Recovery of ethanol-induced *Akkermansia muciniphila* depletion ameliorates alcoholic liver disease

Christoph Grander[1#], Timon E Adolph[1#], Verena Wieser[1], Patrick Lowe[2], Laura Wrzosek[3], Benedek Gyongyosi[2], Doyle V Ward[4,5], Felix Grabherr[1], Romana R Gerner[1], Alexandra Pfister[1], Barbara Enrich[1], Dragos Ciocan[3,8,9], Sophie Macheiner[1], Lisa Mayr[1], Matthias Drach[6], Patrizia Moser[7], Alexander R Moschen[1], Gabriel Perlemuter[3,8,9], Gyongyi Szabo[2], Anne Marie Cassard[3,8] and Herbert Tilg[1]*

1 Department of Internal Medicine I, Gastroenterology, Endocrinology & Metabolism, Medical University Innsbruck, Innsbruck, Austria

2 Department of Medicine, University of Massachusetts Medical School, Worcester MA, United States of America

3 INSERM UMR996 - Inflammation, Chemokines and Immunopathology, Clamart, France

4 Center for Microbiome Research, University of Massachusetts Medical School, Worcester MA, United States of America

5 Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, Massachusetts, United States of America

6 Department of Dermatology, University Hospital Zürich, Zürich, Switzerland

7 Institute of Pathology, Medical University Innsbruck, Innsbruck, Austria

8 Univ Paris-Sud, Univ Paris-Saclay, DHU Hepatinov, Labex Lermit, CHU Bicêtre, Kremlin-Bicêtre, France

9 AP-HP, Hepatogastroenterology and Nutrition, Hôpital Antoine-Béclère, Clamart, France

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MATERIAL AND METHODS

Mice and Ethanol Feeding.

Animal care was performed in accordance with the Institutional Animal Care and Use Committee regulations at the University of Massachusetts Medical School and the Medical University of Innsbruck, respectively. For microbiota analysis of wild-type C57BL/6 mice fed a Lieber-DeCarli ethanol diet (acute on chronic ALD model, see below), mice were purchased from Jackson Laboratories and were cohoused in the University of Massachusetts Medical School Animal Medicine Facility for one week prior to the start of the experiment at which time they were housed in pairs. Mice were exposed to a ten-day Lieber-DeCarli diet containing ethanol as described by Bertola et al. [1]. Briefly, all mice were fed the Lieber-DeCarli pair-fed diet for five days to become acclimated to a liquid diet. Some mice were then switched to the Lieber-DeCarli ethanol diet containing 5% ethanol or maltose. Pair-fed mice were calorie matched with the ethanol-fed mice. On day 10, mice were gavaged with either ethanol (5 g kg^{-1} body weight) or isocaloric maltose dextran. Mice were cheek bled then euthanized 9 hours post-gavage.

All *A. muciniphila* experiments were aligned to ethical principles according to Austrian law (BMWFW-66.011/0019-WF/V/3b/2015) and carried out in the animal facility of the Medical University in Innsbruck. Seven to eight week old female C57BL/6 mice were fed with a Lieber-DeCarli diet (BioServ, Flemington, NJ) [1] containing an increasing ethanol concentration from 1% up to 5 vol% (EtOH-fed) *ad libitum* for 15 days. Control diet was supplemented with an isocaloric amount of maltose (Pair-fed). A subset of ethanol-fed and pair-fed mice was treated with *A. muciniphila* ($1{,}5x10^9$ CFU/200µl PBS) by intragastric infusion with a 24-gauge stainless steel free tube every other day, starting at day 1. Control groups were orally administered an equivalent volume of PBS.

For addressing the therapeutic properties of *A. muciniphila* on ALD, mice were fed with a Lieber-DeCarli diet for 15 days. *A. muciniphila* administration ($1,5x10^9$ CFU/200µl PBS) was carried out only on day 10, 12, and 14, after liver injury was verified on day 9.

In the acute model of ethanol-toxicity, mice were treated with *A. muciniphila* ($1,5x10^9$ CFU/200µl PBS) two days prior to the gavage of 6g ethanol per kg bodyweight. Eight hours after the ethanol application, mice were sacrificed and tissue samples were collected.

Before euthanasia mice were anaesthetized with Xylazin 5 mg/kg (Intervet, Vienna, Austria) and Ketamin 100 mg/kg (AniMedica, Senden, Germany), followed by collection of blood, stool and tissue samples of liver and intestine. Serum was stored at -20 °C. Stool and tissue samples were stored at -80 °C or in RNAlater (Qiagen, Hilden, Germany).

Histology. Liver and gut sections were stained with haematoxylin and eosin (H&E); additionally colon samples were stained with periodic acid-Schiff (PAS) after fixation in Carnoy-solution (6 parts of ethanol abs., 6 parts of acetic acid glacial, 1 part of chloroform). Staining was performed by the Institute of Pathology at the Medical University of Innsbruck. A pathologist analyzed the h&e liver sections in a blinded fashion in regards to hepatic steatosis, inflammation and infiltration of inflammatory cells. Hepatic steatosis was quantified by percentage of cells showing lipid accumulation. For Oil Red O staining, liver samples were frozen in OCT compounds (Sakura, Tissue-Tek, Netherlands) followed by cryo-sectioning (5µm). After air-drying, sections were fixed and stained with an Oil red O (Amresco, Cleveland, OH) – isopropanol solution and counterstained with hematoxylin (Dako, Santa Clara, CA).

Myeloperoxidase immunohistochemistry. Liver sections were deparaffinized in xylene and dehydrated in an ethanol gradient. Antigen was unmasked with 2 % citrate-buffer (pH = 6; Vector Laboratories, Burlingame, CA) in a conventional steamer. Endogenous peroxidase activity was inactivated with peroxidase (Dako, Santa Clara, CA) for 10 min. Protein blocking was performed using a ready-to-use kit (MP-740; Dako, Santa Clara, CA). Rabbit MPO antibodies (Dako, Santa Clara, CA) and secondary anti-rabbit antibodies (Vector Laboratories, Burlingame, CA and Dako, Santa Clara, CA) were applied. ImmPACT AMEC (Vector Laboratories, Burlingame, CA) was used to visualize immunoreactivity. Sections were stained with DAB (Dako, Santa Clara, CA) for 2 minutes and counterstained with hematoxylin (Dako, Santa Clara, CA) for 20 seconds. MPO positive cells were counted in five randomly selected high-power fields by a blinded observer.

Immunofluorescence staining. Slides were deparaffinized in xylene and dehydrated in an ethanol gradient. Antigen was unmasked with 2% citrate-buffer (pH = 6; Vector Laboratories, Burlingame, CA) in a conventional steamer, followed by protein blocking with 1% bovine serum albumin (in PBS). Primary antibodies Claudin-3 (monoclonal rabbit antibody; Cell signalling technology, Cambridge, UK), Muc-2 (polyclonal rabbit antibody, Santa Cruz, TX) and Occludin (polyclonal rabbit antibody, abcam, UK) were diluted in 1% BSA-PBS and incubated for 90 minutes in a humid chamber, followed by incubation with secondary goat anti-rabbit antibodies (AF488, life technologies, Carlsbad, CA) for 30 minutes. Slides were mounted with Prolong® Diamond Antifade Mountant supplemented with DAPI (4′,6′-diamidino-2-phenylindole, life technologies, Carlsbad, CA) and analyzed with a 340 confocal microscope (Zeiss, Oberkochen, Germany). Intensity was quantified by two independent, blinded observers in three randomly picked fields of view.

Mucus thickness and goblet cell counting. Carnoy-fixed and PAS stained colon samples were analyzed with Pannoramic Viewer (3DHISTECH, Budapest, Hungary). Mucus thickness was measured in colon sections right angle to the epithelial surface. For each section, 60 to 150 single measurements were taken. To quantify goblet cells in the colon, PAS positive cells per colonic crypt were counted. For each section at least 12 crypts were analyzed.

ALT measurement. For measuring the ALT concentrations in the serum, an enzymatic assay (BQ-Kit, San Diego, CA) was used. To 10 µl serum 100 µl of reagent were added, followed by absorbance measurement at 37 °C, five times in intervals of one minute. Means of absorbance changes per minute were calculated to analyze the activation of ALT.

RNA isolation in tissue and stool samples. Purification of RNA (tissue and r stool) was achieved by homogenization of samples in TRIzol® reagent (Thermo Fisher Scientific, Waltham, MA). Reverse transcription was accomplished with the Reverse Transcription System (Thermo Fisher Scientific, Waltham, MA), followed by qPCR. For qPCR SybrGreen (Eurogentec, Seraing, Belgium) and the Mx3000 qPCR cycler (Stratagene California, CA) were used. PCR primers sequences are available upon request.

Triglyceride measurement. Frozen liver tissue samples were homogenized in PBS (volume was adjusted to the liver tissue weight) and incubated for 30 minutes at 60°C, followed by centrifugation (12.000g, 10min, room temperature). Supernatant was taken and triglyceride concentration was measured with TG-Reagent (Roche, Switzerland). For isolation of triglycerides only fatty-free BSA (Sigma, St. Louis, MO) -coated vials were used.

ELISA. Frozen liver tissue were homogenized using a adapted Tris-buffer (200mM NaCl, 5mM EDTA, 10mM Tris, 10% glycerin; pH 7,4) with PMSF (Sigma-Aldrich, St. Louis, MO), Leupeptin (Sigma-Aldrich, St. Louis, MO), Aprotinin (Sigma-Aldrich, St. Louis, MO). Commercial available ELISA kits for Il-1ß and Tnf-α were purchased from BD (NJ) and used according to the manufacture instructions.

Intestinal permeability assays. Intestinal permeability was measured by intragastric infusion of fluorescein isothiocyanate-dextran (FD4) (4kDa; Sigma-Aldrich, St. Louis, MO). Mice were gavaged with 200 µl FD4 (120 mg/ml) 4 hours before euthanization. Blood was collected and subsequently centrifuged (5000g, 4°C, 10 minutes). Quantification of FD4 levels was assessed by photometric measurement using PHERAstar (BMG Labtech, Ortenberg, Germany). As control groups normal chow fed mice were used.

In-vitro assay for measuring ethanol metabolism and growth by *A. muciniphila*. To exclude a possible enhanced growth of *A. muciniphila* in the presence of ethanol, BHI media (Sigma-Aldrich, St. Louis, MO) was supplemented with ethanol (0mM, 1mM, 50mM, 100mM) and inoculated with *A. muciniphila.* After 30 hours the abundance of *A. muciniphila* was measured by OD600 photometric absorption.

SUPPLEMENTARY TABLES

Supplementary Table 1: Patient characteristics

	Severe ASH	ASH	Healthy controls
Age [yr]	55.1 ± 11,95	50.9 ±10.04	41.1 ± 2.6
BMI	26.4 ± 6.4	24.6 ± 3.4	23.5 ± 1.1
Sex (f/m)	2/13	4/17	9/7

Supplementary Table 2: Clinical characteristics of the ASH cohort

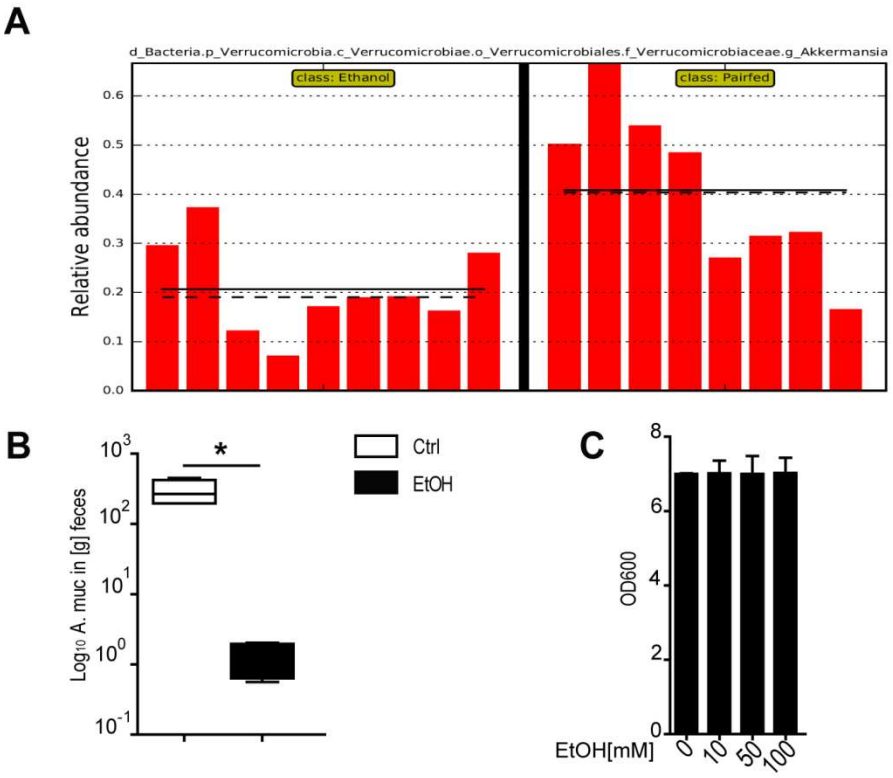
	ASH (n=21)		severe ASH (n=15)	
	mean	sd	mean	sd
Age [yr]	50,9	10,04	55,13	11,95
sex (M/F)	17/4		13/2	
Body hight [kg]	1,7	0,08	1,73	0,09
Body weight [m]	71,67	13,05	78,54	21,25
BMI [kg/m2]	24,57	3,37	26,37	6,41
alcohol_intake [g/d]	179,24	93,83	91,43	33,71
alc_time [yr]	19,53	13,54	24,08	11,06
Smoking (yes/no)	15/6		11/4	
smoking [yr]	22,25	17,65	15	15,27
diabetes (yes/no)	2/19		2/13	
Creatinine [µmol/L]	72,2	14,5	89,87	44,38
CRP [mg/L]	17,68	20,66	31,36	32,49
AST [IU/L]	159,48	128,04	290,2	661,64
ALT [IU/L]	79,38	69,42	79,53	116,54
Total bilirubin [µmol/L]	45,52	45,57	216,33	200,16
GGT [IU/L]	911,62	1129,48	371	275,73
Triglycerides [g/L]	1,67	1,64	1,15	0,52
Blood glucose [mmol]	5,75	2,03	5,63	1,19
Albumin [g/L]	35,87	7,41	27,62	4,48
PT [%]	159,33	105,35	111,53	105,58
INR	79,43	22,93	34,93	9,94
Factor V	1,31	0,58	2,56	0,56
Maddrey discriminant function	89,81	25,64	48,8	20,05
Fibrotest-Score	23,84	18,16	54,85	18,02
Elastometry	0,64	0,3	0,95	0,06
Albumin	16,26	9,71	20	NA
Child-Pugh-Score (A/B/C)	2/2/3		0/2/12	
AHT (yes/no)	3/18		3/12	
PPI (yes/no)	6/14		5/10	
Lactulose (yes/no)	2/19		0/15	

Supplementary Table 3: Correlations of fecal *A. muciniphila* abundance with patient characteristics

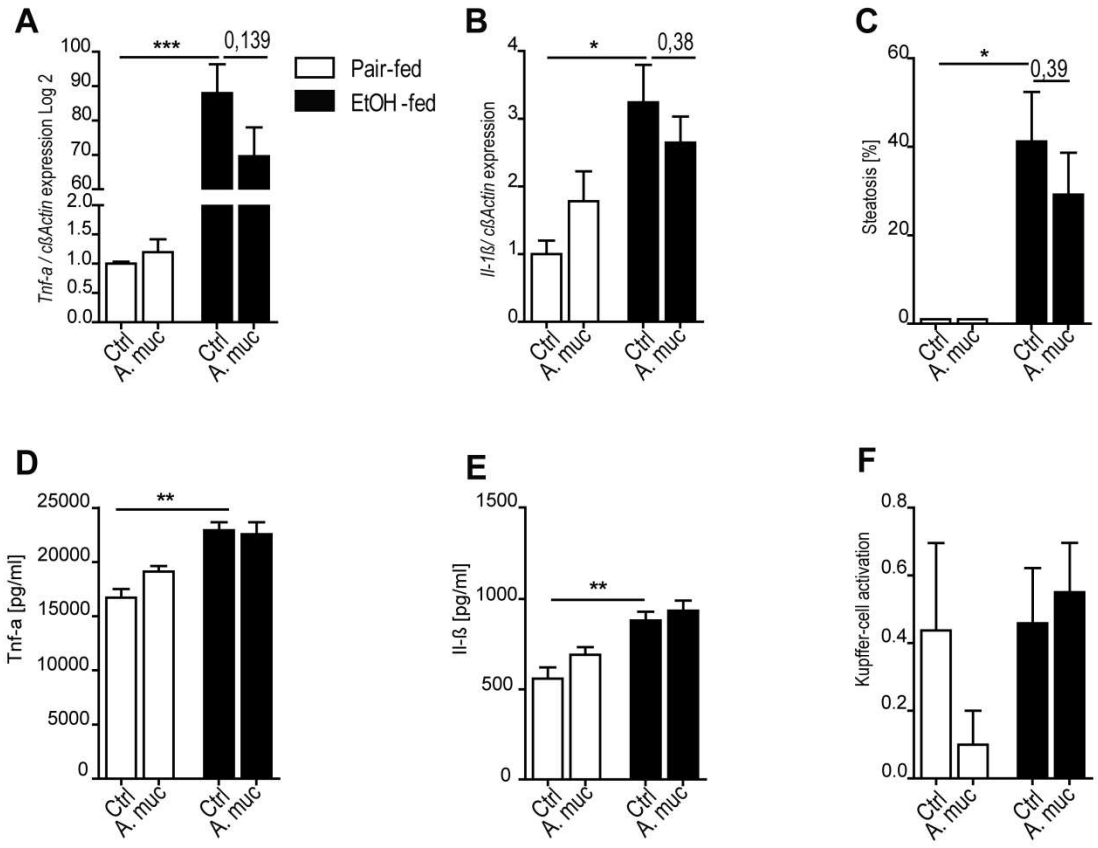
	Severe ASH (n=15)		ASH (n=21)		severe ASH + ASH	
	r	**p**	**r**	**p**	**r**	**p**
Age	0.01	0.96	-0.07	0.76	-0.10	0.56
BMI	0.00	1.00	0.62	*0.00*	0.27	0.12
Alcohol intake	-0.21	0.47	-0.09	0.70	0.06	0.72
alcohol_time	-0.25	0.43	-0.02	0.93	-0.08	0.68
Na^+	-0.12	0.68	0.01	0.97	0.12	0.49
Creatinine	0.42	0.12	-0.04	0.88	-0.04	0.80
CRP	0.01	0.96	0.46	0.05	0.19	0.30
AST	-0.14	0.63	0.00	0.98	-0.05	0.76
ALT	-0.15	0.60	0.08	0.75	0.03	0.86
Total bilirubin	0.53	*0.04*	-0.22	0.35	-0.16	0.34
GGT	0.03	0.91	-0.22	0.34	-0.11	0.51
Triglycerides	0.37	0.24	0.08	0.73	0.13	0.48
LDL	0.31	0.45	0.55	*0.02*	**0.56**	***0.00***
HDL	-0.33	0.39	0.56	*0.01*	**0.58**	***0.00***
Total cholesterol	-0.22	0.50	-0.15	0.53	-0.03	0.89
Blood glucose	-0.23	0.48	-0.14	0.55	-0.13	0.50
Albumin	0.01	0.97	0.19	0.40	0.28	0.11
PT	0.57	0.03	0.11	0.63	0.29	0.08
INR	-0.27	0.36	-0.10	0.65	-0.27	0.12
Factor V	-0.10	0.72	0.10	0.68	0.24	0.16
Maddrey-Score	-0.35	0.22	-0.24	0.50	-0.35	0.10
Fibrotest-Score	0.38	0.36	-0.46	*0.06*	**-0.50**	***0.01***
Elastometry			-0.37	0.26	-0.39	0.22

BMI, body mass index; CRP, C reactive protein; AST, aspartate aminotransferase; ALT alanine aminotransferase; GGT, gamma glutamyl transferase; LDL, low density lipoprotein; HDL, high density lipoprotein; PT, prothrombin time as percentage of control; INR, international normalized ratio; AHT, atrial hypertension; PPI, proton pump inhibitor;

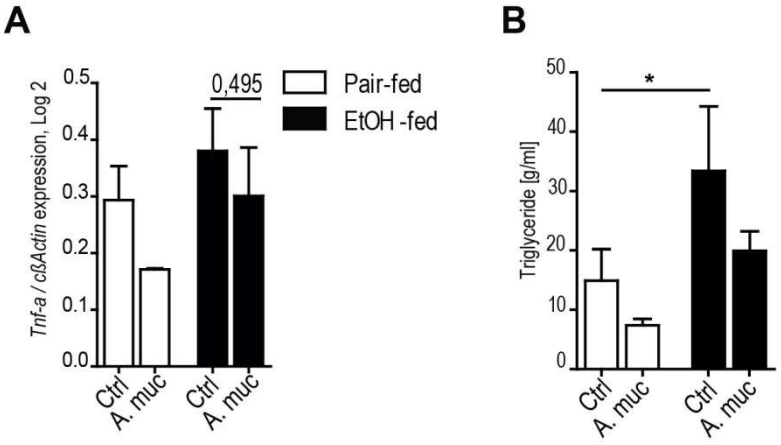
SUPPLEMENTARY FIGURES



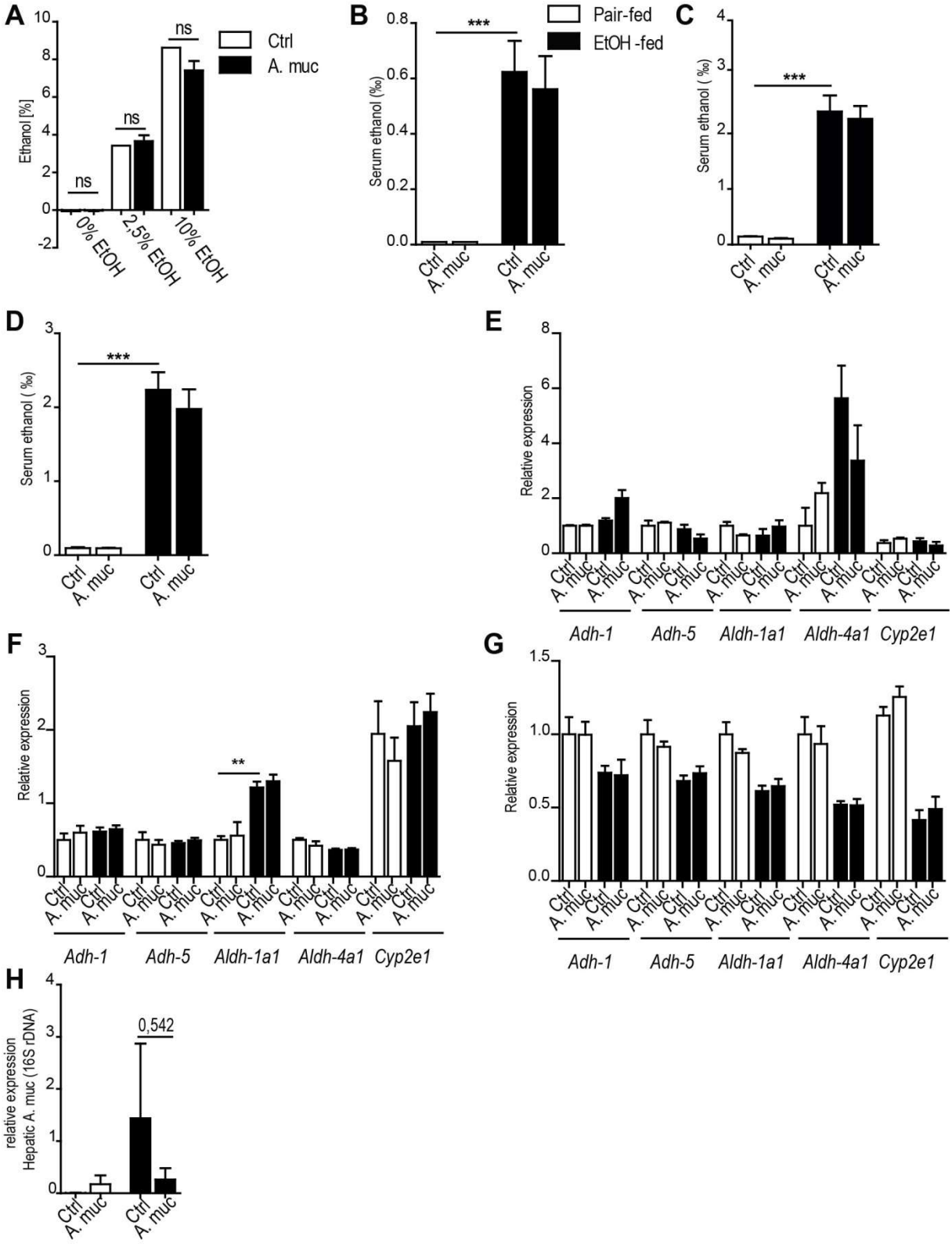
Supplementary Fig. 1: Ethanol depletes fecal *A. muciniphila* abundance. (A) Analysis of taxonomic abundances determined by LEfSe of 16S rDNA sequencing in mice fed the Lieber-DeCarli diet with or without ethanol. Each bar represents a single experimental mouse. (B) *A. muciniphila* quantification in feces of ethanol fed mice, quantified by qPCR. (C) *A. muciniphila* abundance determined by OD600 absorption was analyzed with or without stimulation with indicated EtOH concentrations *in vitro* n: EtOH-fed = 9; Pair-fed = 8, (B) EtOH-fed = 5; Pair-fed = 4, (C) 3 per group. *P < 0.05; **P<0.01; ***P<0.001 according two-tailed Student's *t*-test (B).



Supplementary Fig. 2: Hepatic inflammation in the chronic ALD model. Expression of *Tnf-α* (A) and *Il-ß* (B) relative to *β-Actin* determined by qPCR. (C) Quantification of lipid droplet accumulation based on histology. Quantification of Tnf-α (D) and Il-1ß (E) by ELISA. (F) Kupffer-cell activation based on histology. Data are shown as means ± SEM; n: (A-C): EtOH-fed = 10, EtOH-fed + A.muc = 10, Pair-fed = 4, Pair-fed + A.muc =4. *P < 0.05; **P<0.01; ***P<0.001 according to One-way ANOVA followed by Newman-Keuls multiple comparison test.

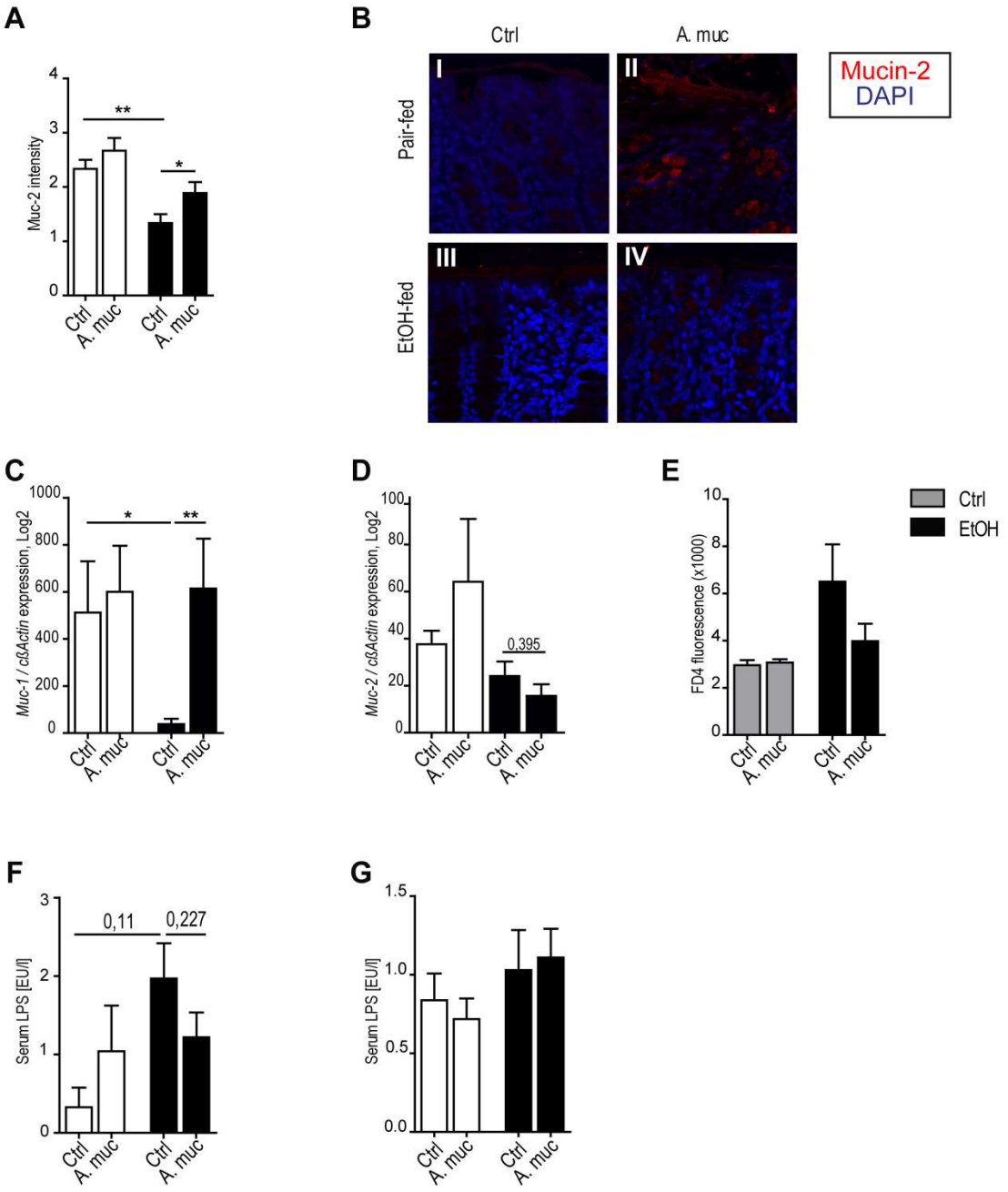


Supplementary Fig. 3: Effects of three oral *A. muciniphila* gavages on steatosis and cytokine expression. (A) Fold expression of *Tnf-α* relative to *β-Actin* determined by qPCR. (B) Biochemical quantification of hepatic triglyceride concentration. Data are shown as means ± SEM; n: (A, B): EtOH-fed = 10, EtOH-fed + A.muc = 10, Pair-fed = 4, Pair-fed + A.muc =4. *P < 0.05; **P<0.01; ***P<0.001 according to One-way ANOVA followed by Newman-Keuls multiple comparison test.



Supplementary Fig. 4: *A. muciniphila* does not metabolize ethanol. (A) *A. muciniphila* was exposed to indicated ethanol concentrations and ethanol turn-over was determined by an enzymatic assay. (B) Serum ethanol concentration in the acute model, (C) preventive chronic

ALD model and (D) therapeutic-chronic ALD model. (E-G) Fold expression of indicated genes involved in ethanol metabolism relative to *β-Actin* in the therapeutic model (E) preventive chronic ALD model (F) and acute model (G). (H) Quantification of *A. muciniphila* 16S rDNA in livers determined by qPCR. Data are shown as means ± SEM; n: (A) 3 per group; (B, G) EtOH = 11, EtOH + A.muc = 12, Ctrl = 5, Ctrl + A.muc =6; (C, F) EtOH-fed = 10, EtOH-fed + A.muc = 10, Pair-fed = 4, Pair-fed + A.muc =4; (D, E) EtOH-fed = 10, EtOH-fed + A.muc = 10, Pair-fed = 4, Pair-fed + A.muc =4. *P < 0.05; **P<0.01; ***P<0.001 according to One-way ANOVA followed by Newman-Keuls multiple comparison test.



Supplementary Fig. 5 Characterisation of gut barrier after *A. muciniphila* administration. (A, B) Quantification and representative confocal images of Muc-2 immunoreactivity (red) from murine colon samples. Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI), blue. (C) Fold expression of *Muc-1* and (D) *Muc-2* in colonic tissue normalized to *β-Actin*. (E) Serum FD4 (fluorescein isothiocyanate dextran 4) concentration of

the *in vivo* gut permeability assay. (F, G) Systemic LPS levels in the therapeutic setting during the Lieber-DeCarli ALD model (F) and the acute model (G). Data are shown as means ± SEM; n: (A-B) EtOH-fed = 10, EtOH-fed + A.muc = 10, Pair-fed = 4, Pair-fed + A.muc =4; (C-D) EtOH-fed = 5, EtOH-fed + A.muc = 6, Pair-fed = 5, Pair-fed + A.muc =5; (E, G) EtOH = 11, EtOH + A.muc = 12, Ctrl = 5, Ctrl + A.muc =6; (F) EtOH-fed = 10, EtOH-fed + A.muc = 10, Pair-fed = 4, Pair-fed + A.muc =4. *P < 0.05; **P<0.01; ***P<0.001 according to One-way ANOVA followed by Newman-Keuls multiple comparison test.

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