Reduced-fat Cheddar and Swiss-type cheeses harboring exopolysaccharide-producing probiotic *Lactobacillus mucosae* DPC 6426

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ABSTRACT

Exopolysaccharide-producing *Lactobacillus mucosae* DPC 6426 was previously shown to have promising hypocholesterolemic activity in the atherosclerosis-prone apolipoprotein-E-deficient (apoE^{-/-}) murine model. The aim of this study was to investigate the suitability of reduced-fat Cheddar and Swiss-type cheeses as functional (carrier) foods for delivery of this probiotic strain. All cheeses were manufactured at pilot-scale (500-L vats) in triplicate, with standard commercially available starters: for Cheddar, *Lactococcus lactis*; and for Swiss-type cheese, *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii*. *Lactobacillus mucosae* DPC 6426 was used as an adjunct culture during cheese manufacture, at a level of $\sim 10^6$ cfu·mL⁻¹ cheese milk (subsequently present in the cheese curd at $> 10^7$ cfu·g⁻¹). The adjunct strain remained viable at $> 5 \times 10^7$ cfu·g⁻¹ in both Swiss-type and Cheddar cheeses following ripening for 6 mo. Sensory analysis revealed that the presence of the adjunct culture imparted a more appealing appearance in Swiss-type cheese, but had no significant effect on the sensory characteristics of Cheddar cheeses. Moreover, the adjunct culture had no significant effect on cheese composition, proteolysis, pH, or instrumentally quantified textural characteristics of Cheddar cheeses. These data indicate that low-fat Swiss-type and Cheddar cheeses represent suitable food matrices for the delivery of the hypocholesterolemic *Lactobacillus mucosae* DPC 6426 in an industrial setting.

Key words: reduced fat, Cheddar, Swiss type, *Lactobacillus mucosae*, exopolysaccharide

INTRODUCTION

Lactic acid bacteria (LAB) are essential to biochemical processes such as acidification and proteolysis, and selected microbes have also found uses as adjunct cultures, conferring technofunctional attributes to the product containing them, and health-promoting properties for consumers. Whereas starter LAB such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* have earned claims in aiding host lactose digestion (EFSA, 2010), many products claiming probiotic status lack the requirements of a clinically proven health benefit, in conjunction with the ability to survive gastrointestinal tract (GIT) transit (Hill et al., 2014). *Lactobacillus mucosae* DPC 6426 is an exopolysaccharide (EPS)-producing strain, previously isolated from the mammalian GIT, which has demonstrated hypocholesterolemic activity in atherosclerosis-prone mice on a high-fat diet (London et al., 2015b). Furthermore, the heteropolysaccharide that the strain synthesizes appears to afford the strain higher tolerance to heat, acid, bile, and salt stresses in vitro (London et al., 2014). These properties make *Lb. mucosae* DPC 6426 an ideal candidate for industrial exploitation by the functional food sector (Ryan et al., 2015b). Previous work in our group has identified the potential of *Lb. mucosae* DPC 6426 as a technofunctional adjunct strain in yogurt manufacture, reducing syneresis (London et al., 2015a). As a species, *Lb. mucosae* are acknowledged for their GIT adhesion capabilities, expressing mucus binding protein (Mub) homologs and other adhesin-like proteins, such as the ABO blood group histone H3-binding Lam29 of *Lb. mucosae* ME-340 (Roos et al., 2000; Lee et al., 2012; Watanabe et al., 2012; Valeriano et al., 2014). Such adherence is theorized to aid in the competitive exclusion of attaching and effacing enteric pathogens in vivo, thus reducing the opportunity for infection (Ramiah et al., 2008; Dicks et al., 2014). In addition, *Lb. mucosae* has demonstrated an extended transient occupation of the host when compared with

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allochthonous *Lactobacillus* spp., and so is more likely an indigenous species of the mammalian GIT (Frese et al., 2012).

Exopolysaccharide-synthesizing LAB, such as *Lactococcus lactis* ssp. *cremoris* DPC 6532, have previously been investigated as technofunctionally beneficial adjuncts (Costa et al., 2010). The use of these adjuncts in reduced-fat cheeses generally provides enhanced water-binding capacity, leading to higher cheese yield and a cheese more similar in texture and quality to its full-fat counterpart (Awad et al., 2005; Dabour et al., 2006; Lynch et al., 2014). Furthermore, cheese has the potential to act as a favorable matrix for *Lb. mucosae* DPC 6426 survival, due to the near anaerobic conditions created by the primary starter bacteria over the initial weeks of ripening and the relatively high fat content of the cheese structure (van den Tempel et al., 2002; Lavermicocca, 2006).

In a recent study, we have shown that dietary intervention with EPS-producing *Lactobacillus mucosae* DPC 6426 resulted in modulation of lipid metabolism in an animal model of lipid-driven atherosclerosis. Apo-lipoprotein E deficient (*apoE*^{-/-}) mice fed a high-fat/high-cholesterol diet, supplemented with *Lactobacillus mucosae* DPC 6426, resulted in significantly lower serum cholesterol and triglyceride levels compared with unsupplemented controls (London et al., 2015b). These data indicate that dietary intervention with food products containing live EPS-producing probiotic strains is a promising strategy for managing lipid metabolism. Therefore, the aim of this study was to investigate the performance of the cholesterol-lowering EPS-producing strain, *Lactobacillus mucosae* DPC 6426, as adjunct culture during manufacture of reduced-fat Cheddar and Swiss-type cheeses. We hypothesized that reduced-fat cheese would be an ideal food matrix for delivering this probiotic and also that the EPS produced by *Lb. mucosae* DPC 6426 might improve functional properties, such as cheese texture and yield recovered, in the final product.

MATERIALS AND METHODS

Strains and Growth Conditions

The ropy EPS-producing adjunct used for both cheese types, *Lactobacillus mucosae* DPC 6426, was selected from the Teagasc Food Research Centre Moorepark culture collection and had originally been isolated from the mammalian GIT (Ryan et al., 2015a). The strain was rifampicin-tagged to allow enumeration in the presence of other nonstarter lactic acid bacteria (NSLAB). *Lactobacillus mucosae* DPC 6426 was cul-

tured directly from a -20°C stock on de Man Rogosa Sharpe (MRS, Difco, Unitech Ltd., Dublin, Ireland) agar anaerobically in jars with Anaerocult A gas packs (Merck KGaA, Darmstadt, Germany) for 48 h at 37°C and all subsequent cultures were grown under the same conditions in MRS broth with $250\text{ mg}\cdot\text{L}^{-1}$ rifampicin (Sigma Aldrich, Wicklow, Ireland) to select for the resistant derivative.

The day before cheese manufacture, *Lb. mucosae* DPC 6426 was inoculated at 1% in 1,000 mL of MRS, containing 5% (wt/vol) sucrose to facilitate EPS production, and grown to stationary phase overnight. The cells were pelleted by centrifuging at $16,900 \times g$ for 15 min at 4°C (Sorvall Legend RT, Thermo Scientific, Dublin, Ireland, with SLA3000 rotor) and washed twice with PBS (Sigma-Aldrich). The cells were then resuspended in 100 mL of PBS and maintained on ice until cheese-making. The adjunct culture was added at $10^6\text{ cfu}\cdot\text{mL}^{-1}$ of cheese milk or 100 mL of PBS for control cheeses.

The starter cultures used were *Lactococcus lactis* R604 ($0.1\text{ g}\cdot\text{L}^{-1}$, Chr. Hansen, Little Island, Co. Cork, Ireland) for Cheddar cheese and *Streptococcus thermophilus* Th3 ($0.1\text{ g}\cdot\text{L}^{-1}$, Chr. Hansen), *Lactobacillus helveticus* LHBO2 ($0.05\text{ g}\cdot\text{L}^{-1}$, Chr. Hansen), and *Propionibacterium freudenreichii* DPC 6451 (PAB, 0.007% vol/vol) for Swiss-type cheese.

Cheese Manufacture

Production. Trials were conducted in triplicate over a 6-mo period. Raw milk was standardized to a protein:fat ratio of 2.19:1 or 3.48:1 for Swiss-type and Cheddar cheeses, respectively. Cheese milk was pasteurized at 72°C for 15 s, and cooled to 31°C before cheese-making. Starter cultures were stored at -80°C until required for use and adjunct cultures were kept on ice until use. Reduced-fat Cheddar cheeses were produced according to the methods of Rynne et al. (2004), and Swiss-type cheeses were manufactured according to the method of Sheehan et al. (2008), with minor modifications.

The control Swiss-type coagula were cut at gel firmness standard for the production of such cheeses as observed visually, whereas the coagula of the Swiss-type with adjunct vats were cut at the same duration of coagulation. Gel firmness was measured instrumentally using the method reported by Costa et al. (2010). Swiss-type vats were then cut to $\sim 5\text{-mm}^3$ curds and allowed a 10-min healing period before cooking. Swiss-type curds and whey were cooked from 32 to 45°C at $0.5^{\circ}\text{C}\cdot\text{min}^{-1}$ and then from 45 to 53°C at $1^{\circ}\text{C}\cdot\text{min}^{-1}$.

Swiss-type curds were drained to a pre-press vat at pH 6.3, molded, and pressed at pressures increasing incrementally to 35.2 kPa before brining for 10 h in a saturated brine solution of 23% NaCl and 0.56% CaCl₂ (10°C, pH 5.2).

Ripening. Cheddar was vacuum-packed and ripened at 8°C for 6 mo. Swiss-type cheese was vacuum-packed in CO₂-permeable bags and kept at 9 to 10°C for 10 d, then moved to 22°C for 3 wk to facilitate eye formation and back down to 8°C for the remaining 5 mo to retard the PAB fermentation.

Enumeration of Starter, PAB, NSLAB, and Adjunct

Cheese samples were aseptically removed at 0, 1, 7, 21, 35, 56, 70, 91, 119, 154, and 182 d of ripening. The samples were placed in a stomacher bag, diluted 1:10 with sterile trisodium citrate (2% wt/vol), and homogenized in a stomacher (Stomacher, Lab-Blender 400, Seward, Thetford, Norfolk, UK) for 5 min. Further dilutions were prepared as required in maximum recovery diluent (MRD, Oxoid, Fannin Healthcare, Dublin, Ireland). *Lactobacillus mucosae* DPC 6426 was enumerated on MRS supplemented with rifampicin (100 mg·L⁻¹, Sigma-Aldrich, Wicklow, Ireland). Random amplified polymorphic DNA (RAPD) PCR was performed on selected colonies to confirm identity of *Lb. mucosae* DPC 6426, using the primer sequence (5'-GCTCGTATGTTGTGTGG-3') described in Navidghasemizad et al. (2013). Viable *S. thermophilus* and *Lb. helveticus* cells and NSLAB were enumerated in duplicate on M-17 agar supplemented with 0.5% lactose (42°C; Terzaghi and Sandine, 1975), MRS pH 5.4 (30°C), and *Lactobacillus*-selective agar (LBS, 30°C), respectively. The PAB were enumerated on sodium lactate agar supplemented with kanamycin (40 mg·L⁻¹, Merck) after incubation at 30°C for 7 d (Drinan and Cogan, 1992). Finally, *Lb. mucosae* DPC 6426 were also enumerated in the whey at various time points during processing.

Cheese Composition

Grated cheese samples were analyzed in duplicate for salt content by a potentiometric method (International Dairy Federation, 1981), for fat content by the international standard method (International Dairy Federation, 1996) and for moisture content by oven-drying at 102°C for 5 h (International Dairy Federation, 1982). The pH of cheese was assessed by blending 12 mL of H₂O with 20 g of grated cheese and measured by using a standard pH meter (Mettler Toledo MP220; British Standards Institution, 1976).

Proteolysis

% pH 4.6 Soluble N/Total N. Concentration of nitrogen soluble at pH 4.6 was determined in duplicate by the method of Fenelon et al. (2000a) using the macro-Kjeldahl method (International Dairy Federation, 1993) and was expressed as a percentage of total nitrogen (% pH 4.6-SN/TN).

Free Amino Acids. Individual free AA (FAA) were determined on the pH 4.6-soluble N extracts as described by Fenelon et al. (2000b, 2002) using a Jeol JLC-500V AA analyzer fitted with a Jeol Na⁺ high performance cation exchange column (Jeol Ltd., Tokyo, Japan). The chromatographic analyses were conducted at pH 2.2 on samples from d 1, 91, and 182. Results are expressed as parts per million (mg·kg⁻¹).

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) was performed as per London et al. (2014) with modifications for sample type. Briefly, the protein matrix was fluorescently dyed using Fast Green FCF (excitation 633 nm; Sigma-Aldrich), polysaccharide was labeled by lectin Wheat Germ Agglutinin Alexafluor 555 conjugate (excitation 561nm; WGA; Invitrogen, BioSciences, Dublin, Ireland), and finally SYTO9 (excitation/emission maxima of 480/500 nm) was employed to label viable and nonviable forms of bacteria. The excitation wavelengths and emission detection were set in accordance to the maximum excitation/emission value of the fluorescent dyes. Mature Cheddar cheese was sampled and sectioned using a scalpel into roughly 10 mm × 10 mm × 2 mm sections and incubated at room temperature with the aforementioned labeling dyes applied to the surface. Images were acquired using the Leica TCS SP5 CLSM (Heidelberg GmbH, Mannheim, Germany), objective 63× (oil, Na 1.4) objective of a Leica TCS SP3 CLSM (Leica Microsystems, Heidelberg GmbH).

Texture Profile Analysis

Two-bite texture profile analysis (TPA) was performed on uniformly cubed Cheddar cheeses on d 7, 70, 119, 154, and 182, as described by Costa et al. (2010). Swiss-type cheeses were excluded from TPA analysis because eye formation would confound results.

Cheese Yield Calculation

Sampling of milk, whey, and cheese were conducted as per Fenelon and Guinee (1999) and Guinee et al.

(2006). In the case of Swiss-type cheeses, the individual components lost in the white whey were calculated by measuring moisture, fat, protein, and salt contents and the weights of cheeses pre- and postbrining and also allowing for the salt uptake during brining.

Yield analysis was undertaken as per Guinee et al. (2006) and included the following: Actual cheese yield (Y_a), a unit of gross cheese conversion (i.e., kilograms of cheese obtained from 100 kg of cheese milk). This is calculated by weighing milk before cheese making and weight of cheese obtained following pressing for Cheddar and following brining for Swiss-type cheeses. In addition, Y_a was normalized to levels of fat and protein present (Y_{afpam}) to negate intertrial variation. Y_{afpam} negates the differences of different milk compositions to yield and thus allows comparisons between trials. Y_{macy} is a calculation adjusted for moisture content, allowing the yields of cheeses with different moistures to be compared. Finally, $Y_{macyfpam}$ is the combined measurement of cheese yield adjusted for moisture and normalized for the fat and protein content of the cheese milk.

Sensory Analysis

Sensory analysis of both Swiss-type and Cheddar cheeses was performed by a trained sensory panel by affective evaluation and ranking descriptive analysis. Thirty Swiss cheese consumers and 26 Cheddar cheese consumers, aged 21 to 48 yr, were recruited for affective evaluation. Selection criteria for consumers were availability and motivation to participate on all days of the experiment. Consumers used the sensory hedonic descriptors in Appendix Table A1 for 2 different Swiss cheeses and 3 different Cheddar cheeses, presented in duplicate and triplicate, respectively. A further 10 Swiss cheese consumers and 12 Cheddar consumers, aged 21 to 48 yr, were recruited for ranking descriptive analysis. All panelists had previously participated in cheese descriptive profiles and were well versed in the sensory experimental protocol. Panelists were briefly trained using the sensory intensity descriptors in Appendix Table A1.

Sensory analysis was carried out in panel booths conforming to international standards (ISO 8589:2007). All samples were blast frozen to -20°C then stored at -20°C until analysis. Samples were then held at refrigeration temperatures overnight (4°C), before monadic presentation to the naïve assessor panel at ambient temperatures ($\sim 21^{\circ}\text{C}$) and coded with a randomly selected 3-digit code. The cheese was immediately served to consumers. Each assessor was provided with deionized water and instructed to cleanse their palates between tastings. Additionally, each assessor was asked

to indicate their degree of liking on a 10-cm line scale ranging from 0 (extremely dislike) at the left to 10 (extremely like) at the right and rating subsequently scored in centimeters from the left. The order of the presentation of all test samples was randomized to prevent first-order and carryover effects. The appearance attributes of Swiss-type cheeses were assessed in a separate session by 9 assessors, in triplicate.

Statistical Analysis

A split plot design, generated in SAS v9.3 (SAS Institute Inc., Cary, NC), was used to determine the effects of the adjunct culture on all textural, sensory, and biochemical variables at each time point throughout the ripening period. A time and treatment ANOVA was undertaken for all split plot data with a Tukey-Kramer adjustment, and differences were considered significant at an adjusted $P < 0.05$. Regression and correlation analyses were performed on data using PASW Statistics v18.0 (IBM Corp., Chicago, IL).

RESULTS AND DISCUSSION

Starter, PAB, NSLAB, and Adjunct Viability During Ripening

***Lactococcus lactis*.** All starter and adjunct cultures were subject to a ~ 10 -fold concentration from cheese milk during curd formation and draining of whey. Mean viable counts of *L. lactis* were $10^{9.5}$ to 10^{10} cfu·g $^{-1}$ following Cheddar cheese manufacture and decreased significantly to $\sim 10^8$ cfu·g $^{-1}$ during ripening ($P < 0.0001$; Figure 1a). The co-inoculation with *Lb. mucosae* DPC 6426 had no significant effect on the growth of *L. lactis* at any time throughout ripening.

***Streptococcus thermophilus*.** *Streptococcus thermophilus* was found at $\sim 10^9$ cfu·g $^{-1}$ in both Swiss-type cheeses on d 1 (Figure 1b). Following this, *St. thermophilus* viability significantly decreased in both cheeses by d 21 ($P < 0.05$), after which point enumeration was ceased due to increased prevalence of NSLAB, which could confound counts. *Lactobacillus mucosae* DPC 6426 had no significant effect on the growth of *St. thermophilus* during ripening.

***Lactobacillus helveticus*.** Mean viable counts of *Lb. helveticus* were 5×10^8 cfu·g $^{-1}$ at d 1 and decreased significantly to $\sim 5 \times 10^6$ cfu·g $^{-1}$ during ripening of Swiss-type cheeses ($P < 0.0001$; Figure 1c). Co-inoculation with *Lb. mucosae* DPC 6426 had no significant effect on the growth of *Lb. helveticus* at any time during ripening.

Propionic Acid Bacteria. Propionic acid bacteria were inoculated to Swiss-type cheese milk to achieve a

Figure 1. Starter and nonstarter lactic acid bacteria (NSLAB) counts. (A) Cheddar starter (*Lactococcus lactis* R604) counts of control (black circles) and adjunct (gray squares) over the ripening period. (B) Swiss-type *St. thermophilus* counts of control (black circles) and adjunct (gray squares) for the first 21 d of the ripening period. (C) Swiss-type *Lb. helveticus* counts of control (black circles) and adjunct (gray squares) for the first 119 d of the ripening period. (D) Propionic acid bacteria (PAB) counts of control (black circles) and adjunct (gray squares) Swiss-types over the ripening period. (E) NSLAB development in Swiss-type control (black circles and dashed line), adjunct (gray squares and dashed line), and Cheddar control (black circles and solid line) and adjunct (gray squares and solid line) cheeses. Shaded area represents counts during manufacture.

mean viable count of $\sim 5 \times 10^5$ cfu·mL⁻¹ in cheese and remained unchanged from d 1 to 7 (Figure 1d). Thereafter, the cheeses entered hot-room ripening, resulting in >2.5 log increase for both control and adjunct cheeses to $\sim 10^8$ cfu·g⁻¹ by d 56 ($P < 0.0001$). Co-inoculation with *Lb. mucosae* DPC 6426 had no significant effect on the viability of PAB at any time during ripening.

NSLAB. Nonstarter lactic acid bacteria were present in all cheeses ($<10^4$ cfu·g⁻¹) at d 7 and increased significantly over the ripening period to $\sim 5 \times 10^8$ cfu·g⁻¹ ($P < 0.0001$; Figure 1e). Although not significant, NSLAB counts increased more dramatically between d 21 and 56 in Swiss-type cheeses than Cheddar, most likely due to the aforementioned hot step period. The presence of *Lb. mucosae* DPC 6426 had no significant effect on NSLAB growth in comparison with control cheeses of both Swiss-type and Cheddar cheeses.

***Lactobacillus mucosae* DPC 6426.** After inoculation into cheese milk, mean viable counts of *Lb. mucosae* DPC 6426 were 10^6 cfu·mL⁻¹ of cheese milk, with a subsequent 10-fold concentration in curd formation. During ripening, mean viable counts of the strain increased by a further log in Cheddar ($P < 0.05$) and remained viable at these levels after 6 mo of ripening at 8°C (Figure 2a). No significant change was found in mean viable counts of *Lb. mucosae* DPC 6426 in Swiss-type cheese during the entire ripening period. The milk fermentation and acidification potential of the adjunct strain, as assessed by iCinac (Unity Scientific, Brookfield, CT), was weak (data not shown), suggesting that *Lb. mucosae* DPC 6426 cannot grow well with lactose as the primary carbon source and may also have poor proteinase activity. This is in agreement with a previous *Lb. mucosae* DPC 6426 yogurt study, in which the strain was inoculated to milk at 4×10^8 cfu·mL⁻¹ and demonstrated no significant increase in mean viability over the 28-d 4°C storage period (London et al., 2015a).

Overall, Cheddar rather than Swiss-type cheeses appeared a marginally more suitable matrix for *Lb. mucosae* DPC 6426 as an adjunct culture. Whereas *L. lactis* growth was the most substantial of all primary and secondary starter bacteria, the starter has a reasonably narrow spectrum of metabolism (McSweeney and Sousa, 2000). In addition, galactose is known to be expelled during *L. lactis* lactose fermentation, left to be metabolized by the *L. lactis* population when lactose is depleted (Benthin et al., 1994). This may have given *Lb. mucosae* DPC 6426 a period in the first 21 d of Cheddar ripening where galactose was available, allowing modest growth. Conversely, Swiss-type cheese, which was manufactured with *St. thermophilus*, *Lb. helveticus*, and PAB, has a far more diverse microbial metabolic profile (Hutkins and Morris, 1987; Piveteau, 1999), indicat-

Figure 2. Adjunct survival counts. (A) DPC 6426 survival during ripening in Swiss-type (squares) and Cheddar (circles) cheese: shaded area represents counts during manufacture. (B) DPC 6426 survival in cheese whey during processing: shaded area represents counts in cheese milk.

ing competition for a more diverse selection of carbon sources. Finally, the high cooking temperature involved in Swiss-type cheese manufacture may have been responsible for injury and ultimately reducing viability of *Lb. mucosae* DPC 6426 (Sheehan et al., 2007a).

The influence of whey processing, including clarification, pasteurization, evaporation, and drying, on mean viable counts of *Lb. mucosae* DPC 6426 was evaluated (Figure 2b). As has previously been reported for cheese

starter bacteria (Jeanson et al., 2011; Doolan et al., 2014), less than 5% of the viable *Lb. mucosae* DPC 6426 found in the cheese milk partitioned with the whey. It has previously been hypothesized that this is due to an affinity that bacteria show for milk fat globule and casein-fat interface (Laloy et al., 1996). Clarification of the whey is a centrifugal process in which much of the remaining milk fat globules will be drawn out (Byrne et al., 2002). As a result, this step incurred a log depletion of mean viable *Lb. mucosae* DPC 6426 counts. The adjunct culture subsequently proved unable to survive pasteurization at any detectable levels.

Cheese Composition

Cheese composition at d 7 of ripening is shown in Table 1. Full-fat Swiss-type and Cheddar cheese generally contain 32 and 30% fat, respectively. Swiss-type control and adjunct cheeses in the present study both achieved a 55% reduction in fat content compared with standard Swiss-type cheeses (Sheehan et al., 2008). Both control and adjunct-containing Cheddar cheese fat content were found to be 44% reduced from their full-fat counterparts (Chen et al., 1998). Fat content was dependent on cheese type, with Cheddar containing significantly more fat than Swiss-type ($P < 0.0001$). Protein content was similar to what has been previously reported for Swiss-type and Cheddar cheeses (Fenelon and Guinee, 2000; Costa et al., 2010). The presence of *Lb. mucosae* DPC 6426 had no significant effect on the protein or fat content of either cheese type.

Although both of the cheeses containing *Lb. mucosae* DPC 6426 appeared to retain a slightly higher moisture content than control cheeses manufactured without the

adjunct culture, our results indicate this was not statistically significant; despite the ability of the adjunct to produce up to ~0.25 g of EPS per liter of yogurt (London et al., 2015a). However, this was not an entirely unexpected outcome. Previous research regarding the EPS-producing potential of *Lb. mucosae* DPC 6426 has indicated a significantly reduced EPS production efficacy when grown in the presence of lactose as the carbon source, compared with sucrose or glucose (London et al., 2011). The aforementioned yogurt was manufactured with 5% (wt/vol) sucrose in reconstituted skim milk, which was not possible in cheese manufacture, as this addition would greatly affect starter and NSLAB growth and metabolism.

The salt content and salt-in-moisture levels of reduced Cheddar and Swiss-type cheeses were similar to those reported previously (Sheehan et al., 2008; Costa et al., 2010), with Cheddar cheeses containing significantly more salt than Swiss-type cheeses ($P < 0.01$). The presence of *Lb. mucosae* DPC 6426 did not significantly influence salt content or salt-in-moisture levels.

Lactobacillus mucosae DPC 6426 had no effect on the pH profiles of either cheese type both at d 7 (Table 1) and during ripening (Figure 3). The pH for both control and adjunct Cheddar cheeses did not change significantly over the entire ripening period, whereas pH of both control and adjunct Swiss-type cheeses rose significantly ($P < 0.0001$). A positive correlation between pH and ripening time was observed for both adjunct-containing ($R^2 = 0.99$; $P < 0.0001$) and control ($R^2 = 0.98$; $P < 0.0001$) Swiss-type cheeses. This linear increase in pH is due to the metabolism of L-lactate by the growing PAB population producing propionate, acetate, and CO₂ (McSweeney and Sousa, 2000).

Table 1. Mean composition of Swiss-type and Cheddar cheeses

Composition ¹	Swiss-type		Cheddar	
	Control	Adjunct	Control	Adjunct
Fat (% wt/wt)	13.64 ^a	13.51 ^a	18.23 ^b	18.05 ^b
Moisture (% wt/wt)	39.31 ^a	39.51 ^a	39.57 ^a	40.31 ^a
FDM (% wt/wt)	24.59 ^a	24.37 ^a	32.16 ^b	31.70 ^b
MNFS (% wt/wt)	51.05 ^a	51.40 ^a	52.89 ^a	53.32 ^a
Protein (% wt/wt)	35.66 ^a	35.38 ^a	33.43 ^a	33.62 ^a
Salt (% wt/wt)	1.13 ^a	1.24 ^a	1.69 ^b	1.70 ^b
S/M (% wt/wt)	2.57 ^a	2.78 ^a	3.92 ^b	4.10 ^b
pH at d 20	5.56 ^a	5.52 ^a	5.24 ^b	5.23 ^b
Y _a (%)	6.147 ^a	6.057 ^a	7.800 ^a	7.857 ^a
Y _{macy} (%)	5.593 ^a	5.467 ^a	7.200 ^b	7.173 ^b
Y _{macy} pfam (%)	9.187 ^a	8.930 ^a	10.60 ^a	10.55 ^a
Y _{afam} (%)	10.10 ^a	9.897 ^a	11.48 ^a	11.55 ^a

^{a,b}Means in a row with common superscripts do not differ ($P \geq 0.05$).

¹FDM = fat dry mass; MNFS = moisture in the nonfat substance; S/M = salt in moisture; Y_a = gross yield; Y_{macy} = moisture adjusted. Y_{macy}pfam = moisture-adjusted cheese yield adjusted for protein and fat; Y_{afam} = cheese yield adjusted for protein and fat. Data displayed are the means of triplicate trials.

Figure 3. Mean pH profile of Cheddar control (black circles and solid line) and adjunct (gray squares and solid line) and Swiss-type control (black circles and dashed line) and adjunct (gray squares and dashed line) cheeses over 182 d of ripening.

Proteolysis

% pH 4.6-SN/TN. Overall, the addition of *Lb. mucosae* DPC 6426 had no significant effect on levels of pH 4.6-SN/TN during ripening. A significant increase ($P < 0.0001$) in % pH 4.6-SN/TN, which is indicative of primary proteolysis, was recorded for all cheeses over the ripening period (Figure 4). All cheeses finished ripening with between 20 and 25% pH 4.6-SN/TN, which is comparable with previously reports for such cheeses (Sheehan et al., 2008; Costa et al., 2010).

Figure 4. Ripening index. Percentage pH 4.6 soluble nitrogen in Cheddar control (black circles and solid line) and adjunct (gray squares and solid line) and Swiss-type control (black circles and dashed line) and adjunct (gray squares and dashed line). Expressed as a percentage of total nitrogen.

Total FAA. A significant increase occurred in total FAA for both Swiss-type ($P < 0.0001$; Figure 5a) and Cheddar ($P < 0.001$) cheeses during 6 mo of ripening from baseline. Total FAA levels in the ripened cheeses were comparable with previously reported values for each cheese type (Lawlor et al., 2002; Costa et al., 2010). Swiss-type cheeses were produced with *Lb. helveticus*, a species known to harbor FAA-liberating intracellular peptidases, likely to account for the significant differences in total FAA between the 2 cheese types (P

Figure 5. (A) Sum of total free AA in Cheddar control (black circles and solid lines) and adjunct (gray squares and solid lines) and Swiss-type control (black circles and dashed line) and adjunct (gray squares and dashed lines). Expressed as micrograms of AA per gram of cheese. (B) Free AA profile of Swiss-type control (black) and adjunct (gray) at 6 mo ripening. Expressed as microgram of AA per gram of cheese. (C) Free AA profile of Cheddar control (black) and adjunct (gray) at 6 mo ripening. Expressed as micrograms of AA per gram of cheese.

< 0.0001 ; Kenny et al., 2006). However, no significant differences were observed resulting from the addition of *Lb. mucosae* DPC 6426.

Individual FAA. The draft genome of *Lb. mucosae* DPC 6426 implies several predicted peptidases, including putative methionine aminopeptidases and proline iminopeptidases (Ryan et al., 2015a). However, this was not reflected in the FAA profile, with no significant increases in methionine or proline at any time point in either adjunct-containing cheese types (Figures 5b and 5c). Leucine, an AA associated with bitter taste, was previously found to be increased in cheeses manufactured with an EPS-producing adjunct (Awad et al., 2005); however, this was not noted in either adjunct containing Cheddar or Swiss-type cheeses. Arginine was found to be higher in the control Swiss-type cheese at 1 mo ($P < 0.05$; data not shown). By mo 3, this difference had attenuated as arginine was almost entirely depleted in both cheeses, a process thought to be caused by NSLAB metabolism of the AA into NH_3 , ornithine, and citrulline (Laht et al., 2002). At mo 3, lysine was increased in the adjunct Swiss-type cheese compared with the control ($P < 0.05$; data not shown). No significant differences were recorded between the Swiss-type cheeses at 6 mo (Figure 5b) or Cheddar cheeses at any time point ($P > 0.05$; Figure 5c).

CLSM

Although previous studies using CLSM have shown the presence of polysaccharide (EPS) in the crevices within the cheese protein matrix (Hassan et al., 2002), no significant accumulation was observed in this study (Figure 6). This is in agreement with the similarities found in moisture content of control and adjunct-containing cheese, and indicates that *Lb. mucosae* DPC 6426 did not produce significant amounts of EPS within the cheese matrix in the presence of lactose. It is hypothesized that the cholesterol-reducing effects of *Lb. mucosae* DPC 6426 observed by London et al. (2015b) were at least in part due to EPS production. However, it should be noted that in this animal study the probiotic underwent several washing steps to eliminate non-microbial nutrients, a process that most likely removed much of the loosely associated EPS. Taken together, this suggests that much of the beneficial effects observed were the result of EPS or metabolites produced by the strain in vivo, in its native GIT ecosystem.

Texture Profile

Mean levels of hardness and chewiness decreased in both Cheddar control and adjunct-containing cheeses

over the ripening period ($P < 0.0001$; Figure 7a), as did cheese springiness ($P < 0.0001$; Figure 7b). However, no significant differences were observed between control Cheddar cheeses and those containing *Lb. mucosae* DPC 6426 in texture parameters at any time during ripening.

The general trend toward reduction in all TPA metrics during ripening may be due to the proteolytic breakdown of casein by remaining chymosin and the enzymes of starter, nonstarter, and adjunct LAB (Sousa et al., 2001; O'Mahony et al., 2005; Sheehan et al., 2007b). Reducing the fat content of cheeses invariably leads to an inherently harder and chewier product (Agrawal and Hassan, 2008; Lteif et al., 2009), which is the result of the tighter casein calcium-phosphate network which is formed (Mistry, 2001). The addition of EPS-producing adjunct cultures during cheese manufacture has previously been reported as a successful strategy in reducing this undesirable attribute (Awad et al., 2005; Dabour et al., 2006; Costa et al., 2010). Although moisture retention, as a result of an increase in cheese structure openness, has previously been implicated as the mechanism through which EPS-producing strains reduce hardness and chewiness (Dabour et al., 2006), no significant effect on moisture content was recorded for adjunct-containing cheeses in this study. The EPS cultures harnessed in previous research were of dairy origin and were solely intended to improve cheese quality; however, heart health promoting *Lb. mucosae* DPC 6426 is native to the mammalian GIT and, as a result, does not favor lactose as a substrate for EPS production.

Cheese Yields

Cheddar Y_a , Y_{macypfam} , and Y_{afpam} were all found to be comparable or slightly higher than what has previously been reported for reduced-fat Cheddars (Costa et al., 2010). However, no Cheddar cheese yield metric was significantly altered by the presence of *Lb. mucosae* DPC 6426. The Y_{macy} of Swiss-type cheeses were significantly higher than that of Cheddar cheeses. Nonetheless, inoculation with *Lb. mucosae* DPC 6426 also had no effect on any Swiss-type cheese yield parameter.

Sensory Analysis

Cheddar. Hedonic sensory analysis of Cheddar cheeses revealed that control cheeses scored significantly higher for liking of aroma ($P < 0.05$; Figure 8a). However, ranking descriptive analysis attributes were not significantly different for any of the attributes assessed (data not shown).

Swiss-Type. Figure 8b displays Swiss-type hedonic analysis, in which the control cheese was scored significantly higher for overall acceptability ($P < 0.05$), although the magnitude of the difference was small. However, the *Lb. mucosae* DPC 6426 containing cheese was found to have a significantly more appealing texture ($P < 0.05$). Descriptive analysis (Table 2) revealed that the adjunct Swiss-type cheese scored significantly ($P < 0.05$) higher than control for the bitter taste attribute, whereas no other descriptors were significantly different. However, as previously discussed, no individual AA associated with a bitter flavor (i.e., phenylalanine, tyrosine, and leucine) were significantly higher in either of the cheese types containing *Lb. mucosae* DPC 6426. Appearance ranking descriptive data revealed that the

adjunct-containing Swiss-type cheese was rated higher than control for eye quality and also eye distribution ($P < 0.05$; Figure 8c). *Lactobacillus mucosae* are known obligate heterofermentative lactobacilli and, as such, can have an effect on eye formation through gas production (Marth and Steele, 2001; Quiberoni et al., 2008). None of the other descriptors were significantly different.

CONCLUSIONS

Two types of reduced-fat, semi-hard cheeses were manufactured in triplicate with and without the addition of an EPS-producing adjunct strain with beneficial heart health attributes, *Lb. mucosae* DPC 6426, and monitored for culture viability and product quality over

Figure 6. Confocal laser scanning microscopy. Adjunct Cheddar protein matrix stained with Fast Green and polysaccharide-staining Alexa Fluor 555, demonstrating lack of EPS in matrix pores or surrounding live (green) and dead (red) stained bacteria. Scale bar is 7.5 μm .

Figure 7. Cheddar texture profile. (A) Hardness of control (black circles and dashed line) and adjunct (gray squares and dashed line) and chewiness of control (black circles and solid line) and adjunct (gray squares and solid line) Cheddars over ripening period. (B) Cheddar control (black circles and dashed line) and adjunct (gray squares and dashed line) springiness over ripening period.

a 6-mo ripening period. Although *Lb. mucosae* DPC 6426 remained viable at high levels ($>5 \times 10^7$ cfu·g⁻¹) over the 6-mo ripening period without imparting any negative attributes, the presence of the strain was not associated with any effect on sensory or textural quality of the cheeses. Whereas the beneficial heart health effects of *Lb. mucosae* DPC 6426 have been at least partly attributed to the EPS production ability of the strain, previous research implicates in vivo EPS production for efficacy. We can infer from this that, although the adjunct culture had little or no effect on cheese texture and composition, the probiotic cheese may still confer a heart health effect to the host. This study demonstrates that both reduced-fat Swiss-type and Cheddar cheeses are suitable functional food matrices for delivery of cholesterol-lowering *Lb. mucosae* DPC 6426.

Figure 8. (A) Hedonic sensory analysis of control (black) and adjunct (gray) Cheddars. (B) Hedonic sensory analysis of control (black) and adjunct (gray) Swiss types. (C) Appearance analysis of control (black) and adjunct (gray) Swiss types.

Table 2. Descriptive flavor of reduced-fat Swiss-type cheeses at 6 mo of ripening

Item	Control	Adjunct
Sweaty/sour	4.49 ^a	4.91 ^a
Dairy sweet	4.44 ^a	4.10 ^a
Nutty aroma	3.10 ^a	2.81 ^a
Pungent	4.02 ^a	3.68 ^a
Off	2.12 ^a	2.39 ^a
Diacetyl	3.90 ^a	3.61 ^a
Fruity estery	3.24 ^a	3.24 ^a
Creamy	2.84 ^a	3.22 ^a
Sweet taste	3.81 ^a	3.66 ^a
Salt	2.47 ^a	2.75 ^a
Sour taste	3.42 ^a	3.74 ^a
Bitter taste	2.61 ^a	3.26 ^b
Firmness in mouth	3.99 ^a	4.02 ^a
Rubbery texture	2.02 ^a	2.08 ^a
Smooth texture	1.06 ^a	0.93 ^a
Oily texture	1.94 ^a	1.94 ^a
Sticky texture	1.17 ^a	1.17 ^a
Nutty flavor	3.57 ^a	3.13 ^a
Off flavor	2.67 ^a	2.77 ^a
Dairy sweet flavor	3.82 ^a	3.87 ^a
Diacetyl flavor	3.73 ^a	4.02 ^a
Buttery flavor	3.45 ^a	3.31 ^a
Fruity estery flavor	3.17 ^a	3.31 ^a

^{a,b}Means in a row with common superscripts do not differ ($P \geq 0.05$).

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APPENDIX

Table A1. Sensory terms for the assessor evaluation of Swiss-type cheese

Attribute	Definition	Scale
Hedonic		
Appearance-liking	The liking of appearance	0 = extremely dislike; 10 = extremely like
Flavor-liking	The liking of flavor	0 = extremely dislike; 10 = extremely like
Aroma-liking	The liking of aroma	0 = extremely dislike; 10 = extremely like
Texture-liking	The liking of texture	0 = extremely dislike; 10 = extremely like
Overall acceptability	The acceptability of the product	0 = extremely unacceptable; 10 = extremely acceptable
Descriptive		
Sweaty/sour aroma	The aromatics reminiscent of perspiration, foot odor. Sour, stale, slightly cheesy, moist, stained, or odorous with sweat	0 = none, 10 = extreme
Dairy sweet aroma	Dairy sweet aroma intensity. The aromatics associated with sweetened cultured dairy products such as fruit yogurt	0 = none, 10 = extreme
Nutty aroma	Nutty aroma intensity—The nonspecific nut aromatic that is characteristic of different nuts (e.g., peanuts, hazelnuts, pecans)	0 = none, 10 = extreme
Pungent aroma	Physically penetrating sensation in the nasal cavity. Sharp smelling or tasting, irritant	0 = none, 10 = extreme
Off aroma	Off aroma (rancid)	0 = none, 10 = extreme
Diacetyl aroma	Diacetyl aroma-buttery, butterscotch, popcorn	0 = none, 10 = extreme
Fruity estery aroma	Fruity estery aroma-short-chain fatty acid esters	0 = none, 10 = extreme
Creamy aroma	The smell associated with creamy/milky products	0 = none, 10 = extreme
Sweet taste	Fundamental taste sensation of which sucrose is typical	0 = none, 10 = extreme
Salt taste	Fundamental taste sensation of which sodium chloride is typical	0 = none, 10 = extreme
Sour taste	Fundamental taste sensation of which lactic acid is typical	0 = none, 10 = extreme
Bitter taste	Fundamental taste sensation of which caffeine or quinine in soda water is typical	0 = none, 10 = extreme
Firmness in the mouth	Firm texture in the mouth	0 = none, 10 = extreme
Rubbery texture	Rubbery texture in the mouth	0 = none, 10 = extreme
Smooth texture	Smooth, silky texture	0 = none, 10 = extreme
Oily texture (greasy)	Oily texture (greasy)	0 = none, 10 = extreme
Sticky texture	Sticky texture	0 = none, 10 = extreme
Nutty flavor	The nonspecific nut flavor that is characteristic of several different nuts (e.g., peanuts, hazelnuts, pecans)	0 = none, 10 = extreme
Off-flavor	Off-flavor (rancid)	0 = none, 10 = extreme
Dairy sweet flavor	The flavors associated with sweetened cultured dairy products such as fruit yogurt	0 = none, 10 = extreme
Diacetyl flavor	Diacetyl flavor-buttery, butterscotch, popcorn	0 = none, 10 = extreme
Buttery flavor	Of the nature of, or containing butter	0 = none, 10 = extreme
Fruity estery flavor	Fruity estery flavor-short-chain fatty acid esters	0 = none, 10 = extreme
Appearance-color	Appearance-ivory to yellow color	0 = Ivory 10 = Yellow
Smooth/shiny appearance	Smooth/shiny appearance	0 = none, 10 = extreme
Eye quality	Regular shaped eyes	0 = none, 10 = extreme
Eye distribution	Eye distribution	0 = none, 10 = extreme
Irregular eyes	Irregular shaped eyes	0 = none, 10 = extreme
Defects	Slits/cracks	0 = none, 10 = extreme
Rind	Visible rind layer	0 = none, 10 = extreme