Effect of modified atmosphere packaging on the growth of spoilage microorganisms and *Listeria monocytogenes* on fresh cheese

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ABSTRACT

Queso Fresco has a limited shelf life and has been shown to support the rapid growth of *Listeria monocytogenes* during refrigerated storage. In addition to improving quality and extending shelf life, modified atmosphere packaging (MAP) has been used to control the growth of pathogenic microorganisms in foods. The objectives of this study were to determine the effects of MAP conditions on the survival and growth of spoilage microorganisms and *L. monocytogenes* during storage of Queso Fresco manufactured without starter cultures. For *L. monocytogenes* experiments, cheeses were surface inoculated at $\sim 4 \log_{10}$ cfu/g before packaging. Inoculated and uninoculated (shelf life experiments) cheeses were placed in 75- μ m high-barrier pouches, packaged under 1 of 7 conditions including air, vacuum, or combinations of N₂ and CO₂ [100% N₂ (MAP1), 30% CO₂:70% N₂ (MAP2), 50% CO₂:50% N₂ (MAP3), or 70% CO₂:30% N₂ (MAP4), 100% CO₂ (MAP5)], and stored at 7°C. Samples were removed weekly through 35 d of storage. *Listeria monocytogenes* counts were determined for inoculated samples. Uninoculated samples were assayed for mesophilic and psychrotolerant counts, lactic acid bacteria, coliforms, and yeast and mold. In general, cheeses packaged under conditions consisting of higher contents of CO₂ had lower pH levels during storage compared with those stored in conditions with lower levels or no CO₂ at all. Similarly, the antimicrobial efficacy of MAP in controlling spoilage microorganisms increased with increasing CO₂ content, whereas conditions consisting of 100% N₂, vacuum, or air were less effective. Mean *L. monocytogenes* counts remained near inoculation levels for all treatments at d 1 but increased $\sim 2 \log_{10}$ cfu/g on cheeses packaged in air, vacuum, and 100% N₂ (MAP1) conditions at d 7 and an additional $\sim 1.5 \log_{10}$ cfu/g at d 14 where they remained through 35 d. In contrast, treatments

consisting of 70% CO₂ (MAP4) and 100% CO₂ (MAP5) limited increases in mean *L. monocytogenes* counts to $< 1 \log_{10}$ cfu/g through 14 d and $\sim 1.5 \log_{10}$ cfu/g by d 21. Mean *L. monocytogenes* counts increased to levels significantly higher than inoculation (d 0) on cheeses stored in MAP2 and MAP3 on d 21, on d 28 for MAP4, and on d 35 for cheeses stored under MAP5 conditions. Overall, significant treatment $\times$ time interactions were observed between air, vacuum, and MAP1 when each was compared with MAP2, MAP3, MAP4, and MAP5. These data demonstrate that packaging fresh cheese under modified atmospheres containing CO₂ may be a promising approach to extend shelf life while limiting *L. monocytogenes* growth during cold storage.

Key words: *Listeria monocytogenes*, cheese, modified atmosphere packaging, Queso Fresco

INTRODUCTION

The Hispanic population in the United States increased by 43% to 50.5 million people between 2000 and 2010, which was 4 times the growth rate of the total US population at 10% (Ennis et al., 2011). With the growing Hispanic population and the incorporation of Hispanic cuisine into the American diet, per capita consumption of Hispanic-style cheese has more than doubled over the same period reaching 0.70 lb. (0.32 kg) in 2010 (Gould, 2017b). Similarly, Hispanic-style cheese production in the United States has grown from 96.3 million lb. in 2000 to more than 266 million lb. in 2016 (Gould, 2017a).

Queso Fresco (QF) is a soft, unripened, Hispanic-style cheese with a mild, slightly salty flavor and crumbly texture that is popular in ethnic markets and is often a staple in Hispanic households. Although a formal definition and federal standard of identity do not exist, QF has been generally characterized as having a high moisture content ($> 50\%$), low levels of sodium chloride, and low acidity (pH > 5.8 ; Van Hekken and Farkye, 2003). Such conditions provide a favorable growth environment for contaminants including bacterial pathogens (Genigeorgis et al., 1991a; Kasrazadeh and Genigeorgis

Received November 29, 2017.

Accepted May 11, 2018.

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1994, 1995). Without a national standard, considerable variation in composition exists. Depending on the microbial and physicochemical compositions of the cheese, the shelf life is typically less than 1 mo and can be as short as 7 d (Villar et al., 1999; Renye et al., 2008; Bermúdez-Aguirre and Barbosa-Cánovas, 2010).

Increasing consumer demand for preservative-free, “clean label” foods favors the use of modified atmosphere packaging (MAP) to extend the shelf life of a variety of foods. Modifying the composition of the gases that surround a food product during storage, including N₂ and CO₂, can reduce physiological changes, oxidation reactions, and microbial growth (Mastromatteo et al., 2014). However, extending the shelf life of refrigerated foods raises concerns about the growth of the foodborne pathogen *Listeria monocytogenes*, which can reach high levels rapidly in QF when stored at refrigeration temperatures due to the cheese variety’s relatively low acidity and high moisture content (Kozak et al., 2018). According to the Centers for Disease Control and Prevention’s National Outbreak Reporting System, 11 listeriosis outbreaks between 2009 and 2015 were linked to cheeses knowingly produced from pasteurized milk, 6 of which were associated with the consumption of Mexican- or Hispanic-style soft cheeses including QF (CDC, 2018). Outbreaks linked to cheese produced from pasteurized milk highlight the need for strategies for controlling *L. monocytogenes* contamination of postlethality exposed ready-to-eat foods.

The potential of MAP to extend the shelf life of different dairy products has been demonstrated (Khoshgozaran et al., 2012). However, MAP is an under investigated approach for extending shelf life and controlling the growth of *L. monocytogenes* on QF and similar cheeses. Given the relatively hospitable composition of QF for supporting microbial growth, this cheese also serves as a worst-case scenario model for other cheeses. Therefore, the objectives of this study were to determine the effects of MAP conditions on the survival and growth of (1) spoilage organisms and (2) *L. monocytogenes* as surface contaminants on QF during refrigerated storage at 7°C.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Inoculum Preparation

A cocktail of 8 *L. monocytogenes* strains was prepared as previously described (Kozak et al., 2018) using strains associated with outbreaks linked to soft cheeses or isolated from cheese processing environments, which included F5069/ATCC 51414 (milk-related outbreak),

CWD 675–3 (Hispanic-style cheese-related outbreak), CWD 1567 (Hispanic-style cheese-related outbreak), Scott A (milk-related outbreak), 2012L-5323 (Ricotta salata cheese-related outbreak), 2014L-6025 (Hispanic-style cheese-related outbreak), DJD 1 (washed-rind cheese-related outbreak), and CWD 193–10 U5–2 (dairy plant food contact surface). The cocktail was serially diluted in Butterfield’s phosphate buffer (BPB), pelleted through centrifugation (30 min, 4,000 × *g* at 4°C; Thermo Scientific Sorvall Legend X1R, Thermo Fisher Scientific, Waltham, MA), and resuspended in BPB to attain ~7 log₁₀ cfu/mL.

Cheese Manufacture and Analysis

For *L. monocytogenes* growth experiments, QF was manufactured in the University of Connecticut Creamery according to a standard protocol using 50 gallons (189.3 L) of cow milk standardized to 3.5% fat using pasteurized skim milk and cream (Garelick Farms, Franklin, MA) as previously described (Kozak et al., 2018). Briefly, after filling the vat, calcium chloride was added to the milk at a level of 0.02% (vol/vol) and thoroughly mixed. Milk temperature was raised to 32°C and the pH was adjusted to 6.45 with dilute lactic acid (50% vol/vol in sterile water, 85% lactic acid, Sigma-Aldrich, St. Louis, MO). Chymosin (CHY-MAX DCI Star double strength microbial rennet, Dairy Connection, Madison, WI) was added at a rate of 15.83 mL/100 L, and milk was stirred for approximately 45 s to mix. Once desired firmness was reached, the coagulum was cut into 1 × 1 cm curds using sanitized stainless-steel curd knives. Curds were allowed to rest for 5 min and then stirred for 5 min, which was repeated for 3 cycles for a total of 30 min. After an additional 5 min rest, the majority of the whey was removed by draining. Curds were then transferred to standard 20# Wilson hoops lined with disposable cheese cloth and pressed at 68.95 kPa for 20 min. Pressed curds were then removed from the hoops, broken into individual curds, and kosher salt (Diamond Crystal, Cargill, Wayzata, MN) was added to obtain a target salt (as sodium chloride) concentration of ~2% in the final cheese. Salted curds were returned to the hoops and pressed at 68.95 kPa for an additional 75 min. For shelf life experiments, QF was manufactured in the laboratory using a laboratory-scale cheese vat (Labtronix Inc., Philomath, OR) according to the same standard protocol using 6 gallons (22.7 L) of cow milk standardized to 3.5% fat using pasteurized skim milk and cream (Price Chopper, Schenectady, NY). The only other change was that curds from laboratory-scale production were transferred to plastic hoops (15 × 10.5 cm; M605MTF066, FromageX, Rimouski, Quebec,

Canada) and pressed at a reading of 60 lb. for both pressings. Cheese blocks from both scales of production were vacuum sealed (Ultravac 250, UltraSource LLC, Kansas City, MO) in pouches (3 mil, oxygen transmission rate: 50–70 mL/m²·24 h; UltraSource LLC) and stored at 4°C before cutting into experimental units.

Physiochemical analysis was conducted on representative samples from each independent batch of cheese, in duplicate, at the time of cutting, including pH (potentiometric method; Hooi et al., 2004; Accumet AB150 with microtip electrode, Fisher Scientific International Inc., Hampton, NH), DM (forced draft oven method; Hooi et al., 2004), fat (Babcock method; Hooi et al., 2004), and salt (Quanttab Chloride, Hach, Loveland, CO). The pH of homogenized samples was also measured in duplicate for each technical replicate at each microbiological sampling point in the shelf life experiments before buffer addition using a sanitized electrode. Salt in moisture (SM), moisture in the non-fat substance (MNFS), and fat in dry matter (FDM) were determined using the following formulae: % SM = [% salt/(100 – % DM)] × 100; % MNFS = [100 – % DM/(100 – % fat)] × 100; and % FDM = (% fat/% DM) × 100. Compositional targets based on previous work included ~52% moisture, ~22% fat, ~2.0% salt, SM ~4%, MNFS ~45%, and pH of ~6.4 (Genigeorgis et al., 1991b; Clark et al., 2001; Kozak et al., 2018).

Cheese Sample Preparation, Inoculation, and Packaging

Cheeses were cut into 25 ± 2 g samples using sterile knives for use in their respective experiments (*L. monocytogenes* vs. shelf life) within 48 h of manufacture. For *L. monocytogenes* growth experiments, cheeses were inoculated with 100 µL of the *L. monocytogenes* suspension in BPB, which was spread over a single surface of the cheese sample with a sterile spreader to attain a target contamination level of ~4 log₁₀ cfu/g of cheese. Inoculated cheese samples were allowed a 30-min drying period to enable bacterial attachment before packaging.

Inoculated (for *L. monocytogenes* experiments) and uninoculated (for shelf life experiments) cheese samples were packaged under 1 of 7 different conditions in high-barrier pouches (nylon/ethylene-vinyl alcohol/polyethylene; 75 µm; oxygen transmission rate 1.2 mL/m²·24 h; moisture vapor transmission 4 g/m²·24 h; DELI*1, Winpak, Winnipeg, Manitoba, Canada) based on previous studies in the literature and industry recommendations (Dansensor, 2017). Conditions included air, vacuum, 100% N₂ (MAP1), 30% CO₂:70% N₂ (MAP2), 50% CO₂:50% N₂ (MAP3), 70% CO₂:30% N₂ (MAP4), and 100% CO₂ (MAP5). Pouches were

evacuated, flushed, and sealed in a vacuum packager (Ultravac 250, UltraSource LLC) with gas injection. Gases (Airgas, North Franklin, CT) were mixed using a WITT gas mixer (KM 100–3, WITT Gas Controls LP, Alpharetta, GA). Packager program settings included 99% vacuum for vacuum and MAP treatments and 10% vacuum for air conditions. Packaging under MAP conditions (MAP1–5) required the addition of non-ambient gas with the gas setting of the packaging program set to 5%. Dry anaerobic indicator strips (BD BBL, Becton, Dickinson and Company, Sparks, MD) were included with each sample in each package to monitor the presence/absence of O₂ over time. Any blue coloration, regardless of strength, was considered positive. All samples were randomly assigned a sampling date (e.g., d 7) and stored at 7°C to mimic retail storage and mild temperature abuse along the food supply chain (Kennedy et al., 2005).

Sampling and Enumeration

In addition to initial counts on d 0, duplicate samples from each condition were removed from storage on d 1 (for *L. monocytogenes* only), 7, 14, 21, 28, and 35 post-packaging. The BPB was added to samples at a sample to medium ratio of 1:4 (~100 mL of BPB) and homogenized in a Smasher stomacher (Biomerieux, Marcy-l'Étoile, France) for 1 min at 560 strokes/min. Following serial dilutions in BPB, homogenates were plated onto media specific for each target microorganism or groups of microorganisms based on standard methods (Frank and Yousef, 2004; Laird et al., 2004). When applicable, 1 mL of the homogenate was plated over 4 agar plates (250 µL per plate) to achieve a limit of detection of ≥5 cfu/g. Briefly, mesophilic microorganisms were enumerated on trypticase soy agar with yeast extract (Difco, Detroit, MI) and incubated at 32°C ± 1°C for 48 h. Psychrotolerant microorganisms were enumerated on trypticase soy agar with yeast extract incubated at 7°C ± 1°C for 10 d. Total lactic acid bacteria (LAB) were enumerated on de Man, Rogosa, and Sharpe (Difco) agar incubated anaerobically at 32°C ± 1°C for 48 h. Samples for yeasts and mold were inoculated onto dichloran rose bengal chloramphenicol agar (DRBC; Difco), incubated at 25°C ± 1°C, and examined after 3, 4, and 5 d. Reported counts are those enumerated on d 5. Homogenates for coliforms were plated onto Petrifilm Coliform Count plates (3M Microbiology, St. Paul, MN) and incubated at 32°C ± 1°C for 24 h. None of the colonies recovered were confirmed by catalase reaction or Gram stain. *Listeria monocytogenes* counts were enumerated on modified Oxford agar (Difco) following incubation at 37°C ± 1°C for 48 h. Two inoculated

samples were processed after the bacterial attachment period to verify initial *L. monocytogenes* inoculation levels. Two uninoculated negative controls were also included to verify the absence of *L. monocytogenes* in uninoculated QF based on this enumeration procedure.

Statistical Analysis

Completely randomized designs with 7×6 (7 conditions at 6 time points) and 7×7 (7 conditions at 7 time points) factorial treatment structures were followed for shelf life and *L. monocytogenes* studies, respectively. Two cheese samples were included for each microbiological target/condition/time point/trial. *Listeria monocytogenes* growth experiments and shelf life experiments were repeated 4 and 3 times, respectively, using independently produced batches of cheese. Counts from technical replicates for each of the biological replicates were averaged, and data are presented as means $\pm$ standard deviation of the biological replicates. Data from only 2 replicate batches of cheese were included in the analysis of yeast and mold counts due to the presence of rapidly growing mold colonies on DRBC plates in a single replicate that impeded the growth and enumeration of other colonies. Pooled data were analyzed using the PROC GLM procedure of SAS (ver. 9.4, SAS Institute, Cary, NC). To compare changes in pH or microbial counts between treatments over time, the model included treatment and time as main effects and the interaction of treatment $\times$ time. pH values and *L. monocytogenes* counts between treatments at individual time points and *L. monocytogenes* counts between time points within a treatment were also compared using one-way ANOVA. All pairwise comparisons were performed using LSMEANS with the Tukey or Tukey–Kramer method. Significance was defined as $P < 0.05$. Inhibition was defined as $<1 \log_{10}$ cfu/g increase in mean *L. monocytogenes* counts at a given time point compared with the initial inoculum level (NACMCF, 2010).

RESULTS AND DISCUSSION

Physicochemical Parameters

The composition of cheese produced for each of the 2 experiments was similar with the exception of the lower salt content achieved in laboratory-scale cheeses manufactured for shelf-life studies (Table 1). This could be attributed to the differences in production equipment and volume, to differences in personnel making the cheeses in separate locations, or to both. Regardless, the physicochemical properties of experimental QF batches were within target ranges determined to be acceptable

to both traditional and nontraditional consumers while also considering the composition of cheeses associated with the presence of *Listeria* (Genigeorgis et al., 1991b; Clark et al., 2001). As shown in Table 2, the pH of uninoculated cheeses decreased after 7 d in MAP conditions containing CO₂ (MAP2–MAP5). The observed increase in acidity was most likely due to carbonic acid formation, as observed in previous studies (Farber, 1991; Gonzalez-Fandos et al., 2000; Olarte et al., 2002). Carbon dioxide is highly soluble in fat and water and forms carbonic acid, which, in its undissociated form, has antimicrobial activity attributed to alterations in intracellular pH. Treatment and time effects were observed for pH over 35 d of storage ($P < 0.001$). The pH levels of cheeses stored under air, vacuum, MAP1, MAP2, and MAP3 were similar throughout the study, whereas the pH levels of cheeses stored under MAP4 and MAP5 conditions were only different than air ($P = 0.016$ and $P < 0.001$, respectively). We also observed differences in pH through the duration of the study between cheeses stored under MAP5 conditions when compared with vacuum ($P = 0.012$), MAP1 ($P = 0.007$), and MAP2 ($P = 0.022$). When comparing pH values between treatments at individual time points, we only observed differences in mean pH between conditions at d 7 and 14 (Table 2). Overall, the changes in pH are similar to those reported for fresh goat cheese produced without starter culture and stored under varying MAP conditions (Olarte et al., 2002).

Monitoring additional changes in physicochemical parameters was outside the scope of the present study, but we noted that cheese packaged under air showed signs of moisture loss (whey off) by d 7 compared with by d 14 for all other treatments in both experiments (data not shown), but this was not quantified. Gonzalez-Fandos et al. (2000) found that measured moisture loss was similar between fresh cheese packaged under vacuum and air, which differed significantly from cheeses packaged under different atmospheres containing CO₂.

Table 1. Composition (mean $\pm$ SD) of Queso Fresco for experiments 1 (shelf life, $n = 3$) and 2 (growth of *Listeria monocytogenes*, $n = 4$)

Item ¹	Experiment 1	Experiment 2
DM (%)	46.7 $\pm$ 0.7	46.7 $\pm$ 0.8
Moisture (%)	53.3 $\pm$ 0.7	53.3 $\pm$ 0.8
Fat (%)	21.3 $\pm$ 1.0	20.7 $\pm$ 1.2
Salt (%)	1.6 $\pm$ 0.2	2.1 $\pm$ 0.1
MNFS (%)	67.0 $\pm$ 0.5	67.2 $\pm$ 1.6
SM (%)	3.3 $\pm$ 0.3	3.9 $\pm$ 0.2
FDM (%)	43.8 $\pm$ 2.3	44.2 $\pm$ 2.7
pH	6.49 $\pm$ 0.07	6.45 $\pm$ 0.02

¹MNFS = moisture in the nonfat substance; SM = salt in moisture; FDM = fat in DM.

Table 2. pH of uninoculated Queso Fresco during storage at 7°C for 35 d

Treatment ¹	pH (mean ± SD)					
	0 d	7 d	14 d	21 d	28 d	35 d
Air	6.49 ± 0.07	6.49 ± 0.02 ^{AB}	6.36 ± 0.07 ^A	6.37 ± 0.12	6.35 ± 0.07	6.41 ± 0.07
Vacuum	6.49 ± 0.07	6.50 ± 0.09 ^{AB}	6.34 ± 0.06 ^{AB}	6.25 ± 0.22	6.31 ± 0.17	6.27 ± 0.19
MAP1	6.49 ± 0.07	6.55 ± 0.15 ^A	6.40 ± 0.05 ^A	6.23 ± 0.33	6.24 ± 0.23	6.30 ± 0.37
MAP2	6.49 ± 0.07	6.33 ± 0.10 ^{ABC}	6.31 ± 0.02 ^{AB}	6.26 ± 0.12	6.34 ± 0.08	6.38 ± 0.07
MAP3	6.49 ± 0.07	6.28 ± 0.04 ^{BC}	6.32 ± 0.10 ^{AB}	6.23 ± 0.10	6.29 ± 0.05	6.34 ± 0.08
MAP4	6.49 ± 0.07	6.16 ± 0.12 ^C	6.17 ± 0.07 ^{BC}	6.16 ± 0.06	6.31 ± 0.19	6.32 ± 0.06
MAP5	6.49 ± 0.07	6.12 ± 0.06 ^C	6.09 ± 0.03 ^C	6.11 ± 0.07	6.17 ± 0.05	6.28 ± 0.15

^{A-C}Values within a column with different superscripts differ ($P < 0.05$).

¹MAP1 = 100% N₂; MAP2 = 30% CO₂:70% N₂; MAP3 = 50% CO₂:50% N₂; MAP4 = 70% CO₂:30% N₂; MAP5 = 100% CO₂.

Table 3 shows the detection of oxygen in packages for the shelf life experiments based on anaerobic indicator test strips. Similar trends were observed in the *L. monocytogenes* experiments (data not shown). The number of positive strips in packages decreased during storage for air conditions. This is most likely the result of the consumption of O₂ and production of CO₂ by microbial activity and cheese respiration (Olarde et al., 2002; Khoshgozaran et al., 2012). Observed increases in the presence of O₂ in samples during storage under vacuum may be the result of residual oxygen, packaging permeability, or incomplete seals. Positive indicators were typically not detected in MAP conditions with the exception of slight color changes (light blue indicators) recorded for single technical replicates of MAP1, MAP5, and MAP4 at d 14, 28, and 35, respectively, as well as 2 samples of MAP5 at d 21. This could have resulted from test strip failures, packaging permeability, or the presence of small amounts of O₂ from incomplete seals. Regardless, the presence of positive test strips in MAP samples does not appear to have affected mean microbial counts as described below. According to a review by Khoshgozaran et al. (2012), CO₂ levels may decrease as CO₂ dissolves into the cheese matrix, is

consumed by anaerobic microorganisms, or permeates out of the package. Losses of 2 to 5% CO₂ have been reported for MAP packaged cheese (CO₂ permeability of less than 13 mL/m²·24 h and O₂ permeability of 5 mL/m²·24 h) over its shelf life (Olarde et al., 2002).

Effect of MAP on Mesophile Counts

Overall, we observed treatment and time effects ($P < 0.001$) on mesophile counts (Figure 1). Counts over the duration of the study for cheeses packaged under air differed from MAP3 ($P = 0.047$), MAP4 ($P = 0.017$), and MAP5 ($P < 0.001$) conditions. Counts for cheeses packaged under vacuum or 100% N₂ (MAP1) also differed from MAP3 ($P = 0.022$ and 0.023 , respectively), MAP4 ($P = 0.007$ and $P = 0.007$, respectively), and MAP5 ($P < 0.001$ and $P < 0.001$, respectively) over the duration of the study. No differences in mean counts were detected between treatments based on pairwise comparisons at individual time points (Figure 1). As shown in Figure 1, mean counts increased ~2 log₁₀ cfu/g at d 7 in cheeses stored under air (4.47 log₁₀ cfu/g), vacuum (4.27 log₁₀ cfu/g), and 100% N₂ (MAP1; 4.22 log₁₀ cfu/g) conditions. Mean mesophile

Table 3. Results of anaerobic test strips in uninoculated Queso Fresco sample packages (including technical replicates) under modified atmospheres during storage at 7°C for 35 d

Condition ¹	No. of positive samples/total no. of samples ²				
	7 d	14 d	21 d	28 d	35 d
Air	6/6	3/4 ³	3/6	3/6	2/6
Vacuum	4/6	3/4	6/6	6/6	6/6
MAP1	0/6	1/4	0/6	0/6	0/6
MAP2	0/6	0/4	0/6	0/6	0/6
MAP3	0/6	0/4	0/6	0/6	0/6
MAP4	0/6	0/4	0/6	0/6	1/6
MAP5	0/6	0/4	2/6	1/6	0/6

¹MAP1 = 100% N₂; MAP2 = 30% CO₂:70% N₂; MAP3 = 50% CO₂:50% N₂; MAP4 = 70% CO₂:30% N₂; MAP5 = 100% CO₂.

²Indicator strip results not recorded after packaging on d 0.

³Results not recorded for 1 biological replicate of each condition at 14-d time point.

Figure 1. Mean mesophilic count ($\pm$ SD) in Queso Fresco packaged under modified atmospheres during storage at 7°C for 35 d. MAP1 = 100% N₂; MAP2 = 30% CO₂:70% N₂; MAP3 = 50% CO₂:50% N₂; MAP4 = 70% CO₂:30% N₂; MAP5 = 100% CO₂. The dashed line indicates initial contamination level at d 0 ($2.68 \pm 0.54 \log_{10}$ cfu/g).

counts exceeded 6 log₁₀ cfu/g in the MAP1 treatment (100% N₂) on d 28 and the air treatment on d 35. In contrast, mean counts remained <4 log₁₀ cfu/g through 28 d under the MAP3 conditions (3.68 log₁₀ cfu/g) and 35 d for both the MAP4 and MAP5 conditions, with growth inhibited to <1 log₁₀ cfu/g through 35 d (3.19 log₁₀ cfu/g) in cheeses held under MAP5 (100% CO₂) conditions. A similar trend was observed in mesophilic counts in fresh goat cheese packaged under similar conditions (Gonzalez-Fandos et al., 2000; Olarte et al., 2002). Similarly, total viable counts in low-moisture Mozzarella cheese samples packaged in atmospheres having high CO₂ concentrations (50% CO₂:50% N₂ and 75% CO₂:25% N₂) were less than those packaged in air or with low CO₂ levels and high N₂ levels (25% CO₂:75% N₂) through 28 d of storage (Mastromatteo et al., 2014). Alam and Goyal (2011) also reported that the plate counts in Mozzarella cheese stored for 12 wk were lowest in samples packed under 100% CO₂ and highest in vacuum-packaged samples.

Coliform Counts

No coliforms were detected in any batch of cheese produced for the shelf life experiments. However, previous research suggests that the growth of coliforms would be suppressed when exposed to higher concentrations of CO₂ (Rosenthal et al., 1991; Gonzalez-Fandos et al., 2000).

Effect of MAP on Psychrotolerant Counts

Initial mean psychrotolerant counts in QF (5.40 log₁₀ cfu/g) were just below the threshold of 6 log₁₀ cfu/g used in previous studies based on detectable organoleptic changes in milk (Figure 2; Bishop and White, 1986). Mean counts reached or exceeded this threshold in the air (7.53 log₁₀ cfu/g), vacuum (6.17 log₁₀ cfu/g), and MAP1 (6.03 log₁₀ cfu/g) conditions at d 7. Mean counts remained below this threshold for 28 d under MAP3 conditions (5.42 log₁₀ cfu/g) and 35 d for both MAP4 (5.22 log₁₀ cfu/g) and MAP5 conditions (4.98 log₁₀ cfu/g). Mean counts in the latter 2 conditions at 35 d did not exceed initial counts at d 0. In contrast, mean counts exceeded 8 log₁₀ cfu/g in the air treatment at d 14 and remained at this level through 35 d. Overall, we observed a treatment effect on psychrotolerant counts ($P < 0.001$). Psychrotolerant counts through the duration of the study differed between air and MAP2–5 treatments ($P < 0.001$) and counts in cheeses under MAP1 conditions were higher than QF samples under MAP3 ($P = 0.032$), MAP4 ($P < 0.001$), and MAP5 ($P < 0.001$) conditions. Counts in QF stored under vacuum also differed from both MAP4 and MAP5 ($P = 0.004$ and <0.001 , respectively) through the duration of the study. Differences in counts between treatments at specific time points were only observed on d 14 (air vs. MAP5) as shown in Figure 2. These results are in agreement with those previously

Figure 2. Mean psychrotolerant count ($\pm$ SD) in Queso Fresco samples packaged under modified atmospheres during storage at 7°C for 35 d. MAP1 = 100% N₂; MAP2 = 30% CO₂:70% N₂; MAP3 = 50% CO₂:50% N₂; MAP4 = 70% CO₂:30% N₂; MAP5 = 100% CO₂. The dashed line indicates initial contamination level at d 0 ($5.40 \pm 0.51 \log_{10}$ cfu/g). ^{a,b}Values within a time point with different letters differ ($P < 0.05$).

reported for whey cheese (Dermiki et al., 2008), fresh goat cheese (Gonzalez-Fandos et al., 2000; Olarte et al., 2002), and Mozzarella (Alam and Goyal, 2011) cheese, where psychrotolerant growth was lower when the CO₂ concentration increased, with 100% CO₂ conditions being the most effective for inhibiting growth. These observations are consistent with the suggestion that most psychrotolerant bacteria in dairy products are aerobic, gram-negative bacteria, which are generally more sensitive to CO₂ than gram-positive bacteria (Rosenthal et al., 1991). It is important to note that not all colonies enumerated for psychrotolerant counts in the present study were verified as bacteria nor were their Gram reactions determined. High psychrotolerant counts could be explained by the use of pasteurized milk purchased at retail, but data are not available to support this suggestion.

Effect of MAP on Lactic Acid Bacteria Counts

Because no starter culture was used in the manufacture of QF in the present study, initial LAB counts were relatively low ($1.59 \log_{10}$ cfu/g). However, populations increased by $>4 \log_{10}$ cfu/g at 35 d for cheeses packaged with air. Counts also increased under all MAP conditions (Figure 3), which is in agreement with the literature as LAB are typically facultative anaerobes (Khoshgozaran et al., 2012). No differences in mean

counts were detected between treatments based on pairwise comparisons at individual time points (Figure 3). We observed overall treatment and time effects ($P < 0.001$) on counts; however, no significant interaction between terms was observed. The LAB counts in QF stored in air differed from MAP5 (100% CO₂; $P = 0.001$) through the duration of the study. Mean LAB counts in cheese under MAP5 conditions reached $2.70 \log_{10}$ cfu/g after 35 d. The LAB counts in cheeses under MAP3, MAP4, and MAP5 differed from vacuum ($P = 0.043$, $P = 0.067$, and $P < 0.001$, respectively) and MAP1 ($P = 0.040$, $P = 0.063$, and $P < 0.001$, respectively) through the duration of the study. Despite the increase in counts, corresponding reductions in pH were not observed. Because we did not conduct additional tests to confirm if isolates produced acid, it is possible that they were not significant acid producers in QF. It is also possible that the yeast growth resulted in metabolism of lactic acid, which is supported by the lower pH of vacuum-packaged samples that inhibited yeast growth compared with those packaged under air, whose pH remained relatively stable.

Effect of MAP on Yeast and Mold Counts

Colonies enumerated on DRBC were consistently identified as yeast but are reported as yeast and mold for comparison with previous studies. Similar to psy-

Figure 3. Mean lactic acid bacteria count ($\pm$ SD) in Queso Fresco samples packaged under modified atmospheres during storage at 7°C for 35 d. MAP1 = 100% N₂; MAP2 = 30% CO₂:70% N₂; MAP3 = 50% CO₂:50% N₂; MAP4 = 70% CO₂:30% N₂; MAP5 = 100% CO₂. The dashed line indicates initial contamination level at d 0 (1.59 ± 1.51 log₁₀ cfu/g).

chroloerant counts, a treatment effect on yeast and mold counts was observed ($P < 0.001$). Modified atmospheres, as well as packaging under vacuum, inhibited the growth of yeasts and molds on QF to increases of <1 log₁₀ cfu/g through 35 d of storage. In contrast, mean counts in cheeses packaged in air increased ~ 3 log₁₀ cfu/g at d 7 (Figure 4). Yeast and mold counts in cheeses packaged in air conditions were different than all MAP treatments ($P < 0.001$) through the duration of the study. Differences were also observed between vacuum packaged cheeses when compared with MAP5 ($P = 0.005$). Counts in cheeses stored under 100% N₂ (MAP1) differed from conditions containing higher CO₂ levels, including MAP4 ($P = 0.011$) and MAP5 ($P < 0.001$). The MAP2 QF samples also had higher yeast and mold counts compared with QF packaged under MAP5 conditions ($P = 0.033$) through the duration of the study. The inhibition and reductions in yeast and mold counts in QF packaged under modified atmospheres compared with growth when packaged under air are consistent with previous reports for Mozzarella stored under similar conditions (Alam and Goyal, 2011). The inhibitory effect of CO₂ on yeast and mold counts is also in agreement with those reported previously (Alves et al., 1996; Eliot et al., 1998; Dermiki et al., 2008), including reports of reduced counts in vacuum treatments compared with air (Sarais et al., 1996).

Effect of MAP on *Listeria monocytogenes* Counts

Listeria monocytogenes contamination can occur at any point or at multiple points in production including milk, curds, and finished cheese. The present study focused on postmanufacture surface contamination of QF. No listeriae were detected in uninoculated samples from each of the 4 batches of cheese. Mean *Listeria monocytogenes* counts remained near inoculation levels (d 0 = 4.27 log₁₀ cfu/g) for all treatments at d 1 but increased ~ 2 log₁₀ cfu/g on cheeses packaged in air (6.49 log₁₀ cfu/g), vacuum (6.29 log₁₀ cfu/g), and MAP1 (6.23 log₁₀ cfu/g) conditions at d 7 (Table 4). In contrast, mean counts in treatments containing CO₂ (MAP2–5) increased ≤ 0.4 log₁₀ cfu/g by d 7 of storage. However, differences in mean counts between treatments at d 7 were not statistically significant due to the variability in counts at this time point resulting from differences in growth of *L. monocytogenes* between replicates. Mean *L. monocytogenes* counts increased >3 log₁₀ cfu/g for air, vacuum, and MAP1 treatments at d 14 and remained at ~ 8 log₁₀ cfu/g through 35 d. Treatments consisting of 70% (MAP4) and 100% CO₂ (MAP5) limited increases in mean *L. monocytogenes* counts to <1 log₁₀ cfu/g higher than initial inoculation through 14 d (5.25 and 4.86 log₁₀ cfu/g, respectively) and ~ 1.5 log₁₀ cfu/g through 21 d (5.8 and 5.6 log₁₀ cfu/g, respectively).

As shown in Table 4, *L. monocytogenes* counts were significantly higher than initial inoculation levels (d 0 = 4.27 log₁₀ cfu/g) on cheeses stored in air, vacuum, and MAP1 conditions at d 7 (6.49, 6.29, and 6.23 log₁₀ cfu/g, respectively). Counts within each of these treatments did not increase significantly through subsequent time points after d 14. In contrast, *L. monocytogenes* counts increased to levels significantly higher than inoculation (d 0) on cheeses stored in MAP2 and MAP3 on d 21 ($P = 0.022$ and $P = 0.019$, respectively), on d 28 for MAP4 ($P = 0.012$), and on d 35 for cheese stored under MAP5 ($P = 0.045$) conditions (Table 4). Although no statistically significant differences in counts between treatments were observed at d 1 and 7, between treatment differences in *L. monocytogenes* counts were observed on d 14, 21, and 28 (Table 4). Counts were similar for all treatments again at d 35 (Table 4). Overall, treatment and time effects as well as the treatment $\times$ time interaction were significant ($P < 0.001$ each). Significant treatment $\times$ time interactions were observed between air, vacuum, and MAP1 when each were compared with MAP2, MAP3, MAP4, and MAP5. Treatment and time effects were observed when MAP2 was compared with MAP5 ($P < 0.05$ and $P < 0.001$). In terms of listeristatic treatments, mean *L. monocytogenes* counts increased >1 log₁₀ cfu/g by d 7 for air, vacuum, and MAP1 conditions; d 14 for

MAP2 and MAP3; and d 21 for MAP4 and MAP5 conditions. Previous research on surface inoculated cheese has also reported that increasing the CO₂ content of MAP conditions (0–20%) increased the inhibition of *L. monocytogenes* growth on cheese (Whitley et al., 2000). Olarte et al. (2002) investigated the effect of MAP on the growth of *L. monocytogenes* in fresh goat cheese produced from contaminated milk, which has a similar composition to QF (no starter culture, near-neutral pH, low salt content, and high moisture content), and found that MAP conditions containing 100% CO₂ reduced *L. monocytogenes* growth rates but did not inhibit growth altogether even when stored at refrigeration temperatures (Olarte et al., 2002). Based on the similarities in *L. monocytogenes* growth in the present study and those of Olarte et al. (2002), it is possible that the MAP conditions used here would be similarly effective against *L. monocytogenes* present in QF as the result of contaminated milk or curd. However, additional studies would be needed to test this assumption. Although increasing CO₂ content inhibits the outgrowth of spoilage microorganisms, thereby increasing the shelf life of QF, the growth of the facultative anaerobic pathogen *L. monocytogenes* presents an increasing food safety hazard as shelf life progresses.

In agreement with previous studies (Eliot et al., 1998; Alam and Goyal, 2011), we found that the efficacy of

Figure 4. Mean yeast and mold counts ($\pm$ SD) in Queso Fresco samples packaged under modified atmospheres during storage at 7°C for 35 d. MAP1 = 100% N₂; MAP2 = 30% CO₂:70% N₂; MAP3 = 50% CO₂:50% N₂; MAP4 = 70% CO₂:30% N₂; MAP5 = 100% CO₂. The dashed line indicates initial contamination level at d 0 (4.25 ± 0.53 log cfu/g). ^{a,b}Values within a time point with different letters differ ($P < 0.05$).

MAP in controlling spoilage microorganisms increases with increasing CO₂ content, whereas conditions consisting of 100% N₂, vacuum, or air were less effective. The antimicrobial properties of CO₂ are thought to be due to several factors including malfunctions in cell membrane, direct physicochemical changes of membrane-located proteins and lipids, inhibition of enzyme systems directly or depression in the rate of enzymatic reactions, inhibition of cell division, and alteration of cell morphology (Khoshgozaran et al., 2012). In addition to the suppression of spoilage microorganisms, Dermiki et al. (2008) concluded that increasing CO₂ concentration increased shelf life of whey cheese based on sensory analysis, with CO₂ inhibiting lipolysis, proteolysis, and lipid oxidation. Gonzalez-Fandos et al. (2000) also reported increased fat acidity and proteolysis in cheeses packaged under air and vacuum, with the lowest values among CO₂-packaged cheeses. Increased fat acidity and proteolysis were attributed to the increases in microbial populations, which were highest in cheeses packaged in air.

With that, MAP is generally effective in prolonging the sensory acceptability of cheeses in terms of taste and odor (Khoshgozaran et al., 2012). Carbon dioxide adsorption may also lead to lower pH at the surface of products and can potentially alter flavor. For example, Gonzalez-Fandos et al. (2000) reported that 100% CO₂ negatively affected the quality of fresh Cameros cheese and that packaging in 50% CO₂:50% N₂ and 40% CO₂:60% N₂ were the most effective conditions for extending the shelf life of cheese while maintaining good sensory characteristics (Gonzalez-Fandos et al., 2000). Therefore, in cases where CO₂ may negatively increase the acidic taste of products, altering the CO₂:N₂ ratio may be needed to balance flavor with microbial control (Scott and Smith, 1971; Kosikowski and Brown, 1973).

Although MAP conditions containing CO₂ aid in the control of *L. monocytogenes*, the absence of oxygen

in MAP conditions may also support the growth of nonproteolytic psychrotolerant strains of *Clostridium botulinum* (Farber, 1991). Determining the fate of *C. botulinum* in QF stored under different atmospheric conditions was outside the scope of the present study, but previous studies and pathogen modeling suggest that control of nonproteolytic *C. botulinum* growth and toxin production relies on a combination of substrate, redox potential, pH, water activity, as well as head-space composition, temperature, and the presence of preservatives (Farber, 1991; Chen and Hotchkiss, 1993). Although maintaining cold storage temperatures is important for controlling *C. botulinum* growth and toxin production, increasing QF storage temperature would likely result in increased acid development, which would further suppress pathogen growth (Chen and Hotchkiss, 1993) or result in marked spoilage due to growth of contaminants that would deter consumption. It has been suggested that *C. botulinum* incidence in milk is low (Franciosa et al., 1999) and that toxin production in raw and pasteurized milk does not occur because of the product's short, refrigerated shelf life and the inability of this organism to readily compete with the native background flora (Glass et al., 1999). Therefore, additional studies are needed to determine the effect of various MAP conditions on the growth of nonproteolytic *C. botulinum* in cheeses using different production methods and with varying composition. According to a review by Farber (1991), the presence of starter LAB would enhance the safety of products stored in MAP conditions by keeping background microflora and any pathogenic microorganisms that may be present at very low levels. The manufacture and resulting composition as well as the storage temperatures of the experimental QF in the present study represent conservatively permissive estimates of commercial practice and excluded the use of starter cultures. Therefore, similar cheeses with higher acidity or produced with starter cultures

Table 4. *Listeria monocytogenes* counts on Queso Fresco samples packaged under modified atmospheres during storage at 7°C for 35 d

Treatment ¹	<i>L. monocytogenes</i> count (log ₁₀ cfu/g ± SD)						
	0 d	1 d	7 d	14 d	21 d	28 d	35 d
Air	4.27 ± 0.38 ^a	4.28 ± 0.18 ^a	6.49 ± 0.91 ^b	7.98 ± 0.51 ^{A,c}	8.24 ± 0.42 ^{A,c}	8.16 ± 0.33 ^{A,c}	8.11 ± 0.64 ^c
Vacuum	4.27 ± 0.38 ^a	4.42 ± 0.10 ^a	6.29 ± 0.94 ^b	7.69 ± 0.26 ^{A,c}	7.91 ± 0.26 ^{AB,c}	7.95 ± 0.25 ^{AB,c}	7.74 ± 0.61 ^c
MAP1	4.27 ± 0.38 ^a	4.44 ± 0.11 ^a	6.23 ± 1.15 ^b	7.77 ± 0.33 ^{A,c}	8.04 ± 0.11 ^{AB,c}	8.04 ± 0.11 ^{A,c}	7.86 ± 0.48 ^c
MAP2	4.27 ± 0.38 ^a	4.34 ± 0.14 ^a	4.57 ± 0.79 ^{ab}	5.68 ± 1.01 ^{B,abc}	6.63 ± 1.19 ^{ABC,bc}	6.92 ± 1.06 ^{AB,c}	7.03 ± 0.95 ^c
MAP3	4.27 ± 0.38 ^a	4.52 ± 0.36 ^{ab}	4.56 ± 0.81 ^{ab}	5.76 ± 0.93 ^{B,abc}	6.25 ± 0.80 ^{BC,bc}	6.56 ± 0.94 ^{AB,c}	6.91 ± 0.79 ^c
MAP4	4.27 ± 0.38 ^a	4.49 ± 0.15 ^a	4.67 ± 0.72 ^{ab}	5.25 ± 1.05 ^{B,abc}	5.80 ± 0.90 ^{C,abc}	6.48 ± 0.94 ^{AB,bc}	6.97 ± 1.00 ^c
MAP5	4.27 ± 0.38 ^a	4.24 ± 0.16 ^a	4.59 ± 0.86 ^{ab}	4.86 ± 1.14 ^{B,ab}	5.60 ± 1.19 ^{C,ab}	6.06 ± 1.23 ^{B,ab}	6.38 ± 0.85 ^b

^{A-C}Values within a column with different superscripts differ ($P < 0.05$).

^{a-c}Values within a row with different superscripts differ ($P < 0.05$).

¹MAP1 = 100% N₂; MAP2 = 30% CO₂:70% N₂; MAP3 = 50% CO₂:50% N₂; MAP4 = 70% CO₂:30% N₂; MAP5 = 100% CO₂.

or both would be more likely to suppress growth of unwanted contaminants.

Although the MAP conditions in this study containing $\geq 30\%$ CO₂ (MAP2–5) were effective in limiting *L. monocytogenes* growth, the effects of MAP alone on *L. monocytogenes* growth in other cheeses is under-researched. Combining MAP conditions with other antimicrobial interventions, including antimicrobials and active coatings, may also enhance the antimicrobial activity of MAP against several pathogens (Szabo and Cahill, 1998; Del Nobile et al., 2009; Govaris et al., 2011), warranting further studies.

ACKNOWLEDGMENTS

This project was supported in part by the National Dairy Council (Rosemont, IL) and the United States Department of Agriculture (USDA) National Institute of Food and Agriculture (NIFA, Washington, DC), Multistate project S1056, accession no. 1003888. Any opinions, findings, conclusions, or recommendations expressed in this manuscript are those of the author(s) and do not necessarily reflect the view of the NIFA, the USDA, or any other sponsors.

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