SUPPLEMENTAL INFORMATION

Figure S1. MTX alters community composition in individually housed mice, related to Figure 1.

(A) Alpha diversity of Observed ASVs after treatment with 50 mg/kg MTX (n=4) or PBS (n=3) by oral gavage for 4 days in mice that were individually housed (Wilcoxon rank sum).

(B-C) PCA of Euclidean distances using clr-transformed values at multiple time points after treatment with either PBS (n=3) or MTX (50 mg/kg; n=4) by oral gavage for 4 days in individually housed mice.

(B) Data from multiple days are shown together and lines connect samples from the same mouse; each point is labeled by day of treatment. ANOSIM statistical results comparing Day 0 vs. Day 4 within each treatment group are shown.

(C) Data are split by day, and ANOSIM results comparing PBS vs. MTX are shown for each time point in (C).

(D) The Euclidean distance of clr-transformed abundances between each mouse's fecal community (in individually housed mice) on days 7-18 after colonization with a human microbiome (day 0) compared to itself 3 days after colonization. The biggest differences in the community composition occurs within the first week which is depicted at days 3 and 7 (at day 0 , the mice are germ-free, which is why that time point is not included). Oral gavage with MTX or vehicle control was started on day 14 after colonization. The biggest changes in the MTX treated community occur 1-2 days after treatment.

(E) Twenty-three ASVs were differentially abundant in MTX treated mice relative to PBS control (p_{adj}<0.01, DESeq). Colors indicate log2 fold change.

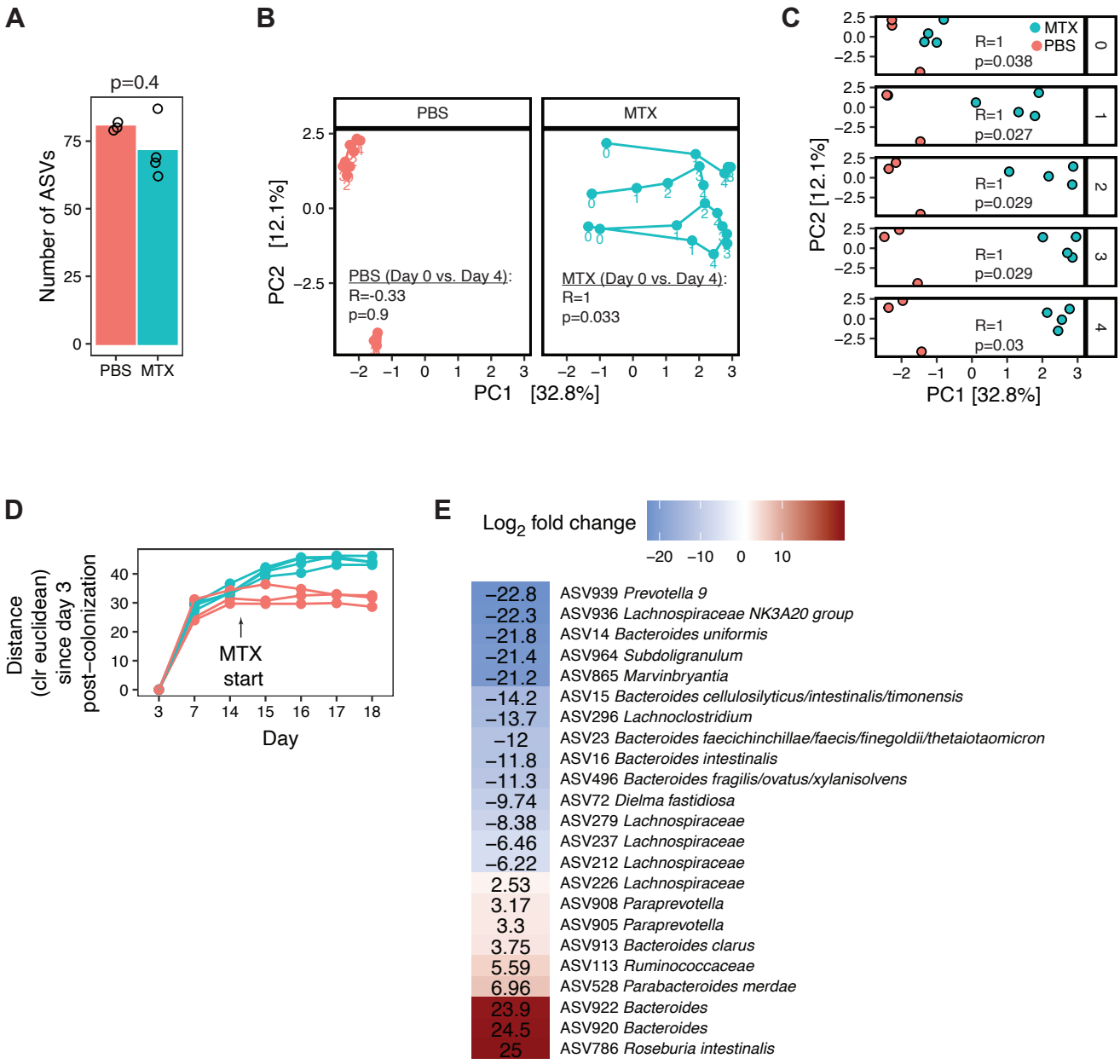

A
p=0.4
Number of ASVs
75
50
25
0
PBS MTX
B
PBS
MTX
PC2 [12.1%]
2.5
0.0
−2.5
PBS (Day 0 vs. Day 4):
R=-0.33
p=0.9
MTX (Day 0 vs. Day 4):
R=1
p=0.033
−2 −1 0 1 2 3
PC1 [32.8%]
C
MTX
PBS
R=1
p=0.038
p=0.027
p=0.029
p=0.029
p=0.03
0
1
2
3
4
PC2 [12.1%]
PC1 [32.8%]
D
Distance
(clr euclidean)
since day 3
post−colonization
40
30
20
10
0
MTX
start
3 7 14 15 16 17 18
Day
E
Log2 fold change
−20 −10 0 10
−22.8 ASV939 Prevotella 9
−22.3 ASV936 Lachnospiraceae NK3A20 group
−21.8 ASV14 Bacteroides uniformis
−21.4 ASV964 Subdoligranulum
−21.2 ASV865 Marvinbryantia
−14.2 ASV15 Bacteroides cellulosilyticus/intestinalis/timonensis
−13.7 ASV296 Lachnoclostridium
−12 ASV23 Bacteroides faecichinchillae/faecis/finegoldii/thetaiotaomicron
−11.8 ASV16 Bacteroides intestinalis
−11.3 ASV496 Bacteroides fragilis/ovatus/xylanisolvens
−9.74 ASV72 Dielma fastidiosa
−8.38 ASV279 Lachnospiraceae
−6.46 ASV237 Lachnospiraceae
−6.22 ASV212 Lachnospiraceae
2.53 ASV226 Lachnospiraceae
3.17 ASV908 Paraprevotella
3.3 ASV905 Paraprevotella
3.75 ASV913 Bacteroides clarus
5.59 ASV113 Ruminococcaceae
6.96 ASV528 Parabacteroides merdae
23.9 ASV922 Bacteroides
24.5 ASV920 Bacteroides
25 ASV786 Roseburia intestinalis

Figure S2. MTX alters community composition in mice with altered route of MTX or rescue with folic acid, related to Figure 1.

(A-B) Alpha diversity of Observed ASVs (A) and PCA of Euclidean distances using clr-transformed values (B) at multiple time points after treatment with 50 mg/kg MTX either IP (n=3) or PO (n=3) by oral gavage for 2 days in mice that were co-housed by treatment. ANOSIM statistical results are shown for each time point comparing IP vs. PO treatment.

(C-D) Alpha diversity of Observed ASVs (C) and PCA of Euclidean distances using clr-transformed values (D) at multiple time points after treatment with 50 mg/kg MTX with vehicle control (n=3) or 50 mg/kg folic acid (n=3) by oral gavage for 2 days in mice that were co-housed by treatment. ANOSIM statistical results are shown for each time point comparing vehicle control vs. folic acid supplementation treatment.

(E-F) Phylum-level trends over time (in days) in the route and rescue experiments, where lines depict the median for each treatment group. Shown are significance values from DESeq for significant phyla (Day 0 vs. Day 2, $p_{adj}<0.01$).

(G) 41 ASVs were differentially abundant in both route and rescue experiments (Day 0 vs. Day 2, $p_{adj}<0.01$). Colors indicate log2 fold change.

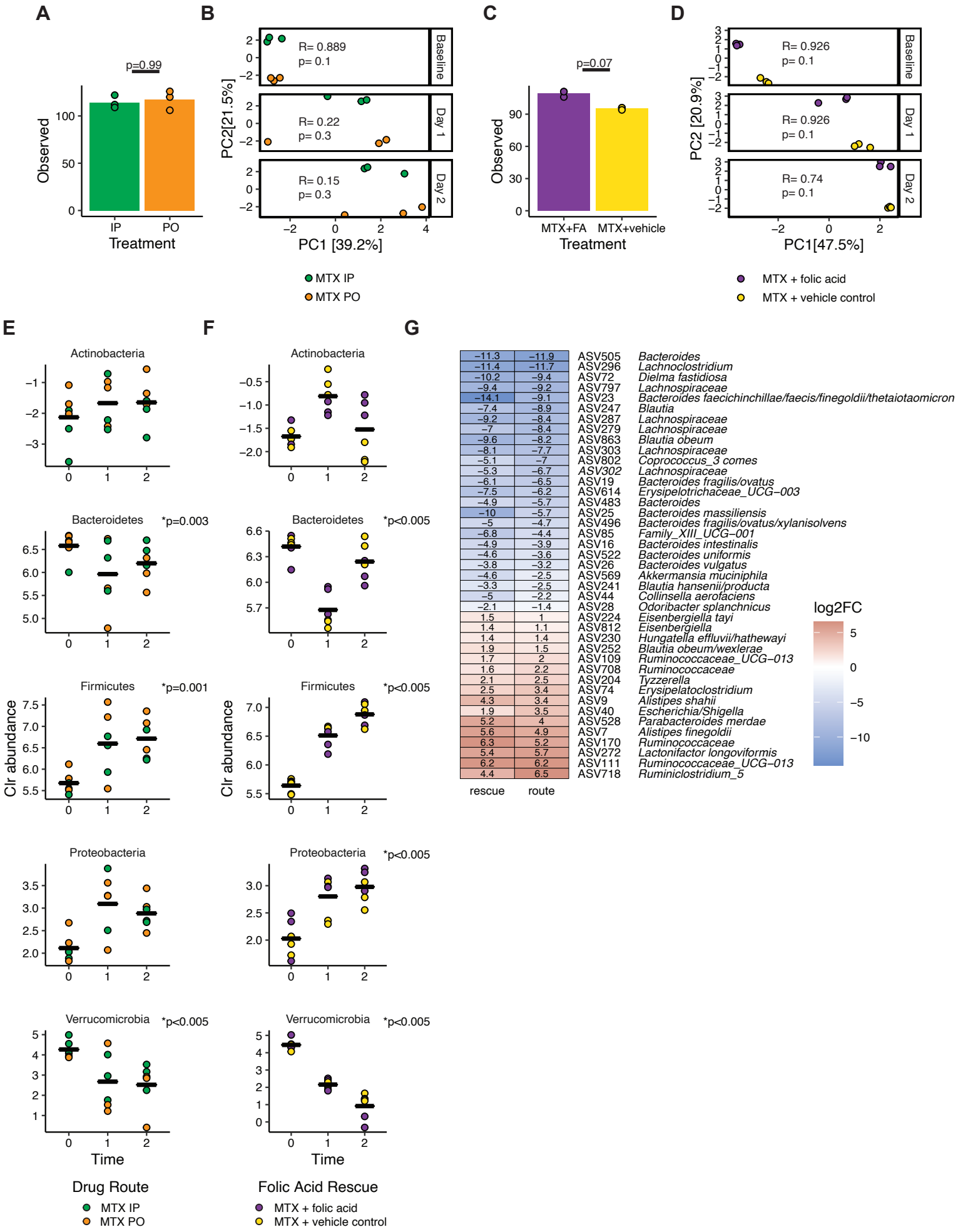

A
p=0.99
Observed
Treatment
IP
PO
B
R= 0.889
p= 0.1
R= 0.22
p= 0.3
R= 0.15
p= 0.3
Baseline
Day 1
Day 2
PC2[21.5%]
PC1 [39.2%]
MTX IP
MTX PO
C
p=0.07
Observed
MTX+FA
MTX+vehicle
Treatment
D
R= 0.926
p= 0.1
R= 0.926
p= 0.1
R= 0.74
p= 0.1
PC2 [20.9%]
PC1[47.5%]
MTX + folic acid
MTX + vehicle control
E
Actinobacteria
Bacteroidetes
*p=0.003
Firmicutes
*p=0.001
Proteobacteria
Verrucomicrobia
*p<0.005
Clr abundance
Time
Drug Route
MTX IP
MTX PO
F
Actinobacteria
Bacteroidetes
*p<0.005
Firmicutes
*p<0.005
Proteobacteria
*p<0.005
Verrucomicrobia
*p<0.005
Clr abundance
Time
Folic Acid Rescue
MTX + folic acid
MTX + vehicle control
G
rescue
route
ASV505 Bacteroides
ASV296 Lachnoclostridium
ASV72 Dielma fastidiosa
ASV797 Lachnospiraceae
ASV23 Bacteroides faecichinchillae/faecis/finegoldii/thetaiotaomicron
ASV247 Blautia
ASV287 Lachnospiraceae
ASV279 Lachnospiraceae
ASV863 Blautia obeum
ASV303 Lachnospiraceae
ASV802 Coprococcus_3 comes
ASV302 Lachnospiraceae
ASV19 Bacteroides fragilis/ovatus
ASV614 Erysipelotrichaceae_UCG–003
ASV483 Bacteroides
ASV25 Bacteroides massiliensis
ASV496 Bacteroides fragilis/ovatus/xylanisolvens
ASV85 Family_XIII_UCG–001
ASV16 Bacteroides intestinalis
ASV522 Bacteroides uniformis
ASV26 Bacteroides vulgatus
ASV569 Akkermansia muciniphila
ASV241 Blautia hansenii/producta
ASV44 Collinsella aerofaciens
ASV28 Odoribacter splanchnicus
ASV224 Eisenbergiella tayi
ASV812 Eisenbergiella
ASV230 Hungatella effluvii/hathewayi
ASV252 Blautia obeum/wexlerae
ASV109 Ruminococcaceae_UCG–013
ASV708 Ruminococcaceae
ASV204 Tyzzerella
ASV74 Erysipelatoclostridium
ASV9 Alistipes shahii
ASV40 Escherichia/Shigella
ASV528 Parabacteroides merdae
ASV7 Alistipes finegoldii
ASV170 Ruminococcaceae
ASV272 Lactonifactor longoviformis
ASV111 Ruminococcaceae_UCG–013
ASV718 Ruminiclostridium_5
log2FC

Figure S3. MTX alters community composition in a combined analysis of gnotobiotic mouse experiments, related to Figure 1.

(A) Alpha diversity of Observed ASVs from a combined analysis of the dose-response, individually housed, route and rescue gnotobiotic experiments. Mice treated with low-dose MTX were excluded. Significance assessed by two-factor ANOVA.

(B-C) PCA of Euclidean distances using clr-transformed values showing the first vs. second principal components (B) and the second vs. third principal components (C). ANOSIM statistical results are shown for donor effect (B) and MTX treatment effect (C).

(D) Phylum-level trends with MTX treatment, where lines depict the median for each treatment group. Shown are significance values from DESeq for significant phyla ($p_{adj}<0.05$).

(E) A phylogenetic tree of 61 ASVs were differentially abundant in the combined analysis (with vs. without MTX, $p_{adj}<0.05$).

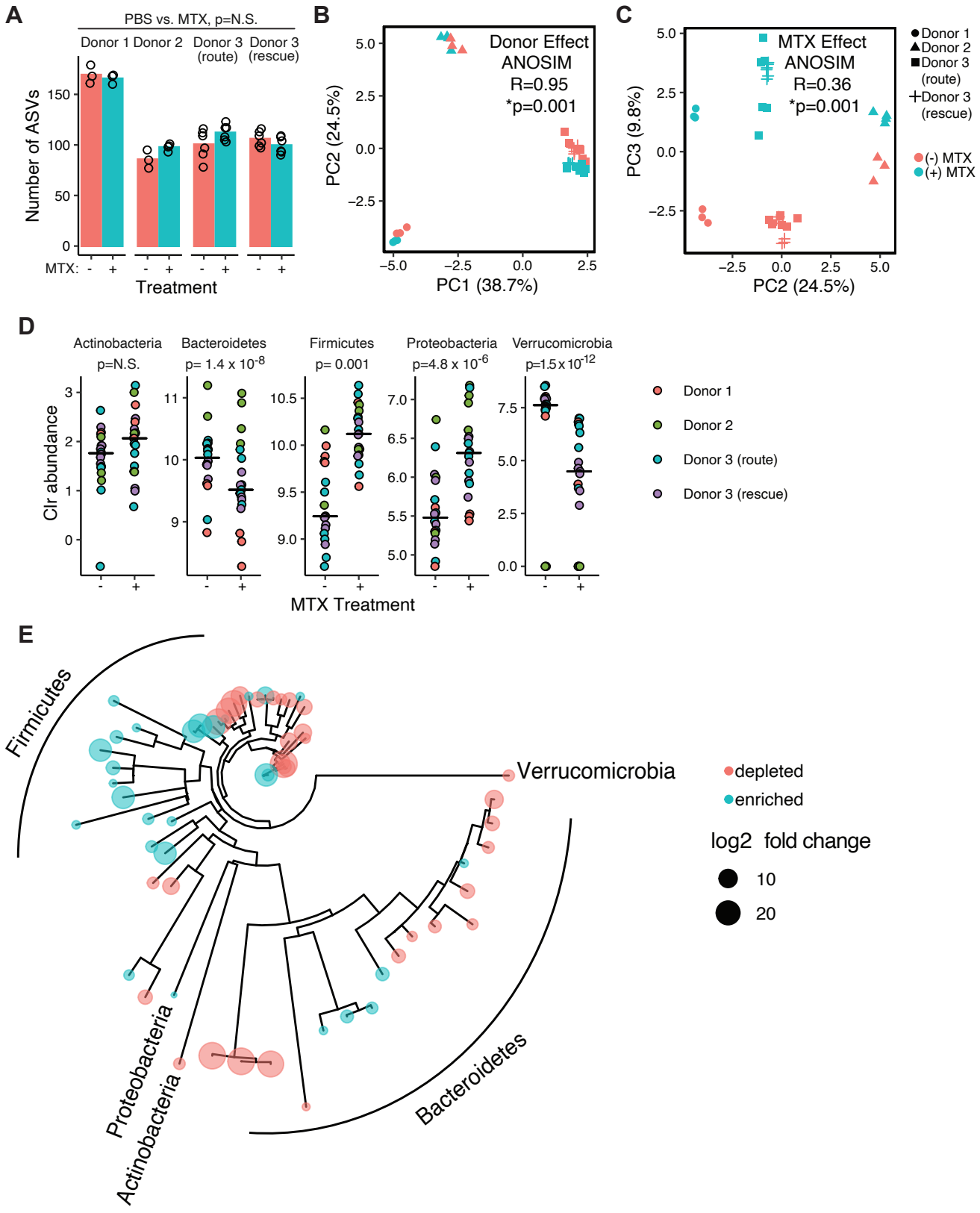

A
PBS vs. MTX, p=N.S.
Donor 1
Donor 2
Donor 3 (route)
Donor 3 (rescue)
Number of ASVs
MTX:
Treatment
B
Donor Effect
ANOSIM
R=0.95
*p=0.001
PC2 (24.5%)
PC1 (38.7%)
C
MTX Effect
ANOSIM
R=0.36
*p=0.001
PC3 (9.8%)
PC2 (24.5%)
(-) MTX
(+) MTX
D
Actinobacteria
p=N.S.
Bacteroidetes
p= 1.4 x 10-8
Firmicutes
p= 0.001
Proteobacteria
p=4.8 x 10-6
Verrucomicrobia
p=1.5 x 10-12
Clr abundance
MTX Treatment
E
Firmicutes
Verrucomicrobia
Proteobacteria
Actinobacteria
Bacteroidetes
depleted
enriched
log2 fold change
10
20

Figure S4. MTX alters growth and community composition in complex gut microbial communities from RA patients, related to Figure 5.

(A) Carrying capacity was significantly decreased among the 30 patient fecal suspensions *ex vivo* (paired Student's *t*-test) treated with MTX (100 μg/ml).

(B) Time to mid-exponential was significantly increased among the 30 patient fecal suspensions *ex vivo* (paired Student's *t*-test).

(C) Alpha diversity of Observed ASVs in *ex vivo* samples from 4 patients treated with either MTX or vehicle control (tested in quadruplicate; 24 hours after treatment). Linear mixed effects modeling revealed significant decrease in alpha diversity with MTX treatment.

(D) PCA of Euclidean distances on clr-transformed microbial abundances before ("pre", circles) and 1 month after ("post", triangles) initiation of MTX treatment based on 16S-seq. Each patient's samples are connected by a line. ANOSIM statistical results are shown for MTX treatment effect.

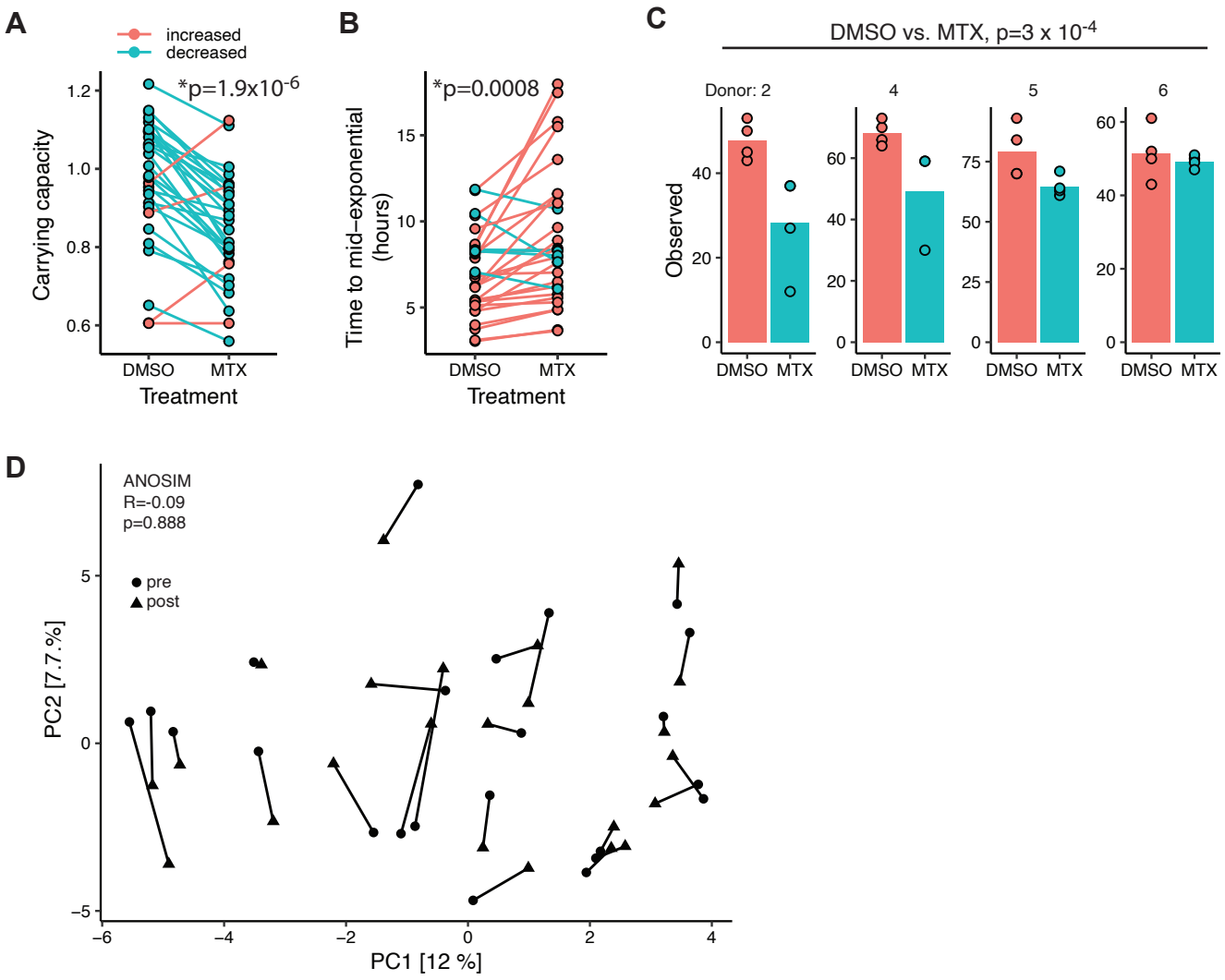

A
increased
decreased
*p=1.9x10-6
Carrying capacity
1.2
1.0
0.8
0.6
DMSO
MTX
Treatment
B
*p=0.0008
Time to mid−exponential (hours)
15
10
5
DMSO
MTX
Treatment
C
DMSO vs. MTX, p=3 x 10-4
Donor: 2
4
5
6
Observed
40
20
0
60
40
20
0
75
50
25
0
60
40
20
0
DMSO MTX
D
ANOSIM
R=-0.09
p=0.888
pre
post
PC2 [7.7.%]
5
0
−5
−6
−4
−2
0
2
4
PC1 [12 %]

Figure S5. Transplantation of RA patient microbiota in germ-free mice, related to Figure 6 and 7.

(A) Design of a gnotobiotic transplant experiment in which fecal microbiota from a patient before (Pre) and 1 month after MTX treatment (Post) were transferred into germ-free C57BL/6J mice. A subset of mice were challenged with dextran sodium sulfate (DSS). This design was used to evaluate 3 donor transplantations.

(B) Representative colonic histology from mice colonized with pre-MTX or post-MTX microbiota with or without DSS in the drinking water with H&E staining at 8x magnification.

(C-E) Percent change in weight (C), endpoint colitis score (D) and colon length (E) in mice challenged with DSS after transplantation with pre-MTX and post-MTX microbiota from Donor 2. Significance assessed using Wilcoxon rank sum test.

(F) PCA of Euclidean distances of clr-transformed values for each transplant experiment, showing that mice receiving microbiota from each donor (gavage fluid) generally resemble the donor and are distinct from each other approximately 1 week after colonization.

(G) Relative abundance of each phylum in gavage fluid and in recipient mice (averaged over 10-12 mice per group). Phyla with relative abundance >0.01 are annotated.

(H-J) PCA of Euclidean distances using clr-transformed values of samples collected on days 3, 7, and 14 for transplanted mice from each donor. Each point represents a fecal sample. ANOSIM R and p values reported comparing pre-MTX and post-MTX groups on day 14.

(K) There were 41 ASVs that were differentially abundant in pre-MTX vs. post-MTX across the three donor FMT experiments (p_{adj}<0.05, DESeq). Colors represent log2 fold change.

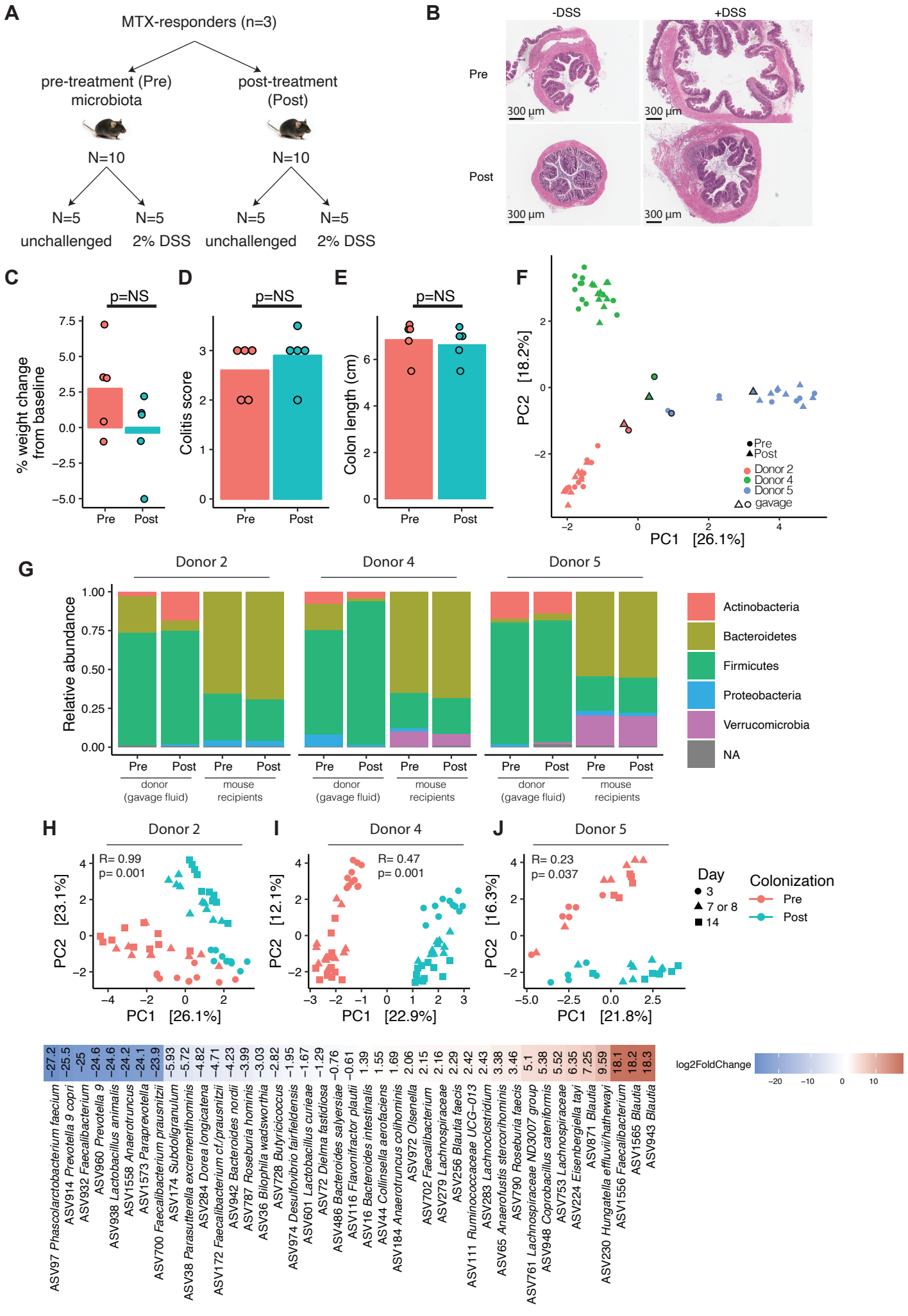
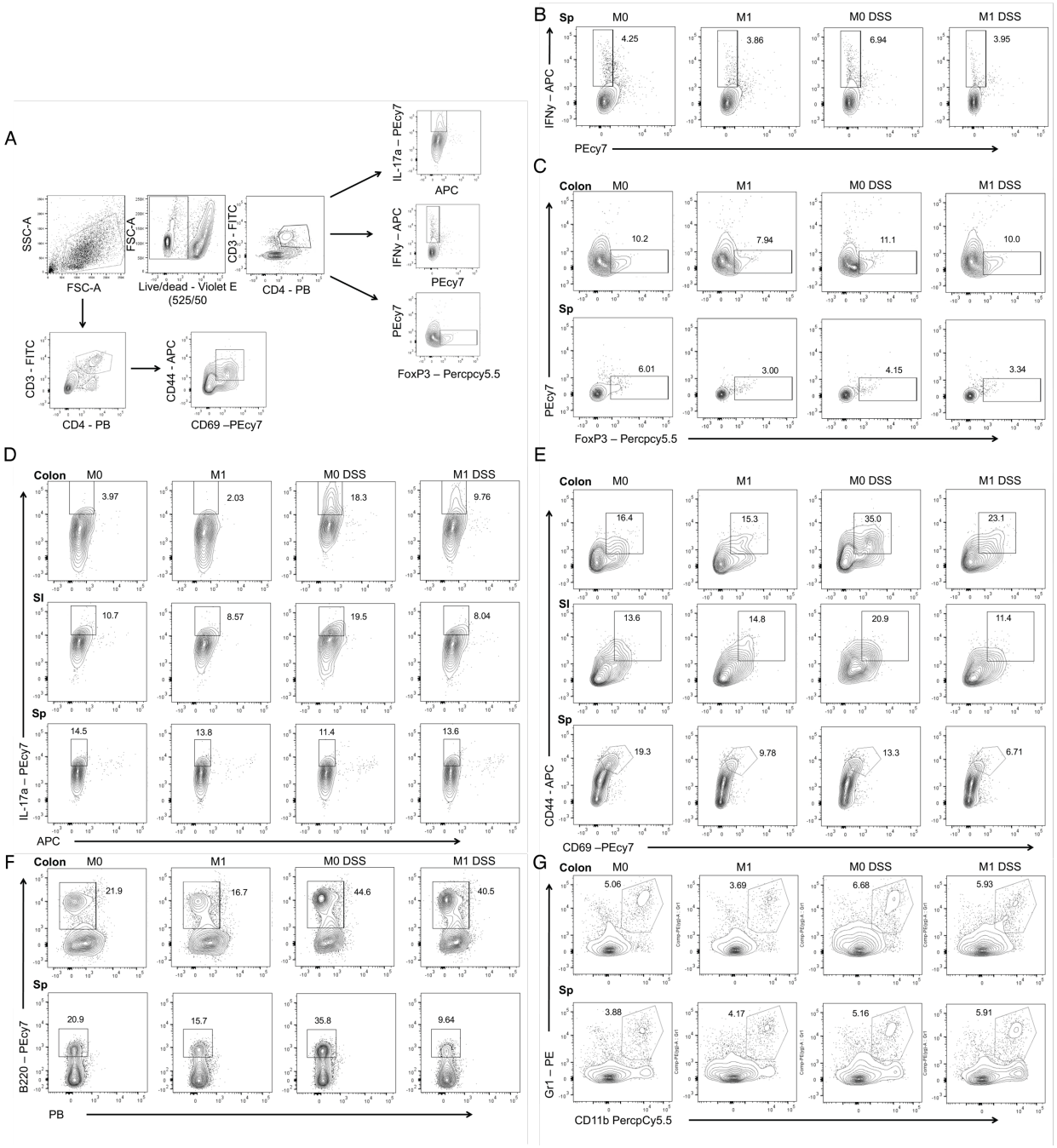

A
MTX-responders (n=3)
pre-treatment (Pre) microbiota
post-treatment (Post)
N=10
N=10
N=5
N=5
N=5
N=5
unchallenged
2% DSS
unchallenged
2% DSS
B
-DSS
+DSS
Pre
Post
300 µm
C
p=NS
% weight change from baseline
Pre
Post
D
p=NS
Colitis score
E
p=NS
Colon length (cm)
F
PC2 [18.2%]
PC1 [26.1%]
Pre
Post
Donor 2
Donor 4
Donor 5
gavage
G
Donor 2
Donor 4
Donor 5
Relative abundance
donor (gavage fluid)
mouse recipients
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Verrucomicrobia
NA
H
Donor 2
R= 0.99
p= 0.001
PC2 [23.1%]
PC1 [26.1%]
I
Donor 4
R= 0.47
p= 0.001
PC2 [12.1%]
PC1 [22.9%]
J
Donor 5
R= 0.23
p= 0.037
PC2 [16.3%]
PC1 [21.8%]
Day
3
7 or 8
14
Colonization
Pre
Post
K
log2FoldChange
ASV97 Phascolarctobacterium faecium
ASV914 Prevotella 9 copri
ASV932 Faecalibacterium
ASV960 Prevotella 9
ASV938 Lactobacillus animalis
ASV1558 Anaerotruncus
ASV1573 Paraprevotella
ASV700 Faecalibacterium prausnitzii
ASV174 Subdoligranulum
ASV38 Parasutterella excrementihominis
ASV284 Dorea longicatena
ASV172 Faecalibacterium cf.prausnitzii
ASV942 Bacteroides nordii
ASV787 Roseburia hominis
ASV36 Bilophila wadsworthia
ASV728 Butyricicoccus
ASV974 Desulfovibrio fairfieldensis
ASV601 Lactobacillus curieae
ASV72 Dielma fastidiosa
ASV486 Bacteroides salyersiae
ASV116 Flavonifractor plautii
ASV16 Bacteroides intestinalis
ASV44 Collinsella aerofaciens
ASV184 Anaerotruncus colihominis
ASV972 Olsenella
ASV702 Faecalibacterium
ASV279 Lachnospiraceae
ASV256 Blautia faecis
ASV111 Ruminococcaceae UCG-013
ASV283 Lachnoclostridium
ASV65 Anaerofustis stercorihominis
ASV790 Roseburia faecis
ASV761 Lachnospiraceae ND3007 group
ASV948 Coprobacillus catenaformis
ASV753 Lachnospiraceae
ASV224 Eisenbergiella tayi
ASV871 Blautia
ASV230 Hungatella effluvii/hathewayi
ASV1556 Faecalibacterium
ASV1565 Blautia
ASV943 Blautia

Figure S6. Gating strategy and representative flow plots for immune populations in mice transplanted with microbiota from a patient before MTX treatment (M0, pre-MTX) and 1 month after treatment (M1, post-MTX) with or without DSS treatment, related to Figure 6 and 7.

(A) Colonic and small intestinal lamina (SI) propria lymphocytes as well as splenocytes (Sp) were isolated and stained for flow cytometry analysis. Shown is the gating strategy.

(B-G) Representative flow plots are shown for the following: percentage of IFN-γ+ (B), Foxp3 (C), IL17-A+ (D), and CD69+ CD44+ (E) cells within the CD3+CD4+ T cell compartment are displayed. Representative flow plots for B220 B cells (F) and Gr1+CD11b+ (G) myeloid cell populations are shown. Sp, spleen; SI, small intestine.


A
SSC-A
FSC-A
FSC-A
Live/dead - Violet E (525/50
CD3 - FITC
CD4 - PB
IL-17a – PEcy7
APC
IFNy – APC
PEcy7
PEcy7
FoxP3 – Percpcy5.5
CD3 - FITC
CD4 - PB
CD44 - APC
CD69 –PEcy7
B
Sp
M0
M1
M0 DSS
M1 DSS
4.25
3.86
6.94
3.95
IFNy – APC
PEcy7
C
Colon
M0
M1
M0 DSS
M1 DSS
10.2
7.94
11.1
10.0
Sp
6.01
3.00
4.15
3.34
PEcy7
FoxP3 – Percpcy5.5
D
Colon
M0
M1
M0 DSS
M1 DSS
3.97
2.03
18.3
9.76
Sl
10.7
8.57
19.5
8.04
Sp
14.5
13.8
11.4
13.6
IL-17a – PEcy7
APC
E
Colon
M0
M1
M0 DSS
M1 DSS
16.4
15.3
35.0
23.1
Sl
13.6
14.8
20.9
11.4
Sp
19.3
9.78
13.3
6.71
CD44 - APC
CD69 –PEcy7
F
Colon
M0
M1
M0 DSS
M1 DSS
21.9
16.7
44.6
40.5
Sp
20.9
15.7
35.8
9.64
B220 – PEcy7
PB
G
Colon
M0
M1
M0 DSS
M1 DSS
5.06
3.69
6.68
5.93
Sp
3.88
4.17
5.16
5.91
Gr1 – PE
CD11b PercpCy5.5

SUPPLEMENTAL DATA FILES

Data File S1. Growth curves of bacterial isolates with MTX with or without rescue agents, related to Figures 2 and 3.

(A-D) Representative growth curves from isolates treated with 10 concentrations of MTX. Included are growth and sterile controls as well. Each concentration was assessed in duplicate and both duplicates are plotted on the same graph. Bumps seen in the sterile controls (and in treatments without any growth) deviating from 0 are technical artifacts. Isolates (e.g., *E. rectale, D. longicatena*) with clumpy growth tend to produce variability in OD600 readings.

(A) Members of the Bacteroidetes phylum.

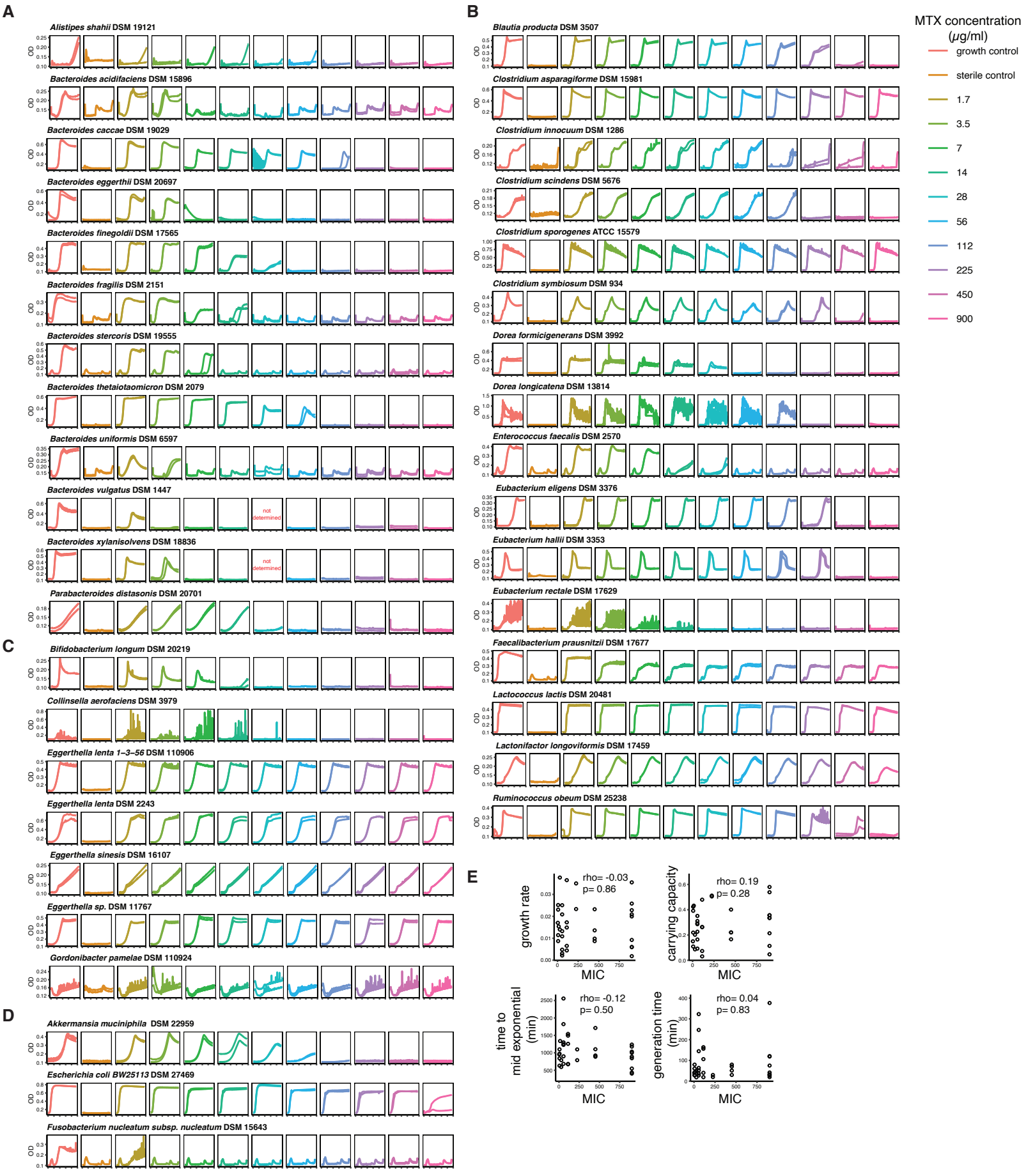
(B) Members of the Firmicutes phylum.

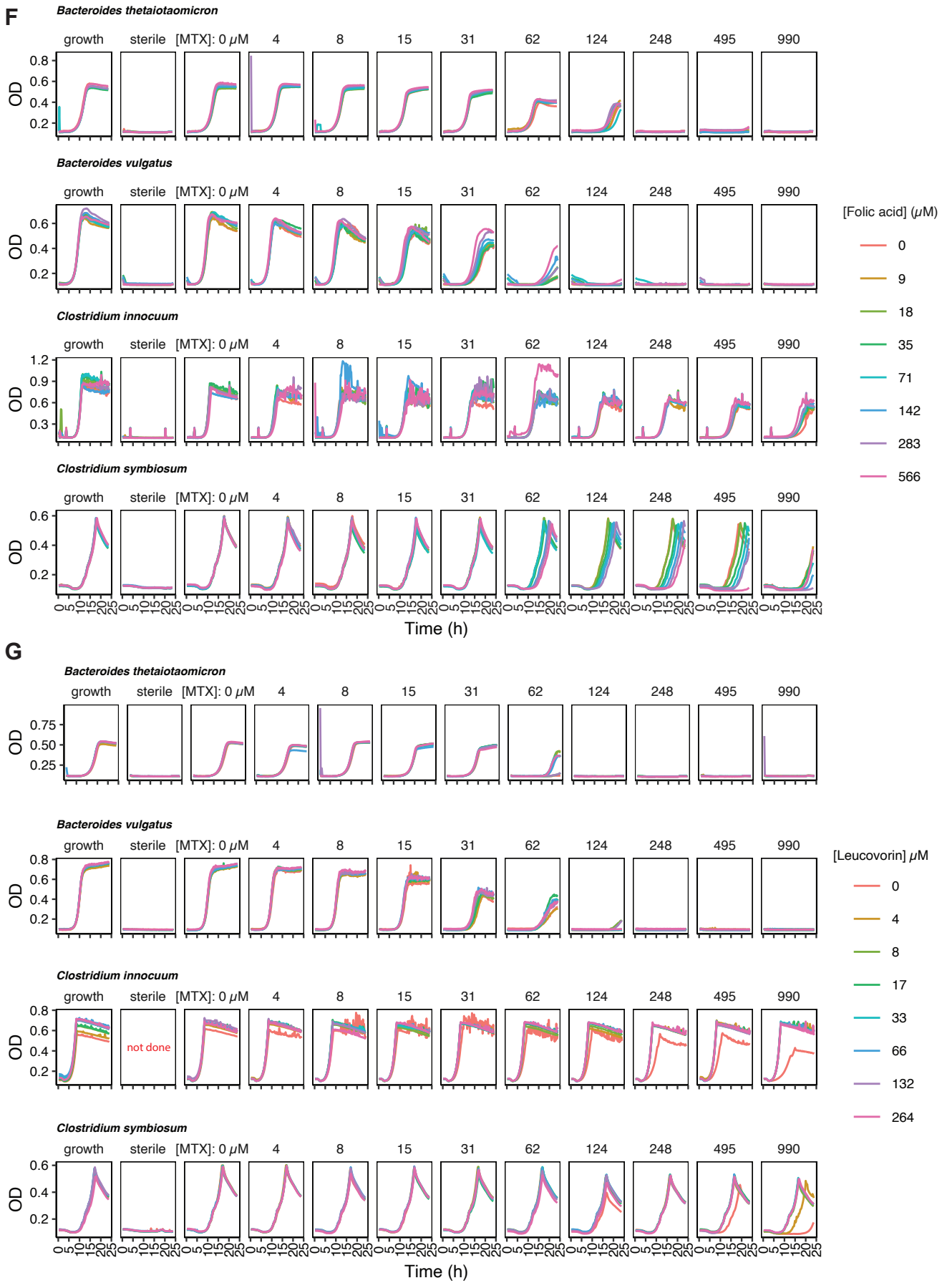
(C) Members of the Actinobacteria phylum.

(D) Members of multiple other phyla (Verrucomicrobia, Fusobacteria, Proteobacteria).

(E) Lack of a detectable correlation between MIC and estimated growth curve parameters of isolates grown in BHI+ media (in the absence of MTX).

(F-G) Bacterial isolates were treated with 9 concentrations of MTX and 7 concentrations of folic acid (F) or leucovorin (G) and growth was measured by optical density at OD600 every 15 minutes. Transient deviations from the baseline are technical artifacts.


A
Alistipes shahii DSM 19121
Bacteroides acidifaciens DSM 15896
Bacteroides caccae DSM 19029
Bacteroides eggerthii DSM 20697
Bacteroides finegoldii DSM 17565
Bacteroides fragilis DSM 2151
Bacteroides stercoris DSM 19555
Bacteroides thetaiotaomicron DSM 2079
Bacteroides uniformis DSM 6597
Bacteroides vulgatus DSM 1447
not determined
Bacteroides xylanisolvens DSM 18836
not determined
Parabacteroides distasonis DSM 20701
B
Blautia producta DSM 3507
Clostridium asparagiforme DSM 15981
Clostridium innocuum DSM 1286
Clostridium scindens DSM 5676
Clostridium sporogenes ATCC 15579
Clostridium symbiosum DSM 934
Dorea formicigenerans DSM 3992
Dorea longicatena DSM 13814
Enterococcus faecalis DSM 2570
Eubacterium eligens DSM 3376
Eubacterium hallii DSM 3353
Eubacterium rectale DSM 17629
Faecalibacterium prausnitzii DSM 17677
Lactococcus lactis DSM 20481
Lactonifactor longoviformis DSM 17459
Ruminococcus obeum DSM 25238
MTX concentration (μg/ml)
growth control
sterile control
1.7
3.5
7
14
28
56
112
225
450
900
C
Bifidobacterium longum DSM 20219
Collinsella aerofaciens DSM 3979
Eggerthella lenta 1–3–56 DSM 110906
Eggerthella lenta DSM 2243
Eggerthella sinesis DSM 16107
Eggerthella sp. DSM 11767
Gordonibacter pamelae DSM 110924
D
Akkermansia muciniphila DSM 22959
Escherichia coli BW25113 DSM 27469
Fusobacterium nucleatum subsp. nucleatum DSM 15643
OD
E
growth rate
rho= -0.03
p= 0.86
MIC
carrying capacity
rho= 0.19
p= 0.28
MIC
time to mid exponential (min)
rho= -0.12
p= 0.50
MIC
generation time (min)
rho= 0.04
p= 0.83
MIC


F
Bacteroides thetaiotaomicron
Bacteroides vulgatus
Clostridium innocuum
Clostridium symbiosum
growth
sterile
[MTX]: 0 μM
4
8
15
31
62
124
248
495
990
OD
Time (h)
[Folic acid] (μM)
0
9
18
35
71
142
283
566
G
Bacteroides thetaiotaomicron
Bacteroides vulgatus
Clostridium innocuum
Clostridium symbiosum
not done
[Leucovorin] μM
0
4
8
17
33
66
132
264