Supplementary Material for

Stearidonic-enriched soybean oil modulates obesity, glucose metabolism, and fatty acid profiles independently of *Akkermansia muciniphila*

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Supplemental Methods

1. RNA Isolation and Real-time RT-PCR Analysis

Mice were euthanized by CO_2 asphyxiation and blood collected by cardiac puncture. Liver, cecal contents, and epididymal adipose tissue (EAT) were collected, flash frozen and stored at -80°C until use in various assays. Tissue RNA extraction and RT-PCR were performed as previously described using RPL4 as the house-keeping gene.[1] Primers for C-C motif chemokine ligand 2 (CCL-2) and stearoyl-CoA desaturase-1 (SCD-1) were described by Caesar et. al. and Tan et. al., respectively.[2,3]

2. DNA Extraction, qPCR and Microbiome Analysis

DNA was isolated from fecal samples collected immediately prior to test diet introduction and at the end of the experiment using the phenol/chloroform/isoamyl alcohol method described previously.[4] DNA pellets were resuspended in 100 uL of TE buffer, vortexed, kept on ice for 30 min, vortexed again and stored at -80C until use.[4] A 2 uL aliquot was used to quantify DNA using Qubit dsDNA HS kit (Invitrogen, Carlsbad, CA). The V4 region of the bacterial 16S-ribosomal RNA (rRNA) encoding gene was amplified using the dual-indexing sequencing strategy[5] in a realplex2 thermocycler (Eppendorf, Hamburg, Germany). The amplified PCR products were purified and normalized by using a SequalPrep™ Normalization Plate kit (Applied Biosystems, Waltham, MA). Equal volumes of the normalized PCR products were pooled together. Quality of the pooled library was checked by Agilent High Sensitivity D1000 ScreenTape system (Agilent, Santa Clara, CA). Quantification of the library was checked by qPCR using a Library Quantification Kit (Kapa Biosystems, Wilmington, MA). The library

was sequenced on the Illumina MiSeq platform using a MiSeq Reagent Kit v2 500 cycles (Illumina, San Diego, CA) according to the manufacturer's instructions.

After sequencing, fasq.gz files were checked for quality and sequences trimmed using TrimGalore (Babraham Bioinformatics). The resulting files including "_val_" in their file names were processed in QIIME1.9 software[6] to merge forward and reverse reads, to pick open reference OTUs and perform core diversity analysis. Complementary analyses of diversity, visualization and statistical inference were performed in R3.4 using the phyloseq package.[7]

For quantification of *A. muciniphila* levels, DNA extracted from a pure culture of *A. muciniphila* BAA-835 of known concentration (CFU/mL) was utilized for standard curves; qPCR was performed using primers from Schneeberger et al.[8]

3. Fatty Acid Profiles

To measure fatty acid (FA) profiles in soybean oils and test diets, 20 µg of tissue was mixed in a 500 µL mixture containing hexane and 50 µL trimethylsulfonium hydroxide in a gas chromatography (GC) vial and incubated with shaking for 30 min prior to injecting 0.2 µL into a GC flame ionization detector. Values presented in tables are the mean percentage of every FA. Standard FA were run for reference, and literature was consulted for specific validation of FA.[9,10] FA levels were obtained by analyzing peak intensity as previously described.[11]

Supplemental References

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Table S1. Fatty acid profile of oils used in feeding study 1. Data is presented as percentage of fatty acids from extracted oils.

Fatty Acid	Palmitic	Stearic	Oleic	Linolenic	GLA	ALA	SDA	EPA	DHA		
length:double bonds	16:0	18:0	18:1	18:2	18:3	18:3	18:3	20:5	22:6		
Oil Type										**n-6**	**n-3**
Wild Type	10.8	3.9	24.1	52.6		7.2	--	--	--	52.6	7.2
SDA	10.5	3.4	24.8	8	6.2	23.3	21.7	--	--	14.2	45
EPA	10.4	4.1	20.6	6.3	4.7	23.8	16.3	5.43	--	11	46.73

Blue color highlights n-6 fatty acids. Green color highlights n-3 fatty acids.

Table S2. Fatty acid profile of diets used in Feeding Study 1. Data is presented as percentage of fatty acids.

Fatty Acid	Palmitic	Stearic	Oleic	Linolenic	GLA	ALA	SDA	EPA	DHA			
length:double bonds	16:0	18:0	18:1	18:2	18:3	18:3	18:3	20:5	22:6			
Diet										**n-6**	**n-3**	**Ratio n3:n6**
LF+WT	14.5	4.5	27.5	37.1	--	5.5	--	--	--	37.1	5.5	**1:7**
HF+WT	15.1	9.7	35.4	24.8	--	2.5	--	--	--	24.8	2.5	**1:10**
HF+SDA	13.7	9.7	34.9	19.1	1.6	4.4	3.2	--	--	20.7	7.6	**1:3**
HF+EPA	11	7	32.7	18.3	2.3	4.7	1.1	4.9	--	20.6	10.7	**1:2**

Blue color highlights n-6 fatty acids. Green color highlights n-3 fatty acids.

Table S3. Diet formulations used in feeding study 1.

	HF diets		LF diet	
	gm%	*kcal%*	gm%	*kcal%*
Protein	23.7	*20*	19.2	*20*
Carbohydrate	41.4	*35*	67.3	*70*
Fat	23.6	*45*	4.3	*10*
Total		*100*		*100*
kcal/gm	4.73		3.85	
Ingredient	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	200	*800*
L-Cystine	3	*12*	3	*12*
Corn Starch	72.8	*291*	550	*2200*
Maltodextrin 10	100	*400*	150	*600*
Sucrose	172.8	*691*	0	*0*
Cellulose	50	*0*	50	*0*
Soybean Oil*	**25**	*225*	**25**	*262*
Lard	177.5	*1598*	20	*180*
Mineral Mix S10026	10	*0*	10	*0*
Dicalcium Phosphate	13	*0*	13	*0*
Calcium Carbonate	5.5	*0*	5.5	*0*
Potassium Citrate, 1 H2O	16.5	*0*	16.5	*0*
Vitamin Mix V10001	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*
FD&C Dye	0.05	*0*	0.05	*0*
TOTAL	**858.15**	***4057***	**1055.05**	***4057***

*Wild Type or Transgenic (SDA- or EPA-enriched)

Table S4. Diet formulations used in feeding study 2.

	HF diets		LF diet	
	gm%	*kcal%*	gm%	*kcal%*
Protein	23.7	*20*	19.2	*20*
Carbohydrate	41.4	*35*	67.3	*70*
Fat	23.6	*45*	4.3	*10*
Total		*100*		*100*
kcal/gm	4.73		3.85	
Ingredient	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	200	*800*
L-Cystine	3	*12*	3	*12*
Corn Starch	72.8	*291*	550	*2200*
Maltodextrin 10	100	*400*	150	*600*
Sucrose	172.8	*691*	0	*0*
Cellulose	58	*0*	50	*0*
Soybean Oil*	**63**	*567*	**31.5**	*284*
Lard	139.5	*1256*	20	*180*
Mineral Mix S10026	10	*0*	10	*0*
Dicalcium Phosphate	13	*0*	13	*0*
Calcium Carbonate	5.5	*0*	5.5	*0*
Potassium Citrate, 1 H2O	16.5	*0*	16.5	*0*
Vitamin Mix V10001	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*
FD&C Dye			0.05	*0*
TOTAL	**858.15**	***4057***	**1055.05**	***4057***

*Wild Type or Transgenic (SDA- or EPA-enriched)

Table S5. Fatty acid profile of oils used in feeding study 2. Data is presented as percentage of fatty acids from extracted oils.

Fatty Acid	Palmitic	Stearic	Oleic	Linolenic	GLA	ALA	SDA	EPA	DHA	n-6	n-3
length:double bonds	16:0	18:0	18:1	18:2	18:3	18:3	18:3	20:5	22:6		
Oil Type											
Wild Type	14.8	3.7	23.8	44.9	--	11.1	--	--	--	44.9	11.1
SDA	13.8	2.5	19.8	8.4	7.9	20.2	24.2	--	--	16.3	44.4

Blue color highlights n-6 fatty acids. Green color highlights n-3 fatty acids.

Table S6. Fatty acid profile of diets used in Feeding Study 2. Data is presented as percentage of fatty acids.

Fatty Acid	Palmitic	Stearic	Oleic	Linolenic	GLA	ALA	SDA	EPA	DHA			
length:double bonds	16:0	18:0	18:1	18:2	18:3	18:3	18:3	20:5	22:6			
Diet										**n-6**	**n-3**	**Ratio n3:n6**
LF+WT	13.78	6.17	28.19	42.59	--	5.94	--	--	--	37.1	5.5	**1:7**
HF+WT	16.63	8.35	34.57	30.98	--	3.7	--	--	--	24.8	2.5	**1:10**
HF+SDA	16.74	7.93	34.82	15.16	2.41	8	8.79	--	--	20.7	7.6	**1:3**

Blue color highlights n-6 fatty acids. Green color highlights n-3 fatty acids.

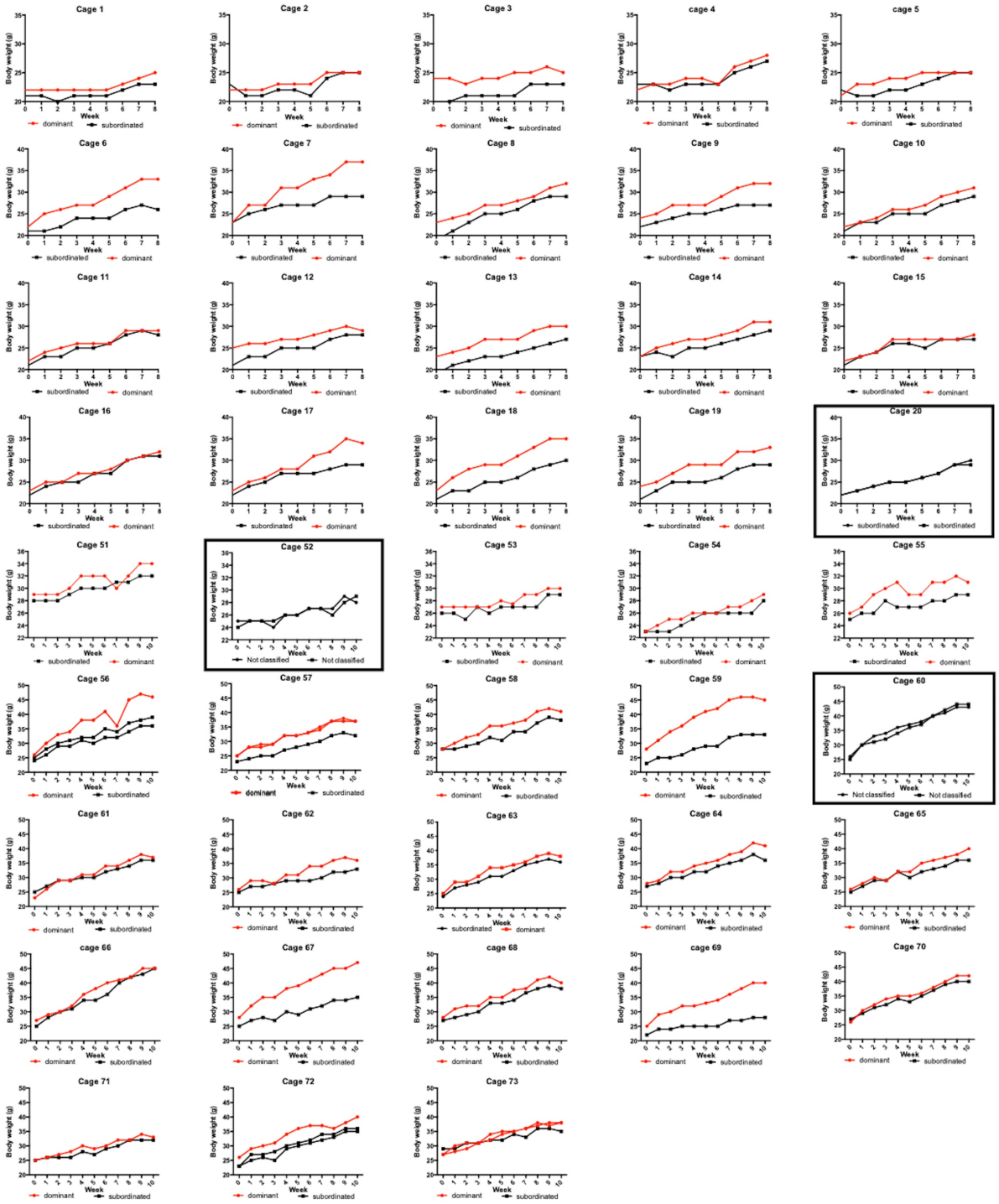

Figure S1. Dominant mice were defined as having the heaviest body weight in a cage throughout the study. Each plot presents body weight of mice over time for a given cage. The heaviest mouse in a cage was identified at 5 time points, over the first 7 weeks of feeding, and designated as the dominant mouse (red lines). The other mice were considered subordinated (black lines) mice. Cages 20, 52, and 60 contained mice that did not meet the dominance criteria and thus were not considered when data were stratified by dominance for analysis.

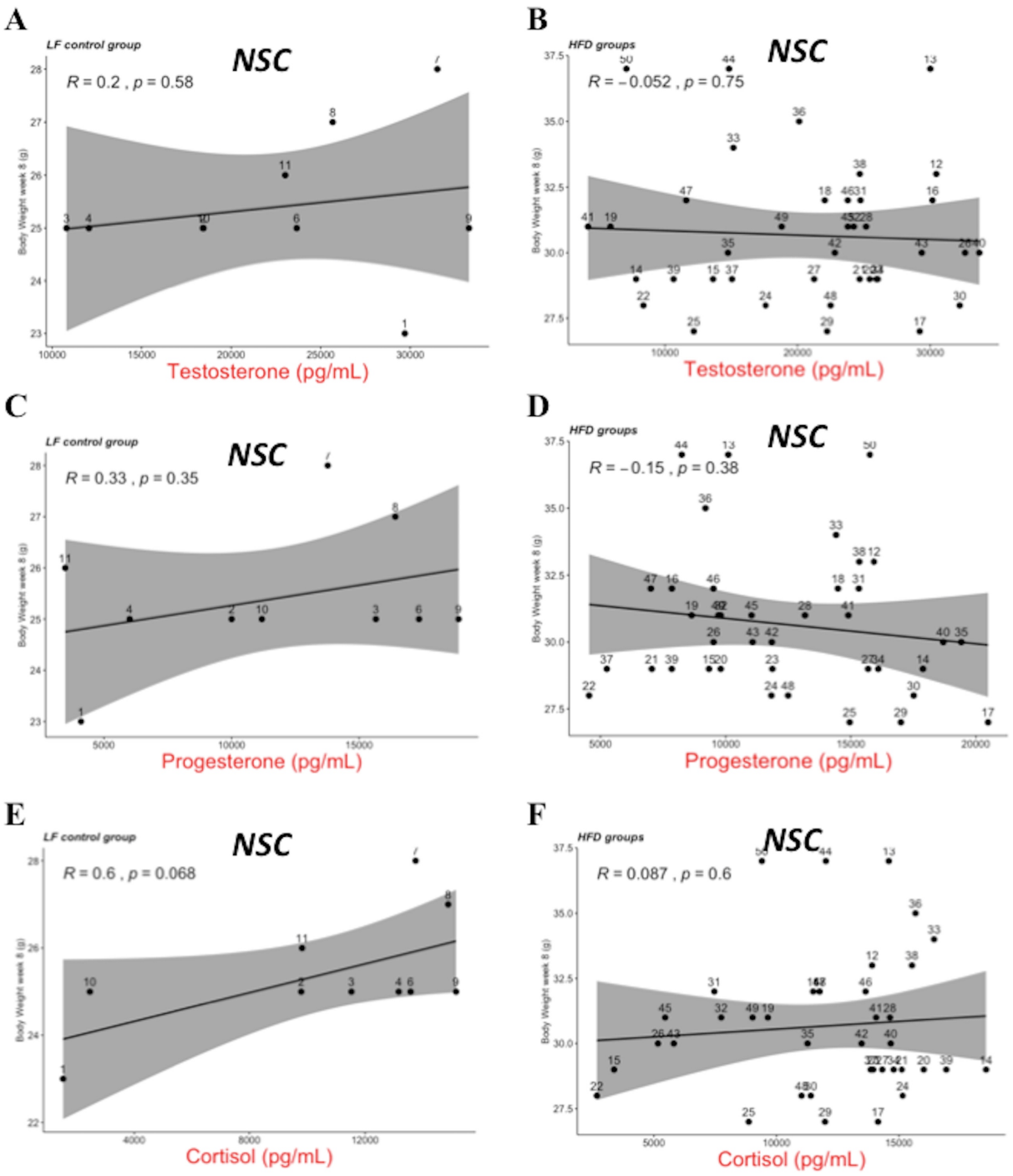

Figure S2. Hormone levels were not correlated with mouse body weight. Correlation of body weight with testosterone levels in mice fed A) a low fat control diet and B) all mice fed high fat-containing diets. Correlation of body weight with progesterone levels in mice fed C) a low fat control diet and D) all mice fed high fat-containing diets. Correlation of body weight with cortisol in mice fed E) a low fat control diet and F) all mice fed high fat-containing diets. NSC refers to no significant correlations.

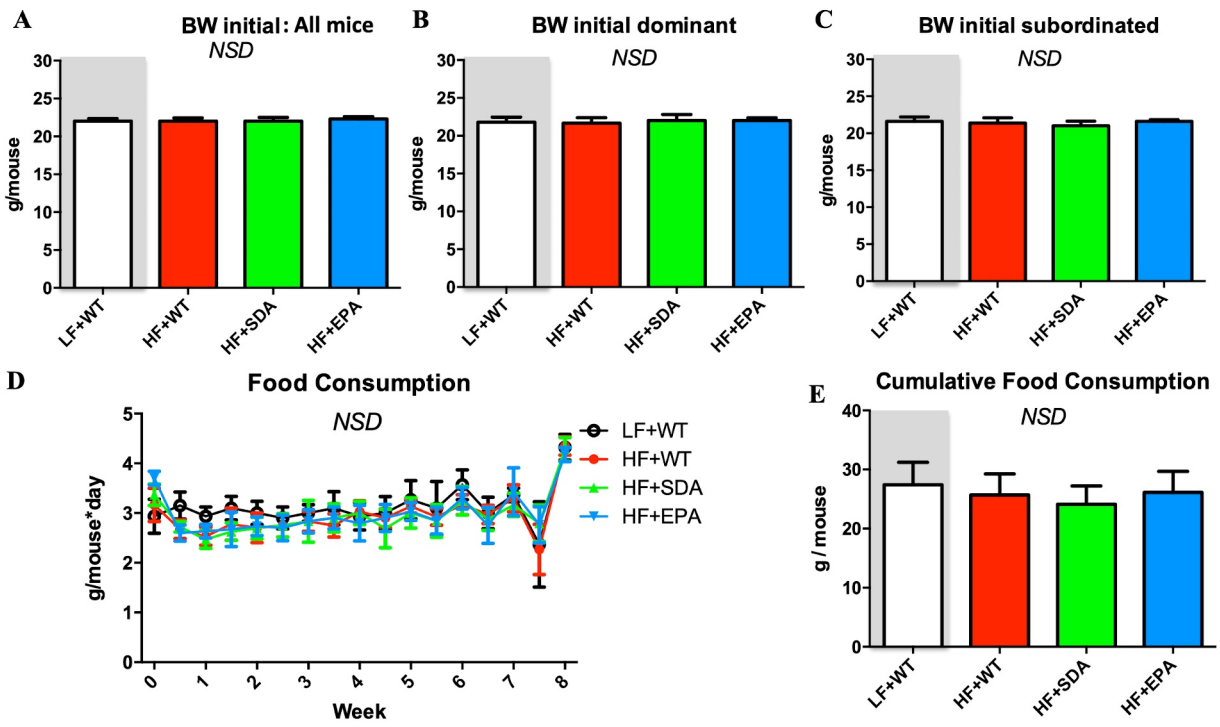

Figure S3. Initial body weights and food consumption throughout the study did not differ across treatments. Initial body weights for A) all mice, B) dominant mice and C) subordinated mice. Shaded area corresponds to low fat diet control. Analysis via one-way ANOVA and Tukey test multiple comparisons. D) Food consumed during the study. Analysis via two-way ANOVA with repeated measures and Tukey Test multiple comparisons. E) Total food consumed throughout the study. *NSD* means no significant differences were observed.

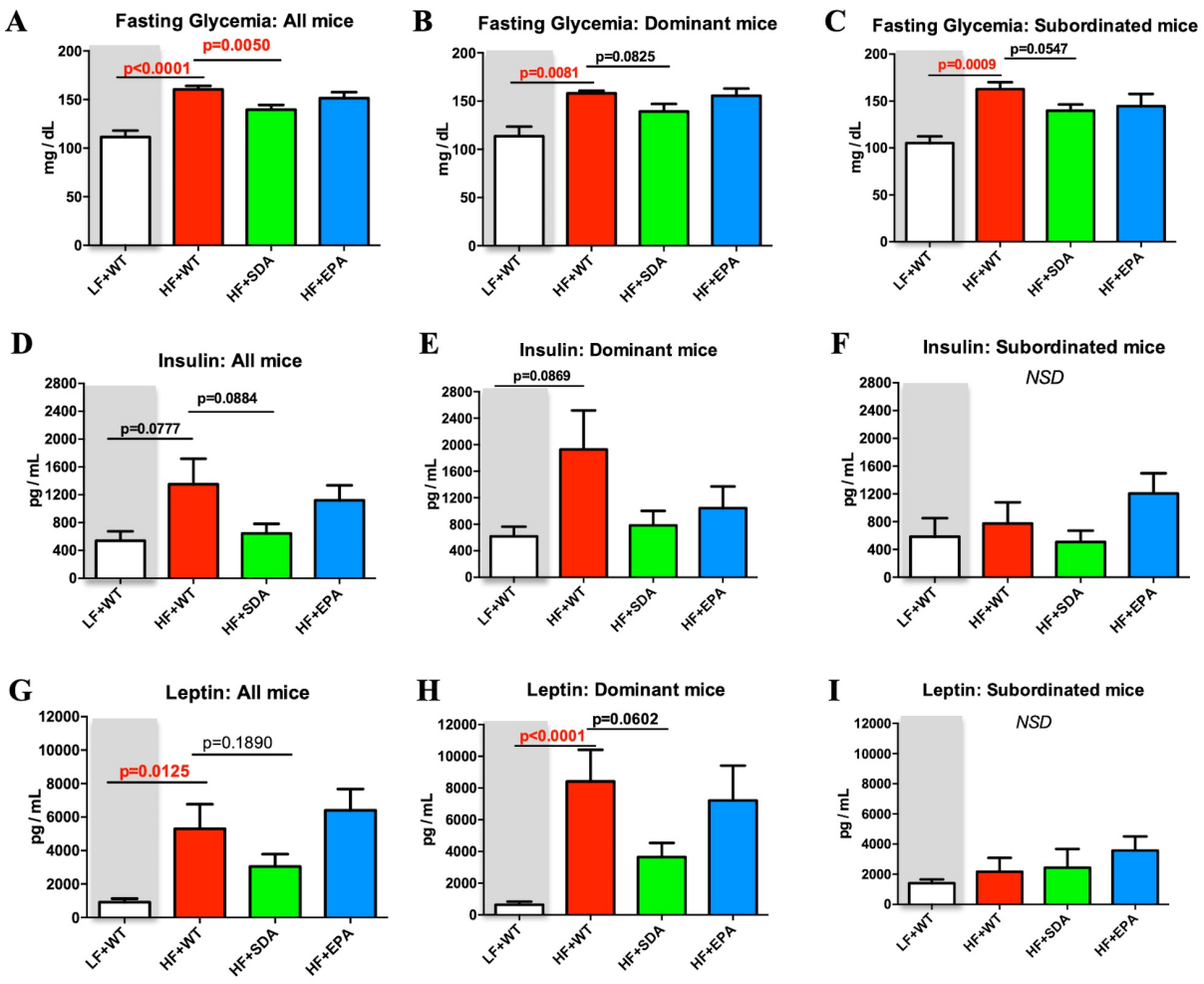

Figure S4. Feeding SDA-enriched soybean oil decreased fasting glucose. Fasting plasma glycemia in A) all mice, B) dominant mice and C) subordinated mice. Fasting plasma insulin in D) all mice, E) dominant mice and E) subordinated mice. Plasma leptin collected at necropsy from G) all mice, H) dominant mice and I) subordinated mice. Shaded area corresponds to low fat diet control. Significant (red numbers, $p<.05$) or marginal (black numbers, $.05<p<.08$) p-values are shown for specific pairwise comparisons. *NSD* means no significant differences were observed.

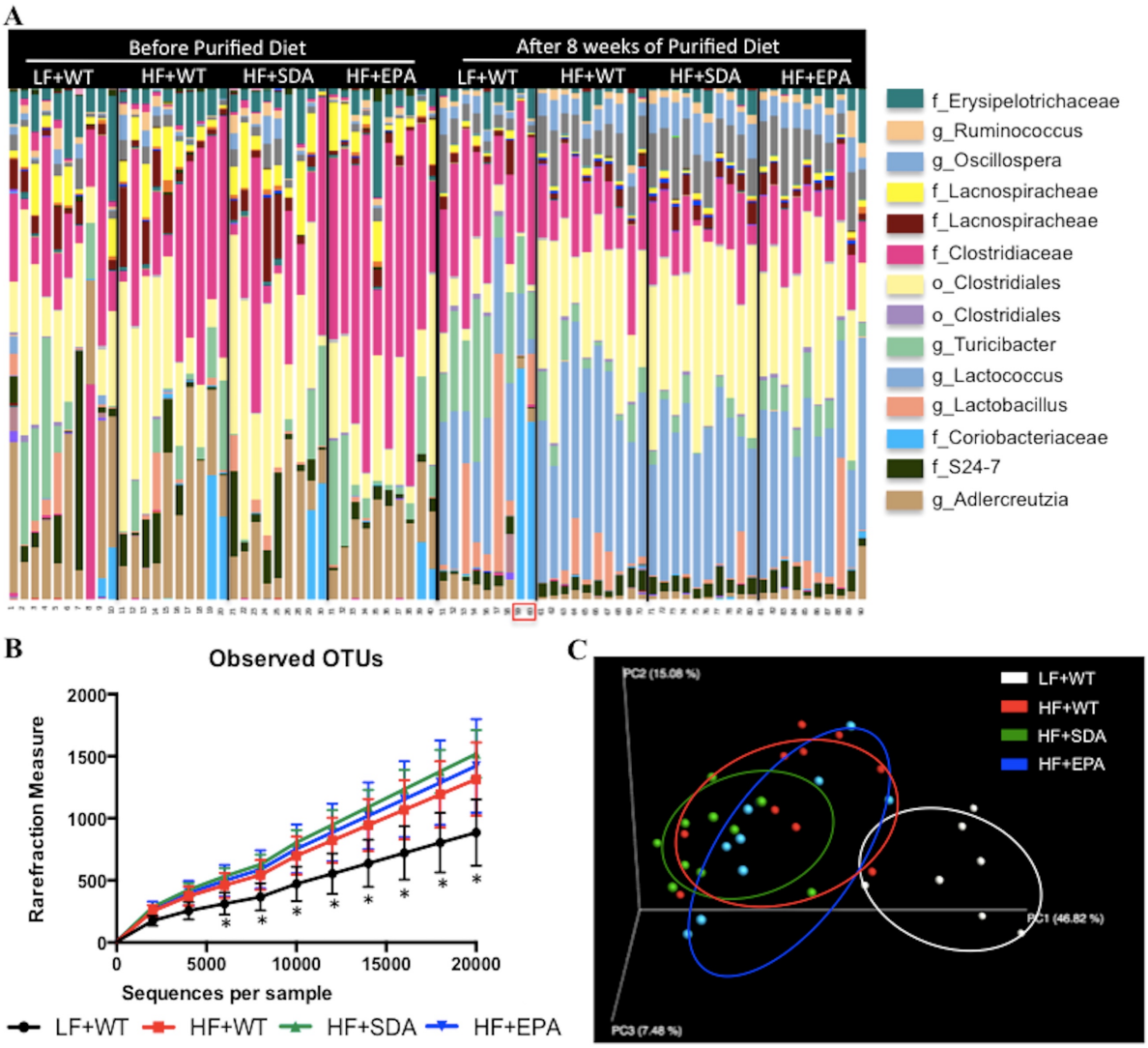

Figure S5. Microbiome composition of mice before and after introduction of test diets for Feeding Study 1. Fecal samples collected one day prior to test diet introduction and after 8 weeks of feeding the test diet were subjected to DNA extraction, PCR and Illumina sequencing. A) Relative microbiome composition of each sample. Each column represents a sample from an individual mouse and colors refer to bacterial taxa. The red square highlights two mice in an outlier cage. B) Alpha diversity as measured by number of observed OTUs after feeding test diets. C) Beta diversity as assessed by weighted UniFrac distances did not show clustering of microbiomes after feeding test diets for 8 weeks.

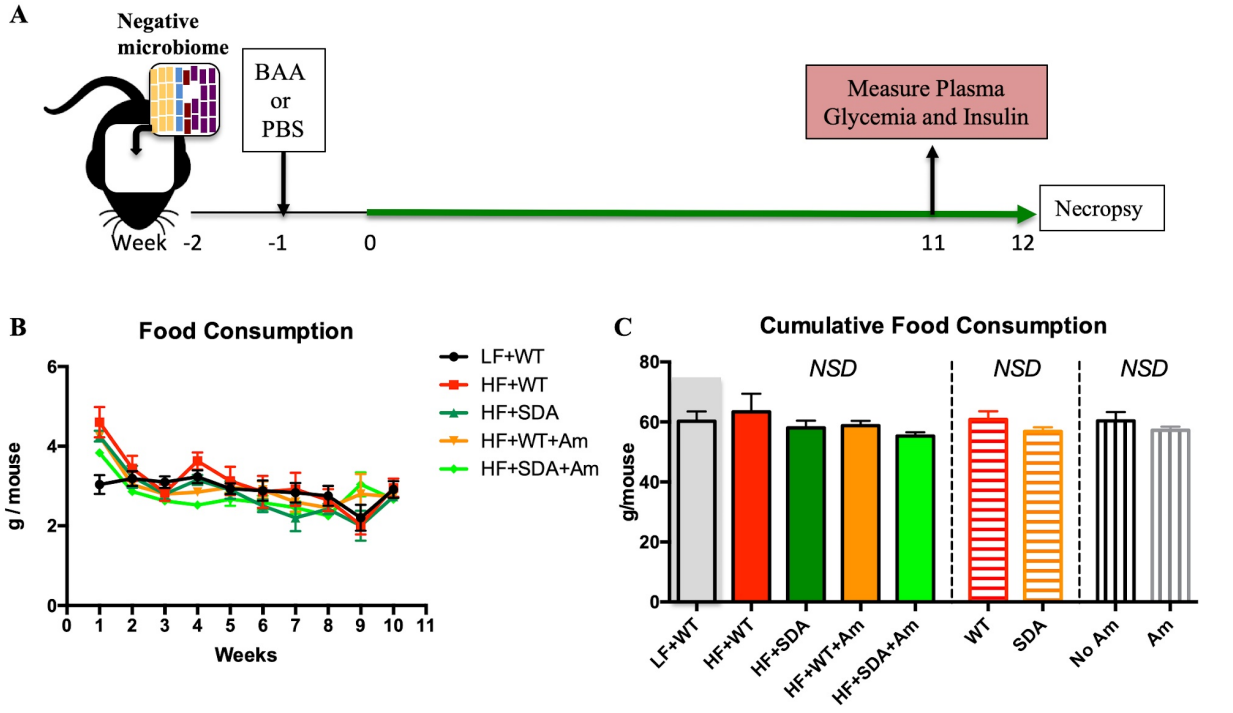

Figure S6. Experimental design and food consumption for experiments testing the role of *A. muciniphila* in mediating the metabolic benefits of feeding an SDA-enriched soybean oil diet for 12 weeks. A) Germ-free mice were first colonized with an *A. muciniphila*-negative microbiome and then colonized with *A. muciniphila* BAA-835 prior to the introduction of HF diet for 12 weeks (green line). B) Food consumed during the study. Analysis via two-way ANOVA repeated measures and Tukey Test multiple comparisons. C) Total food consumed throughout the study. Treatments with different letters are significantly different from one another by Tukey Test. Shaded area corresponds to low fat diet control. Factorial analysis was only performed with HF diet-fed treatments by Two-Way ANOVA. p-values from this analysis are presented for main effects and interactions (Oil X Am). *NSD* means no significant main effects were observed.

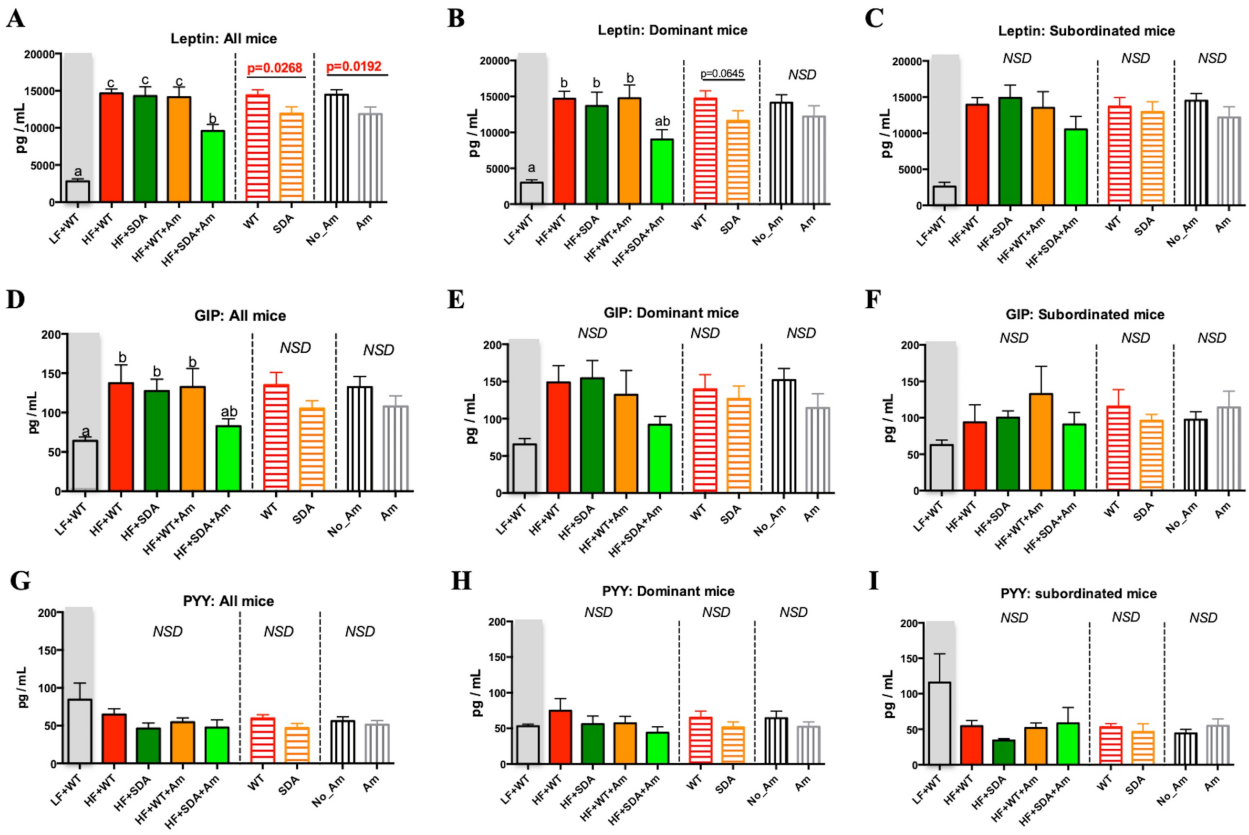

Figure S7. Plasma levels of leptin but not other satiety hormones were reduced in mice colonized with *A. muciniphila* and fed an SDA-enriched diet. Plasma levels at necropsy of leptin in A) all mice, B) dominant mice and C) subordinated mice. Plasma levels of gastric inhibitory polypeptide (GIP) in D) all mice, E) dominant mice and F) subordinated mice. Plasma levels of peptide YY (PYY) in G) all mice, H) dominant mice and I) subordinated mice. Treatments with different letters are significantly different from one another by Tukey Test. Shaded area corresponds to low fat diet control. Factorial analysis was only performed with HF diet-fed treatments by Two-Way ANOVA. From this analysis, p-values are presented for main effects and interaction (Oil X Am). Only significant interactions are shown. Significant p-values (<.05) are shown in red. Marginal p-values (.05-.07) are shown in black. *NSD* means no significant main effects were observed. A specific comparison of interest is highlighted in yellow in panel (B).

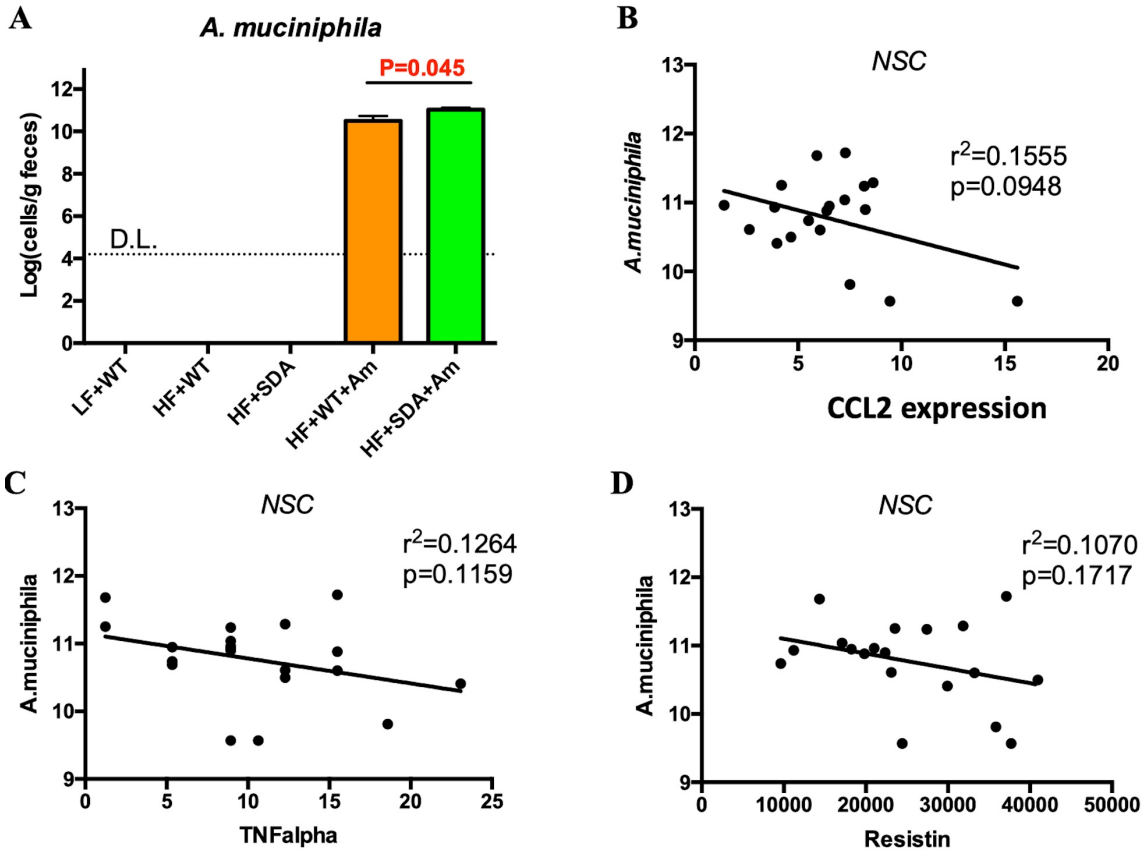

Figure S8. *A. muciniphila* abundance was not correlated with CCL2 expression or plasma levels of either TNF-α or resistin. A) Fecal levels of *A. muciniphila* by qPCR. Correlation between *A. muciniphila* levels and B) CCL2 expression in epididymal adipose tissue, C) plasma TNF-α levels and D) plasma resistin levels. *NSC* means no significant correlations were observed ($p<.05$).

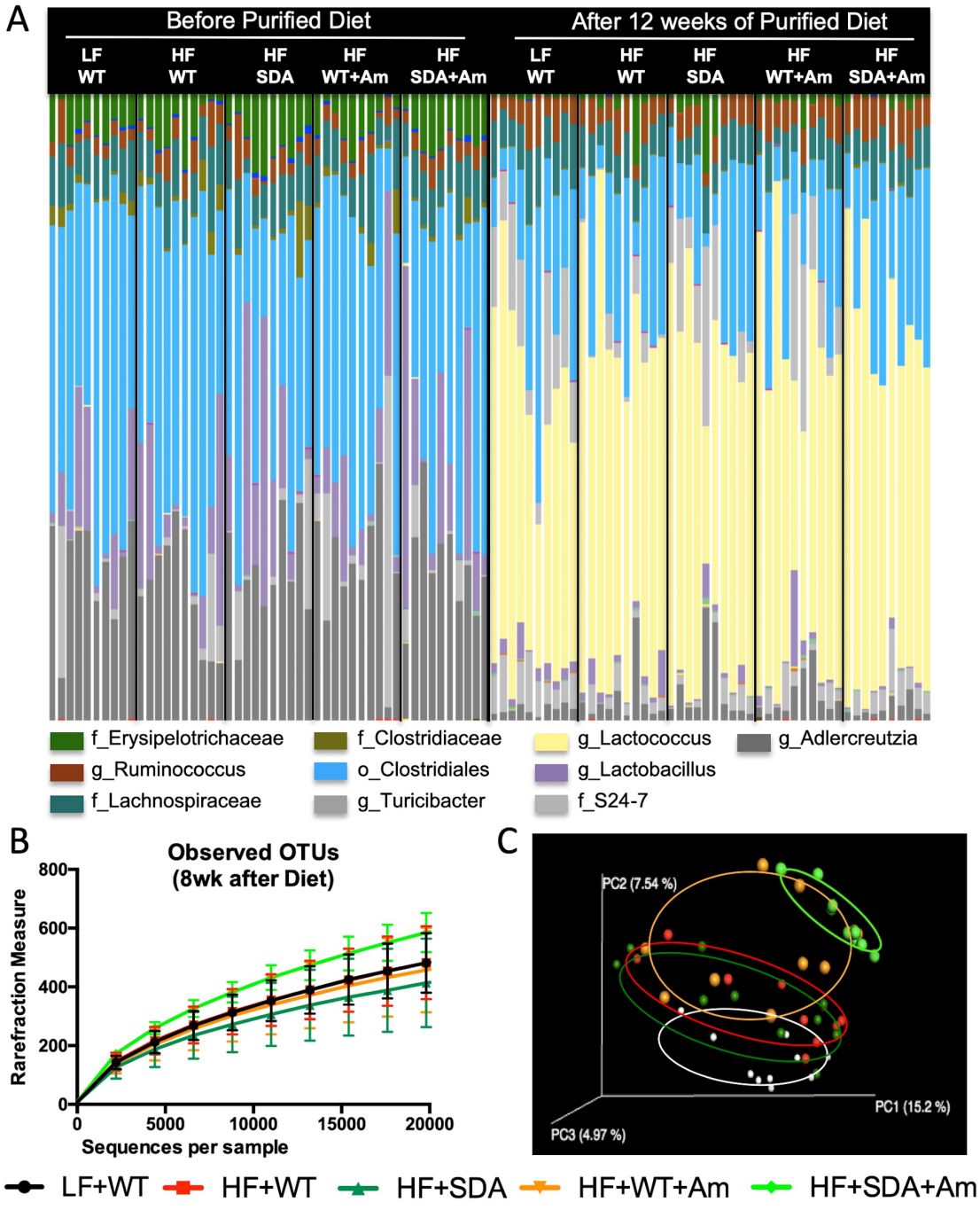

Figure S9. Microbiome composition of mice before and after introduction of test diets for Feeding Study 2. Fecal samples collected one day prior to test diet introduction and after 12 weeks of feeding the test diet were subjected to DNA extraction, PCR and Illumina sequencing. A) Relative microbiome composition of each sample. Each column represents a sample from an individual mouse and colors refer to bacterial taxa. The red square highlights two mice in an outlier cage. B) Alpha diversity as measured by number of observed OTUs after feeding test diets for 12 weeks. C) Beta diversity as assessed by weighted UniFrac distances did not show clustering of microbiomes after feeding test diets for 12 weeks.

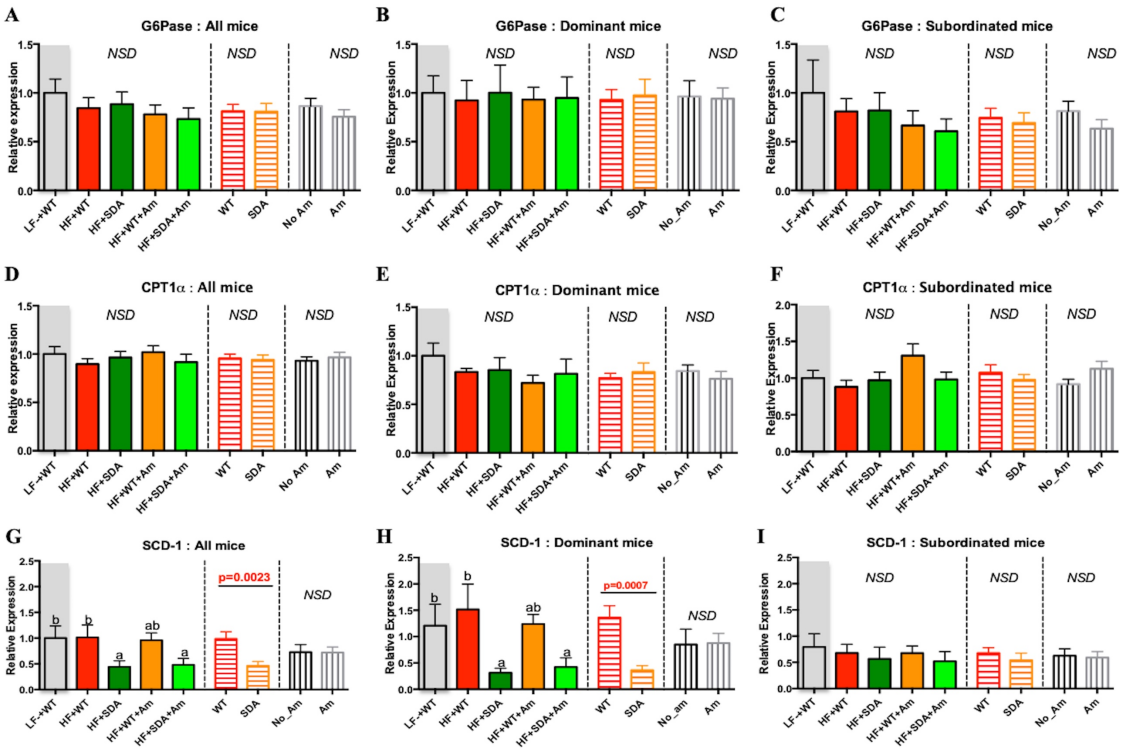

Figure S10. Feeding an SDA-enriched diet decreased hepatic expression of SCD-1 but not G6Pase or CPT1α. Relative expression of glucose-6 phosphatase (G6Pase) in liver of A) all mice, B) dominant mice and C) subordinated mice. Relative expression of carnitine palmitoyltransferase I alpha (CPT1α) in liver of D) all mice, E) dominant mice and F) subordinated mice. Relative expression of stearoyl-CoA desaturase I (SCD-1) in liver of G) all mice, H) dominant mice and I) subordinated mice. Treatments with different letters are significantly different from one another by Tukey Test. Shaded area corresponds to low fat diet control. Factorial analysis was only performed with HF diet-fed treatments by Two-Way ANOVA. From this analysis, p-values are presented for main effects and interactions (Oil X Am). Only significant interactions are shown. Significant p-values (<.05) are shown in red. Marginal p-values (.05-.07) are shown in black. *NSD* means no significant main effects were observed.

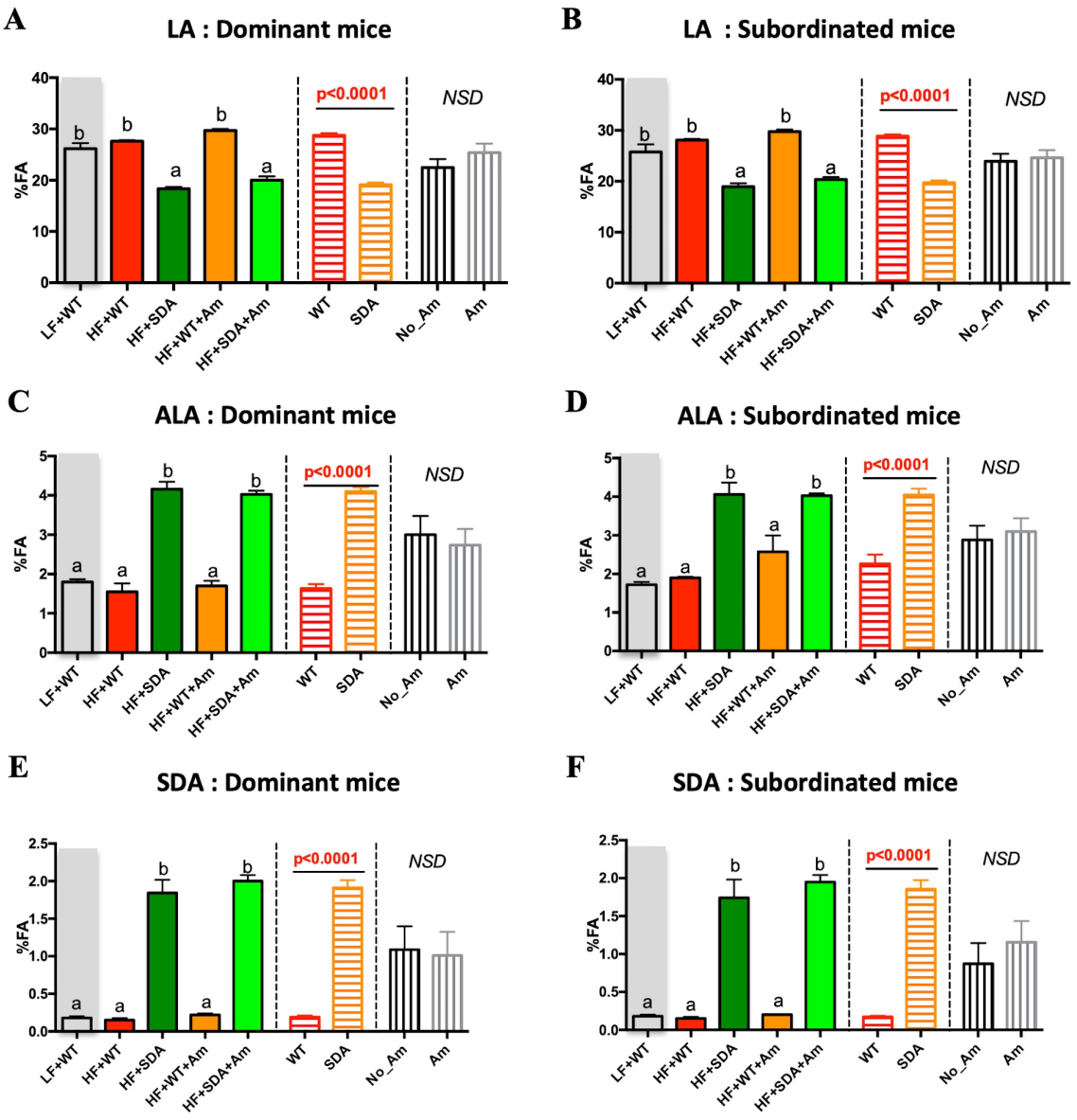

Figure S11. Feeding an SDA-enriched diet increased levels of n-3 PUFA in adipose tissue. Values are presented as percentages of total fatty acids in the epididymal adipose tissue (EAT). Linoleic acid in A) dominant and B) subordinated mice. Alpha-linolenic acid in C) dominant and D) subordinated mice. Stearidonic acid in E) dominant and F) subordinated mice. Treatments with different letters are significantly different from one another by Tukey Test. Shaded area corresponds to low fat diet control. Factorial analysis was only performed with HF diet-fed treatments by Two-Way ANOVA. From this analysis, p-values are presented for main effects and interactions (Oil X Am). Only significant interactions are shown. Significant p-values (<.05) are shown in red. Marginal p-values (.05-.07) are shown in black. *NSD* means no significant main effects were observed. All values are presented as percentages of all fatty acids.

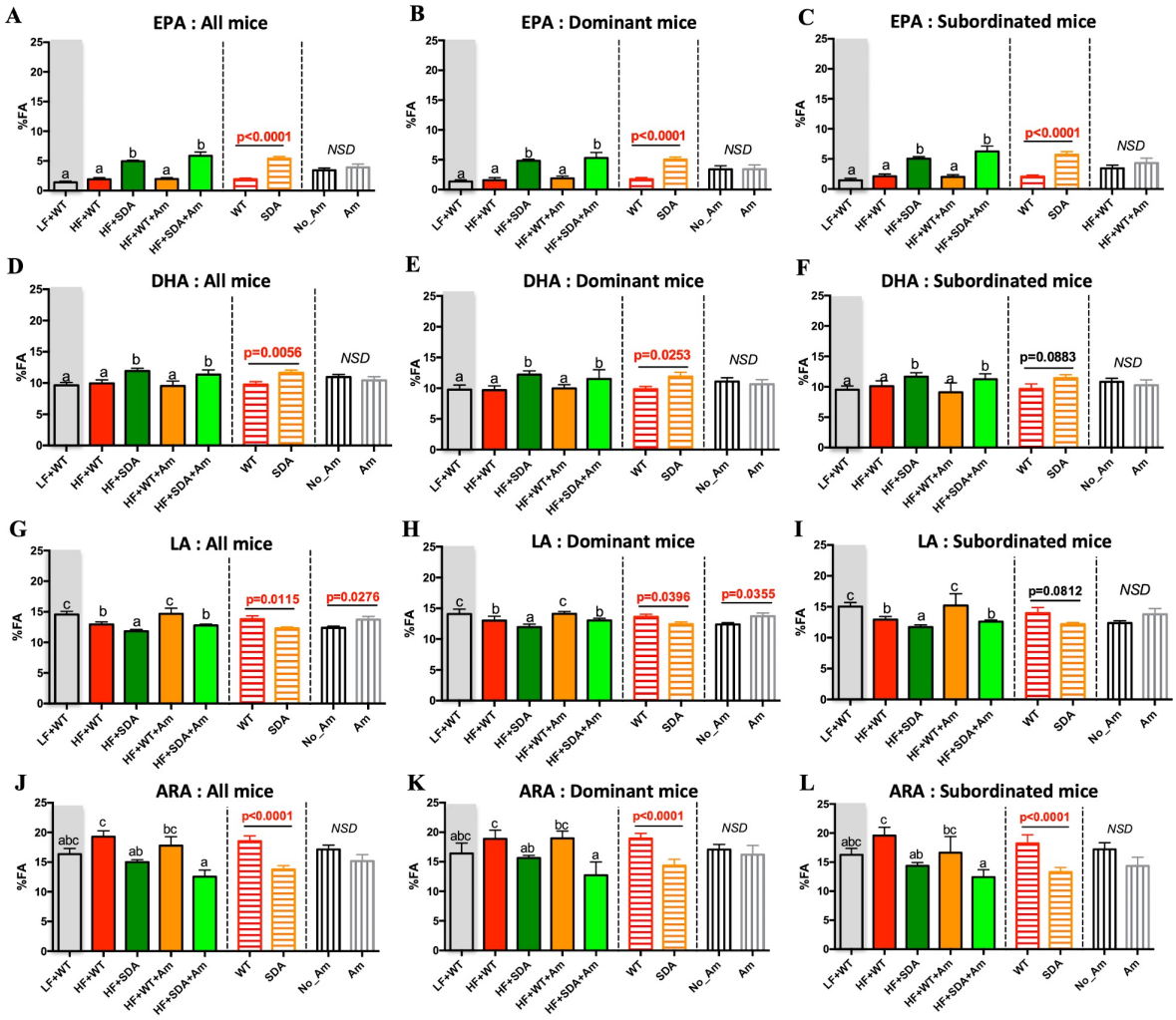

Figure S12. Feeding an SDA-enriched diet increased n-3 and decreased n-6 PUFA in the liver. Eicosapentaenoic acid in A) all mice, B) dominant and C) subordinated mice. Docosahexaenoic acid in D) all mice, E) dominant and F) subordinated mice. Linoleic acid in G) all mice, H) dominant and I) subordinated mice. Arachidonic acid in J) all mice, K) dominant and L) subordinated mice. Treatments with different letters are significantly different from one another by Tukey Test. Shaded area corresponds to low fat diet control. Factorial analysis was only performed with HF diet-fed treatments by Two-Way ANOVA. From this analysis, p-values are presented for main effects and interactions (Oil X Am). Only significant interactions are shown. Significant p-values (<.05) are shown in red. Marginal p-values (.05-.07) are shown in black. *NSD* means no significant main effects were observed. All values are presented as percentages of all fatty acids.

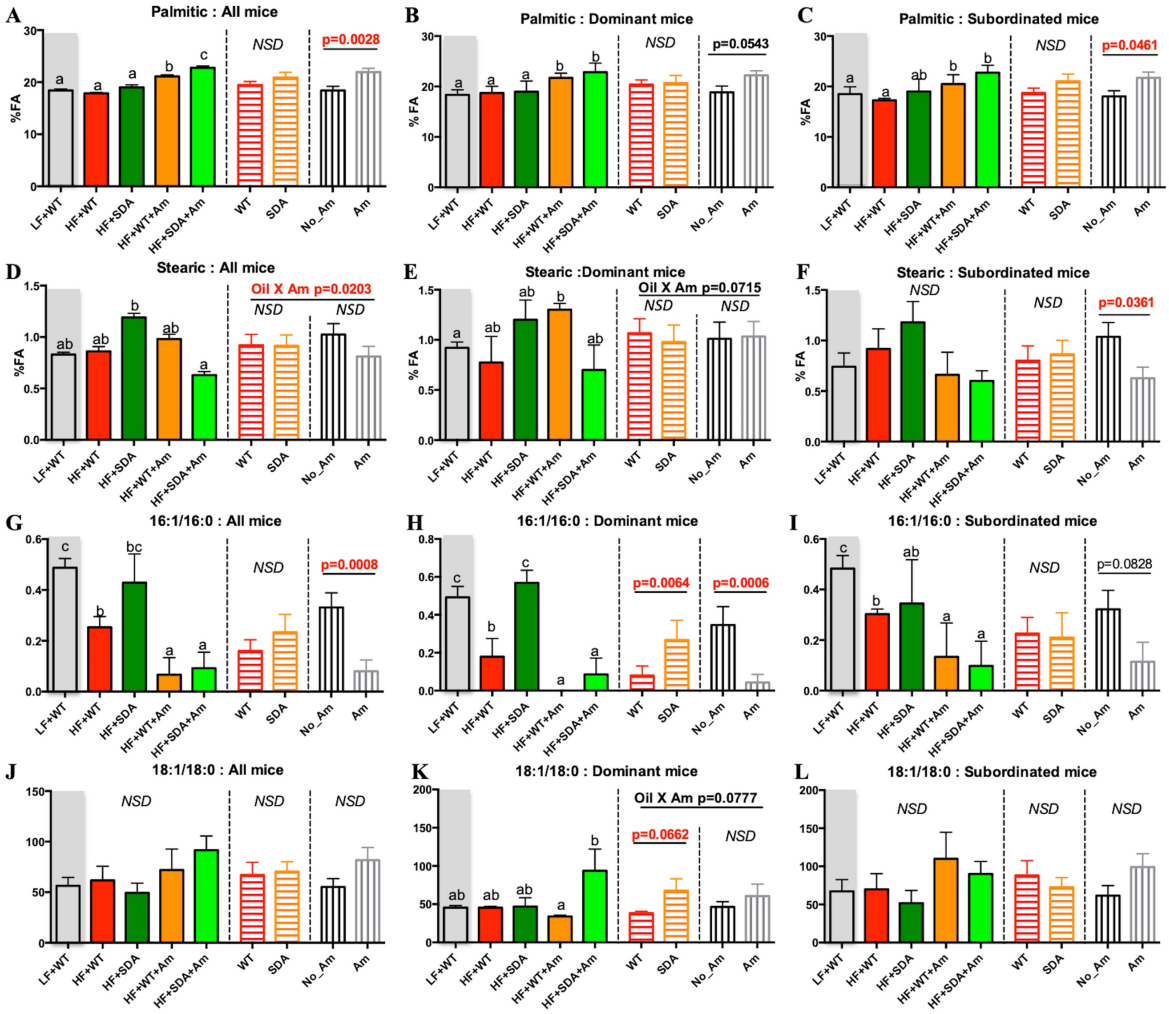

Figure S13. *A. muciniphila* presence and SDA intake influenced SFA levels and desaturation index in adipose tissue. Fatty acid levels are presented as percentages of total fatty acids in epididymal adipose tissue (EAT). Palmitic acid in A) all mice, B) dominant and C) subordinated mice. Stearic acid in C) all mice, D) dominant and E) subordinated mice. Ratio of palmitoleic (C16:1) to palmitic (C16:0) acid in G) all mice, H) dominant and I) subordinated mice. Ratio of oleic (C18:1) to stearic (C18:0) acid in J) all mice, K) dominant and L) subordinated mice. Treatments with different letters are significantly different from one another by Tukey Test. Shaded area corresponds to low fat diet control. Factorial analysis was only performed with HF diet-fed treatments by Two-Way ANOVA. From this analysis, p-values are presented for main effects and interactions (Oil X Am). Only significant interactions are shown. Significant p-values (<.05) are shown in red. Marginal p-values (.05-.07) are shown in black. *NSD* means no significant main effects were observed. All values are presented as percentages of all fatty acids.

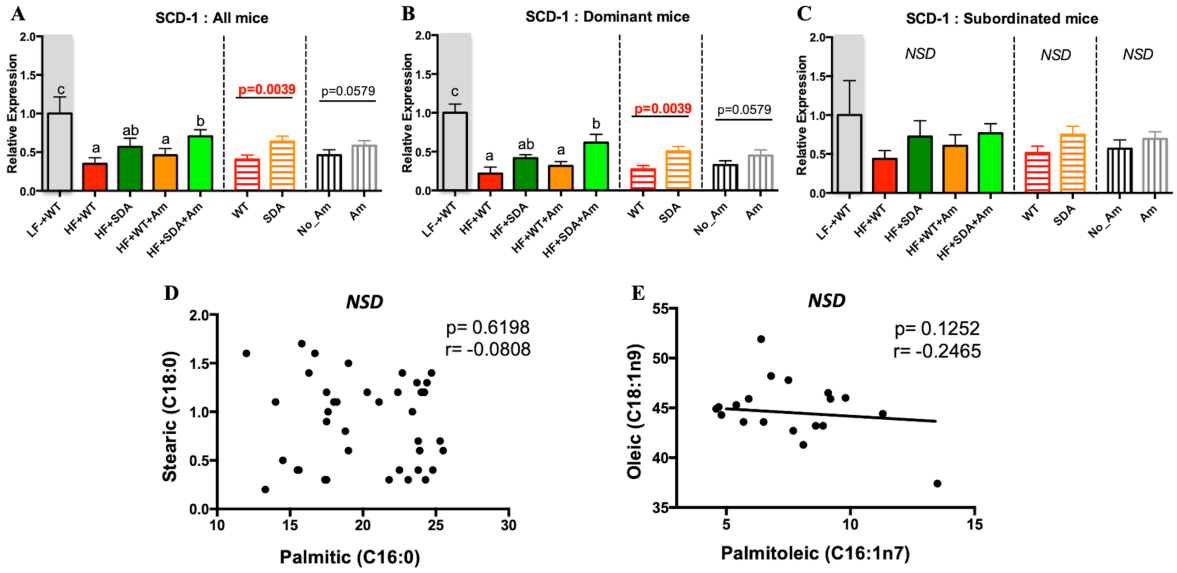

Figure S14. Changes in palmitic and palmitoleic acid were not associated with changes in markers of desaturation and elongation in adipose tissue. Relative expression of SCD-1 in epididymal adipose tissue (EAT) from A) all mice, B) dominant mice and C) subordinated mice. Correlations between stearic and palmitic acids (D) and oleic and palmitic acids (E) in EAT. NSD refers to no significant pairwise comparisons or correlations. Treatments with different letters are significantly different from one another by Tukey Test. Shaded area corresponds to low fat diet control. Factorial analysis was only performed with HF diet-fed treatments by Two-Way ANOVA. From this analysis, p-values are presented for main effects and interactions (Oil X Am). Only significant interactions are shown. Significant p-values (<.05) are shown in red. Marginal p-values (.05-.07) are shown in black.

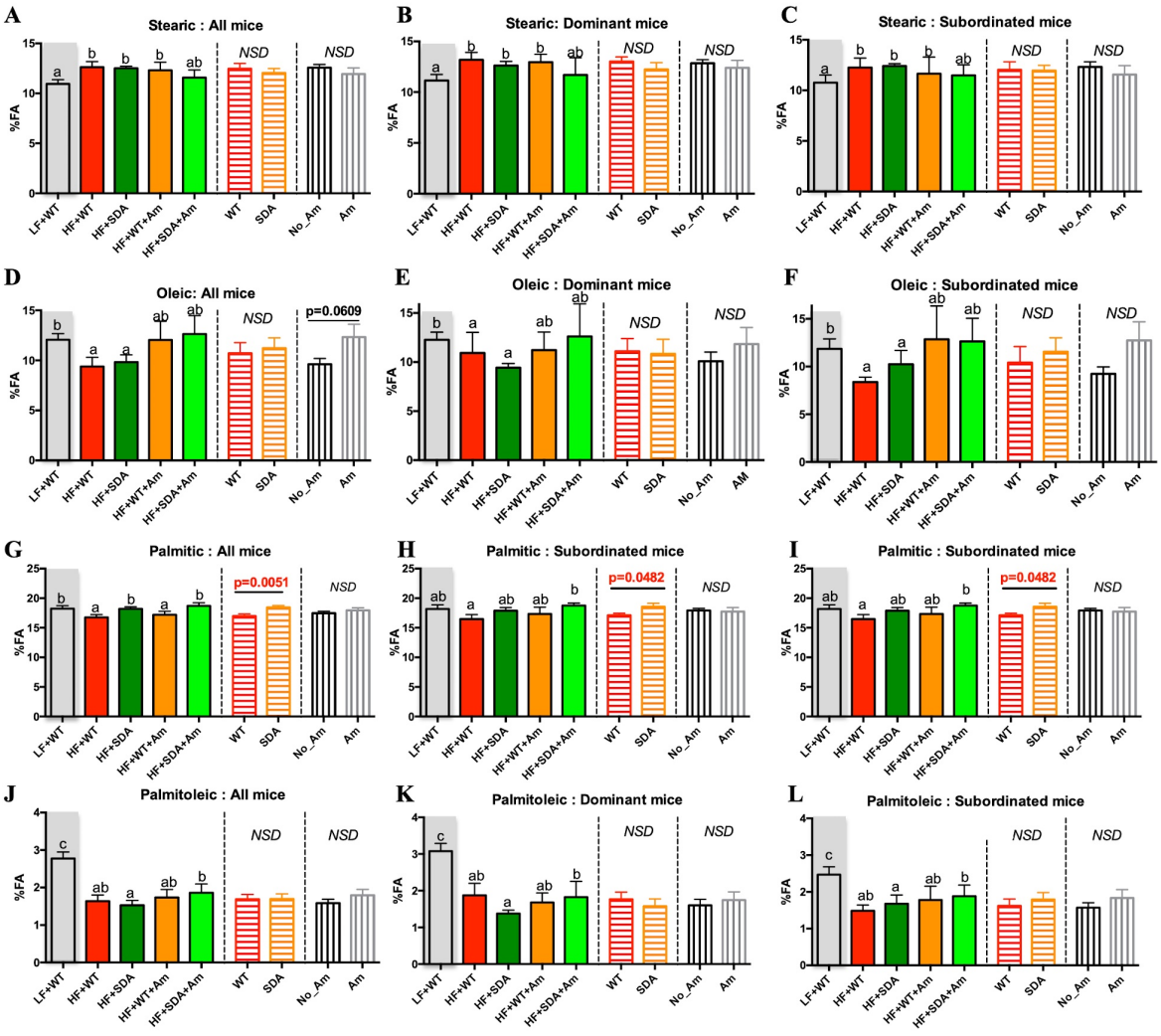

Figure S15. Feeding SDA-enriched diet increased hepatic levels of palmitic acid. Stearic acid in liver from A) all mice, B) dominant mice and C) subordinated mice. Oleic acid in liver from D) all mice, E) dominant mice and F) subordinated mice. Palmitic acid in liver from G) all mice, H) dominant mice and I) subordinated mice. Palmitoleic acid in liver from J) all mice, K) dominant mice and L) subordinated mice. Treatments with different letters are significantly different from one another by Tukey Test. Shaded area corresponds to low fat diet control. Factorial analysis was only performed with HF diet-fed treatments by Two-Way ANOVA. From this analysis, p-values are presented for main effects and interactions (Oil X Am). Only significant interactions are shown. Significant p-values (<.05) are shown in red. Marginal p-values (.05-.07) are shown in black. *NSD* means no significant main effects were observed. All values are presented as percentages of all fatty acids.