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Abstract. The antibacterial activity of cinnamaldehyde was evaluated against *S. aureus* experimentally inoculated (10^3 CFU/mL) in UHT-pasteurized milk, which was treated with different concentrations of the cinnamaldehyde (0.1% and 0.05%) and stored at 4 °C for 12 days. The MIC of cinnamaldehyde was 160 µg/ml. During the storage period, *S. aureus* counts in milk were reduced by 0.35-2.77 log CFU/mL. Significantly greater decreases were observed when cinnamaldehyde was added, regardless of the concentration used, compared with the control. A triangle test showed that panellists could detect the difference between milks with different concentrations of cinnamaldehyde ($P<0.01$). These results suggest that by adding 0.05% cinnamaldehyde to milk, the safety of the milk can be increased and a pleasant, desirable flavour can be obtained.

1. Introduction

Foodborne diseases caused by contamination with *Staphylococcus aureus* and enterotoxin production are still a relevant food safety issue, as the numbers of reported cases and outbreaks continue to increase worldwide. Rich in macro- and micro-nutrients, milk is a convenient medium for *S. aureus* growth. Due to the favourable conditions during storage and preparation, staphylococcal enterotoxin can be produced [1] [2].

The dairy industry is improving processing techniques to prolong the shelf life and ensure the safety of milk and at the same time, meet consumers' needs and demands for attractive and more natural products. In recent years, plant essential oils and their major components have been used to improve the safety, quality and sensory attributes of drinks and food. Milk drinks flavoured with cinnamon, cloves and other

spices have become popular in some countries, including Spain and Latin American countries [3], while in Egypt there is a trend to add these flavouring agents to milk intended for manufacturing different types of dairy products. These plant-based antimicrobials can be used as natural preservatives [4] [5]. Essential oils are low molecular weight liquids, limpid, rarely coloured, volatile mixtures that are lipid soluble and soluble in organic solvents [6] [7] [8].

Cinnamaldehyde is the most abundant component of cinnamon essential oil, which is isolated from bark and possesses a wide spectrum of antimicrobial activity against different microorganisms. Cinnamaldehyde is categorised as GRAS (Generally Recognized as Safe) by the Food and Drug Administration, and previous studies demonstrated this compound could be used in the food industry due to its noteworthy antibacterial activity [9].

The aim of this study was to investigate the antimicrobial effect of cinnamaldehyde in different concentrations (0.05% and 0.1%) against *S. aureus* in UHT-pasteurized milk, as well as its impact on the sensory characteristics of the milk.

2. Materials and Methods

2.1. Materials and culture

UHT-pasteurized milk containing 1.5% fat was bought from a local supermarket. *Staphylococcus aureus* was from the American Type Culture Collection (ATCC) 25923. Cinnamaldehyde (98% purity) was purchased from Carl Roth, Germany.

2.2. Determination of minimum inhibitory concentration

Susceptibility of *S. aureus* ATCC 25923 to cinnamaldehyde was investigated by the broth microdilution method. The broth microdilution method was performed in sterile U-bottom microtitre plates. The inoculum density was set to 0.5 on the McFarland scale, then further diluted 10 times in sterile saline and 5 µL of this suspension was inoculated in 0.1 mL of Cation Adjusted Mueller-Hinton Broth (CAMHB; Becton, Dickinson and Company, Sparks, USA) to reach a final inoculum of 5×10^4 CFU/well. Cinnamaldehyde was diluted in dimethyl sulphoxide (Serva, Heidelberg, Germany) and added to CAMHB in levels from 2560 µg/mL to 1.25µg/mL by two-fold dilution in 96-well microtitre plates. After inoculation, plates were incubated at 37°C for 24 h. The minimum inhibitory concentration (MIC) was the lowest concentration of cinnamaldehyde that prevented visible growth of *S. aureus*.

2.3. Milk preparation, storage condition and microbiological analysis

Milk was analysed for *S. aureus* on day 0 in order to determine the presence or absence of this pathogen. Approximately 3 log CFU/mL of *S. aureus* was inoculated into *S. aureus*-free milk. Then, experimentally contaminated milk was divided into thirds. Cinnamaldehyde at different concentrations (0.1% and 0.05%, respectively) was added to the first (C1-0.1%) and second part (C2-0.05%), while the third part (C-control) remained without cinnamaldehyde. For bacterial enumeration, 25 mL of milk was transferred into a sterile Stomacher bag and 225 mL of Buffered Peptone Water (BPW) (Merck, Germany) was added. The contents of each bag were homogenized in a Stomacher blender (Stomacher 400 Circulator, Seward, UK) for 2 min. Serial decimal dilutions were prepared and 0.1 mL of appropriately diluted suspension was plated on Baird Parker agar (Oxoid CM 275, Basingstoke, Hampshire, UK) with egg yolk tellurite emulsion (Oxoid CM 275, Basingstoke, Hampshire, UK) and incubated at 37°C for 24 h according to EN ISO 6888-1 [10]. All milks were stored at refrigerator temperature (4±1°C) for 12 days and examined on day 0 and on days 3, 6, 9, and 12 of storage. Number of colonies was counted, and results were recorded as colony forming units per ml (CFU/ml).

2.4. Sensory analysis

Sensory analysis was performed according to the ISO standard for triangle tests [11], using the UHT-pasteurized milk with two different concentrations (0.05%, 0.1%) of cinnamaldehyde. The aim of using the triangle test was to determine the sensory differences in the attributes most susceptible to modification after addition of cinnamaldehyde (odour, colour and taste). Two sets of three milks, of which two were identical, were offered to each of 12 semi-trained panellists. The panellists were asked to identify the different milk in each set. Milks were presented in 30 mL volumes, served at room temperature in white plastic cups, and coded using three-digit numbers chosen randomly. Water and bread were served to the panellists to clean the palate between the sets. Results were compared with tables of the minimum number of correct responses required for significant differences in this triangle testing [12].

2.5. Statistical analysis

The bacterial counts (mean±standard deviation of log CFU/mL) were analysed by one-way analysis of variance (ANOVA) and individual counts were compared on a 0.05-level of significance by Tukey's multiple comparison test using GraphPad Prism 6 (GraphPad Software, San Diego, California USA, www,graphpad.com). Data from the triangle tests were analysed by counting the number of correct responses (correctly identified different sample) and the number of total responses. These numbers were compared with critical values found in Table 18 in Baltić [12] to determine significant differences.

3. Results and Discussion

Cinnamaldehyde showed good antimicrobial activity with an obtained MIC of 160 μg/ml for *S. aureus*. However, despite the good antibacterial effect *in vitro*, higher concentrations are needed to exhibit antimicrobial activity in food model media due to their interactions with food matrix components e.g. fat and proteins [13]. Gutierrez et al. [14] found that for inhibition of *L. monocytogenes* and *P. fluorescens*, approximately 10-fold higher concentrations oregano or thyme were needed in milk than in their control medium. Thus, in the present study, we used approximately 4- and 9-fold higher concentrations of cinnamaldehyde than the MIC we measured.

Initial *S. aureus* counts ranged between 3.31 (control) and 2.29 (0.1% cinnamaldehyde) log CFU/mL and decreased during storage in all milk groups (Table 1).

Table 1. Antibacterial activity of essential oil cinnamaldehyde on *S. aureus* counts (log CFU/mL) in milk during storage at 4°C

Group	Days				
	0	3	6	9	12
C	3.31±0.22 ^{a}	2.87±0.27 ^{a}	2.91±0.25 ^{a}	3.14±0.29 ^{a}	2.96±0.24 ^{a}
C1	2.29±0.31 ^{b}	2.37±0.26 ^{b}	1.93±0.35 ^{b}	1.77±0.28 ^{b}	0.54±0.08 ^{b}
C2	2.45±0.45 ^{b}	2.46±0.26 ^{b}	2.22±0.32 ^{b}	1.91±0.22 ^{b}	1.62±0.20 ^{c}

Mean±SD with different lower-case superscript letters in the same column indicates differences (P<0.05); C – control; C1 – 0.1% cinnamaldehyde; C2 – 0.05% cinnamaldehyde.

This finding can be attributed to low temperature storage below 5°C. Although *S. aureus* can survive freezing, the minimum temperature for growth is about 7°C [15]. At the beginning of the study (day 0), initial significant ($P < 0.05$) reductions of *S. aureus* counts were observed in the groups with added cinnamaldehyde. In the milk with 0.1% cinnamaldehyde, *S. aureus* was reduced by 1.02 log CFU/mL, and in the milk with 0.05% cinnamaldehyde, the count of these bacteria decreased by 0.86 log CFU/mL, indicating the immediate antibacterial effect of cinnamaldehyde. Cinnamaldehyde is an aldehyde which, along with terpenes and phenols, is mainly responsible for the antibacterial effect of essential oils [16].

Being hydrophobic, aldehydes interrupt the microbial cytoplasm membrane due to their influence on the unsaturated fatty acids in the membrane [16,17]. Zhang et al. [18] suggested the mechanism behind the antibacterial effect of cinnamon essential oil containing 92.40% cinnamaldehyde. These authors showed that cinnamon essential oil led to leakage of small electrolytes, causing rapid increase in the electric conductivity and leading to decrease in bacterial metabolic activity. These changes happened within the first hours, which could be linked to the decrease in the number of *S. aureus* on day 0 in our study. The decrease in *S. aureus* numbers slowed down in the milks with added cinnamaldehyde during the first three days of storage, and numbers did not change from those on day 0. After that, *S. aureus* numbers decreased during the following days until the end of the storage.

The *S. aureus* count was lower ($P<0.05$) in the milks with added cinnamaldehyde than in the control milk during the 12 days of storage. An interesting observation is that although the *S. aureus* count was lower in milk with 0.1% cinnamaldehyde than in the milk with 0.05% cinnamaldehyde, no significant differences ($P>0.05$) were observed between these two milks during storage except on day 12, when *S. aureus* counts were significantly lower ($P<0.05$) in the milk with the higher concentration of cinnamaldehyde. At the end of the storage period, the *S. aureus* count was 2.96 log CFU/mL in the milk without cinnamaldehyde, 0.54 log CFU/mL in milk with 0.1% cinnamaldehyde and 1.62 log CFU/mL in milk with 0.05% cinnamaldehyde.

These results indicate that cinnamaldehyde could be effective as an anti-staphylococcal substance in the milk or dairy products. However, regardless of antibacterial activity, in general, the use of essential oils and their major components as food preservatives has been limited due their effect on organoleptic properties of food.

The triangle test is a useful method to compare two samples for which differences, especially in flavour, are difficult to detect [19]. Since the control sample obviously differed, we used a triangle test (12 panellists) to clarify the distinction in odour and flavour under normal lighting conditions between milk with 0.1% or 0.05% cinnamaldehyde added. The results (Table 2) showed that in Set II, where the higher concentration of cinnamaldehyde was different, 75% of the panellists marked their ballots correctly, and this number was the critical value 9 at $P<0.01$. When the milk with 0.05% cinnamaldehyde differed (Set I), only 50% of the panellists gave the correct answer, which was not enough to indicate a significant difference (critical value 8, $P>0.05$). Having in mind that cinnamaldehyde is a yellow oily liquid, its addition in milk at these concentrations did not affect the milk colour (data not shown). However, on addition of cinnamaldehyde to the milk, in which defects and deviations from the characteristic quality are easy to detect, its strong cinnamon odour and sweet taste [20] led to noticeable changes in the sensory properties. The intensity of characteristic odour was equal in milk with both cinnamaldehyde levels, while the sweet taste was more pronounced at the higher concentration of cinnamaldehyde, which made it easier to distinguish this milk. Additionally, panellists indicated milk with 0.1% cinnamaldehyde was less acceptable than milk with 0.05% cinnamaldehyde.

To the best of our knowledge, there are no available data on cinnamaldehyde use in milk and other dairy products or its effect on sensory quality. However, Olmedo et al. [21] used oregano and rosemary essential oils as a preservative agent in cream cheese and found that the inclusion of these essential oils increased bitterness and sourness that significantly changed cream cheese's typical flavour and aroma. The effect of cinnamaldehyde on meat's sensory properties has been examined in studies where it was supplemented in lamb diets as a feed additive [22, 23].

Considering this lack of data and the many difficulties in introducing novel bioactive ingredients, mostly as preservative agents, in dairy products, [24], the results of the present study point to the possibility of designing a new, acceptable, dairy product by adding cinnamaldehyde into the uniform system of fluid milk.

Table 2. Scores obtained in the triangle test (triangle testing for difference) comparing milks with different levels of cinnamaldehyde

	Odour	Flavour
Set I: C1 Vs. C2		
Correct replies	6 (ns)	6 (ns)
Incorrect replies	6	6
Set II: C2 Vs. C1 samples		
Correct replies	9 (P<0.01)	9 (P<0.01)
Incorrect replies	3	3

For n=12 panellists, the number of correct answers to conclude that a perceptible differences exist between samples was 8 (P<0.05), 9 (P<0.01) or 10 (P<0.001); ns = not significant; C – control; C1 – 0.1% cinnamaldehyde; C2 – 0.05% cinnamaldehyde.

4. Conclusion

The results of the present study indicate that addition of cinnamaldehyde significantly reduces the number of *S. aureus* in UHT-pasteurized milk. Taking into account this antibacterial effect and the results of the sensory analysis that showed the obvious difference between milks with higher and lower concentrations of cinnamaldehyde, further research should focus on finding optimal concentrations acceptable to consumers. Also, further studies should be focused on application of cinnamaldehyde under abusive temperature conditions and the antibacterial effect of cinnamaldehyde should be assessed against higher levels of *S. aureus* contamination.

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