

# Salivary and Gut Microbiomes Play a Significant Role in *In Vitro* Oral Bioaccessibility, Biotransformation, and Intestinal Absorption of Arsenic from Food

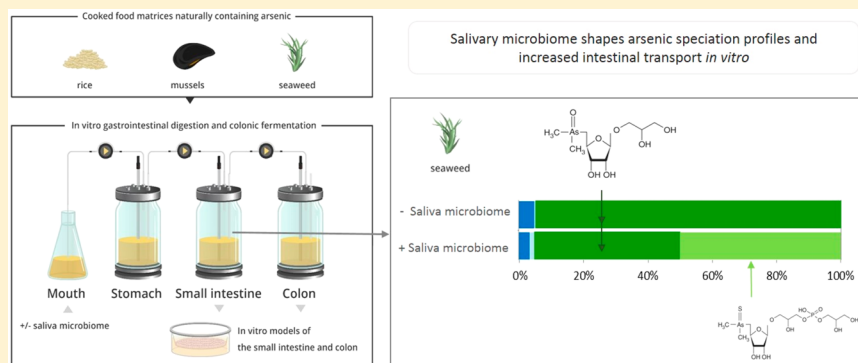
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## Supporting Information



**ABSTRACT:** The release of a toxicant from a food matrix during the gastrointestinal digestion is a crucial determinant of the toxicant's oral bioavailability. We present a modified setup of the human simulator of the gut microbial ecosystem (SHIME), with four sequential gastrointestinal reactors (oral, stomach, small intestine, and colon), including the salivary and colonic microbiomes. Naturally arsenic-containing rice, mussels, and nori seaweed were digested in the presence of microorganisms and *in vitro* oral bioaccessibility, bioavailability, and metabolism of arsenic species were evaluated following analysis by using HPLC/mass spectrometry. When food matrices were digested with salivary bacteria, the soluble arsenic in the gastric digestion stage increased for mussel and nori samples, but no coincidence impact was found in the small intestinal and colonic digestion stages. However, the simulated small intestinal absorption of arsenic was increased in all food matrices (1.2–2.7 fold higher) following digestion with salivary microorganisms. No significant transformation of the arsenic species occurred except for the arsenosugars present in mussels and nori. In those samples, conversions between the oxo arsenosugars were observed in the small intestinal digestion stage whereupon the thioxo analogs became major metabolites. These results expand our knowledge on the likely metabolism and oral bioavailability of arsenic during human digestion, and provide valuable information for future risk assessments of dietary arsenic.

## INTRODUCTION

Humans are exposed to arsenic, a highly toxic element, primarily through dietary sources such as drinking water, rice, and seafood.<sup>1</sup> Epidemiological and toxicological research over many years has established the toxic nature of inorganic arsenic (iAs), even at low levels, and led to regulations in many countries that set maximum permissible concentrations of arsenic in water and rice.<sup>1–6</sup>

*In vitro* oral bioaccessibility testing has been adopted as a conservative estimator of contaminant bioavailability. Most of the current *in vitro* methods incorporate physicochemical parameters (e.g., temperature, pH, enzymatic activity),<sup>7–11</sup> however, the impact of the microbial component, widely present in the colonic, but also in the oral environments, is not

typically considered in *in vitro* models. Earlier work showed that anaerobic microbiota from mouse or human origin converted aqueous standards or iAs in soils into simple methylated oxyarsenicals and thioxo analogs.<sup>12–14</sup> Thioxoarsenicals show toxicity toward human cells,<sup>15–18</sup> in particular, thioxo-DMA has shown strong cytotoxic effects in cultured human bladder cells.<sup>18</sup> Furthermore, thioxo-arsenosugars have higher intestinal bioavailability compared to the oxo-arsenosugars.<sup>17</sup> Thus, a risk assessment of dietary arsenic should

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consider not only the amount of arsenic and its chemical form, but also its bioaccessibility and metabolism. Moreover, the gut microbiome is involved in maintaining the intestinal barrier integrity, which is an essential factor in the arsenic bioavailability process.<sup>19</sup> Thus, the gut microbiome through its interaction with the food matrix and with the host, may play a substantial role in arsenic toxicokinetics.

The main aim of this research was, therefore, to optimize *in vitro* gastrointestinal model systems by incorporating microbiota from the oral and colonic environment and evaluate to what extent microbial presence is an important determinant of oral arsenic bioaccessibility, bioavailability, and arsenic speciation profiles. We use HPLC/mass spectrometry to follow the biotransformation of the natural arsenic compounds in the foods in order to evaluate the metabolic potential of the microbiome and the influence of microbiota as determinants of oral arsenic bioaccessibility and intestinal transport.

## MATERIALS AND METHODS

Reagents used in the investigation were of analytical or reagent grade. Reagents used in this research were purchased from Merck KGaA, Darmstadt, Germany, unless otherwise stated. Water used for experiments was purified (18.2 M $\Omega$  cm) with a Millipore purification system (Millipore GmbH, Vienna, Austria or Millipore Inc., Belgium).

**Arsenic Quantification.** Plastic and glassware material was treated with 10% HNO<sub>3</sub> (v/v) for 24 h, and then rinsed with deionized water before use. Reagents and standard solutions of arsenic used for identification and quantification of arsenic species are described in [SI Methods S1](#). For quality control, we used the certified reference material (CRM) IAEA 407 (homogenized fish tissue) from International Atomic Energy Agency (Vienna, Austria), CRM 7405-a (Hijiki) from National Metrology Institute of Japan (Tsukuba, Ibaraki, Japan), and ERM-BC211 (rice flour) from Sigma-Aldrich (Vienna, Austria) ([SI Table S1](#)).

Total arsenic measurements were performed on the microwave-assisted acid mineralized samples (UltraCLAVE IV Microwave Reactor; MLS GmbH, Leutkirch, Germany) by using an Agilent 7900 ICP-MS (Agilent Technologies, Waldbronn, Germany). Arsenic speciation was performed by HPLC (Agilent 1260 Infinity HPLC system) coupled in parallel with an inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7900) and electrospray ionization tandem mass spectrometer (ESI-MS-MS, Agilent 6460). Detailed information about determination of total arsenic, extraction procedure, and determination of arsenic species is provided as [SI Methods S2–S4](#). For the structures of typical arsenic species see [SI Figure S1](#). Full details of the operating conditions for the analyses by HPLC-ICP-MS/ESI-MS-MS were provided in [SI Table S2](#). MRM transitions and optimum conditions for determining arsenic species by MS/MS analysis were listed in [SI Table S3](#).

**Food Samples.** Three batches of brown rice (*Oriza sativa*), mussels (*Mytilus edulis*), and laminated nori seaweed (*Porphyra tenera*) were purchased at different supermarkets in Ghent (Belgium). Rice was washed twice with 1:10 (w/w) water and once drained, it was cooked in a stainless steel stewpot with 1:3 (w/w) water for 30 min. Mussels were rinsed with water (1:10, w/w) two times and steamed in a stainless steel stewpot for 10 min without adding cooking water. Nonedible portions (valves) were removed and liquid and edible parts were mixed. Nori seaweed was roasted 15 s in a preheated Teflon

pan. After cooking, food matrices were homogenized by using a Thermomix food processor (Vorwerk, Spain M.S.L, S.C) at highest speed until obtaining a soft texture (mussels, rice) or a powder (nori).

**In Vitro Gastrointestinal Digestion and Fermentation.** Foods were digested using a semicontinuous simulation of the digestion process, modified from the SHIME with four stages: oral, gastric, small intestine, and colon. A schematic representation of the *in vitro* digestion is given in the [SI Figure S2](#).

The prepared samples of rice (90 g), mussels (50 g), and nori (2.5 g) were added to double-jacketed reactors maintained at 37 °C under continuous stirring. As a control, sterile water samples (50 g) were used. The amount of food was estimated on representative quantities of daily intakes for the European population.<sup>24</sup> The *in vitro* digestions were run in parallel and tested in duplicate. Samples were mixed with a simulated salivary fluid (SSF) in a ratio of 1:1 (w/w) for rice, mussels and control, and in a ratio of 1:20 (w/w) for nori. The higher ratio of SSF/sample for nori was selected to mimic the dilution of the 2.5 g of sample with digestive fluids in a real theoretical scenario. Oral digestion was performed for 2 min. The SSF was used either supplemented with salivary bacteria or not supplemented. SSF with bacteria was obtained by resuspending the pellet obtained after centrifugation<sup>25,26</sup> (15 min, 9000g) of 100 mL of saliva from a pool of 5 donors with 100 mL of SSF.

To emulate the gastric stage of the digestion, the pH was adjusted to 3 with 1 M HCl by using a pH electrode coupled to a pH controller (Consort R301) and a Master Flex pump drive (Cole-Parmer Instrument Company, LLC). Then, gastric simulated fluid (GSF) was added (1:1, w/w). The gastric digestion was maintained for 2 h. For the intestinal digestion, the pH value was raised to 6.5 by addition of 1 M NaOH. Then, the simulated intestinal fluid (SIF) was added (1:1, w/w). Incubation at 37 °C and continuous stirring were maintained for 2 h. Colonic digestion was performed by adding anaerobic nutritional medium (1:1, v/v) and flushing the reactors with N<sub>2</sub> for 15 min to create anaerobic conditions. After flushing, fecal inoculum (20%, w/v) diluted in anaerobic PBS (pool of five donors, the same as for the saliva pool) was added to the vessels in a proportion of 1:10 (v/v). Information about the origin of the salivary and fecal samples is shown in [SI Table S4](#). Detailed methodology for inocula preparation is described in [SI Method S5](#).

The pH was adjusted to 5.6–5.9 with 1 M HCl and maintained in this range by adding 0.5 M HCl or 0.5 M NaOH. The system was flushed with N<sub>2</sub> for 15 min more and incubated 24 h at 37 °C and constant stirring.

Detailed composition of SSF, GSF, SIF, and nutritional medium for colonic fermentation is described in [SI Methods S6](#).

At the end of each of the four digestion step, samples were obtained and pH values recorded. Samples were centrifuged 9509g/10 min at 4 °C and the supernatant was filtered (Whatman qualitative filter paper, grade 1, 11  $\mu$ m, Millipore, Belgium). Samples for bioaccessibility and arsenic biotransformation assays were freeze-dried (Heto Powerdry PL3000 Thermo, Denmark) for further analysis. We defined bioaccessible arsenic as the amount of arsenic released from the matrix and which is soluble after the centrifugation and filtration steps. We calculated the % of bioaccessible arsenic considering the total arsenic quantification. The values above 100% may be most likely caused by the complexity of the experimental

**Table 1. Arsenic Species [Arsenic in  $\mu\text{g}$ ; % (Arsenic  $\mu\text{g}$  of Each Species/Sum of Species)] in Initial and Gastrointestinal Digested Mussel Samples**

food matrix stage salivary bacteria unit	initial mussel (244 $\mu\text{g}$ As)		gastrointestinal digested mussel											
			gastric				small intestinal				colonic			
			without		with		without		with		without		with	
			$\mu\text{g}$	%	$\mu\text{g}$	%	$\mu\text{g}$	%	$\mu\text{g}$	%	$\mu\text{g}$	%	$\mu\text{g}$	%
AB	61	60	40	53	67	53	62	53	59	52	62	51	80	51
Oxo-AsSug-glycerol	10	9	11	15	18	14	16	13	15	13	18	14	30	19
unknown(1)	2	2	1	1	2	2	4	3	2	2	5	4	5	3
AC	1	1	1	1	1	1	3	2	3	3	2	2	3	2
unknown(2)	2	2	2	3	3	3	3	3	4	3	3	3	3	2
TETRA	2	2	3	4	5	4	2	2	3	3	1	1	2	2
DMA	2	2	2	2	3	3	4	3	3	3	4	3	5	3
Oxo-AsSug-phosphorylglycerol	8	8	11	15	19	15	14	12	15	13	16	13	19	12
As(V)	1	1	1	1	1	1	2	1	2	1	1	1	1	1
unknown(3)	1	1	1	1	2	1	1	1	1	1	2	1	2	1
thioxo-AsSug-Glycerol	4	4	<0.2		<0.2		2	2	1	1	1	1	<0.2	
unknown(4)	2	2	2	3	4	3	3	2	3	3	4	3	5	3
thioxo-AsSug-phosphorylglycerol	5	5	<0.2		<0.2		2	2	1	1	2	2	<0.2	
sum of species	103	100	75	100	126	100	118	100	113	100	122	100	157	100

model, combined human samples and food matrices in a complex mixture of gastrointestinal fluids. The sampling, subsampling, and processing (e.g., freeze-drying) could introduce an experimental variation which causes the observed deviations.

Samples for cell culture assays were filter sterilized (0.22  $\mu\text{m}$ ; Millipore, Belgium), and pH and osmolarity were adjusted to  $7.2 \pm 0.2$  and  $290 \pm 15$  mOsM/kg, respectively. All gastrointestinal digestion stages containing salivary bacteria will be referred to as bacteria-conditioned and those without salivary bacteria as nonbacteria-conditioned. Control samples will be considered as digestive fluids without food matrices.

**Cell Cultures.** Caco-2 (ECACC 86010202) and HT29-MTX-E12 (ECACC 12040401) cells were obtained from the European Collection of Authenticated Cell Cultures. Cell maintenance was carried out routinely as described in Calatayud et al. 2011.<sup>20</sup> All the cultures were used between passages 50 and 60.

Cell differentiation and the posterior tests were carried out in double chamber wells (Corning HTS Transwell-24 well, pore size 0.4  $\mu\text{m}$ ; Costar Corp., NY). The cells were seeded at a density of  $7.5 \times 10^4$  cells/cm<sup>2</sup> in a proportion of 90/10 and 70/30 Caco-2/HT29-MTX for resembling the small intestine and colon epithelium, respectively, and maintained with Dulbecco's Modified Eagle's Medium-high glucose (4.5 g/L) (DMEM), supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (iFBS, Greiner Bio-One, Wemmel, Belgium), 1% (v/v) GlutaMAX (Gibco, Life Technologies Europe BV), and 1% penicillin/streptomycin (Life Technologies, Merelbeke, Belgium) until differentiation (15 days). Then, DMEM was removed and cells were washed twice with 0.2 mL of Hanks Balanced Salt Solution (HBSS). The cell monolayer resembling the colon was covered by 50  $\mu\text{L}$  of a biosimilar mucus layer prepared as described in Boegh et al. 2014.<sup>21</sup>

**Apparent Permeability Coefficient (Papp) of Arsenic and Arsenic Cellular Uptake.** One mL of HBSS was added to the basolateral compartment and the filter-sterilized supernatants from the small intestine and colonic digestion were diluted in HBSS (v/v) 1:2 (rice samples, small intestine),

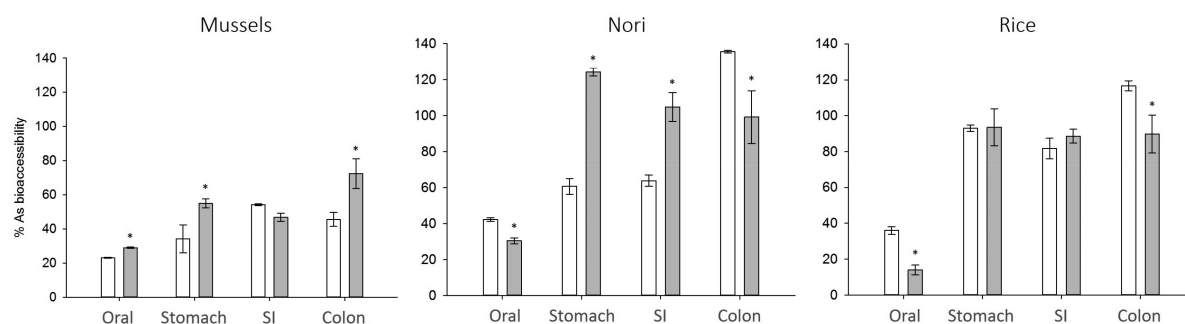
1:5 (rice samples, colon), 1:10 (nori and control samples, small intestine), 1:20 (nori, mussel, and control samples, colon), and added to the apical chambers (0.2 mL).

The control condition consisted of the simulated digestion fluids (small intestine or colon), not containing any dietary matrix, and in absence or presence of salivary bacteria, spiked with As(V) 100  $\mu\text{g/L}$ .

For permeability assays, samples from the basolateral compartment (0.5 mL) were obtained at 30, 60, and 120 min (small intestine) and at 60, 240, 1440 min (colon), and replaced with HBSS. During the transport assays, cell monolayers were kept under stirring conditions (60 rpm) in a shaker (ROCKER 3D basic, IKA, Belgium), at 37 °C, 90% humidity and 10% CO<sub>2</sub>. At the end of the permeability assay (small intestine model: 2 h; colon model: 24 h), cell monolayers were washed twice with HBSS. The apical and basal media and the cells were recovered, digested with 65% HNO<sub>3</sub>/30% H<sub>2</sub>O<sub>2</sub> (2:1 v/v) 90 °C, 4h, in a proportion to the sample of 1:1 (v/v), and filtered (0.45  $\mu\text{m}$ , PTFE; Metrohm Belgium N.V.) before analysis. Tests were evaluated independently at least in triplicate. Percentage of cell uptake and cell uptake + transport (total uptake) was calculated with respect to the total arsenic content added to the apical compartment. The Papp of arsenic was calculated as described in Calatayud et al., 2010.<sup>22</sup>

**Assessment of the Epithelial Barrier Function: TEER and Papp of LY.** During the period of growth and differentiation, cell monolayer integrity was monitored every 2–3 days, measuring the transepithelial electrical resistance (TEER) with a Millicell-ERS (Merck KGaA, Darmstadt, Germany). The reported values were calculated as described by Srinivasan et al., 2015.<sup>23</sup> The cell monolayer was considered completely formed when stable TEER values were obtained ( $\geq 80 \Omega \text{ cm}^2$ ). Monolayer integrity was also evaluated by calculating the Papp of the paracellular transport marker Lucifer Yellow (LY) as described in Calatayud et al., 2010,<sup>22</sup> using a microplate fluorescence reader (Spectramax Gemini XS Microplate Reader, Molecular devices, Orleans, CA).

Results of TEER are expressed as a percentage of TEER after 2 h (small intestine) or 24 h (colon) of exposure to



**Figure 1.** Bars represent the percentage of arsenic bioaccessibility from mussels, nori, and rice in the different digestion steps [oral, stomach, small intestine (SI), and colon] in absence (white bars) or presence (gray bars) of salivary bacteria. The percentage of arsenic solubilized from the food matrix was calculated with respect to the total arsenic content in the original sample (mean  $\pm$  standard deviation;  $n = 2$ ). Significant differences ( $p < 0.05$ ) comparing the absence and presence of salivary bacteria are marked by an asterisk (\*).

different digests, compared to the initial TEER values in the individual wells.

**Mitochondrial Metabolic Activity Assay.** The cells were grown in Transwell inserts and exposed to the supernatants from the small intestine and colonic digestion as previously described. After 2 h (small intestine model) or 24 h (colon model) the cells were washed with phosphate buffered saline (PBS) (Gibco, Belgium). Resazurin test (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) was performed as described by Calatayud et al. 2013<sup>27</sup> and a Spectramax Gemini XS Microplate Reader was used for quantification of resorufin (S60Ex/S90Em) in the apical media. The results were expressed as percentages of resazurin reduction with respect to the fluorescence from cells exposed to the simulated digestion fluids without food matrix.

**Protein Quantification.** The cellular protein content was evaluated by the Bradford dye-binding method (BioRad, Belgium), following the instructions of the manufacturer.

**Statistical Analysis.** The statistical analysis was performed on SigmaPlot 13 software (Systat Software Inc., UK). The significance level was set at 0.05. Normality of the data set was tested with the Kolmogorov–Smirnov test. In case of normality, mean values of two different groups were compared with an independent samples  $t$  test. Significant differences between treatments were tested with one way ANOVA in case of normality. Homogeneity of variances was tested with the Modified Levene test. Depending on the outcome of the Levene test, Bonferroni or Dunnett T3 were used as post hoc tests to determine  $p$ -values. In case of non-normal distributions, differences were tested with nonparametric Mann–Whitney U test. Pearson Product Moment Correlation coefficient was calculated to assess the possible linear correlation between different variables.

## RESULTS AND DISCUSSION

**Salivary Bacteria Significantly Modify Arsenic Bioaccessibility Dependent on Food Matrix and Digestion Stage. Mussels.** The total arsenic content found in the mussel sample ( $23.4 \pm 3.0 \text{ mg kg}^{-1}$  dry weight, dw) was higher than previously reported values  $9.15\text{--}17.48 \mu\text{g g}^{-1}$  dw.<sup>28</sup> The initial mussel sample contained mainly arsenobetaine and arsenosugars (Table 1, SI Figure S3 and S4), which was the expected arsenic species pattern based on many previous studies.<sup>29–31</sup> High concentrations of iAs (up to  $5.8 \text{ mg kg}^{-1}$  ww) and other organic arsenicals (e.g thio-arsenosugars) have

also been described occasionally in fresh and processed mussel samples.<sup>29,32</sup>

Arsenic bioaccessibility values for digested mussels in the absence of salivary bacteria ranged from 23 to 54% (Figure 1). The presence of salivary bacteria significantly ( $p < 0.05$ ) increased the arsenic bioaccessibility in the oral ( $29 \pm 0.3\%$ ), gastric ( $55 \pm 3\%$ ), and colon ( $72 \pm 9\%$ ) reactors by a factor of 1.3, 1.6, and 1.7 (Figure 1). Independent of the effect of the salivary bacteria, the high solubility of arsenic in the oral digestion (14 to 42%) indicates a significant release from food at an early digestion stage. Our findings agree with Leufroy et al., 2012 who demonstrated that the arsenic released by saliva represents at least half of the bioaccessible arsenic in seafood certified reference materials and real seafood samples.<sup>33</sup> Despite the fast transit time of the food in the oral cavity, high bioaccessibility values in the mouth could lead to increased absorption in the proximal sections of the gastrointestinal tract.

Mussels contain collagenous molecules,<sup>34</sup> which can be partially degraded by  $\alpha$ -amylase.  $\alpha$ -Amylase is one of the principal enzymes of the saliva<sup>35</sup> and that this enzyme releases acid glycosaminoglycan from various connective tissues.<sup>36</sup> The removal of glycoprotein fraction of the collagen is required for further collagenase digestion. We removed the host  $\alpha$ -amylase during the centrifugation process but previous research has reported  $\alpha$ -amylase activity in different bacterial strains from human and environmental samples.<sup>37,38</sup> The initial process of the mussels digestion in the oral reactor may affect food matrix structure and further digestion in the lower compartments. Moreover, recent research has shown that human salivary amylase gene copy number impacts oral and gut microbiomes,<sup>35</sup> supporting the hypothesis of a close interplay between the oral cavity and gut microbiomes and the host.

It has been reported that mussel consumption could result in the provisional tolerable weekly intake for iAs being exceeded.<sup>29</sup> Despite the high arsenic concentration in the tested mussels, we found the % of iAs to be quite low (0.43% of total arsenic). An increased release of arsenobetaine in the oral cavity may thus cause a larger absorption in the small intestine, but there are no known consequences for risk assessment up to now.

**Nori Seaweed.** In our study, the total arsenic content in nori samples was  $18.3 \pm 2.9 \text{ mg/kg}^{-1}$  dw. The percentage of total arsenic bioaccessible varied from  $30 \pm 2\%$  (oral reactor, bacteria-conditioned) to  $136 \pm 0.7\%$  (colon reactor, non-conditioned) (Figure 1). Previous research reported in vitro

**Table 2.** Arsenic Species [Arsenic in  $\mu\text{g}$ ; % (As  $\mu\text{g}$  of Each Species/Sum of Species)] in Initial and Gastrointestinal Digested Nori Samples

food matrix		gastrointestinal digested nori													
stage		initial nori (45 $\mu\text{g}$ As)		gastric				small intestinal				colonic			
salivary bacteria				without		with		without		with		without		with	
unit		$\mu\text{g}$	%	$\mu\text{g}$	%	$\mu\text{g}$	%	$\mu\text{g}$	%	$\mu\text{g}$	%	$\mu\text{g}$	%	$\mu\text{g}$	%
oxo-AsSug-glycerol		0.7	3	0.7	4	1.5	4	0.5	3	1.5	5	6.7	16	1.0	4
DMA		0.3	1	0.2	1	0.3	1	0.3	2	0.3	1	0.8	2	0.1	0.4
oxo-AsSug-phosphorylglycerol		6.5	30	11	61	28	70	7.0	43	30	92	<0.1		<0.1	
As(V)		0.1	1	0.2	1	1.5	4	0.2	1	0.4	1	<0.1		0.3	1
thioxo-AsSug-glycerol		0.4	2	0.3	2	0.4	1	0.2	1	0.2	1	34	81	23	94
thioxo-AsSug-phosphorylglycerol		14	62	5.6	31	7.9	20	7.7	48	<0.1		<0.1		<0.1	
sum of species		22	100	18	100	40	100	16	100	33	100	41	100	25	100

total arsenic bioaccessibility in *Porphyra* spp. between 67 and 87% (raw) and 80–106% (cooked).<sup>39,40</sup> For dialyzable arsenic in the red alga (*Porphyra umbilicalis*), however, Garcia-Sartal et al.<sup>33</sup> observed much lower values of 17.0% (raw) and 15.3% (cooked).

Interestingly, the arsenic bioaccessibility from nori was increased by salivary bacteria in the stomach and small intestine from  $\sim 60$  to  $\sim 100\%$  (Figure 1).

Previous research has reported carbohydrate active enzymes in the human gut,<sup>41,42</sup> involved in the agarolytic pathway<sup>43</sup> which allows for agarose saccharification into 3-O- $\beta$ -D-galactose (GAL) and 4-O- $\alpha$ -3,6-anhydro-L-galactose (AHG). Despite the bacterium containing this specific enzymatic activity was only isolated in Japanese and Chinese individuals, it is possible that this enzymatic activity was also present in the microbiomes of one or multiple donors of this study. The degradation of complex polysaccharides from nori to GAL and AHG may cause the release of arsenic in the form of inorganic arsenic.

Moreover, in the presence of salivary bacteria, the amount of iAs in the gastric, small intestine and colon compartments in nori samples increased 8, 2, and 3 times respectively (Table 2). It has been shown that iAs induces oxidative stress, inflammation, cell cycle alterations, changes in protein expression in intestinal cells, and epithelial barrier impairment,<sup>27,44</sup> besides being a well-recognized human carcinogen.<sup>45</sup>

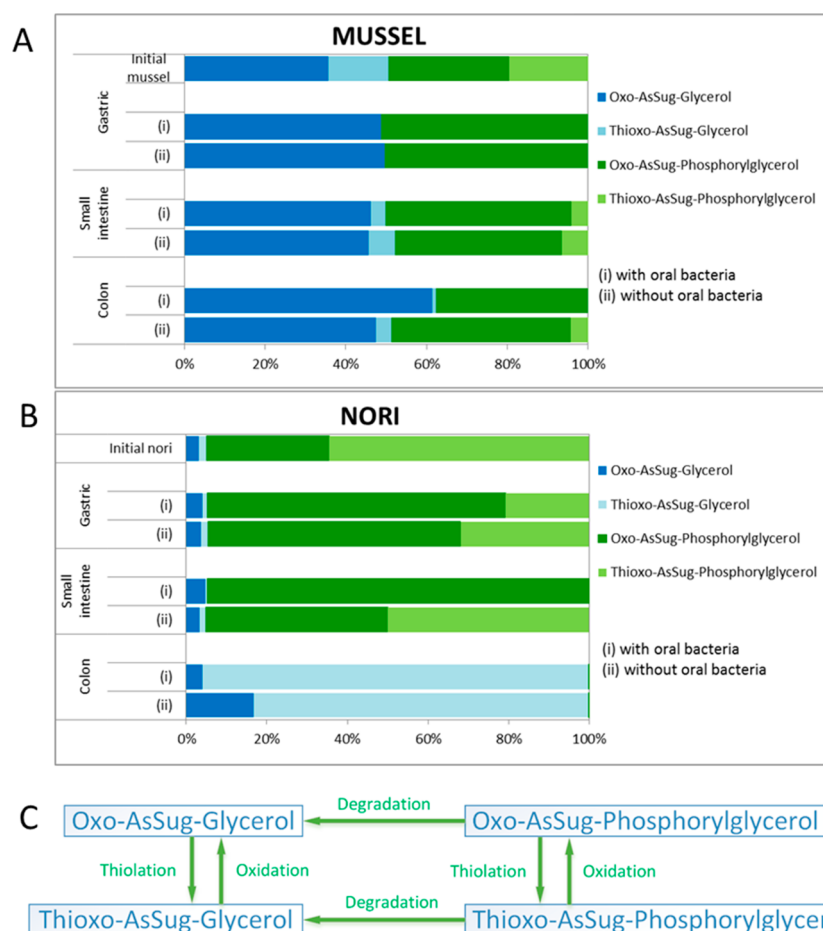
Previous data showed that conventional mice methylate and absorb iAs to the same extent as germfree mice,<sup>46</sup> thus gut microorganisms may have a negligible effect on biotransformation and absorption of iAs in an in vivo mice model. However, the bioaccessible arsenic in the lumen of the stomach, small intestine, and colon can be affected by the presence of microorganisms. Taking into account the small intestine as the main site of arsenic absorption, the increase in bioaccessibility may result in higher internal exposure after nori intake, but also in a higher local exposure of intestinal cells and the mucosal niche to different arsenic species. Previous research has shown that As(III) induced erosion of bacterial biofilms adjacent to the mucosal lining and changes in the diversity and abundance of morphologically distinct species indicated changes in microbial community structure.<sup>47</sup> The effect of other arsenical species in the host–microbiome interface is still unknown.

**Rice.** Total arsenic content in the rice used in our studies was  $0.22 \pm 0.01 \text{ mg/kg}^{-1} \text{ dw}$ . The percentage of arsenic bioaccessible at different digestion stages varied from  $14 \pm 3\%$  (oral reactor, bacteria-conditioned) to  $117 \pm 3\%$  (colon reactor,

nonbacteria-conditioned). Trenary et al. 2012 found that the bioaccessible arsenic in cooked brown rice during synthetic gastrointestinal extraction ranged from 58% to 64%;<sup>48</sup> Laparra et al. 2005 found that the bioaccessible fraction accounted for more than 90% of the total arsenic content of cooked whole grain rice after simulated gastrointestinal digestion;<sup>49</sup> and He et al. 2012 found the extractable arsenic ranged from 53% to 102% after rice was treated with *in vitro* artificial gastrointestinal fluid.<sup>50</sup>

In the presence of salivary bacteria along the gastrointestinal digestion, the % of arsenic bioaccessible decreased from  $36 \pm 2\%$  to  $14 \pm 3\%$  (oral), and from  $117 \pm 3\%$  to  $89 \pm 10\%$  (colon), a trend that was also observed in nori samples (Figure 1). Salivary bacteria did not affect the arsenic bioaccessibility in the gastric and small intestinal digestion. A possible explanation for our findings is that iAs has chemical similarity to substrates of membrane transporter proteins of bacterial cells. The uptake of iAs by aquaglyceroproteins (e.g., GlpF)<sup>51</sup> or phosphate transporters (e.g., Pit and Pst) has been previously described.<sup>52</sup> The centrifugation of the samples (9509 g, 10 min) can remove the microorganisms containing arsenic from the bioaccessible fraction, which results in lower bioaccessibility values. In a highly dense ( $>10^8$  viable cells/mL) and complex community as the saliva or fecal microbiome, the “trapping” effect caused by the bacterial cells could be significant. To corroborate this hypothesis, a batch test incubating 100  $\mu\text{g/L}$  of arsenate with saliva and fecal samples was performed, and the percentage of arsenic retention by the bacterial cells was found to be quite substantial, ranging from 35 to 54% (SI Method S7; data not shown). Sun et al., 2012 also observed a significant drop in arsenic bioaccessibility from rice in a simulated colon digestion model. Authors suggested that this drop is probably due to the higher amount of organic matter that is introduced in the colon suspension under the form of microbial biomass.<sup>53</sup>

Other factors related to food sample preparation, as the cooking processes, may affect bioaccessibility of arsenic. Laparra et al., 2004 that bioaccessible inorganic arsenic in raw seaweed (54–67%) increased after cooking (78–84%).<sup>54</sup> Other study reported comparable values of arsenic species in raw and cooked seaweed.<sup>55</sup> These both studies used boiling water as the cooking method, which may cause a different effect on the food matrix than the roasting process applied in this research. Zhuang et al., 2006 observed that cooking process of rice (30 min in water, 2:1 w/v ratio) reduced bioaccessibility of arsenic.<sup>56</sup>



**Figure 2.** Panel A and B: transformation of the arsenosugars in initial samples and gastrointestinal digested fluids of mussel and nori. The bar graphs are color-coded to represent the two arsenosugars (blue and green) as their oxo (dark shading) and thioxo (light shading) forms. Panel C: overview of the interrelationships between the four major arsenosugars found in this study.

Moreover, Alava et al., 2013 showed that the particle size of rice had a major influence on arsenic extraction from the food matrix.<sup>57</sup> In this research, the grinding process and the solid to volume ratios might affect the arsenic solubility from the food matrices.

Thus, the presence of salivary and colonic bacteria decreases the bioaccessible arsenic (potentially available for intestinal absorption) in rice and nori, possibly acting as a symbiotic/mutualistic protective mechanism against internal arsenic exposure.<sup>54–56,58</sup> The disruption of the microbial ecosystems by antibiotic intake or infections could change the behavior of arsenic in the gastrointestinal tract, and such an outcome would require further research.

Individual microbial signatures of salivary and stool samples have previously been demonstrated<sup>59–61</sup> but have not been considered in this study because of the use of a single spot sample of pooled saliva and feces from five donors. The effect of the interindividual variability on arsenic bioaccessibility and speciation may require further research. Moreover, sample processing may affect the salivary and fecal microorganisms, reducing the representativeness of the microbiomes. Regarding the saliva, flow cytometry counts of viable cells before and after the centrifugation steps gave similar results ( $\sim 10^8$  viable cells/mL). Fecal samples are routinely used to investigate the intestinal microbiome and have been demonstrated to be a useful proxy of distal colon microbiome,<sup>62</sup> although prepara-

tion of the fecal inoculum can affect the viability of strictly anaerobic microorganisms.

**Biotransformation of Arsenic from Food during the Gastrointestinal Passage.** Arsenic speciation analyses were performed on extracts of the initial samples and on the fluid from each of the gastrointestinal digestive compartment by HPLC-ICP-MS under both anionic and cationic chromatographic conditions (SI Figure S3).

**Mussels.** The *in vitro* digestion did not transform the arsenobetaine present in the original sample; the soluble arsenobetaine initially constituted 60% of total arsenic, and the values were 51–53% for all three gastrointestinal digested fluids (gastric, small intestinal and colonic) for nonbacteria and bacteria-conditioned treatments (Table 1). This result is consistent with metabolic studies of arsenobetaine with mice<sup>63</sup> and humans,<sup>64</sup> which showed that arsenobetaine is excreted mostly unchanged in the urine after oral intake.

The original pattern of arsenosugars slightly changed in the gastrointestinal digestive compartments with the oxo-form generally becoming more dominant (Figure 2A). We did find small amounts of thioxo arsenosugars in the small intestinal and colonic stages, as well as in the initial mussels but not in the gastric digestion stage (Table 1; SI Figures S3 and S4). There was no clear change in the ratio between the glycerol arsenosugar and the phosphorylated arsenosugar going from initial mussel extract to the colon fluid.

**Table 3. Arsenic Species [Arsenic in  $\mu\text{g}$ ; % (As  $\mu\text{g}$  of Each Species/Sum of Species)] in Initial and Gastrointestinal Digested Rice Samples**

food matrix		gastrointestinal digested rice												
stage	initial rice (6.0 $\mu\text{g}$ As)		gastric				small intestinal				colonic			
salivary bacteria			without		with		without		with		without		with	
unit	$\mu\text{g}$	%	$\mu\text{g}$	%	$\mu\text{g}$	%	$\mu\text{g}$	%	$\mu\text{g}$	%	$\mu\text{g}$	%	$\mu\text{g}$	%
As(III)	3.4	71	3.1	70	3.2	72	2.8	68	3.3	68	3.8	63	3.0	62
DMA	0.5	11	0.6	15	0.6	14	0.5	11	0.6	11	1.0	17	0.9	18
As(V)	0.9	18	0.7	15	0.7	15	0.9	21	1.0	21	1.2	20	1.0	20
sum of species	4.8	100	4.3	100	4.5	100	4.1	100	4.9	100	6.0	100	4.9	100

Few studies have assessed the toxicity and toxicokinetics of arsenosugars. To our knowledge, the only study in vivo showed that high doses (20–50 mg synthetic oxo-arsenosugar-glycerol/kg body weight) induced blood and brain oxidative stress, DNA damage and neurobehavioral impairments.<sup>65</sup> Few in vitro studies showed that oxo-arsenosugars were non-cytotoxic or genotoxic to Caco-2 cells.<sup>66</sup>

Thioxo-AsSug-Glycerol was detected by Molin et al. 2012 in human urine after blue mussel consumption, which accounted for about 1.5% of the total excreted arsenic species.<sup>67</sup> The metabolism of arsenosugars from mussels might occur in the gastrointestinal tract due to biotransformation by the human tract microbiota. Previous research supports these findings, as the anaerobic microbiota of mouse cecum material can produce sulfur-containing analogs of arsenosugars.<sup>68,69</sup> Because of the in vitro intestinal permeability of thio-AsSugar-Gly was found to be twice as high as its oxo- analog, the preabsorptive metabolism in the digestive tract can change the toxicokinetics and toxicodynamics of arsenic, but also affect the gastrointestinal barrier functionality.<sup>66</sup>

The minor arsenicals found in mussel (e.g., DMA, TETRA) were also present at comparable levels in the digest fluids, a result suggesting that these compounds, like arsenobetaine, were not greatly affected by the gastrointestinal digestive process or by the presence of salivary bacteria.

**Nori Seaweed.** By far, the major compounds in initial samples were the thioxo (62% of total arsenic) and oxo (30%) forms of the phosphorylated arsenosugar (Table 2; SI Figures S3 and S6). This result is consistent with previous studies on nori,<sup>70</sup> but differs in two ways. First, the related (dephosphorylated) Oxo-AsSug-Glycerol was present in trace amounts only, whereas it usually occurs at levels comparable to the phosphorylated compound. For example, Li et al. 2003 found that the distribution of Oxo-AsSug-Phosphorylglycerol and Oxo-AsSug-Glycerol (SI Figure S6) in five red alga samples (*Porphyra*) obtained in Beijing varied from 13–68% and 19–86%, respectively.<sup>31</sup> Second, the thioxo, rather than the oxo form of the arsenosugar was the dominant compound. Although thioxo forms of arsenosugars are commonly reported in algae,<sup>71</sup> they are usually minor compounds compared to the oxo analogs. We note that the nori used in our experiments was briefly roasted before gastrointestinal digestion, and this treatment may have influenced the observed speciation pattern.

Quite different from the outcome with mussels, in nori samples we observed marked changes in the ratios of both the oxo/thioxo forms and the glycerol/phosphoryl arsenosugars, which also depended on the absence or presence of oral bacteria (Table 2, Figure 2B). In the gastric fluids, the oxo form predominated, a clear change from the initial nori, which contained mainly the thioxo arsenosugar. In the colon, the

phosphorylated arsenosugars originally present in the nori were completely degraded to the glycerol arsenosugar, which was then mainly present as the thioxo form. This large change was not evident after the gastric and small intestinal stages, and thus had been elicited only at the colon stage. In the colon environment, microbial sulfate reduction to hydrogen sulfide is a common process which can trigger the formation of thioarsenosugars.<sup>13,68</sup> Conklin et al. 2006 reported that the anaerobic microflora from mouse gastrointestinal tract can readily convert an oxo-arsenosugar to its thioxo analogue.<sup>68</sup> In contrast, Chavez-Capilla et al. 2016 showed that oxo-arsenosugars were not changed when seaweed was exposed to physiologically based extraction.<sup>72</sup>

The presence or absence of salivary bacteria did not appear to play a significant role in the observed transformations, except for the small intestine fluid where the thioxo form was present only in trace amounts in the bacteria-conditioned treatments.

Sulfate reducing bacteria (SRB) can produce  $\text{H}_2\text{S}$ , which is necessary to induce arsenic thiolation.<sup>73</sup> Heggendorf et al., 2013 have detected SRB in human saliva samples and identified *Desulfovibrio fairfieldensis*, *Desulfovibrio desulfuricans*, and *Raoultella ornithinolytica* as SRB inhabitants of the oral cavity.<sup>74</sup> It is feasible that the saliva samples used in this research also contain SRB.

Because of the high oral bioavailability of arsenosugars and the cellular toxicity of their metabolites,<sup>75,76</sup> the possible risks from ingesting arsenosugars cannot be fully excluded. Experiments with the Caco-2 intestinal barrier model, which mimics human intestinal absorption, indicated that the thioxo-arsenosugars have higher bioavailability and toxicity as compared to the oxo-arsenosugars.<sup>16,18</sup> The observed differences between mussel and nori samples during the gastrointestinal digestive process suggest that the composition of the food matrix might be a significant factor in the transformation of arsenosugars. Previous research shows that diet has a significant impact in shaping the gut microbiome, even after short-term (24 h) of dietary alterations.<sup>77</sup> The same research showed that foodborne microbes can survive the transit through the digestive system and be present in a metabolically active form in the distal gut. This phenomenon described in vivo, can occur in the in vitro system, affecting the microbial communities in the colonic reactor and, in consequence, the metabolic potency toward arsenic. Lu et al., 2014 observed that the metabolic profile of arsenic in urine significantly differ in dysbiotic mice induced by IL-10 knockout, compared to wild type, supporting the relevance of gut microbiome impact on arsenic biotransformations.<sup>78</sup> Transformations between the various forms of arsenosugars under simulated gut conditions

could have direct implications for toxicity studies and risk assessment.

In our *in vitro* system, both the digestion stage and the food source influenced the transformation between the two major arsenosugars and between their oxo- and thioxo forms (Figure 2C).

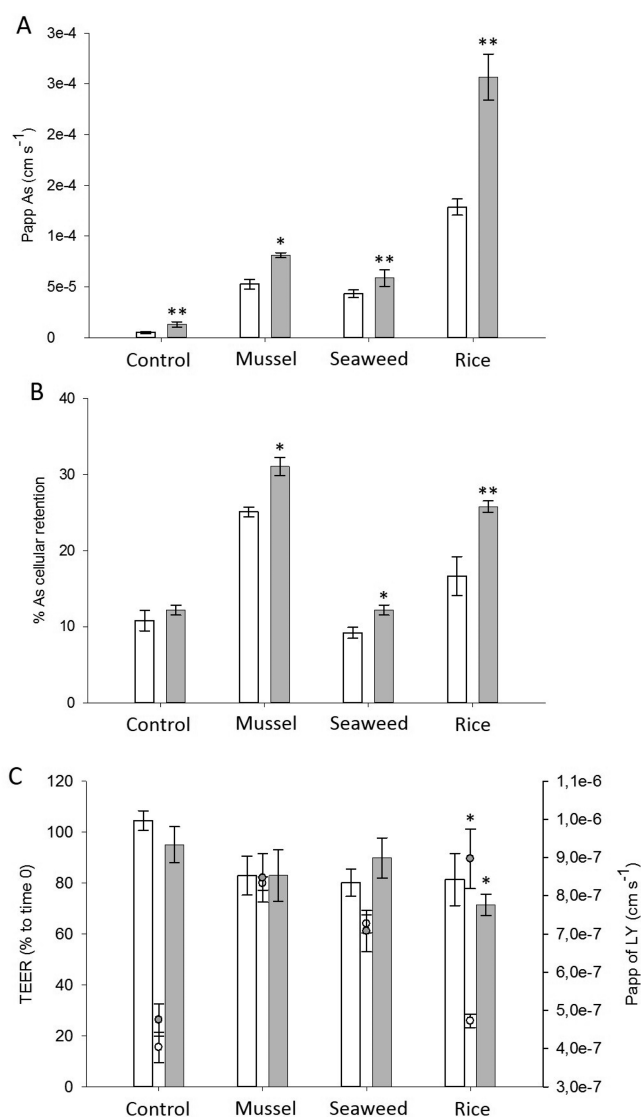
**Rice.** The rice in our study contained mainly iAs (ca. 70% of extractable arsenic) and DMA (8%); about 20% was not extractable and hence could not be assigned by our method (Table 3; SI Figure S3). There were no clear differences between the nonbacteria or bacteria-conditioned treatments for any of the arsenic species. A study by Sun et al. 2012 found similar results for iAs bioaccessibility in cooked white rice where the soluble iAs stayed between 77% and 87% in the stomach, intestine and colon treatments.<sup>79</sup> Contrarily to this study, Sun et al., 2012 found biotransformation of iAs from rice to MMA(III) in a simulated colonic fermentation. Differences in rice composition, microbial inoculum, composition of the media used for the digestion can be some of the factors causing discrepancies between studies.

The *in vitro* model applied in this research allows the investigation of arsenic transformations occurring in specific simulated gastrointestinal digestive processes, and when combined with HPLC/mass spectrometry it can provide a more complete profile of the bioaccessibility of the various arsenic species found in food. Our results indicate that salivary bacteria could increase the soluble arsenic species, especially in the gastric digestion, and show that arsenosugars readily interchange between their oxo and thioxo forms during the different stages of the gastrointestinal simulation. This signals a possible modification in the risk estimation of arsenic exposure when considering salivary microorganisms *in vitro*. The system is ideal to further investigate the effects of salivary treatment, and the possible risk of arsenosugar-rich food consumption, also considering the interindividual variability of the human digestive microbiome.

**Salivary Bacteria Increase the Papp and Cellular Uptake of Arsenic from Digested Food Matrices in the Small Intestine.** Small intestinal digests of food matrices displayed arsenic Papp values of  $1 \pm 0.1 \times 10^{-4} \text{ cm s}^{-1}$  while control digests without a food matrix showed significantly ( $p < 0.001$ ) lower arsenic Papp ( $1 \pm 0.3 \times 10^{-6} \text{ cm s}^{-1}$ ). This was independent of bacterial presence. Yet, within the small intestinal digests of the three food matrices, bacterial presence increased arsenic Papp by a factor 2 ( $2.6 \pm 0.02 \times 10^{-4} \text{ cm s}^{-1}$ ,  $p < 0.001$ ), 1.5 ( $8.1 \pm 0.3 \times 10^{-5} \text{ cm s}^{-1}$ ,  $p < 0.001$ ), and 1.4 ( $5.9 \pm 0.8 \times 10^{-5} \text{ cm s}^{-1}$ ) for rice, mussels, and nori, respectively (Figure 3A). The highest Papp value was observed for rice digests, both nonconditioned ( $1.3 \pm 0.08 \times 10^{-4} \text{ cm s}^{-1}$ ) and bacteria-conditioned ( $2.6 \pm 0.02 \times 10^{-4} \text{ cm s}^{-1}$ ) (Figure 3A).

The Papp values for digested rice, mussels and nori ( $5.3\text{--}25.7 \times 10^{-5} \text{ cm s}^{-1}$ ) correspond to compounds that are considered well-absorbable. Previous research compared the Papp values in the Caco-2 model with *in vivo* absorption data of several drugs in humans.<sup>80,81</sup> Papp values  $< 1 \times 10^{-6} \text{ cm s}^{-1}$ , between 1 and  $10 \times 10^{-6} \text{ cm s}^{-1}$  and  $> 10 \times 10^{-6} \text{ cm s}^{-1}$  have been classified as poorly (0–20%), moderately (20–70%), and well (70–100%) absorbed compounds, respectively.

Our Papp values, in the presence of food matrices, are higher than previously reported values for aqueous standards of inorganic and methylated arsenicals in the trivalent and pentavalent state ( $0.3\text{--}10.6 \times 10^{-6} \text{ cm s}^{-1}$ ).<sup>20,22</sup> Low Papp



**Figure 3.** Effect of salivary bacteria on the apparent permeability coefficient and cellular retention of arsenic in the small intestine. Figure 3A: Papp values of arsenic; Figure 3B: % of arsenic cellular retention; Figure 3C: Transepithelial electrical resistance (bars, left axis) and Papp values of Lucifer Yellow (dots, right axis), at the small intestine model after exposure to nonconditioned (white bars) or bacteria-conditioned (gray bars) digests of food matrices or digestive fluids (control) (average  $\pm$  standard deviation,  $n \geq 4$ ). Significant differences comparing each food matrix in nonconditioned and bacteria-conditioned treatment are marked by an asterisk (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ).

values for aqueous standards of arsenobetaine ( $0.76 \pm 0.02 \times 10^{-6} \text{ cm s}^{-1}$ ) and arsenosugar metabolites have been described ( $0.05\text{--}1.66 \times 10^{-6} \text{ cm s}^{-1}$ ).<sup>82,83</sup>

Standard of arsenobetaine had a low cell retention (3%) and transport (1.7–3.4%) in *in vitro* models.<sup>82</sup> Laparra et al. 2007 already showed a higher efficiency of arsenobetaine transport (12%) when certified protein fish material (DORM-2) was digested and tested in a Caco-2 *in vitro* model.<sup>84</sup> In our study, the cell uptake and transport of total arsenic from mussels was  $38 \pm 2\%$ , which is a closer value to *in vivo* data.

Compared with previous *in vitro* studies of arsenic intestinal absorption, we applied relevant concentrations for a food exposure scenario (1.4–37.1  $\mu\text{g/L}$ ) and the food matrices were



digested in the presence of microorganisms, both key factors in physiological intestinal absorption processes. The presence of a complex mixture of food components and microbial metabolites may stress the *in vitro* intestinal epithelium, increasing the transport of arsenic to the basolateral (blood-resembling) compartment. We only observed a significant impairment of the epithelial barrier in the conditioned-bacteria digest of rice, compared to the nonconditioned digests. Thus, the increased arsenic Papp, at least for mussels and nori, may be linked to specific food components or bacterial metabolites originated during the digestion.

Furthermore, our results are close to *in vivo* assessments of oral arsenic bioavailability. Swine model studies indicated that up to 94% of arsenic from rice was bioavailable.<sup>85</sup> Human intervention studies showed that between 44 and 60% of the dietary dose of arsenic from rice intake is absorbed and quickly eliminated in urine within 24 h of ingestion.<sup>86,87</sup> Other human studies demonstrated a high absorption of organic arsenic aqueous standards (~70–80%), synthetic arsenosugars (70–88%), or organic arsenic species from food items (cod, salmon, mussels, and seaweed) (56–99%) measured as a percentage of total arsenic excreted in urine.<sup>88–92</sup> The values reported for mussels (41–86%)<sup>93</sup> and nori seaweed samples (>90%, a proxy from Le et al., 1999) in human studies fit with those obtained in this research (70–100%).<sup>92</sup>

It is remarkable that, even though small intestinal bioaccessibility of arsenic from mussels and rice was not affected by salivary microorganisms, the Papp of arsenic was further significantly increased by the salivary bacteria. Because the salivary microorganisms made only minimal changes to the speciation of arsenic, we assume that other factors in the bacteria-host-arsenic interplay affect the higher arsenic transport rates. For example, unknown bacterial metabolites or food matrix composition, as phytic acid content in rice<sup>94</sup> or bioactive proteins, peptides, and amino acids derived from mussels<sup>95</sup> might explain the increase of arsenic transport.

In addition to the paracellular route, intestinal absorption of arsenic can occur via the transcellular pathway.<sup>20,96</sup> Averaged out for all food sources, cellular retention of arsenic in the small intestine model was in general higher (18 ± 8%), than in the colon model (9 ± 6%). While cellular uptake from small intestine digests ranged from 8 to 26%, the presence of bacteria further increased the cellular uptake to 12–31%. (1.2–2.7 fold) (Figure 3B). In contrast, this increasing effect from bacterial presence toward cellular arsenic uptake was not observed in the absence of a food matrix, using the aqueous standard of As(V) (10.8 ± 0.4% and 12.4 ± 1.2%, respectively). The same trend was observed in the total uptake (cellular uptake + transport to the basolateral compartment), with a 2-fold increase for nori and rice (SI Table S6).

Specifically for rice, the total uptake (cell retention + transport) reached 47 ± 3% and 93 ± 14% for the nonbacteria conditioned and bacteria-conditioned digests, respectively (SI Table S6). These values were significantly higher than those previously reported for cooked rice (3.9–17.8%)<sup>97</sup> or aqueous standards of pentavalent arsenic species (≤0.5%) and As(III) (≈1.4%)<sup>20,22</sup> using *in vitro* models. Changes on tight junctions or modifications of transporters of the cell membranes can be the cause of these results. Glucose permeases, which are also involved in inorganic arsenic uptake by intestinal cells<sup>96</sup> are up regulated by microbial metabolites as butyrate.<sup>98</sup>

**Bacteria-Conditioned Digests of Rice Impaired the Intestinal Epithelial Barrier Function without Affecting**

**Cellular Viability in the Small Intestine Model.** During the course of the assay (2 h, small intestine), the TEER values were maintained above 80% of the initial TEER, except for the conditioned-bacteria digest of rice, which caused a drop in TEER values to 71 ± 4% ( $p = 0.02$ ) (Figure 3C). Accordingly, the Papp of LY was increased by conditioned-bacteria digests from rice ( $9 \pm 0.8 \times 10^{-7} \text{ cm s}^{-1}$ ), compared to nonbacteria conditioned digests ( $4 \pm 0.2 \times 10^{-7} \text{ cm s}^{-1}$ ). Independently of the salivary bacteria, the presence of food matrices increased the Papp of LY ( $6.8\text{--}8.4 \times 10^{-7} \text{ cm s}^{-1}$ ) compared to the controls ( $4\text{--}4.7 \times 10^{-7} \text{ cm s}^{-1}$ ) (Figure 3C).

Previous research showed that none of the aqueous standards of pentavalent arsenic species, including arsenosugar metabolites, affected the barrier integrity in a Caco-2 model at concentrations below 100 μg/L for 48–72 h.<sup>16,27</sup> Moreover, we found a moderate positive correlation between the cellular uptake of arsenic and the Papp of LY in both small intestine ( $r = 0.527$ ,  $P = 0.008$ ) and colon ( $r = 0.712$ ,  $P < 0.001$ ) models (SI Figure S7). The reason for the drop in TEER and increased Papp of LY for rice digests may be related to iAs toxicity, but also to specific food constituents. For example, phytic acid is a naturally occurring compound in brown rice,<sup>99</sup> and it can decrease the integrity of Caco-2 cell monolayers by modulating the expression levels and localization of tight junction proteins.<sup>94</sup> The effect of the digests on the epithelial barrier integrity may cause a weak intestinal epithelium and expose the lamina propria to antigenic compounds, triggering an inflammatory response, or increasing the absorption of certain molecules. The paracellular route is involved in the absorption of some arsenic species,<sup>20,22,100</sup> thus an impairment of the tight junctions could cause higher absorption of the toxicant. This effect could be of relevance for iAs, which is the main specie in the rice.

The concentrations of arsenic from rice in the cells (1.7–2 ng arsenic/mg prot) and the time of exposure (2 h) did not decrease the resazurin reduction ability, a biological marker of cell survival and mitochondrial activity<sup>101</sup> (SI Figure S8). This result indicates that the cells were not under toxic stress during the assays. Moreover, the bacteria-conditioned digest of nori and rice significantly increased the resorufin production (144 ± 3% and 127 ± 9%, respectively,  $p < 0.01$ ), compared to the nonconditioned digests. Previous studies have supported the crosstalk between microbiota and the host through bacterial metabolites; for example, butyrate reduced the mitochondrial production of reactive oxygen species and positively modulated mitochondrial function.<sup>102,103</sup>

These findings reinforce the relevance of considering the food matrix in arsenic risk assessment.<sup>104</sup> We suggest that a reevaluation of the standardized models for oral bioaccessibility, bioavailability, and furthermore, risk assessment of metal(oid)s or other xenobiotics from food matrices be carried out taking into account the human microbiome as a relevant factor in the process.

**The Colonin Vitro Model Behaves Differently than the Small Intestine Model When Exposed to Food Matrices Containing Arsenic. Papp and Cellular Retention of Arsenic.** In the colon model, contents of arsenic in the basolateral compartment were below the limit of quantification. The absence of transport to the blood-resembling compartment indicates that the biocompatible mucus layer and/or the presence of mucus-producing cells could interfere with the arsenic absorption, acting as a protective barrier against arsenic uptake. Our research applied a complex food

matrix combined with digestion fluids, and independent of the matrix, the protective effect of the mucus against arsenic absorption was evidenced; however, the entrapment of arsenic by the mucus could affect the resident microbiota.<sup>47</sup>

While no arsenic transport was observed in the colon model, the cellular uptake was 0.8–5 ng arsenic/mg protein. The bacteria-conditioned digestion increased the cellular retention of arsenic by 1.4 and 2.8 times for control (non-conditioned:  $7.2 \pm 0.2\%$ ; bacteria-conditioned:  $10.2 \pm 0.4\%$ ;  $p = 0.01$ ) and nori (non-conditioned  $3.8 \pm 0.4\%$ ; bacteria-conditioned  $10.7 \pm 0.8\%$ ;  $p = 0.03$ ), whereas the cells exposed to bacteria-conditioned digests of mussel or rice had a 30–60% lower arsenic uptake, compared to values nonconditioned digests (Figure 4A).

Previous reports showed a link between bacterial metabolites and protein expression levels (CYP3A4, Pgp, MRP2, PepT1, MCT4) in intestinal cells. Butyrate increased the CYP3A4 protein levels by 40-fold and the MRP2 expression was decreased by 10-fold in Caco-2 cells.<sup>105</sup> The effect of different food digests naturally containing arsenic in the expression of transporters and enzymes in intestinal cells is still unknown.

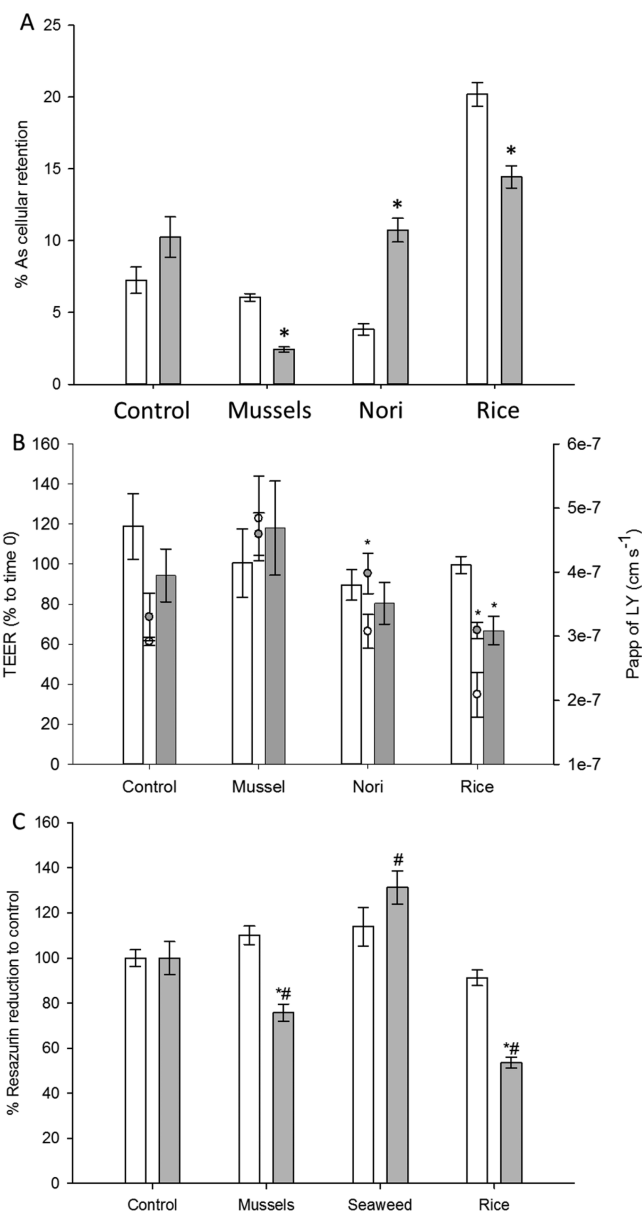
It would be unexpected to find high levels of arsenic in the colonic environment, as most of the toxicant is absorbed in the small intestine, however, when arsenic is bound to a food matrix, it could reach the colon. For example, arsenic in rice is accumulated in the husk and bran,<sup>106</sup> which are not digested in the upper gastrointestinal tract. Nondigestible polysaccharides can be fermented by gut bacteria causing the release of arsenic to the colonic lumen. In a theoretical scenario of an adult with a daily intake (0.3 kg/day) of brown rice (0.2 mg arsenic/kg), assuming 50% bioaccessibility and 0.5 L of colonic volume, up to 60  $\mu\text{g}$  arsenic/L could be released in the colon. This estimated value can be even higher if considering the arsenic bound to soils.<sup>107</sup> Due to the complexity of the food digest matrix, there are several possible explanations for the observed changes in cellular uptake such as increased arsenic release, food constituents, the microorganisms or their metabolites, or a combination of different factors.

#### Epithelial Barrier Integrity and Mitochondrial Activity.

The biocompatible mucus layer and the different cellular composition of the small intestine and colon models did not affect the epithelial barrier properties in basal conditions, as is shown by the similar values of TEER after the differentiation period (average  $n = 24$ , small intestine:  $111 \pm 12 \Omega \text{ cm}^2$ ; colon:  $114 \pm 20 \Omega \text{ cm}^2$ ). The Papp of LY (average  $n = 24$ ) was lower in the colon model ( $3.5 \pm 0.9 \times 10^{-7} \text{ cm s}^{-1}$ ) than in the small intestine ( $6.7 \pm 1.9 \times 10^{-7} \text{ cm s}^{-1}$ ) ( $p < 0.001$ ) probably due to the presence of the mucus layer on top of the epithelial cells impeding the passage of the LY by the paracellular route.

During the course of the assay, the cells exposed to conditioned-bacteria digest of rice decreased the TEER values to  $67 \pm 7\%$  ( $p = 0.03$ ) (Figure 4B), while the other conditions maintained the TEER above 80% of the initial values.

There have been no previous published studies using a biosimilar mucus membrane to estimate arsenic intestinal transport. We found a lack of correlation between the TEER and LY transport (SI Figure S9), which may be caused by the presence of a mucus layer. Glycosylated regions of mucins are densely coated with negative charges,<sup>108</sup> the same as LY in solution and the electrostatic forces could impede the LY to permeabilize through the simulated epithelium.<sup>109</sup> Consequently, the Papp of LY could not be an accurate marker for



**Figure 4.** Cellular retention, epithelial barrier function, and mitochondrial activity of the colon in vitro model after exposure to food digests. Percentage of cellular arsenic retention (4A), percentage of transepithelial electrical resistance (TEER) values (bars, left axis) and Papp of Lucifer Yellow (dots, right axis) (4B), and resazurin reduction (4C) of the colon in vitro model after exposure (24 h) to nonconditioned (white) or bacteria-conditioned (gray) digests. TEER percentages were calculated compared to the initial TEER values at the start of the assay. Significant differences ( $p < 0.05$ ) comparing the nonconditioned and bacteria-conditioned digestion are marked with an asterisk (\*). Only in the graph 4C significant differences ( $p < 0.05$ ) comparing nonconditioned and controls are marked by a pad symbol (#). Values are expressed as mean  $\pm$  standard deviation ( $n \geq 3$ ).

assessing the epithelial barrier function when applying a mucus layer on top of the cells.

The bacteria-conditioned digest of mussels, nori and rice decreased the resorufin in the colon model (54–114%; 1.2–1.6 times), compared to the nonconditioned digests (84–131%) (Figure 4C). Reduction in resorufin levels may indicate an impaired mitochondrial activity and therefore toxicity of the colonic digests toward the colonic epithelium in vitro. Longer

exposure (24 h) and longer contact of food matrices with gut microorganisms could cause the differences observed between small intestine and colon models. Thus, not only concentration but also time of exposure should be considered when using in vitro tests to assess the toxic effects of arsenic to small intestine and colon epithelium.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b04457.

Supplementary methods: Method S1. Reagents for arsenic quantification. Method S2. Extraction of arsenic species. Method S3. Determination of total arsenic. Method S4. Determination of arsenic species. Method S5. Preparation of salivary and fecal microbiomes. Method S6. In vitro gastrointestinal digestion and fermentation. Method S7. Batch assay. Supplementary figures: Figure S1. Structures of arsenic species found in food (mussel, nori and rice) samples. Figure S2 Schematic representation of the in vitro gastrointestinal digestion and cell culture assays. Figure S3. HPLC-ICP-MS chromatography of the extracts from three food matrix. Figure S4. HPLC-ICP-MS/ESI-MS-MS of cation retain arsenic species in 100 ppb mixed standards and initial mussel sample. Figure S5. HPLC-ICP-MS/ESI-MS-MS of anion retain arsenic species in 100 ppb mixed standards and Hijiki reference material. Figure S6. HPLC-ICP-MS/ESI-MS-MS of anion retain arsenic species in 100 ppb mixed standards and initial nori sample. Figure S7. Correlation plot of Papp of LY and cellular uptake of As. Figure S8. Effect of the small intestine digests on the mitochondrial activity of the intestinal cells. Figure S9. TEER and Papp of LY are negatively correlated only in the small intestine model. Supplementary tables: Table S1. Total As (dry weight) in reference material. Table S2. Instrument settings and parameters. Table S3. MRM transitions and optimum conditions used for MS/MS. Table S4. Characteristics of the donors of salivary and fecal samples used in this study. Table S5. Arsenic species ( $\mu\text{g/L}$ ) in colonic digested food samples with salivary bacteria treatment. Table S6. Percentage of total As uptake (cellular retention + basolateral transport) in cells of the small intestine and colon models exposed to non-conditioned and bacteria-conditioned food digests. (PDF)

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## Notes

The authors declare no competing financial interest.

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