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Cultural Isolation and Characteristics of the Blood Microbiome of Healthy Individuals

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Abstract

Background: On the analogy of the non-pathogenic microbiota found in oral cavity, skin and gastrointestinal tract, existence of blood microbiota was confirmed by DNA sequencing, but never deeply characterized. Hypothesis for the existence of dormant blood microbiota in healthy humans have been arisen and single species have been isolated. The aim of our study was to resuscitate and investigate the biodiversity of bacterial and fungal dormant blood microbiota in healthy individuals by blood culturing and NGS DNA sequencing. **Results:** Twenty eight blood samples of healthy individuals, seven for each blood type, were studied. Several culture media were tested. Blood microbiota resuscitation was performed in BHI broth supplemented with vitamin K 1 mg/ml, 2% sucrose, 0.25% sodium citrate and 0.2% yeastolate at 43°C for 72 h. All tested blood samples were culture positive, as confirmed by Gram staining and TEM. TEM images demonstrated well defined cell structures. Analysis for bacterial and eukaryotic species was performed by 16S rRNA and ITS2 targeted sequencing. The obtained sequences were clustered (≥97% identity) in Operational Taxonomic Units (OTUs). Among cultured and uncultured samples we identified OTUs similarity with 47 bacterial orders belonging to 15 phyla and 39 fungi orders blonging to 2 phyla. For the first time we demonstrated isolation and sequencing identification of fungal blood microbiota in healthy individuals. Blood-group differences were identified among the bacterial microbiome compositions. **Conclusion:** The dormant blood microbiome is innate of the healthy individuals. Interventional strategies to bind the host blood microbiome with the states of health and disease remain an unmet research goal.

Keywords

Blood Microbiota, Targeted Next Generation Sequencing, Operational

Taxonomic Unit

1. Introduction

In the human and animal evolution many microbial species successfully adapted to the macroorganism. Most of them could not be cultured and are proven indirectly by DNA sequencing. On the bases of sampling 242 healthy adults at 18 different anatomical sites of the human body and sequencing the 16S rRNA genes the presence of 5177 microbial taxonomic profiles was proven, but only 800 of them could be cultured [1]. The observation of cell-free DNAemia is well described feature of the healthy blood [2] [3], but the presence of transient culturable blood microbiota in the blood of healthy individuals could also be supposed. We tested whether the presence of DNA in healthy blood is associated with DNAemia or it is due to existing blood microbiota. It is proven that in the blood of clinically healthy individuals microorganisms could persist for many years without causing illness. The most investigated examples being the latent tuberculosis.

Nevertheless the blood microbiome is still an enigma, its existence was proven during the last 50 years. Indirect evidences for existence of bacteria residing in erythrocytes have been predicted in the past by radiometric methods [4]. In 1969 Tedeschi *et al.* reported on incorporation of nucleosides in human erythrocyte attributed to the metabolic activity of mycoplasma or bacterial L-forms [4]. In 1977, Domingue and Schlegel identified in 7% of the blood specimens from supposedly healthy individuals novel bacterial structures [5]. In 1993 the Bulgarian scientist Emil Kalfin experimentally proved by culturing and electron microscopy that microorganisms are multiplying in the erythrocytes of healthy people [6]. Kalfin reported 100% positivity of the blood cultures. Subsequently several other authors questioned the existence of the blood microbiome or DNAemia in healthy individuals, Domingue (1977) [5], (1997) [7], Nikkari *et al.* (2001) [8], Mc Laughlin *et al.* (2002) [9], Moriyama *et al.* (2008) [10], Markova (2015) [11], Damgaard (2015) [12] Dimova *et al.* (2017) [13], Gosiewski *et al.* (2017) [2] and Kowarsky *et al.* (2017) [3]. The authors reported supporting electron microscopy, cultural and molecular data in favour of the existence of the blood microbiota in healthy individuals. Hypothesis on the bacterial structure of the blood microbiota in healthy humans have been arisen [5] [6] [12], but cultures for exhaustive microbiota analysis remain a target. Rich bacterial diversity in the blood of healthy individuals was confirmed by 16S rRNA genes sequencing [14] and total RNA sequencing [15]. In 2016 Paise *et al.* demonstrated that a diversified microbiome exists in healthy blood. Most of the blood bacterial DNA was found located in the BC (93.74%), while RBCs contain more bacterial DNA (6.23%) than the plasma (0.03%) [14]. A significant number of bacterial species were also detected in the blood of healthy chickens [16] and cats

by NGS analysis [17]. Resuscitation of dormant blood microbiota in healthy individuals has been tested and single bacterial species have been isolated on agar plates [12]. However, presence of eukaryotic microbial cells in the blood of healthy individuals has never been challenged. Goal of our study was to resuscitate the blood microbiota of healthy individuals and apply cultural, microscopic and NGS targeted sequencing methods for their characterization. Here, we demonstrate that bacteria and fungi constitute a rich microbial diversity in the blood of healthy humans by applying a successful culture resuscitation strategy, DNA isolation and NGS targeted sequencing analysis. First preliminary results of the study have been presented as poster at the ASM Microbe congress 2017 [18].

2. Materials and Methods

Ethical committee approval of the study (decision 38/14.07.2016) by the Institute of Neurobiology, Bulgarian Academy of Sciences (BAS) and individual written consent were obtained.

2.1. Culturing

Blood of 28 healthy volunteers was collected in Vacutainer tubes with K_3EDTA as anticoagulant (Vacutainer K3E, BD, USA), 7 samples per blood group. The blood samples were divided in two parts. One part for culturing (3 mL) and another part for direct DNA isolation (7 mL). All blood samples were tested for sterility by growing on Sabouraud and blood agar.

We applied a modified resuscitation strategy previously developed by Emil Kalfin [6]. Three ml of blood sample were added to 22 ml of culture medium. Culturing was performed in sterile 50 ml polypropylene Falcon tubes (Corning Inc, USA). The culture base medium was composed by Brain Hearth Infusion (BHI, Difco, USA) medium and 0.2% yeastolate (Difco) adjusted at pH 6.8 and sterilized. Sterile (D+) sucrose at 10% final concentration and water-soluble form of vitamine K_3 - menadione sodium bisulfite (Sigma-Aldrich, USA) in concentration of 1 mg/ml sterilized by filtration were added to the base medium. Resuscitation growth was induced at 43°C at 24 h, but 72 h was found to be optimal time for growing blood microbiota in liquid cultures. The agar medium for subculturing contained 1.2% Noble agar (Difco, BD, USA). Isolated blood microbiota was confirmed by Gram staining and 16S rRNA genes PCR analysis. Gram staining was directly applied on the cultured sample.

2.2. Transmission Electron Microscopy (TEM)

Blood samples for TEM were processed within 1 hour after collection. Sterile heparinized blood collected in Vacutainer tube (BD, USA) was diluted 1:3 with PBS pH 7.4 (Thermo Fisher Scientific, USA) and centrifuged at 400 g for 30 min on Histopaque-1077 (Sigma-Aldrich, USA) density gradient. The peripheral blood mononuclear cells were removed from the gradient, washed 3 times in

PBS, then washed in RPMI 1640 (Sigma-Aldrich, USA) medium with 10% FCS (Sigma-Aldrich, USA) and processed for TEM. The cells were resuspended in 3% low gelling temperature agarose (Sigma-Aldrich, USA) and put on ice. The solidified agarose was cut into 1 mm^3 cubes, then fixed in 2.5% glutaraldehyde (Sigma-Aldrichm USA), postfixed in 1% osmium tetraoxide (Sigma-Aldrich, USA) and dehydrated in increasing concentrations of 30, 50, 70, 96% of ethanol for 10 min each, 100% ethanol for 2 × 20 min and propylene oxide for 2 × 20 min. Specimens were impregnated in propylene oxide: Durcupan ACM (Sigma-Aldrich, USA) 1:1 and embedded in Durcupan ACM. Polymerisation was carried out at 60°C for 18 h in an oven. The samples were thin cut to 20 30 nm and observed under transmission electron microscope Opton EM 109 (Zeiss, Germany).

2.3. Physico-Chemical Analysis and Radioresistance

Disruption of cells with 0.1 mm glass beads with beat beater machine (Mini-beatbeater, Biospec Products, USA), treatment in microwave oven at 850 W and disintegration with ultrasound (MSE, UK) on ice was applyed. Integrity of the cells in fresh cultures was tested by treatments with 4 M guanidine thiocianate, 10% NaOH, 10% KOH, 10% CH_3COOH, 10% HCl and 10% H_2SO_4 for 30 min at 37°C.

To test the level of the ionizing radiation that the microbiota are able to withstand we applied gamma irradiation with ^{60}Co (Isledovatel MPX-γ-25M, TENEX, Moscow, RU) at 15, 20 and 25 kGy to 5 ml of blood from three healthy donors and erythrocyte concentrate of blood group A, Rh(+). The erythrocyte concentrate was a sample produced by collecting blood from several blood donors at the National Center for Transfusiology and Hematology in Sofia, Bulgaria. In order to control the quality of irradiation two control blood samples were spiked with *Candida guiliermondii* and *Candida albicans* with 10^9 cells. After irradiation with dose of 15 kGy, fungi inoculated control samples were tested for growth on Sabouraud agar. Control plates were negative after two weeks of culturing at 30°C.

2.4. DNA Isolation and Sequencing Analysis

Seven commercial kits were tested for DNA isolation of the cultured blood microbiota: Tissue and cells genomic Prep kit and Blood genomic Prep kit, (GE, USA); Genomic DNA purification kit (Thermo Fisher Scientific, USA); QIAamp DNA isolation kit (Qiagen, Germany); NucleoSpin Soil and NucleoSpin DNA Stool kit (Macherey-Nagel, Germany); and RIBO-prep kit (Ecoli s.r.o., Slovakia). Most of the DNA extraction kits: Tissue and cells genomic Prep kit and Blood genomic Prep kit, (GE, USA); Genomic DNA purification kit, (Thermo Fisher Scientific, USA); QIAamp DNA isolation kit, (Qiagen, Germany) and the standard CTAB phenol/chlorophorm DNA extraction protocol were not successful for DNA extraction of the cultured blood microbiota. Successful DNA extrac-

tions were obtained with NucleoSpin Soil and NucleoSpin DNA Stool kits (Macherey-Nagel, Germany) and RIBO-prep kit (Ecoli s.r.o., Slovakia) applying the following essential modifications: After 72 h of culturing the tubes were centrifuged at 5000 g for 15 min. The pellet was washed by vortexing with 40 ml of DNase I 1 U/ml (Thermo Fisher Scientific, USA) in ddH$_2$O and incubated for 30 min at 37˚C in order to eliminate residual DNA contaminations associated with lysed nuclear blood cells, DNA from the culture medium or DNA traces in water. Centrifugation and washings were repeated three times. These steps lyse the blood cells, wash away medium and human blood cells DNA/RNA contaminations. The cell pellet was resuspended in 2 ml of TE buffer (pH 7.5). The same procedure was applied for direct microbiota isolation from whole blood. Briefly, 7 mL of blood were lysed by vortexing with 40 ml of DNase I 1 U/ml in ddH$_2$O and incubated for 30 min at 37˚C. After centrifugation at 5000 g for 15 min the same procedure was repeated three times. The pellet from the lysed whole blood was resuspended in 400 μl of TE buffer. One hundred μl of cell suspension was processed according to the manufacturer instructions and modifications. The cell suspension was subjected eight times to freeze-thawing in liquid nitrogen and dry bath at 96˚C for 10 min combined with vigorous vortexing or optional homogenization with ceramic/zirconium 0.6 - 0.8 mm beads on a bead beater for 5 min (Biospec Products, USA). The extracted DNA was resuspended in 100 μl of TE and repurified by addition of 5 μl of 20% Chelex 100 (Bio Rad, USA). Suspension was vigorously vortexed for 1 min and centrifuged for 10 min at 15.000 × g. The supernatant, ~95 μl was recovered in a new tube without touching the Chelex 100 pellet. DNA typical yield was >150 ng/μl with a 260/280 nm ratio of ~1.7.

For DNA analysis we applied 16S rRNA genes and ITS2 targeted sequencing on Illumina MiSeq (Illumina Inc., USA). DNA sequencing was performed at IMGM Laboratories GmbH (Martinsried, Germany). For bacteria identification V3 - V4 hypervariable regions of the 16S rRNA genes were amplified with universal primers 314F and 805R. For fungi identification ITS2 region was amplified with universal primers ITS3 and ITS4. The obtained sequences were clustered (≥97% identity) into Operational Taxonomic Units (OTUs). OTUs were analyzed for sequence similarities against reference sequence databases; SILVA database for 16S rRNA gene (https://www.arb-silva.de/) and UNITE for ITS2 (https://unite.ut.ee/). Common sequences between the samples and the negative (dH$_2$0) and culture medium DNA controls were excluded from analysis. These were considered as contaminants from reagents and culture medium. For taxonomic affiliation the interference created by the MiSeq sequencer as well as those created between samples was also considered.

3. Results and Discussion

All tested blood samples were culture positive, as confirmed by Gram staining and TEM (Figure 1). Optimal growing temperature was at 43˚C. At 37˚C growth of the blood microbiota was suppressed. However, to our knowledge
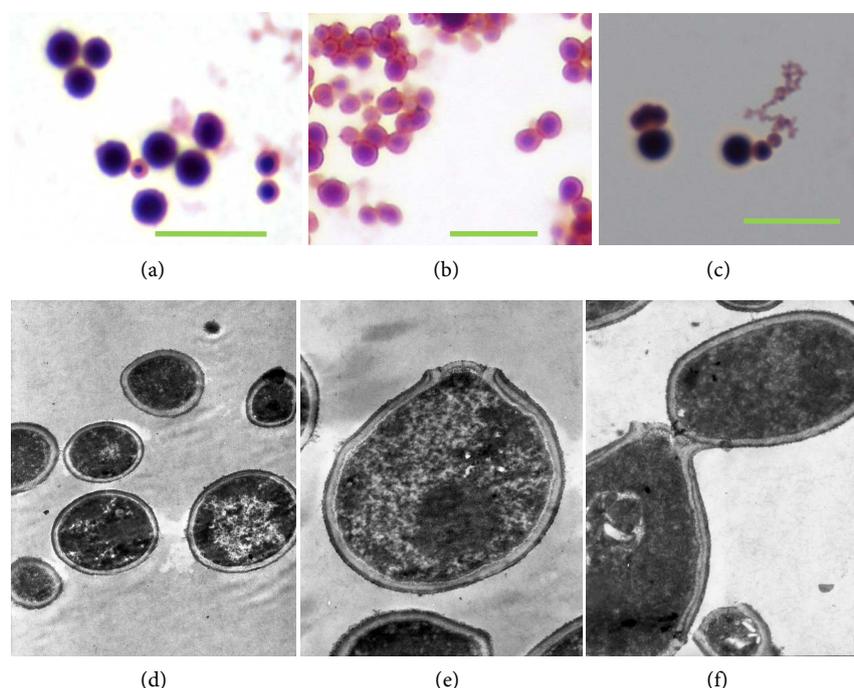
Figure 1. Microscopic examinations of blood microbiota. (a) Gram stained normal blood microbiota of cultured blood at 43˚C for 48 h, 10 × 100. Several morphological forms could be observed; (b) "Dense body" of Gram positively stained microbial core structure surrounded by Gram negative coat. (b), (c) The "dense body" could divide by simple budding, and/or (c) produce in chains Gram negatively stained "primary cells". Green bar 10μm; (d) (e), (f), TEM of blood microbiota in stages of maturation and division. The blood sample was processed within 1 hour after collection. Magnifications: Originally (d) × 7600; (e) × 12700; (f) × 12700.

evidences completely lacking that microbiological isolation of the blood microbiome in healthy subjects is possible within 48 hours. The applied methodology of isolation in liquid culture media is technically affordable and repeatable, although we did not found appropriate conditions to maintain viable the blood microbiota in subcultures. In subcultures the blood microbiota revert to their dormant state. We did not identified the factors responsible of this process.

We observed several morphological forms of Gram stained blood microbiota, previously described by Domingue [5] [7] as "dense body" of Gram positively stained microbial core structure surrounded by Gram negative coat and chains of Gram negatively stained "primary cells" (**Figure 1**). TEM images demonstrated well defined cell structures (**Figure 1**). TEM and culture results confirmed that the blood microbiome represents viable structures rather than debris resulting from degradation of blood elements, such as lipids or haemoglobin complexes. We suppose that the "dense body" is a compact mass composed by "primary cells". Microscopic evidences are shown on **Figure 3(c)**. Microscopic slides have poor stainability for DNA with Acridine Orange (AO). Impermeability is probably due to difficult stain penetration even after 15 min of staining in 1 mg/ml of AO.

After optimizing growth media and temperature conditions for liquid cultures

next step was to develop agar media for isolation. Before subculturing on agar plates all liquid cultures were tested for contamination on Sabouraud and sheep blood agar. Cultures were negative for contaminations. Subculturing on agar was performed from 48 h liquid culture. Blood agar plates were prepared by adding to the optimized liquid medium 1.2% Noble agar (Difco, BD, USA) and 10% sheep blood. We found that sheep blood agar was not suitable for isolation of human blood microbiota. Growth was not visible with naked eye even after prolonged cultivation for more than two months. Isolation strategy was changed by preparing agar plates with homologous human blood. Agar plates prepared with 10% blood of the same individual used for inoculation of liquid culture gave partial growth. Plates were sealed in plastic bags to diminish drying of agar and culturing was performed at 43°C. After 30 days of culturing brown colonies, with no odour and diameter of 0.5 - 1 mm were observed (Figure 2). Longer periods of culturing do not give increase in colony size, due to the fact that agar is drying and somehow growth seems inhibited or much slower. Agar plates with 10%, 20% and 80% of homologous blood were tested and there was no difference in growth efficiency. The bacterial colonies were confirmed visually and by Gram. Isolation of colonies on agar media of the blood microbiota from healthy individuals was partly successful and still challenging. Subcultures are unstable.
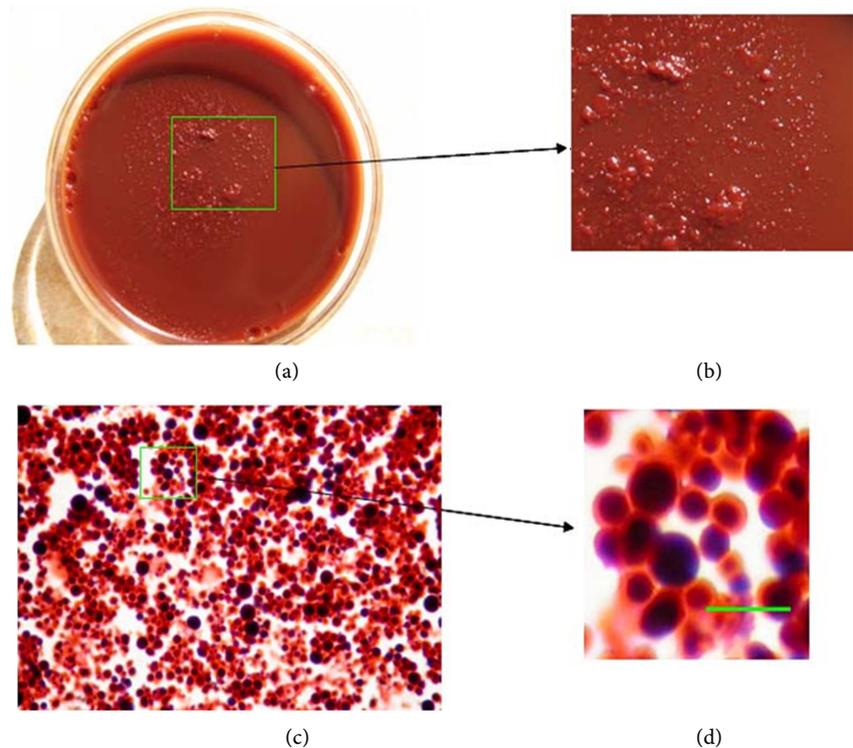

(a)	(b)
(c)	(d)

Figure 2. Colonies of normal blood flora on agar plate. (a) Colonies of blood microbiota on agar plate. Culture is performed for 30 days at 43°C; (b) zoom in of the sector plate; (c) Gram stained single colony; (d) Several bacterial "dense body" forms of Gram positive, Gram negative and forms with Gram positive center and Gram negative coat could be observed. Green bar 10μm.

Several physicochemical characteristics were examined. Disruption of cells for 15 min with 0.1 mm glass beads with beat beater machine (Mini-beatbeater, Biospec Products), treatment in microwave oven at 850 W for 3 min and disintegration with ultrasound (MSE, UK) for 1 h performed on ice applying maximum power pulsations, *i.e.* 15 s of disintegration and 15 s at rest were without success. Evaluation of microbial cell lysis or disintegration was performed microscopically after Gram staining. On the slides intact "dense bodies" were observed. Increasing the time for disintegration with ultrasound for 2 h brought to unexpected finding. Cells observed with yeast like shape on light microscopy and on electron microscopy as "dense bodies" at different stages of maturation were disintegrated to single "elementary bodies" with streptococcal appearance (Figures 3(a)-(c)). We conclude that the "dense bodies" give origin to elementary structures of "primary cells" with the size of 0.1 - 0.2 μm. Integrity of the "dense body" cells in fresh cultures was tested by treatments with 4M guanidine thiocianate, 10% NaOH, 10% KOH, 10% CH$_3$COOH, 10% HCl and 10% H$_2$SO$_4$ for 30 min at 37˚C, 0.5 M EDTA for 4 days at room temperature and freeze-thawing
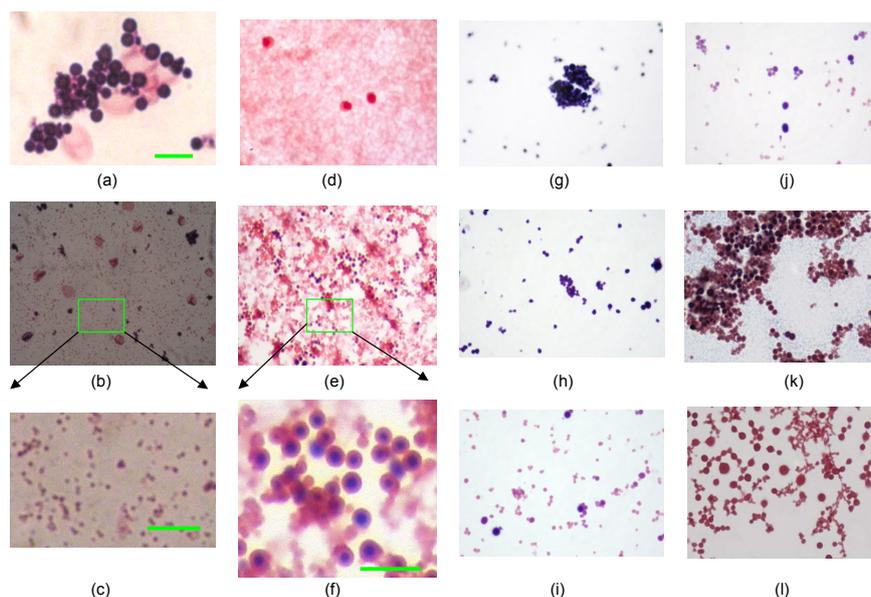

Figure 3. Resistance of the blood microbiota to physical and chemical treatments. (a) Gram stained microbial structures of blood microbiota grown on agar for 30 days at 43˚C; (b), (c) Disintegration with ultrasound at maximum power for one hour with pulsations of 30 s of ultrasound and 30 s of rest. After disintegration with ultrasound "dense bodies" significantly decrease in number. "Cells" with streptococcal appearance dominate on the microscopy field; (d) Non-cultured blood sample after gamma irradiation with 25 + 25 KGy dose. Only blood cells could be observed. After the first irradiation with 25 KGy growth was observed after 14 days of culture (not shown). Second dose of 25 KGy was applied to the same sample; (e) 25 + 25 KGy irradiated sample cultured for 14 days at 43˚C; (f) Sector zoom-in of the blood microbiota. Cells seem not affected by this extreme dose of irradiation; (g) Blood microbiota treated with 10% KOH; (h) with 10% NaOH; (i) with 10% CH$_3$COOH and (j) with 10% H$_2$SO$_4$ for 30 min at 37˚C; (k) 0.5 M EDTA for 4 days at room temperature and (l), freeze-thawing resistance in liquid nitrogen and 96˚C. Green bar 10 μm.

resistance in liquid nitrogen and 96˚C. We observed that only Gram-positive microbial structures were seen after treatment with bases. The treatment with acids decreased the number of observed Gram-negative structures. Microbial cells were partially affected by these coarse treatments as observed on microscopy (Figures 3(g)-(k)).

Blood microbiota resisted to high dose of 25 + 25 kGy gamma irradiation with [60]Co (Figures 3(d)-(f)). Growth of radioresistant blood microbiota was detected after two weeks. This is not surprising, because the majority of soil bacteria are killed by 20 kGy, however dose higher than 70 kGy may be required to kill certain radio-resistant bacteria [19].

For DNA analysis we applied 16S rRNA gene targeted sequencing of V3-V4 hypervariable region on Illumina MiSeq (Illumina Inc., USA). DNA sequencing was performed at IMGM Laboratories GmbH (Martinsried, Germany). The resulting 2×300 bp reads were demultiplexed, quality controlled and merged into continuous reads. Further bioinformatics analysis including clustering, phylogenetic analysis and alpha and beta diversity calculation was performed with the CLC genomics workbench and its microbial genomics module. In order to correctly describe the microbial biodiversity on phylum and order level, four filtering steps were applied: 1) only Operational Taxonomic Units (OTUs) exceeding similarity of >97% on genus level were included in the analysis, 2) only OTUs exceeding a combined abundance among all tested samples of 100 were included, 3) OTU reads per sample below 1% abundance at order level were excluded and 4) exclusion of common sequence reads originating from exogenous DNA. The sources of these contaminants are reagents used in DNA extraction, culture medium, PCR reagents, and next-generation sequencing library preparation. The sequences, available after trimming and merging showed a high amount of high quality reads, suitable for downstream analysis. Among the cultured and non-cultured samples sequencing analysis identified rich biodiversity of OTUs similarity (>97% on genus level) with 47 bacterial orders belonging to 15 phyla and 39 fungi orders blonging to 2 phyla. The bacterial orders were predominantly of phylum *Proteobacteria.* Among non-cultured samples *Proteobacteria* were 93%, *Actinobacteria* 2%, *Planctomycetes* 2% and *Firmicutes* 2%, while among cultured samples *Proteobacteria* were 46%, *Firmicutes* 25%, *Actinobacteria* 14%, *Bacteroidetes* 6%, *Fusobacteria* 3% and *Cyanobacteria* 2%. The fungi phyla *Basidiomycota*, *Ascomycota* and unidentified fungi were 64%, 22% and 14% respectively among the non-cultured samples, while among the cultured samples they were 27%, 31% and 41% respectively. We note, that some phyla are enriched by the applied resuscitation strategy according to the OTUs combined abundancy (Table 1). Other studies also identified *Proteobacteria* as predominant in the blood of healthy individuals [14], healthy broilers [16] and cats [17].

Our results demonstrate that isolation of the blood microbiota is technically affordable by conventional means. We identified, that in 100% of the blood

Table 1. OTUs abundances of identified bacterial and fungi taxa among non-cultured and cultured blood samples.

BACTERIA	N: of reads in non-cultured samples (%)	N: of reads in cultured samples (%)
Phylum: Acidobacteria	44 (<1%)	421 (1%)
Phylum: Actinobacteria	820 (2%)	9754 (14%)
Phylum: Armatimonadetes	422 (1%)	239 (<1%)
Phylum: Bacteroidetes	73 (<1%)	4580 (6%)
Phylum: Chlamydiae	0	129 (<1%)
Phylum: Chloroflexi	5 (<1%)	99 (<1%)
Phylum: Cyanobacteria	179 (<1%)	1652 (2%)
Phylum: Firmicutes	1159 (2%)	17635 (25%)
Phylum: Fusobacteria	30 (<1%)	2284 (3%)
Phylum: Planctomycetes	730 (2%)	297 (<1%)
Phylum: Proteobacteria	43138 (93%)	32272 (46%)
Phylum: Saccharibacteria	7 (<1%)	432 (1%)
Phylum: Spirochaetae	0	262 (<1%)
Phylum: SR1 (Absconditabacteria)	0	250 (<1%)
Phylum: Synergistetes	0	198 (<1%)
FUNGI		
Fungi unidentified	45118 (14%)	187419 (41%)
Phylum: Ascomycota	70401 (22%)	142425 (31%)
Phylum: Basidiomycota	204113 (64%)	122503 (27%)

samples the blood microbiota resuscitate in 24 - 48 hours under stress by culturing at 43˚C in high concentrations of vitamin K. In vitro we identified by microscopy that the most common mechanism of formation and reproduction of the "dense body" is by production in chains Gram negatively stained "primary cells", as previously described by Domingue (1977) [5], (1997) [7]. We observed loss of the Gram negative component of the "dense body" after treatment with 10% NaOH. Evidences for intraerythrocytic presence of normal blood microbiota were reported by Kalfin in 1997 [6]. We identified free circulating microbiota, "primary cells", by electron microscopy in blood samples processed within the first hour after collection (Figures 1(e)-(g)). We suppose that the "electron dense body" is composed by Gram negative and Gram positive 'primary cells'. Currently applied approaches for biodiversity analysis of blood microbiota use microbe-derived DNA or RNA that may have crossed into the blood from the gut. The proposed by us culture resuscitation approach overcomes these limitations. We showed that blood DNAemia in healthy individuals is associated wtih the presence of culturable microbiota in the blood. To prove this, cell-free DNA circulating in the blood (DNAemia) was eliminated by

applying a triple DNAse I treatment, centrifugation and washing of the cell pellets with 40 ml dH_2O in cultured and uncultured blood. Our approach included the use of positive and negative controls. Contaminating sequences associated with reagents were excluded from analysis. Among cultured and non-cultured blood samples certain orders replicate well as demonstated by increase in OTUs abundance (Table 1). It could be concluded that the applied resuscitation culture conditions enrich specific orders while others remain unchanged. Moreover we predict that applying other growth conditions or media will change the composition of the cultured microbiota in the sample.
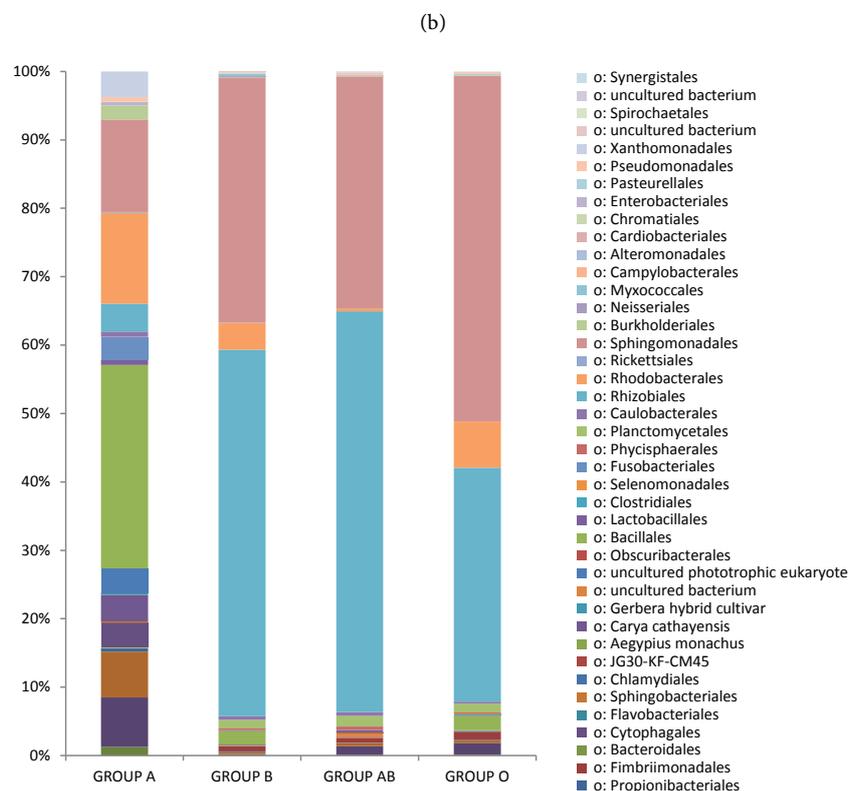
When grouping the samples according the blood type we identified significant differences. In non-cultured samples of individuals of blood type A we identified less number of reads but higher bacterial diversity at order level as compared to the other blood types (Table 2).

The identified taxa in the blood have been reported in previous studies of the blood microbiota in healthy individuals or animals. Mangul *et al.* (2016) identified 23 bacterial taxa at pylum level applying total RNA sequencing [16]. In a recent study Chen *et al.* (2017) showed that the uterus and the fallopian tubes, which are generally believed to be sterile, are home of rich microbiota indicative of a non-sterile environment [20].

Table 2. Bacterial blood microbiota according to the blood group. (a) Number of reads according to blood group and order in non-cultured blood samples; (b) Graphical distribution of the bacterial orders.

(a)

Taxonomy	Group A	Group B	Group AB	Group O
p: Actinobacteria, o: Actinomycetales	11	0	0	0
p: Actinobacteria, o: Corynebacteriales	64	0	95	346
p: Actinobacteria, o: Micrococcales	59	0	0	0
p: Armatimonadetes, o: Fimbriimonadales	0	131	0	238
p: Bacteroidetes, o: Cytophagales	32	0	0	0
p: Cyanobacteria, o: Carya cathayensis	34	0	0	0
p: Cyanobacteria, o: uncultured bacterium	31	0	0	0
p: Firmicutes, o: Bacillales	262	343	0	432
p: Fusobacteria, o: Fusobacteriales	29	0	0	0
p: Planctomycetes, o: Planctomycetales	0	216	113	275
p: Proteobacteria, o: Rhizobiales	36	9187	4309	7152
p: Proteobacteria, o: Rhodobacterales	117	668	0	1412
p: Proteobacteria, o: Sphingomonadales	119	6151	2495	10570
p: Proteobacteria, o: Burkholderiales	18	0	0	0
p: Proteobacteria, o: Xanthomonadales	33	0	0	0
TOTAL	**845**	**16696**	**7012**	**20425**

(b)

The bacterial and fungi composition of the blood microbiota is highly diverse and each individual or even blood group has its own specificities (Table 2, Table 3). The abundance of given OTUs is indicative for high prevalence and not contamination.
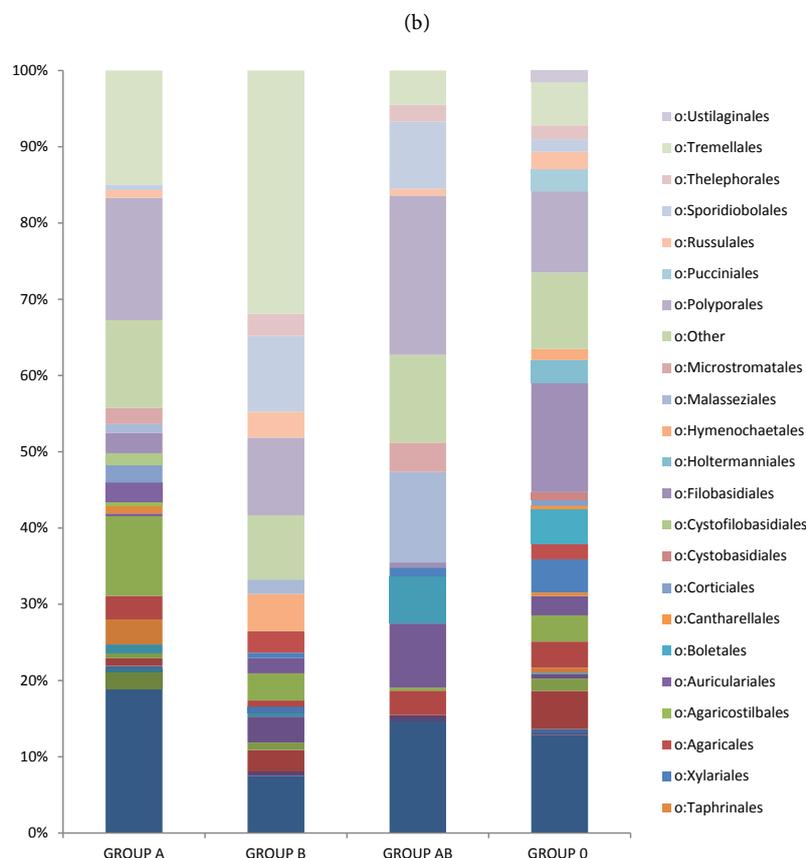
4. Conclusion

Here we demonstrated rich biodiversity of naturally occurring bacterial and fungal blood microbiota in healthy individuals. We developed a successful strategy for analysis of the blood microbiome by total microbial resuscitation by blood culturing, microscopy and NGS DNA sequencing. The total microbial resuscitation was proved by comparative analysis of cultured and non-cultured blood samples. Blood-group differences were identified among the bacterial microbiome composition. For the first time we identify rich fungiome in the blood. We showed that blood DNAemia in healthy individuals is also associated with the presence of culturable microbiota in the blood. Our results confirm that the blood of healthy humans is not as sterile as previously supposed by other authors [4]-[15] [20]. This work brings evidences, that the resident blood microbiota in healthy individuals should be considered non-pathogenic and a normal feature of the healthy blood. Furthermore, we anticipate our resuscitation strategy and sequencing approach to be a model for studying the numerous chronic diseases, such as obesity, diabetes, cardiac failure, liver diseases, hematologic disorders, neurodegenerative diseases and chronic infections, like latent tuberculosis. Beyond cataloguing the species of the blood environment,

Table 3. Fungal blood microbiota according to blood group. (a) Abundance of OTU reads according to blood group and fungi orders in non-cultured blood samples; (b) Graphical distribution of the fungi orders.

(a)

Taxonomy	Group A	Group B	Group AB	Group O
Fungi unidentified	19213	3657	4598	17650
o:Caliciales	2291	0	0	0
o:Capnodiales	0	284	240	293
o:Chaetosphaeriales	328	0	0	0
o:Diaporthales	458	0	0	779
o:Dothideales	1005	1377	0	6914
o:Erysiphales	658	471	0	2227
o:Eurotiales	0	1677	0	839
o:Helotiales	1173	213	0	219
o:Hypocreales	3339	0	14	881
o:Microascales	0	420	0	0
o:Other	3151	399	1015	4722
o:Pleosporales	10624	1745	130	4741
o:Saccharomycetales	330	970	2637	3492
o:Sordariales	0	0	1925	0
o:Taphrinales	1053	0	0	637
o:Xylariales	0	358	383	5976
o:Agaricales	0	1377	0	2818
o:Agaricostilbales	536	0	0	0
o:Auriculariales	2580	0	0	0
o:Boletales	0	0	0	6289
o:Cantharellales	0	0	0	556
o:Corticiales	2355	0	0	1022
o:Cystobasidiales	0	0	0	1433
o:Cystofilobasidiales	1551	0	0	0
o:Filobasidiales	2709	0	205	19742
o:Holtermanniales	0	0	0	4102
o:Hymenochaetales	0	2382	0	1982
o:Malasseziales	1151	937	3730	0
o:Microstromatales	2187	0	1207	0
o:Other	11674	4115	3619	13801
o:Polyporales	16349	4990	6562	14683
o:Pucciniales	0	0	0	3968
o:Russulales	1005	1654	280	3153
o:Sporidiobolales	740	4869	2777	2249
o:Thelephorales	0	1411	683	2523
o:Tremellales	15209	15610	1411	7848
o:Ustilaginales	0	0	0	2046
TOTAL	101669	48916	31416	137585

(b)

the blood microbiome field will focus on defining the mechanisms underpinning the interactions between microbes and host, mechanisms influencing the initiation and progression of diseases with a view towards personalization of patient's diagnosis and treatment.

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