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Research article

Potential values of formalin-fixed paraffin-embedded tissues for intratumoral microbiome analysis in breast cancer

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ABSTRACT

Breast cancer (BC) tissues have been proved to harbor microorganisms, which could potentially contribute to oncogenesis. Formalin-fixed paraffin-embedded (FFPE) tissues are the most widespread clinical samples in BC research. To verify the potential of FFPE tissues in microbiological analysis, we analyzed the microbial communities of FFPE and fresh frozen (FF) tumor samples from 30 participants diagnosed with BC deploying 16S rRNA sequencing. The operational taxonomic units (OTUs) analysis showed that 78.55% of OTUs in FFPE samples were consistent with FF samples. The composition of core bacteria did not change much, and there is also no difference in alpha diversity between FFPE and FF (without unclassified bacteria). Taxonomic variation results show that *Firmicutes* and *Bacteroidota* phyla, and their major classes, maintained the same proportion under two preservation methods. In addition, the major class *Gammaproteobacteria*, as well as its dominant orders *Burkholderiales* and *Pseudomonadales* all showed no significant difference in paired analysis. Moreover, the *Proteobacteria* and *Actinobacteriota* phyla showed no significant difference between FFPE and FF samples after subtracting unclassified bacteria. Therefore, premised with the intrinsic tumor heterogeneity and unclassified bacteria, there are potential values of FFPE tissues for intratumoral microbiome analysis in breast cancer.

1. Introduction

As an indispensable part of the human body, the microbiome has been shown to interact with the host and affect its physiological state and disease susceptibility [1]. In addition to the trillions of microbes found in the skin, mouth, and gut [2], a low-biomass microbiome can also be detected in breast [3], pancreas [4], lung [5], and other organs [6]. However, the microbiome of breast tumors is richer and more diverse than that of other tumor types [7]. Such microbial communities have been proved to contribute to cancer onset or progression by promoting metabolic function and regulating immunity [8]. For example, it has been reported that

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specific bacterial taxa are closely related to the clinical prognosis of breast cancer (BC) [3], and intratumoral microorganisms also play an important role in the process of metastasis and colonization of BC [9]. To deepen the research on the microbiome and BC, next-generation sequencing (NGS) based on the 16S rRNA gene provides a mainstay for bacterial analysis [10]. It is common to amplify the hypervariable regions (V1–V9) of the 16S rRNA gene by PCR and then sequence, compare, and classify the sequenced reads with the microbial gene database [10,11].

Fresh frozen (FF) samples which have high-quality nucleic acids [12], are considered to be the best choice for sequencing-based tumor microbial research. Nevertheless, FF tissues are subjected to immediacy and availability [13]. On the contrary, formalin-fixed paraffin-embedded (FFPE) samples, the most common samples in clinical cancer research, are convenient to obtain as long-term preserved samples promptly and conduct retrospective analysis. However, there are obstacles to FFPE samples being used in NGS technology. Formalin fixation and long-term storage of paraffin may compromise the integrity of DNA [14], for example, the length of detectable fragments in FFPE samples decreases with the storage time [15]. In addition, the embedding process of FFPE samples is not completely sterile, which may affect the microbiome analysis data [16]. Therefore, the accuracy and clinical validity of microbiome profiling in FFPE tumor tissues are uncertain.

To evaluate the feasibility of FFPE samples in microbiome analysis, some scholars analyzed the microbial composition of the same sample under the above two different storage methods by NGS. In gastric studies, Pinto-Ribeiro analyzed the differences of microorganisms in FF and FFPE samples of gastric tissue obtained from five patients [17]. The results show that the number of operational taxonomic units (OTUs) in paired FFPE samples decreased significantly compared with FF samples, and there was a significant difference in taxonomic variation in the relative abundance of phyla and orders. Borgognone studied the microbial composition of colorectal cancer tissues obtained from ten different individuals by 16S rRNA sequencing and RNA-ISH [18]. They found that there were differences in microbial diversity and composition, and FFPE samples were rich in typical bacterial contaminants compared with FF samples. In addition to human tissue studies, the alpha diversity of microorganisms was found to be different in rainbow trout, but there was no significant difference in Beta-diversity [19]. Although these studies provide some ideas, they are limited by sample capacity and the results are easily affected by individual differences.

Here we analyzed FF and FFPE tumor tissues of 30 participants diagnosed as BC. First, the DNA of FF tumor tissues and matched FFPE tumor tissues was detected by 16S rRNA sequencing. After subsampling, we compared the number of OTUs and the alpha diversity of microorganisms. Finally, the microbial composition at different taxonomic levels was also analyzed, and the dominant microorganisms in each sample were compared separately.

2. Materials and methods

2.1. Sample collection

We studied tumor samples from 30 participants who were diagnosed with BC at the Affiliated Cancer Hospital of Zhengzhou University (Henan Province, China) in 2021. For each participant, two adjacent fragments of the same tumor tissue were used: one was sharp frozen with liquid nitrogen and stored at −80 °C (abbreviated as FF), and the other was fixed in formalin and then embedded in paraffin for the storage (abbreviated as FFPE).

This study was approved by the institutional review board. All participants signed written informed consent after the study protocol was fully explained, as well as informed consent agreeing to deliver their anonymous information for future studies.

2.2. DNA extraction

FF samples were stored in sterile EP tubes. To reduce environmental pollution of FFPE samples, the surface of FFPE blocks was removed and the internal tissue was obtained by drilling from FFPE blocks on a clean bench. The detailed protocol of sampling from FFPE blocks refers to the previous article [20]. Microbial total DNA of FFPE samples was extracted using the QIAamp DNA FFPE Tissue Kit (QIAGEN, CA) according to the manufacturer's protocol, and that of FF samples was extracted using cetyltrimethylammonium bromide (CTAB). After being checked on 1% agarose gel, the concentration and purity of DNA extracts were determined with NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA).

2.3. Library preparation for Illumina MiSeq sequencing

The bacterial 16S rRNA gene hypervariable region V3–V4 was amplified using specific primers with the barcode (338F: 5′-ACTCCTACGGGAGGCAGCAG-3'; 806R: 5′-GGACTACHVGGGTWTCTAAT-3′) by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). After purification with AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantification with Quantus™ Fluorometer (Promega, USA), purified amplicons were mixed in equimolar amounts and paired-end sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA) by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

2.4. 16S rRNA sequence analysis

The raw reads outputted by the Illumina MiSeq platform were demultiplexed. According to the specific primers with barcode and overlapping information, paired-end reads were merged by FLASH (version 1.2.11) [21], and high-quality filtering of raw reads was conducted by fastp (version 0.19.6) to obtain the filtered sequences [22].

Non-repetitive sequences extracted from filtered sequences were clustered with 97% similarity using UPARSE (version 7.1) [23], which were defined as an operational taxonomic unit (OTU). Meanwhile, the chimeric sequences were identified and removed by USEARCH (version 11). RDP Classifier (version 11.5) was used to analyze the OTU taxonomies against the 16S rRNA database Silva v138, with a confidence threshold of 70% [24]. At each taxonomic level, namely domain, kingdom, phylum, class, order, family, genus, and species, the community species composition of each sample was counted. OTUs that were not matched in the database were defined as unclassified or no rank group. After OTU clustering, we took the minimum number of sample sequences as the threshold (sample F597094T: 47289 reads) for subsampling of sequences using mothur (version 1.30.2). Alpha diversity of each sample was

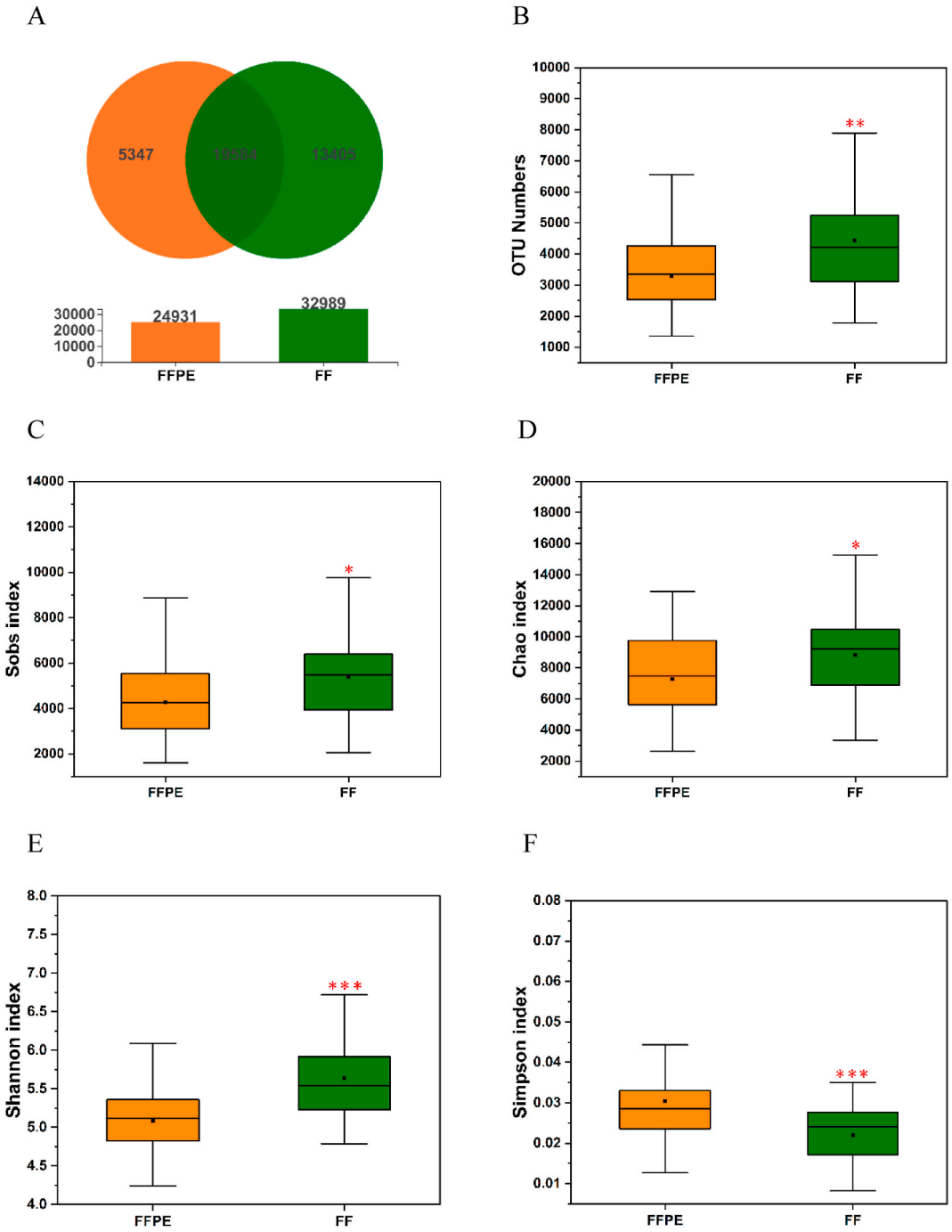

Fig. 1. (A) &(B)Number of OTU of FF and FFPE samples, respectively; (C) alpha analysis of Sobs index, (D) Chao index, (E) Shannon index; and (F) Simpson index. *$p < 0.05$, ***$p < 0.001$. OTU, operational taxonomic unit.

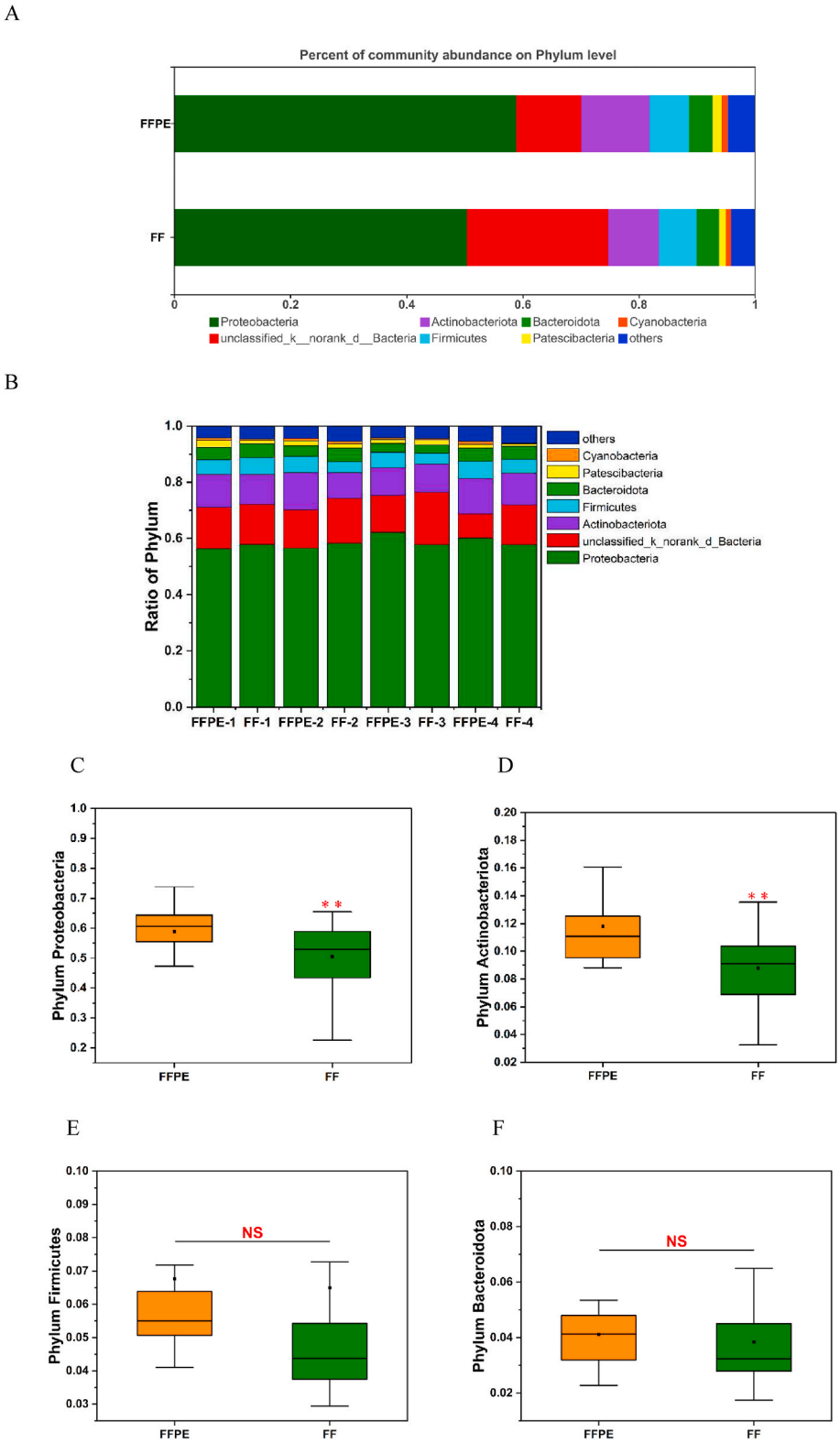

Fig. 2. (A) Relative abundance of phyla in FF and FFPE samples; (B) relative abundance of four tumor tissues under two different storage methods at phylum level; (C) relative abundance of the phylum Proteobacteria; (D) relative abundance of the phylum Actinobacteria; (E) relative abundance of the phylum Firmicutes; (F) relative abundance of the phylum Bacteroidota; **$p < 0.01$.

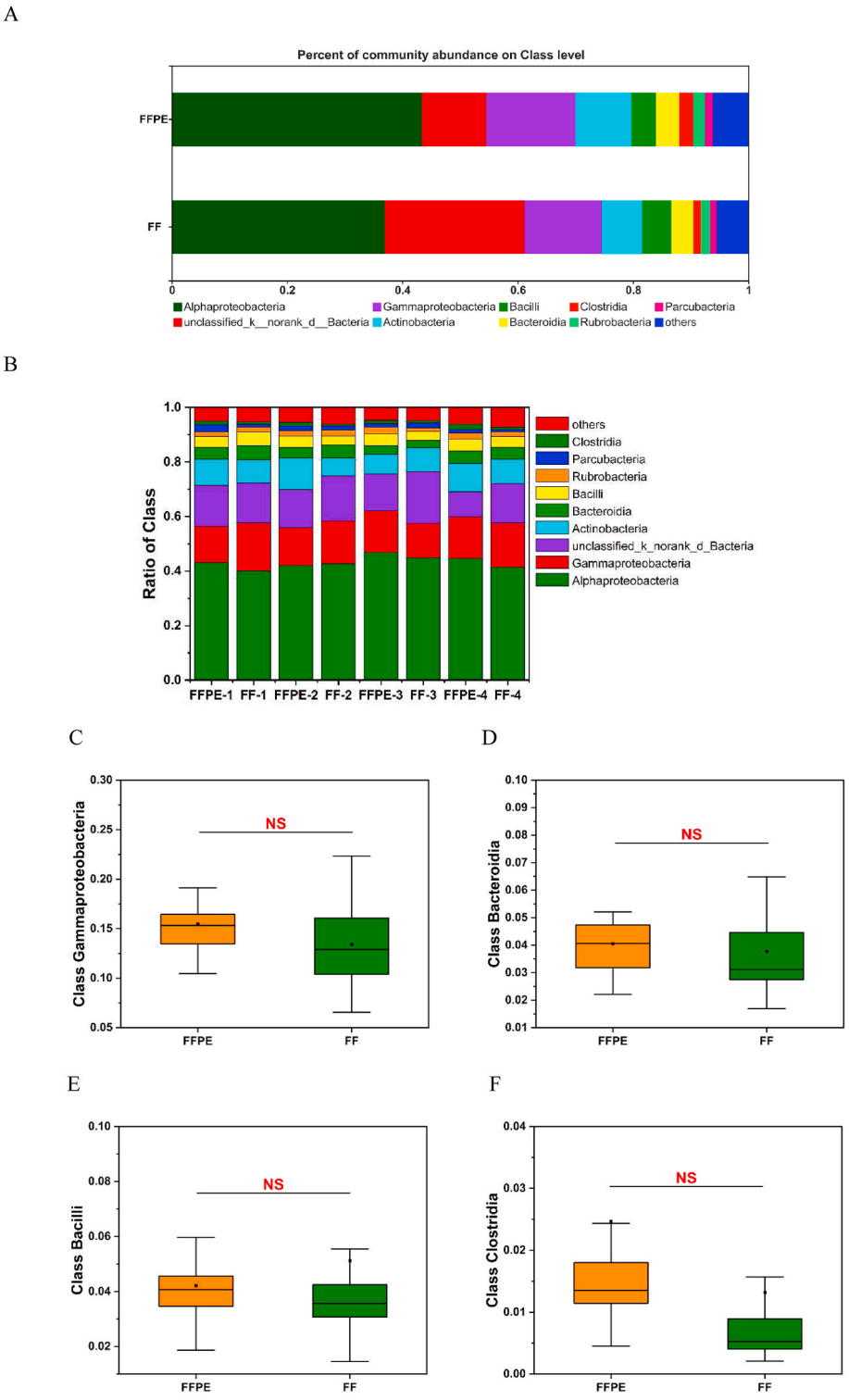

Fig. 3. (A) Relative abundance of classes in FF and FFPE samples; (B) relative abundance of four tumor tissues under two different storage methods at class level; (C) relative abundance of the class Gammaproteobacteria; (D) relative abundance of the class Bacteroidia; (E) relative abundance of the class Bacilli; (F) relative abundance of the class Clostridia.

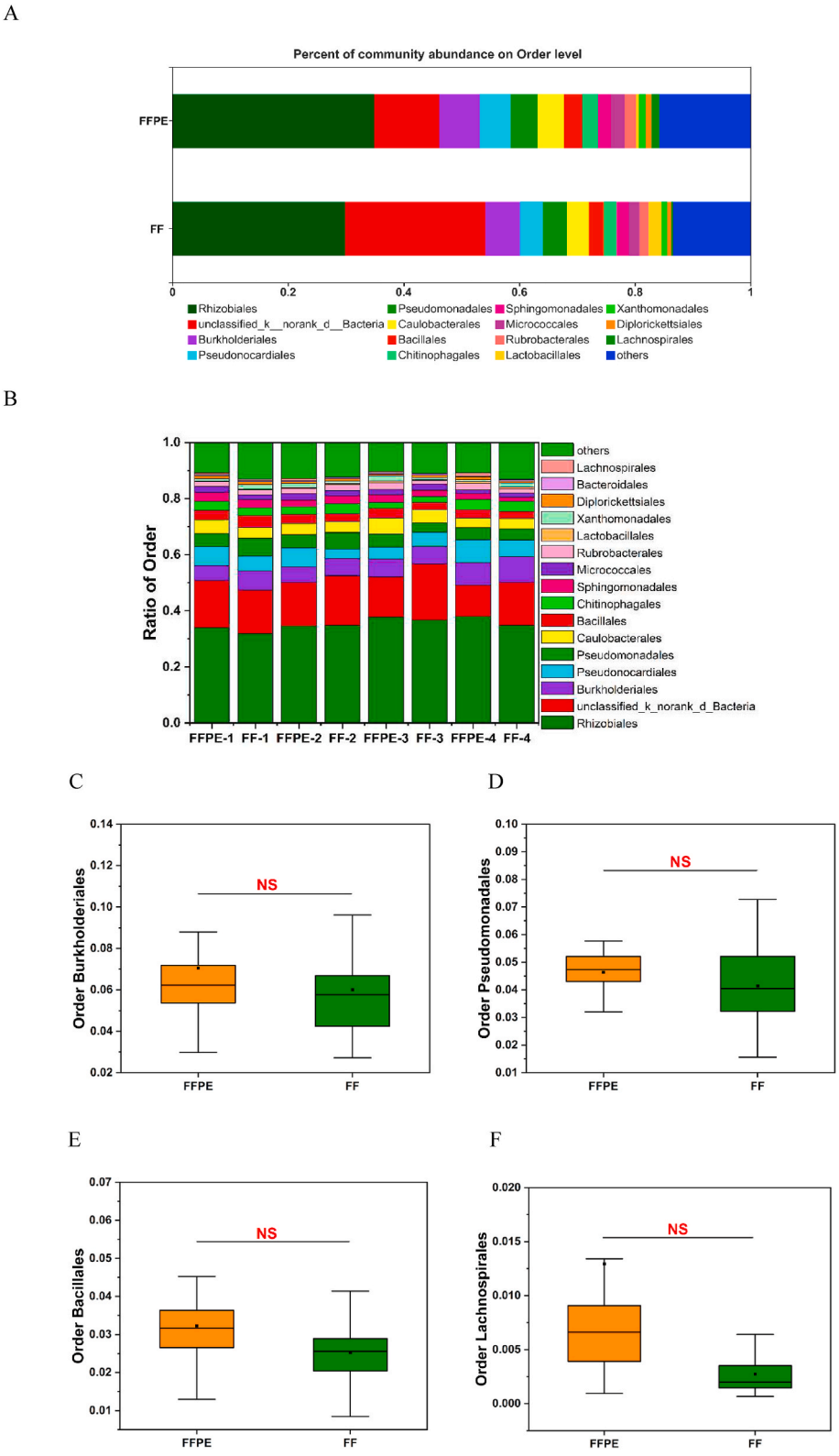

Fig. 4. (A) Relative abundance of orders in FF and FFPE samples; (B) relative abundance of four tumor tissues under two different storage methods at order level; (C) relative abundance of the order Burkholderiales; (D) relative abundance of the order Pseudomonadales; (E) relative abundance of the order Bacillales; (F) relative abundance of the order Lanchnospirales.

determined by analyzing OTU composition using mothur (version 1.30.2), and presented as Sobs index, Chao index, Shannon index, and Simpson index.

2.5. Statistical analysis

OriginPro 2022 SRO was used to perform statistical analyses. OTU number and alpha diversity differences between FF and FFPE samples were assessed using paired sample *t*-test. The bar-plots of community abundance at different levels were drawn with the R program package vegan (version 3.3.1). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Comparison of OTUs and microbial diversity between FF and FFPE tissues

After 16S rRNA gene sequencing and quality filtering, 5,233,202 filtered reads (2,770,724 for the FFPE group, and 2,462,478 for the FF group) were identified (Supplementary Table 1). To minimize the effects of sequencing depth on alpha diversity measure, the number of 16S rRNA gene sequences from each sample was rarefied to 47,289. Then 38,336 operational taxonomic units (OTUs) were identified from whole samples (Fig. 1A). Among these OTUs, the FFPE sample contained 24,931 OTUs, of which 19,584 OTUs were shared with the FF sample, accounting for 78.55% of the FFPE samples. The paired sample *t*-test results show that the OTU number of

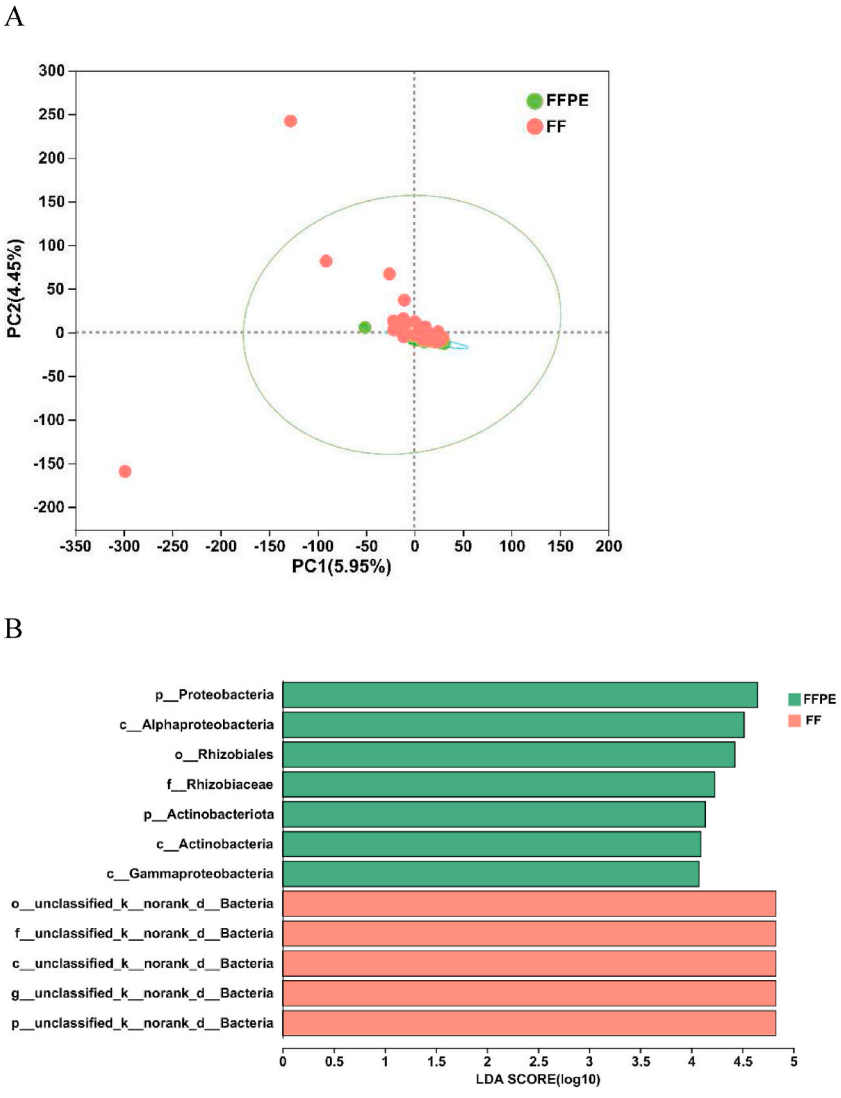

Fig. 5. (A) Beta diversity analysis of FF and FFPE tissue samples. (B) LEfSe rank plot of microbial differences between FF and FFPE tissue samples (LDA>4).

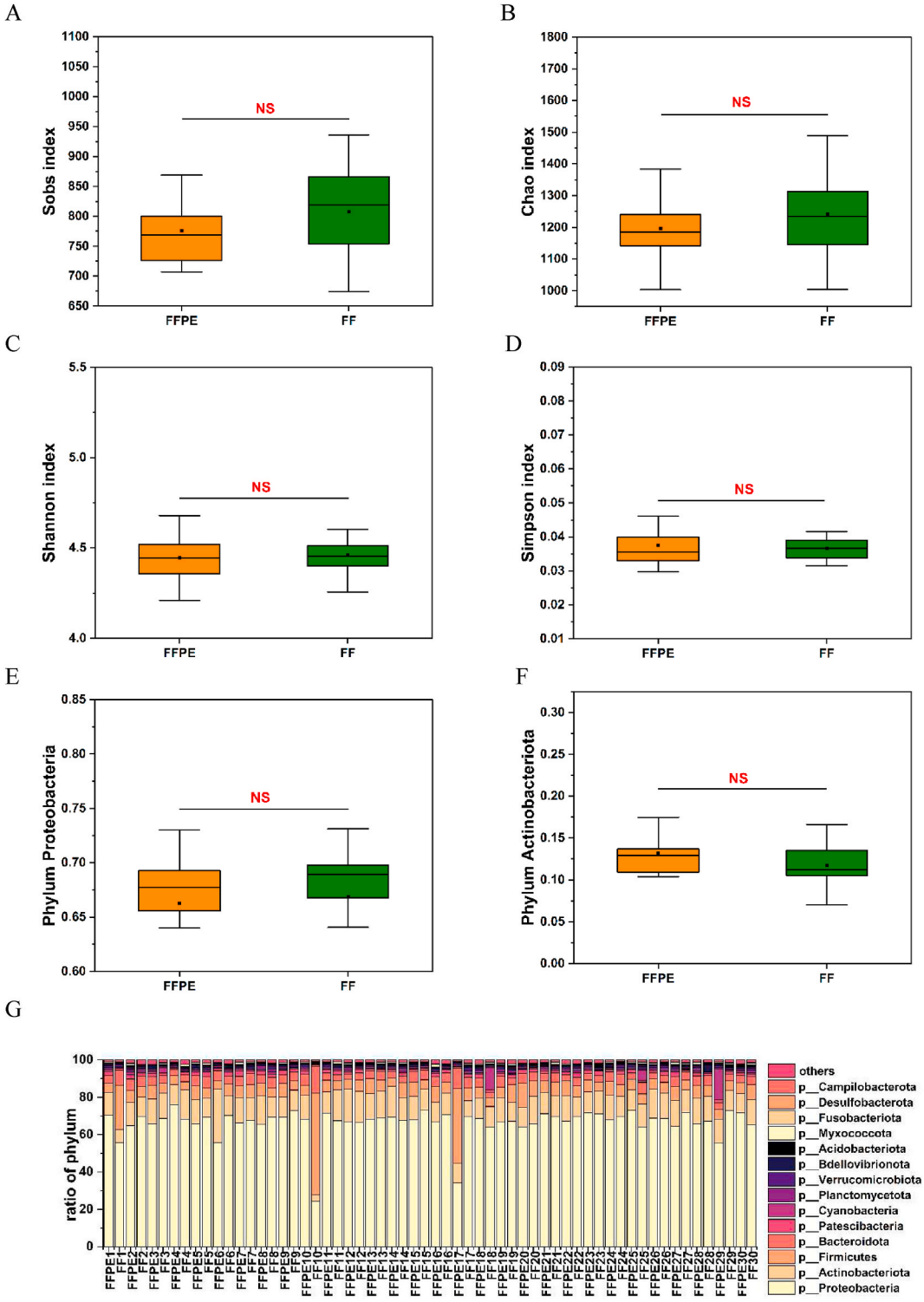

Fig. 6. (A) alpha analysis of Sobs index, (B) Chao index, (C) Shannon index; and (D) Simpson index without unclassified bacteria. Relative abundance of the phyla Proteobacteria (E) and Actinobacteria (F) without unclassified bacteria. (G) relative abundance of 30 tumor tissues under two different storage methods at phylum level without unclassified bacteria.

each FFPE sample decreased significantly (Fig. 1B).

The trends of Shannon–Wiener and rarefaction curves (Supplementary Fig. 1&2) of all samples supported the adequacy of the sampling efforts. In addition, the rank abundance distribution curves (Supplementary Fig. 3) indicated decreased richness and a relative imbalance of bacterial community in the FFPE group compared with the FF group. Compared with FF samples, the community richness of FFPE samples was significantly reduced, as estimated by the Sobs index (Fig. 1C) and Chao index (Fig. 1D). Community diversity of FFPE samples was significantly lower than that of paired FF samples, estimated by the Shannon index (Fig. 1E), and Simpson index (Fig. 1F). The Simpson index of tumor tissue FFPE samples was higher than that in FF samples, indicating that some microorganisms occupy a dominant position in the tissue after paraffin embedding.

3.2. Taxonomic profiling between FF and FFPE tissue samples

To further explore the impact of the paraffin embedding process on the microbial composition of a single sample, we analyzed the community composition of different groups (Fig. 2A). There was no significant difference in the microbial composition of four representative tumor tissues under two different storage methods (Fig. 2B). Hierarchical clustering based on euclidean distance showed no significant difference between FFPE and FF samples, and FFPE samples and FF samples of the same tissue were clustered together (Supplementary Fig. 4). The abundance of *Proteobacteria* (Fig. 2C) and *Actinobacteria* (Fig. 2D) phyla in FFPE samples was higher than that in FF samples, while the microbial levels of phyla *Firmicutes* (Fig. 2E) and *Bacteroidota* (Fig. 2F) did not differ significantly under the two different storage methods.

We further analyzed the community abundance at the class level (Fig. 3A). What excites us is that the four representative tumor tissues that did not have significant differences at the phylum level also had the same trend at class level (Fig. 3B). *Alphaproteobacteria*, unclassified bacteria, *Gammaproteobacteria*, *Actinobacteria*, and *Bacilli* classes accounted for more than 80%. *Gammaproteobacteria*, belonging to the phylum *Proteobacteria*, had no significant difference between the two groups (Fig. 3C). In addition, the class *Bacteroidia* (Fig. 3D), the main class of *Bacteroidota*, is also consistent with FF samples. We also noticed that the classes *Bacilli* (Fig. 3E) and *Clostridia* (Fig. 3F) of FFPE samples, which account for a large proportion of *Firmicutes*, were not significantly different from FF samples. More importantly, the abundance of *Gammaproteobacteria* and *Bacilli* ranked in the top five in the class level analysis.

Next, we analyzed the abundance at the order level (Fig. 4A). The four tumor tissues, which had no significant differences at the phylum and class levels, also had similar proportions at the order level (Fig. 4B). *Burkholderiales* (Fig. 4C) and *Pseudomonadales* (Fig. 4D), both belonging to the class *Gammaproteobacteria* had no difference after paraffin embedding. The abundance of the orders *Bacillales* (belonging to the class *Bacilli*) (Fig. 4E) and *Lachnospirales* (belonging to the class *Clostridia*) (Fig. 4F) of FFPE samples still maintained consistency with FF samples. Moreover, order *Bacteroidales* (belonging to the class *Bacteroidia*) (Supplementary Fig. 5) also showed the same tendency.

When performing taxonomic analysis at different levels, we found that there were differences in microbial composition at the level of phylum and class. To assess whether differences exist between FF samples and FFPE samples, we performed an analysis of beta diversity (Fig. 5A). PCA results showed that there was a significant difference in beta diversity between the two groups ($p = 0.001$). To further analyze the sources of differences, we performed LEfSe analysis again. The results showed that the difference microorganisms between the two groups were unclassified Bacteria, *Gammaproteobacteria*, *Actinobacteria*, *Actinobacteriota*, *Rhizobiaceae*, *Rhizobiales Alphaproteobacteria*, *Proteobacteria* (Fig. 5B).

3.3. Comparison of microbial diversity between FF and FFPE tissues without unclassified bacteria

The LEfSe results suggested that unclassified bacteria may be responsible for alpha diversity and some differences in bacterial composition. In order to exclude the influence of unclassified bacteria, we subtracted unclassified bacteria for analysis.

After deducting unclassified bacteria, the results of alpha diversity showed that there was no significant difference in Sobs (Fig. 6A), Chaos (Fig. 6B), Shannon (Fig. 6C), and Simpson (Fig. 6D) between FFPE and FF samples. In addition, the *Proteobacteria* (Fig. 6E) and *Actinobacteriota* (Fig. 6F) phyla showed no significant difference between FFPE and FF samples after subtracting unclassified bacteria. Except for individual tissues, the microbial composition of FFPE and FF samples had a high consistency after deducting unclassified bacteria in most tissues (Fig. 6G).

4. Discussion

There is increasing evidence that the research on BC-associated microbiota can reveal key aspects of cancer progression and treatment response. We used BC tissues as a model to evaluate the feasibility of using FFPE tissue samples to characterize the microbiota through the NGS. After 16S rRNA gene sequencing and subsampling, we analyzed the OTU number and alpha diversity of FFPE and FF samples. Although the number of OTU and alpha diversity in the FFPE sample group were lower than those in the FF sample group, 78.55% of OTUs in the FFPE sample group were consistent with those in the FF group. Previous research results also confirm the decrease of the OTU number of FFPE samples [17]. The major reason that has charge of the change in the counts of OTUs may be the intrinsic tumor heterogeneity, which leads to different microbial compositions in different tumor microenvironment.

By comparing the microbiota composition of FFPE and its paired FF tissues, the feasibility of using FFPE tissues was further analyzed. Taxonomically, there were consistency in the abundance of taxa between FF tissues and FFPE tissues, which could be detected at the phylum, class, and order levels. In combination with OTU results, we speculated that in addition to new microorganisms being introduced into FFPE samples, some microorganisms already existing in tissues have also been introduced. The abundance of the

phyla *Firmicutes* and *Bacteroidota* did not change, and the abundance of the classes *Bacilli*, *Clostridia*, *Bacteroidia*, and *Gammaproteobacteria* did not change at a deeper taxonomic level. Based on the analysis of the order level, it was found that there was no significant difference in *Bacillales*, *Lachnospirales*, *Burkholderiales*, *Pseudomonadales*, and *Bacteroidales*. The taxonomic results further support the influence of tumor heterogeneity on intratumoral microbiome composition.

Previous studies have shown that targeting the V3–V4 region for sequencing may amplify host sequences and that human DNA is more prevalent as a contaminant in FFPE tissues [25,26]. Combined with the fact that most of the differential flora were unclassified bacteria in our LEfSe analysis, we speculated that there may be host sequences in unclassified bacteria. Of course, these unclassified bacteria are not necessarily all host sequences, because the database we compared when annotating OTUs does not perfectly contain all bacterial species sequences. However, after deducting unclassified bacteria, the difference in alpha diversity between FFPE and FF samples disappeared. This result further illustrates that the major taxa associated with tumor tissue appear to be unaffected by the use of FFPE and FF tissues.

As for the obstacle that the length of detectable fragments will decrease, the current strategy of microbial sequencing is to detect the hypervariable region v3–v4 of 16S rRNA, with a length of about 460 bp [27]. The size of DNA fragments isolated from the FFPE samples still meets the requirements of microbiome analysis, as Cruz Flores identified that FFPE samples within two years can still amplify about 1500bp products by PCR [28].

Previous studies have shown that FFPE samples will be contaminated by bacteria in the process of paraffin embedding and manipulation [29]. To reduce the influence of microorganism's contaminants, we cut off the surface of paraffin blocks and utilized new blades between samples as recommended in the literatures [20,30]. There is a weakness of this study. Although we tried to reduce microbial contamination in FFPE samples through standardized operation, we did not use a separate paraffin sample as the negative control group as described in the previous study [17]. In this way, we could deduct the microbial contaminants introduced during the FFPE sample operation, to overcome the obstacle that the embedding process of FFPE samples was not completely sterile.

Actually, there have been some discussions about the application of FFPE samples in microbial composition. In a study on the relationship between pancreatic ductal adenocarcinoma (PDAC) microorganisms and long-term survival, Riquelme et al. confirmed that there was no statistically significant difference in microbial composition between FFPE and FF samples by 16S rRNA sequencing [4] Masi et al. investigated the pancreatic microbiome in PDAC of FFPE samples from endoscopic ultrasound fine-needle biopsy [31]. Their results demonstrate the feasibility of FFPE samples in studying the pancreas microbiome. Qu et al. also applied it to the research on primary liver cancer (PLC) [32]. Their research revealed that differences in microbial populations exist not only in cancer tissues and matched adjacent non-tumor tissues, but also in different histopathological subtypes, and even in PLC patients with different prognoses.

In addition to the potential in 16S rRNA sequencing, FFPE tissues have also been used in metagenomic analysis. Debesa-Tur used metagenomics to detect FFPE samples of tumor and normal mucosa in Lynch syndrome patients with colorectal cancer [13]. The results show that the microbial diversity in tumor tissues decreased, suggesting the availability of FFPE samples in metagenomics. To assess the etiology detection ability of metagenomic on FFPE samples, Sun et al. used metagenomics to quickly detect fungi and mycobacteria in infectious granuloma FFPE samples [33]. Salian et al. applied FFPE samples to metagenomics, and the results show that the application can make up for the deficiency that histopathology cannot distinguish between *Mycobacterium tuberculosis* and non-Mycobacterium tuberculosis [34].

The detection of FFPE samples with NGS can be used not only to analyze microorganisms, but also to detect fungi and viruses. Sun et al. showed that FFPE samples can also detect fungi with metagenomics [33]. Compared with histopathology and staining, fungi can be analyzed at genus level. Bodewes et al. detected viruses of FFPE samples with NGS [35], which can detect the sequences of known and unknown viruses, although with relatively low sensitivity.

NGS with FFPE samples is also expected to provide convenience for the analysis of metabolomics. Gomez-Gomez used targeted metabolomics to compare tumor and matched normal FFPE tissues from colorectal cancer patients [36]. Their results demonstrated that targeted metabolomics analytes in FFPE samples may serve to validate known potential cancer biomarkers and discover potential novel biomarkers. Microorganisms in tumor tissues have been proved to affect tumor tissues through their own metabolites or derivatives. For example, stimulators of the interferon gene derived from the microbiota have been shown to program mononuclear phagocytes in the tumor microenvironment into immunostimulatory monocytes and dendritic cells (DCs) [37]. Therefore, FFPE samples hold promise for use in the joint analysis of microbiome and metabolomics, which would help to further explore the role of microorganisms in the tumor microenvironment.

In conclusion, considering the intrinsic tumor heterogeneity and unclassified bacteria, there are potential values of FFPE tissues for intratumoral microbiome analysis in breast cancer.

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Data availability statement

The original contributions presented in this study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16267.

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