FOOD & FUNCTION

Effects of ellagitannin-rich berries on blood lipids, gut microbiota, and urolithin production in human subjects with symptoms of metabolic syndrome

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Ellagitannins are polyphenols abundant in strawberries, raspberries, and cloudberries. The effects of a mixture of these berries were studied in a randomized controlled trial with subjects having symptoms of metabolic syndrome. The study focused on serum lipid profiles, gut microbiota, and ellagitannin metabolites. The results indicate that bioavailability of ellagitannins appears to be dependent on the composition of gut microbiota.

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Both clinical observations and epidemiological studies have shown that consumption of berry constituents, such as polyphenols, micronutrients, and dietary fiber is associated with reduced cardiovascular risk [1, 2]. However, in vivo evidence of the biological activity of berry ellagitannin compounds is not yet well established [3]. In humans, ellagitannins are not absorbed in the small intestine but the colon microbiota converts them to urolithins [4], which in turn are absorbed and circulated in plasma at concentrations that may reach millimolar levels.

To get a more profound estimation of the effects of ellagitannin-rich berry consumption on humans with metabolic syndrome, we used a randomized, controlled 16-wk intervention trial. The study followed the principles recently published by Kolehmainen et al. [5]. Intervention was preceded by a 4-wk baseline period with the restriction of berry consumption (maximum 1 dL/day). The dietary intervention lasted for the following 8 wk, and it was followed by a 4-wk recovery period, during which subjects in the berry group returned to their normal diet with the restriction of berry consumption. In the trial, the subjects consumed daily an equal dose of 300 g fresh berries comprising of 100 g of strawberry purée, 100 g of frozen raspberries, and 100 g of frozen cloudberries. The berry consumption substituted evenly the other carbohydrates in their habitual diet. In the control group, berry consumption was restricted. There were a total of five laboratory visits, during which 12-h fasting blood samples, urine and fecal samples were collected and body composition measurements and compliance checks were made (for more details of dietary intervention, see Supporting Information 1). Description of statistical methods as

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Abbreviations: ChoE, cholesteryl ester; **DGGE**, denaturing gradient gel electrophoresis; **PC**, phosphatidyl choline; **TGs**, triacylglycerols

This study is registered at ClinicalTrials.gov as NCT01414647.

Table 1. Clinical biomarkers (mean ± SD), berry group $n = 20$, and control group $n = 12$. Non (p) and Bonferroni (p') corrected statistical significances are indicated. Dashes indicate that data were not quantified. Time points correspond to the start (week 0), midpoint (week 4), and end (week 8) of the intervention period and end (week 12) of recovery period

	Week 0	Week 4	Week 8	Week 12	Group (p)	Time (p)	Group × time (p)	Group (p')	Time (p')	Group × time (p')
Systolic blood pressure (mm/Hg)										
Berry	139.2 ± 18.9	137.6 ± 13.6	136.4 ± 15.2	134.4 ± 18.1	>0.05	0.01	>0.05	>0.05	>0.05	>0.05
Control	144.3 ± 12.3	136 ± 16.1	138.2 ± 12.6	136.1 ± 9.8						
Diastolic blood pressure (mm/Hg)										
Berry	91.5 ± 9	90.5 ± 7.4	89.2 ± 8.7	88.7 ± 8.5	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Control	92.4 ± 8.4	90.1 ± 8.4	89.9 ± 8.5	89.5 ± 6.8						
Cholesterol (mmol/L)										
Berry	5.6 ± 0.9	-	-	5.3 ± 0.9	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Control	5.5 ± 0.5	-	-	5.4 ± 0.6						
LDL cholesterol (mmol/L)										
Berry	3.7 ± 0.8	-	-	3.4 ± 0.7	>0.05	0.03	>0.05	>0.05	>0.05	>0.05
Control	3.7 ± 0.5	-	-	3.5 ± 0.6						
HDL cholesterol (mmol/L)										
Berry	1.4 ± 0.3	-	-	1.4 ± 0.3	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Control	1.3 ± 0.3	-	-	1.4 ± 0.2						
8-isoprostane (pg/mL)										
Berry	15.2 ± 5.6	13.7 ± 5.5	10.9 ± 3.4	10.7 ± 5.5	>0.05	0.00	>0.05	>0.05	0.00	>0.05
Control	12.5 ± 5.4	11.7 ± 4.2	9.6 ± 4.5	10 ± 5.6						
TRAP (μM)										
Berry	1394 ± 238	1429 ± 237	1362 ± 233	1394 ± 216	>0.05	0.00	>0.05	>0.05	>0.05	>0.05
Control	1406 ± 198	1456 ± 270	1355 ± 221	1448 ± 191						
Leptin/BMI (pg/mL)										
Berry	720 ± 439	730 ± 467	766 ± 507	699 ± 498	>0.05	0.03	0.03	>0.05	>0.05	>0.05
Control	896 ± 456	728 ± 327	888 ± 381	836 ± 463						
Resistin/BMI (pg/mL)										
Berry	369 ± 95	380 ± 92	395 ± 95	381 ± 97	>0.05	0.00	>0.05	>0.05	>0.05	>0.05
Control	393 ± 128	412 ± 151	423 ± 124	408 ± 125						

TRAP = total radical-trapping antioxidant parameter

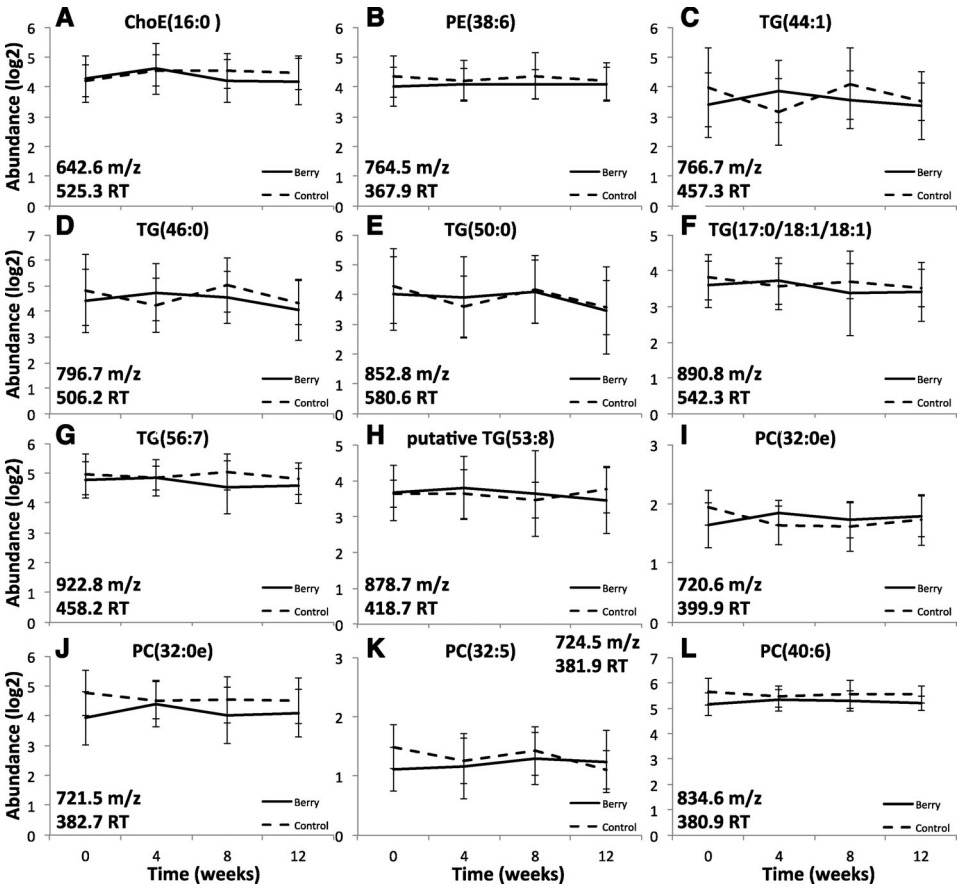

Figure 1. Diagrams showing the average $\log_2$ abundance estimates of selected discriminating lipids with variable importance in the projection score > = 2.0. Shown are the 12 lipids that could be characterized. *m/z* and retention time (RT) are given for each lipid within the panel. (ChoE = cholesteryl ester; PE = phosphatidyl ethanolamine; TG = triacylglycerols; and PC = phosphatidyl choline). Time points are presented as in Table 1.

well as anthropometric measurements, body composition, blood pressure measurements, serum lipids, plasma glucose and insulin, plasma fatty acids, plasma antioxidant capacity, oxidative stress and inflammatory markers, and adipokines have been described in the Supporting Information 2.

All the subjects in the berry group ($n = 20$) completed the study, but there were five drop-outs in the control group (final $n = 12$). The reason for discontinuation was lack of motivation as the subjects did not get the berries. No significant differences were found between the groups at the baseline, but there was a significant difference in sucrose ($p < 0.05$, Bonferroni corrected $p > 0.05$) and fiber ($p < 0.001$, Bonferroni corrected $p < 0.001$) intakes between the groups at the end of berry intervention (Supporting Information 3 and Supporting Information Table 2). In the berry group, the daily intakes of ellagitannins, anthocyanins, and flavonols from the berries were 789 ± 27 mg, 70.7 ± 52 mg, and 4.1 ± 0.4 mg, respectively. Estimated ellagitannin intake in the control group was close to zero. Compliance to the berry diet, calculated on the basis of dietary records, was excellent.

Surprisingly, most measured variables did not differentiate between the two study groups. Most notably, there was a slightly significant difference in leptin concentrations ($p = 0.03$, Bonferroni corrected $p > 0.05$, Table 1). In the berry group, leptin concentration constantly increased throughout the 8-wk substitution period, while no such trend was seen in the control group. It should, however, be noted that overall changes in leptin levels were small and that variation was high. As leptin is playing a major role in regulating energy intake and expenditure, including appetite and metabolism, we speculate that consumption of berries could enhance leptin levels, which in turn could result in more controlled food intake. Group-wise, no other measured variable differed at the 0.05 level, although there were some borderline positive effects on systolic blood pressure, LDL cholesterol, 8-isoprostane, total radical-trapping antioxidant parameter, and resistin (Table 1).

Serum lipidomic profiles were determined by an UPLC-QTOF-MS method in ESI+ mode [6], and the data were processed by using MZmine software [7] (for more details of the methods, see Supporting Information 4). Partial least squares discriminant analysis accompanied with the variable importance in the projection feature selection identified 20 lipids discriminating between controls and berry consumers. One of these lipids was cholesteryl ester (Fig. 1A), one was phosphatidyl ethanolamine (Fig. 1B), four of these lipids were phosphatidyl cholines (PCs) (Fig. 1I–L), and six were triacylglycerols (TGs) (Fig. 1C–H). Notably, one of these lipids, PC32:5 (Fig. 1K), was significantly differentially present at the week 8 sampling point, when tested with moderated

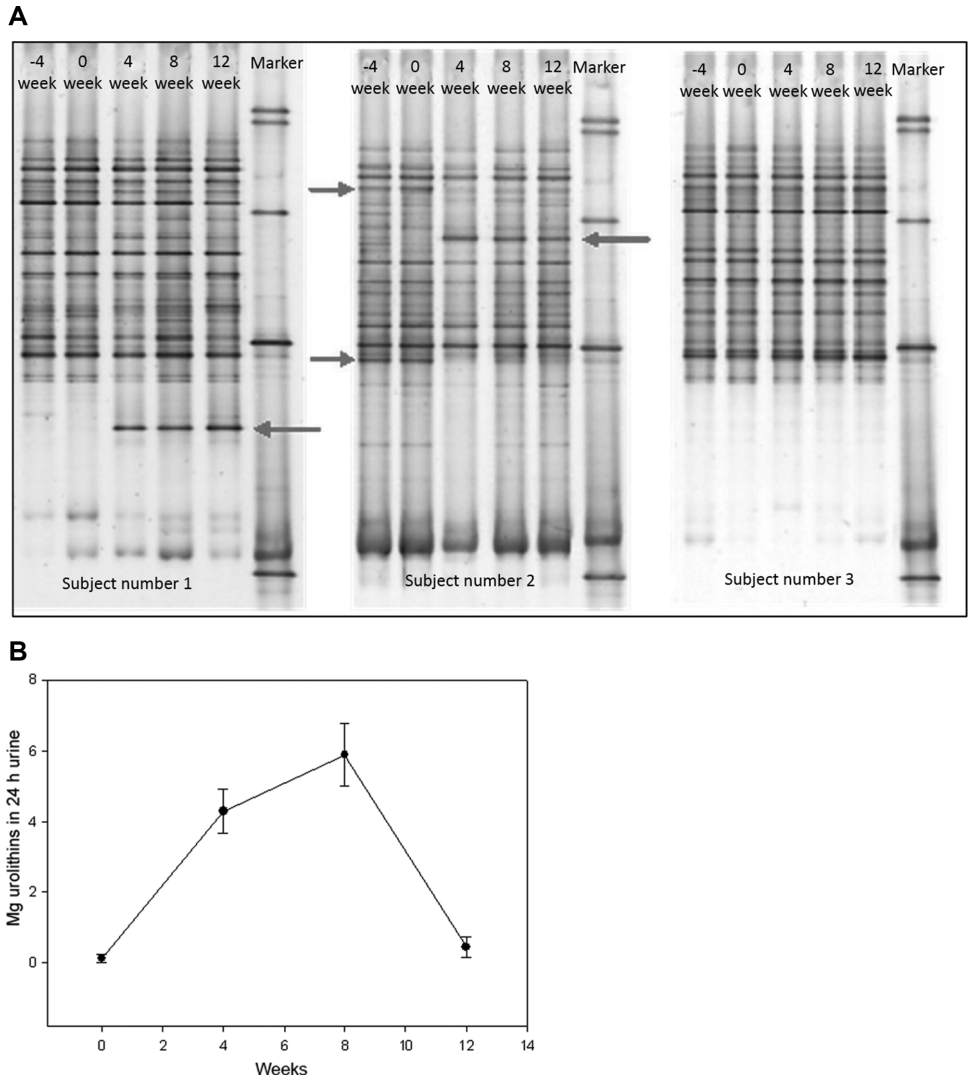

Figure 2. (A) DGGE profiles of three subjects from the berry group. Time points are presented as in Fig. 1. In addition, time point week – 4 corresponds to the start of baseline period. (B) Urolithin excretion curve in the berry group (means ± SEM). Time points are presented as in Fig. 1.

t-test (*Q*-value corrected *p*-value $\leq$ 0.05) [8]. Interestingly, there was a slight trend toward more unsaturated PCs. Saturated and monounsaturated TGs, in turn, decreased in the control group but reached the berry group levels by the end of substitution. In general, PCs, TGs, phosphatidyl ethanolamines, and cholesteryl esters were the lipid classes that showed some response to the ellagitannin-rich diet, and the results suggest that such a diet may have a positive effect on these lipids and on these lipid categories. Plasma fatty acid compositions remained almost unchanged during substitution, which was expected due to low amount of fat in the berries.

PCR denaturing gradient gel electrophoresis (DGGE) was used for the analysis of the fecal predominant bacterial populations in order to compare the stability and bacterial diversity between the two groups using the methods of Maukonen et al. [9] and Mättö et al. [10] (Supporting Information 5). There were no significant differences in similarity values or diversity of predominant bacterial populations between the groups measured at different time points. Furthermore, no grouping was observed based on the interventional study group (Supporting Information 6 and Supporting Information Tables 3 and 4 and Supporting Information Fig. 2).

In visual inspection, there were four subjects in the berry group whose predominant bacterial DGGE profile changed during the intervention and the altered profile did not return to the original profile (Fig. 2A, 1 and 2), Nevertheless, 13 subjects of the berry group (65%) showed no differences in the predominant bacterial profile during the berry intervention (e.g. Fig. 2A, 3), or the predominant bacterial DGGE profiles were also unstable during the time period before the berry intervention. Detailed results from the sequencing of the amplicons from the berry group are presented in Supporting Information 7 and Supporting Information Table 5. All the sequenced amplicons belonged to either Family Ruminococcaceae (Clostridial cluster IV; *Clostridium leptum* group) or

Family Lachnospiraceae (Clostridial cluster XIV; *Eubacterium rectale* group). The predominant bacterial DGGE targets those bacteria that represent more than 1% of the total studied population. Since Ruminococcaceae and Lachnospiraceae have been shown to constitute 40–60% of the total Finnish fecal microbiota, it is expected that clear changes in these groups may also be detected with the predominant bacterial DGGE. The families Ruminococcaceae and Lachnospiraceae contain the majority of butyrate-producing isolates from human fecal samples. Since butyrate is known to play an important role in the metabolic welfare of colonocytes and is also implicated in providing protection against cancer and ulcerative colitis [11], a more detailed analysis of these two clostridial groups would provide more insight into the impact of berry diets on the beneficial part of the gastrointestinal microbiota.

According to recent reports of Vendram et al. [12] and Hidalgo et al. [13] anthocyanins seem to increase the number of *Bifidobacterium* spp. and *Lactobacillus* spp. in the human gut microflora. Hidalgo et al. [13] also investigated the effect of gallic acid, which is a structural component of ellagitannins, on human gut microflora, and showed clear inhibition of the growth of potentially harmful gut bacteria of the *Clostridium histolyticum* group. In addition, gallic acid significantly enhanced the growth of total bacterial count and also the number of *Atopobium* spp. bacteria, which have been shown to have a potential positive effect on gut health. Also, a clear effect of donor microbiota on the variation in metabolism of anthocyanins and their metabolites was shown. These results of Hidalgo et al. [13] suggested that different types of phenolic compounds affect the growth of different bacterial families in the colon, and on the other hand, that individual's microbiota composition affects bioavailability of phenolics.

Ellagitannin metabolites were detected in urine using an HPLC-DAD-MS/MS analysis [14, 15]. Urolithin A, B, C, and D glucuronides, methyl-urolithin C and D glucuronides as well as dimethyl-ellagic acid glucuronide were detected. The compounds showed the same MS spectra and chromatographic behavior as those reported earlier in Iberian pigs and humans [14, 16]. In the berry group, 15 subjects out of 20 produced urolithins after eating berry and five subjects were identified as nonproducers (Fig. 2B). None of the subjects in the control group produced urolithins. There were marked qualitative and quantitative variations in the individual urolithin profiles in the berry group, indicating differences in colonic microbiota responsible for ellagitannin degradation. This phenomenon has also been shown in a recent bioavailability study in which subjects ate 300 g of raspberries as a single portion [17]. In our study, one subject in the berry group already produced urolithins before berry intake, and in this case, the increase in excretion after the berry intake was much larger. Two subjects (subjects 1 and 2 in Fig. 2A) did not produce urolithins before berry intake, and started producing urolithins several weeks after berry supplementation. Changes in the microbiota were observed in both cases, which may be associated with the growth of bacteria responsible for the degradation of ellagitannins and production of urolithins. Interestingly, all the subjects whose microflora changed were also urolithin producers. Our results indicate that bioavailability of berry ellagitannins appears to be dependent on the composition of the gut microbiota. In more general terms, this also means that microbiota may have a central role in the biological effects of ellagitannin-containing berries.

As a conclusion, more clinical studies with a large number of subjects are needed to establish the potential relation between consumption of ellagitannin-rich berries, urolithin production, microbiota, and lipid profiles.

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The authors have declared no conflict of interest.

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Supplementary information 1

DIETARY INTERVENTION

Subjects and study design

Thirty-seven male and female volunteers were recruited with announcements in local newspapers. At screening, the health status and medical history of the volunteers were examined by an interview and by laboratory measurements including routine hematological parameters, thyroid function (serum thyroid stimulating hormone), liver function (serum alanine aminotransferase, gamma glutamyltransferase and alkaline phosphatase), and kidney function (serum creatinine and urine albumin). Height, weight, waist circumference, blood pressure, and fasting plasma glucose and lipids were measured. Volunteers with lipid-lowering medication were excluded. The inclusion criteria were overweight (BMI 26-39 kg/m^2), elevated fasting plasma glucose in the absence of diabetes (5.6-6.9 mmol/l) and/or abnormal serum lipid concentration (fasting serum triglyceride concentration $\geq$1.7 mmol/L, fasting serum HDL cholesterol <1.0 mmol/L (males) or <1.3 mmol/L (females)), waist circumference >102 cm (males) or >88 cm (females), and blood pressure $\geq$130/85 mmHg (Adult Treatment Panel III criteria with AHA modification 12;13). In the berry group there were 6 subjects and in the control group 9 subjects with regular medication.

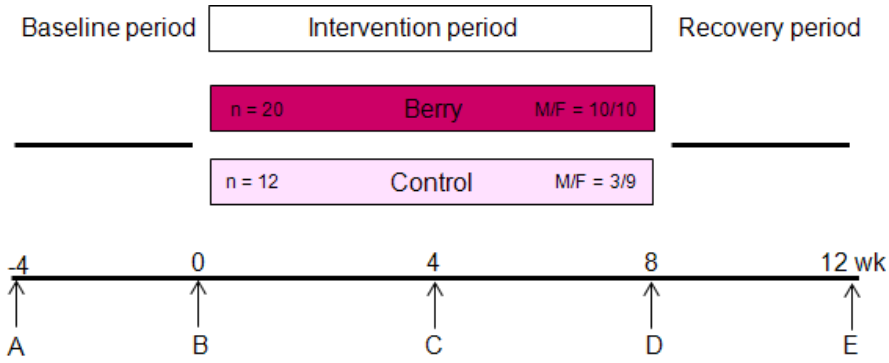
The participants of the present study were abdominally obese and most of them were slightly dyslipidemic and hypertensive. No statistically significant differences were found between the groups. The baseline values of the subjects that completed the study are presented in Supporting Information Table S1.

Supporting Information Table S1. Selected baseline characteristics of the subjects. Data are presented as mean ± SD.

Variable	Berry	Control	*p*-value	Q-value
Number of subjects(M/F)	20 (10/10)	12 (3/9)	-	-
Age (yr)	53.0 ± 6.5	49.8 ± 7.1	0.21	1.00
Body weight (kg)[1]	92.4 ± 14.4	93.1 ± 10.8	0.88	1.00
BMI (kg/m^2)	31.8 ± 4.4	32.9 ± 3.4	0.44	1.00
Waist circumference (cm)	103.9 ± 10.8	105.0 ± 8.1	0.76	1.00
Body fat (%)	33.3 ± 7.2	36.0 ± 6.8	0.30	1.00
Fasting serum cholesterol (mmol/l)	5.6 ± 0.9	5.5 ± 0.5	0.79	1.00
Fasting serum LDL cholesterol (mmol/l)	3.7 ± 0.8	3.7 ± 0.5	0.73	1.00
Fasting serum HDL-Chol (mmol/l)	1.4 ± 0.3	1.3 ± 0.3	0.58	1.00
Fasting serum triglycerides (mmol/l)	1.6 ± 0.6	2.1 ± 0.9	0.09	1.00
Systolic blood pressure (mmHg)[1]	139.2 ± 18.9	144.3 ± 12.3	0.36	1.00
Diastolic blood pressure (mmHg)[1]	91.5 ± 9.0	92.4 ± 8.4	0.77	1.00
Fasting plasma glucose (mmol/l)	5.7 ± 0.4	5.8 ± 0.5	0.55	1.00

The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human participants were approved by the Research Ethics Committee of the Hospital District of Northern Savo (Finland). Written informed consent was obtained from all participants.

The study was a randomized controlled 16-week intervention trial with 2 treatment groups berry and control group [1] (Supporting Information Figure S1). The randomization was carried out according to gender, age, BMI and fasting plasma glucose concentration. Intervention was preceded by a 4-week baseline period, during which the subjects followed their normal diet, with the exception that only 1 dl of berries/day was allowed. The dietary intervention lasted for the following 8 weeks. The dietary modification was followed by a 4-week recovery period, during which subjects in the berry group returned to their normal diet with the restriction of berry consumption. There were a total of five laboratory visits: at the beginning of the baseline period (week -4, A), at the start of the intervention period (week 0, B), at the midpoint (week 4, C) and at the end of intervention period (week 8, D) and at the end of recovery period (week 12, E). During these laboratory visits 12 h fasting blood samples, urine and faecal samples were collected and body composition measurements and compliance checks were made.



Supporting Information Figure S1. Study design (n = number of subjects, M/F = male/female).

Dietary intervention

This study followed the principles published elsewhere [1], but is described here shortly. The subjects of the berry group ate their normal daily diet, except that an eq. dose of 300 g of fresh berries was used to substitute other sources of carbohydrates normally consumed. The diet was counseled by a trained nutritionist based on the habitual diet of each participant. The berry consumption comprised of 100 g of strawberry purée, 100 g of frozen raspberries and 100 g of frozen cloudberries, which were included in the diet. It was recommended that the berries were eaten as such, and not cooked. A moderate amount of sugar sweetening was permitted.

The strawberry purée contained 15% granulated sugar and 1% of a mixture of guargum and carob as precipitants, with no other ingredients. Raspberries and strawberry purée were obtained from Pakkasmarja Ltd. (Suonenjoki, Finland), and cloudberries from Lapin Liha Ltd. (Rovaniemi, Finland). The subjects of the control group consumed their normal diet, which was allowed to include occasionally not more than 1 dl berries per day, corresponding to 30-60 g of fresh berries.

For measurement of compliance the subjects kept daily records of the number of portions of test berries eaten and the quantity, quality, and frequency of other berries that were eaten. Four-day food records, which included one weekend day, were kept by the subjects before the intervention and twice during weeks 3 and 6. The 4d food records were analysed on the

basis of the food records and daily records of berry consumption using the Micro Nutrica® program version 3.1 (Finnish Social Insurance Institute, Turku, Finland), which includes a database of Finnish foods.

Phenolic content and composition of the berries and berry purée were quantified by HPLC combined with a diode array detector after acid hydrolysis using the method of [2, 3]. Ellagitannins were quantified as ellagic acid, flavonols as aglycones and anthocyanins as cyanidin-3-glucoside.

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Supporting information 3

DAILY AVERAGE NUTRIENT INTAKE

Supporting Information Table S2. Daily average nutrient intake. Data are presented as mean ± SD or as median and interquartile range*. Non (p) and Bonferroni (p') corrected statistical significances are indicated. Time points correspond to the start (week 0, B), midpoint (week 4, C) and end (week 8, D) of intervention period.

		week 0	week 4	week 8	Group (p)	Time (p)	Group × time (p)	Group (p')	Time (p')	Group × time (p')
Energy (kJ)										
	Berry	8382±2415	8613±1583	8797±1898	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
	Control	8401±1571	7721±1932	9215±3082						
Protein (g)										
	Berry	79±21	84±16	85±20	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
	Control	79±14	84±24	85±19						
Fat (g)										
	Berry	76±26	72±20	75±20	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
	Control	71±17	65±20	77±36						
Carbohydrate (g)										
	Berry	230±73	248±53	249±65	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
	Control	218±44	208±59	253±78						
Sucrose (g)										
	Berry	42±32	56±24	54±21	0.01	0.04	0.04	>0.05	>0.05	>0.05
	Control	33±14	27±11	38±21						
Fibre (g)										
	Berry	25±8	37±10	36±10	0.02	0.00	0.00	>0.05	0.00	0.00

Control	24±8	26±10	25±8						
Alcohol (g)*									
Berry	5±12	5±7	4±9	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Control	7±25	4±15	5±24						
Vitamin A (mg)*									
Berry	864±530	915±631	857±515	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Control	745±310	797±594	751±522						
Vitamin D (mg)									
Berry	6±4	6±5	7±5	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Control	5±2	7±3	7±3						
Vitamin C (mg)									
Berry	111±30	86±56	85±41	>0.05	>0.02	>0.05	>0.05	>0.05	>0.05
Control	109±66	91±50	84±35						

Supporting information 6

DATA OF MICROBIOLOGICAL PROFILING BY PCR-DGGE

Similarity values and diversity of the predominant bacterial DGGE profiles are presented in Supporting Information Tables S3 and S4, respectively. Three-dimensional presentation of clustering of the similarity values of different study groups are presented in Supporting Information Figure S2. There were no significant differences in similarity values measured at different time points between the groups (Supporting Information Table S3). In addition, there were no differences regarding the diversity of predominant bacterial population between the berry group and the control group (Supporting Information Table S4). Furthermore, when all the predominant bacterial DGGE profiles of all samples were compared to each other, no grouping was observed for the intervention study group (Supporting Information Figure S2).

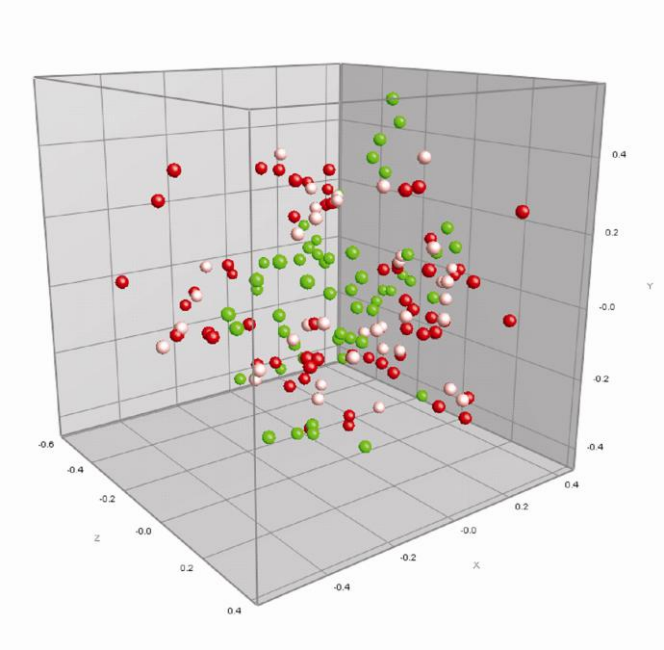
Supporting Information Table S3. Similarity values of predominant bacterial DGGE profiles – calculated using BioNumerics 4.50 software – of human faecal samples obtained at five different time points (100% equals to identical DGGE profiles and 0% equals to completely different DGGE profiles). Samples A and B were taken before the berry intervention, samples C and D during the berry intervention, and sample E after the berry intervention ended.

Study group	**Similarity (all) average**	**Similarity A vs B**	**Similarity A vs C**	**Similarity A vs D**	**Similarity A vs E**
berry group	84,5	85,9	84,6	83,2	80,4
control group	84,5	86,7	85,2	83,1	80,4
			Similarity B vs C	**Similarity B vs D**	**Similarity B vs E**
berry group			84,7	82,9	81,3
control group			88,3	85,2	83,1
				Similarity C vs D	**Similarity C vs E**
berry group				88,2	85,0
control group				84,6	83,1
					Similarity D vs E
berry group					86,0
control group					85,4

Supporting Information Table S4. Diversity of predominant bacterial DGGE-profiles (number of amplicons), as calculated using the BioNumerics 4.50 software, obtained at five different time points.

	Number of amplicons						
	Sample A	**Sample B**	**Sample C**	**Sample D**	**Sample E**	**Average**	**stdev**
berry group	35,1	36,7	33,8	33,3	36,3	34,9	6,1

control group	31,6	32,1	32,5	32,5	31,2	32,0	4,2



Supporting Information Figure S2. Three-dimensional presentation of clustering of the similarity values of different study groups (Similarity values calculated by BioNumerics software). Green dots represent samples from control group subjects and red dots samples from the berry group (samples week -4, week 0 and week 12; no berry consumption), and pink dots samples from the berry group (samples week 4 and week 8; during consumption of strawberry, raspberry, and cloudberry).

Supporting information 4

SERUM LIPIDOMICS AND STATISTICAL ANALYSIS OF LIPID PROFILES

Serum samples for lipidomics were taken on four occasions B-E (week 0 – week 12). The lipidomic profiles were determined by a UPLC-QTOFMS method in ESI+ mode [1], and the data were processed by using MZmine software [2]. Briefly, Plasma samples (10 µl) were spiked (0.2-0.6 µg/sample) with an internal standard (IS) mixture containing 9 lipid compounds and extracted with chloroform: methanol (2:1). The extracts were further spiked with another standard mixture containing 3 labeled lipid compounds (0.1-0.2 µg/sample) and analysed on a Waters Q-Tof Premier mass spectrometer combined with an Acquity UPLC. The column (at 50 °C) was an Acquity UPLC BEH C18 1×50 mm with 1.7 µm particles and the solvent system included A) ultrapure water (1% 1 M NH4Ac, 0.1% HCOOH) and B) LC/MS grade acetonitrile/isopropanol (5:2, 1% 1M NH4Ac, 0.1% HCOOH). The gradient started from 35% B, reached 100% B in 6 min and remained there for the next 7 min. The flow rate was 0.200 mL/min and the injected amount 1.0 µl. The lipid profiling was carried out using ESI+ mode and the data was collected at mass range of m/z 300-1200 with scan duration of 0.2 sec, reserpine was used as the lock spray reference compound. The data was processed by using MZmine software (version 6.0) and the lipid identification was based on an internal spectral library.

Lipidomics data were $\log_2$-transformed and subjected to quality analysis. Lipids quantified as present in less than 10% of samples in either group, known standards, compounds originating from the background matrix and compounds with retention times over 13 minutes were removed. Samples deviating from the normal were also discarded. Differentially abundant lipids were identified with mixed model two-way factorial ANOVA for repeated measurements and moderated t-test [3]. For the moderated t-test, data were baseline adjusted by subtracting the baseline abundances from succeeding time points. P-values were adjusted for multiple comparisons by the Q-value approach [4]. Additionally, the baseline-adjusted data were analysed with partial least square discriminant analysis [5] (PLS-DA) using the PLS package in R [6] and repeated double cross-validation function (10 and 4 segments for splitting the data into training and test data and for selecting the optimal component number, respectively) implemented in the chemometrics package for R [7]. In these analyses, dependent variables were chosen so that they represented either sample groups, sampling points or a combination of both. Final PLS-DA and subsequent variable importance in the projection (VIP) analyses were made in MatLab using the settings producing the best prediction performance in R, *i.e.* the dependent variable represented sample grouping and with five latent variables.

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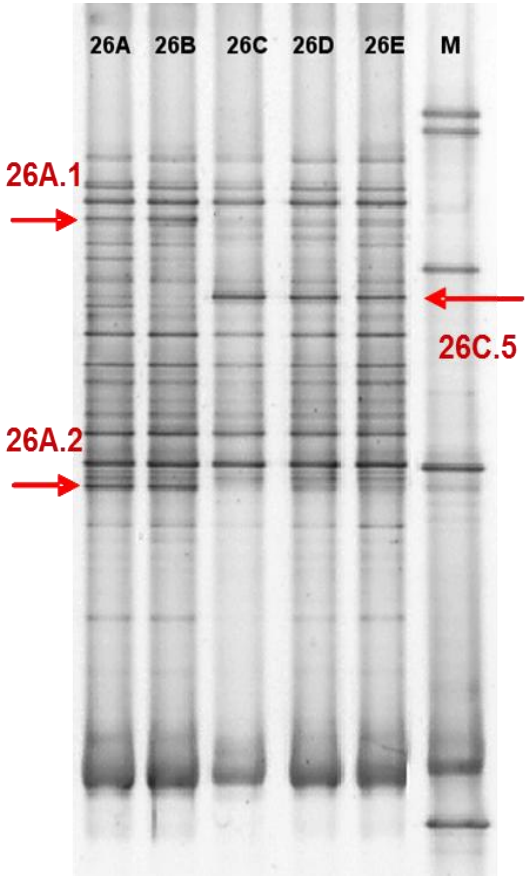
Supporting information 7

RESULTS FROM THE SEQUENCING OF THE DGGE-AMPLICONS FROM THE BERRY GROUP

Supporting Information Table S5. Identity of the amplicons excised from subjects of the berry study group whose predominant bacterial DGGE profile altered during the intervention. Amplicons that disappeared during the intervention diet are marked with A and B (amplicons were present in the first 2 samples but not in the following 3 samples). Amplicons that appeared during the intervention diet are marked with C, D and E (amplicons were present in the last 3 samples but not in the first 2 samples). Sample number corresponds to the subjects in question (i.e. 26C indicates a sample taken from subject 26 during the berry intervention; samples A and B were taken before the berry intervention, samples C and D during the berry intervention, and sample E after the berry intervention ended). Subject numbers 23, 26 and 45 correspond to subject numbers 1, 2 and 3 in the main text.

Sample	Blast identification	similarity	Phylum / Family
23C= 23D= 23E appearance	*Ruminococcus bromii*, type strain ATCC 27255	93.5 %	Firmicutes / Ruminococcaceae
	Clostridium leptum, type strain DSM 753T	93.3 %	
24C= 24D= 24E appearance	*Eubacterium hallii*, type strain ATCC 27751	98 %	Firmicutes / Lachnospiraceae
26A-1 = 26B.3 disappearance	*Roseburia faecis*, type strain JCM 17581	95 %	Firmicutes / Lachnospiraceae
26A.2 = 26B.4 disappearance	*Blautia glucerasea*, type strain JCM 17039	95 %	Firmicutes / Lachnospiraceae
	Blautia schinkii, type strain DSM 10518	95 %	
26C= 26D = 26E appearance	*Roseburia faecis*, type strain JCM 17581	99 %	Firmicutes / Lachnospiraceae
50C = 50D = 50E appearance	*Ruminococcus obeum*, type strain ATCC 29174	99 %	Firmicutes / Lachnospiraceae
50A = 50B disappearance	*Eubacterium eligens*, type strain ATCC 27750	97 %	Firmicutes / Lachnospiraceae,
61A.2 = 61B.4 disappearance	*Faecalibacterium prausnitzii*, type strain ATCC 27768	96 %	Firmicutes / Ruminococcaceae
61A.1 = 61B.3 disappearance	*Ruminococcus bromii*, type strain ATCC 27255	87 %	Firmicutes / Ruminococcaceae



Supporting information 2

STATISTICAL METHODS, ANTROPOMETRIC MEASUREMENTS, BODY COMPOSITION, BLOOD PRESSURE AND BIOCHEMICAL ANALYSES OF THE BLOOD SAMPLES

Statistical methods

Baseline characteristics between the subjects of the berry and control groups at the beginning of the intervention period were compared using the Fisher-Freeman-Halton test for categorical variables and student's t-test for numeric variables. P-values were adjusted for multiple comparison using Q-values [1]. Mixed model two-way factorial analysis of variance (ANOVA) for repeated measurements was used for the remaining variables. The ANOVA model contained fixed effects (*time* and *group*) and one random effect (*subject*). Of these, *time* was modeled as repeated within factor and *group* as a between factor. For a particular variable, subjects with missing values were rejected. P-values were adjusted for multiple comparisons using Bonferroni correction. Statistics were calculated using R, a system for statistical computation and graphics [2].

Antropometric measurements and body composition

Body weight was measured with a standardized electronic scale in light clothing. Height was measured at the beginning of the study in a Frankfurt position. The waist circumference was measured halfway between the lowest rib and the iliac crest. Body composition was measured by a bioelectrical impedance STA/BIA Body Composition Analyzer (Akern Bioresearch Srl, Florence, Italy).

Blood pressure

Blood pressure was measured by trained study nurses using a standard sphygmomanometer twice on the right arm after 10 minutes of rest with the subject in a sitting position. The means of systolic and diastolic BP were calculated from two measurements obtained.

Biochemical analyses of the blood samples

The blood samples were collected into EDTA-tubes, and serum and plasma were separated by centrifugation. All samples were stored at -70 ºC before analyses.

Concentrations of total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides and apolipoproteins A1 and B were analyzed using Konelab System Reagents and a Konelab 20 XTi clinical chemistry analyzer (Thermo Electron Corp, Vantaa, Finland).

Plasma glucose concentrations were analyzed by the hexokinase method using Konelab System Reagents and a Konelab 20 XTi clinical chemistry analyzer (Thermo Fisher Scientific, Vantaa, Finland). Plasma insulin was determined with ACS 180 Plus Automated Chemiluminescence System (Bayer Diagnostics, Tarrytown, NY, USA).

Total antioxidant capacity (total peroxyl radical-trapping antioxidant potential, TRAP) of plasma samples was measured by a chemiluminescent method as described earlier [3,4]. 8-isoprostane concentrations in plasma were measured by immunoassay (Cayman Chemical Company, Ann Arbor, Michigan, USA).

High sensitive CRP (hsCRP) was analysed with the Image Immunochemistry System (Beckman Coulter Inc., USA).

ELISA kits were used for the detection of adiponectin, adipsin, leptin, resistin, TNF-α (R&D Systems Inc., MN, USA), high molecular weight (HMW) adiponectin (St. Charles, Missouri, USA) and IL-6 (Sanquin, Amsterdam, The Netherlands).

Plasma fatty acid compositions were determined as methyl esters by gas chromatography [5]. Identification of fatty acids was based on retention times of reference compounds and on information from the literature.

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Supporting information 5

MICROBIOLOGICAL PROFILING BY PCR-DGGE

Predominant bacterial PCR-DGGE

Faecal samples were placed in an anaerobic atmosphere immediately after defecation and stored in cool temperature (+4 to +10 °C). The samples were frozen within 4 h after defecation and stored at -70 °C until further analysed.

PCR-DGGE was used for the analysis of the predominant bacterial population of 20 subjects on the berry diet and 12 control subjects in order to compare the temporal stability and bacterial diversity between the two groups. DNA was extracted from frozen faecal samples using FastDNA Spin Kit for Soil (QBIOgene, Carlsbad, CA, USA) as described by Maukonen *et al.* [1]. Partial 16S rRNA gene was PCR-amplified for the detection of predominant bacteria using primers U968-f+GC (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAAC CTTA) and U1401-r (CGGTGTGTACAAGACCC) and thereafter PCR products were separated in polyacrylamide gels with a denaturing gradient of 38-60% (where 100% is 7 M urea and 40% (vol/vol) deionized formamide) as described by Mättö *et al.* [2]. Comparison of the profiles was performed by visual inspection of the gels and by calculating the similarity percentage using BioNumerics software version 4.50 (Applied Maths BVBA, Sint-Martens-Latem, Belgium) as described earlier [1].

The DGGE-bands of interest were carefully excised and thereafter treated as described earlier [3]. Sequencing reactions of the PCR amplicons were performed with the ABI PRISM BigDye terminator Cycle sequencing kit v.3.1 (Applied Biosystems, USA) with primers U968-f+GC and U1401-r as previously described [3]. Sequences were checked and edited with the Chromas program (Technelysium Pty Ltd, Helensvale, Australia) and thereafter identified through the GenBank database (www.ncbi.nlm.nih.gov) using the BLASTN (Basic Local Alignment Seach Tool) algorithm [4] and RDP Classifier from Ribosomal Database Project II [5] (RDPII). DNAMAN 4.1 (Lynnon BioSoft) was used for sequence alignment. The sequences were deposited in the GenBank database and are available under accession numbersKC789797-KC789814. Original subject numbering has been used in the database. Subject numbering has been changed in the main text to avoid confusion. Subject numbers 1, 2 and 3 in the main text correspond to subject numbers 23, 26 and 45, respectively, in the GenBank database. Mean and standard deviation was calculated for each experiment. Student's *t*-test (two-sample assuming unequal variances) was used for statistical analysis of the results.

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