ORIGINAL ARTICLE

MOLECULAR CHARACTERIZATION OF FECAL MICROBIOTA OF HEALTHY CHINESE TOBACCO SMOKER SUBJECTS IN SHAANXI PROVINCE, XI'AN CHINA

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Background: Tobacco Smoking, most commonly, can cause the diseases affecting the lungs and heart. Human gut microbiota plays a key role to decide the health status of the host. Current study aimed to characterize the gut microbiota of healthy Chinese tobacco smokers and to study the alteration in diversity and similarity of gut microbiota, with comparison of healthy non-smokers. **Methods:** Fecal samples were collected from fourteen healthy tobacco smokers and six from healthy non-smoker individuals. PCR-denaturing gradient gel electrophoresis, with universal primers focusing V3 region of the 16S rRNA gene, was done to characterize the overall gut microbial composition of healthy tobacco smokers in comparison with healthy non-smoker subjects and some strongly dominant gel bands were excised for sequencing. Real time PCR was also performed to evaluate the copy numbers of some dominant bacteria of intestinal flora. **Results:** The results indicated that gut microbial diversity in tobacco smoker group was lower than non-smoker controls. Furthermore, similarity index comparison also indicated that it was lower in inter-group than intra-group, which showed that gut microbial composition was changed in tobacco smoker group. Sequencing results also indicated a change in bacterial composition between both groups. We also observed that in tobacco smoker group, there was a significant reduction in *Bifidobacterium* and non-significant increase in *Bacteroides vulgatus*, while non-significant decrease in *Lactobacillus and clostridium leptum sub group,* respectively. **Conclusion:** It can be concluded that in healthy Chinese tobacco smoker group, there is a notable alteration in the molecular characterization of gut microbiota.

Keywords: Tobacco smokers; Gut microbiota; Characterization; DGGE

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INTRODUCTION

Human gut microbiota is considered to be an important factor in determining the health status of an individual. Human gut flora protects the body against many diseases by performing metabolic, trophic and protective function.[1] Its functions and composition are stable over the time but may be modulated by different factors including age, disease, and diet.[2] The human gut microbiota contains, approximately, hundred trillion bacterial cells and plays an important role in human physiology, i.e., metabolism, nutrition, absorption, and immune function.[3]

Gut microbiota plays a key role in immune homeostasis of the host.[4] China is the world's largest tobacco producer and consumer country. There are more than 350 million tobacco smokers and china also produces 42% of total world's cigarette.[5] Tobacco smoking can cause cancers at 20 organ sites and also can cause pleotropic physiologic effects.[6] Alteration of gut microbial composition has been linked with many disease conditions, i.e., Crohn's disease, inflammatory bowel disease, viral diarrhoea, hyperthyroidism, colitis, metabolic diseases such as type II diabetes and obesity.[7–9]

PCR–DGGE with combination of image analysis, was used to study the microbial similarity and diversity, while dendrogram (UPGMA) construction and sequencing were done to analyse the disease associated DGGE motifs and taxa.[10] Real time PCR was done to quantify the *clostridium leptum* sub group, *Bacteroides vulgatus, Lactobacillus* and *Bifidobacterium* genus, to determine the changes in gut microbial composition of tobacco smoker group.[7]

Current study aimed to characterize the fecal microbiota of healthy tobacco smoker group, also to study the alteration in the diversity and similarity of gut flora of healthy tobacco smoker group, with comparison of healthy non-smoker volunteers.

MATERIAL AND METHODS

In this cross-sectional study fecal samples were collected from 14 healthy tobacco smokers (Chinese men, in Shaanxi province Xi'an China) having the history of 10-year tobacco smoking (aged between 35 to 50 years) and 6 healthy non-smoker volunteers (Chinese men, aged between 35–50 years) in a sterile cup. A questionnaire was filled regarding age, gender, dietary habits, body weight, and health status of healthy tobacco smokers as well as healthy non-smoker volunteers. Two

healthy tobacco smoker subjects were tracked for about three weeks to test out the stability of gut microbiota (samples collected on day 1, 7, 14 and 21 days). All the fecal samples were delivered on ice, within 4 hour of defecation. In the laboratory, fecal sample were stored at -80 ºC until DNA extraction. Neither of the healthy tobacco smoker group and healthy non-smoker group had any previous history of gastrointestinal diseases nor taken probiotics, antibiotics, and prebiotics 60 days prior to sampling.

All fecal samples were thawed and DNA extraction was performed by using the QIAGEN (Hilden, Germany) Mini Stool kit, according to the manufacturer's instructions, with initial bead-beating step of 30 s. at 5000 rpm. DNA concentration was estimated by using a Nano-Photometer (IMPLEN, Germany).[11]

For PCR–DGGE, fecal bacterial DNA was used as templates for bacterial community fingerprinting. V3 region of 16SrRNA was amplified by using universal primers shown in (Table-1). 50 µl PCR (reaction mixture) had 20 pmol of each primer, 200 mM of deoxy nucleotide triphosphate (dNTP), 2U of Taq DNA polymerase (Promega, USA), 2.5 mM MgCl2, 2.5 mM 10×buffer and fecal bacterial DNA 2 µl, (120 ng approximately). Amplification of PCR was performed in a thermo cycler (automated, ABI2720, USA) by using touchdown PCR program.[12] Final PCR products were electrophoresed in 1.5% agarose gel and stained in Ethidium bromide solution to visualize under UV illumination.

DCodeTM Universal Mutation Detection System (Bio-Rad, USA) was used to perform denaturing gradient gel electrophoresis. Briefly, PCR product that were amplified from total bacteria of each fecal sample, was loaded in 8% (w/v) (acrylamide-bis, 37.5:1) polyacrylamide gels in 1×TAE buffer tank, having 30~65% linear denaturing gradient. The DGGE gel was allowed to run at 90 V. for 14 h. on constant temperature at 60 ºC.[13]

Physically, a sterilized scalpel was used to excise the dominant bands of interest from the DGGE gel. The polyacrylamide gel piece was kept in a tube having 50 ml of sterilized water and incubated for 30 min. at 37 ºC. 8 µl of this was used, after centrifugation, as a template for PCR re-amplification of 16sRNA gene focusing V3 region with the same primers without GC-clamps, as previously were used for DGGE analysis.[14] The re-amplified PCR products were sequenced by using ABI 3500xL.[15] Real-time PCR detection and quantification were performed in a Bio-Rad CFX96 (Bio-Rad, USA). Each 20 µl reaction mixture contained 1 µl of each primer (5uM) shown in (Table-2**),** 10 µl of 2×SYBR Green PCR Master Mix (TOYOBO, Japan), 2 µl of DNA fecal sample and 6 µl of sterilized H_2O. Primers used in real time PCR shown in (Table-2).[7,10] Standard curve was generated, with standard DNA, in the same experiment. *Bacteroides vulgatus* (CICC 22938), *Clostridium leptum sub group* (YIT 6169), *Bifidobacteriaum* (CICC 6186), NWS *Lactobacillus* (from our lab), were used as standard strains. Real time PCR was done thrice and mean was taken in results. Data were reported as the average estimate of logarithms of fecal PCR target genetic amplicon, copy numbers present in 1g of feces.

Real time PCR and DGGE experiments were performed thrice; Statistics software SPSS 17 was applied for statistical analysis. The *p*-values were calculated by applying the *U* test where ($p<0.05$) was considered as statistically significant.

RESULTS

Denaturing gradient gel electrophoresis analysis was done with amplified PCR product with universal primers (targeting V3 region of 16S rRNA gene), in both tobacco smoker and non-smoker groups. Figure-1 (S1- S14) indicates samples from tobacco smokers and (C1- C6) non-smoker control. As the position, number and bands intensity were different among samples, which indicated the complex fingerprints of gut microbiota. For gut microbial diversity analysis between tobacco smoker and non-smoker group, Mann-Whitney *U* test was used to compare the *(H^1)* Shannon weaver index of diversity. The diversity results indicated (1.98±0.39 vs. 2.20±0.58) a non-significant ($p<0.167$) difference between the two groups. But, when we compared the Shannon weaver index of diversity (H^1) between two groups, it was to be lower in tobacco smoker group as compare to non-smoker group. Similarity levels of all DGGE profiles (UPGMA dendrogram and Dice similarity coefficient) were determined by using Quantity one software, shown in (Figure-2).[13] The band-based values of Dice similarity coefficient of tobacco smoker and non-smoker group, with mean similarity index, were (0.232±0.203) and (0.225±0.164) respectively, shown in (Table-3). When all values of statistical samples of tobacco smoker and non-smoker groups were compared by Dice similarity coefficient, mean similarity index between the two groups was (0.189±0.108), which indicated that it was lower in inter-group than intra-group, which shows that gut microbiota of smoker group was different from non-smoker control group. To check the stability of gut microbiota, two tobacco smoker subjects were tracked over a period of 3 weeks. DGGE microbial fingerprinting analysis was shown high similarity in band patterns of gut microbiota. Dice similarity coefficient was ranging between 94–96%.

Shannon-Weaver index *(H^1)* was calculated by following equation.

Shannon-Weaver index *(H^1)* = $-\sum_{i=1}^{s}(Pi)(\ln Pi)$

In Figure-1, 10 dominant DGGE gel bands were excised for quantity analysis. In order to verify the resolution ability of DGGE Bands in different lanes but in same positions (S8a and S10b) were cut for sequencing. Bands S8a and S10b were identified as *Bacteroides vulgatus* with 97% similarity. Taxonomic identities of other bands have been shown in (Table.4). Sequencing results were analyzed by using BLAST. Sequencing results show that phylum Bacteroidetes, Firmicutes, and Proteobacteria were dominant.

The *Bacteroides vulgatus, Clostridium leptum* sub group, *Bifidobacterium* and *Lactobacillus* genus was quantified by real time PCR. Results showed that copy numbers of *Bifidobacterium* were decreased (4.90±1.10 vs. 6.32±1.30) significantly (*p<0.010), while *Lactobacillus* (3.92±2.13 vs. 4.61±2.42) was non-significantly decreased in tobacco smoker group as compared to non-smoker subjects. Copy numbers of *Bacteroides vulgatus* (2.60±2.23 vs. 1.83±1.98) and *Clostridium leptum sub group* (1.90±1.80 vs. 3.11±1.12) were increased and decreased non-significantly, respectively. All the results are summarized in (Table-5).

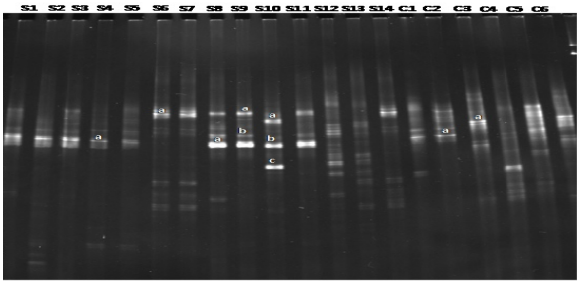

Figure-1: DGGE profile erected by using universal primer targeting V3 region and cluster analysis of tobacco smoker group (S1-S14) and non-smoker control group (C1-C6)

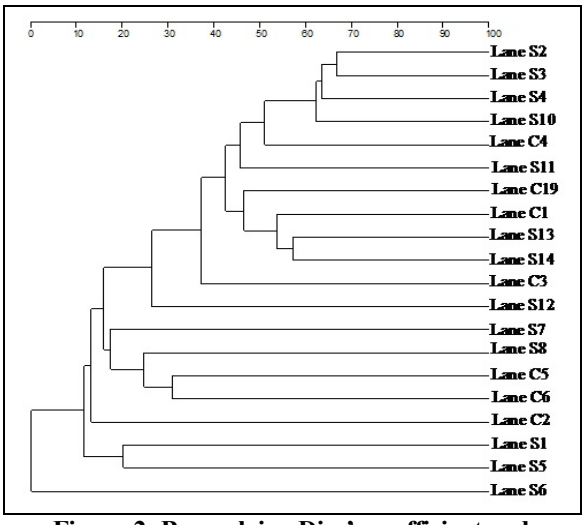

Figure-2: By applying Dice's coefficient and UPGMA

Table-1: Primer used in PCR-DGGE[25]

Primer	Sequence (5^1–3^1)
341-F	CCTACGGGAGGCAGCAG
534-R	ATT ACCGCGGCTGCTGG
341FGC	CGCCCGCCGCGCGCGGCGG GCGGGGCGGGGGCACGGG GGGCCTACGGGAGG CAG CAG

Table-2: Primers that used in Real Time PCR[25–28]

Target bacteria	Primer Sequence (5^1–3^1)	
Bifidobacterium (550 bp)	Bifid F	CTC CTGGAAACGGGTGG
	Bifi-R	GGTGTTCTTCCCGATATCTACA
Lactobacillus (250 bp)	Lact F	CTC AAA ACT AAACAAAGTTTC
	Lact R	CTC AAA ACT AAACAAAGTTTC
Bacteroides vulgatus (287bp)	BV- F	GCATCATGAGTCCGCATGTTC
	BV-R	TCC ATA CCC GACTTT ATT CCTT
Clostridium leptum sub group (239bp)	C.lep-F	GCACAAGCAGTG GAG T
	C.lep-R	CTTCCTCCGTTTTGTCAA

Table-3: Gut microbial diversity and similarity of smoker and non-smoker control group

Groups	Diversity		Similarity	
	Number of bands[a]	Shannon index[b]	Intra-similarity[c]	Inter-similarity[d]
Smokers group	5.64 ± 1.90	1.98 ± 0.39	0.232 ± 0.203	0.189 ± 0.108
Non-smokers group	6.66 ± 1.86	2.20 ± 0.58	0.225± 0.164	
p-value	0.141	0.167		

Results that are significantly different through, Mann–Whitney *U* test, where p<0.05. **a.** DGGE bands number produced by each sample. **b.** Shannon Weaver diversity index *(H^1)* was calculated by using (relative) intensities of all DGGE bands in each sample. **c.** Comparing of DGGE band profiles with Dice similarity coefficients within individual of a given group. d. Comparing of DGGE band profiles by using Dice similarity coefficients between members of tobacco smoker and non-smoker group.

Table-4: Sequencing of PCR Amplicons of excised gel bands from DGGE profile and identities based on BLAST database

Selected excised bands	Bacteria with highest % homology	Sequence Accession number	Bacterial phyla	Gene bank number
S4a	*Bacteroides oleiciplenus* (90)	YIT 1205.	Bacteroidetes	NZ_JH992946
S6a	*Bacillus sp.* (98)	FJAT-25496	Firmicutis	NZ_LMBY01000086.1
S8a	*Bacteroides vulgatus* (97)	ATCC 8482.	Bacteroidetes	NC_009614.1.
S9a	*Escherichia coli* (98)	IAI39.	Proteobacteria	NC_011750.1
S9b	*Clostridium leptum* (86)	DSM 753.	Firmicutis	NZ_DS480348.1
S10a	*Prevotella copri* (90)	DSM 18205.	Bacteroidetes	NZ_GG703855.1
S10b	*Bacteroides vulgatus* (97)	ATCC 8482.	Bacteroidetes	NC_009614.1
S10c	*Bacteroidetes oral taxon* (86)	274 str.	Bacteroidetes	NZ_GG774892.1
C2a	*Prevotella dentalis* (91)	DSM 3688.	Bacteroidetes	NC_019968.1
C3a	*Phascolarctobacterium sp.* (89)	YIT 12067.	Firmicutis	NZ_GG774892.1

Table-5: Real time PCR quantification (Mean±SD) of different bacteria

Bacteria	Non-smokers	Smokers	*p*-value
Bifidobacterium (10^4)	6.32±1.30	4.90±1.10	0.0109*
Bacteroides vulgatus (10^8)	1.83±1.98	2.60±2.23	0.2376
Lactobacillus (10^4)	4.61±2.42	3.92±2.13	0.2656
Clostridium leptum sub group (10^7)	3.11±1.12	1.90±1.80	0.0739

Data were presented as the average estimate of logarithms of fecal PCR target genetic amplicon, copy numbers present in 1 g of feces. *indicates (p<0.05).

DISCUSSION

As gut flora plays a significant role in human life. Tobacco smoking can cause vascular lung cancer, heart attack, stenosis and chronic obstructive pulmonary disease.[16] In current study, our focus is to characterize the gut microbiota of tobacco smoker (Chinese men) and non-smoker subjects. Bacterial similarity and diversity of the gut microbiota in tobacco smoker and non-smoker group were analysed by DGGE profile of 16S rRNA gene with imaging and sequencing of dominant bands of PCR amplicons, along with statistical analysis. DGGE fingerprinting techniques with 16S rRNA gene and quantification of some major bacteria through real time PCR, have been used to study the complex bacterial profiles.[7,8] To evaluate the gut microbial diversity, we calculated the Shannon–Weaver index *(H')* and number of bands from DGGE profile, which indicated that the gut microbial diversity in tobacco smoker group was non-significantly lowered when compared to the non-smoker control group. Also, Dice similarity coefficient was calculated to analyse the differences in gut microbiota of tobacco smoker and non-smoker subjects. Similarity Index comparison also indicated that it was lower in inter-group than intra-group, which shows that gut microbiota of tobacco smoker group was different from non-smoker control group. In our study, gut microbial similarity index and Shannon–Weaver diversity index *(H')* results are consistent with previous study.[7] It shows that intestinal flora of tobacco smoker subjects varies to different degrees as a result of tobacco smoking. Hence, tobacco smoking may alter the gut microbial composition. These outcomes also indicate that tobacco smoking may cause some changes in the physiology of the intestine, which may result in the modulation of gut microbial composition.[3]

In consistent with previous study, our results also showed the same phyla Bacteroidetes Firmicutes and Proteobacteria in gut microbiota of tobacco smoker group.[8,10,17,18] According to sequence results, excised gel bands show that opportunistic bacteria got increased in tobacco smoker group and dysbiosis occurred. Though, DGGE is a semi-quantitative experimental technique, band density estimation results may not relate the target abundance accurately. Hence, there is a chance of subtle associations between species abundance, and diseases may not necessarily be identified.[19] In our study, some basic and significant characterization of gut microbial composition were established by combining similarity and diversity analysis with dominant bands excision from DGGE profile and with PCR re-amplification of excised bands and sequencing.

Real time PCR was performed to study the quantitative changes of gut microbiota[20] and results indicated that a significant reduction of *Bifidobacterium* and non-significant reduction of *Lactobacillus* in tobacco smoker group, which are aligned with previous work.[7,8] Moreover, there was non-significant increase of *Bacteroides vulgatus* and non-significant decrease in *Clostridium leptum sub group* in tobacco smoker group. This non-significant decrease in *Clostridium leptum sub group* and non-significant increase of *Bacteroides vulgatus* in tobacco smoker group is also in line with previous studies.[10,21] In our study, though, there is a non-significant reduction of *Clostridium leptum* sub group in tobacco smoker group but it has been documented in previous study that low levels of *Clostridium leptum* shows high risk factor of asthma development. Exposure of *Clostridium leptum* has shown to alter the (adaptive) immunity which leads to failure of asthma development.[22]

There is a high risk of colon cancer which is linked with the presence of *Bacteroides vulgatus* and low risk factor in the presence of *lactobacillus.*[1] In daily life, the most frequently used probiotics are *Lactobacillus* and *Bifidobacterium* genera and shows health benefits in the body[23], and they have also shown anti-atherogenic, anti-inflammatory and anti-obesity effects in number of studies[24].

Conflict of interest: We hereby disclose that we do not have any conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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CONCLUSION

In this study, DGGE analysis shows that gut microbial diversity in healthy tobacco smoker group are lower than healthy non-smoker controls. While, similarity index comparison also shows that it is lower in inter-group than intra-group, which indicates that gut

microbial composition has been changed in healthy tobacco smoker group. Real time PCR results reveal that *Clostridium leptum* sub group, *Bacteroides vulgatus,* and *Lactobacillus* have shown the changes of different degree in healthy tobacco smoker group, while Copy numbers of *Bifidobacterium* are significantly reduced. So, further studies are needed with large sample numbers of both healthy tobacco smoker and healthy non-smoker subjects to understand the process and mechanism in gut microbial dysbiosis.

AUTHORS' CONTRIBUTION

HMI has performed all experimental work and written the manuscript. MS performed part of data analysis, proof-reading, editing and submission of the manuscript. XW and CM both helped in data analysis. JX has conceived the idea of this study and overall supervised it.

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