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Bioactivity of food melanoidins is mediated by gut microbiota

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ABSTRACT

Melanoidins are an important component of the human diet (average consumption 10g/day), which escape gastrointestinal digestion and are fermented by the gut microbiota. In this paper melanoidins from different food sources (coffee, bread, beer, balsamic vinegar, sweet wine, biscuit, chocolate, and breakfast cereals) were submitted to an *in vitro* digestion and fermentation process, and their bioactivity was assessed. Some melanoidins were extensively used by gut microbes, increasing production of short chain fatty acids (mainly acetate and lactate) and favoring growth of the beneficial genera *Bifidobacterium* (bread crust, pilsner and black beers, chocolate and sweet wine melanoidins) and *Faecalibacterium* (biscuit melanoidins). Quantification of individual phenolic compounds after *in vitro* fermentation allowed their identification as microbial metabolites or phenolics released from the melanoidins backbone (specially pyrogallol, 2-(3,4-dihydroxyphenyl)acetic and 3-(3,4-dihydroxyphenyl)propionic acids). Our results also showed that antioxidant capacity of melanoidins is affected by gut microbiota fermentation.

KEYWORDS: melanoidins, gut microbiota, short chain fatty acids, polyphenols, antioxidant capacity.

1. Introduction

Melanoidins are end products of Maillard reaction (MR), which occurs among the amino group of an amino acid, protein, or vitamin and the carbonyl group of a reductive sugar or oxidized lipid (Rufián-Henares & Pastoriza, 2016). The MR is very common in foods since it happens during heating but also during storage at room temperature (Rufián-Henares, Guerra-Hernández & García-Villanova, 2006; Tagliazucchi & Verzelloni, 2014). Melanoidins are therefore high molecular weight products responsible for the brown color of thermally treated foods like bread, coffee, cocoa, etc. The detailed structure of melanoidins remains unknown due to the varied nature of the foods in which melanoidins are formed. Thus, in biscuits and bread, melanoidins are mainly composed of polysaccharides and proteins (melanoproteins), while in coffee and cocoa melanoidins are usually smaller and incorporate phenolic compounds such as chlorogenic acids or catechins (Morales, Somoza, & Fogliano, 2012).

Melanoidins make an important contribution to our diet: on average, around 10 g of melanoidins per day could be ingested (Fogliano & Morales, 2011; Pastoriza and Rufián-Henares, 2014). Therefore, knowing the possible benefits or risks of melanoidins to human health is important. Accordingly, many different biological effects have been attributed to melanoidins. They can behave as prebiotic (Helou et al., 2015; Jiménez-Zamora, Pastoriza & Rufián-Henares, 2015), they have an important antioxidant capacity (Delgado-Andrade & Morales, 2005; Carvalho, Correia, Lopes, & Guido, 2014; Pastoriza& Rufián-Henares, 2014; de la Cueva, Seiquer, Mesías, Rufián-Henares, & Delgado-Andrade, 2017) and antimicrobial activity (Rufián-Henares & de la Cueva, 2009), and they can possess

inhibitory activity against angiotensin I-converting enzyme (Rufián-Henares & Morales, 2007).

In the gut, dietary melanoidins escape gastrointestinal digestion (similarly to fiber) and reach the colon where they become substrates for the gut microbiota (Wang, Qian, & Yao, 2011). However, the potential bioactivity of melanoidins after being exposed to the gut microbiota has not been extensively studied. It has been proposed that the colon is the main region of the gut where melanoidins exert their effects since they escape digestion (Rufián-Henares & Pastoriza, 2015). However, fermentation of melanoidins by gut microbes and resulting production of short chain fatty acids (SCFAs) have not been yet deeply studied. SCFAs are the main microbial metabolites and have been attributed to several health effects. For example, decreasing the luminal pH is one of the most obvious effects of SCFAs release in the gut, which can hinder the growth of pathogenic bacteria. Butyrate is used as a substrate by the epithelial cells, and the three main SCFAs (acetate, propionate and butyrate) are important for the maintenance of the gut barrier (Ríos-Covián et al., 2016). In addition, all these SCFAs have a protective role in diet-induced obesity (Lin et al., 2012) since butyrate and propionate have been related to the production of gut hormones and therefore reduction of food intake (Ríos-Covián et al., 2016). SCFAs (mostly butyrate) could also have an important role in colorectal cancer protection via reducing inflammation and increasing cell apoptosis (Donohoe et al., 2014).

Taking all this information into account, the objective of this research was to investigate the fate of melanoidins during simulated gastrointestinal digestion and subsequent fermentation by human gut microbiota. We assessed how melanoidins shaped gut microbial community structure and its functionality (SCFAs production), and we

measured the release of phenolic compounds and antioxidants as consequence of microbial metabolism.

2. Materials and methods

2.1. Reagents.

For the antioxidant assays, 2,4,6-Tri(2-pyridyl)-*s*-triazine (TPTZ), potassium persulfate, iron (III) chloride hexahydrate, 2,2-Diphenyl-1-picrylhydrazyl, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), and methanol were purchased from Sigma-Aldrich (Germany). Short chain fatty acids and phenolic standards were from Sigma-Aldrich (Germany).

For the *in vitro* digestion and fermentation, the following reagents were used: potassium di-hydrogen phosphate, potassium chloride, magnesium chloride hexahydrate, sodium chloride, calcium chloride dihydrate, sodium mono-hydrogen carbonate, ammonium carbonate, hydrochloric acid, all obtained from Sigma-Aldrich (Germany). The enzymes – salivary alpha-amylase, pepsin from porcine, and bile acids (bile extract porcine) – were purchased from Sigma-Aldrich, and porcine pancreatin was fromAlfa Aesar (United Kingdom). The fermentation reagents (sodium di-hydrogen phosphate, sodium sulfide, tryptone, cysteine, and resazurin) were obtained from Sigma-Aldrich (Germany).

For individual phenolic quantification the following standards were used: hydroxymethyl-furan-carbaldehyde, 3,4-Dihydroxybenzoic acid, 3,5-Dihydroxybenzoic acid, 3,4-Dihydroxyphenilacetic acid, 3,4-Dihydroxybenzaldehyde, p-Hydroxyphenilacetic

acid, cafeic acid, chlorogenic acid, vanillina, 4-O-Cafeoylquinic acid, 3,4-Hydroxyphenilpropionic acid, trans-hydroxycinnamic acid, p-Cumaric acid, epicatechin, epicatechin gallate, ferulic acid, 3,4-Dimethoxybenzaldehide, o-Cumaric acid, pyrogallol, resveratrol, daidzein, glicitein, genistein and formonotein, were purchased from Sigma-Aldrich (Germany). Moreover, diethyl ether for phenolic compounds extraction was purchased from Sigma-Aldrich (Germany).

2.2. Melanoidins preparation

Melanoidins were isolated from ground coffee "Cafés Cumbal", pilsner beer "Alhambra" and Irish black beer "Guinness", corn breakfast cereals "Corn Flakes", bread crust separated from French bread by grinding, Marie biscuits, 85% black chocolate "Lindt", balsamic vinegar "Borges", and sweet wine "Málaga Vírgen". All products were purchased in Carrefour in Granada (Spain). Spent coffee grounds were obtained after brewing the same coffee used for melanoidins extraction in an espresso coffee machine. In the case of melanoproteins (biscuit and bread crust) they were obtained after *in vitro* digestion with Pronase E and subsequent diafiltration (Pastoriza, Roncero-Ramos, Rufián-Henares, & Delgado-Andrade, 2014). *In vitro* digestion of bread crust and biscuits were carried out in sodium tetraborate buffer 0.1M and pH 8.2 at a ratio of 150 g of food/L of buffer during 72h at 37ºC. Pronase E concentration was 100 mg/L of buffer. The digested solution was submitted to diafiltration for melanoidin isolation using a 5 kDa membrane from Sartorius (United Kingdom) and a peristaltic pump from Cole Parmer (USA). Melanosaccharides (obtained from coffee, beers, chocolate, balsamic vinegar and sweet wine) were directly isolated by diafiltration using the same membrane and pump as before

as described in Rufián-Henares & de la Cueva, (2009). In the case of chocolate, it was first dissolved in Milli-Q water. Melanoidins were stored at -80 °C until analysis.

2.3. Spent coffee grounds preparation

Spent coffee grounds were included in the experiments since they still retain melanoidins from coffee and other bioactive compounds (Jiménez-Zamora et al., 2015). Spent coffee grounds were obtained after brewing coffee in an expresso coffee maker as described in Pérez-Burillo et al. (2019). Spent coffee grounds were afterwards freeze-dried and stored at -80 °C until further analysis.

2.4. In vitro gastrointestinal digestion and fermentation

All samples were subjected to an *in vitro* digestion and fermentation to mimic physiological processes in the human gut. The *in vitro* digestion method was carried out with alpha-amylase, pepsin, pancreatin and bile salts according to the protocol described by Pérez-Burillo, Rufián-Henares & Pastoriza (2018). The *in vitro* fermentation was carried out according to the protocol described by Pérez-Burillo et al. (2018). Briefly, the solid residue obtained after *in vitro* digestion plus a 10% of the digestion supernatant was fermented by gut microbiota from faecal samples obtained from three healthy adult donors (mean age range 27.3 years, not taking antibiotics, mean Body Mass Index = 21.3).

2.5.High-throughput amplicon sequencing

High-throughput sequencing of microbial 16S rRNA genes was carried out as previously described (Pérez-Burillo et al., 2019). Bacterial genomic DNA was isolated from each sample using ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research). DNA

was amplified using two pairs of primers, one targeting 16S rDNA V1-V2 region [forward primer 16S gene complementary sequence AGRGTTYGATYMTGGCTCAG and reverse primer 16S gene complementary sequence GCWGCCWCCCGTAGGWGT], and another targeting V4 region [forward GCCAGCMGCCGCGG and reverse GGACTACHVGGGTWTCTAAT complementary sequences, respectively]. 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). High throughput sequencing was performed on Ion Torrent Personal Genome Machine. We obtained an average of 17,490 sequence reads per sample. Sequence reads were processed in QIIME (Caporaso et al., 2010). Sequence read counts for each 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, Agans, Foy, & Paliy, 2011), and the resulting cell counts were sub-sampled to the lowest value among samples. The cell counts obtained independently for each sample based on the sequencing of V1-V2 and V4 16S rRNA gene regions were merged together into a single taxon abundance estimate via $A_{CUM} = \sqrt{(A_{V1V2}^2 + A_{V4}^2)/2}$ calculation, where A is an abundance value for each taxon.

2.6. Analysis of short chain fatty acids

SCFAs determination was carried out by UV-HPLC according to the procedure described in Panzella et al. (2017). After the fermentation process, 1 mL of melanoidin 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. Analysis of phenolic compounds

Phenolic compounds were analyzed through UV-HPLC as described in Moreno-Montoro et al. (2015). In brief, one mL of melanoidin fermentation supernatant was mixed with 1 mL of diethyl ether and kept in the dark at 4ºC for 24 hours. The organic phase was then collected and another two extractions with diethyl ether were performed. These 3 mL of diethyl ether were dried in a rotary evaporator set at 30ºC and the solid residue was resuspended in 1 mL of methanol:water (50:50 v/v) mix. The mixture was then ready to be injected into HPLC system. Identification and quantification were carried out by comparing retention times obtained from pure standards (listed in *reagents* section).

2.8. Antioxidant assays

The antioxidant capacity of melanoidins before and after *in vitro* digestion-fermentation was measured using three different approaches.

$TEAC_{ABTS}$ assay. The radical scavenging activity of samples was performed following the method described by Re et al. (1999) adapted to a microplate reader (Jiménez-Zamora, Delgado-Andrade & Rufián-Henares, 2016). The results were expressed as μmol Trolox equivalents per g of sample.

$TEAC_{FRAP}$ assay. The reducing capacity of iron was analyzed by the method described by Benzie & Strain (1996) adapted to a microplate reader (Rufián-Henares, García-Villanova & Guerra-Hernández, 2006). The results obtained were expressed as μmol Trolox equivalents per g of sample.

$TEAC_{DPPH}$ assay. This method was carried out according to the procedure described by Yen & Chen (1995). Results were expressed as μmol equivalents of Trolox per g of sample.

2.9.Multivariate statistical analyses

Unconstrained principal coordinates analysis (PCoA) utilizing phylogenetic weighted UniFrac distance as a measure of sample dissimilarity was performed on the genus-level microbial abundance dataset. Principal component analysis was carried out to assess sample similarity based on the levels of short chain fatty acids and phenolic compounds. Multivariate analyses were run in R and Matlab (Paliy & Shankar, 2016).

3. Results and discussion

Melanoidins are bioactive compounds generated during the thermal processing of foods (Rufián-Henares, Delgado-Andrade & Morales, 2006) with bioactive effects potentially similar to those of fiber-enriched foods (Delgado-Andrade, Rufián-Henares & Morales, 2007). Thus, in order to assess the bioactivity of food melanoidins and the effect of the gut microbiota over such bioactivity, ten melanoidins were isolated from different foods and subjected to an *in vitro* digestion-fermentation process designed to mimic natural digestion in the human oral, gastric, and intestinal chambers. Melanoidins bioactivity was measured as their ability to (i) modify the gut microbial community (gut microbiota composition) and activity (production of SCFAs), (ii) release phenolic compounds, and (iii) provide antioxidant capacity.

3.1. Fermentation of melanoidins from different foods by human fecal microbiota promotes different community structures

Using high-throughput sequencing of 16S rRNA gene, microbiota community structure was determined in all fermented samples. As depicted in Figure 1A, samples could be separated into 4 distinct groups based on microbiota composition. Digested melanoidins from bread crust, cereals, and pilsner and black beers promoted a remarkable expansion of members of genus *Bifidobacterium*, which represented on average 92% of all microbial cells in these samples. Microbiota that was fed with digested chocolate and sweet wine melanoidins also contained significant amounts of bifidobacteria, albeit at a lower level (68% on average). In contrast, biscuit melanoidins promoted the expansion of *Faecalibacterium* (30% relative abundance in this community vs 5% average abundance in all other samples). The remaining three melanoidin samples maintained microbial communities more similar to that of the baseline inocula (represented by the blank sample that only received inocula and buffer, but no additional nutrient sources). This baseline community was relatively abundant in members of genera *Blautia*, *Roseburia*, *Citrobacter* and *Enterobacter*.

The separation of communities into 4 distinct groups was confirmed by the phylogenetic PCoA ordination analysis (Figure 1B). Abundances of *Bifidobacterium* and *Faecalibacterium* were the two main drivers of sample distribution in the PCoA space (see Figures 1C and 1D). Based on the community structure analysis, it appears that melanoidins from spent coffee grounds were largely not fermented by human fecal microbiota, because the community structure was very similar to that maintained in the buffer medium. Abundances of *Bifidobacterium* and *Faecalibacterium* displayed a reciprocal relationship (Spearman rank correlation R_s= -0.97), likely indicating different

enzymatic capacities of these bacteria to break down different melanoidin structures. *Bifidobacterium* expansion resulted in a remarkable reduction in community diversity (Shannon H' index of 0.5 for group 1 samples vs 2.3 for the baseline group communities), possibly indicating that only members of this genus were able to utilize melanoidins from bread crust, cereals, and pilsner and black beers for growth. Note that both *Bifidobacterium* and *Faecalibacterium* are considered beneficial to human health (Murri et al., 2013; Flint, Duncan, Scott, & Louis, 2015), thus their expansion can be viewed as a positive effect and these melanoidins could be used to formulate functional foods.

3.2. SCFAs production from melanoidins fermentation

Short chain fatty acids are the main end products of microbial fermentation in the gut. Thus, the concentrations of four major SCFAs (acetate, butyrate, lactate, and propionate) were measured in all fermented samples (Flint et al., 2015). Overall, acetate and lactate were the main end products of fermentation of melanoidins, with levels of butyrate and propionate being significantly lower (Figure 2A). Acetate and lactate are the main end products of *Bifidobacterium* fermentation (Flint et al., 2015), and their levels correlated with this genus abundance (Spearman rank correlation R_s = 0.75 and 0.78, respectively). Thus, the SCFAs measurements fit well with the gut microbiota analysis described above. As anticipated, blank sample had very low levels of all four SCFAs, and other samples in group 4 also generated lower SCFAs levels, indicating poor melanoidin fermentation. In contrast, melanoidins from bread crust, cereals, pilsner and black beers (group 1) were highly fermented and produced the largest SCFAs amounts (see Figure 2A). Since melanoidins from these foods promoted *Bifidobacterium*, they can be considered possible prebiotics for improvement of gut health.

The output of the principal component analysis of SCFAs abundance dataset also corroborated the separation of samples into four groups (Figure 3A). Samples from group 4 where SCFAs production was low were well split from other samples, which were more tightly clustered. Co-inertia analysis (Shankar et al., 2017) was used to assess if sample distribution was similar between SCFAs-based PCA and genus-based PCoA ordinations. As shown in Figure 3C, samples were indeed distributed similarly in both ordination spaces ($p = 0.03$), indicating that microbiota and short chain fatty acids measurements corroborated each other.

3.3. Release of phenolic compounds during melanoidins fermentation

Melanoidins can contain different phenolics depending on the food source (Tagliazucchi & Bellesia, 2015). Therefore, the metabolization of melanoidins by gut microbes could result in a release of phenolic compounds, which could elicit anti-oxidant properties. Moreover, these phenolics could be further metabolized by the microbes, usually yielding smaller compounds (Selma, Espín, & Tomás-Barberán, 2009). UV-HPLC was used to measure the concentrations of 26 phenolic compounds in all profiled samples (Figure 2B and Table 1). In contrast to the SCFAs production, PCA analysis of phenolic compounds dataset failed to distribute samples according to the four groups defined above. Instead, most samples clustered together, with an exception of chocolate and coffee samples that were significant outliers (Figure 3B). Most samples differed significantly in their metabolite profiles, and the detected compounds in each sample are described below.

Bakery products melanoidins: Biscuit, bread crust, and breakfast cereal melanoidins (melanoproteins) released similar amounts of polyphenols during fermentation (2.08-2.44 μg/g). Most of them (60-82%) have been identified as microbial metabolites from other

phenolic compounds (Rowland et al., 2018). These melanoidins showed high amounts of pyrogallol (**Table 1**), which could indicate the presence of epicatechin or gallic acid in the melanoidin backbone (Rowland et al., 2018). **Biscuit melanoidins** also released noticeable amounts of vanillin and daidzein, probably coming from the food source. **Bread crust melanoidins** showed high amounts of 2-(3,4-dihydroxyphenyl)acetic acid and 3-(3,4-dihydroxyphenyl)propionic acid (**Table 1**), microbial metabolites of quercetin, myricetin, epicatechin, or hydroxycinnamic acids (Selma et al., 2009; Marín, Miguélez, Villar, &Lombó, 2015; Rowland et al., 2018). **Breakfast cereals melanoidins** showed high amounts of p-hydroxyphenylacetic acid (**Table 1**), a potential microbial metabolite of hydroxycinnamic acids (Rowland et al., 2018). Relatively high amounts of ferulic acid were also found, probably from the food sources.

Beer melanoidins: Black and pilsner beer melanoidins released similar amounts of polyphenols during *in vitro* fermentation, 7.38 and 7.42 μg/g respectively. However, the percentage of possible microbial metabolites was higher (52%) in black beer than in pilsner (35%). Both contained notable amounts of the microbial metabolites 2-(3,4-dihydroxyphenyl)acetic acid, 3-(3,4-dihydroxyphenyl)propionic acid and pyrogallol. Both of them also showed high amounts of 3,5-dihydroxybenzoic acid, which could come from the beer itself (Wannenmacher, Gastl & Becker, 2018), since it has not been related to microbial metabolism. **Black beer** was characterized by high amounts of 4-O-caffeoylquinic acid and epigallocatechin gallate and the microbial metabolite 3,4-dihidroxybenzoic acid, metabolite from hydroxycinnamic acids and cyanidin (Rowland et al., 2018) (**Table 1**). **Pilsner beer,** on the other hand, was characterized by high amounts of

ferulic acid and the microbial metabolite 3,4-dihydroxybenzaldehyde, which, according to (Selma et al., 2009), results from microbial degradation of quercetin (**Table 1**).

Balsamic vinegar melanoidins: Released the lowest amount of polyphenols (1.09 μg/g), almost exclusively 3,5-dihydroxybenzoic acid and the microbial metabolite 2-(3,4-Dihydroxyphenyl)acetic acid.

Sweet wine melanoidins: The amount of polyphenols released from these melanoidins was similar to that of bakery products, 1.93 μg/g. Most of them (74%) were possible microbial metabolites: 2-(3,4-dihydroxyphenyl)acetic acid, 3,4-dihydroxybenzaldehyde, p-hydroxyphenylacetic acid, 3-(3,4-dihydroxyphenyl)propionic acid, and pyrogallol. Regarding non-microbial metabolites, the higher amounts were showed by orto- and meta-coumaric acids.

Chocolate, and coffee melanoidins, and spent coffee grounds: Released the highest amounts of polyphenols, 60.15, 48.76, and 58.45 μg/g, respectively. Phenolic profile of chocolate melanoidins and spent coffee grounds were similar, with most phenols being non-microbial metabolites. Especially large amounts of 3,5-dihydroxybenzoic acid were found in both samples. **Chocolate melanoidins** showed also noticeable amounts of the microbial metabolites 3,4-dihidroxybenzoic acid, 2-(3,4-dihydroxyphenyl)acetic acid, 3-(3,4-dihydroxyphenyl)propionic acid, and pyrogallol. S**pent coffee grounds** were largely not metabolized, since 90% of the phenolics were represented by 3,5-dihydroxybenzoic acid, which is not described as microbial metabolite. On the other hand, 51% of **coffee melanoidins** phenolics were represented by microbial metabolites, especially p-hydroxyphenylacetic acid (probably coming from hydroxycinnamates metabolism), and

pyrogallol (from epicatechin or gallic acid metabolism) (**Table 1**). A large concentration of chlorogenic acid was also released from coffee melanoidins.

3.4. Melanoidins possess significant antioxidant capacity

According to Tagliazucchi & Verzelloni (2014) there is a strong correlation between the phenolic content of melanoidins and their antioxidant capacity. Thus, three different methods were used to estimate the antioxidant capacity of fermented and digested samples: ABTS (measures most antioxidants including phenolics and thiols), FRAP (measures Fe^{3+} reduction) and DPPH (measures antiradical activity). A strong correlation among the three antioxidant methods was found ($R_s \geq 0.95$). All three measurements also showed a moderate correlation with the total amounts of phenolics detected in each sample ($0.35 \leq R_s \leq 0.44$), though the relationships were not statistically significant ($p \geq 0.2$). In all three assays, coffee and spent coffee melanoidins showed the highest antioxidant capacity after fermentation, followed by sweet wine melanoidins and balsamic vinegar melanoidins (Figure 2C). On the other hand, pilsner beer, breakfast cereals, and bread crust melanoidins were always those with the lowest values. Our results are in agreement with Verzelloni, Tagliazucchi & Conte (2010) who reported that coffee melanoidins had more antioxidants than black beer and balsamic vinegar melanoidins. Even though the structure of melanoidins varies significantly, coffee melanoidins as well as sweet wine and chocolate melanoidins contain phenolic moieties that could make them more antioxidant than others (Morales et al., 2012).

Measurements of antioxidant capacity of digested but not fermented melanoidins similarly revealed that coffee melanoidins had the highest capacity, whereas the bread crust melanoidins had the lowest (**Table 2**). For biscuit, breakfast cereals, and balsamic vinegar

melanoidins the antioxidant capacity increased after fermentation; for other melanoidins it was opposite. According to Wang et al. (2011) melanoidins antioxidant capacity is thought to be partly related to their metal chelating ability because of their anionic nature and partly due to their scavenging activity. After fermentation, melanoidins are degraded to some extent by the gut microbiota, thus decreasing their chelating capacity. Moreover, scavenging capacity is directly related to the presence of phenolic moieties in melanodin structure (Wang et al., 2011), and the biotransformation of some of them by gut microbes could result in a decreased antioxidant capacity. Accordingly, a statistically significant positive correlation was found between the ratio of antioxidant capacities prior to/after fermentation and the total of identified phenolic compounds ($R_s = 0.70$, $p < 0.05$). These data potentially indicate that transformation of melanoidins by gut microbiota can reduce their antioxidant capacity. This was true for black and pilsner beer, chocolate, coffee and sweet wine melanoidins (**Table 2**). This hypothesis was supported by a negative correlation found between antioxidant capacity of the fermented samples and the total SCFAs produced in those samples ($R_s = -0.47$, $p < 0.05$).

4. Conclusions

Melanoidins are an important part of our diet, reaching average amounts of 10 g/day. Here we have demonstrated that melanoidins can be used by gut microbes resulting in SCFAs production and also shaping their communities. Many melanoidins favored the growth of beneficial genera such as *Bifidobacterium* and *Faecalibacterium*. Gut microbes, by fermenting melanoidins, were able to release some phenolics initially linked to the melanoidin backbone, which in turn could increase phenolics absorption. Such analysis of polyphenols could be used to investigate melanoidins structure and also to investigate

microbial pathways. Therefore, these results suggest that melanoidins should be considered as potential prebiotic agents.

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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 legends

Figure 1. Fermentation of melanoidin preparations by human fecal microbiota promotes different community structures. Panel **A** shows relative abundances of the top nine most abundant microbial genera across all samples. Each column represents a community derived from an independent microbiota-based fermentation of particular melanoidin preparation. Abundances of other genera were summed and are represented cumulatively as "other genera". Sample names were abbreviated as follows: BRCR - bread crust; CERL – cereal; BLBR - black beer; PLBR - pilsner beer; BISC – biscuits; CHOC – chocolate; SWWN - sweet wine; BLVN - balsamic vinegar; COFF – coffee; SPCF - spent coffee; BLNK – blank. Four groups of samples with similar genus abundance profiles within group are separated by dotted lines. Panel **B** displays the output of the unconstrained PCoA ordination analysis of microbial genus abundance dataset among all profiled samples. Phylogenetic weighted UniFrac distance was used to calculate the sample dissimilarity matrix. The percent of dataset variability explained by each principal coordinate is shown in parentheses in axis titles. Samples are colored based on groups defined in Panel A. Panels **C** and **D** show the same PCoA output as in panel B, but with samples colored according to the abundance of *Bifidobacterium* (panel C) or *Faecalibacterium* (panel D) genera.

Figure 2. Metabolite measurements. Concentrations of short chain fatty acids (in mM), phenolic compounds (in parts per million), and antioxidants (defined as μmol of Trolox equivalent/g of sample) and are displayed in panels **A**, **B**, and **C**, respectively. Concentrations are visualized in log-scale and are represented by color gradient as shown

next to each heatmap. Four groups of samples defined in Figure 1A are separated by horizontal black lines. Sample abbreviations are defined in Figure 1 legend.

Figure 3. Ordination analysis of metabolite data. Panels **A** and **B** display the output of Euclidean-distance based principal component analysis of short chain fatty acids and phenolic compounds, respectively. Due to vast differences in concentrations among different compounds, all values for each compound were standardized across samples. Sample abbreviations are defined in Figure 1 legend. Co-inertia analysis depicted in panel **C** reveals congruency of sample dispersal in ordination space based on SCFAs and genus abundance profiles. The distance between each sample position on two ordination plots is indicated by a connecting line. Shorter lines represent similar sample positioning in each plot. The statistical significance and the relative 'fit' of the ordinations were assessed by p-value and RV coefficient, respectively.

Table 1. Microbial phenolic metabolites, parent phenolic compounds and microbial types (Data obtained from Selma et al., 2009; Marín et al., 2015; Rowland et al., 2018).

Phenolic compound group	Phenolic compound sub-group	Parent compound	Principal metabolites	Microbial types
Phenolic acids	**Benzoic acids**	Gallic acid	Urolithins A & B, isourolithins A & B	*Gordonibacterurolithinfaciens, Gordonibacterpamelaeae*
		Ellagitannins	Pyrogallol	
	Hydroxycinnamic acids	Chlorogenic acid	3-(3,4-Dihydroxyphenyl)-propionic acid	*Escherichia coli, Bifidobacterium lactis, Lactobacillus gasseri*
		Hydroxycinnamates	3-(3-hydroxyphenyl)-propionic acid	
			3-(4-hydroxyphenyl)-propionic acid	
			Hydroxyphenyl-ethanol	
			Vanillin	
			Phenylacetic acids and Benzoic acids	
Flavonoids	**Flavonols**	Kaempferol	2-(4-Hydroxyphenyl)propionic acid	*Clostridium orbiscidens, Enterococcus casseliflavus*
		Kaempferol	2-(3,4-Dihydroxyphenyl)acetic acid	*C. orbiscidens, Eubacterium*
		Kaempferol	2-(3-hydroxyphenyl)acetic acid	Clostridium strains
		Quercetin	2-(3,4-dihydroxyphenyl)acetic acid	*Clostridium orbiscindens, Eubacteriumoxidoreducens, Butyrivibrio spp.*

	Quercetin	2-(3-hydroxyphenyl)acetic acid	
	Quercetin	3-(3,4-dihydroxyphenyl)propionic acid	
	Quercetin	3,4-dihydroxybenzaldehyde	
	Quercetin	3,4-dihydroxybenzoic acid	
	Quercetin	3-methoxy-4-hydroxybenzoic acid (vanillic acid)	
	Quercetin	3-(3,4-Dihydroxyphenyl)-acetic acid	*Clostridium orbiscidens, Eubacterium.oxidoreducens*
	Myricetin	2-(3-Hydroxyphenyl)acetic acid	Clostridium strains
		3-(3,4-Dihydroxyphenyl)propionic acid	*Eubacteriumramulus*
		3-(3-Hydroxyphenyl)propionic acid	*Clostridium orbiscidens, E. oxidoreducens*
		2-(3,5-Dihydroxyphenyl)acetic acid	
		2-(3-Hydroxyphenyl)acetic acid	
Flavanones	Naringenin	3-(4-Hydroxyphenyl)propionic acid	Clostridium strains, *Eubacteriumramulus*
		3- phenylpropionic acid	
	Isoxanthohumol (from hops)	8-Prenyl-naringenin	*Eubacteriumlimosum*

	Flavan-3-ols	Catechin	3-(3-Hydroxyphenyl)propionic acid	*Clostridium coccoides, Bifidobacterium infantis*
			5-(3′,4′-Dihydroxyphenyl)--valero-lactone	
		Epicatechin	5-(3,4-Dihydroxyphenyl)valeric acid	
			3-(3,4-Dihydroxyphenyl)propionic acid	
			Pyrogallol	
		Epigallocatechin	5-(3′,4′-Dihydroxyphenyl)-γ-valero-lactone	
			5-(3′,5′-Dihydroxyphenyl)-γ-valero-lactone	
	Flavones	Luteolin	3-(3,4-Dihydroxyphenyl)-propionic acid	*Clostridium. orbiscindens, Enterococcus avium*
		Apigenin	3-(4-hydroxyphenyl)-propionic acid	*Eubacteriumramulus, Bacteroidesdistasonis*
			3-(3-hydroxyphenyl)-propionic acid	
			4-hydroxycinnamic acid, phloretin	
	Isoflavones	Daidzein	Equol	*Bacteroidesovatus, Streptococcus intermedius, Ruminococcusproductus*
			O-desmethylangolensin	

			Eggerthellasp.Julong 732, Slakiaequolifaciens, Adlercreutziaisoflavoniconvertens, Slakiaequolifaciens
			Consortium of Lactobacillus, mucosae *Enterococcus faecium, Finegoldia magna, Veillonella spp.*
			Clostridium spp. HGHA136, Eubacteriumramulus
	Formononetin	Daidzein	
	Genistein	6′-OH-O-desmethylangolensin	*Eubacteriumramulus*
		2-(4-hydroxyphenyl)propionic acid	
Anthocyanidins	Cyanidin	3,4-Dihydroxybenzoic acid	*Clostridium saccharogumia, Eubacteriumramulus, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus acidophilus LA-5, Bifidobacterium lactis BB-12*
	Peonidin	3-Methoxy4-hydroxybenzoic acid	
	Pelargonidin	3-hydroxycinnamic acid	
	Malvidin	4-Hydroxybenzoic acid	
		3,4-Dimethoxybenzoic acid	

Lignans	**Lignans**	Secoisolaricinresinoldiglucoside	Enterodiol	*Bact. distasonis, Bact. fragilis, Bact. ovatus, Clostridium cocleatum, Clostridium.sp SDG-MT85-3Db, Butyribacteriummethylotrophicum, Eubacteriumcallanderi, Eubacteriumlimosum, Peptostreptococcusproductus, Clostridium scindens*
			Enterolactone	*Eggerthellalenta, ED-Mt61/PY-s6*
Secoiridoids	**Secoiridoids**	Oleuropein	Tyrosol	
		Ligstroside	Hydroxytyrosol	

Table 2. Antioxidant capacity of melanoidins prior and after *in vitro* fermentation. Sample names were abbreviated as follows: BRCR - bread crust; CERL – cereal; BLBR - black beer; PLBR - pilsner beer; BISC – biscuits; CHOC – chocolate; SWWN - sweet wine; BLVN - balsamic vinegar; COFF – coffee; SPCF - spent coffee grounds.

Sample	FRAP (μmol Trolox equivalent/g of sample)			ABTS (μmol Trolox equivalent/g of sample)			DPPH (μmol Trolox equivalent/g of sample)			FRAP (μmol Trolox equivalent/g of sample)			ABTS (μmol Trolox equivalent/g of sample)			DPPH (μmol Trolox equivalent/g of sample)		
	Digested melanoidins									Fermented melanoidins								
BRCR	17.4	±	0.5	65.4	±	2.0*	25.6	±	0.8	16.9	±	0.9	52.2	±	2.0	15.5	±	1.9
CERL	3.05	±	0.07*	12.8	±	0.3*	1.64	±	0.04*	29.1	±	1.0	65.4	±	5.0	4.8	±	0.3
BLBR	63.6	±	2.9*	347	±	14.9*	52.9	±	2.4	47.3	±	2.3	203	±	3.0	46.0	±	1.8
PLBR	36.6	±	1.8*	175	±	9.3*	42.0	±	2.1*	21.7	±	1.5	98.7	±	3.6	22.2	±	1.1
BISC	43.1	±	1.5*	154	±	5.8*	12.0	±	0.5	103	±	3.6	319	±	1.3	25.5	±	1.9
CHOC	164	±	11.2	511	±	32.0*	97.9	±	6.7	108	±	2.7	232	±	12.9	103	±	5.6
SWWN	464	±	17.1	946	±	35.1*	539	±	19.0*	433	±	5.3	688	±	9.9	263	±	5.2
BLVN	219	±	3.2**	541	±	7.8**	139	±	2.0**	339	±	3.9	628	±	8.2	225	±	3.5
COFF	1475	±	41.0**	2700	±	78.1*	1595	±	46.1*	674	±	21.1	1065	±	13.4	628	±	5.6
SPCF	126	±	2.8*	198	±	4.6*	168	±	3.8	1048	±	19.7	1511	±	71.3	735	±	29.0

*whithin the column means statistical significance between digested and fermented melanoidins.

Figure 1

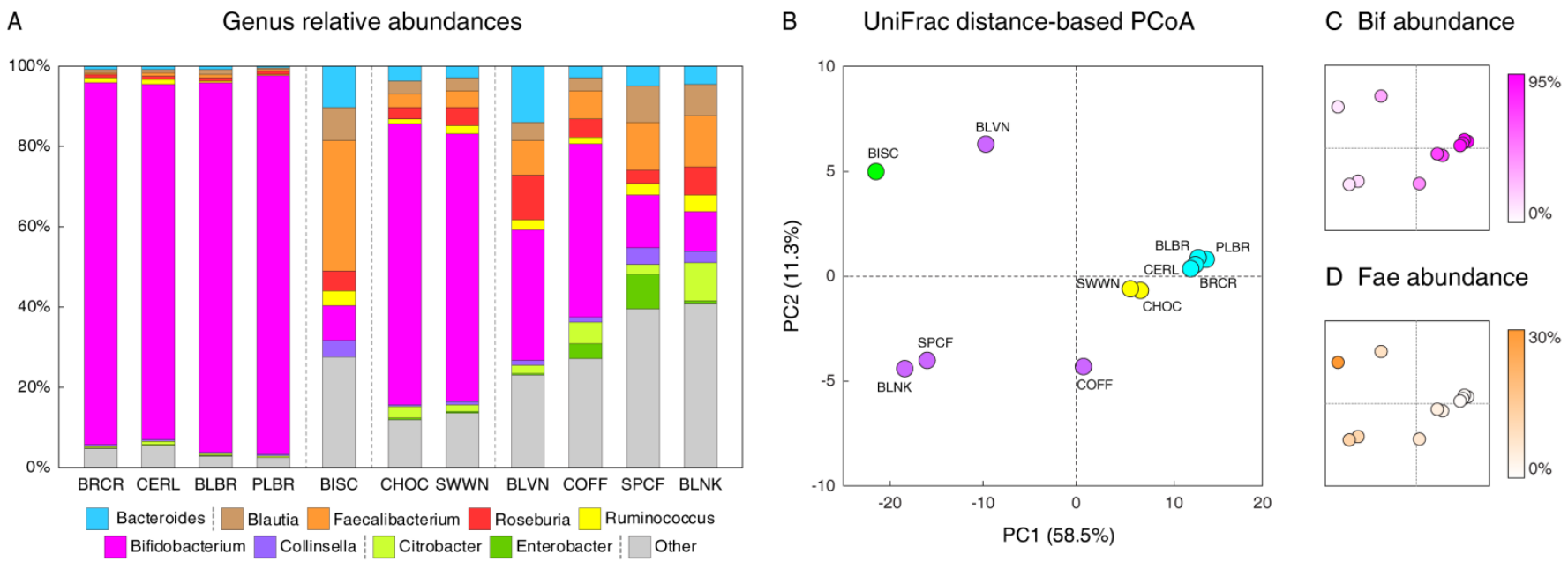

A
Genus relative abundances
100%
80%
60%
40%
20%
0%
BRCR CERL BLBR PLBR BISC CHOC SWWN BLVN COFF SPCF BLNK
Bacteroides
Blautia
Faecalibacterium
Roseburia
Ruminococcus
Bifidobacterium
Collinsella
Citrobacter
Enterobacter
Other
B
UniFrac distance-based PCoA
PC2 (11.3%)
PC1 (58.5%)
BISC
BLVN
BLBR
PLBR
CERL
BRCR
SWWN
CHOC
SPCF
BLNK
COFF
C Bif abundance
95%
0%
D Fae abundance
30%
0%

Figure 2

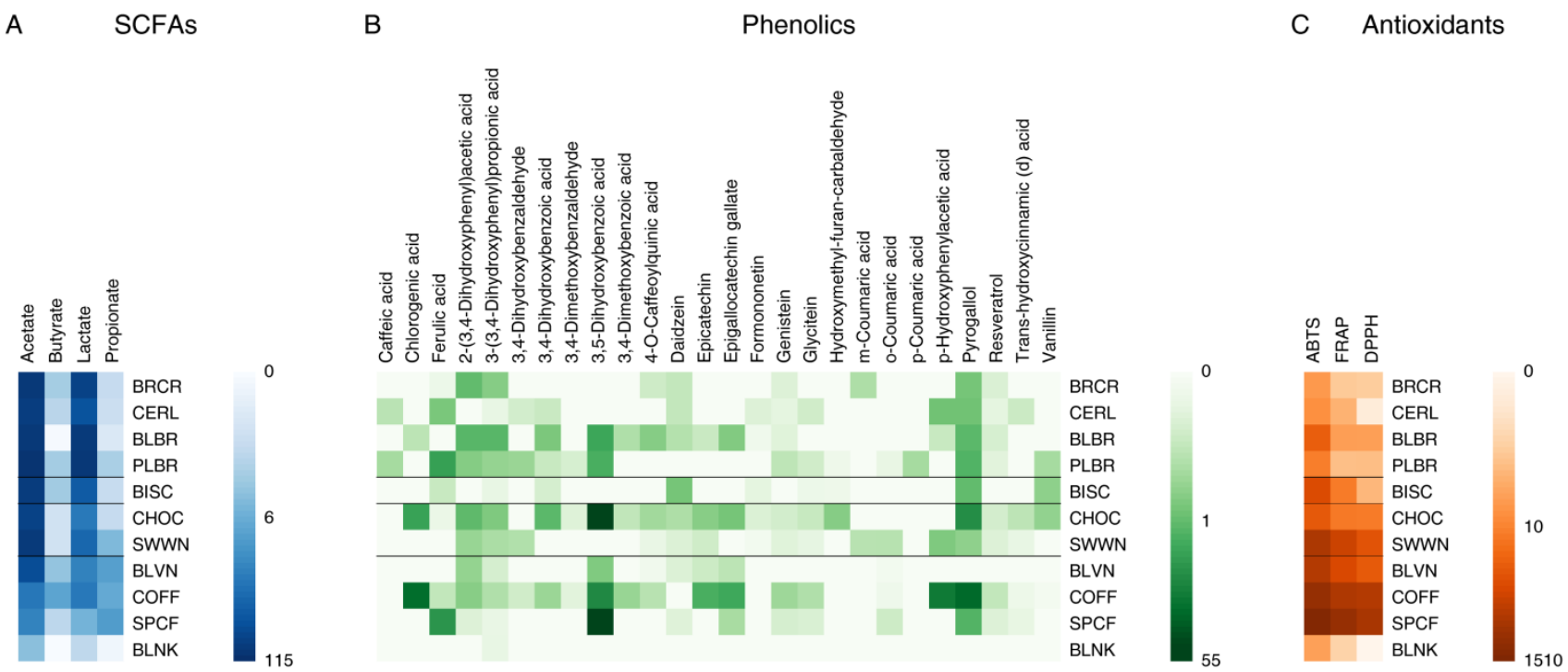
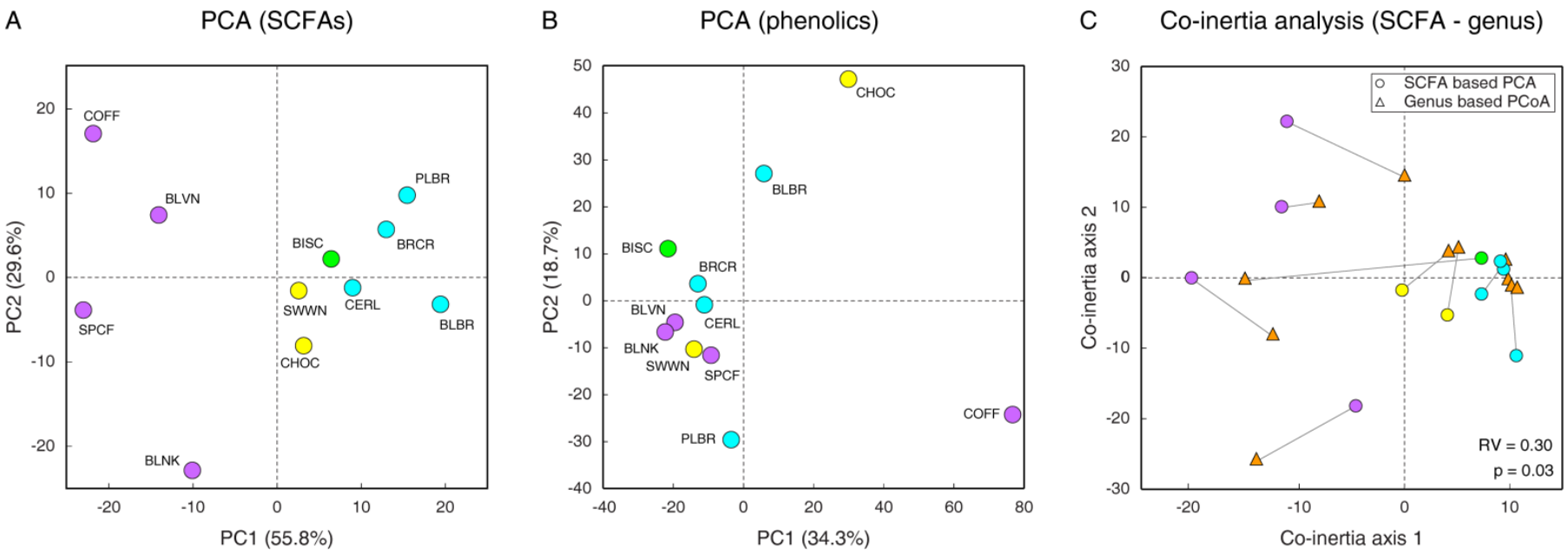

A
SCFAs
Acetate
Butyrate
Lactate
Propionate
BRCR
CERL
BLBR
PLBR
BISC
CHOC
SWWN
BLVN
COFF
SPCF
BLNK
0
6
115
B
Phenolics
Caffeic acid
Chlorogenic acid
Ferulic acid
2-(3,4-Dihydroxyphenyl)acetic acid
3-(3,4-Dihydroxyphenyl)propionic acid
3,4-Dihydroxybenzaldehyde
3,4-Dihydroxybenzoic acid
3,4-Dimethoxybenzaldehyde
3,5-Dihydroxybenzoic acid
3,4-Dimethoxybenzoic acid
4-O-Caffeoylquinic acid
Daidzein
Epicatechin
Epigallocatechin gallate
Formononetin
Genistein
Glycitein
Hydroxymethyl-furan-carbaldehyde
m-Coumaric acid
o-Coumaric acid
p-Coumaric acid
p-Hydroxyphenylacetic acid
Pyrogallol
Resveratrol
Trans-hydroxycinnamic (d) acid
Vanillin
0
1
55
C
Antioxidants
ABTS
FRAP
DPPH
0
10
1510

Figure 3


A
PCA (SCFAs)
PC2 (29.6%)
PC1 (55.8%)
COFF
BLVN
PLBR
BISC
BRCR
SWWN
CERL
BLBR
SPCF
CHOC
BLNK
B
PCA (phenolics)
PC2 (18.7%)
PC1 (34.3%)
CHOC
BLBR
BISC
BRCR
BLVN
CERL
BLNK
SWWN
SPCF
COFF
PLBR
C
Co-inertia analysis (SCFA - genus)
SCFA based PCA
Genus based PCoA
Co-inertia axis 2
Co-inertia axis 1
RV = 0.30
p = 0.03