Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry xx (2015) xxx–xxx

Journal of Nutritional Biochemistry

Reshaping faecal gut microbiota composition by the intake of *trans*-resveratrol and quercetin in high-fat sucrose diet-fed rats

U. Etxeberria[a,b], N. Arias[c,e], N. Boqué[d], M.T. Macarulla[c,e], M.P. Portillo[c,e], J.A. Martínez[a,b,e,*], F.I. Milagro[a,b,e]

[a]Department of Nutrition, Food Science and Physiology, University of Navarra, C/Irunlarrea s/n, 31008 Pamplona, Spain
[b]Centre for Nutrition Research, University of Navarra, Irunlarrea St. E-31008 Pamplona, Spain
[c]Nutrition 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
[d]Nutrition and Health Research Group, Technological Center of Nutrition and Health (CTNS), TECNIO, CEIC S. Avinguda Universitat 1, 43204 Reus, Spain
[e]Physiopathology of Obesity and Nutrition, CIBERobn, Carlos III Health Research Institute, 28029 Madrid, Spain

Received 24 June 2014; received in revised form 25 November 2014; accepted 7 January 2015

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 they 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 randomised into four groups fed an HFS diet supplemented or not with *trans*-resveratrol [15 mg/kg body weight (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 the intestinal level, altering the mRNA expression of tight-junction proteins 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 supports 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), has 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 characterisation is helping to elucidate the role of gut microorganisms in the development of metabolic diseases [13]. Obesity has been characterised by a specific "bacterial trademark" represented by enlarged *Firmicutes* and reduced *Bacteroidetes* phyla [14], even if the association with the latter still remains 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, nondigestible 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].

Abbreviations: HFS, high-fat sucrose; TJPs, tight-junction proteins; HOMA-IR, homeostasis model assessment of insulin resistance; LPS, lipopolysaccharide; LBP, LPS-binding protein; IL-18, interleukin- 18; NFκβ, nuclear factor kappa β; TNF-α, tumour necrosis factor α; TLR-2, toll-like receptor 2; TLR-4, toll-like receptor 4; TJP-1, tight-junction protein 1; TJP-2, tight-junction protein 2; Ocln, occludin; SCFA, short-chain fatty acid; GC/MS, gas-chromatography-mass spectrometry; SEM, standard error of the mean.

* Corresponding author. Department of Nutrition, Food Science and Physiology, University of Navarra, C/Irunlarrea s/n, 31 008 Pamplona, Spain. Tel.: +34 948425600 (80 6424).
E-mail address: jalfmtz@unav.es (J.A. Martínez).

http://dx.doi.org/10.1016/j.jnutbio.2015.01.002
0955-2863/

Natural bioactive compounds are widely recognised to have potential biological properties [22,23], such as antiadiposity [24] and antidiabetic effects [25,26]. Within the existing plant secondary metabolites, *trans*-resveratrol (stilbene) and quercetin (flavonoid) have been described to exert anti-inflammatory and antiobesity 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 reaches the colonic region unabsorbed, being the subject of 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 substudy 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°C±2°C) under a 12-h: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 a.m. and switched on at 9:00 p.m. 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 6 days. Afterwards, animals were randomly distributed into four groups and changed to an 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 body weight (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, the 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 (≥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, Strasburg 1986, Directive 2003/65/EC and Recommendation 2007/526/EC). Fasting serum glucose was measured using a glucose oxidase/peroxidase kit (ref. 110504; BioSystems, Barcelona, Spain), and insulin levels were determined by using a specific enzyme-linked immunosorbent assay (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)×insulin levels (mU/L)]/22.5. Inflammatory-related markers were determined in serum with commercial ELISA kits. Tumour necrosis factor (TNF)-α levels were determined with the TNF-alpha Platinum ELISA Kit (EBioScience, Vienna, Austria); lipopolysaccharide (LPS) levels, with the Limulus Amebocyte Lysate QCL-1000 chromogenic assay (Lonza, Walkersville, MD, USA); and LPS-binding protein (LBP) levels, with the Mouse LBP ELISA Kit (Cell Sciences, Canton, MA, USA).

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 15-ml 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, on one hand, to ensure 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 a.m. to 12:00 p.m. 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, bar-coded polymerase chain reaction (PCR) and bacterial 16S rDNA pyrosequencing

Faecal DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), following supplier's instructions. DNA was measured by NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Samples for 454 pyrosequencing were amplified for the 16S rDNA hypervariable regions (V4 to V6) using 16S-0515F (5'-TGYCAGCMGCCGCGGTA-3') and 16S-1061R (5'-TCACGRCACGAGCTGACG-3') universal primers. With the Roche 454 FLX chemistry, read lengths up to 550 nt are achieved on PCR products which are in the range of 600–800 nt when using the off-instrument in amplicon mode. This mode favourises 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. With the V4–V6 amplicon being 560 nt 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" 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 three 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 not only achieves full-length coverage but also increases 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 postprocessing

The off-instrument 454 downstream processing software was used in amplicon mode to optimise read length as outlined above and to demultiplex 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 520 nt, with a modal of 510 nt.

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 400 nt. 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.