Microbial diversity and component variation in Xiaguan Tuo Tea during pile fermentation

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Abstract

Citation: Haizhou Li,Min Li,Xinrui Yang,Jiuyun Chu,Changwang He,Weitao Wang,Feng Han,Ping Li Microbial diversity and component variation in Xiaguan Tuo Tea during pile fermentation. **protocols.io**

dx.doi.org/10.17504/protocols.io.kc8cszw

Published: 19 Oct 2017

Protocol

Tea sample collection

Step 1.

Xiaguan Tuo Tea was made from the leaves of the plant Camellia sinensis and obtained from Xiaguan, Yunnan, China (25°34’55.88’’N, 100°12’41.88’’E). The tea was produced by Yunnan Xiaguan Tuo Tea (Group) Co., Ltd in April 2015. We collected unfermented tea, first turn tea (6 d), second turn tea (11 d), third turn tea (19 d), fourth turn tea (27 d), fifth turn tea (34 d), sixth turn tea (40 d), seventh turn tea (47 d), and fermentation termination tea (56 d) samples.

Fermentation process characterization

Step 2.

At the beginning of Xiaguan Tuo tea fermentation, water was scattered on the leaves until it reached approximately 30-35%. The wet tea was placed into a fermentation room. During tea fermentation, the tea was turned over seven times to control the fermentation temperature. When leaves were turned over, the temperature was measured in the core of the tea pile. The water content in the tea was calculated after drying the tea at 105°C for 1 hour. Then, 1 g of tea was suspended in 10 mL of deionized water by using a homogenizer at 150 rpm for 10 min, and the pH of the water was measured. Each sample was analyzed in triplicate, and the values were expressed as the mean (n=3). When leaves were turned over, tea samples were removed for analysis of microorganisms and chemical components.

Cultivation conditions of microorganisms in the Xiaguan Tuo Tea

Step 3.

First, 1 g of fermented tea was placed into 10 mL of sterile water. Then, the flask was shaken several times to break up microorganisms that had adhered to the tea. Then, 100 µL of water was transferred into 900 µL of sterile water. The procedure was repeated five times, and each time resulted in a ten-fold dilution of the previous tube. Finally, 100 µL of water from each dilution was transferred to plates of different culture media. The bacterial culture medium contained 5 g of peptone, 1.5 g of beef extract and 15 g of agar in 1000 mL of water; fungal culture medium contained 10 g of glucose, 15 g of agar, and 100 g of peeled potato in 1000 mL of water; yeast culture medium contained 10 g of glucose, 5 g of peptone, 2.5 g of yeast extract and 10 g of agar in 1000 mL of water. Ampicillin was added to the fungal and yeast media (10µg/mL). The incubation temperature and period were 30°C and 7 d, respectively. Each sample was done in triplicate, and the values were expressed as the mean (n=3).

Isolation of total DNA

Step 4.

The isolation of DNA from bacteria, fungi, and yeasts from tea samples was done using the Fast DNA SPIN Kit for Soil (MP Biomedical, USA), finally eluting in 50 µL of MQ water. The DNA was then stored at −20°C.

Next-generation sequencing and sequence analysis

Step 5.

We amplified the ITS region using the Miseq-ITS primers ITS1FI2 and ITS2. ITS1 primer: 5’-CCCTACACGACGCTCTTCCGATCTN(barcode)CTTGGTCATTTAGAGGAAGTAA, ITS2-Rev primer 5’-GTGACTGGAGTTCCTTGGCACCCGAGAATTCCAGCTGCGTTCTTCATCGATGC-3’. PCRs were set up to run at 3 min at 95°C, followed by 5 cycles of 20 s at 95°C, 30 s at 65°C, 20 cycles of 20 s at 94°C, 20 s at 55°C, and 30 s at 72°C. A final elongation was done at 72°C for 5 min. Then, we used combinatorial primer labeling to identify samples after the first PCR. The second PCR conditions were 30 s at 95°C, followed by 5 cycles of 15 s at 95°C, 15 s at 55°C, and 30 s at 72°C. A final elongation was done at 72°C for 5 min. Amplifications were carried out in a total volume of 50 µL, using 20 ng of DNA, Taq polymerase (Thermo, USA), 100 mM KCl, 500 µM each dNTP, 3 mM MgCl2, 20 mM Tris-HCl (pH 8.3), and 0.4 µM each primer and PCR-enhancing substances. Purification was done with an Agencourt AMPure XP system (Beckman, USA). We normalized PCR products after quantifying them with a Qubit 2.0 Fluorometer(Invitrogen). Paired-end sequencing (2×150 bp) was carried out on an Illumina MiSeq sequencer at the Sangon Genome Center (Shanghai, China) using NGS. We assembled paired-end reads using PEAR[18]. The quality of the reads was checked by using PRINSEQ[19]. Chimera detection was performed with the USEARCH[20]. OTUs were picked at the 97% sequence identity level by USEARCH. One sequence from each OTU was selected to be representative, and the closest reference sequences (GenBank: http://www.ncbi.nlm.nih.gov & RDP) were pooled and aligned using CLUSTAL X[21]. Phylogenetic analysis was performed using the distance-based maximum likelihood method with MEGA 7.0[22]. Bootstrap analysis was performed using 1000 replications. The Shannon-Weaver and Chao1 diversity indices were calculated using MOTHUR[23]. Rarefaction curves were calculated using MOTHUR. Sequence data are publicly available via the NCBI Sequence Read Archive database (SRP091015).

Microorganism counts in fermentation using FISH

Step 6.

All samples for FISH were collected at different time of tea fermentation and stored on dry ice during transportation. For detection, 0.5 g of tea sample was used. Then, 320 µL of 25%(w/v) particle free paraformaldehyde solution (4% final concentration) was added, flled up with 1× PBS, mixed up completely, and the suspension was stored at 4 °C for 24 h. The fixed samples were washed twice with 1× PBS, centrifuged at 10,000×g for 5 min at 4 °C after each washing, and stored in PBS/ethanol (1:1) at −20 °C for further processing. Then, 100 µL of the fixed sample was diluted with 900 µL of PBS/ethanol, and the mixture was dispersed by ultrasound with an ultrasonic probe at minimum power for 10 s using 1-s sonication pulses. Then, 20 µL of the sample was diluted in 10 mL of MQ water. This suspension was filtered through polycarbonate filters (0.2 mm pores, 25 mm in diameter). If the signal intensity is low, the tea sample dilution rate was reduced accordingly. After filtration, the filters were dipped in 0.1% low-melting point agarose and dried in an incubator at 46°C. The cell walls were permeabilized by addition of proteinase K solution (15 µg/mL, Roche) and were then subsequently incubated in 3% H2O2 to inactivate endogenous peroxidases. Air-dried filters and cut filter sections were used for hybridization. Filter sections were placed in a 1.5-mL tube and mixed with 300 µL of hybridization buffer (10% (w/v) dextran sulfate, 2% (w/v) blocking reagent (Roche, Germany), 20 mM Tris–HCl [pH 8.0], 0.1% (w/v) sodium dodecyl sulfate, 0.9 M NaCl, and 55% (v/v) formamide) and 1 µL of probe working solution (final concentration 0.028 µM). The probes and their sequences are shown in Table 1. The nonsense probe NONEUB was used as a control. After

hybridization at 46°C for at least 90 min on a rotor, the filters were transferred to prewarmed washing buffer (20 mM Tris-HCl [pH 8.0], 5 mM EDTA [pH 8.0], and 3 mM NaCl, 0.01% (w/v) SDS) and incubated for 15 min at 48°C; the samples were then mixed with 1000 µL of amplification buffer (1×PBS [pH 7.4], 0.1% (w/v) blocking reagent, and 0.0015% H2O2,) and 1 µL of Alexa488 Tyramide (molecular probes, Life TechnologiesTM). Then, the filter sections were incubated in amplification buffer at 46°C for at least 20 min in the dark. Afterwards, the filters were stained with DAPI and mounted with 5 µL droplets of antifade reagent (Molecular Probes, Life TechnologiesTM). Cell counting was performed on 10 randomly selected micrographs that were taken with 20× objectives (150,415 µm2), and the results were extrapolated to 1 g of tea. Automated counting was performed on micrographs that exhibited a high contrast between stained cells and background fluorescence with the image analysis software ImageJ [24-28].

Determination of tea polyphenol content

Step 7.

The total polyphenol content in Xiaguan Tuo Tea was measured by using the Folin–Ciocalteu method. The Folin regent was made by adding 5 g of phosphomolybdic acid, 25 g of sodium tungstate, and 12.5 mL of phosphoric acid to 180 mL of distilled water and boiling the solution for 2 h. Then, the volume was made up to 1 L with distilled water. Next, 1 g of tea was boiled in 100 mL of distilled water for 1 h, and the solution was filtered to remove residual solids. In total, 5 mL of the extract was mixed with the same volume of Folin regent and left for 3 min. Then, 5 mL of sodium carbonate was added, and the solution was left for 1 h. The reaction mixture was centrifuged at 3000 rpm for 5 min, and the absorbance at 700 nm of the supernatant was measured. Each sample was done in triplicate, and the values were expressed as the mean (n=3)[13].

Determination of caffeine content

Step 8.

In total, 1 g of tea was extracted with 100 mL of boiling water for 60 min. 25 mL of the above solution was mixed with equal volume of chloroform (Sinopharm Chemical Reagent Co., Ltd, China) to extract caffeine from the tea. Caffeine was subsequently extracted into chloroform from the solution by using a separatory funnel. Finally, the absorbance of the solution was measured by using a UV spectrophotometer at 276 nm against the corresponding reagent blank. Each sample was done in triplicate, and the values were expressed as the mean (n=3).

Determination of free amino acid and theanine content

Step 9.

Free amino acid and theanine content was determined using an Agilent HPLC instrument (Agilent Technologies, USA). HPLC separation was carried out using a C18 analytical column (250 mm×4.6 mm, 5 µm, Agilent, USA) maintained at 30°C. Gradient elution was used to obtain adequate separation. The mobile phase consisted of solvents A (0.1 M NaAc:ACN 97:3, v/v, pH 6.5) and B (ACN:water 4:1, v/v). The flow rate was 2 mL/min. Absorbance at 254 nm was measured using a UV detector. The total run time was 35 min. The sample injection volume was 2.0 µL. The analytical data were processed using Agilent software. Each sample was done in triplicate, and the values were expressed as the mean (n=3).