1 **Stable Engraftment of a Human Gut Bacterial Microbiome in Double Humanized BLT-mice**

2 Lance Daharsh[1,2], Amanda E. Ramer-Tait[3], *Qingsheng Li[1,2]

3

4 [1]Nebraska Center for Virology, [2]School of Biological Sciences, [3]Department of Food Science and

5 Technology, University of Nebraska-Lincoln, Lincoln, NE 68583, USA

6

7 Corresponding author: Qingsheng Li (qli@unl.edu)

8

9 **Abstract**

10 **Background**

11 Humanized mice featuring a functional human immune system are an important pre-clinical

12 model for examining immune responses to human-specific pathogens. This model has been

13 widely utilized to study human diseases that are otherwise impossible or difficult to investigate

14 in humans or with other animal models. However, one limitation of using humanized mice is

15 their native murine gut microbiome, which significantly differs from the one found in humans.

16 These differences may be even greater for mice housed and bred in specific pathogen free

17 conditions. Given the importance of the gut microbiome to human health and disease, these

18 differences may profoundly impact the ability to translate the results from humanized mice

19 studies to human disease. Further, there is a critical need for improved pre-clinical models to

20 study the complex *in vivo* relationships of the gut microbiome, immune system, and human

21 disease. We therefore created double humanized mice with both a functional human immune

22 system and stable human-like gut microbiome.

23   **Results**

24   Surgery was performed on NOD.*Cg-Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice to create bone-marrow,

25   liver, thymus (BLT) humanized mice. After immune reconstitution, mice were treated with

26   broad spectrum antibiotics to deplete murine gut bacteria and then transplanted with fecal

27   material from healthy human donors. Characterization of 173 fecal samples obtained from 45

28   humanized mice revealed that double humanized mice had unique 16S rRNA gene profiles

29   consistent with those of the individual human donor samples. Importantly, transplanted

30   human-like gut microbiomes were stable in mice for the duration of the study, up to 14.5 weeks

31   post-transplant. Microbiomes of double humanized mice also harbored predicted functional

32   capacities that more closely resembled those of the human donors compared to humanized

33   mice.

34   **Conclusions**

35   Here, we describe successful engraftment of a stable human microbiome in BLT humanized

36   mice to further improve this preclinical humanized mouse model. These double humanized

37   mice represent a unique and tractable new model to study the complex relationships between

38   the human gut microbiome, human immune system, and human disease *in vivo*.

39

40   **<u>Background</u>**

41   The complex ecosystem of the gut microbiome plays a critical role in human health and

42   disease [1-5]. Specifically, the gut microbiome has a highly reciprocal and dynamic relationship

43   with the immune system. Antigens derived from the gut microbiome influence host immune

44   responses, and the immune system in turn contributes to shaping the spatial distribution and

45   composition of the gut microbiota [6-8]. Humanized mice (hu-mice) with an engrafted human

46   immune system have facilitated important advancements in the study of human cancer,

47   autoimmune diseases, hematopoiesis, and infectious diseases [9-17]. However, gut

48   microbiomes of hu-mice are murine in origin and are often not well-characterized in

49   translational studies. The murine gut microbiome differs substantially in composition and

50   function from that of humans [18], primarily due to anatomical differences as well as other

51   factors such as diet [19]. Considering the importance of the gut microbiota to proper

52   immunological development and influencing immune responses, the murine origin of the

53   microbiome harbored by hu-mice could affect translational study outcomes. Consequently, a

54   need exists to not only characterize the gut microbiomes of hu-mice, but also impart these mice

55   with a more human-like gut microbiome to improve the translational aspects of the model for

56   human medicine.

57   The creation of a new pre-clinical hu-mice model to study the human immune in the

58   context of a human microbiome offers numerous benefits over existing options. Many aspects

59   of human disease are difficult or impossible to study directly in humans due to practical or

60   ethical concerns. Non-human primate models are informative but are genetically outbred, and

61   large studies are often resource and cost prohibitive. Many important discoveries in the

62   microbiome field have been made using mouse models; however, translating results from

63   mouse studies to humans has often proved difficult. The use of germ-free mice reconstituted

64   with human-like gut microbiomes has been the gold standard in studying the relationship of the

65   gut microbiome to human health and disease [20, 21]. However, working with or deriving germ-

66   free animals requires expertise and facilities that are not always available. Further, many

3

67   immunodeficient mouse strains commonly used to reconstitute a human immune system, such

68   as NOD.Cg-$Prkdc^{scid}Il2rg^{tm1Wjl}$/SzJ (NSG), are currently not commercially available as germ-free.

69   We therefore created a double hu-mice model featuring both a functional human immune

70   system and a stable human-like gut microbiome under specific pathogen free (SPF) conditions.

71   Here, we show that double hu-mice had unique 16S rRNA gene profiles based on the individual

72   human donor sample with which they were colonized. Importantly, the transplanted human-

73   like microbiome was stable in the mice for the duration of the study, up to 14.5 weeks post-

74   transplant. Double hu-mice also harbored gut microbiomes with a more human-like predicted

75   functional capacity compared to their hu-mice counterparts.

76

77   **Results**

78   **Gut microbiomes of double hu-mice are distinct and more human-like compared to hu-mice.**

79   To create double hu-mice, surgery was performed on NSG mice to create bone-marrow,

80   liver, thymus (BLT) hu-mice. Hu-mice were then pre-treated with a cocktail of broad-spectrum

81   antibiotics and administered fecal transplants using fecal material from healthy human donors

82   (see Methods and Daharsh et al.[22] for detailed descriptions and demonstrations). Multiple

83   cohorts of double hu-mice were created using fecal material from one of three unique healthy

84   human donors or an equal mixture of all three (Table 1). We used 16S rRNA gene sequencing

85   and characterized the gut bacterial microbiome of 100 fecal samples from 16 double hu-mice

86   and compared them with 67 fecal samples representing the pre-existing murine gut bacterial

87   microbiomes of hu-mice and the profiles of the 4 human fecal donor samples. To visualize beta-

88   diversity relationships between hu-mice, double hu-mice, and the human donor samples, non-

89   metric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA) plots were

90   created (Fig. 1). Using NMDS, the gut microbiome profiles of the three groups separated into

91   distinct clusters (Fig. 1a). Using PCoA, the human donor samples fell within the double hu-mice

92   hull, which was distinct from the hu-mice profiles (Fig. 1b). Both dimensionality reduction

93   methods showed that after engraftment of a human gut microbiome, double hu-mice

94   represented a distinct population that clustered closer to the human donor samples compared

95   to hu-mice harboring murine gut microbiomes. Importantly, there was no reversion to hu-mice

96   profiles post-transplant. Displaying the relatedness of the samples through a hierarchical

97   dendrogram based on Bray-Curtis distances further confirmed the similarity of double hu-mice

98   microbiomes to the human donor samples and differentiated them from those of hu-mice (Fig.

99   2).

100   **Table 1. Experimental summary of double hu-mice cohorts**

Cohort	Antibiotic start	Antibiotic duration	Number of fecal transplants	Human fecal donor	Maximum weeks post-transplant	Double hu-mice	Double hu-mice fecal samples	Hu-mice	Hu-mice fecal samples
Antibiotic Pilot	After immune reconstitution	9 days	0	NA	0	0	0	6	9
Donor 65 Cohort 1	After immune reconstitution	14 days	2 (24 & 48 hr post antibiotics)	65	13.5	3	35#	3	23
Donor 65 Cohort 2	After immune reconstitution	14 days	2 (24 & 48 hr post antibiotics)	65	14.5	2	16#	8	8
Donor 74	After immune reconstitution	7 days	2 (24 & 48 hr post antibiotics)	74	6	3	17#	3*	3*

Donor 82	After immune reconstitution	7 days	2 (24 & 48 hr post antibiotics)	82	6	4	27#	3*	3*
Donor Mix	3 days after hu-mice surgery	14 days	2 (24 & 48 hr post antibiotics)	Equal mixture of 65, 72, & 84	9	4	21#	9	10

\# Includes 1 pre-fecal transplant sample for each of the double hu-mice
* Control hu-mice were the same for Donor 74 and Donor 82 groups

101

102   We also observed that double hu-mice maintained the pre-existing relationships

103   between gut microbiome profiles of the human fecal donors (Fig. 1c & 1d). Importantly, pre-

104   treatment samples from corresponding double hu-mice in each cohort had similar gut

105   microbiome profiles as untreated control hu-mice. After engraftment, double hu-mice

106   resembled the individual human donor that was transplanted as demonstrated by the

107   relationships between the human donor microbiome profiles. Human donors 72 and 84 had

108   more similar profiles to one another than to human donor 65. This relationship was maintained

109   in the double hu-mice after engraftment. We also prepared an "un-biased" human sample by

110   mixing equal parts of the three human donor fecal samples, designated hereafter as donor mix.

111   The microbiome profile of this mixed sample resembled a mixture of the three individual

112   human donor profiles. Specifically, the mixed donor sample more closely resembled individual

113   donors 72 and 84, and this observation was mirrored in the double hu-mice engrafted with the

114   donor mix sample.

115   We also investigated the impact of antibiotic treatment duration on the engraftment of

116   human gut microbiome. The double hu-mice engrafted with human donors 74 and 82 were

117   generated after only 7 days of antibiotic pre-treatment. Their microbiome profiles were less

118   similar to the human donor profiles than those from cohorts pre-treated with antibiotics for 14

119   days prior to fecal transplant (Fig. 1c & 1d). We found the 2 weeks of antibiotic treatment was

120   optimal for the creation of double hu-mice. Together, these results demonstrate that our

121   approach to generating double hu-mice is reproducible and able to create hu-mice with unique

122   16S rRNA gene profiles based on the individual human fecal donor.

123

124   **Gut microbiomes of double hu-mice have increased levels of alpha diversity compared to hu-**

125   **mice.**

126   Due to the highly reciprocal nature of the gut microbiome and immune system, we

127   hypothesized that highly immunodeficient mice, such as NSG, would have low pre-existing gut

128   microbiome diversity, especially when the mice were housed under SPF conditions with limited

129   exposure to outside sources of microbes. We tested this hypothesis and found that the gut

130   microbial diversity of double hu-mice with a functional immune system significantly increased

131   to the levels observed in our human donor samples compared to hu-mice. Several alpha

132   diversity measurements confirmed that hu-mice had very low measures of alpha diversity

133   compared to our human donor samples (Fig. 3). However, after engraftment, double hu-mice

134   had increased species richness compared to hu-mice (P<.001) and did not differ significantly

135   from the human donor samples (Fig. 3a). Further, the Shannon index values of the human

136   donor samples were significantly higher than hu-mice (P<.05) but were not significantly

137   different from the double hu-mice samples. Double hu-mice had increased Simpson index

138   values compared to hu-mice, but the human donor samples were significantly higher than both

139   the double hu-mice (P<.05) and hu-mice (P<.05). As expected, samples taken during antibiotic

140   treatment had the lowest measures of all three diversity metrics tested. Alpha diversity metrics

141   were also measured based on the human donor sample engrafted (Supplemental Figure –

142   Alpha_Diversity.pdf). Double hu-mice engrafted after 14 days of antibiotics (donor 65 cohorts 1

143   & 2, donor mix) had higher levels of alpha diversity compared to double hu-mice engrafted

144   after only 7 days of antibiotics (donors 74 & 82). Overall, double hu-mice had increased alpha

145   diversity compared to hu-mice that was more similar to the levels observed in the human donor

146   samples. The shorter antibiotic treatment duration (7 days versus 14 days) was associated with

147   lower alpha diversity measurements in double hu-mice engrafted with human donors 74 or 82.

148

149   **The relative abundance of gut bacteria in double hu-mice is similar to that found in human**

150   **donor samples.**

151   We next hypothesized that if bacteria from the human donors were successfully

152   engrafted into double hu-mice, then we would see more taxonomic similarities between human

153   donor gut microbiome profiles and double hu-mice versus hu-mice. Several differences were

154   observed in the relative abundances of double hu-mice based on the length of antibiotic

155   treatment, the human fecal donor sample engrafted, and mouse cohort (Supplemental Figure –

156   Relative_Abundances.pdf). At the Phylum level, both double hu-mice and hu-mice samples

157   were largely represented by *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Verrucomicrobia*.

158   Interestingly, we found that hu-mice had high proportions of *Verrucomicrobia* that remained

159   high even after antibiotic treatment and engraftment with human donor samples that had low

160   abundances of *Verrucomicrobia*. At the Family level, hu-mice samples had a higher relative

161   abundance of *S24-7* than double hu-mice and human donor samples (Fig. 4). Both hu-mice and

162   double hu-mice samples had higher relative abundances of *Lactobacillaceae* and

163   *Verrucomicrobiaceae* than human donor samples. The human donor and double hu-mice

164   samples had higher relative abundances of *Bacteroidaceae* than hu-mice samples. The human

165   donor samples also had a much higher relative abundance of *Lachnospiraceae*.

166   We next compared the taxonomic differences between the microbiomes using Kruskal-

167   Wallis testing with FDR adjusted P values below .05 (Supplemental File – KW_Testing.xlsx). We

168   found 195 significant differences between the hu-mice and human donor samples and 170

169   significant differences between hu-mice and double hu-mice. However, only 108 significant

170   differences were observed between double hu-mice and human donor samples. Both the

171   human donor and double hu-mice samples had significantly higher relative abundances of the

172   genus *Bacteroides*, while hu-mice had a significantly higher relative abundance of the family

173   *S24-7*. Human donor samples had a higher relative abundance of the Phylum *Firmicutes*

174   compared to both hu-mice and double hu-mice. Human donor samples had lower relative

175   abundances of the class *Bacilli* and family *Lactobacillaceae* compared to both hu-mice and

176   double hu-mice. Human donor samples also had a higher relative abundance of the class

177   *Clostridia* than both hu-mice and double hu-mice samples. Significant differences found within

178   this class were the family *Lachnospiraceae*, genus *Blautia*, genus *Roseburia*, family

179   *Ruminococcaceae*, genus *Ruminococcus*, and species *Faecalibacterium prausnitzii*. Some of

180   these taxa do increase in abundance in double hu-mice as compared to hu-mice, as observed

181   with significant differences in the genus *Blautia* and genus *Ruminococcaceae*. Levels of the

182   species *Akkermansia muciniphila* are significantly higher in hu-mice and double hu-mice

183   samples compared to human donor samples. However, double hu-mice have significantly less

184   relative abundance of this species than hu-mice samples.

9

185   To further determine differences in relative abundances among microbiomes from the

186   various treatments, we used Linear discriminant analysis Effect Size (LEfSe) with a P value < .05

187   and LDA score > 2 (Supplemental File – LefSe.xlsx)[23]. Taxa with LDA scores higher than 4

188   were plotted to show significant differences between double hu-mice and human donor

189   samples and double hu-mice and hu-mice samples (Fig. 5). Human donor samples were

190   associated with higher relative abundances of several types of *Clostridia* including

191   *Lachnospiraceae, Blautia, Coprococcus, Roseburia, Facalibacterium*, and *Ruminococcus*

192   compared to double hu-mice, while double hu-mice samples were associated with *Lactobacillus*

193   and *Akkermansia muciniphila* (Fig. 5a). Double hu-mice were associated with higher relative

194   abundances of *Bacteroides* and several types of *Clostridia* including *Blautia, Coprococcus*, and

195   *Ruminococcaceae*, while hu-mice samples were associated with *S24-7* and *Mogibacteriaceae*

196   (Fig. 5b). By characterizing the relative bacterial abundance in double hu-mice, we

197   demonstrated that certain taxa, like members of *Bacteroides,* readily engrafted while others

198   such as *Clostridia* were more difficult to transplant. Further, several species such as those found

199   in the phylum *Verrucomicrobia* were highly prevalent in hu-mice, and antibiotic treatment

200   followed by fecal transplant did not fully diminish or replace this population based on relative

201   abundances. Altogether, these results demonstrate that engraftment of human donor samples

202   significantly changed the taxonomic profile of double hu-mice and that their human-like gut

203   microbiomes were statistically more similar to human donor profiles compared to hu-mice.

204

205   **The engrafted human-like gut microbiome in double hu-mice is stable.**

206   To evaluate the stability of the engrafted human-like gut microbiome in double hu-mice

207   after fecal transplant, the proportion of shared amplicon sequence variants (ASVs) with the

208   human donor sample was calculated (Fig. 6). After engraftment, double hu-mice had increased

209   proportions of shared ASVs with their respective human donor samples, and those proportions

210   remained higher than pre-treatment and control levels for the duration of the study. The first

211   cohort of double hu-mice engrafted with human donor 65 had an average shared ASV

212   proportion of 13.70% after transplant, while the pre-treatment samples had 1.06% and control

213   samples levels had 1.42% (Fig. 6a). This increased proportion of shared ASVs was maintained

214   for the duration of the study, up to 14 weeks post-transplant. A second cohort of double hu-

215   mice engrafted with human donor 65 was created and a similar increase in the proportion of

216   shared ASVs was observed (Fig. 6b). The average proportion of shared ASVs was 17.65% post-

217   transplant, while the pre-treatment samples had 2.60% and the control samples had 0.96%.

218   This increased proportion of shared ASVs was maintained for the duration of the study, up to

219   14.5 weeks post-transplant. Double hu-mice transplanted with the mixture of all three human

220   donors had an average shared ASV proportion of 10.91% after transplant, while the pre-

221   treatment samples had 0.31% and the control samples had 0.61% (Fig. 6d).

222   The proportion of shared ASVs was then calculated for cohorts of double hu-mice only

223   treated with 7 days of antibiotics prior to fecal transplant. Double hu-mice engrafted with

224   human donor 74 had an average shared ASV proportion of 11.85% after transplant, while the

225   pre-treatment samples had 1.02% and the control samples had 0.86% (Fig. 6c). Double hu-mice

226   transplanted with human donor 82 had an average shared ASV proportion of 10.56% after

227   transplant, while the pre-treatment samples had 1.33% and the control samples had 0.95% (Fig.

228   6c).

229   To further evaluate the stability of the engrafted human-like microbiome after

230   transplant into double hu-mice, the contributions of the human donor and pre-treatment

231   sample to the post-transplant samples were determined using SourceTracker (Fig. 7)[24]. The

232   first cohort of mice transplanted with human donor 65 had an average donor contribution

233   percentage of 18.76% after transplant, while the pre-treatment samples had 0.00% and the

234   control samples had 0.05% (Fig. 7a). At the final time point collected at 14 WPT, the donor

235   contribution was consistent at 18.10%. The second cohort of mice transplanted with human

236   donor 65 had an average donor contribution percentage of 29.01% after transplant, while the

237   pre-treatment and controls samples had no donor contribution (Fig. 7b). The donor

238   contribution percentage was 34.10% at 14.5 WPT, thus demonstrating the stable engraftment

239   of donor bacteria. Double hu-mice engrafted with donor 74 had an average donor contribution

240   percentage of 12.71% after transplant, while the pre-treatment samples had 0.00% and the

241   control samples had 0.00% (Fig. 7c). At the final time point collected at 6 WPT, the average

242   donor contribution was 18.75%.

243   The SourceTracker algorithm was unable to clearly distinguish donor 82 contributions as

244   both pre-treatment and control samples were assigned high human donor contribution

245   percentages. Mice transplanted with human donor 82 had an average donor contribution

246   percentage of 10.18% after transplant, while the pre-treatment samples had 11.95% and the

247   control samples had 20.62% (Fig. 7c). SourceTracker also assigned very high donor contribution

248   percentages to the pre-treatment and control samples in the study of double hu-mice

249	transplanted with a mixture of all three human donors. Mice transplanted with the mixture of

250	all three human donors had an average donor contribution percentage of 14.88% after

251	transplant, while the pre-treatment samples had 21.04% and the control samples had 15.89%

252	(Fig. 7d).

253	The high donor contribution percentages of donor 82 to the pre-treatment and control

254	samples originated from an ASV with taxonomic assignment to *Akkermansia muciniphila*. This

255	ASV was highly abundant in both hu-mice and double hu-mice and was much more prevalent in

256	human donor 82 and the mixed donor sample compared to donors 65 or 74. To get a more

257	accurate account of the stability of the human-like gut microbiome in post-transplant samples,

258	we removed this ASV that was resulting in false positive donor contributions and once again

259	used SourceTracker (Supplemental Figure – SourceTracker_82_Mix.pdf). After the removal of

260	the ASV, the double hu-mice engrafted with human donor 82 had an average donor

261	contribution percentage of 12.64% after transplant and double hu-mice engrafted with the

262	mixture of all three human donors had an average donor contribution percentage of 18.11%

263	after transplant, while all pre-treatment control samples were at 0.00%. Using both a

264	percentage of shared ASVs and SourceTracker, we have demonstrated that double hu-mice had

265	a stable human-like gut microbiome for the duration of the study, up to 14.5 weeks post-

266	transplant.

267

268	**Double hu-mice have increased human-like predicted metagenome functional content.**

269	In addition to evaluating microbiome classification, we also sought to assess the

270	functional capacity of the microbiomes in double hu-mice. PICRUSt was used to predict the

13

271   metagenome functional content from the 16S rRNA data after ASV inference [25], and the

272   predicted KO features were graphed using both NMDS and PCoA plots (Fig. 8). Many of the

273   double hu-mice samples clustered closer to the human donor samples than the hu-mice

274   samples (Fig. 8a & 8b). When color-coded by donor and cohort, the microbiomes that clustered

275   closest to the human donor samples belonged to mice from the second cohort of double hu-

276   mice generated by engrafting bacteria from human donor 65 (Fig 8c & 8d). Similarly, several

277   samples from the other double hu-mice cohorts also separated themselves from the hu-mice

278   cluster and were closer to the human donor samples. (Fig. 8c & 8d).

279   We next tested for differences of each predicted KO feature among the three different

280   groups of mice (Supplemental File – KO_Significant_Differences.xlsx). In total, there were 4,513

281   non-zero predicted KO features. Using Kruskal-Wallis testing and an FDR adjusted p-value of <

282   0.05, we found 35.54% (1,604/4,513) significantly different predicted KO features between hu-

283   mice and human donor samples. There were 39.09% (1,764/4,513) significantly different

284   predicted KO features between double hu-mice and hu-mice samples. However, when we

285   compared double hu-mice and the human donor samples, there were only 1.35% (61/4,513)

286   significantly different predicted KO features. To clarify what functional aspects were missing

287   from the double hu-mice gut microbiomes, we determined the predicted ASV contribution for

288   each of the 61 significantly different KO features between the double hu-mice and human

289   donor samples (Supplemental File – KO_Metagenome_Contribution.xlsx). This analysis provided

290   insight into which functionally significant bacteria were not successfully engrafted from the

291   human donor samples to the double hu-mice. A total of 95 ASVs with 73 unique taxonomic

292   assignments were found to contribute to the 61 significantly different KO features

14

293   (Supplemental Figure – KO_Contributions.pdf). Family level taxa with the highest levels of

294   contribution included *Actinomycetaceae* (10.46%), *Bifidobacteriaceae* (8.76%),

295   *Streptococcaceae* (20.74%), *Lachnospiraceae* (23.95%), and *Peptococcaceae* (8.66%).

296   *Bifidobacterium adolescentis* was the highest contributing species (7.85%), and the highest

297   contributing ASV (13.01%) had the taxonomic assignment of an unclassified *Streptococcus*

298   species. Collectively, these analyses demonstrate that the predicted functional capacity of

299   double hu-mice is more similar to human donor samples compared to hu-mice.

300

301   **Discussion**

302   The goal of this study was to investigate the establishment and stability of an engrafted

303   human gut microbiome after antibiotic pre-treatment in hu-mice. We call these mice double

304   hu-mice as they have both a functional human immune system and human bacterial gut

305   microbiomes similar to human donor samples. Our approach created highly reproducible and

306   donor-specific human-like gut microbiomes across multiple cohorts of double hu-mice. Further,

307   we showed that double hu-mice had increased measures of diversity and increased functional

308   capacity compared to hu-mice. The engrafted human-like gut microbiomes were also stable for

309   the duration of the study, up to 14.5 weeks post-transplant. Further, we demonstrated that the

310   predicted functional capacity of double hu-mice is also more similar to the human donor

311   samples than hu-mice.

312   One of the most significant aspects of the study was that the double hu-mice had gut

313   microbiome profiles that were unique to the human donor engrafted. Each of the 4 different

314   donor samples resulted in a distinct population of double hu-mice resembling the human

315 donor. Double hu-mice create a huge opportunity for implementing personalized medicine and

316 translational research. Potential applications for double hu-mice include testing for

317 personalized responses of patient microbiomes to drug regimens, therapies, or dietary

318 interventions. These mice could also be used to identify mechanisms underlying observations

319 from human studies establishing a connection between the gut microbiome and disease.

320 In this study, we created a cohort of double hu-mice by engrafting a mixture of all three

321 human donors to create an 'un-biased' human gut microbiome profile. Further study is needed

322 to determine whether a mixed sample is beneficial in creating an un-biased human-like profile

323 or if mixing samples creates an un-natural or unstable community of gut bacteria after

324 engraftment. It may be advantageous to use a mixture of fecal samples derived from a large

325 population to determine the broad impact of different treatments or diets to the human gut

326 microbiome. This un-biased mix of a large population of donors could be used to add additional

327 information to double hu-mice generated with individual donor profiles.

328 We found that double hu-mice had increased measures of alpha diversity compared to

329 hu-mice. Several studies have highlighted the importance of microbiome diversity within the

330 gut and have linked low gut microbiome diversity with several disease conditions[26, 27]. While

331 not all low diversity conditions are detrimental, specifically when there is enrichment of

332 potentially beneficial bacteria through prebiotic or probiotic treatment, the low pre-existing

333 diversity found within the hu-mice was far below the levels observed in our human donor

334 samples. After engraftment, the double hu-mice had increased levels of alpha diversity and

335 maybe more importantly, had increased predicted functional capacities. This increased diversity

336   may also offer a more realistic gut environment as it allows for diverse reciprocal interactions

337   with the engrafted human immune system.

338   We also found that the engrafted human-like microbiome was very stable in our model

339   for the length of the study, up to 14.5 weeks after transplant. We used several methods to

340   determine the engraftment level and stability of the gut microbiome after transplant and found

341   no reversion to the pre-existing murine profile. This stability allows study of the role of the gut

342   microbiome in many human diseases such as HIV-1 and cancer. One outstanding question is

343   whether the unique presence of human immune cells plays a role in stabilizing or enhancing

344   engraftment of the human-like gut microbiome in our model compared to other non-

345   humanized mouse models. Our data showed no reversion to the pre-existing murine gut

346   microbiome profile, perhaps due to some enhanced stability or selection by the reconstituted

347   human immune system. Further studies are needed to determine the relationship between the

348   engrafted gut microbiome and human immune system.

349   Many different methods and antibiotic regimens have been used for preconditioning of

350   mice prior to fecal transplantation[28-30]. Different combinations and durations of antibiotic

351   treatments may increase the efficiency of the fecal transplant into the host[29]. While the

352   combination of Metronidazole, Ampicililn, Neomycin, and Vancomycin is widely used due to its

353   broad spectrum of bacterial targets, the best methods are still being investigated. We found

354   that the very rigorous method of gavaging antibiotics twice daily for 14 days used by Hintze et

355   al. was too invasive for our NSG hu-mice and resulted in increased mortality[31]. Providing the

356   antibiotics in the drinking water proved to be less stressful with improved health and survival of

357   the mice. Meanwhile, we also found that 14 days was the optimum duration of antibiotic pre-

358   treatment to generate double hu-mice.

359   As expected, there is not a complete reconstitution of the human fecal donor profile in

360   our double hu-mice due to several hypothesized reasons. There are major differences between

361   the human and mouse digestive tract including structure, function, and pH[19]. Our mice are

362   not germ-free to begin with, and the broad-spectrum antibiotic treatment can only reduce the

363   prevalence of pre-existing murine gut bacteria. There were several key differences in the

364   reconstituted mice compared to the human donors. Double hu-mice had significantly lower

365   levels of several types of *Clostridia* including *Lachnospiraceae, Blautia, Coprococcus, Roseburia,*

366   *Faecalibacterium,* and *Ruminococcus* compared to human donor samples. Many of these

367   bacteria are well documented to be difficult to reconstitute within germ-free and SPF mouse

368   models[20, 31, 32]. Similar to fecal transplants in humans designed to treat *C. difficile*

369   infections, the engrafted human-like gut microbiome in our double hu-mice is the result of a

370   combination of host, donor, and environmental bacteria[33]. Despite these previously known

371   limitations, our double hu-mice model reproducibly results in a donor specific, stable, human-

372   like gut microbiome in the presence of a human immune system.

373   Germ-free animals are the gold standard for studying the gut microbiome. Using germ-

374   free mice to study the impact of the gut microbiome has been well-documented[1, 20]. Germ-

375   free animal models may allow for a more complete reconstitution of a human-like gut

376   microbiome following fecal transplant, however these models often do not have human

377   immune system. Studying human immune reconstitution in hu-mice and pathogenesis of

378   human specific diseases in a gnotobiotic environment could reveal important clues about the

379 role of the gut microbiome. Nevertheless, many important mouse strains are not commercially

380 available as germ-free, including NSG mice. Several studies have also shown that gnotobiotic

381 mice may have long-lasting immune deficiencies, even after gut microbiome reconstitution[34-

382 36]. Further, working with germ-free animals requires gnotobiotic facilities and equipment that

383 is expensive and has limited availability. Lundberg et al., and Kennedy et al., nicely review the

384 advantages and disadvantages of using antibiotic-treated versus germ-free rodents for

385 microbiota transplantation studies [37, 38].

386 Our double hu-mice have the advantage of requiring only SPF housing conditions, which

387 are widely available and less expensive compared to germ-free facilities. It also does not

388 perturb the complex surgical procedures in generating BLT hu-mice because there is no need

389 for a completely germ-free environment. Our NSG mice are housed and bred under SPF

390 conditions and the diversity of their murine gut microbiota is low. Their immunodeficiency may

391 contribute to their pre-existing low diversity gut microbiome status before human fecal

392 material transplant. In a study by Zhou et al., NSG and C57BL6/J mice whose native microbiota

393 were depleted by antibiotics followed by FMT had no significant differences in diversity but did

394 observe significant differences in which species colonized[39]. A study done by Ericsson et al.

395 showed that it is easier to transfer high diversity fecal donor samples into low diversity

396 recipients, which could help to explain the success of engraftment and stability in our

397 model[30]. Many questions remain as to the best antibiotic preconditioning regimen, the timing

398 of fecal transplants, the total number of fecal transplants, the route of administration (oral

399 versus rectal), the use of antacids, and preconditioning with osmotic laxatives such as

400 polyethylene glycol, diet, and housing. Methods to optimize murine bacterial depletion along

401 with reconstitution and stability of human specific bacteria are currently being explored.

402

403 **Conclusion**

404 Here, we describe successful and stable transplantation of human fecal microbiomes

405 into immunodeficient NSG mice surgically engrafted with a functional human immune system

406 to create double hu-mice with human donor-specific human gut microbiomes. Double hu-mice

407 will be beneficial to many applications of personalized medicine to test the impact of the

408 human gut microbiome on human health and disease in the presence of a human immune

409 system.

410

411 **Methods**

412 **Generation of humanized BLT mice**

413 All methods described here were conducted as we previously reported in accordance

414 with Institutional Animal Care and Research Committee (IACUC)-approved protocols at the

415 University of Nebraska-Lincoln (UNL)[22, 40-42]. The IACUC at the University of Nebraska-

416 Lincoln (UNL) has approved two protocols related to generating and using humanized BLT (hu-

417 BLT) mice, including Double Hu-Mice. Additionally, the Scientific Research Oversight Committee

418 (SROC) at UNL has also approved the use of human embryonic stem cells and fetal tissues,

419 which are procured from the Advanced Bioscience Resources for humanized mice studies

420 (SROC# 2016—1-002).

421	Briefly, 6- to 8-week-old female NSG mice (NOD.Cg-$Prkdc^{scid}Il2rg^{tm1Wjl}$/SzJ, catalog

422	number 005557; (Jackson Laboratory) were housed and maintained in individual microisolator

423	cages in a rack system capable of managing air exchange with prefilters and HEPA filters. Room

424	temperature, humidity, and pressure were controlled, and air was also filtered. Mice were fed

425	irradiated Teklad global 14% protein rodent chow (Teklad 2914) and were given autoclaved

426	acidified drinking water. The second cohort of double hu-mice engrafted with fecal material

427	from Donor 65 were supplemented with a high calorie gel (DietGel Boost). On the day of

428	surgery, mice received whole-body irradiation at the dose of 12 cGy/gram of body weight with

429	the RS200 X-ray irradiator (RAD Source Technologies, Inc., GA) and were then implanted with

430	one piece of human fetal thymic tissue fragment sandwiched between two pieces of human

431	fetal liver tissue fragments within the murine left renal capsule. Within 6 hours of surgery, mice

432	were injected via the tail vein with 1.5×10^5 to 5×10^5 CD34$^+$ hematopoietic stem cells isolated

433	from human fetal liver tissues. Human fetal liver and thymus tissues were procured from

434	Advanced Bioscience Resources (Alameda, CA). After 9 to 12 weeks, human immune cell

435	reconstitution in peripheral blood was measured by a fluorescence-activated cell sorter (FACS)

436	Aria II flow cytometer (BD Biosciences, San Jose, CA) using antibodies against mCD45-APC,

437	hCD45-FITC, hCD3-PE, hCD19-PE/Cy5, hCD4-Alexa 700, and hCD8-APC-Cy7 (catalog numbers

438	103111, 304006, 300408, 302209, 300526, and 301016, respectively; BioLegend, San Diego,

439	CA). Raw data were analyzed with FlowJo (version 10.0; FlowJo LLC, Ashland, OR). All mice used

440	in this study had high levels of human immune cell reconstitution with an average of 85%

441	hCD45+ cells in peripheral blood 10 weeks post-surgery. The mice were randomly assigned into

442	experimental groups with similar immune reconstitution levels.

443

Antibiotic treatment

445   A broad-spectrum antibiotic cocktail was prepared fresh daily consisting of

446   Metronidazole (1 g/L), Neomycin (1 g/L), Vancomycin (0.5 g/L), and Ampicillin (1 g/L). The

447   antibiotic cocktail was given to the mice ad libitum in the drinking water along with grape

448   flavored Kool-Aid to improve palatability. Control group mice were given only grape flavored

449   Kool-Aid in the drinking water. During antibiotic treatment, cages were changed daily to limit

450   re-inoculation of pre-existing bacteria to the mice due to their coprophagic behavior.

451   Antibiotics were given for 14 days for double hu-mice reconstituted with Donor 65 and Donor

452   Mix and for 7 days in double hu-mice reconstituted with Donor 74 and Donor 82. Mice in the

453   Pilot Study were given antibiotics via oral gavage. Mice were first given three days of anti-fungal

454   Amphotericin B treatment (1 mg/kg) twice daily via oral gavage. Mice were then given the

455   antibiotic cocktail along with Amphotericin B via twice daily via oral gavage. After 4 days of

456   treatment, the Amphotericin B was stopped due to toxicity concerns and after 10 days of

457   treatment oral gavage was reduced to once daily. Post-antibiotic treatment, mice were given

458   autoclaved non-acidified deionized drinking water.

459   During the first few days of antibiotic treatment, the mice lost a considerable amount of

460   body weight (10-20%). The weight loss plateaued at 3-4 days and remained steady for the

461   remainder of antibiotic treatment. Body weight was carefully monitored during this time and If

462   needed, mice were treated with Intraperitoneal (IP) injections of Ringer's solution to mitigate

463   any effects of dehydration. After fecal transplant, the mice began to regain weight and returned

464   to their pre-existing weight within 2 weeks post-transplant. During antibiotic treatment, there

465   was a large reduction in spleen size and a large increase in cecum size compared to controls.

466   This is similar to the morphology observed in germ-free mice, providing further evidence for the

467   efficacy of the antibiotic regimen[35].

468

469   **Donor samples and Fecal transplant**

470   At 24 and 48 hours after the completion of antibiotic pre-treatment, mice were given

471   200 ul of human fecal material via oral gavage. OpenBiome supplied 3 FMT Upper Delivery

472   Microbiota Preparations from 3 different healthy human donors (Donor 65, Donor 74, Donor

473   82). Samples were thawed once before fecal transplant to aliquot the samples within an

474   anaerobic chamber. During this step, an equal portion of each of the samples were mixed

475   together to create an unbiased human donor sample (Donor Mix). 16S rRNA sequencing data

476   on the three donors was also supplied by OpenBiome (Supplementary data).

477

478   **Mouse fecal collection and DNA extraction**

479   Individual mice were placed into autoclaved paper bags within a biosafety hood until

480   fresh fecal samples were produced. Fecal samples were stored in 1.5 ml Eppendorf tubes at -80

481   °C until DNA extraction. DNA was extracted from the fecal samples using the

482   phenol:chloroform:isoamyl alcohol with bead beating method described previously [43]. Briefly,

483   fecal samples were washed three times with 1 ml PBS buffer (pH 7). After the addition of 750 ul

484   of lysis buffer, samples were transferred to tubes containing 300 mg of autoclaved 0.1 mm

485   zirconia/silica beads (Biospec). 85 ul of 10% SDS solution and 40 ul of Proteinase K (15mg/ml,

486   MC500B Promega) were added and samples were incubated for 30 minutes at 60° C. 500 ul of

487   Phenol:Chloroform:Isoamyl alcohol (25:24:1) was added and then samples were vortexed.

488   Samples were then put into a bead beater (Mini-beadbeater 16 Biospec) for 2 minutes to

489   physically lyse the cells. The upper phase of the sample was collected and an additional 500ul of

490   Phenol:Chloroform:Isoamyl alcohol (25:24:1) was added. After samples were vortexed and spun

491   down, the DNA in the upper phase was further purified twice with 500 ul of

492   Phenol:Chloroform:Isoamyl alcohol (25:24:1). and was then precipitated with 100% Ethanol (2.5

493   x volume of sample) and 3M Sodium acetate (.1 x volume of sample) overnight at -20° C.

494   Samples are then centrifuged and dried at room temperature. DNA was resuspended in 100 ul

495   of Tris-Buffer (10mM, pH8) and stored at -20° C. DNA samples were quality checked by

496   nanodrop (ND-1000 Nanodrop) .

497

498   **16S rRNA gene sequencing**

499   16S rRNA gene sequencing was performed at the University of Nebraska Medical Center

500   Genomics Core Facility using xxx (detailed Illumina instrument here). DNA normalization and

501   library prep were performed followed by V3-V4 16S rRNA amplicon gene sequencing using a

502   MiSeqV2 (Illumina) The following primer sequences were used: (Primer sequences: Forward

503   Primer = 5'

504   TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 16S Amplicon PCR

505   Reverse Primer = 5'

506   GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

24

507   Illumina overhangs: Forward overhang: 5'

508   TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locusspecific sequence] Reverse overhang: 5'

509   GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locusspecific sequence]).

510

511   **Generation of the amplicon sequence variant table and data analysis**

512   Illumina-sequenced paired-end fastq files were demultiplexed by sample and barcodes

513   were removed by the sequencing facility. The University of Nebraska Holland Computer Center

514   Crane cluster was used to run the DADA2 v1.8 R package in order to generate an amplicon

515   sequence variant (ASV) table[44]. An example of the script used to generate the ASV table is

516   provided in the supplementary materials. The DADA2 pipeline was performed as follows,

517   sequences were filtered and trimmed during which any remaining primers, adapters, or linkers

518   were also removed. The sequencing error rates were estimated using a random subset of the

519   data. Dereplication of the data combined all identical sequencing reads into unique sequences

520   with a corresponding abundance. The core sample inference algorithm was then applied to the

521   dereplicated data. The forward and reverse reads were then joined to create the full denoised

522   sequences and an initial ASV table was generated. Any sequences outside the expected length

523   for the V3-V4 amplicon were then filtered from the table. Chimeric sequences were then

524   removed and a final ASV table was generated. Taxonomy was assigned using the Greengenes

525   13.8 database and RDP Classifier with a minimal confidence score of 0.80[45, 46].

526

527   **Data analysis**

528   Analysis was performed using R package mctoolsr (https://github.com/leffj/mctoolsr/).

529   and samples were rarified to 13,000 ASVs for downstream analysis. Additional testing of

530   differences between groups was performed using LEfSe[23]. SourceTracker was used to

531   evaluate the stability of the transferred donor microbiome in the double hu-mice[24].

532   GraphPad Prism 5 were used to create some figures. DADA2 generated ASVs were used to

533   predict the functional metagenome capacity using PICRUSt[25] via the following pipeline

534   (https://github.com/vmaffei/dada2_to_picrust).

535

536   **Data availability**

537   The datasets generated during the current study are available in the NCBI SRA repository,

538   [https://www.ncbi.nlm.nih.gov/bioproject/PRJNA507247].

539

540

541

542   <u>**References**</u>

543   1.   Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI: **An obesity-**
544   **associated gut microbiome with increased capacity for energy harvest.** *Nature* 2006,
545   **444**:1027-1031.
546   2.   Gopalakrishnan V, Spencer CN, Nezi L, Reuben A, Andrews MC, Karpinets TV, Prieto PA,
547   Vicente D, Hoffman K, Wei SC, et al: **Gut microbiome modulates response to anti-PD-1**
548   **immunotherapy in melanoma patients.** *Science* 2018, **359**:97-103.
549   3.   Routy B, Le Chatelier E, Derosa L, Duong CPM, Alou MT, Daillere R, Fluckiger A,
550   Messaoudene M, Rauber C, Roberti MP, et al: **Gut microbiome influences efficacy of**
551   **PD-1-based immunotherapy against epithelial tumors.** *Science* 2018, **359**:91-+.
552   4.   Clemente JC, Manasson J, Scher JU: **The role of the gut microbiome in systemic**
553   **inflammatory disease.** *Bmj-British Medical Journal* 2018, **360**.
554   5.   Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez
555   F, Yamada T, et al: **A human gut microbial gene catalogue established by metagenomic**
556   **sequencing.** *Nature* 2010, **464**:59-65.
557   6.   Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI: **Human nutrition, the gut**
558   **microbiome and the immune system.** *Nature* 2011, **474**:327-336.

559   7.   Hooper LV, Littman DR, Macpherson AJ: **Interactions Between the Microbiota and the**
560   **Immune System.** *Science* 2012, **336:**1268-1273.
561   8.   Maynard CL, Elson CO, Hatton RD, Weaver CT: **Reciprocal interactions of the intestinal**
562   **microbiota and immune system.** *Nature* 2012, **489:**231-241.
563   9.   Simpson-Abelson MR, Sonnenberg GF, Takita H, Yokota SJ, Conway TF, Kelleher RJ,
564   Shultz LD, Barcos M, Bankert RB: **Long-term engraftment and expansion of tumor-**
565   **derived memory T cells following the implantation of non-disrupted pieces of human**
566   **lung tumor into NOD-scid IL2R gamma(null) mice.** *Journal of Immunology* 2008,
567   **180:**7009-7018.
568   10.   Bankert RB, Balu-Iyer SV, Odunsi K, Shultz LD, Kelleher RJ, Barnas JL, Simpson-Abelson
569   M, Parsons R, Yokota SJ: **Humanized Mouse Model of Ovarian Cancer Recapitulates**
570   **Patient Solid Tumor Progression, Ascites Formation, and Metastasis.** *Plos One* 2011, **6.**
571   11.   Vudattu NK, Waldron-Lynch F, Truman LA, Deng SY, Preston-Hurlburt P, Torres R,
572   Raycroft MT, Mamula MJ, Herold KC: **Humanized Mice as a Model for Aberrant**
573   **Responses in Human T Cell Immunotherapy.** *Journal of Immunology* 2014, **193:**587-596.
574   12.   Whitfield-Larry F, Young EF, Talmage G, Fudge E, Azam A, Patel S, Largay J, Byrd W, Buse
575   J, Calikoglu AS, et al: **HLA-A2 Matched Peripheral Blood Mononuclear Cells From Type 1**
576   **Diabetic Patients, but Not Nondiabetic Donors, Transfer Insulitis to NOD-scid/gamma**
577   **c(null)/HLA-A2 Transgenic Mice Concurrent With the Expansion of Islet-Specific CD8(+)**
578   **T cells.** *Diabetes* 2011, **60:**1726-1733.
579   13.   Yi GH, Xu XQ, Abraham S, Petersen S, Guo H, Ortega N, Shankar P, Manjunath N: **A DNA**
580   **Vaccine Protects Human Immune Cells against Zika Virus Infection in Humanized Mice.**
581   *Ebiomedicine* 2017, **25:**87-94.
582   14.   Stary G, Olive A, Radovic-Moreno AF, Gondek D, Alvarez D, Basto PA, Perro M, Vrbanac
583   VD, Tager AM, Shi JJ, et al: **A mucosal vaccine against Chlamydia trachomatis generates**
584   **two waves of protective memory T cells.** *Science* 2015, **348.**
585   15.   Sun ZF, Denton PW, Estes JD, Othieno FA, Wei BL, Wege AK, Melkus MW, Padgett-
586   Thomas A, Zupancic M, Haase AT, Garcia JV: **Intrarectal transmission, systemic**
587   **infection, and CD4(+) T cell depletion in humanized mice infected with HIV-1.** *Journal*
588   *of Experimental Medicine* 2007, **204:**705-714.
589   16.   Wang LX, Kang GB, Kumar P, Lu WX, Li Y, Zhou Y, Li QS, Wood C: **Humanized-BLT mouse**
590   **model of Kaposi's sarcoma-associated herpesvirus infection.** *Proceedings of the*
591   *National Academy of Sciences of the United States of America* 2014, **111:**3146-3151.
592   17.   Ernst W: **Humanized mice in infectious diseases.** *Comparative Immunology*
593   *Microbiology and Infectious Diseases* 2016, **49:**29-38.
594   18.   Xiao L, Feng Q, Liang SS, Sonne SB, Xia ZK, Qiu XM, Li XP, Long H, Zhang JF, Zhang DY, et
595   al: **A catalog of the mouse gut metagenome.** *Nature Biotechnology* 2015, **33:**1103-+.
596   19.   Nguyen TLA, Vieira-Silva S, Liston A, Raes J: **How informative is the mouse for human**
597   **gut microbiota research?** *Disease Models & Mechanisms* 2015, **8:**1-16.
598   20.   Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI: **The Effect of Diet on the**
599   **Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice.**
600   *Science Translational Medicine* 2009, **1.**
601   21.   Hazenberg MP, Bakker M, Verschoor-Burggraaf A: **Effects of the human intestinal flora**
602   **on germ-free mice.** *J Appl Bacteriol* 1981, **50:**95-106.

603 22.   Daharsh L ZJ, Ramer-Tait A, Li Q: **A Double Humanized BLT-mice Model Featuring a**
604   **Stable Human-Like Gut Microbiome and Human Immune System.** *Jove-Journal of*
605   *Visualized Experiments* 2019.
606 23.   Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C:
607   **Metagenomic biomarker discovery and explanation.** *Genome Biol* 2011, **12**:R60.
608 24.   Knights D, Kuczynski J, Charlson ES, Zaneveld J, Mozer MC, Collman RG, Bushman FD,
609   Knight R, Kelley ST: **Bayesian community-wide culture-independent microbial source**
610   **tracking.** *Nat Methods* 2011, **8**:761-763.
611 25.   Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC,
612   Burkepile DE, Vega Thurber RL, Knight R, et al: **Predictive functional profiling of**
613   **microbial communities using 16S rRNA marker gene sequences.** *Nat Biotechnol* 2013,
614   **31**:814-821.
615 26.   Willing BP, Dicksved J, Halfvarson J, Andersson AF, Lucio M, Zheng Z, Jarnerot G, Tysk C,
616   Jansson JK, Engstrand L: **A Pyrosequencing Study in Twins Shows That Gastrointestinal**
617   **Microbial Profiles Vary With Inflammatory Bowel Disease Phenotypes.**
618   *Gastroenterology* 2010, **139**:1844-U1105.
619 27.   Chang JY, Antonopoulos DA, Kalra A, Tonelli A, Khalife WT, Schmidt TM, Young VB:
620   **Decreased diversity of the fecal microbiome in recurrent Clostridium difficile-**
621   **associated diarrhea.** *Journal of Infectious Diseases* 2008, **197**:435-438.
622 28.   Reikvam DH, Erofeev A, Sandvik A, Grcic V, Jahnsen FL, Gaustad P, McCoy KD,
623   Macpherson AJ, Meza-Zepeda LA, Johansen FE: **Depletion of murine intestinal**
624   **microbiota: effects on gut mucosa and epithelial gene expression.** *PLoS One* 2011,
625   **6**:e17996.
626 29.   Staley C, Kaiser T, Beura LK, Hamilton MJ, Weingarden AR, Bobr A, Kang J, Masopust D,
627   Sadowsky MJ, Khoruts A: **Stable engraftment of human microbiota into mice with a**
628   **single oral gavage following antibiotic conditioning.** *Microbiome* 2017, **5**.
629 30.   Ericsson AC, Personett AR, Turner G, Dorfmeyer RA, Franklin CL: **Variable Colonization**
630   **after Reciprocal Fecal Microbiota Transfer between Mice with Low and High Richness**
631   **Microbiota.** *Frontiers in Microbiology* 2017, **8**:1-13.
632 31.   Hintze KJ, Cox JE, Rompato G, Benninghoff AD, Ward RE, Broadbent J, Lefevre M: **Broad**
633   **scope method for creating humanized animal models for animal health and disease**
634   **research through antibiotic treatment and human fecal transfer.** *Gut Microbes* 2014,
635   **5**:183-191.
636 32.   Wos-Oxley M, Bleich A, Oxley AP, Kahl S, Janus LM, Smoczek A, Nahrstedt H, Pils MC,
637   Taudien S, Platzer M, et al: **Comparative evaluation of establishing a human gut**
638   **microbial community within rodent models.** *Gut Microbes* 2012, **3**:234-249.
639 33.   Smillie CS, Smith MB, Friedman J, Cordero OX, David LA, Alm EJ: **Ecology drives a global**
640   **network of gene exchange connecting the human microbiome.** *Nature* 2011, **480**:241-
641   244.
642 34.   Chung HC, Pamp SJ, Hill JA, Surana NK, Edelman SM, Troy EB, Reading NC, Villablanca EJ,
643   Wang S, Mora JR, et al: **Gut Immune Maturation Depends on Colonization with a Host-**
644   **Specific Microbiota.** *Cell* 2012, **149**:1578-1593.

645 35.   Smith K, McCoy KD, Macpherson AJ: **Use of axenic animals in studying the adaptation**
646   **of mammals to their commensal intestinal microbiota.** *Seminars in Immunology* 2007,
647   **19:**59-69.
648 36.   Hansen CHF, Nielsen DS, Kverka M, Zakostelska Z, Klimesova K, Hudcovic T, Tlaskalova-
649   Hogenova H, Hansen AK: **Patterns of Early Gut Colonization Shape Future Immune**
650   **Responses of the Host.** *Plos One* 2012, **7**.
651 37.   Lundberg R, Toft MF, August B, Hansen AK, Hansen CHF: **Antibiotic-treated versus**
652   **germ-free rodents for microbiota transplantation studies.** *Gut Microbes* 2016, **7:**68-74.
653 38.   Kennedy EA, King KY, Baldridge MT: **Mouse Microbiota Models: Comparing Germ-Free**
654   **Mice and Antibiotics Treatment as Tools for Modifying Gut Bacteria.** *Frontiers in*
655   *Physiology* 2018, **9**.
656 39.   Zhou W, Chow KH, Fleming E, Oh J: **Selective colonization ability of human fecal**
657   **microbes in different mouse gut environments.** *ISME J* 2018.
658 40.   Li QS, Tso FY, Kang GB, Lu WX, Li Y, Fan WJ, Yuan Z, Destache CJ, Wood C: **Early Initiation**
659   **of Antiretroviral Therapy Can Functionally Control Productive HIV-1 Infection in**
660   **Humanized-BLT Mice.** *Jaids-Journal of Acquired Immune Deficiency Syndromes* 2015,
661   **69:**519-527.
662 41.   Destache CJ, Mandal S, Yuan Z, Kang G, Date AA, Lu W, Shibata A, Pham R, Bruck P,
663   Rezich M, et al: **Topical Tenofovir Disoproxil Fumarate Nanoparticles Prevent HIV-1**
664   **Vaginal Transmission in a Humanized Mouse Model.** *Antimicrob Agents Chemother*
665   2016, **60:**3633-3639.
666 42.   Yuan Z, Kang G, Ma F, Lu W, Fan W, Fennessey CM, Keele BF, Li Q: **Recapitulating Cross-**
667   **Species Transmission of Simian Immunodeficiency Virus SIVcpz to Humans by Using**
668   **Humanized BLT Mice.** *J Virol* 2016, **90:**7728-7739.
669 43.   Martinez I, Wallace G, Zhang C, Legge R, Benson AK, Carr TP, Moriyama EN, Walter J:
670   **Diet-induced metabolic improvements in a hamster model of hypercholesterolemia**
671   **are strongly linked to alterations of the gut microbiota.** *Appl Environ Microbiol* 2009,
672   **75:**4175-4184.
673 44.   Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP: **DADA2: High-**
674   **resolution sample inference from Illumina amplicon data.** *Nat Methods* 2016, **13:**581-
675   583.
676 45.   Wang Q, Garrity GM, Tiedje JM, Cole JR: **Naive Bayesian classifier for rapid assignment**
677   **of rRNA sequences into the new bacterial taxonomy.** *Applied and Environmental*
678   *Microbiology* 2007, **73:**5261-5267.
679 46.   Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N,
680   Pena AG, Goodrich JK, Gordon JI, et al: **QIIME allows analysis of high-throughput**
681   **community sequencing data.** *Nature Methods* 2010, **7:**335-336.
682

683

684 <u>**Acknowledgements**</u>

697

698   Author contributions

699   LD and QL designed the experiments and wrote the manuscript. LD performed experiments and

700   analyzed the data. ART provided input on experimental design and manuscript preparation.

701

702   Competing interests

703   The author(s) declare no competing interests.

704

705   Figure Legends

706   **Figure 1. Gut microbiomes of double hu-mice are distinct and more human-like compared to**

707   **hu-mice and feature donor specific profiles.** A) Non-metric multidimensional scaling (NMDS)

708   plot displaying double hu-mice as a distinct cluster between the human donor samples and pre-

709   treatment or untreated control hu-mice. B) Principal coordinate analysis (PCoA) plot showing

710   the double hu-mice cluster with the human donor samples distinct from the pre-treatment or

711   untreated control hu-mice. C) NMDS plot displaying human donor specific profiles in the double

712   hu-mice. D) PCoA plot showing human donor specific profiles in the double hu-mice.

713

714   **Figure 2. The gut microbiomes of double hu-mice cluster with that of human donor fecal**

715   **samples.** Dendrogram based on Bray-Curtis distances for the gut microbiome profiles of pre-

716   treatment and untreated control hu-mice (Pre-treatment or Control Hu-Mice), double hu-mice

717   (Double Hu-Mice), antibiotic treated hu-mice (Antibiotic Treated Hu-mice), and human donor

718   fecal samples (Human Donor).

719

720   **Figure 3. The gut microbiomes of double hu-mice have increased alpha diversity measures**

721   **compared to that of pre-treatment or untreated control hu-mice.** A) Species richness, B)

722   Shannon index, and C) Simpson index. Data are shown for pre-treatment or untreated control

723   hu-mice (Hu-Mice), antibiotic treated mice (Antibiotics), double hu-mice (Double Hu-Mice), and

724   human donor fecal samples (Human Donor).

725

726   **Figure 4. Comparison of relative abundance of taxa grouped by Family**. The 11 most abundant

727   taxa by relative percent abundance grouped by Family are shown for pre-treatment and

728　　untreated control hu-mice (Hu-mice), double hu-mice (Double), and human fecal donor

729　　samples (Donor).

730

731　　**Figure 5. Engraftment of human fecal donor bacteria in double hu-mice as shown by LEfSe.** A)

732　　All significant features with a linear discriminant analysis (LDA) score > 4.0 between human

733　　fecal donor samples (Donor) and double hu-mice (Double). B) All significant features with an

734　　LDA score > 4.0 between double hu-mice (Double) and pre-treatment or untreated control hu-

735　　mice (Hu-Mice).

736

737　　**Figure 6. Engraftment and stability of a human-like gut microbiome in double hu-mice as**

738　　**determined by shared amplicon sequence variants (ASVs).** A) Proportion of shared ASVs with

739　　the donor in the first cohort of double hu-mice created using fecal material from human donor

740　　65 (Donor 65). B) Proportion of shared ASVs with donor in the second cohort of double hu-mice

741　　created using fecal material from human donor 65 (Donor 65). C) Proportion of shared ASVs

742　　with donor in in double hu-mice created using fecal material from human donor 74 (Donor 74)

743　　or 82 (Donor 82). D) Proportion of shared ASVs with donor in double hu-mice created using a

744　　mixture of fecal material from all three human donors (Donor Mix). X-axis numbers represent

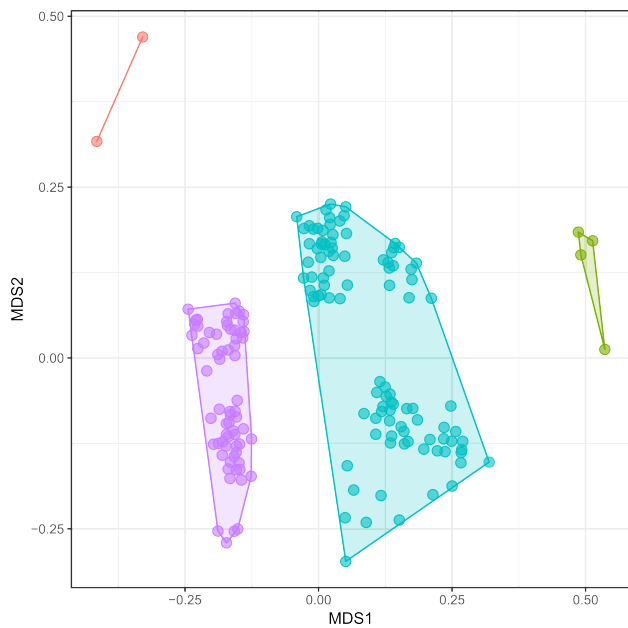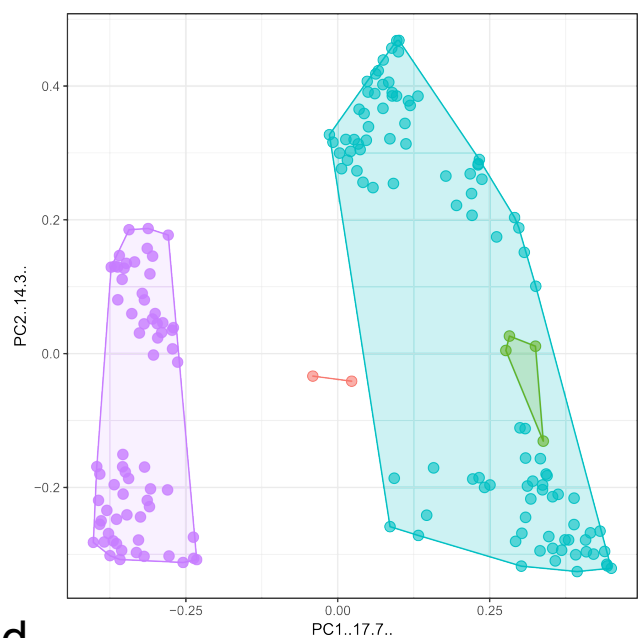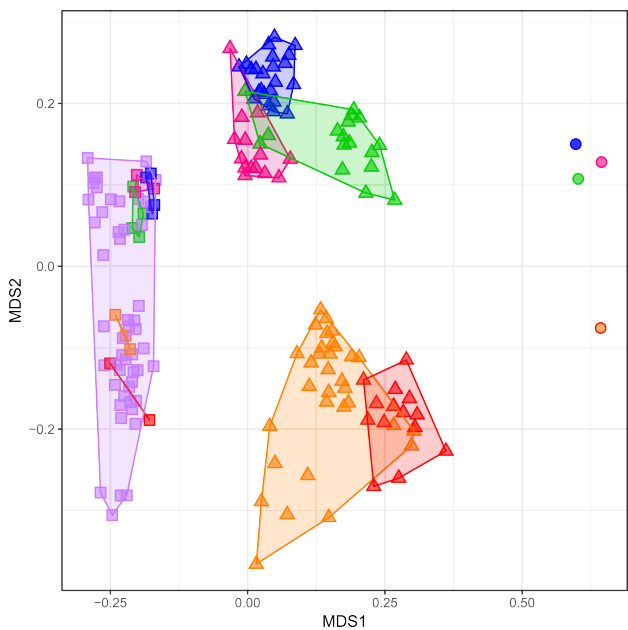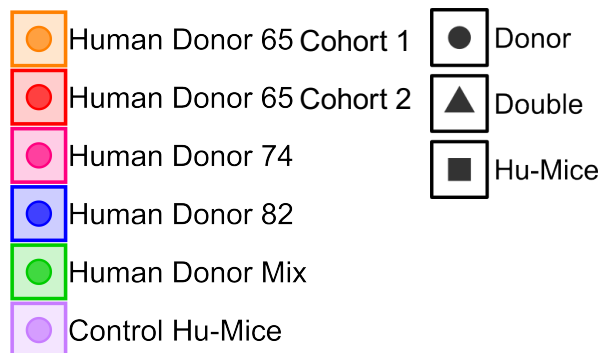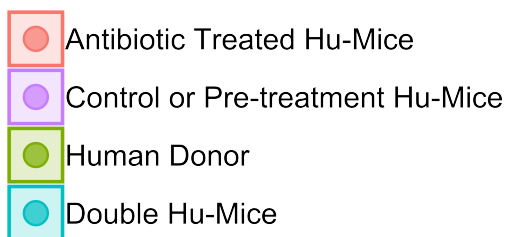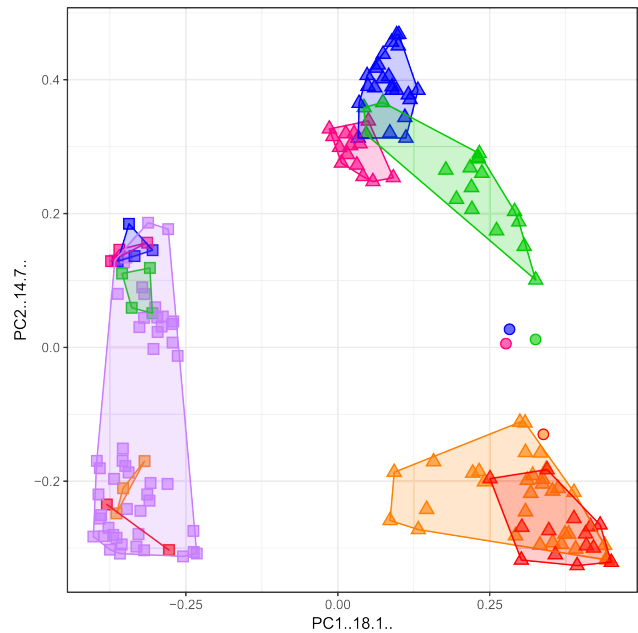745　　number of weeks post fecal transplant.

746

747　　**Figure 7. Stability of the engrafted human-like gut microbiome in double hu-mice as**
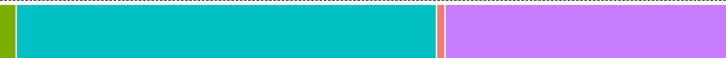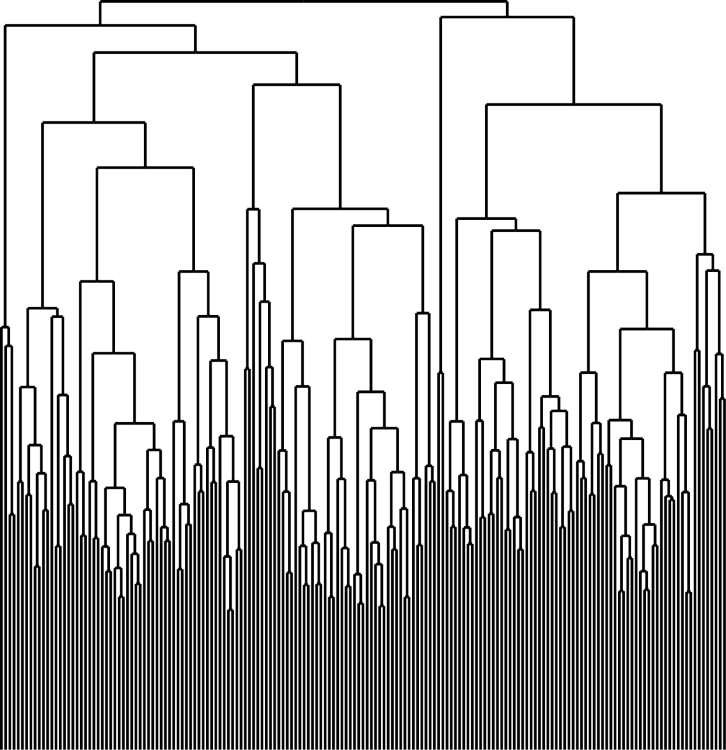
748　　**determined by SourceTracker.** A) Contributions of the human fecal donor sample and pre-

749　　treatment sample in the first cohort of double hu-mice created using fecal material from

750   human donor 65 (Donor 65). B) Contributions of the human fecal donor sample and pre-

751   treatment sample in the second cohort of double hu-mice created using fecal material from

752   human donor 65 (Donor 65). C) Contributions of the human fecal donor sample and pre-

753   treatment sample in double hu-mice created using fecal material from human donor 74 (Donor

754   74) or 82 (Donor 82). D) Contributions of the human fecal donor sample and pre-treatment

755   sample in double hu-mice created using a mixture of fecal material from all three human donor

756   (Donor Mix). X-axis numbers represent number of weeks post fecal transplant.

757

758   **Figure 8. Double hu-mice have increased human-like predicted metagenome functional**

759   **content.** A) NMDS plot displaying the double hu-mice as a distinct cluster between the human

760   fecal donor samples and pre-treatment or untreated control hu-mice. B) NMDS plot displaying

761   human donor specific functional profiles in double hu-mice. C) PCoA plot displaying the double

762   hu-mice as a distinct cluster between the human fecal donor samples and pre-treatment or

763   untreated control hu-mice. D) PCoA plot displaying human donor specific functional profiles in

764   double hu-mice.

a

b

c

d

Antibiotic Treated Hu-Mice

Control or Pre-treatment Hu-Mice

Human Donor

Double Hu-Mice

Human Donor 65 Cohort 1

Human Donor 65 Cohort 2

Human Donor 74

Human Donor 82

Human Donor Mix

Control Hu-Mice

Donor

Double

Hu-Mice

Human Donor

Double Hu-Mice

Antibiotic Treated Hu-Mice

Pre-treatment or Control Hu-Mice

a

Donor   Double

k__Bacteria.p__Firmicutes.c__Bacilli
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Lactobacillaceae
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Lactobacillaceae.g__Lactobacillus
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Lactobacillaceae.g__Lactobacillus.s__
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales
k__Bacteria.p__Verrucomicrobia.c__Verrucomicrobiae.o__Verrucomicrobiales.f__Verrucomicrobiaceae
k__Bacteria.p__Verrucomicrobia.c__Verrucomicrobiae.o__Verrucomicrobiales.f__Verrucomicrobiaceae.g__Akkermansia
k__Bacteria.p__Verrucomicrobia
k__Bacteria.p__Verrucomicrobia.c__Verrucomicrobiae
k__Bacteria.p__Verrucomicrobia.c__Verrucomicrobiae.o__Verrucomicrobiales
k__Bacteria.p__Verrucomicrobia.c__Verrucomicrobiae.o__Verrucomicrobiales.f__Verrucomicrobiaceae.g__Akkermansia.s__muciniphila

k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Ruminococcus_
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Coprococcus.s__
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Roseburia.s__
k__Bacteria.p__Firmicutes.c__Erysipelotrichi.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Eubacterium_.s__biforme
k__Bacteria.p__Actinobacteria.c__Coriobacteriia.o__Coriobacteriales.f__Coriobacteriaceae.g__Collinsella
k__Bacteria.p__Actinobacteria.c__Coriobacteriia.o__Coriobacteriales.f__Coriobacteriaceae.g__Collinsella.s__aerofaciens
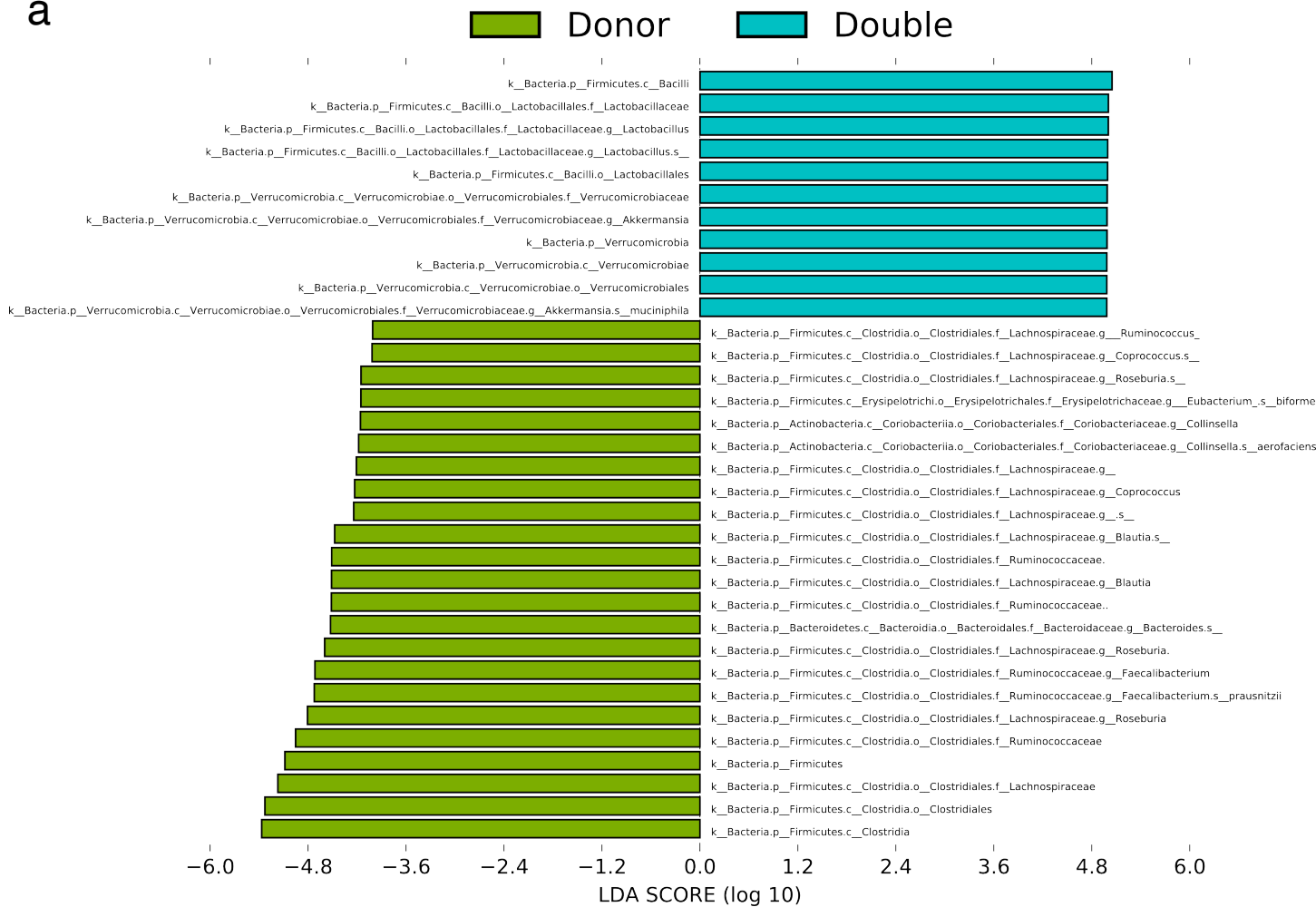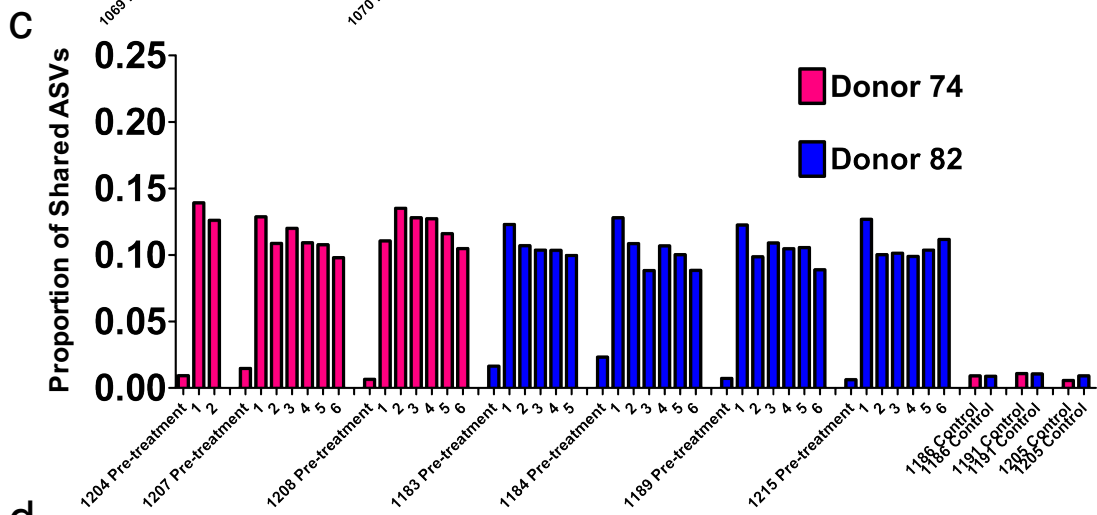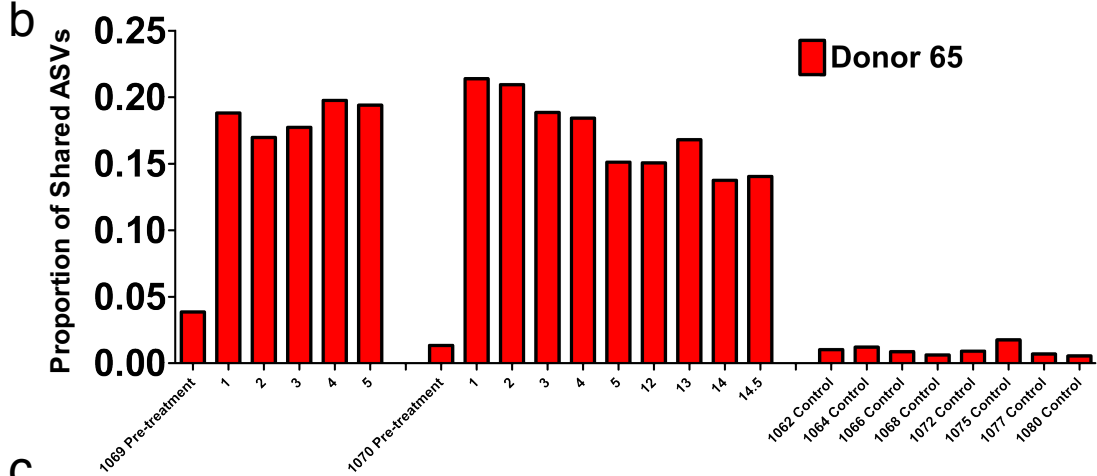k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Coprococcus
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__.s__
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Blautia.s__
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Blautia
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae..
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.s__
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Roseburia.
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__Faecalibacterium
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__Faecalibacterium.s__prausnitzii
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Roseburia
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae
k__Bacteria.p__Firmicutes
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales
k__Bacteria.p__Firmicutes.c__Clostridia

-6.0   -4.8   -3.6   -2.4   -1.2   0.0   1.2   2.4   3.6   4.8   6.0
LDA SCORE (log 10)

b

Double   Hu-Mice

k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__S24_7
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__S24_7.g__.s__
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__S24_7.g__
k__Bacteria.p__Verrucomicrobia.c__Verrucomicrobiae
k__Bacteria.p__Verrucomicrobia.c__Verrucomicrobiae.o__Verrucomicrobiales.f__Verrucomicrobiaceae
k__Bacteria.p__Verrucomicrobia.c__Verrucomicrobiae.o__Verrucomicrobiales
k__Bacteria.p__Verrucomicrobia
k__Bacteria.p__Verrucomicrobia.c__Verrucomicrobiae.o__Verrucomicrobiales.f__Verrucomicrobiaceae.g__Akkermansia.s__muciniphila
k__Bacteria.p__Verrucomicrobia.c__Verrucomicrobiae.o__Verrucomicrobiales.f__Verrucomicrobiaceae.g__Akkermansia

k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Porphyromonadaceae.g__Parabacteroides
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Porphyromonadaceae
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.s__uniformis
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.s__
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides

-6.0   -4.8   -3.6   -2.4   -1.2   0.0   1.2   2.4   3.6   4.8   6.0
LDA SCORE (log 10)

a

b

c

d

Antibiotic Treated Hu-Mice

Control or Pre-treatment Hu-Mice

Human Donor

Double Hu-Mice

Human Donor 65 Cohort 1

Human Donor 65 Cohort 2

Human Donor 74

Human Donor 82

Human Donor Mix

Control Hu-Mice

Donor

Double

Hu-Mice