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Inhibitory effect of combinations of caprylic acid and nisin on *Listeria monocytogenes* in queso fresco

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ABSTRACT

Queso fresco (QF), a fresh Hispanic cheese has been linked to outbreaks and recalls caused by *Listeria* contamination. The use antimicrobial treatments may be a potential solution. The goal of this research was to test the addition of nisin (N), caprylic acid (CA) and *trans*-cinnamaldehyde (CN) as anti-listerial ingredients in QF. QF batches were inoculated with approx. 10^4 CFU/g of 5- or 6-strain mixtures of *Listeria monocytogenes* and treated with antimicrobials. Samples were stored at 4 °C for three weeks and *Listeria* counts were determined by plating on PALCAM agar. The impact on the QF's natural indicator microorganisms was also assessed during refrigerated storage. All N and CA combinations (≥0.4 g/kg each) were effective against *L. monocytogenes* and reduced the final counts by at least 3 log CFU/g after 20 days of storage compared to controls. The levels of most strain mixtures were reduced immediately after treatment and their numbers remained below 10^3 CFU/g during storage. CN (1.2 g/kg) was bacteriostatic against *L. monocytogenes*, but it did not reduce initial counts. The addition of CN to the combination of N and CA did not enhance their antimicrobial effect. Results indicated that combinations of N and CA could control *L. monocytogenes* in QF with little impact on the natural flora of the cheese, providing a solution to control post processing *L. monocytogenes* contamination of QF.

1. Introduction

According to the U.S. Census Bureau agency, the Hispanic population increased 43% from 2000 to 2010, making Hispanics the fastest-growing minority group in the U.S. (Census Bureau, 2011). The rapid growth of the Hispanic population in the United States combined with the exposure of people to Latin American culinary culture has resulted in a greater demand for traditional cheeses. There is a wide diversity of Hispanic cheeses, and queso fresco (QF) is one of the most popular soft Hispanic cheeses that is manufactured with little or no starter culture and subsequently does not undergo a fermentation or aging period.

Due to the absence of an aging period, the QF cheese is normally consumed within 14 days of manufacture (Renye et al., 2008). Fresh Hispanic cheeses are typically white, have relatively high moisture content (>50%) and have pH values greater than 5.8 (Van Hekken and Farkye, 2003). With near neutral pH, high moisture content and low salt concentration, fresh unripen Hispanic-style cheeses like QF are very susceptible to growth of spoilage organisms and pathogenic bacteria (Lin et al., 2006). The major food safety concern related to QF stems from the linkage of this cheese to several foodborne disease outbreaks associated with *Listeria monocytogenes*.

Five of 11 cheese associated outbreaks reported to the Centers for Disease Control between 1973 and 1992 were associated with soft cheeses such as queso fresco (Altekruse et al., 1998). The first documented queso fresco outbreak occurred in 1985, in Los Angeles, CA where contaminated QF caused more than 140 listeriosis cases (Linnan et al., 1988). The risk of *L. monocytogenes* in Hispanic fresh cheeses is based on its ubiquitous occurrence in the environment of dairy plants and its ability to survive and grow on these products. Improvements in the safety of QF need to be effective inhibiting the growth of *L. monocytogenes*, while maintaining the properties and characteristics of this type of cheese. The use of antimicrobial ingredients may offer a viable solution to this problem.

Nisin is a low molecular weight bacteriocin produced by certain strains of *Lactococcus lactis* subsp, *lactis* (Delves-Broughton et al., 1996). This antimicrobial is very effective against Gram-positive bacteria such as *L. monocytogenes* (Boziaris and Adams, 2000). Nisin inhibits target cells by forming pores in the membrane, depleting the trans-membrane potential and the pH gradient, resulting in the leakage of cellular materials (Cleveland et al., 2001). Under U.S. federal regulation, nisin has been affirmed generally-

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recognized-as-safe (GRAS) status and is allowed in foods at a maximum level of 250 ppm (CFR, 2013). Caprylic acid (CA), or octanoic acid, is an eight-carbon fatty acid naturally present in breast milk, bovine milk and coconut oil (Jensen, 2002). Under federal regulation, CA has GRAS status and is allowed in cheese at levels of 0.04% (CFR, 2013). The exact mechanism of action of CA on bacteria is unknown, however, hypotheses have been suggested to explain the mode of antimicrobial activity of free fatty acids and their monoglycerides (Bergsson et al., 1998).

Cinnamaldehyde, or *trans*-cinnamaldehyde (CN), is the main component of cinnamon flavor (*Cinnamomum zeylandicum*) and naturally occurs in the bark of cinnamon trees (Burt, 2004). CN has GRAS status, is classified as a flavoring substance, and no usage levels are established by the FDA (CFR, 2013). The mode of action of *trans*-cinnamaldehyde is still uncertain; studies have shown that the composition of unsaturated fatty acids from the cytoplasmatic membrane of bacterial cells change drastically when exposed to high levels of CN, suggesting that this compound acts on the membrane, altering its lipid profile and structure (Di Pasqua et al., 2007; Di Pasqua et al., 2006). The goal of this research project was to test a combination of GRAS antimicrobial treatments to control a diverse collection of *L. monocytogenes* strains in queso fresco. GRAS food additives used in this study included nisin (N), caprylic acid (CA) and *trans*-cinnamaldehyde (CN).

2. Materials and methods

2.1. Bacterial strains

The strains used in this research are listed on Table 1. Most of the *L. monocytogenes* strains are part of the International Life Sciences Institute collection housed at Cornell University provided by Dr. Martin Wiedmann.

2.2. GRAS antimicrobial ingredients

The antimicrobial ingredients used in this project included nisin (N; Nisaplin®, kindly provided by Rex Infanger from Danisco, Inc., Copenhagen, DK), caprylic acid (CA; Sigma–Aldrich, Saint Louis, MO), and *trans*-cinnamaldehyde (CN; Sigma–Aldrich).

Table 1
L. monocytogenes strains used in this study and their origin.

ID	Ribotype	Serotype	Source
N3-013	1042B	4b	Food, epidemic, UK, pate, 1988
J1-158	10142	4b	Animal, goat
J1-169	1052A	3b	Human, sporadic
J1-049	1042C	3c	Human, sporadic
C1-056	1030A	1/2a	Human, sporadic
J1-177	1051D	1/2b	Human, sporadic
M1-004	1039B	NA	Human, sporadic
J2-020	1039C	1/2a	Animal, cow
J1-031	1059A	4a	Human, sporadic
J1-168	116-110-S2	4a	Human, sporadic
J2-031	1039E	1/2a	Animal, cow
R2-501	1042B	4b	Human, epidemic, North Carolina, cheese, 2000
NA[b]	ATCC 15313	NA	Rabbit, Cambridge, England
J1-094	116-1501-S4	1/2c	Human, sporadic
NA	ATCC 51775	1/2a	Cheese, Belgium
NA	H7762	4a	Franks, Sara Lee outbreak, 1998
NA	2349	4a	Environmental isolate, FSML[a]
NA	3528	4a	Environmental isolate, FSML
NA	2422	4a	Environmental isolate, FSML
J2-064	1052	1/2b	Animal, cow

[a] Food Safety Microbiology Laboratory, University of Minnesota.
[b] NA: Not available.

2.3. Inoculum preparation

Stock cultures were stored in glycerol at −55 °C and working cultures were grown on tryptic soy agar (TSA) (Neogen Corp., Lansing, MI) at 37 °C for 48 h. One colony was picked from each plate, transferred to a tryptic soy broth (TSB) (Neogen Corp.) tube and incubated at 37 °C for 18 h. After streaking each culture onto TSA slants and incubating at 37 °C for 48 h, working cultures were stored at 4 °C for no more than a month. New culture slants were prepared each month. For inoculation of cheese curds, TSB tubes were inoculated individually with working cultures of each strain and incubated at 37 °C for 18 h. Aliquots of 2 mL culture samples from each bacterial strain were mixed, serially diluted and applied to 2.3 kg portions of cheese curds in order to obtain an initial count of approximately 10^4 CFU/g. Groups of five to six strains of *L. monocytogenes* were used for inoculation.

2.4. Cheese manufacture, inoculation and treatment

Approximately 110 L batches of milk were pasteurized for each experimental trial. Non-fat dried milk (2%) was added to the milk before pasteurization. Pasteurized milk was transferred to a sanitized vat and heated to a temperature of 30–33 °C in a food processing pilot plant. Calcium chloride (0.02%) was mixed evenly into the milk. The milk was set by adding chymosin (Chymax®, Christian Hansen, Hørsholm, Denmark) (0.01%) and allowing the curd to form for 30 min. Cheese knives that produced 1/2 inches cubes were used to cut the curds. After cutting, the curds were stirred for 1 h and the temperature was increased to 35 °C. Whey was drained to about 50% of the original volume and the curds were salted (2%). The curds were bagged and transferred to a biosafety level 2 (BSL2) laboratory.

N was weighed to the appropriate amount to obtain a concentration of 0.49 g/kg of curds. CA and CN were measured volumetrically to deliver concentrations between 0.36–0.72 g/kg and 0.3–1.2 g/kg respectively, before curd mixing. The weighed amounts of these compounds were individually mixed with 100 mL aliquots of wet curds and added separately to a total of 2.3 kg portions of cheese curds before inoculation and mixed for 2 min by hand in sealed bags. Binary combinations of N and CA with or without CN were tested. CN was also tested individually. Curds were molded and allowed to drain for 1 h and the resulting queso fresco portions were bagged and stored at 4 °C for 20 days.

2.5. Microbiological analysis

During storage time, 11 g composite samples were taken from QF pieces every 2–3 days for microbiological analysis. Samples were homogenized with 99 mL buffered peptone water (BPW) (Neogen Corp.), serially diluted 10-fold and spread plated in duplicate on PALCAM (Neogen Corp.) agar plates. Plates were incubated at 12 °C for 10 days, typical colonies were counted and the values were transformed into log CFU/g of cheese sample. The choice of low incubation temperature reduced the interference of natural background bacteria capable of growing on PALCAM producing non-typical *Listeria* colonies. Non-inoculated control samples were also included in every trial and no presumptive colonies were observed in any of those plates. The pH of samples was also measured directly using a pH electrode (Beckman Instruments, Inc., Fullerton, CA). The lower limit of detection was 100 CFU/g; when no plates had colonies at 10^{-1} dilution, a value of 10 CFU/g was assigned.

2.6. Microbiological analysis of natural flora

Cheese batches were manufactured following the protocol described above, except that no inocula were used. During storage,