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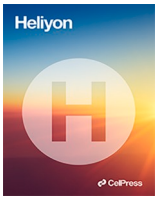

Research article

Fortification of yogurt with mulberry leaf extract: Effects on physicochemical, antioxidant, microbiological and sensory properties during 21-days of storage

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ABSTRACT

Mulberry leaves are medicinal and edible, with many physiological functions. To improve the potential function of yogurt, the effects of mulberry leaf extract (MLE) incorporation on the fermentation kinetics, physicochemical, antioxidant properties, and sensory parameters of yogurt were evaluated. The results showed that 0.1–0.3 % MLE improved the acidification rate and shortened the fermentation process. The addition of MLE significantly increased the values of total titratable acids, water holding capacity (WHC), total phenolic content and antioxidant capacities of the yogurt ($p < 0.05$). Specifically, the WHC values of 0.1 % MLE added yogurt were 1.33–1.41 times that of the control over 21 days of storage. In addition, MLE changed the texture and sensory quality of yogurt, resulting in light green, more stable products. Compared to the control, the yogurt with an appropriate concentration of MLE (0.1 % and 0.2 %) showed stable microbiological properties, and the survival of lactic acid bacteria in the yogurt was able to maintain a stable probiotic count of 10^8 CFU/g over 21 days of shelf life. The yogurt containing 0.1 % MLE achieved a good balance between the physicochemical and sensory qualities of the yogurt, and the use of MLE as an ingredient in yogurt production was a step towards the development of healthier dairy products.

1. Introduction

Mulberry trees are commonly cultivated in China, South Korea, Thailand, and India. Mulberry leaves are a traditional food for local people, and are also an important medicinal plant in Asia. In traditional Chinese medicine, mulberry leaves are used to treat fever and diabetes [1,2]. The leaves have been found to be rich in phenolic compounds with powerful antioxidant properties [1,3]. Zou et al. investigated the digestion and absorption characteristics of polyphenols and polysaccharides from mulberry leaves, identifying their

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synergistic regulatory effects on glucose and lipid metabolism [4]. Natural organic compounds in mulberry leaves have also been reported to play roles in anti-inflammatory [5] and neuroprotective activities [6]. At present, mulberry leaves are widely used in the development of various health foods, such as mulberry leaf tea and mulberry leaf carbonated beverages [7].

Yogurt is a fermented milk product typically made from cow's milk and produced through pasteurization, followed by the addition of a starter culture, fermentation, post-ripening, and other processes. Fermented dairy products are excellent carriers of probiotics and serve as effective sources of these beneficial bacteria for consumers. In addition, yogurt is rich in micronutrients, effective in reducing or preventing diseases associated with nutritional deficiencies [8]. The increase in yogurt consumption in the diet is accompanied by a sufficient intake of lactic acid bacteria and a nutrient-rich matrix. Recent research [9] has shown that yogurt improves immune function through gut-mediated prevention. From the perspective of the food-gut health axis, non-digestible carbohydrates and bioactive ingredients (e.g., quercetin, kaempferol, apigenin, catechins, etc.) have been added to yogurt as functional ingredients. Traditional fermented dairy products are gradually evolving into plant-based functional alternatives. Noori et al. [10] investigated the potential prebiotic activity of rye sprout extract and its antimicrobial activities and sensory characteristics in synbiotic yogurt. Nazari et al. [11] prepared yogurt using an extract of germinated black cumin (*Nigella sativa* L.) seeds to improve the quality and bioactive properties. Tian et al. [12] prepared functional yogurt added with insoluble dietary fiber from soya bean dregs, which demonstrated significant potential for sodium cholate adsorption capacity and antioxidant activity. Sharifi et al. [13] prepared and incorporated carrot waste extract powder into yogurt production. The carrot extract increased the total phenolic content and antioxidant capacity of yogurt. Recently, mulberry leaves have attracted increasing attention as functional plant materials. Zhao et al. [14] reported that mulberry leaf polyphenols improved gut microbiota, and Yang et al. [15] found that mulberry leaf polysaccharides acted as prebiotics to promote the proliferation of plant lactobacilli. Currently, few studies have explored the use of mulberry leaf or mulberry leaf extracts in yogurt preparation, indicating that mulberry leaf has potential for development in functional milk products.

Normally, the addition of natural plant ingredients, such as dried grape juice powder [16], pineapple pomace powder [17], and lentil (*Lens culinaris*) flour [18], enhances the flavor of yogurt. However, mulberry leaves have a strong grassy odor and astringent taste; therefore, the direct addition of mulberry leaf powder will have an adverse effect on the sensory quality of the yogurt. Our previous research found that yogurt supplemented directly with mulberry leaf powder showed significant whey separation after two weeks of storage at 4 °C. To improve flavor and antioxidant properties, Wang et al. [19] incorporated an aqueous extract obtained from steamed or roasted mulberry leaves into yogurt. However, ethanol extraction was reported as a more effective method to obtain polyphenols, flavonoids, and other compounds from mulberry leaf [20]. For maximum recovery of phenolics and other functional components, an ethanol extract was prepared from mulberry leaf powder and incorporated into yogurt in this study. Thus, this study aims to evaluate the changes in the physicochemical properties, antioxidant capacity, microbiological characteristics, and sensory attributes of yogurt supplemented with mulberry leaf ethanol extract over a 21-day storage period.

2. Materials and methods

2.1. Materials and chemicals

Mulberry leaf powder was purchased from Anhui Huaiqitang Pharmaceutical Co., Ltd. (Bozhou, Anhui, China). Skimmed milk powder (protein content: 32.9 %) was purchased from New Zealand Anchor Milk Brand Co., Ltd (Shanghai, China). The yogurt strains (YO-MIX 863) were purchased from Danisco Co., Ltd. (Shanghai, China). 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) was purchased from TCI Chemical Industry Development Co., Ltd. (Shanghai, China). Other analytical-grade chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China)

2.2. Preparation of MLE

MLE was prepared according to Katsube et al. with minor modifications [20]. 25 g of mulberry leaf powder (200 mesh) was added to 500 mL of 40 % ethanol solution, and then fully mixed. The suspension was continually treated by ultrasonic at 40 kHz frequency, 110 W constant powers and 40 °C for 30 min. Then the suspension was centrifuged at 8000 rpm for 10 min, and the separates was retained, while the precipitate was extracted again. The twice supernatant was firstly rotary evaporated at 40 °C and then freeze-dried. The freeze-dried extract was ground into powder, sealed, and stored at −20 °C for later use.

2.3. Preparation of yogurt

Twelve grams (12 g) of skimmed milk powder was dissolved in 100 mL of distilled water by gentle stirring until fully dissolved. The 12 % (m/v) milk solution was sterilized at 121 °C for 10 min, then cooled. The bacterial powder containing *Streptococcus thermophiles* (*S. thermophillus*) and *Lactobacillus bulgaricus* (*L. bulgaricus*) was added to the above milk solution according to the instructions from Danisco Co., Ltd. (Shanghai, China). The mixture was incubated at 42 °C until the milk was fully curdled, then placed at 4 °C as starter culture. Reconstituted milk containing 12 % (m/v) milk powder, 8 % (m/v) sucrose, and different concentrations of mulberry leaf extract (0.1 %, 0.2 %, and 0.3 % (m/v)) was prepared using distilled water as a solvent. The reconstituted milk was gently stirred until fully dissolved and then pasteurized at 95 °C for 5 min. After cooling to 40–45 °C under sterile conditions, the reconstituted milk was added with 2.5 % starter culture to ferment at 42 °C for 4.5 h and then kept it at 4 °C for post-ripening for about 20 h to obtain MLE yogurt. The sample without MLE was the control yogurt. All samples were stored at 4 °C for further analysis over 1, 5, 9, 13, 17 and 21 days of storage.

2.4. Determination of pH during acid solidification

The pH was determined every 10 min for 10 h during the yogurt acid solidification by Rex pHS-25 pH-meter (INESA Scientific Instrument Co., Ltd. Shanghai, China).

2.5. Physicochemical characteristics of yogurt during the storage

2.5.1. Determination of pH and titratable acidity (TA)

The pH of different groups' yogurt was measured by Rex pHS-25 pH-meter.

The TA of yogurt was determined according to AOAC standard (AOAC, 2005) [21] with minor modifications. Briefly, 1.00 g of yogurt (M_1) mixed with 20 mL of distilled water and two drops of phenolphthalein indicator, and then was titrated with NaOH standard solution (0.1 mol/L, C_1) in a conical flask. During the titration, the conical flask was shaken while adding NaOH standard solution dropwise until the solution changed from milky white to light red (without fading within 30 s), which can be regarded as the end point of the titration. The volume of NaOH standard solution consumed was recorded as V_1. Finally, 20 mL of distilled water was titrated in the same way and the volume of NaOH standard solution consumed was record as V_0.

The TA was calculated as Eq (1):

$$TA\ (^{\circ}T) = C_1 \times (V_1 - V_0) \times 100/(M_1 \times 0.1) \quad (1)$$

2.5.2. Determination of water-holding capacity (WHC)

The determination of WHC of yogurt was according to the method described by Nazari et al. [11]. A yogurt sample was weighed and recorded as a_1, then was centrifuged at 4000 rpm, 4 °C for 15 min to obtain the sediment, and the weight of sediment was recorded as a_2. The WHC was calculated as Eq (2):

$$WHC(\%) = (a_2/a_2) \times 100 \quad (2)$$

2.5.3. Texture profile analysis (TPA)

The texture of yogurt was evaluated by TA.XTC-18 Texture Analyzer (Bosin Tech. Co., Ltd. Shanghai, China). The method was according to Meena et al. with minor modifications [17,22]. The gelatin probe (TA/0.5) was used with parameters as follows: trigger force was 3.00 gf, deformation degree was 10 %, initial height was 50.00 mm, pre-test speed was 3.00 mm/s, test speed and post-test speed were 0.50 mm/s. Hardness refers to the maximum force value perceived by the probe during the initial compression cycle. Adhesion refers to the negative area under the compression curve, indicating the sample's ability to adhere to the probe. Springiness denotes the ability of semi-solid yogurt samples to deform under external force and return to their original state upon removal of the force. Gumminess describes the force necessary to break down a semi-solid yogurt sample into a swallowable state. The above parameters are automatically calculated by the instrument's built-in software.

2.5.4. Determination of color parameters

The color parameters were measured by NS800 spectrophotometer (3nh Tech. Co., Ltd. Shenzhen, China). The results were recorded as L*, a* and b*. L* indicates the brightness of the image, a* indicates red (positive values) and green (negative values), and b* indicates yellow (positive values) and blue (negative values) [23].

2.5.5. Determination of total phenolic content

The determination of total phenolic content referred to method described by Zhuo et al. [24] with minor modifications. Quantitative determination was performed based on the standard curve, and the standard curve was made of gallic acid (GA) solutions (0, 0.025, 0.05, 0.10, 0.15, 0.20 mg/mL). The regression equation: $y = 2.2034x + 0.0066$, $R^2 = 0.999$. The final phenolic content was expressed in mg/100 g (yogurt).

2.6. Evaluation of antioxidant capacity

2.6.1. Determination of total reducing power

The total reducing power was determined according to the method of Zhuo et al. [24] with slight modification. 3.00 g yogurt was added to 7.00 mL distilled water, and then well mixed and centrifuged at 4000 rpm for 15 min (4 °C), and the supernatant was obtained as yogurt extract. 1.00 mL of yogurt extract was mixed with 2.50 mL of 0.2 mol/L sodium phosphate buffer (pH 6.6) and 2.50 mL of 1 % potassium ferricyanide. The mixture was incubated at 50 °C for 30 min. Next, 2.50 mL of 10 % trichloroacetic acid was added to the mixture and centrifuged at 4000 r/min for 5 min. The upper layer (2.00 mL) of the solution was mixed with 2.00 mL distilled water and 0.40 mL of 0.1 % of ferric chloride. After that, the absorbance of the yogurt sample (A_S) was measured at 700 nm. The absorbance of the blank control (A_0) was measured at 700 nm by the same method with distilled water replaced yogurt sample. Each assay was performed in triplicate. The total reducing power was calculated using Eq (3):

$$Total\ reducing\ power = A_S - A_0 \quad (3)$$

2.6.2. Determination of superoxide anion radical scavenging capacity

The superoxide anion radical scavenging capacity was determined by method of Hao et al. with slightly modified [25]. Firstly, 4.50 mL of 50 mmol/L Tris-HCl buffer (pH 8.2) was in a water bath at 25 °C for 10 min, then 1.00 mL of yogurt extract and 0.40 mL of 25 mmol/L o-benzyltriphenol were added and well mixed, and then the reaction was continued for 15 min in a water bath at 25 °C, and 1 mL of 0.1 mol/L HCl was added to terminate the reaction. Shaking well and standing for 3 min, then the mixture was centrifuged at 4000 r/min for 15 min, and the absorbance of the supernatant was measured at 420 nm (A_S). The absorbance with distilled water replaced yogurt extract was measured at 420 nm by the same method was A_{max}, and the absorbance of the supernatant of yogurt sample at 420 nm was A_0. The superoxide anion scavenging rate was calculated using Eq (4):

$$Rate\ of\ superoxide\ anion\ radical\ (\%) = \left(1 - \frac{As - A_0}{A_{max}}\right) \times 100 \tag{4}$$

2.6.3. Determination of DPPH-radical scavenging capacity

DPPH-radical scavenging ability was measured by the method described by Jin et al. with minor modifications [26]. Briefly, DPPH standard solution, and yogurt extract were mixed together. The mixture was reacted for 30 min in the darkness, and then measured at 517 nm, and the absorbance was recorded as A_i. The absorbance of the DPPH solution plus methanol was recorded as A_0, and the absorbance of the yogurt extract plus methanol was recorded as A_j. The DPPH-radical scavenging rate was calculated as Eq (5):

$$DPPH\ radical\ scavenging\ (\%) = \left(1 - \frac{A_i - A_j}{A_0}\right) \times 100 \tag{5}$$

2.7. Total viable bacteria count

The method of Lactobacillus colony count was referred to Padilha et al. [27] with some modification. *L. bulgaricus* was counted on MRS agar (Merck, Darmstadt, Germany) and incubated under anaerobic conditions at 37 °C for 72 h. Anaerocult (Merck) was used to generate anaerobic conditions. *S. thermophilus* was counted on M 17 agar and incubated aerobically at 37 °C for 48 h (Merck). Bacterial counts were determined in triplicate and expressed as lg CFU/g.

2.8. Sensory evaluation

The sensory evaluation criteria referred to the description of Noori et al. [10] with minor adjustments, mainly from the five aspects of yogurt flavor, taste, texture, color and overall acceptability. Each item is set to 1 (extremely disliked) - 10 (extremely liked), and the details of the reference for sensory evaluation was listed as a supplementary material. The sensory evaluation team consisted of 16 pre-trained adults (8 males and 8 females) and the yogurt evaluated was made the day before.

2.9. Statistical analysis

All experiments were carried out in triplicate (n = 3) and results were expressed as mean ± SD. Statistical analysis was performed using unpaired 2-tailed Student's t-tests or ANOVA (S-N-K test) by SPSS 23.0 software. Correlations were analyzed with Pearson's correlation coefficient.

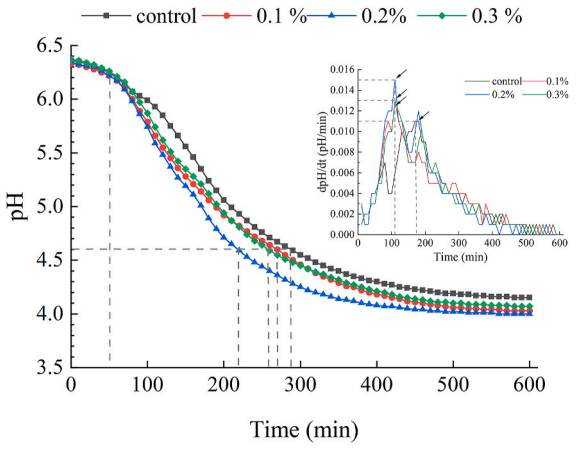

Fig. 1. Effects of MLE on yogurt pH during fermentation.

3. Results and discussion

3.1. Effects of MLE on the fermentation kinetics of yogurt

The yogurt with different concentrations of MLE was prepared according to the method described in section 2.3. The pH changes were measured every 10 min during yogurt production, and the results showed in Fig. 1.

As shown in Fig. 1, the initial pH of all groups was about 6.35, and then the pH of the yogurt with 0, 0.1 %, 0.2 %, and 0.3 % MLE were all decreased during yogurt preparation process. The pH of yogurt with 0.2 % MLE decreased fastest, and reached 4.6 in 220 min which was the isoelectric point of casein. The time of the yogurt with 0, 0.1 %, and 0.3 % MLE reached pH 4.6 was 270 min, 260 min and 290 min, respectively. Compared with the control, the pH of the yogurt with MLE decreased faster, and the maximum value of dpH/dt of 0.1 %, 0.2 %, and 0.3 % MLE yogurt was 0.013, 0.015, and 0.013 respectively, which appeared at the 110th min (that of the control yogurt was 0.011 at the 170th min). The dpH/dt value represents the acidification rate of yogurt fermentation. Compared with the control, the Vmax values of 0.1 %, 0.2 %, and 0.3 % MLE-added yogurt increased by18.2 %, 36.3 %, and 18.2 %, respectively. Due to the presence of organic acids, phenolic acids and flavonoids in the plant extracts which promote the growth of lactobacilli and facilitate a rapid decrease in pH, the addition of plant extracts such as chia seed [28] and moringa [29] resulted in a rapid decrease in pH during yogurt fermentation. These results were similar with our results. Additionally, excessive addition of extracts led to a decrease in Vmax. The Vmax value of yogurt with 0.3 % MLE was smaller than that of samples added 0.2 % MLE, which was consistent with Ning's research on passion fruit juice yogurt [22]. In this study, after 10 h' fermentation, the pH of yogurt with 0, 0.1 %, 0.2 %, 0.3 % MLE was 4.15, 4.03, 4.00, and 4.07, respectively. The addition of MLE improved the fermentation of the yogurt, and accelerated the acidification of yogurt.

3.2. Effects of MLE on quality characteristics and functional properties of yogurt during the storage

3.2.1. pH and titratable acidity (TA)

Yogurt was prepared after 4.5 h' fermentation and 20 h' post-ripening at 4 °C. The pH and titratable acidity of yogurt containing different concentrations MLE were determined. The results showed that the pH were 4.62, 4.50, 4.46, 4.48 when the concentration of MLE were 0, 0.1 %, 0.2 %, 0.3 %, respectively, and the titratable acidity were 75.1 °T, 80.8 °T, 87.2 °T, 104.5 °T, respectively (Fig. 2). The yogurt was stored at 4 °C for 21days, and the pH of the yogurt decreased throughout the storage. The pH of yogurt added MLE was significantly lower than that of the control yogurt ($p < 0.05$) (Fig. 2a). The more MLE was added, the lower the pH of yogurt. The TA of the yogurt increased throughout the storage and consistently remained above the general requirement of 70°T for yogurt acidity. The TA of the yogurt added MLE was significantly higher than that of the control yogurt ($p < 0.05$), and the TA increased with the increase of MLE content (Fig. 2b). The TA of yogurt with 0.3 % MLE reached 104.5°T. The decrease in pH and increase in TA of yogurt by adding MLE may be attributed to the improvement of MLE on the growth of lactic acid bacteria (LAB). It was reported that MLE has potential to improve the growth of LAB to increase the acid content of yogurt, which also inhibits the growth of miscellaneous bacteria in yogurt [30].

3.2.2. Water-holding capacity (WHC)

The changes in WHC of yogurt during the storage were showed in Fig. 3. During the storage, the WHC of all MLE-fortified yogurt remained higher than that of the control till 17 days. However, 0.3 % MLE-added yogurt decreased to be lower than the control. The

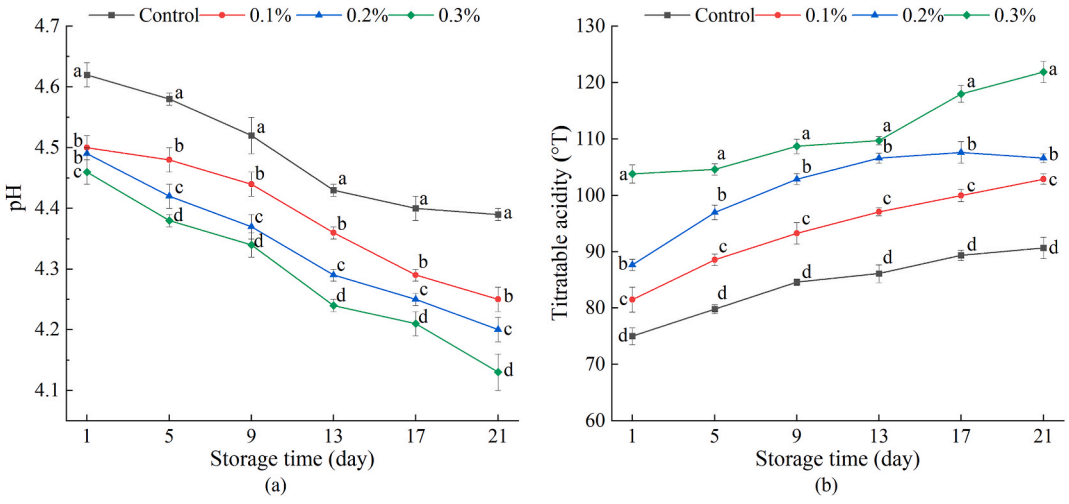

Fig. 2. Effects of MLE on the pH (a) and acidity (b) of yogurt during storage. Different letters within the same column represent statistically significant differences ($p < 0.05$).

addition of MLE increased WHC of the yogurt on day 1 of storage. The highest WHC was observed with 0.1 % MLE yogurt (64.6 %), which was 15.9 percentage units higher than the control (48.7 %). On the 21st day of storage, the WHC values of the yogurt fortified with 0.1 % and 0.2 % MLE were 53.8 % and 44.9 %, respectively, while that of the control and 0.3 % MLE were 39.6 % and 36.6 %, respectively. Similarly, Mohamed Ahmedet al. [31] reported that the WHC value of yogurt containing 0.1 % argel leaf extract was greater than that of yogurt containing 0.2 % extract and the control yogurt, respectively. The enhancement of WHC by MLE may be due to the interaction of certain components in MLE with yogurt protein, which could make the yogurt gel matrix stronger and hold more whey. MLE contains mulberry leaf polysaccharides composed of rhamnose, galactose, glucose, galacturonic acid and glucuronic acid [32]. Polysaccharide molecules readily bind with water molecules, thereby reducing the water content within the casein gel network and increasing water holding capacity. Additionally, polysaccharides may increase the water holding capacity of casein micelles by interacting with casein [33]. MLE is rich in phenolics, which may react with proteins in yogurt to improve the affinity between milk proteins. Dönmez et al. reported that green coffee seeds were added to yogurt to stabilize the gel structure to improve syneresis [34]. Chia seed is rich in phenolics, which was significantly increased the viscosity and WHC in yogurt [28]. However, an excess of these components may play a negative role in the stability of the protein gel network. Moreover, the low pH of high concentration MILE yogurt will weaken the gel structure of the yogurt, resulting in a lower water holding capacity compared to low concentration MILE yogurt. In the later stages of storage, the pH of 0.3 % MLE yogurt deviated significantly the isoelectric point of casein, causing an increase in electrostatic repulsion forces and destabilizing the gel network, leading to a rapid decrease in water holding capacity. WHC is an important indicator of structural stability of yogurt, which represents the ability to hold various small molecules in the system after high-speed centrifugation. Therefore, supplementation with MLE improves the physical characteristics of yogurt by increasing the content of active components and stabilizing the gel matrix in yogurt.

3.2.3. *Texture profile analysis (TPA)*

MLE significantly affected the texture profile (Hardness, Adhesiveness, Springiness, and Gumminess) of yogurt. Specifically, the hardness of yogurt was negatively correlated with the amount of MLE. Both hardness and adhesion can reflect the force felt in the mouth during chewing. In particular, hardness is an important parameter for the texture of yogurt, indicating the strength of the yogurt structure. As indicated in Table 1, during 21 days of storage, the hardness of yogurt in the same group gradually increased, likely due to the decrease in pH leading to the contraction of the gel structure. The hardness of the control yogurt was significantly higher than that of the MLE-added yogurt throughout the storage ($p < 0.05$). These results are similar to those observed in yogurt with added quince seed mucilage powder [35], argel leaf extract [31], and Pu-Erh tea extract [36]. Dietary fiber, organic acid and other substances in these exogenous substances may weaken the gel structure of yogurt protein [22]. The high water-holding capacity of MLE yogurt also makes the texture of the protein gel soft [31].Both adhesiveness and springiness are positively correlated with the amount of MLE added. Adhesiveness is also an important parameter of yogurt texture, representing the adsorption force experienced by the probe during the process of detachment from the yogurt. A high adhesiveness parameter indicates a strong binding strength of internal chemical bonds in yogurt, which may be due to the formation of protein-polyphenol hydrogen bonds between polysaccharides, polyphenols and milk proteins in MLE. Springiness represents the ability of yogurt to recover its shape, indicating the integrity of its texture. The interaction between MLE polysaccharides and polyphenols and proteins helps to rearrange the protein network [31].

3.2.4. *Color parameter analysis*

The color of yogurt is related to the freshness of the product and also influences consumer recognition. The results of color changes of yogurt during the storage were showed in Table 2. Compared with the control yogurt, the color properties of MLE-added yogurt were significantly different. Due to the obvious green color of MLE, the greenness of the MLE-added yogurt increased, and the color

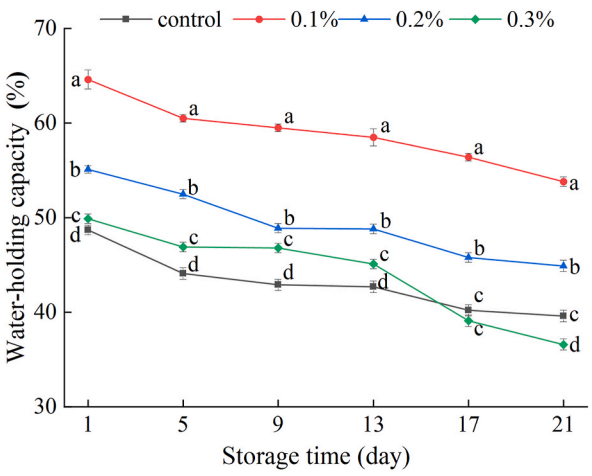

Fig. 3. Effects of MLE on the water-holding capacity of yogurt during storage. Different letters within the same column represent statistically significant differences ($p < 0.05$).

Table 1
Effects of MLE on the textural parameters of yogurt during storage.

Parameters	Storage time (day)	Treatment			
		Control	0.1%MLE	0.2%MLE	0.3%MLE
Hardness (g)	1	$5.97 \pm 0.16^{D,a}$	$4.16 \pm 0.09^{C,b}$	$3.11 \pm 0.07^{D,c}$	$2.62 \pm 0.06^{D,d}$
	5	$6.63 \pm 0.11^{A,a}$	$4.22 \pm 0.03^{C,b}$	$3.42 \pm 0.11^{C,c}$	$3.02 \pm 0.11^{B,C,d}$
	9	$6.47 \pm 0.13^{A,B,a}$	$4.52 \pm 0.08^{B,b}$	$4.29 \pm 0.09^{A,c}$	$3.29 \pm 0.06^{A,d}$
	13	$6.20 \pm 0.06^{B,C,D,a}$	$5.09 \pm 0.26^{A,b}$	$4.00 \pm 0.04^{B,c}$	$2.68 \pm 0.22^{D,d}$
	17	$6.29 \pm 0.08^{B,Ca}$	$4.17 \pm 0.13^{C,b}$	$3.48 \pm 0.26^{C,c}$	$2.84 \pm 0.03^{C,D,d}$
	21	$6.15 \pm 0.16^{B,C,D,a}$	$3.96 \pm 0.09^{C,b}$	$3.78 \pm 0.12^{C,b}$	$3.08 \pm 0.02^{C,c}$
Adhesiveness (g s)	1	$-0.33 \pm 0.01^{A,a}$	$-0.52 \pm 0.03^{B,C,b}$	$-0.60 \pm 0.01^{B,c}$	$-1.34 \pm 0.03^{D,d}$
	5	$-0.35 \pm 0.01^{A,a}$	$-0.40 \pm 0.02^{A,B,b}$	$-0.48 \pm 0.03^{A,c}$	$-1.28 \pm 0.05^{D,d}$
	9	$-0.34 \pm 0.03^{A,a}$	$-0.64 \pm 0.12^{C,b}$	$-0.77 \pm 0.05^{C,b}$	$-0.89 \pm 0.02^{B,c}$
	13	$-0.28 \pm 0.05^{A,a}$	$-0.55 \pm 0.03^{C,b}$	$-0.81 \pm 0.04^{C,c}$	$-0.97 \pm 0.05^{C,d}$
	17	$-0.33 \pm 0.02^{A,a}$	$-0.36 \pm 0.03^{A,a}$	$-0.59 \pm 0.06^{B,b}$	$-0.82 \pm 0.06^{A,B,c}$
	21	$-0.27 \pm 0.07^{A,a}$	$-0.51 \pm 0.04^{B,C,b}$	$-0.61 \pm 0.03^{B,c}$	$-0.80 \pm 0.02^{A,d}$
Springiness	1	$0.17 \pm 0.01^{B,d}$	$0.39 \pm 0.01^{C,c}$	$0.61 \pm 0.01^{A,b}$	$0.92 \pm 0.01^{A,a}$
	5	$0.12 \pm 0.01^{C,d}$	$0.41 \pm 0.01^{B,C,c}$	$0.61 \pm 0.01^{A,b}$	$0.92 \pm 0.05^{A,a}$
	9	$0.29 \pm 0.04^{A,d}$	$0.38 \pm 0.04^{C,c}$	$0.64 \pm 0.04^{A,b}$	$0.90 \pm 0.04^{A,a}$
	13	$0.18 \pm 0.03^{B,d}$	$0.44 \pm 0.02^{A,B,b}$	$0.60 \pm 0.02^{A,b}$	$0.90 \pm 0.01^{A,a}$
	17	$0.26 \pm 0.03^{A,d}$	$0.46 \pm 0.02^{A,b}$	$0.62 \pm 0.02^{A,b}$	$0.94 \pm 0.03^{A,a}$
	21	$0.25 \pm 0.01^{A,d}$	$0.44 \pm 0.02^{A,B,b}$	$0.65 \pm 0.03^{A,b}$	$0.96 \pm 0.03^{A,a}$
Gumminess (g)	1	$2.64 \pm 0.06^{C,a}$	$1.85 \pm 0.06^{C,a}$	$1.53 \pm 0.08^{A,b}$	$1.36 \pm 0.05^{B,c}$
	5	$2.71 \pm 0.10^{C,a}$	$1.96 \pm 0.08^{B,C,b}$	$1.62 \pm 0.06^{C,c}$	$1.19 \pm 0.07^{B,d}$
	9	$3.26 \pm 0.21^{A,a}$	$2.06 \pm 0.10^{B,b}$	$1.80 \pm 0.03^{B,c}$	$1.34 \pm 0.05^{A,d}$
	13	$2.93 \pm 0.02^{B,C,a}$	$2.55 \pm 0.06^{A,b}$	$2.04 \pm 0.05^{A,c}$	$1.32 \pm 0.08^{A,B,d}$
	17	$3.10 \pm 0.12^{A,B,a}$	$1.93 \pm 0.06^{B,C,b}$	$1.58 \pm 0.04^{C,c}$	$1.29 \pm 0.05^{A,B,d}$
	21	$2.95 \pm 0.21^{B,C,a}$	$1.83 \pm 0.03^{C,b}$	$1.59 \pm 0.04^{C,c}$	$1.25 \pm 0.02^{A,B,d}$

Data are expressed as mean ± standard deviation. Different capital (A-D) in the same column indicate significant differences between data in that column ($p < 0.05$). Different lowercase (a-d) in the same row indicate significant differences between data in that row ($p < 0.05$).

parameter L* (light-ness to darkness, 100 to 0), a* (redness to greenness, 0 to 100 = red; −80 to 0 = green) and b* (yellowness and blueness, 0 to +70 = yellow; −100 to 0 = blue) of MLE-added yogurt changed. When increasing the addition of MLE, the a* values decreased, and the L* values slightly increased, b* values significantly increased. During the storage period, all L*, a*, b* values of different groups of yogurt gradually increased, and the color became more stable as the amount of MLE added increased. On the 21st day of storage, the L* value of the control reached 88.73, and that of 0.1 %, 0.2 % and 0.3 % MLE-added samples were 81.31, 80.03, 76.01, respectively. The a* values of all groups were progressively and smoothly increased, whereas b* values were significantly increased as storage time increased in most samples. As a whole, the changes in yogurt color brought about by MLE addition were an increase in L* and b* values and a decrease in a* values. The yogurt samples with MLE appeared light green, which did not reduce consumer acceptability. Many reports indicated that the color parameters of yogurt containing plant additives was changed, which not only increased the nutritional value but also had no effects on consumer acceptance of the product [17,28,37].

Table 2
Changes in color parameters of yogurt during storage.

Color Parameters	Storage time (day)	Treatment			
		Control	0.1%MLE	0.2%MLE	0.3%MLE
L*	1	$75.22 \pm 0.55^{E,d}$	$78.43 \pm 0.02^{F,a}$	$77.81 \pm 0.09^{F,b}$	$76.70 \pm 0.03^{D,c}$
	5	$81.36 \pm 0.15^{D,a}$	$79.68 \pm 0.02^{E,b}$	$78.64 \pm 0.05^{E,c}$	$77.98 \pm 0.07^{B,d}$
	9	$84.42 \pm 0.06^{C,a}$	$83.29 \pm 0.02^{B,b}$	$81.15 \pm 0.08^{A,c}$	$78.84 \pm 0.11^{A,d}$
	13	$86.45 \pm 0.84^{B,a}$	$83.61 \pm 0.02^{A,b}$	$80.41 \pm 0.02^{B,c}$	$77.72 \pm 0.05^{C,d}$
	17	$88.56 \pm 0.26^{A,a}$	$82.25 \pm 0.02^{C,b}$	$80.22 \pm 0.04^{C,c}$	$76.37 \pm 0.07^{E,d}$
	21	$88.73 \pm 0.19^{A,a}$	$81.31 \pm 0.02^{D,b}$	$80.03 \pm 0.12^{D,c}$	$76.01 \pm 0.03^{F,d}$
a*	1	$-2.47 \pm 0.05^{F,a}$	$-3.08 \pm 0.02^{E,b}$	$-3.65 \pm 0.03^{E,c}$	$-3.81 \pm 0.07^{E,d}$
	5	$-2.27 \pm 0.02^{E,a}$	$-2.98 \pm 0.04^{D,b}$	$-3.54 \pm 0.03^{D,c}$	$-3.73 \pm 0.06^{D,d}$
	9	$-2.15 \pm 0.05^{D,a}$	$-2.91 \pm 0.01^{C,b}$	$-3.36 \pm 0.04^{C,c}$	$-3.54 \pm 0.07^{C,d}$
	13	$-2.06 \pm 0.05^{C,a}$	$-2.86 \pm 0.03^{C,b}$	$-3.25 \pm 0.04^{B,c}$	$-3.45 \pm 0.03^{B,d}$
	17	$-1.96 \pm 0.03^{B,a}$	$-2.63 \pm 0.05^{B,b}$	$-3.11 \pm 0.06^{A,c}$	$-3.36 \pm 0.03^{A,d}$
	21	-1.86 ± 0.03^{Aa}	$-2.32 \pm 0.04^{A,b}$	$-3.04 \pm 0.03^{E,c}$	$-3.26 \pm 0.03^{F,d}$
b*	1	$-0.34 \pm 0.03^{F,a}$	$6.97 \pm 0.04^{F,b}$	$10.06 \pm 0.06^{F,c}$	$15.17 \pm 0.04^{E,d}$
	5	$0.61 \pm 0.03^{E,a}$	$7.79 \pm 0.04^{E,b}$	$13.01 \pm 0.04^{E,c}$	$15.48 \pm 0.06^{D,d}$
	9	$1.88 \pm 0.07^{D,a}$	$9.55 \pm 0.04^{D,b}$	$14.02 \pm 0.02^{D,c}$	15.54 ± 0.02^{Dd}
	13	$2.31 \pm 0.07^{C,a}$	$10.00 \pm 0.05^{C,b}$	$14.55 \pm 0.03^{C,c}$	$16.03 \pm 0.05^{C,d}$
	17	$3.86 \pm 0.07^{B,a}$	$11.56 \pm 0.06^{B,b}$	$14.87 \pm 0.02^{B,c}$	$16.39 \pm 0.03^{B,d}$
	21	$4.45 \pm 0.03^{A,a}$	$12.21 \pm 0.03^{A,b}$	$15.04 \pm 0.04^{A,c}$	$16.55 \pm 0.04^{A,d}$

Data are expressed as mean ± standard deviation. Different capital (A-F) in the same column indicates significant differences between data in that column ($p < 0.05$). Different lowercase (a-d) in the same row indicate significant differences between data in that row ($p < 0.05$).

3.3. Total phenolic content (TPC) and antioxidant properties of yogurt

Phenolics have been proved to have pharmacological functions and health benefits [38,39]. However, the content of phenolics in plain milk yogurt is relatively low. Mulberry leaves have important medical and edible value, which are rich in phenolics [40]. As shown in Fig. 4a, addition of MLE significantly increased the content of phenolics in yogurt ($p < 0.05$), and the content of phenolics in yogurt was positively proportional to the amount of MLE. On the first day of storage, the content of phenolics in yogurt with 0.3 % MLE reached 14.03 mg/100g, while that of the control was 5.10 mg/100g. With the prolongation of storage, the levels of phenolics decreased in all yogurt samples. Similarly, the reduction of TPC in all yogurt samples was consistent with the results previously reported from green pepper-fortified yogurt [41], green tea-fortified yogurt [42], rose flower-fortified yogurt [43] and coriander and cumin seeds-fortified yogurt [44].This phenomenon is attributed to the formation of complexes between phenolic compounds and proteins, which reduces the recovery rate of phenolic compounds, and the degradation of some environmentally sensitive phenolic compounds during storage [11,31,45]. However, the total phenolic content of MLE-added yogurt was higher than that of the control during the whole storage period.

The total antioxidant capacity of yogurt may be caused by multiple reaction mechanisms, so total reducing power capacity, DPPH radical scavenging ability, and superoxide anion radical scavenging ability were determined to evaluate the antioxidant activity of MLE yogurt. Compared with the control, MLE yogurt had better antioxidant capacity, and presented concentration-dependent effects during the storage period (showed in Fig. 4). The total reducing power of all groups significantly increased during the 1st to 9th day, which reached the highest value on the 9th day. Compared with the 1st day yogurt, the total reducing power values of the yogurt on the 9th day with 0, 0.1 %, 0.2 % and 0.3 % MLE increased to 260 %, 244 %, 313 % and 367 %, respectively (Fig. 4b). The superoxide anion radical scavenging capacity of the yogurt increased firstly and then decreased, which reached the maximum values on the 13th day of storage. The maximum values were 72.70 %, 74.45 %, 81.90 %, 87.07 %, respectively. Compared to the 1st day, the superoxide anion radical scavenging capacity of the control yogurt increased by 1.28 times, while that of the 0.1 %, 0.2 % and 0.3 % MLE yogurts increased by 1.27, 1.36 and 1.39 times, respectively (Fig. 4c). At the 1st day of storage, the DPPH radical scavenging capacity of yogurt with 0, 0.1 %, 0.2 % and 0.3 % MLE were 38.25 %, 48.64 %, 57.08 % and 64.31 %, respectively. Then the DPPH free radical scavenging capacity of the yogurt increased until to the 17th day, which were 61.30 %, 75.00 %, 85.84 % and 98.19 % (Fig. 4d).

These results suggested that mulberry leaf was a good source for providing active ingredients and antioxidants. Mulberry leaves extract, especially extracted by ethanol or methanol solutions containing some water (ranging from 40 % to 80 %), was efficient in the extraction of phenolic compounds [20]. The extract increased the antioxidant capacity of yogurt mainly for the existence of phenolics and flavonoids in mulberry leaves [46], as well as amino acids and peptides produced during the milk fermentation process [47]. In the

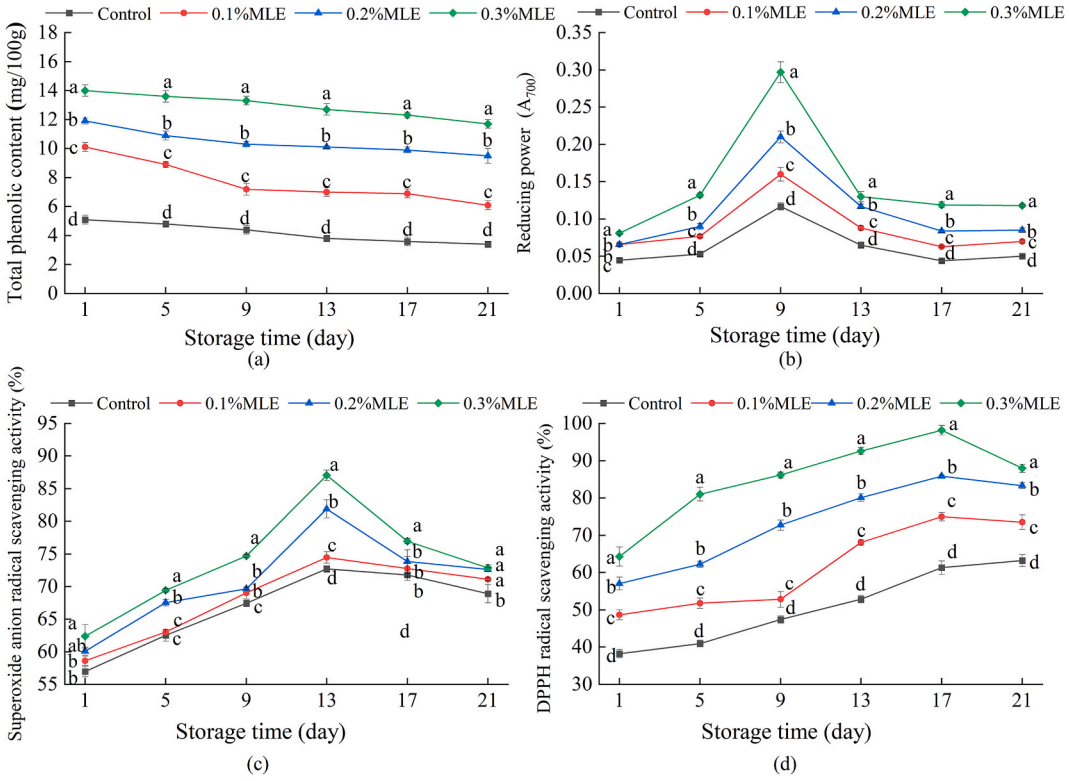

Fig. 4. Effects of MLE on total phenolic contents (a), total reducing power (b), superoxide anion (c) and DPPH (d) radical scavenging capacity of yogurt. Different letters within the same column represent statistically significant differences ($p < 0.05$).

later stages of storage, the decrease in antioxidant capacity may be due to the binding effects of protein and phenolics in yogurt, or a decrease in the amount of free phenolics in yogurt [45].

3.4. Effects of MLE on the growth and viability of LAB in yogurt

The effect of MLE on the growth and viability of LAB in yogurt was showed in Fig. 5. There were L.*bulgaricus* and *S. thermophiles* as the starter culture for the preparation of yogurt. After fermentation, the counts of *L.bulgaricus* in 0, 0.1 %, 0.2 %,0.3 % MLE-added yogurt were 7.56 lg CFU/g, 7.38 lg CFU/g, 8.35 lg CFU/g and 7.345 lg CFU/g,respectively (Fig. 5a). However, the counts of *S. thermophiles* in 0, 0.1 %, 0.2 %, 0.3 % MLE-added yogurt was 8.23 lg CFU/g, 8.44 lg CFU/g, 8.32 lg CFU/g and 8.25 lg CFU/g, respectively (Fig. 5b). There were differences on the effects of MLE on the growth of *S. thermophiles* and *L.bulgaricus*. During the fermentation, MLE improved the growth of *S. thermophiles*, while inhibited the growth of *L.bulgaricus*. The growth of *S. thermophiles* may be promoted by prebiotic components from MLE, including phenolics, flavonoid compounds, alkaloids, polysaccharides, water-soluble polysaccharides, hemicellulose, pectin substances, etc., which were reported increasing the growth of LAB [13,24,29]. The effect of MLE on *L. bulgaricus* was relatively small, with only 0.1 % MLE displayed a positive promoting effect from 5th ~ 21st day of storage. Overall, only 0.1 % MLE addition improved the LAB growth during the fermentation and maintained the promotion during the storage. From the 1st to the 21st day of storage, the number of LAB increased and then decreased in all groups. These results were mainly attributed to acidity of the yogurt, and the phenolic compounds in MLE had dual effects on growth and viability of LAB in yogurt, and their metabolites behaved as activators or inhibitors of bacterial growth depending on their chemical structures and concentrations [22,48]. 0.1 % MLE addition played a positive role in the Lactobacillus counts of yogurt, while 0.2 % and 0.3 % MLE addition presented the opposite results. During storage time, the counts of LAB of all samples decreased significantly, probably due to the decrease of pH, and, consequently, acidity increased. Nevertheless, the survival of LAB of control yogurt and the yogurt with 0.1 %–0.3 % MLE incorporation were able to maintain a significantly higher ($p < 0.05$) level of steady probiotic count of 10^6 CFU/g over 21 days of shelf life, which was the recommended therapeutic minimum value of yogurt.

3.5. Sensory evaluation

The sensory evaluation including assessments of flavor, taste, texture, color and overall acceptability, with the results shown in Fig. 6. Addition of 0.1 % MLE gave the yogurt a pleasant light green color and emitted a plant-specific odor without significantly affecting the texture and viscosity. The chewiness and viscosity of yogurt containing 0.1 % MLE were balanced in the oral cavity, resulting in good taste. However, as the added amount of MLE increased, the acidity and astringency of the yogurt increased significantly, accompanied by increased whey separation and a rapid decline in taste and palatability. The texture of yogurt with 0.3 % MLE appeared denser than other samples, and it was too viscous in the mouth, lacking a refreshing sensation. The inclusion of certain plant extracts, such as in the case of moringa yogurt (moringa extract >0.05 %), was found to have a negative impact on sensory properties

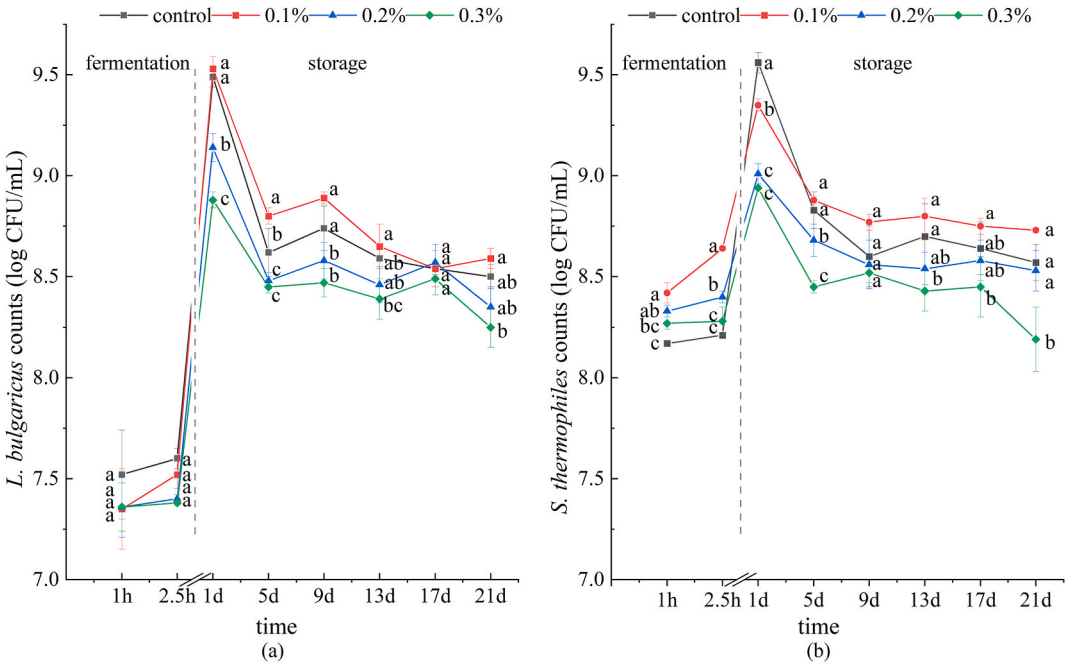

Fig. 5. Effect of MLE on the growth of *L.bulgaricus* (a) and *S. thermophiles* (b) in yogurt. Different letters within the same column represent statistically significant differences ($p < 0.05$).

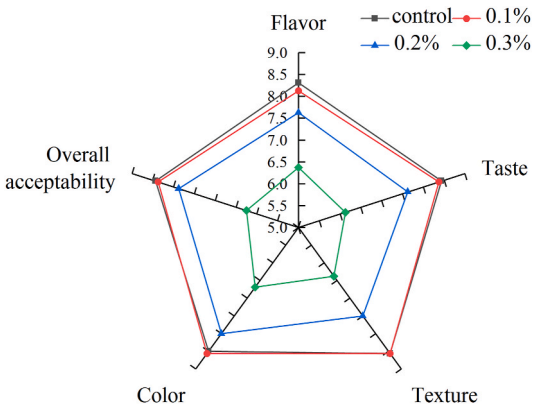

Fig. 6. Effects of MLE on sensory evaluation of yogurt.

[29]. Compared to the control yogurt, the addition of 0.1 % MLE to the yogurt did not significantly affect the overall acceptability ($p > 0.05$). Based on the sensory evaluation data obtained in this study, the addition of 0.1 % MLE significantly improved the quality characteristics of yogurt without causing any significant negative sensory properties.

4. Conclusions

Mulberry leaves are edible and medicinal, rich in various active ingredients such as flavonoids, polysaccharides, polyphenols, and alkaloids Therefore, the preparation of mulberry leaf extract (MLE) and its application in food represents an effective combination of mulberry leaf resource utilization and enhancing the health value of food.

In this study, MLE was prepared with 40 % ethanol/water solutions for more active phenolic compounds, and the incorporation of MLE as an ingredients for the preparation of yogurt shortened the coagulation time, improved the physicochemical and antioxidant properties of the yogurt. The total phenolic content of yogurt with 0.1 % MLE reached 10.10 mg/100 g (1st day of storage) and 6.06 mg/100 g (21st day of storage) respectively, while that of the control was 5.10 mg/100 g (1st day of storage) and 3.44 mg/100 g (21st day of storage) respectively. The total reducing power capacity, DPPH radical and superoxide anion radical scavenging abilities were all significantly increased by the addition of MLE over 21 days storage ($p < 0.05$). The addition of MLE changed the texture properties of the yogurt, the more MLE was added, the more hardness and springiness the yogurt became, and the addition of MLE made these properties more stable. All the results showed that the yogurt products containing the incorporated MLE exhibited a good balance between the physicochemical and sensory quality of the yogurt throughout the entire storage period. The addition of MLE as an ingredient in yogurt production not only incorporates bioactive phenolic compounds from mulberry leaves but also ensures product stability during storage. Nowadays, especially in the post-pandemic era, functional supplements are particularly attractive to the food industry. Further study on the synergistic effect of special fermentation strains and MLE on the health value of the yogurt product will develop the potential for commercialization.

Data availability statement

Data to this article can be found online at https://DOI:10.17632/f9bm437t8k.3.

Ethics approval

This article does not contain any study with human participants or animals performed by any of the authors.

Consent for publication

We declare that the publisher has the author's permission to publish the relevant contribution.

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Institutional review board statement

All participants volunteered to take part in this project. All subjects gave their informed consent for inclusion before they

participated in the study. These materials were safe for sensory research. All the experimental procedures involving volunteers were conducted in accordance with Food Safety Law of the People's Republic of China.

CRediT authorship contribution statement

Jingni Tang: Writing – review & editing, Visualization, Validation. **Wei Zhang:** Writing – original draft, Data curation. **Ru Yuan:** Validation. **Yiying Shu:** Software. **Guanhui Liu:** Visualization, Supervision, Resources, Project administration, Methodology, Conceptualization. **Boqiang Zheng:** Conceptualization. **Jie Tu:** Writing – review & editing, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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