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Black Tea Quality is Highly Affected during Processing by its Leaf Surface Microbiome

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ABSTRACT: Microbiomes can greatly affect the quality of fermented food and beverages, including tea. In this study, microbial populations were characterized during black and green tea manufacturing, revealing that tea processing steps can drive both the bacterial and fungal community structure. Tea leaves were found to mostly harbor Proteobacteria, Bacteriodetes, Firmicutes, and Actinobacteria among bacteria and Ascomycetes among fungi. During processing, tea microbial populations changed especially between sterilized and unsterilized samples. The surface sterilization of fresh leaves before processing can remove many microbes, especially the bacteria of the genera *Sphingomonas* and *Methylobacteria*, indicating that these are mostly phylloplane microbes on tea leaves. The surface sterilization removed most fungi, except the *Debaryomyces*. We also observed a fluctuation in the content of several tea quality-related metabolites during processing. Caffeine and theanine were found in the same quantities in green tea with or without leaf surface sterilization. However, the sterilization process dramatically decreased the content of total catechins and theanine in black tea, indicating that microbes on the surface of tea leaf may be involved in maintaining the formation of these important metabolites during black tea processing.

KEYWORDS: *tea quality, microbial community, tea processing, black tea, surface sterilization*

INTRODUCTION

For thousands of years, leaf infusions from the tea plant *Camellia sinensis* have been a vital component of Asian cultures. Now consumed worldwide, more than one-third of the world's population sips more than two billion cups of tea infusion per day for its relaxation, stimulation, and health benefits.[1] Apart from its beverage properties, evidence concerning the multiple health benefits of tea continues to pour forth, with encouraging data from human studies implying the roles in oral health, in the prevention of cardiovascular disease and some forms of cancer, and for other physiological functions such as antioxidant, antihypertensive effect, and antibacterial activities.[2] Other health associated benefits relate to type 2 diabetes and the control of body weight,[3] a phenomenon typically attributed to the existence of polyphenolic compounds like catechins. Tea leaves contain at least 2000 different phytochemicals, including phenolics, volatiles, and amino acids;[4] among them, the high relative abundance and bioactivity of polyphenols make them especially important constituents of tea leaves. All teas contain certain groups of nonoxidized monomeric flavonols, commonly referred to as catechins. Catechins are a major contributor to the astringent taste and are key compounds associated with the antioxidant capacity of tea.[5,6] Epigallocatechin-3-gallate (EGCG) is one of the most abundant and biologically active catechins. Other catechins include epigallocatechin, epicatechin-3-gallate, gallocatechin, and gallocatechin-3-gallate. Many studies have shown the antiviral activities of tea constituents, like EGCG from green tea and theaflavins from black tea, including against COVID-19 (as reviewed by Mhatre et al.[7]).

On the basis of the type of postharvest processing, tea is available in four major forms: postfermented (dark tea), fermented (usually black tea), nonfermented (green tea), and semifermented (oolong tea).[8] These processing approaches differ dramatically in the quantities of antioxidants present in the final tea product. Among these types, black tea is the most popular, accounting for ∼78% of total tea consumption worldwide, with green tea comprising ∼20%, while most of the rest is oolong tea.[9] The highest quality of green tea is obtained from the tender leaves or leaf buds, which must be harvested by hand plucking. Old leaves are always avoided for green tea production, although oolong and black tea can come from highly processed mature leaves of *Camellia sinensis*.[10] Fresh tea leaves are rich sources of bitter compounds, highly astringent, and with scarce aroma. However, numerous compounds related to flavor are removed or formed as a result of processing, even in the barely processed green teas. Hence, processing plays a vital role in generating the key features of any type of tea.[11] Processing transforms the original composition of tea leaves by exposure to a specific temperature, humidity, oxidative, and enzymatic regimens that are provided by endogenous enzymes as well as microbial enzymatic catalysis. Although many of the key contributors

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to tea quality that are altered by processing are well-known,[8,12−14] it is likely that many more remain to be discovered. Moreover, the relative roles of the microbial and endogenous physiologies to these changes are largely unknown.

The main processing steps for black tea can be described simply as plucking, withering, rolling, oxidation, and drying, among which leaves are withered, rolled, and allowed to ferment when enzyme-mediated oxidation occurs.[8,15−17] During this fermentation process, it is believed that >90% of the catechins are extensively oxidized and oligomerized by endogenous oxidase and peroxidase enzymes, leading to the formation of metabolites including theaflavins, theasinensins, and thearubigins that are primarily responsible for the antioxidant activity of black tea.[5,18] The extensive oxidation produces the red-brown color, distinctive taste, and sweet smell of processed black tea.

In recent years, attention has been paid to the metabolic functions of diverse microorganisms, including possibly beneficial bacteria and fungi, for their roles in altering the composition of tea components.[19−21] Dark teas have been extensively studied in this regard because they are the most heavily processed (with extensive fermentation). For instance, several fungal (e.g., *Cyberlindnera*, *Aspergillus*) and bacterial (e.g., *Klebsiella*, *Lactobacillus*) genera were found to be involved in quality traits in dark tea.[20] This same group reported that microbes of the genera *Aspergillus*, *Bacillus*, *Rasamsonia*, *Lichtheimia*, and *Debaryomyces* were the major flavor-producing microorganisms during the natural solid-state fermentation process in the postfermented (dark) tea called Pu-erh.[20] Members of the fungal genera *Eurotium*, *Debaryomyces*, and *Aspergillus* also were identified as possible beneficial microbes associated with another fermented dark tea, Fuzhuan brick tea, during its manufacture.[22] Other researchers found that *Aspergillus niger* and *Blastobotrys adeninivorans* were the dominant fungi and *Bacillus* and *Enterobacteriaceae* were the primary bacterial genera in Pu-erh tea.[23,24] Studies also showed that the key shift in the diversity and functions of microbial communities was attributed to the tea manufacturing process; for instance, bacterial genera, e.g., *Klebsiella*, *Pseudomonas*, *Lactococcus*, and *Bacillus*, that are mainly involved in the metabolism of carbon and flavor compounds were significantly changed during the tea manufacturing.[25] In another study, during initial stages of primary dark tea manufacturing, fungal genera *Cyberlindnera*, *Aspergillus*, *Uwebraunia*, and *Pleosporales* as well as bacterial genera *Klebsiella* and *Lactobacillus* were predominant, but only *Cyberlindnera* and *Klebsiella* prevailed in the later stages and remained relatively persistent until the end of processing.[20] However, it is still unclear how the microbiome or metabolites change in black and green tea processing and also the correlations between them. Often during tea storage and/or processing in local facilities, tea leaves come in contact with dust and microbes, including possible food-borne pathogens. One of the challenges for food scientists is to eliminate pathogens and spores in food without compromising the quality traits. For many foods, this is achieved by inactivating the pathogenic cells and spores using thermal[26] and nonthermal sterilization techniques.[27] Oxidizing compounds, such as sodium hypochlorite (NaClO), have been frequently used in the food industry due to their broad-spectrum antibacterial activity, high effectiveness, and low cost.[28] Understanding tea leaf microbial responses under sterilized conditions during processing could help to establish effective disinfectant strategies.

Information concerning the effects of black tea and green tea processing are very limited, perhaps due to the notion that the lack of fermentation (green teas) or less fermentation (black teas) compared to dark teas means that microbial action is of little importance to these teas. To date, a few studies have focused either on the tea processing methods that alter black tea biochemical composition and quality[29,30] or the chemical composition of tea components such as polyphenols using a metabolome approach[31] without any investigation of microbial involvement. Other studies have been conducted to investigate the microbial composition changes during the fermentation stage of dark tea production.[19,20,32] Hence, there is a need for the investigation of the bacterial and fungal community compositions and their possible association with tea quality-related metabolites change during the processing stages undertaken for black and green teas. Moreover, we used surface-sterilized fresh leaves to compare the relative contributions of phylloplane versus internal microbes and tea-endogenous activities. This knowledge should provide insights into the manufacturing process and valuable knowledge to improve the quality of black and green teas for both flavor and health-related benefits.

MATERIALS AND METHODS

Tea Sample Processing. Green and black tea processing were undertaken using variety "Fuding Dabaicha" (*Camellia sinensis* (L.) Kuntze) from a local processing unit of the Dongzhi Tea Plantation (latitude 29°36′15.03″ N, longitude 116°45′54.24″ E, Anhui Province, China) according to the routine methods.[14,33] We followed the standard processing steps for black and green teas at this facility and did the treatments inside the facility itself so that the appropriate ambient conditions were present; we then performed them manually in parallel for small experimental aliquots.

The four major steps in black tea processing (withering, rolling, oxidation, drying) were followed. For green tea, two types of green tea processing samples were collected. The first were fresh leaf samples, and the second were leaves after a one-step processing conducted on a fixation machine to produce the dry green tea sample. The details of the conditions during this tea leaf processing are illustrated in Figure S1. In addition, we also performed a leaf surface-sterilization (fresh leaves treated with 1% NaClO for 5 min followed by washing three times with sterilized H_2O) for half of the aliquots of both green and black tea prior to any processing. The processing steps generated a total of 12 sample sets. These were named fresh tea leaves without sterilization (TCF) and with sterilization (TSF), green tea dried samples without/with sterilization (GCD/GSD), black tea dried samples without/with sterilization (BCD/BSD), withered black tea without/with sterilization (BCW/BSW), rolled black tea without/with sterilization (BCR/BSR), and oxidized/fermented black tea without (BCO) and with (BSO) sterilization. These samples were collected on 10 May 2018 with three technical replicates for each. All of the samples during the processing were collected onto dry ice and stored at −80 °C until DNA extraction.

DNA Extraction and Amplicon Sequencing. Total DNAs of all samples were extracted using a modified cetyltrimethylammonium bromide method.[34] The quality and quantity of each DNA preparation were estimated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), gel electrophoresis, and Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA). Samples were used for library construction and subsequent sequencing at the sequencing facility of SAGENE (Guangzhou, China). An equal amount (100 ng) from three total genomic DNA extracts were pooled together as the final DNA template for amplification and sequencing. PCR amplifications were conducted with two sets of primers in the V4 variable regions for 16s rRNA and

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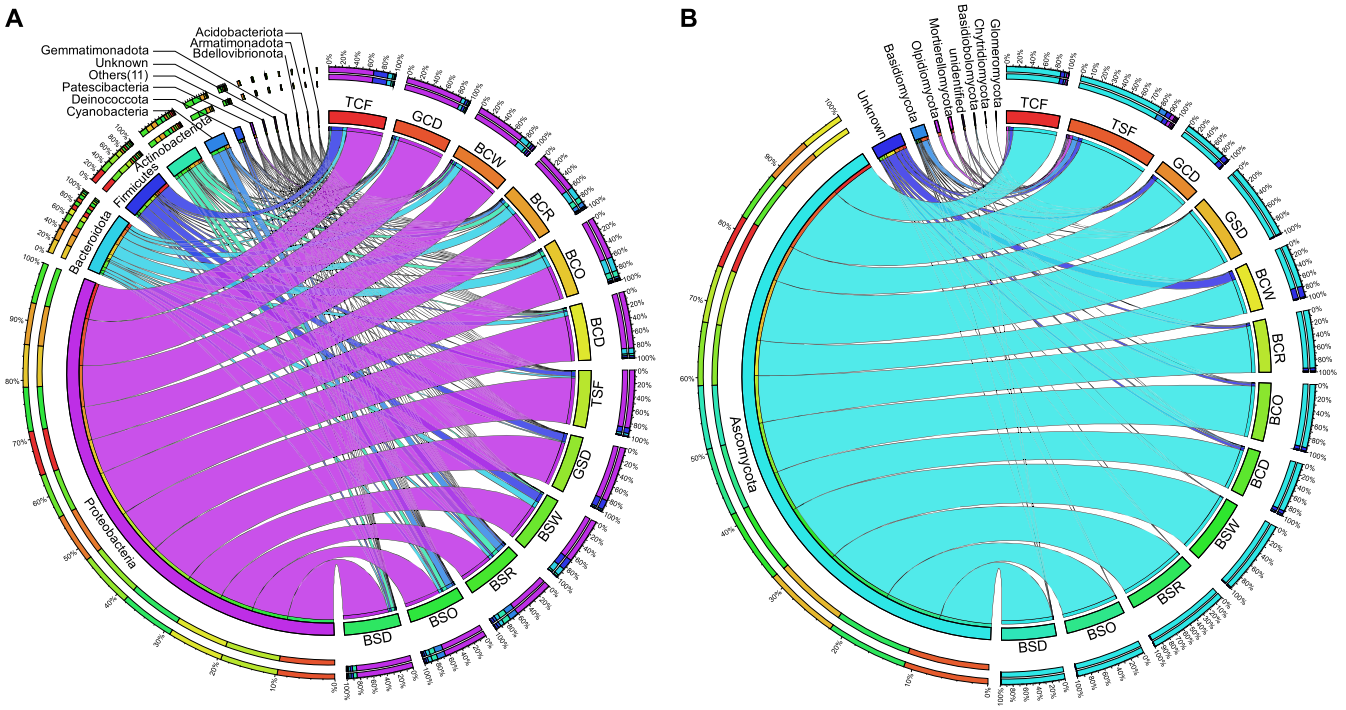

Figure 1. Microbiome communities at the phylum level found in black and green tea processing with and without leaf surface sterilization. (A) Bacterial microbiome communities from 16S amplicon sequencing. (B) Fungal microbiome communities from ITS amplicon sequencing. Each color in the left circle indicates one phylum and the right panel indicates the samples. The outer rings show the composition of each phylum or each sample, by sample type or phylum, respectively. The detail percentage information is also presented in Figure S3 and Tables S4 and S5.

ITS1 region for fungi. The conserved primers for amplifying the bacterial V4 region are 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), the standard primers used in the Earth Microbiome Project.[35] The primers used for amplifying the ITS1 region were ITS-1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′- GCTGCGTTCTTCATCGATGC-3′).[36] Sequencing libraries were generated after PCR with equal amounts using an Illumina Truseq DNA sample preparation kit (Illumina, San Diego, CA) following the manufacturer's recommendations. The PCR reactions were held at 95 °C for 2 min to denature the DNA, with amplification proceeding for 27 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; a final extension of 10 min at 72 °C was added to ensure complete amplification. In order to select DNA fragments of preferentially ∼400 bp in length, agarose electrophoresis was performed (120 V, 40 min, 1.5% agarose gel) and the fragment was excised from the gel. After purification using a spin column (QIAGEN, Dusseldorf, Germany), DNA fragments with ligated adapter molecules on both ends were selectively enriched using Illumina PCR Primer Cocktail in a 10 cycle PCR reaction. Products were purified using an AMPure XP system (Beckman Coulter, Beverly, CA) and quantified using the Agilent high-sensitivity DNA assay on an Agilent Bioanalyzer 2100 system. Libraries were sequenced with the PE250 specification on the Hiseq 2500 platform (Illumina, San Diego, CA).

Bioinformatic Analysis of Amplicon Sequencing Data. Paired end 16S and ITS amplicon reads generated from the Hi-Seq Illumina sequencing platform were imported and analyzed using the open-source pipeline Quantitative Insights Into Microbial Ecology (QIIME2, release-2020.8).[37] Briefly, forward and reverse raw reads were first trimmed with the CutAdapt tool within the QIIME pipeline to remove primers or any additional spacer nucleotide sequences. Filtering, demultiplexing, and denoising of truncated reads were performed using the DADA2 algorithm[38] to identify the ribosomal sequence variants (RSVs), and the resulting RSVs were summarized into feature/OTU tables. High-quality representative sequences were aligned using the MAFFT program,[39] followed by the construction of a phylogenetic tree using FastTree. Taxonomy annotation was performed using a customized Naïve-bayes classifier trained on operational taxonomic units (OTUs) for the 16S rRNA gene. These sequence variants were clustered at 99% similarity against the latest SILVA release 132 database[40] with a 403 bp trimmed length and a pretrained Naïve-bayes classifier trained on UNITE dynamic database release available on the QIIME web site for ITS sequences (https://unite.ut.ee). Mitochondria, chloroplast, and archaeal sequences were removed using a q2-taxa plugin by taxonomy-based filtering of unwanted sequences within the QIIME2 pipeline.

To assess α diversity, which is used to measure the diversity present within a sample or community of the samples, the data set was rarefied to an even sequencing depth, in order to remove sample heterogeneity (Figure S2). The resulting rarefied tables were then used to estimate the α diversity in the tea samples using Faith_PD (richness) and evenness using core metrics analysis within the Qiime2 pipeline. β diversity estimation, which provides a way to compare the diversity or composition between two samples or microbial communities, was performed using Jaccard distance metrics and visualized through principal coordinate analysis (PCoA) emperor plots. In addition, the distribution of taxa among the samples during tea processing steps with and without sterilization treatment was assessed using a reduced feature table retaining only taxa present in >80% (16S) and >50% (ITS) of the samples (further referred to as the tea leaf core microbiome) for the subsequent network construction.

Statistical analysis was carried out through both QIIME2[37] and R software (https://www.r-project.org/). Significant differences between samples with and without sterilization treatments and from different processing steps were assessed using the Kruskal−Wallis test. Analysis of similarity (ANOSIM) with 999 permutations was used to test significant differences between sample groups on the basis of α and β diversity distance matrices. Differential abundance of taxa was assessed through the analysis of composition of microbiomes (ANCOM) test within the QIIME2 pipeline. We also tested for a potential correlation between microbial communities and tea quality-related metabolites using RDA (redundancy analysis).

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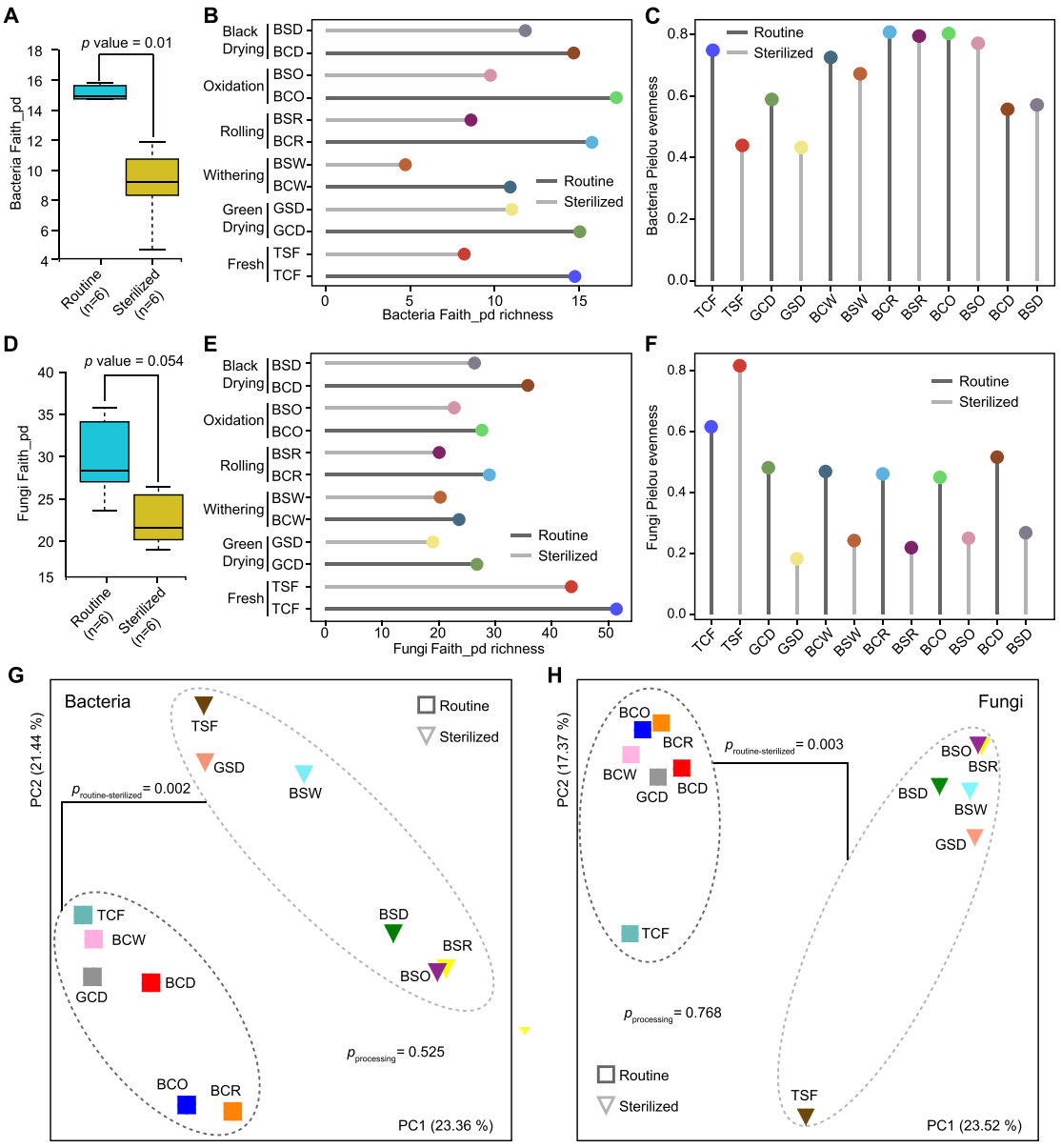

Figure 2. Microbiome diversity during black and green tea processing under routine and sterilization treatments. (A and D) Faith's PD-based α diversity (richness) of routine and sterilized samples. (B and E) α diversity of bacterial and fungal microbiome communities in each sample. (C and F) α diversity (Pielou evenness) in bacterial and fungal amplicon sequencing of all the samples. (G and H) β diversity of bacterial and fungal microbiomes, respectively. Each sample is indicated by one color. The square with dark gray and the triangle with light gray indicate the routine and sterilized samples, respectively.

Quantification of Tea Quality-Associated Secondary Metabolites. The three major secondary metabolites in tea are catechins, theanine, and caffeine. These metabolites are the primary determinants of tea quality and flavor. In the current study, the content of tea quality-related compounds was evaluated using high-performance liquid chromatography (HPLC). Catechins were extracted as described previously by Wei et al. in 2018.[41] Briefly, 0.1 g of freeze-dried tissue was ground in liquid nitrogen and extracted with 3 mL of 80% methanol under sonicating for 10 min at 4 °C. After centrifugation at 6000 rpm for 10 min, the residues were re-extracted twice as described above. Supernatants were combined and diluted with 80% methanol to a volume of 10 mL and filtered through a 0.22 μm organic membrane. The catechin (C), epicatechin (EC), gallocatechin (GC), epigallocatechin (EGC), epicatechin gallate (ECG), gallocatechin gallate (GCG), EGCG, and caffeine contents in the extracts were measured using a Waters 2695 HPLC system equipped with a 2489 ultraviolet (UV)-visible detector. A reverse-phase C18 column (Phenomenex 250 mm × 4.6 mm, 5 μm) was employed, and the samples were eluted at 25 °C at a flow rate of 1 mL min^{-1}. The detection wavelength was set to 278 nm. Then, 10 μL of the filtrate was injected into the HPLC system for analysis. Each experiment was performed in triplicate. Theanine was extracted and detected as described by Wei et al. in 2018.[41] Each experiment was performed in triplicate.

RESULTS

Microbiome Communities Found in Black and Green Teas. After short reads were filtered and then chloroplast and mitochondrial reads were removed, the feature table for the 16S data set contained a total of 342 410 reads (ranging from 55 487 to 8589) with an average of 28 534 reads per sample assigned to 712 RSVs, while the ITS data set includes 802 RSVs with a total abundance of 880 205, with an average of 73 350 (ranging from 111 930 to 55 276) reads per sample

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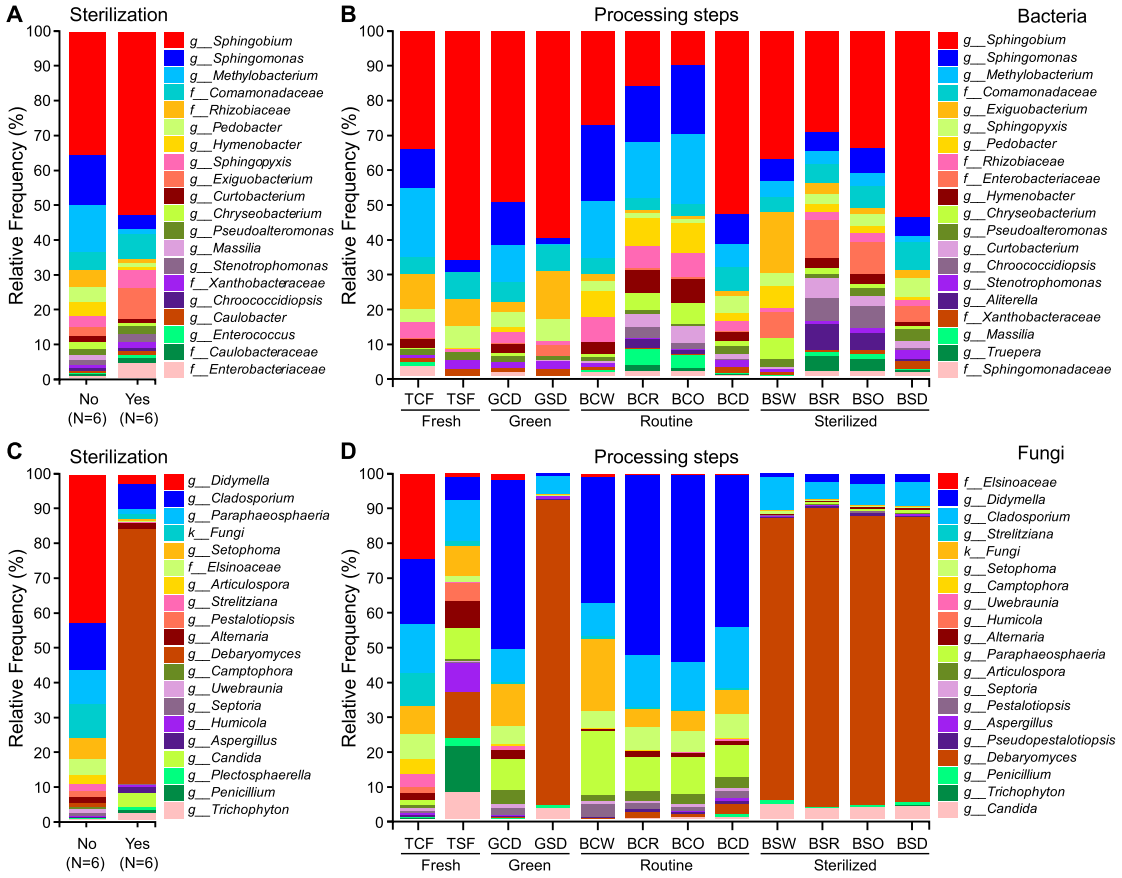

Figure 3. Black and green tea microbiome compositions described at the level of genus. (A) Bacterial composition of routine and sterilized samples. (B) Relative abundance of bacteria in all the black and green tea samples from the processing steps. (C) Relative abundance of fungi between routine and sterilized groups. (D) Sample-wise relative abundances of fungi from the different processing steps. Fresh, fresh leaves before processing; Green, samples of green tea; Routine, routine processing samples of black tea; Sterilized, samples of black tea after leaf surface sterilization.

(Tables S1−S3). The feature tables of both data sets were rarefied to a minimum read number of 8589 for the 16S data set and 55 276 reads for the ITS data set. The results from α rarefaction curves showed that the sequencing depth was sufficient to proceed to the corresponding analysis (Figure S2).

A total of 22 bacterial phyla, 45 classes, 112 orders, 180 families, and 320 genera were identified from the 16S amplicon data set. Protobacteria was the most abundant phylum across all the samples, accounting for >85% of the total community composition, followed by Bacteroidetes (2.1−20.6%), Firmicutes (0.9−20.7%), Actinobacteria (0.7−10.3%), and Cyanobacteria (0.3−13.9%) (Figure 1A, Figure S3, and Table S4). For the fungal communities 8 phyla, 28 subphyla, 72 orders, 155 families, and 252 genera were inferred from the sequence features. Ascomycetes was the most abundant phylum, representing 83.1−96.9% of the total fungal community diversity across the samples, followed by unknown fungi (2.1−8.6%) and Basidiomycetes (0.9−5.5%), while other phyla were present only at low abundances (0.01−2.3%) (Figure 1B, Figure S3, and Table S5).

Overall Microbial Diversity during Black and Green Tea Processing. When assessing the bacterial diversity within the tea leaf samples, we found the α diversity (Faith's PD) to be higher in routinely processed samples compared to the sterilized samples throughout tea processing steps, as shown in Figure 2A,B. The highest α diversity was found for leaves in the oxidation/fermentation step, followed by rolling steps for routinely processed samples, whereas the least diversity was found in the withering step with sterilized samples. When looking at the evenness, overall α diversity was higher in the initial steps of withering, rolling, and the oxidation of black tea and fresh green tea, compared to the last step, which is the drying of both black and green tea samples (Figure 2C). In contrast with the patterns regarding richness, leaf surface sterilization did not change the evenness excessively, especially for black teas (Figure 2C). The fungal communities of the processing steps were less affected in both Faith's PD and evenness (Figure 2D−F) than were the bacterial communities.

On the contrary, β diversity analysis based on the Jaccard indices showed that the bacterial and fungal communities varied significantly for the treatments. With or without leaf surface sterilization, it is noticeable that β diversity for both microbial kingdoms changed dramatically during the treatment steps (Figure 2G−H). This was true for both bacterial and, especially true, for fungal communities. The β diversity differences for the bacteria were evidenced impressively by the PCoA plots, where all the routinely processed samples were clustered separately from the sterilized samples (Figure 2G). Bacterial communities from the processing steps of drying, rolling, and oxidation clustered together, while fresh, withering, and drying samples clustered separately, regardless of sterilization (Figure 2G). Similar results were found for fungal communities under sterilization treatment, wherein routine samples clustered far from the treated samples (Figure

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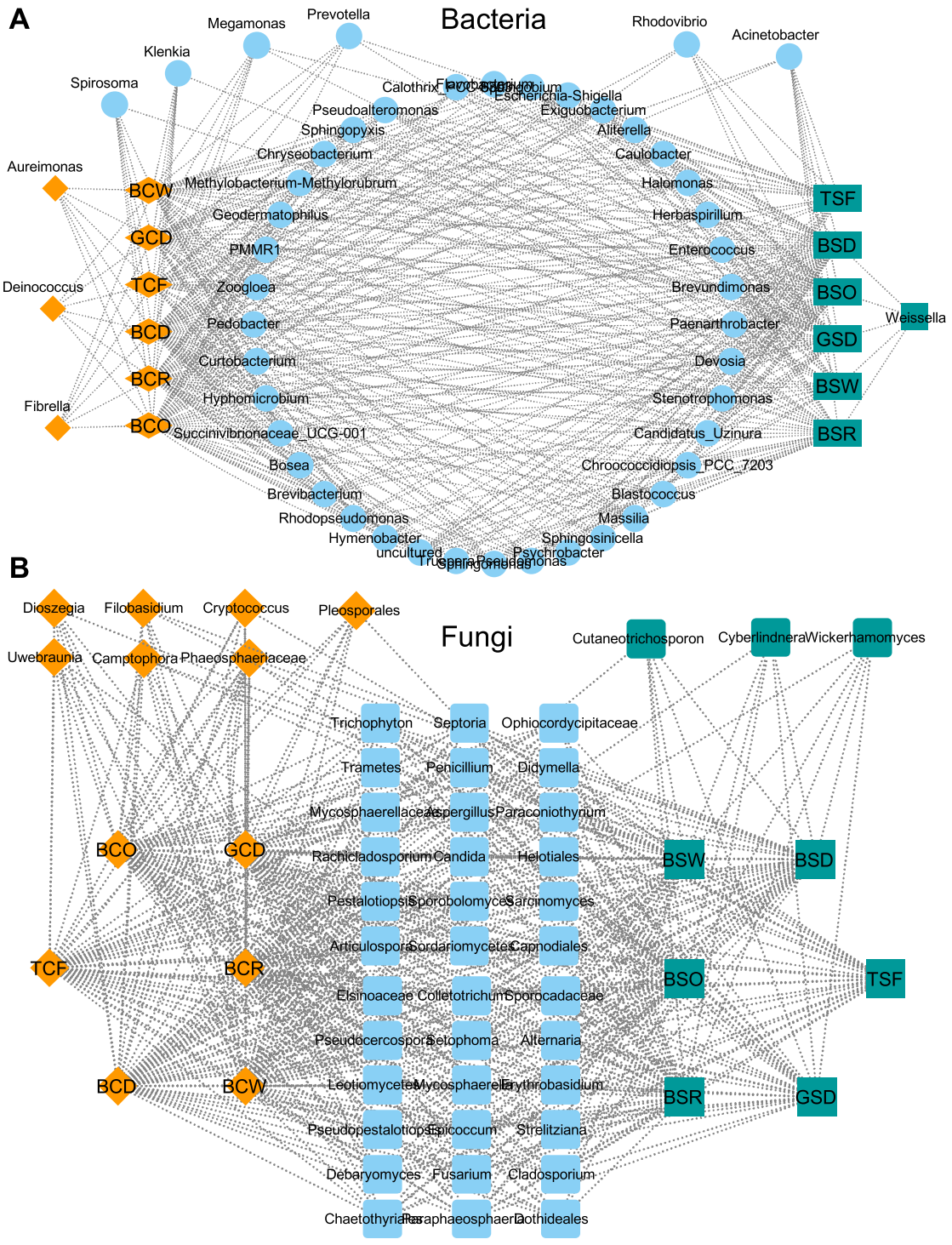

Figure 4. Microbiome network analysis of core communities of bacteria and fungi in tea leaves. (A) Bacterial core microbiomes and their distribution in different samples at the genus level. (B) Core fungal genera in different types across the various samples. The orange and dark green colors indicate the routine and sterilized processed samples, respectively, while the blue color indicates the genera identified in the amplicon sequencing.

2H). Unlike bacteria, the PCoA analysis of the fungal community provided a clear separation of fresh leaves compared to all the other samples under both sterilized and routine conditions (Figure 2H).

Sterilization and Processing Altered Black and Green Tea Microbiome Compositions. By comparing the abundances of microbiota under sterilization treatment, we found that the bacterial genera *Sphingobium* (28.85%), *Sphingomonas* (11.74%), and *Methylobacterium* (11.04%), plus genera belonging to the Comamonadaceae (4.19%) and Rhizobiaceae (3.94%) families, were the most abundant genera in routine processed samples. In contrast, *Sphingobium* (46.12%), *Exiguobacterium* (7.82%), Comamonadaceae (6.25%), and *Sphingopyxis* (4.70%) were most enriched in sterilized samples (Figure 3A and Table S6), suggesting that they are mostly endophytes unaffected by the leaf sterilization

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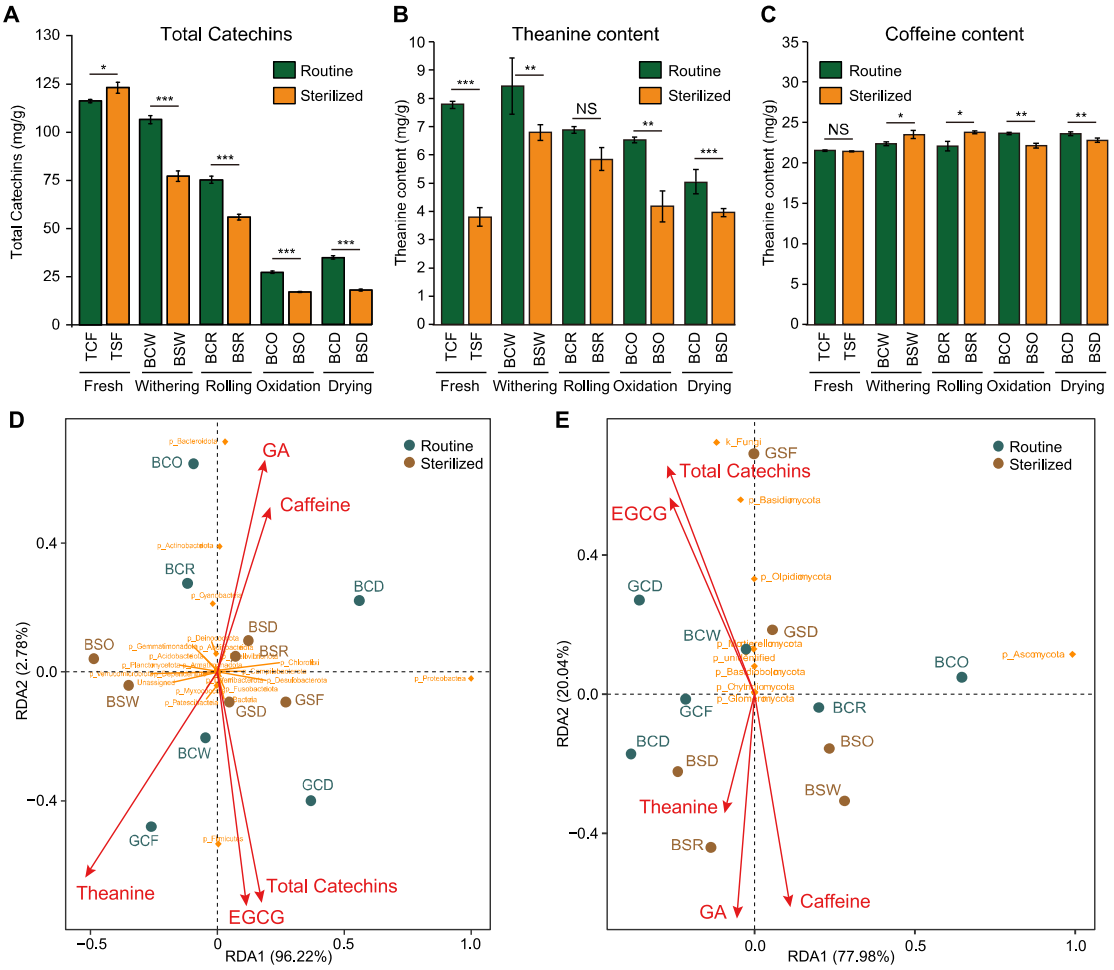

Figure 5. Tea quality-related metabolites change during the processing of black and green teas after leaf surface sterilization. (A) Content of total catechins across the processing steps in routine and sterilized samples of black tea. (B) Theanine content of different types of samples from black tea. (C) Caffeine content of different types of samples from black tea. (D) RDA plot indicates the interactions of the tea bacterial microbiome with tea quality-related metabolites. (E) Interactions of the tea fungal microbiome with tea quality-related metabolites. PC1 and PC2 of RDA were plotted with five major metabolites (GA, caffeine, total catechins, EGCG, and theanine) of black and green teas. "NS" indicates not significant, while "***,**, and *" indicate the significance using *t*-test under *p* values <0.001, < 0.01, and < 0.05.

process. A further comparative analysis at the genus level of black and green tea samples at various processing steps, after sterilization treatment, showed that the abundance of *Sphingobium* starts to gradually decrease in black tea withering, rolling, and oxidation stages but was recovered to a relatively high level after drying in both routine and sterilized samples (Figure 3B and Table S7). Withering, rolling, and oxidation increased the abundance of the genus *Sphingomonas* after the initial stages in routine samples of black tea. Surface sterilization dramatically reduced the relative abundances of the aerobic bacteria, *Sphingomonas* and *Methylobacterium*, compared to routinely processed samples regardless of processing steps in black tea. The genus *Methylobacterium* and members belonging to the Rhizobiaceae family were overrepresented in routine samples, while Enterobacteriaceae were overrepresented in sterilized samples throughout the processing stages in black tea (Figure 3B and Table S7). In green tea processing, genera *Sphingomonas* and *Methylobacterium* were highly represented in fresh leaves but decreased after drying, while the relative abundance of *Exiguobacterium* and genera belonging to the Enterobacteriaceae increased in the green tea drying stage (Figure 3B and Table S7).

The most abundant fungal genera present in routinely processed samples were *Didymella* (38.74%), *Cladosporium* (12.10%), *Paraphaeosphaeria* (8.93%), unknown fungal genera (8.71%), and *Setophoma* (5.62%). After sterilization, members of *Debaryomyces* were very highly represented, accounting for >65% of the total reads, indicating that they are fully internal to the leaf and that most of the other fungi were either fully or mainly external. The other major fungi after sterilization were *Cladosporium* (6.40%), *Candida* (3.60%), *Didymella* (2.24%), and *Trichophyton* (1.71%) (Figure 3C and Table S8).

The fresh tea leaves had large quantities of the genera *Didymella*, *Setophoma*, and *Strelitziana*, plus genera from the Elsinoaceae family, in the routine samples, whereas genera *Debaryomyce*, *Cladosporium*, *Trichophyton*, and *Aspergillus* are the major genera in the sterilized samples (Figure 3D and Table S9). When assessed during the processing steps, fungi of the genus *Debaryomyces* became even more dominant among the sterilized samples during the initial drying process and retained that dominance throughout the many subsequent steps in black tea processing (Figure 3D). *Trichophyton* and *Aspergillus*, in contrast, rapidly disappeared during the drying and all subsequent steps. *Cladosporium* and *Candida* remained relatively constant at low abundances throughout the

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processing stages in black tea sterilized samples. The relative abundances of most fungal genera increased or decreased to at least some degree during both the simple green tea drying step and/or during the more elaborate processing steps that are used to produce black tea (Figure 3D and Table S9). This was true in both samples derived from sterilized leaf surfaces and those derived from unsterilized leaves.

Core Communities and Differential Abundance Analysis. A total of 91 bacterial taxa belonging to 64 genera were represented in most 16S data sample, while 75 fungal taxa belonging to 50 known genera were found in most ITS samples, which were characterized as core microbiome (Figure S4). A network analysis of these core taxa was performed and visualized through Cytoscape (Figure 4). Although the majority of the bacterial and fungal core taxa were present in all types of samples, regardless of treatment and processing steps, some genera differed in presence/absence between routinely processed and sterilized samples. For instance, the genera *Aureimonas*, *Deinococcus*, and *Fibrella* were only present in the routinely treated samples, while *Weissella* was only found in sterilized samples (Figure 4A). Unlike bacteria, most of the fungal core taxa were represented in all types of samples (Figure 4B). The analysis of composition of microbiomes test (ANCOM)[42] was performed to test which, if any, genera discriminated between the black and green tea microbiota and/or of routinely processed teas versus sterilized samples. The results of ANCOM confirmed that a few genera are landmarks for differences between the groups. The three bacterial genera *Spirosoma*, *Aureimonas*, and *Klenkia* were significantly different between the routinely processed and sterilized samples (Figure S5 and Table S10). Similarly, in the case of fungi, genera such as *Wickerhamomyces* and unidentified genera belonging to the Mycosphaerellaceae family were found to be statistically significant in their differential abundances between the sterilized and unsterilized samples (Figure S5 and Table S11).

Manufacturing and Sterilization Alter Metabolite Contents Related to Tea Quality. The content of several tea quality-related secondary metabolites was examined from samples under routine versus leaf surface sterilization treatments at different processing steps. These several important tea metabolites showed significant differences during processing (Figure 5A−C and Table S12). Overall, the total catechin content in black tea gradually decreased as the tea processing proceeded regardless of treatment (Figure 5A). The highest content of this major tea polyphenol was observed in fresh leaves up until the withering step but then started to drop off at the rolling stage. Compared to fresh leaves, ∼36% and ∼55% of the catechins in rolling stage were lost of routine and sterilized samples, while the oxidation step only retained 23% in routine and 14% in sterilized samples (Figure 5A). Both routinely processed and sterilized samples showed significant differences when compared for each processing step, except for fresh leaves. The absence of an effect in fresh leaves shows that the sterilization process itself did not alter detectable catechin amounts directly.

Theanine, a nonprotein amino acid associated with the umami trait in tea, exhibited a relative increase in amounts at the early withering step for both routinely and sterilized samples but was consistently reduced during rolling, oxidation, and final drying step in routine samples (Figure 5B). Interestingly, the theanine content in sterilized samples was lower than in the routine samples of each step and remained stable in at the rolling, oxidation, and drying stages (Figure 5B). In fact, the differences of theanine content between sterilized and routine processed black tea samples was highly significant (t-test $p = 0.0105$) but not in green tea samples (t-test $p = 0.1865$, Figure S6). Compared with the routine processed samples, the caffeine contents also show differences in the relevant sterilized samples during the black tea processing but were not as significant as the observation in total catechins or theanine change (Figure 5C). No significant differences in caffeine contents and total catechins between the routine or sterilized green tea samples were observed (Figure S6).

Association of the Tea Leaf Surface Microbiome with Tea Quality-Related Metabolites. Redundancy analysis (RDA) was performed to study the relationships between tea quality-related metabolites including total catechins, caffeine, theanine, EGCG and gallic acid (GA), and the abundance of different microbes in the tea leaf microbiome. In the RDA of bacteria and fungi, the major tea quality-related metabolites factors explained a great amount of variance in first two RDA axes (>90%), showing that both bacterial and fungal microbiomes affect tea quality-related metabolite production, turnover, or storage (Figure 5D,E). Bacteria of the phylum Firmicutes showed a strong association in their abundance with the levels of total catechins and EGCG, while Bacteroidetes, Actinobacteria, and Cyanobacteria were associated with GA and caffeine (Figure 5D). Similarly, associations were found between the total catechins and EGCG levels with the relative abundances of fungal phyla Olpidiomycota, Basidiomycota, and unknown fungi (Figure 5E). We further undertook the correlations analyses between the abundance of bacterial or fungi phylum and the tea quality-related metabolites content (Figure S7). We found that total catechins (including EGCG) are positively correlated with the abundance of bacterial phylum Firmicutes but significantly negatively correlated with phyla like Actinobacteriota, Deinococcota, Bdellovibrionota, Chloroflexi, and Gemmatimonadota. In contrast with the observation in total catechins, GA is positively correlated with those phyla. Theanine content is negatively correlated with Gemmatimonadota but positively correlated with Verrucomicrobiota. No significant correlations between caffeine and the bacterial abundance were observed. In fungal phyla, only Ascomycota were significantly correlated with the theanine content. Caffeine and total catechins were mostly negatively and positively correlated with the fungal phyla, respectively. The results showed similar correlation patterns as were observed in the RDA results.

DISCUSSION

Diverse populations of bacteria and fungi are associated with health benefits from many different fermented foods and beverages.[43] The degree to which the microbes dwelling on tea leaves are important to the distinctive biochemical characteristics of processed tea are largely unknown. In this study, green and black tea samples were used to investigate the bacterial and fungal communities shift during tea processing. We also sterilized the surface of fresh tea leaves before the processing to investigate how sterilization affects the microbial diversity and tea quality during different processing stages. Our investigations have revealed a rich microbial community present both on the tea leaf surface and inside the leaf. Surprisingly, we found that the tea leaf surface sterilization greatly affected the tea microbial abundances, as well as the major tea quality-

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related metabolites during tea processing. For the entire intact leaf, the phyla Proteobacteria, Bacterioidetes, Firmicutes, Actinobacteria, and Cyanobacteria are the five most abundant, in that respective order. In these same samples, the most abundant fungi belong to the phyla Ascomycota and Basidiomycota. Dominant patterns of these same phyla were also observed in a previous study on dark tea processing, with the exception that Firmicutes were more abundant than Bacteriodetes in an earlier study.[20] At the level of genus, leaf surface sterilization experiments indicated that some microbes were mostly or exclusively on the leaf surface, while others existed primarily as endophytes inside the leaf. These endophytes include the genera *Sphingobium* and *Exiguobacteria*, plus members of the family Comamonadaceae, among the prokaryotes, and *Debaryomyces*, *Trichophyton*, *Aspergillus*, *Candida*, and *Penicillium* among the fungi. Microbial genera found primarily on the leaf surface and thus subject to removal by the surface sterilization step were found to be *Sphingomonas* and *Methylobacterium* among the prokaryotes and *Didymella*, *Cladosporium*, *Setophoma*, and members of the family Elsinoaceae among the fungi. *Sphingomonas* as an endophyte was previously isolated from surface sterilized kernels of maize.[44] The high abundance of *Methylobacterium* among the prokaryotes in routine samples and decreased abundance after surface sterilization are explainable because the leaf stomata are a methanol release site, implying that they can be the primary site for colonization and can serve as the site for bacterial entry into apoplast since *Methylobacterium* are capable of using the plant-derived, reduced one-carbon compound methanol as a sole source of carbon and energy for their growth and development.[45,46]

The α diversity in routine samples was significantly higher than that in the sterilized samples. Both the abundance and α diversity differed to a great extent between the routine and treatment samples than the differentiation between the later stages during manufacturing, especially in the fungi community. Surface sterilization also affects the β diversity of both bacterial and fungal communities, as evidenced in PCoA plots where all the routinely processed samples were clustered separately from the treated samples. This alteration of the community abundance and diversity demonstrated that an initial leaf surface sterilization before the tea manufacturing should affect the microbial community contribution to tea quality, especially in dark teas that undergo extensive fermentation. Interestingly, the most dramatic shifts of both bacterial and fungal communities in green and black tea manufacturing occurred in the fresh tea leaves between routine and sterilized samples. In the black teas, bacterial microbial communities underwent relatively minor compositional changes during the subsequent processing steps and the fungal communities changed hardly at all. The sterilization by sodium hypochlorite treatment created a black tea microbiome that was dominated by bacteria of the genus *Sphingobium* and the fungus *Debaryomyces*. Sphingobia are common soil bacteria that are notable for their capacity to degrade a great variety of environmental chemicals, including many herbicides, but their roles as endophytes are unknown. *Debaryomyces* is a genus of yeasts that consists of 19 recognized species.[47] *Debaryomyces hanseii* provides antifungal activities in a number of environments and has been proposed as a biological control for fungal contaminants in the food industry.[48] A study of Fu brick tea, a type of dark tea, has shown that *D. hanseii* enzymatic activity during fermentation generates the sweet substance xylitol, vitamins, and some important organic acids that have been proven to enhance tea quality.[22] Such bacteria genera as *Aurantimoas* and *Methylobacterium* and the fungal genera *Cyberlindnera*, *Aspergillus*, *Uwebraunia*, and *Debaryomyces* are all present and variable in abundance across the samples in our study. Previously, these bacterial and fungal taxa have been shown to be core microorganisms of dark teas that are responsible for changes in the enzyme activities of polyphenol oxidase, peroxidase, cellulase, and pectinase. These microbes also change the composition of chemical compounds such as polyphenols, amino acids, flavonoids, and soluble sugar during the dark tea fermentation process.[20] Although the majority of these core taxa were present in all of our samples, most were present in very different relatives amounts in sterilized versus routinely processed samples. For instance, *Aureimonas*, *Deinococcus*, and *Fibrella* appeared only in routinely processed samples. Hence, our results suggest that tea quality should be influenced by this surface sterilization.

Our analyses of several known tea quality-related metabolites during black tea processing indicated major differences in their abundances in sterilized versus routinely processed samples and during tea processing steps. In previous research, the declines in such metabolites as catechins were primarily attributed to enzymes secreted by the microbial communities during the fermentation process.[49] As also observed in other studies, the content of total catechins and theanine contents gradually decrease during our routine tea processing.[13,16,17] However, with leaf surface sterilization, the content of total catechins of each processing step was much less than those in routine samples, suggesting that the leaf surface microbes actually induced a higher level of catechin synthesis or retention. Interestingly, we found that theanine was even more reduced in the surface sterilized samples, indicating that the missing phylloplane microbes are also involved in overall theanine retention rather than loss. However, the caffeine contents were not as significant as those observed in total catechins or theanine change during the black tea processing between sterilized and routine processed samples, even slightly dynamic differences were detected. During processing, some other bacterial and fungal taxa were significantly different in their abundances when comparing the routinely processed and sterilized samples These included the bacterial genera *Spirosoma*, *Aureimonas*, and *Klenkia* and fungal genera such as *Wickerhamomyces* and members of the Mycosphaerellaceae family. In green tea, catechin, caffeine, and theanine contents in both fresh and dry leaves showed no excessive differences between quantities regardless of routine or sterilization treatment. The RDA and correlation analyses showed that both the bacterial and fungal microbiomes can affect or can be influenced by the tea secondary metabolites, further supporting the idea that microbiota changes may be involved in the formation of processed tea quality-related metabolites.

This study established that tea processing steps can drive both the bacterial and fungal community structure and composition changes. Moreover, fluctuation in the content of several important tea quality-related metabolites observed in this study indicated that leaf surface microbes protect against the loss of these quality-related compounds rather than cause their loss. Specifically, we found that sterilization could reduce the content of total catechins and theanine in black tea compared to routine manufacturing. It still remains possible that endophytic microbes might increase the turnover of catechins and/or theanine. Further in-depth studies still need

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to be undertaken to validate the current observations in different places/varieties under a more standard processing procedure. Overall, our findings pave the way toward our understanding of the tea microbiome, toward a goal that might help to identify certain beneficial microbiota that could be used as supplements to improve tea quality during and after processing.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c01607.

Figures of sampling and processing steps of black and green tea applied in current report, normalization of amplicon sequencing to an even sequencing depth for estimate the α diversity, microbiome communities at the phylum level found in black and green tea processing with and without leaf surface sterilization, core microbiomes of bacteria and fungi and their abundance in each sample at genera level, ANCOM results, tea quality-related metabolites change during the processing of green tea after leaf surface sterilization, and correlation analysis between the tea quality-related metabolites and the abundance of the identified microbiotas during the tea processing of all the samples (PDF)

Tables of clean reads count of bacteria and fungi amplicon sequencing, absolute abundance of RSVs identified in bacteria amplicon sequencing and fungi amplicon sequencing, absolute abundance of bacterial phyla identified in 16S amplicon sequencing and ITS amplicon sequencing, abundance of top 20 taxa in genera level of bacteria and fungi across between routine and sterilized samples, differential abundance with ANCOM test for bacteria and fungi among routinely processed and sterilization treatment, and tea quality-related secondary metabolites contents in black and green tea (XLSX)

Accession Codes

Raw reads of all the 16S and ITS amplicon sequences reported in this study have been deposited into the National Center for Biotechnology Information BioProject database under accession number of PRJNA703764.

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Author Contributions

[⊥]W.T. and J.Y. contributed equally. J.L.B., A.I.M., and W.T. designed and supervised the study. J.Y., W.T., and C.W. collected the samples. Q.W., L.H., and Y.W. did the HPLC analysis. A.I.M., W.T., J.Y., and D.T. performed amplicon sequencing analyses. A.M., J.Y., and W.T. wrote the manuscript. A.I.M., J.Y., J.L.B., T.L., and W.T. revised the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

EGCG: epigallocatechin-3-gallate
GA: gallic acid
QIIME2: Quantitative Insights into Microbial Ecology
RSV: ribosomal sequence variants
OUT: operational taxonomic units
PCoA: principal coordinate analysis
ANOSIM: analysis of similarity
ANCOM: analysis of composition of microbiomes
RDA: redundancy analysis

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