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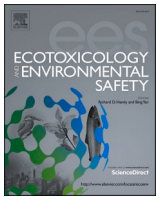

Gut microbiota metabolize arsenolipids in a donor dependent way

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ABSTRACT

Understanding the interplay between the gut microbiome and arsenolipids can help us manage the potential health risk of consuming seafood, but little is known about the bioconversion fate of arsenolipids in the gastrointestinal tract. We use an *in vitro* mucosal simulator of the human intestinal microbial ecosystem (M-SHIME) to mimic the digestive tract of four healthy donors during exposure to two arsenolipids (an arsenic fatty acid AsFA 362 or an arsenic hydrocarbon AsHC 332). The metabolites were analyzed by HPLC-mass spectrometry. The human gut bacteria accumulated arsenolipids in a donor-dependent way, with higher retention of AsHC 332. Colonic microbiota partly transformed both arsenolipids to their thioxo analogs, while AsFA 362 was additionally transformed into arsenic-containing fatty esters, arsenic-containing fatty alcohols, and arsenic-containing sterols. There was no significant difference in water-soluble arsenicals between arsenolipid treatments. The study shows that arsenolipids can be quickly biotransformed into several lipid-soluble arsenicals of unknown toxicity, which cannot be excluded when considering potential implications on human health.

1. Introduction

Arsenic naturally occurs in the environment in various chemical forms that show vastly different toxicities (Bornhorst et al., 2020; Sakurai et al., 1997; Styblo et al., 2000; Stýblo et al., 2002; Witt et al., 2017). Humans are mainly exposed to arsenic through drinking water (Argos et al., 2010), rice (Gilbert-Diamond et al., 2011) and seafood (Al Amin et al., 2020). The major arsenic species in drinking water and most rice strains is inorganic arsenic (iAs) (Mantha et al., 2017), whereas in seafood organoarsenical compounds such as arsenobetaine, arsenosugars, and arsenolipids predominate (Taylor et al., 2017). Arsenic-containing lipids including fatty acids (AsFAs) (Rumpler et al., 2008), hydrocarbons (AsHCs) (Sele et al., 2013) and phospholipids (AsPLs) (Viczek et al., 2016) have been found in oily fish and fish-derived oils, which are common in the human diet.

A risk assessment of dietary arsenic should consider not only the amount of arsenic ingested and its chemical form, but also biotransformation, active metabolism, and bioavailability, as all these processes can significantly affect arsenic species arriving at the target organs (Laparra et al., 2006, 2007; Leffers et al., 2013). Studies showed that arsenolipids, naturally present in cod liver oil, when ingested by humans were metabolized with the production of dimethylarsinate (DMA) and AsFAs (both oxo- and thioxo- forms) as the major urinary metabolites (Schmeisser et al., 2006a, 2006b). That study showed that absorption and metabolism of arsenolipids in the human body had taken place, but it could not shed light on any possible biological role of these compounds.

Recently, it has been shown that salivary and colonic microbiomes play a significant role in *in vitro* arsenic bioaccessibility and bioavailability from various food matrices, while anaerobic microbiota can convert iAs into methylated oxyarsenicals and thioxo-analogs in *in vivo* mice models (Pinyayev et al., 2011; Trenary et al., 2012) and also in human *in vitro* systems (Calatayud et al., 2013; van de Wiele et al., 2010). Similar work on water-soluble organoarsenicals from seaweed confirmed that arsenosugars could be converted into their thioxo-analogs (Calatayud et al., 2018). In a Caco-2 intestinal barrier model, arsenolipids were more available than arsenosugars or arsenobetaine (Meyer et al., 2015a); however the behavior of these

Abbreviations: M-SHIME, Mucosal simulator of the human intestinal microbial ecosystem; AsFA, Arsenic-containing fatty acid; AsHC, Arsenic-containing hydrocarbon; AsPL, Arsenic-containing phospholipid; AsFE, Arsenic-containing fatty ester; MA, Monomethylarsonate; DMA, Dimethylarsinate; iAs, Inorganic arsenic; HPLC, High performance liquid chromatograph; ICPMS, Inductively coupled plasma mass spectrometry; ESMS, Electrospray mass spectrometry.

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organoarsenicals in the human gastrointestinal tract and their interplay with the gut microbiome are not yet fully explored. Toxicological research over recent years has established the toxic nature of arsenolipids at low levels. Meyer et al. found that AsHCs are cytotoxic to human bladder and liver cells with IC_{50} values (AsHC 332) of 13.5 μM and 17 μM, respectively (Meyer et al., 2014). Additionally, they reported that AsFAs are also cytotoxic to human liver cells with IC_{70} values of 96 μM (AsFA 362) (Meyer et al., 2015b). These results, together with the higher intestinal absorption of arsenolipids, suggest that the gut microbiome could be playing an active role in modulating the bioaccessibility, transformation and toxicity of arsenolipids.

The main aim of this research was to evaluate the biotransformation of two types of arsenolipids, namely an arsenic hydrocarbon AsHC 332 and an arsenic fatty acid AsFA 362, by using the *in vitro* mucosal simulator of the human gut microbial ecosystem (M-SHIME). Arsenolipid biotransformation was determined by characterizing the arsenic metabolites using high performance liquid chromatography (HPLC) coupled with both inductively coupled plasma mass spectrometry (ICPMS) and electrospray mass spectrometry (ESMS).

2. Material and methods

2.1. Dynamic simulation of the colon environment

An *in vitro* mucosal simulator of the human intestinal microbial ecosystem (M-SHIME) (van den Abbeele et al., 2012), with three sequential gastrointestinal reactors (gastric, small intestinal, and colonic) was used in our experiment, the M-SHIME vessels were inoculated with fecal slurries during incubation for 70 h from 4 individual healthy donors who did not take any medication, including antibiotics 6 months or more before the assay. Information (age, BMI, exercise habits, living area) about the donors is included in Table S1 (Supporting Information), information about the sampling and inocula preparation is shown in Method S1, and the composition of digestive fluids is shown in Method S2.

To evaluate the interplay between two typical arsenolipids (an arsenic-containing fatty acid 362, AsFA 362 and an arsenic-containing hydrocarbon 332, AsHC 332) and the colonic microbial digestion process, a single dose of the arsenolipid (AsHC 332 or AsFA 362, each 1.33 μmol = 100 μg As) was added to the gastric reactor and pre-digested by the SHIME system, which was then maintained for 70 h. For each donor we used 3 reactors (control, 1.33 μmoles AsHC332 treated, and 1.33 μmoles AsFA362 treated). The detailed feeding time points are listed in Methods S3.

2.2. Sample collection and preparation

Lumen samples (70 mL) were taken 4 times at 6, 22, 46 and 70 h from the colonic vessels just before feed was introduced to the system (Method S4). A fraction of lumen sample (30 mL) was centrifuged (9509 g, 10 min) to separate the bacteria from supernatant for later analysis. Mucin agars (40 beads) were taken two times at 22 and 70 h from the colonic vessels. Mucin agar-covered microcosms were washed with sterile PBS to remove luminal bacteria. Mucin agars (40 beads) were then removed from the microcosms and transferred to 50 mL polypropylene tubes. All the samples were freeze-dried (Christ Alpha 1–4 freeze-drying system; Christ, Osterode am Harz, Germany) and stored at − 80 ℃ until further processing.

2.3. Chemicals, reagents and standards

Milli-Q water (18.2 MΩ cm) was used for all experiments (Millipore GmbH, Vienna, Austria). Methanol (≥ 99.9%, MeOH), methyl tert-butyl ether (≥ 99.5%, MTBE), formic acid (≥ 98%, CH_2O_2) and nitric acid (65%, HNO_3) were obtained from Carl Roth GmbH (Karlsruhe, Germany). Ethanol (≥ 99.9%, EtOH) and malonic acid (>99%) was obtained from Merck (Buchs, Switzerland). Single-element standard solutions of arsenic (1000 mg As L^{-1} ± 0.2%, in 2% HNO_3) and germanium (1000 ± 3 mg Ge L^{-1}, in 2% HNO_3) for total arsenic determinations were purchased from CPI International (Santa Rosa, USA). The certified reference material (CRM) was IAEA 407 (homogenized fish tissue), obtained from the International Atomic Energy Agency (Vienna, Austria). Arsenic-containing fatty acid (AsFA 362) and arsenic-containing hydrocarbon (AsHC 332) (structures were shown in Fig. S3) were previously synthesized in our lab (Taleshi et al., 2014).

2.4. Extraction of arsenic species

Portions of mucus (50 mg), bacteria (50 mg) or supernatant (200 μL diluted to 10 mL in H_2O) were extracted on a rotating arm with 5 mL MTBE at room temperature for 1 h. Then 1.25 mL H_2O was added to effect phase separation. The combined solution was centrifuged (21,380 g, 15 min), the upper layer was removed, and the lower layer was re-extracted with 2 mL of a mixture of MTBE/MeOH/H_2O (10:3:2.5, v/v/v) (Stiboller et al., 2019). The two upper (organic) layers were combined and washed with 3 mL H_2O, which was then added to the previous lower (aqueous) layers. Both the organic and aqueous phases were evaporated to dryness and the resultant residue was re-dissolved in MeOH/H_2O. Prior to analysis by HPLC-ICPMS/ESMS, the residues from the organic phases were dissolved in 200–1000 μL MeOH (depending on arsenic content) and the residues from the aqueous phases were dissolved in 200 μL H_2O.

2.5. Determination of total arsenic and arsenic species

Total arsenic measurements were performed on solutions of the microwave-assisted acid mineralized samples (UltraCLAVE 83 IV Microwave Reactor, MLS GmbH, Leutkirch, Germany) by using an Agilent 7900 ICPMS (Agilent Technologies, Waldbronn, Germany). Details of acid-digestion and total arsenic determination are shown in Method S5. Measurements of water-soluble and lipid-soluble arsenic species were performed by using HPLC (Agilent 1200, Agilent Technologies, Waldbronn, Germany) coupled in parallel with an ICPMS (Agilent 7900) and an ESMS (Agilent 6460). Identification of arsenic species, for which standards were available, was based on retention time matching of both arsenic (ICPMS) and molecular mass (ESMS) chromatograms. Unknown lipid-soluble arsenic species were investigated by HPLC-HR-ESMS. Detailed information about the determination of arsenic species is provided in the supporting information as Methods S6-S8, and the instrument parameters and chromatographic conditions are listed in Table S2.

The total arsenic determination was validated against the certified reference material IAEA 407 with a certified value for arsenic of 12.6 (12.3–12.9) mg As kg^{-1}; we obtained 12.7 ± 0.2 mg As kg^{-1} (n = 3). Mixed calibration standards of AsFA 362 and AsHC 332 were prepared in methanol in the calibration range of 0.1–100 μg of As L^{-1}; quantification of lipid-soluble arsenicals was based on peak areas. The limit of detection (50 μL injection volume, 3σ of blank noise, n = 3) of arsenolipids in the organic fraction was 1 μg As L^{-1} in solution. Quantification of each of the water-soluble arsenic compounds in the samples was based on calibration curves constructed from mixed anionic calibration standards of As(III), DMA, MA and As(V) prepared in H_2O in the calibration range of 0.1–100 μg of As L^{-1}. The limit of detection (5 μL injection volume, 3σ of blank noise, n = 3) of anionic arsenic species in the aqueous fraction was 0.1 μg As L^{-1} in solution. The certification of arsenolipids was validated against Hijiki (CRM 7405-a) extracted with DCM/MeOH, the certified value of AsHC 332 is 1073 ± 44 μg As kg^{-1} (Glabonjat et al., 2014), we obtained 1180 ± 98 μg As kg^{-1} (n = 3).

Lipid-soluble and water-soluble arsenical species in duplicate analyses were performed on two mucus and two bacteria samples (donor 1 at 22 h treated with AsFA 362/AsHC 332) which returned RSD values within 10% of each main compound (those As species present at >10% of the total As). Hence, only one of each sample was subsequently

analyzed.

3. Results

3.1. Human gut bacteria accumulate arsenolipids in a donor-dependent way

The total As in mucus, bacteria, and supernatant samples from the control groups (mucus: 63–127 μg As kg^{-1}, dry mass; bacteria: 50–279 μg As kg^{-1}, dry mass; supernatant: 0.1–0.9 μg As L^{-1}, wet mass; Table S3), and from AsHC 332 and AsFA 362 indicate donor-dependent distribution of arsenic between different compartments (Fig. 1).

Mucus samples exposed to AsFA 362 retained more ($p < 0.01$, n = 4) arsenic (1.20–2.77 mg As kg^{-1}, mean ± SD, 2.03 ± 0.58 mg As kg^{-1}, dry mass, n = 4) than those treated with AsHC 332 (0.65–1.67 mg As kg^{-1}, mean ± SD, 1.08 ± 0.40 mg As kg^{-1}, dry mass, n = 4) (Fig. 1A). Reactors exposed to AsHC 332 showed a significant increase ($p < 0.05$, n = 4) in total arsenic between 22 h (0.78 ± 0.15 mg As kg^{-1}, $n = 4$) and 70 h (1.38 ± 0.34 mg As kg^{-1}, $n = 4$) in the mucosal compartment, however this trend was not observed in the mucus samples treated with AsFA 362. As the dose of AsHC 332 was a single dose, it is expected that the increase in arsenic in the mucus compartment with time can be due to equilibrium. No significant difference was found between donors in the arsenolipids treated mucus samples.

Bacteria samples exposed to AsHC 332 retained more ($p < 0.01$, n = 4) arsenic (7.3–56.1 mg As kg^{-1}, mean ± SD, 22.1 ± 14.7 mg As kg^{-1}, dry mass, n = 4) than those treated with AsFA 362 (2.0–24.1 mg As kg^{-1}, mean ± SD, 9.9 ± 6.9 mg As kg^{-1}, dry mass, n = 4) (Fig. 1B). Total arsenic bacterial uptake decreased with time in AsHC 332-treated samples (6 h = 37.0 ± 15.6, 22 h = 26.6 ± 14.6, 46 h = 12.8 ± 5.4, 70 h = 12.0 ± 4.1 mg As kg^{-1}, $n = 4$) with a significant difference ($p < 0.05$, n = 4) between 6–46 h and between 6 h and 70 h. Total arsenic uptake in bacteria treated with AsFA 362 stayed fairly constant in the first 22 h (6 h = 14.4 ± 6.7, 22 h = 13.1 ± 7.1 mg As kg^{-1}, $n = 4$), then dropped off ($p < 0.05$, n = 4) to as low as ca 40% (46 h = 6.6 ± 5.8, 70 h = 5.4 ± 5.3 mg As kg^{-1}, $n = 4$). In the bacterial samples treated with AsHC 332, donor 3 (27.7 ± 14.3 mg As kg^{-1}, $n = 4$) and donor 4 (32.6 ± 19.4 mg As kg^{-1}, $n = 4$) retained more arsenic than donor 1 (16.2 ± 8.7 mg As kg^{-1}, $n = 4$) and donor 2 (11.7 ± 6.7 mg As kg^{-1}, $n = 4$), although these differences were not significant ($p > 0.05$, n = 4). For bacteria treated with AsFA 362, donor 3 (19.1 ± 5.6 mg As kg^{-1}, $n = 4$) retained more arsenic ($p < 0.05$, n = 4) than donor 1, donor 2 and donor 4 (7.8 ± 4.9, 6.5 ± 3.3, 6.1 ± 4.4 mg As kg^{-1}, respectively; $n = 4$).

Supernatant samples from AsFA 362-treated reactors had more ($p > 0.05$, n = 4) arsenic (10–168 μg As L^{-1}, mean ± SD, 83 ± 50 μg As L^{-1}, wet mass, n = 4) than those treated with AsHC 332 (13–199 μg As L^{-1}, mean ± SD, 56 ± 48 μg As L^{-1}, wet mass, n = 4). In general, total arsenic decreased with time in supernatant samples treated with AsHC 332 (6 h = 110 ± 62, 22 h = 64 ± 23, 46 h = 31 ± 7, 70 h = 17 ± 4 μg As L^{-1}, $n = 4$), with significant differences ($p < 0.05$, n = 4) except between 6 h and 22 h. The same trend was observed in supernatant

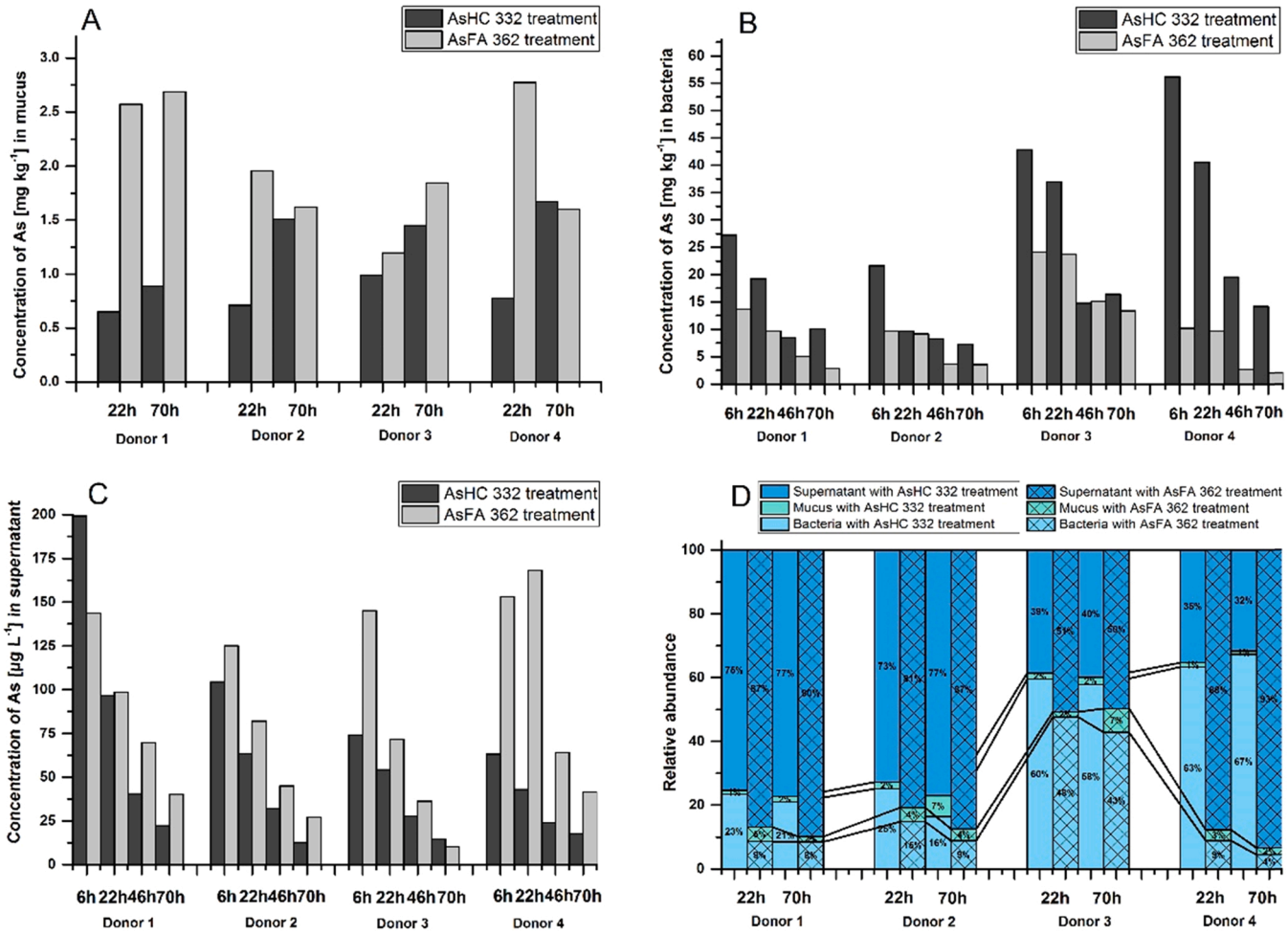

Fig. 1. Effect of arsenolipids treatment on total arsenic uptake in different compartment of (A) mucus, (B) bacteria, (C) supernatant, and (D) the arsenic distribution in mucosal, bacterial, and luminal compartments. Bars represent the total arsenic quantification from n = 1 sample at each time point. Stacked bars in graph D represent the percentage of arsenic in each compartment, calculated to the total arsenic added to the M-SHIME system.

samples treated with AsFA 362 (6 h = 142 ± 12, 22 h = 105 ± 43, 46 h = 54 ± 16, 70 h = 30 ± 14 μg As L^{-1}, $n = 4$). Significant differences ($p < 0.001$, n = 4) were found between 6–46 h and between 6 h and 70 h. No significant difference was found between donors in the arsenolipids treated supernatant samples.

Arsenic distribution in donor 1 and donor 2 was similar under AsHC 332 treatment, with most of the arsenic being found in the supernatant (over 70%) and bacterial (16–25%) compartments, and less than 7% in the mucosal compartment (Fig. 1D). In donor 3 and donor 4, most arsenic was detected in the bacterial compartment (58–67%), whereas 32–40% was found in the supernatant, and less than 2% in the mucus. The arsenic distribution in donors under AsFA 362 treatment was similar for donors 1, 2 and 4, with most of the arsenic occurring in the supernatant (81–93%) and bacterial compartments (4–15%), and less than 5% in the mucosal compartment. In donor 3, half of the arsenic was detected in the bacteria, half in the supernatant, and less than 7% in the mucus.

3.2. Gut mucosal and bacterial niches retain lipid-soluble arsenicals

Because of the low arsenic content in the luminal compartment (< 200 μg As L^{-1}) compared to mucus (0.65–2.77 mg As kg^{-1}) and bacteria (2.0–56.1 mg As kg^{-1}), the lipid-soluble and water-soluble arsenical species were analyzed only in mucus and bacteria samples and discussed below.

Most of the arsenic in mucus samples was lipid-soluble, representing 33–76% of the total arsenic (Table S4). The water-soluble fraction constituted 6–34% of the total arsenic, and < 12% was retained in the mucus pellet. There were significant differences ($p < 0.01$, n = 4) in lipid-soluble constituents between AsHC 332 treated and AsFA 362 treated mucus samples: in AsHC 332, the lipid-solubles constitute 71–76% of the total arsenic, but this value was less (33–57%) in the AsFA 362 treated samples.

Most of the arsenic in bacteria samples was lipid-soluble, representing 40–84% of the total (Table S4). The water-soluble fraction constituted 1–29% of the total arsenic, and only < 13% was retained in the bacteria pellet. Similar to the results from the mucus samples, there were significant differences ($p < 0.01$, n = 4) between AsHC 332 and AsFA 362 treated reactors: in AsHC 332 treated bacteria, the water-solubles constitute < 2% of the total arsenic, but this value was much higher (17–29%) in AsFA 362 treated bacteria.

3.3. Novel metabolites derived from arsenolipids are generated in the gut mucosal and bacterial compartments

The structures of the arsenolipids in the mucus and bacteria samples were assigned based on data from HPLC-ICPMS/ESMS (Fig. 2 & Fig. S1) and HPLC-HR-ESMS measurements (Table 1, Figs. S2 & S3).

AsHC 332 was recovered largely intact from mucus and bacteria samples, comprising 94–99% of the sum of arsenolipids, with the remaining arsenic being present as the closely related sulfur analog thioxo-AsHC 332 (1–6%) and traces of unidentified arsenic (Fig. S2 & Table S5). AsFA 362, on the other hand, was more extensively biotransformed: although most of the total lipid arsenic was present as intact AsFA 362 (77–94%) and its thioxo analog (1–5%) (Fig. S2), eight additional arsenicals were detected collectively constituting 5–19% of the total lipid arsenic. These transformation products included a group of arsenic-containing fatty esters (AsFEs, ca 2%, Fig. S2), and two groups of unknown compounds represented by peaks in Fig. 2 at RT 18.2 min (< 3%, Fig. S3) and RT 19.7 min (< 10%, Fig. S3).

HPLC-HR-ESMS and MS/MS experiments, however, revealed that these two peaks contained at least five compounds containing AsFA 362 (Fig. 2). Based on this information together with accurate mass

Table 1
Arsenolipids in mucus and bacteria samples identified by HPLC-HR-ESMS.

RT [min]	Compounds	Formula	$[M+H]^+$ Calculated	$[M+H]^+$ Measured	△m/m [ppm]
	AsHC 332 treated				
13.7	AsHC 332	$C_{17}H_{37}OAs$	333.2133	333.2133	< 0.1
19.4	Thioxo-AsHC 332	$C_{17}H_{37}AsS$	349.1905	349.1901	-1.1
	AsFA 362 treated				
9.9	AsFA 362	$C_{17}H_{35}O_3As$	363.1875	363.1872	-0.8
11.1	AsFE 376	$C_{18}H_{37}O_3As$	377.2031	377.2024	-1.9
11.8	AsFE 390	$C_{19}H_{39}O_3As$	391.2188	391.2189	0.3
12.6	AsFE 404	$C_{20}H_{41}O_3As$	405.2344	405.2332	-3.0
13.7	AsFE 418	$C_{21}H_{43}O_3As$	419.2500	419.2497	-1.0
16.5	Thioxo-FA362	$C_{17}H_{35}O_2AsS$	379.1646	379.1647	0.3
18.2	Aslipid-586	$C_{33}H_{67}O_3As$	587.4379	587.4383	0.7
18.2	Aslipid-612	$C_{35}H_{69}O_3As$	613.4535	613.4533	-0.3
19.7	Aslipid-732	$C_{44}H_{81}O_3As$	733.5474	733.5467	-1
19.7	Aslipid-746	$C_{45}H_{83}O_3As$	747.5631	747.5613	-2.4
19.7	Aslipid-760	$C_{46}H_{85}O_3As$	761.5787	761.5784	-0.4

For MS/MS spectra of all the compounds see Figs. S2 & S3, and for the structures see Figs. S4 & S5.

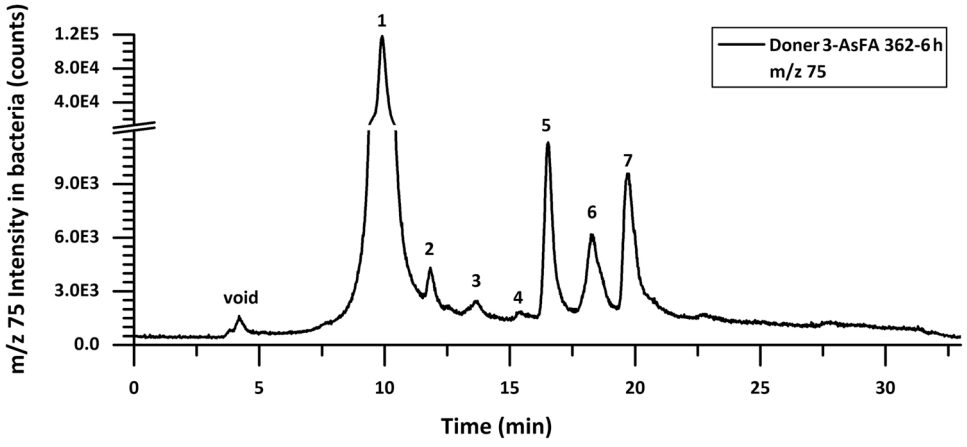

Fig. 2. HPLC-ICPMS chromatogram showing minor arsenolipids (thioxo-arsenolipids, AsFEs & late-eluting compounds) in the lipid-soluble fraction from donor 3 after AsFA362 treatment at 6 h. Arsenolipids identified by HPLC-HR-ESMS (Table 1). 1, AsFA 362; 2, AsFE 376; 3, AsFE 390; 4, AsFE 418; 5, Thioxo-AsFA 362; 6, Aslipid 586 + 612; 7, Aslipid 732 + 746 + 760.

measurements (Table 1), we tentatively propose that the peak at RT 18.2 m (Fig. 2) contains AsFA 362 combined with the fatty alcohols cetyl alcohol ($C_{16}H_{34}O$) or oleyl alcohol ($C_{18}H_{36}O$) giving rise to Aslipid-586 and Aslipid-612, respectively (Table 1). Similarly, we propose that the peak at 19.7 m (Fig. 2) contains three new arsenolipids with AsFA 362 bound to the steroids coprostanol ($C_{27}H_{48}O$), campestanol ($C_{28}H_{50}O$), or stigmastanol ($C_{29}H_{52}O$) giving rise to Aslipid-732, Aslipid-746, and Aslipid-760, respectively.

3.4. Quantification of arsenolipids present in mucus and bacteria samples

The arsenolipids were quantified from the HPLC-ICPMS measurements (Fig. 2, Table S5, & Table S6). The Aslipid-586 and Aslipid-612 were not resolved by HPLC, so quantification was recorded as the sum of these two compounds based on the combined HPLC-ICPMS peak. The same approach was taken to quantify Aslipid-732, Aslipid-746, and Aslipid-760 which were similarly not resolved from each other by HPLC-ICPMS.

Unchanged AsHC 332 was by far the dominant arsenical (> = 94%) in the mucosal and bacterial compartments after AsHC 332 treatment although its thioxo analog was present in all samples ranging from 0.5% to 22% of the total arsenolipids (Fig. 3A, Fig. 3C, & Table S5). The high value resulted from one sample (donor 3, bacteria at 6 h, 22% thioxo), which appeared to be an outlier and is not included in the following brief discussion. The mucus samples contained substantially higher percentages of the thioxo analog (4–5% compared with 1% for bacteria) (Fig. 3). There was no significant difference in thioxo-AsHC 332 between donors or between different time points in the mucus samples ($p > 0.05$, n = 4) (Fig. 3A). The bacteria samples from donor 2 (1.5 ± 0.6%, n = 4) retained higher percentage ($p < 0.05$, n = 4) of thioxo-AsHC 332 than donor 1 (0.5 ± 0.2%, n = 4) and donor 4 (0.7 ± 0.2%, n = 4), but no such trend was found between the time points ($p > 0.05$, n = 4) (Fig. 3C).

The AsFA 362, thioxo-AsFA 362, AsFEs (AsFE 376 and AsFE 390), and two groups of unknown compounds (fatty alcohol Aslipids and steroid Aslipids) were detected in the mucosal and bacterial compartment after AsFA 362 treatment (Fig. 3B, Fig. 3D, & Table S6). The thioxo-AsFA 362 represented < 5% in both mucus and bacteria samples. The bacteria samples accumulate more ($p < 0.01$, n = 4) arsenolipid-derived metabolites (AsFEs, 1.7–2.2%; fatty alcohol Aslipids, 2–3%; steroid Aslipids, 3–10%) than the mucus samples (AsFEs, 0.8%; fatty alcohol Aslipids, 0.1–0.4%; steroid Aslipids, 0.2–0.7%) (Fig. 3D & Table S6). At the end of the assay (70 h) mucus samples retained a higher ($p < 0.05$, n = 4) percentage of thioxo-AsFA 362 (5 ± 1%, n = 4) than at 22 h (1 ± 1%, n = 4), and a higher ($p < 0.05$, n = 4) percentage of fatty alcohol Aslipids (0.5 ± 0.3%, n = 4) compared with values at 22 h (0.2 ± 0.1%, n = 4).

Overall, the bacterial samples from donors 3 and 4 had higher percentages of arsenolipid-derived metabolites than donor 1 or 2. This trend was most marked for the steroid Aslipids, which constituted more

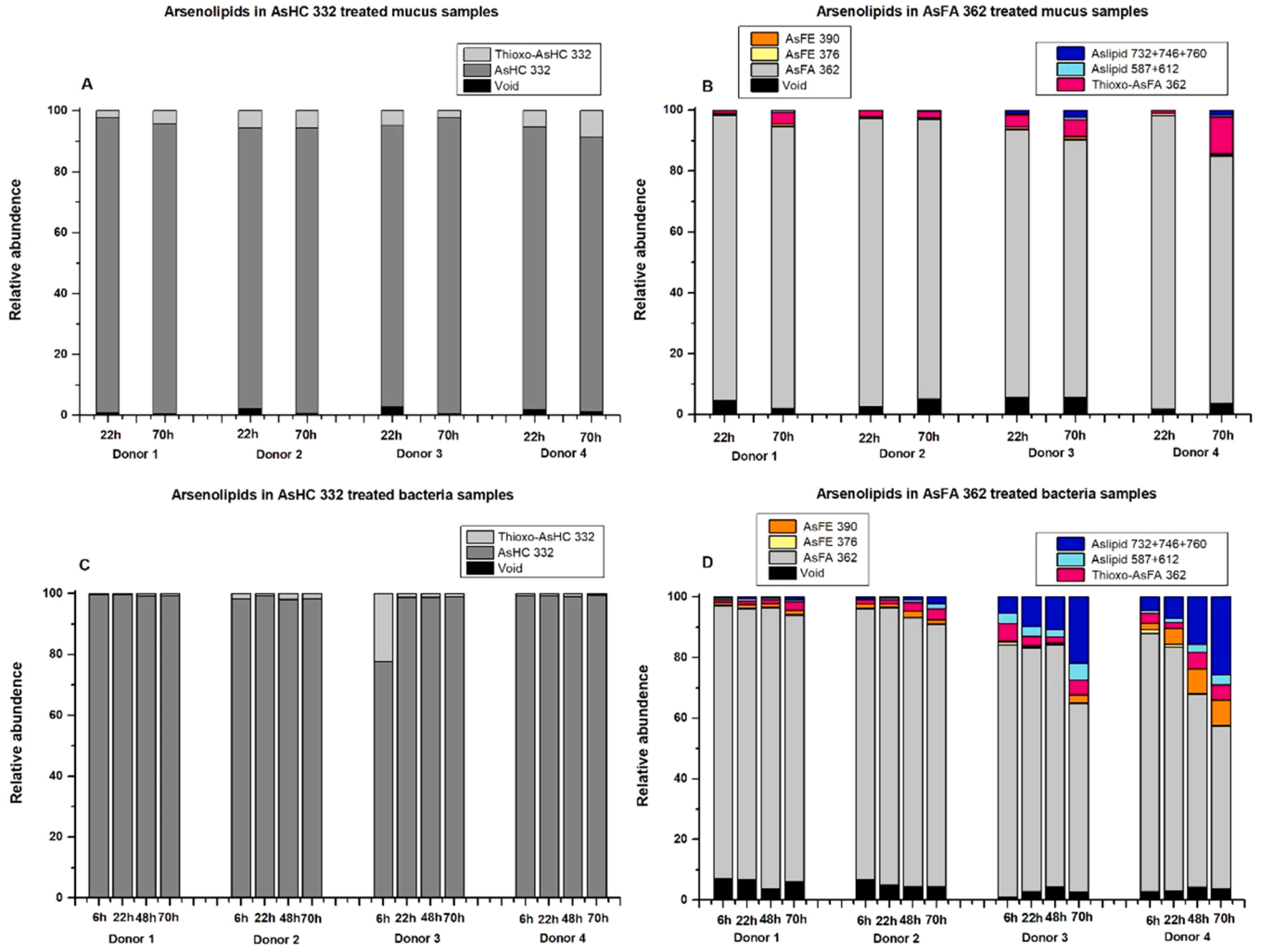

Fig. 3. Arsenolipids present in the samples from 4 donors. (A) AsHC 332 treated mucus, (B) AsFA 362 treated mucus, (C) AsHC 332 treated bacteria, and (D) AsHC 332 treated bacteria. Stacked bars represent the percentage of each arsenolipid calculated to the sum of arsenolipids.

than 10% of the arsenolipids in donors 3 and 4 compared with just 1% in donors 1 and 2 ($p < 0.05$, n = 4, Fig. 3D). A similar trend was observed for thioxo-AsFA 362, with values around 4% for donor 3 and 4 and ca 1% for donors 1 and 2 ($p < 0.05$, n = 4, Fig. 3D). Donor 4 showed the highest levels ($p < 0.05$, n = 4, Fig. 3D) in AsFEs (7 ± 2%, n = 4), compared to the other donors (1–2%), whereas donor 3 had the highest percentage ($p < 0.05$, n = 4, Fig. 3D) of the fatty alcohol Aslipids (4 ± 1%, n = 4) compared to the other donors (ca 1%).

3.5. Water-soluble arsenicals, including carcinogenic arsenate, were found in the mucosal and bacterial compartment of arsenolipid-exposed M-SHIME

Identification of water-soluble arsenicals was based on retention time matching of standards by HPLC-ICPMS (Fig. S6). In the arsenolipids treated mucus samples, the water-soluble arsenical metabolites comprised mainly As(V), which accounted for ca 70% of the sum of water-soluble arsenicals, then MA (14%), DMA (11%), and As(III) (5%) (Table S7). In the arsenolipids treated bacteria samples, the water-soluble arsenical metabolites comprised mainly As(V), which accounted for ca 50% of the sum of water-soluble arsenicals, then DMA (ca 20%), MA (ca 15%), and As(III) (ca 15%) (Table S8). There was no significant difference observed in the water-soluble arsenical metabolites between treatments, donors or time points ($p > 0.05$, n = 4).

4. Discussion

Human gut bacteria can transform arsenic-containing compounds, as previously demonstrated in several *in vitro* and *in vivo* settings (Calatayud et al., 2013, 2018; van de Wiele et al., 2015; Yin et al., 2019; Zhou et al., 2020). These transformations are likely to affect the arsenic toxicity to the host (Coryell et al., 2019), with most of the literature referring to inorganic arsenic, but little is known about the role of gut microbiota in arsenolipid biotransformations and potential implications on human health.

4.1. The microbiome biotransforms arsenolipids in a donor-dependent way

Our study is the first to investigate the bioconversion of arsenolipids in the human gastrointestinal tract, demonstrating that colonic bacterial metabolized both arsenolipids to their thioxo analogs, while additionally metabolizing AsFA 362 to arsenic-containing fatty esters, arsenic-containing fatty alcohols, and arsenic-containing sterol compounds. Fatty alcohols and steroids are common bacteria metabolites during the colonic transit (Oliphant and Allen-Vercoe, 2019), especially coprostanol, campestanol, and stigmastanol were found in human feces as the end products (Juste and Gérard, 2021). No such *in vitro* studies including gut bacteria have been done before for arsenolipids, but previous studies on iAs and water-soluble arsenic showed that the human gut bacteria can metabolize arsenic (van de Wiele et al., 2010; Calatayud et al., 2018). Notably, we found water-soluble metabolites [As(V), As(III), MA, and DMA] derived from arsenolipids in the bacterial and mucosal niche, suggesting a potential toxicity derived from arsenolipid biotransformations.

More interestingly, we found that the total arsenic uptake, the thioxo-compound conversion efficiency, and the production efficiency of novel metabolites in the bacteria samples had a donor-dependent feature. The results indicate that the combination of bacteria from different donors accumulate arsenic and metabolize arsenolipids differently. The differences in bacteria contribute more to the arsenolipid-related metabolites than time, treatments, or compartments. Few studies can be found in this area, but similarly, the interindividual variability of arsenic metabolism by human gut microbiota was previously evaluated by Yin et al. (Yin et al., 2017) showing significant differences on arsenic metabolism (water-soluble organic arsenicals) between adult and child with arsenic methylation ability of the adult 3-fold higher than that of the child. Coryell et al., (Coryell et al., 2018) observed significantly less arsenic excreted in stool and higher arsenic levels in internal organs in antibiotic and germ-free mice than in conventional mice, suggesting that microbiota also retain iAs and protect from toxicant exposure. In this research we demonstrated the protective role of gut microbiota by quantifying the bacterial compartment in the M-SHIME, supporting a bio-accumulating role of gut bacteria to prevent internal arsenic exposure.

4.2. The time-dependent metabolism under arsenolipid exposure

Total arsenic in the bacteria samples decreased over time after both arsenolipid exposures, which indicates the bacteria lose the ability to accumulate arsenic over time. As the experimental setup included a single dose, the dynamic wash-out of the system simulating the intestinal transit ensured the partial elimination of the arsenolipids with time. Constantly feeding the system with arsenolipids would represent a chronic, repeated-dose exposure to the contaminant, whereas we wanted to simulate an acute, single-dose exposure as could occur after a single fish or seafood intake.

Mucus samples accumulated more AsHC 332 at 70 h than at 22 h, but no such trend was found after AsFA 362 exposure. The AsHCs are known to have higher cellular bioavailability than AsFAs (Meyer et al., 2015a), which could be the reason why the AsHCs accumulate in the mucosal environment over time. Higher retention of AsHC 332 in mucosal samples might be linked to higher contact with intestinal cells, potentially increasing bioavailability of the compound through diffusion into the mucus layer. Both mucus and bacterial samples retain more thioxo-AsFA 362 at 70 h than at 22 h, which indicates that the longer fermentation times led to a higher thioxo conversion efficiency. The thioxo-AsFA also has been detected in the *in vivo C. elegans* model after treatment with AsFA 362 by Bornhorst et al. (Bornhorst et al., 2020), but was not previously described by human microbiota. Toxicological profiles of thioxo arsenical compounds have not yet been fully evaluated and would require further characterization to determine potential disruptive effects on the intestinal environment.

4.3. Toxicological considerations

In our study, the thioxo- analogs of AsHC 332 and AsFA 362, and their lipid-soluble arsenolipid-derived metabolites were detected in the colonic compartment, so the potential toxicity should also be considered. Previous toxicity studies of the main compounds revealed that AsHC has cytotoxicity comparable to that of arsenite and much higher than that of AsFA (Meyer et al., 2014; Meyer et al., 2015b). It was also reported for whole-animal studies that AsHCs can cross the blood-brain barrier of the fruit fly (Niehoff et al., 2016) and be accumulated in the brain of tuna fish (Stiboller et al., 2019). Furthermore, AsHCs are transferred to the milk of nursing mothers (Stiboller et al., 2017; Xiong et al., 2020), raising concerns about possible effects on neural development in breast-fed infants. But limited toxicity studies were found for the arsenolipid-derived metabolites. There has been only one study on thioxo-AsHC 332; Ebert et al. (Ebert et al., 2020) found that the oxo- and thioxo- analogs of AsHC 332 affected the cell viability in cultured human liver cells in the same concentration range. For the metabolites of thioxo-AsFA 362 and AsFEs, no equivalent toxicity studies have been done so far. Thus, there is a need expand our knowledge on arsenolipid-derived metabolites toxicity.

The presence of water-soluble metabolites in the bacterial and mucosal niche, including As(III), suggests a potential disruptive effect of arsenolipids on the gut ecosystem. Mucosal environment is colonized by commensal microorganisms, and it is essential for the development of the immune system and host health (Naughton et al., 2014). Mucus layers and resident microbiota through the gastrointestinal tract have a close and complex crosstalk with host-effector cells, like epithelial,

goblet, enteroendocrine, neuronal and immune cells and disruption of this system can induce functional dysbiosis. The toxicological consequences of arsenolipid metabolism on intestinal barrier function and immune response would require further studies.

5. Conclusion

Overall, this study demonstrated that arsenolipids are retained in mucosal and bacterial compartments of the simulated human gut in a donor-dependent way. Gut bacteria mediated biotransformation of arsenolipids to their thioxo analogs and, in the case of the AsFA, to several additional metabolites, the bioaccessibility and toxicity of which need also to be assessed. Further research using more individuals, a repeated-dose approach and food-matrix effect would be needed to evaluate chronic effects and confounding factors affecting the translation to real-life scenarios.

CRediT authorship contribution statement

Chan Xiong: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Marta Calatayud**: Methodology, Data curation, Formal analysis, Writing – original draft. **Tom van de Wiele**: Methodology. **Kevin Francesconi**: Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113662.

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