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Black Currant Anthocyanins Attenuate Weight Gain and Improve Glucose Metabolism in Diet-Induced Obese Mice with Intact, but Not Disrupted, Gut Microbiome

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ABSTRACT: Black currant (*Ribes nigrum* L.) is a rich source of anthocyanins; however, the relationship between their apparently limited bioavailability and significant protection against metabolic pathologies is poorly understood. This study examined the gastrointestinal distribution of black currant anthocyanins and their phenolic acid metabolites in lean and diet-induced obese mice with healthy and antibiotic-disrupted microbiomes. Daily consumption of low- or high-fat diet supplemented with 1% black currant powdered extract (32% anthocyanins) for 8 weeks reduced body weight gain and improved glucose metabolism only in mice with the intact gut microbiome. Administration of antibiotic cocktail resulted in a 16−25-fold increase ($P < 0.001$) in anthocyanin content of feces, and cyanidin-based anthocyanins showed the largest increase in fecal content upon disruption of gut microbiome (92.3 ± 16.3 vs 4719 ± 158 μg/g feces), indicating their high susceptibility to microbial degradation in the gut. A 3-fold enrichment ($P < 0.05$) in gallic over protocatechuic acid was observed in the jejunum of both intact and antibiotic-treated animals, suggesting that this effect was likely independent of their gut microbiome status. Taken together, the data clearly demonstrate that gut microbiome and the type of the anthocyanin aglycone moiety can alter the protective effect of anthocyanins against obesity and associated insulin resistance.

KEYWORDS: *gastrointestinal tract, gut microbiome, obesity, inflammation, functional food*

INTRODUCTION

Among environmental factors, the high-fat diet and sedentary lifestyle common to the Western world are considered as major causes of obesity-associated insulin resistance and impaired glucose tolerance.[1] Changes in physiology and metabolism during the development of metabolic disorders are tightly linked to low-grade inflammatory responses of key metabolic and gastrointestinal tissues.[2]

Anthocyanins, phenolic pigments belonging to the flavonoid family, are known for their antioxidant, anticancer, and anti-inflammatory properties.[3] Recently, anthocyanins have been identified as modulators of lipid metabolism and energy expenditure by reducing fat mass and improving lipid profiles in diet-induced obese rodent models, largely independent of plant source or composition. Anthocyanin-rich extracts from purple corn,[4] black soybean,[5] blueberry,[6] chokeberry,[7] purple sweet potato,[8] mulberry,[9] cherry,[10] grape,[11] or black currant[12] protected against body weight gain and metabolic aberrations observed with a high-fat diet. On the other hand, supplementation with strawberry,[6] jaboticaba,[13] or black raspberry[14] anthocyanins did not alter development of obesity in this model. Results from both cohort studies and randomized trials suggested that anthocyanins from berries might lower the risk of type 2 diabetes and cardiovascular diseases; however, many of these studies are not equivocal.[15] Whereas predominantly delphinidin- and malvidin-containing berries generally improved metabolic and cardiovascular disease risk biomarkers (i.e blueberry,[16] black currant and bilberry,[17,18] or grapes[19]), cyanidin-containing fruits and extracts offered little or no effect on lipid and inflammatory markers in human studies (i.e., elderberry,[20] blood orange,[21] or purple carrot[22]).

The majority of dietary anthocyanins are not absorbed at the upper gastrointestinal level, hence reaching the intestinal microbiome where they are biotransformed into their metabolites and then absorbed.[23] As anthocyanin bioavailability is low, there is insufficient understanding of the difference in anthocyanin metabolism and biotransformation in the gastrointestinal tract of healthy versus obese states, as well as the role of intact versus disrupted gut microbiome in these processes. The apparent discrepancy between biological activities of cyanidin- versus delphinidin- or malvidin-type anthocyanins may therefore be explained by differences in their structure, absorption, and metabolism in the gut. Black currant (*Ribes nigrum* L.) contains as much as 700 mg anthocyanins/100 g fresh fruit in the form of four major anthocyanins that are

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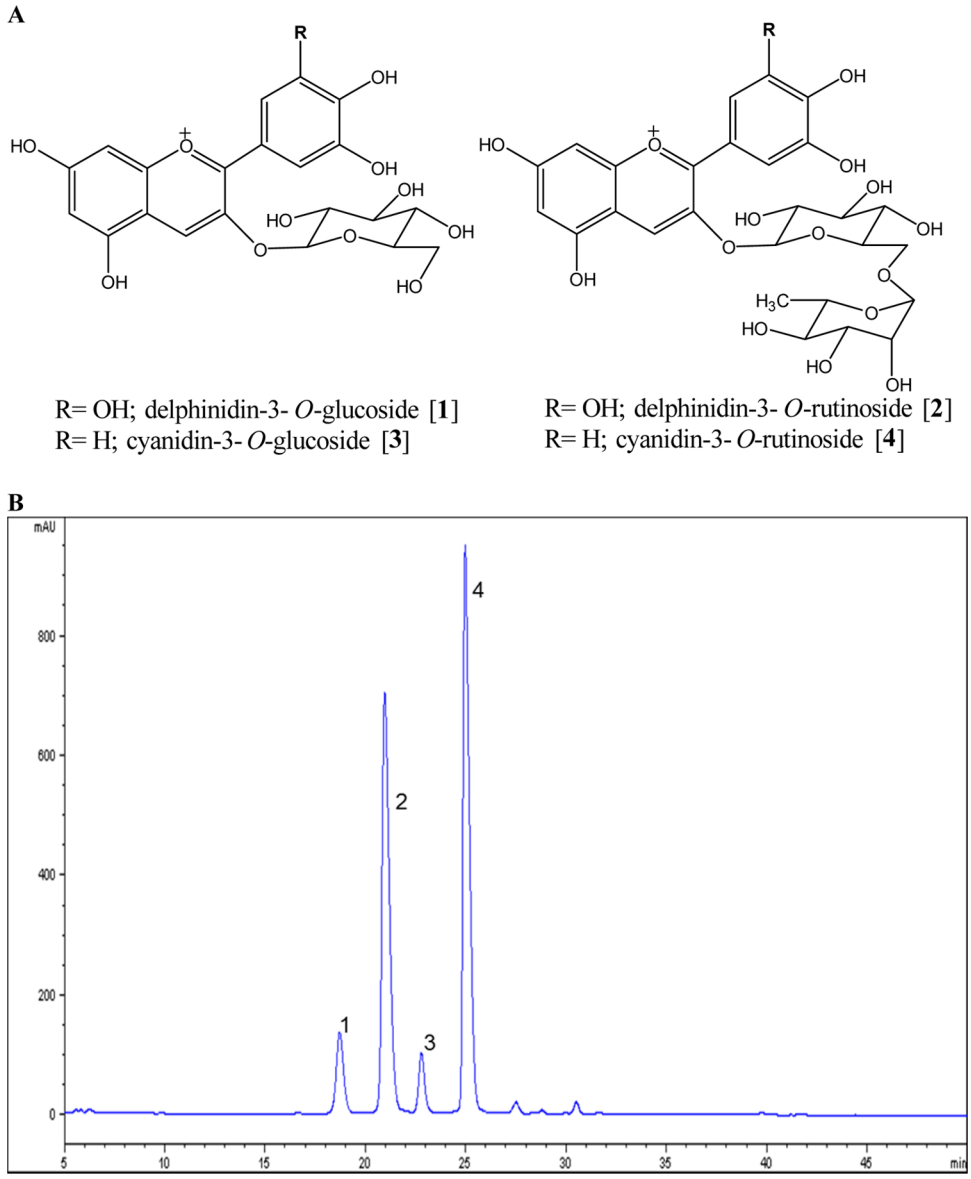

Figure 1. Chemical structures of black currant anthocyanins (A) and their respective HPLC-DAD chromatogram peaks. Peak numbers correspond to Table 1 and 2 labels.

equally distributed between cyanidin and delphinidin aglycones[24] and, therefore, provides an attractive model to investigate these differences. Moreover, anthocyanins represent the most abundant phenolic compounds in black currants, with other minor phenolics contributing to <5% of black currant powdered extract.[25] Protocatechuic and gallic acids are major breakdown metabolites of cyanidin- and delphinidin-type anthocyanins, respectively.[26]

The present study was designed to examine the preventive effect of anthocyanin-rich black currant extract on the development of obesity and hyperglycemia induced by feeding a high-fat diet in C57BL/6 mouse polygenic obesity model with intact and antibiotic-disrupted gut microbiome. Major anthocyanins and their phenolic acid metabolites were identified in feces and gastrointestinal tissue samples from these animals.

MATERIALS AND METHODS

Chemicals. The anthocyanins cyanidin- and delphinidin- 3-*O*-*β*-glucosides (HPLC grade standards) were purchased from Polyphenols Laboratories AS (Sandnes, Norway). All other chemical reagents and solvents were purchased from Sigma (St. Louis, MO, USA). A commercial black currant powdered extract (Active Cassis Extract 30) was kindly provided by Eddie Shiojima (Just the Berries, Palmerston North, New Zealand). Total anthocyanin content of the black currant powdered extract was 32.4%, and its anthocyanin composition was 41.4% delphinidin-3-*O*-rutinoside, 11.2% delphinidin-3-*O*-glucoside, 42.4% cyanidin-3-*O*-rutinoside, and 5.0% cyanidin-3-*O*-glucoside according to the manufacturer's certificate of analysis (Figure 1).

Animals and Diets. All animal experiments were performed according to procedures approved by the NC Research Campus Institutional Animal Care and Use Committee in the David H. Murdock Research Institute, an AAALAC accredited animal care facility.

Male, 6-week-old, C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and housed four animals per cage under controlled temperature (24 ± 2 °C) and light (12 h light−dark cycle, lights on at 7:00 a.m.). Immediately upon arrival, animals were

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allowed to adapt to the new conditions for 7 days, and handling the animals was performed daily during this time to reduce the stress of physical manipulation. Mice were then randomized into ad libitum access to Research Diets (New Brunwick, NJ, USA) low 10 kcal% fat diet D12450J (LFD, 3.85 kcal/g, n = 24) or high 60 kcal% fat diet D12492 (HFD, 5.24 kcal/g, n = 24) and tap water for 6 weeks (weeks 1−6).

Lean and obese mice were further randomized to control low-fat diet (LFD, n = 8), control high-fat diet (HFD, n = 8), and the respective low-fat (LFD+C, n = 16) and high-fat (HFD+C, n = 16) black currant-supplemented treatment groups (1% black currant powdered extract, weight on g/kcal basis, incorporated in LFD or HFD diet by Research Diets). Because typical 24 h food intake for a C57BL/6 mouse is 2.5−3.0 g or 10−12 kcal/g body weight,[27] the animals were expected to consume an average of 25 mg/day of black currant powdered extract or 8 mg/day of black currant anthocyanins. Mice were kept on the respective diets for an additional 8 weeks (weeks 7−14). Animal weight and food intake (accounting for spillage) were recorded weekly for the duration of the study. All animal diets were kept at −80 °C for long-term storage and stability, and freshly thawed food was dispensed to animals every 3−4 days to limit phytochemical degradation in food matrix.

Antibiotic Knockdown of Endogenous Gut Microbiome. An antibiotic cocktail (0.5 g/L vancomycin, 1 g/L neomycin sulfate, 1 g/L metronidazole, 1 g/L ampicillin) previously shown to be sufficient to deplete all detectable commensal bacteria[28] was administered in drinking water ad libitum to half of the animals on LFD+C and HFD +C supplemented with black currant powdered extract (n = 8, LFD +CA and HFD+CA groups, respectively) for the entire duration of the black currant feeding study (weeks 7−14).

Oral Glucose and Insulin Tolerance Tests. For the oral glucose tolerance test, mice were fasted overnight (16 h) and received an oral gavage of D-glucose (1.5 g/kg body weight, Sigma). For the insulin tolerance test, mice were fasted for 4 h and received an intraperitoneal injection of insulin (0.75 U/kg body weight, Santa Cruz Biotechnology). Blood glucose concentrations were measured at 0, 15, 30, 60, and 120 min after glucose or insulin challenge in blood samples obtained from tail-tip bleedings, using a glucometer (Lifescan, Johnson and Johnson).

Sample Collection. Fecal samples from mice were collected, weighed, and pooled by cage midway (week 10) and at the end of the black currant feeding study (week 14) and stored at −80 °C. At the end of the experiment, blood was collected by heart puncture after CO_2 inhalation. Gastrointestinal tissues (stomach, duodenum, jejunum, ileum, cecum, and colon) and luminal digesta were collected and stored at −80 °C to determine the temporal sequence and signaling events that are responsible for changes in physiology and metabolism.

Anthocyanin and Metabolite Extraction. Anthocyanins were extracted from feces, gastrointestinal digesta, and gastrointestinal wall tissue using 60% aqueous methanol (1% trifluoroacetic acid acid) following a previously reported method for the rapid analysis of various anthocyanins in rats.[29]

HPLC and LC-MS Analysis. The phytochemical composition of ACE30 black currant powdered extract was analyzed in triplicate by LC-MS in positive mode (Agilent G1312B-1200 series Infinity Quaternary HPLC system coupled with Agilent 6530A accurate-mass quadrupole time-of-flight MS with Agilent Jet Stream source). Dry samples were reconstituted with 500 μL of 80% methanol, and 5 μL was injected onto an Agilent ZORBAX Eclipse Plus C18 column (3 × 100 mm, 1.8 μm). A gradient from 30% acetonitrile in 0.1% formic acid to 90% acetonitrile in 0.1% formic acid was used for HPLC separation. Mass data were acquired with the following parameters: drying gas temperature, 300 °C; drying gas flow, 7 L/min; nebulizer pressure, 40 psi; sheath gas temperature, 350 °C; sheath gas flow, 10 L/min; capillary voltage, 3500 V; nozzle voltage, 500 V; fragmentor voltage, 150 V; skimmer voltage, 65 V; octopole RF peak voltage, 750 V. Standard curves for compounds of interest were obtained in a linear dynamic range of 10−50000 pg and used for quantitative analysis. Peak area was determined by Agilent MassHunter Qualitative Analysis (version B.05.00) software.

Individual anthocyanins and phenolic acids were quantified by HPLC as described in our previous publication.[30] Briefly, filtered samples were injected (10 μL) into a 1200 HPLC system (Agilent Technologies) equipped with a UV−vis diode array detector (DAD), controlled-temperature autosampler (4 °C), and column compartment (30 °C) using a reversed-phase Supelcosil LC-18 column, 25 mm × 4.6 mm × 5 μm (Supelco, Bellefonte, PA, USA). Standard curves were calculated using peak areas at UV of 520 or 325 nm for anthocyanins (as cyanidin 3-O-glucoside or delphinidin 3-O-glucoside equivalents) or phenolic acids, respectively, and the known injected concentrations for the standards.

Statistics. Data were analyzed by one-way ANOVA followed by Dunnett's multiple-range tests using Prism 6.0 (GraphPad Software, San Diego, CA, USA). Body weight gain and glucose tolerance were analyzed by two-factor repeated-measures ANOVA, with time and treatment as independent variables. All data are presented as means ± SEM. Significant differences were accepted when the P value was <0.05.

RESULTS

Characterization of Black Currant Powdered Extract and Diets. The LC/JS-ESI/Q-TOF/MS was used for initial untargeted characterization and quantification of the major phytochemicals in ACE30 black currant powdered extract prior to food incorporation studies. Five major phytochemicals were identified in the extract (Table 1). Citric acid was a major weak organic acid present in the powdered extract in the amount of 279 mg/g. Total anthocyanin content of the extract was 323 mg/g, together with citric acid this accounted for 60% of components present in the ACE30 extract. Four anthocyanins were routinely quantified in the powdered extracts and tissue samples by subsequent HPLC-DAD analysis (Figure 1). ACE30 extract contained two cyanidin-based anthocyanins and two delphinidin-based anthocyanins in approximately equal amounts (153 vs 170 mg/g). On the other hand, 3-O-rutinosides were 5 times more abundant in the powdered extract as compared to their 3-O-glucoside counterparts (271 vs 52 mg/g). Anthocyanins represented the most abundant phenolic compounds in black currants, with other minor phenolics contributing to <5% of black currant powdered extract as shown by us previously.[25]

Table 1. Characterization and Quantification of Major Components in ACE30 Black Currant Powdered Extract Using LC-MS

peak	phytochemical composition	other name	m/z $[M]^+$	concentration (mg/g extract)
	citric acid		192	279
1	delphinidin-3-O-glucoside	mirtillin	465	36
2	delphinidin-3-O-rutinoside	tulipanin	611	134
3	cyanidin-3-O-glucoside	chrysanthemin	449	16
4	cyanidin-3-O-rutinoside	antirrhinin	595	137

Incorporation of ACE30 extract in animal food at the 1% level was performed at the same grams per kilocalorie basis (Table 2). Anthocyanin levels in the diet remained stable for at least 4 days at room temperature and for the duration of the study when diets were stored at −80 °C (data not shown).

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Table 2. Anthocyanin Composition of ACE30-Incorporated Diets

peak	anthocyanin	concentration[a] (μg/g diet) LFD	HFD
1	delphinidin-3-*O*-glucoside	334 ± 26.4	423 ± 98.0
2	delphinidin-3-*O*-rutinoside	930 ± 65.0	1195 ± 264
3	cyanidin-3-*O*-glucoside	173 ± 14.5	219 ± 46.2
4	cyanidin-3-*O*-rutinoside	1015 ± 68.7	1321 ± 286
	total quantified cyanidins	1189 ± 83.2	1540 ± 332
	total quantified delphinidins	1264 ± 91.4	1618 ± 361
	total quantified 3-*O*-rutinosides	1946 ± 133	2516 ± 549
	total quantified 3-*O*-glucosides	507 ± 41.0	643 ± 143
	total quantified anthocyanins	2451 ± 175	3158 ± 690

[a]LFD, low-fat diet; HFD, high-fat diet.

Changes of Body Weight, Food Intake, and Food Efficiency. Six-week-old mice were fed LFD or HFD for 6 weeks to initiate development of obesity in the HFD animals (Figure 2). Next, the treatment groups were presented with anthocyanin-enriched diets and control (tap water) or antibiotic-supplemented tap water for an additional 8 weeks. There were no abnormal clinical signs throughout the entire study. After 14 weeks, mice fed HFD developed 48% larger body weights as compared to their LFD controls (30.8 vs 45.6 g, Figure 2A). Incorporation of 1% black currant powdered extract into the respective diets for 8 weeks resulted in 8−10% smaller body weights in animals receiving anthocyanin treatment, irrespective of fat content of the diet (LDF+C and HFD+C groups). However, body weight loss was absent in anthocyanin-treated animals with disrupted gut microbiome due to the addition of antibiotic cocktail to their drinking water (LFD+CA and HFD+CA groups, Figure 2B).

Food intake was not significantly different among all treatment groups and varied in the range of 2.4−3.0 g/mouse/day (Figure 2C). The caloric intake was significantly higher in HFD animals; however, neither supplementation with the black currant extract nor antibiotic cocktail affected food intake of the treatment animals respective to their controls (Figure 2D). Therefore, the data suggest that anthocyanin supplementation decreased mouse body weight without affecting the food or caloric intake.

Feed efficiency (%), calculated as (body weight gain/food intake) × 100 ratio, was higher in HFD groups (Figure 3). Supplementation with black currant powdered extract decreased feed efficiency in LFD+C and HFD+C mice. Upon disruption of gut microbiome with antibiotic cocktail in the drinking water, a trend to increase feed efficiency was observed in HFD+CA but not LFD+CA mice.

Anthocyanins in Fecal Samples. We observed the intense red/purple coloration of the small intestine, cecal, and fecal content samples only from anthocyanin-fed mice. HFD diet alone was responsible for a 20% increase in fecal pellet weight as compared to LFD controls. Neither anthocyanin treatment nor antibiotic cocktail supplementation changed the number or size of fecal pellet output in treated animals as compared to the respective LFD or HFD controls (Figure 4). Relative anthocyanins and their phenolic acid metabolite concentrations found in fecal samples after 4 and 8 weeks of treatment are summarized in Table 3.

No significant differences in metabolism or fecal output of anthocyanins or their respective phenolic acids were observed

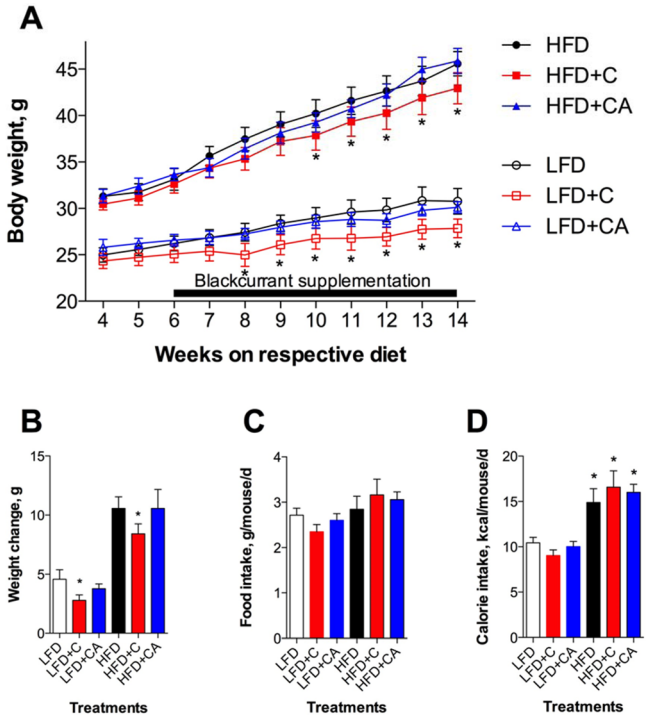

Figure 2. Effects of black currant and gut microbiome on body weight (A), body weight gain (B), food intake (C), and calorie intake (D) in the C57BL/6J mice. Six-week-old male mice were fed a low- or high-fat diet for 6 weeks. Lean and obese mice were further randomized to control low-fat diet (LFD), control high-fat diet (HFD), and the respective low-fat (LFD+C) and high-fat (HFD+C) black currant-supplemented treatment groups (1% black currant powdered extract incorporated in LFD or HFD) and kept on the same diet for an additional 8 weeks. An antibiotic cocktail was administered in drinking water ad libitum to half of the animals on black currant-supplemented treatment groups (LFD+CA and HFD+CA groups, respectively) for the entire duration of the black currant feeding study (weeks 7−14). Animal weight and food intake were recorded weekly for the duration of the study. Results are expressed as means ± SEM, $n = 8$. Body weight gain was analyzed by two-factor repeated-measures ANOVA, with time and treatment as independent variables. (∗) $P < 0.05$ versus respective LFD or HFD control. One-way ANOVA, Dunnett's post hoc test. Body weight gain was analyzed by two-factor, repeated-measures ANOVA, with time and treatment as independent variables.

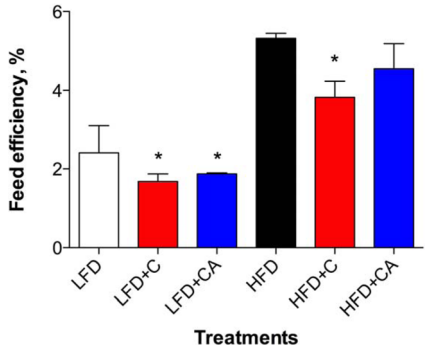

Figure 3. Effects of black currant and gut microbiome on feed efficiency in the C57BL/6J mice fed low- and high-fat diets. Animals received low-fat diet (LFD) or high-fat diet (HFD) or black currant-supplemented treatment groups (1% black currant extract) LFD+C or HFD+C for 8 weeks. Feed efficiency was calculated as (body weight gain/food intake) × 100 ratio. (∗) $P < 0.05$ versus respective LFD or HFD control. One-way ANOVA, Dunnett's post hoc test.

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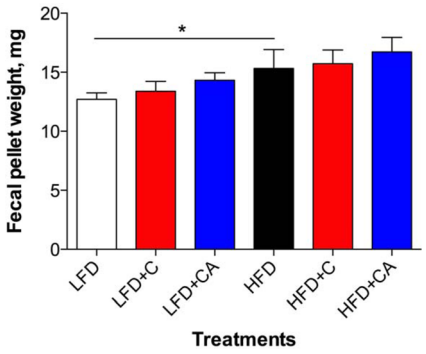

Figure 4. Effects of black currant and gut microbiome on fecal excretion in the C57BL/6J mice fed low- and high-fat diets. Animals received low-fat diet (LFD) or high-fat diet (HFD) or black currant-supplemented treatment groups (1% black currant extract incorporated in LFD or HFD; LFD+C or HFD+C) for 8 weeks. Results are expressed as means ± SEM. (*) $P < 0.05$ versus respective LFD or HFD control. One-way ANOVA, Dunnett's post hoc test.

between LFD+C and HFD+C treatments. Administration of antibiotic cocktail to LFD+CA and HFD+CA groups resulted in marked 16−25-fold increases in anthocyanin content of feces. The effect was more pronounced in lean animals, suggesting that the gut microbiome was more active at biotransformation of anthocyanins than in their obese counterparts. The 3-*O*-rutinosides seemed to be the most susceptible to microbial biotransformation (24−55-fold increases in lean and 16−28-fold increase in obese animals receiving antibiotic cocktail in drinking water for week 10 of the study). Among all molecules tested, cyanidin-type anthocyanins showed the most dramatic increase in fecal content upon disruption of gut microbiome (28−55-fold for week 10; 32−51-fold for week 14 of treatment).

We also observed a parallel decrease in phenolic acid metabolites in feces of antibiotic-treated animals. Both gallic acid (delphinidin-derived metabolite) and protocatechuic acid (cyanidin-derived metabolite) fecal contents decreased 2−4-fold depending on the time of analysis (Table 3).

Anthocyanins in Gastrointestinal Wall Tissues. Jejunum, cecum, and colon gastrointestinal wall tissues collected from each diet group were analyzed to compare anthocyanin and phenolic acid profiles. Anthocyanin ratio profiles from jejunum tissues were very similar to those in the original extract (Table 4). Administration of antibiotic cocktail to LFD+CA and HFD+CA groups resulted in marked 2−5-fold increases in anthocyanin content of jejunum tissues. The effect was more pronounced in lean animals (4−5-fold) versus obese mice (1.5−2-fold), suggesting that, once again, the intact gut microbiome was more active at biotransformation of anthocyanins. Even though cyanidin- and delphinidin-type anthocyanins were present in black currant powdered extract and diet in almost equal concentrations (Tables 1 and 2), a strong preference for selective absorption of gallic acid (delphinidin-derived metabolite) was evident as compared to protocatechuic acid (cyanidin-derived metabolite) levels in the jejunum walls (Table 4). An approximately 3-fold enrichment in gallic acid over protocatechuic acid was observed in the jejunum of lean LFD+C and LFD+CA mice, and this ratio dropped to <2-fold in obese HFD+C and HFD+CA animals. Because similar ratios were observed in both intact and antibiotic-treated animals, this effect was likely independent of gut microbiome.

Smaller amounts of anthocyanins were also observed in cecum (Table 5) and colon (Table 6) walls. A small parallel increase in anthocyanin and phenolic acid metabolite content was observed in the colon as animals LFD+CA and HFD+CA were treated with antibiotic cocktail; however, generally lower amounts of tissue metabolites suggested that these tissues were less likely to absorb the bioactive anthocyanins from diet. Phenolic acid content was increased in cecum tissue compared to jejunum tissue (with the exception of HFD+CA animals)

Table 3. Individual Anthocyanins and Phenolic Acids Recovered from the Feces (Week 10)

	concentration[a] (μg/g feces)			
	LFD+C	LFD+CA	HFD+C	HFD+CA
Week 10				
total anthocyanins	**244 ± 5.82**	**6307 ± 891**	**293 ± 68.9**	**4834 ± 826**
delphinidin-3-*O*-glucoside	39.5 ± 8.69	134 ± 38.6	33.5 ± 7.39	94.2 ± 111
delphinidin-3-*O*-rutinoside	121.4 ± 3.06	2941 ± 1084	160 ± 36.7	2635 ± 850
cyanidin-3-*O*-glucoside	25.7 ± 5.96	60.0 ± 11.8	20.2 ± 4.15	46.0 ± 5.05
cyanidin-3-*O*-rutinoside	57.9 ± 5.77	3172 ± 757	79.9 ± 20.5	2230 ± 813
total phenolic acids	**81.5 ± 55.7**	**32.6 ± 5.91**	**93.3 ± 64.8**	**30.0 ± 0.06**
gallic acid	47.2 ± 33.6	16.3 ± 3.74	59.0 ± 42.0	16.2 ± 0.41
protocatechuic acid	34.4 ± 22.1	16.3 ± 2.17	34.3 ± 22.8	13.7 ± 0.35
Week 14				
total anthocyanins	**251 ± 0.49**	**4203 ± 820**	**347 ± 69.0**	**9906 ± 235**
delphinidin-3-*O*-glucoside	39.0 ± 7.35	45.7 ± 7.92	37.9 ± 7.20	150.0 ± 41.9
delphinidin-3-*O*-rutinoside	126 ± 6.72	2169 ± 216	193 ± 41.7	4939 ± 209
cyanidin-3-*O*-glucoside	26.0 ± 7.15	12.8 ± 0.83	23.2 ± 3.11	98.0 ± 90.2
cyanidin-3-*O*-rutinoside	60.5 ± 7.16	1976 ± 617	92.3 ± 16.3	4719 ± 158
total phenolic acids	**58.8 ± 18.0**	**47.9 ± 20.9**	**77.0 ± 0.90**	**48.6 ± 5.18**
gallic acid	31.6 ± 10.9	19.2 ± 9.2	42.6 ± 3.38	35.6 ± 4.57
protocatechuic acid	27.2 ± 7.05	28.7 ± 11.7	34.4 ± 2.48	12.8 ± 0.62

[a]LFD+C, low-fat diet supplemented with black currant extract; LFD+CA, low-fat diet supplemented with black currant extract and antibiotic cocktail; HFD+C, high-fat diet supplemented with black currant extract; HFD+CA, high -fat diet supplemented with black currant extract and antibiotic cocktail.

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Table 4. Individual Anthocyanins and Phenolic Acids Recovered from the Jejunum (Week 14)

	concentration[a] (μg/g tissue)			
	LFD+C	LFD+CA	HFD+C	HFD+CA
total anthocyanins	**35.2 ± 14.1**	**154 ± 12.3**	**111 ± 74.0**	**195 ± 33.3**
delphinidin-3-*O*-glucoside	6.90 ± 0.86	28.7 ± 13.1	23.8 ± 15.4	27.0 ± 12.7
delphinidin-3-*O*-rutinoside	11.4 ± 5.48	54.8 ± 0.83	37.9 ± 27.7	68.6 ± 46.0
cyanidin-3-*O*-glucoside	6.26 ± 1.54	22.0 ± 7.67	18.6 ± 10.8	25.3 ± 11.9
cyanidin-3-*O*-rutinoside	10.6 ± 6.24	48.2 ± 7.58	30.9 ± 20.2	74.2 ± 46.8
total phenolic acids	**29.0 ± 8.08**	**14.0 ± 5.49**	**21.4 ± 11.0**	**38.1 ± 35.4**
gallic acid	22.2 ± 7.34	10.3 ± 2.81	12.4 ± 9.31	26.3 ± 28.2
protocatechuic acid	6.81 ± 0.74	3.70 ± 2.68	9.32 ± 1.67	11.8 ± 7.17

[a]LFD+C, low-fat diet supplemented with black currant extract; LFD+CA, low-fat diet supplemented with black currant extract and antibiotic cocktail; HFD+C, high-fat diet supplemented with black currant extract; HFD+CA, high-fat diet supplemented with black currant extract and antibiotic cocktail.

Table 5. Individual Anthocyanins and Phenolic Acids Recovered from the Cecum (Week 14)

	concentration[a] (μg/g tissue)			
	LFD+C	LFD+CA	HFD+C	HFD+CA
total anthocyanins	**16.5 ± 8.17**	**52.7 ± 23.9**	**23.7 ± 0.58**	**18.4 ± 17.6**
delphinidin-3-*O*-glucoside	2.16 ± 3.05	7.00 ± 4.06	n/a	3.48 ± 4.91
delphinidin-3-*O*-rutinoside	2.60 ± 3.72	19.8 ± 12.9	6.40 ± 0.76	n/a
cyanidin-3-*O*-glucoside	2.79 ± 3.95	5.38 ± 1.36	5.61 ± 0.05	14.9 ± 12.7
cyanidin-3-*O*-rutinoside	8.97 ± 2.55	20.5 ± 5.50	11.7 ± 0.23	n/a
total phenolic acids	**57.6 ± 5.86**	**53.3 ± 1.10**	**24.3 ± 17.1**	**30.0 ± 0.06**
gallic acid	50.0 ± 4.11	33.1 ± 3.53	14.5 ± 16.4	18.9 ± 12.6
protocatechuic acid	7.60 ± 1.75	20.2 ± 2.43	9.86 ± 0.62	11.0 ± 3.41

[a]n/a, not available; LFD+C, low-fat diet supplemented with black currant extract; LFD+CA, low-fat diet supplemented with black currant extract and antibiotic cocktail; HFD+C, high-fat diet supplemented with black currant extract; HFD+CA, high-fat diet supplemented with black currant extract and antibiotic cocktail.

Table 6. Individual Anthocyanins and Phenolic Acids Recovered from the Colon (Week 14)

	concentration[a] (μg/g tissue)			
	LFD+C	LFD+CA	HFD+C	HFD+CA
total anthocyanins	**15.5 ± 12.8**	**53.9 ± 4.82**	**41.5 ± 9.24**	**61.0 ± 29.2**
delphinidin-3-*O*-glucoside	3.10 ± 4.38	5.51 ± 0.82	2.44 ± 3.44	2.13 ± 3.01
delphinidin-3-*O*-rutinoside	3.56 ± 5.63	25.0 ± 4.31	20.8 ± 8.44	27.4 ± 10.1
cyanidin-3-*O*-glucoside	2.57 ± 3.63	n/d	n/d	2.13 ± 3.01
cyanidin-3-*O*-rutinoside	6.23 ± 0.28	23.4 ± 0.31	18.3 ± 4.25	28.8 ± 12.3
total phenolic acids	**n/d**	**n/d**	**n/d**	**n/d**
gallic acid	n/d	n/d	n/d	n/d
protocatechuic acid	n/d	n/d	n/d	n/d

[a]n/d, not detected LFD+C, low-fat diet supplemented with black currant extract; LFD+CA, low-fat diet supplemented with black currant extract and antibiotic cocktail; HFD+C, high-fat diet supplemented with black currant extract; HFD+CA, high-fat diet supplemented with black currant extract and antibiotic cocktail.

and diminished below detection levels in the colon tissue. These data may reflect increased biotransformation of anthocyanins in the cecum and further degradation of phenolic acid metabolites into simple phenols in the colon.[23]

Effect on Glucose Metabolism and Insulin Sensitivity. Baseline blood glucose levels were measured at week 14 of the study after an overnight fast (Figure 5, time point 0 min). Black currant supplementation or gut microbiome disruption had no effect on blood glucose in animals on LFD (Figure 5A). Baseline blood glucose levels did not differ for HFD and HFD +C groups as well (Figure 5B). However, HFD+CA mice without functional gut microbiome showed a 45% increase in baseline blood glucose (284 mg/dL) versus HFD controls or HFD+C animals with black currant supplementation (193 mg/dL). The HFD group blood glucose levels were significantly higher at 30, 60, and 120 min after oral gavage as compared with the LFD mice, whereas the anthocyanin treatment (HFD +C) significantly reversed peak blood glucose concentration at 60 min (Figure 5B). Despite black currant supplementation, mice with disrupted gut microbiome (HFD+CA) showed high peak blood glucose values similar to those of untreated HFD controls (Figure 5B).

Insulin sensitivity was enhanced in LFD animals receiving black currant supplementation, even when their gut microbiome was suppressed by antibiotic cocktail (Figure 5C). HFD mice developed insulin resistance as expected, whereas black currant-supplemented HFD+C mice showed improved insulin sensitivity at 30, 60, and 120 min after insulin injection (Figure 5D). Suppression of gut microbiome had a dramatic negative effect on insulin sensitivity in HFD+CA animals: despite black

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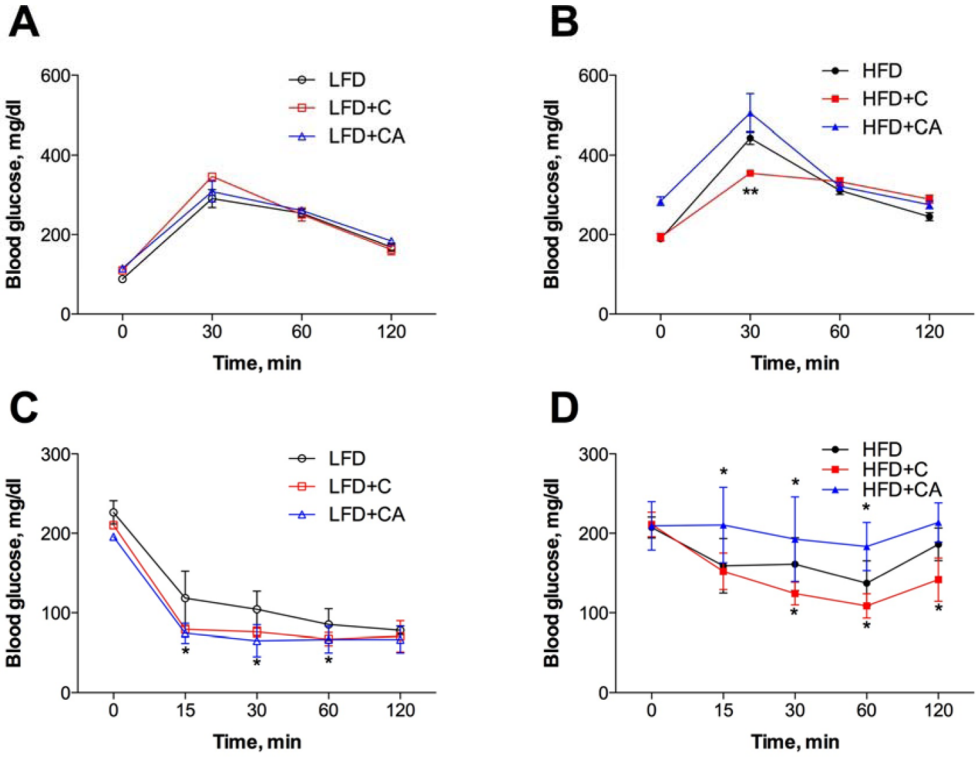

Figure 5. Chronic insulin-sensitizing effect of black currant supplementation on oral glucose tolerance test on low-fat diets (A) and high-fat diets (B) and insulin tolerance test on low-fat diets (C) and high-fat diets (D) in the C57BL/6J mice. Eight-week-old male mice were fed a low- or high-fat diet for 6 weeks. Lean and obese mice were further randomized to control low-fat diet (LFD), control high-fat diet (HFD), and the respective low-fat (LFD+C) and high-fat (HFD+C) black currant-supplemented treatment groups (1% black currant extract incorporated in LFD or HFD diet) and kept on the same diet for an additional 8 weeks. An antibiotic cocktail was administered in drinking water ad libitum to half of the animals on black currant-supplemented treatment groups (LFD+CA and HFD+CA groups, respectively) for the entire duration of the black currant feeding study (weeks 7−14). For oral glucose tolerance test, mice were fasted overnight (16 h) and received oral gavage of D-glucose (1.5 g/kg body weight). For insulin tolerance test, mice were fasted for 4 h and received intraperitoneal injection of insulin (0.75 U/kg body weight). Blood glucose concentrations were measured at 0, 15, 30, 60, and 120 min after glucose or insulin challenge in blood samples obtained from tail-tip bleedings, using a glucometer. Values are means ± SEM. (*) $P < 0.05$ and (**) $P < 0.01$ when compared with LFD or HFD control by one-way ANOVA followed by Dunnett's post hoc test.

currant supplementation, the insulin tolerance curve remained virtually flat for the duration of the testing, showing significant degree of insulin resistance at all time points tested (15−120 min).

DISCUSSION

Bioactive anthocyanins are of substantial health-promoting interest as their daily intake is 180−200 mg in the United States and may be significantly higher in other countries where more fruits and vegetables are consumed on a regular basis.[31] A single serving of anthocyanin-rich berries may contribute in excess of 100−200 mg of anthocyanins to a regular diet,[32] which is 5−10-fold higher than the daily intake of other flavonoids, such as quercetin or apigenin.[33] Anthocyanins are structurally diverse compounds that are present in plants in different concentrations and have various degrees of hydroxylation, methylation, glycosylation, and acylation.[3] This inherent diversity may be responsible for the lack of equivocal results from animal and human studies, because predominantly delphinidin- and malvidin-containing fruits are more likely to improve metabolic and cardiovascular disease risk biomarkers (i.e blueberry,[16] black currant and bilberry,[17,18] or grapes[19]), whereas cyanidin-based supplementation strategies offer less protection (i.e., elderberry,[20] blood orange,[21] or purple carrot[22]). In this study, we focused on the effects of black currant powdered extract on glucose metabolism and insulin sensitivity in diet-induced obese mice with intact and antibiotic-disrupted gut microbiome. The predominant flavonoids in the extract (Tables 1 and 2) were four anthocyanins that are equally distributed between cyanidin and delphinidin aglycones (Figure 1), in agreement with previously published data.[24] Anthocyanins represented the most abundant phenolic compounds in black currants, with other minor phenolics contributing to <5% of black currant powdered extract as shown by our previous work.[25]

Reduced body weight associated with 1% black currant powdered extract supplementation was observed together with no change in food intake (Figure 2). In this study mice consumed on average between 2.5 and 3.0 g food/animal/day, which is equal to ingestion of 8−10 mg/animal/day (300−400 mg/kg/day) of black currant anthocyanins. The human equivalent dose for this study was estimated at 25 mg/kg/day or 1.5 g/day of total anthocyanins for an average adult.[34] This dose was 8 times larger than an average anthocyanin intake in a regular U.S. diet[31] and could be achieved by incorporating 3−4 cups of fresh black currants (250−750 mg anthocyanins/100 g fruit)[35,36] or wild blueberries (350−550 mg anthocyanins/100 g fruit)[37] in the diet. Citric acid present in the black currant powdered extract was not likely to contribute to beneficial effects of black currant anthocyanin supplementation on obesity and metabolic disease, as obese patients show significantly

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higher values in the level of citric acid as a result of natural changes in intermediate metabolism components associated with obesity.[38]

The majority of studies focusing on anthocyanin absorption have shown that the bioavailability of anthocyanins is low. The maximum black currant anthocyanin concentration in plasma reached 0.4 μM (t_{max} = 15 min) and returned to baseline after 7 h.[24] Gastrointestinal tissues, however, receive longer exposure time to anthocyanins that could increase their absorption in the tissue walls. Indeed, in this study we observed concentrations up to 35 and 111 μg/g anthocyanins in the jejunum tissue of LFD and HFD mice, respectively, and these levels increased to 154 and 195 μg/g tissue in antibiotic-treated animals (Table 4).

Anthocyanin concentrations were much lower in cecum and colon tissues, suggesting that black currant anthocyanins are maximally absorbed in the jejunum—a conclusion that is consistent with previous observations.[24] On the other hand, gallic and protocatechuic acid levels were highest in cecum and largely undetectable in colon tissues (Table 5). Even though cyanidin- and delphinidin-type anthocyanins were present in black currant extract and diet in almost equal concentrations, a strong preference for selective absorption of gallic acid was evident as compared to protocatechuic acid in the jejunum (Table 4). Because similar ratios were observed in both intact and antibiotic-treated animals, this effect was likely independent of gut microbiome.

We observed that the gut microbiome was crucial for the protective effect of black currant anthocyanins against obesity and associated insulin resistance (Figure 5). Indeed, germ-free HFD mice gained more weight than controls.[39] At the same time, addition of antibiotic cocktail to drinking water decreased anthocyanin biotransformation in the gut, which resulted in higher anthocyanin concentrations in the gastrointestinal tissues (Tables 4−6) and feces (Table 3). Cyanidin-based anthocyanins showed the most dramatic increase in fecal content upon disruption of gut microbiome, suggesting their high susceptibility to microbial degradation in the gut. At the same time, an approximately 3-fold enrichment in gallic acid (major delphinidin-derived metabolite) over protocatechuic acid (major cyanidin-derived metabolite) was observed in the jejunum of both intact and antibiotic-treated animals. These observations may explain why plants or foods rich in cyanidin-type anthocyanins are less effective in preventing metabolic and inflammatory disorders in animal and human studies. Higher gastrointestinal tissue levels of anthocyanins in antibiotic-treated animals, however, did not correlate with metabolic health outcomes, possibly indicating that not intact anthocyanins but their gut microbiome metabolites are partially responsible for these effects.

In conclusion, consumption of black currant powdered extract decreases weight gain of the LFD and HFD fed C57Bl/6J mice with healthy gut microbiome. Feces of gut microbiome-deficient mice showed an increase in anthocyanins and a decrease in their phenolic acid metabolites, whereas a corresponding increase was observed in the jejunum tissue. Cyanidin-type anthocyanins were much more sensitive to gut microbiome metabolism than delphinidins. Thus, gut microbiome was crucial for the protective effect of black currant anthocyanins against obesity and associated insulin resistance.

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Notes

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