The effect of different drinking water in culture medium on feces microbiota diversity

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ABSTRACT

The human gut harbors trillions of microbes, which are extremely important to the health of the host. However, the effect of drinking water on gut microbiota has been poorly understood. 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 four types of drinking water. 16S rRNA gene analysis was performed. Our results reveal that the microbiota composition is different among culturing with four types of drinking water. As the culture time increases, the number of OTUs significantly decreased, except under the aerobic condition of MW. Under aerobic conditions on the 5th day, the considerable differences of alpha diversity index are found between MW and three others, and these are the most unique taxa in the MW group. Importantly, the LEfSe analysis discovers that the *Bacteroidetes* taxa dominate the differences between MW and the other water types. Our findings demonstrate that the mineral water as a culture medium may lead to a progressive increase of the gut microbiota diversity by providing the growth convenience to *Bacteroidetes*.

Key words | 16S rRNA, drinking water, gut microbiota

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HIGHLIGHTS

- The gut microbiota composition was different by culturing with different types of drinking water.
- Mineral water leads to a progressive increase of the microbiota diversity and provides the growth convenience to Bacteroidetes.

INTRODUCTION

Our gut microbiota contains 100 trillion (10^{14}) of microorganisms, including at least 1,000 different species of known bacteria with more than three million genes (Whitman *et al.* 1998). The microbial associates which reside in and on the human gut constitute our microbiota, and the genes which the microbiota encode are known as our microbiome (Clemente *et al.* 2012). These microorganisms residing in the gut play a fundamental role in the well-being of their host (Clemente *et al.* 2012). Over the last two decades, many studies have 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 (Hsiao *et al.* 2013; Andoh 2016; Feng *et al.* 2018).

Intestinal microbiota–host interactions play a critical role in the regulation of human physiology. Deleterious

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changes to the composition of gut microbiota have been linked to the development and progression of numerous diseases. Many studies have reported that the gut microbiome disorders are part of the etiology of various gastrointestinal diseases, especially Irritable Bowel Disease (IBD) and colorectal cancer (CRC) (Gao *et al.* 2015). Apart from gastrointestinal diseases, the gut microbiota modulate cirrhosis, non-alcoholic fatty liver disease (NAFLD), alcohol liver disease (ALD) and even hepatic carcinoma (Betrapally *et al.* 2017). In childhood asthma, it may be closely associated with the reduced relative abundance and metabolite changes of the genus *Veillonella*, *Lachnospira*, *Faecalibacterium* and *Rothia* (Arrieta *et al.* 2015). Also, Alzheimer's disease, Parkinson's disease and mental illness are all related to gut microbiota (Scheperjans *et al.* 2015; Leue *et al.* 2017). In addition, the gut microbiota is responsible for bone physiology, regulating bone mass and promoting bone formation and resorption through the immune system (Ohlsson & Sjogren 2015).

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 models 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 (Bonder *et al.* 2016; Goodrich *et al.* 2016). 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 (Mueller *et al.* 2006). The use of pharmacological drugs and antibiotics can have a major impact on the gut microbiota (Iizumi *et al.* 2017; de Gunzburg *et al.* 2018). Long-term diet affects the activity and structure of the trillions of microorganisms living in the human gut (Wu *et al.* 2011). Also, the gut microbiome can quickly respond to changes in diet and promote the diversity of human dietary lifestyles (David *et al.* 2014). However, little is known about the effect of drinking water on gut microbiota. Many external factors may affect the quality and quantity of the microbiota, such as diet and personal hygiene, but the effect of drinking water only as a culture medium on gut microbiota has not been elucidated.

In the present study, we wished to determine whether different types of drinking water only as a culture medium could drive the change of taxa within the gut microbiota. The response of the 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.

METHODS

Experimental animals

BALB/c mice were obtained from the Tianjin Institute of Environmental and Operational Medicine (Tianjin, China), and they were provided with standard rodent chow and water ad libitum. They were housed under ambient temperatures of $23 \pm 1\,^{\circ}C$ and 45–60% humidity. 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 the 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, purified, natural and mineral water. Feces of BALB/c mice were collected for the experiment and 0.08 g of feces were dissolved into 8 mL saline, and then 100 ul of the mixture was transferred to 10 mL brain heart infusion broth with different types of water and respectively cultured for 24 hours under both aerobe and anaerobe conditions. The culture medium of brain heart infusion broth with different types of water was sterilized. After 24 hours, 10 ul was pipetted to 10 ml brain heart infusion broth with different types of water and culture was continued for 24 h. The above steps were repeated until the 5th day. The samples were collected on the 1st and 5th day and then stored at −20° until DNA extraction. Three biological replicates were started separately from the fecal suspension under

each of the conditions. A total of 48 biological samples was collected for 16S rRNA sequencing.

DNA extraction, library construction

Total genome DNA from all samples was extracted using the 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.* (2013) 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′-GAC-TACHVGGGTATCTAATCC-3′). All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). An equal volume of 1× loading buffer (contained SYB green) was mixed with PCR products and electrophoresed on a 2% agarose gel for detection. PCR products were mixed in equal-density ratios. Then, mixture PCR products were purified with a GeneJETTM Gel Extraction Kit (Thermo Scientific). Sequencing libraries were generated using Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific) following the manufacturer's recommendations. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific). Finally, the library was sequenced on an IonS5^{TM} XL platform and 400 bp/600 bp single-end reads were generated.

Single-end reads assembly and quality control

Single-end reads were 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 quality control process (V1.9.1, http://cutadapt.readthedocs.io/en/stable/). The reads were compared with the reference database (Gold database, http://drive5.com/uchime/uchime_download.html) using the UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html) to detect chimera sequences, and then the chimera sequences were removed and the effective tags finally obtained.

Operational taxonomic unit (OTU) cluster and species annotation

Sequences analysis were performed by Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/). Sequences with ≥97% similarity were assigned to the same OTUs. The 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 the RDP classifier (Version 2.2 http://sourceforge.net/projects/rdp-classifier/) algorithm annotate taxonomic information. In order to study the phylogenetic relationship of different OTUs, and the difference of the dominant species in different samples (groups), multiple sequence alignment was conducted using MUSCLE software (Version 3.8.31, http://www.drive5.com/muscle/). OTUs abundance information was normalized using a standard of sequence numbers corresponding to the sample with the least sequences. Subsequent analysis of alpha and beta diversity was carried out based on this output normalized data.

Diversity and statistical analysis

Alpha diversity was applied in analyzing the complexity of species diversity for a sample through six 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 obtain principal coordinates and visualize them 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 the first principal coordinate, and the second maximum variation factor 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). An 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 1(a)). The four drinking water types included mineral water (MW), natural water (NW), purified water (PW) and tap water (TW). To illustrate the differences between the four water types, chemical analysis of the water was detected by the Standard Examination Methods for Drinking Water (GB/T 5750-2006/GB 8538-2016). A total of 44 indicators are shown in Supplementary Material, Table 1. Compared with pure water and tap water, metasilicate is higher in mineral water and natural water. The content of strontium in natural water, mineral water (MW) and tap water (TW) is 49.89, 83.70 and 167.6 μg/L, respectively, while the content of barium antimony and sulfate in tap water was higher than the other types of water. Moreover, the content of sodium was the highest in the mineral water. The content of

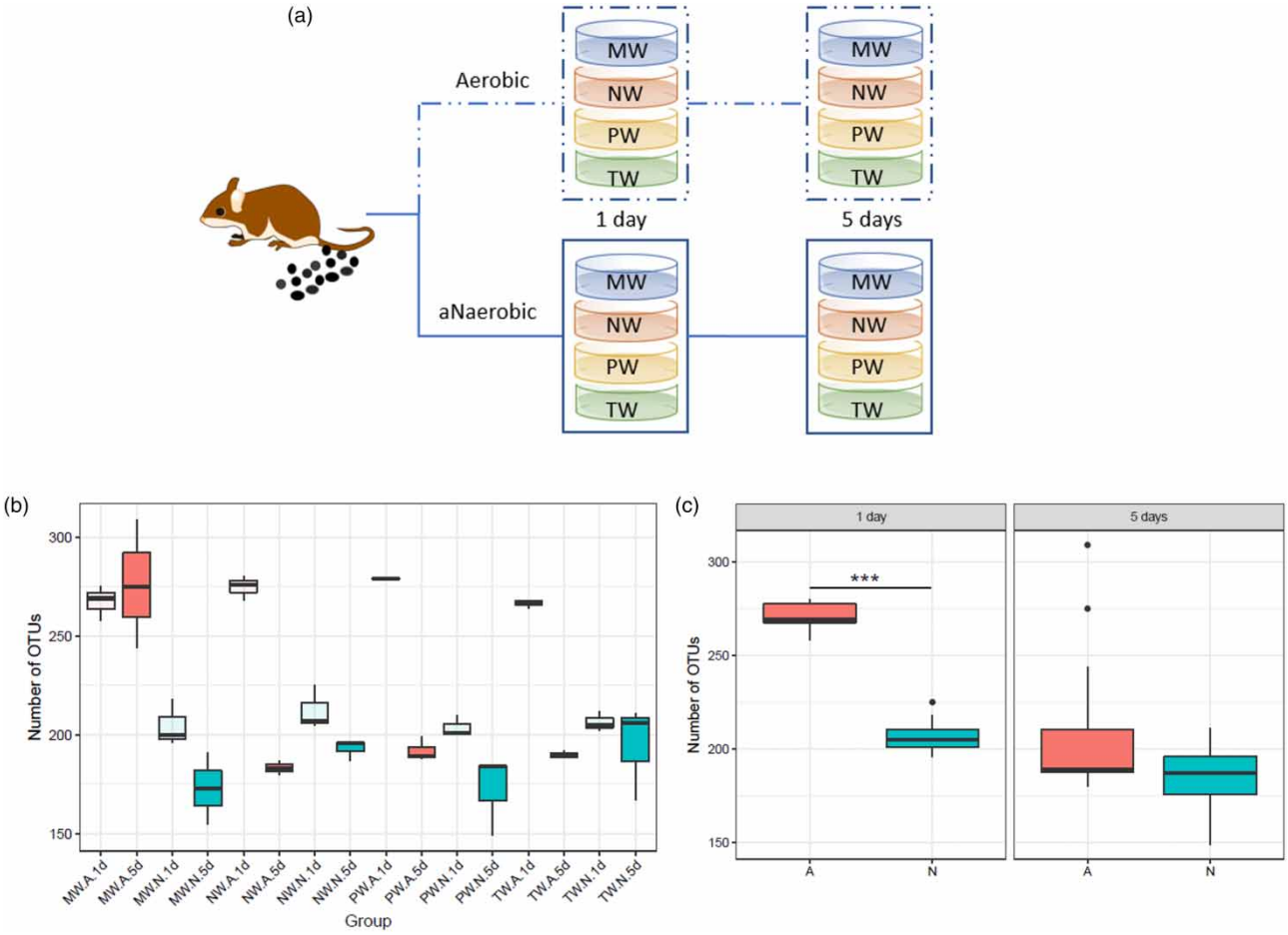

Figure 1 | The experimental design and comparison of OTUs number. (a) Schematic of sample preparation for 16S rRNA sequencing. Mice feces were cultured using brain heart infusion broth with four types of water in both aerobic (A) and anaerobic (N) conditions. Four water types included mineral water (MW), natural water (NW), pure water (PW) and tap water (TW). Microbial samples were collected on days 1 and 5 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-fold of the inter-quartile range. (c) The difference analysis of OTUs number between aerobic (A) and anaerobic (N) conditions on 1st and 5th day. Two-tailed t-test was used (***, $p < 1 \times 10^{-15}$).

magnesium and potassium was the highest in the natural water. The content of calcium in tap water was as high as 48.4 mg/L.

As shown in Figure 1(a), 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 and 5th day respectively. Each experimental group had three 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 Material, Table 2). On average, 226 OTUs (operational taxonomic units) were assigned to each sample (Supplementary Material, Table 3).

Effects of water types on OTUs number

The analysis of OTUs revealed that differential number of microorganisms was caused by four distinct water types under multiple culture conditions (Figure 1(b)). On day 1, the OUTs under aerobic culture were significantly more than that under the anaerobic culture (two-tailed t-test, $p < 1 \times 10^{-15}$; Figure 1(c)). As the culture time increased, the number of OTUs significantly decreased, except under the aerobic condition of MW. For the three water types NW, PW and TW, the average falling number from 1 day to 5 days under aerobic conditions was clearly larger than that under anaerobic conditions, 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 aerobic conditions, which perhaps resulted from the benefit of the minerals contained in MW to the microbial growth.

Effects of water types on the species composition and abundance of microbiota during culture

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 2(a, b), of which Proteobacteria, Firmicutes and Bacteroidetes were the majority. Generally, as the culture time increased, the relative abundance of Proteobacteria decreased and Firmicutes increased in both 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 the MW.A.5d group, the ratio of Bacteroidetes was more than others, while the ratio of *Escherichia Shigella* was significantly decreased in the MW.A.5d and MW.N.5d groups. Next, differential abundance of taxa in distinct samples was observed by the unsupervised clustering for the top 35 most abundant taxa in the genus level (Figure 2(c)). The MW.A.5d group had many unique high-abundance taxa of Proteobacteria phylum type, including *Ralstonia*, *Stenotrophomonas*, *Sphingomonas* and *Zoogloea*. *Bacillus and Staphylococcus* were specifically high in the PW.A.1d group, both of them belong to Firmicutes phylum type.

Mineral water in culture medium leads to a progressive increase of the microbiota diversity

Additionally, the alpha diversity measures were used to evaluate the microbiota diversity in each sample. Significant differences of Shannon and Simpson index were found between MW and three others under aerobic conditions on the 5th day (Figure 3(a, b)), consistent with the results of the taxa number analysis (Figure 3(c)). Under aerobic and 1-day conditions, the PW had the most complex microbial composition, which was also evidenced by the 102 unique taxa number of PW (Figure 3(d)). 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 superiorities in microbiota diversity.

Mineral water as a culture medium provided growth convenience to Bacteroidetes

A comparison of beta diversities between different water type groups was applied to examine the differences of microbiota composition. Principal Component Analysis (PCoA) revealed that the primary differences were dominated by the culture time and the aerobic or anaerobic conditions also distinguished the different groups to a certain extent (Figure 4(a)). Interestingly, MW.A.5d was still distinct from the other groups, revealing that it was important to

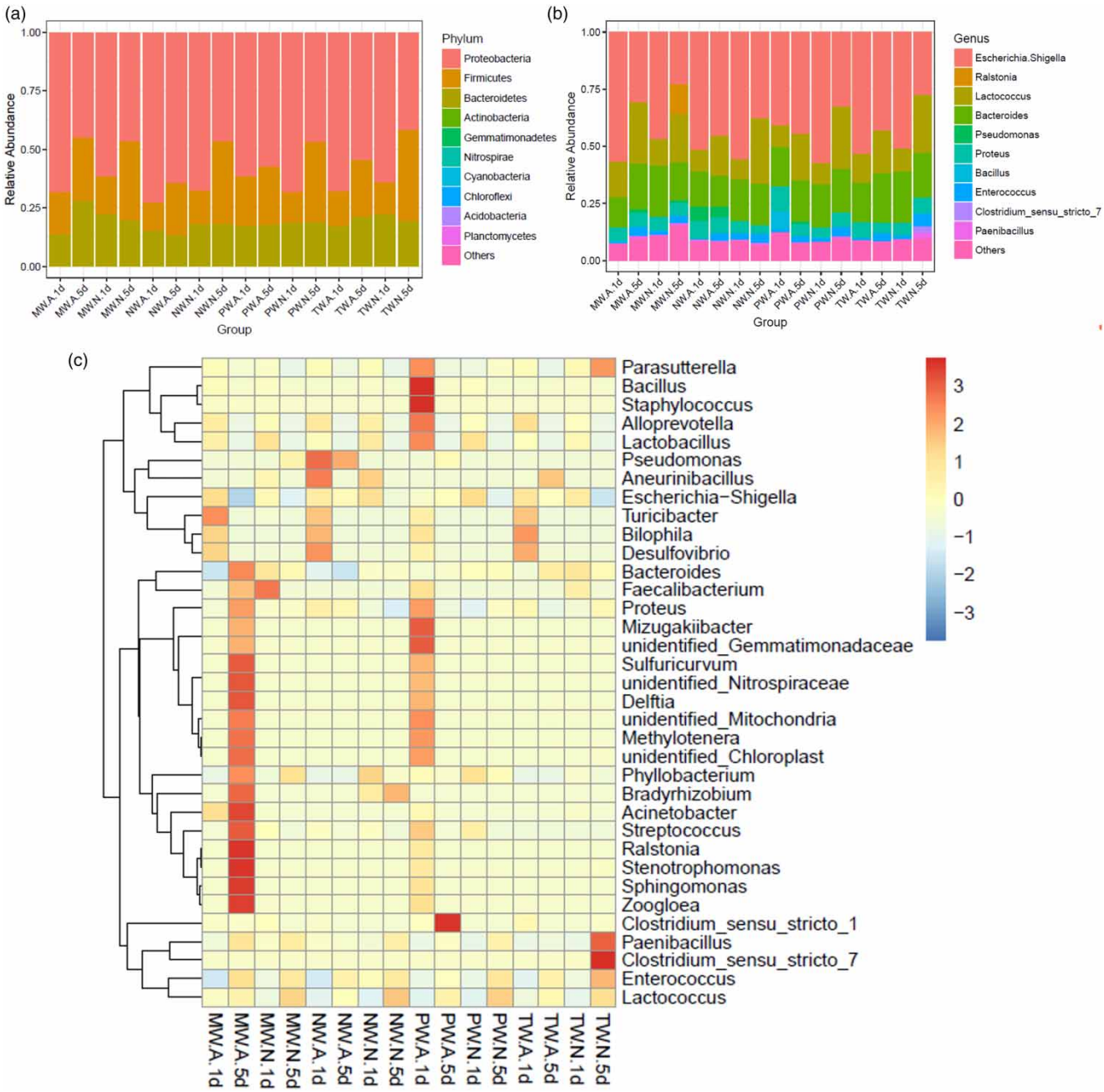

Figure 2 | Microbial composition of distinct water type groups. Bar plots showing the percentage of the relative abundance of top ten taxa in the Phylum level (a) and in the Genus level (b). (c) The unsupervised clustering for top 35 abundant taxa in genus level.

investigate the microbiota taxa specifically present in the MW group. The LDA Effect Size (LEfSe) analysis was performed to discover significantly differential taxa between MW.A.5d and three other water types. Six 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 4(b) and (c)). All these six taxa belong to the Bacteroidetes type in the phylum level. So, under aerobic conditions, mineral water provided the growth convenience

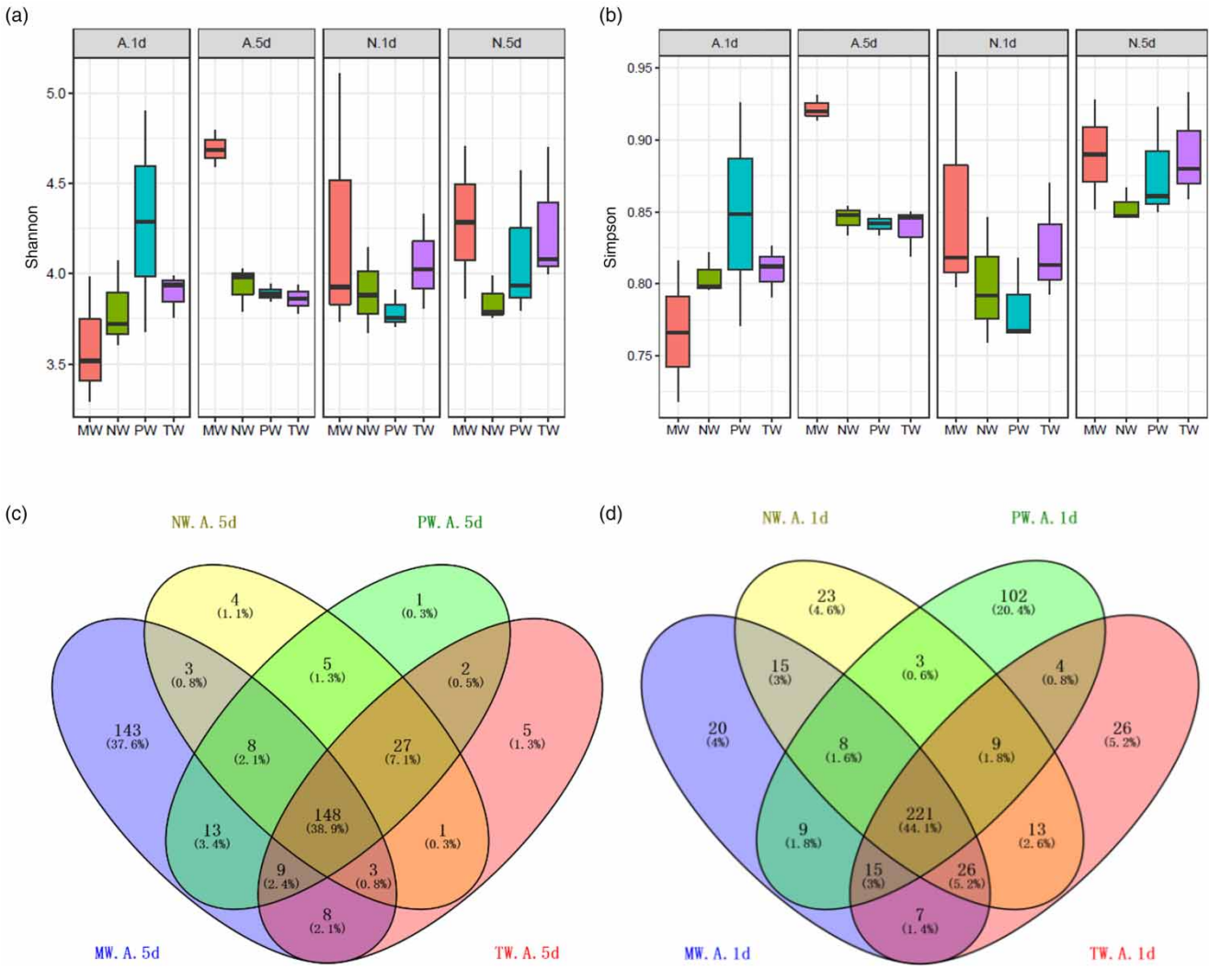

Figure 3 | Microbial community diversity between four water types in different culture conditions. Comparison of microbial community diversity between MW, NW, PW and TW was evaluated by the alpha diversity measures, including Shannon index (a) and Simpson index (b). Four culture conditions were respectively 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-fold of the inter-quartile range. (c) The Venn plot illustrates the overlap of taxa number between four water types on the aerobic condition of the 5th day. (d) The Venn plot illustrates the overlap of taxa number between four water types on the aerobic condition of the 1st day.

to Bacteroidetes. The relative abundance of Bacteroidetes in the MW group was also significantly higher than other groups (two-tailed t-test $p < 0.05$, Figure 4(d)).

DISCUSSION

We are increasingly aware of the human gut microbiota as a contributor and indicator to human health, suggesting that it will play an important role in the diagnosis, treatment and eventual prevention of human disease (Faith *et al.* 2013; Andoh 2016; von Martels *et al.* 2017). Therefore, in recent years, much attention has been paid as to how extrinsic disturbances affect the gut microbiota. Many studies reported that environmental factors, host genetics, diet, stress, disease and some other factors influence the structure of the gut microbiota (Sartor 2010; Partrick *et al.* 2018; Tsilimigras *et al.* 2018; Zhang *et al.* 2018). However, the possible effects of drinking water on gut community composition and diversity is still poorly understood. Our study compared the

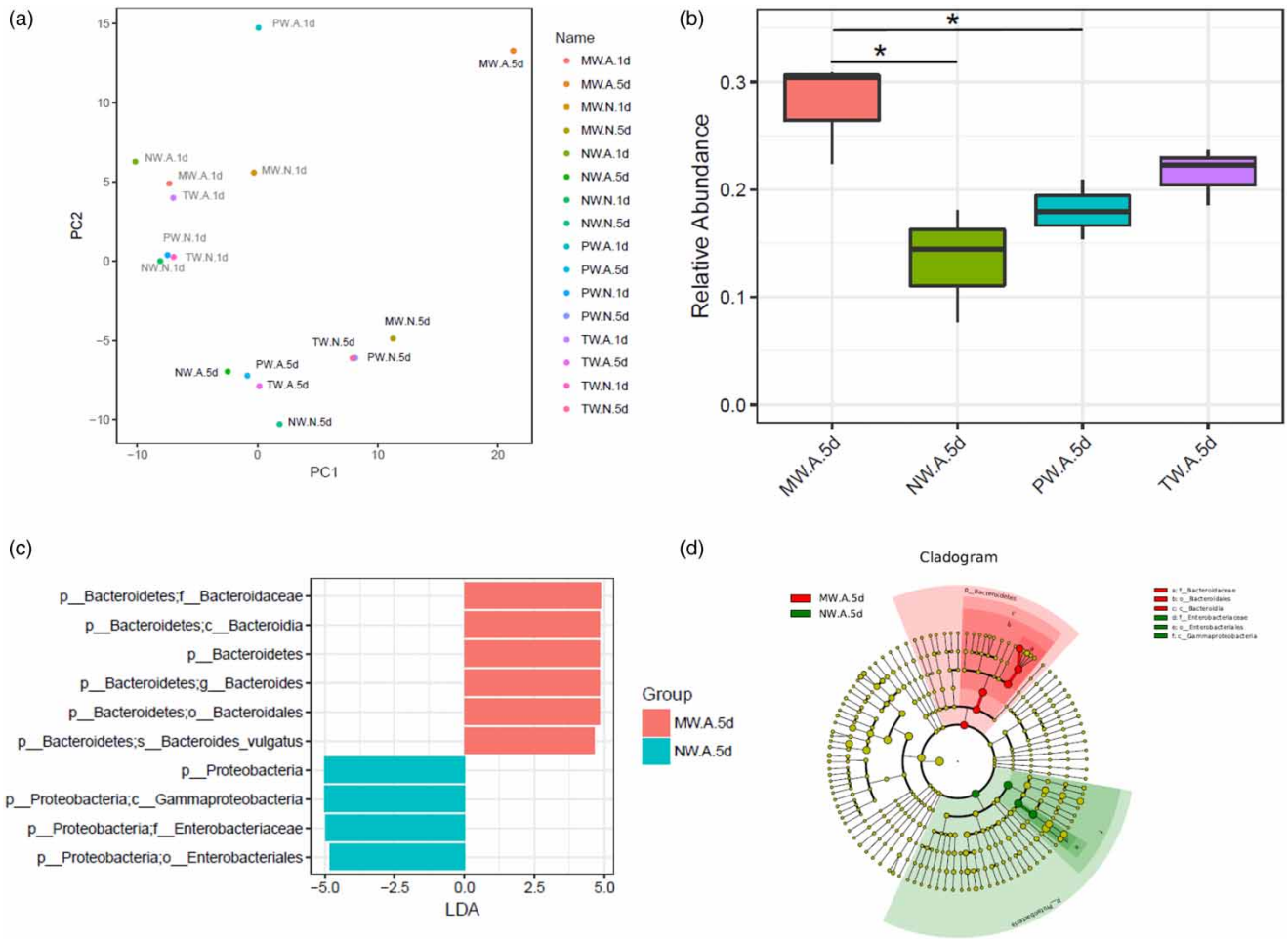

Figure 4 | Over-represented taxa in the mineral water. (a) The principal component analysis of the microbiota for the four water types in different conditions. Each color dot represents the corresponding gut microbiota. (b) Significantly different taxa between MW and three other water types revealed by the LDA effect size analysis. The horizontal axis represented the LDA scores. (c) The relative abundance of Bacteroidetes in the mineral water on the 5th day. (d) The evolutionary cladogram demonstrated the statistically overrepresented taxa in the MW.A.5d group (marked in red) and NW.A.5d (marked in green). 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 is colored yellow. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wh.2020.075.

effects of four types of drinking water on gut microbiota. A key finding of this study is that gut microbiota composition was different when culturing with different types of drinking water. Also, as the culture time increased, mineral water led to a progressive increase of the microbiota diversity.

To our knowledge, only a few studies have so far addressed the impact of drinking water on gut microbiota. They focused on the interaction between water bacteria and gut microbiota. The results showed there are typical drinking water taxa in the intestinal tract, illustrating that water microorganisms played a role in intestinal microbiota dynamics (Dias *et al.* 2018). However, the effect of drinking water only as a culture medium on gut microbiota has not been elucidated. It has been reported that diet affected the activity and structure of the trillions of microorganisms living in the human gut. Like food, drinking water is an essential substance in our life. We drink a lot of water every day, and gut microbiota are always in the drinking water environment. Because biological systems, and gut microbiota in particular, are subjected to many uncontrolled processes, it is frequently difficult to point out a single factor that would be predominantly responsible for observed differences *in vivo*. In order to eliminate the intervention factors, we adopted a preliminary observation *in vitro*. Also, the culture medium of brain heart infusion broth with different types of water was sterilized to eliminate the intervention

of water microorganisms. We found that the number of OTUs was different among culturing with four types of drinking water. As the culture time increased from 1 to 5 days, the number of OTUs significantly decreased, except under the aerobic conditions of MW. Also, Proteobacteria, Firmicutes and Bacteroidetes were the main phyla observed in the samples which is consistent with previous studies (Weldon *et al.* 2015; Xiao *et al.* 2015), while the phylum level and genus level of the samples were different among culturing with four 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* etc., which suggests that MW may have a unique effect on gut microbiota.

The gut microbiota has been a topic of immense interest over the last ten years, as its composition and diversity seem to be intimately linked to health and disease (Cho & Blaser 2012). 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 (Larsen & Claassen 2018). We found that 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 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 (David *et al.* 2014; Brandt 2017; Maida *et al.* 2018). Drinking different types of water may be a new therapeutic route to improve health.

Dysbiosis of the microbiota is associated with a large and growing number of diseases. Recent research has mainly focused on the analysis of the relationship between an aberrant microbiota composition and disease (Feng *et al.* 2018). The relative proportion of some major phyla of gut bacteria, such as Bacteroidetes, Firmicutes and Proteobacteria was associated with type 2 diabetes (Larsen *et al.* 2010). Also, a lower proportion of Bacteroidetes was associated with metabolic syndrome and liver cirrhosis (Chen *et al.* 2011; Xu *et al.* 2017). Moreover, oral treatment of MIA offspring with the human symbiotic Bacteroides fragilis can improve gut permeability, alter microbial composition, and ameliorate communication disorders, stereotyping, anxiety and sensorimotor behaviors deficits (Hsiao *et al.* 2013). We found that six 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 in the prevention and cure of these diseases by inducing the growth convenience to Bacteroidetes. It is noteworthy that the drinking water (MW, PW, NW) used in this experiment is the common commercial bottled water. The chemical analysis of drinking water is shown in Supplementary Material, Table 1. As long as the water composition is almost the same as the mineral water, the same effect may be produced.

CONCLUSIONS

In conclusion, our results suggest that the 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 Bacteroidetes and the microbiota is targeted therapeutically, microbiota reprogramming may need to involve strategies that incorporate drinking water.

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AUTHOR CONTRIBUTIONS

ZK, LWL and CZL contributed equally to this work. SZQ and XQY designed the research; ZK, LWL, CZL, YD, QZG, FH, LC, JM and LJW performed experiments; and 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.

DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

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