

1 **Perturbation of the human gut microbiome by a non-antibiotic drug**  
2 **contributes to the resolution of autoimmune disease**

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15 **Abstract**

16 The trillions of microorganisms (microbiota) found within the human gut play a critical role in  
17 shaping the immune system, yet these complex microbial communities are also highly sensitive  
18 to numerous environmental factors. While much of the focus to date has been on dietary intake,  
19 emerging data has begun to suggest that the use of pharmaceutical drugs, even those that are not  
20 considered to be antibiotics, can alter the human gut microbiota with unknown consequences for  
21 treatment outcomes. Here, we use a combination of *in vitro*, *in vivo*, and *ex vivo* methods to  
22 demonstrate that the first-line therapy for rheumatoid arthritis (RA), methotrexate (MTX), has  
23 off-target effects on the human gut microbiota, resulting in a significant growth advantage for  
24 drug-resistant Firmicutes over the Bacteroidetes, which tend to be more sensitive. Longitudinal  
25 analyses of the gut microbiotas of RA patients revealed that MTX-induced shifts in bacterial  
26 relative abundance are associated with improved drug response and transplant experiments in  
27 gnotobiotic mice show that these shifts lead to reduced inflammation. Together, these results  
28 suggest that the mechanism-of-action of non-antibiotic drugs may be due in part to off-target  
29 effects on the gut microbiota, while providing a critical first step towards explaining long-  
30 standing differences in drug response between patients.

## 31 **Introduction**

32 The human gut microbiota consists of trillions of microorganisms that facilitate nutrient  
33 extraction from food<sup>1</sup>, avoidance of pathogen colonization<sup>2</sup>, and development of the host  
34 immune system<sup>3</sup>. Recent studies have highlighted how the gut microbiota is sensitive to multiple  
35 host and environmental factors<sup>4,5</sup>. While much of the focus has been on diet and host disease  
36 status, emerging data have demonstrated that pharmaceutical drugs can also impact the growth of  
37 human gut bacteria<sup>6</sup>. In a recent screen of 1,197 drugs, 24% were found to directly affect the *in*  
38 *vitro* growth of at least 1 of 40 human gut bacterial isolates<sup>6</sup>. However, the *in vivo* relevance and  
39 clinical significance of these off-target effects of non-antibiotic drugs remains to be determined.

40 Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology that affects  
41 between 0.5% to 1% of the population worldwide, leading to inflammation and destruction of  
42 joints as well as other organs<sup>7</sup>. Nearly all newly diagnosed RA patients are initiated on  
43 methotrexate (MTX)<sup>8</sup>. While about one third of patients demonstrate a favorable response to  
44 MTX<sup>9</sup>, needing little else for the management of their disease, the remaining two thirds require  
45 escalation of therapy with additional agents<sup>10</sup>. Furthermore, some patients may be intolerant of  
46 MTX, incurring side effects such as diarrhea, oral ulcers, liver toxicity or hair loss<sup>11</sup>. Thus, there  
47 is significant variability in patient response to MTX, with some patients experiencing great relief  
48 and others incurring little benefit from the drug<sup>12</sup>.

49 Although MTX is used in the treatment of RA and many other inflammatory diseases, it was  
50 originally developed as an anti-cancer therapy and was designed to target the “Achilles heel” of  
51 proliferating cells – the enzyme dihydrofolate reductase (DHFR)<sup>13</sup>. MTX is a structural analogue  
52 of folic acid, differing from this essential vitamin at only two sites. The DHFR enzyme converts  
53 folic acid into tetrahydrofolate (THF), which in turn serves as a carbon source for the production

54 of purines and pyrimidines that are required for DNA synthesis and cell cycle progression<sup>13</sup>.  
55 Furthermore, THF provides carbon sources for pathways involving protein synthesis and lipid  
56 metabolism. For example, THF donates a methyl group to methionine, which then can be  
57 converted into S-adenosyl-methionine (SAM), a key molecule involved a myriad of pathways  
58 including protein synthesis, lipid methylation, and DNA methylation<sup>14</sup>. Thus, MTX targets a  
59 multitude of cellular functions downstream of DHFR, making it an effective anti-cancer drug.

60 Despite over 60 years of use in patients, the mechanism of action of MTX in modulating the  
61 immune system in RA and other rheumatologic disease is still not fully understood. MTX is used  
62 at far lower doses than those used in cancer<sup>15</sup>. Additionally, folic acid is frequently co-  
63 administered with MTX in order to reduce side effects in patients without an attendant loss in  
64 efficacy<sup>14</sup>, suggesting that MTX may act via other mechanisms besides DHFR inhibition. Here,  
65 we examine the impact of MTX on the gut microbiome, which in recent years has been shown to  
66 be a potent modulator of the host immune system<sup>3,16,17</sup>.

67 We reasoned that while MTX was originally developed to inhibit growth of human cells by  
68 targeting human DHFR, it may have “off-target” effects on human gut bacteria since DHFR is an  
69 enzyme that is conserved across all domains of life. Indeed, researchers have shown that MTX  
70 can bind DHFR homologues in *Escherichia coli* and *Lactobacillus casei*<sup>18</sup>. *E. coli* are resistant to  
71 any growth inhibitory effects of MTX due to the expression of a multi-drug efflux transporter  
72 *TolC*<sup>19</sup>. Overexpression of DHFR in a strain of *E. coli* that lacks *TolC* rescues it from the effects  
73 of MTX, suggesting that MTX interferes with DHFR in the cellular context<sup>19</sup>. Despite the  
74 discovery of this interaction 18 years ago, it remains unclear how generalizable this phenomenon  
75 is among other members of the human gut microbiota. A recent high-throughput screen<sup>6</sup>  
76 confirmed these original findings and demonstrated that 12/40 strains were sensitive to MTX.

77 We reasoned that this variation in the sensitivity of human gut bacteria to MTX may contribute  
78 to inter-individual variability in treatment outcomes.

79 Here, we use *in vitro* culturing, humanized mouse models, and the longitudinal analysis  
80 of human patients to demonstrate that the non-antibiotic drug MTX affects the structure and  
81 function of the human gut microbiome. Consistent with the prior literature in *E. coli*<sup>6,19</sup>, our data  
82 suggests that MTX acts via inhibition of bacterial DHFR, with downstream consequences for  
83 purine and pyrimidine biosynthesis. In treatment naïve RA patients, MTX induces a decrease in  
84 Bacteroidetes, consistent with our *in vivo* and *in vitro* data. However, this shift was only  
85 observed in drug responders, suggesting that MTX may act in part by modulating the gut  
86 microbiome. In support of this, MTX-altered microbiota from an RA patient (a drug responder)  
87 elicited less inflammation when transplanted into gnotobiotic mice. Together, these results  
88 demonstrate that drugs intended to target host pathways can have biologically relevant off-target  
89 effects on the gut microbiome, providing the first step towards addressing long-standing  
90 questions about the inter-individual variation in drug response and the integration of microbiome  
91 data for precision medicine.

92

## 93 **Results**

94 *MTX has a dose-dependent impact on the gut microbiotas of humanized gnotobiotic mice*

95 In order to determine whether MTX alters the community composition of the human  
96 microbiome *in vivo*, we utilized gnotobiotic mice colonized with a fecal aliquot from a healthy  
97 human male. We treated mice with 4 daily oral gavages of MTX at two doses, high (50 mg/kg)  
98 and low (1 mg/kg), selected to span the range of oral doses typically used in cancer<sup>20</sup> and

99 arthritis<sup>21</sup>, respectively. Vehicle controls were also included (n=3 mice/group). Daily stool  
100 samples were collected and processed for 16S rRNA gene sequencing to enable a “within-  
101 subjects” analysis of longitudinal changes in the gut microbiota for each mouse over the course  
102 of drug therapy. Endpoint samples were collected and analyzed from the small and large  
103 intestine (**Supplementary Table 1**).

104 MTX had a significant and dose-dependent impact on the gut microbiota. Total  
105 colonization based on 16S rRNA gene copy number (**Fig. 1a**) and microbial richness were  
106 comparable between treatment groups (**Fig. 1b**). In contrast, MTX treatment induced a  
107 significant shift in gut microbial community structure after a single day (ANOSIM, R=0.6049,  
108  $p=0.004$ ), and this effect persisted to day 4 of treatment (ANOSIM, R=0.7449,  $p=0.003$ ) (**Fig.**  
109 **1c**). There was no significant difference between groups prior to treatment (ANOSIM,  $p=0.109$ ).  
110 Analysis with a second statistical method (PERMANOVA) confirmed these results  
111 (**Supplementary Table 2**). By day 4 of treatment, high-dose MTX significantly decreased the  
112 normalized abundance of the Bacteroidetes phylum (DESeq  $p_{adj}=0.001$ , **Fig. 1d, Supplementary**  
113 **Fig. 1a**) compared to treatment with vehicle control. To take advantage of the “within-subjects”  
114 analysis of longitudinal changes, we used a generalized linear mixed-effects model to evaluate  
115 longitudinal changes<sup>22</sup>, and found that Firmicutes were significantly increased (slope=0.038,  
116  $p=0.005$ ) and Bacteroidetes were decreased (slope=-0.049,  $p=0.057$ ). Eleven bacterial genera  
117 were differentially abundant comparing high-dose treatment to vehicle control (4 decreased and  
118 7 increased, DESeq  $p_{adj} < 0.01$ , day 4). At a finer level, we found that 82 ribosomal sequence  
119 variants (RSVs) were differentially abundant upon treatment with high-dose MTX (DESeq  
120  $p_{adj} < 0.01$ , day 4; **Fig. 1e, Supplementary Table 3**). Low-dose MTX also significantly altered  
121 the gut microbiota, albeit to a lesser extent (20 were affected by low-dose MTX, DESeq  $p_{adj}$

122 <0.01, day 4, **Fig. 1e, Supplementary Table 4**). Fourteen RSVs were altered in both high- and  
123 low-dose MTX relative to vehicle-treated mice (**Fig. 1f**). These RSVs were altered in the same  
124 direction with either treatment, and the probability of this occurring by chance alone is  
125 exceedingly low (hypergeometric test,  $p=7.09 \times 10^{-5}$ ). Together, these results demonstrate that  
126 MTX alters the human gut microbiome in a dose-dependent manner.

127 To test if the changes observed in the fecal samples were reflective of changes in the  
128 small and large intestine, we sequenced samples from three sites (ileum, cecum, and colon). We  
129 were unable to detect significant changes in the ileum. We suspect this may be due to limited  
130 recovery because the ileum has a lower bacterial burden than the distal GI tract<sup>23</sup>, and we  
131 subsequently obtained less sequencing coverage (**Supplementary Table 1**). In contrast, the  
132 cecum and colon demonstrated significant changes in community composition (cecum:  
133 ANOSIM  $R=0.7695$ ,  $p=0.01$ ; colon: ANOSIM  $R=0.7366$ ,  $p=0.005$ ) (**Fig. 1g**). Twenty-one and  
134 23 RSVs were significantly different in the cecum and colon with low-dose treatment,  
135 respectively, and 77 and 68 RSVs were significantly different in the cecum and colon with high-  
136 dose treatment, respectively (DESeq  $p_{adj} < 0.01$ , **Supplementary Table 5, 6**). The majority of the  
137 RSVs (60% or 49/82) that were significant in the fecal samples with high-dose treatment were  
138 also significant in the cecal and colon samples (**Fig. 1h**), consistent with our prior data that fecal  
139 compositional trends reflect changes within the gastrointestinal (GI) tract<sup>24</sup>.

140 MTX is administered subcutaneously or intravenously in some patients and undergoes  
141 enterohepatic circulation, returning a portion of the systemic drug to the gastrointestinal tract<sup>25,26</sup>.  
142 To test the reproducibility of our findings given different dosing methods and in the context of a  
143 disease-associated microbiome, we compared oral (PO) administration of MTX to intraperitoneal  
144 (IP) injection in humanized gnotobiotic mice colonized with stool from a MTX-naïve RA patient

145 (n=6 adult female C57BL/6J recipients; 50 mg/kg daily for 2 days). Analysis of gut microbial  
146 community structure based on 16S rRNA gene sequencing revealed that there was no significant  
147 difference in the gut microbiotas of mice administered PO vs. IP MTX (ANOSIM R=0.111,  
148  $p=0.4$ ). When pooling both treatments and comparing pre- vs. post-treatment samples, both  
149 groups experienced a significant effect on community composition (ANOSIM R=0.4756,  
150  $p=0.019$ ; PERMANOVA  $p=0.001$ ; comparing Day 0 vs. Day 2). The Bacteroidetes phylum  
151 decreased in abundance in both PO and IP treated mice, replicating the effects we saw in our  
152 initial experiment (-1.45 fold-change,  $p_{adj}<10^{-5}$ , DESeq) (**Fig. 1i, Supplementary Fig. 1b**). We  
153 detected a significant increase in two other phyla, Firmicutes (1.65 fold-increase;  $p_{adj}=0.007$ ) and  
154 Proteobacteria (1.35 fold-increase;  $p_{adj}=0.01$ ), accompanied by a significant decrease in  
155 Verrucomicrobia (2.54 fold-decrease;  $p_{adj}=7.27 \times 10^{-4}$ ) (**Fig. 1i**). We also detected a significant  
156 impact of MTX at finer taxonomic levels: 10 genera were significantly changed (4 increased and  
157 6 decreased,  $p_{adj}<0.05$ , DESeq) and 51 RSVs were significantly altered (24 increased and 27  
158 decreased,  $p_{adj}<0.05$ , DESeq, **Supplementary Table 7**). Together, these results confirm that  
159 MTX alters the gut microbiome regardless of the route of delivery.

160 We next asked whether folic acid rescues the effects of MTX on the microbiome, since  
161 this drug is used to rescue the effects of MTX toxicity in humans<sup>27</sup>. We treated mice either with  
162 oral MTX 50 mg/kg or with MTX and folic acid in approximately a 1:1 molar ratio at 50 mg/kg  
163 daily for 2 days. Community composition was altered in both treatment groups to similar extents  
164 and we did not find a significant difference between the two groups (ANOSIM R=0.7401,  
165  $p=0.1$ ), suggesting that folic acid at the dose given in our study was not sufficient to rescue the  
166 effects of MTX on the microbiome. When comparing pre- and post-treatment samples using  
167 DESeq, we saw reproducible trends at the phylum level: a decrease in Bacteroidetes and



168 Verrucomicrobia (fold changes of -1.44 and -12.47 with  $p_{adj} = 3.69 \times 10^{-6}$  and  $p_{adj} = 3.7 \times 10^{-21}$ ,  
169 respectively), and an increase in Firmicutes (1.76 FC;  $p_{adj} = 4.56 \times 10^{-8}$ ) and Proteobacteria (1.39  
170 FC;  $p_{adj} = 0.001$ ) (**Fig. 1i, Supplementary Fig. 1c**). Twenty genera (10 increased and 10  
171 decreased,  $p_{adj} < 0.05$ , DESeq) and 67 RSVs (32 increased and 35 decreased,  $p_{adj} < 0.05$ , DESeq,  
172 **Supplementary Table 8**) were affected by MTX.

173 Taken together, these experiments demonstrate that MTX reproducibly alters the human  
174 gut microbiota in gnotobiotic mice, with reproducible effects across multiple delivery routes and  
175 co-therapies. Next, we sought to test if MTX directly impacts the growth of human gut bacteria  
176 or reflects a downstream consequence of this drug on host tissues.

177

#### 178 *Methotrexate directly inhibits the growth of human gut bacteria*

179 High-dose MTX acts by inhibiting dihydrofolate reductase (DHFR)<sup>13</sup>, an enzyme  
180 conserved across all 3 domains of life<sup>28</sup>. Given the importance of DHFR for bacterial growth  
181 across multiple phyla<sup>29</sup>, we hypothesized that MTX could act as a broad-spectrum antibiotic. To  
182 test for a direct impact of MTX on bacterial cell growth, we incubated MTX with a panel of 43  
183 bacterial isolates from 6 phyla, 40 of which are commonly found in the human gut microbiota  
184 (**Supplementary Table 9**). The exceptions include *Bacteroides acidifaciens* (from mice), and  
185 *Delftia acidovorans* and *Bacillus subtilis* 168 (from soil). The combined average relative  
186 abundance of these bacterial isolates is 43%<sup>30</sup> of the human gut microbiota (**Supplementary Fig.**  
187 **2; Methods**). Each isolate was incubated with a gradient of MTX ranging from 0-900  $\mu\text{g/ml}$  (0-2  
188 mM) and bacterial growth in rich media was quantified by optical density. The concentration  
189 required to prevent  $\geq 90\%$  growth ( $\text{MIC}_{90}$ ) ranged three orders of magnitude: from 2  $\mu\text{M}$  to 2 mM

190 **(Fig. 2a)**. While 11 of 45 isolates (24%) were resistant to the maximum concentration tested, 34  
191 isolates (76%) were variably sensitive to the growth inhibition induced by MTX. On average,  
192 Bacteroidetes tended to be sensitive to the antimicrobial effects of MTX relative to the other  
193 phyla **(Fig. 2b,  $p=0.005$ , Wilcoxon rank-sum test)**.

194 Nineteen (42%) of the MIC<sub>90</sub> values were at or below the predicted concentration of  
195 MTX in the proximal GI tract (250  $\mu$ M, 113  $\mu$ g/ml, *Methods*)<sup>31</sup>, suggesting that MTX is capable  
196 of inhibiting growth of these isolates at physiological concentrations. Among the remaining 26  
197 isolates (58%) whose MIC<sub>90</sub> are above the predicted concentration, we asked whether MTX  
198 could still significantly alter the growth of these isolates by examining carrying capacity, growth  
199 rate, and the duration of lag phase (*i.e.* time to mid-exponential growth). We found that 19 of the  
200 remaining 26 (73%) isolates showed changes in at least one of these three growth parameters in a  
201 dose-dependent manner **(Fig. 2c, Supplementary Fig. 3, see *Methods*)**. Taken together, our  
202 results show that 38 of the 45 (84%) tested isolates exhibit either growth inhibition or alterations  
203 in growth curve parameters upon exposure to MTX at concentrations that are predicted to be  
204 found in the human gut. These results raised the question of whether or not MTX acts through  
205 the canonical mechanism-of-action established in eukaryotic cells or if it has off-target effects on  
206 other aspects of bacterial physiology.

207

### 208 *Methotrexate impacts gut bacterial purine and pyrimidine biosynthetic pathways*

209 Prior studies have revealed that MTX can directly inhibit bacterial DHFR<sup>18</sup>; however, the  
210 downstream consequences of this inhibition for bacterial physiology remain unexplored. To  
211 globally profile the impact of bacterial DHFR inhibition, we used transcriptomics (RNA-Seq) to

212 search for differentially expressed transcripts in the presence or absence of MTX. Based on the  
213 extensive literature in human cells<sup>32,33</sup>, we anticipated that the inhibition of DHFR by MTX  
214 would lead to changes in the expression of purine and pyrimidine pathways, which rely on folate  
215 as a co-factor for multiple key reactions. We selected isolates with varying sensitivity to the  
216 growth-inhibitory effects of MTX: *Clostridium asparagiforme* DSMZ 15981 (MIC<sub>90</sub> > 900  
217 µg/ml), *Clostridium sporogenes* ATCC 15579 (MIC<sub>90</sub> > 900 µg/ml), *Clostridium symbiosum*  
218 DSM 934 (MIC<sub>90</sub> = 450 µg/ml), and *Bacteroidetes thetaiotaomicron* DSMZ 2079 (MIC<sub>90</sub> = 112  
219 µg/ml). Bacteria were treated with MTX 100 µg/ml or vehicle control (DMSO) for 30 minutes  
220 during mid-exponential phase (n=3 per treatment group per isolate).

221 MTX induced a profound shift in gene expression in the isolate that was most sensitive to  
222 MTX, *B. thetaiotaomicron*, affecting 83% of genes in the transcriptome (**Fig. 3a**, 3,735 genes at  
223 FDR<0.2, DESeq). In contrast, two less sensitive isolates, *C. sporogenes* and *C. symbiosum*,  
224 exhibited a more defined shift in gene expression in response to MTX (**Fig. 3a**, 21 and 55 genes,  
225 FDR<0.2, DESeq, respectively). Although *C. asparagiforme* did not show significant growth  
226 inhibition with MTX (**Fig. 2a**), it had a robust transcriptional response to the drug, with 468  
227 genes (8% of expressed transcripts) exhibiting differential expression at 30 minutes. These  
228 results demonstrate that gut bacterial strains vary in their response and sensitivity to MTX at the  
229 transcriptional level. Surprisingly, some drug resistant bacteria still show a significant  
230 transcriptional response to the drug, consistent with our prior observations that MTX can impact  
231 bacterial physiology even below the MIC<sub>90</sub>.

232 To gain insight into the metabolic pathways most impacted by MTX, we used the KEGG  
233 database to search for differentially expressed metabolic pathways and modules. Both purine and  
234 pyrimidine metabolism were significantly changed in *C. asparagiforme* and *B. thetaiotaomicron*.

235 While *B. thetaiotaomicron* exhibited multiple pathway enrichments (57 pathways enriched  
236 among transcripts with FDR<0.2), purine and pyrimidine metabolism were among the top 10  
237 pathways (9<sup>th</sup> and 5<sup>th</sup>, respectively) when ranked by *p*-value ( $p<0.05$  with BH adjustment), and  
238 these enrichments were insensitive to the FDR threshold used in our analyses (**Supplementary**  
239 **Table 10**). *C. asparagiforme* exhibited enrichment of 23 pathways, and purine and pyrimidine  
240 metabolism were 2<sup>nd</sup> and 11<sup>th</sup> among this list (**Supplementary Table 11**). These results suggest  
241 that MTX can exert wide-ranging effects on numerous pathways in sensitive bacteria like *B.*  
242 *thetaitaomicron*, or have more specific effects on metabolic pathways in bacteria like *C.*  
243 *asparagiforme*.

244 To assess the dynamics of sub-MIC<sub>90</sub> MTX response, we performed a time course  
245 experiment on *Clostridium asparagiforme* (MIC<sub>90</sub> >900 µg/ml), comparing transcriptional  
246 profiles at 30 minutes, 4 hours and 20 hours post drug exposure to vehicle controls. We found  
247 that 1,018 genes were affected at 4 hours and 900 affected at 20 hours (**Fig. 3b**; FDR<0.2).  
248 Consistent with our original analyses, purine and pyrimidine metabolism continued to be among  
249 the pathways that were affected at 4 and 20 hours of treatment in *C. asparagiforme*  
250 (**Supplementary Table 11**). Of the 41 transcripts that were differentially expressed at all three  
251 timepoints (FDR<0.2, DESeq), 21 consistently changed in the same direction (5 upregulated, 16  
252 down-regulated), whereas 20 demonstrated more complicated dynamics (**Fig. 3c**).

253 Because MTX is thought to target multiple enzymes, including DHFR<sup>34</sup> and AICAR  
254 transformylase<sup>35</sup> which are both involved in purine metabolism, we examined the homologs of  
255 enzymes involved in purine metabolism in *C. asparagiforme*. While we did not find that DHFR  
256 or AICAR transformylase were differentially expressed, we found that multiple enzymes either  
257 upstream or downstream of these genes were differentially expressed. Enzymes involved in *de*

258 *novo* purine biosynthesis as well as the salvage pathway for purine synthesis showed differential  
259 expression (**Fig. 3d**). For example, adenylosuccinate synthase (ADSS), which is involved in  
260 converting inosine monophosphate (IMP) into adenylosuccinate (AMPS)<sup>36</sup>, is significantly  
261 upregulated. These findings further support the hypothesis that MTX targets bacterial enzymes  
262 involved in purine metabolism; however, it remains possible that other pathways would be more  
263 dramatically affected in later stages of drug exposure.

264         Taken together, these results suggest that while MTX was developed as a drug to target  
265 human cells, it has off-target effects on pathways for purine and pyrimidine metabolism in  
266 bacterial cells. MTX inhibits the growth of several gut bacterial isolates in a dose-dependent  
267 manner. Transcriptionally, it targets pathways that are reliant on folate, including purine and  
268 pyrimidine metabolism, but can also affect more widespread pathways as seen in *B.*  
269 *thetaitaomicron* and the 20-hour time point in *C. asparagiforme*. Our data from *C.*  
270 *asparagiforme* demonstrates that even when MTX does not affect the growth of the bacteria, it  
271 can still affect transcriptional pathways that depend on folic acid.

272

### 273 *Clinical relevance of the interaction between MTX and the human gut microbiome*

274         A major caveat of our isolate-based studies is that human gut bacteria may not be directly  
275 sensitive to MTX in the context of a complex gut microbial community or in the physiochemical  
276 conditions found within the human gastrointestinal tract. To address this concern, we turned to *ex*  
277 *vivo* incubations of human distal gut microbiomes and studies of patient cohorts. First, we  
278 examined whether MTX affects growth in *ex vivo* stool samples obtained from 30 MTX-naïve  
279 patients. We treated fecal suspensions with MTX 100 µg/ml or vehicle control and measured

280 growth by optical density over 48 hours. We found that MTX inhibits growth of these complex  
281 *ex vivo* communities (**Fig. 4a**). Specifically, we found that MTX decreases the carrying capacity  
282 ( $p < 10^{-5}$ , paired Student's *t*-test, **Fig. 4b**) and increases the time to mid-exponential growth  
283 ( $p < 0.001$ , paired Student's *t*-test, **Fig. 4c**) in *ex vivo* samples, supporting the hypothesis that  
284 MTX can have growth inhibitory effects on patient microbiomes *ex vivo*.

285 We next asked whether these findings extended to the *in vivo* setting in RA patients. We  
286 performed 16S rRNA gene sequencing on stool samples collected at baseline and 1 month after  
287 treatment initiation from 23 MTX-naïve patients. Consistent with prior reports<sup>24,37-39</sup>, we  
288 detected a strong signal of individuality in gut microbial community structure ( $R=0.9152$ ,  
289  $p=0.001$ , ANOSIM). Although there were marked shifts in community structure within each  
290 subject following treatment (**Supplementary Fig. 4**), we were unable to detect a reproducible  
291 shift across the entire patient cohort in response to MTX (ANOSIM,  $R=-0.09$ ,  $p=0.888$ ),  
292 consistent with prior studies<sup>40</sup>. We anticipated an individualized microbial community response  
293 to MTX given inter-individual differences in the gut microbiota prior to therapy coupled to the  
294 extensive strain-level variation in drug sensitivity identified in this study.

295 These results led us to hypothesize that differences in the gut microbial response to MTX  
296 could explain some of the differences in clinical response. Given our relatively small sample  
297 size, we used a binary classification of drug response defined as a decrease in DAS28<sup>41</sup> of at  
298 least 1.8 and continued use of oral MTX without addition of other disease-modifying anti-  
299 rheumatic drugs (DMARDs) or biologics. Using these criteria, our cohort was split between 15  
300 MTX non-responders (MTX-NR) and 8 responders (MTX-R). The baseline disease activity  
301 parameters between these two groups did not differ (**Supplementary Table 12**). Among the 8  
302 MTX-R, we observed a significantly decreased relative abundance of Bacteroidetes compared to

303 MTX-NR ( $p=0.01$ , Wilcoxon rank-sum test, **Fig. 4d, 4e**). This decrease in Bacteroidetes  
304 recapitulated results we observed in our *in vitro* and humanized gnotobiotic mouse studies and  
305 led us to ask whether drug-induced shifts in the human gut microbiota contribute to the  
306 resolution of autoimmune disease.

307

### 308 *MTX-altered microbiota dampen host immune responses*

309 To determine whether the anti-inflammatory effects of MTX are mediated in part via the  
310 microbiome, we examined the functional impact of MTX-altered microbiota on mucosal and  
311 peripheral T cell populations in gnotobiotic mice. We identified the MTX-responder among our  
312 patient cohort with the greatest decrease in Bacteroidetes and transferred fecal microbiota  
313 obtained before (M0) and 1 month after MTX treatment (M1) from this donor into C57BL/6J  
314 germ-free mice (N=10 per donor sample, 20 female mice total, **Supplementary Fig. 4b**). Half of  
315 the animals in each group were challenged with an inflammatory trigger using 2% dextran  
316 sodium sulfate (DSS) (N=5 per donor sample), a chemical added to the drinking water used to  
317 induce rapid and robust colitis. Since RA pathophysiology is associated with a dysregulated T  
318 cell response<sup>42</sup>, we focused on the T cell compartment in the spleen and the intestinal lamina  
319 propria of the small intestine and colon using flow cytometry. In unchallenged mice, we found  
320 that splenic populations of activated (CD44+CD69+) T cells were decreased in mice transplanted  
321 with MTX-altered microbiota (M1) compared to M0 microbiota (10.6% vs. 16.8%;  $p=0.04$ ,  
322 Student's *t*-test; **Fig. 4f**). We did not observe significant differences in mucosal T cell  
323 populations (Th17, Th1 or T regulatory cells) or in myeloid cells in unchallenged mice  
324 (**Supplementary Fig. 4c**).

325 In mice challenged with DSS, similar extents of weight loss, clinical colitis, and colonic  
326 shortening were observed between transplant groups (**Supplementary Fig. 4d**). Examination of  
327 immune cell populations confirmed that DSS induced an inflammatory response (**Fig. 4f,g**). T  
328 cell activation in the spleen was decreased in mice colonized with M1 compared to M0  
329 microbiota (6.7% vs. 12%;  $p=0.0001$ , Student's *t*-test; **Fig. 4f**), similar to what was observed in  
330 the unchallenged state. Furthermore, splenic T cells expressing IFN- $\gamma$  were reduced (3.5% vs.  
331 4.8%;  $p=0.01$ , Student's *t*-test; **Supplementary Fig. 4c**). In mice harboring M1 microbiota, Th17  
332 T cells were relatively decreased in the small intestine (15.8% vs. 31.4%;  $p=0.0001$ , Student's *t*-  
333 test) and colon (11.1% vs. 18.5%;  $p=0.01$ , Student's *t*-test) (**Fig. 4g**), suggesting that MTX  
334 treatment leads to a reduction in the Th17-inflammatory potential of human gut microbiota.  
335 Overall, these findings suggest that MTX-induced shifts to the microbiota reduce its  
336 inflammatory potential and may contribute to resolution of autoimmune disease.

337

## 338 **Discussion**

339 While MTX was developed to target human dihydrofolate reductase (DHFR), it was not  
340 designed to be specific for the human enzyme, leading us to ask whether this non-antibiotic drug  
341 may have direct, off-target effects on bacteria. Previous studies demonstrated that community  
342 composition is altered in rheumatoid arthritis patients relative to healthy individuals<sup>43</sup> and that  
343 this alteration is "reversed" with treatment<sup>44</sup>. But in these patient studies, it is unclear whether the  
344 changes to the microbiome are a result of drug treatment, concomitant treatment with other  
345 medications, improvement of the disease process, reduction of inflammation, or other  
346 confounding factors that may be present in observational cohort studies. Our studies in disease-  
347 free gnotobiotic mice, coupled to *in vitro* and *ex vivo* results, demonstrate that MTX



348 directly exerts growth inhibitory effects on a diverse panel of human gut bacterial isolates with  
349 downstream consequences for bacterial physiology and the expression of genes involved in  
350 purine and pyrimidine metabolism. Thus, our findings extend our knowledge of the effects of  
351 MTX on the host to include its effects on the gut microbiome.

352         These results provide a critical foundation to ask whether MTX may exert part of its anti-  
353 inflammatory effects via the gut microbiome. Although MTX has been used for several decades  
354 to reduce inflammation in patients with autoimmune disease, the mechanisms by which it does  
355 so are not fully understood. A leading hypothesis is that MTX directly inhibits an enzyme called  
356 AICAR transformylase, resulting in an increase in extracellular levels of adenosine, a potent  
357 anti-inflammatory mediator<sup>14,45</sup>. Other possible mechanisms that have been investigated suggest  
358 that MTX may affect cytokine production by immune cells, such as by decreasing the production  
359 of the proinflammatory cytokine TNF $\alpha$ <sup>46</sup> or increasing the production of the  $\alpha$ -inflammatory  
360 cytokine IL-10<sup>47</sup>. But it remains unknown whether MTX reduces inflammation by altering the  
361 gut microbiome, which has been shown to be a potent modulator of the host immune system.  
362 Importantly, there is a precedent for a drug's therapeutic effects being mediated via the  
363 microbiome in the context of the treatment of diabetes with metformin<sup>48,49</sup>.

364         We provide evidence for such a mechanism by transferring microbiota from a MTX-  
365 treated RA patient donor (obtained before and 1 month after treatment) into germ-free mice. We  
366 found that MTX results in a decrease in Bacteroidetes, permitting members of other bacterial  
367 phyla to expand in relative abundance, and that these shifts alter the inflammatory potential of  
368 the microbiome. In particular, we found a reduction in Th17 cells in the mucosa and a reduction  
369 in activated T cells and IFN- $\gamma$ + T cells in the periphery. These cell types are thought to play key  
370 roles in RA pathogenesis<sup>42</sup>, and prior studies suggest that MTX decreases IL-17 levels in RA

371 patients<sup>50</sup>. By uncoupling the effect of MTX on the host from its effect on the gut microbiome,  
372 these gnotobiotic results provide causal evidence suggesting that MTX exerts its anti-  
373 inflammatory effects in part by reducing the ability of the gut microbiome to contribute to an  
374 inflammatory response. Our findings advance the current understanding of the  
375 pharmacodynamics of MTX and provide a critical foundation to further investigate the microbial  
376 effectors of these immune responses.

377         The impact of MTX on bacterial biomass are not as pronounced as seen with many  
378 broad-spectrum antibiotics<sup>51</sup>; however, our data shows that MTX can have effects on bacterial  
379 physiology at a sub-inhibitory concentration. These results indicate that we are likely  
380 underestimating the effect of MTX (and possibly other drugs<sup>52</sup>) on the gut microbiome when  
381 examining community composition by DNA sequencing alone. As expected, bacteria sensitive to  
382 the growth inhibitory effects of MTX, such as *B. thetaiotaomicron*, exhibited profound  
383 transcriptomic changes. Surprisingly, we found that *C. asparagiforme*, which is resistant to the  
384 growth inhibitory effects of MTX, experienced pronounced changes at the transcriptional level.  
385 Thus, *in vivo*, MTX may not only affect community composition, but it may also have broader  
386 effects on transcriptional and metabolic programs of human gut microbiota. Further studies are  
387 needed to examine transcriptional and metabolic changes to the human gut microbiome induced  
388 by MTX in both humans and mouse models.

389         While we did not observe a consistent effect of MTX on community composition among  
390 all RA patients when examining 16S rRNA gene sequencing-based community composition, this  
391 may be because there is heterogeneity among RA patients with respect to the drug disposition<sup>53</sup>,  
392 genetic risk factors<sup>54</sup>, and/or microbiome composition<sup>44</sup>. Our data demonstrates that gut bacterial  
393 strains and intact microbial communities can vary dramatically in their drug sensitivity. The

394 underlying determinants of this variability remain to be investigated and are likely multifactorial.  
395 Of note, responders tend to experience a greater decrease in Bacteroidetes upon initiation of  
396 MTX relative to non-responders. Additionally, the transcriptional response to MTX *in vivo* can  
397 be greater than detected by 16S rRNA gene sequencing alone.

398 Our data suggests that the interaction between MTX and the gut microbiome is broadly  
399 relevant to patients even given differences in dosing route and adjuvant therapies. Both  
400 intraperitoneal dosing and oral dosing impacted the gut microbiome, likely due to extensive  
401 enterohepatic circulation<sup>25,26</sup>. Similar microbiome effects were observed with folic acid  
402 supplementation, which is commonly given in RA patients, suggesting that the dose administered  
403 does not markedly rescue *in vivo* bacterial growth inhibition. Follow-on longitudinal analysis in  
404 patients given a variety of treatment regimens would help to further clarify whether or not the  
405 impact of MTX on the gut microbiome depends at all on common clinical confounders.

406 In conclusion, our results emphasize the importance of taking a broader view of  
407 toxicology that encompasses the unintended consequences of non-antibiotic drugs for our  
408 associated microbial communities. Our studies demonstrate the utility of integrated studies *in*  
409 *vitro*, in gnotobiotic mice, *ex vivo*, and in drug naïve patients to begin to elucidate the causality  
410 and mechanism for these complex drug-microbiome-host interactions. Remarkably, the observed  
411 drug-induced changes in microbial community structure were associated with patient response,  
412 providing a useful biomarker for accelerating the stable initiation of therapy and a first step  
413 towards determining which bacterial taxa contribute to or interfere with treatment outcomes.

414

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428

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431 and P.J.T.

432

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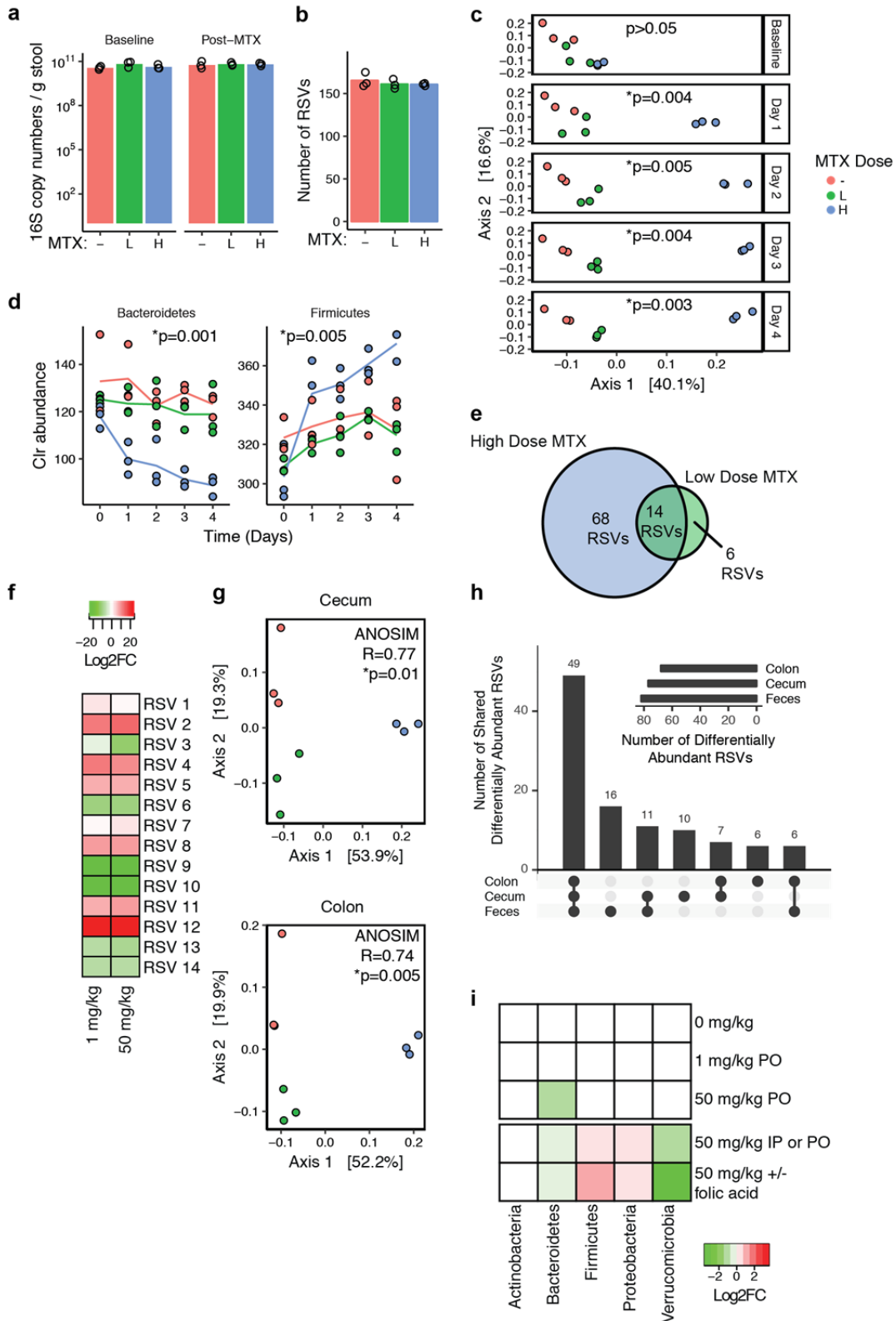
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609

610 **Figures & Legends**

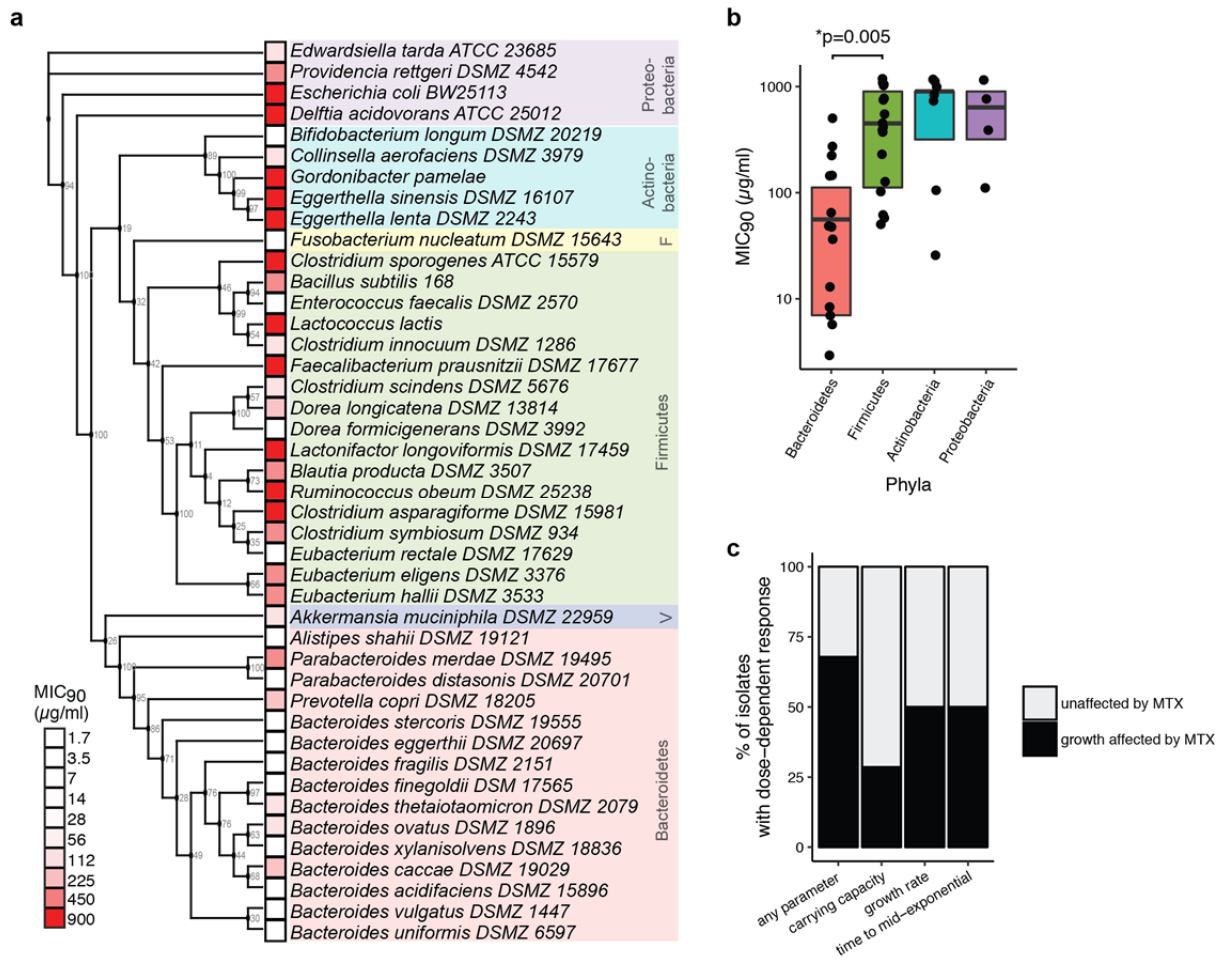


611

612

613 **Figure 1. MTX alters the gut microbiomes of humanized mice.** (a) Bacterial biomass as  
614 assessed by 16S copy number per gram of stool was quantitated using qPCR at baseline (Day 0)  
615 and after MTX treatment (Day 4). Treatments denoted by “-”, vehicle control; “L”, low-dose  
616 MTX (1 mg/kg); “H” high-dose MTX (50 mg/kg) (N=3 per treatment group). (b) Number of  
617 ribosomal sequence variants (RSVs) detected in treatment groups using 16S rRNA gene  
618 amplicon sequencing (N=3 per treatment group). (c) Community composition is altered with  
619 MTX treatment. Principal coordinates analysis (PCoA) of Bray-Curtis distances using clr  
620 transformed values at multiple time points after treatment. ANOSIM testing was performed  
621 comparing MTX at each time point. (d) The Bacteroidetes phylum significantly decreased with  
622 high-dose MTX treatment (DESeq  $p_{adj}=0.001$ , comparing Day 4 vehicle vs. 50 mg/kg MTX).  
623 Firmicutes showed a time-dependent increase with treatment ( $p=0.005$ , generalized linear mixed  
624 modeling, see *Methods*). (e) Eighty-two and 20 RSVs were altered with 4 days of high- and low-  
625 dose MTX, respectively, relative to vehicle control. There were 14 RSVs that overlapped  
626 between these two sets. (f) Among the 14 RSVs that changed with both high- and low-dose  
627 treatment, all changed in the same direction. (g) Sites in the proximal GI tract also revealed  
628 changes in community composition with MTX treatment. (h) An UpSet plot of RSVs that were  
629 differentially abundant when comparing high-dose samples vs. vehicle controls in the cecum,  
630 colon and feces. The majority of RSVs that were differentially abundant in the feces were also  
631 differentially abundant in the cecum and colon. (i) A heatmap summarizing the significant  
632 phylum level changes observed in multiple gnotobiotic mouse experiments. MTX reproducibly  
633 induces a decrease in Bacteroidetes and Verrucomicrobia with a concomitant increase in

634 Firmicutes and Proteobacteria (as assessed using DESeq comparing pre- and post-treatment time  
635 points).

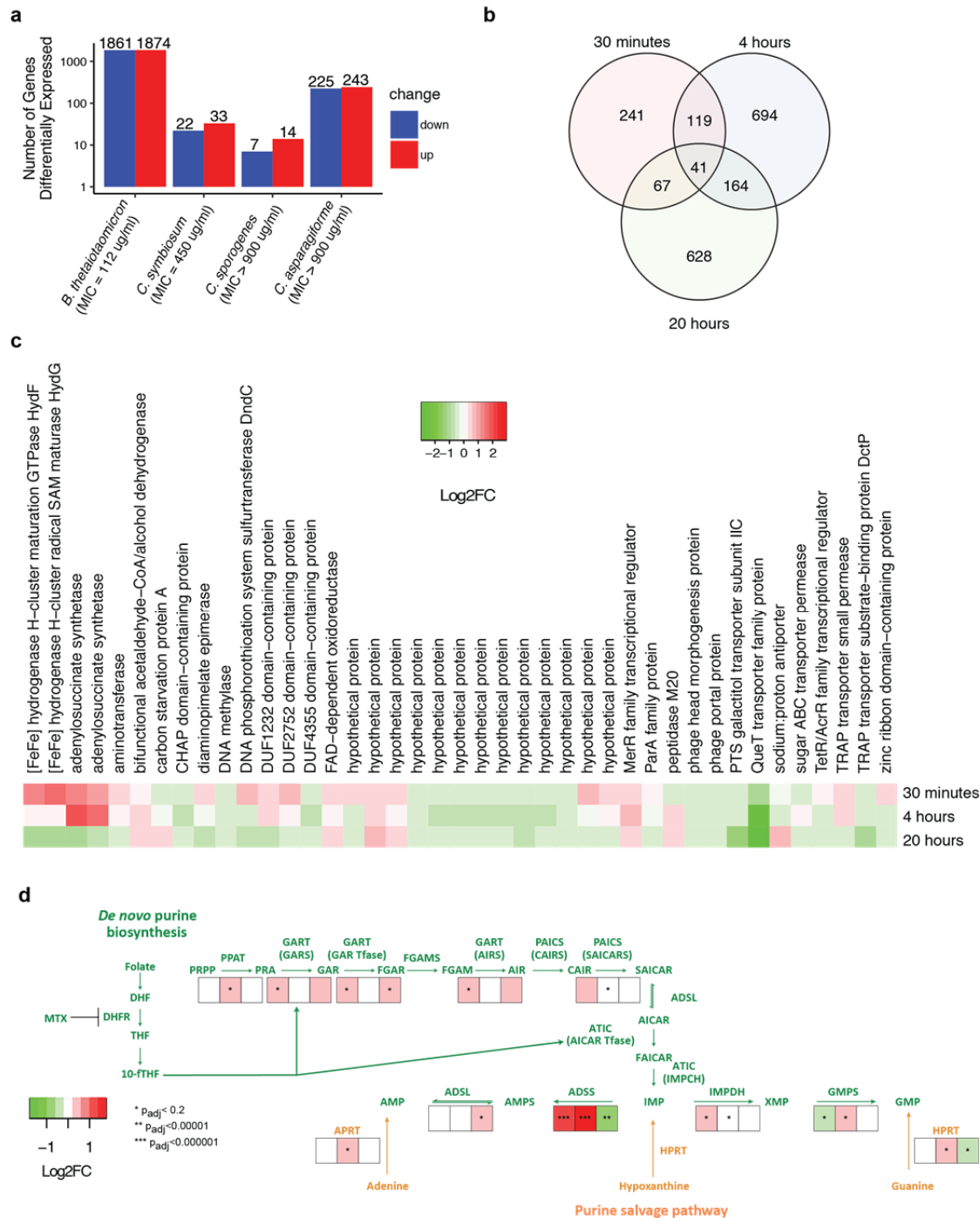


636

637 **Figure 2. MTX directly affects growth of human gut bacteria.** (a) A diverse panel of 43  
 638 isolates were incubated with varying concentrations of MTX and the minimal inhibitory  
 639 concentration (MIC<sub>90</sub>) was measured. F, Fusobacteria; V, Verrucomicrobia. A maximum  
 640 likelihood phylogenetic tree using 16S rRNA gene sequences for each organism was constructed,  
 641 with bootstrap values from 100 iterations shown at each branch point. (b) The MICs of various  
 642 isolates spanning 4 major phyla. Bacteroidetes tended to be sensitive relative to other phyla when  
 643 tested *in vitro* ( $p=0.005$ , Wilcoxon rank-sum test). The top and bottom hinges in the boxplot  
 644 correspond to the first and third quartile, respectively, and the horizontal lines denotes the

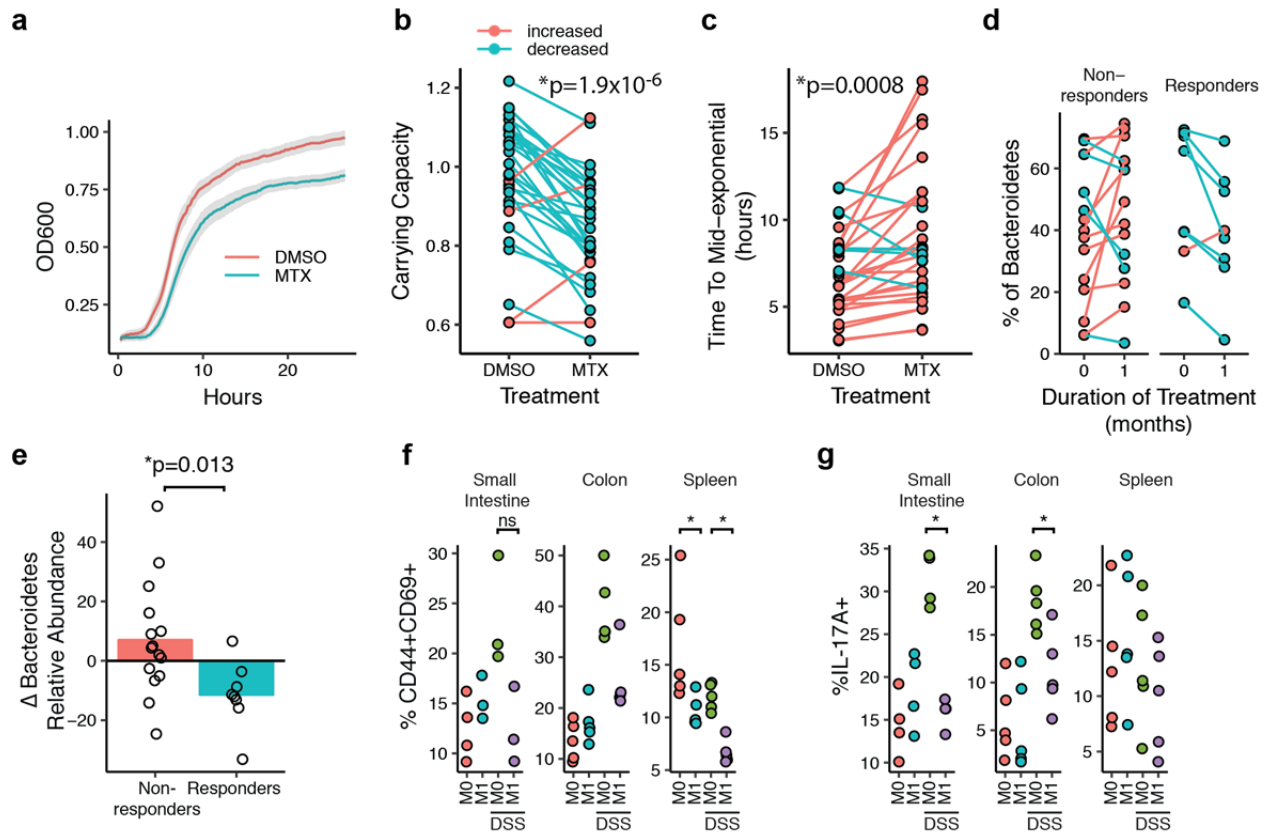


645 median. (c) Carrying capacity, growth rate and lag phase parameters were affected in a dose-  
646 dependent manner by MTX among a significant proportion of bacteria.



648 **Figure 3. MTX affects transcriptional pathways associated with purine and pyrimidine**  
 649 **metabolism in human gut bacteria.** (a) A variable number of transcripts, as determined by  
 650 RNA-Seq, were differentially expressed (FDR<0.2, DESeq) upon 30 minutes of MTX 100 µg/ml  
 651 (compared to growth control) in 4 bacterial isolates with varying sensitivity to the growth

652 inhibitory effects of the drug (n=3 per treatment). *B. thetaiotomicron*, which is sensitive to the  
653 growth inhibitory effects of MTX, exhibited a profound alteration in transcriptional response. *C.*  
654 *asparagiforme*, which is resistant to the growth inhibitory effects of MTX, also manifested a  
655 transcriptional response. **(b)** We performed a time course analysis on *C. asparagiforme*, which  
656 was profiled at 30 minutes, 4 hours and 20 hours of treatment (n=3 per treatment group per time  
657 point). The Venn diagram shows the number of transcripts that were differentially expressed  
658 with each treatment duration. **(c)** A heatmap of the 41 transcripts that were differentially  
659 expressed (FDR<0.2) at all three time points in *C. asparagiforme*. **(d)** Multiple enzymes  
660 involved in purine metabolism<sup>36</sup> were affected at some point during the time course study that  
661 was performed on *C. asparagiforme* (heatmap of log<sub>2</sub> fold change values for enzymes shown,  
662 with asterisks indicating  $p_{adj}$  values). See Supplementary Table 13 for a description of substrate  
663 and enzyme symbols.

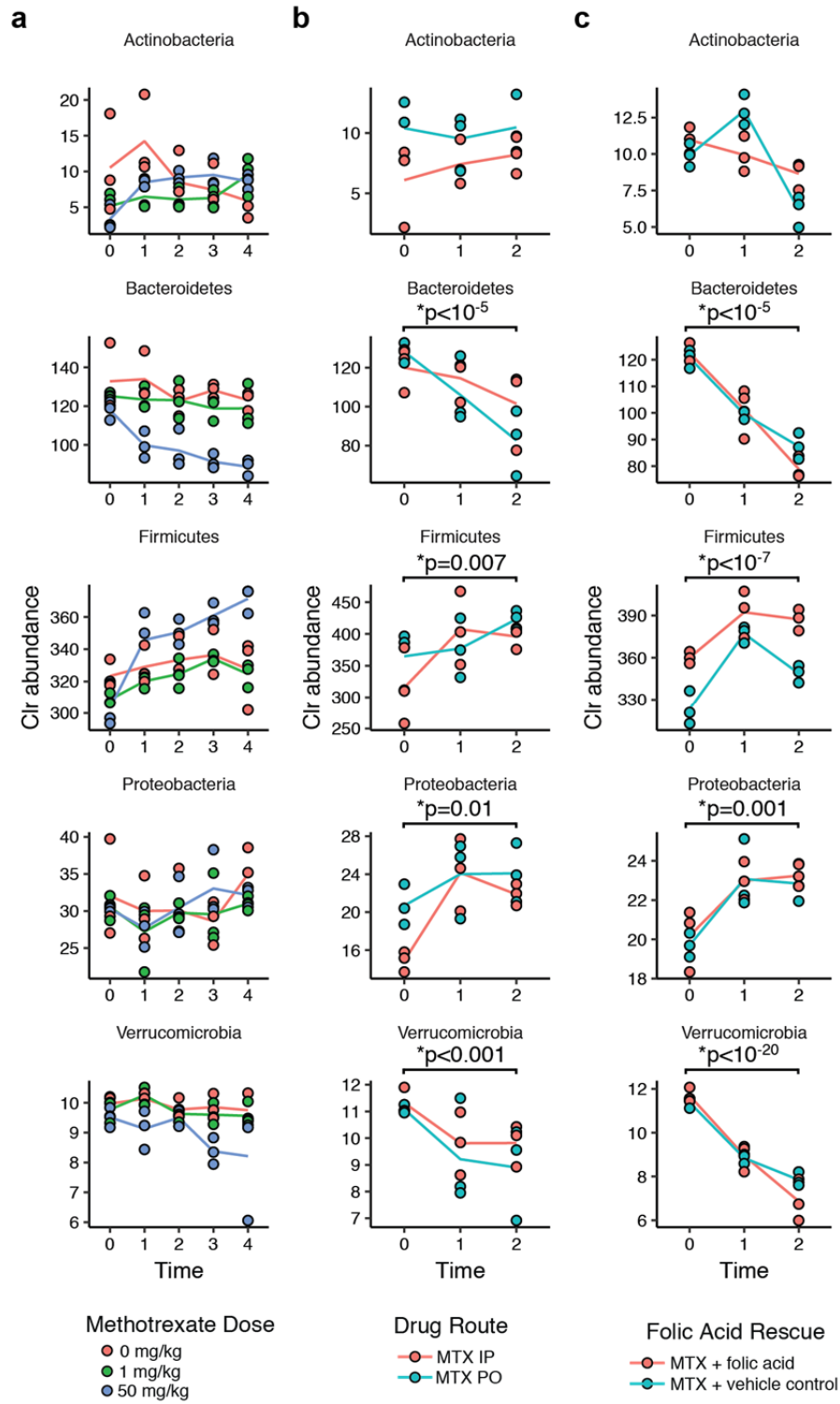


664

665 **Figure 4. Human gut bacteria from rheumatoid arthritis (RA) patients are affected by**  
 666 **MTX *ex vivo* and *in vivo*, and MTX alters their inflammatory potential. (a)** Growth of fecal  
 667 suspensions from treatment-naive RA patients (n=30) was measured using optical density in the  
 668 presence of MTX 100 µg/ml or DMSO. The average growth curves among the 30 patients are  
 669 shown for each treatment. Shaded areas represent ±SEM. **(b)** Carrying capacity was significantly  
 670 decreased among the 30 patient fecal suspensions *ex vivo* (paired Student's *t*-test). **(c)** Time to  
 671 mid-exponential was significantly increased among the 30 patient fecal suspensions *ex vivo*  
 672 (paired Student's *t*-test). **(d)** Fecal samples from 23 RA patients was subjected to 16S sequencing  
 673 before treatment with MTX and 1 month after treatment. Patients that demonstrated a favorable  
 674 clinical response to MTX showed a decrease in relative abundance of Bacteroidetes compared to  
 675 non-responders. **(e)** There is a significant decrease in the relative abundance of Bacteroidetes

676 with MTX treatment among responders compared to non-responders ( $p=0.013$ , Wilcoxon rank-  
677 sum). **(f)** Percentage of CD3+CD4+ T cells with CD44+CD69+ staining (activated T cells) from  
678 the small intestine and colon lamina propria and spleen of mice transplanted with microbiota  
679 from a patient before MTX treatment (“M0”) and 1 month after treatment (“M1”) (N=3-5 per  
680 treatment group). Mice were also treated with dextran sodium sulfate (“DSS”) in their drinking  
681 water. **(g)** Percentage of IL-17A+ cells in the CD3+CD4+ T cell compartment as determined by  
682 flow cytometry in the small intestine and colon lamina propria and spleen (N=3-5 per treatment  
683 group). Panels f,g:  $*p<0.05$ , 2-tailed Student’s *t*-test.

684 **Supplementary Figures & Legends**

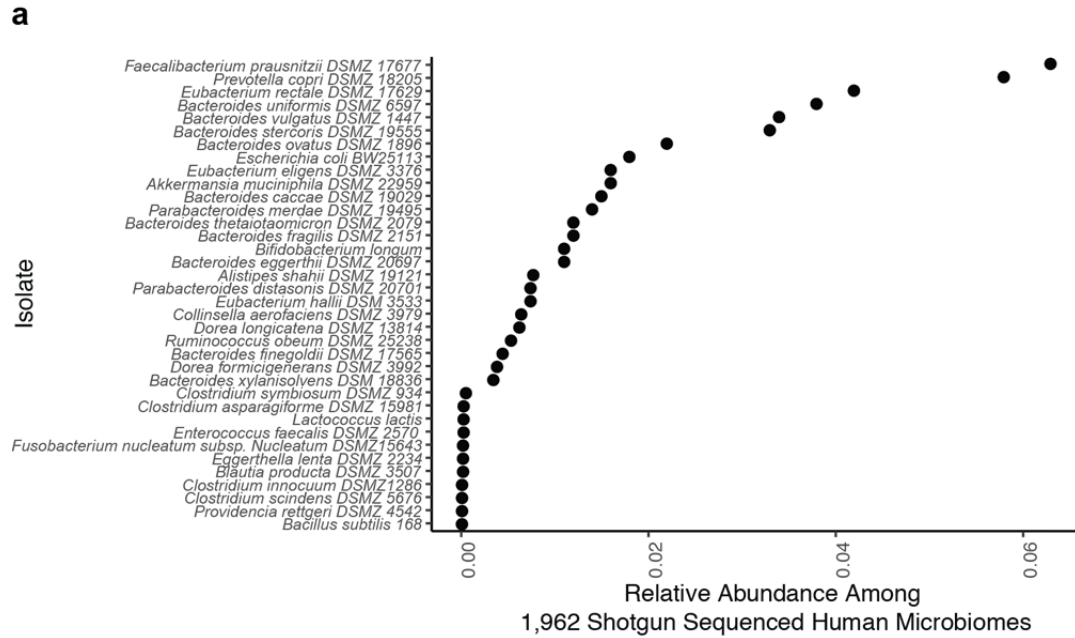


685

686 **Supplementary Figure 1. Phylum-level changes are seen in response to MTX treatment. (a)**

687 Germ-free mice were colonized with a fecal aliquot from a healthy human donor, and treated  
688 with either vehicle control, low-dose MTX (1 mg/kg), or high-dose MTX (50 mg/kg) (n=3 per  
689 treatment group). Stool samples were collected daily and underwent 16S rRNA gene amplicon  
690 sequencing. Shown are the phylum-level trends over time (in days). **(b)** Germ-free mice were  
691 colonized with a fecal aliquot from a treatment-naïve rheumatoid arthritis patient and treated  
692 with either IP or PO MTX (50 mg/kg) (n=3 per treatment group). Shown are the phylum-level  
693 trends. DESeq was used to compare pre- (Day 0) and post-treatment (Day 2) phylum levels  
694 among all mice, regardless of treatment group. Significance values are BH-corrected. **(c)** Germ-  
695 free mice were colonized with a fecal aliquot from a treatment-naïve rheumatoid arthritis patient  
696 and treated with either oral MTX (50 mg/kg) + vehicle control or oral MTX + folic acid (50  
697 mg/kg) (n=3 per treatment group). Shown are the phylum-level trends. DESeq was used to  
698 compare pre- (Day 0) and post-treatment (Day 2) phylum levels among all mice, regardless of  
699 treatment group. Significance values are BH-corrected. For all plots, colored lines connect the  
700 average abundance per treatment group.

701



702

703 **Supplementary Figure 2. Relative abundance of isolates used in our *in vitro* screen among**

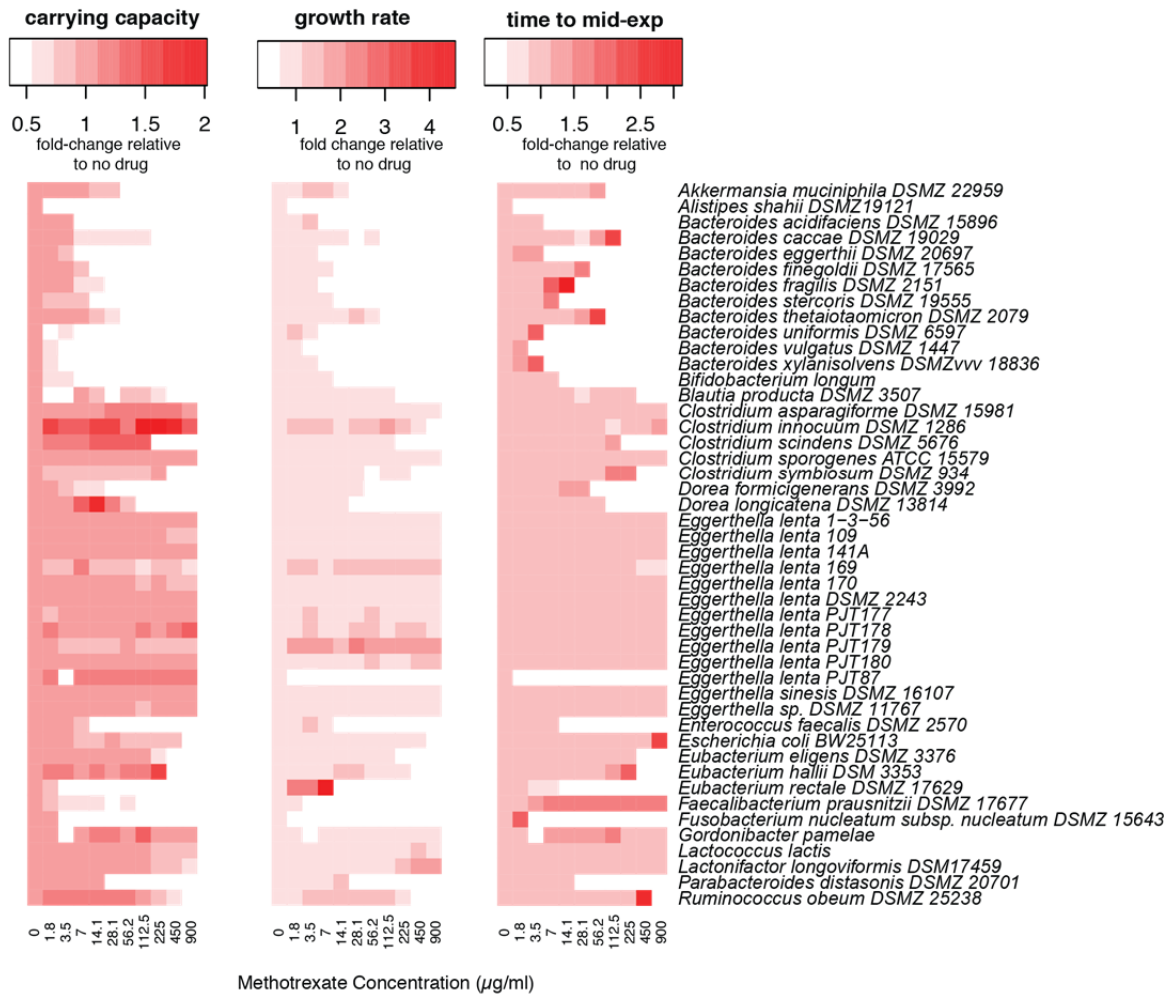
704 **publicly available gut microbiomes. (a) Shown is the mean abundance of each isolate among**

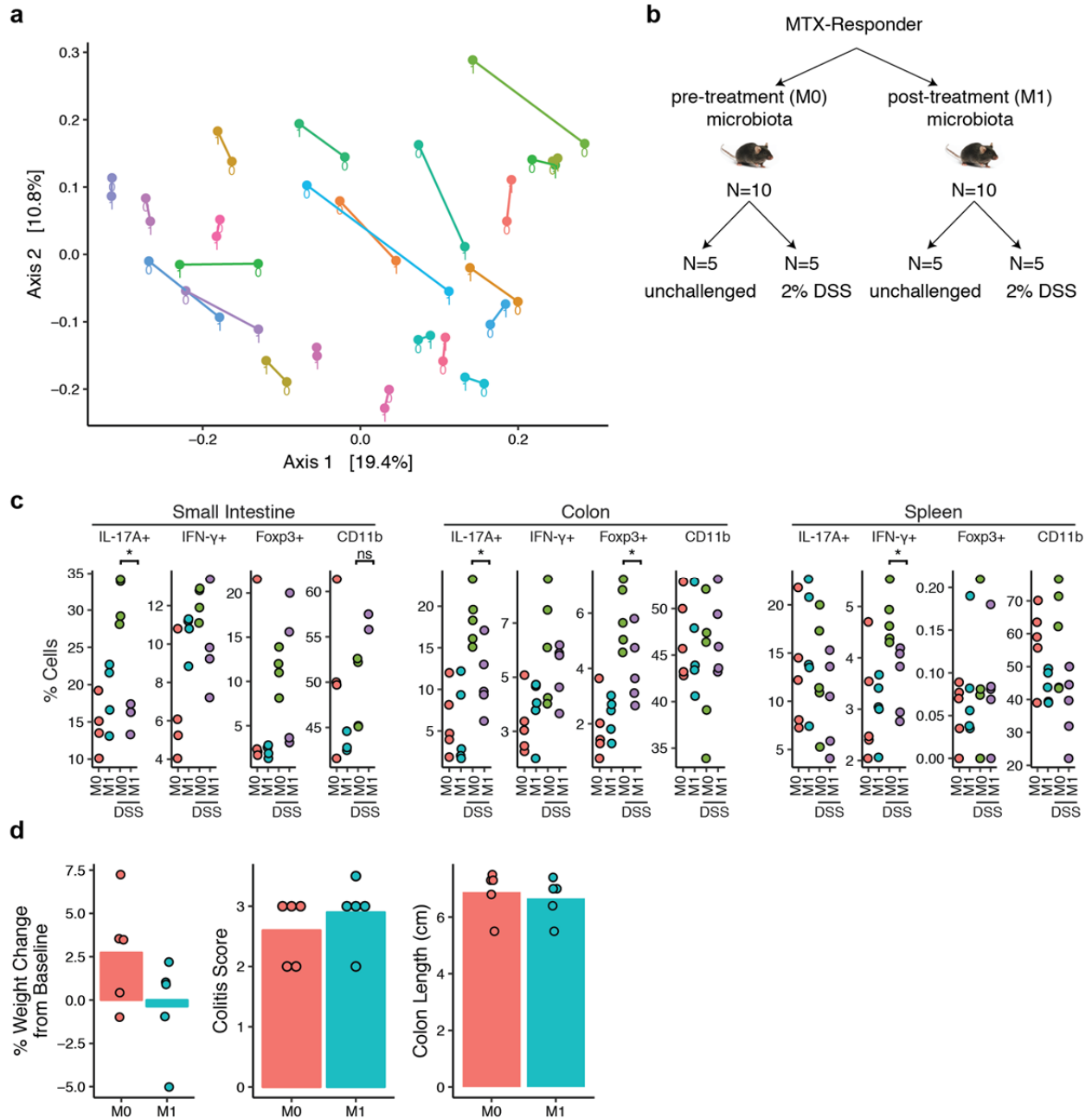
705 **1,962 shotgun sequenced microbiomes from healthy and diseased human individuals.**

706



a





714

715 **Supplementary Figure 4. Changes in community composition among rheumatoid arthritis**

716 **patient microbiomes. (a)** Principal coordinates analysis (PCoA) of Unifrac distances before

717 (“0”) and 1 month after (“1”) initiation of MTX treatment. Each patient is represented by a

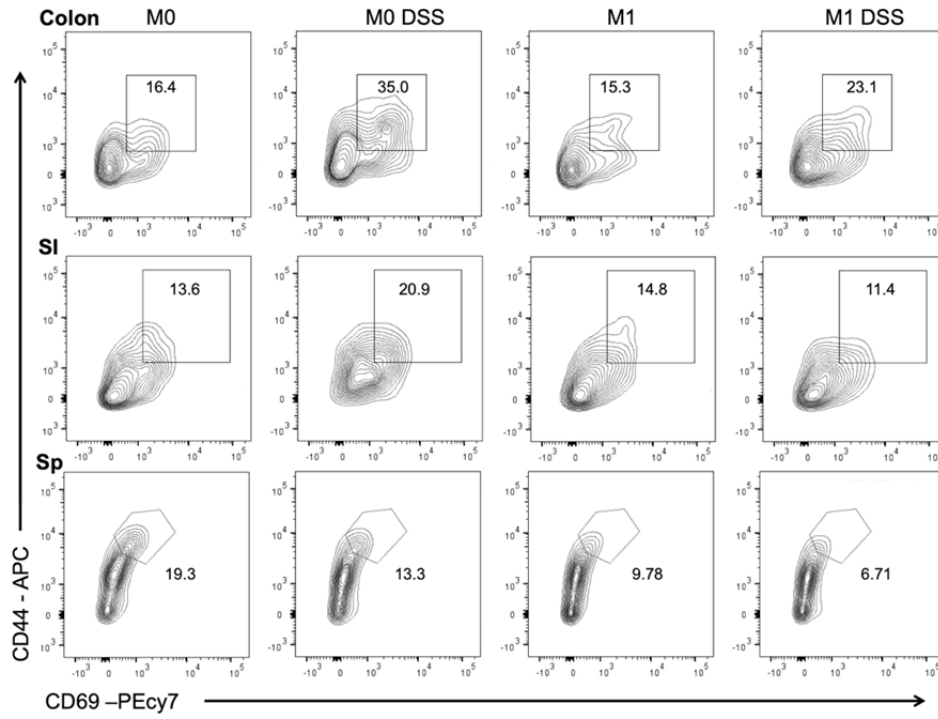
718 unique color and lines connect “0” and “1” time points for that patient. **(b)** Design of a

719 gnotobiotic transplant experiment in which fecal microbiota from a patient before (M0) and 1

720 month after MTX treatment (M1) were transferred into germ-free C57BL/6J mice. A subset of  
721 mice were challenged with dextran sodium sulfate (DSS). **(c)** Percentage of CD11b<sup>+</sup> cells or  
722 CD3<sup>+</sup>CD4<sup>+</sup> T cells with IL-17A<sup>+</sup>, IFN- $\gamma$ <sup>+</sup>, or FoxP3<sup>+</sup> staining from the small intestine and  
723 colon lamina propria and spleen of mice transplanted with microbiota from a patient before MTX  
724 treatment (“M0”) and 1 month after treatment (“M1”) (N=2-5 per treatment group). Some mice  
725 were treated with dextran sodium sulfate (“DSS”) in their drinking water. **(d)** Change in weight,  
726 colitis score or colon lengths from mice treated with DSS. Each circle represents a single mouse.  
727 Each bar represents the mean value among the mice in each transplant group.

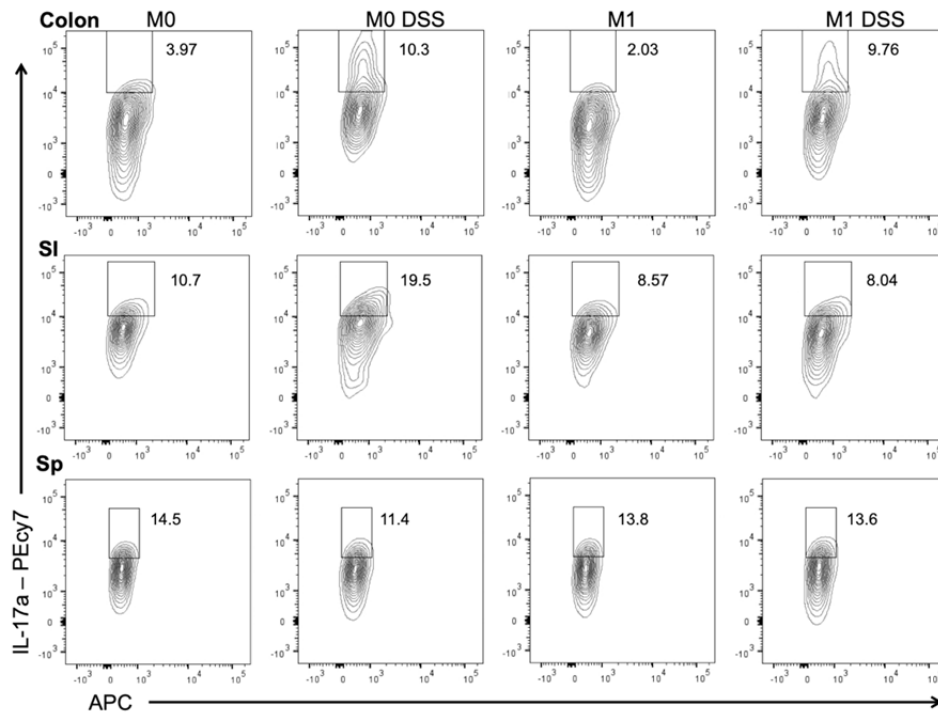
728

729 a



730

731 b



732

733 **Supplementary Figure 5. Representative flow plots for immune populations in mice**  
734 **transplanted with microbiota from a patient before MTX treatment (M0) and 1 month**  
735 **after treatment (M1) with or without DSS treatment. (a)** Colonic and small intestinal lamina  
736 (SI) propria lymphocytes as well as splenocytes (Sp) were isolated and stained for flow  
737 cytometry analysis. Representative flow plots are shown where the percentage of CD69+ CD44+  
738 cells within the CD3+CD4+ T cells compartment are displayed. **(b)** Representative flow plots of  
739 the percentages of Th17 cells as assessed by IL-17A+ within the CD3+ CD4+ compartment.

740 **Methods**

741 **Gnotobiotic mouse studies.** C57BL/6J mice (females, ages 8-16 weeks) were obtained from the  
742 UCSF Gnotobiotics core facility ([gnotobiotics.ucsf.edu](http://gnotobiotics.ucsf.edu)) and co-housed in gnotobiotic isolators  
743 for the duration of each experiment (Class Biologically Clean). Mice were colonized with stool  
744 from human donors, either a healthy male donor or a treatment-naïve female and male donors  
745 with rheumatoid arthritis (as defined by American College of Rheumatology classification  
746 criteria<sup>55</sup>). For colonization with a human microbiome, stool was diluted 1:10 g/mL in reduced  
747 PBS or saline and homogenized in an anaerobic chamber using pre-equilibrated reagents and  
748 supplies. Insoluble material was separated from supernatant by centrifugation at 50g for 1  
749 minute. Aliquots of supernatant (200 ul per mouse) were gavaged into mice at least 1-2 weeks  
750 before initiation of treatment with MTX. Mice were treated either with saline/PBS, MTX 1  
751 mg/kg or 50 mg/kg daily or folic acid 50 mg/kg. Treatment was carried out either by oral gavage  
752 or intra-peritoneal injection. Mice were monitored and weighed daily during treatment. No gross  
753 signs of toxicity and minimal-to-no weight loss were observed for the short MTX treatment  
754 durations used in this study. Stool samples were collected daily during treatment. Following  
755 treatment, mice were euthanized, and contents from the ileum, cecum and colon were collected  
756 for sequencing. For dextran sodium sulfate treatment (DSS) (Alfa Aesar, Cat no. 9011-18-1),  
757 mice were given 2% DSS (w/v) ad libitum in their drinking water. Mice were monitored for  
758 disease progression and weighed daily. Gross signs of toxicity, including hematochezia and  
759 weight loss were observed in this study. Stools were scored as follows: 0 = normal stool  
760 consistency, 1 = soft stool, 2 = blood in stool, 3 = bloody anus, 4 = prolapsed anus, 5 =  
761 moribund/death. All mouse experiments were approved by the University of California San  
762 Francisco Institutional Animal Care and Use Committee.

763

764 **16S rRNA gene sequencing of humanized mouse gut microbiota.** Aliquots of 108 mouse  
765 fecal, ileal, cecal and colon samples (**Supplementary Table 1**) were homogenized with bead  
766 beating for 5 min (Mini-Beadbeater-24, BioSpec) using beads of mixed size and material (Lysing  
767 Matrix E 2mL Tube, MP Biomedicals) using the digestion solution and lysis buffer of a Wizard  
768 SV 96 Genome DNA kit (Promega). The samples were then centrifuged for 10 min at 16,000g  
769 and the supernatant was transferred to the binding plate. The DNA was then purified according  
770 to the manufacturer's instructions.

771 16S rRNA gene PCR was carried out as before using GoLay-barcoded 515F/806R  
772 primers<sup>56</sup> according to the methods of the Earth Microbiome Project ([earthmicrobiome.org](http://earthmicrobiome.org)). 2µL  
773 of DNA was combined with 25 µL of AmpliTaq Gold 360 Master Mix (Life Technologies) 5 µL  
774 of primers (2µM each GoLay-barcoded 515/806R), and 18µL H<sub>2</sub>O. Amplification was as  
775 follows: 10 min 95°C, 25x (30s 95°C, 30s 50°C, 30s 72°C), and 7 min 72°C. Amplicons were  
776 quantified with PicoGreen (Quant-It dsDNA; Life Technologies) and pooled at equimolar  
777 concentrations. Libraries were then quantified (NEBNext Library Quantification Kit; New  
778 England Biolabs) and sequenced with a 600 cycle MiSeq Reagent Kit (251x151; Illumina) with  
779 ~10% PhiX.

780

781 **16S rRNA amplicon analysis of mouse and human fecal samples.** Reads were demultiplexed  
782 using QIIME<sup>57</sup> v1.9.1 (`split_libraries_fastq.py`) before denoising and processing with DADA2<sup>58</sup>  
783 v1.1.5 under MRO v3.2.5. Taxonomy was assigned using the DADA2 implementation of the  
784 RDP classifier<sup>59</sup> using the DADA2-formatted RDP v14 training set

785 (benjjneb.github.io/dada2/assign.html). A phylogenetic tree was constructed using DECIPHER  
786 v2.8.1 and PHANGHORN v2.4.0 as described in Callahan et al<sup>60</sup>. Diversity metrics were  
787 generated using Vegan v2.4-3 and Phyloseq v1.20.0<sup>61</sup> with principal coordinate analysis (PCoA)  
788 carried out with Ape v4.1. Analyses were carried out on either: (1) centered log<sub>2</sub>-ratio (clr)  
789 normalized taxonomic abundances calculated as  $A_{clr}=[\log_2(A_1/g_a), \log_2(A_2/g_a), \dots, \log_2(A_n/g_a)]$ ,  
790 where A is a vector of non-zero read counts and  $g_a$  is the geometric mean of all values of A, or  
791 (2) relative abundance calculated as proportion of reads. ANOSIM and PERMANOVA were  
792 used to detect changes in community composition using counts from rarefied data and Bray-  
793 Curtis distances. DESeq2<sup>62</sup> was used to determine differentially abundant taxa on raw count data.  
794 Significance testing of longitudinal trends was determined using generalized mixed effects  
795 models using the cplm package<sup>22,63</sup> (v. 0.7-7) on clr normalized values. For each sample, fastq  
796 files are available in NCBI's Sequence Read Archive (SRA), accession number SRP5125967.

797

798 **Quantitative PCR for 16S copy number determination.** Quantitative PCR (qPCR) of total  
799 16S rRNA gene copies was carried out in triplicate 10 $\mu$ L reactions with 200nM 891F(5'-  
800 TGGAGCATGTGGTTTAATTCGA-3')/1003R(5'-TGCGGGACTTAACCCAACA-3') primers  
801 using a BioRad CFX384 thermocycler with iTaq<sup>TM</sup> Universal Probes Supermix (BioRad  
802 1725132) and probe 1002P ([Cy5]CACGAGCTGACGACARCCATGCA[BHQ3]) according to  
803 the manufacturer's instructions and an annealing temperature of 60°C. Absolute quantifications  
804 were determined based against a standard curve of 8F/1542R amplified from purified bacterial  
805 DNA. Reactions were performed in triplicate and mean values were taken for further  
806 downstream analyses. Absolute bacterial abundance was derived by adjustments for dilutions



807 during DNA extraction, normalization, and PCR reaction preparation dividing by the total fecal  
808 mass used for DNA extraction in grams.

809

810 ***In vitro* bacterial growth studies.** The isolates used in this study are shown in **Supplementary**  
811 **Table 9.** Each of these strains was obtained from the Deutsche Sammlung von Mikroorganismen  
812 und Zellkulturen (DSMZ) culture collection. A single colony of each isolate was subcultured in  
813 Bacto Brain Heart Infusion (BD Biosciences, 37 g/L) supplemented with *L*-cysteine-HCl (0.05%,  
814 w/v), menadione (1 µg/mL), and hemin (5 µg/mL) (referred to hereafter as BHI+) for 48 hours in  
815 an anaerobic chamber (Coy Laboratory Products) at 37°C with an atmosphere composed of 2-3%  
816 H<sub>2</sub>, 20% CO<sub>2</sub>, and the balance N<sub>2</sub>. This subculture diluted down to an OD<sub>600</sub> of 0.08-0.1, which  
817 was then further diluted 100-fold, and then used to inoculate a microtiter plate with 2-fold serial  
818 dilutions of MTX concentrations ranging from 0 – 900 µg/ml. Plates were incubated at 37°C  
819 with shaking in an Eon Microplate Spectrophotometer (BioTek Instruments, Inc) over a 48 to 72-  
820 hour period in the anaerobic chamber. Growth was monitored every 15 minutes at OD<sub>600</sub> and  
821 corrected for background (no growth control). Data were exported using the Gen5 (v 2.0)  
822 software. The minimal inhibitory concentration (MIC<sub>90</sub>) was measured as the lowest  
823 concentration of MTX that resulting in >90% growth inhibition after 48 hours of incubation.  
824 Growth parameters (carrying capacity, time to mid-exponential and growth rate) were calculated  
825 in R using GrowthCurveR<sup>64</sup> (v 0.2.1). Determination of dose-dependency was done by fitting a  
826 linear regression with methotrexate concentration as the independent variable and the estimated  
827 growth parameter the dependent variable using lm from the stats package (v 3.5.1). Heatmaps of  
828 these parameters were generated using gplots (v 3.0.1), with each parameter normalized to the  
829 parameter derived from growth control curves (i.e. no drug present).

830

831 **Tree Construction.** Ribosomal sequences for each isolate were extracted from the Greengenes<sup>65</sup>  
832 database (May, 2013). Sequences were imported into UGENE<sup>66</sup> (v 1.31.0), and aligned using  
833 MUSCLE<sup>67</sup>. Gaps occurring in > 50% of sequences were removed, and a maximum likelihood  
834 tree was generated using PhyML<sup>68</sup> with 100 bootstraps and the GTR substitution model.

835

836 **Abundance Quantification of Bacterial Isolates from Shotgun Sequencing Data.** Bacterial  
837 abundances were quantified using data from Metaquery<sup>30</sup>, a web-based application that provides  
838 taxonomic abundances from >1,900 publicly available human gut metagenomes. For each  
839 isolate, we queried the “metaphlan2” database and recorded the mean abundance value.

840

841 **Predicted concentration of MTX in the GI tract.** The predicted concentration of MTX in the  
842 proximal GI tract was estimated by taking the oral dose used for rheumatoid arthritis (25 mg) and  
843 dividing it by 250 ml<sup>31</sup>, giving a concentration of 100 µg/ml or 220 uM.

844

845 **MTX Treatment for RNA-Seq.** The bacterial strains used in RNA-Seq are given in  
846 **Supplementary Table 9.** Genomes are available in NCBI’s GenBank Assembly database (see  
847 **Supplementary Table 9** for accession numbers). Culture media was composed of BHI+ and  
848 allowed to equilibrate in an anaerobic environment prior to use. Briefly, bacteria were cultured in  
849 BHI+ at 37 °C in an anaerobic chamber. Cultures for each isolate were grown to mid-exponential  
850 (achieving an OD600 ~ 0.5), aliquoted into triplicates, treated for 30 minutes with either DMSO

851 or MTX 100 µg/ml, and then removed from the anaerobic chamber. For *C. asparagiforme*,  
852 cultures were incubated for 4 and 20 hours as well. Cultures were centrifuged at 2000 rpm for 10  
853 min at 4 °C to facilitate removal of supernatant, and the remaining bacterial pellet was flash-  
854 frozen in liquid nitrogen.

855

856 **Total RNA extraction.** Each bacterial pellet was incubated with 1 ml of Tri reagent (Sigma  
857 Aldrich, catalog #: T9424) at room temperature for 10 minutes. The cell suspension was  
858 transferred into Lysing Matrix E tubes (MP Biomedicals, 116914050), and homogenized in a  
859 bead-beater (Mini-Beadbeater-24, BioSpec) for 5 minutes at room temperature. The sample was  
860 incubated with 200 µL of chloroform at room temperature for 10 minutes, followed by  
861 centrifugation at 16,000 x g for 15 minutes at 4 °C. Next, 500 µL of the upper aqueous phase was  
862 transferred into a new tube and 500 µL of 100% ethanol was added. To isolate RNA, we used the  
863 PureLink RNA Mini Kit (Life Technologies, catalog #: 12183025). This mixture was transferred  
864 onto a PureLink spin column and spun at  $\geq 12,000$  x g for 30 seconds. The column was washed  
865 with 350 µl of wash buffer I as described in the PureLink manual. The column was incubated  
866 with 80 µl of PureLink DNase (Life Technologies, catalog #: 12185010) at room temperature for  
867 15 minutes, and washed with 350 µl of wash buffer I. The column was washed with wash buffer  
868 II twice as described in the PureLink manual. Total RNA was recovered in 50 µl of RNAase-free  
869 water. A second round of DNase treatment was undertaken. The RNA was incubated with 6 µl  
870 of TURBO DNase (Ambion, ThermoFisher, catalog #: AM2238) at 37°C for 30 minutes. To  
871 stop the reaction, 56 µl of lysis buffer from the PureLink kit and 56 µl of 100% ethanol was  
872 added to the sample and vortexed. This suspension was transferred onto a PureLink column, and

873 washed once with 350 ul of wash buffer I and twice with 500 ul of wash buffer II. The RNA was  
874 recovered in 30 ul of RNase-free water.

875

876 **rRNA depletion, library generation, and RNA sequencing.** Total RNA was subjected to  
877 rRNA depletion using Ribo-Zero Bacterial rRNA Depletion (Illumina, catalog #: MRZB12424),  
878 following the manufacturer's protocol. RNA fragmentation, cDNA synthesis, and library  
879 preparation proceeded using NEBNext Ultra RNA Library Prep Kit for Illumina (New England  
880 BioLabs, catalog #: E7530) and NEBNext Multiplex Oligos for Illumina, Dual Index Primers  
881 (New England BioLabs, catalog #: E7600), following the manufacturer's protocol. All samples  
882 were single-end sequenced (1x50 bp) using an Illumina HiSeq2500 platform (High Output, v4  
883 chemistry) at UCSF's Institute for Human Genomics. For each sample, fastq files are available in  
884 NCBI's Sequence Read Archive (SRA), accession number SRP5125967.

885

886 **RNA-Seq analysis.** Reads were mapped to reference genomes using Bowtie2<sup>69</sup>. HTSeq (v 0.8.0)  
887 was used to count the number of transcripts mapping to genes<sup>70</sup>. Differential gene expression  
888 was assessed using DESeq2<sup>62</sup>. KEGG Pathway enrichment was carried out using clusterProfiler<sup>71</sup>  
889 (v3.4.1).

890

891 **Human RA patient samples acquisition.** Consecutive patients from the New York University  
892 Langone Medical Center's rheumatology clinics and offices were screened for the presence of  
893 RA based on ACR criteria<sup>55</sup>. After informed consent was signed, each patient's medical history  
894 (according to chart review and interview/questionnaire), diet, and medications were determined.

895 A screening musculoskeletal examination and laboratory assessments were also performed or  
896 reviewed. All RA patients who met the study criteria were offered enrollment. The criteria for  
897 inclusion in the study required that patients meet the American College of  
898 Rheumatology/European League Against Rheumatism 2010 classification criteria for RA<sup>55</sup>,  
899 including seropositivity for rheumatoid factor (RF) and/or anti-citrullinated protein antibodies  
900 (ACPAs), and that all subjects be age 18 years or older. New-onset RA was defined as disease  
901 duration of a minimum of 6 weeks and up to 6 months since diagnosis, and absence of any  
902 treatment with disease-modifying anti-rheumatic drugs (DMARDs), biologic therapy or steroids  
903 (ever). The exclusion criteria applied to all groups were as follows: recent (<3 months prior) use  
904 of any antibiotic therapy, current extreme diet (e.g., parenteral nutrition or macrobiotic diet),  
905 known inflammatory bowel disease, known history of malignancy, current consumption of  
906 probiotics, any gastrointestinal tract surgery leaving permanent residua (e.g., gastrectomy,  
907 bariatric surgery, colectomy), or significant liver, renal, or peptic ulcer disease. This study was  
908 approved by the Institutional Review Board of New York University School of Medicine  
909 protocols #09-0658 and # as previously published<sup>43</sup>. All new onset rheumatoid arthritis (NORA)  
910 patients (n=30 patients, 60 stool samples) were recruited using established protocols from a  
911 previously described study<sup>43</sup>. Patients received oral MTX at standard of care doses as prescribed  
912 by their treating rheumatologists. Stool samples were collected at baseline and 1 month after  
913 MTX initiation and metadata were obtained at baseline and 4 months after therapy initiation.  
914 Clinical and demographic data was de-identified and recorded in RedCap by the designated  
915 study personnel. Clinical responder status (MTX-R) was defined *a priori* as any NORA patient  
916 whose DAS28 score was greater than 2 at baseline and improved by at least 1.8 by month 4 post-  
917 treatment. DNA was extracted from human fecal samples (n=23 patients, 46 stool samples) as we

918 have previously described<sup>43</sup>, using the MoBio Powersoil DNA extraction kit, based on cell  
919 membrane disruption by high-speed shaking in the presence of beads. The V4 hypervariable  
920 region of bacterial 16S ribosomal RNA (rRNA) was performed using a MiSeq Illumina platform  
921 (150 bp read length, paired-end protocol) at the New York University Genome Technology  
922 Center as previously described<sup>72</sup>. For each sample, fastq files are available in NCBI's Sequence  
923 Read Archive (SRA), accession number SRP5125967.

924

925 ***Ex vivo* incubation of RA patient stool samples.** All work was carried out in an anaerobic  
926 chamber. For each patient, stool was aliquoted into a pre-equilibrated cryovial, diluted in reduced  
927 PBS at 10 ml per 1 gram of stool, and vortexed to homogenize the sample. The sample was spun  
928 at ~20g for 1 minute on a mini-centrifuge to facilitate settling of sediment, and the sediment-free  
929 supernatant was then aliquoted into a new pre-equilibrated cryovial for evaluation of *ex vivo*  
930 growth. Growth was evaluated by inoculating liquid BHI with 1:50 dilution of this fecal slurry,  
931 with OD600 readings performed every 15 minutes for 48 hours with a 2-minute shake prior to  
932 each reading. Samples were treated with MTX 100 µg/ml or an equal volume of DMSO at time  
933 zero. Each patient's fecal slurry and treatment was evaluated in quadruplicate. Growth curves  
934 were averaged by treatment and individual, and growth parameters were estimated using the  
935 GrowthCurveR package (v 0.2.1). Paired Student's *t*-tests were used to determine changes in  
936 growth parameters.

937

938 **Lamina Propria Lymphocyte Isolation.** Lamina propria lymphocytes (LPLs) were isolated  
939 with slight modifications of previously described methods<sup>73-75</sup>. In brief, small intestinal (SI)

940 Peyer's patches were removed and colons and SI tissue were splayed longitudinally with mucus  
941 removed by scraping and stored in complete RPMI (10% fetal bovine serum, 100 units per ml  
942 penicillin and streptomycin,  $\beta$ -mercaptoethanol, glutamate, sodium pyruvate, HEPES and non-  
943 essential amino acids). Supernatants were removed by filtering through a 100 $\mu$ M filter, and  
944 remaining tissue incubated in 1X HBSS (without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) containing 5 mM EDTA  
945 (Promega) and 1 mM DL-Dithiothreitol (DTT) (Bioplus chemicals) for 45 min at 37°C on a  
946 shaker. Supernatant was removed by filtering through a 100  $\mu$ M filter, and remaining tissue was  
947 incubated for 45 min (colon) or 35 min (SI) at 37°C on a shaker in a solution containing 1X  
948 HBSS containing 5% (v/v) fetal bovine serum (GIBCO heat inactivated), 1 U/ml Dispase  
949 (Sigma), 0.5 mg/ml Collagenase VIII (Sigma), and 20  $\mu$ g/ml DNaseI (Sigma). The supernatant  
950 was filtered over a 40 mm cell strainer into ice-cold sterile 1X PBS. Cells were subjected to a  
951 Percoll (VWR) gradient (40%/80% [v/v] gradient) and spun at 2000RPM for 20 min with no  
952 brake and no acceleration. Cells at the interface were collected, washed in PBS and prepared for  
953 flow cytometry analysis.

954

955 **Flow Cytometry.** Lymphocytes were isolated from the colonic and small intestinal lamina  
956 propria as described above. Spleen cells were prepped through gentle mashing with a syringe  
957 plunger. Spleen cells were treated with 1X RBC Lysis Buffer (Biolegend) to lyse and remove red  
958 blood cells. Surface staining for lymphocytes was done in staining buffer (1X HBSS (Corning)  
959 supplemented with 10 mM HEPES (Cellgro), 2 mM EDTA (Promega), and 0.5% (v/v) fetal  
960 bovine serum (GIBCO heat inactivated)) for 20 min at 4°C. Cells were then washed twice in  
961 supplemented 1X HBSS and enumerated via flow cytometry. The following antibodies were  
962 used: anti-CD3 (17A2, Invitrogen), anti-CD4 (GK1.5, Biolegend), anti-CD69 (H1.2F3,

963 Biolegend), anti-CD11b (M1/70, Biolegend), and anti-CD44 (IM7, Tonbo biosciences). For  
964 intracellular staining, cells were first stimulated with ionomycin (1000 ng/ml), PMA (50 ng/ml),  
965 and Golgi Plug (1 µl/sample) (BD Bioscience) overnight at 37°C. Stimulated cells were stained  
966 with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fisher) CD3<sup>+</sup> CD4<sup>+</sup> cells were  
967 assessed within the live population. Cells were surface stained, washed, and then  
968 fixed/permeabilized in 100µl Perm/Fix buffer (BD Bioscience). Cells were washed twice in  
969 Perm/Wash buffer (BD Bioscience) and then stained for intracellular cytokines with the  
970 following antibodies: anti-IFN-γ (XMG1.2, Millipore), anti-IL-17A (ebio17B7, Invitrogen), anti-  
971 Foxp3 (150D, Biolegend). Cells were washed twice in Perm/Wash buffer and then placed in  
972 staining buffer for flow cytometry analysis. Gating cell populations was done using isotype and  
973 single stain controls. Representative gating strategies are provided in **Supplementary Fig. 5**.  
974 These data were collected with a BD LSR Fortessa and analyzed with FlowJo software.

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976 **Data Availability.** All sequencing data generated in the preparation of this manuscript has been  
977 deposited in NCBI's Sequence Read Archive (SRA), accession number SRP5125967

978

979 **Code Availability.** Code used in the analyses presented here are publicly available as described  
980 in Callahan et al<sup>60</sup> and upon request.