Probiotic therapy alleviates hyperuricemia in C57BL/6 mouse model.

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Abstract

Aim: The present study is to investigate differences in gut bacteria especially *bifidobacteria* and *lactobacilli* between C57BL/6 mice with and without hyperuricemia, as well as the effect of probiotic therapy with *bifidobacteria* and *lactobacilli* on hyperuricemia-associated serum markers over time in the mouse model.

Methods: The mouse model of hyperuricemia was established by using C57BL/6 mice and a reported protocol. DNA was extracted from fecal samples collected from C57BL/6 mice. Specific primers for 16S rRNA gene were designed for PCR-based detection of *bifidobacteria* and *lactobacilli*, respectively. Uric acid (UA) level, lipopolysaccharides (LPS) level, and xanthine oxidase (XO) activity in serum were measured.

Results: The amount of *bifidobacteria* and *lactobacilli* in fecal samples from hyperuricemic mice was significantly decreased, while UA level, XO activity, and LPS level were significantly increased in hyperuricemic mice compared to those of normal mice. These changes were attenuated after five weeks of probiotic therapy.

Conclusion: The present study demonstrates the relationship between hyperuricemia and gut microbial changes over time and the effect of probiotic therapy on hyperuricemia in mouse model.

Introduction

Uric acid (UA) is an end product of purine nucleoside metabolism in humans, which is regulated by balancing its production in the liver and urinary excretion [1-4]. Unlike other mammals, hominoids have high serum UA levels due to the lack of the urate-degrading enzyme uricase, which sometimes leads to hyperuricemia [5,6]. It has been suggested that serum UA should be kept below 7 mg/dL to prevent hyperuricemia, which is a clinically important risk factor for cardiovascular diseases, chronic kidney disease and gout [7,8]. Intestinal microorganisms play various roles in human health including metabolism of complex fiber and fats, as well as immune regulation [9]. Intestinal microbial imbalance increases lipopolysaccharide (LPS) levels in blood circulation, induces chronic inflammation [10], and is associated with metabolic syndrome (MS), obesity, and insulin resistance (IR) [11]. Numerous studies support the role of intestinal microbiome in regulating body weight and energy metabolism as well [12-16].

In healthy individuals, about 70% UA is excreted through kidneys and the remaining 30% is excreted or decomposed by intestinal flora [17,18]. As a metabolic disease, hyperuricemia is closely associated with increased production and reduced excretion of UA. However, the impact of reduced excretion of UA on the decomposition of UA by intestinal flora in hyperuricemia patients is unknown. Moreover, little work has been conducted to investigate the relationship between hyperuricemia and intestinal flora. Abnormal level of serum UA is a clinical marker for hyperuricemia. Xanthine oxidase (XO) is a key enzyme in the production of UA, and increased activity of XO can lead to increased UA production. LPS is a metabolite of intestinal flora. The abnormal level of LPS in blood circulation induces inflammation in many diseases accompanied by increased XO activity. Therefore, the pathological relationship between hyperuricemia and intestinal flora could be monitored by measuring UA level, LPS level, and XO activity in serum. In the present study, we employ high purine diet and administer intraperitoneal injection of potassium oxonate to induce consistent high levels of UA in C57BL/6 mice as hyperuricemia model [19,20]. We further use

probiotics to modulate intestinal microbiome. The proportion of *bifidobacteria* and *lactobacilli* in intestinal flora is quantified, while LPS level, UA level and XO activity were measured to investigate the relationship between intestinal flora and hyperuricemia.

Materials and Methods

Animals

C57BL/6 mice (18-22 g) were purchased from the Animal Center of Shandong Gout Laboratory, Qingdao, China. All animals were handled according to the guidelines provided by the Animal Care and Use Committee at Shandong Province. Experimental protocols for animal use were reviewed and approved by the animal ethics committee of Qingdao University. Animals were housed at 26 ± 3°C with a 12 h light/12 h dark cycle.

Thirty mice were randomly divided into three groups (n=10 each) as follows: normal group (NG), hyperuricemia group (HG) and hyperuricemia with probiotic therapy group (HPG). NG was fed a normal diet (laboratory chow and water ad libitum) with saline (0.5 ml) by intragastric administration once a day, while the other two groups were fed high-purine diet (laboratory high-purine chow and water ad libitum) and administered intraperitoneal injection of potassium oxonate 300 mg/kg once a day, with saline (0.5 ml) for HP and probiotics (0.5 ml) for HPG (*bifidobacteria* and *lactobacilli*, 1.0×10^6 CFU each) by intragastric administration once a day.

DNA extraction

Fecal samples were collected into 1.5 ml tubes. To avoid contamination and microbial growth, fecal samples were put on ice immediately and stored at -80°C in less than an hour.

Fecal samples were thawed and centrifuged at 12,000 rpm for 3 min. The pellet was collected for DNA extraction according to the manufacture's instruction (QIAamp Fast DNA Stool Mini Kit; No. 148048456, Qiagen, Hilden, Germany). The quality of all DNA samples was analyzed by using 1% agarose gel electrophoresis. DNA concentration of each sample was quantified by taking 1.5 µL of the extracted DNA sample and measuring the OD using an ultraviolet spectrophotometry (Nanodrop ND2000, Thermo Scientific, Waltham, MA, USA). To ensure the repeatability and stability of PCR analysis, the concentration of all DNA samples were higher than 20 ng/µL according to the suggestions of a published report [21]. The OD values of all DNA samples were between 1.8-2.0, indicating the absence of RNA or protein cross contaminations.

Polymerase chain reaction (PCR)

Primers were designed based on 16S rRNA gene sequences available from GenBank using Primer Premier for Windows, version 5.0 (Premier Biosoft International, Palo Alto, CA) (Table 1). Primer specificities for their target organisms were checked with an online tool provided by the Ribosomal Database Project. Primers were synthesized commercially by Invitrogen Life Technologies (Thermo Fisher Scientific, Waltham, MA, USA). Each reaction mixture for PCR (50 µL) contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM deoxynucleoside triphosphates, 0.5 M primer, 1 µL of bacterial template DNA, and 1 U of Taq DNA polymerase. Optimal annealing temperatures and specificity testing were done by using a Mastercycler gradient PCR instrument and a Techne Genius PCR instrument, respectively. PCR protocol consisted of 35 cycles with DNA denaturation at 95°C (1 min), followed by annealing (1 min) and elongation at 72°C (45 seconds); a final elongation step was performed at 72°C (5 min).

Table 1. PCR primers used in this study.

Target organisms	Sequences (5' to 3')	Annealing temperature (°C)	References
Bifidobacteria	GGGTGGTAATGCCGGATG (forward)	59	26
	TAAGCCATGGACTTTCACACC (reverse)		
Lactobacilli	CATCCAGTGCAAACCTAAGAG (forward)	58	26
	GATCCGCTTGCCTTCGCA (reverse)		

Quantitative real-time polymerase chain reaction (qRT-PCR)

According to references [22,23], iCycler iQ apparatus (Bio-Rad, Hercules, CA, USA) and iCycler Optical System Interface software (version 2.3; Bio-Rad, Hercules, CA, USA) was used for qRT-PCR. Each reaction was performed in duplicate in a volume of 20 µL in 96-well optical-grade PCR plates. Amplification reactions were done with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) containing 3 mM $MgCl_2$, 20 mM Tris HCl (pH 8.4), 50 mM KCl,

deoxynucleoside triphosphate (200 µM), SYBR Green I, 10 nM fluorescein, 0.625 U iTaq DNA polymerase mixed with selected primer, and 1.6 µL of respective template DNA or water. Amplifications were done with the following protocol: one cycle at 95°C (3 min), 35 cycles of denaturation at 95°C (30 s), primer annealing (30 s), and one final cycle at 95 °C (30 s). Finally, melt curve analyses were made by slowly heating PCR mixtures from 55 to 95°C (1°C per cycle of 10 s) with simultaneous measurements of the SYBR Green I signal intensities. Quantification was done by using standard curves

made from known concentrations of plasmid DNA containing respective amplicon for each set of primers.

Table 2. Quantification of bifidobacterium 16S rRNA genes in mouse fecal DNA preparations of normal group (NG), hyperuricemia group (HG) and probiotics-treated hyperuricemia group (HPG) at different time points (logCFU/g, means ± standard deviations, n=10).

	Day 7	Day 14	Day 21	Day 28	Day 35
NG	8.13 ± 0.20	8.14 ± 0.27	8.09 ± 0.32	8.10 ± 0.26	8.09 ± 0.47
HG	8.02 ± 0.33	7.66 ± 0.50#	7.67 ± 0.33#	7.71 ± 0.27#	7.69 ± 0.25#
HPG	8.57 ± 0.35#*	8.76 ± 0.30#*	8.82 ± 0.26#*	8.98 ± 0.19#*	8.94 ± 0.20#*

Note: #P<0.05 compared with NG; *P<0.05 compared with HG.

Quantification of UA level, XO activity and LPS level in serum

Blood samples were collected from tail veins of mice on days 7, 14, 21, 28 and 35. Serum was obtained after centrifugation at 4,000 rpm for 10 min. Serum was used for measuring UA level, XO activity, and LPS level. UA was measured by a clinical automatic biochemical analyzer (AU5800, Beckman Coulter, Brea, CA, USA). Serum XO activity and LPS level were measured according to the manufacturer's instructions (Xanthine Oxidase Activity Assay Kit; NO. MAK078, Sigma-Aldrich, St. Louis, MO, USA) and a previous publication [24], respectively.
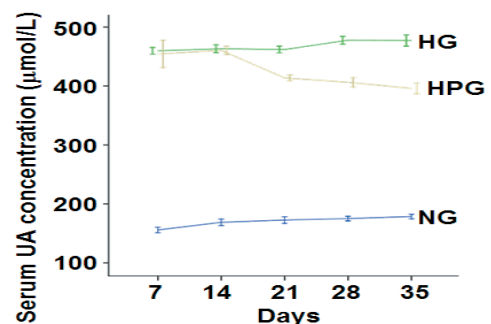
Statistical analyses

Logarithms of fecal 16S rRNA gene copy numbers were used to achieve normal distribution, and means ± standard deviations were calculated. SPSS for Windows (version 10.0.7, IBM, Armonk, NY, USA) was employed for statistical analyses with one-way analysis of variance and Bonferroni multiple comparisons to compare the three groups with each other. P<0.05 was considered as statistical significance.

Results

The numbers of *bifidobacteria* and *lactobacilli* are reduced in hyperuricemic mice, and intragastric probiotic therapy increases the amount of *bifidobacteria* and *lactobacilli* in fecal samples. To determine rRNA gene copies, qRT-PCR was performed. The data showed that the number of rRNA gene copies of *bifidobacteria* from fecal samples in HG was significantly lower than that of NG on days 14, 21, 28 and 35, and that in HPG was significantly higher than that of NG and HG on all days (Table 2). Similarly, the number of rRNA gene copies of *lactobacilli* from fecal samples in HG was significantly lower than that of NG on days 14, 21, 28 and 35, and that in HPG was significantly higher than that of NG and HG on all days (Table 3). These results suggest that the numbers of *bifidobacteria* and *lactobacilli* are reduced in hyperuricemic mice, and intragastric probiotic therapy increases the amount of *bifidobacteria* and *lactobacilli* in fecal samples.

Probiotic therapy reduces serum UA level in hyperuricemic mice

To measure serum UA level, a clinical automatic biochemical analyzer was used. The data showed that UA level was increased by at least 88% in HG mice compared to NG mice on days 7-35 (Figure 1). After intragastric probiotic therapy once a day, serum UA level in HPG mice was decreased by more than 18% compared to HG mice on days 21, 28 and 35 (Figure 1). However, serum UA levels in HPG mice were still significantly higher than that of NG. These results indicate that probiotics reduces serum UA level in hyperuricemic mice even though the UA level is still higher than that in normal mice.
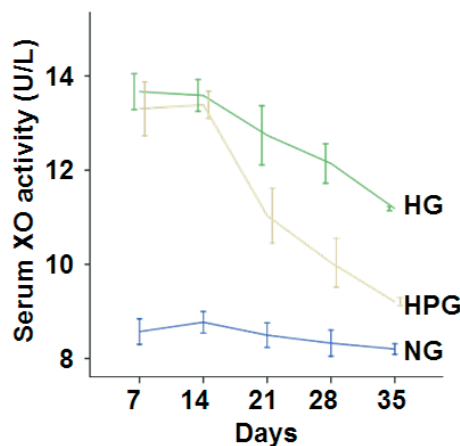

Figure 1. Serum uric acid (UA) concentrations in mice of normal group (NG), hyperuricemia group (HG) and probiotics-treated hyperuricemia group (HPG) at different time points. Blood samples were collected from the tail vein of mice on days 7, 14, 21, 28 and 35. Serum was obtained after centrifugation at 4,000 rpm for 10 min. Serum UA concentration was measured by a clinical automatic biochemical analyzer.
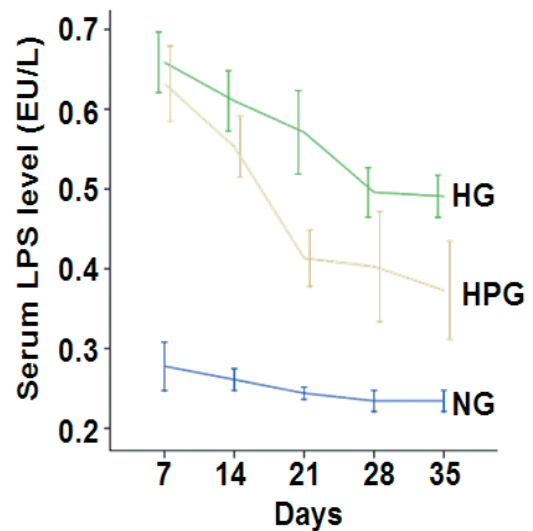
Probiotic therapy decreases serum XO activity in hyperuricemic mice

To determine XO activity, colorimetric assay was performed. The data showed that XO activity in HG mice was significantly increased compared to that in NG mice on days 7-35. Moreover, XO activity in HPG mice was significantly decreased compared to that in HG mice on days 21, 28 and 35. However, serum XO activity in HPG mice was still significantly higher than that in NG mice (Figure 2). The

results suggest that probiotic therapy reduces the activity of XO, a key enzyme in the production of UA.

Figure 2. Serum xanthine oxidase (XO) activity in mice of normal group (NG), hyperuricemia group (HG) and probiotics-treated hyperuricemia group (HPG) at different time points. Blood samples were collected from the tail vein of mice on days 7, 14, 21, 28 and 35. Serum was obtained after centrifugation at 4,000 rpm for 10 min. Serum XO activity was measured using Xanthine Oxidase Activity Assay Kit.

Figure 3. Serum lipopolysaccharide (LPS) level in mice of normal group (NG), hyperuricemia group (HG) and probiotics-treated hyperuricemia group (HPG) at different time points. Blood samples were collected from the tail vein of mice on days 7, 14, 21, 28 and 35. Serum was obtained after centrifugation at 4,000 rpm for 10 min. Serum LPS level was measured according to a published method.

Table 3. Quantification of lactobacillium 16S rRNA genes in mouse fecal DNA preparations of normal group (NG), hyperuricemia group (HG) and probiotics-treated hyperuricemia group (HPG) at different time points (logCFU/g, means ± standard deviations, n=10).

	Day 7	Day 14	Day 21	Day 28	Day 35
NG	7.12 ± 0.28	7.15 ± 0.30	7.19 ± 0.21	7.14 ± 0.23	7.10 ± 0.28
HG	7.09 ± 0.18	6.82 ± 0.23#	6.78 ± 0.28#	6.79 ± 0.24#	6.80 ± 0.30#
HPG	7.79 ± 0.20#*	7.81 ± 0.25#*	7.87 ± 0.19#*	7.88 ± 0.17#*	7.96 ± 0.16#*

Note: #$P<0.05$ compared with NG; *$P<0.05$ compared with HG.

Probiotic therapy decreases serum LPS level in hyperuricemic mice

To investigate the metabolism of intestinal flora, serum LPS level was measured. The data showed that serum LPS level in HG mice was significantly increased compared to that in NG mice on days 7-35. By contrast, serum LPS level in HPG mice was significantly decreased compared to that in HG mice on days 14-35 (Figure 3). These results indicate that probiotic therapy decreases serum LPS level in hyperuricemic mice.

Discussion

Hyperuricemia is a clinically important risk factor for cardiovascular diseases, chronic kidney disease and gout. At present, the clinical medicine used for hyperuricemia treatment has low drug tolerance, high drug dependence and multiple side effects due to lack of understanding of the complicated disease. Interestingly, imbalanced compositions of intestinal flora are associated with human metabolic diseases related to abnormal lipid and sugar metabolism. The amount of probiotics, especially *bifidobacteria* and *lactobacilli*, is

decreased in these diseases [10-12,15,16]. As a metabolic disease, hyperuricemia is associated with increased production and reduced excretion of uric acid. However, the impact of reduced excretion of uric acid on decomposition of uric acid by intestinal flora of hyperuricemic patients is unknown. Therefore, we have hypothesized that hyperuricemia may also be linked to changes of probiotics in gut microbiome. To establish the mouse model of hyperuridemia, a high-purine diet and intraperitoneal injection of potassium oxonate at 300 mg/kg once a day were used to maintain a consistent high level of serum uric acid over 35 days. The data showed the reliability of the hyperuricemic mouse model.

Most of the studies on human gut bacteria use cultivation techniques to monitor bacterial populations, although it has been reported that less than 25% of the intestinal species are cultivable [24]. In the present study, we have used molecular biology techniques based on qRT-PCR analysis of 16S rRNA genes to determine the amount of probiotics, especially *bifidobacteria* and *lactobacilli*. The qRT-PCR technique has enabled the populations of *bifidobacteria* and *lactobacilli* to be directly quantified by using DNA isolated from fecal samples

[25]. We observe that the numbers of rRNA gene copies of both *bifidobacteria* and *lactobacilli* in fecal samples are significantly lower in HG mice on days 14-35 compared to those in NG mice. The observation indicates that the populations of intestinal flora are changed in hyperuricemic mice, mainly in the reduction of the probiotics *bifidobacteria* and *lactobacilli*. Interestingly, probiotic therapy is widely used clinically in a variety of diseases associated with the reduction of probiotics such as *bifidobacteria* and *lactobacilli*. However, little work has been conducted on whether probiotics reduces serum uric acid levels. In the present study, intragastric probiotic therapy with both *bifidobacteria* and *lactobacilli* has restored the amount of the probiotics beyond the normal level. In addition, serum UA level in HPG is decreased by more than 18% compared to that in HG mice after day 21. Therefore, the application of probiotics reduces serum UA level in hyperuricemic mice.

Imbalance of intestinal flora is accompanied by an increase of LPS in blood circulation. High level of serum LPS induces chronic inflammation that is closely associated with many diseases such as metabolic syndrome, obesity, and insulin resistance [10,11]. In addition, serum LPS level is reduced when mice are treated with probiotics in a metabolic disorder model [26]. XO is a key enzyme in the production of uric acid. Chronic inflammation induced by increasing serum LPS level is often accompanied by increased XO activity [27]. In the present study, serum LPS level in HG mice is significantly increased compared to that in NG mice on days 7 to 35. Serum XO activity in HG mice is increased by more than 26% compared to that in NG mice on days 7 to 35. After day 14, serum XO activity and LPS level in HPG mice are decreased by almost 20% compared to those in HG mice after probiotic therapy. Therefore, these results show clearly that serum LPS level is increased in hyperuricemic mice and accompanied by increased serum UA level and XO activity in HG mice. Moreover, probiotic therapy decreases not only LPS level but also serum XO activity and UA level. Therefore, the present study provides novel insights in understanding the relationship between hyperuricemia and intestinal flora.

There are also some limitations in the present study. Firstly, probiotic therapy reduces serum UA level in hyperuricemic mice, but UA concentration is still significantly higher than that in NG mice. Moreover, whether serum UA level is related to the dosage of probiotics therapy is unclear. Secondly, the mouse model resembles high serum UA level in hyperuricemic human patients, but the cause of human hyperuricemia is much more complex than that of mouse model. Therefore, it is still unknown whether probiotics has therapeutic effect on human hyperuridemic patients. In conclusion, the present study demonstrates that hyperuricemia is associated with changed intestinal flora, mainly in the reduction of probiotics such as *bifidobacteria* and *lactobacilli*. Probiotic treatment increases the amount of *bifidobacteria* and *lactobacilli* in intestinal flora, and decreases UA level, LPS level, and XO activity in serum.

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