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Article

Changes of microbial active ingredients and antioxidant capacity during fermentation process of dark tea

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Abstract: Dark tea, a traditional fermented tea in China, is known for its unique flavor and enhanced health potential due to its fermentation process. However, previous studies on the evolution of its active ingredients and antioxidant properties have been limited by inadequate sample collection, single analytical methods, and insufficient data processing. To address these challenges, this study employed a comprehensive strategy to analyze the dynamic changes of active compounds and antioxidant efficacy during dark tea fermentation using refined sampling, diverse assay techniques, and advanced data analysis. A multi-point temporal sampling method was used to capture key stages of fermentation, ensuring comprehensive data. High-Performance Liquid Chromatography (HPLC) and various antioxidant assays (1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Ferric ion reducing antioxidant power (FRAP)) enabled precise quantification of tea polyphenols, catechins, theaflavins, and thearubigins. Multivariate statistical analysis revealed that tea polyphenols and catechins decreased, theaflavins increased then slightly declined, and thearubigins steadily rose as fermentation progressed. These changes were linked to fluctuations in antioxidant capacity, peaking at around 30 mg/g of phenolic compounds. The study also explored optimizing fermentation to enhance the retention of beneficial components, maximizing antioxidant properties and improving product quality. This research advances the understanding of dark tea fermentation and supports the sustainable development of the dark tea industry.

Keywords: dark tea fermentation; active ingredients; antioxidant capacity; high performance liquid chromatography; fermentation process optimization

1. Introduction

The unique fermentation techniques and rich nutritional content of black tea have attracted the attention of academia and the market. During the fermentation process, black tea undergoes profound chemical and biological transformations, which not only form its unique flavor and color, but also enrich its health value. Facing the demand of the healthy beverage market, in-depth research on the changes in active ingredients and antioxidant capacity during the fermentation process of black tea has become an important topic. At present, there are limitations in sample selection, single analysis techniques, and shallow data processing in research, which limit the understanding and application of the quality and health benefits of black tea.

In recent years, scholars have explored the changes in active ingredients and antioxidant capacity during the fermentation process of black tea. Sahara P studied the effect of microbial treatment on the release of polyphenols and antioxidants,

indicating that microbial enzyme secretions play an important role in the release of total phenols, total flavonoids, and antioxidant components during solid-state fermentation [1]. Marazza uses Lactobacillus rhamnosus CRL981 to ferment soybean milk to produce β-glucosidase and increase the content of isoflavone aglycone. This innovative process enhances the biological activity and antioxidant capacity of soy beverages [2]. The recently discovered genus of lactic acid bacteria related to food fermentation or by-products by Khubber et al. focuses on the antibacterial and antioxidant metabolites of lactic acid bacteria, as well as the recovery of these compounds after fermentation in various food systems [3]. Zhu et al. found that fermented feed improved the growth performance, immune function, and antioxidant capacity of laying hens. The proliferation of T cells induced by fermented feed, the production of helper T type 1 and helper T type 2 cytokines, and the regulation of antioxidant activity are related to NF-κ B activation [4]. Liu et al.'s study aims to explore the effects of combined polyphenols on the in vitro and in vivo fermentation and antioxidant properties of carrot dietary fiber. It was found that combined polyphenols have a significant impact on the fermentation and antioxidant properties of carrot dietary fiber [5]. However, most of these studies focus on specific time points or use a single analytical method, which fails to fully reflect the dynamic changes in active ingredients and antioxidant capacity during the fermentation process of black tea. To address this issue, Santos et al. [6] proposed a research strategy that combines comprehensive sampling with multiple methods, but existing research still lacks sufficient data processing and analysis. Previous studies have shown that during the fermentation process of black tea, its main active ingredients such as tea polyphenols, theaflavins, and thearubigins undergo significant changes [7,8], thereby affecting its antioxidant capacity.

Literature shows that multiple analytical methods can solve the problems in current research. For example, high-performance liquid chromatography (HPLC) is widely used in the analysis of tea components, with high resolution and sensitivity. The antioxidant capacity determination methods such as 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and Ferric ion reducing antioxidant power (FRAP) can comprehensively evaluate the antioxidant performance of tea [9–11]. However, these methods also have limitations in application, and the diversity and complexity of data require more systematic statistical analysis methods to handle [12,13]. This article combines advanced technological means and uses HPLC and various antioxidant capacity determination methods to systematically study the changes in active ingredients and their effects on antioxidant properties during the fermentation process of black tea [14,15]. By planning sampling points for key fermentation stages, tracking changes in active ingredients and their antioxidant capacity [16,17]. By utilizing high-resolution HPLC analysis, the core active ingredients and their antioxidant capacity in tea were accurately quantified, providing a data foundation for subsequent research [18,19]. By combining multivariate analysis tools such as Principal Component Analysis (PCA), correlation analysis, and advanced statistical models, the interaction between components and their impact on antioxidant effects were deeply analyzed [20,21],

providing data support for constructing predictive models and comprehensively evaluating various indicators.

The predictive model constructed in this experiment not only reveals the relationship between the dynamic changes of active ingredients and antioxidant capacity during the fermentation process of black tea [22,23], but also has the potential to predict future fermentation effects, providing a basis for process optimization. Based on this model, a refined fermentation process optimization scheme was proposed, focusing on regulating fermentation temperature, humidity, and cycle [24,25] to maximize the accumulation of beneficial components, reduce adverse changes, and improve the quality and health effects of black tea [26,27]. In summary, this study provides a new perspective for the quality improvement and sustainable development of the black tea industry, as well as a reference for process innovation in the fermented food field, highlighting the role of scientific research in promoting the modernization of the traditional food industry.

2. Multi-time point sampling

Accurately capturing and analyzing the dynamic changes in active ingredients and antioxidant capacity during the fermentation process of dark tea is key to understanding its quality formation mechanism and optimizing the fermentation process [28]. To achieve this goal, this study designs a detailed and scientific multi-time sampling scheme to ensure the continuity and representativeness of the data. Firstly, based on the characteristics of the traditional fermentation process of dark tea, several key stages in the fermentation process are identified as sampling points: raw tea (unfermented), initial fermentation (the first day after fermentation begins), mid-term fermentation (the middle stage of fermentation, usually 3–5 days, adjusted according to the fermentation environment), late fermentation (fermentation is close to completion, usually 7 days or longer, until the predetermined fermentation level is reached), and final finished tea (dried after fermentation is completed). The selection of these time points aims to comprehensively cover the main stages of change in the fermentation process. **Figure 1** shows the raw tea samples at four selected fermentation times.

At each sampling time point, a random sampling method is used to select multiple sample points from the fermentation pile. Samples are randomly taken at key time points such as raw tea (unfermented), initial fermentation, mid fermentation, late fermentation, and final tea. Each sampling amount is about 500 grams, quickly packaged in sealed bags, labeled with sampling time, batch number, and environmental conditions, and immediately stored at low temperature (4 °C to reduce component changes caused by oxidation or other environmental factors.

All collected samples undergo a unified preprocessing process in the laboratory: first, coarse impurities are removed, and then appropriately crushed and sieved (usually using a 40 mesh sieve) to obtain uniform tea powder for subsequent analysis. This step ensures the consistency and comparability of the analyzed samples. The sample processing at each time point during the fermentation process of dark tea is shown in **Table 1**.

Figure 1. Raw tea with four fermentation times.

Table 1. Sample processing at each time point during dark tea fermentation process.

Sampling Time Point	Sample ID	Sample Weight (g)	Step 1: Removal of Impurities	Step 2: Crushing	Step 3: Sieving	Storage Conditions
Raw Tea	S1	500	Removal of large impurities	Crushed to fine particles	Passed through 40-mesh sieve	Sealed storage at 4 °C
Initial Fermentation (Day 1)	S2	500	Removal of large impurities	Crushed to fine particles	Passed through 40-mesh sieve	Sealed storage at 4 °C
Mid-term Fermentation (Day 3)	S3	500	Removal of large impurities	Crushed to fine particles	Passed through 40-mesh sieve	Sealed storage at 4 °C
Mid-term Fermentation (Day 5)	S4	500	Removal of large impurities	Crushed to fine particles	Passed through 40-mesh sieve	Sealed storage at 4 °C
Late Fermentation (Day 7)	S5	500	Removal of large impurities	Crushed to fine particles	Passed through 40-mesh sieve	Sealed storage at 4 °C
Final Finished Tea	S6	500	Removal of large impurities	Crushed to fine particles	Passed through 40-mesh sieve	Sealed storage at 4 °C

In **Table 1**, multiple key sampling time points are determined based on the characteristics of traditional fermentation processes for dark tea, and standardized sampling and processing are performed at each time point. Samples are taken from raw tea (unfermented, sample ID S1), initial fermentation (sample ID S2 on the first day of fermentation), mid-term fermentation (sample ID S3 on the third day and sample ID S4 on the fifth day), late fermentation (sample ID S5 on the seventh day), and final finished tea (sample ID S6 after fermentation). The sampling amount is about 500 grams per time. After sampling, it is quickly packaged in sealed bags, marked with information such as sampling time, batch number, and environmental conditions, and immediately stored at a low temperature of 4 °C to reduce the impact of oxidation or other environmental factors on the components.

A unified sample preprocessing scheme is implemented with the aim of effectively removing large particle impurities. Subsequently, fine grinding

technology is adopted and a standardized 40 mesh screening procedure is used to ensure that all tea samples reach a highly uniform particle size. To maintain the stability and activity of the samples, all processed samples are sealed and stored in a constant temperature environment of 4 °C.

3. HPLC

The following provides a detailed explanation of the specific implementation process of HPLC analysis to determine the changes in the content of key active ingredients during dark tea fermentation, the methods used, and how to effectively solve research problems. To ensure the reliability of research results, HPLC analysis accuracy is ensured by regularly replacing consumables such as chromatography columns and filters, as well as calibrating pump pressure and flow rate.

3.1. Optimization of chromatographic conditions

Firstly, HPLC analysis conditions are optimized for the main active ingredients in dark tea, including tea polyphenols, catechins, theaflavins, thearubigins, etc. [29]. This includes that: suitable chromatography columns are selected, such as C_{18} reverse phase chromatography columns, which are suitable for the separation of most tea components; the composition and proportion of gradient elution mobile phase are used, such as acetonitrile water, ethyl acetate methanol water and other systems, to optimize the separation efficiency of different polar components; the flow rate is set at 0.8–1.2 mL/min to ensure good separation and analysis time; the column temperature is controlled at 25–40 °C to reduce the impact of temperature on separation efficiency; the detection wavelength is selected based on the absorption characteristics of the target component, such as 280 nm, which is commonly used for the detection of tea polyphenols.

3.2. Sample preparation

To ensure the accuracy and reliability of HPLC analysis, strict pretreatment is carried out on the collected tea samples. The sample is first subjected to drying, crushing, sieving and other steps to obtain a uniform tea powder. Subsequently, appropriate solvents such as methanol and ethanol are used for extraction, which may involve methods such as ultrasound assisted, heated reflux, or Soxhlet extraction to improve extraction efficiency. After filtration and concentration, the extract is further purified if necessary using solid-phase extraction and membrane filtration to remove impurities, reduce contamination of the chromatographic column, and improve analytical sensitivity. **Table 2** shows the entire process of sample pretreatment.

Table 2. The entire process of sample pretreatment.

Step	Condition
Drying	60 °C, 24 h
Grinding	Mechanical grinder, 5 min
Sieving	40 mesh sieve

Table 2. (*Continued*).

Step	Condition
Extraction	Methanol:Water = 70:30, ultrasonic-assisted, 30 min
Filtration	Microporous filter, 0.45 μm
Concentration	Rotary evaporator, 40 °C, 20 min
Further purification	Solid-phase extraction, C_{18} column, methanol elution

3.3. Implementation of HPLC

The processed sample solution is injected into the HPLC system for separation and detection. Under the set chromatographic conditions, the active ingredients in the sample are effectively separated on the chromatographic column, and their absorption peaks or signal intensities are recorded by detectors such as UV (ultraviolet) visible detectors and fluorescence detectors [30,31]. By comparing the retention time and spectral characteristics of standard samples, the active ingredients in the samples are qualitatively analyzed; the external standard method or internal standard method is used, combined with the standard curve, to calculate the content of each component.

In **Figure 2**, the retention time range is from 0 to 30 min, and the signal intensity shows four main peaks according to the concentration changes of different compounds. This peak corresponds to the characteristic retention time and concentration of a certain compound in HPLC separation. The first peak appearing in the chromatogram is at 5 min, with a peak signal intensity of 2.21 mAU, which is the highest peak of the four peaks. This indicates that the compound concentration in the sample solution with this retention time is high and the separation state is good. The second peak is located at 12 min, with a peak signal intensity of 1.48 mAU. The second peak appearing in the graph is wider than the other three, indicating that the separation effect of the compound is not as good as the concentration and separation state of the compound in the first peak. The third peak and fourth peak are located at 20 min and 25 min, respectively, with peak signal intensities of 0.91 mAU and 0.75 mAU. The compound concentration is not high and the separation effect is average at 20 and 25 min through data and image analysis.

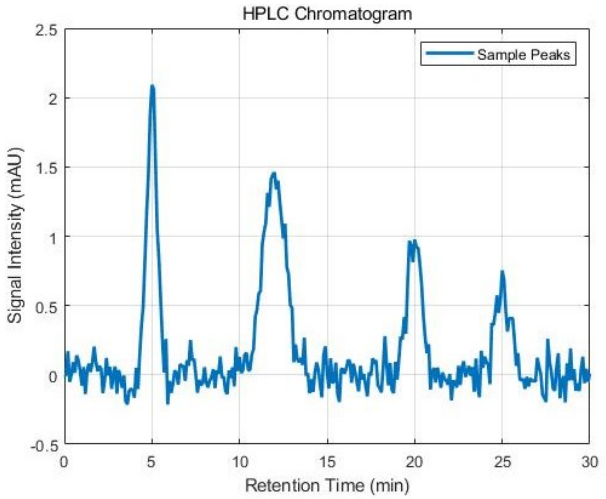

Figure 2. HPLC chromatogram.

4. Determination of antioxidant capacity

4.1. DPPH radical scavenging method

First, prepare tea extract solutions with multiple concentration gradients. Extract the appropriate tea extract solution and dilute it with water. Mix it with an equal volume of 0.10 mm DPPH solution and stir evenly. Leave it in the dark for a predetermined time of 30 min. The mixed liquid is removed and its absorbance is measured at 517 nm using a spectrophotometer. After completing the measurement using the change in absorbance of the blank DPPH solution as a reference, the scavenging rate of DPPH can be calculated to assess the free radical scavenging ability of tea extracts [32].

The absorbance of the sample reaction solution and the blank control are measured first. Based on the absorbance measurements, the DPPH radical scavenging rate (%) is calculated:

$$\text{DPPH Clearance rate (\%)} = \left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}}\right) \times 100\% \quad (1)$$

The relationship between sample concentration and DPPH radical scavenging efficiency is utilized to determine the IC_{50} value, which is the concentration required to achieve 50% radical inhibition. By constructing a model between concentration (C) and clearance rate, and using the curve parameters a and b in Equations (2) and (3), the value of IC_{50} can be calculated by reverse calculation.

$$\text{Scavenging Rate}(\%) = a\log(C) + b \quad (2)$$

$$IC_{50} = 10^{\left(\frac{50-b}{a}\right)} \quad (3)$$

4.2. ABTS cation radical scavenging method

The generation of ABTS+ • is achieved through the reaction of ABTS salt with potassium persulfate, and the resulting ABTS+ • solution is diluted to a specific absorbance for later use. During testing, an equal volume of tea extract solution is added to the diluted ABTS+ • solution, and after a certain reaction time (such as 6 min), the absorbance of the reaction solution is measured at a wavelength of 734 nm. Similarly, antioxidant capacity is evaluated by calculating the clearance rate of ABTS+ • .

4.3. FRAP method

The FRAP method is based on the ability of antioxidants to reduce trivalent ferri ions (Fe^{3+}) to divalent ferri ions (Fe^{2+}) under acidic conditions. Fe^{2+} forms a colored complex with 2, 4, 6-tripyridyltriazine (TPTZ), and its absorbance is measured at 593 nm wavelength. After determining the ferri ion concentration of the sample, the total antioxidant capacity (FRAP value) is further calculated using the following formula:

$$\text{FRAP value}=\left(\frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}}\right) \times C_{\text{standard}} \times V \quad (4)$$

Calculate the absorbance changes of the measured sample and standard solution using Equation (4). In the DPPH and ABTS tests during the fermentation process of black tea, if the extracted sample has a dark color and interferes with absorption, the dark colored sample can be appropriately diluted to reduce the influence of the sample's color on absorbance measurement. The diluted sample should be able to reduce color interference while maintaining sufficient concentration of antioxidant substances for accurate measurement.

5. Data processing

In this study, the purpose of data processing and analysis is to deeply explore the changing law of active ingredients and antioxidant capacity during the fermentation of dark tea. The specific steps are as follows: firstly, the active ingredient content data obtained from HPLC analysis and the raw data for antioxidant capacity determination are organized, and the sampling time point, sample number, detection indicators, and other information are checked to ensure the completeness and accuracy of the data. Next, data cleaning is performed to remove outliers and missing values, and interpolation or exclusion methods are used to ensure the reliability of the analysis. Then, descriptive statistical analysis is conducted on the organized data to calculate statistical measures such as the mean, standard deviation, maximum, and minimum values of each active ingredient content and antioxidant capacity index, in order to gain a preliminary understanding of the distribution characteristics and trends of the data [33].

Pearson correlation coefficient or Spearman's rank correlation coefficient is used to analyze the correlation between different active ingredients and their relationship with antioxidant capacity. Through correlation analysis, which components undergo synergistic changes during the fermentation process and which components make significant contributions to antioxidant capacity are revealed. **Figure 3** shows the correlation analysis results between the content of phenolic compounds in tea and its antioxidant capacity.

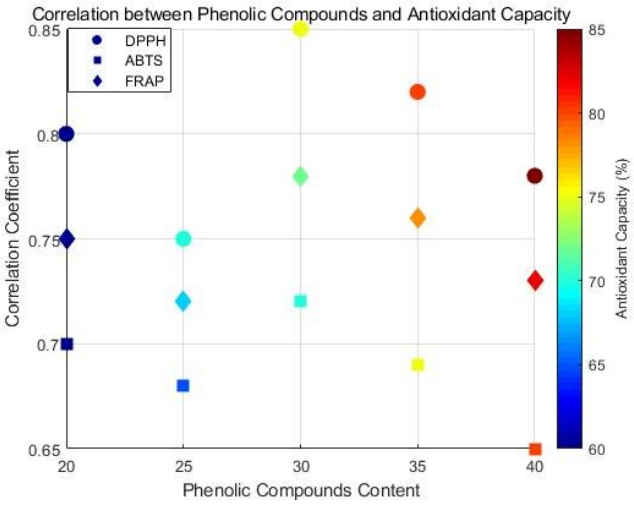

Figure 3. Correlation analysis results.

In **Figure 3**, the horizontal axis (X-axis) represents the content of phenolic compounds in micrograms per gram of tea leaves, and the vertical axis (Y-axis) represents the correlation coefficient of antioxidant capacity, calculated by Pearson correlation coefficient. The data points (color mapping) represent the percentage of different antioxidant capacity measurements obtained using DPPH, ABTS, and FRAP methods.

The positive correlation between the content of phenolic compounds and the antioxidant capacity of tea is shown in **Figure 3**. As the content of phenolic compounds increases, the antioxidant capacity of tea (measured by DPPH, ABTS, and FRAP) also increases accordingly. The data changes between different antioxidant capacity determination methods reflect their sensitivity and specificity, and combined with the trend of changes in phenolic compounds, further reveal the differences in their contributions to the antioxidant capacity of tea. These analysis results contribute to a deeper understanding of the mechanism of action of phenolic compounds in the antioxidant process of tea.

In order to further reduce dimensionality and extract key information, principal component analysis is used to process multiple active ingredient variables. Complex principal component analysis (PCA) converts raw data into a set of new, independent variables (that is, principal components) through linear transformation. These principal components are sorted according to their variance contribution rate, which maximizes the preservation of information in the raw data. Through PCA, several components that play a major role in the changes of active ingredients during the fermentation process of dark tea are identified, providing direction for subsequent process optimization.

Figure 4 shows the complex principal component analysis of antioxidant capacity and phenolic compound content in tea.

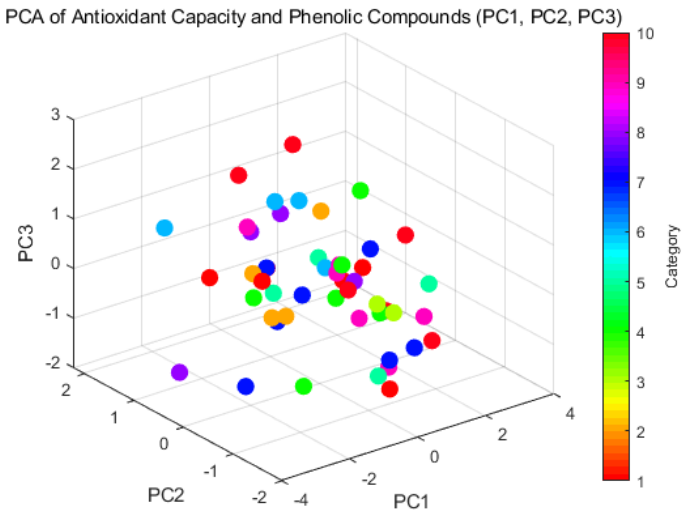

Figure 4. Results of complex principal component analysis.

Figure 4 shows a three-dimensional scatter plot generated using PCA analysis data. The horizontal axis represents the score of the first principal component (PC1); the vertical axis represents the score of the second principal component (PC2); the

Z-axis represents the score of the third principal component (PC3). Each point represents the position of a sample in the original dataset in the PCA transformed space. The position of data points varies based on their scores on the three principal components, which capture the main variability in the data.

The changes in data are reflected in the distribution of samples in three-dimensional space, which may form clusters or exhibit certain trends. These changes indicate in which dimensions the samples in the original data are similar or different, and which features are most important for distinguishing these samples. Through PCA analysis, high-dimensional data can be reduced to a low dimensional space while preserving the main information in the data, thus providing a more intuitive understanding of the structure and relationships of the data.

Figure 4 shows the distribution of samples in the principal component space, reflecting the diversity of antioxidant capacity and phenolic compound content in tea samples and their correlation in the principal component direction. Principal component analysis reveals the comprehensive characteristics of antioxidant capacity and chemical composition among different samples, providing a multidimensional perspective for understanding the differences in antioxidant function among different tea samples.

Regression analysis: in order to investigate the effects of fermentation time, temperature and other process parameters on the content of active ingredients and antioxidant capacity, multiple linear regression method is used. Through regression analysis, a mathematical model is established between process parameters, active ingredients, and antioxidant capacity to quantify the degree of influence and interaction of each parameter. **Figure 5** shows a linear regression graph.

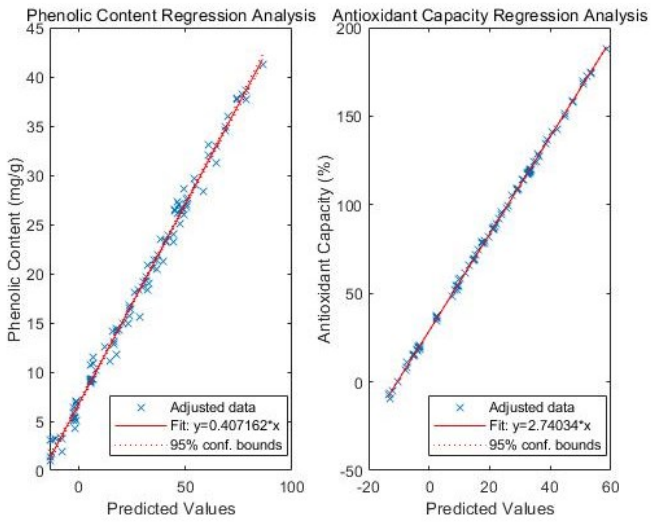

Figure 5. Linear regression plot.

Figure 5 shows the regression analysis of the content of phenolic compounds. The horizontal axis in the left figure represents the predicted value, and the vertical axis represents the content of phenolic compounds (mg/g); the right figure shows the regression analysis of antioxidant capacity, where the horizontal axis represents predicted values and the vertical axis represents antioxidant capacity (%). In **Figure 5**, fit: $y = 0.407162 \times x$ and fit: $y = 2.74034 \times x$ represents the fitted linear equations

in regression analysis, and 95% conf. bounds represents the 95% confidence interval, indicating a 95% confidence that the true regression coefficients fall within this interval. The regression analysis in **Figure 5** shows the results of comparing the content of phenolic compounds and antioxidant capacity in the same graphical window, which can intuitively understand the predictive performance of the model, including the accuracy and possible bias of the prediction, and thus evaluate the reliability of the model.

Based on the data analysis results, problems in the fermentation process of dark tea are identified, such as excessive loss of certain active ingredients and unclear improvement in antioxidant capacity. In response to these issues, specific optimization suggestions are proposed based on the actual operating conditions of the fermentation process, such as adjusting fermentation time, temperature, humidity and other parameters, and applying new fermentation strains or enzyme preparations to improve the quality and health functions of tea. In summary, this study comprehensively utilizes various statistical analysis methods such as descriptive statistical analysis, correlation analysis, principal component analysis, and regression analysis to deeply explore the changes in active ingredients and antioxidant capacity during the fermentation process of dark tea, as well as their interrelationships.

6. Comprehensive evaluation of fermentation effect of dark tea

The biochemical mechanisms involved in the fermentation process of black tea mainly involve microbial activity, enzymatic reactions, and physicochemical changes. Microorganisms such as mold and yeast secrete enzymes to decompose tea components, producing secondary metabolites that promote the oxidative polymerization of polyphenolic compounds such as catechins, forming theaflavins and thearubigins, thereby changing the color and flavor of tea leaves. Meanwhile, physical and chemical factors such as humidity, temperature, and oxygen supply also significantly affect the fermentation process. To optimize fermentation, raw materials can be carefully selected, temperature and humidity can be precisely controlled, excellent bacterial strains can be introduced, staged fermentation processes can be adopted, and monitoring and management can be strengthened. By comprehensively evaluating various indicators and continuously adjusting and optimizing fermentation conditions, we aim to improve the quality and health benefits of black tea, and promote the sustainable development of the black tea industry.

After completing multi-time sampling, HPLC analysis, and antioxidant capacity determination during the fermentation process of dark tea, this study enters a critical stage—comprehensive evaluation of the fermentation effect of dark tea. This section aims to integrate preliminary experimental data, comprehensively analyze the impact of fermentation on the quality and health functions of dark tea through a scientific evaluation system, and propose strategies for optimizing the fermentation process.

Firstly, all active ingredient content data obtained from HPLC analysis, results of antioxidant capacity determination using DPPH, ABTS, FRAP methods, and records of environmental parameters during fermentation (such as temperature, humidity, fermentation time, etc.) are systematically integrated. The active ingredient

content data and antioxidant capacity determination data obtained from HPLC analysis are summarized and statistically analyzed in **Table 3**.

Table 3. Active ingredient content data obtained from HPLC analysis.

Active Ingredient	Mean (mg/g)	Standard Deviation (mg/g)	Maximum (mg/g)	Minimum (mg/g)
Tea Polyphenols	105	15	120	90
Catechins	52	8	60	45
Theaflavins	17	8	25	5
Thearubigins	22	11	35	10

Table 4. HPLC analysis of antioxidant capacity determination data.

Measurement Method	Mean (%)	Standard Deviation (%)	Maximum (%)	Minimum (%)
DPPH	35	5	40	30
ABTS	38	4	42	34
FRAP	36	3	39	33

Tables 3 and **4** show the changes in active ingredients and antioxidant capacity during the fermentation process of dark tea, based on the analysis of the active ingredients and antioxidant capacity. The average content, standard deviation, maximum and minimum values of tea polyphenols, catechins, theaflavins and thearubigins are determined in **Table 3**, and the average contents are 105 mg/g, 52 mg/g, 17 mg/g and 22 mg/g, respectively. The standard deviation of theaflavins during fermentation is 8 mg/g, indicating a significant change in their content, which may be due to enzymatic reactions during fermentation affecting the production of theaflavins. **Table 4** shows the average, standard deviation, maximum, and minimum values of three antioxidant capacity determination methods. The average antioxidant capacities of DPPH, ABTS, and FRAP are 35%, 38%, and 36%, respectively. The ABTS method has the highest antioxidant capacity, with an average of 38% and a standard deviation of 4%.

Using professional data processing software, the data is cleaned to remove outliers, and standardized to ensure comparability between data from different batches and time points. **Figure 6** shows the effect of moderate phenolic compound content on the antioxidant capacity of dark tea during fermentation.

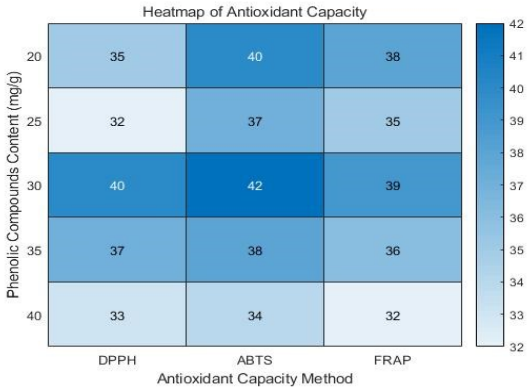

Figure 6. Relevant heatmap.

In **Figure 6**, the horizontal axis represents different antioxidant capacity measurement methods (DPPH, ABTS, FRAP), and the vertical axis represents different phenolic compound contents (mg/g). The color intensity of the heatmap represents the strength of antioxidant capacity, with darker colors indicating stronger antioxidant capacity.

DPPH method: when the content of phenolic compounds is 30 mg/g, the antioxidant capacity of DPPH is the highest (40%). As the content increases or decreases, the antioxidant capacity of DPPH shows a decreasing trend. ABTS method: when the content of phenolic compounds is 30 mg/g, the antioxidant capacity of ABTS also reaches its highest level (42%). FRAP method: the antioxidant capacity of FRAP is also highest (39%) when the content of phenolic compounds is 30 mg/g. Increasing or decreasing within this range leads to a slight decrease in antioxidant capacity.

Through data and heat maps, it is found that there is a strong correlation between the content of different phenolic compounds and various antioxidant capacity determination methods. Especially when the content of phenolic compounds is 30 mg/g, all three antioxidant capacity determination methods show the highest antioxidant capacity. During the fermentation process of dark tea, an appropriate amount of phenolic compounds has a significant effect on improving the antioxidant capacity of tea leaves.

Table 5 shows the quantitative changes of four main microbial species during black tea fermentation and their effects on the characteristics of fermentation products. As fermentation progresses, the number of yeast genera significantly increases, from an initial 10^4 CFU/g to 10^7 CFU/g at the end of fermentation. This process promotes an increase in tea alcohol content and fruit aroma. The number of lactic acid bacteria also increased significantly, especially in the middle stage of fermentation, from 10^3 CFU/g to 2×10^6 CFU/g, and finally stabilized at 5×10^6 CFU/g, mainly promoting the generation of theaflavins and enhancing the antioxidant properties of tea leaves. Although the genera Aspergillus and Penicillium are relatively small in the early stages of fermentation, they also significantly increase during the fermentation process, participating in the synthesis of thearubigins, enriching the color of tea leaves, and endowing them with unique aromas, increasing the diversity of flavors. The dynamic changes of these microorganisms work together in the fermentation process of black tea, ultimately affecting the quality and flavor of the tea leaves.

Table 5. Microbial diversity and its impact on tea fermentation.

Microbial Species	Initial Quantity (CFU/g)	Mid-Fermentation Quantity (CFU/g)	Final Fermentation Quantity (CFU/g)	Effect on Fermentation Product Characteristics
Yeast	10^4	5×10^6	10^7	Enhances body and fruity aroma of the tea liquor
Lactobacillus	10^3	2×10^6	5×10^6	Promotes theaflavin production, enhances antioxidant properties
Aspergillus	10^2	1×10^5	3×10^5	Involved in thearubigin synthesis, enriches color
Penicillium	Not Detected	10^3	1×10^4	Imparts unique aroma, increases flavor complexity

Analysis of changes in active ingredients: in order to visually display the trend of changes in the content of the main active ingredients, tea polyphenols, catechins, theaflavins, and thearubigins, during the fermentation process, **Figure 7** compares and analyzes to identify which components significantly increase or decrease during the fermentation process, as well as the key time nodes of these changes.

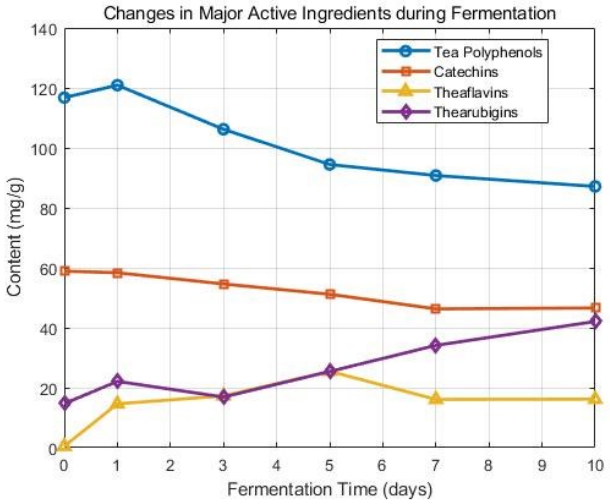

Figure 7. Trend of changes in main active ingredients.

Tea polyphenols: the content decreases from an initial 117 mg/g to 84 mg/g after 10 days of fermentation. Catechins: the content decreases from the initial 60 mg/g to 45 mg/g after 10 days of fermentation. Theaflavins: the content increases from 0 mg/g initially to 25 mg/g after 5 days of fermentation, and then decreases to 18 mg/g after 10 days of fermentation. Thearubigins: the content increases from an initial 18 mg/g to 41 mg/g after 10 days of fermentation.

Change analysis: the content of tea polyphenols and catechins continues to decrease during the fermentation process, indicating that these components are gradually transformed or degraded during the fermentation process. The content of theaflavins significantly increases in the early stages of fermentation and slightly decreases after reaching its peak, indicating that it is first formed during the fermentation process and then partially converted into other components. The content of thearubigins increases continuously with the increase of fermentation time, indicating its continuous accumulation during the fermentation process.

Based on the analysis results of active ingredient content and antioxidant capacity, combined with sensory evaluation data, a comprehensive evaluation index system for the fermentation effect of dark tea is constructed, including color, aroma, and taste. The system should be able to comprehensively reflect the improvement effect of fermentation on the quality and health functions of dark tea. The Analytic Hierarchy Process (AHP) is used to allocate weights to various evaluation indicators. Based on experimental data at various time points, each batch of dark tea is scored or rated to quantitatively evaluate the fermentation effect. Based on the comprehensive evaluation results mentioned above, combined with the biological principles and production practices of dark tea fermentation, targeted fermentation process optimization suggestions are proposed. Using the weight allocation results obtained

from AHP and combined with the actual data of color, aroma, and taste scores of each batch of dark tea, **Figure 8** is created.

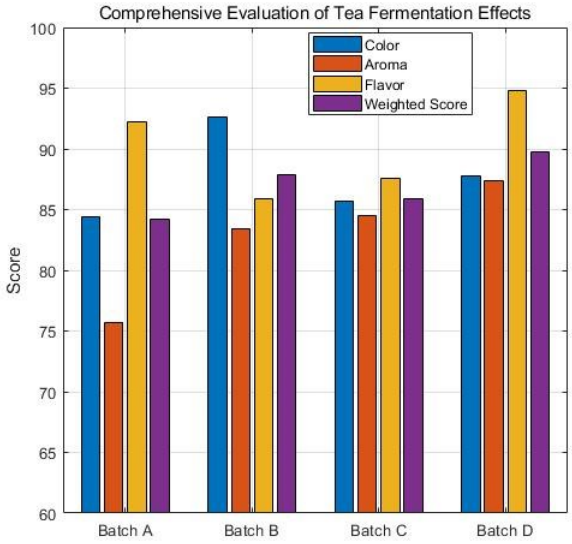

Figure 8. Comprehensive evaluation of fermentation effect of dark tea.

In **Figure 8**, the horizontal axis represents different dark tea batches (Batch A, Batch B, Batch C, Batch D), and the vertical axis represents the score values (Score), ranging from 0 to 100. Color scores: the color scores of each batch of dark tea range from 80 to 100, with Batch B having the highest color score and Batch A having the lowest. Aroma scores: the aroma scores range from 70 to 88, with Batch D having the highest and Batch A having the lowest. Flavor scores: the flavor scores range from 85 to 95, with Batch D having the highest and Batch B having the lowest. Weighted scores: the weighted scores of comprehensive evaluation combine the weights of color, aroma, and flavor, with Batch D having the highest comprehensive score and Batch A having the lowest. The evaluation results show that when the content of phenolic compounds is about 30 mg/g, the antioxidant capacity reaches its peak. The scoring of indicators such as color, aroma, and flavor shows that Batch D performs the best, indicating that it has reached a high level in all aspects, providing a clear direction for optimizing the fermentation process.

Three measures are taken to apply the results of this research to practical production, namely condition adaptability evaluation: before practical application, different fermentation conditions should be evaluated to determine the applicability and effectiveness of optimization strategies in different environments. Flexible adjustment strategy: Based on specific environmental conditions, flexibly adjust the parameter settings in the optimization strategy to ensure that it can maximize its effectiveness. Continuous monitoring and feedback: Continuously monitor the quality and active ingredient content of black tea during the production process, adjust fermentation conditions in a timely manner to ensure product stability and consistency.

7. Conclusions

This study systematically revealed the changes in active ingredients and antioxidant capacity during the fermentation process of black tea through comprehensive sampling, HPLC analysis, multiple antioxidant capacity determinations, and in-depth data analysis. The results showed that during the fermentation process, the content of tea polyphenols and catechins gradually decreased, while theaflavins first increased rapidly and then slightly decreased, while thearubigins continued to increase. These changes in components directly affect the antioxidant capacity of black tea. The results of DPPH, ABTS, and FRAP measurements show that the antioxidant activity reaches its peak when the concentration of phenolic compounds reaches about 30 mg/g. Compared with other studies, this study adopts a multi-point time-series sampling method and multiple antioxidant capacity measurement methods, providing a multidimensional evaluation of the changes in components and antioxidant capacity during the fermentation process of black tea. This method is more comprehensive and in-depth than previous studies on single time points or limited indicators. However, the research also has limitations. In terms of sensory evaluation, more subjective indicators such as taste and texture need to be combined to improve the evaluation system. In addition, new fermentation technologies should be explored in the future, such as temperature and humidity control or microbial co fermentation, to further optimize the quality and health benefits of black tea. Through these improvements, research will provide a more scientific basis for optimizing the fermentation process of black tea, promoting its competitiveness and health benefits in the market.

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References

1. Saharan P, Sadh PK, Duhan S, et al. Bio-enrichment of phenolic, flavonoids content and antioxidant activity of commonly used pulses by solid-state fermentation. Journal of Food Measurement and Characterization. 2020; 14(3): 1497-1510. doi: 10.1007/s11694-020-00399-z
2. Marazza JA, Nazareno MA, de Giori GS, et al. Enhancement of the antioxidant capacity of soymilk by fermentation with Lactobacillus rhamnosus. Journal of Functional Foods. 2012; 4(3): 594-601. doi: 10.1016/j.jff.2012.03.005

3. Khubber S, Marti-Quijal FJ, Tomasevic I, et al. Lactic acid fermentation as a useful strategy to recover antimicrobial and antioxidant compounds from food and by-products. Current Opinion in Food Science. 2022; 43: 189-198. doi: 10.1016/j.cofs.2021.11.013
4. Zhu F, Zhang B, Li J, et al. Effects of fermented feed on growth performance, immune response, and antioxidant capacity in laying hen chicks and the underlying molecular mechanism involving nuclear factor-κB. Poultry Science. 2020; 99(5): 2573-2580. doi: 10.1016/j.psj.2019.12.044
5. Liu S, Yu Q, Huang H, et al. The effect of bound polyphenols on the fermentation and antioxidant properties of carrot dietary fiber in vivo and in vitro. Food & Function. 2020; 11(1): 748-758. doi: 10.1039/c9fo02277e
6. Santos TR, Feitosa PR, Gualberto NC, et al. Improvement of bioactive compounds content in granadilla (Passiflora ligularis) seeds after solid-state fermentation. Food Science and Technology International. 2020; 27(3): 234-241. doi: 10.1177/1082013220944009
7. Bang SI, Gwon GH, Cho EJ, et al. Characteristics of fermented vinegar using mulberry and its antioxidant activity. Korean Journal of Food Preservation. 2020; 27(5): 651-662. doi: 10.11002/kjfp.2020.27.5.651
8. Szutowska J, Rybicka I, Pawlak-Lemańska K, et al. Spontaneously fermented curly kale juice: Microbiological quality, nutritional composition, antioxidant, and antimicrobial properties. Journal of Food Science. 2020; 85(4): 1248-1255. doi: 10.1111/1750-3841.15080
9. Liu W, Dun M, Liu X, et al. Effects on total phenolic and flavonoid content, antioxidant properties, and angiotensin I-converting enzyme inhibitory activity of beans by solid-state fermentation with Cordyceps militaris. International Journal of Food Properties. 2022; 25(1): 477-491. doi: 10.1080/10942912.2022.2048009
10. Değirmencioğlu N, Yıldız E, Sahan Y, et al. Impact of tea leaves types on antioxidant properties and bioaccessibility of kombucha. Journal of Food Science and Technology. 2020; 58(6): 2304-2312. doi: 10.1007/s13197-020-04741-7
11. Cuvas-Limon RB, Nobre C, Cruz M, et al. Spontaneously fermented traditional beverages as a source of bioactive compounds: an overview. Critical Reviews in Food Science and Nutrition. 2020; 61(18): 2984-3006. doi: 10.1080/10408398.2020.1791050
12. Chai KF, Voo AYH, Chen WN. Bioactive peptides from food fermentation: A comprehensive review of their sources, bioactivities, applications, and future development. Comprehensive Reviews in Food Science and Food Safety. 2020; 19(6): 3825-3885. doi: 10.1111/1541-4337.12651
13. Milić MD, Buntić AV, Mihajlovski KR, et al. The development of a combined enzymatic and microbial fermentation as a viable technology for the spent coffee ground full utilization. Biomass Conversion and Biorefinery. 2021; 13(8): 6747-6759. doi: 10.1007/s13399-021-01605-8
14. Liu Z, de Souza TSP, Wu H, et al. Development of Phenolic-Rich Functional Foods by Lactic Fermentation of Grape Marc: A Review. Food Reviews International. 2023; 40(6): 1756-1775. doi: 10.1080/87559129.2023.2230278
15. Jan S, Krishan Kumar KK, Yadav AN, et al. Effect of diverse fermentation treatments on nutritional composition, bioactive components, and anti-nutritional factors of finger millet (Eleusine coracana L.). Journal of Applied Biology & Biotechnology. 2022; 46-52. doi: 10.7324/jabb.2022.10s107
16. Guo Y, Chen X, Gong P, et al. Advances in the in vitro digestion and fermentation of polysaccharides. International Journal of Food Science & Technology. 2021; 56(10): 4970-4982. doi: 10.1111/ijfs.15308
17. Dai J, Sha R, Wang Z, et al. Edible plant Jiaosu: manufacturing, bioactive compounds, potential health benefits, and safety aspects. Journal of the Science of Food and Agriculture. 2020; 100(15): 5313-5323. doi: 10.1002/jsfa.10518
18. Zhou J, He C, Qin M, et al. Characterizing and Decoding the Effects of Different Fermentation Levels on Key Aroma Substances of Congou Black Tea by Sensomics. Journal of Agricultural and Food Chemistry. 2023; 71(40): 14706-14719. doi: 10.1021/acs.jafc.3c02813
19. Tanaka T, Matsuo Y. Production Mechanisms of Black Tea Polyphenols. Chemical and Pharmaceutical Bulletin. 2020; 68(12): 1131-1142. doi: 10.1248/cpb.c20-00295
20. Zhu K, Ouyang J, Huang J, et al. Research progress of black tea thearubigins: a review. Critical Reviews in Food Science and Nutrition. 2020; 61(9): 1556-1566. doi: 10.1080/10408398.2020.1762161
21. Zhang G, Yang J, Cui D, et al. Transcriptome and Metabolic Profiling Unveiled Roles of Peroxidases in Theaflavin Production in Black Tea Processing and Determination of Tea Processing Suitability. Journal of Agricultural and Food Chemistry. 2020; 68(11): 3528-3538. doi: 10.1021/acs.jafc.9b07737

22. Hua J, Wang H, Yuan H, et al. New insights into the effect of fermentation temperature and duration on catechins conversion and formation of tea pigments and theasinensins in black tea. Journal of the Science of Food and Agriculture. 2021; 102(7): 2750-2760. doi: 10.1002/jsfa.11616
23. Yu P, Huang H, Zhao X, et al. Dynamic variation of amino acid content during black tea processing: A review. Food Reviews International. 2022; 39(7): 3970-3983. doi: 10.1080/87559129.2021.2015374
24. Yildiz E, Guldas M, Gurbuz O. Determination of in-vitro phenolics, antioxidant capacity and bio-accessibility of Kombucha tea produced from black carrot varieties grown in Turkey. Food Science and Technology. 2021; 41(1): 180-187. doi: 10.1590/fst.00320
25. Li F, Boateng ID, Yang X, et al. Effects of processing methods on quality, antioxidant capacity, and cytotoxicity of Ginkgo biloba leaf tea product. Journal of the Science of Food and Agriculture. 2023; 103(10): 4993-5003. doi: 10.1002/jsfa.12577
26. Kong J, Yang X, Zuo X, et al. High-quality instant black tea manufactured using fresh tea leaves by two-stage submerged enzymatic processing. Food Science and Human Wellness. 2022; 11(3): 676-685. doi: 10.1016/j.fshw.2021.12.025
27. Ding J, Mei S, Gao L, et al. Tea processing steps affect chemical compositions, enzyme activities, and antioxidant and anti-inflammatory activities of coffee leaves. Food Frontiers. 2022; 3(3): 505-516. doi: 10.1002/fft2.136
28. Tong W, Yu J, Wu Q, et al. Black Tea Quality is Highly Affected during Processing by its Leaf Surface Microbiome. Journal of Agricultural and Food Chemistry. 2021; 69(25): 7115-7126. doi: 10.1021/acs.jafc.1c01607
29. Luo T, Jiang JG. Anticancer Effects and Molecular Target of Theaflavins from Black Tea Fermentation in Vitro and in Vivo. Journal of Agricultural and Food Chemistry. 2021; 69(50): 15052-15065. doi: 10.1021/acs.jafc.1c05313
30. Das AK, Ghosh A, Majumder S, et al. Characterization of tea and tea infusion: A study of marketed black tea samples from some tea growing regions of India. Journal of Pharmacognosy and Phytochemistry. 2020; 9(5): 1532-1540. doi: 10.22271/phyto.2020.v9.i5v.12553
31. Qin C, Lian L, Xu W, et al. Comparison of the chemical composition and antioxidant, anti-inflammatory, α-amylase and α-glycosidase inhibitory activities of the supernatant and cream from black tea infusion. Food & Function. 2022; 13(11): 6139-6151. doi: 10.1039/d2fo00707j
32. Liang Z, Zhang P, Zeng XA, et al. Variations in physicochemical characteristics, antioxidant activity, phenolic and volatile profiles, and sensory attributes of tea-flavored Chardonnay wine during bottle aging. Food & Function. 2023; 14(18): 8545-8557. doi: 10.1039/d3fo03137c
33. Bilge G, Özdemir KS. Synchronous fluorescence spectroscopy combined with chemometrics for determination of total phenolic content and antioxidant activity in different tea types. Journal of the Science of Food and Agriculture. 2020; 100(9): 3741-3747. doi: 10.1002/jsfa.10413