Electronic Supplementary Material (ESI) for Food & Function.

SUPPLEMENTARY INFORMATION

Phenolic-rich beverages reduce bacterial TMA formation in an *ex vivo-in vitro* colonic fermentation model.

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Supplementary materials and methods:

Growth media preparation: Growth media was prepared according to our optimized methodology [9], which was originally adapted from Alqurashi *et al*. [22]. The composition of 1 L of growth medium was 2 g peptone water, 2 g yeast extract, 0.1 g NaCl, 40 mg Na_2HPO_4, 40 mg KH_2PO_4, 10 mg $MgSO_4 \times 7H_2O$, 10 mg $CaCl_2 \times 6H2O$, 2 g $NaHCO_3$, 50 mg hemin, 0.5 g bile salts, 2 mL Tween 80, 10 μL vitamin K_1, 1 mg resazurin, and 0.5 g L-cysteine. Two different 500 mL solutions were prepared at 2X final concentration. Solution A included all components except for resazurin and L-cysteine, which were included in Solution B. The pH of both solutions was adjusted to 6.8, and solutions were filter-sterilized separately through a 0.22 μm sterile filtering system (Corning, Corning, NY, USA). Solution B was then boiled for 10 min. Thereafter, both solutions were sparged overnight (minimum of 8 h) with N_2 (g) under stirring at 250 rpm and were then combined 1:1 in the anaerobic chamber (O_2 ≤10 ppm) to the final 1X concentration growth media solution. Growth media was used to grow bacteria in fecal slurries under different experimental conditions. PBS 1X solution was filter-sterilized (22 μm sterile filtering system, Corning, Corning, NY, USA) and sparged overnight with N_2 (g) to maintain sterile and anaerobic working conditions.

Total polyphenol content: The total polyphenol content of undigested, digested and digested fractions samples was calculated as per the methodology reported in Racine *et al.* [21]. Briefly, in a 96-well plate, 130 μL distilled water were mixed with 20 μL sample followed by 20 μL of the Folin-Ciocalteu reagent. Plates were then vigorously shaken and let react in the dark for 5 min. Then, 30 μL of Na_2CO_3 (25 % w/v) were added, plates were shaken and let incubate 1 h in the dark. Absorbances were then read at 765 nm. For

digested samples and digested fractions, the absorbance of saline digesta and its fractions was removed from the absorbance reported for digested beverages and digested beverage fractions to remove the background signal provided by digesta components unrelated to phenolic compounds. Total polyphenol content of saline digesta and its fractions can be found in Table S1. Gallic acid in water (0 – 300 mg/mL) was used to construct the calibration curve for sample quantification. Results were expressed as gallic acid equivalents (GAE) per liter ± SEM (*n*=3).

Extraction and concentration of phenolic compounds: Prior to extraction, undigested beverages (0.5 mL) and their digesta fractions (1 mL; whole, pellet and supernatant) were freeze-dried and extracted through the methodology described by Dorenkott *et al*. [23] with modifications. Briefly, freeze-dried samples were mixed with 1 mL of methanol:water:formic acid (80:18:2; v:v:v) and sonicated in a water bath for 20 min. Supernatants (3700 x g, 4 min) were collected, and pellets extracted two times with 1 mL methanol:formic acid (98:2; v:v). All supernatants (3700 x g, 4 min) were pooled and evaporated under vacuum at 40 °C in a speed-vac. The resulting dried extract was reconstituted in 1 mL of 0.1% formic acid in water, vortexe, sonicated for 2 min and filtered through a 0.45 μm PTFE syringe filter and frozen at – 80 °C until use. Extracts were purified and concentrated following the procedure described by Mohamedshah *et al.* [24]. Briefly, Strata-X 33 μm polymeric reverse phase 96-well plates (Phenomenex, Torrance, CA, USA) were conditioned with 1 mL of 1% formic acid in methanol and 1 mL of 1% formic acid in water. Then, 1 mL of 1:1 sample diluted in formic acid 1% in water were added, followed by 20 μL of ethyl gallate (0.1 mM). Samples were then washed with 1 mL of 1 % formic acid in water and 1 mL of formic acid 0.1% in water. Phenolic compounds were eluted with 300 μL. This procedure was conducted in a Positive Pressure-96 Processor (Waters). Eluted samples were evaporated at 40 °C in a speed-vac, and reconstituted with 100 μL 0.1 % formic acid in water. Samples were frozen at – 80 °C until injection into the LC-MS system.

Extraction and quantification of TMA-related compounds: Externally added choline-d_9 and produced TMA-d_9 in fermentation samples were quantified according to our previously reported methodology [12]. To extract choline-d_9, 25 μL of fermentation sample were mixed with 10 μL of ZnSO4 solution (5 % w/v in water), 100 μL acetonitrile and 20 μL choline-1-^{13}C-1,1,2,2-d_4 (IS; 10 μM) in 96-well plates. After sonication for 5 min in a water bath, samples were filtered through AcroprepAdv 0.2 μm WWPTFE 96-well filtering plates (Pall Corporation, Port Washington, NY, USA) by centrifugation (10 min, 3,400 x g), collected in a fresh 96-well collection plate and frozen at –80 °C until UHPLC-MS/MS analysis. TMA-d_9 requires a derivatization process to the quaternary amine compound ethyl betaine-d_9 to facilitate LC-MS/MS ionization. Briefly, 25 μL of fermentation sample were mixed with 20 μL of TMA-$^{13}C_3$-^{15}N internal standard solution (10 μM, for

derivatization to ethyl betaine-d_9 or ethtylbetaine-$^{13}C_3$-^{15}N, respectively), 8 μL concentrated ammonia and 120 μL ethyl bromoacetate (20 mg/mL), and let sit for 30 min. Then, 120 μL 50 % acetonitrile/0.025 % formic acid in distilled water were added. TMA-d_9 samples were filtered and stored as described above.

After extraction, TMA and choline compounds were analyzed by UPLC-ES-MS/MS. TMA-d_9 and TMA-$^{13}C_3$-^{15}N were analyzed separately from choline-d_9 and choline-1-^{13}C-1,1,2,2-d_4, but with the same UHPLC-ESI-MS/MS method. Briefly, separation was achieved on a Waters Acquity UPLC system (Milford, MA, USA) with an ACQUITY BHE HILIC column (1.7 μm, 2.1x100 mm) coupled to an ACQUITY BHE HILIC pre-column (1.7 μm, 2.1x5 mm) (Waters). Mobile phases consisted of 5 mM ammonium formate in water (pH 3.5) (A) and acetonitrile (B). The gradient was isocratic at 80 % B for 3 min, with a flow rate of 0.65 mL/min. Colum temperature was 30 °C, and autosampler at 10 °C. Quantification was achieved with a Waters Acquity triple quadrupole mass spectrometer. Source and capillary temperatures were 150 and 400 °C, respectively. Capillary voltage was 0.60 kV, and desolvation and cone gas flows (both N_2) were set at 800 and 20 L/h, respectively. Electrospray ionization (ESI) was operated in positive mode, and data were acquired by multiple reaction monitoring (MRM) in MS/MS mode. MRM fragmentation conditions of analytes and IS compounds can be found in elsewhere [12].

For sample quantification, 45% growth media in PBS 1X was spiked with 0 – 300 μM of choline-d_9 and TMA-d_9 standards to obtain external calibration curves in a relevant background matrix. Samples were quantified by interpolating the analyte/IS peak abundance ratio in the standard curves. Data acquisition was carried out using Masslynx software (V4.1 version, Waters). Method sensitivity was determined by limit of detection (LOD) and limit of quantification (LOQ), respectively defined as the concentration of analyte corresponding to 3 and 10 times the signal/noise ratio. Method detection (MDL) and quantification (MQL) limits were calculated for the analysis of 25 μL of non-diluted fecal fermentation media samples.

Cell viability: To study potential cytotoxic effects of treatments, cell count and cell respiration rate were monitored as measures of viability per our previously published studies with minor modifications [9,12]. The number of cells present in the fermentation media was evaluated by reading the optical density of 50 μL fermentation media at 600 nm in a SpectraMax iD3 plate reader (Molecular Devices, San Jose, CA, USA) at every sampling time (0 – 24 h). Cell respiration rate assay was used to estimate cell viability at 12 and 24 h. Briefly, 20 μL of fermentation mixture was mixed with 80 μL of pre-heated (37 °C) PBS 1X with glucose 0.2% (m/v) and 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide at a concentration of 25 mg/mL in PBS 1X [25]. Samples were allowed to react for 30 min under anaerobic conditions (37 °C and O_2 < 10 ppm), and the

resulting formazan crystals were resuspended to a final volume of 0.6 mL with DMSO by shaking for 30 min. An aliquot of 100 μL was read at 560 nm in a SpectraMax iD3 plate reader (Molecular Devices, San Jose, CA, USA). All reagents were prepared with overnight-sparged PBS 1X and filter-sterilized 22 μm sterile filtering system, Corning) before their use. Results obtained from digested and undigested samples are expressed as percentages of change to their respective digested or undigested control conditions ± SEM (*n*=6).

Supplementary figures:

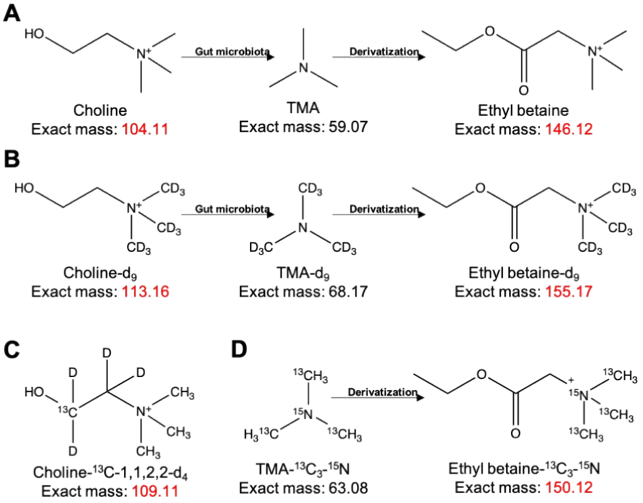

Figure S1. Schematic and structures of the chemical species in this study. The mass difference between each isotope makes the quantification of exogenous species possible without the interference of endogenous (background) species. Background levels of choline are transformed by fecal slurry bacteria into TMA, which is derivatized into ethyl betaine for quantification (**A**). Exogenous choline-d_9 is metabolized by fecal slurry bacteria into TMA-d_9, which is derivatized into ethyl betaine-d_9 (**B**). Choline-^{13}C-1,1,2,2-d_4 is used as an internal standard for LC-MS (**C**). TMA-$^{13}C_3$-^{15}N, used as internal standard for LC-MS, is derivatized into ethyl betaine-$^{13}C_3$-^{15}N (**D**).

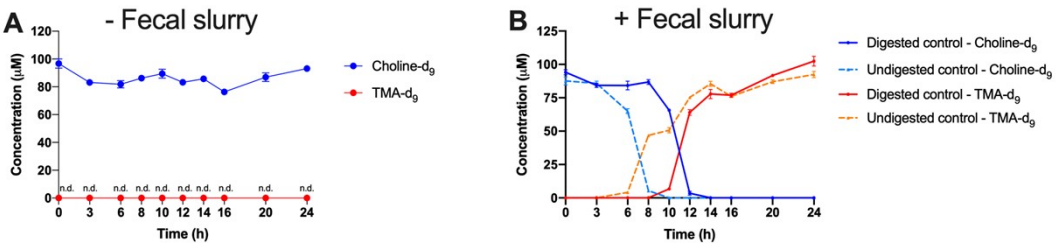

Figure S2. Stability of choline-d_9 and spontaneous formation of TMA-d_9 in fecal slurry-free fermentation media with choline-d_9 100 μM and control saline digesta (30%) (**A**). Changes in choline-d_9 and TMA-d_9 in

fermentations with choline-d_9 100 µM, fecal slurry 1:10 in PBS (20 %), and either with digested saline (digested control; continuous lines) or without digested saline (replaced by PBS; undigested control; dotted lines) (**B**). Results are expressed as µM ± SEM (*n*=6). Abbreviations: n.d., not detected.

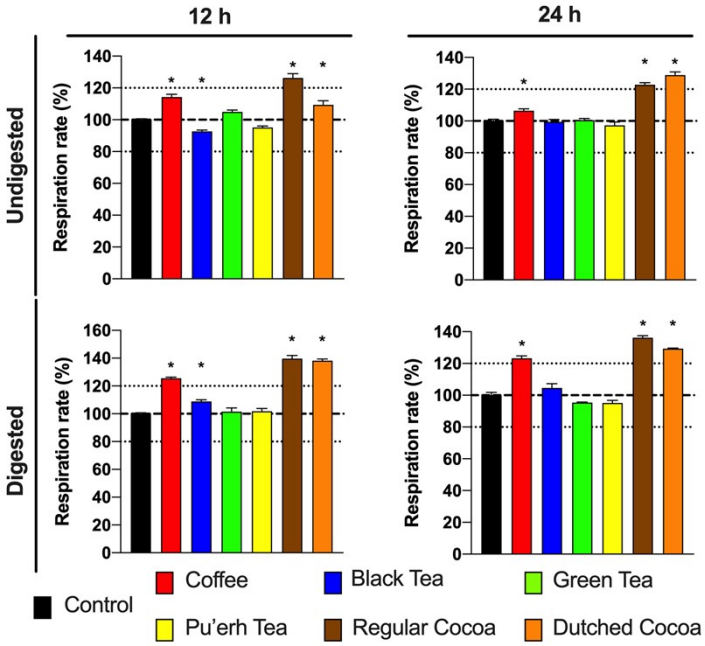

Figure S3. Relative cell respiration rates at 12 and 24 h of digested and undigested treatments. Results are expressed percentage of change against respective digested/undigested control conditions ± SEM (*n*=6). * Indicates statistical differences (p<0.05) in relative cell respiration rate against respective digested/undigested control conditions by One-Way ANOVA (Dunnett's *post hoc* test)**.**

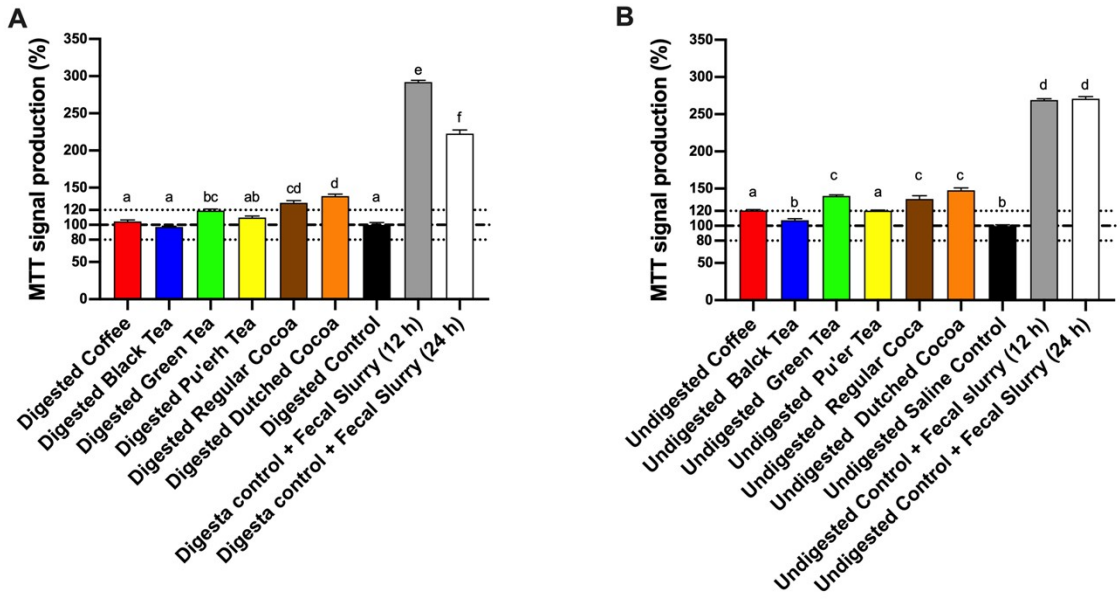

Figure S4: Abiotic MTT signal production of digested (**A**) and undigested treatments (**B**). The cell respiration of control conditions inoculated with fecal slurries at 12 and 24 h is also included in the panels.

All results are relativized to abiotic (either saline digesta control or undigested saline control) control conditions, and expressed as mean of *n* ≥4 ± SEM.

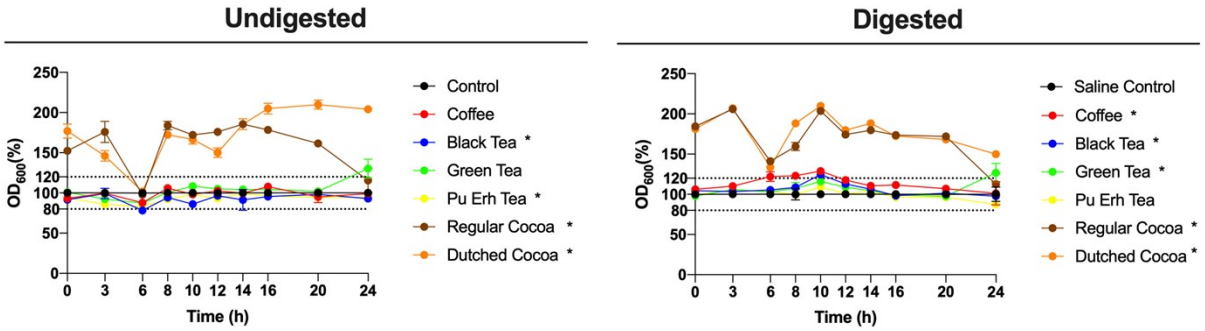

Figure 5S. Relative cell growth evaluated as optical density (OD) at 600 nm in digested and undigested fermentation treatments. Results are expressed percentage of change against respective digested/undigested control conditions ± SEM (*n*=6). * Indicates statistical differences ($p<0.05$; main treatment effect) in relative cell growth against respective digested/undigested control conditions by Two-Way ANOVA.

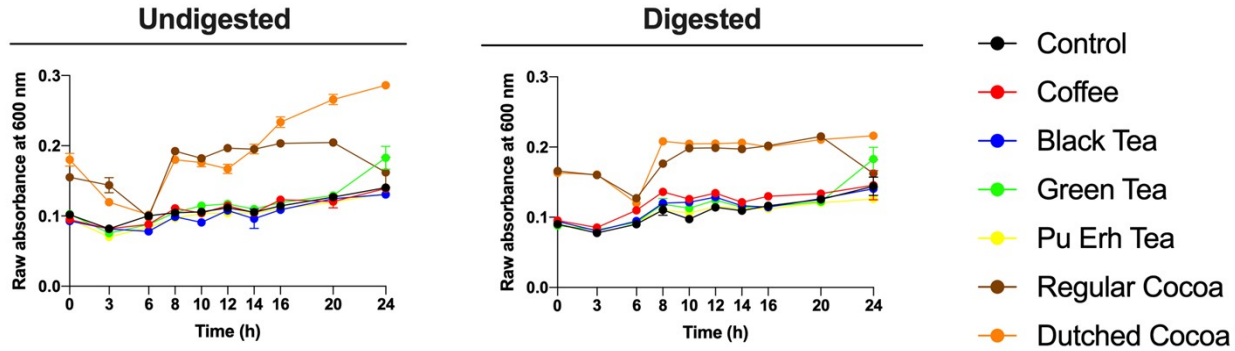

Figure S6: Absolute absorbance of optical density (OD) at 600 nm in digested and undigested fermentation treatments. Results are expressed as mean ± SEM (*n*=6).

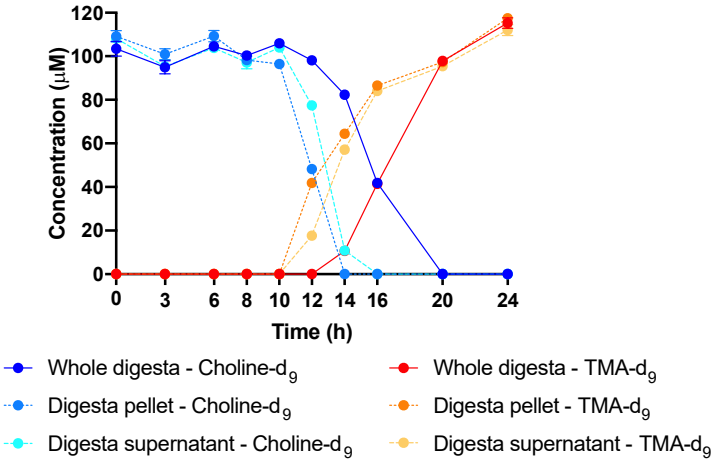

Figure S7: Changes in choline-d_9 and TMA-d_9 in fermentations with choline-d_9 100 µM, fecal slurry 1:10 in PBS (20 %), with the different fractions (whole digesta, digesta pellet and digesta supernatant) of saline digesta control conditions. Results are expressed as µM ± SEM (*n*=6).

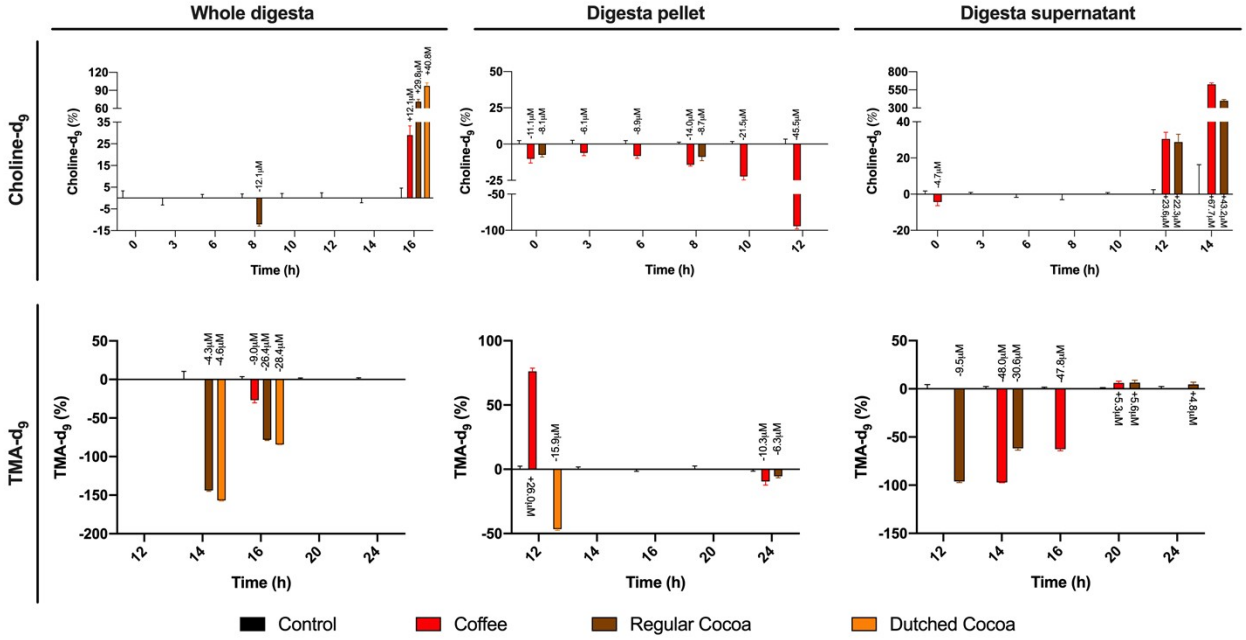

Figure S8: Percentage of change (%) and absolute change (µM) of the levels of choline-d_9 and TMA-d_9 between beverage digesta fractions (whole digesta, digesta pellet and digesta supernatant) and respective saline digesta control fractions (*n*=6). Data is only showed if statistically significant (p<0.05) levels of choline-d_9 and TMA-d_9 were detected in the kinetic curves by Two-Way ANOVA (Sidak's *post hoc* test) between digested beverage fractions and time-matched digesta saline control fractions, and if levels were quantified in both samples.

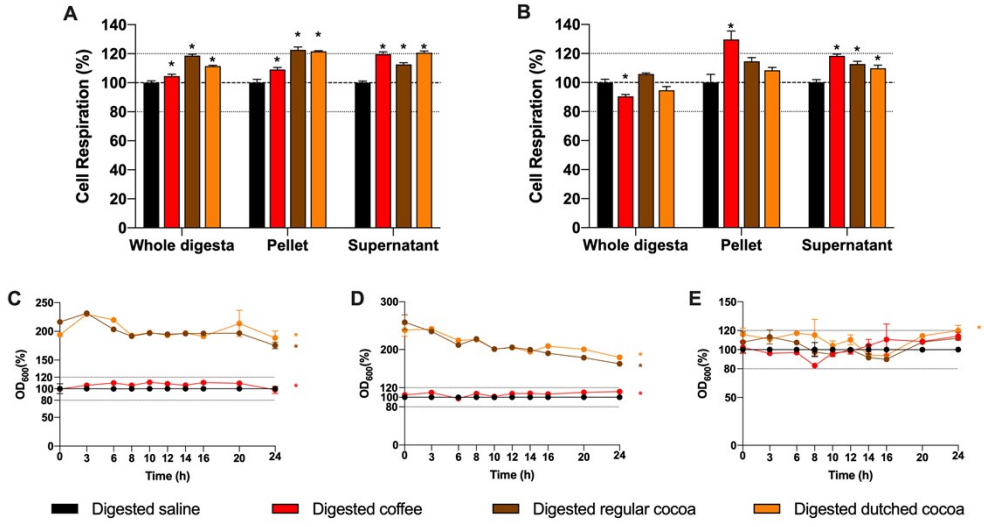

Figure S9. Cell viability assays in digesta fractions. Relative cell respiration rate at 12 (**A**) and 24 (**B**) h after the inoculation of fecal slurry in fermentations including whole digesta, pellet or supernatant fractions. Relative cell growth evaluated as optical density (OD) at 600 nm in whole digesta (**C**), pellet fraction (**D**), and supernatant fraction (**E**). Results are expressed percentage of change against respective control fractions ± SEM (*n*=6). * Indicates statistical differences (p<0.05) against respective fraction control

conditions by either One-Way ANOVA (Dunnett's *post hoc* test; **A – B**) or Two-Way ANOVA (main effect of treatment; **C – E**).

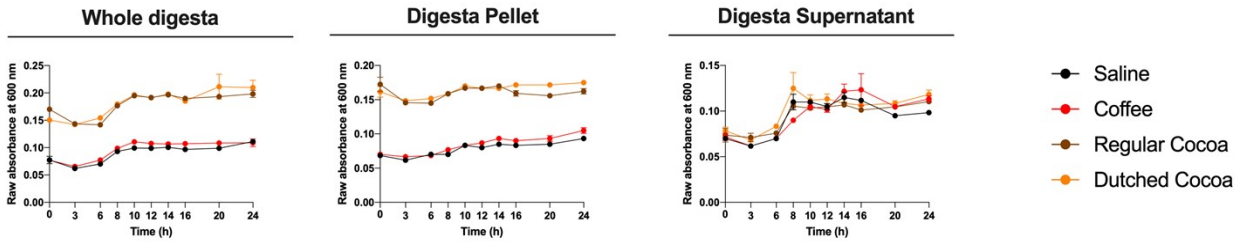

Figure S10: Absolute absorbance of optical density (OD) at 600 nm in whole digesta, pellet fraction, and supernatant fraction samples. Results are expressed as mean ± SEM (*n*=6).

Supplementary Tables:

Table S1: Total polyphenol content (TPC) of undigested and digested beverages, and calculated TPC supplied by undigested and digested beverages at initial (t=0h) fermentation conditions (*n*=3; each).

Beverage	TPC of beverages		Calculated TPC at initial fermentation conditions	
	Undigested (mg GAE/L)	Digested (mg GAE/L)[a]	Undigested (mg GAE/L)	Digested (mg GAE/L)[a]
Coffee	2095.7 ± 89.2	180.5 ± 2.5	74.5 ± 3.2	54.2 ± 0.8
Black tea	367.3 ± 12.4	43.0 ± 7.4	13.1 ± 0.4	12.9 ± 2.2
Green tea	430.9 ± 11.0	44.1 ± 5.0	15.3 ± 0.4	13.2 ± 1.5
Pu'erh tea	196.5 ± 5.2	29.9 ± 8.9	7.0 ± 0.2	9.0 ± 2.7
Regular cocoa	1136.9 ± 60.1	160.7 ± 0.3	40.4 ± 2.1	48.2 ± 0.1
Dutched cocoa	1135.6 ± 36.5	133.3 ± 4.8	40.4 ± 1.3	40.0 ± 1.4

Results are expressed as mean ± SEM (*n*=3). Abbreviations: GAE, gallic acid equivalents. [a] Total polyphenol content after saline digesta background subtraction.

Table S2: Total polyphenol content (TPC) in saline digesta fractions

Saline digesta	TPC (mg GAE/L)
Whole digesta	27.9 ± 0.4
Digesta pellet	5.5 ± 0.5
Digesta supernatant	23.3 ± 0.8

Results are expressed as mg gallic acid equivalents (GAE)/L ± SEM (*n*=4).

Table S3: Multiple reaction monitoring conditions for the quantification of relevant phenolic compounds in coffee and cocoa beverages, and their digesta.

Compound	MW	RT (min)	Quantification			Confirmation		
			MS>MS	CV (V)	CE (eV)	MS>MS	CV (V)	CE (eV)
***p*-Coumaric acid**	164.2	2.6	163.0>93.1	28	28	163.0>119.1	28	17
Gallic acid	170.1	0.7	169.0>125.0	34	16	169.0>79.1	34	16
Caffeic acid	180.2	1.8	179.0>135.1	32	22	179.0>106.9	32	22
Ethylgallate (IS)	198.2	2.8	197.0>123.9	30	30	197.0>125.0	30	30
Catechin	290.3	2.3	289.1>109.0	33	24	289.1>123.0	33	26
Epicatechin	290.3	1.5	289.1>109.0	33	24	289.1>123.0	33	26
Chlogenic acid	354.3	1.6	353.0>190.9	26	18	353.0>84.9	26	40
Chlogenic acid D1	354.3	1.2	353.0>190.9	26	18	353.0>84.9	26	40
Feruloylquinic acid D1	368.3	3.0	367.2>190.7	34	14	367.2>173.1	34	14
Dicaffeoylquinic acid D1	516.4	3.8	515.2>353.1	44	18	515.2>173.1	46	28
Dicaffeoylquinic acid D2	516.4	4.0	515.2>353.1	44	18	515.2>173.1	46	28
Procyanidin B2	578.5	2.2	557.5>289.0	25	25	557.5>407.0	25	30

Abbreviation: MW, molecular weight; RT; retention time; CV, cone voltyage; CE, colision energy; and IS, internal standard.

Table S4: Parameters for the quantification of phenolic compounds in selected beverages and their digesta fractions by UPLC-MS/MS.

Compound	Calibration curve	R^2	Working linear range (µM)	LOD (nM)	LOQ (nM)	MDL (nM) [a]	MQL (nM) [a]
***p*-Coumaric acid**	y=0.0692x + 0.0071	>0.99	0.6 – 12.2	7.4	24.7	37.1	123.5
Gallic acid	y=0.0071x – 0.00847	>0.99	0.6 – 23.5	43.1	143.6	215.5	718.2
Caffeic acid	y=0.0311x – 0.9299	>0.99	0.6 – 16.6	79.8	265.9	398.8	1329.5
Catechin	y=0.0045x – 0.0927	>0.99	0.2 – 17.2	6.2	20.5	30.8	102.6
Epicatechin	y=0.0032x + 0.0302	>0.99	0.2 – 13.8	41.2	137.5	206.2	687.4
Chlorogenic acid	y=0.0131x – 0.325	>0.99	0.3 – 8.5	25.8	86.1	129.2	430.7
Procyanidin B2	y=0.0002x – 0.0065	>0.99	0.2 – 8.6	2.3	7.5	11.3	37.6

Abbreviations: R^2, determination coefficient; LOQ, limit of detection; LOQ, limit of quantification; MDL, method detection limit; and MQL, method quantification limit. [a] Method detection and quantification limits indicating the lowest levels samples need to contain for their detection and quantification.

Table S5: Parameters for the quantification of choline-d_9 and TMA-d_9 in spiked fecal fermentation samples by UPLC-MS/MS.

Compound	Calibration curve	R^2	Working linear range (µM)	LOD (nM)	LOQ (nM)	MDL (nM) [a]	MQL (nM) [a]
Choline-d_9	y=0.1371x + 0.6121	0.9932	1.0 – 200.0	106.9	356.3	213.8	712.6
Ethyl betaine-d_9 [b]	y=0.0884x + 1.4222	0.9989	1.0 – 200.0	71.9	239.5	143.7	479.1

Abbreviations: R^2, determination coefficient; LOQ, limit of detection; LOQ, limit of quantification; MDL, method detection limit; and MQL, method quantification limit. [a] Values for 25 L undiluted fermentation media. [b] TMA-d_9 derivative.

Table S6: Two-way ANOVA *p* values obtained by comparing choline-d_9 and TMA-d_9 kinetic curves of treatments (digested and undigested beverages) against their respective control conditions.

Beverage	Two-way ANOVA *p* values					
	Choline-d_9			TMA-d_9		
	Treatment	Time	Interaction	Treatment	Time	Interaction
Undigested coffee	ns	****	**	****	****	****
Undigested green tea	ns	****	*	*	****	****
Undigested black tea	ns	****	ns	ns	****	****
Undigested pu'erh tea	ns	****	ns	*	****	****
Undigested regular cocoa	ns	****	***	****	****	****
Undigested dutched cocoa	ns	****	*	ns	****	****
Digested coffee	****	****	****	****	****	****
Digested green tea	**ns**	****	**ns**	**ns**	****	***
Digested black tea	**	****	**	**ns**	****	****
Digested pu'erh tea	***	****	****	****	****	****
Digested regular cocoa	****	****	****	****	****	****
Digested dutched cocoa	****	****	****	****	****	****

Significance: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$; ns, $p>0.05$ (not significant).

Table S7: Two-way ANOVA *p* values obtained by comparing choline-d_9 and TMA-d_9 kinetic curves of digesta treatment fractions (whole, pellet and supernatant) against their respective fraction control conditions.

Digested beverage fraction	Two-way ANOVA *p* values					
	Choline-d_9			TMA-d_9		
	Treatment	Time	Interaction	Treatment	Time	Interaction
Whole coffee	***	****	*	*	****	***
Pellet coffee	****	****	****	****	****	****
Supernatant coffee	****	****	****	****	****	****
Whole regular cocoa	ns	****	****	****	****	****
Pellet regular cocoa	***	****	**	**	****	ns
Supernatant regular cocoa	****	****	****	****	****	****
Whole dutched cocoa	****	****	****	****	****	****
Pellet dutched cocoa	ns	****	****	***	****	****
Supernatant dutched cocoa	ns	****	****	ns	****	ns

Significance: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$; ns, $p>0.05$ (not significant).

Table S8: Volumes (µL) of reagents and samples required to test the contribution background abiotic reduction of MTT.

Condition	Sample	Growth media	Sample	Choline-d_9 (mM)	Fecal slurry (1:10)	PBS 1X
Abiotic samples	Digested beverages	405	270	45	0	180
	Undigested beverages	405	32	45	0	418
+ Fecal slurry samples	Digested control	405	270	45	180	0
	Undigested control	405	32	45	180	238

Supplementary discussion:

Phenolic compounds in undigested and digested fermentation samples have a small contribution on abiotic MTT reduction: We previously identified that some phenolic compounds, including chlorogenic acid, are able to produce an abiotic reduction of the MTT reagent, which can generate bias when evaluating cell respiration. Ultimately, this can affect at the perception of whether or not a treatment is cytotoxic or not. In order to evaluate if the phenolic compounds, as well as other samples components, produce an abiotic reduction of MTT reagent, fermentation conditions were reproduced without fecal slurries. Table S8 provides a clear description of the volumes of different fermentation components used to evaluate the abiotic contribution of fermentation background on MTT reduction. MTT assay was preproduced as previously described. Figure S8 clearly show that, although some of the digested treatments statistically increase the reduction of MTT through an abiotic mechanism, these increases in MTT signal are minor when compared to the signal reported by control conditions (digested beverages replaced for saline digesta) when fecal slurries are added to the fermentation mix, both at 12 and 24 h. In this sense, and to be more precise, the raw absorbance means for the fecal slurry-free condition that reports the highest abiotic reduction of MTT (abiotic digested dutched cocoa) is of 0.090, 38 % higher than digested control abiotic production of MTT signal of 0.077. This indicates that, in this particular case, the abiotic contribution to MTT signal (understood as those reactions that lead to MTT reduction due to the presence of different chemicals that do not involve the bacteria within fecal slurries) is of 0.023. Of note, this is a small contribution to MTT's signal for fermentations of digested dutched cocoa with fecal slurries at 12 h (raw absorbance=0.263) and 24 h (raw absorbance=0.187). Thus, we can confidently say that the abiotic MTT reduction contribution of our fermentation mix is minimal (maximum of 9 – 13%). Further, we would like to highlight that the phenolic content found at 12 and 24 h is going to be lower than the one found in this abiotic, chemical experiment due to the metabolizing action of the bacteria within the fecal slurries. Overall, this suggests that the abiotic contribution of phenolic compounds in our real fermentation samples is going to be even lower than 9 – 13 %. A similar rationale can be followed for undigested samples. In this case, for abiotic undigested saline control conditions, the raw absorbance is of 0.059. The abiotic treatment with the higher MTT signal production (+47 %) is also dutched cocoa, with a raw absorbance of 0.087 (+0.03). If we compare this abiotic + 0.03 in raw absorbance to the raw absorbance s of this treatment at 12 h (raw absorbance=0.175) and 24 h (raw absorbance=0.207) when the fecal slurry is present, we can see that the abiotic contribution in MTT's signal of this treatment is, at maximum, of 17 %. Overall, our data suggests that the abiotic contribution of MTT reduction in our fermentation samples is minimal. Thus, MTT results can be interpreted without an abiotic bias component.

Limitations in OD at 600 nm as a measurement of cell viability: The analysis of OD at 600 nm as a measurement of cell viability has its limitations. First, OD measurements are turbidity measurements, and Beer-Lambert law can only be applied only for microbial cultures of low densities. Of note, the fecal slurries from open biome are diluted 10-fold with PBS, and this dilution ends up being only 20 % of the total fermentation media. Thus, the percentage of our human fecal slurry in the final fermentation mix is of 2 %, which is a low percentage. Figure S5 and S10 show the raw absolute absorbance of samples at OD 600 nm, and, in our specific case, the absolute absorbance of all or samples rarely goes above 0.2. Thus, this seems to be a minor incompetent for our samples. However, some samples (especially cocoa ones) provide turbidity due to insoluble matter in the sample. This is discussed in the main manuscript, but the optical density for these samples is not a good estimate on cell viability by the means of DO at 600 nm. However, complementary measures (cell respiration) are applied to ensure lack of toxicity of our treatments. Another general limitation of OD at 600 nm is that bacteria in our fecal slurry are not homogeneous, and different shapes and sizes disperse the light differently. Further, dead cells that are not lysed can also affect the readings. This is another reason why cell viability is not only tracked by OD at 600 nm, and why cell respiration rates (by MTT) is also evaluated. Overall, the

combination of both OD at 60 nm and cell respiration by MTT provide sufficient evidence to support a lack of toxicity of our treatments.