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Effects of Bilberry (*Vaccinium myrtillus*) in Combination with Lactic Acid Bacteria on Intestinal Oxidative Stress Induced by Ischemia–Reperfusion in Mouse

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ABSTRACT: Intestinal ischemia–reperfusion (I/R) results in oxidative stress, inflammation, and tissue injuries. The present study investigates the antioxidative and anti-inflammatory effects of a dietary supplement of bilberry, either alone or in combination with *Lactobacillus plantarum* RESO56, *L. plantarum* HEAL19, or *Pediococcus acidilactici* JAM046, in an I/R-induced model for oxidative stress in mice. A bilberry diet without addition of bacteria significantly decreased both lipid peroxidation ($p = 0.001$) and mucosal injury in the ileum. Of 14 anthocyanins identified in bilberry, anthocyanin arabinosides were the most resistant to absorption and microbial degradation in the intestines. Cyanidin-3-glucoside and delphinidin-3-glucoside seemed to be mostly absorbed in the stomach and upper part of the small intestine, while malvidin-3-galactoside, peonidin-3-glucoside, peonidin-3-galactoside, and petunidin-3-galactoside seemed to be digested by the microbiota in the cecum. Bilberry strongly influenced the composition of the cecal microbiota. In conclusion, a food supplement of bilberry protected small intestine against oxidative stress and inflammation induced by ischemia–reperfusion.

KEYWORDS: *bilberry (Vaccinium myrtillus), ischemia–reperfusion, Lactobacillus plantarum, Pediococcus acidilactici, anthocyanins, oxidative stress, ileum, cecum, colon*

INTRODUCTION

Ischemia–reperfusion (I/R) injury occurs when the blood supply returns (reperfusion) to a tissue that temporarily has been deprived of blood supply (ischemia). I/R induces oxidative stress caused mainly by overproduction of reactive oxygen species (ROS) and activated polymorphonuclear leukocytes (PMNLs).[1] ROS and activated leucocytes cause lipid peroxidation, protein oxidation, and DNA damage and are suggested to play a key role in pathophysiology of I/R injury.[1] Intestinal I/R provokes the rupture and increased permeability of the mucosal barrier, causing translocation of bacteria and endotoxin to the extraintestinal sites by the circulation system.[1] Local and systemic inflammatory responses leading to distant organ failure, and in some cases death, are triggered by I/R injury. The intestine is one of the organs most sensitive to I/R injury, commonly seen in intestinal surgical interventions such as strangulated bowel and organ transplantation, hemorrhagic shock, and trauma.[2] The superior mesenteric artery (SMA) has been shown to maintain intestinal perfusion and mucosal integrity in rodents.[3] In the present study, the clinical setting that occurs in critically ill patients has been mimicked by using a mouse model, in which occlusion of the SMA followed by reperfusion results in mucosal damage in the small intestine, cecum, and colon.

The body has a natural defense system against ROS in the form of endogenous antioxidative enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase and antioxidants such as glutathione, α-tocopherol, and ascorbic acid.[4] When ROS is overproduced during I/R injury, the endogenous antioxidant defense systems of the body are not sufficient to prevent oxidative stress, and there is a growing interest in using dietary antioxidants for prevention of the oxidative damage. Bilberry (*Vaccinium myrtillus*) has been used as a food and alternative medicine for centuries. Bilberry is known to be one of the best natural sources of anthocyanins and has been used in various studies to prevent oxidative stress injury in different organs.[5−8] Anthocyanins are not only responsible for the blue, purple, and red colors of the fruits and berries but are suggested to act as antioxidative, anti-inflammatory, and antimicrobial agents.[9−11] The absorption of anthocyanins in the gastrointestinal tract has been reported to be poor.[12] Essential organs for anthocyanin absorption seem to be the stomach and upper part of the small intestine (jejunum).[13,14] Absorbed anthocyanins enter the systemic circulation after passage through the liver, where they may be metabolized by mainly methylation but also glucuronidation reactions.[15] In addition, unmetabolized anthocyanin glycosides can be absorbed and excreted. A large nonabsorbed fraction of anthocyanins reaches the large intestine, where they are exposed to the microbial population and may be degraded to phenolic acids.[16,17] Metabolites derived from the ingested anthocyanins may contribute to the health effects.

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Dietary supplements of lactic acid bacteria (LAB) with the capability to catabolize polyphenols may increase the efficiency of the gut microbiota in converting the nonabsorbed fraction of polyphenols into absorbable metabolites and might increase the health-promoting ability of the polyphenols. Furthermore, it has been shown that *Lactobacillus plantarum* possesses enzymes that transform phenolic acids (*p*-coumaric, caffeic, and ferulic acid) into anti-inflammatory phenyl propionic acids.[18] *L. plantarum* strains are able to degrade hydrolyzable tannins, thereby producing gallic acid and the antioxidant pyrogallol.[19] Certain strains of *L. plantarum* are used as probiotics (bacteria with health beneficial effects after ingestion), and some probiotics can prevent bacterial translocation by stabilizing the intestinal mucosal barrier function and normalizing indigenous microbiota in the intestine damaged by I/R.[20] Some strains of LAB also possess antioxidative ability, scavenge reactive oxygen species, and chelate metal ions, providing protection against oxidative stress and lipid peroxidation.[21] Most studies on dietary prevention of I/R have investigated the effects of either polyphenolic supplements or probiotics.[2,9,22−24] There are only a few reports on the prevention of I/R damage by using a combination of a polyphenol-rich diet and probiotics.[5,25,26]

The aim of the present study was to elucidate the anti-inflammatory and antioxidative effects of the polyphenol-rich bilberry fruit on oxidative stress in mice caused by intestinal ischemia−reperfusion. Bilberry alone and in combination with *L. plantarum* RESO56, *L. plantarum* HEAL19, or *Pediococcus acidilactici* JAM046 was administered as dietary supplements in an attempt to protect from I/R injury. The distribution and amount of anthocyanins originating from the administered bilberry powder were recorded in the ileum, cecum, and colon.

MATERIALS AND METHODS

Chemicals. Dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), trisodium citrate dehydrate ($C_6H_5Na_3O_7{\cdot}2H_2O$), magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$), sodium chloride (NaCl), Tween 80, L-cysteine monochloride monohydrate, violet red-bile-glucose agar, and formic acid were purchased from Merck KGaA (Darmstadt, Germany). Myeloperoxidase was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The MDA-586 colorimetric kit was purchased from Oxis International Inc. (Portland, OR, USA). Glycerol was obtained from VWR International (Braire, France), and bacteriological peptone from Unipath LTD (Basingstoke, Hampshire, England). Rogosa agar was purchased from Oxoid LTD (Basingstoke, Hampshire, England). Cyanidin-3-*O*-*β*-glucopyranoside (glucoside) was purchased from Polyphenols Laboratories AS (Sandnes, Norway). All solvents were of HPLC grade, and water was of Milli-Q quality (Millipore Corp., Bedford, MA, USA).

Animals. Male Balb/cJ mice (Taconic, 8680 Ry, Denmark), weighing approximately 20 g, were kept under standard laboratory conditions with a controlled 12 h light/dark cycle. Animals were acclimatized 1 week before use and had free access to standard animal chow (R3; Lactamin, Stockholm, Sweden) and water. The study was approved by the Ethical Committee for Animal Experimentation at Lund University.

Experimental Diets. Each animal was placed in its own cage with a food dish. After 7 days of acclimatization, animals were fed experimental diets for 10 days. Experimental diets were prepared every day and consisted of standard chow supplemented with bilberry powder (*Vaccinium myrtillus*) alone or together with one of the following strains: *Lactobacillus plantarum* HEAL19, *Lactobacillus plantarum* RESO56 (both of human origin), or *Pediococcus acidilactici* JAM046 (isolated from lactic acid fermented rosehips). Frozen, whole bilberries purchased from Polarica (Haparanda, Sweden) were freeze-dried and ground. The daily dose of bilberry powder was 1.62 g/mouse. In order to provide each animal with the same energy amount, the I/R-control group and sham group were given 9 g of standard chow per mouse per day, while bilberry groups were given 7.16 g of chow per mouse per day. The animal chow R3 was dissolved in water to soften the consistency prior to the addition of the different supplements. The bacterial cultures were kept in a freezing media (0.85 g/L dipotassium hydrogen phosphate, 0.2 g/L potassium dihydrogen phosphate, 0.6 g/L trisodium citrate dihydrate, 0.25 g/L magnesium sulfate heptahydrate, 121 mL of 99.5% glycerol, 879 mL of distilled water) at −80 °C. Dose per day and cage was 1×10^9 cfu. Groups without bacterial supplementation (I/R-control, sham, and bilberry alone) were compensated by adding the same amount of pure freezing media.

Experimental Groups. Animals were randomly divided into the following 6 groups with 8 mice in each group: I/R-control group, fed soft standard chow; sham group, fed soft standard chow; B group, fed standard chow supplemented with bilberry (*V. myrtillus*); B+Lpl56, fed standard chow supplemented with bilberry and *L. plantarum* RESO56 strain (Lpl56); B+LplH19, fed standard chow supplemented with bilberry and *L. plantarum* HEAL19 strain (LplH19); B+Ped group, fed standard chow supplemented with bilberry and *P. acidilactici* JAM046 strain (Ped). Tap water and diet were provided ad libitum. All experimental groups except for the sham group were subjected to intestinal ischemia−reperfusion.

Intestinal Ischemia−Reperfusion Procedure. The mice were anesthetized with 7.5 mg of ketamine (Ketalar 50 mg/mL [Pfizer, UK]) and 2.5 mg of xylazine (Narcoxyl 20 mg/mL [Veterinaria AG, Schweiz]) per 100 g of body weight by intraperitoneal injection. The animal was placed on a 37 °C warming pad to maintain body temperature. A midline abdominal incision was performed and abdominal contents were deflected to the left side. The superior mesenteric artery was identified and occluded with a vessel clamp to obtain ischemia of the small intestine and colon, and the bowel was returned to the abdominal cavity. Ischemia was confirmed when the intestines became pale. The peritoneal cavity was filled with 1 mL of Dulbecco's phosphate-buffered saline (PBS) for fluid resuscitation. The clamp was removed after 40 min of ischemia. Immediate reperfusion was confirmed with the restoration of color. The abdomen was closed using a running Vicryl 5-0 suture (Johnson & Johnson, USA). After 120 min of reperfusion, the animal was anaesthetized again, sampled, and sacrificed. The sham group was subjected to the surgical procedure described above but without clamping of the superior mesenteric artery. Contents from ileum, cecum, and colon were used for analyses of polyphenols. Cecal tissue was used for determination of microbial diversity and number of viable cells of lactobacilli and Enterobacteriaceae. Ileal tissue was rinsed in ice-cold Dulbecco's PBS and was used for determination of lipid peroxidation by malondialdehyde (MDA), myeloperoxidase, and histological examination. Colonic tissue was rinsed in ice-cold Dulbecco's PBS and used for the determination of lipid peroxidation (MDA) and number of viable cells of lactobacilli and Enterobacteriaceae. Segments of mucosal tissues used for viable counts were placed in 3 mL of freezing media (0.85 g/L dipotassium hydrogen phosphate, 0.2 g/L potassium dihydrogen phosphate, 0.6 g/L trisodium citrate dihydrate, 0.25 g/L magnesium sulfate heptahydrate, 121 mL of 99.5% glycerol, 879 mL of distilled water). All samples were weighed, frozen in liquid nitrogen, and stored at −80 °C until analysis. Surgery was performed with attention to sterile technique.

Viable Count. Samples taken from cecal and colonic mucosal tissue were sonicated for 5 min and vortexed for 2 min. A 1 mL amount of the sample was mixed with 9 mL of dilution liquid (8.5 g/L sodium chloride, 1 g/L bacteriological peptone, 1 g/L Tween 80, and 0.2 g/L L-cysteine monochloride monohydrate) and serially diluted. After dilution, 0.1 mL of the samples from appropriate dilutions was spread with glass beads (5 mm diameter) on Rogosa agar plates and anaerobically incubated (Gas Pack System, Becton Dickenson Microbiology Systems, Cockeynsville, MD, USA) for 72 h at 37 °C (lactobacilli count) and on violet red-bile-glucose agar plates aerobically incubated for 24 h at 37 °C (Enterobacteriaceae count).

Lipid Peroxidation Determined by Malondialdehyde. MDA-586, a colorimetric assay, was used to determine malondialdehyde, an indicator of lipid peroxidation, in collected ileal and colonic tissues. Samples were homogenized in 1 mL of cold Dulbecco's phosphate-buffered saline and 10 μL of butylated hydroxytoluene (5 mM). After homogenization samples were centrifuged at 4000*g* for 10 min at 4 °C, and an aliquot of 0.2 mL of the supernatants was mixed with 10 μL of probucol and 640 μL of diluted *N*-methyl-2-phenylindol. Concentrated hydrochloric acid (150 μL of 12 M) was added to the samples before incubation in a water bath at 45 °C for 60 min. The samples were then centrifuged at 10000*g* for 10 min at 4 °C, the supernatant was transferred to a cuvette, and the absorbance was measured at 586 nm. Since MDA is not stable, tetramethoxypropane (TMOP) was used as a MDA standard. During the acid incubation step at 45 °C, the TMOP is hydrolyzed and generates MDA. The unit for expression of MDA was nmol/g of tissue.

Neutrofil Infiltration Determined by Myeloperoxidase. The enzyme myeloperoxidase (MPO) is abundant in neutrophil leucocytes and has been found to be a reliable marker for the detection of neutrophil accumulation in inflamed tissue *in vivo*.[4] Specimens of ileal tissue were collected and weighed prior to storage at −80 °C until the time of the assay for MPO activity. The ileal segments were homogenized in 1 mL of potassium phosphate buffer (20 mM, pH 7.4) for 60 s. The homogenate was centrifuged at 14 000 rpm for 10 min, and the pellets were resuspended in 1 mL of potassium phosphate buffer (50 mM, pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. The samples were then freeze−thawed once, sonicated for 90 s, and kept in a water bath at 60 °C for 120 min. Next, the samples were centrifuged (14 000 rpm, 10 min) and the MPO activity of the supernatant (20 μL) was assessed in 96-well plates (Nunc, Invitrogen A/S, Taastrup, Denmark). The enzyme activity was determined spectrophotometrically at 450 nm. MPO (Sigma Chemical Co., St. Louis, MO, USA) was used as a standard, and values were expressed as MPO units/g of tissue (U/g).

Histopathological Evaluation. Approximately 1 cm segments of proximal ileum were placed in phosphate-buffered 4% formaldehyde, fixed overnight, and dehydrated. The tissues were subsequently embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). All specimens were evaluated in a blinded fashion using light microscopy. Mucosal damage was assessed as described by Chiu et al. The grading was performed according to the following criteria: grade 0, normal mucosa; grade 1, development of subepithelial Gruenhagen's space at the tip of the villus; grade 2, extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria; grade 3, massive epithelial lifting with a few denuded villi; grade 4, denuded villi with exposed dilated capillaries; grade 5, digestion and disintegration of lamina propria, hemorrhage, and ulceration.

DNA Extraction. Samples were taken from the cecal mucosal tissue, and DNA extraction was performed as previously described by Karlsson et al. Samples were sonicated for 5 min and vortexed for 2 min before centrifugation at 9000 rpm for 7 min in sterile UV-treated tubes. After addition of buffer G2 (380 μL) and 30 μL of Proteinas K (Qiagen, Hilden, Germany) to the pellet, samples were kept in a water bath at 56 °C for 3−4 h until totally dissolved. Further disintegration was achieved by adding glass beads (2 mm in diameter) to the samples and shaking for 45 min at 4 °C in an Eppendorf mixer. After centrifugation, the supernatant was used for total DNA extraction in a BioRobot EZ1 with EZ1 DNA tissue card and EZ1 DNA tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was eluted in 200 μL.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis. *PCR Amplification for T-RFLP Analysis.* Primers FAM-ENV1 (5′-AGA GTT TGA TII TGG CTC AG-3′) and ENV2 (5′-CGG ITA CCT TGT TAC GAC TT-3′) were used for amplification of the 16S rRNA genes. The forward primer ENV1 was synthesized and fluorescently labeled with FAM (Applied Biosystems, Foster City, CA, USA) at the 5′ end. The PCR reaction mixture contained 0.4 μM primer FAM-ENV1 and 0.2 μM primer ENV2, 2.5 μL of 10× PCR reaction buffer (500 mM Tris HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, pH 8.3), 0.2 mM of each deoxyribonucleotide triphosphate, 2.5 U of FastStart Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), and 10−30 ng of template DNA, in a final volume of 25 μL. Amplification was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the following program: 95 °C for 3 min, 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 50 °C for 45 s, and 72 °C for 2 min, with an additional extension at 72 °C for 7 min. Triplicate reactions were performed for each sample, and a negative control was included in all PCR runs. PCR products were verified by agarose gel electrophoresis. Products from three PCR reactions were pooled and purified by MiniElute PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 30 μL of sterile water, and the concentration was measured by Nanodrop ND-1000 (Saveen Werner, Limhamn, Sweden).

T-RFLP Analysis. A 200 ng amount of the purified aplicons was digested for 5 h at 37 °C with 15 U of the restriction endonuclease *Msp*I (Fermentas Life Science, Burlington, Canada) in a total volume of 15 μL. The enzyme was inactivated by heating at 65 °C for 20 min. After digestion, aliquots of the products were diluted four times with sterile water in a sterile 96-well plate (Becton Dickinson, Franklin Lakes, NJ, USA) and sent to DNA-lab Malmö University Hospital (UMAS) for the T-RFLP analysis. Analysis was performed on a 3130x1 genetic analyzer (Applied Biosystems), and a DNA size marker, GeneScan LIZ 600 (Applied Biosystems), was included in all samples. Fragment sizes, peak height, and peak area were analyzed with Genemapper software version 4.0 (Applied Biosystems). The local Southern method was chosen for size calling, and the size range was set from 40 to 580 bp. The peak amplitude thresholds were set to 50 relative fluorescence units (rfu) for samples and 10 rfu for standards. The total peak area for each sample was calculated by summarizing the area for all peaks in a sample. The relative area of each peak was calculated as the peak area of the respective T-RF divided by total peak area and was expressed as percentage of the total area.

Extraction of Phenolic Compounds. Approximately 0.2 g of frozen samples of ileum, cecum, and colon contents were extracted with 1.5 mL of 80% methanol in water acidified with 1% formic acid. Samples were homogenized with a Polytron Homogenizer PT3100 (Kinematica AG, Littau, Switzerland) for 20 s and sonicated for 5 min in an ultrasound bath at 4 °C. After centrifugation at 1300*g* for 10 min at 4 °C the supernatant was collected and kept on ice. The pellet was re-extracted with 1 mL of 80% methanol in water acidified with 1% formic acid. Supernatants were pooled, and methanol was removed under nitrogen gas. The weight of the extract was accurately recorded. The weights of the extracts were made up to twice the sample weight by adding acidified Milli-Q water. The extracts were filtered through a Millex HA 0.45 μm filter (Millipore Corp., Cork, Ireland) before HPLC-DAD-ESI-MS^n analysis.

HPLC-DAD-ESI-MS^n Analysis of Anthocyanins. The anthocyanins in extracts of ileal, cecal, and colonic contents were analyzed on an Agilent 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler cooled to 6 °C, a DAD (190−600 nm), and an MSD XCT ion trap mass spectrometer fitted with an ESI interface. The compounds were separated on a Betasil C18 column (250 mm × 2.1 mm i.d, 5 μm particles) equipped with a 5 μm C18 guard column (4.0 mm × 2.1 mm i.d.), both from Thermo Hypersil-Keystone (Bellefonte, PA, USA). The separation was executed with mobile phases consisting of A [formic acid/water (5/95, v/v)] and B [formic acid/acetonitrile (5/95, v/v)] with the following gradient elution: 0−2 min 10% B, 2−17 min 10−20% B, 17−21 min 20−60% B, 21−25 min 60% B, 25−27 min 60−10% B. The column was allowed to equilibrate for 5 min between injections (10 μL). The column temperature was held at 40 °C, and the solvent flow rate was 0.25 mL/min.

After UV−vis detection the effluent was introduced directly, without splitting, to the ESI interface, where ionization was performed in positive mode. The nebulizer pressure was 40 psi; dry gas flow, 10 L/min; dry temperature, 350 °C; and capillary voltage, 3.5 kV. Ions with m/z 100 to 2000 were measured, with a scan speed of

27 000 amu/s. Fragmentations (MS^{2-3}) were carried out in the automatic mode; that is, the two most abundant ions in MS^{1-2} were fragmented. The fragmentations were performed with 50% energy (0.85 V) with helium as the collision gas.

The anthocyanins in the samples were identified based on their UV−vis spectra (190−600 nm), mass spectra, and retention times relative to external standards and comparison with literature reports on anthocyanins in bilberries.[29] The anthocyanins were quantified by an external standard of cyanidin-3-glucoside (at 520 nm) in the concentration range 0.6−16.1 μg/mL, and the concentration of anthocyanins in the samples was expressed as μg/g of ileal, cecal, or colonic contents.

Statistical Analysis. The data were processed in SigmaStat version 3.1 (SPSS Inc., Chicago, IL, USA). The differences between all groups were evaluated by Kruskal−Wallis one-way ANOVA on ranks followed by all-pairwise-multiple-comparison Dunn's test. The differences between two experimental groups were assessed by a Mann−Whitney rank sum test. Results were considered statistically significant when $p < 0.05$. Values are presented as median (25th−75th percentiles).

The number of T-RFs for different groups was evaluated by the statistical software Minitab 16 (State College, PA, USA) using one-way ANOVA. Values are presented as mean ± SEM (standard error of the mean). Values were considered statistically significant when $p < 0.05$.

Principal component analysis (PCA) was applied to the T-RFLP data (a matrix of the relative peak area) in order to clarify the possible cluster-structures in cecal microbiota related to different experimental diets (SIMCA-P+ software, version 12.0.1; Umetrics, Umeå, Sweden).

RESULTS

Intestinal Ischemia−Reperfusion. All but two animals (in I/R-control) survived the I/R procedure, and both ischemia and reperfusion were performed and observed in all the animals (except for sham). Due to intestinal bleeding or abnormal reaction on anesthetics, which changed the time for reperfusion, one animal in the B+LplH19 group, one animal in the B+Ped group, and one animal in the sham group were excluded from the data evaluation.

Viable Count. The lactobacilli count of cecal tissue varied between 10^5 and 10^7 cfu/g. In the B group the count of lactobacilli was significantly lower than in groups B+Lpl56 and B+Ped ($p = 0.030$ and $p = 0.003$, respectively). In addition, the B+LplH19 group showed a significantly lower lactobacilli count than the B+Ped group ($p = 0.028$). The count of Enterobacteriaceae of ceacal mucosal tissue was low and varied between $<10^2$ and 10^4 cfu/g. There were no significant differences in Enterobacteriaceae count between the groups.

The lactobacilli count of colonic tissue varied between 10^5 and 10^6 cfu/g. All groups fed bilberry showed an insignificant decrease in lactobacilli compared to the IR-control and -sham groups. The count of Enterobacteriaceae in colonic mucosal tissue was low and varied between $<10^2$ and 10^4 cfu/g. No significant differences in viable counts were seen between the groups.

Lipid Peroxidation Determined by Malondialdehyde. In the ileal tissue, lipid peroxidation (MDA) was significantly lower in the sham group than in the I/R-control ($p = 0.035$). MDA was significantly decreased in the B group ($p = 0.001$) compared to the I/R-control. The B group also showed a significantly lower MDA value than the B+Lpl56 group ($p = 0.002$; Figure 1). The median values of groups B+LplH19 and B+Ped (24.1 and 20.4 nmol/g, respectively) were also high compared to the B group (10.6 nmol/g), but the difference did not reach statistical significance ($p = 0.054$ for both groups; Figure 1). The distribution between the MDA values of the different animals was strikingly high within the bacterial groups.

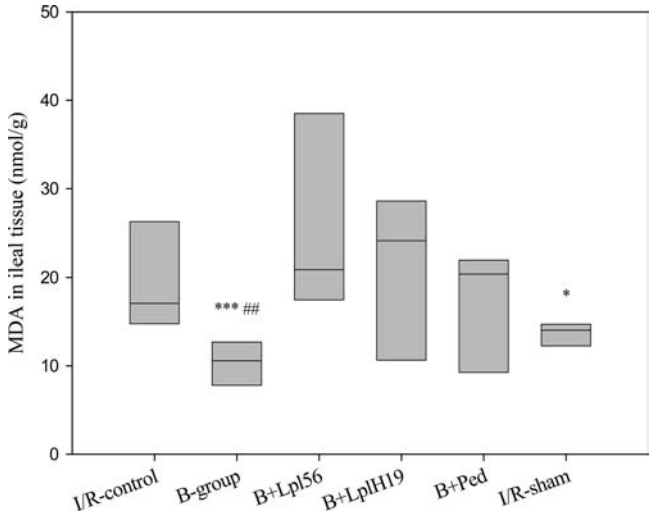

Figure 1. Lipid peroxidation (MDA) of ileum after intestinal ischemia−-reperfusion in mice fed a diet supplemented with bilberry (B), either alone or in combination with different strains of lactic acid bacteria: *Lactobacillus plantarum* RESO56 (Lpl56), *Lactobacillus plantarum* HEAL19 (LplH19), or *Pediococcus acidilactici* JAM046 (Ped). The asterisk (*) denotes $p < 0.05$ and asterisks (***) denote $p < 0.001$ compared to I/R-control. The hashes (##) denote $p < 0.01$ compared to the B+Lpl56 group. Median values are indicated by the transverse line within the box, and the top and bottom lines of each box represent the 25th and 75th percentile.

In the colonic tissue, MDA was significantly higher in the I/R-control group compared to the sham ($p = 0.035$). Lipid peroxidation was reduced with all dietary supplements compared with control, but not significantly ($p = 0.055$). The B group showed almost as low a median value as the sham group (8.9 and 8.2 nmol/g tissue, respectively; Figure 2).

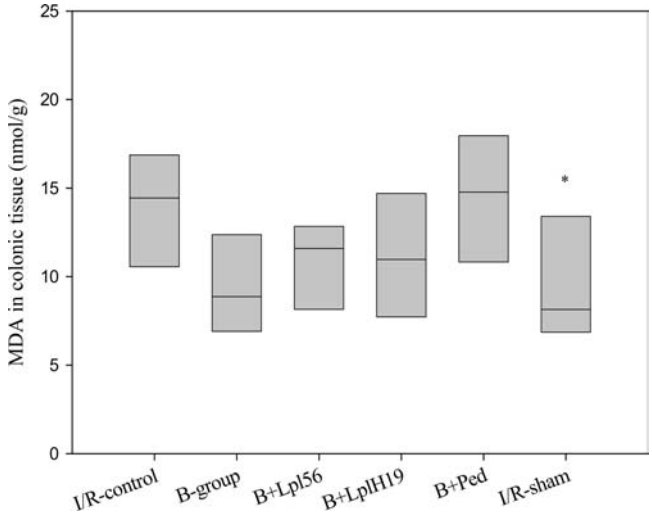

Figure 2. Lipid peroxidation (MDA) of colon after intestinal ischemia−reperfusion in mice fed a diet supplemented with bilberry (B), either alone or in combination with different strains of lactic acid bacteria: *Lactobacillus plantarum* RESO56 (Lpl56), *Lactobacillus plantarum* HEAL19 (LplH19), or *Pediococcus acidilactici* JAM046 (Ped). The asterisk (*) denotes $p < 0.05$ compared to I/R-control. Median values are indicated by the transverse line within the box, and the top and bottom lines of each box represent the 25th and 75th percentile.

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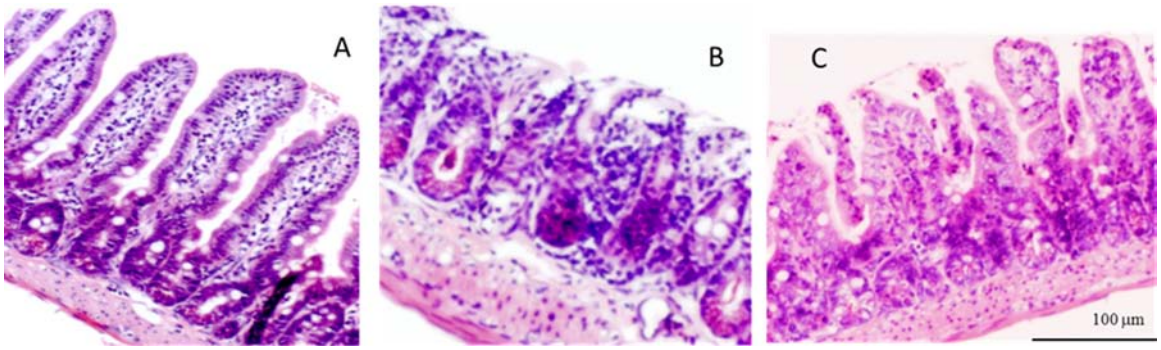

Figure 3. Histological examination of ileal tissue shows normal villi and epithelium in sham (A), severe mucosal damage with complete loss of villi and marked congestion in I/R-control (B), and mild mucosal damage with preserved villi in animals fed bilberry alone (B group) (C).

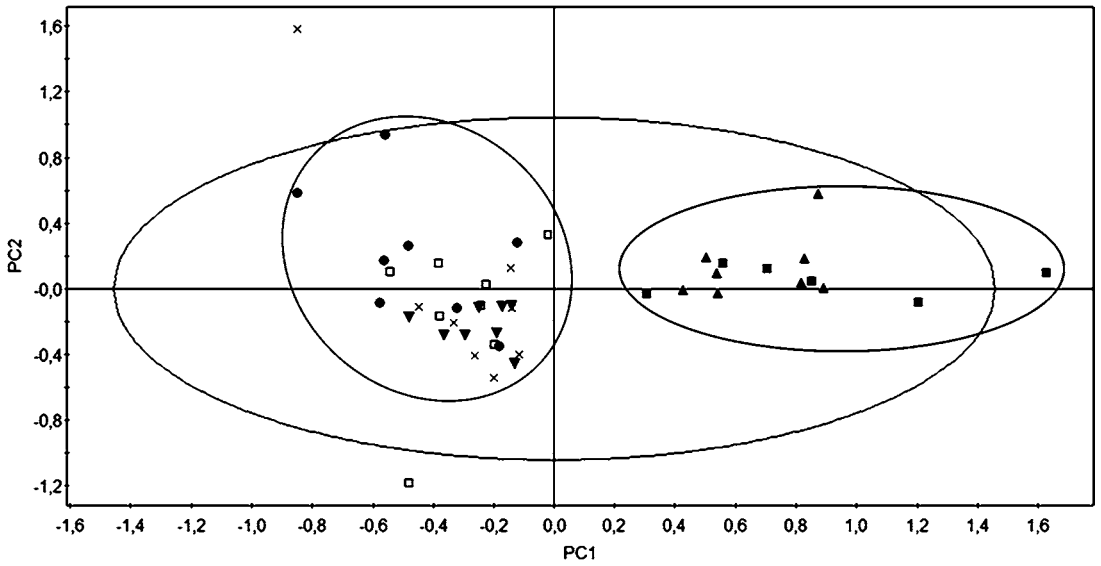

Figure 4. Principal component analysis (PCA) score plot of cecal mucosa-associated microbiota. PC1 explained 25.5% and PC2 explained 12.8% of the variance. Two clusters are distinguished, showing that bacterial populations are influenced by dietary supplementation of bilberry. Black box represents mice in I/R-sham group; black triangle for the I/R-control; white box for the B group; black dot for the B+Ped group; black inverted triangle for the B+LplH19 group; diagonal cross for the B+Lpl56 group.

Neutrofil Infiltration Determined by Myeloperoxidase. In the ileal tissue, MPO was significantly lower in the sham (p = 0.040) compared to the I/R-control. The median values for MPO were 1.44 (1.34−1.82) U/g tissue for the B group, 1.91 (1.84−2.51) U/g tissue for B+Lpl56, 2.04 (1.58−2.55) U/g tissue for B+LplH19, and 1.89 (1.73−3.27) U/g tissue for B+Ped. None of the dietary supplements showed a significant decrease of MPO.

Histopathological Evaluation. The proximal ileum of the sham animals showed intact and clearly visible villi with no evidence of small intestine epithelial disruption (Figure 3A). The median value of the injury scores of this group was 0.25, which was defined as healthy ileum. In the I/R-control, massive destruction of villi and disintegration of lamina propria were observed (Figure 3B). The median value of the mucosal injury score of the I/R-control was 4.5, and this was significantly higher than that of the sham (p < 0.001). In the diet-supplemented groups the median values of the injury scores were 2.5, 3.5, 4.0, and 3.3 for the groups B, B+Lpl56, B+LplH19, and B+Ped, respectively (Figure 3C). No significant differences were recorded.

T-RFLP Analysis. The microbiota of the cecal tissue of each animal was analyzed by T-RFLP. A total of 40 T-RFs were recorded for the whole cohort of animals. The mean values (±SEM) of the number of T-RFs for the different groups were 9.43 (±4.5), 9.38 (±2.97), 10.13 (±4.22), 8.75 (±3.54), 10.25 (±3.85), and 7.13 (±2.85) for sham, I/R-control, B, B+Lpl56, B+LplH19, and B+Ped, respectively. There were no significant differences between the groups.

The bacterial diversity of the microbiota was calculated with the Shannon−Wiener diversity index and was found to be 1.91 (1.87−2.29) for I/R-control, 2.25 (1.8−2.36) for sham, 1.99 (1.82−2.23) for B, 2.09 (1.99−2.21) for B+Lpl56, 2.07 (1.94−2.13) for B+LplH19, and 1.83 (1.71−1.92) for B+Ped. No significant differences were obtained. The differences in T-RFLP patterns between animals were visualized by principal component analysis. The T-RFLP data were scaled with Pareto scaling to not amplify the impact of the occasionally detected T-RFs. Two principle components, PC1 and PC2, explained 25.5% and 12.8% of the variance, respectively. Two clearly distinguished clusters could be seen, formed by the animals of the I/R-control and sham (one cluster) and all the bilberry-supplemented groups (B, B+Lpl56, B+LplH19, and B+Ped; the other cluster) (Figure 4).

Concentration and Recovery of Anthocyanins. In the bilberry powder administered to the animals, 14 different

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anthocyanins were detected and identified, i.e., glucoside (glu), galactoside (gal), or arabinoside (arab) of cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), and malvidin (Mv). Peonidin-3-arabinoside was not detected. Cy-3-gal and Dp-3-arab coeluted in the chromatographic analysis (Table 1).

Table 1. Concentration (μg/g) of Anthocyanins in Freeze-Dried Bilberry Powder Given to Mice

compound	t_R (min)	parent ions ($[M - -H]^+$, m/z)	fragment ions (m/z)	conc in bilberry (μg/g)
delphidin-3-*O*-galactoside	5.0	465	303	169.0
delphidin-3-*O*-glucoside	5.7	465	303	171.4
delphidin-3-*O*-arabinoside + cyanidin-3-*O*-galactoside	7.1	435, 449	303, 287	356.9
cyanidin-3-*O*-glucoside	8.4	449	287	183.7
petunidin-3-*O*-galactoside	9.6	479	317	52.6
cyanidin-3-*O*-arabinoside	10.4	419	287	147.4
petunidin-3-*O*-glucoside	11.3	479	317	110.4
peonidin-3-*O*-galactoside	12.9	463	301	18.0
petunidin-3-*O*-arabinoside	13.2	449	317	42.4
peonidin-3-*O*-glucoside	14.6	463	301	75.1
malvidin-3-*O*-galactoside	15.1	493	331	46.5
malvidin-3-*O*-glucoside	16.4	493	331	127.9
malvidin-3-*O*-arabinoside	18.0	463	331	28.6

Glycosides of Dp and Cy represented 67%, Pt and Mv glycosides represented 13% each, and Pn glycosides represented 6% of the total anthocyanin content. The concentration of the coeluted compounds Cy-3-gal and Dp-3-arab was the highest of all anthocyanins, while Mv-3-arab and *Pn*3-gal concentrations were the lowest.

In the intestinal content of the I/R-control group and sham group no anthocyanins were detected, in accordance with the fact that no bilberry powder was present in the diet.

In the ileum of the animals fed bilberry alone or in combination with different LAB, all 14 anthocyanins from the powder were detected (Table 2). The concentration of coeluted compounds Cy-3-gal and Dp-3-arab was the highest, especially in groups B+LplH19 and B+Ped. Dp-3-glu and Cy-3-glu, together with *Pn*3-gal, were detected in the lowest concentrations in the ileum except for the B+Ped group. In all bilberry-supplemented groups, the concentrations of different anthocyanins, except for Mv-3-arab, in the ileum were significantly higher than in the cecum or colon. The concentration of anthocyanins in the ileum was not significantly different between experimental groups.

The recovery of different anthocyanins in ileum compared to the bilberry powder was the highest in the B+Lpl56 and B +LplH19 groups (Figure 5). Around 30% of Mv-3-arab and Mv-3-gal present in the bilberry powder was recovered in the ileum of the B+Lpl56 and B+LplH19 groups, which was around twice the recovery in the B group. The lowest recovery, less than 3%, was seen for Dp-3-glu and Cy-3-glu in the B, B+Lpl56, and B+LplH19 groups compared to the content of these anthocyanins in bilberry powder. For the B+Ped group the lowest recovery of 5% was seen for Dp-3-gal.

In the cecum of the animals fed bilberry alone or in combination with different LAB, 10 different anthocyanins were detected out of the 14 anthocyanins found in the bilberry powder. Compared to the bilberry powder, the missing anthocyanins in the cecum were Pt-3-gal, Pn-3-gal, Pn-3-glu, and Mv-3-gal. In the cecum, as in the ileum, the concentration of the coeluted Cy-3-gal and Dp-3-arab was the highest of all anthocyanins followed by Dp-3-gal. The lowest concentrations were seen for Cy-3-glu and Pt-3-arab (Table 2). The lowest recoveries (<0.4%) were found for Cy-3-glu and Mv-3-glu. The highest recovery compared to the bilberry powder was observed for Mv-3-arab (7%) followed by Dp-3-gal and Pt-3-arab (around 2%) (Figure 5). The number and the concentration of different anthocyanins were generally lower in the cecum than in the ileum and the colon (Table 2 and Figure 5).

In the colon of the animals fed bilberry alone or in combination with different LAB, 13 different anthocyanins were detected out of the 14 anthocyanins found in the bilberry powder. Pn-3-gal was not detected in the colon of mice, and Pn-3-glu was found in the lowest concentration (Table 2). The concentrations of coeluted Cy-3-gal and Dp-3-arab, followed by Mv-3-arab, were the highest of all anthocyanins in the colon of bilberry-supplemented groups. Mv-3-arab showed the highest recovery in the colon (30−48%) followed by Pt-3-arab (around 7%). The lowest recoveries were observed for glucosides and galactosides of Mv, Pn, Cy, and Pt (Figure 5). It is obvious that Pt-3-arab and Mv-3-arab were relatively poorly degraded by the gut microbiota or absorbed by the body.

B+Lpl56 and B+LplH19 groups showed the highest recovery in the ileum for eight (Dp-3-gal, Pt-3-gal, Pt-3-glu, Pn-3-gal, Pt-3-arab, Mv-3-gal, Mv-3-glu, Mv-3-arab) of the 14 different anthocyanins, and the corresponding number for B+Ped was six (Dp-3-glu, Cy-3-glu, Cy-3-arab, Pn-3-glu, and coeluted Dp-3-arab and Cy-3-gal) of the 14 anthocyanins in the ileum (Figure 5). The differences in anthocyanin recovery between the different bilberry-supplemented groups were negligible in the cecum and colon.

DISCUSSION

Overproduction of ROS during return of oxygenated blood to the ischemic tissue (reperfusion) results in attraction and activation of neutrophils and lipid peroxidation of cell membranes, causing alternations in their structure and permeability.[1] Significantly increased levels of MDA in the ileum and colon, and MPO in the ileum, were observed in the I/R-control compared to the sham of the present study, which was in agreement with other studies.[2,22] Supplementation with bilberry alone (B group) significantly decreased MDA in the ileum. These results are supported by other studies where bilberry successfully reduced lipid peroxidation in inflammatory processes caused by oxidative stress.[5−8]

In bilberries, anthocyanins are especially abundant and comprise about 90% of the phenolic compounds.[30] It has been shown that anthocyanins exhibit anti-inflammatory, antioxidative, and radical-scavenging activities.[10,30,31] In the present study, the decrease of MDA in the B group may be due to the antioxidative and radical-scavenging ability of anthocyanins in bilberry, which may directly reduce ROS in the inflamed tissues and thereby prevent the decrease of endogenous antioxidants and antioxidative enzymes in the tissues. It can also be speculated that bilberry antioxidants may decrease the amount of ROS-generating enzyme xanthine oxidase accumulated in ischemic tissue and thus prevent lipid peroxidation and neutrophil infiltration.[4]

In the colonic tissue, MDA showed a tendency to decrease in the groups fed bilberry together with a single LAB strain. In contrast, in the ileum MDA increased when bilberry was fed

Table 2. Concentrations (μg/g) of Anthocyanins in the Intestines of Mice Administered Bilberry (B), Either Alone or Supplemented with a Lactic Acid Bacterium: Either *Lactobacillus plantarum* RESO56 (Lpl56), *Lactobacillus plantarum* HEAL19 (LplH19), or *Pediococcus acidilactici* JAM046 (Ped)[a]

	B group			B+Lpl56			B+LplH19			B+Ped		
compound	ileum	cecum	colon	ileum	cecum	colon	ileum	cecum	colon	ileum	cecum	colon
Dp-3-gal	**9.22** *** (7.3−18)	**3.48** (2.9−3.6)	**6.61** ** (4.1−7.9)	**17.9** *** ## (10.2−18.5)	**3.33** (1.6−4.9)	**5.1** * (4.2−6.3)	**27.9** * (7.7−40.3)	**3.61** (3.4−4.6)	**6.48** * (6.0−8.0)	**8.22** (2,3−52.3)	**2.6** (2.0−3.1)	**5.15** (3.4−6.1)
Dp-3-glu	**2.57** (1.4−5.2)	**2.1** (1.8−2.4)	**4.22** *** (2.7−5.2)	**3.4** (2.1−4.9)	**2.44** (1.5−2.8)	**3.53** (2.9−4.1)	**5.29** (1.4−8.9)	**2.39** (2.1−3.0)	**4.16** (3.8−5.7)	**25.5** (25.3−25.6)	**1.59** (1.3−2)	**3.24** ** (2.4−4.1)
Dp-3-arab + Cy-3-gal	**23.3**** # (18.7−40)	**4.81** (4.5−6)	**13.8** *** (8.9−19.3)	**34.6** *** ## (21.4−50.2)	**4.7** (3.3−6.8)	**11.7** *** (9.3−15)	**56.4** *** (16.9−88.1)	**5.82** (4.6−6.8)	**17.8** *** (11.6−20.3)	**63.33**** (3.2−117.2)	**4.59** (3.4−5.4)	**12.5**** (5.8−18.7)
Cy-3-glu	**2.83** (0.79−3.5)	**0.8** (0.7−0.82)	**1.4** *** (1.1−1.7)	**3.12** ** # (1.5−5.2)	**0.77** (0.6−0.9)	**1.2** * (0.9−1.3)	**3.62** (0.59−4.2)	**0.76** (0.7−0.83)	**1.59** (1.1−1.9)	**22.1** (21.1−23.1)	**0.61** (0.6−0.8)	**1.06** ** (0.9−1.3)
Cy-3-arab	**13.5** ***## (9.2−20.6)	**1.52** (1.4−1.7)	**3.3** *** (2.8−4.9)	**18.4** ***### (11.2−27.2)	**1.33** (1.0−1.8)	**2.64** ** (2.2−3.2)	**26.3** ***### (7.9−44.5)	**1.79** (1.5−1.83)	**3.36** *** (3.2−5.8)	**30.4** ** (3.8−61.7)	**1.34** (1.2−1.6)	**2.62** ** (1.9−3.1)
Pt-3-gal	**4.46** ### (4.3−8.8)	**ND**	**1.06** (0.9−1.6)	**10.01** ### (4.8−11.1)	**ND**	**0.85** (0.7−1.0)	**12.44** ## (5.1−17.5)	**ND**	**1.04** (0.9−1.1)	**6.53** # (1.9−23.9)	**ND**	**0.85** (0.8−0.9)
Pt-3-glu	**7.9** ***### (7.2−15.5)	**0.85** (0.7−0.9)	**1.61** ** (1.1−1.8)	**17.5** ***### (8.6−18.2)	**0.88** (0.6−1.0)	**1.57** ** (1.3−1.7)	**20.3** **## (8.4−31.2)	**0.95** (0.9−1.0)	**1.67** ** (1.5−2.0)	**7.14** # (2.5−39.7)	**0.92** (0.7−1.3)	**1.13** (0.9−1.6)
Pt-3-arab	**4.14** ***# (4.0−8.4)	**0.68** (0.6−0.8)	**3.0** *** (1.5−3.8)	**9.13** *** (4.2−9.9)	**0.75** (0.5−1.1)	**3.03** *** (2.0−4.1)	**11.4** *** (3.6−16.2)	**0.82** (0.7−1.1)	**2.89** *** (2.5−4.9)	**5.46** ** (2.4−11 −5)	**0.84** (0.5−0.9)	**3.38** (1.1−6.1)
Pn-3-gal	**2.22** (1.9−2.5)	**ND**	**ND**	**3.26** (1.9−4.5)	**ND**	**ND**	**3.8** (2.1−6.9)	**ND**	**ND**	**1.72** (0.65−6.4)	**ND**	**ND**
Pn-3-glu	**4.79** (2.7−7.9)	**ND**	**0.51** (0.5−1.0)	**6.7** (4.7−8.8)	**ND**	**0.47** (0.4−0.49)	**8.72** (3.8−14.3)	**ND**	**0.58** (0.5−0.6)	**11.5** (1.2−21.6)	**ND**	**0.47** (0.4−0.48)
Mv-3-gal	**5.77** ### (5.1−9.0)	**ND**	**0.92** (0.6−0.93)	**11.5** (6−13.9)	**ND**	**0.73** (0.65−0.81)	**13.7** ## (6.2−20.9)	**ND**	**0.75** (0.5−1.3)	**8.06** (2.6−27.4)	**ND**	**0.65** (0.6−0.9)
Mv-3-glu	**10.7** ### (9.8−20.4)	**0.4** (0.3−0.41)	**1.17** (0.6−1.6)	**24.1** ### (11.8−26.4)	**0.44** (0.4−0.5)	**0.67** (0.5−1.2)	**27.0** ### (12.8−39)	**0.45** (0.41−0.5)	**0.94** (0.6−1.3)	**12.3** ## (3.7−49.3)	**0.5** (0.4−0.56)	**0.71** (0.47−1.0)
Mv-3-arab	**3.9** ** (3.8−7.4)	**0.98** (0.6−1.4)	**8.7** *** (5.9−13.2)	**7.7** ** (3.9−9.2)	**2.1** (0.9−2.9)	**13.7** *** (6.6−17.7)	**9.3** ** (3.9−13.3)	**1.2** (0.8−1.7)	**13.7** *** (10.5−17.2)	**5.8** (3.1−16.5)	**0.95** (0.9−1.6)	**10.5** (5.7−14.1)

[a]*, **, *** denote $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to the cecum for the same compound and group; #, ##, ### denote $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to the colon for the same compound and group. Values are expressed as medians (25th and 75th percentile).

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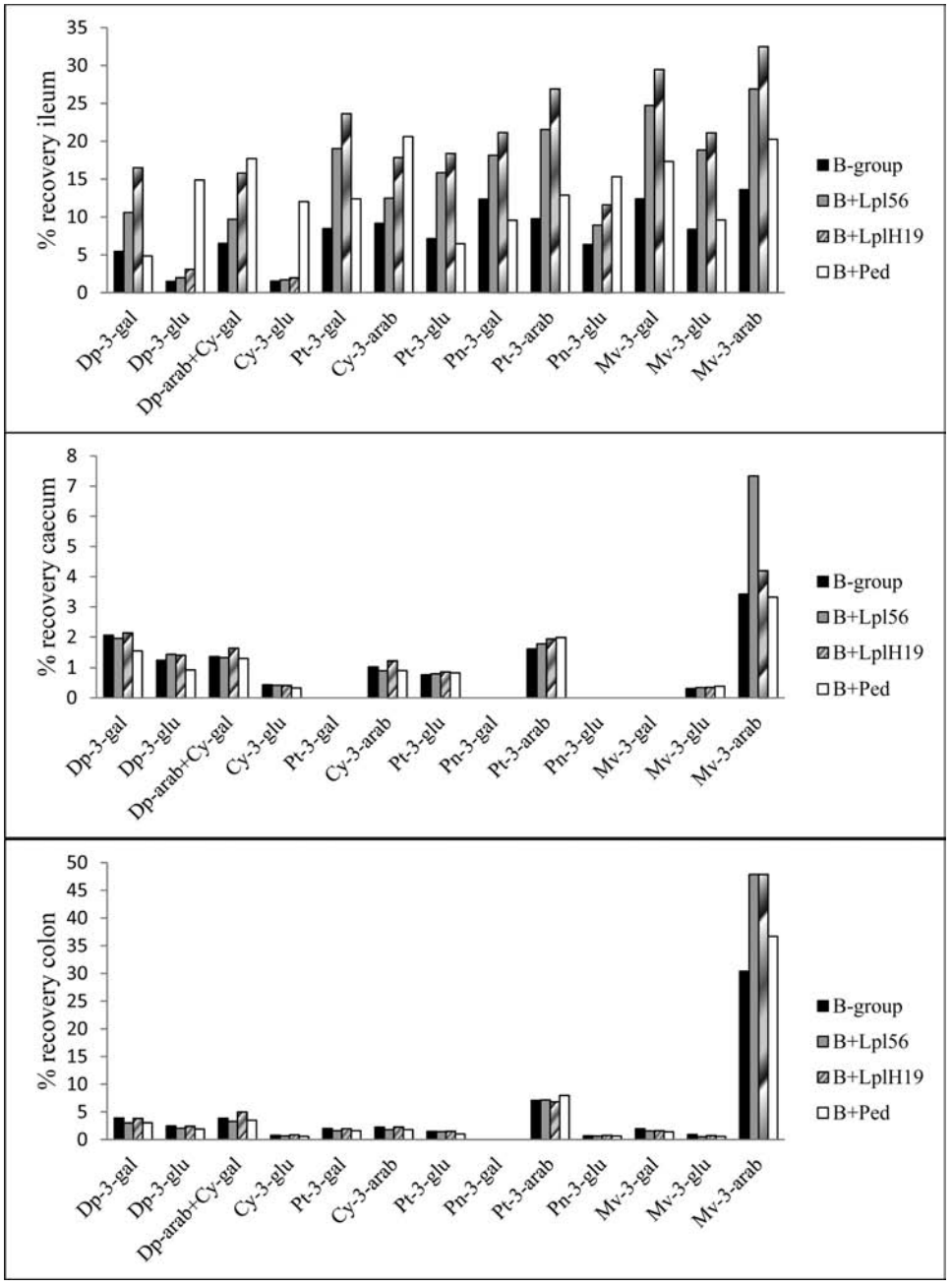

Figure 5. Recovery (%) of different anthocyanins from ileum, cecum, and colon of mice fed bilberry (B), alone or in combination with a lactic acid bacterium: either *Lactobacillus plantarum* RESO56 (Lpl56), *Lactobacillus plantarum* HEAL19 (LplH19), or *Pediococcus acidilactici* JAM046 (Ped).

together with a single LAB strain. The significant MDA increase in the ileum was seen when *L. plantarum* RESO56 was added to the bilberry compared to the bilberry alone (Figure 1). Inability of bilberry in combination with LplH19, Lpl56, or Ped to reduce MDA in the ileum may be due to the aerobic activity of these LAB. When exposed to high oxygen levels during reperfusion of previously oxygen-deprived tissue, *L. plantarum* may overproduce hydrogen peroxide (H_2O_2) from O_2 via NADH-dependent oxidase or pyruvate oxidase, causing oxidative stress.[32,33] Furthermore, gastrointestinal mucosa contains large numbers of phagocytes within the lamina propria.[4] According to Schriffrin et al. administration of probiotic yogurt containing *Bifidobacterium bifidum* and *Lactobacillus acidophilus* activated and increased phagocytic activity in the ileum. In order to destroy invasive bacteria, activated phagocytes produce ROS such as H_2O_2 and superoxide (O_2^-), which could further contribute to the increased oxidative stress and hence lipid peroxidation and inflammation in the ileum upon LAB administration with bilberry. It should be pointed out that there are physiological and environmental differences between the small intestine and colon; for example, the oxygen tension is higher in the small intestine than in the colon. It has been suggested that the small intestine is more susceptible to ischemia−reperfusion injury than the colon.[35]

Histological damage that occurs after intestinal I/R is characterized by shortening and loss of intestinal villi, necrosis, and neutrophil infiltration.[27] In the present study, pretreatment with bilberry (B group) most efficiently preserved the mucosal integrity and morphology by showing the lowest injury score

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and minimizing the tissue injury. This coincides with the observations that the B group also showed a lower MDA value in the ileum than the LAB groups but significantly lower than the B+Lpl56 group (Figure 1). Recent *in vitro* studies[36,37] have shown that anthocyanins offered cell protection and anti-inflammatory activity by upregulation of the HO-1 gene in the oxidative stress model, preventing ROS-induced cell injury (necrosis and apoptosis), and by inhibiting the expression and secretion of pro-inflammatory mediators associated with, for example, inflammatory bowel disease.

The gut microbiota can transform blueberry polyphenols, producing more easily absorbed and more physiologically active metabolites.[38,39] In turn, some polyphenols and their metabolites may influence the composition and function of gut microflora.[11,39] It has been shown that unabsorbed polyphenols and their metabolites can selectively inhibit the growth of pathogens and stimulate the growth of lactobacilli and bifidobacteria.[11,39,40] In this study, the lactobacilli count was not significantly changed by bilberry diet. However, addition of *L. plantarum* RESO56 and *P. acidilactici* JAM046 to bilberry diet seemed to be more efficient in keeping a stable lactobacilli count in cecal tissue than *L. plantarum* HEAL19 and bilberry alone. *Lactobacillus* strains used in the present study were frequently reisolated from the cecum of mice fed a diet supplemented with polyphenol-rich rosehips together with these strains, which indicates their survival and tolerance toward polyphenols.[26] In the same study, *L. plantarum* HEAL19 was less frequently reisolated compared to *L. plantarum* RESO56.[26] It appears that some species or strains of lactobacilli may be more susceptible to polyphenols than others. Results from different studies suggest that several polyphenols, of which some are present in bilberry, showed concentration-dependent inhibitory effects on the growth of *L. plantarum* and reduced adhesion ability of probiotic *L. rhamnosus* to a human gut cell line.[41,42]

In the present study, no significant differences in bacterial diversity were seen. The T-RFLP results did not suggest that cecal microbiota was altered by intestinal I/R injury. However, supplementing the diet with bilberry seems to have changed the composition of the cecal microbiota (Figure 4). There is evidence that consumption of polyphenols may influence the intestinal microbes.[40,43−46] Anthocyanin-rich berries showed *in vitro* ability to inhibit pathogenic bacteria such as *Staphylococcus* spp., *Salmonella* spp., *Helicobacter pylori*, and *Bacillus cereus*.[11]

The bilberry powder had a relatively complex anthocyanin profile consisting of 14 different anthocyanins. The intestinal contents of mice fed bilberry had a dark purple to black color, suggesting a high concentration of anthocyanins in the gut. The highest concentrations of anthocyanins were found in the ileum followed by the colon and finally the cecum. Borges et al. showed that up to 4 h after ingestion of raspberry juice in rats the anthocyanins were found in the highest concentrations in the ileum followed by the colon and cecum. The rational conclusion for this is that the anthocyanins from the ileal content are degraded to their metabolites in the cecum, and the concentration increases in the colon because the nonabsorbed and nondegraded fraction of anthocyanins ends up here.

All anthocyanins detected in the bilberry powder were also recovered from the ileum content of the mice fed bilberry diets. In the cecum and colon 10 and 13 anthocyanins, respectively, were detected. In the bilberry powder, Cy and Dp glycosides were present in the highest concentration and represented 67% of total anthocyanins. In the ileum, higher recoveries were observed for Mv, Pn, and Pt glycosides than for Dp and Cy glycosides. An especially low recovery in the ileum was seen for the Cy-3-glu. The lower recovery of Cy and Dp glycosides may be due to several factors such as the methylation of these anthocyanins in the small intestine resulting in the yield of Pn, Pt, and Mv glycosides under physiological conditions as suggested by others,[12,47] the higher uptake from the stomach and upper GI tract, or metabolism by bacteria and enzymes in the small intestine. Passamonti et al. have shown that anthocyanins, and especially Cy-3-glu, have the ability to permeate the gastric mucosa probably through a bilitranslocase-mediated mechanism. Furthermore, it has been shown that anthocyanins have been efficiently absorbed from the small intestine, with the highest absorption occurring in the jejunum and duodenum, while no absorption occurred from the ileum and colon.[14,48,49] It has been proposed that lactase-phlorizin hydrolase, which is located in the brush border membrane of mature small intestine enterocytes, and β-glucosidase activity of small intestinal microbiota are responsible for hydrolysis and degradation of anthocyanins, and mainly Cy-3-glu, in the small intestine.[49,50]

In the cecum and colon, glucosides and galactosides of Mv, Pn, and Pt showed the lowest recoveries. Pn-3-gal was not detected in the colon and cecum, and additionally Pn-3-glu, Mv-3-gal, and Pt-3-gal were not detected in the cecum. Increased proportions of anthocyanin arabinosides and especially Mv-3-arab were observed. Gastrointestinal uptake and stability of anthocyanins are influenced by the attached sugar moiety. Galactosides and glucosides are, compared with arabinosides, absorbed more efficiently and are also less resistant to degradation by intestinal microbiota.[47,51] In the present study, the disappearance and low concentrations of Mv, Pt, and Pn glucoside and galactoside in the cecum compared to the ileum suggest that they may have been degraded by cecal microflora or absorbed by the animal.

There were no significant differences in the anthocyanin profile and concentrations between groups fed bilberry alone and those supplemented with different LAB strains. Higher concentrations of some anthocyanins in B+Lpl56 and B+LplH19 and in some cases B+Ped compared to the other groups in the ileum are due to the interindividual differences within the same group, with extremely high or low values in some individuals. Besides possible variations in individual microbiota, an important factor for the concentration differences may be that time for the last intake of bilberry-supplemented feed before sample collection differed between the animals, since they had free access to feed during the present study. According to Borges et al., the concentration and recoveries of anthocyanins in the ileum after ingestion of a polyphenol-rich diet are the highest within the first 2−4 h and then they gradually decline.

In summary, a food supplement of bilberry protected against inflammation, oxidative stress, and tissue injury caused by ischemia−reperfusion, and the addition of LAB to the diet did not improve the ileal status. The daily intake of bilberry for 10 days and accumulation of anthocyanins in the intestines may have contributed to the antioxidative and anti-inflammatory protection of the B group. The highest concentration and recovery of anthocyanins were seen in the ileum followed by the colon and finally the cecum. Accumulation of anthocyanin arabinosides, and especially Mv-arab, in the colon confirms that these glycosides were the most resistant to absorption and microbial degradation. Cy-3-glu and Dp-3-glu seemed to be

mostly absorbed in the stomach and upper part of the small intestine, while Mv-3-gal, Pn-3-glu, Pn-3-gal, and Pt-3-gal seemed to be among the ones most efficiently digested by the microbiota in the cecum. The bilberry supplementation strongly influenced the cecum microbiota, while no clear differences could be seen by the additional supplementation with different strains of LAB. However, the composition of the microbiota differed widely between different individuals irrespectively of diet supplementation.

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Notes

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ABBREVIATIONS

cfu, colony-forming units; bp, base pair; DAD, diode array absorbance detector; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; MS, mass spectroscopy; UV−vis, ultraviolet−visible light; GI, gastrointestinal; ANOVA, analysis of variance; SEM, standard error of the mean

REFERENCES

(1) Cerqueira, N. F.; Hissni, C. A.; Yoshida, W. B. Pathophysiology of mesenteric ischemia/reperfusion: A review. *Acta Circ. Bras.* **2005**, *20*, 336−343.

(2) Ozkan, O. V.; Yuzbasioglu, M. F.; Ciralik, H.; Kurutas, E. B.; Yonden, Z.; Aydin, M.; Bulbuloglu, E.; Semerci, E.; Goksu, M.; Atli, Y.; Bakan, V.; Duran, N. Resveratrol, a natural antioxidant, attenuates intestinal ischemia/reperfusion injury in rats. *Tahoku J. Exp. Med.* **2009**, *218*, 251−258.

(3) Leung, F. W.; Su, K. C.; Pique, J. M.; Thiefin, G.; Passaro, E.; Guth, P. H. Superior mesenteric artery is more important than inferior mesenteric artery in maintaining colonic mucosal perfusion and integrity in rats. *Dig. Dis. Sci.* **1992**, *37*, 1329−1335.

(4) Grisham, M. B.; Granger, D. N. Neutrophil-mediated mucosal injury. Role of reactive oxygen metabolites. *Dig. Dis. Sci.* **1988**, *33*, 6S−15S.

(5) Jakesevic, M.; Aaby, K.; Borge, A.; Jeppsson, G.-I.; Ahrné, B.; Molin, S. G. Antioxidative protection of dietary bilberry, chokeberry and *Lactobacillus plantarum* HEAL19 in mice subjected to intestinal oxidative stress by ischemia-reperfusion. *BMC Complementary Altern. Med.* **2011**, *11*, 8.

(6) Bao, L.; Yao, X. S.; Yau, C. C.; Tsi, D.; Chia, C. S.; Nagai, H.; Kurihara, H. Protective effects of bilberry (*Vaccinium myrtillus* L.) extract on restraint stress-induced liver damage in mice. *J. Agric. Food Chem.* **2008**, *56*, 7802−7807.

(7) Bao, L.; Yao, X. S.; Tsi, D.; Yau, C. C.; Chia, C. S.; Nagai, H.; Kurihara, H. Protective effects of bilberry (*Vaccinium myrtillus* L.) extract on $KBrO_3$-induced kidney damage in mice. *J. Agric. Food Chem.* **2008**, *56*, 420−425.

(8) Yao, N.; Lan, F.; He, R.-R.; Kurihara, H. Protective effects of bilberry (*Vaccinium myrtillus* L.) extract against endotoxin-induced uveitis in mice. *J. Agric. Food Chem.* **2010**, *58*, 4731−4736.

(9) Tsuda, T.; Horio, F.; Kitoh, J.; Osawa, T. Protective effects of dietary cyanidin-3-O-*β*-D-glucoside on liver ischemia-reperfusion injury in rats. *Arch. Biochem. Biophys.* **1999**, *368*, 361−366.

(10) Wang, H.; Nair, M. G.; Strasburg, G. M.; Chang, Y.-C.; Booren, A. M.; Gray, J. I.; DeWitt, D. L. Antioxidant and anti-inflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *J. Nat. Prod.* **1999**, *62*, 294−296.

(11) Puupponen-Pimiä, R.; Nohynek, L.; Hartmann-Schmidlin, S.; Kähkönen, M.; Heinonen, M.; Määttä-Riihinen, K.; Oksman-Caldentey, K.-M. Berry phenolics selectively inhibit the growth of intestinal pathogens. *J. Appl. Microbiol.* **2005**, *98*, 991−1000.

(12) Borges, G.; Roowi, S.; Rouanet, J.-M.; Duthie, G. G.; Lean, M. E. J.; Crozier, A. The bioavailability of raspberry anthocyanins and ellagitannins in rats. *Mol. Nutr. Food Res.* **2007**, *51*, 714−725.

(13) Passamonti, S.; Vrhovsek, U.; Vanzo, A.; Mattivi, F. The stomach as a site for anthocyanins absorption from food. *FEBS Lett.* **2003**, *544*, 210−213.

(14) Matuschek, M. C.; Hendriks, W. H.; McGhie, T. K.; Reynolds, G. W. The jejunum is the main site of absorption for anthocyanins in mice. *J. Nutr. Biochem.* **2006**, *17*, 31−36.

(15) Talavéra, S.; Felgines, C.; Texier, O.; Besson, C.; Gil-Izquierdo, A.; Lamaison, J.-L.; Rémésy, C. Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. *J. Agric. Food Chem.* **2005**, *53*, 3902−3908.

(16) Del Bò, C.; Ciappellano, S.; Klimis-Zacas, D.; Martini, D.; Gardana, C.; Riso, P.; Porrini, M. Anthocyanin absorption, metabolism, and distribution from wild blueberry-enriched diet (*Vaccinium angustifolium*) is affected by diet duration in the Sprague-Dawley rat. *J. Agric. Food Chem.* **2010**, *58*, 2491−2497.

(17) Fleschhut, J.; Kratzer, F.; Rechkemmer, G.; Kulling, E. S. Stability and biotransformation of various dietary anthocyanins *in vitro*. *Eur. J. Nutr.* **2005**, *45*, 7−18.

(18) Barthelmebs, L.; Divies, C.; Cavin, J.-F. Knockout of the *p*-coumarate dexarboxylase gene from *Lactobacillus plantarum* reveals the existence of two other inducible enzymatic activities involved in phenolic acid metabolism. *Appl. Environ. Microbiol.* **2000**, *66*, 3368−3375.

(19) Osawa, R.; Kuroiso, K.; Goto, S.; Shimizu, A. Isolation of tannin-degrading lactobacilli from humans and fermented foods. *Appl. Environ. Microbiol.* **2000**, *66*, 3093−3097.

(20) Rolfe, R. D. The role of probiotic cultures in the control of gastrointestinal health. *J. Nutr.* **2000**, *130*, 396S−402S.

(21) Lin, M.-Y.; Yen, C.-L. Antioxidative ability of lactic acid bacteria. *J. Agric. Food Chem.* **1999**, *47*, 1460−1466.

(22) Muiá, C.; Mazzon, E.; Di Paola, R.; Genovese, T.; Menegazzi, M.; Caputi, A. P.; Suzuki, H.; Cuzzocrea, S. Green tea polyphenol extract attenuates ischemia/reperfusion injury of the gut. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2005**, *371*, 364−374.

(23) Nardone, G.; Compare, D.; Liguori, E.; Di Mauro, V.; Rocco, A.; Barone, M.; Napoli, A.; Lapi, D.; Iovene, M. R.; Colantuoni, A. Protective effects of *Lactobacillus paracasei* F19 in a rat model of oxidative and metabolic hepatic injury. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2010**, *299*, G669−G676.

(24) Xing, H. C.; Li, L. J.; Xu, K. J.; Shen, Y.; Chen, Y. B.; Sheng, J. F.; Chen, Y.; Fu, S. Z.; Chen, C. L.; Wang, J. G.; Yan, D.; Dai, F. W.; Zheng, S. S. Protective role of supplement with foreign *Bifidobacterium* and *Lactobacillus* in experimental hepatic ischemia-reperfusion injury. *J. Gastroenterol. Hepatol.* **2006**, *21*, 647−656.

(25) Håkansson, Å.; Stene, C.; Mihaescu, A.; Molin, G.; Ahrné, S.; Thorlacius, H.; Jeppsson, B. Rosehip and *Lactobacillus plantarum* DSM 9843 reduce ischemia-reperfusion injury in the mouse colon. *Dig. Dis. Sci.* **2006**, *51*, 2094−2101.

(26) Jakesevic, M.; Håkansson, Å.; Adawi, D.; Jeppsson, B.; Rumpunen, K.; Ekholm, A.; Ahrné, S.; Molin, G. Antioxidative protection of dietary rosehips and polyphenol active lactobacilli in

mice subjected to intestinal oxidative stress by ischemia-reperfusion. *Microb. Ecol. Health Dis.* **2009**, *21*, 193−202.
(27) Chiu, C. J.; McArdle, A. H.; Brown, R. Intestinal mucosal lesions in low-flow states. A morphological, hemodynamic, and metabolic reappraisal. *Arch. Surg.* **1970**, *101*, 478−483.
(28) Karlsson, C.; Ahrné, S.; Molin, G.; Berggren, A.; Palmquist, I.; Fredrikson-Nordin, G.; Jeppsson, B. Probiotic therapy to men with incipient arteriosclerosis initiates increased bacterial diversity in colon: A randomized controlled trial. *Atherosclerosis* **2010**, *208*, 228−233.
(29) Laaksonen, O.; Sandell, M.; Kallio, H. Chemical factors contributing to orosensory profiles of bilberry (*Vaccinium myrtillus*) fractions. *Eur. Food Res. Technol.* **2010**, *231*, 271−285.
(30) Kähkönen, M. P.; Heinämäki, J.; Ollilainen, V.; Heinonen, M. Berry anthocyanins: isolation, identification and antioxidant activities. *J. Sci. Food Agric.* **2003**, *83*, 1403−1411.
(31) Zheng, W.; Wang, S. Y. Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries and lingonberries. *J. Agric. Food Chem.* **2003**, *51*, 502−509.
(32) De Angelis, M.; Gobbetti, M. Environmental stress responses in *Lactobacillus*: A review. *Proteomics* **2004**, *4*, 106−122.
(33) Murphy, M. G.; Condon, S. Correlation of oxygen utilization and hydrogen peroxide accumulation with oxygen induced enzymes in *Lactobacillus plantarum* cultures. *Arch. Microbiol.* **1984**, *138*, 44−48.
(34) Schriffrin, E. J.; Rochat, F.; Link-Amster, H.; Aeschlimann, J. M.; Donnet-Hughes, A. Immunomodulation of human blood cells following ingestion of lactic acid bacteria. *J. Dairy Sci.* **1995**, *78*, 491−497.
(35) Leung, F. W.; Su, K. C.; Passaro, E.; Guth, P. H. Regional differences in gut blood flow and mucosal damage in response to ischemia-reperfusion. *Am. J. Physiol.* **1992**, *263*, G301−305.
(36) Zhang, B.; Kang, M.; Xie, Q.; Xu, B.; Sun, C.; Chen, K.; Wu, Y. Anthocyanins from Chinese bayberry extract protect β cells from oxidative stress-mediated injury via HO-1 upregulation. *J. Agric. Food Chem.* **2011**, *59*, 537−545.
(37) Triebel, S.; Trieu, H.-L.; Richling, E. Modulation of inflammatory gene expression by a bilberry (*Vaccinium myrtillus* L.) extract and single anthocyanins considering their limited stability under cell culture conditions. *J. Agric. Food Chem.* **2012**, *60*, 8902−8910.
(38) Russell, W. R.; Labat, A.; Scobbie, L.; Duncan, S. Availability of blueberry phenolics for microbial metabolism in the colon and the potential inflammatory implications. *Mol. Nutr. Food Res.* **2007**, *51*, 726−731.
(39) Hidalgo, M.; Oruna-Concha, M. J.; Kolida, S.; Walton, G. E.; Kallithraka, S.; Spencer, J. P. E.; Gibson, G. R.; de Pascual-Teresa, S. Metabolism of anthocyanins by human gut microflora and their influence on gut bacterial growth. *J. Agric. Food Chem.* **2012**, *60*, 3882−3890.
(40) Lacombe, A.; Wu, V. C. H.; White, J.; Tadepalli, S.; Andre, E. E. The antimicrobial properties of the lowbush blueberry (*Vaccinium angustifolium*) fractional components against foodborne pathogens and the conservation of probiotic Lactobacillus rhamnosus. *Food Microbiol.* **2012**, *30*, 124−131.
(41) Rodríguez, H.; Curiel, J. A.; Landete, J. M.; De Las Rivas, B.; López de Felipe, F.; Gómez-Cordovés, C.; Mancheño, J. M.; Muñoz, R. Food phenolics and lactic acid bacteria. *Int. J. Food Microbiol.* **2009**, *132*, 79−90.
(42) Parkar, S. G.; Stevenson, D. E.; Skinner, M. A. The potential influence of fruit polyphenols on colonic microflora and human gut health. *Int. J. Food Microbiol.* **2008**, *124*, 295−298.
(43) Smith, A. H.; Mackie, R. I. Effect of condensed tannins on bacterial diversity and metabolic activity in rat gastrointestinal tract. *Appl. Environ. Microbiol.* **2004**, *70*, 1104−1115.
(44) Tzounis, X.; Vulevic, J.; Kuhnle, G. G. C.; George, T.; Leonczak, J.; Gibson, G. R.; Kwik-Uribe, C.; Spencer, J. P. E. Flavanol monomer-induced changes to the human faecal microflora. *Br. J. Nutr.* **2008**, *99*, 782−792.
(45) Hara, H.; Orita, N.; Hatano, S.; Ichikawa, H.; Hara, Y.; Matsumoto, N.; Kimura, Y.; Terada, A.; Mitsuoka, T. Effect of tea polyphenols on fecal flora and fecal metabolic products of pigs. *J. Vet. Med. Sci.* **1995**, *57*, 45−49.
(46) Dolara, P.; Luceri, C.; De, F. C.; Femia, A. P.; Giovannelli, L.; Caderni, G.; Cecchini, C.; Silvi, S.; Orpianesi, C. Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. *Mutat. Res.* **2005**, *591*, 237−246.
(47) Ichiyanagi, T.; Shida, Y.; Rahman, M. M.; Hatano, Y.; Konishi, T. Bioavailability and tissue distribution of anthocyanins in bilberry (*Vaccinium myrtillus* L.) extract in rats. *J. Agric. Food Chem.* **2006**, *54*, 6578−6587.
(48) Talavéra, S.; Felgines, C.; Texier, O.; Besson, C.; Manach, C.; Lamaison, J.-L.; Rémésy, C. Anthocyanins are efficiently absorbed from the small intestine in rats. *J. Nutr.* **2004**, *134*, 2275−2279.
(49) He, J.; Wallace, T. C.; Keatley, K. E.; Failla, M. L.; Giusti, M. M. Stability of black raspberry anthocyanins in the digestive tract lumen and transport efficiency into gastric and small intestinal tissues in the rat. *J. Agric. Food Chem.* **2009**, *57*, 3141−3148.
(50) Tsuda, T.; Horio, F.; Osawa, T. Absorption and metabolism of cyanidin-3-O-β-D-glucoside in rats. *FEBS Lett.* **1999**, *449*, 179−182.
(51) He, J.; Magnuson, B. A.; Giusti, M. M. Analysis of anthocyanins in rat intestinal contents−impact of anthocyanin chemical structure on fecal excretion. *J. Agric. Food Chem.* **2005**, *53*, 2859−2866.