Healthy Protection Of Bergamot Is Linked To The Modulation Of Microbiota

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Research article

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Abstract

Background: The present study aimed to evaluate the effects of a new food-grade bioavailable delivery system of bergamot on human gut microbiota, in order to demonstrate the potential correlation of microbiota modulation in cardiovascular health.

The identification of human gut microbiota modification was performed after *ex-vivo* incubation with bergamot phytosome (Vazguard™) of individual faecal slurries from healthy women (45–53 years) as follows: after incubation at 37°C in anaerobic condition, DNA was extracted and a 16S Metagenomic Sequencing Analysis performed.

Results: Twenty-five different phyla were identified, among which 4 were modulated: *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria*. The decreased *Firmicutes/Bacteroidetes* ratio and the increase o*f Proteobacteria* were observed indicating a positive modulation of microbiota possibly linked to cardiovascular health. 418 different genera were also identified, among which several of them were mildly modulated.

Conclusions: For the first time, a gut microbiome modulation was associated to the new delivery system of bergamot phytosome, supporting its clinical efficacy for cardiovascular health. New potential applications in weight control and gastrointestinal benefits were suggested.

Background

Intestinal microbiota is a complex ecosystem of microorganisms (such as bacteria, archeaebacteria, fungi and protists) resident in the gut [1]; their density and composition increase from stomach to colon and in the colon, about 10^{11} to 10^{12}/ml are the estimated bacteria content [2, 3]. A great number of studies have been dedicated in the last 15 years to explore human microbiota and its relationship with human diseases, such as cardiovascular and neurological disorders, inflammation, cancer, diabetes, and obesity [4], in order to identify modifications in gut bacterial composition possibly involved in a disease onset.

The high level of a mixture of flavonoids contained in the juice, albedo and flavedo of bergamot fruit is responsible for bergamot antioxidant and antisenescence properties [5]. Other beneficial effects such as hypoglycaemic and hypolipidaemic have been described for bergamot in metabolic syndrome and in cardiovascular diseases (CVD) prevention [6, 7].

A lecithin food-grade delivery system (Phytosome®) containing the Bergamot Polyphenolic Fraction (BPF) from *Citrus Bergamia* Risso et Poiteau from Calabria was developed by Indena SpA to ameliorate the oral absorption of flavonoids. The structural phytochemical composition of bergamot was deeply analysed by HPLC-DAD-MS and LC-NMR [8], and a recent clinical trial in individuals with type 2 diabetes mellitus and hyperlipaemia showed a reduction of cardiovascular risk by modulating total cholesterol

(tChol), low-density Lipoproteins (LDL), triglycerides (TG), high-density Lipoproteins (HDL) and blood glucose after just 30-day supplementation [9].

The aim of the present work is to study for the first time the effect of bergamot phytosome on human gut microbiota to find possible associations with its benefits on cardiovascular health.

Results

The 16S Metagenomic results were analysed in order to highlight the potential gut microbiota modulation produced by incubation of faecal samples with the bergamot phytosome.

Shannon Index as a measure of the entropy of Species-level classifications [10] for both test and control groups (Fig. 1) was comprised between 1.5 and 3.5, indicating a good evenness on samples.

By using Illumina 16S Metagenomics workflow analysis, 25 different phyla were identified, with *Firmicutes, Proteobacteria, Bacteroidetes* and *Actinobacteria* as the four major phyla, as described in Fig. 2. The bergamot phytosome induced a decrease in the *Firmicutes/Bacteroidetes* ratio from 2.27 (Control) to 1.79 (test). Considering that an increase of the F/B ratio is correlated with obesity [3, 11], bergamot phytosome is endowed with a great potential for counteracting body fat accumulation.

A significant increase of *Proteobacteria* in bergamot phytosome treated samples was also observed ($p < 0.01$ vs control), as reported in Fig. 3.

The *Actinobacteria* is the less represented phylum. Also in this case, an increased level was observed after bergamot phytosome incubation.

The analysis allowed identification of 418 different genera. Among them, 8 major genera (representing the 62% of those detected) were modulated, i.e. *Escherichia, Serratia, Bacteroides, Prevotella, Enterococcus, Bifidobacterium, Blautia* and *Faecalibacterium* (Fig. 4). More in detail (Fig. 5), an increase was observed in *Blautia, Bifidobacterium*, *Escherichia, Serratia* and *Bacteroides* while a reduction was observed for *Enterococcus, Prevotella*, and *Faecalibacterium*. Among them the observed difference for *Escherichia (*bergamot-treated 23.65 ± 2.32; control 17.34 ± 2.00) and *Serratia* (bergamot-treated 3.98 ± 0.38; control 2.67 ± 0.24) was statistically significant ($p < 0.05$).

Discussion

For the first time, the effects of a new food-grade bioavailable delivery system of bergamot formulated in phytosome on human gut microbiota were investigated and then correlated to cardiovascular health, obesity and gastrointestinal disorders, thus supporting bergamot phytosome benefits in these areas, due to its potential to modulate related human gut microbiota after incubation with individual faecal slurries from healthy volunteers.

Cardiovascular diseases (CVDs) are the first cause of death at global level: the World Health Organization declared that about 17.9 million people died from CVDs in 2016, corresponding to 31% of all global deaths (https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds).

Several factors, like smoking and alcohol use, poor diet, and obesity represent the major risks for CVDs [12], so prevention measures are of high importance in limiting the onset of diseases.

A large number of studies over ten years [13, 14] suggested a link between obesity and gut microbiota, i.e. the plethora of microorganisms living in the gastrointestinal tract [1].

In animal models, adiposity and weight increase can be affected by intestinal microbes. Mice of identical genotype exposed to a different environments and fed by a high-fat diet develop a distinct arrangement of gut microbiota, which influences the development of obesity and metabolic syndrome [15]. An alteration in the gut barrier induced by microbiota changes due to a high-fat diet in mice was also previously demonstrated [16, 17], and that condition led to metabolic diseases.

A human study conducted in obese and non-obese Danish people analysed genes from gut microbial populations identified individuals with low bacterial richness having more adiposity and dyslipidaemia in respect to high bacterial richness people [18], confirming a link between gut microbiota composition and obesity.

A recent paper reported a decrease in both triglycerides and LDL-C after 8-week treatments of a nutraceutical mixture containing BPF in dislypidemic overweight patients [19]. A significant reduction of serum low density lipoprotein cholesterol (LDL-C) and triglyceride was also demonstrated by a new bergamot phytosome formulation [9]: in that clinical study, the delivery by phytosome allowed increased oral absorption of the naringin, the major component of bergamot, in respect to standard unformulated bergamot. Actually, phytosome techniques have recently demonstrated the successful delivering of other low-soluble natural compounds, like quercetin [20] and Coenzyme Q10 [21].

In this experimental study, we explored the hypothesis that bergamot phytosome would be able to modulate the human gut microbiome. Among the 25 phyla detected, a decrease in the *Firmicutes/Bacteroidetes* ratio was observed, indicating involvement of bergamot phytosome in the field of weight control. In fact, it is well known that the *Firmicutes/Bacteroidetes* ratio value increases in the presence of an obesity status [2, 3].

The significant increase of *Proteobacteria* observed in bergamot phytosome-treated samples ($p < 0.01$ *vs* control) supports a positive effect for health status: a decrease in *Proteobacteria* was associated with an increased risk of cardiovascular diseases [22].

When analysis of genus was performed, an increased modulation in *Blautia* was observed in bergamot-treated samples. *Blautia*, involved in complex carbohydrate digestion, is recognized as a good indicator of the intestinal health, and its increase is beneficial. On the contrary, a *Blautia* reduction was observed in patients affected by heart failure [23], by hepatic and Chron's disease, and cancer [24–26]. Very

interestingly, it has just been published that *Blautia* genus is inversely related with visceral fat accumulation [27].

Data obtained in the present study reported an increase in *Bifidobacterium* in bergamot-treated samples, suggesting again a positive modulation in weight control, because *Bifidobacterium* is decreased in obese subjects [28]. In our experiment, a reduction in *Faecalibacterium* was also observed: in the same paper of Gao the reduction is found in obese subjects, indicating for the latter a non-positive balance. However, in a very recent paper [29] an increase in *Faecalibacterium prausnitzii* is believed to be a good indicator of a health condition. In this regard, we must to underline that a large number of study on microbiome in different conditions (such as healthy or ill population, gender differences) do not allow the formulation of unique conclusions. In the same way *Escherichia* and *Serratia* increase (observed in bergamot-treated samples) would appear a non-positive effect.

The advantage in the present study of using fresh faecal materials from healthy subjects is undoubted, in respect to animal models. The study otherwise has some limitations due to its pilot design: the limited number of subjects, and the treatment administered via 'incubation' in laboratory samples. Further clinical studies are needed to better explore the benefits of bergamot phytosome related to microbiome modulation, even though encouraging preliminary indications in the present study suggested a potential positive modulation of microbiota for CVD by bergamot phytosome and give a strong rational for further exploration.

Conclusions

In conclusion, for the first time, a microbiome modulation was associated to bergamot phytosome, supporting preliminary evidence of its clinical benefit for cardiovascular health and opening interesting new applications.

Methods

Samples collection

Faecal samples from 3 healthy women aged 45–53 years were collected in sterile containers, stored at -20 °C and then transferred at -80 °C until analysis. All subsequent methodologies were performed under anaerobic conditions to preserve the microbiota environmental. Donors had not used antibiotics in the previous 12 months.

Phytosome digestion

Bergamot Phytosome® (Vazguard®, Indena SpA) was used in gut microbiota experiments. A simulated gastric and duodenal human digestion of bergamot phytosome (1000 mg/L) was performed *in vitro* before incubation with faecal slurries, mimicking *in vivo* conditions. Briefly, phytosome was subjected to simulated gastric digestion by incubation at 37 °C for 60 minutes under shaking at pH 2 with HCl 1 M (to

simulate gastric conditions before meal under a fasting state) in the presence of the gastric enzyme pepsin [30]. Further incubation under shaking for 150 minutes at pH 7.0 with Sodium Carbonate 1 M and in the presence of bile extract pancreatin (ratio 6:1) was performed to simulate pancreatic digestion [31]. The bile extract contains glycine, taurine and the main biliar salts, while pancreatin was utilized as source of pancreatic lipases and colipases.

Ex-vivo incubation and samples analysis

Faecal slurries (1% w/v) from each individual were used to inoculate the batch-culture system containing the nutrient medium and the digested bergamot phytosome (test). A batch culture system without bergamot phytosome and a non-treated faecal slurry sample were also included in the experiments as negative background control. After 16 h of incubation at 37 °C under anaerobic condition, samples were centrifuged and DNA extracted. 16S Amplicon barcoded library were prepared and DNA sequence obtained by Next Generation Sequence utilizing the Miseq platform (Illumina Inc, CA, USA).

Bioinformatics Analysis

The 16S Metagenomics analysis were performed by using taxonomic classification of 16S rRNA targeted amplicon reads using an Illumina-curated version of the GreenGenes taxonomic database. The classification was performed by using the Illumina 16S Metagenomics workflow, as described in Wang Q. et al [32].

Statistical Analysis

Data were analysed by Student’s t test.

Abbreviations

HPLC-DAD-MS
High-Pressure Liquid Chromatograph-Diode Array Detector-Mass Spectrometry
LC-NMR
Liquid Chromatography with Nuclear Magnetic Resonance Spectroscopy
tChol
total cholesterol
LDL
low-density Lipoproteins
TG
triglycerides
HDL
high-density Lipoproteins
CVD
Cardiovascular
F/B ratio

Firmicutes/Bacteroidetes ratio
LDL-C
lipoprotein-cholesterol

Declarations

Ethics Approval and Consent to participate

The study was conducted in accordance with the Principles of Good Clinical Practice and the Declaration of Helsinki and was in agreement with the Ethical code of PTP Science Park (Lodi, Italy) (https://www.ptp.it/it/about-us#governance). The collection of fecal samples from donors healthy volunteers was performed after Written informed consent in an anonimyzed way, and any information cannot be directly related to each volunteer [33].

Consent for publication

All authors of the above manuscript, as well as the responsible authorities at both institutes where the work has been carried out, have agreed to this submission, and agree to transfer the ownership of copyright to BMC Microbiology. If accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

Competing Interests

AR, VL, DB, PA, GP are Indena's employees. GM and SB are employees at PTP Science Park.

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Authors' contributions

Conceived and designed experiments: AR, GM, SB, DB, PA, GP. Performed the experiments: GM, SB. Analyze the data: VL, GP. Wrote the paper: AR, VL, DB, SB, GP. All the Authors read and approve the final manuscript.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

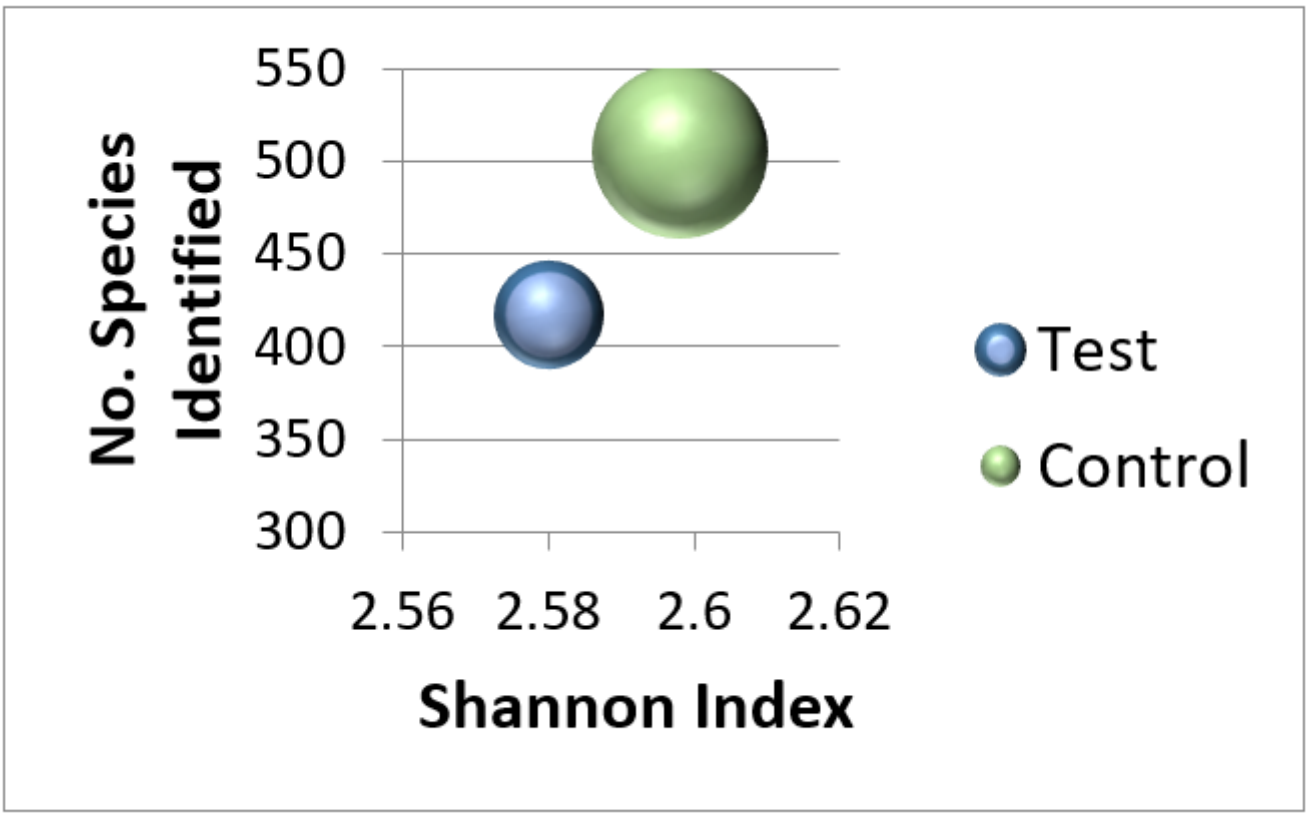

Figure 1

Shannon Index

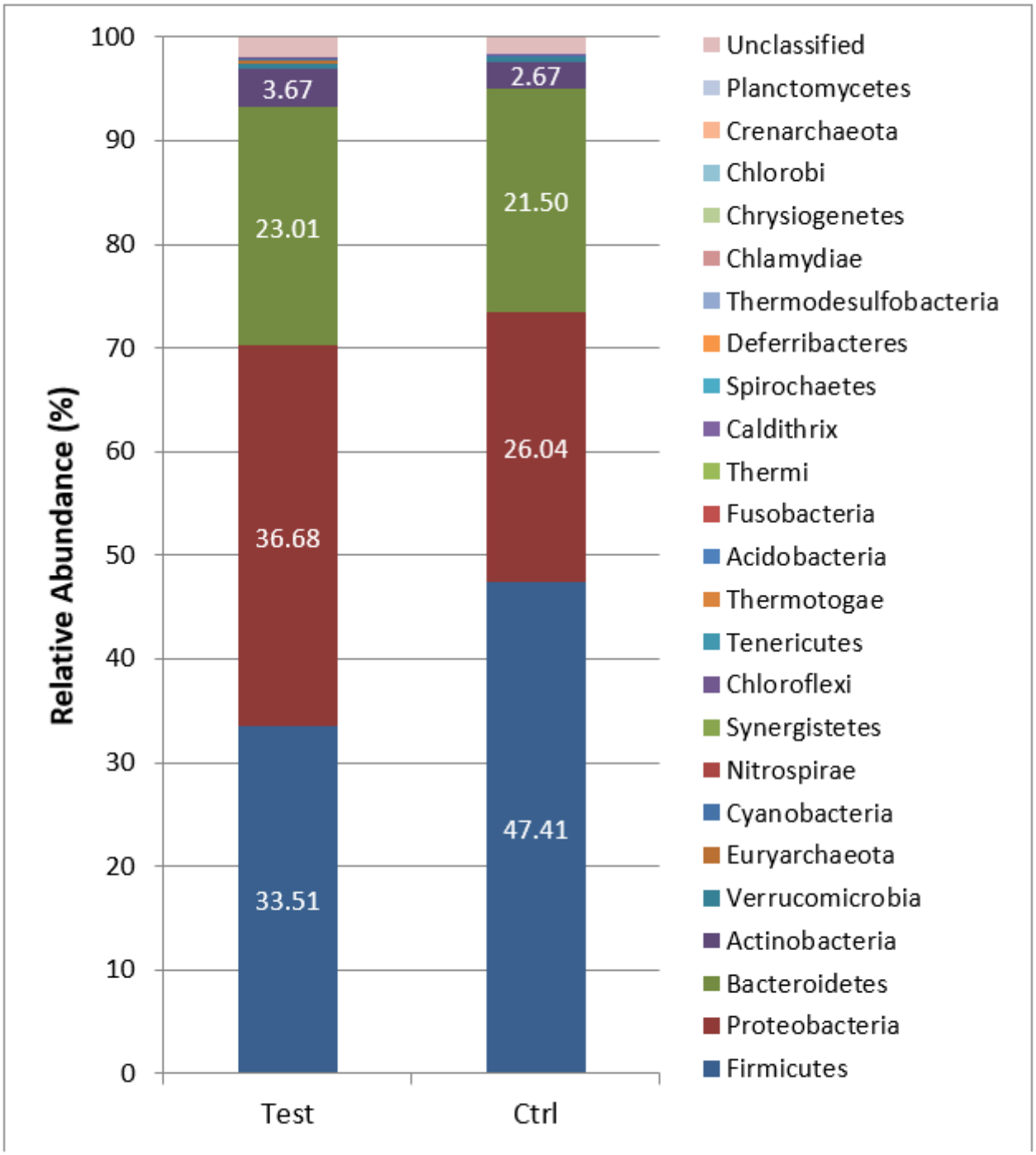

Figure 2

Relative Phyla abundance in control and treated faecal samples.

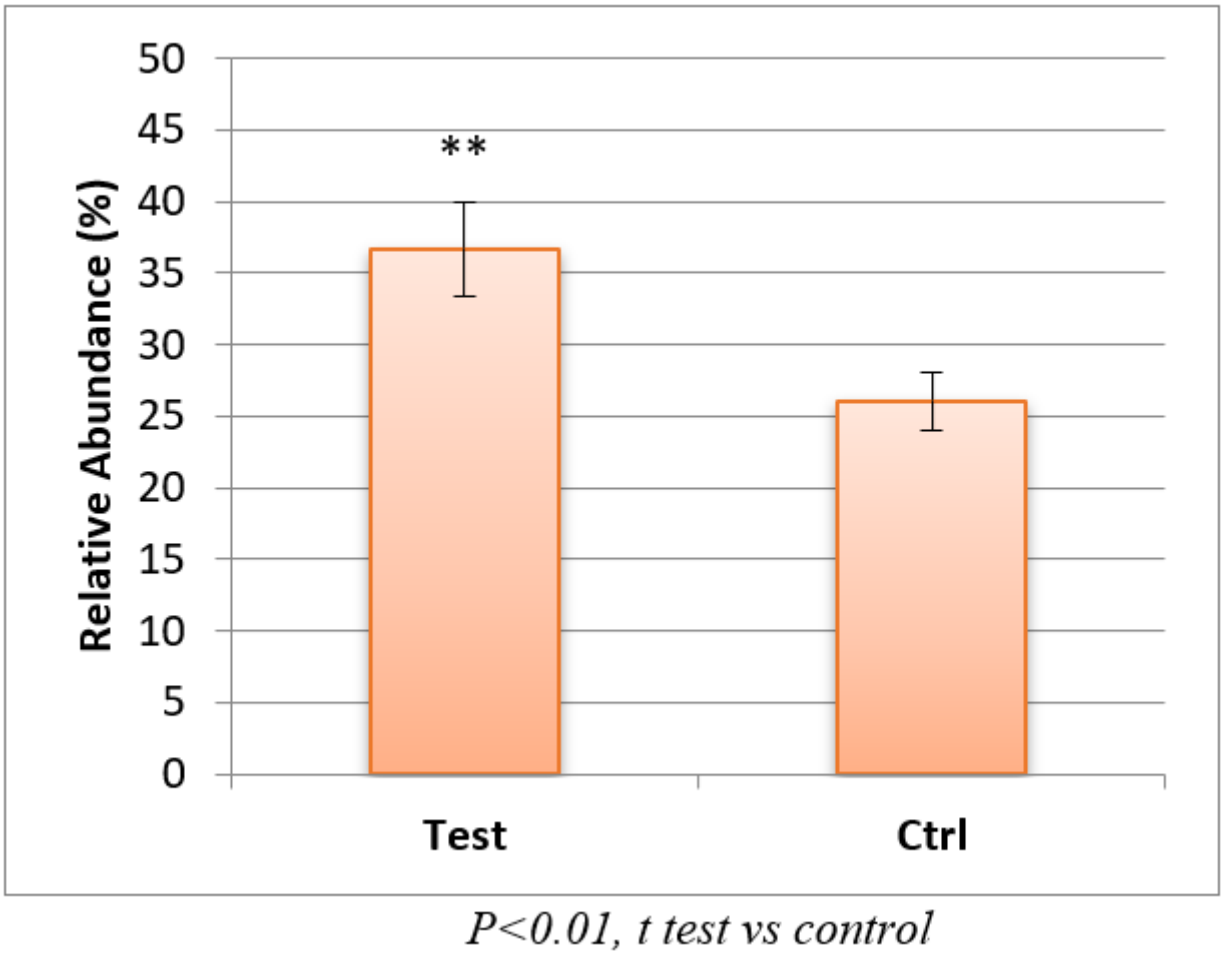

Figure 3

Proteobacteria levels in control and treated faecal samples. P<0.01, t test vs control (Ctrl).

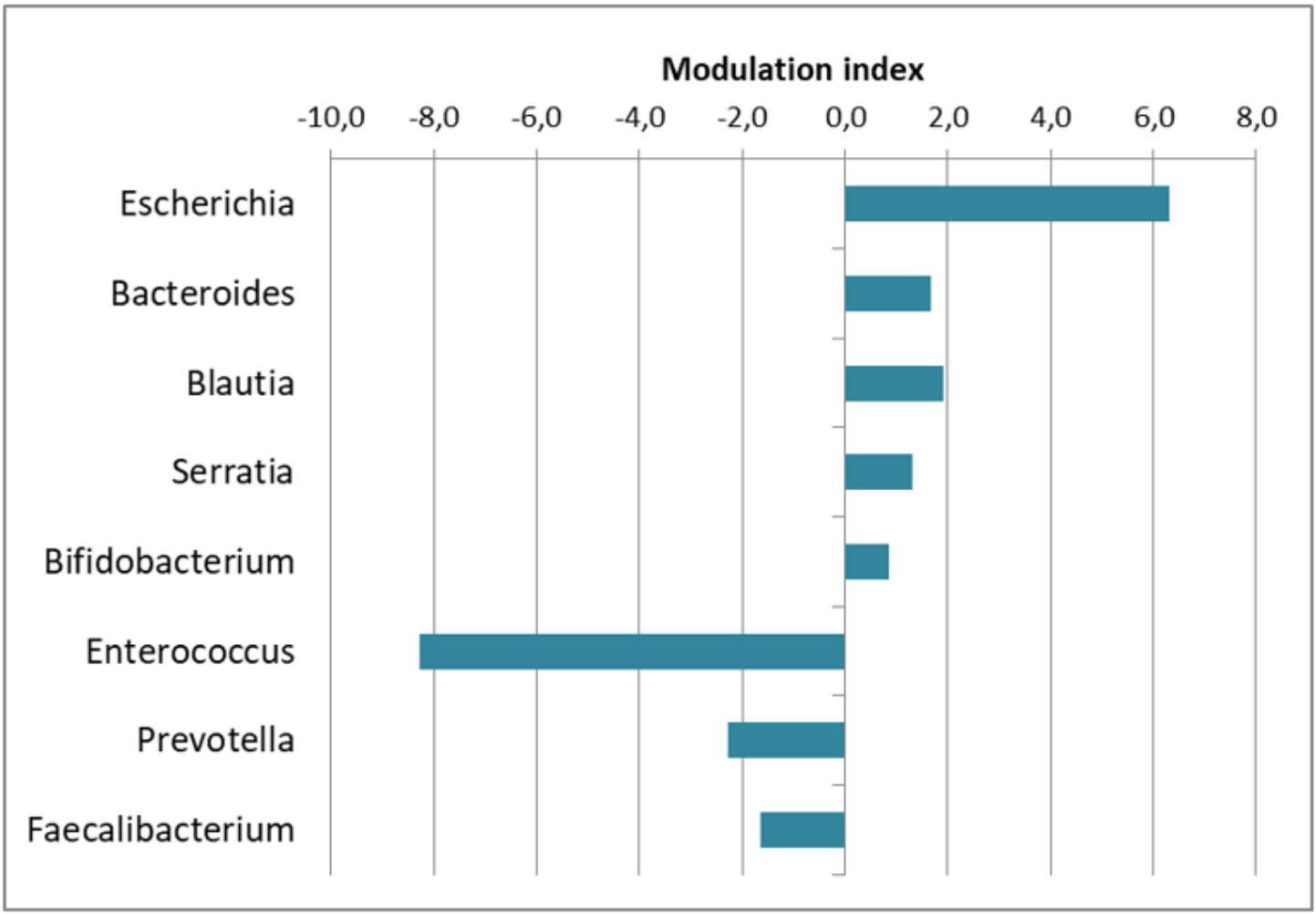

Figure 4

Summary of the Genera modulated by bergamot phytosome.

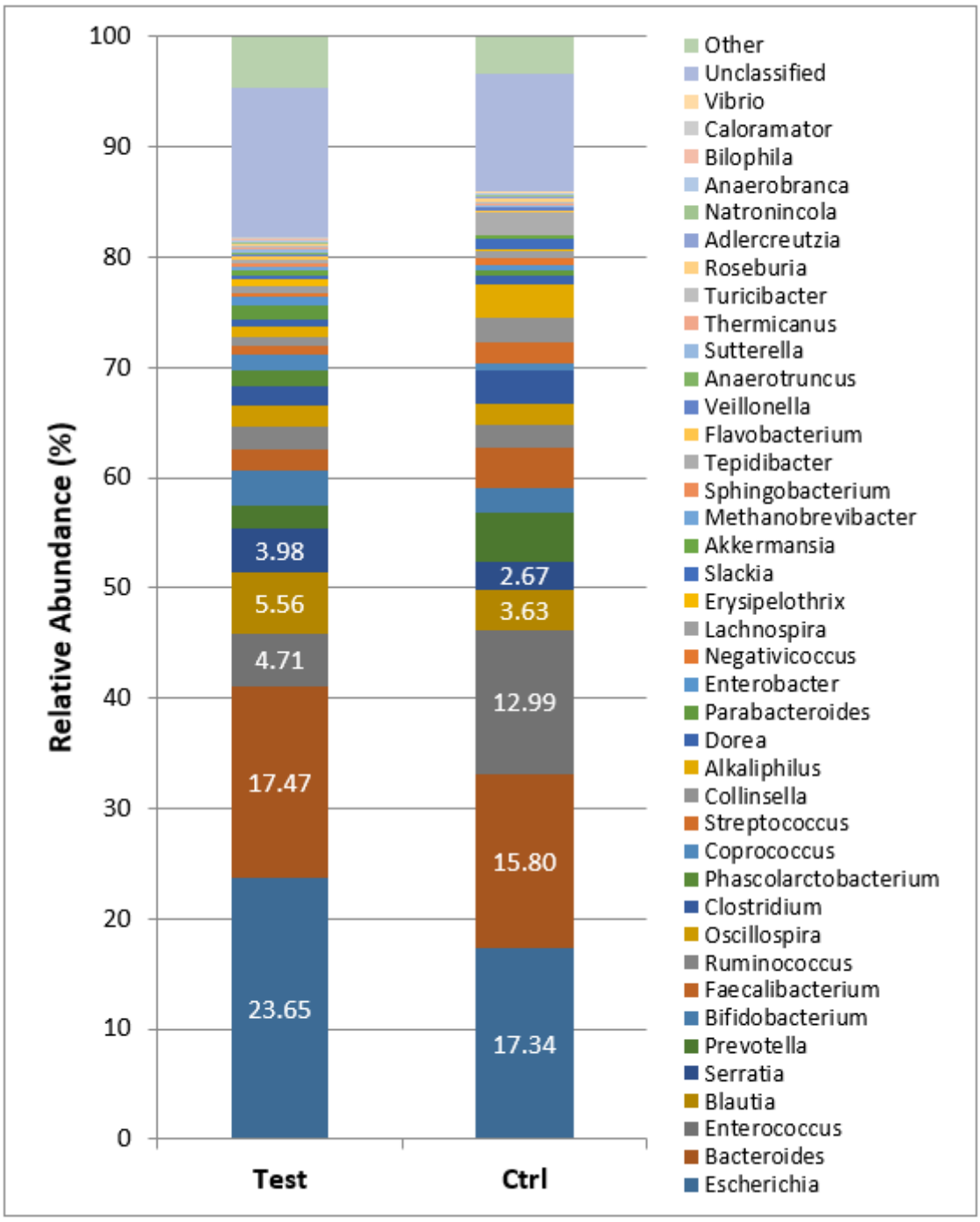

Figure 5

Relative Genera abundance in control and treated faecal samples.