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

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Abstract

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

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

Introduction

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

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

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

Thus, with the aim of clarifying the metabolic consequences of the interaction between phenolic compounds (*trans*-resveratrol and quercetin) and microbiota in the gut, a metabolic profiling of faecal samples at the end of a 6 week dietary treatment was conducted in rats fed a high-fat sucrose (HFS) diet. As far as we know, this is the first study showing a differential metabolomic clustering of animals supplemented with such pure phenolic compounds based on a faecal metabolome analysis.

Experimental

Animals and diets

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

recorded daily. Tissue samples were collected and frozen as described elsewhere.[16] Insulin resistance was assessed by the homeostasis model assessment of insulin resistance (HOMA-IR) formula:[18] [serum glucose levels (mmol/L) x insulin levels (mU/L)]/22.5. All the experiments were performed in agreement with the Ethical Committee of the University of the Basque Country (document reference CUEID CEBA/30/2010), following the European regulations (European Convention- Strasburg 1986, Directive 2003/65/EC and Recommendation 2007/526/EC).

Faeces collection and preparation

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

Chemicals and reagents

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

Gut microbiota composition analysis

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

Metabolite extraction method

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

LC-HRMS analysis

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

LC-MS/MS identification of putative metabolites

The LC-MS/MS analyses were performed using the same LC-HRMS conditions. The precursor ions corresponding to putative metabolites were selected for their selective fragmentation at 10 and 20 eV using nitrogen as collision gas over the *m/z* range 40 – 500 with an acquisition rate of 4 spectra per s. For those compounds for which commercial standards are available (*trans*-resveratrol and quercetin), the identification was done by retention time and MS/MS spectra matching, while for those compounds for which commercial standards are not available and no MS/MS spectra are available in public metabolite databases (dihydroresveratrol and 3,4'–dihydroxybibenzyl (lunularin)), identification was done by theoretical MS/MS fragmentation assignment.

Data processing and statistical analysis

LC-HRMS (ESI+ and ESI− mode) data were processed using the Mass Profiler Professional (MPP) software (Agilent Technologies, Barcelona, Spain) to detect and align features. A feature is defined as a molecular entity with a unique *m/z* and a specific retention time. MPP analysis of these data provided a matrix containing the retention time, *m/z* value, and the integrated peak area of each feature for every sample. Quality control samples (QCs) consisting of pooled faecal samples from every condition were used in UHPLC-(ESI)-HRMS analyses. QCs were injected at the beginning and periodically every 5 samples. Furthermore, samples entering the study were entirely randomized to reduce systematic error associated with instrumental drift. QCs were always projected in a Principal Component Analysis (PCA) model together with the samples under study to verify that technical issues do not mask biological information. PCA-based methods are usually employed as the first step when evaluating metabolomics data. This method is useful for the calculation of linear combinations of the original data (PCs), and identifies the most influential variables reducing the dimensionality of the data set.[20,21] The performance of the analytical platform for each detected feature in faecal samples was assessed by calculating the relative standard deviation of these features on pooled samples

(CV_{QC}). Faecal samples were compared using the integrated peak area of each feature, and assigning a fold value to indicate the level of differential regulation. For the screening of metabolites, the following filters were specified: the *m/z* of metabolites should appear in at least two samples. Subsequently, the detected *m/z* should be present in the 100 % of all samples tested in at least one experimental group. Afterwards, One-Way ANOVA was conducted followed by Tukey range test, and Benjamini-Hochberg multiple correction procedure was used to statistically compare significant metabolites ($p< 0.05$). Differentially regulated metabolites that were statistically significant ($p< 0.05$) after correction were putatively identified by matching the obtained neutral exact mass to those published in the selected databases, such as METLIN,[22] Human Metabolome Database (HMDB)[23], and Kyoto Encyclopedia of Genes and Genomes (KEGG) database[24] within a mass accuracy below 40 ppm. Moreover, in those cases where more than one putative compound was shown, those presenting no difference (Δppm=0) to the detected *m/z* value were chosen.

Results

Phenotypical characteristics

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

Metabolic profiling of the LC-HRMS data

The LC-HRMS method as a tool to assess global faecal metabolite profiling, allowed the detection of 22533 metabolites in the ESI + mode and 4134 metabolites in the ESI – mode (data not shown). These data were statistically analysed, and statistically significant ($p< 0.05$) metabolic changes were found between supplemented groups and the HFS diet-fed control group at the end of the 6 week dietary treatment. From the detected molecules, 38 metabolites were found to significantly differ in the three supplemented groups when compared to the non-treated group in ESI + mode (Supplementary Table 1), while the number of metabolites that were found to be significantly different in ESI – mode was 10 (Supplementary Table 2). When the Log fold-change (Log FC) was calculated, from the total 51 metabolites that reached statistical significance, 11 metabolites were present uniquely in quercetin supplemented group (Log FC> 10 or FC< -5) in ESI + mode, while the *trans*-resveratrol- supplemented group was distinguished (Log FC> 10) by the presence of 5 metabolites (Supplementary Table 1). In contrast, in ESI – mode, the quercetin-treated group was characterized by one singular metabolite (Log FC> 10), while the number of metabolites occurring only in the *trans*-resveratrol group (Log FC> 5) was 4 (Supplementary Table 2). Each of the compounds detected in *trans*-resveratrol and quercetin groups separately were found in the faeces from the animals supplemented with the combination of both polyphenols (Supplementary Table 1 and 2).

Metabolic fingerprinting

The overall metabolic differences between the HFS diet-fed control group and the three experimental groups supplemented with either *trans*-resveratrol, quercetin or the combination of both polyphenols were evaluated by PCA. The LC-HRMS data showed a distinctive clustering of the four experimental groups. In ESI + mode, the PC1 could explain 45.63 % of the total variance, while the PC2 explained the 28.04 % and the PC3 only the 6.09

%. In ESI – mode, the PC1-3 explained 63.02 %, 15.35 % and 5.45 %, respectively. Also, the four clusters were clearly separated representing each experimental group (Fig. 2A and Fig. 2B).

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

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

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

Uridine 3'-monophosphate, or a similar compound related to pyrimidine metabolism, was upregulated (Log FC= 14.1) in the *trans*-resveratrol supplemented group, as well as in faecal samples obtained from the experimental groups that were administered both polyphenols (Log FC= 11.2). 2,4-Dioxotetrahydropyrimidine D-ribonucleotide was also upregulated (Log FC= 11.6) in the *trans*-resveratrol group and in the experimental group treated with both compounds (Log FC= 13.6). Correlations of gut microbial species and putative metabolites identified showed a strong inverse correlation between uridine 3'-monophosphate and *Clostridium hathewayi* ($p< 0.0001$; r= -0.781), *Clostridium aldenense* ($p< 0.001$; r= -0.668) and *Clostridium* sp. MLG661 ($p< 0.0001$; r= -0.767) (Fig. 3A, 3B, 3C). Also, between levels of 2,4-dioxotetrahydropyrimidine D-ribonucleotide and *Clostridium hathewayi* ($p< 0.001$; r= -0.694), *Clostridium aldenense* ($p< 0.01$; r= -0.668) and *Clostridium* sp. MLG661 ($p< 0.001$; r= -0.702) (Fig. 3D, 3E, 3F). These associations were only observed in those animals that were administered *trans*-resveratrol alone or the combination of *trans*-resveratrol and quercetin.

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

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

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

Discussion

The LC-HRMS method performed in faeces detects biomarkers that might reflect the impact that consumed nutrients or ingredients exert on health. Furthermore, faecal metabolomics, despite being in its infancy, represents a feasible source of information about modifications on gut microbiota composition and activity of intestinal bacteria, among others.[14] In the present work, this approach has successfully identified, although putatively, marked metabolome alterations that were profound enough to categorise experimental groups into differentiated clusters based on the administration of specific food components. Interestingly, as far as we know, there are no metabolomic studies carried out in faecal samples where outcomes of animals' dietary exposure to *trans*-resveratrol and/or quercetin have been analysed. In contrast, this pipeline has been previously applied to other studies aiming to identify metabolites derived from wine intake.[25] Accordingly, the MS/MS fragmentation assignment of *trans*-resveratrol and its derived metabolites, allowed the detection of two known microbial *trans*-resveratrol metabolites namely dihydroresveratrol and lunularin. These compounds have been recently described in human intervention studies as products obtained from resveratrol metabolic conversion by intestinal bacteria, but their physiological outcomes need to be explored.[26,27]

Interestingly, the current study shows that animals supplemented with *trans*-resveratrol or quercetin, were characterized by a different set of faecal compounds. Briefly, rats consuming quercetin showed metabolites related to carbohydrates derivatives or conjugates, which might initially reflect an impact of this flavonoid on carbohydrate metabolism as previously postulated.[28] In contrast, samples from *trans*-resveratrol-treated animals seemed to be characterized by compounds involved in nucleotide metabolism. None of the listed putative compounds distinguishing *trans*-resveratrol-supplemented group have been specifically identified previously. Nevertheless, alterations in nucleotide metabolic processes have been

recognized in intestinal inflammatory conditions associated to gut microbiota dysbiosis.[29] In fact, the role of extracellular nucleotides as proinflammatory mediators in intestinal inflammatory conditions is worth mentioning.[30] For instance, uridine diphosphate (UDP) has been described as a mediator of cytokine secretion from immune cells and gastrointestinal epithelial cells reporting the capability to induce neutrophil migration in response to bacterial ligands.[31] In this sense, a previous study conducted by our group observed that *trans*-resveratrol significantly increased the expression levels of genes related to inflammation in colonic mucosa of diet-induced obese rats (i.e. Tlr-2, Tlr-4). Moreover, this stilbene was found to inhibit the growth of *Clostridium hathewayi, Clostridium aldenense and Clostridium sp.* MLG661 when compared to the HFS diet-fed control rats.[19] *Clostridium hathewayi* is classified within *Clostridial* cluster XIVa, which encompasses major butyrate producers.[32] Notably, *Clostridium* strains from cluster XIVa and cluster IV (such as, *Clostridium hathewayi*), have been demonstrated to be T (T_{reg})-cell-inducing bacteria, lacking virulence related genes.[33] Thus, the significantly reduced levels of bacteria belonging to *Clostridia* cluster XIVa[19] perceived in *trans*-resveratrol treated groups, together with the increased levels of nucleotide-related metabolites detected in faeces, may suggest a possible damage present in the colonic tissue of rats supplemented with *trans*-resveratrol alone or combined with quercetin.

Metabolites mostly contributing to the differentiation of animals supplemented with quercetin were 3-phenylpropyl glucosinolate and dTDP-D-mycaminose. Glucosinolates are direct parents of the candidate 3-phenylpropyl glucosinolate compound. These molecules are precursors of isothiocyanates, which have potential chemoprotective properties.[34] Noteworthily, isothiocyanates mainly exist as glucosinolate conjugates in cruciferous vegetables (family *Brassicaceae*) as for instance broccoli, which is known to be rich in flavonoids such as quercetin.[35] Interestingly, dTDP-D-mycaminose is involved in the biosynthesis of polyketide sugar units, a diverse group of natural products commonly found in bacteria, fungi and plants, with carbon skeletons that encompass polyphenols, macrolides, polyenes, enediynes and

polyethers.[36] These compounds represent an important source of novel therapeutics, known for their antibiotic, immunosuppressant, antiparasitic, cholesterol-lowering and antitumor effects.[37] Accordingly, dTDP-D-mycaminose is a deoxyaminosugar that contains a core aglycone of polyketide origin, with a deoxysugar moiety attached.[38] The union of the deoxysugar moiety has been reported to be essential for the bioactivities of these bacterial products, including antibacterial and anti-inflammatory effects.[39,40] Our group previously demonstrated that quercetin supplementation, but especially the combined administration of *trans*-resveratrol and quercetin, increased the abundance of the bacterium *Clostridium methylpentosum* when compared to the HFS diet-fed reference group.[19] Remarkably, *Clostridium methylpentosum* [41] has been described as fermenting pentoses and methyl-pentoses, namely L-rhamnose. This feature has been associated with the presence of α- L-rhamnosidase activity in this bacterium. [42] As a consequence, based on the findings from this study, it might be postulated that the unabsorbed quercetin reaching the colon may be metabolized by α- L-rhamnosidases of bacterial origin.[43]

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

Conclusions

The present work highlights the robustness and reliability of exploratory faecal metabolomics to distinguish indicators of the metabolic effects associated with the intake of pure polyphenols. To our knowledge, this is the first study assessing the impact of *trans*-resveratrol and quercetin on diet-induced obese animal's faecal metabolome. Taken together, these data lead to the conclusion that this approach has the ability to differentiate metabolomic clusters depending on the ingested polyphenols and reveals a faecal metabolic fingerprint of the

overall impact of *trans*-resveratrol and quercetin based on the identification of potential indicators that correlate with specific gut microbiota composition. Indeed, some of the putative metabolites identified were products of metabolic pathways, namely microbial metabolism, which were strongly correlated with the abundance of specific bacterial species affected by the intake of such bioactive compounds. Importantly, the metabolic fate of *trans*-resveratrol was explored and microbial-derived *trans*-resveratrol metabolites were distinguished in faeces. Overall, these results indicate that data from metabolomics analysis in faeces reflect microbial catabolism of polyphenols, an important feature to be considered, since it has been already demonstrated that bioactivity of metabolites might be greater than the parent compounds, thereby, profound health effects might be expected.[44]

Regarding limitations of the study, it must be stated that the exposure of animals to natural compounds might lead to changes in endogenous metabolomes, microbial metabolomes and xenometabolomes. In such cases, despite not venturing to sort metabolites, this technique enabled us to ascertain that the candidate compounds identified were resulted from the specific impact of *trans*-resveratrol or quercetin, yet the lack of commercial standards for the accurate identification of metabolites remains an important limitation, hence a targeted metabolomics analysis which confirms the putative compounds would be of interest. Importantly, although *trans*-resveratrol metabolites produced by intestinal bacteria were detected, the low ionization capability of quercetin impeded the identification of its possible metabolites. In accordance, the impact of diet on whole metabolome was not analysed due to the lack of a standard diet-fed control group. Future studies on humans ingesting these natural compounds would be also useful in order to validate the identified metabolic signatures.

Noteworthily, the outcomes presented here open the door to new associations between gut microbiota and faecal metabolites, which might ultimately help to further understand the impact of bioactive constituents on health. The untargeted screening of metabolic markers in

faeces represents a promising tool to interpret health consequences derived from the intake of foods and beverages rich in *trans*-resveratrol and quercetin and compliance with the treatment.

Abbreviations

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

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Table 1. Weight-related parameters at the end of a 6-week dietary treatment in animals fed 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 group. 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.

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, log 2 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, log 2 value of fold change.

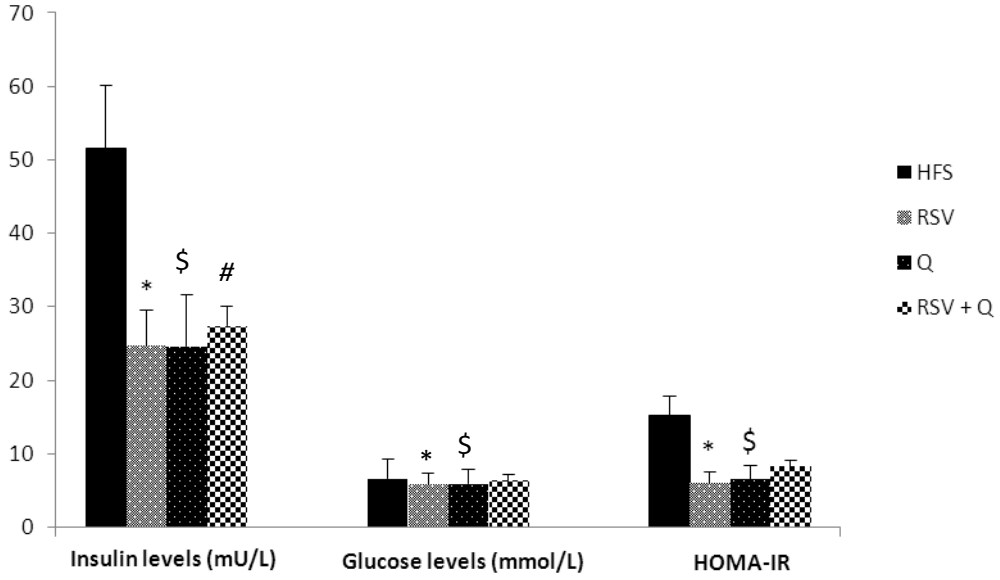
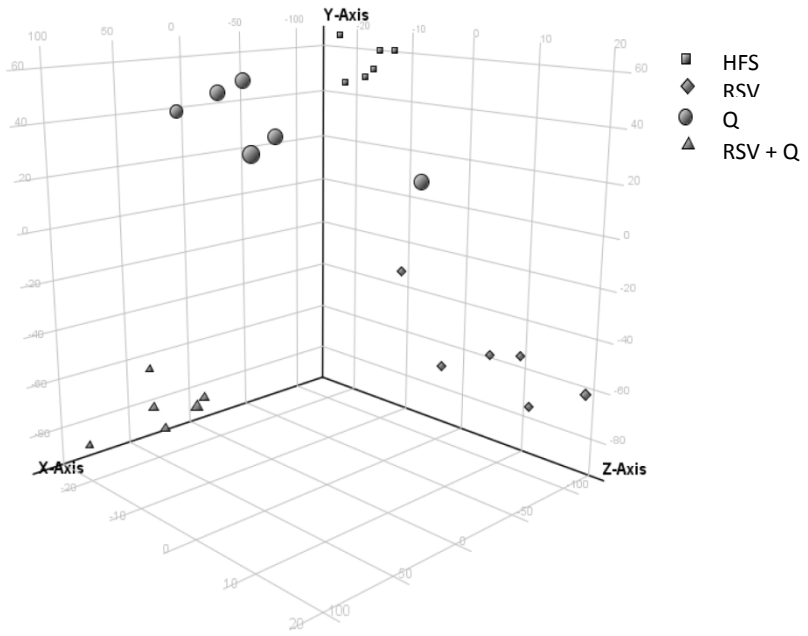


Fig. 1. **Serum biochemical variables of HFS diet-fed rats supplemented or not with *trans*-resveratrol, quercetin or the combination of both polyphenols**. Results are expressed as mean ± SEM. Statistical analyses were performed using One-Way ANOVA followed by Dunnett *post hoc* test. Data with different 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.

A



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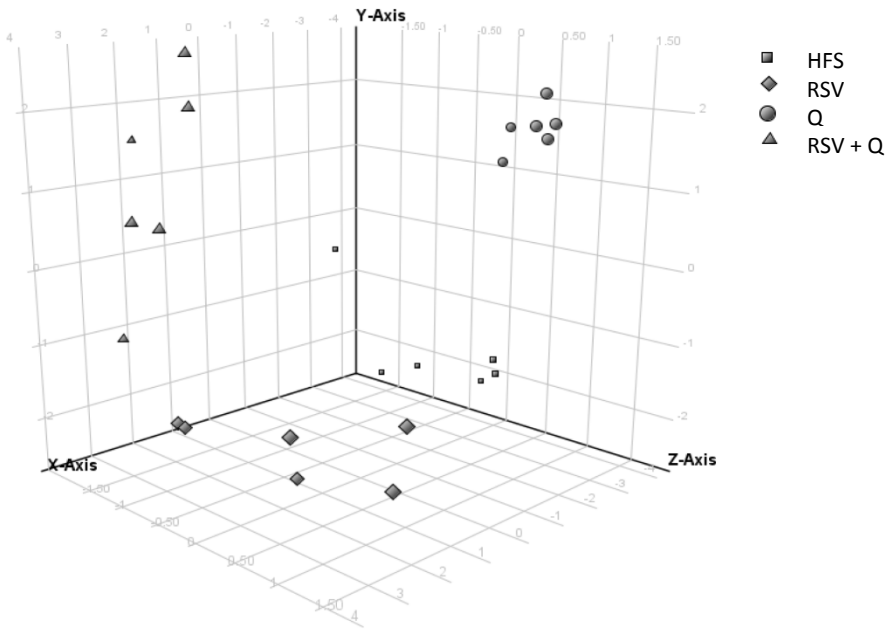


Fig. 2. Principal Component Analysis (PCA) graphs showing faecal metabolomic alterations in HFS diet-fed rats supplemented or not with *trans*-resveratrol, quercetin or the combination of both polyphenols. (A) PCA in ESI + mode with an EVp 79.76 % and (B) PCA in ESI – mode with an EVn 83.82 %. EVp, explained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

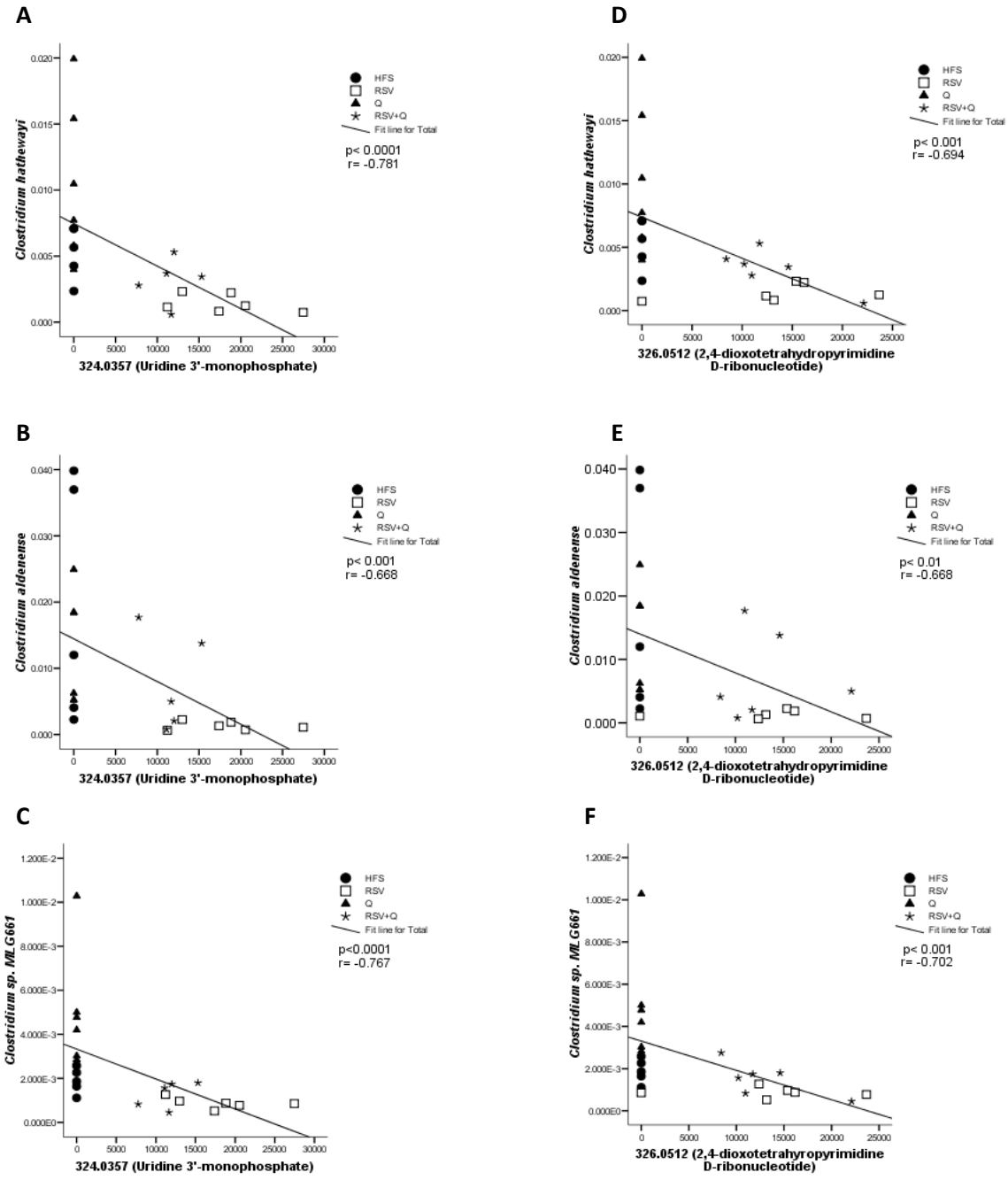


Fig. 3. Correlations between the number of bacteria (taxa frequencies detected in faecal samples) and putative metabolites detected in ESI negative mode. A) Uridine 3'-monophosphate levels and *Clostridium hathewayi* levels, B) *Clostridium aldenense* levels, and C) *Clostridium* sp. MLG661 levels, D) 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.

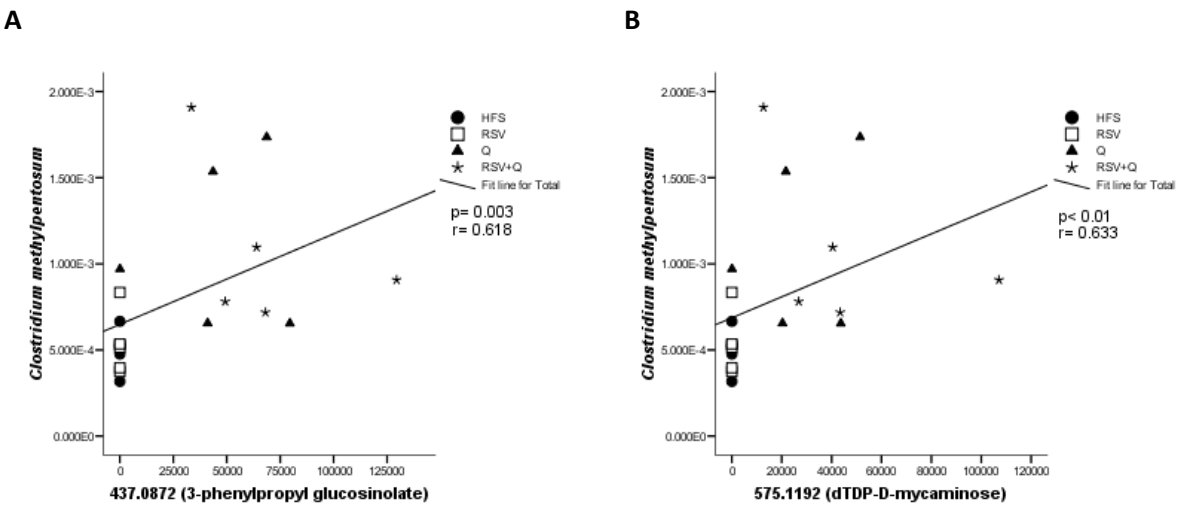
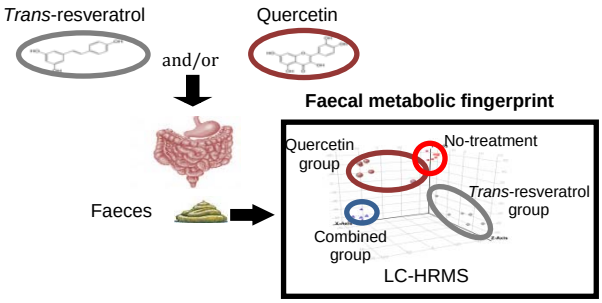


Fig. 4. Correlations between the number of bacteria (taxa frequencies detected in faecal samples) and putative metabolites detected in ESI positive mode. A) 3-Phenylpropyl glucosinolate levels and *Clostridium methylpentosum*, B) dTD-D-mycaminose levels and *Clostridium methylpentosum*. Insert corresponds 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.

Graphical Abstract



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