Metagemonic analysis of bacterial genetic diversity in blood samples of Iranian healthy women

Z. Pourramezan
Pasteur Institute of Iran
M. Oloomi (manaoloomi@yahoo.com)
Pasteur Institute of Iran
S. Bouzari
Pasteur Institute of Iran

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Abstract

Rising evidence proved the existence of dormant blood microbiota in healthy individuals, yet there is no information on the circulating microbiome of Iranian healthy women. The High-throughput Next-Generation Sequencing (NGS) provides a powerful method for the characterization of the blood microbiome. We investigated circulating bacterial composition in healthy individuals by 16S rDNA- based next-generation sequencing technique targeting the V3-V4 hypervariable region on the Illumina platform. We identified Operational Taxonomic Units (OTUs) similarity with various phyla, predominated by Proteobacteria (83%), Firmicutes (8%), Bacteroidetes (6%), and Actinobacteria (2%) phyla in healthy subjects. Based on the sequencing data, a diverse bacterial family was found in the whole blood of the healthy subjects, belongs to Burkholderiaceae (69%) and Enterobacteriaceae (13%) families on average. At the genus level, we illustrated the abundance of *Ralstonia*, *Rhizobium, Ignatzchineria, Lactococcus, Stenotrophomonas*, and *Ideonella*. Given the dominance of the putative blood microbiota within the different niches, it may be of considerable clinical significance and therapeutic strategies potential in the future. The blood microbiota can facilitate both the diagnosis and improved understanding of the onset of numerous human diseases as innovative biomarkers.

Introduction

The human microbiome consists of microorganisms that colonize on or within different bio-fluids and tissues such as the skin, oral mucosa, vagina, and gastrointestinal tract (Castillo et al. 2019). Most environments inside of the body such as blood in healthy individuals expected to be sterile and microbes were supposed to be found only in cases of sepsis. Nonetheless, there is documentation indicative of blood microbiota presence in healthy humans (McLaughlin et al. 2002; Amar et al. 2013; Païssé et al. 2016; Li et al. 2018; Whittle et al. 2019). Potgieter et al. (2015) explained that the blood microbiome is a consequence of a process termed atopobiosis in which organisms translocate from their classical niches such as the oral cavity, gastrointestinal tract, vagina, and skin to the circulation, yet the immune system function did not allow sepsis.

Advancements in high-throughput targeted metagenomics sequencing and bioinformatic tools enabled phylogenetic investigations of the uncultured bacterial community, thereby improving the original perspective on the taxonomic profile of the blood microbiome and dynamic changes of the microbiota (Païssé et al. 2016; Whittle et al. 2019). According to both the 16S rRNA gene and metagenomic sequencing of blood bacterial profiles, the blood-microbiome differed significantly from the gut microbiome (Whittle et al. 2019). The gut microbiota is predominated by Firmicutes and Bacteroidetes (Eckburg et al. 2005), while the blood microbiome is dominated by Proteobacteria followed by Actinobacteria, Firmicutes, and Bacteroidetes (Amar et al. 2013; Païssé et al. 2016; Li et al., 2018; Whittle et al. 2019). Moreover, geographic origin, lifestyle, and behavioral patterns can change the diversity and complexity of microbiota (Senghor et al. 2018).

On the other hand, many studies have reported that changes in human microbial community composition may lead to the onset of diseases (Petersen and Round 2014). In order to associate the human microbiome and medical diseases, we should first get information from multinational cohorts' studies of healthy human microbiota (lacking a disease phenotype) (Vangay et al. 2018). Understanding healthy blood microbiome changes might be analogous to predicting indispose.

In this study, we performed 16S rDNA-based sequencing on the blood of Iranian healthy individuals to profile the microbial landscape in peripheral circulation and estimated its potential connection with the health state. We use "healthy" to refer to the absence of any obvious disease as defined by Aagaard et al. 2012.

Materials And Methods

Blood specimen collection

The study was conducted on healthy blood donors at the Pasteur Institute of Iran, Tehran, Iran. Written informed consent was obtained from all the participants. The criteria for the selection of blood donors are set by the Pasteur Institute of Iran (IR.PII.REC.1397.008). To give blood, volunteers must be between 20 and 70 years old (years old on average); weigh at least 50 kg. Exclusion criteria included a body mass index (BMI) outside the range of 18 to 35 kg/m^2, being pregnant; and suffering from specified chronic diseases and infectious diseases, smoking, and alcohol consumption. Excluded from the donation are volunteers who are under certain medical treatments such as corticosteroids, immunosuppressive agents, antibiotics, or probiotics within the last 6 months. Donors must wait for defined time intervals after dental treatment, surgical intervention, body piercing, or returning from specific countries. All of the samples were given under strict aseptic conditions.

Dna Extraction

DNA extraction of the whole blood (200µL) was performed under a Class II biologic safety cabinet, using a PCR template preparation kit (Roche, Germany) according to the manufacturer's protocol. The DNA extraction was carefully designed to minimize any risk of contamination between samples. The quality and quantity of extracted DNA were examined by 1% agarose gel electrophoresis (in Tris Borate EDTA) and by the microplate reader (BioTek, USA). All DNA samples were stored at – 20°C until further analyses.

Amplicon 16S generation and next-generation sequencing

Amplification of hypervariable regions (V3 and V4) of 16S rRNA was used to characterize taxonomic diversity present in blood samples. A nested amplification was performed using PCR product from the first reaction as a template. The universal external primers for the conservative V3 and V4 regions of 16S rRNA were used that included the overhang adapter sequences attached to the 5' end of prims (Table 1).

Table 1
Used primers in 16S Illumina sequencing

Primer name	Sequences	Reference
341F	CCTAYGGGRBGCASCAG	(Hjelmsø et al. 2014)
805R	GGACTACNNGGGTATCTAAT	
515F	GTGCCAGCMGCCGCGGTAA	(Caporaso et al. 2011)
806R	GGACTACHVHHHTWTCTAAT	

DNA amplification was done by using PCR Master Mix Phusion® (New England Biolabs, US) and amplified following the protocol described previously (Magoč and Salzberg 2011). PCR products with a bright main strip between 400–450 bp were extracted from 2% agarose gels and purified using the Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries generated using NEBNext®, Ultra DNA Library PreKit for Illumina, following manufacturer's recommendations and index codes added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific, USA) and Agilent Bioanalyzer 2100 system. Sequencing procedure performed on the Illumina platform at Novogene Bioinformatics Technology Co., Ltd, Beijing, China.

Sequence Analysis

Pairs of reads from the original DNA fragments were merged by using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/). Sequences analyzed by Quantitative Insights In to Microbial Ecology (QIIME) package V1.7.0, (http://qiime.org/index.html) (Caporaso et al. 2011). The tags are compared with the reference database (Gold database) (http://drive5.com/uchime/uchime_download.html) (Edgar et al. 2011). Sequence analysis was performed by using Uparse software (Uparse version 1.0.1001, http://drive5.com/uparse/) (Edgar, 2013).

Sequences with ≥ 97% similarity were assigned to the same OTU. OTUs abundance information was obtained by normalizing the sequence number corresponding to the sample with the least sequences (OTU) counts rarefied to 103,744 reads per sample.

Data Analysis And Statistics

Frequency of OTUs across samples compared using non-parametric ANOVA (Kruskal-Wallis test). Differential abundance of OTUs among sample groups was analyzed using DESeq2 (negative binomial Wald test). Analysis between age and blood microbiome were calculated using the Spearman test using SPSS version 17.0 statistical software.

Results

A comparison of phylum-level composition indicated similar patterns in the blood microbiome of healthy volunteers (Fig. 1a, b). We observed that Proteobacteria (83%), Firmicutes (8%), Bacteroidetes (6%), and Actinobacteria (2%) were overrepresented in all healthy subjects. The composition of the putative blood microbiota of healthy women at the class levels assessed by 16S targeted metagenomic sequencing was shown in Fig. 2. Betaprotebacteria (47%), Gammaproteobacteria (29%), Bacteroidia (8%), Clostridia (7%), Alphaproteobacteria (4%), and Bacilli (3%) consist the predominant classes on average.

Family-level analyses showed highly consistent changes with those of the aggregate microbiota (Fig. 3), suggesting that shifts of the putative gut-derived organisms contributed predominantly to the blood microbiome that predominated by Burkholderiaceae (69%) and Enterobacteriaceae (13%) families on average.

The abundance of predominant blood microbiota of healthy women at the genus level identified by taxonomic classification presented in Fig. 4, which included *Ralstonia*, *Rhizobium, Ignatzchineria, Lactococcus, Stenotrophomonas*, and *Ideonella* dominated the blood microbiota in Iranian healthy women.

We did not observe the significant relations between ages and the blood microbiota.

Discussion

Although conventional culture-based methods did not approve the existence of a human blood microbiome, development of NGS allowed a complete study of microbial diversity at various niches including blood samples taken from healthy volunteers.

There are some studies about gut microbiota composition in healthy Iranian subjects (Marvasti et al. 2020; Heidarian et al. 2019), but as far as we know, their blood microbiome has not been examined so far. In the current study, we performed 16S rRNA gene NGS sequencing of DNA from the blood of healthy women in Iran and illustrated the bacterial composition of healthy blood subjects.

Similar healthy blood microbiome compositions reported in some studies worldwide imply the presence of a predominant microbiome profile in the bloodstream (Amar et al. 2013; Païssé et al. 2016; Lelouvier et al. 2016), and eliminate the hypothesis of contamination during the collection of blood samples. Here are the key findings of these researches. Amar et al. (2011) stated that Proteobacteria represented 90% of the overall microbiota in human blood samples in France. Shah et al. (2019) compared the blood microbiome of 20 healthy controls and 20 patients with CKD and showed that Proteobacteria (54%), Bacteroidetes (21%), Actinobacteria (18%), Firmicutes (4%) mostly consist of the blood microbiome of healthy controls in the USA. Païssé et al. (2016) illustrated that blood fractions contain bacterial DNA mainly from the Proteobacteria phylum (more than 80%), followed by Actinobacteria (6.7–10%), Firmicutes, and Bacteroidetes. According to Rajendhran et al. (2013), Proteobacteria and Firmicutes were the two major phyla with relative abundances in the blood microbiota of healthy human samples in India. Among non-cultured blood samples examined by Panaiotov et al. (2018), bacterial phyla of Proteobacteria (93%),

Actinobacteria (2%), Planctomycetes (2%), and Firmicutes (2%) predominated in blood samples of healthy individuals from Bulgaria. Whittle et al., (2019) detected 16S rDNA of Proteobacteria (74.9%), Firmicutes (19.5%), Bacteroidetes (0.05%), and Actinobacteria (0.01%) in the blood of the healthy population of the United Kingdom. We confirmed the previous studies and observed the presence of Proteobacteria (83%), Firmicutes (8%), Bacteroidetes (6%), and Actinobacteria (2%) phyla in healthy subjects in the Iranian population (Fig. 1). The difference in the values reported in various studies may be attributed to a difference in geographic factors, such as local cuisines and the ethnicity of healthy humans studied.

Based on the sequencing data, Betaprotebacteria (47%), Gammaproteobacteria (29%), Bacteroidia (8%), Clostridia (7%), Alphaproteobacteria (4%), and Bacilli (3%) consist the predominant classes on average, while Païsséet al. (2016) stated that Alphaproteobacteria (34–54%), Betaprotebacteria 17.35–21.9%), Gammaproteobacteria (10–28%), Actinobacteria (6–10%), Bacilli (2.68–3.74%) are the most predominant classes of bacteria in healthy blood.

According to the results, a diverse bacterial family was found in the whole blood of the healthy subjects that the most abundant of them belong to Burkholderiaceae (69%) and Enterobacteriaceae (13%) families on average.

The relative abundance of a few genera reported in this study turned out to be contrasting to some previous studies on healthy individuals from other geographical areas. This may attribute to the lifestyle of the host, and behavioral patterns of the healthy subjects, or the relatively limited sample size of this study. According to Qian et al. (2018), and Whittle et al. (2019), the major bacterial DNA genera found in the blood samples of healthy controls are *Limnobacter* and *Achromobacter*, respectively. Interestingly, Iranian women appear to have a high blood microbiota diversity, with dominant bacterial genera such as *Ralstonia*, *Rhizobium, Ignatzchineria, Lactococcus, Stenotrophomonas*, and *Ideonella* detected in the blood of healthy individuals. This is the first report of *Ideonella* presence in the blood of healthy individuals.

In the current study, *Ralstonia* by more than 1300 OTU counts is the most predominant genus that closely resembles the oral microbiomes. It showed that damage caused by various daily activities including tooth brushing, potentially as a result of damage caused by leakage across the mucosal surfaces, chewing, and flossing could lead to the translocation of bacteria from the oral cavity into the bloodstream (Parahitiyawa et al. 2010; Horliana et al. 2014). Widmer et al., (2018) showed that pulp spaces of pristine healthy teeth contain detectable bacterial DNA including *Ralstonia*, *Actinetobacter*, and *Staphylococcus* (43–78% of the total community). Whittle (2019) suggests that the blood microbiome community is perhaps more likely to result from the translocation of organisms from the oral cavity and skin, than from organisms that colonize the gastrointestinal tract.

On the other hand, the airborne soil bacteria are continuously inhaled, being constantly devastated in the surface mucosa of the respiratory system, then the released rDNA may enter the bloodstream directly or carry by phagocytes. This may also explain why some of our blood-associated rDNA sequences differed

from those found in studies reported previously from geographically different laboratories (Nikkari et al. 2001; McLaughlin et al. 2002; Brecher and Hay 2005). According to the results, *Rhizobium* and *Stenotrophomonas* are the most abundant soil-derived bacteria in the Iranian blood microbiome of a healthy human, whereas Li et al., (2018) stated that some soil-derived bacteria were dominated the blood microbiota in 17 healthy individuals from China that included *Stenotrophomonas*, *Pseudomonas*, and Corynebacterium. Païssé et al. (2016) detected the presence of *Stenotrophomonas* in RBCs of healthy donors assessed by 16S targeted metagenomic sequencing, too. Besides, Shah et al. (2019) showed the presence of *Stenotrophomonas* in a healthy blood microbiome.

Some variables such as dietary habits, age, and the use of probiotics and antibiotics influence the composition of an individual's microbiota (Azad et al. 2016). As a result, the relative percentage of each taxon varies between individuals. Further study of individuals representing different cultural traditions and ethnic origins will allow us to observe greater diversity in the microbiome composition according to individual provenance (Davenport et al. 2017).

Identification of the blood microbiome provides novel insights into characteristics and diagnostics of transfusion-transmitted bacterial infection through blood transfusion. Further study is required to associate the possible impact of the blood microbiome on health and disease.

Conclusion

The blood microbiome can facilitate both the diagnosis and improved understanding of the onset of numerous human diseases as innovative biomarkers. Given the dominance of the putative blood microbiome within the different niches, it may be of considerable clinical significance and therapeutic strategies potential in the future. Future, larger studies may employ to investigate blood microbiome changes across time.

Declarations

Acknowledgements

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Author contributions

Zahra Pourramezan: Investigation; formal Analysis; writing-original draft; and editing writing manuscript. Mana Oloomi: Project Administration; conceptualization; methodology, and supervision. Saeed Bouzari: Methodology and editing writing manuscript.

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Conflict of interest The authors declare that there were no conflicts of interest.

Ethics approval and consent to participate The study protocols were approved by the institutional ethics review board at Pasteur Institute of Iran (IR.PII.REC.1397.008). Informed written consent was taken from every patient before recruiting in the study.

Consent for publication Not applicable.

Availability of data and materials It will be provided on request and there is no supplementary data provided.

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Figures

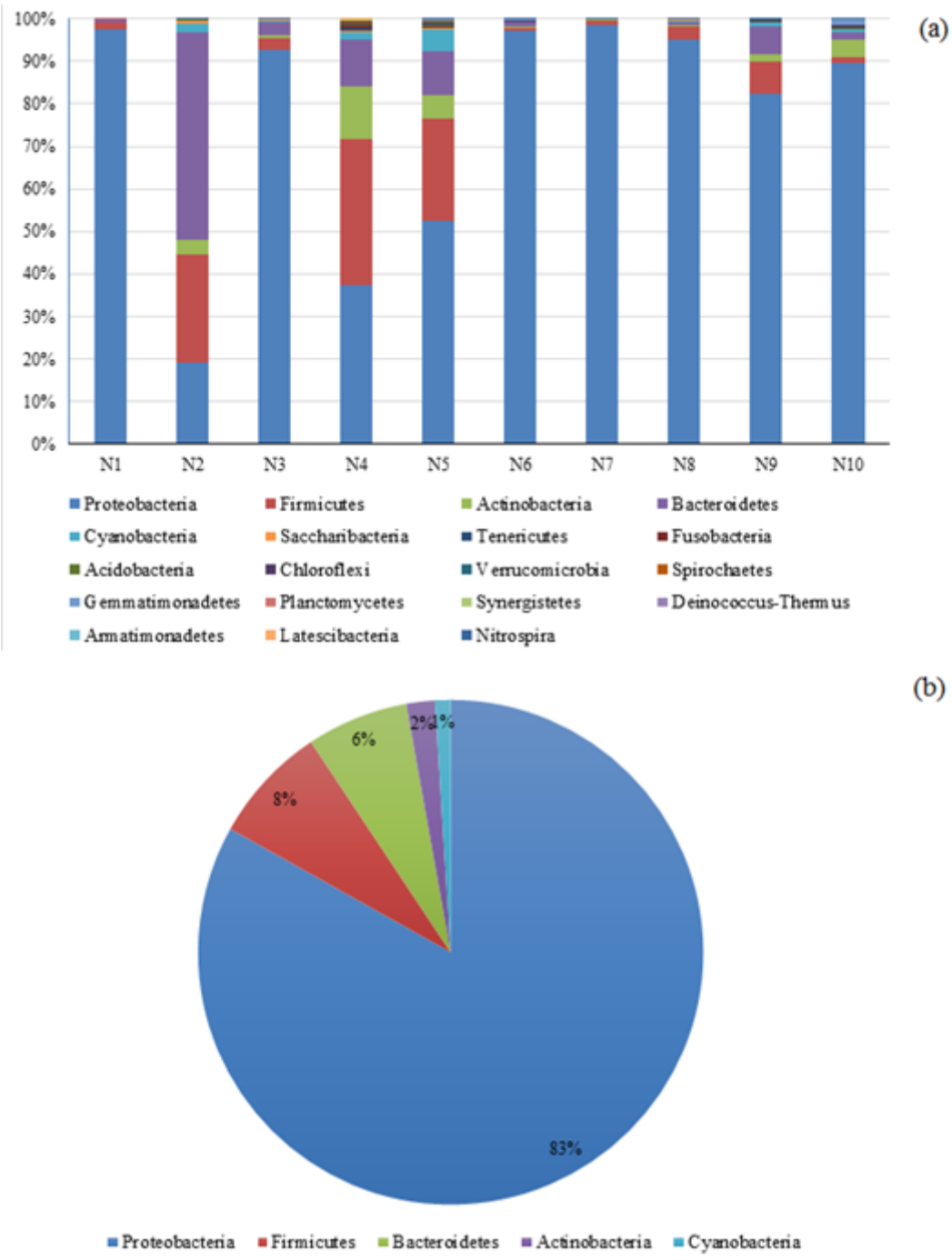

Figure 1

(a) Composition of the putative blood microbiota of healthy women at the phylum levels assessed by 16S targeted metagenomic sequencing. (b) Pie charts representing the mean relative proportions of bacterial phylums in whole blood samples. Data are mean abundance expressed as a percentage of the total bacterial sequence count.

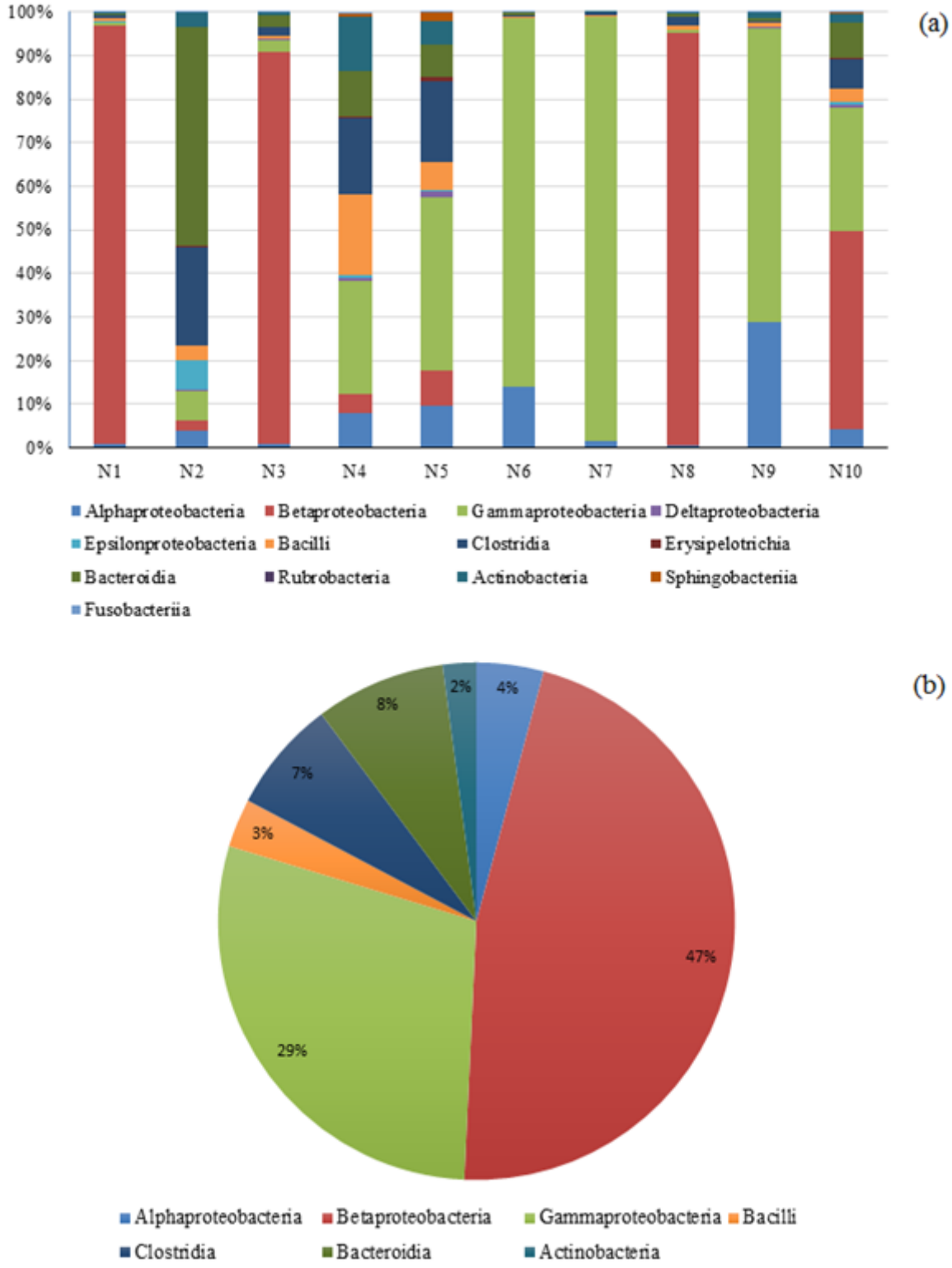

Figure 2

Composition of the putative blood microbiota of healthy women at the class levels assessed by 16S targeted metagenomic sequencing. (b) Pie charts representing the mean relative proportions of bacterial classes in whole blood samples. Data are mean abundance expressed as a percentage of the total bacterial sequence count.

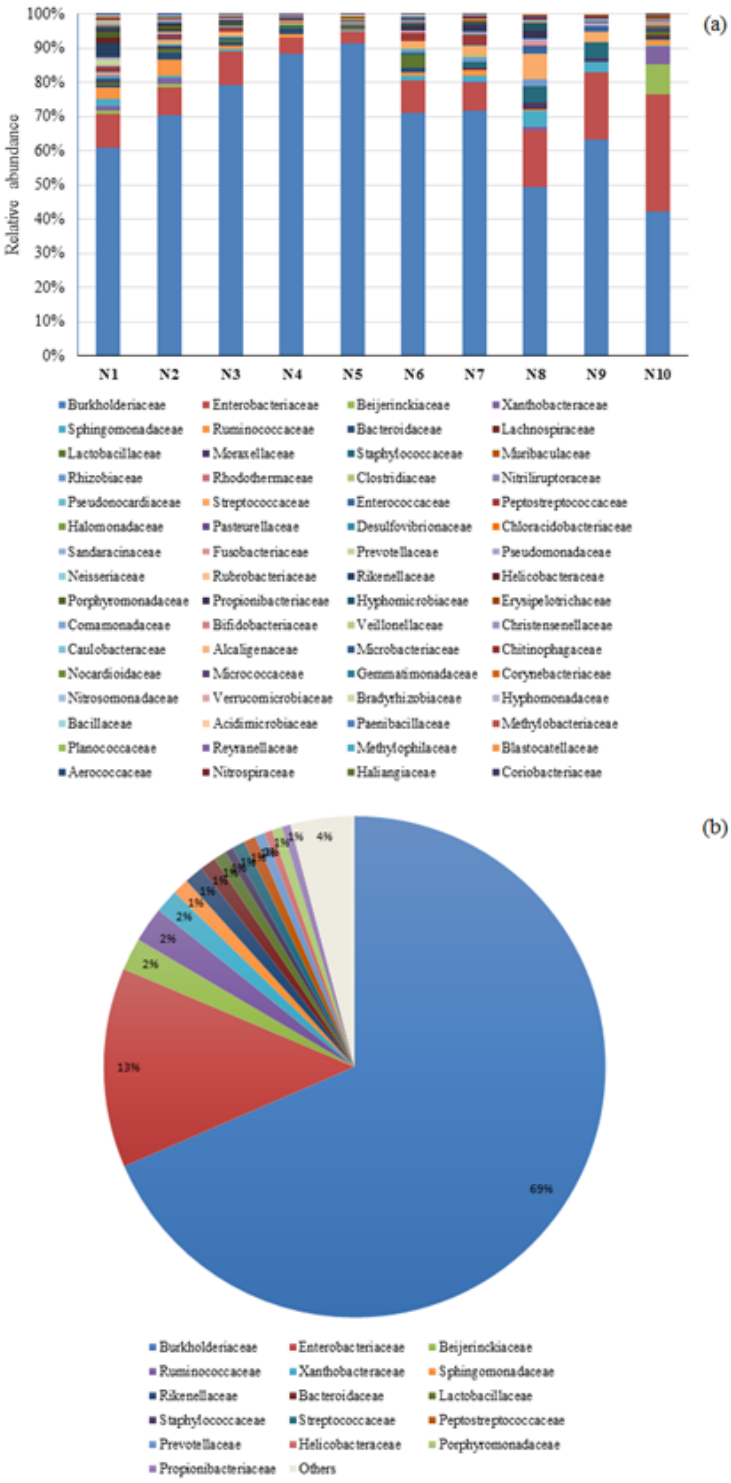

Figure 3

(a) Composition of the putative blood microbiota of healthy women at the family levels assessed by 16S targeted metagenomic sequencing. (b) Pie charts representing the mean relative proportions of bacterial

families in whole blood samples. Data are mean abundance expressed as a percentage of the total bacterial sequence count.

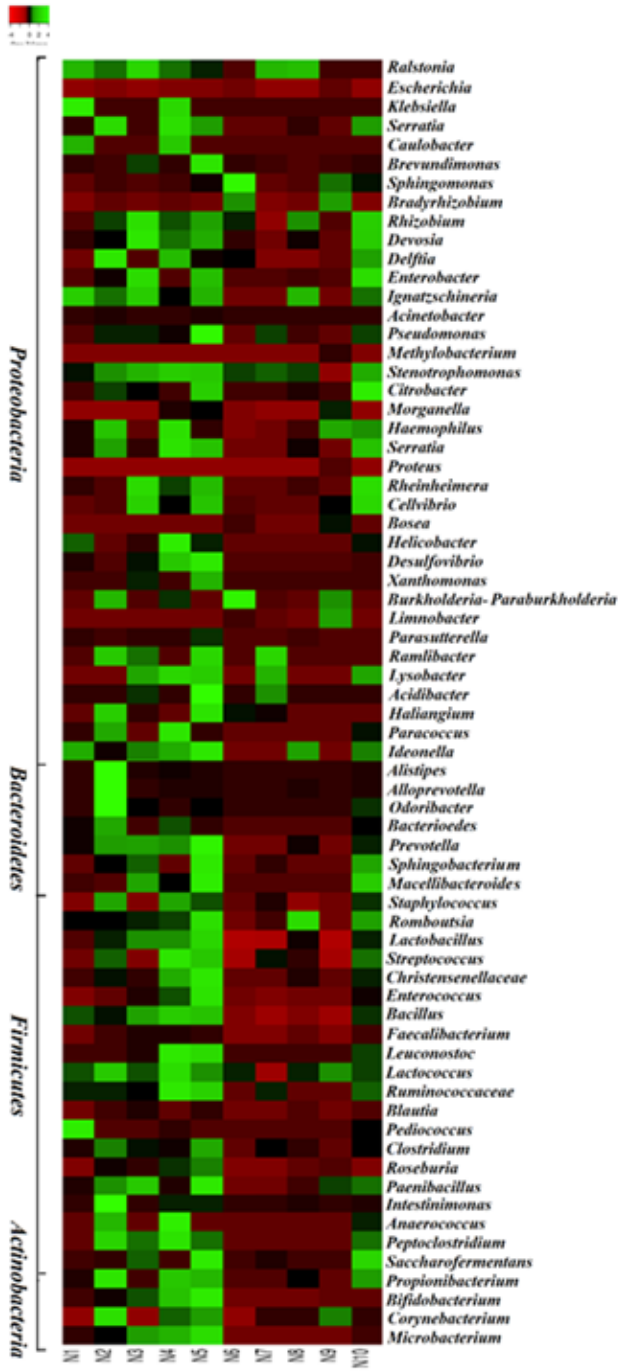

Figure 4

Heatmap showing the abundance of predominant blood microbiota of healthy women at the genus level identified by taxonomic classification. The data represent the Log10 values of the operational taxonomic unit (OTU) counts each genus.