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Short communication: Inhibitory activities of the lantibiotic nisin combined with phenolic compounds against *Staphylococcus aureus* and *Listeria monocytogenes* in cow milk

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ABSTRACT

We aimed to investigate the antibacterial activities of carvacrol, thymol, eugenol, cinnamaldehyde, and lantibiotic nisin against standard bacterial strains of the milk pathogens *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* ATCC 15313 in cow milk. The minimum inhibitory concentrations (MIC) of these substances were recorded. The synergistic effects were also assessed in culture medium (time kill curve) and in a food model (cow milk) during the storage period (4°C for 6 d) after inoculation with *S. aureus* and *L. monocytogenes* individually by combining nisin and the phenolic compounds at proportions of 1/4 + 1/4 the MIC (determined in a previous in vitro assay) in the culture medium and 1/4 + 1/4 of MIC in the food model. Inhibitory activities of nisin and the tested compounds, as well as synergism in the combinations, were found against both bacteria assayed. Bacteriostatic effects were found with all combinations and a significant difference in *L. monocytogenes* reduction was found compared with the control assays. Thus, the antibacterial activity of nisin combined with phenolic compounds was confirmed against these pathogenic bacteria that are important in the milk industry, or more broadly in food science, with potential applications for milk preservation.
Key words: nisin, phenolic compounds, synergism, antimicrobial, milk pathogen

Short Communication

Milk and dairy products can be contaminated with microorganisms that are pathogenic to humans during milking as well as during their transport or processing. Although these products are pasteurized, depending on the care taken in the processing method as well as the nature and initial load of contaminants, this procedure may fail and not completely eliminate the potential pathogens from milk (Oliver et al., 2005). In *Listeria monocytogenes*, for example, persistence in raw milk and processed products is related to its growth from 0.5 to 45°C (Fox et al., 2009).

Staphylococcus aureus is another pathogen found in milk, whose food-borne disease is a result of contamination and ingestion of preformed enterotoxins produced in foods (Aragon-Alegro et al., 2007). Thus, dairy products, such as raw milk and fresh cheese, could be sources of this pathogen and cause human intoxication (Kadariya et al., 2014; Cortimiglia et al., 2015).

A variety of chemical and synthetic compounds have been used as antimicrobials to inhibit pathogens in food models. However, preference for minimally processed food is increasing (Sobrino-Lopez and Martin-Belloso, 2008), but it must be done with no loss of food safety and extend the shelf life and improve the safety of food by natural microbiota or antimicrobial compounds (Samadi et al., 2012).

Essential oils have been studied for their potential against pathogenic microorganisms according reported properties such as antibacterial, antifungal, antiviral, antiparasitic, antitoxigenic, antiseptic, and anesthetic. The characteristic phenolic compounds in essential oils are normally responsible for antimicrobial activity by acting on cell sites, causing the loss of cellular constituents, collapse of the bacterial cytoplasmatic membrane, and subsequent cell death (Burt, 2004; Sacchetti et al., 2004; Sartoratto et al., 2004; Barbosa et al., 2009; Al-Reza et al., 2010). Among phenolic compounds, carvacrol from oregano (*Origanum vulgare*; Lukas et al., 2013), cinnamaldehyde from cinnamon (*Cinnamomum zeylanicum*; Feniman et al., 2012), eugenol from clove (*Syzygium aromaticus*; Moritz et al., 2012), and thymol from thyme (*Thymus vulgaris*; Nikolić et al., 2014) have been studied, among others.

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Table 1. Minimum inhibitory concentrations (μg/mL; ±SD) from culture medium assays with antimicrobial compounds against bacterial strains

Bacteria	Thymol	Carvacrol	Cinnamaldehyde	Eugenol	Nisin
Staphylococcus aureus	155 ± 7.07	60 ± 0	100 ± 0	25 ± 7.07	110 ± 14.14
Listeria monocytogenes	150 ± 14.14	65 ± 7.07	65 ± 7.07	90 ± 14.14	125 ± 7.07

Moreover, the use of antimicrobial peptides in food has also been reported to promote the elimination or reduction of microorganisms and to increase the shelf-life of food (Brul and Coote, 1999; Burt, 2004; Mills et al., 2011). In particular, nisin, a bacteriocin of the lantibiotic class produced by *Lactococcus lactis* ssp. *lactis*, has been approved by the US Food and Drug Administration as a preservative in food and also recognized as generally recognized as safe (Williams and Delves-Broughton, 2003; Cotter et al., 2005; Deegan et al., 2006).

The antibacterial activities of nisin are pronounced against gram-positive food-borne pathogens such as *L. monocytogenes* and *S. aureus* (Campion et al., 2013). However, its effectiveness is often affected by environmental factors such as pH, temperature, composition, structure, and natural microbiota of food (Zhou et al., 2013). Thus, its combined use with other antimicrobial agents for food preservation has been proposed as an alternative to increase its action spectrum (Pol and Smid, 1999; Ettayebi et al., 2000). Therefore, we aimed to investigate the antibacterial synergism of nisin and phenolic compounds that are normally present in essential oils against standard *S. aureus* and *L. monocytogenes* strains by in vitro assays, including a food model (milk) test.

Nisin (from *Lactococcus lactis* N5764) and the phenolic compounds thymol (density = 0.965 g/mL, purity = 99.5%, code T0501), eugenol (density = 1.06 g/mL, purity = 99%, code E51791), carvacrol (density = 0.976 g/mL, purity = 98%, code 282197), and cinnamaldehyde (density = 1.05 g/mL, purity = 93%, code W228613) were purchased from Sigma Aldrich (St. Louis, MO). The working solutions of each product were prepared in solutions (ratio 1:1) of dimethyl sulfoxide (Sigma Aldrich) and water. Standard American Type Culture Collection (ATCC) bacterial strains of *S. aureus* (ATCC 25923) and *L. monocytogenes* (ATCC 15313) were chosen for the antimicrobial assays.

The susceptibility assays were performed according to the Clinical and Laboratory Standards Institute protocol (CLSI, 2012) adapted to resazurin microtiter plate assay on ELISA microplates (96 wells) with Mueller Hinton broth (Difco, Becton Dickinson and Company, Franklin Lakes, NJ) culture medium. The bacterial strains were grown (37°C for 18–24 h) in brain heart infusion (Difco) and, after standardization by 0.5 McFarland scale, were inoculated (around 10^5 cfu/mL) in wells at concentrations previously prepared from 1 to 500 μg/mL with the tested antimicrobial compounds. After 37°C for 18 to 24 h, the MIC of each strain was recorded after adding 50 μL of 0.01% resazurin to the respective wells and then assessing the color change based on the redox state. Assays were performed in triplicate, using positive and negative controls for each bacterium as well as the antimicrobials and dimethyl sulfoxide controls, which were tested at the highest concentrations. The in vitro susceptibility assays against ATCC *S. aureus* and *L. monocytogenes* to provide results for the next stage of this research are presented in Table 1.

Combinations of nisin and antibacterial compounds were assayed (time kill curve) against *S. aureus* 25923 and *L. monocytogenes* ATCC 15313 strains. Tubes with 30 mL of Mueller Hinton broth with combinatorial proportions of one-fourth the previously defined MIC values and tubes containing the MIC of each individual antimicrobial compound and nisin separately were also inoculated with around 10^5 cfu/mL of the standardized bacterial suspensions 0.5 McFarland and maintained at 37°C for 24 h.

Aliquots of 0.1 mL at 0, 2, 4, 6, 8, 12, and 24 h were subcultured on plate count agar (Difco), bacterial cells were recovered and the log of colony-forming units per milliliter were recorded after 37°C for 24 h. Reductions of less than 3 log cfu/mL indicate a bacteriostatic effect, whereas reductions of 3 or more log colony-forming units per milliliter showed a bactericidal effect compared with the initial inoculum of each (NCCLS, 1999; Belley et al., 2008).

Nisin and phenolic compound combination assays were performed at 1/4 and 1/4 MIC combinatory proportions of antimicrobials with pasteurized milk type A samples with 3% fat. The MIC values of each antimicrobial compound and their combinations with nisin were tested in milk inoculated (tubes containing 50 mL of milk for each experiment with around 10^5 cfu/mL of bacteria) with *S. aureus* and *L. monocytogenes* and maintained at ±4°C for 6 d. The first count from milk samples was performed at the time of incubation, and aliquots were subsequently taken after 3 and 6 d of storage. Aliquots from tubes were spread onto petri dishes with plate count agar medium and also with selective medium palcam agar (Oxoid, Basingstoke, UK)