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ARTICLE

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

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The production of pro-atherogenic trimethylamine *N*-oxide (TMAO) is dependent on the gut microbiota metabolism of quaternary amines (*i.e.,* choline) into trimethylamine (TMA). Nutritional strategies that target microbial conversion of choline into TMA could reduce cardiovascular disease and atherosclerosis burden by reducing subsequent formation of TMAO. This study aimed to evaluate 1) whether beverages rich in known inhibitors of TMA production (chlorogenic acid, catechin and epicatechin) can reduce TMA formation and 2) the effect of upper gastrointestinal digestion on efficacy. To do this, either raw or digested coffee, tea and cocoa beverages were evaluated for their TMA-d_9 production inhibition in our *ex vivo-in vitro* fermentation model with human fecal slurries and choline-d_9 substrate. Results showed that digestion was required to unlock the TMA-d_9 production inhibition potential of coffee and cocoa beverages, and that teas did not possess a strong inhibition potential either digested or undigested. By fractionating digested bioactive beverages, we determined that those fractions rich in chlorogenic acid were the most bioactive. Overall, this study suggests that regular cocoa and coffee consumption could be a nutritional strategy able to reduce TMAO levels. *In vivo* studies should be carried out to confirm the potential of these beverages as strategies to inhibit TMA production.

Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide, accounting for 17.9 million deaths a year [1]. Thus, efforts to minimize its incidence could have a huge health and economic impact on society. Different factors can contribute to cardiovascular risk (CVR), including atherosclerosis and thrombosis risk. Atherosclerosis is a disease of the blood vessels where a fibrofatty plaque forms, hindering endothelial function [2]. Thrombosis (formation of blood clots in the vessels) usually occurs after the erosion/rupture of atherosclerotic plaque, potentially leading to ischemia/stroke [3]. Different factors can modify the risk of atherosclerosis development and thrombosis [2,3]. Recently, trimethylamine *N*-oxide (TMAO) has been identified as a risk factor for CVD, atherosclerosis development and thrombosis [4–6].

TMAO is formed through a meta-organism pathway that involves the gut microbiota and the host's detoxification enzymes [4]. Quaternary amines from the diet can be metabolized into trimethylamine (TMA) by bacterial species within the gut that possess TMA lyase activity [7]. Once TMA is formed, it is absorbed and directed to the liver, where flavin-containing monooxygenases (FMOs), mainly FMO3, oxidize it into TMAO [8]. TMAO then enters systemic circulation, where it exerts its pro-atherogenic and pro-thrombotic action [4,6], and finally reaches the kidney, where it is eliminated in urine. The primary substrate for TMA production is choline [4,9,10], a semi-essential nutrient involved in neurotransmitter synthesis and a component of lipoproteins and membrane lipids [11]. Thus, a logical approach to reduce circulating TMAO levels is to inhibit microbial conversion of choline into TMA [4].

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Electronic Supplementary Information (ESI) available: Figure S1: Schematic and structures of the chemical species in this study; Figure S2. Stability of choline-d_9 and spontaneous formation of TMA-d_9 (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); Figure S3: Relative cell respiration rates at 12 and 24 h of digested and undigested treatments; 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; Figure S5: Relative cell growth evaluated as optical density at 600 nm in digested and undigested fermentation treatments; Figure S6: Absolute absorbance of optical density (OD) at 600 nm in digested and undigested fermentation treatments; 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 of saline digesta control conditions; Figure S8: Percentage of change (%) and absolute change (µM) of the levels of choline-d_9 and TMA-d_9 between beverage digesta fractions and control fractions; Figure S9: Cell viability assays in digesta fractions. Relative cell respiration rate at 12 (A) and 24 (B) h. Relative cell growth evaluated as optical density at 600 nm in whole digesta (C), pellet fraction (D), and supernatant fraction (E); Figure S10: Absolute absorbance of optical density (OD) at 600 nm in whole digesta, pellet fraction, and supernatant fraction samples; Table S1: TPC of undigested and digested beverages, and TPC supplied by undigested and digested beverages at initial fermentation conditions; Table S2: Total polyphenol content (TPC) in saline digesta fractions; Table S3: Multiple reaction monitoring conditions for the quantification of phenolic compounds; Table S4: Parameters for the quantification of phenolic compounds; Table S5: Parameters for the quantification of choline-d_9 and TMA-d_9; Table S6: Two-way ANOVA *p* values obtained by comparing choline-d_9 and TMA-d_9 kinetic curves of treatments against control conditions; Table S7: Two-way ANOVA *p* values obtained by comparing choline-d_9 and TMA-d_9 kinetic curves of digesta treatment fractions against control conditions; Table S8: Volumes (µL) of reagents and samples required to test the contribution background abiotic reduction of MTT. See DOI: 10.1039/x0xx00000x

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We recently developed an *ex vivo-in vitro* colonic fermentation model that allows identification of lead compounds with promising TMA-formation inhibition activity [9]. So far, we have identified catechin, epicatechin and chlorogenic acid as phenolic compounds with inhibitory effects at a dose of 2 mM [9,12]. Catechin and epicatechin can be found in foodstuffs such as tea and cocoa [13,14], and one of the main dietary sources of chlorogenic acid is coffee [15]. These compounds have also each reported cardioprotective effects *in vivo* [16,17]. However, their role as TMA-production inhibitors and potential cardioprotective functions through TMAO production inhibition requires further study. These bioactive components are typically consumed within a food matrix (*i.e.,* tea, cocoa, coffee…), and subjected to harsh conditions throughout the gastrointestinal tract, which affect their structure and bioactivity [18,19]. Thus, in this study we aimed to evaluate if physiologically relevant doses of beverages rich in catechin, epicatechin or chlorogenic acid are able to reduce TMA formation in our *ex vivo-in vitro* fermentation model. To account for potential degradation and loss of bioactivity throughout the gastrointestinal track, an *in vitro* gastrointestinal digestion was used, as well as undigested samples supplying the same content of phenolic compounds as the one in digested samples prior to digestion for comparison purposes. Further, bioactive beverages were fractionated into soluble and insoluble components, and each fraction was evaluated for its bioactivity.

Materials and Methods

Chemicals and reagents

Commercially available English breakfast black tea (*i.e.,* theaflavin- and thearubigin-rich), pinhead gunpowder green tea (catechin-rich), pu'erh (theabrownin-rich) (all organic teas from Organic Positively Tea Company, Sunbury, PA), regular and dutched cocoa powder (Hershey's; 100 % cacao, Hershey, PA), ground coffee (medium roast; Three Sisters, Kicking Horse Coffee Company, Invermere, BC, Canada), and coffee filters (#2 cone style) were purchased at the local supermarket (Kannapolis, NC, USA). Glucose, peptone water, yeast extract, KCl, NaCl, Na_2HPO_4, KH_2PO_4, $MgSO_4{\times}7H_2O$, $CaCl_2{\times}6H_2O$, $ZnSO_4{\times}7H_2O$, $NaHCO_3$, HCl, NaOH, ammonium formate, hemin, bile salts, bile extract (porcine), Tween 80, vitamin K1, resazurin, L-cysteine, pepsin (from porcine gastric mucosa; ≥400 U/mg protein), lipase (from porcine pancreas; 100 – 500 U/mg protein), pancreatin (from porcine pancreas), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, ammonia, ethyl bromoacetate, choline, choline-d_9, choline-1-^{13}C-1,1,2,2-d_4, TMA, TMA-d_9,TMA-$^1C_3$-15N, *p*-coumaric acid, gallic acid, caffeic acid, chlorogenic acid, catechin, epicatechin, procyanidin dimer B2, and ethyl gallate were purchased from Sigma-Aldrich/Millipore (St. Louis, MO, USA). Acetonitrile and water (HPLC grade) as well as dimethyl sulfoxide (DMSO; reagent grade) were purchased from VWR International (Suwanee, GA, USA). Fecal samples from different donors were obtained from OpenBiome (Cambridge, MA, USA). These fecal samples were from healthy donors and rigorously screened for 30 infectious diseases by OpenBiome. Health histories, clinical data, pathogen screen results and 16S rDNA sequences are available at OpenBiome. Samples are processed by OpenBiome in sterile 12.5% glycerol and 0.9% saline buffer at 2.5 ml of buffer per gram of stool and filtered through a 330 µm filter to remove large particulate matter, and frozen at −80°C until use.

Beverage preparation and gastrointestinal digestion

Beverages were prepared per standard human consumption in triplicates, and 100 mL of each replicate were pooled together before any other analysis or use. Coffee (20 g) was mixed with 300 g of water at 90 °C, stirred for 4 min at 500 rpm and then filtered through a conventional coffee filter. Filtrates were quickly placed on an ice bath for 5 min before pooling. Black, green and pu'erh teas (2 g each) were mixed with 236 mL (the equivalent of a "cup of tea") of water at 90 °C, and stirred for 4 min at 500 rpm and the liquid was decanted [20]. Samples were then placed on an ice bath for 5 min before decantation and pooling together. Regular and Dutched cocoa (5 g each) were mixed with 236 mL (the equivalent of a "cup of cocoa") of water at 90 °C, and stirred for 4 min at 500 rpm. Samples were then placed on an ice bath for 5 min and pooled together while stirring. Note that cocoas were not filtered or decanted to mimic preparation as typically consumed.

A volume of 5.9 mL of each beverage was digested to a final simulated upper GI volume of 50 mL in screw-cap tubes in triplicates as per Racine *et al*. [21]. This simulates the consumption of 1 cup of beverage by an adult human being with an upper GI volume of 2 L, then and scaled to our 50 mL final upper GI volume. An oral phase was not included in the digestion protocol, as beverages are not chewed, and simulated gastrointestinal digestion started at the gastric phase. Beverages (5.9 mL) were mixed with saline (0.9 % NaCl in water), and pH was adjusted to 2.5 ± 0.1 with HCl (1N). Then, 2 mL of pepsin solution (40 mg pepsin/mL in HCl 0.1M) were added, and samples were brought to a final volume of 40 mL with saline. Tubes were then blanketed with N_2 (g), and digested for 1 h at 85 rpm in a shaking water bath at 37 °C. Every 5 minutes, tubes were inverted to ensure homogeneity in sample digestion. After 1 h, samples were placed on ice immediately and 0.1M NaOH added to reach pH 6.0 ± 0.1. Then, 2 mL pancreatin + lipase solution (10 mg pancreatin/ml + 5 mg lipase/mL in $NaHCO_3$ 100 mM) and 3 mL bile extract solution (40 mg bile extract/mL in $NaHCO_3$ 100 mM) were added. After that, pH was measured and readjusted to pH 6.0 ± 0.1 with either HCl 0.1M or NaOH 0.1M, and samples brought to a final volume of 50 mL with saline. Tubes were then blanketed with N2 (g) and digested over 2 h as described above. Digested beverages (n=3 per beverage) were then pooled together, aliquoted and stored at – 80 °C until use. Three saline digestions (replacement of beverage with saline) were also carried out as controls.

Growth media preparation

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Growth media was prepared according to our optimized methodology [9], which was originally adapted from Alqurashi *et al.* [22]. Further details can be found in Supplementary materials and methods.

Fermentation conditions

All fermentations were carried out inside a 4-glove 855-ACB anaerobic chamber (Plas-Labs, Lansing, MI, USA) filled with a mixed gas composed of 5 % H_2, 5 % CO_2 and 90 % N_2 (Airgas, Durham, NC, USA). H_2 (~2 %) and O_2 (≤10 ppm) levels were monitored with a CAM-12 anaerobic monitor (Coy Lab Products, Grass Lake, MI, USA). Temperature was set at 37 °C, and it was maintained constant (recorded values within 38 – 40 °C) throughout the fermentation procedure. Humidity ranged between 41 and 47 % during fermentation. Due to the presence of endogenous choline and other TMA-lyase substrates which lead to the production of TMA in the absence of exogenous choline [9], choline-d_9 at a final concentration of 100 μM was added as the substrate. The resulting TMA-d_9 was quantified to evaluate the effects of the treatments, which reduces the impact of background TMA production on assay results (Figure S1). Fermentations were carried out in 96-well plates following the optimized procedure described by Iglesias-Carres *et al.* [9] up to 24 h.

Digested vs. undigested samples

A first set of fermentations was carried out to assess the effects of digested and undigested phenolic-rich beverages on choline-d_9 fermentation and TMA-d_9 production per our previous methodology [9]. In 1.1 mL 96-well plates, 405 μL of growth media were mixed with 45 μL of choline-d_9 stock solution (2 mM) in PBS 1X and, either 270 μL of beverage digesta, or 32 μL undigested beverages (*i.e.,* equivalent levels of starting beverage due to the ~8.5-fold dilution during digestion). Both digested and undigested samples were freeze-dried and reconstituted inside the anaerobic chamber with filter-sterilized, overnight-sparged PBS 1X to avoid inclusion of O_2. All wells were brought to 720 μL with filter-sterilized, overnight-sparged PBS 1X, and 180 μL fecal slurry (1:10 in PBS 1X) was added to reach a final well volume of 900 μL. The final reaction composition was: growth media 45 % v/v, original fecal slurry 2 % v/v and choline-d_9 100 μM, as optimized previously [9]. All solutions were pre-heated at 37 °C, and fermentation was considered to start (time 0 h) when fecal slurry was inoculated into the reaction mixture. Aliquots from two different donors were used as fecal slurries, with reference numbers 2449-0005 and 2398-003. An aliquot of 50 μL was sampled from 0 to 24 h at different time points, analyzed for cell density (see section 2.8), then combined with 50 μL acetonitrile and immediately frozen at – 80 °C until used for choline-d_9 and TMA-d_9 analyses. Digested control conditions were defined as growth media (45 %), PBS 1X with choline-d_9 100 μM and saline digesta (270 μL; 30% v/v) in fermentations with 2 % original fecal slurry. Undigested control conditions were defined as growth media (45 %), choline-d_9 100 μM and PBS 1X in fermentations with 2 % original fecal slurry. Choline and choline-d_9 stability in fermentation conditions without digesta have already been reported, with minimal degradation of choline and choline-d_9 or generation of TMA and TMA-d_9 [9,12]. To evaluate stability and chemical degradation of choline-d_9 in fermentations with digesta, a vehicle (VH) condition, consisting of growth media (45 %), choline-d_9 100 μM and saline digesta reconstituted in PBS 1X (30 %), was included. In all cases, growth media volume was 45 % of the total, and the remaining 55 % was achieved by the addition of different components (fecal slurry, choline-d_9, digested and undigested beverages, or PBS 1X) all prepared in PBS 1X. All conditions were fermented at n=6 replicate wells.

Digested beverage fractionation and fermentation

A second set of fermentations was conducted on selected digested beverages with promising TMA-d_9 production inhibition bioactivity in the first experiment (coffee, regular cocoa and Dutched cocoa) in order to differentiate the activities of the soluble (*i.e.,* bioaccessible) vs. insoluble fractions. Saline digesta was included to serve as a control. A 25 mL aliquot of whole digesta was used for aliquoting purposes. A total of 10 mL of unprocessed digested beverages and saline control were aliquoted and used as whole ("crude") digesta. Another 10 mL of the unprocessed digesta were aliquoted and centrifuged for 75 min (3,500 x g, 4 °C) to obtain pellet (insoluble) and supernatant (soluble, bioaccessible) fractions. A total of 9 ml of the supernatant were recollected and stored in a new falcon tube, while the pellet (after removal of remaining supernatant) was kept. Whole digesta, supernatant and pellet fractions were frozen at – 80 °C and later freeze-dried. These samples were then reconstituted inside the anaerobic chamber with filter-sterilized, overnight-sparged PBS 1X to their original volume (10 mL for all), and used as treatments in fermentations carried out as previously described.

Beverage and digesta analyses

Total polyphenol content

The total polyphenol content of undigested, digested, and digested fractions samples was calculated per our reported methodology [21]. Further details are presented in Supplementary materials and methods. The total polyphenol content of digested and undigested samples can be found in Table S1, and the values for saline digesta can be found in Table S2. The estimated TPC provided by the undigested and digested beverages in initial (t=0h) fermentation conditions can be found in Table 1.

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. Extracts were purified and concentrated following the procedure described by Mohamedshah *et al.* [24]. Further details on polyphenol extraction and concentration can be found in Supplementary materials and methods.

Characterization of the phenolic compound profile

The characterization of potentially bioactive phneolic species in selected beverages (coffee, regular cocoa and dutched cocoa) and digesta fractions was performed by LC-MS/MS as described by Mohamedshah *et al.* [24]. Multiple reaction monitring (MRM) conditions for the quantification of phenolic compounds can be found in Table S3. Method quality parameters can be found in Table S4. Concentrations of phenolics in fermentation media are found in Table 2.

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], see Figure S1. Further details can be found in Supplementary materials and methods. Method quality parameters can be found in Table S5.

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]. Further details can be found in Supplementary materials and methods.

Statistics

Prism 8.0 (GraphPad, La Jolla, CA, USA) was used for statistical analyses and graph creation purposes. Two-way ANOVA (main effects: treatment and time) was used to estimate differences in choline-d_9 and TMA-d_9 kinetic curves and cell density values. If a significant main effect or interaction (p<0.05) was reported for choline-d_9 and TMA-d_9 kinetic curves or cell density values, Sidak's *post hoc* test was used to estimate time-matched differences between respective digested or undigested control conditions and digested or undigested treatments. One-way ANOVA was used to estimate differences in choline-d_9 and TMA-d_9 AUCs and cell respiration rate between respective control conditions and treatments (with Dunnetts' *post hoc* test if a significant overall treatment effect was detected). In all cases, statistical significance was stablished a priori as $p < 0.05$.

Results

Control conditions

Our previous work demonstrated the stability of choline-d_9 and essentially zero conversion to TMA-d_9 in fermentation conditions free of fecal sample [12]. Some experimental conditions in this study include the addition of digestive enzymes and other molecules in the fermentation mix (digested samples), which could potentially promote choline-d_9 degradation to TMA-d_9 by non-bacterial mechanisms. Prior to experiments, the stability of choline-d_9 and the spontaneous formation of TMA-d_9 under fermentation including digesta with and without fecal slurry were evaluated over 24 h (Figure S2A). In line with previous results, choline-d_9 concentrations in fermentations with saline digesta (i.e., digestion reagents but no beverage) but not fecal inoculum remained essentially constant throughout the fermentation (24h), while no detectable TMA-d_9 was formed by spontaneous/chemical degradation of choline-d_9. In control conditions (no digested beverages added) inoculated with fecal slurry + saline digesta (Figure S2B), choline-d_9 levels started to decrease at 3 h and at 8 h in controls without and with saline digesta added, respectively. This clearly shows that inclusion of fecal slurry is a requirement for the transformation of choline-d_9 into TMA-d_9, and that choline-d_9 provides ~1:1 stoichiometry of choline-d_9 utilization and TMA-d_9 production. TMA-d_9 started to be quantified in undigested control conditions at 6 h and at 10 h in digested control conditions, indicating that microbial metabolism of choline-d_9 into TMA-d_9 occurs sooner in fermentations with undigested conditions compared to fermentations including digesta (saline digesta in control conditions). This delay is likely due to the microbiome prioritizing metabolism of more-easily fermented materials (digestion reagents) from the matrix.

TMA production inhibition by digested and undigested beverages:

Beverages were selected for their contents of phenolics with observed TMA inhibition activities in our previous study (tea and cocoa with catechin and epicatechin, and coffee with chlorogenic acid). Variations in beverages were selected to examine effects of processing and levels of native bioactives. Dutched cocoa was employed to examine the effects of catechin losses and formation of Dutching (alkalization) products in comparison with catechin-rich natural cocoa (non-Dutched/alkalized). Black and pu'erh teas were employed to examine the effects of catechin losses and successive formation of theaflavins, thearubigins (black tea) and theabrownins (pu'erh tea) during fermentation in comparison with catechin-rich green (unfermented) tea. The kinetic curves of choline-d_9 use and TMA-d_9 formation generated by the digested and undigested beverages and compared to their respective controls are shown in Figure 1. The kinetic curves generated by treatments were compared to their respective controls (digested or undigested control) by Two-way ANOVA (with Sidak's *post hoc* test), and the statistical significance of treatment, time and interaction effects can be found in Table S6.

Compared to digested saline control conditions, all the digested beverages except for digested black and green teas were able to reduce choline-d_9 use (statistically significant treatment effect; p<0.05 by Two-Way ANOVA, Sidak's *post hoc* test) around 10 – 12 h. While green tea produced no effect on choline-d_9 use, black tea increased its use, but only at 10 h. The reduction in choline-d_9 use (*i.e.,* greater remaining choline-d_9 levels) promoted by the digested beverages was usually accompanied by a reduction in TMA-d9 formation compared to saline control conditions (statistically significant treatment effect; p<0.05 by Two-way ANOVA, Sidak's *post hoc* test). Overall, digested coffee, digested regular cocoa and digested

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Dutched cocoa were the digested beverages that produced the highest inhibition of choline-d_9 use and TMA-d_9 production. As a matter of fact, digested cocoa beverage delayed the pattern of choline-d_9 use and TMA-d_9 production seen in digested saline conditions. These effects in the kinetics can clearly be seen in the area under the curve (AUC) of choline-d_9 use and TMA-d_9 in Figure 2A – B, which shows higher choline-d_9 and lower TMA-d_9 AUCs for digested coffee and digested cocoas compared to digested saline control conditions. Of note, by comparing digested and undigested samples it is clear that the digestion matrix itself (*i.e.,* the reagents added for the digestion and/or their digestion products) delays choline-d_9 use and TMA-d_9 production. However, this effect is accounted for in the analysis by the introduction of saline digesta control conditions, shown in Figure 1 by shaded areas, and comparing effects within digested controls and treatments, and within undigested controls and treatments.

Regarding undigested beverages, effects on choline-d_9 use and TMA-d_9 production were not as prominent as for digested samples. No differences in choline-d_9 use between undigested samples and their control conditions were observed for any beverage. Despite that, TMA-d_9 production was modulated by undigested beverages at some time points. Unlike digested beverages, where the effects promoted by digested beverages on TMA-d_9 levels were produced at the time where bacteria were more metabolically active (8 – 14 h), the effects promoted by undigested beverages occurred at later timepoints (10 – 20 h), where TMA-d_9 levels were already reaching a plateau stage. Of note, all undigested treatments (except for Dutched cocoa) reported an increase on TMA-d_9 production compared to controls at 10 h, but also reductions on TMA-d_9 levels in several later timepoints, especially at 14 h. Overall the effects of undigested beverages on choline-d_9 use and TMA-d_9 production were modest and not translated into a significant reduction on the choline-d_9 and TMA-d_9 AUCs (Figure 2A – B) when compared to undigested control conditions (Figure 2C – D).

Cell viability evaluation of digested and undigested treatments

To evaluate whether the effects on choline-d_9 use and TMA-d_9 production were due to a cytotoxic or cytostatic effect of our treatments, cell respiration (Figure S3) and cell growth (Figures S5-S6) were evaluated during fermentation. In all cases, results are reported as % change against respective digested/undigested control conditions. Cell respiration was evaluated mid-fermentation (12 h) and at the end (24 h). At 12 h, digested coffee, digested black tea, digested regular cocoa and digested Dutched cocoa reported a statistical increase in cell respiration when compared to control saline digesta, which was ~120 % for all of them, except for digested black tea. The results reported at 12 h were reproduced at 24 h for all digested beverages, except for black tea, which did not produce an increased cell respiration rate. Undigested coffee, undigested regular cocoa and undigested Dutched cocoa also reported a statistical increase in cell respiration rate, while undigested black tea produced a statistically significant reduction, compared to undigested control conditions at 12 h. However, this reduction did not reach a change > 20 % (*i.e.*, values <80% against control). Overall, these results show that our treatments generally increase cell respiration rate, indicative of a higher growth and activity of bacteria in fermentations with digested and undigested beverages compared to their respective controls. In fact, the inhibition of TMA-d_9 production is even more striking considering that the treatments that lowered TMA-d_9 levels broadly also increased bacterial cell activity. Thus, observed inhibitory effects are unlikely to be due to broad cytotoxicity.

Cell growth kinetic curves of all digested samples were statistically different from saline digesta control conditions, except for digested pu'erh tea. Of note, the kinetic curves of digested black and green tea and digested coffee presented a higher OD at 600 nm generally in the middle of the fermentation (3 – 14 h), indicating that the number of cells were higher than saline digesta control during the time where choline-d_9 was metabolized into TMA-d_9. The kinetic curves of undigested black tea and undigested pu'erh tea were statistically different from their undigested control conditions. In this case, the curves of the undigested beverages were typically below control conditions at times between 6 – 14 h, which includes the timeframe where choline-d_9 is metabolized into TMA-d_9 in fermentations with undigested beverages. Noteworthily, these undigested beverages did not reach reductions in OD at 600 nm indicative of cell density below 80 %. Thus, this can be seen as biological variability rather than a true cytotoxic/cytostatic effect of these undigested treatments. It is important to highlight that the kinetic curves of digested and undigested regular and Dutched cocoa presented a clearly different behavior than their relative controls. Cocoa beverages were not filtered prior to their inoculation to the fermentations, unlike coffee, and thus cocoa samples were cloudy. Various components of the cocoa beverages, such as fiber, could have provided turbidity to the samples, which resulted in higher apparent absorbance at 600nm. Considering that optical density was higher at 0 h, when these treatments had not had time to modulate cell numbers, this is the likely explanation. Thus, cell growth cannot accurately be evaluated in cocoa fermentation samples with the current methodology. Overall, our data suggest that non-specific cytotoxic/cytostatic effects of tested treatments can be ruled out. Figure S4 provides validation that the reducing activity of beverages did not appreciably contribute to MTT values compared to bacterial MTT reduction, and Figure S6 demonstrates that OD turbidity values were sufficiently low to be in the linear range (<0.3) and facilitate their use for approximation of bacterial numbers. These both provide further evidence of the lack of cytotoxic/cytostatic effects of our treatments. These are discussed in Supplementary information.

TMA production inhibition by digesta beverage fractions

Based on the observed TMA-d_9 production inhibitory activities of digested coffee and cocoas, we sought to identify the fraction

responsible for such activities, as soluble components and insoluble compounds behave differently in the GI tract. The kinetic curves for choline-d_9 use and TMA-d_9 production from saline digesta control fractions can be found in Figure S7. The kinetics in whole saline digesta were different from digesta pellet and supernatant fractions. For digesta pellet and supernatant, choline-d_9 started to decrease at 10 h, with a fast, sharp decrease in its levels up to non-detected levels between 12 – 16h. This was accompanied with a fast, sharp increase on TMA-d_9 levels, which started to be quantified by 12 h. For whole digesta control conditions, choline-d_9 also started to decrease after 10 h, but its levels only reached non-detection at 20 h, showing a slower kinetic profile. This was accompanied by a lower speed in the production of TMA-d_9, which started to be quantified at 14 h, and only reached similar values to digesta pellet and supernatant at 20 h. These data suggest that both pellet and supernatant fractions of the digestion background (secretions) contribute to inhibit bacterial conversion of choline-d_9 to TMA-d_9.

The kinetic curves for choline-d_9 use and TMA-d_9 production of the different fractions (whole digesta, digesta pellet and digesta supernatant) of the most promising digested beverages (coffee, regular cocoa and Dutched cocoa) from the first experiment and their respective digested saline control fractions are shown in Figure 3. The kinetic curves generated by treatments were compared to their respective control fractions (by Two-way ANOVA (Sidak's *post hoc* test), and the statistical significance of treatment, time and interaction effects can be found in Table S7. The percentage of change against time-matched control conditions (%) and the overall level of changes (μM) of digesta fractions can be found in Figure S8. In this fermentation batch (fecal slurries from same donors but collected at different times from first fermentation), inhibitory effects of whole digesta on choline-d_9 use and TMA-d_9 were not as marked as its effects reported in the first batch of fermentations (Figure 1). Moreover, fermentations seem to occur at later timepoints. These are likely due to differences in the fecal inoculum used. However, the overall inhibitory effects were conserved between experiments. Whole coffee digesta produced a small significant effect on choline-d_9 levels (+ 30.0 %; + 12.1 μM) and TMA-d_9 levels (−26.4 %; − 9.0 μM) at 16h. The effects of both whole cocoa treatments were more marked. At 16 h, whole regular cocoa digesta inhibited choline-d_9 use by 71.0 %, which represented an increase of choline-d_9 levels of 29.8 μM. This effect was translated in a significant reduction in TMA-d_9 levels at 16 h of − 78.3 % (− 26.4 μM). Whole Dutched cocoa digesta reported a similar effect at 16 h, with a + 97.4 % choline-d_9 levels (40.8 μM) and a − 84.2 % TMA-d_9 levels (−28.4 μM). The effects produced by digesta pellets were different to those reported by whole digesta. Noteworthily, initial apparent choline-d_9 levels for coffee digesta pellet were statistically lower than control condition by ~11 μM. This may be due to choline-d_9 binding to pellet components. Despite that, coffee digesta pellet surprisingly increased TMA-d_9 production at 10 (+19.1 μM) and 12 h (+76.1%; + 26.0 μM). Digested regular cocoa pellet reported lower choline-d_9 levels at initial concentrations of ~8 μM. Another small changes in choline-d_9 level at 8 h was reported, as well as a small change in TMA-d_9 levels at 24 h. No statistically significant effects were reported in the levels of choline-d_9 by digested Dutched cocoa pellet. However, a reduction of TMA-d_9 levels of −46.5% (−15.9 μM) was found at 12 h. The supernatant fraction of digested coffee presented the highest bioactivity of all the treatments tested in the second fermentation batch. Of note, choline-d_9 levels were maintained higher in supernatant coffee digesta fraction between 12 – 16 h, with absolute differences in choline-d_9 levels of +22.3 and +67.7 μM (+ 30 to + 625 %) at 12 and 14 h, respectively. At 16 h, where choline-d_9 levels were not detected in supernatant control conditions, their levels were of 50.2 μM in supernatant coffee digesta. This was translated into a potent inhibition of TMA-d_9 production between 12 and 16 h. For example, while TMA-d_9 levels were not detected in supernatant coffee digesta, the levels of control conditions were of 17.6 μM. Between 14 – 16 h the percentage of inhibition in TMA-d_9 production promoted by supernatant coffee digesta were of 97.0 % (− 48.0 μM) and -62.6 % (−47.8 μM). The effect promoted by supernatant from regular cocoa digesta was much lower, and no effect was reported for supernatant Dutched cocoa digesta. Of note, supernatant from regular cocoa digesta inhibited choline-d_9 use by maintaining + 28.7 % (+23.6 μM) at 12h and + 398.8 % (+ 43.2 μM) at 14 h. This was translated into a n inhibition of TMA-d_9 production of − 96.0 % (−9.5 μM) at 12 h and -61.8 % (-30.6 μM) at 14 h. Overall, the effects reported in the kinetic curves are reproduced in AUCs (Figure 4).

Cell viability evaluation of digesta beverage fractions:

To evaluate that the effects in choline-d_9 use and TMA-d_9 production in fermentations with digesta beverage fractions were not due to a cytotoxic or cytostatic effect of our treatments, cell respiration (Figure S9A-B) and cell growth (Figure S9C-E) were evaluated during the fermentation. Figure 10S shows the absolute absorbance at 600 nm. In all cases, results are reported as percentage of change against respective fraction of saline digesta. Cell respiration was evaluated mid-fermentation (12 h) and at endpoint (24 h). At 12 h, all the digested beverage fractions reported a higher cell respiration rate than their respective saline digesta control fraction, but only regular cocoa digesta pellet reported an increase on cell respiration higher than +20%. This effect was not conserved at 24 h for all treatments. In fact, at 24 h, the supernatant fractions of all beverages reported a higher cell respiration rate compared to the respective control, while only coffee digesta pellet statistically increased cell respiration rate against control conditions and by a > 20%. It is noteworthy that whole coffee digesta at 24 h was the only treatment that reduced cell respiration rate against control conditions, but it did not reach a reduction lower than 20%. Again, it is notable that the inhibition of TMA-d_9 production was associated with unaltered or increased bacterial cell activity, indicating that efficacy was not due to broad reductions in microbial function or viability.

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Cell growth kinetic curves of all beverages and fractions were statistically different from their respective saline digesta control fractions, except for digested coffee and regular cocoa supernatant fractions. Of note, the growth curves of whole digesta and digesta pellet of both cocoa treatments were significantly above their respective controls, potentially due to the contribution of elements in those fractions that contribute to fermentation media turbidity, and thus, OD at 600 nm. As for digested coffee, whole digesta, and digesta pellet, cell growth values were generally above their respective controls and never reaching increases over 20%. This was also generally true for regular cocoa digesta supernatant, which only reached lower cell growth levels than control conditions at 14 – 16h, and never presented changes of ± 20%.

Phenolic compound profiling of beverages and their digesta fractions

The TPC of all digested and undigested beverages is reported in Table S1. The beverage with the higher TPC is coffee. Both regular and Dutched cocoa beverages presented a very similar TPC, and so did green and black teas. Pu'erh tea presented the lowest TPC of all beverages. The TPC of digested samples was lower than the one of undigested samples. However, it should be highlighted that reductions in TPC are mostly due to a dilution effect, as beverages are diluted ~8.5-fold (5.9 mL of beverage in 50 mL of total digesta) during digestion. Of note, the contribution of TPC between undigested and digested beverages in initial fermentation conditions was similar (Table 1). For example, the calculated TPC provided by coffee in undigested and digested initial fermentation conditions was of 74.5 ± 3.2 and 54.2 ± 0.8 mg GAE/L, respectively.

The phenolic profiles of digesta fractions from coffee, regular cocoa and Dutched cocoa were characterized to gain insights into the potential compounds responsible for the TMA-d_9 production inhibition reported by these beverages. The phenolic profile of undigested beverages was also characterized. Table 2 shows the levels of phenolic compounds in fermentation media with different selected undigested, digested and digesta fraction treatments, while Figure 5 shows the distribution of the TPC of whole digesta between digesta fractions (pellet and supernatant). Undigested coffee significantly supplemented fermentation media with relevant concentrations of hydroxycinnamic acids, especially chlorogenic acid. The digestion of coffee reduced the levels of the phenolic compounds provided by whole digested coffee treatment (*i.e.,* reduction of ~60% in the levels of chlorogenic acid between undigested coffee and whole coffee digesta). The separation of whole digesta into bioacessible supernatant faction, and non-bioaccessible pellet fraction revealed that most phenolic compounds were present in the supernatant fraction, with residual levels in the pellet. It should be noted that lower apparent levels reported for some compounds in whole digesta compared to supernatant fractions are likely due to matrix effects (reduced extraction efficiency, etc.). This was in line with the TPC levels distribution of coffee digesta fractions reported in Figure 5A. Undigested regular cocoa supplemented the fermentation media with catechin, epicatechin and procyanidin dimer B2. Although the digestion of regular cocoa produced some reductions in the levels of phenolic compounds provided by whole digesta (*i.e.,* ~840 nM catechin in undigested fermentations to ~736 nM in whole digesta fermentation), digestion of regular cocoa did not produce as extensive of a decrease in phenolics as the one reported by coffee digestion. The separation of whole regular cocoa digesta into digesta pellet and digesta supernatant revelated that the supernatant fraction retained most of the compounds quantified by LC-MS/MS, with residual levels of some compounds in the digesta pellet. This contradicted the TPC results, which indicate that only 38 % of all phenolics are present in the supernatant. This could be attributed to unextracted large procyanidins present in cocoa, which, due to their insolubility, would remain in the pellet. Overall, the same trends reported for regular cocoa and its digesta fractions were reported for Dutched cocoa. Noteworthy, the levels of phenolic compounds in Dutched cocoa treatments are much lower than those of regular cocoa, confirming that the Dutching process has a huge impact in the phenolic content of cocoa.

Discussion

Elevated TMAO circulating levels are associated with CVD, atherosclerosis development and thrombosis risk [4–6]. To date, there does not exist an approved drug to reduce TMAO circulating levels. However, plant bioactive components have been shown to reduce TMA and/or TMAO formation *in vitro* and *in vivo* [4,9,10]. The gut microbiome is the most logical and attractive target to inhibit TMAO formation, achieved through inhibition of choline microbial biotransformation into TMA [4,9]. We recently identified catechin, epicatechin and chlorogenic acid as candidate bioactive phenolics able to reduce microbial conversion of choline into TMA through a non-cytotoxic/cytostatic mechanism in our *ex vivo-in vitro* fermentation model [9,12]. These compounds can be found in high concentrations in foodstuffs such as coffee, tea and cocoa [13–15]. Once phenolic compounds are consumed within a food matrix, their structure, soluble concentrations and potential bioactive effects can be altered throughout the gastrointestinal tract [18,19]. In this sense, phenolic compounds can be metabolized by the gut microbiota into smaller phenolic acids [15], but our recent study shows that native compounds hold greater bioactivity to inhibit TMA production [12]. Thus, the aim of this study was to provide further evidence that catechin, epicatechin and chlorogenic acid and foods containing them are candidates for use to reduce TMAO formation through the study of the TMA production inhibitory effects of digested beverages rich in these compounds in our *ex vivo-in vitro* fermentation model.

To reach this aim, we verified that choline-d_9 was stable under our experimental conditions, and that no TMA-d_9 was formed by non-microbial reactions (Figure S2A), as reported previously [9,12], even in the presence of control digesta. Much like in our previous work [9,12], fecal slurry is required for choline-d_9

transformation into TMA-d_9 (Figure S2B). Thus, TMA-d_9 generated in our fermentations is solely due to microbial biotransformation of choline-d_9 into TMA-d_9 by bacteria present in the inoculated fecal slurry, and not endogenous signal or non-microbial reactions.

We then evaluated the effect of including different digested beverages to the fermentation at 30 % of final fermentation mixture volume. Undigested beverages were included as well, calculated to provide the same volume of beverage as digested samples. A fermentation including saline digesta (no beverage) condition as well as an undigested saline were included as controls for comparison. Coffee was selected as a relevant source of chlorogenic acid [15,19], a caffeoylquinic acid derivate with previously reported *in vitro* TMA production inhibition properties [9]. Moreover, caffeic acid, a microbial metabolite from chlorogenic acid (5-caffeoylquinnic acid) has shown potential to inhibit TMA formation in our *ex vivo-in vitro* fermentation model. This is also true for other structurally related compounds (*i.e.,* ferulic acid and p-coumaric acid) [12]. Chlorogenic acid (200 – 400 mg/Kg) has been shown to inhibit the formation of TMA and TMAO in male mice supplemented with L-carnitine (3 % in food) [25]. Of note, in our previous study, chlorogenic acid inhibited TMA production between 8 – 12 h of ~ 25 – 35 % at concentration of 2 mM, between ~ 11 – 18 % at a concentration of 1 mM, and 8.7 % 100 µM (only at 12 h) [9]. At the timepoint of its maximum TMA-d_9 production inhibition (12 h), digested coffee produced a ~52 % reduction in TMA-d_9 levels. However, undigested coffee, supplying the same amount of beverage as digested conditions, did not inhibit TMA-d_9 production at timepoints where bacteria were more active (6 – 10 h). It is important to note in our previous study the concentrations of chlorogenic acid tested were higher than the ones we are reporting in our study. In this sense, the concentration of total hydroxycinnamic acid derivatives (chlorogenic acid + chlorogenic acid derivatives + feruloylquinic acid derivatives + dicaffeoylquinic acid derivatives) in our undigested coffee approximates to 38 µM (Table 2), which is 2.8-fold times lower than the lowest concentration of pure chlorogenic acid (100 µM) that reports a bioactive effect in our previous work. These levels were even lower in digested coffee fermentations. The levels of phenolic compounds identified by LC-MS/MS provided by digested coffee (whole digesta) was lower than the one provided by undigested coffee, and this was particularly true for compounds with one (or more) quinic acid moieties such as chlorogenic acid (Table 2). This is likely due to degradation, as well as a possible matrix effect (reduced extraction) in the whole digesta. Overall, digestion seemed to be a pre-requisite to unlock the inhibition potential of coffee. Some factors, such as bioaccessibility of phenolic compounds in undigested coffee, could play a role in determining the bioactivity of coffee. However, undigested coffee was able to reduce TMA formation in the fermentation model of Bresciani *et al*. [10], which contradicts our data.

It is worth to note that, in all of our experimental set-ups, pH was not controlled nor set at a specific value beyond the initial pH. Although our fermentation is tamponed, previous literature using our *ex vivo-in vitro* fermentation method has shown that digested spinaches [26] and grape juices [27] can reduce fermentation pH to 6.0 within a 45 h fermentation window of time. This factor could affect the reported bioactivity of undigested beverages, and digested beverages and their fractions. Thus, *in vivo* experiments should be performed to corroborate the bioactivity of these beverages. Further, interactions with the human gut epithelium are not present in our model. Physiologically, as TMA is produced by the gut microbiota, it is absorbed by the gut epithelium, and directed to the liver where it is metabolized into TMAO. Thus, from a physiological point of view, it is preferable to inhibit TMA formation at timepoints where bacteria are more biochemically active than at later timepoints, where most choline has already been converted into TMA. Put simply, the delay in TMA-d_9 production produced by digested coffee, digested regular cocoa, and digested Dutched cocoa produced around 10 – 14 h may have a more relevant impact *in vivo* than the inhibition produced by other treatments at later timepoints. Overall, the inhibition of TMA-d_9 production produced by digested coffee are unlikely to be attributed to a cytotoxic/cytostatic effect. Of note, rather than decreasing cell viability, digested coffee increased cell viability as measured cell respiration and growth. Thus, digested coffee most likely achieves reductions in TMA-d_9 production through non-cytotoxic/cytostatic mechanisms. Coffee and chlorogenic acids consumption have been shown to present other different cardioprotective functions [17,28]. The inhibition of TMAO formation through the inhibition of TMA formation could be another potential cardioprotective mechanism of action of chlorogenic acid and coffee, which will require further studies *in vitro* and *in vivo*.

In our previous study, we also identified catechin and epicatechin as compounds with promising TMA production inhibition activities [12]. To test their efficiency through a more physiological approach, we selected three different teas (black, green and pu'erh) and two cocoas (regular and Dutched), as these are relevant sources of catechin, epicatechin and other flavan-3-ols with relevant cardioprotective functions [13–15]. Of note, green tea is rich in epicatechin and epigallocatechin derivates, such as epigallocatechin gallate (EGCG) and epigallocatechin (EGC), while black tea is rich in epicatechin but generally has a lower polyphenol content than green tea [29]. Pu'erh tea presents an even lower content in polyphenols than black tea compared to green tea [30], confirmed by our TPC analyses. The trends reported in the literature are in line with the TPC reported in our study (Table S1). Overall, tea treatments did not markedly inhibit choline-d_9 use and TMA-d_9 production, especially at time points where bacteria were more metabolically active. Rather, in some cases, they promoted choline-d_9 use and TMA-d_9 production. This was true for both digested and undigested tea treatments, which suggests that catechin and epicatechin provided through tea consumption may not be an effective strategy to reduce TMA, and ultimately TMAO, levels *in vivo*. Moreover, EGCG and EGC are quite unstable under gastrointestinal digestions, while catechin and

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epicatechin are more stable [18]. This could also be an explanation for the lack of general bioactivity of digested teas, especially green tea. Of note, tea treatments have shown different effects in the literature. For example, Bresciani *et al*. [10] found that undigested black tea produced no effect or a low inhibition effect in an *ex vivo-in vitro* fermentation model with either omnivorous or vegetarian fecal starter, and choline as a substrate. However, when L-carnitine was used, black tea promoted the production of TMA in the vegetarian fecal starter and inhibited its production in the omnivorous fecal starter. However, Chen *et al*. [31] showed that oolong tea extract, rich in catechin, epicatechin and (epi)catechin-derived compounds (*i.e.,* gallocatechin, epigallocatechin gallate, gallocatechin gallate and epigallocatechin) reduced TMAO levels in L-carnitine-supplemented mice. Moreover, Liu *et al*. [32] have recently shown that *Ligusrum robustum*, a tea-like plant rich in flavonoids, reduced TMAO formation through modulation of gut microbiota and TMA production in mice. Angiletta *et al*. [33] showed that some individuals responded to green tea administration, and that their TMAO plasma levels were reduced after green tea treatment, while some other individuals were unresponsive. Our current work adds further information on the role of teas as TMA, and potentially TMAO, production inhibitors. Overall the results from our study and others [10,31–33], suggest that the composition of the gut microbiota may play a huge role on the potential of teas as TMA and TMAO production inhibitors. Thus, further research is required to evaluate the potential of teas in TMA and TMAO production inhibition, with special emphasis on gut microbiota composition.

Cocoa is a rich source of epicatechin but its levels drastically decrease due to Dutching [34], as evidenced by our LC-MS/MS analyses (Table 2). The trends in digested/undigested coffee treatments were also seen in cocoa treatments. In our previous study, undigested catechin and epicatechin were able to reduce TMA-d_9 formation up to ~ 72 % and 51 %, respectively, at 8 h [12]. In our current study, at 12 h, time at which the TMA-d_9 production was most inhibited by digested cocoas, digested regular cocoa produced a ~ 88 % decrease in the levels of TMA-d_9, while digested Dutched cocoa produced a ~ 82 % decrease in the levels of TMA-d_9. However, and much like coffee, the undigested cocoa treatments did not produce an inhibition of TMA-d_9 production at timepoints where bacteria were more metabolically active (6 – 10 h). Of note, despite the differences in phenolic compounds provided by regular and Dutched cocoa treatments (Table 2), both digested cocoa treatments reported a similar bioactive effect. This suggests that some other food matrix components (*i.e.,* fibre) may play a role on the bioactivity of cocoas. Although cell growth could not be properly evaluated due to the turbidity provided by cocoa treatments, cell respiration was not reduced due to digested and undigested cocoa treatments. This suggests that the effects promoted by cocoa treatments were not due to a cytostatic/cytotoxic effect. It is important to highlight that, in both cocoa treatments, the levels of phenolic compounds between undigested beverages and digested beverages (whole digesta) were similar. This was true for all compounds except for procyanidin B2, which suffered a relevant decrease (~66 %) between undigested and digested (whole digesta) regular cocoa. Digestion was required to unlock the TMA-d_9 inhibition potential of cocoa treatments. This suggests that bioaccessibility may play an important role in the bioactivity associated to cocoa. *In vivo*, however, Angiletta *et al*. [33] reported that cocoa administration did not generally reduce TMAO circulating levels in obese adults, except for some responding individuals.

Overall, our results suggest that digestion may play an important role in unlocking the bioactivity of the beverages studied, and that this could be related to a higher bioaccessibility of bioactive phenolic compounds. However, it is worth to note that the digesta background could also be shifting the composition and function of the bacteria within our fermentations, promoting a higher effect of the beverages independent of the release of phenolic compounds. This could be relevant for beverages such as teas, in which phenolic compounds are unlikely bound to other food matrix components.

Noteworthily, the treatments that achieved a higher inhibition on choline-d_9 use and TMA-d_9 production were those treatments that could provide other energy-rich substrates for the microbiome than phenolic compounds. For example, filtered coffee can contain around 0.26 – 0.47 g soluble dietary fibre/100 mL beverage [35,36]. According to manufactures, cocoa contains ~40 % dietary fibre in weight, which means that our cocoa beverages can contain up to 2.12 g fibre/100 mL. Dietary fibre can play a significant role in the inhibitory effects promoted by these beverages, as it can interact with the gut microbiome through multiple ways [37,38]. Moreover, fibre could serve as an alternative, more efficient source of energy for bacteria, which could promote a reduction in the use of choline-d_9 and the consequent reduction in the production of TMA-d_9. As a matter of fact, Bresciani *et al.* [10] showed that the most bioactive components of undigested citrus juices in a similar *ex vivo-in vitro* fermentation model were free sugars, which could serve as an alternative, efficient source of energy for bacteria. However, enrichment of Taurisolo®, an extract rich in catechin, epicatechin and other phenolic compounds [39], with fibre (pectin) did not enhance its potential to reduce the circulating levels of TMAO in overweight/obese subjects [40]. In line with this, inulin supplementation has been shown inefficient in reducing TMAO levels in humans at risk of type 2 diabetes [41]. To narrow down the contribution of digested phenolic compounds of digested beverages to the inhibition of TMA-d_9 formation, whole digesta from selected bioactive treatments (coffee, regular cocoa and Dutched cocoa) was fractioned into soluble (supernatant) and insoluble (pellet) constituents. Each fraction was then tested for their potential to inhibit choline-d_9 use and TMA-d_9 production in our *ex vivo-in vitro* model.

It was apparent that the kinetic curves with whole digesta between the first batch of fermentations (Figure 1) and the second batch (Figure 3) present key differences. First, the

kinetics of the second batch of fermentations started later and appeared much slower than the kinetics in the first batch of fermentations with digested coffee, digested regular cocoa and digested Dutched cocoa. Moreover, the effect of the whole digested beverages on the second batch of fermentations seemed much more discrete than the ones reported in the very first fermentation batch. Regardless, the overall inhibitory effect of on choline-d_9 use and TMA-d_9 production of whole beverage digesta between fermentation batches appeared to be conserved between experiments. For example, while changes in choline-d_9 in digested regular cocoa appeared at 10 – 12 h (+18.2 – +64.0 μM) in the first fermentation batch, they appeared at 16 h (+29.8 μM against control) in the second fermentation batch. However, this highlights the fact that each fermentation experiment must be compared to its own control conditions, and that comparisons between different fermentation experiments might be misleading. Of note, the fecal slurries used to conduct these fermentations come from human samples, not isolated, commercial bacterial strains, which is possible factor contributing to this effect. Each fecal slurry batch might contain lower or higher number of bacteria cells, affecting the overall kinetics. Moreover, the use of different fecal slurries between fermentations might involve changes in bacteria species, which can affect both affect the kinetic curves of choline and TMA, as well as the bioactive effects of treatments.

The phenolic content distribution of digested coffee and digested cocoas was different between beverages: 99 % of all phenolics of digested coffee were present in the supernatant fraction, while only 38 % and 31 % of the total polyphenol content was found in the supernatant fraction for digested regular and Dutched cocoas, respectively (Figure 5). While the LC-MS characterization of the phenolic profile of coffee digesta fractions coincided with the distribution of the TPC, there was a discrepancy between TPC and LC-MS characterization in the digesta cocoa fractions. This can be explained by the fact that cocoa is rich in large procyanidins [42], which were not profiled by LC-MS and would remain in the pellet fraction due to their insolubility. Supernatant digesta is traditionally associated with those food compounds that are bioaccessible in the small intestine, available for absorption and thus less likely reach the colon and be subjected to gut microbiota fermentation (although a significant portion of released, water-soluble compounds are not absorbed and are available for colonic metabolism). It should also be noted that significant loads of viable, active bacteria are present in the small intestine, albeit orders of magnitude less abundant than those found in the colon. On the one hand, digesta pellet is traditionally associated with those compounds that are not bioaccessible in the small intestine, and thus more likely to reach the colon and be subjected to gut microbiota fermentation. It is worth noting that the bioavailability of flava-3-ols (*i.e.,* monomeric catechins found in cocoa) is generally low (< 5%) [43,44]. However the bioavailability of cocoa epicatechin can amount up to 25 % of the ingested dose in humans [45]. Also, it has been estimated that more than 2/3 of chlorogenic acids are not absorbed throughout the whole gastrointestinal tract [46]. This low bioavailability of phenolic compounds in the upper gastrointestinal track results in a large proportion of ingested polyphenols reaching the colon where they can be subjected to gut microbiota fermentation. Considering the low bioavailability in the upper tract of phenolic compounds, the fractioning of whole digesta in this study served to remove the interference of non-phenolic compounds (*i.e.,* insoluble fibres) that could mask the effects of the phenolic compounds present in the digested beverages. In this sense, most fibres should remain in the pellet fraction (except soluble fibres), while phenolics were distributed in the different fractions (supernatant or pellet) depending on the beverage (Figure 5).

The most relevant observation in the fractioning trial is that the bioactivity of digested coffee is found in the supernatant fraction, where the majority of their TPC (Figure 5A), and chlorogenic acid and its derivates are present (Table 2). As previously discussed, coffee is a rich source of chlorogenic acid [15,19], which has shown potential to decrease TMA and TMAO formation in different *in vitro* and *in vivo* models [9,10,25]. This result is further evidence of the potential of chlorogenic acid as a lead compound to manage TMA and TMAO formation. However, further research needs to be conducted to identify other TMA production inhibitors in coffee, if any. Although not as marked, the bioactivity of regular cocoa was also mostly present in the supernatant fraction. This fraction was rich in monomeric flavan-3-ols catechin and epicatechin (Table 2), with known ability to inhibit TMA production [12]. The pellet fraction, although richer in total polyphenol content, is presumably rich in larger procyanidins. These might achieve a regulation of TMA and TMAO levels *in vivo* through a probiotic modulation of the gut microbiota rather than a direct enzyme inhibition, a most likely mechanism of action of smaller compounds such as catechin and epicatechin. Digested Dutched cocoa bioactivity was found in whole digesta, while the bioactivity in the pellet (rich in fibre and procyanidins) and supernatant (rich in monomeric flavan-3-ols) fractions was lost. Thus, it seems that a combination of the bioactive substances present in both fractions are required for the bioactivity of Dutched cocoa. As previously mentioned, Dutching produces relevant changes in the composition of cocoa phenolic compounds, which might modulate their bioactive effects.

Overall, the mechanisms of action of the bioactive beverages and their digested fraction that led to the inhibition of TMA production are not elucidated in this study. Potentially, *in vivo* studies could elucidate if the supplementation of phenolic-rich beverages achieves TAMO production inhibition through a modulation of the gut microbiome, potentially by reducing the number of bacteria encoding the *cutC/D* gene cluster. Another potential mechanism of action is a direct TMA-lyase enzyme inhibition by phenolic compounds in beverages, which should be evaluated by the production of TMA lyase enzymes through cell lysates [4]. However, our study shows that chlorogenic acid-rich beverages could be a good strategy to reduce TMAO levels

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in vivo, and that chlorogenic acid seems to be a relevant compound for their bioactivity.

Conclusions

Overall, our data suggests that coffee and regular cocoa are the beverages with the most potential to inhibit TMA formation *in vivo*, which could ultimately affect CVD and atherosclerosis risk and burden through the reduction of TMAO levels. Further studies in *in vivo* models should be performed to confirm the bioactivity of chlorogenic acid and chlorogenic acid-rich foodstuffs. While these beverages are known to possess cardioprotective activities, studies are needed to determine whether reduction of TMAO formation is a contributing mechanism of action.

Author Contributions

Conceptualization, L.I.-C. and A.P.N.; methodology, L.I.-C., K.C.R. and A.P.N.; investigation, L.I.-C. and K.C.R.; data curation, L.I.-C.; writing—original draft preparation, L.I.-C.; writing—review and editing, L.I.-C., K.C.R. and A.P.N.; supervision, A.P.N.; project administration, A.P.N.; funding acquisition, A.P.N. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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Tables:

Table 1: Estimated total polyphenol content provided by undigested and digested beverages in fermentation conditions.

Beverage	Undigested (mg GAE/L)	Digested (mg GAE/L)
Coffee	74.5 ± 3.2	54.2 ± 0.8
Black tea	13.1 ± 0.4	12.9 ± 2.2
Green tea	15.3 ± 0.4	13.2 ± 1.5
Pu'erh tea	7.0 ± 0.2	9.0 ± 2.7
Regular cocoa	40.4 ± 2.1	48.2 ± 0.1
Dutched cocoa	40.4 ± 1.3	40.0 ± 1.4

Abbreviations: GAE, gallic acid equivalents

Table 2: Phenolic compound levels (nM ± SEM; *n*=3) in fermentations media of digested fractions (whole digesta, digesta pellet, and digesta supernatant) and undigested coffee, regular cocoa and Dutched cocoa at the start of the fermentation.

Beverage	Fraction	*p*CA	GA	CA	EC	C	CGA	CGA D1	FQA D1	DCQA D1	DCQA D2	PC B2
Coffee	**Undigested beverage**	n.d.	18.0 ±. 1.1	281.5 ± 22.8	95.6 ± 11.0	n.d.	20106.5 ± 6.7	11164.9 ± 931.3	6460.3 ± 587.2	1341.4 ± 121.1	864.9 ± 69.7	n.d.
	Whole digesta	6.4 ± 0.3	29.5 ± 0.5	270.9 ± 2.3	n.d.	n.d.	7954.4 ± 99.7	4749.7 ± 234.9	1986.4 ± 151.0	178.9 ± 11.7	76.1 ± 8.8	n.d.
	Digesta pellet	n.d.	80.3 ± 17.6	49.8 ± 0.0	n.d.	n.d.	117.2 ± 6.8	64.5 ± 0.5	38.8 ± 1.2	15.4 ± 0.5	14.9 ± 0.2	n.d.
	Digesta supernatant	6.7 ± 0.7	35.2 ± 2.5	292.7 ± 13.9	n.d.	n.d.	9241.3 ± 542.3	6069.8 ± 300.3	2765.1 ± 170.6	165.9 ± 24.8	63.1 ± 10.2	n.d.
Regular Cocoa	**Undigested beverage**	0.8 ± 0.2	7.6 ± 0.0	n.d.	791.2 ± 107.6	841.8 ± 111.1	n.d.	n.d.	n.d.	n.d.	n.d.	1311.9 ± 82.3
	Whole digesta	n.q.	n.q.	n.d.	678.6 ± 71.1	736.2 ± 75.6	n.d.	n.d.	n.d.	n.d.	n.d.	445.1 ± 38.1
	Digesta pellet	n.d.	23.8 ± 1.0	n.d.	95.3 ± 10.6	122.5 ± 5.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Digesta supernatant	n.q.	n.q.	n.d.	571.1 ± 44.0	646.4 ± 44.1	n.d.	n.d.	n.d.	n.d.	n.d.	281.1 ± 36.7
Dutched Cocoa	**Undigested beverage**	n.q.	7.9 ± 0.1	n.d.	156.1 ± 11.3	62.8 ± 3.8	n.d.	n.d.	n.d.	n.d.	n.d.	30.4 ± 1.0
	Whole digesta	n.q.	n.q.	n.d.	82.5 ± 5.6	49.1 ± 1.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Digesta pellet	n.d.	22.3 ± 0.3	n.d.	21.5 ± 1.5	29.8 ± 0.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Digesta supernatant	n.q.	22.4 ± 0.6	n.d.	89.5 ± 12.9	51.4 ± 2.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Abbreviations: *p*CA, *p*-coumaric acid; GA, gallic acid; CA, caffeic acid; EC, epicatechin; C, catechin; CGA, chlorogenic acid; FQA, feruloylquinic acid; DCQA, dicaffeoylquinic acid; PC B2, procyanidin dimer B2; n.q., not quantified; n.d., not detected; and D1 and D2, different derivates

Figure Legends:

Figure 1. Effect of digested and undigested beverages on choline-d_9 use and TMA-d_9 production. Blue and red shading respectively indicates digested saline control or control choline-d_9 and TMA-d_9 levels. Results are expressed as µM ± SEM (*n*=6). # Indicates statistical differences ($p<0.05$) in the levels of choline-d_9 between digested or undigested beverage and time-matched control conditions by Two-Way ANOVA (Sidak's *post hoc* test). * Indicates statistical differences ($p<0.05$) in the levels of TMA-d_9 between digested or undigested beverage and time-matched control conditions by Two-Way ANOVA (Sidak's *post hoc* test).

Figure 2: Area under the curve (AUC) of choline-d_9 (**A** and **C**) and TMA-d_9 (**B** and **D**) kinetic curves obtained from fermentations with choline-d_9 100 µM, fecal slurry 1:10 in PBS (20 %) and digested or undigested beverages and their respective controls. Results are expressed as h x µM ± SEM (*n*=6). * Indicates statistical differences ($p<0.05$) in the AUCs against respective control conditions by One-Way ANOVA.

Figure 3. Effect of the different factions of digested coffee, digested regular cocoa and digested dutched coca, namely whole digesta, digesta pellet and digesta supernatant, on choline-d_9 use and TMA-d_9 production. Blue and red shading respectively indicates digested saline control choline-d_9 and TMA-d_9 levels in the respective fractions. Results are expressed as µM ± SEM (*n*=6). # Indicates statistical differences ($p<0.05$) in the levels of choline-d_9 between digested beverage fraction and time-matched saline digesta control conditions, respectively, by Two-Way ANOVA (Sidak's *post hoc* test). * Indicates statistical differences ($p<0.05$) in the levels of TMA-d_9 between digested beverage fraction and time-matched saline digesta control conditions, respectively, by Two-Way ANOVA (Sidak's *post hoc* test).

Figure 4: Area under the curve (AUC) of choline-d_9 and TMA-d_9 kinetic curves obtained from fermentations with choline-d_9 100 µM, fecal slurry 1:10 in PBS (20 %) and digesta fractions (whole digesta, digesta pellet and digesta supernatant) and their respective saline digesta control fractions. Results are expressed as h x µM ± SEM (*n*=6). * Indicates statistical differences ($p<0.05$) in the AUCs against respective control conditions by One-Way ANOVA. When significant, the percentages of change between control and treatment are expressed.

Figure 5. Distribution of the total polyphenol content in digested coffee (**A**), regular cocoa (**B**) and Dutched cocoa (**C**) pellet and supernatant fractions. Results are expressed as percentage (%) and as mg gallic acid equivalents (GAE)/L ± SEM (*n*=4).

Figure 1

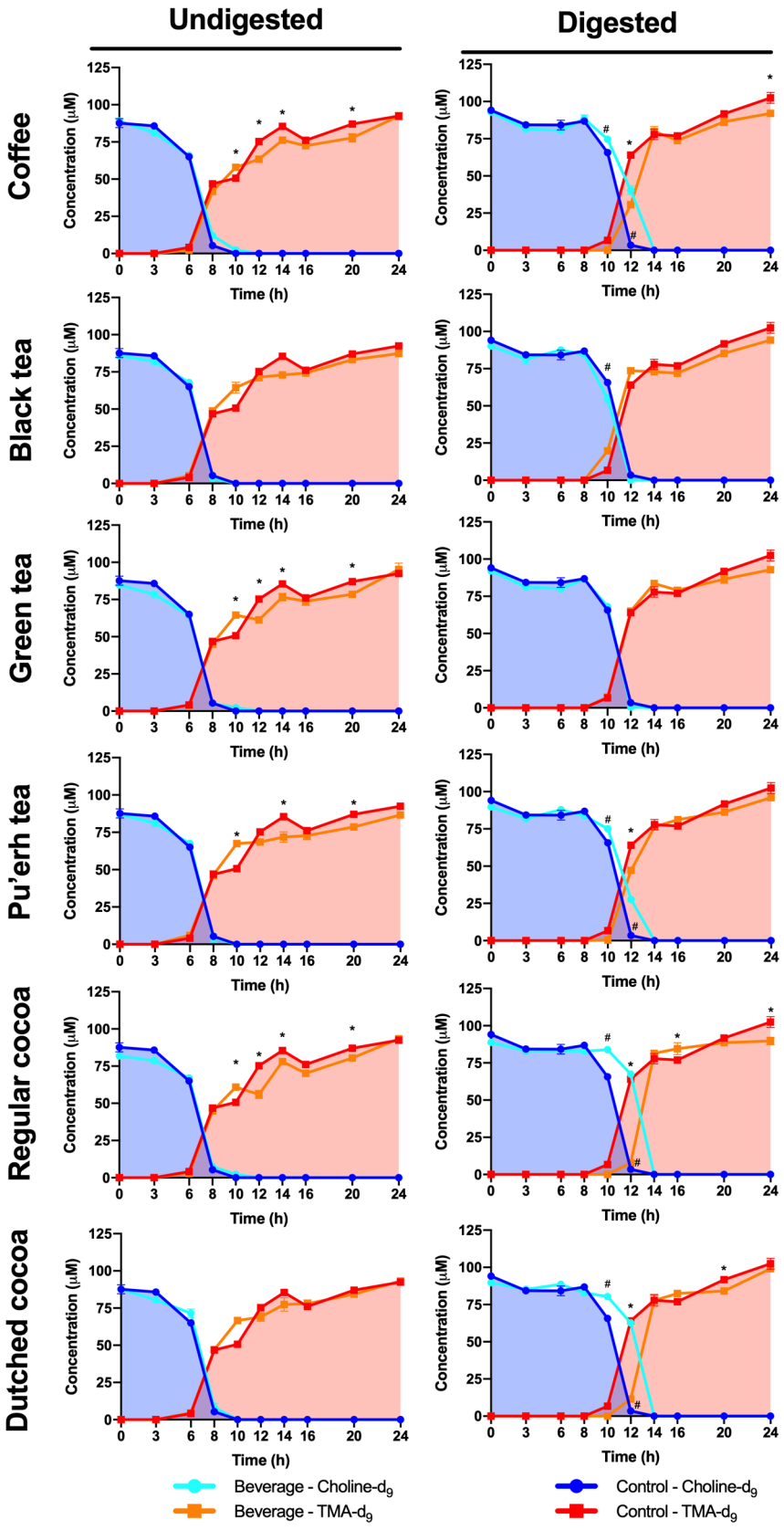

Undigested
Digested
Coffee
Black tea
Green tea
Pu'erh tea
Regular cocoa
Dutched cocoa
Concentration (μM)
Time (h)
Beverage - Choline-d9
Beverage - TMA-d9
Control - Choline-d9
Control - TMA-d9

Figure 2

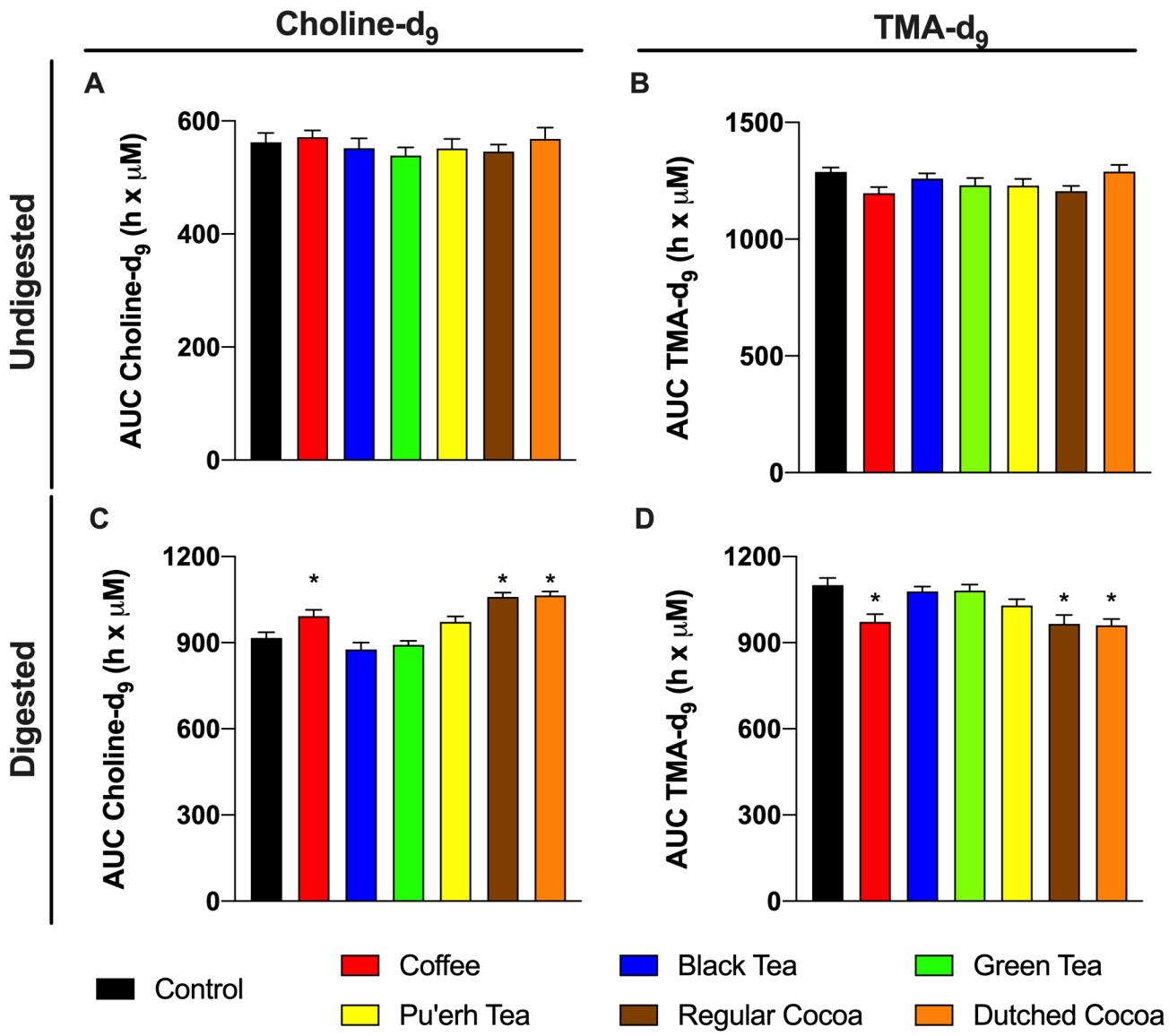

Choline-d9
TMA-d9
Undigested
Digested
A
B
C
D
AUC Choline-d9 (h x μM)
AUC TMA-d9 (h x μM)
600
400
200
0
1500
1000
500
1200
900
600
300
*
Control
Coffee
Pu'erh Tea
Black Tea
Regular Cocoa
Green Tea
Dutched Cocoa

Figure 3

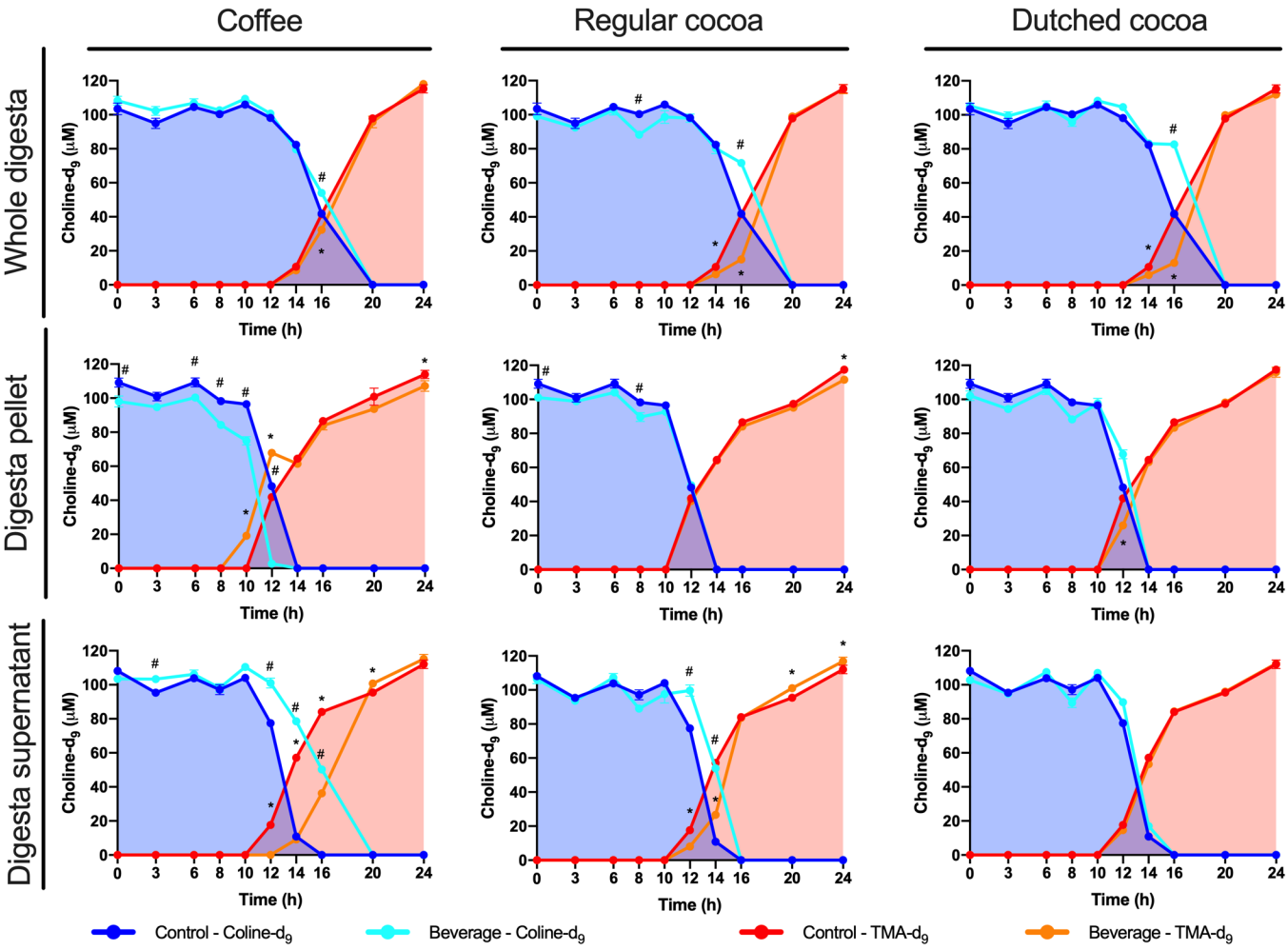

Coffee
Regular cocoa
Dutched cocoa
Whole digesta
Digesta pellet
Digesta supernatant
Choline-d9 (μM)
Time (h)
Control - Coline-d9
Beverage - Coline-d9
Control - TMA-d9
Beverage - TMA-d9

Figure 4

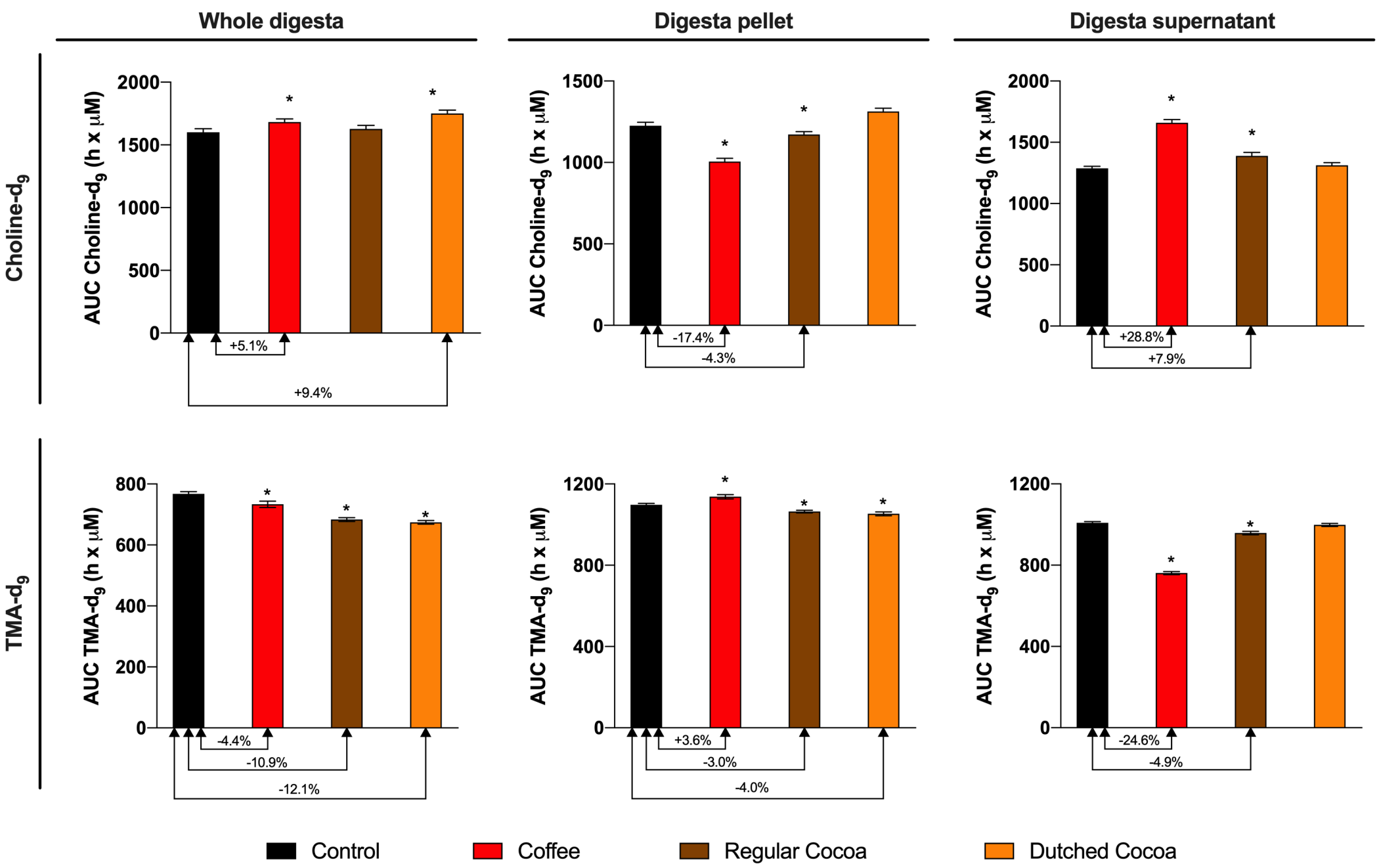
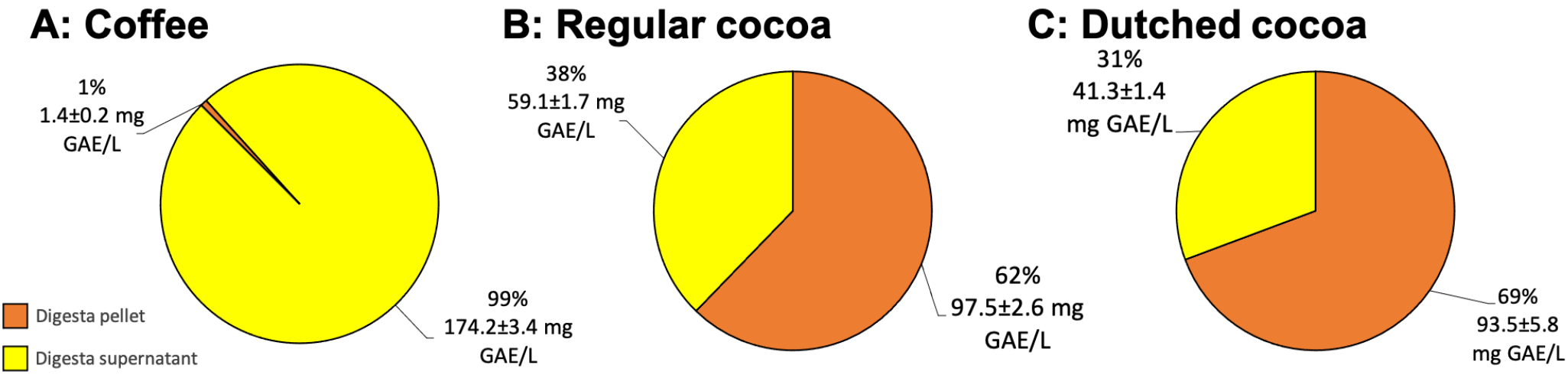

Whole digesta
Digesta pellet
Digesta supernatant
Choline-d9
TMA-d9
AUC Choline-d9 (h x μM)
AUC TMA-d9 (h x μM)
2000
1500
1000
500
0
800
600
400
200
1200
+5.1%
+9.4%
-17.4%
-4.3%
+28.8%
+7.9%
-4.4%
-10.9%
-12.1%
+3.6%
-3.0%
-4.0%
-24.6%
-4.9%
*
Control
Coffee
Regular Cocoa
Dutched Cocoa

Figure 5


A: Coffee
1%
1.4±0.2 mg
GAE/L
99%
174.2±3.4 mg
GAE/L
Digesta pellet
Digesta supernatant
B: Regular cocoa
38%
59.1±1.7 mg
GAE/L
62%
97.5±2.6 mg
GAE/L
C: Dutched cocoa
31%
41.3±1.4
mg GAE/L
69%
93.5±5.8
mg GAE/L