




Dysbiosis of the gut microbiome is associated with thyroid cancer and thyroid nodules and correlated with clinical index of thyroid function

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Abstract

Purpose Thyroid cancer and thyroid nodules are the most prevalent form of thyroid endocrine disorder. The balance of gut microbiome is highly crucial for a healthy human body, especially for the immune and endocrine system. However, the relationship between gut microbiome and the thyroid endocrine disorders such as thyroid cancer and thyroid nodules has not been reported yet.

Methods A cohort of 74 patients was recruited for this study. Among them, 20 patients had thyroid cancer, 18 patients had thyroid nodules, and 36 were matched healthy controls. Gut microbiome composition was analyzed by 16S rRNA (16S ribosomal RNA) gene-based sequencing protocol.

Results We compared the gut microbiome results of 74 subjects and established the correlation between gut microbiome and thyroid endocrine function for both thyroid cancer and thyroid nodules. The results inferred that *alpha* and *beta* diversity were different for patients with thyroid tumor than the healthy controls ($p < 0.01$). In comparison to healthy controls, the relative abundance of *Neisseria* ($p < 0.001$) and *Streptococcus* ($p < 0.001$) was significantly higher for thyroid cancer and thyroid nodules. *Butyrivimonas* ($p < 0.001$) and *Lactobacillus* ($p < 0.001$) displayed notably lower relative abundance for thyroid cancer and thyroid nodules, respectively. It was also found that the clinical indexes were correlated with gut microbiome.

Conclusion Our results indicate that both thyroid cancer and thyroid nodules are associated with the composition of gut microbiome. These results may support further clinical diagnosis to a great extent and help in developing potential probiotics to facilitate the treatment of thyroid cancer and thyroid nodules.

Keywords Gut microbiome · Thyroid cancer · Thyroid nodules · 16S rRNA gene sequencing · Clinical index

Abbreviations

Tg Thyroglobulin

TgAb Thyroglobulin antibody

TSH Thyroid stimulating hormone

FT3 Free triiodothyronine

FT4 Free tetraiodothyronine

TRAb Thyrotropin receptor antibody

TPOAb Thyroid peroxidase antibody

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Introduction

With technological advancement, the relationship between the composition of human microbiome such as the gut microbiome and corresponding diseases is being explored gradually and studied extensively [1]. The intestinal tract, which is the biggest digestive organ in a human body, harbors trillions of bacteria whose genomes are 100 times that of humans [2]. Human intestinal tract fosters a number

of phyla including Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria [3]. Recent studies have reported that the microbiome being an immunity influencer, contributes to the immune system control or escape of distant metastasis, such as liver cancer and pancreatic cancer [4–6]. The variation of gut microbiome was connected with endocrinopathy, like Polycystic Ovarian Syndrome (PCOD/PCOS), obesity, type 1 and 2 diabetes [7–10], etc. Thyroid cancer is the most common endocrine malignant tumor, which is divided into 5 types [11]. Papillary thyroid cancer is another very common thyroid cancer, accounting for approximately 80–85% thyroid cancer patients in developed countries [12]. In the year 2012, about 230,000 new cases of thyroid cancer were estimated among women and men with an age-standardized (world population) rate of 6.10/100,000 for women and 1.90/100,000 for men [13]. In recent years, the patient pool suffering from thyroid disorders has seen a rapid increment worldwide, especially in women [14]. If similar trends are maintained, thyroid cancer may become the fourth most common cancer by 2030 in the United States [15]. The increasing incidence of thyroid cancer in many European countries exhibits a parallel trend as that of the United States followed by China [16]. Thyroid nodules, a form of thyroid endocrine disease, is more prevalent clinically and accounts approximately up to 4 to 7% of the population [17]. 19 to 68% of individual thyroid nodules can be detected with high-resolution ultrasound, especially in women and the elderly [18]. There are many factors that may lead to a certain thyroid disease, including ethnicity, estrogen, BMI (Body Mass Index), radioactive radiation and abnormal iodine intake [19–23]. It is very well-known that any thyroid disease is very closely related to thyroid hormone levels or the hormone functioning, which could influence the composition of gut microbiome [24]. At the same time, the gut microbiome could affect the integration of peripheral and central immune, metabolic and endocrine signals by gut–brain axis [25]. Functional thyroid disorders were associated with excessive bacterial growth and a different microbial composition [24]. Several studies have demonstrated that both the Graves' disease and Hashimoto's thyroiditis are connected with gut microbiome [26–28]. However, no reports of the relationship between gut microbiome, thyroid cancer and thyroid nodules have been validated.

Therefore, in this study, we analyzed the gut microbiome of thyroid cancer, thyroid nodules and healthy controls by sequencing the 16S ribosomal RNA (rRNA) gene. The results discovered effective biomarkers of these diseases while revealing significant information about the variation of the gut microbiome based on structure, composition and function. Several specific microbial biomarkers of thyroid

cancer and thyroid nodules were evaluated and correlated with clinical indices.

Methods

Study subjects

Following the approval by the Institutional Review Boards of the Affiliated Central Hospital of Qingdao University (2016-06-2101), we recruited a cohort of 74 subjects at the Affiliated Central Hospital of Qingdao University, of which 20 patients were suffering from thyroid cancer, 18 patients had thyroid nodules and there were 36 healthy controls. All the subjects signed informed consent forms, and belonged to the same geographical area. Data collected was in the format of a standardized questionnaire that included basic information, medical history and examination results (Table 1). 1) Thyroid nodule: Nodular tumors formed by benign hyperplasia of thyroid follicular epithelium; 2) Thyroid cancer: Thyroid malignant epithelial tumor characterized by follicular epithelial differentiation; 3) All the subjects had not taken antibiotics for the prior three months. All study procedures were approved by the Medical Ethics Committee of the Affiliated Central Hospital of Qingdao University.

Table 1 Characteristics of individuals investigated in this study

	Healthy controls	Thyroid cancer	Thyroid nodule
No. of individuals	36	20	18
Mean age (\pm SD, years)	49 \pm 8.59	53.50 \pm 10.19	50.61 \pm 13.22
Gender (M/F)	22/14	17/3	12/6
<i>Clinical index</i>			
AST(U/L)	18.30 \pm 6.30	17.85 \pm 4.64	20.44 \pm 12.45
ALT(U/L)	18.22 \pm 11.51	16.25 \pm 6.26	23.55 \pm 25.28
R-GT(U/L)	20.80 \pm 14.77	19.35 \pm 7.06	25.22 \pm 19.94
Cr(μ mol/L)	96.50 \pm 56.39	61.55 \pm 16.82	57.05 \pm 16.56
UA(μ mol/L)	304.91 \pm 80.55	307.35 \pm 58.39	310.55 \pm 83.66
TC(mmol/L)	4.82 \pm 0.75	5.41 \pm 1.23	5.13 \pm 1.23
TG(mmol/L)	1.13 \pm 0.78	1.39 \pm 0.91	1.56 \pm 1.02
LDL(mmol/L)	2.10 \pm 0.60	3.35 \pm 1.06	3.21 \pm 1.07
HDL(mmol/L)	1.29 \pm 0.79	1.78 \pm 0.39	1.59 \pm 0.3
GLU	5.20 \pm 0.69	5.93 \pm 2.24	6.43 \pm 3.08
<i>Thyroid function</i>			
FT3(pmol/ml)	N/A	4.45 \pm 0.82	4.63 \pm 0.57
FT4(pmol/ml)	N/A	16.15 \pm 2.92	72.81 \pm 243.38
TSH (μ IU/ml)	N/A	1.86 \pm 0.79	2.06 \pm 1.17
Tg(μ g/L)	N/A	114.3 \pm 175.2	117.16 \pm 167.02
TgAb(μ g/L)	N/A	67.98 \pm 93.32	85.42 \pm 143.29
TRAb(μ g/L)	N/A	32.3 \pm 63.37	41.27 \pm 77.95
TPOAb(μ g/L)	N/A	59.84 \pm 136.27	56.65 \pm 145.24

Biospecimen collection, DNA extraction and sequencing

Fecal specimens were collected in sterilized 2-ml tubes containing pure ethanol on ice, immediately frozen (within 30 min) and stored at -80°C until analysis. Genomic DNA was extracted using CTAB (cetyl trimethylammonium bromide) method [29]. We used Nanodrop 2000 (Thermo Scientific) spectrophotometer to determine the concentration of the extracted DNA. The V1-V2 regions of the 16S rRNA gene were amplified and sequenced on an Illumina HiSeq 2500 system. The PCR was conducted using the bacterial universal primers 27F (5'-AGAGTTTGATCM TGGCTCAG-3') and 338R-I (5'-GCWGCCTCCCGTAG GAGT-3') and 338R-II (5'-GCWGCCACCCGTAGGTGT -3') [30]. Follow-up experiments were carried out according to the sequencing manual.

16S rRNA gene sequence analysis

The 16S sequence paired-end data set was joined and quality filtered using Laser FLASH method, described by Magoč and Salzberg [31]. All sequences were analyzed using the Quantitative Insights into Microbial Ecology (QIIME, version 1.9.1) software suite [32], according to the QIIME tutorial (<http://qiime.org/>) with a few modified methods. Chimeric sequences, where a single organism has distinct genotypes, were removed using Metagenomics tool—usearch61 [33] with *de novo* models. Sequences were clustered against the 2013 Greengenes (13_5 release) ribosomal database's 97% reference data set (<http://greengenes.secondgenome.com/downloads>). Sequences that remained unmatched with any of the entries in this reference were subsequently clustered into *de novo* OTUs at 97% similarity with UCLUST algorithm. Taxonomy was assigned to all OTUs using the RDP classifier [34] within QIIME and the Greengenes reference data set. Rarefaction and rank abundance curves were calculated from OTU tables using alpha diversity and rank abundance scripts within the QIIME pipeline. The hierarchical clustering based on population profiles of most common and abundant taxa was performed using UPGMA clustering (Unweighted Pair Group Method with Arithmetic mean, also known as average linkage) on the distance matrix of OTU abundance. This resulted in a Newick-formatted tree, which was obtained utilizing the QIIME package. Furthermore, QIIME was used to analyze Alpha diversity (Shannon, ACE), beta diversity (weighted UniFrac, Principal Coordinate Analysis (PCoA)), Linear discriminant analysis (LDA) and Effect Size (LEfSe). The Venn diagram using the taxa of the average relative abundance $> 10^{-5}$ was derived using the 'jvenn website' (<http://jvenn.toulouse.inra.fr/app/example.html>).

Statistical analysis

The clinical characteristics of the subjects are represented as the mean \pm SD, which were determined using the Mann-Whitney U test. The diversity categorization of alpha and beta diversity was defined in the OTU table to a sequencing depth of 20,000 per sample. Moreover, Alpha diversity was determined using Mann-Whitney U test and beta diversity was acquired by ANOSIM- Analysis of Similarities. LEfSe combines Kruskal-Wallis test or pair wise Wilcoxon rank-sum test with linear discriminant analysis (LDA), whose threshold value on the logarithmic LDA score equals to 2.0. Spearman's rank correlation method was used to analyze the relationship between the microbiome and clinical index. Analyses were performed using the SPSS statistical package (version 17.0) and R software (version 3.4.4). *p* values < 0.05 were considered statistically significant.

Results

To establish the gut microbiome characteristics of patients with abnormal thyroid endocrine function, we conducted 16S rDNA gene sequencing to analyze 74 fecal samples from 74 individuals (20 patients with thyroid cancer, 18 patients with thyroid nodules and 36 healthy controls). The clinical characteristics of subjects are summarized in Table 1. No significant differences were noticed in either age or gender among the thyroid cancer, thyroid nodules and healthy control groups. Through pre-processing of the sequencing data, 4,153,725 high-quality sequences (Phred \geq Q30) with an average of 56,131 sequences per sample were obtained.

Gut microbiome composition of thyroid cancer, thyroid nodules and healthy control

To characterize the community attributes of gut microbiome of individuals with thyroid disease, relative taxon abundances of thyroid cancer, thyroid nodules and healthy control groups were compared. At the phylum level, three prominent phyla were found to be Bacteroidetes ($50 \pm 12\%$), Firmicutes ($35 \pm 12\%$) and Proteobacteria ($11 \pm 6\%$), which represented over 90% of the total phyla in the three groups (Fig. 1a). At the genus level, genus of *Prevotella* demonstrated significant difference among the three groups ($p = 0.0005$, Fig. 1b). Venn diagrams distinguished between gut microbiome of the three groups. Within the healthy control group, 10 unique species were found, such as *Proteus*, *Acinetobacter*, *Erwinia*, *Psychrobacter* and *Sneathia*. Similarly, 35 unique species like *Thiobacillus*, *Rhodobacter*, *Rheinheimera*, *Mycobacterium* and *Anaerotruncus* were found in the thyroid cancer group; while 36 unique species, including *Rubellimicrobium*, *Propionibacterium*, *Peptostreptococcus*, and *Parvimonas* were found in the thyroid nodules group.

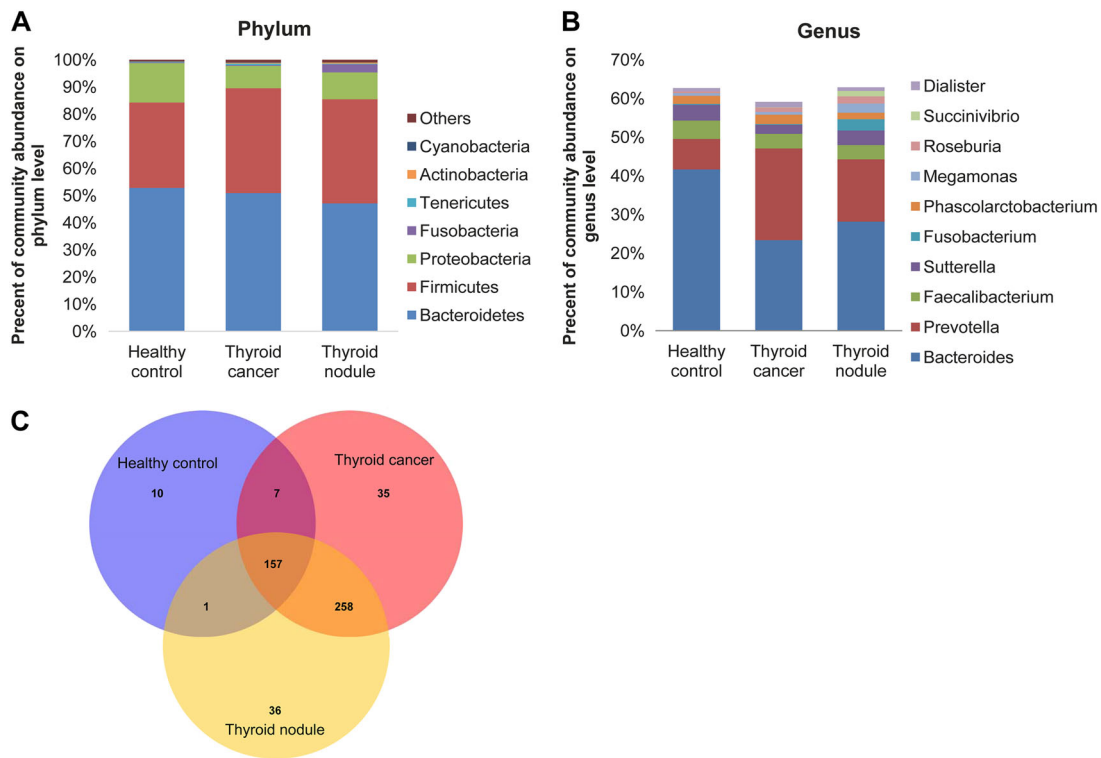


Fig. 1 Comparison of OTUs and relative taxa abundance among thyroid cancer, thyroid nodules, and healthy controls. **a** Comparison of relative abundance among thyroid cancer, thyroid nodules and healthy

controls at phylum level. **b** Comparison of relative abundance among thyroid cancer, thyroid nodules and healthy controls at genus level; **c** Venn diagram

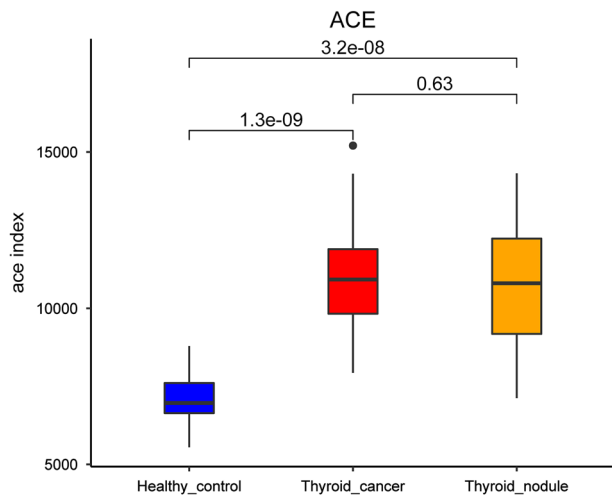


Fig. 2 Phylogenetic diversity of gut microbiome among thyroid cancer, thyroid nodules and healthy controls. Box plots depict microbiome diversity differences according to the ACE index among thyroid cancer, thyroid nodules and healthy controls

Variations of the gut microbiome among the three groups

We analyzed the *alpha* and *beta* diversity to explore the variations in the gut microbiome among the three groups. Although no significant difference was found among the healthy control, thyroid cancer and thyroid nodules groups

with respect to microbial diversity (Shannon index, $p > 0.05$), microbial abundance (ACE index) was higher in both thyroid cancer and nodules than in healthy control group ($p < 0.05$, Fig. 2). In addition, we calculated the unweighted and weighted UniFrac distance to testify the correlation of gut microbiome between the thyroid disease and healthy control group. These calculations revealed that the healthy control group was significantly different from the thyroid cancer group ($r = 0.598$, $p = 0.001$, weighted UniFrac; $r = 0.972$, $p = 0.001$, unweighted UniFrac) and thyroid nodules group ($r = 0.596$, $p = 0.001$, weighted UniFrac; $r = 0.966$, $p = 0.001$, unweighted UniFrac) based on ANOSIM method (Fig. 3). Adding the results, they indicated a significant variation of gut microbiome between thyroid disease and healthy control groups.

Bacterial taxonomic differences among the three groups

In order to select biomarkers from diseased group, we used linear discriminant effect size (LEfSe) to analyze and distinguish the composition of gut microbiome between thyroid disease and healthy control groups. The gut microbiome of the thyroid cancer group was characterized by a dominance of *Prevotella*, *Roseburia*, *Coprococcus*, *Anaerostipes*, *Ruminococcus*, *Neisseria*, *Streptococcus* and

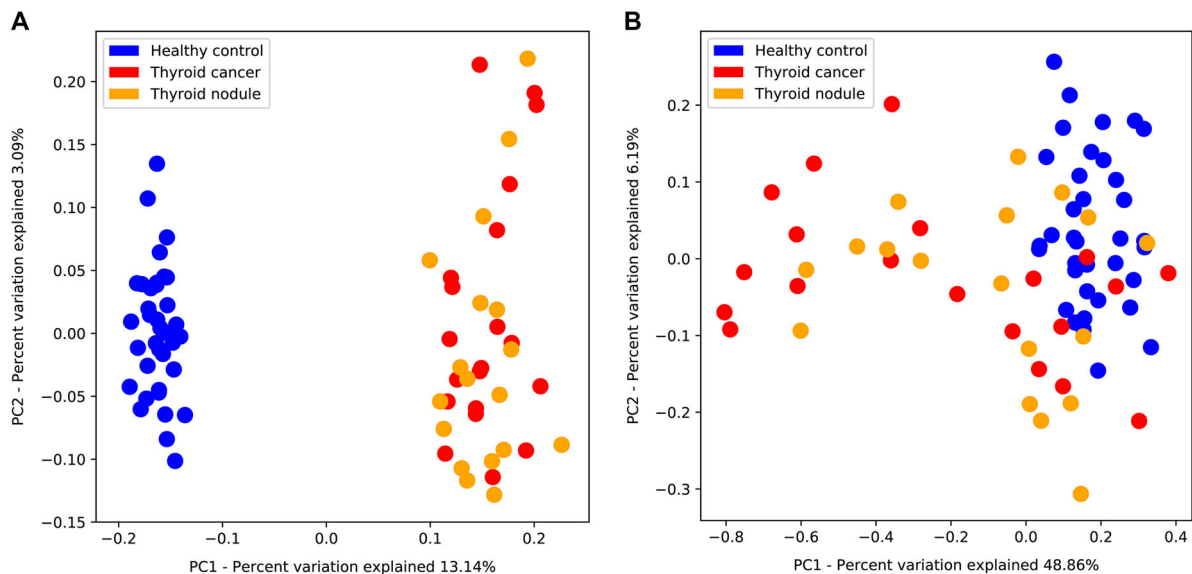


Fig. 3 PCoA analysis of the microbiota among thyroid cancer, thyroid nodules and healthy controls. **a** Unweighted Unifrac PCoA. **b** Weighted Unifrac PCoA

Porphyromonas, whereas the microbiome in healthy control group were dominated by genera of *Bacteroides*, *Sutterella*, and *Butyricimonas* ($p < 0.05$, Fig. 4). The thyroid nodules group's microbiome were characterized by a dominance of *Roseburia*, *Neisseria*, *Streptococcus*, *Anaerostipes* and *Porphyromonas*, whereas the microbiome in healthy control group displayed a dominance of *Bacteroides*, *Sutterella*, *Pseudomonas*, *Lactobacillus* and *Oscillospira* ($p < 0.05$, Fig. 5).

The gut microbiome of thyroid disease was associated with clinical indices

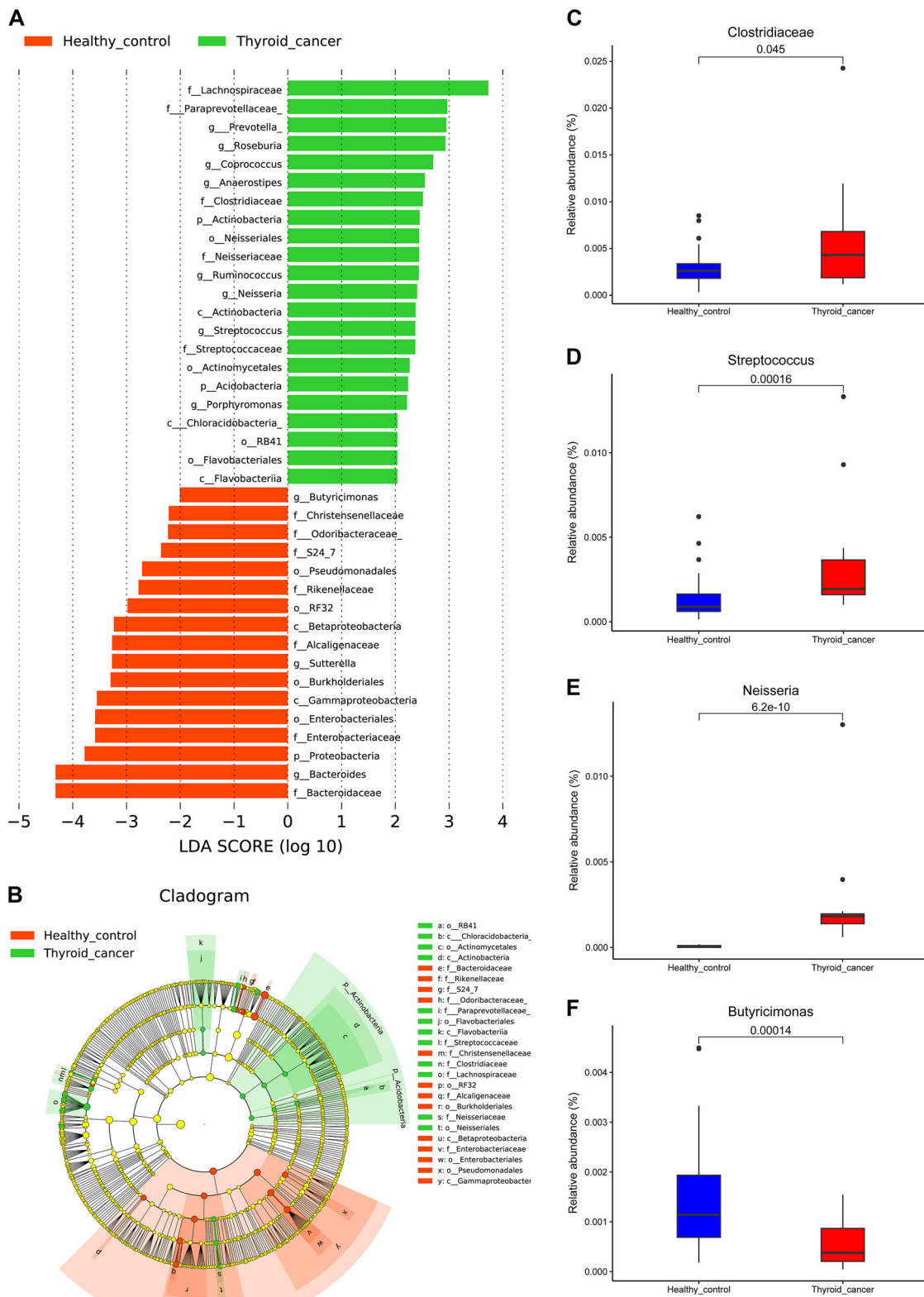
Spearman's rank correlation coefficient method was used to evaluate the correlation between each subject's gut microbiome and clinical indices parameters including FT3 (Free triiodothyronine), FT4 (Free tetraiodothyronine), TSH (Thyroid stimulating hormone), Tg (Thyroglobulin), TgAb (Thyroglobulin antibody), TRAb (Thyrotropin receptor antibody) and TPOA (Thyroid peroxidase antibody) (Fig. 6). Strong correlations (correlation $r > 0.40$ or $r < -0.40$, $p < 0.05$, $n = 20$) were found among 23 taxa and the 7 clinical indices in thyroid nodules. TG exhibited a significant negative correlation with the family Lactobacillaceae ($r = -0.73$, $p < 0.01$) and the genus *Bacteroides* ($r = -0.62$, $p < 0.01$). At the same time, FT3 was negatively correlated with the genera *Bacteroides* ($r = -0.47$, $p < 0.05$) and *Lactobacillus* ($r = -0.47$, $p < 0.05$). Additionally, a distinctive positive association was observed between TSH and the genera *Porphyromonas* ($r = 0.55$, $p < 0.05$) and *Roseburia* ($r = 0.47$, $p < 0.05$). Similarly, the relationship between the biomarkers (22 taxa) and clinical indices (7

indices) in thyroid cancer was established. TSH exhibited a significant positive connection with the genus *Porphyromonas* ($r = 0.57$, $p < 0.01$). At the same time, FT3 was positively correlated with the genera *Streptococcus* ($r = 0.47$, $p < 0.05$). In addition, a significant negative association can be observed between the TRAb and the families Clostridiaceae ($r = -0.54$, $p < 0.05$) and Lachnospiraceae ($r = -0.53$, $p < 0.05$). Furthermore, TPOAB was negatively correlated with the genera *Ruminococcus* ($r = -0.53$, $p < 0.05$).

Discussion

In this study, analysis of gut microbiome composition was performed for stool samples from subjects affected by thyroid cancer, thyroid nodules, and matched healthy controls. It was found that the gut microbiome of patients both with thyroid cancer and thyroid nodules demonstrated higher microbial richness as well as distinct composition compared to the healthy control group. It was further revealed that gut microbiome might be associated with multiple clinical indices. Our pilot study reported that the composition of gut microbiome was correlated with thyroid cancer and thyroid nodules.

Several studies have demonstrated that the dysbiosis of human microbiome could be primarily caused by inflammatory disorders and diverse cancer types throughout the body [4–6, 35, 36]. In our study, we observed that the microbial richness was dominantly higher in both the thyroid cancer group and thyroid nodules group than in the healthy control group. A number of studies suggested that



the microbial diversity was significantly higher in cancer cases [37, 38]. The assembled results indicate that the

opportunistic pathogen may colonize in patients suffering from thyroid endocrine disease.

Fig. 4 Characteristics of microbial community composition in thyroid cancer and healthy control groups. **a** The most differentially abundant taxa between thyroid cancer and healthy control (LDA score above 2), which was generated from LEfSe analysis. **b** The enriched taxa in thyroid cancer and healthy control gut microbiome is represented in the Cladogram. The central point represents the root of the tree (Bacteria) and each ring represents the next lower taxonomic level (phylum to genus: p phylum, c class, o order, f family, g genus). The diameter of each circle represents the relative abundance of the taxon. **c–f** Relative abundance of taxonomy between thyroid cancer and healthy control was compared (Wilcoxon Signed Rank Test)

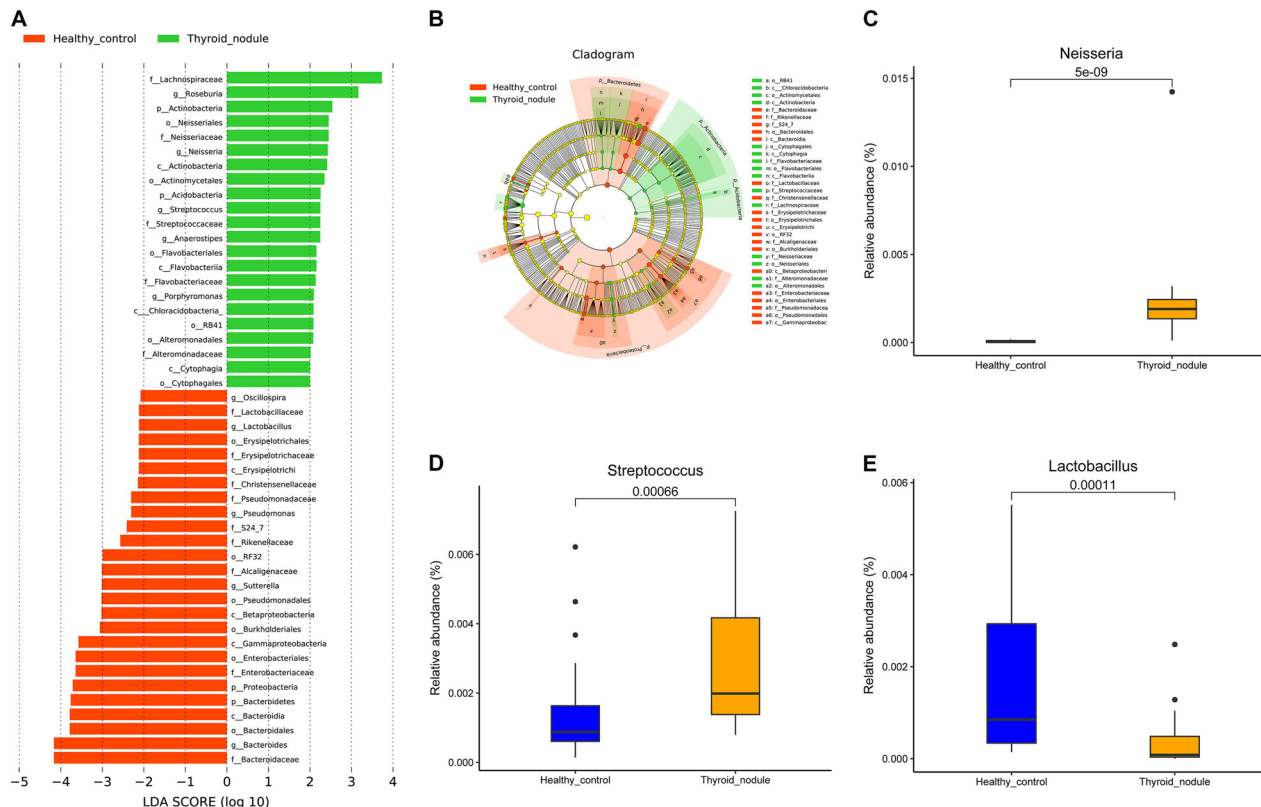


Fig. 5 Characteristics of microbial community composition in thyroid cancer and healthy control groups. **a** The most differentially abundant taxa between thyroid nodule and healthy control (LDA score above 2), which was generated from LEfSe analysis. **b** The enriched taxa in thyroid cancer and healthy control gut microbiome is represented in the Cladogram. The central point represents the root of the tree (Bacteria),

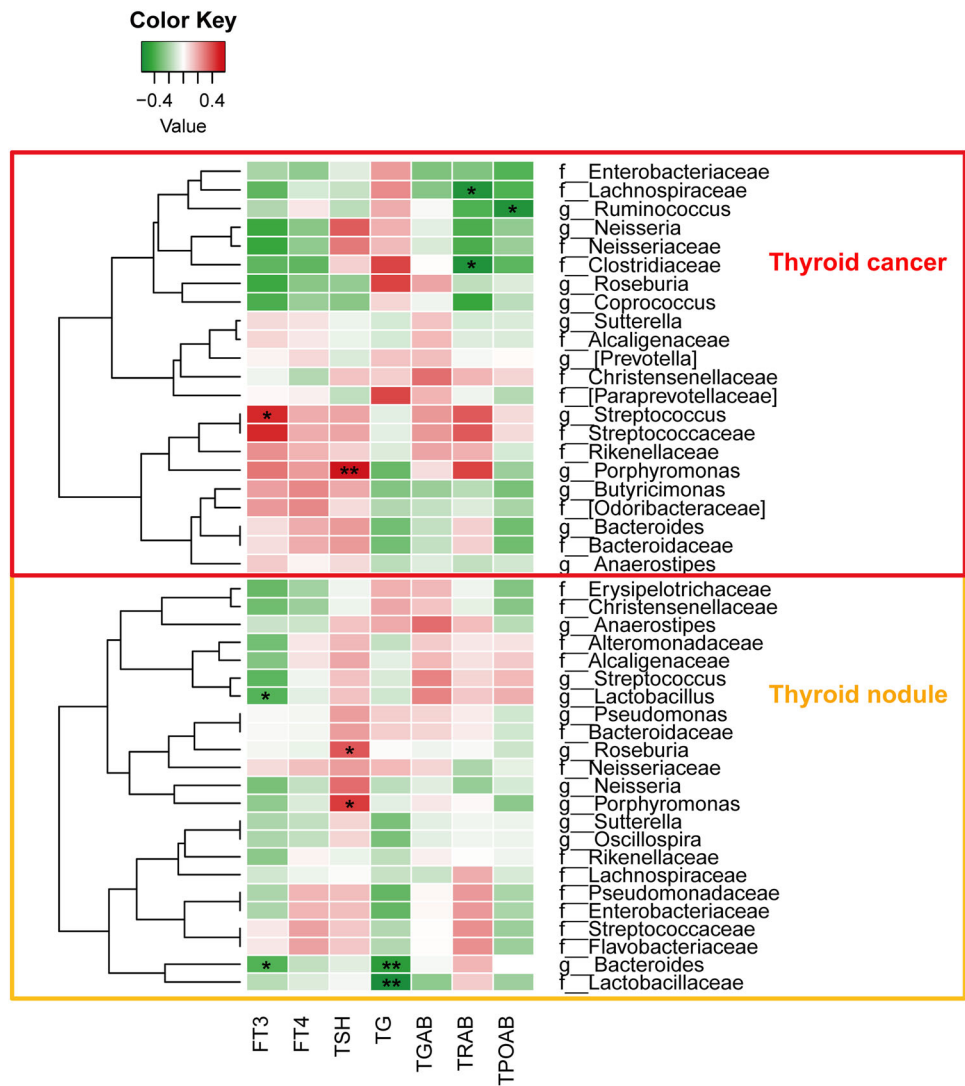
esophageal inflammation, which was correlated to inflammatory disorders [40–42]. Meanwhile, it was also associated with pancreatic disease [43]. Several studies also demonstrated that the increment of both adenomas and carcinomas was associated with the presence of *Streptococcus* [44–46]. Our results showed an increment in these three genera of bacteria, which indicates that these bacteria could be a factor that affects thyroid disease or thyroid functioning.

and each ring represents the next lower taxonomic level (phylum to genus: p phylum, c class, o order, f family, g genus). The diameter of each circle represents the relative abundance of the taxon. **c–e** Relative abundance of taxonomy between thyroid nodule and healthy control was compared (Wilcoxon Signed Rank Test)

With respect to gut microbiome composition, the relative abundance of Clostridiaceae ($p = 0.045$), *Neisseria* ($p < 0.001$) and *Streptococcus* ($p < 0.001$) was significantly higher in the thyroid cancer group, while the abundance of *Neisseria* ($p < 0.001$) and *Streptococcus* ($p < 0.001$) was notably increased in the thyroid nodules group. *Clostridium subterminale* from gut microbiome was detected in the blood of a metastatic gastrointestinal adenocarcinoma patient, which indicated that a few species of Clostridiaceae have carcinogenic effects [39]. *Neisseria* levels were found to be elevated in the setting of

Overall, the relative abundance of *Butyricimonas* ($p < 0.001$) and *Lactobacillus* ($p < 0.001$) was significantly lower in the thyroid cancer group and thyroid nodules group, respectively. As we know, *Butyricimonas* is strictly anaerobic gram-negative bacteria, which could produce SCFAs (Short Chain Fatty Acids) to maintain the health of intestinal tract [47, 48]. Butyrate (BT) is one of the SCFAs, which could regulate innate and adaptive immune cell function. For instance, BT develops an anti-inflammatory effect by inhibiting the recruitment and pro-inflammatory activity of immune cell, such as neutrophils. And it could

Fig. 6 Heat map of Spearman’s correlation analysis between the gut microbiome of thyroid cancer and thyroid nodules and the clinical indices. * $p < 0.05$, ** $p < 0.01$



activate the differentiation of colonic Treg cells to suppress inflammation [49]. *Lactobacillus* is an important genus in the human gut which could improve the concentration of various trace elements in human cells, such as selenium [50]. Selenium is crucial for the transformation of thyroid hormone activation and protects the thyroid gland from oxidative damage during hormone synthesis [51].

The gut microbiome plays a critical role in substance metabolism and influence the essential diagnosis and treatment for various pivotal diseases such as cancer, diabetes and melanoma [52–54]. In this study, our results indicated that gut microbiome was associated with the disease index. TSH was found to have a significant positive association with the *Porphyromonas* ($p < 0.01$) and FT3 was positively connected with the *Streptococcus* in thyroid cancer cases. It is known that TSH and FT3 levels are higher in thyroid cancer cases [55]. In the previous description, *Porphyromonas* and *Streptococcus* were conditional pathogens [56]. Meanwhile, we also found TSH

was positively associated with the *Porphyromonas* ($p < 0.01$) in thyroid nodules, which indicated gut microbiome possessed some of the same mechanisms for affecting thyroid cancer and thyroid nodules. In addition, FT3 was negatively connected with *Lactobacillus* and its relative abundance was slightly lower than that of the healthy controls. *Lactobacillus* could reduce inflammation by producing SCFAs [47, 48]. In summary, our results indicated *Porphyromonas*, *Streptococcus* and *Lactobacillus* could serve as essential biomarkers having potentials in detecting thyroid cancer or thyroid nodules.

Conclusion

This study indicates that the variation of gut microbiome is associated with thyroid cancer and thyroid nodules. It also suggests the potential pathogens active in thyroid cancer and thyroid nodules. These results may be essential for

clinical diagnosis and could be further used to develop potential probiotics that could facilitate the treatment of thyroid cancer and thyroid nodules. However, it still remains unknown if thyroid disorders cause dysbiosis or vice-versa. Further studies need to scrutinize and explore the mechanisms between the gut microbiome and thyroid cancer or nodules using modeled organisms.

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Author Contributions L.Z., J.Y. and M.Y. designed the study. J.Z., C. L., Y.Y., H.W., Y.S., Y.W., X.M., D.Z., and C.Z. performed measurements and data analysis. C.Z. and Q.X. obtained samples. F.Z., J. Z. and L.Z. wrote the manuscript. All authors have read and critically revised the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. The authors have nothing to disclose.

Ethical approval All procedures performed in the above studies involving human participants were in accordance with the ethical standards of the institution and/or national research committee and with the 1964 Declaration of Helsinki along with its later amendments or comparable ethical standards.

Informed consent All the subjects signed informed consent beforehand and belonged to the same geographical area. Data was collected by using a standardized questionnaire including basic information, medical history and examination results.

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