A review of the research on microbial diversity and its effects on components and functions of Kombucha

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Abstract. Kombucha is a healthy beverage rich in functional active ingredients and has multiple health benefits. At present, Kombucha has become a global research hotspot and has very broad market prospect. The diversity of microbial communities significantly affects the health benefits of Kombucha. The development of modern biological research methods, especially the development of independent culture analysis techniques, has provided a comprehensive understanding of the diversity of the microbial community structure in Kombucha. This article reviews the research progress on the diversity of microbial communities in Kombucha, including the population composition and interactions of microorganisms during fermentation, methods and influencing factors of microbial diversity, as well as the artificial regulation and assembly of Kombucha microbial communities. This article will provide theoretical reference for improving the quality and safety of Kombucha and innovating Kombucha products through the microbial communities regulation of Kombucha.

Keywords: Kombucha; fermentation; symbiotic culture of bacteria and yeast; Microbial community diversity; Microbial community regulation.

1. Introduction

Kombucha is a kind of healthy drink, which was fermented by the symbiotic culture of bacteria and yeast (SCOBY) using tea and sugar as raw materials [1]. It originated in China from 220 B.C. and was introduced to the West during World War I. Starting in the 1990s, Kombucha products were gradually produced and marketed around the world [2]. In recent years, the economic trend of Kombucha has grown steadily, and the global market is expected to reach $5.9 billion by 2029 [3].

During the fermentation process of Kombucha, the complex microbial metabolic network generates a large number of active substances, including polyphenols, flavonoids, organic acids, amino acids, vitamins, trace elements, and volatile organic compounds, etc.. Therefore, Kombucha displays powerful probiotic activity and exhibit a wide range of health benefits, such as anti-inflammatory and anti-bacterial properties [4], liver protection [5], and cancer prevention [6], balancing intestinal flora [7]. In conclusion, Kombucha greatly satisfies the growing health needs of consumers in various countries.

The health benefits of Kombucha are mainly attributed to the powerful microbial system. With the development of modern biological research methods, microorganisms in Kombucha have been recognized and isolated, and their interactions and metabolic patterns have been resolved. The dominant bacteria in Kombucha are acetic acid bacteria (AAB), mainly including *Acetobacter*, *Gluconobacter*, *Komagataeibacter* and others [8]. Kombucha also contains a small amount of lactic acid bacteria (LAB), dominating as *Lactobacillus* spp. [9]. Many kinds of yeasts have been isolated from Kombucha, mainly including *Zygosaccharomyces*, Schizosaccharomyces, *Brettanomyces*, *Saccharomyces*, *Pichia*, *Candida*, and so on. The microorganisms most relevant to the function of Kombucha probiotics are *Lactobacillus*, *Bifidobacterium*, *Bacillus cereus*, *Propionibacterium freudenreichii*, and *Saccharomyces* [10]. The mixed fermentation of AAB, LAB and yeast in Kombucha forms a complex metabolic chain of substances through mutual control, coordination and promotion. The interaction between the strains is conducive to the utilization of carbohydrates, which can effectively regulate the content of organic acids and improve the human body's utilization of polyphenol compounds [11]. From this fact, the species and composition of microorganisms have

important impact on the fermentation effect, chemical composition and biological efficacy of Kombucha. Therefore, conducting research on the microorganisms in Kombucha has become an important topic.

In order to improve fermentation efficiency and product stability, it is necessary to study the diversity and complexity of microbial community structure of Kombucha. Non-culture-dependent technology is to take DNA as the research object to detect microorganisms that are resistant to culture or unable to be cultured. This method evolves from independent studies of individual strains to comprehensive studies of microbial community composition. It fills the limitations of traditional cultivation methods and enables the in-depth study of microbial fermentation mechanisms, metabolic pathways, colony dynamics, and other information. Therefore, this method better describes the diversity of microbial phylogeny as well as the interactions between species and their environments [12].

In recent years, non-culture-dependent analyses such as high-throughput sequencing technology, macrogenomics, oligonucleotide mapping technology, and terminal restriction fragment length polymorphism analysis have played an important role in the microbial research of Kombucha [13]. The microbial diversity in Kombucha vary from many factors, such as raw materials, fermentation starter, fermentation parameters, even region and climate, and consequently affects the chemical composition and biological efficacy of Kombucha [14]. This article reviewed the recent research progress on the microbial diversity and its effects on components and functions of Kombucha, including the microbial population composition and interactions during Kombucha fermentation, the research methods and influencing factors of Kombucha microbial diversity, as well as the assembly and regulation of Kombucha fermentation microorganisms. This article will provide theoretical reference for better utilize the roles of functional microorganisms in Kombucha, improving the quality of Kombucha, and innovating Kombucha products.

2. Composition and interactions of microbial population in Kombucha

The core microorganisms in the fermentation system of Kombucha are bacteria and fungi. Their composition is an important factor affects the fermentation process and biochemical properties of Kombucha. At present, the microorganisms contained in Kombucha are continuously being recognized.

Among AAB, *Proteobacteria* has the highest relative abundance. All three genera of *Acetobacter* with a major role in the fermentation process were classified as the *Proteobacteria*, namely Komagataeibacter, *Acetobacter* and *Gluconobacter* [15]. The genus *Acetobacter* mainly includes *A. tropicalis, A. musti, A. lovaniensis, A. okinawensis, and A. peroxydans*, etc. As for *Glucobacterium*, *G. intermedius, G. liquefaciens, G. xylinus, G. potus, G. hansenii and G. oxydans* are the frequently reported species. The genus *Komagataeibacter* mainly consists of *K. hansenii, K. europaeus and K. rhaeticus* [16].

Another important bacteria in Kombucha is LAB, which mainly includes *Lacticaleibacillus casei, Lactiplantibacillus plantarum, Lactobacillus* rhamnosus, *Lactobacillus mali, Pediococcus pentosaceus, P.* acidiliactici, *Liqourilactobacillus nagelii, Oenococcus oeni, and Bifidobacterium* [17]. The superior LAB in Kombucha can also be utilized as potential functional strains. For example, Guo et al. investigated the functionality of three strains of acid-producing and nitrite-degrading *Lactobacillus acidophilus* (identified as *L. plantarum* and *L. casei*). They found that these LAB strains were able to tolerate the physicochemical factors in the gastrointestinal environment and colonize in the human intestines owing to the adhesion ability. Meanwhile, the metabolic process would not produce harmful substances to the human's body [18].

Ascomycota is the dominant fungus of Kombucha. In the diversity study at the genus level, the main yeast genera detected include *Zygosaccharomyces*, *Candida*, *Pichia*, *Brettanomyce*s and *Saccharomyces*. Of those, *Zygosaccharomyces, Brettanomyces* and *Candida* are the dominant typical genera [19]. However, the dominant fungi in Kombucha might vary from the origin. For example,

Zhao et al. reported that Dekkera and *Starmerella* were the dominant yeast genus in Kombucha samples from 12 different regions in China [15]. However, *Cyberlindnera*, *Thermoascus* and *Aspergillus* were detected from the Kombucha from Guangdong, China [19]. It is worth noting that there may be opportunistic pathogen such as *Pseudomonas*, *Acinetobacter*, and *Fusarium* in Kombucha, which are toxic to human beings and have serious threat to human health [15]. Therefore, the microbiological study of Kombucha not only helps identify beneficial microorganisms to achieve controllable fermentation processes, and maximize functional benefits, but also helps to effectively avoid the adverse impacts harmful microorganisms on fermentation.

The microbial diversity has a profound impact on the quality of Kombucha, playing an important role in the organic acids, enzymes, vitamins, flavor, aroma, viscosity, and other important characteristics of Kombucha. Bacteria contribute more to product flavor than fungi. Some specific bacteria strains (e.g. *Nucleobacterium glucosum* and *Acetobacter*) can enhance the concentration of detoxifying acids and increase the viscosity of the product. Different yeasts result in different ethanol content, therefore affecting flavor and nutritional value [20]. For example, *Komagataeibacter* showed a clear positive correlation with the generation of most acids and esters, while, and *Gluconobacter*, *Ralstonia*, and *Stenotrophomonas* had the opposite correlation with the content of some key volatile flavor compounds in the Kombucha [21].

3. Research methods for microbial diversity analysis of Kombucha

In recent years, a number of technologies, such as High-throughput sequencing, Metagenomics, Macro-transcriptome, Metabolomics and Multi-omics integration analysis, have been widely used to study the diversity of the microbiota of Kombucha. These techniques are of great significance for the in-depth analysis of the composition structure and function of the microbes, as well as their impact on the fermentation of Kombucha.

High-throughput sequencing, also known as “next-generation sequencing”, can be used to rapidly and accurately determine the changes in the composition, structure, and abundance ratio of the microbes in the Kombucha. He,X.Y. used 16SrDNA amplicon sequencing and ITS sequencing to analyze the dynamic changes of bacteria and fungi in the Kombucha that fermented by different days. The results showed that bacteria and fungi have stable metabolic functions during the fermentation process, and they could affect the flavor and efficacy of Kombucha through multiple pathways. They found that the diversity of bacteria was relatively higher than fungi during the fermentation initiation stage, but the decline rate of bacterial abundance was faster than fungi as the fermentation progressed. At the same time, they explored the physiological metabolism of Kombucha by using high-throughput targeted metabonomics technology. Based on the target gene prediction, pathway enrichment analysis and functional annotation of the differential metabolites of Kombucha samples with different fermentation times, 106 effective targets and four major target annotation pathways were obtained [22]. Gaggia et al. compared the microbial diversity of Kombucha fermented by three types of tea: black tea, green tea, and rooibos by combined the culture-dependent methods with high-throughput sequencing. *Acetobacteraceae* and *Pichiaceae* were identified as having the greatest abundance in all Kombucha samples, but the composition of yeasts in Kombucha were differed in biofilm phase and liquid phase at 7-14 days of fermentation. After 14 days, yeasts were significantly affected by different fermentation substrates [23]. Li et al. determined the microbial diversity of Kombucha using amplicon sequencing and found *Gluconacetobacter* was the absolutely dominant bacteria genus in the samples, followed by *Pseudomonas* [24]. Harrison et al. used high-throughput sequencing to study the microbial composition of 103 North American Kombucha beverages and the results indicated that *Brettanomyces* and *Komagataeibacter* were the dominant groups in the SCOBY of Kombucha. There was a negative correlation between the abundance of *Lactobacillaceae* and *Komagateibacter*. In addition, *Zygosaccharomyces* could serve as a compensatory group for *Brettanomyces* [25]. Arikan et al. systematically investigated the pattern of change in the diversity of bacteria and fungi in two liquid phase samples of Kombucha from Turkey during the fermentation time of 3-15 days by using

a combination of both macro-genome sequencing and amplicon sequencing (16srrna gene and ITS1). Their results showed that *Proteobacteria* had the highest abundance ratio in all samples throughout the fermentation process. The ratio of bacteria to fungi was increased significantly at 15 days of fermentation, and the dominant bacterial and fungal genus was *Komagataeibacter* and *Zygosaccharomyces*, respectively [9]. Zhao et al. constructed a macro-genome sequence by screened the high-throughput sequencing data, and analyzed the diversity and functional analysis of the microorganisms of Kombucha. They suggested a variety of functional probiotics existed in the Kombucha, mainly including *L. brevis, L. plantarum, L. paraplantarum, L. fermentum*. Among these, *L. brevis* had the highest abundance [26].

4. Factors affecting the microbial diversity of Komucha

There are many factors affecting the microbial community structure and ecological composition of Kombucha, such as fermentation substrate, culture conditions, climate and geographical location.

Fermentation substrate and culture conditions are the most direct factors, especially the type and nutrient composition of substrate, temperature and time of the fermentation[27].The fermentation substrate would affect the microflora composition and biochemical properties of Kombucha. Liu et al. reported that the microbial species and proportions in Kombucha fermented from black tea and green tea are different[28]. The dominant bacteria genus in Kombucha fermented with black tea as substrate was *Gluconobacter* (76.87%), but consisted of *Acetobacter* (31.65%) and *Gluconobacter* (24.27%) when fermented with green tea as substrate. The thickness and number of filamentous fibers of the bacterial cellulose film, and the microbial cell concentration in Kombucha fermented by black tea were greater than those fermented by green tea. Xiao et al. compared the microbe difference in quinoa Kombucha fermentation broth (QFB) and traditional Kombucha fermentation broth (TFB) [29]. They found that the total number of colonies of QFB was significantly greater than that of TFB, and the survival rate of *Saccharomyces cerevisiae* in QFB was 1.42 times higher than that of TFB. These results not only confirmed that the change of fermentation substrate had remarkable influence on the microbial diversity of Kombucha, but also indicated that the addition of quinoa had the benefit for enhancing the cell growth of microbes and improving the fermentation efficiency of Kombucha.

Microbial composition and diversity of Kombucha varied with storage time and temperature. Grassi et al. monitored the microbial dynamics of yeasts and acetic acid bacteria in the Kombucha beverage through PCR-DGGE [30]. They found that although the composition of total yeast would change dynamically under different conditions, *Dekkera anomala* was always the dominant yeast species in all samples. It could be preserved for long time at both room temperature and 4°C and produced organic acids. In contrast, AAB in Kombucha would maintain its biological activity for 90 days at 4°C, but only lasted for 20 days at room temperature. After been kept for 20 days, the composition and physiological metabolism of AAB would be significantly affected by temperature.

Geographic location might also lead to population differences in the distribution of microorganisms in Kombucha. For example, it was reported that the main yeast genus in Chinese Kombucha were *Candida, Zygosaccharomyces*, *Dekkera bruxellensis*. *Schizosaccharomyces*, *Brettanomyces bruxellensis*, while, *Saccharomyces* was dominated in French and Australian Kombucha [31]. By analyzed the species richness level of Kombucha from four different regions of China, Li et al. found that the microbial diversity in Kombucha liquid phase and biofilm phase was varied by region [32]. In the Kombucha from different regions, the abundance of fungi in the liquid phase was much higher than that that in the biofilm phase, but it was opposite for bacteria. Additionally, the relative abundance of metabolic pathways varied significant from different regions, and the changes in upregulated and downregulated gene pathways of bacteria were more abundant.

5. Microbial assembly and regulation for Kombucha fermentation

Currently, based on the in-depth study of microbial diversity in Kombucha, many researchers have devoted themselves to achieving the controllability of the fermentation process and the stabilization of product performance by assembling or regulating the microbes involved in Kombucha fermentation. Tran et al. designed nine microbial combinations for the Kombucha fermentation by selecting three yeasts from the genera of *Brettanomyces*, *Hanseniospora* and *Saccharomyces*, as well as three AAB strains from the genera *Acetobacter* and *Komagataeibacter* [33]. The results indicated that compared with the traditional Kombucha fermentation process, combinations of each yeast with AAB both could enhance the microbial fermentation kinetics. It suggested that AAB could promote yeast to show high invertase activity and enhance yeast metabolism, contributing to the improvement of nutritional value of Kombucha products. Wang et al. constructed a combined microbial agent, including *Candida*, *Komagataeibacter* and *Lodderomyces elongispora*s [11]. It could effectively improve the flavor and quality of Kombucha, since the organic acid content in the fermented Kombucha was increased with the fermentation time and the types of generated volatile and non-volatile substances was also enhanced. Li et al. found that the combination of *Gluconacetobacter* with a variety of non-*Saccharomyces* yeasts (such as Zygosaccharomyces and *Brettanomyces bruxellensis*) in the appropriate proportion could enrich the flavor of Kombucha products [24]. On the other hand, the study of microbial diversity in Kombucha also provides ideas for the innovation of novel Kombucha products. For instance, Fabricio et al. assembled *K. saccharioyans*, *B. anomala* and *K. marxianusw* as microbial symbionts and customized the fermenter medium using forced carbonation techniques [20]. In this way, non-alcoholic Kombucha with probiotic activity was efficiently produced, referred to as probiotic kombucha.

6. Conclusion

The strong symbiotic relationship between microbial communities and the complex metabolic pathways make Kombucha rich in biologically active substances and have potential therapeutic effects on a variety of diseases. The researches on microbial community diversity in Kombucha has always been a focus and hotspot worldwide, as they are the fundamental issues to ensure the quality, stability, and safety of Kombucha products. In recent years, attribute to the introduction of non-culture-dependent microbial diversity analysis technologies and equipment, the composition and interactions of core microorganisms in Kombucha, the microbial population diversity and its influencing factors, as well as the artificial regulation and assembly of microbiota in Kombucha have gained extensive attention of researchers all over the world and gained a lot of achievements. All these achievements promote the rapid development of the basic theory as well as the industrialization of Kombucha and provide theoretical support and technical guarantee for optimizing the fermentation efficiency, improving the health efficacy and guaranteeing the safety of drinking. In the future, further optimizing the artificial assembly of microbial populations and exploring the development of new types of Kombucha products will become the key research directions, which will contribute to enhance the potential of Kombucha in food and non-food industries.

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