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Research Article

Citrus leaf essential oils: chemical composition, antioxidant activity, and selective antibacterial effects on foodborne and starter culture bacteria during fermentation

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Abstract
Natural preservation of fermented foods is increasingly important due to consumer demand for safe, additive-free products. This study evaluated lemon (*Citrus limon*) and mandarin (*Citrus reticulata*) leaf essential oils as natural preservatives for fermented foods. Chemical analysis using GC/MS showed limonene as the major compound in lemon leaf essential oil (57.96%) and dimethyl anthranilate in mandarin leaf essential oil (54.97%). Antioxidant activity was measured via DPPH and ABTS assays, confirming significant radical scavenging capacity. Antibacterial activity was assessed against food spoilage bacteria using disc diffusion and MIC determination. The smallest inhibition zone was observed for *Escherichia coli* ATCC 23922 (6.33 ± 0.76 mm) and the largest for *Bacillus cereus* ATCC 11798 (19.83 ± 0.28 mm), with MICs inversely related to inhibition diameters. Importantly, the impact on fermentation parameters was also investigated. For *Streptococcus thermophilus* LMS8, lactic acid production reached 8.1 $g.L^{-1}$ in control cultures and slightly decreased to 7.5 $g.L^{-1}$ with 50 µL of essential oils, while growth and sugar utilization remained comparable. *Lactiplantibacillus plantarum* LMF6 exhibited unaffected growth and acidification capacity, with lactic acid levels similar to controls. Citrus leaf essential oils selectively inhibit harmful bacteria while preserving normal fermentation, making them promising natural bio-preservatives for fermented foods.

Keywords
citrus essential oils, fermented food, starter cultures, spoilage bacteria, fermentation parameters, antioxidant activity, natural preservatives

Abbreviations
MLEO – Mandarin (Citrus reticulata) Leaf Essential Oil, LLEO – Lemon (Citrus limon) Leaf Essential Oil, GC/MS – Gas Chromatography / Mass Spectrometry, DPPH – 2,2-diphenyl-1-picrylhydrazyl, ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), MIC – Minimum Inhibitory Concentration, ATCC – American Type Culture Collection

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Introduction

The increasing global demand for functional foods has been a driving force behind innovations aimed at improving food quality and sustainability. Foods classified as functional, which provide additional health benefits beyond fundamental nutrition, are increasingly vital in enhancing public health, by lowering the chances of chronic diseases like heart conditions and diabetes (Hasler 2002). One of the key approaches in this field is the valorization of agricultural by-products, especially plant-based materials, for the production of value-added functional ingredients. Citrus leaves, particularly lemon leaves, represent a promising but underutilized resource in this regard, with potential applications in the fermented food (dairy industry), owing to their functional molecules (Li et al. 2022). Citrus leaves, rich in essential oils, have demonstrated significant antioxidant, antimicrobial, and antifungal properties (Bhavaniramya et al. 2019). Such properties make lemon leaves an appealing alternative for application as a natural preservative in dairy goods, replacing synthetic preservatives that are associated with potential health risks and environmental concerns (Chouliara et al. 2007). With consumer preferences shifting towards natural and sustainable ingredients, the use of lemon leaves in the dairy industry aligns well with current market demandrs for cleaner labels and minimally processed foods.

The dairy industry, which represents a significant portion of global food production, is under continuous pressure to enhance the functional properties of its products while ensuring safety and extended shelf life. Traditionally, synthetic additives like sodium benzoate and sorbates have been used to preserve dairy products, but these have been criticized for their long-term toxicological effects, including carcinogenesis and negative environmental impacts (Basak & Guha 2018). In contrast, natural preservatives derived from agro-resources, such as Citrus leaves, offer a safer and more sustainable alternative (Rout et al. 2022).

Furthermore, the integration of agro-resources like lemon leaves into dairy products also promotes circular economy principles. The circular economy seeks to minimize waste by valorizing by-products and enhancing resource efficiency (Rajković et al. 2020). Using lemon and mandarin leaves in dairy production is an innovative way to recycle agricultural by-products, reducing waste and adding economic value to the citrus industry. This approach not only supports sustainability but also opens new economic opportunities for farmers and food producers, fostering a more resilient and eco-friendly food system.

By leveraging the antimicrobial and antioxidant properties of lemon leaves, fermented food products can benefit from extended shelf life, reduced spoilage, and enhanced nutritional profiles. Moreover, this natural preservation method aligns with sustainable food practices and contributes to reducing the ecological footprint of food manufacturing (Ziarno et al. 2021).

This research focuses on evaluating the potential of Citrus (*Citrus limon* and *Citrus reticulata*) leaves as a natural preservative in fermented food products, focusing on their antioxidant activity, their effects on starter cultures and their effectiveness in hindering the proliferation of spoilage bacteria. The outcomes will contribute to the development of functional fermented food products that meet consumer demands for both quality and sustainability.

Materials and Methods

Plant material and essential oil extraction. The Citrus (*Citrus limon* and *Citrus reticulata*) leaves were meticulously cleansed and subsequently sectioned for a 3-hour steam distillation process. Following filtration, the solvent was eliminated using vacuum distillation via a rotary evaporator. The resulting pure essential oil was preserved under refrigeration at 4°C in a dark environment, prior to initiating the essential oil analysis. The yield of extracted oil from each plant sample was quantified using the formula:

$$\text{Oil (\%v/w)} = \text{observed volume of oil (ml)} / \text{weight of sample (g)} \times 100 \quad (1)$$

Gas Chromatography-Mass Spectrometry Analysis of essential oils. A gas chromatograph (Hewlett–Packard co, model 6890) coupled to a mass spectrophotometer (Hewlett–Packard co, model 5973, Palo Alto, CA, USA) with an HP-5Ms capillary column (30.0m×0.25mm×0.25 μm) was used for gas chromatographic analysis of essential

oils. The carrier gas was helium and was used at a flow rate of 1.2 mL.min^{-1}. The initial temperature was 60°C for 8 min and was then increased to 200°C at a rate of 2°C.min^{-1}. The chromatograph was equipped with a split/splitless injector, which was used in split mode, and 2 µL of essential oil was always injected. The essential oil compounds were identified based on the GC-MS retention times, their Kovats indices (calculated using a homologous series of C10-C22 n-alkanes injected under the same conditions) and the mass spectra (authentic chemicals and NIST 05 spectral collection).

Antioxidant Activity

DPPH free radical scavenging activity. The DPPH radical inhibition activity of essential oils was evaluated using the method previously described by Cheung et al. (2003) with modification. The proportion of DPPH free radical inhibition (DPPH %) was determined using the following equation:

$$\text{DPPH}\ (\%) = 100 \times \frac{\text{A blank} - \text{A sample}}{\text{A blank}} \qquad (2)$$

In this procedure, A blank refers to the absorbance of the control reaction, which includes all reagents except the compound being tested, while A sample corresponds to the absorbance of the tested compound itself. The antiradical activity was quantified in terms of IC50 (the concentration required to inhibit 50% of radicals) and ascorbic acid equivalent values (mg AEs.g^{-1} sample). The analysis was carried out in triplicate, with ascorbic acid serving as the positive control.

ABTS radical scavenging potential. The ABTS radical scavenging potential of the essential oil was assessed following the method outlined by Ziarno et al. (2021), with minor adjustments. To prepare the ABTS•+ solution, an aqueous ABTS solution (7 mmol.L^{-1}) was mixed with potassium persulfate (2.45 mmol.L^{-1}), and the resulting solution was kept in the dark at ambient temperature for 12–16 h. Prior to the assay, the ABTS•+ solution was diluted with phosphate-buffered saline (0.01 mol.L^{-1}, pH 7.4) to achieve an absorbance value of 0.7 ± 0.05 at 734 nm. Subsequently, 50 µL of the essential oil or a vitamin C solution was added to 950 µL of the ABTS•+ working solution. After incubation at room temperature in the absence of light, the absorbance at 734 nm was measured precisely 6 min post-incubation. Calibration was conducted using vitamin C solutions with concentrations spanning from 0 to 15 µM.

Antibacterial efficacy of the essential oils against food spoilage pathogens

Microorganisms and growth conditions. The food spoilage bacteria strains used in this experiment are reference strains according to the international standard, the American Type Culture Collection (ATCC): Gram-négative bacteria: *Pseudomonas aerugonosa* ATCC 27453, *Escherichia coli* ATCC 23922; Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 11798. The bacteria to be tested are inoculated on Petri dishes inoculated with Mueller Hinton agar, bacterial cultures were incubated for 24 h to produce a fresh bacterial culture and isolated colonies. From these plates, a few well-isolated and morphologically identical colonies were selected using a platinum loop and transferred into 5 mL of sterile physiological saline solution containing 0.9% NaCl. The bacterial suspension was thoroughly homogenized, and the optical density was measured at 625 nm. The turbidity was then adjusted to the 0.5 McFarland standard, corresponding to an optical density of 0.08–0.1 and approximately 1–2 × 10^{8} CFU. mL^{-1}. The sensitivity of the tested bacteria to the two essential oils (mandarin and lemon) was subsequently assessed using various methods.

Disc diffusion assay. The antibacterial efficacy of essential oils extracted from mandarin and lemon leaves was evaluated using the disc diffusion assay (with modifications) outlined by Goni et al. (2009). For bacterial evaluation, a 1 ml microbial suspension containing approximately 1 × 10^{7} CFU.mL^{-1} was spread evenly on MHA medium. Dimethyl sulfoxide (DMSO) served as a negative control, while (Gentamicin® 30 µg) served as a positive control in similar experiments. A paper disc measuring 6 mm in diameter, infused with 5 µL of the sample, was positioned on the surface of the agar plates and incubated at 37°C for 24 h. After incubation, the regions of suppressed bacterial growth were examined, and the diameter of each inhibition zone was recorded in millimeters.

Assessment of the minimum inhibitory concentration. The minimum inhibitory concentration (MIC) was determined using an adjusted version of the method outlined by Zhao et al. (2020). The sample was diluted in a 0.5% (v/v) Tween 80 solution, and a series of concentrations ranging from 600 to 10 µg.mL^{-1} (including 300, 100, 50, and 25 µg.mL^{-1}) was prepared by direct dilution from the stock solution. A 180 µL aliquot of the diluted solution was then combined with 20 µL of a microbial suspension (approximately 10^7 CFU. mL^{-1}), was added to 96-well plates and then incubated at 37°C for 24 h. Growth controls (medium with inoculum, without essential oil) and sterility controls (medium without inoculum) were included in each assay. The MIC was defined as the lowest concentration of essential oil at which no visible bacterial growth (turbidity) was observed, compared with the growth control. To confirm results, optical density was measured at 625 nm using a microplate reader. All tests were performed in triplicate.

Effect of essential oils against starter culture. The starter culture used in this study consisted of two lactic acid bacteria: *Lactiplantibacillus plantarum* LMF6 and *Streptococcus thermophilus* LMS8, sourced from the collection of the Laboratory of Biology of Microorganisms and Biotechnology (LBMB) (Baghdad Belhadj et al. 2019; Zheng et al. 2020).

To establish the appropriate concentration of essential oils derived from lemon and mandarin leaves, preliminary trials were conducted using two selected doses (50 µL and 250 µL), based on their previously determined antioxidant capacity and MIC values.

These initial assays were carried out using MRS broth for *Lpb. plantarum* LMF6 and M17 broth for *S. thermophilus* LMS8. For each essential oil concentration and bacterial strain, key fermentation parameters; namely pH, Dornic acidity, optical density (OD), and viable cell counts; were measured at five time points to assess microbial growth and metabolic activity.

Following these preliminary evaluations, the 50 µL dose was selected for further experimentation, as it provided a balance between bioactivity and microbial viability. Subsequent assays were conducted to evaluate the effects of essential oils on starter cultures comprising *Lpb. plantarum* LMF6 and *S. thermophilus* LMS8. Parallel fermentations were performed in M17 broth, with and without the addition of 50 µL of essential oil. Fermentation kinetics and biochemical responses were monitored over time, including pH variation, titratable acidity (Dornic), optical density, microbial enumeration, and residual sugar concentration, in order to characterize the modulatory effects of essential oils on the functionality and dynamics of the starter cultures.

pH measurement. pH measurements were recorded every 2 h over a 24-hour incubation period using a pH meter (Hanna Instruments, Romania).

Titratable acidity. Dornic acidity was determined by titrating 10 mL of the culture with 0.1N NaOH in the presence of phenolphthalein (2%, w/v) as a pH indicator. Titration continued until the appearance of a stable pale pink color. The acidity is expressed in Dornic degrees (1°D corresponds to 0.1 g of lactic acid per kilogram of dairy product).

Assessment of total sugars by the Dubois method. The assessment of total sugars was carried out using the method described by Dubois et al. (1956). A volume of 5 mL of sample was combined with 300 mL of distilled water and 3 g of calcium bicarbonate for neutralization. The mixture was heated for 30 minutes while being continuously stirred, cooled and then treated with 1 mL of lead acetate. After dilution to 1000 mL, the solution was filtered to remove precipitated proteins and lead, which was assisted by potassium oxalate. Control samples were prepared with phenol, sulfuric acid and water to calibrate the spectrophotometer, while standard solutions of pentoses and hexoses were employed to develop a calibration curve. Finally, the optical densities of every sample were measured after calibration at a wavelength of 494 nm.

Enumeration of *Lactiplantibacillus plantarum* LMF6 and *Streptococcus thermophilus* LMS8. Bacterial enumeration was standardized by adjusting the initial microbial load to an optical density (OD_{600}) of 0.7. If the load was less than 0.7, a bacterial suspension was added and if not, MRS or M17 broth (depending on the germ to be counted) was added. The calibration of the spectrophotometer was carried out with MRS broth

for *Lpb. plantarum* LMF6 and with M17 broth for *S. thermophilus* LMS8. At each time, the measurement of OD at 600 nm was done.

After optical adjustment, enumeration of each bacterial suspension was performed by the standard plate count method. Serial decimal dilutions were prepared in sterile 0.85% NaCl solution, and 0.1 mL aliquots from appropriate dilutions were spread in duplicate on MRS agar for *Lpb. plantarum* LMF6 and M17 agar for *S. thermophilus* LMS8. Plates were incubated at 37°C for 48 h, and colonies were counted on plates containing 30–300 colonies. Results were expressed as colony-forming units per millilitre (CFU.mL^{-1}) according to ISO 15214:1998, and each enumeration was performed in triplicate.

Determination of kinetic parameters. The kinetic parameters were calculated from the experimental data of cell growth, titratable acidity, and residual sugar concentration during fermentation.

Specific growth rate μ (h^{-1}). The specific growth rate was determined from the slope of the linear portion of the natural logarithm of viable cell concentration (ln CFU.mL^{-1}) versus time curve, according to the equation:

$$\mu = \frac{\ln N_2 - \ln N_1}{t_2 - t_1} \quad (3)$$

where N_1 and N_2 are viable counts (CFU.mL^{-1}) at times t_1 and t_2 (h). The unit of μ is per hour (h^{-1}).

Specific acidity production rate QAc (g. h^{-1}). QAc was calculated as the slope of the titratable acidity curve (expressed as g lactic acid equivalents) versus time during the exponential phase of acidification. The unit of QAc is grams of lactic acid produced per hour (g. h^{-1}).

Specific sugar consumption rate Qs (g. L^{-1}. h^{-1}). Qs was determined from the slope of the residual sugar concentration (g.L^{-1}) versus time curve over the same period, representing the rate of substrate consumption.

Statistical analysis. Statistical analysis was performed using XLSTAT version 2014.5.03. Results were expressed as means ± standard deviation (SD). Statistical significance was established at $p < 0.05$, with significance determined using ANOVA, followed by Tukey's test for multiple comparisons.

Results and Discussion

Chemical composition of the essential oil. The evaluation of both qualitative and quantitative constituents in essential oils is vital due to their antimicrobial properties. The changes in the chemical composition of essential oils can significantly impact their biological activity. The yield of essential oils from mandarin and lemon leaves was 0.28% and 0.49%, respectively. The composition of these essential oils is detailed in Table 1.

In the mandarin leaf essential oil, 22 compounds were identified, accounting for 99.398% of the total volatile components (Table 1). These compounds were grouped into three categories: monoterpenes (43.705%), sesquiterpenes (0.218%), and other compounds, with dimethyl anthranilate being the most abundant (54.973%). Among the monoterpenes, the predominant compounds were γ-terpinene (21.718%), limonene (6.764%), and (Z)-β-Ocimene (6.533%).

These findings are consistent with those of Fahmy et al. (2022), who analyzed mandarin leaf essential oils from six cultivars and identified dimethyl anthranilate as the major component, ranging from 49.06% to 58.77% of the total oil content. Similarly, dimethyl anthranilate was identified as the primary component by Eldahshan (2015) and Luro et al. (2023), representing 65.3% and 49.7–78.8% of the essential oil, respectively, in agreement with the current results. In contrast, Blanco et al. (1995) reported that the predominant constituents in essential oils extracted from Colombian mandarin leaves were linalool (52.66%), limonene (8.32%), and trans-β-ocimene (7.87%).

The variations in essential oil composition underscore the impact of multiple factors, including plant genotype, the stage of fruit ripening, soil characteristics, seasonal and climatic conditions extraction methodologies, and the analytical techniques employed (Bouabdallah et al. 2022).

Table 1. Chemical composition (%) of lemon (LLEO) and mandarin (MLEO) leaves essential oils isolated using GC/MS analysis.

N°	Components	KI[a]	LLEO (%)	MLEO (%)
1	α -Thujene		-	1.236
2	α-Pinene		4.773	2.356
3	Camphene		0.02	2.171
4	β-Pinene		16.29	0.533
5	Myrcene	911	0.405	0.068
6	α -Phellandrene	918	-	0.569
7	3-Carene	965	-	0.047
8	α-Terpinene	976	-	0.308
9	Limonene	981	57.96	6.764
10	trans-β-Ocimene	995	0.03	6.533
11	cis-β-Ocimene	1002	-	0.501
12	γ-terpinene	1017	0.511	21.718
13	trans-sabinene hydrate	1020	0.016	-
14	α -terpinolene	1030	0.054	0.828
15	Linalool	1041	3.07	-
16	Citronellal	1051	1.027	-
17	Terpinen-4-ol	1060	0.086	0.134
18	Anisole	1080	-	0.162
19	(Z)-carveol	1093	0.036	-
20	Neral	1156	6.252	-
21	Cis Citral	1170	0.152	-
22	thymol	1227	-	0.139
23	Carvacrol	1223	-	0.067
24	Neryl acetate	1238	1.478	0.073
25	Geranyl acetate	1248	2.232	-
26	Dimethyl anthranilate	1288	-	54.973
27	(Z)-α-Bergamotene	1292	0.291	-
28	β-Caryophyllene	1361	1.36	-
29	α -Humulene	1381	-	0.100
30	(Z)-β-Farnesene	1401	0.02	-
31	Viridiforene	1410	0.025	-
32	(Z)-α-Bisabolene	1415	0.024	-
33	β-Bisabolene	1452	0.385	-
34	bicyclogermacrene	1481	-	0.070
35	Caryophyllene oxide	1490	0.175	0.048
36	δ-Cadinene	1498	0.019	-
37	α-Bisabolol	1504	0.032	-
		1532		
	Monoterpene hydrocarbons	1578	80.30	41.50
	Oxygenated monoterpenes	1642	10.63	0.34
	Sesquiterpene hydrocarbons	1685	2.13	0.22
	Oxygenated sesquiterpenes		0.21	0.05
	Esters		3.71	55.04
	Aromatic compounds		-	0.16
	Total identified (%)		**96.723**	**99.398**

[a]KI - Kovats Indices on HP-5MS Capillary Column in reference to C10-C22 n-alkanes injected in the same conditions.

The volatile components constituting lemon leaf essential oils are recorded in Table 1. A total of 27 compounds were identified, representing 96.723% of the overall composition. The composition of the lemon leaves essential oils was dominated by monoterpenes (83.06%). Limonene (57.96%) is the

predominant component of monoterpenes, followed by β-pinene (16.29%), and α-pinene (4.77%). Aldehydes are the second group with neral (6.25%) and citronellal (1.02%) as principal components. In addition, tow esters: neryl acetate (1.47 %) and geranyl acetate (2.23%) were found in lemon leaf essential oils. The sesquiterpenes (2.15%) included β-caryophyllene (1.36%), β-bisabolene (0.38%) and (Z)-α-bergamotene (0.29%). The only sesquiterpene oxide detected was caryophyllene oxide (0.17%). The main alcohol detected was linalool (3.07%). Similar findings were reported in a study by Yazgan et al. (2019), which indicated that lemon plant essential oil primarily consists of limonene (52.85%) and β-pinene (13.69%). Wu et al. (2021a) identified citronellal (78.35%), limonene (12.29%), and citronellol (12.18%) as the main components of lemon leaf essential oils.

Additionally, other studies have noted that different lemon leaf essential oils exhibit varying compositions, with limonene, β-pinene, sabinene, and β-ocimene being the predominant volatile compounds (Chi et al. 2019; Mihoubi et al. 2024).

Antioxidant Activity. The antioxidant activity of LLEO and MLEO was evaluated through ABTS and DPPH radical scavenging assays (Table 2). Both LLEO (5.69 ± 0.93 µg. mL^{-1} for DPPH and 3.49 ± 0.75 µg. mL^{-1} for ABTS) and MLEO (4.74 ± 0.15 µg. mL^{-1} for DPPH and 2.99 ± 0.14 µg. mL^{-1} for ABTS) demonstrated significant radical scavenging activity when assessed against the positive controls, BHT and ascorbic acid. Previous studies have established that essential oils from Citrus species, particularly those containing γ-terpinene, α-pinene, β-pinene and limonene, exhibit antioxidant effects (Conforti et al. 2007). β-Pinene has shown strong antioxidant potential in the DPPH assay (Wang et al. 2008), while limonene, a monocyclic monoterpene, has demonstrated noteworthy antioxidant capacity in both ABTS (IC50:603.23 µM) and DPPH (IC50:384.73 µM) assays (Shah and Mehta 2018). Furthermore, according to Ding et al. (2024), compounds such as α-pinene, β-caryophyllene, caryophyllene oxide, nerolidol, α-phellandrene, terpinen-4-ol, α-terpineol and p-cymene also possess antioxidant activity. The antioxidant potential of LLEO and MLEO can be attributed to the presence of these key components (Budiarto et al. 2024).

Table 2. The DPPH and ABTS free radical scavenging capacity of LLEO and MLEO.

	DPPH **IC50 µg. mL^{-1}**	**ABTS** **IC50 µg. mL^{-1}**
Ascorbic Acid	$4.88\pm0.33^{a***}$	$2.76\pm0.1^{a***}$
BHT	$6.39\pm0.45^{c***}$	$4.41\pm0.02^{c***}$
LLEO	$5.69\pm0.05^{b**}$	$3.49\pm0.56^{b**}$
MLEO	$4.75\pm0.58^{a***}$	$2.97\pm0.27^{a***}$

Note: mean ± standard deviation. a,b,c : groups considerably different statistically. NS: Non -Significant, *: P < 0.05; **: P<0.01; ***: P<0.001.

Antimicrobial activity of essential oil against food spoilage bacteria

Disc diffusion assay. The antimicrobial ability of mandarin and lemon leaves essential oil was investigated against several contaminating strains of foods, using the disc diffusion method (Table 3). Gram-positive and Gram-negative bacteria were used for this purpose. All bacterial strains were cultivated on Muller Hinton agar and incubated at 37°C overnight (Wu et al. 2021b).

The results indicated that the essential oils from mandarin and lemon leaves exhibited significantly different antimicrobial activities against the tested microorganisms ($p \leq 0.05$).

It is evident that Gram-positive bacteria were more sensitive to mandarin and lemon leaf essential oils, exhibiting larger inhibition zones ranging from 10.5 ± 0.5 mm for *S. aureus* ATCC 25923 to 19.83 ± 0.28 mm for *B. cereus* ATCC 11798. In contrast, Gram-negative bacteria showed lower sensitivity, with inhibition zones ranging from 6.33 ± 0.76 mm for *E. coli* ATCC 23922 to 11.83 ± 0.28 mm for *P. aeruginosa* ATCC 27453.

Large variations were observed in the diameters of the inhibition zones obtained, ranging from 6.33 ± 0.76 mm to 19.83 ± 0.28 mm. It is also noted that the two essential oils have an antibacterial action on all tested strains, except *E. coli* ATCC 23922. This action significantly varied from one strain to another ($p \leq 0,05$). For all the tested strains, the essential oil of lemon was more effective than that of mandarin ($p \leq 0.05$).

Ben Hsouna et al. (2017) indicated that essential oils exhibit slightly greater effectiveness against Gram-positive bacteria than against Gram-negative bacteria. The reduced susceptibility of Gram-negative bacteria to essential oils is due to the presence of an outer membrane surrounding their cell wall, which impedes the penetration of hydrophobic compounds because of its lipopolysaccharide layer.

Table 3. Diameters of the zones of inhibition of mandarin and lemon leaves essential oils relating to the bacteria tested.

Strains	Inhibition diameter of Gentamicin (mm)	Diameter of inhibition zones (mm)	
		LLEO	MLEO
***Pseudomonas aerugonosa* ATCC 27453**	$20.16 \pm 0.28^{a***}$	$11.83 \pm 0.28^{b***}$	$10.33 \pm 0.57^{b***}$
***Escherichia coli* ATCC 23922**	$23 \pm 0.50^{a***}$	$7 \pm 0.50^{b***}$	$6.33 \pm 0.76^{b***}$
***Staphylococcus aureus* ATCC 25923**	$22.50 \pm 0.80^{a***}$	$14.83 \pm 0.28^{b***}$	$10.50 \pm 0.50^{c***}$
***Bacillus cereus* ATCC 11798**	$21 \pm 0.50^{a***}$	$19.83 \pm 0.28^{a***}$	$12 \pm 0.50^{b***}$

Note: mean ± standard deviation. ^{a,b,c}: groups considerably different statistically. NS: Non -Significant, *: $P < 0.05$; **: $P<0.01$; ***: $P<0.001$.

Determination of MIC. Table 4 presents the MICs of LLEO and MLEO against four different microorganisms after 24 h of incubation. The two strains, *S. aureus* ATCC 25923 and *B. cereus* ATCC 11798, exhibited the highest susceptibility, as their growth was effectively inhibited by lower concentrations of both LLEO and MLEO.

Essential oils are recognized for their antimicrobial properties, with the antibacterial activity of Citrus essential oils largely caused by their chemical composition, particularly the presence of citral. The individual components neral, citral, and geranial have demonstrated antibacterial activity toward both Gram-negative and Gram-positive bacteria. (Valková et al. 2021).

Table 4. Minimum inhibitory concentrations (MIC) expressed as µl. mL^{-1}, of LLEO and MLEO against the tested bacteria determined after 24 h of incubation.

	MIC (µl. ml^{-1})	
Strains	LLEO	MLEO
***Pseudomonas aerugonosa* ATCC 27453**	100	300
Escherichia coli ATCC 23922	ND*	ND*
***Staphylococcus aureus* ATCC 25923**	50	10
***Bacillus cereus* ATCC 11798**	25	50

**ND: Not Determined.*

Additionally, geraniol, which was also identified in our essential oils, has been demonstrated to suppress the growth of various bacterial species (Lira et al. 2020; Song et al. 2020). According to the classification by Ponce et al. (2003), *Bacillus cereus* is highly sensitive to citrus essential oils. This

sensitivity may be attributed to the structural characteristics of Gram-positive bacteria, which tend to exhibit greater susceptibility to essential oils. Although *S. aureus* is also a Gram-positive bacteria, it exhibited a lower sensitivity compared to *B. cereus*. This difference could be due to the clustered arrangement of *S. aureus*, which may hinder the action of essential oil components. Furthermore, staphylococci are known for their antibiotic resistance (Song et al. 2020).

The inhibition zones varied among the strains tested, indicating that antibacterial activity of essential oils is strain-dependent and influenced by both essential oil composition and bacterial traits. It is recognized that different bacterial species display varying degrees of sensitivity to antibacterial agents. Our results indicate antibacterial activity against three of the four strains tested. As noted by Kalemba and Kunicha (2003), the sensitivity of bacteria to essential oils is influenced by both the characteristics of the essential oil and the characteristics of the bacteria themselves. Generally, Gram-positive bacteria are more susceptible to essential oils compared to Gram-negative bacteria. This finding is supported by several studies on the suppressive properties of essential oils (Pool 2001; Cimanga et al. 2002). Additionally, the sensitivity of a bacterial population to essential oils can be affected by various factors, including the concentrations of the antimicrobial agent used, the size of the inoculum, and the resistance or tolerance status of the bacteria (Li et al. 2017).

Antibacterial activity of essential oils against starter culture. The results of the analysis of acidity, pH, optical density, and bacterial counts for the two tested strains, both in the absence of treatment (control) and in the presence of 50 and 250 µL of lemon and mandarin essential oils, are presented in Fig. 1.

From these results, it can be seen that the level of acidity produced by *S. thermophilus* LMS8 varies from 25 ° to 75 °D and for *Lpb. plantarum* LMF6 varies from 27 °D to 76 °D during 5 h of culture. The increase in acidity is linked to a significant bacterial growth of lactic acid bacteria. It appears that LLEO and MLEO have the same effect on both strains. no impact on the growth of *S. thermophilus* LMS8 and a minimal effect on the growth of *Lpb. plantarum* LMF6 in relation to the control.

For acidity, production is practically linear over time in the presence of essential oils, while for the control production stops after 2.5 h of growth. The pH decreases in all crops due to the production of lactic acid. The two essential oils are estimated to have negligible effect on the strains tested. The two doses applied (50 and 250µL) of lemon and mandarin essential oils have practically the same effect.

Effect of adding 50 µL of essential oils of lemon and mandarin essential oils to the fermentation parameters of stater cultures. The results of the parameters of the fermentations of *S. thermophilus* LMS8 and *Lpb. plantarum* LMF8 without (control) and with of 50 µL of lemon and mandarin essential oils are presented in Fig. 2,3 and 4.

For the *Lpb. plantarum* LMF6 strain, the presence or absence of 50µL of the tested essential oils does not affect cell growth or lactic acid production (Fig. 3). Contrary to these results, several authors have found that the addition of certain essential oils retards the proliferation of *Lpb. plantarum* LMF6. Kivanç et al. (1991) observed that at high levels of EO in cumin the growth of *Lpb. plantarum* LMF6 is retarded, on the other hand EO of oregano inhibits the growth of *Lpb. plantarum* LMF6 even at low concentrations.

In another study, Chouliara et al. (2007) reported that oregano essential oil at a concentration of 1% exhibited strong inhibitory effects against lactic acid bacteria, with a reduction exceeding 6 log CFU. In comparison, a concentration of 0.1% resulted in only a 1.7 log CFU reduction. Bukvicki et al. (2023), in their comprehensive review, reported that essential oils and plant extracts in general exert inhibitory effects on lactic acid bacteria. Conversely, Nes and Skjelkvale (1982) demonstrated that coriander extract actually stimulated the growth of *Lactobacillus plantarum.*

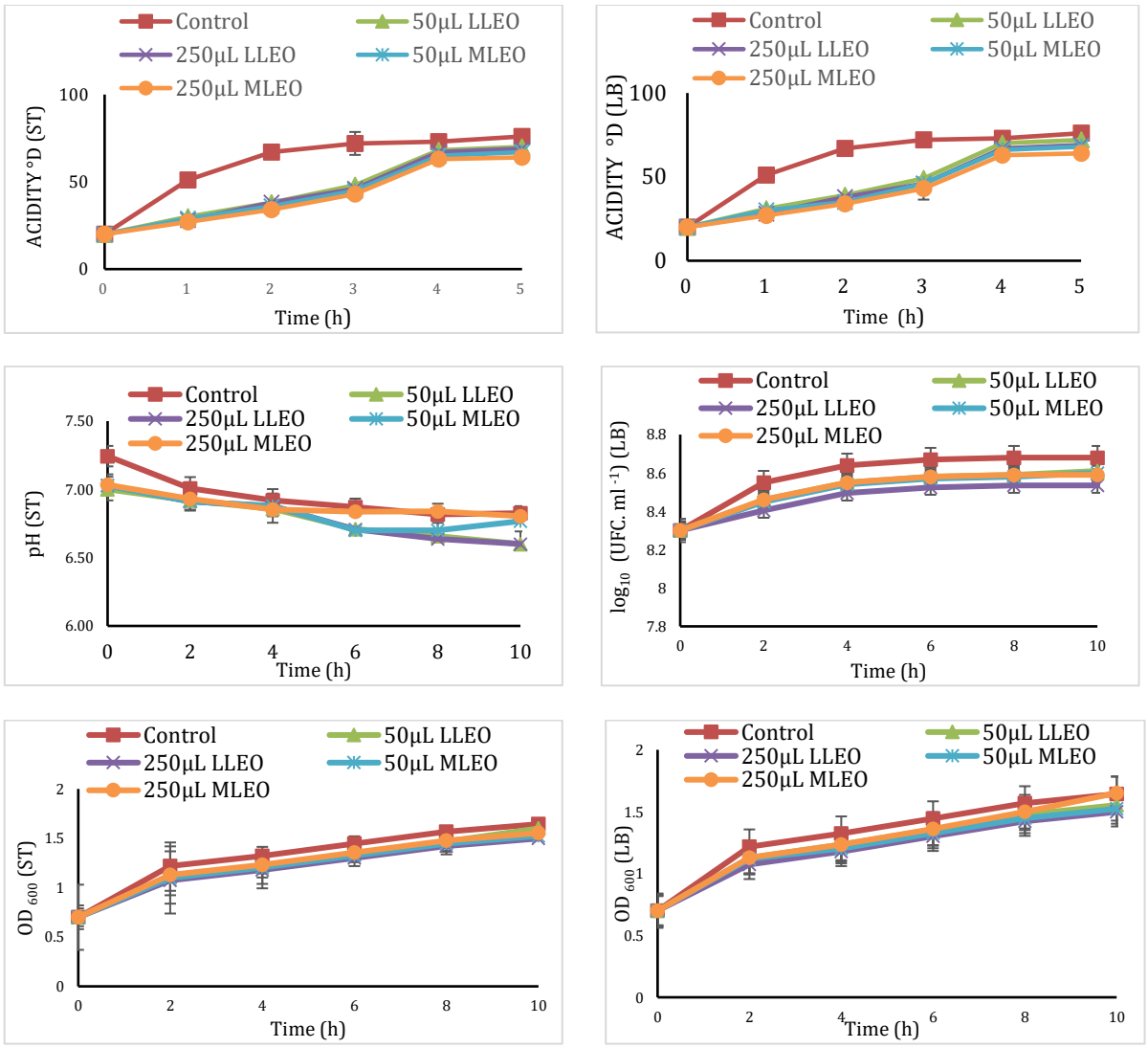


Figure 1. Culture parameters (pH, titratable acidity (1°D= 0.1g.L^{-1}), optical density (OD_{600}), and viable cell counts (log_{10} (CFU. mL^{-1})) of *Streptococcus thermophilus* LMS8 (ST) and *Lactiplantibacillu*

Conversely, Nes and Skjelkvale (1982) demonstrated that coriander extract actually stimulated the growth of *Lactobacillus plantarum*, highlighting that the effects of plant-derived compounds on LAB can be stimulatory, inhibitory, or neutral depending on the plant and context. Garcia-Ruiz et al. (2012) found that the phenolic extract of thyme and Eucalyptus exert an inhibitory effect on *Lactobacillus casei* by recording IC50s of the order of 2.92 g. L^{-1} and 0.24 g. L^{-1}, respectively. Zaika et al. (1983) recorded a reduction in lactic acid bacteria in pure culture of the order of 4 logs CFU after addition of essential oil of Oregano at a rate of 4 g. L^{-1}. In a work carried out by Hayouni et al. (2008), *Lactobacillus delbrueckii* proved to be the most sensitive, showing total inhibition after 3 days of contact with 25, 5 and 2.5µl essential oil of Melaleuca armillaris, followed by *Lactobacillus casei*, which showed no growth after 72 h of contact with 25µl essential oil of the same plant tested. Nair et al. (2015) found that the use of 1% carvacrol (compound of essential oils) exerted an inhibition exceeding 3 log CFU after 14 days of contact and that this inhibition is dose dependent. The essential oils of cinnamon, cardamon and peppermint, studied by Hadad Khodaparast et al. (1999), reduced the growth of lactic acid bacteria in flavored yogurt, while oregano essential oils and its

compounds inhibited *Lactobacillus plantarum* and *Leuconostoc mesenteroides* in liquid media. Our results indicate that the antibacterial effects of LLEO and MLEO on *Lpb. plantarum* LMF6 and *S. thermophilus* LMS8 were significantly lower compared to their effects on spoilage bacteria. This aligns with previous studies, which have shown that certain Citrus essential oil components exhibit selective antimicrobial activity, with stronger inhibition against pathogenic bacteria (e.g., *E. coli*) and weaker effects on probiotics like *Lactobacillus* (Li et al. 2022; Tang et al. 2023). The selective action of Citrus essential oils on pathogenic and lactic acid bacteria is influenced by their chemical composition (Guo et al. 2018; Song et 2020).

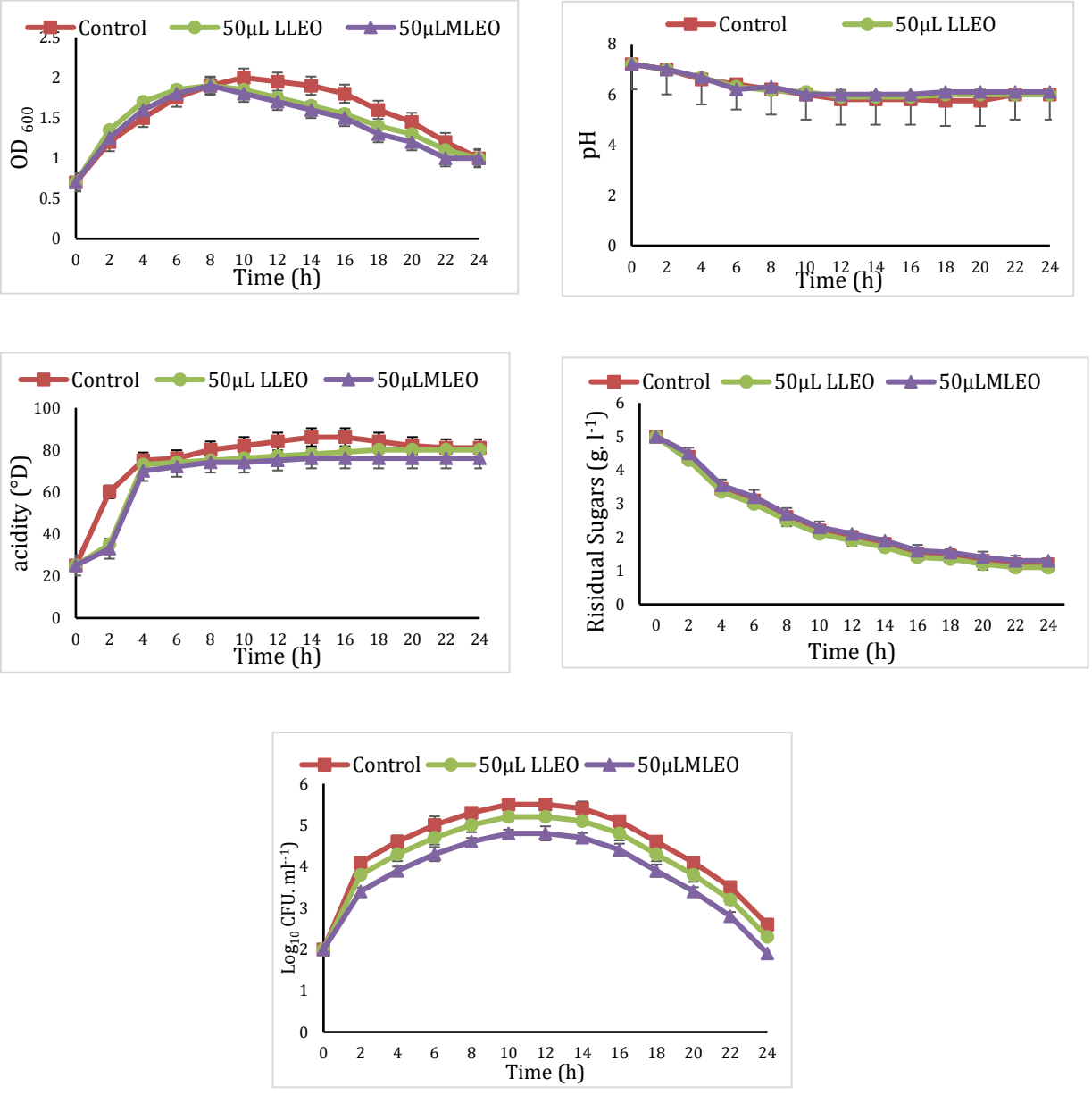


Figure 2. Evolution of culture parameters (pH, titratable acidity (1°D= 0.1g.L^{-1}), optical density (OD_{600}), viable cell counts (log_{10}(CFU. mL^{-1})), and residual sugars (g. L^{-1})) of *S. thermophilus* LMS8 (ST) and *Lpb. plantarum* LMF6 (LB) in the absence and presence of 50 and 250 µL of lemon and mandarin leaf essential oils (LLEO: lemon leaf essential oil; MLEO: mandarin leaf essential oil).

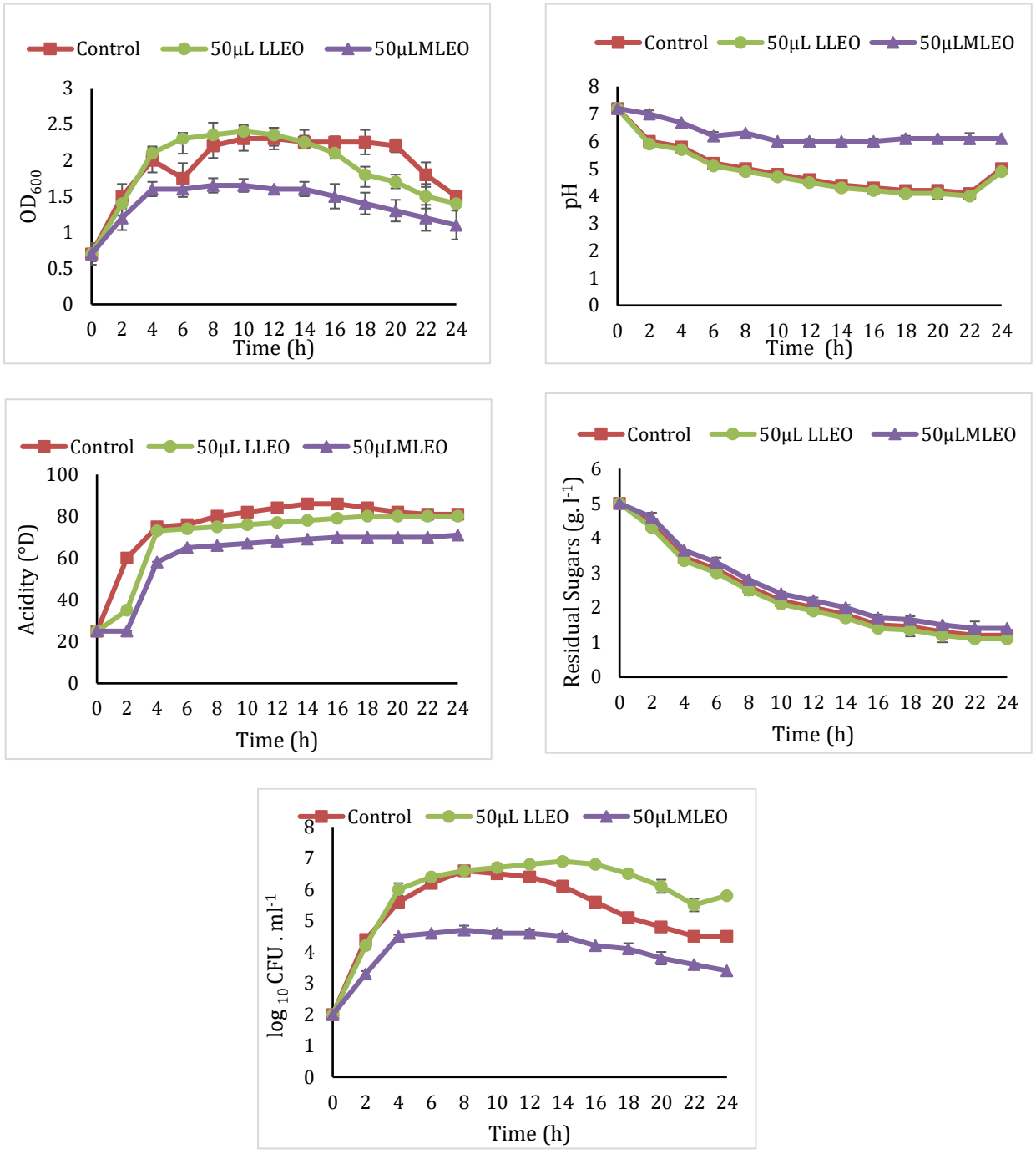


Figure 3. Culture parameter (pH, titratable acidity (1°D = 0.1g.L^{-1}), optical density (OD_{600}), viable cell counts (log_{10}(CFU. mL^{-1})), and residual sugars (g. L^{-1})) of *Lpb. plantarum* LMF8 in the absence (control) and in the presence of 50µL of lemon and mandarin leaves essential oils. (LLEO: lemon leaf essential oil. MLOE: mandari leaf essential oil).

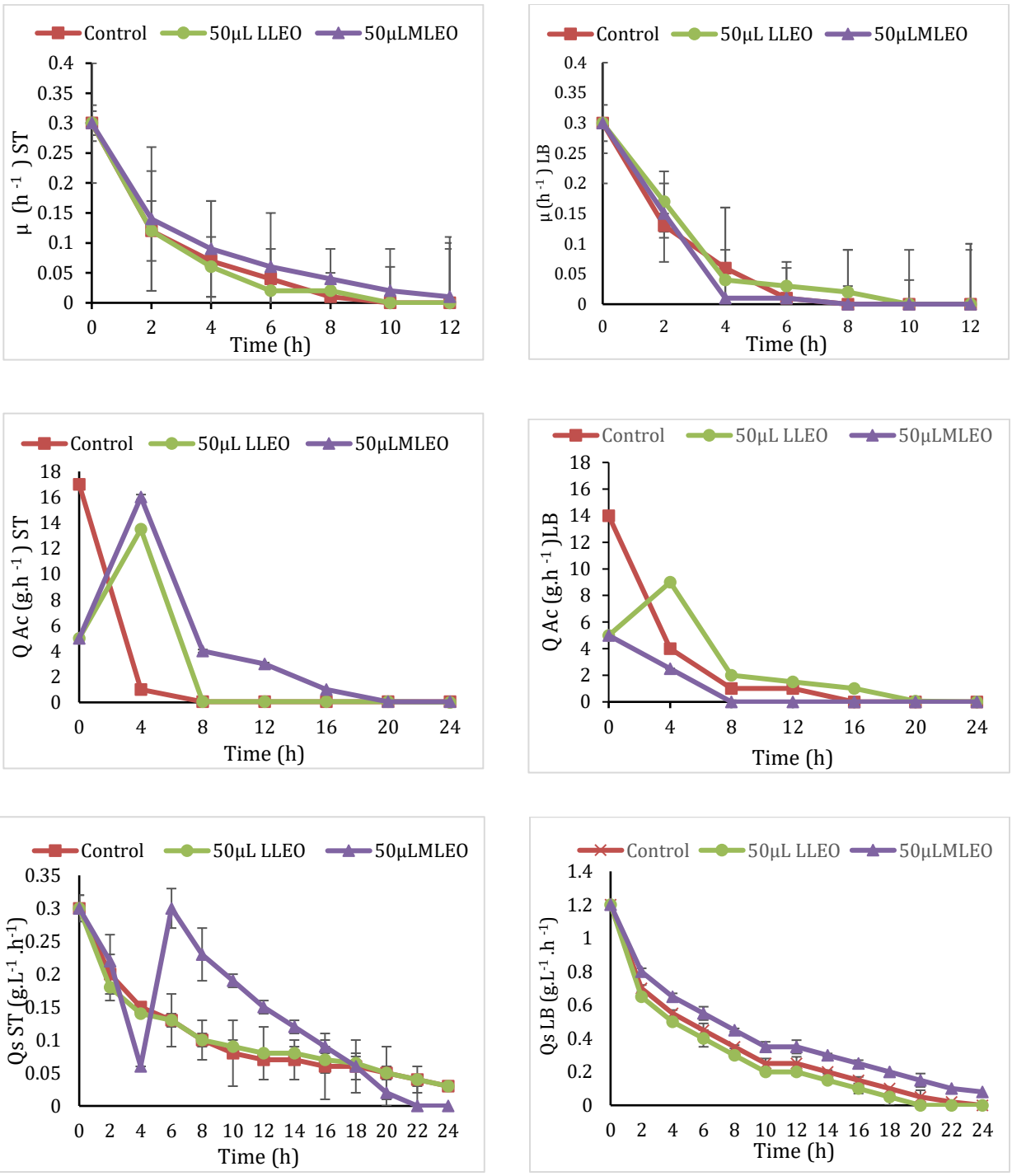


Figure 4. Kinetic parameters (µ: specific growth rate (h^{-1}), QAc: Specific acidity production rate ($g.h^{-1}$), Qs: Specific sugar consumption rate ($g.L^{-1}.h^{-1}$)) of cultures of *Lpb. plantarum* LMF6 and *S. thermophilus* LMS8 in the absence (control) and in the presence of 50 µL of lemon and mandarin leaves essential oil.

The results clearly indicate that the addition of 50µL of lemon and mandarin leaves essential oils exhibited no inhibitory effect on the tested strains.

S. thermophilus LMS8 reaches a final OD of 2 after 12 hours of culture. This growth is reflected in a significant consumption of sugars used for cell development and maintenance, along with lactic acid production (8.1 $g.L^{-1}$ of lactic acid) at the end of fermentation. The incorporation of 50 µL of lemon and mandarin essential oils hardly interferes with cell growth, the final OD being of the order of 1.9 and the final amount of lactic acid obtained being of the order of 7.5 $g. L^{-1}$ of essential oils (Fig. 2).

Conclusions

In conclusion, the incorporation of essential oils derived from citrus leaves, particularly those from lemon and mandarin, into fermented food products presents a promising approach for improving food safety and quality. The bioactive constituents found in these essential oils exhibit strong antimicrobial activity against a range of pathogenic bacteria, encompassing both Gram-positive and Gram-negative strains commonly associated with food spoilage. Notably, the antimicrobial effects of citrus essential oils can suppress the growth of harmful microorganisms without significantly affecting the viability of starter cultures necessary for the fermentation process.

This unique ability to selectively target spoilage bacteria while allowing lactic acid bacteria to thrive is critical for the successful production of fermented food products, including cheese and yogurt. By promoting the growth of beneficial cultures, citrus essential oils serve to both extend the shelf life of fermented food products and enhance their nutritional value and probiotic benefits, meeting consumer demands for healthier, more natural food options.

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Author Contributions

Conceptualization and methodology, M.M.; formal analysis, M.M., F.B. and M.Mh.; resources, L.B.; supervision, L.B.; project administration, M.M.; data analysis, S.C.; writing–original draft preparation, M.M., FB.; writing–review and editing, M.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study did not involve human or animal subjects.

Informed Consent Statement

Not applicable.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflict of interest..

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