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1 METABOLIC FAECAL FINGERPRINTING OF *TRANS*-RESVERATROL AND QUERCETIN ON A HIGH-
2 FAT SUCROSE DIETARY MODEL USING LIQUID CHROMATOGRAPHY COUPLED TO HIGH-
3 RESOLUTION MASS SPECTROMETRY

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20 **Abstract**

21 Faecal non-targeted metabolomics deciphers metabolic end-products resulting from the
22 interactions among food, host genetics, and gut microbiota. Faeces from Wistar rats fed a
23 high-fat sucrose (HFS) diet supplemented with *trans*-resveratrol and quercetin (separately or
24 combined) were analysed by liquid chromatography coupled to high-resolution mass
25 spectrometry (LC-HRMS). Metabolomics in faeces categorised four clusters based on the type
26 of treatment. Tentative identification of significantly differing metabolites highlighted the
27 presence of carbohydrate derivatives or conjugates (3-phenylpropyl glucosinolate and dTDP-D-
28 mycaminose) in quercetin group. *Trans*-resveratrol group was differentiated by compounds
29 related to nucleotides (uridine monophosphate and 2,4-dioxotetrahydropyrimidine D-
30 ribonucleotide). Marked associations between bacterial species (*Clostridium* genus) and the
31 amount of some metabolites were identified. Moreover, *trans*-resveratrol and resveratrol-
32 derived microbial metabolites (dihydroresveratrol and lunularin) were also identified.
33 Accordingly, this study confirms the usefulness of omics-based techniques to discriminate
34 individuals depending on the physiological effect of food constituents and represents an
35 interesting tool to assess the impact of future personalized therapies.

36 **Keywords**: polyphenols; untargeted metabolomics; candidate metabolites; gut bacteria;
37 *Clostridium*.

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38 **Introduction**

39 Protective effects of polyphenols occurring in grapes and fruits-derived products have been
40 reported against diverse metabolic diseases, including non-alcoholic fatty liver disease,
41 cardiovascular disease, obesity, metabolic syndrome and cancer.[1-3] In this context, plant
42 secondary metabolites, such as the stilbene resveratrol and the flavonol quercetin, have
43 attracted much scientific attention[4,5] because of their potential use as bioactive molecules or
44 nutraceuticals.[6] Nevertheless, in order to understand physiological effects of bioactive
45 constituents, the identification of biomarkers of effect clarifying the contribution of
46 polyphenols to the beneficial or detrimental health outcomes is required.[7]

47 In this sense, metabolomics has raised as a high-throughput approach that performs the
48 comprehensive analysis of the metabolome, defined as the collection of low molecular weight
49 molecules produced by cells[8] and has become a promising diagnostic tool for metabolic
50 arrangement of individuals.[9,10] Metabolomic characterization provides the potential to
51 distinguish biomarkers and contribute to the knowledge of the ethio-pathological processes,[11]
52 allowing discovering new targets and tools to be applied in personalized therapies.[12]

53 Importantly, the role of gut microbiota in the conversion of phytochemicals should not be
54 disregarded.[13] Thus, the use of omics approaches in faecal samples might be an effective
55 strategy for further understanding the interactions between phenolic compounds, metabolic
56 processes occurring in the intestine and gut microbiota composition.[14] In addition,
57 employment of these analytical techniques in faecal samples may enable the screening of
58 novel metabolic markers of intake that may correlate with potential health benefits of food
59 constituents.[15]

60 Thus, with the aim of clarifying the metabolic consequences of the interaction between
61 phenolic compounds (*trans*-resveratrol and quercetin) and microbiota in the gut, a metabolic

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62 profiling of faecal samples at the end of a 6 week dietary treatment was conducted in rats fed
63 a high-fat sucrose (HFS) diet. As far as we know, this is the first study showing a differential
64 metabolomic clustering of animals supplemented with such pure phenolic compounds based
65 on a faecal metabolome analysis.

66 **Experimental**

67 **Animals and diets**

68 A sub-cohort of twenty-four Wistar rats, supplied from Harlan Ibérica (Barcelona, Spain), were
69 housed individually in polypropylene cages and kept in an isolated room with a constantly
70 regulated temperature (22 ± 2 °C) under a 12:12-h artificial light/dark cycle. Rats were fed a
71 standard-chow diet (C; 2.9 Kcal/g) from Harlan Ibérica (ref. 2014) during an adaptation period
72 that lasted six days. Subsequently, animals were randomly distributed into four experimental
73 groups and changed to a HFS commercial obesogenic diet (ref. D12451M, OpenSource Diets,
74 Research Diets Inc., New Brunswick, USA) for 6 weeks. The HFS diet provided 4.7 Kcal/g and
75 contained 20 % of energy as proteins, 35 % of energy as carbohydrates (17 % sucrose, 10 %
76 maltodextrin and 7 % corn starch) and 45 % of energy as fat (31.4 % as saturated fats, 35.5 %
77 as monounsaturated fats, 33.1 % as polyunsaturated fats) as described elsewhere.[16] All
78 animals had free access to food and water. The experimental groups were distributed as
79 follows: control group (HFS; n=6), fed the HFS diet; *trans*-resveratrol group (RSV; n=6),
80 supplemented with trans-resveratrol 15 mg/Kg BW/day; quercetin group (Q; n=6),
81 supplemented with quercetin 30 mg/kg BW/day; and *trans*-resveratrol + quercetin group
82 (RSV+Q; n= 6), treated with a mixture of *trans*-resveratrol 15 mg/ kg BW/ day and quercetin 30
83 mg/ kg BW/day. Polyphenols were daily incorporated into the powdered diet in quantities that
84 ensured that each animal consumed the prescribed levels.[17] Body weight and food intake were
85 daily recorded. Tissue samples were collected and frozen as described elsewhere.[16] Insulin
86 resistance was assessed by the homeostasis model assessment of insulin resistance (HOMA-IR)

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87 formula:[18] [serum glucose levels (mmol/L) x insulin levels (mU/L)]/22.5. All the experiments
88 were performed in agreement with the Ethical Committee of the University of the Basque
89 Country (document reference CUEID CEBA/30/2010), following the European regulations
90 (European Convention- Strasburg 1986, Directive 2003/65/EC and Recommendation
91 2007/526/EC).

92 **Faeces collection and preparation**

93 Fresh faecal samples were collected at the end of the intervention period, early in the morning
94 and before the overnight fasting period, by abdominal massage. Samples were immediately
95 frozen at -80° C for future analysis.

96 **Chemicals and reagents**

97 *Trans*-resveratrol (> 98 % purity) was supplied by Monteloeder (Elche, Spain) and quercetin (≥
98 98 % purity) by Sigma-Aldrich (St. Louis, MO, USA). LC/MS grade methanol (MeOH) and
99 acetonitrile (ACN), analytical grade chloroform ($CHCl_3$), formic acid and ammonium fluoride
100 were purchased from Sigma-Aldrich (Steinheim, Germany). Water was produced in an in-house
101 Milli-Q purification system (Millipore, Molsheim, France).

102 **Gut microbiota composition analysis**

103 DNA from faecal samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden,
104 Germany) and quantified by Nanodrop ND-1000 spectrophotometer (Thermo Scientific,
105 Delaware, USA). The 454 pyrosequencing of the faecal microbiota was conducted as described
106 elsewhere[19] and analysed as a custom service by Beckman Coulter Genomics (Danvers, MA,
107 USA).

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108 **Metabolite extraction method**

109 Metabolites were extracted from faecal samples by adding 500 µL of a mixture of
110 methanol/water (8:2, v/v) to 4 mg of lyophilized and milled sample. The resulting suspension
111 was bath-sonicated for 20 sec, incubated at 4ºC for 10 min and centrifuged at 5,000 *g* and 4ºC
112 for 15 min. Supernatants were analysed by liquid chromatography coupled to high-resolution
113 mass spectrometry (LC-HRMS) technique.

114 **LC-HRMS analysis**

115 LC-HRMS analyses were performed using a 1290 infinity UHPLC system (Agilent Technologies)
116 coupled to a 6550 ESI-QTOF (Agilent Technologies) operated in positive (ESI^+) or negative (ESI^-)
117 electrospray ionization mode. When the instrument was operated in a positive ionization
118 mode, metabolites were separated using an Acquity UPLC (HSS T3) C18 reverse phase (RP)
119 column (2.1 x 150 mm, 1.8 µm) and the solvent system was A1 = 0.1% formic acid in water and
120 B1 = 0.1% formic acid in acetonitrile. When the instrument was operated in a negative
121 ionization mode, metabolites were separated using an Acquity UPLC (BEH) C18 RP column (2.1
122 x 150mm, 1.8 µm) and the solvent system was A2 = 1Mm ammonium fluoride in water and B2
123 = acetonitrile. The linear gradient elution started isocratic at 100% B (0-1.5 min) and finished at
124 100% A (12 min). The injection volume was 5 µl. ESI conditions were as follows: gas
125 temperature, 290 °C; drying gas, 13 L min^{-1}; nebulizer, 35 psig; capillary voltage, 3500 V;
126 fragmentor, 120 V; and skimmer, 65 V. The instrument was set out to acquire over the *m/z*
127 range 100 – 1000 with an acquisition rate of 4 spectra/s.

128 **LC-MS/MS identification of putative metabolites**

129 The LC-MS/MS analyses were performed using the same LC-HRMS conditions. The precursor
130 ions corresponding at putative metabolites were selected for their selective fragmentation at
131 10 and 20 eV using nitrogen as collision gas over the *m/z* range 40 – 500 with an acquisition

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132 rate of 4 spectra/s. For those compounds that commercial standards are available (*trans*-
133 resveratrol and quercetin), the identification was done by retention time and MS/MS spectra
134 matching, while for those compounds where commercial standards are not available and no
135 MS/MS spectra is available in public metabolite databases (dihydroresveratrol and 3,4'–
136 dihydroxybibenzyl (lunularin)), their identification was done by theoretical MS/MS
137 fragmentation assignment.

138 **Data processing and statistical analysis**

139 LC-HRMS (ESI+ and ESI− mode) data were processed using the Mass Profiler Professional (MPP)
140 software (Agilent Technologies, Barcelona, Spain) to detect and align features. A feature is
141 defined as a molecular entity with a unique *m/z* and a specific retention time. MPP analysis of
142 these data provided a matrix containing the retention time, *m/z* value, and the integrated peak
143 area of each feature for every sample. Quality control samples (QCs) consisting of pooled
144 faecal samples from every condition were used in UHPLC-(ESI)-HRMS analyses. QCs were
145 injected at the beginning and periodically every 5 samples. Furthermore, samples entering the
146 study were entirely randomized to reduce systematic error associated with instrumental drift.
147 QCs were always projected in a Principal Component Analysis (PCA) model together with the
148 samples under study to verify that technical issues do not mask biological information. PCA-
149 based methods are usually employed as the first step when evaluating metabolomics data.
150 This method is useful for the calculation of linear combinations of the original data (PCs), and
151 identifies the most influential variables reducing the dimensionality of the data set.[20,21] The
152 performance of the analytical platform for each detected feature in faecal samples was
153 assessed by calculating the relative standard deviation of these features on pooled samples
154 (CV_{QC}). Faecal samples were compared using the integrated peak area of each feature, and
155 assigning a fold value to indicate the level of differential regulation. For the screening of
156 metabolites, the following filters were specified: the *m/z* of metabolites should appear in at

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157 least two samples. Subsequently, the detected *m/z* should be present in the 100 % of all
158 samples tested in at least one experimental group. Afterwards, One-Way ANOVA was
159 conducted followed by Tukey range test, and Benjamini-Hochberg multiple correction
160 procedure was used to statistically compare significant metabolites ($p < 0.05$). Differentially
161 regulated metabolites that were statistically significant ($p < 0.05$) after correction were
162 putatively identified by matching the obtained neutral exact mass to those published in the
163 selected databases, such as METLIN,[22] Human Metabolome Database (HMDB)[23], and Kyoto
164 Encyclopedia of Genes and Genomes (KEGG) database[24] within a mass accuracy below 40 ppm.
165 Moreover, in those cases where more than one putative compound was shown, those
166 presenting no difference (Δppm=0) to the detected *m/z* value were chosen.

167 **Results**

168 **Phenotypical characteristics**

169 Administration of the combination of *trans*-resveratrol and quercetin significantly reduced
170 body-weight gain at the end of the treatment period (Table 1). However, supplementation
171 with pure polyphenols did not significantly affect the weight of the different fat depots. The
172 combined administration of both polyphenols significantly decreased serum insulin levels
173 when compared to the HFS diet-fed control rats, but no statistical differences were found for
174 glucose levels and HOMA-IR index (Fig. 1). In contrast, the separate administration of *trans*-
175 resveratrol and quercetin, significantly improved serum insulin and glucose levels, as well as
176 HOMA-IR index values (Fig. 1).

177 **Metabolic profiling of the LC-HRMS data**

178 The LC-HRMS method as a tool to assess global faecal metabolite profiling, allowed the
179 detection of 22533 metabolites in the ESI + mode and 4134 metabolites in the ESI – mode

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180 (data not shown). These data were statistically analysed, and statistically significant ($p< 0.05$)
181 metabolic changes were found between supplemented groups and the HFS diet-fed control
182 group at the end of the 6 week dietary treatment. From the detected molecules, 38
183 metabolites were found to significantly differ in the three supplemented groups when
184 compared to the non-treated group in ESI + mode (Supplementary Table 1), while the number
185 of metabolites that was found to be significantly different in ESI – mode was of 10
186 (Supplementary Table 2). When the Log fold-change (Log FC) was calculated, from the total 51
187 metabolites that reached statistical significance, 11 metabolites were present uniquely in
188 quercetin supplemented group (Log FC> 10 or FC< -5) in ESI + mode, while *trans*-resveratrol-
189 supplemented group was distinguished (Log FC> 10) by the presence of 5 metabolites
190 (Supplementary Table 1). In contrast, in ESI – mode, the quercetin-treated group was
191 characterized by one singular metabolite (Log FC> 10), while the number of metabolites
192 occurring only in the *trans*-resveratrol group (Log FC> 5) was 4 (Supplementary Table 2). Each
193 of the compounds detected in *trans*-resveratrol and quercetin groups separately were found in
194 the faeces from the animals supplemented with the combination of both polyphenols
195 (Supplementary Table 1 and 2).

196 **Metabolic fingerprinting**

197 The overall metabolic differences between the HFS diet-fed control group and the three
198 experimental groups supplemented with either *trans*-resveratrol, quercetin or the
199 combination of both polyphenols were evaluated by PCA. The LC-HRMS data showed a
200 distinctive clustering of the four experimental groups. In ESI + mode, the PC1 could explain
201 45.63 % of the total variance, while the PC2 explained the 28.04 % and the PC3 only the 6.09
202 %. In ESI – mode, the PC1-3 explained 63.02 %, 15.35 % and 5.45 %, respectively. Also, four
203 clusters were clearly separated representing each experimental group (Fig. 2A and Fig. 2B).

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204 **Identification of candidate molecules significantly differing in *trans*-resveratrol and**
205 **quercetin-supplemented groups when compared to the HFS diet-fed control group**

206 The compounds exhibiting the greatest Log FC (Log FC> 10) contributed most to the variance
207 between the experimental groups. Table 2 summarizes metabolites that were putatively
208 identified based on the information obtained from different databases. Accordingly,
209 statistically significantly differing masses that only appeared as a result of the intake of *trans*-
210 resveratrol or quercetin, were subjected to tentative identification. With this purpose,
211 specifically METLIN database was consulted. Consequently, looking at the neutral mass,
212 candidate compounds were detected when the mass difference between the theoretical *m/z*
213 and detected *m/z* did not exceed 40 ppm. In addition, a candidate compound was also
214 suggested in those cases when the *m/z* difference between detected and theoretical *m/z,* was
215 set as 0 or only a unique metabolite was listed in the database. Finally, in those cases where
216 the list of metabolites shown in METLIN was classified within the same chemical class, a
217 putative compound was also designated. As a result, it was discerned that quercetin
218 supplemented group was exclusively distinguished by carbohydrate derivatives or
219 carbohydrate conjugates, while rats that were administered *trans*-resveratrol were found to
220 present particular metabolites related to nucleotides metabolism.

221 In the present study, a total of 2 metabolites were putatively identified in the *trans*-resveratrol
222 supplemented group (Table 2). The candidate metabolites that were largely upregulated were
223 associated to nucleotide metabolism, namely uridine 3'-monophosphate or related
224 compounds and 2,4-dioxotetrahydropyrimidine D-ribonucleotide.

225 Uridine 3'-monophosphate, or a similar compound related to pyrimidine metabolism, was
226 upregulated (Log FC= 14.1) in the *trans*-resveratrol supplemented group, as well as in faecal
227 samples obtained from the experimental groups that were administered both polyphenols
228 (Log FC= 11.2). 2,4-dioxotetrahydropyrimidine D-ribonucleotide was also upregulated (Log FC=

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229 11.6) in the *trans*-resveratrol group and in the experimental group treated with both
230 compounds (Log FC= 13.6). Correlations of gut microbial species and putative metabolites
231 identified showed a strong inverse correlation between uridine 3'-monophosphate and
232 *Clostridium hathewayi* (p< 0.0001; r= -0.781), *Clostridium aldenense* (p< 0.001; r= -0.668) and
233 *Clostridium* sp. MLG661 (p< 0.0001; r= -0.767) (Fig. 3A, 3B, 3C). Also, between levels of 2,4-
234 dioxotetrahydropyrimidine D-ribonucleotide and *Clostridium hathewayi* (p< 0.001; r= -0.694),
235 *Clostridium aldenense* (p< 0.01; r= -0.668) and *Clostridium* sp. MLG661 (p< 0.001; r= -0.702)
236 (Fig. 3D, 3E, 3F). These associations were only observed in those animals that were
237 administered *trans*-resveratrol alone or the combination of *trans*-resveratrol and quercetin.

238 In relation to quercetin supplementation, also two putative indicators were identified, 3-
239 phenylpropyl glucosinolate and dTDP-D-mycaminose. In this sense, a positive correlation was
240 found between the levels of 3-phenylpropyl glucosinolate (p= 0.003; r= 0.618) and dTDP-D-
241 mycaminose (p< 0.01; r= 0.633) and those of *Clostridium methylpentosum* bacterium (Fig. 4A
242 and 4B).

243 The experimental group supplemented with *trans*-resveratrol and quercetin showed the
244 presence in faeces of all the metabolites separately detected in both *trans*-resveratrol and
245 quercetin supplemented groups.

246 Finally, the use of pure standards allowed the targeted screening identification of the parent
247 compound (*trans*-resveratrol) and related metabolites (dihydroresveratrol and lunularin) in
248 faecal samples of resveratrol-supplemented rats (Table 3).

249 **Discussion**

250 The LC-HRMS method performed in faeces detects biomarkers that might reflect the impact
251 that consumed nutrients or ingredients exert on health. Furthermore, faecal metabolomics,
252 despite being in its infancy, represents a feasible source of information about modifications on

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253 gut microbiota composition and activity of intestinal bacteria, among others.[14] In the present
254 work, this approach has successfully identified, although putatively, marked metabolome
255 alterations that were profound enough to categorise experimental groups into differentiated
256 clusters based on the administration of specific food components. Interestingly, as far as we
257 know, there are no metabolomic studies carried out in faecal samples where outcomes of
258 animals' dietary exposure to *trans*-resveratrol and/or quercetin have been analysed. In
259 contrast, this pipeline has been previously applied to other studies aiming to identify
260 metabolites derived from wine intake.[25] Accordingly, the MS/MS fragmentation assignment of
261 *trans*-resveratrol and its derived metabolites, allowed the detection of two known microbial
262 *trans*-resveratrol metabolites namely dihydroresveratrol and lunularin. These compounds have
263 been recently described in human intervention studies as products obtained from resveratrol
264 metabolic conversion by intestinal bacteria, but their physiologic outcomes need to be
265 explored.[26,27]

266 Interestingly, the current study shows that animals supplemented with *trans*-resveratrol or
267 quercetin, were characterized by a different set of faecal compounds. Briefly, rats consuming
268 quercetin showed metabolites related to carbohydrates derivatives or conjugates, which might
269 initially reflect an impact of this flavonoid on carbohydrate metabolism as previously
270 postulated.[28] In contrast, samples from *trans*-resveratrol-treated animals seemed to be
271 characterized by compounds involved in nucleotide metabolism. None of the listed putative
272 compounds distinguishing *trans*-resveratrol-supplemented group have been specifically
273 identified previously. Nevertheless, alterations in nucleotide metabolic processes have been
274 recognized in intestinal inflammatory conditions associated to gut microbiota dysbiosis.[29] In
275 fact, the role of extracellular nucleotides as proinflammatory mediators in intestinal
276 inflammatory conditions worth's mentioning.[30] For instance, uridine diphosphate (UDP) has
277 been described as a mediator of cytokine secretion from immune cells and gastrointestinal
278 epithelial cells reporting the capability to induce neutrophil migration in response to bacterial

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279 ligands.[31] In this sense, a previous study conducted by our group observed that *trans*-
280 resveratrol significantly increased the expression levels of genes related to inflammation in
281 colonic mucosa of diet-induced obese rats (i.e. Tlr-2, Tlr-4). Moreover, this stilbene was found
282 to inhibit the growth of *Clostridium hathewayi, Clostridium aldenense and Clostridium sp.*
283 MLG661 when compared to the HFS diet-fed control rats.[19] *Clostridium hathewayi* is classified
284 within *Clostridial* cluster XIVa, which encompasses major butyrate producers.[32] Notably,
285 *Clostridium* strains from cluster XIVa and cluster IV (such as, *Clostridium hathewayi*), have been
286 demonstrated to be T (T_{reg})-cell-inducing bacteria, lacking virulence related genes.[33] Thus, the
287 significantly reduced levels of bacteria belonging to *Clostridia* cluster XIVa[19] perceived in *trans*-
288 resveratrol treated groups, together with the increased levels of nucleotides-related
289 metabolites detected in faeces, may suggest a possible damage present in the colonic tissue of
290 rats supplemented with *trans*-resveratrol alone or combined with quercetin.

291 Metabolites mostly contributing to the differentiation of animals supplemented with quercetin
292 were 3-phenylpropyl glucosinolate and dTDP-D-mycaminose. Glucosinolates are direct parents
293 of the candidate 3-phenylpropyl glucosinolate compound. These molecules are precursors of
294 isothiocyanates, which have potential chemoprotective properties.[34] Noteworthy,
295 isothiocyanates mainly exist as glucosinolate conjugates in cruciferous vegetables (family
296 *Brassicaceae*) as for instance broccoli, which is known to be rich in flavonoids such as
297 quercetin.[35] Interestingly, dTDP-D-mycaminose is involved in the biosynthesis of polyketide
298 sugar units, a diverse group of natural products commonly found in bacteria, fungi and plants,
299 with carbon skeletons that encompass polyphenols, macrolides, polyenes, enediynes and
300 polyethers.[36] These compounds represent an important source of novel therapeutics, known
301 for their antibiotic, immunosuppressant, antiparasitic, cholesterol-lowering and antitumor
302 effects.[37] Accordingly, dTDP-D-mycaminose is a deoxyaminosugar that contains a core
303 aglycone of polyketide origin, with a deoxysugar moiety attached.[38] The union of the
304 deoxysugar moiety has been reported to be essential for the bioactivities of these bacterial

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305 products, including antibacterial and anti-inflammatory effects.[39,40] Our group previously
306 demonstrated that quercetin supplementation, but especially the combined administration of
307 *trans*-resveratrol and quercetin, increased the abundance of the bacterium *Clostridium*
308 *methylpentosum* when compared to the HFS diet-fed reference group.[19] Remarkably,
309 *Clostridium methylpentosum* [41] has been described to ferment pentoses and methyl-pentoses,
310 namely L-rhamnose. This feature has been associated to the presence of α- L-rhamnosidase
311 activity in this bacterium. [42] As a consequence, based on the findings from this study, it might
312 be postulated that the unabsorbed quercetin reaching the colon may be metabolized by α- L-
313 rhamnosidases of bacterial origin.[43]

314 It should be bear in mind that the four candidate metabolites discovered in the current study
315 have not been confirmed with the use of specific commercial standards. Therefore, there
316 exists the possibility that the tentatively identified neutral masses belong to other compounds,
317 hence, different explanations might be plausible.

318 **Conclusions**

319 The present work highlights the robustness and reliability of exploratory faecal metabolomics
320 to distinguish indicators of the metabolic effects associated to the intake of pure polyphenols.
321 To our knowledge, this is the first study assessing the impact of *trans*-resveratrol and quercetin
322 on diet-induced obese animal's faecal metabolome. Taken together, these data conclude that
323 this approach has the ability to differentiate metabolomic clusters depending on the ingested
324 polyphenols and reveals a faecal metabolic fingerprint of the overall impact of *trans*-
325 resveratrol and quercetin based on the identification of potential indicators that correlate with
326 specific gut microbiota composition. Indeed, some of the putative metabolites identified were
327 products of metabolic pathways, namely microbial metabolism, which were strongly
328 correlated with the abundance of specific bacterial species affected by the intake of such
329 bioactive compounds. Importantly, the metabolic fate of *trans*-resveratrol was explored and

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330 microbial-derived *trans*-resveratrol metabolites were distinguished in faeces. Overall, these
331 results indicate that data from metabolomics analysis in faeces reflect microbial catabolism of
332 polyphenols, an important feature to be considered, since it has been already demonstrated
333 that bioactivity of metabolites might be greater than the parent compounds, thereby,
334 profound health effects might be expected.[44]

335 Regarding limitations of the study, it is remarkable to state that the exposure of animals to
336 natural compounds might lead to changes in endogenous metabolome, microbial metabolome
337 and xenometabolome. In this case, despite we could not adventure to sort metabolites, this
338 technique enabled to ascertain that the candidate compounds identified were resultant from
339 the specific impact of *trans*-resveratrol or quercetin, yet the lack of commercial standards for
340 accurate identification of metabolites remains an important limitation, hence a targeted
341 metabolomics analysis which confirm the putative compounds would be of interest.
342 Importantly, although *trans*-resveratrol metabolites produced by intestinal bacteria were
343 detected, the low ionization capability of quercetin impeded the identification of its possible
344 metabolites. In accordance, the impact of diet on whole metabolome was not analysed due to
345 the lack of a standard diet-fed control group. Future studies on humans ingesting these natural
346 compounds would be also useful in order to validate the identified metabolic signatures.

347 Noteworthy, the outcomes presented here open the door to new associations between gut
348 microbiota and faecal metabolites, which might ultimately help to further understand the
349 impact of bioactive constituents on health. The untargeted screening of metabolic markers in
350 faeces represents a promising tool to interpret health consequences derived from the intake of
351 foods and beverages rich in *trans*-resveratrol and quercetin and compliance to the treatment.

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352 **Abbreviations**

353 HFS, high-fat sucrose; HOMA-IR, homeostasis model assessment of insulin resistance; MeOH,
354 methanol; ACN, acetonitrile; $CHCl_3$, chloroform; LC-HRMS, liquid chromatography coupled to
355 high-resolution mass spectrometry; ESI, electrospray ionization mode; RP, reverse phase; MPP,
356 mass profiler professional; QC, quality control; PCA, principal component analysis; PC, principal
357 component; HMDB, human metabolome database; KEGG, kyoto encyclopedia of genes and
358 genomes; Log FC, Log fold-change; UDP, uridine diphosphate.

359 **Acknowledgements**

360 This study was supported by grants from the Ministerio de Economía y Competitividad
361 (AGL2011-27406-ALI), Instituto de Salud Carlos III (CIBERobn) Fisiopatología de la Obesidad y
362 Nutrición, Centro de Investigación en Nutrición (CIN) de la Universidad de Navarra,
363 Government of the Basque Country (IT-572-13) and University of the Basque Country
364 (UPV/EHU) (ELDUNANOTEK UFI11/32). The authors wish to acknowledge Línea Especial about
365 Nutrition, Obesity and Health (University of Navarra LE/97, Spain) for the financial support and
366 the Department of Education, Language policy and Culture from Government of the Basque
367 Country for the predoctoral grant given to Usune Etxeberria.

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Table 1. Weight-related parameters at the end of a 6-week dietary treatment with a HFS diet supplemented or not with *trans*-resveratrol, quercetin or the combination of both polyphenols.

	HFS (n=6)	RSV (n=6)	Q (n=6)	RSV+Q (n=6)	*ANOVA*
Phenotypic characteristics					
Body-weight gain (g)	180 ± 7	169 ± 6	162 ± 7	144 ± 11*	*p*= 0.014
Visceral adipose tissue (g)	27.95 ± 1.72	26.08 ± 0.48	25.43 ± 2.53	22.00 ± 1.15	*p*= 0.056
Subcutaneous adipose tissue (g)	13.00 ± 0.84	12.43 ± 1.33	11.66 ± 1.14	11.32 ± 0.89	NS
Liver weight (g)	10.14 ± 0.19	10.06 ± 0.11	9.73 ± 0.60	8.82 ± 0.47	NS
Gastrocnemius muscles mass (g)	0.96 ± 0.07	0.93 ± 0.05	0.97 ± 0.07	0.82 ± 0.06	NS

All results are expressed as the mean ± SEM. Statistical analyses were performed using One-Way ANOVA followed by Dunnett post-hoc test, *$p < 0.05$ vs HFS. HFS, high fat sucrose diet-fed control rats; RSV, supplemented with 15 mg/kg BW/day of *trans*-resveratrol; Q, supplemented with 30 mg/kg BW/day of quercetin; RSV + Q, supplemented with a combination of *trans*-resveratrol and quercetin at the same doses. NS, statistically non-significant.

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Table 2. Putative identification of metabolites mostly contributing to the variance between the experimental groups (HFS diet-fed control group, and groups fed the same diet supplemented with *trans*-resveratrol, quercetin or the combination of both polyphenols).

ESI Mode	Detected m/z	Δppm	Rt,min	Putative annotation	Metabolic pathway or Chemical taxonomy	Log FC [RSV vs HFS]	Log FC [Q vs HFS]	Log FC [RSV+Q vs HFS]	Corrected *p*
+	437.0872	13	5.05	3-phenylpropyl glucosinolate	CHO and CHO conjugates	0	13.17	15.91	*p*< 0.001
+	575.1192	15	5.05	dTDP-D-mycaminose	Biosynthesis of 12-, 14- and 16-membered macrolides; polyketide sugar unit biosynthesis; biosynthesis of secondary metabolites	0	12.53	15.12	*p*< 0.001
-	324.0357	0	3.45	Uridine 3'-monophosphate or related compounds	Pyrimidine metabolism	14.1	0	11.2	*p*< 0.001
-	326.0512	0	0.72	2,4-dioxotetrahydropyrimidine D-ribonucleotide	Reaction R04346 substrate or product	11.6	0	13.6	*p*< 0.001

Statistical analyses were performed using One-Way ANOVA followed by Tukey range test and *p* values were corrected by Benjamini-Hochberg procedure. ESI, electrospray ionization mode; Rt, retention time; HFS, high fat sucrose diet-fed control rats; RSV, supplemented with 15 mg/kg BW/day of *trans*-resveratrol; Q, supplemented with 30 mg/kg BW/day of quercetin; RSV + Q, supplemented with a combination of *trans*-resveratrol and quercetin at the same doses; Log FC, log2 value of fold change.

Table 3. *Trans*-resveratrol and resveratrol-derived metabolites detected in faeces by a targeted screening analysis among experimental groups (HFS diet-fed control group, and groups fed the same diet supplemented with *trans*-resveratrol, quercetin or the combination of both polyphenols).

ESI Mode	Detected m/z	Rt,min	Putative annotation	Log FC [RSV vs HFS]	Log FC [Q vs HFS]	Log FC [RSV+Q vs HFS]	Corrected *p*
-	228.0827	4.99	*Trans*- resveratrol	15.8	0.0	17.2	*p*< 0.001
-	230.0983	5.04	Dihydroresveratrol	15.3	4.6	16.4	*p*= 0.002
-	214.1027	5.81	Lunularin	16.4	9.8	15.6	*p*= 0.010

Statistical analyses were performed using One-Way ANOVA followed by Tukey range test and *p* values were corrected by Benjamini-Hochberg procedure. ESI, electrospray ionization mode; Rt, retention time; HFS, high fat sucrose diet-fed control rats; RSV, supplemented with 15 mg/kg BW/day of *trans*-resveratrol; Q, supplemented with 30 mg/kg BW/day of quercetin; RSV + Q, supplemented with a combination of *trans*-resveratrol and quercetin at the same doses; Log FC, log2 value of fold change.

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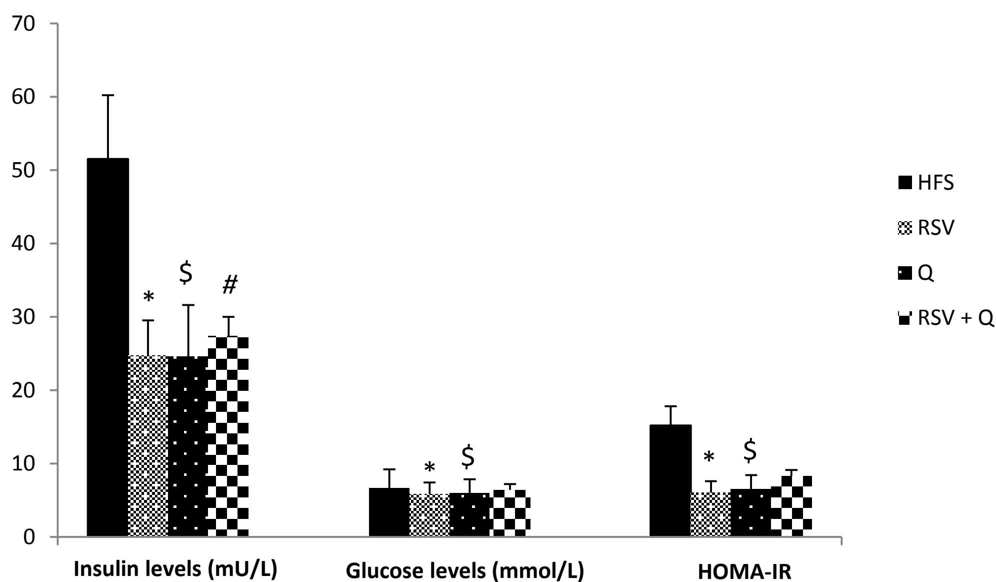

Fig. 1. **Serum biochemical variables of HFS diet-fed rats supplemented or not with *trans*-resveratrol, quercetin or the combination of both polyphenols**. Results are expressed as mean ± SEM. Statistical analyses were performed using One-Way ANOVA followed by Dunnett post-hoc test. Data with different superscript letters are significantly different. *p< 0.05, HFS vs RSV; $^{\$}p$< 0.05 HFS vs Q; $^{\#}p$< 0.05, HFS vs RSV + Q. HFS, high-fat sucrose diet; RSV, supplemented with *trans*-resveratrol 15 mg/kg BW/ day; Q, supplemented with quercetin 30 mg/kg BW/day; RSV+Q, supplemented with a combination of *trans*-resveratrol + quercetin at the same doses.

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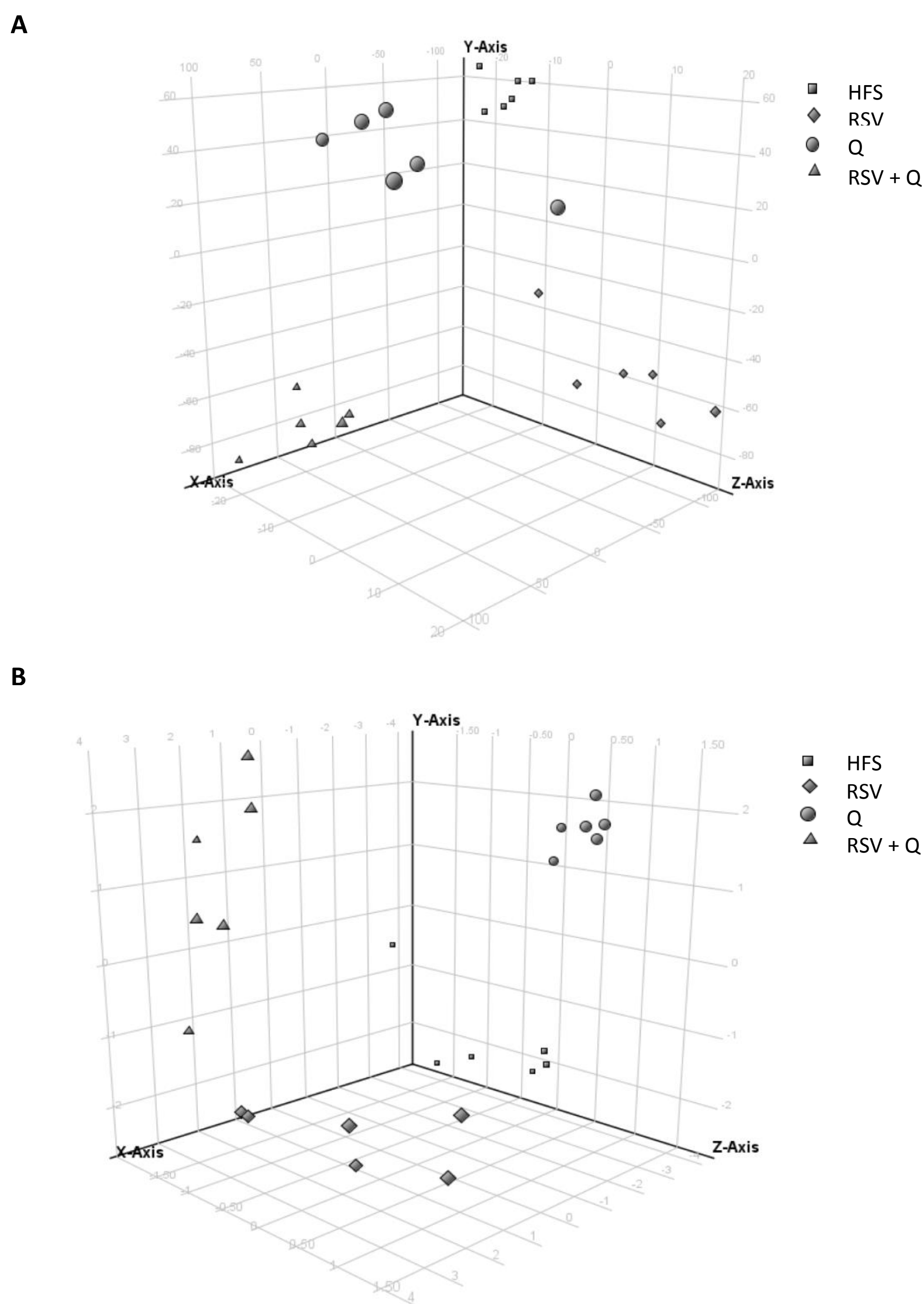

Fig. 2. Principal Component Analysis (PCA) graphs showing faecal metabolomic alterations in HFS diet-fed rats supplemented or not with *trans*-resveratrol, quercetin or the combination of both polyphenols. (A) PCA in ESI + mode with an EVp 79.76 % (B) PCA in ESI – mode with an EVn 83.82 %. EVp, explained variability in positive ionized metabolites; EVn, explained variability in negative ionized metabolites.

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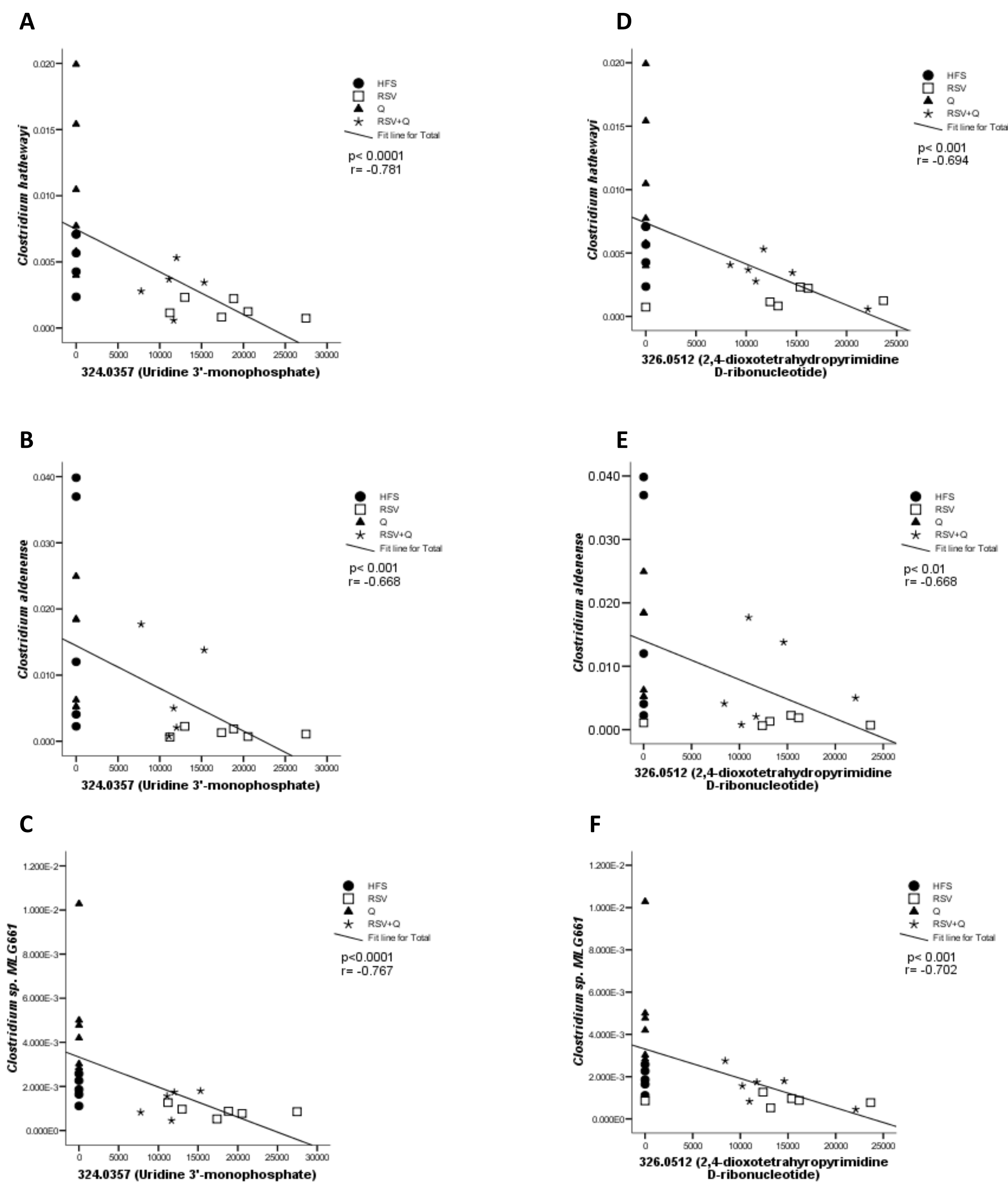

Fig. 3. Correlations between the number of bacteria (taxa frequencies detected in faecal samples) and putative metabolites detected in ESI negative mode. A) Uridine 3'-monophosphate levels and *Clostridium hathewayi* levels, B) *Clostridium aldenense* levels, and C) *Clostridium* sp. MLG661 levels, D) 2,4- Dioxotetrahydropyrimidine D-ribonucleotide and *Clostridium hathewayi* levels, E) *Clostridium aldenense* levels, and F) *Clostridium* sp. MG661 levels. Inserts corresponds to Spearman's correlation and the *p* value. HFS, high fat sucrose diet-fed control rats; RSV, supplemented with 15 mg/kg BW/day of *trans*-resveratrol; Q, supplemented with 30 mg/kg BW/day of quercetin; RSV + Q, supplemented with a combination of *trans*-resveratrol and quercetin at the same doses.

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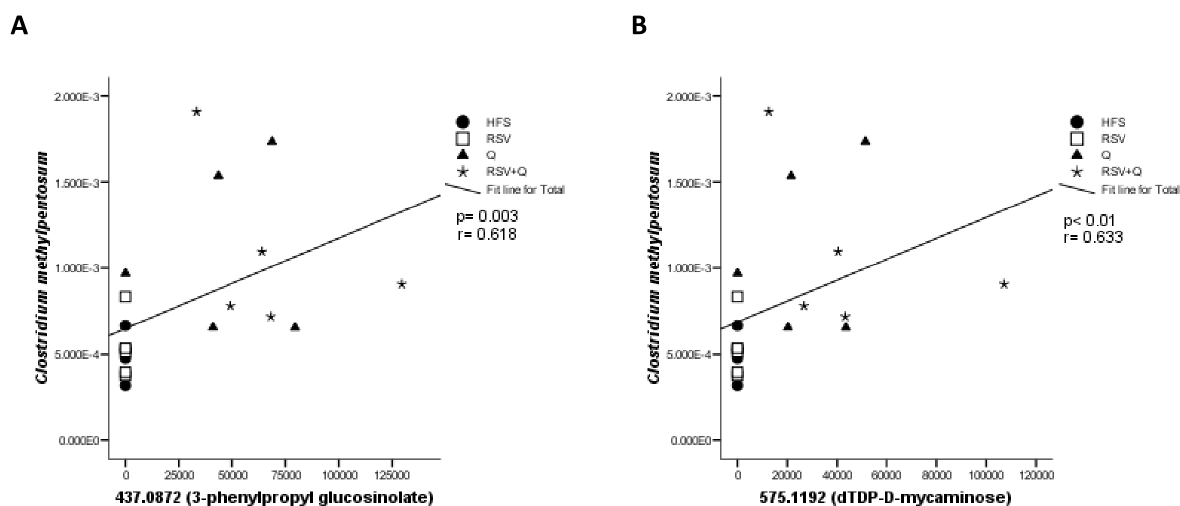

Fig. 4. Correlations between the number of bacteria (taxa frequencies detected in faecal samples) and putative metabolites detected in ESI positive mode. A) 3-Phenylpropyl glucosinolate levels and *Clostridium methylpentosum*, B) dTD-D-mycaminose levels and *Clostridium methylpentosum*. Insert corresponds to Spearman's correlation and the *p* value. HFS, high fat sucrose diet-fed control rats; RSV, supplemented with 15 mg/kg BW/day of *trans*-resveratrol; Q, supplemented with 30 mg/kg BW/day of quercetin; RSV + Q, supplemented with a combination of *trans*-resveratrol and quercetin at the same doses.

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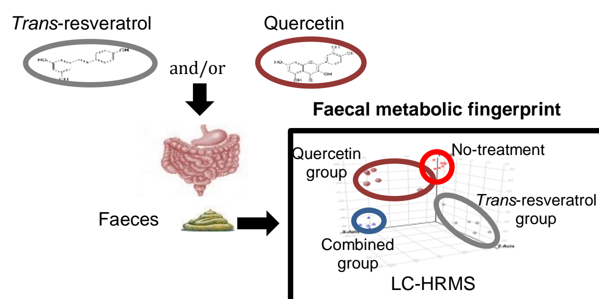
Graphical Abstract



Untargeted metabolomics distinguishes individuals into clusters based on the physiological impact of the dietary treatment they have been subjected to.

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Figure Captions

1. **Figure 1**

Fig. 1. **Serum biochemical variables of HFS diet-fed rats supplemented or not with *trans*-resveratrol, quercetin or the combination of both polyphenols**. Results are expressed as mean ± SEM. Statistical analyses were performed using One-Way ANOVA followed by Dunnett post-hoc test. Data with different superscript letters are significantly different. *p< 0.05, HFS vs RSV; $^{\$}p$< 0.05 HFS vs Q; $^{\#}p$< 0.05, HFS vs RSV + Q. HFS, high-fat sucrose diet; RSV, supplemented with *trans*-resveratrol 15 mg/kg BW/ day; Q, supplemented with quercetin 30 mg/kg BW/day; RSV+Q, supplemented with a combination of *trans*-resveratrol + quercetin at the same doses.

2. **Figure 2**

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Table legends

1. Table 1

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2. Table 2

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