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Analysis of Microbial Populations and Metabolism of Anthocyanins by Mice Gut Microflora Fed with Blackberry Powder

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Abstract

Blackberries contain non-nutritive phytochemicals which are abundant in berry fruits. Among these chemicals, anthocyanins are responsible for the bright colors seen in many fruits and plant as well as have potential health properties against chronic diseases like cancer, cardiovascular disease, diabetes, and obesity. Therefore, two anthocyanins, cyanidin-3-glucoside and cyanidin-3-rutinoside were adopted to evaluate metabolism rate by cecal microflora which were acquired from four treatments 1) lean control (L-CTL), 2) obese control (O-CTL), 3) aged blackberries supplemented at 10% (w/w) (O-AB10), and 4) fresh blackberries supplemented at 10% (w/w) (O-FB10). Furthermore, a non-culture based technique; polymerase chain reaction-based denaturing gradient gel electrophoresis (PCR-based DGGE) and further sequencing were performed to identify microbial populations. There were differences in the metabolism rates of two anthocyanins (cyanidin-3-glucoside and cyanidin-3-rutinoside) between two control groups and two treatment groups while no differences were shown within two control groups. The sequencing results based on DGGE represented that *Lachnospiraceae* bacteria found predominantly in blackberry treatment mice, *Lactobacillus johnsonii* was dominant species in both control and treatment groups. *Bifodobacterium pseudolongum* was identified in only obese control samples. In conclusion, differences in diets and body phenotype can have an effect on gut microflora and subsequently affect the metabolism rate of anthocyanins. Also, blackberry treatment groups demonstrated a more rapid anthocyanin degradation rate than the un-adapted control groups.

Keywords: Blackberry; Anthocyanin; PCR-based DGGE

Introduction

Anthocyanins are a group of naturally occurring phenolic compounds found in plants, and are responsible for the bright colors seen in many fruits, vegetables, and grains [1]. Recently, there has been much interest in the potential health properties and biological activities of anthocyanins, namely in the protection of chronic diseases like cancer, cardiovascular disease, atherosclerosis, diabetes, and obesity [1-3]. In order to understand the potential health effect of anthocyanins, information on their absorption, distribution, metabolism, and excretion must be established. Furthermore, anthocyanins in berry fruits are easily degraded by several environmental factors such as oxygen, light, enzyme, and the presence of beta-glucosidases in the small intestine of humans and animals [4,5]. As a result, aged blackberries represented less bioavailability than fresh ones [4]. There have been several studies conducted which demonstrate that intact anthocyanins are poorly absorbed [6,7]. These studies and others showed that some absorbed anthocyanins were metabolized into sulphated and glucuronidated forms in addition to bacterial metabolism by colonic flora [5,8]. The deconjugating enzyme activities of colon bacteria release aglycones of flavonoids from their glycosides and glucuronides, but the appearance of aglycones is only transient before ring fission occurs and smaller phenolic acids are released [9].

Blackberry contains a large number of phenolic compounds including anthocyanins and ellagitannins [10,11]. Fan-Chiang and Wrolstad [11] reported the anthocyanin content of blackberry-cyanidin 3-glucoside (44-95%), cyanidin 3-rutinoside (trace amounts~53%), cyanidin 3-xyloside (~11%), cyanidin 3-malonyl-glucoside (~5%), and cyanidin 3-dioxolane-glucose (~15%). The major anthocyanins in blackberry are cyanidin 3-glucoside and cyanidin 3-rutinoside.

Different microbial populations in the gut such as *Bacteroidetes* and *Firmicutes* have been explored as possible explanations for increased capacity for energy harvest and obesity in humans [12,13]. In addition to energy harvest, it is also possible that different gut microflora may be more efficient in metabolizing anthocyanins than others, which may explain individual differences in anthocyanins metabolism. Numerous microbial populations in the gastrointestinal tract can metabolize anthocyanin fragments into smaller stable compounds which have anti-inflammatory properties [14,15]. Therefore, the diverse and complex microbial communities in the gut are important for digestion as well as impact many aspects of host health [16].

Previous studies have used the polymerase chain reaction based denaturing gradient gel electrophoresis (PCR-based DGGE) to analyze and compare various microbial populations taken from feces and gut samples [17]. Common regions of the 16S ribosomal DNA (rDNA) sequences are targeted, amplified, and separated on a gel by containing various denaturant concentrations. With this method, amplicons are separated based on their G+C contents and the banding patterns can be used to determine differences between groups. Additionally, bands of interest on a gel can be excised for DNA sequencing to identify specific species.

The hypothesis of the study was that diets with blackberries modulate the composition of the gut microflora to increase degradation of anthocyanins. The aim of this study was to investigate the differences

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in bacterial metabolism of the two anthocyanins (cyainidin-3-glucoside and cyanidin-3-rutinoside) using cecum contents of lean and obese mice fed with rodent diets including blackberries. The disappearance of these two anthocyanins was demonstrated by *in vitro* fermentation and analyzed the resulting *in vitro* products over time using high performance liquid chromatography (HPLC). Furthermore, the culture independent technique, PCR-based DGGE was utilized to analyze the microflora profiles used for anthocyanins metabolism. Finally, selected bands based on DGGE were sequenced to identify specific bacteria.

Materials and Methods

Chemicals and reagents

HPLC grade acetonitrile, methanol, trifluoroacetic acid, tetrahydrofuran, and all other chemicals were purchased from VWR Scientific Products Inc (Fayetteville, AR, USA). The Milli-Q system (Millipore Co., Bedford, MA, USA) for HPLC grade water was used to prepare all aqueous solutions and HPLC solvents. Resorcinol, 4-hydroxyphenylacetic acid and boron trifluoride etherate used in the synthesis of 2,4,4'-trihydroxydeoxybenzoin (THB) were purchased from Alfa Aesar (Ward Hill, MA, USA). Two anthocyanins, cyainidin-3-glucoside and cyanidin-3-rutinoside, were purchased from Quality Phytochemicals LLC (Edison, NJ, USA). A cell culture grade DMSO was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Mice studies

Six weeks old female obese (BKS(D)-Lepr^{db}/J72) and lean (C57BL/6J) mice were purchased from Jackson Laboratory. (Bar Harbor, ME, USA). Each treatment including six mice was housed to a 14"×8" cage containing 100% cellulose fiber bedding which was autoclaved prior to use. There were a total of four treatments in the experiment: lean control (L-CTRL), obese control (O-CTRL), obese mice with aged blackberries supplemented at 10% (O-AB10), and obese mice with fresh blackberries supplemented at 10% (O-FB10). Each treatment group was performed in duplicate with 12 female mice. Both control groups were composed entirely of the lean and obese phenotype mice fed with AIN-93M purified rodent diet. Whole freeze-dried blackberries (Watershed Foods, Gridley, IL, USA) were purchased and ground into powders using a Robot Coupe RSI 6V food processor (Robot Coupe USA Inc., Joliet, IL, USA). The two experimental treatments consisting of AIN-93M purified rodent diet with aged or fresh blackberries at a level of 10% (w/w) fed to groups of mice displaying the obese phenotype. Aged blackberries were prepared after 2 months of storage at 4°C. The treatment groups utilizing obese mice were fed to the mean food intake of the lean control group which had *ad libitum* access to food. All groups had unrestricted access to deionized water through the duration of the study. After 10 weeks of treatment, the mice were fasted for 12 h before being sacrificed by exsanguination via cardiac puncture. The cecum was removed from the mice after sacrifice and transferred to an anaerobic chamber immediately.

In vitro anaerobic cecum incubations

Brain heart infusion (BHI) media (DIFCO Laboratories, Detroit, MI, USA) consisted of 3.7 g BHI, 400 mg of Na_2CO_3, 25 mg of cysteine sulfide (reducing agent), 0.1 mg of resazurin (Aldrich Chemical, Milwaukee, WI, USA), and filled up to 100 ml with distilled water. The media was autoclaved at 121°C for 15 min before experimental use. Under strictly anaerobic conditions, the cecum contents were deposited into CO_2 flushed tubes containing BHI media along with 5 mg of cyainidin-3-glucoside or cyanidin-3-rutinoside which were dissolved in DMSO. The cecum contents of 3 mice were pooled for each group (averaging approximately 400 mg) and added to 8 ml of BHI media. Before being moved to a 37°C incubator, a 1 ml of sample was drawn from the tube using a sterile syringe and transferred to a 2 ml microcentrifuge tube, designated as time point 0, and immediately stored at -80°C for later analysis. Subsequent aliquots were anaerobically obtained at 0.5, 1, 2, 4, 6, and 8 h and stored at -80°C for later analysis.

Anthocyanin extractions

Anthocyanin extractions were performed using a modified method from Hassimotto et al. [18]. Briefly, samples were centrifuged at 16,000 g for 10 min at 4°C and supernatant was transferred to a fresh tube. The supernatant was loaded along with the internal standard THB on a 1 g polyamide column (CC 6; Macherey-Nagel, Germany) which had been equilibrated with 20 ml of methanol followed by 60 ml of distilled water. Impurities were removed with 20 ml of distilled water and the retained anthocyanins were eluted with methanol containing 0.1% of HCl. The anthocyanin containing fractions were evaporated under reduced pressure at 30°C. Samples were dissolved in 200 µl of methanol: acetic acid (95:5) and filtered through a 0.45 µm of PTFE (Polytetrafluoroethylene) filter into HPLC vials.

Anthocyanin analysis using HPLC

The Beckman System Gold HPLC (Beckman, Fullerton, CA, USA) with auto-sampler (model 508), dual pump (model 126), photodiode array detector (model 168), and Beckman System 32 Karat software (version 8) was used to analyze anthocyanin extraction samples. A 50 µl of the sample was injected into the Prodigy 5 µ ODS-3 reversed phase silica (250×4.6 mm ID; Phenomenex Ltd, Torrance, CA, USA) at ambient temperature. The HPLC solvents were composed of both water/tetrahydrofuran/trifluoroacetic acid (WTT, 98:2:0.1, v/v) and acetonitrile. Following injection of 50 µl of sample, the acetonitrile was increased from 4 to 20% in 6 min, the solvent flow rate was reduced from 1 to 0.5 ml/min in 1 min while acetonitrile was increased from 20 to 50% over 20 min. Solvent flow was returned to 1 ml/min, acetonitrile increased to 90% in 2 min, and returned to 4% over the next 6 min. A Beckman photodiode array detector (model 168) was monitored from 200 to 600 nm.

DNA extraction from cecum

Bacterial DNA from cecal samples was extracted using QIAamp DNA stool kit (Qiagen, Valencia, CA, USA) with some modification to increase yields. A 250 mg of cecum was lysed by vortexing using Garnet beads (MO BIO, Carlsbad, CA, USA). The lysed sample was centrifuged at 3,000 g for 1 min and transferred supernatant to bead beating tube (MO BIO). The sample was further lysed by vortexing for 10 min and incubated at 95°C for 6 min. After incubation, the sample was centrifuged at 16,000 g for 1 min and the supernatant was further processed using the kit according to manufacturer's instruction.

PCR-based DGGE 155

All DNA amplifications and DGGE procedures were performed as previously described with modification [19]. Briefly, a 240-bp portion of the 16s rRNA gene was amplified by PCR using primers of DGGE-Hume-F (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCCTAC GGG AGG CAG CAG-3') and DGGE-Hume-R (5-ATT ACC GCG GCT GCT GG-3) (Integrated DNA Technologies, Coralville, IA, USA) [20]. A 50 ng of template DNA, 0.4 µM of primer pair and PCR grade water were mixed with Jump Start Ready Mix (Sigma Chemical Co., St Louis, MO, USA) to total of 25

µl. The amplification was performed by MJ thermocycler consisted of initial denaturation at 95°C for 2 min, then 17 cycles of denaturation at 94°C for 1 min, annealing at 67°C for 45 s decreasing by -0.5°C per cycle to a touchdown temperature of 59°C, and annealing at 72°C for 2 min. Then the reaction followed with 12 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 45 s with a final elongation step at 72°C for 7 min. The products were electrophoresed on 1.5% of agarose gel and visualized using ultraviolet transilluminator (Bio-Rad Laboratories, Richmond, CA, USA).

The 8% polyacrylamide gel consisted of a 35 to 60% of urea-deionized formamide gradient was prepared to perform PCR-based DGGE. A ten microlitre of each PCR product was added to loading buffer and the mixed samples were loaded in each well of polyacrylamide gel. Electrophoresis was carried out using a D-Code Universal Mutation Detection System (Bio-Rad Laboratories) in TAE buffer at 59°C for 17 h at 60 V. The polyacrylamide gel was stained with SYBR Green (1:50,000 dilution, Cambrex Bioscience, Walkersville, MD, USA) in TAE buffer for 40 min and destained in fresh TAE buffer for 10 min. The result was visualized with ultraviolet transilluminator using Quantity One software (Bio-Rad Laboratories).

DNA extraction from excised gel and sequencing

Bands of interest were excised from the polyacrylamide gel for sequencing analysis. Excised gel fragments were disrupted, transferred in 300 µl of TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0) and incubated at 65°C for 15 min to dissolve the DNA. The contents were transferred to a Spin-X® centrifuge tube (Corning, Tewksbury, MA, USA) and centrifuged at 16,000 g for 5 min to purify DNA from acrylamide gel. The filtrate was mixed with 133 µl of 7.5 M ammonium acetate, 900 µl of ethanol and 3 µl of glycogen (20 mg/ml), vortexed and incubated at 80°C for 1 h. The samples were then centrifuged at 16,000 g for 15 min and the pellet was washed twice with 70% of cold ethanol. The purified DNA was sequenced using ABI 3100 capillary analyzing system (Applied Biosystems, Foster City, CA, USA) and the sequences were compared with nr database in GenBank using the basic local alignment search tool (BLAST) algorithm.

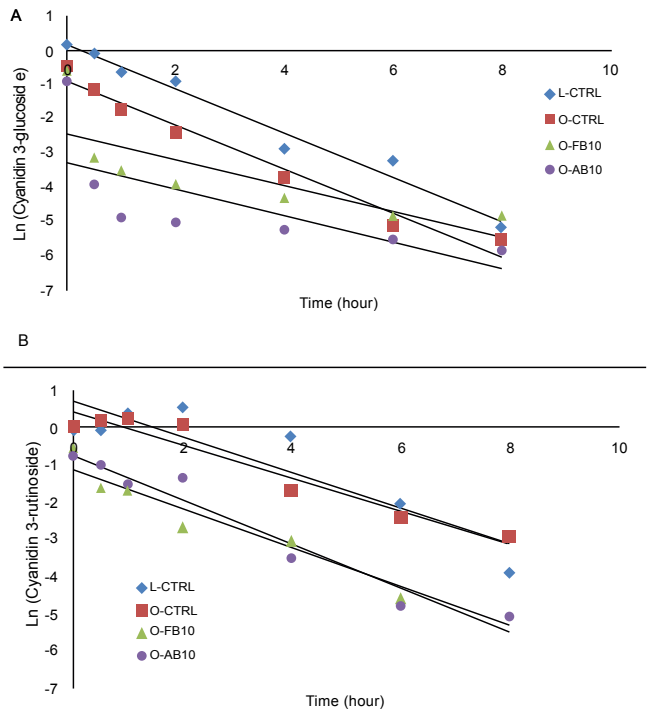

Figure 1: Rate loss of anthocyanins vs incubation time.
(A) Incubation with cyanidin-3-glucoside, (B) Incubation with cyanidin-3-rutinoside.

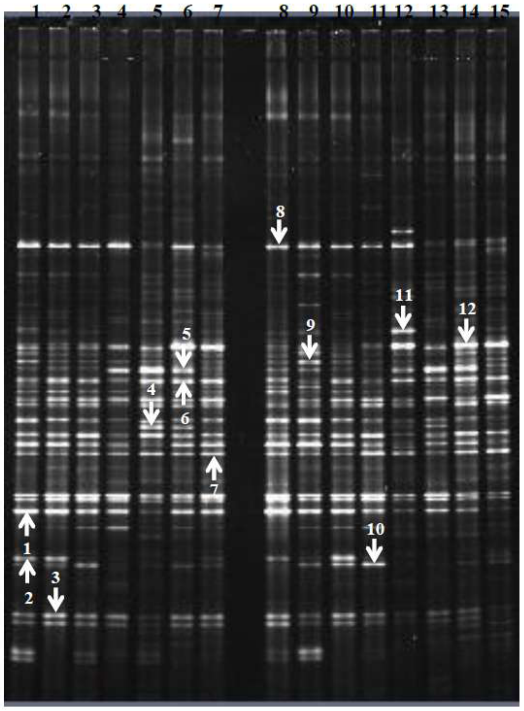

Figure 2: DGGE results of both control and treatment groups using cecal samples before adding cyanidin-3-gluscoside and cyanidin-3-ruinoside.
Lanes 1 to 7 for cyanidin-3-gluscoside and lanes 8 to 15 for cyanidin-3-ruinoside; Lane 1: L-CTRL-II, lane 2: O-CTRL-I, lane 3: O-CTRL-II, lane 4: O-FB10-I, lane 5: O-FB10-II, lane 6: O-AB10-I, lane 7: O-AB10-II, lane 8: L-CTRL-III, lane 9: L-CTRL-IV, lane 10: O-CTRL-III, lane 11: O-CTRL-IV, lane 12: O-FB10-III, lane 13: O-FB10-IV, lane 14: O-AB10-III, lane 15: O-AB10-IV.

Results

Degradation of anthocyanins by gut microflora

The logarithm of the remaining anthocyanin concentrations vs incubation time was plotted as seen in Figure 1. There were differences in the rate of anthocyanin disappearance between control groups (L-CTRL and O-CTRL) and treatment groups (O-AB10 and O-FB10) while no differences were shown within two control groups or two treatment groups. Both lean control (L-CTRL) and obese control (O-CTRL) were not as efficient in metabolizing anthocyanins when compared to the 10% of aged blackberry treatment (O-AB10) and 10% of fresh blackberry treatment (O-FB10). In Figure 1A, most of the cyanidin-3-glucoside was degraded within the first 0.5-2 h while the cyanidin-3-rutinoside (Figure 1B) was slowly degraded. Compared with control groups (Figure 1A), concentration of cyanidin 3-glucoside in blackberry treatment groups initiated to rapidly decline from the time points 0 h. However, both L-CTRL and O-CTRL groups had a slower rate of degradation over incubation time. In Figure 1B, the control groups containing cystidin-3-rutinoside displayed slower rates of anthocyanins degradation than the O-FB10 and O-AB10 groups.

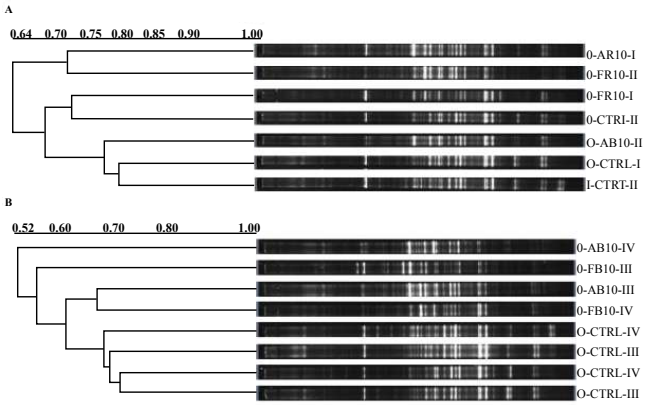

Figure 3: Phylogenetic tree based on DGGE results using cecum samples before adding cyanidin-3-gluscoside (A) and cyanidin-3-ruinoside (B) treatments.

Analysis of microflora in mice cecum

To verify the microbial populations in both controls and treatment groups, a PCR-based DGGE was performed and results were showed in Figure 2. Lanes 1 to 7 represented both controls and treatment groups before adding cyanidin-3-glucoside and lanes 8 to 15 represented before adding cyanidin-3-rutinoside, respectively. Although overall band patterns showed high similarities between control and treatment groups, some of bands were increased or decreased in a specific group. Based on these results, phylogenetic trees were generated by UPGMA algorithm to illustrate the correlation between control and treatment groups and were shown in Figure 3. The bacterial population patterns in the cecum used for cyanidin-3-glucoside metabolism were categorized into three distinct groups (Figure 3A). The control groups, O-CTRL-I and L-CTRL-II represented 80% of relatedness and O-AB10-II was clustered with these having 78% relatedness. The O-AB10-I and O-FB10-II as well as O-FB10-I and O-CTRL-II banding patterns showed approximately 73% relatedness (Figure 3A). The generated phylogenetic tree based on bacterial populations in cecum used for cyanidin-3-rutinoside metabolism is shown in Figure 3B. Interestingly, two obese control groups (O-CTRL-III and O-CTRL-IV) were represented with 73% of relatedness and categorized with two lean control groups (L-CTRL-III and L-CTRL-IV) with 70% relatedness. In addition, other treatment groups varied from 68 to 52% of relatedness.

Sequencing results

Based on DGGE results, common and specific bands (Figure 2, No. 1 to 12) were excised from the polyacrylamide gel and extracted DNA to identify specific bacteria. Many bands were excised from the gel however, some of bands cannot be recovered DNA and other bands were failed for sequencing. Although there were many banding patterns shared by control and treatment groups (Figure 2), recovered DNA showed that specific bacteria was based on BLAST algorithm. Excised bands number and identifications were illustrated in Table 1. There were three (No. 3, 6, and 7) and one bands (No. 1) found in both control and treatment groups were identified as uncultured bacterium and uncultured bacteriodetes, respectively (Table 1). Three bands (No. 4, 5 and 12) were found to be most intense in a selected number of FB10 and AB10 groups while being almost nonexistent in the control groups (Figure 3). All three bands were found to be of the genus *Lachnospiraceae* (Table 1). Of interest, *Lactobacillus johnsonii* (No. 8) was dominated in both control groups than treatments. Uncultured *Firmicutes* and *Clostridiales* (No. 9) were specific for L-CTRL as well as *Enterococcus faecalis* (No. 11) was present in only FB10 samples. *Bifodobacterium pseudolongum* (No. 10) was identified in only O-CTRL samples, not in blackberry treatment groups.

Discussion

The significance of this research was to evaluate metabolism rates of two different anthocyanins by mice gut microflora fed with 10% of aged or fresh blackberries along rodent diets as well as to identify different microbial populations in the mice cecums. Since anthocyanins are abundant in berry fruits and showed anti-obesity effects in animals, consumption of blackberry are associated with increasing overall health conditions [13,21]. Different anthocyanins metabolism rates between cyanidin-3-glucoside and cyanidin-3-rutinoside by mice gut microflora were demonstrated in this study as well as others [9,22]. In a previous study, Aura et al. [9] reported that cyanidin-3-rutinoside is degraded more slowly than cyanidin-3-glucoside which is the intermediate metabolite of cyanidin-3-rutinoside. The degradation rate of cyanidin 3-rutinoside was slower than cyanidin 3-glucoside in the present study.

Gut microflora plays an important role in the gastrointestinal tract by preventing colonization of pathogens as well as generating metabolites such as fermentation acids directly that inhibit other species [23,24]. The cecum has been considered as the primary site of focus because it contains one of the most plentiful and diverse microbial communities in animals, including strict anaerobes [25]. Gut microbial populations in the cecum are susceptible to shifts in the numbers making up their composition in response to diet modifications, as seen in early prebiotic research [26]. The bifidogenic effect of prebiotics such as fructooligosaccharides (FOS) was demonstrated in humans and animals by fecal analysis. Many studies have reported that beneficial bacteria such as *Bifidobacteria* and *Lactobacillus* were increased in the large intestine while decreasing the numbers of *Bacteroides* and *Clostridia* when supplemented with FOS consistently [27,28].

Results from this study and those like it, confirm that our gut microbe populations adapt to the food that we eat. Such is a logical explanation for the differences seen in anthocyanin degradation rates between the control and blackberry treatment groups. Both L-CTRL and O-CTRL mice had never been exposed to anthocyanins during their lifespan, and therefore neither had their gut microflora, which is in stark contrast to the treatment groups whose diets contained 10% of blackberry powders. Lack of adaptation to anthocyanins in the CTRL group mice diet was reflected in the slow rates of anthocyanin degradation by their gut microflora. This implies that different compositions of gut microflora caused by exposure to different diets need time to adapt to new substrates for utilization.

In general, both gut bacteria *Firmicutes* and *Bacteriodes* were present in cecum and identified widely [12]. *Firmicutes* are composed of

Band No.	Identification
1	Uncultured *bacteriodetes*
2,3,6,7	Uncultured bacterium
4,5	*Lachnospiraceae*
8	*Lactobacillus johnsonii*, uncultured *Lactobacillus* species
9	Uncultured *Firmicutes*, uncultured *Clostridiales*
10	*Bifidobacterium pseudolongum*, uncultured *Bifidobacterium*
11	*Enterococcus faecalis*
12	Uncultured *Lachnospiraceae*

Table 1: Sequencing results of selected bands from both control and treatment groups.

over 250 genera including *Clostridium*, *Enterococcus*, *Lactobacillus*, and *Staphylococcus* [12]. In this study, three bands shared by the majority of blackberry treatment groups were found to come from the same genus, *Lachnospiraceae*, of the phylum *Firmicutes*. Previous studies demonstrate that obese humans or animals have higher percentages of *Firmicutes* than their lean counterparts, and that the microbiota in obese mice are more effective at releasing calories from food than their lean counterparts [13,29,30]. These studies suggest that the *Lachnospiraceae* bacteria found predominantly in the obese mice may have played a part in the accelerated anthocyanin metabolism demonstrated by the blackberry treatment groups. Interestingly, *Lachnospiraceae* bacteria were found specifically in the obese treatment groups, but not the obese control groups, meaning that gut microbe populations can be shaped by several factors, not just obesity and diet alone. This result could possibly be explained by a stalled fermentation due to anaerobic conditions being compromised or other factors resulting in bacterial death.

PCR-based DGGE is a popular technique to screen complex bacterial populations in the cecum because they can be separated by G+C contents of a partial 16S rDNA sequence [19]. Although some bacterial species may have similar G+C contents and subsequently represented as one band, sequencing can alleviate this problem and determine specific bacterial species [19,20]. PCR-based DGGE has been identified as a suitable technique for qualitative analysis; however, it has a limitation for quantitative analysis of each bacterial species. Therefore, quantitative analysis based on band intensity should be interpreted considering PCR bias towards amplification of some specific 16S rDNA sequences [16].

Conclusion

In this study, it appeared that differences in diets and body phenotype can have an effect on gut microbial populations and subsequently affect the metabolism rate of anthocyanins cyanidin-3-glucoside and cyanidin-3-rutinoside. A microflora shift in blackberry treatment groups may have resulted in an increase in the populations of microbes with an elevated capacity to metabolize anthocyanins. Due to this adaptation by the gut microflora, blackberry treatment groups demonstrated a more rapid anthocyanin metabolism rate than the un-adapted control groups.

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