SUPPLEMENTAL MATERIAL

The impact of gut microbiota and diet on the development of atherosclerosis in *Apoe-/-* mice

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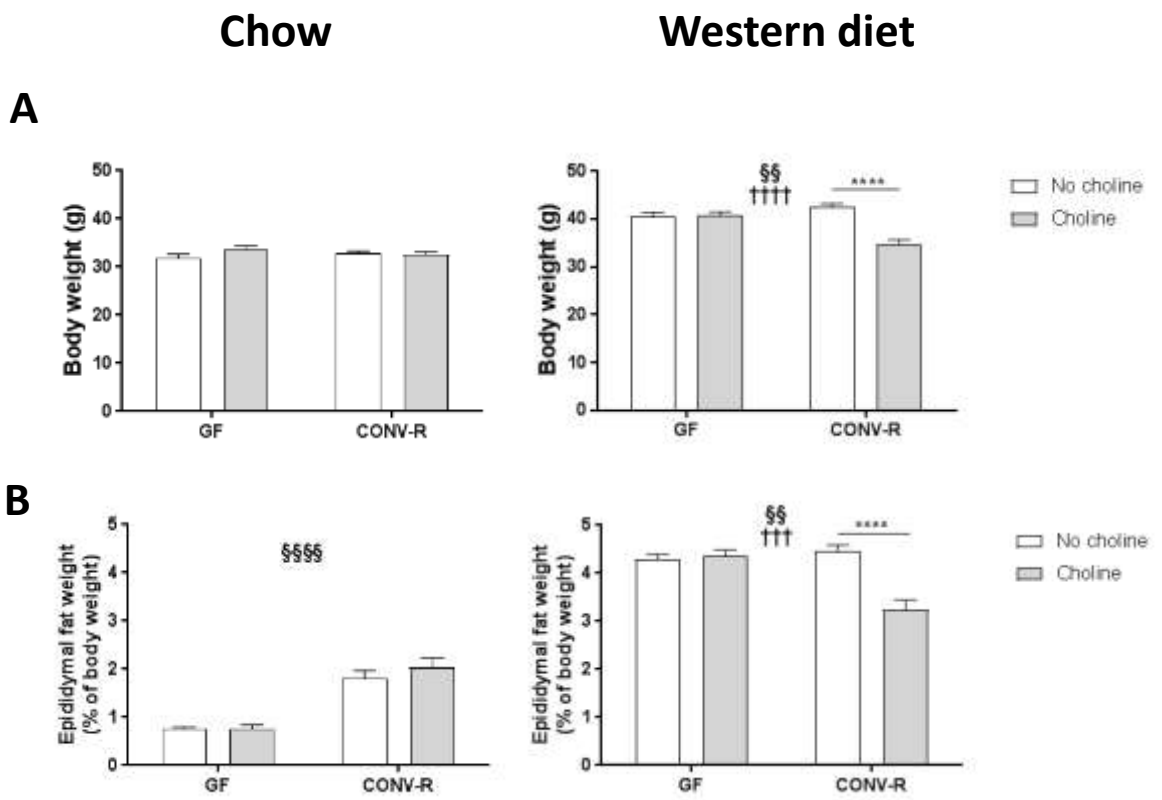
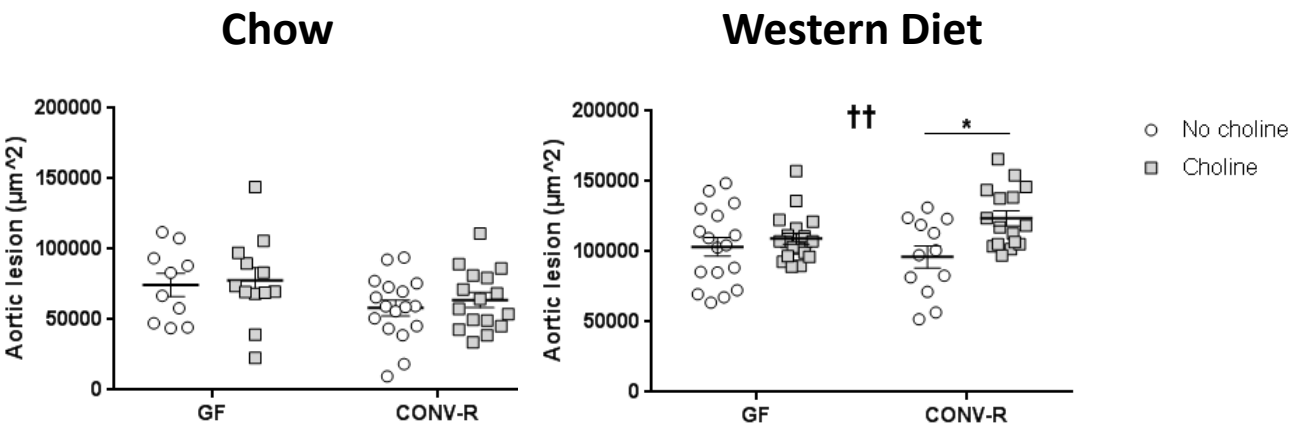
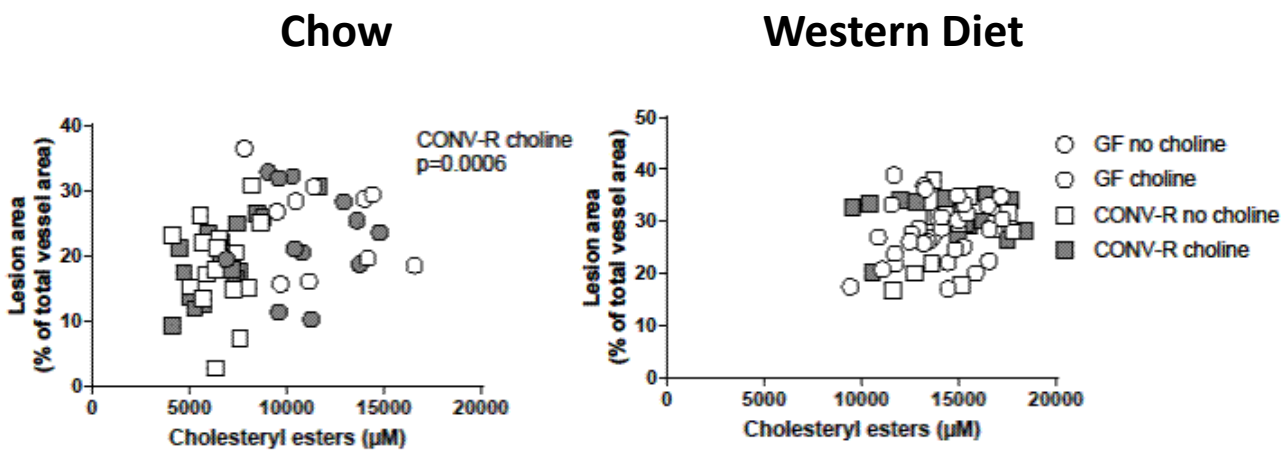
Supplemental Figures and Figure Legends



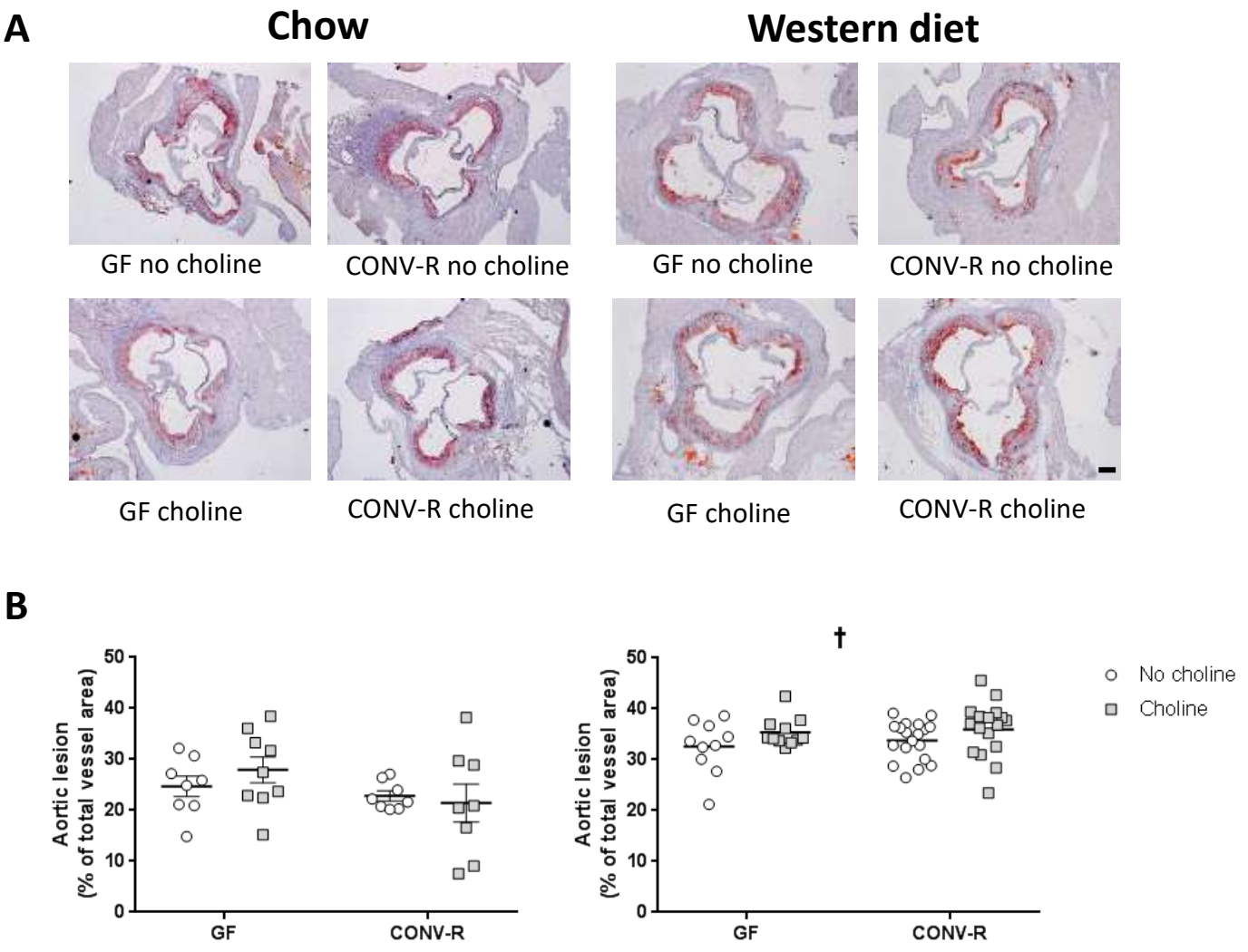
Supplemental Figure I. Physiological parameters in male *Apoe-/-* mice are dependent on microbiota and diet. Body weight (A) and epididymal fat weight (B) in GF (n=15-19) and CONV-R (n=18-20) *Apoe-/-* mice fed chow (left panels) or Western diet (right panels) with or without supplementation of choline for 12 weeks. Data are presented as mean ± SEM. Variation induced by the gut microbiota: $^{\S}p<0.05$, $^{\S\S}p<0.01$, $^{\S\S\S\S}p<0.0001$. Variation induced by choline: $^{\dagger\dagger\dagger}p<0.001$, $^{\dagger\dagger\dagger\dagger}p<0.0001$. Post hoc multiple comparison analysis: ****p<0.0001.



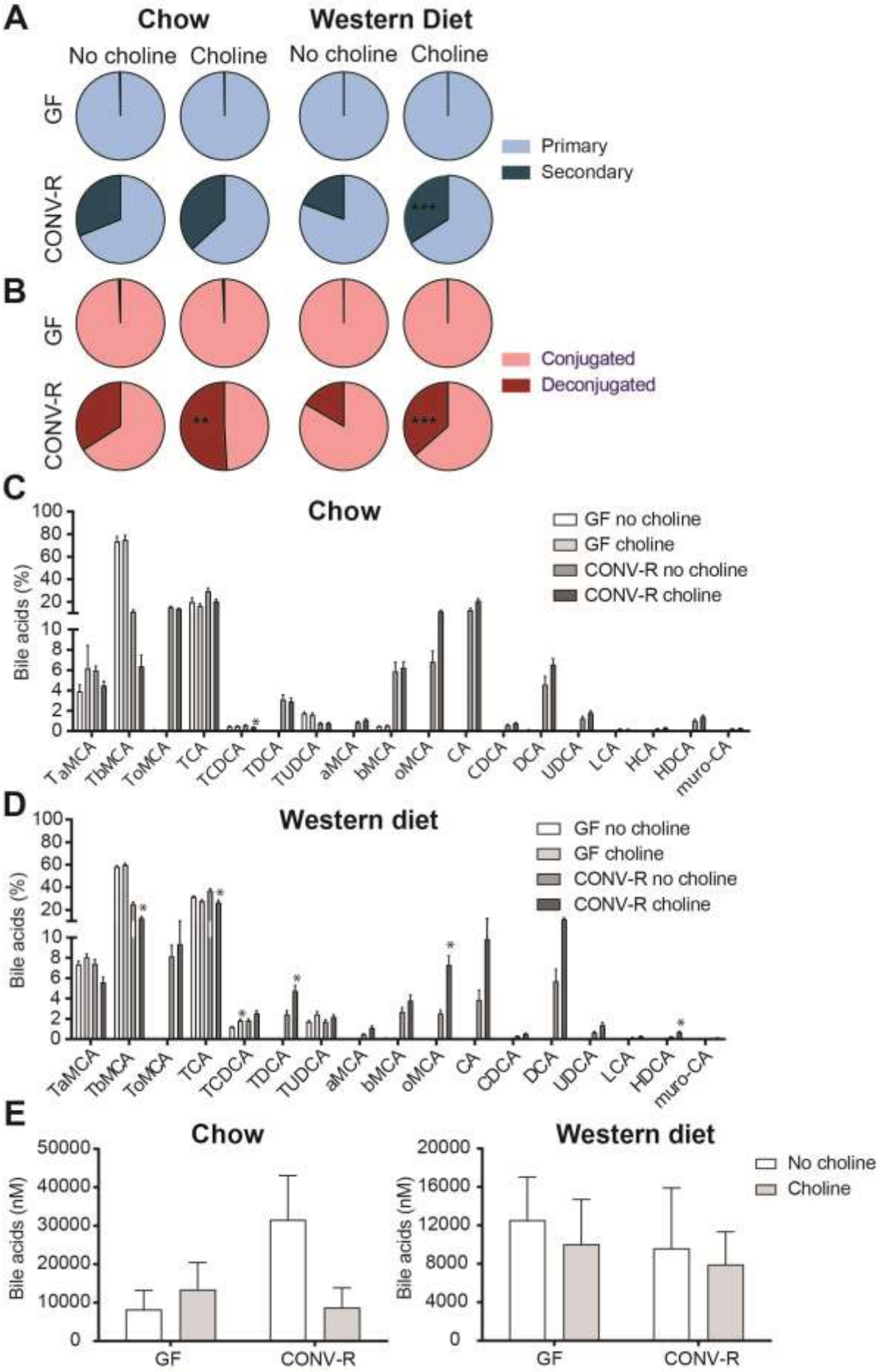
Supplemental Figure II. Development of atherosclerosis is dependent on microbiota and diet. The lesion area in aortic root sections from chow-fed (left panel) and Western diet-fed (right panel) mice. Data are presented as mean ± SEM. Variation induced by the gut microbiota: $^{\S}p<0.05$. Variation induced by choline: $^{\dagger\dagger}p<0.01$. Post hoc multiple comparison analysis: $^{*}p<0.05$.



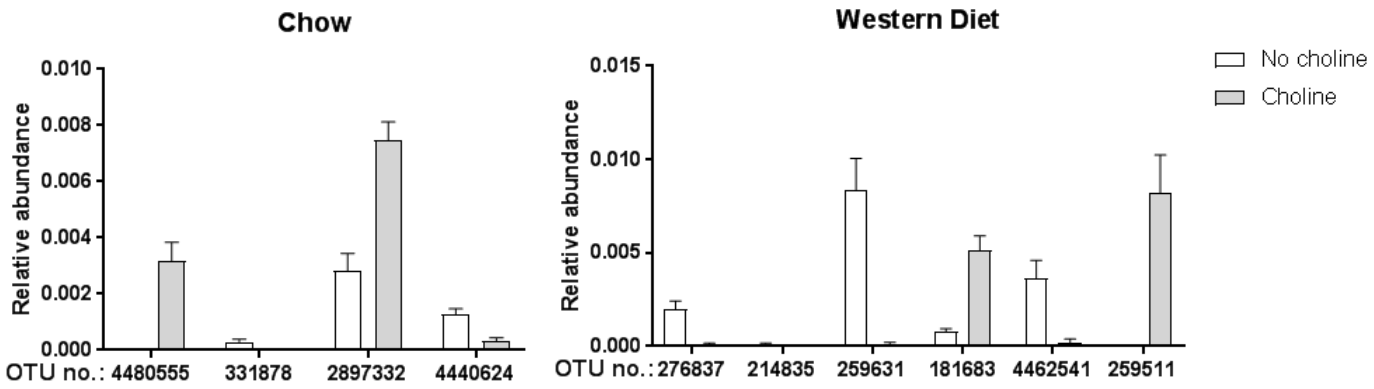
Supplemental Figure III. Correlation between atherosclerosis lesion area and cholesteryl esters. The lesion area in aortic root sections from chow-fed (left panel) and Western diet-fed (right panel) mice plotted against plasma levels of cholesteryl esters.



Supplementary Figure IV. Atherosclerotic lesion size in female *Apoe*$^{-/-}$ mice. Female GF and CONV-R mice were fed chow (n = 8-9, left panel) or Western diet (n = 10-19, right panel) with or without supplementation of choline for 12 weeks. Presented are representative Oil-red-O-stained aortic root lesions (A) and lesion size normalized to vessel area (B). Data are presented as mean ± SEM. Variation induced by choline: $^{\dagger}p<0.05$. Scale bar = 20µm.


A
Chow
Western Diet
No choline
Choline
No choline
Choline
GF
CONV-R
Primary
Secondary
B
GF
CONV-R
Conjugated
Deconjugated
C
Chow
Bile acids (%)
GF no choline
GF choline
CONV-R no choline
CONV-R choline
TaMCA
TbMCA
ToMCA
TCA
TCDCA
TDCA
TUDCA
aMCA
bMCA
oMCA
CA
CDCA
DCA
UDCA
LCA
HCA
HDCA
muro-CA
D
Western diet
Bile acids (%)
GF no choline
GF choline
CONV-R no choline
CONV-R choline
E
Chow
Bile acids (nM)
GF
CONV-R
Western diet
Bile acids (nM)
GF
CONV-R
No choline
Choline

Supplemental Figure V. Microbial status and diet determines bile acid composition. Pie charts showing the distribution of primary and secondary (A) or conjugated and deconjugated (B) bile acids in GF and CONV-R mice fed chow (n=10-18, left panel) or Western diet (n=15-20, right panel). Individual bile acid composition in chow (C) and Western diet (D)- fed mice. Total bile acid concentration in plasma (E). Data are presented as mean relative proportion of bile acid composition ± SEM. In (A) and (B) **p<0.01, ***p<0.001 indicate difference versus CONV-R with no choline supplementation determined by Mann-Whitney test. In (C) and (D) *p<0.05 indicate difference versus no choline determined by PERMANOVA. GF, germ-free; CONV-R, conventionally raised; CA, cholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; MCA, muricholic acid; T, taurine-conjugated species.



Supplemental Figure VI. Relative abundance of operational taxonomic units (OTUs). Analysis of taxa using the pipeline in QIIME revealed that the relative abundance of four (chow, n=17-18) and six (Western diet, n=13-16) OTUs belonging to the order *Clostridiales* were differentially abundant in cecal content from *Apoe-/-* mice that were fed a choline-free or choline-supplemented diet.

Supplemental Table I. Cytokine concentrations in plasma from *Apoe-/-* mice.

	Chow					**Western diet**				
	GF		**CONV-R**		***p***	**GF**		**CONV-R**		***p***
Cytokine	**No choline**	**Choline**	**No choline**	**Choline**		**No choline**	**Choline**	**No choline**	**Choline**	
IFN-γ	0.440 ± 0.04	0.437 ± 0.11	0.408 ± 0.06	0.426 ± 0.07	ns	0.377 ± 0.03	0.425 ± 0.03	0.483 ± 0.05	0.490 ± 0.05	§
IL-1β	0.183 ± 0.02	0.161 ± 0.03	0.622 ± 0.12	0.797 ± 0.28	§§§§	0.162 ± 0.01	0.171 ± 0.02	0.291 ± 0.04	0.347 ± 0.05	§§§
IL-2	1.025 ± 0.11	0.850 ± 0.13	1.215 ± 0.11	1.046 ± 0.21	ns	1.116 ± 0.07	1.087 ± 0.08	1.256 ± 0.10	1.081 ± 0.11	ns
IL-4	0.046 ± 0.02	0.063 ± 0.04	0.029 ± 0.01	0.051 ± 0.02	ns	0.160 ± 0.03	0.095 ± 0.02	0.151 ± 0.04	0.115 ± 0.04	ns
IL-5	5.236 ± 0.85	4.953 ± 0.56	3.365 ± 0.32	4.003 ± 0.66	§	6.232 ± 0.48	6.276 ± 0.64	4.017 ± 1.27	4.569 ± 0.48	ns
IL-12p70	0.978 ± 0.54	3.626 ± 3.44	0.845 ± 0.57	2.106 ± 1.12	ns	1.319 ± 0.92	1.872 ± 0.74	6.734 ± 2.06	7.085 ± 1.48	§§§

All concentrations are in pg/mL and shown in mean ± SEM. For samples where values were below detection range the cytokine concentration was put to 0.
Differences are determined by 2-way ANOVA with Tukey post hoc multiple comparison. Chow n = 11-12, Western diet n = 12-19.
GF, germ-free; CONV-R, conventionally raised, IFN, interferon; IL, interleukin.
Variation induced by the gut microbiota: §p<0.05, §§p<0.01, §§§p<0.001, §§§§p<0.0001.

Supplemental Table II. Proportion of OTUs significantly correlated with plasma TMAO and bile acids.

					TMAO		TCDCA		TbMCA		TCA		ωMCA		TDCA		HDCA	
Diet	**OTU no.**	**Taxa**	**No choline**	**Choline**	**r**	***p***	**r**	***p***	**r**	***p***	**r**	***p***	**r**	***p***	**r**	***p***	**r**	***p***
Chow	4480555	o_Clostridiales; f_; g_; s_	0.001±0.001	0.319±0.065	0.512	0.002	-0.423	0.011										
	331878	f_Lachnospiraceae; g_; s_	0.030±0.009	0.003±0.001	-0.410	0.014	0.003	0.98										
	2897332	g_Ruminococcus; s_gnavus	0.281±0.064	0.748±0.065	0.626	<0.0001	-0.295	0.086										
	4440624	f_Lachnospiraceae; g_; s_	0.128±0.019	0.033±0.011	-0.621	<0.0001	0.256	0.14										
Western diet	276837	o_Clostridiales; f_; g_; s_	0.199±0.045	0.009±0.008	-0.600	0.0006			0.638	0.0002	0.443	0.016	-0.489	0.007	-0.474	0.009	-0.565	0.001
	214835	f_Lachnospiraceae; g_; s_	0.015±0.004	0.000±0.000	-0.573	0.001			0.293	0.12	0.256	0.18	-0.379	0.043	-0.141	0.47	-0.348	0.064
	259631	g_Ruminococcus; s_gnavus	0.834±0.171	0.011±0.009	-0.617	0.0004			0.459	0.012	0.354	0.060	-0.456	0.013	-0.275	0.15	-0.441	0.017
	181683	o_Clostridiales; f_; g_; s_	0.079±0.016	0.514±0.077	0.385	0.039			-0.261	0.17	-0.311	0.10	0.284	0.14	0.282	0.14	0.305	0.11
	4462541	o_Clostridiales; f_; g_; s_	0.366±0.095	0.021±0.019	-0.561	0.002			0.569	0.0013	0.400	0.032	-0.441	0.017	-0.399	0.032	-0.517	0.004
	259511	o_Clostridiales; f_; g_; s_	0.000±0.000	0.822±0.202	0.586	0.0008			-0.446	0.015	-0.369	0.049	0.439	0.017	0.097	0.62	0.503	0.006

Pearson's correlation was used to calculate the correlation coefficient r and *p*-values of the proportion of OTUs and TMAO or bile acids regulated by choline in chow and Western diet-fed mice.

OTU, operational taxonomic unit; TMAO, trimethylamine-*N*-oxide; TCDCA, taurochenodeoxycholic acid; TbMCA, tauro-beta-muricholic acid; TCA, taurocholic acid; ωMCA, omega-muricholic acid; TDCA, taurodeocycholic acid; HDCA, hyodeoxycholic acid.

Different taxonomic levels: o, order; f, family; g, genus; s, species.