Journal of Agricultural and Food Chemistry 2019, 67, 2500-2509

Spent coffee grounds extract rich in manooligosaccharides promote a healthier gut microbial community in a dose dependent manner

Sergio Pérez-Burillo[1], Silvia Pastoriza[1], Alejandro Fernández-Arteaga[2], Germán Luzón[2], Nuria Jiménez-Hernández[3], Giuseppe D'Auria[3], M. Pilar Francino[3,4] & José Ángel Rufián-Henares[1,5]*

[1]*Departamento de Nutrición y Bromatología, Instituto de Nutrición y Tecnología de los Alimentos, Centro de Investigación Biomédica, Universidad de Granada, Granada, Spain.*

[2]*Departamento de Ingeniería Química, Facultad de Ciencias, Universidad de Granada, Granada, Spain.*

[3]*Unitat Mixta d'Investigació en Genòmica i Salut, Fundació per al Foment de la Investigació Sanitària i Biomèdica de la Comunitat Valenciana (FISABIO-Salut Pública) / Institut de Biologia Integrativa de Sistemes (Universitat de València), València, Spain*

[4]*CIBER en Epidemiología y Salud Pública, Madrid, Spain*

[5]*Instituto de Investigación Biosanitaria ibs.GRANADA, Universidad de Granada, Granada, Spain.*

* Corresponding author:

Telephone: +34 958 24 28 41

E-mail: jarufian@ugr.es

ABSTRACT

Coffee is one of the most consumed beverages around the world and as a consequence, spent coffee grounds are a massively produced residue that is causing environmental problems. Reusing them is a major focus of interest nowadays. We extracted mannoligosaccharides (MOS) from spent coffee grounds and submitted them to an *in vitro* fermentation with human feces. Results obtained suggest that MOS are able to exert a prebiotic effect on gut microbiota by stimulating the growth of some beneficial genera such as *Barnesiella, Odoribacter, Coprococcus, Butyricicoccus, Intestinimonas, Pseudoflavonifractor,* or *Veillonella.* Moreover, SCFA production also increased in a dose dependent manner. However, we observed that 5-hydroxymethylfurfural, furfural and polyphenols (which are either produced or released from spent coffee grounds matrix during hydrolysis) could have an inhibitory effect on some beneficial genera such as *Faecalibacterium, Ruminococcus, Blautia, Butyricimonas, Dialister, Collinsella,* or *Anaerostipes* which could affect negatively to the prebiotic activity of MOS.

KEY WORDS: Mannooligosaccharides, mannan, MOS, short chain fatty acids, gut microbiota, prebiotic activity, 5-hydroxymethylfurfural, polyphenols.

Introduction

Coffee brew is consumed all over the world and, in fact, is the food product most consumed in the world, after water.[1] Coffee has been related to health since ancient times and still today is the main object of study in many research projects related to chronic diseases.[2] Coffee is thought to have a protective role on health, mostly due to its phytochemical compounds such as chlorogenic acids and other polyphenolic substances.[3] However, carbohydrates also account for an important part of the coffee bean. In green coffee, carbohydrates account for up to 60% of the dry weight, with sucrose as the main monosaccharide. Among polysaccharides, which can range from 37 to 55% of the dry weight, cellulose, hemicellulose, galactomannans and arabinogalactans are found. Although galactomannans are the main polysaccharide present in beans, in coffee infusion they are not the main ones due to a low solubility.[4] On the other hand, roasting has an important influence on carbohydrate content. Sucrose and low molecular weight oligosaccharides are almost completely degraded whereas polysaccharide content is reduced to around 24-39% of the dry weight. Roasting also partially hydrolyses polysaccharides to a lower degree of polymerization making them more soluble.[5]

Due to its high consumption, coffee by-products are massively produced specially spent coffee grounds. The main problem is that they are not properly disposed of, generating a growing environmental concern.[5,6] Therefore, spent coffee grounds have become a major focus of interest regarding their reutilization as a source of bioactive compounds.[7-12]

Since hot water is not able to extract the whole carbohydrate fraction during coffee brewing, spent coffee grounds are still rich in polysaccharides, with galactomannans

representing around 50% of them and arabinogalactans and cellulose accounting for the other 50%.[4] Therefore, mannanoligosaccharides (MOS) are one of the bioactive compounds that can be obtained from spent coffee grounds. MOS are short chain carbohydrates produced from coffee galactomannans usually through acid, alkaline or enzymatic hydrolysis.[13] Their physiological effects arise from different functional properties: they are able to enhance the growth of beneficial bacteria, decrease blood pressure, reduce fat absorption, or decrease attachment of pathogenic bacteria to the intestinal mucosa.[14-15]

Finding ingredients that can promote the growth of beneficial gut bacteria is becoming a major focus of interest in order to produce functional foods. That is because now it is very clear that the gut microbiota has important implications in different diseases and is therefore closely related to health status.[16] In this sense, the gut microbiota has been linked to inflammatory bowel disease,[17,18] obesity,[19, 20] autism spectrum disorders,[21] and immune system disorders,[22] among others. It has been reported that around 40 g of polysaccharides reach the colon every day, being an important source of nutrients for gut microbes and acting as prebiotic agents that enhance the growth of some of them.[23] The metabolization of fiber takes places specially in the proximal colon resulting in the production of several metabolites such as short chain fatty acids (SCFAs), mainly acetate, propionate and butyrate, which have an important role in host health, not only in the gut.[24] Dietary fiber is the main nutrient for gut microbes and it has been proven to modulate gut microbiota *in vitro* and *in vivo* with beneficial effects for host health.[23]

Taking all this information into account, the objective of this study was to evaluate the effects of MOS produced from spent coffee grounds on gut microbial communities and also on SCFAs production, after *in vitro* fermentation with human fecal samples.

Materials and methods

Reagents

Acetic, formic and propionic acids, 1-phenyl-3-methyl-5-pyrazolone (PMP), chloroform, mannose, manobiose, manotriose, manotetraose, mannopentaose and mannohexaose standards, sodium di-hydrogen phosphate, tryptone, cysteine, sodium sulphide, resazurin, Folin-Ciocalteu reagent, sodium carbonate, gallic acid, 5-(Hydroxymethyl)furfural (HMF), furfural and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich (Darmstadt, Germany).

Samples

A mix of spent coffee grounds and water (10% w/w) was hydrolyzed in a reactor (270 mL of distilled water and 30 g of spent coffee grounds) by applying high temperatures and high pressure for 60 minutes and taking samples after 10 minutes, 30 minutes and 60 minutes. Four experiments were carried out varying only the temperature: experiment A was carried out at 220 ºC, experiment B was carried out at 185 ºC, experiment C was carried out at 170 ºC and experiment D was carried out at 180 ºC. Each experiment was composed of the three samples taken after 10 minutes, 30 minutes and 60 minutes. Aliquots were taken and stored at -80 ºC for MOS quantification, HMF, furfural and total polyphenols quantification and for *in vitro* fermentation.

In vitro fermentation

The *in vitro* fermentation method was carried out according to the protocol described by Pérez-Burillo et al.[25] A Fecal sample from four healthy donors (not taking antibiotics, people with body mass index within the "normal weight range": mean Body

Mass Index = 21.3), was obtained in the morning, placed in sterile containers and stored at 4 ºC until the inoculum was prepared (as an equal mixture of feces from all four donors). Briefly, 0.5 mL of MOS mixture obtained from the reactor was placed in a screw-cap tube. Then, 7.5 mL of fermentation medium (948.75 mL of peptone water (15 g/L), 50 mL of reductive solution (312 mg of cysteine and 312 mg of sodium sulphide in 48 mL of water and 2 mL of NaOH 1M), and 1.25 mL of resazurin (0.1% w/v)) and 2 mL of inoculum (consisting of a solution of 32% faeces in phosphate buffer 100 mM, pH 7.0) were added, to reach a final volume of 10 mL. Nitrogen was bubbled through the mix to produce an anaerobic atmosphere and the mix was then incubated at 37 ºC for 24 hours under oscillation. Immediately afterwards, the samples were immersed in ice, to stop microbial activity. Aliquots were taken for DNA isolation and SCFAs analysis and stored at -80 ºC until analysis.

Short chain fatty acids determination

Short chain fatty acid (SCFAs) determination was performed on the supernatant obtained after *in vitro* fermentation. The analysis was carried out according to the procedure described by Panzella et al.[11] with few modifications. SCFAs determination 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 SCFA standards were prepared in the mobile phase at concentrations ranging from 5 to 10000

ppm. After the fermentation process, 1 mL of MOS 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. Results are expressed as μmol/g of spent coffee grounds. Chromatograms are depicted in **Figures S1A-S1C**.

MOS HPLC determination

Pre-column derivatization: MOS mixtures obtained from the reactor were labeled with PMP following the procedure described by Zang et al.[26] with few modifications. Briefly, 0.1 mL of sample was mixed in a screw-cap pyrex tube with 0.1 mL of NaOH 0.3 M. Right after, 0.12 mL of PMP 0.5 M was added and the mixture was vortexed for 10 seconds and placed at 70 ^{o}C for 60 minutes. Thereafter, 0.1 mL of HCl 0.3 M was added to neutralize the NaOH. The mixture was then mixed with 0.5 mL of chloroform, vortexed, and the aqueous layer collected, discarding the organic one. This step was done two more times. Samples were filtered through a 0.22 μm filter and injected in the HPLC.

HPLC analysis: Determination of MOS was carried out on Accela 600 HPLC (Thermo Scientific) equipped with a pump, an autosampler and a UV-VIS PDA detector set at 245 nm; the mobile phase used was A) Milli-Q water with 0.1% of formic acid, and B) acetonitrile with 0.1% of formic acid running with the following gradient: 2% of B to 20% of B in 25 minutes (0-25 minute), 20% of B for 15 minutes (25-40 minute), 20% of B to 2% of B in 5 minutes (40-45 minute), and 2% of B for 15 minutes (45-60 minute). Standard curves were performed for each sugar in the range of 5-0.15 ppm. The column used was an Accucore C18 reversed phase 150 mm x 2.1 mm and 2.6 μm of particle size. Results are

expressed as mg/g of spent coffee grounds. Chromatograms are depicted in **Figures S1D-S1F**.

Folin-Ciocalteu total phenolics assay

Total phenolic content was estimated following the procedure described by Singleton and Rossi[27] with few modifications and adapted to a microplate reader. The assay was performed on the samples obtained from the reactor. Briefly, sodium carbonate 10% was prepared and then the needed volume was diluted to 2.35%. 30 µL of sample were placed by triplicate in 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. Absorbance lectures at 725 nm were taken for 60 minutes at 37 ºC. The equipment used was a FLUOStar Omega microplate reader (BMG Labtech, Germany). A 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 (GAE) per g of spent coffee grounds.

HMF and furfural assay

HMF and furfural determination was performed according to the method described by Rufián-Henares et al.[28] One mL of the MOS mixture taken from the reactor was centrifuged at 10000 rpm for 5 minutes. Then, it was filtered through a 0.22 µm filter. The sample was ready for HPLC injection. Determination was carried out on Accela 600 HPLC (Thermo Scientific) equipped with a pump, an autosampler and a UV-VIS PDA detector set at 284 nm. The mobile phase was made up of a mixture of acetonitrile in water (5% v/v)

delivered at the flow rate of 1 mL/min under isocratic conditions through C18 reverse phase column. 20 μL of sample were injected. HMF and furfural was quantified by the external standard method within the range 0.01–50.00 mg/L. Results are expressed as mg/g of spent coffee grounds.

DNA extraction and sequencing

DNA extraction was performed using a NucliSENSEasyMAG platform (Biomérieux) following the standard protocol. Microbial genomic DNA was used at a concentration of 5 ng/μL in 10 mMTris (pH 8.5) for the Illumina protocol for 16S rRNA gene Metagenomic Sequencing Library Preparation (Cod. 15044223 Rev. A). PCR primers targeting the 16S rRNA gene V3 and V4 regions were designed as in Klindworth et al.[29] Primer sequences are: Forward 5'TCGTCGGCAGCGTCAGATG-TGTATAAGAGACAGCCTACGGGNGGCWGCA-G3' and Reverse 5'GTCT-CGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC3'. Primers contained adapter overhang sequences added to the gene-specific sequences, making them compatible with the Illumina Nextera XT Index kit (FC-131-1096). After 16S rRNA gene amplification, amplicons were multiplexed and 1 mL of amplicon pool was run on a Bioanalyzer DNA 1000 chip to verify amplicon size (~550 bp). After size verification, libraries were sequenced in an Illumina MiSeq sequencer according to manufacturer's instructions in a 2x300 cycles paired-end run (MiSeq Reagent kit v3 MS-102-3001).

Bioinformatic analysis

Quality assessment of sequencing reads was performed with the prinseq-lite program applying the following parameters:[30] a minimal length (min_length) of 50 nt and a quality score threshold of 30 from the 3'-end (trim_qual_right), using a mean quality score (trim_qual_type) calculated with a sliding window of 10 nucleotides (trim_qual_window). Read 1 and read 2 from Illumina sequencing where joined using fastq-join from the ea-tools suite.[31] Taxonomic affiliations were assigned using the RDP_classifier from the Ribosomal Database Project (RDP).[32] Reads that had an RDP score below 0.8 were assigned to the next higher taxonomic rank, leaving the last rank as unidentified. We assigned 6 taxonomic levels, which were kingdom, phylum, class, order, family and genus.

Statistical analysis

Pearson correlations were carried out through multivariate analysis with Statgraphics Centurion XVI.I. Bacterial abundances between groups, SCFA, HMF and polyphenols were compared by analysis of variance (ANOVA) at $p < 0.05$ confidence level with Statgraphics Centurion XVI.I and R software.

Results and discussion

MOS quantification

MOS quantification was carried out after 10, 30 and 60 minutes of hydrolysis in every experiment. For each sample, mannose, mannobiose, mannotriose, mannotetraose, mannopentaose and mannohexaose were determined. **Table 1** shows the MOS content in each sample. All carbohydrates were detected except mannotetraose. After 10 minutes, temperature was not stable yet and MOS values are similar among the different experiments. However, experiment B had significantly lower ($p < 0.05$) amounts of total

MOS than the other experiments. The highest levels of mannose, mannobiose and mannopentaose were obtained in experiment A, whereas mannotriose and manohexaose were significantly higher in experiment C.

After 30 minutes of hydrolysis, experiment A showed the highest MOS content with statistical significance ($p < 0.05$), followed by experiments B and D, with no significant differences among the two of them. Finally, experiment C showed significantly lower ($p < 0.05$) amounts than the other three experiments. This makes sense since experiment A was carried out at a higher temperature, whereas in experiment C the lowest temperature was used. On the other hand, experiments B and D showed very similar amounts of MOS, probably due to the very close working temperature. Individually, each sugar followed the tendency described above. Finally, at 60 minutes experiment A decreased in all six sugars, probably due to a thermal degradation related with the high temperatures applied; experiment B had significantly higher values than the other three experiments, whereas experiment C had significantly lower values. Individually, each sugar followed the tendency described above.

As a result of these trends, the overall highest amounts of individual and total MOS were obtained after 60 minutes in experiment B, as at this point MOS concentrations reached values beyond those that had been obtained at 30 minutes in experiment A.

Microbial composition

As the objective of this project was to study the possible use of spent coffee grounds rich in MOS (after hydrolysis) as potential prebiotic, an initial *in vitro* fermentation of a pure MOS mix before starting with spent coffee grounds hydrolysis was performed. Accordingly, 30 mg of MOS/mL (5 mg/mL of each compound) were

fermented. Significant stimulation in the growth of some genera, in comparison with basal situation of the microbial community, were found. Therefore, *Bifidobacterium, Barnesiella, Anaerostipes, Blautia, Clostridium XVIb, Coprococcus, Butyricicoccus, Intestinimonas, Ruminococcus and Bilophila* showed significantly higher abundances when MOS where used (**Figure S2A**).

Once the prebiotic effect of pure MOS could have on some bacteria was confirmed, and since MOS have been described as prebiotic compounds, the next step was to unravel whether higher amounts of MOS would lead to a more beneficial microbial community structure. For this purpose, the samples corresponding to 30 minutes were used for several reasons: a) after 10 minutes the temperature was not stable and thus, MOS production was very variable. In this sense, MOS production was not sufficiently different among studies as to be able to make comparisons and therefore to establish clear links to certain microorganisms. b) After 60 minutes, experiment A suffered a decrease in oligosaccharide content (probably due to a thermal degradation) which would yield new compounds with potential effect on microbial growth, making this experiment useless for comparisons. c) Related to the previous reason, during carbohydrate heating there is a production of furanic compounds (such as HMF and furfural) from non-enzymatic browning.[28] In this sense, we observed an increase in the levels of furanic compounds with hydrolysis time and temperature (HMF and furfural, **Table 2**), which have been reported to inhibit some bacterial growth.[33] Therefore we decided to avoid samples obtained after 60 minutes due to the excessive accumulation of furanic compounds; in fact, we detected that some bacteria stopped growing or even decreased in abundance after 30 minutes (described in section 3.2.1.). In addition, comparisons were made between experiments and not

between samples from the same experiment, using only the sample obtained after 30 minutes of hydrolysis.

At phylum level, nearly 100% of the bacterial populations belonged to the phyla *Actinobacteria, Firmicutes, Bacteroidetes* or *Proteobacteria.* Experiment A (with the highest MOS content) had a significant ($p < 0.05$) enrichment in phylum *Firmicutes* compared with the other three experiments. Moreover, *Bacteroidetes* showed a tendency to become more abundant in experiments with higher amounts of MOS. In addition, *Bacteroidetes* abundance was significantly higher ($p < 0.05$) in experiments A, B and D compared with experiment C (the one with the lowest amounts of MOS). These results are in accordance with previously published literature stating that fiber-rich diets increase *Firmicutes* and *Bacteroidetes* phyla.[34] The same authors described that an increase in *Firmicutes* and *Bacteroidetes* is accompanied by a decrease in *Proteobacteria.* Our results also show how *Proteobacteria* abundance is significantly higher ($p < 0.05$) in the experiment with the lowest amounts of MOS (**Figure 1A)**. On the other hand, no significant differences in the *Firmicutes:Bacteroidetes* ratio were observed.

The observed increase in *Firmicutes* could be explained by the higher abundance of some specific genera: *Clostridium XIVb, Coprococcus, Butyricicoccus, Intestinimonas, Pseudoflavonifractor* and *Veillonella.* In addition, statistically significant differences related with MOS content were found in the Bacteroidetes genera *Barnesiella* and *Odoribacter* and in the Proteobacteria *ud-Burkholderiales* and *Bilophila.* Experiment A (with the highest MOS levels) showed significantly higher ($p < 0.05$) abundances of the above mentioned taxa than the other three experiments. On the other hand, experiment C (with the lowest MOS amounts) generally showed significantly lower abundances of such bacteria than the other experiments (**Figure 1B**). These results indicate that a higher supply

of MOS translates into a higher abundance of these bacteria. Moreover, these assumptions are supported by significantly positive correlations between each bacterial abundance and every MOS analyzed (**Figure 2**). Except for *Bilophila*, all of the bacterial taxa associated with MOS have been linked mainly to positive effects on health, summarized in **Table 3.** According to these results, MOS could have a prebiotic effect over gut microbial communities, promoting a healthier microbial structure.

The analysis of microbial α-diversity in terms of the Shannon and Simpson diversity indexes was also performed. In experiment A, the Shannon diversity index was significantly higher than in the other experiments. Moreover, the Simpson diversity index followed the same behavior, being significantly higher in A than in the other three experiments (where no significant differences were found). These results are in accordance with published literature about the effect of fiber-rich diets on the increase of gut microbial diversity.[34] Additionally, statistically significant correlations between Shannon diversity index and mannose, mannobiose, mannotriose, mannopentaose, mannohexaose, and total MOS were found (**Figure 2)**. Finally, the Simpson diversity index was also positively correlated with mannose, mannobiose, mannopentaose and total MOS (**Figure 2)**. According to these results, we observed that a higher MOS concentration led to a richer microbial community in terms of diversity.

Microbial growth inhibitors

The appearance of inhibitors of microbial growth during biotransformation processes is a main issue that has been widely reported.[33] Such potential inhibitors include furanic compounds (like HMF and furfural) and some polyphenols. HMF and furfural are

metabolites produced during non-enzymatic browning and therefore are already present in roasted coffee beans[35] and spent coffee grounds.[36] The inhibition caused by furanic compounds has been widely reported, mostly in relation to the fermentation of lignocellulosic residues.[33] In the case of polyphenols, it is widely known that they are strong antioxidants and therefore play a protective role in health. Some polyphenols, mostly complex ones such as flavonoids, chlorogenic acids, tannins, ellagitannins, etc., can be metabolized by some gut microbial communities, yielding simpler phenolics. These metabolites can be absorbed and exert a beneficial effect either systemically or locally in the colon.[37] However, some other polyphenols have also been described as possible growth inhibitors for some microorganisms.[33,38] In this sense, it has been reported that ferulic acid and quercetin can inhibit or reduce the growth of some fiber degrading bacterial communities in rats, leading to a lower SCFA production and an increased excretion of oligosaccharides in feces.[39]

One of the objectives of the project was to study the potential use of hydrolyzed spent coffee ground extracts without further purification, since they still have some beneficial compounds, such as polyphenols. However, it was hypothesized that along hydrolysis (due to high temperatures) some Maillard compounds (such as HMF and furfural) would appear. Therefore, the possible inhibitory effect of HMF and furfural on microbial growth we tested, by *in vitro* fermentation of a MOS mixture with and without addition of HMF-furfural mix at two concentrations (10 and 1 mg/mL). Growth inhibition of *Barnesiella, Coprobacter, Butyricimonas, Odoribacter, Anaerostipes, Blautia, Clostridium XVIb, Ruminococcus, Clostridium IV, Rumiococcus2, Flavonifractor, Intestinimonas, Oscillibacter, Clostridium XVIII* and *Bilophila* (**Figure S2B**) were observed

under this in vitro fermentation conditions. Therefore, the inhibitory activity of HMF and furfural was demonstrated.

The analysis of bacterial communities showed that the relative abundances of some genera were lower in the fermentations employing coffee grounds hydrolyzed for longer than 30 minutes (**Figure 3)**. These bacteria included some SCFA producers *Collinsella, Ruminococcus* and *Blautia* (acetate producers), *Blautia* and *Dialister* (propionate producers), and *Anaerostipes, Faecalibacterium,* and *Butyricimonas* (butyrate producers).[16] In view of this pattern, it could be hypothesized that some microbial inhibitors that accumulate during hydrolysis, such as furanic compounds and/or polyphenols, can negatively affect such microbial genera. The relative abundance of several of these taxa had increased with coffee grounds hydrolysed for 30 minutes in comparison to those hydrolysed for 10, whereas the relative abundance of *Faecalibacterium, Ruminococcus* and *Blautia* already decreased with coffee grounds hydrolysed for 30 minutes, suggesting a more harmful effect of such inhibitors towards them. However, in experiment C, the relative abundance of these three genera increased between 10 minutes and 30 minutes of coffee grounds hydrolysis. This could be related with the lower HMF and furfural values found in experiment C.

Taking all this information into account, the analysis of HMF and furfural concentrations in the samples collected after 10, 30 and 60 minutes of hydrolysis was performed. As expected, HMF and furfural concentrations increased with hydrolysis time due to longer exposure to high temperature (**Table 2).** HMF and furfural production also depend on the temperature applied, and thus, in those experiments in which the applied temperature was higher, HMF and furfural concentrations were also higher. We found statistically significant negative correlations ($p < 0.05$) between HMF and furfural and

Blautia, Dialister, Faecalibacterium, Ruminococcus, Butyricimonas, and Anaerostipes (**Figure 2)**. Correlation coefficients ranged from -0.3325 (in the case of *Ruminococcus*) to -0.8312 (for *Butyricimonas*). Therefore, HMF and furfural seem to inhibit or decrease the growth of these microorganisms, and accordingly reduce the prebiotic effect of MOS.

The levels of total polyphenols in the samples collected after 10, 30 and 60 minutes of hydrolysis were also analyzed. Experiment A showed, in average, lower amounts of polyphenols than the other three experiments. This could be related with the higher hydrolysis temperature, which could degrade some polyphenols. Experiments B, C and D showed similar polyphenol content in average, with no significant differences (**Table 2)**. However, polyphenol content increased along hydrolysis time, which could be related with the constant release of polyphenols as the degree of hydrolysis of the spent coffee grounds increases. As previously stated, polyphenols could also act as microbial inhibitors. In this sense, statistically significant negative correlations ($p < 0.05$) for *Blautia, Faecalibacterium, Ruminococcus* and *Collinsella* (**Figure 2)** were found. Correlation coefficients ranged from -0.3228 (for *Collinsella*) to -0.7334 (for *Faecalibacterium*). In this sense, polyphenols released by spent coffee grounds could reduce the growth of some gut bacteria and therefore reduce fiber degradation and SCFA production. *Blautia, Faecalibacterium and Ruminococcus* showed significant negative correlations with HMF, furfural and total polyphenols. This could mean that these bacteria are negatively affected by both kinds of inhibitors. Accordingly, as depicted in **Figure 3,** these bacteria seem to be more aggressively affected and their abundances already start decreasing with coffee grounds hydrolyzed for 30 minutes.

Short chain fatty acids production

Several health effects are attributed to SCFAs. Decreasing the luminal pH is one of the most obvious, which can counter the growth of pathogenic bacteria. On the other hand, butyrate is used as substrate by the epithelial cells, being important for their functionality and, overall, the three main SCFAs (acetate, propionate and butyrate) are important for the maintenance of the gut barrier. [24] Moreover, while butyric acid is mostly metabolized by colonocytes, acetate and propionate are mostly absorbed and incorporated to different metabolic routes related to energy balance. Thus, these SCFAs have a protective role in diet-induced obesity.[40] On the other hand, butyrate and propionate have been related to the production of gut hormones and therefore in reducing food intake.[40] Moreover, it is thought that SCFAs could have an important role in colorectal cancer protection (mostly butyrate) by increasing motility, irrigation, reducing inflammation and increasing apoptosis. In fact, it has been suggested that the protective effect of dietary fiber over colorectal cancer depends upon the production of butyrate.[41] Propionate along with butyrate has also been associated with regulation of intestinal inflammation through induction of T-regulatory cell differentiation.[24]

SCFAs production is summarized in **Table 2**. SCFA production showed a similar pattern across experiments as, generally, higher amounts of MOS led to higher SCFAs production though for 60 minutes of coffee grounds hydrolysis the levels of SCFAs decreased (**Table 2**). In experiment A this makes sense since MOS production was lower at 60 minutes. However, this was found even in experiments were MOS production increased until the end. The decrease in SCFAs production with coffee grounds that have undergone more than 30 minutes of hydrolysis could be attributed to the inhibitory effect exerted by HMF, furfural and/or polyphenols. As described in **section 3.2.1,** some SCFAs producers are inversely correlated with HMF, furfural and polyphenol concentrations, which could

mean that they are negatively affected by those substances. As a consequence, SCFAs production is reduced. Moreover, supporting these findings, SCFAs producers *Anaerostipes, Collinsella, Coprococcus, Faecalibacterium, Bididobacterium, Veillonella, Dialister, Butyricimonas, Butyricicoccus* and *Intestinimonas* correlated positively with the corresponding SCFAs (**Figure 2**). Therefore, this could explain, at least in part, SCFAs production.

When comparing experiments, a higher MOS production usually translated into a higher SCFAs production (**Table 2**). Mean acetate values over the three measured time points ranged from 75.05 μmol/g of spent coffee grounds for experiment A to 130.79 μmol/ g for experiment D. Experiment A showed significantly lower production of acetate ($p < 0.05$) than the other three experiments. In the case of propionate, its mean levels ranged from 39.53 μmol/g of spent coffee grounds in experiment A to 63.10 μmol/ g in experiment B. Propionate production was significantly higher ($p < 0.05$) in experiments B and D compared to A and C, both of them with similar values. Finally, mean butyrate production ranged from 34.78 μmol/g of spent coffee grounds in experiment A to 49.07 μmol/ g in experiment D. As in the case of propionate, experiments B and D produced significantly ($p < 0.05$) higher amounts of butyrate than experiments A and C, both of them with similar production. The high amounts of MOS present in experiments B and D likely enabled the high production of SCFAs. In support of this notion, significantly positive correlations (**Figure 2**) among SCFAs production and most of the saccharides analyzed were found: acetate production was positively correlated with mannobiose, mannotriose, mannopentaose, mannohexaose and total MOS production; propionate correlated positively only with mannose production; butyrate production was positively correlated with

mannose, mannobiose, mannotriose, mannopentaose, mannohexaose and total MOS production (**Figure 2**). In contrast, the low mean production of SCFAs in experiment A could be explained by 1) the decrease in MOS production at 60 minutes due to an excessive temperature, 2) the higher concentrations of HMF and furfural (**Table 2)**, and 3) the resulting significant decrease in producers of acetate (such as *Blautia*), propionate (such as *Dialister*) and butyrate (such as *Faecalibacterium* and *Butyricimonas*) in comparison with the other experiments **(Figure 3).**

As conclusions, the prebiotic activity of mannooligosaccharides obtained from spent coffee grounds was tested by studying gut microbial community structure and SCFAs production after *in vitro* fermentation with human feces. Results suggest that MOS have a prebiotic effect increasing gut microbial α-diversity and, on the other hand, promoting the growth of some beneficial bacterial genera. Moreover, a higher dose of MOS translates into a higher production of SCFAs, which reinforces the possible prebiotic effect of MOS. However, during hydrolysis some microbial inhibitors are either produced or released from the spent coffee grounds matrix, mainly HMF and furfural but also polyphenols. Accordingly, the accumulation of these compounds along the hydrolysis period seems to reduce or inhibit the growth of some beneficial bacteria. This should be taken into account for future trials in order to clean the samples from such kind of compounds in order to improve the prebiotic effects of such oligosaccharides.

Abbreviations: HPLC: High Performance Liquid Chromatography, SCFA: Short Chain Fatty Acids, HMF: 5-hydroxymethyl-furfural, MOS: Mannoligosaccharides.

Acknowledgements

This paper will form part of the doctoral thesis by Sergio Pérez-Burillo, conducted within the context of the "Nutrition and Food Sciences Programme" at the University of Granada.

Supporting information

- Chromatograms from MOS and SCFA analysis (Figure S1).

- Barplot of the relative abundance at genus level for those bacteria that were significantly different among basal situation and after adding a mixture of MOS (M1-M6) in equal proportions (Figure S2A).

- Effect of adding HMF and furfural at low (1 mg/mL) and high (20 mg/mL) concentration on gut microbiota growth (Figure S2B).

Conflict of interest

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

Funding

This study was supported by the project AGL2014-53895-R from the Spanish Ministry of Economy and Competitiveness and by the European Regional Development Fund (FEDER) and by a predoctoral fellowship granted by the Spanish Government to Pérez-Burillo (FPU14/01192).

References

(1) Farah, A.; Ferreira dos Santos, T. The Coffee Plant and Beans: An Introduction. In *Coffee in health and disease prevention*; Elsevier: London, UK, 2015; pp 5–10.

(2) Galluzzi Bizzo, M. L.; Farah, A.; Ann Kemp, J.; Berguinins Scancetti, L. Highlights in the History of Coffee Science Related to Health. In *Coffee in health and disease prevention*; Elsevier: London, UK, 2015; pp 11–16.

(3) Kocadağlı, T.; Gökmen, V. Effect of Roasting and Brewing on the Antioxidant Capacity of Espresso Brews Determined by the QUENCHER Procedure. *Food Res. Int.* **2016**, *89*, 976–981.

(4) Campos-Vega, R.; Loarca-Piña, G.; Vergara-Castañeda, H. A.; Oomah, B. D. Spent Coffee Grounds: A Review on Current Research and Future Prospects. *Trends in Food Science & Technology* **2015**, *45* (1), 24–36.

(5) Tian, T.; Freeman, S.; Corey, M.; German, J. B.; Barile, D. Chemical Characterization of Potentially Prebiotic Oligosaccharides in Brewed Coffee and Spent Coffee Grounds. *J. Agric. Food Chem.* **2017**, *65* (13), 2784–2792.

(6) Bravo, J.; Arbillaga, L.; de Peña, M. P.; Cid, C. Antioxidant and Genoprotective Effects of Spent Coffee Extracts in Human Cells. *Food Chem. Toxicol.* **2013**, *60*, 397–403.

(7) Bravo, J.; Juániz, I.; Monente, C.; Caemmerer, B.; Kroh, L. W.; De Peña, M. P.; Cid, C. Evaluation of Spent Coffee Obtained from the Most Common Coffeemakers as a Source of Hydrophilic Bioactive Compounds. *J. Agric. Food Chem.* **2012**, *60* (51), 12565–12573.

(8) Bravo, J.; Monente, C.; Juániz, I.; De Peña, M. P.; Cid, C. Influence of Extraction Process on Antioxidant Capacity of Spent Coffee. *Food Res. Int.* **2013**, *50* (2), 610–616.

(9) Monente, C.; Bravo, J.; Vitas, A. I.; Arbillaga, L.; De Peña, M. P.; Cid, C. Coffee and Spent Coffee Extracts Protect against Cell Mutagens and Inhibit Growth of Food-Borne Pathogen Microorganisms. *J. Funct. Foods* **2015**, *12*, 365–374.

(10) López-Barrera, D. M.; Vázquez-Sánchez, K.; Loarca-Piña, M. G. F.; Campos-Vega, R. Spent Coffee Grounds, an Innovative Source of Colonic Fermentable Compounds, Inhibit Inflammatory Mediators in Vitro. *Food Chem.* **2016**, *212*, 282–290.

(11) Panzella, L.; Pérez-Burillo, S.; Pastoriza, S.; Martín, M. Á.; Cerruti, P.; Goya, L.; Ramos, S.; Rufián-Henares, J. Á.; Napolitano, A.; d'Ischia, M. High Antioxidant Action and Prebiotic Activity of Hydrolyzed Spent Coffee Grounds (HSCG) in a Simulated Digestion–Fermentation Model: Toward the Development of a Novel Food Supplement. *J. Agric. Food Chem.* **2017**, *65* (31), 6452–6459.

(12) Cervera-Mata, A.; Pastoriza, S.; Angel Rufian-Henares, J.; Parraga, J.; Manuel Martin-Garcia, J.; Delgado, G. Impact of Spent Coffee Grounds as Organic Amendment on Soil Fertility and Lettuce Growth in Two Mediterranean Agricultural Soils. *Arch. Agron. Soil Sci.* **2018**, *64* (6), 790–804.

(13) Nguyen, Q. A.; Cho, E. J.; Lee, D.-S.; Bae, H.-J. Development of an Advanced Integrative Process to Create Valuable Biosugars Including Manno-Oligosaccharides and Mannose from Spent Coffee Grounds. *Bioresource Technology* **2019**, *272*, 209–216.

(14) Asano, I.; Ikeda, Y.; Fujii, S.; Iino, H. Effects of Mannooligosaccharides from Coffee on Microbiota and Short Chain Fatty Acids in Rat Cecum. *Food Sci. Technol. Res.* **2007**, *10* (3), 273–277.

(15) Chauhan, P. S.; Sharma, P.; Puri, N.; Gupta, N. Purification and Characterization of an Alkali-Thermostable β-Mannanase from <Emphasis Type="Italic">Bacillus Nealsonii</Emphasis> PN-11 and Its Application in Mannooligosaccharides Preparation Having Prebiotic Potential. *Eur. Food Res. Technol.* **2014**, *238* (6), 927–936.

(16) Flint, H. J.; Duncan, S. H.; Scott, K. P.; Louis, P. Links between Diet, Gut Microbiota Composition and Gut Metabolism. *Proc. Nutr. Soc.* **2015**, *74* (01), 13–22.

(17) Kassinen, A.; Krogius-Kurikka, L.; Mäkivuokko, H.; Rinttilä, T.; Paulin, L.; Corander, J.; Malinen, E.; Apajalahti, J.; Palva, A. The Fecal Microbiota of Irritable Bowel Syndrome Patients Differs Significantly From That of Healthy Subjects. *Gastroenterology* **2007**, *133* (1), 24–33.

(18) Malinen, E.; Krogius-Kurikka, L.; Lyra, A.; Nikkilä, J.; Jääskeläinen, A.; Rinttilä, T.; Vilpponen-Salmela, T.; von Wright, A. J.; Palva, A. Association of Symptoms with Gastrointestinal Microbiota in Irritable Bowel Syndrome. *World J. Gastroenterol. WJG* **2010**, *16* (36), 4532–4540.

(19) Ley, R. E.; Bäckhed, F.; Turnbaugh, P.; Lozupone, C. A.; Knight, R. D.; Gordon, J. I. Obesity Alters Gut Microbial Ecology. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102* (31), 11070–11075.

(20) Million, M.; Maraninchi, M.; Henry, M.; Armougom, F.; Richet, H.; Carrieri, P.; Valero, R.; Raccah, D.; Vialettes, B.; Raoult, D. Obesity-Associated Gut Microbiota

Is Enriched in Lactobacillus Reuteri and Depleted in Bifidobacterium Animalis and Methanobrevibacter Smithii. *Int. J. Obes. 2005* **2012**, *36* (6), 817–825.

(21) Strati, F.; Cavalieri, D.; Albanese, D.; De Felice, C.; Donati, C.; Hayek, J.; Jousson, O.; Leoncini, S.; Renzi, D.; Calabrò, A.; De Filippo, C. New Evidences on the Altered Gut Microbiota in Autism Spectrum Disorders. *Microbiome* **2017**, *5* (1).

(22) Salazar, N.; Ruas-Madiedo, P.; Kolida, S.; Collins, M.; Rastall, R.; Gibson, G.; de los Reyes-Gavilán, C. G. Exopolysaccharides Produced by Bifidobacterium Longum IPLA E44 and Bifidobacterium Animalis Subsp. Lactis IPLA R1 Modify the Composition and Metabolic Activity of Human Faecal Microbiota in PH-Controlled Batch Cultures. *Int. J. Food Microbiol.* **2009**, *135* (3), 260–267.

(23) Poeker, S. A.; Geirnaert, A.; Berchtold, L.; Greppi, A.; Krych, L.; Steinert, R. E.; Wouters, T.; Lacroix, C. Understanding the Prebiotic Potential of Different Dietary Fibers Using an in Vitro Continuous Adult Fermentation Model (PolyFermS). *Sci. Rep.* **2018**, *8* (1), 4318.

(24) Ríos-Covián, D.; Ruas-Madiedo, P.; Margolles, A.; Gueimonde, M.; de los Reyes-Gavilán, C. G.; Salazar, N. Intestinal Short Chain Fatty Acids and Their Link with Diet and Human Health. *Front. Microbiol.* **2016**, *7*.

(25) Pérez-Burillo, S.; Rufián-Henares, J. A.; Pastoriza, S. Towards an Improved Global Antioxidant Response Method (GAR+): Physiological-Resembling in Vitro Digestion-Fermentation Method. *Food Chem.* **2018**, *239* (Supplement C), 1253–1262.

(26) Zang, H.; Xie, S.; Wu, H.; Wang, W.; Shao, X.; Wu, L.; Rajer, F. U.; Gao, X. A Novel Thermostable GH5_7 β-Mannanase from Bacillus Pumilus GBSW19 and Its

Application in Manno-Oligosaccharides (MOS) Production. *Enzyme Microb. Technol.* **2015**, *78*, 1–9.

(27) Singleton, V. L.; Rossi, J. A. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *Am. J. Enol. Vitic.* **1965**, *16* (3), 144–158.

(28) Rufián-Henares, J. A.; García-Villanova, B.; Guerra-Hernández, E. Occurrence of Furosine and Hydroxymethylfurfural as Markers of Thermal Damage in Dehydrated Vegetables. *Eur. Food Res. Technol.* **2008**, *228* (2), 249–256.

(29) Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glöckner, F. O. Evaluation of General 16S Ribosomal RNA Gene PCR Primers for Classical and Next-Generation Sequencing-Based Diversity Studies. *Nucleic Acids Res.* **2013**, *41* (1), e1.

(30) Schmieder, R.; Edwards, R. Quality Control and Preprocessing of Metagenomic Datasets. *Bioinforma. Oxf. Engl.* **2011**, *27* (6), 863–864.

(31) Aronesty, E. Command-Line Tools for Processing Biological Sequencing Data. 2011.

(32) Cole, J. R.; Wang, Q.; Cardenas, E.; Fish, J.; Chai, B.; Farris, R. J.; Kulam-Syed-Mohideen, A. S.; McGarrell, D. M.; Marsh, T.; Garrity, G. M.; Tiedje, J.M. The Ribosomal Database Project: Improved Alignments and New Tools for RRNA Analysis. *Nucleic Acids Res.* **2009**, *37* (Database issue), D141-145.

(33) Monlau, F.; Sambusiti, C.; Barakat, A.; Quéméneur, M.; Trably, E.; Steyer, J.-P.; Carrère, H. Do Furanic and Phenolic Compounds of Lignocellulosic and Algae Biomass Hydrolyzate Inhibit Anaerobic Mixed Cultures? A Comprehensive Review. *Biotechnol. Adv.* **2014**, *32* (5), 934–951.

(34) Simpson, H. L.; Campbell, B. J. Review Article: Dietary Fibre–Microbiota Interactions. *Aliment. Pharmacol. Ther.* **2015**, *42* (2), 158–179.

(35) Rufián-Henares, J. A.; de la Cueva, S. P. Assessment of Hydroxymethylfurfural Intake in the Spanish Diet. *Food Addit. Contam. Part A* **2008**, *25* (11), 1306–1312.

(36) Juarez, G. F. Y.; Pabiloña, K. B. C.; Manlangit, K. B. L.; Go, A. W. Direct Dilute Acid Hydrolysis of Spent Coffee Grounds: A New Approach in Sugar and Lipid Recovery. *Waste Biomass Valorization* **2018**, *9* (2), 235–246.

(37) Selma, M. V.; Espín, J. C.; Tomás-Barberán, F. A. Interaction between Phenolics and Gut Microbiota: Role in Human Health. *J. Agric. Food Chem.* **2009**, *57* (15), 6485–6501.

(38) Poirier, S.; Bize, A.; Bureau, C.; Bouchez, T.; Chapleur, O. Community Shifts within Anaerobic Digestion Microbiota Facing Phenol Inhibition: Towards Early Warning Microbial Indicators? *Water Res.* **2016**, *100*, 296–305.

(39) Zhang, L.; Dong, M.; Guangyong Xu; Yuan Tian; Tang, H.; Wang, Y. Metabolomics Reveals That Dietary Ferulic Acid and Quercetin Modulate Metabolic Homeostasis in Rats. *J. Agric. Food Chem.* **2018**, *66* (7), 1723–1731.

(40) Lin, H. V.; Frassetto, A.; Kowalik Jr, E. J.; Nawrocki, A. R.; Lu, M. M.; Kosinski, J. R.; Hubert, J. A.; Szeto, D.; Yao, X.; Forrest, G.; Marsh, D.J; Brennan, L. Butyrate and Propionate Protect against Diet-Induced Obesity and Regulate Gut Hormones via Free Fatty Acid Receptor 3-Independent Mechanisms. *PLoS ONE* **2012**, *7* (4), e35240.

(41) Donohoe, D. R.; Holley, D.; Collins, L. B.; Montgomery, S. A.; Whitmore, A. C.; Hillhouse, A.; Curry, K. P.; Renner, S. W.; Greenwalt, A.; Ryan, E. P.; Godfrey, V; Haise, M.T.; Threadgill, D.S; Han, A.; Swenberg, J.A.; Threadgill, D.W.; Bultman,

S.J. A Gnotobiotic Mouse Model Demonstrates That Dietary Fiber Protects against Colorectal Tumorigenesis in a Microbiota- and Butyrate-Dependent Manner. *Cancer Discov.* **2014**, *4* (12), 1387–1397.

(42) Bilinski, J.; Grzesiowski, P.; Sorensen, N.; Madry, K.; Muszynski, J.; Robak, K.; Wroblewska, M.; Dzieciatkowski, T.; Dulny, G.; Dwilewicz-Trojaczek, J.; Wiktor-Jedrzejczak, W.; Basak, G.W. Fecal Microbiota Transplantation in Patients With Blood Disorders Inhibits Gut Colonization With Antibiotic-Resistant Bacteria: Results of a Prospective, Single-Center Study. *Clin. Infect. Dis.* **2017**, *65* (3), 364–370.

(43) Gomez-Arango, L. F.; Barrett, H. L.; McIntyre, H. D.; Callaway, L. K.; Morrison, M.; Nitert, M. D. Increased Systolic and Diastolic Blood Pressure Is Associated With Altered Gut Microbiota Composition and Butyrate Production in Early PregnancyNovelty and Significance. *Hypertension* **2016**, *68* (4), 974–981.

(44) Salomäki-Myftari, H.; Vähätalo, L. H.; Ailanen, L.; Pietilä, S.; Laiho, A.; Hänninen, A.; Pursiheimo, J.-P.; Munukka, E.; Rintala, A.; Savontaus, E.; Pesonen, U.; Koulu, M. Neuropeptide Y Overexpressing Female and Male Mice Show Divergent Metabolic but Not Gut Microbial Responses to Prenatal Metformin Exposure. *PLoS ONE* **2016**, *11* (9).

(45) Ling, Z.; Jin, C.; Xie, T.; Cheng, Y.; Li, L.; Wu, N. Alterations in the Fecal Microbiota of Patients with HIV-1 Infection: An Observational Study in A Chinese Population. *Sci. Rep.* **2016**, *6*, 30673.

(46) Eeckhaut, V.; Machiels, K.; Perrier, C.; Romero, C.; Maes, S.; Flahou, B.; Steppe, M.; Haesebrouck, F.; Sas, B.; Ducatelle, R.; Vermeire, S.; Immerseel, F.V.

Butyricicoccus Pullicaecorum in Inflammatory Bowel Disease. *Gut* **2013**, *62* (12), 1745–1752.

(47) Devriese, S.; Eeckhaut, V.; Geirnaert, A.; Van den Bossche, L.; Hindryckx, P.; Van de Wiele, T.; Van Immerseel, F.; Ducatelle, R.; De Vos, M.; Laukens, D. Reduced Mucosa-Associated Butyricicoccus Activity in Patients with Ulcerative Colitis Correlates with Aberrant Claudin-1 Expression. *J. Crohns Colitis* **2017**, *11* (2), 229–236.

(48) Louis, S.; Tappu, R.-M.; Damms-Machado, A.; Huson, D. H.; Bischoff, S. C. Characterization of the Gut Microbial Community of Obese Patients Following a Weight-Loss Intervention Using Whole Metagenome Shotgun Sequencing. *PLOS ONE* **2016**, *11* (2), e0149564.

(49) Sen, T.; Cawthon, C. R.; Ihde, B. T.; Hajnal, A.; DiLorenzo, P. M.; de La Serre, C. B.; Czaja, K. Diet-Driven Microbiota Dysbiosis Is Associated with Vagal Remodeling and Obesity. *Physiol. Behav.* **2017**, *173*, 305–317.

Figure captions.

Figure 1. A) Bacterial relative abundances at phylum level. **B)** Heatmap of relative abundance at genus level for those bacteria that were significantly different among experiments. Statistical labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant. Comparisons are made taking as the reference group Experiment C. Note the log2 scale.

Figure 2. Heatmap of correlation coefficients (R). Statistical labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant.

Figure 3. Relative abundance of some bacteria depending on the hydrolysis time of coffee grounds. Note the log2 scale.

Table 1. Mannooligosaccharides Content (mg/g of Spent Coffee Ground).

	Time, minutes	Mannose	Mannobiose	Mannotriose	Mannotetraose	Mannopentaose	Mannohexaose	Total MOS
Experiment A	10	0.11 ± 0.00^{a}	0.69 ± 0.04^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00	1.34 ± 0.07^{a}	0.45 ± 0.02^{a}	2.59 ± 0.11^{a}
(220 ºC)	30	4.16 ± 0.26^{b}	3.52 ± 0.16^{b}	0.72 ± 0.04^{b}	0.00 ± 0.00	10.02 ± 0.41^{b}	3.33 ± 0.23^{b}	21.75 ± 0.76^{b}
	60	1.11 ± 0.04^{c}	1.81 ± 0.06^{c}	0.15 ± 0.01^{c}	0.00 ± 0.00	2.61 ± 0.07^{c}	1.21 ± 0.05^{c}	6.89 ± 0.14^{c}
	Mean	1.79 ± 0.04	2.01 ± 0.03	0.29 ± 0.01	0.00 ± 0.00	4.66 ± 0.05	1.66 ± 0.04	10.41 ± 0.08
Experiment B	10	0.04 ± 0.00^{d}	1.11 ± 0.05^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00	1.42 ± 0.05^{a}	0.99 ± 0.05^{c}	3.55 ± 0.12^{d}
(185 ºC)	30	0.15 ± 0.00^{a}	2.70 ± 0.22^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00	1.52 ± 0.11^{a}	1.94 ± 0.04^{d}	6.31 ± 0.40^{c}
	60	6.47 ± 0.53^{e}	10.28 ± 0.79^{d}	1.14 ± 0.08^{e}	0.00 ± 0.00	16.89 ± 1.15^{d}	6.57 ± 0.60^{e}	41.35 ± 2.49^{e}
	Mean	2.22 ± 0.16	4.69 ± 0.20	0.38 ± 0.03	0.00 ± 0.00	6.61 ± 0.25	3.17 ± 0.25	17.07 ± 0.48
Experiment C	10	0.04 ± 0.00^{d}	0.83 ± 0.03^{a}	0.07 ± 0.01^{c}	0.00 ± 0.00	2.09 ± 0.06^{c}	1.01 ± 0.06^{c}	4.03 ± 0.09^{f}
(170 ºC)	30	0.02 ± 0.00^{d}	1.32 ± 0.00^{c}	0.05 ± 0.00^{c}	0.00 ± 0.00	1.23 ± 0.00^{a}	0.86 ± 0.04^{c}	3.48 ± 0.08^{d}
	60	0.31 ± 0.02^{a}	2.43 ± 0.03^{b}	0.48 ± 0.02^{f}	0.00 ± 0.00	2.60 ± 0.03^{c}	1.82 ± 0.12^{d}	7.64 ± 0.04^{c}
	Mean	0.12 ± 0.00	1.53 ± 0.12	0.20 ± 0.01	0.00 ± 0.00	1.97 ± 0.13	1.23 ± 0.04	5.05 ± 0.32
Experiment D	10	0.01 ± 0.00^{d}	0.74 ± 0.06^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00	1.06 ± 0.08^{a}	0.77 ± 0.04^{c}	2.58 ± 0.17^{a}
(180 ºC)	30	0.30 ± 0.01^{a}	2.61 ± 0.24^{b}	0.02 ± 0.00^{a}	0.00 ± 0.00	3.14 ± 0.26^{e}	2.52 ± 0.11^{e}	8.59 ± 0.66^{c}
	60	2.38 ± 0.03^{f}	4.33 ± 0.05^{e}	0.34 ± 0.02^{d}	0.00 ± 0.00	8.31 ± 0.09^{f}	4.26 ± 0.06^{f}	19.63 ± 0.05^{b}
	Mean	0.90 ± 0.05	2.56 ± 0.12	0.12 ± 0.01	0.00 ± 0.00	4.17 ± 0.17	2.52 ± 0.15	10.27 ± 0.34

$^{a\text{-}f}$Different letter within the same column means statistically significant ($p < 0.05$).

Table 2. SCFAs Content (μmol/g of spent coffee grounds), HMF and Furfural Content (mg/g of spent coffee grounds) and Total Polyphenols Content (mg GAE/g of spent coffee grounds)

[a-f]Different letter within the same column means statistically significant ($p < 0.05$).

	Hydrolysis time, minutes	Acetate	Propionate	Butyrate	Total SCFAs	HMF	Furfural	Polyphenols
Experiment A	10	68.32 ± 4.97[a]	32.95 ± 1.92[a]	24.67 ± 1.83[a]	125.94 ± 7.09[a]	0.29 ± 0.03[a]	0.01 ± 0.00[a]	16.89 ± 0.40[a]
	30	80.21 ± 4.48[b]	44.28 ± 2.02[b]	43.84 ± 2.04[b]	168.33 ± 13.85[b]	7.99 ± 0.45[b]	0.42 ± 0.02[b]	20.66 ± 1.31[b]
	60	76.61 ± 3.52[b]	41.36 ± 1.46[b]	35.84 ± 2.31[c]	153.80 ± 1.89[c]	16.84 ± 1.27[c]	0.94 ± 0.08[c]	26.14 ± 1.37[c]
	Mean	75.05 ± 1.43	39.53 ± 0.56	34.78 ± 1.09	149.36 ± 6.32	8.37 ± 0.31	0.46 ± 0.03	21.23 ± 0.90
Experiment B	10	97.31 ± 4.92[c]	53.87 ± 2.25[c]	32.50 ± 1.20[c]	183.67 ± 11.66[d]	0.60 ± 0.03[a]	0.03 ± 0.00[a]	30.65 ± 1.95[d]
	30	126.23 ± 12.66[d]	66.21 ± 5.45[d]	58.71 ± 4.73[d]	251.15 ± 20.67[e]	2.56 ± 0.25[d]	0.10 ± 0.01[d]	45.38 ± 3.88[e]
	60	119.03 ± 10.79[d]	69.21 ± 5.21[d]	49.71 ± 3.15[b]	237.95 ± 17.73[e]	9.42 ± 0.74[b]	0.47 ± 0.01[b]	42.55 ± 1.94[e]
	Mean	114.19 ± 6.68	63.10 ± 2.86	46.97 ± 3.58	224.26 ± 20.99	4.19 ± 0.38	0.20 ± 0.01	39.53 ± 2.86
Experiment C	10	105.66 ± 5.58[c]	35.25 ± 1.40[a]	15.79 ± 1.31[e]	156.70 ± 8.20[c]	0.32 ± 0.03[a]	0.01 ± 0.00[a]	33.01 ± 1.77[d]
	30	134.02 ± 2.23[d]	43.84 ± 0.38[b]	49.73 ± 3.45[b]	227.59 ± 0.57[e]	2.28 ± 0.18[d]	0.12 ± 0.00[d]	35.37 ± 3.31[d]
	60	109.31 ± 2.46[c]	43.49 ± 0.68[b]	39.49 ± 1.94[c]	192.29 ± 2.79[d]	6.09 ± 0.34[b]	0.34 ± 0.02[b]	47.81 ± 2.56[e]
	Mean	116.33 ± 9.75	40.86 ± 2.93	35.00 ± 1.19	192.20 ± 12.49	2.90 ± 0.13	0.16 ± 0.01	38.73 ± 1.64
Experiment D	10	108.21 ± 10.62[c]	64.59 ± 5.24[d]	35.67 ± 2.53[c]	208.47 ± 6.78[d]	0.68 ± 0.06[a]	0.03 ± 0.00[a]	31.07 ± 2.56[d]
	30	135.44 ± 14.27[d]	37.40 ± 3.35[a]	63.32 ± 3.15[d]	236.15 ± 19.44[e]	2.73 ± 0.18[d]	0.17 ± 0.02[d]	34.57 ± 1.58[d]
	60	148.71 ± 3.94[e]	44.92 ± 0.78[b]	48.21 ± 3.34[b]	241.83 ± 22.06[e]	11.90 ± 0.94[e]	0.48 ± 0.02[b]	54.32 ± 4.11[f]
	Mean	130.79 ± 7.70	48.97 ± 2.30	49.07 ± 3.02	228.82 ± 16.59	5.10 ± 0.38	0.23 ± 0.01	39.98 ± 2.49

Table 3. Positive and Negative Effects of Microbial Genera Found to Be Significantly Different Depending on MOS Content.

Bacteria		Health effect
Barnesiella	+	It could prevent or treat infections by antibiotic resistant bacteria.
Odoribacter	+	Butyrate producer, lower abundance has been related to higher blood pressure in pregnant women and regulation of blood sugar in the host.[44]
Clostridium XIVb	+	Correlated with systemic inflammatory cytokines in patients with HIV-1.[45]
Coprococcus	+	Acetic and butyric acid producer, and lower amounts of propionic or formic acid.[24]
Butyricicoccus	+	Butyrate producer. Its abundance is reduced in ulcerative colitis patients and patients with inflammatory disease in general.[24 46,47]
Intestinimonas	+	Butyrate producer.[4]
Pseudoflavonifractor	+	It is related to weight loss along with *Alistipes.*
Veillonella	+	Propionate and acetate producer.
Bilophila	+/-	Its abundance is higher in dysbiosis with diets high in sugars or fats being therefore related to obesity and inflammation. It is also reduced in autism spectrum disorders subjects.[21,]

Figure 1

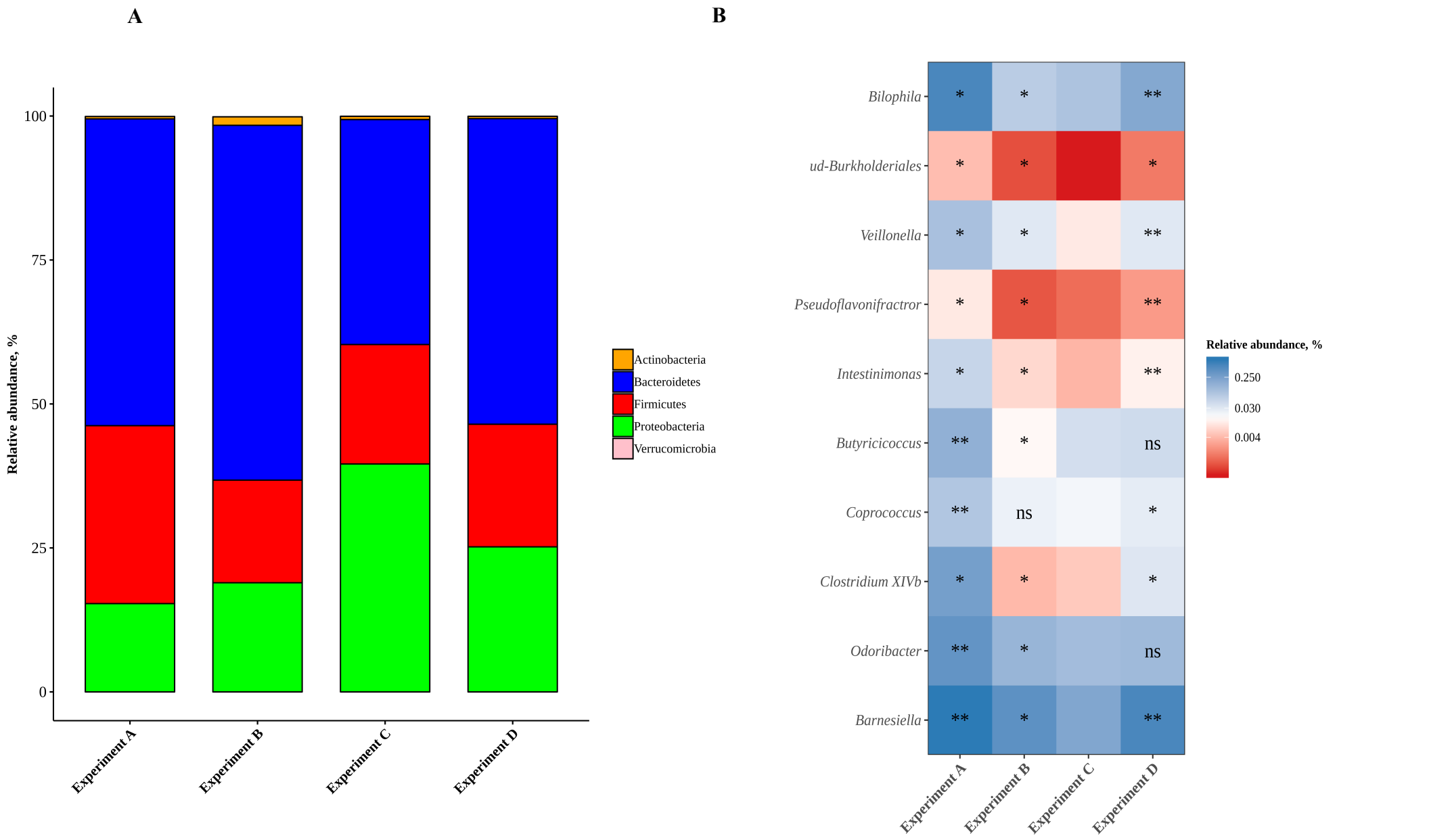

A
B
100
75
50
25
0
Relative abundance, %
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Verrucomicrobia
Experiment A
Experiment B
Experiment C
Experiment D
Bilophila
ud-Burkholderiales
Veillonella
Pseudoflavonifractror
Intestinimonas
Butyricicoccus
Coprococcus
Clostridium XIVb
Odoribacter
Barnesiella
Relative abundance, %
0.250
0.030
0.004

Figure 2

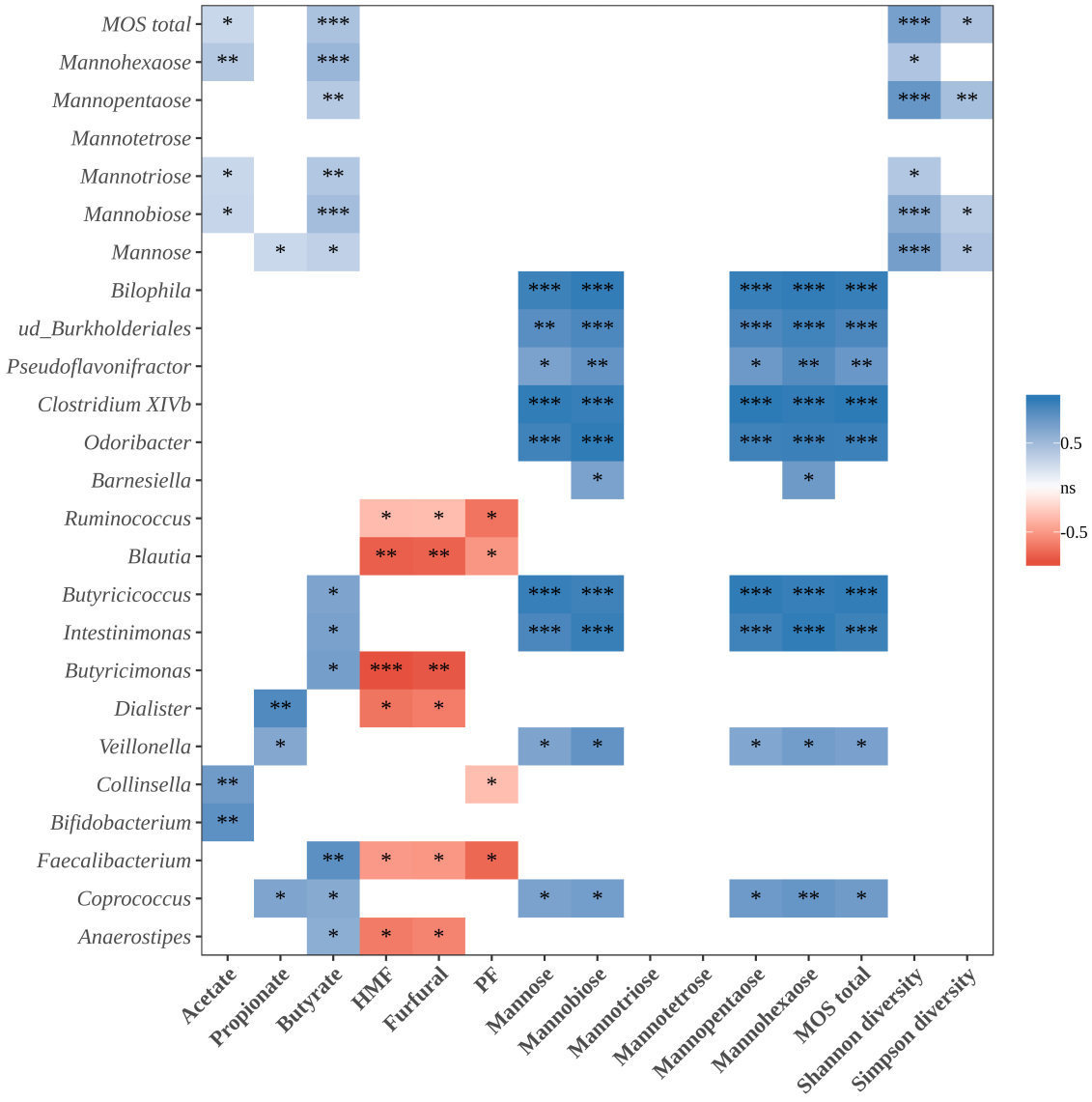
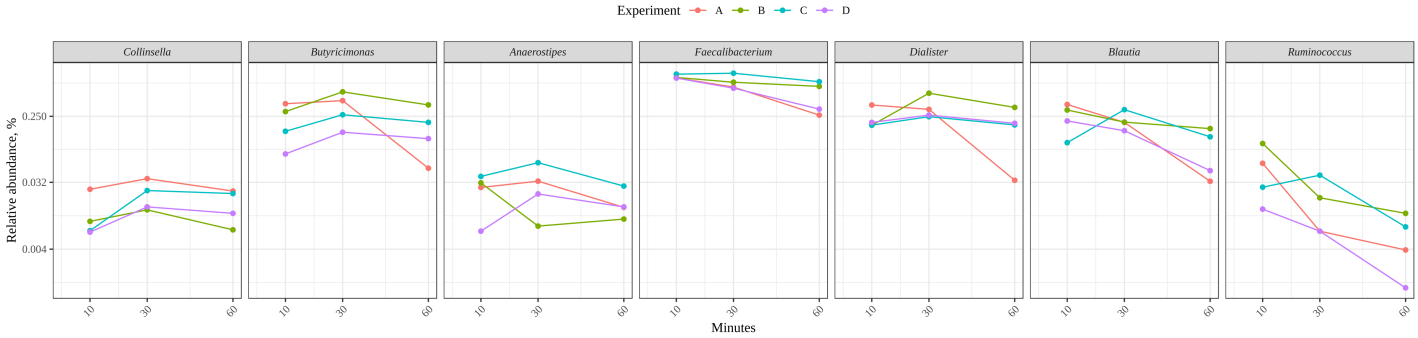

MOS total
Mannohexaose
Mannopentaose
Mannotetrose
Mannotriose
Mannobiose
Mannose
Bilophila
ud_Burkholderiales
Pseudoflavonifractor
Clostridium XIVb
Odoribacter
Barnesiella
Ruminococcus
Blautia
Butyricicoccus
Intestinimonas
Butyricimonas
Dialister
Veillonella
Collinsella
Bifidobacterium
Faecalibacterium
Coprococcus
Anaerostipes
Acetate
Propionate
Butyrate
HMF
Furfural
PF
Mannose
Mannobiose
Mannotriose
Mannotetrose
Mannopentaose
Mannohexaose
MOS total
Shannon diversity
Simpson diversity
0.5
ns
-0.5

Figure 3


Experiment
A
B
C
D
Collinsella
Butyricimonas
Anaerostipes
Faecalibacterium
Dialister
Blautia
Ruminococcus
Relative abundance, %
0.250
0.032
0.004
10
30
60
Minutes

Supplemental figure captions.

Figure S1. **Chromatograms from MOS and SCFA analysis. Figure S1A**: MOS standards chromatogram. **Figure S1B**: Experiment B after 10 minutes of hydrolysis MOS chromatogram. **Figure S1C**: Experiment B after 60 minutes of hydrolysis MOS chromatogram. **Figure S1D**: SCFA standards chromatogram. **Figure S1E**: Experiment A after 10 minutes of hydrolysis SCFA chromatogram. **Figure S1F**:Experiment B after 60 minutes of hydrolysis SCFA chromatogram.

Figure S2. Panel A: Barplot of the relative abundance at genus level for those bacteria that were significantly different among basal situation and after adding a mixture of MOS (M1-M6) in equal proportions. Statistical labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Comparisons are made taking as the reference group "Basal". **Panel B:** Effect of adding HMF and Furfural at low (1 mg/mL) and high (20 mg/mL) concentration on gut microbiota growth.

Figure S1A

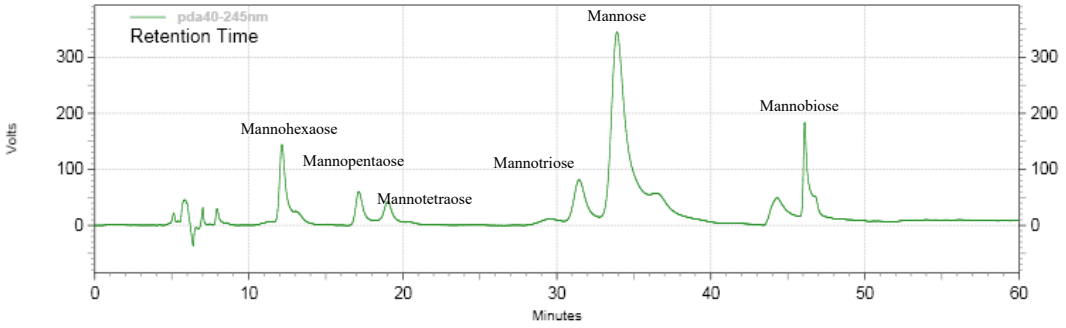


Figure S1B

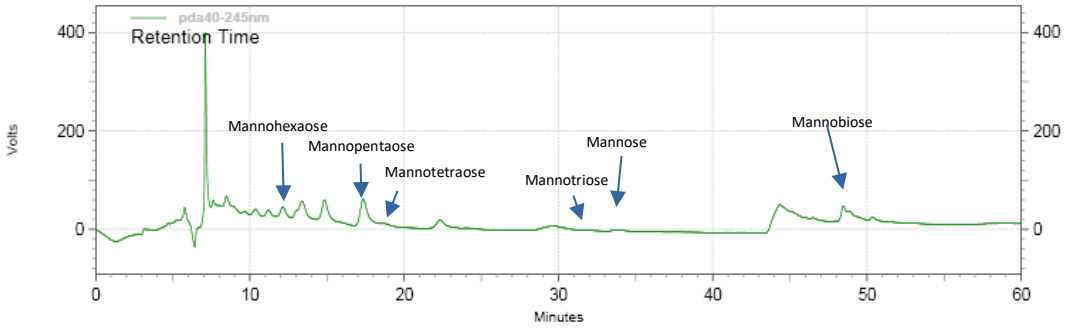


Figure S1C

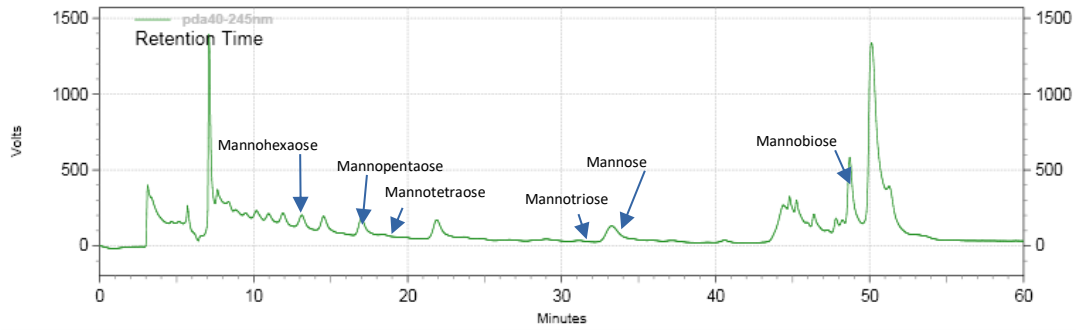


Figure S1D

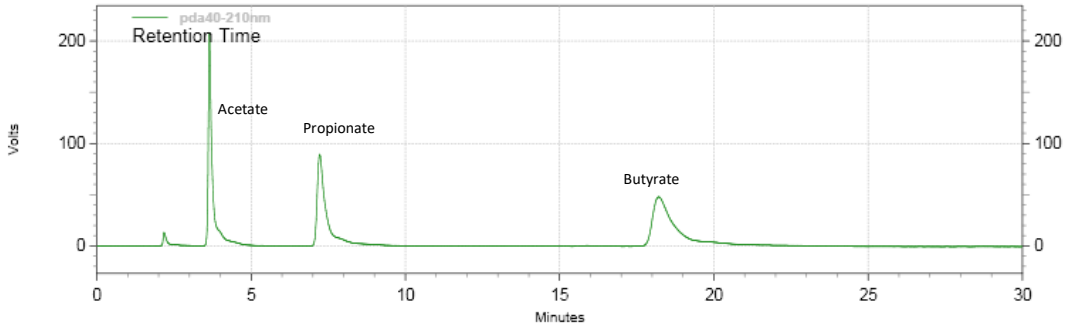


Figure S1E

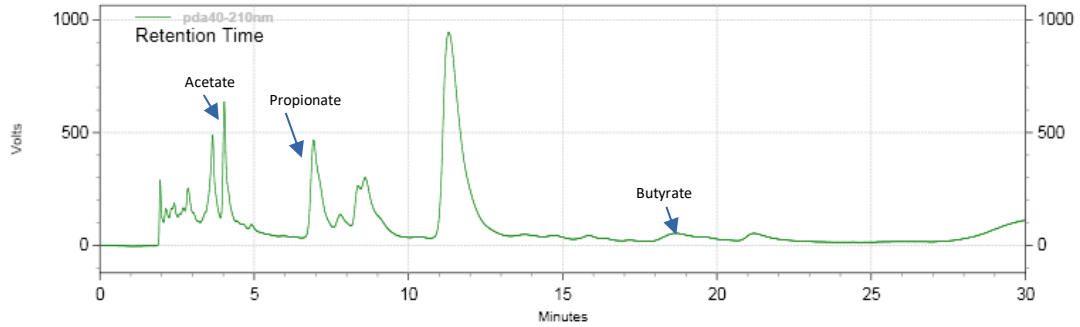


Figure S1F

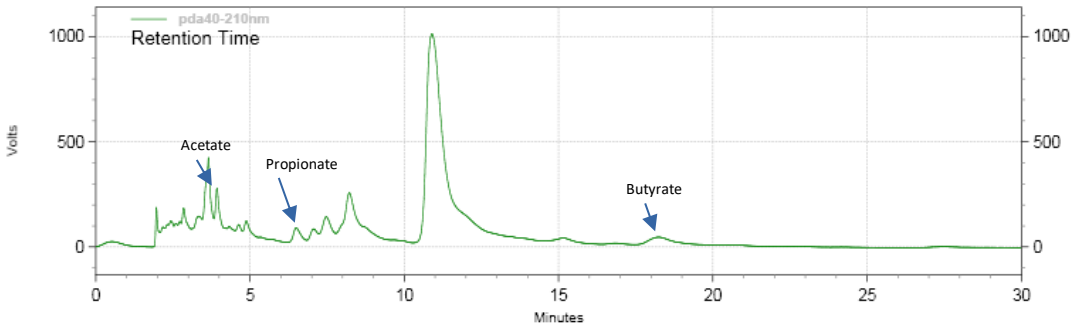


Figure S2A

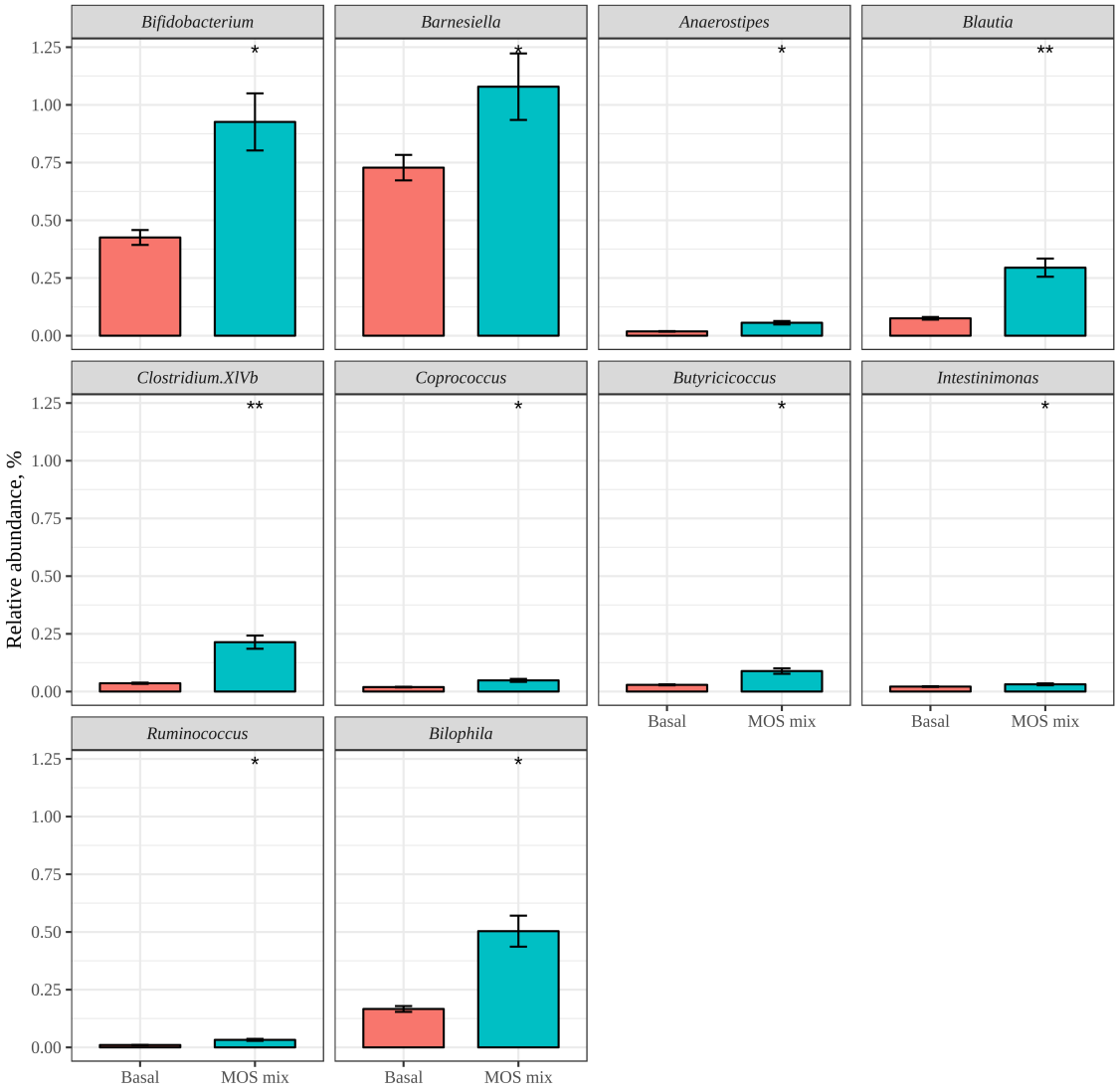

Bifidobacterium
Barnesiella
Anaerostipes
Blautia
Clostridium.XlVb
Coprococcus
Butyricicoccus
Intestinimonas
Ruminococcus
Bilophila
Relative abundance, %
1.25
1.00
0.75
0.50
0.25
0.00
*
**
Basal
MOS mix

Figure S2B

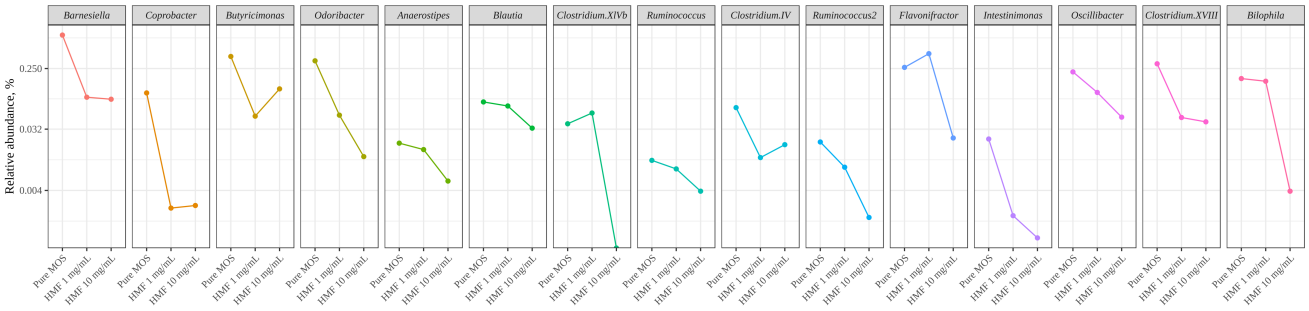

Barnesiella
Coprobacter
Butyricimonas
Odoribacter
Anaerostipes
Blautia
Clostridium.XlVb
Ruminococcus
Clostridium.IV
Ruminococcus2
Flavonifractor
Intestinimonas
Oscillibacter
Clostridium.XVIII
Bilophila
Relative abundance, %
0.250
0.032
0.004
Pure MGS
HMF 1 mg/mL
HMF 10 mg/mL

Table of Contents Graphic (TOC)