Gut Microbial Metabolites Induce Donor Specific Tolerance of Kidney Allografts through SCFA Induction of T Regulatory Cells

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Methods:

Kidney Transplantation:

Heterotopic kidney transplants were performed with the left kidney of the donor animal flushed with heparinized saline and removed together with the ureter and vessels en mass, including a small (1-2mm) bladder cuff attached to the distal ureter. The recipient animal underwent a left sided nephrectomy and the transplanted kidney was placed heterotopically in the left iliac fossa on day zero. Urinary tract reconstruction was established by either inserting the ureter into the bladder (day 14 experiment only) or by suturing the bladder patch to a cystotomy located on the bladder dome (a bladder-to-bladder anastomosis) for survival study and day 100 experiments. All mice received induction and maintenance anesthesia with inhaled isoflurane and were monitored throughout procedures. All recipient allograft mice received a single, intra-peritoneal injection of ampicillin at the time of transplant surgery with the exception of mice on diet experiments or microbiota analysis, which did not receive any antibiotics. No immunosuppressive therapy was administered. The recipient's right native kidney was removed at day 3-7, rendering the graft to be life-sustaining. Animals with technical graft failure or wound infection became overtly ill (and were euthanized) or died within 4 days of the contralateral nephrectomy and were removed from the study.

Histology

Periodic acid-Schiff (PAS) staining was performed on 3 μm paraffin embedded kidney sections to assess tubulitis (day 14 group only), glomerulosclerosis and interstitial fibrosis. Picro-Sirius red (PSR) staining was performed on 5 μm paraffin embedded sections of the kidney (D100 group only) to assess for interstitial collagen deposition. Scoring systems for each histological parameter have been previously described in detail, we summarize them briefly below. All histological analysis was performed in a blinded manner.

Tubulitis was examined on 250 tubular cross-sections per animal. Each tubular cross section was assessed as either, i) normal, ii) mild tubulitis (one infiltrating mononuclear cell per tubular cross-section), iii) moderate

tubulitis (two or three infiltrating mononuclear cells per tubular cross-section and disruption of the basement membrane), or iv) severe tubulitis (defined as ≥ four infiltrating mononuclear cells per tubular cross-section). A score for the degree of tubulitis was calculated for each animal, whereby each normal tubule received a score of 0, with mild tubulitis assigned a value of 1, and the number of tubules affected with mild and severe tubulitis was multiplied by 2 or 3 respectively. The total tubulitis score for each animal was the sum of these figures.

Glomerulosclerosis was quantitated by the presence of PAS-positive staining material involving >30% of each glomerulus. All glomeruli per section were scored to determine the percentage of glomeruli displaying glomerulosclerosis.

Interstitial fibrosis and tubular atrophy was graded following the Banff 97 scoring criteria on a scale of 0 to 3: 1 = mild interstitial fibrosis and tubular atrophy (<25% of cortical area); 2 = moderate interstitial fibrosis and tubular atrophy (26–50% of cortical area); 3 = severe interstitial fibrosis and tubular atrophy/loss (>50% of cortical area). If changes were minimal but not absent, the score of 0.5 was applied. Using an ocular grid, the score of each sample was counted in at least 15-25 consecutive fields across a full section (x 400 magnification) and was averaged for each graft.

Interstitial PSR staining for collagen was assessed by point counting using an ocular grid in at least 15 consecutive fields (x 400 magnification). Only interstitial collagen was counted, with collagen surrounding vessels and glomeruli excluded. The result was expressed as the number of interstitial grid points positive over the total number of interstitial grid points assessed per field.

Immunohistochemistry staining

Acetone-fixed frozen sections (7 μm) were exposed to 0.06% H_2O_2 in PBS for 10 minutes, and subsequently blocked with an avidin-biotin blocking system (DAKO North America Inc. Ca., USA.) followed by 20% normal horse serum in PBS. Primary antibody consisting of rat anti-mouse CD68 antibody (clone FA-11, AbD Serotec MCA1957), CD4 (clone RM4-5, BD Pharmingen 550280), CD8 (clone 53-6.7, BD Pharmingen 550281), FoxP3 (clone FJK-16s eBioscience 14-5773-82), or hamster anti-mouse CD11c (clone HL3, BD Pharmingen 550283) was applied to the sections for 60 min. Concentration-matched IgG was used as an isotype negative control. Sections were incubated with the appropriate biotinylated secondary antibody: anti-

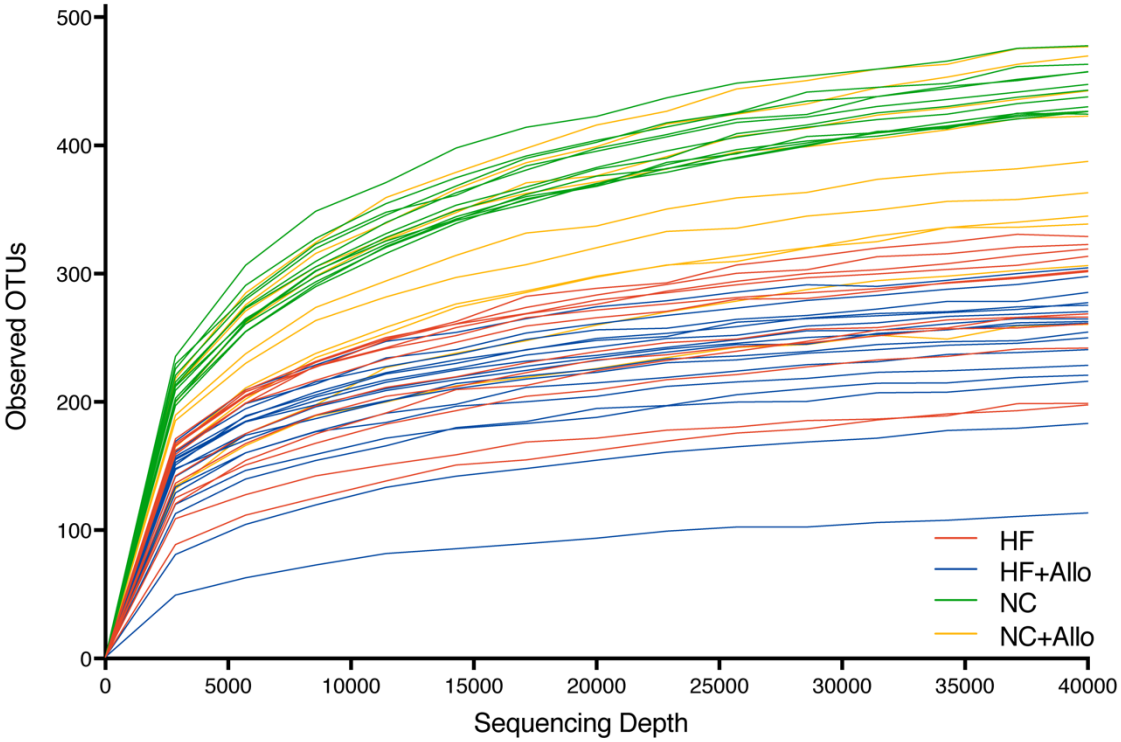
rat IgG or anti-hamster IgG (BD Pharminogen). Vector stain ABC kit (Vector Laboratories Inc.) was applied to the tissue followed by 3,3'diaminobenzidine (DAB) substrate-chromogen solution (DAKO North America Corporation Inc. CA., USA.) Slides were counterstained with Harris' haematoxylin.

Quantification of immunohistochemistry

Analysis of the cellular infiltrates for CD4, CD8 and Foxp3 was performed in a blinded manner, by assessing 20 consecutive high-power fields (HPFs, x 400 magnification) of the cortex in each section. Using an ocular grid, the number of cells staining positively for each antibody was counted and expressed as cells per HPF. Analysis of CD68 and CD11c infiltrates was performed using a digital image analysis program (Image-Pro Premier 9.0, Media Cybernetics). An area of cortex was analyzed for interstitial cellular positive staining versus counter-stained area. The results were expressed as percentage of positive staining per HPF.

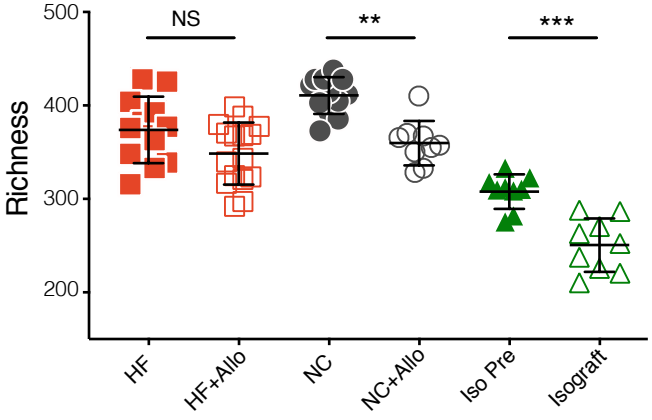
Immunofluorescence

For C4d immunofluorescent staining, frozen sections were blocked with 1% BSA in PBS for 20 minutes and incubated with rat anti-mouse antibodies to C4d (Abcam plc, Cambridge, UK) for 60 min followed by anti-rat IgG conjugated with AlexaFluor 488 (Molecular Probes, Eugene, OR). Staining for C4d was considered positive when the peritubular capillaries were diffusely (all high-power fields) and brightly stained. Scoring of C4d staining was based on the percentage of stained tissue on immunofluorescence that had a linear, circumferential staining pattern in PTCs following the Banff 97 scoring criteria on a scale of 0 to 3: 0 = Negative: 0%; 1 = Minimal C4d stain/detection: 1<10%; 2 = Focal C4d stain/positive: 10–50%; 3 = Diffuse C4d stain/positive: >50%.

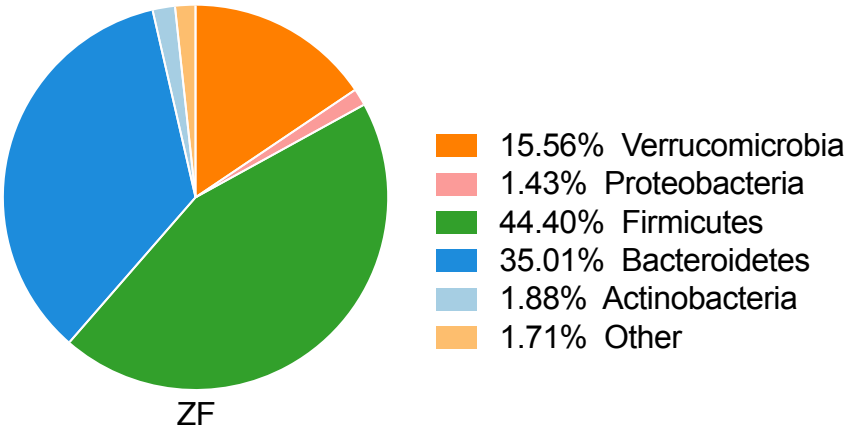


Supplemental Figure 1. Multiple sample rarefaction curve based on 16S rRNA gene sequencing.

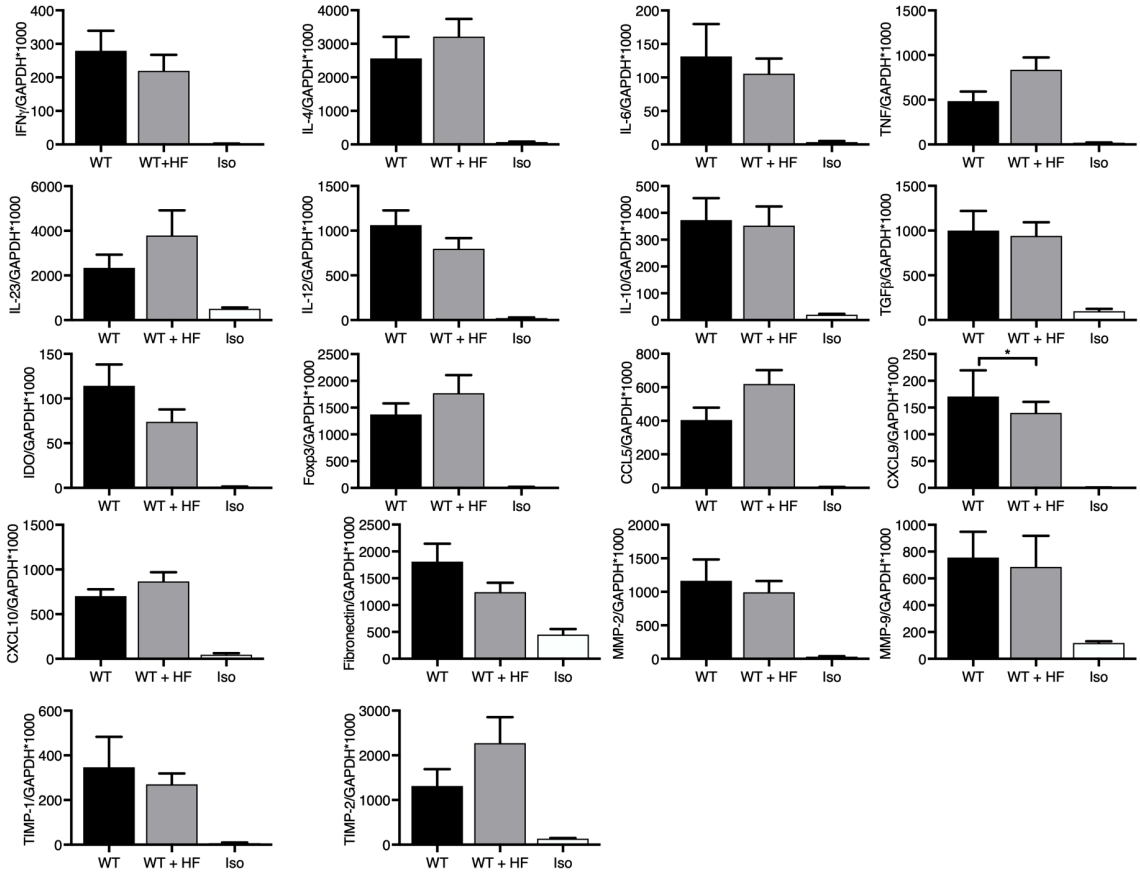
HF n=12; NC n=12; NC+Allo n=10; HF+Allo n=16



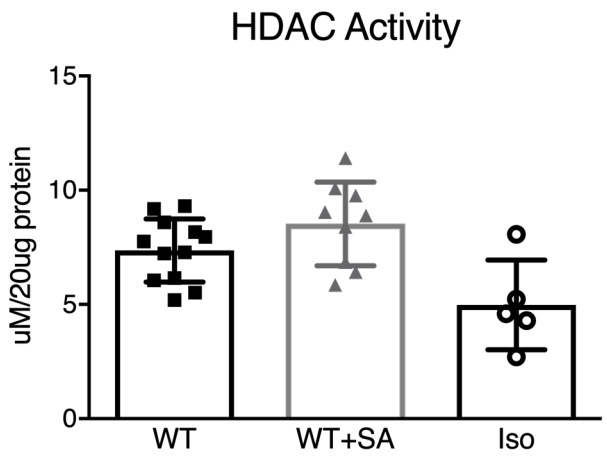
Supplemental Figure 2. Richness of gut microbial communities in allograft and isograft mice.



Supplemental Figure 3. Dominant phylum of WT C57BL/6 mice fed a zero-fiber diet. WT mice fed a fiber restricted diet develop dysbiosis with expansion of the pylum Verrucomicrobia. (n=10)



Supplemental Figure 4. Cytokine and Chemokine mRNA expression in WT and WT+HF allografts, and WT isografts at day 100 post-transplant. Similar to WT-allografts, HF fed allograft mice demonstrated a marked upregulation of cytokines, chemokines, and genes involved in tissue remodeling as compared to isografts. WT+HF mice demonstrated a decrease in the expression of chemokine CXCL9 as compared to WT allograft mice ($P<0.05$). WT n=9, WT+HF n=9, isografts n=5. P values by one-way ANOVA. *$P<0.05$



Supplemental Figure 5. HDAC activity in transplanted kidneys was not upregulated by SA supplementation . Compared to WT allograft mice, WT+SA allograft mice did not demonstrate a significant change in HDAC activity (P=0.2731). WT n=12, WT+SA n=9, Iso n=5. P values by one-way ANOVA.

Nutritional Parameter	Normal Chow	High-Fiber
Protein (%)	19	13.2
Total Fat (%)	4.6	4.5
Crude Fiber (%)	5.2	35.0
Acid Detergent Fiber (%)	-	35.0
Digestible Energy (MJ/kg)	14.2	11.0
Total Calculated Energy from Carbohydrate (%)	59.9	58.7
Total Calculated Energy from Protein (%)	23	19.7
Total Calculated Energy from Lipids (%)	12	15.0

Supplemental Table 1. Nutritional parameters of high-fiber and normal mouse chow used in experiments.

Deseq2: Significant OTUs Pre-Isograft vs Isograft Mice

	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Rank2	Rank3	Rank4	Rank5	Rank6
37d7	269.7341	-1.4682	0.33194	-4.42308	9.73E-06	0.00035	p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Porphyromonadaceae	g__Parabacteroides

Supplemental Table 2. DESeq2 analysis demonstrating differential abundance of significant OTUs at the genus level (FDR adjusted p value < 0.01) between isograft recipients, pre and 2 weeks following isograft-placement.

Deseq2: Significant OTUs NC v HF

	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Rank2	Rank3	Rank4	Rank5	Rank6
180107	337.997638	1.25712714	0.41221822	3.04966421	0.00229097	0.00646863	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Ruminococcaceae	g__Ruminococcus
323024	37.5128584	6.44311967	1.27498867	5.0534721	4.34E-07	2.97E-06	p__Tenericutes	c__Mollicutes	o__RF39	f__	g__
263705	8.84691163	4.82531286	0.83718449	5.76373896	8.23E-09	9.87E-08	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Peptococcaceae	g__
363731	4901.67386	-4.0497423	0.66609898	-6.0797906	1.20E-09	1.93E-08	p__Verrucomicrobia	c__Verrucomicrobiae	o__Verrucomicrobiales	f__Verrucomicrobiaceae	g__Akkermansia
180869	41.5894473	4.16699017	1.22476158	3.40228683	0.00066824	0.00229112	p__Firmicutes	c__Erysipelotrichi	o__Erysipelotrichales	f__Erysipelotrichaceae	g__
444791	719.772719	2.34417242	0.62334656	3.7606246	0.00016949	0.00062581	p__Cyanobacteria	c__4C0d-2	o__YS2	f__	g__
780650	72.0221425	4.52100366	0.68796399	6.57157021	4.98E-11	1.19E-09	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Clostridiaceae	g__
1684221	283.245117	-1.5515791	0.49489642	-3.1351593	0.00171761	0.00515282	p__Proteobacteria	c__Deltaproteobacteria	o__Desulfovibrionales	f__Desulfovibrionaceae	g__Desulfovibrio
OTU220	283.016401	1.89250587	0.55987963	3.38020134	0.00072433	0.00231785	p__Proteobacteria	c__Alphaproteobacteria	o__RF32	f__	g__
1136443	27.1575525	-4.9111887	1.03285481	-4.7549653	1.98E-06	1.19E-05	p__Deferribacteres	c__Deferribacteres	o__Deferribacterales	f__Deferribacteraceae	g__Mucispirillum
22668	15.9244237	4.72749781	1.05258323	4.49132923	7.08E-06	3.40E-05	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Clostridiaceae	g__Candidatus Arthromitus
1107027	5549.48278	-2.5278564	0.54581097	-4.6313772	3.63E-06	1.94E-05	p__Firmicutes	c__Bacilli	o__Lactobacillales	f__Lactobacillaceae	g__Lactobacillus
997439	8487.29121	-3.6404419	0.68481853	-5.3159221	1.06E-07	8.49E-07	p__Actinobacteria	c__Actinobacteria	o__Bifidobacteriales	f__Bifidobacteriaceae	g__Bifidobacterium
338644	48.4806212	1.26437036	0.30396756	4.15955682	3.19E-05	0.00013914	p__Actinobacteria	c__Coriobacteriia	o__Coriobacteriales	f__Coriobacteriaceae	g__Adlercreutzia
589277	5686.25973	-3.5039549	0.41566534	-8.42975	3.46E-17	1.66E-15	p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Bacteroidaceae	g__Bacteroides
839200	200.959355	3.09877931	0.56380796	5.49616099	3.88E-08	3.73E-07	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Lachnospiraceae	g__Dorea
372622	158.313804	1.66402245	0.43192768	3.85254873	0.00011689	0.00046758	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Lachnospiraceae	g__Coprococcus

Supplemental Table 3. DESeq2 analysis demonstrating differential abundance of significant OTUs at the genus level (FDR adjusted p value < 0.01) between NC and HF fed mice

Deseq2: Significant OTUs NC+Allo v HF+Allo

	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Rank2	Rank3	Rank4	Rank5	Rank6
696563	76.412471	-26.48346386	3.068907	-8.62960562	6.16E-18	1.48E-16	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Lachnospiraceae	g__Blautia
187768	8867.6480	-1.915591554	0.361279	-5.30223721	1.14E-07	6.86E-07	p__Firmicutes	c__Clostridia	o__Clostridiales	f__	g__
180107	337.99763	1.418430243	0.402489	3.52414028	0.000424859	0.0014566	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Ruminococcaceae	g__Ruminococcus
264240	1464.7510	-1.681575223	0.363969	-4.6201005	3.84E-06	1.84E-05	p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Rikenellaceae	g__
OTU554	61.153891	9.339921041	0.971742	9.61152050	7.15E-22	3.43E-20	p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Rikenellaceae	g__Rikenella
323024	37.512858	6.678376416	1.301158	5.13263856	2.86E-07	1.52E-06	p__Tenericutes	c__Mollicutes	o__RF39	f__	g__
263705	8.8469116	2.747841117	0.742125	3.70266291	0.000213348	0.0007877	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Peptococcaceae	g__
363731	4901.6738	2.269838261	0.649579	3.49432151	0.000475268	0.0015208	p__Verrucomicrobia	c__Verrucomicrobiae	o__Verrucomicrobiale	f__Verrucomicrobiacea	g__Akkermansia
OTU152	115.85461	4.825780024	0.774636	6.22973761	4.67E-10	4.49E-09	p__Firmicutes	c__Bacilli	o__Bacillales	f__Staphylococcaceae	g__Staphylococcus
780650	72.022142	3.797212847	0.662794	5.7290925	1.01E-08	6.92E-08	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Clostridiaceae	g__
1136443	27.157552	-7.97276023	1.098077	-7.26065628	3.85E-13	4.62E-12	p__Deferribacteres	c__Deferribacteres	o__Deferribacterales	f__Deferribacteraceae	g__Mucispirillum
22668	15.924423	4.757650907	1.050405	4.52934675	5.92E-06	2.58E-05	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Clostridiaceae	g__Candidatus Arthromitus
OTU45	40.638197	10.70153101	2.645877	4.0446050	5.24E-05	0.0002096	p__Firmicutes	c__Bacilli	o__Turicibacterales	f__Turicibacteraceae	g__Turicibacter
997439	8487.2912	-2.14056513	0.668522	-3.2019329	0.001365088	0.0040952	p__Actinobacteria	c__Actinobacteria	o__Bifidobacteriales	f__Bifidobacteriaceae	g__Bifidobacterium
342873	807.37847	-3.130335277	0.509779	-6.14056337	8.22E-10	6.58E-09	p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Porphyromonadacea	g__Parabacteroide
589277	5686.2597	-3.198392568	0.405763	-7.88239948	3.21E-15	5.14E-14	p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Bacteroidaceae	g__Bacteroides

Supplemental Table 4. DESeq2 analysis demonstrating differential abundance of significant OTUs at the genus level (FDR adjusted p value < 0.01) between NC+Allo and HF+Allo mice