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Characteristics of gastric cancer gut microbiome according to tumor stage and age segmentation

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Abstract

With the development of 16S rRNA technology, gut microbiome evaluation has been performed in many diseases, including gastrointestinal tumors. Among these cancers, gastric cancer (GC) exhibits high morbidity and mortality and has been extensively studied in its pathogenesis and diagnosis techniques. The current researches have proved that the gut microbiome may have the potential to distinguish GC patients from healthy patients. However, the change of the gut microbiome according to tumor node metastasis classification (TNM) has not been clarified. Besides, the characteristics of gut microbiome in GC patients and their ages of onset are also ambiguous. To address the above shortcomings, we investigated 226 fecal samples and divided them according to their tumor stage and onset age. The findings revealed that surgery and tumor stage can change the characteristic of GC patients' gut microbiota. In specific, the effect of surgery on early gastric cancer (EGC) was greater than that on advanced gastric cancer (AGC), and the comparison of postoperative microflora with healthy people indicated that EGC has more differential bacteria than AGC. Besides, we found that *Collinsella*, *Blautia*, *Anaerostipes*, *Dorea*, and *Lachnospiraceae_ND3007_group* expressed differently between EGC and AGC. More importantly, it is the first time revealed that the composition of gut microbiota in GC is different between different onset ages.

Key points

•Gut microbiota of gastric cancer (GC) patients are either highly associated with TNM stage and surgery or not. It shows surgery has more significant changes in early gastric cancer (EGC) than advanced gastric cancer (AGC).
•There existed specific gut microbiota between EGC and AGC which may have potential to distinguish the early or advanced GC.
•Onset age of GC may influence the gut microbiota: the composition of gut microbiota of early-onset gastric cancer (EOGC) and late-onset gastric cancer (LOGC) is significantly different.

Keywords Gastric cancer · 16S rRNA · Gut microbiota · Tumor stage · Tumor biomarkers · Onset age

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Introduction

Gastric cancer (GC), a high-risk malignancy of the digestive tract, is ranked sixth for incidence and third for the number of deaths among cancers (Wroblewski and Peek 2016). It can be divided into gastric adenocarcinoma and gastroesophageal junction adenocarcinoma based on anatomy and diffuse type (undifferentiated type) and intestinal type (well-differentiated type) based on histology (Van Cutsem et al. 2016). The incidence of gastric cancer is believed to rise with age, reaching a plateau between 55 and 80 years of age, and showing variation by gender to a certain extent; its frequency is 2–3 times of that in males than females (Thrift and El-Serag 2020).

The human intestine is colonized by approximately 10^{14} microorganisms (Dicks et al. 2018). The gut microbiome has been heavily studied and determined to show an association with GC (Qi et al. 2019; Liang et al. 2019). In our previous studies, we collected and analyzed gut microbiota from GC patients and found that certain gut microbiota could distinguish GC patients from healthy individuals (Zhang et al. 2021; Chen et al. 2021). Besides, specific members of microbiota were linked to therapy, such as the eradication of *Helicobacter pylori* can decrease GC and GC-related precancerous lesion incidence (Chiang et al. 2020), although it does not change the high fatality rate. The main reason for this lower prognosis is the low efficacy of tools used in early diagnosis. Recently, several screening approaches have been proposed, including indirect atrophy detection by measuring pepsinogen in the circulation; however, none of them have been implemented in the clinic. Therefore, more data are required to justify any practical applications (Pasechnikov 2014).

TNM is one of the most widely used indicators for tumor status assessment and prognosis prediction based on the depth of tumor invasion (T), regional lymph nodes (N), and distant metastases (M) (Edge and Compton 2010). According to the T category, T1 tumors can be seen as early gastric cancer (EGC) regardless of the lymph node or metastasis stage (Japanese Gastric Cancer Association 2011). The definition of advanced gastric cancer (AGC) is still unclear, while most articles define T3/T4 as AGC (Blackshaw et al. 2003; Tuttle et al. 2016). A survey of long-term survival for EGC patients after surgery in Japan showed that the overall 5- and 10-year survival rates were 84% and 64%, respectively. The disease specific 5- and 10-year survival rates were both 99% (Uedo et al. 2006), while the 5-year survival rate in AGC was only 5–20% (Japanese Gastric Cancer Association 2011), indicating the significance of early diagnosis and intervention in GC.

Besides, the frequency of early-onset gastric cancer (EOGC) increases significantly among the young population worldwide (He et al. 2021). EOGC patients present low survival rates, poor prognosis, rapid disease progression, a low degree of differentiation (signet–ring cell tumors are common), and rapid lymph node and distant metastasis, compared to older counterparts (Bergquist et al. 2019). The pathogenesis and mechanism of EOGC is believed to be considerably different from traditional GC (Ma et al. 2021) and late-onset gastric cancer (LOGC) (Zhao and Hu 2020).

Our study explored the relationship between the gut microbiota of GC patients and their TNM stage. Besides, we investigated the differences of gut microbiota among different onset ages. We hypothesized the existence of several gut microbiota among different stages of GC, which may benefit early GC diagnosis or screening.

Material and methods

Study population

In total, 226 individuals including 196 GC patients and 30 healthy individuals were recruited, and their fecal samples were collected at Zhejiang Provincial People's Hospital from April 2018 to December 2021. Among them, part of the fecal data came from our previous studies (Zhang et al. 2021; Chen et al. 2022). All of the GC patients were diagnosed by pathological examination and gastroscopy. Patients with the following diseases were excluded: complicated blood disease; immune diseases; combination with other tumors; kidney disease, acute/chronic infection, etc.; previous history of gastric cancer; treatment with antibiotics within 1 month. Regarding preoperative medication for GC patients, antibiotics are routinely used 2 h before the operation of GC. Acid-inhibiting and hemostatic drugs will be used for patients with severe gastric ulcers and bleeding, and nutritional support will be used for patients with anemia. In order to avoid the influence of medication, we collected samples from the patients when they were just admitted to the hospital and had not received treatment. Healthy individuals were recruited by the Department of Health Examination Center of our hospital. Participants' clinical data were collected by reviewing the medical records.

According to the purpose of this research, we preliminarily classified the collected samples by tumor stage and age as main factors. After the initial classification of tumor stage and age, the samples were further divided based on whether the patient had surgery or not.

Thus, to explore the connection between tumor stage and GC patients' gut microbiota, we divided patients into 5 groups, including early gastric cancer without surgery group (EGCNS, $n = 35$), early gastric cancer with surgery group (EGCS, $n = 29$), advanced gastric cancer without surgery

group (AGCNS, $n=52$), advanced gastric cancer with surgery group (AGCS, $n=51$), and healthy control group (HC, $n=30$).

To verify the relationship between EOGC and LOGC, we divided patients into 5 groups, including early-onset gastric cancer without surgery group (EOGCNS, $n=30$), early-onset gastric cancer with surgery group (EOGCS, $n=25$), late-onset gastric cancer without surgery group (LOGCNS, $n=70$), late-onset gastric cancer with surgery group (LOGCS, $n=71$), and healthy control group (HC, $n=30$). At present, the age limit of EOGC and LOGC is unclear. Some scholars believe that 45 years of age can be used as a dividing value, while some scholars use 60 as the cutoff age between EOGC and LOGC (Mun et al. 2019; Bergquist et al. 2019; MacArthur et al. 2021). After referring to the above articles, we chose to use 55 years as the cutoff age in this work.

Patients' information includes gender, age, TNM stage, surgery or not (Table 1). Fecal samples were collected from every individual and frozen at − 80 ℃ for 16S rRNA sequence.

This study was approved by the Ethics Committee of Zhejiang Provincial People's Hospital (No. 2022QT010). All samples were anonymously given.

DNA extraction and PCR amplification

The Guhe Stool Mag DNA Kit (Guhe Info Technology Co., Ltd, Zhejiang, China) was used to extract microbial DNA from fecal samples. All procedures were performed as per the manufacturer's instructions. The concentration and purity of extracted DNA was tested by a NanoDrop 2000 UV–Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used as primer to amplify the V4 regions of 16S rRNA. A barcode was synthesized into the sequence using a specific 7-bp sequence. The PCR cycling conditions were as follows: pre-denaturation at 98 ℃ for 30 s, followed by 25 cycles consisting of denaturation at 98 ℃ for 15 s, annealing at 58 ℃ for 15 s, and extension at 72 ℃ for 15 s, with a final extension of 1 min at 72 ℃. The PCR amplicons were purified and quantified by using Agencourt AMPure XP Beads (Beckman Coulter, Indianapolis, IN) and the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). The Illumina Novaseq 6000 platform with 2 × 150 bp (Illumina, San Diego, CA, USA) was employed to pool after quantification at Guhe Info Technology Co., Ltd (Hangzhou, China).

Table 1 Clinical features of participants enrolled in this study

Characteristics	Health	Gastric cancer
Sex	30	196
Male	20 (67%)	147 (75%)
Female	10(33%)	49 (25%)
Age (years)	54.67 ± 2.18	62.46 ± 0.89
≤ 55	15 (50%)	55 (28%)
> 55	15 (50%)	141 (72%)
Tumor stage		
I	–	64 (33%)
II	–	11 (6%)
III, IV	–	109 (56%)
Unknown	–	12 (6%)
Surgery		
Yes	–	96 (49%)
No	–	100 (51%)

Measurement data are expressed as mean ± SEM, and quantitative data are expressed as number (percentage, %)

Sequencing and bioinformatics analysis

The raw tag data were obtained by splicing the reads of each sample using Vsearch Version 2.4.4 (https://github.com/torognes/vsearch) after truncating the barcode and primer sequences. At the same time, quality control, the filtering of sequence quality, and the removal of chimera sequences were performed to obtain effective tags. The criteria for screening low-quality sequences were sequence length < 150 bp, average Phred scores of < 20, the sequence containing ambiguous bases, and the single nucleotide repeat sequence containing > 8 bp.

After effective tags were obtained, the tags would be clustered into operational taxonomic units (OTUs), and an OTU was commonly defined based on the diversity of 16S rRNA (Auer et al. 2017). Usually, 97% similarity can be accepted as an OTU (Gill et al. 2006). The QIIME Version 1.8.0 (http://qiime.org/) toolkit was used to annotate OTU and database using SILVA128 database (http://www.arb-silva.de), and the output files were statistically analyzed using STAMP package Version 2.1.3 (http://kiwi.cs.dal.ca/Software/STAMP).

Sequence data analysis was primarily performed using QIIME and the R package Version 3.2.0 (https://www.r-project.org/). The ACE metric (abundance-based coverage estimator) and Simpson index were calculated using the OTU table in QIIME. Besides beta diversity, visualized via principal coordinate analysis (PCoA), principal component analysis (PCA), random forest, and linear discriminant analysis (LDA) effect size (LEfSe) were applied to analyze the consistency and difference among samples. The R package and MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/) were used to perform the data visualization.

Results

EGC shows significant difference compared to AGC in gut microbiota before and after surgery

To explore the influence of surgery on different TNM stages, we compared the gut microbiota between the ECGNS, ECGS, AGCNS, and AGCS groups. Two indicators, Simpson and ACE (Fig. 1 a and b), were used to evaluate the diversity and richness of the gut microbiome in these groups. The results showed no clear difference in diversity among these groups ($P=0.063$), but there was a statistically significant difference in abundance ($P=0.037$). Principal component analysis (PCoA) (Fig. 1c) based on the genus level was used to compare the composition of gut microbiota among these groups. The analysis revealed a clear difference in the composition of gut microflora among the groups ($P<0.003$).

To further understand the composition of the examined groups, we analyzed the relative abundance of microbiota at the family and genus levels (Supplemental Fig. S1a and b), which showed a varied distribution among different groups. Therefore, we compared the preoperative and postoperative gut microbiota changes in EGC and AGC. In the EGCS and EGNS groups, the Simpson and ACE index (Fig. 2 a and b) revealed that surgery did not change the diversity and abundance of gut microbiota in GC patients ($P=0.17$ and $P=0.74$, respectively). PCoA (Fig. 2c) based on the genus level illustrated that the composition of gut microbiota in EGCS and EGCNS was different ($P<0.002$). In the AGCS and AGCNS groups, the Simpson and ACE index (Fig. 3 a and b) revealed that the diversity and the abundance of gut microbiota changed significantly after surgery ($P=0.037$, $P=0.0196$). However, PCoA (Fig. 3c) based on the genus level did not show difference in the composition of gut microbiota in AGCS and AGCNS ($P=0.32$).

In order to explain the differences in the composition of gut microbiota before and after surgery in different stages of TNM, we used LEfSe to analyze and compare the significantly different microbiota present in ECGS and EGCNS (Supplemental Fig. S2) and AGCS and AGCNS (Supplemental Fig. S3). We found that *Enterococcus*, *Parabacteroides*, *Hungatella*, *Clostridium_innocuum_group*, *Eggerthella*, *Eisenbergiella*, *Anaerotruncus*, *TM7x*, *Anaeroglobus*, *Roseburia*, *Lachnospiraceae_NK4A136*, *Odoribacter*, and *Lachnospira* exhibited different abundances between EGCS and EGCNS ($P<0.01$). Moreover, 11 gut microbiota at the genus level between AGCS and AGCNS were significantly different, including *Enterococcus*, *Holdemania*, *Lachnospiraceae_NK4A136_group*, *Saccharimonadaceae*, *Lachnospiraceae_UCG_008*, *Eubacterium_hallii_group*, *Blautia*, *Haemophilus*, *Ruminococcus_gauvreauii_group*, *Oscillibacter*, and *Dorea*.

Specific gut microbiomes can be used as potential biomarkers to distinguish AGC

To explore the relationship between gut microbiota and the TNM stage of GC, the Simpson and ACE index were used, which showed that the diversity and richness of gut microbiota in EGCNS and AGCNS were not obviously different ($P=0.090$ and $P=0.133$, respectively) (Fig. 4 a and b). Besides, PCoA (Fig. 4c) showed no significant difference between EGC and AGC ($P=0.629$).

However, we used LEfSe to list the specific gut microbiomes existing in EGCNS and AGCNS (Supplemental Fig. S4). Five gut microbiota were highly abundant in AGCNS at the genus level, including *Collinsella*, *Blautia*, *Anaerostipes*, *Dorea*, and *Lachnospiraceae_ND3007_group*.

EGC and AGC gut microbiota are obviously different after surgery, and EGC has more specific microflora than AGC in comparison to HC

To clarify the alteration of gut microbiota after surgery in patients with different TNM stages, we compared the EGCS and AGCS with HC by using Simpson, ACE, and PCoA. The abundance of gut microbiota in GC didn't show significant difference between different tumor stage of GC after surgery ($P=0.079$) (Fig. 5a), but the diversity of gut microbiota has shown significant differences among these groups ($P<0.05$) (Fig. 5b). Besides, the PCoA result illustrated the different compositions of EGCS, AGCS, and HC ($P<0.001$) (Fig. 5c).

Based on the differences observed among EGCS, AGCS, and HC, we first compared EGCS with AGCS by the above indexes. The Simpson and ACE indexes revealed that the abundance and richness of gut microbiota did not have a significant difference between EGCS and AGCS ($P=0.12$ and $P=0.73$, respectively) (Fig. 6 a and b). However, the PCoA showed that the composition of gut microbiome in EGCS and AGCS was significantly different ($P<0.002$) at the genus level (Fig. 6c). To further identify the influence of surgery on different stages of GC in patients, we compared EGCS and AGCS fecal microbiota with health control samples by using PCoA at the genus level (Fig. 7 a and b). The composition of gut microbiota was different between both EGCS and HC ($P<0.001$), and AGCS and HC ($P<0.049$). Moreover, we used LFfSe to observe the similarity between EGCS, AGCS, and HC. A total of 31 gut microbiota showed different abundances between EGCS and HC ($P<0.01$) (Supplemental Fig. S5), including *Enterococcus*, *Clostridium_innoccum_group*, *Parabacteroides*, *Erysipelatoclostridium*, *Peptostreptococcus*, *Lachnospiraceae_FC2020_group*, *Streptococcus*, *Anaeroglobus*, *Saccharimonadaceae*, *Veillonella*, *Lachnospiraceae_ND3007_group*, *Rothia*, *Hungatella*, *Butyricicoccus*, *Anaerotruncus*, *Eubacterium_ventriosum_group*, *Agathobacter*,

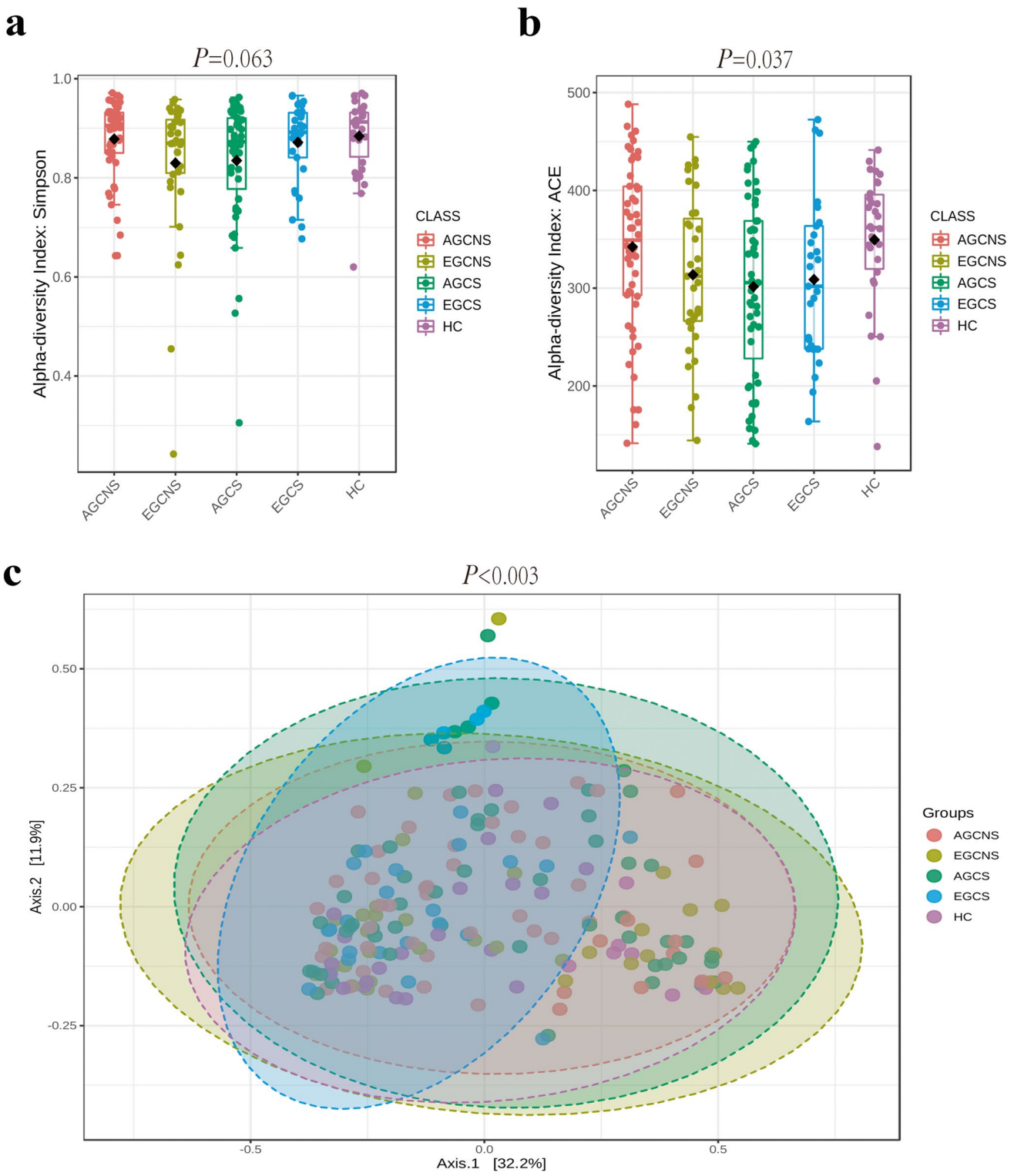

Fig. 1 Comparison of gut microbiota between the AGCNS, EGCNC, AGCS, EGCS, and HC groups. The Simpson index (**a**) showed that the diversity of the gut microbiome was not significantly different, and the ACE index (**b**) showed a statistically significant difference in abundance. PCoA (**c**) was applied to compare the microbiota space between AGCNS, EGCNC, AGCS, EGCS, and HC and it reflected the differences in the composition of gut microbiome in these groups

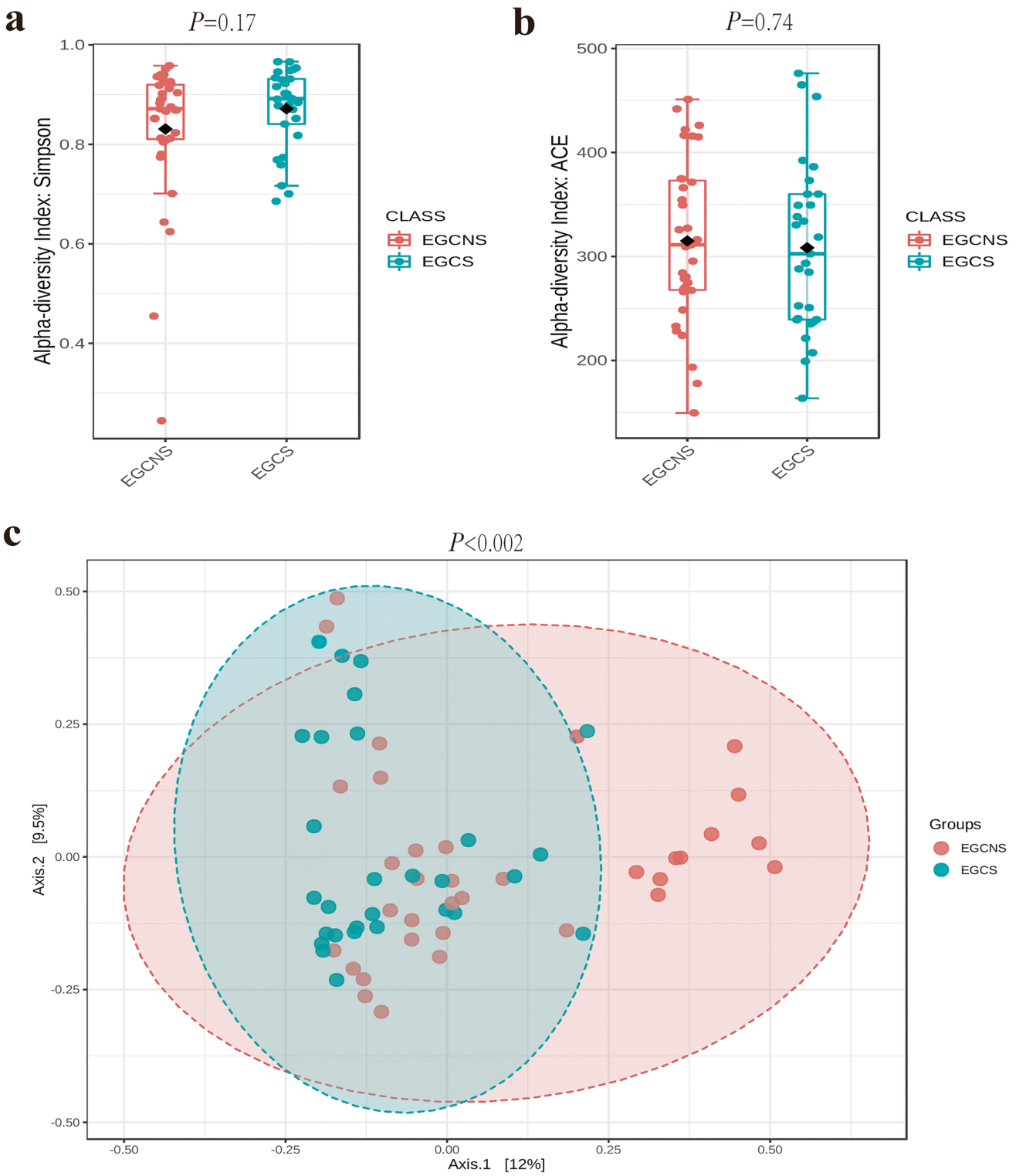

Fig. 2 The comparison of gut microbiota between the EGCS and EGCNS groups. Simpson (**a**) and ACE (**b**) did not show significant differences in diversity and abundance between the EGCS and EGCNS groups. PCoA (**c**) showed that the composition of EGCS and EGCNS's gut microbiota was different

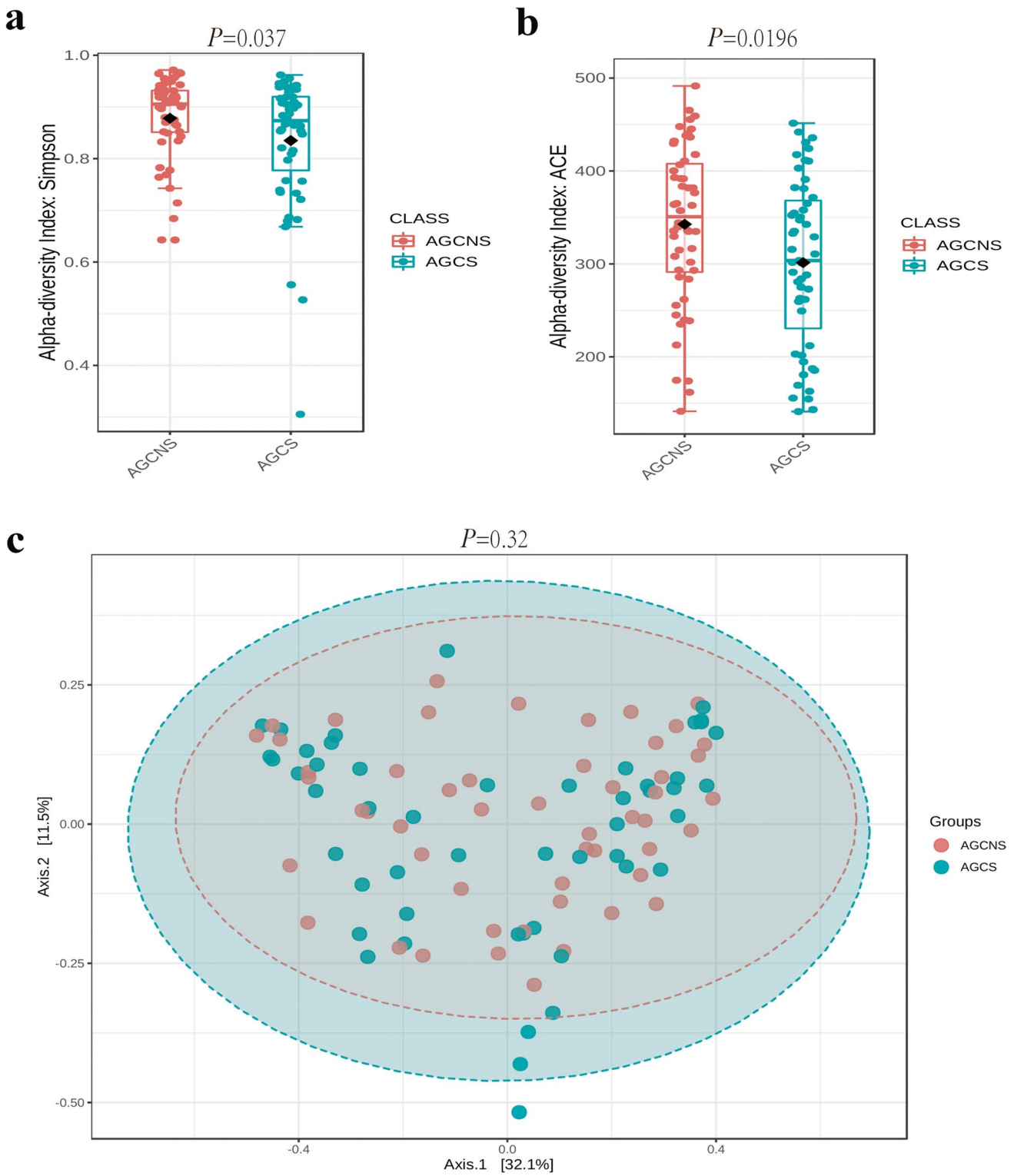

Fig. 3 Comparison of gut microbiota between the AGCS and AGCNS groups. The Simpson index (**a**) and ACE (**b**) showed significant difference in both the abundance and diversity of the gut microbiome. PCoA (**c**) did not show differences in the gut microbiota composition between the AGCS and AGCNS groups

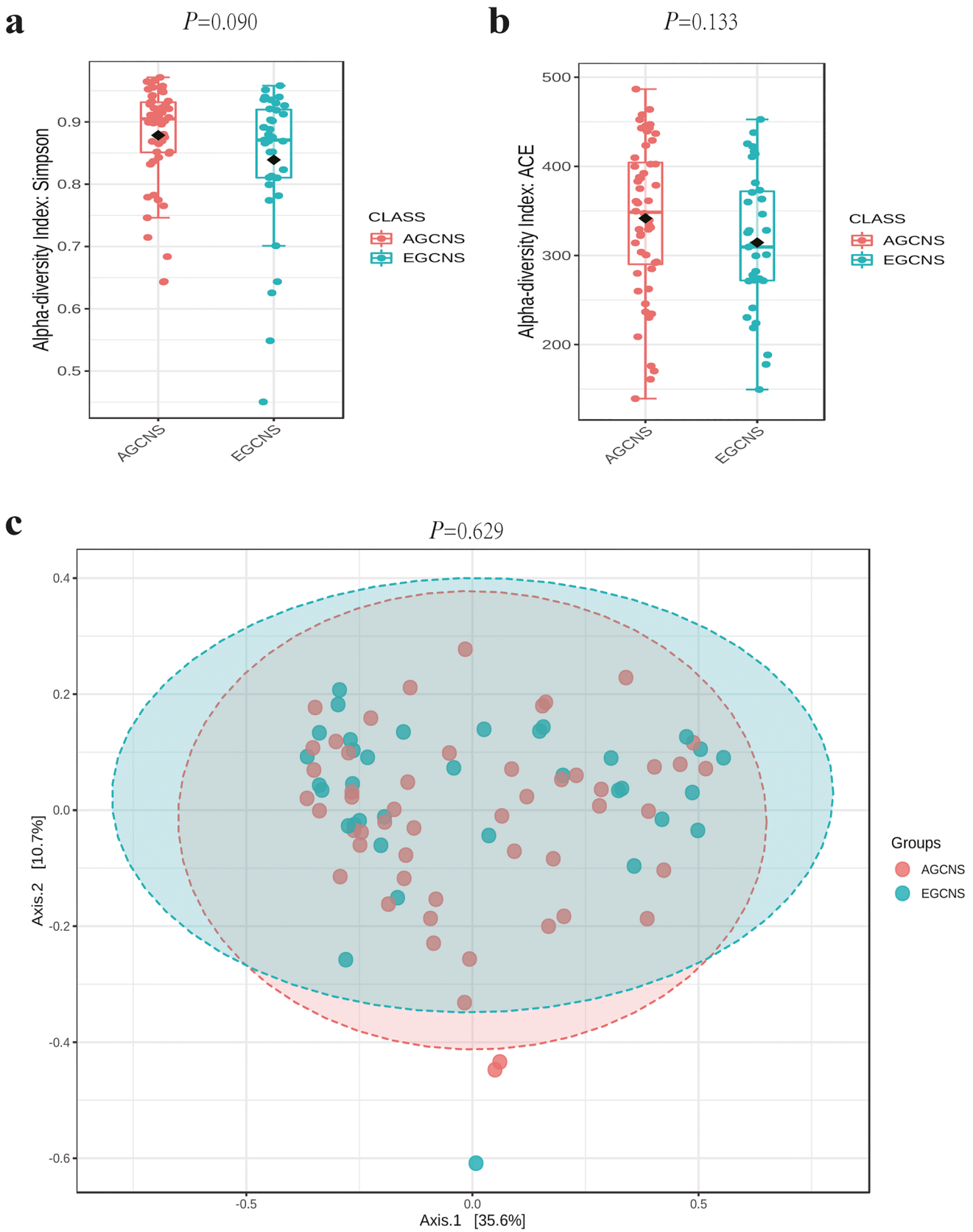

Fig. 4 The comparison of gut microbiota between the AGCNS and EGCNS groups. The Simpson index (**a**) and ACE (**b**) didn't showed significant difference both in diversity and abundance. Meanwhile, PCoA (**c**) did not show differences in the gut microbiota composition between AGCNS and EGCNS

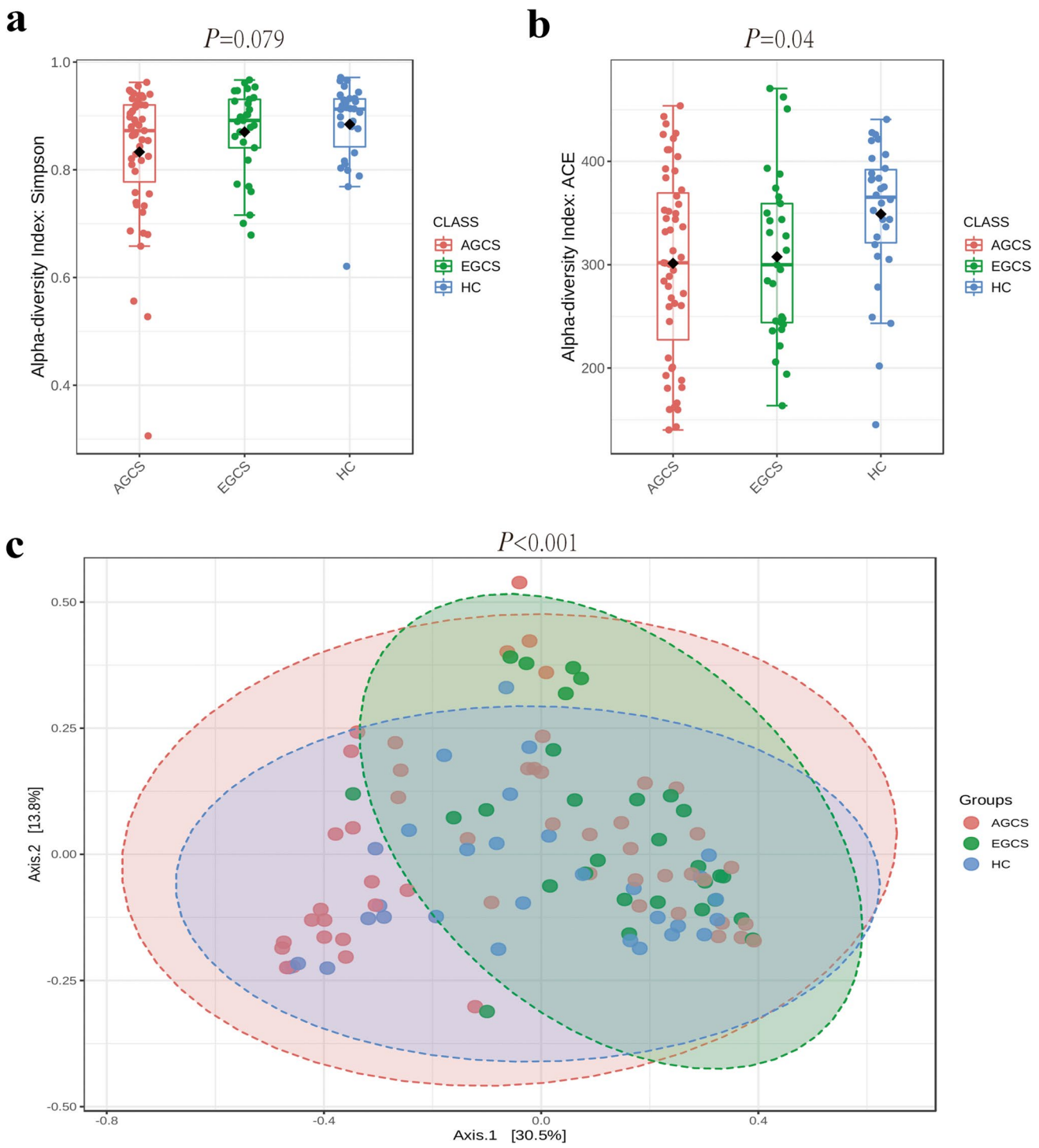

Fig. 5 Comparison of gut microbiota between the AGCS, EGCS and HC groups. The Simpson (**a**) and ACE (**b**) indexes show the abundace of gut microbiota is not different between these groups but the diversity show significant differences between these groups. PCoA (**c**) showed that the composition of gut microbiota between these groups was significantly different

Dialister, *Erysipelotrichaceae_UCG_003*, *Eggerthella*, *Lachnosraceae_NK4A136_group*, *Lachnospiraceae_UCG_004*, *Eubacterium_hallii_group*, *Eisenbergiella*, *TM7X*, *Fenollaria*, *Colidextribacter*, *Escherichia_Shigella*, *GCA_90066575*, *Coprococcus*, and *Romboutsia*. Eighteen gut microbiota were detected in the AGCS and HC group ($P < 0.01$) (Supplemental Fig. S6), including *Veillonella*, *Streptococcus*, *Anaerogolobus*, *Enterococcus*, *Holdmania*,

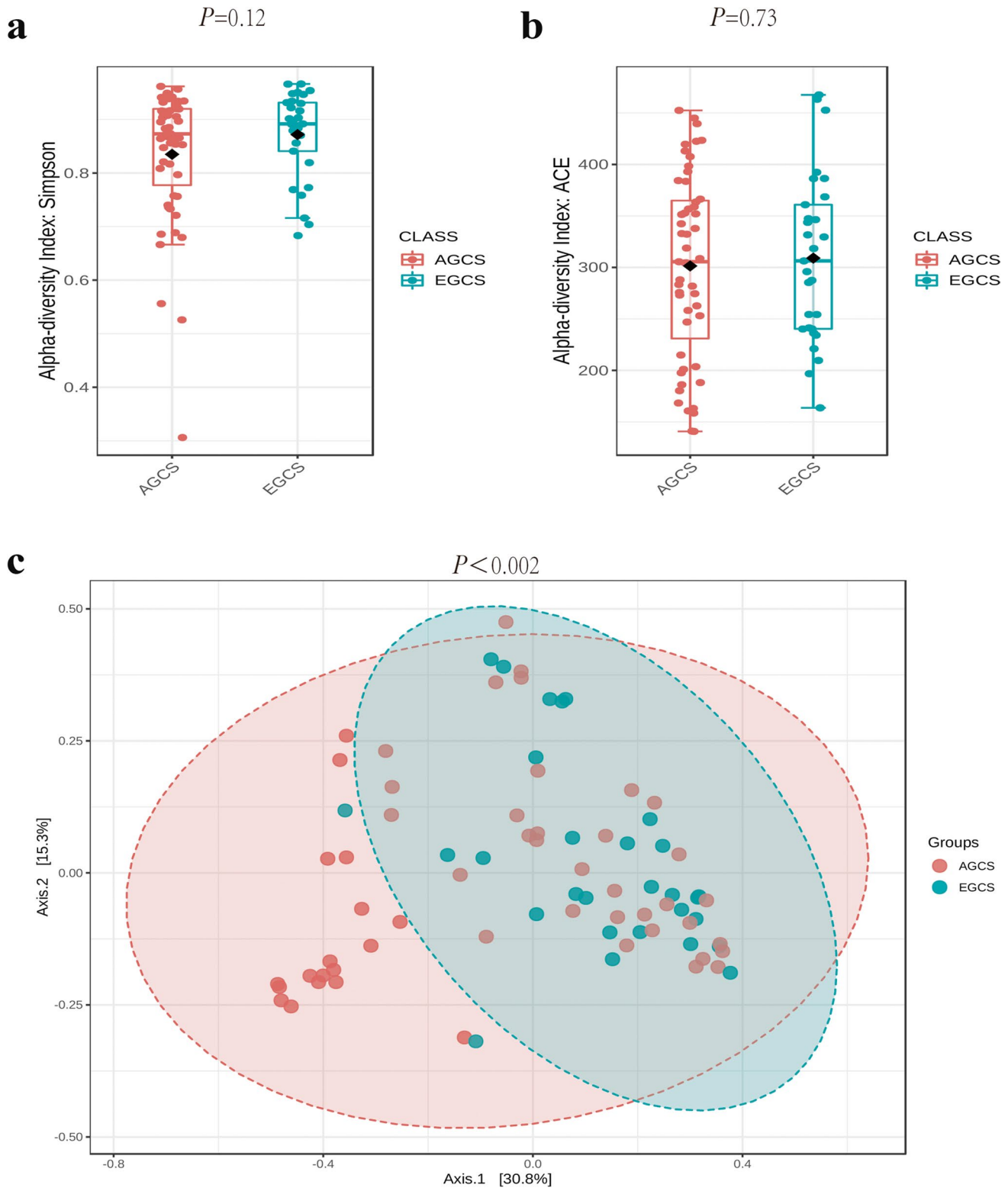

Fig. 6 Comparison of gut microbiota between the AGCS and EGCS groups. The Simpson (**a**) and ACE (**b**) indexes showed no significant difference in diversity and abundance between AGCS and EGCS. PCOA (**c**) showed that the composition of AGCS and EGCS's gut microbiota was significantly different

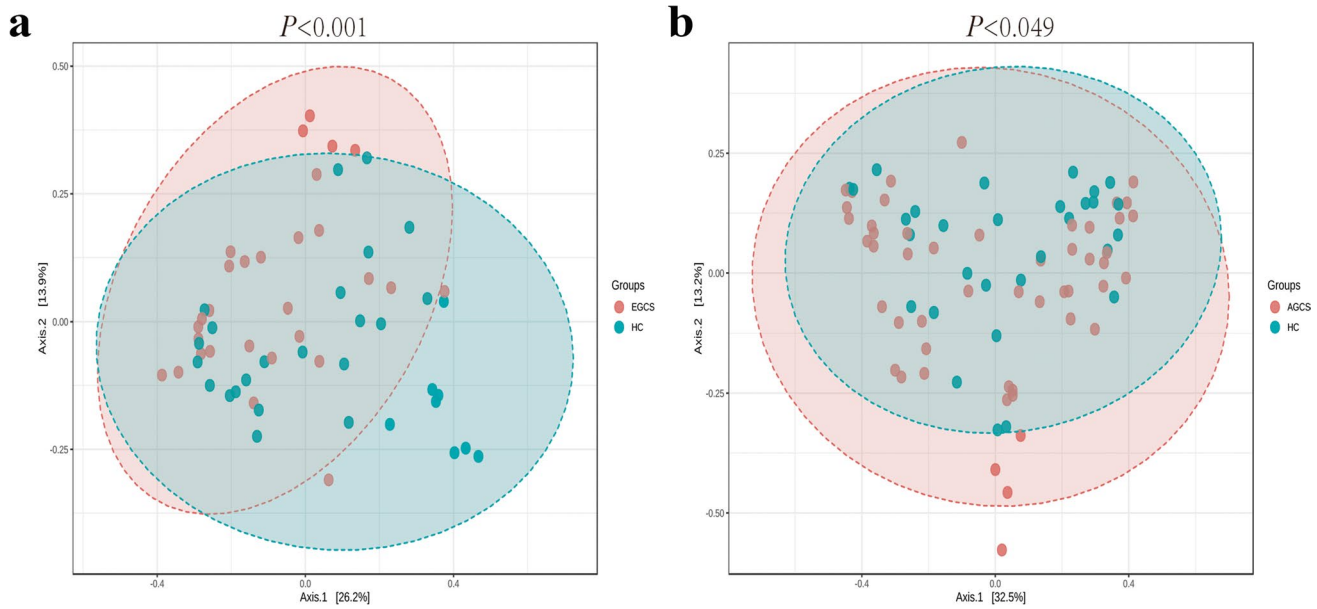

Fig. 7 Comparison of gut microbiota in patients with different stages of GC after surgery and the HC group. PCoA illustrated the difference in the composition of gut microbiota between the EGCS (**a**), AGCS (**b**), and HC group

Peptostreptococcus, *Saccharimonadaceae*, *Rothia*, *Campylobacter*, *Erysipelotrichaceae_UCG_003*, *GCA_900066575*, *Oscillibacter*, *Parasutterella*, *Lachnospiraceae_NK4A136_group*, *Lachnospiraceae_UCG_008*, *Romboutsia*, *Incertae Sedis*, and *Eubacterium_ventriosum_group*. In addition, 13 gut microbiota had different levels between the EGCS and AGCS groups ($P<0.01$) (Supplemental Fig. S7), including *Parabacteroides*, *Clostridium_innoccum_group*, *Paraprevotella*, *Ruminococcus_gnavus_group*, *Erysipelatoclostridium*, *UBA1819*, *Hungatella*, *Coprobacillus*, *Negativibacillus*, *Bilophila*, *Bacteroides*, and *Lachnospira*.

Age of onset affects the distribution of intestinal flora in gastric cancer

To explore the influence of onset age on the gut microbiota of GC patients, we divided patients into EOGCS, EOGCNS, LOGCS, and LOGCNS groups according to onset age and surgery intervention. Simpson and ACE were used to evaluate the diversity and abundance of these groups (Fig. 8 a and b), and we found that gut microbiota diversity among these groups did not show a significant difference ($P=0.254$), whereas the abundance of gut microbiota was significantly different ($P=0.033$). The PCoA (Fig. 8c), suggested different gut microbiota composition within these groups ($P<0.001$). Moreover, the relative abundance analysis at the family and genus levels showed the composition of gut microbiomes among these groups (Supplemental Fig. S8a and b).

Based on the above results, we explored EOGCNS and LOGCNS to clarify the influence of onset age to gut microbiota with GC. The Simpson index showed that the diversity of EOGCNS and LOGCNS might be different between these two groups ($P=0.05$) (Fig. 9a), and ACE showed that the abundance of the two groups was not different ($P=0.243$) (Fig. 9b). In addition, upon the further analysis of the similarity of these groups, the PCoA result showed a significant difference ($P=0.046$) (Fig. 9c).

Next, we used LefSE to compare the specific gut microbiomes among EOGCNS, LOGCNS, and HC. At the genus level, we found that 6 gut microbiomes were different between EOGCNS and HC ($P<0.01$) (Supplemental Fig. S 9), including *Streptococcus*, *Veillonella*, *Odoribacter*, *Tyzzerella*, *Eubacterium_ventriosum_group*, and *Aggregatibacter*. Moreover, there were 7 gut microbiomes with different levels between LOGCNS and HC ($P<0.01$) (Supplemental Fig. S10), including S*treptococcus*, *Veillonella*, *Campylobacter*, *Peptostreptococcus*, *Rothia*, *Clostridium_sensu_stricto_1*, and *Lactobacillus*.

Discussion

The role of TNM stage in EGC and AGC, which are characterized by specific microbiomes

The TNM stage has always been the most significant parameter in clinical tumor diagnosis and treatment (Nakajima 2002). Meanwhile, the existing researches did not explore the relationship between gut microbiome and gastric cancer. In the original research trail of colorectal cancer (CRC), Sheng and his colleagues showed no obvious difference in the abundance

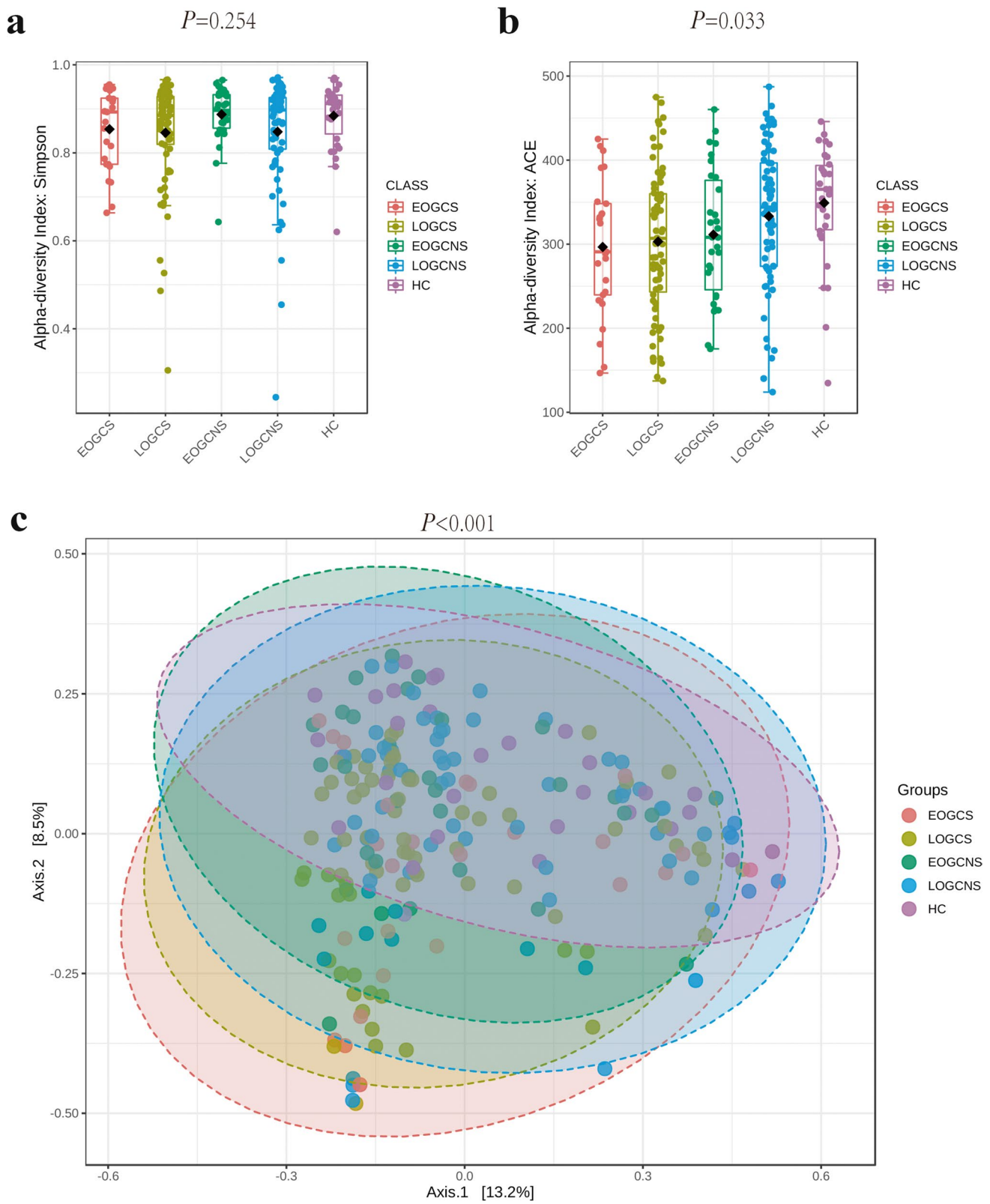

Fig. 8 Comparison of gut microbiota between the EOGCS, LOGCNS, EOGCNS, LOGCNS, and HC groups. The Simpson index (**a**) showed that the diversity of the gut microbiome was not significantly different, and ACE (**b**) showed the difference in abundance. PCoA (**c**) was applied to compare the microbiota space between these groups, and it reflected the difference in the composition of gut microbiome among these groups

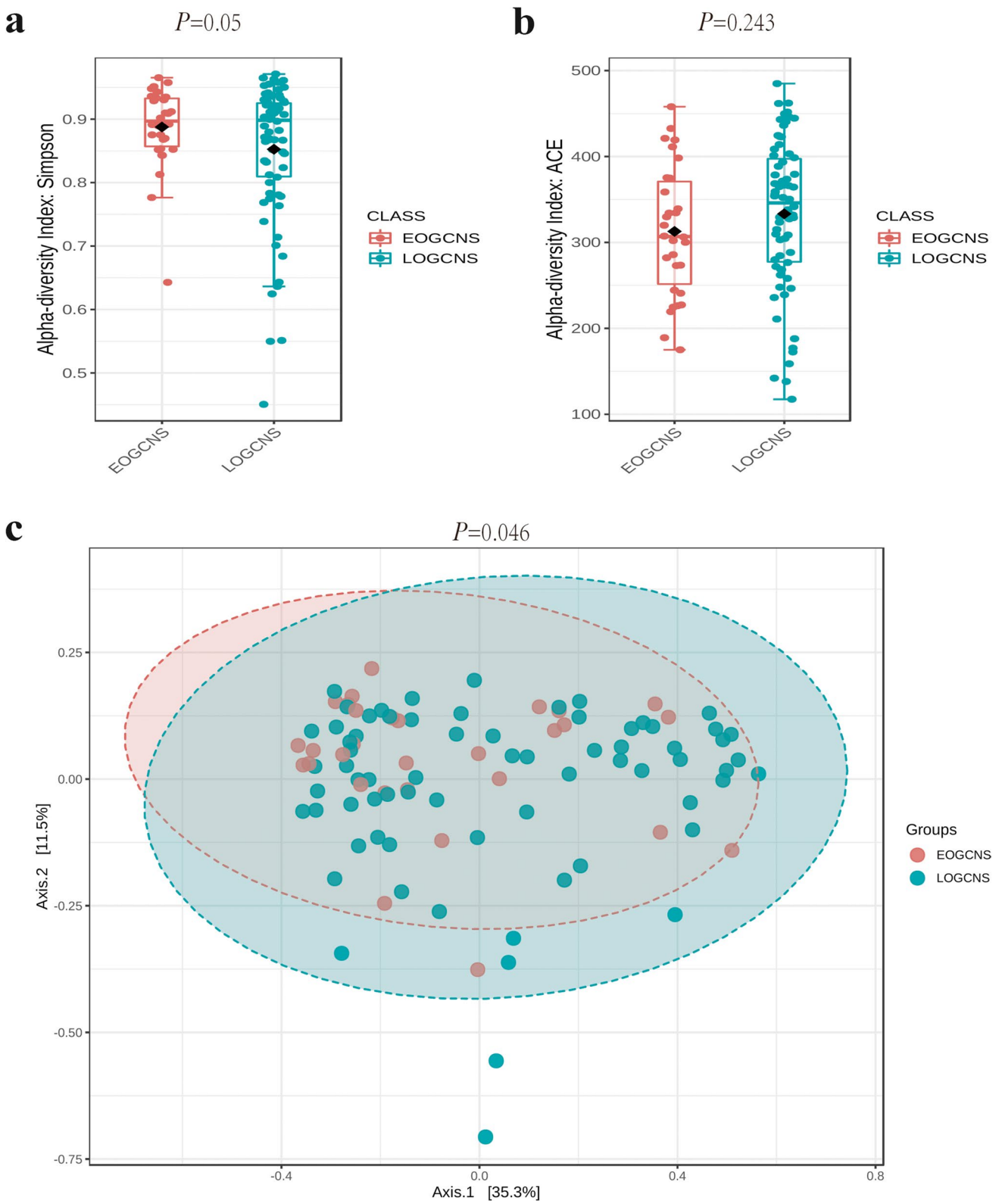

Fig. 9 Comparison of gut microbiota between the EOGCNS and LOGCNS groups. The Simpson (**a**) and ACE (**b**) indexes did not show significant differences in diversity and abundance between EOGCNS and LOGCNS. PCoA (**c**) showed that the gut microbiota composition of EOGCNS and LOGCNS was significantly different

and diversity of gut microbiome in CRC patients (Sheng et al. 2020). Due to the current lack of research on GC, this study explored the relationship between different TNM stages and GC patients' gut microbiota.

According to our results, the PCoA showed no difference between EGCNS and AGCNS ($P > 0.05$). However, the composition of gut microbiota was significantly different between EGCS and AGCS ($P < 0.002$). Accordingly, we believe that there is a difference between EGC and AGC gut microbiota before surgery, since the characteristics of the main components were not obvious during comparison, covering up the differences in the characteristic bacteria of the low-abundance flora.

Moreover, we found specific gut microbiomes between EGNC and AGNC ($P < 0.01$), including *Collinsella*, *Blautia*, *Anaerostipes*, *Dorea*, and *Lachnospiraceae_ND3007_group*. These microbiota show potential in distinguishing the stages of GC.

High levels of the genus *Collinsella* in the family *Coriobacteriaceae* and the phylum *Actinobacteria* are usually seen as abnormal as they can affect metabolism by altering intestinal cholesterol absorption, reducing glycogenesis in the liver and enhancing triglyceride synthesis (Gomez-Arango et al. 2018). Recently, this genus was found highly abundant in CRC stage I (Sheng et al. 2019). In addition, *Collinsella* was also verified as an enriched microbiota in GC patients' gastric mucosa (Li et al. 2021). *Blautia* belongs to the family *Lachnospiraceae*, which can degrade complex polysaccharides to short-chain fatty acids including acetate, butyrate, and propionate that can be used as energy sources by the host (Eren 2015). It was also found enriched in GC patients' gastric mucosa (Li et al. 2021). *Anaerostipes* was proved to have the ability to convert D,L-lactate plus acetate to butyrate, and it was indicated to have benefits to the gut environment for it can appropriately utilize lactate (Shetty et al. 2020). *Dorea* is considered harmful bacteria in the gut microbiome; current research suggests that it is inversely related to insulin resistance and positively related to gas production. Besides, it is considered to be one of the inflammatory biomarkers and colorectal cancer risk biomarkers in patients with irritable bowel syndrome (Mortaş et al. 2020). The *Lachnospiraceae_ND3007_group* is a microbiota that seems to possess the potential for producing short-chain fatty acids (SCFAs) (Nishiwaki et al. 2020). It is difficult to explain why *Anaerostipes* and *Lachnospiraceae_ND3007_group*, as beneficial bacteria, are highly abundant in the intestinal flora of patients with advanced gastric cancer; hence, this phenomenon is worth exploring.

The role of surgery in EGC vs AGC: preoperative and postoperative differences

Our previous study has revealed that surgery is an essential factor which can alter the gut microbiota of GC (Chen et al. 2022). In these studies, we found that the preoperative and postoperative outcomes of patients with early gastric cancer were significantly different from those of patients with advanced gastric cancer. *Enterococcus*, *Parabacteroides*, *Hungatella*, *Clostridium_innocuum_group*, *Eggerthella*, *Eisenbergiella*, *Anaerotruncus*, *TM7x*, *Anaeroglobus*, *Roseburia*, *Lachnospiraceae_NK4A136*, *Odoribacter*, and *Lachnospira* had different abundances between EGCS and EGCNS ($P < 0.01$). Meanwhile, the levels of *Enterococcus*, *Holdemania*, *Lachnospiraceae_NK4A136_group*, *Saccharimonadaceae*, *Lachnospiraceae_UCG_008*, *Eubacterium_hallii_group*, *Blautia*, *Haemophilus*, *Ruminococcus_gauvreauii_group*, *Oscillibacter*, and *Dorea* exhibited differences between AGCS and AGCNS ($P < 0.01$). It was interesting to find that *Enterococcus* and *Lachnospiraceae_NK4A136* repeatedly appeared in the comparison of preoperative and postoperative GC patients, whether early stage or not. Combining the data, we found that *Enterococcus* was abundant after surgery, and *Lachnospiraceae_NK4A136* had higher levels before surgery. *Enterococcus* is phylogenetically a member of *Enterococcaceae* together with the genera *Catellicoccus*, *Melissococcus*, *Pilibacter*, *Tetragenococcus*, and *Vagococcus*. Current studies have shown that *Enterococci* can cause bacteremia, endocarditis, and other human infections (Švec and Franz 2014). Thus, the high abundance of *Enterococcus* may be correlated with postoperative infection. *Lachnospiraceae_NK4A136*, which belongs to the family *Lachnospiracea*, has ability to produce SCFA, such as *Lachnospiraceae_ND3007_group* (Wu et al. 2020). This result suggests that surgery can alter the composition of the gut microbiota and cause instability in the gut environment.

The role of surgery in EGC vs AGC: similarity of gut microbiota compared with healthy people at different TNM stages

Another phenomenon worth discussing is that the difference in gut microbiome between before and after surgery for advanced GC is greater than that for early GC. According to current research, as the depth of tumor invasion increases, the probability of lymph node metastasis and hematogenous metastases also rises. Besides, lymph node metastasis is the main cause of cancer recurrence (Kodera 2016). However, based on the difference in microbiota between early or advanced preoperative patients and healthy people, the LFfSe were used to explore the differences in gut microbiome between the EGCNS, AGCNS, and HC groups, respectively. We found that there were 10 gut microbiome genera showing different abundance ($P < 0.01$) between the EGCNS and HC group (Supplemental Fig. S11), including *Streptococcus*, *Erysipelotrichaceae_UCG_003*, *Clostridium_sensu_stricto_1*, *Campylobacter*, *Rothia*, *Odoribacter*, *Lachnospiraceae_ND3007_group*, *Anaerostipes*, *GCA_900066575*, and *Veillonella*. Besides, 7

gut microbiome genera had variations between the AGCNS and HC group (Supplemental Fig. S12), including *Streptococcus*, *Veillonella*, *Rothia*, *Peptostreptococcus*, *Campylobacter*, *Anaeroglobus*, and *Aggregatibacter*.

We could establish that *Erysipelotrichaceae_UCG_003*, *Clostridium_sensu_stricto_1*, *Odoribacter*, *Lachnospiraceae_ND3007_group*, *Anaerostipes*, and *GCA_900066575* had different abundances in EGC. *Erysipelotrichaceae_UCG_003* had a high abundance in CRC (Park et al. 2021). Current research shows that *Erysipelotrichaceae* can affect cholesterol and lipid metabolism, but the definite role of *Erysipelotrichaceae_UCG_003* is still unclear (Singh et al. 2019). *Clostridium_sensu_stricto_1* is considered as a pathogenic gut microbiome because it contains *C. perfringens* and other true *Clostridium* species (Rajilić-Stojanović and de Vos 2014). *Odoribacter* is a genus that could produce SCFA (Liu et al. 2020), and it was found abundant in CRC both in the early and advanced stages (Park et al. 2021). *GCA-900066575* belongs to the *Lachnospiraceae* family, which is considered as a pathogenic genus and has been identified as significantly correlated with obesity-related indicators in mice (Mills et al. 2021), and it is also purified to be associated with functional bowel disorder (FBD) (Kumbhare et al. 2021). It was interesting to find that in the early stage of GC, the abundance of pathogenic microbiomes increases, and most of them also increase in CRC. However, by combining the aforementioned data, we found that *Lachnospiraceae_ND3007_group* and *Anaerostipes* as beneficial microbiota had lower abundance at the early stage and higher abundance at the late stage, which is a remarkable point to characterize the trend of tumor staging from early to late based on the gut microbiome.

Moreover, we found that *Peptostreptococcus*, *Anaeroglobus*, and *Aggregatibacter* had different levels in AGC. *Peptostreptococcus* is an anaerobic bacterium selectively enriched in the fecal and mucosal microbiota of patients with CRC, and a recent study showed that *Peptostreptococcus anaerobius* drives CRC via the PCWBR2-integrin α2/β1-PI3K–Akt–NF-κB signaling axis (Long et al. 2019). *Anaeroglobus* belongs to the family *Veillonellaceae*, whose major metabolic end products are acetic, propionic, isobutyric, butyric, and isovaleric acid (Carlier 2015). *Aggregatibacter* species exhibit a dominant etiology of infective endocarditis caused by fastidious organisms (Nørskov-Lauritsen 2014). *Aggregatibacter* was not correlated with gastrointestinal disease, but *Peptostreptococcus* and *Anaeroglobus* were all correlated with CRC.

Effect of age of onset on the microbiota in gastric cancer: differences in the microbiota composition between EOGCNS and LOGCNS

The influence of onset age on the intestinal flora of gastric cancer patients is currently lacking. However, in the study of colorectal cancer, scholars have proved the influence of onset age on the intestinal flora of patients with colorectal cancer (Yang et al. 2021). Therefore, we used this parameter as the classification basis to analyze the gut microbiota of gastric cancer patients. We found that 6 gut microbiomes showed differences between EOGCNS and HC, including *Streptococcus*, *Veillonella*, *Odoribacter*, *Tyzzerella*, *Eubacterium_ventriosum_group*, and *Aggregatibacter*. Furthermore, 7 gut microbiomes were different among LOGCNS and HC, including S*treptococcus*, *Veillonella*, *Campylobacter*, *Peptostreptococcus*, *Rothia*, *Clostridium_sensu_stricto_1*, and *Lactobacillus*. This result proved that the onset age of GC has a strong influence on GC patients' gut microbiome.

On the whole, gut microbiota is easier to obtain and monitor than tissue flora of stomach, and its application in the diagnosis and treatment of digestive tract diseases, especially tumors, should be paid more attention. This paper improves the knowledge of the intestinal flora in gastric cancer. Moreover, the effect of surgery on the intestinal flora of gastric cancer is elaborated, and the effect of different tumor stages on the intestinal flora of gastric cancer patients is comprehensively analyzed. For the first time, it is proposed that there are differences in the microbiota composition among gastric cancer patients of different onset ages, and this conclusion may suggest that we can provide a potential tool for the diagnosis of EOGC through microflora. However, this article also has some limitations. In the control group of this paper, although we tried our best to collect the corresponding normal specimens, the age and quantity were still not well matched. In addition, due to the lack of information on the mechanism of gut microbiota, we were unable to make clear explanations and investigate some of the phenomena that we have discovered. In the follow-up research, we aim to further study these phenomena.

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Author contribution LJL and JS designed and supervised this study, CCC and YQD drafted this manuscript, YFN and GLJ provided technical and material support and data analysis, and YKS and YXL provided the samples and clinical data. YQD and JXL revised this manuscript critically for important intellectual content.

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Data availability Sequences of this study have been uploaded on NCBI under BioProject ID PRJNA817689 (available at https://dataview.ncbi.nlm.nih.gov/object/PRJNA817689).

Declarations

Ethics approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the national research committee and this study was approved by the Ethics Committee of Zhejiang Provincial People's Hospital (No. 2022QT010). All the samples were anonymous.

Conflict of interest Author Yaofang Niu and Gulei Jin are employed by the Hangzhou Guhe Information and Technology Company. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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