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Full Paper

Effects of blackcurrant extract on indole and ammonia productions in an *in vitro* human fecal culture model

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Blackcurrant is available as a traditional medicine in Europe. However, the detailed effects of blackcurrant on the human gut microbiota remain unknown. In this study, we investigated the prebiotic effects of a blackcurrant extract using a human fecal culture model in six healthy subjects. Feces were individually inoculated into a medium with or without the blackcurrant extract and then fermented for 48 hr under anaerobic conditions. The results obtained from analysis of samples from the fermented medium demonstrated that after 48 hr of fermentation, the pH of the medium with the blackcurrant extract was significantly decreased (control, 6.62 ± 0.20; blackcurrant extract, 6.41 ± 0.33; p=0.0312). A 16S rRNA gene sequencing analysis of the microbiota of the fermented medium showed a significant increase in the relative abundance of *Bifidobacteriacea*e. In measuring the concentrations of putrefactive components in the fermented medium, we found that the blackcurrant extract significantly reduced ammonia levels and displayed a tendency toward reduced indole levels. Our results suggest that blackcurrant extract could be a potential ingredient for relief of putrefactive components in the gut.

Key words: blackcurrant extract, ammonia, indole, fecal fermentation

INTRODUCTION

Blackcurrant contains flavonoids, anthocyanidins, and vitamin C and exhibits anti-oxidative, anti-inflammatory [1–3], and anti-obesity effects [4, 5]. Blackcurrant has been reported to alter the gut microbiota. Animal experiments, wherein a blackcurrant extract was supplemented to C57BL/6J female mice, revealed the upregulation of Bacteroidetes and downregulation of Firmicutes/Bacteroidetes; the Actinobacteria phylum, which includes bifidobacteria, was scarcely detected [6]. On the other hand, Molan *et al.* evaluated the effect of blackcurrant extract powders on male 8-week-old Sprague Dawley rats [7] and healthy adult human volunteers [8] and observed an increase in the abundance of lactobacilli and bifidobacteria and a decrease in the abundance of Bacteroides and Clostridia. The effects of blackcurrant on the gut microbiota composition seem to differ depending on the experimental model. Therefore, an investigation of the direct effects of blackcurrant on the human gut microbiota is essential.

Metabolites derived from gut microbiota fermentation exert various effects. Indole and ammonia are putrefactive products from the microbial breakdown and fermentation of dietary proteins. An excess amount of ammonia may be harmful, as observed in studies on the lack of productive performance in laying ducks [9] and the progression of inflammation in fattening pigs [10]. Additionally, it was identified as a risk factor for dementia in a cross-sectional study [11]. Suppression of the production of putrefactive products is also important for maintaining a healthy intestinal condition. In an *in vitro* fecal model, a culture medium seeded with feces can be used to investigate alterations in the composition and metabolism of the gut microbiota over time [12]. In this study, we evaluated the effects of a blackcurrant extract on the production of beneficial and putrefactive products and its impact on bacterial composition using a human fecal culture model.

MATERIALS AND METHODS

Blackcurrant extract

We used a blackcurrant extract powder that was provided by Morishita Jintan Co., Ltd. (Osaka, Japan), and contained 350 mg of anthocyanins per 1 g of the product. The dosage of anthocyanins of 50 mg per day is recommended for the regulation of eye focusing function [13]. The capacity of the large intestine in Japanese adults is approximately 3–3.5 L (length, approximately 1.7 m; diameter, approximately 5 cm [14]), whereas a fecal

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culture has the capacity to hold a volume of 100 mL of Gifu anaerobic medium (GAM). Therefore, the dosage of blackcurrant extract we adopted, 4.76 mg, contained 1.67 mg anthocyanins, which was equivalent to one-thirtieth of the recommended daily allowance.

Fecal samples

Fresh fecal samples were obtained from six healthy human volunteers who provided informed consent. Donors 1, 2, 4, and 5 were female and 22, 47, 21, and 21 years old, respectively. Donors 3 and 6 were male and 27 and 22 years old, respectively. We confirmed that the volunteers had not been treated with antibiotics for at least two months before the trial. After collection, the fecal samples were stored in anaerobic culture swabs (BD Biosciences, Franklin Lakes, NJ, USA) at 4°C until culture and used within 48 hr. The experimental procedures were reviewed and approved by the Medical Ethics Committee at Gifu University (Reference number: 29-382, 1/10/2018).

In vitro *fermentation*

Fecal fermentation was performed once per donor using a multi-fermenter BME (ABLE, Tokyo, Japan) as previously described by Takagi *et al.* [12] with some modifications. Briefly, 100 mL of GAM (Nissui Pharmaceutical, Tokyo, Japan) was added to each vessel. Before the start of fermentation, the dissolved oxygen in the GAM was removed by bubbling a gas mixture of N_2 and CO_2 (80:20) through a 0.2 µm sterile filter. The 4.76 mg dosage of Blackcurrant extract was added immediately before the start of fermentation. After dissolving the fecal sample in 2 mL of 0.9% NaCl with 1.0% L-ascorbic acid, 100 µL of the fecal suspension was inoculated, and the pH of the culture medium was monitored and recorded continuously. During fermentation, the vessel was maintained at 37°C with stirring at 300 rpm, and the anaerobic state was sustained by aerating continuously with the abovementioned gas mixture. Fermentation samples for bacterial DNA analysis were stored at −30°C, and those for use in additional analyses were centrifuged at 22,000 × g for 5 min at 4°C, and the supernatants were stored at −30°C.

Measurement of short-chain fatty acids (SCFA)

The SCFA concentration was measured using a high-performance liquid chromatography system (HPLC, Shimadzu Corporation, Kyoto, Japan) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA). The column was equilibrated with 5 mM H_2SO_4 and used at 65°C with a flow rate of 0.6 mL/min. The refractive index was measured using a refractive index detector (RID-10A, Shimadzu Corporation).

Measurement of indole

The indole concentration was measured using a gas chromatograph-mass spectrometer (GCMS-QP 2010, Shimadzu Corporation) equipped with an Agilent J&W DB-5MS column (30 m × 0.25 mm × 1.0 µm; Agilent Technologies, Santa Clara, CA, USA). The gas phases of the fermented samples were then measured. In brief, 4 mL of fermented sample was packed in a glass vial with octadecyl silica resin (MonoTrap RSC18; GL Science Inc., Tokyo, Japan) and then absorbed with stirring at 400 rpm for 90 min at 37°C. The absorbate was then dissolved in 200 µL of diethyl ether. Subsequently, the sample solution was injected and analyzed. After splitless injection (1 µL) at 250°C, analytes were separated on the aforementioned column using He as a carrier gas (2.15 mL/min). The temperature gradient started at 40°C (following a 2 min hold) with an increase of 5°C/min until reaching 250°C (12 min hold).

Measurement of ammonia

The ammonia concentration was determined using an ammonia assay kit (Cell Biolabs, San Diego, CA, USA), with ammonium chloride as the standard. The procedure was performed according to the manufacturer's protocol, and absorbance was measured at 660 nm.

Extraction of bacterial DNA

Bacterial DNA was prepared according to the methods described by Takagi *et al.* [12], with one minor change. The disruption of bacterial membranes was performed by glass bead-beating using a µT-12 bead homogenizer (Taitec Corporation, Saitama, Japan) at 3,000 rpm for 2 min.

Quantitative analysis of Bifidobacterium

Copy numbers of *Bifidobacterium* were measured using quantitative real-time polymerase chain reaction (qPCR) with TB Green® Premix Ex Taq™ II (Takara Bio Inc., Kusatsu, Japan) and a StepOne™ System (Thermo Fisher Scientific Inc., Waltham, MA, USA). The PCR mixture was prepared as a 20 µL reaction containing 2 µL of DNA template and 200 nM of each primer, according to the manufacturer's protocol. Quantitative real-time PCR of *Bifidobacterium* was performed using the forward and reverse primers g-Bifid-F 5′-CTCCTGGAAACGGGTGG-3′ and g-Bifid-R 5′-GGTGTTCTTCCCGATATCTACA-3′ (Eurofins Genomics K.K., Tokyo, Japan), respectively, as previously described by Matsuki *et al.* [15]. Copy numbers of Bifidobacterium were quantified using a standard curve that was constructed using a pure culture of B. longum BB536 and calculated as the log per milliliter of the fermented sample. Quantitative real-time PCR of *Lactobacillus* was performed using the forward and reverse primers 5′-AGCAGTAGGGAATCTTCCA-3′ and 5′-CACCGCTACACATGGAG-3′ (Eurofins Genomics K.K., Tokyo, Japan), respectively, as previously described by Rinttilä *et al.* [16]. Copy numbers of *Lactobacillus* were quantified using a standard curve that was constructed using a pure culture of L. acidophilus GAI91366 and calculated as the log per milliliter of the fermented sample.

Microbiota composition by 16S rRNA amplicon sequencing

A 16S rRNA library was prepared according to the manufacturer's instructions (Illumina, San Diego, CA, USA) using amplicon and index PCRs with KAPA HiFi HotStart ReadyMix (Roche Diagnostics, Mannheim, Germany). First, the bacterial DNA extract was diluted with TE solution to a concentration of 5 ng/µL. Amplicon PCR of the V3–V4 region was performed using the forward primer 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and reverse primer 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′ (Eurofins Genomics) [17]. PCR amplification was performed as follows: 95°C for 3 min, followed by 25 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, and then 72°C for 5 min. After clean-up using AMpure XP beads (Beckman Coulter, Brea, CA, USA), the individual DNA products were tagged using index

PCR primers (Nextera® XT Index kit, Illumina). The quality and concentration of each DNA library was measured using an Agilent 2100 Bioanalyzer (Agilent Technology Inc., Santa Clara, CA, USA) and Qubit dsDNA HS assay kit (Thermo Fisher Scientific). The concentration of each DNA library was adjusted to an equal concentration (4 nM) with TE buffer. The 16S rRNA genes were subjected to paired-end sequencing using the MiSeq platform with MiSeq Reagent Kit v3 (Illumina). Primer regions of paired-end reads were removed using Cutadapt [18]. The quality of read sequences was guaranteed above a Q score of 25 by using sickle (version 1.33) [19]. The reads were assembled into single sequences using the FLASH software [20]. The assembled reads were processed and clustered into operational taxonomic units (OTUs) using mothur [21] in accordance with the MiSeq SOP (https://mothur.org/wiki/miseq_sop/). Amplicon OTUs were clustered and classified into groups with 97% sequence similarity; this was done based on non-redundant SILVA datasets (release 132) [22]. For all samples, the numbers of reads were unified to be equal to the minimum sample size using the phyloseq R package [23]. Experimental information and amplicon sequence data are available under BioProject accession no. PRJDB14563. Alpha diversity and beta diversity analyses were performed using the gplots, phyloseq, and vegan R packages [24].

Statistical analysis

Statistical analyses were performed using the Wilcoxon matched pairs signed rank test using GraphPad Prism 9 (GraphPad, San Diego, CA, USA). The data are presented as medians and ranges. Differences were considered statistically significant when p-values were less than 0.05. The paired samples Wilcoxon test was performed to compare counts at different taxonomic ranks in the two groups using the Wilcox.test function and microbiomeMarker R packages [25].

RESULTS

pH fluctuation during in vitro fermentation

Blackcurrant extract was added immediately before fecal fermentation; the control culture did not receive this treatment. Considering that pH is one of the indexes associated with the conditions of the colon, fluctuation in the pH of the fermentation medium affects fermentation progression in vitro. The pH values of the samples fermented with blackcurrant extract gradually decreased compared with the control 12 hr after fermentation was initiated, and the low pH environment was maintained for a further 26 hr (Fig. 1).

Effects of blackcurrant extract on SCFA production

SCFA concentrations were measured 48 hr after fermentation (Fig. 2). Acetate and butyrate production tended to decrease (p=0.0938 and p=0.0625, respectively). There were differences in the effects of the blackcurrant extract on propionate production among the donors.

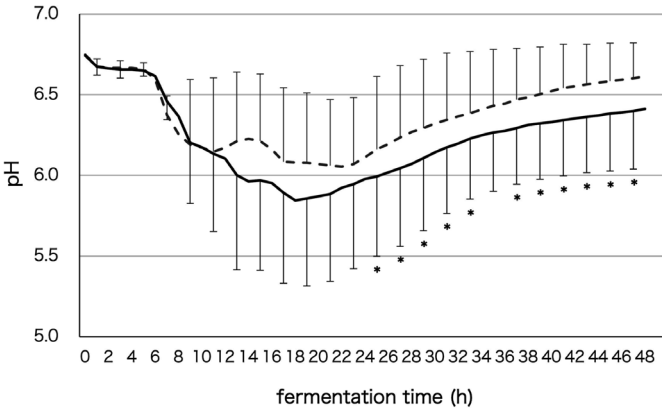

Fig. 1. Effects of blackcurrant extract on the mean pH value in the fecal fermented samples. Blackcurrant extract was added immediately before fecal fermentation; it was not added for the control. The mean pH ± standard deviation values for the blackcurrant extract (solid line) and control (dotted line) were plotted against time (n=6). The pH values observed every 2 hr after the start of fermentation were subjected to statistical analysis. *p<0.05 indicates a significant difference from the control.

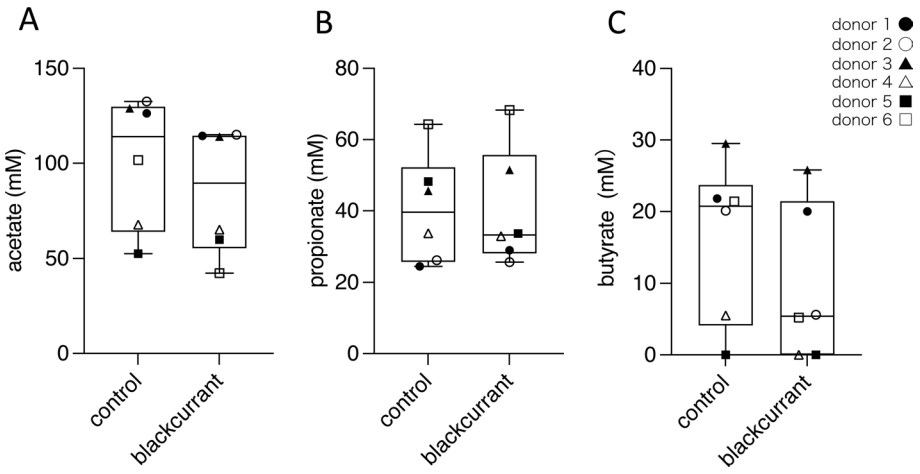

Fig. 2. Effects of blackcurrant extract on short-chain fatty acid (SCFA) production. Acetate (A), propionate (B), and butyrate (C) in the fermented samples were measured at 48 hr using high-performance liquid chromatography system (HPLC). Results are expressed as the median and range.

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Effects of blackcurrant extract on putrefactive products, indole and ammonia

The concentrations of putrefactive products 48 hr after fermentation were measured. Blackcurrant extract tended to decrease indole production, and this was more effective for donors whose indole levels were above 10 μg/mL in the control culture (p=0.0625, Fig. 3A). Ammonia production was significantly suppressed (p=0.0312, Fig. 3B). These results suggested that blackcurrant extracts moderated the incidence of putrefactive products.

Effects of blackcurrant extract on the growth of Bifidobacterium and Lactobacillus

We assessed the effects of blackcurrant extract on the growth of *Bifidobacterium* and *Lactobacillus*, well-known genera of beneficial bacteria. The copy numbers of *Bifidobacterium* in the control and blackcurrant extract fermentation samples were $8.05 \pm 6.38 \times 10^8$ and $1.58 \pm 0.84 \times 10^9$ copies/mL, respectively (p=0.0938, Fig. 4A). The copy numbers of *Lactobacillus* in the control and blackcurrant extract fermentation samples were $2.15 \pm 4.82 \times 10^4$ and $1.98 \pm 3.95 \times 10^6$ copies/mL, respectively (p=0.0625, Fig. 4B).

Impact of blackcurrant extract on the microbiota composition

Microbiota diversity was calculated using the vegan R package. Analysis of alpha-diversity in this study showed no significant difference between the control and blackcurrant extract groups (for the Chao1 and Shannon indexes, p=0.094 and p=0.313, respectively; control, 99.7 ± 32.25 and 2.58 ± 0.38, and blackcurrant extract, 81.1 ± 23.7 and 2.45 ± 0.46, respectively).

The population of the *Escherichia-Shigella* genus dominated in the *Enterobacteriaceae* family and was highly abundant, compared with their feces. Similar observations (relative abundance of *Escherichia* of ~40%) of the have been reported by other researchers using a similar fecal culture method [12, 26]. There was a significant increase in the relative abundance of *Bifidobacteriaceae* (control, 7.1 ± 5.9, and blackcurrant extract, 14.1 ± 8.3; p=0.03125) and decrease in the relative abundance of *Lachnospiraceae* (control, 8.0 ± 4.6, and blackcurrant extract: 4.5 ± 4.0; p=0.03125) (Fig. 5). Blackcurrant extract exerted differing effects on the relative abundances of Bacteroidetes (control, 27.5 ± 9.0, and blackcurrant extract, 25.9 ± 11.9; p>0.999) and Clostridia (control, 18.8 ± 16.0, and blackcurrant extract, 16.0 ± 17.5; p=0.1562) among the inoculum donors.

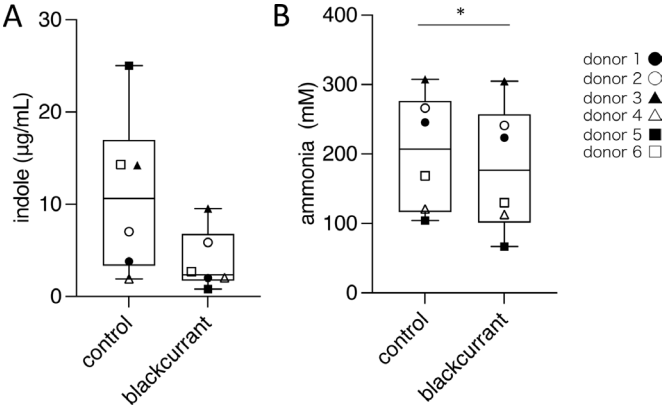

Fig. 3. Effects of blackcurrant extract on putrefactive products. Indole (A) and ammonia (B) in the fermented samples were measured at 48 hr with a gas chromatograph-mass spectrometer equipped with a non-polar column using phenyl arylene polymer and an ammonia assay kit. Results are expressed as the median and range. *p<0.05 indicates a significant difference from the control.

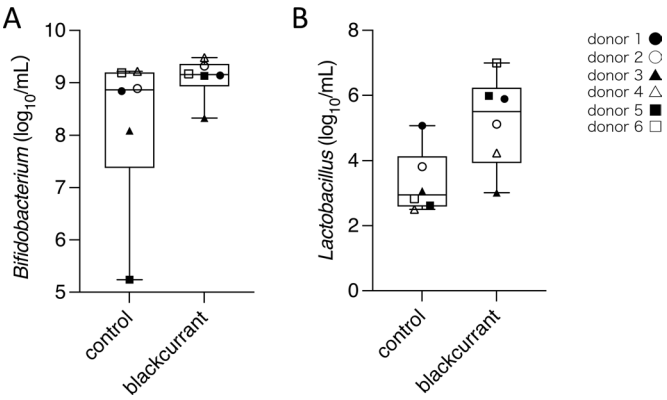

Fig. 4. Effects of blackcurrant extract on the copy numbers of *Bifidobacterium* and *Lactobacillus*. The DNAs of *Bifidobacterium* (A) and *Lactobacillus* (B) bacteria were extracted from the fermented fecal samples and quantified using quantitative real-time PCR (qPCR). Results are expressed as the median and range of the $\log_{10}$ of the absolute bacterial copy number.

DISCUSSION

In vitro culture can help assess the response to food sources in each gut environment. This study evaluated the prebiotic effects of a blackcurrant extract on the gut microbiota using an *in vitro* fecal fermentation model by analyzing pH, SCFAs, indole, ammonia, copy numbers of *Bifidobacterium*, and microbiota composition.

In this study, we observed that the blackcurrant extract significantly reduced the pH of the fermentation samples after 26 hr of fermentation (Fig. 1). The extract increased *Bifidobacterium*, as demonstrated by the results of analyses of copy numbers using qPCR (Fig. 4) and relative abundance using 16S rRNA amplicon sequencing (Fig. 5). These simulated results in fecal culture were consistent with those observed in a study of interventions with blackcurrant extract products in healthy humans by Molan *et al.* [8], which showed that the intake of blackcurrant extracts prompts an increase in bifidobacteria and a decrease in fecal pH. A recent report has shown that anthocyanins significantly increase the growth of *Bifidobacterium* in mice with high-fat diet-induced oxidative stress [27]. The anthocyanins in blackcurrant extract may be the primary compound promoting *Bifidobacterium* growth.

One of the objectives of this study was to assess indole and ammonia production. The analysis of indole was performed in the gas phase derived from the fermented sample, and the detected indole level was in agreement with the previous report by Nakata *et al.* [28], who conducted a colorimetric assay with Kovacs reagent on a human fecal culture [29]. Although *Bacteroides uniformis* and *Bacteroides ovatus* are known indole producers [30], there was no correlation between these indole-producing bacteria and the levels of indole detected. In addition, as indole is produced from sources of protein during some steps of protein fermentation

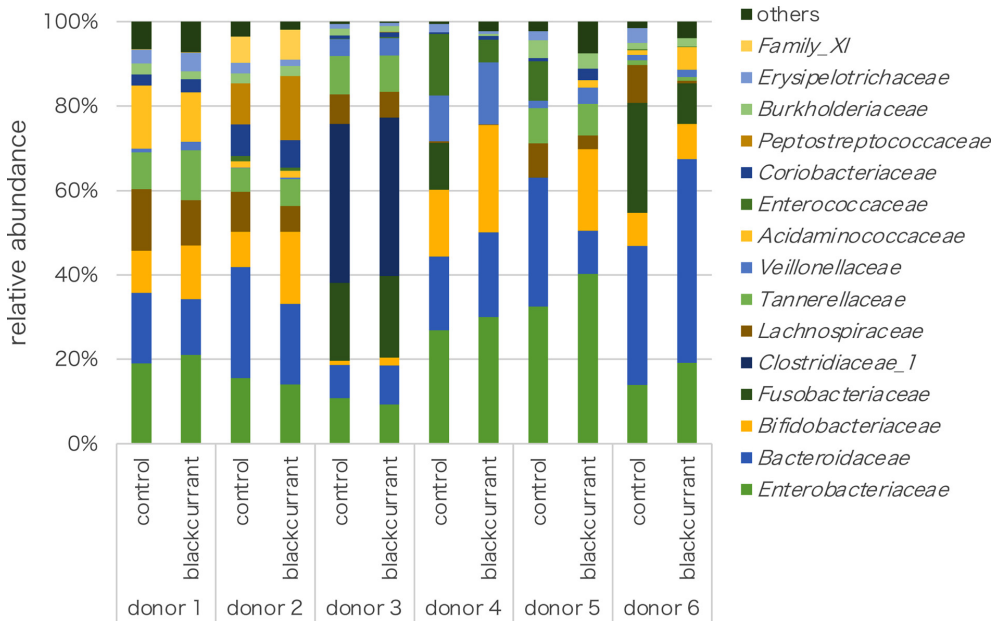

Fig. 5. Effects of blackcurrant extract on microbiota in the fermented samples at 48 hr. The relative abundances of the constituent microbes at the family level based on sequencing of the V3–V4 region of bacterial 16S rRNA are displayed. Families with collective relative abundance <1% are grouped under "others".

by unique gut microbiota [31], the mechanism of suppression of indole production cannot be easily explained by a decrease in the relative abundance of indole-producing bacteria. Indole has been reported to play a role in improving barrier function in intestinal epithelial cells [32] and to play a role as a signaling molecule between bacteria (e.g., in quorum sensing) [33]. This suggests that indole may be one of the critical molecules for maintaining an equilibrium within the gut microbiota. Therefore, we believe that the implications of indole for the microbiome and quantity of it required should be discussed with caution.

The blackcurrant extract also significantly suppressed ammonia production after 48 hr of fermentation (Fig. 3B). This suppressive effect on ammonia production has also been reported in rabbits fed a high-fat diet [34]. In intervention studies, the administration of *Bifidobacterium* decreased in healthy humans decreased the ammonia content [35, 36] and urease activity [35] in feces. Some reports have also suggested a negative correlation between ammonia production and *Lactobacillus* [37, 38]. Blackcurrant extract may suppress ammonia production via moderate increases in *Bifidobacterium* and *Lactobacillus* (Fig. 4).

Similar to blackcurrant extract (anthocyanin), other polyphenols might also suppress the effects of pH and ammonia. For example, Sepperer *et al.* [39] reported that tannins and tannin-derivative polymers induce considerable reductions in pH and ammonia in cattle manure. Approximately 95% of ingested polyphenols are estimated to reach the colon, where they are metabolized by gut microbiota [40]. In the colon, polyphenols are metabolized by gut bacteria and converted to various derivatives; hence, the availability and activity of polyphenols depend on the individual gut microbiota [41, 42]. Blackcurrant is used as a traditional medicine in Europe [43]. Most naturally derived herbal medicines do not always show efficacy. This may imply that natural medicines need to intimately match the gut microbiota of an individual for the individual to receive the benefits of them.

Future research will hopefully elucidate the details of the blackcurrant-derived polyphenol modifications caused by gut microbiota fermentation and the activity and availability of blackcurrant polyphenol derivatives.

CONFLICT OF INTEREST

There is no conflict of interest to declare.

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