



## Ex Vivo Fecal Fermentation of Human Ileal Fluid Collected After Wild Strawberry Consumption Modulates Human Microbiome Community Structure and Metabolic Output and Protects Against DNA Damage in Colonic Epithelial Cells

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Ex vivo faecal fermentation of human ileal fluid collected after wild strawberry consumption modulates human microbiome community structure and metabolic output and protects against DNA damage in colonic epithelial cells.

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## ABSTRACT

Scope: Wild strawberries (*Fragaria vesca*) are richer in (poly)phenols than common commercial strawberry varieties e.g. *Fragaria* × *ananassa*. (Poly)phenols and their microbiota-derived metabolites are hypothesised to exert bioactivity within the human gut mucosa. To address this, the effects of wild strawberries were investigated with respect to their bioactivity and microbiota-modulating capacity using both *in vitro* and *ex vivo* approaches.

Methods and results: Ileal fluids collected pre- (0h) and post-consumption (8h) of 225 g wild strawberries by ileostomates (n = 5) and also *in vitro* digested strawberry varieties (*Fragaria vesca* and *Fragaria* × *ananassa* Duchesne) supernatants were collected. Subsequent fermentation of these supernatants using an *in vitro* batch culture proximal colon model

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revealed significant treatment-specific changes in microbiome community structure in terms of alpha but not beta diversity at 24 h. Nutri-kinetic analysis revealed a significant increase in the concentration of gut microbiota catabolites, including 3-(4-hydroxyphenyl)propionic acid, 3-(3-hydroxyphenyl)propanoic acid and benzoic acid. Furthermore, post-berry ileal fermentates (24 h) significantly ( $p < 0.01$ ) decreased DNA damage (% Tail DNA, COMET assay) in both HT29 cells (~45%) and CCD 841 CoN cells (~25%) compared to untreated controls.

Conclusions: Post berry consumption fermentates exhibited increased overall levels of (poly)phenolic metabolites which retained their bioactivity, reducing DNA damage in colonocytes.

Keywords: ileostomy, microbiota, (poly)phenols, short chain fatty acids, wild strawberry

## 1. INTRODUCTION

The consumption of fruit and vegetables as part of an overall healthy diet is associated with reduced risk of all-cause mortality (1,2). The inverse association between fruit and vegetable consumption and reduced risk of premature death is shown in previous meta-analysis (3,4). According to Wang et al., up to five additional servings a day of fruit (equivalent to 70 g) and vegetables (equivalent to 80 g), reduces the risk of all-cause mortality by 5-6% (5). It is likely that (poly)phenols are, in part, responsible for these effects given their relatively high daily intake levels (~800 mg/day) (6) and pleiotropic bioactivity in the context of "healthy diets" (7-9).

Strawberries, a rich source of polyphenols, represent one of the most important fruit crops worldwide, with an overall global production of 8.3 million tons per year (10). In the European context, 1.64 kg/year per person are consumed as raw fruit and processed food (10) which is of interest given emerging evidence supporting their role as a functional food with beneficial health effects (11,12). Strawberries are rich in proanthocyanidins, anthocyanins, ellagitannins and flavonol glycosides, with wild varieties like *Fragaria vesca* having up to twice the (poly)phenol content (324.3-448.2 mg/100g) of cultivated varieties (200.6-288.9 mg/100g) (13). In particular, *F. vesca* is characterized by higher levels of ellagitannins ranging from 26 to 40% of the total phenolic content, contrasting with the common strawberry at ~10% (13) whereas flavonoid (26 mg/100g vs 20 mg/100g) and anthocyanin (41.39 mg/100g v 24-44.47 mg/100g) contents remain comparable (14).

While consumption of richer sources of (poly)phenols is desirable, it is important to note that the bioavailability of many (poly)phenol classes has generally been considered to be low

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(15). However, the realization that the human gut microbiota metabolises unabsorbed (poly)phenols, catabolising them to more biologically available components, is now changing the way we measure the nutri-kinetics of plant macromolecules (16–18). Microbial transformation of native plant (poly)phenols may also impact on their biological activities both within the gut and systemically once absorbed across the intestinal wall (19,20). This two-way (poly)phenol microbiota interaction is determined by inter individual variability leading to different (poly)phenol-metabolising phenotypes or metabotypes which can, in turn, influence the effects of dietary (poly)phenols. For example, metabolism of ellagitannins to urolithins via gut microbiota is dependent upon the host metabotype (UM-A, -B, or -0) which have been reported to predict individual responsiveness to a (poly)phenol-rich diet (21). The variability in metabotypes has been associated with an increase of all-cause mortality (22), however the inherent stability of these metabotypes over time remains poorly understood (23).

Many studies have addressed the impact of secondary plant metabolite on gastrointestinal (GI) tract using a variety of *in vitro* models (24). While they are effective at comparing potential in-gut availability, they do not comprehensively mimic the dynamic and active processes associated with the digestion of food *in vivo* (25). Often, it has been demonstrated that (poly)phenols have variable stabilities and differ in the amounts necessary to exert physiological processes. In contrast to the experimental approach, ileostomy-based bioavailability studies provide a unique means of assessing the *in vivo* effects on the intestinal tract, facilitating identification of compounds which, in individuals with an intact colon, would pass from the small to the large intestine and therein exert their biological effects (26–28). While numerous studies have reported bioactivities for (poly)phenols from strawberries, especially anti-genotoxicity (26), most lack physiological relevance as the impact of the digestive process and gut microbiota activity on the composition, structure and bioactivity of the compounds present has largely been ignored (29).

In this study we hypothesized that physiologically relevant levels of wild strawberry (*F. vesca*) metabolites would be colon-available following consumption of a *F. vesca* puree and would be both bioavailable and biologically active.

## 2. EXPERIMENTAL SECTION

**2.1 Chemicals and reagents:** Enzymes for *in vitro* digestion and chemicals for the batch culture basal nutrient medium were purchased from Sigma-Aldrich (St. Louis, MO, USA), Applichem (Darmstadt, Germany), Thermo Fischer Scientific (Waltham, MA, USA) unless otherwise stated. For the chemical standards of (poly)phenols and microbial metabolites as

well as the LC-MS reagents please refer to Vrhovsek et al.(2012) and Gasperotti et al. (2014) (30,31).

**2.2 Ileostomy feeding study:** The study (11/0284) was conducted with the prior approval of Ulster University Ethical Committee and the Office for Research Ethics Committees Northern Ireland (ORECNI) and the and with the informed consent of participants who were recruited from Clinics at Altnagelvin Area Hospital. Prior to the beginning of the study, 5 ileostomates (3 males, 2 females) mean age  $44 \pm 12$ , followed a diet low in (poly)phenolic compounds for 48 h as described by Gonzalez-Barrio et al. (27). After an overnight fast, they provided a 0 h ileal fluid sample (baseline). Then, they consumed 225 g of homogenized wild strawberries (*F. vesca*) and continued the low (poly)phenolic diet.

A second ileal fluid sample was collected 8 h post-consumption (+ wild strawberry 8h). The ileal fluid samples were processed as described previously McDougall et al. (2014) (32), and stored at  $-80^{\circ}\text{C}$  until use.

The selection of 225g was based on the amounts of wild strawberries available, and quantities used in our previous raspberry study where participants were fed 300g (32). Which equated to similar concentrations of anthocyanins ( $200.887 \pm 23.48 \mu\text{mole}/225\text{g}$ ) and ellagitannins ( $301.42 \pm 55.90 \mu\text{mole}/225\text{g}$ ) reported to be associated with reduced risk of chronic disease (CVD) inhibiting inflammation, improving both endothelial function and plasma lipid profile (33,34).

**2.3 Faecal donors:** Five healthy volunteers, (3 males, 2 females), mean age  $38 \pm 8$  y, were used as faecal donors for the *in vitro* fermenter study. They had not received antibiotic treatment within 3 months of stool collection, had no history of bowel disorders, and had not consumed pre- or pro-biotic supplements prior to experiment. All five donors were informed of the study aims and procedures and provided their verbal consent for their faecal matter to be used for the experiments, in compliance with the ethics procedures required at Fondazione Edmund Mach and APSS (Azienda provinciale per i servizi sanitari), Trento (TN), Italy.

**2.4 *In vitro* digestion of strawberry puree:** Fresh commercial strawberries (*Fragaria × ananassa Duchesne*) (S) and wild strawberries (*Fragaria vesca*) (WS) were pureed and *in vitro* digested (IVD). A 2-step procedure including both gastric and duodenal phases was performed as described by Mandalari et al. (2008) (35). A phospholipid vesicles suspension was prepared according to Koutsos et al. (2017) (36), prior to the *in vitro* digestion. To mimic bolus absorption, the final suspensions were transferred to 1kDa MWCO (molecular weight

cut off) cellulose dialysis tubing (Spectra/Por® 6, Spectrum Europe, Breda, Netherlands) and dialyzed overnight against NaCl (10 mmol/L) at 4 °C. Finally, samples were freeze-dried and stored at -80 °C until use. Inulin and cellulose were also digested using the same protocol.

**2.5 Faecal batch-culture fermentation of ileal fluids and *in vitro* digests:** To mimic the environment of the proximal colon, twenty four hour pH controlled faecal batch-cultures were performed at 37 °C under anaerobic condition according to Koutsos et al. (2017) (36). The following substrates were used; a pooled baseline ileal fluid (S1, S2, S3, S4, S5) from each participant, (Baseline(Pooled)), ileal fluid samples 8 h post-wild strawberry consumption (+ wild strawberry) and *in vitro* digested samples of S and WS. Inulin and cellulose were used as a readily and a poorly fermentable plant fibre respectively. Glass water-jacketed vessels (200 mL) were heat sterilized and filled aseptically with 90 mL of pre-sterilized basal nutrient medium according to Sanchez-Patan et al. (2012) (37). Throughout the 24 h fermentation, temperature was set at 37 °C using a circulating water bath, anaerobic conditions were supplied through an O<sub>2</sub>-free N<sub>2</sub> (15mL/min) flow bubbled in the medium, and the pH was kept and adjusted to 5.5–6.0 range through an automatic addition of NaOH (0.5 M) or HCl (0.5 M). Fresh human faecal samples, from 5 healthy donors, were collected in an anaerobic jar, and within 1 h, homogenized, and diluted 1:10 using sterile pre-reduced phosphate-buffered saline (PBS). Each vessel was inoculated with 10 mL of faecal slurry and dosed with the appropriate substrate/treatment, for a final concentration of 1 % w/v. A 1mL aliquot of a 10 % w/v filtered, freshly prepared, solution of L-cysteine hydrochloride (Applichem) was added prior to inoculation of each vessel, as oxygen reducing agent. Samples were collected at 4 time points (at T0, T5, T10 and T24 h) and centrifuged at 18 000 × g at 4 °C for 5 min. Pellets were stored at -80 °C and subsequently used for Illumina sequencing analysis, whereas the supernatant was first filtered through 0.22 µm filters and then stored at -80 °C until required for LC-MS and GC-MS analysis.

## 2.6 DNA extraction, amplification and sequencing

Amplicon library preparation, quality and quantification of pooled libraries and high throughput sequencing by Illumina technology were performed at the Sequencing Platform, Fondazione Edmund Mach (FEM, San Michele all'Adige, Italy). DNA was extracted from sample pellets obtained from the *in vitro* fermenter experiments (0, 10 & 24 h time points) using the FastDNA Spin Kit for Faeces (MP Biomedicals, UK). Nucleic acid purity was tested on NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific). Total genomic DNA was then subjected to PCR amplification targeting a ~460 bp fragment of the 16S rRNA

variable region V3-V4 using the specific bacterial primer set 341F (5' CCTACGGGNGGCWGCAG 3') and 806R (5' GACTACNVGGGTWTCTAATCC 3') with overhang Illumina adapters. PCR amplification of each sample was carried out using 25  $\mu$ L reactions with 1  $\mu$ M of each primer, following the Illumina Metagenomic Sequencing Library Preparation Protocol for 16S Ribosomal RNA Gene Amplicons. The PCR products were checked by electrophoresis on 1.5 % agarose gel and cleaned from free primers and primer dimers using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions. Subsequently, dual indices and Illumina sequencing adapters Nextera XT Index Primer (Illumina) were attached by 7 cycles of PCR (16S Metagenomic Sequencing Library Preparation, Illumina). The final libraries, after purification by the Agencourt AMPure XP system (Beckman), were analysed on a Tytestation 2200 platform (Agilent Technologies, Santa Clara, CA, USA) and quantified using the Quant-IT PicoGreen dsDNA assay kit (Thermo Fisher Scientific) by the Synergy2 microplate reader (Biotek). Finally, all the libraries were pooled in an equimolar way in a final amplicon library and analysed on a Tytestation 2200 platform (Agilent Technologies, Santa Clara, CA, USA). The Barcoded library was sequenced on an Illumina® MiSeq (PE300) platform (MiSeq Control Software 2.0.5 (Illumina, San Diego, CA, USA) and Real-Time Analysis software 1.16.18 (Illumina, San Diego, CA, USA)) generating a total of 6,022,557 reads with  $140,059 \pm 27.939,45$  read per sample (mean  $\pm$  SD). After filtering the sequences and removing the chimera, using QIIME2 pipeline (38) (version 2018.2) the total number of reads was 4,494,974 with an average of  $35,813 \pm 6.413,81$  read per sample (mean  $\pm$  SD).

## 2.7 Analytical methods

**2.7.1 LC–MS/MS analysis of precursors and phenolic acid catabolites in fermented samples.** The determination (poly)phenols and phenolic microbial metabolites was performed according to (30) and (31). Briefly, batch culture supernatants were thawed at 4 °C, centrifuged at 13,000 rpm for 5 min, and filtered (0.22  $\mu$ m) (Millipore). Trans-cinnamic acid-D5 (5 $\mu$ g/mL) was added to the filtrate as the first internal standard. Then, samples were dried under nitrogen and reconstituted in methanol:water (1:1 v/v) containing rosmarinic acid (1  $\mu$ g/mL) as the second internal standard. Finally, samples were shaken for 10 min in an orbital shaker, centrifuged for 5 min at 16,000 rpm to clarify and a 2  $\mu$ L aliquot injected into the UHPLC–MS/MS system. Details of the liquid chromatography and mass spectrometry are reported are described in Vrhovsek et al.(2012) and Gasperotti et al. (2014) (30,31).

**2.7.2 LC–MS/MS analysis of (poly)phenols in ileal fluids:** Ileal fluid samples were extracted as described by Brown et al. (2014) with some modifications. Briefly, 2.5 g of ileal

fluid was homogenized in 10 mL of 1 % formic acid in 50 % (v/v) aqueous methanol, for 1 min at 24,000rpm using an Ultra-Turrax homogenizer and then shaken for 30 min at 400 rpm. Samples were centrifuged for 20 min at 5600 × g at 4 °C and supernatants collected. The pellet was re-extracted and the two supernatants were pooled before being reduced to dryness *in vacuo*. The residues were re-suspended in 2 mL of 1 % formic acid in 50 % (v/v) aqueous methanol, and 2 µL aliquots were analysed by UHPLC- MS/MS. Details of the liquid chromatography and mass spectrometry are reported are described in Vrhovsek et al. (2012) and Gasperotti et al. (2014) (30,31).

**2.7.3 Analysis of short chain fatty acids (SCFA):** Analysis of SCFA in fermented samples was performed as previously described by (40). Briefly, 10 µL of acidified water (15 % v/v phosphoric acid), 20 µL of internal standard (IS) (acetic d4 45 mM; propionic d6 and butyric D7 10mM; 2-ethyl butyric and decanoic acid-d19 2 mM) and 100 µL of faecal suspension, previously filtered, were mixed in a 2 mL tubes. Consecutively, a liquid-liquid extraction (LLE) was performed using 980 µL of methyl-t-butyl ether (MTBE). The extraction was assisted by an orbital shaker (Multi RS-60; BioSan, Latvia) for 5 min with the following cycle program: 90 rpm of orbital rotation for 5 s followed by reciprocal motion at 20 °C (from the vertical plane) for 15 s. At this point, tubes were centrifuged at 25,314 × g at 5 °C for 5 min. Finally, the organic phase aliquot was transferred into 2 mL glass vial and subjected to GC–MS analysis in a split ratio 10:1 (total run-time of 6,5 min). Details of the liquid chromatography and mass spectrometry are reported are described in Lotti et al. (2017) (40).

## 2.8 Biological activity assays.

**2.8.1 Tissue culture:** HT 29 cells and CCD 841 CoN were acquired from European Collection of Cell Cultures (ECACC) and American Type Culture Collection (ATCC) respectively, incubated at 37 °C with 5 % CO<sub>2</sub> and grown as monolayers in Roux flasks. Both cells were sub-cultured every 3-4 days by the addition of trypsin (0.25 % trypsin-EDTA) at 37 °C for 5 min. Cells were centrifuged at 1200 rpm for 3 min, the supernatant decanted and cells re-suspended in the appropriate medium and divided in flasks <sup>[35]</sup>.

**Exposure to treatments:** The effects of ileal fluid fermentates and *in vitro* digested fermentates extract on HT 29 and CCD 841 CoN cells were determined using the MTT assay according to Hansen et al. (1989) (41) (at 20 % v/v in media) to establish the sub-cytotoxic dose that has been used in the treatments (data not shown). Cells were grown in 96 multi-well plates (Costar, Cambridge, MA, USA) at a concentration of 1.5 × 10<sup>4</sup> and 3.0 × 10<sup>4</sup> per well for HT29 and CCD841 CoN cells, respectively. After 2 days incubation at 37 °C,

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cells were separately treated for 24 h with 20 % v/v ileal fluids fermentates *in vivo* (S1-S5 and baseline(POOLEDT0) and *in vitro* (S, WS, inulin and cellulose). Untreated cells were used as control.

**2.8.2 Genotoxicity assay (COMET assay):** The assay was performed as described previously (26), using the well-established HT29 cell model for colonic DNA damage and the normal colonocyte cell line, CCD841. Briefly, both cell lines (HT29 and CCD841) were incubated for 24 h with either 20 % v/v of the ileal fluid fermentates, or baseline(POOLED), or 20 % v/v of the *in vitro* digested fermentates. To assess the anti-genotoxic potential of the treatments, the cells were treated with hydrogen peroxide (75  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for HT29 and 25  $\mu\text{M}$ ,  $\text{H}_2\text{O}_2$  for CCD841) for 5 min at 4 °C, then centrifuged for 5 min at 258  $\times$  g. The supernatant was discarded, and the cell pellet re-suspended in 85  $\mu\text{L}$  of 0.85 % low melting point agarose (LMPA) in PBS (previously maintained in a water bath at 40 °C). The percentage DNA tail was recorded using Komet 5.0 image analysis software (Kinetic Imaging Ltd, Liverpool, UK) and for each slide, 50 cells were scored. Data represents the mean percentage tail DNA of triplicate gels per treatment from three independent experiments. To assess the genotoxic potential of the compounds, cells were treated as above, omitting the  $\text{H}_2\text{O}_2$  challenge. Positive ( $\text{H}_2\text{O}_2$ ) and negative (PBS) controls were included in all experiments.

## 2.9 Statistical analysis

QIIME2 pipeline version 2018.2 was used for 16S rRNA data analysis, whereas the rest of the analysis was performed using R software (version 3.6.3). The mean of five independent experiments  $\pm$  standard deviation (SD) was calculated for each type of dataset and used for the analysis. Data were checked for normality using the Kolmogorov–Smirnov and Shapiro–Wilk tests. Alpha and beta-diversity estimates were determined using phyloseq R package (R version 3.6.3). Differences in alpha diversity indices (Observed, Chao1, Shannon index) between treatments were tested using one-way analysis of variance (ANOVA). The p values were corrected with Bonferroni adjustment and  $p < 0.05$  was considered statistically significant. Principal component analysis (PCA), using the ade4 package in R (version 3.6.3) was performed to check whether different fermentation treatments formed distinct clusters. A Null hypothesis that clusters were not significantly different from each other was tested using analysis of similarities (ANOSIM) employing Euclidean distances and 9999 permutations. Percentage relative abundance of taxa from different fermentation treatments were compared using nonparametric Wilcoxon statistical test.

Missing values of phenolic compounds and metabolites datasets were imputed with a random value between zero and LOQ. A One-sample Wilcoxon signed-rank test was performed to evaluate significant differences between phenolic compounds and metabolites at the baseline and 8 h post-wild strawberry consumption (significance level,  $p < 0.05$ ). Kruskal–Wallis test and Dunnett’s Multiple comparison post-hoc test, was used for COMET ileal fluid results and to determine differences between fermentation treatments (Inulin, Cellulose, S1, S2, S3, S4, S5, baseline(POOLED), S, and WS) at the same time points (T0, T5, T10 or T24 h) and to explore the differences within the same treatment/vessel at all time points. The p values were corrected with Bonferroni adjustment and  $p < 0.05$  was considered statistically significant. Correlation between bacterial taxa at genus level and SCFA and (poly)phenols was performed by Spearman correlation analysis. False discovery rate FDR adjustment was applied to correct for multiple testing and  $p < 0.05$  was considered statistically significant.

### 3. RESULTS

#### 3.1 Dynamics of bacterial communities during fermentation: alpha and beta diversity

Diversity of the gut microbiota community (alpha diversity - Observed, Chao1, Shannon) in the fermenter models was assessed over time (Figure S1) in the response to the various substrate treatments (ileal fluids, berry digestates).

Comparison between treatment found significant differences at T5h for Chao1 index and in the number of Operational Taxonomic Units (OTUs). Post-hoc analysis and adjustment of the p-value did not confirm the significance for Chao index whereas Operational Taxonomic Units (OTUs) diversity revealed significant effects between S2 and Strawberry ( $p = 0.02$ ) and Strawberry and baseline (POOLED) ( $p = 0.02$ ) (Figure S1). Whereas within treatment comparison to baseline, revealed significantly lower alpha diversity indexes at T24h for inulin, S3, S5. While S2 showed lower diversity for Chao1 index at T24h compared to T0h ( $p < 0.05$ ) (Figure S1.2).

Beta diversity was analysed based on Bray-Curtis dissimilarities (Figure S2A), unweighted (qualitative) (Figure S2B) and weighted (quantitative) phylogenetic UniFrac distance matrices. Figure 1, based on a weighted (quantitative) phylogenetic UniFrac distance matrix shows clustering according to faecal donor at T0 h (ADONIS test,  $p = 0.004$  ( $R^2 = 10\%$ )). Over time, this clustering was less distinct and was no longer significant at T24 h (ADONIS test,  $p = 0.06$  ( $R^2 = 4\%$ )). In fact, after comparing treatments, we found significant differences in community structure as assessed by weighted UniFrac (ADONIS test,  $p =$

0.002  $R^2 = 33\%$ ) at T24 h (Figure 1), but no difference for Bray-Curtis (ADONIS test,  $p = 0.81$ ,  $R^2 = 17\%$ ) nor unweighted UniFrac (ADONIS test,  $p = 0.99$ ,  $R^2 = 15\%$ ), indicating that the microbial communities were phylogenetically similar among donors (Figure S5). Neither were there any treatment effects on beta diversity at T0 h and T10 h, for weighted and unweighted UniFrac distance matrices.

### 3.2 Faecal bacterial relative abundance at the phylum and genus level

The total sequence reads obtained in this study were classified into 12 phyla. OTUs represented by an average relative abundance of more than 1% belong to Firmicutes (50–62%), Bacteroidetes (28–41%), Actinobacteria (2–6.9%), Proteobacteria (2–6.7%) and Verrucomicrobia (0–2.2%) at phylum level (Figure 2A). Treatments did not have any significant effect on the relative abundance of phylum level during the 24 h fermentation. Only Actinobacteria relative abundance showed an overall statistically significant difference between treatments and timepoints ( $p = 0.037$ ). However post-hoc analysis did not confirm this result. At genus level, *Bacteroides*, *Acidamidococcus*, *Megamonas*, *Sutterella* were the major representative taxa comprising almost 70% of the total microbiome (Figure 2B). However, different treatments did not have any significant effect at genus level throughout the fermentation. Detailed relative abundant composition at order, class, family and species level is presented in Table S3.

### 3.3 Quantification of (poly)phenolic compounds of *F. vesca* and ileal fluids prior to faecal fermentation.

The (poly)phenolic composition of the ingested 225 g of raw wild strawberries is reported in Table 1. A total of 55 (poly)phenols were analysed in the ileal fluids collected 0–8 h after wild strawberry consumption. Forty-five (poly)phenols were identified and quantified (Table S1) at baseline. The most abundant (poly)phenols present, were phenylpropanoids ( $1.96 \pm 1.00 \mu\text{mol}$ ), followed by benzoic acid derivatives ( $0.45 \pm 0.43 \mu\text{mol}$ ), flavonoids ( $0.29 \pm 0.21 \mu\text{mol}$ ) and ellagitannins ( $0.20 \pm 0.16 \mu\text{mol}$ ), whereas a smaller amount was represented by anthocyanins ( $0.01 \pm 0.01 \mu\text{mol}$ ). In samples taken 8 hours post-consumption of the wild strawberries, fifty-one (poly)phenols were quantified and an increase in the proportion of ellagitannins was observed, reaching a total amount of  $52.62 \pm 21.28 \mu\text{mol}$  and increasing from 20% to almost 90%, becoming the most abundant component at this point. Five ellagitannins increased significantly ( $p < 0.05$ ), ellagic acid, methyl ellagic rhamnoside, agrimoniin and casuarictin and sanguin H6, among which ellagic acid was the most abundant ( $34.16 \pm 19.81 \mu\text{mol}$ ).

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### 3.4 Characterization of the ileal fluids before and after faecal fermentation

The changes in the amounts of key ellagitannins, flavonoids and anthocyanins after small intestine digestion (+ wild strawberry 8 h) and after the faecal fermentation (+ wild strawberry 24 h) of ileal fluids collected 0-8 h after wild strawberry consumption are presented in Table 2. At T8 h, ellagic acid ( $34.16 \pm 19.81 \mu\text{mol}$ ) and methyl ellagic acid rhamnoside ( $16.89 \pm 7.72 \mu\text{mol}$ ), accounting for the majority of phenolic compounds, followed by catechin ( $1.67 \pm 1.22 \mu\text{mol}$ ) and kaempferol ( $0.54 \pm 0.60 \mu\text{mol}$ ) which were the most representative flavonoids. The amount of total anthocyanin was very low, with the concentration of single compounds being  $<0.10 \mu\text{mol}$ . In context of the original quantity of wild strawberry (poly)phenols consumed, less than 1 % of the ingested dose of all (poly)phenols was recovered at the end of the *in vitro* fermentation, with the exception of ellagic acid where the recovery was 2.27 % (Table 2). During faecal fermentation, the quantities of all compounds detected in the wild strawberry ileal fluids progressively decreased over the four timepoints with the exception of ellagic acid which, in ileal fluids S2 and S5, showed the lowest amounts at T5 h (TableS2). In contrast to the considerable decrease in the amounts of most (poly)phenols, total (poly)phenol catabolites increased over the 24 h fermentation (Table 3). Notably, the molar concentration of catabolites in the ileal fluids collected 0-8 h after wild strawberry consumption ranged from 0.00-1.13  $\mu\text{M}$  (mean  $2.94 \pm 0.59 \mu\text{M}$ ) while the 24 h post-fermented samples contained 0.20-72.22  $\mu\text{M}$  (mean  $123.82 \pm 15.74 \mu\text{M}$ ). Moreover, comparing the concentration of the wild strawberry ileal fluids and pooled ileal fluids collected at the baseline we also observed an increase between the start of fermentation (T0 h) and the end of the fermentation (T24 h) (Figure 3). However, this increment was significantly higher ( $p < 0.05$ ) only in the wild strawberry ileal fluids, becoming 2.61 times higher than the initial value. In the pooled ileal fluids, this increase was less and not statistically significant (Figure 3).

### 3.5 (Poly)phenolic profile changes during the *in vitro* fermentation of *in vitro* and *in vivo* digested strawberries

Global changes of native (poly)phenols over the 24 h fermentation is visualized by scatter plot PCA (Figure 4A). Different clustering of the *in vitro* digested fermentates and ileal fluids fermentates was observed over time. Wild strawberry tended to overlap more with the ileal fluid clusters than strawberry (*Fragaria × ananassa*), and this is particularly evident at T10 h ( $R = 0.241$ ,  $p = 0.007$ ). At level of single (poly)phenols, amongst the ellagitannins, methyl ellagic acid rhamnoside decreased significantly over the 24 h fermentation, whereas the concentration of ellagic acid slightly decreased with respect to initial concentrations.

Catechin and kaempferol, which accounted for the majority of flavonoids along with taxifolin, were almost absent at the end of the fermentation. Finally, all anthocyanins detected at the start of the fermentation were observed after 24 h fermentation, except cyanidin-3-galactoside + cyanidin-3-glucoside (Table S2) but in greatly reduced amounts. Differences between *in vitro* and *in vivo* digested fermentates were observed for pelargonidin 3 glucoside, peonidin 3 galactoside, cinnamic acid, salicylic acid and ellagic acid (Figure 5A and 5B).

### 3.6 Biotransformation of (poly)phenols during the *in vitro* fermentation of *in vitro* and *in vivo* digested strawberries

The overall changes of phenolic metabolites over the 24 h fermentation is shown in the scatter plot PCA (Figure 4B). Ileal fluid fermentates tended to cluster together over time, whereas the *in vitro* digested fermentates showed a different behaviour and diverged over time. Differently, the wild strawberry treatment followed the same trend of ileal fluid samples, reaching the maximum orbital overlap at time 24 h ( $R = 0.1235$ ,  $p = 0.042$ ). Looking at the single compounds after 24 hours of fermentation, a substantial and general increase was observed for 3-hydroxyphenyl propanoic acid, 3-hydroxyphenyl propanoic acid, benzoic acid, and catechol whereas a decrease in concentrations was found for 3,5-diOH-benzoic acid, caffeic acid, ferulic acid and sinapic acid (Table S3).

Differences between *in vitro* and *in vivo* digested substrates were observed for m-coumaric acid, caffeic acid, isoferulic acid, 2,4-diOH-benzoic acid and 3,5-diOH-benzoic acid (Figure 5B).

### 3.7 SCFA production

Concentrations of SCFAs in batch culture fermentation (24 hours) varied by substrate (Table 4). With the exception of hexanoic acid and valeric acid concentrations of all measured SCFAs ( $p < 0.05$ ) increased upon fermentation of all substrates. Acetic, butyric and propionic acid ( $p < 0.05$ ), were produced in large amounts by all the substrates except cellulose. Only ileal fluid fermentates exhibited significantly increased concentrations of Valeric acid ( $p < 0.05$ ). Significant changes in acetic acid, between the treatments at the same time point (0, 5, 10 or 24 h), were observed at T5 h between cellulose and Strawberry ( $p < 0.05$ ), cellulose and Wild strawberry ( $p < 0.05$ ), between Inulin and baseline(POOLED) ( $p < 0.05$ ) and Inulin and Strawberry ( $p < 0.05$ ) and at T24 h between cellulose and strawberry ( $p < 0.05$ ) and cellulose and baseline(POOLED) ( $p < 0.05$ ) (data not shown).

### 3.8 Correlations between native (poly)phenol compounds, catabolites and faecal microbiota

The gut microbiota is known to have a pivotal role in the transformation of plant (poly)phenols and production of catabolites. We further investigated this relationship, correlating the relative abundance of 26 faecal bacteria taxa (genus > 1%) with the profile of compounds present in the ileal berry fermentates. Native (poly)phenols, phenolic catabolites and SCFA were correlated with 16S rRNA profiles of faecal bacteria at the genus level, using Spearman correlation analysis (Figure 6) and heatmaps to visualize the data. Looking at the native (poly)phenol correlation of the ileal fluids fermentates (Figure 6A), only *Coprococcus* was significantly negatively correlated with methyl ellagic acid rhamnoside. Phenolic catabolites of ileal fluid fermentates showed several significant correlations with different bacterial taxa (Figure 6B). Negative correlations were detected for *Dorea* and hydroferulic acid, *Lachnospira* 3–4hydroxyphenyl propionic acid, and *Desulfovibrio* and hydroferulic acid. In contrast, *Bilophila* was positively correlated with p-coumaric acid. Contrary to the ileal fluid fermentates, *in vitro* digested fermentates did not show any significant correlation with relative abundance of microbiota at genus level (Figure 6C and 6D). Finally, in the case of SCFAs, *Dialister* correlated positively with valeric acid (Figure 6E).

### 3.9 Bioactivity of *in vitro* and *in vivo* digested fermentates on HT 29 and CCD 841 CoN cells.

The effects of ileal fluid & *in vitro* digested fermentates were determined on HT 29 and CCD 841 CoN cells using the MTT assay. A concentration of 20 % v/v was established to be a sub-cytotoxic dose (data not shown) and was used subsequently in the COMET assay. The fermented wild strawberry ileal fluids, in both the cell lines, exerted a stronger anti-genotoxic activity than the *in vitro* digested fermentates (Figure 7). In HT 29 cells ileal fluid fermentates reduced by ~40 %, the % tail of DNA (Figure 7A). *In vitro* digested fermentates also demonstrated a significant decrease in DNA damage both for strawberries ( $p < 0.001$ ) and wild strawberries ( $p < 0.01$ ) compared to the untreated control (Figure 7B). Similar effects were observed in the CCD 841 CoN cells where there was a significant decrease in the DNA damage in the ileal fluid fermentates compared to the controls (Figure 7C). However, the effects were not as prominent as the HT 29 cells. A significant reduction of DNA damage was also observed for the *in vitro* digested fermentates of wild strawberry and strawberry on CCD 841 CoN cells (Figure 7D).

#### 4. DISCUSSION

In this study, we characterized the bioavailability and bioactivity of wild strawberry (*Fragaria vesca*) by comparing *in vivo* and *in vitro* digestion in the context of the gut microbiome and protection from intestinal DNA damage. To date, the majority of the studies investigating the bioactive potential of plant components on human health have focused on native plant compounds and their direct effects on the model of interest, leading to an overestimation of the bioactivity, without considering the metabolism of the enteric tissue and the gut microbiota. In our study, the contribution of ileostomates was vital since we were able to obtain an *in vivo* digestion of wild strawberries through the ileal fluids. We firstly examined the variation of the bacterial community composition and its metabolic output. Formation and disappearance of phenolic compounds and production of SCFA upon fermentation were then monitored over a 24 h incubation period. We further investigated the potential of the fermentation to protect against DNA damage, a key process thought to underpin the anticarcinogenic effects of fruit and vegetables, in two model cell lines.

Strawberry is one of the major sources of ellagitannins. In the present study, the recovery of ingested ellagitannins, in particular methyl ellagic acid rhamnoside ( $16.89 \pm 7.72 \mu\text{mol} \sim 14.6\%$ ) in *F. vesca* ileal fluids, was consistent with the recovery rates reported for other ileostomy bioavailability studies on berries (8.2-30.5 %)(26,27). Ellagitannins are hydrolyzed to ellagic acid under physiological conditions and ellagic acid is then gradually metabolized by the intestinal microbiota to produce different types of urolithins. A previous study investigating the recovery of ellagic acid in ileal fluid after consumption of raspberry in ileostomy patients found an increase of more than 2-fold. In our study with *F. vesca* we did not observe this phenomenon. According to Daniel et al., 87.8 % of the ellagic acid of raspberry is present in the seeds, whereas in strawberries, 95.7 % of the ellagic acid is in the pulp (42). For this reason, it is likely that the ellagic acid in strawberries is more bioavailable and easily absorbed during the first stages of digestion. Contrary to ellagitannins, anthocyanins were almost absent after 8 hours ingestion. Similar results were also observed by Mueller et al. who studied the bioavailability of anthocyanins from bilberries (43). After 8 h, no excretion of anthocyanins was found in 8 out 10 ileostomy subjects. Generally, the recovery of anthocyanins in ileal fluid after consumption of berries over 8 h corresponded to only one fifth of the anthocyanins. However, the majority of the excretion occurs between 0-4 h post-intake, therefore at T8 h almost all content of anthocyanins has reached the large intestine (43).

After 24 h faecal fermentation of these samples, a marked catabolism of the wild strawberry (poly)phenols was observed with a total mean recovery of only 0.01 % of anthocyanins, 0.99 % of flavonoids and 2.29 % of ellagitannins compared with the amounts in the 225 g of ingested wild strawberry. Our findings revealed that the remaining wild strawberry (poly)phenols in ileal fluids were strongly catabolized by faecal microbiota, and thereby contributing to the significant increase in the total concentration of microbial phenolic compounds from  $2.94 \pm 0.59 \mu\text{M}$  to  $123.82 \pm 15.74 \mu\text{M}$ .

The *in vitro* fermentation revealed several differences between *in vitro* and *in vivo* digestion approaches which became particularly evident when comparing the concentration of (poly)phenols derived from strawberry. Derivates of pelargonidin-3-O-glucoside, generally high in strawberry, were present at very low concentration after *in vivo* digestion, becoming irrelevant at the end of the fermentation process. On the contrary however, these compounds persisted after *in vitro* digestion becoming barely detectable only after microbial fermentation. The same observation may be applied for taxifolin, a flavonoid characteristic of *F. vesca* (13). This might be related to the fact that flavonoids are transformed first by the mammalian  $\beta$ -glucosidases in the small intestine and then transported in the bloodstream (44). However, the levels of aglycons in the bloodstream are usually low, due to the activity of phase I and II metabolism which may confound the kinetics of their metabolism (45).

(Poly)phenols that escape the enteric digestive phase are metabolized by the gut microbiota. In our study, we found degradation of precursor (poly)phenols, which started as early as 5 h of fermentation and was complete throughout the 24 h for most of the (poly)phenols. In general, we observed a marked inter-individual variability in human gut microbiota metabolism which might explain the lack of statistical significance of relative abundances at all taxonomic levels and in particular diversity measurements. Each individual's microbiota seemed to produce a distinct catabolite profile as previously reported in the catabolism of ellagitannins (27,46). However, beta diversity analyses revealed treatment-associated changes in gut microbiota composition ( $p < 0.01$ ), despite the variability between donors.

Microbial ellagitannin derivatives urolithin A and B were produced in higher concentrations by wild strawberry ileal fluids. Urolithin B was recovered in a lower concentration compared to Urolithin A (0.00-0.02  $\mu\text{mol}$  vs 0.26-1.41  $\mu\text{mol}$ ). This observation may depend on the urolithin metabotype of the single donor which will be closely related to gut microbiota composition (21). García-Mantrana et al. reported *Coriobacteriaceae* family being representative of urolithin metabotype-B, whereas members of the *Lachnospiraceae* family predominated in urolithin metabotype-A. The increase in Urolithin A at the end of the

fermentation was typical of all the wild strawberry substrates which in some cases significantly differ from strawberry samples. Urolithin A has been extensively studied for its antioxidant, anti-inflammatory and anti-cancer activities(47). It is involved in the induction of detoxification enzymes and in the phase I and II enzymes expression and with a reduced risk of developing colon cancer *in vitro*(48) and in a rat colitis model(49). Recently, it has been demonstrated that Urolithin A ameliorates colitis in pre-clinical models by upregulating epithelial tight junction protein through activation of aryl hydrocarbon receptor (AhR)- nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent pathways(20).

The gut microbiota is known to have a pivotal role in the transformation of plant (poly)phenols and production of catabolites. We correlated the composition of gut microbiota with the profiles of *in planta* (poly)phenols and microbial catabolites to explore this relationship considering *in vitro* digested and wild strawberry ileal fluid fermentates. Significant correlations were found only in the second. Particularly, negative statistical correlations were found with bacteria taxa associated with pathogenic conditions, as in the case of Lachnospiraceae family. The evidence from different studies shows that diverse chronic diseases including metabolic syndrome, obesity, diabetes, liver diseases and IBD present with aberrant relative abundance of Lachnospiraceae family compared to non-disease control groups(50). The present study found a negative correlation between Methyl ellagic acid rhamnoside and hydroferulic acid with *Coprococcus* and *Dorea* and 3-4 hydroxyphenylpropionic acid and *Lachnospira*. *Dorea* is a mucin degrading bacterium and some studies have shown that certain species of *Dorea* might promote the inflammation inducing interferon gamma (IFN $\gamma$ ) production, metabolizing sialic acids and degrading mucin (51,52). Another negative correlation was observed between the sulfate reducing bacterium, *Desulfovibrio*, and hydroferulic acid. *Desulfovibrio* has been generally associated to negative effects on human health. In fact, it has been proven to be increased in type II diabetes mellitus (T2DM) patients (53), and it has generally high levels in patients with inflammatory bowel diseases such as ulcerative colitis (54). Other studies have observed lowered levels of *Desulfovibrio* in overweight and obese children, suggesting an age-dependent effects(55). Finally, we also found a positive correlation with *Dialister* genus and benzoic acid. *Dialister* abundance has been previously associated with impaired glucose metabolism(56). However, higher abundance of *Dialister* has also been reported to have beneficial effects on host physiology by reducing plasma interleukin-6 (IL-6)<sup>[ 52]</sup>. Depletion of *Dialister* and *Coprococcus* were found in people suffering from depression (58). *Dialister* has been shown to be capable of producing acetate, lactate and propionate but not butyrate(59). In the present study *Dialister* was positively correlated with Valeric acid.

Our results have shown that fermented ileal fluids exhibited significant anti-genotoxic and anti-cytotoxic effects in human cell culture models of colorectal cancer, anti-inflammatory activity is also a relevant mechanism to study but were beyond the scope of this study. The effects of several berries on human colon cancer-derived cell lines have been previously studied. Focusing on *in vitro* studies, digestion and fermentation of raspberries, strawberries and blackcurrants retained their anti-genotoxic, anti-mutagenic and anti-invasive activities on colon cells compared to their native compounds/plant extracts, but the catabolites resulting from the fermentation showed lower growth inhibition activity on the human colon cancer cells (19). Similar results were found by Correa-Betanzo et al. using wild blueberry(60). In the present study, fermented ileal fluids induced a higher protection to DNA damage compared to *in vitro* digested samples, as previously found by Brown et al. using ileal fluids from lingonberry digestion. Our observations confirm the persistence of anticancer activity found in berry extracts post-intestinal digestion and contradicts the hypothesis that gut microbiota may suppress the biological activity of dietary phytochemicals (19). Our measurements suggest phenolic compounds as the best candidate to exert the protective effects as also previously stated by Ferguson et al. (61). In fact, although SCFA significantly increased throughout the fermentation process, the positive control inulin showed a greater production of acetic, propionic and butyric acids but had reduced anti-DNA damage activity compared to the strawberry fermentates.

In conclusion, we have shown that the (poly)phenolic composition of both wild strawberry and strawberry undergoes large but similar intestinal digestion and microbial transformation, with some differences between *in vivo* (ileostomate) and *in vitro* digested materials. However, the *in vivo* digestion resulted in greater protection against DNA damage with respect to *in vitro*, contradicting the hypothesis that gut microbiota may suppress the biological activity of polyphenolic metabolites. This reinforces the need for studies to consider the impact of digestion when investigating bioactivity of dietary phytochemicals. Moreover, the observation that the fruit fermentates, rich in phenolic acids and SCFA had a greater anti-DNA damage activity compared to the inulin fiber fermentates with higher concentrations of SCFA supports the whole plant food approach to gut health. It also highlights the need for further studies examining the direct effects of polyphenols within the gut, both on the gut microbiota and their metabolic output and on the gastrointestinal mucosa.

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**Author Contributions**

C.I.R.G and K.M.T were involved in study design. G.O'C, K.P. and R.L. in study conduct. C.D., L.C., M.G., A.A., C.L, M.B and F.F carried out the experiments. C.D. and M.F. were involved in data analysis and writing the manuscript and. C.I.R.G, N.G.T. and K.M.T critically revised the manuscript. All authors approved the submitted version.

**Conflict of Interest**

The authors declare no conflict of interest.

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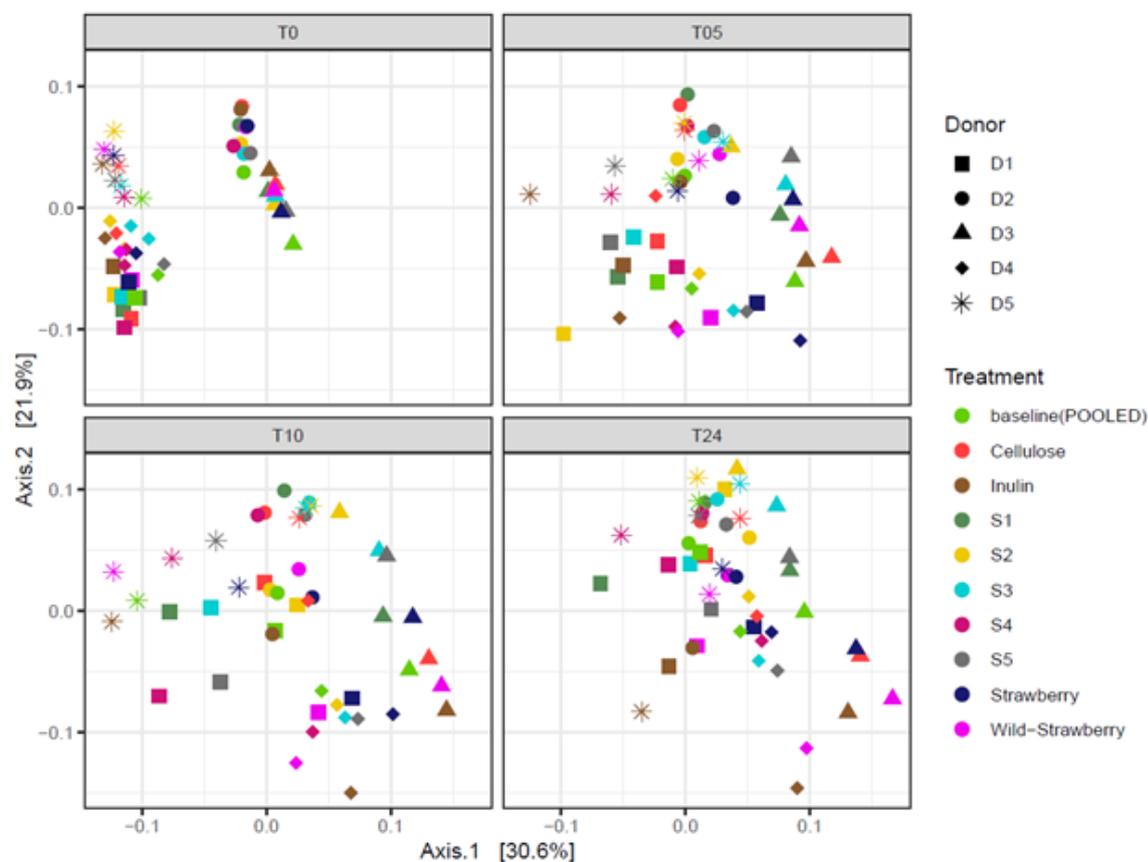
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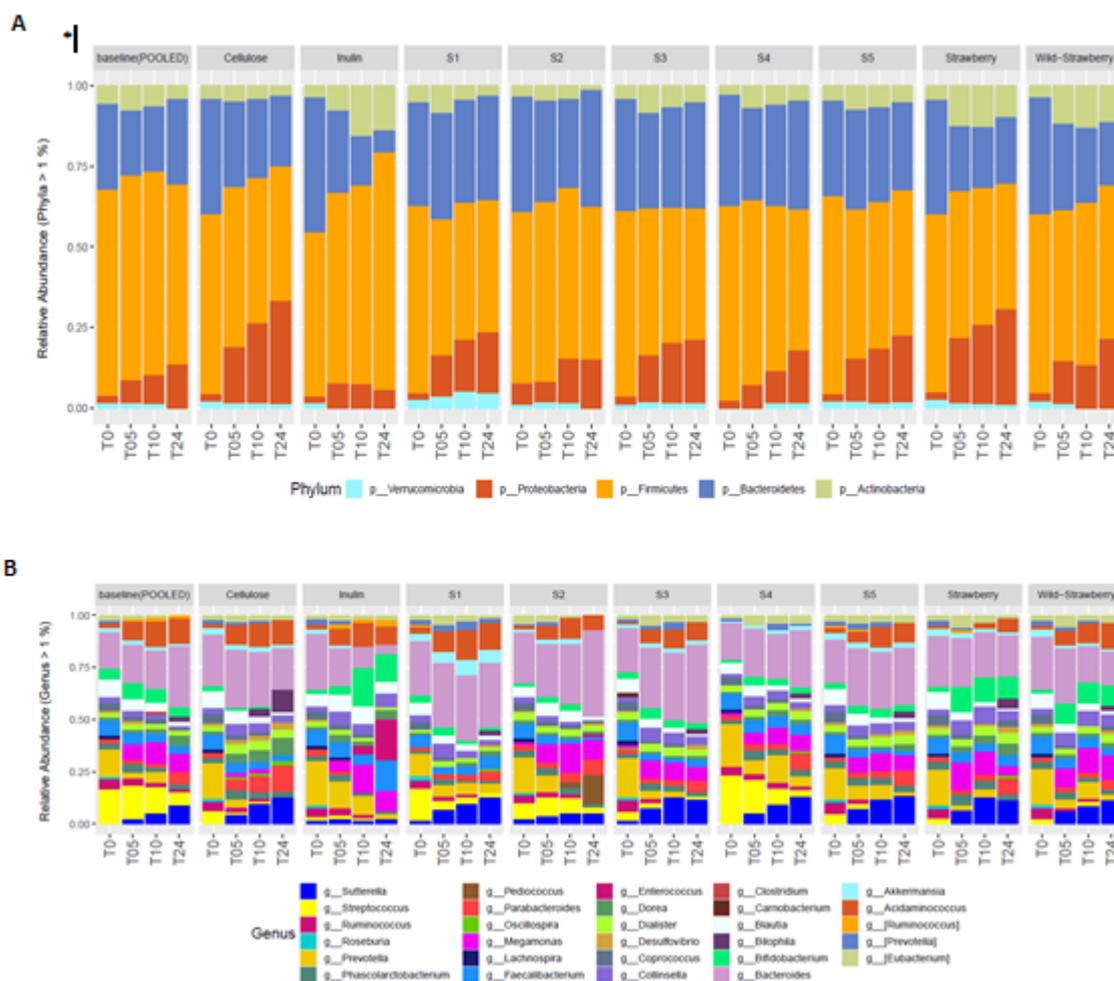
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**Figure 1:** Beta-diversity of the gut microbiome using weighted unifrac distances.

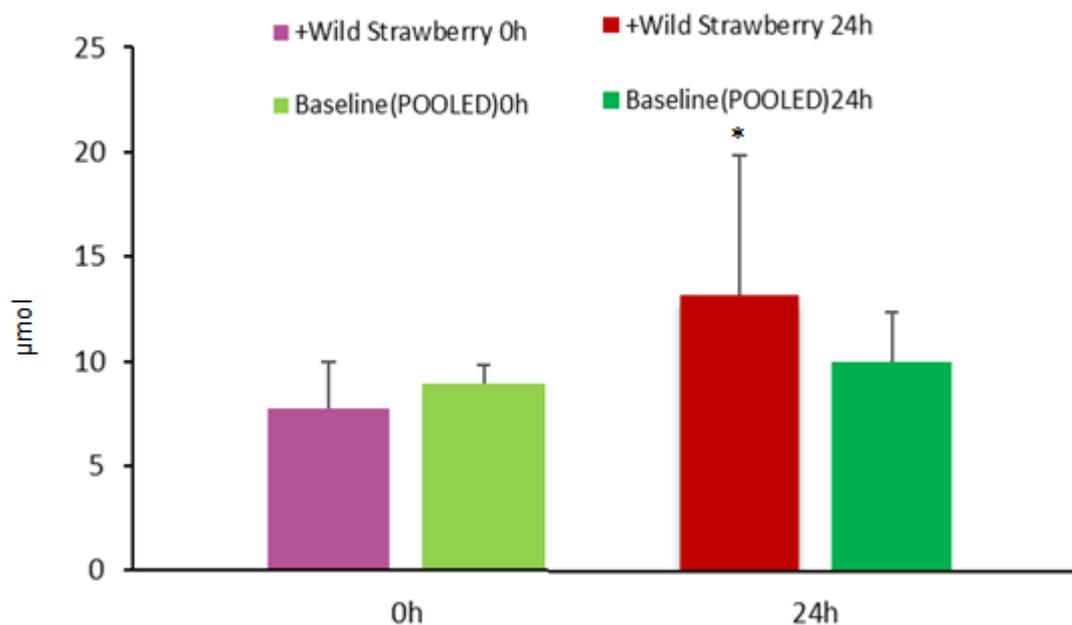
*In vitro* batch-culture fermentations with wild strawberry ileal fluids (S1, S2, S3, S4, S5), baseline(POOLED), *in vitro*-digested strawberry, wild strawberry, inulin and cellulose as treatments were inoculated with human feces (pooled, from n=5 healthy donors). Samples were analysed at 0, 5, 10 and 24 h. Each colour represents a different treatments and different shape represents different timepoint. Display of Principal coordinate analysis (PCoA) plots of the samples in two-dimension (PC1=32.7%, PC2=18.1%).



**Figure 2:** Changes in relative abundance (%) of OTUs at A) phylum B) genus level throughout 24 h *in vitro* batch culture fermentations inoculated with human faeces. Data represent mean values of 5 donors. Only OTUs represented by an average relative abundance of more than 1% of sequences in any sample are shown.



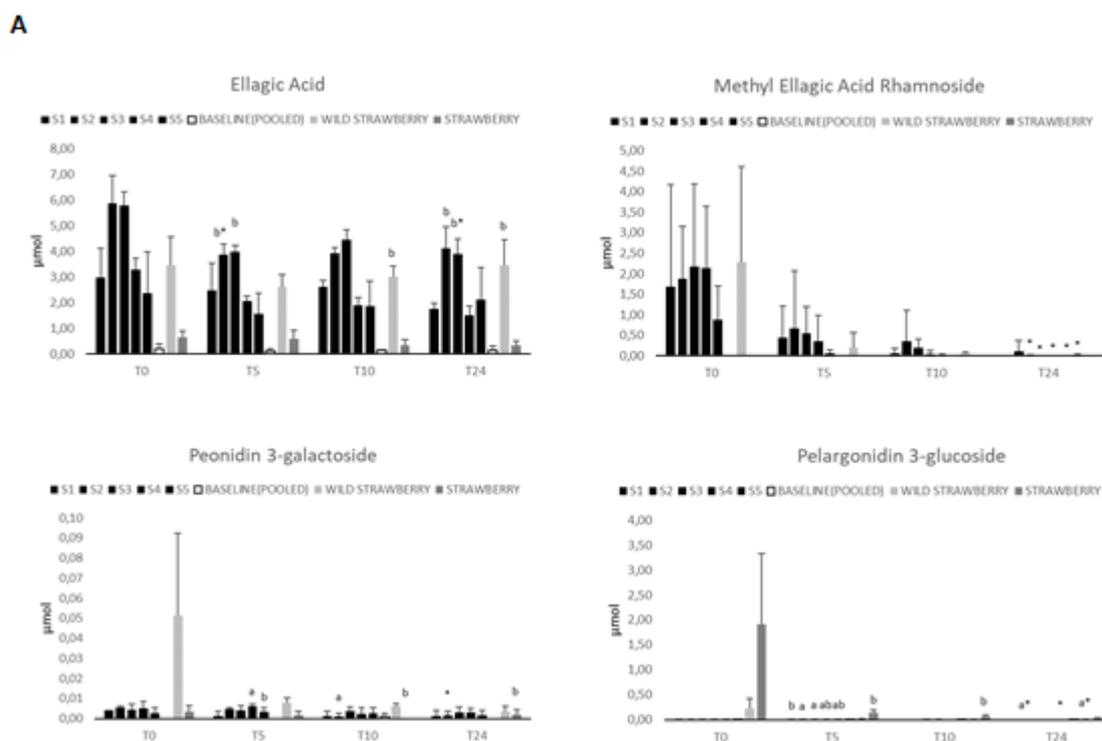
**Figure 3.** Total amount of phenolic acid catabolites presents in wild strawberry ileal fluids of all subjects (n=5) and the baseline pooled ileal fluids (n=5) analysed before (0 h) and after (24 h) fecal fermentation. Data expressed as  $\mu\text{mol} \pm \text{SD}$ . Significant differences from the 0 h time point are indicated with an asterisk (\*p < 0.05).



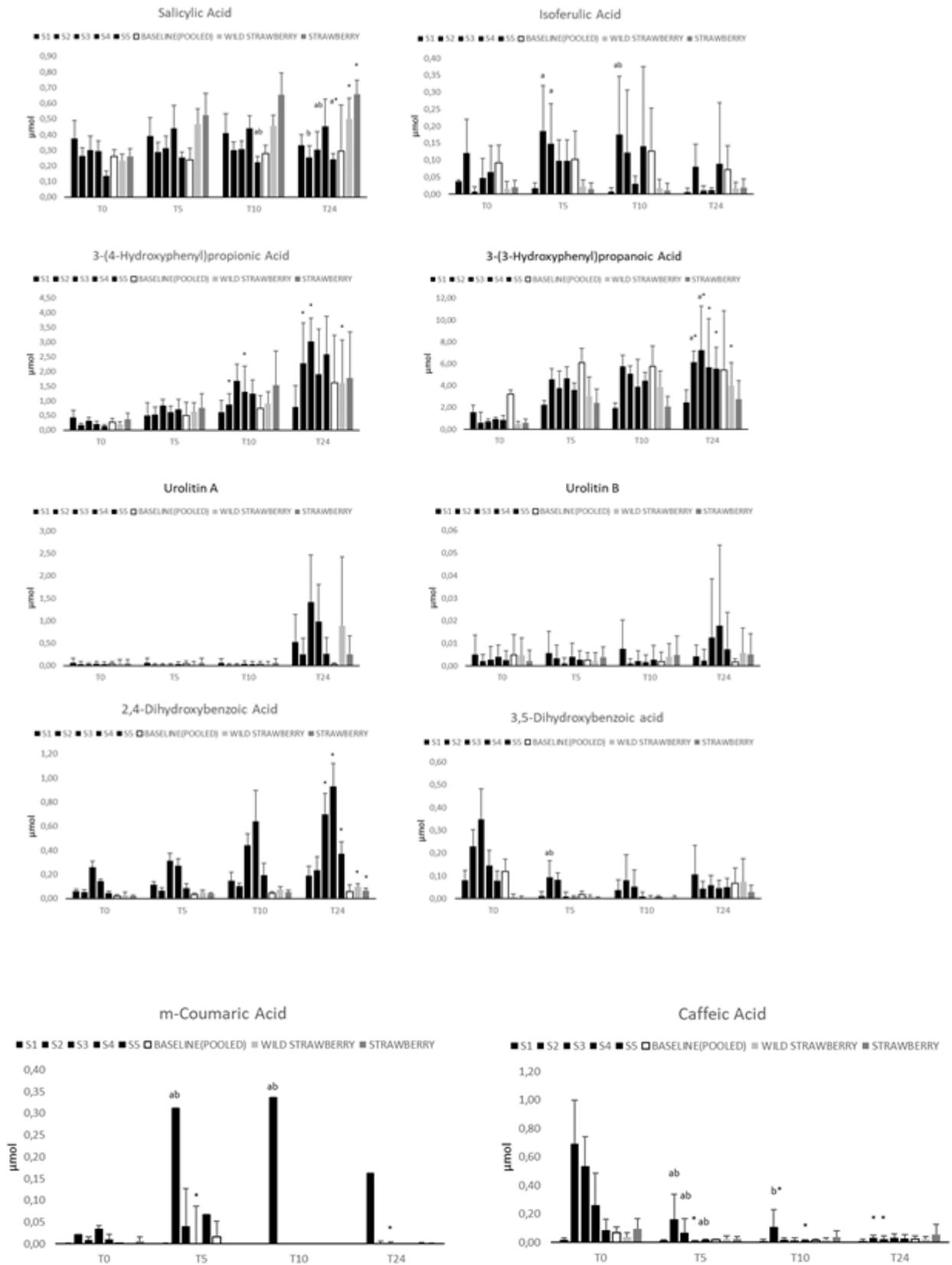
**Fig.4:** PCA scatterplots of A) parent polyphenol and B) phenolic catabolites throughout the 24 h fecal fermentation. Each treatment is identified by different colors and every sample is represented by a dot. The ANOSIM statistic was applied to test for differences between treatments at the same timepoint, with corresponding R and p-values shown.



**Figure 5:** Changes in A) parent polyphenols and B) catabolites throughout *in vitro* strawberry fermentations. 24-h *in vitro* batch-culture fermentations containing wild strawberry ileal fluids (S1, S2, S3, S4, S5), baseline(POOLED), *in vitro*-digested strawberry and wild strawberry, inulin, or cellulose as treatments, and inoculated with human feces. Results are expressed as  $\mu\text{mol} \pm \text{SD}$  the five fermentations. Samples were collected at 0, 5, 10 and 24 h. Significant differences between treatments at the same time point are indicated with different letters (one letter,  $p < 0.05$ ; two letters,  $p < 0.01$ ). Significant differences from the 0 h time point within the same treatment are indicated with an asterisk (\* $p < 0.05$ , \*\* $p < 0.01$ ).

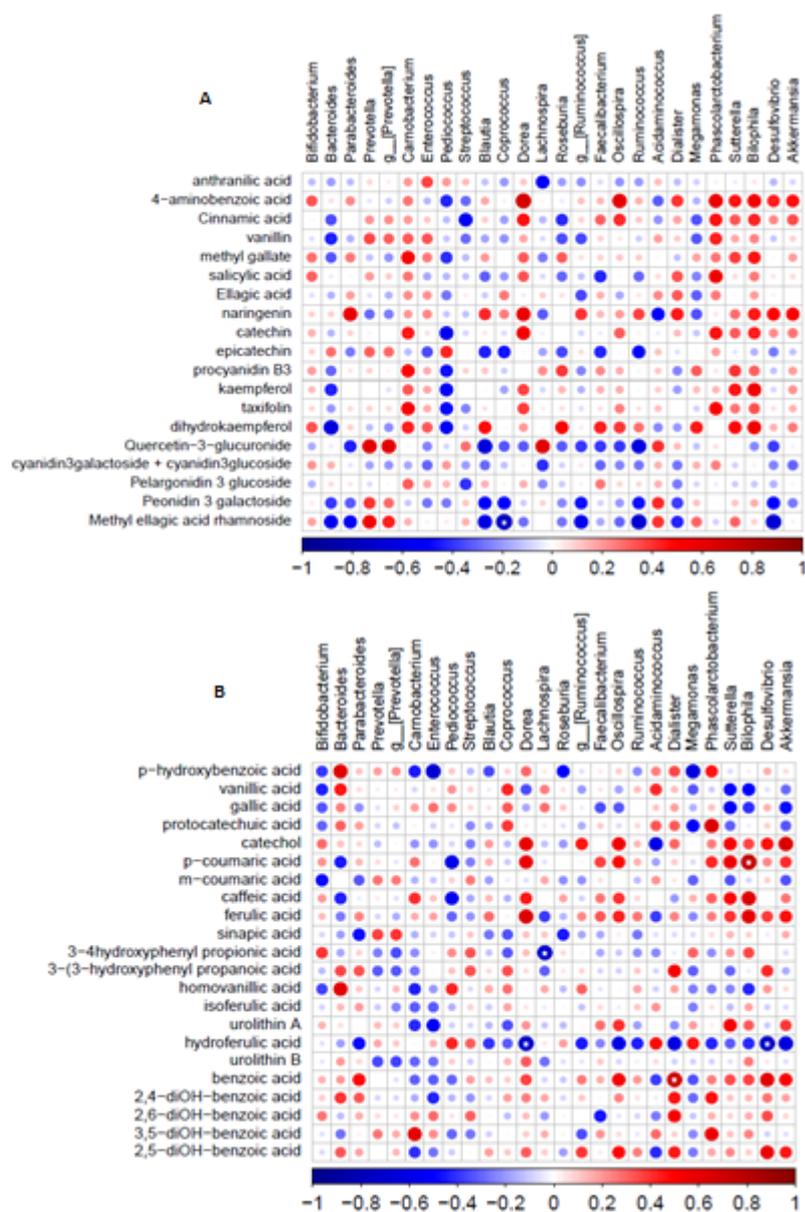


B



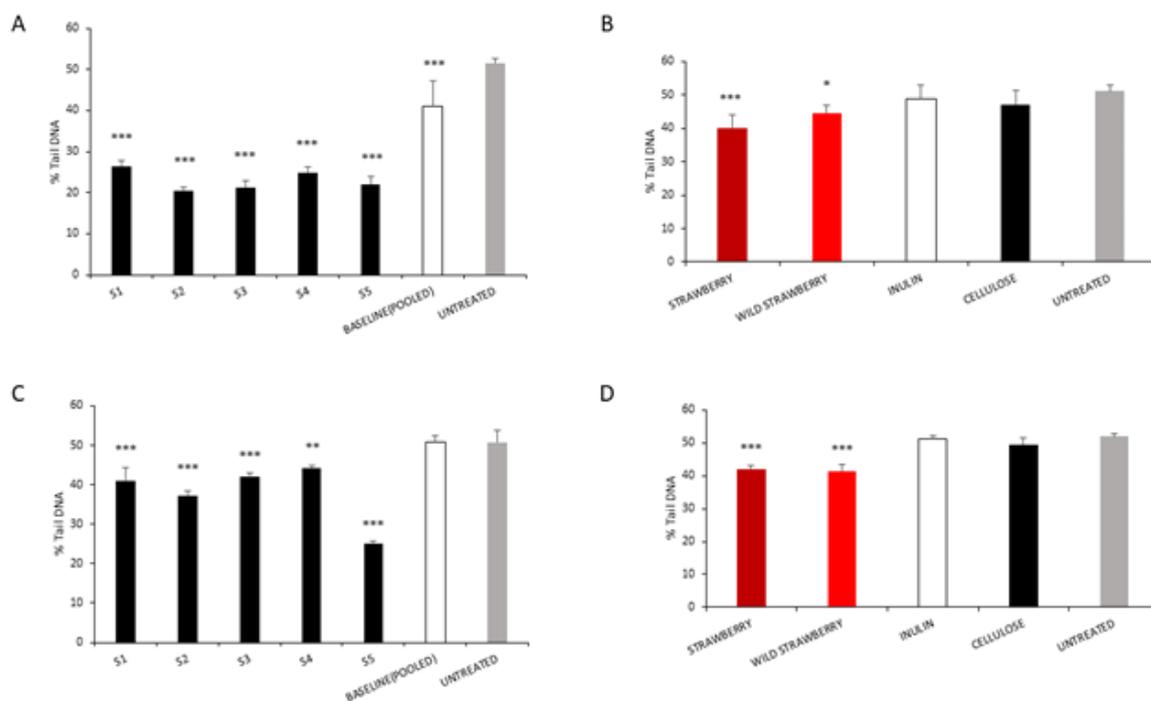
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**Figure 6:** Heatmap of Spearman's correlation between genus level 16S rRNA relative abundance greater than 1 % of faecal microbiota present in each subject and A) native polyphenols of wild strawberry ileal fluids B) phenolic catabolites of wild strawberry ileal fluids C) native polyphenols of samples digested *in vitro* (Strawberry and Wild Strawberry) D) phenolic catabolites of samples digested *in vitro* (Strawberry and Wild Strawberry) E) SCFAs. Correlation was performed on the values of area under the curve (AUC) of the datasets. Dark red indicates positive correlation, while dark blue represents negative correlation. Statistical significance: \*  $p < 0.05$ .





**Figure 7:** Anti-genotoxic effects of the fermented ileal fluids and *in vitro* digested fermentates. A) HT29 cells pre-treated for 24h with ileal fluid fermentates (S1, S2, S3, S4, S5) or a baseline pooled of ileal fluid fermentates (baseline(POOLED)). B) HT29 cells pre-treated for 24h with *in vitro* digested fermentates of strawberries, wild strawberry, inulin and cellulose. After 24h of incubation at 37°C HT29 cells were treated with 75µM H<sub>2</sub>O<sub>2</sub>. The same treatment was used for CCD 841 cells treated with C) ileal fluid fermentates and with D) *in vitro* digested fermentates. Data expressed as mean of 3 independent experiments ± SD. Statistical significance: \* p < 0.05, \*\* p < 0.01, p < 0.001\*\*\*.



**Table 1:** Identification and quantification of phenolic compounds detected in Wild Strawberry.

Compounds	Concentration μmole/225g	RT (min)	ESI mode	cone voltage (V)	Q1 m/z	collision energy (V)	Q2 m/z	Collision energy (V)	Q2 m/z
Cy 3 arabinoside	0.167±0.02	3.09	+	24	419.3	287.2	24	137.2	56
Pg 3 glucoside	130.3±20.69	3.45	+	24	479.3				
Cy 3 glucoside + cy 3 galactoside	54.46±10.39	2.54	+	26	449.3	287.2	22	137.2	66
Pn 3 galactoside	15.91±3.956	3.63	+	24	463.3	301	24	286	42
Cy 3 sambioside	0.05±0.011	2.81	+	30	581.3	287.2	26	137.2	72
<b>TOTAL ANTHOCYANINS</b>	<b>200.887±23.48</b>								
ellagic acid	118.3±35.44	4.38	-	52	301	34	145	30	185
Methyl ellagic acid rhamnoside	115.7±42.56	6.97	-	30	460.8	18	314.8	20	299.8
Agrimoniin	46.04±6.786	5.06	-	32	934.1	22	632.8	46	300.8
Casuarictin + sanguin H6	21.38±3.453	3.27	-	66	934.7	26	632.7	46	300.8
<b>TOTAL ELLAGITANNINS</b>	<b>301.42±55.90</b>								
luteolin	0.317±0.099	7.37	+	52	287	32	153	30	135
catechin	40.53±19.23	2.8	-	32	289	20	203	32	123
epicatechin	1.943±1.287	3.32	-	34	289	20	203	30	123
Procyanidin B1	6.848±3.036	2.4	-	32	577	26	289	16	425
procyanidin B2 + B4	0.962±0.792	3.01	-	30	577	24	289	16	425
procyanidin B3	6.372±2.944	2.72	-	34	577	22	289		
kaempferol	0.132±0.07	8.45	+	50	287	32	153	26	165
Kaempferol-3-	0.15±0.087	5.45	+	18	463	16	287	44	165

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glucuronide									
isorhamnetin-3-Glc	0.686±0.4	5.69	+	16	479	14	317	30	302
dihydrokaempferol	0.155±0.078	6.07	+	18	289	18	153	16	243
quercetin-3-Glc acetyl	0.35±0.108								
<b>TOTAL FLAVONOIDS</b>	<b>58.445±19.75</b>								
Cinnamic acid	0.417±0.089	7.55	+	16	149	12	131	10	103
p-coumaric acid	0.547±0.48	4.04	+	16	165	24	91	26	119
t-ferulic acid	0.18±0.017	4.52	+	12	195	16	145	24	117
<b>TOTAL PHENYLPROPANOIDS</b>	<b>1.144±0.48</b>								
anthranilic acid	0.09±0.05	3.92	+	14	138	26	65	34	92
vanillin	0.286±0.094	3.23	+	18	169	14	93	18	110
p-hydroxybenzoic acid	1.209±0.376	2.84	+	20	139	18	77	26	65
<b>TOTAL BENZOIC ACIDS DERIVATIVES</b>	<b>1.585±0.39</b>								

Data are expressed as mean values (n = 3)

**Table 2:** Quantities of flavonoids, ellagitannins, anthocyanins, ellagic acid recovered in A) ileal fluids collected 0-8 h after wild strawberry consumption and in B) wild strawberry ileal fluid after 24 h faecal fermentation.

Compounds	A-Recovery after small intestine		B-Recovery after faecal fermentation	
	$\mu\text{mol} \pm \text{SD}$	% Intake	$\mu\text{mol} \pm \text{SD}$	% Intake
Epicatechin	0.18±0.15	9.5	0.00±0.00	0.49
Catechin	1.67±1.22	4.11	0.00±0.00	0.01
Procyanidin B3	0.39±0.33	6.1	0.00±0.02	0.02
Kaempferol	0.54±0.6	45.46	0.00±0.00	0.36
Dihydrokaempferol	0.03±0.04	17.95	0.00±0.00	0.1
<b>Total flavonoids</b>	<b>2.81±1.41</b>	<b>83.13</b>	<b>0.01±0.00</b>	<b>0.99</b>
Ellagic acid	34.16±19.81	28.87	0.23±0.09	2.27
Methyl ellagic acid rhamnoside	16.89±7.72	14.6	0.00±0.00	0.02
<b>Total ellagitannins</b>	<b>51.05±21.26</b>	<b>43.46</b>	<b>0.23±0.09</b>	<b>2.29</b>
Pelargonidin 3-glucoside	0.06±0.03	0.05	0.00±0.00	0
Cy 3 glucoside + cy 3 galactoside	0.01±0.00	0.01	0.00±0.00	0
Peonidin 3 galactoside	0.03±0.01	0.16	0.00±0.00	0.01
<b>Total anthocyanins</b>	<b>0.09±0.03</b>	<b>0.22</b>	<b>0.00±0.00</b>	<b>0.01</b>

Data are expressed as  $\mu\text{mol} \pm \text{SD}$  (n=5).

**Table 3:** Concentration of phenolics catabolites recovered in ileal fluids collected 0-8 h after wild strawberry consumption and in wild strawberry ileal fluid after a 24 h faecal fermentation.

Compounds	A-Concentration in ileal fluid		B-Concentration in ileal fluid after faecal fermentation	
	$\mu\text{M} \pm \text{SD}$	Range ( $\mu\text{M}$ )	$\mu\text{M} \pm \text{SD}$	Range ( $\mu\text{M}$ )
2,4-diOH-benzoic acid	0.1 $\pm$ 0.11	0.02-0.24	4.85 $\pm$ 3.18	1.19-9.30
2,6-diOH-benzoic acid	0.02 $\pm$ 0.01	0.00-0.02	0.96 $\pm$ 0.23	0.80-1.30
3,5-diOH-benzoic acid	0.68 $\pm$ 0.29	0.38-1.13	0.62 $\pm$ 0.26	0.40-1.10
2,5-diOH-benzoic acid	0.22 $\pm$ 0.14	0.04-0.39	5.91 $\pm$ 1.63*	3.61-8.12
catechol	0.14 $\pm$ 0.14	0.00-0.30	10.30 $\pm$ 2.67*	7.10-12.34
3-(3-hydroxyphenyl) propanoic acid	n.d.	n.d.	60.09 $\pm$ 7.33*	24.76-72.22
3,4-dihydroxyphenyl acetic acid	n.d.	n.d.	21.13 $\pm$ 8.47*	24.8-72.3
p-hydroxybenzoic acid	0.63 $\pm$ 0.28	0.17-0.72	8.24 $\pm$ 2.71*	5.64-12.71
vanillic acid	0.31 $\pm$ 0.22	0.07-0.58	2.83 $\pm$ 1.12	5.60-12.7
gallic acid	0.49 $\pm$ 0.33	0.00-0.90	11.84 $\pm$ 7.61*	6.00-24.1
protocatechuic acid	n.d.		2.39 $\pm$ 0.30	2.01-2.70
p-coumaric acid	0.35 $\pm$ 0.23	0.02-0.62	0.27 $\pm$ 0.13	0.20-0.53
<b>Total phenolics</b>	<b>2.94<math>\pm</math>0.59</b>	<b>0.00-1.13</b>	<b>123.82<math>\pm</math>15.74**</b>	<b>0.20-72.22</b>

Data are expressed as  $\mu\text{M} \pm \text{SD}$  (n = 5).

**Table 4:** SCFA concentrations before and after the 24 h *in vitro* batch culture fermentations containing wild strawberry ileal fluids (S1, S2, S3, S4, S5), baseline(POOLED), *in vitro*-digested strawberry and wild strawberry, inulin, or cellulose as treatments, and inoculated with human feces. Results are expressed as mmol/L  $\pm$  SD of the five fermentations.

	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5
	T0h	T24h	T0h	T24h	T0h	T24h	T0h	T24h	T0h	T24h
acetic acid	3.28 $\pm$ 1.04	27 $\pm$ 3.03*	2.87 $\pm$ 0.49	28.65 $\pm$ 8.44**	3.18 $\pm$ 0.7	35.1 $\pm$ 3.69**	2.87 $\pm$ 0.67	33.87 $\pm$ 4.33*	2.93 $\pm$ 0.48	35.44 $\pm$ 2.6**

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propionic acid	0.62±0.36	6.22±1.3*	0.42±0.19	9.36±3.81**	0.63±0.29	9.99±0.69*	0.56±0.3	9.75±0.97*	0.5±0.25	9.65±1.69**
isobutyric acid	0.03±0.01	0.13±0.05*	0.03±0.01	0.08±0.04**	0.03±0.01	0.12±0.03*	0.03±0.01	0.14±0.04*	0.03±0.01	0.12±0.04*
butyric acid	0.38±0.28	5.65±1.85*	0.17±0.16	4.1±2.3	0.17±0.12	6.04±1.57*	0.18±0.12	9.53±0.16*	0.29±0.17	6.32±1.84*
isovaleric acid	0.03±0.01	0.16±0.08*	0.02±0.01	0.11±0.07*	0.02±0.01	0.16±0.03*	0.02±0.01	0.14±0.02*	0.02±0.01	0.15±0.05*
2-methylbutyric acid	0.02±0.01	0.13±0.06*	0.02±0.01	0.09±0.06*	0.02±0.01	0.12±0.03*	0.02±0.01	0.14±0.04*	0.02±0.01	0.12±0.05*
2methylbutyric+isovaleric	0.05±0.01	0.28±0.13*	0.04±0.02	0.19±0.12*	0.05±0.02	0.26±0.06*	0.04±0.02	0.27±0.07*	0.04±0.02	0.25±0.1*
valeric acid	0.03±0.01	1.41±1.16*	0.04±0.02	0.23±0.27	0.04±0.02	1.09±1.36*	0.04±0.02	2.43±1.61*	0.04±0.02	1.4±1.37**
hexanoic acid	0.02±0.01	0.04±0.03	0.02±0.01	0.03±0.01	0.02±0.01	0.04±0.02	0.02±0.01	0.04±0.01	0.03±0.01	0.04±0.02

	Baseline (POOLED) T0	Baseline POOLED T24	Inulin T0h	Inulin T24h	Cellulose T0h	Cellulose T24h	WS T0	WS T24	S T0h	S T24h
acetic acid	8.56±1.14	37.95±3.26**	1.33±0.48	42.27±15.73*	1.61±0.61	19.99±2.42*	1.69±0.67	34.48±2.86**	1.82±0.62	37.09±4.54**
propionic acid	0.89±0.42	8.78±2.76**	0.32±0.16	3.74±3.36*	0.43±0.18	4.13±0.96*	0.41±0.22	7.96±3.97*	0.39±0.22	9.3±4.81*
isobutyric acid	0.04±0.01	0.1±0.04**	0.03±0.01	0.06±0.03*	0.03±0.01	0.12±0.07*	0.03±0.01	0.07±0.03**	0.03±0.01	0.07±0.02*
butyric acid	1.18±0.35	9.71±1.98*	0.21±0.17	6.92±5.33*	0.22±0.13	4.02±1.21*	0.21±0.18	7.75±2.81*	0.18±0.12	7.41±3.33*
isovaleric acid	0.03±0.01	0.12±0.05*	0.02±0.01	0.05±0.04*	0.02±0.01	0.14±0.07*	0.02±0.01	0.07±0.03*	0.02±0.01	0.08±0.04*
2-methylbutyric acid	0.02±0.01	0.1±0.03*	0.01±0.01	0.05±0.04*	0.02±0.01	0.12±0.09*	0.02±0.01	0.06±0.03*	0.02±0.01	0.06±0.02*
2methylbutyric+isovaleric	0.06±0.02	0.2±0.07*	0.03±0.02	0.09±0.05*	0.05±0.02	0.24±0.16*	0.04±0.02	0.12±0.06*	0.05±0.02	0.13±0.04*
valeric acid	0.04±0.02	1.08±0.98*	0.04±0.02	0.9±1.28*	0.05±0.02	1.16±0.74*	0.04±0.02	0.6±0.49*	0.04±0.01	0.9±0.7*
hexanoic acid	0.02±0.01	0.03±0.01	0.02±0.01	0.05±0.04	0.02±0.01	0.04±0.01	0.02±0.01	0.03±0.01	0.03±0.01	0.04±0.01

Significant differences from the 0 h time point within the same treatment are indicated with an asterisk (\* $p < 0.05$ , \*\*  $p < 0.01$ ).

### Graphical abstract

Wild strawberry (*Fragaria vesca*) contains high content and variety of polyphenols, principally ellagitannins and anthocyanins. After either *in vitro* or *in vivo* digestion, strawberry phytochemicals, were available for colonic fermentation and significantly stimulated the production of phenolic metabolites by gut microbiota. After 24h fermentation, post berry fermentates were demonstrated exert protective effects by reducing DNA damage in colonocytes.

