Gut-Thyroid axis: How gut microbial dysbiosis associated with euthyroid thyroid cancer

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Abstract

Thyroid cancer in humans has a fast-growing prevalence, with the most common lethal endocrine malignancy for unknown reasons. The current study was aimed to perform qualitative and quantitative investigation and characterization of the gut bacterial composition of euthyroid thyroid cancer patients. The fecal samples were collected from sixteen euthyroid thyroid cancer patients and ten from healthy subjects. The PCR-DGGE was conducted by targeting the V3 region of 16S rRNA gene, as well as real-time PCR for Bacteroides vulgatus, E.coli Bifidobacterium, Clostridium leptum and Lactobacillus were carried. High-throughput sequencing of V3+V4 region of 16S rRNA gene was performed on Hiseq 2500 platform on 20 (10 healthy & 10 diseased subjects) randomly selected fecal samples. The richness indices and comparative diversity analysis showed significant gut microbial modification in euthyroid thyroid cancer than control. At phylum level, there was significant enrichment of Firmicutes, Verrucomicrobia, while a significant decrease in Bacteroidetes was detected in the experimental group. At family statistics, significant high levels Ruminococcaceae and Verrucomicrobiaceae, while the significant lower abundance of Bacteroidaceae, Prevotellaceae, Porphyromonadaceae, and Alcaligenaceae was after observed. It also found that the significantly raised level of Escherichia-Shigella, Akkermansia [Eubacterium]_coprostanoligenes, Dorea, Subdoligranulum, and Ruminococcus_2 genera, while significantly lowered genera of the patient group were Prevotella_9, Bacteroides and Klebsiella. The species-level gut microbial composition showed a significantly raised level of Escherichia coli in euthyroid thyroid cancer. Thus, this study reveals that euthyroid thyroid cancer patients have significant gut microbial dysbiosis. Moreover, Statistics (P<0.05) of each gut microbial taxa were significantly changed in euthyroid thyroid
cancer patients. Therefore, the current study may propose new approaches to understanding thyroid cancer patients' disease pathways, mechanisms, and treatment.

**Key words:** Euthyroid thyroid cancer; DGGE; High-throughput sequencing; Characterization; Gut microbiota.

**Introduction**

Human gut microbiota is defined as a crucial factor in determining an individual's normal body functioning and health status. The body physiological mechanism depends on the configuration of gut microbiota remains consistent over the period of time. It may be modulated by different factors like ageing, food, and sickness [1]. The human gut microbiota constitutes approximately 100 trillion bacteria contributing to the immune function, metabolism, nutrition, absorption [2], and defence mechanism against pathogens [3]. The modulation of gut microbial composition act as a predisposing factor for different disease condition like colitis, inflammatory bowel disease, Crohn's disease, metabolic disorders including diabetes mellitus, asthma, smoking and obesity [4-6].

Cancer is characterized by abnormal growth of cells and is among the deadliest diseases [7-10], with an estimated 11.5 million deaths by 2030 worldwide [11]. The etiological findings of thyroid cancer are commonly manifested with thyroid nodules exhibiting significant clinical signs in diagnosis. Recently, the number of patients with thyroid problems has been observed a sharp rise in worldwide, particularly in women [12]. The recent epidemiological data suggested thyroid dysfunction is the 5th leading cancer diagnosed in women [13]. If the same developments are continued, thyroid malignancy may perhaps develop into fourth leading carcinoma of the United States in 2030 [15]. The occurrence of thyroid malignancy in several European states
shows a similar tendency to the United States and followed in China [14]. Thyroid cancer is commonly found in malignant endocrine carcinoma classified into 5 distinct types [15]. The most prevailing thyroid cancer is papillary thyroid carcinoma (PTC), which is approximately about (80–85%) thyroid malignant patients in well-advanced nations [16]. In PTC, the genetic modulations predispose the activation of [17] serum TSH (thyroid-stimulating hormone), thus generating malignant thyroid nodules. Many factors may cause many thyroid disorders, including estrogen, BMI ethnicity, abnormal iodine consumption and radioactivity [18-22]. It was described that the microbial composition of the gut could be altered by abnormal thyroid function [23]. Numerous experimental works have been established that both Graves' disease and Hashimoto's thyroiditis are linked with intestinal microbiota [24, 25].

Furthermore, the traditional role of the thyroid gland in the immune system plays a vital role in the development of thyroid cancer. The recent experimental investigations have elaborated on the intestinal microbiota contribution to host immunity and body homeostasis [26]. The current study was planned to determine the modification in diversity and similarity of intestinal microbiota qualitatively and quantitatively in euthyroid thyroid cancer patients with normal circulating antibodies compared to healthy subjects. Gut microbial diversity and similarity of euthyroid thyroid cancer patients were monitored by applying metagenomic High-throughput sequencing, PCR-DGGE, and qPCR. The findings revealed the significant difference in gut microbial composition with some distinctive gut bacteria portraying significantly elevated or lowered richness against healthy subjects. However, the association between gut microbiota and euthyroid thyroid cancer patients with normal level thyroid circulating antibodies has not been reported yet. The current study thus facilitates the explanation of the overall gut microbial composition of euthyroid thyroid cancer patients, as described in Scheme 1.
Scheme 1. The whole study methodology and results clearly indicate gut bacterial dysbiosis in euthyroid thyroid cancer patients.

Methodology

Ethics statement

The volunteer wrote down a semi-structured detailed questionnaire as per the rules of Xi'an Jiaotong University (School of Medicine), the Ethics Committee.
Sample collection

Fecal samples were collected in a sterilized cup from 16 thyroid cancer patients (8 males + 8 females) having euthyroid (aged linking 30 to 50 years) and 10 healthy volunteers (5 males + 5 female) (having the same age between 30 to 50 years). The patients with euthyroid thyroid cancer were identified by set protocols of metabolic disease and endocrinology department, attached hospital of Xian Jiatong university medical school [27]. The standard antibodies and serum thyroid levels are T3 (0.78-2.20 ng/ml), TSH (0.25-5 μIU/ml), T4 (4.2-13.5μg/dl), Anti-TGAb (< 30%) TMAb (< 20%), and Anti-TPOAb (<15 IU/ml). The information sheet for every volunteer was formatted according to their medical status, nutritional habits, lifestyle, and thyroid cancer disease. This inquiry Performa also includes individual physical weight, age, and gender. The fecal samples were transported on ice almost four hours after defection. The samples were stored at -80 °C freezer in the lab for further protocols like DNA extraction. In the last 60 days of sample collection, neither the patients nor the healthy volunteers had taken any antibiotics, probiotics, and prebiotics. Our study subjects were also free of any GIT disease.

DNA extraction from the fecal sample

DNA was extracted from all thawed fecal samples of diseased and control using the QIAGEN Stool kit (Germany) according to established protocol. The first step of bead-beating was conducted for ½ min with 5000 rpm. Concentration of DNA was evaluated using Nano Photometer TM (IMPLEN, Germany) [28].

PCR Amplification for DGGE

The fecal extracted bacterial DNA was used for analyzing PCR–DGGE. The linkage primers used in the V3 region were applied to enhance the 16S rRNA gene (Table 1). A total of 50 μl PCR mixture (thermocycler ABI2720 USA) was used to amplify the DNA sequence through
touchdown PCR programming: Initial start was done with denaturation of PCR mixture at 95 ºC for 5 min that further include 10 extra cycles and followed by a final extension. The gene bands assessed using 1.5% agarose gel through electrophoresis, the amplified PCR mixture, and envisaged under UV light after dipping in the Ethidium bromide solution [29].

Table 1. Primers used in Real-time PCR and PCR-DGGE

<table>
<thead>
<tr>
<th>Target bacteria</th>
<th>Primer Sequence (5¹–3¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR-DGGE Primer</td>
<td></td>
</tr>
<tr>
<td>341-F</td>
<td>CCTACGGGAGGCAGCAG</td>
</tr>
<tr>
<td>534-R</td>
<td>ATT ACCGCCGCTGCTGG</td>
</tr>
<tr>
<td>341FG</td>
<td>CGCACCAGCAGCGCCGCGACGGCGGGGCGGGCGGGGCGGG</td>
</tr>
<tr>
<td></td>
<td>GGCACGGGAGGCGCTACGAGCGAGCAG</td>
</tr>
<tr>
<td>Real-Time PCR Primer</td>
<td></td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td></td>
</tr>
<tr>
<td>(550 bp)</td>
<td></td>
</tr>
<tr>
<td>Bifid F</td>
<td>CTC CTGGAACCGGGTGG</td>
</tr>
<tr>
<td>Bifi-R</td>
<td>GGTGTTCCTCCGATATCTACA</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td></td>
</tr>
<tr>
<td>(250 bp)</td>
<td></td>
</tr>
<tr>
<td>Lact F</td>
<td>CTAAAAACTAAACAAAGTTTC</td>
</tr>
<tr>
<td>Lact R</td>
<td>CTAAAAACT AAACAAAGTTTC</td>
</tr>
<tr>
<td>Bacteroides vulgaris</td>
<td></td>
</tr>
<tr>
<td>(287bp)</td>
<td></td>
</tr>
<tr>
<td>BV- F</td>
<td>GCACTAGTGAGTCGCCATAGTC</td>
</tr>
<tr>
<td>BV-R</td>
<td>TCCATACCCGACTTTATCCT</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td></td>
</tr>
<tr>
<td>(287bp)</td>
<td></td>
</tr>
<tr>
<td>E.coli-F</td>
<td>CATTGACGTTACCAGAGAAGGCG</td>
</tr>
<tr>
<td>E.coli-R</td>
<td>CTCTACGAGACTCAAGCTCT</td>
</tr>
<tr>
<td>Clostridium leptum</td>
<td></td>
</tr>
<tr>
<td>(239bp)</td>
<td></td>
</tr>
<tr>
<td>C.lep-F</td>
<td>GCACAGCAGTGAGGT</td>
</tr>
<tr>
<td>C.lep-R</td>
<td>CTTCTCCGTTTTATCA</td>
</tr>
</tbody>
</table>

Denaturing gradient gel electrophoresis

The DGGE experimental protocol of universal mutational (Bio-Rad, USA) analysis was followed. In brief, Amplified PCR microbial DNA was loaded in 8% acrylamide DGGE gels in a container with 1xTAE buffer solution (linear denaturant grade 30–65 %) with the constant optimal temperature at 60 ºC. DGGE gel was run for 13 hours at 90V. The syngene Genetool (4.3.14) software was used to determine each sample's fecal microbial diversity and intensity.
strength by analyzing the total number of bands in DGGE. The Dice similarity coefficient was also assessed by using a similarity index. The algorithm clusters and arithmetic means (UPGMA) were applied to develop the unweighted euthyroid thyroid cancer pair group dendrogram [30].

**DGGE statistical analysis with band configuration**

Bands intensity strength of DGGE profiles along with bands number were evaluated by computed Syngene software. Bacterial diversity in DGGE profiles was estimated by \( H' \) Shannon Weaver diversity index [31, 32]. Similarity index and DGGE profile's cluster analysis were done through the UPGMA method (band-based Dice similarity coefficient) [33]. GraphPad 7 prism and Microsoft Excel (2010) were used to analyse the Shannon-Weaver and similarity index, where statistically (P<0.05) was considered as significant. Also, the Similarities among DNA samples were indicated by the dendrogram, shown in Figures 1B and 1D.

Calculation of \( H' \) Shannon Weaver diversity index was done by using the undermentioned formula.

\[
\text{Shannon-Weaver index } (H') = - \sum_{i=1}^{S} (Pi)(\ln Pi)
\]

**Excision of bands and sequencing**

Manually, prominent curiosity bands were cut with a sterile scalpel blade from the DGGE gel profile. The excised DNA gel bands (polyacrylamide) were kept in a 2 ml tube. 50 \( \mu l \) of distilled water was added to the tube and placed for 30 min. at 37 °C. After centrifugation, 8 \( \mu l \) of DNA water was added as a template and similar primers (without GC-clamps) were used for amplification of V3 region 16S rRNA gene [34]. The sequencing of the amplified PCR-DNA mixture was evaluated through ABI-3500xL. Obtained sequences were carefully analyzed by applying BLAST to identify the species or genus.
**Real-time PCR**

The Bio-RAD CFX96 (USA) protocol kit was followed for real-time PCR. A total of 20 μl reaction mixture was used for Real-time PCR. The sample includes 2 μl of genomic DNA, 1 μl each of forward and reverse primer along with 10 μl of Sybr green adding in 6 μl of water, thus making the 20 μl of reaction mixture for loading. The details of linkage primers for Real-time PCR are shown in Table 1 [35]. The bacterial species *Bifidobacterium* (CICC.6186), *E.coli*, NWS *Lactobacillus* (taken from our lab), *Clostridium leptum* (YIT.6169), *Bacteroides vulgatus*, (CICC.22938) were regarded as standard indicative bacterial strains. The average mean values were retrieved after running Real-time PCR three times. The resultant data was an estimation of the mean logarithm in the fecal sampling of genomic PCR amplicon, which is a replica count in 1g of the fecal mass.

**High-throughput sequencing and data analysis**

The Ilumina Hiseq protocol was followed for sequencing the paired-end. The data was retrieved and aligned by using QIIME and FLASH software. The QIIME(V1.7.0) software was used for diversity analysis like Simpson and Shannon diversity index and Good's coverage, ACE and chaol. The metagenomic high-throughput sequencing procedure was conducted based on the random reaction of 20 fecal samples. These include 10 samples collected from euthyroid thyroid cancer patients and the other 10 received from healthy subjects. The 16S rRNA gene alongside the V3+V4 region was adjoined with linker primers: 806R (GGACTACHVGGGTWTCTAAT) 515F (GTGCCAGCMGCCGCGGTAA) primers for the construction of amplicon taxonomic libraries [36]. The Ilumina Hiseq protocol was followed for sequencing the paired-end. The data was retrieved and aligned by using QIIME [37] and FLASH [38] software. The UCLUST procedure [37] was employed to aggregate the bacterial DNA sequences in operational
taxonomic units (OTUs) at the level of 97% identity threshold. The taxonomic position of each OTU was allocated by using the RDP classifier [39]. The QIIME software was used for diversity analysis like Simpson as well as Shannon diversity index along with chaol, Good's coverage and ACE. Moreover, the OUTs data tables retrieved through QIIME pipeline which were incorporated with MEGAN4 softwear and mapped with taxonomic database of NCBI [40]. The gut microbial population texture is witnessing significant fluctuations. UniFrac distances were calculated by applying QIIME. PCA (Principal component analysis) and NMDS (non-metric multi-dimensional scaling) estimation were done to observe the dissimilarities and similarities of variables which are represented in paired wise distances between study and control groups. It showed through stat packages, ggplot2 package and WGCNA packages, in software R (Version 2.15.3). The differences of alpha diversity were compiled by using a nonparametric unpaired t-test with the help of graph pad prism 7 (statistic software) and Microsoft Excel (2010).

Results

Statistics and DGGE profiles analysis in euthyroid thyroid cancer and healthy group

Analytical and experimental process of DGGE (denaturing gradient gel electrophoresis) was performed by using amplified PCR mixture employing universal primers of 16S rRNA gene (V3 region) in patients with euthyroid thyroid cancer and normal control groups as well. In figure 1A, D1–D8 shows samples of euthyroid thyroid cancer patients and C1–C5 healthy controls. Similarly, in Figure 1C, the D9–D16 shows the samples of euthyroid thyroid cancer patients and C6–C10 healthy controls. Since the location, bands' strength intensity, and numbers were diverse among all fecal DNA samples that depicted the multifarious intestinal microbial fingerprints. A total of 225 bands were identified by using the Syngene software [41], in 16 tracks of euthyroid thyroid cancer patients with an average band (14.1 ± 3.21). Moreover, a total of 86 DGGE bands were identified in 10 tracks of healthy subjects averaging (8.6 ± 2.55), which was significantly
different (P< 0.001) between euthyroid thyroid cancer and healthy control groups. These results indicate that the increased band numbers demonstrate the increased diversity as well as bacterial overgrowth in the group of euthyroid thyroid cancer patients. To analyze, the diversity of stool microbiota in euthyroid thyroid cancer patients and healthy group, Shannon weaver ($H'$) diversity index depicted (3.225 ± 0.422 vs 2.542 ± 0.432) as significant (P< 0.003) in intestinal bacterial diversity changes between euthyroid thyroid cancer and healthy subjects. ($H'$) Shannon Weaver diversity index values were elevated in euthyroid thyroid cancer patients compared to healthy subjects, depicting significant gut bacterial overgrowth in euthyroid thyroid cancer patients. The Similarity level of all the gut bacteria in DGGE gel profiles was assessed through the Dice similarity coefficient (UPGMA) dendrogram explained in Figures 1 B and 1D). The band intensity-based numeral values of the Dice similarity coefficient between euthyroid thyroid cancer and healthy groups, along with mean similarity index (0.319 ± 0.141) and (0.288 ± 0.130) respectively, are depicted in Table 2. When all the statistical numerals of each sample of euthyroid thyroid cancer and healthy control were calculated and analyzed through mean similarity index and Dice similarity coefficient, they were (0.269 ± 0.125). The findings illustrated that it was exhibited lesser in intergroup compared to intragroup, thus presenting dissimilarity of gut microbial texture in euthyroid thyroid cancer patients in contrast to the control subjects.
Table 2. Gut microbial similarity and diversity of euthyroid thyroid cancer patients and healthy group

<table>
<thead>
<tr>
<th>Groups</th>
<th>Diversity</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>The number of Bands</td>
<td>Shannon Index</td>
</tr>
<tr>
<td>Disease group</td>
<td>14.1 ± 3.21</td>
<td>3.225 ± 0.422</td>
</tr>
<tr>
<td>Control group</td>
<td>8.6 ± 2.55</td>
<td>2.542 ± 0.432</td>
</tr>
<tr>
<td>P. Value</td>
<td>0.001</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Significantly different results (unpaired t-test), with P<0.05

a. DGGE bands number produced by each sample.
b. Shannon diversity index \( (H') \) was calculated with the help of all DGGE bands (relative intensities) in each sample.
c. Comparing DGGE band profiles with Dice similarity coefficients within the individual of a given group.
d. Comparing DGGE band profiles with Dice similarity coefficients between members of euthyroid thyroid cancer and the healthy group.

Dominant bands sequencing results analysis

The sums of 22 gel bands were excised by two DGGE gel profiles. From Figure 1A, 13 gel bands were cut from the DGGE profile for quantitative analysis. The resolution capability of DGGE profile bands was confirmed in different tracks, but in a similar location, the gel bands D5a and C5a were sequenced after excision that was identified as \textit{Prevotella copri} with 96% similarity. Furthermore, as in Figure 1C, 9 bands were cut and assessed the resolution capability of DGGE gel composition, bands D16b and C6a were sequenced and detected as \textit{Bacteroides vulgatus} having 98% similarity. The taxonomic identity of other bands of the DGGE profile has depicted in Table 3. Sequencing outcomes were evaluated and analyzed by deploying BLAST software, and findings have confirmed the prevalence of phylum Firmicutes, Proteobacteria, and Bacteroidetes as prominent presence. Sequencing results of excision band from two DGGE gel profiles, also depicted in Table 3, opportunistic bacteria are prevalent (\textit{Escherichia coli, Proteus mirabilis, Pseudomonas cremoricolorata, Prevotella oulorum, Faecalibacterium prausnitzii,}}
Phascolarctobacterium sp, Alistipes putredinis, Shigella dysenteriae, Bacteroides pyogenes, Bacillus sp. Klebsiella sp, Enterobacter sacchari, Parabacteroides distasonis) in euthyroid thyroid cancer patients.

Figure 1. (A) Assembling the DGGE profile of euthyroid thyroid cancer patients (D1-D8) and control subjects (C1-C5). (B) UPGMA application for Cluster analysis between diseased (D1-D8) and controlled (C1-C5) groups. (C) DGGE depicts constructed between euthyroid thyroid cancer (D9-D16) and control groups (C6-C10). (D) assembly analysis of euthyroid thyroid cancer(D9-D16) and control (C6-C10) groups by applying UPGMA. “a” and “b” in figure (A) and (C) constitute the dominant bands of different patients. D or C represents the euthyroid thyroid cancer patients and control subjects, respectively.
Table 3. Sequencing of re-amplified PCR Amplicons excised bands from DGGE gel and identities were checked by BLAST database.

<table>
<thead>
<tr>
<th>Selected Excised bands</th>
<th>Bacteria with the highest % homology</th>
<th>Sequence Accession number</th>
<th>Bacterial phyla</th>
<th>Gene bank number</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3a</td>
<td>Escherichia coli (93).</td>
<td>IAI39.</td>
<td>Proteobacteria</td>
<td>NZ_JH114216.1</td>
</tr>
<tr>
<td>D3b</td>
<td>Proteus mirabilis (94).</td>
<td>HI4320.</td>
<td>Proteobacteria</td>
<td>NC_010554.1</td>
</tr>
<tr>
<td>D3c</td>
<td>Pseudomonas cremoricolorata (98).</td>
<td>ND07.</td>
<td>Proteobacteria</td>
<td>NZ_CP009455.1</td>
</tr>
<tr>
<td>D3d</td>
<td>Prevotella ouserum (93).</td>
<td>F0390.</td>
<td>Bacteroidetes</td>
<td>NZ_JH114216.1</td>
</tr>
<tr>
<td>D5a</td>
<td>Prevotella copri (96).</td>
<td>DSM 18205.</td>
<td>Bacteroidetes</td>
<td>NZ_GG703862.1</td>
</tr>
<tr>
<td>D5b</td>
<td>Faecalibacterium prausnitzii (96).</td>
<td>TDY5834930.</td>
<td>Firmicutes</td>
<td>NZ_CZBH01000014.1</td>
</tr>
<tr>
<td>D6a</td>
<td>Phascolarctobacterium sp (94).</td>
<td>YIT 12067.</td>
<td>Firmicutes</td>
<td>NZ_GL830850.1</td>
</tr>
<tr>
<td>D7a</td>
<td>Alistipes putredinis (99).</td>
<td>DSM 17216.</td>
<td>Bacteroidetes</td>
<td>NZ_DS499580.1</td>
</tr>
<tr>
<td>C2a</td>
<td>Bacteroides oleicplenus (92).</td>
<td>YIT 12058.</td>
<td>Bacteroidetes</td>
<td>NZ_JH992946.1</td>
</tr>
<tr>
<td>C3a</td>
<td>Bacteroides uniformis (90).</td>
<td>CL03T00C23.</td>
<td>Bacteroidetes</td>
<td>NZ_JH724260.1</td>
</tr>
<tr>
<td>C4a</td>
<td>Barnesiella intestinhominis (98).</td>
<td>YIT 11860.</td>
<td>Bacteroidetes</td>
<td>NZ_JH815205.1</td>
</tr>
<tr>
<td>C5a</td>
<td>Prevotella copri (96).</td>
<td>DSM 18205.</td>
<td>Bacteroidetes</td>
<td>NZ_GG703862.1</td>
</tr>
<tr>
<td>C5b</td>
<td>Bacteroides stercoris (90).</td>
<td>ATCC 43183.</td>
<td>Bacteroidetes</td>
<td>NZ_DS499675</td>
</tr>
<tr>
<td>D10a</td>
<td>Shigella dysenteriae (98).</td>
<td>Sd197.</td>
<td>Proteobacteria</td>
<td>NC_007606.1</td>
</tr>
<tr>
<td>D10b</td>
<td>Bacteroides pyogenes (88).</td>
<td>JCM 10003.</td>
<td>Bacteroidetes</td>
<td>NZ_BAIU01000058.1</td>
</tr>
<tr>
<td>D10c</td>
<td>Bacillus sp. (94).</td>
<td>FJAT-25496.</td>
<td>Firmicutes</td>
<td>NZ_LMBY01000086.1</td>
</tr>
<tr>
<td>D15a</td>
<td>Klebsiella sp. (94).</td>
<td>NODE14.</td>
<td>Proteobacteria</td>
<td>NZ_LGIT01000014.1</td>
</tr>
<tr>
<td>D15b</td>
<td>Enterobacter sacchari (94).</td>
<td>SP1.</td>
<td>Proteobacteria</td>
<td>NZ_CP007215.2</td>
</tr>
<tr>
<td>D16a</td>
<td>Parabacteroides distasonis (97).</td>
<td>ATCC 8503.</td>
<td>Bacteroidetes</td>
<td>NZ_JH815205.1</td>
</tr>
<tr>
<td>D16b</td>
<td>Bacteroides vulgatus (98).</td>
<td>ATCC 8482.</td>
<td>Bacteroidetes</td>
<td>NC_009614.1</td>
</tr>
<tr>
<td>C6a</td>
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<td>ATCC 8482.</td>
<td>Bacteroidetes</td>
<td>NC_009614.1</td>
</tr>
<tr>
<td>C10a</td>
<td>Bacteroides paurosaccharolyticus (91).</td>
<td>JCM 15092.</td>
<td>Bacteroidetes</td>
<td>NZ_BAJR01000054.1</td>
</tr>
</tbody>
</table>
Real-time PCR Amplification

The *Bacteroides vulgatus, Bifidobacterium, Lactobacillus Clostridium leptum*, were enumerated by Real-time PCR. The replica counts of *Bifidobacterium* \((5.75 \pm 0.87 \text{ vs. } 6.73 \pm 0.87)\) lessened significantly \((P < 0.005)\), also copy numbers of *Lactobacillus* \((6.19 \pm 0.98 \text{ vs. } 6.98 \pm 0.99)\) were lowered significantly \((P < 0.029)\) in the disease group. Conversely, *Bacteroides vulgatus* \((5.77 \pm 0.86 \text{ vs. } 6.59 \pm 0.82)\) were found significantly \((P < 0.011)\) reduced in euthyroid thyroid cancer patients. Copy numbers of *Escherichia coli* \((5.60 \pm 0.78 \text{ vs. } 4.89 \pm 0.74)\) were significantly \((P < 0.016)\) increased in patients. Moreover, the copy numbers of *Clostridium leptum* \((4.05 \pm 1.07 \text{ vs. } 3.75 \pm 1.11)\) \((P < 0.249)\) had a non-significant rise in the fecal samples of euthyroid thyroid cancer patients in contrast with healthy subjects. The results are shown in Table 4.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Healthy Subjects</th>
<th>Patients</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bifidobacterium</em> (10^9)</td>
<td>6.73±0.87</td>
<td>6.30±0.90</td>
<td>0.123</td>
</tr>
<tr>
<td><em>Bacteroides vulgatus</em> (10^9)</td>
<td>6.59±0.82</td>
<td>5.77±0.86</td>
<td>0.011</td>
</tr>
<tr>
<td><em>Lactobacillus</em> (10^5)</td>
<td>6.98±0.99</td>
<td>6.19±0.98</td>
<td>0.029</td>
</tr>
<tr>
<td><em>Clostridium leptum</em> (10^7)</td>
<td>3.75±1.11</td>
<td>4.05±1.07</td>
<td>0.249</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (10^6)</td>
<td>4.89±0.74</td>
<td>5.60±0.78</td>
<td>0.016</td>
</tr>
</tbody>
</table>

Results were presented as the average estimate of logarithms of fecal PCR target genetic amplicon copy numbers present in 1 g of feces, where \((P < 0.05)\).

High-throughput sequencing of gene analysis

Comparative sequencing amplicons of PCR were computed with 1,731,168 at the site of V3+V4 of the 16S ribosomal RNA genome, from 10 euthyroid thyroid cancer and also 10 from healthy controls. High-throughput sequencing reads 1, 438,586 (control 736,933 and disease 701,653) were passed, having an average per sample (72,169) for quality assurance and analysis. The
taxon tag was estimated (Ave. 68796.2) in both euthyroid thyroid cancer and healthy control groups. Total unique tag counted in the study and healthy groups were 10, 656 and 7,686, respectively (Ave. 917.1 in entire samples). OUT numbers were assigned which is 52,12 (healthy 24,80 and patients 2,732) average/sample (260.6) in current study. The high-throughput unique tag was 18,342 from study and healthy groups, demonstrating the entire phylotypes of current experimental work. The OTU clustering and annotation results of each sample are comprehensively calculated. The results are shown in Figure 2. The average length of the sequence was estimated 418 bp after removal of linkage primers.

![Bar chart](image)

**Figure 2.** Euthyroid thyroid cancer observation of Tag number and OTUs analysis with comparison of control, Tag number and OTUs were estimated at the level of (97 %) similarity

**Intestinal bacterial diversity analysis**

Bacterial community diversity and richness were estimated at a similarity level of 97%. Alpha diversity, as computed by Simpson and Shannon diversity, PD Tree, algorithm ACE, observed species, and Chao1, were found significantly higher in euthyroid thyroid cancer with a comparison of healthy volunteers. The level of bacterial diversity assessment in study and control is described in Table 5. Additionally, the analysis of alpha diversity exhibits an elevated
level in euthyroid thyroid cancer patients compared to controls. The elevated diversity depicted a strong intestinal bacterial overgrowth in the patients' group compared to healthy volunteers. Samples of intestinal bacterial DNA in each group were distributed in two distinct clusters, constructed on weighted UniFracs distance shown in Figure 3, which is also analogous to the arrangement of PCR-DGGE of euthyroid thyroid cancer and normal volunteers.
Figure 3. Diversification among euthyroid thyroid cancer samples of High-throughput sequencing. UPGMA is based on weighted UniFrac distances. D and C denotes the euthyroid thyroid cancer patients and controlled group, respectively.

To determine the microbial diversity between the healthy and study groups, the beta diversity was estimated. NMDS (Non-metric dimensional scaling) and OTU number based PCA Principal-
component analysis were performed, which clearly illustrate the alteration of the intestinal bacterial composition of two groups, shown in Figures 4A and 4B.

Figure 4

**Figures 4.** (A) Beta diversity between diseased and healthy subjects. PCA plots which are obtained from Highthroughput sequencing of fecal microbial DNA samples. (B) NMDS plot between study and control bacterial DNA samples. Each dot in the plot indicates an individual fecal bacterial DNA Samples of patient and control group.

**Table 5. Gut bacterial richness and diversity index, based on 97% similarity through High-throughput analysis.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Observed Species</th>
<th>OTUs</th>
<th>Shannon</th>
<th>Simpson</th>
<th>Chao1</th>
<th>ACE</th>
<th>PD Tree</th>
<th>Evenness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>255</td>
<td>248</td>
<td>4.62</td>
<td>0.876</td>
<td>273.50</td>
<td>279.39</td>
<td>22.02</td>
<td>0.357</td>
</tr>
<tr>
<td>Control</td>
<td>226.5</td>
<td>273.2</td>
<td>3.85</td>
<td>0.770</td>
<td>240.24</td>
<td>244.54</td>
<td>19.44</td>
<td>0.296</td>
</tr>
<tr>
<td>P. Value</td>
<td>0.042</td>
<td>0.067</td>
<td>0.011</td>
<td>0.0321</td>
<td>0.036</td>
<td>0.030</td>
<td>0.037</td>
<td>0.014</td>
</tr>
</tbody>
</table>

**Phyla level intestinal bacteria**

Intestinal bacterial taxa had a percentage over 0.5% - 1%, considered in the present study, at the phylum level; family; genus and species.
At the level of phylum, a total of 15 phyla were found in sequencing; among the 10 topmost phyla, significantly increased phyla abundance in the diseased group were Firmicutes and Verrucomicrobia while non-substantially raised in Proteobacteria and Actinobacteria. However, Phylum Bacteroidetes in the experimental group was significantly reduced compared to healthy volunteers, depicted in Figure 5A. Statistics of the 10 most prevalent phyla in Table 6A illuminated significant quantitative disparity between the two groups.

**Intestinal bacterial composition at a family level**

Family level sequencing, 75 diverse families were sequenced through Illumina based sequencing, in 10 topmost families, taxa richness of Ruminococcaceae, and Verrucomicrobiaceae were significantly elevated while non-significantly increased in families Enterobacteriaceae, Lachnospiraceae and Rikenellaceae in the study group with the comparison of control, depicted in Figure 5B. Among all these families, the community abundance of Bacteroidaceae and Prevotellaceae was significantly reduced in the study group compared to healthy volunteers. Percentage data statistics in euthyroid thyroid cancer displayed a significant quantitative variation of families shown in Table 6A.

**Table 6A. Gut microbial taxa at phyla and families level from High-throughput results**

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Mean D</th>
<th>Mean C</th>
<th>p.value</th>
<th>q value</th>
<th>%D</th>
<th>% C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phylum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>0.2224</td>
<td>0.6605</td>
<td>0.0010</td>
<td>0.0040</td>
<td>22.24</td>
<td>66.05</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>0.2420</td>
<td>0.0941</td>
<td>0.1039</td>
<td>0.1662</td>
<td>24.20</td>
<td>9.41</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>0.4305</td>
<td>0.2345</td>
<td>0.0050</td>
<td>0.0160</td>
<td>43.05</td>
<td>23.45</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>0.0862</td>
<td>0.0009</td>
<td>0.0010</td>
<td>0.0040</td>
<td>8.62</td>
<td>0.09</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>0.0167</td>
<td>0.0092</td>
<td>0.2947</td>
<td>0.3929</td>
<td>1.67</td>
<td>0.92</td>
</tr>
<tr>
<td>Tenericutes</td>
<td>0.0015</td>
<td>0.0000</td>
<td>0.0060</td>
<td>0.0160</td>
<td>0.15</td>
<td>0.00</td>
</tr>
<tr>
<td>Fusobacteria</td>
<td>0.0000</td>
<td>0.0008</td>
<td>0.0290</td>
<td>0.0515</td>
<td>0.00</td>
<td>0.08</td>
</tr>
<tr>
<td>Saccharibacteria</td>
<td>0.0002</td>
<td>0.0000</td>
<td>0.0070</td>
<td>0.0160</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>Synergistetes</td>
<td>0.0001</td>
<td>0.0000</td>
<td>0.0010</td>
<td>0.0040</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>0.0002</td>
<td>0.0000</td>
<td>0.0250</td>
<td>0.0500</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Genus level intestinal bacterial distribution

Genera level hightrough-put sequencing characterized the abundance of 211 diverse genera. Among Sequenced 30 topmost genera, there was significantly elevated taxa prevalence of *Escherichia-Shigella*, [Eubacterium]_coprostanoligenes*, Subdoligranulum*, and *Ruminococcus_2* in the study group with the comparison of healthy group depicted in Figure 5C. Conversely, significantly lowered genera in the patients’ group were *Bacteroides, Klebsiella* and *Prevotella_9*. Dissimilarity genera abundance statistics in euthyroid thyroid cancer group were gathered in Table 6B. Euthyroid thyroid cancer has a specific effect on intestinal bacteria, in particular, Phylum Firmicutes Verrucomicrobia, Proteobacteria, Bacteroidetes and Actinobacteria, families Ruminococcaceae, Verrucomicrobiaceae, Prevotellaceae and Bacteroidaceae genera *Escherichia-Shigella*, [Eubacterium]_coprostanoligenes*, Subdoligranulum* *Ruminococcus_2*, *Prevotella_9*, *Bacteroides* and *Klebsiella*. The disease also dramatically influences the intestinal bacteria, which may change the health status of an individual due to the alteration of intestinal bacterial composition.
Figure 5. (A) Configuration of Gut microbiota at phyla levels from High-throughput sequencing results. The excessive occurrence of the most prevalent phyla in euthyroid thyroid cancer and control D and C designated as euthyroid thyroid cancer patients and control group, respectively, (B) High-throughput sequencing findings of gut bacterial conformation at family level. The relative plentiful of the most profoundly found families in euthyroid thyroid cancer and healthy controls. D and C represent euthyroid thyroid cancer and control group, respectively, (C) The genera levels gut bacterial compositions from High-throughput sequencing results. The relative abundance of the most prevalent genera in euthyroid thyroid cancer and healthy control. D and C represent euthyroid thyroid cancer and control group, respectively, (D) LDA (linear discriminant analysis) value distribution histogram was applied to find the most altered gut bacterial taxa abundance between euthyroid thyroid cancer patients D and control subjects C.
**Table 6B. Gut microbial phylotypes at genus level from High-throughput results**

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Mean D</th>
<th>Mean C</th>
<th>p.value</th>
<th>q value</th>
<th>% D</th>
<th>% C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia-Shigella</td>
<td>0.1941</td>
<td>0.0265</td>
<td>0.0140</td>
<td>0.0824</td>
<td>19.41</td>
<td>2.65</td>
</tr>
<tr>
<td>Prevotella_9</td>
<td>0.0125</td>
<td>0.2568</td>
<td>0.0020</td>
<td>0.0193</td>
<td>1.25</td>
<td>25.68</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>0.1260</td>
<td>0.3238</td>
<td>0.0160</td>
<td>0.0869</td>
<td>12.60</td>
<td>32.38</td>
</tr>
<tr>
<td>Akkermansia</td>
<td>0.0862</td>
<td>0.0009</td>
<td>0.0010</td>
<td>0.0125</td>
<td>8.62</td>
<td>0.09</td>
</tr>
<tr>
<td>Klebsiella</td>
<td>0.0031</td>
<td>0.0351</td>
<td>0.0340</td>
<td>0.1412</td>
<td>0.31</td>
<td>3.51</td>
</tr>
<tr>
<td>[Eubacterium]_coprostanoligenes</td>
<td>0.0564</td>
<td>0.0178</td>
<td>0.0290</td>
<td>0.1253</td>
<td>5.64</td>
<td>1.78</td>
</tr>
<tr>
<td>Subdoligranulum</td>
<td>0.0888</td>
<td>0.0105</td>
<td>0.0010</td>
<td>0.0125</td>
<td>8.88</td>
<td>1.05</td>
</tr>
<tr>
<td>Dorea</td>
<td>0.0377</td>
<td>0.0038</td>
<td>0.0010</td>
<td>0.0125</td>
<td>3.77</td>
<td>0.38</td>
</tr>
<tr>
<td>Citrobacter</td>
<td>0.0179</td>
<td>0.0020</td>
<td>0.4525</td>
<td>0.7260</td>
<td>1.79</td>
<td>0.20</td>
</tr>
<tr>
<td>Ruminococcus_2</td>
<td>0.0459</td>
<td>0.0109</td>
<td>0.0180</td>
<td>0.0953</td>
<td>4.59</td>
<td>1.09</td>
</tr>
<tr>
<td>Alistipes</td>
<td>0.0619</td>
<td>0.0377</td>
<td>0.2388</td>
<td>0.4907</td>
<td>6.19</td>
<td>3.77</td>
</tr>
<tr>
<td>Ruminococcaceae_UCG-013</td>
<td>0.0280</td>
<td>0.0024</td>
<td>0.0020</td>
<td>0.0193</td>
<td>2.80</td>
<td>0.24</td>
</tr>
<tr>
<td>Ruminococcaceae_UCG-002</td>
<td>0.0168</td>
<td>0.0060</td>
<td>0.1948</td>
<td>0.4302</td>
<td>1.68</td>
<td>0.59</td>
</tr>
<tr>
<td>[Eubacterium]_rectale_group</td>
<td>0.0181</td>
<td>0.0248</td>
<td>0.5335</td>
<td>0.7440</td>
<td>1.80</td>
<td>2.47</td>
</tr>
<tr>
<td>Parabacteroides</td>
<td>0.0092</td>
<td>0.0314</td>
<td>0.0080</td>
<td>0.0529</td>
<td>0.91</td>
<td>3.14</td>
</tr>
<tr>
<td>Faecalibacterium</td>
<td>0.0137</td>
<td>0.0385</td>
<td>0.0030</td>
<td>0.0254</td>
<td>1.37</td>
<td>3.84</td>
</tr>
<tr>
<td>Desulfovibrio</td>
<td>0.0112</td>
<td>0.0004</td>
<td>0.0010</td>
<td>0.0125</td>
<td>1.25</td>
<td>0.04</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>0.0074</td>
<td>0.0143</td>
<td>0.2358</td>
<td>0.4900</td>
<td>0.73</td>
<td>1.42</td>
</tr>
<tr>
<td>Ruminococcus_1</td>
<td>0.0041</td>
<td>0.0082</td>
<td>0.5654</td>
<td>0.7684</td>
<td>0.40</td>
<td>0.81</td>
</tr>
<tr>
<td>Parasutterella</td>
<td>0.0018</td>
<td>0.0203</td>
<td>0.0240</td>
<td>0.1105</td>
<td>0.17</td>
<td>2.06</td>
</tr>
<tr>
<td>Megamonas</td>
<td>0.0003</td>
<td>0.0079</td>
<td>0.0010</td>
<td>0.0125</td>
<td>0.03</td>
<td>0.79</td>
</tr>
<tr>
<td>Dialister</td>
<td>0.0006</td>
<td>0.0061</td>
<td>0.0999</td>
<td>0.2750</td>
<td>0.06</td>
<td>0.60</td>
</tr>
<tr>
<td>Phascolarctobacterium</td>
<td>0.0021</td>
<td>0.0044</td>
<td>0.5604</td>
<td>0.7665</td>
<td>0.21</td>
<td>0.44</td>
</tr>
<tr>
<td>Paraprevotella</td>
<td>0.0026</td>
<td>0.0053</td>
<td>0.3516</td>
<td>0.6318</td>
<td>0.26</td>
<td>0.52</td>
</tr>
<tr>
<td>Ruminococcaceae_NK4A214_group</td>
<td>0.0051</td>
<td>0.0020</td>
<td>0.1369</td>
<td>0.3297</td>
<td>0.50</td>
<td>0.19</td>
</tr>
<tr>
<td>Lachnoclostridium</td>
<td>0.0052</td>
<td>0.0098</td>
<td>0.0420</td>
<td>0.1435</td>
<td>0.52</td>
<td>0.98</td>
</tr>
<tr>
<td>Christensenellaceae_R-7_group</td>
<td>0.0035</td>
<td>0.0009</td>
<td>0.1129</td>
<td>0.2919</td>
<td>0.34</td>
<td>0.87</td>
</tr>
<tr>
<td>Blautia</td>
<td>0.0081</td>
<td>0.0039</td>
<td>0.0619</td>
<td>0.1960</td>
<td>0.80</td>
<td>0.39</td>
</tr>
<tr>
<td>Coprococcus_2</td>
<td>0.0012</td>
<td>0.0043</td>
<td>0.0519</td>
<td>0.1721</td>
<td>0.12</td>
<td>0.43</td>
</tr>
<tr>
<td>Ruminococcaceae_UCG-014</td>
<td>0.0005</td>
<td>0.0026</td>
<td>0.2238</td>
<td>0.4697</td>
<td>0.04</td>
<td>0.26</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12.30</td>
<td>8.77</td>
</tr>
</tbody>
</table>

Euthyroid thyroid cancer D and Control C, (P < 0.05)

**Gut bacterial composition at the species level**

Species-level intestinal bacterial community patterns are demonstrated in Table 7. These findings of species illustrate the significant differences between euthyroid thyroid cancer and healthy
volunteers. However, the levels of *Escherichia coli* in euthyroid thyroid cancer patients have been raised significantly when compared to healthy volunteers.

The LDA (linear discriminant analysis) value distribution histogram demonstrates the taxa differences between the two groups. Taxa with significant differences in abundance in euthyroid thyroid cancer and control groups, shown in Figure 5D, and the length of the histogram represents the size and impact of the different Taxa.

The final data obtained from results through the analysis of metagenomic DGGE and High-throughput sequencing confirms the similar bacterial taxa prevalence. However, High-throughput sequencing is a highly sensitive, authentic, and much reliable technique than DGGE to study the intestinal bacterial taxa. Trend-wise High-throughput sequencing and qPCR quantitative endorsed each other result findings in the whole gut bacterial composition. In conclusion, the results agree and affiliate the intestinal bacterial data generated by three molecular procedures.

**Table 7. High-throughput differential intestinal bacterial phylotypes between euthyroid thyroid cancer D and Control C at the species level**

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Mean C</th>
<th>Mean D</th>
<th>p value</th>
<th>q value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>0.026521</td>
<td>0.194062</td>
<td>0.016983</td>
<td>0.079437</td>
</tr>
<tr>
<td><em>Bacteroides vulgatus</em></td>
<td>0.166316</td>
<td>0.033518</td>
<td>0.004995</td>
<td>0.03812</td>
</tr>
<tr>
<td><em>Klebsiella_pneumoniae</em></td>
<td>0.03508</td>
<td>0.003094</td>
<td>0.022977</td>
<td>0.104115</td>
</tr>
<tr>
<td><em>Dorea longicatena</em></td>
<td>0.003479</td>
<td>0.035789</td>
<td>0.000999</td>
<td>0.013169</td>
</tr>
<tr>
<td><em>Bacteroides stercoris</em></td>
<td>0.065741</td>
<td>0.005255</td>
<td>0.001998</td>
<td>0.019314</td>
</tr>
<tr>
<td><em>Bacteroides uniformis</em></td>
<td>0.028511</td>
<td>0.006969</td>
<td>0.00999</td>
<td>0.060356</td>
</tr>
<tr>
<td><em>Akkermansia muciniphila</em></td>
<td>0.00379</td>
<td>0.01261</td>
<td>0.001998</td>
<td>0.019314</td>
</tr>
<tr>
<td><em>Parabacteroides distasonis</em></td>
<td>0.018982</td>
<td>0.004001</td>
<td>0.015984</td>
<td>0.079437</td>
</tr>
<tr>
<td><em>Bacteroides cellulosilyticus</em></td>
<td>0.004009</td>
<td>0.015244</td>
<td>0.032967</td>
<td>0.119505</td>
</tr>
<tr>
<td><em>Dialistersuccinatilphilus</em></td>
<td>0.003622</td>
<td>0.000106</td>
<td>0.042957</td>
<td>0.144855</td>
</tr>
<tr>
<td><em>Bacteroides coprophilus;</em></td>
<td>0.0039</td>
<td>0.000248</td>
<td>0.008991</td>
<td>0.059259</td>
</tr>
<tr>
<td><em>Bacteroides plebeius</em></td>
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<td>0.000388</td>
<td>0.002997</td>
<td>0.025563</td>
</tr>
<tr>
<td><em>Bacteroides massiliensis</em></td>
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<td>0.002997</td>
<td>0.025563</td>
</tr>
<tr>
<td><em>Roseburia inulinivorans;</em></td>
<td>0.010472</td>
<td>0.002868</td>
<td>0.001998</td>
<td>0.019314</td>
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<td><em>Bacteroides thetaiotaomicron;</em></td>
<td>0.007045</td>
<td>0.002454</td>
<td>0.026973</td>
<td>0.111745</td>
</tr>
</tbody>
</table>
**Discussion**

Thyroid cancer is an endocrine system disorder leading to malignancy, which is progressively rising in the last few decades that is to be believed due to better diagnostic facilities [42]. Recent studies have been illustrated that gut microbiome has great importance and role in driving the different types of malignancies, including lung, breast, intestine and esophageal cancer [43, 44]. The human gut microbiota role is also critical in protecting the body's defense mechanism by employing trophic and preventative activity [45]. The gut microbiota and its metabolites like short-chain fatty acids have great impact in normal functioning of thyroid gland. It shows an existence of gut–thyroid axis and gut bacterial dysbiosis in autoimmune thyroid diseases such as Hashiomet’s thyroiditis and Graves’ disease [24, 25, 46].

The intestinal bacterial confirmation has been underlined in various disease situations, i.e., melanoma, diabetes [4]. However, thyroid cancer cancer is a fatal malignancy. During the last few decades, its rate it increased dramatically, that is to be believed due to better diagnostic facilities. Recent studies have been illustrated that gut microbiome has great importance and key
role in driving the different types of malignancies, including lung, breast intestine and esophageal cancer [43]. The DGGE gel profile findings were elucidated by illuminating the sequencing of prominent bands, High-throughput sequencing data analysis, and Real-time PCR. The statistical data in α diversity, nonparametric Simpson, Shannon, Chao1, observed species, and the ACE algorithm were found significantly elevated in the patients’ group compared to the control group [47]. Likewise, the diversity of gut bacterial population assessment in DGGE banding profiles and High-throughput sequencing analysis were found elevated in euthyroid thyroid cancer patients. Therefore, this increased level of gut microbiota evident the notable overgrowth in patients compared to the control group. However, these raised findings and interpersonal variations parallel preceding microbial results of skin, vagina, and gastrointestinal tract [48, 49].

The statistical data interpretation of intestinal microbial similarity index of euthyroid thyroid carcinoma group in DGGE banding profile configuration of intra-groups was measured to be significantly elevated; this drew the bacterial overgrowth in the gut of study group. The comparative data analysis of diversity similarity index found lesser in intergroup with the comparison of intra-group that are aligned with preceding research literature [50], indicating the variation in the composition of intestinal microbiota in euthyroid thyroid cancer patients with comparison of normal control. Hence, the diversity as mentioned above, findings elucidate a significant dissimilarity in the composition of intestinal bacteria between patients and healthy control groups. The data statistics denote the significant quantitative and qualitative alteration between study and healthy groups.

At the level of phylum study, Firmicutes exhibited a significantly higher trend while low in Bacteroidetes in euthyroid thyroid cancer patients with a comparison of control that is
compatible with reported research in gut microbial alteration, endocannabinoid tone system and chronic pain with vitamin D mediated deficiency [51]. The reported meta-analysis of intestinal bacteria related to IBD and obesity showed that the percentage of Firmicutes to Bacteroidetes is not a constant feature that is distinct between lean and obese intestinal bacteria [52]. The current study illustrates a higher level of Proteobacteria, which is in accordance with prior work of proteobacteria as risk-factor abdominal pain in patients of the post-cholecystectomy syndrome [53]. It has also been reported that Proteobacteria has a crucial role in inflammatory bowel disease, metabolic disorders, asthma, and obstructive pulmonary diseases. [54] Our work indicates the significantly increased abundance of phylum Verrucomicrobia which agrees with the reported literature of abnormalities of blood pressure associated and gut microbiota of children with anomalies of the urinary tract and kidney [55]. Furthermore, *Verrucomicrobia* is related to blood pressure abnormalities in the initial stage of CKD children [55].

At the family level, Our study showed an increased level of Enterobacteriaceae, agreeing with the reported work of altered gut microbiota in vitamin D deficiency-mediated chronic pain [56]. It has been documented that pathogens of family Enterobacteriaceae involve in nosocomial pneumonia, approximately 1/3 of reported cases [57]. Our work showed a significant reduction of family Bacteroidaceae which is aligned with published literature of intestinal microecology in primary Sjogren's Syndrome patients [58]. The current study showed a significantly higher level abundance of family Ruminococcaceae, while significantly decreased family of Prevotellaceae, which is parallel with reported work of diabetes type 2 and changes in intestinal bacteria with supplementation of diet [59].

Family Verrucomicrobiaceae is significantly enriched in our study, which is aligned with previous work of variation of the intestinal microbiota of the Chinese population with
Parkinson's disease [60]. Our study depicted a significant abundance of *Eubacterium* and Akkermansia, which aligns with the existing work of altered intestinal microbiota in chronic pain endocannabinoid tone systems with vitamin D deficiency [56]. However, in advanced periodontal disease, *Eubacterium* may contribute to making about half of the microbiota and express a significant relationship with the disease [61]. In numerous reported studies of humans and mice, increased abundances of *Akkermansia* are linked with patients of post-RYGB [62].

Moreover, genera *Prevotella_9* was significantly diminished in the study group. Decreased level of *Prevotella* has been publicized in type 1 diabetes and autism with intestinal bacteria [63, 64], while *Escherichia-Shigella* genera were increased in the study group, which agrees with reported preliminary research of gut bacterial relationship with autism problems [65]. Current study outcomes depicted the lowered level of the *Prevotella* genus; however, the existing research literature shows the *Prevotella* dominance in intestinal microbial texture, which has a positive effect on the host's metabolism [66]. Prevalence of the *Prevotella* genus is considered a useful gut bacteria which help in digestion of plant-based food material. Also, the gut microbiota has been associated with numerous inflammatory conditions and diseases [67, 68]. Our results exhibited a significant raised in *Escherichia-Shigella* in euthyroid thyroid cancer patients with a comparison of healthy control. Previously, it was documented that *Escherichia-Shigella* can release Shiga-toxin, which may cause thrombocytopenia septicaemia, hemorrhagic colitis, gastrointestinal inflammations, particularly in the ileocolonic region, hemolytic uremic syndrome (HUS), problems in urinary duct passages [69]. The current study indicated a significantly higher prevalence of genus *Escherichia-Shigella* ge, particularly *Escherichia coli* species, which could be the strongest contributing agent in intestinal bacteria of euthyroid thyroid cancer.
patients. Besides, *Escherichia coli* (ubiquitous) is responsible for triggering predominant infections, i.e. (UTIs) urinary tract infections and foodborne illnesses [70].

Current research work described significant predominance fluctuations in the abundance of the phylum to genus and species-level taxa of fecal samples trails, which demonstrated the clear disparity of euthyroid thyroid cancer patients and control groups. Furthermore, the bacterial community and species-level comparison also unveiled a significant variation of the gut bacterial texture of study as compared of healthy control [71]. These research findings further elaborate that euthyroid thyroid cancer plays a critical role in changing intestinal physiology changes that may cause alteration in the composition of gut microbiota. Likewise, such variations in intestinal microbial patterns may trigger disease complications [72].

The clinical signs of thyroid cancer manifested with thyroid nodules. However, Serum circulating antibodies, i.e., anti-thyroglobulin antibodies, anti-thyroid peroxidase and thyroid hormones in euthyroid thyroid cancer patients and healthy volunteers, are shown in (Table S1 and Table S2). The normal serum results of thyroid cancer patients in Table S1 showed euthyroid in thyroid cancer patients. So, it may be hypothesized that euthyroid thyroid carcinoma might alter the intestinal bacterial configuration, in particular, Phylum Bacteroidetes, Firmicutes, Verrucomicrobia, family Enterobacteriaceae, Prevotellaceae, Bacteroidaceae, Verrucomicrobiae Ruminococcaceae, genera *Escherichia-Shigella, Prevotella_9, Bacteroides, Akkermansia, Klebsiella, Eubacterium and Escherichia coli* species, also mainly disturb the intestinal bacteria. The current study on bacterial intestinal alterations between the euthyroid thyroid cancer group and healthy counterparts was very interesting because there was no direct connection and the straight relationship between euthyroid thyroid cancer and gut bacteria. Thus, the current study results further intricate the diverse intestinal bacteria makeup between
euthyroid thyroid cancer and normal healthy counterparts. These bacterial alterations may disturb the host's health status. Nevertheless, disease progress has no direct linkage with the gastrointestinal tract [73].

The Real-time PCR experiment was done to investigate the intestinal bacterial quantitative changes [74]. Statistics elucidate a significant reduction of Lactobacillus in the study group, hence parallel with previously reported research [75]. The food supplement probiotics belong to genera Lactobacillus and incorporate physiological health benefits in body function [76]. Moreover, Lactobacillus has been observed a reduced trend in diseases like colorectal cancer [77]. In the human gut, Lactobacillus has great importance in the maintenance of selenium levels inside the cell. Selenium plays a crucial role in producing thyroid hormone and avoiding the oxidative destruction of the thyroid gland [78, 79]. Many studies also exhibited good effects against anti-atherogenic anti-obesity and anti-inflammatory body response [80]. Different strains of lactobacillus have excellent antimicrobial characteristics in the human body to protect against uropathogens [81].

There was a significantly decreased level of Bacteroides vulgates in the study group, which is steady with the reported literature of viral diarrhea with intestinal bacteria, while a significant increase of Escherichia coli [82, 83]. Numerically Bacteroides vulgatus species is the predominant Bacteroides of human intestinal bacteria, which comprise a beneficial but complex association with its host and the avoidance of gut colonization [84, 85]. Data generated from PCR-DGGE and Illumina-based High-throughput sequencing analysis are suitable for characterizing intestinal bacteria. However, PCR-DGGE has been observed as a semi-quantitative technique; banding profile assessment may not accurately illustrate the targeting taxa abundance of intestinal bacterial community [32]. Moreover, Illumina-based High-
throughput sequencing is a highly sensitive, most advanced, and pretty much reliable procedure to study and investigate the intestinal bacterial ecology [73]. Furthermore, an experimental technique like PCR-DGGE might be applied as routine basic laboratory testing to detect the significant modulation of gut bacterial taxa due to the least time-taking and low-cost experimental method.

**Conclusion**

A difference in composition of gut microbiota was found between euthyroid thyroid cancer patients and healthy counterparts. More precisely, there is a significant alteration in intestinal bacterial taxa abundance of the study group compared to healthy groups. The bacterial estimation analysis of taxa diversity exhibits a higher level of presence of intestinal bacteria in the euthyroid thyroid cancer group than controls, which shows the bacterial dysbiosis and overgrowth in the euthyroid thyroid cancer patients group. Consequently, the additional multicentre study approach has been needed to apprehend the basic underlying process and mechanism of bacterial dysbiosis in the intestine of euthyroid thyroid cancer patients.

**Competing Interests**

All authors declare no conflict of interest.

**Acknowledgment**

This study was supported by National Natural Science Foundation of China (NSFC 81730056). The authors pay thanks to Dr. Hui Guo (Department of Endocrinology 1st affiliated Hospital Xi'an Jiaotong University, China) for helping in sample collection for current experimental study. The authors also thankful to NCBI for sequence analysis.
References


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### Table S1. Serum thyroid antibodies and hormone in euthyroid thyroid cancer patients.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age/Gender</th>
<th>T3 (ng/ml)</th>
<th>T4(μg/dl)</th>
<th>FT3(pmol/L)</th>
<th>FT4(pmol/L)</th>
<th>TSH(μIU/ml)</th>
<th>Anti-TPO (U/ml)</th>
<th>Anti-TG Ab(%)</th>
<th>TM-Ab (IU/L)</th>
<th>Type of Thyroid Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38/Female</td>
<td>0.85</td>
<td>8.09</td>
<td>5.19</td>
<td>15.90</td>
<td>2.81</td>
<td>&lt;15.0</td>
<td>1.22</td>
<td>2.21</td>
<td>Papillary Thyroid cancer</td>
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<tr>
<td>2</td>
<td>35/Female</td>
<td>1.25</td>
<td>8.23</td>
<td>4.67</td>
<td>11.88</td>
<td>2.07</td>
<td>&lt;15.0</td>
<td>4.06</td>
<td>1.10</td>
<td>Papillary Thyroid cancer</td>
</tr>
<tr>
<td>3</td>
<td>48/Male</td>
<td>0.95</td>
<td>5.90</td>
<td>4.30</td>
<td>12.70</td>
<td>1.90</td>
<td>&lt;15.0</td>
<td>3.03</td>
<td>2.70</td>
<td>Papillary Thyroid cancer</td>
</tr>
<tr>
<td>4</td>
<td>50/Female</td>
<td>0.92</td>
<td>7.30</td>
<td>6.26</td>
<td>13.12</td>
<td>3.20</td>
<td>12</td>
<td>2.30</td>
<td>3.40</td>
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<tr>
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<td>1.70</td>
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<td>3.42</td>
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<tr>
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<tr>
<td>9</td>
<td>49/Male</td>
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<td>7.20</td>
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<td>15.30</td>
<td>1.80</td>
<td>6</td>
<td>2.90</td>
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<td>1.90</td>
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<tr>
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<td>5.40</td>
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<td>6</td>
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<td>1.40</td>
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<tr>
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<td>8.06</td>
<td>5.10</td>
<td>12.10</td>
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<td>7</td>
<td>3.60</td>
<td>2.10</td>
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<tr>
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<td>2.11</td>
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<tr>
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Table S2. Serum thyroid antibodies and hormone in healthy subjects.

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<th>T4(μg/dL)</th>
<th>FT3(pmol/L)</th>
<th>FT4(pmol/L)</th>
<th>TSH(μIU/ml)</th>
<th>Anti-TPO (U/ml)</th>
<th>Anti-TG Ab(%)</th>
<th>TM-Ab (IU/L)</th>
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