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Evaluation of the addition of *Moringa oleifera* L. and *Salvia hispanica* L. on the antioxidant activity and viability of lactic acid bacteria in yogurt during refrigerated storage

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Abstract

Moringa oleifera L. and *Salvia hispanica* L. are plants known to possess nutritional and medicinal properties, especially in bioactive compounds (antioxidants), which could be used to generate functional foods. The present study investigated the effect of *M. oleifera* leaves *and S. hispanica* seeds on yogurt's antioxidant activity and microbial viability during 21 days of storage at 4 °C. Peptide content, pH changes, lactic acid content, and the microbial viability of lactic acid bacteria were analyzed on days 0, 7, 14, and 21. Antioxidant activity was determined by the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods. Total phenolic compounds were determined by the Folin-Ciocalteu technique. During storage, treatments showed post-acidification, with lactic acid accumulation (1.30 to 1.80 lactic acid percentages) and pH values close 4.0. Compared to the other treatments, the mix treatment showed significantly ($p < 0.05$) elevated antioxidant activity, with 97.03 ± 1.2% free radical scavenging capacity in the ABTS assay at the end of storage. Regarding phenolic compounds, the Mix treatment obtained a concentration of 313.80 ± 15.71 mg GAE/100 g of yogurt, compared to the other treatments, which presented lower concentrations at the end of storage. In the DPPH assay, the treatment added with *M. oleifera* presented higher antioxidant activity on day 14 of cold storage with a free radical scavenging capacity of 23.15 ± 0.92% compared to the other treatments. The highest peptide concentration shown during cold storage of the treatments was in the Mix treatment during days 7, 14, and 21, with values of 26.86 ± 2.19, 37.34 ± 5.94, and 34.71 ± 1.33 mg leucine/100 g yogurt, respectively. The added treatment with *M. oleifera* showed increased lactic acid bacteria viability up to day 14 of refrigerated storage with a maximum value of 4.75×10^7 (CFU/ml) compared to the other treatments. These findings indicate that *M. oleifera* and *S. hispanica* influences the physicochemical properties of yogurt during storage for 21 days at 4 °C, affecting parameters such as pH, titratable acidity, LAB viability, peptide content and antioxidant capacity. These modifications can impact the stability and functionality of yogurt, making it a potential candidate studies in the field of functional foods.

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Highlights

- Yogurts enriched with *Moringa oleifera* L. leaf extract and *Salvia hispanica L.* flour demonstrated enhanced antioxidant properties, as evidenced by increased total phenolic compounds and ABTS radical scavenging activity.
- The yogurt with the *M. oleifera* and *S. hispanica* mixture exhibited the highest phenolic content (313,80 mg GAE/100 g) and ABTS inhibition (97.03%) after 21 days of storage, compared to control.
- The pH of the enriched yogurts decreased over time, with *S. hispanica* enriched yogurt showing the highest titratable acidity (1.84% lactic acid).
- The viability of lactic acid bacteria was significantly affected by enrichment, with *M. oleifera* enriched yogurt showing increased LAB content during storage.
- Peptide concentration were highest in the yogurt with *M. oleifera* and *S. hispanica* mixture (34.71 mg leucine/ 100 g) after 21 days of storage, indicating potential proteolytic activity.

Keywords *Moringa oleifera*, Antioxidant, Yogurt, Storage, Peptides

Introduction

Yogurt is a widely consumed fermented dairy product recognized for its nutritional and probiotic benefits, particularly in improving gut health [33, 67]. However, although yogurt possesses numerous health and nutritional effects, it is known that milk and its derivatives are not usually considered a rich source of bifunctional properties like the antioxidant activity [24, 42]. Additionally, another important aspect or yogurt is the viability of lactic acid bacteria (LAB), a critical factor influencing its health benefits; LAB survival is affected by various factors, including the bacterial strain used, pH level, and the presence of metabolites such as lactic and acetic acids [69]. Research has shown that while adding certain fruit juices can enhance the sensory qualities of yogurt, their acidity and antimicrobial properties may negatively impact the LAB viability, suggesting that careful ingredient selection is essential to mitigate these adverse effects [52]. In this regard, there has been growing interest in developing yogurt to improve functional properties for the benefit of consumer, efforts have been made to enrich yogurt with natural ingredients that serve as sources of bioactive compounds while also supporting the LAB survival and activity. Various studies have suggested incorporating ingredients such as plant extracts, plant powders and seeds [64]. For instance, research has shown that incorporating leaf extracts, such as those from *Diospyros kaki* THUNB leaf and *Nelumbo nucifera*, during yogurt fermentation can enhance the metabolic activity of yogurt bacteria, potentially improving its nutritional and functional properties [25]. Additionally, soybean, has been used in yogurt production to increase LAB viability and antioxidant activity during storage [2]. Another example of incorporating bioactive sources into yogurt is the study by Kim et al. [27], which found that adding lotus (*Nelumbo nucifera*) leaf powder can enhance both the quality and health benefits of yogurt. With these studies in mind, the importance of the implementation of bioactive compounds that are from natural resources and practical and economical sources such as plants, seeds, or fruits has been given.

Moringa oleifera L. is recognized for its rich nutritional profile, which includes high levels of proteins, vitamins (A, C, and E) and minerals (calcium, potassium, and iron), this plant has been increasingly integrated into various food products, due to its health-promoting properties associated with anti-inflammatory, antioxidant and hypolipidemic effects [11, 44, 63]. In addition, *M. oleifera* leaves contain flavonoids such as kaempferol, quercetin, and rhamnetin, which are known for their potent antioxidant properties; furthermore, they contain phenolic compound such as caffeic acid and gallic acid [51, 57, 58]. The incorporation of *M. oleifera* into bakery and dairy products has been shown to improve their quality, sensory attributes and shelf life, research indicates that Moringa extracts possess antimicrobial properties which can help in preserving products by inhibiting the growth of spoilage microorganisms and the same time making them more appealing to consumers [5, 6, 45]. On the other hand, *Salvia hispanica* L., commonly known as chia are rich in omega-3-fatty acids, fiber, and antioxidants, making this seed valuable addition to dairy formulations which enhances the nutritional profile and contributes to improve the texture and stability [29, 36]. The seeds of chia also contain a variety of polyphenolic antioxidants, which contribute to their health benefits and play a significant role in reducing oxidative stress [49]. As a background, according to Kwon et al. [30] and Derewiaka et al. [15], chia seed extracts and chia seed oil enhance the physicochemical properties of yogurt, including viscosity and syneresis, which are critical for maintaining the quality.

Both plants, *Moringa oleifera* and *Salvia hispanica*, have rich historical backgrounds that enhance their unique, Moringa has been utilized in traditional medicine for centuries valued for its therapeutic properties [63]. Similarly, chia was a staple food for ancient Mesoamericans cultures, who recognized its nutritional benefits and used it in various culinary and medicinal application [8, 38]. Therefore, this study explores the combined use of *M. oleifera* and *S. hispanica*, in yogurt fortification, and approach that has received limited attention. While both ingredient have been individually studied in dairy products, their combined effects on yogurts physicochemical properties, microbial stability, and potential heal benefits remain largely unexplored. To address this gap, this study examines the influence of *M. oleifera* leaf extract and *S. hispanica* flour —both rich in polyphenols—on key quality parameters of yogurt. Specifically, it assesses their impact on pH, titratable acidity, antioxidant activity, peptide concentration, and microbiological viability (LAB) during cold storage at 4 °C during 21 days.

Materials and methods

Materials

Dry extract of *M. oleifera.* Fresh leaves were collected in the peripheral area of the city of Mérida, Yucatán (21° 00′ 08.4"N 89° 33′ 39.1"W); the tree was identified according to the morphological characteristics described by [62]. The collected leaves were dried and processed in a grinder until flour was obtained and stored in dark plastic containers under refrigeration until use. To make the dry extract of *M. oleifera*, 5 g of powder was mixed with 200 mL of distilled water. The mixture was heated to boiling point for 5 min, then cooled for another 5 min and filtered. The filtrate was evaporated for 3 days at 50 °C until the dry extract was obtained [10].

S. hispanica flour. Commercial seeds of the Xiomega Superfoods + brand was used. The seeds were pulverized in a grinder for about 1 min, sieved with a kitchen strainer, and stored refrigerated until used in a plastic container in the dark.

Preparation of the yogurts to be stored

Yogurts were prepared according to the Official Mexican Standard NOM-181-SCFI-2010 specifications. Commercial whole milk (3.3 g total fat and 8.6 g of SNF; according to the nutritional specifications of the product), preheated to 60 °C, was used with the addition of powdered milk as stabilizer (6.5%), to help to prevent syneresis and increase viscosity [26, 41]. It was homogenized until it reached a temperature of 45 °C (inoculation temperature). Once the inoculation temperature was reached, 0.1% dry extract of *M. oleifera* and 0.5% *S. hispanica* flour (%/100 mL of milk) were added separately, and a mixture was made with both components, denoting it"Mix"(0.1% *M. oleifera* + 0.5% *S. hispanica*) (percentages were based on preliminary tests). A control was prepared. The lyophilized starter culture YOGURMET (*Lactobacillus bulgaricus* and *Streptococcus thermophilus* was added directly. It was maintained at a temperature of 42 ± 3 °C (according to manufacturer's specifications of the starter culture for an incubation period of 5 h) treatments were stabilized at 4℃ for 12 h after completion of fermentation and stored at 4℃ until analyses at 0, 7, 14, and 21 days of storage for each treatment.

Preparation of the aqueous extract of yogurts in refrigerated storage

Ten grams of yogurt from each treatment was homogenized with distilled water in a 1:0.25 w/v ratio. The pH of the solution was adjusted to 4.0 with 1.0 M HCl to reduce the solubility of casein present in the milk. The solution was incubated at 45 °C for 10 min. It was centrifuged at 5000 rpm at 4 °C for 10 min to remove precipitated proteins from the milk. The sediment was discarded, and 0.5 M NaOH was added to neutralize the supernatant to a pH of 7.0. It was centrifuged to achieve further precipitation of proteins and salts. The supernatant from the second centrifugation was used in the analyses of phenolic compound content and antioxidant activity (DPPH and ABTS) and for the determination of peptide content [56]. This procedure was performed during days 0, 7, 14, and 21 of storage at 4 °C for each treatment.

Determination of pH and titratable acidity during refrigerated storage

pH

The pH was measured with a HANNA HI220 potentiometer and calibrated with the buffer solutions pH 7 and pH 4. This procedure was carried out during days 0, 7, 14, and 21 of storage at 4 °C for each treatment.

Titratable acidity

The measurement of acidity content was carried out according to that described in Mexican Official Standard NOM-185-SSA1-2002, products and services, butter, creams, sweetened, fermented, and acidified condensed milk products, and milk-based sweets. Sanitary specifications. This procedure was performed during days 0, 7, 14, and 21 of storage at 4 °C for each treatment.

Analysis of total phenolic compounds during refrigerated storage

The content of phenolic compounds was determined using the method of Wei et al. [66], with slight modifications. 100 µL of the aqueous yogurt extract was mixed with 100 µL of 1 N Folin-Ciocalteu reagent, and the

mixture was allowed to react at room temperature for 3 min. 300 µL of 1 N Na_2CO_3 was added to the mixture, vortexed, and incubated at room temperature for 90 min. 1 mL of distilled water was added to complete the reaction. The samples were read at an absorbance of 725 nm using a UV–Vis spectrophotometer. The results were expressed as mg of gallic acid equivalents (GAE) per 100 g sample. Gallic acid was used as a standard (to prepare the standard solution of gallic acid, a stock solution of 200 mg/mL was prepared, from which dilutions were made to obtain 50, 100, 150 and 200 mg/mL) [12]. This procedure was performed during days 0, 7, 14, and 21 of storage at 4 °C.

In vitro antioxidant activity during refrigerated storage

Measurements were performed on days 0, 7, 14, and 21 of storage for each treatment.

DPPH (1-Diphenyl-2-picryhydrazyl) assay

The DPPH + radical was formed with 2.5 mg of the radical dissolved in 90 mL of pure methanol. The solution was adjusted to an absorbance of 0.7 ± 0.02 with the same solvent at a wavelength of 515 nm. As for the chemical reaction of DPPH, the radical was reacted with the aqueous yogurt extract of each treatment. The blank was adjusted with methanol. 3.9 mL of the DPPH solution and 100 µL of aqueous extract were placed in a tube. The results were expressed as the sample's percent free radical scavenging capacity [7, 17, 35]. Radical scavenging activity was calculates using the following formula:

$$\% \text{ inhibition} = [(A_B - A_E)/A_B] \times 100 \quad (1)$$

where A_B = absorbance of the blank sample, and A_E = absorbance of the aqueous yogurt extract.

ABTS (2,2′-azinobis(3-ethylbenzothiazolin)−6-sulfonic acid) assay

To release the ABTS + radical, 10 mL of the ABTS solution was taken, and 40 µL of 70 mM $K_2S_2O_8$ was added to form the ABTS reagent. The mixture was kept in the dark at room temperature for 16 h to prevent radical formation. The ABTS solution was diluted with ethanol to obtain an absorbance of 0.81 at 734 nm. It is achieved by mixing 2.5 mL of the radical solution with approximately 100 mL of ethanol. The standard curve was performed with the Trolox reagent at different concentrations. Readings were taken after 7 min of interaction between the ABTS and the sample (from the solution stock of Trolox, dilution were made to obtain the concentration of 0.01, 0.03, 0.05, 0.1, 0.2, 0.3 and 0.4 mmol/mL). 1900 µL of ABTS were used per 100 µL of aqueous yogurt extract from each treatment [48]. The results were expressed as the sample's percent free radical scavenging capacity.

The methods of DPPH and ABTS have been consistently utilized in yogurt studies to measure antioxidant activity, demonstrating their relevance and effectiveness in food research, and the combination of this assays allows more comprehensive assessment of antioxidant activity [19, 27, 28, 31, 69].

Microbial count of viable lactic acid bacteria (LAB) during refrigerated storage

3 M Petri film plates were used for counting lactic acid bacteria, a system that eliminates oxygen, creating an anaerobic environment to recover homofermentative and heterofermentative LAB. Serial dilutions (10^{-7}) were prepared for inoculation using 0.9% saline. Ten grams of yogurt from each treatment was mixed with 90 mL of sterile saline solution. 1 mL was transferred to a tube with sterile saline, and serial dilutions were continued. 1 mL of dilution was placed in the center of the Petri film dish so that the pipette was perpendicular to the inoculation area; the top film was carefully lowered, avoiding the formation of air bubbles. The diffuser was placed in the center of the plate and pressed gently to distribute the sample uniformly. To wait without moving the plate for the gel to solidify. The plates were incubated with the transparent side up at 37 °C for 48 h. The colonies formed were expressed in colony-forming units per milliliter of sample (CFU/mL) [56]. Measurements were performed on days 0, 7, 14, and 21 for the storage of the yogurts under study.

$$\frac{CFU}{mL} = \frac{N^{\circ}\ of\ colonies\ formed\ \times\ dilution\ factor}{mL\ of\ sample}$$

Cadmium-ninhydrin colorimetric method for the assay of peptides during refrigerated storage

The modified ninhydrin colorimetric method was used to determine peptide concentration in the yogurts under study [18]. Fifty µL of aqueous extract of the yogurt were taken and placed in a test tube (previously shaking the extract in a vortex); 950 µL of distilled water were placed in the same test tube and proceeded to shake in the vortex for approximately 1 min; and 2 mL of the cadmium-ninhydrin reagent were placed (constant shaking). Finally, it was heated to 84 °C for 10 min; it was then allowed to cool to room temperature, and an intense pink color appeared, which shows the reaction. The samples were read at an absorbance of 507 nm with a UV–visible absorption spectrum. In parallel, a blank assay was performed. The results were calculated from the standard curve obtained with known concentrations of leucine, expressed in mg of leucine per 100 g of sample. Measurements were performed on days 0, 7, 14, and 21 to store the yogurts under study.

Statistical analysis

The experiment was carried out using three different batches of yogurt ($n = 3$). Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Duncan's test for comparison of means. The criterion for statistical significance was $p < 0.05$.

Results

Changes in pH and titratable acidity (%) during yogurt storage

Figure 1 shows the changes in pH and titratable acidity of the treatments added with *M. oleifera*, *S. hispanica*, the mixture (Mix), and the control. According to the results, the pH of all treatments decreased as storage time progressed. The pH of the control yogurt during storage ranged from 4.63 ± 0.06 on day zero to 4.14 ± 0.02 on day twenty-one. The pH of yogurts added with *S. hispanica* and *M. oleifera* showed the lowest values at the end of storage (4.10 ± 0.02 and 4.07 ± 0.00, respectively) compared to the control. The Mix treatment showed higher values than the control, and the treatments added with *M. oleifera* and *S. hispanica* at the end of storage time (4.89 ± 0.03 to 4.4 ± 0.01). As for titratable acidity, the results were expressed as the percentage of lactic acid in the yogurt. The control and the yogurt added with *M. oleifera* presented a similar tendency as storage time progressed, with 1. 39% ± 0.05 and 1.42% ± 0.00 of lactic acid, respectively. On the other hand, the treatment with *S. hispanica* presented the highest percentage of lactic acid (1.84% ± 0.02 of lactic acid), with a statistical difference ($p < 0.05$) between these treatments.

Effect of *M. oleifera* and *S. hispanica* on the content of total phenolic compounds in yogurt during storage

The content of total phenolic compounds in the treatments during the storage period is summarized in Table 1. From the beginning of the storage (day 0), marked differences were observed between treatments, with the yogurt added with the mixture of *M. oleifera* and *S. hispanica* (Mix) showing the highest phenolic compound content (196.25 ± 1.64 mg GAE/100 g yogurt), followed by *S. hispanica*, *M. oleifera*, and finally the control, which had lowest initial value (102.92 ± 2.81 mg GAE/100 g yogurt). During storage, most treatments exhibited an increase in phenolic compound concentration, though with different variation patterns. In the Control and *M. oleifera* treatments, values peaked at 14 days (212.46 ± 3.64 and 194.31 ± 2.02 mg GAE/100 g yogurt, respectively), followed by a slight decrease at 21 days. In contrast, *S. hispanica* and Mix treatments showed a continues increase through the storage period, with the Mix treatment reaching the highest vale at 21 days (313.78 ± 15.71 mg GAE/100 g yogurt). This results suggest that the addition of *S. hispanica* and *M. oleifera* to yogurt influences the stability and accumulation of phenolic compounds during storage, with a more pronounced effect in their combination.

Table 1 Total phenolic content of yogurt added with *M. oleifera*, *S. hispanica*, and Mix during different periods of storage

Storage time (days)	Total phenolic content (mg GAE/100 g yogurt)			
	Control	*M. oleifera*	*S. hispanica*	Mix
0	102.92 ± 2.81^{a}	133.70 ± 3.04^{b}	176.45 ± 1.95^{c}	196.25 ± 1.64^{d}
7	148.15 ± 0.16^{b}	161.15 ± 1.67^{b}	187.74 ± 8.42^{c}	232.33 ± 0.60^{d}
14	212.46 ± 3.64^{b}	194.31 ± 2.02^{b}	162.84 ± 10.28^{a}	280.96 ± 6.57^{d}
21	168.57 ± 3.97^{a}	174.35 ± 1.86^{a}	259.57 ± 6.15^{b}	313.78 ± 15.71^{c}

The values are presented as the mean value ± standard deviation. The significance level was present at $p < 0.05$

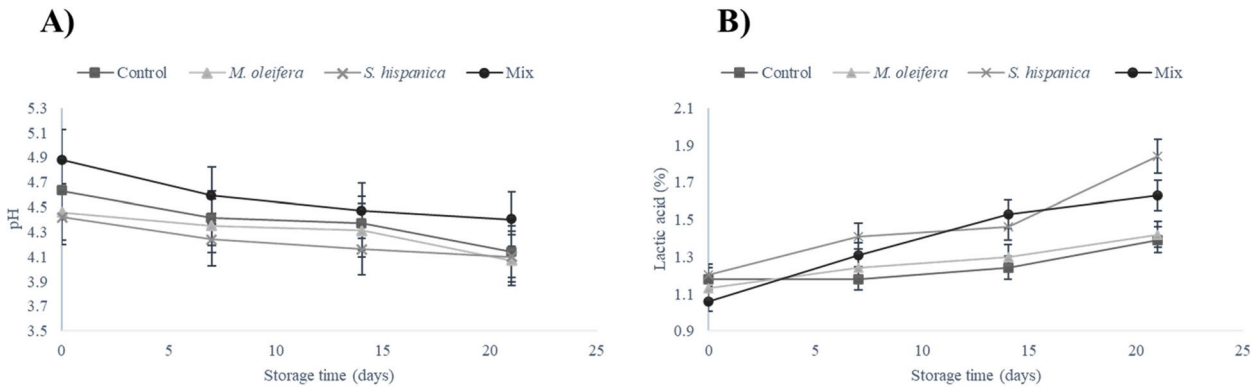

Fig. 1 **A** Changes in pH in yogurt added with *M. oleifera* and *S. hispanica* during storage. **B** Changes in acidity in yogurt added with *M. oleifera* and *S. hispanica* during storage. The value is presented as the mean value ± standard deviation. The level of significance was present at $p < 0.05$

Effect of *M. oleifera* and *S. hispanica* on in vitro antioxidant activity during yogurt storage

The antioxidant activity by the ABTS method of the treatments under study increased significantly ($p < 0.005$) during the storage time of 21 days (Fig. 2). In particular, the Mix yogurt obtained an inhibition of 97.03% at the end of the storage time, followed by the yogurt added with *S. hispanica* (93.35%), compared to the control and the yogurt added with *M. oleifera*, which obtained values of 66.03% and 90.40%, respectively.

The DPPH + radical inhibition activity presented significant differences ($p < 0.005$) during the storage period of the treatments (Fig. 3). At the beginning of storage, the treatments added with *M. oleifera* and *S. hispanica*, and the Mix treatment presented a higher percentage of radical inhibition compared to the control (15%, 7.20%, and 18.00%, respectively). During the 21 days of storage of the treatments, the highest values of radical inhibition were presented in the yogurt added with *M. oleifera*, which showed a percentage of 23.15% on day 14 of storage, while the Mix yogurt obtained a percentage of 20.50% on day 7 of storage. After 21 days of storage, the radical inhibition activity was reduced in the treatments with *M. oleifera* and *S. hispanica* and in the control.

Effect of *M. oleifera* and *S. hispanica* on the viability of lactic acid bacteria (LAB) during storage

The results obtained for lactic acid bacteria during the storage period of the yogurts under treatment are shown in Fig. 4. The viability of lactic acid bacteria was significantly ($p < 0.05$) affected by adding *M. oleifera* and *S. hispanica* during yogurt storage. The yogurt added with *M. oleifera* presented an increase in lactic acid bacteria content from 2×10^7 CFU/mL on day 0 of storage to 3.0×10^7 CFU/mL on day 14, compared to the control. The yogurt added with *S. hispanica* and the Mix yogurt showed a decrease in CFU after day 14 of storage with values of 3×10^{-6} and 9×10^{-6} CFU/mL, respectively, on day 21 of storage. As for the control, it did not present significant differences ($p < 0.05$) during the 21 days of refrigerated storage.

Effect of *M. oleifera* and *S. hispanica* on peptide concentration during storage

Regarding the concentration of peptides in Control, *M. oleifera*, *S. hispanica*, and Mix yogurts, the results were expressed in leucine concentration in mg leucine/100 g yogurt sample, as observed in Fig. 5. According to the results presented, the leucine values in the control, *M. oleifera*, *S. hispanica*, and Mix treatments on day 0 of storage were 19.42 ± 1.04, 25.67 ± 1.17, 23.95 ± 1.41 and 27.09 ± 2.95 mg leucine/100 g yogurt, respectively. The control yogurt and the yogurt added with *M. oleifera* presented low values of leucine concentration (22.31 ± 1.17 and 19.03 ± 0.24 mg leucine/100 g yogurt sample, respectively) at the end of storage, while the Mix yogurt and the yogurt added with *S. hispanica* presented higher concentrations of leucine (34.71 ± 1.33 and 27.94 ± 0.43 mg leucine/100 g yogurt, respectively).

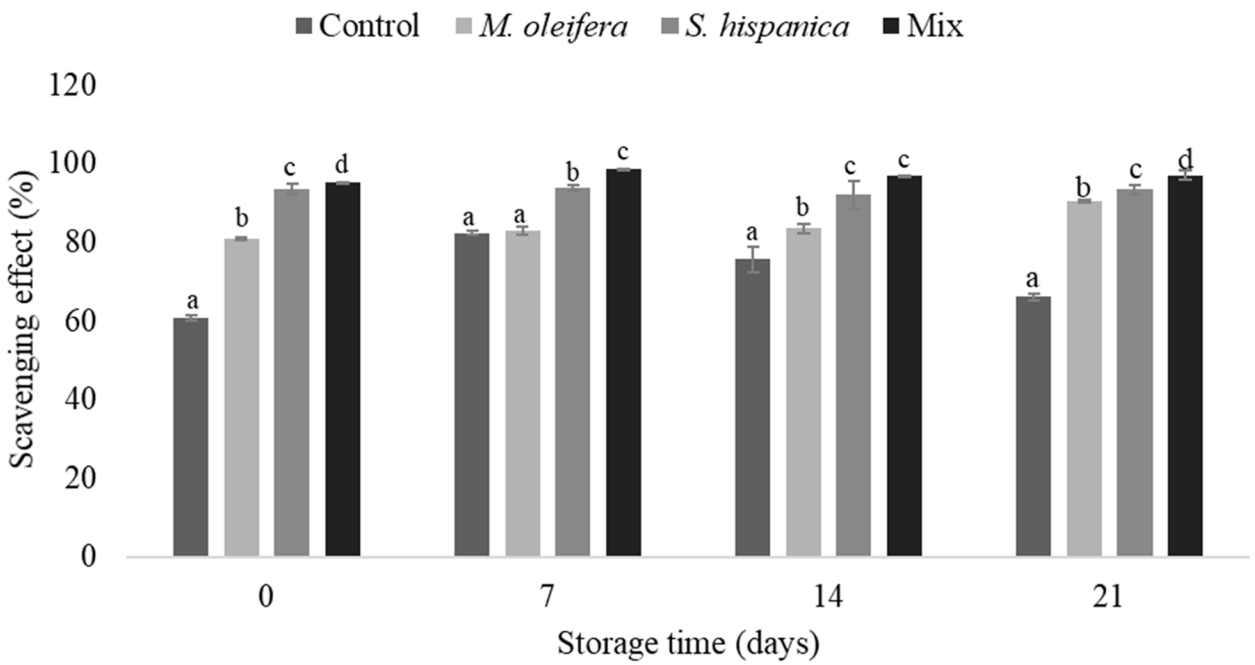

Fig. 2 Total antioxidant activity (ABTS %) of plain yogurt and yogurt with *M. oleifera* and *S. hispanica* during 21 days of storage. The value is presented as the mean value ± standard deviation. The level of significance was present at $p < 0.05$

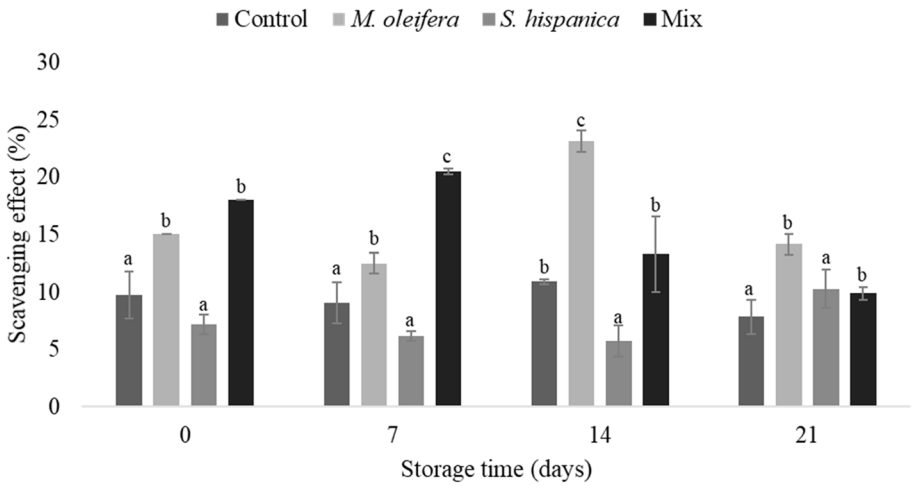

Fig. 3 Total antioxidant activity (DPPH %) of plain yogurt and yogurt with *M. oleifera* and *S. hispanica* during 21 days of storage. The value is presented as the mean value ± standard deviation. The level of significance was present at $p < 0.05$

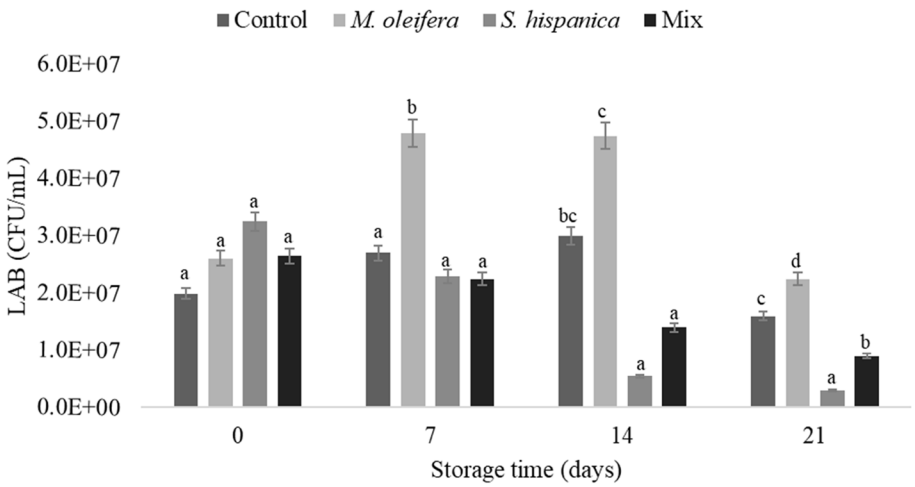

Fig. 4 Changes in lactic acid bacteria count in plain yogurt and the yogurt added with *M. oleifera* and *S. hispanica* during 21 days of storage. The value is presented as the mean value ± SD. The level of significance was present at $p < 0.05$

Discussion

Changes in pH and lactic acid content (%) during yogurt storage

During the storage period, a post- acidification was observed (Fig. 1), characterized by an increased in lactic acid content and a decrease in pH over time. The treatment added with *S. hispanica* reached the highest lactic acid concentration compared to the other treatments. According to Shori [55] and Shori et al. [56], this phenomenon occurs due to the residual metabolic activity of lactic acid bacteria (LAB) in yogurt. The decrease in pH aligns wo the findings of Robinson [47], who reported that after 3 to 4 weeks of cold storage, yogurt reaches pH values around 4.3 to 4.5, at which point the growth and metabolism of *S. thermophilus* are inhibited by acidic conditions. This behavior is consistent with other studies on yogurts enriched with plant extracts, such as those by Aydemir & Altun [4] and Tamjidi et al. [60], who attribute

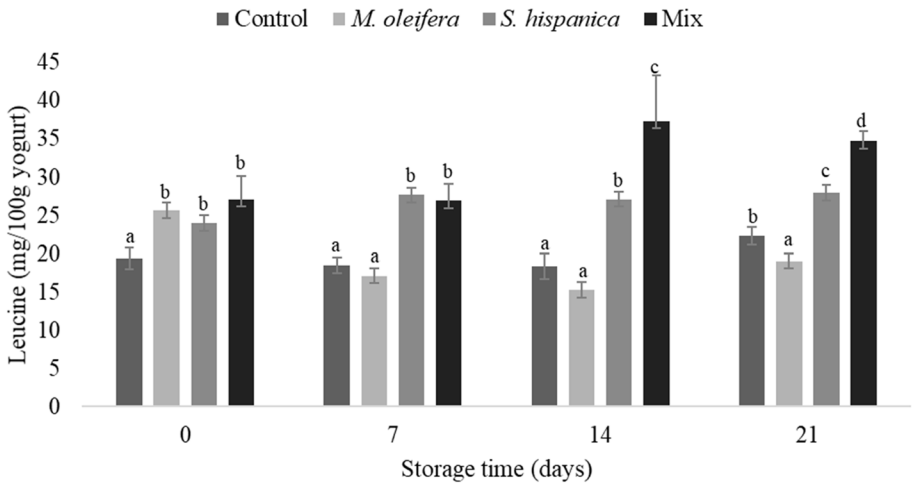

Fig. 5 Peptide concentration of plain yogurt and yogurt added with *M. oleifera* and *S. hispanica* over 21 days of storage. The value is presented as the mean value ± standard deviation. The level of significance was present at $p < 0.05$

this effect to the continuous production of lactic acid by yogurt cultures. Similarly, [50] observed the same pattern in his study on yogurt fortification with *M. oleifera* When using leaf powder, a significant pH decrease was recorded after 15 days of storage, a trend comparable to our findings with dry *M. oleifera* extract.

Effect of *M. oleifera* and *S. hispanica* on the content of total phenolic compounds in yogurt during storage

The yogurt Mix exhibited the highest total phenolic compound content, followed by yogurt supplemented with *S. hispanica* at the end of the storage period (Table 1). The increase in phenolic compounds in yogurts enriched with non-dairy ingredients (*M. oleifera* and S. *hispanica*) can be attributed to the bioactive compounds naturally present in these ingredients, such as flavonoids (quercetin, kaempferol) and hydroxycinnamic acids (gallic, caffeic, and chlorogenic acid) [29, 40]. Similar studies have reported that yogurt fortified with herbal extracts enhances and stabilizes the phenolic compound content, particularly during storage [14]. Helal et al. [23] found that coffee-fortified yogurt exhibited a significant increase in phenolic compound content compared to the control, which was attributed to the presence of these bioactive compounds. On the other hand, some studies have reported a decrease in total phenolic compound stability during storage., this decline appears to be associated with yogurts supplemented with fruit juices, as certain extracts may be more susceptible to degradation under cold storage conditions [16, 61]. Therefore, the use of *S. hispanica* and *M. oleifera*, either individually or in combination, appears to be a suitable strategy for increasing the phenolic compound content in yogurt.

In the case of the control yogurt, the increase in phenolic compounds during storage is likely due to the presence of compounds resulting from milk protein breakdown. Specifically, the amino acid tyrosine has a phenolic side chain in its structure, which contributes to the total phenolic compound content in yogurt [59]. Several studies have also reported that the Folin-Ciocalteu reagent reacts with molecules beyond polyphenols [46] including proteins, carbohydrates, amino acids, nucleotides, certain fatty acids, aldehydes, and ketones [20].

Effect of *M. oleifera* and *S. hispanica* on antioxidant activity in vitro during yogurt storage

Antioxidant activity increased in the treatments supplemented with *M. oleifera* and *S. hispanica*, with the yogurt Mix showing the highest inhibition. *M. oleifera* contains bioactive metabolites such as flavonoids (catechin, rutin and quercetin) and hydroxycinnamic acids (gallic, caffeic, and chlorogenic acid) [9, 22, 43], while *S. hispanica* is rich in omega-3 fatty acids and phenolic compounds [29, 37, 70]. This suggests that the increased antioxidant activity observed in treated yogurts is due to these bioactive properties. Several authors suggest that this increase in antioxidant activity is due to the presence of plant-derived secondary metabolites, with similar findings reported by Farag et al. [21] and Kwon et al. [30]. These studies demonstrated that the incorporation of *S. hispanica* seeds and *M. oleifera* leaves enhanced and enriched the antioxidant properties of yogurt, particularly during cold storage. Similarly, Amirdivani & Baba [3] and [53] found that yogurts supplemented with plant extracts exhibited significantly higher antioxidant activity from the seventh day of storage. However, variations in

antioxidant activity measured by the DPPH assay depend on several factors. One key aspect is that high-molecular-weight compounds present in the yogurt matrix may not effectively react with the DPPH radical, whereas low-molecular-weight molecules have lower steric hindrance, allowing for greater interaction [46]. Additionally, [12] reported a decrease in radical inhibition activity after 14 days of yogurt storage, which aligns with the findings of this study, specifically the observed that DPPH radical inhibition in yogurt supplemented with grape and stem extracts declined between 1.16- to 3.78-fold during storage.

Effect of *M. oleifera* and *S. hispanica* on the viability of lactic acid bacteria (LAB) during storage

The viability of LAB in yogurt can be influenced by the type of natural extract added. Mediza Romero et al. [34] reported that the addition of green tea did not negatively impact LAB viability, maintaining levels above the recommended threshold of 10^7 CFU/mL throughout storage. Similarly, the addition of *S. hispanica* powder and *M. oleifera* dry extract in this study did not adversely affect LAB viability for most of the storage period, also maintaining the recommended 10^7 CFU/mL threshold. In fact, an increase in microbial viability was observed in yogurt supplemented with *M. oleifera*, which may be attributed to the ability of certain lactic cultures to utilize plant-derived compounds during fermentation and storage. These include small peptides, vitamins, minerals, reducing sugars, and some acetates, which can enhance bacterial viability in dairy products [54, 55]. Similarly, Ye et al. [68] found that fortifying yogurt with Korean *Litsea* tea significantly increased LAB counts and even improved sensory characteristics.

On the other hand, the observed decline in bacterial viability in yogurt supplemented with *S. hispanica* and the yogurt mix may be attributed to the accumulation of organic acids (such as lactic acid), dissolved oxygen levels, and hydrogen peroxide [32]. According to Robinson [47], *S. thermophilus* viability tends to decrease when pH levels drop to 4.3–4.5 by the third or fourth week of storage. Therefore, the reduction in LAB counts in yogurt supplemented with *S. hispanica* and the yogurt mix is likely due to post-acidification during storage. This aligns with previous results in this study, where both yogurt mix and yogurt containing *S. hispanica* exhibited high lactic acid concentrations (Fig. 1B) and low pH values (Fig. 1A).

Effect of *M. oleifera* and *S. hispanica* on peptide concentration during storage

Control yogurt and yogurt supplemented with *M. oleifera* exhibited lower leucine concentrations at the end of storage, whereas yogurt Mix and yogurt with *S. hispanica* showed higher leucine levels (Fig. 5). Muniandy et al. [39] and Citta et al. [13] reported that yogurt is a source of bioactive peptides, formed during the fermentation process, which may contribute to antioxidant activity, these peptides can act as"sacrificial"antioxidants. For instance, Abdel-Hamid et al. [1] demonstrated that yogurts fortified with *Rubus suavissimus* extract exhibited increased bioactive peptide concentrations and improved antioxidant activity. Likewise, Wang et al. [65] suggested that certain plant extracts may enhance the overall peptide profile of yogurt during storage. However, it is important to note that peptide concentration can be influenced by several factors, including storage conditions, pH, and the specific natural extracts used. This was evident in the treatment containing *M. oleifera*, where a lower peptide concentration was observed.

Conclusions

This study evaluated the effects of *Moringa oleifera* and *Salvia hispanica* (chia) additions on yogurt during 21 days of storage. The results showed that both ingredients enhanced total phenolic content, antioxidant activity, and peptide concentration, while also affecting pH levels and lactic acid bacteria (LAB) viability. Notably, the combination of both ingredients (Mix) exhibited the highest antioxidant activity and phenolic content, along with increased peptide concentrations and improved LAB viability, particularly up to day 14. These findings suggest that the incorporation of *M. oleifera* and *S. hispanica* into yogurt can significantly enhance its functional benefits, contributing to both its nutritional quality and stability during storage. The potential for the commercial production of functional yogurts enriched with *M. oleifera*, *S. hispanica*, or their Mix lies in the growing consumer demand for functional foods that offer added health benefits. These ingredients, with their bioactive compounds, could provide a valuable contribution to the development of new functional foods. Future research should focus on in vivo trials to further validate the antioxidant effects and explore the optimization of the production process. This would help to bring these biofunctional yogurts to a broader market, supporting the ongoing interest in functional foods that improve health and well-being.

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Authors' contributions

The first and second authors wrote the main text of the manuscript. The third and fourth authors have prepared figures and tables. Fifth and sixth authors monitored the results of experiments. All authors reviewed the manuscript.

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