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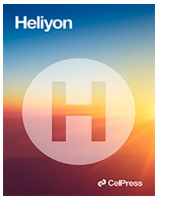

Research article

The gut-brain-axis: A positive relationship between gut microbial dysbiosis and glioblastoma brain tumour

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A B S T R A C T

The glioblastoma brain tumour (GBM) stands out as the most aggressive and resistant-to-treatment malignancy. Nevertheless, the gut-brain connection plays a pivotal role in influencing the growth and activation of the central nervous system. In this particular investigation, we aimed to assess and characterize the gut microbial ecosystem in GBM patients, both quantitatively and qualitatively. We collected faecal samples from 15 healthy volunteers and 25 GBM patients. To delve into the microbial content, we employed PCR-DGGE, targeting the V3 region of the 16S rRNA gene, and conducted qPCR to measure the levels of crucial intestinal bacteria. For a more in-depth analysis, high-throughput sequencing was performed on a selection of 20 random faecal samples (10 from healthy individuals and 10 from GBM patients), targeting the V3+V4 region of the 16S rRNA gene. Our findings from examining the richness and diversity of the gut microbiota unveiled that GBM patients exhibited significantly higher microbial diversity compared to healthy individuals. At the phylum level, Proteobacteria saw a significant increase, while *Firmicutes* experienced a noteworthy decrease in the GBM group. Moving down to the family level, we observed significantly elevated levels of *Enterobacteriaceae*, *Bacteroidaceae*, and *Lachnospiraceae* in GBM patients, while levels of *Veillonellaceae*, *Rikenellaceae*, and *Prevotellaceae* were notably lower. Delving into genera statistics, we noted a substantial increase in the abundance of *Parasutterella*, *Escherichia-Shigella*, and *Bacteroides*, alongside significantly lower levels of *Ruminococcus 2*, *Faecalibacterium*, and *Prevotella_9* in the GBM group compared to the control group. Furthermore, when examining specific species, we found a significant increase in *Bacteroides vulgatus* and *Escherichia coli* in the GBM group. These observations collectively indicate a marked dysbiosis in the gut microbial composition of GBM patients. Additionally, the GBM group

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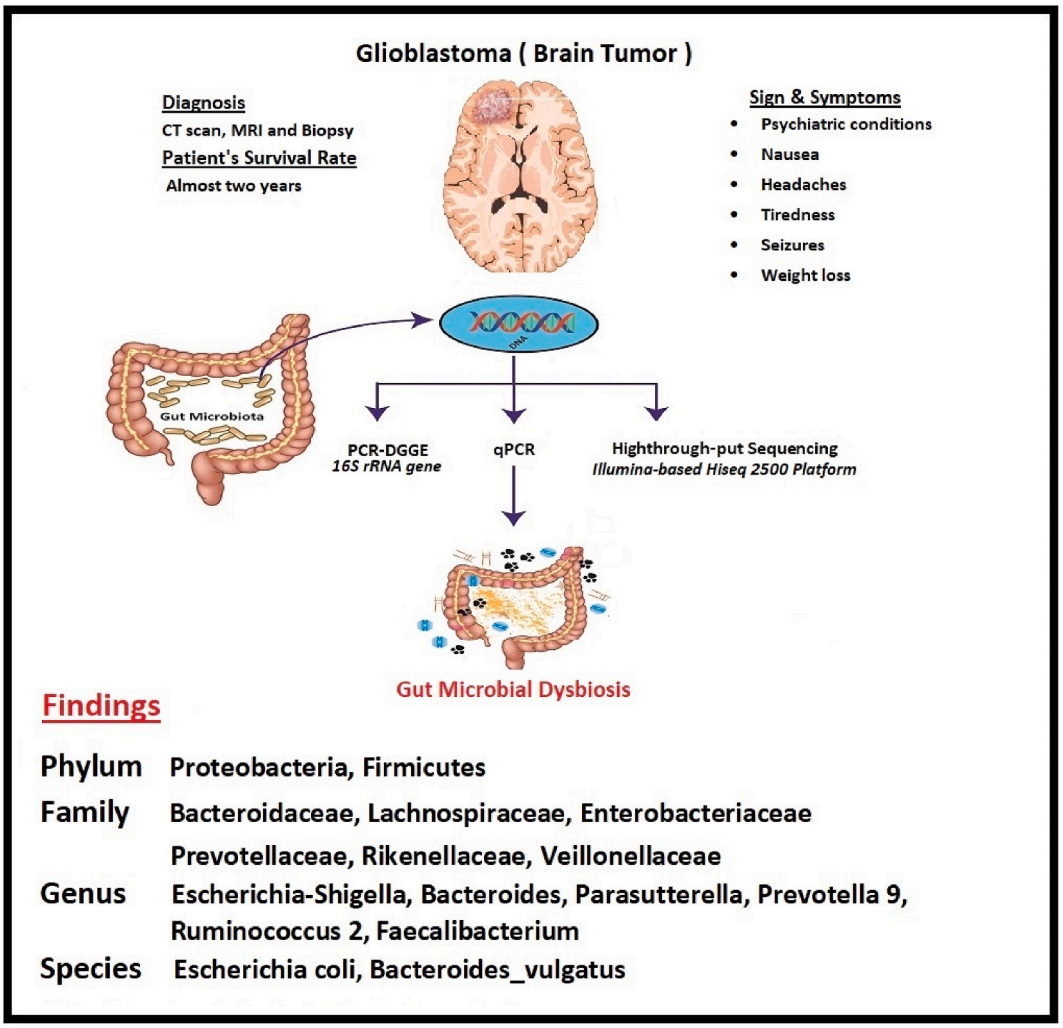
exhibited notably higher levels of alpha diversity when compared to the control group. This increase in diversity suggests a significant bacterial overgrowth in the gut of GBM patients in contrast to the controls. As a result, this research opens up potential avenues to gain a better understanding of the underlying mechanisms, pathways, and potential treatments for GBM, stemming from the significant implications of gut microbial dysbiosis in these patients.

1. Introduction

The human intestinal microbiota is a crucial factor in maintaining an individual's health, playing a significant role in normal body functions. The gut microbiota remains relatively stable over time but can be influenced by factors like disease, diet, and age [1]. Comprising over 100 trillion bacteria, the intestinal microbiota carries out metabolic, trophic, absorption, and nutritional functions [2,3] while safeguarding the body from pathogens [4]. An imbalance in the gut microbial composition can exacerbate various conditions, including asthma, colitis, Crohn's disease, inflammatory bowel disease, diabetes mellitus, obesity, thyroid cancer and smoking-related disorders [5–8]. Among these, brain tumours, particularly Glioblastoma (GBM), are highly malignant and often fatal, with a low survival rate. Approximately two-thirds of the adult population have been diagnosed with GBM cancer. It is the most lethal and aggressive malignancy, and the patient may die within two years of diagnosis [9,10]. It is the most common brain carcinoma reported in adults (Glioblastoma WHO grade IV). GBM has been treated through surgical removal and followed by chemotherapy and radiotherapy with poor response. It has the lowest survival rates among all cancers [11].

GBM, the most lethal brain malignancy, is challenging to treat due to the complex physiology and anatomy of the brain, leading to



Scheme 1. The whole study findings clearly show that GBM patients have gut bacterial dysbiosis.

high relapse rates and poor prognoses [12]. The causes of brain tumours are not well-defined, but mutations in suppressing genes like P53 and celiac disorder have been suggested as risk factors for carcinoma [13,14]. These tumours can manifest various psychiatric and neurological symptoms, making diagnosis challenging [15].

Globally, approximately 321,000 individuals are diagnosed with lethal brain malignancies each year, with a significant portion succumbing to the disease. Brain cancer represents a small percentage of all malignancies and tumour-related deaths but is highly dangerous, especially if not detected early [12]. Benign brain tumours can also disrupt brain function and daily life [12]. These tumours affect both adults and children, making early diagnosis a significant challenge due to nonspecific psychological symptoms [15].

The gut-brain axis, a communication pathway between the gut microbiota and the brain, may play a role in brain tumour development. Microbiota can impact immunity, cause inflammation, and influence various factors involved in tumorigenesis [16]. Research has documented the gut-brain axis's effects on immune function, treatment response, and inflammation. It encompasses immune cells, glands, the brain, gut, and intestinal microbes, all connected by the lymphatic system. Disruptions in this system might contribute to brain cancer [17]. Recent studies have highlighted how the gut microbiota influences brain function and behaviour through endocrine, immunological, and neuronal pathways [18]. Furthermore, the gut microbiome is vital for central nervous system development [19]. Research on lymphatic channels in the brain has also suggested a potential link between brain tumour inflammation and the immune system [20–22].

Hence, it is hypothesized that specific intestinal microbiota may influence the host's immune system and metabolism, potentially contributing to tumour development [2]. These interactions could modify and selectively suppress the immune system or affect metabolic functions, promoting cancer cell growth [1]. As a result, various intestinal microbial changes could facilitate the growth and establishment of brain tumours [16].

The current study aimed to assess the differences in gut microbial diversity and similarity between GBM patients and healthy controls. To achieve this, the researchers employed Metagenomic PCR-DGGE, Illumina-based Hiseq 2500 Highthrough-put sequencing, and Real-time PCR. Their findings revealed significant variations in the intestinal microbial ecology of GBM patients, with specific bacterial taxa showing differences in richness compared to healthy individuals. This research contributes to a better understanding of the overall intestinal microbial composition in GBM patients, as illustrated in Scheme 1.

2. Material and methods

2.1. Collection of faecal samples

Stool specimens were collected from 25 individuals diagnosed with Glioblastoma (GBM) using sterile containers, comprising 13 males and 12 females, all aged between 45 and 55. In a parallel manner, 15 samples from healthy control volunteers were also gathered in the same age range of GBM patients, including 8 males and 7 females for the current study. The GBM patients were diagnosed following the established protocols of the oncology department at the School of Medicine, Xian Jiaotong University, China. A structured questionnaire was designed to capture data related to dietary habits, lifestyle, medical history, body weight, gender, and age of the patients and healthy volunteers. In the current study, we enrolled those patients and healthy volunteers who did not use any prebiotics, probiotics or antibiotics before 30 days of sample collection The faecal specimens were collected approximately within 30 min of defecation and promptly transported in an ice-cold container to the laboratory. In the laboratory, these samples were stored at −80 °C till DNA extraction was done.

2.2. Extraction of DNA from all faecal samples

All fecal samples were thawed, and bacterial DNA was extracted by using the QIAGEN Stool kit (Germany) according to the manufacturer's protocol. Initially, the bead-beating procedure was performed at 5000 rpm for ½ min. DNA concentration was

Table 1
Primers deployed for PCR-DGGE and Real-time PCR.

Target bacteria		Primer Sequence (5^1–3^1)
PCR-DGGE Primer		
	341-F	CCTACGGGAGGCAGCAG
	534-R	ATT ACCGCGGCTGCTGG
	341FG	CGCCCGCCGCGCGCGGCGGCGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG
Real-Time PCR Primer		
Bifidobacterium(550 bp)	Bifid F	CTC CTGGAAACGGGTGG
	Bifi-R	GGTGTTCTTCCCGATATCTACA
Lactobacillus(250 bp)	Lact F	CTCAAAACTAAACAAAGTTTC
	Lact R	CTCAAAACT AAACAAAGTTTC
Bacteroides vulgatus(287bp)	BV- F	GCATCATGAGTCCGCATGTTC
	BV-R	TCCATACCCGACTTTATTCCTT
Escherichia coli(287bp)	*E.coli*-F	CATTGACGTTACCGCAGAAGAAGC
	E.coli-R	CTCTACGAGACTCAAGCTTGC
Clostridium leptum (239bp)	C.lep-F	GCACAAGCAGTGGAGT
	C.lep-R	CTTCCTCCGTTTTGTCAA

evaluated using NanoPhotometer TM (Germany) [23].

2.3. DNA amplification of PCR-DGGE

The bacterial DNA obtained from faecal samples served as the starting material for the PCR-DGGE (Polymerase Chain reaction-Denature Gradient Gel Electrophoreses) analysis. To specifically target the V3 region of the 16S rRNA gene, linking primers listed in Table 1 were utilized. The PCR reaction mixture, totalling 50 μl, facilitated the replication of the DNA sequence and followed a touchdown PCR program. In the initial step, genomic material was denatured at approximately 95 °C for 5 min. Additionally, 10 extra cycles were incorporated following the final extension step. The resulting gene-amplified bands were visualized using gel electrophoresis, employing a 1.5 % agarose gel. To confirm successful gene amplification, the gel containing the amplified genomic DNA was immersed in Ethidium bromide and then examined under UV light [24].

2.4. PCR-DGGE

The DGGE (Denature Gradient Gel Electrophoresis) analysis was conducted as per the mutational experimental system protocol (Bio-Rad, USA). Specifically, the amplified bacterial DNA was denatured on an 8 % acrylamide gel with a linear denaturant gradient ranging from 30 % to 65 %. This denaturation was performed in a chamber filled with a 1TAE buffer solution, maintained at a temperature of 60 °C. The DGGE gel was subjected to an electrical current of 90 V for 14 h. Microbial diversity in the DGGE gel profile was analyzed using Genetool Syngene software (version 4.3.14), which calculated the total band intensity. Additionally, the similarity index was determined using the Dice similarity coefficient. To establish the relationship between GBM patients and healthy participants, an unweighted pair group dendrogram was constructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm [25].

2.5. DGGE statistical analysis with band configuration

The Syngene software was utilized to assess the number of bands and their respective intensities in the DGGE gel. To explore the diversity of intestinal bacteria in the DGGE profiles, the Shannon Weaver diversity index (H^1) was employed for this purpose [26,27]. On the other hand, the UPGMA method, utilizing the band-based Dice similarity coefficient, was adopted for determining the similarity index and conducting cluster analysis of the DGGE gel profiles [28]. The Shannon-Weaver diversity and similarity indices were computed using Microsoft Excel (2013) and GraphPad Prism 7, with statistical significance established at $P < 0.05$. The dendrogram also visualized the relationships between the DNA samples, with Fig. 1B illustrating the similarities among the samples.

The Shannon Weaver diversity index (H') was computed by employing the below equation Shannon-Weaver index $(H^1) = -\sum_{i=1}^{s}(Pi)(\mathrm{In}Pi)$.

2.6. Excision of bands and sequencing

The distinct polyacrylamide DNA gel bands were carefully cut out using a sterile scalpel blade and transferred into 2 ml centrifuge tubes. Subsequently, the tubes were filled with 50 μl of pure distilled water and allowed to incubate at 37 °C for 30 min. After this

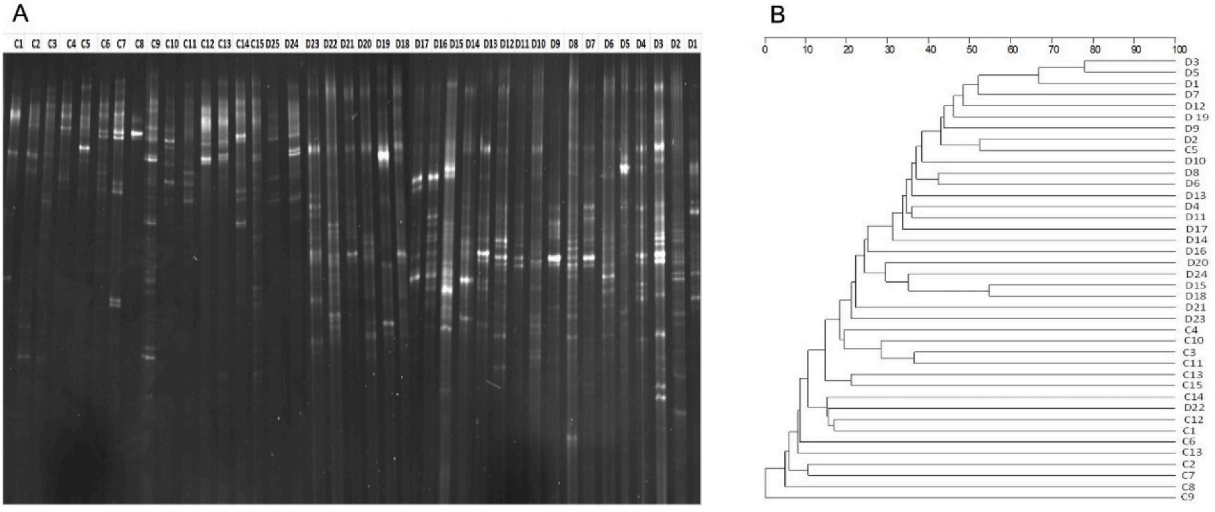


Fig. 1. (A) Constructing the DGGE profiling of GBM patients (D1-D8) and healthy controls (C1–C5). (B) Cluster analysis using UPGMA compared diseased (D1-D25) and control (C1–C15) groups. The letters D and C signify the GBM and control, respectively.

incubation period, 8 μl of the DNA water served as the template for the subsequent PCR procedure. In this second PCR run, the objective was to amplify the V3 region of the 16S rRNA gene, excluding the use of GC clamps [29]. The resulting PCR-amplified DNA sequence was then analyzed using the ABI-3500xL platform. The sequence data were further scrutinized through BLAST analysis to determine the genus or species.

2.7. Real-time PCR

The real-time PCR analysis was conducted following the protocol provided by Bio-RAD CFX96 (USA). In this procedure, a total volume of 20 μl for the PCR reaction mixture was prepared. This mixture included 1 μl each of the forward and reverse primers, 2 μl of DNA, 10 μl of Sybr green, and 6 μl of water, summing up to 20 μl for the PCR experiment] [30]. The primer sequences for the linkage are detailed in Table 1 [30]. To establish standard strains for gut bacteria, such as *Bifidobacterium* (CICC.6186), *Clostridium leptum* (YIT.6169), *Bacteroides vulgatus* (CICC.22938), NWS *Lactobacillus*, and *E. coli* (from our lab) were utilized. Real-time PCR was conducted three times, and the average of these runs was taken as the final result. The resulting data represented the average logarithm of the genetic PCR amplicon of faecal bacteria, approximating the copy number found in 1 g of faecal material.

2.8. Highthrough-put-2500 methodology and data computation

In this study, we employed the Illumina-based Hiseq2500 Highthrough-put sequencing paired-end technique. Data acquisition and alignment were carried out using QIIME [31] and FLASH [32] software. Highthrough-put sequencing was applied to 20 randomly selected faecal samples (10 from patients and 10 from control participants) targeting the V3+V4 region of the 16S rRNA gene with the primer pairs 806R (GGACTACHVGGGTWTCTAAT) and 515F (GTGCCAGCMGCCGCGGTAA) primers [33]. The UCLUST software [31] was utilized to categorize microbial DNA sequences into operational taxonomic units (OTUs), applying a 97 % threshold criterion. Taxonomic classification of each OTU was accomplished using the RDP classifier [34]. QIIME software was utilized to assess various diversity indices, including Chaol, ACE, Simpson, Good's coverage, and Shannon. Additionally, the OTU dataset was processed using QIIME integrated into the MEGAN4 software and mapped to the NCBI taxonomy database [35]. As a result, significant differences were observed in the taxonomy of gut microbial communities. To quantify dissimilarities, UniFrac distances were calculated using QIIME. Principal Component Analysis (PCA) and Non-metric multi-dimensional scaling (NMDS) were conducted to visualize the variations and similarities between patient and control groups, represented as paired distances [31]. These analyses were carried out using R software (Version 2.15.3) through the stat programs, WGCNA packages, and ggplot2 packages. To assess alpha diversity, statistical analyses were performed using Microsoft Excel (2013) and an unpaired *t*-test (nonparametric) with GraphPad Prism 10.

3. Results

3.1. Evaluation of DGGE profiles in GBM and control groups with statistical analysis

Denaturing Gradient Gel Electrophoresis (DGGE) was employed to analyze bacterial DNA from both GBM patients and healthy controls through PCR amplification. Fig. 1A shows the samples from GBM patients (labelled as D1-D25) and healthy controls (labelled as C1–C15). These DNA samples revealed differences in location, band count, and band intensity, indicating variations in the fingerprint of gut bacteria. The Syngene software was utilized to identify 376 bands within the 25 GBM samples, with an average band count of (10.72 ± 3.9) [36]. Similarly, 15 control samples displayed 108 DGGE bands on average (7.2 ± 2.88). Notably, a significant difference ($P < 0.04$) was observed between GBM patients and controls, suggesting that higher band counts in GBM patients indicate increased bacterial diversity and overgrowth. To further investigate the diversity of intestinal flora in both groups, the Shannon Weaver diversity index (H^1) was examined, resulting in values of (3.97 ± 0.53) for GBM patients and (3.64 ± 0.48) for healthy controls. These results displayed significant disparities ($P < 0.027$) in gut bacteria between GBM patients and healthy controls, with the Shannon Weaver diversity index values being higher for GBM patients, indicating notable intestinal microbial overgrowth. A Dice similarity coefficient (UPGMA) dendrogram was constructed to assess the similarity of samples within the DGGE gel profiles, as

Table 2
Gut microbial similarity and diversity of GBM cancer patients and healthy group.

Groups	Diversity		Similarity	
	The number of Bands A	Shannon Index B	Intra-similarity C	Inter-similarity D
Disease group	10.72 ± 3.90	3.97 ± 0.53	0.391 ± 0.172	0.263 ± 0.114
Control group	7.2 ± 2.88	3.64 ± 0.48	0.278 ± 0.190	
P. Value	0.041	0.027	0.037	/

Results that differ significantly (unpaired *t*-test), with $P < 0.05$.
a. DGGE bands count that each sample produces.
b. The Shannon diversity index (H^1) was computed using all DGGE band's relative intensities in each sample.
c. Comparing Dice similarity coefficients and DGGE band patterns between individuals within a given group.
d. Evaluating DGGE band patterns between individuals with GBM and control groups using Dice similarity coefficients.

depicted in Fig. 1B. The band intensity-based values of the Dice similarity coefficients for GBM and control groups yielded average similarity indices of (0.391 ± 0.172) and (0.278 ± 0.190), respectively ($P < 0.037$), as shown in Table 2. Statistical evaluation of the mean similarity index between GBM and control group samples revealed a value of (0.263 ± 0.114), signifying that it is less prevalent in the intergroup than in the intragroup. In essence, this indicates that the composition of gut bacteria differs between GBM patients and the control group in this study.

3.2. Evaluation of dominant bands sequencing findings

In the DGGE experiment, a total of 63 gel bands were excised from the DGGE gel profile. Specifically, 21 bands were retrieved from the healthy subjects (depicted as C1–C15 in Fig. 1A), while 42 bands were collected from the patient group (labelled as D1-D25) for quantitative examination (Fig. 3). The findings revealed the prominence of *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* as the prevailing taxa. The sequencing results of the bands cut from the DGGE gel profile are detailed in Table 3. Notably, within GBM, opportunistic bacteria were more prevalent compared to the control group, with percentages as follows: *Escherichia coli* (21 % vs. 5 %), *Bacteroides vulgates* (19 % vs. 10 %), *Enterobacter aerogenes* (7 % vs. 5 %), *Klebsiella oxytoca* (7 % vs. 5 %), *Clostridium botulinum* (7 % vs. 5 %), *Prevotella copri* (12 % vs. 10 %), and *Bacteroides uniformis* (7 % vs. 5 %). Conversely, GBM exhibited a lower percentage level of *Dialister succinatiphilus* (2 % vs. 10 %), *Ruminococcus flavefacien* (2 % vs. 10 %), *Bacillus pumilus* (2 % vs. 10 %), *Faecalibacterium prausnitzii* (2 % vs. 10 %), *Clostridium leptum* (2 % vs. 10 %), and *Alistipes putredinis* (7 % vs. 10 %) compared to the control group. Further details on the taxonomic identification of the DGGE band profile percentages can be found in Table 3. The sequencing results were analyzed using the NCBI BLAST database.

3.3. Gut bacterial quantification through real-time PCR

Real-time PCR was employed to measure the quantity of *Clostridium leptum*, *Bifidobacterium*, *Bacteroides vulgates*, *Escherichia coli*, and *Lactobacillus* bacteria. Notably, the copy numbers of *Bifidobacterium* and *Lactobacillus* (4.77 ± 0.75 compared to 5.48 ± 0.72) exhibited a significant decrease ($P < 0.012$) in GBM patients. Conversely, the copy number of *Bacteroides vulgatus* (6.59 ± 0.86 versus 5.77 ± 0.86) showed a significant increase ($P < 0.019$) in GBM patients compared to the control group. Similarly, the copy counts of *Escherichia coli* (4.60 ± 0.82 versus 3.89 ± 0.78) were significantly elevated ($P < 0.018$) in the GBM group. However, *Clostridium leptum* (2.15 ± 2.07 versus 3.98 ± 1.88) copy numbers in GBM patients were notably reduced ($P < 0.016$) compared to healthy subjects. You can refer to Table 4 for a visual representation of these findings.

3.4. Illumina-based Highthrough-put 2500 sequencing and data computation

Highthrough-put sequencing was carried out on a total of 20 individuals, including 10 patients with Glioblastoma (GBM) and 10 control subjects. This sequencing process yielded a substantial dataset consisting of 2,068,201 sequences. The sequences were generated by targeting the V3+V4 region of the 16S ribosomal RNA gene. Following a thorough quality control analysis, 1,592,745 Highthrough-put sequencing reads were retained (788,000 from control subjects and 804,745 from GBM patients), resulting in an average of 79,637.26 sequences per sample. Both the control and GBM groups exhibited the average taxon tag count, approximately 74,750.45 tags. However, the GBM group had 13,525 unique tags, while the control group had 11,207 unique tags, with an overall average of 1236.6 unique tags across all samples. In this study, the OUT numbers were assigned, with 55,66 for patients and healthy subjects, averaging 2844.0 and 27.22, respectively, per sample. There were a total of 24,732 unique Highthrough-put tags identified in

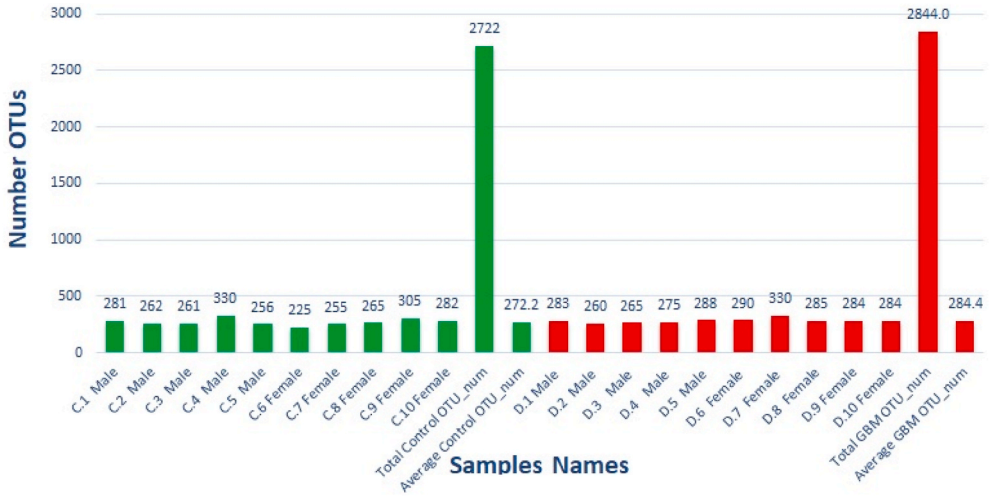


Fig. 2. Depicts the OTUs numbers of GBM and control groups sample-wise, average and total numbers.

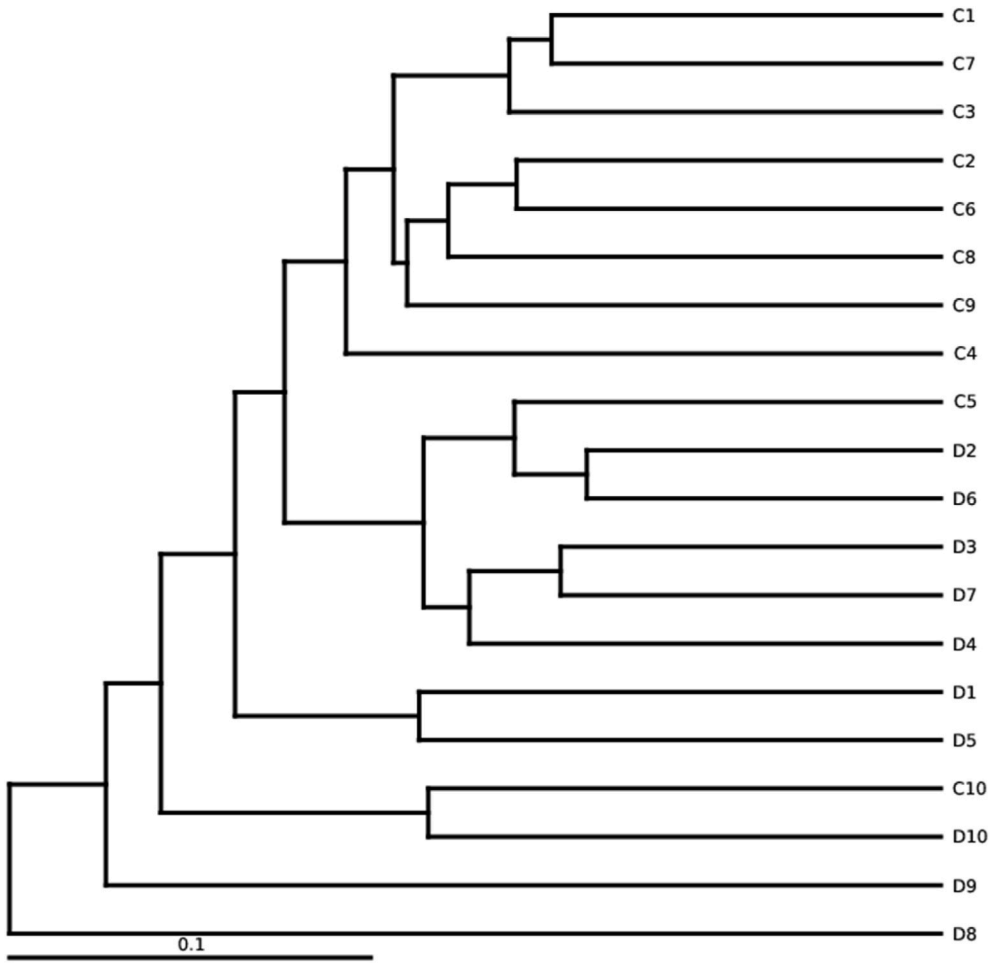


Fig. 3. Highthrough-put sequencing reveals the glioblastoma cancer sample diversity. The UPGMA algorithm is established on weighted UniFrac distances. The letters D and C represent GBM and control groups, respectively. PCA (principal component analysis) and NMDS (Non-metric dimensional scaling) based on OTU numbers were carried out, showing how the gut bacterial composition differed between the two groups, as depicted in Fig. 4A and B.

Table 3
Re-amplified PCR amplicons were sequenced, and the band's identities were verified by using the BLAST database.

Bacteria with Highest % Homology	Phylum	(C)Healthy Group n = 21	(D) Disease Group n = 42	C %	D %	Gene Bank Number
Dialister succinatiphilus (87)	*Firmicutes*	2	1	10	2	NZ_JH591188.1
Ruminococcus flavefaciens (92)	*Firmicutes*	2	1	10	2	NZ_JNKE01000007.1
Bacillus pumilus (91)	*Firmicutes*	2	1	10	2	NZ_CP012329.1
Faecalibacterium prausnitzii (96)	*Firmicutes*	2	1	10	2	NZ_CZBH01000014.1
Clostridium leptum (91)	*Firmicutes*	2	1	10	2	NZ_DS480348.1
Clostridium botulinum (88)	*Firmicutes*	1	3	5	7	NC_009495.1
Bacteroides vulgates (97)	*Bacteroidetes*	2	8	10	19	ATCC 8482
Bacteroides uniformis (97)	*Bacteroidetes*	1	3	5	7	NZ_JH724260
Prevotella copri (96)	*Bacteroidetes*	2	5	10	12	NZGG703855.1
Alistipes putredinis (90)	*Bacteroidetes*	2	3	10	7	NZ_DS499580.1
Escherichia coli (98)	*Proteobacteria*	1	9	5	21	NC_011750.1
Enterobacter aerogenes (92)	*Proteobacteria*	1	3	5	7	NC_015663.1
Klebsiella oxytoca (92)	*Proteobacteria*	1	3	5	7	NZ_CP011636.1

Table 4
Real-time PCR quantification results in different gut bacteria.

Bacteria	Healthy Subjects	Patients	P value
Bifidobacterium (10^3)	5.48 ± 0.72	4.77 ± 0.75	0.012
Bacteroides vulgatus(10^8)	5.77 ± 0.86	6.59 ± 0.86	0.011
Lactobacillus (10^3)	6.89 ± 0.92	5.88 ± 0.87	0.005
Clostridium leptum (10^5)	3.98 ± 1.88	2.15 ± 2.07	0.016
Escherichia coli (10^7)	3.89 ± 0.78	4.60 ± 0.82	0.019

The average estimate of the logarithm of fecal qPCR targeting genetic amplicon copy numbers present in 1 g of fecal material shown in the results, where ($P < 0.05$).

the disease and control groups, encompassing all the phylotypes examined in the current study. After removing the primer sequences, the average length of the gene sequences was found to be 420.15 base pairs.

3.5. Assessment of intestinal bacterial diversity

The study assessed the richness and diversity of gut microbial communities at a 97 % similarity level. When analyzing alpha diversity in GBM patients, including metrics like PD Tree, observed species, Chao1, Simpson, Shannon diversity, and the ACE algorithm, the results revealed significantly higher values compared to those in healthy control subjects. The details of the microbial diversity estimation in the GBM and control groups are provided in Table 5. Additionally, the GBM group exhibited notably higher levels of alpha diversity when compared to the control group. This increase in diversity suggests a significant bacterial overgrowth in the gut of GBM patients in contrast to the controls. The UniFracs distance analysis divided the gut microbial DNA samples of each group into two distinct clusters, as depicted in Fig. 2. This pattern resembled the PCR_DGGE pattern seen in both the GBM and control groups. The researchers also calculated beta diversity to compare the diversity of intestinal bacteria between the control and GBM groups (see) shown in Fig. 4A & B.

3.6. Intestinal bacteria at the phyla level

In the analysis conducted through Highthrough-put sequencing, a total of 16 phyla were detected. Among the top ten phyla, it was observed that *Proteobacteria* exhibited a notably higher abundance in GBM patients, while *Bacteroidetes* showed a marginally increased presence, but this increase was not statistically significant. Additionally, Fig. 5A visually represents the marked decrease in Phylum *Firmicutes* in the GBM group in comparison to the control group. The quantitative data for these top ten phyla displayed a significant disparity between the GBM and control groups, as detailed in Table 6.

3.7. Composition of intestinal microbes at the family level

Highthrough-put sequencing techniques were employed to produce a dataset encompassing 82 distinct family categories. The taxonomic distribution of *Lachnospiraceae*, *Enterobacteriaceae*, and *Bacteroidaceae* exhibited a notable increase in GBM patients when contrasted with the control group, as depicted in Fig. 5B. Conversely, the presence of *Veillonellaceae*, *Rikenellaceae*, and Prevotellaceae showed a significant decrease in GBM patients when compared to the control subjects. The family percentage data statistics demonstrate a substantial quantitative distinction between the GBM and control cohorts, as outlined in Table 6.

3.8. Genera-level intestinal bacterial distribution

The Highthrough-put sequencing data revealed the presence of 205 different genera in both the study and control groups. Notably, GBM patients showed significantly higher taxonomic abundance of *Parasutterella*, *Escherichia-Shigella*, and *Bacteroides* compared to the control group, as illustrated in Fig. 5C, which displays the top 10 sequenced genera. Conversely, *Prevotella 9*, *Ruminococcus 2*, and *Faecalibacterium* were notably reduced in GBM patients compared to the control group. Detailed statistical differences between the GBM and control groups can be found in Table 6.

GBM cancer has a distinct impact on gut microbiota, particularly affecting Phylum *Firmicutes*, *Proteobacteria*, *Fusobacteria*, and

Table 5
Intestinal microbial diversity and richness index calculated using Highthrough-put testing with 97 % similarity.

Group	Observed Species	OTUs	Shannon	Simpson	Chao1	ACE	PD Tree	Evenness
Patients	287.50	284.4	4.82	0.876	300.85	307.32	23.82	0.362
Control	249.15	272.2	4.05	0.780	264.47	271.00	20.54	0.311
P. Value	0.019	0.089	0.018	0.031	0.037	0.036	0.013	0.030

The table shows the average values for each group. The unpaired *t*-test t was computed to denote the results where ($P < 0.05$). The Shannon-based evenness was determined by using equation $E = H/\ln(S)$, in which H stands for the Shannon index of diversity while S indicates the entire amount of sequences in a specific group.

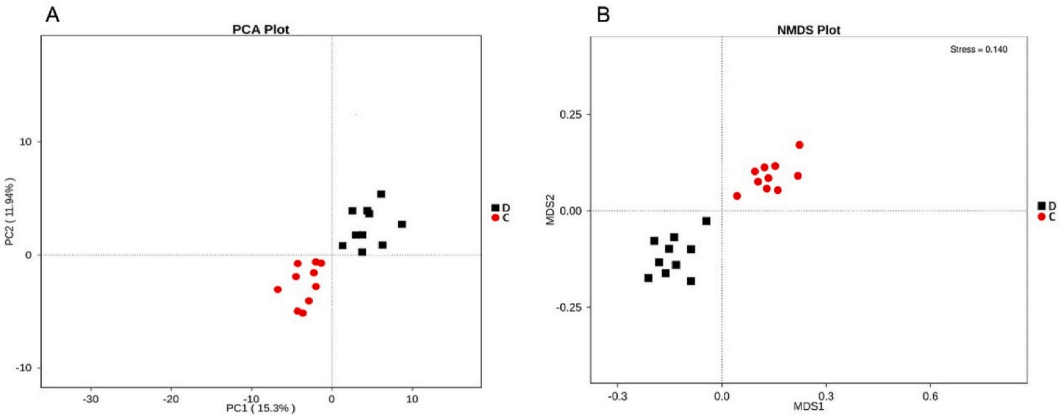


Fig. 4. Beta diversity between healthy and diseased subjects. (**A)** PCA plots generated by Highthrough-put sequencing of faecal bacterial DNA samples. (**B)** NMDS plot of bacterial DNA samples from experimental and control groups. Each faecal bacterial DNA sample from the study and control groups is represented by dots in the plot.

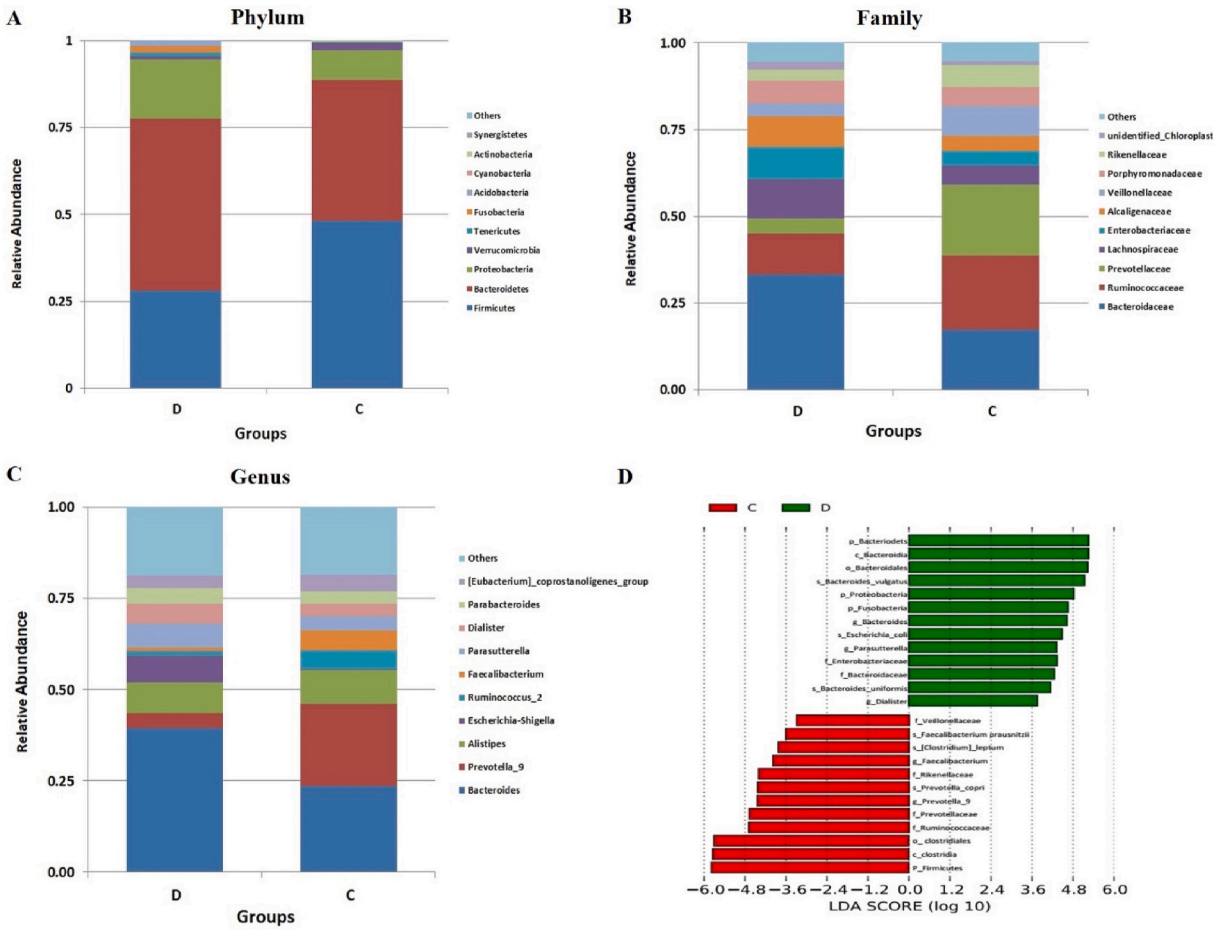


Fig. 5. (A) Highthrough-put sequencing findings show the phyla-level gut microbiome. The prevalence of the most common phyla in GBM patients is higher than in controls. **(B).** Findings from Highthrough-put sequencing of intestinal microbial ecology at the family level. The relative abundance of the most prevalent families in GBM patients and healthy controls. **(C).** Highthrough-put sequencing data revealed the genus levels of gut microbial assemblages. The comparative occurrence of the most common genera in patients with GBM versus healthy controls. **(D**) applying the linear discriminant analysis (LDA) scores distribution histogram, the most altered intestinal microbial taxon abundance was identified between GBM and control groups. The letters D and C represent the GBM and the control groups, respectively.

Table 6
Highthrough-put data reveal the gut bacterial taxa at the phylum, family, and genus levels.

Taxa	Mean D	Mean C	P. value	Q. value	% D	% C
Phylum						
Firmicutes	0.28156	0.48318	0.02437	0.66487	28.11	48.32
Bacteroidetes	0.49317	0.40233	0.24247	0.78317	49.31	40.23
Proteobacteria	0.16978	0.08572	0.03442	0.65223	16.98	8.57
Verrucomicrobia	0.01009	0.02209	0.12275	0.77439	1.01	2.21
Tenericutes	0.01057	0.00164	0.27843	0.83146	1.06	0.16
Fusobacteria	0.02050	0.00059	0.06812	0.77364	2.05	0.06
Acidobacteria	0.02004	0.00021	0.13514	0.88244	1.00	0.02
Cyanobacteria	0.00125	0.00010	0.65814	0.88418	0.13	0.01
Actinobacteria	0.00221	0.00389	0.32434	0.92240	0.22	0.39
Synergistetes	0.00103	0.00002	0.14357	0.68880	0.10	0.00
Others					0.02	0.02
Family						
Bacteroidaceae	0.33258	0.17419	0.02431	0.63447	33.25	17.41
Ruminococcaceae	0.11761	0.21259	0.10341	0.78653	11.76	21.26
Prevotellaceae	0.04244	0.20437	0.02533	0.54320	4.24	20.44
Lachnospiraceae	0.11379	0.05437	0.02177	0.63123	11.38	5.43
Enterobacteriaceae	0.09376	0.04288	0.04355	0.64495	9.38	4.29
Alcaligenaceae	0.08844	0.04461	0.03241	0.62157	8.84	4.46
Veillonellaceae	0.03732	0.08433	0.01121	0.62343	3.73	8.43
Porphyromonadaceae	0.06513	0.05431	0.67534	0.89758	6.51	5.43
Rikenellaceae	0.03175	0.06453	0.02409	0.58866	3.18	6.45
unidentified_Chloroplast	0.02232	0.01041	0.09415	0.78693	2.23	1.04
Others					5.48	5.34
Genus						
Bacteroides	0.39533	0.23643	0.03368	0.72458	39.53	23.64
Prevotella_9	0.04157	0.22444	0.03263	0.62182	4.16	22.44
Alistipes	0.08436	0.09344	0.75637	0.79738	8.44	9.34
Escherichia-Shigella	0.07134	0.00178	0.01430	0.62269	7.13	0.18
Ruminococcus_2	0.01354	0.05383	0.03421	0.57328	1.35	5.38
Faecalibacterium	0.00935	0.05387	0.04327	0.51149	0.93	5.38
Parasutterella	0.06529	0.03844	0.03372	0.62116	6.52	3.84
Dialister	0.05443	0.03453	0.09435	0.63127	5.44	3.45
Parabacteroides	0.04368	0.03378	0.65427	0.89451	4.37	3.37
[Eubacterium]_coprostanoligenes_group	0.03444	0.04558	0.58537	0.83373	3.44	4.56
Others					18.68	18.42

GBM and Control groups are denoted by D and C letters, respectively. $P < 0.05$.

Bacteroidetes, as well as various families and genera such as *Veillonellaceae*, *Enterobacteriaceae*, *Bacteroidaceae*, *Prevotellaceae*, *Lachnospiraceae*, and specific genera like *Escherichia-Shigella*, *Bacteroides*, *Parasutterella*, *Prevotella_9*, *Ruminococcus_2*, and *Faecalibacterium*. This disease has a significant influence on the composition of intestinal microorganisms, potentially impacting the overall health of GBM patients.

3.9. Species-level gut microbiota distribution

Table 7 illustrates the trends in species-level intestinal bacterial communities, revealing significant differences between individuals with GBM malignancy and healthy subjects. Notably, GBM patients showed elevated levels of *Escherichia coli* and *Bacteroides vulgatus* but reduced levels of *Clostridium leptum*, *Bifidobacterium longum*, *Ruminococcus gnavus*, *Lactobacillus* gasseri, and *Lactobacillus casei* compared to the control group.

The LDA values histogram, as seen in Fig. 5D, visually presents the taxonomic distinctions between GBM patients and control participants, highlighting the microbial taxa with noteworthy variations in abundance between the two groups. The length of the histogram bars indicates the magnitude and impact of these gut microbial taxa.

To ensure data accuracy, three molecular techniques were employed: metagenomic Highthrough-put sequencing, qPCR, and PCR-DGGE. The results obtained from these techniques are consistent and mutually support the findings of the gut microbial data, reinforcing their reliability.

4. Discussion

Recent findings have unveiled bidirectional communication pathways between the gut and the brain, facilitated by the intestinal microbiota [37]. Malfunctions in the gut-brain axis can exacerbate various central nervous system disorders, including chronic cerebral hypoperfusion, which can be attributed to abnormal production of short-chain fatty acids (SCFAs) metabolites [38]. Emerging research highlights the vital role of the intestinal microbiota in the development of various malignancies such as breast, lung, esophageal, and intestinal cancers [39,36]. The human microbiome also plays a crucial role in bolstering the host's immune system

Table 7
At the species level, Highthrough-put variations in intestinal microbial phylotypes between GBM (D) and controls (C).

Taxa	D	C	P value	Q value
Escherichia_coli	0.087570	0.004743	0.011375	0.524422
Bacteroides_vulgatus	0.077271	0.001147	0.030141	0.513325
Haemophilus_parainfluenzae	0.032022	0.012007	0.143846	0.781262
Bacteroides_uniformis;	0.047558	0.021524	0.081389	0.725367
Proteus_mirabilis;	0.034724	0.021127	0.111029	0.794457
Prevotella_copri;	0.009477	0.011654	0.081349	0.735467
[Clostridium]_leptum;	0.001126	0.099833	0.033441	0.896800
Coprobacillus_cateniformis;	0.008416	0.00017	0.425574	0.702323
Bacteroides_fragilis	0.013456	0.005174	0.076723	0.645337
Akkermansia_muciniphila	0.000356	0.007624	0.025874	0.426573
[Ruminococcus]_gnavus;	0.006807	0.000486	0.068022	0.563348
Faecalibacterium prausnitzii	0.001285	0.084638	0.011418	0.567112
Bifidobacterium_kashiwanohense	0.019487	0.021242	0.084486	0.612162
Campylobacter_concisus	0.021180	0.004875	0.127606	0.723423
Bifidobacterium_longum;	0.001645	0.009418	0.025854	0.674353
[Ruminococcus]_gnavus;	0.001245	0.009889	0.020559	0.693323
Phascolarctobacterium_faecium;	0.004485	0.003819	0.151518	0.712223
Sutterella_wadsworthensis	0.002496	0.001672	0.788125	0.237637
Ruminococcus_bicirculans	0.003461	0.004153	0.118783	0.748188
Bifidobacterium_kashiwanohense	0.005149	0.001014	0.05856	0.601323
Alistipes_onderdonkii	0.004145	0.003128	0.210899	0.814323
Dialister_pneumosintes;	0.009985	0.003805	0.093284	0.743519
Alistipes_shahii	0.004680	0.003107	0.147942	0.772519
Proteus_mirabilis;	0.000274	0.00361	0.024699	0.554448
Lactobacillus_gasseri;	0.002966	0.052533	0.022258	0.673843
Lactobacillus_casei;	0.001042	0.009879	0.032627	0.502323
Flavonifractor_plautii	0.000930	0.00219	0.852148	0.929725
Lactobacillus_fermentum;	0.000879	0.01360	0.001985	0.481098
Pseudomonas_sp._108Z1;	0.001892	0.000685	0.243157	0.789725

$P < 0.05$.

through trophic mechanisms [40].

The myriad of microbiomes within the intestinal flora has garnered significant attention recently due to their far-reaching impacts on various aspects of human physiology and pathophysiology. Given the pivotal role of gut microbial composition in health and disease, innovative microbiome-based therapeutic approaches, including prebiotics and probiotics, have been applied to enhance clinical outcomes in the treatment of different malignancies [41]. The interaction within the gut-brain axis is mediated through immune, endocrine, and neural pathways [42]. Additionally, it has been observed that metabolites of short-chain fatty acids produced by intestinal bacteria can substantially influence the function of the thyroid gland, indicating the existence of a gut-thyroid axis and gut microbial dysbiosis in autoimmune thyroid disorders like Hashimoto's thyroiditis and Graves' disease [43–45].

Assessing the alpha diversities, including Chao1, nonparametric Simpson, ACE, observed species, and Shannon, revealed significantly higher values in GBM patients compared to controls [46]. Similarly, the diversity indices of gut microbiota obtained through both Highthrough-put sequencing and DGGE were elevated in GBM patients. Consequently, these heightened diversity levels indicate a substantial overgrowth of gut microbiota in GBM patients when compared to controls. Importantly, these findings align with previous research in cutaneous, vaginal, and gastrointestinal systems [47,48], supporting the notion of increased diversity in these microbial ecosystems.

In the GBM group, the statistics of gut microbial similarity index of DGGE banding pattern was significantly higher in intra-groups. This suggests that GBM patients exhibit abnormal bacterial overgrowth in their gut. The results of the comparative similarity index revealed that it was lower in the intergroup than in the intragroup, which is aligned with the previously reported literature [49]. The statistical analysis shows a significant qualitative and quantitative alteration between GBM and control groups.

In the patient group, there was a significantly higher prevalence of the *Proteobacteria* phylum, while *Firmicutes* were less abundant compared to the control group. This finding corresponds to previous research on changes in gut microbiota associated with chronic pain and vitamin D deficiency within the endocannabinoid tone system [50]. The existing meta-analysis on gut bacteria related to inflammatory bowel disease (IBD) and obesity has shown that the *Bacteroidetes* to *Firmicutes* ratio in the intestinal microbiota of obese and lean individuals is not a constant feature [51]. The current study revealed a significantly increased prevalence of *Proteobacteria* in the GBM group, consistent with previous research suggesting that *Proteobacteria* may be a risk factor for post-cholecystectomy syndrome-related stomachache [52]. It has also been reported that the microenvironment of GBM tissue is predominantly enriched with Phylum *Proteobacteria*. As the brain is a sterile organ, numerous studies have been reported on non-etiological and pathological human brain specimens which unveil the high abundance of phylum *Proteobacteria* [53]. It's worth noting that *Proteobacteria* has also been linked to inflammatory bowel disease, asthma, metabolic abnormalities, and obstructive pulmonary disease [54].

At the family level, there was a significant rise in *Bacteroidaceae*, *Lachnospiraceae*, and *Enterobacteriaceae* in the GBM group, in line with previous research [55,56]. Pathogens belonging to the *Enterobacteriaceae* family have been associated with one-third of reported cases of nosocomial pneumonia [57]. The current findings indicated a significant decrease in the *Bacteroidaceae* family, consistent with

research on Sjogren's Syndrome patients and their gut ecology [58]. The study also revealed that the *Prevotellaceae, Rikenellaceae*, and *Veillonellaceae* families were significantly less abundant in the GBM group, which is consistent with research on diabetes and dietary supplementation [59]. The gut microbiota in particular phylum *Proteobacteria* and family *Enterobacteriaceae* may have an important role in the progression of glioma by regulating of metabolic and immune microenvironment [60]. In most cancers which are located at different locations, Enterobacteriaceae is the most prevalent taxa in the microenvironment of tumours [61]. Immune suppression has been observed at the time brain neoplasia occurs which provides a suitable environment for tumour development [62].

In the current study, the family *Veillonellaceae* shows a significantly lowered trend in the GBM patients as compared to healthy subjects. Moreover, *Veillonellaceae* is a beneficial commensal that plays a role in promoting the development of immune T regulatory cells and is closely associated with *Clostridium* [63]. Gut bacteria produce metabolites and other substances that can enter the bloodstream, thereby influencing the immune response significantly [64].

Moreover, the GBM group exhibited a significant decrease in the genera *Prevotella 9*, *Ruminococcus 2*, and *Faecalibacterium*, as noted in previous studies [56,65,66]. In contrast, the study group displayed a notably higher abundance of *Escherichia-Shigella*, *Bacteroides*, *Dialister,* and *Parasutterella* genera, aligning with existing research on gut bacteria and autism issues [67]. The presence of *Parasutterella*, particularly, was significantly elevated in the gut of GBM patients, a genus previously associated with conditions like Crohn's disease in the ileum submucosa and hypertriglyceridemia with acute necrotic pancreatitis in rodents [68,69]. It's worth noting that *Faecalibacterium*, specifically *Faecalibacterium prausnitzii,* has been suggested to have immune-boosting and anti-inflammatory properties; however, it was markedly reduced in GBM patients compared to the control group [70]. Reduced levels of *F. prausnitzii* have also been linked with inflammation in various diseases, including inflammatory bowel disease [71].

The current investigation uncovered a diminished abundance of the Prevotella genus in GBM patients, which is consistent with prior literature suggesting that *Prevotella* dominance in the gut bacterial community is beneficial for host metabolism [72]. *Prevotella* is believed to be a helpful gut microbe involved in the digestion of plant-based foods [73,74]. Additionally, GBM patients exhibited significantly higher levels of *Escherichia-Shigella* compared to healthy subjects. *Escherichia-Shigella* is known for releasing the Shiga toxin, which can lead to gastrointestinal inflammation, hemorrhagic colitis, thrombocytopenia, hemolytic uremic syndrome (HUS), septicemia, and urinary tract issues [75]. The genus *Escherichia-Shigella*, with a notable increase in the species *Escherichia coli*, may be a crucial factor in GBM development [76]. *Escherichia coli*, a ubiquitous bacterium, is notorious for causing common ailments such as foodborne diseases and urinary tract infections (UTIs) [76]. The exact invasion mechanism of gut microbe in the brain tumour through the blood-brain barrier is still unclear. It is assumed that circulating bacteria could enter the tumour environment from the bloodstream through leaky blood vessels [77].

The present study identified significant variations in microbial abundance at the phylum, family, genus, and species levels, indicating a distinct alteration of gut microbiome between GBM patients and the control group. This analysis highlights a considerable shift in gut microbial composition in GBM patients compared to their healthy counterparts [78]. These findings support the idea that GBM malignancy may influence gut physiology, ultimately leading to changes in the gut bacterial composition. These alterations in the gut microbiota may exacerbate the disease condition [79], leading to significant shifts in the microbial equilibrium. Additionally, the gut plays a role in the production of neuromodulators and central nervous system-related neurotransmitters. The intestinal microbiota is also capable of generating short-chain fatty acid metabolites (SCFAs), which help to prevent the production of proinflammatory cytokines [80].

Clinical manifestations of Glioblastoma (GBM) often include fatigue, nausea, headaches, and weight loss, which are typically observed before its diagnosis. Consequently, there is a hypothesis that GBM may have an impact on the gut's bacterial composition, particularly within Phylum, *Firmicutes*, *Proteobacteria*, families like *Lachnospiraceae*, *Bacteroidaceae*, *Veillonellaceae*, *Prevotellaceae*, and *Enterobacteriaceae*, as well as genera including *Dialister*, *Escherichia-Shigella*, *Parasutterella*, *Prevotella_9*, *Bacteroides*, *Ruminococcus_2*, *Faecalibacterium*, and specific species like *Bacteroides vulgates* and *Escherichia coli*, *Clostridium_leptum*, *Bifidobacterium_longum*, *Ruminococcus_gnavus*, *Lactobacillus_gasseri*, *Lactobacillus_casei*. This suggests that GBM may induce significant disruptions in the gut microbiota. The investigation into alterations in gut microbial profiles in GBM patients compared to healthy individuals was particularly intriguing since there was no established and direct connection between GBM and gut bacteria. Consequently, the study's findings unveiled a notable divergence in gut bacterial composition between GBM patients and their healthy counterparts. As a result, it is proposed that modifications in the gut microbiota can have an impact on the overall health of the host [81].

To gauge the changes in intestinal bacteria, real-time PCR was conducted [82]. The data revealed a marked reduction in *Lactobacillus* within the GBM group, aligning with prior research findings [83]. Probiotics, which are commonly associated with the *Lactobacillus* genera, are frequently used as dietary supplements due to their positive effects on the host's physiological well-being [84]. Moreover, it's worth noting that *Lactobacillus* is less prevalent in certain disease conditions, such as colorectal cancer [85]. *Lactobacillus* plays a crucial role in maintaining selenium levels within human intestinal cells. Deficiencies in selenium have been reported in patients with brain malignancies, thus highlighting its significance in GBM treatment [86]. Additionally, selenium is essential for the production of thyroid hormones, which serve to safeguard the thyroid gland against oxidative damage [87,88]. Several strains of *Lactobacillus* found in the human gut exhibit strong antibacterial properties against uropathogenic infections [89].

Clostridium leptum has been associated with maintaining gut mucosa balance in patients with rheumatoid arthritis by promoting the growth of regulatory T cells, leading to increased levels of growth factor-β and the activation of transcription factor Foxp3+ [90]. In our research, we observed a noteworthy decrease in *Clostridium leptum* levels among GBM patients when compared to the control group [91]. In contrast, *Bacteroides vulgatus* and *E. coli* levels were significantly higher in the GBM group, consistent with previous studies on intestinal bacterial and viral diarrhoea [92,93]. *Bacteroides vulgatus* is quantitatively the most prevalent species within the *Bacteroides* genus in the human gut microbiota. It engages in a complex, host-beneficial relationship and aids in preventing the colonization of the gut by other microbes [94,95].

The data was generated by using Illumina-based Hiseq2500 Highthrough-put sequencing, Real-Time PCR, and PCR-DGGE, which are suitable methods for depicting and characterizing gut microbiota. It is important to note that PCR-DGGE is a semi-quantitative technique, and its banding profile analysis may not provide a comprehensive understanding of the specific microbial taxa within the gut ecosystem [27]. In contrast, Highthrough-put sequencing represents the most advanced, sensitive, and reliable approach for investigating and analyzing gut microbial ecology [81]. Additionally, PCR-DGGE can be employed as a cost-effective and less time-consuming routine laboratory method for monitoring changes in gut microbiota.

5. Conclusion

GBM patients exhibited a marked change in their gut bacterial composition compared to individuals without the condition. Specifically, there was a significant shift in the abundance of various bacterial taxa within the gut microbiome when comparing GBM patients to healthy subjects. The analysis of gut bacterial diversity revealed a notably higher level in GBM patients compared to normal individuals, indicating an overgrowth of gut microbes and an imbalance in the GBM group. Consequently, additional multicenter research is needed to gain a deeper understanding of the underlying pathways and mechanisms that lead to intestinal bacterial dysbiosis in GBM patients.

Ethical approval

Current study was started in accordance with the guidelines of the Research Ethics Review Committee of School of Medicine, Xi'an Jiaotong University China. Approval No. JIOTONG MED/563. Research was also conducted according to the rules of World Medical Association and Declaration of Helsinki.

Informed consent

Written informed consent were also obtained from both patients and healthy volunteers participated in this study.

Data availability statement

The data sets generated for this study can be found in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI), Accession number. SUB14318512.

CRediT authorship contribution statement

Hafiz Muhammad Ishaq: Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Riffat Yasin:** Writing – review & editing, Validation, Formal analysis. **Imran Shair Mohammad:** Writing – review & editing, Software, Formal analysis. **Yang Fan:** Writing – review & editing, Software, Formal analysis. **Huan Li:** Writing – review & editing, Software, Investigation, Formal analysis. **Muhammad Shahzad:** Writing – review & editing, Software, Formal analysis. **Jiru Xu:** Writing – review & editing, Validation, Supervision, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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