Journal of Agricultural and Food Chemistry, 2018, 66, 11-500-11509

Effect of food thermal processing on the composition of the gut microbiota

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ABSTRACT

Cooking modifies food composition due to chemical reactions. Additionally, food composition shapes the human gut microbiota. Thus, the objective of this research was to unravel the effect of different food cooking methods on the structure and functionality of the gut microbiota. Common culinary techniques were applied to five foods, which were submitted to *in vitro* digestion-fermentation. Furosine, HMF (5-hydroxymethyl-furfural) and furfural were used as Maillard reaction indicators to control the heat treatment. Short chain fatty acids production was quantified as indicator of healthy metabolic output. Gut microbial community structure was analyzed through 16S rRNA. Both food composition and cooking methods modified the microbiota composition and release short chain fatty acids. In general, intense cooking technologies (roasting and grilling) increased the abundance of beneficial bacteria like *Ruminococcus spp*. or *Bifidobacterium spp*. compared to milder treatments (boiling). However, for some foods (banana or bread) intense cooking decreased the levels of healthy bacteria.

KEYWORDS: Cooking, food processing, gut microbiota, Maillard reaction, metagenomics.

Introduction

The human intestine is inhabited by a massive number of microorganisms and, although microbes are present throughout the gastrointestinal tract, the distal colon population is the most studied. It has been estimated that around 10^{13}-10^{14} microorganisms are present, most of them belonging to the phyla Firmicutes and Bacteroidetes.[1] It is now very clear that the gut microbiota has important implications in different diseases and is therefore closely related to health status. [2] In this sense, the gut microbiota has been linked to inflammatory bowel disease, obesity, autism spectrum disorders and immune system disorders, [3] among others.

The gut microbiota is influenced by several factors such as age and antibiotics intake, but diet is likely to be one of the most influential factors.[1] Accordingly, several studies have demonstrated important differences in gut microbiota among populations of different regions.[4] However, not only long-term dietary patterns shape the gut microbiota composition and functionality, but also shifts from protein-rich to vegetable-rich diet can rapidly affect the gut microbiota.[5] On the other hand, a significant amount of nutrients escape digestion and absorption at the small intestine and reach the colon, including fibers, resistant starch, some proteins and fats as well as bile acids and some phytochemicals like polyphenols.[1] Such molecules become substrates for microbial transformations, producing molecules with beneficial (e.g. short chain fatty acids, SCFAs) or detrimental effects (e.g. trimethylamine).[4] Therefore, microbial metabolites are some of the main the bioactive molecules that play a role in human health, and thus it is imperative to analyze how their production will be influenced by diet.[2]

Since gut microbiota can be rapidly affected by diet, it is important to unravel the specific effects of foodstuffs from different groups like vegetables, fruits, meat, legumes or cereals, among others. However, the gut microbiota ability to use substrates

could also be influenced by the culinary-heat treatment undergone by the foodstuffs prior to ingestion.[6] Upon cooking, many different compounds will be generated, most of them derived from the Maillard reaction.[7] All these neo-formed compounds could have some effect over the gut microbiota and, in fact, melanoidins are known to behave as fiber, and therefore as substrate for gut microbes.[8] The Maillard reaction (MR), and thus thermal damage, can be monitored along cooking through the formation of furosine (early stage indicator) and 5-hydroxymethylfurfural (HMF) or furfural (intermediate stage indicators).[9]

Only a few studies have been carried out on the effect of cooking conditions on gut microbiota composition, being mostly focused on meats and grain legumes.[6] Therefore, our main objective was to shed light on gut microbiota changes produced after fermentation of meat, fruits, vegetables, cereals and legumes while paying special attention to the influence of cooking techniques and heat damage. In order to achieve this goal, we selected chicken, banana, red pepper, bread and chickpeas and exposed them to the most common culinary techniques for each of them (frying, boiling, grilling, roasting, toasting *Vs.* raw). Then, we linked microbial changes to the type of food, culinary treatment or heat intensity (in terms of thermal damage monitored through furosine and HMF-furfural content).

Materials and methods

Reagents

Furosine was obtained from NeoMPS (Strasbourg, France). Furfural, 5-(Hydroxymethyl)furfural, hydrochloric acid, formic, acetic, propionic and butyric acids, potassium ferrocyanide, zinc acetate, potassium chloride, potassium di-hydrogen phosphate, sodium mono-hydrogen carbonate, sodium chloride, magnesium chloride

hexahydrate, ammonium carbonate, calcium chloride dihydrate, sodium di-hydrogen phosphate, salivary alpha-amylase, pepsin from porcine, bile acids (bile extract porcine), tryptone, cysteine, sodium sulphide, resazurin, hydrochloric acid, methanol and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Pancreatin from porcine pancreas was purchased from Alpha Aesar (Lancashire, United Kingdom).

Samples

Samples were representative foods from 5 different groups of foodstuffs: chicken (meat), chickpeas (legumes), wheat bread (cereals), red pepper (vegetables) and banana (fruits). The main criteria to choose these foods was to have some food representing each of the main consumed groups of foodstuffs. Therefore, we would have high protein food (chicken), starchy foods (banana and bread [fruit and cereal], fiber rich food (pepper), and high carbohydrate and protein food (chickpeas). Each one was submitted to the different types of cooking that are usual for them: chicken was boiled, fried, grilled and roasted; wheat bread was used raw and toasted (low toasting and high toasting degree), red pepper was used raw, fried and roasted; chickpeas were boiled and grilled; banana was used raw, roasted and fried. Boiling was carried out at 100 ºC for 20 minutes (N) with a proportion water:food of 5:1. Extra virgin olive oil (EVOO) was used as liquid medium for grilling and frying. Grilling was carried out at 220-250 ºC for 3 minutes on each side (N) with a proportion oil:food of 0.5:1. Frying was carried out at 180 ºC for 8 minutes (N) with a proportion oil:food of 5:1. Roasted was carried out at 180 ºC for 8 minutes. Toasted bread was prepared in a toaster at two different degrees: low and high. After cooking, samples were homogenized and stored at -80 ºC until analysis.

In vitro gastrointestinal digestion

All samples were subjected to an *in vitro* digestion process followed by an *in vitro* fermentation to mimic physiological processes in the human gut. The *in vitro* digestion method was carried out according to the protocol described by Perez-Burillo et al.[10] The gastrointestinal *in vitro* digestion was composed of an oral phase (5 minutes at 37 ºC with alpha-amylase 75 U/mL, pH 7.0), a gastric phase (2 hours at 37 ºC with pepsin 2000U/mL at pH 3.0) and an intestinal phase (2 hours at 37 ºC with pancreatin 13.37 mg/mL, bile salts at a concentration of 10 mM and $CaCl_2$ at a concentration of 0.3 mM, at pH 7.0).

In vitro fermentation

The *in vitro* fermentation was carried out according to the protocol described by Perez-Burillo et al.[10] *In vitro* fermentation was carried out using faecal samples from three healthy donors (not taking antibiotics, people with body mass index within the "normal weight range", mean Body Mass Index = 21.3). The faecal samples from the donors were pooled together and the pool used as the inoculum. After *in vitro* digestion, the solid residue (fraction not available for absorption) that is left after removing the supernatant, plus 10% of such digestion supernatant are used as substrate for fermentation. The amount of solid residue used is 500 mg.

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

Furosine assay

Furosine determination was performed following the method described by Rufian-Henares et al.[11] Briefly, samples were hydrolysed with 7.95 M HCl at 120 °C for 23 h. The hydrolisate was purified with a Sep-pack C_{18} cartridge (Millipore, MA) and the resulting solution was analysed by ion-pair RP-HPLC. The analysis was performed in duplicate and the data are the mean values expressed as μg per g of food.

HMF and furfural assay

HMF determination was performed according to the method described by Rufian-Henares et al.[9] The ground sample was suspended in deionised water, clarified with Carrez I (potassium ferrocyanide, 15% w/v) and Carrez II (zinc acetate 30% w/v) solutions. The resulting solution was analysed with RP-HPLC. The analysis was performed in duplicate and the data are the mean values expressed as μg per g of food.

Short chain fatty acids determination

SCFAs determination was carried out according to the procedure described in Panzella et al.[12] with few modifications. SCFAs were determined in fermentation supernatant. After the fermentation process, the supernatant was centrifuged filtered through a 0.22 μm nylon filter and analysed by means of a HPLC system. The analysis was performed in duplicate and the data are the mean values expressed as μmol per g of food.

DNA extraction and sequencing

DNA extraction was performed using a NucliSENS easyMAG platform

(Biomérieux) following the standard protocol. Microbial genomic DNA was used at a concentration of 5 ng/μL in 10 mM Tris (pH 8.5) for the Illumina protocol for 16S rRNA gene Metagenomic Sequencing Library Preparation (Cod. 15044223 Rev. A). PCR primers targeting the 16S rRNA gene V3 and V4 regions were designed as in Klindworth et al.[13] Primer sequences are: Forward 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCA-G3' and Reverse 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTA-CHVGGGTATCTAATCC3'. Primers contained adapter overhang sequences added to the gene-specific sequences, making them compatible with the Illumina Nextera XT Index kit (FC-131-1096). After 16S rRNA gene amplification, amplicons were multiplexed and 1 ml of amplicon pool was run on a Bioanalyzer DNA 1000 chip to verify amplicon size (~550 bp). After size verification, libraries were sequenced in an Illumina MiSeq sequencer according to manufacturer's instructions in a 2x300 cycles paired-end run (MiSeq Reagent kit v3 MS-102-3001).

Bioinformatic analysis

Quality assessment of sequencing reads was performed with the prinseq-lite program,[14] applying the following parameters: a minimal length (min_length) of 50 nt and a quality score threshold of 30 from the 3'-end (trim_qual_right), using a mean quality score (trim_qual_type) calculated with a sliding window of 10 nucleotides (trim_qual_window). Read 1 and read 2 from Illumina sequencing where joined using fastq-join from the ea-tools suite.[15] Taxonomic affiliations were assigned using the RDP_classifier from the Ribosomal Database Project (RDP).[16] Reads that had an RDP score below 0.8 were assigned to the next higher taxonomic rank, leaving the last rank

as unidentified. We assigned 6 taxonomic levels, which were kingdom, phylum, class, order, family and genus.

Statistical analysis

Correlations between bacterial abundance and Maillard reaction products were carried out through multivariate analysis with Statgraphics Centurion XVI.I. Bacterial abundances between groups were compared by analysis of variance (ANOVA) with Statgraphics Centurion XVII and R software. Principal Coordinates Analysis (PCoA) with unifrac distance was carried out with R software.

Results and discussion

Furosine content of cooked foods

Furosine (ε–N-(furoylmethyl)-L-lysine) is an artificial amino acid obtained from the acidic hydrolysis of Amadori compounds derived from the MR of heat-processed foods;[7] thus, furosine can be used as an indicator of the early stages of the MR. Furosine was detected in all the food analysed except in chicken (**Table 1**), which could be explained by the very low amount of reducing sugars available for the development of the MR. Furosine content ranged from 809 to 7.61 μg/g in toasted bread and raw pepper, respectively. Results were in accordance with other previously reported.[7,9,17] The highest levels were found in bread, followed by pepper, banana, chickpeas and chicken (**Table 1**). There were statistically significant differences ($p < 0.05$) in the furosine content among the different types of cooking procedures within the same type of food. In the case of chickpeas, furosine values were significantly higher for grilling than for boiling (56.9 *Vs.* 12.0 μg/g), most likely due to a higher temperature during cooking. Such was also the case for bread, in which furosine increased significantly

with toasting, and further when the degree of toasting was high. Regarding red pepper and banana, furosine values were low in the raw food and increased significantly ($p < 0.05$) in fried and roasted preparations. Furosine is therefore a sensitive indicator of heat damage during cooking in all the assessed foods except for chicken.

HMF and furfural content of cooked foods

HMF and furfural appear during the 1,2-enolisation of Amadori compounds under acidic conditions and sugar degradation at high temperature, known as caramelisation;[9] thus, HMF and furfural are related with intermediate stages of the MR, so that they can be used as indicators of thermal damage.[18] HMF values ranged from 57.2 to 0.04 μg/g in well-done toasted bread and raw banana respectively and from 235 to 0.11 μg/g in well-done toasted bread and raw pepper respectively (**Table 1**). Results were in accordance with other previously reported.[7,9,17] These indicators behaved similarly to furosine, in that significant differences in the values of these compounds among the different types of foods were found: bread > pepper > banana > chickpeas > chicken. This situation could be related with food composition, since bread (an excellent medium for the MR due to its content in sugars and proteins) reached the highest HMF and furfural values, whereas chicken (with no available reducing sugars) had no detectable amounts. Moreover, we also observed that HMF and furfural amounts were closely linked to the applied temperature (**Table 1**), with statistically significant differences ($p < 0.05$) among cooking methods within the same food. In this sense, whereas boiled chickpeas had no HMF, grilled chickpeas HMF value was 2.44 μg/g of food. As expected, raw bread showed very low amounts of either HMF or furfural, but when it was submitted to toasting the levels increased, being higher with longer toasting time. Regarding uncooked red pepper, it showed the lowest amounts of furfural, with

increasing HMF levels during roasting and furfural content during frying. This could be related with the degradation of HMF to furfural during frying, due to the high temperatures used during this type of coking. Bananas behaved similarly to the other samples, with raw banana showing low amounts of HMF and furfural, whereas either roasted or fried banana showed significantly higher amounts, with the highest amounts in fried banana. Therefore, according to our results, HMF and furfural were dependent upon composition and type of foodstuff, as well as upon the applied temperature.

Short chain fatty acids generated after microbial fermentation of cooked foods

Several health effects are attributed to SCFAs; decreasing the luminal pH is one of the most obvious, which can impede the growth of pathogenic bacteria.[19] On the other hand, butyrate is used as a substrate by epithelial cells and is important for their functionality, while all three main SCFAs (acetate, propionate and butyrate) are important for the maintenance of the gut barrier.[19] Moreover, while butyric acid is mostly metabolized by colonocytes, acetate and propionate are mainly absorbed and incorporated into different metabolic routes. The participation in different metabolic routes related to energy balance could link these SCFAs to the control of the metabolic syndrome. In accordance, all three have a protective role in diet-induced obesity.[20] On the other hand, butyrate and propionate have been related to production of gut hormones and therefore reduction of food intake. It is thought that SCFAs, and mostly butyrate, could have an important role in colorectal cancer protection, via increasing motility, irrigation, apoptosis, and reducing inflammation. In fact, it has been suggested that the protective effect of dietary fiber over colorectal cancer depends upon the production of butyrate.[21] Propionate has also been associated with the regulation of intestinal inflammation through induction of T-regulatory cell differentiation.[19] Accordingly, it is

important to know that the production of SCFAs derives from different foodstuffs that are a main part of the Western diet.

Accordingly, we first studied SCFAs production (**Table 1**) depending on the **type of food** (bread, chickpeas, chicken, red pepper and banana). In this sense, regarding acetic acid, bread produced the highest amount, 74.8 μmol/g, whereas chicken produced the lowest one, 5.9 μmol/g. All types of foodstuff produced significantly higher amounts of acetic acid than chicken; bread produced significantly higher amounts than banana, red pepper and chickpeas, and the levels of acetic acid after banana fermentation were higher than those of chickpeas and red pepper. In the case of propionic acid, chickpeas where the highest producers with 52.1 μmol/g whereas chicken showed the lowest value, 12.2 μmol/g. Every kind of food produced significantly more propionic acid than chicken, while chickpeas, red pepper and banana produced significantly higher amounts than bread, with chickpeas producing significantly higher amounts than any other foodstuff. Finally, the levels of butyric acid ranged from 51.5 to 9.3 μmol/g for red pepper and bread, respectively. Red pepper produced significantly higher amounts than any other foodstuff, whereas the production by banana and chickpeas was significantly higher than that by chicken and bread. Overall, banana produced the highest amounts of SCFAs followed by chickpeas, whereas chicken yielded the lowest amounts.

Secondly, we studied the possible influence of the **culinary treatment** over SCFAs production **(Table 1)**. Regarding boiled and grilled chickpeas, there were no statistically significant differences in acetic acid or propionic acid production. However, butyric acid production was significantly higher in grilled chickpeas. One possible explanation could be the formation of melanoidins, which can behave as fibre in the gut,[8] therefore increasing butyric acid production. In the case of bread, there were no

significant differences between raw or toasted bread regarding acetic or propionic acid production. However, butyric acid production was, in this case, significantly higher in raw bread. On the other hand, red pepper showed a significantly higher production of acetic acid when roasted and of butyric acid after frying; however, no significant differences were observed in propionic acid production. In this sense, products that appear as a consequence of the thermal treatment, such as melanoidins, could be responsible for the different SCFAs production. In the case of banana, no differences were found between acetic acid production for roasting or frying, but both had a significantly higher production than raw banana. Propionic acid production was also significantly higher in roasted banana, though there were no differences among raw or fried fruit. Regarding butyric acid, both roasted and fried banana yielded significantly higher amounts than raw banana, while the fried fruit had significantly higher values than the roasted one. In this case, it also seems that the culinary treatment has some effect on SCFAs production and that, as in the case of chickpeas and red pepper, cooking favours SCFAs production. Finally, roasted, fried and grilled chicken showed a significantly higher production of acetic and butyric acids than boiled chicken. However, no differences were found in the case of propionic acid. Overall, it seems that cooking has an influence over SCFAs production, which can be positive or occasionally negative, as observed in bread. However, much more research is needed in order to generate conclusive statements.

Finally, we tried to unravel whether or not there were any correlations among SCFAs production and **bacteria** known to be producers of such fatty acids (**Table 2**). Regarding acetic acid, we found positive significant correlations with *Ruminococcus spp*., *Bifidobacterium spp*. and *Collinsella spp*. In the case of propionic acid, it correlated significantly with the phylum Bacteroides and with *Ruminococcus spp.,*

Butyricimonas spp., Blautia spp., Roseburia spp., and *Veillonella spp.* Finally, butyric acid correlated significantly with *Butyricimonas spp., Anaerostipes spp., Intestimonas spp., Roseburia spp., and Faecalibacterium spp.* In this sense, our results are in accordance with existing bibliography about SCFAs producers.[2,19] Accordingly, bread and banana yielded the highest acetic acid productions and also had significantly higher abundances of *Bifidobacterium spp., Collinsella spp.,* and *Ruminococcus spp* (**figure 2)**. On the other hand, chicken, which showed the lowest acetic acid production, had also significantly lower abundance of *Bifidobacterium spp.,* and *Collisella spp.* Chickpeas, which had the highest propionic acid production, showed also, along with banana, higher abundances of *Blautia spp.* and *Roseburia spp.,* both related to production of this acid.[2,19] However, we did not find this kind of correlation with butyric acid.

Microbial community composition after fermentation of cooked foods

Cooking is known to modify food composition due to the development of different chemical reactions such as the Maillard reaction.[7] Distinct cooking technologies with different heat transfer media and heating intensities will modify food composition in a different way. Therefore, it is expected that cooking conditions could modify the composition of the gut microbiota. Under our experimental conditions, we observed statistically significant differences in the abundance of Firmicutes and Bacteroidetes between food groups **(Figure 1)** at phylum level. We found the highest abundance of Bacteroidetes in chicken and the lowest in bread and banana. In the case of Firmicutes, the highest abundance was found in bread and banana and a very low abundance was detected in pepper. The Firmicutes/Bacteroidetes ratio was statistically higher ($p < 0.05$) in bread and banana in comparison with chicken and pepper.

Moreover, we observed a high abundance of Proteobacterias, most probably due to donors' microbial composition.[22]

At genus level, we found statistically significant ($p < 0.05$) differences among foodstuffs for several beneficial bacteria **(Figure 2)**: *Bifidobacterium spp., Collinsella spp., Gordonibacter spp., Barnesiella spp., Blautia spp., Fusicatenibacter spp., Roseburia spp.*, *Pseudoflavonifractor spp., Alistipes spp., Anaerostipes spp., Coprococcus spp., Butyricimonas spp., Intestinimonas spp., Butyricicoccus spp.*, *Clostridium XIVa spp., Clostridium XIVb spp., Ruminococcus spp., Eggerthella spp., Oscillibacter spp. and Parasutterella spp.* All these genera have been related with a decreased risk or severity of different pathologies like inflammatory bowel disease, cancer, diabetes, obesity, etc. (**Table 3**).

Therefore we found that banana had higher abundances of *Anaerostipes spp., Blautia spp., Collinsella spp., Fusicatenibacter spp., Roseburia spp., Ruminococcus spp., Butyricicoccus spp., Clostridium XIVa spp., Gordonibacter spp.,* and *Pseudoflavonifractor spp.* However, it showed lower abundance of *Eggerthella.* Chickpeas showed higher abundance of *Coprococcus spp.* Bread on the other hand, showed higher abundance of *Barnesiella spp., Bifidobacterium spp., Oscillibacter spp.,* and *Parasutterella spp.* However, it showed lower abundance of *Anaerostipes spp., Butyricimonas spp.,* and *Intestinimonas spp.* Red pepper showed higher abundance of *Butyricimonas spp.,* and *Eggerthella spp.* On the other hand, it showed lower abundance of *Blautia spp., Coprococcus spp., Fusicatenibacter spp., Oscillibacter spp., Parasutterella spp., Roseburia spp., Ruminococcus spp.,* and *Gordonibacter spp.* Finally, chicken showed lower abundance of *Barnesiella spp., Bifidobacterium spp., Collinsella spp., Butyricicoccus spp., Clostridium XIVa spp.,* and *Pseudoflavonifractor spp.*

Principal Coordinates Analysis (PCoA) of microbiota composition with phylogenetic weighted UniFrac (**Figure 3**) showed a clear separation among high starch-content foods (banana and bread) and non-starchy foods (chicken and pepper). Permutational multivariate analysis of the variance (PERMANOVA) showed that in fact, different foods produced significantly different ($p < 0.05$) microbial communities. The main difference among them was the higher content of Firmicutes in bread and banana whereas in chicken and pepper Bacteroidetes levels were higher. Therefore, the provision of a specific kind of food (legume, cereal, vegetable, fruit or meat) can change microbial community composition and consequently affect health. Moreover, we observed differences among distinct types of cooking techniques. Accordingly, boiled chickpeas were closer to banana and bread whereas grilled chickpeas were closer to chicken and pepper (**Figure 3**). A possible explanation could be the participation of starchy carbohydrates in the Maillard reaction during chickpeas grilling, so that they are not available for gut microbiota fermentation, whereas during boiling the degree of the Maillard reaction is quite low, so that starch suffers a low degree of modification and is easily available to bacteria. In the case of bread, toasting also showed some effects, especially when toasting was carried out for a longer time. We observed lower abundance of *Roseburia spp., Coprococcus spp, Blautia spp., Butyricimonas spp., Anaerostipes spp., Clostridium XIVa spp., Clostridium XIVb spp.,* and *Ruminococcus spp* in toasted bread, and even lower levels in the well done form. However, *Collinsella spp.* and *Parasutterella spp.* behaved differently, being higher in toasted bread. Banana displayed higher abundance of *Bifidobacterium spp., Barnesiella spp., and Butyricimonas spp.* in the raw form. However, the abundance of *Roseburia spp., Oscillibacter spp., Coprococcus spp.* and *Parasutterella spp.* was higher in fried and roasted banana. Although bread and banana are starchy foods, the different behaviour of

gut microbiota after fermentation could be related with both differences in carbohydrate content (bread 55% - banana 20%) and protein content (bread 11% - banana 1%). Finally, as **Figure 3** shows, little separation was detected among different types of cooking in chicken or pepper. In this sense, this finding could be important when it comes to choosing the most adequate cooking method.

Moreover, we also found significant correlations among furosine, HMF and furfural and some bacterial taxa (**Table 2**). At the phylum level, Actinobacteria was positively correlated with all three indicators whereas Verrucomicrobia was only correlated with HMF and furfural. At the genus level, we detected several correlations with one or more indicators in different foodstuffs. In chickpeas, bread, pepper and banana, we found positive correlations among *Akkermansia* and HMF-furfural. *Akkermansia muciniphila* (the only known species of the genus) has been found to be reduced in obese and diabetic type II mice and the treatment with such bacteria reversed high-fat-diet related metabolic disorders.[23] Further, we found positive correlations between *Prevotella spp.* and the three indicators in all four foodstuffs. *Prevotella spp.* are also quite important for human health since they are susceptible to dietary changes and have been related to inflammatory phenotypes.[24]

Additionally, we also detected positive correlations among *Butyricicoccus spp.* and HMF-furfural (**Table 2**). In chickpeas, bread and pepper (not in banana) we found positive correlations among furosine and *Bifidobacterium spp.* and *Pseudoflavonifractor spp.*, and between furfural-*Collinsella spp.* In bread, pepper and banana positive correlations among *Intestinibacter spp.* and all three indicators were also found. This bacterium has been found to increase in some neurological diseases such as Parkinson´s disease.[25] In chickpeas, bread and banana, *Eggerthella spp.* was positively correlated with HMF and furfural. In bread and pepper, the Firmicutes phylum, *Collinsella spp.*

and *Oscillibacter spp.* were positively correlated with furosine. In banana and pepper, *Parasutterela spp.* was positively correlated with furfural. Further, in chickpeas and pepper we detected a correlation between *Christensenella spp.,* which is associated with weight reduction in mice,[26] and furfural. Finally, we also found a single negative correlation between furosine and *Blautia spp.* in bread and banana.

These correlations, most of them positive, could indicate that the Maillard reaction provides substrates for the growth of these bacteria and thus favors their thriving. In fact, it has been already stated that melanoidins, the end-product of the Maillard reaction, are fiber-like products;[8] they escape digestion and absorption, so that they reach the large intestine where they could be substrates for the gut microbiota. However, we also detected one negative correlation, indicating that some of the Maillard reaction products act as inhibitors for certain types of bacteria.

Finally, we performed a distance-based Redundancy Analysis (db-RDA) with Bray-Curtis distance (**Figure 4**). This multivariate analysis allowed us to relate each microbial genus to the samples and therefore to observe in the same plot which foodstuff was richer in which genus. As it can be observed in **figure 4,** there is a clear and significant ($p < 0.05$) separation of the foodstuffs as it happened with UniFrac PcoA. The distance between the foodstuffs and the genus in the plot is indicative of their abundance, the closer they are the higher the abundance in such food. Therefore, we could conclude that bread, raw banana and boiled chickpeas showed higher abundances in beneficial bacteria such as *Roseburia, Ruminococcus, Bifidobacterium, Dialister, Collinsella,* or *Barnesiella.* This would indicate that such foods would result in a healthier gut microbial community. On the other hand, the arrows of **figure 4** represent SCFA and they point to where higher amounts are found. Thus, bread and banana produced higher amounts of acetate, chickpeas propionate and pepper butyrate.

As conclusions, Maillard reaction indicators like furosine, HMF and furfural are sensitive indicators to control heat damage during cooking, especially for grilling, roasting and frying. However, their behaviour depends on the composition and type of foodstuff. When cooked foods are submitted to *in vitro* digestion and fermentation, they are readily metabolized by the gut microbiota, increasing the levels of beneficial bacteria like *Ruminococcus spp*. or *Bifidobacterium spp*., among others. In addition, different healthy short chain fatty acids like acetic, propionic and butyric acids are released. Although SCFAs concentration depended on food type, cooking methods and heat intensity had a definitive influence over SCFAs production and microbial composition. The influence of culinary technologies on gut microbiota composition and functionality could be derived from the production of Maillard reaction compounds like melanoidins, which escape digestion and can be fermented by the gut microbiota. According to our results, bread, raw banana and boiled chickpeas produced a healthier gut microbial community characterized by higher abundance of some beneficial bacteria such as *Roseburia, Ruminococcus, Bifidobacterium, Dialister, Collinsella,* or *Barnesiella.* One way or another, it is likely that the gut microbiota can be modulated not only by the type of food but also by the type of cooking and the thermal treatment applied. Therefore, more studies are needed in order to unravel the specific effect of cooking techniques on food composition and their direct effect over the gut microbiota.

Abbreviations: HPLC: High Performance Liquid Chromatography, SCFA: Short Chain Fatty Acids, HMF: 5-hydroxymethyl-furfural, MR: Maillard Reaction,

Acknowledgements

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

Founding

This study was supported by project AGL2014-53895-R from the Spanish Ministry of Economy and Competitiveness and by the European Regional Development Fund (FEDER). It was also supported by a Spanish predoctoral fellowship F.P.U. for S. Perez-Burillo from the Spanish Government.

Conflict of interest

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

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Figure captions.

Figure 1. Relative bacterial abundance (at Phylum level) after *in vitro* fermentation of the assessed foodstuffs.

Figure 2. Relative abundances (at genus level) of bacteria with significant differences between the assessed foodstuffs. Differences were assessed by ANOVA and compared with the mean relative abundance of the five different foodstuffs. Statistic labels: ns: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. (+): significantly higher than the mean; (-): significantly lower than the mean.

Figure 3. PCoA ordination analysis of genus abundance among all profiled samples. Phylogenetic weighted UniFrac distance was used to calculate the sample dissimilarity matrix.

Figure 4. Distance-based Redundancy Analysis (db-RDA) triplot with Bray-Curtis distance.

Figure 1.

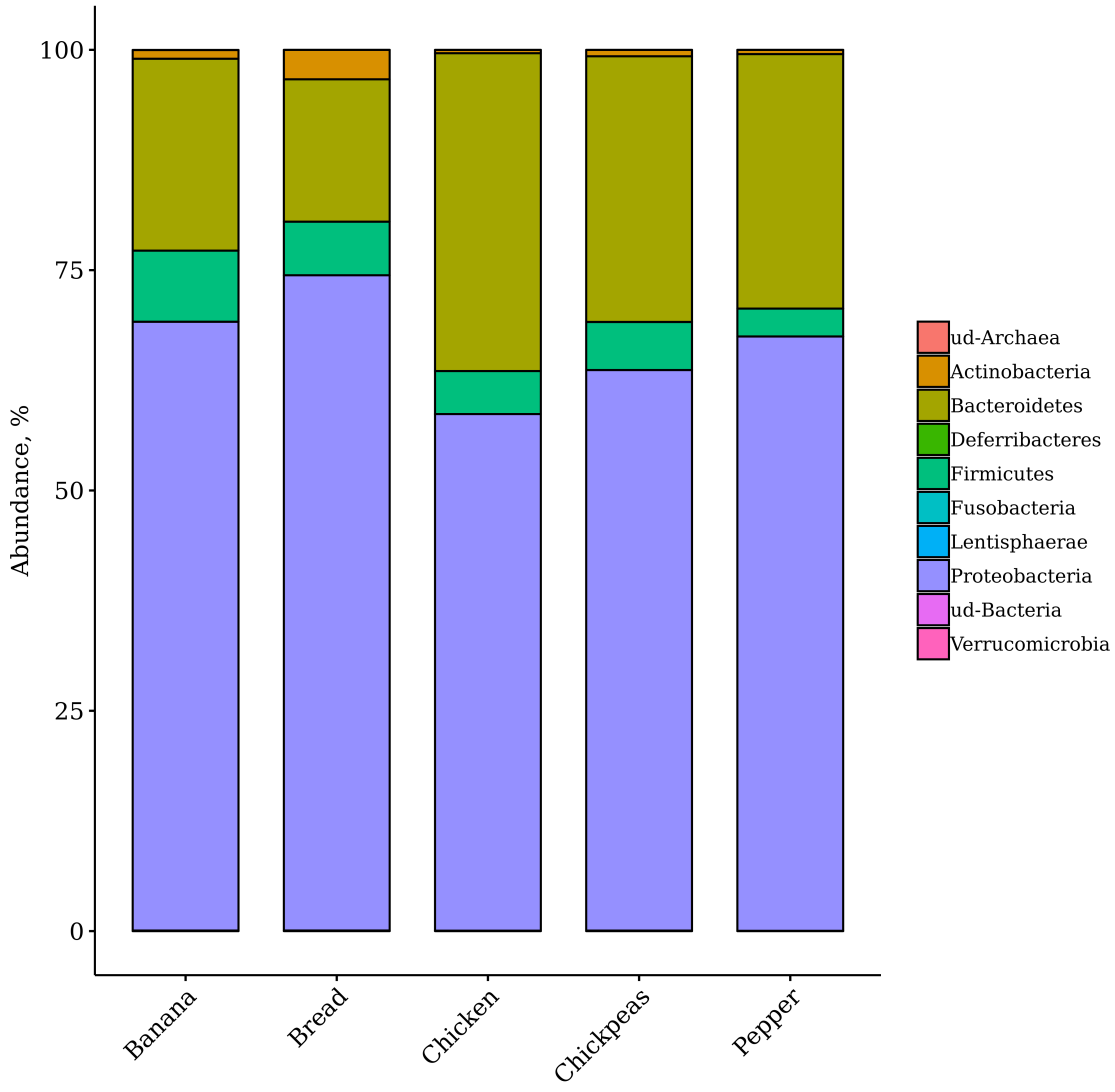

100
75
50
25
0
Abundance, %
Banana
Bread
Chicken
Chickpeas
Pepper
ud-Archaea
Actinobacteria
Bacteroidetes
Deferribacteres
Firmicutes
Fusobacteria
Lentisphaerae
Proteobacteria
ud-Bacteria
Verrucomicrobia

Figure 2.

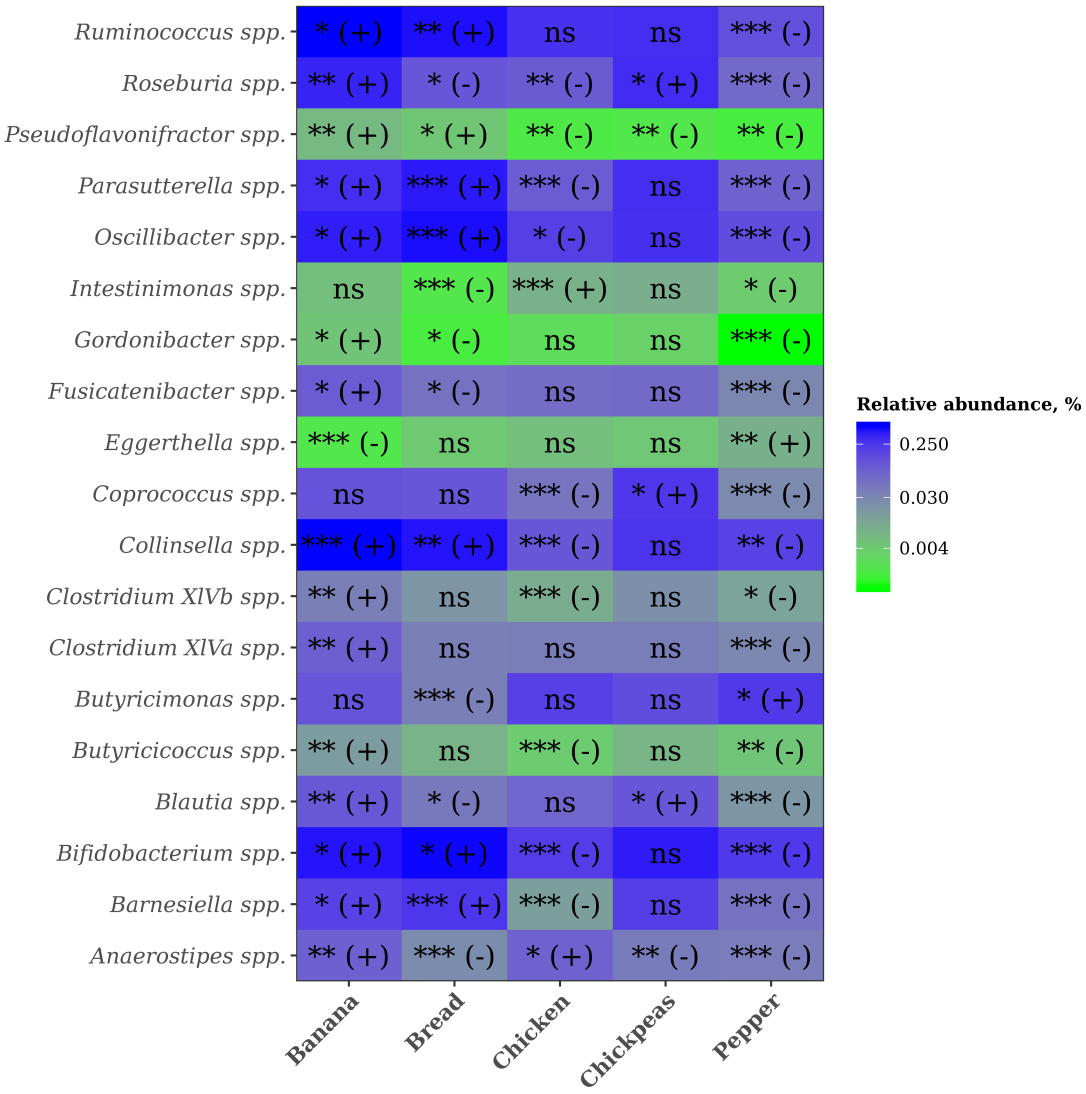

Ruminococcus spp.
Roseburia spp.
Pseudoflavonifractor spp.
Parasutterella spp.
Oscillibacter spp.
Intestinimonas spp.
Gordonibacter spp.
Fusicatenibacter spp.
Eggerthella spp.
Coprococcus spp.
Collinsella spp.
Clostridium XlVb spp.
Clostridium XlVa spp.
Butyricimonas spp.
Butyricicoccus spp.
Blautia spp.
Bifidobacterium spp.
Barnesiella spp.
Anaerostipes spp.
* (+) ** (+) ns ns *** (-)
** (+) * (-) ** (-) * (+) *** (-)
** (+) * (+) ** (-) ** (-) ** (-)
* (+) *** (+) *** (-) ns *** (-)
* (+) *** (+) * (-) ns *** (-)
ns *** (-) *** (+) ns * (-)
* (+) * (-) ns ns *** (-)
* (+) * (-) ns ns *** (-)
*** (-) ns ns ns ** (+)
ns ns *** (-) * (+) *** (-)
*** (+) ** (+) *** (-) ns ** (-)
** (+) ns *** (-) ns * (-)
** (+) ns ns ns *** (-)
ns *** (-) ns ns * (+)
** (+) ns *** (-) ns ** (-)
** (+) * (-) ns * (+) *** (-)
* (+) * (+) *** (-) ns *** (-)
* (+) *** (+) *** (-) ns *** (-)
** (+) *** (-) * (+) ** (-) *** (-)
Banana
Bread
Chicken
Chickpeas
Pepper
Relative abundance, %
0.250
0.030
0.004

Figure 3.

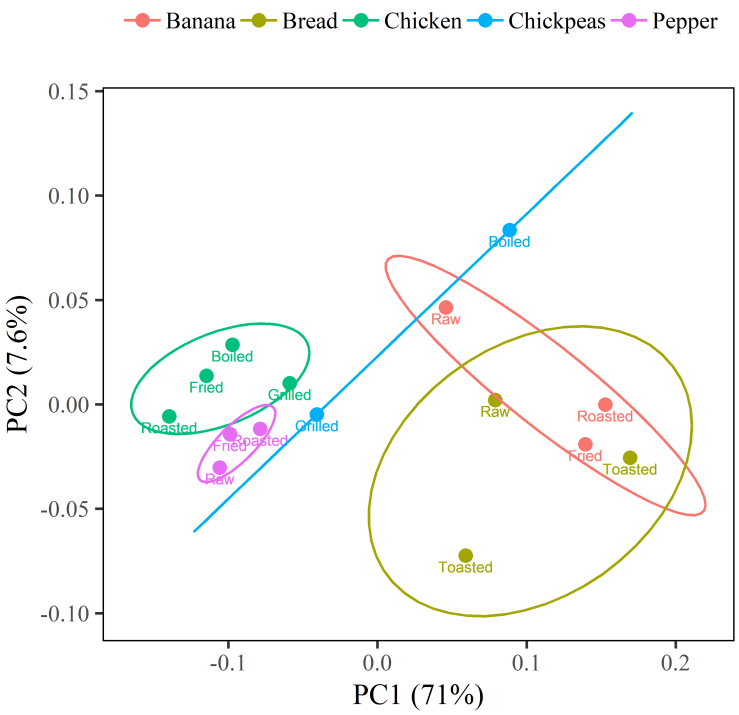
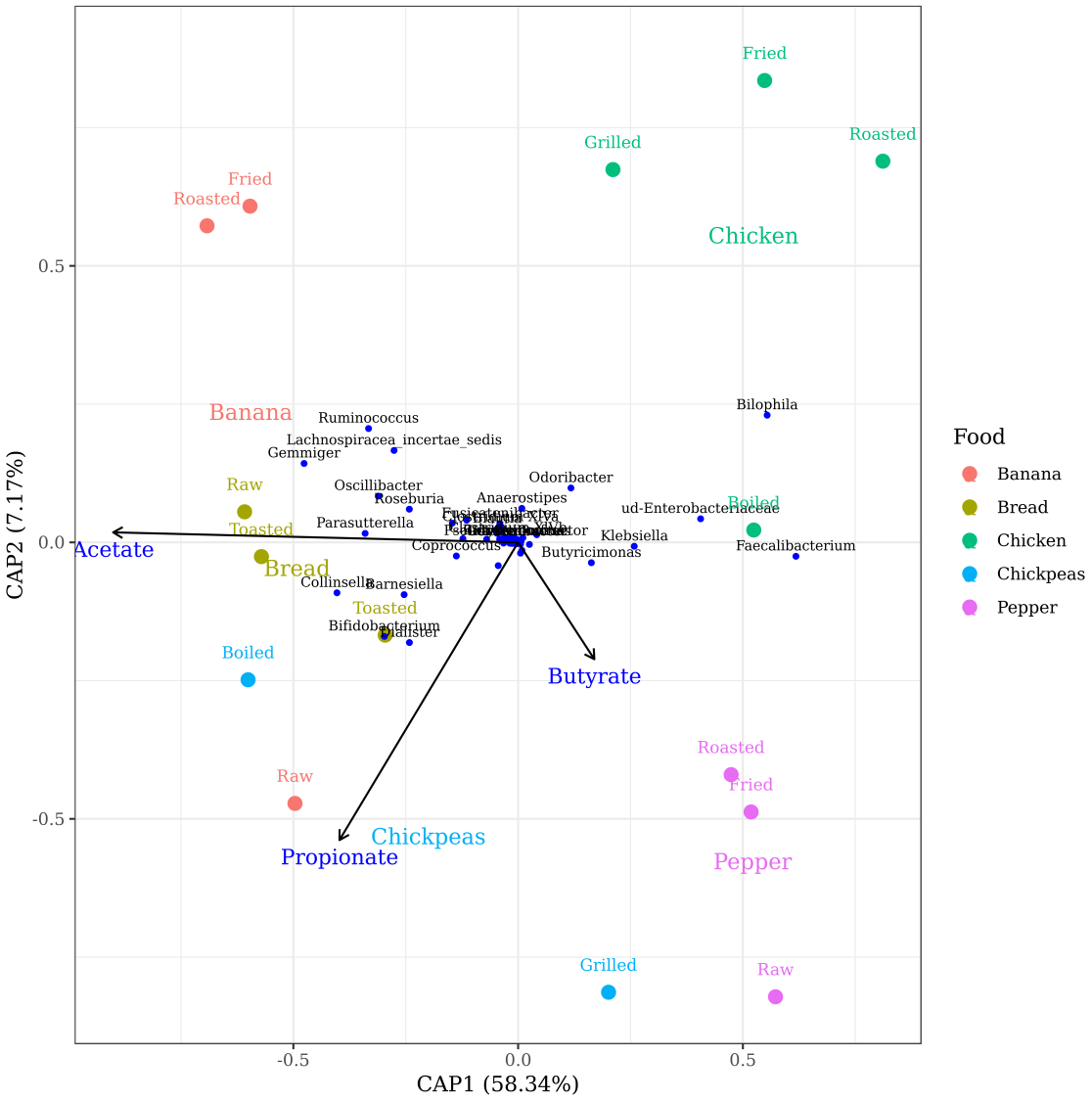

Banana
Bread
Chicken
Chickpeas
Pepper
0.15
0.10
0.05
0.00
-0.05
-0.10
PC2 (7.6%)
-0.1
0.0
0.1
0.2
PC1 (71%)
Boiled
Fried
Grilled
Roasted
Raw
Boiled
Raw
Raw
Roasted
Fried
Toasted
Toasted

Figure 4.


Fried
Grilled
Roasted
Fried
Roasted
Chicken
0.5
Banana
Ruminococcus
Bilophila
Lachnospiracea_incertae_sedis
Gemmiger
Raw
Oscillibacter
Odoribacter
Roseburia
Anaerostipes
Boiled
ud-Enterobacteriaceae
Toasted
Parasutterella
Klebsiella
0.0
Acetate
Coprococcus
Butyricimonas
Faecalibacterium
Bread
Collinsella
Barnesiella
Toasted
Bifidobacterium
Boiled
Butyrate
Roasted
Raw
Fried
-0.5
Chickpeas
Propionate
Pepper
Grilled
Raw
-0.5
0.0
0.5
CAP1 (58.34%)
CAP2 (7.17%)
Food
Banana
Bread
Chicken
Chickpeas
Pepper

104 **Table 1**. Furosine, HMF, Furfural and SCFAs Content of Processed Foods.

Sample	Furosine (μg/g of food)	HMF (μg/g of food)	Furfural (μg/g of food)	Acetic acid (μmol/g of food)	Propionic acid (μmol/g of food)	Butyric acid (μmol/g of food)	Total SCFAs (μmol/g of food)
Boiled chickpeas	12.0[a] ± 0.6	0.00[a] ± 0.00	0.79[a] ± 0.03	36.8[a] ± 1.9	54.7[a] ± 0.6	21.3[a] ± 0.4	113[a]
Grilled chickpeas	56.9[b] ± 3.5	2.44[b] ± 0.15	1.13[b] ± 0.06	30.1[a] ± 1.8	49.6[a] ± 2.9	51.6[b] ± 2.1	131[a]
Chickpeas (mean)	*34.4 ± 2.1*	*1.22 ± 0.08*	*0.96 ± 0.04*	*33.5 ± 1.8*	*52.1 ± 1.7*	*36.4 ± 1.2*	*122*
Raw bread	20.5[a] ± 1.1	0.56[a] ± 0.01	0.75[a] ± 0.03	69.6[a] ± 4.8	29.8[a] ± 2.2	13.4[a] ± 0.5	113[a]
Well done toasted bread	809[b] ± 34	57.2[b] ± 4.7	235[b] ± 15	60.4[a] ± 1.6	46.8[a] ± 1.0	5.3[b] ± 0.3	113[a]
Normal toasted bread	537[c] ± 18	6.22[c] ± 0.46	17.1[c] ± 1.2	94.2[a] ± 4.4	33.8[a] ± 2.9	9.5[b] ± 0.5	137[b]
Bread (mean)	*456 ± 14*	*21.3 ± 1.3*	*84.7 ± 4.1*	*74.8 ± 3.2*	*36.8 ± 2.0*	*9.4 ± 0.6*	*121*
Roasted pepper	176[a] ± 15	35.5[a] ± 1.5	0.35[a] ± 0.03	40.8[a] ± 3.8	43.9[a] ± 0.2	29.6[a] ± 1.0	114[a]
Raw pepper	7.61[b] ± 0.53	0.85[b] ± 0.02	0.11[a] ± 0.01	9.5[b] ± 0.8	48.6[a] ± 2.0	35.8[a] ± 1.7	94[a]
Fried pepper	175[a] ± 14	19.2[c] ± 0.2	0.72[c] ± 0.01	15.0[b] ± 0.8	38.9[a] ± 2.5	89.1[b] ± 1.6	143[b]
Pepper (mean)	*120 ± 11*	*18.5 ± 0.8*	*0.39 ± 1.0*	*21.8 ± 2.1*	*43.8 ± 1.7*	*51.5 ± 1.2*	*117*
Roasted banana	73.8[a] ± 3.1	5.71[a] ± 0.31	1.18[a] ± 0.05	71.3[a] ± 7.5	67.6[a] ± 2.8	22.0[a] ± 0.2	161[a]
Raw banana	9.21[b] ± 0.22	0.04[b] ± 0.00	0.41[b] ± 0.02	42.4[b] ± 2.9	26.6[b] ± 1.2	2.5[b] ± 0.0	72[b]
Fried banana	216[c] ± 19	13.1[b] ± 0.5	1.78[c] ± 0.11	68.6[a] ± 4.1	22.1[b] ± 0.5	70.8[c] ± 5.0	162[a]

Banana (mean)	*100 ± 8*	*6.28 ± 0.39*	*1.23 ± 0.30*	*60.8 ± 4.2*	*38.8 ± 1.5*	*31.8 ± 1.6*	*131*
Roasted chicken	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	7.3[a] ± 0.2	13.9[a] ± 0.4	11.5[a] ± 0.7	33[a]
Fried chicken	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	7.8[a] ± 0.1	13.6[a] ± 0.8	23.6[b] ± 1.0	45[a]
Boiled chicken	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	2.0[b] ± 0.1	10.7[a] ± 0.4	2.5[c] ± 0.1	15[b]
Grilled chicken	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.5[a] ± 0.5	10.6[a] ± 0.1	16.7[a] ± 0.3	34[a]
Chicken (mean)	*0.00 ± 0.00*	*0.00 ± 0.00*	*0.00 ± 0.00*	*5.9 ± 0.2*	*12.2 ± 0.4*	*13.6 ± 0.5*	*32*

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

106 **Table 2**. Lineal Correlations (r Values) among Bacteria and SCFAs Content and

107 Maillard Reaction Products.

	Acetic acid	**Propionic acid**	**Butyric acid**
Phylum Bacteroidetes		0.6586[i]	
Butyricimonas spp.		0.8026[e]	0.8154[a]
Ruminococcus spp.	0.5288[a]	0.9114[a]	
Anaerostipes spp.			0.9282[g]
Clostridium XIVa spp.			
Blautia spp.		0.7550[g]	
Roseburia spp.		0.7801[g]	0.9187[e]
Intestimonas spp.			0.6827[e]
Faecalibacterium spp.			0.7999[a]
Veillonella spp.		0.9512[g]	
Bifidobacterium spp.	0.8162[a]		
Collinsella spp.	0.7589[a]		
	Furosine	**HMF**	**Furfural**
Phylum Actinobacteria	0.8503[a]	0.6954[a]	0.9758[a]
Plylum Verrucomicrobia		0.7541[a]	0.9694[a]
Phylum Firmicutes	0.9173[c]		
Akkermansia spp.		0.7534[a]	0.7366[a]
Butyricicoccus spp.		0.7487[a]	0.8328[a]
Bifidobacterium spp.	0.8272[b]		
Pseudoflavonifractor spp.	0.8462[b]		
Collinsella spp.	0.8629[c]		0.7081[b]
Blautia spp.	-0.8265[e]		
Christensenella spp.			0.9598[d]
Prevotella spp.	0.7638[a]	0.7889[a]	0.9562[a]
Intestinibacter spp.	0.7289[f]	0.7342[f]	0.8084[f]
Eggerthella spp.		0.7130[g]	0.8023[g]
Parasutterella spp.			0.7138[h]
Oscillibacter spp.	0.8954[c]		

[a]Chickpeas, bread, pepper and banana; [b]Chickpeas, bread and pepper; [c]Bread and pepper; [d]Chickpeas and pepper; [e]Bread and banana; [f]Bread, pepper and banana; [g]Chickpeas, bread and banana; [h]Banana and pepper; [i]All

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Table 3. Beneficial or Detrimental Effects of Bacteria on Human Health. (+) positive health effect; (-) negative health effect.

Bacteria		Health effect
Akkermansia spp.	+	It helps to control diet-induced obesity and associated metabolic disorders. [23]
Christensenella spp.	+	Associated with lower body mass index. [26]
Faecalibacterium spp.	+	Produces butyrate, it helps to regulate the immune system, it could exert a positive role on Chron´s disease. [2]
Veillonella spp.	+	Produces propionate. [2]
Bifidobacterium spp.	+	Reduced in colorectal cancer and in type I diabetes. [27]
Collinsella spp.	+	Reduced in irritable bowel syndrome patients with more severe symptoms. [28]
Gordonibacter spp.	+	Produce anti-inflammatory urolithins from ellagic acid. [29]
Barnesiella spp.	+	May prevent or treat infections by antibiotic resistant bacteria. [30]
Blautia spp.	+	Related to decreased inflammation in cirrhosis and hepatic encephalopathy, reduced in colorectal cancer and type I diabetes. [27], [31]
Fusicatenibacter spp.	+	Reduced in ulcerative colitis patients and probable anti-inflammatory function. [32]
Roseburia spp.	+	Associated with weight loss and decreased glucose intolerance in mice, reduced in ulcerative colitis patients, differs in abundance between type II diabetes patients and non-diabetic people. [33]
Pseudoflavonifractor spp.	+	Related to weight loss along with *Alistipes spp.* [34]
Anaerostipes spp.	+	Produces acetic, lactic and butyric acid. [2],
Coprococcus spp.	+	Produces acetic and butyric acid, and lower amounts of propionic or formic acid. [2],
Butyricimonas spp.	+	Produces butyric acid. [2],
Intestinimonas spp.	+	Produces butyric acid. [2],
Butyricicoccus spp.	+	Produces butyric acid, reduced in ulcerative colitis patients and patients with inflammatory disease in general. [2],
Clostridium XIVa spp.	+	Produces butyric acid. [2],
Ruminococcus spp.	+	Key role in degradation of resistant starch. [35]
Clostridium XIVb spp.	+	Correlated with systemic inflammatory cytokines in patients with HIV-1. [36]
Eggerthella spp.	-	Related to ulcerative colitis, hepatic abscesses and systemic bacteraemia. [37]
Oscillibacter spp.	-	Increased in depression and in high-fat diet. [38]
Parasutterella spp.	-	Related with Crohn´s disease and with dysbiosis in hypertriglyceridemia associated to necrotizing pancreatitis. [39]
Prevotella spp.	-	It outgrows in autoinflammatory disease. [24]
Intestinibacter spp.	-	It has been found to be increased in patients with neurological disorders other than Parkinson´s

disease. [25]

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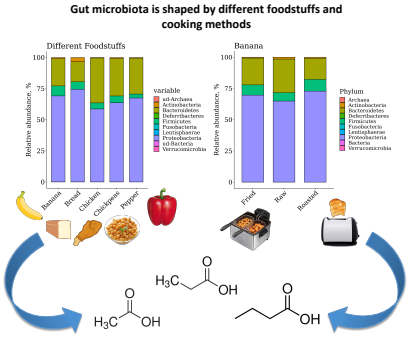

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