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Effect of *in vitro* digestion-fermentation on green and roasted coffee bioactivity: the role of the gut microbiota

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ABSTRACT

Coffee is one of the most consumed beverages and has been linked to health in different studies. However, green and roasted coffees have different chemical composition and therefore their health properties might differ as well. Here, we study the effect of *in vitro* digestion-fermentation on the antioxidant capacity, phenolic profile, production of short-chain fatty acids (SCFAs), and gut microbiota community structure of green and roasted coffee brews. Roasted coffees showed higher antioxidant capacity than green coffees, with the highest level achieved in fermented samples. Polyphenol profile was similar between green and roasted coffees in regular coffee brews and the digested fraction, but very different after fermentation. Production of SCFAs was higher after fermentation of green coffee brews. Fermentation of coffee brews by human gut microbiota led to different community structure between green and roasted coffees. All these data suggests that green and roasted coffees behave as different types of food.

KEYWORDS: coffee, polyphenols, Maillard reaction, antioxidant capacity, short chain fatty acids, gut microbiota.

1. Introduction

Coffee is the food product most consumed in the world after water (Farah & Ferreira dos Santos, 2015). Coffee has been related to health since ancient times and still today is studied frequently in many research projects related to chronic diseases (Galluzzi Bizzo, Farah, Ann Kemp, & Berguinins Scancetti, 2015). The protective role of coffee on health is thought to be mostly related to its high antioxidant capacity and is also linked to its high consumption (Saura-Calixto & Goñi, 2006). Coffee brews have a wide range of antioxidant compounds such as different types of phenolics (hydroxycinnamic acids such as caffeic, ferulic, coumaric and chlorogenic acids), aromatic compounds and Maillard reaction products such as melanoidins (Pastoriza & Rufián-Henares, 2014). Polyphenols have been reported to exert a protective role against several diseases such as cardiovascular or neurodegenerative diseases and cancer thanks to their ability to protect cells against oxidation (Galluzzi et a., 2015). Further, 5-caffeoylquinic acid (a chlorogenic acid) has been shown to have a protective role against neurodegenerative diseases (Jeszka-Skowron, Stanisz, & De Peña, 2016). Melanoidins which are produced during roasting process, are also potent antioxidants (Jiménez-Zamora, Pastoriza & Rufián-Henares, 2015).

During the roasting step, most of the aromatic compounds of coffee are generated while others are affected (mostly chlorogenic acids) by the heat treatment. During this process, some other compounds (like melanoidins or quinic acid) appear, which contributes to coffee's antioxidant capacity (Ludwig, Bravo, De Peña, & Cid, 2013). Generally, the main changes in composition involve loss of polysaccharides, oligosaccharides, chlorogenic acids, and trigonelline (Gniechwitz, Reichardt, Blaut, Steinhart & Bunzel, 2007). Moreover, the lactones of the chlorogenic acids are formed during roasting, but such formation depends on the degree of roasting.

Dietary polyphenols are mostly utilized by the gut microbiota, since most escape digestion and have low absorption rate in the small intestine (Selma, Espín, & Tomás-Barberán, 2009). Phenolic compounds feed the human gut microbiota, and microbial metabolites of polyphenols are absorbed in the large intestine and reach the blood stream (Russell & Duthie, 2011). It has been reported that coffee is able to change some microbial communities and modify the end-products of community metabolism (Cowan et al., 2014). On the other hand, roasted coffee is also able to modify the gut microbiota due to the high content of melanoidins, which can behave as fiber-like compounds in the gut (Jiménez-Zamora et al., 2015).

Taking all this information into account, green and roasted coffee could be considered as two different products due to the different chemical composition they have, which in turn will shape the composition and functionality of the gut microbiota. Therefore, the aim of the present paper is to unravel the differences between green and roasted coffee after *in vitro* digestion-fermentation process. To do that, the differences in antioxidant capacity, individual polyphenols and gut microbiota structure of digested-fermented extracts of three green and roasted coffees from different locations were studied.

2. Materials and methods

2.1. Chemicals

Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 2,4,6-Tri(2-pyridyl)-*s*-triazine (TPTZ), potassium persulphate, 3,5-dicaffeoylquinic acid, caffeic acid, dimethyl caffeic, chlorogenic acid, ferulic acid, coumaric acid, gallic acid, tyrosol, p-hydroxybenzoic

acid, m-hydroxyphenylacetic acid, acetic acid, propionic acid, butyric acid, Folin-Ciocalteu reagent, sodium hydroxide, hydrochloric acid, iron (III) chloride hexahydrate, sodium acetate, potassium chloride, potassium di-hydrogen phosphate, sodium mono-hydrogen carbonate, sodium chloride, magnesium chloride hexahydrate, ammonium carbonate, calcium chloride dihydrate, sodium di-hydrogen phosphate, tryptone, cysteine, sodium sulphide, resazurin, salivary alpha-amylase, pepsin from porcine, bile acids (porcine bile extract) and ethanol were from Sigma-Aldrich (Darmstadt, Germany). Pancreatin from porcine pancreas was purchased from Alpha Aesar (United Kingdom).

2.2. Samples

Coffee beans, green and roasted, were supplied by the local company Cafes Cumbal (Granada, Spain). Green coffee beans from three different locations (Brazil, Colombia and Vietnam) and their respective commercial roasted coffees were coded as follows: Brazil Arabica green coffee (BA), Brazil Arabica roasted coffee (BAR), Colombia Arabica green coffee (CA), Colombia Arabica roasted coffee (CAR), Vietnam Robusta green coffee (VR) and Vietnam Robusta roasted coffee (VRR). Coffee beans were ground using a coffee mill and coffee infusions were prepared using a mocha coffeemaker. Right after milling, 40g of ground coffee were extracted with 500 mL of distilled water using a mocha coffee machine. Aliquots were taken for individual phenolics, *in vitro* digestion-fermentation, and antioxidant capacity assays. For antioxidant capacity and individual phenolics assays three different fractions for each sample were collected: one coming from the coffee brew prior to digestion-fermentation, another one coming from the *in vitro* digestion and the last one coming from the *in vitro* fermentation. These fractions will be called from now on “coffee brew”, “digested coffee brew” and “fermented coffee brew”, respectively.

2.3. In vitro gastrointestinal digestion

All samples were subjected to an *in vitro* digestion process followed by an *in vitro* fermentation to mimic physiological processes in the human gut. The *in vitro* digestion method was carried out according to the protocol described by Pérez-Burillo, Rufián-Henares, & Pastoriza (2018). The gastrointestinal *in vitro* digestion was composed of an oral phase (5 minutes at 37ºC with alpha-amylase 75 U/mL, pH 7.0), a gastric phase (2 hours at 37ºC with pepsin 2000U/mL at pH 3.0) and an intestinal phase (2 hours at 37ºC with pancreatin 13.37 mg/mL at pH 7.0).

2.4. In vitro fermentation

The *in vitro* fermentation was carried out according to the protocol described by Pérez-Burillo et al. (2018). *In vitro* fermentation was carried out using faecal samples from three healthy donors (not taking antibiotics, people with body mass index within the "normal weight range", mean Body Mass Index = 21.3). The solid residue obtained after *in vitro* gastrointestinal digestion plus 10% of the digestion supernatant was fermented (500 mg).

After *in vitro* gastrointestinal digestion and *in vitro* fermentation three different fractions were obtained: digestion supernatant (fraction available for absorption at the small intestine), fermentation supernatant (fraction available for absorption at the large intestine) and fermentation solid residue (fraction not available for absorption and excreted with feces).

2.5. Antioxidant assays

The antioxidant capacity of the undigested, digested, and fermented (supernatant) coffee brews was measured with three different methods:

- *$TEAC_{ABTS}$ assay:* The antioxidant capacity was estimated in terms of radical scavenging activity, following the procedure described by Re et al. (1999). Briefly, ABTS was produced by reacting ABTS stock solution (7 mM) with potassium persulphate (2.45 mM) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS solution was diluted with an ethanol and water mixture (50:50) to an absorbance of 0.70 ± 0.02 at 730 nm. After placing 20 µL of sample or Trolox standard and 280 µL of diluted ABTS solution on a transparent 96-well polystyrene microplate (Biogen Científica, Spain), absorbance readings were taken every 60 seconds for 20 minutes on a FLUOStar Omega microplate reader (BMG Labtech, Germany) with temperature control (37ºC). Calibration was performed with a Trolox stock solution ranging from 0.01 to 1.00 mg/mL. The results obtained are expressed as µmol Trolox equivalents per mL of sample.

- *$TEAC_{FRAP}$ assay:* The ferric reducing ability of each sample solution was estimated according to the procedure described by Benzie & Strain (1996) and adapted to a microplate reader. Briefly, 280 µL of FRAP reagent, prepared freshly and warmed at 37ºC, were mixed in each well of a transparent 96-well polystyrene microplate (Biogen Científica, Spain) with 20 µL of sample or water to provide appropriate blank reagent. The FRAP reagent contained 2.5 mL of a TPTZ solution (10 mM) in HCl (40 mM), plus 2.5 mL of $FeCl_3 \cdot 6H_20$ (20 mM), and 25 mL of acetate buffer (0.3 M; pH 3.6). Readings of maximum absorbance (595 nm) were taken every 60 seconds using a FLUOStar Omega microplate reader (BMG Labtech, Germany). The temperature was maintained at 37ºC and the reaction was monitored for 30 min. Trolox stock solutions ranging from 0.01 to 1.00

mg/mL were used to perform the calibration curves. The results obtained are expressed as μmol Trolox equivalents per mL of sample.

- *Folin-Ciocalteu (total phenolics assay):* The Folin-Ciocalteu method is widely used to measure the amount of total polyphenols in foods, although it also measures the antioxidant capacity of foods due to the similar mechanism involved in the chemical reaction (as in the case of the FRAP and ABTS methods). The procedure described by Singleton & Rossi (1965) was used with few modifications, and adapted to a microplate reader. Briefly, sodium carbonate 10% was prepared and then the needed volume was diluted to 2.35%. 30 μL of sample were placed into the wells of a transparent 96-well polystyrene microplate (Biogen Científica, Spain), followed by 255 μL of the sodium carbonate and finally 15 μL of the Folin-Ciocalteu reagent. All tests were run in triplicate. Absorbance measurements at 725 nm were taken for 60 minutes at 37ºC. The equipment used was a FLUOStar Omega microplate reader (BMG Labtech, Germany). Calibration curve was performed using Gallic acid as the standard with concentrations ranging from 0.01 to 1.00 mg/mL. The results obtained are expressed as μmol Gallic acid equivalents per mL of sample.

2.6. *Short chain fatty acids determination*

The production of short chain fatty acids (SCFAs) as a measure of the gut microbiota functionality was assessed according to the procedure described in by Delgado-Andrade et al. (2017) with few modifications. The analysis of SCFAs was carried out on Accela 600 HPLC (Thermo Scientific) equipped with a pump, an autosampler and a UV-VIS PDA detector set at 210 nm; the mobile phase used was 0.1 M phosphate buffer (pH 2.8)/acetonitrile 99:1 v/v delivered at a 1.25 mL/min flow rate; the column used was an

Aquasil C18 reverse phase (Thermo Scientific) (150 × 4.6 mm, 5 μm), with a total run-time of 30 min. The sample did not require any pretreatment before injecting. Briefly, the SCFAs standards were prepared in the mobile phase at concentrations ranging from 5 to 10000 ppm. After the fermentation process, 1 mL of fermentation supernatant was centrifuged to remove solid particles, filtered through a 0.22 μm nylon filter and finally transferred to a vial for HPLC analysis.

2.7. Individual phenolic analysis

The profile of individual polyphenols was obtained by means of UPLC-MS/MS-QTOF equipment (Esteban-Muñoz, Barea-Álvarez, Oliveras-López, Giménez-Martínez, Rufián-Henares & Olalla-Herrera, 2018).

- *Extraction procedure.* One mL of sample was mixed with 1 mL of diethyl ether in a 2 mL tube and was kept with agitation at 5ºC wrapped in foil for 24 hours. Then the supernatant was recovered and poured into a 10 mL tube. Right after, 1 mL of diethyl ether was added again to the 2 mL tube, vortexed and the supernatant was recovered and poured into a 10 mL tube. This step was repeated once. Sodium sulphate anhydrous was added to remove any humidity and the 3 mL aliquot was vacuum-evaporated at 30ºC. Finally, the solid residue was resuspended in 1 mL of a water-methanol mix (50:50 v/v) and the sample was filtered through a 0.20 μm filter, making it ready for UPLC analysis.

- *UPLC-MS/MS-QTOF equipment and operating conditions.* The ESI-MS2 experiments were performed on a liquid chromatography system hybrid mass spectrometer UPLC-MS/MS-QTOF Synapt G2 HDMS (Waters, Mildford, USA). The UPLC separation was performed using in an ACQUITY UPLC™ system, equipped with a chromatographic

column HSS T3 2.1 x 100 mm, 1.8 mm. The elution program was set with a binary gradient consisting of (A) water with 0.5% acetic acid and (B) acetonitrile, as follows: 0.0–15.0 min, 5% (B); 15.0–15.1 min, from 5 to 95% (B); and 15.1–18.0 min, from 95% to 5% (B). Ten microliters of sample were injected and the flow rate was set at 0.4 mL/min. The TOF conditions consisted of a full MS, and data-dependent scanning was performed in negative mode with electrospray ionisation (ESI).

- *Identification and quantification.* Individual phenolic compounds were identified by comparing the negative masses recorded in previous research, using the MassLynx V4 software (Waters Laboratory Informatics, Mildford, USA) for instrument control, data acquisition and data analysis. Individual phenolic compounds were quantified by obtaining a series of solutions, with a concentration of 0.1-40ppm of standard with different retention times. For each phenolic compound selected, a five-point calibration curve was performed (with $R^2 \geq 0.9$) to ensure the linearity of the method. The standards were analysed under the same working conditions as the samples. Identification was performed by means of the retention times and mass spectrum of the corresponding standards.

2.8. High-throughput amplicon sequencing.

Bacterial genomic DNA was isolated from each fermented sample using ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, Irvine, USA) as previously stated (Rigsbee, Agans, Foy, & Paliy, 2011). The V4 region of the 16S rRNA gene was amplified using primers complementary to flanking conserved sequences (forward primer 16S gene complementary sequence GCCAGCMGCCGCGG and reverse primer 16S gene complementary sequence GGACTACHVGGGTWTCTAAT). Forward primers also contained Ion Torrent P1 adapter sequence and 6-nucleotide barcode. PCR amplification

was performed with 25ng of starting DNA material and included 10 cycles of linear elongation with only the forward primers used, followed by 25 cycles of traditional exponential PCR (Paliy & Foy, 2011). Inclusion of linear PCR step decreased the stochasticity of first few PCR reaction steps (Rigsbee et al., 2011) and allowed the use of a single PCR amplification reaction per sample. Purified amplicons were pooled equimolarly and sequencing libraries were prepared with the Ion PGM Template OT2 400 kit (Life Technologies, Inc.) according to the manufacturer's protocol. High throughput sequencing was performed on Ion Torrent PGM using Ion PGM Sequencing 400 kit and Ion 316 chip. An average of 16,954 sequence reads per sample were obtained and were processed in QIIME as previously described (Shankar *et al.*, 2017). Sequence read counts for each operational taxonomic unit (OTU) were adjusted by dividing them by known or predicted number of 16S rRNA gene copies in that organism's genome following a previously described approach (Rigsbee et al., 2011). Thus derived cell counts were sub-sampled (rarefied) to the lowest value among all samples.

Statistical analysis

Statistical significance of the data and differences among samples were tested by Student's t-test at $p < 0.05$ significance level. Evaluation of the relationship among different assays was carried out by computing the Pearson correlation coefficient. These statistical analyses were performed using Statgraphics Plus software (Statpoint Technologies, Inc., The plains, USA), version 5.1, 2001 and R version 3.4.0.

Multivariate statistical analyses were performed on the genus-level microbial abundance dataset generally following the approaches previously described (Paliy & Shankar, 2016). These included unconstrained principal coordinates analysis (PCoA)

utilizing phylogenetic weighted UniFrac distance as a measure of sample dissimilarity, and orthogonal projections to latent structures discriminant analysis (OPLS-DA). The statistical significance of the separation of green and roasted coffee samples in PCoA space was determined by a permutation test of the Davies-Bouldin index measure ran with 1,000 iterations. DB index compares the intra-cluster distances among samples to the distance between cluster centroids.

3. Results and discussion

3.1. Antioxidant capacity

ABTS assay. The antiradical antioxidant capacity of coffees measured with the ABTS assay showed values in the same range for the regular coffee brew, digested and fermented coffee brews (**Figure 1A**). Regular coffee brews had values ranging from 18.1 to 30.1 mmol Trolox equivalents/L for BA and VRR, respectively. In general, undigested coffee brews obtained from roasted coffees were statistically more antioxidant ($p < 0.05$) than their respective green coffees, with an exception of Brazilian coffee. The effect of roasting over the antioxidant capacity of coffee has been previously studied, though there are some discrepancies in the matter. For example, some reports demonstrated an increase on antioxidant capacity with roasting (Liang, Xue, Kennepohl, & Kitts, 2016), while others described a decrease in antioxidant activity (Perrone, Farah, & Donangelo, 2012). During roasting some compounds, mainly phenolic acids like chlorogenic acids, are either incorporated into melanoidins or degraded, which could result in a loss of antioxidant capacity (Perrone et al., 2012). However, the high temperatures used during coffee roasting allow the generation of other compounds (like melanoidins) via the Maillard reaction; these compounds can also contribute to antioxidant capacity of the coffee brew, compensating for

the degradation of polyphenols (Ludwig et al., 2013). Another potential explanation to such differences could be related with the amount of extracted solids (Duarte, Abreu, Menezes, dos Santos & Gouvea, 2005): roasting degree influences solids extraction, so that roasting increases yield of extraction, and a roasted coffee brew (with more solids) may have higher antioxidant activity than green coffee brew (with a lower amount of solids).

When coffee brews were subjected to *in vitro* digestion, the antioxidant capacity ranged from 13.8 to 36.4 mmol Trolox equivalents/L for BA and VRR, respectively. In the case of fermentation, the antioxidant activity was between 21.3 and 36.4 mmol Trolox equivalents/L for VR and BA, respectively **(Figure 1A)**. Digestion of roasted coffees resulted in higher antioxidant capacity ($p < 0.05$) compared to green coffees except for Colombian coffees (as in the case of regular coffee brews). Surprisingly, no differences were found for fermented coffee brews except for Brazilian green coffee, which was more antioxidant ($p < 0.05$) than its roasted counterpart. Altogether, these results point out to a low effect of the digestion process on the antioxidant capacity of coffee brews, while the activity of microorganisms during fermentation could release more antioxidant compounds (or modify their structure allowing then a higher antioxidant activity) from green coffees. This could be related to the microbial metabolism of coffee polysaccharides present in the green coffee (Gniechwitz et al., 2007) or polyphenolic compounds that are not present in roasted coffee (Selma et al., 2009; Russell & Duthie, 2011; Liang et al., 2016).

FRAP assay. The analysis of the reducing capacity of samples with the FRAP assay showed higher values ($p < 0.05$) for digested and fermented coffee brews than in the case of the ABTS assay (**Figure 1B**). For regular coffee brews the reducing capacity was significantly higher ($p < 0.05$) in roasted coffees, which is in accordance with the results obtained from the ABTS method. In this case the values ranged from 14.0 to 24.8 mmol

Trolox equivalents/L for BA and VRR coffees, respectively. When the samples were subjected to digestion, only the Vietnamese coffee showed significant differences between green and roasted varieties, being again roasted coffee higher than green coffee ($p < 0.05$). The antioxidant capacity values of digested samples were noticeably higher and ranged from 41.6 to 76.8 mmol Trolox equivalents/L for VR and CAR, respectively. The results obtained with the FRAP method about the higher antioxidant capacity of roasted coffees could reinforce the idea that some compounds (like polyphenols) are lost during roasting, while others appear (Liang et al., 2016). It is noteworthy to mention that the reducing capacity of fermented samples was very high, ranging from 99.7-120 mmol Trolox equivalents/L (**Figure 1B**). However, no significant differences were found among the assessed coffees. Such large values of antioxidant capacity could be related to the generation of new molecules arising from the microbial fermentation of melanoidins (Jiménez-Zamora et al., 2015), polysaccharides (Asano, Ikeda, Fujii, & Iino, 2007) and phenolic compounds (Selma et al., 2009).

Folin-Ciocalteu assay. The measurements of total phenolic content showed a similar tendency to that revealed with the FRAP assay. The content of polyphenols was higher in the digested and fermented coffee brews ($p < 0.05$) than in regular coffee brews (**Figure 1C)**. In this sense, values ranging from 1802 to 2587 mg of gallic acid/L were obtained for regular coffees, from 3153 to 4693 mg of gallic acid/L for digested coffees and from 4692 to 7034 mg of gallic acid/L in the case of fermented ones. No statistically significant differences ($p < 0.05$) were found between green and roasted coffees except for digested BAR and fermented VRR (see **Figure 1C**). These results suggest that roasted brews have somewhat higher content in phenolic compounds, which could be explained by the degradation of chlorogenic acids to their corresponding hydroxycinnamates such

caffeic, ferulic, and quinic acids (Liang et al., 2016). In addition, it has been shown that the content of benzoic acids is higher in roasted coffee than in green coffee (Köseoğlu-Yılmaz & Kolak, 2017). Even more, the analysis of individual polyphenols (**Table 1; Figure 2**) reflects a higher content of m-hydroxybenzoic and p-hydroxybenzoic acids in roasted coffees than in green ones, which could lead to a higher value in the Folin-Ciocalteu assay.

3.2. Individual phenolic compounds

Nine individual phenolic compounds were quantified in the analyzed coffee brews: 3,4-di-O-Caffeoylquinic acid, caffeic acid, dimethyl caffeic acid, chlorogenic acid, ferulic acid, coumaric acid, tyrosol, m-hydroxybenzoic acid and p-hydroxyphenylacetic acid. The polyphenolic profile of regular and digested coffee brews was quite similar (**Table 1; Figure 2**) although the amounts of all the studied phenols in regular coffee brews was slightly higher (not statistically significant) than those found in digested coffee brews. The concentration of individual polyphenols ranged from 0.81 to 11315 mg/L for tyrosol and p-hydroxyphenyl acetic acid, respectively. In addition, the levels of the chlorogenic acids fraction were much higher in green coffees than in their respective roasted ones (**Table 1**). This is in agreement with the findings reported by other authors (Clifford, 2000; Perrone et al., 2012; Ludwig et al., 2013; Liang et al., 2016) and is explained by the degradation of such compounds during roasting. On the other hand, higher amounts of caffeic and ferulic acid were found in roasted coffees, which could be related to the degradation of chlorogenic acids during roasting, yielding their corresponding cinnamates (Clifford, 2000). Moreover, m-hydroxybenzoic and p-hydroxyphenylacetic acids were found only in roasted coffees. These phenolic compounds could be products of the degradation of more complex polyphenols during roasting, since m-hydroxybenzoic acid was not detected in green coffee

and the levels of p-hydroxyphenylacetic acid were 100-fold lower in green coffee samples compared to roasted coffees.

The phenolic profile found in fermented coffee brews was different from either digested or regular coffees (**Table 1; Figure 2**). Caffeic and ferulic acids were detected, but in much lesser amounts than in regular or digested coffee brews. Hydroxycinnamates are extensively metabolized by the gut microbiota, which could explain the decrease (even disappearance) of these polyphenols in fermented samples (Clifford, 2000; Selma et al., 2009; Russell & Duthie, 2011). The main products are usually hydroxybenzoic acids such as m-hydroxybenzoic and p-hydroxyphenylacetic acids (Selma et al., 2009). Thus, the higher amount of these metabolites found in fermented samples could be related to the microbial metabolism of other phenolic compounds.

The antioxidant activity of phenolic compounds depends on their redox potential. Both FRAP and ABTS are able to measure compounds in the same range of redox potential (<0.7 V) and this ability is related, in the case of polyphenols, to the degree of hydroxylation and conjugation. Higher ability to donate electrons (more OH groups and/or lower redox potential) leads to higher antioxidant capacity. According to Šeruga & Tomac, (2016), chlorogenic acids have a higher redox potential than caffeic acid and di-chlorogenic acids have higher potential than chlrogenic acids. On the other hand, caffeic acid has a lower redox potential than ferulic and coumaric acid (ferulic < coumaric) (Kilmartin, 2001). Hydroxycinnamic acids usually have lower redox potential than chlorogenic acids. Accordingly, higher hydroxycinnamic acids content should lead to higher antioxidant capacity. Positive and significant ($p < 0.05$) spearman correlations (r values 0.81 and 0.88 respectively) were found between hydroxycinnamic acids concentration in coffee brews and FRAP method values. However, no correlations were obtained with the ABTS method.

A possible explanation is the complexity of antioxidant capacity, depending on many different compounds such as Maillard reaction products, and not only polyphenols. Moreover, no correlations were found with individual phenolics, only with the sum of the hydroxycinnamic acids.

3.3. Short chain fatty acids production

Coffee brews contain polysaccharides that can be fermented by the gut microbiota (Gniechwitz et al., 2007). This polysaccharide fraction is mainly composed of arabinogalactans, galactomannans, and manooligosaccharides (Asano et al., 2007). However, during roasting some of these carbohydrates are degraded, taking part in the Maillard reaction and sugar caramelization. This is the reason for the almost 2-fold higher carbohydrate content of green coffee compared to roasted coffee (Gniechwitz et al., 2007). In addition, different Maillard reaction products are readily metabolized by the gut microbiota (behaving as prebiotics), leading to the formation of SCFAs (Delgado-Andrade et al., 2017).

We have measured the levels of three main short-chain fatty acids (acetate, butyrate, propionate) in all fermented samples (see **Figure 1D)**. Overall, the SCFAs levels are in line with those reported by other authors (Gniechwitz et al., 2007; Delgado-Andrade et al., 2017). The results obtained for acetic acid ranged from 7.81 to 8.37 mM for regular coffee brews. In addition, no statistically significant differences were found among green or roasted coffees ($p > 0.05$); this could probably be due to the presence of melanoidins in roasted coffees, which generate SCFAs upon fermentation (Jiménez-Zamora et al., 2015), compensating for the breakdown of polysaccharides during roasting process. Secondly, the levels of propionic acid were comprised between 2.92 and 5.15 mM. For this SCFA,

statistically higher ($p > 0.05$) levels were found in Brazilian and Vietnamese green coffee fermented beverages, compared to their roasted coffees brews (**Figure 1D**). In the case of butyrate the values ranged from 6.42 to 7.79 mM, being higher ($p > 0.05$) only for the Brazilian green coffee. According to these results, green coffee has a tendency to produce higher amounts of SCFAs, though the differences are relatively small (up to 20%). Fermentation of coffee melanoidins by bacterial species able to produce SCFAs (Jiménez-Zamora et al., 2015) could explain the observed differences between green and roasted coffee SCFAs production.

In order to unravel the overall effect of processing (green *vs.* roasted coffee) or *in vitro* metabolization (regular *vs.* digested *vs.* fermented brew) on SCFAs production, antioxidant capacity and polyphenolic content, a principal component analysis was carried out. As depicted in **Figure 3A**, a clear separation between green and roasted coffee samples was observed, since only two components were able to explain 90.8% of the variability. Regular coffee and digested coffee brews were very similar, while fermented coffee samples were separated in the PCA space, likely because of the significant effect of gut microbiota metabolism **(Figure 3B)**. This makes sense since the properties of the later depends on the gut microbiota metabolism, which in turn change the original profile of bioactive compounds. In this case, a 69.2% of the differences were explained with two principal components.

3.4. Fermentation of green and roasted coffee by human gut microbiota promotes different community structures.

The effect of the different coffee brews on the microbiota community structure was determined after the fecal microbiota fermented coffee samples for 20 hours. Overall, the

community composition was comparable among all samples, with *Escherichia*/*Shigella*, *Bifidobacterium* and *Bacteroides* being the most abundant genera. Among the top nine genera, only *Bacteroides* was statistically differentially abundant between green and roasted coffee samples (**Figure 4A**). However, the differences among other genera were sufficient to separate these two types of samples in PCoA space with statistical significance (**Figure 4B**). OPLS discriminant analysis of microbiome dataset (**Figure 4C**) similarly separated the sample groups well (R^2 = 0.998), though the predictive power of the generated model was modest (Q^2 = 0.528). The top discriminating genera included *Escherichia*/*Shigella*, *Lactobacillus,* several *Clostridium* clusters, *Providencia*, *Enterobacter*, and *Bacteroides* (**Figure 4C**). Many of the discriminating genera play a role on human health. The beneficial-detrimental effects of different bacteria are described in **Table 2**.

4. Conclusions

This manuscript reports the effect of *in vitro* digestion-fermentation of green and roasted coffees on the antioxidant capacity, phenolic profile, SCFAs production and microbial community structure. In many cases, roasted coffee brew showed higher antioxidant values than green coffees. Moreover, in FRAP and Folin-Ciocalteu assays, digested coffee brews displayed significantly higher values than regular coffee brews, and even higher values were obtained for fermented coffees. On the other hand, the phenolic profile of regular and digested coffee brews were very similar, while the profile of fermented coffees differed greatly from the others, probably due to microbial metabolism. SCFAs production was higher for microbial communities fermenting green coffee brews. All these data allowed the separation of green and roasted coffees through PCA ordination

analysis. In addition, the metabolite profiling also allowed the differentiation of regular-digested coffee brews from fermented ones with PCA. Finally, the microbial community structure also differed between fermented green and roasted coffee samples. Therefore, green and roasted coffees behave in many ways as different types of food and therefore they should probably be considered as such, especially taking into account the effect of the gut microbiota on human health. More studies to link specific compounds from green or roasted coffee with individual microbial taxa are needed.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Figure captions

Figure 1. Antioxidant capacity (**1A**, **1B**), total polyphenols (**1C**) and SCFAs (1**D**) of regular, digested and fermented green and roasted coffee brews. Abbreviations: BA (green coffee beans from *Brassica arabica*), CA (green coffee beans from *Colombia arabica*), VR (green coffee beans from *Vietnam robusta*), BAR (roasted coffee beans from *Brassica arabica*), CAR (roasted coffee beans from *Colombia arabica*), and VRR (roasted coffee beans from *Vietnam robusta*). Ns - not significant, * - $p \leq 0.05$), ** - $p \leq 0.01$.

Figure 2. Heatmap representation of polyphenols amounts in different coffee brews. Amounts are represented by a color gradient as shown in the legend; note the log2-scale of the gradient.

Figure 3. PCA ordination analysis of the dataset of measured metabolites and antioxidant capacities. Panel **A** shows the separation between green and roasted coffee. Panel **B** shows the separation between regular coffee brew, digested coffee brew and fermented coffee brew. Different groups are denoted by colors as shown in the legend. Group clouds represent areas of three standard errors around the group centroid (diamond). P_{DB} values denote the statistical significance of the separation of green and roasted coffee samples in PCoA space based on the permutation analysis of Davies-Bouldin index as described (Shankar et al., 2015). The percent of dataset variability explained by each principal coordinate is shown in parentheses in axis titles.

Figure 4. Fermentation of green and roasted coffee by human fecal microbiota promotes different community structures. Panel **A** displays relative abundances of the

top nine most abundant microbial genera. Each column represents a community derived from an independent fermentation of green or roasted coffee sample. Abundances of other genera were summed and are represented cumulatively as "other genera". Star represents the statistically significant difference (at $p < 0.05$ level) in the abundance of *Bacteroides* genus between sample types as determined by paired samples T-test. Panel **B** shows the output of the unconstrained PCoA ordination analysis of genus abundance dataset among all profiled samples. Phylogenetic weighted UniFrac distance was used to calculate the sample dissimilarity matrix. Different groups are denoted by colors as shown in the legend. Group clouds represent areas of three standard errors around the group centroid (diamond). P_{DB} value denotes the statistical significance of the separation of green and roasted coffee samples in PCoA space based on the permutation analysis of Davies-Bouldin index as described (Shankar et al., 2015). The percent of dataset variability explained by each principal coordinate is shown in parentheses in axis titles. Panel **C** displays the T-vs-$T_{orthogonal}$ scatter plot of the OPLS discriminant analysis of the genus abundance dataset. R^2Y denotes the amount of group-related variation in the data explained by the model. The genera with the largest discriminating weights are listed in the tables. IS – incertae sedis.

Figure 1

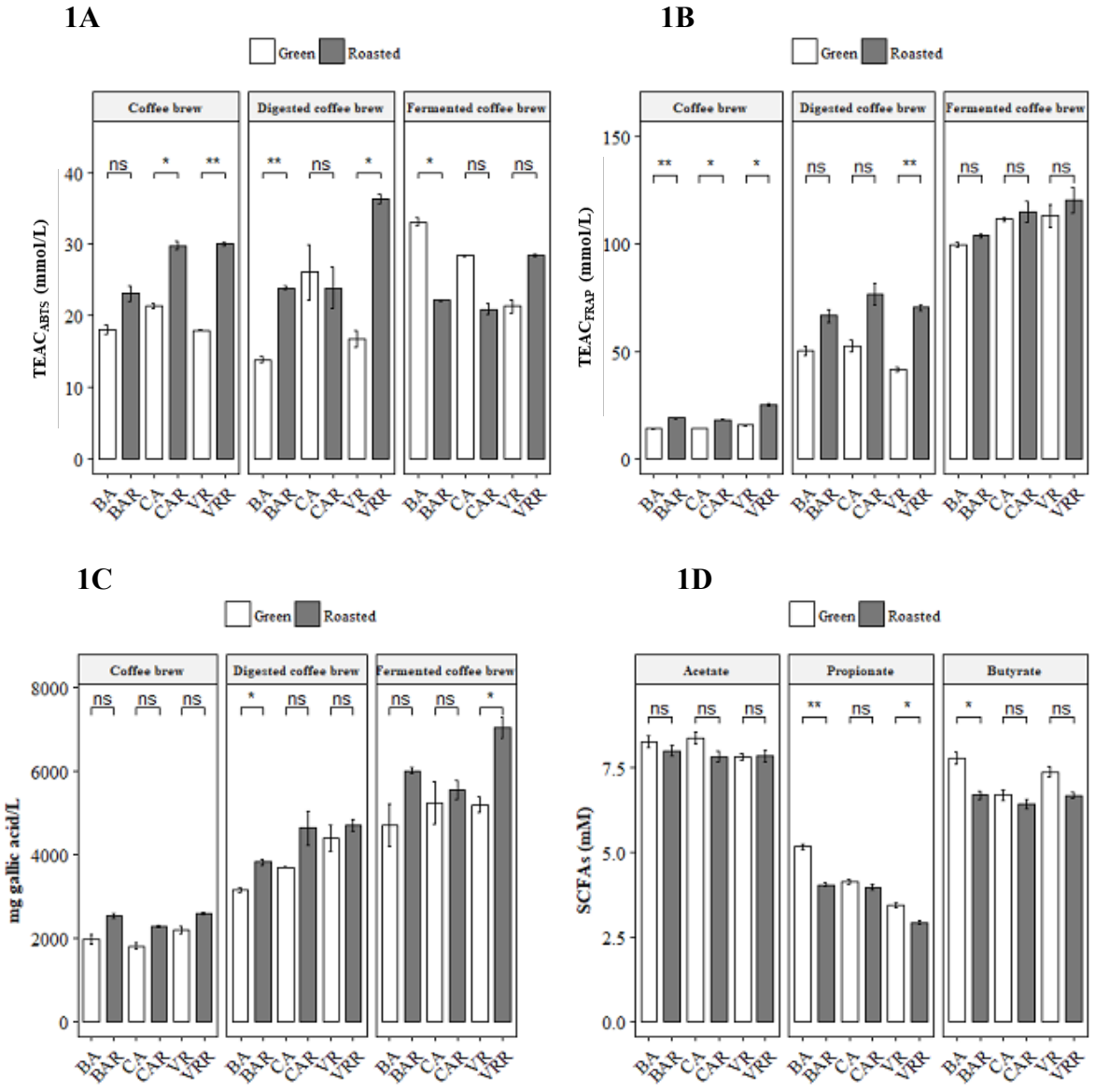
1A
Green
Roasted
Coffee brew
Digested coffee brew
Fermented coffee brew
TEAC$_{ABTS}$ (mmol/L)
ns
*
**
BA
BAR
CA
CAR
VR
VRR
1B
TEAC$_{FRAP}$ (mmol/L)
1C
mg gallic acid/L
1D
Acetate
Propionate
Butyrate
SCFAs (mM)

Figure 2

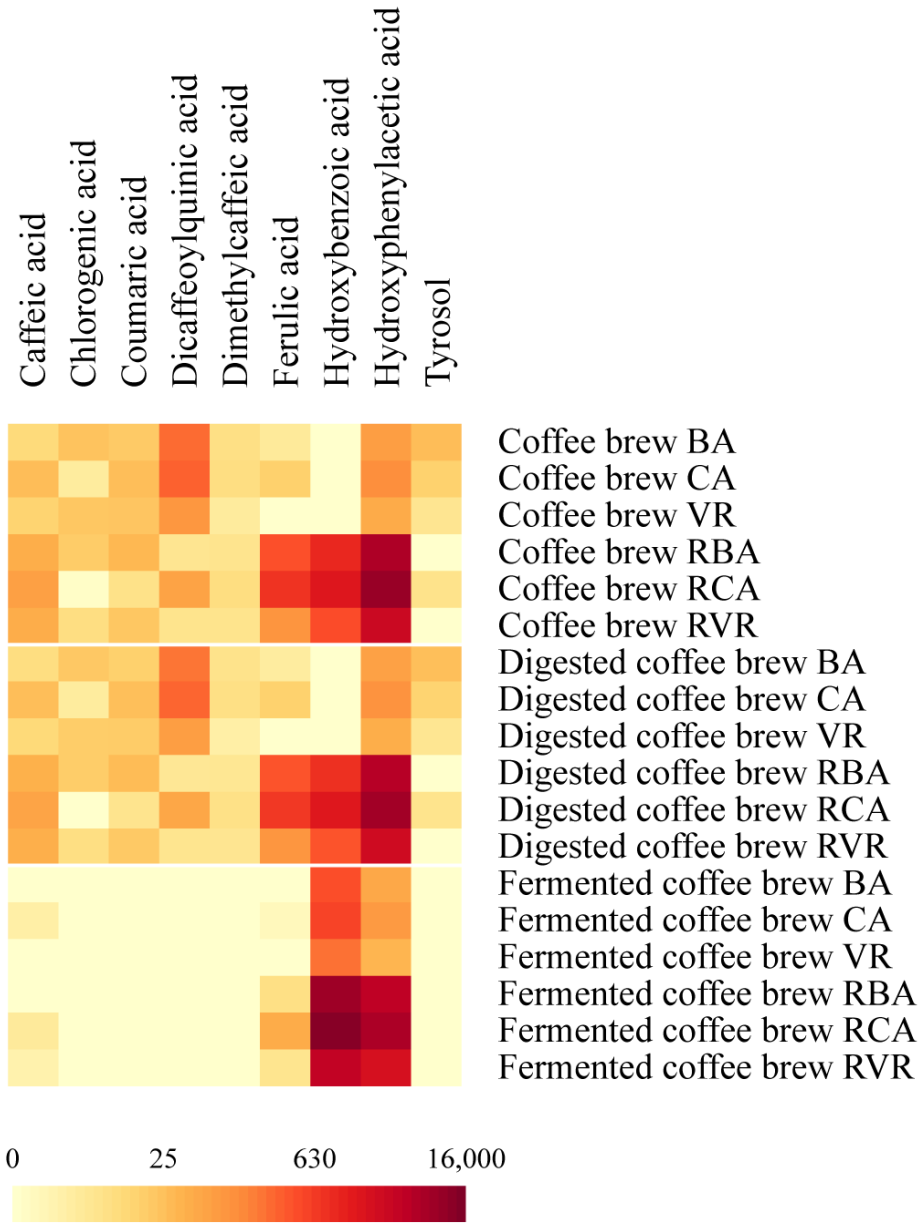
Caffeic acid
Chlorogenic acid
Coumaric acid
Dicaffeoylquinic acid
Dimethylcaffeic acid
Ferulic acid
Hydroxybenzoic acid
Hydroxyphenylacetic acid
Tyrosol
Coffee brew BA
Coffee brew CA
Coffee brew VR
Coffee brew RBA
Coffee brew RCA
Coffee brew RVR
Digested coffee brew BA
Digested coffee brew CA
Digested coffee brew VR
Digested coffee brew RBA
Digested coffee brew RCA
Digested coffee brew RVR
Fermented coffee brew BA
Fermented coffee brew CA
Fermented coffee brew VR
Fermented coffee brew RBA
Fermented coffee brew RCA
Fermented coffee brew RVR
0
25
630
16,000

Figure 3

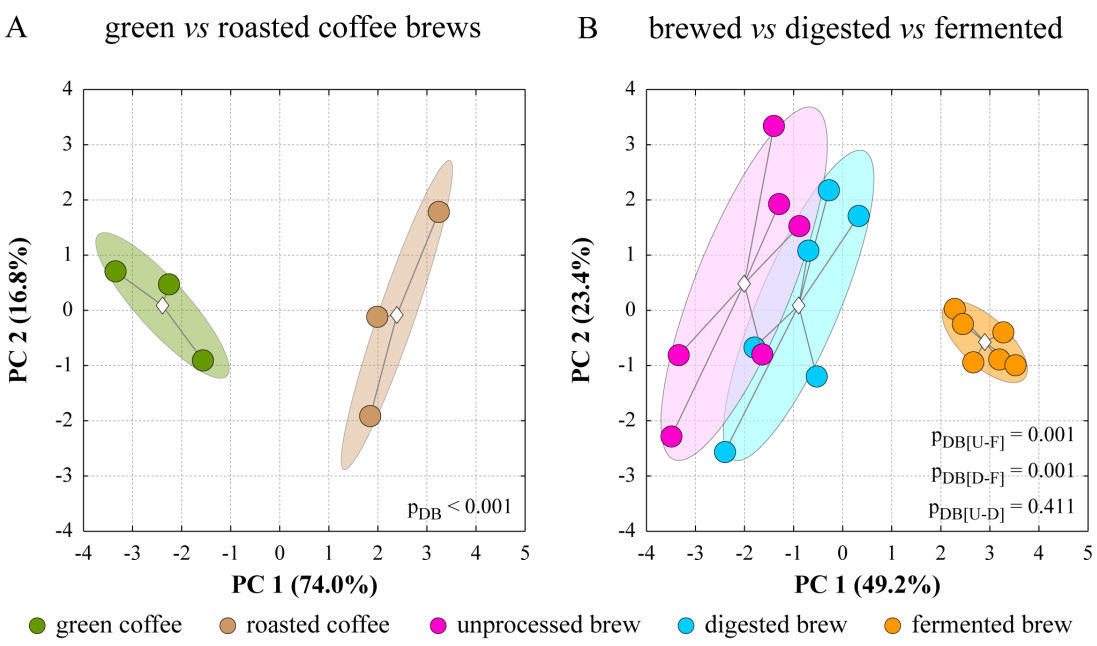
A
green *vs* roasted coffee brews
B
brewed *vs* digested *vs* fermented
PC 2 (16.8%)
PC 1 (74.0%)
$p_{DB} < 0.001$
PC 2 (23.4%)
PC 1 (49.2%)
$p_{DB[U-F]} = 0.001$
$p_{DB[D-F]} = 0.001$
$p_{DB[U-D]} = 0.411$
green coffee
roasted coffee
unprocessed brew
digested brew
fermented brew

Figure 4

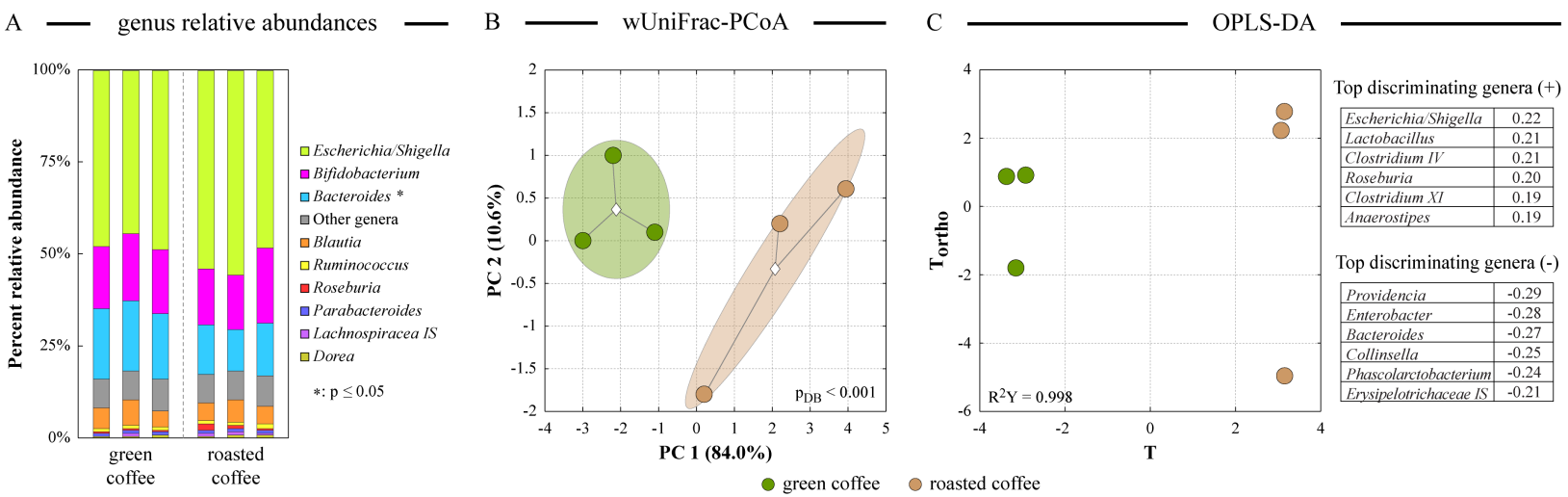
A
genus relative abundances
B
wUniFrac-PCoA
C
OPLS-DA
Percent relative abundance
100%
75%
50%
25%
0%
green coffee
roasted coffee
Escherichia/Shigella
Bifidobacterium
Bacteroides *
Other genera
Blautia
Ruminococcus
Roseburia
Parabacteroides
Lachnospiracea IS
Dorea
*: p ≤ 0.05
PC 2 (10.6%)
PC 1 (84.0%)
pDB < 0.001
Tortho
T
R2Y = 0.998
green coffee
roasted coffee
Top discriminating genera (+)
Escherichia/Shigella 0.22
Lactobacillus 0.21
Clostridium IV 0.21
Roseburia 0.20
Clostridium XI 0.19
Anaerostipes 0.19
Top discriminating genera (-)
Providencia -0.29
Enterobacter -0.28
Bacteroides -0.27
Collinsella -0.25
Phascolarctobacterium -0.24
Erysipelotrichaceae IS -0.21

Table 1. Individual phenolic compounds (mg/L) in coffee brews and digestion-fermentation extracts.

Samples	3,4-Di-O-Caffeoylquinic acid	Caffeic acid	Dimethyl Caffeic acid	Chlorogenic acid	Ferulic acid	Coumaric acid	Tyrosol	m-Hydroxybenzoic acid	p-Hydroxyphenyl acetic acid
BA brew	268 ± 6.32^{a}	9.91 ± 0.24^{a}	7.67 ± 0.18^{a}	23.3 ± 0.47^{a}	4.09 ± 0.09^{a}	18.3 ± 0.51^{a}	28.0 ± 0.57^{a}	ND	78.6 ± 2.37^{a}
CA brew	317 ± 3.90^{b}	28.4 ± 0.36^{b}	8.19 ± 0.10^{a}	3.63 ± 0.04^{b}	15.1 ± 0.17^{b}	27.0 ± 0.39^{b}	14.6 ± 0.15^{b}	ND	126 ± 1.98^{b}
VR brew	99.1 ± 4.19^{c}	13.5 ± 0.59^{c}	3.76 ± 0.16^{b}	20.7 ± 0.74^{c}	1.01 ± 0.04^{c}	21.9 ± 1.09^{a}	5.31 ± 0.19^{c}	ND	51.0 ± 2.75^{c}
BAR brew	5.43 ± 0.34^{d}	45.3 ± 2.91^{d}	5.80 ± 0.36^{c}	18.1 ± 0.96^{c}	463 ± 25.7^{d}	32.9 ± 2.41^{c}	0.87 ± 0.05^{d}	1161 ± 61.5^{a}	7383 ± 321^{d}
CAR brew	65.3 ± 2.69^{e}	72.9 ± 3.09^{e}	8.41 ± 0.35^{a}	1.15 ± 0.04^{d}	870 ± 32.0^{e}	6.72 ± 0.33^{d}	6.30 ± 0.22^{c}	1910 ± 66.9^{b}	11315 ± 594^{e}
VRR brew	6.01 ± 0.15^{d}	46.9 ± 1.23^{d}	6.00 ± 0.15^{c}	8.11 ± 0.17^{e}	106 ± 2.41^{f}	20.7 ± 0.62^{a}	ND	499 ± 10.8^{c}	3609 ± 116^{f}
BA Digested	222 ± 3.02^{a}	8.42 ± 0.12^{a}	6.21 ± 0.08^{a}	20.3 ± 0.23^{a}	3.52 ± 0.04^{a}	15.4 ± 0.25^{a}	26.7 ± 0.31^{a}	ND	67.6 ± 1.17^{a}
CA Digested	301 ± 7.10^{b}	27.6 ± 0.67^{b}	7.62 ± 0.18^{b}	3.59 ± 0.07^{b}	14.8 ± 0.31^{b}	25.9 ± 0.72^{b}	13.7 ± 0.28^{b}	ND	109 ± 3.30^{b}
VR Digested	78.2 ± 4.20^{c}	10.9 ± 0.60^{a}	2.90 ± 0.16^{c}	17.1 ± 0.78^{c}	0.83 ± 0.04^{c}	17.6 ± 1.11^{a}	5.10 ± 0.23^{c}	ND	45.4 ± 3.10^{c}
BAR Digested	4.83 ± 0.16^{d}	41.3 ± 1.38^{c}	5.05 ± 0.16^{d}	16.8 ± 0.46^{c}	426 ± 12.3^{d}	29.6 ± 1.13^{b}	0.81 ± 0.02^{d}	952 ± 26.3^{a}	6054 ± 251^{d}
CAR Digested	56.2 ± 2.36^{e}	64.1 ± 2.78^{d}	7.06 ± 0.30^{b}	1.04 ± 0.04^{d}	774 ± 29.1^{e}	5.85 ± 0.29^{c}	5.92 ± 0.21^{c}	1814 ± 65.0^{b}	916 ± 492^{e}
VRR Digested	5.53 ± 0.06^{d}	44.1 ± 0.48^{c}	5.40 ± 0.06^{a}	7.79 ± 0.07^{e}	101 ± 0.95^{f}	19.2 ± 0.24^{d}	ND	419 ± 3.74^{c}	3139 ± 42.1^{f}
BA Fermented	ND	0.52 ± 0.02^{a}	ND	ND	0.22 ± 0.01^{a}	ND	ND	481 ± 5.23^{a}	54.8 ± 0.56^{a}
CA Fermented	ND	2.90 ± 0.16^{b}	ND	ND	1.55 ± 0.07^{b}	ND	ND	593 ± 54^{c}	88.4 ± 1.89^{c}
VR Fermented	ND	0.02 ± 0.00^{c}	ND	ND	0.00 ± 0.00^{c}	ND	ND	237 ± 10.22^{e}	36.1 ± 1.06^{e}
BAR Fermented	ND	0.74 ± 0.01^{a}	ND	ND	7.64 ± 0.07^{d}	ND	ND	10026 ± 432^{d}	5038 ± 289^{d}
CAR Fermented	ND	3.96 ± 0.16^{d}	ND	ND	47.8 ± 1.72^{e}	ND	ND	15680 ± 475^{d}	7680 ± 412^{d}
VRR Fermented	ND	2.42 ± 0.06^{b}	ND	ND	5.55 ± 0.13^{f}	ND	ND	4732 ± 247^{f}	2530 ± 111^{f}

Different letters within the same column and type of food indicate statistically significant differences ($p < 0.05$).

Table 2. Beneficial or detrimental effects of bacteria in human health.

Bacteria		Health effect	References
Anaerostipes	+	Produce acetic, lactic and butyric acid	Ríos-Covián et al. (2016)
Bacteroides	-/+	*B. fragilis* is involved in inflammatory processes that could lead to colorectal cancer; associated with western diets and low fiber intake	Simpson & Campbell (2015)
Bifidobacterium spp.	+	Reduced in colorectal cancer and in type- I diabetes	Murri et al. (2013)
Clostridium IV	-/+	Reduced in dysbiosis in IBD, seems to be increased in children with food sensitization;	Chen, Chen, Kong, Chang, & Huang (2016)
Clostridium XI	+	Decreased in Budd-Chiari symdrom and increases after treatment along with *Bacteroides*	Sun et al. (2017)
Collinsella	+	Reduced in irritable bowel syndrome patients with more severe symptoms	Malinen et al. (2010)
Enterobacter	-	Member of the ESKAPE pathogens	Pendleton, Gorman & Gilmore (2013)
Escherichia/shigella	-	Responsible for gastrointestinal disorders such as constipation, diarrheic symptoms.	Verbeke, Boesmans, & Boets (2014)
Lactobacillus	+	Represents the largest and most diverse genera of all lactic acid bacteria with uses in biotechnology, medical field, probiotic, etc.	Stefanovic, Fitzgerald, & McAuliffe (2017)
Phascolarctobacterium	+	SCFA producer. It has been found increased in rats fed with inulin	Zhang et al. (2018)
Providencia	-/+	Participates in communication between bacteria and host	Hughes & Sperandio (2008)
Roseburia	+	Associated with weight loss and decreased glucose intolerance in mice, reduced in ulcerative colitis patients, differ in abundance between type II diabetes patients and non-diabetic people	Ryan et al. (2014)