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Molecular Estimation of Alteration in Intestinal Microbial Composition in Hashimoto's Thyroiditis Patients

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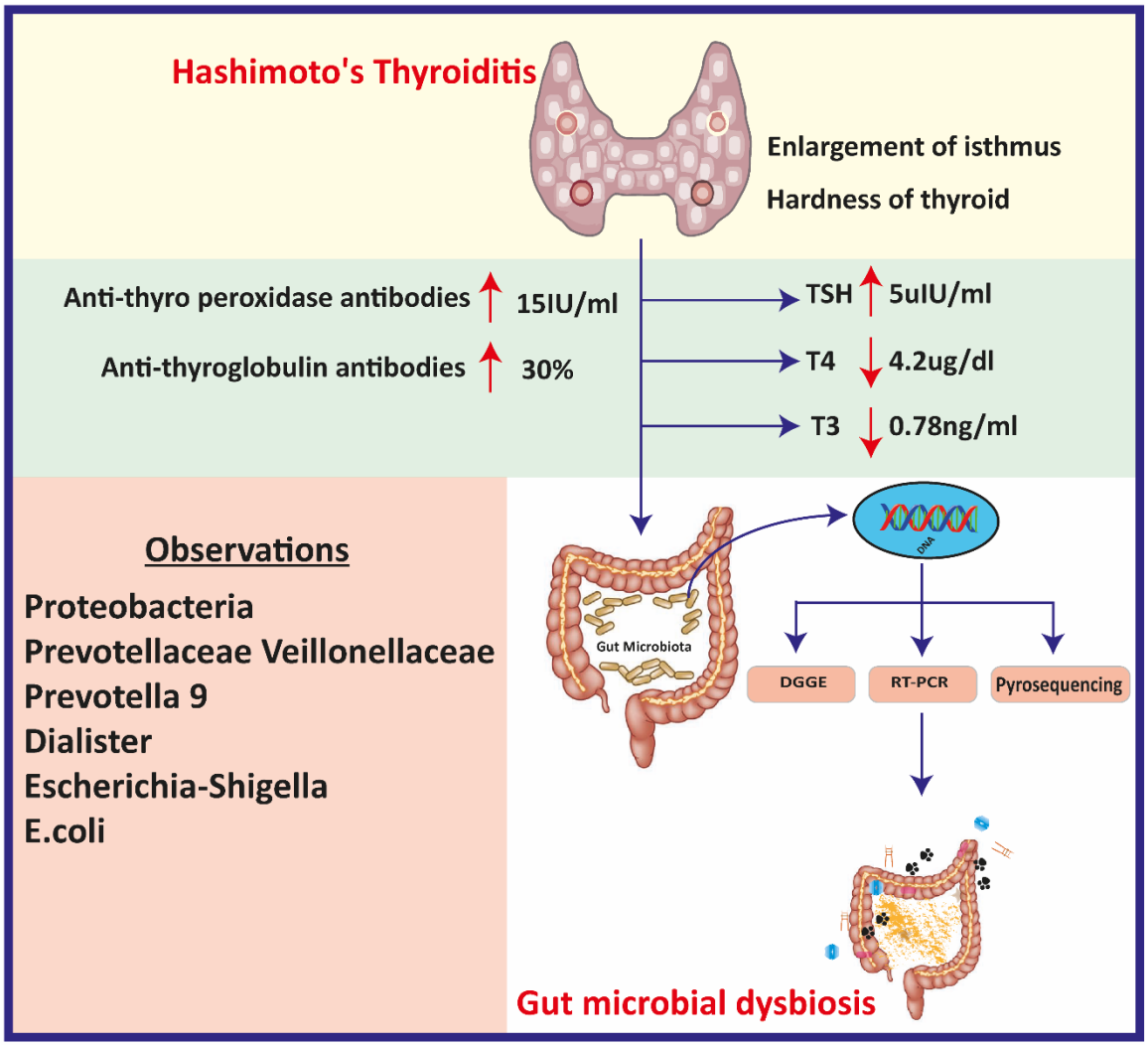
Abstract

The gut microbiotahasa crucial effect on human health and physiology. Hypothyroid Hashimoto's thyroiditis(HT) is an autoimmune disorder manifested with environmental and genetic factors. However, it is hypothesized that intestinal microbes might play avital role in the pathogenesis of HT. The aim of current study was to investigate and characterize the gut microbial composition of HT patients both quantitatively and qualitatively. The fecal samples from 29 HT patients and 12 healthy individuals were collected. The PCR-DGGE targeted V3 siteof 16S rRNA gene and real time PCR for *Bifidobacterium Lactobacillus, Bacteroides vulgatus and Clostridium leptum*wereperformed. Pyrosequencing of 16S rRNA gene with V4 location was performedon 20 randomly selected samples. The comparative analysis of diversity and richness indices revealed diversification of gut microbiota in HT as compared to control. The statistical data elucidate the alterations in phyla of HT patients which was also affirmed at the family level. We observed the declined abundance of*Prevotella_9* and *Dialister,* while elevated genera of the diseased group included *Escherichia-Shigella* and *Parasutterella*. The alteration in gut microbial configuration was also monitored at the species level,which showed an increased abundance of *E.coli*in HT. Therefore, the current study is in agreement with the hypothesis that HT patients have intestinal microbial dysbiosis. The taxa statistics at species-level along with each gut microbial community were modified in HT. Thus, the current study may offer the new insights into the treatment of HT patients, disease pathway, and mechanism.

Keywords: Hashimoto’s thyroiditis, Gut microbiota, Hyporthyroidism, DGGE, Pyrosequencing

At a glance



1. Introduction

The human gut microbiotais a major factor for host health status, and its contribution is crucial for normal body mechanism, thus considered as vital aspect for influencing the health grading of an individual [1]. The complexion of the human gut microbiota is quite diverse with approximately 100 trillion microbes in the body serve as a metabolic, nutrition, absorption and immune function against pathogens [2]. The abnormality in the body homeostasis can, in turn, affect the composition pattern of gut microbiota,therefore resulting in diseases implications[3].

HT is specifically organ linked autoimmune disease characterized by thyroid gland chronic inflammation. The disease was first reported in 1912 by Hakaru Hashimoto and was referred as autoimmune thyroid deficient disease (AITD).The exact pathogenicity of the disease still needs to be unraveled under the intense phase of most probable mechanisms[4]. The disease is now believed to be the autoimmune [5] endocrine disorder [6] considered as the contributing factor of hypothyroidism [7].

This autoimmune disorder manifested with no unusual clinical symptoms but with the gradual deterioration of thyroid gland, characterized by goiter, hypothyroidism, weight gain, constipation, and depression [8].

The epidemiological data depicted the prevalence of disease frequency eight times higher in females as compared to males [9]. The most common cause of hypothyroidism is iodine deficiency[10-12].The body's innate immune mechanism permits the binding of specific receptors thus identifying the molecules related to gut bacteria. The specificity of bound receptors activate the immune response of the host and release the defensive cytokines, white blood cells and peptides [13].

The recent molecular studies performed on 250 Chinese HT patients identified that single nucleotide polymorphism (SNP) in STAT3 gene has an association with HT [14]. The bacteriocins production by intestinal bacteria competing for nutrients and clinging of gut lining thus averting any colonization by pathogens [15].

The modulation in gut floral configuration has been linkedto numerous disease disorders, including colitis, Crohn's disease, viral diarrhea, metabolic diseases like obesity, and diabetes type II [16]. Mori K *et al.* described the possible relationship between thyroid autoimmunity and gut with weak evidence having very few studies consolidating such link.

Furthermore, the review emphasizes to validate the hypothesis of gut microbial dysbiosis in HT with further research[17].

The current study aim was to estimate the alteration, similarity, and diversity of gut microbiota quantitatively and qualitatively in HT patients in comparison to healthy controls. By using PCR-DGGE and sensitive metagenomic pyrosequencing, we have monitored the gut microbial similarity and diversity in patients suffering from HT disease. The investigations demonstrated the variation in bacterial taxa richness in contrast to controls, with some distinct gut microbes depicting significantly higher or lower abundance against the control. The significance of these alterations in the gut microbiota of HT was notably high-pitched as never reported before regarding gut microbial characterization in HT. Current findings thus help to illustrate the overall composition of gut microbiota in HT patients.

2. Material and methods

2.1. Ethics statement

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

2.2.Sample collection

Fecal samples from 29 HT patients (20 females and 9 males, aged between 40 to 60 years) and 12 healthy volunteers (8 females 4 males, aged between 40 to 60 years) were collected in a sterile cup. The patients with HT were diagnosed according to the standard protocol of department of endocrinology and metabolic diseases, 1st affiliated hospital, school of

medicine Xi'an Jiaotong University. It includes goiter, especially the enlargement of the isthmus, medium hardness of the enlarged thyroid. The Thyroid stimulating hormone(TSH) was higher than 5μIU/ml, T4 was lower than 4.2 μg/dl, T3 was lower than 0.78 ng/ml, anti-thyroid peroxidase antibodies level was higher than 15 IU/ml, and anti-thyroglobulin antibodies was more than 30%[18]. Normal range of serum thyroid hormones and antibodies are TSH (0.25-5 μIU/ml), T4 (4.2-13.5μg/dl), T3 (0.78-2.20 ng/ml), Anti-TPOAb (<15 IU/ml) and Anti-TGAb(< 30%).A questionnaire for each participant was prepared based on the information about gender, age, body weight, health and dietary habits. All the samples were delivered on ice, usually within 4 hours of defecation. Upon arrival in the laboratory, the fecal samples were stored at -80ºC until DNA extraction. Neither of the patients and healthy individuals had any history of gastrointestinal diseases nor taken antibiotics, probiotics, and prebiotics, 60 days before sampling.

2.3. DNA extraction from fecal sample

QIAGEN (Hilden, Germany) Stool kit was used for DNA extraction after thawing the fecal samples, with the first step of bead-beating at 5000 rpm for 30 s. Nano Photometer (IMPLEN, Germany) was used to estimate the DNA concentration [19].

2.4. PCR Amplification for DGGE

The fecal bacterial DNA was used for PCR–DGGE. Universal linkage primers **table 1**was deployed to augment 16S rRNA gene focusing V3 region. 50 μl PCR reaction mixture was amplified through PCR touchdown programming by using thermocycler (ABI2720 USA): final PCR products were electrophoresed on1.5% agarose gel and stained with ethidium bromide for visualization under UV illumination [20].

2.5. Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis was performed by using the DCodeTM Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). Briefly, amplified PCR product from total bacteria was loaded in 8% (w/v)polyacrylamide (acrylamide-bis, 37.5:1) gels in the1×TAE buffer, containing 30~65 % linear denaturant gradient. The gel was allowed to run for 14hours at 90V at a constant temperature of 60ºC[21]

2.6.Statistical analysis of DGGE band pattern

Bacterial diversity was estimated by the number of bands and band intensity of DGGE profiles by applying Syngene software (Bio-Rad, USA). The diversity of taxa was evaluated by Shannon–Weaver index of diversity*(H¹)*[22, 23]. Similarity matrix and cluster analysis of DGGE profiles were computed by using the UPGMA method based on the Dice similarity coefficient (band-based). Microsoft Excel 2010 and GraphPad 7 prism were applied, whereas ($P<0.05$) was considered as statistically significant. Similarities among the samples were shown through graphical dendrogram (Fig.1.B and Fig.1.D). Clustering algorithm and (UPGMA) arithmetic averages weredeployed to estimate unweighted pair group dendrograms [24].

Shannon Weaver diversity index *(H¹)* was estimated with the help of the following equation.

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

Excision of bands and sequencing

Physically a sterilized scalpel was used to excise the dominant band of interest from the gel with care. The polyacrylamidegel piece was placed in a 2 ml tube containing50 µlof water and incubated at 37ºC for 30 min. After centrifugation,8µl of this was used as a template for PCR re-amplification (targeting V3 region) with the same primers (without

149 GC-clamps) as previously used for DGGE analysis[25]. ABI 3500xL was used to
150 sequencing the reamplified PCR products. Sequences were studied and analyzed by using
151 BLAST and Seqmatch software for identification of species or genus.

152 **Real time PCR execution**

153 Real time PCR was performed in Bio-Rad CFX96 (USA) system. Total 20 µl PCR reaction
154 combination possessed 1 µl of two linkage primer (5 uM), 10 µl 2× SYBR Green
155 (TOYOBO, Japan), 2 µl sample DNA and 6 µl sterilized H_2O[26]. Real time PCR Primers
156 are shown in (Table 1). *Clostridium leptum*(YIT.6169), *Bacteroides vulgatus* NWS
157 *Lactobacillus*, (from our lab), (CICC.22938) and *Bifidobacteriaum*(CICC.6186) were
158 taken as standard strains. Real time PCR was performed in thrice and mean was calculated.
159 The outcome data were considered as the estimate of average logarithms in afecal sample
160 of PCR genomic amplicons, replica counts in 1 g of fecal mass.

161 **2.7.Pyrosequencing and data analysis**

162 Twenty fecal samples were randomly selected for metagenomic pyrosequencing analysis
163 (10 samples from HT and 10 samples from healthy control). According to a previously
164 described method, 16S rRNA gene along with V4 location was augmented with linkage
165 primer: 515F (GTGCCAGCMGCCGCGGTAA) 806R
166 (GGACTACHVGGGTWTCTAAT) primers to develop the amplicon libraries [27]. The
167 sequencing along with paired-end was performed on the platform with an Illumina Miseq
168 based on a standard protocol from the manufacturer. Raw data were screened and
169 assembled by QIIME [28] and FLASH [29] software packages. The UCLUST method
170 [28]was applied in clustering the bacterial sequences in OTUs (Operational Taxonomic
171 Units) at an identity threshold of 97%. Meanwhile, RDP Classifier [30] was applied to allot

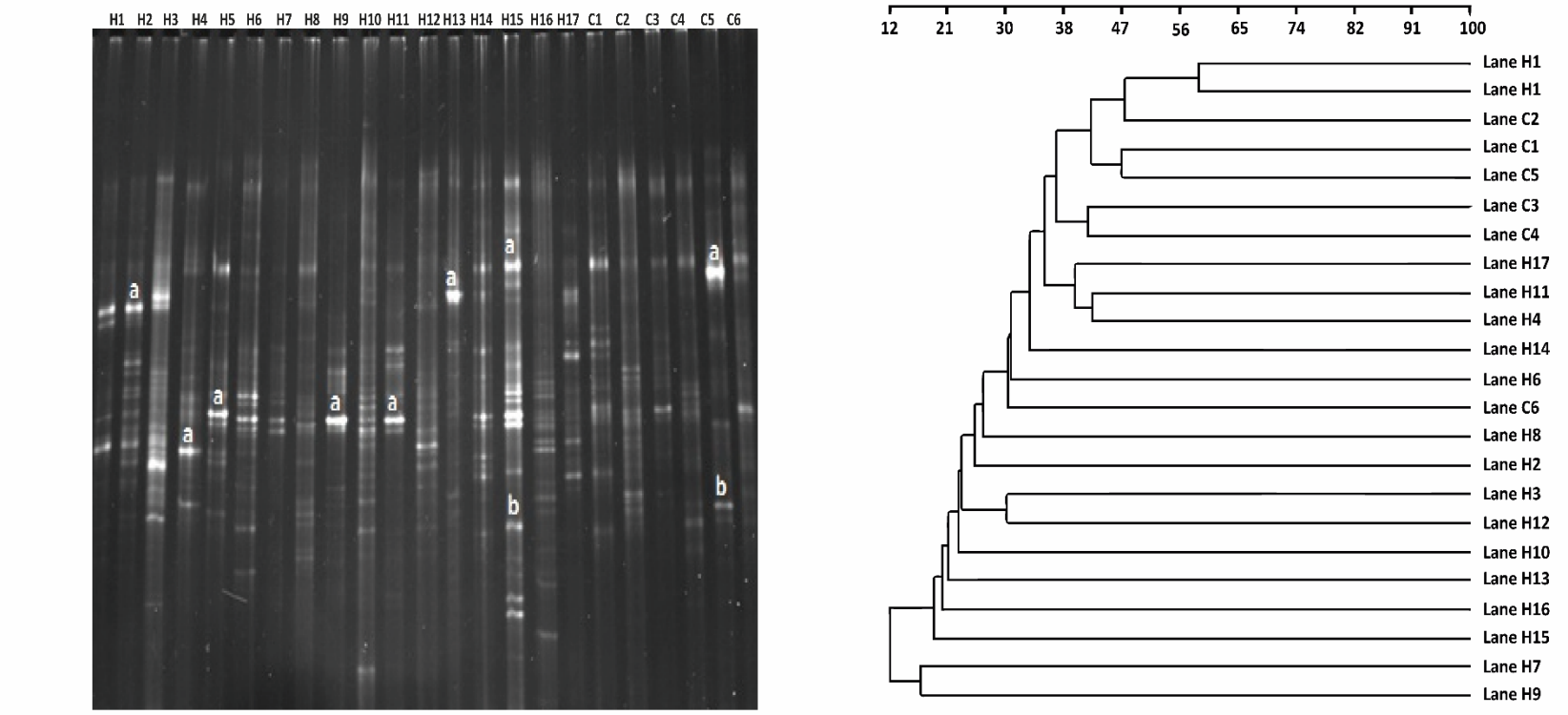
each OTU to a taxonomic level. Diversity analysis, such as Shannon and Simpson diversity index, Chao1, ACE and Good's coverage, was carried out with QIIME. In addition, the OTU table produced by the QIIME pipeline was imported into MEGAN 4 and mapped on the NCBI taxonomy database [31]. The significant statistical differences of gut microbial community texture along with Simpson and Shannon index between DNA sample batches were estimated by computing (unpaired nonparametric *t* test) Microsoft Excel 2010 and statistic software GraphPad Prism 7.

3. Results

3.1. Statistical DGGE characterization of bacterial population in HT

The Denaturing Gradient Gel Electrophoresis (DGGE) was deployed with amplified PCR product targeting 16S rRNA gene along with specific primers at the site of V3 region in both HT patients and control. The findings in **figure 1 panel A (H1–H17),** indicate samples from HT and (C1-C6) healthy control, while **figure 1 panel C (H18–H29)**, with samples from HT and (C7- C12) healthy control. The band's intensity, location, and number were diverse among samples indicating diverse intestinal microbial fingerprints. Syngene software detected the sums of 278 bands in 29 tracks of HT with an average band of (9.2 ± 3.75). A total 96 bands were detected in 12 tracks of the control group with an average of (8.00 ± 2.04). Stool microbial diversity among patients and normal control group were analyzed by applying nonparametric unpaired *t* test to evaluate the (H^1) Shannon Weaver diversity index. The (H^1) diversity outcomes depicted (2.72 ± 0.621 vs. 2.64 ± 0.45) insignificant (P = 0.299) gut microbial diversity difference between HT and healthy control groups. Conversely while comparing the Shannon Weaver index (H^1), it was found to be higher in HT group in contrast to control group that denoted the bacterial overgrowth in

the diseased group. The DGGE profiles similarity levels of all samples were determined by (UPGMA) dendrogram and Dice similarity coefficient **(figure 1 panel B, D)**. The value of band based assessment along with Dice similarity coefficient in HT and healthy control, through mean similarity index, were (0.300 ± 0.280) and (0.290 ± 0.121), respectively shown in **(table.2)**. Comparative statistical samples estimated values in HT and control groups were evaluated by using Dice similarity coefficient as well as mean similarity index linking between the two groups. The results indicated the lower level of similarity index in inter-group in contrast to intra-group that demonstrated the gut microbiota of HT patients were differentfrom healthy control group.


A
H1 H2 H3 H4 H5 H6 H7 H8 H9 H10 H11 H12 H13 H14 H15 H16 H17 C1 C2 C3 C4 C5 C6
B
12 21 30 38 47 56 65 74 82 91 100
Lane H1
Lane H1
Lane C2
Lane C1
Lane C5
Lane C3
Lane C4
Lane H17
Lane H11
Lane H4
Lane H14
Lane H6
Lane C6
Lane H8
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Lane H16
Lane H15
Lane H7
Lane H9
C
H18 H19 H20 H21 H22 H23 H24 H25 H26 H27 H28 H29 C7 C8 C9 C10 C11 C12
D
9 18 27 36 45 54 63 73 82 91 100
Lane C8
Lane C9
Lane C11
Lane C12
Lane C7
Lane H29
Lane H18
Lane H25
Lane H24
Lane H22
Lane C10
Lane H19
Lane H20
Lane H26
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Lane H23
Lane H27
Lane H28

214

Figure 1., (A) DGGE profile constructed betweenHT (H1-H17) and control groups (C1-C6). (B) Cluster analysis between HT(H1-H17) and control (C1-C6) groups by applying UPGMA. (C) DGGE profile erected between HT (H18-H29) and control groups (C7-C12).(D) Cluster analysis between HT (H18-H29) and control (C7-C12) groups by applying UPGMA."a" and "b"in figure (A) and (C) related to dominant bands from different patients. H or C represent Hashimoto and control group, respectively.

3.2. Sequence results analysis in DGGE

A total of 18 bands were excised from two DGGE gels. From DGGE gel **figure 1panel A**, 10 bands were cut for gut bacterial quantity estimation. To endorse the resolution competence of DGGE genomic bands in the same positions but indifferent lanes (H9a and H11a) were excised and sequenced. Bands H9a and H11a were identified as *Shigella dysenteriae*with98% similarity. Similarly, from **(figure 1 panel C)**, 8 bands were cut. Also to check the resolution ability of DGGE gel, bands H25a, H26a were sequenced and identified as *Shigella flexneri*with 99%similarity. Taxonomic identity of other genomic bands are shown in **table.3**. The results were demonstrating the phylum Proteobacteria Firmicutes and Bacteroidetes were prevalent in all samples. The findings of two DGGE gel profile were also illustrated, the prevalence of opportunistic gut bacteria (*Bacteroides uniformis, Bacteroides pyogenes, Bacteroides vulgates, Shigella dysenteriae, Bacteroides intestinalis, Escherichia coli, Sporomusa ovate, Bacillus sp., Shigella flexneri)*in HT patients.

3.3. Real time PCR

By applying real time PCR, *Bifidobacterium, Clostridium leptum,Bacteroides vulgatus* and *Lactobacillus* were quantified, the resultant copy number of *Bifidobacterium* and *Lactobacillus* were significantly ($P < 0.05$) reduced in the diseased group as compared to healthy subjects. On the other hand, the replica count of *Bacteroides vulgatus*elevated non-significantly and *Clostridium leptum*was lowered non-significantly in the patients while comparing to control group, respectively. All these results were summarized in **table.4**.

3.4. Pyrosequencing analysis of gene sequence

The PCR sequence amplicons comparative statistics were estimated with 1,843,183 at theV4 site of 16S rRNA gene from 10 HT and 10 from a normal control. Among these sum of pyrosequencing reads 1,541,154 (control 767,213 and disease 773,941, with an average per sample 77,058) were passed for quality control and were processed for further analysis. Taxon tag was (Ave. 72810.35) in all samples of both HT and control group and the total unique tag count detected in diseased and control group was12241 and10457, respectively (with Ave. 1134.9 in all samples).The total number of OUT were assigned 5509 (control 2644 and disease 2865, with an average per sample 275.45) in this study. The sum of the unique tag from the two groups was 22698 that exhibited the whole phylotypes in the current study, after deletion of linkage primers;the length of the average sequence was 418 bp.

3.5.Gut microbial diversification and conformational analysis

The richness and diversity of bacterial community were calculated at the 97% similarity level. Alpha diversities, as estimated by nonparametricalgorithm ACE, Chao1 and observed species were significantly elevated in HT ($P = 0.042$,$P = 0.039$) and$P=$

0.045respectively)as comparedto the normal individuals. However, Good's coverage was significantly higher in control group (P = 0.012). Conversely, there were no statistically significant differences in Simpson and Shannon diversity index between the two groups. The degree of diversity estimation in all thesame groups of bacterial community is shown in **table 5**. Furthermore, the alpha gut bacterial diversity analysis demonstrates the raised level in HT patients as compared to control group. The diversity elevation indicates a clear gut microbial overgrowth in patients group in contrast to healthy control. The bacterial DNA samples within each group or individual samples were divided into two clusters, based on weighted UniFrac metrics depicted in **figure 2** corresponding to samples of HT and normal control group analogous to the pattern of PCR-DGGE.

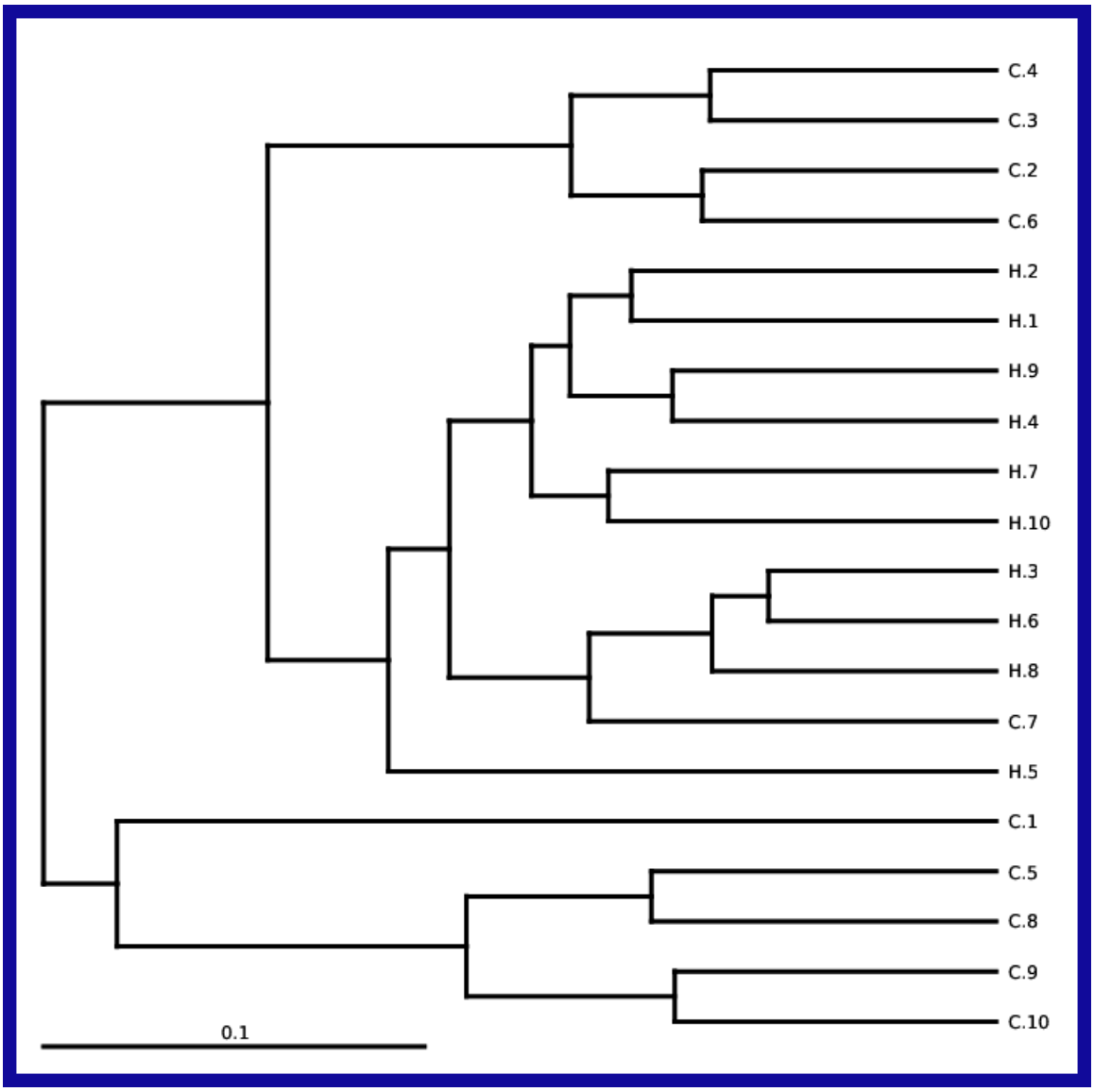

Figure 2. Diversity among HT samples of pyrosequencing. UPGMA based on weighted UniFrac distances.H or C represent Hashimoto and control group, respectively.

a) Gut microbiota population at phyla level

The gut microbial taxa composed of more than (1%-0.5%) were focused, and composition was assessed on the taxonomic basis at phylum, family, genus and species level.

At Phylum level, total 13 phyla were sequenced;depicted in**table S1**, among the 10 top most phyla, the more phyla abundance of Proteobacteria and Cyanobacteria and less Firmicutes and Bacteroidetes in the study group as compared to the normal healthy controls, shown in **figure 3**. The data statistics in **table6** demonstrated that dissimilarity was quantitatively different in top 10 phyla between study and control groups.

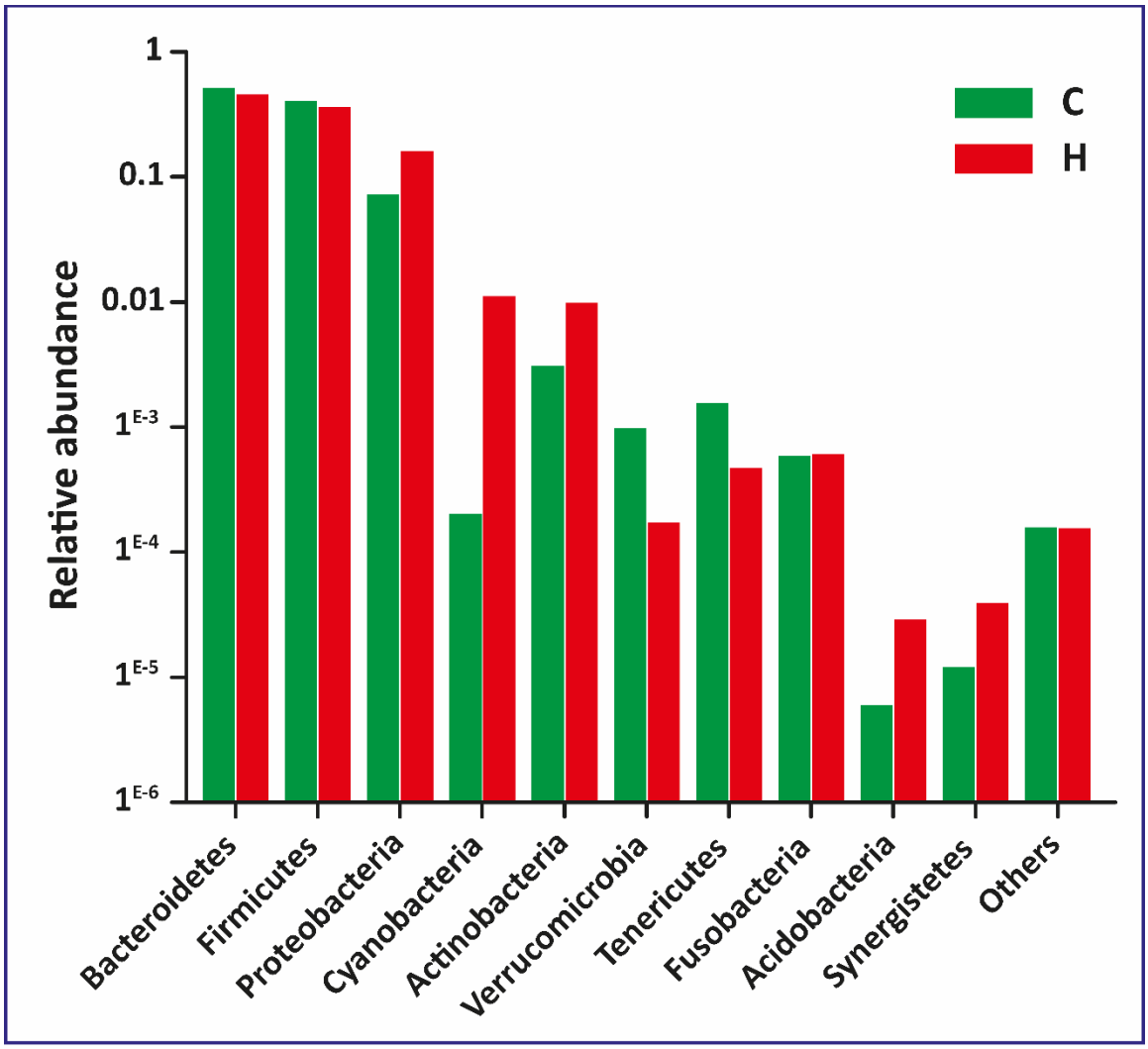

Figure 3. Gut microbial composition at phyla levels from Pyrosequencing results. Relative abundance of most prevalent phyla in HT and healthy control.H or C represent Hashimoto and control group, respectively

b) Gut microbial organization at family level

At the family level, 83 different families were sequenced, among 10 top most families, the prevalence of Bacteroidaceae, Enterobacteriaceae and Alcaligenaceae were higher in the diseased group as compared to control, shown in **figure4**. In these families, the relative abundance of Prevotellacea, Ruminococcaceae, and Veillonellaceae was lowered in patients group in contrast to healthy control. The family level data statistics with the percentage in HT illustrates the quantitative difference displayed in **table 6**.

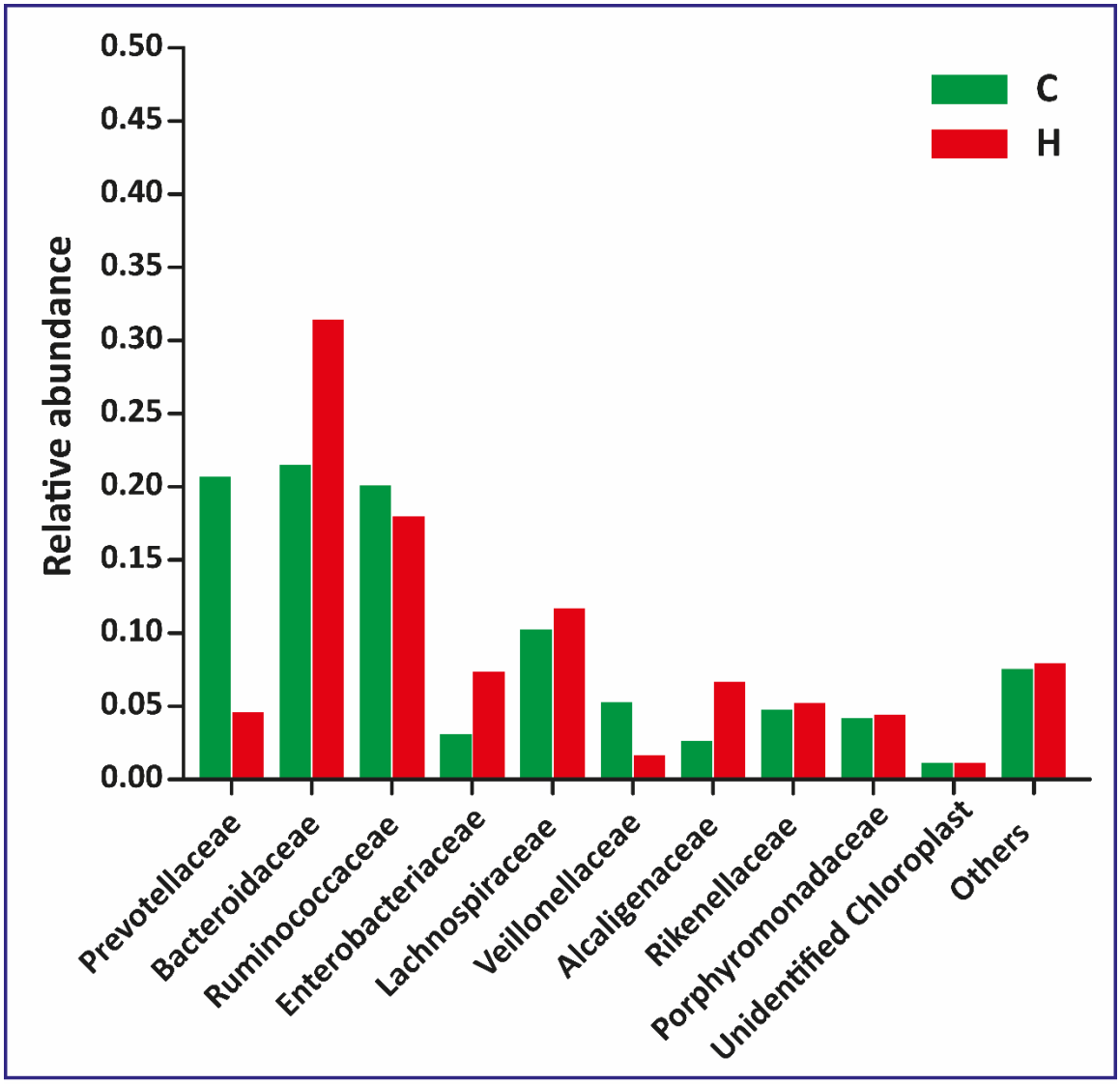

Figure 4. Pyrosequencing results of gut microbial compositions at family levels. Relative abundance of most dominant families in HT and healthy controls.H or C represent Hashimoto and control group, respectively

c) Gut microbiota distribution at Genus level

The genus level sequence represented with 194 different genera. In 10 top most genera, there was raised anabundance of *Bacteroides, Escherichia-Shigella* and *Parasutterella* genera in the diseased group in contrast to control group shown in **figure 5**. However, decreased genera in disease group were *Prevotella_9* and *Dialister*. The Statistics dissimilarity at genera level in HT group findings were compiled in **table 6**.

HT has an impact on specific groups of gut microbiota, in particular, the Phylum Proteobacteria, family Prevotellaceae, Veillonellaceae, genera*Prevotella_9, Dialister,* and *Escherichia-Shigella* and also largely influences the gut flora, which diverges as of the general normal healthy status based on the intestinal microbial composition of the individual.

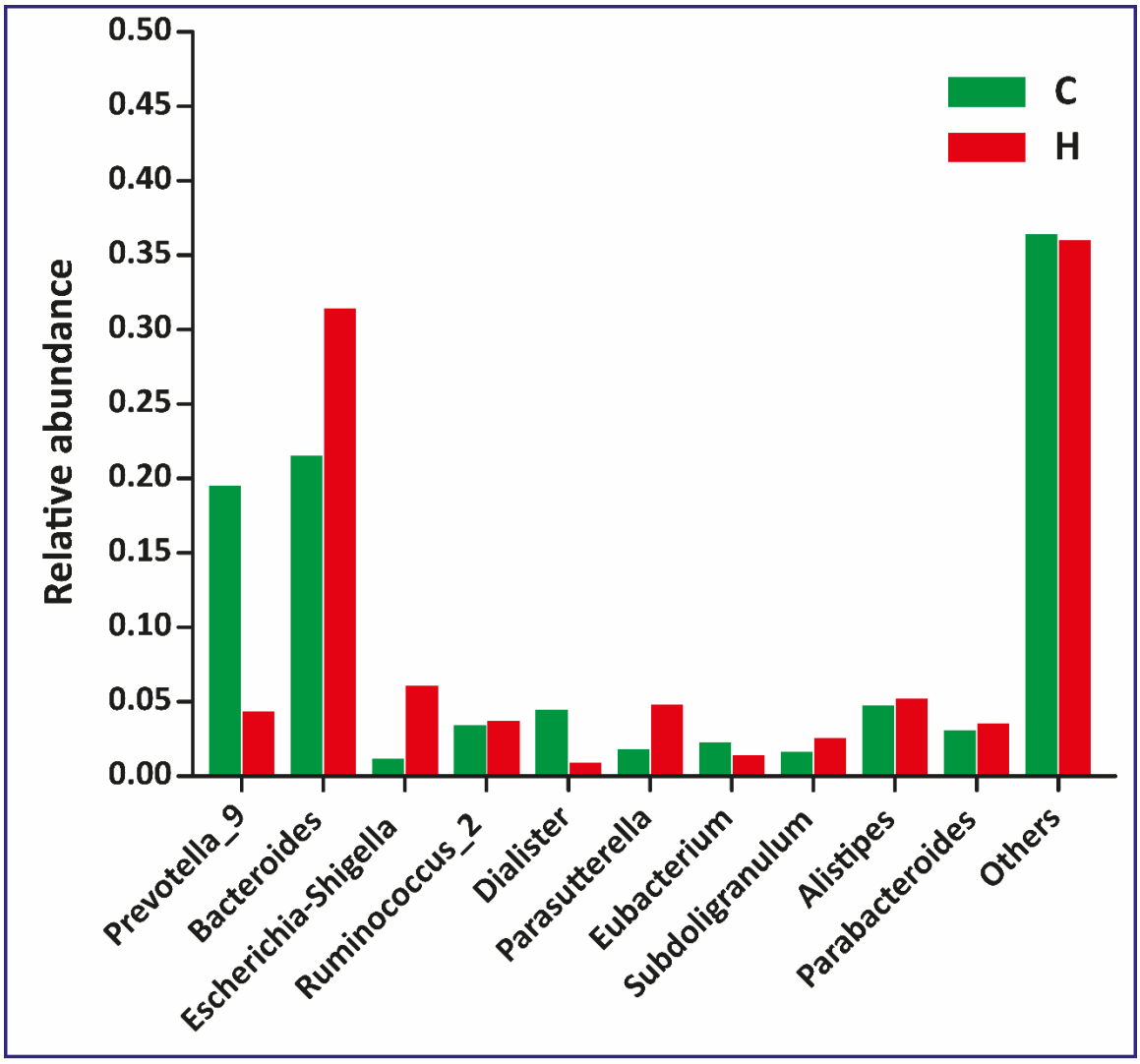

Figure 5. Gut microbial compositions at genera levels from Pyrosequencing results. Relative abundance of most prevalent genera in HT and healthy control.H or C represent Hashimoto and control group, respectively

d) Gut microbiota distribution at species level

The patterns of gut microbial community monitored at species level shown in **table 7**. These findings demonstrate the considerable dissimilarity at species level between HT and control group.However, *E.coli* has raised the level in HT patients in comparison to their healthy controls.

The concluding data from these findings by applying the metagenomic analysis of DGGE and pyrosequencing validate the same prevalent bacterial taxa. Nevertheless, the much reliable and sensitive pyrosequencing procedure affirm the authenticity of enhanced diversified bacterial population than DGGE. Quantitatively, trend wise metagenomic pyrosequencing and Real time PCR results ratified each other in the whole bacterial community. Therefore, in conclusion, the whole result findings were thus aligning the gut microbiota data produced by the three molecular methods.

4. Discussion

Human gut microbiota plays a critical role inbody protection through metabolic, trophic and protective function [32]. The gut microbial composition can be altered in disease conditions like Crohn's disease, malnutrition, inflammatory bowel disease, colitis, obesity and type II diabetes [16]. The experimental findings have elaborated that there is a distinct difference between the gut microbial composition of HT patients and healthy controls. The results were validated by revealing the dominant bands sequencing of DGGE profile, pyrosequencing analysis as well as real time PCR. The statistical analysis of α –diversity, nonparametric Chao1, algorithm ACE, and observed species was significantly elevated in the diseased group as compared to healthy control, while good's coverage was significantly higher in control group. Conversely, no significant distinction was found in diversity index like Shannon and Simpson, which was aligned with recently reported work[33]. Moreover, the bacterial community diversity estimation by DGGE profiles and pyrosequencing analysis was higher in HT patients. This elevation denotes the overgrowth of the gut flora in patients than in healthy control, although there was raised interpersonal difference that

corresponds to previous microbial finding in the vagina, gastrointestinal tract and skin [34, 35].

The statistical interpretation of similarity index of gut microbiota in HT patients in DGGE profiles pattern in intra-groups was found to be higher; this rise clearly demarcated the intestinal bacterial overgrowth in patients group. The estimation of similarity index comparison i.e. less in inter- group as compared to intra-group that by previous research findings[36], demonstrating the dissimilarity of gut microbial composition in HT patients as compared to control group. Therefore, all these diversity above outcomes illustrate that there is a significant disparity of gut microbial texture between diseased and control group. The statistical data represent the important quantitative difference between diseased and control groups. At the phylum level, Actinobacteria showeda raised level in the diseased group as compared to control which is consistent with previous work of physiological stress and gut microbiota [37]. The relative abundance of families Prevotellaceae and Veillonellaceae was lowered in patients group in contrast to healthy control which is aligned with previous work[33, 38].Veillonellaceae has beneficial commensal role i.e.very closely interrelated to*Clostridium* and isinvolved inthe induction of immune T regulatory cells [39].While Enterobacteriaceae and Alcaligenaceae were higher in the diseased group in comparison to healthy control.A Higher level of Enterobacteriaceae is aligned with previous work of type 2 diabetic patients and gut flora [40].However, decreased genera in the diseased group were *Prevotella_9* and Dialister. The diminished *Prevotella* presence has been shown in a disease like autism and types1 diabetes with intestinal microbiota [41, 42], while augmented genera in the diseased group were *Escherichia-Shigella* and *Parasutterella*. The raised level of *Escherichia-Shigella*is reported in preceding work of

autism spectrum disorders related to intestinal flora [43], while a higher level of *Parasutterella* aligned with preceding findings[44].

The current meta-analysis on intestinal microbiota linked with obesity and IBD revealed that percentage between Bacteroidetes to Firmicutes: may not be a steady characteristic that is distinctive between obese to lean gut flora[45]. Also, the current study findings demonstrate the raised level of Actinobacteria. It has been documented that Actinobacteria has the capability to settle in gingival crevices and is responsible for dental plaque accumulation, also produces the acid that results in infections or cavities [46].Our findings denoted the decreased values of genus *Prevotella*;nevertheless, the literature evidence describes the dominance of *Prevotella* in agut microbial composition exhibiting the positive impact on host metabolism[47]. *Prevotella* prevalence is considered as abeneficial bacteria in connection with plant based diet, and thus its intestinal flora has linked with many diseases and inflammatory conditions [48, 49]. The results showed a raised level of *Escherichia-Shigella* in HT patients as compared to healthy subjects. It has been documented that *Escherichia-Shigella* is Shiga-toxin producing bacteria that can cause septicemia, hemorrhagic colitis, thrombocytopenia, severe gastrointestinal tract inflammations in particular ileo-colonic area, spiteful problems of urinary duct channels and (HUS) hemolytic uremic syndrome.[50]. The current experimental study depicted a raised level of genus *Escherichia-Shigella*, in particular, species (*E.coli)* that might be the causative gut microbe in HT. Furthermore, ubiquitous *Escherichia coli* are responsible for causing the prevalent infections like (UTIs) urinary tract infections and food borne illnesses [51].

The evidence of current work elaborated the relative predominance of phylum, family, genus as well as species level taxa in stool samples, which also illustrated a clear disparity between HT patients and normal healthy subjects. Moreover, species level phylotypes with community comparison also divulged a clear demarcation of intestinal microbial texture between diseased and control groups [52]. These investigations further unravel the HT role in physiological intestinal changes that in turn contributes in alteration of the gut microbial composition. Moreover, these fluctuations in gut microbial configuration may lead to the complication of diseases[53].

The clinical signs of autoimmune HT manifested with hypothyroidism, goiter along with circulating antibodies to thyroid antigens. The results of the serum thyroid hormones level and circulating antibodies, anti-thyroglobulin anti-thyroid peroxidase in both HT and control, shown in **table S3 and table S2** respectively. The findings of HT patientsin **table S3** demonstrate the extremely raised level of aforesaid antibodies in HT as compared to healthy subjects. The increased level of antibodies in HT patients might change the gut microbial composition, in particular, the PhylumProteobacteria, family Prevotellaceae Veillonellaceae, genera*Prevotella_9*, *Dialister,Escherichia-Shigella* and *E.coli* species, and also broadly affect the gut flora. The current work on gut microbial differences between HT patients and healthy subjects was found pretty motivating as there was no straight and direct established relationship between HT and intestinal flora. Therefore, current findings further elaborate the diversification of gut microbial compositions between HT diseased and healthy groups. These fluctuations may change the health status of the host even though disease development is not associated with the intestinal tract [54].

The Real time PCR was performed to observe the gut microbial quantitative alterations [55], the data represent a significant decrease in *Bifidobacterium* and *Lactobacillus* in the diseased group thus aligning with preceding work [56]. The probiotics used in food most recurrently, generally belong to *Bifidobacterium* and *Lactobacillus* genera and encompass physiological benefits in the body[57]. *Bifidobacterium* and *Lactobacillus*are constantly reduced in colorectal cancer [58]. In addition to that, they also showed anti-obesity, anti-inflammatory and anti-atherogenic influential effects in many studies [59]. Various *lactobacillus* strains possess fair antimicrobial behavior to shield the body in opposition to uropathogens[60].

The current study findings generated from DGGE and pyrosequencing are reliable to analyze the gut microbiota. However, an experimental technique like PCR-DGGE is a semi-quantitative procedure; outcomes of bands density evaluation might not narrate the target abundance of gut flora correctly. [23]. However, pyrosequencing is a more advanced, sensitive and more reliable method to investigate and analyze the gut microbial ecology [54]. On the other hand, thePCR-DGGE technique could be used as a basic test to examine the notable shift of gut microbial community due to inexpensive and less time-consumingprocedure.

5. Conclusion

The current study demonstrates that gut microbial composition is different between HT patients and the normal control groups. More precisely, there is an important dissimilarity of gut microbial taxa richness as compared to control group. Furthermore, thelevel of certain intestinal microbes was either lowered or elevated profusion in HT patients in comparison to their healthy counterparts. The diversity of bacterial community estimation

analysis demonstrates an elevated level of gut flora in HT patients as compared with controls,which is an indication of bacterial overgrowth in HT patients. Therefore, the further multicenter approach is obligatory to comprehend the underlying mechanism and process of intestinal bacterial dysbiosis in Hashimoto's thyroiditis.

Disclosure

All authors disclose that they do not have any conflict of interest.

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