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Randomized controlled-feeding study of dietary emulsifier carboxymethylcellulose reveals detrimental impacts on the gut microbiota and metabolome

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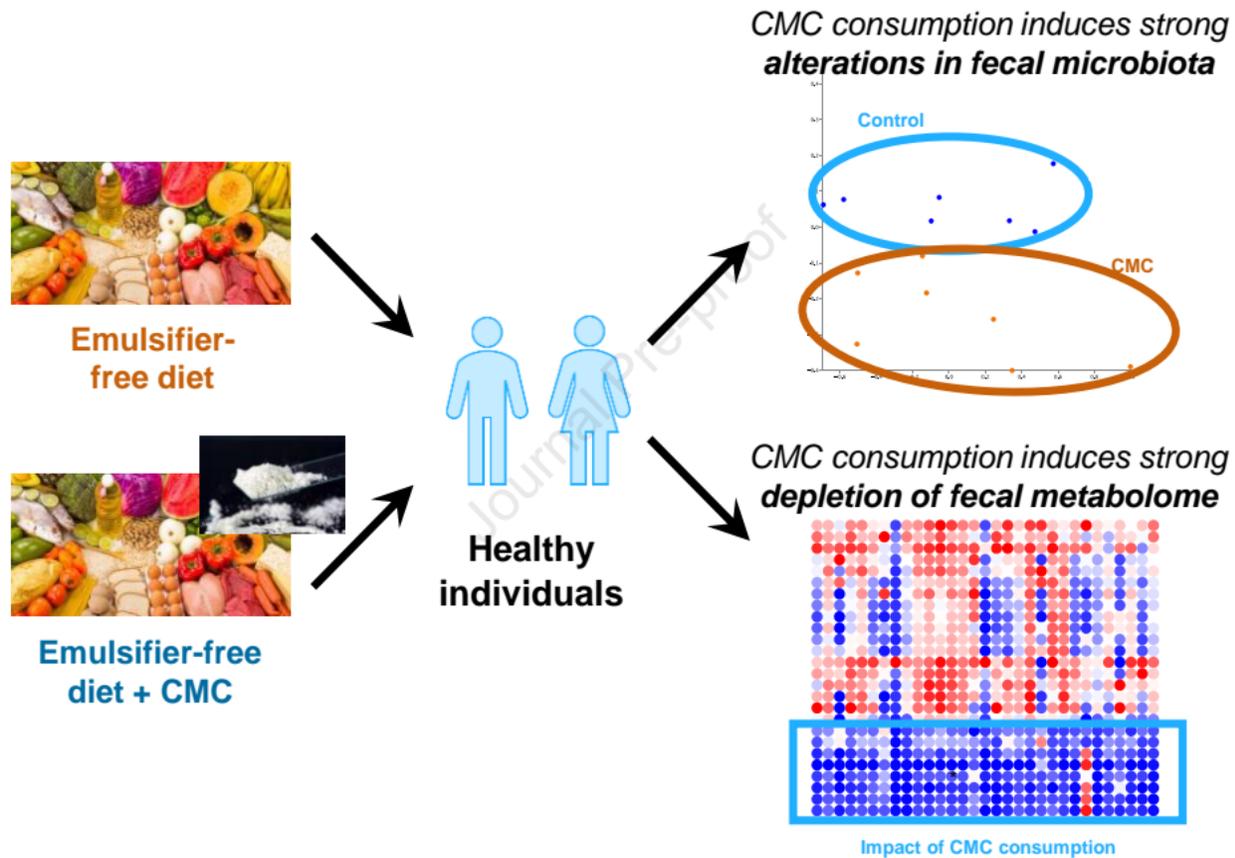
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*CMC consumption induces strong **alterations in fecal microbiota***

Control

CMC

*CMC consumption induces strong **depletion of fecal metabolome***

Impact of CMC consumption

Emulsifier-free diet

Emulsifier-free diet + CMC

Healthy individuals

Gastroenterology

1 **Randomized controlled-feeding study of dietary emulsifier carboxymethylcellulose reveals**

2 **detrimental impacts on the gut microbiota and metabolome**

3

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49   **Abstract**

50   **Background & Aims**: Epidemiologic and murine studies suggest that dietary emulsifiers

51   promote development of diseases associated with microbiota dysbiosis. While the detrimental

52   impact of these compounds on the intestinal microbiota and intestinal health have been

53   demonstrated in animal and *in vitro* models, impact of these food additives in healthy humans

54   remains poorly characterized.

55   **Methods**: To examine this notion in humans, we performed a double-blind controlled-

56   feeding study of the ubiquitous synthetic emulsifier carboxymethylcellulose (CMC) in which

57   healthy adults consumed only emulsifier-free diets (n=9) or an identical diet enriched with 15

58   grams per day of CMC (n=7) for 11 days.

59   **Results**: Relative to control subjects, CMC consumption modestly increased postprandial

60   abdominal discomfort and perturbed gut microbiota composition in a way that reduced its

61   diversity. Moreover, CMC-fed subjects exhibited changes in the fecal metabolome, particularly

62   reductions in short-chain fatty acids and free amino acids. Furthermore, we identified 2 subjects

63   consuming CMC who exhibited increased microbiota encroachment into the normally sterile inner

64   mucus layer, a central feature of gut inflammation, as well as stark alterations in microbiota

65   composition.

66   **Conclusions**: These results support the notion that the broad use of CMC in processed

67   foods may be contributing to increased prevalence of an array of chronic inflammatory diseases

68   by altering the gut microbiome and metabolome.

69

70   **Keywords:** Emulsifier, Metabolism, Microbiota, Metabolome.

71   **Introduction**

72   Consumption of highly processed foods has increased dramatically since the mid-20th

73   century and is associated with increased incidence of several chronic inflammatory diseases.

74   Among these are inflammatory bowel disease [1] and metabolic syndrome [2], both of which are

75   associated with, and thought to be promoted by, alterations in gut microbiota [3-5]. A common feature

76   of highly processed foods is the use of one or more emulsifiers or thickeners (referred hereafter as

77   emulsifiers), which are added to enhance texture and extend shelf-life. Some of the emulsifiers

78   that are commonly added to foods, such as lecithin, are a natural component of unprocessed foods,

79   while others, such as carboxymethylcellulose (CMC), are synthetic. Despite lack of extensive

80   safety testing, CMC was approved in the 1960s for use in foods at concentrations up to 2% (wt/wt)

81   by regulatory agencies, including the United States Food and Drug Administration and European

82   Commission based on the GRAS (generally regarded as safe) designation developed by these

83   agencies. Part of the basis for presuming that CMC, and some other emulsifiers, are safe is that

84   they are not well absorbed and thus mostly eliminated in feces. However, such passage through

85   the intestine allows these products to directly interact with gut microbiota and the intestinal

86   mucosa. For example, CMC has been shown to impact gut transit time [6] and alter fecal bile acid

87   profiles [7]. More recent studies show that CMC impacts human microbiota composition and gene

88   expression *in vitro,* and in mice, wherein its impacts on gut microbiota promote the development

89   of colitis or metabolic syndrome [8-12]. These findings compelled us to investigate the extent to which

90   CMC impacts intestinal-microbiota interactions in humans.

91   Examination of how an individual food component impacts human microbiota is

92   complicated by inter-individual heterogeneity in factors such as quantity of the food consumed,

93   background diet quality and composition, and gut microbiota composition. To minimize the

94   potential confounding impact of these factors, we performed an in-patient (domiciled) study that

95   assured protocol adherence, identical background diets, and enabled daily monitoring and

96   specimen collections before, during, and after CMC consumption, or lack thereof.

97   **Methods**

98

99   *Study design*

100   *General information:* This randomized, controlled-feeding study took place in the

101   University of Pennsylvania's Center for Human Phenomic Science (CHPS) and was registered at

102   https://clinicaltrials.gov as trial no. NCT03440229. The first 3 days of the study were as an

103   outpatient followed by 11 days as an inpatient, as presented **figure 1A**. Once admitted to the CHPS

104   unit, participants were not allowed to leave the unit unless accompanied by study staff. The study

105   included 16 healthy volunteers between the ages of 18 and 60 years.

106

107   *Study endpoint and objectives:* There were no pre-specified efficacy or safety endpoints

108   for this study. The objectives were to 1) establish a tractable and physiologic means of measuring

109   CMC consumption and its metabolic impact in healthy volunteers; 2) examine the extent to which

110   CMC consumption impacts human gut microbiota composition, gene expression, and/or

111   localization; and 3) explore effects of CMC consumption on a range of inflammatory and

112   metabolic parameters that characterize metabolic syndrome. These included concentration of

113   lipocalin in feces and IFN-γ, IL-17, IL-8 and, IP-10 in serum. Additionally, insulin sensitivity was

114   assessed with a 2.5 hour oral glucose tolerance testing performed after an overnight fast on

115   inpatient days 1 and 11. Insulin sensitivity was measured as change in insulin divided by change

116   in glucose from time 0 to 30 minutes.

117

118   *Sample size calculation:* Power calculation was based on measure of bacterial-epithelial

119   distance, which provides a quantitative parameter whose diminution is associated with disease

120   (colitis and metabolic syndrome) in both mice and humans [12, 13]. Specifically, the difference in

121   mean distance of the nearest bacteria to the epithelium between patients with and without diabetes

122   was 19.13 μm. The within group standard deviation (SD) for patients without diabetes was

123   7.17 μm. The within group SD for those with diabetes was even smaller. With a sample size of 8

124   subjects per group and assuming a within group SD of 7.17 μm, we projected to have 90% and

125   80% power to detect a difference in the distance of the nearest bacteria to the epithelium between

126   the treatment groups (CMC vs. no CMC) that is 35% and 44% smaller than the difference observed

127   between patients with and without diabetes, respectively.

128

129   *Changes to methods after trial commencement:* As fully detailed **Table S1**, the study

130   design was modified in order to improve participant recruitment. More specifically, while the first

131   three participants stayed at CHPS for the washout period, the remaining 13 participants were

132   allowed to go back home with provided in-house cooked food for the washout period. Moreover,

133   while the CMC treatment duration was 14 days for the three first participants, it was 11 days for

134   the remaining 13 participants. Importantly, the only data from days 12, 13 and 14 for the three first

135   participants utilized in the analysis were the mucosal biopsies which were collected on day 14.

136

137   *Recruitment:* Participants were recruited *via* advertising the study on an online system at

138   the University of Pennsylvania from 4/12/2018 to 1/16/2019.

139

140   *Early withdrawal of participants:* No participant was withdrawn from the study.

141

7

142   *Eligibility criteria for participants:* Inclusion criteria were ability to give informed consent

143   and age 18 to 60 years. Exclusion criteria were: diagnosis with IBD, celiac disease, or other chronic

144   intestinal disorders; baseline bowel frequency less than every 2 days or greater than 3 times daily;

145   current smoker; body mass index (BMI) <18.5 or >40 at screening; more than two of the criteria

146   for metabolic syndrome (waist circumference >89 cm for women or 102 cm for men, diagnosis of

147   diabetes mellitus or baseline HbA1c > 6.4% or a fasting glucose level of greater than 100mg/dL;

148   systolic blood pressure >130 mmHg or diastolic blood pressure >85 mmHg or treated with

149   medications for hypertension; fasting triglycerides >149 mg/dl or treated with medications for

150   hypertriglyceridemia; fasting HDL cholesterol <40 mg/dl in men or <50 mg/dl in women or treated

151   with medications for hypercholesterolemia); known substance abuse disorder or consumption of

152   illicit drugs or alcohol in the 24 hours prior to admission; prior bowel resection surgery other than

153   appendectomy; WBC less than 3,500 per µL or an absolute neutrophil count of less than 1,000 per

154   µL; platelet count of less than 100,000 per µL or an INR greater than 1.2; estimated

155   GFR<60ml/min/1.73m2; pregnant or lactating women; use of antibiotics in the 6 months prior to

156   screening; use of laxatives or anti-diarrhea medications in the 2 weeks prior to screening; use of

157   anticholinergic medications, narcotics, antacids, NSAIDs, or dietary supplements in the week prior

158   to screening; HIV infection, AIDS, or other known conditions resulting in immunosuppression;

159   allergies or intolerance to the components of the study diets; following a vegan or vegetarian diet;

160   and experienced diarrhea within the two weeks prior to screening.

161

162   *Blinding:* The study employed concealed allocation with neither the participants nor the

163   research team being aware of the treatment assignment during the screening phase and until all

164   data were collected. The research team remained blinded to treatment assignment until all biopsies

165   had been reviewed to assess for bacteria distance from the epithelium and data were analyzed for

166   this outcome together with the oral glucose tolerance tests and inflammatory markers.

167

168   *Intervention:* All food was prepared within the CHPS metabolic kitchen without

169   emulsifiers (unless specifically added). All participants followed the same Western style diet (the

170   only difference being portion size). The macronutrient percentages of calories for the study diet

171   were 55% carbohydrate, 30% fat, and 15% protein. The diet provided is considered healthy with

172   a Healthy Eating Index (HEI) score of 75 [14-16]. The diet was composed of two menus that were

173   consumed on alternating days. Water, black coffee, and plain tea were provided as desired.

174   Participants had access to additional servings of food beyond the meals provided. However, the

175   entire serving of the previous meal must have been consumed to receive additional servings.

176   For the three days prior to admission, participants ate an emulsifier free diet at home with

177   food provided by the CHPS metabolic kitchen. After admission to CHPS, all participants

178   consumed the same emulsifier-free diet until dinner on the first day of the inpatient stay. Thus, all

179   participants had approximately 80 hours of emulsifier free washout time prior to administration of

180   the food containing CMC (source: Modernist Pantry) or matched CMC-free food.

181   Participants were randomly assigned to receive 0 or 15 gm per day of CMC (9 and 7

182   participants in each arm of the study) using concealed allocation by Dr. Hongzhe Li. Because of

183   the small sample size, we used block randomization with a block size of 4 participants. Beginning

184   with the dinner meal on inpatient Day 4, all participants consumed three servings of brownie and

185   three servings of sorbet per day, each containing 0 or 2.5 gm CMC per serving. The brownie and

186   sorbet servings were provided at three scheduled meals and three scheduled snacks. Prior to eating

187   any other food on the study menu, participants were required to consume the brownie and sorbet

188   servings. Neither the participant nor the investigators were aware of which diet participants were

189   assigned until the analyses of metabolic parameters, inflammatory parameters, microbiome

190   composition and bacteria-mucosa distance assessment had been performed.

191   Physical activity was monitored during the 3-days prior to admission to CHPS through the

192   use of a FitBit Flex. During the inpatient portion of the study, participants were required to attain

193   within 10% of the average number of daily steps that they took in the 3 days prior to admission.

194

195   *Sample collection*: Urine was collected prior to starting the outpatient study diet and each

196   morning of the inpatient stay after an overnight fast and aliquoted and frozen at minus 80°C. Blood

197   was collected after an overnight fast prior to breakfast at the screening visit, at a post-screening

198   visit prior to admission, and on Days 1-4, 8, 10, and 11 of the inpatient study, and 1 month after

199   discharge. Plasma was separated from the blood samples and stored frozen at minus 80°C for use

200   in metabolomic studies. Stool samples were collected without preservatives or stabilizers prior to

201   starting the outpatient diet, daily during the inpatient stay, and at 1 and 3 months after discharge.

202   The first stool sample of the day was aliquoted and frozen at minus 80°C. All other stool samples

203   during the inpatient stay were weighed and then discarded. On inpatient days 1 and 11 (or 14 for

204   the first 3 participants), each participant underwent a sigmoidoscopy to obtain biopsies from the

205   area of approximately 15 cm from the anal verge, which correlates with approximately the

206   rectosigmoid junction. No bowel preparation was utilized prior to the sigmoidoscopy. Biopsy

207   samples were placed in Carnoy solution for non-denaturing confocal microscopy.

208

209   *Additional data collection:* We collected information on the participant's usual diet

210   utilizing the Diet History Questionnaire II (DHQ II), a food frequency questionnaire developed by

211   the National Cancer Institute. On inpatient days 2, 3, 5, 6, 9, and 10, following lunch, participants

212   completed a standard food satiety questionnaire utilizing a 150mm visual analog scale to measure

213   satiety and hunger as per Doucet [17, 18]. Diet quality was assessed using the Healthy Eating Index

214   2015 [14]. On days 1 and 11, participants completed the PROMIS scales for belly pain (version 1.0

215   – 5a) and gas/bloating (version 1.0 – 13a).

216

217   *Measurements of circulating metabolic parameters and cytokines*

218   Serum cytokines were assayed using the Luminex™ 100 Multi-analyte System by

219   University of Maryland's Cytokine Core Laboratory.

220

221   *Serum lipopolysaccharide- and flagellin-specific immunoglobulins.*

222   *Cf.* supplemental methods section.

223

224   *Microbiota analysis by 16S rRNA gene sequencing using Illumina technology*

225   *Cf.* supplemental methods section.

226

227   *16S rRNA gene sequence analysis*

228   *Cf.* supplemental methods section.

229

230   *Microbiota analysis by shotgun sequencing using Illumina technology*

231   *Cf.* supplemental methods section.

232

233

234 *Bacterial density quantification by 16S rRNA qPCR*

235   *Cf.* supplemental methods section.

236

237 *Quantification of fecal lipocalin-2 (Lcn-2) by ELISA*

238   For quantification of fecal Lcn-2 by ELISA, frozen fecal samples were reconstituted in

239 PBS containing 0.1% Tween 20 to a final concentration of 100 mg/mL and vortexed for 20 min to

240 get a homogenous fecal suspension [19]. These samples were then centrifuged for 10 min at 14 000

241 g and 4°C. Clear supernatants were collected and stored at −20°C until analysis. Lcn-2 levels were

242 estimated in the supernatants using Duoset Human Lcn-2 ELISA kit (R&D Systems, Minneapolis,

243 MN, USA) using the colorimetric peroxidase substrate tetramethylbenzidine, and optical density

244 (OD) was read at 450 nm (Versamax microplate reader).

245

246 *Fecal flagellin and lipopolysaccharide load quantification*

247   *Cf.* supplemental methods section.

248

249 *Immunostaining of mucins and localization of bacteria by FISH*

250   *Cf.* supplemental methods section.

251

252 *Metabolomic analysis of stool and urine samples*

253   Stool and urine sample preparation for NMR were performed as previously described [20].

254 ^{1}H NMR spectra were acquired on a Bruker Avance NEO 600 MHz spectrometer equipped with

255 an inverse cryogenic probe (Bruker Biospin, Germany) at 298 K. A typical 1D NMR spectrum

256 named NOESYPR1D was acquired for each sample. The metabolites were assigned on the basis

257 of published results [21]and confirmed with a series of 2D NMR spectra. All ^{1}H NMR spectra were

258 adjusted for phase and baseline using Chenomx (Chenomx Inc, Canada). The chemical shift of ^{1}H

259 NMR spectra were referenced to sodium 3-trimethylsilyl [2,2,3,3-d4] propionate (TSP) at δ 0.00.

260 **Table S2** is listing all the quantitated metabolites and their characteristics (Moieties, δ ^{1}H (ppm)

261 and δ ^{13}C (ppm). The relative contents of metabolites were calculated by normalizing to the total

262 sum of the spectral integrals. The quantification of metabolites, including CMC, in stool was

263 calculated by NMR peak area against trimethylsilylpropanoic acid using Chenomx. The lower

264 limit of CMC detection using the NMR approach is about >1μM for pure CMC and 1-10 μM for

265 CMC in stool and urine samples. For CMC absolute quantification, five concentrations were used

266 in triplicates, with a lower limit of detection of 0.5 mg/ml, as presented Figure S12.

267

268 *AccQ•Tag Amino Acid Analysis of Stool Samples*

269 Amino acids were extracted from stool samples with 1 mL of ice-cold methanol/water (2:1)

270 solution (contain 2.5 uM of Norvaline), followed by homogenization (Precellys, Bertin

271 Technologies, Rockville, MD) with 1.0-mm-diameter zirconia/silica beads (BioSpec, Bartlesville,

272 OK), three freeze–thaw cycles and centrifugation (Eppendorf, Hamburg, Germany). Supernatant

273 was collected, evaporated to dryness (Thermo Scientific, Waltham, MA) and then resuspend in 50

274 uL 0.1N HCl solution. Amino acid derivation with AccQ•Tag reagents (Waters, Milford, MA) was

275 conducted according to the manufacturer's protocol. Briefly, 10 μL of stool extract were mixed

276 with 70 μL of AccQ•Tag Ultra borate buffer and 20 μL of AccQ•Tag Ultra reagent in Total

277 Recovery Vial. The vials were capped and vortex for several seconds and proceed for 10 min at

278 55 °C. Amino Acid were detected by Waters Xevo TQS coupled with PDA, an AccQTag Ultra

279 Column (C18 1.7 um 2.1 x 100 mm) with in-line filter (Waters, Milford, MA) were used for

280 separation [22]. Results were quantified by comparing integrated peak areas against a standard curve.

281

Statistical analysis

283   Significance was determined using *t*-tests, Mann-Whitney test, one-way ANOVA

284 corrected for multiple comparisons with a Bonferroni post-test, two-way ANOVA corrected for

285 multiple comparisons with a Bonferroni post-test (or mixed-effect analysis when some values were

286 missing), or repeated *t*-tests corrected with the false discovery rate approach where appropriate

287 (GraphPad Prism software, version 6.01). Differences were noted as significant at $P \leq 0.05$.

288   **Results**

289   We enrolled 16 subjects, deemed healthy based on lack of disease history or current

290   evidence of metabolic syndrome (see methods), who were randomly assigned with concealed

291   allocation to the CMC-containing (n=7) or control (n=9) diets with both investigators and subjects

292   blinded to assignments (**Figure 1A and Table S1**). The groups were similar in terms of age,

293   gender, body mass index, and blood pressure (**Figure 1B**). At the time of screening, subjects in

294   both groups were consuming similar diets as indicated using principal coordinate analysis (PCoA)

295   to visualize the varied food recall responses provided by subjects upon study enrollment. (**Figure**

296   **S1**). On study days 4-14, all subjects consumed 3 servings of brownies and 3 servings of sorbet

297   that lacked or contained 2.5 g CMC per serving. Both groups of subjects exhibited reductions in

298   body weight of about 1 kg and had modest improvements in glycemic control over the course of

299   study, the extent of which did not vary significantly between the 2 groups except that a modest

300   decrease in serum insulin levels was seen in the CMC-fed group (**Figure 1C-D**). CMC

301   consumption was not associated with severe adverse events or alterations in serum levels of

302   inflammatory cytokines, nor did it have an appreciable impact on appetite, food consumption, or

303   bloating (**Figure 1E and S2**). Moreover, levels of anti-lipopolysaccharide and anti-flagellin IgG

304   antibodies, which have been used as an indirect measure of gut permeability [23, 24], did not change

305   over the course of the study in control or CMC-fed subjects (**Figure S3**). CMC consumption did

306   associate with a modestly significant increase in postprandial abdominal pain (**Figure 1F,** $P =$

307   .019).

308

309

310

311   *Microbiota composition*

312   Microbiota composition of daily-collected fecal specimens was characterized by 16S

313   rRNA gene sequencing. In accord with previous studies[25], PCoA of the pairwise distances

314   (unweighted UniFrac) between samples revealed strong clustering within subjects, indicating that

315   extent of inter-individual variations in gut microbiota composition exceeds impacts of short-term

316   alterations in diet (**Figure 2A,** Permanova P = .001). Consequently, as a means of focusing on the

317   potential impact of CMC on each individual subject, we used samples collected the morning of

318   day 4, the day on which the subjects began consuming CMC in the study, to normalize all

319   microbiota composition data. This approach revealed that subjects fed CMC displayed greater

320   changes in microbiota composition during the intervention period, resulting in PCoA plots

321   showing clear treatment-based clustering after 10 days of CMC consumption (**Figure 2B**,

322   Permanova Day 0 P = .928, Day 9 P = .228, Day 14 P = .002). Moreover, analysis of BrayCurtis

323   distance changes from the morning of day 4 revealed a trend toward greater microbiota alterations

324   during the intervention period in the CMC group compared to the control group (**Figure 2C**, P =

325   .102). These relative shifts in microbiota composition occurred without significant alterations in

326   daily fecal weight (**Figure S4)** or fecal bacterial density between the control and CMC groups

327   (**Figure 2D**, diet effect P = .503). Phyla and order level analysis did not reveal significant

328   differences in the CMC and control groups during the intervention period, **Figure S5**).

329   Investigation of the most significantly differentially abundant sequence variants (SVs) between

330   CMC and control groups revealed SVs that were generally stably represented in control subjects

331   on day 14 compared to day 4, with relative values being very close to 1 (**Figure S6**), while the

332   relative abundance of these SVs were markedly impacted by CMC consumption, including

333   decreases in *Faecalibacterium prausnitzii* and *Ruminococcus sp.*, and increases in *Roseburia sp.*

16

334   and Lachnospiraceae (**Figure S6).** While it is difficult to reliably ascribe functional consequences

335   to these alterations, we note that CMC consumption induced loss of *F. prausnitzii*, which is

336   associated with health and known to mediate production of beneficial metabolites such as short-

337   chain fatty acids [26-28].

338   CMC also reduced microbiota richness, which is a hallmark of various diseases states [29],

339   as revealed by decrease in the evenness (**Figure 2E**, diet effect P = .070, with P = .059 at day 9

340   and P = .032 at day 14) and Shannon indices (diet effect P = .151 with P = .091 at day 14). To

341   further investigate impacts of CMC on microbiota composition, we next performed shotgun

342   metagenomic sequencing on fecal samples collected shortly before or after 10 days of CMC

343   consumption (day 4 and 14, respectively). Quality filtered reads were assigned to taxa and

344   function. Use of PCoA analysis of the Bray-Curtis distances to compare all of the samples (*i.e.*,

345   pre- and post-CMC) showed within subject clustering both taxonomically and functionally

346   (**Figures 3A and S7A**), reflecting patterns observed using 16S rRNA gene sequence data.

347   Nonetheless, there was clear post-treatment clustering of samples from control and CMC-fed

348   subjects based on taxonomic (**Figures S7B-C**), and, especially, function-based analysis (**Figures**

349   **3B-C**, PCoA analysis of the Bray-Curtis distances, *cf.* method section for details). The significantly

350   altered functional categories that drove such clustering, identified via Maaslin2, comprised a

351   variety of microbial metabolic pathways, suggesting that CMC-induced alteration in microbiota

352   composition might have broad impacts on microbiota function (**Fig 3D).**

353

354   ***Changes in fecal metabolome***

355   To investigate the functional consequences of CMC's impacts on microbiota, we first

356   measured fecal levels of molecules known to mediate host-microbiota interactions. Use of TLR4

17

357   and TLR5 reporter cells revealed, respectively, that fecal levels of lipopolysaccharide and flagellin

358   were not impacted by CMC consumption (**Figure 4A, B**, mixed-effects analysis with Bonferroni

359   multiple comparisons tests, diet effect $P = .413$ for 4A and $P = .220$ for 4B, Bonferroni corrected

360    $P > .1$ for all days). There was no significant change in levels of fecal lipocalin-2, an inflammatory

361   marker (**Figure 4C,** mixed-effects analysis with Bonferroni multiple comparisons tests, diet effect

362    $P = .258$, Bonferroni corrected $P > .1$ for all days). Next, we sought to broadly examine the extent

363   to which CMC altered the fecal metabolome, which is both shaped by gut microbiota and mediates

364   many of its impacts on the host. We used a ^{1}H NMR-based targeted assay capable of quantitating

365   about 40 metabolites that are reliably detected in stools of a healthy person, many of which can be

366   influenced by the gut microbiota. In accord with the notion that, in general, there is far less inter-

367   person heterogeneity in microbiota metabolic function than in species composition [30], we

368   compared fecal metabolomes between control and CMC-fed subjects, without normalization to

369   correct for basal variation amongst subjects. Accordingly, prior to CMC consumption (day 4), no

370   significant clustering by study group was evident for the fecal metabolome (**Figure 4D**,

371   Permanova Day 0 $P = .573$). In contrast, following CMC consumption, this approach showed a

372   clear ability to distinguish fecal metabolomes of control versus CMC-fed subjects (**Figure 4D**,

373   Permanova Day 9 $P = .001$, Day 14 $P = .001$). Concomitantly, display of individual values of each

374   metabolite for each subject on day 14 (**Figure S8**), as well as viewing mean values for each group

375   over time *via* a heat-map (**Figure 4E**), demonstrated that fecal metabolomes of CMC-fed subjects

376   were, on average, depleted in an array of microbiota-related metabolites, including short-chain

377   fatty acids and essential amino acids. Such changes were clearly evident by 3 days after initiating

378   CMC consumption and remained throughout the period of CMC consumption and had resolved

379   when subjects were re-sampled about 1 month later (day 48) (**Figure 4E**). Moreover, NMR-based

18

380   detection of fecal amino acids concentration demonstrated a decrease in the fecal amounts of

381   numerous amino acids, as presented **figure S9**. The depletion of metabolites in feces of CMC

382   subjects occurred despite lack of significant difference in fecal bacterial density (**Figure 2D**,

383   adjusted P = .503) or change in total stool mass produced per subject (**Figure S4,** within group

384   change in stool weight P = .903 for control group and P = .990 for CMC group), arguing against

385   it reflecting loss of bacteria or stool dilution. Nor did CMC directly inhibit NMR-based detection

386   of amino acids (**Figure S10**), indicating that the reductions these metabolites did not reflect a

387   technical artifact but rather that CMC feeding depleted an array of microbiota-related metabolites.

388

389   *A new assay for CMC quantification*

390   Animal studies using radiolabeled CMC indicate that most of the label is eliminated in

391   feces, suggesting that this compound is poorly absorbed [31]. Hence, we developed a new ^{1}H NMR-

392   based assay which detected copious amounts of seemingly intact CMC in feces of subjects

393   receiving the CMC-containing diet compared to participants consuming the control diet and

394   compared to their usual diet **(Figure 4F and S11)**. While the non-zero levels of CMC measured

395   by this assay may reflect background (*i.e*., another fecal metabolite with spectral properties similar

396   to CMC), the significant decreased level in the participants consuming an additive-free diet (P <

397   .05 for all time points except day 13) and subsequent increase at day 48 and 107 after the study

398   suggests that the readout is capturing CMC contained in processed foods that were consumed

399   before or after participation in our study (**Figure 4G**). In further accord with the notion that CMC

400   is not absorbed, it was undetectable in urine, nor were alterations in the urinary metabolome

401   associated with CMC consumption (**Figure S12**). Thus, our results comport with the notion that

402   CMC is non-absorbed but significantly altered the host-microbiota relationship.

403 *Distance between the intestinal mucosa and the microbiota and identification of CMC-sensitive*

404 *subjects*

405   A characteristic of altered host-microbiota interactions in a range of chronic inflammatory

406 diseases, including IBD, metabolic syndrome, and cancer, is encroachment of gut microbiota into

407 the normally near-sterile inner mucus layer. Hence, we hypothesized that CMC consumption might

408 result in microbiota reduce bacterial-epithelial distance as measured via confocal microscopy in

409 distal colonic biopsies preserved in Carnoy's solution collected before or after the intervention

410 period. On average, bacterial epithelial distance did not change over the course of the study in the

411 control or CMC group. However, 2 individual subjects within the CMC group showed a marked

412 reduction in this parameter, such that their biopsies showed bacteria in very close proximity to the

413 epithelium following CMC exposure (**Figure 5A and S13**), reminiscent of observations made in

414 patients with IBD [32]. Application of Fisher's Exact Test to the observation that 2 of 7 CMC-fed

415 subjects and 0 of 9 control subjects displayed this phenotypic change over the course of the study

416 yielded a 2-tailed *P* value of 0.175, which does not meet common standards of being statistically

417 significant but nonetheless suggests a reasonable likelihood it was a consequence of CMC

418 treatment. Accordingly, we examined if any of the clinical and/or microbiota parameters might

419 give insight into these seemingly CMC-sensitive subjects. Although these subjects did not respond

420 differently in terms of clinical parameters or inflammatory markers, they had significantly greater

421 relative changes in microbiota composition in response to CMC consumption relative to other

422 participants in the CMC group (**Figure 5B-C,** group effect *P* = .004). Moreover, these subjects

423 displayed significantly increased levels of fecal LPS (**Figure 5D,** group effect *P* = .005). Analysis

424 of the metagenomic data at the functional level using beta diversity measurement of the BrayCurtis

425 distance revealed that these two participants had striking greater relative changes in microbiota

426 function in response to CMC consumption relative to the other participants of the CMC group

427 (**Figure 5G**, P = .0002). Analysis of morphometric characteristics taken at the beginning of the

428 clinical trial revealed that CMC-sensitive subjects are both males and are older compared with

429 other members of the CMC group, without any other significant differences (weight, height, BMI,

430 SBP, DBP, **Figure 5H**). Collectively, these results suggest that some individuals may be prone to

431 develop alterations in the host-microbiota interactions in response to CMC consumption, and

432 future studies are warranted to investigate the long-term consequences on intestinal health.

Discussion

433

434   That the post-mid-20th century increased incidence of chronic inflammatory diseases has

435   been roughly paralleled by increased consumption of highly processed foods has long suggested

436   the possibility that some components of such foods promote inflammation. Appreciation of the

437   role of the intestinal microbiota in driving inflammation led to interest in food additives capable

438   of perturbing the host-microbiota relationship. Our previous findings that some dietary emulsifiers

439   can impact microbiota *in vitro* and in animal models, whereby they promote inflammatory

440   diseases, suggest that these compounds might be one specific example of this notion [9-12]. However,

441   the extent to which such substances actually increase risk of disease in the doses and frequency in

442   which they are consumed by humans remains far less clear. Our findings reported herein that

443   consumption of one widely used food additive, namely the synthetic emulsifier

444   carboxymethylcellulose (CMC), impacted microbiota in humans in a seemingly detrimental

445   manner are a step toward filling this knowledge gap.

446   Epidemiologic-based studies of food additives have limited power to assess consequences

447   of specific food additives for numerous reasons. For one, concentrations of these components in

448   commercially prepared foods are not widely reported, making extremely challenging to

449   quantitatively estimate food additives consumption in humans [33]. Furthermore, processed foods

450   often contain multiple potentially detrimental ingredients making the driver of associations

451   difficult to identify. Randomized control trials to assess the impact of food additives on disease

452   incidence are very challenging due to the long period of follow-up required. Nonetheless, they

453   remain the gold standard means to identify impact specific ingredients, for example artificial

454   sweeteners [34]. Indeed, controlled feeding studies, such as ours, are ideal to study the physiologic

455   response of humans to short-term dietary exposures in a tightly controlled setting. Our design

22

456   allowed us to focus on microbiota changes that are associated with chronic diseases, where a role

457   in causation has been proposed. We observed stark changes in gut microbiota, fecal metabolome

458   and, in a subset of the participants, encroachment of microbiota upon the gut epithelium. The

459   predominant changes in the fecal metabolome upon CMC feeding was loss of purportedly

460   beneficial metabolites. We envision this change likely reflected loss of key taxa and/or general

461   disruption of microbial community homeostasis. We also demonstrate that CMC consumption can

462   be assayed by quantitating its level in feces, thus providing a tool to facilitate longer term studies

463   that could address extent to which CMC exposure promotes chronic diseases increasingly

464   prevalent in developed countries.

465   The dose of CMC (15 g per person per day) used in this study likely exceeds CMC intake

466   of most individuals but might approximate the total amount of emulsifier consumption by persons

467   whose diets are largely comprised of highly processed foods that contain numerous emulsifiers,

468   many of which appear to detrimentally impact human microbiotas *in vitro* [8]. While this study

469   focused on one specific food additive, CMC, the results obtained support the need to apply this

470   paradigm to other dietary emulsifiers, and mixtures thereof, at lower concentration, thus better

471   mimicking their use in processed foods. Further, we view it as important to discern the extent to

472   which the highly heterogenous impact of emulsifier on human microbiota *in vitro* is recapitulated

473   *in vivo* [8]. Finally, while our study was not powered to discover CMC-sensitive/CMC-insensitive

474   participants, our results nonetheless suggest that microbiota responsiveness to this food additive

475   may be highly personalized. While follow-up studies are needed to better understand such inter-

476   individual variability and assess its role in driving microbiota-mediated disease states, our

477   observations argue that a particular food additive might perturb the host-microbiota relationship

478   to promote disease in a subpopulation of individuals. If our results are confirmed in larger studies

479   with longer term follow up, the identified mechanism(s) may inform healthy food choices and

480   enable the development of healthier processed foods.

Figure legends

Figure 1: Effect of carboxymethylcellulose consumption on metabolic parameters. A. Schematic representation of the in-patient study that enabled daily monitoring and specimen collections before, during, and after CMC consumption, or lack thereof, and presenting timing of oral glucose tolerance tests, intestinal biopsies and feces collection. **B.** Biomorphometric characterization of study's participants at the beginning of the study. **C.** Effect of dietary emulsifier CMC consumption on various metabolic parameters, measured both pre- and post- intervention. **D-F.** Effect of dietary emulsifier CMC consumption on weight (**D**), PROMISE gas/bloating (**E**) and belly pain (**F**) scores, measured both pre- and post- intervention. OGTT, Oral Glucose Tolerance Test. Significance was determined using Mann-Whitney test; *$P < 0.05$ compared to control group.

Figure 2: Effect of carboxymethylcellulose consumption on microbiota composition. A. Principal coordinates analysis of the unweighted UniFrac distance matrix of study's participants microbiota assessed by 16S rRNA gene sequencing. All time points are included in the representation, and samples are colored by participants. **B.** Principal coordinates analysis of the BrayCurtis distance matrix at days 0, 9 and 14 of study's participants microbiota composition after normalization of every SVs based on day 4 value, with samples colored by group. **C.** Changes in the microbial community structure over time, as measured by BrayCurtis distance from day 4 to subsequent days, for each group. **D.** Fecal bacterial load assessed by 16S qPCR. **E.** Changes in Evenness and Shannon alpha diversity measures for CMC intervention versus control groups, at days 0, 9 and 14. Significance was determined using two-way ANOVA corrected for multiple

519 comparisons with a Bonferroni post-test (panel **E**), multiple t-tests (panel **E**) or PERMANOVA

520 analysis (panels **A-B**).

521

522 **Figure 3: Effect of carboxymethylcellulose consumption on fecal metagenome. A.** Principal

523 coordinates analysis of the BrayCurtis dissimilarity of study's participants metagenome (uniref90

524 categories) assessed by shotgun sequencing. Days 4 and 14 are included in the representation, and

525 samples are colored by participants. **B-C.** Principal coordinates analysis of the BrayCurtis distance

526 matrix at day 4 (**B**) and 14 (**C**) of study's participants metagenome assessed by shotgun

527 sequencing, with samples colored by group.

528

529 **Figure 4: Effect of carboxymethylcellulose consumption on the fecal metabolome. A.** Changes

530 of the fecal level of bioactive LPS from day 0 to subsequent days measured with HEK-TLR4

531 reporter cells. **B.** Changes of the fecal level of bioactive flagellin from day 0 to subsequent days

532 measured with HEK-TLR5 reporter cells. **C.** Changes of the fecal level of the inflammatory marker

533 Lipocalin-2 from day 0 to subsequent days. **D.** Principal coordinates analysis of the Euclidean

534 distance at days 0, 9 and 14 of study's participants' fecal metabolome, with samples colored by

535 group. **E.** Heatmap presenting participants fecal metabolome over the course of the study. **F.**

536 Changes of the fecal level of carboxymethylcellulose from day 0 to subsequent days in both control

537 and CMC-treated groups. **G.** Changes of the fecal level of carboxymethylcellulose from day 0 to

538 subsequent days in control group. Significance was determined using two-way ANOVA corrected

539 for multiple comparisons with a Bonferroni post-test or repeated *t*-tests corrected with the false

540 discovery rate approach for panel G; *$P < 0.05$ compared to control group for panel F, *$P < 0.05$

541 compared to day 0 for panel G.

542

543 **Figure 5: Intersubject variability in the response to carboxymethylcellulose consumption. A.**

544 Effect of dietary emulsifier CMC consumption on microbiota localization (distance of the closest

545 bacteria from the surface of the epithelium), measured both pre- and post- intervention. **B.** Changes

546 of the BrayCurtis distance matrix, for each study's participant from the CMC-treated group, from

547 day 4 to subsequent days. **C.** Changes of the BrayCurtis distance matrix, for the CMC – Insensitive

548 and the CMC- Sensitive groups, from day 4 to subsequent days. **D.** Changes of the fecal level of

549 bioactive LPS from day 0 to subsequent days measured with HEK-TLR4 reporter cells. **E.** Changes

550 of the fecal level of bioactive flagellin from day 0 to subsequent days measured with HEK-TLR5

551 reporter cells. **F.** Changes of the fecal level of the inflammatory marker Lipocalin-2 from day 0 to

552 subsequent days. **G.** Effect of dietary emulsifier CMC consumption on fecal metagenome

553 measured through BrayCurtis distance. **H.** Biomorphometric characterization of study's

554 participants at the beginning of the study and according to CMC sensitivity status. Significance

555 was determined using one-way ANOVA corrected for multiple comparisons with a Bonferroni

556 post-test (panels A and G) or two-way ANOVA corrected for multiple comparisons with a
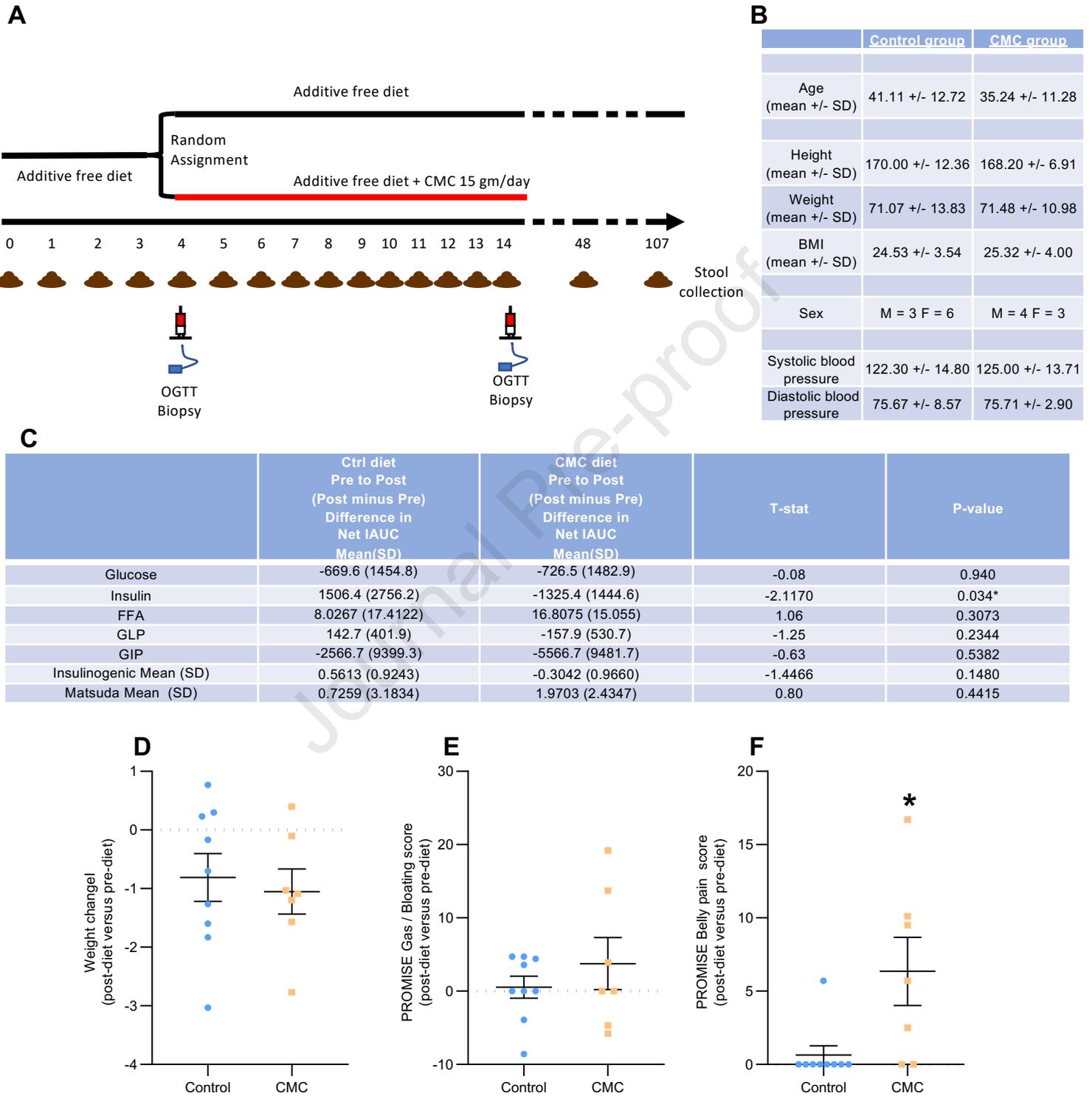
557 Bonferroni post-test (panels C, D, E and F). NS, not statistically significant.

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643

A

Additive free diet

Random Assignment

Additive free diet

Additive free diet + CMC 15 gm/day

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14   48   107

Stool collection

OGTT Biopsy

OGTT Biopsy

B

	Control group	CMC group
Age (mean +/- SD)	41.11 +/- 12.72	35.24 +/- 11.28
Height (mean +/- SD)	170.00 +/- 12.36	168.20 +/- 6.91
Weight (mean +/- SD)	71.07 +/- 13.83	71.48 +/- 10.98
BMI (mean +/- SD)	24.53 +/- 3.54	25.32 +/- 4.00
Sex	M = 3 F = 6	M = 4 F = 3
Systolic blood pressure	122.30 +/- 14.80	125.00 +/- 13.71
Diastolic blood pressure	75.67 +/- 8.57	75.71 +/- 2.90

C

	Ctrl diet Pre to Post (Post minus Pre) Difference in Net IAUC Mean(SD)	CMC diet Pre to Post (Post minus Pre) Difference in Net IAUC Mean(SD)	T-stat	P-value
Glucose	-669.6 (1454.8)	-726.5 (1482.9)	-0.08	0.940
Insulin	1506.4 (2756.2)	-1325.4 (1444.6)	-2.1170	0.034*
FFA	8.0267 (17.4122)	16.8075 (15.055)	1.06	0.3073
GLP	142.7 (401.9)	-157.9 (530.7)	-1.25	0.2344
GIP	-2566.7 (9399.3)	-5566.7 (9481.7)	-0.63	0.5382
Insulinogenic Mean (SD)	0.5613 (0.9243)	-0.3042 (0.9660)	-1.4466	0.1480
Matsuda Mean (SD)	0.7259 (3.1834)	1.9703 (2.4347)	0.80	0.4415

D

Weight changel (post-diet versus pre-diet)

Control CMC

E

PROMISE Gas / Bloating score (post-diet versus pre-diet)

Control CMC

F

PROMISE Belly pain score (post-diet versus pre-diet)

*

Control CMC

Figure 1. Effect of carboxymethylcellulose consumption on metabolic parameters.

Figure 2. Effect of carboxymethylcellulose consumption on microbiota composition.

Figure 3. Effect of carboxymethylcellulose consumption on the fecal metagenome.

Figure 4. Effect of carboxymethylcellulose consumption on the fecal metabolome.

A

B

16S rRNA

C

P = .004

D

E

F

G

Metagenomic

H

	Control	CMC – Insensitive	CMC - Sensitive	Statistic
Age (mean +/- SD)	41.11 +/- 12.72	29.85 +/- 7.95	48.72 +/- 1.46	**0.0251**
Height (mean +/- SD)	170.00 +/- 12.36	167.02 +/- 7.87	170.80 +/- 4.60	0.5886
Weight (mean +/- SD)	71.07 +/- 13.83	68.52 +/- 11.79	78.88 +/- 3.70	0.2982
BMI (mean +/- SD)	24.53 +/- 3.54	24.62 +/- 4.68	27.05 +/- 0.19	0.5186
Sex	M = 3 F = 6	M = 2 F = 3	M = 2 F = 0	
SBD	122.30 +/- 14.80	121.60 +/- 15.11	133.5 +/- 3.54	0.3438
DBP	75.67 +/- 8.57	76.20 +/- 7.16	74.50 +/- 8.50	0.8176

Figure 5. Intersubject variability in the response to carboxymethylcellulose consumption.

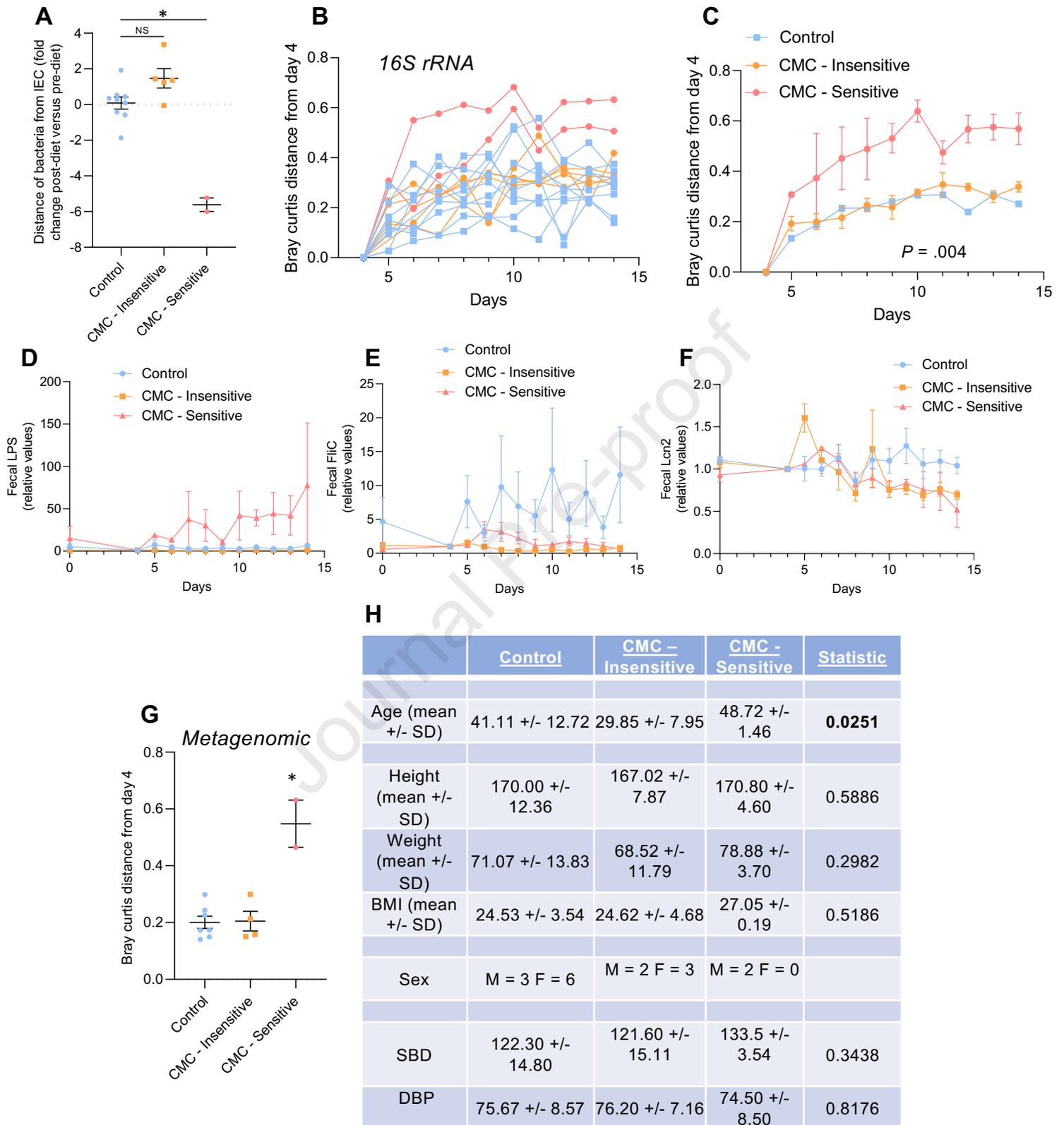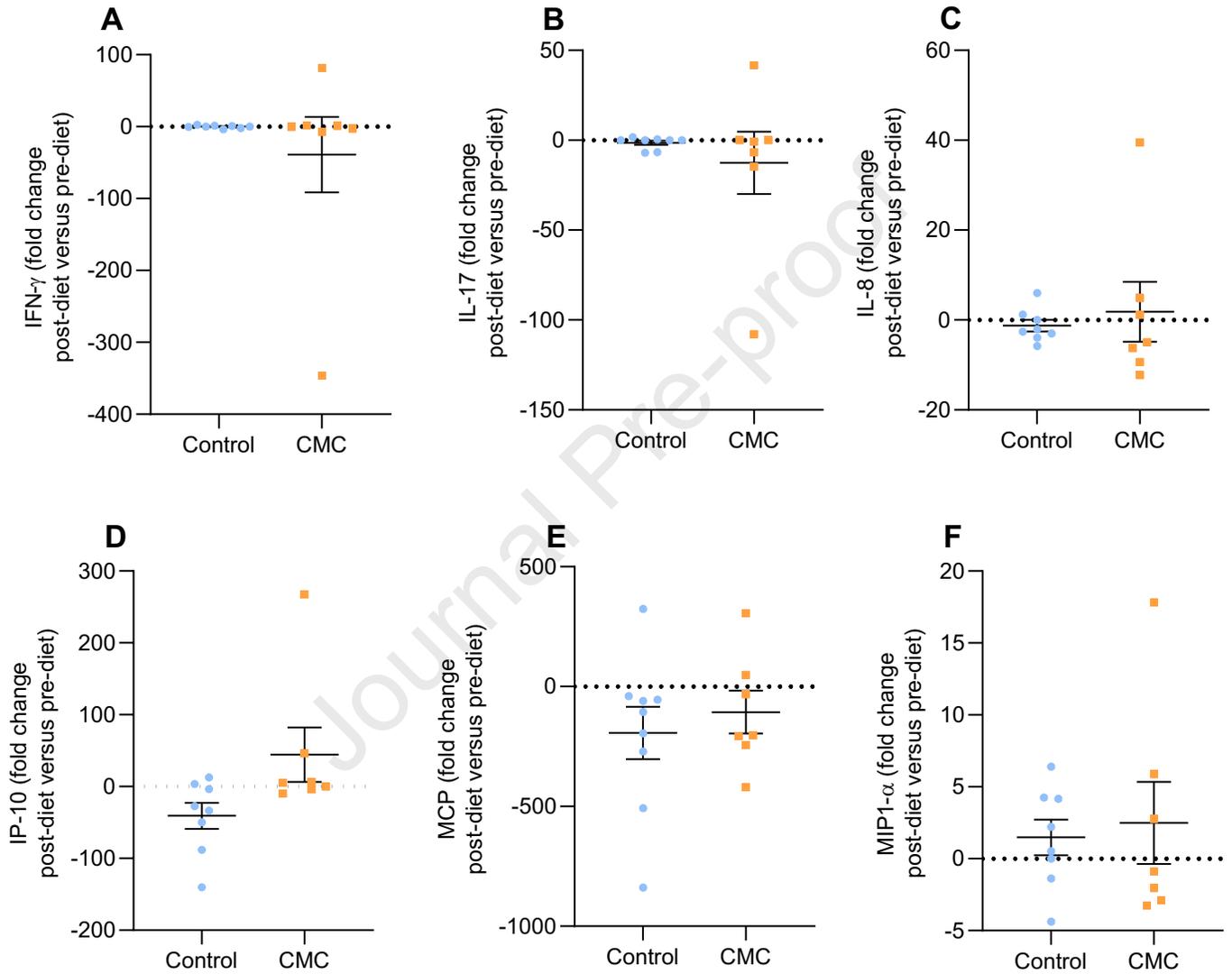|	Timepoints for the 1st three participants	Timepoints for other participants
Prior to washout period - collected at home	Day 0 - stool	Day 0 - stool
Washout period	Day 1 - stool, urine, plasma, buffy coat, serum	
Washout period	Day 2 - stool, urine, plasma, buffy coat, serum	
Washout period	Day 3 - stool, urine, plasma, buffy coat, serum	
End of washout period, last samples collected before Randomized diet phase (CMC-containing vs Emulsifier-free diet)	Day 4 - stool, urine, plasma, buffy coat, serum, OGTT, biopsies	Day 4 - stool, urine, plasma, buffy coat, serum, OGTT, biopsies
CMC exposure	Day 5 - stool, urine, plasma, buffy coat, serum	Day 5 - stool, urine, plasma, buffy coat, serum
CMC exposure	Day 6 - stool, urine	Day 6 - stool, urine, plasma, buffy coat, serum
CMC exposure	Day 7 - stool, urine	Day 7 - stool, urine, plasma, buffy coat, serum
CMC exposure	Day 8 - stool, urine, plasma, buffy coat, serum	Day 8 - stool, urine
CMC exposure	Day 9 - stool, urine	Day 9 - stool, urine
CMC exposure	Day 10 - stool, urine, plasma, buffy coat, serum	Day 10 - stool, urine
CMC exposure	Day 11 - stool, urine	Day 11 - stool, urine, plasma, buffy coat, serum
CMC exposure	Day 12 - stool, urine, plasma, buffy coat, serum	Day 12 - stool, urine
CMC exposure	Day 13 - stool, urine	Day 13 - stool, urine, plasma, buffy coat, serum
CMC exposure	Day 14 - stool, urine	Day 14 - stool, urine, plasma, buffy coat, serum, OGTT, biopsies
CMC exposure	Day 15 - stool, urine, plasma, buffy coat, serum	
CMC exposure	Day 16 - stool, urine	
CMC exposure	Day 17 - stool, urine, plasma, buffy coat, serum, OGTT, biopsies	
Post study samples	Day 48 - stool, plasma, buffy coat, serum	Day 48 - stool, plasma, buffy coat, serum
Post study samples	Day 107 - stool	Day 107 - stool

Table S1: Timeline and list of samples collected during the study.

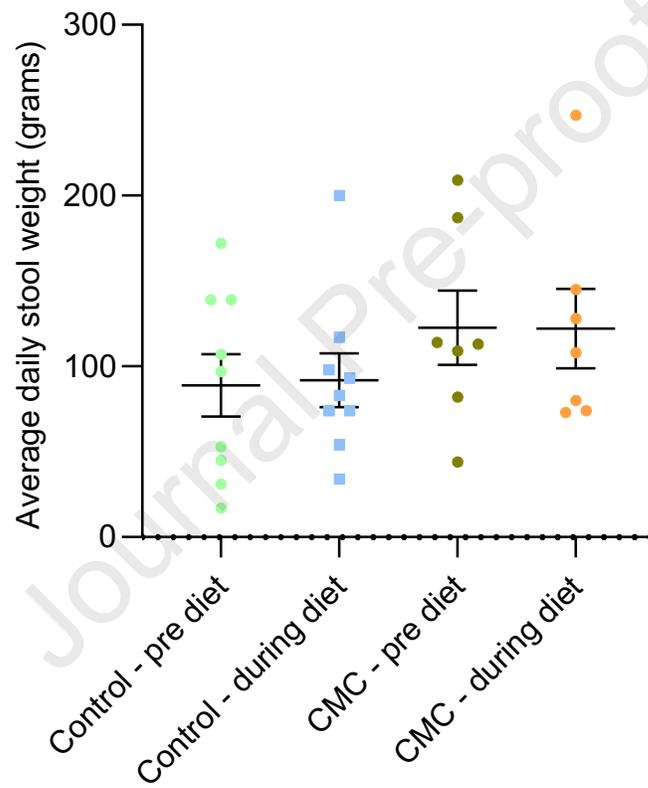**Figure S1: Dietary habits did not significantly differ between groups at the beginning of the study.**

Figure S2: Impact of carboxymethylcellulose exposure on circulating cytokines.

Figure S3: Impact of carboxymethylcellulose exposure on circulating anti-lipopolysaccharide and anti-flagellin IgG.

Figure S4: Impact of carboxymethylcellulose consumption on stool weight.
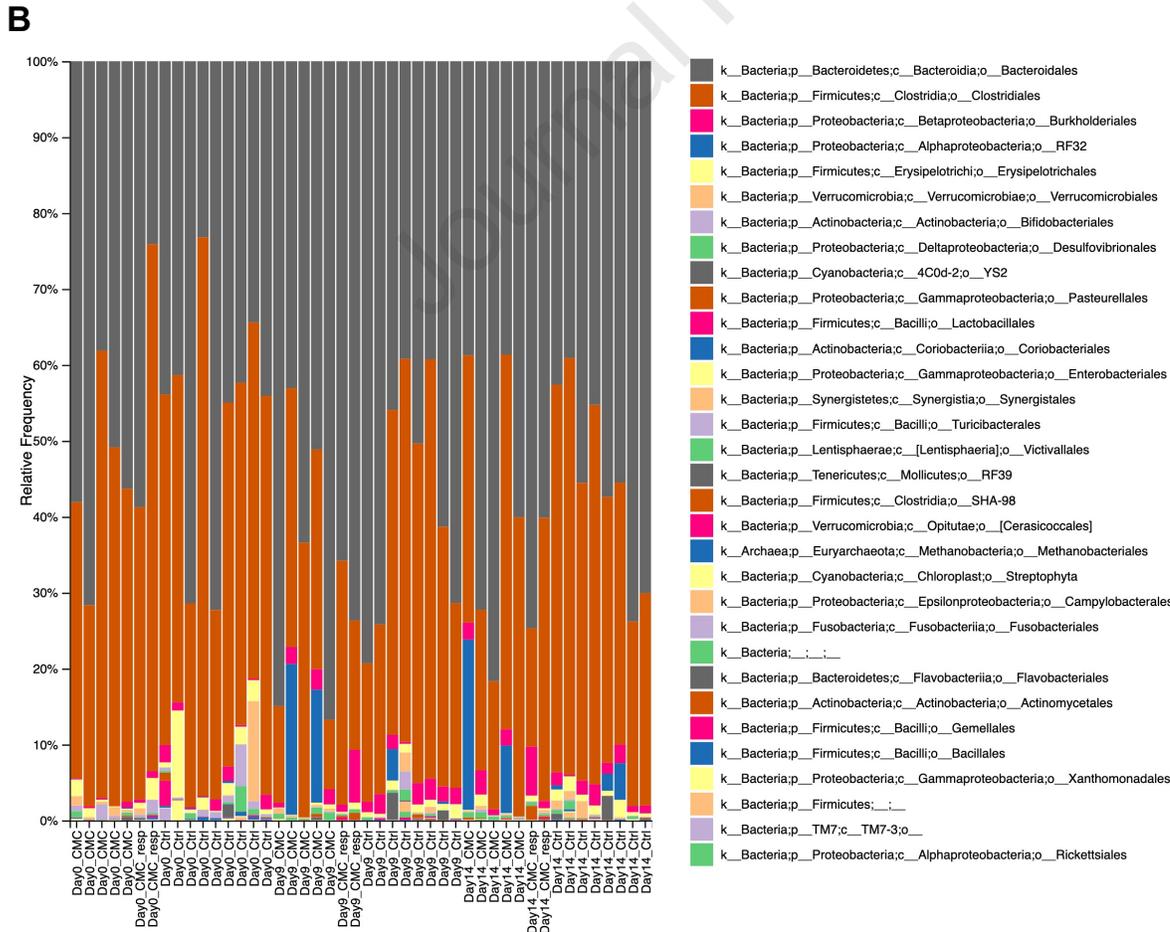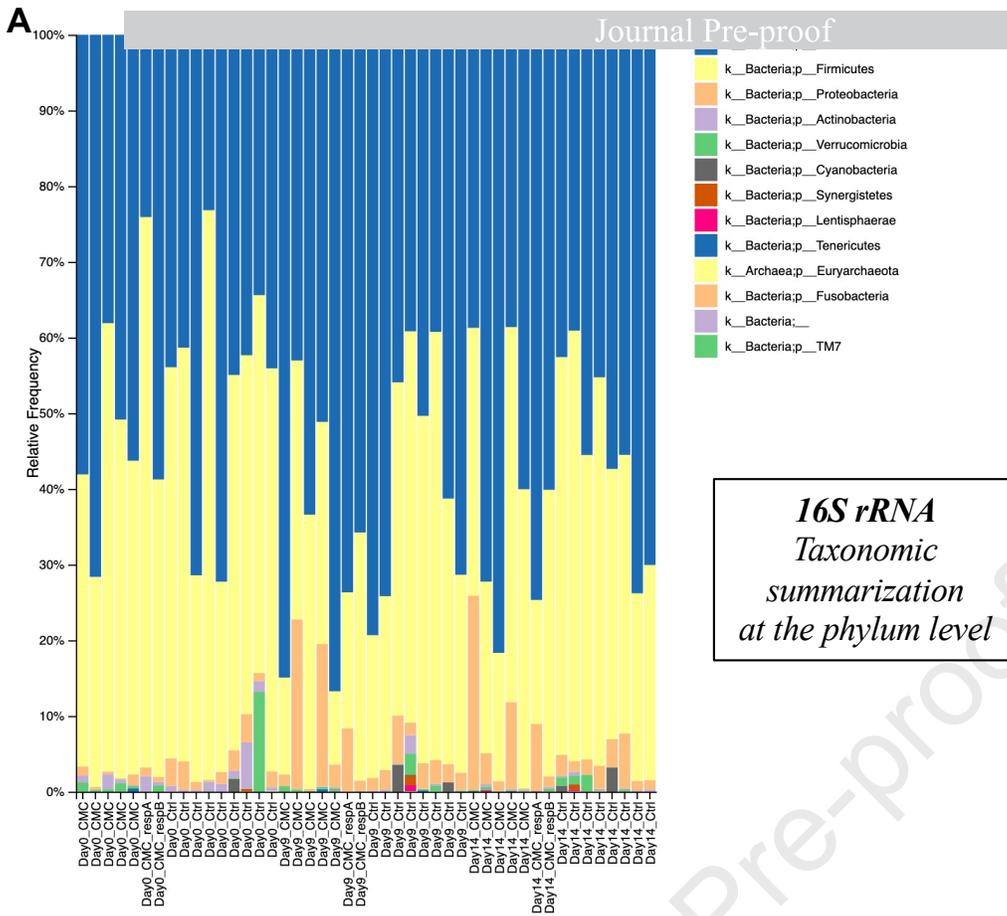
A

16S rRNA
Taxonomic
summarization
at the phylum level

Legend (phylum level):
- k__Bacteria;p__Firmicutes
- k__Bacteria;p__Proteobacteria
- k__Bacteria;p__Actinobacteria
- k__Bacteria;p__Verrucomicrobia
- k__Bacteria;p__Cyanobacteria
- k__Bacteria;p__Synergistetes
- k__Bacteria;p__Lentisphaerae
- k__Bacteria;p__Tenericutes
- k__Archaea;p__Euryarchaeota
- k__Bacteria;p__Fusobacteria
- k__Bacteria;__
- k__Bacteria;p__TM7

B

16S rRNA
Taxonomic
summarization
at the order level

Legend (order level):
- k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales
- k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
- k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales
- k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__RF32
- k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales
- k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales
- k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales
- k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales
- k__Bacteria;p__Cyanobacteria;c__4C0d-2;o__YS2
- k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales
- k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales
- k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales
- k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales
- k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales
- k__Bacteria;p__Firmicutes;c__Bacilli;o__Turicibacterales
- k__Bacteria;p__Lentisphaerae;c__[Lentisphaeria];o__Victivallales
- k__Bacteria;p__Tenericutes;c__Mollicutes;o__RF39
- k__Bacteria;p__Firmicutes;c__Clostridia;o__SHA-98
- k__Bacteria;p__Verrucomicrobia;c__Opitutae;o__[Cerasicoccales]
- k__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales
- k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Streptophyta
- k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales
- k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales
- k__Bacteria;__;__;__
- k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales
- k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales
- k__Bacteria;p__Firmicutes;c__Bacilli;o__Gemellales
- k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales
- k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales
- k__Bacteria;p__Firmicutes;__;__
- k__Bacteria;p__TM7;c__TM7-3;o__
- k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales

Figure S5: Effect of carboxymethylcellulose consumption on microbiota composition.
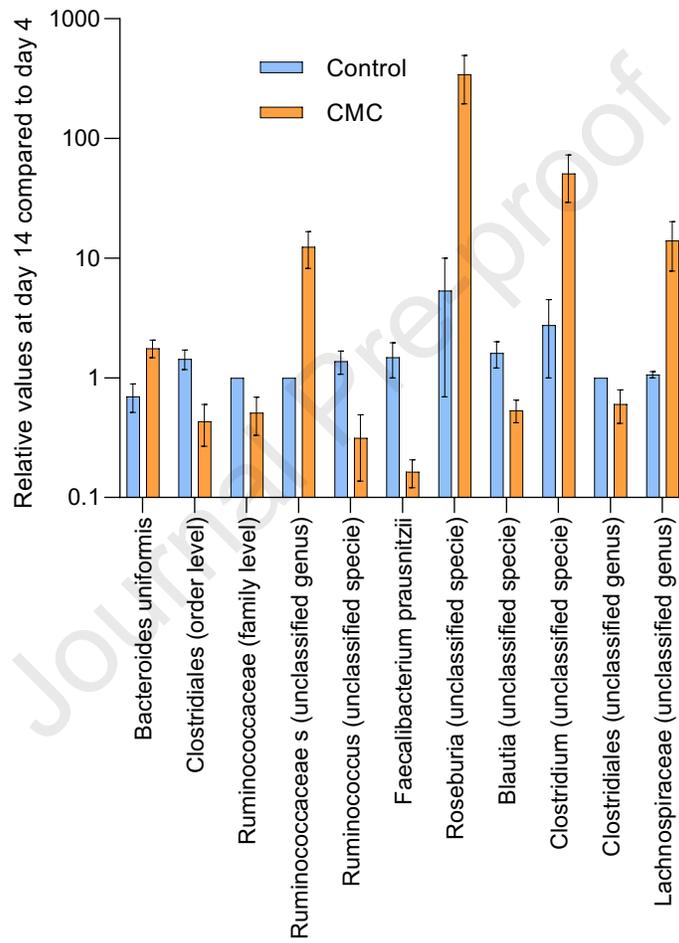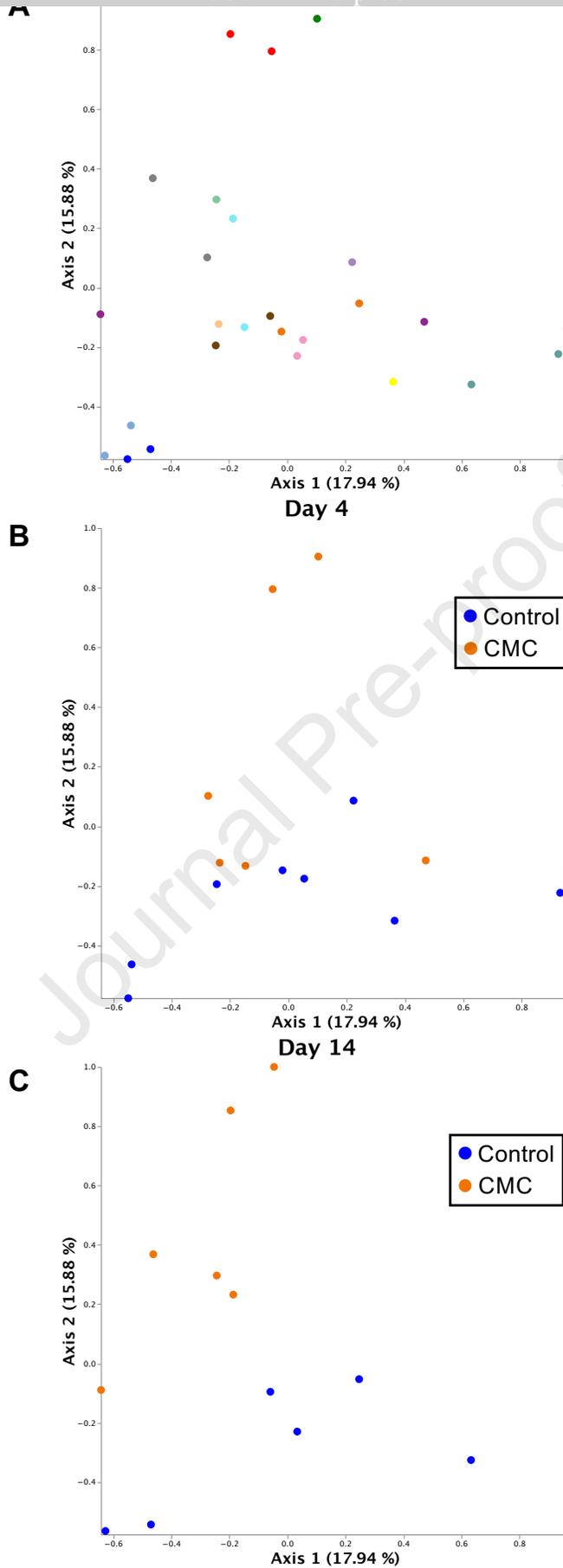
Figure S6: Effect of carboxymethylcellulose consumption on microbiota composition.

Figure S7: Effect of carboxymethylcellulose consumption on microbiota taxonomic composition based on metagenomic data.
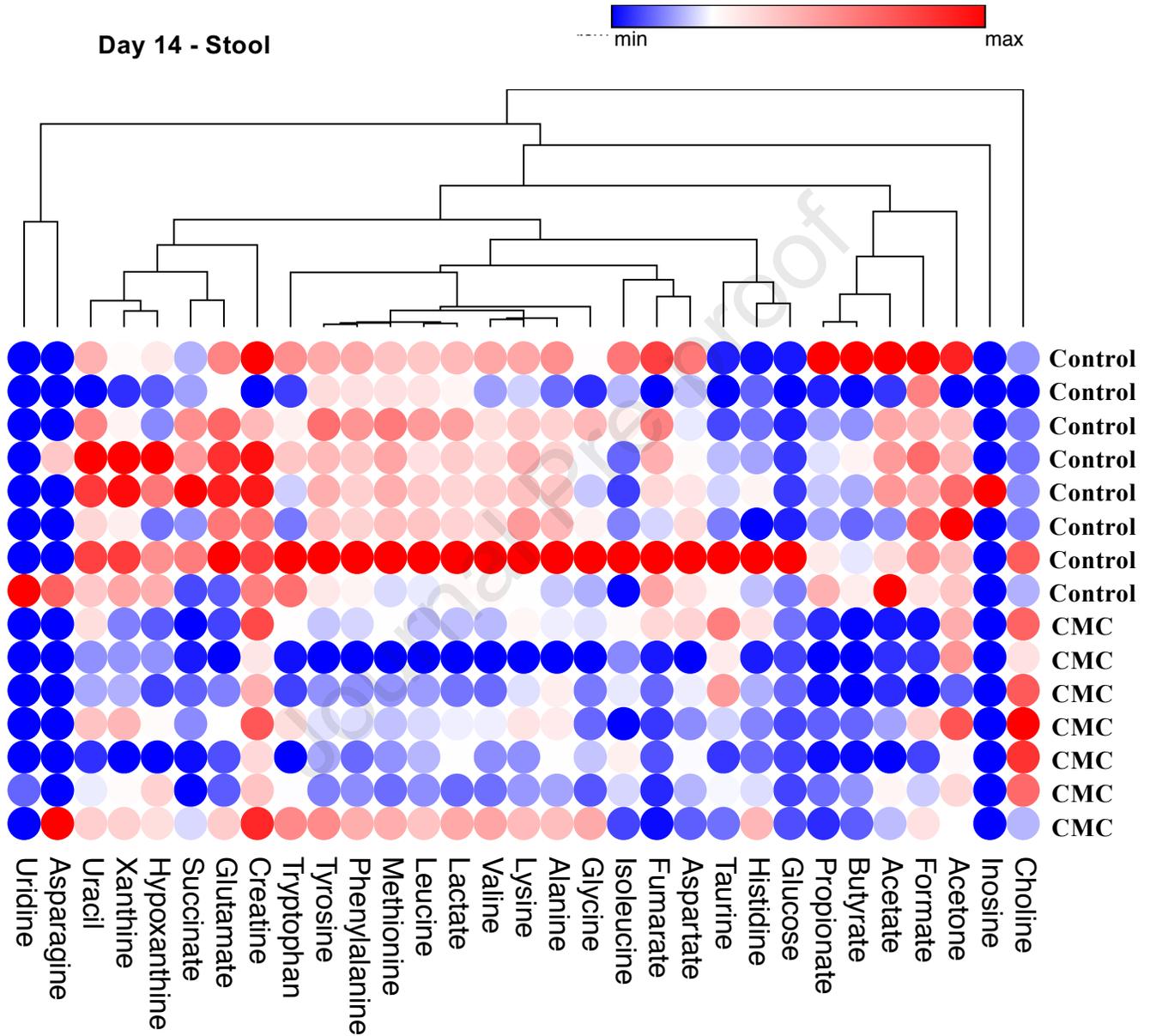
Day 14 - Stool

min max

Figure S8: Effect of carboxymethylcellulose consumption on the fecal metabolome.

Figure S9: Effect of carboxymethylcellulose consumption on the fecal metabolome

Figure S10: Impact of carboxymethylcellulose on AccQ•Tag-based detection of various amino acids.

Figure S11: NMR-based detection of carboxymethylcellulose in fecal samples.

Figure S12: Effect of carboxymethylcellulose consumption on the urine metabolome.

Microbiota - IEC = 14.16μm

Microbiota - IEC = 2.23μm

Microbiota - IEC = 0.00μm

Microbiota - IEC = 38.91μm

Microbiota - IEC = 23.86μm

Microbiota - IEC = 22.69μm

Figure S13: Effect of carboxymethylcellulose consumption on microbiota localization

What You Need to Know

BACKGROUND AND CONTEXT
Some widely used food additives, including dietary emulsifiers, alter gut microbiota and promote inflammation in *in vitro* and animal models, but applicability of such observations to humans remains poorly characterized. To begin to fill this knowledge gap, we investigated the impact of the synthetic dietary emulsifier carboxymethylcellulose (CMC) on healthy human volunteers.

NEW FINDINGS
Addition of CMC to a healthy additive-free diet increased postprandial abdominal discomfort and altered intestinal microbiota composition. Moreover, CMC consumption starkly impacted the fecal metabolome, including depletion of health-promoting metabolites such as short-chain f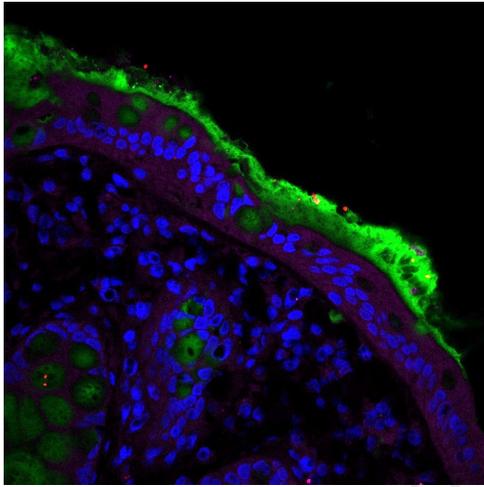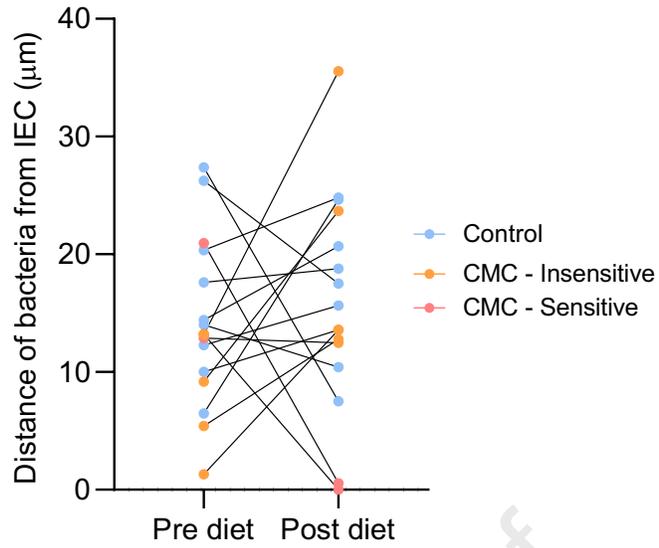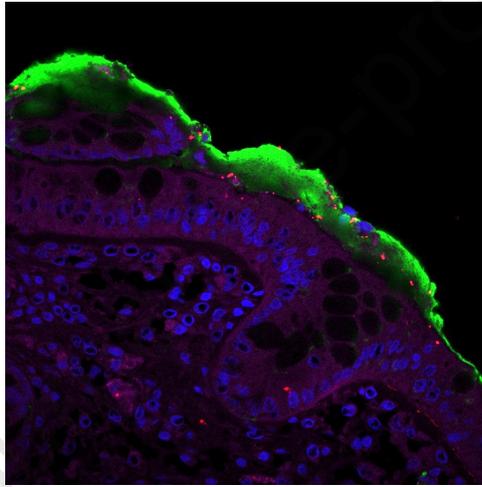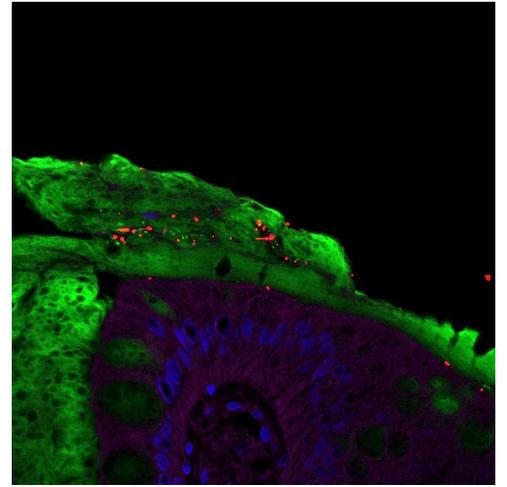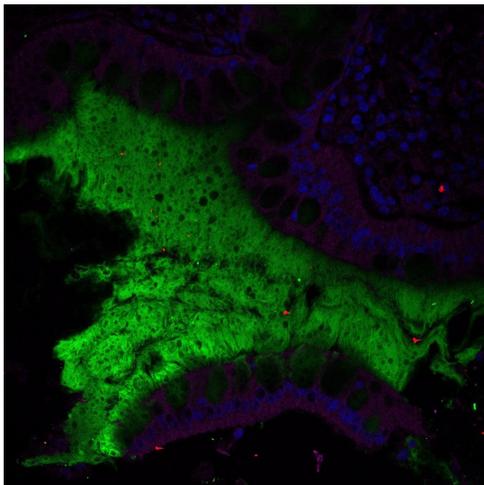atty acids and free amino acids. Furthermore, some individuals displayed microbiota encroachment into the normally sterile inner mucus layer following CMC consumption.

LIMITATIONS
This study was focused on CMC's short-term impacts, particularly on gut microbiome. Assessing the extent to which these changes would persist in states of long-term consumption of CMC and/or other emulsifiers, and determining their phenotypic consequences, would require additional studies.

IMPACT
That CMC consumption by humans impacted the microbiome supports the notion that wide use of this compound, and perhaps other dietary emulsifiers, in processed foods may have contributed to increased incidence of chronic inflammatory diseases.

Lay Summary

Dietary emulsifier carboxymethylcellulose increased abdominal discomfort and altered microbiota composition and fecal metabolome, supporting the notion that its consumption may be promoting development of chronic inflammatory diseases.

1 **Supplemental methods**

2

3 ***Serum lipopolysaccharide- and flagellin-specific immunoglobulins.***

4 Flagellin- and LPS-specific IgG levels were quantified by ELISA, as previously described

5 [1]. Microtitre plates were coated overnight with purified *E. coli* flagellin (100 ng per well) or LPS

6 (2 μg per well). Serum samples diluted 1:100 or 1:200 were then applied. After incubation and

7 washing, wells were incubated with anti-human IgG. Quantification was performed using the

8 colorimetric peroxidase substrate tetramethylbenzidine. Data are reported as optical density

9 corrected by subtracting background (determined by readings in samples lacking serum) and

10 normalized to the post-washout pre-intervention sample.

11

12 ***Microbiota analysis by 16S rRNA gene sequencing using Illumina technology***

13 16S rRNA gene amplification and sequencing utilized the Illumina MiSeq technology

14 following the protocol of Earth Microbiome Project with their modifications to the MOBIO

15 PowerSoil DNA Isolation Kit procedure for extracting DNA (www.earthmicrobiome.org/emp-

16 standard-protocols) [2, 3]. Bulk DNA was extracted from frozen feces using a PowerFecal-HT kit

17 from Qiagen with mechanical disruption (bead-beating). The 16S rRNA genes, region V4, were

18 PCR amplified from each sample using a composite forward primer and a reverse primer

19 containing a unique 12-base barcode, designed using the Golay error-correcting scheme, which

20 was used to tag PCR products from respective samples [3]. We used the forward primer 515F 5'-

21 *AATGATACGGCGACCACCGAGATCTACACGCT*XXXXXXXXXXXX**TATGGTAATT*GT*G**

22 TGYCAGCMGCCGCGGTAA-3': the italicized sequence is the 5' Illumina adapter, the 12 X

23 sequence is the golay barcode, the bold sequence is the primer pad, the italicized and bold sequence

1

24   is the primer linker and the underlined sequence is the conserved bacterial primer 515F. The

25   reverse primer 806R used was 5'-*CAAGCAGAAGACGGCATACGAGAT***AGTCAGCCAG***CC*

26   <u>GGACTACNVGGGTWTCTAAT</u>-3': the italicized sequence is the 3' reverse complement

27   sequence of Illumina adapter, the bold sequence is the primer pad, the italicized and bold sequence

28   is the primer linker and the underlined sequence is the conserved bacterial primer 806R. PCR

29   reactions consisted of Hot Master PCR mix (Quantabio, Beverly, MA, USA), 0.2 µM of each

30   primer, 10-100 ng template, and reaction conditions were 3 min at 95°C, followed by 30 cycles of

31   45 s at 95°C, 60s at 50°C and 90 s at 72°C on a Biorad thermocycler. PCRs products were

32   quantified using Quant-iT PicoGreen dsDNA assay, a master DNA pool was generated from the

33   purified products in equimolar ratios and subsequently purified with Ampure magnetic purification

34   beads (Agencourt, Brea, CA, USA). The pooled product was quantified using Quant-iT PicoGreen

35   dsDNA assay and then sequenced using an Illumina MiSeq sequencer (paired-end reads, 2 x 250

36   bp) at Cornell University, Ithaca, NY.

37

38   ***16S rRNA gene sequence analysis***

39   16S rRNA sequences were analyzed using QIIME2 – version 2019 [4]. Sequences were

40   demultiplexed and quality filtered using Dada2 method [5] with QIIME2 default parameters in order

41   to detect and correct Illumina amplicon sequence data, and a table of Qiime 2 sequence variants

42   (SVs) was generated. A tree was next generated, using the align-to-tree-mafft-fasttree command,

43   for phylogenetic diversity analyses, and alpha and beta diversity analysis were computed using the

44   core-metrics-phylogenetic command. In order to normalize for inter-individual variations in

45   microbiota composition, day4 data were normalized at 1 for every SV identified, and the data for

46   the other days were expressed, for each individual patient, as relative values compared to day 4

47   data. Principal coordinates analysis (PCoA) of the Unweighted Unifrac and Bray-Curtis metrics

48   was visualized to assess the variation between experimental groups (beta diversity). For taxonomy

49   analysis, taxonomies were assigned to SVs with a 99% threshold of pairwise identity to the

50   Greengenes reference database 13_8 [6]. Unprocessed sequencing data are deposited in the European

51   Nucleotide Archive under accession number XXXXXX.

52

53   ***Microbiota analysis by shotgun sequencing using Illumina technology***

54   Bulk DNA, extracted as described above for 16S rRNA gene amplicon sequencing, was

55   processed for Illumina HiSeq sequencing in an approach similar to that described by Baym *et al*.

56   [7]. Briefly, DNA concentrations were normalized, and 5ng of DNA in 10μl H_2O were added to 10

57   μl of tagmentation mix (2ul of TAPS-DMF buffer 0.25 ul Tn5 enzyme, and 7.75 ul H_2O), then

58   held for 10 min at 55°C. Next, pairwise combined Nextera indexes (N7+S5) were added to each

59   well, followed by 25 ul of PCR reaction mix. PCR thermocycler settings were as follows: step 1

60   72°C, 5min, step 2 98°C 30sec, step 3 98°C, 15sec, step 4 67°C, 30sec, step 5 72°C, 1min 30sec,

61   repeat steps 3-5 13x. Samples were next pooled, and cleaned with a Zymo DNA cleanup kit.

62   Samples were then size selected with a BluePippin on a 1.5% gel for a 350-700 base pair size

63   range. Finally, the combined sample was diluted to 2.5nM with Illumina resuspension buffer, and

64   sequenced on an Illumina HiSeq 3000 system at the Max Planck Institute for Developmental

65   Biology in Tuebingen, Germany. On average, 4.9 millions paired raw reads were obtained per

66   samples (minimum = 2.7 millions paired raw reads, maximum = 13 millions paired raw reads).

67   Sequencing adapters were removed from the resulting sequences *via* the bbduk module of

68   BBMap, version 37.78. BBMap (align2.BBMap) was additionally used to detect and remove

69   human sequences [8]. The skewer v0.2.2 software was used to detect any remaining adapters, as well

70   as filter reads for degeneracy, and truncate and/or filter reads for low quality scores [9]. These

71   quality-filtered reads were then grouped via humann2 v0.11.2 into functional categories (the

72   chocophlan v0.1.1 nucleotide reference database, the Sep-12-2016 uniref90.ec_filtered.1.1 protein

73   reference file, and the Bowtie mpa_v20_m200 database were used as reference files for these

74   queries) [10].

75

76   ***Bacterial density quantification by 16S rRNA qPCR***

77   Extracted DNAs were diluted 1/10 with sterile DNA-free water and amplified by

78   quantitative PCR using the 16S V4 specific primers 515F 5'-GTGYCAGCMGCCGCGGTAA-3'

79   and 806R 5'-GGACTACNVGGGTWTCTAAT-3' or using the using the AIEC LF82 PTM

80   specific primers PTM-F 5'- CCATTCATGCAGCAGCTCTTT -3' and PTM-R 5'-

81   ATCGGACAACATTAGCGGTGT -3' on a LightCycler 480 (Roche) using QuantiFast SYBR®

82   Green PCR Kit (Qiagen). Amplification of a single expected PCR product was confirmed by

83   electrophoresis on a 2% agarose gel, and data are expressed as relative values normalized with

84   feces weight used for DNA extraction.

85

86   ***Fecal flagellin and lipopolysaccharide load quantification***

87   Levels of fecal bioactive flagellin and lipopolysaccharide (LPS) were quantified as

88   previously described [11] using human embryonic kidney (HEK)-Blue-mTLR5 and HEK-

89   BluemTLR4 cells, respectively (Invivogen, San Diego, CA, USA) [11]. Fecal material was

90   resuspended in PBS to a final concentration of 100 mg/mL and homogenized for 10 s using a Mini-

91   Beadbeater-24 without the addition of beads to avoid bacteria disruption. Samples were then

92   centrifuged at 8000 g for 2 min and the resulting supernatant was serially diluted and applied on

4

93   mammalian cells. Purified *E. coli* flagellin and LPS (Sigma-Aldrich) were used for standard curve

94   determination using HEK-Blue-mTLR5 and HEK-Blue-mTLR4 cells, respectively. After 24 h of

95   stimulation, the cell culture supernatant was applied to QUANTI-Blue medium (Invivogen) and

96   the alkaline phosphatase activity was measured at 620 nm after 30 min.

97

98   ***Immunostaining of mucins and localization of bacteria by FISH***

99   Mucus immunostaining was paired with fluorescent *in situ* hybridization (FISH), as

100   previously described [12], in order to analyze bacteria localization at the surface of the intestinal

101   mucosa [13, 14]. Briefly, colonic biopsies collected during rectosigmoidoscopy were placed in

102   methanol-Carnoy's fixative solution (60% methanol, 30% chloroform, 10% glacial acetic acid) for

103   a minimum of 3 h at room temperature, and then stored at 4C. Tissues were then washed in

104   methanol 2 x 30 min, ethanol 2 x 15 min, ethanol/xylene (1:1) 15 min and xylene 2 x 15 min,

105   followed by embedding in Paraffin with a vertical orientation. Five μm sections were performed

106   and dewax by preheating at 60°C for 10 min, followed by xylene 60°C for 10 min, xylene for 10

107   min and 99.5% ethanol for 10 minutes. Hybridization step was performed at 50°C overnight with

108   EUB338 probe (5'-GCTGCCTCCCGTAGGAGT-3', with a 5' labeling using Alexa 647) diluted

109   to a final concentration of 10 μg/mL in hybridization buffer (20 mM Tris–HCl, pH 7.4, 0.9 M

110   NaCl, 0.1% SDS, 20% formamide). After washing 10 min in wash buffer (20 mM Tris–HCl, pH

111   7.4, 0.9 M NaCl) and 3 x 10 min in PBS, PAP pen (Sigma-Aldrich) was used to mark around the

112   section and block solution (5% fetal bovine serum in PBS) was added for 30 min at 4°C. Mucin-2

113   primary antibody (rabbit H-300, Santa Cruz Biotechnology, Dallas, TX, USA) was diluted 1:1500

114   in block solution and apply overnight at 4°C. After washing 3 x 10 min in PBS, block solution

115   containing anti-rabbit Alexa 488 secondary antibody diluted 1:1500, Phalloidin-

5

116 Tetramethylrhodamine B isothiocyanate (Sigma-Aldrich) at 1μg/mL and Hoechst 33258 (Sigma-

117 Aldrich) at 10μg/mL was applied to the section for 2h. After washing 3 x 10 min in PBS slides

118 were mounted using Prolong anti-fade mounting media (Life Technologies, Carlsbad, CA, USA).

119 Observations were performed with a Zeiss LSM 700 confocal microscope with software Zen 2011

120 version 7.1. This software was used to determine the distance between bacteria and epithelial cell

121 monolayer, as well as the mucus thickness.

122

123 ***Metabolomic analysis of stool and urine samples***

124 Stool and urine sample preparation for NMR were performed as previously described [15].

125 ^{1}H NMR spectra were acquired on a Bruker Avance NEO 600 MHz spectrometer equipped with

126 an inverse cryogenic probe (Bruker Biospin, Germany) at 298 K. A typical 1D NMR spectrum

127 named NOESYPR1D was acquired for each sample. The metabolites were assigned on the basis

128 of published results [16] and confirmed with a series of 2D NMR spectra. All ^{1}H NMR spectra were

129 adjusted for phase and baseline using Chenomx (Chenomx Inc, Canada). The chemical shift of [1]H

130 NMR spectra were referenced to sodium 3-trimethylsilyl [2,2,3,3-d4] propionate (TSP) at δ 0.00.

131 The relative contents of metabolites were calculated by normalizing to the total sum of the spectral

132 integrals. The quantification of metabolites including CMC in stool was calculated by NMR peak

133 area against TSP using Chenomx.

134

135 ***AccQ•Tag Amino Acid Analysis of Stool Samples***

136 Amino acids were extracted from stool samples with 1 mL of ice-cold methanol/water (2:1)

137 solution (contain 2.5 uM of Norvaline), followed by homogenization (Precellys, Bertin

138 Technologies, Rockville, MD) with 1.0-mm-diameter zirconia/silica beads (BioSpec, Bartlesville,

139   OK), three freeze–thaw cycles and centrifugation (Eppendorf, Hamburg, Germany). Supernatant

140   was collected, evaporated to dryness (Thermo Scientific, Waltham, MA) and then resuspend in 50

141   uL 0.1N HCl solution. Amino acid derivation with AccQ•Tag reagents (Waters, Milford, MA) was

142   conducted according to the manufacturer's protocol. Briefly, 10 μL of stool extract were mixed

143   with 70 μL of AccQ•Tag Ultra borate buffer and 20 μL of AccQ•Tag Ultra reagent in Total

144   Recovery Vial. The vials were capped and vortex for several seconds and proceed for 10 min at

145   55 °C. Amino Acid were detected by Waters Xevo TQS coupled with PDA, an AccQTag Ultra

146   Column (C18 1.7 um 2.1 x 100 mm) with in-line filter (Waters, Milford, MA) were used for

147   separation [17]. Results were quantified by comparing integrated peak areas against a standard curve.

148

149   ***Statistical analysis***

150   Significance was determined using *t*-tests, Mann-Whitney test, one-way ANOVA

151   corrected for multiple comparisons with a Bonferroni post-test, two-way ANOVA corrected for

152   multiple comparisons with a Bonferroni post-test (or mixed-effect analysis when some values were

153   missing), or repeated *t*-tests corrected with the false discovery rate approach were appropriate

154   (GraphPad Prism software, version 6.01). Differences were noted as significant at $P \leq 0.05$.

7

Supplementary figure legends

Table S1: Timeline and list of samples collected during the study.

Table S2: NMR data for the metabolites found in stool (S) and urine (U).

Figure S1: Dietary habits did not significantly differ between groups at the beginning of the study. Principal coordinates analysis of the Euclidean distance matrix based on the Dietary History Questionnaire II, a semi-quantitative food frequency questionnaire developed by the NCI. Samples are colored by group.

Figure S2: Impact of CMC exposure on circulating cytokines. A-F. Effect of dietary emulsifier CMC consumption on circulating IFN-γ (**A**), IL-17 (**B**), IL-8 (**C**), IP-10 (**D**), MCP-1 (**E**) and MIP-1α (**F**) scores, measured both pre- and post- intervention. Significance was determined using *t*-test. IFN, Interferon; IL, Interleukin; IP, Inducible protein; MCP, Monocyte Chemoattractant Protein ; MIP, Macrophage inflammatory protein.

Figure S3: Impact of CMC exposure on circulating anti-lipopolysaccharide and anti-flagellin IgG. Effect of dietary emulsifier CMC consumption on circulating anti-lipopolysaccharide (**A**, **C**, **E**) and anti-flagellin (**B**, **D**, **F**) IgG levels, measured both pre- and post- intervention with data normalized to 1 for the pre-intervention value. In **A** and **B**, individual participants are represented. In **C**, **D**, **E** and **F** values are averaged by group.

178 **Figure S4: Impact of carboxymethylcellulose consumption on stool weight.** Daily stool weight

179 production of study's participants measured before and during the study.

180

181 **Figure S5: Effect of carboxymethylcellulose consumption on microbiota composition.**

182 Microbiota composition of study's participants at days 0, 9 and 14 at the phylum (**A**) and order (**B**)

183 levels.

184

185 **Figure S6: Effect of carboxymethylcellulose consumption on microbiota composition.** List of

186 the most significantly altered microbiota members between control and CMC-treated study's

187 participants after normalization of every SVs based on day 4 value. These microbiota members

188 were the most significant between groups when using repeated *t*-tests corrected with a false

189 discovery rate approach (q-values < .05).

190

191 **Figure S7: Effect of carboxymethylcellulose consumption on microbiota taxonomic**

192 **composition based on metagenomic data. A.** Principal coordinates analysis of the BrayCurtis

193 dissimilarities of study's participants microbiota metagenome assessed by shotgun sequencing. All

194 time points are included in the representation, and samples are colored by participants. **B.** Principal

195 coordinates analysis of the BrayCurtis dissimilarities of study's participants day 4 microbiota

196 metagenome assessed by shotgun sequencing. Samples are colored by group. **C.** Principal

197 coordinates analysis of the BrayCurtis dissimilarities of study's participants day 14 microbiota

198 metagenome assessed by shotgun sequencing. Samples are colored by group.

199

200   **Figure S8: Effect of carboxymethylcellulose consumption on the fecal metabolome.** Heatmap

201   presenting day 14 participants fecal metabolome.

202

203   **Figure S9: Effect of carboxymethylcellulose consumption on the fecal metabolome.** Evolution

204   of fecal concentration of the various amino acids over the course of the study. Significance was

205   determined using two-way ANOVA corrected for multiple comparisons with a Bonferroni post-

206   test

207

208   **Figure S10: Impact of CMC on AccQ•Tag-based detection of various amino acids.**

209   Significance was determined using one-way ANOVA corrected for multiple comparisons with a

210   Bonferroni post-test.

211

212   **Figure S11: NMR-based detection of carboxymethylcellulose in fecal samples. A.**

213   Representative spectra obtained using feces from a control participant, feces from a CMC-treated

214   participant, and CMC standard. **B.** Spectra obtained using various concentration of CMC (0.5, 1.0,

215   2.0, 3.0 and 4.0 mg/ml). Each concentration was analyzed in triplicate. **C.** Standard curve obtained

216   using CMC standard solutions and ^{1}H NMR data (ppm 3.976-4.02). CMC integral data were

217   normalized to the internal standard TSP.

218

219   **Figure S12: Effect of carboxymethylcellulose consumption on the urine metabolome. A.**

220   Heatmap presenting participants fecal metabolome over the course of the study. **B.** Heatmap

221   presenting day 14 participants urine metabolome.

222

223 **Figure S13: Effect of carboxymethylcellulose consumption on microbiota localization.** Effect

224 of dietary emulsifier CMC consumption on microbiota localization (distance of the closest bacteria

225 from the surface of the epithelium), measured both pre- and post- intervention, for each participants

226 colored by group. Representative images are presented.

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278

Table S2. NMR data for the metabolites found in stool (S) and urine (U).

NO.	Metabolites	Moieties	δ ^1H (ppm)	δ ^{13}C (ppm)	Location	Source*
1	acetone	CH_3	2.23(s)[a]	33.2	S	
2	butyrate	CH_3	0.90(t)	16.3	S	microbial
		βCH_2	1.56(m)	21.5		
		αCH_2	2.15(t)	42.7		
		COOH		186.8		
3	isoleucine	δCH_3	0.94(t)	14.2	S	
		γCH_3	1.01(d)	17.7		
		γCH_2	1.25(m)	27.5		
		$\gamma' CH_2$	1.48(m)	27.5		
		βCH	1.98(m)	37.7		
		αCH	3.67(d)	62.4		
		COOH		177.1		
4	leucine	δCH_3	0.96(d)	24.5	S	
		δCH_3	0.97(d)	23.5		
		γCH	1.69(m)	27.3		
		βCH_2	1.71(m)	42.8		
		αCH	3.74(t)	56.4		
		COOH		178.3		
5	valine	γCH_3	0.99(d)	19.6	S	
		γCH_3	1.04(d)	20.7		
		βCH	2.27(m)	32.0		
		αCH	3.62(d)	63.3		
		COOH		177.1		
6	propionate	CH_3	1.06(t)	13.2	S	microbial
		CH_2	2.19(q)	33.7		
		COOH		187.4		
7	lactate	CH_3	1.33(d)	22.5	S, U	
		CH	4.11(q)	71.9		
		COOH		185.3		
8	alanine	βCH_3	1.48(d)	19.2	S, U	
		αCH	3.79(q)	53.4		
		COOH		178.8		
9	lysine	γCH_2	1.48(m)	23.9	S	
		δCH_2	1.72(m)	29.4		
		βCH_2	1.90(m)	33.0		
		εCH_2	3.03(t)	42.2		
		αCH	3.76(t)	57.6		
		COOH		177.5		
10	acetate	CH_3	1.92(s)	26.2	S, U	microbial
		COOH		184.2		
11	glutamate	βCH_2	2.10(m)	30.1	S	
		$\beta' CH_2$	2.09(m)	30.1		
		γCH_2	2.36(m)	36.4		
		αCH	3.77(m)	57.6		

		C=O		184.0	
		COOH		177.5	
12	methionine	δCH₃	2.14(s)	16.8	S
		βCH₂	2.16(m)	33.2	
		γCH₂	2.65(t)	31.6	
		αCH	3.86(m)	56.9	
		COOH		176.6	
13	succinate	CH₂	2.41(s)	37.6	S, U
		COOH		184.4	
14	citrate	CH₂	2.54(d)	46.5	U
		'CH₂	2.66(d)	46.5	
		C-OH		76.4	
		COOH		181.5	
		COOH		183.9	
15	aspartate	βCH₂	2.68(m)	39.5	S
		β'CH₂	2.82(m)	39.5	
		αCH	3.91(m)	55.3	
		βCOOH		180.5	
		αCOOH		176.9	
16	asparagine	βCH₂	2.86(dd)	37.6	S
		β'CH₂	2.96(dd)	37.6	
		αCH	4.00(m)	54.3	
		C=O		177.1	
		COOH		176.3	
17	dimethylamine (DMA)	CH₃	2.72(s)	39.4	U
18	creatine	CH₃	3.04(s)	40.0	S
		CH₂	3.93(s)	57.1	
		C=NH		159.4	
		COOH		177.2	
19	choline	N(CH₃)₃	3.21(s)	56.8	S, U
		NCH₂	3.52(m)	58.5	
		OCH₂	4.07(m)	70.2	
20	taurine	CH₂SO₃	3.25(t)	50.7	S, U
		NCH₂	3.43(t)	38.5	
21	glycine	CH₂	3.57(s)	44.6	S, U
		COOH		175.2	
22	α-glucose	4CH	3.42(dd)	72.7	S
		2CH	3.54(dd)	74.9	
		3CH	3.73(dd)	76.2	
		5CH	3.83(dd)	74.4	
		6CH₂	3.83(dd)	63.7	
		1CH	5.24(d)	95.4	

23	β-glucose	2CH	3.26(dd)	77.5	S
		4CH	3.40(dd)	72.9	
		5CH	3.47(dd)	79.0	
		3CH	3.50(dd)	79.0	
		6CH	3.74(dd)	63.7	
		6CH'	3.90(dd)	63.9	
		1CH	4.45(d)	99.3	
24	uracil	CH	5.81(d)	103.9	S
		CH	7.54(d)	146.5	
		C=O		170.6	
		C=O		155.9	
25	fumarate	CH	6.53(s)	138.1	S
		COOH		179.2	
26	tyrosine	βCH$_2$	3.06(dd)	38.3	S
		β'CH$_2$	3.15(dd)	38.3	
		αCH	3.94(dd)	59.2	
		3 or 5CH	6.91(d)	118.8	
		2 or 6CH	7.20(d)	132.4	
		C(ring)		129.4	
		C-OH(ring)		157.7	
		COOH		177.1	
27	tryptophan	βCH$_2$	3.31(dd)	29.5	S
		β'CH$_2$	3.49(dd)	29.5	
		αCH	4.06(dd)	58.5	
		5CH	7.21(t)	122.5	
		6CH	7.29(t)	125.0	
		2CH	7.33(s)	128.2	
		7CH	7.55(d)	114.9	
		4CH	7.74(d)	121.5	
		COOH		177.4	
28	phenylalanine	βCH$_2$	3.13(dd)	38.4	S
		β'CH$_2$	3.29(dd)	38.4	
		αCH	3.98(dd)	59.3	
		2 or 6CH	7.33(m)	130.7	
		4CH	7.38(m)	131.9	
		3 or 5CH	7.43(m)	132.0	
		C(ring)		139.4	
		COOH		176.4	
29	histidine	βCH$_2$	3.14(dd)	30.8	S
		β'CH$_2$	3.25(dd)	30.8	
		αCH	3.99(dd)	58.7	
		5CH	7.08(s)	120.1	
		3CH	7.83(s)	138.3	
		C(ring)		133.6	
		COOH		176.4	
30	formate	CH	8.45(s)	172.4	S

31	hypoxanthine	8CH	8.20(s)	145.6	S	
		6CH	8.22(s)	149.2		
32	inosine	CH₂	3.85(dd)	63.8	S	
		'CH₂	3.92(dd)	63.8		
		5H'	4.28(q)	88.6		
		4H'	4.44(t)	73.4		
		2H'	6.10(d)	91.4		
		8H	8.24(s)	150.1		
		2H	8.34(s)	143.3		
33	xanthine	8CH	7.89(s)	144.0	S	
34	uridine	CH₂	3.81(d)	64.3	S	
		'CH₂	3.92(d)	64.3		
		4H'	4.14(q)	86.6		
		3H'	4.24(t)	73.1		
		2H'	4.36(t)	78.0		
		5H	5.90(d)	95.2		
		6H	5.91(d)	90.8		
		1H'	7.87(d)	144.1		
35	creatinine	CH₃	3.05(s)	33.2	U	
		CH₂	4.06(s)	59.2		
36	trimethylamine N-oixide (TMAO)	N-CH₃	3.27(s)	62.5	U	microbial
37	hippurate	αCH₂	3.97(s)	47.2	U	
		3 or 5 CH	7.56(dd)	132.1		
		4CH	7.64(t)	135.5		
		2 or 6 CH	7.83(dd)	130.2		
		NH	8.56(brs)			
		C=O		173.3		
		COOH		180.0		
38	phenylacetylglycine (PAG)	CH₂	3.65(s)	45.2	U	
		2 or 6 CH	7.36(m)	132.0		
		4CH	7.36(m)	119.3		
		3 or 5 CH	7.42(m)	132.0		
		C=O		167.8		
39	p-hydroxyphenylacetate	CH₂	3.45(s)	46.9	U	microbial
		2 or 6 CH	6.87(d)	118.2		
		3 or 5 CH	7.16(d)	133.4		
		C-OH		156.9		
		COOH		182.7		
40	indoxyl sulfate	5CH	7.20(m)	123.0	U	microbial
		6CH	7.27(m)	125.2		
		2CH	7.36(s)	118.7		
		7CH	7.50(m)	115.0		
		4CH	7.70(m)	120.3		

41	1-methylnicotinamide	CH$_3$	4.48(s)	51.3	U
		5CH	8.18(m)	130.9	
		4CH	8.89(dt)	146.4	
		6CH	8.96(m)	150.0	
		2CH	9.27(m)	147.9	
42	*scyllo*-inositol	CHOH	3.35(s)	74.3	U

ª s, singlet; d, double; t, triplet; q, quartet; m, multiplet; dd, double of doubles; dt, double of triplet.
*Only the microbial metabolic products are indicated. Other metabolites have more complicated sources [18-20].

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