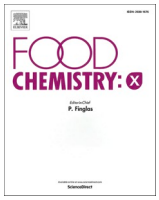
Food Chemistry: X 19 (2023) 100863

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Variations in microbial diversity and chemical components of raw dark tea under different relative humidity storage conditions

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ARTICLE INFO

Keywords:
Raw dark tea
Metabolomics
Microbiome
Storage
Relative humidity

ABSTRACT

Raw dark tea (RDT) usually needs to be stored for a long time to improve its quality under suitable relative humidity (RH). However, the impact of RH on tea quality is unclear. In this study, we investigated the metabolites and microbial diversity, and evaluated the sensory quality of RDT stored under three RH conditions (1%, 57%, and 88%). UHPLC-Q-TOF-MS analysis identified 144 metabolites, including catechins, flavonols, phenolic acids, amino acids, and organic acids. 57% RH led to higher levels of *O*-methylated catechin derivatives, polymerized catechins, and flavonols/flavones when compared to 1% and 88% RH. The best score in sensory evaluation was also obtained by 57% RH. *Aspergillus*, *Gluconobacter*, *Kluyvera*, and *Pantoea* were identified as the core functional microorganisms in RDT under different RH storage conditions. Overall, the findings provided new insights into the variation of microbial communities and chemical components under different RH storage conditions.

1. Introduction

Tea (*Camellia sinensis*) is one of the world's top three non-alcoholic beverages due to its excellent sensorial properties and numerous health benefits (Zhu, Li, Zhao, Yu, & Wu, 2017). Previous studies have indicated that sensory qualities are influenced by the content and composition of compounds in dark tea (DT) (Yang et al., 2021). In particular, bitterness and astringency are believed to be mainly attributed to polyphenols and alkaloids (Liu, Zhang, Liu, Ma, Shi, & Ruan, 2016). In addition, health benefits are also related to major compounds, especially the formulas and components of dimeric catechins (Fraser et al., 2014). Different from green tea, the quality of DT is believed to improve with increasing storage time under proper conditions (Li, Shen, Fu, Liu, & Huang, 2016). Moreover, because the primary and refining processes of DT are usually discontinuous, there is long-distance transportation and prolonged storage for raw dark tea(RDT). Similarly, customers or investors often purchase large quantities of DT and store them in warehouses for several years to improve the quality or increase profits (Xu, Zhao, Jiang, Wu, & Zhu, 2018).

DT tends to absorb moisture at high RH, providing a favorable environment for rapid microbial reproduction and mycotoxin production in the absence of strict warehouse management measures (Xu et al., 2018). Ning et al. (2019) investigated the effects of storage environment on the main chemical components of raw pu-erh tea and revealed that the aging rate of raw pu-erh tea was faster when stored in Guangdong than in Xinjiang, but its quality was inferior to that in Xinjiang, and different RH was the main factor that caused variation.

Several investigations on changes in chemical components in storage DT have been reported. Cheng et al. (2021) conducted a study on the metabolites and taste quality of Qingzhuan Tea during the aging process and indicated that alterations in sensory qualities were primarily attributed to the methylation of catechins, glycosylation of flavonoids, degradation of flavoalkaloids, biosynthesis of triterpenoids, and formation of theabrownins. Zhou et al. (2020) revealed the quality and chemical changes of raw Pu-erh tea during storage, indicating a reduction in ester catechins and parts of L-amino acid, while an increase was observed in gallic acid (GA), kaempferol, and quercetin content. These studies usually focused on the major compounds or the total contents of

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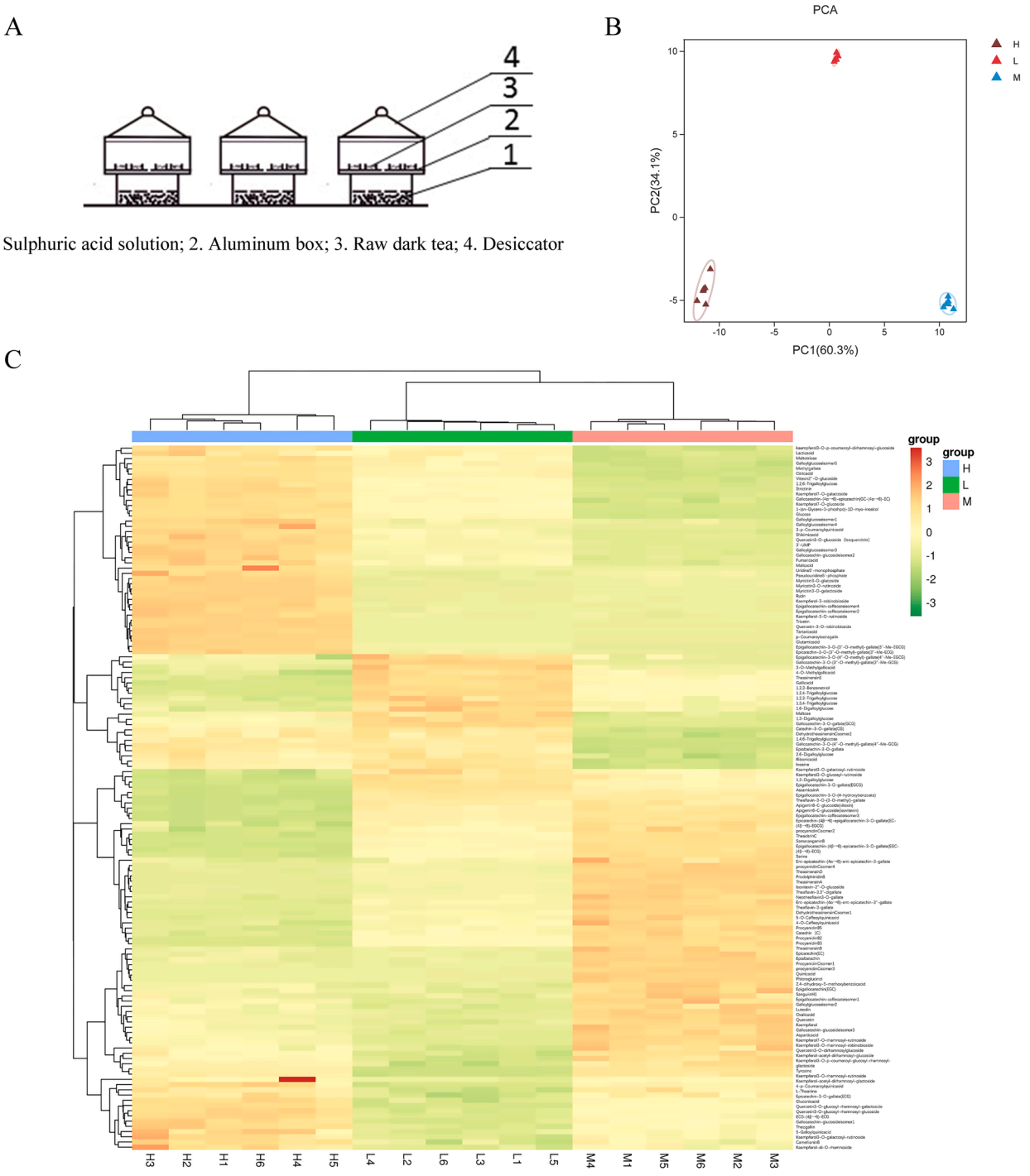
https://doi.org/10.1016/j.fochx.2023.100863
Received 25 June 2023; Received in revised form 29 August 2023; Accepted 1 September 2023
Available online 2 September 2023

tea polyphenols, theaflavins, thearubigins and theabrownine in DT during different storage times or areas. Generally, RH is the main factor that affects the quality change of DT in storage. Therefore, a comprehensive characterization of DT metabolomics during storage under different RHs is urgently needed. In addition, there is a lack of a survey on the different biological marker components in different RHs.

Studies have shown that microorganisms and their extracellular enzymes contribute to the quality formation of DT (Wen, Zhang, Wu, & He, 2010; Zhou, Li, Zhao, Han, & Yang, 2004). Furthermore, research has demonstrated a correlation between variations in microorganisms and humidity levels in DT. Metabolomics, with its unique advantage of surveying the endogenous compound compositions in food, has been widely used in tea research (Dai et al., 2018; Zhu, Li, Zhao, Yu, & Wu, 2017). RDT is produced by microbial fermentation and typically requires long-term storage to improve its quality. Since the packaging of DT usually employs breathable materials, the quality of DT is significantly affected by RH. Different storage areas of products are usually considered important factors for evaluating the quality of aged DT, and aged DT that has been stored for a long time is generally recognized as having a better taste under conditions of suitable RH. However, there is no systematic study on the chemical components of RDT under different RH storage conditions.



1. Sulphuric acid solution; 2. Aluminum box; 3. Raw dark tea; 4. Desiccator

Fig. 1. Diagrammatic sketch of RDT treated in different RH(A). The compound patterns of each group of RDT with different storage RH(B). Heat map of the compound contents in RDT samples with different RH storage conditions(C).

In this study, we investigated the chemical components and microorganisms in RDT samples that were stored under different RH conditions. We illuminated shifts in the chemical components and microorganisms under different RH conditions, identified core fungi, and illuminated their correlations with metabolites. Additionally, we surveyed the potential biomarker compounds related to the stored DT under different RH conditions and investigated the main influence of RH on sensory evaluation during the storage period. To our knowledge, this is the first report to investigate the effect of different RH conditions on the variations of chemical components and the quality of RDT.

2. Materials and methods

2.1. Chemicals and stored RDT sample preparation

MS grade acetonitrile, methanol, water, and formic acid (FA) were purchased from Merck (Darmstadt, Germany). Ethanol was provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

The RDT samples were obtained in Taojiang County, China. From June 2019 to June 2021, 2.0 g of RDT was stored in each aluminum box and placed in different desiccators with different water activity (aw) at varied sulfuric acid concentrations (Fig. 1A and Table S1). The water activity is equal to RH at a given temperature (Botheju, Amarathunge & Mohamed, 2008). Finally, the samples were stored at −80 ℃ for further analysis. Six replications were used for each sample. According to our previous study, the grouping is as follows: group H, (88 ± 0.1)%RH; group M, (57 ± 1.3)%RH; and group L, (1 ± 0.1)%RH.

2.2. UHPLC-DAD-QTOF-MS analysis

The UHPLC-DAD-Q-TOF-MS analysis was carried out according to our previously described method, with slight modifications (Zhu et al., 2017). 0.1 g of freeze-dried and crushed sample was ultrasonicated at 60 kHz with 5 mL of 70% methanol containing 0.1% FA for 40 min. The extract was centrifuged at 12,000 g for 10 min, and the supernatant was diluted with 70% methanol containing 0.1% FA. The solution was filtered through a 0.22 μm membrane (ANPEL Laboratory Technologies Inc., Shanghai, China), and then analyzed using an UHPLC-DAD-Q-TOF-MS system. Each sample was analyzed six times in duplicate. In addition, a quality control (QC) sample was created by blending an equal volume of extract from each biological sample, and the QC sample was measured after every 10 samples to evaluate the stability of the UHPLC-DAD-QTOF-MS system.

2.3. DNA extraction and sequencing processing

Microbial genomic DNA was obtained from different samples according to the manufacturer's instructions using the TGuide S96 Magnetic DNA Kit (Tiangen Biotech Co., Ltd., China). The conserved intergenic transcribed spacer region of the eukaryotic (fungal) small-subunit rRNA gene was amplified using primers ITS1F and ITS4. The 16S ribosomal RNA gene was amplified using primers 27F and 1492R, the sequencing and analysis process is consistent with that described by Zhao et al. (2022). Following purification and quantification, the mixed sample amplicons were sequenced using the PacBio system by Biomaker Technology Co., Beijing, China.

2.4. RDT sensory evaluation

The sensory evaluation of tea was performed by our previously described method with slight modifications (Zhu et al., 2017). The tea infusion was evaluated by 10 professional assessors (3 professors, 5 associate professors, and 2 lecturers; 5 females and 5 males, 35–60 years of age), all of whom hold the sensory evaluation qualification certification for tea. Briefly, 3 g of tea samples were steeped in 150 mL of boiled water in special tea cups for 7 min. The tea infusion was then poured immediately into a tea bowl and evaluated by the professional assessors. Sequentially evaluate the aroma, soup color, taste, and residual leaf in the bottom cup. Each sample was repeated three times. To minimize interference during each individual tasting, participants were asked to rinse their mouths with warm boiled water three times before tasting the next sample.

2.5. Data analysis

The data were preprocessed by mean centering and scaling prior to analysis. The principal component analysis (PCA), analysis of variance (ANOVA), and OPLS-DA were performed by SIMCA (Version12.0, Sweden). The alpha diversity and the beta diversity were analyzed or generated using cloud tools on the BMKCloud platform. The microbial composition was used as the X variable and the volatile composition as the Y variable for O_2PLS analysis. Origin (v9.8.0) was used for data processing and figure generation.

3. Results and discussion

3.1. Metabolites in RDT under different RH storage conditions

The metabolites in RDT samples were separated within 40 min by using UHPLC-Q-TOF-MS, which resulted in the detection of 144 ion features by peak alignment. Then, 144 metabolites were identified (Table S2). To the best of our knowledge, this is the first study report on the components of RDT that were stored in different RH air conditions.

PCA statistic model was constructed to investigate the influence of the RH condition on the storage of RDT compounds (Fig. 1B). The group samples were clustered together, indicating good reproducibility of the compound extraction and LC − MS/MS analysis in the metabolomics investigations. Samples from groups H, M, and L were significantly separated, which indicated that the RH storage condition of RDT has a larger influence on the non-volatile chemical components.

A total of 144 chemical compounds were identified (Fig. 1C & Table S2) based on the comparison of retention times, MS, and MS/MS spectra with the standards, and the metabolome databases that had been set up by previous work (Zhu et al., 2017). These metabolites included 21 monomeric catechins and their derivatives, 31 polymerized catechins and their derivatives, 38 flavonols/flavones and their glycosides (FGs), 24 phenolic acids, 5 amino acids, 6 organic acids, 5 nucleosides, and 14 carbohydrates and their derivatives. The tea polyphenol levels are one group of characteristics and the most abundant compounds in teas, which contain approximately 10%, and the flavonoids and theabrownins are considered the cellular antioxidant activity constituents in dark tea (Lv, Zhang, Shi, & Lin, 2017). Ning et al. (2019) reported that the EGCG, EGC, and EC contents in Pu-erh tea were significantly different between Guangdong province (high air RH areas) and Xinjiang province (low air RH areas) after 7 years of storage duration ($p < 0.05$). In this study, monomeric catechin derivatives, and all O-methylation products measured in this analysis, including Epigallocatechin-3-O-(4″-O-methyl)-gallate(4″Me-EGCG), Epigallocatechin-3-O-(3″-O-methyl)-gallate(3″Me-EGCG), Gallocatechin-3-O-(4″-O-methyl)-gallate(4″Me-GCG), Gallocatechin-3-O-(3″-O-methyl)-gallate(3″Me-GCG) and Epicatechin-3-O-(3″-O-methyl)-gallate(3'Me-ECG), were extremely significant higher between the group samples that were stored in higher RH conditions (group H, $p < 0.01$) (Table S2). These phenomena indicated that the O-methylation rate is accelerated under high humidity. The O-methylated derivatives of catechins were synthesized by O-methyltransferase in tea plants (Kirita et al., 2010). However, these results may be due to O-methyl-transferase from microorganisms.

The O-methylated derivatives have much higher biological and pharmacological activities than their substrates, especially antiallergic biological functions (Zhang et al., 2013). Surco-Laos et al. (2012) reported that the 200 μM methylated epicatechin derivatives (3′-O-Methyl-epicatechin, 4′-O-Methylepicatechin) increased the mean

lifespan of the nematode by 6–12%, whereas catechin and epicatechin did not influence its life duration. Lu et al. (2006) and Li et al. (2018) reported that the content of 3″Me-EGCG is related to the variety and leaf positive of *Camellia sinensis* (L.) and came to the conclusion that the fermentation of black tea or pile-fermentation of dark tea will reduce the content of 3″Me-EGCG. Our results do not agree with the findings. Before this, the research team found that there is a risk of mildew in dark tea when RH is above the 87% (Xu et al., 2018). So, the increasing content of 3″Me-EGCG and 4″Me-EGCG in stored RDT may benefit from the microorganisms. The extremely significant ($P < 0.01$) higher level of O-methylated derivatives of EGCG, GCG, and ECG may partially explain the better health-promoting effects of tea stored at higher RH (group H), and the content of O-methylated derivatives of catechins may be a kind of important chemistry marker for good quality and better health benefits.

31 types of polymerized catechin derivative components were identified based on the characteristic fragmentation ions by LC-MS/MS. Usually, polymerized catechins and their derivatives are transformed from monomeric catechins and their derivatives. The high levels of polymerized catechins indicated a deeper degree of oxidation, which may be based on natural or enzymatic oxidation. Different from green tea (Liu et al., 2016; Zhu et al., 2017), the high content of polymerized catechins usually implies the good taste of dark tea (Zhu et al., 2020). The levels of gallocatechin-(4α → 8)-epicatechin, theaflavin-3-O- (3-O-methyl)-gallate, *ent*-epicatechin-(4α → 8)-*ent*-epicatechin-3″-gallate and *ent*-epicatechin -(4α → 8)-*ent*-epicatechin-3-gallate are higher in the highest RH conditions (group H) than others (Table S2). The content of prodelphinidin B was slightly higher in low-humidity conditions (group L).

38 kinds of FGs are also major phenolic compounds in teas, with a series of bioactivities and low astringent taste thresholds. In this study, the contents of 3 different types of myricetin-O-glycosides (myricetin 3-O-rutinoside, myricitin 3-O-galactoside, and myricitin 3-O-glucoside), 6 different types of quercetin-O-glycosides (quercetin 3-O-glucosyl -rhamnosyl-galactoside, quercetin 3-O-glucosyl-rhamnosyl-glucoside, quercetin-3-O- robinobioside, quercetin 3-O-dirhamnosyl- glucoside, hyperoside, and isoquercitrin), and 5 different types of kaempferol-O-glycosides (kaempferol 7-O-galactoside, kaempferol 7-O-glucoside, kaempferol 3-O-galactosyl- rutinoside, kaempferol 3-O-glucosyl-rutinoside, and kaemp-ferol-acetyl-dirhamnosyl- glucoside) changed significant differences between the groups during RDT storage. We can conclude that the contents of most FGs are lower under low RH conditions than under high RH conditions.

A total of 24 kinds of phenolic acids were identified in these samples. Gallic acid is usually created by the degradation of ether- type catechins and their derivatives. Gallic acid is significantly higher in the group with a high RH condition. Zhao et al. (2019) reported that the content of phenolic compounds was degraded during the pilefermentation for microbial purposes, and the gallic acid increased. In this study, the phenomenon of pinions is proven. 1,2,3-benzenetriol was also found to have the highest content in the group with a high RH condition. Some research reports that 1,2,3-benzenetriol can be produced by the decarboxylation of gallic acid through microorganisms (Xu, Wang, & Zhang, 2018). In this study, we have found that the RDT has the highest content in the high RH condition. These results also give us a message: under conditions of high air humidity, some factors may contribute to the decarboxylation of gallic acid.

5 kinds of amino acids (serine, aspartic acid, glutamic acid, L-theanine, and tyrosine) were identified. Except for the aspartic acid, the contents of the identified amino acids were significantly ($P < 0.01$) higher under the high air RH (above 88 ± 0.1 %RH). Usually, amino acids might be induced by the Maillard reaction with reducing sugars during thermal processing or long storage (Gupta, Gupta, Sharma, Das, Ansari, & Dwivedi, 2018). Ning et al.(2019) reported that the contents of L-Theanine in raw pu-erh tea in Guangdong (high air RH district) samples were lower than those in Xinjiang (low air RH district) samples, and the degradation rate was also faster than that in Xinjiang samples. Our results differ from Ning's, and we suppose the main reason is that microorganisms synthesized some amino acids during the storage period.

6 kinds of organic acids (oxalic acid, tartaric acid, fumaric acid, malic acid, lactic acid, and citric acid) were identified. Some results have shown that a daily intake of moderate organic acids can be beneficial for reducing the risk of some diseases, such as cardiovascular, metabolic, and digestive diseases (Nimse & Pal, 2015). The levels of these components were significantly higher in the high RH condition (88 ± 0.1 % RH), especially tartaric acid and citric acid.

In addition to the compounds discussed above, consisting of monomeric catechin derivatives, polymerized catechin derivatives, FGs, phenolic acids, anthocyanins, amino acids, nucleosides, and carbohydrates (Fig. 1), we have found that several compounds showed strong positive correlations with the high RH condition. As shown in Fig. 2, methylated catechins are typical fold change metabolites in group H samples, in contrast with other group samples. The levels of six out of 21 monomeric catechin derivatives in group H samples were higher than those in the other groups. These six monomeric catechin derivatives are: 4-O-methylgallic acid, 3-O-methylgallic acid, 4″Me-EGCG, 3″Me-EGCG, 3″Me-ECG, and 3″Me-GCG. Compared with other groups of samples, p-coumaroylthreonine showed the highest content in group H. Some studies have shown that p- coumaroyl-stilbene can be used as a biomarker characteristic of tea leaf pubescence (Zhu et al., 2017). At the same time, some studies have also found that this substance has good biological activity (Lv, Zhu, Tan, Guo, Dai, & Lin, 2015). In this experimental system, p-coumaroyltraugalin has a higher content in group H samples, and the reason needs to be further studied.

Through the volcano plot, we can quickly view the difference in the expression level of metabolites between the two groups and the statistical significance of the difference. As shown in Fig. 2, The levels of 10 metabolites in the group H samples were higher than those in the other groups. They are tricetin, 3″Me-GCG, EGC-caffeoate isomer 1, kaempferol-3-o-rutinoside, uridine-5′-monophosphate, myricetin, kaempferol-di-o-rhamnoside, kaemp- ferol-7-o-rhamnosyl-rutinoside, and EGC-caffeoate isomer 2, 1,3,4-trigalloylglucose. These results show that FGs and the monomeric catechin derivatives are the main biomarker metabolites for the storage of RDT in high RH.

3.2. RDT sensory evaluation under different RH storage conditions

During storage, RH is considered an important factor for the storage of RDT, and tea stored under appropriate RH conditions will have a better taste. In this study, the sensory evaluation of tea stored under different RH conditions was conducted (Table S3). These results of these sensory evaluations showed that the chemical components of RDT were influenced by RH conditions, and the group M samples obtained the highest score, which indicates that (57 ± 1.3) % RH may be the most suitable RH condition for RDT. In group M, the RDT exhibits a pure aroma, while in group L, the aroma is less pure. However, in group H, the aroma is described as harsh and coarse. As for the taste, RDT in group M is perceived as stale and normal, while RDT in group H has a moldy taste.

As shown in Fig. 2, polymerized catechin derivatives and FGs significantly predominate in the upper group of group M samples. FGs not only serve as the main astringent substances in tea, but also have a certain synergistic effect on the bitterness of caffeine. Although the content of FGs in tea is relatively low, their taste threshold is very low (Jiang, Engelhardt, Thräne, Maiwald, & Stark, 2015). Among the 31 polymerized catechin derivatives, the levels of 11 in the group M samples were higher than those in the other groups. These 11 polymerized catechin derivatives were procyanidin C isomers 1, theasinensin A, theasinensin D, neotheaflavin 3-o-gallate, theaflavin-3-gallate, prodelphinidin B, theaflavin-3,3″- digallate, dehydrotheasinensin C isomer 1, samarangenin B, camellianin B, and punicacortein A. Generally, the

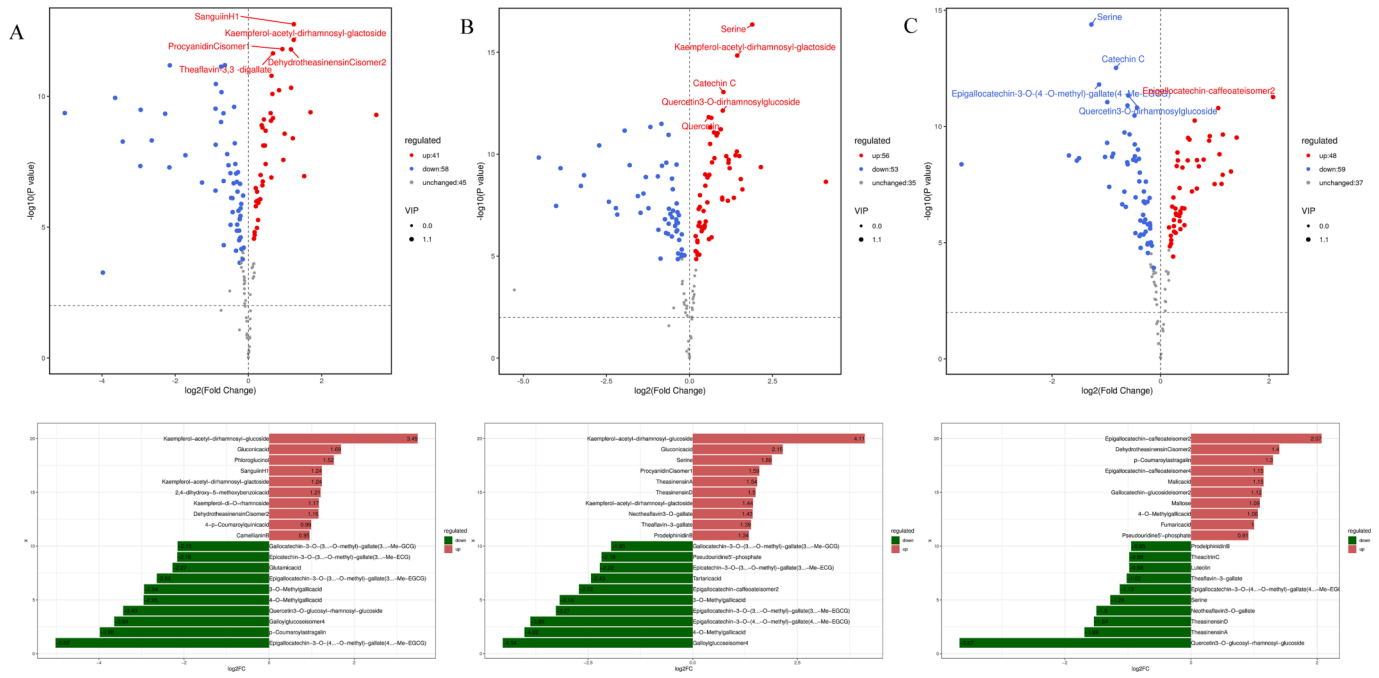


Fig. 2. Volcano Plot and Fold Change between groups. H vs L(A), H vs M(B), M vs L(C).

oxidation of catechins means that the color of tea soup changes from yellow to red, showing the quality characteristics after aging. The results of the comparison between the group M samples and the chemical components in other groups showed a significant increase in the content of catechin polymers and their derivatives, which may be an important reason for the good sensory evaluation score of group M samples.

The levels of 11 metabolites in the group M samples were higher than those in the other groups. They are serine, kaempferol-acetyl-dirhamnosyl-glactoside, catechin, quercetin-3-o-dirhamnosyl-glucoside, quercetin, theacitrin C, kaempferol, dehydrotheasinensin C siomer 1, theaflavin-3-gallate, uridine-5′-monophosphate, and kaempferol-di-o-rhamnoside. These results have shown that FGs and the polymerized catechin derivatives are the main reasons for the good sensory evolution of RDT stored at (57 ± 1.3) %RH.

3.3. Microorganisms in RDT under different RH storage conditions

The microorganism community of 18 samples was analyzed at the phylum, order, and genus levels. Principal-component analysis (PCA) analyses were used to investigate overall differences in fungal and bacterial community structure among the groups. Based on the relative abundance (RA) of operational taxonomic units (OTUs), principal component analysis (PCA) revealed significant differences in fungal and bacterial communities among 18 dark tea samples stored under different RH conditions. α-diversity (based on the OTUs) of the microorganism communities in RDT under different RH storage conditions was measured by the Shannon, Simpson, Chao1, and ACE indices (Figure S1). The results indicated that the bacterial diversity and richness of group M were significantly higher than those of groups H and L, according to the Kruskal-Wallis test followed by Dunn's multiple comparisons ($P < 0.05$). However, there was no significant difference between the indexes of groups H and L($P > 0.05$). The results indicated that the fungal diversity and richness of groups M and L were significantly higher than those of group H.

In total, fungi belonging to 4 phyla, 13 orders, and 15 genera were identified in RDT under different RH storage conditions. The most dominant phylum was *Ascomycota*, accounting for 91.90–99.97% of all the valid sequences. *Basidiomycota* and *Mucoromycota* were also identified in groups M and L but were almost undetectable in group H. At the order level, *Eurotiales* predominated, accounting for 90.97–99.87% of all the valid samples. *Cystofilobasidiales* were also identified in groups M and L, accounting for 6.31% and 4.28%, respectively. *Mortierellales* and *Glomerellales* were also identified in groups M and L. At the genus level, *Aspergillus*, *Tausonia*, and *Mortierella* are the three dominant genera that constituted 98.55–99.88% of all sequences in each sample (Fig. 3), and *Aspergillus* is the predominant fungus in these genera. *Tausonia* and *Mortierella* were not detected in group H but were detected in groups L and M, demonstrating that these genera of fungi are suitable for growth in conditions of low relative moisture content.

In total, bacteria belonging to 14 phyla, 51 orders, and 162 genera were identified across all samples (Fig. 3). At the phylum level, *Proteobacteria* was the most important phylum in all samples, accounting for 80.70–84.61% of the total sequence number, followed by *Firmicutes* (6.81–9.18%), *Bacteroidetes*(5.09–5.97%), *Cyanobacteria*(0.90–2.38%), *Actinobacteria*(0.77–1.61%). At the order level, the six most dominant bacterial orders constituted 85.34–86.33% of all sequences in each sample. *Acetobacterales* and *Pseudomonadales* were the main bacterial orders, and their relative abundance ranges were 34.60–54.80% and 15.71–20.40%, respectively. At the genus level, *Gluconobacter, Acinetobacter, Kluyvera,* and *Pantoea,* are the four dominant genera, constituting 69.09–75.40% of all samples. However, *Kluyvera* was only detected in groups M and L. Different RHs in RDT had a relatively significant effect on the relative abundance of bacteria.

To obtain a measure of microbial association, two OTU co-occurrence networks were constructed (Fig. 4). In the fungal network, most of the OTUs assigned to *Aspergillus* have negative correlations with other genera, whereas the OTUs corresponding to *Tausonia*, *Mortierella,* and others cooccur with each other. In the bacterial co-occurrence network, most of the OTUs corresponding to *Acinetobacter*, *Bacillus*, *Gluconobacter*, and *Pantoea* were negatively correlated with other bacteria. OTUs assigned to *Klebsiella*, *Mycoplasma*, and *Vibrio* cooccurred with others.

The relative abundance of fungi was not significantly altered at different humidity conditions; *Aspergillus* predominated in all the groups. However, there was a significant change in the relative abundance of the bacteria; *Gluconobacter* and *Acinetobacter* showed a decreasing trend with decreasing RH, whereas *Kluyvera* and *Pantoea* increased with decreasing RH. *Aspergillus* spp. fungi are the dominant

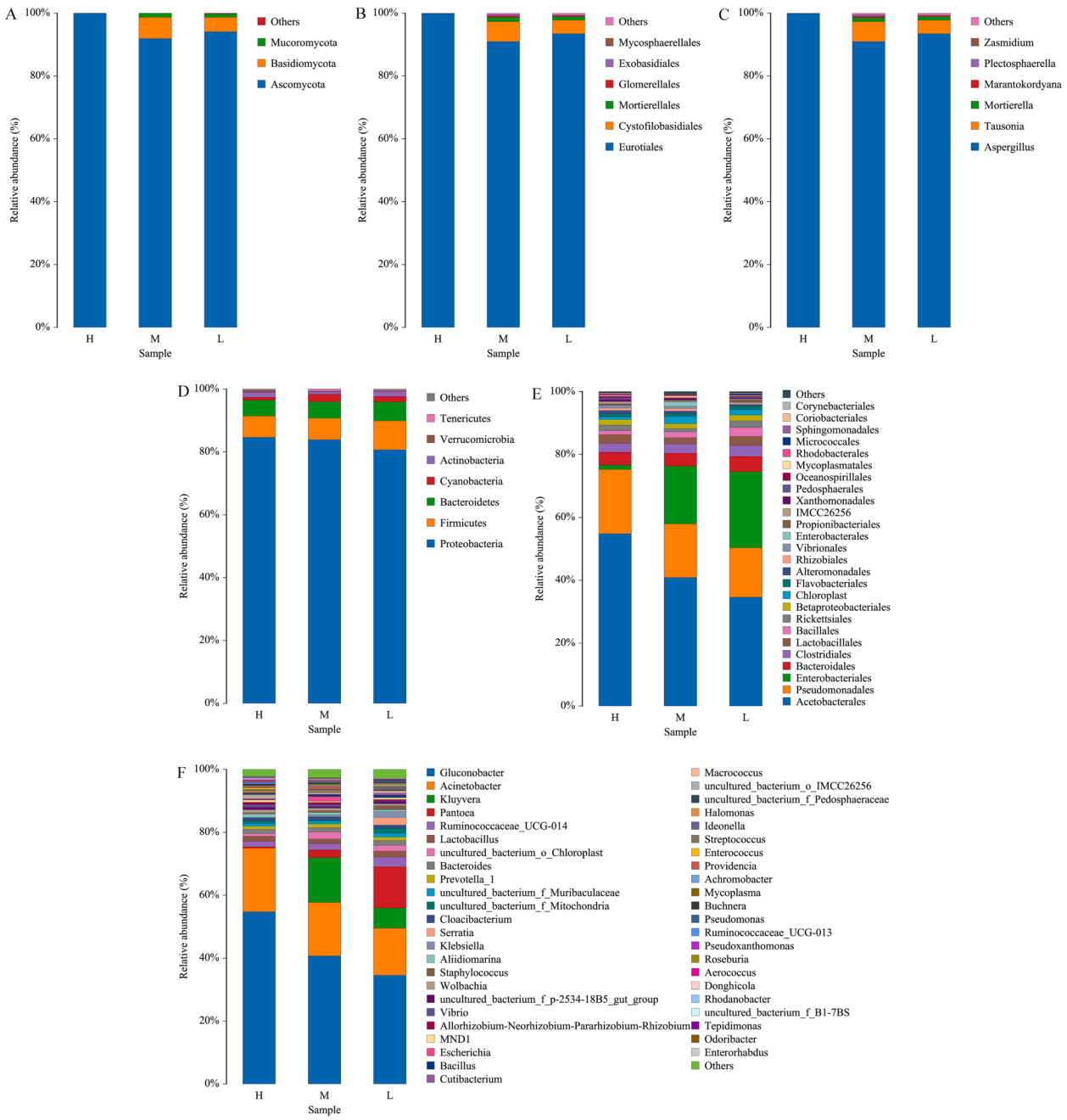


Fig. 3. Fungal (A,B, and C) and bacterial (D,E, and F) taxonomic compositions showing the bacterial successions at phylum, order, and genus level in RDT samples with different RH storage conditions. The taxonomic abundance < 1% was classified as "others".

fungal group in the production and storage of many types of dark tea (Li et al., 2017; Li et al., 2020). Studies have shown that there are differences in the fungal communities of Fu brick tea products from different regions (Zhao, Xu, Wu, Jiang, & Zhu, 2017). Some studies have shown that bacteria are an important flora in Fu brick tea, and Liu et al. (2014) investigated the relationship between the bacterial community and the symbiosis of Corynebacterium during the processing. Zhao et al. (2017) found some differences in the bacterial communities of porcupine tea from different regions. Collectively, the geographical environment and processing processes are the main factors affecting the microbial community structure of dark tea. Our study was carried out using the same raw materials and in the same environment, and the results showed that the fungal community did not vary much under different RH conditions, while the bacterial community varied significantly.

To identify classified microorganism taxa with significantly abundant differences among the RDT under different RH storage conditions, we performed LEfSe analysis. As shown in Fig. 4, the analysis revealed 3 fungal and 21 bacterial clades that exhibited statistically significant differences, with a LDA threshold of 4.0. In total, 1 fungal and 7 bacterial genera were identified as the biomarkers in RDT under different RH storage conditions, including *Aspergillus*, *Kluyvera*, *Bacillus*, *Bacteroides*, *Pantoea*, *Staphylococcus*, *Gluconobacter*, and *Moraxella*. *Gluconobacter* was significantly enriched in group H; *Kluyvera, Bacillus,* and *Bacteroides* were significantly enriched in group M;and *Pantoea, Staphylococcus,* and *Moraxella* were significantly enriched in group L.

3.4. Correlation analysis of major microorganism communities and biochemical components of RDT under different RH storage conditions

3 fungal genera, *Aspergillus, Tausonia,* and *Mortierella,* and 10

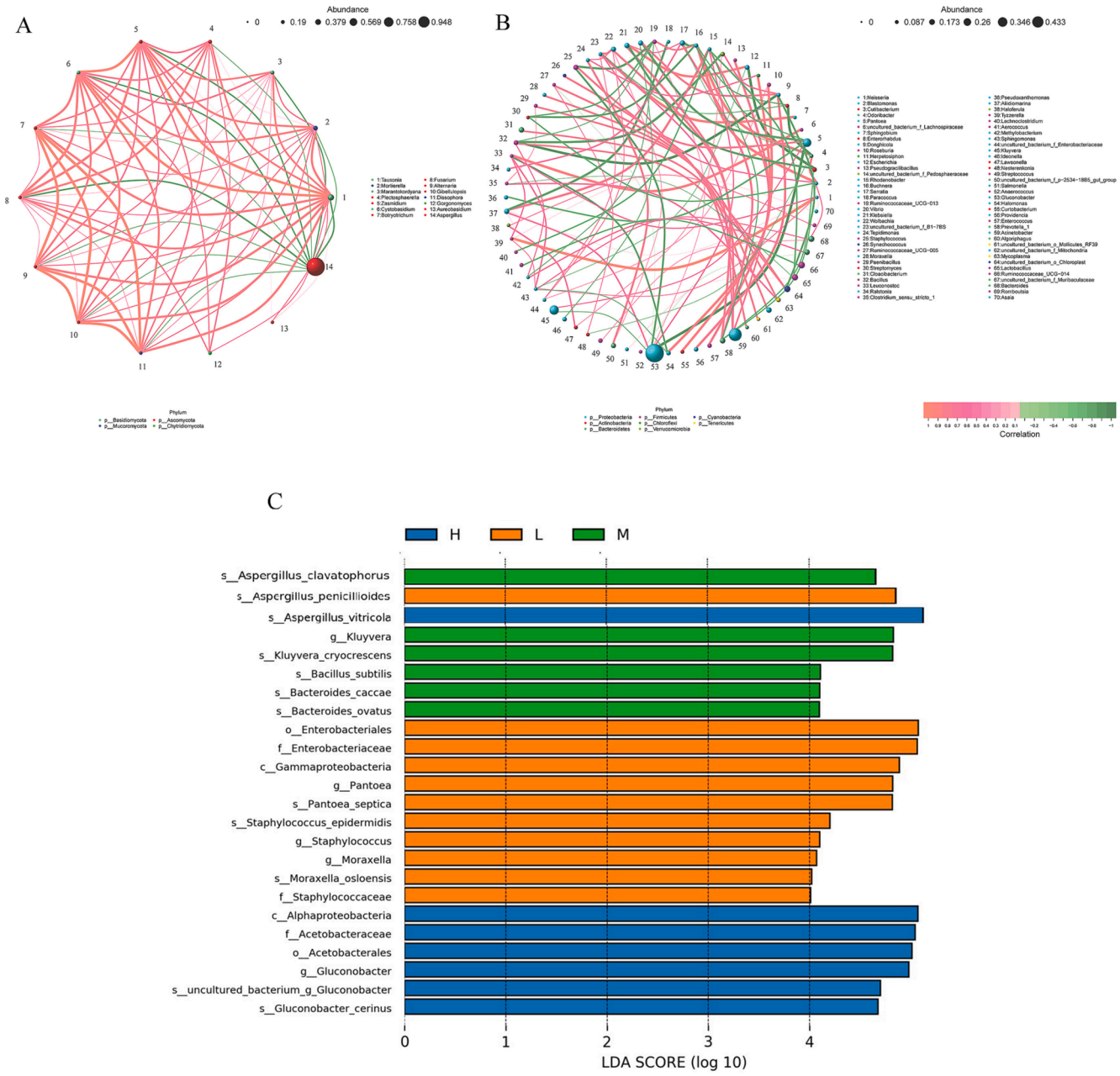


Fig. 4. Interaction networks in the fungal(A) and bacterial(B) communities of RDT samples with different air RH storage conditions. LDA score showed the significant abundance differences of fungal taxa by LEfSe analysis(C).

bacterial genera *Gluconobacter, Acinetobacter, Kluyvera, Pantoea, Ruminococcaceae_UCG014, Lactobacillus, uncultured_bacterium_o_Chloroplast, Bacteroides, Prevotella_1,* and *uncultured_bacterium_f_Muribaculaceae* were the dominant fungi in RDT under different RH storage conditions. Combining the microbial and metabolite data, we investigated the association between microbiome and biochemical compounds in RDT under different RH storage conditions. According to the values of VIPpred and the correlation coefficients, 12 genera were identified as important microorganisms (Fig. 5). Furthermore, three conditions were considered to identify the core functional microorganisms in RDT under different RH storage conditions: (a) VIPpre value ≥ 1.0; (b) correlation coefficient $|r| \geq 0.8$ and $P < 0.05$; (c) the relative content of microbial genera must be $> 1\%$. Based on these criteria, 4 genera were identified as the core functional microorganisms, including the genus of *Aspergillus*, *Gluconobacter, Kluyvera,* and *Pantoea.*

In this study, *Aspergillus* predominates in all the groups. *Aspergillus* fungi are a group of filamentous fungi with a variety of species, are rich in metabolites and applications, and are increasingly being used in various fields such as food fermentation, medicine, and agricultural production (Du, Yang, Yang & Yang, 2022). *Aspergillus* spp., a common dominant fungal genus in RDT processing and storage, play a significant role in promoting the quality of RDT. Under humidity and heat conditions, microorganisms, to satisfy their demands for carbon and nitrogen, secrete extracellular enzymes for enzymatic action, decomposition, oxidation, reduction, and esterification reactions to convert cellulose, pectin, terpenes, proteins, and other substances into various taste and aroma components. Some studies show that *Aspergillus* fungi are capable of producing proteases, tannases, and various hydrolytic enzymes, which play an important role in the quality of dark tea (Zhou et al., 2004). *Aspergillus Eurotium* is associated with the formation of volatile compounds with floral, fruity, and minty aromas (Wang et al., 2021). Studies by Li et al. (2017) have shown that the unique aromatic

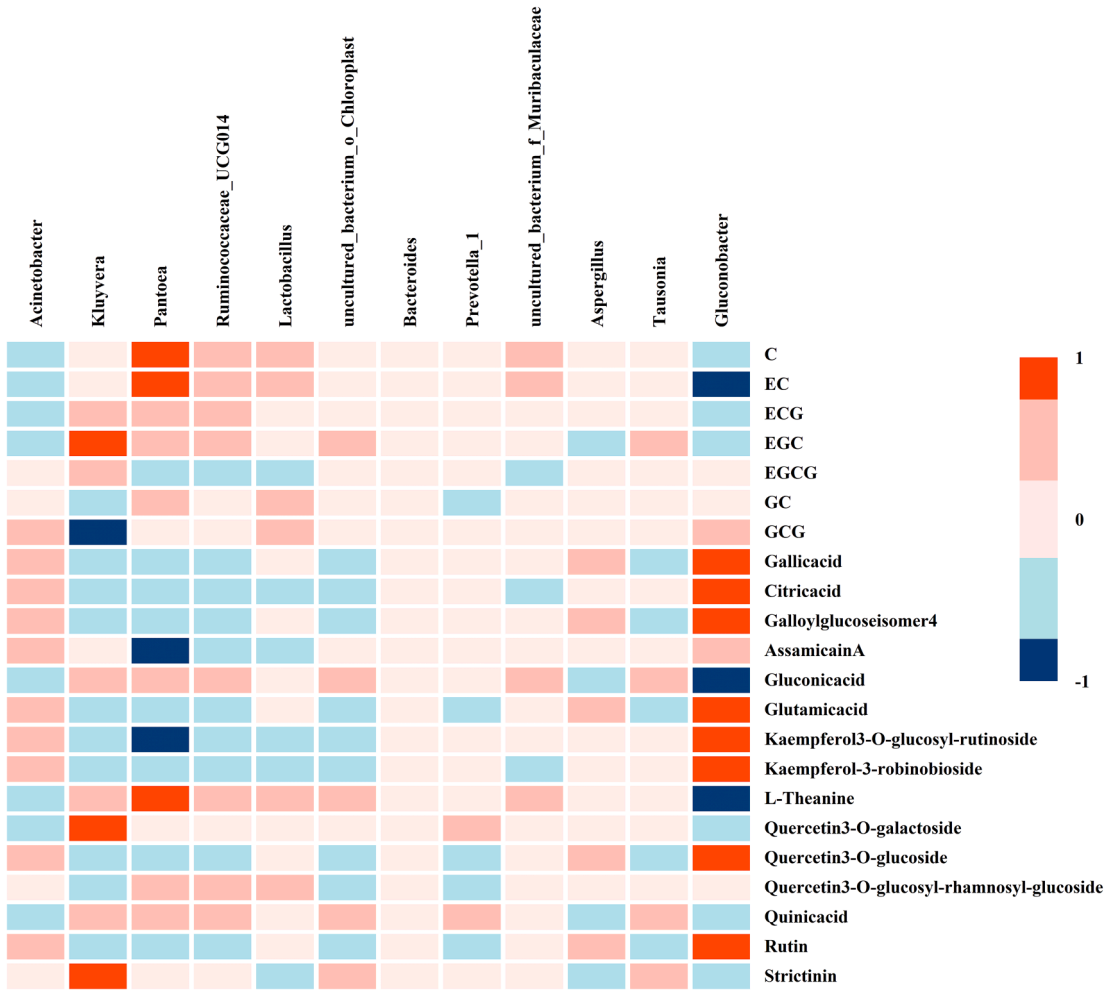


Fig. 5. Correlation between the core functional genera and major compounds variables.

substances and major components of Fu Brick tea are also related to *Aspergillus*. We found that the relative abundance of fungi in the genus *Aspergillus* was essentially constant at different water contents. However, we found a slight increase in *Tausonia* and *Mortierella* in the M and L groups, but the relative abundance content was still very low. The relative abundance of the fungi did not significantly change under different RH conditions, which possibly implies that changes in RH had less influence on the fungal community in RDT.

Gluconobacter constitutes a genus of the acetic acid bacteria, *Acetobacteraceae*, of the class *Alphaproteobacteria*. *Gluconobacter* is widespread in products made from plant materials (Gupta, Singh, Qazi, & Kumar, 2001). *Gluconobacter* is a prominent representative of oxidative bacteria that can conduct efficient oxidative fermentation (Hommel, 2014). *Gluconobacter* strains are involved in a large number of natural fermentations and have great capability for other applications in commercial processes (Deppenmeier, Hoffmeister, & Prust, 2002). Cocoa fermentation is a combination of external microbial processes and autolytic processes involving cocoa bean enzymes. *Gluconobacter oxydans* dominates the second stage of spontaneous fermentation (Hommel, 2014). *Gluconobacter* is also a member of the microbial community in kombucha (tea bacteria), which produces the detoxifying properties of glucuronide and the scavenging of the free radical d-glycolic acid-1,4-lactone(Diez-Ozaeta & Astiazaran, 2022). Nguyen's study found the optimal ratio of symbiosis between the isolated yeasts and bacterial strains, which can produce the high-level glucuronic acid in kombucha (Nguyen, Nguyen, Nguyen, & Le, 2015). In our study, the relative abundance of *Gluconobacter* decreased with decreasing RH, with relative abundances of 54.75%, 40.70%, and 34.54%, respectively. However, gluconic acid was the highest in group M, and we speculate that 57 %RH is more suitable for *Gluconobacter* to produce gluconic acid in RDT. *Pantoea* is widely isolated from soil, water, and plants. *Pantoea* has been found to play an aggressive role in some spontaneous fermentations, such as those of cocoa beans (Lefeber, Gobert, Vrancken, Camu, & De Vuyst, 2011) and coffee (Evangelista et al., 2014). *Pantoea* is the dominant microbiota in the early stages of spontaneous cocoa bean fermentation, where it is thought to be responsible for the production of gluconic acid.

4. Conclusion

This study explored the variations in microbial community, chemical components, and sensory quality of RDT stored under different RH conditions. The best score in sensory evaluation was also obtained by 57% RH. UHPLC-Q-TOF-MS analysis identified 144 metabolites under different RH storage conditions, revealing significant differences in chemical composition, particularly in the monomeric catechin derivatives, polymerized catechins, and flavonols/flavones. Dominant fungal genera were *Aspergillus*, *Tausonia*, and *Mortierella*, while dominant bacterial genera included *Gluconobacter*, *Acinetobacter*, *Kluyvera*, and *Pantoea*. Furthermore, *Aspergillus*, *Gluconobacter*, *Kluyvera*, and *Pantoea* were identified as the core functional microorganisms, contributing to the variations in chemical composition, and ultimately affecting the sensory attributes of RDT. Thus, the RH conditions influenced the microbial community, ultimately affecting the chemical

composition of RDT. Understanding the impact of RH conditions on the microbial community and the chemical composition contributes to better storage practices. Future research could explore the impact of RH conditions on the microbial community, chemical components, and sensory quality of RDT at an industrial scale.

CRediT authorship contribution statement

Wei Xu: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Funding acquisition. **Yiqiao Zhao:** Writing – original draft, Software, Data curation. **Yating Lv:** Visualization, Software, Data curation. **Tunyaluk Bouphun:** Writing – review & editing. **Wenbao Jia:** Data curation, Software. **Si-yu Liao:** Data curation, Software. **Mingzhi Zhu:** Data curation, Writing – review & editing. **Yao Zou:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by National Key Research and Development Program of China (2022YFD2101103), Natural Science Foundation of China (32002095), Sichuan Province S&T Project (2021ZHFP0021, 2022ZHXC0022, 2023YFH0025, and 2023YFN0010), Hunan "Three Top" Innovative Talents Project (2022RC1142), Natural Science Foundation of Hunan Province for Outstanding Young Scholars (2022JJ20028), Changsha City Outstanding Innovative Youth Training Program (kq2107015).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100863.

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