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BRIEF REPORT

Anaerobic fermentation of tea leaves and spent tea residues: Effects on phenolic and flavonoid contents, antioxidant capacity, and antibacterial activity

[version 1; peer review: 2 approved with reservations]

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Abstract

Abstract

Background

Tea (*Camellia sinensis*) and its brewing by-products are promising sources of phenolic-rich extracts that may serve as natural antioxidants in food systems. However, the extent to which anaerobic fermentation modifies the bioactive properties of different processed teas remains unclear. This study evaluated the effects of anaerobic fermentation on the phenolic content, antioxidant capacity, and antibacterial activity of several tea types cultivated in Indonesia.

Methods

A 2 × 2 × 4 factorial experiment with five replicates was conducted using three factors: fermentation status (unfermented vs. anaerobically fermented), material fraction (tea leaf vs. spent residue), and processed tea type (green, black, oolong, and white). Methanolic extracts prepared with 70% methanol were analyzed for total phenolic content (TPC), total flavonoid content (TFC), antioxidant capacity based on DPPH IC₅₀, and antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* expressed as MIC₅₀.

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Results

Across tea types, oolong and white teas exhibited higher TPC and stronger antioxidant capacity than black tea ($p < 0.05$). Tea leaves consistently showed higher TPC and stronger antioxidant activity than spent residues ($p < 0.05$). Anaerobic fermentation increased TPC in most teas and enhanced TFC mainly in leaf samples. However, improved antioxidant capacity following fermentation was observed only in spent residues, as indicated by lower IC_{50} values. In contrast, fermentation generally increased MIC_{50} values, suggesting reduced antibacterial effectiveness.

Conclusions

Anaerobic fermentation differentially affected the bioactive properties of tea materials depending on tea type and material fraction. While this process was effective in enhancing the antioxidant potential of spent tea residues, it was not beneficial for improving antibacterial activity. These findings suggest that anaerobic fermentation may be a useful strategy for valorizing tea brewing by-products as antioxidant-rich functional ingredients.

Keywords

anaerobic fermentation, antibacterial, antioxidant, phenolics, tea



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Introduction

Tea (*Camellia sinensis*) is widely consumed and is recognized as a rich dietary source of phenolic compounds and flavonoids, which contribute to antioxidant and antimicrobial properties and have been associated with various health-related benefits (Nibir et al., 2017; Wang and Kang, 2020). The antioxidant and antibacterial effects of tea are mainly attributed to catechins, including epigallocatechin gallate (EGCG), epicatechin (EC), and epicatechin gallate (ECG) (Zhao et al., 2014). These compounds work by neutralizing free radicals through metabolic processes in the body, thereby preventing oxidative stress associated with various degenerative diseases, including cancer, diabetes, and cardiovascular disorders (Wang and Kang, 2020). Beyond the brewed beverage, tea processing and consumption also generate substantial quantities of spent tea residues. Although these residues are often discarded, they may retain appreciable amounts of bioactive compounds and therefore represent potential resources for the development of functional ingredients or natural antioxidants for food applications (Çakmak et al., 2024).

Green, white, oolong, and black teas originate from the same plant, i.e., *Camellia sinensis*, but differ markedly in chemical composition because they undergo different degrees of enzymatic oxidation and processing (Alves et al., 2025). These differences influence the profile and extractability of phenolic compounds, which can translate into variation in antioxidant capacity and antibacterial activity across tea types (Tanaka & Matsuo, 2020; Esposito et al., 2023). Likewise, converting dried leaves into a spent residue by brewing can remove a portion of water-soluble phenolics, often lowering the functional potency of residue extracts compared with leaf extracts (Saklar et al., 2015). Furthermore, prolonged storage of these teas may reduce their bioactive compound content (Hazra et al., 2020), thereby decreasing their antimicrobial and antioxidant activities.

Fermentation is a food-relevant bioprocessing approach that can stabilize wet plant matrices and modulate phytochemical composition through microbial and enzymatic transformations. In sealed anaerobic systems, fermentation may alter the concentration, molecular forms, and extractability of tea phenolics; for example, changes in catechins and antioxidant activity during anaerobic storage have been reported (Nishino et al., 2007). From a food-processing perspective, a simple sealed-pouch anaerobic fermentation step may be particularly attractive for wet tea residues because it can serve as a low-input stabilization and valorization strategy. However, evidence remains limited regarding how anaerobic fermentation affects the bioactive potential of both tea leaves and spent residues across different processed tea types. Therefore, this study aimed to evaluate the effects of anaerobic fermentation, material fraction (leaf vs. spent residue), and processed tea type on total phenolic and flavonoid contents, antioxidant capacity, and antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.

Methods

Sample preparation

Dried tea leaves representing four processed tea types (green, black, oolong, and white) were sourced as commercial products from the Indonesian Research Institute for Tea and Cinchona (PT Perkebunan Nusantara III, Bandung, West Java, Indonesia). The experiment followed a $2 \times 2 \times 4$ factorial arrangement with three factors: fermentation status (unfermented vs. anaerobically fermented), material fraction (tea leaf vs. spent tea residue), and processed tea type (green, black, oolong, and white). For each processed tea type, two material fractions were prepared. The tea leaf fraction consisted of the original dried leaves. The spent tea residue fraction was produced by brewing the dried leaves with warm water, followed by oven-drying at 60 °C.

For the anaerobic fermentation treatment, 250 g of each material was packed in polyethylene bags and vacuum-sealed. Water was added to the leaf fraction at a 1:2 ratio for the fermented treatment, whereas unfermented leaves were prepared without added water. All bags were stored at -20°C prior to fermentation. Samples were then subjected to anaerobic fermentation in the sealed pouches for 30 days. After fermentation, bags were opened and each sample was divided into two equal subsamples. All materials were freeze-dried and ground to pass a 1-mm sieve before analysis.

Extraction and analyses of bioactive compounds

Extraction followed ISO 14502-1 using 70% methanol (Merck, Sigma Aldrich, catalogue no. 109534). Briefly, 200 mg of ground sample was mixed with 5 mL of preheated 70% methanol and incubated in a water bath for 10 min with vortexing every 5 min. After cooling to room temperature, the mixture was centrifuged at 3,500 rpm for 10 min. The supernatant was collected and the pellet was re-extracted once using the same procedure. Combined extracts were adjusted to a final volume of 10 mL with cold 70% methanol.

Total phenolic content (TPC) was determined using the Folin–Ciocalteu method (Sari et al., 2023). A diluted extract (20 μL) was mixed with 20 μL Folin–Ciocalteu reagent (Merck, Sigma Aldrich, catalogue no. 1.09001) and vortexed for 1 min. After 5 min, 200 μL of 7% sodium carbonate (Merck, Sigma Aldrich, catalogue no. 1.06392) and 10 μL deionized

water were added and mixed. The reaction was incubated in the dark at room temperature for 120 min and measured at 750 nm (Thermo Multiskan GO Microplate Reader, Massachusetts, USA). Gallic acid ($10\text{--}60\text{ mg L}^{-1}$, Merck, Sigma Aldrich, catalogue no. 8.42649) was used for calibration. Results are reported as mg gallic acid equivalents (GAE) per g dry sample (mg GAE/g).

Total flavonoid content (TFC) was measured following [Sari et al. \(2023\)](#). In each well, 50 μL of extract or standard solution was mixed with 100 μL methanol (blank: 150 μL methanol). Then 20 μL of 10% AlCl_3 (Merck, Sigma Aldrich, catalogue no. 8.01081) was added and incubated for 3 min, followed by 20 μL of 1 M sodium acetate (Merck, Sigma Aldrich, catalogue no. 1.06268) and 60 μL methanol. After incubation in the dark at room temperature for 40 min, absorbance was measured at 430 nm. Quercetin (Merck, Sigma Aldrich, catalogue no. Q4951) was used as the calibration standard, and results are reported as mg quercetin equivalents (QE) per g dry sample (mg QE/g).

Antioxidant and antibacterial assays

The radical-scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was evaluated based on [Cheng et al. \(2006\)](#) with minor modifications. Extract (80 μL) was mixed with 20 μL of DPPH solution (Merck, Sigma Aldrich, catalogue no. D9132) in a 96-well microplate, and absorbance was read at 517 nm. Ascorbic acid (Merck, Sigma Aldrich, catalogue no. 1.00468) and quercetin were used as positive controls and analyses were performed in triplicate. Antioxidant activity was expressed as IC_{50} (ppm), defined as the extract concentration required to scavenge 50% of DPPH radicals.

Antibacterial activity was evaluated against *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) using a microdilution assay adapted from [Nabila et al. \(2025\)](#). Mueller-Hinton broth (MHB, Merck, Sigma-Aldrich, Catalogue No. 1.10293) was used as the growth medium and inocula from 24-h cultures were adjusted to 1×10^6 McFarland. In a 96-well microplate, 100 μL sterile MHB was added per well, followed by two-fold serial dilutions of the extracts from 400 to 25 ppm. Each well received 10 μL bacterial suspension (except the media control). Optical density (OD) was recorded at 540 nm at 0 and 24 h (Thermo Multiskan GO, Massachusetts, USA). Growth inhibition was calculated from OD changes relative to the growth control, and MIC_{50} (ppm) was defined as the extract concentration producing 50% growth inhibition.

Statistical analysis

Data were analyzed using three-way analysis of variance (ANOVA) in CoStat software (version 6.400, CoHort software, Monterey, CA, USA), including main effects of fermentation status, material fraction, processed tea type, and their interactions. Least Significant Difference (LSD) tests were used for post hoc mean comparisons. Differences were considered significant at $p < 0.05$.

Results

Across tea types, oolong and white teas showed higher TPC than black tea ($p < 0.01$), whereas black tea had the highest mean TFC ($p < 0.01$; [Tables 1–2](#)). Tea leaves contained substantially higher TPC than spent residues. The effects of

Table 1. Effects of fermentation, tea material, and tea types on total phenolic content.

Treatment		Tea types							
		Green tea		Black tea		Oolong tea		White tea	
Fermentation	Material	-----mgGAE/g sampel-----							
Non-fermented	Leaf	111.01 ± 0.32 ^c	65.89 ± 0.56 ^d	114.81 ± 2.89 ^a		115.26 ± 1.11 ^b			
	Residue	48.61 ± 0.03 ^c	31.15 ± 0.10 ^d	79.38 ± 0.57 ^a		62.85 ± 0.13 ^b			
Fermented	Leaf	118.46 ± 0.42 ^c	73.65 ± 0.15 ^d	122.88 ± 0.10 ^a		128.36 ± 0.37 ^b			
	Residue	46.54 ± 0.37 ^c	39.83 ± 0.83 ^d	99.12 ± 0.93 ^a		104.86 ± 0.07 ^b			
	Average	81.15 ± 0.18 ^c	52.63 ± 0.34 ^d	104.05 ± 1.23 ^a		102.83 ± 0.48 ^b			
Significance of treatment factors		Fermentation	Material	Tea	F × M	F × T	M × T	F × M × T	
(p value)		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	

Note: Different lowercase letters within the same column indicate significant differences ($p < 0.05$) based on the Least Significant Difference (LSD) test. Abbreviations: F, fermentation; M, tea material; T, processed tea type; F $\times$ M, interaction between fermentation and tea material; F $\times$ T, interaction between fermentation and processed tea type; M $\times$ T, interaction between tea material and processed tea type; F $\times$ M $\times$ T, interaction among fermentation, tea material, and processed tea type. Significance levels: highly significant, $p < 0.01$; significant, $p < 0.05$; ns, not significant.

Table 2. Effects of fermentation, tea material, and tea types on total flavonoid content.

Treatment		Tea types						
		Green tea		Black tea		Oolong tea		White tea
Fermentation	Material	-----mg QE/g sampel-----						
Non-fermented	Leaf	14.95 ± 0.12 ^b	18 ± 0.10 ^a		14.79 ± 0.51 ^b		12.28 ± 0.16 ^c	
	Residue	13.05 ± 0.15 ^b	13.65 ± 0.19 ^a		15.07 ± 0.95 ^b		12.16 ± 0.12 ^c	
Fermented	Leaf	17.66 ± 0.33 ^b	22.93 ± 0.50 ^a		13.72 ± 0.23 ^b		12.88 ± 0.13 ^c	
	Residue	12.27 ± 0.24 ^b	14.68 ± 0.10 ^a		14.06 ± 01.44 ^b		12.07 ± 1.35 ^c	
	Average	14.48 ± 0.10 ^b	17.32 ± 0.19 ^a		14.41 ± 0.53 ^b		12.35 ± 0.61 ^c	
Significance of treatment factors		Fermentation	Material	Tea	F × M	F × T	M × T	F × M × T
(p value)		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Note: Different lowercase letters within the same column indicate significant differences ($p < 0.05$) based on the Least Significant Difference (LSD) test. Abbreviations: F, fermentation; M, tea material; T, processed tea type; F × M, interaction between fermentation and tea material; F × T, interaction between fermentation and processed tea type; M × T, interaction between tea material and processed tea type; F × M × T, interaction among fermentation, tea material, and processed tea type. Significance levels: highly significant, $p < 0.01$; significant, $p < 0.05$; ns, not significant.

Table 3. Effects of fermentation, tea material, and tea types on inhibitory concentration (IC₅₀).

Treatment		Tea types						
		Green tea		Black tea		Oolong tea		White tea
Fermentation	Material	----- (ppm) -----						
Non-fermented	Leaf	32.91 ± 0.63 ^b	46.14 ± 0.71 ^a	26.95 ± 0.21 ^d		33.78 ± 1.18 ^c		
	Residue	76.48 ± 0.65 ^b	93.91 ± 0.72 ^a	48.91 ± 0.87 ^d		53.95 ± 2.16 ^c		
Fermented	Leaf	36.9 ± 1.50 ^b	59.8 ± 0.98 ^a	29.3 ± 1.07 ^d		30.6 ± 2.28 ^c		
	Residue	63.68 ± 0.78 ^b	79.47 ± 3.58 ^a	40.68 ± 3.95 ^d		35.27 ± 1.28 ^c		
	Average	52.49 ± 0.42 ^b	69.82 ± 1.39 ^a	36.46 ± 1.65 ^d		38.39 ± 0.58 ^c		
Significance of treatment factors		Fermentation	Material	Tea	F × M	F × T	M × T	F × M × T
(p value)		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Note: Different lowercase letters within the same column indicate significant differences ($p < 0.05$) based on the Least Significant Difference (LSD) test. Abbreviations: F, fermentation; M, tea material; T, processed tea type; F × M, interaction between fermentation and tea material; F × T, interaction between fermentation and processed tea type; M × T, interaction between tea material and processed tea type; F × M × T, interaction among fermentation, tea material, and processed tea type. Significance levels: highly significant, $p < 0.01$; significant, $p < 0.05$; ns, not significant.

anaerobic fermentation on TPC and TFC depended on both material fraction and tea type. Fermented leaves showed a slight increase in TPC across all tea types ($p < 0.01$), whereas fermentation increased TFC only in green tea and black tea leaves ($p < 0.01$). In the residue fraction, fermentation consistently increased TPC and TFC in black tea residues, whereas the same treatment led to a consistent decline in green tea residues ($p < 0.01$).

The DPPH IC₅₀ values indicated stronger antioxidant activity, reflected by lower IC₅₀ values, in oolong and white teas than in black tea (Table 3). Tea leaves exhibited substantially lower IC₅₀ values than spent residues across all tea types and fermentation statuses ($p < 0.01$). Fermentation increased IC₅₀ values in the leaf fraction in almost all tea types ($p < 0.01$), with white tea leaf being the only exception. In contrast, the residue fraction showed a consistent reduction in IC₅₀ after fermentation across all tea types ($p < 0.01$), indicating improved antioxidant capacity following fermentation.

Regarding antibacterial activity, white tea showed the lowest MIC₅₀ values against *S. aureus* and *E. coli* ($p < 0.01$), while oolong tea showed a lower MIC₅₀ only against *E. coli* ($p < 0.01$; Tables 4–5). Fermentation increased the MIC₅₀ against *S. aureus* in the leaf fraction across all tea types ($p < 0.01$). A similar trend was observed in the residue fraction, in which fermentation increased the MIC₅₀ of green tea, white tea, and oolong tea ($p < 0.01$). For antimicrobial activity against

Table 4. Effects of fermentation, tea material, and tea types on antimicrobial activity against *Staphylococcus aureus* (MIC₅₀).

Treatment		Tea types						
		Green tea		Black tea		Oolong tea		White tea
Fermentation	Material	----- (ppm) -----						
Non-fermented	Leaf	280.56 ± 6.55 ^b	150.86 ± 6.56 ^{bc}	57.36 ± 1.10 ^a		54.12 ± 1.58 ^c		
	Residue	288.31 ± 3.73 ^b	471.25 ± 24.46 ^{bc}	80.55 ± 8.10 ^a		205.82 ± 7.41 ^c		
Fermented	Leaf	482.48 ± 100.97 ^b	236.03 ± 15.82 ^{bc}	116.26 ± 0.94 ^a		143.57 ± 3.91 ^c		
	Residue	608.31 ± 110.42 ^b	264.93 ± 13.21 ^{bc}	8934.07 ± 775.25 ^a		272.18 ± 38.63 ^c		
	Average	414.91 ± 58.19 ^b	280.77 ± 7.40 ^{bc}	2297.06 ± 385.95 ^a		188.53 ± 12.34 ^c		
Significance of treatment factors		Fermentation	Material	Tea	F × M	F × T	M × T	F × M × T
(p value)		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Note: Different lowercase letters within the same column indicate significant differences ($p < 0.05$) based on the Least Significant Difference (LSD) test. Abbreviations: F, fermentation; M, tea material; T, processed tea type; F × M, interaction between fermentation and tea material; F × T, interaction between fermentation and processed tea type; M × T, interaction between tea material and processed tea type; F × M × T, interaction among fermentation, tea material, and processed tea type. Significance levels: highly significant, $p < 0.01$; significant, $p < 0.05$; ns, not significant.

Table 5. Effects of fermentation, tea material, and tea types on antimicrobial activity against *Escherichia coli* (MIC₅₀).

Treatment		Tea types						
		Green tea		Black tea	Oolong tea		White tea	
Fermentation	Material	----- (ppm) -----						
Non-fermented	Leaf	158.96 ± 33.81 ^a	232.35 ± 5.17 ^a	139.27 ± 8.10 ^b	183.35 ± 8.18 ^b			
	Residue	211.50 ± 7.27 ^a	340.67 ± 9.21 ^a	244.49 ± 3.88 ^b	176.67 ± 3.29 ^b			
Fermented	Leaf	387.89 ± 68.08 ^a	213.97 ± 3.02 ^a	165.04 ± 27.58 ^b	135.77 ± 0.14 ^b			
	Residue	382.86 ± 15.38 ^a	367.42 ± 2.06 ^a	254.54 ± 2.94 ^b	350.62 ± 28.52 ^b			
	Average	285.30 ± 27.02 ^a	288.60 ± 3.17 ^a	200.83 ± 11.52 ^b	211.60 ± 12.76 ^b			
Significance of treatment factors		Fermentation	Material	Tea	F × M	F × T	M × T	F × M × T
(p value)		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Note: Different lowercase letters within the same column indicate significant differences ($p < 0.05$) based on the Least Significant Difference (LSD) test. Abbreviations: F, fermentation; M, tea material; T, processed tea type; F × M, interaction between fermentation and tea material; F × T, interaction between fermentation and processed tea type; M × T, interaction between tea material and processed tea type; F × M × T, interaction among fermentation, tea material, and processed tea type. Significance levels: highly significant.

E. coli, green tea and oolong tea leaves showed higher MIC₅₀ values after fermentation ($p < 0.01$). In the residue fraction, fermentation consistently increased MIC₅₀ values across all tea types ($p < 0.01$), indicating reduced antibacterial effectiveness.

Discussion

The higher TPC observed in oolong and white teas than in black tea may reflect differences in leaf maturity and processing intensity. In particular, the high TPC in white tea may be attributed to the use of young leaves, which are naturally rich in epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) (Esposito et al., 2023). By contrast, the substantially higher TPC in tea leaves than in spent residues is consistent with the removal of water-soluble phenolics during brewing (Saklar et al., 2015). The slight increase in TPC after fermentation, especially in the leaf fraction, may reflect degradation of native phenolic compounds such as catechins into smaller molecules that still retain phenolic properties rather than being converted into non-phenolic acids (Nishino et al., 2007). These breakdown products may continue to react with the Folin–Ciocalteu reagent, thereby contributing to a slight apparent increase in measured total phenols.

The stronger antioxidant activity of oolong and white teas, as indicated by lower IC₅₀ values, is in agreement with their higher phenolic concentrations. Dudonné et al. (2009) reported a strong correlation between total phenolic content and radical-scavenging activity ($R = 0.939$ for DPPH), and this activity is mediated through mechanisms such as hydrogen atom transfer, single-electron transfer, sequential proton loss electron transfer, and transition metal chelation (Zeb, 2020). Although black tea contained more flavonoids than the other tea types, its antioxidant activity was weaker, as shown by its higher IC₅₀ values. This may be explained by the transformation of catechins into theaflavins and other polymeric compounds during full oxidation in black tea processing (Tanaka & Matsuo, 2020).

The lower IC₅₀ values in the leaf fraction than in the residue fraction are also consistent with the substantially higher TPC and TFC of the leaves. Conversely, the higher IC₅₀ values in tea residues likely resulted from the brewing process, which extracted most of the water-soluble phenolic compounds (Saklar et al., 2015). Interestingly, fermentation had contrasting effects on antioxidant capacity depending on the material fraction. In leaves, fermentation generally increased IC₅₀ values and thus reduced antioxidant capacity. In residues, however, fermentation consistently reduced IC₅₀ values across tea types, indicating improved antioxidant activity. This contrasting response may be related to the presence of bioactive compounds that remain bound to the fibrous matrix of tea residues (Çakmak et al., 2024). Fermentation may facilitate the release of these bound phenolics, thereby increasing the availability of antioxidant compounds in the residue fraction.

With respect to antibacterial activity, the lower MIC₅₀ values of white tea against both *S. aureus* and *E. coli*, and of oolong tea against *E. coli*, indicate a stronger antibacterial potential of these tea types. This finding is consistent with their higher phenolic content, as phenolic compounds can disrupt microbial cell membranes, inhibit metabolic enzymes, and interfere with biofilm formation in pathogenic bacteria such as *E. coli* and *S. aureus* (Huang et al., 2022). Nevertheless, fermentation generally increased MIC₅₀ values in both leaf and residue fractions, indicating reduced antibacterial effectiveness after fermentation. Although TPC appeared to increase after fermentation, antimicrobial activity against both *S. aureus* and *E. coli* declined. This reduction may be attributed to polymerization of phenolic compounds during fermentation or to their degradation by microbial activity, producing derivatives with weaker antibacterial effects (Romero et al., 2025).

Ethical considerations

This study was conducted without utilizing any human or animal resources.

Data availability

Underlying data

Figshare: Dataset of phenolic and flavonoid contents, antioxidant capacity, and antibacterial activity in tea leaves and spent tea residues. <https://doi.org/10.6084/m9.figshare.32030247> [Jayanegara A, 2026].

The project contains the following underlying data:

- Dataset of phenolic and flavonoid contents, antioxidant capacity, and antibacterial activity in tea leaves and spent tea residues. (Raw dataset supporting the results of this article, including phenolic and flavonoid contents, antioxidant capacity, and antibacterial activity in tea leaves and spent tea residues following anaerobic fermentation).

Extended data

No extended data are associated with this article.

Data are available under the terms of the [Creative Commons Attribution 4.0 International license \(CC BY 4.0\)](#).

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Vaibhavi Savalia

RK University, Rajkot, Gujarat, India

The paper examines whether 30 days of anaerobic fermentation changes the TFC, TPC, antioxidant, and antibacterial activity (against *S. aureus* and *E. coli*) of four Indonesian tea types (green, black, oolong, white), comparing whole leaves versus spent (already-brewed) residues. It's a solid factorial design (5 replicates), and the headline findings are believable: oolong and white teas beat black tea on phenolics and antioxidant strength; leaves beat residues; and fermentation helped antioxidant capacity in residues but generally hurt antibacterial activity.

On the statistical analysis (marked Partly)

The tables (1–5) all have superscript letters (a, b, c, d) next to the mean values, which normally signal a post-hoc significance test — but nowhere in the paper is it explained what's actually being compared.

Clarity regarding whether the $\pm$ values in the tables are SD or SEM: the paper needs to state which one it is.

Table 4 has one value- fermented oolong residue, 8934 ppm - that is very different from most values under 600 ppm. It's not addressed anywhere in the text. This needs to be checked and explained.

Following correction recommended

- Add a footnote to each table spelling out exactly what the superscript letters compare and what test was used.
- State clearly whether $\pm$ is SD or SEM.
- Explain in text or (correct) the 8934 ppm in Table 4.

A few other things noticed

In the discussion, specific compounds like catechins, EGCG, and theaflavins has been discussed, but the study never actually measured these directly (only total phenolics/flavonoids), so those explanations are speculation and should be flagged as such.

No proof of real microbial fermentation actually happened in the sealed bags for e.g., no pH measurements, no microbial counts, no metabolite data. So it makes it hard to tell whether the observed changes reflect true fermentation or are simply the result of prolonged anaerobic storage of a wet, sealed plant matrix. Since the paper's whole framing is built around

"fermentation" effects, this distinction matters and should be either demonstrated or acknowledged as a limitation.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Herbal drug technology, nutraceutical, traditional medicines

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 03 August 2026

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Pitchaporn Wanyo

Kalasin University, Kalasin, Kalasin, Thailand

This study is interesting and addresses an important issue regarding sustainable utilization of spent tea residues. The manuscript is generally well written and the experimental design is logical. However, several scientific issues require strengthening to improve rigor and impact. The Discussion mainly repeats the observed results without providing sufficient mechanistic explanations. More comprehensive interpretation supported by recent literature would considerably improve the manuscript.

Major Comments

1. Lack of fermentation characterization

Provide key parameters to confirm successful anaerobic fermentation: pH (before/after), microbial counts or dominant species, lactic acid or other metabolite concentrations, moisture content, and evidence that true fermentation (not mere storage) occurred.

2. Justification of fermentation conditions

The chosen conditions (30 days, 20 °C, vacuum-sealed polyethylene bags) lack rationale. Explain why these were selected, whether preliminary optimization was done, and whether similar conditions are reported in the literature.

3. Mechanism of TPC increase

The Folin-Ciocalteu assay measures reducing capacity, not phenolics specifically. The observed increase could arise from release of bound phenolics, formation of smaller reducing compounds, degradation products, reducing sugars, or Maillard reaction products. The Discussion should acknowledge these limitations rather than implying actual synthesis of phenolic compounds.

4. Absence of individual phenolic profiling

Only TPC and TFC were measured, yet the Discussion frequently refers to catechins, EGCG, ECG, and theaflavins. If these compounds were not verified analytically (e.g., HPLC-MS), the Discussion must clearly state that such explanations remain hypothetical.

5. Limited antioxidant evaluation

Only DPPH was used. Including additional assays (ABTS, FRAP, ORAC) would strengthen the conclusions. If unavailable, this limitation should be explicitly acknowledged.

6. Antibacterial assay validation

Clarify: Were positive antibiotic and solvent controls included? Were MIC endpoints visually confirmed? Were MBC values determined? Were experiments independently repeated? Without these controls, the antibacterial conclusions are less convincing.

7. Statistical analysis

The study uses a 2×2×4 factorial design. Although interaction terms are reported, the discussion focuses mainly on main effects. Given significant three-way interactions, interpretation should emphasize interaction effects; interaction plots would greatly aid clarity.

8. Abnormally high MIC value

Table 4 reports an MIC₅₀ of 8934 ppm for fermented oolong residue—far higher than all other treatments. Please verify whether this is a typo, an outlier, or has a biological explanation, and discuss it specifically.

9. Missing correlation analysis

Given the suggestion that phenolics drive antioxidant and antibacterial activity, perform Pearson correlation between TPC, TFC, DPPH IC₅₀, and MIC₅₀ for both bacterial strains. A correlation matrix or PCA would **substantially improve interpretation**.

10. Practical significance

The conclusion that anaerobic fermentation improves antioxidant potential lacks practical context. Please discuss potential applications (food, nutraceuticals, natural preservatives, shelf-life, industrial feasibility).

Minor Comments

1. Introduction

- Include more recent references (2024–2026) on fermented tea waste valorization.
- Strengthen the novelty statement by clearly differentiating this work from previous studies on fermented tea residues.

2. Methods

- Specify exact brewing conditions (water temperature, time, tea-to-water ratio) for preparing spent residues.
- Clearly state the number of biological and technical replicates separately.
- Justify the choice of 70% methanol.
- Report calibration curve data (R^2 , linear range) for TPC and TFC assays.
- Provide bacterial strain ATCC numbers.

3. Results

- Use figures (bar graphs or interaction plots) to improve readability over dense tables.
- Consistently present units (e.g., mg GAE/g sample) and specify whether values are on a dry-weight basis.
- Report exact p-values wherever possible instead of only "<0.01".

4. Discussion

- Expand mechanistic explanations beyond restating results; consider discussing release of cell-wall bound phenolics, microbial enzymatic hydrolysis, oxidation/polymerization, and structural changes.
- Deepen the discussion on the counterintuitive observation of increased TPC but decreased antibacterial activity.

5. Conclusion

The conclusion is generally supported by the data but should avoid implying causal mechanisms that were not experimentally demonstrated.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: functional foods, bioactive compounds, food processing, product development

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have

significant reservations, as outlined above.

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