Journal of Food Science and Technology (Iran)

Homepage:www.fsct.modares.ir

Scientific Research

Evaluation of Microbiological and Physicochemical Properties of Caspian Sea Yogurt Supplemented with Mint Leaves *(Mentha spicata* L.) and *Cinnamon (Cinnamomum burmanii* B.)

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ARTICLE INFO

Article History:

Received: 2024/03/13
Accepted: 2025/10/13

Keywords:

Antioxidant,

Antibacterial,

Caspian Sea Yogurt,

Mint Leaves,

Cinnamon

DOI: 10.48311/fsct.2025.115606.0

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ABSTRACT

The current study evaluated the microbiological and physicochemical properties of yogurt supplemented with mint leaves (Mentha spicata L.) or Cinnamon (Cinnamomum burmanii B.). Both mint leaves (ML) and cinnamon (Cn) were prepared for 5, 10, and 15% in Caspian Sea yogurt (CSY). The samples were evaluated for their physicochemicals (pH, total phenolic contents (TPC), antibacterial, and antioxidant) and the viability of lactic acid bacteria (LAB) in CSY. The antibacterial properties used *Escherichia coli* and *Bacillus cereus* as pathogenic bacteria. The statistical research design used was the Factorial Randomized Block Design arranged with two factors, particularly the herb types (ML and Cn) and various concentrations tested (5%, 10%, 15%). The optimum supplementation was chosen using the Zeleny method to assess the quality of yogurt. The results demonstrated that supplementation of Cn and ML could increase the antioxidant activity of CSY, but there were no significant differences in TPC and antibacterial activity. The total LAB in CSY was improved significantly in addition to ML and Cn. The best treatment was CSY with 15% cinnamon at pH 4.02 and a total LAB of 3.18 x10^7 CFU/ml. The antibacterial activity in *B. cereus* and *E. coli* was 12.50 and 12.0 mm, respectively, while total phenol and antioxidants were 0.06% and 46.72%, respectively. Adding ML and Cn might be a promising candidate to be incorporated into the CSY product in the future.

1.Introduction

The development of food and beverages has progressed rapidly, and as a result, many have been identified as having required various modifications. Multi-functional foods and beverages, including yogurt, a fermented milk type, were obtained to relieve hunger and maintain health and body fitness [1–3]. Yogurt is a processed drink produced through fermentation and has a sour taste due to lactic acid bacteria activity, particularly *Lactobacillus bulgaricus* and *Streptococcus thermophilus* [4]. Although yogurt has many beneficial effects on health, the sour taste of yogurt is less accepted by the public [5]. An innovative version of Caspian sea yogurt (CSY) has been invented in Japan, featuring a plain flavor characterized by low acidity and high viscosity.

Caspian Sea yogurt (CSY) is the fermented milk distributed domestically in Japan. Despite conventional yogurt, CSY contains *Lactobacillus cremoris* and *Acetobacter orientalis* as starters. Also, CSY has low acidity, and the final texture is thicker than conventional yogurt [6]. The advantage of CSY production is that it can fermented at room temperature [7]. CSY has been reported to have a health effect on the digestive system of healthy young women [8]. Thus, incorporating herbs into yoghurt may enhance the flavour and nutritional profile of CSY, affecting their nutraceutical characteristics and attractiveness to consumers [9,10].

Several types of herbs have been fortified in yogurt to add their nutritional values, including Moringa leaves [11], cinnamon [12], and tea [13], all of which are known to have health benefits. Besides that, cinnamon (Cn) is a spice commodity well-known as a rich source of antioxidants [14]. A previous study demonstrated that Cn supplementation could increase the probiotic viability, which is stimulated by Cn essential compounds [15]. Cn contains essential oils, cinnamaldehyde, eugenol, cinnamic acid, catechins, and epicatechins. Therefore, cinnamaldehyde is the main compound in Cn, with the largest range of 60-70%, and is considered the most important antioxidant compound [16,17].

In addition, mint leaves (ML) are often used in food preparation due to their unique and fresh smells. ML contains 1-2% essential oil, 80-90% menthol, menthone, d-pipirition, hexanolphenyl-acetate, ethyl amylcarbinol, and neomentol. A previous study reported that essential oils from *Mentha* species have good antibacterial and antioxidant effects [18]. ML has antioxidant properties because it contains active components such as mentone, menthol, rosmarinic acid, and carvone. Furthermore, the content of essential oils of 1-2% inhibits the growth of bacteria [19].

However, there is little information about whether adding Cn and ML could enhance the positive effects of CSY. Therefore, this study aimed to evaluate the supplementation of Cn or ML in the viability, antibacterial, and physicochemical properties of CSY. Thus, the result also suggests the optimum Cn or ML supplementation, which might provide useful information for developing CSY with functional properties in the future.

2.MATERIAL AND METHODS

2.1 CSY Herbal Formulation

The Cn or ML was boiled in the water at a ratio of 1:10. On the other hand, milk was pasteurized and poured into glass jars that had been sterilized previously. After that, Cn or ML with 5%, 10%, or 15% (v/v), 20% sugar, and 10% (v/v) starter from CSY was added. The incubation was performed at room temperature (±27°C) for 12 hours. Finally, the physicochemical and microbiological analysis of the yogurt was performed.

2.2 Physicochemical Analysis

1.2.1. *pH measurement*

pH of CSY with or without herb addition was measured using a pH meter at 0, 2, 4, 6, 8, and 24 h after CSY preparation.

1.2.2. *Antioxidant activity evaluation*

The DPPH radical scavenging and antioxidant activity were evaluated as described in the previous study with minor modifications [20,21]. DPPH was freshly prepared by mixing it in ethanol (0.125 μM). Then, 0.5 mL samples were added with 2 mL DPPH solution and kept at room temperature for 30 min at dark conditions. Samples were then measured at

517 nm, and the inhibitory activity (%) was calculated using the following equation.

$$\% \text{ inhibition} = \frac{\text{Abs control-Abs sample}}{\text{Abs control}} \times 100$$

***1.2.3.** Total phenolic contents (TPC) determination*

TPC was measured by the Folin-Ciocalteu method [21]. Briefly, gallic acid as standard was prepared at different concentrations (20-100 μL/mL). The sample (1 mg/mL) was dissolved in ethanol, and 10 μL was mixed with 160 μL distilled water. Then, 10 μL of Folin-Ciocalteu and 20 μL of Na_2CO_3 (10%) were added. The mixed solution was then incubated for 30 min at room temperature and measured at 775 nm.

2.3 Microbiological Analysis

2.4.1 Total LAB measurement

The total viability of LAB was counted using the total plate count. Briefly, CSY with or without herb addition was diluted in sterile distilled water at a ratio of 1:9. Then, the 1 mL sample at dilution 10^{-4}-10^{-6} was plated on MRS agar and incubated at 37°C for 48 h. The viable bacteria were then counted using a colony counter.

2.4.2 Antibacterial test

The antibacterial activity was measured using the agar diffusion method using *Escherichia coli* and *Bacillus cereus*. A paper disk (Φ 6 mm) was soaked with a sample and added to the Petri dish, previously planted with *E. coli* or *B. cereus*. The samples were then incubated at 37°C for 24 h, and the formed clear zone was measured [22].

2.4 Study Design

The statistical design used was a Randomized Block Design arranged in a factorial manner using two factors. The first factor is the type of herb incorporated in yogurt, such as mint leaves and cinnamon, while the second is the variation of the concentration of herbs used, 5%, 10%, and 15%, with three replications, 18 experimental units, and one control. The Zeleny method was used to determine the best treatment (Zeleny, 1982).

2.5 Statistical Analysis

The data were analyzed using Analysis of Variance (ANOVA) using a Randomized Block Design. The Least Significant Difference (LSD) was performed with a 95% confidence interval (*α=0.05*) when there was a significant difference between the experimental factors.

2. RESULTS AND DISCUSSION

3.1 Antioxidant Analysis between CSY and CSY with Herb

Antioxidant analysis was performed using the DPPH method. Table 1 shows changes in the antioxidant activity of Cn and ML. For the CSY, the observations of antioxidant activity were evaluated on the 12th and 24th days. Interestingly, the antioxidant activity was increased on the 24th day (Table 1).

Table 1. Antioxidants Analysis of CSY with/without herb addition. CnP = cinnamon powder, MLP = mint leaves powder, CSY = Caspian Sea yogurt, Cn = cinnamon, ML = mint leaves. The different letters indicated a statistical difference ($p<0.05$) based on the Least Significant Difference (LSD) test.

Sample(s)	Hour	
	12	24
CSY	$15.07^a \pm 3.55$	$29.30^b \pm 5.84$
CSY + Cn 5%	$28.32^b \pm 6.31$	$29.52^{bc} \pm 4.30$
CSY + Cn 10%	$46.67^c \pm 4.07$	$41.56^c \pm 6.65$
CSY + Cn 15%	$44.84^c \pm 7.19$	$45.46^c \pm 6.25$
CSY + ML 5%	$16.06^a \pm 3.59$	$37.12^b \pm 6.50$
CSY + ML 10%	$44.42^c \pm 3.65$	$39.55^{bc} \pm 9.15$
CSY + ML 15%	$28.02^b \pm 8.85$	$45.77^c \pm 11.45$

The addition of Cn or ML did not have a significant effect (*p>0.05*) on the antioxidant activity of CSY (Table 1). However, each concentration slightly increased antioxidant activity after 24 hours of fermentation. The antioxidant activity increases up to 46.72% and 45.72% on Cn and ML addition, respectively, after 24 h fermentation. We suggested that Cn and ML contain phytochemicals that are antioxidants and increase during fermentation due to LAB activity in CSY. Cinnamaldehyde is the most dominant phytochemical compound in cinnamon, and it acts as an antioxidant by inhibiting aldose reductase, an enzyme that plays a role in the polyol pathway, thereby inhibiting the formation of oxidative stress [23,24]. Cinnamon contains several antioxidants, including cinnamaldehyde, cinnamyl acetate, eucalyptol, and eugenol [14]. Meanwhile, ML contains 30-66% menthol, depending on its genotype [25]. In addition, the antioxidant properties of ML come from other biochemicals, such as ascorbic acid, rosmarinic acid, δ-terpinene, α-terpinene, p-cymene, 1,8-cineole, cis-carveol, carvone, rosmarinic acid, cynaroside, crypto chlorogenic acid, naringin [26]

3.2 The Comparison of TPC between CSY and CSY with Herb

TPC was analyzed using the Folin-Ciocalteu method. Table 2 demonstrates the changes in the TPC of CSY added with Cn or ML. CnP has the highest TPC of around 0.24 µg GAE/g than MLP and CSY.

Table 2. TPC analysis of CSY with/without herb addition. CnP = cinnamon powder, MLP = mint leaves powder, CSY = Caspian Sea yogurt, Cn = cinnamon, ML = mint leaves.

Sample(s)	Total Phenol (µg GAE/g sample)
CnP	0.24
MLP	0.18
CSY	0.05 ± 0.03
CSY + Cn 5%	0.02 ± 0.00
CSY + Cn 10%	0.03 ± 0.02
CSY + Cn 15%	0.06 ± 0.03
CSY + ML 5%	0.08 ± 0.04
CSY + ML 10%	0.07 ± 0.05
CSY + ML 15%	0.05 ± 0.02

However, adding Cn or ML had no significant effect (*p>0.05*) on TPC in CSY. Surprisingly, there was an increase in the CSY with Cn and a decrease in the CSY with ML. The decrease of TPC in fortified yogurt might be due to milk protein that can bind and precipitate phenolic compounds from Cn and ML, resulting in low recovery of phenolic compounds when incorporated in CSY [27]. Also, the decrease of polyphenols in CSY might be due to insoluble complexes formed between cinnamon tannins and milk proteins [28]. These conditions result in a loss of polyphenols because of precipitation during yogurt preparation [27].

3.3 pH Comparison Between CSY and CSY with Herb

Changes in pH are an indicator of the quality of yogurt. The acidification is in line with fermentation time. Table 3 showed the decrease in pH during the fermentation process.

Table 3. The pH comparison of Caspian Sea Yogurt with various concentrations of cinnamon and mint leaves at various times. CSY = Caspian Sea yogurt, Cn = cinnamon, ML = mint leaves. * = significant at *p<0.05* compared to 0 h.

Groups	pH					
	0 h	**2 h**	**4 h**	**6 h**	**8 h**	**24 h**
CSY	6.45 ± 0.20	6.36 ± 0.18	5.45 ± 0.20	5.22 ± 0.32	4.92 ± 0.37	4.24* ± 0.20
CSY + Cn 5%	6.28 ± 0.32	5.66 ± 0.02	4.98 ± 0.01	4.70 ± 0.18	4.51 ± 0.11	4.42* ± 0.11
CSY + Cn 10%	6.69 ± 0.07	6.51 ± 0.02	5.70 ± 0.01	5.53 ± 0.153	4.76 ± 0.01	4.36* ± 0.12
CSY + Cn 15%	6.36 ± 0.01	6.20 ± 0.03	5.76 ± 0.01	4.77 ± 0.13	4.39 ± 0.10	4.02* ± 0.32
CSY + ML 5%	6.74 ± 0.02	5.89 ± 0.04	5.68 ± 0.01	4.95 ± 0.45	4.26 ± 0.15	4.31* ± 0.07
CSY + ML 10%	6.30 ± 0.22	6.04 ± 0.00	5.66 ± 0.01	5.44 ± 0.02	4.63 ± 0.50	4.25* ± 0.17
CSY + ML 15%	6.45 ± 0.09	5.90 ± 0.01	5.87 ± 0.04	5.56 ± 0.02	4.75 ± 0.02	4.17* ± 0.19

The addition of Cn or ML in CSY has a significant effect (*p<0.05*) during fermentation. The pH obtained was 6.74 - 4.02 with a fermentation time of 0, 2, 4, 6, 8, and 24 hours. The fermentation is inversely proportional to the pH. CSY with Cn or ML might synergize with LAB to produce organic acids and decrease the pH. The organic acids produced during fermentation were dissociated in the form of H^+ ions. Also, the decrease in pH is related to the total acid produced during fermentation.

Additionally, As acidity increases in yogurt, H^+ ion formation increases, decreasing the pH value [29]. In CSY, *L. cremoris* produces lactic acid, while *A. orientalis* remodels milk lactose into lactobionic acid and other organic types of acetic acid with potential as prebiotics [7]. The two bacteria form a combination that lowers the pH of yogurt. This high lactic acid prevents the growth of spoilage bacteria such as *Clostridium* [30].

3.4 Total LAB Comparison Between CSY and CSY with Herb

The total LAB is expressed in CFU (Colony Forming Unit)/mL. Furthermore, the total plate count method was used to calculate the total number of viable bacteria. Table 4 showed the average value of Total LAB in yogurt**.**

Table 4. Total LAB in CSY with/without herb addition

Types of Yoghurt	Number of Colonies (10^7 CFU/mL)
CSY	$1.28^{e} \pm 6.11$
CSY + Cn 5%	$2.21^{cd} \pm 1.62$
CSY + Cn 10%	$2.55^{b} \pm 3.51$
CSY + Cn 15%	$2.70^{a} \pm 2.81$
CSY + ML 5%	$1.89^{d} \pm 1.62$
CSY + ML 10%	$2.24^{b} \pm 1.78$
CSY + ML 15%	$2.32^{bc} \pm 2.07$

Table 4 demonstrated that the Cn or ML addition in CSY had a significant effect (*p<0.05*) on the total LAB. The highest increase in total LAB was discovered in CSY with the addition of 15% Cn. Meanwhile, CSY without Cn or ML addition has the smallest total LAB of 1.28 x 10^7 CFU/mL. We assumed that Cn or ML addition could stimulate LAB growth in CSY. A previous study reported that the extract of Cn added to yogurt could increase the viability of LAB cells due to the presence of bioactive compounds from Cn, such as vitamin B3, B6, folate, and small amounts of minerals such as manganese, magnesium, and pantothenic acid [15]. Additionally, the essential oils in ML were reported to support LAB's viability. ML contains high essential oils that supports the viability of probiotic bacteria [31].

3.5 Antibacterial Test Between CSY and CSY with Herb

Antibacterial activity was performed using *B. cereus* and *E. coli*. Table 5 demonstrated no significant effect (*p<0.05*) between CSY and CSY added with Cn or ML on *B. cereus* and *E. coli* inhibition. However, the antibacterial activity was increased following the time. Overall, the largest diameter of the inhibition zone occurred in *B. cereus* at 15% Cn with a diameter of 12.50 mm. Meanwhile, the diameter of the inhibition zone in *E. coli* was 12% mm after five days of treatment with 15% Cn. We assumed that the size of the inhibition zone is influenced by the metabolites' ability from yogurt to penetrate the cell walls of pathogenic *B. cereus* and *E. coli.*

Table 5 Inhibition diameter of *B. cereus* and *E. coli* growth after treated by CSY with or without herb addition. CSY = Caspian Sea yogurt, Cn = cinnamon, ML = mint leaves.

Types of Yogurt	The diameter of the inhibition zone (mm)					
	Day 1		Day 3		Day 5	
	B. cereus	*E. coli*	*B. cereus*	*E. coli*	*B. cereus*	*E. coli*
CSY	$11.50^a \pm 0.40$	$10.72^a \pm 1.31$	$12.16^a \pm 1.31$	$11.00^a \pm 0.00$	$12.33^a \pm 0.47$	$11.33^a \pm 0.23$
CSY + Cn 5%	$11.50^a \pm 0.40$	$11.00^a \pm 0.40$	$12.00^a \pm 0.40$	$11.33^a \pm 0.23$	$12.33^a \pm 0.47$	$11.50^a \pm 0.40$
CSY + Cn 10%	$11.83^a \pm 0.23$	$11.50^a \pm 0.40^a$	$12.16^a \pm 0.62$	$11.83^a \pm 0.62$	$12.33^a \pm 0.62$	$11.83^a \pm 0.70$
CSY + Cn 15%	$12.00^a \pm 0.70$	$11.50^a \pm 0.81$	$12.00^a \pm 0.70$	$12.00^a \pm 0.70$	$12.50^a \pm 1.08$	$12.00^a \pm 0.84$
CSY + ML 5%	$11.66^a \pm 0.47$	$10.00^a \pm 0.00$	$12.00^a \pm 0.00$	$10.50^a \pm 0.00$	$12.16^a \pm 0.23$	$11.00^a \pm 0.00$
CSY + ML 10%	$11.83^a \pm 0.94$	$10.00^a \pm 0.40$	$12.00^a \pm 0.81$	$10.66^a \pm 0.47$	$12.16^a \pm 0.23$	$11.00^a \pm 0.00$
CSY + ML 15%	$11.83^a \pm 0.47$	$10.33^a \pm 1.31$	$12.00^a \pm 0.40$	$10.66^a \pm 0.84$	$12.33^a \pm 0.23$	$11.33^a \pm 0.94$

CSY is well known to contain *L. cremoris* and *A. orientalis*, which benefit health. *L. cremoris* could secrete major antimicrobials such as lactic acid, acetic acid, caproic acid, diacetyl, reuterin, hydrogen peroxide, 3-hydroxy fatty acids, phenyl lactic acid, proteinaceous compounds, and cyclic dipeptides [32]. *L. cremoris* also produced bacteriocin that performed strong antibacterial activity on *Listeria monocytogenes, Staphylococcus aureus,* and *E. coli* [33]. Another study reported that *L. cremoris* produced nisin that could permeabilize the bacterial cytoplasmic membranes of gram-positive and gram-negative bacteria [34]. Cinnamaldehyde from Cn is an electronegative molecule that influences the cellular biological process, including protein and nucleic acids. Cinnamaldehyde inhibits cell division, ATPase, and biofilm formation to inhibit bacterial growth [35–37]. Meanwhile, the ML contains essential oils that possess antimicrobial activity. A previous study reported that ML essential oils were more sensitive to gram-positive than gram-negative bacteria [18]. ML phytochemicals are hydrophobic, which allows them to interact directly with membrane cells of bacteria, leading to cell lysis [38]. Both Cn and ML positively impact when incorporated into CSY and improve its functional values.

3.6 The Best Treatment

The selection of the best treatment for CSY, adding 5%, 10%, and 15% Cn or ML, was analyzed using the Multiple Attribute Zeleny method [37]. The Zeleny method is a general procedure used to determine the sample quality through the sedimentation volume of samples in a lactic acid solution [39]. Table 7 summarizes the best treatment according to the present result. CSY, with the addition of 15% Cn, could improve its functional values than other treatments. According to these results, it is possible to develop a functional CSY with the addition of Cn to boost their beneficial effects on health.

Table 7. The characteristics of CSY with the addition of 15% Cn have superior results than other treatments.

Parameter	CSY	CSY + Cn15
pH	4.24	4.02
Total LAB (10^7CFU/ml)	1.28	2.70
Antibacterial activity (mm)		
• *E. coli*	11.33	12.00
• *B. Cereus*	12.33	12.50
Total Phenol (mg GAE/gr)	0.05	0.06

4.GENERAL CONCLUSION

The results demonstrated that 15% Cn incorporated in CSY had better results than other treatments through pH, total LAB, antibacterial activity, and TPC. Adding Cn to CSY might be promising in improving their physicochemical and antimicrobial properties, which could further influence human health.

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