

Gut bacterial nutrient preferences quantified in vivo

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Abstract

Great progress has been made in understanding gut microbiome's products and their effects on health and disease. Less attention, however, has been given to the inputs that gut bacteria consume. Here we quantitatively examine inputs and outputs of the mouse gut microbiome, using isotope tracing. The main input to microbial carbohydrate fermentation is dietary fiber, and to branched-chain fatty acids and aromatic metabolites is dietary protein. In addition, circulating host lactate, 3-hydroxybutyrate and urea (but not glucose or amino acids) feed the gut microbiome. To determine nutrient preferences across bacteria, we traced into genus-specific bacterial protein sequences. We find systematic differences in nutrient use: Most genera in the phylum Firmicutes prefer dietary protein, *Bacteroides* dietary fiber, and *Akkermansia* circulating host lactate. Such preferences correlate with microbiome composition changes in response to dietary modifications. Thus, diet shapes the microbiome by promoting the growth of bacteria that preferentially use the ingested nutrients.

37 INTRODUCTION

38 The gut microbiome possesses an enormous diversity of enzymes, exceeding the number in
39 mammals' genomes by more than 100-fold (Qin et al., 2010). This enzymatic capacity enables the
40 processing of incoming dietary nutrients into a broad spectrum of microbial metabolites. Some of
41 these reach the host circulation at substantial concentrations (Lai et al., 2021; Quinn et al., 2020).
42 Microbial metabolites can play important roles in host pathophysiology. For example, short-chain
43 fatty acids (SCFAs; acetate, propionate, butyrate) (Dalile et al., 2019; Koh et al., 2016),
44 trimethylamine N-oxide (Tang et al., 2013), secondary bile acids (Arab et al., 2017; Funabashi et
45 al., 2020), indole-3-propionate (Wikoff et al., 2009), and imidazole propionate (Koh et al., 2018)
46 affect immune maturation (Campbell et al., 2020; Hang et al., 2019), insulin sensitivity (Koh et
47 al., 2018), cancer growth (Garrett, 2015; Yoshimoto et al., 2013), and cardiovascular disease
48 (Nemet et al., 2020; Wang et al., 2011).

49 Both to replicate themselves and to release metabolic products, gut bacteria require nutrient inputs.
50 These come in forms including ingested food, host-synthesized gut mucus (Desai et al., 2016;
51 Sicard et al., 2017), and host circulating metabolites (Scheiman et al., 2019). The availability of
52 dietary nutrients to gut microbiota depends on the extent of host absorption: nutrients that are
53 absorbed in the small intestine, like starch, are not available to the colonic microbiome. In contrast,
54 nutrients that are poorly digested in the upper gastrointestinal tract, like fiber, can be key
55 microbiome feedstocks (Lund et al., 2021; Wong and Jenkins, 2007).

56 Isotope tracing enables the measurement of the inputs to metabolites and biomass in a quantitative
57 manner. Such studies, employing radioactive tracers, defined the basics of mammalian metabolism
58 (Wolfe, 1984). Recent work has increasingly relied on stable isotope tracers coupled to mass
59 spectrometry detection, which enables the measurement of labeling in specific downstream
60 products (Fernández-García et al., 2020; McCabe and Previs, 2004). This approach has revealed
61 fundamental features of host metabolism, such as circulating lactate being a major TCA fuel
62 (Faubert et al., 2017; Hui et al., 2017). In addition, it has provided important insights into host-
63 microbiome metabolic interplay. For example, it revealed that dietary fructose is processed by the
64 microbiome into acetate, which fuels hepatic lipogenesis (Jang et al., 2018; Zhao et al., 2020).

65 In principle, stable isotope tracing coupled to mass spectrometry can also be applied to determine
66 the metabolic inputs to specific microbes, based on measuring labeling in bacteria-specific peptide
67 sequences (Berry et al., 2015; Holmes et al., 2017; Oberbach et al., 2017; Reese et al., 2018; Zhang
68 et al., 2016a, 2016b). By infusing nitrogen-labeled threonine to label host mucus, investigators
69 were able to compare the contribution of dietary versus mucus protein to the gut microbiome and
70 observed a shift towards more mucus contribution in a low-protein diet condition (Holmes et al.,
71 2017).

72 Here, we perform the first large-scale, quantitative assessment of the metabolic inputs to the gut
73 microbiome and its products. We examine the contributions from dietary starch, fiber, and protein,
74 host mucus, and most major circulating host nutrients, finding that lactate, 3-hydroxybutyrate, and
75 urea stand out for passing from the host to the gut microbiome. Moreover, based on the
76 measurement of bacteria-specific peptide sequences, we assess the nutrient preferences of different
77 bacterial genera and show that these preferences align with microbiome composition changes in
78 response to altered diet.

79 **RESULTS**

80 **Microbiome consumes less digestible dietary components**

81 A major mechanism by which the microbiome may impact host physiology is via secreted
82 metabolic products. As the intestine and colon drain into the portal circulation, metabolites
83 produced by the gut microbiome should be enriched in the portal relative to systemic blood. We
84 measured, in the portal, systemic circulation and the cecal contents, the absolute concentrations of
85 more than 50 metabolites characterized in the literature as microbiome-derived (Campbell et al.,
86 2020; De Vadder et al., 2014; Han et al., 2021; Hang et al., 2019; Koh et al., 2018; Mager et al.,
87 2020; Ridlon et al., 2014; Wikoff et al., 2009) (**Figure 1A, S1A, Table 1, S1-2**). Most microbiome
88 metabolites were elevated in the portal circulation relative to systemic blood, and all but two
89 (inosine and N-acetyl-tryptophan, which are apparently mainly host derived) were depleted by
90 antibiotics treatment.

91 The dominant excreted products on a molar basis (0.4 – 2 mM in the portal blood) are SCFAs.
92 Other relatively abundant microbiome products (10 - 30 uM) are aromatic amino acid fermentation
93 products (phenol, indoxyl sulfate, and 3-phenylpropionate) and branched-chain fatty acids
94 (valerate, isovalerate, 4-methylvalerate, isobutyrate, 2-methylbutyrate). While primary bile acids
95 were also present in the portal circulation at up to ~ 10 uM concentration, these are produced
96 by the host and accordingly were not included in Table 1. Secondary bile acids, which are produced
97 from primary bile acids by the microbiome, were lower in absolute concentration, the most
98 abundant being tauroursodeoxycholic acid (3 uM in portal vein).

99 To probe the dietary inputs to gut microbial products, we began by feeding mice, by oral gavage,
100 starch (readily digestible glucose polymer) and inulin (slowly digestible fructose polymer, i.e.,
101 soluble fiber) (**Figure 1B**). Following ¹³C-starch gavage, labeled glucose, lactate, and alanine
102 quickly appeared in the portal circulation (**Figure 1C, S1B**). In contrast, after ¹³C-inulin gavage,
103 substantial labeled fructose, glucose, lactate, and alanine were not observed, and instead labeled
104 portal metabolites slowly appeared in the form of SCFAs (**Figure 1C, S1C**). Quantitative analysis
105 based on the portal-systemic differences in serum metabolite labeling (Jang et al., 2018) revealed
106 that most starch carbons (~75%) become circulating glucose, lactate, and alanine, with no

107 discernible labeling of SCFAs. In contrast, ~ 40% of inulin carbons become SCFAs (**Figure S1C**),
108 with the remainder being undigested and excreted in the feces (**Figure S1D**). Measurement of
109 cecal content revealed that dietary inulin, but not starch, extensively labeled glycolytic and TCA
110 intermediates and amino acids in the cecal content (**Figure 1D**).

111 We next carried out similar experiments, comparing the gavage of a free amino acid mixture to
112 algal protein, both uniformly ^{13}C -labeled (**Figure 1B**). The free amino acid feeding resulted in the
113 rapid appearance of labeled amino acids in portal circulation, while the algal protein did not
114 (**Figure 1E**). Instead, the algal protein, but not free amino acids, substantially labeled amino acids
115 within the cecal contents (**Figure 1F**). Moreover, the algal protein copiously labeled microbiome-
116 derived portal vein metabolites: SCFAs, branched-chain fatty acids, and aromatics (indole, indole-
117 3-propionate, 3-phenylpropionate) (**Figure S1E-F**). Thus, poorly digestible carbohydrates and
118 protein feed the microbiome directly, and the host indirectly via microbiome-derived products.

119 **Few circulating metabolites reach the microbiome**

120 Next, we examined the possibility that nutrients in host circulation feed the gut microbiota. We
121 infused deuterated water and eighteen major circulating nutrients (^{13}C -labeled) into the systemic
122 circulation of pre-catheterized mice (**Figure 2A**). The infusion rates were selected to achieve
123 modest but readily measurable labeling without substantially perturbing circulating concentrations.
124 Circulating labeling reached a steady-state by 2.5 h, at which time we collected serum and feces
125 to quantitate the carbon contributions of each circulating nutrient to the corresponding fecal
126 metabolites. Upon intravenous infusion of ^{13}C -lactate, fecal lactate labeled rapidly (**Figure 2B**).
127 Most infused circulating nutrients, however, did not penetrate the feces (**Figure 2C-D**). Indeed,
128 while water fully exchanged with the feces, among abundant circulating carbon carriers, only
129 lactate and 3-hydroxybutyrate penetrated. Glucose, amino acids, TCA intermediates and fatty
130 acids did not. Both lactate and 3-hydroxybutyrate are substrates of monocarboxylate transporters
131 (MCTs), which are highly expressed in the colonic epithelium (Halestrap and Price, 1999, p. 1).
132 Thus, in contrast to most host circulating metabolites, which do not reach the colonic microbiome,
133 monocarboxylic transporters render circulating lactate and 3-hydroxybutyrate accessible to gut
134 microbes.

135 **Circulating urea is a microbiome nitrogen source**

136 In addition to carbon, nitrogen is a fundamental constituent of all living cells. To assess nitrogen
137 sources of the gut microbiome, we infused twelve abundant circulating nutrients in ^{15}N -labeled
138 form. Nitrogen from circulating urea and ammonia, but not amino acids, penetrates the feces and
139 contributes to microbiome amino acids (**Figure 2E, S2A-B**).

140 The liver converts ammonia into urea. Accordingly, we were curious if ammonia contributes to
141 the microbiome directly, or only indirectly after being converted by the host into circulating urea
142 (Bartman et al., 2021). Ammonia's contribution is quantitatively explained by the product of its
143 contribution to circulating urea and urea's microbiome contribution (**Figure S2C-D**). Thus, urea
144 is the main circulating host metabolite that provides nitrogen to the gut microbiome.

145 **Microbiota synthesize amino acids from fiber and urea**

146 To determine the physiological sources of microbiome metabolites, we measured their labeling
147 after *ad libitum* feeding of isotopically enriched food. To this end, we fed mice standard chow with
148 a portion of the fiber, fat, or protein ^{13}C -labeled. To account for circulating nutrient inputs, we also
149 infused ^{13}C -lactate or 3-hydroxybutyrate (**Figure 3A, S3A**). These studies identified a majority of
150 the carbon feeding into most microbiome central metabolites, with glycolytic and pentose
151 phosphate metabolites labeling almost exclusively coming from dietary fiber (inulin), while
152 pyruvate and TCA metabolites are also labeled from dietary algal protein and circulating lactate
153 (**Figure 3B**).

154 We next examined inputs to microbiome amino acids, tracing also with ^{15}N -labeled dietary protein
155 and infused urea. Unlike mammals, most gut bacteria have the biosynthetic capacity to make all
156 20 proteogenic amino acids. Nevertheless, we observed that “essential amino acids,” which cannot
157 be made by mammals and require the expression of extensive biosynthetic pathways in bacteria,
158 are derived mainly from dietary proteins. In contrast, “non-essential amino acids” are primarily
159 synthesized within the gut microbiome, using dietary inulin and circulating lactate as carbon
160 sources (**Figure 3C**). Dietary protein was the main nitrogen source for both essential and non-
161 essential amino acids, with host urea also contributing substantially to the non-essential amino
162 acids (**Figure 3D**). Importantly, the amino acids synthesized by the microbiome, stay in the
163 microbiome: We do not observe discernible labeling of these amino acids in the host (**Figure S3B**).
164 Consistent with the gut microbiome synthesizing amino acids from fiber carbon and urea nitrogen,
165 across amino acids, urea's nitrogen contribution correlated with inulin's carbon contribution
166 (**Figure 3E**). Thus, the microbiome obtains amino acids from a blend of dietary protein catabolism
167 and *de novo* synthesis fed by dietary fiber and urea.

168 **Diverse microbiome products come from dietary protein**

169 We next examined, using isotope tracing, the carbon inputs to the microbiome products, especially
170 the ones excreted into the portal circulation (**Table 1**). SCFAs, the most abundant microbial
171 metabolites, come mainly from dietary fiber with minor contributions from dietary protein and
172 host circulating lactate. Many less abundant ones, however, are almost exclusively derived from
173 dietary protein (**Table 1**).

174 In addition to classical microbiome products, we also observed metabolites that are made in a
175 collaborative manner, with the host carrying out the final synthesis using microbiome-derived
176 inputs. For example, a wide range of microbiome-derived carboxylic acids are conjugated to
177 glycine in the liver and the kidneys to make different acyl-glycines (**Figure S4A-D**) (Wikoff et al.,
178 2009).

179 We also examined the host clearance mechanisms of microbiome metabolites, based on arterial-
180 venous gradients across the liver and kidney and levels in the urine. SCFAs and branched-chain
181 fatty acids were avidly consumed by the liver, consistent with their much greater abundance in the
182 portal than systemic circulation. Most microbiome-derived metabolites were excreted by the
183 kidney into the urine, with the notable exception of SCFAs, which are actively reabsorbed (**Table**
184 **S1**). Thus, we establish dietary protein as a major precursor to many microbiome metabolites and
185 identify host-microbiome interplay in the metabolism of SCFAs, including their renal reabsorption
186 and use by liver and kidney for the synthesis of acyl-glycines.

187 **Gut bacterial growth is synchronized with host feeding**

188 Thus far, we have reported inputs and outputs of the gut microbiome as a whole. We now shift to
189 examining the growth and metabolism of specific bacterial genera. To this end, we deployed
190 proteomics to measure gut microbial peptides and their labeling, focusing on peptide sequences
191 specific to a single bacterial genus (**Figure 4A**).

192 To quantify protein synthesis in different gut microbial genera, we used D₂O tracing (Holmes et
193 al., 2015; O'Brien et al., 2020). To achieve steady-state labeling of body water, we gave mice D₂O
194 by bolus injection followed by mixing it into drinking water. Peptide labeling in the cecal contents
195 was then measured by proteomics (**Figure 4B**).

196 A key technical challenge in using proteomics to read out metabolic activity is the complexity,
197 arising from natural isotope abundances, of peptide mass spectra. We used liquid chromatography-
198 high resolution mass spectrometry to obtain the full scan (MS1) mass isotope distribution for each
199 peptide of interest, with MS/MS analysis of the unlabeled form used to determine the peptide's
200 identity. We then calculated, based on the mass isotope distribution, the fraction of peptide that
201 was newly synthesized (θ). To this end, first, we calculated the mass isotope distribution of
202 unlabeled peptides based on natural isotope abundances ("old"). Second, we calculated the
203 expected mass isotope distribution of a newly-synthesized peptide generated from cecal free amino
204 acids, whose labeling we experimentally measured by metabolomics. Then, we determined the
205 fraction of newly synthesized (θ) by linear interpolation between the "old" and "newly synthesized"
206 spectra (**Figure 4C**).

207 For each bacterial genus, we measured the newly synthesized fraction (θ) for a minimum of 5
208 peptides, with abundant gut bacteria yielding θ for over 100 characteristic peptides. Irrespective
209 of their intracellular location, different peptides from the same bacterial genus tended to label at a
210 similar rate, suggesting that the peptide labeling rate largely reflects bacterial growth rate (**Figure**
211 **4D, S5A**). Labeling rate varied across bacterial genera, with a half doubling time ranging from 2.5
212 h for *Akkermansia* to 8 h for *Lactobacillus*, which still markedly exceeded the labeling rate of host
213 intestinal proteins (> 24 h half doubling time) (**Figure 4E-F, Figure S5B**).

214 Our prior analyses revealed that the microbiome is fed substantially by dietary components.
215 Accordingly, we hypothesized that microbial growth synchronizes with physiological feeding,
216 which in mice occurs mainly during the nighttime. To assess the diurnal rhythm of gut bacterial
217 protein synthesis, mice were given D₂O for 6 h intervals throughout the diurnal cycle, followed by
218 proteomic analysis of their cecal contents. Every measured bacterial genus showed greater protein
219 synthesis during nighttime than daytime (**Figure 4G**). Thus, gut bacteria synthesize protein in sync
220 with the physiological feeding patterns of the host.

221 **Preferred carbon sources differ across gut bacteria**

222 Next, we quantitated the carbon feedstocks of different microbes, by combining ¹³C-nutrient
223 labeling and proteomics. Each ¹³C-labeled nutrient (dietary inulin, dietary algal protein, or
224 circulating lactate) was provided for 24 hours, which is sufficient to achieve steady-state labeling
225 in the gut bacteria. Our analysis strategy involved two steps: first, we calculated, based on each
226 genus-specific peptide's observed mass isotope distribution, its relative ¹³C-enrichment (γ)
227 compared to that of cecal free amino acids (**Figure 5A**). Mathematically, this calculation is
228 identical to the calculation of θ in the D₂O case, except here, the tracer is a particular ¹³C-labeled
229 nutrient, which unlike D₂O is used preferentially by certain bacterial genera. The observed
230 peptide's relative ¹³C-enrichment multiplied by the average contribution of that ¹³C-tracer to the
231 gut microbial amino acids pool ($L_{AA_avg \leftarrow nutrient}$) gives a quantitative measure of the tracer's
232 contribution to the observed genus-specific peptide. Averaging across such peptides gives a
233 fractional contribution of the ¹³C-labeled nutrient to protein synthesis in a bacterial genus.

234 Using this method, we measured feedstocks of the bacterial genera that were detected in every
235 proteomics experiment. We observed marked differences in nutrient preferences across members
236 of the microbiota. For example, *Bacteroides* and *Clostridium* use over four-fold more inulin than
237 *Akkermansia*, *Muribaculum*, or *Alistipes* (**Figure 5B**). Overall, bacteria from the phylum
238 Firmicutes, used more dietary protein than did Bacteroidetes (Firmicutes 0.237 ± 0.052 ;
239 Bacteroidetes 0.175 ± 0.031 , $p = 0.02$).

240 *Akkermansia*, which is generally considered a health-promoting gut microbe, used among the least
241 dietary inulin and protein (**Figure 5B-C**). In contrast, it used by far the most circulating lactate
242 from the host (**Figure 5D**).

243 We were curious whether these bacterial nutrient preferences predict microbiome composition
244 changes upon dietary changes. To explore this possibility, we fed mice an inulin-enriched or algal
245 protein-enriched diet for two days and measured microbiome composition by 16S rRNA
246 sequencing. Major bacteria genera with a relative abundance > 0.5% in 16S rRNA sequencing
247 were examined. *Bacteroides*, the top consumer of ¹³C-inulin, increased by 4-fold after high inulin
248 diet (**Figure 5E-G**). *Clostridium*, another high inulin consumer, also increased by 2-fold. Other
249 genera that use less inulin carbon were either unchanged or slightly decreased. Similar consistency
250 between microbes' nutrient preference and relative abundance changes was observed in mice fed
251 the algal protein-enriched diet (**Figure 5H-J**). Carbon-source preference measured by proteomics
252 ($f_{genus \leftarrow nutrient}$) correlates with abundance change following a diet shift measured by 16S
253 sequencing, for both the inulin and algal protein conditions (**Figure 5G, J**). Thus, the nutrient
254 preferences of different gut bacteria help explain microbiome compositional changes following
255 dietary manipulations (David et al., 2014).

256 **Firmicutes consume dietary protein while Bacteroidetes consume secreted host protein**

257 Lastly, we turned to the nitrogen source preferences of different gut bacteria, comparing ¹⁵N-
258 labeled dietary protein feeding to ¹⁵N-urea infusion. The analytical approach was identical to that
259 employed above for carbon source preferences. Bacterial genera that highly use carbon from
260 dietary protein also highly use nitrogen from dietary protein, consistent with amino acids from
261 dietary protein being assimilated intact in bacterial proteomes (**Figure 6A, S6A**).

262 Conversely, among members of the phylum Firmicutes, genera preferring urea nitrogen tended to
263 be avid inulin users, i.e. to synthesize their own amino acids using inulin and urea (**Figure 6B,**
264 **S6B**). Finally, again among Firmicutes, we also saw the expected trade-off where some genera
265 prefer nitrogen from dietary protein, and others from circulating urea (**Figure S6C**). The most
266 intriguing observation, however, was that bacteria from the phylum Firmicutes used more nitrogen
267 both from dietary proteins and from circulating urea than did Bacteroidetes (Dietary proteins:
268 Firmicutes 0.263 ± 0.044 ; Bacteroidetes 0.135 ± 0.006 , $p = 7.7 \times 10^{-5}$; Urea: Firmicutes
269 0.076 ± 0.019 ; Bacteroidetes 0.033 ± 0.012 , $p = 0.0001$) (**Figure 6A-B**).

270 The low use of both dietary protein and circulating urea nitrogen by Bacteroidetes raised a key
271 question: How do Bacteroidetes get nitrogen? It has been shown that some members of gut
272 microbiome (e.g. *Bacteroides* and *Akkermansia*) are capable of digesting host secreted proteins
273 such as mucins (Berry et al., 2013; Reese et al., 2018). We hypothesized that host secreted proteins

274 are a key source of Bacteroidetes nitrogen. To probe this possibility, we performed long-term (36
275 h) ¹⁵N-labeled lysine and arginine infusions to label host proteins in the colon (**Figure 6C and**
276 **Figure S7A-D**). Lysine and arginine do not directly feed the microbiome (**Figure 1E**) but did
277 make a discernible contribution after the long-term infusion, consistent with the labeling occurring
278 via host proteins. Such labeling occurred preferentially in Bacteroidetes peptides (**Figure 6D**).
279 *Akkermansia*, consistent with its mucin degrading capability, is another user of host secreted
280 proteins. The nitrogen contributions from dietary and secreted host proteins were anti-correlated,
281 consistent with some gut bacteria preferentially consuming dietary protein, and others host protein
282 (**Figure 6E**). Interestingly, bacterial genera with a higher preference for dietary protein, which is
283 dependent on host feeding, tend to grow more differently between daytime and nighttime, while
284 genera that prefer host secreted proteins, grow at a similar rate throughout a day. (**Figure S6D-E**).
285 Thus, dietary proteins and circulating urea are the major nitrogen feedstock of Firmicutes, while
286 secreted host proteins provide nitrogen to Bacteroidetes.

287 Discussion

288 As for most microbial communities, the composition of the gut microbiome is shaped by nutrient
289 availability. Here we developed quantitative isotope tracing approaches to measure the nutrient
290 preferences of gut bacteria. In addition to dietary fiber and secreted host proteins, we establish
291 dietary protein and circulating host lactate, 3-hydroxybutyrate, and urea as important nutrients
292 feeding gut bacteria. Importantly, we rule out direct contributions from other circulating host
293 nutrients, like glucose and amino acids, to the colonic microbiome.

294 A key technical achievement is enabling tracing from different carbon and nitrogen sources into
295 bacteria-specific peptides, thereby revealing the nutrient preferences of different bacteria within
296 the complex and competitive gut lumen environment. We find that Firmicutes and Bacteroidetes
297 differ systematically in their utilization of host secreted protein versus dietary protein: Firmicutes
298 tend to acquire amino acids from dietary protein, while Bacteroidetes rely more on secreted host
299 protein (**Figure 6F**). This may relate to different localization of bacteria within the colon, either in
300 terms of central versus peripheral (closer to host mucus) or distal versus proximal (closer to
301 incoming food remnants) (Albenberg et al., 2014; Li et al., 2015; Yasuda et al., 2015).

302 Within these two major families of gut bacteria, we found marked disparities in the use of dietary
303 fiber as a carbon source. The most abundant Bacteroidetes' genus is *Bacteroides*, and it was the
304 most avid assimilator of fiber (inulin). In contrast, other types of bacteria in the same phylum
305 hardly consumed inulin. Likewise, some Firmicutes like *Clostridium* avidly used fiber, while
306 others did not. Strikingly, feeding a fiber-enriched diet led to an increased abundance of
307 *Bacteroides* and *Clostridium*, the precise genera that most actively assimilate fiber based on
308 isotope tracing.

309 A similar trend was observed in the case of dietary supplementation with algal protein: Firmicutes,
310 which actively use such protein, tended to increase in abundance. Algal protein (the only type
311 commercially available in bulk in ^{13}C -labeled form) may be particularly hard for mammals to
312 digest. This is reflected in the limited appearance of ^{13}C -labeled amino acids from algal protein in
313 the portal circulation, and instead extensive passage from the intestine into the colon. This influx
314 of dietary protein to the microbiome was a major contributor to secreted microbiome metabolites:
315 dietary algal protein provided a portion of the carbon in the most abundant microbiome products
316 (e.g., SCFAs) and was the main source of most other microbiome-derived metabolites. An
317 important future question is whether the nature of dietary protein (e.g. plant or animal-based)
318 impacts passage through the small intestine to the colonic microbiome and thereby shapes
319 microbiome composition or metabolite secretion (Madsen et al., 2017; Wali et al., 2021).

320 Host circulating metabolite levels may also impact microbiome nutrient access and ultimately
321 composition. Here we show such effects are likely limited to the few host metabolites that
322 meaningfully penetrate the microbiome: urea, 3-hydroxybutyrate, and lactate. Among them,
323 lactate was recently shown to feed the gut microbiome in human marathon runners (Scheiman et
324 al., 2019). Among gut bacteria, *Akkermansia* most avidly use circulating lactate. *Akkermansia* are
325 mucin degraders, and their proximity to the gut epithelial wall may augment their access to lactate
326 from the host circulation. *Akkermansia* are more abundant in athletes, and exercise increases their
327 levels in mice and human (Liu et al., 2017; Munukka et al., 2018). A possible mechanism involves
328 increased circulating lactate levels following exercise directly feeding *Akkermansia*. Whether
329 lactate-induced *Akkermansia* growth in part mediates beneficial effects of exercise is an important
330 open question.

331 Ultimately, manipulating the microbiome requires understanding which nutrients different bacteria
332 consume, and how such consumption impacts microbiome composition and product secretion.
333 Through isotope tracing, including proteomic measurements that offer bacterial genus specificity,
334 we provide foundational knowledge about which nutrients feed the gut microbiome, and which
335 bacteria prefer which nutrients. Currently, our measurements are limited to young mice, fed typical
336 chow with or without fiber or algal protein supplementation. Furthermore, they are limited to the
337 genus level and to genera that we detect by proteomics and/or sequencing. Nevertheless, we cover
338 many important gut microbiome genera. Moreover, the methodologies developed here are poised
339 for broader application, to eventually contribute to the holistic and quantitative understanding of
340 the diet-microbiome-health connection.

341 **Methods**

342 **Mouse gavage and labeled nutrient feeding.** Mouse studies followed protocols approved by the
343 Princeton University Animal Care and Use Committee. Unless otherwise indicated, mice were

344 group-housed on a normal light-dark cycle (8:00-20:00) with free access to water and chow. For
345 the ^{13}C -nutrient gavage experiments, 7-9-week-old male C57BL/6NCrI mice (strain 027; Charles
346 River Laboratories) were fasted at 9 am and received a 1:2:4 mixture of inulin, protein/amino acids,
347 and starch (0.5 g kg^{-1} inulin, 1 g kg^{-1} protein/amino acids 2 g kg^{-1} starch dissolved in water) at 3
348 pm via oral gavage with a plastic feeding tube (Instech Laboratories). Food was given back at 8
349 pm.

350 For the experiments involving labeled nutrient feeding, the labeled diet was prepared by adding
351 $^{13}\text{C}/^{15}\text{N}$ -nutrients to a diet mixture premix (modified from normal diet with reduced protein, inulin,
352 and starch content, Research diets Inc, D20030303). The final enrichment for each labeled dietary
353 nutrient was 10% - 25% (with observed labeling corrected by dividing by the fraction dietary
354 nutrient labeled). The contribution of each dietary nutrient to metabolites is calculated by the
355 metabolite labeling enrichment normalized to the final enrichment of each labeled dietary nutrient.
356 All diets shared the same final macronutrient composition (40% starch, 20% protein or amino acids,
357 7.5% inulin and 2.5% cellulose). Male C57BL/6NCrI mice (7-9-week-old, strain 027, Charles
358 River Laboratories) were first adapted to a non-labeled diet (of identical composition to the
359 subsequent labeled diet) for 10 days, and then fed labeled diet for 24 h prior to sacrifice.

360 For the deuterium water drinking experiment, mice were administered a bolus intraperitoneal
361 injected of D_2O (1.26 % w/w relative to body weight), followed by having ad lib access to 3%
362 D_2O drinking water.

363 **Intravenous infusions.** To quantify contribution of circulating nutrients to microbiota metabolism,
364 9-11-week-old C57BL/6 mice were catheterized in house in the right jugular vein. The mice were
365 infused with carbon or nitrogen-labeled tracer starting at 3:30 pm without any fasting. Infusion
366 rate was 0.1 ul/min/g . Infusion solutions are described in **Table S3**. Overnight (24 h) infusions
367 both started and finished around 9 am. The contribution of circulating nutrient to each metabolite
368 is calculated by the metabolite labeling enrichment normalized to the average tracer serum
369 enrichment throughout 24 hr.

370 **Antibiotics treatment.** To deplete the mouse resident microbiome, an antibiotic drinking water
371 protocol was used. In brief, mice were treated with a cocktail of antibiotics (1 g/L ampicillin, 1
372 g/L neomycin, 1 g/L metronidazole, and 1 g/L vancomycin) in both their drinking water 14 days.
373 To make the drinking water more palatable, 5% aspartame was added. The effectiveness of
374 antibiotics treatments were verified by observing much lower SCFAs in the feces by LC-MS.

375 **Sample collection.** Systemic blood samples ($\sim 6 \mu\text{l}$) were collected by tail bleeding. For sampling
376 from tissue-specific draining veins, a mouse was put under anesthesia and different tissue veins
377 were exposed, and blood samples were pulled with an insulin syringe (BD insulin syringes, #
378 SY8290328291) insertion into the vein. Successful isolation of portal vein was confirmed by much

379 higher (> 10x) concentrations of SCFAs and secondary bile acids (deoxycholic acid and lithocholic
380 acid) than systemic vein; hepatic vein was confirmed by much lower secondary bile acids, SCFAs
381 and higher glucose, 3-hydroxybutyrate levels compared to portal vein. Mouse urine was collected
382 from the urinary bladder using a syringe. All serum samples were placed on ice without
383 anticoagulant for 15 min, and centrifuged at 16,000 x g for 15 min at 4 C.

384 Tissues were harvested by quick dissection and snap freezing (<5 sec) in liquid nitrogen with a
385 pre-cooled Wollenberger clamp; intestinal contents were removed before clamping. For cecal
386 content sampling, the mouse cecum was first removed and cut on the surface, then the cecal content
387 was squeezed out using a tweezer followed by freeze clamping. Whole liver, intestine, and
388 intestinal contents were collected and grounded to homogenous powder. To sample fresh feces,
389 the mouse belly was gently massaged to induce defecation and fresh feces were freeze clamped.
390 For long-term feces collection, a mouse was transferred to a new cage and mouse fecal pellets on
391 the bedding were collected every 1~2 h and freeze clamped. Serum, tissue, and feces samples were
392 kept at -80 °C until further analysis.

393 **16S rRNA gene amplicon sequencing and analysis.** Extraction of Bacterial DNA from cecal or
394 fecal samples was performed using the Power Soil DNA Isolation kit (QIAGEN). A section of the
395 16S rRNA gene (~250 bp, V4 region) was amplified, and Illumina sequencing libraries were
396 prepared from these amplicons according to a previously published protocol and primers
397 (Caporaso et al., 2012). Libraries were further pooled together at equal molar ratios and sequenced
398 on an Illumina HiSeq 2500 Rapid Flowcell or MiSeq as paired-end reads. These reads were 2x150
399 bp with an average depth of ~20,000 reads. Also included were 8 bp index reads, following the
400 manufacturer's protocol (Illumina, USA). Pass-Filter reads were generated from raw sequencing
401 reads using Illumina HiSeq Control Software. Samples were de-multiplexed using the index reads.
402 The DADA2 plugin within QIIME2 version 2018.6 was used to infer Amplicon sequencing
403 variants (ASVs) from the unmerged paired-end sequences (Bolyen et al., 2019; Callahan et al.,
404 2016). The forward reads were trimmed at 150 bp and the reverse reads trimmed at 140 bp, with
405 all other DADA2 as default. Taxonomy was assigned to the resulting ASVs with a naïve Bayes
406 classifier trained on the Greengenes database version 13.8, with only the target region of the 16S
407 rRNA gene used to train the classifier (Bokulich et al., 2018; McDonald et al., 2012). Downstream
408 analyses were performed MATLAB (Hunter, 2007; McKinney, 2010).

409 **Metabolite extraction.** For serum samples, 3 ul serum was added to 90 ul methanol and incubated
410 on ice for 10 min, followed by centrifugation at 17,000 × g for 10 min at 4°C. The supernatant was
411 transferred to an MS vial until further analysis. For tissues and feces samples, frozen samples were
412 first ground at liquid nitrogen temperature with a cryomill (Restch, Newtown, PA). The resulting
413 tissue powder was extracted with 40:40:20 methanol: acetonitrile: water (40 ul extraction solvent

414 per 1 mg tissue) for 10 min on ice, followed by centrifugation at 17,000 x g for 10 min, the
415 supernatant was transferred to a MS vial until further analysis.

416 **Measurements of metabolites, protein, and polysaccharides.** To measure metabolites in serum,
417 tissue and feces samples, a quadrupole orbitrap mass spectrometer (Q Exactive; Thermo Fisher
418 Scientific) was coupled to a Vanquish UHPLC system (Thermo Fisher Scientific) with
419 electrospray ionization and scan range m/z from 60 to 1000 at 1 Hz, with a 140,000 resolution. LC
420 separation was performed on an XBridge BEH Amide column (2.1×150 mm, 2.5 µm particle size,
421 130 Å pore size; Waters Corporation) using a gradient of solvent A (95:5 water: acetonitrile with
422 20 mM of ammonium acetate and 20 mM of ammonium hydroxide, pH 9.45) and solvent B
423 (acetonitrile). Flow rate was 150 µl/min. The LC gradient was: 0 min, 85% B; 2 min, 85% B; 3
424 min, 80% B; 5 min, 80% B; 6 min, 75% B; 7 min, 75% B; 8 min, 70% B; 9 min, 70% B; 10 min,
425 50% B; 12 min, 50% B; 13 min, 25% B; 16 min, 25% B; 18 min, 0% B; 23 min, 0% B; 24 min,
426 85% B; and 30 min, 85% B. Injection volume was 5-10 µl and autosampler temperature was set at
427 4°C. For cysteine measurement, samples were derivatized before measurement as follows: Serum,
428 cecal content or feces samples were extracted and centrifuged. To the supernatant, 2 mM N-
429 ethylmaleimide was added and incubated at room temperature for 20 min. The resulting mixture
430 was transferred to a MS vial. Derivatized cysteine has a m/z at 245.06015 in negative mode.

431 To quantify the metabolite concentration in serum and tissue samples, either isotope spike-in or
432 standard spike-in was performed. For isotope spike-in, known concentrations of isotope-labeled
433 standard were added to the serum or tissues extraction solution, then the concentration was
434 calculated by the ratio of labeled and unlabeled metabolites. When isotope standard is not available,
435 a serially diluted non-labeled standard was added, and a linear fitting between measured total ion
436 count and added concentration of standard was generated. Then, the concentration of endogenous
437 metabolite was determined by the x intercept of the fitting line.

438 Starch and inulin were measured by acid hydrolysis and LC-MS. In brief, 5-10 mg sample was
439 mixed with 10 µl 2 M hydrochloric acid, and samples were incubated at 80°C for 2 h. After cooling
440 down, the resulting mixture was neutralized with 12 µl saturated sodium bicarbonate, followed
441 with 88 µl 1:1 acetonitrile: methanol solution. After centrifugation at 17,000 × g for 10 min at 4°C,
442 the supernatant was transferred to a MS vial. Inulin and starch concentration in samples was
443 inferred from total ion count of fructose and glucose, respectively.

444 SCFAs and BCFAs were derivatized and measured by LC-MS. Serum (5 µl) or tissue samples
445 (~10 mg) were added to 100 µl derivatizing reagents containing 12 mM 1-Ethyl-3-(3-
446 dimethylaminopropyl) carbodiimide, 15 mM 3-Nitrophenylhydrazine hydrochloride acid and
447 pyridine (2% v/v) in methanol. The reaction was incubated at 4°C for 1 h. Then, the reaction
448 mixture was centrifuged at 17,000 g for 10 min. 20 µl supernatant was quenched with 200 µl 0.5

449 mM beta-mercaptoethanol in 0.1% formic acid water. After centrifugation at 17,000 g for 10 min,
450 the supernatant was transferred to MS vials until further analysis. The measurement of SCFAs and
451 BCFAs are performed using the same Q Exactive PLUS hybrid quadrupole-orbitrap mass
452 spectrometer with different column and LC setup. LC separation was on Acquity UPLC BEH C18
453 column (2.1 mm x 100 mm, 1.7 5 µm particle size, 130 Å pore size, Waters, Milford, MA) using
454 a gradient of solvent A (water) and solvent B (methanol). Flow rate was 200 µL/min. The LC
455 gradient was : 0 min, 10% B; 1 min, 10% B; 5 min, 30% B; 11 min 100% B; 14 min, 100% B;
456 14.5 min 10% B; 22 min 10 % B. Autosampler temperature was 5 °C, column temperature was
457 60 °C and injection volume was 10 µl. Ion masses for derivatized acetate, propionate, butyrate,
458 iso-butyrate, valeric acid, isovaleric acid, 2-methylbutyrate, 4-methylvaleric acid were 194.0571,
459 208.0728, 222.0884, 222.0884, 236.1041, 236.1041, 236.1041, 250.1197 in negative mode,
460 respectively.

461 **Proteomics sample preparation.** Proteomics samples were prepared mostly as previously
462 described (Gupta et al., 2018; Wühr et al., 2014). Mouse cecal samples (10 mg each) were
463 dissolved in 400 µl lysis buffer (6M guanidium chloride, 2% cetrimonium bromide, 5 mM
464 dithiothreitol, 50 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), pH 7.2).
465 Then the sample mixture was put on ice and sonicated for 10 cycles (30 s on and 30 s off cycle,
466 amplitude 50%) by a sonicator (Qsonica), followed by centrifugation at 20,000 × g for 20 min at
467 4 °C. The supernatant was taken and alkylated with 20 mM N-ethylmaleimide for 20 min at room
468 temperature, 5 mM dithiothreitol was added to quench the excessive alkylating reagents. Proteins
469 were purified by methanol-chloroform precipitation. The dried protein pellet was resuspended in
470 10 mM EPPS (N-(2-Hydroxyethyl) piperazine-N'-(3-propanesulfonic acid)) at pH 8.5 with 6 M
471 guanidine hydrochloride. Samples were heated at 60°C for 15 min and protein concentration was
472 determined by BCA assay (Pierce BCA Protein Assay Kit, Thermo Scientific). The protein mixture
473 (30~50 µg) was diluted with 10 mM EPPS pH 8.5 to 2 M GuaCl and digested with 10 ng/µL LysC
474 (Wako) at room temperature overnight. Samples were further diluted to 0.5 M GuaCl with 10 M
475 EPPS pH 8.5 and digested with an additional 10 ng/µL LysC and 20 ng/µL sequencing grade
476 Trypsin (Promega) at 37°C for 16 h. Samples were desalted using a SepPak cartridges (Waters)
477 and then vacuum-dried and resuspended in 1% formic acid before mass spectrometry analysis.

478 **Proteomics peptide measurement.** Samples were analyzed on an EASY-nLC 1200 (Thermo
479 Fisher Scientific) HPLC coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher
480 Scientific) with Tune version 3.3. Peptides were separated on an Aurora Series emitter column (25
481 cm × 75 µm ID, 1.6 µm C18) (Ionopticks, Australia) and held at 60°C during separation using an
482 in-house built column oven over 180 min, applying nonlinear acetonitrile gradients at a constant
483 flow rate of 350 nL/min. The Fusion Lumos was operated in data dependent mode. The survey
484 scan was performed at a resolution setting of 120k in orbitrap, followed by MS2 duty cycle of 1.5
485 s. The normalized collision energy for CID MS2 experiments was set to 30%.

486 Solvent A consisted of 2% DMSO (LC-MS-grade, Life Technologies), 0.125% formic acid (98%+,
487 TCI America) in water (LC-MS-grade, OmniSolv, VWR), solvent B of 80% acetonitrile (LC-MS-
488 grade, OmniSolv, Millipore Sigma), 2% DMSO and 0.125% formic acid in water. The following
489 120 min-gradient with percentage of solvent B were applied at a constant flow rate of 350 nL/min
490 after thorough equilibration of the column to 0% B: 0% – 6% in 5 min; 6 – 25% in 160 min; 25%
491 –100% in 10 min; 100% for 5 min. For electrospray ionization, 2.6 kV were applied between
492 minutes 1 and 113 (or minutes 1 and 83 for fractionated samples) of the gradient through the
493 column. To avoid carry-over of peptides, 2,2,2-trifluoroethanol (>99% Reagent plus, Millipore
494 Sigma) was injected in a 30 min wash between each sample.

495 **Proteomics data analysis.** The data was analyzed using GFY software licensed from Harvard
496 (Nusinow et al., 2020). Thermo Fisher Scientific. raw files were converted to mzXML using
497 ReAdW.exe. MS2 spectra assignment was performed using the SEQUEST algorithm v.28 (rev.
498 12) by searching the data against the combined reference proteomes for *Mus Musculus*, *Bos Taurus*,
499 and all the abundant bacterial families detected in 16S rRNA sequencing (*Bacteroidaceae*,
500 *Porphyromonadaceae*, *Prevotellaceae*, *Rikenellaceae*, *Muribaculaceae*, *Lachnospiraceae*,
501 *Ruminococcaceae*, *Erysipelotrichaceae*, *Oscillospiraceae*, *Clostridiaceae*, *Eubacteriaceae*,
502 *Lactobacillaceae* and *Verrucomicrobiaceae*) acquired from Uniprot on Jan 2021 (SwissProt +
503 Trembl) along with common contaminants such as human keratins and trypsin. The target-decoy
504 strategy was used to construct a second database of reverse sequences that were used to estimate
505 the peptide false discovery rate (Elias and Gygi, 2007). A 20 ppm precursor ion tolerance with the
506 requirement that both N- and C- terminal peptide ends are consistent with the protease specificities
507 of LysC and Trypsin was used for SEQUEST searches, two missed cleavage was allowed. NEM
508 was set as a static modification of cysteine residues (+125.047679 Da). An MS2 spectral
509 assignment false discovery rate of 0.5% was achieved by applying the target decoy database search
510 strategy. Linear Discriminant analysis was used for filtering with the following features:
511 SEQUEST parameters XCorr and unique Δ XCorr, absolute peptide ion mass accuracy, peptide
512 length and charge state. Forward peptides within three standard deviations of the theoretical m/z
513 of the precursor were used as positive training set. All reverse peptides were used as negative
514 training set. Linear Discriminant scores were used to sort peptides with at least seven residues and
515 to filter with the desired cutoff. Furthermore, we performed a filtering step on the protein level by
516 the “picked” protein FDR approach (Savitski et al., 2015). Protein redundancy was removed by
517 assigning peptides to the minimal number of proteins which can explain all observed peptide, with
518 above-described filtering criteria.

519 To quantify the intensities of all the isotopic peaks of the peptides, we used raw intensity. Missed
520 cleavage peptides (more than one K or R in the peptide) and low signal to FT-noise peptides (M_0
521 $S/N < 20$) were removed. Peptide phylogenetic assignment was performed using Unipept (Gurdeep

522 Singh et al., 2019), ‘Equate I and L’ and ‘Advanced missed cleavage handling’ were not selected.
523 Only peptides that are specific at a genus level were used for further analysis.

524 **Quantification of newly-synthesized fraction of peptide.** To determine the newly synthesized
525 fraction of a bacterial peptide in D₂O drinking water experiment, we first measured the cecal
526 content free amino acids deuterium labeling pattern using metabolomics. Then, for each peptide,
527 we simulated the expected isotope envelope pattern if the peptide were old, i.e., unlabeled with
528 deuterium (I_{old}), versus if it were newly synthesized by taking up free amino acids from the cecal
529 content (I_{new}). I_{old} was calculated based on the peptide’s molecular formula and ¹³C, ¹⁵N, ²H, ¹⁷O,
530 ¹⁸O, ³²S, ³³S and ³⁶S natural abundance. I_{new} was calculated based on the peptide’s sequence and
531 experimentally observed labeling of the corresponding cecal free amino acids (after natural isotope
532 correction), and the natural isotope abundance of the unlabeled atoms in the peptide’s formula.
533 The simulation of expected peptide isotope distribution and fitting was performed using a
534 MATLAB code: https://github.com/xxing9703/pepMID_simul. Exact mass isotopic peaks with
535 appreciable abundances were bundled by nominal mass into fraction M+0, M+1, ...M+n,
536 constituting the final simulated spectrum. A least square fit was used to find the scalar θ that best
537 fit the measured peptide isotopic distribution ($I_{measured}$) to a linear combination of I_{old} and I_{new} :

538
$$I_{measured} = I_{old} \times (1 - \theta) + I_{new} \times \theta$$

539 The root mean square error was determined for each peptide fitting, and any fitting with a root
540 mean square error > 1% was removed. For genus-level turnover quantification, only genera with
541 more than two measurements were kept in the analysis, with the median value across peptides
542 reported.

543 **Quantification of contribution of labeled nutrient to peptide.** To determine the contribution of
544 a ¹³C- or ¹⁵N-labeled nutrient to a bacterial peptide, similar to the above approach, we first
545 measured the cecal content free amino acids ¹³C- or ¹⁵N-labeling using metabolomics. Then, for
546 each peptide, we simulated the expected isotope envelope pattern if the peptide were unlabeled
547 ($I_{unlabeled}$) versus if it were synthesized from free cecal amino acids (I_{free}). A scalar γ (analogous
548 to θ above) can then be determined by fitting the measured peptide isotope distribution ($I_{measured}$)
549 to a linear combination of $I_{unlabeled}$ and I_{free} . Note that γ will exceed 1 when a bacterial genus
550 uses a particular nutrient in excess of that nutrient contribution’s to cecal free amino acids. Because
551 the ¹³C- and ¹⁵N-labeling patterns are simpler than the D₂O labeling patterns, in lieu of carrying
552 out this fitting, we instead determined γ (with the same conceptual and mathematical meaning)
553 using simple algebraic equations.

554 Specifically, we measured γ for each peptide as follows:

555
$$\gamma = \frac{\varphi_{\text{measured}} - \varphi_{\text{unlabeled}}}{\varphi_{\text{free}} - \varphi_{\text{unlabeled}}}$$

556 where (with the exception of ^{13}C -protein feeding data, discussed immediately below) φ is the
557 average number of extra neutrons in a given peptide (or simulated peptide), relative to the M+0
558 form. This was calculated based on the experimentally observed (or simulated, as above) fraction
559 of M+0, M+1, M+2, and M+3, which account for > 90% of the isotopes for each peptide (with
560 more heavily labeled forms too low abundance and noisy to contribute productively to the
561 measurements):

562
$$\varphi = \frac{\sum_{i=0}^3 i \cdot M_i}{\sum_{i=0}^3 M_i}$$

563 For the ^{13}C -protein feeding experiments, the most readily detected labeled forms involve
564 incorporation of a single midsized U- ^{13}C -amino acid, which manifests as M+5 or M+6 peptide
565 labeling. Other isotopic forms were sufficiently noisier, as to render their inclusion unhelpful.
566 Accordingly, we calculated γ based on φ' :

567
$$\varphi' = \frac{M_5 + M_6}{M_0 + M_5 + M_6}$$

568 The above equations give nearly identical values for γ as fitting (as done to determine θ).

569 For genus-level measurements of feedstock contributions, only genera with more than 3 peptides
570 measured per mouse was kept in the analysis, with the median value across peptides reported as
571 γ_{genus} . Only genera that were consistently detected in proteomics, and the family of that genera
572 detected (>0.5%) in 16S rRNA sequencing were analyzed. The product of γ_{genus} and the
573 contribution of each nutrient to cecal free amino acids ($L_{AA_avg \leftarrow \text{nutrient}}$) was used to determine
574 the contribution of each nutrient to bacterial genus ($f_{\text{genus} \leftarrow \text{nutrient}}$):

575
$$f_{\text{genus} \leftarrow \text{nutrient}} = \gamma_{\text{genus}} \times L_{AA_avg \leftarrow \text{nutrient}}$$

576 where the contribution of each nutrient to bacterial protein pool ($L_{AA_avg \leftarrow \text{Nutrient}}$) was calculated
577 as the average labeling across amino acids, weighted based on their abundance in that genus'
578 protein and corrected for fraction of the nutrient interest labeled (T):

579
$$L_{AA_avg \leftarrow \text{nutrient}} = \sum f_{\text{Cecal_AA} \leftarrow \text{nutrient}} \times w\%_{AA, \text{bacteria}} / T$$

580 with $w\%_{AA,bacteria}$ taken from literature (Purser and Buechler, 1966).

581 **Statistical analysis.** A two-tailed, unpaired student's t-test was used to calculate P values, with
582 $P < 0.05$ used to determine statistical significance.

583 **Author contributions and information.** X.Z., and J.D.R came up with general approach and X.Z.
584 performed most of the experiments and data analysis. C.J. worked intensively with X.Z. to develop
585 the experimental strategy. M.W. designed and enabled the proteomic measurements. X.X. wrote
586 the MATLAB code. M.G., F.C.K., and M.D.N. contributed to proteomics method development.
587 J.G.L. and M.S.D. provided microbiome expertise and performed 16S rRNA sequencing. A.R.
588 assisted with isotope tracing. W.L. performed ammonia measurement. X.Z., C.J., and J.D.R. wrote
589 the paper. All of the authors discussed the results and commented on the paper.

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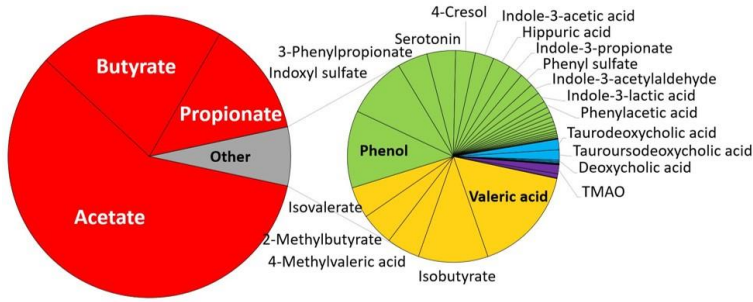
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A Microbiome metabolites in Portal Vein



B

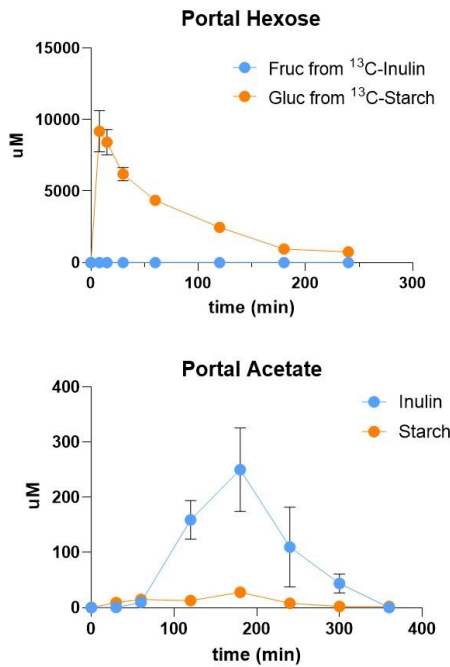
Diet 1	¹³ C-Starch	Inulin	Protein
Diet 2	Starch	¹³ C-Inulin	Protein
Diet 3	Starch	Inulin	¹³ C-AA
Diet 4	Starch	Inulin	¹³ C-protein



Portal blood, cecal content time course LC-MS

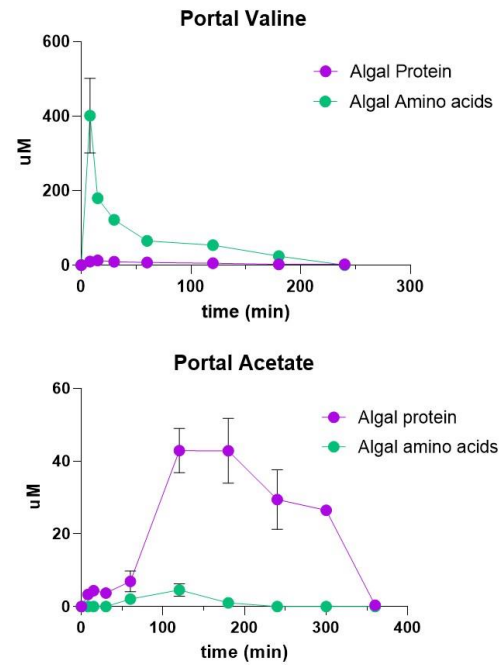
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Inulin vs Starch

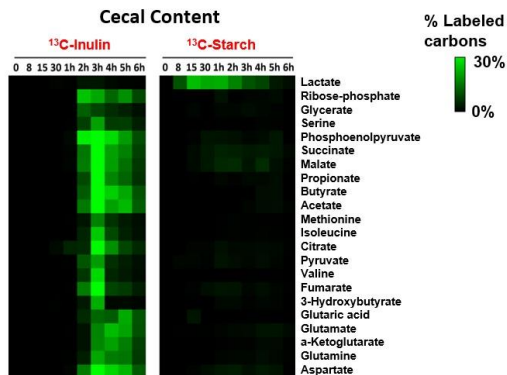


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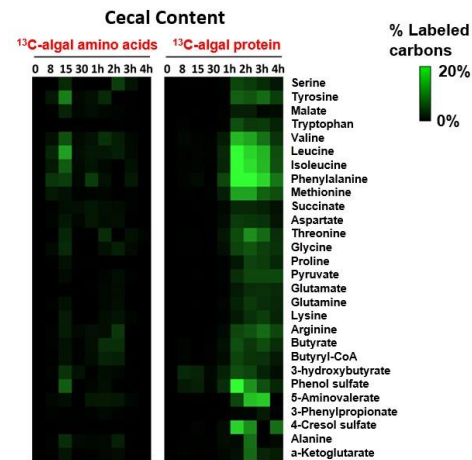
Amino acids vs. Algal Protein



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Figure 1. Microbiome consumes dietary fiber and protein (legend on next page)

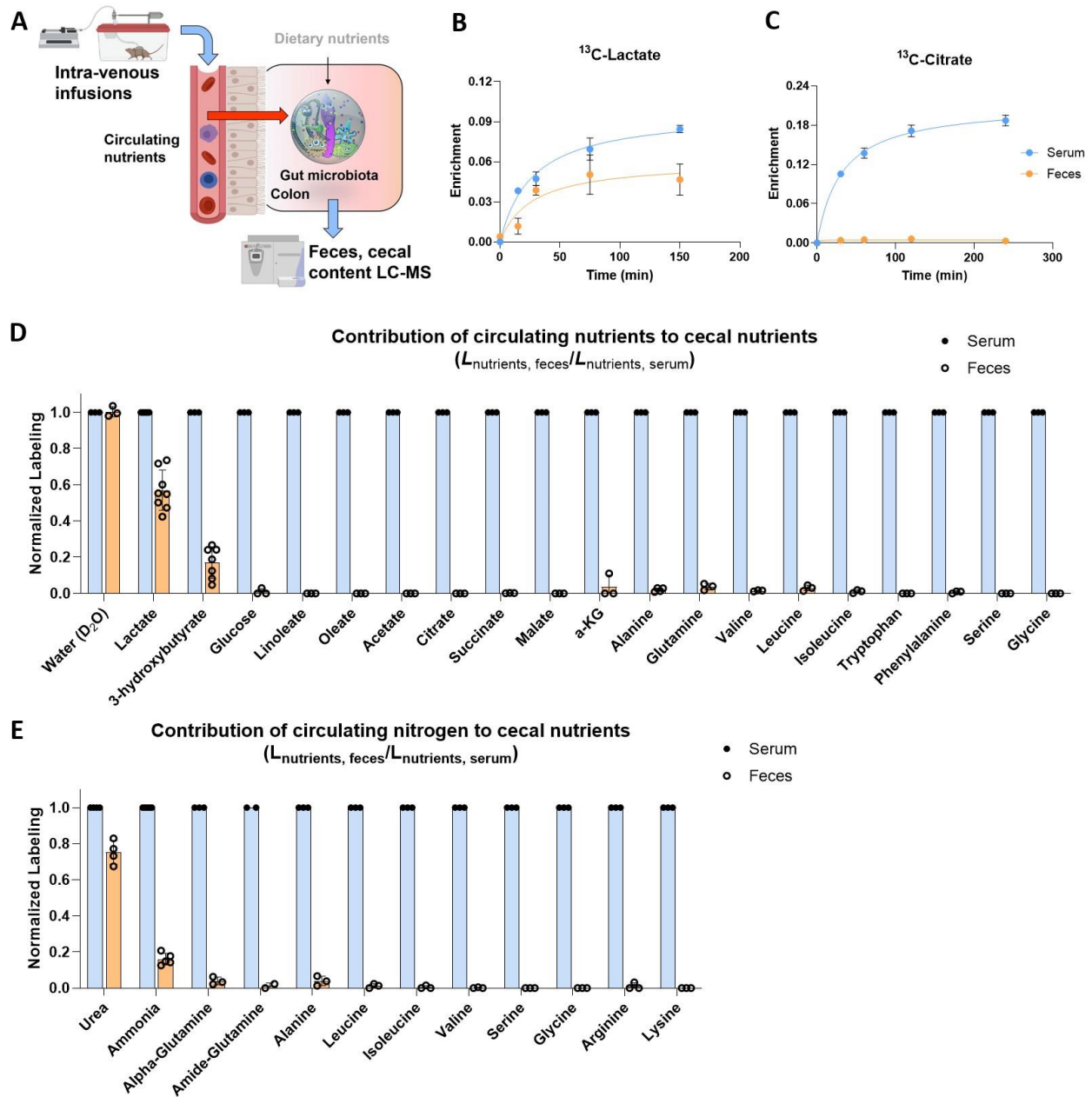
880 **Figure 1. Microbiome consumes dietary fiber and protein.**

- 881 (A) Composition of the measured portal microbial metabolome. The pie charts show the relative
882 molar abundance of different gut microbiota-associated metabolites in mice (N = 6 mice).
- 883 (B) Experimental scheme. Mice received an oral gavage of 4:2:1 starch: protein (or free amino acids):
884 inulin by weight. In each dietary condition, one component was ¹³C-labeled. After gavage of the
885 labeled diet, tissue and serum metabolite labeling were measured over time by LC-MS.
- 886 (C) Dietary starch feeds the host, while dietary inulin feeds the microbiome. The data shows
887 concentrations of labeled carbons in hexose and acetate in portal circulation (mean ± s.e., N = 3
888 mice).
- 889 (D) Inulin is a major microbiome feedstock. Heatmap shows the percentage of labeled carbon atoms
890 in the indicated metabolites in cecal content. Each data point is median of N = 3 mice.
- 891 (E) Dietary amino acids feed the host while dietary algal protein feeds the microbiome. The data
892 shows concentrations of labeled carbons in valine and acetate in portal circulation (mean ± s.e., N
893 = 3 mice).
- 894 (F) Protein but not free amino acids are a major microbiome feedstock. Heatmap shows the
895 percentage of labeled carbon atoms in the indicated metabolites in cecal content. Each data point
896 is median of N = 3 mice.
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Metabolite	Systemic Concentration(μM)	Portal/Systemic (Log 2)	Abx/Conv (Log 2)	Source	Dietary Fiber	Dietary Protein	Circulating lactate	Source
Acetate	210.0	3.1	-1.9		41%	8%	7%	Fiber
Phenol	23.0	0.1	-4.3		0%	41%	0%	Protein
Indoxyl sulfate	22.0	-0.2	<-5.0		0%	68%	0%	Protein
Serotonin	12.0	-0.5	-1.4		0%	15%	0%	Protein
Propionate	9.9	>5.0	<-5.0		28%	4%	6%	Fiber
4-Methylvaleric acid	7.3	0.5	-0.1		6%	43%	2%	Fiber
Indole-3-acetic acid	7.2	-0.3	-0.9		0%	66%	0%	Protein
Phenyl sulfate	6.6	-0.5	<-5.0		0%	42%	0%	Protein
Acetylglycine	5.4	0.2	-0.2		29%	22%	0%	Fiber
4-Ethylphenol	5.2	0.2	-0.9		0%	23%	0%	Protein
Indole-3-propionate	4.6	0.0	<-5.0		0%	39%	0%	Protein
Hippuric acid	4.2	0.3	-4.4		0%	33%	0%	Protein
4-Cresol	4.2	0.6	-0.4		0%	26%	0%	Protein
Indole-3-acetylaldehyde	4.1	-0.3	-0.2		0%	13%	0%	Protein
Trimethylamine-N-oxide	3.8	0.3	-3.8	Others	0%	0%	0%	Others
Butyrate	3.6	>5.0	<-5.0		54%	13%	7%	Fiber
Isobutyrate	2.8	2.8	-4.1		5%	35%	1%	Protein
2-Methylbutyrate	2.8	1.4	-3.0		5%	54%	1%	Protein
Catechol	2.8	0.0	<-5.0	Others	0%	0%	0%	Others
5-Aminovaleric acid	2.0	0.4	-0.3		0%	14%	0%	Protein
Indole-3-lactic acid	1.9	0.8	-0.7		0%	27%	0%	Protein
3-Hydroxycinnamic acid	1.6	-0.1	-0.6		0%	64%	0%	Protein
3-Phenylpropionate	1.5	2.4	-5.0		0%	22%	0%	Protein
Isovaleric acid	1.2	2.9	-3.7		1%	45%	1%	Protein
Benzoic acid	1.2	0.3	<-5.0		0%	32%	0%	Protein
Valerate	1.1	4.9	-3.8		6%	37%	5%	Protein
Cinnamoylglycine	1.1	0.7	-1.1		0%	39%	0%	Protein
Inosine	1.1	-3.3	0.4		12%	11%	0%	Fiber
Phenylacetic acid	0.79	0.6	-2.8		0%	50%	0%	Protein
4-Hydroxyphenylpropionate	0.76	0.2	-2.0		0%	72%	0%	Protein
Propionylglycine	0.75	1.2	-0.1		21%	24%	0%	Fiber
Phenylpropionylglycine	0.75	0.6	-0.3		2%	22%	0%	Protein
Heptanoic acid	0.74	0.6	-0.3		6%	11%	0%	Protein
Indole	0.53	0.2	-2.6		0%	69%	0%	Protein
Butyrylglycine	0.39	1.1	-1.4		37%	28%	0%	Fiber
4-Hydroxyphenylacetate	0.37	0.7	-1.0		0%	32%	0%	Protein
N-Acetyl-Tryptophan	0.37	0.1	0.5		0%	13%	0%	Protein
Tauroursodeoxycholic acid	0.33	2.9	-2.2		0%	0%	0%	Bile acids
4-Hydroxybenzoic acid	0.25	0.7	-1.0		0%	9%	0%	Protein
Cinnamic acid	0.20	2.5	-2.6		0%	61%	0%	Protein
Taurodeoxycholic acid	0.11	4.8	-3.1		0%	0%	0%	Protein
2-Hydroxyhippuric acid	0.062	1.5	-0.7		0%	22%	0%	Protein
Imidazole propionate	0.051	0.7	-0.7		0%	53%	0%	Protein
Deoxycholic acid	0.048	3.1	<-5.0		0%	0%	0%	Bile acids
Indole-3-ethanol	0.032	1.6	-0.3		0%	81%	0%	Protein
Ursodeoxycholic acid	0.026	2.5	<-5.0		0%	0%	0%	Bile acids
4-Cresol sulfate	0.026	-0.2	-2.8		0%	26%	0%	Protein
Phenylacetylglucine	0.017	0.2	-0.3		0%	32%	0%	Protein
Equol	0.013	3.7	<-5.0	Others	0%	0%	0%	Others
Hyodeoxycholic acid	0.009	3.0	-3.7		0%	0%	0%	Bile acids
Lithocholic acid	0.008	1.8	<-5.0		0%	0%	0%	Bile acids
Taroulithocholic acid	<0.001	>5.0	<-5.0		0%	0%	0%	Bile acids
Isoallothocholic acid	<0.001	>5.0	<-5.0		0%	0%	0%	Bile acids
Glycoursodeoxycholic acid	<0.001	>5.0	-4.5		0%	0%	0%	Bile acids
Glycodeoxycholic acid	<0.001	>5.0	-1.3		0%	0%	0%	Bile acids

899 **Table 1. Absolute concentrations and sources of microbiota-associated metabolites.**

900 Data are from ad lib fed state (ZT0); for ad lib fasted state (ZT12), see Supplementary Table S1. Absolute
901 concentration is mean, N = 5 mice. Portal/systemic = fold-change in concentration between the portal vein
902 and tail vein (median, N = 5 mice). Abx/Conv refers to fold-change in portal blood concentration between
903 mice treated with antibiotics cocktail versus not (median, N = 5 mice/group). Source bar indicates the
904 relative contribution to the indicated metabolite from dietary inulin, algal protein and circulating lactate
905 (based on isotope tracing). Percentages indicate quantitative relative contributions from those nutrients
906 (median, N = 4). Numbers typically add up to less than 100%, as other sources (e.g. mucins) contribute.



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908 **Figure 2. Circulating lactate and 3-hydroxybutyrate feed the gut microbiome.**

909 (A) Schematic of intravenous infusion of isotope-labeled nutrients to identify circulating metabolites
910 that feed gut microbiome.

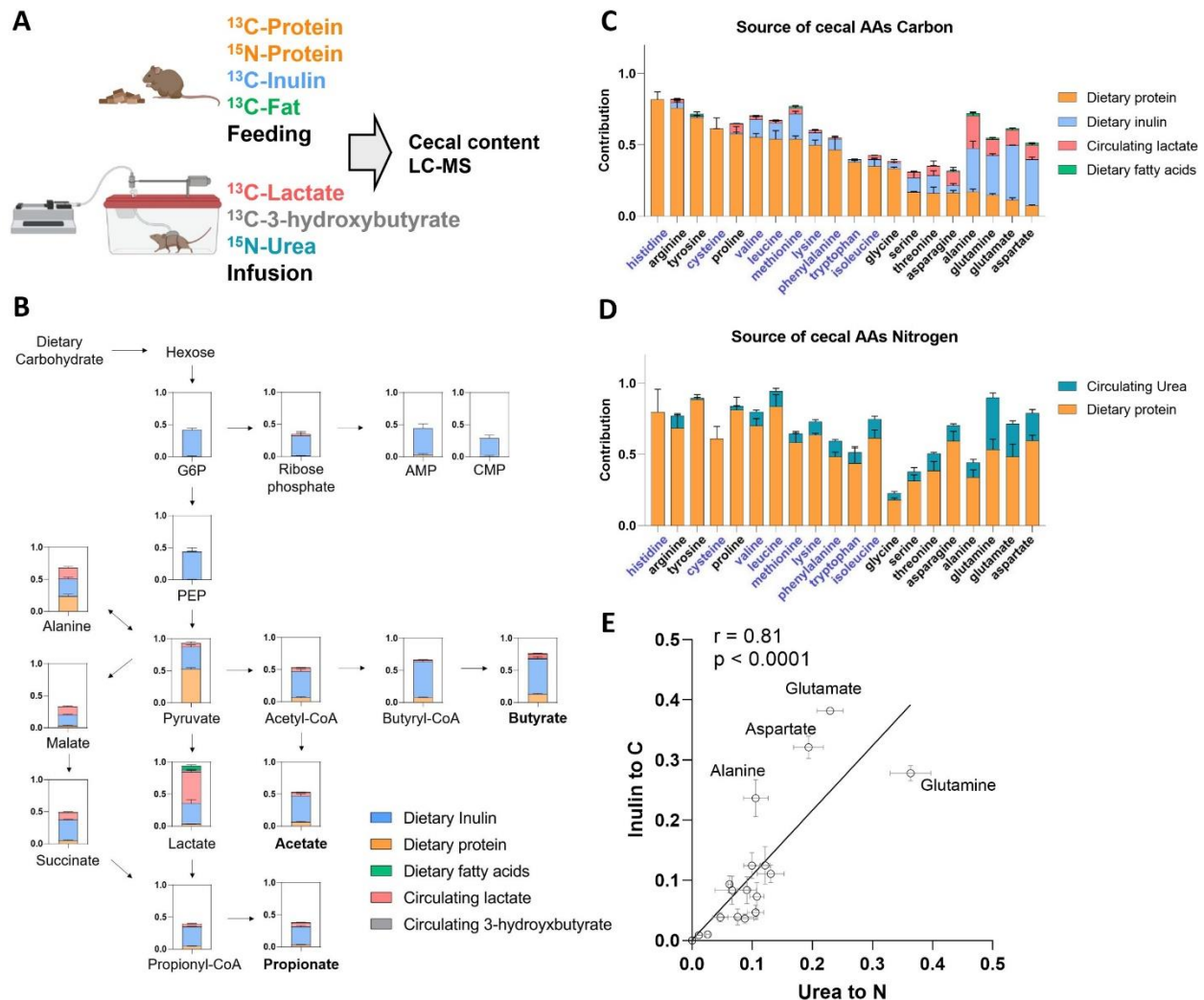
911 (B) Circulating lactate rapidly enters the feces. Mice were infused with ^{13}C -lactate and serum and fresh
912 feces enrichment were compared. Mean \pm s.e. N = 3.

913 (C) Circulating citrate does not enter the feces. As in (B), for ^{13}C -citrate.

914 (D) Passage of circulating ^{13}C -labeled nutrients into the feces. Mice were infused with labeled nutrients
915 for 2.5 h, and labeling fraction in feces was normalized to labeling fraction in serum. Mean \pm s.e.
916 N = 3 except for lactate (N = 8) and 3-hydroxybutyrate (N = 7).

917 (E) Passage of circulating ^{15}N -labeled nutrients into the feces. As in (D), for ^{15}N -labeling. Mean \pm s.e.
918 N = 3 except for urea (N = 4) and ammonia (N = 5).

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922 **Figure 3. Quantitative analysis of dietary and circulating nutrient contributions to gut microbiome.**

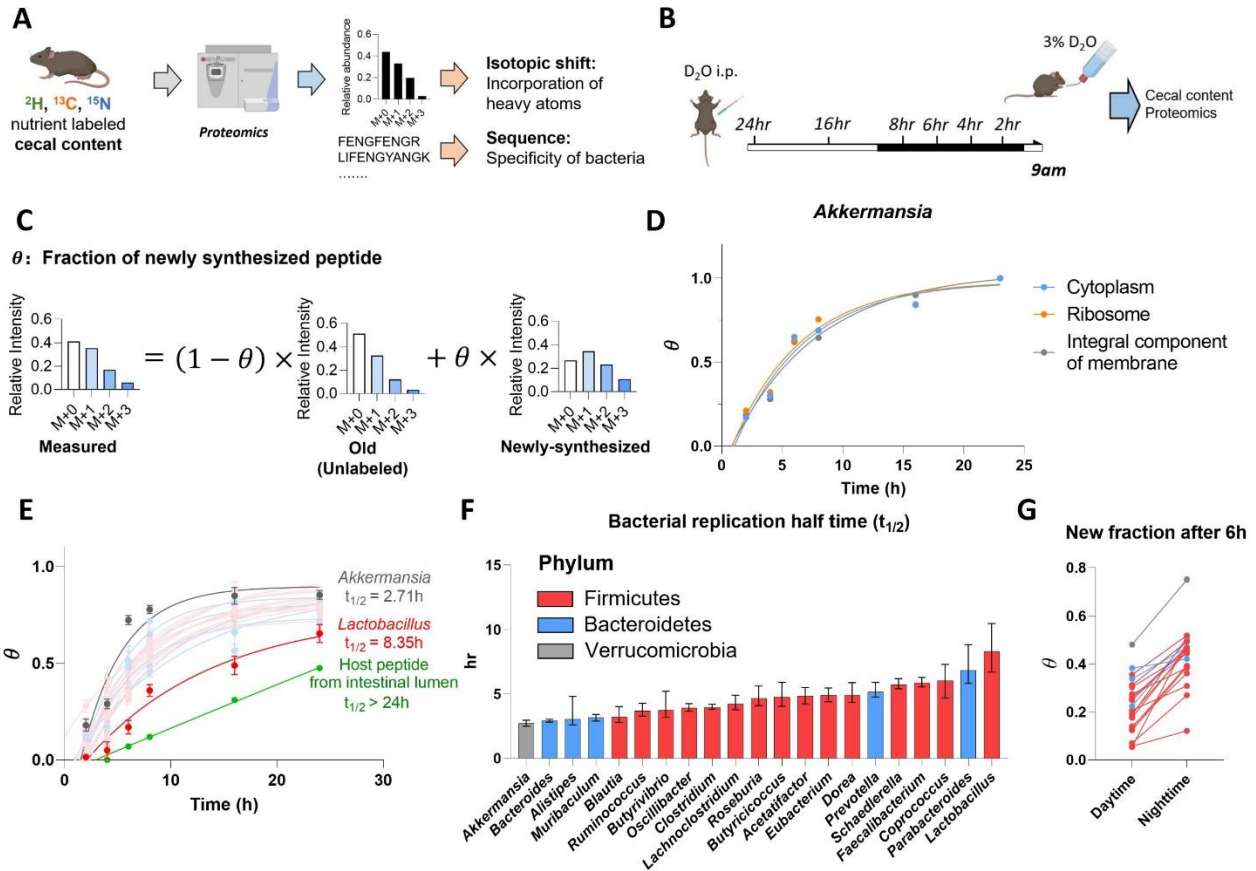
923 (A) Experimental design. Mice were fed chow containing ^{13}C -protein, ^{13}C -inulin, ^{13}C -fatty acids, or ^{15}N -
 924 protein for 24 h. Alternatively, mice were intravenously infused with ^{13}C -lactate, ^{13}C -3-hydroxybutyrate or
 925 ^{15}N -urea for 24 h. The labeling of cecal content metabolites was analyzed by LC-MS.

926 (B) Contribution of dietary and circulating nutrients to carbohydrate fermentation pathways in gut
 927 microbiome. Mean \pm s.e. N = 4.

928 (C) Contribution of dietary and circulating nutrients to cecal amino acid carbon. The names of essential
 929 amino acids (EAA) are written in blue and non-essential amino acids (NEAA) in black. Mean \pm s.e. N = 4.

930 (D) Contribution of dietary and circulating nutrients to cecal amino acid nitrogen. As in (C), for nitrogen.

931 (E) Positive correlation, across amino acids in the cecal contents, of carbon contribution from dietary inulin
 932 and nitrogen contribution from circulating urea. Mean \pm s.e. N = 4.



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934 **Figure 4. Growth rate of different gut bacterial genera quantified by isotope tracing.**

935 (A) Experimental approach for isotope tracing into specific gut bacteria. Only peptides that are specific to
 936 a particular bacterial genus were examined.

937 (B) Growth rate quantification using D₂O. Mice received D₂O by i.p. injection followed by D₂O drinking
 938 water and cecal content labeling was measured over time by proteomics and metabolomics.

939 (C) Calculation of newly synthesized peptide fraction (θ). The experimentally observed peptide mass
 940 isotope distribution was fit to a linear combination of unlabeled peptide (“old,” heavy forms from natural
 941 isotope abundance) and newly synthesized peptide (“new,” heavy forms from isotope labeling pattern of
 942 free cecal amino acids and from natural isotope abundance).

943 (D) Different cellular compartments from the same bacterial genus show similar labeling rate.

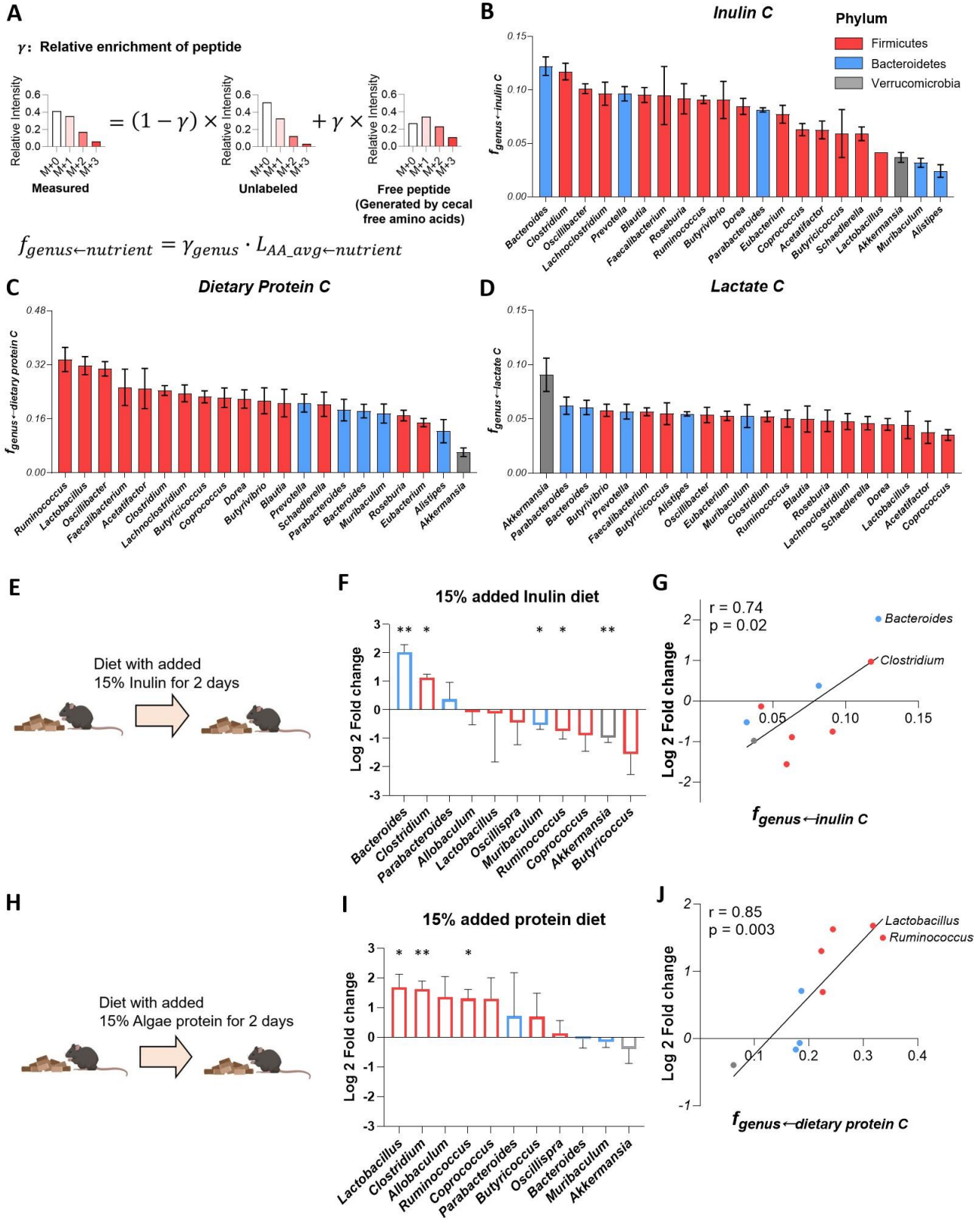
944 (E) Genus-specific growth rates were determined by a single exponential fitting, as a function of time, of θ
 945 (mean across both different peptides measured from that genus and replicate mice). Mean \pm s.e. N=5 mice
 946 for each time point.

947 (F) Bacterial replication half time of different gut bacteria. Data are exponential fits \pm s.e.

948 (G) The gut bacteria synthesize protein in sync with the physiological feeding patterns of the host. The
 949 figure shows the average newly synthesized peptide fraction (θ) for different gut bacterial genera after
 950 D₂O labeling during daytime vs nighttime. Each line connects the daytime and nighttime measurements
 951 for one genus. Mean, N = 5 mice for daytime and for nighttime.

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955 **Figure 5. Preferred carbon sources differ across gut bacteria (legend on next page).**

956 **Figure 5. Preferred carbon sources differ across gut bacteria**

957 (A) Calculation of peptide relative ^{13}C -enrichment (γ) and carbon contribution from the tracer to a bacterial
958 genus ($f_{\text{genus} \leftarrow \text{nutrient}}$). First, the experimentally observed peptide mass isotope distribution was fit to a linear
959 combination of unlabeled peptide (heavy forms from natural isotope abundance) and a peptide made from
960 free cecal amino acids (heavy forms from isotope labeling pattern of free cecal amino acids and from natural
961 isotope abundance), yielding γ . Then, $f_{\text{genus} \leftarrow \text{nutrient}}$ was determined by correcting for the fractional
962 contribution of that tracer to the cecal free amino acid pools.

963 (B) Carbon contribution of dietary inulin across bacterial genera. Mean \pm s.e. N=4 mice.

964 (C) Carbon contribution of dietary algal protein across bacterial genera. Mean \pm s.e. N=6 mice.

965 (D) Carbon contribution of circulating lactate across bacterial genera. Mean \pm s.e. N=7 mice.

966 (E) Experimental scheme of high-inulin diet feeding followed by 16S ribosomal RNA sequencing.

967 (F) Genus-level microbiota composition changes after high-inulin diet. The genera increased after high-
968 inulin diet prefer inulin in (B). Mean \pm s.e. N=3 mice. *P<0.05 and **P<0.01 by two-sided Student's t-test.

969 (G) Correlation between genera abundance changes and carbon-source preference.

970 (H- J) As in (E - G), for algal protein-supplemented diet.

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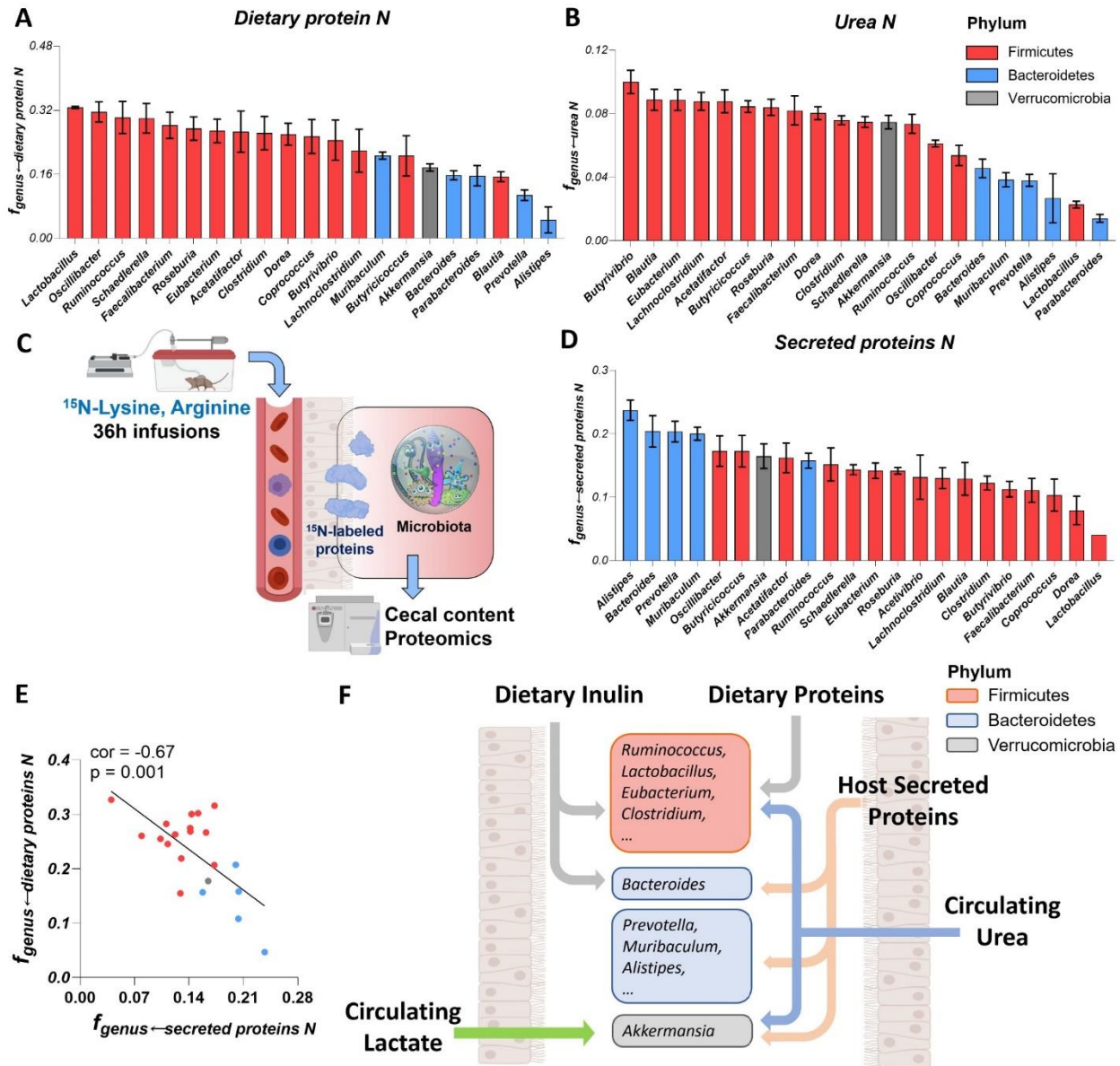
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988 **Figure 6. Firmicutes favor dietary protein while Bacteroidetes prefer secreted host protein**

989 (A) Nitrogen contribution of dietary algal protein across bacterial genera. Mean±s.e. N=6 mice.

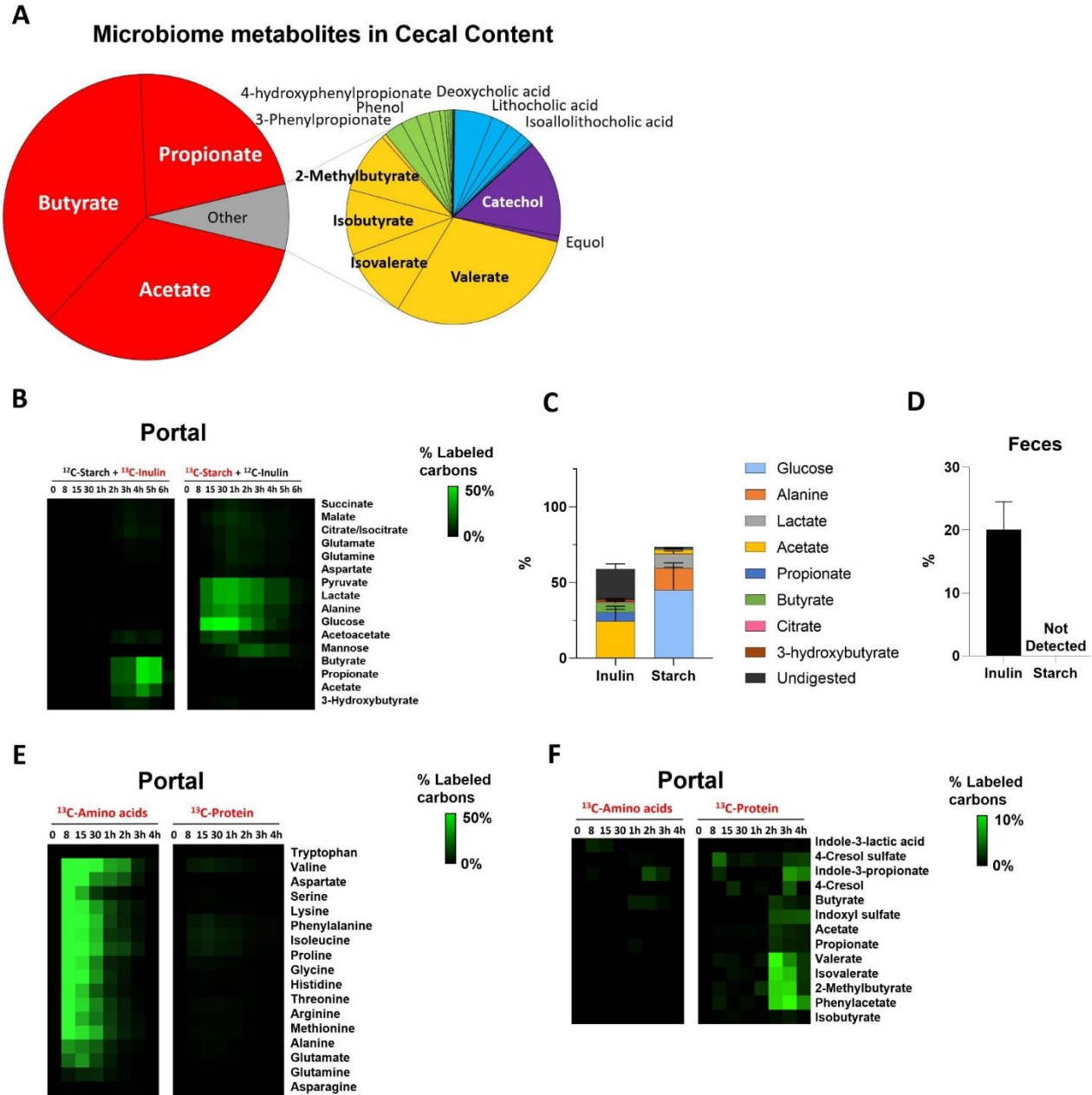
990 (B) Nitrogen contribution of circulating urea across bacterial genera. Mean±s.e. N=6 mice.

991 (C) Experimental schematic of long-term ¹⁵N-lysine and ¹⁵N-arginine infusion to probe the contribution of
 992 secreted host proteins to different bacterial genera.

993 (D) Nitrogen contribution of secreted host proteins across bacterial genera. Mean±s.e. N=5 mice.

994 (E) Negative correlation between $f_{genus \leftarrow secreted\ proteins\ N}$ and $f_{genus \leftarrow dietary\ proteins\ N}$.

995 (F) Summary of carbon and nitrogen inputs to different gut bacteria. Firmicutes prefer dietary carbon
 996 sources (fiber and protein) and nitrogen from host circulating urea. Bacteroidetes heavily use dietary fiber,
 997 while using on host secreted proteins for nitrogen. Verrucomicrobia prefers host secreted nutrients, both
 998 protein and circulating small molecules (lactate, urea).



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1000 **Figure S1. Microbiome consumes dietary fiber and protein.**

1001 (A) Composition of the measured cecal microbial metabolome. The pie charts show the molar
1002 abundance of different gut microbiota-associated metabolites (N = 6 mice).

1003 (B) Heatmap showing the percentage of labeled carbon atoms in the indicated metabolites in portal
1004 circulation, following gavage of 4:2:1 starch: protein (or free amino acids): inulin, with the
1005 indicated nutrient labeled. Each data point is median of N = 3 mice.

1006 (C) Metabolic fate of inulin and starch. Stacked bars show the fraction of gavaged inulin and starch
1007 that is converted into each of the indicated metabolic products, with the undigested fraction being
1008 excreted in the feces (mean \pm s.e., N = 3 mice).

1009 (D) Undigested carbohydrate in the feces. Graph shows the fraction of labeled hexose after cecal
1010 content hydrolysis, 12 h following gavage as above (mean \pm s.e., N = 3 mice).

1011 (E) Heatmap showing the percentage of labeled carbon atoms in the indicated amino acids in portal
1012 circulation, following gavage of 4:2:1 starch: protein (or free amino acids): inulin, with the
1013 indicated nutrient labeled. Each data point is median of N = 3 mice.

1014 (F) As in (E), for microbiota-associated metabolites.

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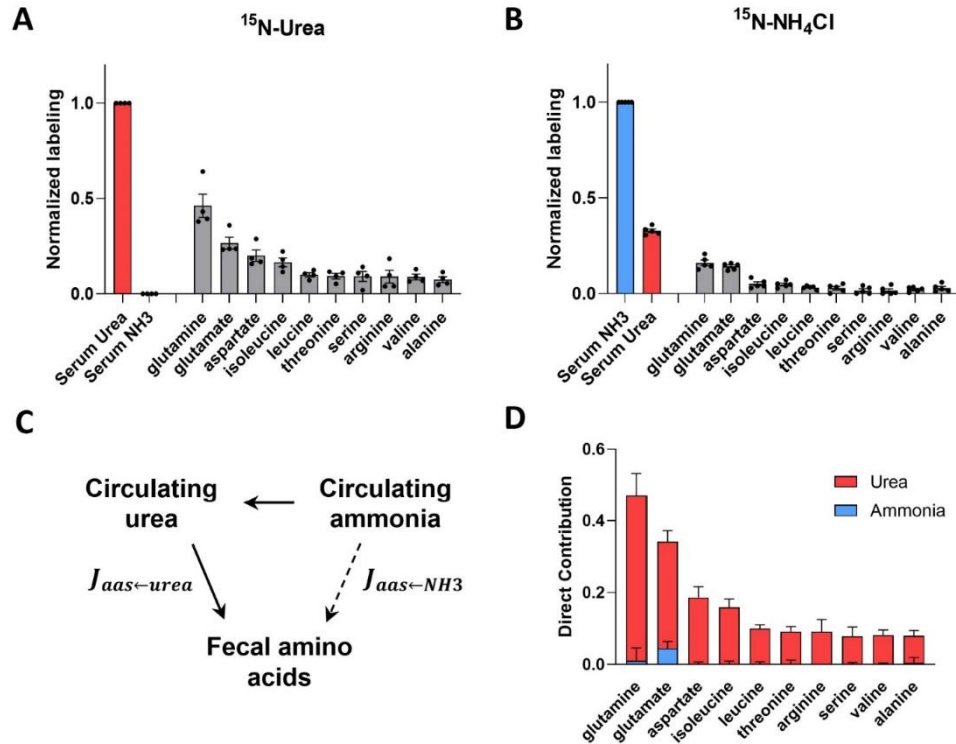
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1036 **Figure S2. Circulating ammonia contributes to microbiota metabolism via circulating urea.**

1037 (A) Normalized labeling of serum urea, ammonia and cecal amino acids after ¹⁵N-urea infusion (mean±
1038 s.e., N = 4 mice). Feces labeling fraction is normalized to serum infused tracer (urea) labeling fraction.

1039 (B) As in (A), for ¹⁵N-ammonia infusion (mean± s.e., N = 5 mice),

1040 (C) Model of direct contribution calculation from circulating urea and ammonia to fecal amino acids.

1041 (D) Direct contributions of circulating urea and ammonia to cecal amino acids (mean± s.e., N = 4
1042 mice).

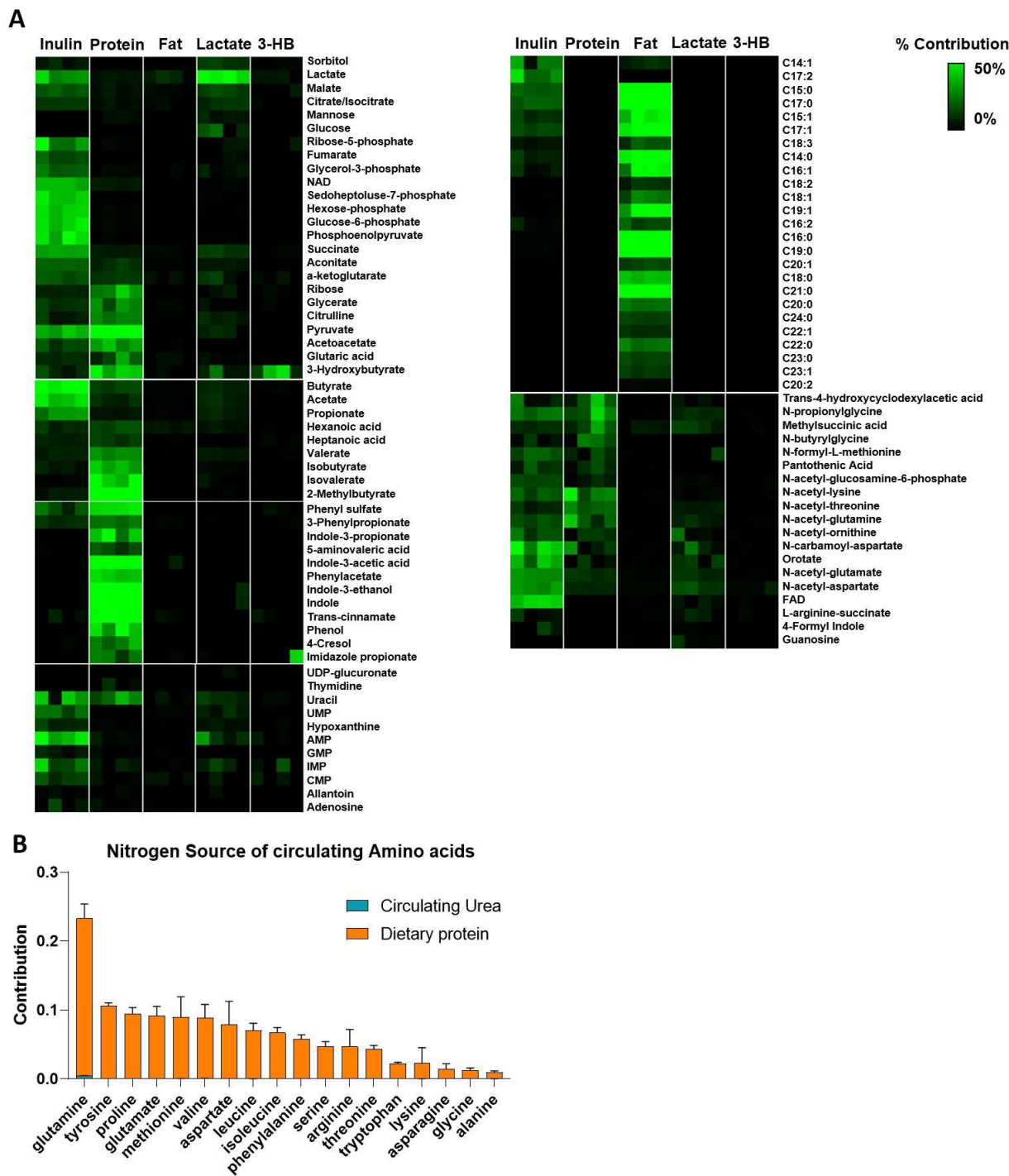
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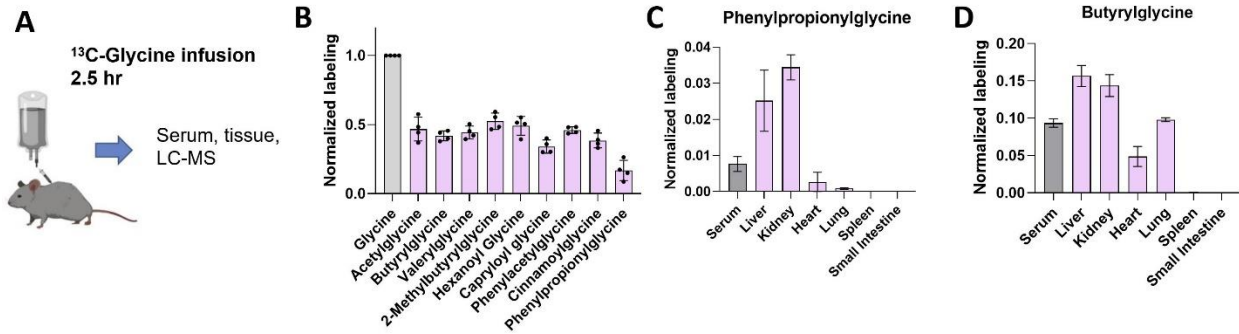


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1049 **Figure S3. Quantitative analysis of dietary and circulating nutrient contributions to gut**
 1050 **microbiome metabolism.**

1051 (A) Heat maps showing the contribution of dietary or circulating nutrients to cecal metabolites. For
 1052 experimental design, see Figure 3. N = 4 mice.

1053 (B) Amino acids synthesized in the gut microbiome, stay in the microbiome, as urea contributes to
 1054 microbiome amino acids but not host circulating amino acids. Mean \pm s.e. N = 4 mice.



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1057 **Figure S4. Liver and kidney use circulating glycine to synthesize acyl-glycines.**

1058 (A) Experimental design. Mice were intravenously infused with [U- ^{13}C]glycine for 2.5 h and tissue
1059 and serum glycine and acyl-glycine labeling were measured.

1060 (B) Circulating acyl-glycines are made from circulating glycine. Mean \pm s.e. N = 4 mice.

1061 (C) Tissue phenylpropionylglycine labeling (normalized to circulating glycine labeling). Mean \pm s.e.
1062 N = 4 mice.

1063 (D) Tissue butyrylglycine labeling (normalized to circulating glycine labeling). Mean \pm s.e. N = 4
1064 mice.

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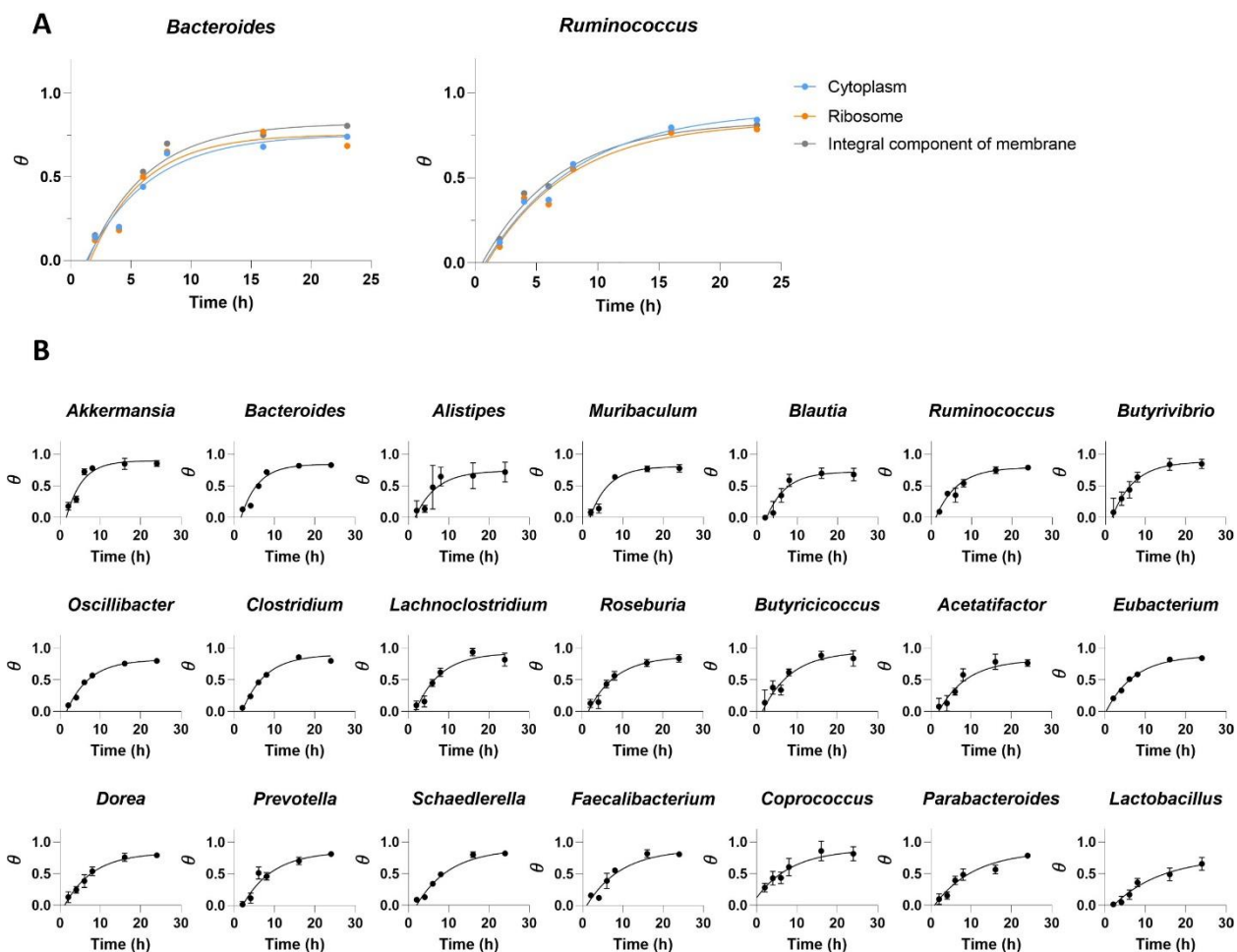
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1079 **Figure S5. Single exponential fit of newly synthesized fraction of microbial peptides over time.**

1080 (A) Different cellular compartments from the same bacterial genus show similar labeling rate.

1081 (B) Single exponential fit was applied to determine genus-level microbial turnover. Data are mean \pm s.e.

1082 N = 5 mice.

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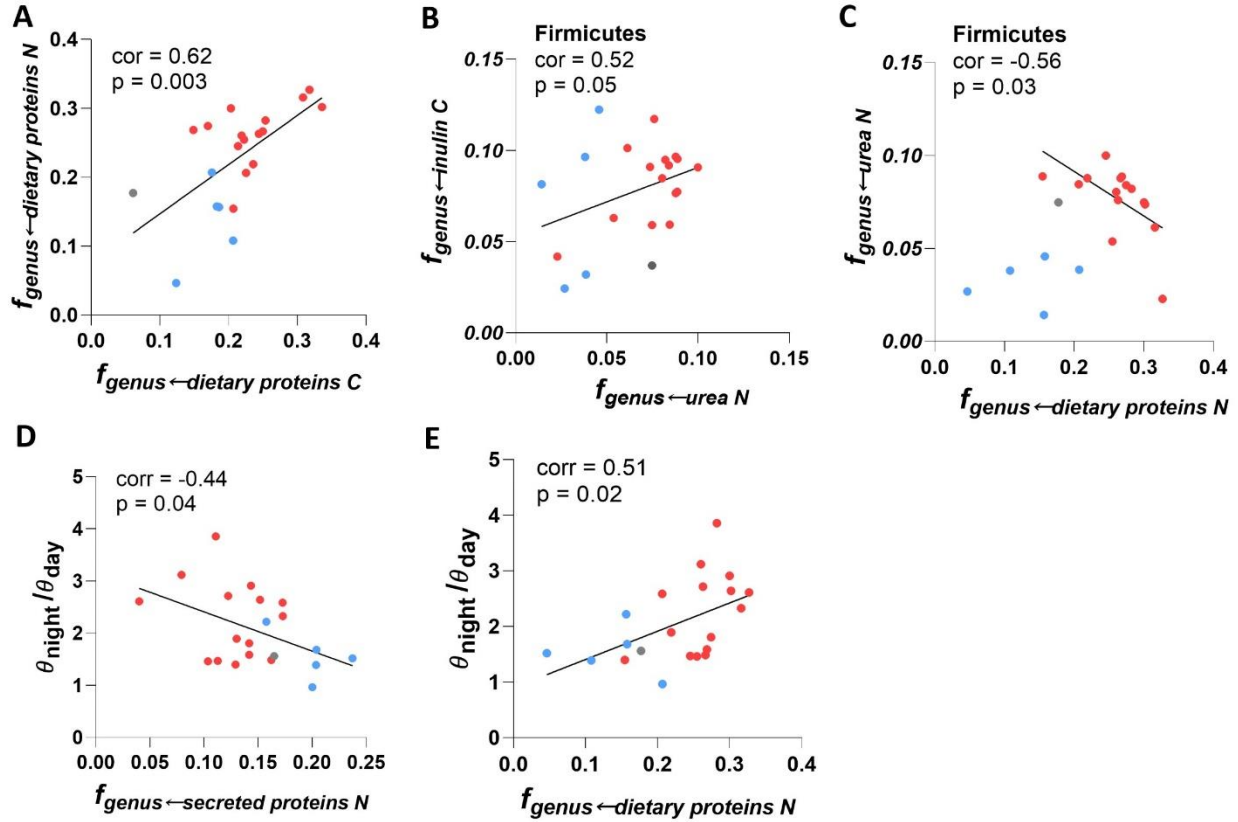
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1091 **Figure S6. Correlation analysis of nutrient preferences across different gut bacterial genera.**

1092 (A) Positive correlation between $f_{\text{genus} \leftarrow \text{dietary proteins C}}$ and $f_{\text{genus} \leftarrow \text{dietary proteins N}}$.

1093 (B) Positive correlation between $f_{\text{genus} \leftarrow \text{inulin C}}$ and $f_{\text{genus} \leftarrow \text{urea N}}$ in Firmicutes.

1094 (C) Negative correlation between $f_{\text{genus} \leftarrow \text{urea N}}$ and $f_{\text{genus} \leftarrow \text{dietary proteins N}}$ in Firmicutes.

1095 (D) Negative correlation between $\theta_{\text{night}}/\theta_{\text{day}}$ and $f_{\text{genus} \leftarrow \text{secreted proteins N}}$.

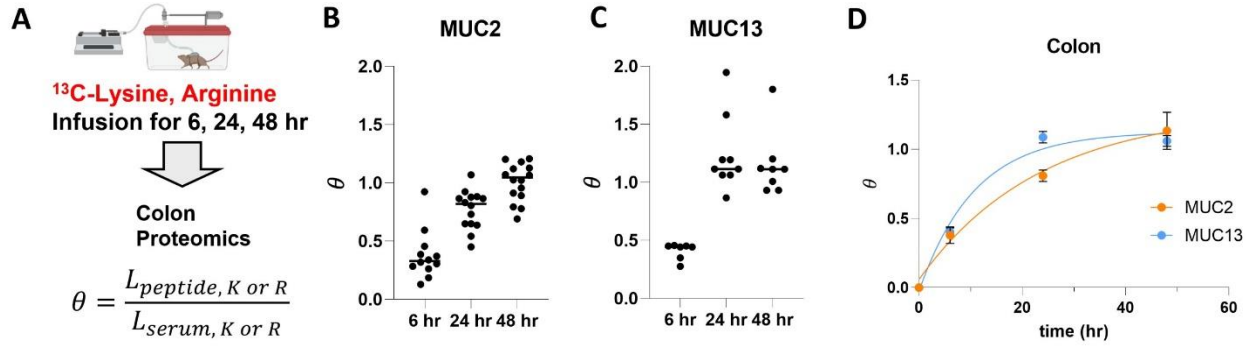
1096 (E) Positive correlation between $\theta_{\text{night}}/\theta_{\text{day}}$ and $f_{\text{genus} \leftarrow \text{dietary proteins N}}$.

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1103 **Figure S7. Host secreted proteins are synthesized from host circulating amino acids.**

1104 (A) Experimental design. Mice were infused with ¹³C-lysine and ¹³C-arginine and mouse colon protein
1105 was analyzed by proteomics.

1106 (B) MUC2 labeling (multiple different MUC2 peptides). N = 3 mice.

1107 (C) MUC13 labeling (multiple different MUC13 peptides). N = 3 mice.

1108 (D) Single exponential fit of labeling fraction of MUC2 and MUC13 over time. Mean±s.e. N = 3 mice.

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