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Research article

Biochemical composition, antioxidant, and antimicrobial properties of fermented plant-based beverages from *Ceratonia siliqua* (Carob) supplemented with *Thymus capitatus*

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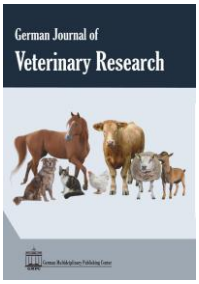

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Abstract

The growing interest in functional beverages has led to the exploration of plant-based formulations enriched with bioactive compounds. This study investigates the biochemical, antioxidant, antimicrobial, and volatile profile modifications resulting from the lactic fermentation of *Ceratonia siliqua* (carob) combined with the aromatic plant *Thymus capitatus* using two probiotic strains: *Lactobacillus plantarum* (LP) and *Lactobacillus acidophilus* (LA). The fermentation process led to a significant drop in pH (to ~3.5–4.0) and an increase in titratable acidity, with *Lactobacillus acidophilus* showing a more pronounced acidification capacity. Phenolic and flavonoid contents increased markedly, particularly in thyme-enriched samples, reaching up to 200 mg GAE/100 mL. Simultaneously, sugar content decreased, reflecting effective microbial metabolism. Volatile compound analysis via GC-MS revealed major fermentation metabolites (lactic acid, acetic acid, ethanol) and the presence of thymol and carvacrol in thyme-supplemented formulations, contributing to both sensory enhancement and biological activity. Antioxidant assays (DPPH, ABTS, and reducing power) confirmed the enhanced radical-scavenging capacity of fermented beverages, especially those fermented with *Thymus capitatus* and *L. acidophilus*, with IC_{50} values comparable to those of synthetic standards. Antimicrobial activity was observed against both Gram-positive and Gram-negative pathogens, with inhibition zones up to 24 mm and MICs as low as 0.5 mg/mL in thyme-acidophilus samples. These effects are attributed to the synergistic action of organic acids and aromatic terpenes. Overall, the combination of aromatic plants and probiotic fermentation significantly improves the functional, antioxidant, and antimicrobial properties with promising applications in veterinary medicine as natural alternatives to promote gut health, enhance disease resistance, and reduce reliance on synthetic antimicrobials.

Keywords: Antimicrobial properties, Antioxidant activity, Bioactive compounds, Functional beverages, Lactic acid bacteria, Plant-based beverages

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Introduction

The growing consumer interest in health-promoting foods has driven demand for plant-based beverages, as they offer natural bioactive compounds and nutritional benefits. The rising consumer demand for natural, health-promoting foods has driven a surge in research on functional beverages derived from plant materials. These products not only provide basic nutrition but also offer bioactive compounds that exert antioxidant, antimicrobial, and anti-

inflammatory effects, contributing to the prevention of chronic diseases and the enhancement of general well-being (Gadhoumi et al., 2024; Granato et al., 2010). In this context, the lactic fermentation of plant-based substrates is gaining momentum as a sustainable and effective biotechnological approach to enhance the nutritional and functional qualities of raw botanical materials (Gadhoumi et al., 2025).

Fermented foods and beverages have attracted growing scientific and industrial interest in recent years, due to their potential health benefits and their ability to enhance the nutritional value of raw plant materials. Numerous international bodies in the fields of food, nutrition, and health have recognized fermentation as a valuable tool to improve the functional quality of food products (Marco et al., 2017; Tamang et al., 2016). During fermentation, complex biochemical transformations occur, leading to the degradation, modification, or synthesis of various bioactive molecules. These changes significantly affect the organoleptic characteristics, such as aroma and functional properties, of the end product by forming new volatile and non-volatile compounds (Filannino et al., 2018). Fermentation not only enriches the nutritional profile of plant matrices but also enhances their digestibility, reduces anti-nutritional factors such as phytates, trypsin inhibitors, and tannins, and improves the bioavailability of micronutrients (Poutanen et al., 2009; Swain et al., 2014).

Lactic acid bacteria (LAB), particularly, play a central role in vegetable and fruit fermentations. Through their metabolic activity, LAB produce health-promoting compounds, including short-chain organic acids, isoflavones, exopolysaccharides, water-soluble vitamins (such as the B-complex), and menaquinones (vitamin K2), which exert various physiological effects in the human body (Wang et al., 2021). Moreover, the rapid acidification of the food matrix through the conversion of fermentable carbohydrates into lactic acid, along with the synthesis of bacteriocins and hydrogen peroxide, suppresses the growth of spoilage and pathogenic microorganisms, thus enhancing food safety and extending shelf-life (Cuvas-Limon et al., 2021).

Aromatic and medicinal plants represent particularly interesting substrates for fermentation due to their rich composition in phenolic compounds, flavonoids, anthocyanins, carotenoids, and tocopherols, all known for their strong antioxidant and antimicrobial activities (Bourgou et al., 2017). These compounds not only improve the functional attributes of fermented beverages but also support the growth and metabolic performance of LAB by providing fermentable sugars, vitamins, and prebiotic fibers (Filannino et al., 2018; Gadhoumi et al., 2021). The interactions between LAB and plant bioactive compounds enhance probiotic activity and increase the bioconversion of polyphenols into more bioavailable and bioactive derivatives. As such, the fermentation of aromatic and medicinal plants is a promising strategy for developing functional beverages and nutraceutical products. Beyond human health, fermented extracts derived from such systems may also play a valuable role in biological control applications, offering a natural alternative for pathogen suppression in food and agricultural systems.

Incorporating aromatic and medicinal plants into carob-based fermentations can further enhance the beverage's functional potential. Plants such as *Thymus capitatus* were rich sources of essential oils, phenolic acids (e.g., caffeic, rosmarinic, and ferulic acids), and flavonoids with well-documented antioxidant and antimicrobial activities (Burt, 2004; El Omari et al., 2023; Matan et al., 2006). During fermentation, these bioactive molecules may be further transformed into more potent derivatives or released from bound forms, increasing their bioactivity and efficacy (Filannino et al., 2018). Plants are rich sources of vitamins, minerals, dietary fiber, and phytochemicals, including phenolic compounds, which are well documented for their health-promoting properties, such as anti-inflammatory, antioxidant, and antimicrobial activities (Gadhoumi et al., 2025, 2022). As consumers become increasingly aware of the importance of functional foods in disease prevention and overall well-being, plant-based beverages are gaining popularity for their role in supporting a healthy lifestyle and enhancing physiological functions (Gadhoumi et al., 2025).

Ceratonia siliqua L., commonly known as carob, is a Mediterranean leguminous tree whose pulp is rich in sugars, dietary fibers (especially insoluble fibers such as cellulose and hemicellulose), and phenolic compounds such as gallic acid, catechins, and flavonoid glycosides (Bourgou et al., 2017; Ginwala et al., 2019). These constituents contribute to its antioxidant,

anti-inflammatory, and gastroprotective activities. However, the full bioactive potential of carob may be limited by factors such as poor solubility, low bioavailability, or the presence of polymeric phenolics that are poorly physiologically accessible (Gómez et al., 2013).

Lactic acid fermentation, carried out by LAB, particularly *Lactiplantibacillus plantarum* and *Lactobacillus acidophilus*, offers a promising method to improve the functional profile of carob-based products. LAB can hydrolyse complex phenolic structures, releasing simpler, more bioavailable phenolics, and synthesize bioactive metabolites such as bacteriocins, organic acids (e.g., lactic and acetic), and exopolysaccharides (Di Cagno et al., 2013; Filannino et al., 2018). These transformations may enhance antioxidant activity and antimicrobial properties, as well as improve shelf stability and consumer acceptability (Petcu et al., 2023).

One of the most notable outcomes of LAB fermentation is the enhancement of antioxidant activity, a crucial mechanism for reducing oxidative stress and preventing chronic diseases associated with free radical damage, such as cardiovascular disease, diabetes, and cancer (Cuvas-Limon et al., 2021). During fermentation, the enzymatic activities of LAB, such as esterases, decarboxylases, and glycosidases, can hydrolyse complex polyphenols into simpler, more bioavailable forms, leading to the depolymerization and transformation of these molecules and a consequent increase in antioxidant capacity (Di Cagno et al., 2013; Filannino et al., 2018).

Additionally, LAB fermentation enhances the microbial safety of plant-based beverages by producing antimicrobial metabolites, including organic acids (e.g., lactic and acetic acids), hydrogen peroxide, diacetyl, and bacteriocins. These compounds lower pH and inhibit the growth of spoilage and pathogenic microorganisms, thus extending shelf-life and reducing the risk of foodborne illnesses (Davies et al., 2017; Pessione, 2012). This natural antimicrobial action positions LAB-fermented beverages as a sustainable alternative to synthetic preservatives while preserving nutritional quality.

Fermented plant-based beverages can be used as natural feed supplements for farm animals to improve their overall health and productivity (Ferreira et al., 2022). These beverages, rich in organic acids, antioxidants, and beneficial microorganisms, help balance the gut microbiota, enhance digestion, and strengthen the immune system (Zhang et al., 2024). When included in animal feed, they can also reduce harmful intestinal bacteria and lower the need for synthetic antibiotics (Park et al., 2019). Moreover, their natural bioactive compounds, such as polyphenols and essential oils, support better nutrient absorption and protect animals against infections and oxidative stress. Therefore, fermented beverages represent a sustainable and safe alternative to chemical additives in animal nutrition (Lian et al., 2024).

The synergistic combination of *Ceratonia siliqua* (carob) with selected aromatic and medicinal plants, followed by lactic fermentation, presents a promising strategy for developing multifunctional beverages with enhanced bioactivities. The unique composition of carob, including dietary fiber, gallic acid, catechins, and other polyphenols, combined with the essential oils and flavonoids of aromatic herbs such as *Thymus capitatus*, may offer synergistic antioxidant and antimicrobial effects (El Omari et al., 2023; Ubeda et al., 2011). However, despite the well-documented individual health benefits of these ingredients, few studies have explored their integration in fermented formulations. Furthermore, the impact of fermentation on the modulation of their volatile compounds, phenolic profiles, and biological properties remains underexplored, necessitating further investigation.

Therefore, this study aims to explore the effect of lactic acid fermentation on the functional and microbiological properties of plant-based beverages composed of *Ceratonia siliqua* pulp supplemented with aromatic and medicinal plants. Specifically, the research focuses on (i) the kinetics and biochemical composition (content of total phenolic compounds, sugar and proteins) and volatile compounds of fermented; (ii) antioxidant potential through multiple assays; and (iii) antimicrobial activities against pathogenic strains for potential veterinary applications aimed at promoting gut health, strengthening disease resistance, and reducing the use of synthetic antimicrobials.

Materials and methods

Plant materials and fermentation

The plant materials used in this work were harvested from two regions of Tunisia. *Punica granatum* L. and *Thymus capitatus* were purchased from a commercial central market in Tunisia. A voucher specimen was deposited in the laboratory of aromatic and medicinal plants at the Center of Biotechnology of Borj-Cédria and identified by Pr. Moufida Saidani Tounsi. Two batches were prepared from *Ceratonia siliqua L.* and fermented with *Lactobacillus acidophilus* ATCC 4356 (LA) and *Lactiplantibacillus plantarum* SGL07 (LP) (Gadhoumi et al., 2022), named FBcs-AC and FBcs-PL, respectively. Also, two batches were prepared with *Ceratonia siliqua* L. supplemented with *Thymus capitatus* using LA and (LP) supplemented with *Thymus capitatus*, named FBcsthym-AC and FBcsthym-PL, respectively. Concerning the fermentation process, the selected plant materials were washed with distilled water and then submerged in 1-liter bottles containing 30 g/L of organic sugarcane molasses dissolved in sterilized distilled water. The solution was pasteurized (80 °C for 15 min). The cultures of *Lactobacillus acidophilus* ATCC 4356 and *Lactiplantibacillus plantarum* were used for fermentation. A ready-to-use inoculum with an activated suspension containing approximately 5×10^6 to 7×10^8 colony-forming units (CFU) at pH = 3.5 was added to each batch at a rate of 6 % (v/v) (Gadhoumi et al., 2021). After fermentation, the different beverages were sterilized by microfiltration through 0.2 µm filters and preserved at +4 °C until use.

Fermentative parameters

Physicochemical parameters were determined using the methods described by Gadhoumi et al. (2021). The pH was measured with a pH meter XS series pH 70 (Bormac srl, Carpi, Italy), and titratable acidity (TTA) was determined on homogenized samples and expressed as the volume (mL) of 0.1 N NaOH required to reach pH 8.3 (Ercolini et al., 2013). Total sugars were assessed by an automatic temperature compensation refractometer (Brix 0–32%). The determination of reducing sugars was assessed using the dinitrosalicylic acid (DNS) method described by Song et al. (2016). Reducing sugar was determined using the DNS reagent: 250 µL of each sample was mixed with 250 µL of 0.2 M Tris–HCl buffer (pH 7.0) and 500 µL DNS reagent. The reaction mixture was incubated for 10 min at 95 °C in a boiling water bath, then cooled, and the absorbance was recorded at 560 nm. A standard maltose concentration curve was used to determine the total reducing sugar content.

Total polyphenol content

The polyphenol content was determined using the Folin-Ciocalteu method described by Tlili et al. (2013). A volume of 0.5 mL of Folin-Ciocalteu reagent and 1.25 mL of Na_2CO_3 (7% w/v) was added to 0.125 mL of each fermented beverage. The absorbance of each sample was measured at 765 nm after incubation in the dark for 90 min. The total polyphenols content expressed as µg Gallic acid equivalents per mL of fermented beverages (mg GAE/mL).

Total flavonoid content

The determination of the total flavonoid contents was carried out according to the method reported by Tlili et al. (Tlili et al., 2013). The experiments were performed in triplicate. The flavonoid content, determined by the Quercetin standard curve (concentration range: 100-750 µg/mL), was estimated as mg of Quercetin equivalent per mL of fermented beverages (ml QE/mL).

Volatile compounds analysis by GC-MS headspace

The volatile compounds were assessed using Headspace Solid-Phase Microextraction (HS-SPME) in conjunction with Gas Chromatography Mass Spectrometry (GC-MS), following the method outlined by Navarini et al. (1999). Ion classification was based on their mass/charge ratio (m/z). The analysis utilized an Agilent mass spectrometer (5975C inert XL MSD) coupled to a chromatograph, employing electron impact ionization (70 eV). A capillary column (HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness), coated with 5% phenyl methyl silicone and 95% dimethylpolysiloxane (Agilent Technologies, Palo Alto, CA, USA), was employed. Identification of volatile compounds was performed by matching their mass spectra against entries in the NIST mass spectral library. The Kovats retention index (RI) was determined using a homologous series of n-alkanes (C6-C28) under conditions consistent with the sample analyses. Volatile compounds with an odor activity value exceeding 1 were considered contributors to the aroma of fermented beverages, according to the criteria established by Tian et al. (2017) and You et al. (2013).

Determination of antioxidant activity

DPPH scavenging ability (DPPH assay)

The DPPH radical-scavenging activity of the extract was determined according to the method of Gadhoumi et al. (2021). A mixture of 0.5 mL of the extract at different concentrations and 3.8 mL of a methanolic solution of DPPH (6 mM) was shaken vigorously, then incubated for 30 min in the dark at room temperature. Absorbance was measured at 517 nm. The percentage inhibition (I%) of free radical DPPH was calculated using the formula: I% = (A_blank - A_sample) / A_blank ×100. The DPPH scavenging activity was expressed as the IC50 value, defined as the concentration of the sample required to scavenge 50% of the free radicals in the test solution. Butylhydroxytoluene served as the reference compound.

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS+) free radical scavenging activity

The ABTS+ assay was conducted following the method described and reported by Gadhoumi et al. (2021). ABTS+ radical cation was generated by mixing 7 mM ABTS solution with 2.45 mM potassium persulphate, and the resulting mixture was left to stand in the dark at room temperature. The ABTS+ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. Then, 25 µL of the sample or Trolox standard was added to 2 mL of diluted ABTS+ solution, and the absorbance at 734 nm was measured. Trolox was employed as the reference standard.

Reducing power assay (RPA)

The RPA was determined by the colorimetric methods reported by Gadhoumi et al. (2021), which evaluate the electron-donating capacity of antioxidants by measuring the reduction of Fe^{3+} to Fe^{2+}. In this assay, 1 mL of sample is mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide, then incubated at 50 °C for 20 min. After incubation, 2.5 mL of 10% trichloroacetic acid is added to stop the reaction, and the mixture is centrifuged at 3000 rpm for 10 min. Next, 2.5 mL of the supernatant is combined with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride, and the appearance of the blue-green color is measured at 700 nm on a UV-VIS spectrophotometer. Increased absorbance indicates stronger reducing power. Ascorbic acid is used as a standard antioxidant. All assays are performed in triplicate, using blanks and controls to ensure accuracy.

Antimicrobial activities

Antimicrobial activity: Disk diffusion assay

The antimicrobial activities of the fermented extracts were evaluated using the agar disk diffusion method, as described by Çeliktaş et al. (2007) with minor modifications. This technique was used to assess the inhibitory effects of the extracts on selected pathogenic bacterial strains. Briefly, Mueller Hinton Agar (MH) plates were uniformly inoculated with 100 µL of microbial suspensions: *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923, *Klebsiella aerogenes* ATCC 13048, *Bacillus cereus* ATCC 11778, adjusted to a concentration of 10^5 to 10^7 CFU/mL. After inoculation, the plates were held at 4 °C for 1 hour to facilitate microbial adhesion. Sterile filter paper disks (6 mm diameter) were impregnated with 20 µL of each fermented extract at the test concentration and carefully placed onto the agar surface. For positive control, disks containing 50 µg of streptomycin (Sigma-Aldrich) were used with pathogenic bacteria. The inoculated plates were then incubated for 24 hours at 37 °C under appropriate atmospheric conditions: a standard aerobic environment for non-fastidious bacteria and a 5% CO_2-enriched atmosphere for capnophilic strains. Following incubation, the diameter of the inhibition zones (in mm) was measured. All assays were performed in triplicate, and results were reported as mean ± standard deviation.

Determination of the minimum inhibitory concentration (MIC)

The antibacterial activity of fermented extracts was also evaluated by determination of MIC according to the methods described by Duarte et al. (2012) with slight modifications, using the microdilution broth in 96-well microplates method. In each well of these plates, a volume of 170 µL of sterile Mueller-Hinton broth was dispersed, 10 µL of blue colored resazurin, 10 µL of the inoculum previously prepared (0.5 McFarland), and 10^5 to 10^6 CFU/mL of each bacterium. Then, various fermented extracts at concentrations ranging from 10 µg/mL to 50 µg/mL (diluted in 10% DMSO, 1% Tween 80, DW)

were added. Plates were then incubated for 12 to 24 h at 37 ◦C for the bacterial strain growth. The wells without bacteria and those without extract were used as negative and positive controls, respectively. MIC was recorded as the lowest concentration of essential oil that didn't give a visible growth, at which the blue colour of resazurin was maintained. Each assay was done in duplicate.

Statistical analyses

All analyses were conducted in triplicate, and results were expressed as mean values ± SD (standard deviations). One-way and multivariate analysis of variance (ANOVA) followed by Duncan's multiple-range tests were used to compare means. Differences in individual mean values at $p<0.05$ were deemed significant. Statistical analysis was performed by using the statistical program package Statistica 16.0 (Hassan et al., 2024).

Results and discussion

Evolution of pH and titratable acidity during fermentation

The evolution of pH and titratable acidity during fermentation provides key insights into the metabolic dynamics of LAB and their ability to acidify plant-based substrates. As shown in Figure 1, all formulations initially exhibited a pH above 6.0, which progressively decreased over time, accompanied by a corresponding increase in titratable acidity, an expected outcome of lactic acid fermentation (Di Cagno et al., 2017; Swain et al., 2014). This acidification reflects the conversion of fermentable carbohydrates into organic acids, predominantly lactic acid, a hallmark of LAB metabolism (Filannino et al., 2018). *Lactobacillus acidophilus* demonstrated a more rapid and significant reduction in pH than *Lactiplantibacillus plantarum*, reaching values of 3.5-4.0 by day 15. The more pronounced pH reduction suggests an enhanced acidification potential, probably associated with higher synthesis of organic acids such as lactic and acetic acids (Rollán et al., 2019). In contrast, the *Lactiplantibacillus plantarum* fermented beverages showed a more gradual decrease in pH, stabilizing slightly above 4.0, suggesting a milder acidification profile. While less aggressive, this acidification was still sufficient to ensure microbial safety and inhibit spoilage organisms (Pessione, 2012; Vuyst et al., 2008). The parallel increase in titratable acidity confirms the active metabolism of both strains and their ability to accumulate organic acids. After day 15, both pH and acidity values tended to stabilize, indicating a likely reduction in bacterial activity due to substrate depletion or the inhibitory effects of accumulated acids, a common phenomenon in LAB-driven fermentations (Marco et al., 2017). The slowdown in acid production at this stage may also reflect the transition from active growth to the stationary phase, during which maintenance metabolism predominates.

Functionally, these changes support the roles of LA and LP in producing safe, stable fermented beverages. The low pH enhances preservation, inhibits pathogenic growth, and can increase the bioavailability of phenolic compounds and other micronutrients by promoting enzymatic hydrolysis (Choi et al., 2002; Poutanen et al., 2009). Notably, the choice of LAB strain influences the kinetics and extent of acidification, which in turn affects the sensory attributes and potential nutraceutical value of the final product. Their differing fermentation dynamics underscore the importance of targeted strain selection in designing functional fermented beverages with specific health-promoting and sensory attributes.

Total phenolics, total flavonoids content, and total sugar content

Before fermentation, the total polyphenol content (expressed as mg GAE/100 mL) ranged between 110 and 120 mg GAE/100 mL across all samples, as shown in Figure 2. After fermentation, a significant increase in TPC was observed in all formulations ($p<0.05$), particularly in those fermented with *Lactiplantibacillus plantarum*. The FBcsthym-AC formulation showed the highest TPC (200 mg GAE/100 mL), followed by FBcsthym-PL and FBcs-AC (Figure 2). This increase suggests that the fermentation process facilitated the release or biotransformation of bound phenolic compounds into more soluble forms (Filannino et al., 2018; Swain et al., 2014). The enzymatic activity of LAB, including β-glucosidase and esterase, is known to hydrolyse complex polyphenol sugar conjugates, enhancing their extractability and bioavailability (Hur et al., 2014).

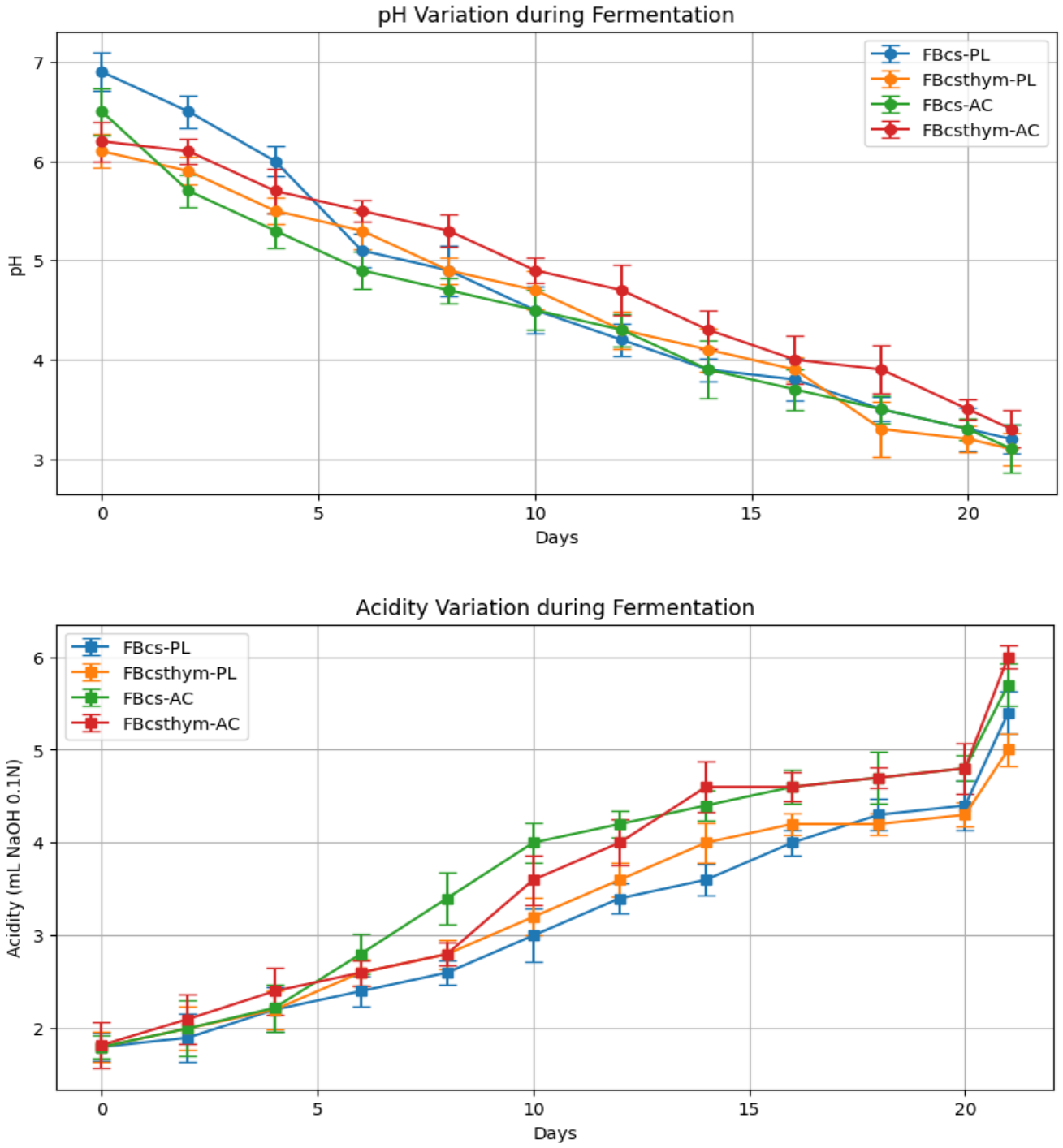

Figure 1: Acidity and pH parameters of fermented plants using lactic acid bacteria during 21 days. Results are expressed as the means of 3 experiments of Fermented plants beverages.

A similar trend was observed in total flavonoid content (expressed as mg RE/100 mL) (Figure 2). Post-fermentation values increased significantly in all samples, with the FBcsthym-AC formulation again showing the greatest enhancement (Figure 2). This could be attributed to the greater liberation of flavonoid aglycones during microbial hydrolysis, particularly in polyphenol-rich formulations (Granato et al., 2010). The higher values in LA-fermented beverages compared to LP suggest a stronger hydrolytic or biotransforming capacity of LA in this context. In contrast, total sugar concentrations markedly decreased after fermentation ($p<0.05$), confirming active carbohydrate metabolism by LAB (Figure 2). This reduction is consistent across all formulations, with final sugar levels falling below 10 g/L (Figure 2), indicating efficient consumption of fermentable sugars during lactic acid production.

The rapid depletion of sugars is critical for acidification, shelf-life extension, and reduced glycemic potential of the final product (Vuyst et al., 2008). These results clearly demonstrate the role of lactic fermentation in enhancing the nutraceutical potential of plant-based beverages. The observed increases in polyphenol and flavonoid concentrations reflect microbial-mediated release and conversion of bioactive compounds, while the sugar reduction ensures lower energy content and microbial safety.

The superior performance of LA in increasing TPC and flavonoid levels highlights its suitability for functional beverage development targeting high antioxidant activity.

These findings align with previous studies showing that the composition of the plant substrate and the LAB strain used both play critical roles in shaping the final biochemical profile (Di Cagno et al., 2013; Poutanen et al., 2009).

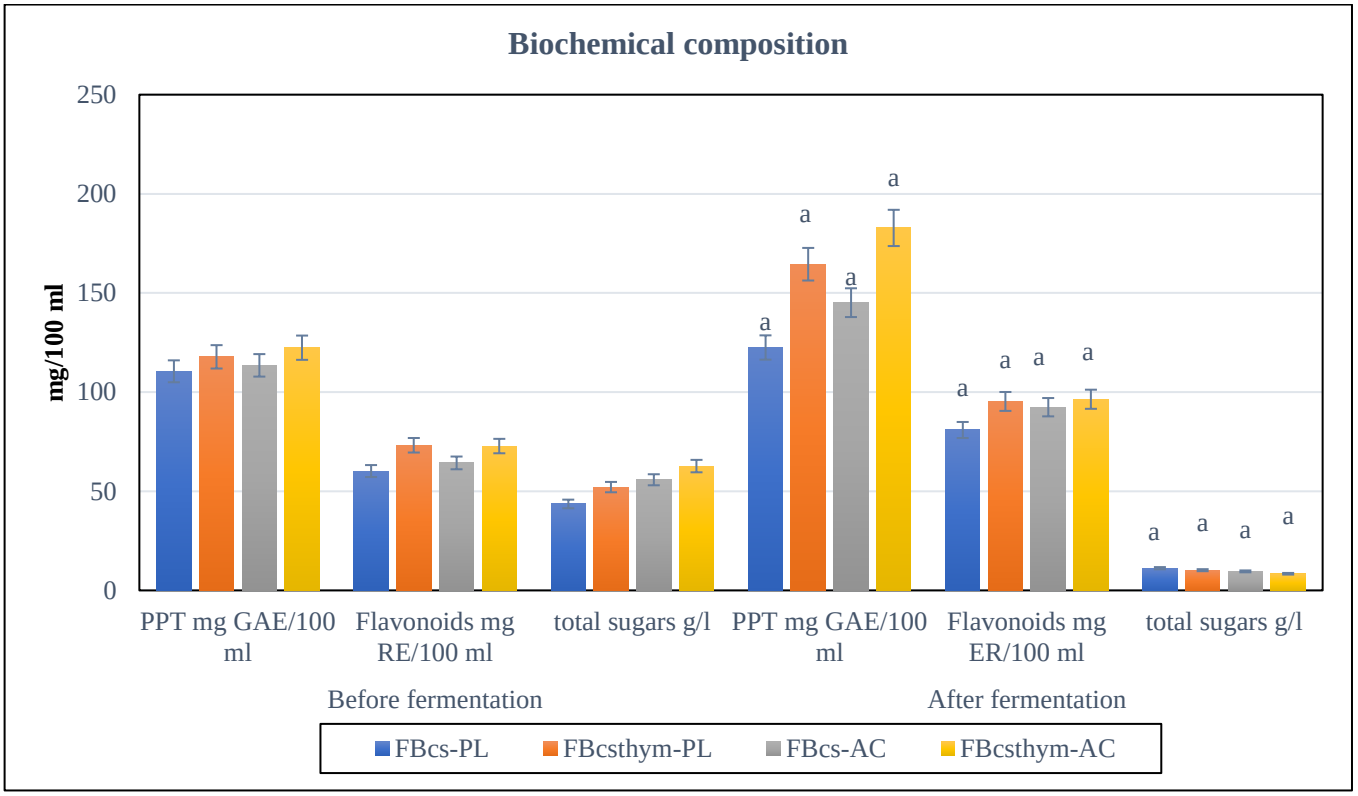

Figure 2: Biochemical composition before and after fermentation of the medicinal plants. Results are expressed as the means of 3 experiments of fermented extracts. Data bearing different lowercase letters indicate a significant difference between the beverages ($p < 0.05$).

Assessment of volatile compounds

The volatile profiles of fermented plant-based beverages showed significant differences, influenced by both the bacterial strain used (LA or LP) and the presence of aromatic plants (*Thymus capitatus*) (Table 1). Across all samples, major compounds such as lactic acid, acetic acid, ethanol, and ethyl acetate dominated the volatile fraction, consistent with previous findings in fermented plant matrices (Di Cagno et al., 2017; Filannino et al., 2018). These metabolites are typical end-products of hetero- and homofermentative LAB metabolism, contributing to both the acidification and the flavour profile of the beverage (Alonso et al., 2004). The higher abundance of lactic and acetic acids in FBcs-AC and FBcs-PL formulations suggests robust fermentative activity. It confirms their role in lowering pH and enhancing microbial safety. Interestingly, the incorporation of *Thymus capitatus* into the formulations (FBcsthym-AC and FBcsthym-PL) resulted in the appearance of distinct volatile terpenes, including thymol, carvacrol, p-cymene, and γ-terpinene, which were absent in carob-only formulations. These compounds are well-documented as the primary

active constituents of Thymus essential oil and are known for their strong antimicrobial and antioxidant properties (Burt, 2004; Celiktas et al., 2007). Their presence in the volatile profile post-fermentation suggests that the fermentation process preserves or even facilitates the release of these bioactive substances, possibly through enzymatic hydrolysis or cell wall disruption induced by LAB metabolism (Rollán et al., 2019). The detection of flavour-active compounds such as diacetyl, acetoin, benzaldehyde, and linalool further support the formation of a complex aromatic profile, which enhances the sensory quality of the beverages (Gadhoumi et al., 2021). Diacetyl and acetoin, both derived from pyruvate metabolism by LAB, contribute creamy and buttery notes and were found in higher amounts in FBcs-PL and FBcsthym-PL, possibly due to strain-specific pathways (Ronie et al., 2024).

Table 1: Volatile flavor compounds were determined by gas chromatography–mass spectrometry (GC–MS) of FPBs.

N°	Composition	R.T (min)	FBcs-AC	FBcs-PL	FBcsthym-AC	FBcsthym-PL
1	Ethanol	3.25	13.42	12.54	10.44	9.82
2	Acide acétique	4.12	18.95	17.78	15.15	13.21
3	Acétate d'éthyle	4.90	10.26	9.04	8.08	7.14
4	Acide lactique	5.34	22.63	21.87	17.51	15.89
5	3-Méthylbutanol	6.15	8.16	7.58	6.57	6.07
6	Diacétyle	6.70	3.95	5.25	3.70	4.64
7	Acétoïne	6.85	6.05	7.29	4.71	5.54
8	Hexanal	7.35	4.21	5.54	2.36	2.68
9	Furfural	8.10	5.53	5.25	4.21	4.11
10	Benzaldéhyde	8.60	3.16	4.08	2.19	2.50
11	Linalol	9.20	3.68	3.79	2.53	2.50
12	γ-Terpinène	9.40	0	0	2.69	3.21
13	p-Cymène	9.85	0	0	3.03	3.57
14	Thymol	10.20	0	0	8.92	10.18
15	2-methyl-5-propan-2-ylphenol	10.45	0	0	7.91	8.93

Overall, the volatile composition suggests that the synergistic effect of LAB fermentation and the addition of *Thymus capitatus* not only modulates microbial stability and acidity but also enhances the functional and aromatic qualities of the beverages. The higher levels of thymol and carvacrol in FBcsthym-PL suggest that *Lactiplantibacillus plantarum* may more effectively preserve or release aromatic compounds during fermentation, likely due to its diverse enzymatic system and strong tolerance to phenolic compounds. These enzymatic reactions can either enhance desirable aromas by releasing terpenes, aldehydes, and phenolic volatiles or reduce off-flavours by degrading undesirable compounds (Filannino et al., 2018). As a result, lactic fermentation not only improves the sensory quality of fermented products but also contributes to the stabilization or even the generation of new aromatic and bioactive molecules, thereby enhancing their functional and nutraceutical value.

Antioxidant Activity

The antioxidant potential of the fermented beverages was evaluated using three complementary in vitro assays: DPPH radical scavenging activity, ABTS•+ radical cation decolorization, and reducing power. The results in Table 2 show that all beverages exhibit notable antioxidant activity, with lower IC_{50} or EC_{50} values indicating stronger antioxidant potential. Among the samples, those fermented with LA (FBcs-AC and FBcsthym-AC) consistently exhibited higher antioxidant activity than their LP-fermented counterparts, particularly in the DPPH and ABTS assays. Moreover, the addition of *Thymus capitatus* significantly enhanced the antioxidant performance of both strains, as evidenced by lower IC_{50} and EC_{50} values in the FBcsthym samples. The FBcsthym-AC formulation showed the most potent activity (DPPH IC_{50}=17.6 µg/mL; ABTS IC_{50}=18.8 µg/mL), approaching the activity of the synthetic antioxidant BHT (DPPH IC_{50}=13.5 µg/mL) and Trolox (ABTS IC_{50}=10.2 µg/mL), respectively. These findings are consistent with previous studies demonstrating that LAB fermentation enhances the antioxidant capacity of plant matrices by releasing or transforming polyphenolic compounds, thereby improving their bioavailability and radical-scavenging

ability (Filannino et al., 2018; Pyo et al., 2004). The superior performance of LA-fermented beverages may be attributed to this strain's greater enzymatic capacity to hydrolyse complex phenolic structures, thus generating more active antioxidant compounds (Ferreira et al., 2022). In addition, the inclusion of *Thymus capitatus*, rich in phenolic terpenes such as thymol and carvacrol, likely contributed to the observed enhancement, given the well-documented antioxidant properties of these bioactive substances (Burt, 2004). The reducing power assay results followed a similar trend, further confirming the improved electron-donating capacity of FBcsthym formulations. The EC_{50} value of FBcsthym-AC (14.2 µg/mL) was significantly lower than that of FBcs-PL (24 µg/mL), underscoring the synergistic effect of lactic fermentation and plant bioactive compounds. Although all fermented beverages demonstrated significant antioxidant activity, the data suggest that both the choice of bacterial strain and the addition of medicinal herbs are crucial in maximizing the functional properties of plant-based fermented products. These enhancements are particularly relevant for the development of nutraceutical beverages, aligning with consumer demand for functional foods with health-promoting effects (Di Cagno et al., 2017; Filannino et al., 2018).

Table 2: Antioxidant activity of three fermented beverages from aromatic and medicinal plants FPBs.

	FBcs-PL	**FBcsthym-PL**	**FBcs-AC**	**FBcsthym-AC**
DPPH IC50 (µg/ml)	32.6 ± 2	20.5 ± 1.5	30.4 ± 2	17.6 ± 1.5
ABTS•+ IC50(µg/ml)	38.5 ± 1	25.2 ± 2	39.8 ± 0.5	18.8 ± 2.5
Reducing power EC50 (µg/ml)	24 ± 2	16 ± 0.8	19.2 ± 1.5	14.2 ± 1.6

Results are expressed as the means of 3 experiments of three different FPBs. $ABTS^{\bullet+}$ IC50(µg/ml) for Trolox was 10.2 ± 1 DPPH IC50 (µg/ml) for BHT was 13.5 ± 0.5. Reducing power EC50 (µg/ml) for vitamin C was 8.5 ± 0.6.

The antimicrobial activity

Antimicrobial Activity: Disk Diffusion Assay

The antibacterial activity of the different beverages, as illustrated in Figure 3, showed that all formulations exhibited measurable inhibitory effects, with inhibition zones ranging from 9.0 mm to 24.0 mm, depending on the microorganism and fermentation conditions. The reference antibiotic, streptomycin, consistently produced the largest inhibition zones (25–32 mm), confirming its efficacy as a positive control. Among the formulations, those supplemented with *Thymus capitatus* FBcsthym-PL and FBcsthym-AC demonstrated significantly superior antimicrobial activity compared to the non-thymus samples (FBcs-PL and FBcs-AC). Notably, the FBcsthym-AC formulation exhibited the highest inhibition zones, particularly against *Klebsiella aerogenes* (24 ± 2 mm), *Bacillus cereus* (22 ± 1 mm), and *Pseudomonas aeruginosa* (24 ± 2 mm), approaching the activity of streptomycin (26–32 mm) in those cases. Similarly, FBcsthym-AC showed strong inhibition against *Staphylococcus aureus* ATCC 25923 (18 ± 2 mm) and its clinical isolate A9003225 (20 ± 0.5 mm), underlining its broad-spectrum effectiveness.

The enhanced antimicrobial properties of the thyme-enriched formulations can be attributed to the presence of thymol and carvacrol, phenolic monoterpenes well known for their ability to disrupt microbial membranes and inhibit respiratory enzymes (Burt, 2004; Celiktas et al., 2007). Their synergistic interaction with fermentation metabolites likely boosts efficacy. Furthermore, beverages fermented with LA (FBcs-AC and FBcsthym-AC) consistently outperformed those fermented with LP. For example, FBcs-AC inhibited *Bacillus cereus* by 15 ± 2 mm and *S. aureus* ATCC 25923 by 12 ± 2 mm, which is similar to 11 ± 1 mm and 14 ± 3.5 mm, respectively, for FBcs-PL. This difference likely stems from LA's greater acidification capacity and its ability to produce bacteriocins, hydrogen peroxide, and higher levels of organic acids (Gänzle, 2015; Vuyst et al., 2008). A consistent trend was observed across all formulations: Gram-positive bacteria (*B. cereus*, *S. aureus*) were generally more susceptible than Gram-negative strains (*K. aerogenes*, *E. coli*, *P. aeruginosa*). For instance, *E. coli* showed a modest inhibition zone of 10.5 ± 2 mm with FBcs-PL and 22 ± 1 mm with FBcsthym-AC. In contrast, *Bacillus cereus* exhibited 22 ± 1 mm inhibition with the same

thyme-supplemented extract. This aligns with established findings that the outer membrane of Gram-negative bacteria serves as a barrier to many natural antimicrobials (Mancini and Pucciarelli, 2020; Nascimento et al., 2000). These results highlight the effectiveness of combining lactic acid fermentation with supplementation with aromatic and medicinal plants to enhance antimicrobial activity in nutraceutical beverages. The dual contribution of microbial metabolites and plant-derived bioactive compounds, such as thymol, provides a promising strategy for developing safe, natural, and functional antimicrobial beverages.

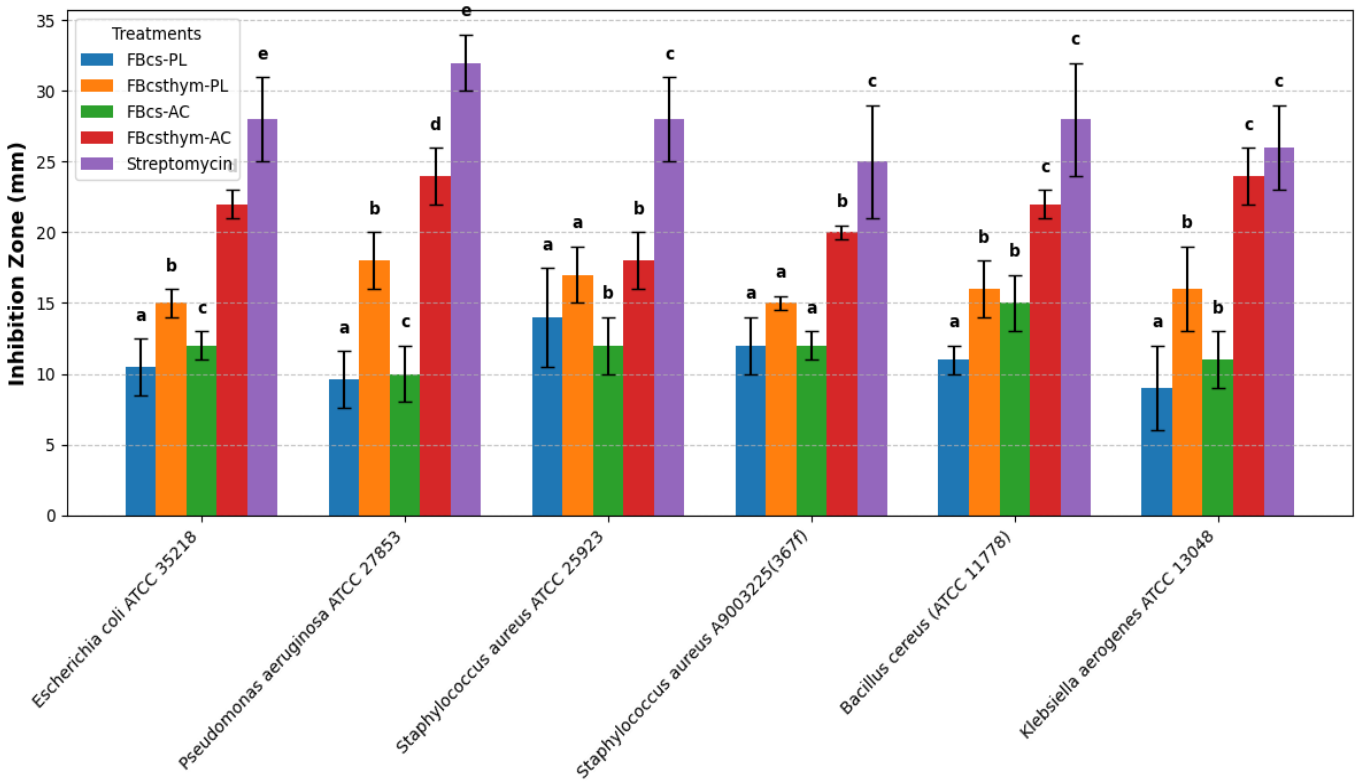

Figure 3: Antimicrobial activity assay using the disc diffusion method of the different fermented plant extracts. Results are expressed as the means of 3 experiments of fermented extracts. Data bearing different lowercase letters indicate a significant difference between the beverages ($p < 0.05$).

Determination of the minimum inhibitory concentration (MIC)

The MIC data presented in Table 3 further illustrate the antimicrobial potential of the fermented plant-based beverages, particularly those enriched with *Thymus capitatus*. MIC values ranged from 0.5 to 5.6 mg/mL, depending on the bacterial strain and the formulation. Among all treatments, the FBcsthym-AC formulation showed the lowest MICs, with values as low as 0.5 mg/mL against *E. coli* and 1 mg/mL against *P. aeruginosa* and *B. cereus*, indicating a strong inhibitory capacity at relatively low concentrations. In contrast, FBcs-PL and FBcs-AC, which lack the aromatic supplementation, demonstrated higher MIC values across all strains, ranging from 1.5 to 5.5 mg/mL, suggesting that the presence of bioactive terpenes such as thymol and carvacrol in *Thymus capitatus* is a critical factor enhancing antimicrobial activity. These compounds are known to alter membrane permeability and disrupt enzymatic functions in microbial cells (Burt, 2004; Celiktas et al., 2007). Moreover, fermentation by LA generally produced extracts with lower MIC values than those fermented by LP.

Table 3: Antibacterial activity of the fermented plant beverages and determination of minimum inhibitory concentration (MIC) (mg/mL).

Bacteria	FBcs-PL MIC (mg/mL)	FBcsthym-PL MIC (mg/mL)	FBcs-AC MIC (mg/mL)	FBcsthym-AC MIC (mg/mL)
Escherichia coli ATCC 35218	3	1.5	1.5	0.5
Pseudomonas aeruginosa ATCC 27853	4.5	1.5	2.5	1
Staphylococcus aureus ATCC 25923	3.5	2.5	1.5	1.5
Staphylococcus aureus A9003225	5.5	3.5	3.5	1.5
Bacillus cereus ATCC 11778	4	2	2	1
Klebsiella aerogenes ATCC 13048	3.5	2.5	3	2

For example, *S. aureus* ATCC 25923 had MIC values of 1.5 mg/mL for FBcs-AC and FBcsthym-AC compared to 3.5 and 2.5 mg/mL, respectively, for the PL-fermented equivalents. This trend supports the hypothesis that LA has a superior capacity to generate antimicrobial metabolites such as lactic acid, bacteriocins, and hydrogen peroxide, contributing to stronger bacteriostatic effects (Celiktas et al., 2007; Gänzle, 2015). On the other hand, Gram-negative bacteria such as *P. aeruginosa* and *K. aerogenes* required higher concentrations to inhibit growth than Gram-positive bacteria. aligning with previous studies indicating that the outer membrane of Gram-negative strains presents a barrier to many natural antimicrobial agents (Gadhoumi et al., 2022; Mancini and Pucciarelli, 2020; Nascimento et al., 2000). Despite this, the thyme-supplemented and acidophilus-fermented beverages still achieved low MIC values even against these more resistant species. Overall, the MIC data reinforce the synergistic antimicrobial effect of combining lactic acid fermentation with aromatic plant enrichment, particularly with *Thymus capitatus*, and highlight their potential use as natural preservatives or therapeutic adjuncts in functional food formulations. The significant increase in total phenolic and flavonoid contents following fermentation further enhances the nutraceutical value of these plant-based formulations. Polyphenols and flavonoids are well-documented for their antioxidant, anti-inflammatory, and immunomodulatory properties, which can contribute to improved gut integrity, reduced oxidative stress, and enhanced immune response in livestock. When included as dietary supplements, these bioactive compounds may help mitigate stress-induced metabolic disorders and inflammatory conditions common in intensive animal production systems. Future research should focus on in vivo trials to assess the safety, bioavailability, and physiological effects of these fermented plant-based formulations in different animal species. Particular emphasis should be placed on evaluating their influence on gut microbiota composition, immune modulation, growth performance, and resistance to enteric infections. Moreover, exploring synergistic formulations combining multiple plant fermentation and probiotic strains could pave the way for next-generation phytogenic probiotic functional feeds tailored to specific veterinary applications.

Conclusions

This study demonstrates that lactic acid fermentation with *Lactobacillus plantarum* (LP) and *Lactobacillus acidophilus* (LA) can significantly enhance the nutritional, functional, and aromatic properties of plant-based beverages formulated from *Ceratonia siliqua* and *Thymus capitatus*. Fermentation increased acidity, improved phenolic and flavonoid content, and reduced sugar levels, especially in LA-fermented samples. Beverages enriched with *Thymus capitatus* exhibited superior antioxidant and antimicrobial activities, largely due to the presence of thymol and carvacrol. The combination of aromatic plants and lactic fermentation significantly improved the volatile profile, bioactivity, and microbial safety of the beverages, supporting their application as natural functional drinks with enhanced health benefits. They contain a rich content of bioactive compounds, such as polyphenols, flavonoids, and tannins, which possess strong antioxidant, anti-inflammatory, and antimicrobial activities. These properties make them promising candidates for use as natural feed additives or phytobiotics in animal nutrition. Incorporating such compounds into livestock diets can help support gut health, modulate the microbiota, and enhance immune response, thereby improving overall animal health and productivity. Moreover, their antimicrobial potential can help reduce the use of synthetic antibiotics, addressing one of the major challenges in modern veterinary medicine, the growing problem of antimicrobial resistance.

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