Mineral water leads to a progressive increase of the microbiota diversity

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Abstract

Background: The human gut harbors trillions of microbes, strongly bearing great importance for the health of the host. However, the effect of drinking water on gut microbiota has been poorly understood. **Results:** In this study, we explored the response of BALB/c mice gut bacterial community (feces) to the different types of drinking water, including commercial bottled mineral water(MW), natural water(NW), purified water(PW) and tap water(TW). Feces were cultured with Brain Heart Infusion Broth dissolved in 4 types of drinking water. 16S rRNA sequencing analysis was performed. Our results reveal that the gut microbiota composition is different among culturing with 4 types of drinking water. As the culture time increases, the number of OTUs significantly decreased except under the aerobic condition of MW. Under the aerobic condition in the 5th day, the considerable differences of alpha diversity index are found between MW and 3 others, consistent to that there are the most unique taxa in MW group. Importantly, the LEfSe analysis discovers that the Bacteroidetes taxa dominate the differences between MW and the other water types. **Conclusion:** our findings demonstrate that the mineral water can lead to a progressive increase of the microbiota diversity by providing the growth convenience to Bacteroidetes.

Background

Our gut microbiota contains 100 trillion (10^{14}) of microorganisms, including at least 1000 different species of known bacteria with more than 3 million genes[1]. The microbial associates which reside in and on the human gut constitute our microbiota, and the genes which the microbiota encode is known as our microbiome[2]. These microorganisms residing in the gut plays a fundamental role in the well-being of their host[2]. Over the last two decades, many studies have been confirmed that there is a strong effect of human health status on the composition of this microbiota. Also, a strong impact of microbiota composition on host physiology has also been found [3-5].

The microbiota plays an important role in human disease and health; In fact, it is sometimes called for our “forgotten organ”[6]. The gut microbiota resides in the gastrointestinal tract, its role of microbiota in regulating intestinal diseases is not surprising. Many studies have been reported that the gut microbiome disorders are part of the etiology of various gastrointestinal diseases, especially Irritable Bowel Disease(IBD) and colorectal cancer (CRC) [7]. Moreover, Emerging data reveal that the gut microbiota is also involved in diverse diseases via gut–liver axis[8],gut–brain axis[9, 10], via gut-lung axis[11, 12], gut–bone axis[13, 14], gut–vascular axis[15], and other axes. The gut microbiota may modulate cirrhosis, non-alcoholic fatty liver disease (NAFLD), alcohol liver disease (ALD) and even hepatic carcinoma[16]. In childhood asthma, it may be closely associated with the reduced relative abundance and metabolite changes of the genus *Veillonella*, *Lachnospira*, *Faecalibacterium*, and *Rothia* [17]. Also, Alzheimer’s disease, parkinson's disease and mental illness are all related to gut microbiota[18, 19]. In addition, the gut microbiota is responsible for bone physiology, regulates bone mass and promotes bone formation and resorption through the immune system [20]. High salt may also promote autoimmunity by inducing T helper 17 (TH17) cells, which can also lead to hypertension. The induction of TH17 cells depends on gut microbiota[21].

The establishment and composition of gut microbiota are affected by multiple factors, including host genotype, age, pharmacological drugs, use of antibiotics, diet and stress. Recent studies of human populations and mouse model have shown that the abundance of gut microbiota is partly regulated by host genotype, and there is a significant consistency between the abundance of gut microbiota and host genotype [22, 23]. Available data showed that there is an increase in the number of enterobacteria and a decrease in the number of anaerobic bacteria, including bifidobacterial, in the elderly[24]. the use of Pharmacological drugs and antibiotics can have a major impact on the gut microbiota[25, 26]. Long-term diet affects the activity and structure of the trillions microorganisms living in the human gut[27]. Also, the gut microbiome can quickly respond to the changes in diet and promote the diversity of human dietary lifestyles[28].However, little is known about the effect of drinking water on gut microbiota.

In the present studies, we wished to determine whether different types of drinking water could drive the change of taxa within the gut microbiota. The response of mouse gut bacterial profile to four different types of drinking water were detected by 16S rRNA gene deep sequencing. This may provide a new insight into the influence factors of gut microbiota. Maybe we can regulate the establishment and composition of gut microbiota by drinking different types of water, in order to improve human health.

Methods

Experimental animals.

The BALB/c mice were obtained from the Tianjin Institute of Environmental and Operational Medicine (Tianjin, China), which were provided with standard rodent chow and water ad libitum. They were housed under ambient temperature ($23\pm1^{\circ}\text{C}$) and humidity (45%–60%) conditions. All animal experimental procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). The protocols were performed in accordance with approved guidelines specified by the Animal and Human Use in Research Committee of the Tianjin Institute of Environmental Medicine and Occupational Medicine. The mice were euthanized through CO_2 asphyxiation followed by cervical dislocation.

Experimental design and sampling

The four water types used in this study included tap water, purified water, natural water and mineral water. Feces of BALB/c mice were collected for the experiment. 0.08g Feces were dissolved into 8 ml saline, and then transfer 100 ul to 10 ml Brain Heart Infusion Broth with different types of water and respectively cultured for 24 hours in two conditions of both aerobe and anaerobe. After 24 hours, pipette 10 ul to 10 ml Brain Heart Infusion Broth with different types of water and continue to culture for 24h. Repeat the above step until 5th day. The samples were collected in the 1st day and 5th day and then stored at -20°C until DNA extraction. Three biological replicates started independently from the fecal suspension under each of the conditions. A total of 48 biological samples were collected for 16S rRNA sequencing.

DNA extraction, library construction

Total genome DNA from all samples was extracted using CTAB/SDS method. DNA concentration and purity were monitored on 1% agarose gels. The V4 region of the 16S rRNA gene was amplified using the dual-index primers described by Kozich et al. with a few modifications to the PCR assay. Each of these dual-index primers contains an Illumina adapter, an 8-nt index sequence, a 10-nt pad sequence, a 2-nt linker, and the V4 primers F515 and R806(5'-GTGY-CAGCMGCCGCGGTAA-3' and 5'-GACTACHVGGGTATCTAATCC-3'). All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Mix same volume of 1X loading buffer (contained SYB green) with PCR products and operate electrophoresis on 2% agarose gel for detection. PCR products was mixed in equal-density ratios. Then, mixture PCR products was purified with GeneJET™ Gel Extraction Kit (Thermo Scientific). Sequencing libraries were generated using Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific) following manufacturer's recommendations. The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific). At last, the library was sequenced on an IonS5™ XL platform and 400 bp/600 bp single-end reads were generated.

Single-end reads assembly and quality control

Single-end reads was assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Quality filtering on the raw reads were performed under specific filtering conditions to obtain the high-quality clean reads according to the Cutadapt V1.9.1, <http://cutadapt.readthedocs.io/en/stable/quality> controlled process. The reads were compared with the reference database (Gold database, http://drive5.com/uchime/uchime_download.html) using UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html) to detect chimera sequences, and then the chimera sequences were removed. Then the Effective Tags finally obtained.

OTU cluster and Species annotation

Sequences analysis were performed by Uparse software (Uparse v7.0.1001, <http://drive5.com/uparse/>). Sequences with $\geq 97\%$ similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. For each representative sequence, the Silva Database (<https://www.arb-silva.de/>) was used based on RDP classifier (Version 2.2 <http://sourceforge.net/projects/rdp-classifier/>) algorithm annotate taxonomic information. In order to study phylogenetic relationship of different OTUs, and the difference of the dominant species in different samples(groups), multiple sequence alignment was conducted using the MUSCLE software (Version 3.8.31, <http://www.drive5.com/muscle/>). OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed basing on this output normalized data.

Diversity and statistical analysis.

Alpha diversity is applied in analyzing complexity of species diversity for a sample through 6 indices, including Observed-species, Chao1, Shannon, Simpson, ACE, Good-coverage. All these indices in our samples were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). Beta diversity on both weighted and unweighted unifrac were calculated by QIIME software (Version 1.7.0). Cluster analysis was preceded by principal component analysis (PCA), which was applied to reduce the dimension of the original variables using the FactoMineR package and ggplot2 package in R software (Version 2.15.3). Principal Coordinate Analysis (PCoA) was performed to get principal coordinates and visualize from complex, multidimensional data. A distance matrix of weighted or unweighted unifrac among samples obtained before was transformed to a new set of orthogonal axes, by which the maximum variation factor is demonstrated by first principal coordinate, and the second maximum one by the second principal coordinate, and so on. PCoA analysis was displayed by WGCNA package, stat packages and ggplot2 package in R software (Version 2.15.3). Unweighted Pair-group Method with Arithmetic Means (UPGMA) Clustering was performed as a type of hierarchical clustering method to interpret the distance matrix using average linkage and was conducted by QIIME software (Version 1.7.0).

Results

Study setup and data generation

The main purpose of this study was to evaluate the effects of different water types on gut microbial diversity (Figure 1A). 4 drinking water types included mineral water (MW), natural water (NW), purified water (PW) and tap water (TW). To illustrate the differences between the 4 water types, the chemical analysis of water was detected by the Standard Examination Methods for Drinking Water (GB/T 5750-2006/GB 8538-2016). A total of 44 indicators were showed in Supplementary Table 1. Compared with pure water and tap water, metasilicate is higher in mineral water and natural water. The content of strontium is respectively 49.89 $\mu\text{g/L}$, 83.70 $\mu\text{g/L}$, 167.6 $\mu\text{g/L}$ in natural water, mineral water (MW) and tap water (TW). While, the content of barium antimony and sulfate in tap water were higher than the other types of water. Moreover, the content of sodium is the highest in the mineral water than the other types of water. The content of magnesium and potassium is highest in the natural water. The content of cadmium in tap water is as high as 48.4.

As shown in Figure 1A, mice feces were cultured using Brain heart infusion broth with four types of water on both the aerobic (A) and anaerobic (N) conditions. Microbial samples were collected on the 1st day and 5th day respectively. Each experimental group have 3 biological replicates. A total of 48 samples were performed on 16S rRNA sequencing, resulting in 3,215,183 clean reads after quality filtering and chimera removal (Supplementary Table 2). On average 226 OTUs (Operational Taxonomic Units) were assigned to each sample (Supplementary Table 3).

The effects of water types on OTUs number

The analysis of OTUs revealed that differential number of microorganisms caused by 4 distinct water types under multiple culture conditions (Figure 1B). At the time point of 1 day, the OTUs under aerobic culture were significantly more than that under the anaerobic culture (two-tailed t-test $p < 1E-15$; Figure 1C). As the culture time increased, the number of OTUs significantly decreased except under the aerobic condition of MW. For the 3 water types of NW, PW and TW, the average falling number from 1 day to 5 days under the aerobic condition was clearly bigger than that under the anaerobic condition, suggesting that aerobic culture was more detrimental to long-term growth of gut microbiota. Notably, MW had the ability to maintain the OTUs number under the aerobic condition, which maybe resulted from the benefit of the minerals contained in MW to the microbial growth.

The effects of water types on the species composition and abundance of gut microbiota.

To further investigate the species composition and abundance in different samples, each OTU was assigned to species annotation. First, the top 10 abundant taxa (phylum level and Genus level) were shown in Figure 2A and 2B, among of which Proteobacteria, Firmicutes and Bacteroidetes accounted the majority. Generally, as the culture time increased, the relative abundance of Proteobacteria decreased and Firmicutes increased on both the aerobic and anaerobic conditions. Also, as the culture time increased, the relative abundance of *Escherichia Shigella* decreased and *Ralstonia* increased in the Genus level. Moreover, in MW.A.5d group, the ratio of Bacteroidetes was clearly more than others. While, the ratio of *Escherichia Shigella* was significantly decreased in MW.A.5d and MW.N.5d group. Next, differential abundance of taxa in distinct samples was observed by the unsupervised clustering for top 35 abundant taxa in genus level (Figure 2C). The “MW.A.5d” group had many unique high-abundance taxa of Proteobacteria phylum type, including *Ralstonia*, *Stenotrophomonas*, *Sphingomonas*, *Zoogloea* and et. al. *Bacillus* and *Staphylococcus* were specifically high in the “PW.A.1d” group, both of them belong to Firmicutes phylum type.

Mineral water results in a progressive increase of the microbiota diversity

Additionally, the alpha diversity measures were used to evaluate the microbiota diversity in each sample. The significant differences of Shannon and Simpson index were found between MW and 3 others under the aerobic condition in the 5th days (Figure 3A and B), consistent to the results of the taxa number analysis (Figure 3C). Under the aerobic and 1 day condition, the PW had the most complex microbial composition, which was also evidenced by the 102 unique taxa number of PW (Figure 3D). The pure water could provide the appropriate and clear environment for aerobic taxa in the initial phase, however,

this advantage gradually disappeared with the increase of cultivation time. While under the anaerobic culture, the MW and TW displayed the superiorities in microbiota diversity.

Mineral water provided the growth convenience to Bacteroidetes

The comparison of beta diversities between different water type groups was applied to examine the differences of microbiota composition. The Principal Component Analysis (PCoA) revealed that the primary differences were dominated by the culture time and the aerobic or anaerobic condition also distinguished the different groups to a certain extent (Figure 4A). Interestingly, MW.A.5d was still distinct from the other groups, revealing that it was important to investigate the microbiota taxa specifically present in MW group. The LDA Effect Size (LEfSe) analysis was performed to discover significantly differential taxa between MW.A.5d and 3 other water types. 6 taxa were identified to be enriched in MW.A.5d ($p < 0.05$), including *f__Bacteroidaceae*, *c__Bacteroidia*, *p__Bacteroidetes*, *g__Bacteroides*, *o__Bacteroidales*, and *s__Bacteroides_vulgatus* (Figure 4B and C). All these 6 taxa belong to the Bacteroidetes type in the phylum level. So, under the aerobic condition, mineral water provided the growth convenience to Bacteroidetes. The relative abundance of Bacteroidetes in MW group was also significantly higher than others (two-tailed t-test $p < 0.05$, Figure 4D). As the relative proportion of Bacteroidetes was associated with metabolic syndrome, liver cirrhosis and behavioral and physiological abnormalities, mineral water may be used to the prevention and cure of many disease by inducing the growth convenience to Bacteroidetes.

Discussion

We are increasingly aware of the human gut microbiota as an contributor and indicator to human health, suggesting that it will play an important role in the diagnosis, treatment and eventual prevention of human disease [3, 29, 30]. Therefore, it has been paid more and more attention to how extrinsic disturbances affect the gut microbiota in recent years. Many studies reported that environmental factors, host genetics, diet, stress, disease and some other factors influence the structure of the gut microbiota [31-34]. But, the possible effects of drinking water on gut community composition and diversity is still poorly understood. Our study compared the effects of 4 types of drinking water on the gut microbiota, A key finding of this study is that gut microbiota composition was different by culturing with different types of drinking water. Also, as the culture time increased, Mineral water leads to a progressive increase of the microbiota diversity.

To our knowledge, only a few study so far has addressed the impact of drinking water on gut microbiota by some experiment [35]. They think that gut microbiota interact with water bacteria to promote the spread of mobile genetic elements through horizontal gene transfer. But The effect of drinking water only as a culture medium on gut microbiota has not been elucidated. Also, many external factors may affect the quality and quantity of the microbiota. Special factors such as diet and personal hygiene. In our

studies, all of the drinking water are sterilized by vertical pressure steam sterilizer. We found that the number of OTUs is different among culturing with 4 types of drinking water. As the culture time increases from 1 day to 5 days, the number of OTUs significantly decreased except under the aerobic condition of MW. Also, Proteobacteria, Firmicutes and Bacteroidetes are the main phyla observed in the samples which is consistent with the previous studies[36, 37]. While the phylum level and Genus level of the sample were different among culturing with 4 types of drinking water. Moreover, the “MW.A.5d” group had many unique high-abundance taxa of Proteobacteria phylum type, including *Ralstonia*, *Stenotrophomonas*, *Sphingomonas*, *Zoogloea* and et. al, which suggest that MW may have the unique effect on gut microbiota.

The gut microbiota has been a topic of immense interest over the last years, as its composition and diversity seem to be intimately linked to health and disease[38] . A higher diversity of the microbial composition leads to a more efficient system, providing a mechanistic base of the general notion that a more diverse microbiota is associated with improved health status[39]. We found that the pure water could provide the appropriate and clear environment for aerobic taxa in the initial phase, however, this advantage gradually disappeared with the increase of cultivation time. While under the anaerobic culture, the MW and TW displayed the superiorities in microbiota diversity. Regulating microbiota through external factors such as fecal transplants and dietary interventions has proven to be a promising therapeutic approach to improve multiple health conditions. [28, 40, 41]. Drinking different types of water may be a new therapeutic route to improve numerous health.

Dysbiosis of the microbiota are associated with a large and growing number of diseases. Recent researches have mostly focused on the analysis of the relationship between an aberrant microbiota composition and disease [4]. The relative proportion of some major phyla of gut bacteria, such as Bacteroidetes–Firmicutes and proteobacteria (a lower proportion of Firmicutes, higher abundance Bacteroidetes and proteobacteria), was associated with type 2 diabetes[42]. Also, a lower proportion of Bacteroidetes was associated with metabolic syndrome and liver cirrhosis[43, 44]. Moreover, Oral treatment of MIA offspring with the human symbiotic *Bacteroides fragilis* can improve gut permeability, alters microbial composition, and ameliorates communication disorders, stereotyping, anxiety and sensorimotor behaviors deficits [5]. We found that 6 taxa were identified to be enriched in MW.A.5d ($p < 0.05$), including *f__Bacteroidaceae*, *c__Bacteroidia*, *p__Bacteroidetes*, *g__Bacteroides*, *o__Bacteroidales*, and *s__Bacteroides_vulgatus*, which belong to the Bacteroidetes type in the phylum level. These results demonstrated that mineral water provided the growth convenience to Bacteroidetes. As the relative proportion of Bacteroidetes was associated with many kinds of disease, mineral water may be used to the prevention and cure of these disease by inducing the growth convenience to Bacteroidetes.

Conclusion

In conclusion, our results suggest that the gut microbiota composition was different by culturing with different types of drinking water. And, more importantly, our findings demonstrate that Mineral water leads to a progressive increase of the microbiota diversity and provides the growth convenience to

Bacteroidetes. As more diseases are linked to the Bacteroidetes and the microbiota is targeted therapeutically, microbiota reprogramming may need to involve strategies that incorporate drinking water.

Abbreviations

MW: mineral water; NW: natural water; PW: purified water; TW: tap water; IBD: Irritable Bowel Disease; CRC: colorectal cancer; NAFLD: non-alcoholic fatty liver disease; ALD: alcohol liver disease; PCA: principal component analysis; PCoA: Principal Coordinate Analysis; UPGMA: Unweighted Pair-group Method with Arithmetic Means; LEfSe: LDA Effect Size.

Declarations

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Availability of data and materials

The datasets used and analysed during this study are available from the corresponding author on reasonable request.

Author Contributions

SZQ, XQY and LWL designed research; ZK, LWL, CZL, YD, YZW, FH, LC, JM, YJ, WHR and LJW performed experiments; LWL and SZQ wrote the paper.

Ethics approval and consent to participate

All animal experimental procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). The protocols were performed in accordance with approved guidelines specified by the Animal and Human Use in Research Committee of the Tianjin Institute of Environmental Medicine and Occupational Medicine.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The experimental design and comparison of OTUs number. (A) Schematic of sample preparation for 16S rRNA sequencing. Mice feces were cultured using Brain heart infusion broth with four types of water on both the aerobic (A) and anaerobic (N) conditions. 4 water types included mineral water (MW), natural water (NW), pure water (PW) and tap water (TW). Microbial samples were collected on 1 day and 5 days respectively. (B) The number of OTUs in the different groups. Box plots showed median number and the first and third quartile, whiskers showed 1.5 folds of the inter-quartile range. (C) The difference analysis of OTUs number between the aerobic (A) and anaerobic (N) conditions on 1st and 5th day. Two-tailed t-test was used (***, $p < 1e-15$).

Figure 2

Microbial composition of distinct water type groups. Bar plots showed the percentage of the relative abundance of top 10 taxa in the Phylum level (A) and in the Genus level (B). (C) The unsupervised clustering for top 35 abundant taxa in genus level.

Figure 3

Microbial community diversity between 4 water types in different culture conditions. Comparison of microbial community diversity between MW, NW, PW and TW was evaluated by the alpha diversity measures, including Shannon index (A) and Simpson index (B). 4 culture conditions were respectively the combination of aerobic or anaerobic and 1st or 5th day. Box plots showed median number and the first and third quartile, whiskers showed 1.5 folds of the inter-quartile range. (C) The venn polt illustrated the overlap of taxa number between 4 water types on the aerobic condition of 5th day. (D) The venn polt illustrated the overlap of taxa number between 4 water types on the aerobic condition of 1st day.

Figure 4

Overrepresented taxa in the mineral water. (A) The principal component analysis of the microbiota for the 4 water types on the different conditions. Each color dot represented the corresponding gut microbiota. (B) Significantly different taxa between MW and 3 other water types revealed by the LDA Effect Size analysis. The horizontal axis represented the LDA scores. (D) The relative abundance of Bacteroidetes in the mineral water on 5th day. (D) The evolutionary cladogram demonstrated the statistically overrepresented taxa in MW.A.5d group (red color marked terms) and NW.A.5d (green color marked terms). The circles from inside to outside represented the classification levels from Phylum, Class, Order, Family, Genus to Species. Each dot in the circle was a subtype in the corresponding level. The dot of taxa without significant difference was colored by yellow.

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