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Taxonomic diversity and succession of bacterial consortium from Kombucha

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Abstract

The aim of the present work was to analyze the taxonomic diversity in the Kombucha bacterial consortium during long-term cultivation in the North (Arctic) of the European part of Russia. The high-performance sequencing showed that 99.1 % of the bacterial fraction of the fresh 7-d culture was *Proteobacteria* of mostly cellulose-producing genera with the order *Acetobacterales* being dominant and represented by the *Komagataeibacter* (87.3 %) and *Gluconobacter* (6.3 %). Aging of the Kombucha bacterial consortium from 20 to 90-d cultivation lead to the reduction of number of cellulose-producing bacteria and intense growth of cellulolytic bacteria including *Clostridiales*, *Bacteroidetes*, *Actinomyces*. Also the acidophilic microorganisms have been detected. The long-term growth of the Kombucha bacterial consortium can be considered as a succession of microbial communities.

Introduction

Kombucha is a mutualistic symbiotic community of bacteria and yeast known for several centuries in the areas of Europe, Asia and Africa. The liquid brew obtained as a result of the microbial community development is consumed as a healthy drink of domestic production (Matei *et al*. 2018; Villarreal-Soto *et al*. 2018; Ivanišová *et al*. 2019; May *et al*. 2019; Khaleil *et al*. 2021). The second product of the microbiome activity, bacterial cellulose, has been intensively studied due to its valuable properties and potential applications (Campano *et al*. 2016; Jozala *et al*. 2016). The drink quality depends mostly on starting nutrient medium and the taxonomic diversity of the microbiome. Recent molecular and genetic approaches to the taxonomic study allow an accurate quantitative analysis of the species diversity (Chirak *et al.* 2013). Culture-independent approaches, such as high-performance sequencing, eliminate the difficulties associated with cultivation, and possible contamination with non-cultivated species. In contrast to controlled laboratory conditions, domestic conditions do not ensure the stability of the culture, and the household geographical location affects the formation of unique Kombucha communities. Microbial consortiums formed in geographically remote territories differ significantly in taxonomic diversity (Safak *et al.* 2002; Ramadani *et al.* 2010; Marsh *et al.* 2014).

For example, significant differences have been described for Kombucha samples from 32

households in Germany (Mayser *et al.* 1995). The Kombucha microbiome forms in specific conditions of a particular geographical area, thus, the influence of environmental factors is also significant. It is important to note that previously taxonomic changes in the Kombucha consortium were only registered during cultivation for less than 21 days (Marsh *et al.* 2014; Chakravorty *et al.* 2016). Our preliminary studies on Kombucha long-term cultivation have shown that near the 90th day the redistribution of dominant species took place. Thus, changes in the trophic structure and diversity of the community, accompanied by a change in the dominant species, i.e. the succession of the microbiome is of great interest. The aim of this work was to study changes in the taxonomic diversity of the bacterial fraction of Kombucha community during microbiome cultivation up to 90 days in households in the North (Arctic) of the European part of Russia.

Experimental

Kombucha preparation and sampling

Five samples of the Kombucha consortium were cultivated at five different households (Russia, Arkhangelsk region). The starting Kombucha inoculum was the same for all household samples. It was divided into five equal portions and placed in different households. The microbiome cultivation was carried out at 20 – 25 °C in 3 L glass vessel for 7 d (household A), 10 d (household B), 15 d (household C), 20 d (household D) and 90 d (household E) on a semi-synthetic medium. The media contained sucrose as the main C-source and black tea extract. Black tea infusion was prepared by adding of dry black tea leaves to hot water at the concentration of 10 – 12 $g.L^{-1}$. After steeping for 15 min infusion, the infusion was filtered and cooled to room temperature and then 20 $g.L^{-1}$ of sucrose was added to the infusion. Cultivation was carried out under static conditions by the periodic method. Samples of the Kombucha consortium harvested in cellulosic matrix were separated from the cultural medium and frozen to preserve the genetic material.

DNA isolation, PCR reaction and high-performance sequencing of the bacterial community

A NucleoSpin Soil kit (Macherey-Nagel, Germany) was used for DNA isolation from Kombucha samples according to the protocol. The cell membranes were destroyed by milling of 100 – 200 mg of a sample with 0.1 mm diameter ceramic balls in a lysing buffer twice for 20 min at 6,000 rpm using a Precellys 24 homogenizer (Bertin Technologies, France).

The taxonomic composition of the community was determined by high-performance sequencing based on the analysis of amplicon libraries of ribosomal operon fragments. Universal primers F515/R806 for a variable section of the 16SpPHKv3-v4 gene (GTGCCAGCMGCCGCGGTAA/GGACTACVSG GGTATCTAAT) specific to a wide range of microorganisms, including bacteria and archaea were used (Bates *et al*. 2010).

PCR reaction was carried out in 15 μL mixture containing 0.5 – 1 unit of Q5® High-Fidelity DNA Polymerase (New Englang BioLabs, USA), 5 pM of forward and reverse primers, 10 ng of DNA matrix, and 2 nM of each dNTP (LifeTechnologies). The mixture was denatured at 94 °C for 1 min, followed by 35 cycles for 30 seconds at 94 °C, for 30 seconds at 50 °C, and for 30 seconds at 72 °C. The final elongation was carried out at 72 °C for 3 min. PCR products were purified using AMPureXP (Beckman Coulter, USA) following the Illumina recommended protocol.

The libraries were prepared in accordance with the MiSeq Reagent Kit Preparation Guide (Illumina Inc. San Diego, USA). The libraries were sequenced in accordance with the instructions on an Illumina MiSeq device (Illumina Inc. San Diego, USA) with MiSeq ® Reagent Kit v3 reagents with a double-sided reading. There were received at least 10,000 reads for each library. Data obtained from sample sequencing were processed using Trimomatic (Bolger *et al*. 2014) and QIIME (Caporaso *et al*. 2010) software packages (available at: http://quiime.sourceforge.net/).

The procedures for denoising and chimeras deleting were carried out using the DADA2 R package as described in (Callahan *et al*. 2016) to obtain a table

of frequencies of unique sequences (ASV, Amplicon Sequence Variant). Primary bioinformatic processing of the obtained data was performed with the results presented as a diagram of bacteria distribution. The results were processed using the Microsoft Office Excel software package.

Scanning electron microscopy analyses

Morphology of the bacterial cellulose matrix was visualized using the high-resolution scanning microscope SEM Sigma VP ZEISS (Germany) equipped within-lens detector at 10 kV accelerating voltage. Prior to the microscopic study samples of bacterial cellulose were subjected to lyophilization using Labconco equipment (FreeZone 2.5 L, USA).

The microscopy of the 7-d biofilm sample showed the bacteria and yeasts microbial cells entrapped in the cellulose matrix (Fig. 1).

The cellulose-producing bacteria in the Kombucha microbiome provide the cellulose biosynthesis and structuration of consortium matrix (Fig. 1B) (Bolotova *et al*. 2016). The formation of a polysaccharide matrix may involve bacteria that produce non-cellulose exopolysaccharides, such as lactic acid bacteria. Yeasts convert media sucrose to ethanol and carbon dioxide in the process of alcoholic fermentation, while bacteria provide assimilation of ethanol by converting it to acetic acid (Campano *et al*. 2016). Fig. 1A represents yeast budding forms and rod-shaped bacterial cells in the structure of the cellulose matrix.

Results and Discussion

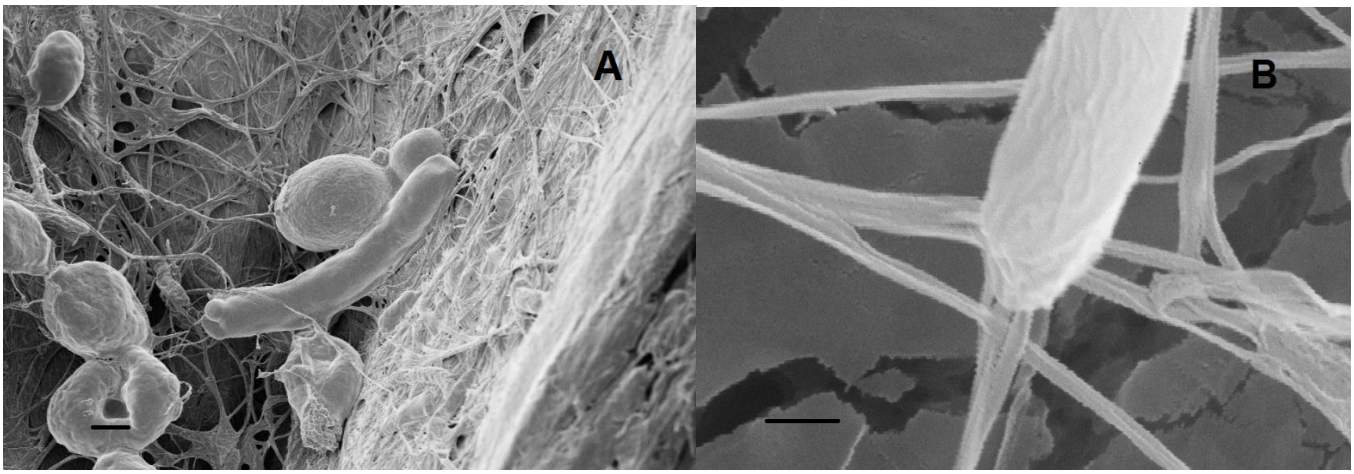

Fig. 1. Morphology of bacteria and yeasts in the cellulose nanofibrous network (**A**) and spatial localization of cellulose-synthases and cellulose fibrils at the surface of the cellulose-producing cell (**B**). Bars: a – 1 µm; b – 200 nm.

Metagenomic studies of the bacterial fraction showed that the content of unclassified bacteria varied in the studied samples from 0 to 13.8 % (Table 1).

The following bacterial phyla were found in all Kombucha samples, regardless of the cultivation period: *Actinobacteria* (0.3 – 27.7 %), Firmicutes (0.3 – 23.3 %) and Proteobacteria (22.1 – 99.1 %). The Bacteroidetes were absent at early stages of culture growth (7-d culture); as the cultivation proceeded the Bacteroidetes biomass increased and it reached 8.3 % as the community ages.

At the initial cultivation stages (7-d culture), Kombucha microbiome bacteria consisted of 99.1 % Protobacteria including members of cellulose-producing species. The phylum was represented by the Alphaproteobacteria (93.6 %) and Gammaproteobacteria (5.5 %) classes. The order Acetobacterales is the dominant one in the class Alphaproteobacteria and is represented by the genera *Komagataeibacter* (87.3 %) and *Gluconobacter* (6.3 %). The number of Lactobacillales members also varied depending on the cultivation time from 0.2 – 2.5 % (7 – 20 d) to 14 % (90 d) (data are not presented in Table 1). The Lactobacillales is included in Table 1 as the representatives of phylum Firmicutes.

Table 1. Composition of the Kombucha bacterial microbiome.

Phylum	Percentage in the bacterial microbiome [%]				
	7 d (hous.A)	10 d (hous.B)	15 d (hous.C)	20 d (hous.D)	90 d (hous.E)
Proteobacteria	99.1	86.1	97.4	95.1	22.1
Actinobacteria	0.3	2.4	0.5	2.0	27.7
Firmicutes	0.3	5.8	1.5	1.3	23.3
Bacteroidetes	–	3.8	0.5	0.7	8.3
Patescibacteria	–	1.1	–	–	0.3
Fusobacteria	–	0.5	–	–	0.6
Verrucomicrobia	–	–	–	–	1.1
Epsilonbacteraeota	–	–	–	–	0.7
Planctomycetes	–	–	–	0.1	0.5
Cyanobacteria	–	–	–	–	0.6
Acidobacteria	–	–	–	0.1	0.4
Deinococcus-Thermus	–	–	0.1	–	0.1
Spirochaetes	–	–	–	–	0.2
Chlamydiae	–	–	–	–	0.1
Synergistetes	–	–	–	–	0.1
Archaea: Thaumarchaeota	–	–	–	–	0.1
Unclassified	0.3	0.3	–	0.7	13.8

Earlier metagenomic studies of Kombucha microbial communities (Jayabalan *et al*. 2014; Reva *et al*. 2015) also revealed the dominance of two acidobacterial genera, *Komagataeibacter* (*Gluconacetobacter*) and *Gluconobacter*. The composition of the community has been shown to depend on the growing conditions (Reva *et al*. 2015). Although, the bacterial community was relatively stable, in some cases it included additionally Lactobacillus.

Similar studies (Coton *et al*. 2017) have found that the dominant bacterial species belonged to the families Acetobacteraceae and, to a lesser extent, to the Lactobacteriaceae. The *Gluconacetobacter* species were isolated from the commercial Kombucha Kombu Australia and were used to produce cellulose (Nguyen *et al*. 2008). In most cases, the *Gluconacetobacter* bacteria composed more than 85.0 % of the bacterial community, while the *Acetobacter* members were represented in a small amount (2.0 %) (Marsh *et al*. 2014). Lactobacillus and some other subdominant species were also found in Kombucha consortium in previous works (Marsh *et al*. 2014; Coton *et al*. 2017). Therefore, literature data show the relative homogeneity of the Kombucha bacterial community. Our results obtained for households in the North of the European part of Russia for the microbiome cultured up to 20 d correspond to previous works. In all the samples for cultivation period of 7 – 20 d, Proteobacteria specifically acetic acid bacteria, dominated. The accumulation of acetic acid and ethanol in the Kombucha microbiome prevents the development of foreign microflora. In addition, ethanol can reduce the growth rate and aging of the bacterial population in the microbial community.

With an increase in the cultivation time up to 90-d, the composition of the microbiome changed and three divisions dominated in almost equal proportions: Actinobacteria (27.7 %), Firmicutes (23.3 %) and Proteobacteria (22.1 %). As the microbiome ages Micrococcales, Corynebacteriales, Pasteurellales and Betaproteobacteriales (fam. Burkholderiaceae), Sphingomonadales developed in it (Fig. 2).

The content of acetic acid bacteria, the key species for the formation of cellulose and creating optimal conditions for the development of the microbiome, was reduced to 1 % by 90 d. The dominant genera of the aged culture (Fig. 2) included lactic acid bacteria of the genus *Lactobacillus* (6.3 %) and some *Streptococcus* species. Acetobacteria and lactobacteria were shown to be in symbiotic relationship; lactic acid bacteria metabolites, xylitol and acetic acid, promoted the growth of acetic acid bacteria (Yang *et al*. 2010). During prolonged Kombucha fermentation organic acids, such as gluconic, lactic, succinic, etc. are accumulated in the media (Chen *et al*. 2000; Greenwalt *et al*. 2000). In 90-d culture minor acid-producing genera were detected, specifically *Cutibacterium* or

Propionibacterium (7.6 %) and *Veillonella* (1.1 %), which are involved in the synthesis of propionates and acetates, as well as Corynebacteria (2.0 %), facultative anaerobes with redox-enzymatic metabolism, participating in the synthesis of glutamic acid (Fig. 2). Futhermore microbiome, bacterial cellulose became a substrate for microbial enzymes. Among the detected genera Clostridiales (3.6 %) in phylum Firmicutes, Bacteroidetes and Actinomyces (1.9 %) in phylum Actinobacteria represented the most active cellulose destructors observed at the early stages of development of the microbial community and not detected in a 90-day-old culture.

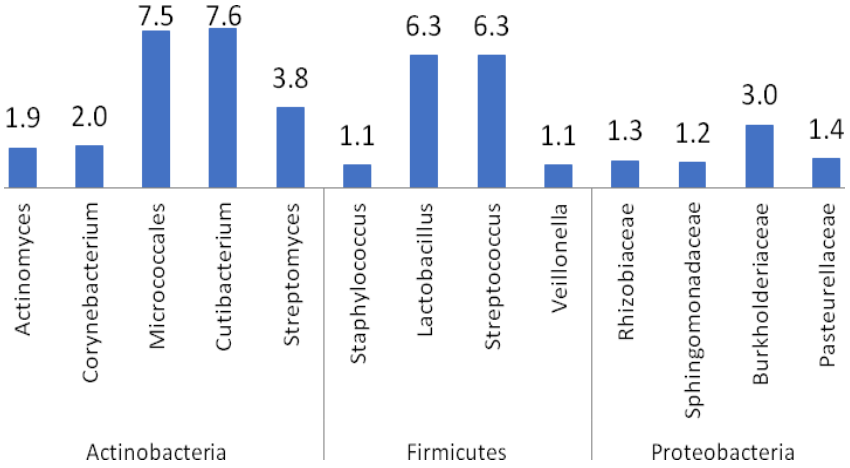

Fig. 2. Percent of Actinobacteria, Firmicutes, and Proteobacteria phylum that predominate in the 90-day-old culture community, % of the bacterial microbiome (genera that are less than 1 % present in the community are not shown in the diagram).

The substrate depletion, the accumulation of acidic metabolites, and the formation of local anaerobic zones in the cellulose matrix during long-term cultivation lead to the development of microorganisms that are completely uncharacteristic of the Kombucha symbiosis. Unique genera were identified in Arctic region Kombucha. Those were nitrogen fixers of the Rhizobiaceae family, Ensifer (0.6 %) and Rhizobium (0.1 %), which are able to assimilate air nitrogen when the nutrient medium is depleted. Previously, members of the family Acetobacteraceae isolated from Kombucha also demonstrated the ability to nitrogen fixation (Dutta *et al*. 2010). Cyanobacteria or blue-green algae, representing the division of photosynthetic gram-negative bacteria, were identified in an amount of 0.5 %. Archaea of the Nitrososphaeraceae family, ammonia-oxidizing chemolithotrophs, were also found in the sample. Their adaptation in the environment can be associated with autolytic processes and proteolysis accompanied by ammonification.

Thus, the development of Kombucha bacterial consortium over time can be considered as a succession of microbial communities resulted in changes in its composition. During Kombucha fermentation, acetic acid bacteria assimilated sugars and produced exopolysaccharides and acidic metabolites. Then microorganisms that assimilate organic compounds produced in autolytic processes and enzymatic hydrolysis of biomass, use specific metabolic schemes, adapted and developed. Hydrolytic bacteria provided cellulose destruction. Monomers and metabolites were consumed by dissipotrophs. Anaerobic hydrolytics and dissipotrophs constitute the primary group of anaerobes. The primary hydrogen-producing anaerobes as well as secondary anaerobes consuming unfermentable compounds were detected.

Conclusion

The taxonomic diversity was analysed for Kombucha samples harvested in households located in Northern European Russia. High-

performance sequencing revealed qualitative and quantitative differences in the composition of the microbiome during long-term cultivation. The microbiome cultivated up to 20 d showed a relative uniformity of the bacterial community. During the initial period (7-d culture), cellulose-producing Proteobacteria represented 99.1 % of the consortium bacteria. The dominant genera were *Komagataeibacter* (87.3 %) and *Gluconobacter* (6.3 %). When the microbiome aged (90-d cultivation), the number and variety of cellulose-synthesizing bacterial species significantly decreased. Bacterial cellulose became a carbon source for the microbial community. Microorganisms secreting cellulolytic enzymes, Clostridiales, members of Bacteroidetes and Actinomyces, started to grow. Along with acetic acid bacteria, acid-producing Propionibacterium, Veillonella, Corynebacterium adapted and developed. The nitrogen-fixing genera of the Rhizobiaceae family, archaea of the Nitrososphaeraceae, as well as cyanobacteria were identified in 90-d culture of the Arctic region Kombucha microbiome.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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