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Valorization of Olive Leaf Biomass through Fermentation: Metabolite Production and Bioactive Characterization

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Abstract

Purpose

This study aimed to enhance the availability of bioactive metabolites from olive leaves through fermentation using a symbiotic culture of bacteria and yeasts (SCOBY), and to evaluate the fermentative potential of olive leaves as an alternative substrate to green tea.

Methods

Olive leaves were used as partial (50%) and complete (100%) substitutes for green tea, the conventional substrate employed in SCOBY-based fermentation. Fermentation performance and metabolite profiles were compared with those of green tea over 14 days of fermentation. Physicochemical and microbiological parameters were monitored throughout the process, while bioactive compounds were identified and quantified by direct infusion electrospray ionization triple quadrupole mass spectrometry (DI-ESI-MS).

Results

Partial substitution with olive leaves (50% green tea/olive leaves) preserved pH, titratable acidity, and antioxidant activity at levels comparable to those observed in the traditional green tea fermentation. In contrast, complete substitution (100% olive leaves) significantly modified fermentation kinetics, leading to changes in acidity, antioxidant capacity, and microbial activity, thereby indicating limitations associated with higher replacement levels. Metabolomic analysis revealed the presence of 32, 32, and 30 bioactive compounds in the Control, 50%, and 100% substitution treatments, respectively. The identified metabolites consisted predominantly of phenolic compounds and flavonoids related to antioxidant and antimicrobial properties. Overall, the 50% substitution treatment demonstrated promising potential as a sustainable strategy for the production of functional metabolites.

Conclusion

The valorization of olive leaves, an underutilized agricultural by-product, represents a sustainable alternative for the development of innovative fermented products with prospective applications in functional foods, beverages, nutraceuticals, and cosmetic formulations.

HIGHLIGHTS

- Olive leaves were converted into high-value bioactive metabolites via fermentation.
- SCOBY fermentation increased phenolic availability and antioxidant potential.

- The 50% olive leaf formulation preserved microbial activity and fermentation kinetics.
- Olive-derived phenolics influenced metabolite profiles and selective microbial adaptation.
- Olive leaf valorization supports circular economy and sustainable bioprocessing strategies.

1. INTRODUCTION

During the fermentation of green tea (*Camellia sinensis*) by a symbiotic culture of bacteria and yeasts (SCOBY), bioactive compounds including organic acids, water-soluble vitamins, tea polyphenols, amino acids, minerals, ethanol, and hydrolytic enzymes are produced [1]. Although many of these compounds are naturally present in plant leaves, the fermentation process can enhance their concentration, bioavailability, and chemical diversity through microbial biotransformation [2, 3]. These compounds possess health benefits, including antioxidant, antimicrobial, anti-inflammatory, antidiabetic, and anticancer properties [4]. However, the extent of metabolite production varies depending on the composition of the raw materials, fermentation conditions, and microbial consortium [5].

In recent years, there has been growing interest in the use of agro-industrial residues as substrates for microbial fermentation, in line with circular economy principles [6, 7]. The valorization of these residues through fermentation represents a sustainable approach for the production of high-value compounds, contributing to waste reduction and resource efficiency. In addition, this strategy minimizes environmental impacts associated with waste disposal while enabling the generation of bioactive metabolites through sustainable bioprocesses [8].

Among agro-industrial residues, olive leaves derived from *Olea europaea* L. cultivation represent a significant and underexploited biomass [9]. Olive trees are widely cultivated in Mediterranean regions and increasingly in other parts of the world, generating substantial amounts of leaves during pruning, harvesting, and olive processing. These residues are often discarded, burned, or used as low-value animal feed, contributing to environmental concerns and representing a loss of potentially valuable bioresources [10].

Olive leaves are naturally rich in bioactive compounds, particularly phenolic compounds such as oleuropein, hydroxytyrosol, flavonoids, and secoiridoids, which are associated with strong antioxidant, antimicrobial, anti-inflammatory, and cardioprotective activities [11]. However, the direct utilization of olive leaves is limited due to factors such as low bioavailability, structural complexity of compounds, and the presence of antinutritional factors [9]. Therefore, strategies that enhance the extraction, transformation, and bioactivity of these compounds are highly desirable.

Fermentation is an effective approach to improve the functional properties of agro-industrial residues. Through microbial enzymatic activity, complex molecules can be degraded into simpler and more bioavailable forms, leading to the release or transformation of phenolic compounds and the formation of new metabolites [2]. In SCOBY-based systems, the synergistic interaction between yeasts and bacteria

plays a key role in metabolite production, influencing both the kinetics of fermentation and the final chemical composition.

During fermentation, microbial enzymes such as β-glucosidases, esterases, and tannases catalyze the hydrolysis of glycosylated phenolics and complex polyphenols, leading to the release of low-molecular-weight metabolites with enhanced antioxidant activity and bioavailability [11]. In olive-derived matrices, this biotransformation may promote the conversion of oleuropein into hydroxytyrosol and related secoiridoids, compounds associated with high biological activity.

While green tea remains the most widely used substrate for SCOBY fermentation, the exploration of alternative substrates such as olive leaves is still limited. Olive leaves have previously demonstrated potential as sustainable sources of bioactive compounds for biotechnological applications [6]. However, little is known about how their incorporation affects SCOBY-driven fermentation dynamics, microbial adaptation, and metabolite diversification when replacing *Camellia sinensis* in kombucha-like systems. We hypothesized that partial substitution of green tea with olive leaves would maintain fermentation performance while modulating microbial metabolism and promoting the generation of distinct phenolic metabolites. Therefore, this study aimed to enhance the availability and diversification of bioactive metabolites from olive leaves through fermentation using a symbiotic culture of bacteria and yeasts (SCOBY). For this purpose, olive leaves were used as partial (50%) and complete (100%) substitutes for green tea, the conventional substrate employed in kombucha fermentation, and their effects on fermentation performance, metabolite production, and bioactive compound composition were evaluated.

2. MATERIAL AND METHODS

2.1 Material

The leaves were collected from non-bearing trees of the Arbequina cultivar (23°16'36.6"S 51°52'57.4"W). The olive leaves were dried at 55°C for 24 hours until a constant mass was reached; they were then crushed, sieved to 60 *mesh* and stored in bottles protected from light. The starter culture used for the fermentation was a symbiotic culture between yeast and acetic bacteria (SCOBY) from Kefirlife Company (SP, Brazil). Green tea and sugar were from the local market. Folin-Ciocalteu, sodium carbonate, gallic acid, 2,2-azinobis radical (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate and 2,2, -Diphenyl-1-Picrylhydrazyl free radical (DPPH) were from Sigma Aldrich (Spruce Street, St Louis, USA). The other reagents used were of analytical grades.

2.2 Methods

Characterization of green tea (GT) and olive leaves (OL)

Preparation of Extracts for Phenolic Compound Analysis

To determine phenolic compounds, the GT extract was prepared using 100% methanol at a ratio of 1:300 m/v. OL extract was prepared at a ratio of 1:100 m/v, followed by homogenization for 1 min,

centrifugation (Centrifuge CT-0603, Parsec, Brazil) at 3000 rpm for 15 minutes. The supernatants were collected for analysis.

Total phenolic compounds

Total phenolic compounds (TPC) were determined by the Folin-Ciocalteu method [12]. The methanolic extracts were mixed with Folin-Ciocalteu and sodium carbonate, homogenized, and after 30 minutes, protected from light, the samples were read in a spectrophotometer (UV-Vis spectrophotometer model IL-592, Kasuaki, Brazil) at 725 nm. The absorbance results were compared with the gallic acid curve, expressed in mg gallic acid equivalent per gram of sample mg GAE g^{-1}.

Antioxidant activity

Antioxidant activity analyses were performed by capturing the free radicals ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) [13] and DPPH (2,2-Diphenyl -1-Picrylhydrazyl) [14]. ABTS free radical scavenging was determined by mixing 5 mL of ABTS (7mM) with 88µL of potassium persulfate (140 mM) and after 16 hours protected from light, the $ABTS^{+}$ solution was diluted in ethyl alcohol until reaching the absorbance of 0.70 ± 0.05. Aliquots of 40 µL of extracts were mixed with 1.96 mL of $ABTS^{+}$ solution, homogenized and absorbances were read after 6 minutes protected from light, in a spectrophotometer at 734 nm. DPPH free radical scavenging was determined by mixing 100 µL of extract with 1.90 mL of DPPH radical (0.06 mM). After homogenization, the samples were kept in the dark for 30 min and after that, the reading was performed in a spectrophotometer at 515 nm. The ABTS and DPPH readings were calculated, and the results are expressed as percentages.

Color

The color was determined by direct reading of the GT and OL powder using a colorimeter (Konica Minolta, CR400, Japan) and the reflectance of coordinates L* (+white; - black), a* (+red; -green) and b* (+yellow; -blue) using the CIELAB scale.

Fermentation with olive leaves

To activate starter culture, 1.2 liters of infusion was prepared by combining 7.5 g L^{-1} of green tea into filtered and sterilized water, which was heated to 80 ºC. After cooling to room temperature, the infusion was filtered, and 80 g L^{-1} of sugar was dissolved into the mixture. The preparation was divided into three glass vessels, each containing 10% of the starter culture and the SCOBY pellicle. The glass vessel was covered with clean cloth and incubated for 14 days at 25 ± 2 ºC.

The fermentation process was conducted using three treatments: Control (100% green tea), 50-OL (made with 50% green tea and 50% olive leaves) and 100-OL (made using 100% olive leaves) Table 1. The 50% substitution level was selected to evaluate whether olive leaves could partially replace green tea without compromising SCOBY metabolism while still contributing relevant phenolic compounds. For

each treatment, three glass vessels were prepared, each with a round cover, cleaned, and incubated at 25 ± 2 °C for 14 days.

Fermentation characterization

Table 1. Formulation for different treatments (100 mL).

Ingredients	Control	50-OL	100-OL
Green tea (g)	0.75	0.37	-
Olive leaves (g)	-	0.37	0.75
Water (mL)	90	90	90
Sugar (g)	8	8	8
Starter culture (mL)	10	10	10
SCOBY pellicle (g)	4	4	4

*50-OL: made with 50% green tea and 50% olive leaves; 100-OL: made with 100% olive leaves.

pH, titratable activity, and total soluble solids

The pH during fermentation time was measured using a glass electrode pH meter (Tecnopon, mPA-210), while total soluble solids (°Brix) were determined with a digital refractometer (HI 96801 – Nusfalau, Romania) every 5 days of fermentation in three samples for treatment. Titratable acidity (TA) of the samples fermented for 14 days was determined by titration with 0.1 M sodium hydroxide until reaching pH 8.2. The volume of NaOH used was recorded and the results were expressed as acetic acid equivalents (g acetic acid L^{-1}) [15].

Determination of bioactive compounds

Extracts were prepared using distilled water at a 1:15 m/v ratio. Total phenolic compounds (TPC) were determined using a gallic acid calibration curve and another using oleuropein by the Folin-Ciocalteu method as previously described. The results were expressed in mg gallic acid equivalent per gram of sample mg GAE g^{-1} and mg Eq Oleuropein g^{-1}.

Antioxidant activity

Antioxidant activity analyses were performed in extracts by capturing the free radicals ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), and DPPH (2,2-Diphenyl -1-Picrylhydrazyl) as described above.

Electrospray ionization with triple quadrupolar mass spectrometry (DI-ESI-MS)

Mass spectra were acquired following the methodology described by Sinosaki et al. [16] using a Premier XE (Águas, Milford, MA, USA) mass spectrometer with electrospray ionization (ESI) and a triple quadrupole analyzer. Multiple reaction monitoring (MRM) analysis under two different conditions was used as reported in previous studies [16–20]. In a 10 mL volumetric flask, 0.01 g of fermented liquid (just autoclaving) extract was weighed, and the volume was completed with HPLC-grade methanol (Merck, Germany). The solution was placed in an ultrasound bath for 10 minutes to completely solubilize the sample. 100 µL of the supernatant was collected and diluted in 900 µL of mobile phase (dilution 1). The dilution procedure was repeated twice more, yielding a final concentration of 1 µg mL-1 (dilution 3). A 25 mL glass syringe was coupled to a PVDF filter (diameter 25 mm, pore size 22 um), and the diluted solution was filtered. The filtered sample was placed in the injection system and performed analysis. For mass analysis, a mobile phase of methanol and ammonium hydroxide 0.1 % (V/V) was used, with a flow of 0.2 mL/min, a capillary voltage of 2 kV, a cone voltage of 20 V, a source temperature of 110 °C, a dissolution temperature of 200 °C, a cone gas flow of 50 L/h, a dissolution gas flow of 200 L/h, and a collision energy of 15. The data were analyzed using the software MassLynx version 4.4.

Color

Color was determined by direct measurement of the fermented liquid at the end of the fermentation period using the CIELAB color space (L*, a*, b*), as previously described.

Microbiology analysis

From each sample, a series of dilutions was prepared. For each dilution, three Petri dishes were used. The analysis focused on the determination of yeasts using Potato Dextrose Agar (PDA), and acetic bacteria by Hestrin e Schramm medium (HS g L^{-1}: glucose (20), yeast extract (5), peptone (5), Na_2HPO_4 (2.7), citric acid monohydrate (1.15) at pH 5.7 [21]. The procedure involved inoculating 0.1 mL of each diluted sample into sterile peptone water using the pour plate method. The Petri dishes were incubated at 30 °C for 36 hours.

Statistical Analysis

The experiment was conducted in three independent rounds (n = 3). In each round, three flasks were prepared per treatment. For pH, titratable acidity, total soluble solids, and color, one measurement was obtained from each individual flask, resulting in nine observations per treatment. For microbiological counts, total phenolic compounds, and antioxidant activity, the contents of the three flasks from each treatment were pooled within each experimental round, and analyses were performed in triplicate.

Data were expressed as mean ± standard deviation. Prior to statistical analysis, data normality and homoscedasticity were evaluated using the Shapiro–Wilk and Levene tests, respectively. Two-way analysis of variance (ANOVA) was applied considering treatment and fermentation time as fixed factors, including their interaction effects. When significant differences were observed, Tukey's post hoc test was

performed for multiple comparisons at a significance level of $p < 0.05$. Statistical analyses were carried out using SISVAR 5.6 software (UFLA, MG, Brazil).

3. RESULTS AND DISCUSSION

Characterization of green tea and olive leaves

Table 2 compares green tea and olive leaves regarding biochemical and colorimetric parameters. GT exhibited significantly higher total phenolic content (TPC) and DPPH radical scavenging activity than OL (approximately two-fold). On the other hand, there was no significant difference between GT and OL for the antioxidant activity determined by ABTS, suggesting that both raw materials may present similar antioxidant potential depending on the mechanism evaluated.

Table 2
Characterization of raw materials green tea (GT) and olive leaves (OL).

TPC (mg GAE g^{-1})	**GT**	**OL**
	39.8 ± 0.7^{A}	**14.59 ± 0.5^{B}**
DPPH (%)	91.83 ± 0.37^{A}	49.74 ± 0.41^{B}
ABTS (%)	62.03 ± 1.24^{A}	65.10 ± 0.36^{A}
L*	41.58 ± 0.56^{B}	50.93 ± 0.46^{A}
a*	-1.39 ± 0.15^{B}	-3.12 ± 0.41^{A}
b*	4.88 ± 0.83^{B}	12.46 ± 0.36^{A}

The results were expressed as mean ± standard deviation. Means with different capital letters differ between lines ($P < 0.05$). TPC: total phenolic content.

Polyphenols are recognized as potent antioxidants and free radical scavengers [22]. Green tea's most abundant polyphenols are catechins, representing around 30–40% of the tea leaf's dry weight. In GT, they can be found in several forms, the most abundant being (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epigallocatechin-3-O-gallate (EGCG) [23]. In OL, hydroxytyrosol, oleuropein, and luteolin-7-*O*-glucoside are the most abundant phenolic antioxidants [24].

Although GT exhibited greater DPPH radical scavenging activity than OL, both materials showed similar ABTS scavenging capacity. This difference may be associated with the distinct reaction mechanisms involved in antioxidant assays, which can occur through hydrogen atom transfer (HAT) and/or single electron transfer (SET) pathways depending on the assay conditions and the chemical nature of the antioxidant compounds [25, 26].

In terms of color, OL showed higher lightness (L*) and a more negative a* value than GT, indicating a more pronounced greenish hue. Since these differences were observed in the raw materials, they are

mainly attributed to intrinsic compositional characteristics, particularly the higher chlorophyll content in olive leaves. Additionally, OL exhibited significantly higher b* values, suggesting a more intense yellow component, which may be related to the presence of carotenoids. In contrast, the lower greenness and lightness observed in GT may be associated with processing steps such as thermal stabilization, which can promote partial chlorophyll degradation and pigment transformation [27].

Characterization of the fermentation process to obtain metabolites

pH and total soluble solids

The progression of fermentation was monitored by measuring pH and soluble solids content at the beginning and end of the process, and the results are presented in Fig. 1. A progressive decrease in pH was observed in all treatments, confirming the occurrence and progression of fermentation and indicating the production of organic acids through microbial metabolism. On day 14, the pH decreased in all formulations, ranging from 2.30 to 2.47. The 100-OL treatments showed significantly higher pH values than the others, suggesting lower organic acid accumulation and reduced microbial metabolic activity during fermentation. These findings were consistent with the titratable acidity results obtained at the end of the fermentation period, where the Control samples presented the highest acidity (16.63 ± 0.38 g acetic acid L^{-1}), followed by 50-OL (10.73 ± 0.13 g L^{-1}) and 100-OL (8.40 ± 0.24 g L^{-1}).

Similar trends have been reported in fermentation processes using alternative substrates, where pH reduction is associated with organic acid production and microbial metabolism [4]. According to the literature, the main organic acids formed during the fermentation of green tea or olive leaves are acetic and gluconic acids [28, 29]. The lower acidity observed in OL-containing formulations may indicate differences in substrate composition and microbial adaptation, which can influence acid production during SCOBY fermentation.

The soluble solids content on day 0 ranged from 8.54 to 9.55 °Brix, with significant differences among treatments. On day 14, there was a reduction in the Control and 50-OL treatments, indicating greater sugar consumption in these cases. In contrast, the lower reduction in soluble solids observed in the 100-OL treatment may indicate reduced carbohydrate utilization by the microbial consortium. This behavior suggests that olive leaf phenolics, particularly oleuropein and related secoiridoids, may have partially inhibited yeast glycolytic activity and ethanol production, consequently limiting acetic acid biosynthesis by acetic acid bacteria. This interpretation is consistent with the higher final pH observed in the 100-OL treatment and the lower acetic acid content determined by titratable acidity analysis, indicating lower organic acid accumulation during fermentation. Previous studies have demonstrated that high concentrations of olive-derived phenolics may exert selective antimicrobial pressure and influence microbial adaptation during fermentation processes [11].

During the tea fermentation, leaves serve as a nitrogen source for microbial fermentation, while sucrose provides the carbon required by the SCOBY throughout the process [30]. In an anaerobic environment,

yeasts break down sucrose into glucose and fructose, generating ethanol and carbon dioxide [31]. Acetic acid bacteria (AAB) then oxidize ethanol into acetaldehyde, which can be further converted into acetic acid. Additionally, glucose oxidation leads to the production of various organic acids, including gluconic acid, glucuronic acid, 2-ketogluconic acid, 5-ketogluconic acid, and 2,5-diketogluconic acid. Certain bacterial genera, such as *Acetobacter, Gluconacetobacter*, and *Komagataeibacter*, can fully oxidize organic acids, such as acetic and lactic acids, to carbon dioxide [30, 32].

The fermentation dynamics and final metabolite composition depend on several factors, including the microbial interactions within the SCOBY, the type of tea or substrate used, the initial sugar concentration, and key fermentation parameters such as time and temperature [30]. Interestingly, the 50-OL treatment exhibited physicochemical behavior closer to the Control, suggesting that partial replacement of green tea with olive leaves maintained the main metabolic pathways involved in SCOBY fermentation while still contributing phenolic substrates for microbial biotransformation. As a result, variations in these conditions can alter the metabolic pathways used by the microorganisms, ultimately influencing the composition of the final product.

Bioactive compounds and antioxidant activity

Table 3 compares the total polyphenol content (TPC) (mg EAG g^{-1}) and the antioxidant activity (by DPPH and ABTS methods, in %) of different treatments at two time points (day 0 and day 14 of fermentation).

Table 3
Total polyphenol content (mg EAG g^{-1}) and antioxidant activity (%) of green tea and olive leaves over 14 days of fermentation

TPC mg EAG g^{-1}		
	0 Days	**14 Days**
Control	5.05 ± 1.01^{Ab}	8.66 ± 0.21^{Aa}
50-OL	4.45 ± 0.71^{Ab}	6.07 ± 0.60^{Ba}
100-OL	2.57 ± 0.37^{Bb}	3.73 ± 1.08^{Ca}
TPC mg Eq Oleuropein g^{-1}		
	0 Days	14 Days
Control	ND	ND
50-OL	$1223{,}35 \pm 51{,}28^{Bb}$	$2180{,}21 \pm 76{,}54^{Ba}$
100-OL	$2776{,}30 \pm 135{,}67^{A}$	$3023{,}35 \pm 35{,}29^{A}$
ABTS (%)		
	0 Days	14 Days
Control	41.78 ± 3.48^{A}	45.80 ± 3.37^{A}
50-OL	31.27 ± 2.14^{B}	32.47 ± 4.49^{B}
100-OL	13.02 ± 1.46^{Cb}	17.38 ± 3.15^{Ca}
DPPH (%)		
	0 Days	14 Days
Control	56.82 ± 5.23^{A}	65.05 ± 3.06^{A}
50-OL	44.63 ± 3.73^{B}	44.27 ± 5.05^{B}
100-OL	21.43 ± 3.62^{C}	23.99 ± 3.30^{C}

The results were expressed as mean ± standard deviation. Means with different capital letters differ between columns. Means with different lowercase letters differ between rows ($P < 0.05$). TPC: total phenolic compounds. Control: 100% green tea; 50-OL: 50% green tea and 50% olive leaves; 100-OL: 100% olive leaves. ND: Not Detected.

Total polyphenol content (TPC) increased significantly after 14 days of fermentation in all treatments ($p < 0.05$), with the Control showing the highest values, followed by 50-OL and 100-OL. An increase in total phenolic content after fermentation has also been reported in studies using alternative substrates,

including olive leaves, where microbial activity promotes the release and transformation of phenolic compounds [28].

Significant differences were also observed among treatments at both time points ($p < 0.05$). During fermentation, enzymes such as β-glucosidase, cellulase, and pectinase, produced by bacteria and yeasts, contribute to the degradation of plant tissue. This process promotes the release of bound phenolic compounds, which leads to an increase in total phenolic content and enhanced DPPH radical scavenging activity. In addition, these enzymes catalyze the transformation of high-molecular-weight phenolic compounds into smaller and more bioactive forms, further contributing to the overall increase in polyphenol content [2, 3]. Similar behavior has been observed in fermentation processes using agro-industrial residues, reinforcing the role of microbial metabolism in enhancing bioactive compound availability [4].

Oleuropein is the main phenolic compound present in OL [33]. As expected, it was not detected in the Control samples at either time point, whereas treatments containing olive leaves exhibited substantially higher oleuropein-equivalent values. The 100-OL treatment showed the highest concentrations throughout the fermentation process due to the greater proportion of olive leaf biomass in the formulation. However, a significant increase after 14 days of fermentation ($p < 0.05$) was observed only in the 50-OL treatment, while the 100-OL treatment remained statistically unchanged.

These results suggest that moderate olive leaf supplementation may favor microbial biotransformation and the release of oleuropein-related compounds during SCOBY fermentation. In contrast, the high concentration of olive-derived phenolics in the 100-OL formulation may have exerted inhibitory effects on microbial metabolism, limiting enzymatic hydrolysis and metabolite conversion. Previous studies have demonstrated that elevated concentrations of secoiridoids and related phenolic compounds can affect microbial adaptation and fermentation activity, particularly in mixed microbial systems [11, 34]. Therefore, although the 100-OL treatment contained the highest oleuropein-equivalent content overall, fermentation-induced enhancement of detectable phenolic compounds was more pronounced in the 50-OL formulation.

Regarding antioxidant activity, ABTS radical scavenging decreased with increasing proportions of olive leaves, with the Control showing the highest values and 100-OL the lowest at both time points. However, a slight increase was observed after fermentation, particularly in the 100-OL treatment, indicating a modest improvement in antioxidant activity. Similarly, DPPH radical scavenging activity decreased with olive leaf addition, following the trend Control > 50-OL > 100-OL. Although the Control treatment showed a slight increase after fermentation, no significant differences were observed over time, suggesting a limited effect of fermentation on DPPH activity.

The lower antioxidant activity observed in olive leaf-containing formulations may be attributed to differences in phenolic composition between substrates. Green tea is rich in catechins, particularly epigallocatechin-3-gallate (EGCG), which exhibits strong radical scavenging capacity due to its multiple hydroxyl groups and high electron-donating ability [35]. In contrast, olive leaves are characterized by

oleuropein and related secoiridoids as predominant phenolic compounds [24, 28]. Although oleuropein also exhibits antioxidant activity, its molecular structure differs from catechins, which may partially explain the lower antioxidant capacity observed in treatments with higher OL proportions.

The slight increase in ABTS radical scavenging activity after fermentation, especially in the 100-OL treatment, suggests that microbial metabolism promoted the release or biotransformation of phenolic compounds into more active antioxidant forms. This effect may be associated with the enzymatic hydrolysis of oleuropein into hydroxytyrosol, which possesses enhanced antioxidant potential due to its catechol structure and greater hydrogen-donating capacity [11]. In contrast, the limited variation observed for DPPH activity may be related to the distinct reaction mechanisms of the assays [36].

Metabolite Characterization by DI-ESI-MS

Table 4 summarizes the metabolites identified by DI-ESI-MS analysis. In the Control treatment (100% green tea), 31 bioactive compounds were detected, including 9 phenolic compounds, 20 flavonoids, and 2 anthocyanins. Quercetin-3-O-rutinoside (rutin) was exclusively detected in this treatment. Hesperetin-O-rutinoside and luteolin-7-O-rutinoside were detected only in the Control and 50-OL treatments.

Table 4
Identification of compounds present in MLK by the MRM method.

Bioactive Compound	**[M-H]- (*m/z*)**	**Fragmentation (*m/z*)**	**Control**	**50-OL**	**100-OL**
Phenolic Compounds					
p-Hydroxybenzoic acid	137	93	D	D	D
Syringic acid	197	182	D	D	D
Ferulic acid	193	134	D	D	D
Protocatechuic acid glucoside	315	153	D	D	D
Chlorogenic acid	353	135	D	D	D
Chlorogenic acid	353.4	191	D	D	D
Salvianolic Acid A	493	295	D	D	D
3-O- and 5-O-caffeoylquinic acid	353.2	191	D	D	D
Rosmarinic acid	359	161	D	D	D
Flavonoids					
Catechin	289	245	D	D	D
Eriodictyol-O-hexoside	499	287	D	D	D
Isorhamnetin	315	300	D	D	D
Myricetin	317	151	D	D	D
Kaempferol	258	161	D	D	D
Kaempferol	285	145	D	D	D
Apigenin	269	107	D	D	D
Myricetin-O-glucoside	479	317	D	D	D
Naringenin	217	151	D	D	D
Luteolin	285	151	D	D	D
Luteolin	285	259	D	D	D
5,7,3',4'-Flavan-3-OL (Quercetin)	301	151	D	D	D
5,7,3',4'-Flavan-3-OL (Quercetin)	301	299	D	D	D
Hesperetin	301	286	D	D	D

MRM Multiple reaction monitoring, ND: Not Detected, D: detected

Bioactive Compound	[M-H]- (*m/z*)	Fragmentation (*m/z*)	Control	50-OL	100-OL
Luteolin-7-O-Glucuronide	461	285	D	D	D
Kaempferol-hexoside	447	285	D	D	D
Kaempferol-3-Malonilhexoside	533	285	D	D	D
Hesperetin-O-Rutinoside	609	301	D	D	ND
Luteolin-O-Diglucuronide	637	285	ND	ND	D
Quercetin-3-O-Rutinoside (Rutin)	609	300	D	ND	ND
Luteolin-7-O-Rutinoside	593	285	D	D	ND
Anthocyanins					
Cyanidine-3-O-arabinoside	418	287	D	D	D
Cyanidine-3-O-Glucoside	448	287	D	D	D
MRM Multiple reaction monitoring, ND: Not Detected, D: detected					

In the 50-OL treatment, 30 compounds were identified, comprising 9 phenolic compounds, 19 flavonoids, and 2 anthocyanins. Luteolin-O-diglucuronide and quercetin-3-O-rutinoside (rutin) were not detected under these experimental conditions.

The 100-OL treatment resulted in the detection of 29 compounds, including 9 phenolic compounds, 18 flavonoids, and 2 anthocyanins. Luteolin-O-diglucuronide was exclusively detected in this treatment, whereas hesperetin-O-rutinoside and luteolin-7-O-rutinoside were not detected.

The MRM analysis demonstrated that the fermentation process preserved a broad spectrum of phenolic compounds and flavonoids across all treatments. Most metabolites detected in the fermented green tea (Control) were also identified in samples containing olive leaves. Differences among treatments were expected, since green tea and olive leaves are chemically distinct plant matrices with inherently different phenolic and flavonoid compositions [23, 24, 28].

The exclusive detection of luteolin-O-diglucuronide in the 100-OL treatment suggests a specific contribution of olive leaves to the metabolite profile of the fermented beverage. Conversely, the absence of certain flavonoids in the 100-OL treatment may reflect their lower natural occurrence in olive leaves and/or substrate-dependent biotransformation during fermentation. Luteolin-O-diglucuronide has been associated with antioxidant, anti-inflammatory, antibacterial, antitumor, and analgesic activities, as well as with the therapeutic potential of polyphenols in the management of metabolic disorders [37, 38].

In addition, quercetin-3-O-rutinoside (rutin), exclusively identified in the Control treatment, is a flavonoid widely recognized for its antioxidant and anti-inflammatory properties, including its potential role in

reducing insulin resistance and improving hyperglycemia [5]. Overall, the metabolites identified in the fermented beverages have been associated with important biological activities, such as antimicrobial, antioxidant, anti-inflammatory, and anticarcinogenic effects [29, 34].

Overall, the metabolite profile obtained by DI-ESI-MS demonstrated that replacing green tea with olive leaves significantly influenced the diversity and occurrence of phenolic compounds and flavonoids during SCOBY fermentation. While green tea-based formulations exhibited a greater diversity of flavonoids, olive leaf incorporation contributed specific metabolites associated with relevant biological activities. These findings reinforce the potential of SCOBY fermentation as a promising strategy for modulating the phenolic composition of plant-based beverages and highlight olive leaves as a sustainable alternative substrate for the development of functional fermented products with potential industrial applications.

Color

Table 5 shows the color values of the treatments at the end of 14 days of fermentation. For luminosity, there was no difference between the formulations ($P > 0.05$). Control presented the lightest color among the samples. There was no significant difference between the formulations for the red/green axis. All samples presented similar values of green indicating comparable colors in this aspect. These color variations reflect both the different leaves for tea and the proportion of olive leaves used, as well as the transformations that occur throughout the fermentation process. This analysis is relevant for evaluating whether color may influence potential industrial applications of the fermented products.

Table 5
Color of green tea and olive leaves over 14 days of fermentation.

Color (14 Days)			
	L*	**a***	**b***
Control	75.59 ± 1.39^{A}	-082 ± 0.61	3.93 ± 0.29^{B}
50-OL	71.92 ± 1.19^{B}	-0.95 ± 0.09	4.36 ± 0.33^{AB}
100-OL	69.81 ± 1.82^{C}	-1.10 ± 0.11	4.91 ± 0.95^{A}

The results were expressed as mean ± standard deviation. Means with different capital letters differ between columns ($P < 0.05$). Control: 100% green tea; 50-OL: 50% green tea and 50% olive leaves; 100-OL: 100% olive leaves.

Microbiology analysis

Figure 2 shows that treatments containing higher proportions of olive leaves exhibited a reduction in yeast and acetic acid bacteria populations after fermentation, particularly in the 100-OL samples. This

behavior may be associated with the presence of oleuropein, the main phenolic compound in olive leaves, which has been reported to exert antimicrobial activity against a wide range of microorganisms [24]. In addition, the progressive decrease in pH observed during fermentation, resulting from organic acid production, may have further influenced microbial dynamics by creating a more selective environment [39]. The reduction in microbial populations may also be related to other bioactive compounds present in olive leaves, such as flavonoids, including luteolin derivatives, which have demonstrated antibacterial activity against several microorganisms.

The microbiological results are consistent with the metabolomic profile identified by DI-ESI-MS, suggesting that olive leaf phenolics not only modulated microbial growth but also influenced metabolic pathways during fermentation. Treatments containing higher proportions of olive leaves, particularly 100-OL, showed lower microbial counts together with the detection of olive-derived metabolites such as luteolin-O-diglucuronide and increased oleuropein-related compounds. These findings suggest that the antimicrobial properties of olive phenolics may have exerted selective pressure on the SCOBY consortium, altering enzymatic activity and substrate biotransformation pathways.

Microbial enzymes produced during fermentation, including β-glucosidases and esterases, are known to catalyze hydrolysis and deglycosylation reactions, promoting the release of phenolic compounds from plant matrices. In olive leaf-rich treatments, these reactions may have contributed to the transformation of oleuropein and flavonoid glycosides into lower-molecular-weight metabolites with enhanced bioactivity, such as hydroxytyrosol derivatives and luteolin-related compounds. Therefore, even under reduced microbial growth, the fermentation system remained metabolically active and capable of generating bioactive metabolites through selective microbial biotransformation.

The lower microbial populations observed in the 100-OL treatment may also explain the reduced diversity of flavonoids detected in this formulation compared with the Control and 50-OL treatments. Since microbial metabolism directly affects phenolic release, oxidation, and structural modification, reduced yeast and acetic acid bacteria activity may have limited metabolite diversification and substrate turnover. In contrast, the 50-OL treatment maintained microbial populations closer to the Control while still providing olive-derived phenolic substrates, which may explain the higher diversity of detected metabolites and the maintenance of antioxidant activity. This behavior suggests that partial replacement created a more balanced fermentation environment, supporting both microbial activity and phenolic biotransformation.

Despite this selective pressure, fermentation still promoted the release and transformation of phenolic compounds, as reflected by the increase in total phenolic content and the maintenance or slight improvement in antioxidant activity. These findings suggest that, although olive leaves may modulate microbial growth, the fermentation process remains effective in generating bioactive compounds with potential functional properties.

Overall, the integration of microbiological and metabolomic data indicates that olive leaf incorporation modulated the metabolic flux of the SCOBY consortium, influencing both microbial adaptation and the

formation of specific phenolic metabolites. This interaction between microbial dynamics and substrate composition appears to play a central role in determining the final bioactive profile of the fermented products.

However, the absence of microbial community sequencing limits a deeper understanding of substrate-dependent microbial shifts during fermentation. Future studies employing metagenomic or amplicon-based approaches could provide further insights into the interactions between microbial dynamics and metabolite production.

4. CONCLUSION

This study demonstrated that the fermentation of olive leaf biomass is a viable strategy for the production of high-value bioactive metabolites, reinforcing its potential within a circular economy framework. The substitution of green tea with olive leaves significantly influenced fermentation dynamics, microbial behavior, and metabolite composition. Partial substitution (50-OL) maintained fermentation performance, including pH reduction, microbial activity and antioxidant capacity while enhancing the diversity of bioactive compounds. In contrast, total replacement (100-OL) affected microbial growth and SCOBY formation, likely due to the presence of phenolic compounds such as oleuropein. Despite these limitations, fermentation promoted the release and transformation of phenolic compounds with antioxidant and antimicrobial activities. From an application perspective, partial substitution represents a balance between process efficiency and metabolite production, highlighting olive leaves as a promising and sustainable substrate for industrial applications.

As a pilot study, these findings provide an initial understanding of the effects of olive leaf incorporation on SCOBY fermentation and metabolite formation. However, further studies are necessary to achieve a more comprehensive characterization of the fermentation process, including the identification and quantification of organic acids such as acetic, gluconic, and glucuronic acids, as well as ethanol production and sugar metabolism. In addition, microbial community characterization using culture-independent approaches, such as metagenomic or amplicon sequencing, would provide deeper insights into substrate-dependent microbial adaptation and its relationship with metabolite production. Future studies integrating metabolomics, microbiome analysis, and fermentation kinetics may contribute to optimizing olive leaf-based fermentations and expanding their potential applications in functional foods, nutraceuticals, and sustainable bioprocesses.

Declarations

Statement of contribution by CrediT

Jessyca Caroline Rocha Ribas contributed to conceptualization, investigation, methodology, formal analysis, data curation, and writing of the original draft; Jaqueline Ferreira Silva contributed to investigation, methodology, formal analysis, review and editing of the manuscript; Évelin Lemos de Oliveira contributed to investigation; Danieli Suzan Valerio contributed to investigation; Eduardo Cesar

Meurer contributed to investigation and resources; Hélène Tormo contributed to conceptualization; Paula Toshimi Matumoto Pintro contributed to conceptualization, as well as review, resources, supervision and acquisition of financing.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Ethics Approval and Consent to Participate

Not applicable

Funding Declaration

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Figures

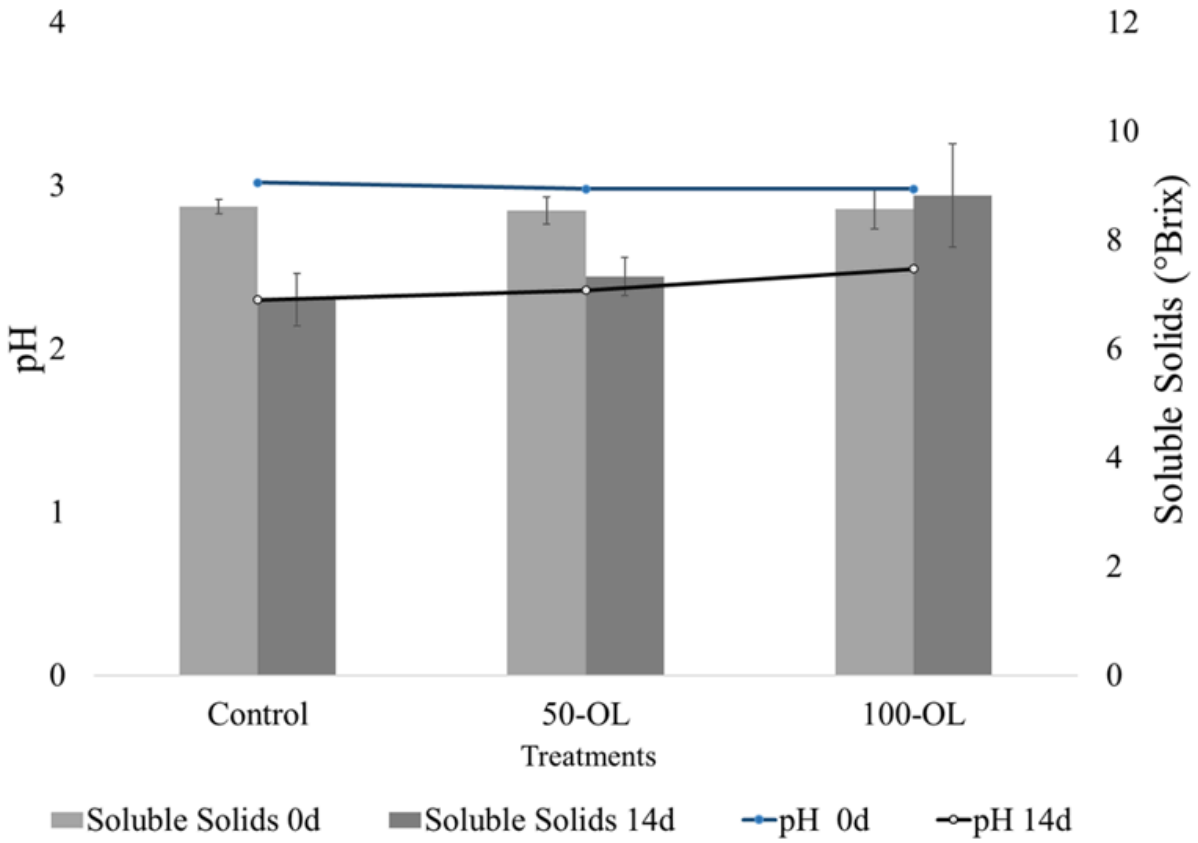


Figure 1

pH and soluble solids of green tea and olive leaves over 14 days of fermentation. *Control: 100% green tea; 50-OL: 50% green tea and 50% olive leaves; 100-OL: 100% olive leaves.

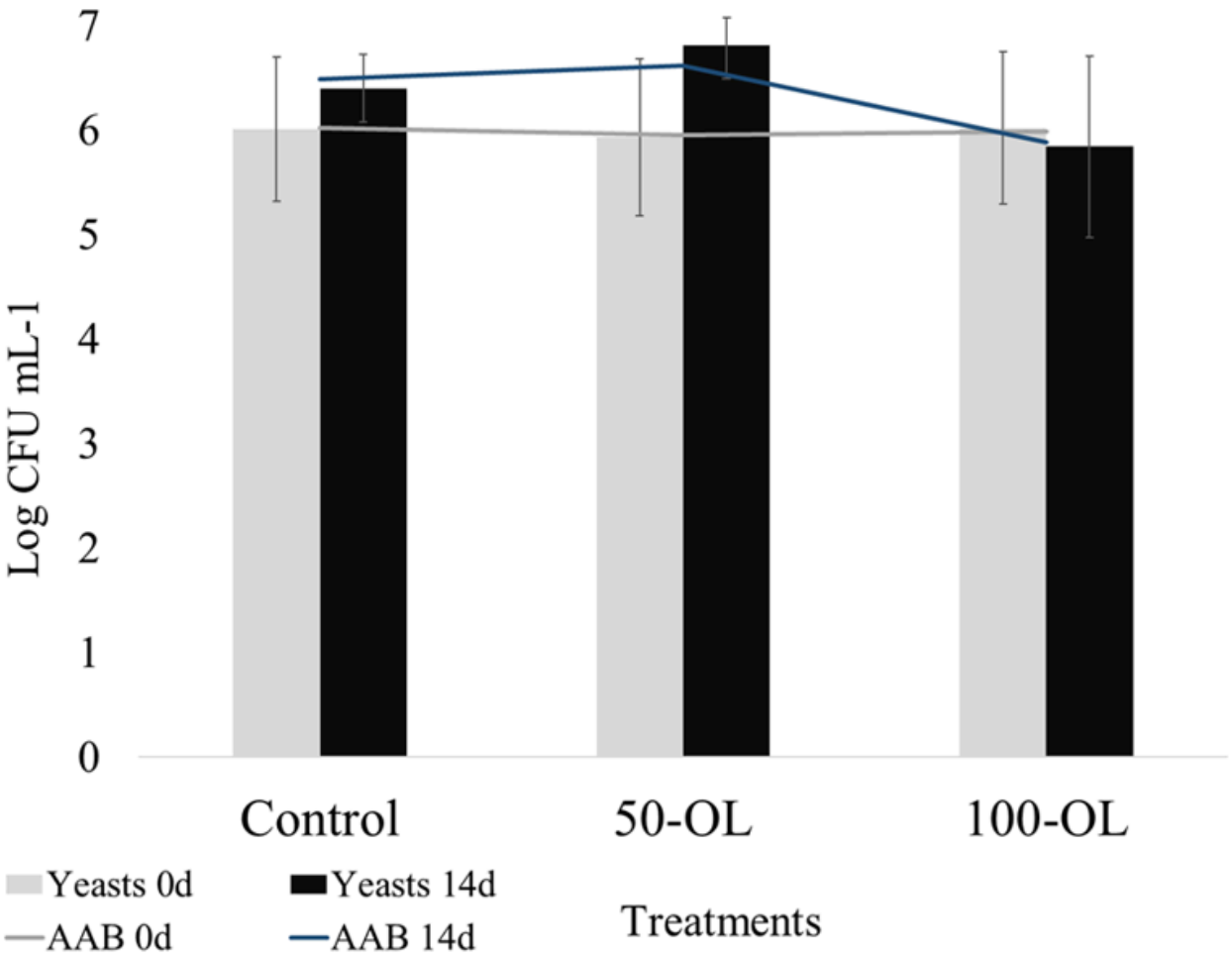


Figure 2

Yeast and acetic bacteria count in green tea and olive leaves over 14 days of fermentation expressed in log CFU mL-1 (Control: 100% green tea; 50-OL: 50% green tea and 50% olive leaves; 100-OL: 100% olive leaves)

Supplementary Files

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- GA.png