



# Measuring the effect of Mankai® (*Wolffia globosa*) on the gut microbiota and its metabolic output using an *in vitro* colon model

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## ABSTRACT

Mankai® is a cultivated strain of *Wolffia globosa* an aquatic plant of the family Lemnaceae commonly known as Duckweeds. Recent studies suggest that consumption of a Mankai® enriched diet may provide positive health effects by decreasing body weight and improving glucose homeostasis and plasma lipid profiles. However, the effects of Mankai® alone on the composition and metabolic output of the human gut microbiota has not been fully investigated. Here, Mankai® was digested and fermented *in vitro* using a batch culture model of the proximal colon. Inulin and cellulose were used as readily and poorly fermentable control fibers respectively. Mankai® significantly stimulated the production of phenolic metabolites and short chain fatty acids by the gut microbiota ( $p < 0.05$ ). Three major microbial metabolites, 3-4-hydroxyphenyl propionic acid, 3-3-hydroxyphenyl propanoic acid and protocatechuic acid were significantly increased after 24 h fermentation. Moreover, Mankai® treatment lowered the overall microbial diversity ( $p < 0.05$ ), in line with a selective microbiome modulation.

## 1. Introduction

Evidence suggests that plant-based dietary patterns, low in animal protein and ultra-processed foods, protect against chronic human disease reducing morbidity and all-cause mortality (Willett et al., 2019). Mankai®, a cultivated strain of *Wolffia globosa*, belongs to the Duckweed family (Lemnaceae) and is considered the smallest plant on earth (Lock, 2013). It is a single flat oval leaf, phytomorphology includes a simple, rootless thallus 0.4–0.9 mm long, without veins, with a budding pouch at its basal end. The nutritional value of *Wolffia globosa* has long been recognized in the culinary traditions of South East Asia. Indeed, in Thailand the plant is called “Khai-nam”, or “Egg of the water” in English (Appenroth et al., 2018). However, the nutritional potential of Mankai®, especially as a healthy, plant based alternative to meat, with a much reduced carbon footprint has only recently been recognized by Western science (Kaplan et al., 2019).

Proteins represent more than 45 % of Mankai® plant dry matter, and Mankai® protein contains all 9 essential amino acids (AAs) and 6 conditional AAs, giving it a similar amino acid profile to egg proteins (Kaplan et al., 2019). Recently, the DIRECT-PLUS trial (ClinicalTrials.gov identifier (NCT number): NCT03020186) evaluated whether

consumption of a Green-Mediterranean diet (GMD), low in meat diet and with elevated polyphenol content, including 100 g of frozen Mankai® shake/day could provide improved benefit to metabolic health over and above healthy eating guidelines or a standard Mediterranean style diet in 300 prediabetic patients over an 18 month period. All groups received free gym membership and physical activity guidelines. After 6 and 18 months, the Mankai® fortified GMD group reduced the risk of Type 2 Diabetes (T2D) and metabolic syndrome risk factors by improving the glycemic control (Zelicha et al., 2019), plasma lipid profile (Tsaban et al., 2020) and promoted weight loss (Rinott et al., 2020). The GMD also increased serum B12 concentrations confirming Mankai®'s potential as a source of bioavailable Vitamin B12 (VitB12) (Sela et al., 2020). Moreover, the GMD was found to be helpful in maintaining iron and folic acid status in humans and completely reversed iron deficiency anemia in an experimental rat model (Yaskolka Meir et al., 2019).

There is a growing awareness that the human gut microbiome contributes significantly to the health effects of plant rich traditional diets, such as the Mediterranean style diet, specifically by producing bioactive compounds like short chain fatty acids (SCFAs) and small phenolic acids from complex plant fiber and polyphenols respectively (Diotallevi, Fava,

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Gobbetti, & Tuohy, 2020). In a sub-study of the DIRECT-PLUS trial, Rinott et al. (2020) demonstrated that not only could the Mankai® fortified GMD modulate the gut microbiota but that autologous fecal microbiota transplantation (aFMT) from patients receiving the GMD, at the nadir of weight loss after 6 months of Mankai® ingestion, could significantly reduce weight regain and help retain improvements in metabolic health compared to those receiving aFMT from either standard Mediterranean diet or health eating guidelines groups (Rinott et al., 2020). The most prominent microbiota changes were an increase in the relative abundance of *Bacteroides massiliensis* and *Paraprevotella clara*. Similarly, Yaskolka Meir et al. (2021) found statistically significant correlations between improved hepatic lipid profiles in patients receiving the Mankai® fortified GMD and plasma concentrations of microbial metabolites of plant polyphenols.

However, although Mankai® has been ascribed several health-promoting effects, the plant components responsible for such effects have not yet been investigated. There is an extensive literature linking favorable effects of plant-based diets to their high concentrations of biologically active polyphenols and dietary fibers, the major source of energy for the human gut microbiota (Cardona, Andrés-Lacueva, Tuli-pani, Tinahones, & Queipo-Ortuño, 2013; Luca et al., 2020; Makki, Deehan, Walter, & Bäckhed, 2018). Similarly, about 90–95 % of dietary polyphenols are very poorly absorbed and together with dietary fiber reach the large intestine where they encounter the colonic microbiota (Loo, Howell, Chan, Zhang, & Ng, 2020). A two-way interaction occurs whereby the gut microbiota produces smaller phenolic catabolites and short chain fatty acids that are more easily absorbed, while both fibers and polyphenols have the potential to modulate the composition of the gut microbiota, stimulating certain taxa and inhibiting others. Biologically active small phenolic acids and the SCFAs (acetate, propionate and butyrate), are important cell signaling molecules capable of regulating host metabolic and inflammatory pathways responsible for metabolic health and controlling the risk of metabolic disease (Chambers, Preston, Frost, & Morrison, 2018; Loo et al., 2020).

Using a pH controlled anaerobic batch culture fermentation system, mimicking the environmental conditions of the proximal colon, the current work investigated the impact of Mankai® on human fecal microbiota composition and its metabolic activity. Disappearance of Mankai® polyphenols, formation of microbial-derived metabolites, changes in microbiota community structure and production of SCFAs were monitored over a 24 h incubation period and targeted metabolomic analysis of polyphenol content of the plant was performed.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Enzymes for *in vitro* digestion, inulin, cellulose and chemicals for the batch culture basal nutrient medium were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Thermo Fischer (Waltham, MA, USA), unless otherwise stated. For the chemical standards of polyphenols, microbial metabolites and SCFAs as well as the LC-MS and GC-MS reagents please refer to Vrhovsek et al. (2012), Gasperotti, Masuero, Guella, Mattivi, and Vrhovsek (2014) and Lotti et al. (2017). Mankai® plant was provided by Hinoman Ltd, (Hevel Shalom, Israel).

### 2.2. Fecal donors

Four healthy volunteers, (N = 4, aged 20–40, 2 males, 2 females), were used as fecal donors. They had not received antibiotic treatment within 3 months of stool collection, had no history of bowel disorders, and had not consumed pre or probiotic supplements prior to experiment. All four donors were informed of the study aims and procedures and provided their written consent for their fecal 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).

### 2.3. Preparation of phospholipid vesicles

A protocol was followed according to Minekus et al. (2014), with adaptations, for the preparation of the phospholipid vesicles and the simulation of the *in vitro* digestion as described below. An aliquot of L- $\alpha$ -Lecithin, Egg Yolk, Highly Purified (50 mg/mL) was placed into a round-bottom flask and dried overnight under rotary evaporation to make a thin phospholipid film. The day after, warm simulated gastric solution fluid (SGF) (pepsin (25000 U/mL),  $\text{CaCl}_2(\text{H}_2\text{O})_2$  (0.3 M), HCl (1 M),  $\text{H}_2\text{O}$ ) was added to reach the final concentration of 10 mg/mL phospholipids in SGF. The solution was kept cold on ice and sonicated until clear to the eye. Then, to remove any debris deposited by sonicator, solution was filtered through a 0.22  $\mu\text{m}$  nylon syringe filter, kept it at 4 °C and used the same day.

### 2.4. In vitro digestion

The *in vitro*-simulated gastrointestinal digestion was performed as described by Minekus et al. (2014) whereas, the concentration of bile acid extract was based on Tullberg et al. (2016). The protocol consists of three sequential steps: an initial step simulating the oral phase in the mouth, followed by digestion with pepsin/HCl to simulate conditions in the stomach, and a third step with bile salts/pancreatin to simulate conditions in the small intestine. Briefly, in the oral step, 30 g of dried Mankai® leaf was mixed with 60 mL of simulated salivary fluid (SSF) (alfa-amylase solution (1500 U/mL),  $\text{CaCl}_2(\text{H}_2\text{O})_2$  (0.3 M),  $\text{H}_2\text{O}$ ) in a glass bottle. The mixture was then incubated for 2 min at 37 °C on a rotary shaking plate (250 rpm). In the gastric digestion, 120 mL of SGF together with the phospholipid vesicles were added to the previous bottle and the pH was adjusted to 3.0 using HCl (1 M). The bottle was incubated for 2 h at 37 °C on a rotatory shaking plate (250 rpm). Following gastric digestion, intestinal digestion was performed with the addition of 240 mL of simulated intestinal phase fluid (SIF) (Pancreatin (800 U/mL), bile extract porcine (20 mg/mL),  $\text{CaCl}_2(\text{H}_2\text{O})_2$  (0.3 M), NaOH (1 M) and the pH was adjusted to 7.0 using NaOH (1 M). Then, the digesta were transferred to 1 kDa 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 to remove low molecular mass digestion products. Finally, Mankai® was lyophilised until the sample were completed dried. Inulin and cellulose were also digested and dialyzed using the same protocol. The final ratio of the *in vitro* digestion was ~ 1:1.67, obtaining 18 gr of digested Mankai®. Finally, sample was stored at -80 °C until use.

### 2.5. Fecal batch culture fermentation

Controlled pH fecal batch cultures were performed at 37 °C under anaerobic conditions. After *in vitro* digestion, Mankai®, inulin and cellulose were used as substrate. A vessel without any substrate was used as blank. Glass water-jacketed vessels (200 mL) were sterilized and filled aseptically with 180 mL of pre-sterilized basal nutrient medium, and 20 mL of fecal slurry (10 % w/v of fresh human feces) to a final concentration of 10 % (w/v). Basal nutrient medium contained per liter was prepared as follow: 2 g Peptone, 2 g Yeast extract, 2 g  $\text{NaHCO}_3$ , 2 mL Tween 80, 0.5 g Bile Salts, 0.1 g NaCl, 0.04 g  $\text{K}_2\text{HPO}_4$ , 0.04 g  $\text{KH}_2\text{PO}_4$ , 0.01 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01 g  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.005 g Hemin dissolved in 1 mL of 1 M NaOH, 10  $\mu\text{L}$  Vitamin K, 0.5 g L-Cysteine HCl, and 1 mL of Resazurin (0.1 g/100 mL). To mimic the proximal colon conditions the pH was adjusted to 5.5–6.1 and kept between this range throughout the experiment with the automatic addition of NaOH or HCl (0.5 M). Anaerobic conditions were maintained through a  $\text{O}_2$ -free  $\text{N}_2$  (15 mL/min) flow overnight. Temperature was set at 37 °C using a circulating water bath. Before the inoculation, each of the vessels was dosed with the appropriate substrate/treatment to a final concentration of 1 % (w/

v) and added with 1 mL of 10 % filtered, freshly prepared, solution of L-cysteine chlorhydrate (Applichem) as oxygen reducing agent. Fresh human fecal samples were collected in an anaerobic jar and fecal slurry was prepared by homogenizing the feces in pre-reduced phosphate buffered saline (PBS) (0.1 M, pH 7.0) within 1 h. Each fermentation was conducted once with fecal inoculum from each of the fecal donors ( $n = 4$ ). Batch cultures were run for 24 h and samples collected at 4 time points (0, 5, 10 and 24 h) for sequencing, SCFAs, polyphenol precursors and polyphenol microbial metabolites detection. Samples and pellets for DNA extraction were stored at  $-80^{\circ}\text{C}$  until analyzed.

## 2.6. Analytical methods

### 2.6.1. LC–MS/MS analysis of polyphenol in Mankai®

Phenolic compounds in Mankai® plant and in lyophilized *in vitro* digested sample, were determined according to [Vrhovsek et al. \(2012\)](#), with some modifications. Briefly, 1.6 mL of chloroform and 2.4 mL of methanol: water (2:1) were added to 100 mg of previously ground dried Mankai® leaf. A 20  $\mu\text{L}$  aliquot of gentisic acid (50 mg/L) and rosmarinic acid (50 mg/L) were added as internal standards. The extraction mixture was shaken for 15 min in an orbital shaker, then centrifuged for 5 min at 15,000g at  $4^{\circ}\text{C}$ . The upper aqueous-methanolic phase was collected. The extraction was repeated by adding 2.4 mL of methanol and water (2:1 v/v) and 0.8 mL of chloroform. The samples were centrifuged for 5 min at 15,000g at  $4^{\circ}\text{C}$ . The aqueous-methanolic phase was collected and combined with the previous one. The merged fractions were then evaporated to dryness under a gentle stream of  $\text{N}_2$ . Samples were, finally, re-suspended in 500  $\mu\text{L}$  of methanol and water (1:1 v/v), and transferred carefully into an HPLC vial. Data processing was performed using Waters MassLynx 4.1 (Waters, Milford, CT, USA) and TargetLynx software (Waters, Milford, CT, USA). Details of the liquid chromatography and mass spectrometry are described in [Vrhovsek et al. \(2012\)](#) and [Gasperotti et al. \(2014\)](#).

### 2.6.2. LC–MS/MS analysis of polyphenols and phenolic microbial metabolites

The determination of polyphenol metabolites was carried out according to [Vrhovsek et al. \(2012\)](#) with some modifications. Briefly, a previously developed targeted metabolomic method was performed with an ultra-performance liquid chromatographic system coupled to tandem mass spectrometry system with electrospray ionization (UHPLC-ESI-MS/MS). Before injection, a SPE clean-up procedure for sample purification was performed. Samples were thawed at  $4^{\circ}\text{C}$  and centrifuged at 13,000 rpm for 5 min at  $4^{\circ}\text{C}$ . Then, an aliquot of supernatant (1 mL) was reconstituted with 10 mL of  $\text{H}_2\text{SO}_4$  0.01 N in water, containing 30  $\mu\text{g}$  cinnamic acid D5 (5  $\mu\text{g}/\text{mL}$ ) as internal standard. The conditioning of the cartridges was done with 20 mL of methanol and 20 mL of  $\text{H}_2\text{SO}_4$  0.01 N in water. After loading the samples, the cartridges were washed with 10 mL of Milli-Q water, dried under a stream of nitrogen and eluted with 20 mL of methanol. Eluates were evaporated to dryness with a rotavapor, and the samples were dissolved in 500  $\mu\text{L}$  of methanol/water (50:50 v/v), containing rosmarinic acid as external standard (1  $\mu\text{g}/\text{mL}$ ) to monitor quantitative recovery during sample reconstitution. Data processing was performed using Waters MassLynx 4.1 (Waters, Milford, CT, USA) and TargetLynx software (Waters, Milford, CT, USA). Details of the liquid chromatography and mass spectrometry are described in [Vrhovsek et al. \(2012\)](#) and [Gasperotti et al. \(2014\)](#).

### 2.6.3. GC–MS/MS analysis of short chain fatty acids (SCFAs)

Analysis of SCFAs was performed as previously described by [Lotti et al. \(2017\)](#). Briefly, 10  $\mu\text{L}$  of acidified water (15 % phosphoric acid), 20  $\mu\text{L}$  of internal standard (IS) (acetic d4 45 mM; propionic d6 and butyric d7 10 mM; 2-ethyl butyric and decanoic acid-d19 2 mM) and 100  $\mu\text{L}$  of fecal suspension, previously filtered, were mixed in a 2 mL tubes. A liquid–liquid extraction (LLE) was then performed using 980  $\mu\text{L}$  of metil-t-butyl ether (MTBE). The extraction was assisted by an orbital

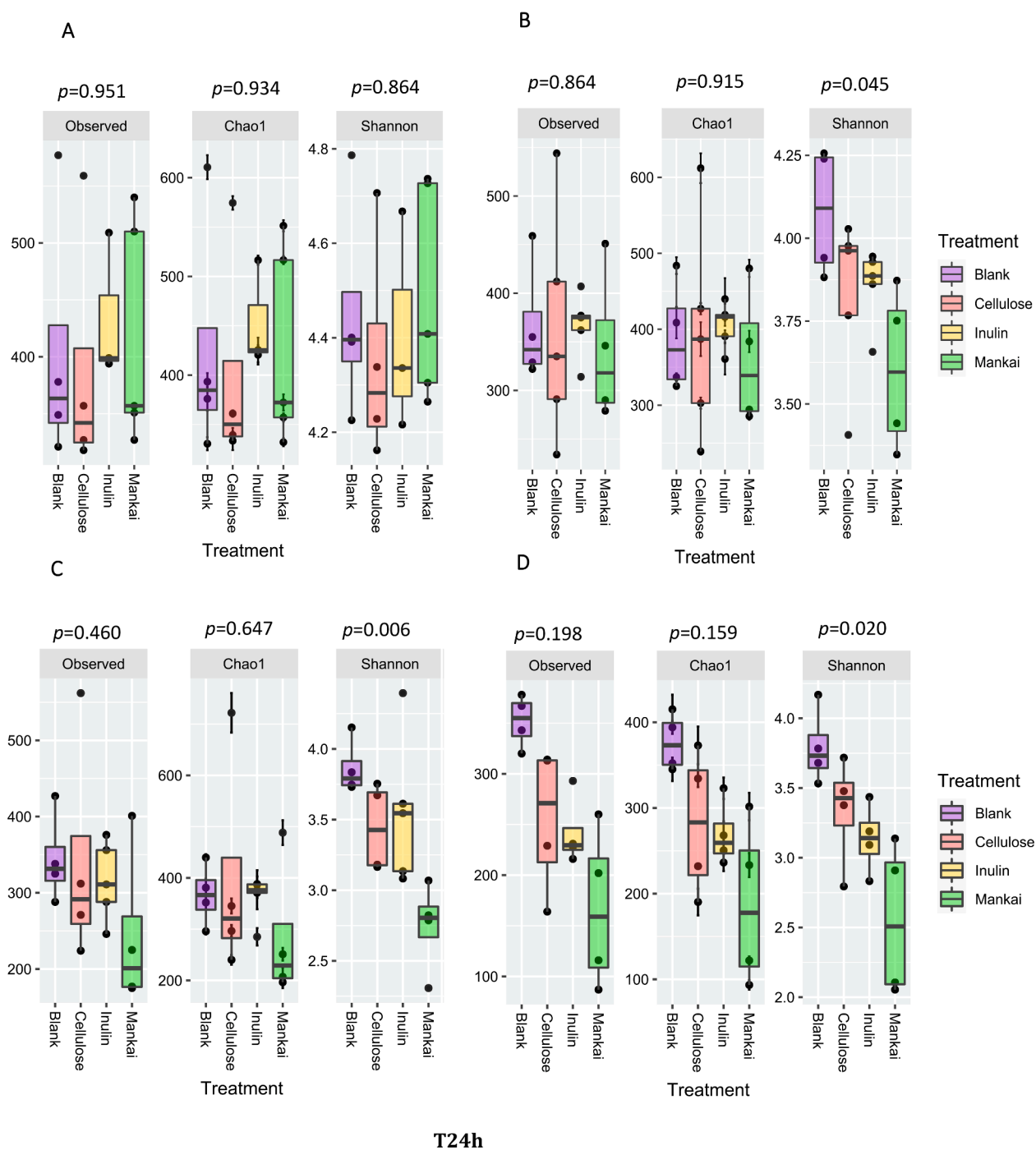
shaker (Multi RS-60; BioSan, Latvia) for 5 min with the following cycle program: 90 rpm of orbital rotation for 5 s followed by reciprocal motion at  $20^{\circ}$  (from the vertical plane) for 15 s. At this point, tubes were centrifuged at 25,314g at  $5^{\circ}\text{C}$  for 5 min. Finally, the organic 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 6,5min). Details of the gas chromatography and mass spectrometry are described in [Lotti et al. \(2017\)](#).

## 2.7. DNA Extraction, amplification and sequencing

DNA was extracted from samples collected at 0, 5, 10 and 24 h time points from each fermentation using the QIAamp PowerFecal DNA Kit (QIAGEN, Germany). Nucleic acid purity was tested on NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific). Total genomic DNA was then subjected to PCR amplification by targeting a  $\sim 460$ -bp fragment of the 16S rRNA variable region V3-V4 using the specific bacterial primer set 341F (5' CCTACGGGNGGCWGCAG 3') and 806R (5' GAC-TACNVGGGTWTCTAATCC 3') with overhang Illumina adapters. PCR amplification of each sample was carried out using 25  $\mu\text{L}$  reactions with 1  $\mu\text{M}$  of each primer, following the Illumina Metagenomic Sequencing Library Preparation Protocol for 16S Ribosomal RNA Gene Amplicons. The PCR products were checked on 1.5 % agarose gel and cleaned from free primers and primer dimer using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA) following the manufacturer's instructions. Subsequently dual indices and Illumina sequencing adapters Nextera XT Index Primer (Illumina) were attached by 7 cycles PCR (16S Metagenomic Sequencing Library Preparation, Illumina). The final libraries, after purification by the Agencourt AMPure XP system (Beckman), were analyzed on a Typestation 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 analyzed on a Typestation 2200 platform (Agilent Technologies, Santa Clara, CA, USA). 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 10,032,600 reads with  $525,270 \pm 86,931.80$  read per sample, mean  $\pm$  standard deviation. After filtering the sequences and removing the chimerae, using QIIME2 pipeline (version 2018.2) ([Bolyen et al., 2019](#)) the total number of reads was 6,840,265 with an average of  $104,534.28 \pm 20,883.03$  read per sample, mean  $\pm$  SD.

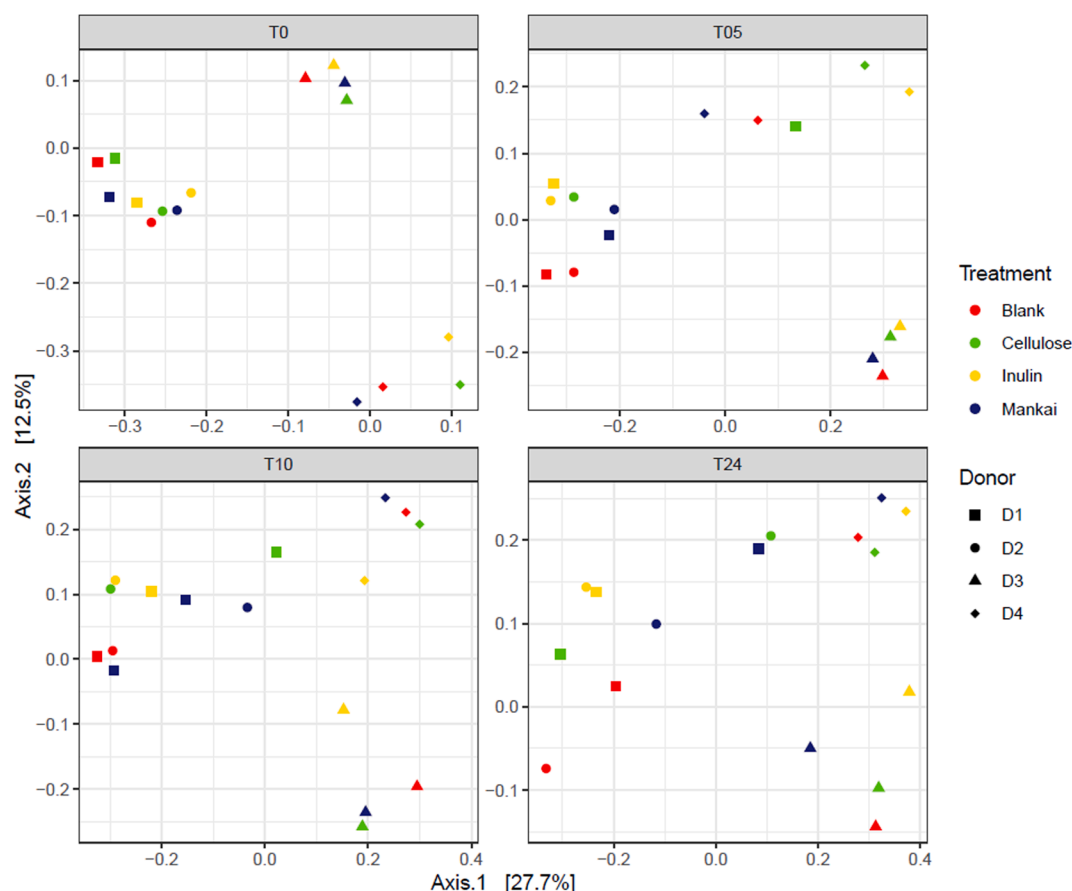
## 2.8. Statistical analysis

Statistical analysis was performed using R software (version 3.6.3). The mean of four 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. We have followed the “80 % rule” for metabolomic data. Missing values of phenolic compounds and metabolites datasets, when less than 20 %, were imputed with a random value between zero and LOQ. Kruskal–Wallis test and Dunnett's Multiple comparison post-hoc test, was then used to determine differences between fermentation treatments (Mankai®, Inulin, Cellulose and Blank) at the same time point (0, 5, 10 or 24 h) and to explore the differences within the same treatment/vessel (Mankai®, Inulin, Cellulose and Blank) with all the time points (0, 5, 10 and 24 h).  $p$  values were corrected with Bonferroni adjustment and  $p < 0.05$  was considered statistically significant. QIIME2 (version 2018.2) was used for 16S rRNA data analysis. Percentage relative abundance of taxa from different fermentation treatments were compared using nonparametric Wilcoxon statistical test. 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



**Fig. 1.** Bacterial alpha diversity. Alpha diversity measured by means of observed Operational Taxonomic Units (OTUs), and Chao1, Shannon indexes of the bacterial communities in 24-h *in vitro* batch culture fermentations inoculated with human feces ( $n = 4$  healthy donors) and administrated with with inulin, cellulose, Mankai® as the substrates (treatments) and blank (without treatment). Box plots show diversity and richness profiles at each sampling point A (T0h), B (T5h), C (T10h), D (T24h). Plotted in the graphic are the interquartile ranges (IQRs) boxes, medians (lines in the boxes), and lowest and highest values for the first and third quartiles. Each treatment is identified by colors, as indicated on the right side of the figure. Every sample is represented by a black dot. \* Significant differences from Mankai® treatment at the same timepoint (\* $p < 0.05$ , \*\* $p < 0.001$ ).





**Fig. 2.** Bacterial beta diversity. Display of Principal coordinate analysis (PCoA) plots of the samples in two-dimension of beta-diversity of the gut microbiome using weighted unifrac distances for the whole data set (24-h *in vitro* batch culture fermentations inoculated with human feces ( $n = 4$  healthy donors) and administrated with inulin, cellulose, Mankai® as the substrates (treatments) and blank (without treatment). Samples were analyzed at 0, 5, 10 and 24 h). Each color represents a different donor and different shape represents different timepoint.

of variance (ANOVA). 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. Correlation between bacterial taxa at genus level and SCFAs and polyphenols was performed by Spearman correlation analysis. False discovery rate FDR adjustment was applied on all tests 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

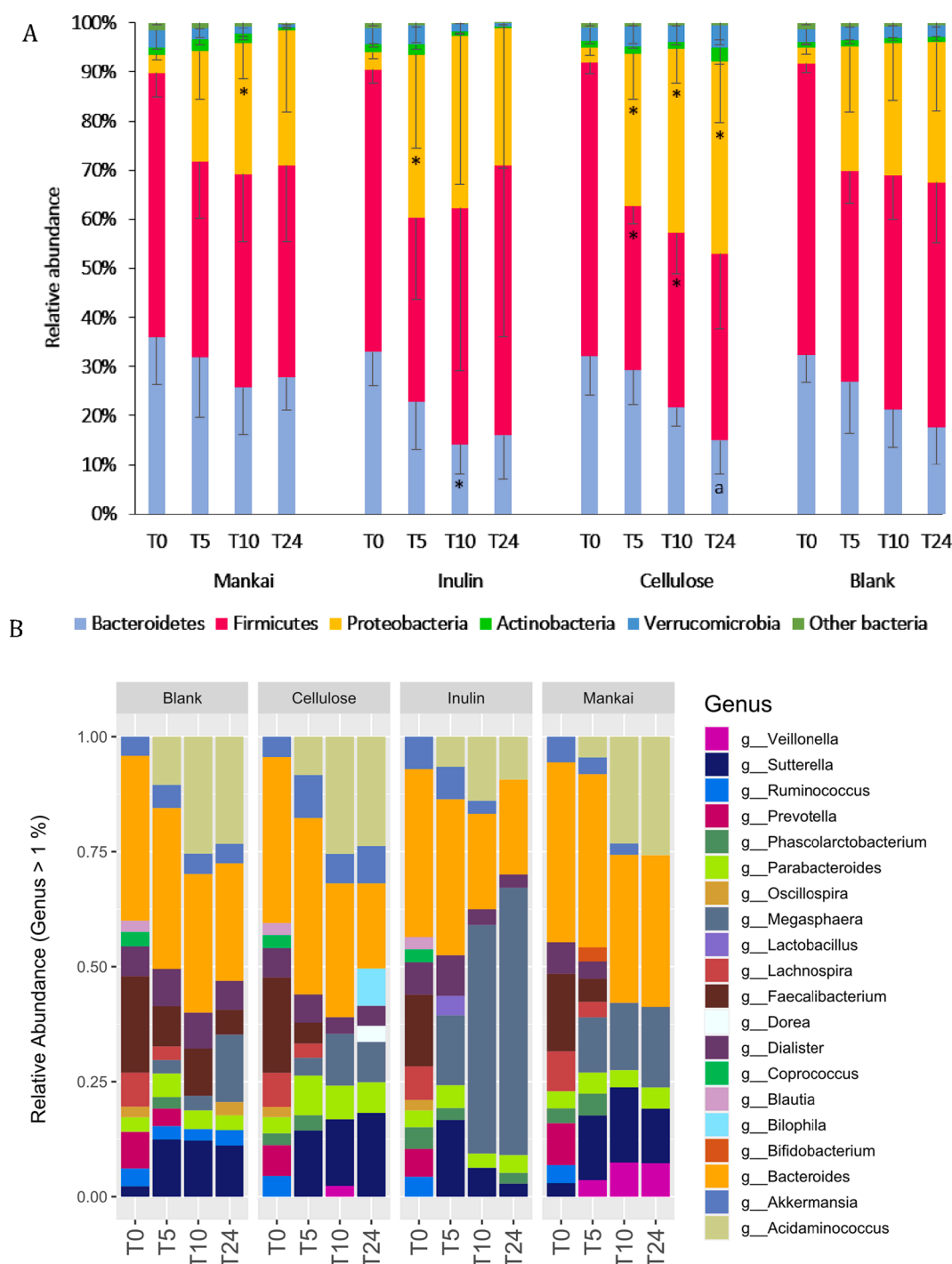
Alpha diversity indices (Observed, Chao1, Shannon) were used to measure the diversity and richness of gut microbiota within the community of each batch culture over the time (Fig. 1). Shannon entropy index revealed significant differences between treatments at T5h, T10h and T24h (Fig. 1). In particular, at T5h the overall diversity of Mankai® was significantly different and lower than the blank ( $p = 0.037$ ), whereas at T10h Mankai® remained lower compared to the Blank ( $p = 0.006$ ) and Inulin ( $p = 0.040$ ) (Fig. 1). At T24h, distinction between treatments was kept for Mankai® and Blank ( $p = 0.004$ ) and became significant between cellulose and Mankai® ( $p = 0.080$ ). Moreover, at the end of the fermentation, differences in the overall diversity was observed also for Chao index and in the number of species observed. In

fact, in both cases Mankai® was significantly lower and different respect to the Blank (Chao  $p = 0.013$ , Observed  $p = 0.004$ ). All alpha diversity indices decreased over time during the fermentation of all the substrates ( $p < 0.05$ ).

Beta diversity was analyzed based on Bray-Curtis dissimilarities and unweighted (qualitative) and weighted (quantitative) phylogenetic UniFrac distance matrices (Fig. 2). According to three matrices, the adonis test with 999 permutations showed clustering according to fecal donor and time point, but no significant effects were observed by treatments on beta diversity.

#### 3.2. Changes in relative abundance

At the beginning of the fermentation, the composition of the bacterial communities at phylum level was numerically dominated by Firmicutes (53–59 %), Bacteroidetes (32–36 %), Proteobacteria (2.9–2.8 %) and lower amount of Actinobacteria (1.2–1.7 %), Verrucomicrobia (2.5–3.2 %) and Elusimicrobia (0.6, 1.5 %) comprising  $>1$  % of relative abundance in all the substrates despite substantial inter-individual variation. At T24h the level of Bacteroidetes tended to decrease in all the treatments except for Mankai®, where bacterial relative abundance remained similar to the T0h level and was significantly higher compared to cellulose ( $p < 0.05$ ) (Fig. 3A). At genus level, *Bacteroides*, *Parabacteroides*, *Acidamidococcus*, *Megasphaera*, *Veillonella*, *Sutterella* were the major representative taxa comprising almost 70 % of the total microbiome in all treatments (Fig. 3B). With respect to the others, Mankai® was higher in *Bacteroides*, *Acidamidococcus* and *Veillonella* and lower in *Blautia* genus. However, these differences were not statistically



**Fig. 3.** Relative abundances at phylum and genus level. Changes in relative abundance (%) of OTUs at A) phylum B) genus level throughout 24-h *in vitro* batch culture fermentations inoculated with human feces ( $n = 4$  healthy donors) and administrated with inulin, cellulose, Mankai® as the substrates (treatments) and blank (without treatment). Samples were analyzed at 0, 5, 10 and 24 h. In Fig. 3A values are mean with SD (the negative error value is shown). Only OTUs represented by an average relative abundance of more than 1 % of sequences in any sample are shown. \* Significant differences from the 0 h time point within the same treatment ( $p < 0.05$ , FDR corrected). <sup>a</sup> Significant difference from Mankai® treatment at the same time point ( $p < 0.05$ ).

significant. Detailed relative abundance composition at order, class, and family level is presented in the Supplementary File, Fig. S1.

### 3.3. Change of Mankai® phenolic compounds during the *in vitro* digestion

The main representative phenolic compounds of Mankai® plant were identified by Flavonoids and Cinnamic acid derivatives (Fig. 4); Meir et al. have provided further detail relative composition of polyphenol in

Mankai® and how the metabolites by the gut microbiota and the human body (Yaskolka Meir et al., 2021). After the *in vitro* digestion, the amount of polyphenol compounds was monitored as shown in Table 1. The glycosylated forms of luteolin and quercetin decreased significantly in concentration whereas their respective aglycones increased ( $p < 0.05$ ). The concentration of cinnamic acid derivatives did not show a similar pattern between the different compounds. In fact, p-coumaric, caffeic acid and catechol decreased after the *in vitro* digestion ( $p < 0.05$ ) and,

■ Flavones ■ Flavonols ■ Flavanols ■ Cinnamic acid derivatives

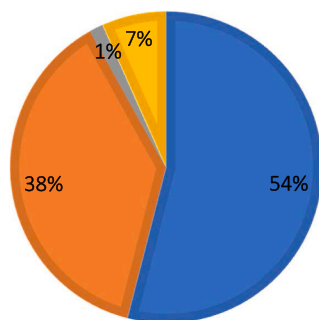


Fig. 4. Relative proportion of polyphenols detected in Mankai® plant, by subclass.

ferulic acid increased ( $p < 0.05$ ). Finally, only flavanols were no longer detectable after the *in vitro* gastrointestinal digestion.

### 3.4. Polyphenol catabolism and formation of polyphenol microbial metabolites during *in vitro* fermentation

A total of 20 parent polyphenols and 20 polyphenol metabolites were detected and quantified over 24 h (Table 2), sampling at four different time points: at the baseline (0 h), after 5, 10 and 24 h. In contrast to the substantial decrease in the amounts of most polyphenols, total phenolic catabolites increased significantly (~25 %,  $p < 0.05$ ) in the Mankai® treatment after 24-h fermentation (Fig. 6). Looking at the compounds individually, the main polyphenols detected in Mankai®, including luteolin-7-O-Glc, caffeic acid and p-coumaric acid, decreased significantly during fermentation together with luteolin, while quercetin-3-Gal, quercetin-3,4-diglucoside were almost absent at the beginning of the fermentation (Table 2). On the other hand, significant increases were observed over time for low molecular weight phenolic acids including protocatechuic acid, 3-3-hydroxyphenyl propanoic acid, dihydroferulic acid, 3-4-hydroxyphenyl propionic acid, 3-hydroxyphenyl acetic acid, 3-4-dihydroxyphenyl acetic acid, and pyrocatechol. Significant differences ( $p < 0.05$ ) were observed mainly at T24h with respect to T0h (Fig. 5).

Table 1

Total amount of polyphenol present in Mankai® after the *in-vitro* digestion. Phenolic quantification is expressed as  $\mu\text{g/g} \pm \text{SD}$  ( $n = 3$ ). Significance difference from polyphenols in the Mankai plant (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). † refers to increase; ‡ refers to decrease.

Compounds	Recovery after <i>in-vitro</i> Digestion ( $\mu\text{g/g}$ )	RT	ESI mode	Precursor ion ( $m/z$ )	Quantifier Product ion ( $m/z$ )	Qualifier Product ion ( $m/z$ )
<b>Flavones</b>						
Luteolin	$27.07 \pm 1.85^* \uparrow$	7.43	+	287	153	134
Luteolin-7-O-Glc	$31.35 \pm 0.25^{***} \downarrow$	4.68	–	447	285	150
Apigenin-7-glc	$0.35 \pm 0.06$	5.85	+	433	271	153
Apigenin	n.d.	8.25	–	269	117	151
<b>Flavonols</b>	$58.77 \pm 2.43^{**}$					
Quercetin-3-Glc	$1.45 \pm 0.06^* \downarrow$					
Quercetin-3,4-diglucoside	$13.39 \pm 0.77^{***} \downarrow$					
Quercetin-3-Gal	$163.64 \pm 1.11^* \downarrow$	5.9	+	449	303	129
Quercetin	$4.39 \pm 0.37$	7.45	+	303	152	228
Isorhamnetin-3-Glc	$0.07 \pm 0.01$	5.79	+	479	317	302
Rutin	$0.07 \pm 0.01$	4.25	+	611	303	465
<b>Flavanols</b>	$183.01 \pm 1.23^*$					
Catechin	<LOQ	2.85	–	288	202	122
Epicatechin	<LOQ	3.39	–	288	202	122
Epigallocatechin	<LOQ	2.74	–	304	124	178
<b>Total Flavonoids</b>	$247.78 \pm 5.67^*$					
<b>Cinnamic acid derivatives</b>						
p-coumaric acid	$8.68 \pm 0.20^* \downarrow$	4.08	+	164	91	65
Caffeic acid	$9.41 \pm 0.36^{**} \downarrow$	3.24	+	180	145	117
Ferulic acid	$5.56 \pm 0.44^* \uparrow$	4.56	+	194	144	117
Catechol	n.d.	2.88	–	109	80	53
<b>Total Cinnamic acid derivatives</b>	$23.65 \pm 0.76^*$					

### 3.5. SCFA production

Changes in SCFA concentrations at the baseline and after 24-h batch culture fermentation with the different treatments are shown in Table 3.

All SCFAs reveal a slight increase over the 24-h fermentation. Acetic, propionic and butyric acids the main SCFAs produced upon fiber fermentation by the gut microbiota, showed significant differences between T0h and T24h. All treatments significantly increased the concentration of acetic acid, with Mankai® and inulin showing the highest production. Fermentation of inulin gave the highest production of butyric acid ( $p < 0.05$ ), followed by Mankai®, blank and cellulose. Mankai® resulted in the highest production of propionate, although this was not statistically significant compared to the other treatments.

### 3.6. Correlations between phenolic compounds and fecal microbiota

The gut microbiota is known to play a pivotal role in the transformation of plant polyphenols and production of catabolites (Fava, Rizzetto, & Tuohy, 2019). We further investigated this relationship correlating the values of area under the curve (AUC) of the phenolic metabolites measured over 24-h Mankai® fermentation, and in order to correlate production of metabolites with changes with microbial profile across time period (T0h-T24h), we used the AUC of the change of relative abundance of microbiota over the time. We applied Spearman correlation for the analysis (Fig. 7) and heatmap to visualize the data. Looking at the main native Mankai® polyphenol compounds (Fig. 6A), caffeic acid and p-coumaric showed the same positive correlation with *Ruminococcus*, whereas quercetin-3-Glc was positively correlated with *Coprococcus* and *Oscillospira*. Negative correlations were reported for quercetin-3-Gal and *Bacteroides*, and for luteolin with *Coprococcus* and *Oscillospira*. Not surprisingly, the phenolic catabolite dataset showed more correlations with bacterial taxa than in the native polyphenols (Fig. 6B). Pyrocatechol and dihydroisoflavanic acid were positively associated with *Oscillospira* and *Dorea* respectively. Protocatechuic acid, 3-4-hydroxyphenyl propionic acid, hippuric acid and hydroferulic acid showed positive correlations with *Faecalibacterium* and *Sutterella*. These same phenolic compounds showed negative correlations with *Prevotella*, *Ruminococcus* and *Bilophila*. Finally, *Coprococcus*, *Dialister* and *Veillonella* reported negative correlations with vanillin.

**Table 2**

Changes in precursor polyphenols of 24-h *in vitro* batch culture fermentations inoculated with human feces (n = 4 healthy donors) and administrated with inulin, cellulose, Mankai® as the substrates (treatments) and Blank (without treatment). Samples were collected at 0, 5, 10 and 24 h. Results are expressed as ng/mL of batch culture medium and values are mean  $\pm$  SD of the four fermentations. \*Significant differences from the 0 h time point within the same treatment (\*p < 0.05, \*\*p < 0.01).

	T0	T5	T10	T24
<b>Flavonoids</b>				
Quercetin-Gal	0.16 $\pm$ 0.20	0.31 $\pm$ 0.25	0.21 $\pm$ 0.22	0.20 $\pm$ 0.14
Luteolin	0.15 $\pm$ 0.04	0.05 $\pm$ 0.03	0.02 $\pm$ 0.01*	<LOQ
Luteolin-7-O-Glc	0.02 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00*
Catechin	0.03 $\pm$ 0.07	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Epicatechin	0.04 $\pm$ 0.08	<LOQ	<LOQ	<LOQ
Quercetin	0.14 $\pm$ 0.05	0.06 $\pm$ 0.07	0.02 $\pm$ 0.040	0.01 $\pm$ 0.02**
Isorhamnetin	<LOQ	<LOQ	<LOQ	<LOQ
Quercetin-3,4-diglucoside	0.02 $\pm$ 0.03	0.01 $\pm$ 0.01	<LOQ	<LOQ
Quercetin-3-Glc	<LOQ	<LOQ	<LOQ	<LOQ
Isorhamnetin-3-Glc	<LOQ	<LOQ	<LOQ	<LOQ
<b>Cinnamic acid derivatives</b>				
p-coumaric acid	0.15 $\pm$ 0.06	0.12 $\pm$ 0.13	0.02 $\pm$ 0.04	<LOQ
Caffeic acid	3.57 $\pm$ 2.02	3.23 $\pm$ 2.41	2.71 $\pm$ 1.46	0.09 $\pm$ 0.18*

Regarding SCFAs, butyric, isobutyric, isovaleric, 2-methylbutyric, 2-methylbutyric isovaleric acids showed the same correlation patterns with *Bifidobacterium*, *Bacteroides*, *Coprococcus*, *Oscillospira*, *Dialister*. However, *Bifidobacterium* and *Bacteroides* showed positive correlations whereas *Coprococcus*, *Oscillospira*, *Dialister* accounted for the negative correlations. Other positive correlations were observed for *Prevotella* and propionic acid and *Veillonella* and acetic acid. Negative correlations were also reported for *Lactobacillus*, *Akkermansia*, *Sutterella* and acetic acid, and for *Faecalibacterium* and propionic acid (Fig. 6C).

#### 4. Discussion and conclusion

The importance of providing the evidence base for healthy alternatives to animal derived dietary protein has been clearly stated at the highest scientific levels (Willett et al., 2019). There is an onus on the scientific community and agrifood industry to develop new foods and ingredients which promote health, reduce the risk of chronic disease and also reduce the environmental impact of our modern food chains. In this context, *Wolffia globosa* (Mankai®) may provide a novel source of proteins and health promoting bioactive compounds. In the present study we measured the ability of Mankai® whole plant to influence the composition and metabolic activity of human gut microbiota *in vitro*.

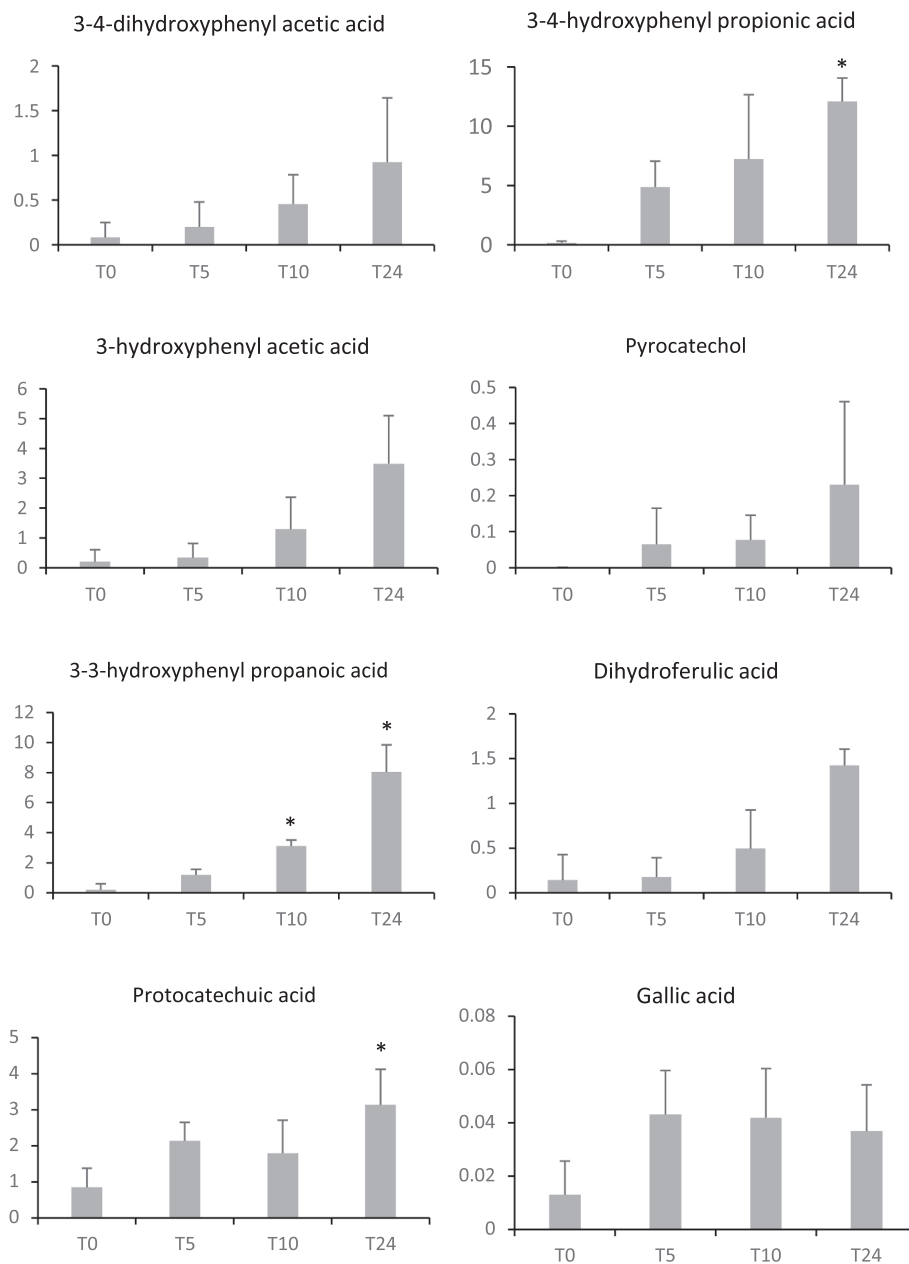
Effects on the bacterial community at the OTU level, were observed for the alpha diversity of gut microbiota when comparing the treatments. Mankai® showed lower overall diversity compared to controls and blank at T5h, T10h and T24h. This may indicate the selective nature of the Mankai® fermentation towards particular species, since it represented the only source of fermentable material in the batch culture. A similar impact on microbial diversity was observed for inulin, again showing that fermentation of a single carbohydrate or even food by the gut microbiota can select for particular bacteria able to use it as a growth substrate at the expense of other species which lack the capability to derive energy or carbon sources from the test substrate. These results in increase in a relative abundance of the microorganisms involved and also a resultant reduction in alpha diversity over time. The beta diversity

analysis were not affected by treatments, but donor and time showed clustering over the fermentation, which indicates that individuals possess a specific starting population of gut bacteria, contributing to high variability. Relative abundance of Bacteroidetes, known to be the main contributors to propionate, was higher in Mankai® to cellulose at T24h (p < 0.05). All other taxonomic levels were not modified significantly by Mankai®, however a strong significant correlation with phenolic compounds at genus level were found, suggesting an active role of microbiota in the metabolism of phenolic compounds as well as SCFAs production.

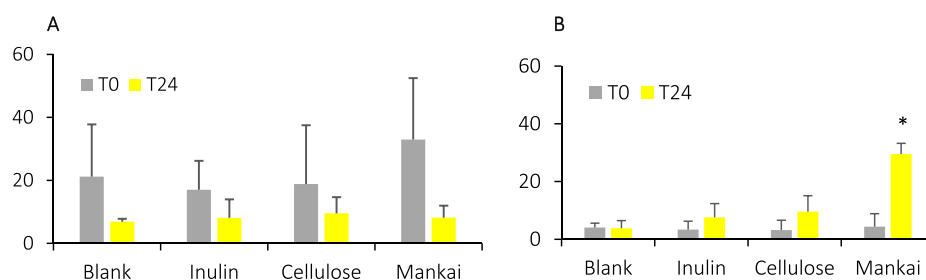
The targeted metabolic analysis revealed a substantial quantity of Cinnamic acids derivatives, that make Mankai® more similar to herbs and spices, and this is especially evident in caffeic acid content (Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010). However, luteolin and quercetin present in their glycosylated form are the most abundant native polyphenol found, that have been extensively associated to multiple biological properties, including antioxidant, anti-inflammatory and anti-allergic effects (Mlcek, Jurikova, Skrovankova, & Sochor, 2016; Seelinger, Merfort, & Schempp, 2008). Both have shown potential chemo-preventive properties in colorectal cancer (Darband et al., 2018; Lin, Shi, Wang, & Shen, 2008; Pandurangan & Esa, 2014) and multiple cardio-protective effects (Luo, Shang, & Li, 2017; Patel et al., 2018). However, evidence on the effects of their glycosylated forms are scarce. Bioavailability and bioactivity of numerous flavonoids depend primarily on the type and position of sugar moiety, as well as dietary source. So far, one study has shown anticancer activity of luteolin-7-Glucoside in keratinocyte (Palombo et al., 2019), but evidence on the effect of quercetin-3,4-diglucoside has not been reported. Therefore, studying the transformation occurring within the gut microbiota will help to understand their physiological metabolism.

Our results reported protocatechuic acid, 3-4-hydroxyphenyl propionic acid, hippuric acid and hydroferulic acid as catabolites showing more correlations with microbiota at genus level. Moreover, they also exhibit the same pattern of correlation with *Prevotella*, *Ruminococcus*, *Bilophila*, *Faecalibacterium* and *Sutterella*. Particularly, positive correlations involved *Faecalibacterium* and *Sutterella* genera. *Faecalibacterium* genus, is an abundant commensal that synthesizes mainly butyrate, a SCFA implicated in driving immunoregulation (Blaak et al., 2020). This genus has been shown to be reduced in several inflammatory conditions such as Crohn's disease, obesity, and psoriasis. Moreover, *Faecalibacterium* is an indicator of higher quality of life being depleted in depression (Jiang et al., 2015; Valles-Colomer et al., 2019). Contrary to *Faecalibacterium*, little is reported about *Sutterella*, but it seems to be associated with gastrointestinal diseases (Kaakoush, 2020). Negative correlations, where instead found for *Prevotella*, *Ruminococcus* and *Bilophila*. Generally high level of *Bilophila* is associated with animal-based diet (David et al., 2014), whereas *Ruminococcus* is responsible for carbohydrate catabolism, especially starch breakdown have been found to be increased in subjects with high carbohydrate diets (Walker et al., 2011). As reported by Gurung et al. (2020), the genera of *Ruminococcus*, along with *Coprococcus*, was indeed positively associated with T2D and impaired glucose metabolism. Reduced relative abundance of *Ruminococcus* has also recently been observed in obese patients following a hypocaloric Mediterranean style diet (Pisanu et al., 2020). On the other hand, negative associations were also found with *Prevotella* which is an indicator of plant-based dietary patterns, playing an important role in preventing weight gain by reducing serum cholesterol and decreasing hepatic lipogenesis (Precup & Vodnar, 2019). However, *Prevotella* is a genus with high genetic diversity within and between species and certain strains have shown pathobiontic properties (Ley, 2016; Precup & Vodnar, 2019). Therefore, although the single correlations between microbiota and polyphenols might be controversial, it is important to highlight that the efflux of metabolites from the colon is determined by dynamic interactions between the community of microorganisms present, with final concentrations of metabolites determined by competitive, collaborative and mutualistic relationships between





**Fig. 5.** Changes in polyphenol metabolites: 3-4-dihydroxyphenyl acetic acid, 3-4-hydroxyphenyl propionic acid, 3-hydroxyphenylacetic acid, Pyrocatechol, 3-3-hydroxyphenyl propionic acid; Dihydroferulic acid; Protocatechuic acid, Gallic acid; throughout 24-h *in vitro* batch culture fermentations inoculated with human feces ( $n = 4$  healthy donors) and administered with Mankai® as the substrate (treatment). Samples were collected at 0, 5, 10 and 24 h. Results are expressed as of batch culture medium and values are mean  $\pm$  SD of the four fermentations. \* Significant differences from the 0 h time point within the same treatment ( $p < 0.05$ ).



**Fig. 6.** Total amount of A) phenolic acids and B) catabolites present in Mankai® plant at the baseline (T0) and the end (T24) of the *in-vitro* fermentation. Data expressed as ng /mg  $\pm$  SD ( $n = 4$ ). Significant differences from the 0 h time point within the same treatment (\*  $p < 0.05$ ).

different taxa and indeed the high degree of cross-feeding recognized within the gut microbiome (Michalak et al., 2020).

During the simulated *in vitro* digestion, the change of polyphenol profile was monitored, and a significant decrease of phenolic

compounds was observed. Loss of small metabolites during *in vitro* digestion and dialysis is likely, a limitation of this *in vitro* digestion method. However, as the *in vitro* digesta was destined for *in vitro* fermentation by the gut microbiota, it was important to eliminate as

**Table 3**

Table reports the SCFA concentrations (mmol/L) in fecal batch cultures at 24 h fermentation with respect to the baseline (time 0) of inulin, cellulose, Mankai® (treatments) and blank (without treatment). Values are mean  $\pm$  SD of the four fermentations. \*Significant differences from the 0 h time point within the same treatment (\* $p < 0.05$ ).

	Blank		Cellulose		Inulin		Mankai®	
	T0h	T24h	T0h	T24h	T0h	T24h	T0h	T24h
Acetic acid	1.25 $\pm$ 0.48	11.59 $\pm$ 1.74*	0.82 $\pm$ 0.43	10.09 $\pm$ 2.44*	0.94 $\pm$ 0.46	19.25 $\pm$ 11.59*	1.23 $\pm$ 0.36	16.39 $\pm$ 7.46*
Propionic acid	0.28 $\pm$ 0.12	2.48 $\pm$ 0.42	0.22 $\pm$ 0.10	2.13 $\pm$ 0.39	0.23 $\pm$ 0.10	4.47 $\pm$ 24.28	0.26 $\pm$ 0.07	4.61 $\pm$ 2.11
Isobutyric acid	0.03 $\pm$ 0.01	0.54 $\pm$ 0.59	0.02 $\pm$ 0.01	0.52 $\pm$ 0.55	0.02 $\pm$ 0.00	0.21 $\pm$ 0.21	0.03 $\pm$ 0.00	0.93 $\pm$ 1.03
Butyric acid	0.24 $\pm$ 0.10	4.37 $\pm$ 1.48	0.23 $\pm$ 0.16	3.29 $\pm$ 1.70	0.18 $\pm$ 0.12	13.29 $\pm$ 5.65*	0.24 $\pm$ 0.18	8.32 $\pm$ 3.87
Isovaleric acid	0.02 $\pm$ 0.01	0.59 $\pm$ 0.65	0.01 $\pm$ 0.00	0.49 $\pm$ 0.54	0.02 $\pm$ 0.00	0.25 $\pm$ 0.27	0.02 $\pm$ 0.00	1.16 $\pm$ 1.28
2-methylbutyric acid	0.02 $\pm$ 0.00	0.37 $\pm$ 0.40	0.01 $\pm$ 0.00	0.27 $\pm$ 0.40	0.01 $\pm$ 0.00	0.13 $\pm$ 0.13	0.01 $\pm$ 0.00	0.67 $\pm$ 0.74
2methylbutyric + isovaleric	0.01 $\pm$ 0.00	0.37 $\pm$ 0.40	0.01 $\pm$ 0.00	0.25 $\pm$ 0.18	0.02 $\pm$ 0.00	0.13 $\pm$ 0.10	0.01 $\pm$ 0.00	0.67 $\pm$ 0.74
Valeric acid	0.04 $\pm$ 0.02	1.38 $\pm$ 1.58	0.03 $\pm$ 0.01	0.88 $\pm$ 1.03	0.04 $\pm$ 0.01	2.48 $\pm$ 3.09	0.04 $\pm$ 0.01	2.26 $\pm$ 2.06
Hexanoic acid	0.04 $\pm$ 0.01	0.29 $\pm$ 0.30	0.03 $\pm$ 0.01	0.13 $\pm$ 0.15	0.03 $\pm$ 0.01	4.10 $\pm$ 4.80	0.03 $\pm$ 0.01	0.56 $\pm$ 0.69
Total SCFAs	1.93 $\pm$ 1.02	22.56 $\pm$ 3.17*	1.41 $\pm$ 0.47	18.85 $\pm$ 3.46*	1.51 $\pm$ 0.49	44.62 $\pm$ 19.28*	1.89 $\pm$ 0.41	33.60 $\pm$ 9.44*

much simple sugar from the intended substrate as possible to mimic small intestinal sugar absorption but also to avoid in-due microbiome modulation by sugars which would not reach the colon in vivo. This result could also be due to the metabolic conversions that occur in the digestive tract. Flavanol monomers, such as catechin and epicatechin, are easily degraded in the small intestine, as well as glucose-bound polyphenols that are de-glucosylated to aglycone forms by enzymatic activity of intestinal epithelial cells which are lacking in the *in vitro* model used here. However, previous studies found similar decrease in polyphenol glucosides during an *in vitro* digestion. Commonly, in the acidic pH (pH 2, gastric phase), phenolic compounds, such as caffeic acid and quercetin along with its glycosylated forms, are highly stable (Bermudez-Soto, 2007; Tagliazucchi, 2010), but the neutral or slightly basic pH found in the duodenal environment can promote chemical transformations, compromising their structure. Afterwards, the remaining unabsorbed polyphenols are transformed by the gut bacteria into smaller phenolic acids with physiological significance, not only locally in the gut, but also systematically after their absorption in the colon and their appearance in circulating blood. In the present study, we found degradation of precursor polyphenols, which started as early as 5 h of fermentation and was complete throughout the 24 h for most of the polyphenols. Mankai® was mainly associated with the production of protocatechuic acid, 3-3-hydroxyphenyl propanoic acid, dihydroferulic acid, 3-4-hydroxyphenyl propionic acid, 3-hydroxyphenyl acetic acid, 3-4-dihydroxyphenyl acetic acid, and pyrocatechol. According to previous studies the identified phenolic acids are in line with *in vitro* fermentation by human fecal microbiota. In particular, previous evidence has reported protocatechuic acid as a widely distributed fermentation metabolite. Urpi-Sarda et al. identified protocatechuic acid as a metabolic product of flavanol-3-ols in rat kidney. Other findings suggest that protocatechuic acid formation is based on the dehydroxylation of caffeic, ferulic acids, and gallic acid whose presence was detected in all treatment in the present study. Phenylpropionic acids are gut bacteria-derived metabolites that may arise from the conversion of Flavonoids and Cinnamic acid derivatives (Mosele, Macià, & Motilva, 2015). In particular, 3-4-dihydroxyphenyl acetic acid is the main colonic metabolite produced in vivo from orally administration of quercetin. This metabolite appears to play an important role in improving glucose homeostasis (Fernández-Millán et al., 2014). Carrasco-Pozo et al. (2015) showed that 3-4 dihydroxyphenyl acetic acid protects against pancreatic  $\beta$ -cells dysfunction induced by high cholesterol, improving insulin secretion, and preventing mitochondrial impairment and cell apoptosis. Further metabolism of this compound deriving from the loss of a hydroxyl group on the benzene ring leads to the formation 3-hydroxyphenyl acetic acid and 4-hydroxyphenyl acetic acid (Di Pede et al., 2020) that, however, has not been detected by our analysis. Differently to quercetin, microbial metabolism of luteolin has not been deeply studied therefore information on specific phenolic microbial metabolites is lacking. Another Mankai® polyphenol that decreased in concentration

during fermentation was caffeic acid. Caffeic acid may be further metabolized to 3-4-dihydroxyphenylpropionic, 3-3-hydroxyphenyl propionic acid and benzoic acids and the last two have been identified as the major microbial metabolites in *in vitro* study with human fecal inoculum. Moreover, 3-3-hydroxyphenyl propionic acid has been shown to act as a potent vasodilator by decreasing arterial blood pressure in rats (Najmanová et al., 2016).

Mankai® treatment showed a significant increase in acetate concentration after 24 h of fermentation. Moreover, it showed also a higher amount of total SCFA production compared to cellulose and the blank fermentation suggesting a high fermentable fiber content. However, inulin produced more SCFA. Previous study has reported the nutritional composition of 11 species of the genus *Wolffia* including *Wolffia globosa* (Appenroth et al., 2018). The content of fiber was estimated to be ~25 % of the freeze-dry weight, however distinction between soluble and insoluble fiber was not investigated. SCFAs are a dependent product of dietary fiber fermentation by the gut microbiota, and soluble fibers are preferentially fermented by microbiota to produce SCFAs.

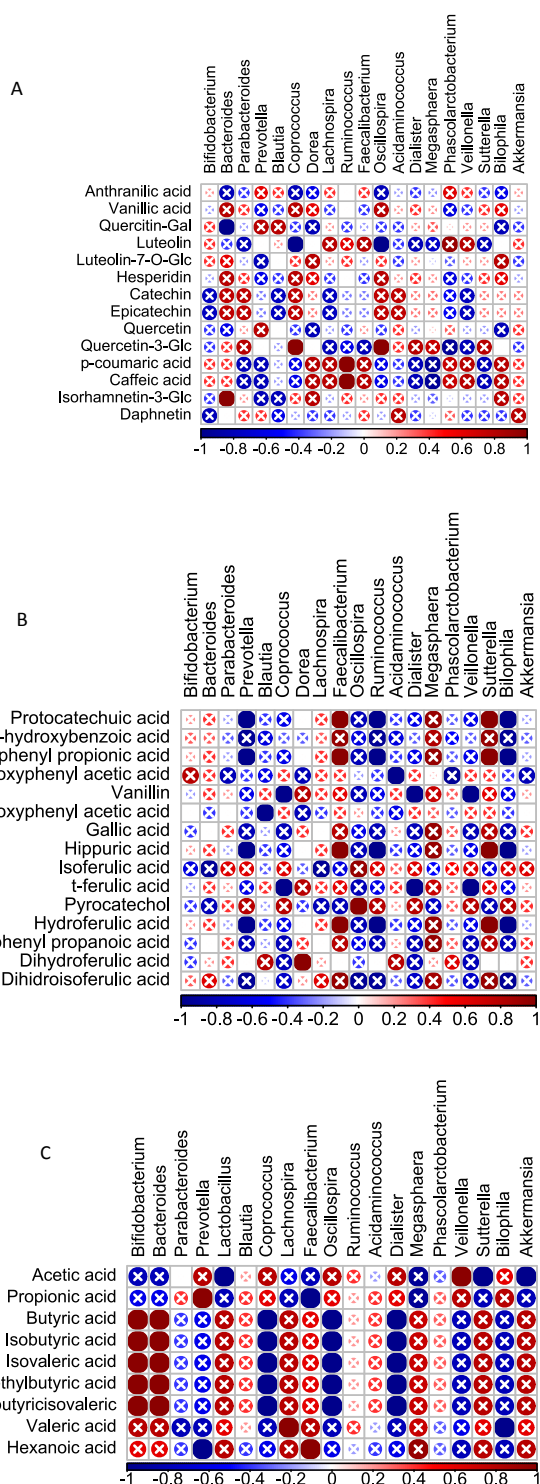
In this study, we demonstrated that Mankai® could modify the gut microbiota and affect the extent of degradation of polyphenols through the production of smaller phenolic acids and SCFAs produced upon Mankai® fiber fermentation. A limitation of the study that might be considered is its reduced complexity of human host-microbiome interactions that exit in vivo. A relevant fraction of the dietary polyphenols undergoes phase II enzyme action to generate a wide pattern of possible circulating metabolites. These microbial metabolites are generated hours after food intake and reach the colon as methyl, glucuronide, or sulfated derivatives. This aspect might modify the fermentation process to an extent that was not measurable in the present work. The sample size ( $n = 4$ ) is consistent with similar studies investigating dietary polyphenols, fibers, and gut microbiota interactions (Koutsos et al., 2017; Pham et al., 2017; Wang et al., 2019), and the observed changes are consistent with outcomes of human intervention studies (Marín, Miguélez, Villar, & Lombó, 2015). However, characteristics that may potentially impact the gut microbiota such as dietary habits, the level of physical activity and stress conditions were not recorded and may have influenced the observed results.

## 5. Ethics statement

The authors declare that no human or animal subjects were included in this study.

## CRedit authorship contribution statement

**Camilla Diotallevi:** Conceptualization, Formal analysis, Resources, Validation, Data curation, Writing - original draft, Visualization. **Giulia Gaudio:** Resources, Validation. **Francesca Fava:** Conceptualization, Methodology. **Andrea Angeli:** Validation. **Cesare Lotti:** Validation,



**Fig. 7.** Heatmap of Spearman's correlation, between genus level 16S rRNA relative abundance greater than 1 % of fecal microbiota present in each subject and A) parent polyphenols B) phenolic catabolites C) SCFAs. Correlation was performed on the values of area under the curve (AUC) of the Mankai® datasets. Dark red indicates positive correlation, while dark blue represents negative correlation. Dots without X are statistically significant:  $p < 0.001$ .

Writing - review & editing. **Urska Vrhovsek**: Writing - review & editing. **Ehud Rinott**: Writing - review & editing, Visualization. **Iris Shai**: Writing - review & editing. **Marco Gobbetti**: Writing - review & editing. **Kieran Tuohy**: Conceptualization, Methodology, Writing - review & editing, Supervision, Visualization, Project administration, Funding

acquisition.

## Declaration of Competing Interest

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

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2021.104597>.

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