Wild blueberry (poly)phenols can improve vascular function and cognitive performance in healthy older males and females: a double-blind randomized controlled trial

Wild Blueberry (Poly)phenols can Improve Vascular Function And Cognitive Performance In Healthy Older Males And Females: A Double-Blind Randomized Controlled Trial

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Conflict of interest

The funders of this study had no input on the design, implementation, analysis or interpretation of the data. The authors received, by way of a gift, the experimental test products from the Wild Blueberry Association of North America. None of the authors declared any other conflicts of interest.

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Author Contributions

EW, SH, CW & ARM designed the study and drafted the manuscript; EW, SH and NA carried out data collection, EW and SH conducted the analysis of the vascular and cognitive behavioral data; RM conducted the bioinformatic analysis of the gut microbiota and the correlation analysis between outcomes; FF provided support and training for the analysis of TCD; YX, EW and SH conducted the analysis of plasma and urine (poly)phenols; ZZ conducted the analysis of (poly)phenols in the wild blueberry intervention;; LB provided support with data analysis and contributed to the editing of the manuscript; ARM and CW had primary responsibility for final content. All authors read and approved the final manuscript.

Data described in the manuscript, code book, and analytic code will be made available upon request pending application and approval.

Keywords

Wild blueberry, polyphenol, cognition, vascular function, gut microbiota, flow-mediated dilation, cerebral blood flow, metabolomics, nutrition, older adults

***Running Title**:*

Blueberry (poly)phenols and healthy aging

Abbreviations

Ambulatory blood pressure (ABP)

Auditory Verbal Learning Task (AVLT)

Blood pressure (BP)

Augmentation index (Aix)

Blood flow velocity (BFV)

Body mass index (BMI)

Cardiovascular disease (CVD)

Cell preparation tubes (CPT)

Cerebral blood flow (CBF)

Diastolic blood pressure (DBP)

Flow-mediated dilation (FMD)

Liquid chromatography mass spectrometry (LC-MS)

Linear mixed modelling (LMM)

Metabolic research unit (MRU)

Micro elution Solid-Phase Extraction (μ-SPE)

Middle cerebral artery (MCA)

Negative Affect (NA)

Positive Affect (PA)

Positive and Negative Affect Schedule (PANAS-NOW)

Pulsatility index (PI)

Pulse wave velocity (PWV)

Randomized control trials (RCTs)

Statistical Package for Social Sciences (SPSS)

Systolic blood pressure (SBP)

Task switching task (TST)

Wild blueberry (WBB)

Abstract

Background: Evidence suggests that intake of blueberry (poly)phenols is associated with improvements in vascular function and cognitive performance. Whether these cognitive effects are linked to increases in cerebral and vascular blood flow or changes in the gut microbiota is currently unknown.

Methods: A double-blind, parallel randomized controlled trial was conducted in 61 healthy older individuals aged 65-80 y. Participants received either 26g of freeze-dried wild blueberry (WBB) powder (302 mg anthocyanins) or a matched placebo (0 mg anthocyanins). Endothelial function measured by flow-mediated dilation (FMD), cognitive function, arterial stiffness, blood pressure (BP), cerebral blood flow (CBF), gut microbiome and blood parameters were measured at baseline and 12 weeks following daily consumption.

Plasma and urinary (poly)phenol metabolites were analyzed using micro-elution solid phase-extraction coupled with LC-MS.

Results: A significant increase in FMD and reduction in 24 h ambulatory systolic BP were found in the WBB group compared to placebo (0.86%; 95% CI 0.56, 1.17, $p<0.001$; -3.59 mmHg; 95% CI -6.95, -0.23, $p=0.037$; respectively). Enhanced immediate recall on the auditory verbal learning task, alongside better accuracy on a task-switch task were also found following WBB treatment compared to placebo ($p<0.05$). Total 24 h urinary (poly)phenol excretion increased significantly in the WBB group compared to placebo. No changes in CBF or gut microbiota composition were found.

Conclusions: Daily intake of WBB powder, equivalent to 178 g fresh weight, improves vascular and cognitive function, and decreases 24h ambulatory systolic BP in healthy older individuals. This suggests that WBB (poly)phenols may reduce future cardiovascular disease (CVD) disease risk in an older population, and may improve episodic memory processes and executive functioning in older adults at risk of cognitive decline.

Clinical Trial Registration number in clinicaltrials.gov: NCT04084457

Introduction

The risk of developing both cardiovascular and neurodegenerative diseases increases during aging. Adults aged 60 and above, have a particularly high increased rate of cognitive decline (1). In parallel, endothelial function is known to decrease with increasing age and endothelial dysfunction is associated with cardiovascular disease development (2). Growing evidence from epidemiological and human intervention trials indicates that (poly)phenols may have cardioprotective properties as well as the ability to improve cognitive function (3-5). Blueberries are high in a subgroup of (poly)phenols known as anthocyanins, as well as other phenolic compounds such as procyanidins, flavonols and phenolic acids (6, 7). Previous randomized control trials (RCTs) have shown beneficial effects of daily blueberry consumption on executive functioning and episodic memory following at least 6 weeks of daily consumption in healthy older adults (8-12). Sustained improvements in FMD have also been shown after 4- and 24-weeks daily consumption of the equivalent to 200 and 150 g fresh blueberries, in healthy males and individuals with the metabolic syndrome (13, 14). It has been hypothesized that improvements in vascular function may influence cognitive performance, for example through changes in cerebral blood flow (CBF) (15-21). Previous research has shown increase in grey matter perfusion in the parietal and occipital lobes was measured using fMRI, following 12-weeks daily blueberry supplementation in older adults (22). Similarly, following 4-months WBB consumption, in older adults with MCI, an increase in neural activity during a memory task was observed (23).

Maintaining a healthy gut microbiota may be another important factor influencing CVD risk and cognitive function, in particular in older populations (24-26). Growing evidence suggests that (poly)phenol-rich foods influence gut-microbiota composition (27), gut microbiota significantly impact (poly)phenol metabolism, and gut microbial metabolites may influence cognition (28, 29). However, very little is known on whether blueberry consumption can modulate gut microbiota composition. A small study in 20 healthy men found an increase in the abundance of *Bifidobacterium* spp. following consumption of 25 g daily freeze-dried WBB powder for 6-weeks (27).

To our knowledge, no study has investigated the effects of daily blueberry (poly)phenol consumption on cognition and vascular function simultaneously in a healthy older population, whilst investigating the potential mechanisms of action by measuring changes in cerebral blood flow and gut microbiota diversity and composition. Therefore, the aim of this study was to investigate the effects of daily WBB (poly)phenol consumption on vascular function and cognitive performance in healthy older individuals. To gain mechanistic insight on potential mechanisms underlying these effects, we assessed the relationship between changes in clinical parameters, gut microbiota diversity, plasma and urinary (poly)phenol metabolites.

Subjects and Methods

Intervention study subjects

Sixty-one healthy older adults were recruited from London, inclusion criteria: healthy males and females aged 65-80 y; BMI 18-35 kg/m^2, able to understand the nature of the study and give written informed consent. Exclusion criteria were: manifest cardiovascular diseases including coronary artery diseases, cerebrovascular diseases and peripheral arterial disease; hypertensive (BP > 140/90 mmHg); diabetes mellitus; metabolic syndrome, as defined by the WHO (30); acute inflammation (i.e., increases in cytokines, acute phase proteins, and chemokines), end-stage renal disease or malignancies; have any known cognitive impairments, dyslexic or unable to complete the cognitive function tasks for any reason (i.e., visual impairments); lost more than 10% of their weight in the past 6 months; allergies to berries of other foods provided during the study (i.e., the standardized breakfast); taking blood pressure (BP) or lipid altering medication (or any other relevant medications); subjects already taking vitamin or minerals at a dose <200% of the UK RNI, or evening primrose/algal/fish oil supplements were asked to maintain habitual intake patterns and advised not to stop taking or begin new supplements during the study. Female participants were postmenopausal and not taking hormone replacement therapy (HRT).

Study design

A randomized, double-blinded, placebo-controlled parallel design study was conducted in 61 healthy older individuals to investigate the effects of daily WBB (poly)phenol consumption on vascular and cognitive function. Randomization was conducted using a computerized research randomizer (www.randomizer.org), generated by one of the researchers conducting the study, using blinded treatment codes provided by the sponsor. All research staff involved in the collection and the analysis of the data remained blinded to the treatment randomization until all aspects of the study were complete, including the statistical analysis. No blocking or stratification was used. Participants received 26 g freeze-dried WBB powder (equiv. to 178 g fresh WBB) containing 302 mg anthocyanins, or an appearance, taste and macro-nutrient, fiber and vitamin C matched placebo containing 0 mg anthocyanins (**Table 1**). Treatment powders were given to participants in an opaque sachet, to consume mixed with water once a day. Participants were asked to keep the sachets in their freezer once they arrived home with them, to minimize (poly)phenol degradation, compliance was assessed using empty sachet returns. Participants were asked to maintain their normal dietary and exercise habits throughout the duration of the study, diet was assessed using food diaries throughout the study.

Because of the mechanistic nature of this study, aiming to measure vascular function and cognition within the same volunteers at the same time, we conducted an RCT with multiple primary outcomes. The primary outcomes were endothelial function, measured by flow-mediated vasodilation, using high-resolution ultrasound, and cognitive function, measured as a battery of 5 tasks (Reys Auditory Verbal Learning Task (AVLT), Corsi blocks task, Serial 3s and 7s subtraction tasks, and Switching task). Secondary outcomes were arterial stiffness, measured as carotid-femoral pulse wave velocity (PWV) and augmentation index (AIx) using applanation tonometry (Sphygmocor), 24 h ambulatory and office BP, and CBF measured using non-imaging transcranial Doppler ultrasound, plasma lipids, (cholesterol, glucose, and other safety parameters such as liver function, kidney function, full blood count), plasma and urine polyphenol metabolites, mood measured as the Positive and Negative Affect Schedule (PANAS), gut microbiota diversity and composition.

The day prior to coming in for their study visit (24 h before), participants attended the metabolic research unit (MRU) to be fitted with a 24 h ambulatory BP monitor and were given a urine collection kit (3 L opaque

container in a cool bag with ice blocks). They were also asked to collect a fecal sample before their study visit, using a collection kit provided (OMNIgene®·GUT, DNA Genotek, Canada). The following day, once participants 24h monitors were removed, they rested supine for 10 minutes then measurements of BP, FMD, arterial stiffness and blood samples were collected. Participants were then given breakfast before completing the cognitive battery along with CBF measurements. The low-fat and low-(poly)phenol breakfast consisted of two slices of medium white toast spread thinly with low-fat Philadelphia® with a 120g low-fat Activia® vanilla yogurt container (Activia, Danone UK). CBF was measured for 10 minutes in a resting, seated position, participants then completed the cognitive battery lasting around 45 minutes, during one of the tasks (task-switching) CBF was measured for 10 minutes. Participants then went home and consumed the intervention treatment, placebo or WBB, for 12-weeks. At the end of the 12 weeks participants returned and all measurements were repeated. In addition, participants attended a follow up visit 1-month after completing the treatment, involving the same procedures stated above, to investigate whether any effects of the treatment remain without consumption (**Figure 1**). The study was conducted from December 2018 until March 2020, and it is registered at clinicaltrials.gov (NCT04084457). The study was conducted according to the guidelines laid down in the Declaration of Helsinki, with all volunteers providing informed consent. All procedures involved were approved by King's College London Research Ethics Committee (RESC reference: HR-18/19-9091).

Dietary Assessment of Background Diet

To assess habitual dietary intake, EPIC (European Prospective Investigation on Cancer; University of Cambridge) 7-day food diaries were completed. Participants were asked to record all food and drink consumed over the 7-day period in as much detail as possible. The food diaries were put into food codes following a standardized protocol by trained coders using Nutritics software (Nutritics Professional Diet Analysis, version 3.74; Nutritics Ltd). Average daily macro- and micro-nutrient composition of participant's diets were analyzed with data from the McCance and Widdowson's "The Composition of Foods Integrated Dataset (CoFID) 2015" (https://www.gov.uk/government/publications/composition-of-foods-integrated-

dataset-cofid). (Poly)phenol intakes were then assessed using an existing comprehensive database compiled at King's College London, including data from Phenol-Explorer (http://phenol-explorer.eu/) and USDA database (https://fdc.nal.usda.gov/) by matching up the food codes generated from Nutritics software to the available food content data in the (poly)phenol content database.

Biochemistry Analysis

Blood samples were collected by venepuncture using a 21G butterfly needle (Beckton Dickinson, Plymouth, UK). The blood sample was collected into vacutainer tubes including; green top heparin tubes (6 ml, for plasma (poly)phenols- spares), purple top EDTA tubes (10 ml, for plasma (poly)phenols), grey top fluoride oxalate tubes (3 ml, for blood glucose levels), purple top EDTA (3 ml, for full blood count), red top serum separator tubes (8.5 ml, for blood lipids and liver function), PAXgene® tubes (2.5 ml, for intracellular RNA analysis), glass cell preparation tubes (CPT) (8 ml, for peripheral blood monocytes). Plasma samples for (poly)phenol analysis were spiked with 2% formic acid and frozen at −80°C. All clinical parameters, including total cholesterol, LDL and HDL cholesterol, TAG, glucose, glycated hemoglobin, and whole blood count, were analyzed according to standard procedures in an accredited laboratory (Affinity Biomarker Labs, White City, London).

Wild Blueberry Powder and Placebo Interventions

The WBB consists of 100% freeze-dried WBB. The placebo is an appearance, taste and macro-nutrient, fiber and vitamin C matched powder containing blueberry flavoring and aroma, coloring (1.05% purple lake, 0.75% red lake, 0.45% blue 2 lake, 0.03% red dye, 0.008% blue 2 dye), glucose, fructose, citric acid, ascorbic acid, cellulose, fibersol-2, xanthan gum, pectin, and silica (**Table 1**). Methods used for anthocyanin and chlorogenic acid (5-O-caffeoylquinic acid) quantification of the WBB powder were previously described (31), with some minor modifications. Briefly, 2 g freeze-dried WBB powder was weighed and extracted 3 times with acidified methanol (0.1% HCL in MeOH). Samples were vortexed for 5 min, sonicated for 5 min in an ultrasonic bath (Fisher Scientific), and centrifuged at room temperature for 14 min at 1800 g. Supernatants

were combined and filtered. Samples were then analyzed using HPLC-DAD (High Pressure Liquid Chromatography Diode-Array Detector) using a method previously described, with some modifications (32). Individual anthocyanins were separated by an Agilent 1100 series HPCL system (Agilent Technologies, Cheshire, UK) equipped with a diode array detector and a Poroshell 120 EC-C18 column (100 x 2.1 mm, 2.7 µm particle size; Agilent Technologies, Cheshire, UK). The separation was accomplished at 40°C with the injection volume of 5 µL. The mobile phase A and B was acidified water (1% formic acid, v/v) and acidified acetonitrile (1% formic acid, v/v). The gradients were as followed: 0-5 min, 5% B; 5-35 min, 5-17% B; 35-50 min, 17-27% B; 50-60 min, 27-90% B; 60-65 min, 90% B; 65-70 min, 90-5% B; 70-80 min, 5% B, with a flow rate of 0.2 mL/min. The eluate was monitored at 520 nm for all samples. Calibration curves were obtained using authentic standards.

Flow-Mediated Dilation

FMD of the brachial artery was the primary outcome of the study, along with cognitive performance. FMD was measured as previously described (33) and analyzed using a semi-automated edge-detection software (Brachial Analyzer, Medical Imaging Applications, Iowa City, USA). In short, participants rested in a supine position for 15-minutes, in a temperature-controlled room. The brachial artery was imaged longitudinally at 2-10 cm proximal to the antecubital fossa. After baseline images were recorded, a BP cuff placed around the forearm was inflated to 180 mmHg. After 5 minutes of occlusion the pressure was released to induce reactive hyperemia, with image collection at 20, 40, 60 and 80 s post-occlusion. A single researcher, blinded to the treatments, analyzed all FMD images. FMD was calculated at each time point as maximal relative diameter gain relative to baseline and expressed as:

$$[(\text{diameter}_{\text{post-deflation}} - \text{diameter}_{\text{baseline}})/(\text{diameter}_{\text{baseline}})] \times 100.$$

Arterial Stiffness and Blood Pressure

PWV and AIx were measured using a SphygmoCor CPV Arterial Tonometry system (ScanMed Medical), to assess arterial stiffness. PWV was determined through measurements taken at the carotid and femoral

artery as described by Van Bortel (34). Central BP was also measured using applanation tonometry. Ambulatory BP was measured using TM2430 ABP monitors (A&D inc) worn for 24-hours before each study day. Readings were taken every 30 minutes during the day and 60 minutes at night (19:00-7:00). Participants self-reported their physical activity at the times readings were obtained and noted the times they were asleep. A&D Professional Analysis software was used to analyze the average 24-hour systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse.

Cognitive Testing

E-prime (Psychology Software Tools, Pittsburgh, PA) was used to display the stimuli and record participants' responses for all cognitive tasks. The AVLT assesses short-term verbal memory through word list learning and requires participants to recall lists of 15 nouns being presented audibly (35). AVLT includes various sub-measures of verbal memory and interference, calculated according to previously published methods (36). The Corsi Blocks task measures visual memory and targets short term spatial episodic memory (37). Participants observe a random sequence of blocks lighting up (ranging from 2-9 blocks) and the task is to repeat the sequence back in the same order. Serial 3's and serial 7's task was used to assess working memory. Here, participants were required to mentally subtract 3 from a randomly generated starting number between 800-999 and continue subtracting 3 from the answer for 2 minutes, this was then repeated with 7. Lastly, participants completed the switching task (TST) which assesses executive functioning, attention and reaction time, as previously described (38). Briefly, participants are given a circle with 8 segments, 4 above and 4 below a bold line. A stimulus digit between 1–9 (excluding 5) appears in each segment in turn in a clockwise direction, participant's task is to determine if the stimulus is odd or even when the number is above the bold line, or higher or lower than 5 when below the bold line. Outcome measures were % accuracy and reaction time (ms). Finally, subjective mood scores were also collected at the beginning and end of the cognitive battery using the Positive and Negative Affect Schedule (PANAS-NOW) (39). The PANAS-NOW self-report measure of Positive Affect (PA) and Negative Affect (NA) with 10 positive and 10

negative mood states. Participants were asked to rate the degree to which they were currently experiencing each item, on a five-point Likert scale.

Assessment Of Cerebral Blood Flow Using Transcranial Doppler Ultrasound

CBF was measured using non-imaging transcranial Doppler ultrasound (EZ-Dop, DWL, ScanMed medical instruments). We placed a 2MHz ultrasound probe into an adjustable Diamon® probe holder (DWL, Compumedics Germany CmbH, Singen) and placed securely onto the participant's head on top of the temporal bone acoustic window. Firstly, the signal was found on the right side of the middle cerebral artery (MCA) using the waveforms interpretation at a depth between 50-56 mm. We took a CBF reading of mean blood flow velocity (BFV) and pulsatility index (PI) every minute for 10-minutes whilst the participant was in a resting state. Subsequently, an active CBF measurement was taken whilst the participants were performing the cognitive task TST.

Quantification Of Plasma and Urinary (Poly)Phenol Metabolites Using LC-MS

Plasma samples were obtained by whole blood centrifugation with EDTA vacutainers (10 mL) at 1800g for 15 min at 4 °C and spiked with 2% formic acid. For the 24 h urine collection, plastic containers (3 L) were used, and the volume was measured using a volumetric cylinder. Formic acid was added to the urine samples to yield a 2% concentration. Samples were stored at −80 °C until analysis. These samples were thawed and processed using Micro elution Solid-Phase Extraction (μ-SPE) as previously described (40). Once the samples were washed and the compounds eluded in a collection plate (Waters, Eschborn, Germany), samples were run through a triple-quadruple mass spectrometer (SHIMADZU 8060, Shimadzu, Kyoto, Japan) coupled with a UPLC system (Shimadzu, Kyoto, Japan) and the (poly)phenol metabolites were identified and quantified using authentic standards as previously described (40). A list of the (poly)phenol metabolites investigated, as well as details on the chromatographic and mass spectrometry conditions used in presented in **Supplementary Table 8**.

16S rRNA Sequencing Of Fecal Microbiota

DNA was extracted from 0.25g of fecal material using the PowerFecal protocol (Qiagen, Hilden, Germany) as per the manufacturer's protocol. Extracted DNA was quantified using both spectrophotometry, Nanodrop (Thermo Fisher Scientific, MA,USA) and fluorimetry, Qubit (Thermo Fisher Scientific, MA,USA). DNA. DNA was standardized to a 5ng/ml using an automated protocol on a BiomekFX liquid handling robot (Beckman Coulter, CA, USA).

PCR

Five ng of DNA was amplified in a reaction volume of 10ml using with 0.5U of FastStart High Fidelity Taq (Roche), 4.5mM MgCl2, 0.1mM forward and reverse primer. The size of amplified products was checked on an 2% agarose. 1 ml of a 1 in 100 dilution of PCR product was used in a second round of PCR to add CS adapters (Fluidigm, CA, USA) 0.5U of FastStart High Fidelity Taq (Roche), 4.5mM MgCl2, 0.1mM forward and reverse primer. Barcode addition was QCed using the Tapestation D1000 tape (Agilent, CA, USA). The primers for the amplification of the 16S V3-V4 region were: ACACTGACGACATGGTTCTACACCTACGGGNGGCWGCAG (forward) and TACGGTAGCAGAGACTTGGTCTGACTACHVGGGTATCTAATCC (reverse).

Sequencing

An equal volume of each barcoded PCR product was pooled and the final pool diluted to 4nM. Pooled library was loaded at 7pM onto a 300bp paired end MiSeq (Illumina, CA, USA), as per manufacturer's instructions generating an average of 57,000 reads per sample.

Power Calculation and Statistical Analysis of Vascular and Behavioral Data

Power calculations were performed for FMD and episodic memory, based on previous human intervention trials using similar WBB treatments (8). The power for FMD was based on the interindividual variability of the operator (SD = 1%). Assuming a power of 80% and significance level 0.05, the total number of subjects required to provide sufficient power to detect a 1% difference in FMD in a two-arm parallel study is 40 (n=20 per arm). In a previous study, this sample size was enough to see significant effects in FMD after 4 weeks of

daily supplementation with similar amounts of WBB powder (13). Assuming a 10% drop out (based on previous studies from our group), 22 participants per arm should be recruited. For cognitive function, a medium effect size of d=0.640 requires a total of 60 participants (n=30 per arm) to achieve a statistical power of 80%. Our power calculation was based on our previous study that was conducted with similar study design, participant demographic, and wild blueberry treatment (8).

Statistical analysis of the vascular and cognitive endpoints was performed using Statistical Package for Social Sciences (SPSS) v.27. (IBM, UK). Z-scores analysis was conducted to identify outliers within the data set ($Z>3.29$). Linear mixed modelling (LMM) analysis was used to determine any post-treatment differences between the WBB and Control groups. The models included subjects as a random factor, treatment as a fixed factor, and baseline as a covariate. Effects were deemed significant at $p< 0.05$. Normality of residuals was confirmed for each significant LMM model using Q-Q Plots, and Shapiro-Wilk analysis. In some cases, the distribution of residuals was observed to deviate slightly from normality, however LMM is reported to be a robust model (41). Any observed deviations are reported alongside the LMM result and are further considered in the discussion. Spearman correlations were performed in R package version 2.5-6 along with heatmaps and correlation graphs.

Bioinformatics

DNA sequences of 16S rRNA amplicons were analyzed using QIIME 2 Core 2021.2 distribution within a conda environment (42). Samples were denoised using Dada2. Sequences were trimmed by 19 bp and truncated to 290 and 260 bp for forward and reverse reads, respectively. After denoising and chimera removal, a total of 15,394 ± 5,175 reads were available to assign the taxonomy of each sample. A classifier was trained on the V3/V4 region from the Silva 132 QIIME-compatible release database using qiime naive-bayes feature-classifier. The classification of sequence variants, at 99% similarity, was performed with VSEARCH, reducing the number of spurious taxonomic assignments ultimately reducing alpha error inflations (43, 44). We further removed sequence variants of chloroplasts or mitochondria (not bacterial or archaeal taxa), and

sequences only found in 1 sample. The output of the preprocessing with QIIME2 was then aggregated as a Phyloseq object for analysis in R.

Alpha diversity was calculated with Phyloseq. Differences in alpha diversity were evaluated using LMM, accounting for the personal differences as a random effect with lmerTest. As major factors affecting variation in the data, sex, ethnicity, and age were used as covariates in the models. Beta diversity was evaluated using an non-metric multidimensional scaling ordination of bray-curtis distance calculated from total sum scaled data. Significance of ordination was evaluated using Permutational Multivariate Analysis of Variance with R package vegan 2.5-6. To evaluate differences in taxonomic composition, we performed multivariable associations with MaAsLin 2 (Microbiome Multivariable Associations with Linear Models).

Random Forest classification of was performed using R package Caret (version 6.0-84). Since the two classes were not balanced, down-sampling was done prior to processing. Input variables were scaled and centered. Accuracy was estimated using repeated cross-validation (5 fold, repeated 10 times). The model was trained using 2/3 of the dataset, the quality of this model was evaluated using predicted sample classification of the remaining 1/3 of the dataset. The quality control metrics were calculated using the confusion Matrix function from Caret, which calculates the overall accuracy along a 95% confidence interval, with statistical significance of this accuracy evaluated with a one-side test comparing the experimental accuracy to the ‘no information rate’.

Results

Baseline Characteristics of The Study Population

A total of 81 volunteers were screened for recruitment in the study with 66 of these included and randomly allocated a treatment (35 received the WBB powder and 31 the placebo) (**Figure 1**). Three volunteers withdrew during their first week due to a dislike of their allocated treatment, though no adverse side effects were reported in any volunteer throughout the study. Two volunteers started taking medications towards the end of their intervention and so were excluded, one for high BP and the other for pre-diabetes symptoms. Overall, 61 participants completed the study; however, due to COVID-related university closures, 7 participants were unable to complete their 12-week visit and associated data collection. Therefore data for 54 participants were analysed on a per protocol basis. The baseline characteristics of the study population were all within normal limits (**Table 2**). No adverse effects from daily consumption of the treatments were reported throughout the study. There were significant changes in the overall protein intake (-6.61 g/day; p=0.034) and vitamin A (-414.2 µg/day; p=0.046) intake of the placebo group between baseline and visit 2 (12 weeks later). No other dietary changes were found between the visits.

Wild Blueberry Intervention Improved Vascular Function

The primary outcome was differences in FMD following 12 weeks of daily supplementation with either placebo or WBB. FMD was significantly higher in the WBB group compared to the placebo after 12 weeks by 0.86 % (95% CI 0.56, 1.17, p<0.001) (**Figure 2A**). In addition, there was a significant reduction in 24 h systolic BP of -3.59 mmHg following daily WBB consumption for 12 weeks, when compared to the placebo (95% CI -6.95, -0.23; p=0.037) (**Figure 2B**). No significant differences in other secondary outcomes were found including arterial stiffness, 24 h diastolic BP, CBF, blood lipids or office BP (**Figure 2C and Supplementary Table 2**).

Wild Blueberry Intervention Improved Some Aspects of Cognitive Function

Significant differences in immediate word recall (R1) was seen following WBB treatment for 12 weeks (F(1,46)=4.321, p=0.043; **Figure 2D**) with WBB-treated participants recalling 5.92 words compared to 5.28 words after placebo treatment. Contrary to previous work, no benefits to delayed memory recall were found following WBB-treatment, and in fact, the placebo group demonstrated significantly better delayed recall than those treated with WBB (F(1,47)= 5.042, p=0.029; 9.43 vs 7.48 words respectively) (**Figure 2E**). No significant differences in performance were observed for any other AVLT measure. In the TST, 12 weeks of daily WBB treatment led to a significant improvement in overall accuracy score, equivalent to an 8.5% increase in performance, relative to placebo (F(1,46)=5.05, p=0.029; **Figure 2F**). However, it should be noted that the residuals for the TST model deviated slightly from the assumption of normality (Shapiro-Wilk p<0.001). No significant differences were seen for other cognitive outcomes or on the mood measure (**Supplementary Table 3**).

Plasma And Urinary (Poly)phenol Metabolites

A total of 87 phenolic metabolites, potentially related to blueberry consumption, were quantified at baseline and after consumption of the treatments for 12-weeks. No significant differences were found at 12 weeks in fasting plasma total (poly)phenol metabolites between the WBB treatment and the placebo (p>0.05) (**Figure 3A**). Total 24 h urinary (poly)phenol excretion levels were significantly higher in the WBB group compared to the placebo by 1235 µmol (95% CI 600, 1870; F(50,1)=13.62, p=0.001) (**Figure 3B**). However, it should be noted that the residuals for the 24h urine model deviated slightly from the assumption of normality (Shapiro-Wilk p=0.003). At 12-weeks, 5 (poly)phenol metabolites including pyrogallol-*O*-sulfate (p=0.017), 2 methylpyrogallol-*O*-sulfate (p=0.042), 4-methylcatechol-*O*-sulfate (p=0.028), 4-methylcatechol (p=0.011) and isoferulic acid (p<0.001) were significantly higher in plasma in the WBB group compared to the placebo (**Supplementary Table 4**) In addition, 2 compounds were significantly lower in the WBB group when compared to the placebo: vanillic acid (p=0.034) and phenylacetic acid (p=0.015). Changes in urinary (poly)phenol metabolites are presented in **Supplementary Table 5**.

Effects of WBB Consumption on Gut Microbiota Diversity and Composition

Fecal samples were collected at baseline and 12 weeks post-intervention and analyzed using 16s rRNA sequencing. Observed alpha diversity significantly increased from baseline in the whole cohort (p=0.04). However, when the analysis was done individually per treatment group, there were no significant differences (p>0.05) (**Figure 4A**). Beta diversity did not differ significantly at baseline between the treatment groups (p>0.05), and this did not change after 12 weeks following either of the treatments (**Figure 4B**). Taxonomic composition was typical of fecal microbiota from Western individuals with average abundances of 52% Firmicutes (**Figure 4C**), 34% Bacteroidetes (**Figure 4D**), 9% Proteobacteria (**Figure 4E**) and 2% Verrucomicrobiota (**Figure 4F**). Taxonomy at the phylum level was not statistically different between the different visits although there was a trend suggesting an increase in the abundance of Firmicutes in the WBB concomitant to a decrease in the abundance of Bacteroidetes and Proteobacteria. We then agglomerated ASV at the genus levels to obtain more details about the effects of WBB (**Figure 4G**). This provided the best resolution for taxonomic evaluations given the number of samples and the DNA sequencing strategy used in this study. The profiles were highly individualized which suggests that additional factors could influence whether or not an individual will present fecal microbiome composition changes after the intervention. A group of individuals had high *Prevotella* and low *Bacteroides,* while *Prevotella* was not detected for some individuals with the highest *Bacteroides* levels. Multivariable association between clinical metadata and taxonomic abundances showed 3 genus which had their abundance increased by the intervention. These included increases in *Ruminiclostridium 9* (p = 0.0007, q = 0.06), *Ruminiclostridium 5* (p = 0.002, q = 0.11) and *Parabacteroides* (p = 0.003, q = 0.20). While the increase in *Parabacteroides* abundance was observed in the placebo arm, the increase in *Ruminiclostridium 5* and *Ruminiclostridium 9* were due to changes in the WBB arm. Other changes were observed following WBB consumption, such as an increase in the levels of *Christensenellaceae* (p = 0.04, q = 0.80), *Eggerthellaceae* (p = 0.007, q = 0.36), or *Intestinibacter* (p = 0.02, q = 0.64) (**Supplementary Table 6**). Note that false discovery rates were high and these results will have to be confirmed by other studies.

Correlations between Clinical Parameters and Plasma Polyphenol Levels

Mechanistic insights on the circulating metabolites responsible for the effect on blood vessel function, BP and cognition observed in this study were investigated using correlational analysis. A total of 6 (poly)phenol metabolites correlated with changes in FMD, however only 2 of these were positive correlations, including 3'-hydroxy-4'-methoxycinnamic acid (isoferulic acid) and 2,3-dihydroxybenzene-1-sulfate (pyrogallol-O-sulfate) (**Figure 5 and Suppl Figure 1**). Changes in 24 h SBP correlated with changes in 12 (poly)phenol metabolites, including hippuric acid, 3-methoxybenzoic acid-4-sulfate (vanillic acid-4-O-sulfate), 3'-hydroxyhippuric acid, 3-(3',5'-dihydroxyphenyl)propanoic acid, benzoic acid, 3(2',4'-dihydroxyphenyl)propanoic acid, cinnamic acid, 3-(2'-hydroxyphenyl)propanoic acid, quercetin, 4'-methoxycinnamic acid-3'-sulfate (isoferulic acid 3-O-sulfate), 2-Hydroxy-4-methylbenzene-1-sulfate (4-methylcatechol-O-sulfate), and 4'-hydroxyhippuric acid, all of which were negatively correlated, implying that reductions in 24 h SBP may correlate with increases in circulating (poly)phenol metabolites (**Figure 5 and Supplementary Figure 1**).

For cognitive function it was found that 8 (poly)phenol metabolites correlated with changes for immediate recall score, with 7 out of the 8 being positive correlations. These metabolites included 2,6-dihydroxybenzene-1-sulfate (pyrogallol sulfate), 2,6- dihydroxybenzoic acid, 3'-hydroxy-4'-methoxycinnamic acid (isoferulic acid), 3-(2',3'-dihydroxyphenyl)propanoic acid, benzoic acid, 2,5-dihydroxybenzoic acid, phenylacetic acid, and 2-hydroxy-3-methoxybenzene-1-sulfate (1-methylpyrogallol-O-sulfate). For delay recall, correlations with 6 plasma metabolites were found, with 4 being positive correlations. These included 3'-hydroxy-4'-methoxyphenyl)propanoic acid-3'-glucuronide (dihydroisoferulic acid 3-glucuronide), 3-(4'-methoxyphenyl)propanoic acid-3'-sulfate (dihydroisoferulic acid 3-O-Sulfate), 4-methoxvbenzoic acid-3-sulfate (ferulic Acid 4-Glucuronide), and 2-hydroxyhippuric acid. For TST accuracy, correlations with 3 plasma metabolites were found 1 of which was positive, including quercetin-7-glucuronide. All correlations with cognitive outcomes are shown in **Figure 6** and **Supplementary Figure 1**.

Correlations between gut microbiota and clinical outcomes

A number of correlations were found between bacteria and clinical outcomes (for full heatmap please see **Supplementary Figure 2**). A total of 4 correlations were found with FMD (negative with *Parabacteroides* and *Ruminococus UCG.003*, positive with *Cocoprocus* and *Family XIII AD03011*), while no correlations were found with 24h SBP. For TST Accuracy, 2 positive correlations were found (*Anaerostipes and Eucbacterium Xylanophilum*), and same for AVLT immediate recall 2 (1 negative with *Ruminoccocus* and 1 positive with *Butyricicoccus*). Finally, AVLT delayed recall had 4 positive correlations with *Lachnospiracea UCG.004, Ruminocuccus UCG.005 and UCG.010, and Parabacteroides.*

Machine Learning Discriminates WBB Consumption Based On Changes In Plasma Or Urine Polyphenols

We tested whether the changes in plasma or urine total polyphenols could predict whether a participant has received the placebo or the blueberry-treatment using a machine learning approach. Despite the relatively low number of individuals, the model appropriately classified 80% of the plasma (poly)phenol profiles as belonging to the placebo or the blueberry-treatment group (**Supplementary Table 7)**. This was largely driven by the changes in plasma isoferulic concentrations, although this parameter alone was not sufficient to appropriately classify the samples. The urine polyphenols profiles or the gut microbiome genera profiles were not sufficient to classify the placebo and the blueberry-treatment group.

Discussion

To our knowledge, this is the first study to investigate the impact of blueberry consumption on cognitive and cardiovascular function simultaneously in a group of healthy older adults. We observed that 12 weeks daily WBB consumption improved FMD by 0.85% and ambulatory systolic BP decreased by -3.59 mmHg with respect to the control, while no effects were found in arterial stiffness and blood lipids. This is consistent with our previous study in younger healthy males where a 1.5% increase in FMD and systolic BP decreased by 5.6 mmHg after 4-week consumption of similar amounts of WBB (13). Overall, the changes in FMD and SBP found here are lower, which may be due to the different study population but also other methodological differences. In the present study, the WBB treatment was consumed once daily in the morning for 12 weeks, while in the previous study it was consumed bi-daily for 4 weeks, and the placebo was not matched for fiber. Improvements in endothelial function and BP after blueberry consumption have also been reported in individuals with metabolic syndrome (45, 46) and hypertension (47), although mixed results exist and a small meta-analysis of 6 RCTs failed to show significant effects in BP after blueberry consumption (48). It is important to note that most studies measured office blood pressure, and very few studies used ambulatory BP, which is considered the gold standard method to assess an individual's BP due to the multiple datapoints collected throughout 24 hours, leading to much more reliable and accurate data than a single BP measurement, which is highly variable within individuals (49).

We also found improvements in episodic memory and executive function, in particular better immediate recall of a word list and improvement in switching accuracy, similarly to findings from other studies recruiting older adults and supplementing with blueberry treatments over periods ranging from 12-48 weeks (8, 9, 11, 50). However, a notable observation from the current study was a lack of any significant difference between WBB and placebo on our delayed recall measure, which contradicts previous findings using the methodologically similar California Verbal Learning Task in older adults >60 years of age (12). The methodological demands of our study comprising of battery of 4 cognitive tasks, including a demanding TST, alongside a delay of 40 minutes between the list-learning and delayed recall components of the AVLT may

explain the overall performance and why no significant differences in delayed recall performance were seen. To date, the array of study designs, dosages, and (poly)phenol content of blueberry interventions hinder between-study comparison and further work on these behavioral domains are required to confirm their sensitivity, or otherwise, to blueberry interventions. Despite the positive effects of WBB treatment on cognitive and cardiovascular parameters, and the predicted mechanistic link between the two outcomes, no changes were seen in CBF following the WBB treatment. The data obtained from our participants in the 'resting' phase aligns with other published TCD studies as we saw a reduced BFV with increasing age. However, in our 'active' phase (where participants were involved in completing the cognitive task) we saw only 2-3% increase in BFV compared to studies that have used an exercise intervention, where increases in the range of 7-24% have been seen or the 8-10% increase in BFV seen following administration of 900 mg cocoa polyphenols in a similar population of healthy older volunteers (16, 51, 52). To our knowledge, this is the first study that has used TCD as a measure of cerebral blood flow velocity to test the effects of blueberry (poly)phenols and may indicate that TCD may not have the sensitivity to detect small changes in vasculature arising from blueberry intervention given the intrinsic noisiness of the methods (53, 54) and cerebral autoregulation (55).

In the present study, total 24 h urinary (poly)phenol metabolites significantly increased in the WBB group when compared with placebo following 12-weeks daily consumption. Total plasma metabolites did not significantly change after 12 weeks, although 5 individual metabolites increased significantly. As the blood collection took place 24 h after consumption of the last blueberry sachet, many of the blueberry derived metabolites had likely already disappeared from circulation. This could explain why we only saw positive correlations between changes in FMD and plasma isoferulic acid and pyrogallol-O-sulfate. However, overall, there were 13 correlations between plasma metabolites and changes in 24 h SBP and 11 metabolites correlating with the improvements in cognition after WBB consumption. Interestingly, the metabolites that correlated with FMD, SBP and cognitive outcomes were different, except for pyrogallol sulfate, that correlated both with FMD and TST accuracy. In our previous work we showed that a mixture of the blueberry

derived plasma metabolites correlating with improvements in FMD after 4 weeks daily consumption, of which 7 metabolites were also correlated with BP and cognitive outcomes in this study, improved vascular function in an FMD animal model (13). Mechanistic studies are needed to understand whether mixtures and individual metabolites are the key bioactive compounds improving vascular function and cognition after blueberry consumption.

The potential mechanisms by which blueberry (poly)phenols may positively affect vascular function and cognitive performance, as reported in this study, are still largely unknown. In the present study, no significant changes in gut microbiota diversity and composition were observed in the blueberry group when compared to the placebo. However, observed alpha diversity significantly increased from baseline in the whole cohort, likely driven by the blueberry arm. Increases in beneficial bacteria such as *Ruminiclostridium* and *Christensellenacea* were also found among volunteers in the wild blueberry arm. Furthermore, most of the bacteria correlating positively with the improvements in FMD and cognition belong to the butyrate producer Clostridium cluster of the phylum Firmicutes, including *Coprococcus* and *Family XIII AD03011,* which correlated positively with FMD*;* Anaerostipes*, Eucbacterium Xylanophilum*, *Butyricicoccus*, *Lachnospiracea UCG.004, Ruminocuccus UCG.005* and *UCG.010,* which correlated with cognitive outcomes including TST accuracy and AVLT. A key proposed mechanism of action related to the improvements in vascular function by (poly)phenol rich foods is by mediation of nitric oxide (NO) bioavailability (56, 57). In our previous work, acute improvements in FMD after blueberry consumption correlated with decreased neutrophil NADHP oxidase activity, and increases in blueberry derived (poly)phenol metabolites were independent predictors of changes in gene expression linked to biological processes involved in cell adhesion, migration, immune response, and cell differentiation (7, 13). Similarly, a number of mechanistic studies also suggest that butyrate may improve endothelial function via a NO-mediated mechanism, including improvements in monocyte–endothelial interactions, macrophage lipid accumulation, smooth muscle cell proliferation and migration, and lymphocyte differentiation and function (58,59). Butyrate has also been shown to improve

cognitive function and exert neuroprotective effects in experimental models (60,61). Future work is needed to investigate whether butyrate may be a key player in the mechanisms of action of blueberry (poly)phenols.

The improvements seen here in vascular function have clinical significance, as according to recent meta-analyses, an 0.85% increase in FMD translates to a 8.5-11% decreased risk of developing CVD (62-64), while a decrease in BP of 3.6 mmHg would translate into a 7% lower risk of CVD events (65). The relatively modest improvements in episodic memory and executive function seen in our study highlights the need for further substantiation of these effects before firm conclusion on risk reduction from flavonoid-rich interventions could be drawn.

Machine learning algorithms have recently been shown to be very useful in stratifying patients or predicting the success of nutritional interventions from individual characteristics. Our results provide a proof of principle that this could be the case for (poly)phenol intake, with the changes in the plasma (poly)phenol metabolome being able to predict whether participants were enrolled on the blueberry or placebo arm in our study, despite the small sample size. No such predictive power was found for the urine (poly)phenols or gut microbiome data, indicating that plasma may be a better predictor of (poly)phenol consumption.

There are some key limitations of this research, including that the results are limited to a healthy older population, therefore cannot be directly extrapolated to all segments of the general population. We did not have access to an MRI machine which would have potentially been a more sensitive measure of CBF. We did not investigate the factors affecting the high inter-individual variability in response to the intervention we observed, which could be due to differences in absorption, metabolism or gut microbiota composition across our population. We also acknowledge the inflated risk of type 1 error because of the number of statistical tests conducted. While LMM is known to be a robust statistical test, residuals were observed to deviate from the assumption of normality is some cases which may limit the statistical reliability of those outcomes. Following the per protocol exclusions, the low number of participants in the cognitive analyses means that the study may have been slightly underpowered. In conclusion, long-term consumption of a dietary achievable amount of WBB was observed to enhance vascular and cognitive function in older adults and may

be a plausible and cost-effective dietary-based strategy to tackle the burden of age-related cognitive decline and vascular dysfunction. Our study findings indicate that gut microbiota and vascular blood flow may play important roles in mediating the cognitive benefits shown by consumption of (poly)phenol rich foods. Further large-scale studies are needed to confirm the findings of this small-scale investigation, and to explore the exact mechanisms of action.

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Tables

Table 1. Nutritional and phytochemical content of the freeze-dried WBB and placebo powder

	Wild blueberry powder (26g)	Placebo powder (26g)
Total fat (g)	1	0
Protein (g)	0.55	0
Total carbohydrates (g)	23.6	17.6
Fructose	9.01	9.23
Glucose	8.70	8.32
Calories (kcal)	106	100
Dietary fiber, Total (g)	4.06	5.17
Insoluble fiber (g)	3.02	4.16
Soluble fiber (g)	1.05	1.01
Vitamin C (mg)	87	90
Anthocyanins (mg)	302	0
Chlorogenic acid (mg)	202.1	0

Placebo treatment information from Nieman et al. (62), WBB information provided by WBANA. The 26 g of freeze-dried WBB is equivalent to 178 g of fresh WBB.

Table 2. Baseline characteristics for both placebo and wild-blueberry treatment groups

	Placebo group Mean (SD) (N=29)	WBB group Mean (SD) (N=32)
Sex (male/female)	12/17	12/20
Age	70.76 ± (3.81)	69.44 ± (3.48)
BMI (kg/m^2)	23.16 (2.59)	24.57 (2.7)
Body Fat %	26.13 (8.27)	29.36 (7.92)
Systolic BP (mmHg)	128.36 (10.01)	128.52 (11.63)
Diastolic BP (mmHg)	79.59 (5.59)	81.05 (7.86)
Heart rate (bpm)	65.71 (8.86)	65.94 (9.65)
HDL-cholesterol (mmol/L)	2.12 (0.78)	1.83 (0.47)
LDL-cholesterol (mmol/L)	3.80 (1.07)	3.90 (1.17)
Fasting Glucose (mmol/L)	4.72 (0.41)	4.50 (0.58)
FMD (%)	4.11 (1.14)	3.62 (1.53)
PWV (m/s)	8.47 (2.35)	7.94 (3.07)
AIx @ HR75(%)	29.4 (11.0)	27.8 (7.11)
Blood flow velocity (cm/s)	53.6 (7.95)	54.9 (7.23)
Pulsatility index (cm/s)	1.18 (0.27)	1.02 (0.17)

AIx; augmentation index, BMI; body mass index, BP; blood pressure, FMD; flow-mediated dilation, PWV; pulse-wave velocity, SD; standard deviation, WBB; wild blueberry.

Legends for figures

Figure 1. A) Study design; *AIx; augmentation index, BP; blood pressure, CBF; cerebral blood flow, FMD; flow-mediated dilation, PWV; pulse-wave velocity.* **b) Flow diagram outlining study activity and participant numbers throughout process.** *Due to Covid-19 pandemic and research disruptions 7 out of 61 participants did not complete their 12-week follow up study visit.

Figure 2. Differences in vascular and cognitive function at 12 weeks following consumption of the control and wild blueberry (WBB) treatment (n=27 on each group). Flow-mediated dilation (FMD) change **(A)** evaluated by linear mixed modelling analysis (P<0.001 for an overall WBB treatment effect compared to placebo, adjusted for baseline FMD values as a covariate. Total 24-hour **(B)** systolic blood pressure (SBP) and **(C)** diastolic blood pressure (DBP) change from baseline. Linear mixed modelling analysis revealed no significance for DBP, and an overall treatment effect in SBP p=0.037 when compared to the placebo. Baseline blood pressure values were used as a covariate; **D**) Analysis for Mean words recalled for immediate recall (R1) revealed significantly improved performance following WBB consumption in comparison to placebo (*p*=0.043). **(E)** Analysis for mean delayed word recall (R8) revealed significantly improved performance following placebo consumption relative to WBB (*p*=0.029) and **(F)** Analysis for overall TST accuracy scores revealed significant effect of treatment, with higher overall accuracy for WBB compared to placebo (*p*=0.026). *AVLT; auditory visual learning task, DBP; diastolic blood pressure, FMD; flow-mediated dilation, SBP; systolic blood pressure, TST; task switching task, WBB; wild blueberry.*

Figure 3. Total A) plasma and B)24 h urinary polyphenol metabolites after wild blueberry or placebo consumption for 12 weeks, evaluated by linear mixed modelling analysis (n=27 on each group). No significant differences were found in fasting plasma total (poly)phenols 12 weeks after daily consumption of WBB or placebo, however WBB group had significantly higher total excreted (poly)phenols in 24 h than the placebo group (p=0.001).

Figure 4. Differences in fecal microbiota composition between the placebo and the blueberry-treatment group (n=27 on each group). Alpha (A) and beta (B) diversity are compared, as well as relative abundances for the major phyla quantified in this study: Firmicutes (C), Bacteroidetes (D), Proteobacteria (E), Verrucomicrobiota (F). The 10 most abundant bacteria genera are presented in (G). . *WBB; wild blueberry.*

Figure 5. Correlations between plasma metabolites and vascular outcomes (n=27). Plots show correlations between plasma (poly)phenol metabolites and changes in main cardiometabolic outcomes (FMD and 24 h ambulatory SBP) showing significant changes from WBB treatment; FMD and 24h SBP. *p<0.05, **p<0.001. *FMD; flow-mediated dilation, SBP; systolic blood pressure.*

Figure 6. Correlations between plasma metabolites and cognitive outcomes (n=27). Plots with correlations between Plasma (poly)phenol metabolites and changes in AVLT immediate recall performance showing significant changes from WBB treatment *p>0.05, **p>0.001.

AVLT; auditory visual learning task, TST; task switching task.

Figure 1

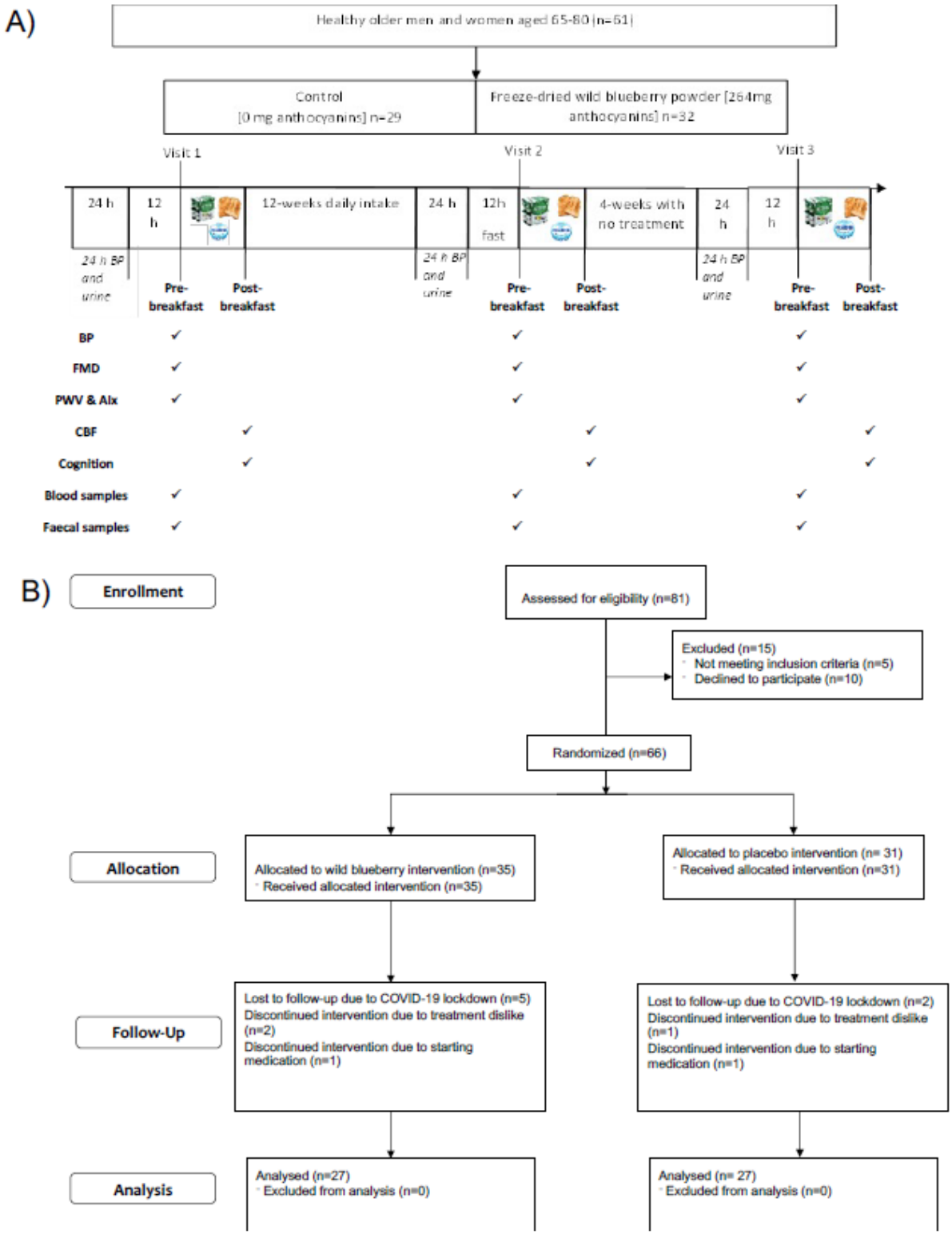

A)
Healthy older men and women aged 65-80 (n=61)
Control
[0 mg anthocyanins] n=29
Freeze-dried wild blueberry powder [264mg anthocyanins] n=32
Visit 1
Visit 2
Visit 3
24 h
12 h
12-weeks daily intake
24 h
12h fast
4-weeks with no treatment
24 h
12 h
24 h BP and urine
Pre-breakfast
Post-breakfast
BP
FMD
PWV & AIx
CBF
Cognition
Blood samples
Faecal samples
B)
Enrollment
Assessed for eligibility (n=81)
Excluded (n=15)
Not meeting inclusion criteria (n=5)
Declined to participate (n=10)
Randomized (n=66)
Allocation
Allocated to wild blueberry intervention (n=35)
Received allocated intervention (n=35)
Allocated to placebo intervention (n= 31)
Received allocated intervention (n=31)
Follow-Up
Lost to follow-up due to COVID-19 lockdown (n=5)
Discontinued intervention due to treatment dislike (n=2)
Discontinued intervention due to starting medication (n=1)
Lost to follow-up due to COVID-19 lockdown (n=2)
Discontinued intervention due to treatment dislike (n=1)
Discontinued intervention due to starting medication (n=1)
Analysis
Analysed (n=27)
Excluded from analysis (n=0)
Analysed (n= 27)
Excluded from analysis (n=0)

Figure 2

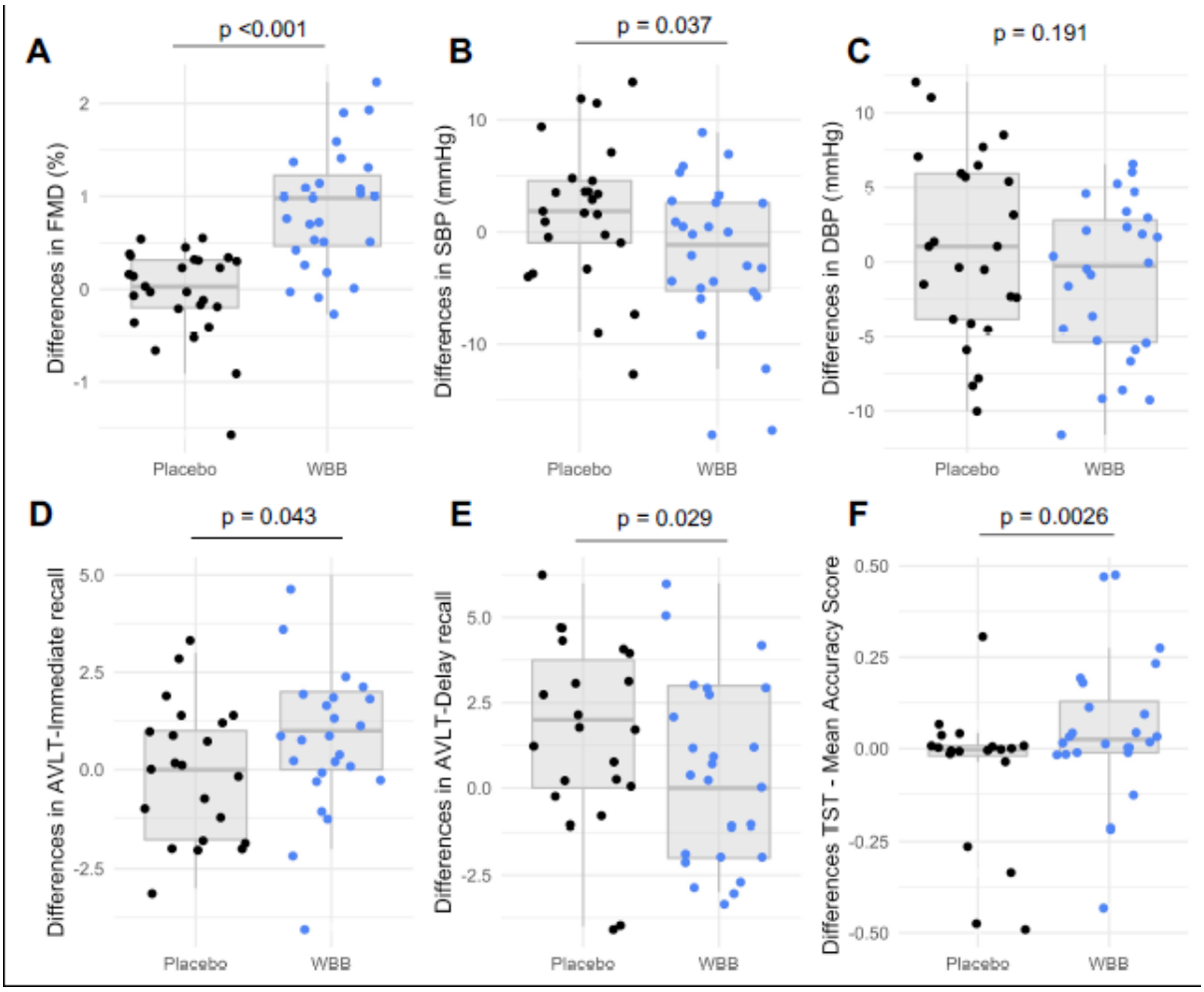

A
p <0.001
Differences in FMD (%)
Placebo
WBB
B
p = 0.037
Differences in SBP (mmHg)
Placebo
WBB
C
p = 0.191
Differences in DBP (mmHg)
Placebo
WBB
D
p = 0.043
Differences in AVLT-Immediate recall
Placebo
WBB
E
p = 0.029
Differences in AVLT-Delay recall
Placebo
WBB
F
p = 0.0026
Differences TST - Mean Accuracy Score
Placebo
WBB

Figure 3

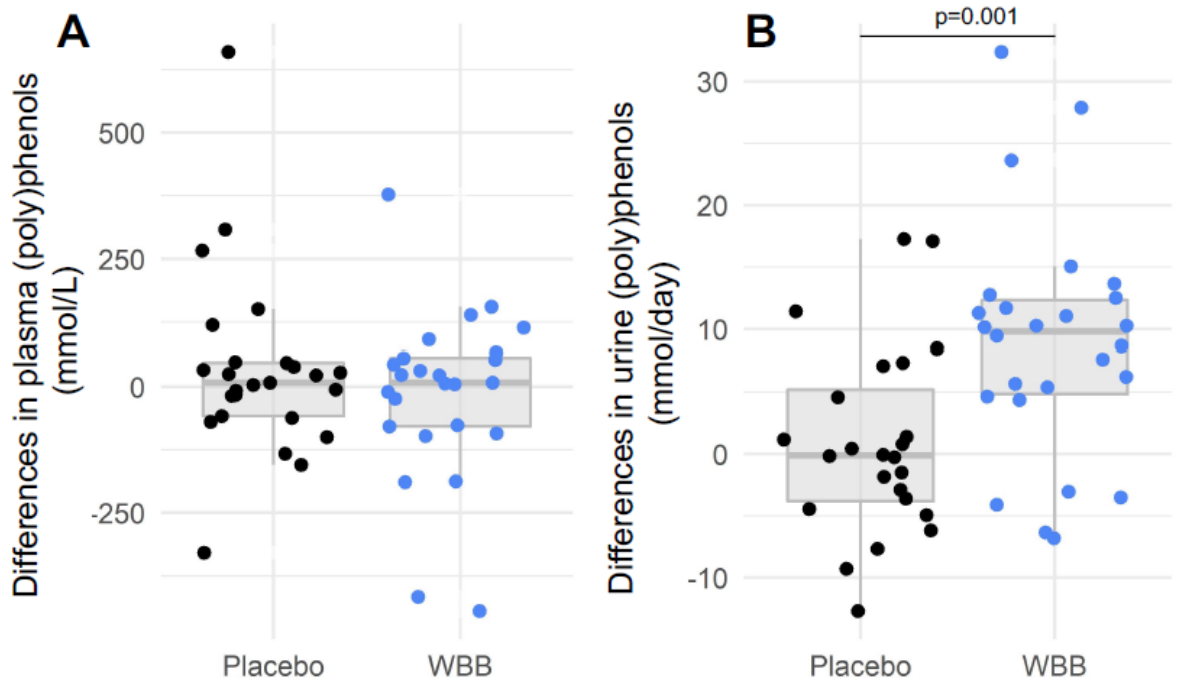

A
Differences in plasma (poly)phenols (mmol/L)
500
250
0
-250
Placebo
WBB
B
p=0.001
Differences in urine (poly)phenols (mmol/day)
30
20
10
0
-10
Placebo
WBB

Figure 4

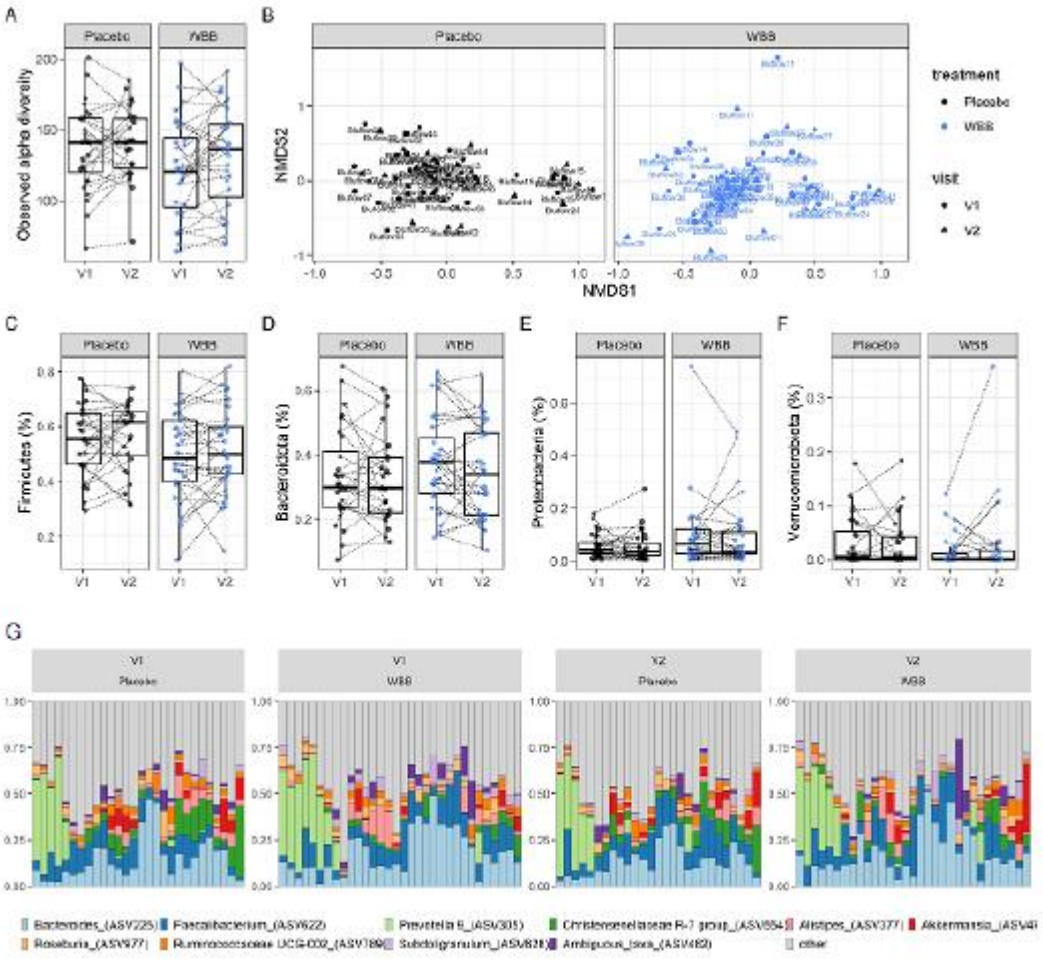

A
Placebo
WB8
Observed alpha diversity
V1
V2
B
NMDS2
NMDS1
treatment
Placebo
WB8
visit
V1
V2
C
Firmicutes (%)
D
Bacteroidota (%)
E
Proteobacteria (%)
F
Verrucomicrobiota (%)
WB5
G
Bacteroides_(ASV225)
Faecalibacterium_(ASV622)
Prevotella 9_(ASV305)
Christensenellaceae R-7 group_(ASV654)
Alistipes_(ASV377)
Roseburia_(ASV677)
Ruminococcaceae UCG-002_(ASV189)
Subdoligranulum_(ASV828)
Ambiguous_taxa_(ASV482)
other

Figure 5

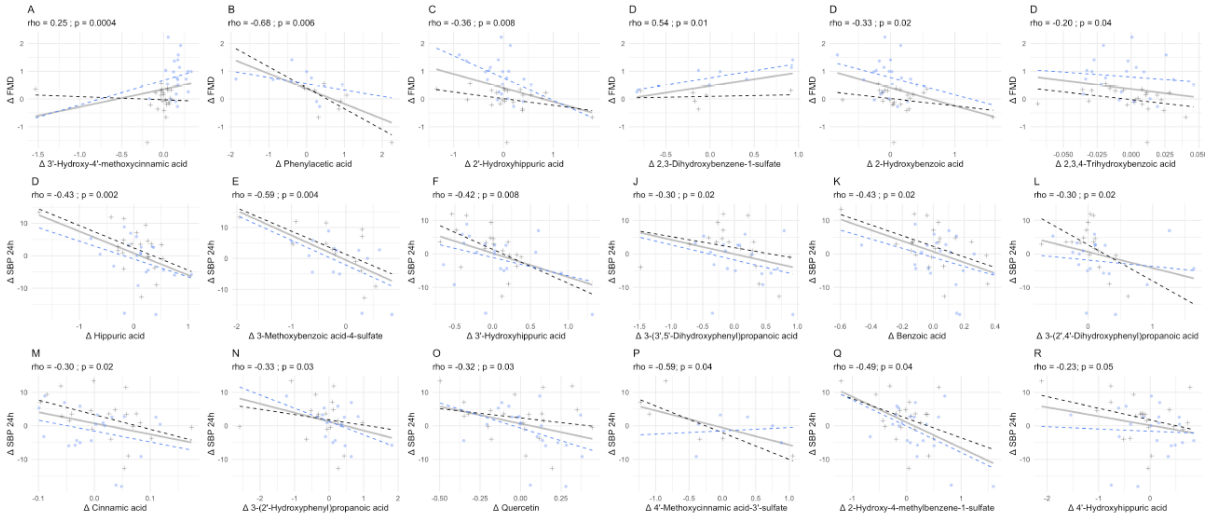
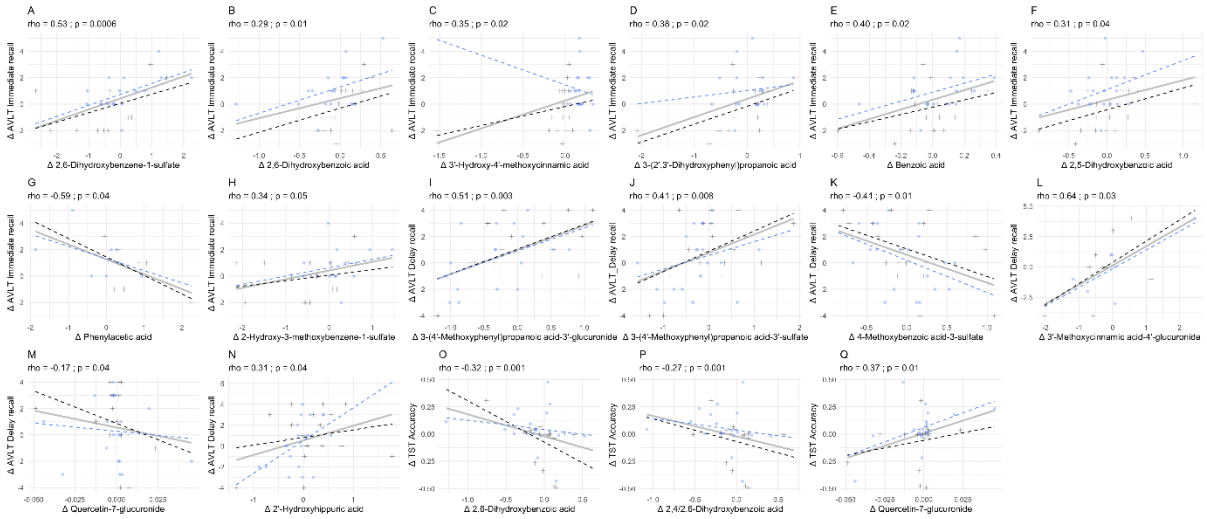

A
rho = 0.25 ; p = 0.0004
Δ FMD
Δ 3'-Hydroxy-4'-methoxycinnamic acid
B
rho = -0.68 ; p = 0.006
Δ FMD
Δ Phenylacetic acid
C
rho = -0.36 ; p = 0.008
Δ FMD
Δ 2'-Hydroxyhippuric acid
D
rho = 0.54 ; p = 0.01
Δ FMD
Δ 2,3-Dihydroxybenzene-1-sulfate
D
rho = -0.33 ; p = 0.02
Δ FMD
Δ 2-Hydroxybenzoic acid
D
rho = -0.20 ; p = 0.04
Δ FMD
Δ 2,3,4-Trihydroxybenzoic acid
D
rho = -0.43 ; p = 0.002
Δ SBP 24h
Δ Hippuric acid
E
rho = -0.59 ; p = 0.004
Δ SBP 24h
Δ 3-Methoxybenzoic acid-4-sulfate
F
rho = -0.42 ; p = 0.008
Δ SBP 24h
Δ 3'-Hydroxyhippuric acid
J
rho = -0.30 ; p = 0.02
Δ SBP 24h
Δ 3-(3',5'-Dihydroxyphenyl)propanoic acid
K
rho = -0.43 ; p = 0.02
Δ SBP 24h
Δ Benzoic acid
L
rho = -0.30 ; p = 0.02
Δ SBP 24h
Δ 3-(2',4'-Dihydroxyphenyl)propanoic acid
M
rho = -0.30 ; p = 0.02
Δ SBP 24h
Δ Cinnamic acid
N
rho = -0.33 ; p = 0.03
Δ SBP 24h
Δ 3-(2'-Hydroxyphenyl)propanoic acid
O
rho = -0.32 ; p = 0.03
Δ SBP 24h
Δ Quercetin
P
rho = -0.59; p = 0.04
Δ SBP 24h
Δ 4'-Methoxycinnamic acid-3'-sulfate
Q
rho = -0.49; p = 0.04
Δ SBP 24h
Δ 2-Hydroxy-4-methylbenzene-1-sulfate
R
rho = -0.23; p = 0.05
Δ SBP 24h
Δ 4'-Hydroxyhippuric acid

Figure 6


A
rho = 0.53 ; p = 0.0006
Δ AVLT Immediate recall
Δ 2,6-Dihydroxybenzene-1-sulfate
B
rho = 0.29 ; p = 0.01
Δ AVLT Immediate recall
Δ 2,6-Dihydroxybenzoic acid
C
rho = 0.35 ; p = 0.02
Δ AVLT Immediate recall
Δ 3'-Hydroxy-4'-methoxycinnamic acid
D
rho = 0.38 ; p = 0.02
Δ AVLT Immediate recall
Δ 3-(2',3'-Dihydroxyphenyl)propanoic acid
E
rho = 0.40 ; p = 0.02
Δ AVLT Immediate recall
Δ Benzoic acid
F
rho = 0.31 ; p = 0.04
Δ AVLT Immediate recall
Δ 2,5-Dihydroxybenzoic acid
G
rho = -0.59 ; p = 0.04
Δ AVLT Immediate recall
Δ Phenylacetic acid
H
rho = 0.34 ; p = 0.05
Δ AVLT Immediate recall
Δ 2-Hydroxy-3-methoxybenzene-1-sulfate
I
rho = 0.51 ; p = 0.003
Δ AVLT Delay recall
Δ 3-(4'-Methoxyphenyl)propanoic acid-3'-glucuronide
J
rho = 0.41 ; p = 0.008
Δ AVLT_Delay recall
Δ 3-(4'-Methoxyphenyl)propanoic acid-3'-sulfate
K
rho = -0.41 ; p = 0.01
Δ AVLT Delay recall
Δ 4-Methoxybenzoic acid-3-sulfate
L
rho = 0.64 ; p = 0.03
Δ AVLT Delay recall
Δ 3'-Methoxycinnamic acid-4'-glucuronide
M
rho = -0.17 ; p = 0.04
Δ AVLT Delay recall
Δ Quercetin-7-glucuronide
N
rho = 0.31 ; p = 0.04
Δ AVLT Delay recall
Δ 2'-Hydroxyhippuric acid
O
rho = -0.32 ; p = 0.001
Δ TST Accuracy
Δ 2,6-Dihydroxybenzoic acid
P
rho = -0.27 ; p = 0.001
Δ TST Accuracy
Δ 2,4/2,6-Dihydroxybenzoic acid
Q
rho = 0.37 ; p = 0.01
Δ TST Accuracy
Δ Quercetin-7-glucuronide

Supplementary material

Wild Blueberry (Poly)Phenols Improve Vascular Function And Cognitive Performance Independently Of Changes In Cerebral Blood Flow Or Gut Microbiota Composition In Healthy Older Males And Females: A Double-Blind Randomized Controlled Trial

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Supplementary Table 1. Average daily macro- micronutrient and (poly)phenol intakes from 7-day food diaries at baseline

	Placebo group Mean (SD) (n=27)	WBB group Mean (SD) (n=27)
Energy and nutrient		
Energy (Kcal)	1685 (534)	1777 (393)
Carbohydrates (g)	177 (85.8)	182 (48.5)
Protein (g)	75.8 (15.1)	74.3 (15.9)
Fat (g)	64.1 (29)	75.3 (16.8)
Monounsaturated	27.2 (1.6)	27.7 (1.3)
Polyunsaturated	13.6 (1.4)	12.7 (0.8)
Saturated	25.4(1.9)	27.4 (1.6)
Alcohol (g)	4.8 (7.2)	6.3 (15.9)
Fibre (g)	19.6 (6.8)	21.2 (12.1)
Total Cholesterol (mg)	250 (92)	212 (78.8)
Vitamin A ((totalRE)µg)	1006 (577)	947 (705)
Vitamin E (mg)	8.7 (4.7)	9.8 (5)
Vitamin C (mg)	109 (66)	105 (69.6)
Sodium (mg)	1644 (875)	1767 (594)
Caffeine (mg)	140 (95.6)	148 (72.3)
(Poly)phenols and main sources		
Total (Poly)phenols (mg)	1342 (546)	1248 (767)
Main sources (%)		
Tea	31	33
Coffee	26	24
Apples	8.3	3.7
Cocoa	4.4	6.1
Wine	1.8	2.6
Blueberries	2.3	1.2
Other	26	29.3
Total Anthocyanins (mg)	47.3 (85.8)	51.8 (66.7)
Main sources (%)		
Wine	25	36
Raspberries	17	10
Strawberries	6.3	4.6
Blueberries	12	5.7
Other berries	17	8.6
Grapes	4.2	7.7
Other	19	27

Supplementary Table 2. Main outcomes of vascular function and cerebral blood flow at baseline and 12-weeks following daily wild blueberry (WBB) or placebo treatment. Linear mixed modelling analysis presented as difference from placebo at 12-weeks, using baseline vascular as a covariate.

Outcome	Wild blueberry powder (n=27)		Placebo powder (n=27)		LMM analysis		
	Baseline Mean (± SD)	12-weeks Mean (± SD)	Baseline Mean (± SD)	12-weeks Mean (± SD)	Mean WBB-Mean Placebo at 12 weeks (± SEM)	95% CI Lower; upper	p-value
FMD (%)-	3.77 (± 1.52)	4.630 (± 1.45)	4.16 (± 1.13)	4.125 (± 1.19)	0.86* (± 0.15)	0.56, 1.17	<0.001
PWV (m/s)-	7.95 (± 3.20)	7.61 (± 2.32)	8.62 (± 2.34)	8.03 (± 2.46)	-0.15 (± 0.70)	-1.56, 1.26	0.835
AIx@75 (%)-	29.04 (± 6.41)	29.0 (± 7.93)	29.63 (± 11.48)	28.5 (± 7.11)	2.01 (± 1.89)	-1.79, 5.81	0.293
CSBP (mmHg)-	122.88 (± 12.46)	123.4 (± 13.4)	123.42 (± 11.25)	121.4 (± 11.7)	1.49 (± 2.75)	-4.05, 7.02	0.592
CDBP (mmHg)-	82.75 (± 8.92)	82.1 (± 7.31)	80.83 (± 5.96)	79.5 (± 7.00)	1.61 (± 1.62)	-1.66, 4.88	0.326
24h SBP awake (mmHg)	134.67 (± 10.56)	133.2 (± 11.4)	132.83 (± 11.76)	135.1 (± 10.1)	-2.88 (± 1.90)	-6.69, 0.92	0.135
24h SBP asleep (mmHg)	112.53 (± 11.58)	112.7 (± 10.8)	113.41 (± 15.33)	112.1 (± 12.0)	-1.54 (± 2.52)	-6.63, 3.56	0.546
Total 24h SBP (mmHg)	130.52 (± 11.80)	128.7 (± 11.4)	128.59 (± 11.32)	131.1 (± 9.8)	-3.59* (± 1.67)	-6.95, -0.23	0.037
24h DBP awake (mmHg)	80.40 (± 7.63)	79.12 (± 8.01)	79.20 (± 7.39)	80.6 (± 6.98)	-2.08 (± 1.71)	-5.51, 1.36	0.231
24h DBP asleep (mmHg)	65.38 (± 7.89)	66.4 (± 7.04)	65.95 (± 8.92)	64.2 (± 8.31)	1.02 (± 1.94)	-2.90, 4.94	0.602
Total 24h DBP (mmHg)-	77.43 (± 7.36)	76.5 (± 7.66)	76.47 (± 7.17)	77.8 (± 6.29)	-1.93 (± 1.45)	-4.85, 0.99	0.191
Mean BFV resting (cm/s) ~-	55.59 (± 5.90)	56.4 (± 6.26)	53.96 (± 8.55)	54.9 (± 9.59)	0.95 (± 2.68)	-4.57, 6.47	0.726
Mean PI resting (cm/s) ~-	1.05 (± 0.16)	1.07 (± 0.20)	1.19 (± 0.29)	1.12 (± 0.20)	0.02 (± 0.07)	-0.12, 0.17	0.749
Mean BFV active (cm/s) ~-	55.67 (± 5.08)	58.1 (± 4.88)	55.55 (± 11.22)	60.9 (± 10.29)	0.08 (± 6.70)	-15.80, 15.90	0.991
Mean PI active (cm/s) ~-	1.16 (± 0.17)	1.46 (± 0.26)	1.11 (± 0.21)	1.11 (± 0.22)	0.29 (± 0.13)	-0.02,0.60	0.065
Office SBP (mmHg)-	128.59 (± 12.15)	129.7 (± 13.5)	128.37 (± 10.44)	127.2 (± 12.2)	2.04 (± 2.48)	-2.93, 7.01	0.414
Office DBP (mmHg)-	81.35 (± 8.48)	81.9 (± 8.24)	79.22 (± 5.61)	78.8 (± 6.70)	1.06 (± 1.44)	-1.84, 3.96	0.466
HDL-Cholesterol (mmol/L)-	1.79 (± 0.44)	1.79 (± 0.36)	2.23 (± 0.81)	2.10 (± 0.53)	0.01 (± 0.80)	-0.15, 0.17	0.892
LDL-Cholesterol (mmol/L)-	4.07 (± 1.26)	4.34 (± 1.32)	3.75 (± 0.97)	3.92 (± 1.14)	0.10 (± 0.14)	-0.19, 0.39	0.502
Fasting plasma glucose (mmol/L)-	4.95 (± 0.60)	5.04 (± 0.63)	4.80 (± 0.45)	4.82 (± 0.58)	0.10 (± 0.13)	-0.17, 0.37	0.457

Augmentation index, AIx; Blood flow velocity, BFV; Flow-mediated dilation; Central diastolic blood pressure, CDBP; Central systolic blood pressure, CSBP; FMD; Diastolic blood pressure, DBP; High-density lipoprotein, HDL; Low-density lipoprotein, LDL; Pulsatility index, PI; Pulse wave velocity, PWV; Systolic blood

*pressure, SBP. *Significant difference from placebo in the WBB group analysed using LMM ($p<0.05$). ~ data collected measuring CBF was lower due to measurements limitations for both resting (n=20 placebo and n=18 WBB) and active cerebral blood flow (n= 16 placebo group and n=10 WBB group).*

Supplementary Table 3. Main outcomes of cognition at baseline and 12-weeks following daily wild blueberry (WBB) or placebo treatment. Linear mixed modelling analysis presented as difference from placebo following at 12 weeks, using baseline vascular as a covariate.

Outcome	Wild Blueberry Powder (n=27)		Placebo Powder (n=27)		LMM Analysis		
	Baseline Mean (± SD)	12-weeks Mean (± SD)	Baseline Mean (± SD)	12-weeks Mean (± SD)	Mean WBB- Mean Placebo (± SEM)	95% CI Lower; upper	*p*-value
AVLT							
Total Acquisition	44.72 (± 7.63)	45.50 (± 8.03)	44.95 (± 9.52)	46.75 (± 8.24)	-0.338 (± 1.80)	-3.95, 3.27	0.851
Immediate Recall	5.28 (± 1.67)	5.92 (± 1.71)	5.23 (± 1.88)	5.28 (± 1.49)	0.854* (± 0.41)	0.03, 1.68	0.043
Proactive Interference	-0.16 (± 2.21)	0.04 (± 1.87)	-0.45 (± 2.28)	-0.48 (± 1.58)	0.618 (± 0.49)	-0.36, 1.60	0.211
Retroactive Interference	3.27 (± 2.09)	2.64 (± 1.98)	2.88 (± 1.92)	2.12 (± 1.86)	0.449 (± 0.49)	-0.53, 1.42	0.360
Delayed Recall	6.81 (± 3.16)	7.48 (± 3.14)	7.83 (± 3.13)	9.43 (± 2.57)	-1.507* (± 0.67)	-2.86, -0.16	0.029
Word Recognition- (out of 15)	12.65 (± 1.65)	13.12 (± 2.17)	13.25 (± 1.39)	13.48 (± 1.69)	-0.087 (± 0.51)	-1.11, 0.94	0.886
Corsi Blocks							
Correct Sequence	15.27 (±3.38)	14.92 (±3.45)	16.09 (±3.87)	15.40 (±4.17)	2.53 (±0.87)	-1.49, 2.00	0.772
Correct Blocks	26.88 (±3.33)	26.42 (±4.76)	27.59 (±3.80)	26.52 (±4.89)	0.482 (±1.11)	-1.75, 2.71	0.666
Initial Reaction Time (ms)	1862.09 (±477.60)	1734.22 (±433.26)	1871.85 (±383.79)	1976.82 (±684.76)	-164.85 (±137.85)	-441.86, 113.09	0.239
Serials Subtraction Task							
Serial 3s Accuracy	21.10 (±9.72)	21.16 (±9.11)	21.74 (±9.69)	25.17 (±6.37)	-1.700 (±1.97)	-5.68, 2.28	0.601
Serial 7s Accuracy	14.56 (±7.70)	14.80 (±7.40)	17.32 (±8.04)	16.79 (±6.94)	-0.049 (±1.22)	-2.51, 2.41	0.968
Task-Switching Test							
Overall Accuracy	0.82 (±0.23)	0.89 (±0.17)	0.88 (±0.17)	0.81 (±0.25)	0.096* (±0.043)	0.12, 0.18	0.026
Overall Reaction Time (ms)	1106.86 (±400.39)	1078.14 (±359.73)	987.54 (±330.06)	1063.39 (±364.67)	-24.97 (±42.53)	-109.47, 59.53	0.559

*LMM; linear mixed modelling. * The mean difference is significant at p< 0.05 analysed using linear mixed modelling.*

Supplementary Table 4. Plasma (poly)phenol metabolites with baseline and 12-week averages for each treatment, and linear mixed modelling (LMM) results with difference from placebo, baseline values as a covariate. Only compounds with significant LMM findings are reported (p<0.05).

(Poly)phenol metabolite (nM)	Wild blueberry powder (n=27)		Placebo powder (n=27)			LMM analysis	
	Baseline (Mean ±SD)	12-weeks (Mean ±SD)	Baseline (Mean ±SD)	12-weeks (Mean ±SD)	WBB- Placebo (Mean ±SEM)	95 % CI Lower; upper	p-value
Pyrogallol-O-sulfate mixture	1567 (±4922)	2105 (±6036)	2595 (±4225)	1025 (±1936)	1973.8 (±795)	367; 3581	0.017
2-Methylpyrogallol-O-sulfate	657 (±1876)	915 (±2740)	441 (±541)	266 (±323)	206.4 (±98.4)	7.4; 405	0.042
4-Methylcatechol-O-sulfate	13057 (±19003)	16764 (±17137)	9769 (±6959)	9196 (±7134)	7805.8 (±3435)	882; 14729	0.028
Vanillic acid	856 (±41)	873 (±45)	1036 (±498)	885 (±34)	-91.9 (±31.9)	-174; -9.9	0.034
4-Methylcatechol	1646 (±1918)	2124 (±1736)	1226 (±1001)	1266 (±1044)	896.9 (±339)	213; 1581	0.011
Isoferulic acid	407 (±83)	576 (±115)	847 (±2215)	398 (±99)	173.4 (±32.2)	108; 238	<0.001
Phenylacetic acid	2786 (±2550)	3171 (±2942)	3355 (±5650)	5242 (±2075)	-3207.5 (±1175)	-5698; -717	0.015

LMM; linear mixed model analysis SD; standard deviation, SEM; standard error mean. Significant difference from placebo in the WBB group whereby p<0.05.

Supplementary Table 5. Urine (poly)phenol metabolites with baseline and 12-week averages for each treatment, and linear mixed modelling (LMM) results with difference from placebo, baseline values as a covariate. Only compounds with significant LMM findings are reported (p<0.05).

(Poly)phenol metabolite (mmol/day)	Wild blueberry powder		Placebo powder		LMM analysis
	Baseline (Mean ±SD)	12-weeks (Mean ±SD)	Baseline (Mean ±SD)	12-weeks (Mean ±SD)	p-value
Isovanillic acid 3-O-sulfate	27.2 (±34.3)	36.9 (±23.4)	43.5 (±26.2)	37.7 (±28.1)	0.001
4-Feruloylquinic acid	37.6 (±30.4)	26.7 (±26.7)	17.0 (±17.1)	25.5 (±28.5)	0.001
Homovanillic acid sulfate sodium salt	58.7 (±33.0)	53.2 (±39.4)	40.2 (±26.1)	44.3 (±22.2)	0.002
(R)-(+)-2-(4-hydroxyphenoxy)-propionic acid	48.4 (±44.6)	47.2 (±33.6)	28.9 (±34.0)	34.5 (±33.7)	0.003
3-(2,3-Dihydroxyphenyl)Propionic Acid	8.5 (±6.3)	6.8 (±5.8)	6.1(±8.6)	5.4 (±5.6)	0.003
Isoferulic acid	661.7 (±313.5)	3267.3 (±1646.8)	623.1 (±1066.4)	558.3 (±425.2)	0.02
3-Feruloylquinic acid	23.4 (±11.0)	25.2 (±10.2)	20.2 (±12.2)	20.8 (±12.9)	0.02
4-Methylcatechol	59.9 (±33.1)	80.4 (±62.7)	40.2 (±26.2)	46 (±27.6)	0.02
Ferulic Acid 4-O-β-D-Glucuronide	285.8 (±221.8)	325.6 (±179.6)	249.9 (±409.9)	227.4 (±185.3)	0.03
Catechol-O-1-glucuronide	10.9 (±5.6)	13.5 (±9.3)	9.0 (±8.6)	8.2 (±6.5)	0.03

LMM; linear mixed model analysis SD; standard deviation. Significant difference from placebo in the WBB group whereby p<0.05.

Supplementary Table 6. Bacteria genera which are the most affected by the intervention. Effect size, their standard deviations and their statistical significance are presented for all the individuals grouped together or separated by intervention arm (placebo or WBB).

	effect size	sd	p all	q all	p placebo	q placebo	p WBB	q WBB
Ruminiclostridium 9	0.009	0.003	0.0007	0.06	0.09	0.79	0.002	0.15
Ruminiclostridium 5	0.009	0.003	0.002	0.11	0.02	0.63	0.04	0.80
Parabacteroides	0.02	0.005	0.003	0.20	0.00002	0.02	0.78	1.0
Eggerthella	0.002	0.0008	0.03	0.75	0.08	0.76	0.18	0.42
Butyrivibrio	0.02	0.008	0.03	0.77	0.02	0.63	0.71	1.0
Faecalibacterium	0.04	0.02	0.03	0.78	0.03	0.65	0.45	1.0
Mollicutes RF39 *metagenome*	0.003	0.001	0.04	0.83	0.13	0.83	0.14	1.0
Victivallaceae *uncultured*	0.001	0.0004	0.04	0.84	0.10	0.80	0.18	1.0
Lachnoclostridium	0.01	0.005	0.04	0.92	0.02	0.63	0.63	1.0
Moryella	0.002	0.001	0.05	1.0	0.07	0.74	0.42	1.0
Intestinibacter	0.0045	0.004	0.19	1.0	0.68	0.93	0.02	0.64
Eggerthellaceae *uncultured*	0.001	0.001	0.48	1.0	0.20	0.83	0.007	0.36
Clostridiales Family XIII *AD3011*	0.003	0.003	0.29	1.0	0.47	0.86	0.01	0.48
Barnesiellaceae *uncultured*	0.003	0.001	0.06	1.0	0.98	1.0	0.03	0.71
Christensenellaceae *uncultured*	0.0008	0.002	0.61	1.0	0.23	0.83	0.04	0.80

p= p-value; q= False Discovery Rate (FDR); WBB=Wild Blueberry Intervention, SD=Standard deviation

Supplementary Table 7. Random forest discrimination of WBB from placebo consumption. We compared the predictive ability of urine or plasma polyphenols or microbiome profiles. The confusion matrix presents the proportion of samples for which treatment was accurately predicted. Summary statistics for each model include accuracy and their 95% confidence interval (95% CI), a p-value testing if the accuracy is different from the no-information rate.

		Plasma polyphenols		**Urine polyphenols**		**Microbiome**	
Confusion matrix		WBB	Placebo	WBB	Placebo	WBB	Placebo
Prediction	WBB	6	2	5	3	5	4
	Placebo	1	6	1	8	3	7
Accuracy		0.8		0.76		0.63	
95% CI		0.52, 0.95		0.5, 0.93		0.38, 0.83	
P-Value		0.03		0.22		0.41	

WBB; wild blueberry

Supplementary Table 8. (Poly)phenol metabolite standards used to quantify metabolites present in plasma and urine samples, including limit of quantification values (LOQ), transitions, collision energy and recommended names.

			Transitions (m/z)			Collision energy (V)				
Compound common name	**Recommended name**	**Parent ion (m/z)**	**Quantifier**	**Qualifier 1**	**Qualifier 2**	**Quantifier**	**Qualifier 1**	**Qualifier 2**	**RT (min)**	**LOQ (nM)**
Flavanols										
(-)-Epicatechin	(-)-Epicatechin	289.05	245.1	203.15	123.15	15	18	31	4.68	16.7
(-)-Epicatechin-3'-sulfate	(-)-Epicatechin-3'-sulfate	369.05	289	97	231.1	31	18	20	4.15	56.9
Flavonols										
Quercetin	Quercetin	301.05	151.1	179.15	107.05	22	19	27	12.20	11.5
Quercetin-3-sulfate	Quercetin-3-sulfate	381.3	301.1	179.1	-	17	29	-	6.20	8.4
Quercetin-3-glucuronide	Quercetin-3-glucuronide	477.05	301	-	-	22	-	-	7.20	57.5
Quercetin-7-glucuronide	Quercetin-7-glucuronide	477.05	301.05	-	-	23	-	-	7.79	16.7
Kaempferol-3-glucuronide	Kaempferol-3-glucuronide	461.05	285.05	257.05	-	20	32	-	9.09	1.8
Benzene diols and triols										
4-Methylcatechol	1,2-Dihydroxy-4-methylbenzene	123.15	108.15	95.1	-	21	15	-	3.52	16.8
4-Methylcatechol-1/2-sulfate	2-Hydroxy-4/5-methylbenzene-1-sulfate	203	123.15	122.2	80.1	22	32	21	3.50	167.0
Catechol-O-1-glucuronide	2-Hydroxybenzene-1-glucuronide	285.05	109.15	113.1	-	30	13	-	3.38	59.1
Pyrogallol-1-sulfate	2,3-Dihydroxybenzene-1-sulfate	205	125.1	80	123.1	18	27	13	0.70	52.5
Pyrogallol-2-sulfate	2,6-Dihydroxybenzene-1-sulfate	205	125.1	80	123.1	18	27	13	1.00	11.1
1-Methylpyrogallol-2/3-sulfate	2-Hydroxy-6/3-methoxybenzene-1-sulfate	219	124.05	139.1	-	25	15	-	2.82	33.5
2-Methylpyrogallol-1-sulfate	3-Hydroxy-2-methoxybenzene-1-sulfate	219	124.1	-	-	25	-	-	1.47	16.4
Benzaldehydes										
3,4-Dihydroxybenzaldehyde	3,4-Dihydroxybenzaldehyde	177	108.1	92.05	81.1	23	25	21	3.26	54.2
4-Hydroxybenzaldehyde	4-Hydroxybenzaldehyde	121	92	93.1	-	24	22	-	4.14	15.9
Vanillin	4-Hydroxy-3-methoxybenzaldehyde	151.15	136.15	92.1	108.05	16	22	23	5.06	518.7
Hydroxybenzoic acids										
Benzoic acid	Benzoic acid	121.05	77.05	-	-	11	-	-	5.52	586.9
2-Hydroxybenzoic acid	2-Hydroxybenzoic acid	137	93.05	65.05	-	18	28	-	5.17	8.3
3-Hydroxybenzoic acid	3-Hydroxybenzoic acid	137	93	-	-	13	-	-	3.64	116.8

4-Hydroxybenzoic acid	4-Hydroxybenzoic acid	137	93.05	65	-	17	30	-	3.06	275.1
2,3-Dihydroxybenzoic acid	2,3-Dihydroxybenzoic acid	153	109.05	108.1	-	15	24	-	3.59	166.7
2,4/2,6-Dihydroxybenzoic acid	2,4/2,6-Dihydroxybenzoic acid	153	67.05	65.1	109.1	16	17	21	3.36	118.7
2,5-Dihydroxybenzoic acid	2,5-Dihydroxybenzoic acid	153	109	108.1	-	15	21	-	2.88	33.4
2,6-Dihydroxybenzoic acid	2,6-Dihydroxybenzoic acid	153.1	135.15	65.1	109.1	17	21	18	3.29	18.5
Protocatechuic acid	3,4-Dihydroxybenzoic acid	153	108.15	109.15	-	15	24	-	1.71	58.3
3,5-Dihydroxybenzoic acid	3,5-Dihydroxybenzoic acid	153	109.1	65	-	14	13	-	1.37	34.1
2,3,4-Trihydroxybenzoic acid	2,3,4-Trihydroxybenzoic acid	169	151.15	107.1	123.1	16	21	21	1.84	57.0
2-Hydroxy-4-methoxybenzoic acid	2-Hydroxy-4-methoxybenzoic acid	167.05	108.15	123.15	80.05	21	16	24	8.95	8.3
Protocatechuic acid-4-sulfate	3-Hydroxybenzoic acid-4-sulfate	233	109.05	153.1	108.05	27	14	45	1.00	8.3
Protocatechuic acid-3-sulfate	4-Hydroxybenzoic acid-3-sulfate	233	109.05	153.1	108.05	27	14	45	1.20	8.3
Protocatechuic acid-3-glucuronide	4-Hydroxybenzoic acid-3-glucuronide	329.05	109.1	153.15	113.15	34	17	14	2.13	16.7
Syringic acid	4-Hydroxy-3,5-dimethoxybenzoic acid	197.05	182.2	123.05	-	12	21	-	4.52	55.3
Gallic acid	3,4,5-Trihydroxybenzoic acid	169	125.15	79.15	81.05	15	21	21	0.75	4.4
4-Methylgallic acid-3-sulfate	3-Hydroxy-4-methoxybenzoic acid-5-sulfate	263	168.2	183.15	124.2	22	15	30	1.63	8.2
Vanillic acid	4-Hydroxy-3-methoxybenzoic acid	167	152.15	108.1	123.15	17	18	15	4.02	557.7
Vanillic acid-4-sulfate	3-Methoxybenzoic acid-4-sulfate	247	167.05	152.1	-	15	25	-	2.32	8.3
Isovanillic acid-3-sulfate	4-Methoxybenzoic acid-3-sulfate	247	167.15	152.15	108	15	23	31	3.23	278.1
Hippuric acids										
Hippuric acid	Hippuric acid	178.05	134.25	77.1	-	14	18	-	3.91	1163.2
2’-Hydroxyhippuric acid	2’-Hydroxyhippuric acid	194.05	93.1	150.2	65.25	23	15	42	4.65	56.4
3’-Hydroxyhippuric acid	3’-Hydroxyhippuric acid	194	150.2	93.05	92	14	17	30	3.08	287.6
4’-Hydroxyhippuric acid	4’-Hydroxyhippuric acid	194	100.1	93.1	74.2	11	18	22	2.52	55.5
α-hydroxyhippuric acid	α-hydroxyhippuric acid	194.05	73.1	-	-	10	-	-	3.03	41.9
Cinnamic acids										
Cinnamic acid	Cinnamic acid	148.7	103.25	131.2	77.2	-15	-22	-32	11.15	115.2
Caffeic acid	3’,4’-Dihydroxycinnamic acid	179.05	134.15	135.2	-	17	25	-	3.97	12.3

Caffeic acid-4′-sulfate	3′-Hydroxycinnamic acid-4′-sulfate	259	179.1	135.1	134.1	15	26	40	3.19	8.3
Caffeic acid-3′-sulfate	4′-Hydroxycinnamic acid-3′-sulfate	259	179.1	135.1	134.1	15	26	40	3.47	16.7
Caffeic acid-4′-glucuronide	3′-Hydroxycinnamic acid-4′-glucuronide	355	179.1	135.25	-	16	35	-	3.49	11.5
Caffeic acid-3′-glucuronide	4′-Hydroxycinnamic acid-3′-glucuronide	355	135.1	179.15	-	35	20	-	3.85	11.5
trans-Ferulic acid	4′-Hydroxy-3′-methoxycinnamic acid	193.05	134.15	178.25	133.05	17	14	25	3.98	550.4
Ferulic acid-4′-sulfate	3′-Methoxycinnamic acid-4′-sulfate	273	193.2	178.2	134.15	14	23	29	3.98	33.3
Ferulic acid-4′-glucuronide	3′-Methoxycinnamic acid-4′-glucuronide	369.1	134.2	193.15	113.2	35	17	14	4.74	60.2
Isoferulic acid	3′-Hydroxy-4′-methoxycinnamic acid	193.05	134.3	-	-	15	-	-	5.97	83.3
Isoferulic acid-3′-sulfate	4′-Methoxycinnamic acid-3′-sulfate	273	193.2	178.1	134.1	16	22	28	4.46	8.1
Isoferulic acid-3′-glucuronide	4′-Methoxycinnamic acid-3′-glucuronide	369.1	113.2	193.2	178.25	16	20	29	4.10	8.6
Cryptochlorogenic acid	4-O-Caffeoylquinic acid	353.2	173.25	179.25	191.2	16	17	23	4.01	10.1
Chlorogenic acid	5-O-Caffeoylquinic acid	353.1	191.2	85.15	93.05	19	43	42	3.97	3.3
3-O-Feruloylquinic acid	3-O-Feruloylquinic acid	367.35	191.2	93.1	134.2	15	27	34	5.38	1.7
4-O-Feruloylquinic acid	4-O-Feruloylquinic acid	367.35	173.3	193.2	134.2	16	17	34	5.17	54.4
Sinapic acid	4′-Hydroxy-3′,5′-dimethoxycinnamic acid	223.05	208.15	121.05	193.15	12	29	21	6.54	11.6
m-Coumaric acid	3′-Hydroxycinnamic acid	163	119.15	92.85	90.9	15	31	25	5.59	10.1
p-Coumaric acid	4′-Hydroxycinnamic acid	163.05	119.15	93	-	16	33	-	4.96	3.3
o-Coumaric acid	2′-Hydroxycinnamic acid	163	119.1	93.05	117.1	14	29	24	6.81	8.5
p-Coumaric acid-4′-sulfate	Cinnamic acid-4′-sulfate	243	119.15	-	-	25	-	-	3.69	17.0
p-Coumaric acid-4′-glucuronide	Cinnamic acid-4′-glucuronide	339.05	119.2	113.05	162.95	39	13	17	3.69	8.3
Phenylacetic acids										
Phenylacetic acid	Phenylacetic acid	135.05	91.1	-	-	9	-	-	5.67	166.1
Homoprotocatechuic acid	3′,4′-Dihidroxyphenylacetic acid	167.05	123.15	-	-	10	-	-	2.80	33.2
Homovanillic acid-sulfate	3′-Methyoxyphenylacetic acid-4′-sulfate	261	181.15	137.15	122.1	16	22	25	3.26	11.4
Phenylpropanoic acids										

2-(4′-Hydroxyphenoxy)propanoic acid	2-(4′-Hydroxyphenoxy)propanoic acid	181.05	109.15	-	-	15	-	-	3.67	33.3
3-(2′-Hydroxyphenyl)propanoic acid	3-(2′-Hydroxyphenyl)propanoic acid	165	121.15	106.15	-	15	21	-	5.47	56.2
3-(3′-Hydroxyphenyl)propanoic acid	3-(3′-Hydroxyphenyl)propanoic acid	165.05	121.1	118.95	-	14	16	-	4.91	59.4
3-(2′,3′-Dihydroxyphenyl)propanoic acid	3-(2′,3′-Dihydroxyphenyl)propanoic acid	181.15	137.2	122.1	163.2	17	24	15	4.19	7.5
3-(2′,4′-Dihydroxyphenyl)propanoic acid	3-(2′,4′-Dihydroxyphenyl)propanoic acid	183.15	123.2	165.15	55.15	-11	-15	-16	3.63	65.7
Dihydrocaffeic acid	3-(3′,4′-Dihydroxyphenyl)propanoic acid	181.05	137.2	-	-	15	-	-	3.61	58.2
3-(3′,5′-Dihydroxyphenyl)propanoic acid	3-(3′,5′-Dihydroxyphenyl)propanoic acid	183.15	165.15	137.2	-	-12	-17	-	3.32	9.7
2-Hydroxy-3-(4′-hydroxyphenyl)propanoic acid	2-Hydroxy-3-(4′-hydroxyphenyl)propanoic acid	181.15	163.2	135.15	-	16	17	-	2.89	66.0
Dihydroferulic acid	3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid	195.05	136.25	120.95	-	15	27	-	5.15	560.7
Dihydrocaffeic acid-3′-glucuronide	3-(4′-Hydroxyphenyl)propanoic acid-3′-glucuronide	357.1	181.15	-	-	21	-	-	3.76	16.7
Dihydrocaffeic acid-3′-sulfate	3-(4′-Hydroxyphenyl)propanoic acid-3′-sulfate	261	181.15	137.25	-	17	23	-	3.41	59.1
Dihydroferulic acid-4′-glucuronide	3-(3′-Methoxyphenyl)propanoic acid-4′-glucuronide	275.1	195.35	136.15	135.3	17	23	35	3.89	12.2
Dihydroferulic acid-4′-sulfate	3-(3′-Methoxyphenyl)propanoic acid-4′-sulfate	371.1	113.15	195.25	-	16	20	-	4.22	51.7
Dihydroisoferulic acid-3′-glucuronide	3-(4′-Methoxyphenyl)propanoic acid-3′-glucuronide	274.8	195.3	136.25	135.25	18	25	35	4.19	1.6
Dihydroisoferulic acid-3′-sulfate	3-(4′-Methoxyphenyl)propanoic acid-3′-sulfate	371.1	113.1	195.05	-	17	21	-	4.66	11.0
Phenyl-γ-valerolactones										
(4R)-5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate	(4R)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone-4′-sulfate	287	207.05	163.2	122.1	20	28	33	4.74	274.8

Internal standard										
Taxifolin	Taxifolin	303.05	285.1	125.1	177.15	11	22	12	6.367	-

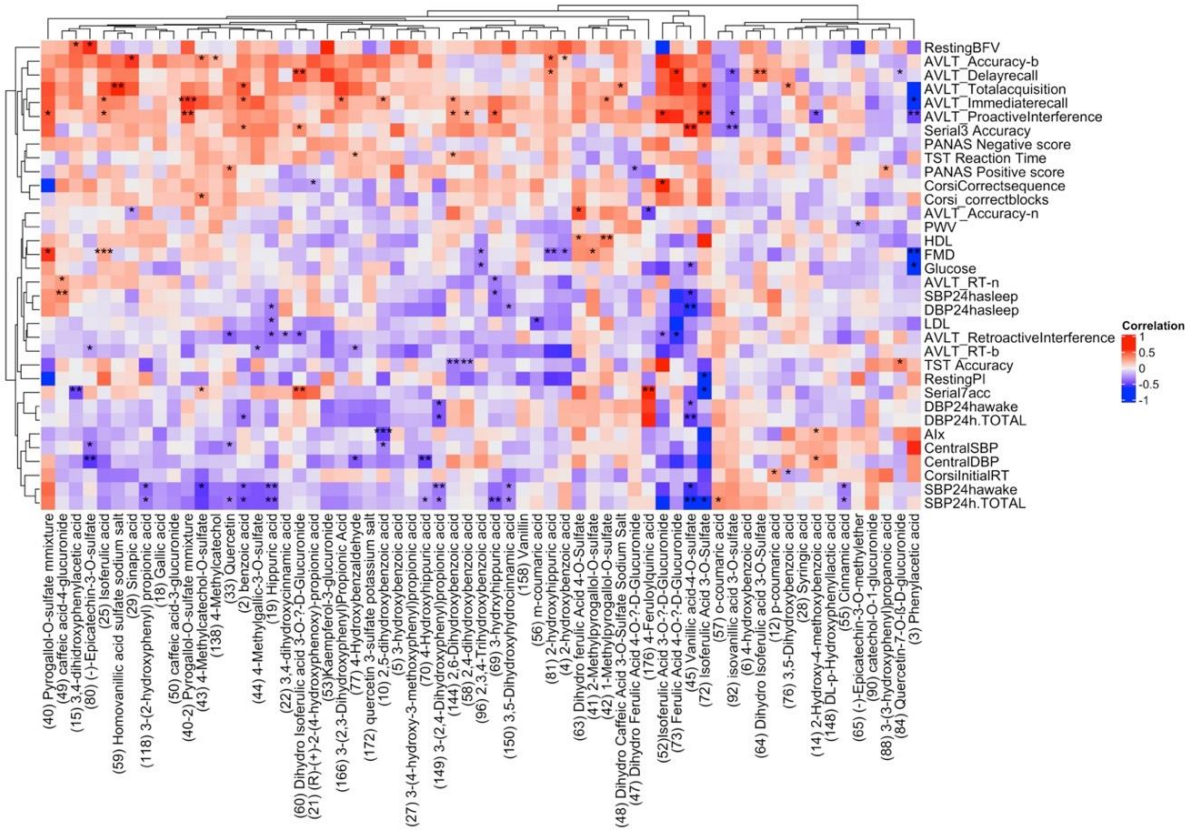
LOQ; limit of quantification, RT; retention time.

Supplementary figure legends

Supplementary Figure 1. Correlations between changes in plasma polyphenol concentrations and all the clinical parameters measured in this study. *AIx; augmentation index, AVLT; auditory visual learning task, DBP; diastolic blood pressure, FMD; flow-mediated dilation, HDL; high density lipoprotein, LDL; low density lipoprotein, PANAS; positive and negative affect score, PI; pulsatility index, PWV; pulse wave velocity, RT; reaction time, SBP; systolic blood pressure, TST; task switching task, WBB; wild blueberry.*

Supplementary Figure 2. Correlations between changes in gut microbiota composition and all the clinical parameters measured in this study. *AIx; augmentation index, AVLT; auditory visual learning task, BFV; blood flow velocity, DBP; diastolic blood pressure, FMD; flow-mediated dilation, HDL; high density lipoprotein, LDL; low density lipoprotein, PANAS; positive and negative affect score, PI; pulsatility index, PWV; pulse wave velocity, RT; reaction time, SBP; systolic blood pressure, TST; task switching task, WBB; wild blueberry.*

Supplementary Figure 1.



Supplementary Figure 2.