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Gender Based Compositional Fluctuations in Gut Microbiota in Hypertension

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Abstract

Background: Emerging evidence suggests that human gut microbiota plays critical role in maintaining physiological homeostasis, and gut dysbiosis is involved directly or indirectly in cardiovascular diseases including hypertension.

Aim: The aim of present study was to investigate whether there were sex based compositional differences in gut microbiota of patients with hypertension.

Methods: Fecal samples were collected from male and female hypertensive patients, and healthy individuals from Xi'an, Shaanxi province of China. Touchdown PCR and denaturing gradient gel electrophoresis (PCR-DGGE) with primers specifically targeting V3 region of 16S rRNA, and real time quantitative PCR (qPCR) were performed to characterize all the samples. High-throughput sequencing of the V3–V4 regions was performed on Illumina HiSeq 2500.

Results: Diversity and richness indices for the gut microbiome were compared among the four groups. The relative abundance of Firmicutes was found increased in hypertensive groups (both female and male patients) while that of Bacteroidetes was decreased in male hypertensive patients only, as compared to the healthy male subjects. The most differentially abundant bacterial taxa belonged to the genera *Prevotella* and *Megasphaera* in female hypertensive patients and *Megamonas* in male hypertensive patients. The female and male hypertensive patients also showed different dominant phylotypes.

Conclusion: These results demonstrated that there were sex based differences in gut microbial composition of patients with hypertension. Our findings indicate *Prevotella*, *Megasphaera* and *Megamonas* as the potential sex-specific biomarkers of hypertension, and need to be further evaluated.

Keywords: Hypertension; Gut microbiota; DGGE; Real Time qPCR; High-throughput sequencing

Introduction

Hypertension (HTN) or high blood pressure nowadays is emerging as a threatening public health issues as it is a modifiable risk factor for diseases like stroke and cardiovascular anomalies [1,2]. Efforts to lower down the blood pressure (BP) become fruitless in elderly people with the habits of taking unhealthy diet, excessive use of alcohol, less physical activity, obesity and depression [3-6]. Strategic management and training is needed to overcome hypertension related mortality [7]. It may differ in lieu of age, sex and other factors [6,7-9]. It is observed that both in humans and animals, gender based differences in hypertension involve biological and behavioral factors [10]. Overall, hypertension is less prevalent in females than males, and the biological factors including sex hormones and other chromosomal and biological sex differences safeguard women against hypertension. These differences vividly become prominent during adolescence and continue to the adulthood until the women reach menopause. At this stage these gender based differences become almost insignificant [10,11]. It has been observed that gut microbiota regulate sex hormones mediated autoimmunity more effectively in women than men [12]. These sex hormones are known to play critical role in the development of cardiovascular diseases [13,14]. Other studies have highlighted the association of intestinal microbiota with metabolism of phosphatidylcholine and cardiovascular risk [15].

Earlier, a couple of reports proposed that gut microbiota may be involved in the maintenance of blood pressure homeostasis and that a compositional fluctuation in the gut microbiome may probably be implicated to the increased risk of developing hypertension [16-18]. In addition, hypertension is associated with increased risk of arterial thrombotic disease. Among other factors, enhanced platelet activity contributes significantly to this phenomenon [19]. Recently, it was reported that gut microbioa directly contribute to platelet hyperreactivity and enhanced thrombosis potential in carotid artery thrombosis animal models (*In vivo*) [20]. Besides, the compositional fluctuation in the individuals' gut microbial community is linked with the environmental as well as host genetic factors [21,22]. Certainly, diet is considered as one of the major factors that can affect gut microbiota composition, individually and chronologically [23,24]. Sex is another important factor and there is no other view point that it has a significant impact on physiology and behavior [25], however there are controversial reports on sex based differences in the gut microbial composition. Some studies suggest that gender has no or very limited effect on gut microbiota [26-28], while others emphasize that there

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are significant differences in microbiota arrangement between sexes [29-31], and the commensal microbial population may influence sex hormone levels [12,32].

Recently, we have reported the gut microbiota compositional analysis and the efficacy of 5-fluorouracil in colorectal cancer rat model [33]. Currently we are working on 'role of gut microbiota in the development and management of hypertension, and related diseases', evaluating multiple factors including sex. In the present study, we have analyzed the gut microbial community of the hypertensive patients between the two sexes comparing with their healthy counterparts and have identified sex-specific microbial biomarkers for the diagnosis of hypertension. The current study uses the modern metagenomic techniques including High-throughput sequencing and Real-Time qPCR to evaluate the gender based gut microbial diversity and similarity in the human hypertensive patients along with the healthy controls.

Materials and Methods

Study participants and design

This study included 30 male hypertensive patients (MHT) and 25 female hypertensive patients (FHT), ages ranging between 40 y and 75 y. 30 healthy volunteers included 15 males (Male Control Group-MCG) and 15 females (Female Control Group-FCG), aged between 40 y and 70 y. A full written consent from all the participants was taken and a questionnaire regarding gender, age, body weight and health status of the study participants was filled at the time of sampling. BP of the individuals was noted with a digital sphygmomanometer and three consecutive values were taken with a 5 min gap. Fecal samples were collected in sterile cups and were stored immediately at -80°C until the DNA extraction was carried out. None of the patients and healthy individuals had diabetes, obesity and any record of chronic metabolic diseases. In addition, the participants had no history of antibiotics, probiotics and prebiotics usage from last 30 days, at the time of sampling.

Ethical statement

The current study was performed under the guidelines of World Medical Association and Declaration of Helsinki. The sampling and experimental procedures were performed with the approval of Ethical Committee of the Xi'an Jiaotong University, School of Medical Sciences and an informed written consent was obtained from all of the participants including male and female hypertensive patients as well as healthy volunteers.

DNA extraction

After thawing the fecal sample, bacterial DNA was extracted using the Qiagen QIAamp MiniStool Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions and the extracted DNA concentration was estimated by absorbance at 260 nm (A260) with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Analysis of gut microbiota by PCR-DGGE

Universal primers for V3 region of 16S rRNA gene were used to amplify the intestinal microbiota of the study samples by using total fecal DNA as a template for PCR-DGGE analysis. Each 50 µl PCR reaction mixture contained 1 µl of each primer (Table 1), 1 µl of deoxynucleotide triphosphate (dNTPs) mix (10 mM), 5 µl of $MgCl_2$ (25 mM), 5 µl of 10x buffer, 0.4 µl of Taq DNA polymerase (TaKaRa, Japan), and 2 µl of total fecal DNA. PCR amplification was performed in an automated thermocycler (ABI2720, USA) using touchdown PCR program for increasing the specificity of PCR reactions. The PCR thermal profile conditions include; initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min. The annealing temperature was decreased by 1°C every second cycle until a touchdown 55°C reached, at which 10 additional cycles were carried out, followed by final extension at 72°C for 10 min [34]. Post-PCR gel electrophoresis was performed to confirm the amplicons. Negative controls included all the components of the reaction mix except the DNA template was run along with each reaction.

After PCR amplification, 15 µl of each PCR product was mixed with 6 µl of loading dye. Denaturing gradient gel electrophoresis (DGGE) was performed using the DCode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) on 16 cm × 10 cm × 1 mm gels. The sequence-specific separation of the amplicons was obtained with 10% (w/v) polyacrylamide (acrylamide-bis, 37.5:1) gel in 1 × Tris-acetate EDTA (TAE) buffer, containing 65-35% linear denaturant gradient. The 100% denaturing solution contained 7.0 M urea and 40% (vol/vol) deionized formamide. Electrophoresis was performed at a constant voltage of 90 V at 60°C for 13 h. After completion of the run, the gel was stained with 5 µg/ml ethidium bromide solution for 30 min, washed with deionized water and viewed under BIO-RAD Gel Doc 2000 system. DNA Marker (DL2000) was used as standard reference to compare the bands in the gel. Quantity One software (Bio-Rad, USA) was used to evaluate the bacterial diversity of the study groups by analyzing the number of bands and the bands intensities of DGGE profiles. The diversity of bacterial taxa was determined by the Shannon–Weaver index ($H^{'}$). The similarity score and cluster analysis of DGGE profiles were determined by the UPGMA method based on the Dice similarity coefficient (band-based). Nonparametric statistical analysis

Groups	Target Gene	Primer	Sequence (5'-3')	Product Size (bp)	Ref.
PCR-DGGE	Bacteria 16s rRNA gene V3 region	341 F[a]	CCTACGGGAGGCAGCAG	193	[34]
		534 R	ATT ACCGCGGCTGCTGG		
Real time PCR	*Bacteroides*	Bac F	AAGGGAGCGTAGATGGATGTTTA	193	[57]
		Bac R	CGAGCCTCAATGTCAGTTGC		
	Prevotella	Pre F	ACAGTAAACGATGGATGCC	513	[58]
		Pre R	GGTCGGGTTGCAGACC		
	Clostridium	Clos F	CGGTACCTGACTAAGAAGC	429	[59]
		Clos R	AGTTTGATTCTTGCGAACG		
	Escherichia coli	E.coli F	CATTGACGTTACCGCAGAAGAAGC	190	[59]
		E.coli R	CTCTACGAGACTCAAGCTTGC		

[a]A 5′ GC-clamp (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G) was added for DGGE analysis.

Table 1: Primers deployed in PCR-DGGE and Real-time PCR.

was performed using IBM SPSS Statistics (v20) software. Results are expressed as mean values and standard deviations.

Sequencing of selected bands from DGGE gels

The distinct, prominent and common bands were cut from the gel with a sterile razor, washed with RNAse Free water, re-suspended in 20 µl of the RNAse Free water and stored at 4°C overnight for the diffusion of DNA. PCR was performed again with the diffused DNA using V3 region primers 341F/534R this time without GC clamp (initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min.). Re-amplified PCR products were sequenced by Shanghai Sangon Biological Engineering Technology Service Co. Ltd. (Shanghai, China). Sequences were analyzed with Chromas v2.23 (Technelysium, Tewantin, Australia) and their similarity was confirmed by using BLAST and Seqmatch softwares.

Real time quantitative PCR analysis

Real-time PCR quantification was performed using the StepOne v2.3 (ABI, USA) system. Real-time PCR primers are depicted in Table 1. To calculate the absolute copy number of the *Bacteroides* spp., *Prevotella* spp., *Clostridium* spp., and *Escherichia coli* within the test samples, QuantiFast SYBR Green PCR kit (Qiagen) was used and a standard curve was prepared from a serially diluted plasmid DNA (of known concentration) ranging from 10^2 to 10^8 copies/g of feces to quantify the test samples. The resultant bacterial populations were expressed as Log10 bacterial replica counts in 1 gram of the fecal mass. Statistical analysis was performed by using Student's *t*- test.

High-throughput sequencing and data analysis

30 samples were randomly selected for metagenomic high-throughput sequencing analysis. 515-F (GTGCCAGCMGCCGCGGTAA) 806-R (GGACTACHVGGGTWTCTAAT) primers were used to develope the amplicon libraries [35]. The libraries were sequenced on an Illumina HiSeq 2500 platform according to the manufacturer's instructions. The Raw data obtained, were screened and assembled by QIIME [36] and FLASH [37] software packages. The UCLUST method [38] was used to cluster the sequences into Operational Taxonomic Units (OTUs) at an identity threshold of 97%. Meanwhile, the RDP classifier [39] was used to assign each OTU to a taxonomic level. Alpha Diversity analysis, including Shannon and Simpson diversity index, Chao 1, ACE and Good's coverage, was performed with QIIME (Version 1.7.0). Student's *t*-test was used to calculate the significant differences in alpha diversity between the groups. The relative abundance of phyla, family, genera and species in each sample was calculated and compared between the study groups. All statistical analysis was carried out by IBM SPSS (v20) software.

Results

Biochemical measurements of the study participants

Other than systolic and diastolic blood pressure, female patients had higher fasting blood glucose (P=0.02) and triglycerides (P=0.01) levels. On the other hand, male hypertensive patients showed increased levels of fasting blood glucose (P=0.02), triglycerides (P=0.03) as well as low-density lipoproteins (P = 0.04) as compared to their healthy groups (Table 2).

Sequencing and statistical analysis of DGGE profile

Dominant bands from different positions within the DGGE profiles were further analyzed through sequencing. Bacteroidetes and Firmicutes were appeared as the dominant bacterial phyla within the study groups; however, the percentage of different genera was different among the four groups (Table S1). The detailed results from DGGE analysis are shown in (Figures 1A and 2A). The DGGE profiles band number and the Shannon diversity index (H′) analysis, in order to estimate the gut microbial diversity within the study groups is summarized in Table 3. The resultant distinguishable findings elucidate that gut microbial diversity showed no significant difference in the hypertensive groups as compared to their healthy counterparts. Furthermore, Dice similarity coefficient and UPGMA, to determine the extent of similarity of all the DGGE profiles, were applied (Figures 1B and 2B). The assessed values of the similarity coefficient of female hypertensive patients and healthy controls with mean index values were 44.78 ± 12.46 and 41.78 ± 12.91, respectively. The similarity coefficient of male hypertensive patients and healthy control with mean index values was 42.69 ± 11.29 and 40.32 ± 8.52, respectively (Table 3). The compiled statistical data of the samples from hypertensive and control groups analyzed by Dice coefficient showed lesser values among the groups, that in turn affirms that gut microbiota of hypertensive patients (male and female) varies from the healthy controls (male and female) respectively.

Real-time PCR

Real-time PCR analysis was performed and evaluation of the mean index value demonstrated that the copy number of *Bacteroides* spp. and *Clostridium* spp. were significantly decreased and increased, respectively in the male hypertensive patients as compared to the male healthy controls (P <0.05). While the *Prevotella* spp. was significantly

	Female		P-value	Male		P-value
	HTN (n=25)	CG (n=15)		HTN (n=30)	CG (n=15)	
Age (yrs)	64.21 ± 11.52	58.85 ± 12.42	0.47	65.1 ± 11.4	61.93 ± 9.77	0.75
Weight (kg)	55.5 ± 5.86	62.7 ± 5.02	0.63	63.61 ± 4.35	67.56 ± 5.98	0.18
SBP, mmHg*	160.15 ± 16.63	120 ± 9.23	<0.0001	175.45 ± 9.23	118.13 ± 6	<0.0001
DBP, mmHg*	100.84 ± 11.2	80 ± 10.1	<0.0001	108 ± 10.1	77.19 ± 7.36	<0.0001
FBG, mmol/L	4.78 ± 0.63	4.19 ± 0.64	0.02	5.17 ± 1.35	4.25 ± 0.66	0.021
TC, mmol/L	4.22 ± 0.92	4.42 ± 1.01	0.5	4.07 ± 0.95	4.26 ± 0.83	0.19
TG, mmol/L	1.62 ± 0.84	1.05 ± 0.32	0.01	1.32 ± 0.49	1.14 ± 0.29	0.03
HDL, mmol/L	1.10 ± 0.25	1.18 ± 0.23	0.8	1.09 ± 0.23	1.03 ± 0.16	0.46
LDL, mmol/L	2.46 ± 0.70	1.79 ± 0.45	0.12	2.17 ± 0.86	1.95 ± 0.59	0.04

The data for age, Weight, SBP, DBP, FGB, TG, TC, HDL and LDL were presented as mean ± SD. P-values for age, weight, SBP, DBP, FGB, HDL, LDL, TG, and TC were calculated using Student's t-test,(P <0.05). SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, triglycerides; and TC, total cholesterol. *Grade 2: current blood pressure 160/100~179/109; Grade 3: current blood pressure ≥ 180/110.

Table 2: Characteristics of study participants.

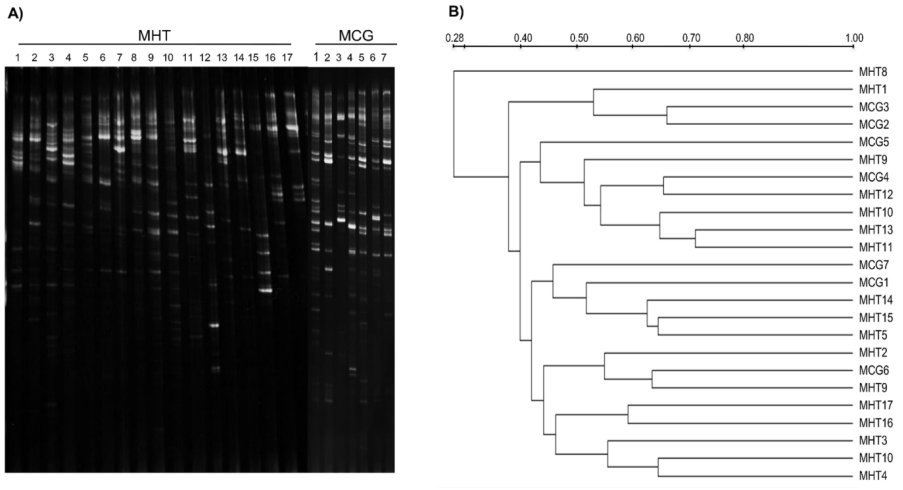

Figure 1: (A) DGGE profiles constructed by linkage primer with V3 region of 16S rRNA gene of MHT and MCG groups. **(B)** Cluster analysis of MHT and MCG by applying UPGMA and Dice coefficient.

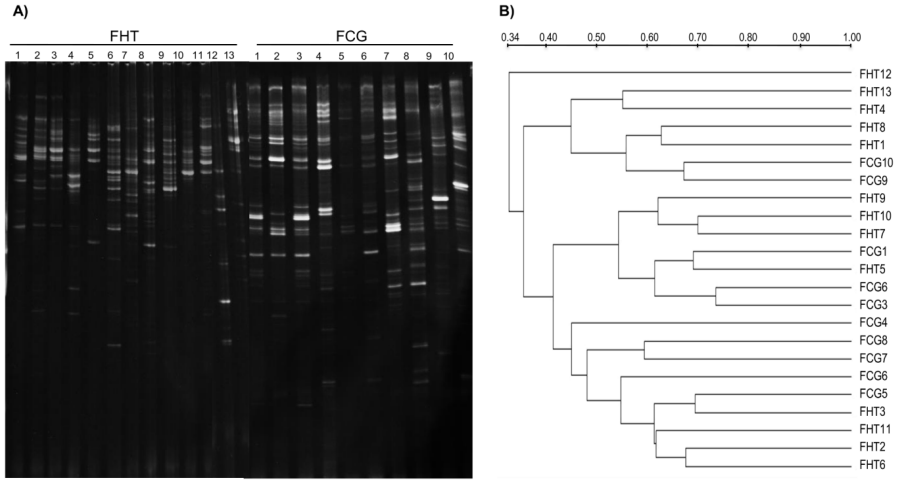

Figure 2: (A) DGGE profile constructed by linkage primers with V3 region of 16S rRNA gene of FHT and FCG groups. **(B)** Cluster analysis of FHT and FCG by applying UPGMA and Dice coefficient.

Group	Microbiota diversity		Microbiota similarity	
	DGGE bands[a] (mean ± SD)	Shannon index[b] (mean H'/H' max ± SD)	Intra-group[c]	Inter-group[d]
FHT	16.23 ± 3.71	2.66 ± 0.25	44.78 ± 12.46	38.07 ± 11.46
FCG	19.8 ± 5.20	2.97 ± 0.29	41.78 ± 12.91	/
MHT	15.64 ± 4.74	2.91 ± 0.30	42.69 ± 11.29	36.21 ± 9.46
MCG	13.14 ± 5.39	2.67 ± 0.07	40.32 ± 8.52	/
P value FHT-FCG	0.06	0.64	/	/
P value MHT-MCG	0.13	0.25	/	/

Values are presented as means ± SD. Data were analyzed using the Student's *t*-test. [a]Number of DGGE bands produced by each sample analyzed. [b]Shannon Diversity Index as calculated using the relative intensities of all DGGE bands in each sample and expressed as a ratio of H' to H'_{max}, where H'_{max} is the maximum value of the Shannon index for a given sample. [c]Dice similarity coefficients comparing DGGE band profiles within individuals of the same group. [d]Dice similarity coefficients comparing DGGE band profiles between members of the study groups.

Table 3: Microbiota diversity and similarity of hypertensive patient groups compared with control groups.

increased in the female hypertensive patients as compared to the healthy counterparts. No other significant differences were observed among the study groups. The above generated data is summarized in Table 4. The triplicate samples were used to calculate the mean value in each experiment.

High-throughput sequencing analysis of gene sequence

The comparative statistics of the PCR sequences were estimated with 1,523,644 amplicons at the V3-V4 site of 16S rRNA gene from 20 hypertensive patients (10 females and 10 males) and 10 healthy controls (5 females and 5 males). Among these, sum of high-throughput sequencing reads 1,395,074 with an average of 46,502.47 per sample were passed for quality control and processed for further analysis. Taxon tag was 36,249.26 (average) in all samples. The total number of OTUs assigned was 5786 with an average of 193 per sample in our study. The sum of the unique tags from the four groups was 307593 with an Avg. of 10,253.1 for all samples; that revealed the total phylotypes in this study. After deletion of linkage primers, the length of the average sequence was 422 bp.

Gut microbial diversity and composition

The total gut microbiota was evaluated for richness and diversity, as computed at 97% resemblance level. Rarefaction curves analysis indicated that the microbial communities were well represented for most of the samples and there were no obvious differences in the rarefaction curves of the four groups under study (Figure 3). Alpha diversity, as measured by Shannon index was significantly higher in the female control group than those in the female hypertensive group (P=0.03), while the Simpson diversity in the male hypertensive patients was significantly higher as compared to the male control group (P=0.01). Good's coverage was significantly decreased in the female hypertensive group while increased in the male hypertensive group as compared to their control groups (female and male, respectively). However, we could not observe the statistically significant differences in ACE and Chao 1 algorithms (Table 5).

The relative abundance of gut microbiota in study groups

The bacterial composition was assessed on the taxonomic basis at six levels and the taxa that composed of more than 1-0.5% were considered. The total usable sequences were classified into 15 phyla, 32 classes, 51 orders, 78 families, 174 genera and 110 species. Among the 15 phyla sequenced, composition of gut microbiota was changed in-between the study groups. The majority of the obtained OTUs belonged to four phyla (female *vs.* male): Bacteroidetes (58% *vs.* 64.2%), Firmicutes (31.9% *vs.* 28.8%), Proteobacteria (8.7% *vs.* 4.2%) and Actinobacteria (2.1% *vs.* 2.1%). The relative abundance of Firmicutes was significantly increased in the fecal microbiota of male and female hypertensive patients as compared to their control groups. The relative abundance of the phylum Bacteroidetes was decreased in the both hypertensive groups but the only statistically significant quantitative difference was observed between male hypertensive patients and male control group (P=0.03) (Figure 4). The statistical data of the top 10 phyla is depicted in Table S2.

Gut microbiota population at family level

Bacteria	Female			Male		
	HTN	CG	*P* value	HTN	CG	*P* value
Bacteroides	6.69 ± 0.98	7.14 ± 0.48	0.09	5.86 ± 1.75	6.78 ± 1.45	0.04
Prevotella	4.82 ± 1.84	3.43 ± 1.01	0.03	5.84 ± 2.13	5.73 ± 1.85	0.22
Clostridium	5.71 ± 0.71	5.54 ± 0.39	0.07	5.97 ± 0.67	5.37 ± 0.39	0.04
Escherichia coli	4.39 ± 1.24	3.71 ± 0.76	0.19	4.38 ± 1.04	4.12 ± 1.23	0.60

Data were reported as the average estimate of Logarithms of fecal PCR target genetic amplicon copy numbers present in 1 g of feces. Results which are significantly different (Student's *t*-test), with P <0.05.

Table 4: Quantitative analysis of bacterial groups in fecal samples by real-time PCR (qPCR).

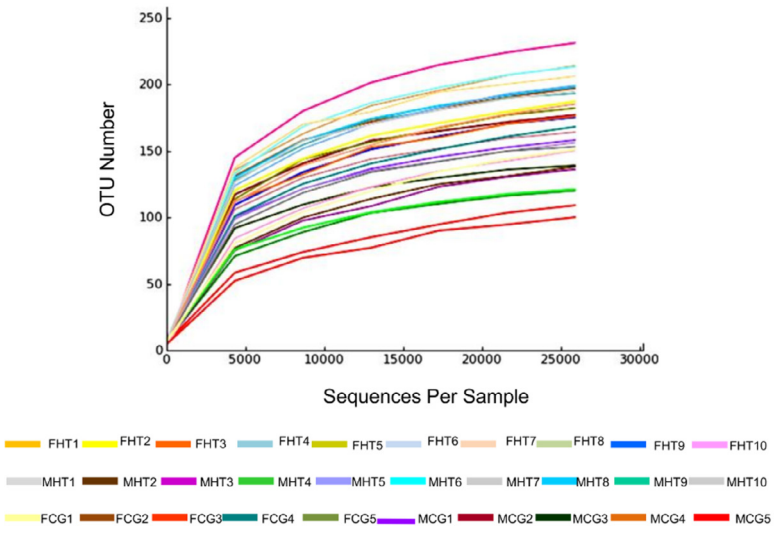

Figure 3: Rarefaction curves used to estimate richness among the study groups at 97% similarity level.

Among the 10 top most families, the prevalence of *Prevotellaceae*, *Veillonellaceae*, *Peptostreptococcaceae* were higher in the female hypertensive patients. On the other hand, *Bacteroidaceae* and *Ruminococcaceae* were significantly decreased and increased respectively, in the male hypertensive patients (Figure 5). The family level data statistics which illustrates the quantitative difference are displayed in Table S2.

Gut microbiota distribution at genus level

Among the 10 top most genera, a significantly increased abundance of *Prevotella_9* (P=0.012) and *Megasphaera* (P=0.017) was observed in female hypertensive patients, while significant increase and decrease of *Megamonas* (P=0.013) and *Bacteroides* (P=0.013) respectively, were observed in male hypertensive patients as compared to their control group (Figure 6). The dominant phylotypes in female and male hypertensive patients were also different. The quantitative differences at genera level between the study groups are compiled in Table S2.

Gut microbial distribution at species level

A total of 110 sequences were classified at the specie level and among these, some of the species revealed significant differences in their relative abundance in the hypertensive and control groups (Table S3).

Correlation of molecular protocols

The data obtained from this study using the molecular techniques

Group	Observed species	Shannon	Simpson	Chao1	ACE	Good
FHT	170.4	3.90	0.86	193.90	197.12	0.9987
FCG	165.5	4.45	0.91	185.37	183.78	0.999
MHT	183.5	4.20	0.96	200.43	205.48	1.0899
MCG	172.5	4.12	0.86	197.63	202.44	0.9986
***P* values**						
FHT-FCG	0.23	0.03	0.07	0.95	0.73	0.01
MHT-MCG	0.58	0.11	0.01	0.58	0.57	0.001

The number shown in this table is the mean value of each group. Results which are significantly different (Student's *t*-test), with P <0.05.

Table 5: Gut bacterial richness and diversity index that were based on 97% similarity by high-throughput sequencing analysis.

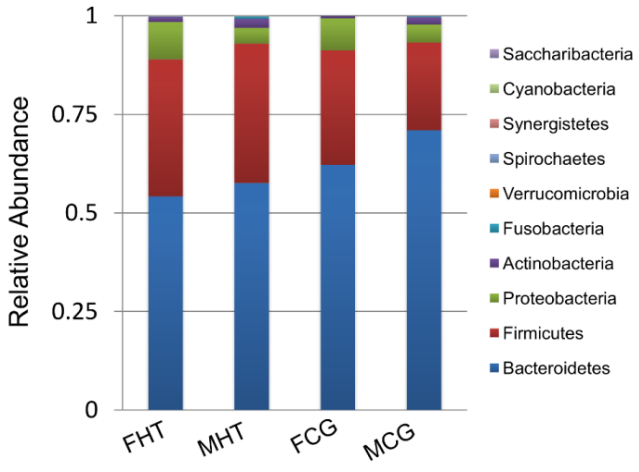

Figure 4: Gut microbial composition at phylum level obtained from high-throughput sequencing results.

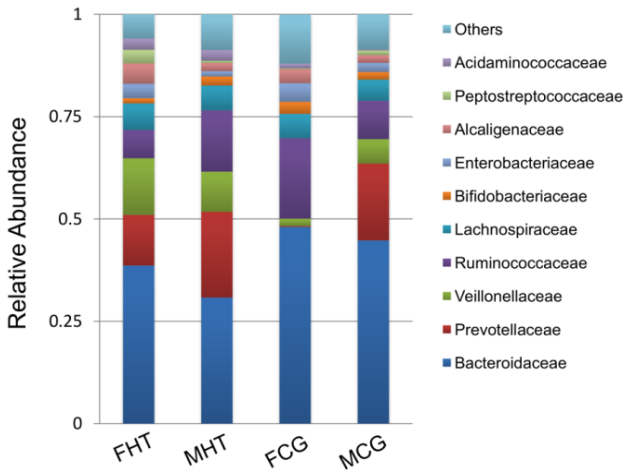

Figure 5: Gut microbial composition at family level obtained from high-throughput sequencing results.

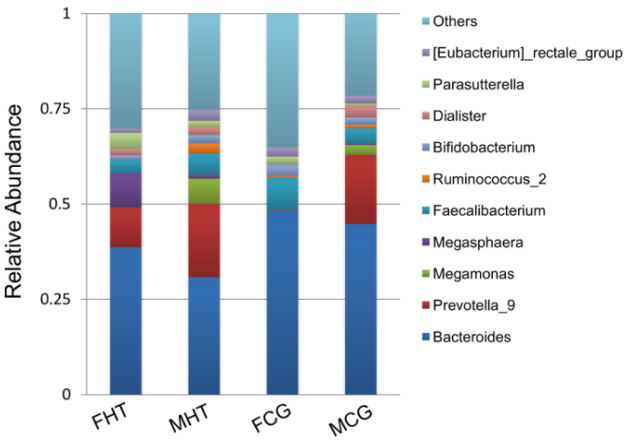

Figure 6: The relative abundance of 10 highly prevalent genera in the study groups obtained from High-throughput sequencing analysis.

including DGGE and High-throughput sequencing revealed the most prevalent bacterial phyla in the gut i.e. Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria. The PCR-DGGE analysis is economical and less time consuming, and is considered a basic technique to scrutinize the substantial shift of gut bacterial populations. On the other hand, High-throughput Illumina sequencing is much reliable, sensitive and authentic technique to validate the diverse bacterial communities. The findings of the PCR-DGGE analysis were aligned with the High-throughput sequencing results.

Discussion

Human gut microbiota plays critical role in body defense in response to various anomalies mediated by metabolic malfunctions and pathologies caused by multiple diseases. The experimental data from current study establish the substantial alterations between gut microbial populations of male and female hypertensive patients and healthy subjects, as evident by DGGE profiling, High-throughput sequencing and Real-time PCR analysis. The α- diversity calculated by Shannon and Simpson indexes was significantly decreased and increased in female hypertensive and male hypertensive patients

respectively, as compared to their healthy controls. Good's coverage was also significantly decreased and increased in female and male hypertensive patients, respectively. Conversely no significant differences were found by statistical analysis of nonparametric Chao 1, algorithm ACE and observed species. Moreover, the bacterial population diversity as estimated by the DGGE profiling and validated by High-throughput sequencing was lower in female patients but higher in male patients. We determined the gut microbiota distribution at the phyla, family, genera and specie levels and observed the variations among the study groups.

The relative abundance of Firmicutes and Bacteroidetes was increased and decreased in female and male hypertensive patients respectively, as compared to their healthy. Moreover, the most differentially abundant bacterial taxa belonged to the genera *Prevotella* and *Megasphaera* in female hypertensive patients and *Megamonas* in male hypertensive patients. Further, the female and male hypertensive patients also showed different dominant phylotypes comparing in between them and to their healthy counterparts. These results demonstrate that there are sex based differences in the gut microbial composition of individuals with hypertension, and categorize *Prevotella*, *Megasphaera* and *Megamonas* as the sex-specific bacteria associated with hypertension. Recently we have found that the relative abundance of Bacteroidetes and Firmicutes is significantly decreased and increased, respectively, in hypertensive patients as compared to the healthy controls (data not published yet). In our last study we investigated the gut microbiota dysbiosis and its causal role in the development of hypertension, and the sex-based evaluation was not taken into consideration. However, sex-based differences are prominent in hypertension as reported by earlier studies [8,9]. Analyzing the differential gut microbiota in female and male hypertensive patients and their healthy counterparts, we found that the Bacteroidetes were decreased significantly only in the male hypertensive patients while Firmicutes were increased significantly, in male and female (both) hypertensive groups.

Clinically, hypertension and metabolic-diseases such as diabetes and obesity are considered as interrelated, associated with common etiological factors [15]. Previous studies have shown that low Bacteroidetes levels were associated with obesity [40]. Therefore, we excluded the obese and diabetic subjects to rule out the potential influence of these factors in our current study. In addition, the BMI difference between hypertensive patients and healthy controls was almost negligible. So, the obesity or diabetes could not be the confounding factors in this study. Although no significant variability in the overall abundance of Bacteroidetes between female hypertensive patients and female healthy controls were identified, some Bacteroidetes OTUs in female patients were increased, while others were decreased. Therefore, the fluctuated Bacteroidetes could still be a hallmark in female hypertensive patients. On the other hand, male patients group showed significant decrease in the relative abundance of *Bacteroides* as compared to the male control group. *Bacteroides* are the beneficial bacteria and maintain a complex relationship with host in the gut, acting as commensals [41]. These bacteria are known to cause the fermentation of a wide range of sugar derivatives from plant materials (which are common in the human colon and are potentially toxic) making them beneficial for humans. *Bacteroides* and other intestinal bacteria cause carbohydrates fermentation and produce volatile fatty acids that are reabsorbed through the large intestine and utilized by the host as an energy source, meeting the considerable proportion of our daily energy requirement [42].

We observed significantly raised levels of *Prevotella* in female hypertensive patients. *Prevotella* plays a central role in digesting plant food. It has also been reported that *Prevotella* participates in urinary tract infections, arthritis, osteomyelitis and brain abcessess [43,44]. *Ruminococcus* spp., *Faecalibacterium* spp., and *Roseburia* spp. are significantly decreased in inflammatory bowel disease (IBD) and acute colitis, suggesting that these bacteria are imperative to maintain the intestinal homeostasis [45]. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium and was found significantly reduced in patients with active IBD rather than those in remission [46] and patients with recurrent IBD [47]. Additionally, *F. prausnitzii* was reported to stimulate peripheral blood mononuclear cells (PBMCs) for the secretion of anti-inflammatory cytokine IL-10, whereas it inhibited the production of pro-inflammatory cytokines, such as IL-12 and IFN-γ [47]. Our data showed a significant reduction of *Ruminococcus and Roseburia* in female hypertensive patients only. On the other hand, *F. prausnitzii* was significantly decreased in both male and female hypertensive patients, suggesting the significant role of these bacteria in sex related hypertension.

Phascolarctobacterium spp. and *Veillonellaceae*, both produce high amounts of short chain fatty acids, acetate and propionate, and belong to the same class within the phylum Firmicutes [48,49]. The butyrate is mainly used by colonocytes, while acetate and propionate are largely taken up by the liver. Acetate promotes hypercholesterolemia, hypertriglyceridemia and results in liver steatosis as it required for cholesterol and fatty acid production. Propionate inhibits cholesterol synthesis in liver tissue and acts as a substrate for hepatic gluconeogenesis. Propionate is also reported to play a role in decreasing the plasma lipid levels [50] however, its anti-lipogenesis effects are divisive. Besides, propionate promotes inhibition of lipolysis and adipocyte differentiation, mediated by the activation of G protein-coupled receptor 43 or free fatty acid receptor 2, causing increased adiposity [51,52]. The members of the *Veillonellaceae* family are usually responsible for polymicrobial and rarely for monomicrobial infections in humans such as osteoarticular infections or endocarditis [53]. In the present study we observed an increased abundance of family *Veillonellaceae* in male and female hypertensive patients as compared to the normal male and female groups.

Many of the taxonomies identified in the current study however, are novel and are reported for the first time. The genus *Bacteroides* and family *Enterobacteriaceae* are both negatively associated with triglycerides and play role in bile acid metabolism [11]. Bile acid activity of commensal bacteria is involved in a complex interplay with host hepatic enzymes, and together they promote digestion and absorption of dietary lipids [54]. Triglycerides levels were found raised in the participants of both male and female hypertensive patients groups of our study, which indicates the role of bacterial bile acids in lipid metabolism. Diet is considered an important factor influencing gut microbiota community. In the present study we could not evaluate the relationship of gut microbiota with dietary habits of the study groups because of the incomplete nutritional information. However, our conclusions are unlikely to be affected by this potential confounding factor, due to almost similar dietary habits and lifestyle of the enrolled subjects from Xian city of China.

The Firmicutes and Bacteroidetes (F/B) ratio has been reported to be increased in hypertension, and the oral administration of minocycline has been found to normalize the F/B ratio and blood pressure in hypertension [17]. Further, manipulating gut microbiota using pre- and probiotics might prove a valuable adjuvant to traditional antihypertensives. Pre- and probiotics modulate cholesterol, inflammation, blood glucose levels, and the renin–angiotensin system to lower BP and risk for HTN [55]. A meta-analysis suggested that the continuous use of probiotics for ≥ 8 weeks may lower BP by a modest

degree, with potentially greater effect when BP is elevated [56-59]. However, a detailed understanding of gut microbial composition in relation to BP, ethnicity etc must be evaluated to fully recognize the implications of pre- and probiotics in HTN therapeutics.

Conclusion

This study elaborates the gender based compositional differences in the gut microbiota and identifies sex-specific bacterial populations in human hypertensive patients, comparing with their healthy counterparts. The most distinctive and differentially abundant bacterial taxa belonged to *Prevotella* and *Megasphaera* in female, while *Megamonas* in male hypertensive patients, respectively. These results provide novel insights for developing new strategies to manage hypertension and its related diseases. However, the suitability of *Prevotella*, *Megasphaera* and *Megamonas* may be further evaluated as the gender-specific biomarkers for diagnosing hypertension.

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Supplementary Material

The data for sequencing of PCR amplicons from DGGE gel profile and recognition linking to BLAST database; Gut microbial phylotypes from High-throughput sequencing; and the significantly divergent gut microbial species from High- throughput sequencing, is given in a separate file (Supplemental File) as Table S1; Table S2; and Table S3, respectively.

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