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Impact of cooking methods of red-skinned onion on metabolic transformation of phenolic compounds and gut microbiota changes†

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Herein, we investigated the stability and bioaccessibility of phenolics in differently cooked red-skinned onion (RSO) and consequently their impact on the gut microbiota and metabolism of phenolics. In fact, the different processes used to cook vegetables can modify and re-arrange the molecular profiles of bioactive compounds, such as phenolics in phenolic-rich vegetables, such as RSO. Fried and grilled RSO were compared to raw RSO and a blank control and subjected to oro-gastro-intestinal digestion and subsequent colonic fermentation. For upper gut digestion, the INFOGEST protocol was used, and for lower gut fermentation, a short-term batch model, namely, MICODE (multi-unit *in vitro* colon gut model), was employed. During the process, phenolic compound profile (through high-resolution mass spectrometry) and colon microbiomics (qPCR of 14 core taxa) analyses were performed. According to the results, the degradation driven by the colon microbiota of RSO flavonols resulted in the accumulation of three main metabolites, *i.e.*, 3-(3′-hydroxyphenyl)propanoic acid, 3-(3′-hydroxyphenyl)acetic acid and 3-(3′,4′-dihydroxyphenyl)acetic acid. Also, colonic fermentation of raw onions resulted in a substantial increase in beneficial taxa, which was larger compared to the heat-treated onions, particularly *Lactobacillales* and beneficial clostridia. Also, a higher level of inhibition of opportunistic bacteria was seen for the raw onion samples, namely, *Clostridium perfringens* group and *Escherichia coli*. Thus, our results showed that RSO, and especially the raw one, is an excellent dietary source of flavonols that are strongly metabolized by gut bacteria and can positively modulate the gut microbiota. Although additional *in vivo* studies are necessary, this work is one of the first to explore how RSO processed with different cooking methods can differently impact the phenolic metabolism and microbiota composition in the large intestine of humans, fine-tuning the antioxidant nature of foods.

1. Introduction

Onion (*Allium cepa* L.) is one of the most consumed vegetables worldwide and is also traditionally part of the Mediterranean diet. Besides its typical taste and flavour, onion is considered a

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healthy food because it contains several classes of phenolic compounds such as flavonols (mainly quercetin and its glycosylated derivatives), hydroxycinnamic acids, and in red-skinned onion, anthocyanins.[1–3] Recently, onion consumption has been associated with a reduction in the onset of some chronic diseases such as cardiovascular diseases, type-2 diabetes, and gastro-intestinal tract cancers.[4,5]

Generally, onion can be consumed in the diet daily, either raw or cooked through different methods such as grilling and frying, which are the two most usual cooking procedures applied to onion. Several studies evidenced that heat treatments during cooking may modify the phenolic profile and structures of vegetables foods resulting in a different and sometimes higher bioactivity and improved bioaccessibility after *in vitro* gastro-intestinal digestion.[1,6,7] Additionally, these modified compounds undergo an ulterior transformation when they enter the colon, where they can feed the colon microbiota to foster beneficial bacteria and inhibit opportunis-

tic microbes.[8] Unfortunately, this improvement can also have the opposite impact on the colon microbiota, adversely affecting sensitive beneficial bacteria, such as bifidobacteria.[9] Moreover, it has been shown that both food composition and thermal treatments alter the composition of the gut microbiota.[10] In addition, previous studies highlighted that different cooking procedures may affect the profile and concentration of phenolic metabolites derived from the gut microbiota metabolic activity during *in vitro* fermentation of several vegetables.[10–13]

The two-way interaction between the gut microbiota and phenolic compounds is considered pivotal in determining the beneficial effect of phenolic compounds in human health.[14] In this sense, the colon bacteria can metabolize phenolic compounds in low molecular weight phenolic metabolites, which are better absorbed and often more active than the parent compounds as well as phenolic compounds may modulate the gut microbiota population.[15,16] In particular, phenolic compounds may increase the relative abundance of beneficial bacteria involved in the protection of the intestinal epithelium (such as *Lactobacillus* and *Bifidobacterium* species) or that displaying anti-inflammatory effects (such as *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*).[10,17–19] In turn, colonic bacteria transform the structure of parent phenolic compounds in metabolites with improved biological activities and more bioavailable to the host.[10,15,20]

In vitro gut models are considered suitable to study the complex relationships between the chemical constituents of food and the colon microbiota because they can clarify the effect of prebiotics such as phenolic compounds on the human gut microbiota through the analysis of the shift in the core microbial groups or selected species, as well as assessing the modification in the produced metabolites by individual bacterial species.[21] In this study, we employed a gut model based on healthy adults, coupling the INFOGEST static protocol to simulate the oro-gastro-duodenal digestion[22] to the colon model MICODE (Multi-Unit *in vitro* Colon Model) to simulate proximal colonic fermentation, previously used with success to study food matrices rich in phenolic compounds.[8–10] Therefore, the aim of this study was to assess the bioaccessibility of phenolic compounds of both raw and cooked (grilled and fried) red-skinned onion as well as to elucidate the principal metabolic pathways and produced metabolites after *in vitro* colonic fermentation. Furthermore, the changes in fecal microbiota groups and selected bacterial taxa were also investigated.

2. Materials and methods

2.1. Red-skinned onion (RSO) thermal treatments and *in vitro* gastro-intestinal digestion

Red-skinned onion (RSO) was subjected to two distinct thermal treatments to mimic the most common home cooking methods to which onion is subjected, *i.e.*, grilling and frying. The cooking procedures and the cooking parameters were fully described previously.[1] Thereafter, raw (RRSO), grilled (GRSO) and fried (FRSO) red-skinned onion samples were subjected to *in vitro* gastro-intestinal digestion using the INFOGEST protocol[22] as previously described for onion.[1] Finally, an aliquot of sample was collected at the end of the digestion procedure, centrifuged (10 min, 10 000g, 4 °C) and the supernatant stored at −80 °C for further analysis, whereas the remaining material (denser portion) was used for the *in vitro* colonic fermentation trials as described in Section 2.2. The cooking treatments and digestions were carried out in triplicate. Afterwards, for colonic *in vitro* fermentation, the triplicates of a given digestate were pooled.

2.2. Colonic fermentation

2.2.1 Human colon microbiota. For this study, stool donations obtained from three lean healthy individuals who respected the inclusion criteria as previously reported[8–10,23–25] were used. Human colon microbiota (HCM) was extracted from the stool samples, which were donated two times (at an interval of seven days) for the two biological replicas. The stool samples were collected with a collection kit, maintained at 37 °C under anaerobiosis and processed within two hours as previously described.[8–10] Briefly, to obtain the HCM, a faecal slurry (FS) was prepared by homogenizing 6 g of stool (2 g of each donation) in 54 mL of O_2 reduced phosphate-buffered saline (PBS). The three healthy donors were told of the aims and procedures of this study and they gave verbal consent for their faecal matter to be used in the experiments, in agreement with the ethics procedures required at the University of Bologna.

2.2.2 Colonic fermentation and sampling. Colonic fermentations were conducted for 24 h in independent vessels using an *in vitro* gut model, MICODE,[8–10,26,27] obtained by the assembly of Minibio Reactors (Applikon Biotechnology BV, Delft, NL) and controlled by the Lucullus PIMS software (Applikon Biotechnology BV, NL). The formulation for the experiments was made according to published procedures,[8–10,23,27,28] and recently described in detail.[9] Briefly, four fermentation bioreactors were filled with 90 mL of basal medium,[23] and then each one loaded as follows: (i) with 10 mL of a mixture of FS and 1 g of the solid portion of *in vitro* digested raw onion (RRSO); (ii) with 10 mL of FS including 1 g of the solid portion of *in vitro* digested grilled onion (GRSO); (iii) with 10 mL of FS including 1 g of the solid portion of *in vitro* digested fried onion (FRSO), and (iv) with 10 mL of FS including 1 mL of digestive enzymes, as the blank control (BC). Batch cultures were run under controlled conditions for a period of 27.48 h including the baseline (BL) (3.48 ± 0.22 h) as previously described.[9] The BL represents the time of adaptation of the HCM to the *in vitro* conditions. The BL was defined as the first pH changes detected by Lucullus® (1 read per 10 s) *via* the pH sensors (Applisense, Applikon Biotechnology BV, NL) of MICODE. Sampling was performed at different intervals, specifically BL; T1 = intermediate time point (18 h of fermentation); and EP = endpoint (24 h of fermentation), as previously reported.[9] The samples from the

different time points were used for UHPL/HR-MS and qPCR. Technical replicas of the analyses were conducted in triplicate from two independent biological experiments.

2.3. High-resolution mass spectrometry analysis of phenolic compounds and fecal metabolites

2.3.1 Extraction of phenolic compounds from undigested, digested and fermented red-skinned onion samples. Phenolic compounds were extracted from raw, fried, and grilled undigested RSO following a previously reported protocol.[6] RSO samples (15 g) were homogenised (6000 rpm for 2 min) with an Ultra-Turrax (Heidolph DIAX900, Sigma-Aldrich, Milan, Italy) after mixing with 30 mL a water/methanol/formic acid (28 : 70 : 2, v/v/v) solution. Then, the mixtures were incubated for 30 min at 37 °C, and after centrifugation (20 min, 6000g, 4 °C), the supernatants were collected and analysed by mass spectrometry for the identification and quantification of the phenolic compounds.

The phenolic compounds in the *in vitro* digested samples and faecal metabolites were extracted as previously described.[12,13] Briefly, 500 μL of extraction solution (water/ methanol/formic acid; 19.9 : 80 : 0.1, v/v/v) was mixed with 500 μL of digested or fermented samples. After vortexing for 1 min, the mixtures were centrifuged (10 min; 12 000g; 4 °C). Then the supernatants were used for mass spectrometry identification and quantification of phenolic compounds and metabolites.

2.3.2 Phenolic compound profiles of undigested, digested and fermented red-skinned onion samples. The identification and quantification of the phenolic compounds were performed by high-resolution mass spectrometry analysis using a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Chromatographic separation was carried out using an UHPLC Ultimate 3000 separation module equipped with a C18 column (Acquity UPLC HSS C18 reversed phase, 2.1 × 100 mm, 1.8 μm particle size, Waters, Milan, Italy). The chromatographic and mass spectrometry parameters were previously reported in detail.[29] Quantification was carried out by building external calibration curves with the available standard phenolic compounds[2] and faecal metabolites[10] as previously reported. Mass spectral data of phenolic compounds[2] and faecal metabolites[10] can be found in the literature. For the phenolic metabolites, the nomenclature rules previously suggested[30] were applied.

2.4. Microbiota analyses

2.4.1 Enumeration of bacterial groups and qPCR prebiotic index (qPI). Bacterial DNA was extracted after sampling (Purelink Microbiome DNA Purification Kit by Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) and quantified (Biodrop, Biochrom Ltd, Cambridge, UK). Enumeration of the bacterial groups was done by qPCR to evidence the changes in the microbiota after fermentation[31–34] and expressed as cells per mL (obtained from values in gene copy numbers per mL multiplied by three for multicopy genes, as *V3–V4 16S*, and by one for monocopy genes, as *RecA*), following previous

protocols.[8–10,26,27,35] Specifically, 14 different bacterial taxa (*Eubacteria*, *Firmicutes*, *Bacteroidetes*, *Lactobacillales*, *Bifidobacteriaceae*, *Enterobacteriaceae*, *Clostridium* group I, *Clostridium* group IV, *Bacteroides–Prevotella–Porphyromonas* (BPP) group, *Atopobium–Collinsella–Eggerthella* (ATOP) group, *Bifidobacterium longum*, *Escherichia coli*, *Faecalibacterium praus-nitzii*, and *Akkermansia muciniphila*) (Table S1†) were assessed by qPCR on a QuantStudio 5 System (Applied Biosystem, Thermo Fisher, USA) and SYBR Green I chemistry. The qPCR prebiotic index (qPI) was calculated as previously reported.[9,10] Briefly, qPI is based on the qPCR data of the bacterial targets entered in the following equation:

$$\begin{aligned} \text{qPI} = \; & (Bifidobacteriaceae/Eubacteria) \\ & - (Enterobacteriaceae/Eubacteria) \\ & + (Lactobacillales/Eubacteria) \\ & - (Clostridium \text{ group I}/Eubacteria). \end{aligned}$$

2.5. Data processing and statistical analysis

The shifts in the abundance of the bacterial groups were calculated as $\log_2(\text{F/C})$ using the raw data[36] obtained from sextuplicates. Also, based on the raw data, the qPI was obtained after normalization with the mean centering method. Normality and homoscedasticity of the datasets were considered with Levene's and Shapiro–Wilk's test, respectively. To compare the "time points" and "samples" with each other, the two factors used to categorize the MANOVAs were "time" and "food matrix", respectively. MANOVA for "time" and "food matrix" as categorical descriptors and followed by a Duncan's multiple range *post-hoc* test was applied using Statistica v 8.0 (Tibco, Palo Alto, CA, USA). The differences were considered significant with $p < 0.05$. To address specific correlations among bacteria and metabolites, two independent datasets were merged and computed by Spearman rank analysis and visualized with a two-way joining heatmap, including Pearson dendrograms with complete linkage for both variables.[8–10] The dataset of correlations was performed with Statistica v. 8.0 (Tibco, USA) and the heatmap was produced with the Expression tool at **https://www.heatmapper.ca**[37] (last accessed on 15/12/2022).

3. Results and discussion

3.1. Effect of cooking and *in vitro* digestion on red-skinned onion phenolic compounds bioaccessibility

A total of 54 phenolic compounds belonging to different classes were identified and quantified in the red-skinned onion (RSO) samples (Table 1). The most representative class of phenolic compounds in RSO were flavonols, and in particular quercetin and quercetin-derivatives. In the raw sample (RRSO), flavonols represented the 85.3% of total phenolic compounds. Among the flavonols, the quercetin-derivatives accounted for 74.2% of the total phenolic compounds. The individual compound found at the highest concentration in RRSO was quercetin that represented the 50.1% of the total phenolic compounds. Two glycosylated-derivatives of querce-

Table 1 Amount of phenolic compounds in raw and cooked red-skinned onion determined by high-resolution mass spectrometry. Results are expressed in μmol of phenolic compound per 100 g of onion. Bioaccessibility index (BI), expressed as percentage value, is the ratio between the concentration of each individual phenolic compound after *in vitro* digestion and the concentration in the extract

Compound	RRSO Before digestion	RRSO After digestion	BI (%)	FRSO Before digestion	FRSO After digestion	BI (%)	GRSO Before digestion	GRSO After digestion	BI (%)
Hydroxycinnamic acids									
Di-hydro-coumaric acid	n.d.	0.30 ± 0.02[a]	n.f.	0.17 ± 0.01[b]	0.35 ± 0.03[a]	205.9	0.13 ± 0.01[c]	0.29 ± 0.02[a]	223.1
Caffeic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.03 ± 0.00	n.d.	0
Sinapic acid	0.08 ± 0.00[a]	0.03 ± 0.00[b]	37.5	0.02 ± 0.00[b]	0.14 ± 0.01[c]	700.0	0.04 ± 0.00[b]	0.03 ± 0.00[b]	67.7
Ferulic acid-4-O-hexoside	0.18 ± 0.01[a]	0.18 ± 0.01[a]	100.0	0.35 ± 0.03[b]	0.11 ± 0.01[c]	31.4	0.19 ± 0.01[a]	0.14 ± 0.01[d]	75.0
Sinapic acid-O-hexoside isomer 1	0.70 ± 0.03[a]	0.40 ± 0.02[b]	57.1	2.05 ± 0.15[c]	1.86 ± 0.09[d]	90.7	0.40 ± 0.02[b]	0.35 ± 0.03[b]	87.5
Sinapic acid-O-hexoside isomer 2	1.59 ± 0.08[a]	0.12 ± 0.01[b]	7.5	1.42 ± 0.08[a]	0.73 ± 0.03[c]	51.4	0.53 ± 0.03[d]	0.15 ± 0.01[b]	28.3
Sinapic acid-O-hexoside isomer 3	1.00 ± 0.10[a]	0.15 ± 0.01[b]	15.0	1.09 ± 0.09[a]	0.54 ± 0.04[c]	49.5	0.10 ± 0.00[d]	n.d.	0
Sinapic acid-O-hexoside isomer 4	0.45 ± 0.04[a]	0.08 ± 0.00[b]	17.8	0.49 ± 0.03[a]	0.54 ± 0.04[a]	110.2	n.d.	n.d.	n.d.
Total hydroxycinnamic acids	**4.00 ± 0.14[a]**	**1.26 ± 0.03[b]**	**31.5**	**5.59 ± 0.20[c]**	**4.27 ± 0.12[a]**	**76.4**	**1.42 ± 0.04[d]**	**0.96 ± 0.04**	**67.6**
Hydroxybenzoic acids									
Hydroxybenzoic acid isomer 1	0.08 ± 0.01[a]	0.04 ± 0.00[b]	50.0	0.17 ± 0.01[c]	0.12 ± 0.01[d]	70.6	0.14 ± 0.01[d]	0.12 ± 0.01[d]	85.7
Hydroxybenzoic acid isomer 2	0.04 ± 0.00[a]	0.06 ± 0.00[a]	150.0	0.05 ± 0.00[a]	0.06 ± 0.00[a]	120.0	0.04 ± 0.00[a]	0.06 ± 0.00[a]	150.0
Protocatechuic acid	0.18 ± 0.01[a]	1.00 ± 0.09[b]	555.6	0.22 ± 0.01[c]	0.86 ± 0.05[b]	390.9	0.24 ± 0.01[c]	1.26 ± 0.07[d]	525.0
Di-hydroxybenzoic acid	0.15 ± 0.01[a]	0.04 ± 0.00[b]	26.7	0.16 ± 0.01[a]	0.05 ± 0.00[b]	31.3	0.17 ± 0.01[a]	0.03 ± 0.00[b]	17.6
Vanillic acid	0.12 ± 0.01[a]	0.01 ± 0.00[b]	8.3	0.07 ± 0.00[c]	n.d.	0	0.12 ± 0.01[a]	n.d.	0
Di-hydroxybenzoic acid-hexoside isomer 1	0.14 ± 0.01[a]	3.51 ± 0.25[b]	2507.1	0.26 ± 0.01[c]	1.45 ± 0.09[d]	557.7	0.51 ± 0.04[e]	2.35 ± 0.12[f]	460.8
Di-hydroxybenzoic acid-hexoside isomer 2	0.03 ± 0.00[a]	0.08 ± 0.00[a]	266.7	0.05 ± 0.00[a]	0.05 ± 0.00[a]	100.0	0.07 ± 0.00[a]	0.07 ± 0.00[a]	100.0
Di-hydroxybenzoic acid-hexoside isomer 3	0.03 ± 0.00[a]	n.d.	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total hydroxybenzoic acids	**0.77 ± 0.02[a]**	**4.74 ± 0.27[b]**	**615.6**	**0.98 ± 0.02[c]**	**2.59 ± 0.10[d]**	**264.3**	**1.29 ± 0.04[e]**	**3.89 ± 0.14[f]**	**308.7**
Flavonols									
Quercetin	74.14 ± 3.61[a]	n.d.	0	50.01 ± 2.57[b]	23.98 ± 1.77[c]	48.0	56.59 ± 3.10[b]	0.38 ± 0.01	0.67
Quercetin-3-O-hexoside	n.d.	n.d.	n.d.	0.04 ± 0.00[a]	n.d.	0	0.04 ± 0.00[a]	n.d.	0
Quercetin-3-O-glucoside	0.96 ± 0.05[a]	0.61 ± 0.03[b]	63.5	0.79 ± 0.01[c]	1.90 ± 0.09[d]	240.5	1.87 ± 0.11[d]	1.76 ± 0.07	94.1
Quercetin-4′-O-glucoside	18.28 ± 1.03[a]	17.50 ± 1.11[a]	95.7	45.11 ± 1.80[c]	18.88 ± 1.21[a]	41.9	50.60 ± 3.24[b]	10.60 ± 0.81[c]	20.9
Quercetin-7-O-hexoside-4′-O-hexoside	0.03 ± 0.00[a]	0.17 ± 0.01[b]	566.7	0.14 ± 0.01[b]	0.17 ± 0.01[b]	121.4	0.22 ± 0.01[c]	0.07 ± 0.00[a]	31.8
Quercetin-3-O-glucoside-4′-O-glucoside	16.21 ± 0.99[a]	15.93 ± 0.99[a]	98.3	31.12 ± 1.51[b]	23.13 ± 1.56[c]	74.3	46.65 ± 2.39[d]	39.76 ± 2.09[e]	85.2
Quercetin-tri-O-hexoside	0.05 ± 0.00[a]	0.04 ± 0.00[a]	80.0	0.12 ± 0.00[b]	0.06 ± 0.00[a]	50.0	0.25 ± 0.02[c]	0.07 ± 0.00[a]	28.0
Quercetin-O-hexoside-O-malonyl-hexoside isomer 1	0.04 ± 0.00[a]	0.04 ± 0.00[a]	100.0	0.03 ± 0.00[a]	0.03 ± 0.00[a]	100.0	0.05 ± 0.00[a]	0.05 ± 0.00[a]	100.0
Quercetin-O-hexoside-O-malonyl-hexoside isomer 2	0.05 ± 0.01[a]	0.03 ± 0.00[a]	60.0	0.04 ± 0.00[a]	0.02 ± 0.00[a]	50.0	0.06 ± 0.00[a]	0.04 ± 0.00[a]	66.7
Quercetin-7-O-acetyl-hexoside	0.07 ± 0.00[a]	0.03 ± 0.00[a]	42.9	0.05 ± 0.00[a]	0.03 ± 0.00[a]	60.0	0.06 ± 0.00[a]	0.03 ± 0.00[a]	50.0
Quercetin-O-hexoside-O-acetyl-hexoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.01 ± 0.00	0.01 ± 0.00	100.0
Isorhamnetin	9.14 ± 0.52[a]	n.d.	0	8.66 ± 0.51[a]	8.30 ± 0.60[a]	95.8	9.78 ± 0.61[a]	n.d.	0
Isorhamnetin-3-O-hexoside	0.09 ± 0.00[a]	0.13 ± 0.01[b]	144.4	0.20 ± 0.01[c]	0.12 ± 0.01[b]	65.0	0.27 ± 0.01[d]	0.16 ± 0.01[b]	66.7
Isorhamnetin-4′-O-hexoside	5.63 ± 0.20[a]	3.49 ± 0.14[b]	62.0	9.73 ± 0.63[c]	5.48 ± 0.35[a]	56.3	5.84 ± 0.67[a]	2.68 ± 0.16[d]	45.9
Isorhamnetin-O-hexoside-O-pentoside	0.02 ± 0.00[a]	n.d.	0	0.03 ± 0.00[a]	0.02 ± 0.00[a]	66.7	0.02 ± 0.00[a]	0.02 ± 0.00[a]	100.0
Isorhamnetin-3-O-hexoside-4′-O-hexoside	0.40 ± 0.02[a]	0.73 ± 0.03[b]	182.5	1.43 ± 0.08[c]	1.38 ± 0.06[c]	96.5	1.40 ± 0.06[c]	1.04 ± 0.03[d]	74.3
Kaempferol-7-O-hexoside-isomer 1	0.35 ± 0.02[a]	n.d.	0	0.32 ± 0.02[a]	0.03 ± 0.00[b]	9.4	0.91 ± 0.07[c]	0.63 ± 0.04[d]	69.2
Kaempferol-7-O-hexoside-isomer 2	0.01 ± 0.00[a]	0.01 ± 0.00[a]	100.0	0.02 ± 0.00[a]	0.01 ± 0.00[a]	50.0	0.01 ± 0.00[a]	0.01 ± 0.00[a]	100.0
Kaempferol-3-O-hexoside	0.10 ± 0.00[a]	0.05 ± 0.00[b]	50.0	0.07 ± 0.00[b]	0.03 ± 0.00[a]	42.9	0.04 ± 0.00[a]	0.02 ± 0.00[a]	50.0
Kaempferol-O-hexoside-O-hexoside isomer 1	0.71 ± 0.05[a]	0.01 ± 0.00[b]	1.4	0.33 ± 0.02[c]	0.03 ± 0.00[b]	9.1	0.81 ± 0.06[a]	0.02 ± 0.00[b]	2.5
Kaempferol-O-hexoside-O-hexoside isomer 2	0.08 ± 0.00[a]	0.05 ± 0.00[b]	62.5	0.05 ± 0.00[b]	0.04 ± 0.00[b]	80.0	0.04 ± 0.00[b]	0.03 ± 0.00[b]	75.0
Total flavonols	**126.36 ± 3.92[a]**	**38.82 ± 1.50[b]**	**30.7**	**148.29 ± 3.58[c]**	**83.65 ± 2.74[d]**	**56.4**	**175.52 ± 5.16[e]**	**57.39 ± 2.25[f]**	**32.7**
Other flavonoids									
Epicatechin-O-hexoside isomer 1	0.02 ± 0.00[a]	n.d.	0	0.01 ± 0.00[a]	n.d.	0	0.01 ± 0.00[a]	n.d.	0
Epicatechin-O-hexoside isomer 2	0.01 ± 0.00[a]	n.d.	0	n.d.	n.d.	n.d.	0.01 ± 0.00[a]	n.d.	0

Table 1 (Contd.)

Compound	RRSO			FRSO			GRSO		
	Before digestion	After digestion	BI (%)	Before digestion	After digestion	BI (%)	Before digestion	After digestion	BI (%)
Epicatechin-O-hexoside isomer 3	0.01 ± 0.00^a	n.d.	0	n.d.	n.d.	n.d.	0.01 ± 0.00^a	n.d.	0
Taxifolin-O-hexoside isomer 1	0.06 ± 0.00^a	0.01 ± 0.00^b	16.7	0.09 ± 0.00^a	0.05 ± 0.00^a	55.6	0.02 ± 0.00^b	0.01 ± 0.00^b	50.0
Taxifolin-O-hexoside isomer 2	0.16 ± 0.01^a	n.d.	0	0.20 ± 0.01^a	0.08 ± 0.00^b	40.0	0.11 ± 0.01^b	n.d.	0
Taxifolin-O-hexoside isomer 3	0.11 ± 0.01^a	n.d.	0	0.11 ± 0.01^a	n.d.	0	0.15 ± 0.01^b	n.d.	0
Taxifolin-O-hexoside isomer 4	0.06 ± 0.00^a	0.01 ± 0.00^b	16.7	0.10 ± 0.01^c	0.04 ± 0.00^a	40.0	0.04 ± 0.00^a	0.01 ± 0.00^b	25.0
Total other flavonoids	$\mathbf{0.43 \pm 0.01^a}$	$\mathbf{0.02 \pm 0.00^b}$	**4.7**	$\mathbf{0.51 \pm 0.02^c}$	$\mathbf{0.17 \pm 0.00^d}$	**33.3**	$\mathbf{0.35 \pm 0.01^e}$	$\mathbf{0.02 \pm 0.00^b}$	**5.7**
Anthocyanins									
Cyanidin-3-O-hexoside isomer 1	1.86 ± 0.15^a	0.02 ± 0.00^b	1.1	1.40 ± 0.11^c	0.11 ± 0.01^d	7.9	2.11 ± 0.18^a	0.09 ± 0.01^d	4.3
Cyanidin-3-O-hexoside isomer 2	0.09 ± 0.01^a	0.05 ± 0.00^b	55.6	0.06 ± 0.00^b	0.02 ± 0.00^c	33.3	0.06 ± 0.00^b	0.02 ± 0.00^c	50.0
Peonidin-3-O-hexoside	0.13 ± 0.01^a	n.d.	0	0.19 ± 0.02^b	0.02 ± 0.00^c	10.5	0.16 ± 0.01^b	0.09 ± 0.00^d	56.3
Cyanidin-O-malonyl-hexoside	8.99 ± 0.74^a	0.19 ± 0.02^b	2.1	6.15 ± 0.51^c	0.86 ± 0.07^d	14.0	7.51 ± 0.48^c	0.43 ± 0.03^e	5.7
Peonidin-O-malonyl-hexoside	0.16 ± 0.01^a	0.01 ± 0.00^b	6.3	0.14 ± 0.01^a	0.03 ± 0.00^b	21.4	0.14 ± 0.01^a	0.01 ± 0.00^b	7.1
Cyanidin-O-hexoside-O-hexoside isomer 1	0.10 ± 0.01^a	0.01 ± 0.00^b	10.0	1.01 ± 0.09^c	n.d.	0	0.21 ± 0.02^d	0.03 ± 0.00^b	14.3
Cyanidin-O-hexoside-O-hexoside isomer 2	1.63 ± 0.12^a	0.03 ± 0.00^b	1.8	0.04 ± 0.00^b	0.09 ± 0.01^c	225.0	1.52 ± 0.11^a	0.07 ± 0.00^c	4.6
Cyanidin-O-hexoside-O-hexoside isomer 3	0.05 ± 0.00^a	0.05 ± 0.00^a	100.0	0.03 ± 0.00^a	0.03 ± 0.00^a	100.0	0.04 ± 0.00^a	0.03 ± 0.00^a	75.0
Cyanidin-O-hexoside-O-malonyl-hexoside isomer 1	n.d.	n.d.	n.d.	0.05 ± 0.00^a	0.01 ± 0.00^b	20.0	0.10 ± 0.01^c	0.01 ± 0.00^b	10.0
Cyanidin-O-hexoside-O-malonyl-hexoside isomer 2	3.49 ± 0.29^a	0.06 ± 0.00^b	1.7	1.55 ± 0.10^c	0.20 ± 0.01^d	12.9	2.58 ± 0.19^e	0.16 ± 0.01^d	6.2
Total anthocyanins	$\mathbf{16.50 \pm 0.82^a}$	$\mathbf{0.42 \pm 0.02^b}$	**2.5**	$\mathbf{10.62 \pm 0.54^c}$	$\mathbf{1.37 \pm 0.07^d}$	**12.9**	$\mathbf{14.43 \pm 0.56^a}$	$\mathbf{0.94 \pm 0.03^e}$	**6.5**
Total	$\mathbf{148.06 \pm 4.01^a}$	$\mathbf{45.26 \pm 1.52^b}$	**30.6**	$\mathbf{165.99 \pm 3.62^c}$	$\mathbf{92.05 \pm 2.75^d}$	**55.5**	$\mathbf{193.01 \pm 5.19^e}$	$\mathbf{63.21 \pm 2.25}$	**32.7**

Different superscript letters (a–c) within the same row mean significant different ($p < 0.05$) values; n.d. = not detected compound; n.f. = newly formed compound; RRSO = raw red-skinned onion; GRSO = grilled red-skinned onion; and FRSO = fried red-skinned onion.

tin, namely, quercetin-4′-O-glucoside and quercetin-3-O-glucoside-4′-O-glucoside were also present in an appreciable amount in RRSO, representing the 12.3% and 10.9% of total phenolic compounds, respectively. The second most important class of phenolic compounds in RRSO was represented by anthocyanins, which accounted for 11.2% of total phenolic compounds. A similar phenolic profile in RRSO was previously reported.[2,38]

Both applied thermal treatments induced an increase in the amount of extractable total phenolic compounds in RSO, mainly as a consequence of the matrix softening effect and the loss of water, which occurred during cooking, as previously suggested.[1,2] Specifically, grilling resulted in a 30.4% increase in total phenolic compounds, whereas the recorded increase was 12.1% in fried RSO. The increase in phenolic compounds in both the grilled (GRSO) and fried (FRSO) red-skinned onion is attributed to the increase in the concentration of quercetin-4′-O-glucoside and quercetin-3-O-glucoside-4′-O-glucoside. Conversely, the concentration of quercetin decreased in both GRSO and FRSO in comparison with the raw sample. Similar results were previously reported.[1,2,39,40]

Both grilling and frying also positively affected the release of onion phenolic compounds during *in vitro* gastro-intestinal digestion (Table 1). The highest amount of released phenolic compounds after *in vitro* digestion was found for the FRSO sample (92.05 ± 2.75 µmol per 100 g), which also showed the highest bioaccessibility index (55.5%). The RRSO and GRSO samples displayed a similar bioaccessibility index for total phenolic compounds (30.6% and 32.7%, respectively) although

in vitro digestion of GRSO resulted in a significantly higher amount of bioaccessible phenolic compounds compared to RRSO (63.21 ± 2.25 and 45.26 ± 1.52 µmol per 100 g, respectively).

The highest bioaccessibility index of FRSO was mainly due to the high bioaccessibility of quercetin (48.0%), which was present in a trace amount or not detected in the *in vitro* digested RRSO and GRSO. As previously suggested, the highest concentration of quercetin in *in vitro* digested FRSO compared to RRSO and GRSO may be due to the presence of oil, which favoured the solubility and stability of quercetin during digestion.[2,41] Similar behaviour was also observed for the aglycone isorhamnetin, which displayed a bioaccessibility index close to 100% in FRSO, whereas not detected in the digested RRSO and GRSO. Considering the behaviour of the other two most important flavonols, quercetin-3-O-glucoside-4′-O-glucoside was characterized by a high bioaccessibility index in all the samples (from 74.3% to 98.3% in FRSO and RRSO, respectively), whereas quercetin-4′-O-glucoside only showed a high bioaccessibility index in RRSO (Table 1). In all the RSO digested samples, the lowest bioaccessibility index was found for anthocyanins (from 2.5% in RRSO to 12.9% in FRSO), whereas the class of hydroxybenzoic acids was always characterized by a bioaccessibility index of 100%. This indicates the occurrence of the oxidative degradation of quercetin-mono-glucosides and quercetin during *in vitro* digestion, which resulted in the production of protocatechuic acid and di-hydroxybenzoic acid-hexosides.[1,42,43]

3.2. Red-skinned onion phenolic compound metabolism during *in vitro* colonic fermentation

The absorption and bioavailability of quercetin and glycosylated quercetin-derivatives have been fairly studied in the past few years.[44,45] In general, quercetin-mono-glucosides (such as quercetin-4′-O-glucoside and quercetin-3-O-glucoside) are quite well absorbed in the small intestine, reaching 50% of the ingested dose.[46] The absorption pathway involves the hydrolysis of the glucose moiety by brush-border glucosidases (such as lactase phlorizin hydrolase) and the absorption of the quercetin aglycone, which is sufficiently hydrophobic to cross the membrane of enterocytes by passive diffusion.[47] Differently, the bioavailability of quercetin aglycone is much lower than that of the glucoside-derivatives (about 20% of the ingested doses) mainly due to its low bioaccessibility and solubility in the intestinal fluid.[48] Therefore, most of the quercetin and its derivatives present in onion are supposed to reach the colon, where they are thoroughly metabolized by the colon microbiota. The fecal metabolism of quercetin (and its derivatives) involves deglycosylation reaction, followed by C-ring fission, which leads to the production of several low-molecular weight phenolic acids.[49,50] Although there are some *in vitro* studies on the elucidation of colonic quercetin metabolism, there are no studies in the literature on the investigation of the effect of cooking on the colonic metabolism of onion quercetin and the changes in the microbiota. Therefore, the *in vitro* digested RSO samples were further subjected to *in vitro* faecal fermentation and the generated metabolites were identified and quantified by means of high-resolution mass spectrometry at BL, T1 and EP of fermentation (Tables 2–4).

In all the RSO samples, the quercetin-glucosides and isorhamnetin-glucosides were rapidly hydrolysed to the corresponding aglycones given that they almost completely disappeared already at the BL of fermentation. These results are in agreement with previous studies that showed the complete hydrolysis of O-glucosides of flavonols in the first hours of colon fermentation.[39,51] In addition to quercetin and isorhamnetin aglycones, luteolin aglycone also appeared at the BL of fermentation in all the RSO samples. Given that luteolin aglycone and luteolin-derivatives were not identified in the RSO samples, we speculate that the luteolin aglycone was derived from quercetin dehydroxylation at the C3 position in the C-ring, as already suggested.[39] The concentration of quercetin and isorhamnetin aglycones strongly decreased at T1 of fermentation and quercetin was no longer detectable at the EP of fermentation (Tables 2–4).

The metabolite found at the highest concentration in all the RSO samples at the BL of fermentation was 3-(3′-hydroxyphenyl)propanoic acid, which represented 57.8%, 48.7% and 52.3% of the total metabolites in RRSO, GRSO and FRSO, respectively. The additional metabolites identified in an appreciable amount at the BL of fermentation were 3-(3′-hydroxyphenyl)acetic acid, 3,4-dihydroxybenzoic acid and 3-(3′,4′-dihydroxyphenyl)acetic acid. At the BL of fermentation, only quantitative differences in the amount of total metabolites were found among the samples. The highest concentration of metabolites was found in GRSO, followed by FRSO and RRSO. In all the RSO samples, the concentration of metabolites at the BL of fermentation strongly exceeded the amount of parent phenolic compounds identified in onion, suggesting the presence of a high amount of bound phenolic compounds in

Table 2 Changes in phenolic compound metabolite concentration during *in vitro* colonic fermentation of raw red-skinned onion (RRSO). Results are expressed in μmol of phenolic compound per 100 g of onion

Compound	Time points of colonic fermentation		
	BL	T1	EP
Hydroxybenzoic acid isomer 1	0.47 ± 0.02^a	1.18 ± 0.09^b	1.17 ± 0.11^b
Hydroxybenzoic acid isomer 2	n.d.	0.71 ± 0.05^a	0.70 ± 0.05^a
3-(3′-Hydroxyphenyl)acetic acid	7.51 ± 0.61^a	7.93 ± 0.59^a	12.49 ± 1.00^b
3-(4′-Hydroxyphenyl)acetic acid	n.d.	n.d.	n.d.
3,4-Dihydroxybenzoic acid	1.89 ± 0.11^a	1.98 ± 0.15^a	2.13 ± 0.11^a
Dihydroxybenzoic acid isomer 1	0.31 ± 0.01^a	n.d.	n.d.
Dihydroxybenzoic acid isomer 2	n.d.	n.d.	n.d.
3-(3′-Hydroxyphenyl)propanoic acid	183.31 ± 11.29^a	32.66 ± 2.36^b	39.50 ± 1.63^c
3-(4′-Hydroxyphenyl)propanoic acid	0.20 ± 0.01^a	2.69 ± 0.19^b	6.12 ± 0.48^c
4-Hydroxy-3-methoxybenzoic acid	0.21 ± 0.01^a	0.27 ± 0.02^b	0.29 ± 0.01^b
3-(3′,4′-Dihydroxyphenyl)acetic acid	0.68 ± 0.02^a	1.79 ± 0.16^b	3.05 ± 0.11^c
3′,4′-Dihydroxycinnamic acid	0.41 ± 0.02^a	0.27 ± 0.02^b	0.29 ± 0.02^b
(Dihydroxyphenyl)propanoic acid	0.22 ± 0.01^a	n.d.	0.05 ± 0.00^b
3-(3′,4′-Dihydroxyphenyl)propanoic acid	0.14 ± 0.01^a	1.25 ± 0.09^b	0.19 ± 0.01^c
3-(4′-Hydroxy-3′-methoxyphenyl)acetic acid	0.02 ± 0.00^a	0.02 ± 0.00^a	0.08 ± 0.00^b
3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid	0.68 ± 0.04^a	0.66 ± 0.05^a	1.16 ± 0.09^b
Luteolin	7.51 ± 0.51^a	3.96 ± 0.28^b	3.39 ± 0.21^b
Quercetin	96.41 ± 5.66^a	1.09 ± 0.10^b	n.d.
Isorhamnetin	17.39 ± 1.08^a	0.43 ± 0.03^b	0.20 ± 0.02^c
Total	317.36 ± 12.70^a	56.89 ± 2.47^b	70.81 ± 1.99^c

Different superscript letters (a–c) within the same row mean significant different ($p < 0.05$) values; n.d. = not detected compound; BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); and EP = endpoint (27.48 h).

Table 3 Changes in phenolic compound metabolite concentration during *in vitro* colonic fermentation of fried red-skinned onion (FRSO). Results are expressed in μmol of phenolic compound per 100 g of onion

Compound	Time points of colonic fermentation		
	BL	T1	EP
Hydroxybenzoic acid isomer 1	0.44 ± 0.03^a	1.24 ± 0.10^b	1.08 ± 0.09^b
Hydroxybenzoic acid isomer 2	0.11 ± 0.00^a	1.01 ± 0.10^b	1.09 ± 0.09^b
3-(3′-Hydroxyphenyl)acetic acid	7.83 ± 0.58^a	11.69 ± 0.99^b	12.84 ± 1.13^b
3-(4′-Hydroxyphenyl)acetic acid	n.d.	15.53 ± 0.81^a	8.58 ± 0.72^b
3,4-Dihydroxybenzoic acid	1.45 ± 0.11^a	6.83 ± 0.49^b	1.52 ± 0.11^a
Dihydroxybenzoic acid isomer 1	0.24 ± 0.02^a	n.d.	n.d.
Dihydroxybenzoic acid isomer 2	n.d.	n.d.	n.d.
3-(3′-Hydroxyphenyl)propanoic acid	170.43 ± 12.84^a	49.70 ± 2.92^b	44.20 ± 2.66^b
3-(4′-Hydroxyphenyl)propanoic acid	0.17 ± 0.01^a	4.13 ± 0.28^b	4.13 ± 0.36^b
4-Hydroxy-3-methoxybenzoic acid	0.19 ± 0.01^a	0.24 ± 0.02^a	0.19 ± 0.01^a
3-(3′,4′-Dihydroxyphenyl)acetic acid	1.16 ± 0.09^a	10.68 ± 0.61^b	14.40 ± 0.97^c
3′,4′-Dihydroxycinnamic acid	0.37 ± 0.01^a	0.41 ± 0.03^a	0.35 ± 0.03^a
(Dihydroxyphenyl)propanoic acid	0.29 ± 0.02^a	2.01 ± 0.11^b	0.61 ± 0.04^c
3-(3′,4′-Dihydroxyphenyl)propanoic acid	0.12 ± 0.00^a	0.09 ± 0.00^b	0.15 ± 0.01^c
3-(4′-Hydroxy-3′-methoxyphenyl)acetic acid	0.01 ± 0.00^a	0.11 ± 0.00^b	0.12 ± 0.01^b
3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid	0.13 ± 0.01^a	0.63 ± 0.05^b	n.d.
Luteolin	7.97 ± 0.60^a	7.62 ± 0.45^a	4.39 ± 0.31^b
Quercetin	121.16 ± 8.22^a	12.44 ± 1.02^b	n.d.
Isorhamnetin	37.99 ± 2.18^a	4.48 ± 0.36^b	1.58 ± 0.09^c
Total	350.06 ± 15.42^a	128.84 ± 3.41^b	95.23 ± 3.09^c

Different superscript letters (a–c) within the same row mean significant different ($p < 0.05$) values; n.d. = not detected compound; BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); and EP = endpoint (27.48 h).

Table 4 Changes in phenolic compound metabolite concentration during *in vitro* colonic fermentation of grilled red-skinned onion (GRSO). Results are expressed in μmol of phenolic compound per 100 g of onion

Compound	Time points of colonic fermentation		
	BL	T1	EP
Hydroxybenzoic acid isomer 1	0.47 ± 0.03^a	1.08 ± 0.08^b	1.15 ± 0.10^b
Hydroxybenzoic acid isomer 2	n.d.	1.14 ± 0.09^a	2.21 ± 0.12^b
3-(3′-Hydroxyphenyl)acetic acid	8.45 ± 0.78^a	11.86 ± 1.28^b	13.53 ± 0.97^b
3-(4′-Hydroxyphenyl)acetic acid	n.d.	n.d.	n.d.
3,4-Dihydroxybenzoic acid	1.74 ± 0.14^a	2.22 ± 0.11^b	2.12 ± 0.09^b
Dihydroxybenzoic acid isomer 1	0.99 ± 0.10^a	n.d.	n.d.
Dihydroxybenzoic acid isomer 2	0.31 ± 0.03^a	n.d.	n.d.
3-(3′-Hydroxyphenyl)propanoic acid	203.89 ± 11.64^a	76.01 ± 4.58^b	91.32 ± 6.64^c
3-(4′-Hydroxyphenyl)propanoic acid	0.20 ± 0.01^a	1.49 ± 0.09^b	1.88 ± 0.12^c
4-Hydroxy-3-methoxybenzoic acid	0.27 ± 0.02^a	0.27 ± 0.02^a	0.31 ± 0.02^a
3-(3′,4′-Dihydroxyphenyl)acetic acid	0.94 ± 0.08^a	2.56 ± 0.19^b	3.37 ± 0.19^c
3′,4′-Dihydroxycinnamic acid	0.40 ± 0.03^a	0.32 ± 0.02^b	0.29 ± 0.02^b
(Dihydroxyphenyl)propanoic acid	0.28 ± 0.02^a	1.85 ± 0.11^b	1.54 ± 0.10^c
3-(3′,4′-Dihydroxyphenyl)propanoic acid	0.15 ± 0.01^a	0.03 ± 0.00^b	0.02 ± 0.00^b
3-(4′-Hydroxy-3′-methoxyphenyl)acetic acid	0.02 ± 0.00^a	0.01 ± 0.00^a	0.01 ± 0.00^a
3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid	0.20 ± 0.01^a	n.d.	n.d.
Luteolin	7.81 ± 0.57^a	6.64 ± 0.46^b	5.97 ± 0.32^b
Quercetin	144.91 ± 7.31^a	8.50 ± 0.71^b	n.d.
Isorhamnetin	18.92 ± 1.04^a	1.23 ± 0.09^b	0.32 ± 0.03^c
Total	389.95 ± 13.82^a	115.21 ± 4.84^b	124.04 ± 6.73^b

Different superscript letters (a–c) within the same row mean significant different ($p < 0.05$) values; n.d. = not detected compound; BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); and EP = endpoint (27.48 h).

onion, which can be released from fiber or other macromolecules by the action of the gut bacteria.

At the end of the fermentation process, a decline in the concentration of phenolic metabolites was observed and GRSO once again was the sample releasing the highest amount of phenolic metabolites. In all the samples, 3-(3′-hydroxyphenyl) propanoic acid was still the metabolite present at the highest concentration although with a different incidence depending on the sample. In GRSO, 3-(3′-hydroxyphenyl)propanoic acid accounted for 73.6% of the total metabolites, whereas in FRSO and RRSO its incidence was lower (46.4% and 55.8%, respectively). In addition, the other important metabolites were found

in different proportions depending on the thermal treatment at the end of the fermentation process. RRSO was the richest in terms of percentage of incidence of 3-(3′-hydroxyphenyl) acetic acid (17.6% of total phenolic compounds) followed by FRSO (13.4% of total phenolic compounds) and GRSO (10.4% of total phenolic compounds). Moreover, in the FRSO sample, the metabolite 3-(3′,4′-dihydroxyphenyl)acetic acid was found in high proportion, representing the 15.1% of total metabolites, whereas it represented only 2.7% and 4.3% of the total metabolites in GRSO and RRSO, respectively. Generally, the three metabolites 3-(3′-hydroxyphenyl)propanoic acid, 3-(3′-hydroxyphenyl)acetic acid and 3-(3′,4′-dihydroxyphenyl)acetic acid tended to accumulate in a high amount in the fermented samples but with a different proportion depending on the sample. Accordingly, cooking may modulate the metabolite profile after fermentation both from a quantitative and qualitative point-of-view.

Fig. 1 displays the colonic microbiota metabolic pathway of red-skinned onion flavonols. The first step is the de-glycosylation of quercetin-glucosides and isorhamnetin-glucosides into the corresponding aglycones quercetin and isorhamnetin.[39,51] The appearance of luteolin, which was not present as aglycone or any derivatives, suggested the possible de-hydroxylation of quercetin.[39] The next step in the flavonols metabolism is ring fission, leading to the production of 3-(3′,4′-dihydroxyphenyl) propanoic acid in the case of quercetin and luteolin or 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid in the case of isorhamnetin.[49] Ring fission of quercetin may also result in the production of 3,4-dihydroxybenzoic acid.[52] The metabolic intermediate 3-(3′,4′-dihydroxyphenyl)propanoic acid may have different fates, as depicted in Fig. 1. The major metabolic pathway for this intermediate was dehydroxylation to 3-(3′-hydroxyphenyl)propanoic acid, which was the major metabolite found at any time in each RSO sample during fermentation.[10] Alternatively, 3-(3′,4′-dihydroxyphenyl)propanoic acid may undergo an α-oxidation step, producing 3-(3′,4′-dihydroxy-phenyl)acetic acid, which can be further de-hydroxylated to 3-(3′-hydroxyphenyl)acetic acid or subjected to a second α-oxidation step, releasing 3,4-dihydroxybenzoic acid.[13] Additional metabolic pathways involve the direct β-oxidation of 3-(3′,4′-dihydroxyphenyl)propanoic acid, producing 3,4-dihydroxybenzoic acid.[53] All these metabolites were found in a high amount in the fermented RSO samples, suggesting that the described pathways are predominant during the fermentation of flavonols. Regarding isorhamnetin metabolism, the C-ring fission product 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid may undergo β-oxidation or two steps of α-oxidation to produce 4-hydroxy-3-methoxybenzoic acid. Otherwise, 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid may be demethoxy-lated, producing 3-(4′-hydroxyphenyl)propanoic acid, which can subsequently be subjected to α-oxidation, generating 3-(4′-hydroxyphenyl)acetic acid.

However, all these metabolites were found in a low concentration in the fermented samples, suggesting a possible alternative metabolic pathways for 3-(4′-hydroxy-3′-methoxy-phenyl)propanoic acid. Indeed, this last intermediate may be demethylated, resulting in the formation of 3-(3′,4′-dihydroxy-phenyl)propanoic acid.[10,54,55]

Phenolic metabolites generated by the action of gut microbiota are generally considered to be better absorbed and responsible for the beneficial effects following the consumption of phenolic compound-rich food.[15] Several in vitro and in vivo studies demonstrated the biological potential of these metabolites. For example, 3-(3′-hydroxyphenyl)propanoic acid displayed in vitro anti-inflammatory and anti-diabetic activities.[55–57] Furthermore, 3-(3′-hydroxyphenyl)propanoic acid and 3-(3′-hydroxyphenyl)acetic acid exhibited a blood pressure-reducing effect ex vivo and in vivo.[58,59] Moreover, 3-(3′,4′-dihydroxyphenyl)acetic acid was reported to inhibit colon cancer cell proliferation more efficiently than 3-(3′-hydroxyphenyl)propanoic acid and 3-(3′-hydroxyphenyl)acetic acid.[60] Finally, all three metabolites showed neuroprotective activity in vitro.[61]

3.3. Shift of total bacteria and microbiota eubiosis

qPCR absolute quantification targeted 14 different bacterial taxa (Table S1†) related to the core microbiota, including total Eubacteria and the two main phyla of the human healthy colon, i.e., Bacteroidetes and Firmicutes. Considering the total Eubacteria, compared to the abundances at the baseline (BL) and besides the values of the blank control (BC), just RRSO at T1 and EP was significantly overrepresented up to 1.43 folds more at the EP ($p < 0.05$) (Table 5). Conversely, both samples of cooked onions, independently from the time points of fermentation, were underrepresented, in particular, GRSO had slight not significant reduction ($p > 0.05$), but FRSO had a 1.68 fold significant reduction. These evident results indicated that the cooked onion samples, similar to the BC, were limited or not able to support the growth of total colon Eubacteria. The quantification of the Bacteroidetes phylum showed significant changes at each time point for all the samples tested, including the BC, with respect to the BL ($p < 0.05$) (Table 5). In par-
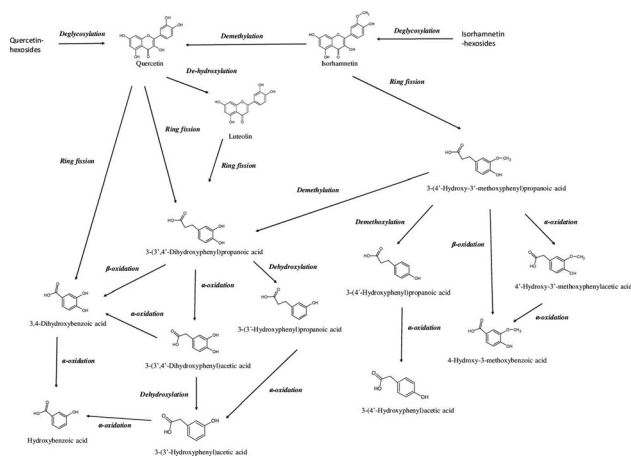

Fig. 1 Proposed metabolic pathways for phenolic compounds microbial metabolism after in vitro gastro-intestinal digestion and colonic fermentation of red-skinned onion.

Table 5 Absolute quantification and changes in the abundance of bacterial taxa of the core-microbiota of colonic fermentation of red-skinned onion by qPCR

Target & sample	Quantifications at baseline (cells per mL)		Changes in log₂(F/C)		MANOVA[a]	
	BL raw values[b]	BL ± SD[c]	T1	EP	p (time)	$-\log_{10}(p)$
Eubacteria						
RRSO	5.13×10^8	$7.42 \times 10^8 \pm 2.08 \times 10^{8\ b}$	0.47^{abA}	1.43^{aA}	0.001469	2.833075
GRSO	6.30×10^8	$7.42 \times 10^8 \pm 2.08 \times 10^{8\ a}$	-0.36^{abB}	-0.81^{bB}	0.000122	3.930721
FRSO	8.59×10^8	$7.42 \times 10^8 \pm 2.08 \times 10^{8\ a}$	-0.55^{abB}	-1.68^{bB}	0.000970	3.013253
BC	9.67×10^8	$7.42 \times 10^8 \pm 2.08 \times 10^{8\ a}$	-0.71^{aB}	-4.21^{bC}	0.002812	2.551055
p (food matrix)	0.726777		0.002947	0.000004		
Firmicutes						
RRSO	4.80×10^7	$5.25 \times 10^7 \pm 6.86 \times 10^{6\ b}$	0.66^{abA}	1.00^{aA}	0.029213	1.534484
GRSO	6.20×10^7	$5.25 \times 10^7 \pm 6.86 \times 10^6$	0.27^{B}	0.28^{B}	0.272088	0.565290
FRSO	4.69×10^7	$5.25 \times 10^7 \pm 6.86 \times 10^{6\ b}$	0.39^{aAB}	0.27^{aB}	0.000182	3.750942
BC	5.32×10^7	$5.25 \times 10^7 \pm 6.86 \times 10^{6\ b}$	0.44^{abA}	0.76^{aAB}	0.001091	2.962952
p (food matrix)	0.589376		0.000126	0.000002		
Bacteroidetes						
RRSO	2.37×10^8	$1.74 \times 10^8 \pm 6.44 \times 10^{7\ a}$	-1.36^{bA}	-1.54^{bA}	0.017556	1.755573
GRSO	2.21×10^8	$1.74 \times 10^8 \pm 6.44 \times 10^{7\ a}$	-6.05^{bC}	-5.94^{bB}	0.002734	2.563179
FRSO	1.05×10^8	$1.74 \times 10^8 \pm 6.44 \times 10^{7\ a}$	-6.30^{bC}	-6.44^{bB}	0.002727	2.564311
BC	1.35×10^8	$1.74 \times 10^8 \pm 6.44 \times 10^{7\ a}$	-3.46^{bB}	-4.16^{bB}	0.003134	2.503885
p (food matrix)	0.972251		0.000018	0.000003		

Upperscore letters (A–C) are for statistical difference within "food matrix" and lowerscore letters (a–c) are for statistical difference within "time ", according to Duncan's multiple range *post hoc* test ($p < 0.05$); $-\log_{10}(p)$ = significance of log₂(F/C); BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); EP = endpoint (27.48 h); RRSO = raw red skinned onion; GRSO = grilled red skinned onion; FRSO = fried red skinned onion; and BC = blank control. [a] Multivariate ANOVA model with category "time" on column and category "food matrix" on rows ($p < 0.05$). [b] BL raw values are the mean of sextuplicates from each sample at the beginning of its colonic fermentations. [c] BL ± SD is BL mean ± standard deviation and obtained from BL raw values.

ticular, fermentation of RRSO generated the lowest underrepresentation of *Bacteroidetes*, up to 1.54-fold less at the EP, and FRSO generated the highest underrepresentation, up to 6.44-fold less at the EP. In detail, FRSO brought a reduction of 4.18 times higher than that brought by RRSO. The relatively low loads of *Bacteroidetes* after FRSO fermentation indicate that this cooking process can particularly reduce the availability of fiber for fermentation, given that many genera of this phylum are fibrolytic.[62] Considering *Firmicutes* (Table 5), overrepresentations were seen for each sample at both time points of fermentation, although almost all not significantly with respect to the BL ($p > 0.05$). The sole time sample and time point that scored a slight increase, but significant in abundance was RRSO at the EP with just 1.00 log₂(FC). This latter increase was significantly higher than that of GRSO and FRSO at the EP ($p > 0.05$), but not than the BC ($p > 0.05$). These outputs indicate opposite scenarios depending on which group of *Firmicutes* was the most fostered, where the positive outlook could be due to the increase in *Lactobacillales*, while the negative due to the increase in *Clostridiaceae*.

As a general parameter for microbiota eubiosis, we chose the *Firmicutes/Bacteroidetes* (F/B) ratio (Table 6), where a value of over two is usually linked to microbiota dysbiosis.[8,10,63–66] The fecal samples at the baseline had an F/B ratio of 0.30 and this eubiosis condition was strongly maintained after 24 h just by RRSO (0.88), while the cooked onion samples failed. In particular, GRSO had a rise in the ratio up to 1.90, and FRSO overcame significantly with respect to the BL the dysbiotic value of 2.00 ($p < 0.05$). These results indicate that during the 24 h of

Table 6 *Firmicutes* to *Bacteroidetes* ratio (F/B) of colonic fermentation of red-skinned onion, by qPCR

Target & sample	*Firmicutes* to *Bacteroidetes* ratio (F/B)			MANOVA[a]
	BL ± SD[b]	T1	EP	p (time)
RRSO	0.30 ± 0.11^{b}	0.51 ± 0.45^{ab}	0.88 ± 0.77^{aC}	0.002296
GRSO	0.30 ± 0.11	1.64 ± 0.25	1.90 ± 0.69^{B}	0.230897
FRSO	0.30 ± 0.11^{b}	1.96 ± 0.90^{a}	2.05 ± 2.20^{aB}	0.004958
BC	0.30 ± 0.11^{c}	1.46 ± 0.15^{b}	4.08 ± 0.45^{aA}	0.016104
p (food matrix)	0.124932	0.328928	0.020129	

Upperscore letters (A–C) are for statistical difference within "food matrix" and lowerscore letters (a–c) are for statistical difference within "time", according to Duncan's multiple range *post hoc* test ($p < 0.05$). BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); EP = endpoint (27.48 h); RRSO = raw red skinned onion; GRSO = grilled red skinned onion; FRSO = fried red skinned onion; and BC = blank control. [a] Multivariate ANOVA model with category "time" on column and category "food matrix" on rows ($p < 0.05$). [b] BL ± SD is BL mean ± standard deviation and is obtained from BL raw values.

fermentation, just the raw onion samples did not perturb the colon core microbiota of the healthy donors and could provide a nutritionally adapted substrate. Oppositely, fermentation of FRSO triggered a dysbiosis condition, which mirrored the lower *Bacteroidetes* content.

3.4. Shift in selected beneficial bacterial taxa

To evidence the shifts in colon core microbiota, we quantified the abundance of selected bacterial taxa known to be active responders to fiber substrate fermentation and modulators of

Table 7 Absolute quantification and changes in the abundance of beneficial bacterial taxa of colonic fermentation of red-skinned onion, by qPCR

Targets & samples	Quantifications at baseline (cells per mL)		Changes in $\log_2$(F/C)		MANOVA[a]	
	BL raw values[b]	BL ± SD[c]	T1	EP	p (time)	$-\log_{10}(p)$
Lactobacillales						
RRSO	7.70×10^5	$8.79 \times 10^5 \pm 1.32 \times 10^5$ [b]	1.05^a	1.90^{aA}	0.014742	1.831452
GRSO	1.05×10^6	$8.79 \times 10^5 \pm 1.32 \times 10^5$ [b]	0.46^{ab}	0.97^{aAB}	0.000859	3.065816
FRSO	7.81×10^5	$8.79 \times 10^5 \pm 1.32 \times 10^5$	0.38	1.03^{AB}	0.084676	1.072238
BC	9.16×10^5	$8.79 \times 10^5 \pm 1.32 \times 10^5$	−0.49	-0.65^B	0.924597	0.034047
p (food matrix)	0.709415		0.369291	0.004476		
Bifidobacteriaceae						
RRSO	1.22×10^5	$5.08 \times 10^4 \pm 5.24 \times 10^4$ [a]	-5.17^{bC}	-5.06^{bC}	0.031567	1.500769
GRSO	5.77×10^4	$5.08 \times 10^4 \pm 5.24 \times 10^4$ [a]	-5.11^{bC}	-5.26^{bC}	0.012853	1.891000
FRSO	1.55×10^4	$5.08 \times 10^4 \pm 5.24 \times 10^4$ [a]	-2.40^{bB}	-3.27^{bB}	0.000041	4.388389
BC	7.85×10^3	$5.08 \times 10^4 \pm 5.24 \times 10^4$	0.19^A	0.28^A	0.777184	0.109475
p (food matrix)	0.683959		0.000962	0.014112		
Bacteroides–Prevotella–Porphyromonas (BPP) group						
RRSO	2.34×10^7	$2.09 \times 10^7 \pm 3.41 \times 10^6$ [a]	-0.34^{bA}	-0.98^{bA}	0.001932	2.714073
GRSO	2.39×10^7	$2.09 \times 10^7 \pm 3.41 \times 10^6$ [a]	-3.45^{bB}	-3.17^{bB}	0.000005	5.324022
FRSO	1.95×10^7	$2.09 \times 10^7 \pm 3.41 \times 10^6$ [a]	-2.58^{bB}	-3.69^{bB}	0.000008	5.081399
BC	1.67×10^7	$2.09 \times 10^7 \pm 3.41 \times 10^6$	-1.32^{bA}	-1.10^{bA}	0.015634	1.805925
p (food matrix)	0.509081		0.000009	0.033771		
Clostridium IV (CLOS IV) group						
RRSO	1.25×10^7	$1.79 \times 10^7 \pm 6.42 \times 10^6$	0.25^B	5.65^A	0.072745	1.138197
GRSO	2.72×10^7	$1.79 \times 10^7 \pm 6.42 \times 10^6$ [b]	1.76^{aA}	2.53^{aB}	0.012853	1.891000
FRSO	1.57×10^7	$1.79 \times 10^7 \pm 6.42 \times 10^6$ [b]	1.16^{bA}	2.99^{aB}	0.000511	3.291724
BC	1.61×10^7	$1.79 \times 10^7 \pm 6.42 \times 10^6$ [a]	-2.28^{bC}	-1.35^{bC}	0.021311	1.671394
p (food matrix)	0.643268		0.008103	0.009921		
Bifidobacterium longum						
RRSO	1.76×10^6	$2.53 \times 10^6 \pm 1.91 \times 10^6$ [a]	-0.17^{Ab}	-0.69^{Bb}	0.014742	1.831452
GRSO	5.04×10^6	$2.53 \times 10^6 \pm 1.91 \times 10^6$ [b]	0.39^{abA}	0.71^{aA}	0.000859	3.065816
FRSO	5.25×10^5	$2.53 \times 10^6 \pm 1.91 \times 10^6$	0.04^A	0.39^A	0.084676	1.072238
BC	2.78×10^6	$2.53 \times 10^6 \pm 1.91 \times 10^6$	-2.59^B	-3.55^C	0.924597	0.034047
p (food matrix)	0.036929		0.042285	0.004473		
Akkermansia muciniphila						
RRSO	6.04×10^5	$5.26 \times 10^5 \pm 8.23 \times 10^4$ [b]	0.51^{aA}	0.81^{aA}	0.000201	3.695973
GRSO	5.85×10^5	$5.26 \times 10^5 \pm 8.23 \times 10^4$ [b]	0.45^{abA}	0.95^{aA}	0.000181	3.743186
FRSO	4.80×10^5	$5.26 \times 10^5 \pm 8.23 \times 10^4$ [a]	0.06^{aA}	-0.53^{bAB}	0.018797	1.725910
BC	4.34×10^5	$5.26 \times 10^5 \pm 8.23 \times 10^4$ [a]	-4.47^{bB}	-3.88^{bB}	0.000004	5.370729
p (food matrix)	0.645485		<0.000001	<0.000001		
Faecalibacterium prausnitzii						
RRSO	1.06×10^7	$2.22 \times 10^7 \pm 7.79 \times 10^6$	0.59^A	0.93^A	0.272796	0.564162
GRSO	2.52×10^7	$2.22 \times 10^7 \pm 7.79 \times 10^6$	-0.32^B	-1.60^B	0.098563	1.006284
FRSO	2.59×10^7	$2.22 \times 10^7 \pm 7.79 \times 10^6$ [a]	-1.45^{bBC}	-2.50^{bB}	0.019097	1.719023
BC	2.71×10^7	$2.22 \times 10^7 \pm 7.79 \times 10^6$ [a]	-1.82^{bC}	-4.74^{cC}	0.011480	1.940064
p (food matrix)	0.300806		0.001150	0.007717		

Upperscore letters (A–C) are for statistical difference within "food matrix" and lowerscore letters (a–c) are for statistical difference within "time", according to Duncan's multiple range *post hoc* test ($p < 0.05$). $-\log_{10}(p)$ = significance of $\log_2$(F/C); BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); EP = endpoint (27.48 h); RRSO = raw red skinned onion; GRSO = grilled red skinned onion; FRSO = fried red skinned onion; and BC = blank control. [a] Multivariate ANOVA model with category "time" on column and category "food matrix" on rows ($p < 0.05$). [b] BL raw values are the means of sextuplicates from each sample at the beginning of its fermentation. [c] BL ± SD is BL mean ± standard deviation and is obtained from BL raw values.

host wellbeing (Table 7). The first set was related to beneficial microbes, while the second set was related to opportunistic and dysbiotic microbes. *Lactobacillales* and *Bifidobacteriaceae* with the exceptions of few species are related to the host's well-being and eubiosis of the colon microbiota.[67] *Lactobacillales* also include *Lactobacillaceae* and *Enterococcaceae*. These three families are implicated in the biotransformation of phenolic compounds, for example, *Lactobacillaceae* can increase the content of epicatechin as well as gallic, caffeic, and ferulic acids[68] and *Enterococcaceae* can promote *O*-deglycosylation and C-ring fission of several flavonols,[17] whereas *Bifidobacteriaceae* can metabolize homovanillic acids[69] and hydrolyze chlorogenic acids.[70] However, in our experiments, only the class of *Lactobacillales* was fostered by any onions fermentation, while targets such *Bifidobacteriaceae* and also specific *Bifidobacterium longum* underwent a large depletion (Table 7). In detail, the *Lactobacillales* were overrepresented in any sample, and still the raw onions almost doubled in abundance than both cooked onions. Thus, all the onion substrates once fermented by the colon microbiota increased the abundance of *Lactobacillales* significantly or not with respect to the BL and the BC. Similarly, in the case of long-term fermentation of phenolic compounds on gut models, other authors found that *Enterococcaceae* was the most fostered family.[34,71]

Alternatively, according to our results, *Bifidobacteriaceae* and the *B. longum* taxa were not fostered by any of the onion

substrates. In particular, the fact that a significant reduction with respect to the BL was achieved only on the onion substrates and not in the BC indicates that the high flavonol content of the samples somehow inhibited the sensitive taxon of *Bifidobacteriaceae* (Table 7). It has been reported that quercetin is inhibitory with respect to bifidobacteria,[72] while other studies in similar *in vitro* environments reported that of this vital group was fostered.[50] This scenario on beneficial core microbiota suggests that *Lactobacillales* are more specific in metabolizing onion flavonol compounds than *Bifidobacteriaceae*, and the latter group is more sensible to some detrimental compounds generated by the pyrolysis of cooked foods. It was reported that *Bifidobacteriaceae* are somehow inhibited by a certain class of phenolic compounds, *e.g.*, that from black tea[71] rather than others, *e.g.* that from olives.[8] Additionally, it is known that *Lactobacillales* can resist a high content of phenolic compounds[73] because they possess an enzymatic arsenal with a wide target range, enabling the de-esterification, hydrolysis, or conversion of phenolics to individual acids (gallic, quinic, caffeic, *p*-coumaric, ferulic, dihydrocaffeic, dihydroferulic acid, vinylcatechol, and vinylguaiacol).[73,74] Also, Date and colleagues[75] reported that onion can foster the growth of three probiotic lactobacilli after *in vitro* fermentation with fecal inoculum. Among the beneficial taxa, *Clostridium* group IV and *Faecalibacterium prausnitzii* are reported to be involved in the biotransformation of phenolic compounds.[76] According to our results (Table 7), *Clostridium* group IV increased significantly at the EP in all the onion fermentations, but not in the BC. However, its resident *F. prausnitzii* was only fostered by the raw onions and limited by the cooked samples, even if less than twice than the BC.

The *Clostridium* group IV and its resident *F. prausnitzii* are important SCFA producers and their presence is associated with a healthy gut. A decrease in the amount of these bacteria is observed in patients with inflammatory bowel disease.[77] Consistent with our results, these taxa were reported to increase after a dietary clinical trial with phenolic compounds from red wine.[78,79]

Lastly, *Akkermansia muciniphila* has been reported to be elevated in the presence of phenolic compounds, similar to some phenolic compounds acting as prebiotics.[16] According to our results (Table 7), this taxon was fostered both at T1 and at EP by the RRSO and GRSO substrates but not significantly, while it was limited at the EP by both FRSO and by BC, which in comparison three times the strength by the latter. Another commensal group that is involved mainly in fiber degradation is the *Bacteroides–Prevotella* group, covered by the BPP (*Bacteroides–Prevotella–Porphyromonas*) target. Generally, these bacteria are sensible to both fibers[80] and phenolic compounds. For example, Tamargo and colleagues[34] observed a significant increase in this group after phenolic compound fermentation on a gut model. Alternatively, according to our results, but in line with our findings about the superior phylum of *Bacteroidetes*, the BPP group was significantly underrepresented at the end of all the fermentations, in a strength larger than the BC (Table 7). These results can be

applied as indicators of health-related effects when RSO is consumed and can be translated to the *in vivo* condition.

3.5. Shift in selected opportunistic bacterial taxa

Considering the opportunistic bacterial taxa, we targeted *Enterobacteriaceae*, *Clostridium* group I, the *Atopobium–Collinsella–Eggerthella* (ATOP) group, and *Escherichia coli* (Table 8). Within these groups, we found the most important features on the modulation of gut microbiota by onion fermentation. Indeed, *Enterobacteriaceae* at both time points were inhibited just by RRSO, but the other samples limited their growth more than that in the BC. A similar trend was also detected by the resident *Escherichia coli* taxon. *Clostridium* group I was reduced almost in all cases at any time point, except for the BC, but significantly just for RRSO at the EP ($p < 0.05$). Lastly, considering the ATOP group, slight increases were observed just in the BC, while the onion samples were able to largely reduce the ATOP group loads up to growth inhibition due to the mean reduction of 4.53-fold less for any of the onion substrates with respect to the BL. Additionally, the RRSO and GRSO were better performers in action than FRSO. These groups engaged in the biotransformation of phenolic compounds because *Atopobium parvulum*, for example, possess tannases,[81] while *Atopobium* spp. is correlated with protocatechuic acid production after colonic fermentation of berry phenolic compounds.[82] However, according to our results, this scenario is oppositely described, giving the onion samples high capability in opposing opportunistic bacteria, such as *Atopobium* and *Eggerthella*. This feature is of high relevance given that it indicates that the consumption of RSO can protect the intestinal tract from colonization by opportunistic bacteria and pathogens, limiting possible dysbiosis.

3.6. qPCR prebiotic index

According to the results for qPI (Table 9), the fermented substrate with the best prebiotic activity at the EP of fermentation was RRSO, followed by GRSO and FRSO. Thus, FRSO had the lowest prebiotic index, except for that of the negative control (BC), although there were no significant differences among RSO samples. The different prebiotic indices of the samples can be attributed to the degradation of the structural composition of the onion fibers after cooking or transformation of the onion flavanols with prebiotic capacity. Similar to most known prebiotics (*e.g.*, fructooligosaccharides), it is recognized that soluble fibers are excellent substrates for the production of postbiotics (*e.g.*, short chain fatty acids) in the large intestine.[24] Additionally, in line with the latest definition of prebiotics,[83] there is evidence that some phenolics can foster the growth of beneficial bacteria, thus possessing a prebiotic nature.[16] Anyhow, the prebiotic potential generated by any of the onion samples can be ascribed to the reduction and limitation of opportunistic growth rather than the fostering of beneficial bacteria. A previous study documented the prebiotic potential of onion, which could foster the growth of three main beneficial lactobacilli in just 12 h of faecal *in vitro* fermentation.[75] Therefore, the relevance of our results is the con-

Table 8 Absolute quantification and changes in the abundance of opportunistic bacterial taxa of colonic fermentation of red-skinned onion by qPCR

Targets & sample	Quantifications at baseline (cells per mL)		Changes in $\log_2$(F/C)		MANOVA[a]	
	BL raw values[b]	BL ± SD[c]	T1	EP	p (time)	$-\log_{10}(p)$
Enterobacteriaceae						
RRSO	1.42×10^6	$1.44 \times 10^6 \pm 2.98 \times 10^5$	-0.69^B	-0.87^B	0.199488	0.700083
GRSO	1.81×10^6	$1.44 \times 10^6 \pm 2.98 \times 10^{5\ b}$	0.90^{aAB}	0.72^{aAB}	0.014941	1.825625
FRSO	1.08×10^6	$1.44 \times 10^6 \pm 2.98 \times 10^{5\ b}$	1.13^{abAB}	1.84^{aAB}	0.000012	4.914587
BC	1.44×10^6	$1.44 \times 10^6 \pm 2.98 \times 10^{5\ b}$	2.61^{aA}	3.62^{aA}	0.000011	4.956323
p (food matrix)	0.977079		<0.000001	<0.000001		
Clostridium group I (CLOS I)						
RRSO	8.91×10^3	$1.51 \times 10^4 \pm 9.51 \times 10^3$	0.11	-1.35	0.142556	0.700083
GRSO	5.19×10^3	$1.51 \times 10^4 \pm 9.51 \times 10^3$	-0.12	-0.02	0.844734	1.825625
FRSO	2.17×10^4	$1.51 \times 10^4 \pm 9.51 \times 10^{3\ a}$	-0.10^b	-0.81^b	0.038646	4.914587
BC	2.46×10^4	$1.51 \times 10^4 \pm 9.51 \times 10^{3\ b}$	0.16^b	1.09^a	0.033585	4.956323
p (food matrix)	0.262495		0.084737	0.086195		
Atopobium–Collinsella–Eggerthella (ATOP) group						
RRSO	1.22×10^5	$5.08 \times 10^4 \pm 5.24 \times 10^4$	-5.17^B	-5.06^B	0.072155	1.141731
GRSO	5.77×10^4	$5.08 \times 10^4 \pm 5.24 \times 10^4$	-5.11^B	-5.26^B	0.071840	1.143634
FRSO	1.55×10^4	$5.08 \times 10^4 \pm 5.24 \times 10^4$	-2.40^B	-3.27^B	0.079729	1.098386
BC	7.85×10^3	$5.08 \times 10^4 \pm 5.24 \times 10^4$	0.19^A	1.18^A	0.152951	0.815447
p (food matrix)	0.046520		0.000043	<0.000001		
Escherichia coli						
RRSO	8.70×10^2	$5.25 \times 10^3 \pm 5.37 \times 10^3$	0.30^B	0.02^C	0.829838	0.081006
GRSO	4.43×10^2	$5.25 \times 10^3 \pm 5.37 \times 10^3$	0.55^B	1.61^B	0.279017	0.554369
FRSO	1.09×10^4	$5.25 \times 10^3 \pm 5.37 \times 10^3$	2.81^{AB}	2.22^B	0.093314	1.030053
BC	8.82×10^3	$5.25 \times 10^3 \pm 5.37 \times 10^{3\ b}$	3.92^{aA}	4.57^{aA}	0.005090	2.293282
p (food matrix)	0.040281		0.000067	0.001695		

Upperscore letters (A–C) are for statistical difference within "food matrix" and lowerscore letters (a–c) are for statistical difference within "time", according to Duncan's multiple range *post hoc* test ($p < 0.05$); $-\log_{10}(p)$ = significance of $\log_2$(F/C); BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); EP = endpoint (27.48 h); RRSO = raw red skinned onion; GRSO = grilled red skinned onion; FRSO = fried red skinned onion; and BC = blank control. [a] Multivariate ANOVA model with category "time" on column and category "food matrix" on rows ($p < 0.05$). [b] BL raw values are the means of sextuplicates from each sample at the beginning of its fermentations. [c] BL ± SD is BL mean ± standard deviation and is obtained from BL raw values.

Table 9 qPI (quantitative PCR prebiotic index) of colonic fermentation of red-skinned onion

Sample	qPCR prebiotic index (qPI)[a]		MANOVA[b]
	T1	EP	p (time)
RRSO	0.821 ± 0.01^{bAB}	0.932 ± 0.05^{aA}	<0.000001
GRSO	0.840 ± 0.07^A	0.857 ± 0.041^{AB}	0.431179
FRSO	0.829 ± 0.09^A	0.814 ± 0.011^{AB}	0.084396
BC	0.749 ± 0.014^{aB}	0.664 ± 0.291^{bB}	0.000047
p (food matrix)	<0.000001	0.000002	

Upperscore letters (A–C) are for statistical difference within "food matrix" and lowerscore letters (a–c) are for statistical difference within "time", according to Duncan's multiple range *post hoc* test ($p < 0.05$); BL = baseline (3.48 h); T1 = intermediate time point (21.48 h); EP = endpoint (27.48 h); RRSO = raw red skinned onion; GRSO = grilled red skinned onion; FRSO = fried red skinned onion; and BC = blank control. [a] Normalized means. [b] Multivariate ANOVA model with category "time" on column and category "food matrix" on rows ($p < 0.05$).

firmation of onion prebiotic activity even after cooking and intestinal digestion.

3.7. Correlations among phenolics and microbial taxa

Spearman rank correlations ($p < 0.05$), two-joining-way heatmaps, and Pearson cluster analysis were performed by comparison of two different normalized datasets, each derived from values of absolute quantification (microbes and phenolic compounds) (Fig. 2). According to the left-side dendrograms, two main clusters were acknowledged. Subsequently, the two key clusters were sorted in two different subgroups obtained by the dendrograms related to phenolics. In the resulting joint cluster 1A, the most significant positive correlations were held by *Bacteroides* and associated BPP group, by *Firmicutes* and associated *Clostridium* group IV and associated *F. prausnitzii*, with respect to five different phenolics. All these taxa shared significant correlations with two common variables, *i.e.* hydroxybenzoic acid isomer 1 and 3-(4′-hydroxy-3′-methoxyphenyl) propanoic acid. In cluster 1B, the same phenolics variables had negative correlations, but the overall significance was small. In particular, ATOP had significant negative correlations towards 4-hydroxy-3-methoxybenzoic acid, *E. coli* towards hydroxybenzoic acid isomer 1 and 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid, and *Enterobacteriaceae* towards the former two and 3-(3′,4′-dihydroxyphenyl)propanoic acid. Suggestions indicate that all these metabolites putatively produced by beneficial *F. prausnitzii* play a central role in the containment of opportunistic taxa, as in the RRSO samples. In this regard, our outputs are consistent with a recent result demonstrating the antibiofilm and antiproliferative action of hydroxybenzoic acids *versus E. coli* and other *Enterobacteriaceae*.[84] Also, in the
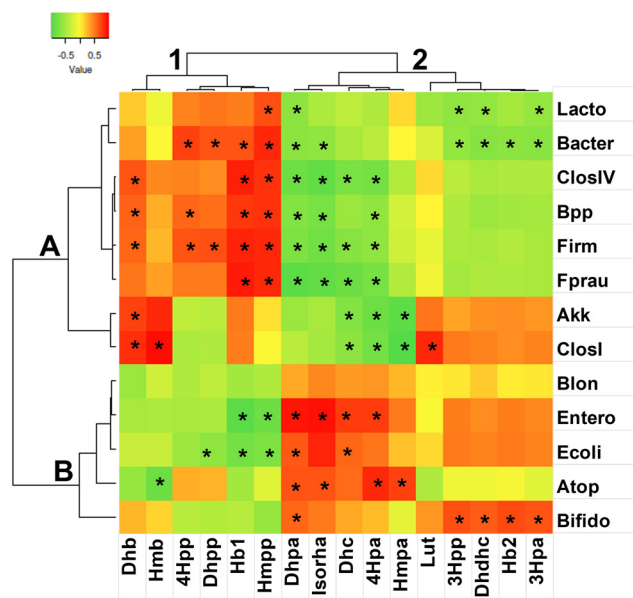
Fig. 2 Two-way joining heatmap of Spearman-rank correlations. (A, B, 1 and 2) Cluster labels from Pearson dendrograms with centroid linkage. (*) Significant correlations ($p > 0.05$). *X* axis labels: Hb1 = hydroxybenzoic acid isomer 1; Hb2 = hydroxybenzoic acid isomer 2; 3Hpa = 3-(3′-hydroxyphenyl)acetic acid; 4Hpa = 3-(4′-hydroxyphenyl)acetic acid; Dhb = 3,4-dihydroxybenzoic acid; 3Hpp = 3-(3′-hydroxyphenyl)propanoic acid; 4Hpp = 3-(4′-hydroxyphenyl)propanoic acid; Hmb = 4-hydroxy-3-methoxybenzoic acid; Dhpa = 3-(3′,4′-dihydroxyphenyl)acetic acid; Dhc = 3′,4′-dihydroxycinnamic acid; Dhdhc = di-hydro-dihydroxycinnamic acid; Dhpp = 3-(3′,4′-dihydroxyphenyl)propanoic acid; Hmpa = 3-(4′-hydroxy-3′-methoxyphenyl)acetic acid; Hmpp = 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid; Lut = luteolin; Isorha = isorhamnetin. *Y* label; Lacto = *Lactobacillales*; Bacter = *Bacteroidetes*; ClosIV = *Clostridium* IV group; BPP = *Bacteroides–Prevotella–Porphyromonas* group; Firm = *Firmicutes*; Fprau = *Faecalibacterium prausnitzii*; Akk = *Akkermansia muciniphila*; ClosI = *Clostridium* group I; Blon = *Bifidobacterium longum*; Entero = *Enterobacteriaceae*; Ecoli = *Escherichia coli*; ATOP = *Atopobium–Collinsella–Eggerthella* group; Bifido = *Bifidobacteriaceae*.

literature, it has been reported that species from *Clostridium* group IV can hydrolyze ester bonds, C-ring cleavage, and dehydroxylation of anthocyanins and proanthocyanidins, releasing hydroxybenzoic acids.[75]

Cluster 2 accounted for a higher number of significant negative correlations compared to cluster 1. Indeed, subcluster 2A was defined by nine variables with significant negative correlations differently shared among seven bacterial variables. In particular, *Firmicutes*, *Clos* IV group and *F. prausnitzii* were negatively correlated with four common variables, namely, 3-(3′,4′-dihydroxyphenyl)acetic acid, isorhamnetin, 3′,4′-dihydroxycinnamic acid, and 3-(4′-hydroxyphenyl)acetic acid. Conversely, in subcluster 2B, the four former phenolics showed positive correlations with respect to the family *Enterobacteriaceae*, and two of these molecules (3-(3′,4′-dihydroxyphenyl)acetic acid and 3′,4′-dihydroxycinnamic acid) also with respect to *E. coli*. It is possible that 3′,4′-dihydroxycinnamic acid and 3-(4′-hydroxyphenyl)acetic acid produced or transformed by *Enterobacteriaceae* could be responsible for the

depletion of *A. muciniphila* during the fermentation of the FRSO samples. Even if it is generally appreciated in the literature that most phenolics protect the gut epithelial barrier function and foster *A. muciniphila* and other beneficial microbes,[85,86] it has also been reported that cinnamic compounds, *e.g.*, cinnamaldehyde, produce side effects on commensals of the gut microbiota.[87]

4. Conclusions

Heat treatment increased the extractability of red-skinned onion phenolic compounds and their release during *in vitro* gastro-intestinal digestion. The grilled and fried red-skinned onions presented the highest amount of phenolic compounds following *in vitro* gastro-intestinal digestion and colonic fermentation. Additionally, specific beneficial taxa of the colon microbiota used in these experiments could transform and modify the chemical structure of onion phenolic compounds employing specific metabolic pathways, consequently gaining energy to multiply and foster in the colon niche. The degradation driven by the colon microbiota of RSO flavonols resulted in the accumulation of three main metabolites, *i.e.*, 3-(3′-hydroxyphenyl)propanoic acid, 3-(3′-hydroxyphenyl)acetic acid and 3-(3′,4′-dihydroxyphenyl)acetic acid, with some qualitative and quantitative differences among the samples.

Colonic fermentation of raw onions resulted in a substantial increase in beneficial taxa, which was larger compared to the heat-treated onions, particularly for *Lactobacillales* and beneficial clostridia. Also, a higher level of inhibition of opportunistic bacteria was seen for the raw onion samples, namely, the *Clostridium perfringens* group and *E. coli*. Eventually, the high flavonol content of the onion raw samples was found to be responsible for this effect, and also negatively seen for the depletion of *Bifidobacteriaceae*, which has been reported to be sensitive to quercetin, slowing metabolic pathways and cell wall synthesis.[50]

Therefore, consuming raw onions is preferable compared to grilled or fried onions in terms of amount of bioaccessible phenolic compounds and metabolites, which also have prebiotic activity fostering the growth of beneficial colonic bacteria and restraining the growth of opportunistic bacteria. Also, the cooked onions failed to maintain an eubiotic condition in the colon, triggering an increase in the F/B ratio to the level of a dysbiotic situation. The results reported in the present study showed that red-skinned onion, and especially raw, is an excellent dietary source of flavonols that are strongly metabolized by gut bacteria and can modulate the gut microbiota, promoting the growth of bacterial groups associated with a healthy status. However, more *in vivo* studies are necessary to validate the observed *in vitro* effect.

Institutional review board statement

The study was conducted according to the guidelines of the ethics procedures required at the University of Bologna.

Informed consent statement

Informed consent has been obtained from the patient(s) to publish this paper.

Data availability

Upon request to the corresponding author.

Author contributions

Conceptualization, L. N., D. T., and A. G.; methodology, A. C., L. N., D. T., and A. G.; software, L. N., F. C., and A. C.; validation, L. N., D. T. and A. G.; formal analysis, A. C., F. C. and L. N.; investigation, A. C., L. N., F. C., D. T. and A. G.; resources, D. T., and A. G.; data curation, A. C., L. N., F. C., D. T., and A. G.; writing—original draft preparation, A. C., L. N., F. C., D. T., and A. G.; writing—review and editing, A. C., L. N., F. C., D. T., and A. G.; visualization A. C., L. N., F. C.; supervision, L. N., D. T., and A. G.; project administration, D. T. and A. G.; funding acquisition, D. T. and A. G. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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