

Research Paper

Molecular Alteration Analysis of Human Gut Microbial Composition in Graves' disease Patients

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Abstract

The gut microbial association with host co-existence is critical for body homeostasis and pathogenicity. Graves' disease (GD) is an autoimmune disease manifested with hyperthyroidism and ophthalmopathy. However, we hypothesized that gut bacteria could affect an important role in GD pathogenicity. The current study aim was to characterize and investigate the intestinal bacterial composition of GD qualitatively and quantitatively. 27 GD and 11 healthy controls were enrolled for fecal sample collection. The PCR-DGGE of 16S rRNA gene by targeting V3 region and Real-time PCR for *Lactobacillus*, *Bifidobacterium*, *Bacteroides vulgatus* and *Clostridium leptum*, were performed. High-throughput sequencing of 16S rRNA gene with the V3+V4 site was performed on Hiseq2500 platform on randomly 20 selected samples. The relative analysis of richness indices and diversity illustrated lesser diversification of intestinal bacteria in GD patients in contrast to controls. The data statistics shows the alteration in phyla of GD as compared to control. At the family taxonomic level, the relative abundance of Prevotellaceae and Pasteurellaceae were significantly higher in patients, while Enterobacteriaceae, Veillonellaceae, and Rikenellaceae were significantly lower in the diseased group as compared to control. At the genus level, a significant raised in genera count of the diseased group were *Prevotella_9* and *Haemophilus*, while significantly decreased in the genera of the GD group were *Alistipes* and *Faecalibacterium*. The modulation in intestinal bacterial composition was checked at species level particularly *H. parainfluenza* abundance was raised in GD. The outcomes of the current study are aligned with the proposed hypothesis of gut microbial dysbiosis in GD. Statistically, alpha indices and differential abundance analyses of each intestinal bacterial community were significantly changed in GD. Therefore, the current study may provide a new insight into the GD pathogenesis and, in turn, explore its contribution in possible treatments.

Key words: GD, Gut microbiota, Ophthalmopathy, Hyperthyroidism, DGGE, High-throughput sequencing

Introduction

Human intestinal bacteria are described as a critical element in determining the standard body mechanism and host health standing. Power et al, described that gut microbial composition and function are stable over time but could be altered by different factors age, disease and diet [1].

The intestinal microbes constitute about 100 trillion gut microbial cells and play a key function in metabolism, absorption, immune functioning [2] along with defense mechanism against pathogen [3]. The regulation of gut microbiota configuration has been compromised in a variety of disorders like inflammatory bowel disease, Crohn's disease,

smoking, obesity, colitis, type II diabetes, Hashimoto's thyroiditis and asthma [4-7]. This gut microbiota contribution is considered as indispensable in host immune homeostasis, and its normal mechanism can be altered by the release of metabolites developing in diseased condition [8].

GD has an indicative signature as an organ-specific autoimmune disease caused due to faulty self-tolerance. Dysfunctional immune system may result in autoimmune diseases. Autoimmune disease like GD is caused by interpreting self-antigens as foreign or harmful antigens. In this situation, the immune system in GD patients cannot differentiate between foreign antigens and thyroid tissue and, resulting in destruction of the thyroid gland by body immune response [9].

Autoantibodies are produced to mimic the TSH against TSHR which is the autoantigen [10]. The epidemiological data demonstrate that GD has a female prevalence with 3% vs 0.5% in male during their lifespan [11]. Thyroid-dependent is redundant since GD is an organ-specific (thyroid) autoimmunity ranging from clinical to genetic variations along with environmental factors which are involved in dysregulate of immune tolerance [12].

The clinical evidence was manifested by an enlarged thyroid, hyperthyroidism, ophthalmopathy, pretibial myxedema muscle fatigue, Irritability, fast heartbeat, heat intolerance, and occasionally diarrhea [13]. Ophthalmopathy is considered an extra-thyroidal symptom and sign of eye disease are present in about 20% [14]. The gut microbiota (*Yersinia enterocolitica*, Gram-positive, Gram-negative, pathogenic and commensal organisms) exhibiting different bacteria have the capability of binding TSH receptor which signifies the production of antibodies from these bacteria with specific TSH receptor. TSH-receptor autoantibodies strongly able to displace ¹²⁵I-labelled TSH from the mammalian TSH receptor differed in their ability to displace the tracer from binding to bacterial extracts from *E. coli* and *Y. enterocolitica* [15]. SNP predisposing the GD were found associated to the TSHR genetic loci, other loci are indeed involved and appeared to be in common with other autoimmune disease [16].

Moreover, recent studies validated environmental factor with less vitamin D in the diet that trigger the autoimmune GD that in turn contribute to the disease progression. Recently, a meta-analysis illustrated that GD patients were more likely to have deficient in vitamin D. [17]. Interestingly, the environmental predisposing factors and genetic triggers, dysregulation of the immune system results in an immune attack on the thyroid [18]. The gut interdependence of lymphoid tissue and

microbiota in the early development of life is essential for growth of mucosal and systemic immunity. In the human fecal analysis, the majority of gut bacteria are overlain with immunoglobulin IgA that are specifically confronting any abnormality thus indicating bacterial contribution in a diseased condition like GD [8].

In this study, by applying PCR-DGGE along with accurate and sensitive High-throughput Illumina-based sequencing, it has been monitored to characterize the intestinal bacterial diversity and similarity in GD patients. Our findings highlighted a gut microbiota profile proper of GD patients that have never been published before, and can serve to pose new insights into the disease pathogenesis.

The aim of current study is to evaluate the diversity and similarity of intestinal microbiota qualitatively and quantitatively in GD as compared to their healthy counterparts.

Material and methods

Ethics statement

The informed written consent was obtained from all the participants of the study including GD patients as well as healthy volunteers. Moreover, the study was approved by an institutional ethical review committee of Xi'an Jiaotong University and performed under the guidelines of the World Medical Association and Declaration of Helsinki.

Sample collection

Stool samples were collected from 27 GD patients (10 males and 17 females) aged 35-50 years and healthy subjects, matched by age, and 11 healthy subjects (4 males and 7 females). A questionnaire was filled up regarding, dietary habits, age, gender, body weight, and health condition of healthy subjects and patients. The GD patients were diagnosed in 1st affiliated hospital (according to the guide line of Chinese Society of Endocrinology) department of endocrinology and metabolic diseases, school of medicine Xi'an Jiaotong University. The symptoms include diffused goiter, thyroid-associated ophthalmopathy, and pretibial myxedema. All the patients enrolled in this study having the disease duration of 1.5 year. Also, the patients selected for this study were taken no medicine for Grave's disease treatment from last 6 month. TSH (Thyroid stimulating hormone) was lesser than 0.25 μ IU/ml, T3 was higher than 2.20 ng/ml, T4 was higher than 13.5 μ g/dl, FT3 was higher than 9.08 pmol/L, FT4 was higher than 25.5 pmol/L, TSH receptor antibodies (TRAb) was higher than 9.31 U/L, anti-thyroglobulin Abs level was more than 30%. level of anti-thyroid peroxidase Abs was higher than 15 IU/ml, and

Normal range of serum antibodies and thyroid hormones are Anti-TGAb (< 30%), Anti-TPOAb (<15 IU/ml), and Thyroid receptor antibodies (TRAb > 9.31). TSH (0.25-5 μ IU/ml), T3 (0.78-2.20 ng/ml), T4 (4.2-13.5 μ g/dl), FT3 (2.91-9.08) and FT4 (9.05-25.5). All the fecal samples were collected in the icebox, in 1 h. of defecation and stored at -80°C for one month. None of the GD patients and normal healthy subjects had any record of gastrointestinal sickness; also there is no history of using prebiotics, probiotics, and antibiotics, two months prior to sampling.

DNA extraction from fecal sample

After thawing of all the fecal samples, DNA was extracted with the help of Qiagen stool Mini Kit (Germany) as per instructions of the manufacturer, with an initial bead-beating step along with 5000 rpm for the duration of 30 sec. [19].

PCR-DGGE

PCR-DGGE was performed by using total genomic DNA and universal primers (shown in table 1) were used to amplify the 16S rRNA gene targeting V3 region by applying touchdown PCR as previously illustrated by Muyzer et al. [20]. The amplified PCR samples were allowed to run on an agarose gel (1.5%) for electrophoresis, and finally, PCR product mixture was dipped in the solution of Ethidium bromide to illuminate and visualize under the UV light.

Table 1. The linker Primer sequence for PCR-DGGE and Real-time PCR

Target bacteria	Primer Sequence (5'-3')
PCR-DGGE Primer	
341-F	CCTACGGGAGGCAGCAG
534-R	ATT ACCGCGGCTGCTGG
341FG	CGCCCGCCGCGCGCGGCGCGGGCGGGGGCGGGG GCACGGGGGGCTACGGGAGG CAG CAG
Real Time PCR Primer	
<i>Bifidobacterium</i> (550 bp)	Bifid-F CTC CTGGAACCGGGTGG Bifid-R GGTGTCTCTCCGATATCTACA
<i>Lactobacillus</i> (250 bp)	Lact F CTC AAA ACT AAACAAGITTC Lact R CTC AAA ACT AAACAAGITTC
<i>Bacteroides vulgatus</i> (287bp)	BV- F GCATCATGAGTCCGCAITTC BV-R TCC ATA CCC GACTTT ATT CCTT
<i>Clostridium leptum</i> (239bp)	C.lep-F GCACAAGCAGTG GAG T C.lep-R CTTCTCCGTTTGCAA

Reference: PCR-DGGE [59]. References: Real-time PCR [60-62]

Denaturing gradient gel electrophoresis

DGGE experimental analysis was performed by using Universal Mutation Detection (Bio-Rad, USA) System. The bacterial amplified PCR product was loaded to acrylamide-bis, 37.5:1, 8% (w/v), gels in a tank of 1×TAE buffer solution, having 30~65 % denaturant linear gradient with the steady temperature after 60 °C DGGE gel profile was permitted to work at 90 V for the duration of 13 h. The

calculation of gut bacterial diversification in each sample was computed by the total numbers and the DGGE bands intensity, with the help of Syngene (USA) software. A similarity index was studied through Dice's similarity coefficient [21]. Moreover, Unweighted Pair Group Method with Arithmetic Mean (UPGMA), was used to establish a dendrogram [22].

Shannon-weaver (H^1) diversity index was determined by applying nonparametric unpaired *t*-test with the aid of Microsoft Excel 20110 and GraphPad Prism 7.

Shannon Weaver diversity was calculated with help of following formula:

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

Excision of bands and sequencing

The excision of the band with the gene of interest from the DGGE gel was cut with the help of a sterilized scalpel. The excised gel band was centrifuged at 37 °C in 50 μ l tube of sterilized water, and 8 μ l of this water was used to re-amplify the V3 region of 16S rRNA gene by using the same primer (with no GC-clamps) which was initially applied for PCR-DGGE experiment [23]. The re-amplified PCR products were sequenced and analyzed by ABI 3500xL and by applying BLAST and Seqmatch software, Sequences were studied and analyzed for detection and identification of genera or species [24].

Real-time PCR

Real-time PCR quantification was performed in a CFX96 Bio-Rad (USA). Primers of Real-time PCR are shown in table 1. The copy number determination of *Bacteroides vulgatus*, *Clostridium leptum*, *Lactobacillus* and *Bifidobacterium* genus in each DNA samples, *Bifidobacterium* (CICC.6186), NWS *Lactobacillus*, (from our lab) *Bacteroides vulgatus* (CICC.22938), and *Clostridium leptum* (YIT.6169) were applied as standard strains. Real-time PCR was carried in triplicates and average was considered in results analysis. For estimation of copy number of *Bacteroides vulgatus*, *Clostridium leptum* sub-group *Bifidobacterium* and *Lactobacillus* genera present in each sample, fluorescent signals detected from six serial dilutions in the linear range of the assay were averaged and compared to a standard curve generated with standard DNA in the same experiment. Data were presented to bacterial copy numbers in 1 g fecal material of GD and healthy control.

High-throughput Illumina-based sequencing and data analysis

High-throughput was performed on twenty randomly selected stool samples in Hiseq 2500

platform (10 samples from healthy control and 10 samples from GD). In a previous study of gut microbial characterization, the same sample size was used by Ishaq et al. [25]. As mentioned before the 16S rRNA gene with V3-V4 regions was amplified with primer: 341F (CCTAYGGGRBGCASCAG) 806R (GGACTACNNGGGTATCTAAT) TruSeq® DNA PCR-Free Sample Preparation Kit was used to construct the amplicons libraries. Sequencing was based on the Illumina HiSeq platform, and used Paired-End sequencing method to construct small fragment library [26]. The data retrieved was assembled and screened by using FLASH [27] and QIIME (V1.7.0) [28] software package. The UCLUST procedure [28] was used to cluster the sequencing into Operational Taxonomic Units (OTUs) at 97% identity threshold. In the process, the RDP Classifier was applied to assign the OTU at taxa level [29]. Diversity analysis of retrieved data, such as Simpson and Shannon index, ACE, Chao1, and Good's coverage, was performed by using QIIME. However, the OTU table was generated by applying QIIME which imported into MEGAN 4 and analyze on the taxonomy database of NCBI [30]. The Shannon measure of evenness was calculated with the formula $E = H/\ln(S)$, where "H" is the Shannon diversity index and S is the total number of sequences in that group.

The statistically gut microbial community composition differences and diversity indices between the samples of GD patients and healthy volunteers were computed nonparametric unpaired t-test ($P < 0.05$) by using Microsoft Excel 2010 along with the aid of GraphPad Prism 7.

Results

DGGE Statistical analysis of gut microbial composition

The amplification of PCR with DGGE assay used to locate the specific primers sequence of the V3 region of 16S rRNA gene in GD patients and controls. The detailed results are shown in **figure 1.A** (G1-G15) demonstrate the GD samples, and (C1-C5) to healthy controls, while **figure 1.C** (G16 - G27) represents GD samples, and (C6-C11) control subjects. The gene fragment location, strength, and variation in the lanes for various samples exemplify the complex fingerprints of intestinal microbiota. The total aggregate of 213 bands was detected by Syngene software in 27 tracks of GD with an average band of (7.67 ± 2.79), other sums of 87 bands were identified in 11 lanes of the control group with a mean of (8.57 ± 2.11). Differences in the diversity of the fecal microbiota of GD patients and healthy controls were

assessed by using the Shannon/Weaver index. The diversity (H') findings stipulated the (2.34 ± 0.44 vs. 2.47 ± 0.42) No significant differences were observed in the (H') index between GD and healthy controls ($P < 0.2$). The resultant distinguishable findings elucidate that gut microbial composition varies with their healthy counterparts. Furthermore, Dice similarity coefficient and UPGMA were applied on (**figure 1.B**, **figure 1.D**) to determine the extent of the resemblance of all the DGGE profiles. Non-significant ($P < 0.159$) assessed values of the similarity coefficient of GD and control with average index values (0.481 ± 0.166) and (0.419 ± 0.183), respectively shown in table 2. The compiled data of statistical samples for GD and the healthy control analogized by Dice coefficient depicted with mean indexed values were (0.339 ± 0.129), thus showing lesser values among the groups that in turn affirm that intestinal microbes of GD patients varies from the normal control group.

Table 2. Gut microbiota diversity and similarity in GD and control

Groups	Diversity		Similarity	
	The number of Bands ^a	Shannon index ^b	Intra-similarity ^c	Inter-similarity ^d
Disease group	7.67±2.79	2.34±0.44	0.481±0.166	0.339±0.129
Control group	8.57±2.11	2.47±0.42	0.419±0.183	
P	0.251	0.211	0.159	/

Results which are significantly different (nonparametric unpaired t test), with ($P < 0.05$)

a. Number of denaturing gel electrophoresis (DGGE) bands produced by each sample.

b. Shannon diversity index (H') was calculated using the relative intensities of all DGGE bands in each sample.

c. Dice similarity coefficients comparing DGGE band profiles within individual of a given group.

d. Dice similarity coefficients comparing DGGE band profiles between members of Graves' disease and healthy group.

Sequence analysis

The sum of 26 bands was cut from two DGGE gels, the figure 1 A, from DGGE profile, 18 bands were cut for quantitative analytical study. The DGGE gel resolution was verified by the excision of bands at the same positions but in different lanes, the bands C1a and C2b, G2a, G5a, and G9a were cut for sequence analysis. The excised gel bands C1a and C2b were sequenced as *Bacteroides uniformis* with 97% homology while, bands G2a, G5a, and G9a were sequenced as *Prevotella copri* with 96% homology. Likewise, from figure 1.C, 8 DGGE gel bands were excised and monitor the DGGE gel resolution capacity, bands G25a, G27a and C10a and C11a were sequenced. The bands at G25a, G27a were identified with *Enterobacter aerogenes* 92% similarity while C10a and C11a were *Bacteroides plebeius* with 91 % homology. Taxonomic identification of other DGGE

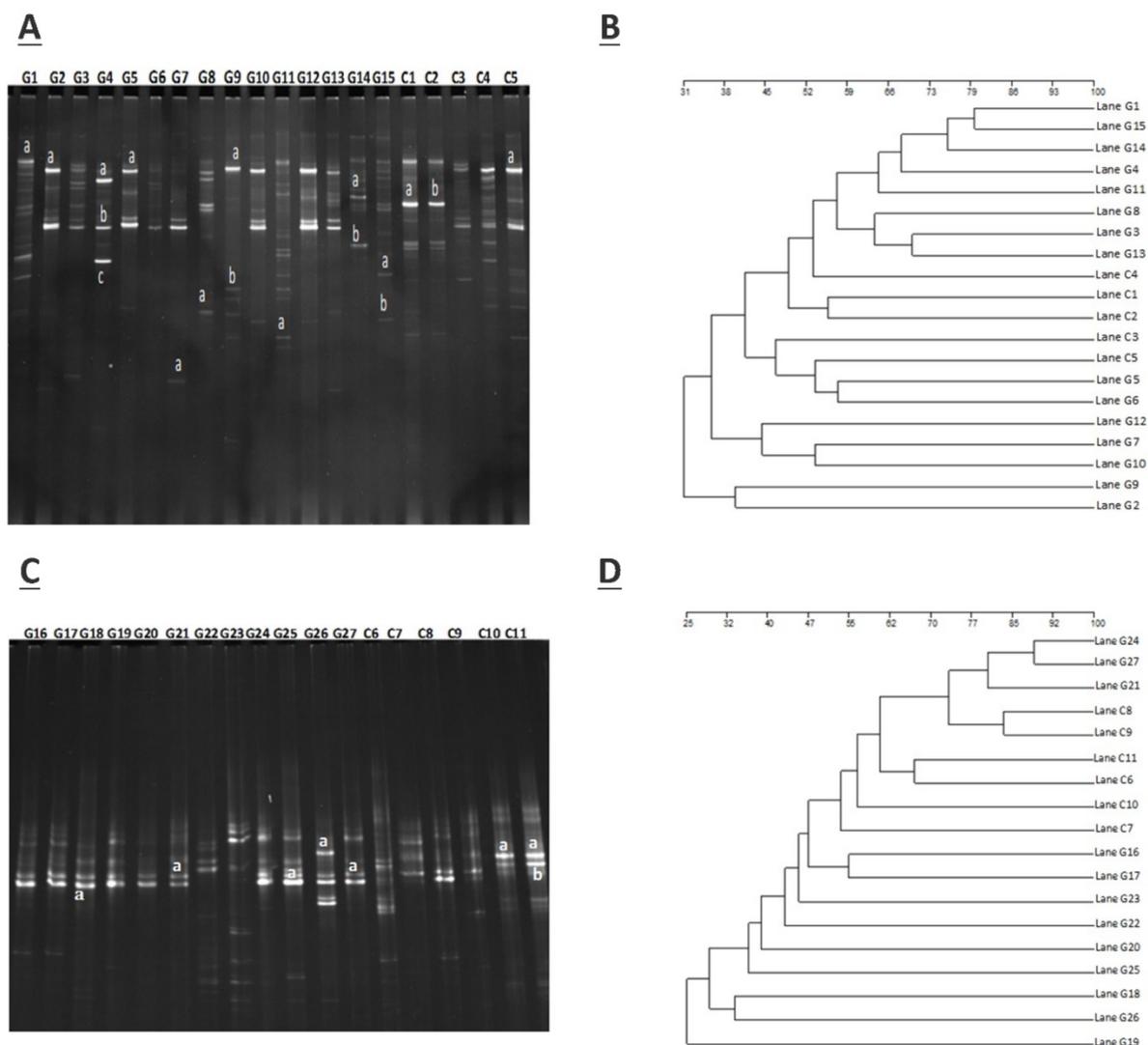


Figure 1. A. DGGE gel profile constructed by linker primer of 16S rRNA gene with V3 region, B. Cluster analysis of GD (G1-G15) and healthy control group (C1-C5) by computing a Dice's coefficient and the UPGMA, C. DGGE gel profile constructed by linker primer of 16S rRNA gene with V3 region, D. Cluster analysis of GD (G16-G27) and control group (C7-C11) by applying the UPGMA and Dice's coefficient.

gel bands has been depicted in table 3. Phyla Firmicutes, Bacteroidetes, and Proteobacteria were dominant. The results findings of two DGGE gel illustrated the more prevalence of opportunistic intestinal bacteria (*Bacteroides intestinalis*, *Shigella dysenteriae*, *Parabacteroides distasonis*, *Alistipes putredinis*, *Bacteroides coprocola*, *Prevotella copri*, *Sporomusa ovata*, *Bacteroides nordii*, *Bacteroides helcogenes*, *Bacteroides paurosaccharolyticus*, *Faecalibacterium prausnitzii*, *Enterobacter aerogenes*, *Klebsiella sp*) in GD patients.

Real-time PCR assay

The real-time PCR was conducted mean value evaluation index demonstrates that *Lactobacillus* and *Bifidobacterium* copy number were reduced in GD compared to controls. Copy number of *Clostridium leptum* and *Bacteroides vulgatus* were reduced and increase, respectively, in GD compared to healthy

controls, however with $P < 0.05$ (Table 4). Unpaired t-test was used for statistical analysis.

High-throughput gene sequencing data

The approximate numbers of amplicon 1,810,600 at V3+V4 location in 16S rRNA gene were sequenced from 10 GD patients and 10 healthy control subjects. A total of combined sequencing reads 1,684,712 (control 851,988 and disease 832,724, Ave. per sample 84,236) were proceeded for quality check thus confining in our analysis. The Taxon tag in both GD and control samples was (Ave.72588). The aggregate number of unique tag revealed in GD and healthy control groups was 7958 and 9569, respectively (Ave. 876) in all experimental samples. The average count of 260 OUTs was attributed in both GD and control group described in figure 2. The gross figures of unique sequence for the two groups were 17527 that exhibit all the phlotypes in this analysis. The current

experimental analysis focused on the gut microbial taxa that were predominantly affluence of more than (0.5-1%) over all. After deletion of the primer, the average sequence length remained 418 bp.

Table 3. Excision of PCR Amplicons from DGGE gel and retrieved sequence from BLAST database

Selected Exised bands	Bacteria with highest % homology	Sequence Accession number	Bacterial phyla	Gene bank number
C1a	<i>Bacteroides uniformis</i> (97)	CL03T00C23	Bacteroidetes	NZ_JH724260
C2b	<i>Bacteroides uniformis</i> (97)	CL03T00C23	Bacteroidetes	NZ_JH724260
C5a	<i>Bacteroides vulgates</i> (97)	ATCC 8482	Bacteroidetes	NC_009614.1
G1a	<i>Shigella dysenteriae</i> (98)	Sd197	Proteobacteria	NC_007606.1
G2a	<i>Prevotella copri</i> (96)	DSM 18205	Bacteroidetes	NZGG703855.1
G4a	<i>Parabacteroides distasonis</i>	ATCC 8503	Bacteroidetes	NC_009615.1
G4b	<i>Clostridium botulinum</i> (88)	ATCC 3502	Fermicuitis	NC_009495.1
G4c	<i>Bacteroides intestinalis</i> (95)	DSM 17393	Bacteroidetes	NZ_ABJL02000006.
G5a	<i>Prevotella copri</i> (96)	DSM 18205	Bacteroidetes	NZGG703855.1
G7a	<i>Alistipes putredinis</i> (97)	DSM 17216	Bacteroidetes	NZ_DS499580.1
G8a	<i>Bacteroides coprocola</i> (96)	DSM 17136	Bacteroidetes	NZ_DS981502.1
G9a	<i>Prevotella copri</i> (96)	DSM 18205	Bacteroidetes	NZGG703855.1
G9b	<i>Sporomusa ovata</i> (87)	DSM 2662	Fermicuitis	NZ_ASXP01000005.1
G11a	<i>Bacteroides nordii</i> (86)	CL02T12C05	Bacteroidetes	NZ_JH724319.1
G14a	<i>Bacteroides fragilis</i> (86)	YCH46	Bacteroidetes	NC_006347.1
G14b	<i>Prevotella dentasini</i> (91)	JCM 15908	Bacteroidetes	NZ_BAKG01000039.1
G15a	<i>Bacteroides helcogenes</i> (91)	P 36-108	Bacteroidetes	NC_014933.1
G15b	<i>Bacteroides paurosaccharolyticus</i> (88)	JCM 15092	Bacteroidetes	NZ_BAJR01000054.1
G18a	<i>Faecalibacterium prausnitzii</i> (96).	TDY5834930.	Firmicutes	NZ_CZBH01000014.1
G21a	<i>Bacillus sp.</i> (94).	FJAT-25496.	Firmicutes	NZ_LMBY01000086.1
G25a	<i>Enterobacter aerogenes</i> (92)	KCTC 2190	Proteobacteria	NC_015663.1
G26a	<i>Klebsiella sp.</i> (94).	NODE14.	Proteobacteria	NZ_LGIT01000014.1
G27a	<i>Enterobacter aerogenes</i> (92)	KCTC 2190	Proteobacteria	NC_015663.1
C10a	<i>Bacteroides plebeius</i> (91)	DSM 17135	Bacteroidetes	NZ_DS990123.1
C11a	<i>Bacteroides plebeius</i> (91)	DSM 17135	Bacteroidetes	NZ_DS990123.1
C11b	<i>Faecalibacterium prausnitzii</i> (96).	TDY5834930.	Firmicutes	NZ_CZBH01000014.1

Table 4. Targeted bacterial load (copy number) quantification by Real-time PCR

Bacteria	Healthy Subjects	GD	P*
<i>Bifidobacterium</i> (10 ³)	4.05 ± 0.83	3.48 ± 0.53	0.008*
<i>Bacteroides vulgatus</i> (10 ⁷)	4.32 ± 0.93	4.59 ± 0.66	0.159
<i>Clostridium leptum</i> (10 ⁶)	4.68 ± 1.21	4.40± 0.74	0.194
<i>Lactobacillus</i> (10 ⁴)	7.44 ±1.08	6.78 ± 0.64	0.012*

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

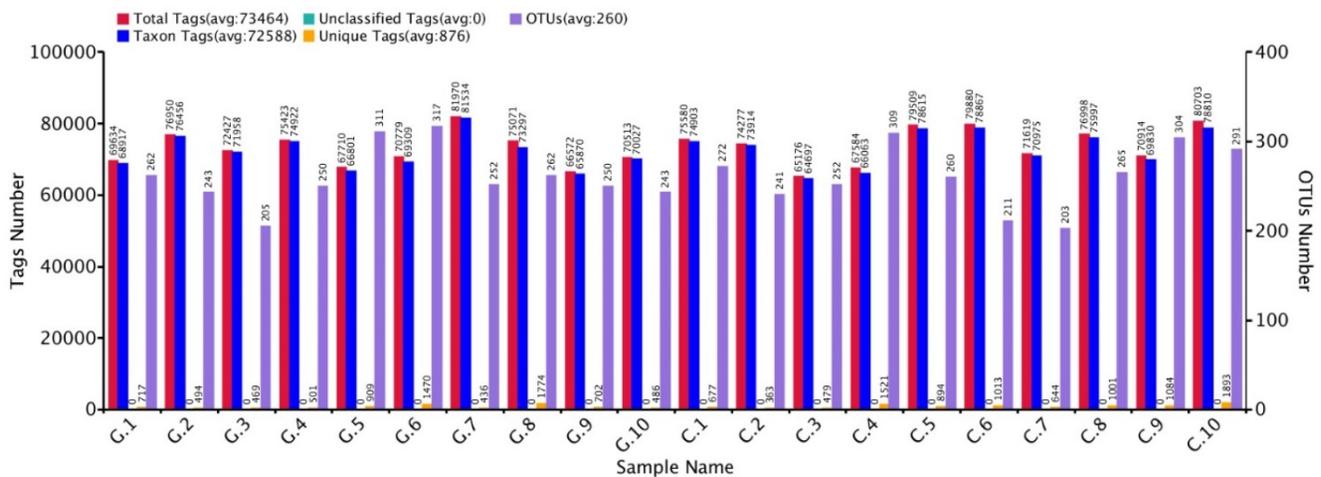


Figure 2. GD observations for OTUs and Tag number versus in contrast to control OTUs and Tag number with 97% similarity level.

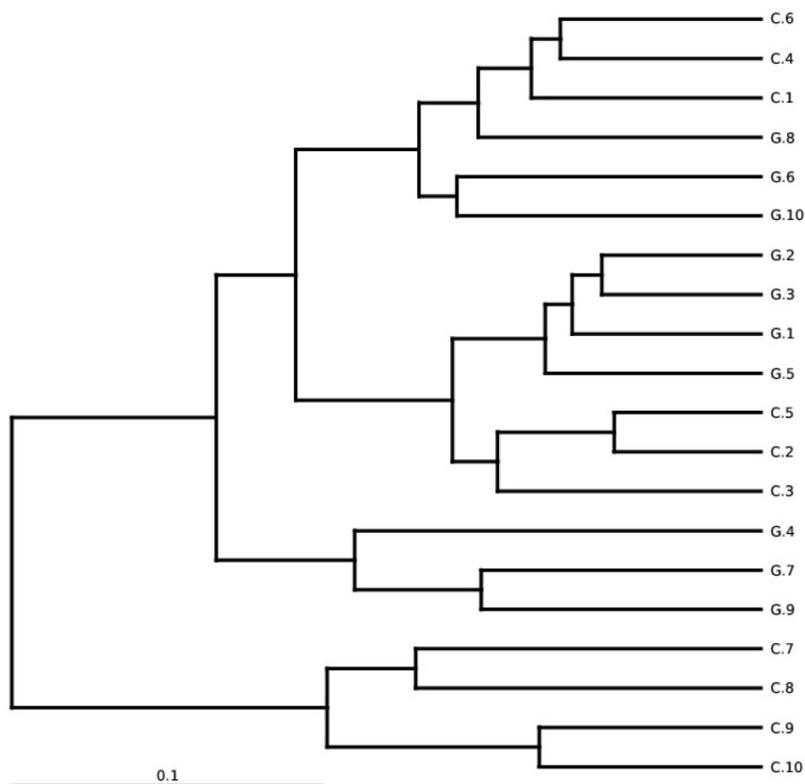


Figure 3. Between-sample diversity, sample G1-G10 from GD patients and C1-C10 controls. UPGMA based on weighted UniFrac distances.

Gut microbial diversity and configuration analysis

The total gut microbial community was explained by diversity and richness as computed at the level of 97% resemblance. The Alpha diversity estimated by the equation of ACE ($P < 0.012$) and Chao1 ($P < 0.016$) observed species ($P < 0.041$) and Good's coverage ($P < 0.037$) that was notably elevated in the controls while contrasting the GD patients, respectively. Alpha diversity indices are within-samples estimation of diversity used to compare two different groups. The level of diversity in each group was described in table 5. The samples were categorized in two batches relying on weighted UniFrac metrics depicted in figure 3. Broadly speaking the gut microbial population was estimated and monitored from phylum to further narrowing down to family, genus and finally at the species level.

Table 5. High-throughput analysis of Gut microbial richness and diversity having 97% similarity index

Group	Observed Species	Shannon	Simpson	Chao1	ACE	Good's coverage	Evenness
Patients	221.80	4.42	0.870	254.66	257.20	0.9994	0.340
Control	244.20	4.63	0.910	282.87	302.42	0.9998	0.356
P*	0.041	0.267	0.092	0.016	0.012	0.037	0.271

The values denoted in the table are the mean values of each group, significant findings were testified with (unpaired t-test) and ($P < 0.05$). The evenness (Shannon) estimation was conducted by applying formula

$E = H / \ln(S)$, where H is the Shannon diversity index and S is the total number of sequences in that group.

Gut microbial composition at phyla level

15 phyla were identified; among 10 topmost phyla in GD was Firmicutes (32.89), Bacteroidetes (57.55%), Proteobacteria (8.47 %), Actinobacteria (0.90 %) Fusobacteria (0.08 %). while Verrucomicrobia, Tenericutes, Cyanobacteria, Synergistetes, and Gemmatimonadetes were sequenced less than (0.5%) and others (around 0.03% of total sequences) in disease group. Likewise in control group, Firmicutes (40.61 %), Bacteroidetes (51.47 %), Proteobacteria (7.26 %), Actinobacteria (0.31 %), Verrucomicrobia, (0.10 %) Tenericutes (0.15%), Fusobacteria (0.06%), while Cyanobacteria, Synergistetes and Gemmatimonadetes found is less than (0.5%) and others (around 0.01 % of total sequences) shown in figure 4. There was more phyla prevalence of Bacteroidetes ($P < 0.153$), Actinobacteria ($P < 0.066$), and Proteobacteria ($P < 0.346$) and less Firmicutes ($P < 0.152$) in the GD patients than in the healthy group. The statistics data estimation of top 10 phyla in table 6 demonstrates the differential abundant phyla.

Gut microbiota distribution at family level

At the level of family, 84 different families were detected in High-throughput sequencing experiment. Among 10 top most family in diseased group were Prevotellaceae (40.74 %), Ruminococcaceae (10.09 %), Bacteroidaceae (13.08 %), Lachnospiraceae (10.14 %),

Pasteurellaceae (9.21 %), Veillonellaceae (1.62 %), Succinivibrionaceae (1.20 %), Enterobacteriaceae (1.14 %), Alcaligenaceae (1.76 %), Rikenellaceae (2.29 %) that constitute approximately (91.27 %) of the entire bacterial community along with (8.73%) others. In addition to the control group, Prevotellaceae (20.09 %), Ruminococcaceae (19.37 %), Bacteroidaceae (20.95 %), Lachnospiraceae (10.04 %), Pasteurellaceae (0.54

%), Veillonellaceae (5.10 %), Succinivibrionaceae (0.98 %), Enterobacteriaceae (4.16 %), Alcaligenaceae (2.57 %), Rikenellaceae (5.76 %) that form the (89.58 %) of the whole bacterial community along with (10.42%) others. Prevotellaceae, Ruminococcaceae, and Bacteroidaceae were enriched values in diseased and controls depicted in figure 5. Nevertheless, the counts of Prevotellaceae ($P<0.023$) and Pasteurellaceae ($P<0.046$) were significantly higher in disease group. On the other hand, Enterobacteriaceae ($P<0.033$), Veillonellaceae ($P<0.040$), and Rikenellaceae ($P<0.0094$) were significantly lowered in the GD group as compared to healthy subjects. The top 10 family level data statistics depicts in table 6.

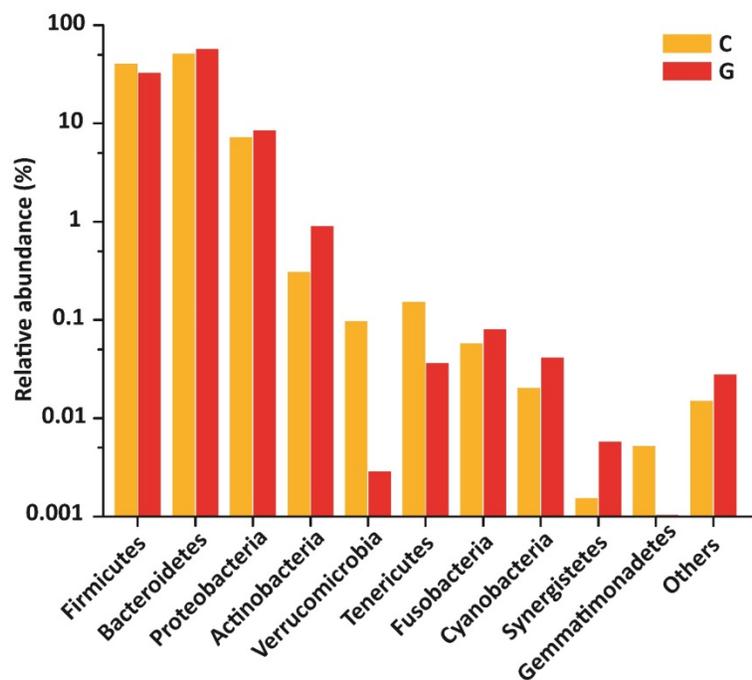


Figure 4. Gut microbial composition differences between GD and C at the phylum level.

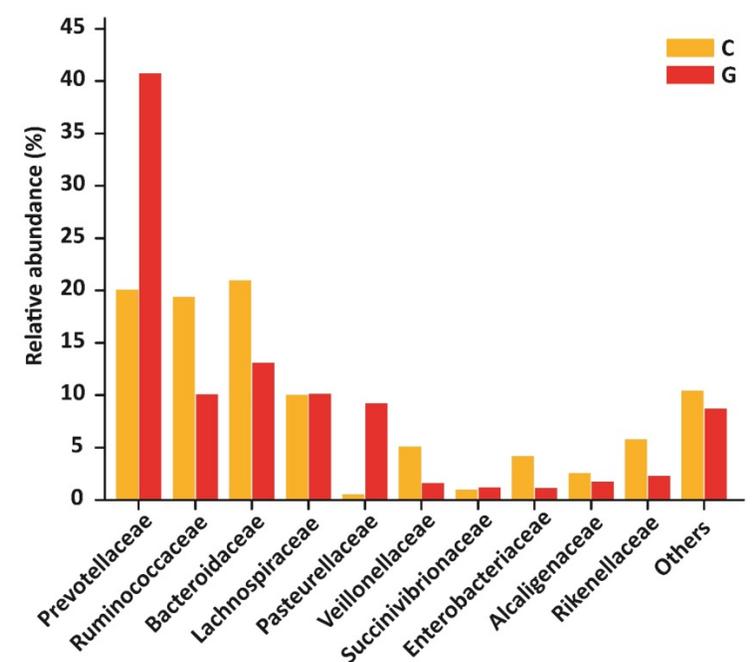


Figure 5. 10 most prevalent families with relative abundance in GD and healthy controls. The relative abundance of Prevotellaceae and Pasteurellaceae were significantly higher in patients, While Enterobacteriaceae, Veillonellaceae, and Rikenellaceae were significantly lower in the diseased group as compared to control. Where (* $P<0.05$).

Gut microbial composition at genera level

At the level of genera, 194 different genera were sequenced in the same experiment. Among 10 top most genera in GD patients were *Prevotella_9* (40.60 %), *Bacteroides* (11.71 %), *Haemophilus* (11.09 %), *Ruminococcus_2* (0.85 %), *Dialister* (0.90 %), *Eubacterium* (1.67 %), *Succinivibrio* (1.16 %), *Alistipes* (1.47 %), *Parabacteroides* (2.06 %), *Faecalibacterium* (2.36 %) that composed of (73.87 %) whole gut bacterial community along with (26.13 %) others. Furthermore, in control group *Prevotella_9* (19.30 %), *Bacteroides* (21.34 %), *Haemophilus* (0.98 %), *Ruminococcus* (3.34 %), *Dialister* (4.40 %), *Eubacterium* (2.23 %), *Succinivibrio* (1.12 %), *Alistipes* (4.68 %), *Parabacteroides* (3.05 %), *Faecalibacterium* (5.56 %) that formed the (66.00%) of gut bacterial population along with (34.00%) others. The OUT counts of *Prevotella_9* ($P<0.033$) and *Haemophilus* ($P<0.048$) genera were significantly raised in GD patients as compared to healthy subjects depicted in the figure 6. While the abundance of *Alistipes* ($P<0.025$) and *Faecalibacterium* ($P<0.014$) genera were significantly reduced in GD patient as compared to control. The 10 topmost genera statistics differential abundance analysis has shown in table 6.

Gut microbiota distribution at species level

We have also examined the organization of gut microbial taxa at species level represented in table 7. The results also determine the alterations at species level between GD patients and healthy control subjects. However, *H. parainfluenzae* species

showed a raised level in GD patient as compared to healthy subjects.

Table 6. Top 10 differentially abundant taxonomy from High-throughput sequencing results.

Taxa	Mean GD	GD. SD	Mean C	C. SD	P*
Phylum					
Firmicutes	0.33546	0.10514	0.40609	0.18348	0.15240
Bacteroidetes	0.58701	0.09408	0.51472	0.19572	0.15320
Proteobacteria	0.08635	0.08921	0.07264	0.06138	0.34680
Actinobacteria	0.00919	0.01203	0.00309	0.00219	0.06610
Verrucomicrobia	0.00003	0.00005	0.00097	0.00285	0.15540
Tenericutes	0.00037	0.00053	0.00153	0.00242	0.07790
Fusobacteria	0.00082	0.00102	0.00058	0.00151	0.33960
Cyanobacteria	0.00042	0.00095	0.00020	0.00030	0.24550
Synergistetes	0.00006	0.00016	0.00002	0.00003	0.21240
Gemmatimonadetes	0.00004	0.00006	0.00005	0.00008	0.36230
Family					
Prevotellaceae	0.48201	0.32681	0.20695	0.24702	0.02390
Ruminococcaceae	0.11944	0.08117	0.19952	0.18935	0.11740
Bacteroidaceae	0.15478	0.05910	0.21579	0.15717	0.13280
Lachnospiraceae	0.11998	0.09688	0.10341	0.05886	0.32470
Pasteurellaceae	0.10899	0.18433	0.00557	0.00549	0.04650
Veillonellaceae	0.01919	0.00646	0.05256	0.05670	0.04050
Succinivibrionaceae	0.01416	0.04423	0.01013	0.03159	0.40860
Enterobacteriaceae	0.01346	0.01892	0.04285	0.04352	0.03310
Alcaligenaceae	0.02087	0.01370	0.02649	0.04033	0.34070
Rikenellaceae	0.02710	0.02760	0.05936	0.03430	0.00940
Genus					
Prevotella_9	0.49699	0.42196	0.19522	0.25092	0.03390
Bacteroides	0.14335	0.10711	0.21579	0.15717	0.12200
Haemophilus	0.13580	0.22717	0.00991	0.01218	0.04860
Ruminococcus_2	0.01044	0.01933	0.03379	0.05251	0.10180
Dialister	0.01101	0.01943	0.04448	0.05659	0.04690
Eubacterium	0.02042	0.02789	0.02255	0.04997	0.45370
Succinivibrio	0.01416	0.04423	0.01132	0.03468	0.43740
Alistipes	0.01795	0.01485	0.04736	0.04185	0.02530
Parabacteroides	0.02523	0.01371	0.03082	0.03580	0.32510
Faecalibacterium	0.02891	0.01750	0.05622	0.03177	0.01430

Nonparametric unpaired t test was applied. Outcomes which are significantly different, where P<0.05

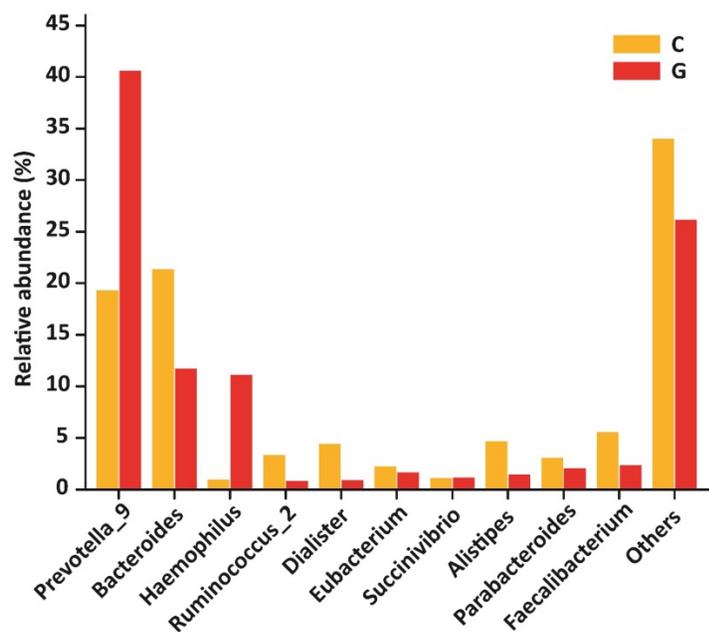


Figure 6. 10 most existed genera with relative abundance observed in GD and healthy controls. Significant increased genera in GD were *Prevotella_9* and *Haemophilus*, while significantly decreased genera of diseased group were *Alistipes* and *Faecalibacterium*. Where (* P<0.05).

Table 7. Differential abundant species in GD v/s C by High-throughput sequencing analysis.

Taxa	mean(GD)	mean(C)	p value	q*
<i>Haemophilus parainfluenzae</i>	0.0322704	0.0099031	0.09254	0.32442
<i>Bacteroides vulgatus</i>	0.0808569	0.0753513	0.86014	0.92973
<i>Bacteroides plebeius</i>	0.0071657	0.0240073	0.15385	0.48126
<i>Bacteroides uniformis</i>	0.0266581	0.0334637	0.61139	0.80204
<i>Bacteroides_stercoris</i>	0.0229439	0.0225265	0.97103	1
<i>Parabacteroides distasonis</i>	0.0072955	0.0138739	0.65135	0.84537
<i>Escherichia coli</i>	0.0075784	0.011543	0.80919	0.9057
<i>Coprobacillus cateniformis</i>	0.0084162	0.00017	0.42557	0.70232
<i>Bacteroides fragilis</i>	0.0123962	0.0040837	0.27672	0.62745
<i>Clostridium leptum</i>	0.0002952	0.006532	0.12587	0.42657
<i>Clostridiales oral-taxon</i>	0.0057081	0.0003972	0.1978	0.56335
<i>Bacteroides ovatus</i>	0.0191771	0.0165495	0.68232	0.86711
<i>Parabacteroides merdae</i>	0.0171971	0.0135416	0.51449	0.72146
<i>Bacteroides massiliensis</i>	0.0101689	0.0059956	0.39361	0.70232
<i>Bacteroides coprophilus</i>	0.0027312	0.0062198	0.45854	0.70232
<i>Campylobacter concisus</i>	0.0007929	1.70E-05	0.005	0.08706
<i>Campylobacter showae</i>	0.0001206	0	0.001	0.02438
<i>Lactobacillus gasseri</i>	0	0.000102	0.001	0.02438
<i>Alistipes sp.</i>	0	8.81E-05	0.001	0.02438
<i>Veillonella sp.</i>	3.86E-05	0	0.001	0.02438
<i>Bifidobacterium_longum</i>	6.18E-06	8.19E-05	0.03397	0.27626
<i>Morganella morganii</i>	2.47E-05	0	0.001	0.02438
<i>Clostridium sp.</i>	1.24E-05	1.55E-06	0.03906	0.29785
<i>Bacteroides coagulans</i>	0	1.24E-05	0.00781	0.1059
<i>Methanobrevibacter smithii</i>	9.27E-06	0	0.03125	0.27232
<i>Lactobacillus iners</i>	0.00E+00	1.39E-05	0.00391	0.07942
<i>Firmicutes bacterium</i>	0	9.27E-06	0.03125	0.27232
<i>Leuconostoc lactis</i>	1.24E-05	0	0.00781	0.1059
<i>Campylobacter ureolyticus</i>	0	9.27E-06	0.03125	0.2723

Where P<0.05

Correlation of molecular protocols

The research findings by applying molecular methods like DGGE and High-throughput sequencing analysis unveiled the most prevalence of bacterial phyla were Bacteroidetes, Firmicutes Proteobacteria, and Actinobacteria. However, the sensitive and more reliable High-throughput Illumina-based sequencing at the platform of Hiseq 2500 confirm the authenticity of the diverse gut microbial population. The PCR-DGGE technique could be applied as a basic routine test to observe the massive changes of intestinal bacterial ecology as less time consuming and economical. In the current study, the data generated from experimental techniques like DGGE, Real-time PCR, and high-throughput sequencing were also aligned. Furthermore, the sensitivity and suitability of high-throughput sequencing are proven in the proceeding methods to analyze and investigate the intestinal bacterial ecology [31].

Discussion

Human intestinal microbes play a vital role in host body defense in response to pathogenesis mediated by protective, trophic, and metabolic functions [8]. The GD patient shows the alterations of intestinal bacterial composition as

compared healthy controls as evident by profiling DGGE, High-throughput sequencing analysis, and Real-time PCR data. So, GD patient's bacterial taxa count testifies the alterations in comparison to healthy controls, with certain gut bacteria corresponds to variable levels.

Indices of richness such as observing species, ACE, Chao1 and Good's coverage significantly elevated in control as compared to GD. Notwithstanding the statistics do not signify the alterations in Shannon index and Simpson index that coordinate with preceding analysis [32]. The intestinal microbial community similarity and diversity estimations by DGGE gel profiles and High-throughput sequencing analysis illustrate that the gut microbiota: compared to healthy control was less varied that was corroborating the previous investigations on gastrointestinal tract, skin, and vagina [33-35]. The similarity index results in DGGE profile showed the lowering values in inter-group distinguishing from intra-group [36].

GD patients showed a notable alteration in gut bacterial composition as compared to healthy control; four phyla (Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria) were the most represented from those identified in both groups. There was more phyla abundance of Bacteroidetes, Proteobacteria and low level of Firmicutes that differentiating diseased and healthy group [31, 37, 38]. Further consistency was attributed with plentifulness of families Prevotellaceae and Pasteurellaceae that were a notably greater in GD with comparison of control volunteers which is aligned with previous work of radiotherapy effect on gut microbiota of gynecological cancer patients [31]. The relative abundance of Enterobacteriaceae Veillonellaceae and Rikenellaceae were significantly decreased in GD with comparison to normal control which is aligned with previous literature of gut microbiota in multiple sclerosis patients [39].

At the level of genera, significantly raised genera in the GD patients were *Prevotella_9* and *Haemophilus*, while significantly decreased genera were *Alistipes* and *Faecalibacterium* which is compatible with previous work [40, 41]. The intestinal bacterial taxa comparison along with species-level community showed a distinct segregation of intestinal bacterial organization between the GD patients and normal healthy volunteers which support the previous investigation on a possible role of the microbiota in the susceptibility or protection against diarrhoea after radiotherapy [42]. Interestingly present findings of gut microbial alterations of diseased and control demonstrated no direct relationship between GD and intestinal microbiota [14, 43, 44]. Thus, our results of

diverse intestinal bacterial composition between the GD and healthy groups may responsible for amending the health status of an individual which is compatible with previous work of gut microbiota of gynecological cancer patients despite that disease development are not related to intestinal tract [31]. Thyroid disease like GD mostly affects the hollow organs with Hyperthyroidism with low gastric acid production. It increases the intestinal motility along with autoimmune gastritis resulting in diarrhea. It endorse that GD may involve in the intestine physiological alterations that further reshape the gut microbial composition [45].

The intestinal microbiota quantitative alterations can be observed by Real-time PCR [46], and the retrieved data signifies the reduction of *Bifidobacterium* and *Lactobacillus* [47]. The *Bacteroides vulgatus* and *Clostridium leptum* copy numbers were raised and lowered in GD patients respectively as compared to healthy control which is also reported in previous literature on metabolic endotoxemia initiates obesity and insulin resistance, early-life exposure to *Clostridium leptum* causes pulmonary immunosuppression [48, 49]. The use of probiotics frequency is increasing due to their health benefits especially the *Lactobacillus* and *Bifidobacterium* genera [50] involved in reducing colorectal cancer and has proven therapeutic role against obesity and inflammation and also exhibit poor probability in the development colon cancer in the existence of *lactobacillus* [8, 51].

As previously reported, low level of *Clostridium leptum* serves as a major predisposing factor for asthma progression. Conversely, *Clostridium leptum* exposure contributes to modulate the adaptive immunity that prevent from asthma development [49]. Our study findings depicted the slightly lower level of *Clostridium leptum* in the diseased group that specifies its role as contributing factor in GD. It has been documented that *Bacteroides vulgatus* is most frequently found in human intestinal microflora that co-exists with the mutual relationship of the host through prevention of intestinal colonization through competing with pathogens [52]. *Bacteroides vulgatus* is associated with a high-risk of colon cancer development while presence of *lactobacillus* constitutes a low-risk [8]. The current study represents the slightly raised level of *Bacteroides vulgatus* in GD patients.

Firmicutes to Bacteroidetes ratio has been previously used to describe the gut microbiota which may alter the host health status [53]. Our findings demonstrated slightly increase/decrease Bacteroidetes, Proteobacteria and Firmicutes thus in fact $P > 0.05$. Future studies with more GD patients and

healthy controls might be helpful to increase the statistical power.

There is a remarkable decrease of Veillonellaceae in GD patients which has a useful commensal function which is belonging to class Clostridia. It plays an important role in the body to induct the T regulatory immune cells [54]. Our results showed that significantly raised levels of *Prevotella* genus in the diseased group. However, *Prevotella* fluctuations play a vital in digesting of plant diet. It has also been documented that it may contribute to urinary tract infection, arthritis brain abscesses and osteomyelitis conditions [55, 56]. Enterobacteriaceae has rarely shown contributing proof in the infective endocarditis [57], and our finding showed an increase level of Enterobacteriaceae in control group as compared to diseased group. *Haemophilus* sub-type contributes in fatal chronic diseases in human especially cancer [41], *H. parainfluenzae* co-existence with host contribute to the infectious diseases involving respiratory, urinary, genital, meningitis, pneumonia along with endocarditis and arthritis [58]. The current experimental findings render rise in genus *Haemophilus* particularly in *H. parainfluenzae* species of GD patients that might be the high possible candidate as a disease-causing microbe.

The gut microbiota and disease inter-linkage have long been under investigation, current experimental data on 16S meta taxonomics approach can highlight association of potential disease-associated bacteria. The GD clinical symptoms like hyperthyroidism, ophthalmopathy, and pretibial myxedema along with circulating antibodies have a crucial role in reshaping the gut microbial configuration that may contemplate being the causative agent. The results findings of blood circulating antibodies anti-thyroglobulin, TRAb and anti-thyroid peroxidase and the level of serum thyroid hormones in GD patients and normal control, depicted in **table S1**, and **table S2**, respectively. The **table S2** results reveal the remarkably raised level of aforementioned antibodies in the GD patients as compared to healthy control group. The higher level of antibodies in GD might alter the composition of intestinal microbes, in particular, the Phyla, Bacteroidetes, Actinobacteria and Firmicutes family Prevotellaceae Pasteurellaceae, Enterobacteriaceae, Veillonellaceae, and Rikenellaceae, genera *Prevotella*, *Haemophilus*, *Alistipes* and *Faecalibacterium* and species *H. parainfluenza*, and also largely influence the intestinal flora. Therefore in case of GD, it can be hypothesized that alteration in gut microbiota has a vital connection with the development of clinical features and further intensifying the disease. The limitations of human clinical work and lack of

substantial data on GD obstruct to establish the possible high connection of understanding the disease pathway and its specific etiology. Therefore, future multi-faceted prospect to unravel the critical question of direct gut microbiota role in GD progression still needs to be elucidated.

Conclusion

Concisely the current study denotes the alteration in intestinal microbial taxa composition in GD patients as compared to controls, with certain gut microbes modulation. The diversity of bacterial community estimation stipulates that the gut flora of GD patients was less diverse in term of richness than in healthy control. These findings provide the basis to elaborate the disease pathway of multifaceted prospective study to illustrate the mechanism of gut microbial dysbiosis in Graves' disease.

Supplementary Material

Supplementary tables.

<http://www.ijbs.com/v14p1558s1.pdf>

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Competing Interests

The authors have declared that no competing interest exists.

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