

Antimicrobial Activity of Clove and Cinnamon Essential Oils against *Listeria monocytogenes* in Pasteurized Milk

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ABSTRACT

The antimicrobial activity of essential oils (EOs) of cinnamon bark, cinnamon leaf, and clove against *Listeria monocytogenes* Scott A were studied in semiskimmed milk incubated at 7°C for 14 days and at 35°C for 24 h. The MIC was 500 ppm for cinnamon bark EO and 3,000 ppm for the cinnamon leaf and clove EOs. These effective concentrations increased to 1,000 ppm for cinnamon bark EO, 3,500 ppm for clove EO, and 4,000 ppm for cinnamon leaf EO when the semiskimmed milk was incubated at 35°C for 24 h. Partial inhibitory concentrations and partial bactericidal concentrations were obtained for all the assayed EOs. The MBC was 3,000 ppm for the cinnamon bark EO, 10,500 ppm for clove EO, and 11,000 ppm for cinnamon leaf EO. The incubation temperature did not affect the MBC of the EOs but slightly increased the MIC at 35°C. The increased activity at the lower temperature could be attributed to the increased membrane fluidity and to the membrane-perturbing action of EOs. The influence of the fat content of milk on the antimicrobial activity of EOs was tested in whole and skimmed milk. In milk samples with higher fat content, the antimicrobial activity of the EOs was reduced. These results indicate the possibility of using these three EOs in milk beverages as natural antimicrobials, especially because milk beverages flavored with cinnamon and clove are consumed worldwide and have been increasing in popularity in recent years.

Listeria monocytogenes is a gram-positive psychrotrophic bacterium that is widely distributed in the environment and can be transmitted to humans through the consumption of infected foods. In recent decades, several outbreaks of listeriosis have been associated with the consumption of dairy products, such as pasteurized milk (1, 8, 12). According to quantitative risk studies of *L. monocytogenes* in ready-to-eat foods that were conducted by the U.S. Food and Drug Administration (FDA) (39), pasteurized milk is a moderate-risk product. Although *L. monocytogenes* is effectively destroyed by pasteurization, its presence in the final product probably is due to faulty thermal treatment or postprocessing contamination, mainly through contact with raw milk (34). In experimental studies, high rates of growth of *L. monocytogenes* were found at refrigeration temperatures in pasteurized milk within its shelf life (39). Effective control of the temperature during transport and storage is very important for ensuring the safety of pasteurized milk; however, these conditions are beyond the direct control of the manufacturer and control measures frequently are inadequate (19). This lack of temperature control can allow *L. monocytogenes* populations to reach levels ($>10^2$ CFU ml⁻¹) considered a grave risk for consumer health (11). The dairy industry is developing minimum processing techniques (16, 22) that can be used to prolong the shelf life and improve the sensorial characteristics of fluid milk. Plant essential oils (EOs), used since antiquity to fla-

vor drinks and food, are being used in recent years for their antimicrobial and antioxidant properties (5, 7, 10, 14, 17, 18, 28). Flavored milk beverages also have increased in popularity, in particular those sold in aseptic packages for individual consumption. This growing trend in the fluid milk industry of incorporating new aromas and flavors has led to the use of natural plant extracts as aroma and flavor enhancers, and these extracts also act as antimicrobial agents. Milk drinks flavored with cinnamon, clove, and other spices are particularly popular in Spain and Latin American countries. The antilisterial activity of cinnamon and clove EOs has been demonstrated in vitro and in foods (2, 13, 14, 24, 35). The objective of this study was to investigate the antimicrobial activity of cinnamon, clove, and cinnamon bark EOs against *L. monocytogenes* in pasteurized whole, semiskimmed, and skimmed milk incubated at different temperatures for different times.

MATERIALS AND METHODS

Microorganisms and culture conditions. *L. monocytogenes* Scott A (FDA, Washington, D.C.) was kept at -80°C in Microbank vials (Pro-Lab Diagnostics, Neston, Wirral, UK). Before each experiment, culture from a vial was activated in Trypticase soy broth (TSB; Cultimed, Barcelona, Spain) for 24 h at 35°C, streaked onto PALCAM agar (Merck, Darmstadt, Germany), and incubated for 48 h at 35°C.

To prepare the inoculum, one colony from the PALCAM agar plate was transferred to TSB and incubated for 24 h at 35°C. The inoculum was standardized until an absorbance of 0.1 at 620 nm was reached. To obtain *L. monocytogenes* concentrations of 2×10^4 to 5×10^4 CFU/ml, serial dilutions were made in fresh TSB.

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Viable counts were measured in petri dishes of Trypticase soy agar (TSA; Cultimed), which were incubated at 35°C for 24 h.

Antimicrobial compounds. The EOs of clove, cinnamon bark, and cinnamon leaf were obtained from Sotéal S.L. (Madrid, Spain). The composition of the EOs was determined in our laboratory by gas chromatography (data not shown). The main compounds in cinnamon bark EO were cinnamaldehyde (67%) and eugenol (4%), those in cinnamon leaf EO were cinnamaldehyde (4%) and eugenol (77%), and those in clove EO were eugenol (86%) and cinnamaldehyde (<0.1%). The EOs were sterilized by filtration before using.

Milk sample preparation. Whole, semiskimmed, and skimmed pasteurized milk (El Prado Cervera S.L., Valencia, Spain) (pH 6.7 to 6.8) with 3.5, 1.5, and 0.3% fat, respectively, was used to prepare the samples. The milk was purchased 1 day before the experiment was performed and 5 to 6 days before its expiration date. Sterile test tubes containing 10 ml of milk were heated by steam for 30 min at 100°C and cooled immediately in an ice bath. The sterility of the samples was confirmed by inoculating 1 ml into petri dishes of TSA and incubating at 35°C for 1 h. The milk samples were used to prepare the different concentrations of antimicrobials and inocula in milk.

PIC, MIC, and PBC. Previous experiments in TSB produced MICs of 400 ppm for cinnamon leaf and clove EOs and 200 ppm for cinnamon bark EO. Based on these results, the clove, cinnamon leaf, and cinnamon bark EOs were assayed in milk at the following concentrations: 4,500, 4,000, 3,000, 2,000, 1,500, 1,000, and 500 ppm for cinnamon leaf and clove EOs and 3,000, 2,000, 1,000, 500, and 250 ppm for cinnamon bark EO. Stock solutions of antimicrobials were prepared in test tubes with 10 ml of sterile milk and double the concentration of EO to be assayed. The tubes were shaken vigorously with a shaker to homogenize the EOs. Inocula were prepared in test tubes with 10 ml of sterile milk and an *L. monocytogenes* population of about 5×10^4 CFU/ml. Experiments were carried out in triplicate in 96-well microplates. Each well received 150 μ l of milk with the EOs to be tested and 150 μ l of milk containing the inoculum. Therefore, each well contained a final volume of 300 μ l of milk with the desired concentration of EO and a population of *L. monocytogenes* ranging from 1×10^4 to 2.5×10^4 CFU/ml. Two microplates were prepared for each assayed antimicrobial; one was incubated at 7°C for 14 days and the other was incubated at 35°C for 24 h. A positive control containing the inoculum without the respective EO and a negative control of sterile milk were prepared for each microplate. The surviving population was enumerated at 0, 2, 4, 6, 8, 10, and 14 days for the microplates incubated at 7°C and at 0, 2, 4, 6, 8, and 24 h for the microplates incubated at 35°C. The surviving population was determined from serial dilutions that were surface plated onto TSA petri dishes and incubated at 35°C for 24 h. Because of the limitations of the spread plate technique, it was not possible to obtain counts below 10 CFU/ml. The MIC was considered the concentration of antimicrobial that maintained the initial population of *L. monocytogenes* without change at the end of the incubation period ($P < 0.05$). The partial inhibitory concentration (PIC) was considered as the antimicrobial concentration lower than the MIC that inhibited growth of *L. monocytogenes* but permitted a microbial population at the end of the incubation period significantly lower ($P < 0.05$) than reached by the positive control without added antimicrobial. The partial bactericidal concentration (PBC) was considered the concentration of antimicrobial that significantly reduced the initial population of *L. monocytogenes* at the end of the incubation period ($P < 0.05$).

MBC. The MBC was defined as the lowest concentration at which 99.9% of the initial inoculum in the milk was killed and no growth was observed when 100 μ l of the milk sample was plated on TSA and incubated at 35°C for 24 h. A range of concentrations between 3,000 and 12,000 ppm for clove, cinnamon bark, and cinnamon leaf EOs was assayed in semiskimmed milk against *L. monocytogenes*. The experimental procedure used was the same as described for determining the MIC, but the samples were tested only at the end of the incubation period to verify the bactericidal effect.

Influence of fat content on the antimicrobial activity of the EOs. The antimicrobial activity of the EOs against *L. monocytogenes* was compared in skimmed milk and whole milk at the following concentrations: 2,000 and 3,000 ppm cinnamon leaf EO, 2,000 and 3,000 ppm clove EO, and 1,000 and 500 ppm cinnamon bark EO. The experiments were carried out in triplicate in 96-well microplates.

Statistical analysis. *L. monocytogenes* growth curves were obtained by representing graphically the log CFU per milliliter versus the incubation period. The microbial populations reached at the end of the incubation periods were compared with an analysis of variance and Duncan's test ($P > 0.05$) from the Statgraphic package version 5.0 (StatPoint, Inc., Herndon, Va.).

RESULTS

PICs, MICs, and PBCs of EOs in semiskimmed milk incubated at 7°C. The growth curves of *L. monocytogenes* after 14 days of incubation at 7°C in semiskimmed milk supplemented with different concentrations of EOs are shown in Figure 1. The PICs, MICs, and PBCs obtained for the same EOs are shown in Table 1. The highest antilisterial activity was exhibited by cinnamon bark EO; 500 ppm (MIC) was sufficient to inhibit the microbial growth compared with 3,000 ppm cinnamon leaf or clove EOs (MICs) necessary to achieve the same effect ($P \geq 0.05$). Concentrations of 1,000 and 2,000 ppm cinnamon bark EO had a partial bactericidal effect (PBCs), reducing the initial microbial population at 14 days by 0.5 and 1 log cycles, respectively (Fig. 1A). A concentration of 250 ppm cinnamon bark EO was considered to be a PIC, reducing the final population of *L. monocytogenes* by 1 log cycle at 14 days compared with the positive control without EO ($P \geq 0.05$). Cinnamon leaf and clove EOs at 4,000 ppm exhibited a partial bactericidal effect (PBC), decreasing the inoculated microbial population by 1 log cycle after 14 days of incubation ($P \geq 0.05$) (Fig. 1B and 1C). Concentrations of 500, 1,000, 1,500, and 2,000 ppm for both EOs had a partial inhibitory effect on the growth of *L. monocytogenes* compared with the positive control ($P \geq 0.05$), and these concentrations were considered PICs.

PICs, MICs, and PBCs of the EOs in semiskimmed milk incubated at 35°C. The growth curves of *L. monocytogenes* after 24 h of incubation at 35°C in semiskimmed milk supplemented with different concentrations of EOs are shown in Figure 2. The PICs, MICs, and PBCs obtained are shown in Table 1. The highest antilisterial activity was exhibited by cinnamon bark EO; 1,000 ppm (MIC) was sufficient to inhibit microbial growth compared with the 4,500 ppm (MIC) cinnamon leaf EO and 4,000 ppm clove

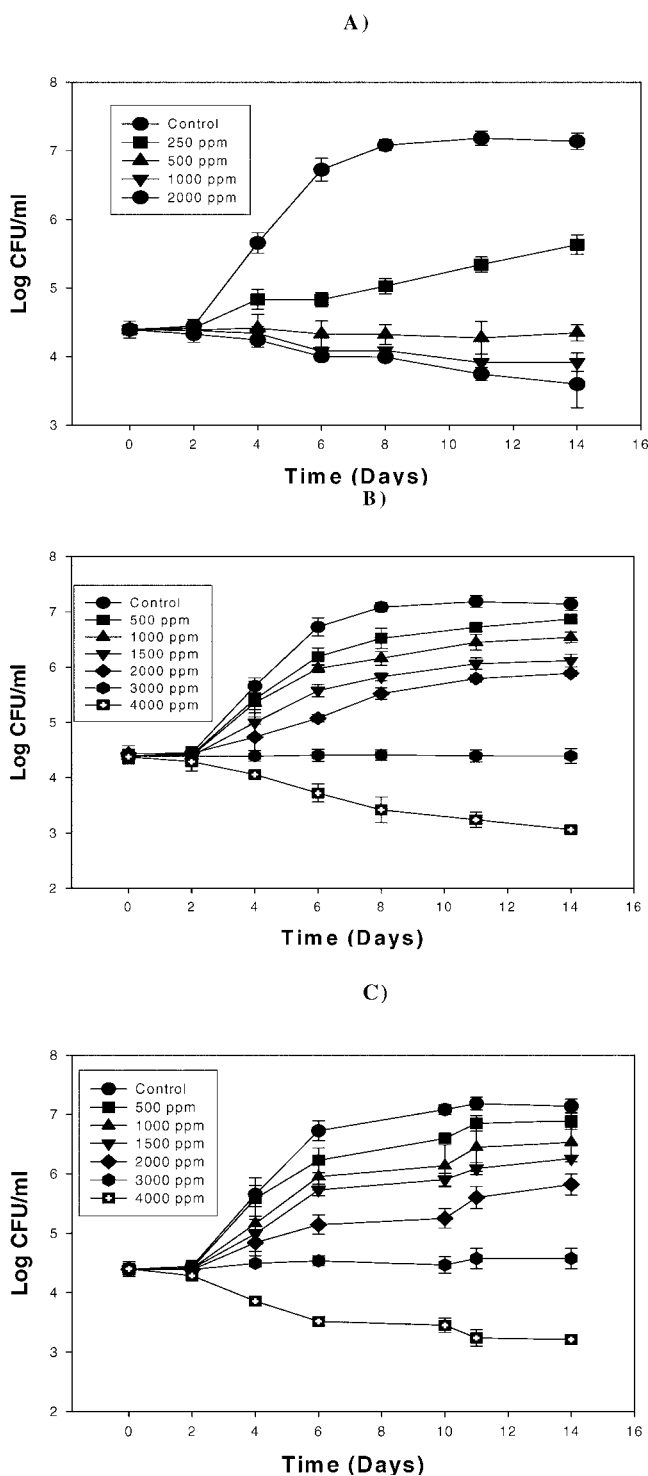


FIGURE 1. Behavior of *L. monocytogenes* in semiskimmed milk incubated at 7°C for 14 days with EOs: (A) cinnamon bark EO; (B) cinnamon leaf EO; and (C) clove EO. Error bars show the standard deviation of triplicate experiments.

EO necessary to achieve the same effect ($P > 0.05$). Concentrations of 250 and 500 ppm cinnamon bark EO were considered PICs, reducing the population of *L. monocytogenes* by approximately 1 and 2 log cycles, respectively, compared with the positive control without antimicrobial ($P > 0.05$) (Fig. 2A). Concentrations of 1,500 and 2,000 ppm had a partial bactericidal effect (PBCs), reducing the

initial population of *L. monocytogenes* ($P > 0.05$), whereas 3,000 ppm had a total bactericidal effect (MBC). Concentrations of 2,000, 2,500, 3,000, 3,500, and 4,000 ppm were PICs for cinnamon leaf EO (Fig. 2B), and concentrations of 2,000, 2,500, 3,000, and 3,500 ppm were PICs for clove EO (Fig. 2C).

MBC of the antimicrobials in semiskimmed milk incubated at 7 and 35°C. The MBCs for cinnamon leaf, cinnamon bark, and clove EOs at 7 and 35°C are shown in Table 1. Concentrations of 3,000 ppm cinnamon bark EO, 11,000 ppm cinnamon leaf EO, and 10,500 ppm clove EOs were sufficient to kill 99.9% of the initial inoculated population of *L. monocytogenes* at 35°C after 24 h and at 7°C after 14 days. The storage conditions did not influence the MBC of these EOs.

Antimicrobial activity of the EOs in whole and skimmed milk. Figure 3 shows the *L. monocytogenes* growth curves obtained in skimmed and whole milk with some of the inhibitory concentrations of EOs assayed in semiskimmed milk. Significant differences ($P > 0.05$) between the whole and skimmed milk were found for the same concentrations of all the antimicrobials tested. A concentration of 500 ppm cinnamon bark EO maintained the *L. monocytogenes* population unchanged for 14 days compared with the initial population in whole milk (MIC) but had some bactericidal effect in skimmed milk (PBC), in which it reduced the population by 1 log cycle ($P > 0.05$) (Fig. 3A). A concentration of 1,000 ppm cinnamon bark EO was partially bactericidal (PBC) in both types of milk; *L. monocytogenes* was reduced by 3 log cycles in skimmed milk but by only 1 log cycle in whole milk. Figure 3B shows the results obtained for cinnamon leaf EO in whole and skimmed milk. The 2,000 ppm concentration decreased bacterial growth by 1 log cycle with respect to the positive control in whole milk (PIC) and had a partial bactericidal effect in skimmed milk, reducing the initial population by 2 log cycles ($P > 0.05$). The 3,000 ppm concentration decreased bacterial growth by 1.5 log cycles with respect to the positive control in whole milk (PIC) but had a partial bactericidal effect in skimmed milk (PBC), reducing the initial population by 3 log cycles ($P > 0.05$). Similar results were obtained for clove EO (Fig. 3C).

DISCUSSION

EOs of clove, cinnamon bark, and cinnamon leaf exhibited an antimicrobial effect against *L. monocytogenes* in pasteurized milk stored at 7 and 35°C. This antimicrobial activity was dependent on the composition and concentration of the EOs, the composition of the food, and the time and temperature of exposure. The cinnamon bark EO had the strongest activity, whereas higher concentrations of clove and cinnamon leaf EOs were needed to completely inhibit the growth of *L. monocytogenes* in semiskimmed pasteurized milk.

The MICs found in this study for all the EOs against *L. monocytogenes* in semiskimmed milk (from 0.05 to 0.3% fat) were lower than the MICs found by other authors in cheese, sausages, and chicken, which were in the range of

TABLE 1. Partial inhibitory concentration (PIC), MIC, partial bactericidal concentration (PBC), and MBC of cinnamon leaf, cinnamon bark, and clove EOs against *L. monocytogenes* in semiskimmed milk incubated at 7 and 35°C

Essential oil	7°C for 14 days (ppm)				35°C for 24 h (ppm)			
	PIC	MIC	PBC	MBC	PIC	MIC	PBC	MBC
Clove	2,000	3,000	4,000	10,500	3,500	4,000		10,500
	1,500				3,000			
	1,000				2,500			
	500				2,000			
Cinnamon leaf	2,000	3,000	4,000	11,000	4,000	4,500		11,000
	1,500				3,500			
	1,000				3,000			
	500				2,500			
Cinnamon bark	250	500	1,000	3,000	500	1,000	1,500	3,000
			2,000		250			

0.5 to 2% (17, 27, 29, 36). However, the MBCs of clove and cinnamon leaf EOs against *L. monocytogenes* in semi-skimmed milk were higher (1.05 to 1.1%) than those found in studies of fruit beverages. For example, Liang et al. (23) found that 0.2% clove powder reduced an inoculated population of *L. monocytogenes* by 5.8 log cycles in apple cider, and Raybaudi-Massilia et al. (33) found that 0.2 and 0.8% clove EOs were bactericidal against *Listeria innocua* in pear juice and melon juice, respectively. Yuste and Fung (41) determined that 0.1% cinnamon in pasteurized apple juice inactivated a 10^4 CFU/ml population of *L. monocytogenes* Scott A to undetectable numbers within 1 h of storage at 5 or 20°C.

The different levels of antimicrobial activity of EOs in foods can be attributed to the influence of intrinsic and extrinsic parameters. Some of the intrinsic parameters that affect the antimicrobial activity of EOs in semiskimmed milk are pH, water activity (a_w), and the fat and protein contents. The antimicrobial activity of EOs is generally supposed to be lower in food with a neutral pH, such as milk, than in acidic foods. The increased hydrophobicity of EOs at low pH enables them to dissolve more easily in the lipid of the cell membrane of target bacteria. The high a_w of milk should increase the antimicrobial activity of EOs because the water content of milk facilitates the movement of antibacterial agents to the target site in the bacterial cell. Protein and fat bind and/or dissolve active compounds, reducing their availability for antimicrobial activity. The influence of these factors on the antimicrobial activity of EOs has been reviewed by Burt (5). The results obtained in the present study also demonstrated that the antimicrobial effect of the natural compounds tested decreases with increasing fat content of milk. Other authors have suggested that fat in foods could form a protective coating around bacterial cells, thereby protecting them from antimicrobial agents (26), and components in the EOs also could migrate to the fatty phase of the food, leaving the aqueous phase and allowing bacteria to develop free of antimicrobials (37).

The MBC of the cinnamon bark EO in pasteurized semiskimmed milk was lower than that of clove and cinnamon leaf EOs. The differences among the antimicrobial

activities of these EOs can be explained by their different compositions. Phenolic compounds found in EOs are responsible for their antimicrobial activity (20, 24, 30). Eugenol and cinnamaldehyde are the active compounds of cinnamon and clove EOs (9, 25). The higher antimicrobial activity of cinnamon bark EO can be attributed to its higher cinnamaldehyde content. Chang et al. (6) also found that the EO of cinnamon with high cinnamaldehyde content had very strong antibacterial activity, and Park et al. (32) reported high insecticidal activity for the same EO.

The antimicrobial activity of the three EOs tested also was influenced by the incubation temperature. Smith-Palmer et al. (35) found a similar effect of incubation temperature on the antimicrobial activity of cinnamon and clove EOs against *L. monocytogenes* in TSB. Bahk et al. (3) and Beuchat et al. (4) also reported that low temperatures enhanced the inhibitory activity of plant extracts. This finding suggests the possibility of applying these EOs at low concentrations in combination with refrigeration temperatures to preserve foods such as pasteurized flavored milk.

The action mechanism of cinnamon and clove EOs is based on the damage they cause in the cell membrane. An important characteristic of these EOs and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing these structures and rendering them more permeable (5). This increased permeability could explain why the assayed EOs are more effective at low temperatures; the phospholipid composition of the cytoplasmic membrane of cells grown at 7°C has a higher degree of unsaturation than does that of cells grown at 35°C to maintain fluidity and function at low temperatures. The increased fluidity would enable the EOs to dissolve more easily into the lipid bilayer of cells grown at 7°C than into that of cells grown at 35°C. Other action mechanisms of clove and cinnamon EOs are based on their capacity to inhibit the production of protease and amylase enzymes (17, 38), inhibit the uptake of glucose (21), and interfere with the proton motive force, electron flow, and active transport (15, 31). They also inhibit enzyme action through their ability to bind with proteins (40). Although all these mechanisms are well docu-

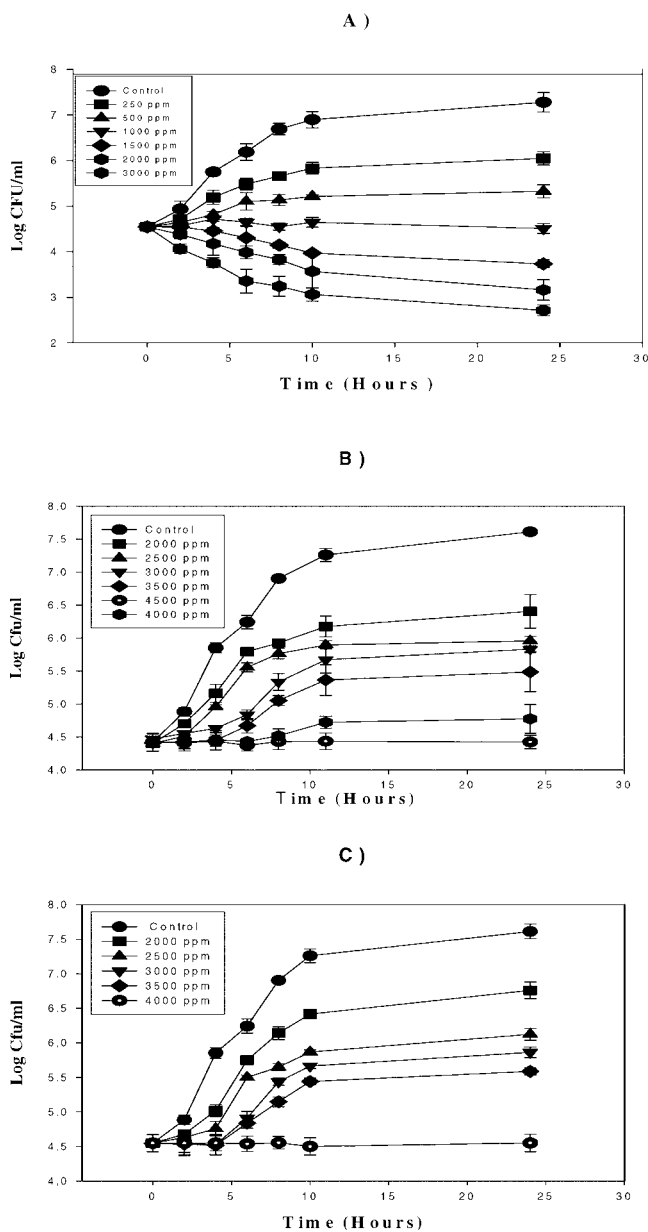


FIGURE 2. Behavior of *L. monocytogenes* in semiskimmed milk incubated at 35°C for 24 h with EOs: (A) cinnamon bark EO; (B) cinnamon leaf EO; and (C) clove EO. Error bars show the standard deviation of triplicate experiments.

mented in the literature, the differences between inhibitory and bactericidal activities are not clear, and research currently is directed at this issue (15).

Generally, the MBC, PBCs, and MIC are too high for EOs to be used as antimicrobials in foods because of the possible negative impact on sensory properties. However, the PICs of the assayed EOs indicate great potential for use as antimicrobials in milk and possibly other foods to increase safety during the product shelf life, provided that the initial pathogen load in the product was low. In the case of milk, the inhibition of microbial growth by just a few log cycles could be sufficient to keep the milk under the recommended microbiological limits. These PICs also could be used to develop synergistic combinations, in which the action of one compound could facilitate the uptake of the

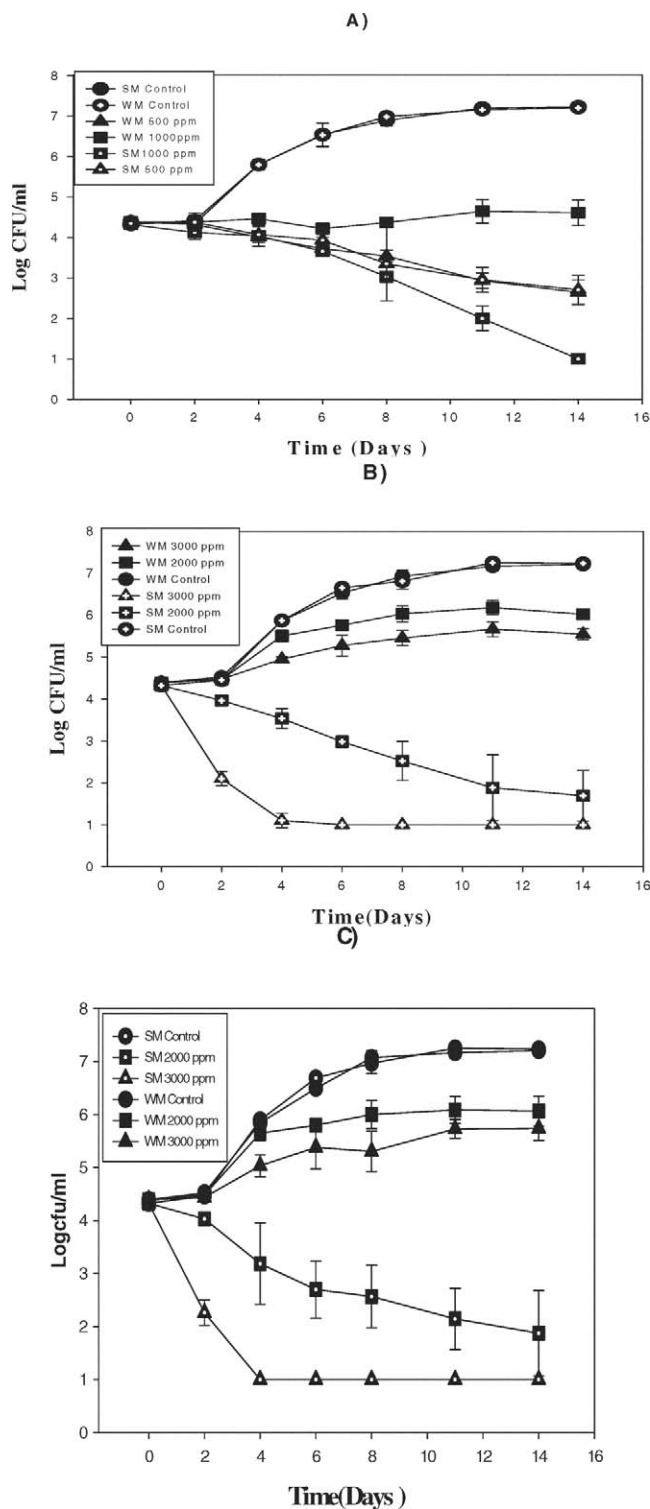


FIGURE 3. Behavior of *L. monocytogenes* in whole milk (WM) and skimmed milk (SM) incubated at 7°C for 14 days with EOs: (A) cinnamon bark EO; (B) cinnamon leaf EO; and (C) clove EO. Error bars show the standard deviation of triplicate experiments.

other in the lipid bilayer of the cytoplasmic membrane. The same antimicrobial effect might then be reached with a lower concentration of EO and therefore with a lower sensory impact on milk.

The data presented here indicate the potential of cinnamon and clove EOs for use as antimicrobials against *L.*

monocytogenes in pasteurized milk. In particular, cinnamon bark EO exhibited high activity at low doses and might be useful as a food preservative because of its possible low sensory impact. The use of these EOs could be an alternative approach to ensuring the safety of flavored pasteurized milk, because refrigeration alone is not totally effective for controlling the growth of *L. monocytogenes*. This finding is especially relevant at a time when there is increasing interest in finding natural alternative flavor enhancers in milk beverages. EOs in general also are recognized as having antioxidant properties and are potentially beneficial for health and for reducing the free radical-mediated deterioration of food. The use of synergistic mixtures of EOS at PIC levels, alone or in combination with other hurdles such as mild heat treatment, represents an exciting potential for future research. The application of these combined preservation methods in milk would result in minimal damage to its nutritive and organoleptic characteristics.

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