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Short communication

Reduction of *Listeria monocytogenes* in queso fresco cheese by a combination of listericidal and listeriostatic GRAS antimicrobials

Kamlesh A. Soni, Monil Desai, Ademola Oladunjoye, Frederick Skrobot, Ramakrishna Nannapaneni *

Department of Food Science, Nutrition and Health Promotion, POB 9805, Mississippi State University, Mississippi State, MS 39762, United States

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ABSTRACT

Single and combined effects of three GRAS (generally recognized as safe) antimicrobials including, bacteriophage P100 (phage P100), lauric arginate (LAE), and potassium lactate–sodium diacetate mixture (PL–SD) were evaluated against *Listeria monocytogenes* cold growth in queso fresco cheese (QFC). The fate of phage P100 when exposed to LAE (200 ppm) or PL–SD (2.8% PL and 0.2% SD) was determined at 4 °C and 30 °C in a broth model. Phage P100 was found to be stable in the presence of these antimicrobial agents as plaque forming units (PFU) did not vary between control, LAE or PL–SD treatments. When 9 log CFU/ml of stationary phase cells of *L. monocytogenes* was exposed to these antimicrobials in tryptic soy broth, there was a 3 to 5 log CFU/ml reduction with phage P100 and a complete 9 log CFU/ml reduction with LAE but no measurable reduction with PL–SD after 24 h at 4 °C or 30 °C. In QFC, the *L. monocytogenes* populations increased from the initial 3.5 log CFU/cm^2 to 7.7 log CFU/cm^2 in 28 days at 4 °C. Treatment with 7.8 log PFU/cm^2 of phage P100 or 200 ppm of LAE showed strong listericidal effect initially by reducing *L. monocytogenes* counts by 2 to 3.5–4 log CFU/cm^2 while there was a subsequent regrowth of *L. monocytogenes* at 4 °C. Treatment with PL–SD showed strong listeriostatic effect without decreasing *L. monocytogenes* counts but growth was prevented for 28 days at 4 °C. Only the combined treatment of listericidal phage P100 or LAE with listeriostatic PL–SD reduced the initial *L. monocytogenes* counts by 2–4 log CFU/cm^2 and also kept the *L. monocytogenes* counts at that reduced level in QFC for 28 days at 4 °C.

1. Introduction

Queso fresco cheese (QFC), one of the popular fresh soft Mexican-style cheeses, has been classified as FDA's cluster 1 of ready-to-eat food products with a highest risk of *Listeria monocytogenes* contamination (Lin et al., 2006). Favorable growth conditions in QFC such as high moisture (45–55%), low salt content and near neutral pH of 6.0–6.5 coupled with the ability of *L. monocytogenes* cells to multiply under refrigeration storage are primary factors contributing to increased *L. monocytogenes* associated food safety risk in this product (Lin et al., 2006; Sandra et al., 2004). *L. monocytogenes* cells are shown to multiply by average of 4 log CFU/g in QFC within 4 weeks of refrigeration storage (Mendoza-Yepes et al., 1999; Soni et al., 2010b).

There are no *Listeria* control measures during actual QFC manufacturing process when cheese curd is processed at 40–45 °C (Sandra et al., 2004). Given the persistence of *L. monocytogenes* in processing environment, the contamination of QFC during post processing packaging is very likely. Moreno-Enriquez et al. (2007) have reported up to 18.5% prevalence of *L. monocytogenes* in QFC samples obtained from Mexico. Recently in 2009 and 2010, consumers in the state of New York were warned not to eat a certain variety of QFC due to the possibility of *L. monocytogenes* contamination (USFDA, 2010; USFDA, 2009). In 2000, a listeriosis outbreak occurred in North Carolina due to the consumption of soft Mexican style cheeses including QFC and resulted in 5 stillbirths, 3 premature deliveries, and 3 infected newborns (MacDonald et al., 2005). In June 1985, an *L. monocytogenes* outbreak associated with QFC resulted in 142 cases of listeriosis that caused 48 deaths (Linnan et al., 1988).

Various measures such as lactic acid starter culture or plant derived essential oil have been previously evaluated for *L. monocytogenes* control in QFC. Use of *Lactococcus lactis* subsp. *diacetylactis* starter culture or essential oils of citrus and rosemary inhibited the *L. monocytogenes* growth for 20 days in QFC but did not decrease the original *L. monocytogenes* levels (Mendoza-Yepes et al., 1997; Mendoza-Yepes et al., 1999). One potential concern with the use of essential oils is also decreased consumer acceptability due to unacquainted flavor and aroma characteristics (Burt, 2004; Ouattara et al., 1997). Nisin has also been evaluated for *L. monocytogenes* control in other soft cheeses such as ricotta and cottage (Davies et al., 1997; Ferreira and Lund, 1996), however, different strains of *L. monocytogenes* vary in sensitivity to nisin and even sensitive strains are shown to develop gradual resistance (Maisnier-Patin and Richard, 1996).

* Corresponding author. Tel.: +1 662 325 7697; fax: +1 662 325 8728.
E-mail address: nannapaneni@fsnhp.msstate.edu (R. Nannapaneni).

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The use of single antimicrobial agents most often does not provide broad spectrum of listericidal and listeriostatic antimicrobial activity. For example, currently a mixture of potassium lactate (PL) or sodium lactate (SL) with sodium diacetate (SD) is the most sought after antimicrobial agent in ready-to-eat food products (Vogel et al., 2006; Yoon et al., 2004). Though highly effective in inhibiting the growth of *L. monocytogenes* cold growth, these preferable antimicrobial agents can only extend the lag phase of *L. monocytogenes* without yielding any listericidal activity (Knight et al., 2007; Nunez de Gonzalez et al., 2004; Vogel et al., 2006). By contrast, some of the other GRAS antimicrobial agents such as lauric arginate or nisin can reduce the *L. monocytogenes* numbers initially but do not prevent the subsequent slow growth in cold storage (Martin et al., 2009; Schillinger et al., 1998; Soni et al., 2010b). Such issues also holds true for the physical intervention processes such as high hydrostatic pressure (HHP), steam pasteurization or irradiation where surviving cells often resuscitate and regrow during storage period (Koseki et al., 2007; Marcos et al., 2008; Niemira et al., 2003). Hence, the goal of the present study is to identify the combinations of GRAS antimicrobial agents that can substantially decrease *L. monocytogenes* numbers in the initial stage and thereafter inhibit the growth of any surviving cells during the subsequent cold storage of QFC. Antimicrobial compounds tested in this study are lauric arginate (LAE), bacteriophage P100 and PL–SD mixture. In the first part of this study, the compatibility of phage P100 with other GRAS antimicrobials (LAE and PL–SD) was evaluated in broth model. In the second part, these antimicrobial agents were evaluated individually or in combinations for controlling *L. monocytogenes* cold growth in QFC.

2. Materials and methods

2.1. L. monocytogenes strains

The serotype and sources of *L. monocytogenes* strains used in this study are as follows: V7 (1/2a, FDA), Bug600 (1/2a, Dr. P. Cossart at Institut Pasteur), F4393 (4b, CDC-cheese isolate), F5069 (4b, CDC-milk isolate) and ATCC 43257 (4b, cheese isolate). These strains were maintained in Tryptic Soy Agar (TSA) slants and cultured in 10 ml of Tryptic Soy Broth (TSB) at 37 °C for 18–24 h to obtain cell concentrations of ~10^9 CFU/ml. Five-strain mixed inoculum of these strains was prepared by mixing equal volumes (2 ml) of overnight grown cell suspensions of each strain. The serial dilutions of the *L. monocytogenes* mixed cell suspensions were further prepared in saline (0.8% NaCl) to obtain the desired cell concentrations.

2.2. Antimicrobial agents

FDA and USDA-FSIS approved (USFDA, 2006; USFDA, 2007) bacteriophage preparation Listex™ P100 (phage P100) was provided by MICREOS Food Safety (formerly EBI Food Safety; Wageningen, Netherlands). Phage P100 stock solution in buffered saline had an approx. concentration of 10^{11} plaque forming units (PFU)/ml. Phage P100 preparation is currently approved by FDA for all raw and ready-to-eat foods at levels not to exceed 10^9 PFU (plaque forming units)/g. Also, we have previously demonstrated that phage P100 is active against diverse strains of *L. monocytogenes* including the five strains used in this study (Soni and Nannapaneni, 2010b). Lauric arginate (LAE) was obtained from Vedeqsa (Vedeqsa inc., New York, NY 10001) which contained 10% active LAE dissolved in propylene glycol (solvent) and polysorbate 20 (emulsifier). LAE is currently approved by FDA for food preservation purpose for concentrations up to 200 ppm on per gram weight basis (USFDA, 2005). The working solution of 10,000 ppm LAE were prepared by diluting (1:10) the 10% active LAE solution in sterile DI water. PURASAL Opti.Form solution which contained mixture of potassium lactate (56%)–sodium diacetate (4%) (referred as PL–SD hereafter) was obtained from the Purac (Purac Inc., Gorinchem, Netherlands).

2.3. Compatibility of phage P100 with LAE and PL–SD

The stability of phage P100 in the presence of LAE (200 ppm) or PL–SD (2.8% PL–0.2% SD) was measured to determine the possibility of combining phage P100 with LAE or PL–SD as a mixed antimicrobial. The phage solution was initially diluted (1:100) in TSB broth to attain 9 log PFU/ml and 900 μl of aliquots were placed in micro-centrifuge tubes. The phage samples were subsequently treated with LAE, PL–SD or control treatment as follows: To achieve a 200 ppm LAE concentration with phage P100, 20 μl of 10,000 ppm of LAE was mixed with 80 μl sterile DI water and then added to 900 μl phage solution. To achieve 2.8% PL–0.2% SD concentration with phage P100, 50 μl of PL–SD stock solution was mixed with 50 μl sterile DI water and then added to the 900 μl phage solution. Control treatments received 100 μl DI water instead of antimicrobial treatment. A total of 6 tubes were prepared for each LAE, PL–SD and control treatment and for each treatment 3 tubes were placed at 4 °C and remaining 3 tubes were placed at 30 °C. The PFU counts of these phage samples were determined at 1, 4 and 24 h using soft agar overlay assay using a previously published protocol (Soni and Nannapaneni, 2010b). Additionally, a triplicate batch of phage samples was also maintained as a lethality control after heat treatment at 90 °C for 5 min and was enumerated for phage viability immediately following the heat treatment. The entire assay was repeated three times with 3 replicates.

2.4. Effect of phage P100, LAE and PL–SD against stationary phase cells of L. monocytogenes in broth

The purpose of this assay was to determine the fate of *L. monocytogenes* cells in the presence of LAE, PL–SD and phage P100. A five-strain mixture of *L. monocytogenes* cells at 9 log CFU/ml was treated with 200 ppm LAE or 2.8% PL–0.2% SD or with phage P100 at 10^8 PFU/ml. These samples were placed at 4 °C and 30 °C and *L. monocytogenes* populations were enumerated at 1, 4 and 24 h. Prior to enumeration, the samples were centrifuged (12,000×*g* for 5 min) and the pellet was resuspended in fresh TSB. This centrifugation step was mainly performed to remove the phage particles from phage P100 challenged *L. monocytogenes* samples and for experimental consistency this step was also performed with LAE and PL–SD treated samples. Enumeration was performed by spread plating 100 μl of original or serially diluted (in 0.8% saline) aliquots on TSA plates and plates were incubated at 37 °C for 24 h. The entire assay was repeated three times independently with three replicates used in each independent trial.

2.5. Effects of phage P100, LAE and PL–SD either alone or in combinations against L. monocytogenes in QFC

Retail samples of QFC (brand: Cacique Ranchero, Cacique Inc., City of Industry, CA, USA; product detail: 340 g round block, 95 mm diameter and 35 mm thickness/height) that contained no chemical preservatives were obtained from a local grocery store and stored at 4 °C (maximum 2 days) prior to experimentation. Each round block of QFC of approx. 340 g was cut diagonally 6 times from the center such that the angle at the center for each cut block was 30° and it resulted into cutting of 12 equal pieces with average weight of 25 g. The exposed surface area of each cut piece was calculated as 54 cm^2 using the following equation: $2\times\pi r^2/12$ (representing area of the top surface and area of the bottom surface) $+2\pi r\times h/12$ (area of front side) $+2\times r\times h$ (area of the two side face).

Subsequently, each piece was placed inside a vacuum packaging bag. On the top of QFC sample, 100 μl of 10^6 CFU/ml of *L. monocytogenes* mixed cell suspension was added to achieve ~4 log CFU/g inoculum on cheese surface and cells were allowed to attach for 15 min. After *L. monocytogenes* inoculation, QFC samples were treated with single or mixed antimicrobial concentrations as described in Table 1. The delivery

Table 1
Preparation of single and combined antimicrobial concentrations used in this study.

Final GRAS antimicrobial concentration per gram of QFC	Antimicrobial solution per 25 g QFC sample			
	Phage P100 at10^{10} PFU/ml	10% LAE (ml)	56% PL–4% SD (ml)	DI water (ml)
Untreated control	0	0	0	1.50
10^8 PFU of phage P100	0.25	0	0	1.25
200 ppm LAE	0	0.050	0	1.45
2.8% PL–0.2% SD	0	0	1.25	0.25
10^8 PFU of phage P100 + 200 ppm LAE	0.25	0.050	0	1.20
10^8 PFU of phage P100 + 2.8% PL–0.2% SD	0.25	0	1.25	0
200 ppm LAE + 2.8% PL–0.2% SD	0	0.050	1.25	1.20

of antimicrobial solution for each treatment was 1.5 ml per 25 g cheese block and it yielded antimicrobial concentrations of 10^8 PFU, 200 ppm LAE or 2.8%PL–0.2%SD per g of cheese either alone or in mixed combinations (Table 1). Antimicrobial solution was added on the surface of QFC sample inside the bag and these were immediately vacuum packed (Berkel-250, Louisville, KY, USA) for storage at 4 °C. Additional samples that contained antimicrobials without *L. monocytogenes* inoculation were also prepared for the pH measurement. After adding antimicrobial solution on the QFC, the samples were homogenized using stomacher and the pH measurement was taken using Accumet pH meter (Model-AB 15, Fisher Scientific, Hampton, NH).

L. monocytogenes enumeration from QFC samples was performed at 0, 1, 7, 14, 21 and 28 days of storage. During vacuum processing, the antimicrobial solution spreads along the product surface as a result of air removal from vacuum packing bags and this method has been successfully employed for antimicrobial delivery at the point of vacuum packaging (Luchansky et al., 2005; Soni et al., 2010b). At each time point, three replicate samples from each treatment were removed from the 4 °C and bags were cut open using sterile scissors. In each bag, 225 ml of peptone water (0.1% peptone containing 0.02% Tween-80) was added and samples were homogenized for 2 min in a stomacher (Seward, Model 400 C) at 230 rpm. From each bag, 1 ml of homogenate was transferred into micro-centrifuge tube for centrifugation at 12,000×*g* for 5 min. After centrifugation, the top supernatant containing the phage P100 was removed and the pellet containing *L. monocytogenes* cells was resuspended in 1 ml of peptone water. This centrifugation step was needed to remove the phage particles that may be present in the supernatant of phage treatments (phage P100, phage P100 + LAE, phage P100 + PL–SD). This centrifugation step was employed for all samples to maintain consistency for all treatments. Sub-sample of 100 µl or 250 µl aliquot of the resuspended pellet was then spread plated on ceftazidime (6 mg/l) containing PALCAM agar (Difco, Detroit, MI). When required, a serial dilution step was performed after resuspending the pellet to yield a countable plate for *L. monocytogenes*. Plates were incubated at 37 °C for 48 h and black *L. monocytogenes* colonies were counted.

Additionally, to ensure that QFC retail samples used in this study did not contain *L. monocytogenes*, 3 randomly selected samples of 25 g each were processed for *L. monocytogenes* enumeration as described above and 1 ml homogenate was spread plated on PALCAM media supplemented with ceftazidime. No typical *L. monocytogenes* colonies were detected (minimum detection limit 1 log CFU/g) and also no QFC background microflora grew in PALCAM plates.

2.6. Stability of phage P100 in QFC

The stability of phage P100 in QFC when applied alone or in combination with LAE or PL–SD was determined during the 28 day storage period at 4 °C. In this assay, QFC samples were not challenged with *L. monocytogenes* inoculum but were only treated with phage P100, phage P100 + LAE and phage P100 + PL–SD antimicrobial treatments. The samples were vacuum packed for storage at 4 °C and phage P100 was enumerated over 28 day storage period as follows: After each time period, the samples were homogenized in 225 ml of peptone water by stomaching. One milliliter of homogenate from each sample was centrifuged and supernatant was filter sterilized with 0.22 µm syringe filter. The centrifugation and filtration step was necessary to remove the background microflora in cheese which would otherwise hamper the plaque formation and visibility on non-selective TSA plates. The filtrate was serially diluted in SM buffer and phage P100 plaques were enumerated using soft agar overlay plaque forming assay (Soni and Nannapaneni, 2010b).

2.7. Statistical analysis

All experiments were repeated three times with three replicates. *L. monocytogenes* and phage P100 counts were converted into the log CFU/cm^2 and log PFU/cm^2, respectively. ANOVA test using SPSS statistical analyses software package (SPSS version 12.0, Chicago, Ill) was performed for finding out the mean significant differences between control and within phage treatments.

3. Results

3.1. Fate of phage P100 in the presence of other GRAS antimicrobials

The plaque formation of phage P100 after exposure with 200 ppm LAE ppm and 2.8%PL–0.2%SD in broth for 24 h at 4 °C and 30 °C is shown in Table 2. Compared to control samples (phage alone), no decrease in PFU count was observed when phage P100 titer of 9 log PFU/ml in TSB broth was challenged with either LAE or PL–SD ($P > 0.05$). Moreover, no temperature dependent effect was observed as the PFU count of phage P100 in control and LAE or PL–SD treated samples were statistically similar at both at 4 °C and 30 °C during 24 h incubation ($P > 0.05$). In contrast to the treatment of phage P100 with LAE or PL–SD, the lethality control with heat treatment of 90 °C/5 min resulted in complete inactivation of 9 log PFU/ml phage titer.

3.2. Comparative efficacy of phage P100, LAE and PL–SD against stationary phase cells of L. monocytogenes at 4 °C and 30 °C in broth

Table 3 shows the single effects of phage P100, LAE and PL–SD against a high dose of *L. monocytogenes* cells at 4 °C and 30 °C for 24 h in TSB broth. Treatment with LAE showed strong listericidal action towards *L. monocytogenes* cells which were decreased to undetectable levels within 24 h (Table 3). Moreover with LAE, there was a time and temperature dependent effect for *L. monocytogenes* reduction. Compared to control samples, *L. monocytogenes* counts were lower by 2.7 log CFU/ml at 4 °C vs. 3.8 CFU/ml at 30 °C after 1 h and by 5.4 log CFU/ml at 4 °C vs. 7.0 log CFU/ml at 30 °C after 4 h. There was also a time and temperature dependent reductions of *L. monocytogenes* cells with phage P100 treated samples with higher reductions observed at 30 °C compared to 4 °C. Overall, phage P100 decreased *L. monocytogenes* counts by 3.1 log CFU/ml at 4 °C and by 5.6 log CFU/ml at 30 °C after 24 h compared to control samples. Treatment of *L. monocytogenes* cells with 2.8%PL–0.2%SD did not show any listericidal effect over 24 h at both 4 °C and 30 °C and *L. monocytogenes* counts remained similar to that of untreated control sample.

3.3. Growth of L. monocytogenes in QFC after surface treatment with phage P100, LAE or PL–SD either alone or in combinations at 4 °C

The efficacy of listericidal or listeriostatic GRAS antimicrobials in reducing the *L. monocytogenes* counts after their surface application either alone or in combinations on QFC is shown in Figs. 1–3. Addition of these antimicrobials did not cause marked alteration in QFC pH. The average pH measurement of QFC samples with different

Table 2
Stability of phage P100 in the presence of LAE or PL–SD in TSB broth at 4 °C and 30 °C.

Treatments*	At 4 °C			At 30 °C		
	1 h	4 h	24 h	1 h	4 h	24 h
Phage**	8.9 ± 0.10	8.8 ± 0.13	9.0 ± 0.11	8.9 ± 0.17	8.4 ± 0.4	8.8 ± 0.22
Phage + LAE**	9.0 ± 0.18	8.8 ± 0.28	8.7 ± 0.25	8.9 ± 0.11	8.8 ± 0.2	8.8 ± 0.17
Phage + PL–SD**	8.9 ± 0.12	9.0 ± 0.21	8.9 ± 0.21	8.9 ± 0.2	8.8 ± 0.31	8.9 ± 0.21

*Concentrations used: P100 titer of 9 log PFU/ml, LAE at 200 ppm; and 2.8% PL–0.2%SD. Phage counts were enumerated at 1, 4 and 24 h of incubation at 4 °C and 30 °C.
**No differences ($P > 0.05$) existed in PFU counts amongst different treatment based on one-way AVOVA test.
In another treatment, the 9 log PFU/ml of phage P100 was reduced to a non-detectable level by heat treatment at 90 °C for 5 min (lethality control).

antimicrobial treatment were as follows: control — 6.56; LAE — 6.48; PL–SD — 6.46; phage P100 — 6.52; LAE + PL–SD — 6.47; LAE + phage P100 — 6.47; PL–SD + phage P100 — 6.46.

In untreated controls, *L. monocytogenes* population increased to 7.7 log CFU/cm^2 from the initial concentration of 3.5 log CFU/cm^2 during 28 day storage at 4 °C, suggesting that QFC is an excellent substrate for *L. monocytogenes* growth under refrigeration temperatures. Treatment of QFC samples with phage P100 reduced the *L. monocytogenes* counts to an undetectable level (minimum detection limit of 5 CFU/cm^2) after 1 day storage at 4 °C, however regrowth in *L. monocytogenes* surviving cells occurred during subsequent cold storage where *L. monocytogenes* cells grew back to ~4 log CFU/cm^2 at the end of 28 days (Fig. 1). Treatment of QFC samples with 200 ppm LAE reduced the *L. monocytogenes* populations by 2 log CFU/cm^2 after 1 day at 4 °C. No further reductions in *L. monocytogenes* counts were observed during subsequent storage of LAE treated samples and instead *L. monocytogenes* counts reached to ~7.0 log CFU/cm^2 at the end of 28 day storage at 4 °C that represent only a marginal reduction of 0.6 log CFU/g compared to that of control (Fig. 1). Treatment with PL–SD did not result in any listericidal activity as *L. monocytogenes* counts did not decline from the original inoculum level of 3.5 log CFU/cm^2 (Fig. 3). By contrast with phage P100 or LAE, PL–SD exerted strong listeriostatic action to completely prevent the growth of *L. monocytogenes* cells during 28 days at 4 °C. Overall, these findings show that the single antimicrobial agents such as phage P100 or LAE have a strong listericidal activity initially while PL–SD has a strong listeriostatic activity towards surviving *L. monocytogenes* cells in QFC for a long time.

Combined treatment with both listericidal agents phage P100 and LAE reduced the *L. monocytogenes* populations by 3 log CFU/cm^2 (Fig. 2). However, similar to their single effects, the combined mixture of phage P100 and LAE also failed to inhibit the regrowth of *L. monocytogenes* in storage and *L. monocytogenes* counts reached to 4.7 log CFU/cm^2 at the end of 28 days at 4 °C. On the other hand, the combined mixture of listericidal phage P100 and listeriostatic PL–SD yielded the initial reduction of *L. monocytogenes* counts by ~3 log CFU/cm^2 and following 28 days storage at 4 °C the *L. monocytogenes* counts were still lower by 3 log CFU/cm^2 compared to the starting inoculum of ~3.5 log CFU/cm^2. Similarly, the combined treatment of listericidal LAE with listeriostatic PL–SD also reduced the initial *L. monocytogenes* counts by ~2 log CFU/cm^2 which were then subsequently kept at that low level throughout the refrigeration storage.

Table 3
Reduction of *Listeria monocytogenes* cells at 4 °C and 30 °C in tryptic soy broth when treated with phage P100, LAE and PL–SD.

Treatments	*L. monocytogenes* counts at 4 °C			*L. monocytogenes* counts at 30 °C		
	1 h	4 h	24 h	1 h	4 h	24 h
Untreated control	9.1 ± 0.2^a	9.0 ± 0.2^a	8.9 ± 0.2^a	9.0 ± 0.1^a	9.0 ± 0.2^a	8.9 ± 0.1^a
Phage P100	7.9 ± 0.1^b	6.8 ± 0.3^c	5.5 ± 0.1^d	6.7 ± 0.2^c	5.0 ± 0.2^e	3.4 ± 0.1^f
LAE	6.4 ± 0.2^c	3.6 ± 0.3^f	ND	5.2 ± 0.1^d	2.0 ± 0.2^g	ND
PL–SD	9.0 ± 0.2^a	8.9 ± 0.2^a	8.8 ± 0.1^a	8.9 ± 0.2^a	9.0 ± 0.3^a	8.6 ± 0.3^a

A five-strain mixture of *L. monocytogenes* at 9 log CFU/ml was used at 9 log CFU/ml. Antimicrobial concentrations were 200 ppm LAE and 2.8%PL–0.2%SD.
L. monocytogenes counts were enumerated in broth at 1, 4 and 24 h of incubation at 4 °C and 30 °C. Letter with different superscript indicates significant differences ($P < 0.05$) based on time and temperature factors.

3.4. Stability of phage P100 in QFC

The stability of phage P100 when present alone or in combination with LAE or PL–SD on the surface of QFC is shown in Fig. 4. The phage P100 was relatively stable on the surface of QFC where the initial treatment level of 7.7 log PFU/cm^2 remained at that similar level throughout 28 days at 4 °C. Also, there were no measurable differences in recovered PFU counts between phage P100 alone, phage P100 + LAE or phage P100 + PL–SD treatments.

4. Discussion

In the initial experiments, the effect of GRAS chemical agents LAE and PL–SD was evaluated against biological agent bacteriophage P where P100 viability was not affected by these GRAS chemical agents (Table 2), suggesting that LAE or PL–SD can be combined with phage P100 as an antimicrobial mixture. The antimicrobial activity of LAE is based on the disruption/instability of the plasma membrane lipid bilayer and thereby it may interfere with the metabolic process (Rodriguez et al., 2004). One possible reason for inability of LAE to kill phage particle could be that phages are obligate parasites that lack such active metabolic processes. Also, the head and tail of phage particle mainly compose of a protein molecule embedded in a capcid which may not have any target plasma membrane-type binding sites for LAE that are abundant on the bacterial cell surface. Also, we observed higher *L. monocytogenes* cell reductions at 30 °C

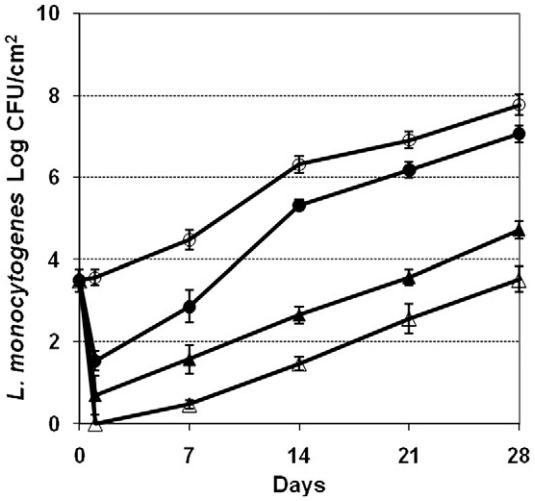

Fig. 1. Reduction of *Listeria monocytogenes* in QFC after surface treatment with bacteriophage P100 (△) or lauric arginate (●) or their combination (▲) compared to untreated control (○). The concentration of phage P100 at 10^8 PFU/g and LAE at 200 ppm/g was used for surface application of QFC.

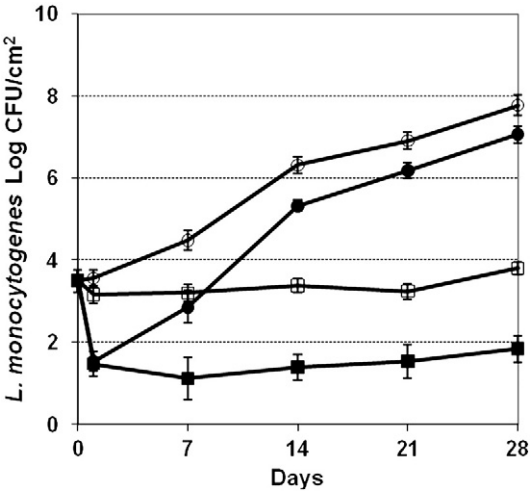

Fig. 2. Reduction of *Listeria monocytogenes* in QFC after surface treatment with lauric arginate (●) or PL–SD (□) or their combination (■) compared to untreated control (○). The concentrations of lauric arginate at 200 ppm and PL–SD (2.8% PL and 0.2% SD) were used for surface application of QFC.

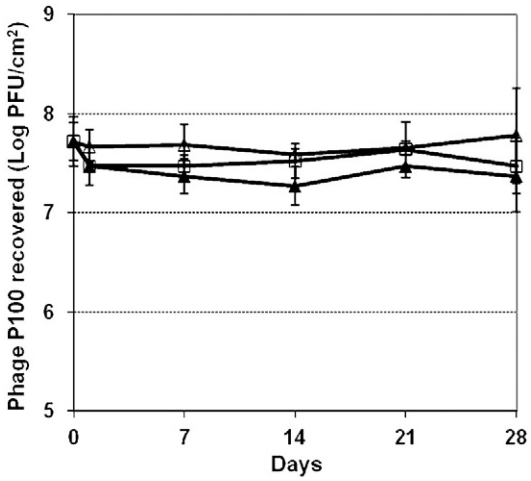

Fig. 4. Stability of phage P100 when alone (△) or in combinations with LAE (▲) or PL–SD (■) in QFC.

compared to 4 °C with LAE which could be correlated to increased cell permeability at higher temperature (Venkitanarayanan et al., 1999). As suggested by Ross et al. (2008), this observation also implies the possibility of using sublethal temperatures to enhance the efficacy of antimicrobial compounds. Other antimicrobial PL–SD did not affect *L. monocytogenes* counts during 24 h test period. The permissible level of PL based on the dry weight of food product is 4.8% whereas SD is approved at 0.1%–0.4% depending on product category (Yoon et al., 2003). On ready-to-eat food products when PL applied at 2–3% in combination with SD applied at 0.2–0.3% appear to extend bacterial lag phase without and demonstrable bactericidal effect (Knight et al., 2007; Nunez de Gonzalez et al., 2004; Vogel et al., 2006). Finally, when phage P100 was tested against *L. monocytogenes* cells, it decreased *L. monocytogenes* counts by 3–5 logs after 24 h (Table 3). This lack of complete *L. monocytogenes* inactivation could be attributed to inaccessibility of phage P100 to *L. monocytogenes* that are trapped within cell debris and dead cell (Soni and Nannapaneni, 2010b). Selection of these five strains used in this work was based on our previous study in which phage P100 was screened against 21 different *L. monocytogenes* strains that represented its 13 known serotype (Soni and Nannapaneni, 2010b). Though all strains were equally sensitive to phage P100, they had different biofilm formation ability with 1/2a and 4b isolates that are of clinical and food safety relevance producing stronger biofilms. Hence, we used strains of these two serotypes which are more food safety relevant and have better biofilm producing capacity.

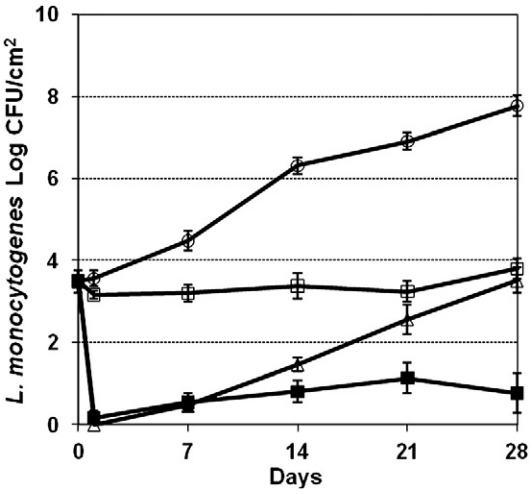

Fig. 3. Reduction of *Listeria monocytogenes* in QFC after surface treatment with bacteriophage P100 (△) or PL–SD (□) or their combination (■) compared to untreated control (○). The concentration of phage P100 at 10^8 PFU/g and PL–SD (2.8% PL and 0.2% SD) was used for surface application of QFC.

In QFC, LAE treatment of 200 ppm provided the initial reductions in *L. monocytogenes* counts by 2 log CFU/cm^2 but regrowth of *L. monocytogenes* cells occurred during subsequent storage. Previous studies elucidating the antimicrobial activity of LAE against *L. monocytogenes* on food products such as ham and frankfurters have reported that the reductions in *L. monocytogenes* populations attained by LAE treatment are only short-term and an additional control measure is needed to prevent the long term growth proliferation during cold storage (Luchansky et al., 2005; Martin et al., 2009; Taormina and Dorsa, 2009). Also, our previous work exploring the LAE efficacy in milk and QFC cheese have revealed that the bactericidal effect of LAE is both concentration and substrate dependent which was highly effective in milk but short lived in QFC (Soni et al., 2010b). In general, we observed that proportionately higher reduction in *L. monocytogenes* counts could be achieved by elevated LAE concentrations with its efficient distribution in liquid medium such as milk over semisolid QFC.

With respect to phage P100, previous studies show its potential antimicrobial efficacy against *L. monocytogenes* cells present on the surface of raw catfish or salmon fillets (Soni et al., 2010a), cheese substrate (Carlton et al., 2005), ready-to eat-meat products (Guenther et al., 2009) and biofilm matrices (Soni and Nannapaneni, 2010b). In the present assay, phage P100 at 10^8 PFU density was highly efficient in reducing *L. monocytogenes* populations by 3.5 log CFU/cm^2 initially. This dose of 10^8 PFU/g for the surface application of QFC was based on our previous experiments with raw salmon fillets where phage density in the range of 10^{7-8} PFU/g is optimum for an efficient kill of host bacterium (Bigwood et al., 2009; Soni and Nannapaneni., 2010a). Also, our previous experimental model using salmon fillet had established that irrespective of inoculum level (i.e. low at 10^2 CFU/g or high at 10^4 CFU/g) the level of phage particle needed to sufficiently cover the product surface area for measurable reduction in *L. monocytogenes* is constant at 10^{7-8} PFU/g (Soni and Nannapaneni, 2010a). Phage P100 particles were also highly stable on QFC surface but were unable to prevent *L. monocytogenes* growth proliferation in subsequent refrigerated storage. Such observation has also been reported in previous literature with phage biocontrol where the host bacteria often regrow during long term storage conditions (Leverentz et al., 2004; Soni et al., 2010a). One possible reason for no long term efficacy with phage applications may be its inaccessibility

to bind to *L. monocytogenes* cells (Guenther et al., 2009; Hagens and Offerhaus, 2008). The resistance development by *L. monocytogenes* cells to phage P100 may also contribute to such growth proliferation phenomenon occurring in long term storage. Carlton et al. (2005) did not observe the resistance development in surviving *L. monocytogenes* cells from cheese samples treated with phage P100. Additionally, O'Flynn et al. (2004) demonstrated that the occurrence of bacteriophage insensitive mutant (BIM) for *Escherichia coli* O157:H7 occur at very low level (1 BIM in 10^6 CFU) and suggested that such low chances for BIM development are not likely to hinder the use of these phages as biocontrol agents. Due to such growth proliferation of *L. monocytogenes* cells during long-term storage after phage treatment, additional control mechanisms are critical.

Treatment with PL–SD did not exert any listericidal activity but kept the *L. monocytogenes* level at the original level. These observations are in consistent with previously published literature which show that these compounds mainly aid in bacterial growth inhibition by prolonging the lag phage (Knight et al., 2007; Nunez de Gonzalez et al., 2004; Vogel et al., 2006). In all practical circumstances, the initial contamination levels of *L. monocytogenes* are expected to be low in various food products (Gram, 2001), hence these compounds can serve the purpose of limiting *L. monocytogenes* associated risk by inhibiting growth. However, considering the current zero-tolerance limit for *L. monocytogenes* contaminations in ready-to-eat food products along with previously identified sporadic chances of initial *L. monocytogenes* levels as high as 4–6 log CFU/g (Gram, 2001), it is imperative to use antimicrobial agent that can provide strong listericidal activity. In our experiments, we were able to combine both listericidal spectrum (by using phage P100 or LAE) and listeriostatic spectrum (by using PL–SD) for *L. monocytogenes* control in QFC for the first time. Both mixtures constituting of either phage P100 with PL–SD or LAE with PL–SD were found to be very efficient in initially reducing *L. monocytogenes* counts by 2–3 log CFU/g and it was then keeping at such reduced levels during subsequent cold storage.

In this study, we used 3.5 log as starting inoculum which is relatively higher than 1–10 CFU/g typically detected in *L. monocytogenes* contaminated ready-to-eat food products. It is usually challenging to quantify the low microbial levels due to inherent limitation associated with the standard microbial enumeration methodology. Considering the 1 ml plating volume from a 250 ml of QFC homogenate in *L. monocytogenes* enumeration assay, the minimum detection limit of our assay was 1 log CFU/g. Previously, we were able to employ centrifugation based concentration of *L. monocytogenes* cells inoculated at 2 log CFU/g on salmon fillet. In that approach, 1 ml of salmon homogenate was first centrifuged and resulting residual pellet was dissolved in 100 µl peptone water for spread plating. The advantage with salmon substrate was that since it is an intact muscle it did not rupture during stomaching and hence the stomached homogenate contained very low salmon residues (Soni and Nannapaneni, 2010a). However with cheese substrate the stomached homogenate became very viscous and hence further concentration with centrifugation step was not practical. Nevertheless, it is important to develop alternative approaches that allow the monitoring of pathogenic organisms challenged at relatively low level. If the sole aim is not to quantify the log scale reductions, the qualitative assessment of the presence or absence of targeted microorganisms as recommended by regulatory agencies for regular screening of food products should be more practical.

In conclusion, our work presented in this study show that combined treatment of listericidal GRAS antimicrobials such as LAE or phage P100 with listeriostatic PL–SD can be an effective tool for control of *L. monocytogenes* cold growth in QFC. In this study, we did not investigate the effects of these antimicrobial treatments on native microflora of cheese because these antimicrobial compounds were applied as a surface treatment and they are not likely to affect the native microflora present inside the cheese mass. Such experiments can be performed by adding these antimicrobial agents as an internal treatment during production of QFC. Moreover, a detailed sensory analysis should be performed to determine the consumer acceptability of QFC after treatment with combined GRAS antimicrobials. The focus of this study was to control the *L. monocytogenes* proliferation using GRAS antimicrobials. However, the other strategies such as pH, water activity, and NaCl concentrations can also be used for predictive growth modeling and such information can be successfully used to optimize the manufacturing process that is sensory acceptable and also limit the growth and survival of *L. monocytogenes* (Mejlholm et al., 2010).

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References

Bigwood, T., Hudson, J.A., Billington, C., 2009. Influence of host and bacteriophage concentrations on the inactivation of food-borne pathogenic bacteria by two phages. FEMS Microbiology Letters 291, 59–64.

Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods — a review. International Journal of Food Microbiology 94, 223–253.

Carlton, R.M., Noordman, W.H., Biswas, B., de Meester, E.D., Loessner, M.J., 2005. Bacteriophage P100 for control of *Listeria monocytogenes* in foods: genome sequence, bioinformatic analyses, oral toxicity study, and application. Regulatory Toxicology and Pharmacology 43, 301–312.

Davies, E.A., Bevis, H.E., Delves-Broughton, J., 1997. The use of the bacteriocin, nisin, as a preservative in ricotta-type cheeses to control the food-borne pathogen *Listeria monocytogenes*. Letters in Applied Microbiology 24, 343–346.

Ferreira, M.A., Lund, B.M., 1996. The effect of nisin on *Listeria monocytogenes* in culture medium and long-life cottage cheese. Letters in Applied Microbiology 22, 433–438.

Gram, L., 2001. Potential hazards in cold-smoked fish: *Listeria monocytogenes*. Journal of Food Science 66, 1072–1081.

Guenther, S., Huwyler, D., Richard, S., Loessner, M.J., 2009. Virulent bacteriophage for efficient biocontrol of *Listeria monocytogenes* in ready-to-eat foods. Applied and Environmental Microbiology 75, 93–100.

Hagens, S., Offerhaus, M.L., 2008. Bacteriophages: new weapons for food safety. Food Technology 4, 46–54.

Knight, T.D., Castillo, A., Maxim, J., Keeton, J.T., Miller, R.K., 2007. Effectiveness of potassium lactate and sodium diacetate in combination with irradiation to control *Listeria monocytogenes* on frankfurters. Journal of Food Science 72, M026–M030.

Koseki, S., Mizuno, Y., Yamamoto, K., 2007. Predictive modelling of the recovery of *Listeria monocytogenes* on sliced cooked ham after high pressure processing. International Journal of Food Microbiology 119, 300–307.

Leverentz, B., Conway, W.S., Janisiewicz, W., Camp, M.J., 2004. Optimizing concentration and timing of a phage spray application to reduce *Listeria monocytogenes* on honeydew melon tissue. Journal of Food Protection 67, 1682–1686.

Lin, C.M., Zhang, L., Doyle, M.P., Swaminathan, B., 2006. Comparison of media and sampling locations for isolation of *Listeria monocytogenes* in queso fresco cheese. Journal of Food Protection 69, 2151–2156.

Linnan, M.J., Mascola, L., Lou, X.D., Goulet, V., May, S., Salminen, C., Hird, D.W., Yonekura, M.L., Hayes, P., Weaver, R., et al., 1988. Epidemic listeriosis associated with Mexican-style cheese. The New England Journal of Medicine 319, 823–828.

Luchansky, J.B., Call, J.E., Hristova, B., Rumery, L., Yoder, L., Oser, A., 2005. Viability of *Listeria monocytogenes* on commercially-prepared hams surface treated with acidic calcium sulfate and lauric arginate and stored at 4 degrees C. Meat Science 71, 92–99.

MacDonald, P.D., Whitwam, R.E., Boggs, J.D., MacCormack, J.N., Anderson, K.L., Reardon, J.W., Saah, J.R., Graves, L.M., Hunter, S.B., Sobel, J., 2005. Outbreak of listeriosis among Mexican immigrants as a result of consumption of illicitly produced Mexican-style cheese. Clinical Infectious Diseases 40, 677–682.

Maisnier-Patin, S., Richard, J., 1996. Cell wall changes in nisin-resistant variants of *Listeria innocua* grown in the presence of high nisin concentrations. FEMS Microbiology Letters 140, 29–35.

Marcos, B., Aymerich, T., Monfort, J.M., Garriga, M., 2008. High-pressure processing and antimicrobial biodegradable packaging to control *Listeria monocytogenes* during storage of cooked ham. Food Microbiology 25, 177–182.

Martin, E.M., Griffis, C.L., Vaughn, K.L., O'Bryan, C.A., Friedly, E.C., Marcy, J.A., Ricke, S.C., Crandall, P.G., Lary Jr., R.Y., 2009. Control of *Listeria monocytogenes* by lauric arginate on frankfurters formulated with or without lactate/diacetate. Journal of Food Science 74, M237–M241.

Mejlholm, O., Gunvig, A., Borggaard, C., Blom-Hanssen, J., Mellefont, L., Ross, T., Leroi, F., Else, T., Visser, D., Dalgaard, P., 2010. Predicting growth rates and growth boundary of *Listeria monocytogenes* — an international validation study with focus on processed and ready-to-eat meat and seafood. International Journal of Food Microbiology 141, 137–150.

Mendoza-Yepes, M.J., Sanchez-Hidalgo, L.E., Maertens, G., Marin-Iniesta, F., 1997. Inhibition of *Listeria monocytogenes* and other bacteria by a plant essential oil (DMC) in Spanish soft cheese. Journal of Food Safety 17, 47–55.

Mendoza-Yepes, M.J., Abellan-Lopez', O., Carrionortega', J., Marin-Iniesta, F., 1999. Inhibition of *Listeria monocytogenes* and other bacteria in Spanish soft cheese made with *Lactococcus lactis* subsp. *diacetylactis*. Journal of Food Safety 19, 161–170.

Moreno-Enriquez, R.I., Garcia-Galaz, A., Acedo-Felix, E., Gonzalez-Rios, I.H., Call, J.E., Luchansky, J.B., Diaz-Cinco, M.E., 2007. Prevalence, types, and geographical distribution of *Listeria monocytogenes* from a survey of retail queso fresco and associated cheese processing plants and dairy farms in Sonora, Mexico. Journal of Food Protection 70, 2596–2601.

Niemira, B.A., Fan, X., Sokorai, K.J., Sommers, C.H., 2003. Ionizing radiation sensitivity of *Listeria monocytogenes* ATCC 49594 and *Listeria innocua* ATCC 51742 inoculated on endive (*Cichorium endivia*). Journal of Food Protection 66, 993–998.

Nunez de Gonzalez, M.T., Keeton, J.T., Acuff, G.R., Ringer, L.J., Lucia, L.M., 2004. Effectiveness of acidic calcium sulfate with propionic and lactic acid and lactates as postprocessing dipping solutions to control *Listeria monocytogenes* on frankfurters with or without potassium lactate and stored vacuum packaged at 4.5 degrees C. Journal of Food Protection 67, 915–921.

O'Flynn, G., Ross, R.P., Fitzgerald, G.F., Coffey, A., 2004. Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7. Applied and Environmental Microbiology 70, 3417–3424.

Ouattara, B., Simard, R.E., Holley, R.A., Piette, G.J., Begin, A., 1997. Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. International Journal of Food Microbiology 37, 155–162.

Rodriguez, E., Seguer, J., Rocabayera, X., Manresa, A., 2004. Cellular effects of monohydrochloride of L-arginine, N-lauroyl ethylester (LAE) on exposure to *Salmonella* Typhimurium and *Staphylococcus aureus*. Applied and Environmental Microbiology 96, 903–912.

Ross, T., Zhang, D., McQuestin, O.J., 2008. Temperature governs the inactivation rate of vegetative bacteria under growth-preventing conditions. International Journal of Food Microbiology 128, 129–135.

Sandra, S., Stanford, M.A., Goddik, L.M., 2004. The use of high-pressure processing in the production of queso fresco cheese. Journal of Food Science 69, E153–E158.

Schillinger, U., Chung, H.S., Keppler, K., Holzapfel, W.H., 1998. Use of bacteriocinogenic lactic acid bacteria to inhibit spontaneous nisin-resistant mutants of *Listeria monocytogenes* Scott A. Applied and Environmental Microbiology 85, 657–663.

Soni, K.A., Nannapaneni, R., 2010a. Bacteriophage significantly reduces *Listeria monocytogenes* on raw salmon fillet tissue. Journal of Food Protection 73, 32–38.

Soni, K.A., Nannapaneni, R., 2010b. Removal of *Listeria monocytogenes* biofilms with bacteriophage P100. Journal of Food Protection 73, 1519–1524.

Soni, K.A., Nannapaneni, R., Hagens, S., 2010a. Reduction of *Listeria monocytogenes* on the surface of fresh channel catfish fillets by bacteriophage Listex P100. Foodborne Pathogens and Disease 7, 427–434.

Soni, K.A., Nannapaneni, R., Schilling, M.W., Jackson, V., 2010b. Bactericidal activity of GRAS lauric arginate in milk and queso fresco cheese against *Listeria monocytogenes* cold growth. Journal of Dairy Science 93, 4518–4525.

Taormina, P.J., Dorsa, W.J., 2009. Short-term bactericidal efficacy of lauric arginate against *Listeria monocytogenes* present on the surface of frankfurters. Journal of Food Protection 72, 1216–1224.

USFDA, 2005. Agency response letter GRAS notice no. GRN 000164. [online] http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/GRASListings/ucm154571.htm (last accessed on March 16, 2011).

USFDA, 2006. Agency response letter GRAS notice no. GRN 000198. [online] http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/GRASListings/ucm154675.htm (last accessed on March 16, 2011).

USFDA, 2007. Agency response letter GRAS notice no. GRN 000218. [online] http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/GRASListings/ucm153865.htm (last accessed on March 16, 2011).

USFDA, 2009. Peregrina Cheese Corporation recalls all products manufactured in its plant because of possible health risk. [online] http://www.fda.gov/Safety/Recalls/ucm177198.htm2009(last accessed on March 16, 2011).

USFDA, 2010. Azteca Linda Corp. recalls queso fresco and queso hebra because of possible health risk. [online] http://www.fda.gov/Safety/Recalls/ucm223977.htm (last accessed on March 16, 2011).

Venkitanarayanan, K.S., Ezeike, G.O., Hung, Y.C., Doyle, M.P., 1999. Efficacy of electrolyzed oxidizing water for inactivating *Escherichia coli* O157:H7, *Salmonella enteritidis*, and *Listeria monocytogenes*. Applied and Environmental Microbiology 65, 4276–4279.

Vogel, B.F., Ng, Y.Y., Hyldig, G., Mohr, M., Gram, L., 2006. Potassium lactate combined with sodium diacetate can inhibit growth of *Listeria monocytogenes* in vacuum-packed cold-smoked salmon and has no adverse sensory effects. Journal of Food Protection 69, 2134–2142.

Yoon, K.S., Burnette, C.N., Whiting, R.C., 2003. Effects of pH and agitation on the growth of *Listeria monocytogenes* Scott A in brain heart infusion broth containing combined potassium lactate and sodium diacetate during storage at 4 or 10 degrees C. Journal of Food Protection 66, 1469–1473.

Yoon, K.S., Burnette, C.N., Abou-Zeid, K.A., Whiting, R.C., 2004. Control of growth and survival of *Listeria monocytogenes* on smoked salmon by combined potassium lactate and sodium diacetate and freezing stress during refrigeration and frozen storage. Journal of Food Protection 67, 2465–2471.