

**RESHAPING FAECAL GUT MICROBIOTA COMPOSITION BY THE INTAKE OF *TRANS*-RESVERATROL
AND QUERCETIN IN HIGH-FAT SUCROSE DIET-FED RATS**

Etxeberria U^{1,2}, Arias N^{3,5}, Boqué N⁴, Macarulla MT^{3,5}, Portillo MP^{3,5}, Martínez JA^{1,2,5,*}, Milagro FI^{1,2,5}

¹Department of Nutrition, Food Science and Physiology, University of Navarra. C/Irunlarrea s/n, 31008 Pamplona, Spain

²Centre for Nutrition Research, University of Navarra. Irunlarrea St. E-31008 Pamplona, Spain

³Nutrition and Obesity group, Department of Nutrition and Food Sciences, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria, Spain

⁴Nutrition and Health Research Group. Technological Center of Nutrition and Health (CTNS), TECNIO, CEIC S. Avinguda Universitat 1, 43204 Reus, Spain

⁵Physiopathology of Obesity and Nutrition, CIBERObn, Carlos III Health Research Institute, 28029 Madrid, Spain

*Corresponding author: Professor J. Alfredo Martínez

Department of Nutrition, Food Science and Physiology, University of Navarra. C/Irunlarrea s/n, 31008 Pamplona, Spain. Tel.: +34 948425600 (80 6424)

E- mail address: jalfmtz@unav.es

Abstract

Diet-induced obesity is associated to an imbalance in the normal gut microbiota composition. Resveratrol and quercetin, widely known for their health beneficial properties, have low bioavailability and, when reach the colon, they are targets of the gut microbial ecosystem. Hence, the use of these molecules in obesity might be considered as a potential strategy to modulate intestinal bacterial composition. The purpose of this study was to determine whether *trans*-resveratrol and quercetin administration could counteract gut microbiota dysbiosis produced by high-fat sucrose diet (HFS) and in turn, improve gut health. Wistar rats were randomized into four groups fed a HFS diet supplemented or not with *trans*-resveratrol (15 mg/kg BW/day), quercetin (30 mg/kg BW/day) or a combination of both polyphenols at those doses. Administration of both polyphenols together prevented body-weight gain and reduced serum insulin levels. Moreover, individual supplementation of *trans*-resveratrol and quercetin effectively reduced serum insulin levels and insulin resistance. Quercetin supplementation generated a great impact on gut microbiota composition at different taxonomic levels, attenuating *Firmicutes/Bacteroidetes* ratio and inhibiting the growth of bacterial species previously associated to diet-induced obesity (*Erysipelotrichaceae*, *Bacillus*, *Eubacterium*

cylindroides). Overall, the administration of quercetin was found to be effective in lessening HFS diet-induced gut microbiota dysbiosis. In contrast, *trans*-resveratrol supplementation alone or in combination with quercetin, scarcely modified the profile of gut bacteria, but acted at intestinal level altering the mRNA expression of tight-junction proteins (TJPs) and inflammation associated genes.

Keywords: flavonol; stilbene; gut bacteria; inflammation; obesity; intestinal permeability

1. Introduction

Obesity is regarded as a major public health issue with an increasing worldwide prevalence [1]. Strong evidence support a direct link between intestinal dysbiosis, namely alterations of the gut microbial composition, and metabolic diseases encompassing obesity [2, 3] diabetes [4], liver disease [5, 6], cardiometabolic complications [7] and even cancer [8]. These findings have driven research interest to converge on making clear the intimate relationships among the gut microbiota, diet, host metabolism and the immune system [9]. In this context, the current use of genomic-based molecular techniques such as transcriptomics, metabolomics and metagenomics, together with the involvement of *in vivo* host models (i.e. germ-free animals) have allowed to boost the studies aiming to identify the most representative and significant gut bacterial groups or species in different disease conditions [10-12]. This specific characterization is helping to elucidate the role of gut microorganisms in the development of metabolic diseases [13]. Obesity has been characterized by a specific “bacterial trademark” represented by enlarged *Firmicutes* and reduced *Bacteroidetes* phyla [14], even if the association with the latter still remains being a matter of debate [15, 16]. Furthermore, dysbiosis produced by high-caloric diets, i.e. high-fat diets [17, 18], has been also demonstrated to be susceptible to modulation by a variety of components such as probiotics and prebiotics [19]. Apparently, non-digestible polysaccharides could come to the aid of proliferating “friendly bacteria” and enhance microbial diversity in the gut [20], which might contribute to the amelioration of obesity and obesity-associated metabolic disorders [21].

Natural bioactive compounds are widely recognized to have potential biological properties [22, 23], such as anti-adiposity [24] and anti-diabetic effects [25, 26]. Within the existing plant secondary metabolites, *trans*-resveratrol (stilbene) and quercetin (flavonoid) have been described to exert anti-inflammatory, anti-obesity effects [27-29] and might be potential candidates for the amelioration of metabolic impairments (i.e. insulin resistance) associated to comorbidities of overweight or obesity [30, 31]. Nevertheless, the health outcomes are intimately dependent on the dose and the form they are provided, and also on their bioavailability within the organism [32]. Overall, it is estimated that around 5-10 % of the total dietary polyphenols are absorbed in the small intestine, while 90-95 % of the total polyphenol intake reach the colonic region unabsorbed being the subject to enzymatic activities of gut microbial ecosystem producing diverse metabolites with a variety of physiological roles [33]. Although the metabolism of these bioactive compounds has been well reported [34-36], few studies have described its bioconversion by gut bacteria [37]. Nevertheless, in view of the direct contact and the dual interaction existing between these natural bioactive compounds and the colonic

gut microbial ecosystem [38], possible composition modifications in intestinal bacteria might be expected as a result of the consumption of these compounds, which might ultimately contribute to functional modifications in the host.

Therefore, the present study was conducted to assess the potential of *trans*-resveratrol, quercetin, and a mixture of *trans*-resveratrol and quercetin supplementation to reverse HFS diet-induced gut microbiota dysbiosis in rats. Furthermore, this research work sought to analyse whether intestinal barrier integrity and inflammation might be affected as a consequence of polyphenol intake.

2. Material and Methods

2.1. Animals, diets and serum biochemical parameters

The experiment was performed with a sub-study of 23 Wistar rats obtained from Harlan Ibérica (Barcelona, Spain). Animals were single housed 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. Concisely, the artificial light/ dark cycle in this experiment was inverted, so that light was switched off at 9:00 am in the morning and switched on at 21:00 pm in the evening so that animals were expected to start eating as soon as the dark cycle began. Rats were fed a standard-chow diet (C; 2.9 kcal/g) from Harlan Iberica (ref. 2014, Barcelona, Spain) during an adaptation period that lasted six days. Afterwards, animals were randomly distributed into four groups and changed to a HFS commercial obesogenic diet (ref. D12451M) OpenSource, (HFS; 4.7 kcal/g) for 6 weeks. The HFS diet contained 20 % of energy as proteins, 35 % of energy as carbohydrates (17 % sucrose, 10 % maltodextrin and 7 % of corn starch) and 45 % of energy as fat (31.4 % as saturated fats, 35.5 % as monounsaturated fats, 33.1 % as polyunsaturated fats). All animals had free access to food and water. The experimental groups were distributed as follows: the control group (HFS; n=5), rats were fed the HFS diet; *trans*-resveratrol group (RSV; n=6), rats were supplemented with *trans*-resveratrol 15 mg/Kg BW/ day; quercetin group (Q; n=6), rats were supplemented with quercetin 30 mg/kg BW/ day; and *trans*-resveratrol + quercetin group (RSV+Q; n= 6), rats were treated with a mixture of *trans*-resveratrol 15 mg/ kg BW/ day and quercetin 30 mg/ kg BW/ day. Polyphenols were daily incorporated to the powdered diet in quantities that ensured that each animal consumed the prescribed levels. Briefly, in a previous study, it was reported that resveratrol was degraded over the feeding period when it was mixed in the diet. Moreover, it was observed that rats started eating immediately upon daily diet replacement at the beginning of the dark period [39]. Thus, based on these assumptions and taking into account that resveratrol and quercetin have limited solubility in water, the compounds were added to the surface of the diet dissolved in ethanol. Variations in the amount of ethanol received by each animal were avoided as ethanol was added to the diet to reach 1

mL/kg BW/day. Although the volume of ethanol provided was negligible, control group was also given the same amount without natural compounds. Body weight and food intake were daily recorded. The doses of polyphenols were chosen based on previous studies [39-41]. *Trans-resveratrol* (> 98 % purity) was supplied by Monteloeder (Elche, Spain) and quercetin (\geq 98 % purity) by Sigma-Aldrich (St. Louis, MO, USA). 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- Strasbourg 1986, Directive 2003/65/EC and Recommendation 2007/526/EC). Fasting serum glucose was measured using a Glucose oxidase/peroxidase kit (Ref. 110504, ByoSystems, Barcelona, Spain) and insulin levels were determined by using a specific ELISA Kit according to the protocol described by the manufacturer (Ref. 10-1250-01, Mercodia AB, Spain). Insulin resistance was assessed by the homeostasis model assessment of insulin resistance (HOMA-IR) formula [42]: [serum glucose levels (mmol/L) X insulin levels (mU/L)]/22.5. Inflammatory related markers were determined in serum with commercial ELISA Kits. TNF- α levels were determined with the TNF-alpha Platinum ELISA Kit (EBioScience, Viena Austria), lipopolysaccharide (LPS) levels by the use of Limulus Amebocyte Lysate (LAL) QCL-1000™ chromogenic assay (Lonza, Walkersville, MD) and LPS-binding protein (LBP) levels using the Mouse LBP ELISA Kit (Cell Sciences, Canton, MA).

2.2. *Faeces collection*

Fresh faecal samples were collected at the end of the intervention period early in the morning, prior to the overnight fasting. Each animal was taken one by one and located in a clean, single cage with the aim of obtaining faeces directly after defecation. A soft abdominal massage was also exerted to the animals in order to facilitate bowel movement and in turn, fresh faeces collection. Samples were gathered in 15ml Falcon tubes and immediately frozen at -80 °C for future analyses.

2.3. *Tissue collection*

At the end of the experimental period (6 weeks), animals were fasted overnight to assure on one hand, that all the animals were sacrificed in similar “feeding” conditions without the bias of the dietary treatment and on the other hand, to guarantee the suitability of biochemical values. Sacrifice was conducted under anaesthesia (chloral hydrate) by cardiac exsanguination between 9:00 and 12:00 am. Liver was collected, weighted and then stored frozen at -80 °C. In all animals colonic mucosa was excised carefully by scratching and frozen immediately in liquid nitrogen for future RNA isolation.

2.4. DNA extraction, barcoded PCR and bacterial 16S rDNA pyrosequencing

Faecal DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), following suppliers instructions. DNA was measured by NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Delaware, USA). Samples for 454 pyrosequencing were amplified for the 16S rDNA hyper-variable regions (V4 to V6) using 16S-0515F (5'-TGYCAGCMGCCGCGGTA-3') and 16S-1061R (5'-TCACGRACGAGCTGACG-3') universal primers. With the Roche 454 FLX chemistry, read lengths up to 550nt are achieved on PCR products which are in the range of 600-800nt, when using the off-instrument in amplicon mode. This mode favors the sequence quality of sequencing wells producing high signals. Since the 16S molecules are very homogeneous and balanced in nucleotide composition, this mode produces better results than the shotgun mode, which is more tolerant to low signal beads, typically carrying GC- or extreme AT rich contents. The V4-V6 amplicon being 560nt in length for most bacteria, the full amplicon sequence can be recovered using a library construction protocol involving blunt ligation of sequencing adapters, the so-called Y-adapters, carrying DNA barcodes referred to as "multiplex identifiers" (MIDs) by the manufacturer. Sequencing on the 454 instrument is single-ended by nature, yet through the blunt ligation approach, approximately as many molecules are sequenced from the PCR 5' end as there are molecules sequenced from the PCR 3' end.

Given the unit read length, the overlap between forward and reverse sequences is in the order of 90% and spanning the 3 variable regions. Thus, a sequence clustering approach using a *de novo* EST assembly process becomes possible without running the risk of generating chimeric sequence consensus. The Mira assembler [43] in EST mode can handle the kind of copy number variation corresponding with relative abundance differences observed among bacterial species in a metagenome sample. At the same time, this assembler can be made tolerant to single base changes often occurring between individual copies of the 16S gene on a bacterial chromosome, which can be in the order of zero to two nucleotide changes over 100 nucleotides. The Mira assembler has a 454 specific error correction model and thus is capable of making the distinction between instrument basecall errors and intrinsic base-pair changes. Furthermore, combining forward and reverse sequences into a single consensus sequence achieves not only full length coverage but also increased sequence reliability, since the reverse sequences have high quality where the forward sequences exhibit quality drop-off and vice versa. Samples with high species diversity will generate a lower proportion of reads clustering than samples with more limited diversity.

2.4.1. *Sequence post-processing*

The off-instrument 454 downstream processing software was used in amplicon mode to optimize read length as outlined above and to de-multiplex the libraries and trim off 3' end adapter sequences, if any. The sequencing of the 23 amplicon libraries produced 16S rDNA reads ranging from 9780 to 71690, generating read lengths from 479 to 520nt, with a modal of 510nt.

The Mira assembler was used at 98% similarity threshold, meaning sequences carrying up to 2 high quality differences out of 100 in any interval were allowed to assemble alongside with similar sequences. Singletons were carried forward into identification, provided their sequence length equalled at least 400nt. The same criterion was applied to contig consensus sequences produced. The proportion of reads producing clusters ranged from 36.1% to 58.5% on a per sample basis.

The singleton and consensus sequence were blasted using NCBI blastall version 2.2.22 in blastN mode, using an expectation value cut-off of 10^{-5} and requesting the 25 best hits produced against the RDP database release 10 update 29, which was curated for redundant entries representing identical sequence and entries with taxonomic classification of less than 4 levels down, counting from the "bacteria" taxon. These hits were duplicated according to the per contig sequence count and kept as-is for singletons, before being provided to the MEGAN classifier, version 4 [44]. MEGAN incorporates alpha rarefaction and assigns operational taxonomic units (OTUs) by comparing taxonomic information associated with equivalent best hits for each input sequence. Construction of libraries and sequencing were all provided as a custom service of Beckman Coulter Genomics.

2.5. *Gene expression in colonic mucosa*

Colonic mucosa RNA was extracted using All-Prep[®] DNA/RNA Mini kit as described in the manufacturer's instructions (Qiagen, Germantown, Md., USA). RNA concentration and quality were measured with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Subsequently RNA (2 µg) was reverse-transcribed to cDNA using MMLV Moloney murine leukemia virus reverse transcriptase (Invitrogen) as described by the suppliers. RT-PCR assays were carried out following the manufacturer's instructions using an ABI PRISM 7000 HT Sequence Detection System, Taqman Universal Master Mix and predesigned TaqMan[®] Assays-on-Demand (Applied Biosystems, Texas, USA). Interleukin 18 (IL-18), Rn01422083_m1; Nuclear factor kappa β (NFκβ), Rn00595794_m1; Tumor necrosis factor α (TNF-α), Rn01525859_g1; Toll-like receptor 2 (TLR2), Rn02133647_s1; Toll-like receptor 4 (TLR4), Rn00569848_m1; Lipopolysaccharide binding protein (LBP), Rn00567985_m1;

Myeloid differentiation primary response 88 (MyD88), Rn01640049_m1; Killer cell lectin-like receptor subfamily K, member 1 (NKG2D or Klrk1), Rn00591670_m1; Tight-junction protein 1 (TJP-1), Rn 02116071_s1; Tight-junction protein 2 (TJP-2), Rn01501483_m1; Occludin (Ocln), Rn 00580064_m1. mRNA levels were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Rn 99999916_s1 as a housekeeping gene (Applied Biosystems). All samples were analysed in triplicate. The relative expression level of each gene was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.6. Short chain fatty acid (SCFA) analysis

Short chain fatty acids were analysed in faeces. Briefly, 500 μ L of acid water, containing internal standard (propionic acid-d6, 98 atom %D), was added to 25 mg of each lyophilized sample. Overnight lyophilization was carried out protecting samples from light. Mixture was shaken for 1 minute. Afterwards, extraction was carried out with 500 μ L of diethyl ether followed by 1 minute of shaking and centrifugation at 4°C at 14000 rpm for 10 min. Supernatants were analysed by gas chromatography-mass spectrometry (GC/MS) in a 7890A Series gas chromatograph coupled to a 7000 GC/MS Triple Quad (Agilent Technologies, Santa Clara, U.S.A.). The chromatographic column was a J&W Scientific HP-FFAP (30 m x 0.25 mm i.d., 0.25 μ m film) (Agilent Technologies). A volume of 1 μ L of sample was automatically injected into a split/splitless inlet (in splitless mode), which was kept at 175°C. Helium (99.999 % purity) was used as a carrier gas, at a flow rate of 1 ml/min in constant flow mode. The oven program was set at an initial temperature of 90°C, increased to 150°C at a rate of 12°C/min, then increased to 240°C at a rate of 20°C/min, and held at 240°C for 5 min. Ionization was done by positive chemical ionization (PCI) with methane gas. SIM mode and GC-QqQ MassHunter software (Agilent Technologies) were used for data acquisition.

2.7. Statistical analysis

Results are expressed as the mean \pm standard error of the mean (SEM). Normality was tested by *Shapiro- Wilk* test. For parametric variables, One-Way ANOVA test followed by Dunnett post- hoc test was carried out. For non-parametric variables, statistical significance of differences among the groups was tested by non-parametric Kruskal- Wallis test followed by Mann Whitney *U*- test when $p < 0.05$, Bonferroni correction test was applied as a correction for multiple comparisons. SPSS 15.0 software (Chicago, USA) was used to perform statistical analysis.

3. Results

3.1. Animal characteristics

Body-weight related measurements and serum biochemical variables in the four experimental groups are reported in Table 1. Animals supplemented with a combination of *trans*-resveratrol and quercetin presented a trend towards prevention of body weight gain induced by a HFS diet intake and showed a slight reduction in liver proportions (% body weight) (Table 1). Moreover, supplementation with *trans*-resveratrol and quercetin alone or in combination significantly reduced serum insulin levels. HOMA-IR was also decreased in the groups supplemented with *trans*-resveratrol or quercetin (Table 1). In addition, serum inflammation- and endotoxin-related markers (LBP, LPS and TNF- α levels) were determined. Although, slightly higher amounts were observed for LPS and TNF- α in *trans*-resveratrol supplemented rats, these results were not statistically significant (data not shown).

3.2. Gut microbiota analysis

At phylum level, quercetin supplementation was found to considerably modify relative percentages of detected phyla when compared to the HFS diet-fed control rats (Table 2). Quercetin supplementation was able to marginally ($p= 0.053$) reduce *Firmicutes* (-34.2 %). This value reflects the percentage of change measured taking into account the mean relative abundance of *Firmicutes* phylum in quercetin supplemented group and the mean relative abundance of this bacterial group in the HFS diet-fed group, which is considered the reference group in the present study. Thus, these data suggest that after quercetin supplementation this bacterial phylum usually up-regulated in rats fed a HF diet [45], was reduced in a 34.2 % when measured against the levels found in the reference group, although this diminution was not statistically significant after correction with the Bonferroni multiple comparison test. Moreover, a considerable decrease (-80.5 %) was detected in *Firmicutes/Bacteroidetes* ratio of animals supplemented with quercetin (Figure 1). In contrast, bacterial composition of faeces from the groups supplemented with *trans*-resveratrol or the combination of *trans*-resveratrol and quercetin, was not significantly affected at phylum level.

At class level, *Erysipelotrichi* (classified within the *Firmicutes* phylum), was the most affected bacterial group after quercetin supplementation, as the mean relative abundance of this bacterial group was significantly ($p= 0.018$) reduced (-83.9 %) compared to the HFS diet-fed control rats (appendix Table A.1).

At family level, groups supplemented with quercetin and the combination of *trans*-resveratrol and quercetin, presented a bacterial profile which substantially differed from the relative abundance of bacterial families detected in the faeces of the HFS diet-fed control and *trans*-resveratrol supplemented rats, being this variation especially notable for the *Erysipelotrichaceae* family (Figure

2). Nevertheless, *trans*-resveratrol supplementation produced a statistically significant inhibition in the *Gracilibacteraceae* family (-57.7 %) compared to the HFS diet-fed control rats. Relative abundance (% of total 16S rDNA) of the most representative families is also reported (Table 3).

Regarding genus level, quercetin supplementation for 6 weeks significantly reduced the levels of *Bacillus* genus (-74.3 %) when compared to the HFS diet-fed rats (appendix table B.1). In contrast, *trans*-resveratrol supplementation alone significantly lessened the abundance of *Parabacteroides* genus (-76.3 %) compared to the mean relative abundance found in the reference group (appendix table B.2). The combination of both polyphenols did not produce any changes at this taxonomic level.

At species level, the group supplemented with quercetin showed a statistically significant inhibition in four bacterial species after multiple adjustments with Bonferroni test (Figure 3). In this regard, it is noteworthy the statistically significant downregulation detected in the mean relative abundance of *Eubacterium cylindroides* (-80.4 %) compared to the relative percentage found in the control group (Figure 4). Moreover, supplementation with *trans*-resveratrol significantly reduced the mean relative abundance of different *Clostridia* species such as *Clostridium aldenense* (-93.1 %), *Clostridium hathewayi* (-73.2 %), *Clostridium* sp. C9 (-76.3 %), *Clostridium* sp. MLG661 (-53.7 %) in opposition to the mean relative abundance of *Clostridium* sp. XB90 (266.6 %) that was notably enhanced when compared to the HFS diet-fed control rats (Figure 3). Furthermore, the percentage of change in the mean relative abundance of *Gracilibacter thermotolerans* (-57.7 %) and *Parabacteroides distasonis* (-77.4 %) was negatively affected by *trans*-resveratrol in comparison to that detected in the HFS diet-fed control group (Figure 3). As far as the effect resulted from the combined consumption of both polyphenols is concerned, the relative abundance of two *Clostridia* species (*Clostridium clariflavum*, 327.0 % and *Clostridium methylpentosum*, 245.6 %), as well as the relative abundance of a member of *Lachnospiraceae* family (*Blautia stercoris*, 321.7 %) were significantly enhanced compared to the percentage determined in the reference group (Figure 3). Moreover, although non-statistically significant, the relative percentage of diverse bacterial species was also found to be differentially affected by the intake of polyphenols (appendix Table C.1, Table C.2 and Table C.3).

In addition, *Akkermansia muciniphila* ATCC BAA-835, the unique bacteria belonging to *Verrucomicrobia* phylum, showed a notable increase (1384.0 %) after the 6 weeks period of quercetin intake when compared to the relative abundance detected in diet-induced overweight control rats (Figure 5). However, these differences were not statistically significant. Furthermore, the relative abundance (% of total 16S rDNA) of bacterial species represented in Figure 6, especially belonging to *Bacteroidia* class, such as *Bacteroides* sp. S-18 (105.2 %), *Bacteroides* sp. dnLKV (87.8 %), *Barnesiella intestinihominis* (127.6 %), *Bacteroides dorei* (295.0 %), *Bacteroides chinchillae* (334.8 %),

Candidatus Prevotella conceptionensis (200.5 %) were boosted by quercetin supplementation, whereas one species belonging to *Deltaproteobacteria* class (*Bilophila wadsworthia*, -36.9 %) was downregulated in comparison to the HFS diet-fed control group. These species, even if the differences were not statistically significant, were found to be the most affected after a 6 weeks dietary treatment with quercetin.

3.3. Gene expression in colonic mucosa

After 6 weeks of supplementation, the rats treated with quercetin showed a significantly higher colonic expression of IL-18. In contrast, the intake of *trans*-resveratrol produced a considerable increase in most of the measured inflammation-related parameters reaching statistical significance with TLR4, LBP and IL-18 gene expression (Figure 7). No statistical differences were found in colonic inflammation-related gene expression in the rats supplemented with the combination of both polyphenols.

Regarding variables related to intestinal permeability, those experimental groups supplemented with *trans*-resveratrol alone or with the mixture of *trans*-resveratrol and quercetin, showed a statistically significant upregulation in the expression levels of TJP-2 and OcIn gene ($p < 0.05$). In contrast, quercetin supplementation did not seem to modify the intestinal barrier permeability associated markers (Figure 8).

3.4. SCFA analysis

SCFA profile detected by GC/MS analysis in faecal contents of the four experimental groups revealed no statistically significant differences. Supplementation with quercetin, although not significant, was found to slightly increase concentrations of acetate (2.00 ± 0.24 mg/g dry weight), propionate (0.51 ± 0.07 mg/g dry weight) and butyrate (0.58 ± 0.11 mg/g dry weight) when compared to the HFS diet-fed control rats (1.68 ± 0.39 , 0.41 ± 0.11 , 0.38 ± 0.11 mg/g dry weight, respectively). In contrast, dietary supplementation with *trans*-resveratrol alone and with the combination of *trans*-resveratrol and quercetin, produced no changes in SCFA levels (appendix Table D.1).

4. Discussion

Therapeutic effects have been attributed to resveratrol in metabolic derangements [46], and also to quercetin, mainly due to allegedly antioxidant and anti-inflammatory properties [47, 48]. Mechanisms surrounding their beneficial effects have attracted research interest [49]. Nevertheless,

studies investigating the interaction within bioactive compounds and gut microbes are rather limited [50-52]. The goal of the present research was to determine whether supplementation with *trans*-resveratrol and/or quercetin was able to modify gut microbiota composition, differing from microbial profile observed in HFS diet-fed control rats. Moreover, we sought to analyse whether these modifications in microbial ecology concur not only with body weight and biochemical changes, but also with alterations at intestinal epithelial level.

In the current research, dietary treatment with pure polyphenolic compounds, especially when jointly administered, decreased body weight gain. The lack of anti-obesity effect of quercetin is in agreement with data in the literature, which suggest the need of treatment periods longer than 9 weeks for this polyphenol to achieve an effective body fat lowering effect [53]. As far as resveratrol is concerned, the dose used in the present experiment was effective in reducing body fat after 6 weeks of treatment, but in a model of genetic obesity (Zucker *fa/fa* rats) [40, 54]. In a model of obesity induced by using the same high-fat high-sucrose diet than that used in the present study, our group previously showed that a dose of 6 mg/kg bw/day resveratrol did not induce changes in adipose tissue weight [39]. By contrast, a dose of 30 mg/kg bw/day was very effective [41]. It seems that in this obesity model a dose higher than 15 mg/kg bw/day is needed to effectively reduce body fat. The dosage used in the current experiment for resveratrol and quercetin was effective to decrease insulin levels and also, to improve insulin sensitivity, these findings support results from previous studies describing resveratrol and quercetin as potential candidates for the treatment of metabolic diseases such as insulin resistance [30, 31]. To our knowledge, few studies have explored the effects of pure resveratrol and quercetin consumption alone or combined, on resident microbiota composition in order to find a possible association between their beneficial properties in the host and specific gut microbial modifications [55]. In contrast, there is strong evidence supporting the role of gut microbiota in several metabolic functions [56]. Furthermore, modifications carried out in microbial ecology have been demonstrated to derive in host metabolic phenotype modulation, influencing host biochemistry and increasing host susceptibility to diseases [57, 58]. The obese metabolic phenotype transmissibility in germ-free mice that were transplanted the gut microbiota from obese (*ob/ob*) mice has been also proven [3]. Nevertheless, gut microbiota modifications have been attributed both, to genetic obesity [2], and to diet-induced alterations independent of obesity [45].

In general, obese gut microbiota has been repeatedly associated with a reduced *Bacteroidetes/Firmicutes* ratio [2, 59]. Our findings agree with this feature as HFS diet-fed control rats exhibited the highest *Firmicutes/Bacteroidetes* ratio. Interestingly, quercetin administration to the HFS diet prominently decreased this ratio. Likewise, animals supplemented with this polyphenol

showed a considerable reduction in *Firmicutes* phylum commonly dominating in obesity and overweight status [14, 60]. The enrichment of *Erysipelotrichaceae* family in diet-induced obesity has been similarly reported in several studies [61, 62]. Indeed, a metagenomic study carried out by Turnbaugh *et al.* [61] in mice that developed obesity by the intake of high-fat high-sucrose diet, it was concluded that the microbiome of HFS diet-fed mice was rich in genes encoding components of a major system, which is essential for the sugar uptake for most intestinal bacteria [61]. Thus, it was suggested that *Erysipelotrichaceae* contributed to the function of these genes leading to a more efficient energy extraction from the diet [61, 63]. In the current investigation, the potential of quercetin to deeply modify general bacterial profile at family level is underlined, indicating an important reduction in the relative abundance of *Erysipelotrichaceae* family, in contrast to what it was found with *trans*-resveratrol administration alone or in combination. Likewise, it was previously demonstrated by our group [17] that *Erysipelotrichi* taxa was profoundly enlarged (1025.4 %) after a HFS diet treatment for 6 weeks [17]. Interestingly, concerning the enrichment found in the *Bacteroidetes* phylum, quercetin supplementation also deeply increased the abundance of *Bacteroidaceae* and *Prevotellaceae* families, which were stated to be negatively influenced in high-fat-fed mice [45]. Besides, quercetin supplementation resulted in the reduction of the genus *Bacillus*, another predominant bacterium in Western diet-fed obese mice [64]. Surprisingly, *trans*-resveratrol solely did not produce significant modifications at the highest division levels, but was detected to follow the opposite pattern to the previously mentioned results. Furthermore, when combining both polyphenols together, gut microbiota modifications emulated quercetin's individual consequences although, these alterations were found to be weaker. Notwithstanding, at species level, *trans*-resveratrol supplementation significantly inhibited several bacterial species, such as *Gracilibacter thermotolerans*, *Parabacteroides distasonis* and species from *Clostridia* class (*Clostridium aldenense*, *Clostridium hathewayi*, *Clostridium* sp. MLG661), whereas it significantly enhanced *Clostridium* sp. XB90. Of especial mention, *Parabacteroides distasonis* has been found to be reduced in obese individuals [65] and suppressions in *Clostridium aldenense* and *Clostridium hathewayi* species, members of *Clostridium* cluster XIVa, have been reported to be major butyrate producers [66]. On the other hand, the differential sensitivity to polyphenols has been postulated indicating a higher sensitivity to phenolic compounds of Gram-positive bacteria rather than the Gram-negative bacteria, possibly attributable to changes in their wall composition [67]. The intake of quercetin positively affected the growth of three species (*Bacteroides vulgatus*, *Clostridium clariflavum* and *Clostridium* sp. MLG661) and strongly inhibited the abundance of *Eubacterium cylindroides*. *Bacteroides vulgatus* and *Clostridium clariflavum* were previously found to be diminished in obese subjects [65, 68] and HFS diet-fed overweight rats [17]. Indeed, *Eubacterium cylindroides* has been detected in faecal microbiota of obese humans [14], it was up-regulated in high-fat diet-induced obesity [14, 61] and

reported to be enlarged (4072.1 %) in rats fed a HFS diet for 6 weeks [17]. *Blautia stercoris*, *Clostridium clariflavum* and *Clostridium methylpentosum* were also up-regulated by the combination of polyphenols. In agreement with these results, *Clostridium* and *Eubacterium* have been considered as the main genera involved in the metabolism of a large variety of phenolic compounds, including isoflavones (daidzein), flavones (naringenin and ixoxanthumol), flavan-3-ols (catechin and epicatechin) and flavonols (quercetin and kaempferol) [69]. According to our data, given the poor absorption of quercetin into the bloodstream from the gut, the mechanisms contributing to the amelioration of insulin resistance might imply a direct gut microbiota modulatory effect. In contrast, resveratrol might act as an anti-microbial compound that counteracts the individual effects of quercetin, suggesting that the improvements in insulin resistance could be due to indirect mechanisms which do not involve big changes in gut microbiota composition.

Given the connection between gut microbiota, metabolism and intestinal epithelium [70], the expression of genes implicated in the permeability of the gut epithelium was analysed. It is widely known that intact mucosal barrier is essential to avoid the development of inflammation [71]. Indeed, impaired intestinal barrier function is regarded as a relevant contributory factor to obesity and associated metabolic disturbances [4, 72]. In the present study, although being aware that the fasting period to which animals were subjected before sacrifice might have an effect on intestinal integrity [73], an upregulation in the expression of TJPs and OcIn was found to be induced by the administration of *trans*-resveratrol alone and when combined with quercetin, which was not perceived with the intake of quercetin alone. Furthermore, the expression levels of inflammation-associated genes were induced by *trans*-resveratrol supplementation such as, the expression of TLR2 and specially, TLR4, LBP and IL-18. TLRs, more specifically TLR2 and TLR4, have been largely implicated in inflammatory states in the gastrointestinal tract [74-76]. TLRs comprise a family of pattern-recognition receptors that identify conserved molecular products of microorganisms, such as lipopolysaccharides and lipoteichoic acid (LTA) [77]. Thus, TLR2 recognizes Gram-positive bacteria derived products, whereas TLR4 is stimulated by LPS derived from Gram-negative bacteria [77]. Their expression is dependent on dysbiosis associated to altered host-bacterial interactions [78]. Indeed, TLRs act as sensors of microbial infection, initiating inflammatory and immune defence responses [79]. However, the bacterial ligands are shared by all classes of bacteria, being also produced by commensal microorganisms [80]. In line with this statement, Rakoff-Nahoum *et al.* demonstrated a non-immune function of TLRs in the maintenance of epithelial homeostasis [80]. Based on the recognition of commensal bacteria as health-promoting “symbionts”, emerging evidence has confirmed the role of luminal microbes to signal the interfacing epithelial layer, to control the turnover rate of enterocytes and to fortify the epithelial turnover regenerative and barrier functions

[81]. Rakoff-Nahoum *et al.* suggested a beneficial role generated from the recognition of commensal bacteria by TLRs [80]. The recognition of commensal bacteria and posterior activation of TLRs was hypothesized to induce generation of protective factors (i.e. cytokines) upon epithelial damage that have been previously reported *in vivo* and *in vitro* to play a beneficial role by initiating repair responses (i.e. process known as restitution) [82] and have been defined as markers of protective responses in colonic tissue [80]. In fact, a growing body of evidence indicates the relevance of certain mediators (IL- α , IL-1 β , IL-18) to preserve mucosal homeostasis, as well as to protect and restore epithelial barrier [83]. In particular, the up-regulation of IL-18 (a family member of IL-1) found in our study might be related with its dichotomous role in the gut, as IL-18 derived from intestinal epithelial cells has been described to facilitate tissue repair and promote mechanisms to induce homeostasis in early phase of inflammation [84]. Experiments conducted by other researchers have concluded the essential role of IL-18 to maintain epithelial integrity and prevent the translocation of bacteria, associating this feature to the activation of TLRs in inducing the signal to repair [85, 86]. From our data, a presumable promotion of intestinal epithelial repair by protective factors (i.e. IL-18) induced by commensal bacteria-derived activation of TLR signalling might be hypothesized, particularly after *trans*-resveratrol administration as a possible mechanism leading to intestinal epithelial homeostasis. It might be speculated that although non-statistically significant, inflammation- and endotoxin-related markers (LBP, TLR4, TLR2 expression levels, LPS serum levels) showed a trend to a subtle increase after *trans*-resveratrol supplementation. This outcome might underlie a slight inflammation and immune response taking place in the intestine [87]. TLRs could be acting upon damage as a defence mechanism to prevent bacteria and bacterial products translocation through rearrangement of tight-junction proteins to enhance intestinal epithelial integrity [88-90]. Nevertheless, these results deserve further research in order to confirm the mechanisms through which polyphenols interact with gut microbiota to cause host gene expression modifications, and also to be able to conclude the overall health consequences of this interactions in health.

Conclusions

From this experiment, it was concluded that the intake of *trans*-resveratrol and quercetin at the same time acted synergistically to reduce body-weight gain. Moreover, administration of both polyphenols alone or in combination improved basal insulin levels and HOMA-IR index. On the other hand, one of the major findings was related to the impact of quercetin supplementation on gut microbiota composition. In fact, treatment with quercetin attenuated the increase in *Firmicutes/Bacteroidetes* ratio in HFS diet-fed rats and notably modified gut microbial pattern at family level, significantly reducing the abundance of *Erysipelotrichaceae* and the genus *Bacillus*,

closely related to the consumption of western diets and body weight gain. Furthermore, supplementation with quercetin produced a significant alteration in specific bacterial species, rising some that have been inversely related to obesity (*Bacteroides vulgatus*, *Akkermansia muciniphila*) and reducing the relative abundance of others associated to diet-induced obesity (*Eubacterium cylindroides*, *Bilophila wadsworthia*). In contrast, after *trans*-resveratrol supplementation no profound effects on gut microbiota composition were identified, especially at the highest division levels. Indeed, from results combining *trans*-resveratrol with quercetin, it can be speculated that individual effects attributed to quercetin were lessened. On the other hand, *trans*-resveratrol supplementation altered the mRNA expression levels of TJPs as well as some inflammation associated markers.

In summary, this study demonstrates the gut microbiota modulatory role of quercetin when administered to HFS diet-fed overweight animals, and left some doubts for future research about the mechanisms of *trans*-resveratrol to exert health benefits due to its uncertain potential to modulate gut microbial population and its impact on host intestinal gene expression. Limitations of this study are that there is no a group fed with a standard diet to identify the modifications induced by the type of diet to conclude whether polyphenols, especially quercetin, reversed diet-induced gut microbiota dysbiosis. On the other hand, this study could not differentiate whether the outcomes discovered were derived from the original compounds provided or from the metabolites produced in the gut. Another issue to be considered is the fact that SCFA measurements were conducted *ex-vivo* in faeces, which could not be an estimation of the real production of SCFA, as rats are caecum fermenters and a great amount of these compounds (95-99 %) are absorbed in the large intestine [91]. In addition, the measurement of the mRNA levels of TJPs is an indirect method to assess the impact of these polyphenols on gut integrity; gene expression levels are not always a reflection of their functional active proteins. As a result, we should be cautious as further analyses are required to be able to draw conclusions about the protective functions of these polyphenols on intestinal epithelial barrier. Furthermore, an important question addressed in this study is that the positive effects derived from the combination of *trans*-resveratrol and quercetin in body weight, were not explained by gut microbiota modifications. Quercetin produced the most profound effect on gut microbial ecology, and this outcome was associated with significant metabolic improvements. In contrast, the administration of *trans*-resveratrol also improved some metabolic derangements, but this effect was not mediated by a modulatory effect on gut microbiota. From the present data, it could be stated that quercetin supplementation, robustly reshapes gut microbiota dysbiosis associated to a westernized dietary intake, which presumably might contribute to beneficial metabolic effects. Besides, from the interaction between polyphenols and bacteria, changes at

intestinal level might be postulated in the host (local inflammatory tone and intestinal mucosal barrier integrity), principally with *trans*-resveratrol administration.

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Abbreviations

HFS, high-fat sucrose; TJPs, tight-junction proteins; HOMA-IR, homeostasis model assessment of insulin resistance; LPS, lipopolysaccharide; LBP, LPS-binding protein; MIDs, multiplex identifiers; OTU, operational taxonomic unit; IL-18, interleukin-18; NF κ B, nuclear factor kappa β ; TNF- α , tumor necrosis factor α ; TLR-2, toll-like receptor 2; TLR-4, toll-like receptor 4; MyD88, Myeloid differentiation primary response 88; NKG2D or Klrk1, Killer cell lectin-like receptor subfamily K, member 1; TJP-1, tight-junction protein 1; TJP-2, tight-junction protein 2; OcIn, occludin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SCFA, short-chain fatty acid; GC/MS, gas-chromatography-mass spectrometry; PCI, positive chemical ionization; SEM, standard error of the mean; LTA, lipoteichoic acid.

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TABLE LEGENDS

1. **Table 1. Body weight-related measurements and serum biochemical parameters of animals fed with a HFS diet supplemented or not with *trans*-resveratrol and quercetin for 6 weeks.**

All results are expressed as the mean \pm 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, 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.

2. **Table 2. Relative abundance (% of total 16S rDNA) of detected bacterial phyla in faeces of HFS diet-fed rats supplemented or not with *trans*-resveratrol and quercetin for 6 weeks.**

All results are expressed as the mean \pm SEM. Statistical analyses were performed using Kruskal-Wallis followed by Mann Whitney *U* test and p values were corrected with the Bonferroni test. $t < 0.1$ after correction with the Bonferroni multiple comparison test was set out as marginal trend. NS, non-significant values after Kruskal-Wallis comparison test. 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.

3. **Table 3. Relative abundance (% of total 16S rDNA) of the most representative families in faeces of HFS diet-fed rats supplemented or not with *trans*-resveratrol and quercetin for 6 weeks.**

Results are expressed as the mean \pm SEM of the relative abundance (% of total 16S rDNA) of different families detected in faecal samples by 16S rDNA pyrosequencing. Statistical analyses were performed using Kruskal-Wallis followed by Mann Whitney *U* test and p values were corrected by Bonferroni test, considering $p < 0.05$ statistically significant after correction with the Bonferroni multiple comparison test. Data with different superscript letters are significantly different. NS, non-significant values after Kruskal-Wallis comparison test. 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.

FIGURE LEGENDS

1. **Figure 1. *Firmicutes/Bacteroidetes* ratio of HFS diet-fed rats supplemented or not with *trans*-resveratrol and quercetin for 6 weeks.** Results are expressed as mean \pm SEM of the relative abundance (% of total 16S rDNA) of each phylum. 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.
2. **Figure 2. Relative bacterial composition at family level (% of total 16S rDNA) in faeces of HFS diet-fed rats supplemented or not with *trans*-resveratrol and quercetin for 6 weeks.** All data are displayed on a relative scale based on the 16S rDNA frequency taxa. 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.
3. **Figure 3. Relative abundance (% of total 16S rDNA) of bacterial species significantly different from the HFS diet-fed control rats.** Results are expressed as the mean \pm SEM. Statistical analyses were performed using Kruskal-Wallis followed by Mann Whitney *U* test and p values were corrected with the Bonferroni test. * $p < 0.05$

after correction with the Bonferroni multiple comparison test. NS, non-significant values after Kruskal-Wallis comparison test. * $p < 0.05$, 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.

4. **Figure 4. Relative abundance (% of total 16S rDNA) of *Eubacterium cylindroides* in faeces of HFS diet-fed rats supplemented or not with *trans*-resveratrol and quercetin for 6 weeks.** Results are expressed as the mean \pm SEM. Statistical analyses were performed using Kruskal-Wallis followed by Mann Whitney *U* test and *p* values were corrected with the Bonferroni test. [§] $p < 0.05$, HFS vs Q after correction with the Bonferroni multiple comparison test. 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.

5. **Figure 5. Relative abundance (% of total 16S rDNA) of *Akkermansia muciniphila* ATCC BAA-835 in faeces of HFS diet-fed control rats supplemented or not with *trans*-resveratrol and quercetin for 6 weeks.** Results are expressed as the mean \pm SEM. Statistical analyses were performed using Kruskal-Wallis followed by Mann Whitney *U* test and *p* values were corrected with the Bonferroni test. 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.

6. **Figure 6. Relative abundance (% of total 16S rDNA) of bacterial species in faeces of HFS diet-fed control rats supplemented or not with *trans*-resveratrol and quercetin for 6 weeks.** Results are expressed as the mean \pm SEM. 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.

7. **Figure 7. Relative mRNA expression of inflammation-related genes in colonic mucosa.** Results are expressed as the mean \pm SEM. Statistical analyses were performed using Kruskal-Wallis followed by Mann Whitney *U* test. * $p < 0.05$, HFS vs RSV; [§] $p < 0.05$, HFS vs Q. TNF α , tumor necrosis factor α ; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4; LBP, Lipopolysaccharide binding protein; NF κ B, Nuclear factor kappa β ; IL-18, Interleukin 18; MyD88, Myeloid differentiation primary response 88; NKGd2, Killer cell lectin-like receptor subfamily K, member 1; 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.

8. **Figure 8. Relative mRNA expression of tight-junction proteins and occludin in colon.** Results are expressed as the mean \pm SEM. Statistical analyses were performed using Kruskal-Wallis followed by Mann Whitney *U* test, *p* values were corrected by Bonferroni test. * $p < 0.05$, HFS vs RSV; [#] $p < 0.05$, HFS vs RSV+Q. TJP-1, tight-junction protein- 1; TJP-2, tight-junction protein- 2; Ocln, occludin; 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.