A randomized trial to evaluate the impact of copra meal hydrolysate on gastrointestinal symptoms and gut microbiome

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The impact of copra meal hydrolysate (CMH) on gut health was assessed by conducting a double-blind, placebo-controlled study. Sixty healthy adult participants aged 18-40 (BMI = 21-22 kg/m^2) were assigned 3 g CMH, 5 g CMH or a placebo of drink powder to consume daily for 21 days. Consumption of CMH at 3 g/d improved defecating conditions by reducing stool size and also relieved flatulence and bloating symptoms. Fecal samples were collected serially at the baseline before treatment, after the treatment period and after a 2-week washout period. The gut microbiomes were remarkably similar across the treatment groups, with, microbial community changes observed within the groups. Intake of CMH at 3 g/d led to increased microbial diversity and richness. Reduction was observed in the ratio between *Firmicutes* to *Bacteroidetes*, that related to reduced risk of obesity. The 3 g/d CMH treatment increased beneficial microbes in the group of fiber-degrading bacteria, especially human colonic *Bacteroidetes*. While induction of *Bifidobacteriaceae* was observed after the washout period. Intake of CMH led to increased lactic acid production, while 3 g/d supplementation promoted Immunoglobulin A (IgA) in stool samples. A 3 g daily dose of CMH led to a potentially beneficial effect on gut health for healthy individuals.

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Abstract

The impact of copra meal hydrolysate (CMH) on gut health was assessed by conducting a double-blinded, placebo-controlled study. Sixty healthy adult participants, aged 18-40 years were assigned to daily consume 3 g of CMH, 5 g of CMH or placebo in the form of drink powder for 21 days. Consumption of CMH at 3 g/d improved defecating conditions by reducing stool size and also relieved flatulence and bloating symptoms. Fecal samples were collected serially at the baseline before treatment, after the treatment and after a 2-week washout period. The gut microbiomes were similar among the treatment groups, with microbial community changes observed within the groups. Intake of CMH at 3 g/d led to increase microbial diversity and richness. Reduction of the ratio between *Firmicutes* to *Bacteroidetes* was observed , although it was not significantly different between the groups. The 3 g/d CMH treatment increased beneficial microbes in the group of fiber-degrading bacteria, especially human colonic *Bacteroidetes,* while induction of *Bifidobacteriaceae* was observed after the washout period. Intake of CMH led to increase lactic acid production, while 3 g/d supplement promoted the present of immunoglobulin A (IgA) in stool samples. The 3 g daily dose of CMH led to the potentially beneficial effects on gut health for healthy individuals.

Keywords: Copra meal hydrolysate (CMH), prebiotic, mannooligosaccharides, human gut microbiome, short-chain fatty acid

Introduction

The human intestine contains trillions of microbes that have been linked to many aspects of health and disease, including metabolic syndrome, infectious diseases and noncommunicable diseases (Jarett et al. 2019). Prebiotics are considered to be functional foods that can modulate the gut microbiome (Prayoonthien et al. 2019). The definition of prebiotics by The International Scientific Association of Probiotics and Prebiotics (ISAPP) is "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al. 2017). Prebiotics increase levels of beneficial bacteria and positively impact the composition of resident microbiota, while also promoting microbe-derived metabolites or signal molecules such as short chain fatty acids (SCFAs) that are important for gut health (Gibson et al. 2017; Markowiak-Kopeć & Śliżewska 2020; Topping 1996). Prebiotics have a mild laxative effect on bowel habits due to indigestible properties. Some prebiotics, especially inulin and oligofructose, alleviate constipation by increasing the fecal bulk (Andersson et al. 2001; Cummings & Macfarlane 2002).

Copra meal contains galactomannan as a byproduct from the coconut milk industry. To improve the functional food properties of copra meal, galactomannan was successfully hydrolyzed to mannooligosaccharides (MOS) by the enzyme β-mannanase (Pangsri et al. 2015; Rungruangsaphakun & Keawsompong 2018; Titapoka et al. 2007). Copra meal hydrolysate (CMH) has been hypothesized as a potential prebiotic. CMH was stable under upper human gastrointestinal tract conditions (Prayoonthien et al. 2019). *In vitro* human fecal fermentation showed that CMH promoted the growth of *Lactobacilli* and *Bifidobacteria* similar to fructooligosaccharides (FOS). Induction of two beneficial microbes enhanced SCFAs production

65   (Prayoonthien et al. 2019). CMH also suppressed pathogenic bacteria such as *Salmonella*,

66   *Escherichia coli*, *Staphylococcus aureus* and *Shigella dysenteriae* (Prayoonthien et al. 2019).

67   Comparing with other prebiotics, documentation of the health-promoting effect of MOS

68   on humans is limited. Impact of MOS from coffee hydrolysate revealed that MOS

69   supplementation over for three weeks promoted the growth of *Lactobacilli* and *Bifidobacteria*

70   (Walton et al. 2010). MOS derived from coffee mannan reduced fat absorption in humans

71   (Asano et al. 2006), while MOS reduced the concentration of triglycerides in serum, and

72   subsequently inhibited absorption of lipids in subjects fed a high-fat diet (Kumao et al. 2006).

73   No official dose recommendation of prebiotics in human diet has been reported. Most

74   prebiotics require an oral dose of 3 - 5 g/d to confer a gut health benefit, depending on prebiotic

75   structure. Daily dosages are suggested at around 5 g for fructooligosaccharides (FOS),

76   galactooligosaccharides (GOS) and plant sources of prebiotics (Gibson et al. 2017). This is the

77   first clinical study to evaluate the impact of daily consumption of CMH over three weeks on the

78   gut microbiome, defecating conditions, and gastrointestinal symptoms in healthy volunteers.

79   Different doses of daily consumption were also investigated.

80   **Materials and Methods**

81   **Preparation of copra meal hydrolysate and placebo drinks**

82   Copra meal hydrolysate (CMH) was prepared following Rungruangsaphakun &

83   Keawsompong (2018). The CMH powder contained 9.7% crude protein, 1% crude fat, 14.5%

84   crude fiber, 12.3% ash and 53% nitrogen-free extract. The nitrogen-free extract was composed of

85   3-6 molecules of MOS, mainly mannose, linking together. Amount of total oligosaccharide of

86   CMH was 14.77%; mainly mannooligosaccharide with 3-6 degrees of polymerization. The CMH

87   drinks were prepared in three formulas: the placebo group (maltodextrin 10 g), the 3 g CMH group

88   (3 g CMH and 7 g maltodextrin) and the 5 g CMH group (5 g CMH and 5 g maltodextrin). The

89   products were packaged in individual daily-use sachets of identical appearance and completely

90   dissolved in room temperature drinking water before consumption.

91   **Study design**

92   This study was a randomized double-blinded placebo-controlled trial with a three-arm parallel

93   group design in 1:1:1 ratio as the placebo group, CMH 3 g (3CMH group) and CMH 5 g (5CMH

94   group) (Figure 1). Healthy adults, aged between 18 and 40 years with a body mass index (BMI) of

95   18.5 to 25.0 kg/m^2, were enrolled (Table S1). This study excluded those who (1) received any

96   antibiotics or anti-viral agents within 3 months before the study, (2) received probiotics/prebiotics

97   within 1 month before the study, and (3) were allergic to coconut and soybean which were components

98   of the study product. Recruitment was conducted through poster and web-based advertising. The study

99   was carried out at the outpatient clinic of King Chulalongkorn Memorial Hospital, Bangkok, Thailand.

100   Physicians and study nurse who involved in the study explained the protocol and enrolled participants

101   to the study. Written informed consent was obtained from all participants. The study was approved by

102   the Ethics Committee of King Chulalongkorn Memorial Hospital, Bangkok, Thailand, under approval

103   reference number 388/61 and registered on the Thai Clinical Trials Registry (TCTR20190426003).

104   **Sample size calculation**

105   The sample size was determined using the tests for two independent means (two-tailed test) as

106   formula described below. On the basis of previously published study of the effect of MOS from coffee

107   mannan on fecal microflora (Asano et al. 2006), the mean change of stool *Bifidobacterium* from

108   baseline of subjects received MOS 3 g (µ1) was 20%, while for those received MOS 1g (µ2) was 10%.

109   The difference (Δ) was used to calculate, with a power of approximately 80% (beta 0.2) and a

110   significance level of alpha 0.05. The ratio (r) was 1. SD was estimated to be 8. The numbers of study

111   participants to be included was 11 subjects per group. With the anticipation of 20% drop out rate, at

112   least 14 subjects per group should be included. So, in our study, we included 20 subjects per group.

$$n_1 = \frac{(z_{1-\frac{\alpha}{2}} + z_{1-\beta})^2 \left[\sigma_1^2 + \frac{\sigma_2^2}{r}\right]}{\Delta^2}$$

$$r = \frac{n_2}{n_1}, \ \Delta = \mu_1 - \mu_2$$

113

114   **Randomization and allocation concealment**

115   Each eligible participant was randomized to 1 of 3 groups and assigned a unique study number.

116   A   blocked   randomization   list   was   created   using   Sealed   Envelope   Ltd.

117   (https://www.sealedenvelope.com/simple-randomizer/v1/lists), with block sizes of 6 by the statistician

118   who was not aware of the code group assigned

119   participants to interventions.

120   **Intervention**

121   After randomization, participants were subjected to a 2-week baseline period, 3-week

122   treatment period and 2-week washout period with instruction to use the products once daily during the

123   treatment period (Figure 2). Participants were asked to fill out a daily stool record chart and a 24-hour

124   diary record 3 times per week (2 weekdays and 1 day during weekend) throughout the study period.

125   Dietary restrictions were imposed throughout the entire study period (total 49 days), with prohibition

126   of consumption of other fermented products, probiotics, prebiotics and synbiotics supplements to limit

127   potential interference with evaluation of the testing product. The participants were excluded from the

128   study if (1) received dietary supplements described above during the study, (2) received any antibiotics

129   or anti-viral agents during the study and (3) failed to collect stool samples according to the study

130   protocol. Each participant visited the clinic weekly throughout the study. Stool samples were collected

131   at the baseline visit, the end of treatment, and washout periods. Subjects and researchers who had

132   access to the outcomes of the study were blinded to the code group assigned to participants.

Stool record chart

134   The stool record charts, adapted from Walton et al. (2010), were recorded daily by all

135   participants throughout the study, in order to investigate whether the products affected the stool

136   frequency, diarrhea, constipation, and gastrointestinal discomfort (for examples bloating symptoms)

137   of the subjects (Table S2). Each stool characteristic was scored as a number and calculated as amean

138   ($\pm$ SD) per week.

24-hour dietary record

140   The 24-hour dietary records were filled in by the subjects 3 times per week (2 weekdays and 1

141   day during weekend) throughout the study period. Energy and nutritive values were calculated using

142   the nutrient calculation computer software INMUCAL-Nutrients V4.0 database NB. 4 (Food

143   Composition Database for INMUCAL Program, 2018) (Table S3).

Stool collection and processing

145   Fresh stools were collected at the baseline, at the end of the treatment period and the end of the

146   washout period for analysis of microbiota, SCFAs and secretory immunoglobulin A (IgA). A stool

147   sample of 20 g was collected in a 76 $\times$ 20 mm sterile container and immediately placed on ice for

148   transfer to storage at -80 °C. Stool samples were prepared following the method of Kisuse et al. (2018)

149   for microbiota and SCFA analyses. The fecal samples were diluted 10-fold with phosphate-buffered

150   saline (pH 8.0) using a stomacher blender (Stomacher® 80 Biomaster, Seward, Worthing, UK) for 5

151   min. A 1 ml sample of fecal slurry was then centrifuged at 13,000$\times g$ for 5 min., and the supernatant

152   was stored at -80 °C for subsequent SCFA analysis. For microbial DNA extraction, 1 ml of fecal slurry

153   was placed in a 1.5 ml centrifuge tube and then stored at -80 °C.

Microbial DNA extraction and 16S rRNA gene sequencing

155   Microbial DNA was extracted according to the modified method of Mirsepasi et al. (2014),

156   utilizing a bead meter and a QIAamp® DNA stool mini kit (Qiagen GmbH, Germany). Qualification

157   and quantification of DNA were determined using a Nanodrop Spectrophotometer (Thermo Fisher

158   Scientific, USA). Extracted DNA samples were immediately stored at -20℃.

159   The variable region of V3-V4 of 16S rRNA genes was amplified using the forward primer

160   Imina-V3-V4-F   (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC

161   WGCAG-3') and reverse primer Imina-V3-V4-R (5'- GTCTCGTGGGCTCGGAGATGTGTAT

162   AAGAGACAGGACTACTACHVGGGTATCTAATCC-3'). Cycling conditions consisted of an

163   initial denaturation at 94 °C for 2 min, followed by 25 cycles of denaturation at 94 °C for 20 seconds,

164   annealing at 57 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C

165   for 10 min. The amplified products were purified using NucleoSpin® Gel and PCR Clean-up

166   (Macherey-Nagel Inc., USA) according to the manufacturer's protocol and sent to the Omics Sciences

167   and Bioinformatics Center (Faculty of Science, Chulalongkorn University, Bangkok, Thailand) for

168   further sequencing using the Illumina MiSeq platform (USA).

Processing of 16S rRNA gene sequences and data analysis

170   The raw sequences were processed using bioinformatics tools. Paired-end reads were first

171   quality trimmed using BBDUK (read quality > 15 at 3'), and the primer at the 5' end was removed

172   using seqtk (https://github.com/lh3/seqtk). Any obtained sequence shorter than 150 bp was

173   excluded along with its pair. Filtered pairs of sequences were corrected, merged and chimeras were

174   removed using the DADA2 pipeline (v1.10) (Callahan et al. 2016). High-quality sequences were

175 obtained at 41,843±10,553 reads per sample. Taxonomy was identified using the QIIME2

176 classifier (v2019.1) (Bolyen et al. 2019) with the Greengenes version 13.8 database. Cutoff

177 confidence was indicated at 0.7.

178   The PICRUSt2 pipeline package (v2.1.0_b) was used to predict the functional potential of

179 gut microbiomes from observable 16S rRNA sequences. The KEGG pathway levels were inferred

180 according to the instructions from the PICRUSt2 website (Douglas & Maffei 2020).

181 **Alpha and beta diversity analysis**

182   Alpha diversity (Chao1, Shannon) and beta diversity were calculated using the Vegan package

183 of R software (version 2.5.6). Statistical differences in the diversity indices among the three groups

184 were identified using the Kruskal-Wallis H test. Beta-diversity among participants was visualized via

185 non-metric multidimensional scaling (NMDS) ordination based on Bray–Curtis dissimilarity matrices.

186 **Short-chain fatty acid analysis**

187   Lactic acid, acetic acid, butyric acid, and propionic acid from the fecal slurries were analyzed

188 using high-performance liquid chromatography (HPLC) (Water 1525, USA). Samples were prepared

189 following the modified method of Wang et al. (2019). The supernatant was collected using

190 centrifugation at 13,000×g at 4 °C for 5 min and mixed with 0.2% v/v tartaric acid as the internal

191 standard (ratio 3:1). Before injection, the mixed solution was filtered through a 0.2 μm PVDF filter

192 (Verical, Thailand). SCFAs were isolated using an Aminex HPX-87H column (300 x 7.8 mm Bio-

193 Rad, USA) at 50 °C, with 8 mM sulfuric acid used as the mobile phase at a flow rate of 0.60 ml/min

194 for 60 min and wavelength 210 nm using a UV detector (Waters 2489, USA). The injector was set

195 with a split ratio of 20:1. Agilent Technologies 7890A equipment (Santa Clara, USA) was used for

196   data collection and calculation of all parameters. Standard solutions were prepared from lactic acid,

197   acetic acid, propionic acid and butyric acid at concentrations of 1, 2, 2.5, 5, 10 and 20 mmol/ml.

198   **Stool secretory IgA**

199   Analysis of secretory immunoglobulin A (IgA) in stool samples was performed in 55 out

200   of the 56 subjects who completed the study (19 in 3CMH, 17 in 5CMH, 19 in placebo group).

201   Samples were collected at the baseline and after the treatment period and stored at -80 °C. All

202   fecal samples were thawed at room temperature and weighed to 0.1 g. Phosphate-buffered saline

203   was added to the samples, then they were mixed in a stomacher machine and centrifuged at

204   14,000 rpm at 4 °C for 15 min. A sample of 200 µl (10X) of supernatant was collected and

205   diluted to 200X for the single plex human isotyping assay. IgA was measured using a Bio-Plex

206   Pro™ Human Isotyping kit (cat no. 171A3101M, Bio-Rad, USA). The Bio-Plex Pro™ assays

207   were essentially immunoassays formatted on magnetic beads by captured antibodies directed

208   against the desired biomarker coupled to the beads. The coupled beads reacted with samples

209   containing the IgA biomarker of interest, and biotinylated detection antibody was added to create

210   a sandwich complex. The final detection complex was formed with addition of streptavidin-

211   phycoerythrin conjugated using the Bio-Plex® 200 system reader (Bio-Rad, USA). The

212   concentration of analyte bound to each bead was proportional to the median fluorescence

213   intensity of the reporter signal.

214   **Statistical analysis**

215   Statistical analyses were performed using the statistical software package SPSS version 17.

216   Statistical significance among the three groups was determined using one way ANOVA and the

217   Kruskal-Wallis test for parametric and non-parametric tests, respectively. Comparisons of the two

218 groups were determined using the independent t-test and Mann-Whitney U-test for parametric and

219 non-parametric tests, respectively.

220   Linear mixed models of bacterial relative abundance were performed for each participant

221 using R package (2.15.0) including age, gender, carbohydrate intake, fiber intake, dose of CMH

222 supplement and sampling period.

223 **Results**

224 **Demographic characteristics of study subjects**

225   Of the 227 subjects screened, 60 were enrolled in the project at visit 1 and 56 completed

226 the study (Figure 1). The drop out occurred randomly due to the use of antibiotics and anti-viral

227 treatment of subjects, which happened to occur in CMH group. Another drop-out subject was

228 due to the non-compliance of stool collection. Therefore, the higher drop out in CMH groups

229 was unlikely related to the testing products. The participants were randomly assigned to the three

230 subgroups, each of which had similar demographic characteristics (Table 1). Participants in the

231 3CMH group were older than those in the placebo group. During the testing period, energy, and

232 nutrient consumption values of the placebo, 3CMH and 5CMH groups were calculated (Table 2).

233 Results showed that all subjects consumed similar carbohydrate, protein, fat, fiber, and sugar

234 contents. Therefore, the energy consumption was also similar in all groups.

235 **Influence of CMH on defecating conditions and gastrointestinal symptoms**

236   Defecating conditions and gastrointestinal symptoms in the baseline period of all groups

237 were similar (Table S4). Interestingly, stool size of participants in the 3CMH group was

238 significantly smaller than that in the placebo group during the testing period ($p < 0.05$). The

239 effect was still apparent even after the products were discontinued (washout period). Stool size

240   from the 5CMH group was also smaller than that from the placebo group but did not reach

241   statistical significance. Participants in the 3CMH and 5CMH groups showed improvement in

242   defecating conditions including less strong smell, softened stools and reduced gastrointestinal

243   discomfort (such as bloating, flatulence and abdominal discomfort) compared to the placebo

244   group during the testing and washout period but without statistical significance (Table S4).

245   Median changes of defecating and gastrointestinal symptoms between the baseline and

246   testing period or washout period were analyzed. Two factors including size of stool and

247   flatulence/bloating symptoms showed significant difference between sample groups (Figure 3).

248   Comparing with the baseline, size of stool and flatulence/bloating symptoms of the 3CMH group

249   reduced and reduction level was significantly lower than the placebo group ($p = 0.059$).

250   Similarly, reduction of stool size and flatulence/bloating symptoms in the 3CMH group was

251   observed in the washout period when compared to the baseline. Median level change was also

252   significantly lower than the placebo group ($p = 0.045$). Unlike the 3CMH group, median changes

253   of stool size and flatulence/bloating symptoms from baseline to testing period in the 5CMH

254   group were similar to the placebo group. Flatulence and bloating symptoms were significantly

255   greater in the 5CMH group at washout period compared with the baseline and significantly lower

256   than the placebo group ($p \leq 0.05$).

257   **Influence of CMH on microbial richness and diversity**

258   Microbial richness indices of stool samples in each group were monitored during the

259   baseline, testing and washout periods (Figure S1). The Chao1 index of the 3CMH group during

260   the testing and washout periods increased compared to the baseline, while the Chao indices for

261   the placebo and 5CMH groups decreased and were stable, respectively.

262   Similarly, the Shannon diversity index for the 3CMH group showed an increasing trend

263   during the testing and washout periods compared to the baseline (Figure S1), while Shannon

264   diversity indices of the 5CMH and placebo groups during the baseline, testing and washout

265   periods were stable. These results suggested that the administration of CMH at 3 g/d promoted

266   microbial diversity and richness.

267   The microbial communities from all groups and time points were intermingled in non-

268   metric multidimensional scaling plots using Bray–Curtis dissimilarity (Figure S1). Similar to the

269   result of alpha diversity, beta-diversity showed no differences or clusters due to CMH intake or

270   sampling period.

271   **Influence of CMH on fecal microbiota**

272   To assess specific changes in gut microbiota caused by CMH, gut microbiota from

273   different taxa were identified from the three sampling periods (baseline, testing, and washout)

274   (Table S5, S6 and S7). Six microbiota phyla (*Actinobacteria, Bacteroides, Firmicutes,*

275   *Lentisphaerae, Proteobacteria* and *Verrucomicrobia*) were found in all groups (Table S8). The

276   most dominant phyla in each period were *Firmicutes, Bacteroides* and *Actinobacteria*. Dynamic

277   changing patterns during the baseline, testing and washout periods of all phyla from the three

278   groups were similar. No significant changes were recorded in the microbial phyla in the CMH

279   treatment. It was observed that *Firmicutes* and *Bacteroidetes* ratios at the testing period for both

280   the 3CMH and 5CMH groups were lower than the placebo, although they were not significantly

281   different (Table S8).

282   At the family level, changes in gut microbiota during testing period were similar in all

283   groups (Figure 4A). Supplementation with 3CMH promoted various microbes including

284   *Desulfovibrionaceae, Bifidobacteriaceae, Barnesiellaceae, Veillonellaceae, Lachnospiraceae,*

285 *Clostridiaceae, Rikenellaceae, Prevotellaceae, Porphyromonadaceae, Paraprevotellaceae,*

286 *Odoribacteraceae, and Bacteroidaceae,* while eight families of gut microbiota in the placebo and

287 5CMH groups were stable (mean change = 0) and six families decreased. *Ruminococcaceae* was

288 reduced in the 3CMH group, whereas it increased in the placebo and 5CMH groups. Median

289 change in *Ruminococcaceae* in the placebo group was significantly greater than in the 3CMH

290 group ($p \leq 0.05$).

291   After the washout period, median changes in gut microbiota were similar in all groups

292 (Figure 4B). However, induction of *Bifidobacteriaceae* was observed in the 3CMH group even

293 after the products were discontinued, while showing reduction in the placebo and 5CMH groups.

294 The amount of *Enterobacteriaceae* reduced in the 3CMH and placebo groups, with significantly

295 different median change compared with the 5CMH group ($p \leq 0.05$), that showed an increase

296 during the washout period.

297   To confirm the relationship of changes of three families including *Ruminococcaceae,*

298 *Bifidobacteriaceae* and *Enterobacteriaceae* with CMH intake, the linear mixed effect model was

299 performed (Table S9). Results revealed that gender, age, sampling period, carbohydrate and fiber

300 consumption had no significant effect on changes in the three families. Supplementation with

301 3CMH showed a significant effect on *Ruminococcaceae* observed during testing period, while

302 with 5CMH significantly affected *Bifidobacteriaceae* and *Enterobacteriaceae* during the

303 washout period ($p \leq 0.05$)

304   At the testing period, predictions of functional analysis of gut microbiota were focused on

305 carbohydrate-related metabolism (Figure 5). Among the fifteen pathways, median change in

306 relative abundance of the two pathways, ascorbate/aldarate and fructose/mannose metabolism

307 showed significant differences between the three groups. The gene function associated with

308   ascorbate/aldarate and fructose/mannose metabolism in the 3CMH group significantly increased

309   more than in the placebo group ($p \leq 0.05$). However, median changes in the 5CMH group were

310   similar to those in the placebo group, indicating that CMH intake enhanced the microbial

311   pathways of ascorbate/aldarate and fructose/mannose metabolism.

312   **Influence of CMH on fecal short-chain fatty acids**

313   The major fecal metabolite in all samples were acetic acid, followed by butyric acid,

314   propionic acid, and lactic acid (Table 3). The SCFAs in all subjects at the baseline were similar.

315   Intake of CMH had no effect on the four SCFAs and they were similar during the testing period;

316   however, significant differences among the three groups were observed during the washout period.

317   Highest abundance of lactic acid was recorded in the 5CMH group followed by the 3CMH and

318   placebo groups ($p<0.05$). The amount of lactic acid in the 5CMH group was significantly higher

319   than in the placebo group but similar to the 3CMH group. The level of butyric acid in the placebo

320   group was significantly greater than in the 3CMH group.

321   **Influence of secretory immunoglobulin A in stools**

322   Results revealed no significant differences in secretory IgA levels in stools among all

323   groups both at the baseline and after the testing period (Table 4). Forty-two percent of the

324   participants who received 3CMH treatment showed increased stool secretory IgA compared to

325   20% of the participants in the 5CMH and placebo groups. The increase in IgA correlated with

326   increased stool frequency ($r = 0.303$ to 0.039) and reduced abdominal discomfort symptoms ($r = -$

327   0.238) during the treatment period.

328   **Discussion**

329   The health effects of dietary fibers have been accepted worldwide (Carlson et. al. 2018).

330   However, health effects of CMH have not been well defined, especially in human. To address

331   this lack of knowledge, a randomized, double-blinded placebo-controlled design was used to

332   determine the impact on gut health of CMH containing mannooligosaccharides. Two different

333   dosages of CMH, 3 and 5 g/d, were investigated for gastrointestinal symptoms and defecating

334   condition. Consumption of CMH 3 g/d improved defecating condition and gastrointestinal

335   symptoms by reducing the size of stools and ameliorating flatulate/bloating symptoms (Table

336   S4). A smaller stool is easier to expel and improves discomfort (Bannister et al. 1987). This

337   effect was still observed after discontinuing CMH consumption. Prebiotics at a dose of $\leq$ 6 g/d

338   reduced flatulence but a higher dose might cause intestinal bloating, pain, flatulence, or diarrhea

339   (Gibson et al. 2010; Moretti et al. 2018; Piemontese et al. 2011; Scholtens et al. 2014; Wilson et

340   al. 2019). In our study, intake of 5 grams of CMH per day did not induce any flatulence/bloating

341   or discomfort, while a dose of 3 g/d had more impact on gastrointestinal symptoms.

342   Prebiotics contribute to change in the gut microbial community by acting as a primary

343   carbon source. Several reports revealed that prebiotics enhanced the intestinal microbial

344   community (Carlson et. al. 2018). Our results suggested that consumption of CMH at both 3 and

345   5 g/d did not significantly change the gut microbial richness and diversity (Figure S1), which

346   tended to increase after the testing period in the 3CMH group, with higher levels still maintained

347   after the washout period.

348   Only a few microbiome phyla changed as the result of CMH treatments (Table S8). The

349   *Firmicutes/Bacteroidetes* ratio of the 3CMH and 5CMH groups after the testing period tended to

350   be lower than in the placebo group. A high *Firmicutes/Bacteroidetes* ratio has been linked with

351   overweight and obesity (Castaner et al. 2018; Verdam et al. 2013). A positive effect of MOS on

352   gut microbiota modulation of obesity and fat metabolism has been reported. Intake of MOS, in

353   mice fed with a high-fat diet, decreased the *Firmicutes/Bacteroidetes* ratio (Wang et al. 2018).

354   Moreover, coffee MOS also inhibited the intestinal absorption of dietary fat and enhanced fat

355   excretion in healthy adults (Kumao & Fujii 2006; Kumao et al. 2005).

356   Enhancement of *Bifidobacteriaceae*, *Lactobacillus, Enterobacteriaceae* and

357   *Enterococcus* spp. was observed in CMH gut model fermentation (Prayoonthien et al. 2018;

358   Prayoonthien et al. 2019). In a human trial, intake of CMH at 3 g/d enhanced human colonic

359   *Bacteroidetes* including *Rikenellaceae, Prevotellaceae, Porphyromonadaceae,*

360   *Paraprevotellaceae, Odoribacteraceae, and Bacteroidaceae.* These bacteria contained

361   hemicellulose-degrading enzymes that hydrolyzed xylans, mannans and galactomannans (Flint et

362   al. 2008; Flint et al. 2012; Holscher et al. 2015). Intake of 3 g/d of CMH promoted several

363   intestinal microbes than the 5CMH group except for *Ruminococcaceae.*

364   Our result was supported by PICRUSt analysis (Figure 5). Changes in genes related to

365   fructose/mannose metabolism in the 3CMH group increased after the testing period, with

366   significantly higher median change than in the placebo group. Change in fructose/mannose

367   metabolism in the 5CMH group also increased after the testing period but was lower than in the

368   3CMH group.

369   Induction of *Bifidobacteriaceae* was not observed after the testing period (Figure 4), but

370   was found in the 3CMH group during the washout period. This result suggested that CMH

371   treatment indirectly introduced the conditions for enrichment of *Bifidobacteriaceae.* This trend

372   was more evident in the washout period. Induction of *Bifidobacteriaceae* during the washout

373   period of 5CMH was not detected, whereas the family *Enterobacteriaceae* increased. A possible

374   explanation for this is selective utilization in small doses but loss of activity when in excess, as

375   other microbes may become involved in fermentation (Walton et al. 2010).

376   Prebiotics are selectively fermented by the gut microbiome to produce SCFAs but effects

377   of CMH on SCFA production remain unclear in human studies (Table 3). SCFA measurement in

378   feces was not reflected the amount within the colon and 95% of SCFAs produced by the gut

379   microbiome were absorbed (Walton et al. 2010). However, levels of lactic acid in feces of the

380   3CMH group were higher than in the placebo.

381   Prebiotics have been reported to boost the immune system through modulation of the gut

382   microbiome (Hemarajata & Versalovic 2013). The daily consumption of 3 g of CMH promoted

383   the increase level of IgA, whereas no induction effect was observed following a high dose of

384   CMH. Moreover, the percentage of subjects whose stool IgA value increased was higher in the

385   3CMH group than in the placebo and 5CMH groups. This result was consistent with other

386   studies reporting that daily consumption of MOS at $1 - 3$ g was more effective on the gut

387   microbiome and immune modification than a higher dosage (Asano et al. 2004; Kumao et al.

388   2006; Umemura et al. 2004a; Umemura et al. 2004b; Walton et al. 2010).

389   Our experimental design showed a high rate of compliance, however there were several

390   limitations. The lack of significant improvement in gut health could be attributed to the small

391   sample size of the intervention study. Participants in the placebo group were mostly younger

392   than the treatment groups. Our research samples were mostly from healthy adult females (77%).

393   Future clinical studies are needed to provide on gender and age associated disparities in the

394   effect of CMH on defecating conditions, gastrointestinal symptoms, and gut microbiome

395   modulation.

Conclusion

396

397   CMH is a novel potential source of prebiotics and has shown health benefits in human

398 studies. Consumption of CMH at 3 g/d improved defecating conditions and gastrointestinal

399 symptoms. Intake of CMH at 3 g/d also improved the gut microbiome by increasing beneficial

400 microbes, their metabolites and host immunity. Our results suggest that CMH has a prebiotic

401 effect when taken at 3 g/d by healthy individuals. This finding can also increase CMH value for

402 functional food industry application.

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403

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Demographic characteristics of study participants at baseline

All values are expressed as mean ± SD. [a,b] Significance between sample groups by the Kruskal-Wallis test are indicated by different lowercase superscripts ($p \leq 0.05$).

1   **Table 1** Demographic characteristics of study participants at baseline.

Variable	Placebo	3CMH	5CMH	_p_ value
Number	20	19	17	0.998
Age (year)	27.7 ± 4^b	32 ± 5^a	31 ± 5^{ab}	0.041
Sex (female/male)	15/5	15/4	13/4	0.958
Body mass index (kg/m^2)	21.6 ± 2.1	22 ± 2	21 ± 2	0.231

3   All values are expressed as mean ± SD

4   [a,b]Significance between sample groups by the Kruskal-Wallis test are indicated by different
5   lowercase superscripts ($p \leq 0.05$).

Energy and nutrient consumption of placebo, 3CMH and 5CMH subjects during testing period.

All values are expressed as median and interquartile range (IQR)

1 **Table 2** Energy and nutrient consumption of placebo, 3CMH and 5CMH subjects during the

2 testing period.

Item	Average intake/day			p value
	Placebo (n=20)	**3CMH (n=19)**	**5CMH (n=17)**	
Carbohydrate (g)	153.95 (135.98, 236.54)	160.89 (163.54, 206.81)	166.46 (143.71, 186.50)	0.404
Protein(g)	59.16 (52.39, 74.36)	61.02 (52.46, 74.32)	61.02 (46.04, 68.38)	0.307
Fat (g)	52.38 (42.93, 69.32)	52.38 (44.35, 60.15)	52.50 (37.77, 55.08)	0.505
Fiber (g)	6.09 (4.89, 9.58)	6.09 (5.4, 9.15)	6.42 (4.92, 8.90)	0.365
Sugar (g)	44.18 (40.16, 93.09)	44.18 (48.38, 91.34)	54.90(37.5, 69.26)	0.396
Saturated fat (g)	16.10 (12.18, 20.56)	16.10 (12.35, 19.27)	15.93 (12.4, 15.61)	0.330
Energy (kcal)	1388.28 (1182.42, 1719.00)	1427.81 (1318.17, 1585.92)	1482.45 (1089.36, 1448.69)	0.341

4 All values are expressed as median and interquartile range (IQR)

Short chain fatty acid concentration of feces at baseline, testing and washout.

*Significant difference between sample groups indicated by the Kruskal-Wallis test (p ≤ 0.05). Descriptive statistic, interquartile range (IQR)

1   **Table 3** Short chain fatty acid concentration of feces at baseline, testing and washout.

SCFA	Time Point	Placebo Median (IQR) (µmol/g feces)	3CMH Median (IQR) (µmol/g feces)	5CMH Median (IQR) (µmol/g feces)	*p*-value			
					P vs 3CMH vs 5CMH	P vs 3CMH	P vs 5CMH	3CMH vs 5CMH
Lactic acid	Baseline	17.15 (11.84, 23.31)	21.35 (18.41, 24.24)	21.89 (17.34, 39.97)	0.125	0.193	0.058	0.347
	Testing	19.68 (15.02, 27.97)	30.67 (20.21, 36.7)	17.69 (16.44, 28.13)	0.123	0.098	0.65	0.068
	Washout	17.7 (4.79, 21)	24.36 (19.11, 31.62)	26.47 (20.87, 29.22)	0.015*	0.057	0.005*	0.664
Acetic acid	Baseline	58.21 (50.51, 79.36)	61.42 (48.41, 79.1)	55.4 (47.16, 66.76)	0.588	0.866	0.377	0.366
	Testing	64.28 (45.18, 70.16)	52.72 (44.39, 69.98)	51.18 (44.13, 62.7)	0.349	0.465	0.135	0.552
	Washout	61.78 (46.2, 75.22)	61.78 (41.43, 77.79)	53.83 (48.95, 71.56)	0.947	0.866	0.927	0.716
Propionic acid	Baseline	23.72 (16.49, 27.18) (n=19)	23.12 (15.86, 28.83)	24.15 (18.71, 34.85)	0.604	0.942	0.311	0.476
	Testing	25.63 (17.58, 30.68)	25.16 (17.11, 29.15)	23.33 (15.82, 26.5)	0.493	0.8	0.273	0.35
	Washout	24.35 (17.16, 28.04)	24.86 (17.95, 31.01)	24.74 (21.76, 28.49)	0.725	0.536	0.465	0.862
Butyric acid	Baseline	29.2 (20.1, 37.1)	29.36 (24.26, 41.67)	29.42 (18.08, 36.87)	0.992	0.955	0.879	1
	Testing	32.48 (24.5, 39.48)	27.55 (22.02, 41.36)	32.94 (24.71, 45)	0.656	0.339	0.784	0.623
	Washout	35.69 (25.48, 40.45)	26.24 (21.54, 32)	28.34 (23.84, 38.21)	0.2	0.049*	0.537	0.476

3   *Significant difference between sample groups indicated by the Kruskal-Wallis test ($p \leq 0.05$).

4   Descriptive statistic, interquartile range (IQR)

Quantity of Immunoglobulin A (IgA) in stool samples.

* indicates significant (p ≤ 0.05) difference. Descriptive statistic, interquartile range (IQR)

1   **Table 4** Quantity of Immunoglobulin A (IgA) in stool samples.

Stool IgA	Placebo (n=19) Median (pg/mL) (IQR)	3CMH (n=19) Median (pg/mL) (IQR)	5CMH (n=17) Median (pg/mL) (IQR)	p-value
Baseline	79069 (13216, 331439.01)	22441.1 (7789.4, 332368.98)	33852.5 (13573.8, 137549.8)	0.654
Testing	50919.7 (13580.8, 332101.51)	37519.6 (10274.7, 331891.65)	14500.4 (3156.4, 39546.6)	0.147
Median change (IQR)	-2914.7 (-241278.22, 3065.58)	2322.3 (-11346.5, 11236.6)	-10800.2 (-88488.5, -1495.07)	0.190
p-value $^{\neq}$	0.198	0.687	0.013	
Change of stool IgA	Placebo (n=19) N (%)	3CMH (n=19) N (%)	5CMH (n=17) N (%)	p-value
Increase	4 (21.1%)	8 (42.1%)	3 (17.6%)	0.195
Decrease	8 (42.1%)	5 (26.3%)	12 (70.6%)	0.027*
Same	7 (36.8%)	6 (31.6%)	2 (11.8%)	0.211

3   * indicates significant ($p \leq 0.05$) difference.

4   Descriptive statistic, interquartile range (IQR)

Figure 1

Flow chart design of the copra meal hydrolysate (CMH) study.
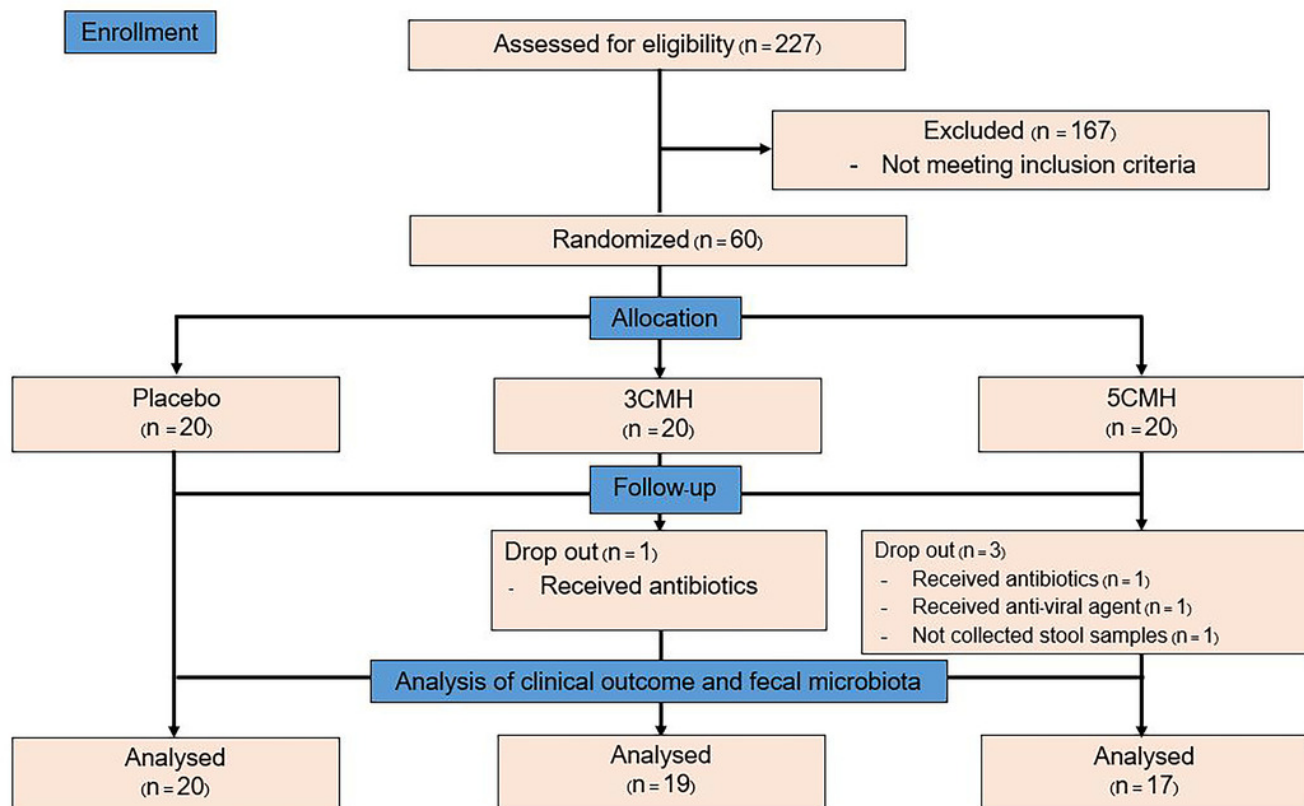
Figure 2

Timeline of copra meal hydrolysate (CMH) studied, including sample groups and fecal collection.
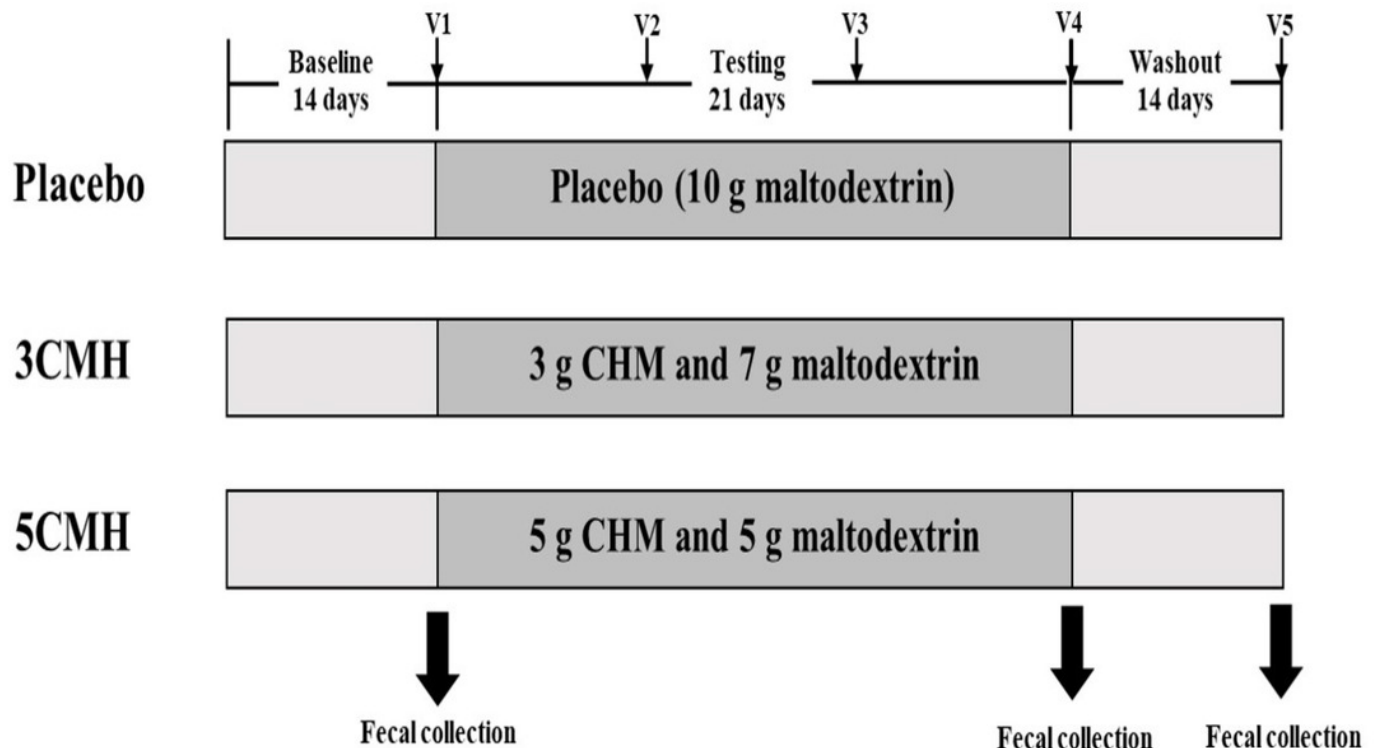
Figure 3

Significant change of defecating condition and gastrointestinal symptoms of placebo, 3CMH and 5CMH groups (p ≤ 0.059).

Comparison between the three groups, different letters (a, b) show significant difference (p ≤ 0.05).
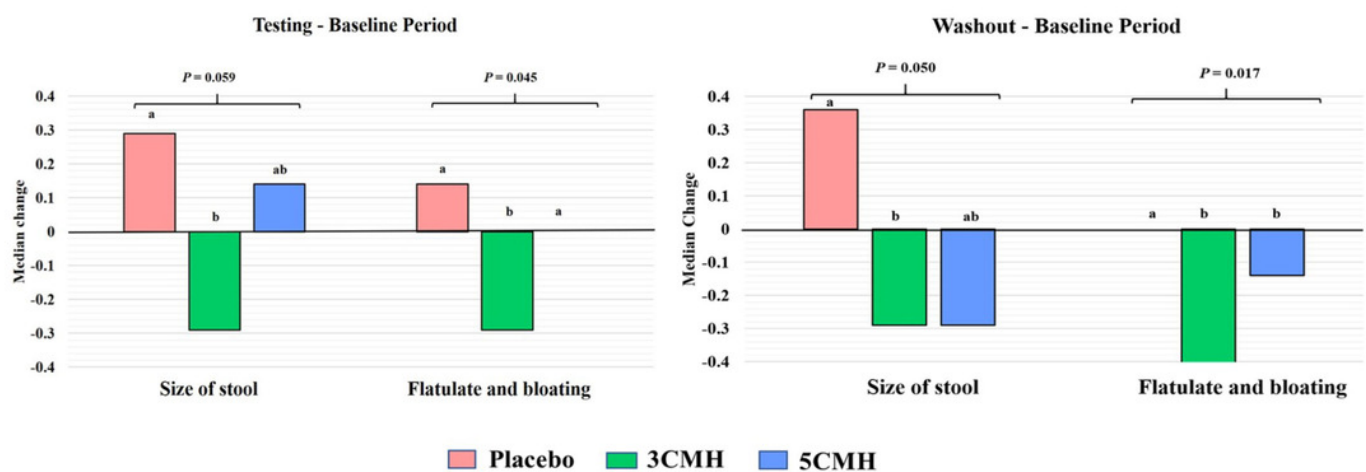
Figure 4

Change of gut microbiota at family level for testing (A) and washout period (B) of placebo, 3CMH and 5CMH groups compared to the baseline.

* Significant difference between placebo, 3CMH and 5CMH groups is shown by different letters (p ≤ 0.05).
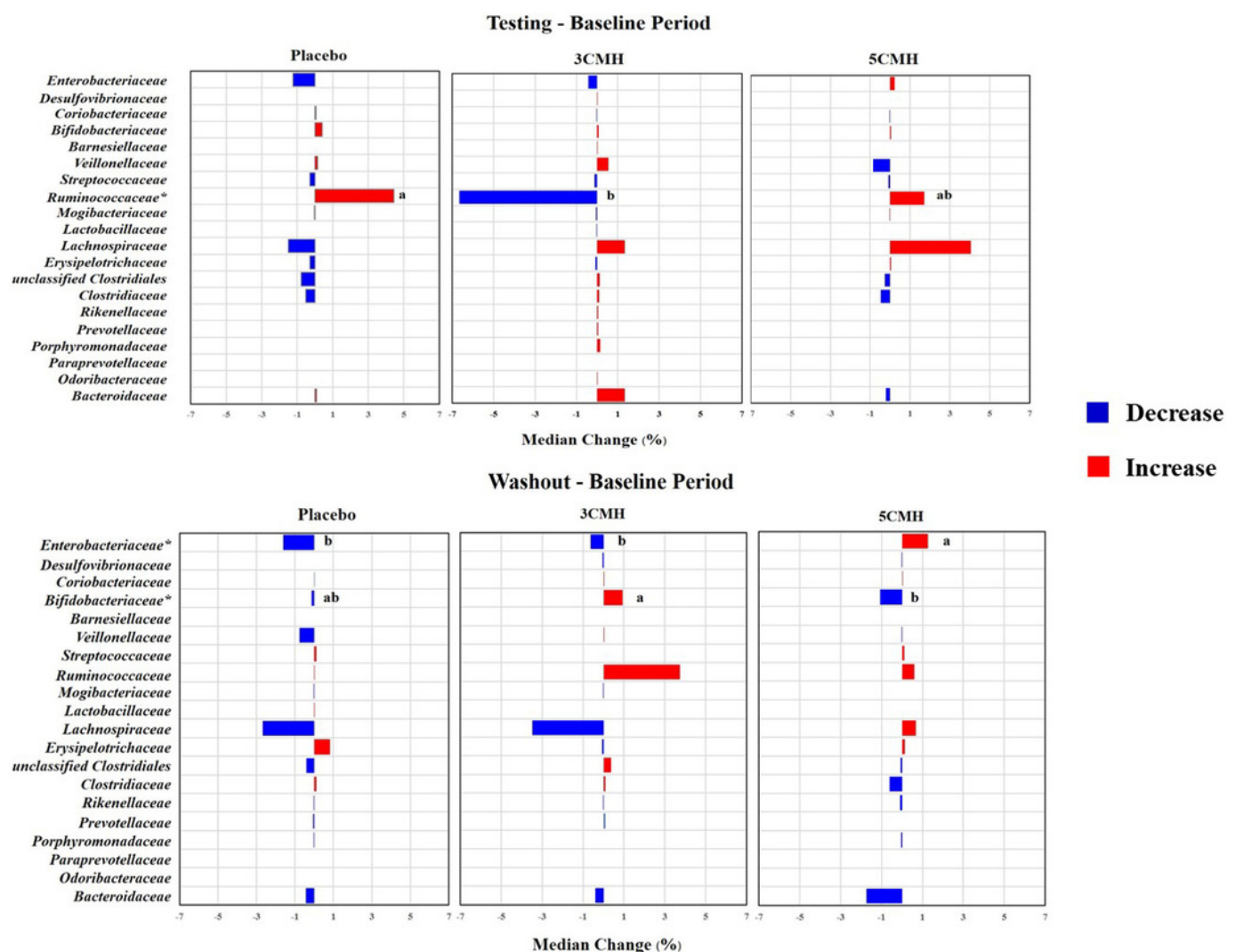
Figure 5

Prediction of change in carbohydrate related metabolism by PICRUSt analysis from baseline to testing period between placebo (white), 3CMH (gray) and 5CMH (black) groups.

* Significant difference between sample groups is shown by different letters (p ≤ 0.05).