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INORGANIC COMPOUNDS

Systemic effects and impact on the gut microbiota upon subacute oral exposure to silver acetate in rats

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Abstract

Context The addition of silver (Ag) to food items, and its migration from food packaging and appliances results in a dietary exposure in humans, estimated to 70–90 µg Ag/day. In view of the well-known bactericidal activity of Ag ions, concerns arise about a possible impact of dietary Ag on the gut microbiota (GM), which is a master determinant of human health and diseases. Repeated oral administration of Ag acetate (AgAc) can also cause systemic toxicity in rats with reported NOAELs of 4 mg AgAc/b.w./d for impaired fertility and 0.4 mg AgAc/b.w./d for developmental toxicity.

Objective The objective of this study was to investigate whether oral exposure to AgAc can induce GM alterations at doses causing reproductive toxicity in rats.

Methods Male and female Wistar rats were exposed during 10 weeks to AgAc incorporated into food (0, 0.4, 4 or 40 mg/kg b.w./d), and we analyzed the composition of the GM (α- and β-diversity). We documented bacterial function by measuring short-chain fatty acid (SCFA) production in cecal content. Ferroxidase activity, a biomarker of systemic Ag toxicity, was measured in serum.

Results and conclusions From 4 mg/kg b.w./d onwards, we recorded systemic toxicity, as indicated by the reduction of serum ferroxidase activity, as well as serum Cu and Se concentrations. This systemic toxic response to AgAc might contribute to explain reprotoxic manifestations. We observed a dose-dependent modification of the GM composition in male rats exposed to AgAc. No impact of AgAc exposure on the production of bacterial SCFA was recorded. The limited GM changes recorded in this study do not appear related to a reprotoxicity outcome.

Keywords Silver · Dysbiosis · Ferroxidase · Ceruloplasmin · Reprotoxicity

Introduction

Silver is a transition metal, existing in the Earth's crust as a sulfide in association with other metals, and is refined as a secondary by-product from the production of Cu, Pb or Zn. The industrial applications of Ag include electrical components, plating, soldering and jewelry. Silver is also used as an antibacterial agent in consumer products, pharmaceuticals, clothes and food items. The antibacterial activity of silver is related to its free monovalent ion (Ag^{+1}) which penetrates the bacterial wall and inhibits bacterial growth (Barras et al. 2018). Different Ag compounds can release Ag^{+1} ions, including highly soluble salts (Ag acetate or nitrate), insoluble compounds such as silver sulfadiazine, and Ag metal nanoparticles which currently appear as an attractive antibacterial form. Food items containing or treated with Ag include water purification systems, toothpaste,

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food packaging material, storage containers, cutting boards and food supplements. The addition of Ag to human food items, and its occurrence in food packaging and appliances results in a dietary exposure, estimated to 70–90 µg Ag/day, approximatively equivalent to 1 µg Ag/kg b.w./day for an adult (Wijnhoven et al. 2009). The US EPA reference dose for Ag is 5 µg/kg b.w./d (EPA 1991). The presence of a bactericidal chemical such as Ag in human diet immediately raises the question of a possible impact on the gut microbiota (GM), which controls human health and diseases. The low intestinal absorption rate (< 10%), and the biliary excretion of Ag (Das et al. 2014) further contribute to the plausibility of this concern.

Several investigators examined the possible impact of Ag compounds on the GM using diverse in vitro and in vivo experimental models, different doses and durations of exposure (Table 1). Most of these studies examined the impact of Ag nanoparticles (Ag NP) of different sizes, with or without coating, at doses varying by several orders of magnitude. The GM alterations recorded in these studies were highly variable, and their overall health significance remains uncertain. A possible health risk might not be excluded based on significant alterations of the GM in mice fed during 28 days with Ag NP at doses relevant for human exposure (µg/kg b.w./d) (van den Brule et al. 2016). Few studies examined the effects of soluble Ag compounds on the GM, mainly included as a soluble reference compound in studies investigating the effect of Ag NP. Hadrup et al. (2012) recorded systemic toxicity as evidenced by weight loss and altered clinical chemistry, but no alteration of the proportions of GM phyla, in rats receiving Ag acetate by gavage (9 mg Ag/kg b.w./d) during 28 days. Williams et al. (2015) reported a reduction of the *Firmicutes*/*Bacteroidetes* ratio in rats gavaged with 100 mg Ag acetate/kg b.w./d during 13 weeks. Wilding et al. (2016) did not observe alterations of the GM in rats gavaged with 10 mg Ag acetate/kg b.w./d during 28 days.

Repeated oral administration of Ag acetate (AgAc) can also cause systemic toxicity, including reprotoxicity. Decreased fertility and developmental toxicity has been recorded in rats exposed to 0.4, 4 or 40 mg AgAc/kg b.w./d via drinking water for 10 weeks, prior to and during mating (both sexes), and during the gestation and lactation periods for females (Sprando et al. 2017).The NOAELs in this study were 4 mg AgAc/b.w./d for impaired fertility and 0.4 mg AgAc/b.w./d for developmental toxicity.

A role of the GM in mediating or influencing the (repro) toxic activity of chemicals has been suggested (Wang et al. 2020). Therefore, the aim of this study was to investigate whether oral exposure to AgAc can induce GM alterations at doses that caused reproductive toxicity in rats. We exposed male and female rats during 10 weeks to AgAc incorporated into food, and analyzed the composition of the GM (α- and β-diversity) as well as bacterial function (short-chain fatty acid production). We also documented the possible systemic impact of AgAc exposure by measuring serum ferroxidase activity, a biomarker of Ag toxicity (Hirasawa et al. 1994).

Materials and methods

Test chemical

Silver(I) acetate (AgAc, purity > 99.5%) was obtained from Heraeus (Hanau, Germany).

Animals

Four-week-old specific pathogen-free Wistar rats (RjHan:WI, 40 females and 40 males) were obtained from Janvier (Saint-Berthevin, France) and acclimatized to the local animal facility under standard conditions (22 ± 2 °C, 50% relative humidity, 12-h/12-h light cycles, fed Altromin standard diet 1324 [Carfil, Turnhout, Belgium] and water ad libitum).

Study design

After acclimation (1 week) and homogenization of the GM (2 additional weeks), the rats were exposed for 10 weeks to AgAc at target intake doses of 0, 0.4, 4 or 40 mg/kg b.w./d. AgAc was incorporated by the food producer to Altromin standard diet 1324 pellets. Considering an average b.w. of 200 or 300 g and an average daily consumption of 20 or 30 g food/d for females or males, respectively, AgAc was added to the diet at fixed concentrations of 4, 40 and 400 mg AgAc/kg food for both sexes. These pellet concentrations were verified by measuring Ag in each preparation by ICP-MS, which were close to nominal concentrations (≥ 90%). As Ag in food can be transformed into insoluble sulfides, we verified the stability of AgAc in the pellets according to van den Brule et al. (2016). No loss of water-soluble Ag in food pellets was recorded up to 7 weeks after preparation, excluding AgS formation. The food producer prepared a fresh batch of AgAc pellets at the start of the exposure, and after 4 and 8 weeks of exposure, so that each batch was used during maximum 1 month after preparation.

The experimental groups were assembled by randomly allocating two animals in five cages per sex and per dose (10 animals/dose/sex). The animals were identified by an ear tag, and followed individually throughout the experiment. The initial body weight of the animals was homogeneous over the different dose groups (Supplemental file—Fig. S1). Animals were fed the experimental diet, received water ad libitum, and were monitored daily for health status by an

Table 1 Summary of experimental studies that examined the possible impact of Ag compounds on the gut microbiota

Ag form			Species or model	Exposure			Effects			Technique	References
Chemical composition	Coating	Size (nm)		Mode	Dose	Duration	Bw	Organs	Microbiota		
Colloidal Ag	No	60–100	Pigs	Diet	0.59 & 1.4 mg/kg bw/d	14 d	↗	No effect (ileal mucosa)	↘ Total bacteria	Bacterial counts + FISH w/ 16S RNA oligont	(Fondevila et al. 2009)
			Pig ileal content	In vitro	25–100 µg/g	4 h			↘ Coliforms and lactobacilli		
AgNP	PVP	14	Rats	Gavage	2.25, 4.5 & 9 mg/kg bw/d	28 d	=	No effect	No effect	qPCR degenerated phyla primers	(Hadrup et al. 2012)
AgAc					9 mg/kg bw/d	28 d	↘	ALP ↗	No effect		
AgNP			Zebrafish	Diet	500 mg/kg food	14 d	ND	No effect (intestinal epithelium)	Weak effect (not clear)	DGGE of 16S RNA	(Merrifield et al. 2013)
AgCl					500 mg/kg food	14 d	ND	No	Weak effect (not clear)		
AgNP	PVP	10	Human stools	In vitro	25, 100 & 200 mg/l	48 h			↘ Bacteroidetes ↗ Proteobacteria	16S RNA sequencing (Roche 454)	(Das et al. 2014)
AgCl					2.5, 10 & 20 mg/l				Weaker effects		
AgNP		20	Drosophila larvae	Diet	450 µg/ml	?	↘ Development		↗ Lactobacillales (Firmicutes) ↘ Rhodospirillales, Sphingomonadales, Rhizobiales (Proteobacteria)	16S RNA sequencing (Roche 454)	(Han et al. 2014)
AgNP	SDS	1–27	Oreochromis niloticus L. (tilapia)	In water	0.8 and 0.4 mg/l	21 d	ND	Thinning of intestinal wall, swelling on mucosal layer	Dose-dependent depletion	Bacterial cell count	(Sarkar et al. 2015)

Table 1 (continued)

Ag form			Species or model	Exposure			Effects			Technique	References
Chemical composition	Coating	Size (nm)		Mode	Dose	Duration	Bw	Organs	Microbiota		
AgNP	Citrate	10, 75 and 110	Rats	Gavage	9, 18 & 36 mg/kg bw/d	13 w	ND#	ND#	Variable results not related to sex, dose or AgNP size ↘ Firmicutes/Bacteroidetes w/ 10 nm AgNP	qPCR	(Williams et al. 2015)
AgAc					100 mg/kg bw/d				↘ Firmicutes/Bacteroidetes		
AgNP	PVP citrate	20 and 110	Mice	Gavage	10 mg/kg bw/d	28 d	ND	ND	No effect	16S RNA sequencing (Roche 454)	(Wilding et al. 2016)
AgAc							ND	ND	No effect		
AgNP	PVP	55	Mice	Diet	11.4, 114 & 1140 µg/kg bw/d	28 d	=	No effect	↗ Firmicutes/Bacteroidetes ↘ in Odoribacteraceae, Bacteroidaceae and S24-7 ↗ Lactobacillaceae and Lachnospiraceae	16S RNA sequencing (Ion Torrent)	(van den Brule et al. 2016)
AgNP spheres	PVP	50	Rats	Gavage	3.6 mg/kg bw/d	14 d	=	Effect on behavior	↘ Oscillospira spp., Dehalobacterium spp., Peptococcaceae, Corynebacterium spp., Aggregatibacter pneumotropica	16S RNA sequencing (Illumina)	(Javurek et al. 2017)

Table 1 (continued)

Ag form			Species or model	Exposure			Effects			Technique	References
Chemical composition	Coating	Size (nm)		Mode	Dose	Duration	Bw	Organs	Microbiota		
AgNP cubes	PVP	45							↘ Clostridium spp., Bacteroides uniformis, Christensenellaceae, and Coprococcus eutactus		
AgNP		12.2	Mice	Gavage	2.5 mg/kg bw/d	7 d	↘	Inflammation, colitis	↘ Firmicutes/Bacteroidetes ↘ Lactobacillus ↗ Alistipes, Bacteroides and Prevotella	16S RNA sequencing (Roche 454)	(Chen et al. 2017)
AgNP		122 and 294	Mice (colitis model)	Gavage	50 mg/d	4 d	ND	↘ Colitis	↘ E. coli and Clostridium perfringens, ↗ Lactobacillus sp.	Bacterial cell count	(Siczek et al. 2017)

[#]In a companion study from the same research group, conducted with similar materials, administration mode and doses, significant reduction of b.w. in male and female rats gavaged with AgAc were recorded, no effet on b.w. was observed with AgNP. Altered RBC parameters were recorded in rats treated with AgAc suggestive of disrupted Fe homeostasis. Intestinal and thymus histopathological lesions were noted in the highest-AgAc dose group. (Cani 2013)

ND not detailed

accredited technician. Body weight was recorded weekly and food consumption was measured weekly per cage.

After 28 days of exposure, blood was collected by puncture of the venous tail under anesthesia to analyze Ag in whole blood, and ferroxidase activity, Cu and Se in serum. Blood samples were collected in an EDTA-anticoagulated tube (Microvette 200 K3E, Sarstedt), and in a dry tube for serum measurements. Blood and serum were stored at − 20 °C until analyses. Feces were also collected in the cages at day 28 to measure Ag content.

At sacrifice, blood and serum were also collected to measure internal Ag exposure (whole blood Ag) and related biochemical parameters (ferroxidase activity, Cu and Se in serum). The thymus, liver, spleen, right kidney, right testis, right ovary + uterus (as reliable separation of the ovary from the uterus was not possible) were dissected and weighed. Relative organ weights were calculated based on b.w. at sacrifice. Cecal content was collected to analyze the GM composition (16S sequencing) and function (short-chain fatty acid content).

The overall experimental design is sketched in Fig. 1.

Sample preparation and Ag analyses

The method to reliably measure Ag in blood, feces and food samples by ICP-MS (Agilent 7050ce), using the ^{107}Ag isotope with helium and ^{103}Rh as internal standard was adapted from Loeschner et al. (2011). A calibration curve (0, 10, 50, 250, 500, 5000 µg Ag/L) was included in each run. Whole blood tubes were thawed at room temperature with the addition of 1% Triton X100, and an exact weight of blood was mineralized in 6 mL 65% HNO_3 in a microwave (Multiwave GO, Anton Paar, The Netherlands). For food and feces, a sample of approximately 250 mg was mineralized with the same procedure as for blood. Six mL 30% HCl was added after mineralization (blood, food, feces), and the total was finally diluted fourfold with 2% HNO_3 before ICP-MS measurement. If appropriate, the samples were further diluted with 2% HNO_3 to fit the calibration range. The proficiency of this procedure was validated with a certified reference

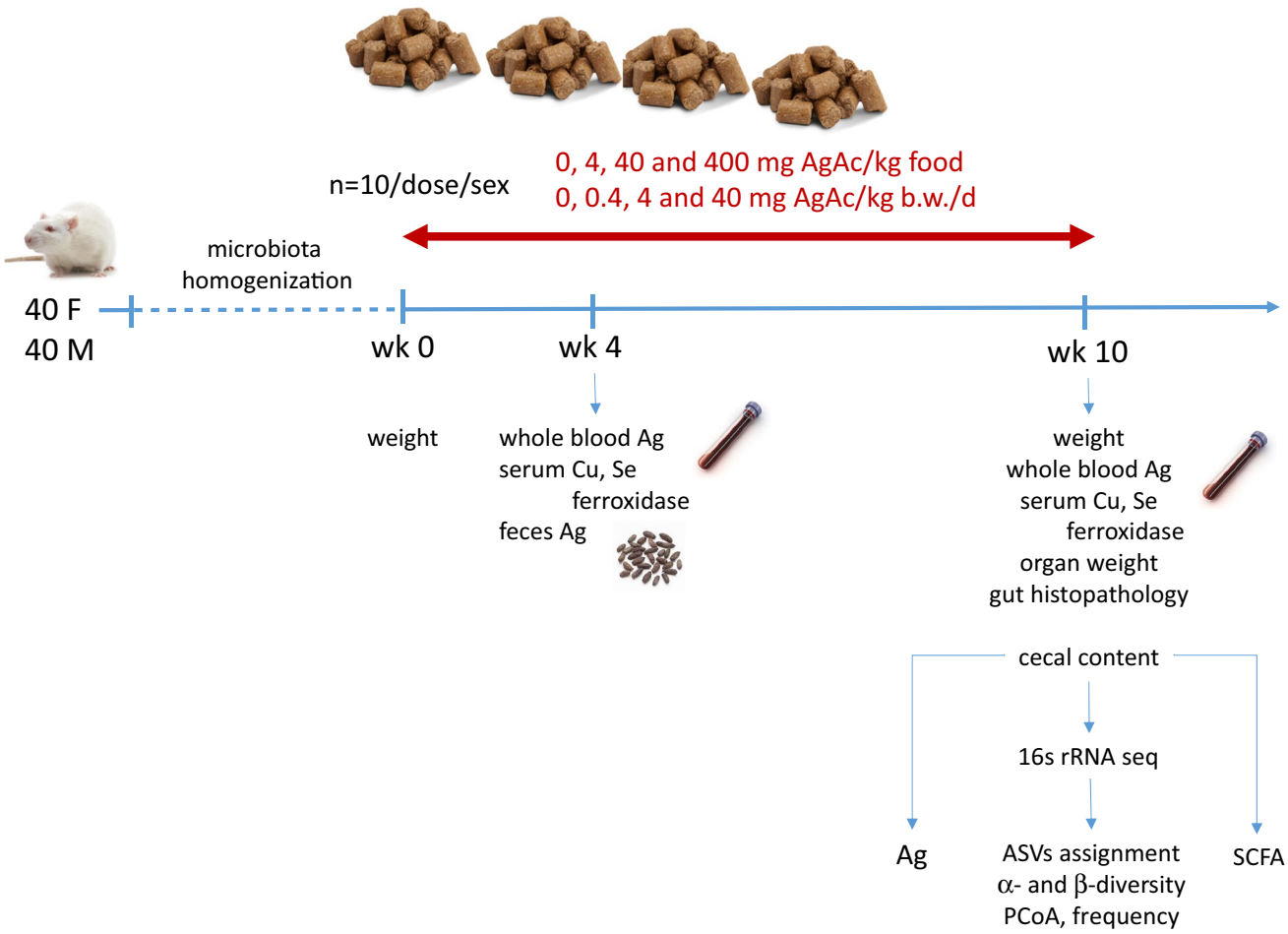

Fig. 1 Outline of the experimental design

material (Dogfish liver DOLT-5 from NRC-CNRC, Canada) with a target concentration of 2.05 ± 0.08 mg Ag/kg. The accuracy of the method was excellent, with a recovery in three replicates close to 100%. The repeatability of the measurement was checked by measuring the same sample 30 times, yielding a variation coefficient < 1.3%.

Cu and Se were measured in serum samples collected at day 28 and at sacrifice by means of a method using an Agilent 7500cx instrument with helium collision mode and H2 reaction mode for Cu and Se quantification, respectively (ISO15189 certified method for both trace elements in plasma/serum matrix). Briefly, serum samples (500 µL) were diluted quantitatively (1 + 9) with a solution of 1-butanol (2%w/v), EDTA (0.05%w/v), Triton X-100 (0.05%w/v), NH_4OH (1%w/v) containing Sc, Ge, Rh and Ir as internal standards.

Ferroxidase activity was measured in serum with a commercial colorimetric assay by following the instructions of the manufacturer (Invitrogen).

Cecal DNA extraction and 16S rRNA gene sequencing

Gut microbial populations were analyzed by Next Generation Sequencing (NGS) of DNA extracted from cecal contents (QIAmp Fast DNA stool kit, Qiagen #51604). The DNA concentration of each sample was measured spectrophotometrically, and the samples were sequenced at MRDNA lab (Shallowater, TX) on an Illumina MiSeq platform with specific primers for the variable region of the bacterial 16S rRNA gene (2 × 300 bp PE Illumina 20 K sequence diversity assay).

Sequencing data analysis

All NGS reads were processed using R 3.6.3 and the DADA2 Bioconductor package (Callahan et al. 2016). Sequences were trimmed and filtered to tolerate a maximum of 3 expected errors per paired ends read. Amplicon Sequence Variants (ASV) were inferred using the high-resolution DADA2 method, which distinguishes sequencing errors from real biological variation. Chimeras and low abundance ASV making up < 0.002% of reads were subsequently removed from the data set (Bokulich et al. 2013). Taxonomy was assigned with a naive Bayesian classifier implemented in the DADA2 package, using the Silva (v138) training set. Sequencing data were rarefied at sequencing depth of 5000 using the phyloseq Bioconductor package. The α-diversity indexes (richness, Simpson, and Shannon) in the microbial communities were calculated using the phyloseq Bioconductor package. The impact of AgAc dose on α-diversity indexes was assessed through linear models with AgAc dose introduced as a continuous variable. To investigate differences in community composition (β-diversity), weighted UniFrac distances were calculated and plotted using principal coordinates analysis with the GUniFrac R package. The linear effect of AgAc dose on β-diversity was assessed through a permutational multivariate analysis of variance (PERMANOVA) with AgAc dose introduced as a continuous variable, using the ADONIS function of the vegan package in R. To identify the bacterial genera, families and phyla impacted by AgAc exposure, ASV were grouped at the family and phylum levels. The counts were then normalized to their relative abundance before applying centered log-ratio (CLR) transformation for compositional data, as described previously (Kong et al. 2020). Linear models were built on the transformed data with AgAc dose introduced as a continuous variable. When the effect of AgAc dose was assessed on multiple outcomes (e.g., relative abundance of several families), *p* values were corrected (*Q* values) using the Benjamini–Hochberg methodology to control the false discovery rate (FDR) at 10% (Benjamini and Hochberg 1995).

Quantification of SCFA

For SCFA analysis, after addition of valproic acid (used as internal standard), the cecal content was homogenized in water and proteins precipitated with acetonitrile. Following centrifugation, the supernatant was recovered and reacted with 3-nitrophenylhydrazine in the presence of 1-ethyl-3-(3′-dimethyl-aminopropyl)carbodiimide hydrochloride (EDC) and pyridine. Following liquid–liquid extraction to remove excess reagents, the final residue was analyzed on an LTQ Orbitrap XL mass spectrometer coupled to an Accela HPLC system (ThermoFisher Scientific). A Hypersil GOLD PFP (100 × 2.1 mm; 1.9 µm) column and a gradient of water–acetonitrile–acetic acid and acetonitrile–acetic acid was used to separate the different isomers. For ionization, an APCI probe was used in positive mode. Calibration curves were prepared using the same conditions. Xcalibur® software was used for data analysis. For each cecal content, an aliquot was freeze-dried to determine a dry residue weight that was used for data normalization.

Statistics

With the exception of sequencing data, graphs and statistical analyses were computed with GraphPad Prism 8.4.2 (GraphPad Software, San Diego, USA). Results are expressed as arithmetic means or arithmetic means ± standard error on the mean (SEM). Differences between control and treated groups were evaluated by analysis of variance (ANOVA) or mixed-effect model (missing values in male rats) followed by a Dunnett's multiple comparison and/or a test for linear trend as appropriate. Statistical significance was considered at *P*value < 0.05.

Results and discussion

General health, body weight and food consumption monitoring.

Two animals died during the course of exposure. One rat (male 400 mg AgAc/kg food) was found dead at the end of week 3. Necropsy did not reveal any evident abnormality, and death was considered incidental. The second rat (male 40 mg AgAc/kg food) died directly after the blood sampling procedure on day 28, and death was attributed to the procedure. No overt sign or symptom of toxicity was recorded in any dose group during the exposure period. No specific alteration of the feces (quantity, aspect or color) was noted.

In female rats, AgAc exposure slightly affected the body weight gain (< 5% change compared to controls), but a dose-dependent response was not evident (Fig. 2a). In male rats, body weight gain was dose-dependently reduced by the exposure; the values were significantly reduced in the high-dose group compared to controls (average effect, Dunnett's test $p = 0.0087$).

Food consumption per animal (not adjusted to b.w.) in female (Fig. 3a) and male rats (Fig. 3b) was consistent with assumptions for calculating food concentrations (see materials and methods; i.e., 20 and 30 g/d, respectively). AgAc exposure did not affect food consumption.

Based on measured body weight and food consumption, we calculated actual intakes using individual b.w. and cage food consumption, both averaged over the 10 week of exposure (Table 2). Actual intakes were 25–35% lower than target doses, reflecting the progressive b.w. increase but stable (or even decreasing) food consumption over the exposure period. Elemental Ag intake was approximately 25-fold higher than in the study of van den Brule et al. (2016) with metallic Ag nanoparticles.

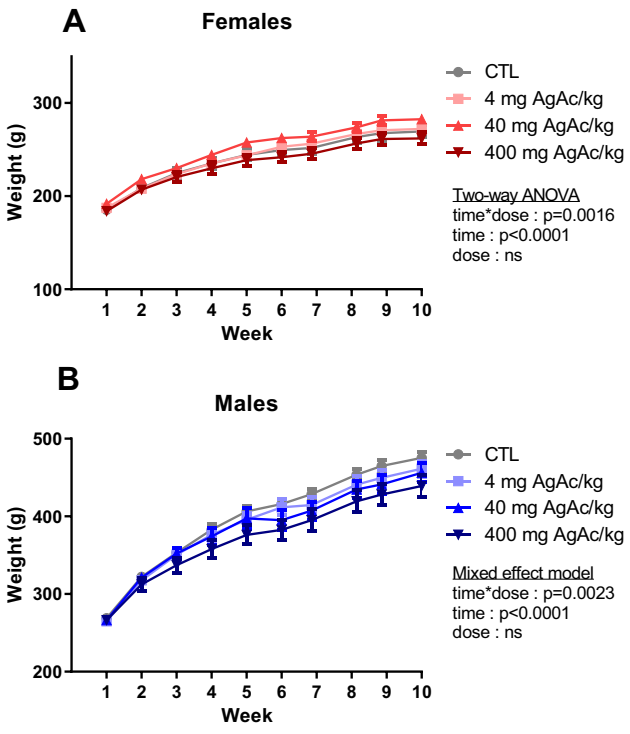

Fig. 2 Body weight gain in female (**a**) and male (**b**) rats orally exposed to Ag acetate (means ± SEM, small SEM are not visible)

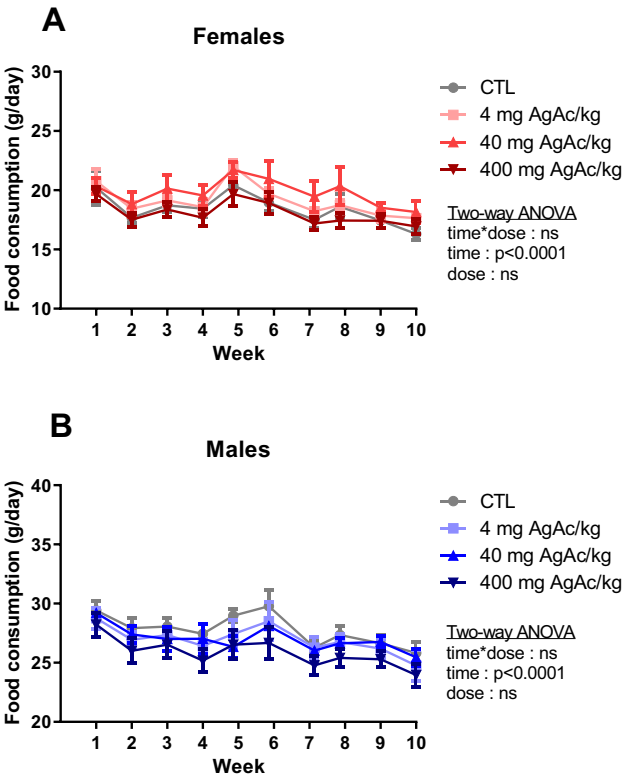

Fig. 3 Food consumption in female (**a**) and male (**b**) rats orally exposed to Ag acetate (means ± SEM; small SEM are not visible)

Table 2 Comparison of target and actual Ag intake in female (F) and male (M) rats

Target intake (mg AgAc/kg bw/d)		0.4	4	40
Actual intake[1] (mg AgAc/kg bw/d)	F	0.3	3.1	27.8
	M	0.3	2.8	25.4

[1]Based on food consumption and body weight averaged over the 10 weeks

AgAc exposure did not significantly affect absolute or relative organ weights, except a reduction of absolute, but not relative liver weight at the highest dose in males (not shown)

Exposure measurements

The Ag impregnation of the animals was documented early after the initiation of exposure (4 weeks) and at the end of the experiment (10 weeks).

At day 27, cage beddings were renewed, and Ag content was measured in cage feces collected at day 28. Cecal contents were collected at sacrifice and analyzed for Ag content. Both at day 28 and at sacrifice (Supplemental file—Fig. S2), fecal and cecal Ag concentrations were dose-dependently increased (not statistically significant at the lowest dose).

Figure 4 depicts blood Ag concentrations at day 28 (upper panel). Female and male controls had similar Ag blood concentrations (t test, $p = 0.531$). In the AgAc-treated groups, females had slightly higher blood concentrations than males; this difference was already statistically significant at the lowest dose (0.0083 versus 0.0050 µg/g blood; t test, $p = 0.0024$). A dose-dependent increase of whole blood Ag concentration was recorded in both sexes. Statistically significantly increased concentrations relative to controls were recorded in groups exposed to 40 and 400 mg AgAc/kg food, in both sexes.

At sacrifice (Fig. 4 bottom), Ag blood concentrations in the AgAc-exposed groups were slightly higher than at day 28 (+30% in males, +65% in females), suggesting a saturation phenomenon (Supplemental file—Fig. S3). Trends for dose–effect were similar at sacrifice and at day 28. At the highest dose, Ag blood concentrations were statistically higher in females than in males. Individual blood Ag concentrations strongly correlated with cecal Ag content at sacrifice ($R = 0.887$; $p < 0.001$; Pearson correlation). The sex-related difference in Ag blood concentrations observed in the present study is consistent with previous toxicokinetic studies with Ag compounds reporting higher blood Ag concentrations in female than in male rats (Boudreau et al. 2016).

Ceruloplasmin is the major copper-carrying protein in the blood of mammals, and promotes Fe^{2+} oxidation (ferroxidase activity), thus contributing to iron transport in the plasma. As Ag exposure can alter Cu homeostasis, and decreases blood ferroxidase activity without affecting ceruloplasmin protein concentration (Hirasawa et al. 1994), we measured ferroxidase activity in the serum of rats exposed to AgAc (Fig. 5). The volume of serum was insufficient in 10 tubes collected at day 28. As expected (Xu et al. 2017), ferroxidase activity was higher in females than in males at both time points. A dose-dependent reduction of ferroxidase activity was recorded in both sexes, somewhat stronger at sacrifice than at day 28. Individual ferroxidase activity

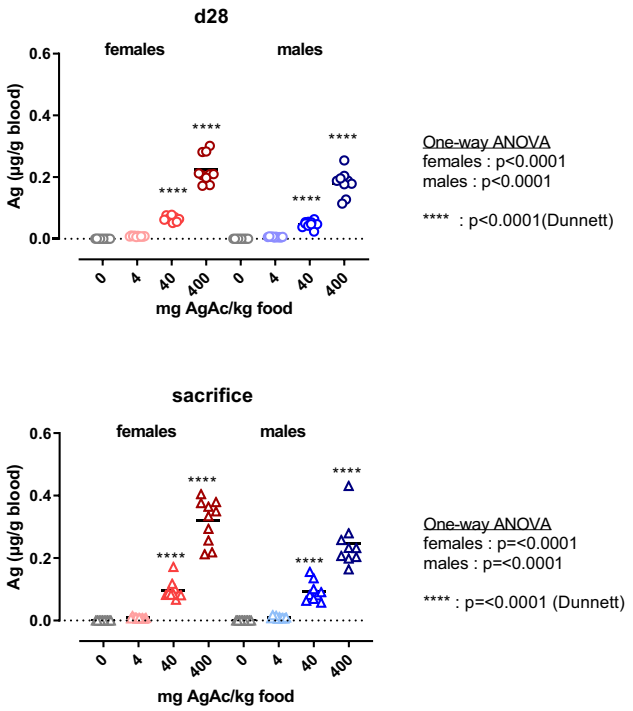

Fig. 4 Blood Ag concentrations at day 28 and at sacrifice. Each symbol represents an animal, horizontal bars reflect group means. Differences are calculated relative to the controls of the same sex

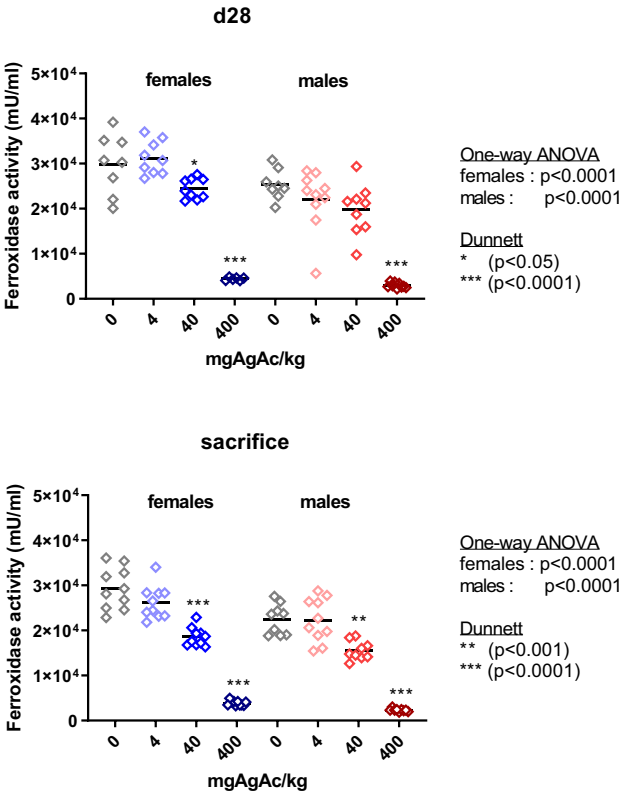

Fig. 5 Serum ferroxidase activity at day 28 (top) and at sacrifice (bottom). Each symbol represents an animal, horizontal bars reflect group means, and differences are calculated relative to the control group of the same sex

values negatively correlated with blood Ag concentrations both at day 28 and at sacrifice ($R = -0.819$, $p < 0.0001$ and $R = -0.623$, $p < 0.0001$, respectively).

Cu and Se were measured in serum samples collected at day 28 and at sacrifice (Fig. 6). As expected (Milne and Johnson 1993), control females had higher serum Cu values than males at both time points. A significant reduction of Cu values (40–50% of control values) was observed in the highest dose group, in both sexes and at both time points. Control females had lower serum Se values than males at both time points. A significant reduction of Se values (50–70% of control values) was observed in the highest dose group, in both sexes and at both time points. Individual ferroxidase activity values (M + F) strongly correlated with serum Cu and Se concentrations at day 28 ($R = 0.933$, $p < 0.0001$ and $R = 0.688$, $p < 0.0001$, respectively) and at sacrifice ($R = 0.921$, $p < 0.0001$ and $R = 0.629$, $p < 0.0001$, respectively).

Thus, these results confirm that high exposure to Ag can affect the metabolism of Cu in rats as reported previously (Ilyechova et al. 2014). This reduction of serum Cu concentration appears to result from the competition between Ag and Cu ions for binding to apo-ceruloplasmin. This Cu depletion of apo-ceruloplasmin affects its transformation into holo-ceruloplasmin, and leads to a strong reduction of its ferroxidase activity (Sugawara and Sugawara 2000). Previous studies on the impact of Ag on Cu metabolism and ferroxidase activity generally used a single acute dose administered by injection (generally i.p.). We report here the effect of a subacute oral administration, with a dose–response relationship, including a NOAEL (4 mg AgAc/kg food, approximatively equivalent to 0.4 mg AgAc/kg b.w./d). This systemic toxicity in male and female rats is important because it can contribute to explain the reprotoxic effects observed by Sprando et al. (2017) at the same dose levels. Indeed, defective ferroxidase activity and Cu deficiency have been reported to cause developmental toxicity. Embryotoxic effects have been observed in rats following dietary administration of Ag chloride throughout the entire period of gestation. It was suggested that this may involve a mechanism whereby an Ag-modified, functionally inactive ceruloplasmin unable to transport copper or oxidize Fe, can lead to secondary embryotoxicity, as a result of a disruption of maternal copper homeostasis (Shavlovski et al. 1995).

Along with Cu, Se is an essential trace element for normal reproduction (Pal 2015). Yoshida (1993) has previously described a depression of plasma and tissue Se levels following 6-week exposure to AgAc (250 µg/kg food). Similar

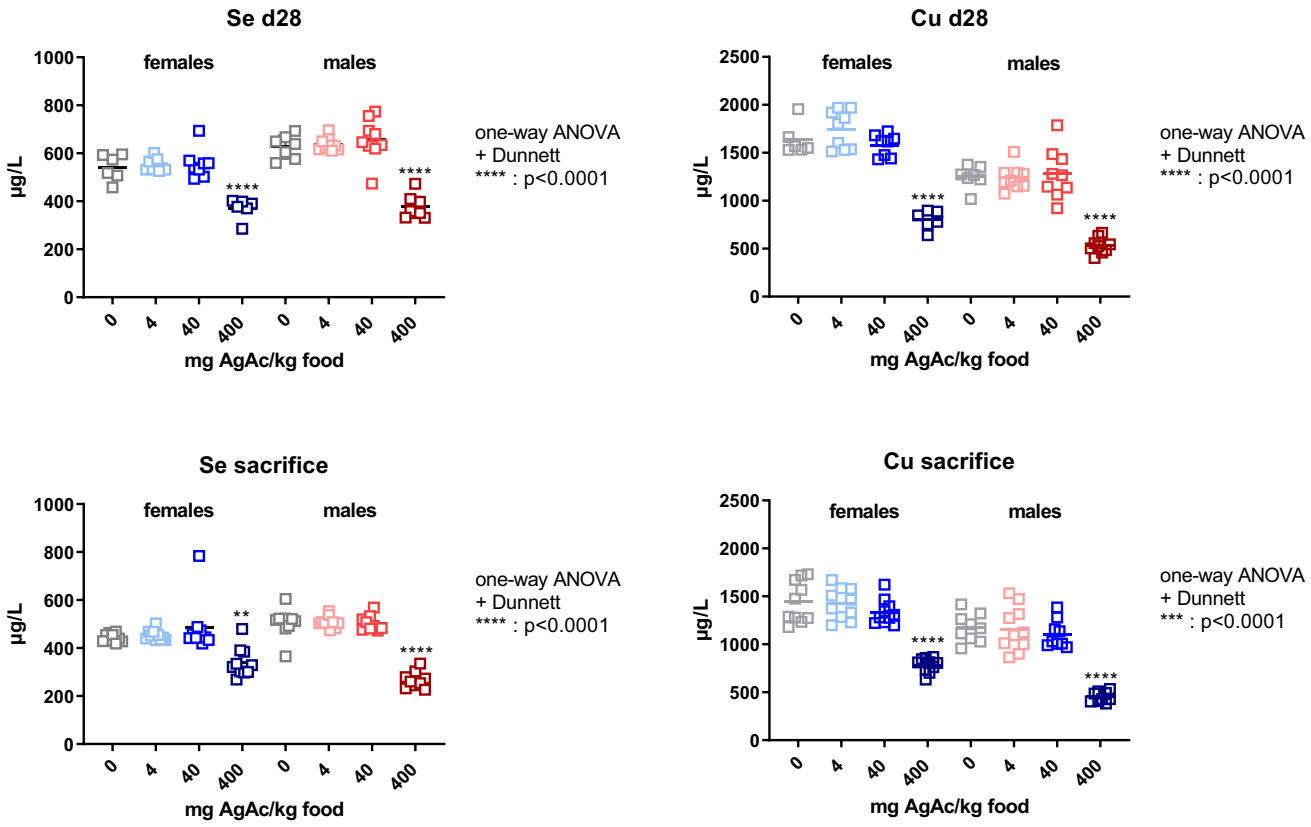

Fig. 6 Serum Cu and Se concentration at day 28 (top) and at sacrifice (bottom). Each symbol represents an animal ($n = 10$ per group), horizontal bars reflect group means; differences are calculated relative to the control group of the same sex

depression of the Se-containing GSH peroxidase activity was recorded in the same tissues. No effect on animal growth or food consumption was reported, but reproductive functions were not examined in this study.

Gut microbiota analyses

Alpha-diversity

We first analyzed the richness and evenness of the microbial communities (within groups). Richness, i.e., the number of ASVs in a sample, is the simplest metric used to represent diversity. Simpson and Shannon indices account for richness and evenness in the populations. Evenness represents the degree to which individuals are split among ASVs (Morris et al. 2014). While no significant influence of AgAC exposure on α-diversity was recorded in female rats, dose-dependent reductions of richness and Simpson indices were recorded in male rats (Fig. 7).

Beta-diversity analysis

Beta-diversity accounts for the degree of variation in ASV composition among groups (between-group diversity). We assessed diversity with the UniFrac procedure, which calculates a distance metric to compare individuals. It incorporates information on the relative relatedness of ASVs by including phylogenetic distances between ASVs in the computation. A principal coordinate analysis (PCoA) was then applied to visualize proximity among the samples (Fig. 8). ADONIS (Permutational Multivariate Analysis of Variance) and linear trend tests were applied to assess the statistical significance of differences in distance among experimental groups. ASV composition was dose-dependently affected by AgAc exposure in male but much less significantly in female rats.

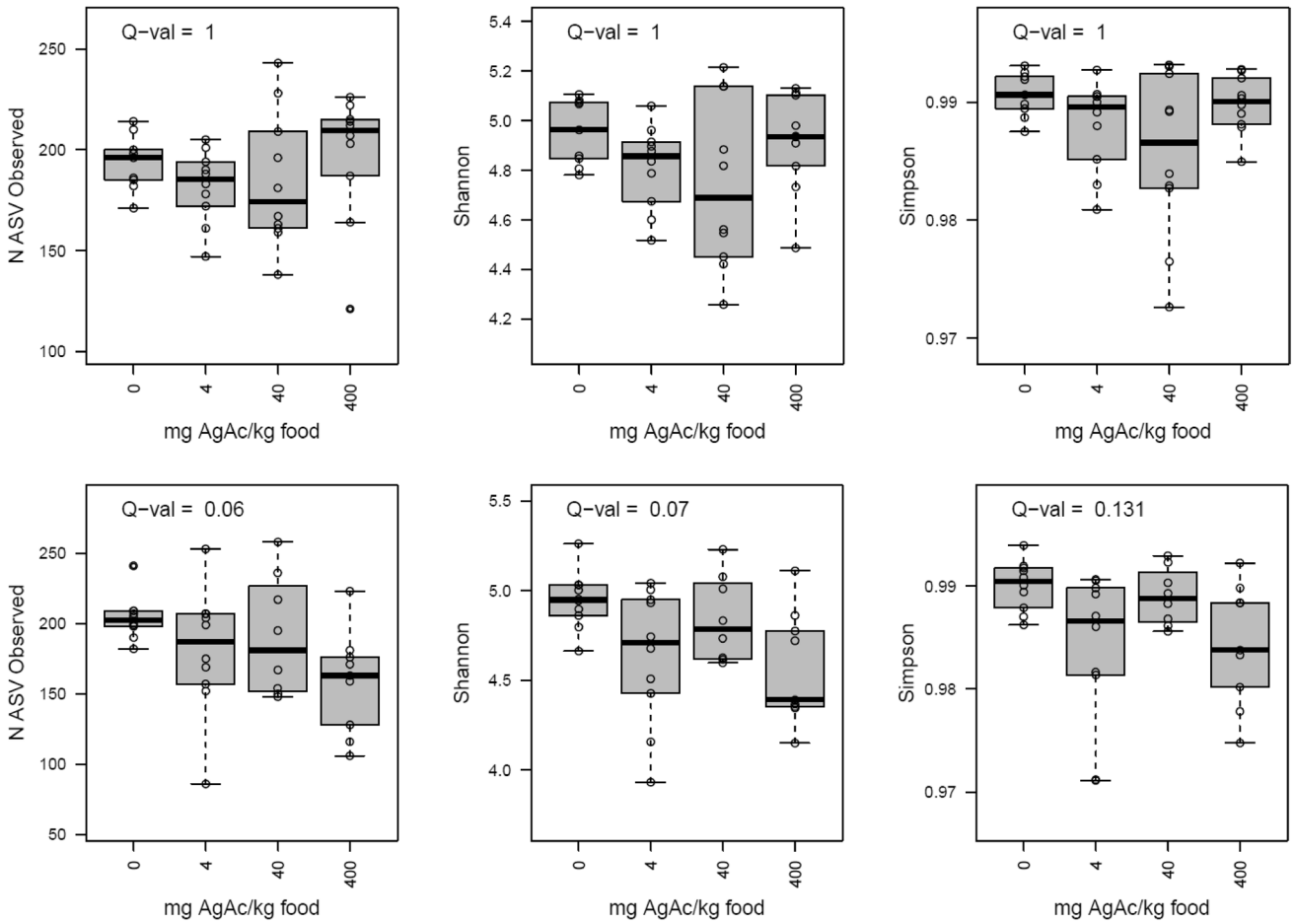

Fig. 7 Alpha-diversity of gut microbiota in female (top) and male (bottom) rats after 10-week exposure to AgAc in food. Boxes represent 25th and 75th percentiles with the median in the middle, whiskers show minimum and maximum values. *Q* values indicate the significance levels of the AgAc dose, introduced as a continuous variable in the linear models

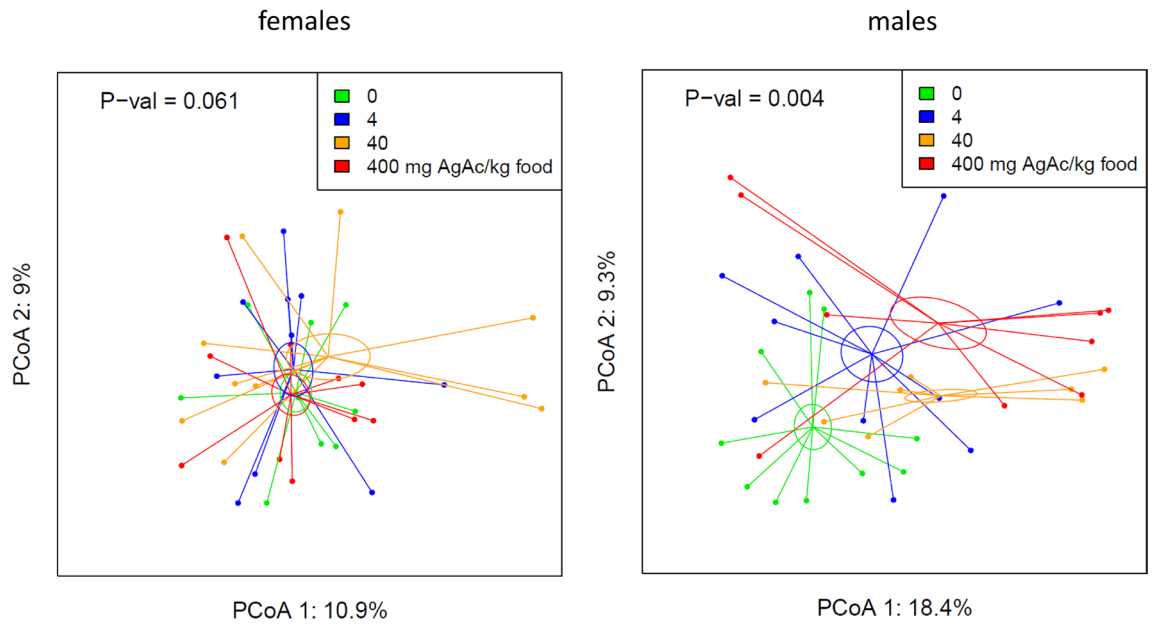

Fig. 8 Beta-diversity of gut microbiota in female (left) and male (right) rats after 10-week exposure to AgAc in food. The dose-dependent effect was assessed by a permutational multivariate analysis of variance (ADONIS) of the weighted UniFrac distance matrix assuming a linear trend effect of AgAc dose in food on the microbial structure

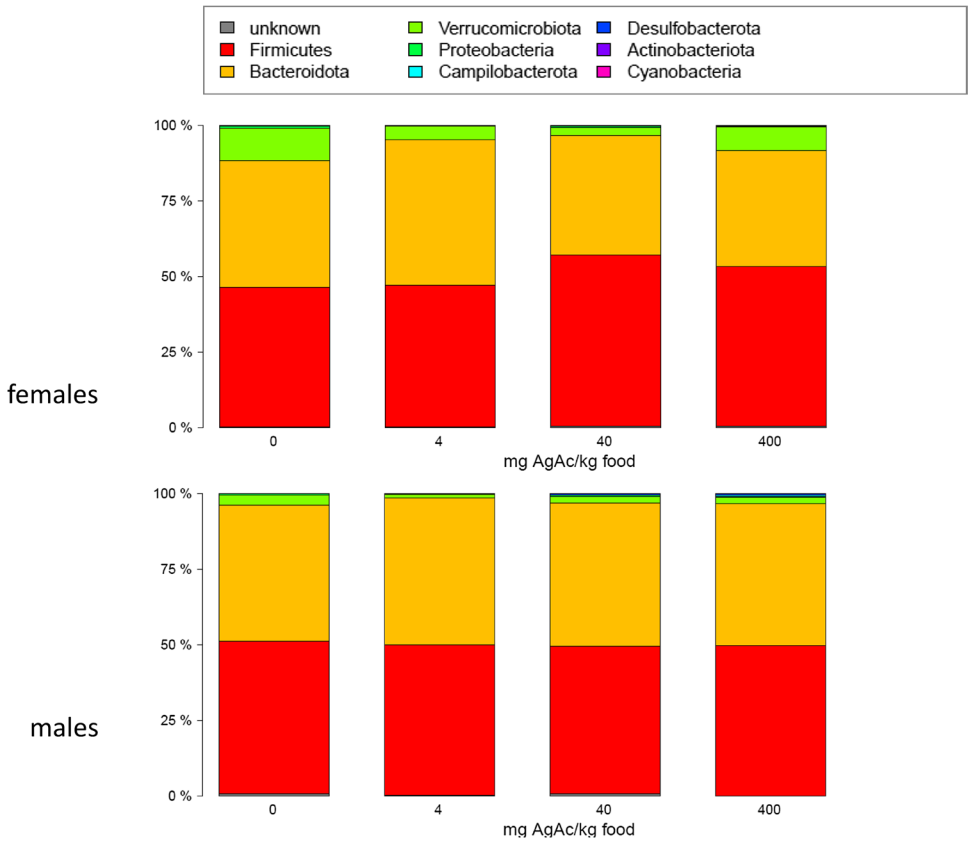

Fig. 9 Relative abundance of phyla in rats exposed during 10 weeks to increasing doses of AgAc in food. Female (top) and male rats (bottom). The relative abundance of phyla was calculated based on ASVs and taxonomic classification derived from the SILVA(v138) database

Phylum composition

Next, we compared the repartition of identified ASVs among the different phyla in experimental groups (Fig. 9).

Statistical differences among the dose groups were assessed for each phylum separately by linear models. In female rats, no statistically significant difference was recorded (Supplemental file—Fig. S4). In male rats, a statistically significant trend towards increased proportions of Desulfobacterota with increasing AgAc dose was noted (Supplemental file—Fig. S5).

Family composition

A similar analysis was conducted at the family level (Fig. 10).

In female rats, no statistically significant influence of AgAc exposure on the relative abundance of families was recorded (Supplemental file—Fig. S6). In male rats, a dose-dependent increase of Desulfovibrionaceae and a dose-dependent decrease of Peptostreptococcaceae were recorded (Supplemental file—Fig. S7).

The interpretation of the sexual dimorphism in the GM response to AgAc exposure (α- and β-diversity), and possible health consequences, is not immediately evident. It appears that each gender has a different susceptibility to environmental factors that shape de GM after birth (Laukens et al. 2016), but little information is available regarding the mechanism(s) involved.

The toxicological relevance of an increased prevalence of Desulfovibrionaceae (also reflected at the Desulfubacterota phylum level) recorded in male rats exposed to AgAc is not obvious. The Desulfovibrionaceae family is part of the normal GM, but its increased relative abundance may contribute to inflammation, possibly related to hydrogen sulfide production (Millien et al. 2018). *Desulfovibrionaceae* are strongly correlated with obesity, metabolic syndrome and inflammation in mouse models (Cani 2013). Increased relative abundance of *Desulfovibrionaceae* has also been reported in patients affected by myasthenia gravis (Moris et al. 2018), non-alcoholic steatohepatitis (NASH) (Panasevich et al. 2018), and celiac disease patients with poly-autoimmune manifestations (Bibbo et al. 2020). No information on a possible implication of *Desulfovibrionaceae* in reprotoxic outcomes could be located. The possible

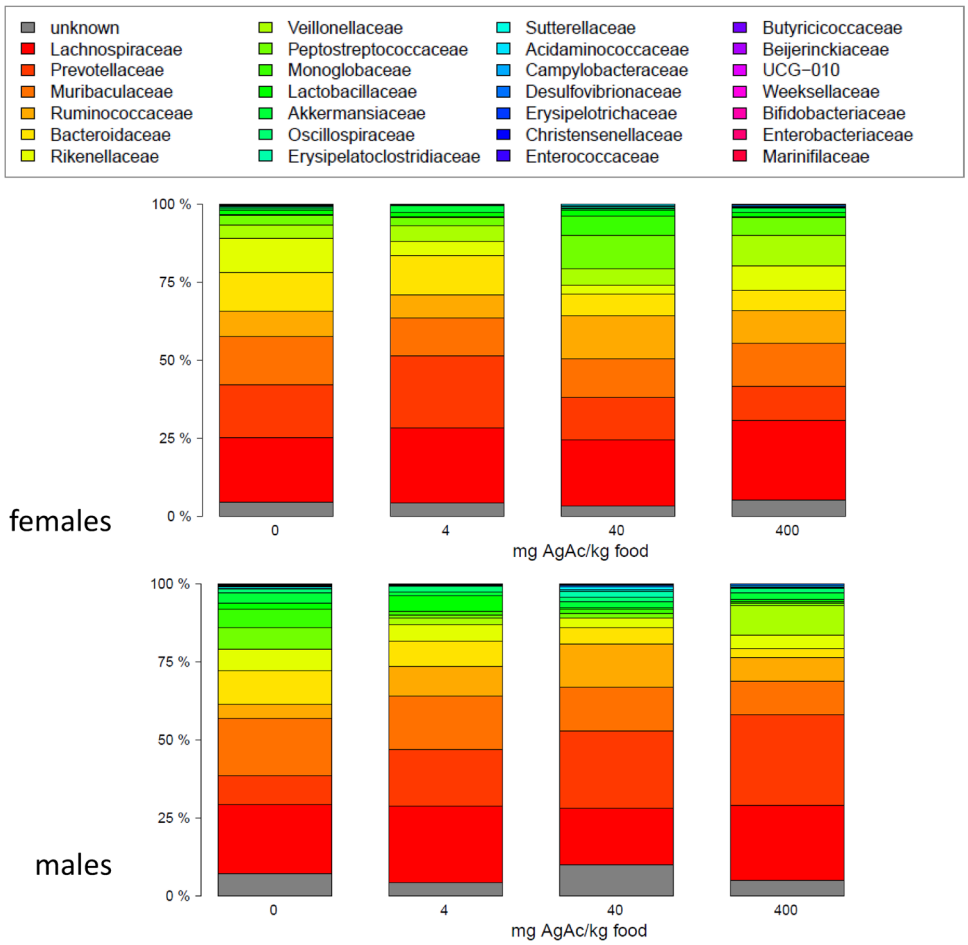

Fig. 10 Relative abundance of families in rats exposed during 10 weeks to increasing doses of AgAc in food. Female (top) and male rats (bottom). The relative abundance of families was calculated based on ASVs and taxonomic classification derived from the SILVA(v138) database

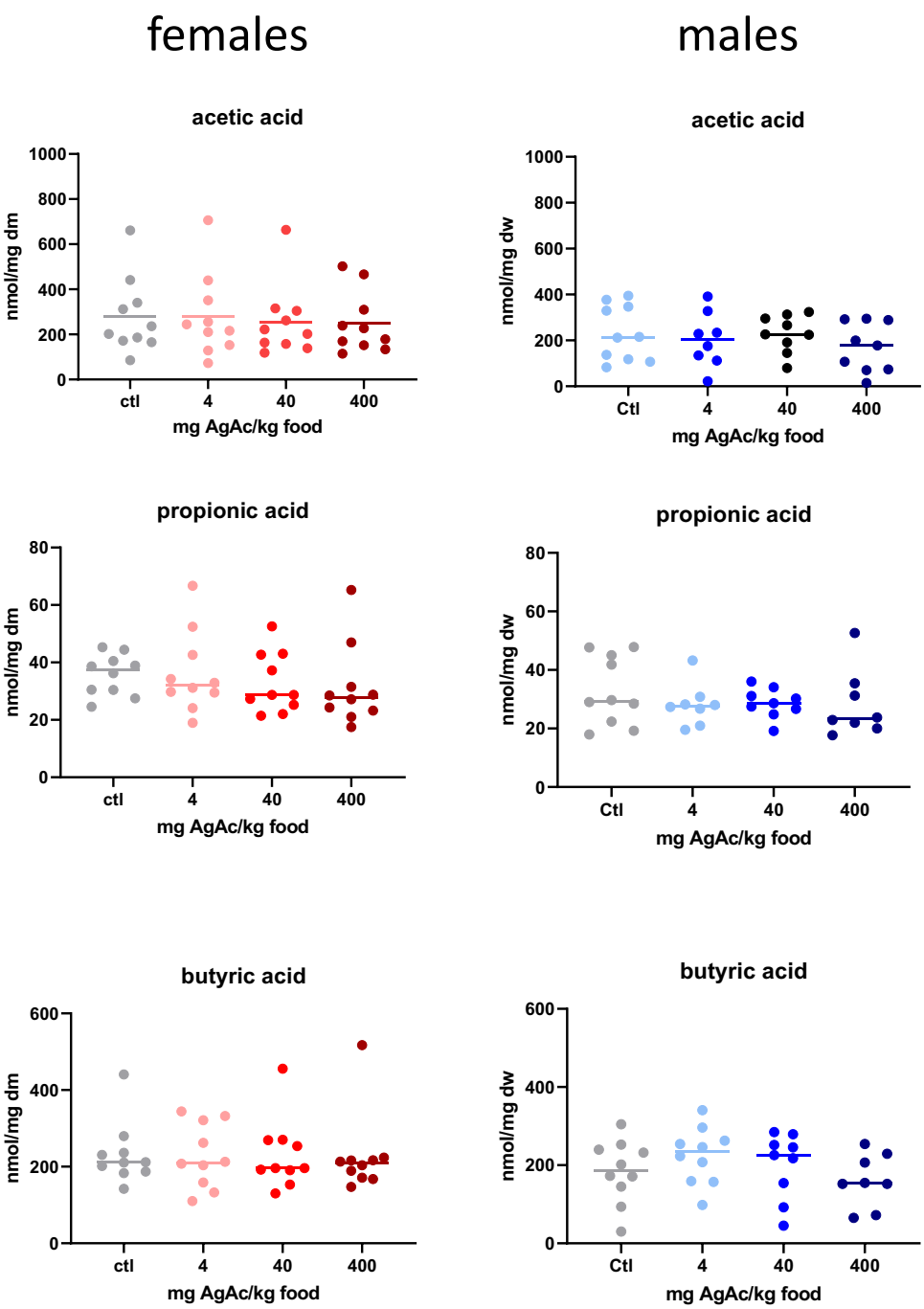

Fig. 11 Short-chain fatty acid content in cecal feces collected at sacrifice. Each symbol represents an animal ($n = 10$ per group), horizontal bars reflect group means. *dm* dry matter. ANOVA did not reveal any statistically significant difference among the experimental groups

toxicological significance of the reduction in the relative abundance of Peptostreptococcaceae recorded in male rats exposed to AgAc is not more evident. Members of the Peptostreptococcaceae family (Firmicutes phylum) are Gram positive, anaerobes with fermentative type of metabolism, and belong to the class Clostridia. Peptostreptococcaceae belong to bacteria clusters of enterotypes II (Rinninella et al. 2019). In a study investigating the possible relation between the hygiene hypothesis of allergic disease and the GM, exposure to pets was associated with an overrepresentation of Peptostreptococcaceae; whereas, infants leaving with older siblings exhibited under-representation of Peptostreptococcaceae (Azad et al. 2013). No information on a possible implication of Peptostreptococcaceae in reprotoxic outcomes was located. Overall, there is no available evidence that the alterations of the GM composition recorded in the present study might contribute to the reprotoxic outcomes observed by Sprando et al. (2017).

Short-chain fatty acid profiles

We next examined the impact of AgAc exposure on the GM function by analyzing the bacterial SCFA content in cecal feces at sacrifice. No significant influence of AgAc exposure on the production of acetic, propionic or butyric acids was recorded neither in male nor in female rats (Fig. 11). Isobutyric, 2-methylbutyric, isovaleric, valeric, 4-methyl valeric or hexanoic acid contents were not affected either by exposure (not shown). These results suggest that the alterations in the composition and structure of the GM induced by AgAc exposure, mainly in male rats, did not significantly affect the function of the bacterial communities. Overall, the SCFA profiling in the present study does not provide evidence that the GM might contribute to mediate the reprotoxic outcomes observed by Sprando et al. (2017).

Conclusion

This study reveals that oral exposure of rats to AgAc during 10 weeks induced systemic toxicity, as indicated by the reduction in b.w. (males), and of serum ferroxidase activity in both sexes. This systemic toxic response to AgAc might contribute to explain reprotoxic manifestations recorded previously (Sprando et al. 2017). AgAc exposure affected the composition and structure of the GM in male rats. No impact of AgAc exposure on the function of the GM, as assessed by the production of bacterial SCFA, was recorded. The limited GM changes recorded in this study do not appear related to a reprotoxicity outcome.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00204-021-02998-1.

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Data availability Data and material are available from the authors upon reasonable request.

Compliance with ethical standards

Conflict of interest The authors do not have conflict of or competing interests.

Ethical approval The Louvain centre for Toxicology and Applied Pharmacology (LTAP) is accredited to conduct animal experiments (reference LA12312, Bruxelles Environnement). The experimental design and procedures for this work were approved by the local ethical committee for biomedical research at UCLouvain (reference 2018/UCL/MD/012).

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