Use of a miniature laboratory fresh cheese model for investigating antimicrobial activities

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ABSTRACT

Hispanic-style fresh cheeses, such as queso fresco, have relatively low salt content, high water activity, and near neutral pH, which predisposes them to growth of *Listeria monocytogenes*. Biosafety constraints limit the incorporation of *L. monocytogenes* into cheeses manufactured via traditional methods in challenge studies, so few have focused on in situ testing of novel antimicrobials in fresh cheeses. We have developed a modular, miniaturized laboratory-scale queso fresco model for testing the incorporation of novel antilisterials. We have demonstrated the assessment of the antilisterials nisin and ferulic acid, alone and in combination, at various levels. Our results support the inhibitory effects of ferulic acid in cheese, against both *L. monocytogenes* and its common surrogate *Listeria innocua*, and we provide preliminary evaluation of its consumer acceptability.

Key words: queso fresco cheese, *Listeria monocytogenes*, nisin, ferulic acid, minimum inhibitory concentration

INTRODUCTION

Most styles of cheese are not commonly associated with foodborne pathogens. Exceptions generally include soft-ripened, unripened, and raw-milk or improperly pasteurized cheeses, which have been associated with the majority of outbreaks in cheeses. Most of the risk associated with cheeses in general is due to outbreaks of *Listeria monocytogenes* in Hispanic-style fresh cheeses such as queso fresco (Batz et al., 2011). Such noncultured, unripened cheeses have high moisture content, low salt content, and near neutral pH, so proper refrigeration is required to maintain shelf life. However, *L. monocytogenes* can proliferate at refrigeration temperatures, leaving few hurdles for its inhibition.

Recent multi-state outbreaks of listeriosis highlight ongoing concerns regarding contamination of Hispanic-style fresh cheeses (Centers for Disease Control and

Prevention, 2014a,b). Outbreaks from illicitly produced queso fresco make headlines (MacDonald et al., 2005) and emphasize that a growing demand for fresh cheeses is outpacing the supply by current markets, because many manufacturers hesitate to capitalize on such demand due to liability concerns. Strategies are needed to help prevent *Listeria* outbreaks and allow for safer expansion of the market for Hispanic-style fresh cheeses. Outbreaks continue to occur, despite pasteurization of raw ingredients, due to listerial contamination during manufacturing processes. What research is available on *Listeria* control measures in fresh cheeses has confirmed survival and growth of *L. monocytogenes* during manufacturing as well as during storage under refrigeration (Leggett et al., 2012). Because postmanufacture processes such as high-pressure or additional heat treatment can unacceptably alter the delicate cheese structure (Hnosko et al., 2012), limited measures are available to eliminate postpasteurization contamination. This suggests that direct incorporation of antimicrobial compounds into fresh cheeses may be necessary for *Listeria* control.

A wide variety of plant essential oils, extracts, and their derivatives (Lis-Balchin and Deans, 1997; Smith-Palmer et al., 1998), as well as organic acids (Shin et al., 2007; Silva et al., 2012), have been shown to exhibit antilisterial activity in vitro, but few have been incorporated into a fresh cheese challenged with pathogens. This may be due in part to the potential effect of such additives on the subtle flavor of fresh cheeses, but the lack of a convenient laboratory-scale fresh cheese-manufacturing model for use in pathogen challenge studies may also present a hurdle to the investigation of novel antimicrobials. In the manufacturing processes of commercial cheese, antimicrobial compounds may be incorporated into the pasteurized milk or the curd to inhibit listerial growth, because listerial contamination could occur at many points throughout the production process. *Listeria* could be present from the raw or insufficiently pasteurized milk or be introduced at various later stages, including coagulation, curd cutting, draining, transfer, and packaging (Silva et al., 2003). However, most studies of antilisterial compounds

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Table 1. Strains of *Listeria*

<i>Listeria</i> strain
<i>Listeria innocua</i> ATCC 33090
<i>Listeria monocytogenes</i> ATCC 19112
<i>Listeria monocytogenes</i> ATCC 7644
<i>Listeria monocytogenes</i> 10403S
<i>Listeria monocytogenes</i> SLCC-5764
<i>Listeria monocytogenes</i> ATCC 13932

in fresh cheese obtain retail products to which *Listeria* and the treatments are then surface-applied (Soni et al., 2010, 2012; Malheiros et al., 2012; Silva et al., 2012). The use of a convenient and accurate small-scale model for production of fresh cheeses could allow for higher-throughput screening of novel additives than in pilot-scale manufacturing, which is cumbersome and impractical in most settings. Consequently, a small-scale model would be both more economical and more amenable to biocontainment during work with pathogens.

Nisin exhibits broad-spectrum inhibition of gram-positive organisms, including *L. monocytogenes*. It is the most commonly used bacteriocin in the food industry, and is often used in processed cheeses to inhibit spore germination. As with many bacteriocins, however, nisin has limited stability and efficacy in neutral pH environments (Delves-Broughton et al., 1996). Several novel antimicrobial additives have been explored as alternatives. For example, ferulic acid is a hydroxycinnamic acid that has been investigated for its antioxidant properties, but it is also capable of inhibiting the growth of *L. monocytogenes* in ready-to-eat foods (Takahashi et al., 2013). Although nisin alone may not be sufficient to mitigate the risk of *L. monocytogenes* in conditions of relative instability, its combination with other antimicrobial compounds may result in synergies that ultimately lead to products safe from listerial contamination. However, investigation within a model that appropriately reflects the food of interest would be necessary to effectively screen for novel antimicrobials and their combinations.

The objective of this study was to develop a flexible, small-scale fresh cheese that accurately reflects the composition of commercial preparations and to demonstrate its use to explore methods for prevention of microbial growth. Such a cheese-preparation model would ideally be contained entirely within a biosafety cabinet for investigation of antimicrobial effects on pathogens and to flexibly model different microbial contamination and intervention scenarios. In this study, we demonstrate applications of such a miniaturized laboratory queso fresco (**MLQF**) and explore methods for prevention of listerial growth in fresh cheese. We specifically

investigate the in situ dose response of the common food-grade bacteriocin nisin and assess the effect of a novel antilisterial, ferulic acid, on *Listeria innocua* and *L. monocytogenes* in fresh cheese.

MATERIALS AND METHODS

Microorganisms and Culture Conditions

Listeria strains (Table 1) were recovered from frozen glycerol stock (-20°C) by subculturing twice in brain heart infusion (**BHI**; Difco, Becton Dickinson and Co., Sparks, MD) broth. Cultures were incubated for 24 h at 37°C with 200-rpm agitation. Enumeration of cultures was carried out on *Listeria* Oxford Agar (Difco) supplemented with 20 $\mu\text{g}/\text{mL}$ ceftazidime (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) and incubated for 24 to 48 h at 37°C .

Minimum Inhibitory Concentrations

Minimum inhibitory concentrations for antimicrobials were tested via the broth microdilution method as described by Patel et al. (2011), with minor modification. Overnight cultures of *Listeria* were inoculated at approximately 10^5 cfu/mL into 96-well microtiter plates containing wells with serial 2-fold dilutions of nisin, in the form of Nisaplin (Danisco, New Century, KS), or ferulic acid (Sigma-Aldrich, St. Louis, MO) in BHI broth, starting from 5 or 10 mg/mL, respectively. Plates were prepared in triplicate and the MIC for each was recorded as the lowest concentration that visibly prevented growth after overnight incubation at 37°C .

Traditional-Scale Queso Fresco Production

Pasteurized whole milk was warmed in stainless steel, steam-jacketed cheese vats to 35°C in batches of 20 L, and 4 g of CaCl_2 was added to promote firm curd formation. Three milliliters of commercial bovine rennet was added to the milk after 1:10 dilution in deionized water. The curd formed over 45 min and was then cut into small cubes. The curds were stirred slowly and cooked for 30 min, while the temperature was increased by 1°C every 6 min until reaching 40°C . Two-thirds of the total whey was drained and 160 g of NaCl was added to the curds and stirred for 10 min at 40°C . Residual whey was drained and the curds were transferred to cheese molds and then stored at 4°C .

MLQF Production

Pasteurized whole milk was warmed to 35°C in batches of 50 mL. The milk was combined with 67 μL

of 0.75 g/mL CaCl_2 and 15 μL of commercial rennet diluted to 1 mL in deionized water. The milk was divided into twenty-five 2-mL portions in 2-mL microcentrifuge tubes, which were then incubated at 35°C for 45 min in a water bath. The curds were then cut via several vertical strokes, at regularly alternating angles, with a sterile inoculating needle featuring a tip bent to conform to the bottom of the microcentrifuge tube. After cutting, curds were returned to the water bath and warmed for another 30 min, while the temperature was increased 1°C every 6 min until reaching 40°C. Tubes were centrifuged at $6,000 \times g$ for 15 s, and then 100 μL of whey was removed from each tube and replaced with 100 μL of 0.16 g/mL NaCl. The curds were stirred gently with a micropipette tip and returned to the water bath for an additional 20-min incubation at 40°C. The tubes were then centrifuged at $6,000 \times g$ for 10 min and whey was completely removed. Cheeses were then stored at 4°C until further analysis.

Dose-Response of Nisin in MLQF

Two independent batches of queso fresco were prepared as described above with 3.15, 6.25, 25, 31.25, 50, and 100 mg/mL Nisaplin added into the milk before renneting. This corresponded to approximately 12.5 to 400 μg of nisin/g of cheese, based on the nisin content of Nisaplin. After salting the curd, 200 μL of whey was removed from each tube and 200 μL of overnight culture of *L. innocua* was added, for a final concentration of approximately 10^5 cfu/g. The tubes were left at room temperature for 15 min to allow bacterial attachment before pressing the curd via centrifugation. Cheeses were sampled after storage at 4°C for 0, 3, 6, and 28 d. At each time point, 2 cheeses from each batch were sampled by mixing into 1:10 dilutions (wt/vol) of PBS using a pipet tip and then vortexing to further disrupt curd structure. Suspensions were then serially diluted for enumeration and spread-plated in triplicate on *Listeria* Oxford Agar supplemented with 20 μg /mL ceftazidime. Plates were incubated at 37°C for 24–48 h.

Effects of Nisin and Ferulic Acid on High-Load Listerial Challenge in MLQF

Batches of queso fresco were manufactured as described above, but with variation in starting inocula and antimicrobial included. Duplicate, independent batches of cheeses were prepared with starting inocula of approximately 10^7 cfu/g *L. innocua*. Antimicrobials were added at the following concentrations: 12.5 μg /g nisin, 2.5 mg/g ferulic acid, or 12.5 μg /g nisin combined with 1.0 mg/g ferulic acid. Cheeses were stored at 4°C and sampled after 0, 1, 3, 7, 14, 21, and 28 d.

Effects of Nisin and Ferulic Acid on Low-Load Listerial Challenge in MLQF

Batches were manufactured as above, but with a lower starting inoculum of *L. innocua* at approximately 10^3 cfu/g and treated with ferulic acid at concentrations of 2.5 or 4 mg/g with or without 12.5 μg /g nisin. This was then followed up by another 2 independent batches treated with 4.0 mg/g ferulic acid and prepared with an inoculum of approximately 10^3 cfu/g of a *L. monocytogenes* cocktail. The cocktail was prepared by standardizing the optical density of all 5 *L. monocytogenes* strains (Table 1) and then combining them in equal proportions by volume and serially diluting to the appropriate concentration in sterile water.

In all batches, ferulic acid was incorporated before centrifugation of the curd, rather than directly into the milk, as follows: after inoculation and a 15-min incubation to allow *Listeria* to attach to the curd, tubes were centrifuged at $6,000 \times g$ for 5 min for incomplete pressing. Accessible whey was removed, the antimicrobial treatments were added, and the curd was centrifuged for another 7 min. The remaining whey was removed and all cheese samples were stored at 4°C for up to 28 d until sampled.

Compositional Analysis

The MLQF samples from 3 separate manufacturing batches, in addition to a traditional-scale manufactured batch, were analyzed in triplicate. Dry matter was determined by heating samples at 105°C to constant weight (method 926.08; AOAC International, 2002). Fat content was determined by hydrolysis first with ammonia and then with hydrochloric acid before triple-extraction in a Mojonnier-style flask with ethyl ether and ethyl ether/alcohol (method 933.05; AOAC International, 2002). Total nitrogen was measured via the Dumas combustion method (method 992.15; AOAC International, 2002), and a protein factor of 6.38 was used to convert total nitrogen to protein (method 2001.14; AOAC International, 2002).

Texture Profile Analysis

Texture profile analysis (TPA) was carried out in traditional-scale cheeses manufactured with the addition of either no antimicrobial or 4.0 mg/g ferulic acid. From each cheese, 2 portions (20 mm diameter, 20 mm in height) were extracted with a circular mold. Using a 50-mm cylindrical probe, each sample was compressed twice in a TA-plus texture analyzer with NEXYGEN-Plus 3.0 software (Lloyd Instruments, Hants, UK). A cross-head operating speed of 1 mm/s, 25% compres-

sion, and 0.3 N contact force were selected. Hardness (N), cohesiveness (adimensional), springiness (mm), gumminess (N), and chewiness (mJ) were calculated via the instrument's software. For each treatment, TPA was conducted twice on 3 separate batches of cheese.

Sensory Evaluation

Sensory evaluation of the queso fresco samples from the same batch prepared for TPA was performed 2 d after manufacturing. An affective test with untrained panelists ($n = 60$; 28 males and 32 females, 18–46 yr old) was conducted to evaluate product acceptability of queso fresco samples treated with and without 4.0 mg/g ferulic acid. Experimental cheese samples were cut into approximately 1.5-cm cubes and allowed to reach room temperature for 30 min before sensory evaluation. The cheese cubes were identified using randomly generated 3-digit codes and randomly presented to the untrained panelists. Each panelist was asked to evaluate the 2 cheese samples for appearance, color, aroma, and texture using a 9-point hedonic scale of the following values: 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely. During sensory evaluation, panelists were asked not to evaluate flavor of the samples, because food-grade ferulic acid could not be obtained.

Statistical Analysis

Experiments were analyzed as completely randomized designs; ANOVA was performed using JMP 7.0.1 (SAS Institute Inc., Cary, NC). Cheese composition, TPA, and sensory results were evaluated using Student's *t*-test to determine the statistical significance of mean differences between treatments.

RESULTS

Compositional Analysis

Production of MLQF is analogous to traditional manufacturing methods in numerous ways, primarily the raw ingredients and time-temperature treatments (Figure 1). It varies considerably only in scale, with some handling steps necessarily adjusted, including cutting of the curd with an inoculating needle instead of a curd knife and centrifugation instead of pressing or molding to remove whey. The resulting cheeses closely resemble traditional queso fresco visually, but are smaller, weighing approximately 0.3 g each. To ensure miniature-scale cheeses were compositionally equivalent to traditional queso fresco, cheeses were subjected to analyses to determine DM, fat, and protein contents. The MLQF did

not differ statistically from the traditional-scale cheese in any of these parameters (Table 2). All samples fell within the range of compositional values expected for DM, fat, and protein, as measured in commercial fresh cheeses (Caro et al., 2014).

Minimum Inhibitory Concentrations

The MIC of nisin for *L. innocua* in BHI was determined to be 12.5 $\mu\text{g/mL}$. The MIC of ferulic acid for *L. innocua* and each of the *L. monocytogenes* strains in BHI was determined to be 2.5 mg/mL.

Dose-Response of Nisin in MLQF

To assess the inhibition of *Listeria* in fresh cheese, nisin was added to a level comparable to its MIC in BHI, and a range of greater levels, in the MLQF model. The use of *Listeria*-selective agar confirmed that no *Listeria* were present in the milk or uninoculated controls and allowed for proper enumeration of surviving listerial cells in the presence of nonlisterial dairy microorganisms. Over 28 d of storage at 4°C, *L. innocua* in the untreated control cheeses grew nearly 2 log cfu/g (Figure 2). Treatment with nisin reduced survival of *L. innocua* during the first week in a dose-dependent fashion. The cheeses treated with 12.5 and 25 $\mu\text{g/g}$ nisin showed *L. innocua* levels reduced by approximately 1 log cfu/g, whereas those with 100 to 400 $\mu\text{g/g}$ nisin had counts reduced by approximately 2 log cfu/g. Counts in cheeses treated with 200 and 400 $\mu\text{g/g}$ nisin remained low through the first week of storage, whereas counts of the other treated cheeses had recovered up to 1 log cfu/g. By the end of the 28-d storage, all treated cheeses had recovered to levels comparable to that of the control.

Effects of Nisin and Ferulic Acid on High-Load Listerial Challenge in MLQF

The MIC of ferulic acid for the listerial strains tested in BHI was used on a cheese-weight basis for inclusion in MLQF. Figure 3 shows the antimicrobial effect against *L. innocua* of nisin, ferulic acid, and their combination when added to MLQF. Over 28 d of storage at 4°C, the *L. innocua* present in untreated control cheeses grew approximately 1.5 log cfu/g. Ferulic acid showed an inhibitory effect, preventing *L. innocua* from growing more than approximately one-quarter log cfu/g over the 28-d storage. Nisin demonstrated an approximately one-half log cfu/g reduction over the first 3 d, but regrowth occurred shortly thereafter and the load of *L. innocua* remained similar to that of the control at the end of the 28-d storage period. The combination of nisin and ferulic acid showed the greatest initial reduc-

Figure 1. Flow diagram of miniaturized laboratory queso fresco (MLQF) production. Asterisks indicate potential points of microbial contamination or antimicrobial treatment: in the milk before or during renneting (A), during the curd cutting (B), during salting, during holding of the curd (C), during “pressing” via centrifugation (D), or on the cheese surface during storage (E).

tion, approaching a decrease of 1 log cfu/g after 7 d of storage, but regrowth also occurred and by the end of storage, the levels of *L. innocua* had risen just above that of the cheeses with ferulic acid alone.

Effects of Nisin and Ferulic Acid on Low-Load Listerial Challenge in MLQF

In the next set of cheeses, featuring a lower starting inoculum, nisin alone provided minor inhibition of *L. innocua* growth only in the first week and grew

from approximately 3 to >8 log cfu/g, much like the untreated control. Both additions of nisin in combination with ferulic acid showed reductions of almost 1 log cfu/g of *L. innocua* after only 1 d of storage, but after 3 d, the levels of *L. innocua* were similar to those treated with ferulic acid alone (Figure 4). However, after 28 d of storage, cheeses treated with 2.5 or 4.0 mg/g ferulic acid remained approximately 2.5 and 3.5 log cfu/g lower than that of the control, respectively.

After establishing and validating model parameters with *L. innocua* as a surrogate organism for extra safety during initial antimicrobial testing, we then confirmed the observed antimicrobial activity of ferulic acid against *L. monocytogenes*. Follow-up with a cocktail of different *L. monocytogenes* strains in the fresh cheese model (Figure 5) showed a slower growth rate in the control cheese and a greater level of inhibition by treatment with 4.0 mg/g ferulic acid; less than one half-log cfu/g growth occurred in the presence of ferulic acid after 21 d, remaining 3 log cfu/g lower than the untreated control.

Table 2. Dry matter, protein, and fat contents of miniaturized laboratory queso fresco (MLQF) and a traditional-scale queso fresco¹

Item	MLQF	Traditional scale
DM (%)	44.43 ± 1.16 ^a	46.14 ± 0.56 ^a
Protein (%)	18.25 ± 0.31 ^a	18.34 ± 0.23 ^a
Fat (%)	22.35 ± 1.14 ^a	23.54 ± 0.17 ^a

^aMeans with dissimilar letters within each row are significantly different ($P < 0.05$).

¹Values are means ± SEM.

Figure 2. Effect of nisin on the survival of *Listeria innocua* in a fresh cheese model stored at 4°C. Nisin was added to cheeses by weight, with final concentrations of 0 (○), 12.5 (●), 25 (□), 100 (■), 125 (△), 200 (▲), and 400 (◇) µg/g. Values are means ± SEM.

Figure 3. Effect of nisin and ferulic acid on survival of *Listeria innocua* in fresh cheese after storage at 4°C. Nisin and ferulic acid were added to cheeses by weight, with final concentrations of 0 (○) or 12.5 (●) µg/g nisin, 2.5 mg/g ferulic acid (□), and 12.5 µg/g nisin + 1 mg/g ferulic acid (■). Values are means ± SEM.

Texture Profile Analysis

Texture profile analysis of traditional-scale queso fresco was performed to assess the effect of treating cheeses with 4.0 mg/g ferulic acid on the structural quality of cheese samples. Relative to the control, springiness and chewiness were significantly lower in cheese treated with ferulic acid (Table 3). However, no differences in cohesiveness, gumminess, or hardness were observed; each cheese demonstrated the characteristically crumbly texture of queso fresco.

Sensory Evaluation

Ferulic acid is not currently an approved food additive in its purified form, so food-grade ferulic acid was unavailable to allow consumers to assess the flavor of treated cheeses. No significant differences in consumer acceptability of appearance, color, aroma, or texture were detected between the control and samples of queso fresco treated with ferulic acid (Table 4).

DISCUSSION

Our MLQF model generates cheeses from batches of milk that can be scaled to suit any experimental replication. This is accommodated by incorporating antimicrobial treatments and rennet before distribution into microcentrifuge tubes for incubation. The produc-

Figure 4. Effect of nisin and different concentrations of ferulic acid on survival of *Listeria innocua* in fresh cheese after storage at 4°C. Nisin and ferulic acid were added to cheeses by weight with final concentrations of 0 (○) or 12.5 (●) µg/g nisin; 2.5 (□) or 4.0 (■) mg/g ferulic acid; 12.5 µg/g nisin + 2.5 mg/g ferulic acid (△); and 12.5 µg/g nisin + 4.0 mg/g ferulic acid (▲). Values are means ± SEM.

Table 3. Texture profile analysis properties of queso fresco treated with ferulic acid¹

Item	Control	Ferulic acid
Hardness (N)	4.19 ± 0.16 ^a	4.62 ± 0.12 ^a
Cohesiveness	0.77 ± 0.02 ^a	0.84 ± 0.02 ^a
Springiness (mm)	3.36 ± 0.24 ^a	2.15 ± 0.32 ^b
Gumminess (N)	8.67 ± 0.35 ^a	8.78 ± 0.24 ^a
Chewiness (mJ)	11.75 ± 0.89 ^a	7.71 ± 1.19 ^b

^{a,b}Means with dissimilar letters within each row are significantly different ($P < 0.05$).

¹Values are means ± SEM.

tion process for each cheese is carried out entirely in a single vessel, including centrifugation of the curd to replace conventional cheese pressing methods that are less feasible at the miniature scale. Similar small-scale production has previously been shown to have little effect on the quality and microstructure of cheese relative to pilot- and commercial-scale manufacturing (Bachmann et al., 2009). We have demonstrated that our MLQF model is compositionally analogous to traditionally produced fresh cheese, with equivalent fat, protein, and moisture contents.

Nisin has been used as a preservative in the food industry for decades. A broad-spectrum bacteriocin produced by *Lactococcus lactis*, nisin inhibits many gram-positive organisms, including the food-borne pathogen *Listeria monocytogenes* (Gálvez et al., 2007). This has made nisin particularly useful for inhibiting growth of *L. monocytogenes*, among others, in processed foods ranging from cheeses to sausages and smoked meats. We found that the MIC of nisin for *L. innocua* ATCC 33090 in laboratory media is comparable to that of numerous *L. monocytogenes* strains (Davies and Adams, 1994; Techathuvanan et al., 2014), consistent with its common use as a model organism for investigating antilisterials. However, elaboration of antimicrobial activity in laboratory media may not always be an appropriate indicator of activity when incorporated into a food matrix. For example, the activity of nisin has been shown to be affected by the milk-fat composition of dairy (Jung et al., 1992), possibly due to adsorption to protein or fat globules or heterogeneous distribution of the compound

Figure 5. Effect of ferulic acid on survival of a *Listeria monocytogenes* cocktail in a fresh cheese after storage at 4°C. Ferulic acid was added to cheeses by weight, with final concentrations of 0 (○) or 4.0 (●) mg/g ferulic acid. Values are means ± SEM.

and bacteria. Nutrient composition may also result in significant discrepancies between antimicrobial activity observed in laboratory media and in cheese (Collins et al., 2011). Additionally, antilisterial activity of nisin has been shown to be more effective at low pH compared with neutral or alkaline conditions (Benkerroum and Sandine, 1988; Davies et al., 1997). Therefore, nisin may exhibit reduced activity in near-neutral pH fresh cheeses. As such, we wished to compare the inhibitory activity of nisin in laboratory media with its incorporation within a queso fresco matrix.

We observed a dose-dependent inhibition of *L. innocua* in queso fresco (Figure 2) at levels of nisin comparable to the concentrations tested in BHI, relative to the volumes of milk used and final yield of the cheeses. Above 100 µg/g, there appeared to be diminishing returns. Even at a treatment of 400 µg/g, which exceeds the US regulatory limit of 250 ppm (US Food and

Table 4. Consumer acceptability of queso fresco with added ferulic acid

Antimicrobial	Acceptability ¹			
	Appearance	Color	Aroma	Texture
None	7.08 ± 0.17 ^a	7.32 ± 0.13 ^a	6.40 ± 0.20 ^a	6.62 ± 0.21 ^a
Ferulic acid	7.17 ± 0.16 ^a	7.13 ± 0.14 ^a	6.13 ± 0.21 ^a	6.75 ± 0.19 ^a

^aMeans with dissimilar letters within each column are significantly different ($P < 0.05$).

¹Consumer acceptability was based on a 9-point scale (1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely). Values are means ± SEM.

Drug Administration, 1988), inhibition of *L. innocua* did not exceed 2 log cfu/g under the tested conditions. Outgrowth of surviving organisms resulted in no treatments displaying considerable inhibition relative to the control after 3 wk of storage. Nisin has been known to alter the cell membrane lipid composition of listerial populations, decreasing membrane fluidity and preventing further pore formation, thereby limiting further activity by bacteriocins (Ming and Daeschel, 1995; Mazzotta and Montville, 1997). However, regrowth may not have been due to the emergence of resistant *L. innocua* strains, because several isolates of *L. innocua* that were recovered after 28 d in MLQF demonstrated MIC identical to those of the starting inocula (data not shown). Nisin may simply not be stable enough in fresh cheese, particularly due to its near-neutral pH, to remain active for extended periods or its activity may be inherently limited by the cheese matrix. Although nisin was somewhat effective at preventing the growth of *Listeria*, queso fresco would likely benefit from additional hurdles to promote further inhibition and avoid the potential development of resistance to nisin.

Ferulic acid is a phenolic phytochemical that has been shown to exert antilisterial preservation in some food matrices (Takahashi et al., 2013). Ferulic acid is abundant in cell walls of cereals such as wheat, corn, and rice, making it a relatively accessible antimicrobial additive for the food industry (Ou and Kwok, 2004). Phenolic acids, such as ferulic acid, generally inhibit bacterial growth by increasing the permeability of cell membranes, often driving leakage of cytoplasmic components (Campos et al., 2009; Borges et al., 2013). Furthermore, Takahashi et al. (2015) demonstrated that *L. monocytogenes* is less likely to develop resistance to the effects of ferulic acid than to nisin. Ferulic acid thereby seemed an appropriate adjunct to pair with nisin in a combined antilisterial approach for queso fresco.

Initial tests began with a high level of listerial contamination to determine the maximum antimicrobial efficacy of the treatments against a bacterial load far greater than that likely encountered during cheese production. Nisin and ferulic acid, in combination, initially resulted in greater reduction in the survival of *L. innocua* than did either alone. However, the inhibition observed in cheeses treated with 2.5 mg/g ferulic acid led to a lower listerial load than the combination treatment after 28 d of storage (Figure 3), whereas nisin-treated cheeses recovered to levels similar to those of the control. The lower dose of ferulic acid used in combination with nisin could account for the lesser inhibitory effect, which called for testing additional concentrations of ferulic acid, alone and in combination with nisin.

We tested these at lower starting inocula to reflect contamination levels more likely to be encountered dur-

ing industrial cheese production, based on what has been measured in contaminated retail products (Beckers et al., 1987; Pini and Gilbert, 1988), and to better assess the inhibitory effects of ferulic acid on cell growth. Interestingly, the combinations of nisin and ferulic acid were no more effective than ferulic acid alone after the first 3 d of storage (Figure 4). Nisin alone demonstrated minimal inhibition of listerial growth under these conditions. Similarly, ferulic acid was less inhibitory than in the previous trial, although it remained significantly more inhibitory than nisin. Perhaps reduced bacterial saturation provided improved conditions for *L. innocua* to resist injury by these compounds, either due to the antimicrobial doses chosen or differing effects on membrane permeability given the conditions of the cheese matrix. Regardless, the inhibition by ferulic acid alone suggests that its combination with nisin demonstrated additive inhibition that could warrant future investigation into combinations with other antimicrobials.

Due to its promising inhibitory activity, we then confirmed that ferulic acid was inhibitory toward a cocktail of *L. monocytogenes* strains (Figure 5). We worked primarily with *L. innocua* for safety and expediency during development of the cheese model, but *L. innocua* is not always considered an appropriate surrogate for *L. monocytogenes* without critical evaluation of their behaviors, particularly when exposed to complex environments and stressors (Milillo et al., 2012). *Listeria innocua* demonstrated a similar, but higher growth rate and tolerance to ferulic acid relative to the *L. monocytogenes* cocktail, which confirms that *L. innocua* is an appropriate surrogate for screening ferulic acid against *L. monocytogenes* in fresh cheese. To our knowledge, this also constitutes the first comparison between the growth of *L. innocua* and *L. monocytogenes* under antimicrobial stress within a cheese matrix. Following our challenge studies, we explored the feasibility of such ferulic acid supplementation as a preventative measure in commercial queso fresco with preliminary assessments of its effect on acceptability.

Plant extracts and essential oils have been known to negatively affect sensory attributes of some cheeses (Tornambé et al., 2008). We tested the inhibitory activity of a pure form of ferulic acid to minimize the potential of affecting the flavor of the cheeses. Ferulic acid is approved as a food additive for its antioxidant properties in Japan, but in most countries it only holds regulatory approval in the form of natural extracts and essences (Ou and Kwok, 2004). We could therefore not assess the effect of ferulic acid on cheese flavor but proceeded with various texture and sensory characterizations. We tested the inclusion of the highest concentration of ferulic acid as constructed in the laboratory to maximize the likelihood of noticeably altering the

product. Consumers demonstrated no difference in preference between the cheeses concerning appearance, color, or aroma. These results are encouraging for the future use of ferulic acid as a commercial preservative and suggest that more-rigorous sensory analysis may be appropriate once food-grade ferulic acid is available.

In summary, we have demonstrated a facsimile of traditional queso fresco at such a scale that it can be manufactured in complete biocontainment, that uses minimal amounts of antimicrobial, and that allows for artificial contamination or treatment of the production process at any stage. Such flexibility warrants further exploration of which production factors may affect antimicrobial efficacy. Future work could focus on further varying microbial contamination and antimicrobial treatment levels, as well as the timing of their introduction. Similarly, processing variables such as the duration and temperature of each step could be adjusted to assess their roles in the proliferation of spoilage organisms and pathogens. Once food-grade ferulic acid is available, the MLQF model may prove useful for identifying synergistic combinations with other common or novel antimicrobials.

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