottle-fed animals (B and BF) and green signifies
529 suckled animals (S and SF). (C) Principal component analysis and (D) heat map of lipids
530 selected for Method 2; within the hierarchical analysis, red signifies fat supplemented (SF and
531 BF) and green signifies not supplemented (S and B) animals. In the heatmap, blue indicates
532 lower ion intensities and red indicates higher ion intensities. MRMs are on the right and animal
533 ID is on the bottom.

534

535 Figure 3. Mean ion intensity of precursor-product ion MRMs of (A) cholesteryl ester 425.4-
536 >369; (B) ceramide 557.4->282.2; (C) glycerolipid containing eicosapentaenoic acid residue
537 631.2->283.9; and (D) glycerolipid containing eicosapentaenoic acid residue 780.2->432.9
538 measure in lipid extracts of vaginal swabs taken from bottle fed (B), bottle fed and fat
539 supplemented (BF), suckled by sow (S) and suckled by sow plus fat supplemented (SF) for 48 h
540 postnatal. Differing letters indicate statistical difference at $p<0.05$; and (*) indicates an overall
541 fat supplement treatment effect.

542

543 Figure 4: Comparison of relative mean intensity of PE (34:4), 712.5->495.19 in (A) nutrition
544 source (24 h colostrum sample versus milk replacer-MR); (B) serum of suckled-S versus bottle
545 fed milk replacer-B; and (C) vaginal swab samples in S versus B gilts. Relative mean intensity of

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546 PE (36:5), 738.5->521.19 in (D) nutrition source (24 h colostrum sample versus milk replacer-
547 MR); (E) serum of suckled-S versus bottle fed milk replacer-B; and (F) vaginal swab samples in
548 S versus B gilts. Relative mean intensity of PG (34:6), 739.6->522.29 in (G) nutrition source (24
549 h colostrum sample versus milk replacer-MR); (H) serum of suckled-S versus bottle fed milk
550 replacer-B; and (I) vaginal swab samples in S versus B gilts. Differing letters indicate statistical
551 difference at p<0.005.

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552 **Supplemental Data Legends**

553 **Supplemental Figure S1.** Study design and work flow.

554

555 **Supplemental Figure S2.** Comparison of relative mean intensity of TAG(46:1) in (A) nutrition
556 source (24 h colostrum sample versus milk replacer-MR); (B) serum of suckled-S versus bottle
557 fed milk replacer-B; and (C) vaginal swab samples in S versus B gilts. Relative mean intensity of
558 TAG(44:0) in (E) nutrition source (24 h colostrum sample versus milk replacer-MR); (F) serum
559 of suckled-S versus bottle fed milk replacer-B; and (G) vaginal swab samples in S versus B gilts.
560 Relative mean intensity of PE (38:5) in (H) nutrition source (24 h colostrum sample versus milk
561 replacer-MR); (I) serum of suckled-S versus bottle fed milk replacer-B; and (J) vaginal swab
562 samples in S versus B gilts. Differing letters indicate statistical difference at $p<0.005$.

563

564 **Supplemental Table S1:** Neutral Loss and Precursor Scan Modes Used for Discovery of MRMs

565

566 **Supplemental Table S2**: MRMs Selected After Discovery Step For 8 Methods

567

568 **Supplemental Table S3**. Selected MRMs for Method 1 (Bottle-fed versus Suckled)

569

570 **Supplemental Table S4.** Selected MRMs for Method 2 (Fat supplemented versus Not fat
571 supplement)

572

573 **Supplemental Table S5.** Tentative Attributions for Method 1

574

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Table 5. Pearson correlation coefficients of 38 MRMs with highest AUC values. Bold values indicate either r > |0.60| or P-value < 0.01.

		Serum vs Nutrition Source		Serum vs Swab		Swab vs Nutrition Source	
MRM ID	**Tentative ID Attribution**	***r***	***P*-value**	***r***	***P*-value**	***r***	***P*-value**
442.4-59.1	hydroxy octadecanoyl carnitine	-0.15	0.56	0.36	0.16	-0.43	0.09
514.5-131.2	Not identified	-0.04	0.89	0.12	0.67	-0.22	0.42
640.4-339.1	Not identified	0.15	0.58	-0.29	0.26	0.09	0.73
678.6-184.2	PC(28:0)/PS(28:1)	0.28	0.28	0.13	0.63	**0.59**	**0.01**
712.5-495.2	PE(34:4)	**0.75**	**0.001**	**0.51**	**0.04**	**0.76**	**0.001**
738.5-521.2	PE(36:5)	**0.66**	**0.005**	0.43	0.09	**0.71**	**0.002**
739.6-522.3	PG(34:6)	**0.69**	**0.003**	0.28	0.29	**0.66**	**0.005**
766.5-521.2	TAG(44:1)	**0.71**	**0.002**	0.39	0.13	**0.61**	**0.01**
766.5-549.2	PE(38:5)	**0.76**	**0.0006**	0.42	0.11	**0.71**	**0.002**
768.4-495.1	TAG(44:0)	**0.67**	**0.005**	0.27	0.32	**0.67**	**0.005**
768.5-523.2	TAG(44:0)	**0.61**	**0.01**	0.11	0.71	**0.63**	**0.01**
776.1-184.2	PC(36:7)	0.14	0.59	0.13	0.62	**0.73**	**0.001**
793.7-520.4	Not identified	**0.49**	**0.05**	0.25	0.34	**0.59**	**0.01**
794.5-521.2	TAG(46:1)	**0.63**	**0.008**	0.38	0.14	**0.62**	**0.01**
794.5-549.2	TAG(46:1)	**0.57**	**0.02**	0.42	0.11	**0.67**	**0.004**
818.8-519.8	TAG(48:3)	0.43	0.09	0.42	0.11	**0.51**	**0.05**
820.7-521.4	TAG(48:2)	0.32	0.22	0.32	0.23	0.36	0.17
822.8-521.8	TAG(48:1)	-0.26	0.32	0.22	0.42	-0.15	0.57
400.4-101.1	Not identified	0.06	0.81	0.22	0.42	0.13	0.62
428.4-85.1	Stearoylcarnitine, hexadecanedioic acid mono-L-carnitine ester	-0.12	0.65	0.07	0.78	**0.69**	**0.003**
476.5-177.2	Not identified	0.26	0.32	0.04	0.87	-0.19	0.48
638.5-339.2	Not identified	-0.11	0.69	-0.39	0.13	-0.31	0.24
640.29-340.99	Not identified	0.35	0.18	-0.03	0.91	0.43	0.1
706.7-184.2	PC(30:0)/PS(30:1)	0.43	0.09	**0.49**	**0.05**	**0.61**	**0.01**
766.5-495.2	TAG(44:1)	0.42	0.11	0.14	0.61	**0.69**	**0.003**
767.1-549.8	TAG(44:1)	**0.54**	**0.03**	-0.07	0.78	**0.49**	**0.05**
774.5-184.2	PC(36:8)/PE(38:1)	0.34	0.19	0.11	0.68	**0.53**	**0.03**
775.8-184.2	PG(36:2)/PI(30:4)	0.11	0.67	-0.02	0.93	**0.65**	**0.006**
792.5-521.2	TAG(46:2)	-0.45	0.08	0.11	0.69	-0.16	0.53
795.5-550.2	TAG(48:6)/PG(38:6)	0.11	0.69	0.22	0.42	**0.54**	**0.03**
796.1-522.8	Not identified	0.29	0.27	214	0.67	0.15	0.58
796.5-579.2	TAG(46:0)	0.45	0.07	- 0.007	0.97	0.31	0.23
808.6-509.3	PC(38:5)	0.26	0.34	-0.01	0.95	0.25	0.35
818.8-521.8	TAG(48:3)	**-0.62**	**0.01**	0.26	0.32	-0.23	0.41

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579

Figure 4.

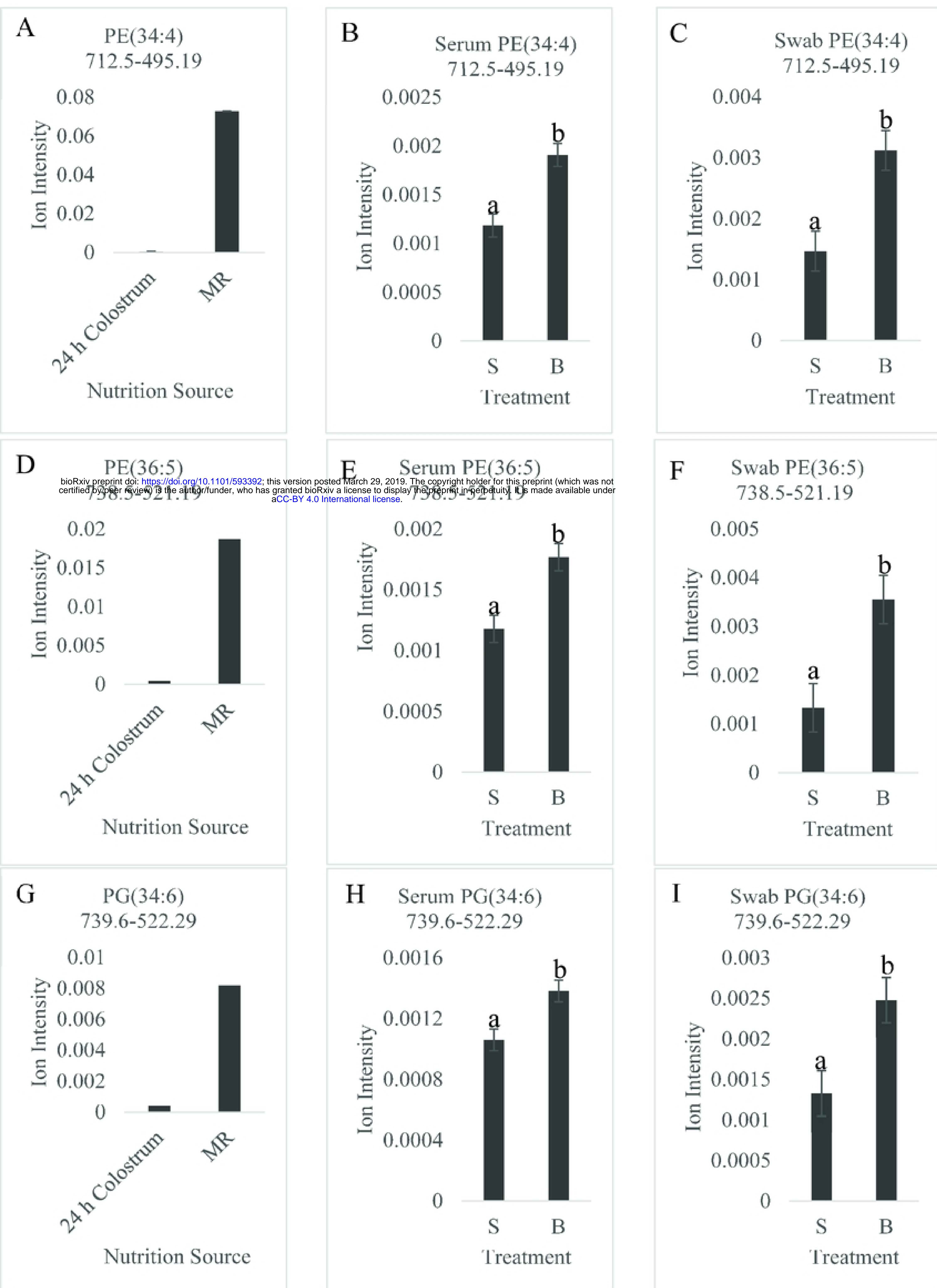

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Figure 4

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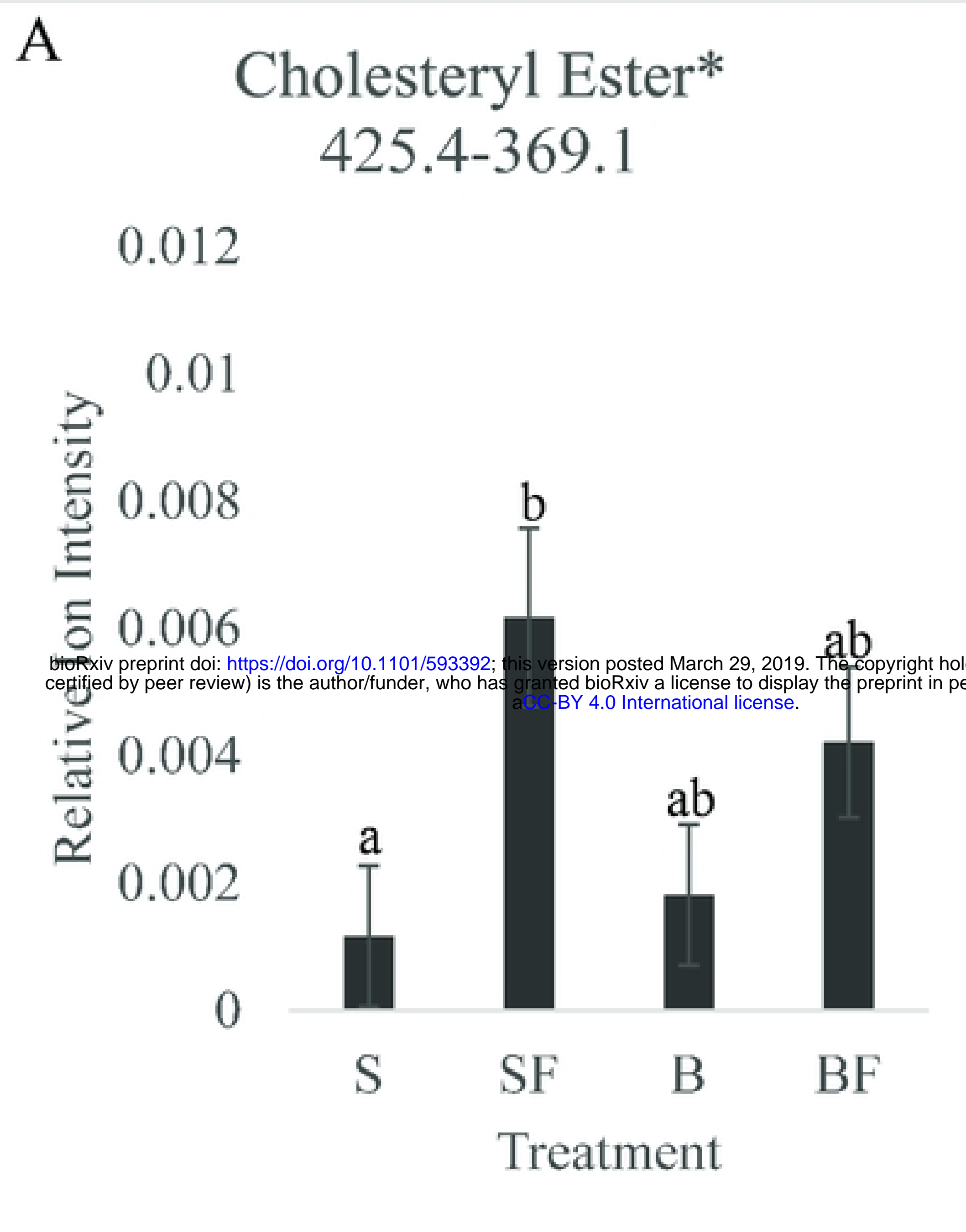

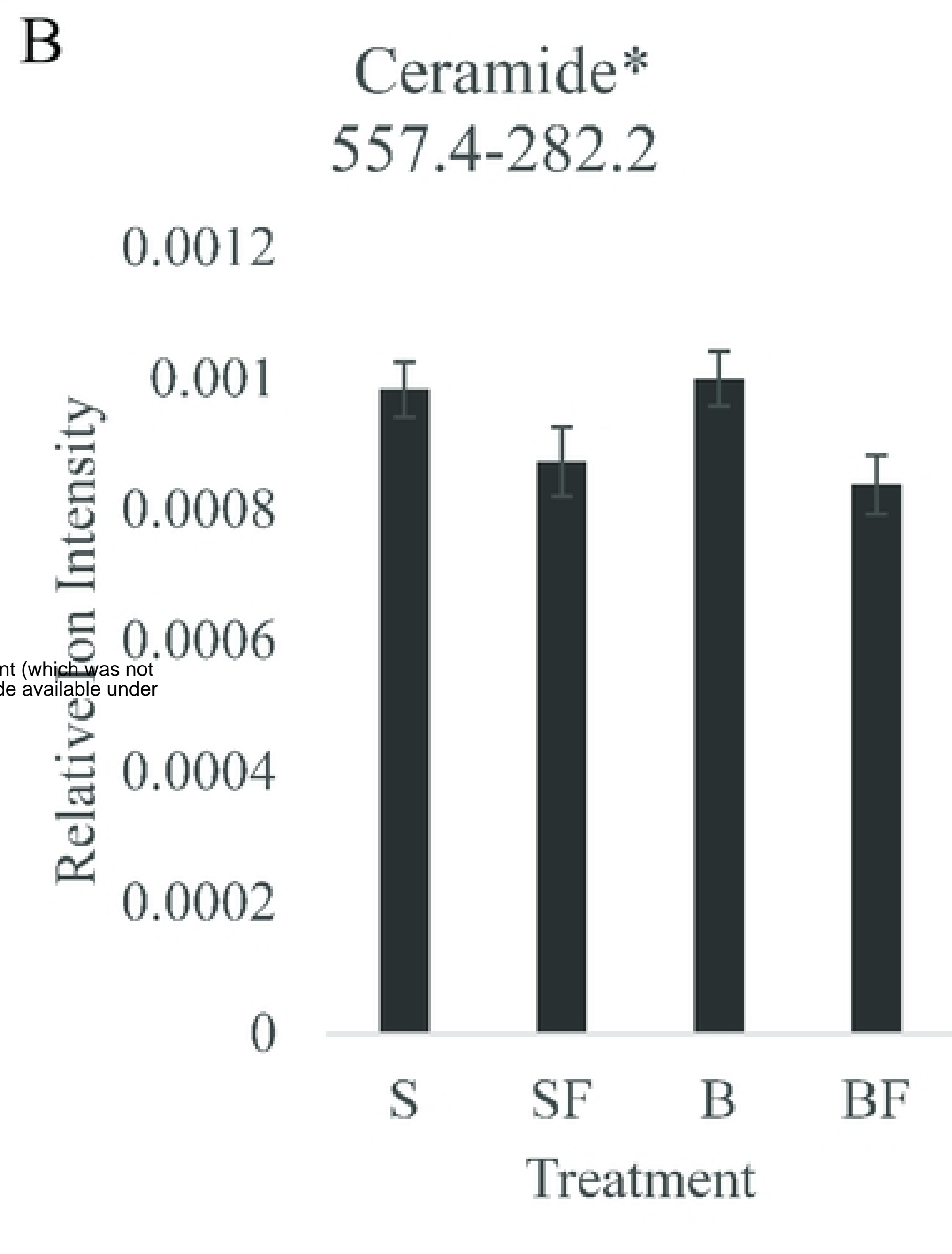

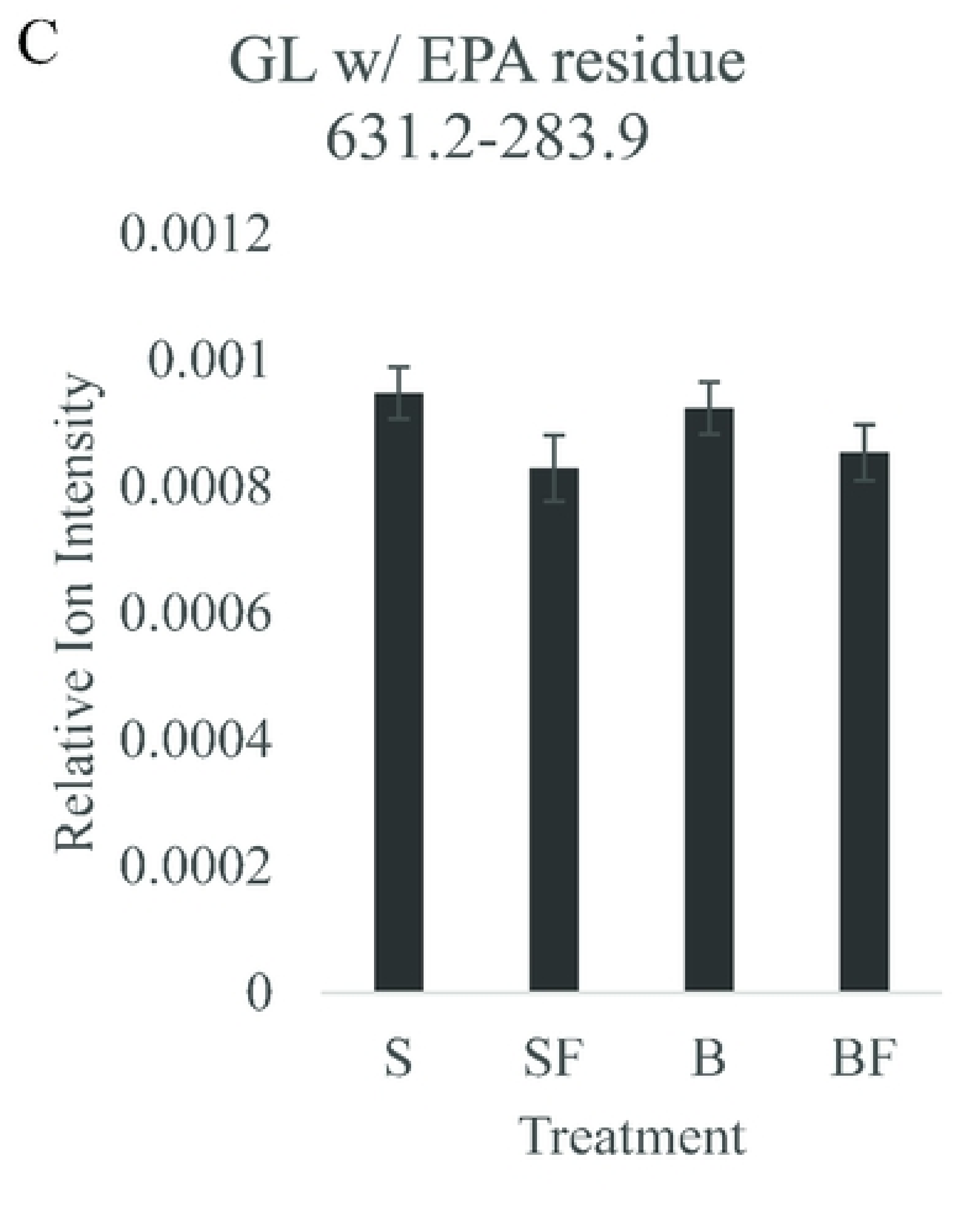

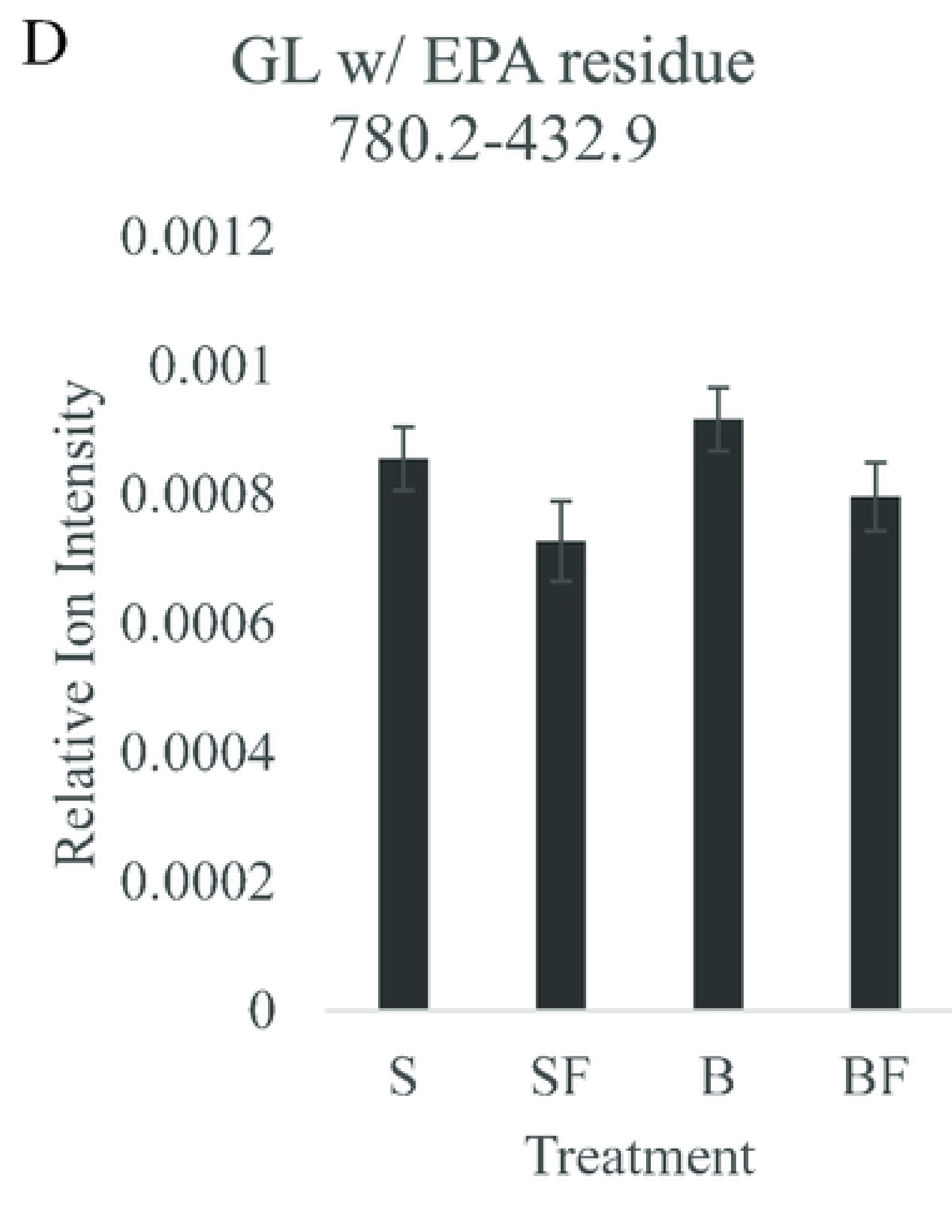

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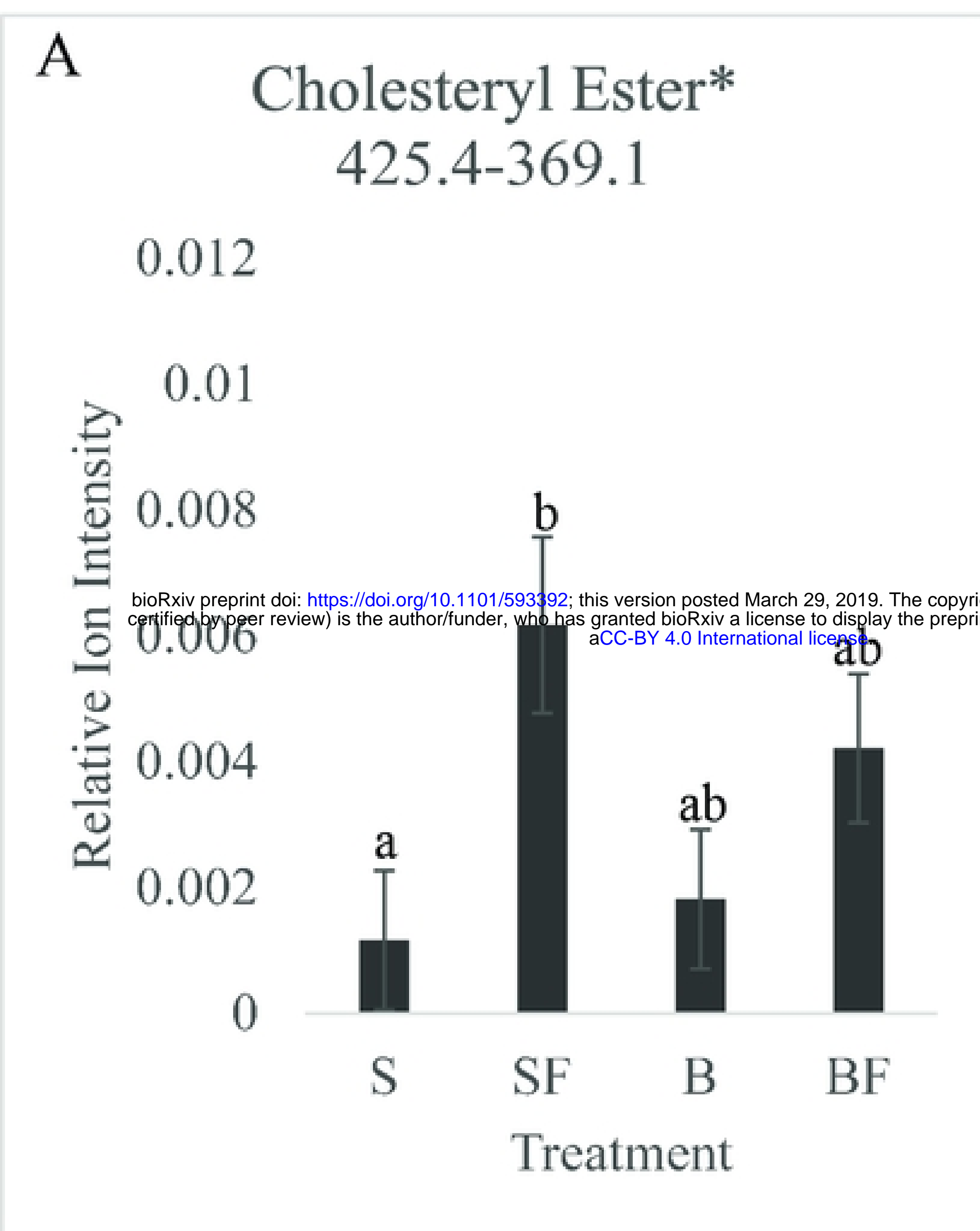
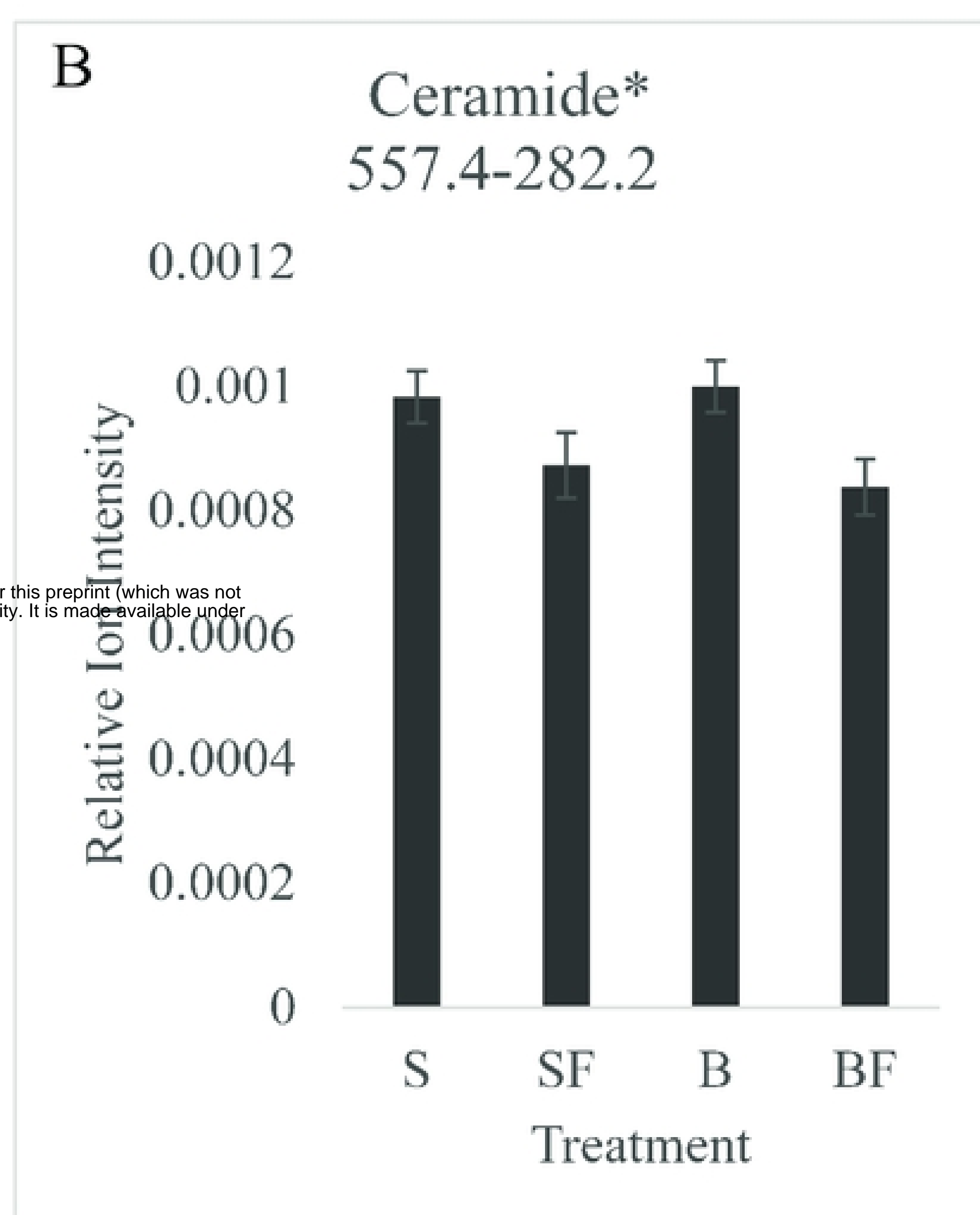
Figure 3.





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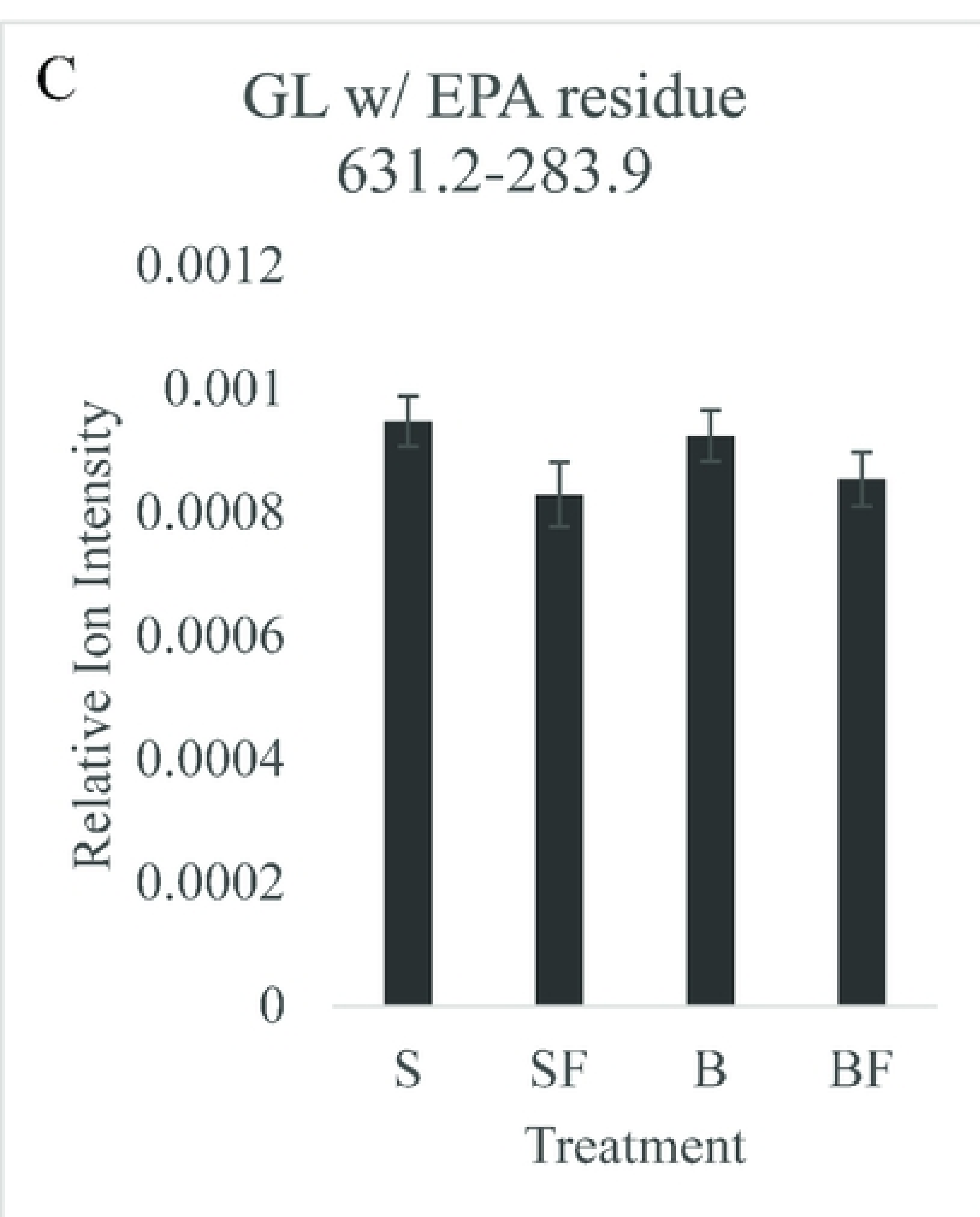

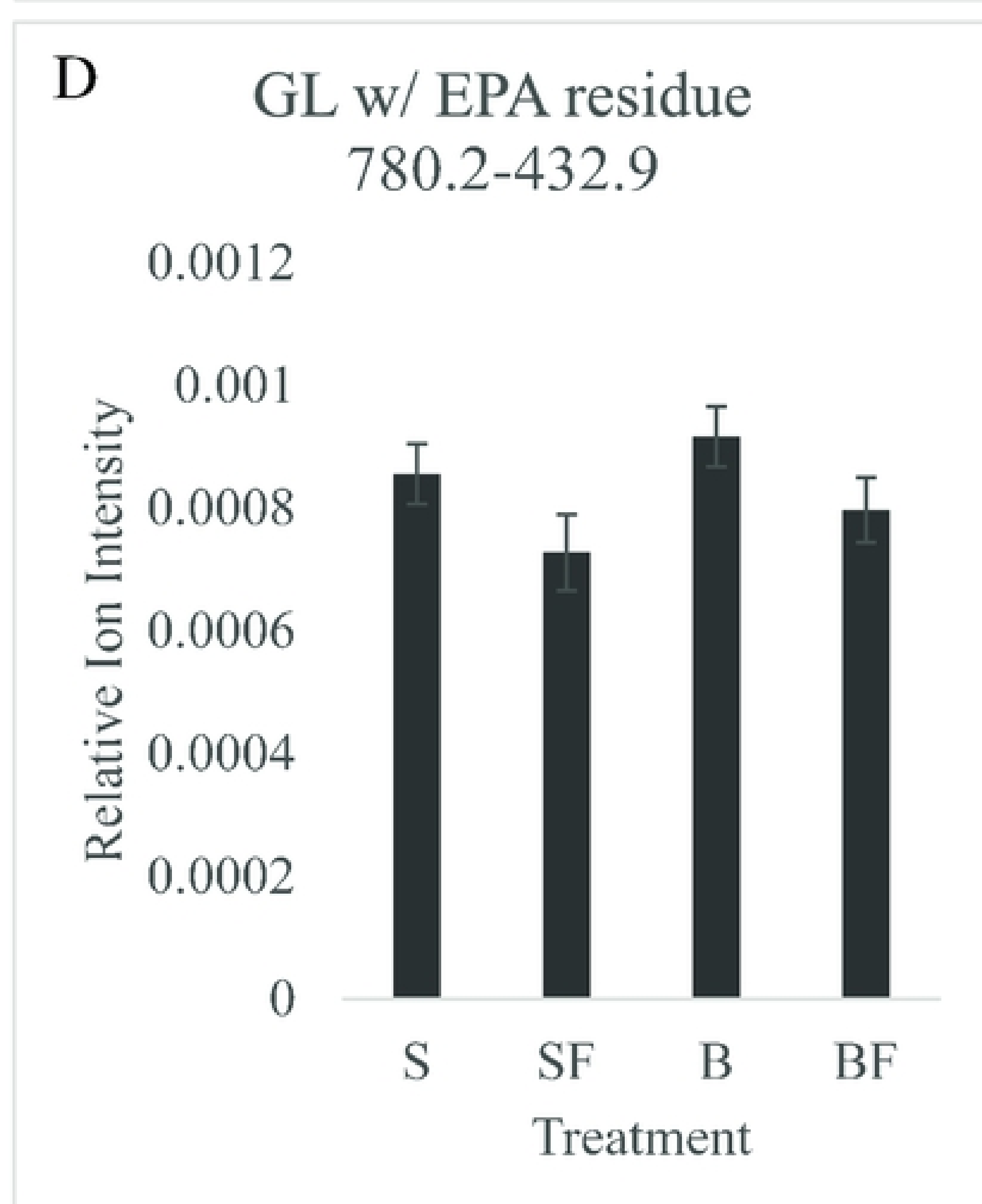

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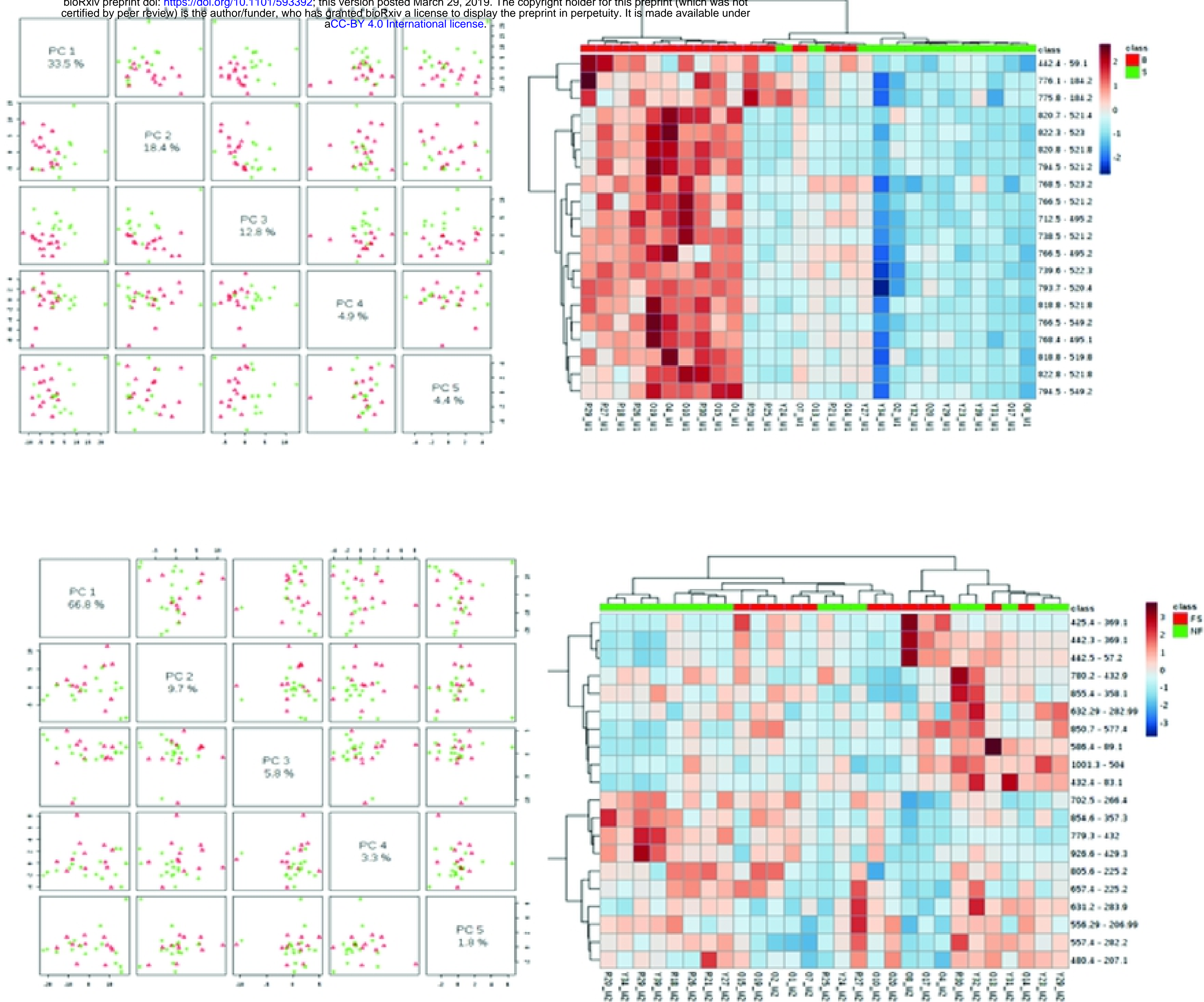
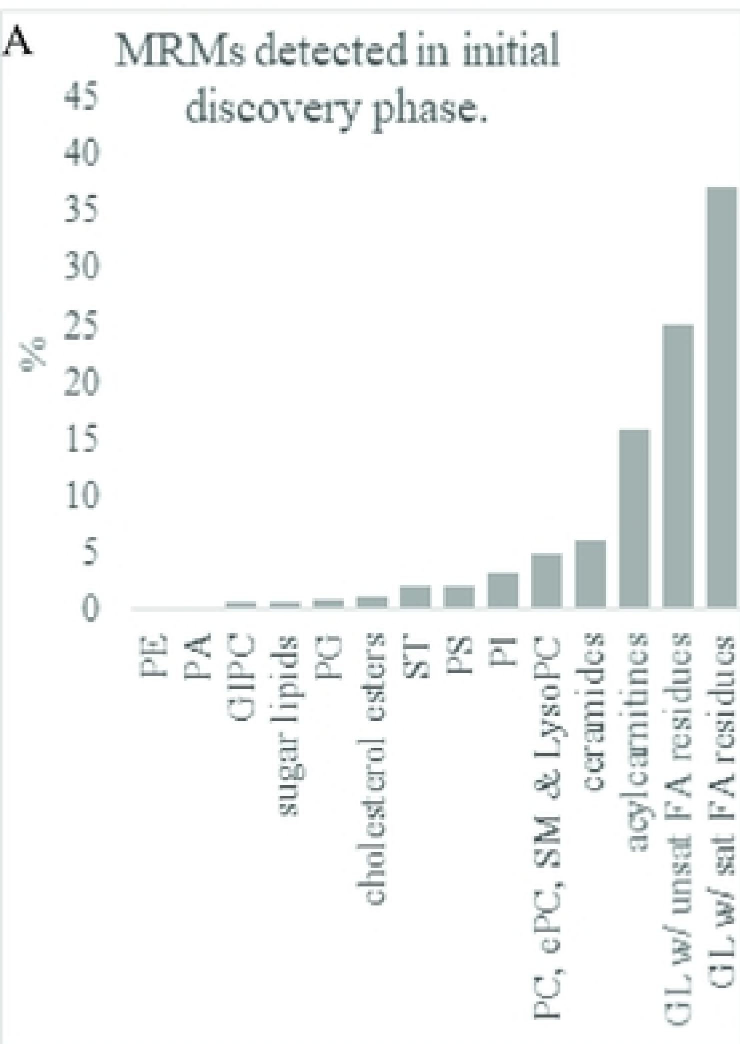
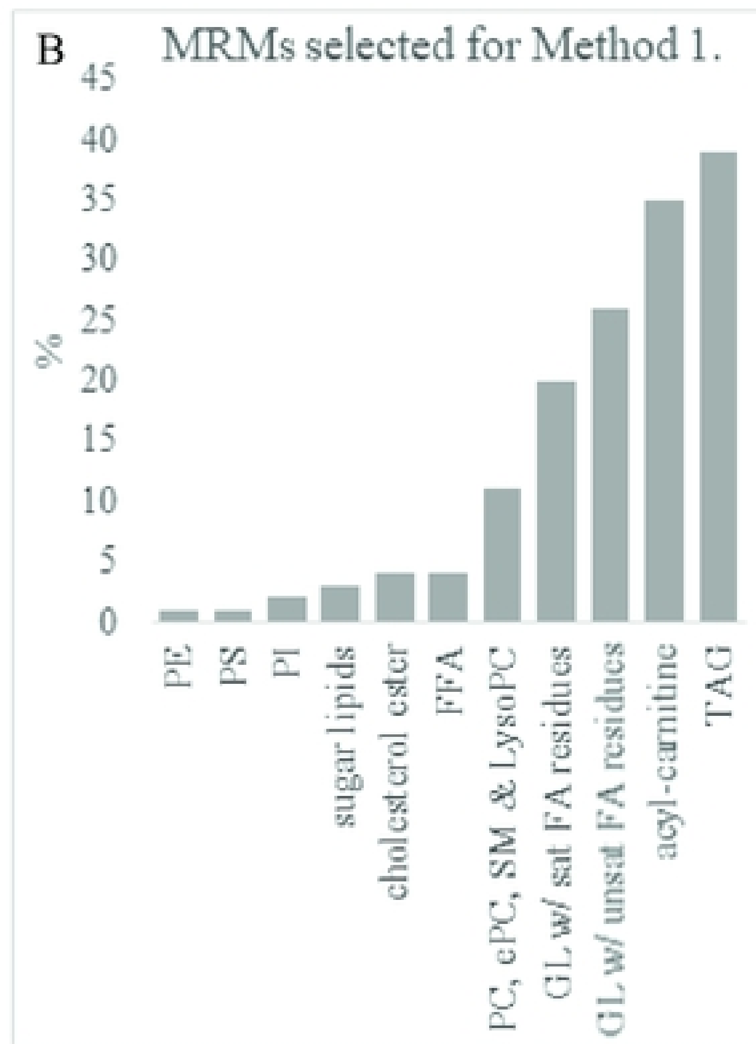


Figure 2





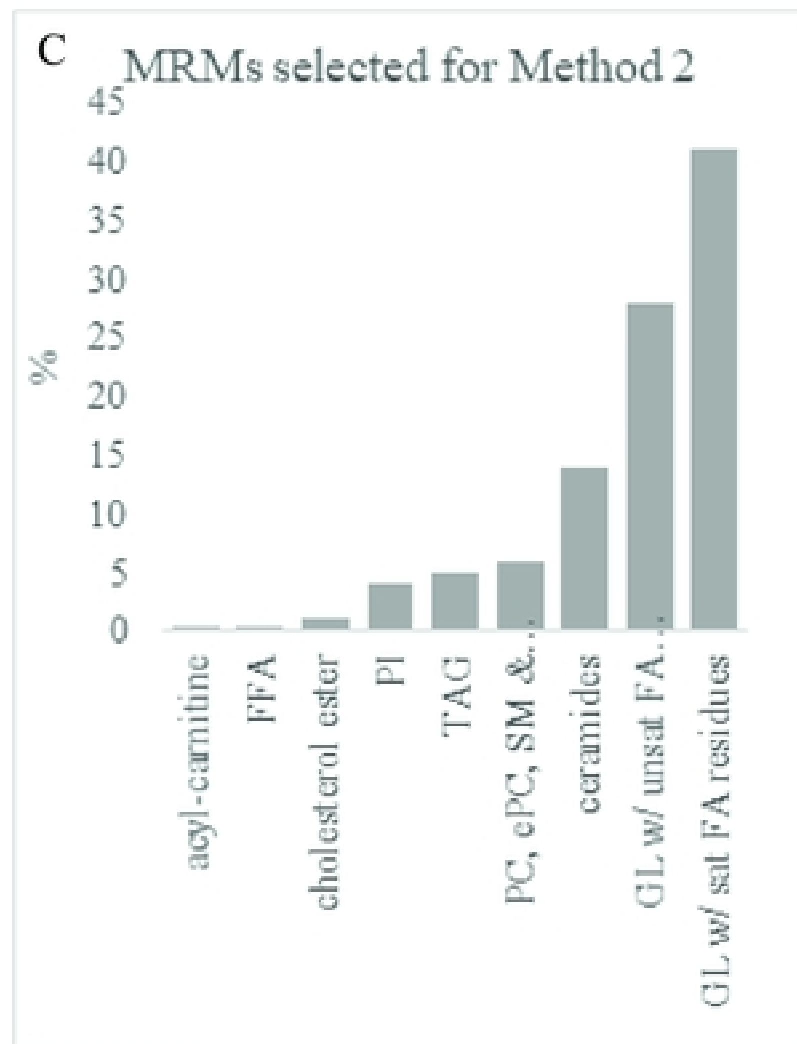


Figure 1