Effects of different foods and cooking methods on the gut microbiota: an in vitro approach

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Research Article

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Abstract

Background

Dietary interventions are likely tools for modulation of the gut microbiota but the large inter-individual variability in gut microbiota composition leads to different host responsiveness and the impact of a particular food cannot be assessed. In contrast, *in vitro* fermentation models allow characterization of the fecal microbiota when fermenting a large number of different foods. Furthermore, cooking methods also directly influence the effects of food on gut microbiota composition. The aim of this study was to investigate the gut microbiota growing on representative foods of the Mediterranean and Western diets as well as the influence of cooking methods using *in vitro* fermentations.

Results

We performed *in vitro* digestions and fermentations of 55 foods, raw or cooked using up to 5 cooking methods, for a total of 159 combinations, employing fecal material from three healthy adults as inoculum. The composition of the bacterial communities was determined by sequencing the 16S rRNA gene. Foods derived from plants or animals had significantly different impacts on the abundances of bacterial taxa. Animal and vegetable fats, fish and dairy products led to the greatest shifts in microbial composition. Specifically, an increase in the beneficial bacteria *Faecalibacterium*, *Blautia* and *Roseburia* was identified in animal and vegetable fats. However, butter, dairy products and fish also resulted in higher abundances of *Lachnoclostridium*, which has been associated to several diseases. With respect to cooking methods, only frying and roasting had strong and common effects across all food categories. In general, fried foods showed more differences than other cooking methods, and *Ruminococcus* was particularly responsive to the cooking method employed.

Conclusions

Despite substantial differences in baseline microbiota composition, some shared effects were detected across the three analyzed individuals, such as the substantial impact of high-fat foods on the abundance of health-relevant bacteria. Cooking methods effects on the gut microbiota resulted to be highly individualized and food-dependent, making them challenging to investigate and integrate into personalized diet. Further characterization of the responses of the fermentative microbiota to food-cooking method combinations will enable the refinement of dietary interventions aimed at gut microbiota modulation, paving the way towards personalized nutrition.

Background

In recent years, convincing evidence has been accumulated on the role of the gut microbiota as a mediator of the impact of diet on the physiology and metabolic status of the host[1, 2]. Long and short-
term dietary intake influence the structure and activity of the gut microbiota, as confirmed by the shifts in microbial community structure caused by short-term animal or plant-based diets[3], and by the strong association between enterotypes and long-term diets[4]. Thus, dietary patterns such as the “Western” diet alter the composition and metabolic activity of the human microbiome, inducing changes suspected of contributing to growing epidemics of chronic illnesses such as obesity and type 2 diabetes[5–8].

Although there are only a few studies to date, there is growing evidence that not only food composition but also cooking methods may play an important role in the modulation of the gut microbiota due to chemical composition alterations during the cooking process[9–12]. Thermal processing can alter the chemical composition of the food, for instance through the well-known Maillard reaction. These changes in chemical composition will be dependent on both the cooking method and the food, highlighting the fact that the effects of cooking on the gut microbiota need to be studied extensively and separately for each food[10]. Therefore, exploring the potential influence of cooking methods on the composition of the gut microbiota emerges as a promising field of study.

Dietary interventions are likely tools for modulation of the gut microbiota. However, the large inter-individual variability[13] in gut microbiota composition, due to differences in diet and other factors, leads to different host responsiveness to dietary interventions. Furthermore, the responsive bacterial taxa may differ among individuals. Therefore, host and microbiota response to an intervention are difficult to predict; and this variability may influence the results of intervention studies and interfere with reproducibility[14]. Ongoing research focuses on finding dietary manipulations aimed at promoting beneficial microorganisms that take into account host-microbiota relationships, with the aim of using personalized nutrition in therapeutic interventions and as a solution to tackle the variability challenge[1].

Nevertheless, intervention studies focused on gut microbiota modifications are difficult to perform, and the impact of a particular food cannot be assessed. For this reason, a variety of in vitro digestion and fermentation models that mimic human processes have been developed in the last decades, allowing for the assessment of the direct effects of certain compounds and foods on the microbiota[15]. Each approach has its advantages and limitations, as, although in vivo studies provide more relevant physiological information, in vitro models are key for testing specific foods and for initial screenings[16]. Furthermore, batch fermentation models enable short time parallel cultures to carry out many fermentations simultaneously. Therefore, this is the best approach to characterize the behavior of the gut microbiota when fermenting a large number of different foods, which can be tested one by one without the confounding factors of other foods.

The aim of this work is to further study how the composition of gut microbiota communities responds to a wide variety of foods representative of “Western” and “Mediterranean” diets, as well as the influence of cooking methods. In this context, 55 different foods raw or cooked using up to 5 cooking methods have been assessed. We performed in vitro digestion and fermentation assays, using fecal material from healthy adults as inoculum. The characterization of the resulting fermentative microbiota, by 16S rRNA gene sequencing, revealed which bacteria tended to increase or decrease when exposed to specific food
items. This knowledge will be useful to refine dietary interventions with the aim of modulating the composition of the gut microbiota and moving towards the goal of personalized nutrition as a therapeutic treatment.

Methods

Study design

Fifty-five different foods, raw or cooked with up to 5 cooking methods, resulting in 159 total combinations, were in vitro digested and fermented to study their effect on the gut microbiota. Stools from three healthy adults were used as separate inocula and in vitro fermentations were performed in duplicates. Water was used as fermentation control.

Due to the large number of foods used, for the analysis of the results they were grouped at three different levels: i) Animal-based and plant-based foods, ii) Food categories and iii) Individual foods. The foods included at each level are further described in Table 1.
Table 1
Foods employed in this study during *in vitro* digestions and fermentations.

<table>
<thead>
<tr>
<th>Plant based/animal-based</th>
<th>Food category</th>
<th>Food</th>
<th>Raw</th>
<th>Fried</th>
<th>Boiled</th>
<th>Roasted</th>
<th>Grilled</th>
<th>Toasted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal-based</td>
<td>Meat</td>
<td>Chicken</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Beef</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Lamb</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pork</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dairy Product</td>
<td>Milk</td>
<td>●</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>Yogurt</td>
<td>●</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Gouda</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Egg</td>
<td>Egg</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fish</td>
<td>Salmon</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Cod fish</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Animal and Vegetable Fats</td>
<td>Butter</td>
<td>●</td>
<td>●</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plant-based</td>
<td>Animal and Vegetable Fats</td>
<td>Olive oil</td>
<td>●</td>
<td>●</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Sunflower oil</td>
<td>●</td>
<td>●</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Fruit</td>
<td>Apple</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Banana</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Orange</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grapes</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plum</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peach</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Olives</td>
<td>●</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Grain-based product</td>
<td>Bread</td>
<td>●</td>
<td>●</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bread whole grain</td>
<td>●</td>
<td>●</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Different cooking methods performed with each food are indicated with a dot. Information is provided on which foods belong to which categories of the two food classifications employed. These two classifications as well as individual foods will be used in subsequent analyses.
<table>
<thead>
<tr>
<th>Food Group</th>
<th>Example Foods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penne</td>
<td>●</td>
</tr>
<tr>
<td>Penne whole grain</td>
<td>●</td>
</tr>
<tr>
<td>Rice longo</td>
<td>●</td>
</tr>
<tr>
<td>Rice longo whole grain</td>
<td>●</td>
</tr>
<tr>
<td>Biscuits</td>
<td>●</td>
</tr>
<tr>
<td>Biscuits whole grain</td>
<td>●</td>
</tr>
<tr>
<td>Breakfast Cereal whole grain</td>
<td>●</td>
</tr>
<tr>
<td>Breakfast Cereal</td>
<td>●</td>
</tr>
<tr>
<td>Legumes</td>
<td>Beans Kidney●●●●</td>
</tr>
<tr>
<td>Lentils</td>
<td>●●●●</td>
</tr>
<tr>
<td>Nuts</td>
<td>Nut mix●●●●</td>
</tr>
<tr>
<td>Peanuts</td>
<td>●●●●</td>
</tr>
<tr>
<td>Starchy Tubers</td>
<td>Potato●●●●</td>
</tr>
<tr>
<td>Sweet potato</td>
<td>●●●●</td>
</tr>
<tr>
<td>Sugar</td>
<td>Dark Chocolate●</td>
</tr>
<tr>
<td>Nutella</td>
<td>●</td>
</tr>
<tr>
<td>Vegetable</td>
<td>Zucchini●●●●●</td>
</tr>
<tr>
<td>Capsicum</td>
<td>●●●●●●</td>
</tr>
<tr>
<td>Carrot</td>
<td>●●●●●●</td>
</tr>
<tr>
<td>Eggplant</td>
<td>●●●●●●</td>
</tr>
<tr>
<td>Onion</td>
<td>●●●●●●</td>
</tr>
<tr>
<td>Cauliflower</td>
<td>●●●●●●</td>
</tr>
<tr>
<td>Spinach</td>
<td>●●●●●●</td>
</tr>
</tbody>
</table>

Different cooking methods performed with each food are indicated with a dot. Information is provided on which foods belong to which categories of the two food classifications employed. These two classifications as well as individual foods will be used in subsequent analyses.
Food and cooking methods

The foods and cooking methods employed are representative of Western and Mediterranean diets[17, 18]. A total of 42 plant foods were studied, belonging to the following categories: alcoholic drinks (beer, red wine), cereals (regular biscuits, whole-grain biscuits, bread, whole-grain bread, breakfast cereals, whole-grain breakfast cereals, penne, whole-grain penne, rice, whole-grain rice), cocoa (dark chocolate, Nutella), coffee (regular coffee, instant coffee), fruits (apple, banana, grape, olive, orange, peach, plum), legumes (kidney beans, lentils), nuts (nut mixture, peanuts), oils (olive oil, sunflower oil), tubers (potato, sweet potato) and vegetables (cabbage, carrot, cauliflower, eggplant, lettuce, onion, pepper, spinach, tomato, zucchini). On the other hand, 11 animal foods were investigated belonging to the following categories: dairy (milk, yogurt, gouda, butter), egg, fish (cod fish and salmon), and meat (chicken, lamb, pork, and beef). In addition, the beverages cola and light cola were also investigated. All foods were bought in three different supermarkets (Carrefour, Dani and El Corte Inglés, Granada, Spain) and stored at room temperature or under refrigeration for a maximum of 2 days before cooking.

The samples were submitted to different culinary treatments: boiling, grilling, roasting, frying, and toasting. In addition, some foods were also investigated in raw form, as this is the common way they are consumed. Finally, milk was commercially processed by ultra-high temperature (UHT).

Extra virgin olive oil (EVOO) was used as a cooking medium for grilling and frying. Boiling was performed at 100°C for 20 min at a water/food ratio of 5:1. Grilling was performed at 220–250°C for 3 min on each side at an oil/food ratio of 0.5:1. Roasting was performed at 180°C for 10 min. Fried foods were obtained
at 180°C for 8 min at an oil/food ratio of 5:1. Toasting was performed in a Grunkel TS140H toaster at the fourth level at 200°C for 3 min at 900 W following the manufacturer’s instructions. The other utensils used for sample preparation were the following: a transportable oven (1500 W), fryer, frying pan and saucepan and forks, knives, and spoons.

Cooking times and food/medium ratios were based on Ramírez-Anaya et al.[19] and Olmedilla-Alonso et al.[20] and adapted to our equipment and laboratory conditions. Once cooked, all samples were homogenized and stored under nitrogen atmosphere at -80 °C in order to avoid oxidation.

**Fecal material collection**

Fecal samples were obtained from three healthy donors, who had not taken antibiotics, prebiotics or probiotics for three months prior to the assay, with a mean Body Mass Index of 21.3. At least three fecal samples of each donor were obtained (each one from a different fecal movement); the fecal samples were pooled together to reduce intra-individual daily variability. Fecal donors followed a regular Mediterranean diet the day before sample collection[21].

Fecal material was delivered in a sterile recipient by the volunteers, stored in a home refrigerator and transported to the laboratory in a cooler bag within 4 h. Upon arriving at the laboratory, the feces were mixed with a water:glycerol solution (20% vol/vol) and stored at -80°C[22].

**In vitro gastrointestinal digestion and fermentation**

All foods were submitted to *in vitro* batch digestion-fermentation in order to mimic physiological processes in the human gut, according to the protocols previously described[21, 22]. For each sample, 5g of food were submitted (in duplicate) to *in vitro* gastrointestinal digestion followed by *in vitro* fermentation. The food was added to Falcon tubes and three digestion phases were performed: oral (adding α-amylase for 2 min under agitation at 37°C), gastric (adding pepsin for 2 h with agitation at pH 2–3 at 37°C) and intestinal (adding bile salts and pancreatin for 2 h under agitation at pH 7 and 37°C). The *in vitro* fermentation was performed at 37°C for 20 h using the fecal samples from healthy donors described above[22]. A control fermentation was performed using only the fecal fermentation solution (peptone, cysteine, and resazurin) and water. The samples were then centrifuged, and the solid residues were taken for analysis.

**DNA extraction**

The solid residues derived from *in vitro* fermentation were used to obtain the bacterial suspensions, which were lysed with lysozyme at a final concentration of 0.1mg/ml. The genomic DNA extraction was then performed with the MagNaPure LC JE379 platform (Roche) and DNA Isolation Kit III (Roche), following the manufacturer's instructions. DNA was quantified with a Qubit 3.0 Fluorometer (Invitrogen),
while agarose gel electrophoresis (0.8% w/v agarose in Tris-borate-EDTA buffer) was used to determine DNA integrity. Finally, the DNA was stored at -20 °C until further processing.

**16S rRNA gene amplicon sequencing**

The V3-V4 hypervariable region of the 16S rRNA gene was amplified using as template 12ng of microbial genomic DNA, following the Illumina protocol for 16S Metagenomic Sequencing Library Preparation. PCR primers were as described by Klindworth et al. (2013)\[23\] with the forward primer (5’-TCGT CGGC AGCG TCAG ATGT GTAT AAGA GACA GCCT ACGG GNGG CWGCA-G3’) and the reverse primer (5’-GTCT CGTG GGCT CGGA GATG TGTA TAAG AGAC AGGA CTAC HVGG GTAT CTAA TCC3’). Primers were fitted with adapter sequences to make them compatible with the Illumina Nextera XT Index kit. Amplicon libraries were pooled and sequenced in an Illumina Miseq sequencer in 2 x 300 cycles paired-end runs (MiSeq Reagent kit v3). The data for the present study were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB51719.

**Bioinformatic analyses**

The DADA2 (v1.8.0) package as implemented in R (v3.6.0) was employed for 16S rRNA gene raw sequence read processing and forward and reverse merging as well as clustering into Amplicon Sequence Variants (ASVs)\[24\]. Filtering and trimming parameters were as follows: maxN = 0, maxEE = c(2,5), truncQ = 0, trimLeft = c(17,21), truncLen = c(270,220), and rm.phix = TRUE. A minimum overlap of 15 nucleotides and a maximum mismatch of 1 were required for read merging. Reads were aligned against the human genome (GRCh38.p13) using Bowtie2 (v2.3.5.1) \[25\] and matches were discarded.

Once the ASV table was obtained, species-level taxonomic identification was assigned to each ASV with DADA2, applying a 100% identity matching between ASVs and the reference sequences in the SILVA v.138 reference database \[26\]. Furthermore, the MegaBLAST tool from BLAST v(2.10.0) \[27\] was used for those ASVs that were identified with DADA2 at genus level but not at species level, requiring at least 97% identity to be species-level assigned. In addition, we only considered ASVs with the same genus assignment from the DADA2 and MegaBLAST methods as well as with a minimum difference of 2% between the first- and second-best matches. Finally, ASVs with total number of counts lower than 10 were removed.

**Statistical Analysis**

Alpha and beta diversity measures (Shannon diversity index, Chao1 and Bray-Curtis dissimilarity index) were computed using the Vegan package (v2.5-2)\[28\] in the R platform. Differences in alpha-diversity were tested using the Wilcoxon rank-sum test with the Benjamini-Hochberg procedure for false discovery rate control. The Bray-Curtis dissimilarity index was used in permutational multivariate analysis of variance (PERMANOVA) and Principal Coordinates Analysis (PCoA). PERMANOVA were performed using the adonis function from Vegan with 600 permutations and the Benjamini-Hochberg procedure for false discovery rate control.
Analysis of the composition of microbiomes (ANCOM) was used to identify differentially abundant taxa among samples and test significance was determined using the Benjamini-Hochberg procedure for false discovery rate control, as described in [29]. ANCOM was performed at the level of individual foods and at the two different levels of food classification for each individual. Those results found to be significant (q < 0.05) in all individuals were collected. In order to reduce abundance table sizes and computational cost, ANCOM analyses were performed only with those taxa whose abundance was above 5 times the lowest non-zero abundance for at least 20% of the samples in a given group.

Results

The 55 foods selected in this study were employed raw or cooked in one to five different ways resulting in 159 combinations that were subsequently in vitro digested and fermented using fecal samples from 3 individuals as separate inocula. 16S rRNA gene amplicon sequencing of the samples after fermentation yielded a total of 89,826,329 reads, averaging 93,277 reads per sample, 76,205,997 of which were assigned at genus-level. Taxonomic assignation identified reads belonging to 10 phyla, 65 families, 175 genera and 147 species.

Microbiota diversity and overall composition

Significant differences in both composition (Fig. 1A) and diversity (Shannon index and Chao1 estimator, q < 0.001) were detected among the microbiotas of the three different individuals analyzed. Therefore, we decided to study the effect of foods and cooking methods separately for each individual.

In all analyses, water was used as a control for the in vitro fermentation. No differences in alpha diversity were detected between the control and the different food categories, while few significant differences were detected for beta diversity (for water-based beverages, legumes, eggs and dairy products, in one individual only). On the contrary, numerous differences between food categories existed for both alpha and beta diversity (Fig. 1B,C). This may be due to different foods modifying the microbiota composition in different directions, resulting in larger differences between two foods than between each and the water control.

When comparing plant vs animal-based foods, we detected clear differences in Chao1-estimated richness, with the fermentation of animal-based foods leading to higher richness than that of plant-based foods in all three individuals (q < 0.0001). However, there was no concordance among individuals when comparing plant-based and animal-based foods in terms of the Shannon index. In fact, there were significant differences between the two food categories (q < 0.0001) in both individual 2 (I2) and individual 3 (I3), but they occurred in opposite directions, while no significant difference was present in individual 1 (I1).

For the 91 comparisons at food category level, diversity analyses showed several significant differences, for both the Chao1 estimator and the Shannon index (Additional file 1: Figure S1 and Figure S2). Most differences were found in one or two individuals, with only a few present in all three (Fig. 1B,C). Among
those food categories that showed common results across individuals, we can highlight that of animal and vegetable fats, which produced in all a higher diversity than grain-based products (Chao1 and Shannon), vegetables (Chao1) and fish (Shannon). More generally, even if not in all 3 individuals, animal and vegetable fats resulted in a higher diversity than other categories in many comparisons, particularly in terms of the Shannon index, for which a higher diversity was detected in 22 out of 39 (56%) comparisons, including comparisons with every food category in I1. In terms of Chao1, fermentations with meat, dairy products, and fish resulted in a richer microbiota than fruits, grain-based products, starchy tubers and vegetables in at least 2 out of 3 individuals.

Regarding microbiota composition, PERMANOVA with the Bray Curtis dissimilarity index revealed that plant and animal-based foods were significantly different for each individual (q < 0.01). In addition, the comparison of food categories for each individual highlighted a large number of significant differences in microbiota composition, several of which were found in all individuals (Fig. 1D). The food categories for which the largest number of comparisons were significant in all three individuals were starchy tubers, fish and animal and vegetable fats.

**Differential abundance of taxa**

Of 175 taxa identified at genus level, 78 were found in all individuals. Because most of the variance in microbiota composition was caused by differences among individuals, we evaluated the differences in relative taxon abundance between animal-based and plant-based foods, between food categories and between individual foods for each individual.

Comparing animal-based to plant-based foods, most of the differences in taxon abundance were only found in a single individual, whereas 21 differential taxa were shared by 2 individuals and 6 were shared by all individuals (Fig. 2A). Among the taxa found as differential in only one individual, most were present in the microbiota of the other two, whereas 23.1% were present in one and 12.3% were not found in either. The 6 genera that were differentially abundant in all individuals (Fig. 2A) were *Faecalibacterium, Lachnoclostridium*, Lachnospiraceae UCG 004, *Fusicatenibacter, Romboutsia* and *Actinomyces*, which were more abundant after animal-based food fermentation.

After all possible comparisons between food categories in each individual, 25 and 59 genera were detected to be differentially abundant for a given food comparison in all or in 2 individuals respectively. In addition, 49 other genera were differentially abundant for a given comparison in a single individual (Fig. 2B). Of those taxa found to be differentially abundant in a single individual, 33.9% were present only in the microbiota of one of the other individuals and 37.3% were not found in either. Of the 25 genera in which differences were detected for a given comparison in all individuals, the following stand out for their higher abundance: *Bacteroides, Faecalibacterium, Lachnoclostridium, Ruminococcus, Bifidobacterium* and *Agathobacter* (Additional file 1: Figure S3).

A genus-level summary of the food category comparisons significant in all the individuals is shown in Fig. 3; also, all significant comparisons can be found in further detail in Additional file 2: Figure S1-S4.
Generally, the food categories with the highest number of differentially abundant taxa were fruits (17), dairy products (12), animal and vegetable fats (11), vegetables (11) and fish (10). Animal and vegetable fats, fish and dairy products tended to result in higher abundances of many genera compared to other foods, while fruits, vegetables and grain-based products rather resulted in lower abundances.

Effect of animal and vegetable fats

The food category that resulted in the most significant differences in taxon abundance was animal and vegetable fats. The abundance of the Lachnospiraceae genera, Blautia, Lachnoclostridium, Lachnospira and Roseburia, and of the Oscillospiraceae genus Faecalibacterium (F. prausnitzii) increased with animal and vegetable fats compared with fruits, legumes, grain-based products (excluding Roseburia) and water-based beverages (excluding Faecalibacterium). Particularly, butter was the food with the highest number of significant comparisons, in all of which the abundance of the Lachnospiraceae genera Blautia, Agathobacter, Fusicatenibacter, Roseburia, Lachnoclostridium and Lachnospira was higher than in the other foods.

The main taxon found at higher abundances in olive oil was Lachnospira, which was increased compared with many foods. Also, we found a higher abundance of Faecalibacterium in olive oil in comparison to apple, beer, whole-grain bread and garlic. Furthermore, Agathobacter abundance was decreased compared with butter. Regarding sunflower oil, the number of significant comparisons was lower than for the other fats, with Faecalibacterium and Agathobacter being the main taxa found at higher abundances with this oil.

Effect of fish

Fish fermentation led to higher abundances of Lachnoclostridium, Ruminococcus and 7 other genera with respect to more than 5 food categories. Also, Clostridium sensu stricto 1 tended to have lower abundances in fish fermentations.

At food level, both salmon and cod fish induced higher abundances of Lachnoclostridium and Ruminococcus, as seen at food category level. Also, we found higher abundances of Faecalibacterium, Monoglobus and Bifidobacterium in cod fish compared with apple, bread, whole-grain bread, carrot, garlic, onion and peanuts.

Effect of dairy products

The use of dairy products resulted in a higher abundance of Bifidobacterium compared with animal and vegetable fats, meat, and nuts. Dairy products also often resulted in higher abundances of Erysipelotrichaceae UCG 003, the Lachnospiraceae Agathobacter and Lachnoclostridium and of other Eubacteriales, such as Ruminococcus (R. bromii) or Romboutsia, in comparison to several plant-based food categories, including fruit and grain-based products, and, to a lesser extent, vegetables and legumes. In particular, all of these genera were more abundant in dairy product fermentations than in those involving fruits. At food level, Lachnoclostridium, R. bromii, Erysipelotrichaceae UCG 003 and Romboutsia were identified as increased in abundance with gouda cheese, while no significant comparisons were
common among the three individuals for milk and only *Roseburia* was detected in lower abundance in yogurt compared with butter.

**Effect of fruit**

Fruit had the largest number of genera with abundances significantly different to those found in other categories (17). However, most of the taxa differed only with respect to one or two categories, in contrast to animal and vegetable fats, which led to fewer significantly different genera but a greater number of significant comparisons for each of them. *Lachnoclostridium* stands out as the only taxon yielding three significant comparisons between fruit and other food categories. All these differences implied lower abundances of genera in fruit fermentations in comparison to other foods.

Apple was the food resulting in most differences, all of them representing reduced abundances. The taxon differing in the largest number of comparisons was *Agathobacter*, followed by *Lachnoclostridium*, *Faecalibacterium*, *Fusicatenibacter*, *Dorea* and *Butyricicoccus*. Furthermore, the comparisons of apple against butter and gouda resulted in more than 12 different taxa being reduced. Similar results were obtained for bananas, which differed against butter and gouda in 7 taxa, with lower levels of *Lachnoclostridium* in comparison to several foods. Also, oranges and grapes differed from butter in at least 8 taxa. Plums and peaches responded similarly to all other fruits in relation to butter; however, plums induced higher abundances of *Bacteroides* and *Ruminococcus*, while peaches induced a higher abundance of *Lachnoclostridium*, contrary to other fruits.

**Effect of grain-based products**

This food category encompasses many foods and most of them, similarly to fruits, differed in many taxa in relation to butter and gouda; also, but to a lesser degree, in relation to salmon, cod fish and olive and sunflower oils. *Lachnoclostridium* was the taxon that was found at lower abundances in most food category comparisons, followed by *Faecalibacterium*.

**Effect of vegetables**

The vegetables food category encompasses the largest number of foods. We found 11 genera with 1 or 2 significative comparisons with animal and vegetable fats, dairy products or fish, mostly at low abundances in comparison to the other food groups.

At food level, results were similar to those obtained with fruits and grain-based products; most of the significant comparisons were against butter, gouda and salmon. Onion resulted in lower abundances of *Lachnoclostridium*, *Ruminococcus* and *Faecalibacterium* compared with butter, gouda, cod fish and salmon. *Lachnoclostridium* was also found at lower abundances in cauliflower and tomato compared with gouda, cod fish and salmon. Furthermore, tomato also resulted in lower abundances of *Lachnoclostridium*, as well as *Dorea*, in comparison to various other foods, such as butter, beef and peach. Garlic is a singular case as it showed differences against a large number of foods, in particular lower abundances of *Ruminococcus* and *Fusicatenibacter*, among others, and higher abundances of
In particular, *Bacteroides* increased in garlic in relation to other plant foods such as capsicum, apple, nut mix and sweet potato, as well as animal foods such as gouda, and salmon.

### Effects of cooking methods on the fermentative microbiota

When analyzing the effects of cooking methods across all food categories, only the comparison between fried foods vs. roasted foods yielded a significant difference in the abundance of one taxon, the low-abundance Ruminococcaceae UBA1819 group, which was significantly reduced in fried foods in all individuals against roasted foods. Since the effects of cooking methods could be masked by the effects of the different food categories, they were analyzed separately within each food category.

Comparing cooking effects within each food category, no significant abundance differences were found in all individuals, but a few common differences were found in two individuals and many differences in single individuals. In general, fried foods showed more differences than other cooking methods. Fried grain-based products were found to differ from those boiled, raw and toasted. In particular, *Ruminococcus* and *Bifidobacterium* were present at higher and lower abundances, respectively, in fried grain-based products vs boiled, raw and toasted. Also, *Subdoligranulum* was present at higher abundance in fried vs. boiled and *Romboutsia* in fried and raw vs. boiled. Regarding vegetables, a Ruminococcaceae Incertae Sedis was decreased in fried vegetables vs boiled, raw and roasted, suggesting that frying vegetables has a strong effect on this taxon. The Ruminococcaceae UBA1819 group was also decreased in fried and raw vegetables vs raw. Among animal-based foods, fried meat produced lower *Lactococcus* abundances compared with grilled meat, while fried fish resulted in higher *Bacteroides* abundances compared with boiled.

Other cooking methods also resulted in significant differences for certain foods. Grilling decreased the abundance of the Anaerovoracaceae *Family XIII AD3011* group in fruits, when compared to roasting, and it increased the abundance of *Ruminococcus* in dairy products, compared to raw. Similarly, roasting increased *Ruminococcus* and *Anaerostipes* in dairy products compared to raw, as well as *Monoglobus* and *Lachnoclostridium* in fish compared to boiling. Finally, boiling reduced *Bacteroides* in fish and increased the Anaerovoracaceae *Family XIII AD3011* group in grain-based products, compared to the raw foods. All significant comparisons can be found in further detail in Additional file 2: Figure S5.

### Discussion

It is well known that the food we eat has an effect on our gut microbial communities, influencing their structural and functional capacity[3, 4]. Here, we examined in which specific way representative foods of Mediterranean and Western diets can alter microbial communities in a rapid manner. The experimental design employed has certain limitations, such as the fact that we can only observe those changes that occur rapidly and not in the long term; however, the power of diet to alter the composition of the gut microbiota in the short term has already been demonstrated[3].
Regarding alpha diversity, the initial composition of the microbiota played an important role in the changes that occurred during the fermentations in terms of the Shannon diversity index. However, a clear trend was detected for the Chao1 richness estimator, with higher richness detected in animal-based vs. plant-based foods for all three individuals, as well as in the fermentations of meat, dairy products, and fish vs. those of fruits, grain-based products, starchy tubers and vegetables for at least two individuals. In contrast, David et al. [3] reported that in vivo there were no significant alpha diversity differences between entirely animal and plant based diets.

In accordance with previous studies [30–32], PCoA and PERMANOVA (p = 0.00166) analysis evidenced that most of the compositional variability in the data was due to the initial composition of the microbiota from the three individuals. Nevertheless, compositional differences between the plant-based and animal-based food fermentations could be detected for each individual (q < 0.01), in accordance with the in vivo results of David et al.[3]. Similarly, in comparisons at food category level (Fig. 1D), most differences detected in all three individuals were also found when comparing specific plant-based and animal-based categories.

Animal and vegetable fats was the food category that produced the most changes in the gut microbiota, generally resulting in higher abundances of bacteria of the phylum Firmicutes and lower abundances of the genera Bacteroides and Bifidobacterium, in accordance with the results of Hildebrandt et al. for mice fed a high-fat diet [33]. Although there are not many studies on the effects of dietary fatty acids on the microbiota in vitro, the ability of the microbiota to grow on fatty acids has been reported, as well as a large effect of these substrates on microbiota composition [34]. Our results showed that the use of fats (especially butter) as substrates induced an increase in the abundance of potentially beneficial taxa such as Faecalibacterium (mainly F. prausnitzii), Roseburia (R. inulinivorans) and Blautia, as previously described in in vivo studies with a medium to low percentage of fatty acids in the diet[35, 36]. F. prausnitzii has been reported as one of the main butyrate producers in the human gut and has been attributed significant anti-inflammatory properties [37, 38]. Evidence on the effect of diet on the modulation of F. prausnitzii is still conflicting [39], although there are findings that certain types of fatty acids such as monounsaturated fatty acids are associated with an increase in its abundance [35, 36, 40, 41], supporting our results. Blautia was found to have a negative relationship with visceral fat accumulation [42] and to decrease in some diseases such as diabetes in children, Crohn's disease or colorectal cancer [43–45]. Some authors have reported an increase in Blautia abundance in high-fat diets[41, 46, 47], in accordance with our in vitro results; however, there is no consensus, as other research suggests the opposite[41, 48]. The taxon that is elevated in animal and vegetable fats vs. the largest number (9) of food categories in our in vitro experiments is Lachnospira, a butyrate producer mainly reported to increase with high-fiber foods[49, 50]. Nevertheless, some studies have reported that unsaturated fats upregulate Lachnospira, also correlating it with vegetable fats like olive oil in vegan and vegetarian people[47, 50].

There are many studies that have assessed the effects of high fat diets and even the effects of saturated and unsaturated fats on the gut microbiota, as comprehensively reviewed in Yang et al. [51]. Broadly,
saturated and unsaturated fats were found to have opposite effects on gut microbiota modulation, with the latter producing an increase in the abundance of beneficial taxa such as *Akkermansia* and *Bifidobacterium*, and a decrease in potentially detrimental taxa such as *Streptococcus* and *Escherichia*. Furthermore, these effects can be classified according to the proportion of calories the fat represents in the diet, but it is difficult in these studies to distinguish between the effect of a specific food and the rest of the diet. In contrast, a strength of our *in vitro* model is the ability to study the interactions between specific animal and vegetable fats and the microbiota. Our analysis of specific fats indicated some differences in the effects of saturated and unsaturated fats on the gut microbiota. Olive and sunflower oil, which contain mainly unsaturated fatty acids, resulted in moderately higher abundances of *Lachnospira*, *Faecalibacterium*, *Fusicatenibacter* and *Agathobacter*. On the other hand, butter, which is a clear example of a food that contains predominantly saturated fatty acids, stands out as producing some of the greatest effects of all foods, resulting in higher abundances of the Lachnospiraceae *Agathobacter*, *Blautia*, *Fusicatenibacter*, *Lachnoclostridium*, *Lachnospira* and *Roseburia*, among others.

Numerous differences in the fermentative microbiota were also detected in comparisons involving dairy products or fish. Fish fermentations produced high abundances of *Lachnoclostridium* and *Ruminococcus*. *Lachnoclostridium* has been previously reported to increase in high-fat diets, as well as to be positively correlated with visceral fat through decreasing circulating acetate levels [47, 52]. *Ruminococcus* is a known Short Chain Fatty Acid (SCFA) producer, although it has been correlated with trimethylamine N-oxide, which is generated by the gut microbiota from choline and carnitine (from sources such as eggs, beef, pork and fish), and it has been associated with risk of atherosclerosis and cardiovascular disorders[53, 54]. Also, *Ruminococcus* has been associated with long term fruit and vegetable consumption as well as with low dietary fiber intake [3, 55, 56]. Hence, *Ruminococcus* abundance is influenced by animal and plant-based diets, as previously described by De Filippis et al., who associated it to omnivore diets[53]. There is not much evidence about the effect of specific fish on the gut microbiota, as most studies assay the effects of fish oil, which is enriched in PUFAs (such as eicosapentaenoic acid and docosahexaenoic acid), without considering the contribution of the whole fish. These studies have observed an increase in Bacteroidetes, Lachnospiraceae family, *Roseburia* and *Bifidobacterium*. In our results we detect an abundance increase of *Lachnoclostridium* and *Ruminococcus* with salmon, which has a high content of PUFAs[51, 57, 58].

In the case of dairy products, considerable effects on the microbiota revealed by our results included increases in the abundances of the genera *Lachnoclostridium*, *Bifidobacterium*, *Agathobacter*, *Ruminococcus*, *Erysipelotrichaceae UCG 003* and *Romboutsia*. The systematic review carried out by Aslam et al. [59] reported a generalized increase with dairy consumption of *Bifidobacterium* and *Lactobacillus*, which are widely considered to be beneficial to the host. *Bifidobacterium* has beneficial effects on human physiology and in diseases such as irritable bowel syndrome due to its anti-inflammatory effects [60, 61]. However, Aslam et al. highlighted the lack of sufficient evidence due to the wide variety of dairy products with very different nutritional composition. On the other hand, members of the *Erysipelotrichaceae* have also been positively associated with dairy intake [62, 63]. However, elevated abundances of these organisms are likely detrimental since they are enriched in the intestines of obese
humans and mice, have been associated with symptoms of the metabolic syndrome and promote obesity in gnotobiotic mice\[64\]. Our study showed that the dairy food that produced the greatest variation in the microbiota was gouda cheese, which, like the foods we have discussed above, has a high fatty acid content. This further supports a fundamental role for fatty acids in modifying the composition of the gut microbiota.

In fact, animal and vegetable fats, fish and dairy products are all characterized by a high percentage of fat compared to other foods, which suggests that this may be the reason for their producing similar responses. Overall, fermentations of animal and vegetable fats, fish and dairy products often produced higher abundances of taxa of the Lachnospiraceae family, with *Lachnoclostridium* standing out as detected in high abundance in the three food categories. The Lachnospiraceae family is a phylogenetically and morphologically heterogeneous taxon that contains many members described as having the ability of hydrolyzing starch and other carbohydrates to produce butyrate and other SCFAs\[42, 65\]. However, *Lachnoclostridium* is a known protein degrader\[66\] and is the most abundant gut bacterium capable of metabolizing the choline obtained from high-fat foods, producing trimethylamine\[67\]. Choline and choline phospholipids, like phosphatidylcholine, are particularly enriched in high-fat dairy products such as butter and gouda cheese, which likely contributed to the significant effect of these two foods on *Lachnosclostridium* abundances. In addition, *Roseburia* as well as the Oscillospiraceae genera *Faecalibacterium* and *Ruminococcus* were also found at high abundance in at least two of these mainly animal-based food categories, suggesting that these foods also favor the increase of these taxa.

It is remarkable that most elevated taxon abundances in the microbiota were detected in fermentations with animal and vegetable fats, dairy products, and fish. In contrast, significant differences involving fruits, vegetables and grain-based products mostly represented reduced abundances of genera in comparison to animal-based food categories. This may suggest that the short fermentation processes used in our experiments were not sufficient to favor increases of specific genera when carbohydrate-rich foods were employed. As many taxa present in the inoculum may be able to take advantage of these substrates, a short fermentation may not have provided enough time to select for taxa having relatively small growth advantages with respect to other carbohydrate fermenters. Longer fermentation times may thus be needed to amplify growth rate differences among taxa with specific carbohydrate-rich foods. In contrast, the ability to grow on proteins and, especially, fats is likely more unevenly distributed across gut bacteria, so that short fermentations with foods rich in these substrates may more readily select for subsets of specialized bacteria, resulting in significant increases in their abundances.

David et al. \[3\] reported that animal-based diets had a greater impact on gut microbiota composition, particularly an increase in the abundance of bile-tolerant microorganisms, presumably due to increased bile acid secretion as a result of the high fat intake linked to such diets\[68\]. However, in our *in vitro* experiments there is no regulation of bile acid level depending on the food, suggesting that the effects of animal-based products on gut microbiota composition and diversity are also influenced by other factors in addition to a higher production of bile acids. In accordance, the taxa favored by animal-based diets *in vivo* in the work by David et al. are the bile-resistant *Bilophila, Bacteroides* and *Alistipes*, which are not
increased by fatty foods in our *in vitro* experiments. As mentioned above, an additional explanation could relate to the fact that most intestinal bacteria are mainly adapted to growth in the presence of the complex polysaccharides and fibers that reach the colon, whereas fermentation with foods high in fats and proteins will only stimulate the growth of the subset of bacteria able to metabolize these nutrients, resulting in a larger impact on microbiota diversity and on relative taxon abundances.

We would like to insist on the fact that all of the significant composition differences associated to different foods that have been discussed above occurred in all individuals, so they likely represent the most robust effects. Besides these, many other changes may be composition-dependent and thus the conditions that enable such modulations are still to be revealed. Previous studies have suggested that the response of the gut microbiota to dietary interventions presents a high inter-individual variability, and that certain microbial taxa can be responsive or resistant to dietary changes [30–32]. It is therefore necessary to expand the number of individuals examined to determine which of the changes are composition-dependent, which taxa drive these dependencies and how to use them to modify the gut microbiota.

Regarding cooking methods, it is well established that they alter the chemical composition of foods[9, 11] affecting their bioaccessibility and digestibility. Food chemical and structural changes due to cooking may impact extensively the gut microbiota community structure as well as its functionality[10]. On one hand, cooking methods that apply high temperatures, such as frying or grilling, favor cellular break-down and, with it, release of chemicals into the environment[69]. This may be beneficial since it facilitates the absorption or use of healthy bioactive compounds. However, there is also evidence of some phytochemicals being harmful for some bacterial species[70, 71]. At the same time, other microbial species have the enzymatic equipment to metabolize such phytochemicals and therefore grow on them[72]. On the other hand, cooking favors specific chemical reactions, such as chemical browning in protein and sugar-rich foods. Chemical browning involves a plethora of chain reactions that yield several compounds, such as furanic compounds, that have inhibitory effects on some bacterial species[73]. Chemical browning can also yield melanoidins, known to have a fiber-like behavior and therefore promote the growth of some beneficial species[74]. Accordingly, cooking may have a complex effect on gut microbial communities. Therefore, it is necessary to take cooking methods into account when designing personalized nutrition approaches to provide not only suggestions of beneficial foods but also of how each food should be cooked to maximize the health benefits. Furthermore, it would be specially interesting to collect information on the cooking methods used in observational studies to further analyze their interactions with the intestinal microbiota.

However, to date, few studies have been conducted on the effect of cooking methods on the composition and diversity of the gut microbiota. Some of these studies have evaluated the effect of different cooking processes on meats, legumes, vegetables, cereals and fruits[9, 10, 12, 75, 76], as well as the production of new compounds by thermal processing, such as via the Maillard reaction. It has been observed that many of these compounds behave as fiber, being able to reach the gut microbiota and affect some bacteria. The production of these compounds, and therefore their effect on the microbiota, is directly related to the
composition of the food; to our knowledge, there are still few studies that address these changes on a variety of foods at a large scale[10].

Here, we have been able to identify that frying seems to have the most differential effect on the microbiota. Pérez-Burillo et al. (2018) identified frying as the cooking method that produced the highest amounts of furosine and furfural (used as indicators of the Maillard reaction), and found it to be correlated with the abundance of different taxa depending on the food[10]. In our experiments, frying resulted in a lower abundance of Ruminococcaceae UBA1819 in comparison with roasted foods for all food categories considered together. This is particularly remarkable in that one could expect the effects of cooking methods to be masked by those of food composition, suggesting that frying and roasting have strong and common effects across food categories. Moreover, fried foods also resulted in significant taxon abundance differences with other cooking methods within many specific food categories, such as grain-based products, vegetables, fish and meat. However, these results were not consistent across individuals, suggesting that cooking method effects may be highly personalized.

Nevertheless, despite the limited number of individuals analyzed and the high interindividual variability, our results suggest that the family Ruminococcaceae and the genus *Ruminococcus* are particularly responsive to cooking methods. *Ruminococcus* was often seen to vary in abundance depending on the cooking method applied in a given food category. Specifically, it was highly abundant in fermentations with grain-based products that had been fried, as well as in fermentations with grilled or roasted dairy products. This represents the type of detailed information regarding the effects of specific food preparations on specific taxa that will facilitate the elaboration of personalized nutrition programs, as similar information is gathered in future work for a larger number of components of the gut microbiota.

**Conclusions**

The *in vitro* approach followed in this work has revealed the short-term effects of specific foods on the gut microbiota of different individuals. The experimental set up employed may have the limitation of reflecting mainly the punctual effects of drastically limiting the substrates available to the microbiota to those present in a single food. Although observed effects on overall parameters such as alpha diversity may indeed mostly reflect this immediate change, nevertheless, the fermentation experiments reveal those bacterial taxa that are most affected by the availability of a given food.

Many of the detected effects after food fermentation have been particular to single individuals, highlighting likely dependencies on initial gut microbiota composition and the highly personalized nature of responses to food. However, some shared trends across individuals have also been manifested. Our results show that foods derived from plants and those derived from animals have significantly different impacts on the abundances of bacterial taxa. Importantly, the fact that this is detected *in vitro* implies that bile-acid levels can not be the only factor affecting the composition of the gut microbiota when following an animal-based diet. Our findings also highlight the substantial effects of vegetable and animal fats, especially butter, which have a positive impact on the abundance of potentially
advantageous taxa including *Faecalibacterium*, *Roseburia*, and *Blautia*. However, butter and other high-fat animal foods, such as dairy products and fish, also resulted in higher abundances of *Lachnoclostridium*, which has been associated to several diseases.

Our results also identified frying as the cooking method producing the most distinct effects on the microbiota when contrasted to other methods of cooking a particular food, maybe due to its high generation of Maillard reaction products. In addition, we also identified that members of the family Ruminococcaceae, including *Ruminococcus*, are often present amongst the taxa that respond the most to the use of different cooking methods. However, overall, the impact of cooking methods on the effects of foods was highly varied across individuals. This highlights the need for further research on the role of cooking methods in modulating the ability of different foods to alter the gut microbiota, while taking into consideration that such effects are likely to be highly individualized. This knowledge will pave the way to a personalized modulation of the microbiota for precision nutrition.

**Abbreviations**

ENA: European Nucleotide Archive, EMBL-EBI: European Molecular Biology Laboratory-European Bioinformatics Institute; ASV: Amplicon Sequence Variant; PERMANOVA; Permutational Multivariate Analysis of Variance; PCoA: Principal Coordinates Analysis; ANCOM: Analysis of the Composition of Microbiomes; SCFA: short chain fatty acid.

**Declarations**

**Ethics approval and consent to participate**

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University of Granada (1080/CEIH/2020). Subjects were provided with an information sheet explaining the study. All study details were also orally explained to the participants, and written informed consent was obtained.

**Consent for publication**

All authors have consented to the final version of the manuscript.

**Availability of data and material**

The datasets generated during the current study are available in the EBI database with the accession number PRJEB51719.

**Conflicts of interest/Competing interests**

The authors have no relevant financial or non-financial interests to disclose.
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Authors’ Contributions

Conception or design of the work: J.Á.R.H., M.J.G. and M.P.F.


Drafting the article: A.L.A., M.J.G. and M.P.F.


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**Figures**
Figure 1

Analysis of growing microbiota in *in vitro* fermentations
Figure 2

Venn diagram of differentially abundant genera according to ANCOM test.
Figure 3

Genus level abundance differences between food categories.

Supplementary Files

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