

Supplementary Information for

Acute infection of mice with *Clostridium difficile* leads to

eIF2 α phosphorylation and pro-survival signalling as part of the

mucosal inflammatory response

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Supplementary Text

Materials and Methods for Figures S1 and S2

DNA isolation, amplicon library preparation and 454 pyrosequencing- The DNeasy blood and tissue kit (Qiagen) was used to extract genomic DNA from the caecal and colonic contents obtained from untreated and *C. difficile*-infected mice. The extraction was performed according to the manufacturer's instructions except for the following modifications: adding a bead-beating step using UltraClean fecal DNA bead tubes (Mo Bio Laboratories, Carlsbad, CA); doubling the amount of ATL buffer and the proteinase K used in the protocol; and decreasing by half the amount of the AE buffer used to elute the DNA. Subsequently, the V3, V4 and V5 hyper-variable regions of the 16S ribosomal RNA gene in each of the samples were targeted for amplification with the 357F and 929R primer sets [1]. Amplicons were purified with the Agencourt AMPure XP PCR purification system (Beckman Coulter, Indianapolis, IN), and quantified with the Quant-iT PicoGreen dsDNA kit (Life Technologies) to obtain an equal pool for pyrosequencing. They were then sequenced on a Roche 454 GS Junior Titanium platform according to the manufacturer's specifications.

Microbiome community analysis- Bacterial 16S sequences were first processed using the microbial ecology software suite *mothur* [2] to generate operational taxonomic units (OTUs) at a 3%, i.e. species level of difference. These data, in the form of the *.shared* file, were then imported into the R software and analysed using the R-package *vegan* [3].

The inverse Simpson diversity measure was calculated using the function *diversity()*, whereas rarefaction was calculated using the function *rarefy()*. Rank abundance plots were generated by selecting for the OTUs that contributed to >0.5% of the population and had an OTU abundance >10. The content of each infected tissue was ordered according to the average rank order of its untreated counterpart. Taxonomic classification of an OTU was assigned within *mothur* by identifying the consensus sequence of the OTU and assigning taxonomy using the Bayesian classifier trained on an RDP training set (*classify.otu*).

Histopathology- Intact caecal and colonic tissue were placed in histology cassettes and fixed with 10% neutral buffered formalin for 24 hours and then transferred to 70% ethyl alcohol. The cassettes were then paraffin-embedded, sectioned and stained with haematoxylin and eosin (H & E) for histopathological evaluation (McClinchey Histology Lab, Stockbridge, MI).

Results for Figure S1

We analysed the caecal and colonic bacterial microbiota of the mice on day 2 by culture-independent 454 pyrosequencing of bacterial 16S rRNA gene amplicon libraries. Analysis of the bacterial communities, following assignment of operational taxonomic units (OTU) to the sequencing data, indicated that in untreated mice, there was a marked difference in the ratio of *Firmicutes* to *Bacteroidetes* between the caeca and colons, lower diversity in the colons compared to the caeca, and differences in the rank abundances of the various OTUs (Figure S1). However, despite differences in the bacterial microbiota of caeca and colons prior to the use of antibiotics and infection, there were significant reductions in the diversity of the bacterial microbiota in the caeca and colons of *C. difficile*-infected mice, as assessed by two metrics of community diversity, Inverse Simpson Index (Figure S1A) and rarefaction (Figure S1B). Taxonomic assignments of the OTUs identified (1) a marked loss of the *Lachnospiraceae* OTUs, (2) a bloom of *Bacteroides*, *Escherichia*, *Akkermansia*, *Lactobacillus*, and *Coprobacillus*, and (3) the detection of *C. difficile* in the caeca and colons of the infected mice (Figure S1D-G and Table S1). Thus, antibiotics altered both the caecal and colonic bacterial microbiota, and the restricted diversity of the bacterial microbiota of the caeca and colons were comparable following treatment with antibiotics and *C. difficile* infection. Therefore, the loss of total species diversity- including *Lachnospiraceae* [4]- at both sites was accompanied by outgrowth of the same set of lesser abundant organisms- including *Enterobacteriaceae*- in both organs, which are typically associated with disrupted communities and mucosal inflammation [5-8].

References

- 1 **Zhao, J., Carmody, L. A., Kalikin, L. M., Li, J., Petrosino, J. F., Schloss, P. D., Young, V. B. and Lipuma, J. J.**, Impact of enhanced Staphylococcus DNA extraction on microbial community measures in cystic fibrosis sputum. *PLoS One* 2012. **7**: e33127.
- 2 **Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J. and Weber, C. F.**, Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009. **75**: 7537-7541.
- 3 **Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., O'Hara, R. B., Simpson, G. L., Solymos, P., Henry, M., Stevens, H. and Wagner, H.**, vegan: Community Ecology Package. R package version 1.17-3, 1.17-3 Edn. 2010.
- 4 **Reeves, A. E., Koenigsnecht, M. J., Bergin, I. L. and Young, V. B.**, Suppression of *Clostridium difficile* in the gastrointestinal tracts of germfree mice inoculated with a murine isolate from the family Lachnospiraceae. *Infect Immun* 2012. **80**: 3786-3794.
- 5 **Reeves, A. E., Theriot, C. M., Bergin, I. L., Huffnagle, G. B., Schloss, P. D. and Young, V. B.**, The interplay between microbiome dynamics and pathogen dynamics in a murine model of *Clostridium difficile* Infection. *Gut Microbes* 2011. **2**: 145-158.
- 6 **Gilliland, M. G., 3rd, Erb-Downward, J. R., Bassis, C. M., Shen, M. C., Toews, G. B., Young, V. B. and Huffnagle, G. B.**, Ecological Succession of Bacterial Communities during Conventionalization of Germ-Free Mice. *Appl Environ Microbiol* 2012. **78**: 2359-2366.
- 7 **Lupp, C., Robertson, M. L., Wickham, M. E., Sekirov, I., Champion, O. L., Gaynor, E. C. and Finlay, B. B.**, Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe* 2007. **2**: 119-129.
- 8 **Lau, H. Y., Huffnagle, G. B. and Moore, T. A.**, Host and microbiota factors that control *Klebsiella pneumoniae* mucosal colonization in mice. *Microbes Infect* 2008. **10**: 1283-1290.

Titles and legends for Supplementary Figures and Table

Figure S1. Pyrosequencing analysis of the effect of antibiotic treatment and *C. difficile* infection on intestinal microbiota.

The bacterial communities of caecal and colonic contents from untreated and *C. difficile*-infected (CDI) mice were compared by 454 pyrosequencing of bacterial 16S rRNA gene amplicon libraries generated from the contents. Operational taxonomic units (OTUs), based on 3% difference, were assigned using the software suite *mothur*. Two metrics of community diversity were applied to the OTUs: (A) Inverse Simpson index, and (B) rarefaction analysis (solid lines represent the mean rarefied community and dashed lines the SEM). A p value of ≤ 0.05 indicates a significant difference between the untreated and CDI samples and is marked with a ★. (C) displays the relative abundance of the bacterial phyla. (D) and (E) are the rank abundance plots of bacterial OTUs (based on 3% difference) of untreated and *C. difficile*-infected (CDI) caecal contents respectively. (F) and (G) respectively depict the rank abundance plots of bacterial OTUs (based on 3% difference) of untreated and *C. difficile*-infected (CDI) colonic contents. The x-axis lists individual OTUs, which are assigned taxonomic identities in Table S1. Six specific OTUs of interest (a-f) and their taxonomic assignments are provided in the figure.

Figure S2. Histopathology of *C. difficile* infection.

Representative histology from untreated and *C. difficile*-infected (CDI) mice, showing marked oedema and cellular infiltration in the caecum (B) and colon (D) of the infected mouse in comparison to the caecum (A) and colon (C) of its untreated counterpart.

Figure S3. The number of dendritic cells and cells of the monocyte/macrophage lineage in the spleens and mesenteric lymph nodes of *C. difficile*-infected mice.

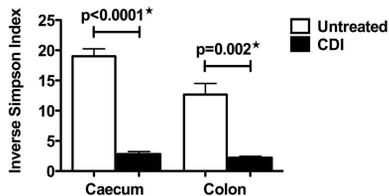
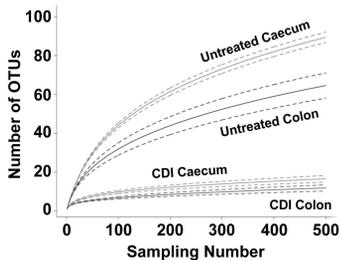
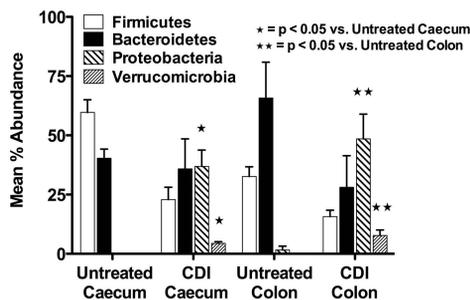
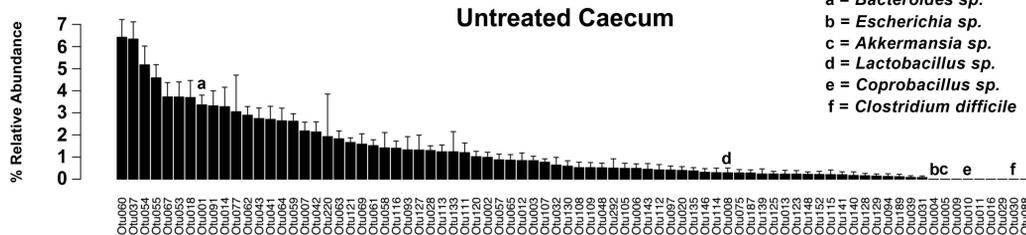
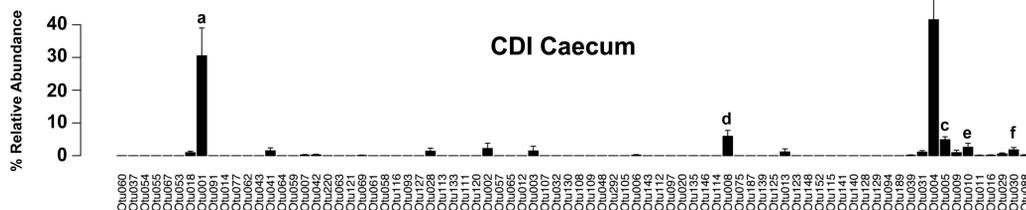
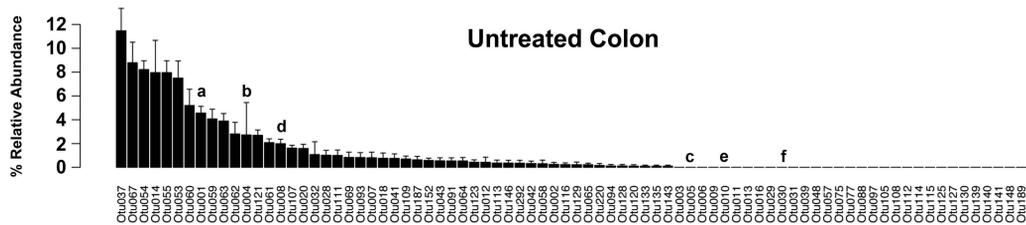
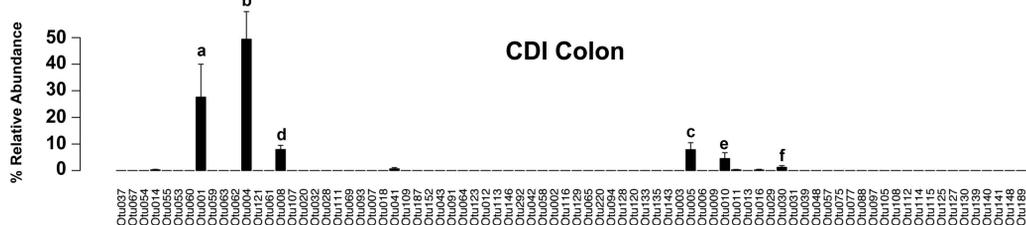
Bar graphs showing the number of dendritic cells (A), and cells of the monocyte/macrophage lineage (B), in the spleens and mesenteric lymph nodes of *C. difficile*-infected (CDI) mice in comparison to untreated mice. The bars represent the mean \pm SEM of 12 pairs of spleens and mesenteric lymph nodes. A p value of ≤ 0.05 indicates a significant difference between the untreated and CDI samples and is marked with a ★.

Figure S4. The number of various lymphocyte subsets in the spleens and mesenteric lymph nodes of *C. difficile*-infected mice.

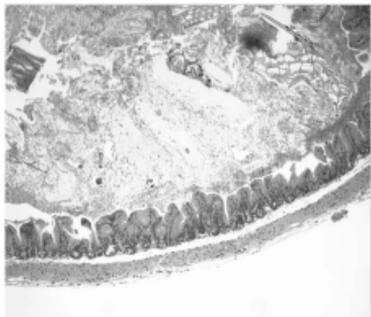
Bar graphs showing the number of B cells, CD4 T cells and CD8 T cells in the spleens and mesenteric lymph nodes of *C. difficile*-infected (CDI) mice in comparison to untreated mice (A). The bars represent the mean \pm SEM of 12 pairs of spleens and mesenteric lymph nodes. A p value of ≤ 0.05 indicates a significant difference between the untreated and CDI samples and is marked with a ★. (B) and (C) respectively show histograms of CD69 and CD25 expression levels in the CD4 T cells of spleens and mesenteric lymph nodes of *C. difficile*-infected (CDI) mice in comparison to untreated mice.

Table S1. The list of operational taxonomic units (OTUs) and their taxonomic identification.

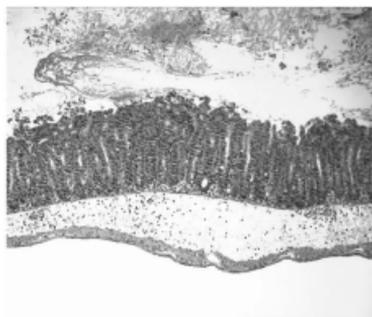
Taxonomic identities were assigned within *mothur* by identifying the consensus sequence of the OTU and assigning taxonomy using the Bayesian classifier trained on an RDP training set (classify.otu).

(A)**(B)****(C)****(D)****(E)****(F)****(G)****Figure S1**

(A)

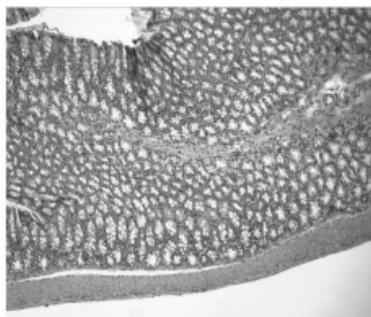


(B)

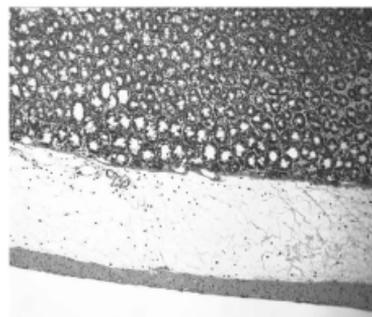


Caecum

(C)



(D)



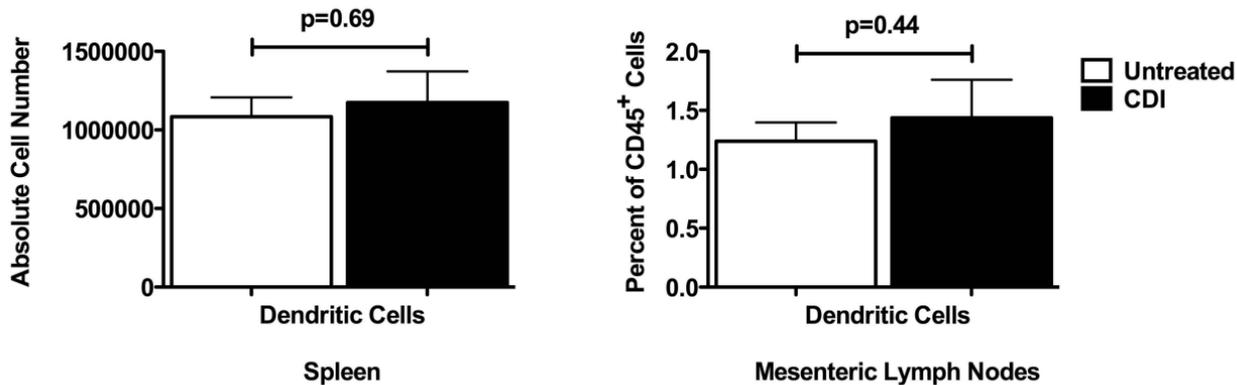
Colon

Untreated

CDI

Figure S2

(A)



(B)

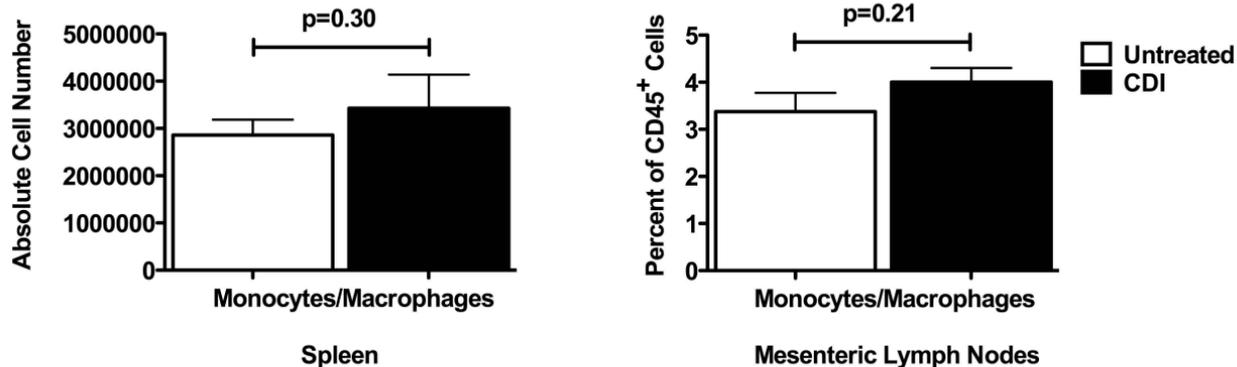
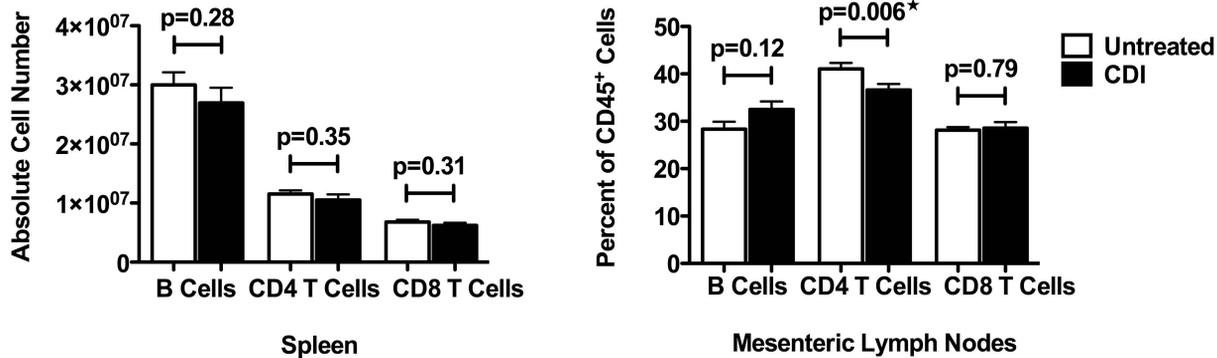
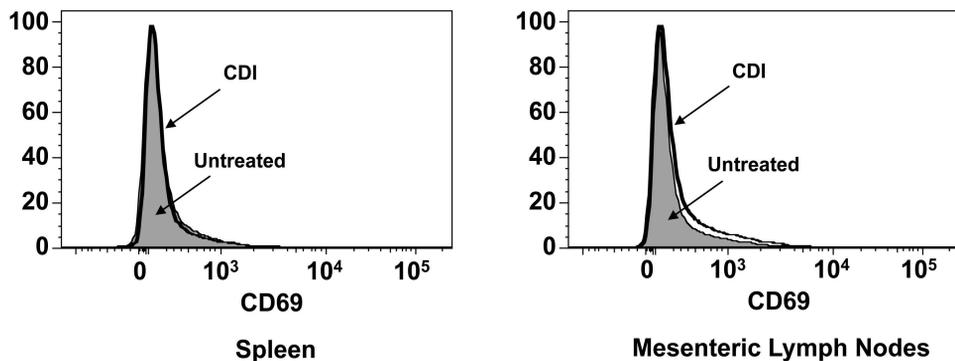


Figure S3

(A)



(B)



(C)

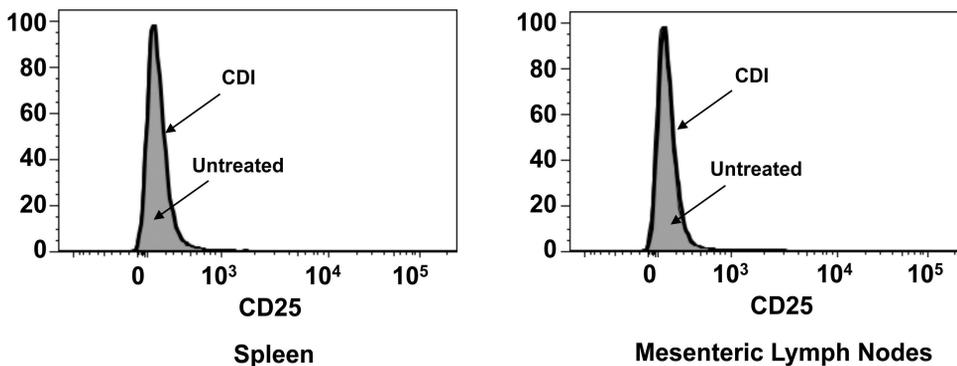


Figure S4

Table S1

OTU_Number	Kingdom	Phylum	Class	Order	Family	Genus
Otu001	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Otu002	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu003	Bacteria	Firmicutes	Clostridia	Clostridiales	unclassified_Clostridiales	unclassified
Otu004	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unclassified_Enterobacteriaceae
Otu005	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia
Otu006	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu007	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillibacter
Otu008	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus
Otu009	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu010	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Coprobacillus
Otu011	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Clostridium_XIVb
Otu012	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu013	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Clostridium_IV
Otu014	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus
Otu016	Bacteria	Firmicutes	unclassified_Firmicutes	unclassified	unclassified	unclassified
Otu018	Bacteria	Firmicutes	Clostridia	Clostridiales	unclassified_Clostridiales	unclassified
Otu020	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus
Otu028	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Turicibacter
Otu029	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus
Otu030	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Clostridium_XI (C. difficile)
Otu031	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified_Ruminococcaceae
Otu032	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu037	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	unclassified_Porphyromonadaceae
Otu039	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu041	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu042	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu043	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu048	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu053	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	unclassified_Porphyromonadaceae
Otu054	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	unclassified_Bacteroidales	unclassified
Otu055	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	unclassified_Porphyromonadaceae
Otu057	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu058	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu059	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Barnesiella
Otu060	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Alistipes
Otu061	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	unclassified_Porphyromonadaceae
Otu062	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	unclassified_Porphyromonadaceae
Otu063	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	unclassified_Porphyromonadaceae
Otu064	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu065	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae

Otu067	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	unclassified_Porphyromonadaceae
Otu069	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu075	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu077	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	unclassified_Erysipelotrichaceae
Otu088	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified_Ruminococcaceae
Otu091	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu093	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu094	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified_Ruminococcaceae
Otu097	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified_Ruminococcaceae
Otu105	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu107	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	unclassified_Bacteroidales	unclassified
Otu108	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu109	Bacteria	unclassified_Bacteria	unclassified	unclassified	unclassified	unclassified
Otu111	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	unclassified_Porphyromonadaceae
Otu112	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Clostridium_XIVa
Otu113	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillibacter
Otu114	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu115	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified_Ruminococcaceae
Otu116	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu120	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu121	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	unclassified_Bacteroidales	unclassified
Otu123	Bacteria	Firmicutes	Clostridia	Clostridiales	unclassified_Clostridiales	unclassified
Otu125	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu127	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu128	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified_Ruminococcaceae
Otu129	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu130	Bacteria	Firmicutes	Clostridia	Clostridiales	unclassified_Clostridiales	unclassified
Otu133	Bacteria	Firmicutes	Clostridia	Clostridiales	unclassified_Clostridiales	unclassified
Otu135	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Barnesiella
Otu139	Bacteria	Firmicutes	Clostridia	Clostridiales	unclassified_Clostridiales	unclassified
Otu140	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified_Ruminococcaceae
Otu141	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu143	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae
Otu146	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	unclassified_Bacteroidales	unclassified
Otu148	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified_Ruminococcaceae
Otu152	Bacteria	Firmicutes	Clostridia	Clostridiales	unclassified_Clostridiales	unclassified
Otu187	Bacteria	Firmicutes	Clostridia	Clostridiales	unclassified_Clostridiales	unclassified
Otu189	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Butyricoccus
Otu220	Bacteria	Firmicutes	Clostridia	Clostridiales	unclassified_Clostridiales	unclassified
Otu292	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified_Lachnospiraceae

Table S1. The list of operational taxonomic units (OTUs) and their taxonomic identification.

Taxonomic identities were assigned within mothur by identifying the consensus sequence of the OTU and assigning taxonomy using the Bayesian classifier trained on an RDP training set (classify.otu).