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Effect of Applying Clove and Cinnamon Essential Oils to Milk Rice Pudding in Controlling *Bacillus cereus* and *Bacillus subtilis* Growth with Respect to the Sensory Traits

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ABSTRACT

Milk rice pudding (MRP) is a commercial and popular dairy dessert, but owing to its characteristics and valuable ingredients, it may be contaminated by many pathogenic and spoilage microorganisms. So, this study aimed to improve the quality and safety of MRP by using cinnamon and clove essential oils. Concerning the evaluation of the minimum inhibitory concentration (MIC) for both oils with 0.2, 0.5, and 1% concentrations, *B. cereus and B. subtilis* were sensitive (+) to cinnamon and clove 0.5%, with inhibition zones of 13.3 and 14 mm for cinnamon and 11.3 and 12 mm for clove EO, respectively. While both bacteria were very sensitive (++) to cinnamon 1% (18.8 and 19.5 mm) and clove 1% (17.3 and 18.7 mm), respectively. Therefore, MRP was prepared by adding cinnamon and clove EOs at 0.6%. Treatments containing EOs showed a significant reduction of tested microorganisms compared to controls. *B. cereus* wasn't detected in clove and cinnamon EO treatments at day 21 of the storage period, while *B. subtilis* vanished on day 14 for the cinnamon treatment and on day 21 for clove MRP. Moreover, the results revealed the enhancement of sensory characteristics of MRP supplemented with EOs without any significant alteration in their pH values. This study recommends the addition of cinnamon and clove EOs (0.6%) to MRP, as it isn't only an excellent substitution of chemical preservatives with powerful antibacterial efficiency but also improves the overall acceptance of the product.

Keywords: *B. cereus*, *B. subtilis*, Cinnamon, Clove, Milk Rice Pudding.

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INTRODUCTION

Milk-based desserts are broadly consumed all over the world. Milk Rice Pudding (MRP) is the most appealing one in comparison to other desserts. It is prepared by cooking rice in milk with sugar; other constituents may also be added **(Hussein *et al.*, 2023)**. Milk rice pudding is considered a healthy dessert, as adding rice to milk raises the nutritional value of the final product. It is suitable for all age groups, including children and elderly people **(Ozcan *et al.*, 2010).** MRP is a non-fermented dairy product characterized by having a neutral pH, plentiful nutrients, and high-water activity, so it may constitute a favorable environment for microbial growth. Since the nature of the product makes it vulnerable to many foodborne pathogens **(Hussein *et al.*, 2023)**.

Bacillus species are broadly distributed in the environment. They have often been implicated in numerous outbreaks worldwide and product spoilage. *Bacillus cereus* was extensively isolated from milk and rice **(Hetta *et al.*, 2020).** Moreover, owing to its resistance to heat treatment, it had been recovered from improperly pasteurized dairy products. It causes two types of food poisoning syndromes: emetic and diarrheal. Its effect is not limited to human illness; it also may result in product spoilage by producing lipase and protease enzymes **(Elafify *et al.*, 2023).** These thermos-resistance enzymes are mainly associated with the deterioration of the deterioration of dairy products, as indicated by distinct sensorial defects **(Shawki *et al.*, 2024).** *Bacillus subtilis*, another Bacillus ssp., was recorded as the dominant spoilage spore-former microorganism in dairy desserts **(Moschonas *et al.*, 2021).**

The utilization of synthetic chemical preservatives to minimize microbial growth has resulted in many threatening side effects, such as cancer, neurological disorders, hypersensitivity, and asthma **(Nasrollahzadeh *et al.*, 2022; Elhennawy *et al.*, 2023)**.

Therefore, natural applicants with potent antibacterial activities like essential oils (EOs) have received growing interest as an alternative to synthetic preservatives **(Bakr *et al.*, 2024).** EOs are generally recognized as safe (GRAS) by the United States Food and Drug Administration **(FDA, 2016).** They are used in many foods, including dairy products, to enhance their safety and extend their shelf life owing to their inhibitory effect against both gram-positive and gram-negative microorganisms. Moreover, they improve the sensory characteristics and nutritional value of products **(Ahmed *et al.*, 2021).**

Clove (*Syzygium aromaticum* or *Eugenia caryophylata*) and cinnamon *(Cinnamomum zeylanicum)* EOs have uncountable therapeutic values; they are considered examples of the most significant herbs used in traditional medicine. They have powerful antibacterial and antioxidant activity; moreover, they can protect against diabetes, inflammation, cancer, Alzheimer's, ulcers, and heart diseases **(Choobkar *et al.*, 2022; Sethunga *et al.*, 2023).**

Currently, owing to the wide resistance of most microorganisms against different antibiotics, this demand focuses on the exploitation of the antimicrobial properties of plant extracts as EOs and using them in dairy products **(Chandra *et al.*, 2017; Atef *et al.*, 2019).** However, fewer studies were conducted for mixing EOs with MRP; therefore, the purpose of this study was to develop a MRP formulation containing cinnamon or clove EOs to assess their effect on *B. cereus* and *B. subtilis* growth as well as their influence on pH and sensory characteristics during 21-day storage. Moreover, new-flavored MRP products were produced with enhanced safety and quality.

MATERIALS AND METHODS

Plant preparation and essential oil (EO) extraction

The clove and cinnamon EOs were extracted from dried buds of *Syzygium aromaticum* flower and the bark of *Cinnamomum zeylanicum*, respectively, and were obtained from native markets in Giza, Egypt. The plants were properly cleaned and dried, then ground into a very fine powder. Finally, the extraction was done by hydro-distillation using Clevenger with complete dryness via sodium sulfate. The produced EOs were kept inside a refrigerator during the experimental work **(Omar *et al.*, 2023).**

Assessment of the minimum inhibitory concentration (MIC) of extracted clove and cinnamon EOs

The concentrations of 0.2, 0.5, and 1% of both oils were tested using the well diffusion assay against *B. cereus* (ATCC® 14579) and *B. subtilis* (ATCC® 6633) strains. The procedures were performed based on guidelines pronounced by **CLSI (2018).** Each bacterial suspension was overnight incubated at 37°C, then their turbidity was adjusted using a saline solution compared to McFarland 0.5 for reaching an 8 $\log_{10}$ CFU/ml count before being inoculated on MH (Mueller Hinton agar, Himedia-1084). About 6 mm wells were prepared on inoculated plates and filled with different oil concentrations with 100 µl. The plates were incubated for 16–20 h at 35 ±2 °C, and the inhibition zones were measured three times via a sliding ruler. The sensitivity results were stated according to **Ponce *et al.*, (2003) and Moreira *et al.*, (2005)**. As for a total diameter less than 8 mm, the microorganism is considered not sensitive (-) to the antimicrobial agent; for a total diameter of 9–14 mm, it is regarded as sensitive (+), while it is very sensitive (++) when the total diameter is from 15 to 19 mm. Finally, it is extremely sensitive (+++) for a total diameter of >20 mm.

Preparation of selected strains

The bacterial strains were added to 5 ml of BHI (Brain Heart Infusion Broth, Himedia-M210) for growth at 37 °C for 24 h. Then each strain was centrifuged for ten minutes at 3500 rpm, and the peptone water (Himedia-M028) was used for washing the obtained bacterial pellets in triplicate. In addition to the peptone, water was utilized for cell re-suspension. It achieved final concentrations of 8 $\log_{10}$ CFU/ml with the use of a serial dilution for the inoculation of the produced rice pudding.

Processing of milk rice pudding

MRP was produced according to **Hussein *et al.*, (2023).** The produced pudding was aseptically packaged in sterile plastic cups with a cover and divided into three trials of uninoculated (control sample without any additives, MRP with clove oil 0.6%, and MRP with cinnamon oil 0.6%) for sensory assessment and pH determination. For the determination of *B. cereus* and *B. subtilis* survival, three trials for each bacterium were prepared (positive control, with clove oil at 0.6% and cinnamon oil at 0.6%). Inoculation of MRP with *B. cereus* and *B. subtilis* in a final count of 6.60 $\log_{10}$ and 6.50 $\log_{10}$ CFU/g, respectively, was done. Finally, the prepared trials were stored in the refrigerator at 5 °C for further examination periodically during 21 days of storage at the following intervals (zero-day, 3rd, 7th, 10th, 14th, and 21st).

Sensory assessment of MRP samples

The evaluation of MRP samples (control without additives, MRP with clove oil at 0.6%, and MRP with cinnamon oil at 0.6%) for their sensory acceptability was performed during the storage period in accordance with the dairy products judging scorecard that was set by the American Dairy Science Association. A total of ten panelists were selected for analysis and filled out the scorecard in the following manner: (1-10) points for flavor, (1-5) points for body and texture, and (1-5) points for color and appearance, with an overall final grade equal to 20 points. The analysis and scoring were completed according to **Lawless and Heymann (2010).**

Recording of pH values of MRP samples during storage

It was determined by dipping a pH meter probe (Hanna Instruments HI 2211, USA) in mixed MRP samples at 20 ˚C three times, with references to **AOAC (2000).**

Enumeration of *Bacillus cereus* and *Bacillus subtilis* (CFU/g) for inoculated samples during storage

The samples (25 g) were diluted with 225 ml of peptone water (Himedia-M028, 0.1%), and then decimal serial dilutions were prepared. Thereafter complete homogenization, 100µl of the appropriate prepared dilutions were inoculated and spread on duplicate plates of Mannitol-Egg-Yolk-Polymyxin agar (MYP, Himedia-M636F). However, 1 ml (100, 300, 300, 300 µl) of each sample (in case of a low count on the 14th and 21st days) was spread on four plates of MYP agar. All plates were kept at 32 ±2 ˚C in an incubator for 24 h **(Bennett *et al.,* 2015).**

Statistical analysis

Each measurement was performed three times; the mean ± SD was used to represent the findings. Data were statistically analyzed by one-way ANOVA using SPSS 23 for Windows **(SPSS, 2017).**

RESULTS

Minimum inhibitory concentration (MIC) of clove and cinnamon EOs

Results in **Table 1** showed the MIC of cinnamon and clove EOs against *B. cereus* and *B. subtilis*; different concentrations were tested (0.2, 0.5, and 1%) using the agar-well diffusion method. *B. cereus* and *B. subtilis* were sensitive (+) to cinnamon 0.5% (13.3, 14 mm) and clove 0.5% (11.3, 12 mm), respectively. Moreover, at a concentration of 1%, *B. cereus* was found to be very sensitive (++) to cinnamon (18.8 mm) and clove (17.3 mm), while *B. subtilis* showed higher sensitivity to cinnamon and clove 1% (19.5 and 18.7 mm), respectively.

Table 1: MIC for clove and cinnamon EOs by expression diameter of inhibition zone in mm (mean ±SD) against the studied bacterial strains.

Strains	Diameter of inhibition zone (mm)					
	Clove oil concentration (%)			Cinnamon oil concentration (%)		
	0.2%	0.5%	1%	0.2%	0.5%	1%
Bacillus cereus	7±0.10	11.3±0.20	17.3±0.10	7.3±0.10	13.3±0.02	18.8±0.10
Bacillus subtilis	7.4±0.20	12±0.01	18.7±0.30	7.9±0.05	14±0.20	19.5±0.01

Effect of cinnamon and clove EOs on the viability of artificially inoculated bacteria in MRP

As shown in **Fig. 1**, the *B. cereus* log count decreased gradually in clove and cinnamon EOs treatments starting from the 3rd till it vanished on day 21 of the storage period. *B. cereus* log count was found to be significantly higher in control throughout storage time compared to clove and cinnamon treatments. After 14 days of storage, the *B. cereus* log count in control was 8.25, while in clove and cinnamon EOs treatments was 1.52 and 1.31, respectively. There was a significant (P < 0.05) difference alon the study between the control and MRP supplemented with clove or cinnamon oils *B. subtilis* log count during the storage period starting on the third day.

Concerning results in **Fig. 2**, the *B. subtilis* log count was 6.50 at zero time; the count increased in the control treatment with storage; in contrast, it decreased in the EOs treatments till it wasn’t detected on the 14th day for the cinnamon treatment and on day 21 in clove MRP. On day 10 of storing the *B. subtilis* log count in control, it was 8.54, while in clove and cinnamon EOs treatments, it declined significantly to 3.81 and 2.93, respectively. The survival results of *B. subtilis* showed a significant statistical difference (P < 0.05) that started from day 3 of storage until the end of the incubation between the control and MRP treatments.

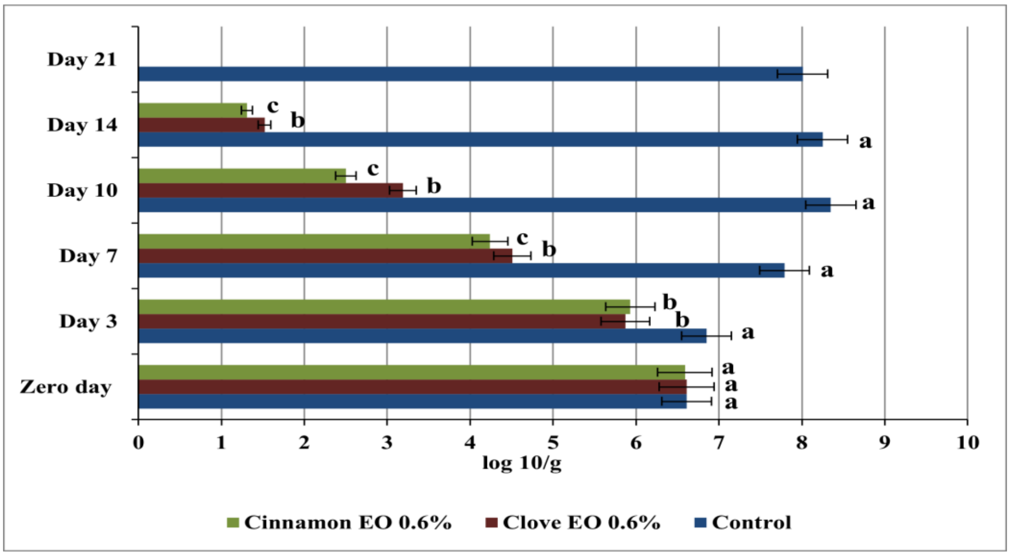

Fig. 1: Survival of *Bacillus cereus* (log_{10} count) in MRP (mean ±SD)
Different letters represented a significant difference ($P < 0.05$) within each storage period.

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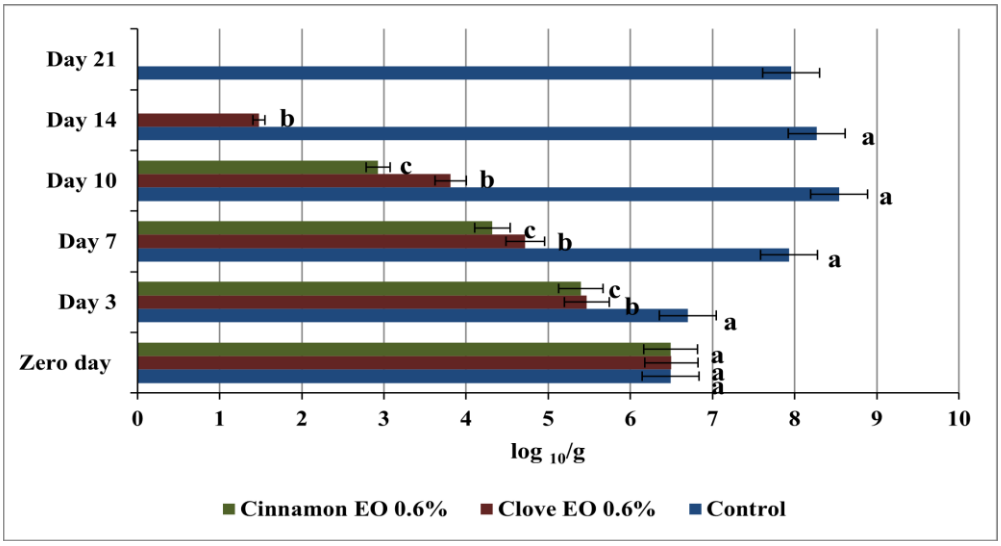

Fig.2: Survival of *Bacillus subtilis* (log_{10} count) in MRP (mean ±SD).
Different letters represented a significant difference ($P < 0.05$) within each storage period.

pH evaluation of MRP treatments

The pH values of the examined treatments were presented in **Table 2;** it was found to be nearly neutral (6.9) at zero time in all treatments, but it decreased during storage (6.8-6.5) in different treatments. During the full storage period, the control and tested treatments didn't show a significant difference ($P > 0.05$) in their determined pH values.

Table 2: pH values (mean ±SD) for milk rice pudding treatments.

	Control	clove oil 0.6%	cinnamon oil 0.6%
Zero day	$6.9±0.01^a$	$6.9±0.01^a$	$6.9±0.01^a$
Day 3	$6.9±0.10^a$	$6.8±0.03^a$	$6.8±0.01^a$
Day 7	$6.8±0.01^a$	$6.8±0.06^a$	$6.8±0.02^a$
Day 10	$6.7±0.20^a$	$6.6±0.01^a$	$6.7±0.01^a$
Day 14	$6.6±0.01^a$	$6.5±0.01^a$	$6.6±0.02^a$
Day 21	$6.6±0.03^a$	$6.5±0.06^a$	$6.5±0.06^a$

Same superscript letters in the same row indicate non-significant differences ($p > 0.05$).

Sensory evaluation of MRP fortified with EOs

The addition of EOs didn't adversely affect the sensorial characteristics of MRP; however, on the contrary, there was a noticeable improvement in treatments containing oils. The cinnamon EO treatment received higher flavor scores during storage as compared with the control and clove treatments. At zero-day, MRP with cinnamon has a score of 9.6 compared to control and clove (9); at the end of the storage period, it was significantly higher, as it received 8.5 while control and clove were 7.2 and 7.7, respectively. The MRP with cinnamon oil flavor scores exhibited a higher significant (P < 0.05) difference than other treatments from the first testing **(Fig.3a)**.

The EOs improve the body and texture of MRP as compared to the control **(Fig.3b)**. The body and texture score of the control treatment decreased within storage from 4.8 at zero time to 2.9 at day 21, where the body was described by panelists as weak with the presence of syneresis. In clove and cinnamon, MRP body and texture scores weren't significantly different at zero time (5 and 4.9) till the end of the storage period (4.3 and 4.4), respectively, but they were significantly higher than control.

Concerning color and appearance as presented in **Fig. 3c**, clove MRP received lower scores than cinnamon; this could be owed to some of the panelists who reported that clove oil gives MRP a yellowish color. At the final storage duration, the color and appearance scores of the control, clove, and cinnamon treatments were 3.2, 3.7, and 4.2, respectively, at which the cinnamon MRP was the highest. As declared in **Fig.3d**, overall acceptability decreased gradually with storage in all treatments, but the cinnamon treatment received higher marks significantly (P < 0.05) difference starting from zero time (19.4) till day 21 (17) compared to other treatments.

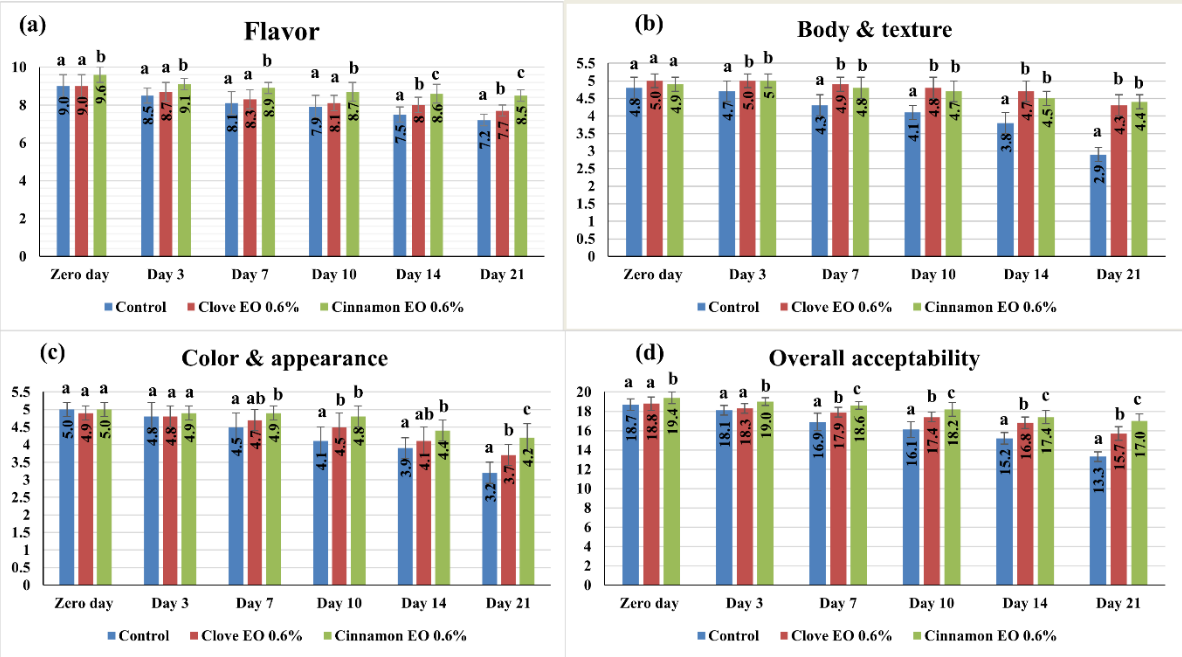

Fig. 3: Sensory evaluation (mean ±SD) for MRP treatments during the storage period.
Different letters represented a significant difference (P < 0.05) within each storage period.

DISCUSSION

B. cereus is regarded as a potential pathogen; several studies have reported that *B. cereus* is responsible for a large number of foodborne intoxication cases. In addition, it produces different virulent toxins in food, causing severe gastrointestinal symptoms that may sometimes be lethal **(ElSherif *et al.*, 2021).** *B. subtilis* has been linked with the spoilage of dairy products as it possesses both proteolytic and lipolytic enzymes that result in lowering product quality and shelf life **(Moschonas *et al.*, 2021).** The presence of *B. cereus* and *B. subtilis* requires the implementation of different food protection strategies, such as the application of essential oils. Our results noticed a directly proportional relationship between EOs concentrations and the diameter of inhibition zones; as concentrations increased, inhibition zones became larger. The inhibition zones formed by cinnamon oil against *B. cereus* were larger than those formed by clove oil at different concentrations, as they ranged from (7.3–18.8 mm) and (7–17.3 mm), respectively. *B. subtilis* was likewise found to be more affected by cinnamon oil than clove oil (7.9–19.5 mm) and (7.4–18.7 mm), respectively.

The obtained results were comparable with the results of **Babu *et al.*, (2011),** who used different concentrations of clove and cinnamon oils to inhibit the growth of *B. cereus*. Clove oil at a concentration of

0.6% gives an inhibition zone diameter of 14–16 mm. While concerning cinnamon oil, the study used concentrations ranging from 0.6 to 0.14%, with inhibition zones varying from 10 to 16 mm. Lower results were obtained by **Prabuseenivasan *et al.*, (2006)**, who mentioned that the lowest concentrations of oils inhibiting visible growth of *B. subtilis* on the agar plate were >1.6 mg/ml of cinnamon oil (0.16%) and >3.2 mg/ml of clove oil (0.32%). Higher results were obtained by **Gupta *et al.*, (2008),** who found that cinnamon oil 2.5% inhibited both *B. cereus* and *B. subtilis* with inhibition zones 29 and 16 mm, respectively. While the 5% clove oil zone of inhibitions was 24 and 18 mm, respectively. This study conducted by **Gupta *et al.*, (2008)** also proved that cinnamon and clove oils displayed a broad range of antimicrobial activities against different gram-positive and gram-negative pathogens.

EOs derived from spice and medicinal plants act as chemical weapons inside the plant; they limit the growth of pathogenic and spoilage microbes due to their antimicrobial properties. Eugenol is the chief antimicrobial compound found in cloveshigh content; it is responsible for the essential oil's' antibacterial properties **(Babu *et al.*, 2011).** Concerning cinnamon EO, cinnamaldehyde is the primary antibacterial compound; other active elements may be present as cinnamate, caryophyllene, linalool, and other terpenes. The mode of action of different EOs depends mainly on membrane disruption or cell lysis, as well as the inactivation of enzymes and genetic material **(Sethunga *et al.*, 2023). Kwon *et al.*, (2003)** and **Elumalai *et al.*, (2010)** proved that *B. cereus* and *B. subtilis* showed sensitivity towards cinnamon oil. Moreover, the studies by **Javed *et al.*, (2012)** and **Fagere and Al Magboul (2016)** documented the inhibition of *B. subtilis* by clove oil.

Numerous publications have stated that milk and dairy products, as well as foods rich in carbohydrates such as rice, have the highest risk of contamination with spore formers, especially *B. cereus*, owing to the ability of spores to attach to equipment and form biofilms **(Adam *et al.*, 2021**; **Rahnama *et al.*, 2023;** and **Adam *et al.*, 2024)**. The high count of aerobic spore formers (32-2.8 x 10^6 CFU/g) stated in MRP by **Abdel-Latif and Saad (2016)**. A great incidence of *B. cereus* in rice pudding samples of 52 and 84% was reported by **ElSherif *et al.*, (2021)** and **El-Kholy *et al.*, (2022)**, respectively**.**

Moreover, **Ibraheim *et al.*, (2023)** isolated *B. cereus* from rice pudding as isolates found to have different toxigenic genes in diverse ratios. *B. subtilis* was documented as the sole microorganism responsible for neutral-pH dairy dessert deterioration (**Moschonas *et al.*, 2021).** Owing to the protective effect of dairy products' protein and fat on bacterial cells against antimicrobial agents, higher EOs concentrations than those tested in vitro should be used **(Ahmed *et al.*, 2021).**

Referring to the previous studies that proved the high incidence of *B. cereus* and *B. subtilis,* as well as their importance to human health and product spoilage, a model to study the antibacterial effect of EOs in MRP has been established. Therefore, depending on MIC results, cinnamon and clove EOs with a 0.6% concentration were used in the preparation of MRP. The concentration of both essential oils exhibited a significant prevention effect on the examined bacteria, with no growth observed at the end of the study.

The study conducted by **El-Kholy *et al.*, (2022)** concluded that cinnamon powder 1% in MRP is more potent against *B. cereus* than 0.5%. Moreover, **Lianou *et al.*, (2018)** found that cinnamon extract enhanced the microbiological quality of vanilla cream pudding. **Cetin-Karaca and Morgan (2018)** stated that trans-cinnamaldehyde and high pressure could be incorporated into infant formula to reduce *B. cereus*. **Salman *et al.*, (2020)** found that coliform, yeast, and mold counts were lower in kishk samples containing cinnamon and clove EOs than in the control. All these previously mentioned research papers achieved nearly similar results to those found in this study, proving the effective usage of both cinnamon and clove EOs in controlling microbial growth in various milk products. Also, cinnamon and clove EOs are applied in products other than dairy products, where **Abd El-Wahaab *et al.*, (2018)** used them to inhibit *B. cereus* in minced meat. Moreover, **Siddiqua *et al.*, (2015)** showed that cinnamaldehyde and clove oils possess antibacterial activity against *B. cereus* in watermelon juice.

Hussein *et al.*, (2023), owing the reduction in pH values along the study period in all treatments to lactose fermentation in MRP. It was also noticed that the addition of EOS reduces pH in MRP slightly compared to the control, as clove and cinnamon have some acid power. The study by **Hammad (2016)** measured the impact of cinnamon and clove on the pH of milk; both herbs at a concentration of reduced the pH of milk slightly to 6.5. When supplying dairy products with EOs, it is important to take into consideration their sensory impact, as EOs frequently have a strong flavor that can change the product's flavor. Since milk and its products are a prime part of consumer diets for achieving nutritional needs, it is essential to optimize the lowest concentrations of EOs that give antibacterial activity without negatively affecting organoleptic acceptability **(Ahmed *et al.*, 2021; Ibrahim *et al.*, 2023)**.

The control treatment's overall acceptability was found to be the lowest, as it took a score of 13.3 at the end of storage compared to clove (15.7) and cinnamon (17). Several studies reported the positive effect of adding clove EO to dairy products, as **Saleh (2018)** added it to Karish cheese at 0.75 and 1% concentrations and found that it improved its sensorial characteristics. **Antigo *et al.*, (2017),** who reported that the produced Dulce de Leche (heat-treated milk + sugar + starch) with cinnamon or clove essential oils (0.01%) showed overall sensorial acceptability of more than seventy percent, nearly similar to the control product. However, the score of Dulce de Leche with cinnamon EO was higher than the one produced by applying clove oil, which agreed with the records in this study.

Chon *et al.*, (2020) proved that market milk with clove oil (0.5%) demonstrated the preferable sensorial total score compared to the control. **Salih *et al.*, (2021)** confirmed that soft cheese supported with cinnamon EOs (0.2 or 0.4%) had flavor characteristics that were more satisfying for consumers than an unfortified treatment. Our results revealed that producing MRP supplemented with clove or cinnamon EOs at a concentration of 0.6 would be salable and acceptable commercially.

CONCLUSION

Essential oils are being reproducibly used in the food industry owing to their inherent antimicrobial potential to control pathogenic and spoilage microorganisms, thereby preventing the emergence of microbial resistance. Therefore, cinnamon and clove EOs were tested in vitro with various concentrations to show their effect on the growth of *B. cereus* and *B. subtilis*. This study concluded that fortifying MRP with essential oils such as cinnamon and clove in concentrations of 0.6% improved its safety and quality; they significantly reduced the tested inoculated bacteria (*B. cereus* and *B. subtilis*) and enhanced the sensory characteristics.

Conflict of interest

No competing interests to declare regarding the research tools and data used.

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