Exploration of Immunology

Open Exploration

Open Access Perspective

Utilization of formalin-fixed paraffin-embedded specimens for microbiota characterization in cancer: utility and concern

Leandro Di Gloria[1], Elena Niccolai[2]*

[1]Department of Biomedical, Experimental and Clinical Sciences "Mario Serio", University of Florence, 50134 Florence, Italy
[2]Department of Experimental and Clinical Medicine, University of Florence, 50134 Florence, Italy

***Correspondence:** Elena Niccolai, Department of Experimental and Clinical Medicine, University of Florence, 50134 Florence, Italy. elena.niccolai@unifi.it
Academic Editor: Dominique J. Charron, Hôpital Saint-Louis, France
Received: May 21, 2022 **Accepted:** July 28, 2022 **Published:** October 31, 2022

Cite this article: Di Gloria L, Niccolai E. Utilization of formalin-fixed paraffin-embedded specimens for microbiota characterization in cancer: utility and concern. Explor Immunol. 2022;2:723–30. https://doi.org/10.37349/ei.2022.00078

Abstract

Microbiome research has enormous potential in cancer research and the use of formalin-fixed paraffin-embedded (FFPE) tissues could offer many advantages. The tumor microenvironment represents a suitable niche for specific microbes and evidence proves the presence of an endogenous tumor microbiota, here referred to as *oncobiota*. Awareness of the oncobiota role in tumorigenesis could have a large influence on cancer care, in terms of diagnosis, prevention, and treatment. Moreover, understanding the microbial-related tumor microenvironment, and its influence on tumor immune response and cancer cells will help define important pathogenetic mechanisms in cancer starting or progression. Routine collection of histopathological FFPE samples provides a large availability of specimens essential for affordable and impactful retrospective analyses and for getting robust statistical results. The FFPE tissues are common in the analysis of tumor biopsies including the tumor microbiota characterization which has an important role in the modulation of our immune system and consequently of tumor cells. However, the microbiota analysis starting from FFPE tissues presents methodological pitfalls and limits that may negatively affect the oncobiota research. After examining the methodological and analytical difficulties of this approach, this work seeks to offer workable solutions to promote that research area.

Keywords

Microbiome, formalin-fixed paraffin-embedded, cancer, oncobiota

Introduction

In the last decade, owing to next-generation sequencing (NGS) techniques, our knowledge about the microbiota implications in health and disease has rapidly increased. As widely recognized, humans live in symbiosis with trillions of microorganisms (bacteria, fungi, viruses, and others) that colonize their different body sites, forming the so-called human microbiota, an adjunct 'virtual organ' that plays essential functions for healthy human physiology. The deep interconnection between host and microbiome has reconceived the human

being as a superorganism, whose features can be understood in the context of the composite phenotype imparted by both host and microbial genomes [1]. The symbiotic relationship between host and microbial communities helps maintain homeostasis and control immune response, while many diseases, such as cancer, immune-dysregulated disorders, and cardiovascular diseases, can result from microbial dysbiosis [2]. As a result, to better understand human pathology, the study of the microbiome is currently considered to be an important component of the molecular pathologic epidemiology (MPE) research frameworks [3, 4]. Of note, considerable research has focused on the role of the host microbiome in human cancer development, a field that has been called "oncobiome" [5]. The tumor microenvironment represents a suitable niche for specific microbes and recent studies have launched the concept of an endogenous tumor microbiota, here referred to as oncobiota [6]. For instance, the vascularized surface of the tumors is suitable for aerobic species while the low oxygen levels inside solid cancers allow colonization of anaerobic species. Overall, despite the nourishment belonging from necrotic eukaryotic cells, the restricted access to nutrients and oxygen determines low biomass [7], with few exceptions as pancreatic cancer, which displays a higher microbial abundance than the normal tissue [8]. Besides the well-known infective etiology of some cancer [9], commensal bacteria may favor cancer initiation and progression in several ways, including the conversion of consumed substances into harmful metabolites, the direct secretion of toxic substances, and modulation of the anti-cancer immune response. Indeed, the microbiota can produce enzymes, microbe-associated molecular patterns (MAMPs), short-chain fatty acids (SCFAs), and other metabolites, capable of conditioning host cytokines secretion, cell cycle and survival, metabolic pathways, and DNA reparation [10]. Among others, bacteria like *Fusobacterium nucleatum* (*F. nucleatum*), *Bilophila wadsworthia*, and *Desulfovibrio desulfurican* [11] cause DNA oxidative stress by producing reactive oxygen species, while *Campylobacter jejuni* [12] generate double-stranded DNA breaks, driving tumorigenesis. What is more, intratumoral microorganisms can influence local immunity and halt anti-tumor response. For instance, *F. nucleatum* has been proven to inhibit the T lymphocyte (T-cell) mediated immune responses through the expressions of fibroblast activation protein 2 (Fap2) which binds and activates the inhibitory T cell immunoreceptor with Ig and ITIM domains (TIGIT) on natural killer (NK) cells and T cells [13]. Bacteria colonizing tumors not only influence their growth but also the response to treatment, for example transforming chemotherapy drugs, reprogramming tumor immunity, and influencing immunotherapy response [14, 15].

According to the METAgenomics of the human intestinal tract (MetaHit) project results, about 99% of the microbiome genes are bacterial [16]. Hence, the fine characterization of the oncobiota composition, in terms of which bacteria are present and what they do inside the tumor microenvironment, is important for the development of more tailored therapy regimens for a variety of malignancies, boosting the anti-cancer effect while reducing patient damage. Overall, awareness of the oncobiota role in cancer development could have a significant impact on cancer management, in terms of diagnosis, prevention, and treatment. Besides, it will be possible only as long as this field will develop a thorough understanding of the microbial-related tumor microenvironment and the causative links between cancer and bacteria.

In many cases, the oncobiota characterization is hampered by various technical problems, such as the analysis of samples with low microbial biomass, the problem of samples contamination, and most importantly, the availability of tumor patient-related samples. Moreover, way before the microbiota sequencing analysis, the first crucial step of the workflow is represented by sample choice, such as the tissue area, and its preservation method. Formalin-fixed paraffin-embedded (FFPE) tissue represents a precious source of cancer patient-related samples, that, if correctly employed, could increase the sample sizes and sites available for the oncobiota studies. Briefly, specimen preservation in FFPE consists in submerging in 10% neutral buffered formalin overnight followed by serial dehydration in gradually more concentrated alcohol ending with inclusion in paraffin [17]. The FFPE storage preserves the general integrity of the sample at room temperature allowing easier and cheaper handling, transport, and storage, compared to other storing methods representing the ideal choice in clinical routine [17, 18].

The formaldehyde, which is the main component of the formalin solution, has fixative proprieties, due to its chemical features: such a small aldehyde has a strong electrophilic behaviour, because of the low dispersal of the partially positive charge of the carbon engaged in double bond with oxygen. This charge

leads first to formalin transformation in methylol and then to a covalent bond of dehydrated methylol with another molecule [19, 20]. Being nucleophiles both the initiators and the final targets of this reaction, the formalin acts as a cross-linker between nucleophilic molecules such as amino acids and DNA bases resulting in sample fixation and long-term preservation. Moreover, the inclusion of paraffin wax block allows an easy cut of slices of desired size and the choice of specific areas for histological, proteomic, genomic, or microbial examinations. The possibility to choose a well-defined tissue area is crucial in oncobiota characterization because the oncobiota inside the tumor mass may be dissimilar from the outer surface [10].

Given its usefulness, many kits regarding nucleic acid extraction from FFPE are available and specifically designed for purifying DNA or RNA from cross-linkers caused by formaldehyde. Studying microbial composition and functionality starting from FFPE tumor tissues would enable us to efficiently deepen our understanding of microbial implications in tumor events thanks also to the possibility of comparing specimens collected even at a distance of years. However, there are some drawbacks when using FFPE specimens that can't be fully avoided leading to technical challenges which sum up to the ones already related to microbiota analysis.

Limits and concerns in using FFPE sample tissues

The FFPE storage of tumor tissues can bring many biases in microbiota characterization due to 1) the low biomass environment [21]; 2) the degradation and mutation to the DNA undergoes during the fixing and embedding process; 3) the contamination inherent to this biobanking process.

The microbiota identification in low biomass specimens runs into complications due to the strong contamination from host DNA and other microbes located in the reagents or the working place. For example, in this context, among the possible sequencing strategies for microbiota characterization, the whole genome sequencing (WGS) method could be less effective because 97% of generated reads will map on the human genome [7, 22] and solely the 3% will reflect the searched microbiota. Conversely, the 16S amplicon targeted sequencing can outperform WGS in bacterial microbiota studies, even if lacking species level resolution, detection of rare species, and other kingdom organisms (viruses and fungi) [23]. That said, also amplicon sequencing should follow certain precautions, since the use of primer pairs targeting the variable (V)3–V4 regions has been associated with an off-target amplification of human DNA, that can be avoided using primer pairs targeting the V1–V2 regions [24]. Nevertheless, the formalin damages to microbial DNA lead to other adv that, as explained below, directly counteract the last one.

In presence of formaldehyde, the DNA bases establish cross-linkers with other bases, histones, and proteins. In addition, formaldehyde reactions generate products which decrease the pH and raise both the DNA depurination rate and the C > U transition after C deamination leads to misplaced thymine (T) during polymerase extension [19, 25]. As a result, the DNA stability is considerably corrupted and errors are committed during polymerase chain reaction (PCR) and sequencing. Moreover, it causes the progressive fragmentation of the sample's DNA sugar-phosphate backbone, which is a crucial issue in the amplicon sequencing method [26, 27]. Of note, the DNA damages increase over time, significantly after four years of storage [27], potentially affecting the microbial community inferred from the sequenced reads. Amplicon sequencing targeting the 16S V4 region, being shorter than other setups (about 254 bp), could mitigate the probability of stochastic mutations and fragmentation in the DNA region [26], but, as said above, can lead to human DNA amplification in low microbiota environment.

In addition, the microbiota characterization in FFPE samples is also complicated by contamination of microbes that normally grow in paraffin [26]. Indeed, together with kit reagents' contaminants, they may hide the true microbial composition of the specimen with low biomass [23, 26]. While the contaminants from kit reagents and fresh frozen storage are usually water and soil associated and then easily recognizable [28], microbial taxa deriving from FFPE storage, such as Proteobacteria and Actinobacteria, can overlap with those truly populating the tumor sample [29]. Pinto-Ribeiro and colleagues [29] found that 12.9% of operational taxonomic units (OTUs) in their analysis were shared in paraffin controls and FFPE tissues and their removal significantly altered the inferred tissue taxonomic profile.

Conflicting results are reported in the literature about the comparison of paired microbiota between FFPE and fresh frozen preserved samples. In the same work, Pinto-Ribeiro et al. [29] did not find significant differences in alpha- or beta-diversity between the two methods of sample preservation, even if the microbial community present in FFPE tissues 12 months post-collecting was slightly different from that of frozen and FFPE tissues preserved for 6 months and 18 months. Also, DebesaTur and colleagues [30] reported comparable data between fresh frozen and FFPE tissues of colorectal cancer patients. Conversely, in a recent study Hockney et al. [26] showed that the fetal membranes-associated microbiota of FFPE and frozen stored samples were not comparable, and additionally that the microbiota of FFPE membranes was indistinguishable from the surrounding paraffin wax, highlighting the impact and biases of the processing methods on microbiota characterization. Likewise, Borgognone et al. [28] reported significant differences between the inferred microbiota compositions of paired FFPE and frozen colon rectal cancer tissues, which, following the authors' suggestion, greatly depends on contamination issues. Despite every potential measure taken to ensure results displaying only the true oncobiota profile, the analysis of microbiota from FFPE tumor tissues is often associated with inevitable background noises.

Advantages and clinical perspectives in FFPE specimens' utilization

Despite the above presented technical limits and concerns, the use of FFPE samples represents an extremely beneficial resource in oncobiome studies. First of all, FFPE preservation allows for the maintenance of the tissues at room temperature for a long time, requiring far less space and costs compared to other methods, and an easy transfer between facilities. Routine collection of histopathological FFPE samples provides a large availability of specimens essential for affordable and impactful retrospective analyses and to obtain robust statistical results. As already stated, the tumor microbiota could vary along the tumor mass, and FFPE samples offer the possibility to easily dissect tumor areas to compare the oncobiota associated with more or less vascularized and necrotic regions. These kinds of studies could help define the tumor-associated microbiota, allowing the understanding of microbes' implication in carcinogenesis and treatment response. Finally, applying *in situ* hybridization approaches, FFPE samples offer the possibility of studying the spatial localization of intratumoral microorganisms, unveiling their interaction with host cells in the tumor microenvironment and suggesting the mechanisms used to drive cancer promotion [31].

Attempts to overcome the challenges

In order to mitigate the existing issues and to successfully infer the oncobiota composition, many precautions should be taken by researchers during their workflows and here they are presented as a unique hypothetical protocol. First of all, the sample size and the sequencing depth should be increased in research projects using FFPE samples compared to the number necessary when utilizing fresh tissue [30]. Debesa-Tur and colleagues [30] suggest comparing when it is possible, the metagenomic analysis of paired FFPE and frozen tissue samples to assess the validity of the results from FFPE specimens, since they may be not optimal as a first choice, but complementary when fresh samples are unavailable.

Then, it will be necessary to include in the analysis a variety of negative controls to assess the degree of contamination introduced during processing steps (i.e. DNA extraction blank control, no-template control, sampling blank control) [32]. In particular, the inclusion of paraffin wax block controls, surrounding the analyzed tissue, is fundamental to detecting and removing paraffin contaminants [26, 29]. Increasing the sample size and including negative controls, could statistically reduce the bias from human and environmental contamination and then automatically refine results arising from the true comparison of the interest.

In addition, analyses such as quantitative PCR (qPCR), digital PCR, and electrophoresis gel would be helpful to control the DNA integrity before amplicon sequencing analysis [19, 26, 33]. Specific kits to repair DNA damage can also be employed [23].

To specifically avoid contaminant DNA, a number of precautions should be taken: 1) personnel should wear protections to cover exposed human surfaces; 2) the first few scrolls of the FFPE blocks should be discarded, the microtome regularly cleaned, and the selected slides collected in sterile tubes; 3) the DNA

extraction must be performed under the sterile condition through specific extraction kit for FFPE sample designed to solve the cross-linkers and mutations [28, 29, 33]. In particular, protocols based on uracil DNA glycosylase (UDG) in combination with heat denaturation and lesion repair enzymes should be applied to further solve formaldehyde-induced mutation [8, 23]. In addition, the frequency of formaldehyde inducted C > T transitions can be evaluated through global imbalance value (GIV) score which is computed comparing forward and reverse reads assuming that *in vitro* damages to DNA are more probably present only in one strand [34].

The DNA sequencing method should be carefully evaluated, since amplicon sequencing, compared to WGS, could avoid the problem of the proportionally more abundant human DNA in low biomass samples [23]. Nevertheless, the fragmentation caused by formaldehyde could make the choice of hypervariable target region difficult [24]. Alternatively, if using the WGS sequencing, host DNA depletion strategies are strongly recommended [35, 36]. The amplicon sequenced DNA reads should be clustered as OTUs and not as amplicon sequence variants (ASVs) that are distinguishable by a single nucleotide difference, since the punctiform mutations in DNA, occurring if formalin preservation, would increase the data noise [23, 37].

Finally, specific bioinformatics approaches will help detect and remove contaminants *in silico* after the sequencing as long as the number of filtered OTUs is acceptable and comparable among samples [30]. For example, host DNA can be aligned to the relative reference database and then removed [24, 29] while bacterial contaminants should be identified through direct OTU comparison with negative samples [37] or using tools such as SourceTracker and Decontam [28, 38, 39].

Conclusions

Microbiome research has enormous potential in health and diseases, and the use of FFPE specimens could be helpful, especially in oncobiome research. Anyway, methodological pitfalls and limits of this approach may negatively affect this research field. For this reason, it is imperative to adopt a precaution and a standardized protocol to acceptably reduce the related bias and exploit the potential behind this storage method.

Abbreviations

FFPE: formalin-fixed paraffin-embedded

OTUs: operational taxonomic units

PCR: polymerase chain reaction

WGS: whole genome sequencing

Declarations

Acknowledgments

We thank Prof. Amedeo Amedei and Prof. Matteo Ramazzotti for intellectual assistance to this paper.

Author contributions

LDG wrote the original draft. EN contributed to conceptualization, manuscript revision, and editing. Both authors read and approved the submitted version.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent to publication

Not applicable.

Availability of data and materials

Not applicable.

Funding

Not applicable.

References

1. Sleator RD. The human superorganism-of microbes and men. Med Hypotheses. 2010;74:214–5.
2. Hou K, Wu ZX, Chen XY, Wang JQ, Zhang D, Xiao C, et al. Microbiota in health and diseases. Signal Transduc Target Ther. 2022;7:135.
3. Mima K, Kosumi K, Baba Y, Hamada T, Baba H, Ogino S. The microbiome, genetics, and gastrointestinal neoplasms: the evolving field of molecular pathological epidemiology to analyze the tumor-immune-microbiome interaction. Hum Genet. 2021;140:725–46.
4. Ogino S, Nowak JA, Hamada T, Milner DA Jr, Nishihara R. Insights into pathogenic interactions among environment, host, and tumor at the crossroads of molecular pathology and epidemiology. Annu Rev Pathol. 2019;14:83–103.
5. Thomas RM, Jobin C. The microbiome and cancer: is the 'Oncobiome' mirage real? Trends Cancer. 2015;1:24–35.
6. Roche B, Ujvari B, Thomas F. Fourth international biannual evolution and cancer conference (Resistance, resilience, and robustness: Can we target cancer's evolutionary and ecological nature?). Evol Appl. 2018;11:829–35.
7. Zhang C, Cleveland K, Schnoll-Sussman F, McClure B, Bigg M, Thakkar P, et al. Identification of low abundance microbiome in clinical samples using whole genome sequencing. Genome Biol. 2015;16:265.
8. Pushalkar S, Hundeyin M, Daley D, Zambirinis CP, Kurz E, Mishra A, et al. The pancreatic cancer microbiome promotes oncogenesis by induction of innate and adaptive immune suppression. Cancer Discov. 2018;8:403–16. Erratum in: Cancer Discov. 2020;10:1988.
9. Pagano JS, Blaser M, Buendia MA, Damania B, Khalili K, Raab-Traub N, et al. Infectious agents and cancer: criteria for a causal relation. Semin Cancer Biol. 2004;14:453–71.
10. Khan AA, Sirsat AT, Singh H, Cash P. Microbiota and cancer: current understanding and mechanistic implications. Clin Transl Oncol. 2022;24:193–202.
11. Barrett M, Hand CK, Shanahan F, Murphy T, O'Toole PW. Mutagenesis by microbe: the role of the microbiota in shaping the cancer genome. Trends Cancer. 2020;6:277–87.
12. He Z, Gharaibeh RZ, Newsome RC, Pope JL, Dougherty MW, Tomkovich S, et al. *Campylobacter jejuni* promotes colorectal tumorigenesis through the action of cytolethal distending toxin. Gut. 2019;68:289–300.
13. Gur C, Ibrahim Y, Isaacson B, Yamin R, Abed J, Gamliel M, et al. Binding of the Fap2 protein of fusobacterium nucleatum to human inhibitory receptor TIGIT protects tumors from immune cell attack. Immunity. 2015;42:344–55.
14. Cheng WY, Wu CY, Yu J. The role of gut microbiota in cancer treatment: friend or foe? Gut. 2020;69:1867–76.

15. Bartolini I, Risaliti M, Tucci R, Muiesan P, Ringressi MN, Taddei A, et al. Gut microbiota and immune system in liver cancer: promising therapeutic implication from development to treatment. World J Gastrointest Oncol. 2021;13:1616–31.
16. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al.; MetaHIT Consortium, Bork P, Ehrlich SD, Wang J. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464:59–65.
17. Gaffney EF, Riegman PH, Grizzle WE, Watson PH. Factors that drive the increasing use of FFPE tissue in basic and translational cancer research. BiotechHistochem. 2018;93:373–86.
18. Berg D, Malinowsky K, Reischauer B, Wolff C, Becker KF. Use of formalin-fixed and paraffin-embedded tissues for diagnosis and therapy in routine clinical settings. Methods Mol Biol. 2011;785:109–22.
19. Do H, Dobrovic A. Sequence artifacts in DNA from formalin-fixed tissues: causes and strategies for minimization. Clin Chem. 2015;61:64–71.
20. Hoffman EA, Frey BL, Smith LM, Auble DT. Formaldehyde crosslinking: a tool for the study of chromatin complexes. J Biol Chem. 2015;290:26404–11.
21. York A. Tumour-specific microbiomes. Nat Rev Microbiol. 2020;18:413.
22. Lam SY, Ioannou A, Konstanti P, Visseren T, Doukas M, Peppelenbosch MP, et al. Technical challenges regarding the use of formalin-fixed paraffin embedded (FFPE) tissue specimens for the detection of bacterial alterations in colorectal cancer. BMC Microbiol. 2021;21:297.
23. Walker SP, Tangney M, Claesson MJ. Sequence-based characterization of intratumoral bacteria-a guide to best practice. Front Oncol. 2020;10:179.
24. Walker SP, Barrett M, Hogan G, Flores Bueso Y, Claesson MJ, Tangney M. Non-specific amplification of human DNA is a major challenge for 16S rRNA gene sequence analysis. Sci Rep. 2020;10:16356.
25. Kim S, Park C, Ji Y, Kim DG, Bae H, van Vrancken M, et al. Deamination effects in formalin-fixed, paraffin-embedded tissue samples in the era of precision medicine. J Mol Diagn. 2017;19:137–46.
26. Hockney R, Orr CH, Waring GJ, Christiaens I, Taylor G, Cummings SP, et al. Formalin-fixed paraffin-embedded (FFPE) samples are not a beneficial replacement for frozen tissues in fetal membrane microbiota research. PLoS One. 2022;17:e0265441.
27. Guyard A, Boyez A, Pujals A, Robe C, Tran Van Nhieu J, Allory Y, et al. DNA degrades during storage in formalin-fixed and paraffin-embedded tissue blocks. Virchows Arch. 2017;471:491–500.
28. Borgognone A, Serna G, Noguera-Julian M, Alonso L, Parera M, Català-Moll F, et al. Performance of 16S metagenomic profiling in formalin-fixed paraffin-embedded versus fresh-frozen colorectal cancer tissues. Cancers (Basel). 2021;13:5421.
29. Pinto-Ribeiro I, Ferreira RM, Pereira-Marques J, Pinto V, Macedo G, Carneiro F, et al. Evaluation of the use of formalin-fixed and paraffin-embedded archive gastric tissues for microbiota characterization using next-generation sequencing. Int J Mol Sci. 2020;21:1096.
30. Debesa-Tur G, Pérez-Brocal V, Ruiz-Ruiz S, Castillejo A, Latorre A, Soto JL, et al. Metagenomic analysis of formalin-fixed paraffin-embedded tumor and normal mucosa reveals differences in the microbiome of colorectal cancer patients. Sci Rep. 2021;11:391.
31. Serna G, Ruiz-Pace F, Hernando J, Alonso L, Fasani R, Landolfi S, et al. *Fusobacterium nucleatum* persistence and risk of recurrence after preoperative treatment in locally advanced rectal cancer. Ann Oncol. 2020;31:1366–75.
32. Eisenhofer R, Minich JJ, Marotz C, Cooper A, Knight R, Weyrich LS. Contamination in low microbial biomass microbiome studies: issues and recommendations. Trends Microbiol. 2019;27:105–17.
33. Flores Bueso Y, Walker SP, Tangney M. Characterization of FFPE-induced bacterial DNA damage and development of a repair method. Biol Methods Protoc. 2020;5:bpaa015.

34. Chen L, Liu P, Evans TC Jr, Ettwiller LM. DNA damage is a pervasive cause of sequencing errors, directly confounding variant identification. Science. 2017;355:752–6.
35. Nelson MT, Pope CE, Marsh RL, Wolter DJ, Weiss EJ, Hager KR, et al. Human and extracellular DNA depletion for metagenomic analysis of complex clinical infection samples yields optimized viable microbiome profiles. Cell Rep. 2019;26:2227–40.e5.
36. Marotz CA, Sanders JG, Zuniga C, Zaramela LS, Knight R, Zengler K. Improving saliva shotgun metagenomics by chemical host DNA depletion. Microbiome. 2018;6:42.
37. Callahan BJ, McMurdie PJ, Holmes SP. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. ISME J. 2017;11:2639–43.
38. Knights D, Kuczynski J, Charlson ES, Zaneveld J, Mozer MC, Collman RG, et al. Bayesian community-wide culture-independent microbial source tracking. Nat Methods. 2011;8:761–3.
39. Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome. 2018;6:226.