J. Dairy Sci. 99:2625–2640
http://dx.doi.org/10.3168/jds.2015-10503

Compromised *Lactobacillus helveticus* starter activity in the presence of facultative heterofermentative *Lactobacillus casei* DPC6987 results in atypical eye formation in Swiss-type cheese

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ABSTRACT

Nonstarter lactic acid bacteria are commonly implicated in undesirable gas formation in several varieties, including Cheddar, Dutch-, and Swiss-type cheeses, primarily due to their ability to ferment a wide variety of substrates. This effect can be magnified due to factors that detrimentally affect the composition or activity of starter bacteria, resulting in the presence of greater than normal amounts of fermentable carbohydrates and citrate. The objective of this study was to determine the potential for a facultatively heterofermentative *Lactobacillus* (*Lactobacillus casei* DPC6987) isolated from a cheese plant environment to promote gas defects in the event of compromised starter activity. A Swiss-type cheese was manufactured, at pilot scale and in triplicate, containing a typical starter culture (*Streptococcus thermophilus* and *Lactobacillus helveticus*) together with propionic acid bacteria. *Lactobacillus helveticus* populations were omitted in certain vats to mimic starter failure. *Lactobacillus casei* DPC6987 was added to each experimental vat at 4 log cfu/g. Cheese compositional analysis and X-ray computed tomography revealed that the failure of starter bacteria, in this case *L. helveticus*, coupled with the presence of a faculatively heterofermentative *Lactobacillus* (*L. casei*) led to excessive eye formation during ripening. The availability of excess amounts of lactose, galactose, and citrate during the initial ripening stages likely provided the heterofermentative *L. casei* with sufficient substrates for gas formation. The accrual of these fermentable substrates was notable in cheeses lacking the *L. helveticus* starter population. The results of this study are commercially relevant, as they demonstrate the importance of viability of starter populations and the control of specific nonstarter lactic acid bacteria to ensure appropriate eye formation in Swiss-type cheese.
Key words: Swiss-type cheese, gas defects, heterofermentative lactobacilli, X-ray computed tomography

INTRODUCTION

Swiss- and Dutch-type cheeses are hard or semihard brine-salted cheeses, containing characteristic eyes resulting from the metabolism of various substrates (Reinbold, 1972; van den Berg et al., 2004; Fröhlich-Wyder and Bachmann, 2007). With respect to Swiss-type cheeses, propionic acid fermentation, due to the presence of environmental or, more typically, deliberate inoculation of propionic acid bacteria (**PAB**), results in the production of propionate and acetate, which contribute to the characteristic nutty flavor, and CO_2, which is responsible for eye formation (Kerjean et al., 2000; Daly et al., 2010). Carbon dioxide production, via lactate metabolism, typically occurs during the hot room (20–23°C) phase of ripening, when the cheese curd is sufficiently elastic to accommodate stretching (Guggisberg et al., 2015). Contrastingly, in Dutch-type cheese, eye formation is primarily due to citrate metabolism by mesophilic lactic acid bacteria (**LAB**; Walstra et al., 1993; Smit et al., 2005).

Factors essential for desirable eye formation, in both Dutch- and Swiss-type cheese, include sufficient quantities of gas-producing microbiota, the presence of fermentable substrates, favorable environmental conditions (pH, salt in moisture, temperature), the presence of nuclei, and a suitably elastic cheese texture (McSweeney, 2007; Porcellato et al., 2015). Regular eye formation is dependent on the amount of CO_2 produced and its diffusion throughout the cheese matrix, which in turn depends on the solubility and pressure of the gas (solubility is temperature- and pH-dependent) within the cheese (Fröhlich-Wyder and Bachmann, 2007; Daly et al., 2010).

Received October 9, 2015.
Accepted December 5, 2015.
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Undesirable or overproduction of gas in brine-salted cheeses can manifest as splits, cracks, secondary fermentations, or excessive eye formation within the cheese. This generally results in downgrading or rejection of the product (Daly et al., 2010; Sheehan, 2011). The extent to which brine-salted cheese suffers from excessive gas production depends on the gas type (CO_2 or H_2), the amount and solubility of the gas produced, the texture of the cheese, and the ripening temperatures employed. Gas formation can be further subdivided depending on the stage of ripening that it occurs [i.e., early gas production (24–48 h) or late gas (later-stage ripening; Sheehan, 2011)]. The presence of coliforms, yeast, and citrate-positive starter bacteria are common causes of early gas defects, primarily due to the lactose metabolism (O'Sullivan et al., 2013). For late gas formation, butyric acid bacteria, such as *Clostridium* spp., are of particular concern because of their ability to produce H_2 or CO_2, which is poorly soluble in the cheese matrix. Adventitious streptococci, in particular CO_2-producing, heat-resistant strains that survive pasteurization and colonize heat exchangers, can also contribute to openness defects in several cheese varieties (Sheehan, 2011).

Lactobacilli and PAB are of particular interest as culprits of gas defects in Swiss-type cheese. Nonstarter lactic acid bacteria (**NSLAB**), including obligately and facultatively heterofermentative lactobacilli (**FHLb**), although recognized as contributors to ripening and flavor development, are commonly implicated in undesirable gas formation in several varieties, including Cheddar, Dutch-, and Swiss-type cheeses (Fröhlich-Wyder et al., 2013; O'Sullivan et al., 2013; Ortakci et al., 2015; Porcellato et al., 2015). The NSLAB populations contaminate cheese via survival of pasteurization or through manufacturing equipment or personnel, and by the end of ripening are the dominant microbiota present in the cheese matrix (Martley and Crow, 1993; Beresford et al., 2001). Salt-tolerant obligately and facultatively heterofermentative lactobacilli, such as those contaminating brine tanks, are further sources of adventitious NSLAB capable of CO_2 formation from substrates present late in ripening, such as AA (van den Berg et al., 2004; Sheehan, 2011). Of NSLAB populations, FHLb are commonly encountered in Dutch- and Swiss-type cheeses and include *Lactobacillus casei*, *Lactobacillus curvatus*, and *Lactobacillus plantarum*. These lactobacilli occur at high numbers ($>10^7$ cfu/g) during cheese ripening (Mullan, 2000; Beresford et al., 2001; Porcellato et al., 2015). Carbohydrates, particularly lactose and galactose, as well as lactate, citrate, and urea have all been proposed as potential substrates used by these microbes for gas formation (Martley and Crow, 1993; Mullan, 2000). Lactose is usually rapidly metabolized by starter bacteria at the start of ripening, liberating glucose and galactose, which together with lactose can provide the carbohydrate source for the growth of gas-producing FHLb (McSweeney, 2004). For this reason *Lactobacillus helveticus* is frequently added with *Streptococcus thermophilus* as a mixed starter to metabolize residual carbohydrates and thereby prevent the growth of undesirable gas-producing microbes (Beresford et al., 2001). Factors such as bacteriophage activity, inadequate starter storage, or elevated salt concentrations may, however, affect the composition or activity of starter bacteria, resulting in the presence of greater than normal amounts of fermentable carbohydrates (Mullan, 2000; Porcellato et al., 2015; Spus et al., 2015). In addition to carbohydrates, citrate can also be metabolized by various FHLb to produce gas (Mullan, 2000; Gagnaire et al., 2001, Adamberg et al., 2005).

Excessive propionic acid fermentation, either during the hot room stage or near the end of ripening, may also result in secondary or late fermentation defects, particularly in Swiss-type cheeses (Fröhlich-Wyder and Bachmann, 2007). The PAB species with high aspartase activity are capable of producing more CO_2 per mole of lactate than those with lower activity (Wyder et al., 2001; Daly et al., 2010). Certain PAB are also capable of growth at low temperatures allowing for further gas production during the later phase (6–8°C) of ripening (Hettinga et al., 1974). Evidence of an interactive effect between LAB, thermophilic LAB in particular, and PAB also exists. Prior studies, using various experimental conditions, have examined the stimulatory effect of various LAB on the growth and metabolism of PAB strains (Piveteau et al., 1995; Baer and Ryba, 1999; Thierry et al., 1999; White et al., 2003).

The size, shape, and distribution of eyes within the cheese matrix is of key importance (Caccamo et al., 2004; Lee et al., 2012; Guggisberg et al., 2015). Assessment of eye formation in Swiss-type cheese is generally done by experienced cheese graders and involves a visual examination of the cheese using a cheese trier, tapping of the cheese surface for a hollow sound, or by cutting the cheese into sections for visual examination. These methods are subjective or involve destructive sampling of the cheese and are often not indicative of eye formation throughout the entire block (Lee et al., 2012; Guggisberg et al., 2015). Noninvasive or nondestructive imaging technology, relying on methods such as ultrasound, magnetic resonance imaging, X-ray, and X-ray computed tomography (**CT**) have recently been applied to profile eye formation in Swiss-type cheeses (Guggisberg et al., 2015). A prior study to determine the quantitative power of CT led to cheese manufacture using hollow balls to represent artificial eyes. In

this study, an accurate correlation between actual and determined volume via CT analysis was observed (Guggisberg et al., 2013).

The objective of this study was to determine the potential for a facultatively heterofermentative *L. casei* isolated from a cheese plant environment to promote gas defects in the event of compromised starter activity. The combined effect of *L. casei* and PAB populations on the pattern of openness in the cheeses was also investigated. X-ray CT was employed as a nondestructive method of imaging defective gas formation.

MATERIALS AND METHODS

Starter Cultures

A mixed culture of *S. thermophilus* (DPC6986) was selected from the Teagasc Moorepark (Fermoy, Co. Cork, Ireland) culture collection for the purpose of this study. The DPC6986 culture was grown on heat-treated 10% reconstituted skim milk (100°C for 90 min) and incubated at 42°C until a pH of 4.5 was reached, before inoculation into cheese milk. *Lactobacillus helveticus* DPC6865 was sourced from the culture collection of Teagasc Moorepark and grown on heat-treated 10% reconstituted skim milk at 42°C until a pH of 5.1 was reached, before inoculation into cheese milk. *Propionibacterium freudenreichii* DPC6451, from the Teagasc Moorepark culture collection, was grown in sodium lactate broth [1 L containing 10 g of tryptone (Oxoid, Hampshire, UK), 10 g of yeast extract (Merck, Cork, Ireland), 5 g of KH_2PO_4 (VWR, Dublin, Ireland), 18.9 g of 50% wt/wt sodium lactate solution (Merck), and 5 mL of NaOH (VWR)] for 7 d at 30°C under anaerobic conditions before inoculation into cheese milk. *Lactobacillus casei* DPC6987 was isolated using de Man, Rogosa, Sharpe (**MRS**; BD, Oxford, UK) supplemented with 6% NaCl from a cheese plant environment. Species verification was carried out via 16S rDNA sequencing before use. *Lactobacillus casei* DPC6987 was maintained on MRS agar. The DPC6987 cultures were grown in MRS broth and concentrated by centrifugation (4,000 × *g*, 20 min, 4°C) before cheese manufacture. Cell concentrations of 4 log cfu/g of cheese milk were selected to achieve 3.8 log cfu/g cheese at 1 d postproduction (Fröhlich-Wyder et al., 2002). *Lactobacillus casei* DPC6987 was also tested for carbohydrate utilization using the API CH50 kit (BioMerieux, Basingstoke, Hampshire, UK).

Cheese Manufacture

Experimental cheeses were produced in triplicate, over a 12-mo period, and corresponded to 4 treatment groups: control (**CTL**; containing *S. thermophilus*, *L. helveticus*, and *P. freudenreichii*), treatment 1 (**SPC**; without *L. helveticus*), treatment 2 (**SLC**; without *P. freudenreichii*), and treatment 3 (**SLPC**; containing *S. thermophilus*, *L. helveticus*, *L. casei*, and *P. freudenreichii*).

Raw milk was obtained from a local dairy farm and standardized to a protein-to-fat ratio of 1.01:1. Milk was held overnight at <10°C before being pasteurized at 72°C for 15 s and pumped into cylindrical, jacketed vats. Each vat contained automated variable speed cutting and stirring equipment (APV Schweig AG, Worb, Switzerland). Milk (454 kg/vat) was inoculated, as per experimental protocols (Table 1), with 500 mL of *S. thermophilus*, 25 mL of *L. helveticus*, 4 mL of *P. freudenreichii*, and 4 log cfu/g of *L. casei* where indicated. A calcium chloride (34% wt/vol) solution was added at 100 mL/454 kg of cheese milk to each respective vat. Rennet (Thermolase from *Cryphonectria parasitica*, Chr. Hansen Ltd., Hoersholm, Denmark) was added at 16.85 mL (diluted in 2 L of water) per 454 kg of milk after a 40-min ripening period at 30°C. Coagulation was achieved over 30 min before a 5-min cut program producing a curd size of approximately 5 mm^2. The curd and whey mixture was then allowed to heal for 5 min before stirring and cooking at a rate of 1°C/3 min from 31 to 33°C and at 2°C/3 min from 33°C to a maximum scald of 50°C. After cooking, curds were prepressed under whey with the resultant curds placed in 10-kg molds. The molded cheeses were then pressed under increasing pressure to 19.2 to 28.8 kPa. Cheeses were held under pressure until a pH of 5.3 was reached before being transferred to a saturated brine solution (23% wt/wt NaCl, 0.56% $CaCl_2$, pH 5.2, and 18°C) for 24 h. After brining, cheese was dried at room temperature for 4 h before being vacuum packed in CO_2-permeable bags and transferred to the ripening room

Cheese Ripening

Cheeses were ripened at 9 to 10°C for 10 d before being transferred to a hot room (22°C) for 35 d. Finally, cheeses were matured at 6°C for a further 50 d.

Enumeration of Starter, Nonstarter, Propionic Acid Bacteria and L. casei

Cheese was sampled aseptically using a cheese trier at 1, 10, 35, 45, and 95 d of ripening. The samples were placed in a sterile stomacher bag, diluted 1:10 with sterile 2% trisodium citrate buffer (VWR) and homogenized using a stomacher (Iul Instruments, Barcelona, Spain) for 10 min. Independent duplicate samples were taken at each time point and dilutions were prepared as required. Viable *S. thermophilus* cells were enumer-

Table 1. Description of the treatments, starter cultures, and ripening regimens used in the study[1]

Treatment	CTL cheese	SPC cheese	SLC cheese	SLPC cheese
Milk volume	454 kg	454 kg	454 kg	454 kg
Starter cultures	*Streptococcus thermophilus*	*S. thermophilus*	*S. thermophilus*	*S. thermophilus*
	Lactobacillus helveticus	—	*L. helveticus*	*L. helveticus*
	Propionibacterium freudenreichii	*P. freudenreichii*	—	*P. freudenreichii*
	—	*Lactobacillus casei*, 10^4 cfu/g	*L. casei*, 10^4 cfu/g	*L. casei*, 10^4 cfu/g
Manufacturing method	Rindless Swiss-type	Rindless Swiss-type	Rindless Swiss-type	Rindless Swiss-type
Ripening regimen	10°C × 10 d	10°C × 10 d	10°C × 10 d	10°C × 10 d
	22°C × 35 d	22°C × 35 d	22°C × 35 d	22°C × 35 d
	6°C × 45 d	6°C × 45 d	6°C × 45 d	6°C × 45 d

[1]CTL cheese: control cheese containing *S. thermophilus*, *L. helveticus* and *P. freudenreichii*; SPC cheese: contains *S. thermophilus, P. freudenreichii*, *L. casei*, and no *Lb. helveticus*; SLC cheese: contains no *P. freudenreichii* populations; SLPC cheese: contains *S. thermophilus*, *L. helveticus*, *P. freudenreichii*, and *L. casei*.

ated aerobically on Ellikers (BD) agar supplemented with 0.5% beef extract (BD) after 3 d of incubation at 42°C. *Lactobacillus helveticus* cells were enumerated anaerobically on MRS agar (BD) at pH 5.4 after 3 d at 45°C. *Lactobacillus casei* cells were plated on MRS medium supplemented with vancomycin (Sigma, Arklow, Ireland) as per Ong et al. (2006). Total NSLAB were enumerated anaerobically on *Lactobacillus* selective agar (BD) for 5 d at 30°C. Coliforms were plated on violet red bile agar (BD) at 30°C for 1 d. Propionic acid bacteria were enumerated on sodium lactate agar after 7 d incubation at 30°C (Drinan and Cogan, 1992).

Cheese Compositional and Biochemical Analysis

Cheese samples were taken at 1, 10, 35, 45, and 95 d of ripening and stored at −20°C for biochemical analysis. Fresh samples, at 10 d postmanufacture, were grated for salt, protein, moisture, and calcium as described by Sheehan et al. (2007a).

Primary proteolysis was determined using the macro-Kjeldahl method (IDF, 1993), and was expressed as a percentage of total N soluble at pH 4.6. Secondary proteolysis was determined by measuring the free amino acid (**FAA**) content of the pH 4.6-soluble extracts according to the methods described by Fenelon et al. (2000) and expressed as a percentage of total N. The FAA were separated using ion-exchange chromatography with postcolumn ninhydrin derivatization and colorimetric detection. Represented values are means of triplicate trials.

Citrate content of the cheeses was determined using an enzyme assay kit (Megazyme International, Wicklow, Ireland). Contents of D-, L-, and total lactic acid were also determined using enzymatic kits (Megazyme International). Samples were prepared for analysis as per the method described by Bouzas et al. (1993). Short-chain volatile acids (acetate, propionate, and *n*-butyrate) were determined using the ligand exchange, ion-exclusion HPLC method as described by Kilcawley et al. (2001).

X-ray CT Measurement and Image Analysis of CT Data

X-ray CT measurement of control and experimental cheeses was carried out at 95 d of ripening using a CT scanner [VTOMEX L 300 – Microfocus (300kV), General Electric Company, Wunstorf, Germany] with the following scan parameters: 255 kV, 180 μA, 105.5 μm (voxel resolution), and 10.5 mm slice thickness. Image analysis of CT data was carried out using the VG StudioMax 2.2 (Volume Graphics, Heidelberg, Germany), using the defect detection module and default parameters.

Statistical Analysis

Three replicate cheese trials were conducted in which the effects of 4 treatments were tested. A randomized complete block design incorporating the 4 treatments and 3 blocks (replicate trials) was used for data analysis. An ANOVA was carried out using a SAS protocol (version 9.3; SAS Institute Inc., Cary, NC) protocol. Tukey's multiple comparison test was used as described by Hou et al. (2012), and the level of significance was determined at $P < 0.05$.

A split-plot design was used to determine the effects of the experimental variations on response variables, including *L. helveticus* counts, *S. thermophilus* counts, pH 4.6-soluble N, total plus free AA, pH, L-, D-, and total lactate, citrate levels, and short-chain volatile acids. Analysis of variance was carried out using SAS (version 9.3) as per Hou et al. (2012).

RESULTS AND DISCUSSION

In our study a Swiss-type cheese was manufactured to investigate the potential for a facultatively heterofermentative *L. casei* to promote gas defects in the event of compromised starter activity. A description of treatments, cultures, and ripening regimens is present in Table 1.

Growth and Viability of Bacteria During Cheese Manufacture

Viable counts of *S. thermophilus* remained constant up to 10 d of ripening before decreasing significantly ($P < 0.0001$), to approximately 7.2 log cfu/g at d 95 (Figure 1A). No significant effect of treatment or interaction between treatment and time on *S. thermophilus* levels (Table 2) was observed. In addition, viable *S. thermophilus* numbers were similar to those encountered in Swiss-type cheeses manufactured using similar starter bacteria and ripening conditions (Sheehan et al., 2008).

Mean viable numbers of *L. helveticus*, enumerated on MRS pH 5.4 agar, were 6.3 log cfu/g after 1 d of ripening in the CTL as well as the SLC and SLPC cheeses (Figure 1B). As expected, no *L. helveticus* was detected in the SPC cheeses. A significant effect ($P < 0.05$) was observed with respect to both treatment and time over the 35 d monitored. Between d 10 and 35, viable counts of *L. helveticus* increased from 0 to 5.8 log cfu/g in the SPC cheese. This was unexpected and is most likely due to *L. casei* growth on MRS pH 5.4 agar, which is not solely selective for *L. helveticus* and can support the growth of *L. casei* (data not shown). Viable counts in the SLC and SLPC cheeses decreased to 2.3 and 2.2 log cfu/g, respectively, possibly indicating lysis of *L. helveticus*. Alternatively, prior studies have shown that *Lactobacillus delbrueckii*, often used as an alternative to *L. helveticus*, cell numbers decrease in the presence of FHLb adjuncts (Bouton et al., 2009). With respect to this, a similar effect may have affected *L. helveticus* populations. In the control cheeses, counts of 7.3 log cfu/g were observed at d 10 and decreased to 6.0 log cfu/g by d 35. Cell counts of *L. helveticus* were not enumerated beyond 35 d, as increased NSLAB numbers, *L. casei* in particular, precluded accurate counts on MRS pH 5.4 agar. Viable counts on MRS agar were lower than those previously encountered in Swiss-type cheese (Sheehan et al., 2008).

Mean viable counts of *P. freudenreichii* were 4.2 log cfu/g in the CTL, SPC, and SPLC cheeses after 1 d of ripening (Figure 1C). *Propionibacterium freudenreichii* populations increased significantly ($P < 0.0001$) during hot room ripening to reach 7.9 log cfu/g by d 35, and

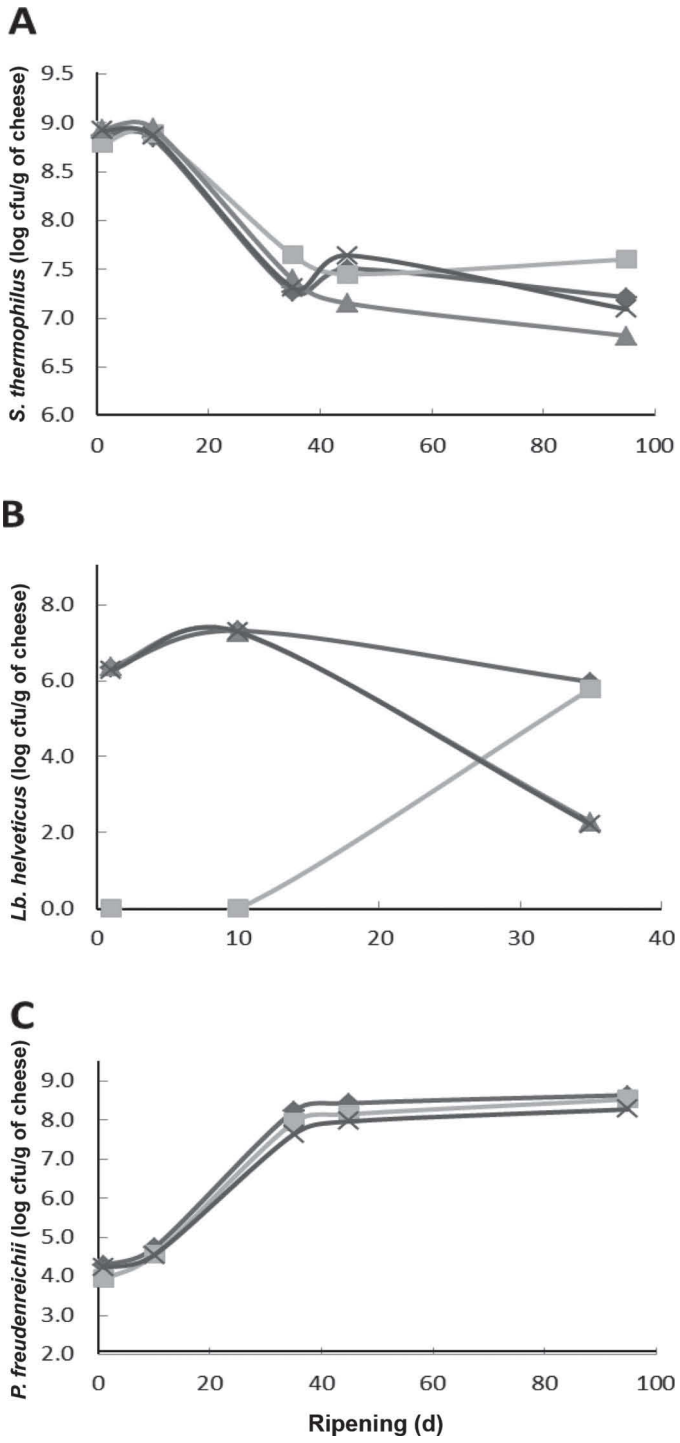

Figure 1. Effect of the respective treatments on mean viable counts of (A) *Streptococcus thermophilus*, (B) *Lactobacillus helveticus*, and (C) *Propionibacterium freudenreichii*, enumerated on Ellikers agar (BD, Oxford, UK), de Man, Rogosa, Sharpe agar pH 5.4 (BD), and sodium lactate agar, respectively. Cheeses included control (CTL, ◆; containing *S. thermophilus*, *L. helveticus*, and *P. freudenreichii*), cheese without *L. helveticus* (SPC, ■), cheese without *P. freudenreichii* (SLC, ▲), and cheese with *S. thermophilus*, *L. helveticus*, *P. freudenreichii*, and *Lactobacillus casei* (SLPC, ×). Values presented are means of 3 replicate trials.

Table 2. Statistical summary for the effect of respective treatment, time, and their interaction in a Swiss-type cheese[1]

Parameter	Treatment	Time	Interactive effect (treatment × time)
Streptococcus thermophilus	NS	***	NS
Lactobacillus helveticus	*	*	***
Propionic acid bacteria	***	***	***
Lactobacillus casei	***	***	***
NSLAB[2]	**	***	NS
pH	NS	**	NS
Lactose	*	***	*
Galactose	*	***	**
Citrate	***	***	***
Total lactate	*	*	NS
D-Lactate	*	***	*
L-Lactate	NS	**	NS
Propionate	NS	**	NS
Acetate	NS	***	NS
Butyrate	***	***	***
Total FAA[2]	**	***	NS
Individual FAA	*	***	**
%pH4.6SN/TN[2]	NS	***	NS

[1]Description of the various treatments given in Table 1.

[2]Nonstarter lactic acid bacteria (NSLAB), free amino acids (FAA), soluble nitrogen at pH 4.6 as a percentage of total nitrogen (SN/TN).

*$P < 0:05$, **$P < 0:01$, ***$P < 0:001$, NS = $P > 0.05$.

eventually to 8.5 log cfu/g by the end of ripening. As expected, *P. freudenreichii* was not detected throughout ripening in the SLC cheeses. The PAB growth was comparable to that seen in similar studies (Noel et al., 1999; Kocaoglu-Vurma et al., 2008; Sheehan et al., 2008). Although prior studies have reported that PAB growth is reduced by 0.4 to 1 log cycles in cases where adjunct cultures, such as *L. casei*, are added (Kocaoglu-Vurma et al., 2008), this effect was not observed in our study, as PAB growth was consistent in control, SPC, and SLPC cheeses.

Growth and Viability of L. casei and Total Lactobacilli During Cheese Manufacture

A citrate-positive strain of *L. casei* (DPC6987) was added to each treatment vat at approximately 4 log cfu/g. It was established, using a BioMerieux Api 50 CH kit, that the strain used in our study was capable of metabolizing a variety of carbohydrates, including lactose, galactose, glucose, fructose, mannose, and ribose (data not shown). As expected, *L. casei* was not detected in the CTL cheeses, at the early stages of ripening (1–10 d; Figure 2A). Mean viable numbers of *L. casei* increased significantly ($P < 0.0001$) in all cheeses during hot room ripening, eventually reaching levels of 8.6 log cfu/g in the SPC, SLC, and SLPC cheeses by d 95. Increased cell numbers observed during hot room ripening resembled that of total *Lactobacillus* counts. Mean levels of *L. casei* were significantly lower ($P < 0.0001$) in the CTL cheeses in comparison to the treatment cheeses in the initial stages of ripening (d 1–10), where *L. casei* was not detected. Levels of *L. casei* were consistently lower in the CTL cheeses, although not significantly for the remainder of ripening. This 1 to 2 log cfu/g difference between control and cheeses manufactured with a mesophilic adjunct has been observed in similar studies (Kocaoglu-Vurma et al., 2008). Viable cells were isolated in CTL cheeses at d 35 and eventually reached levels of 7.4 log cfu/g by the end of ripening. The detection of *L. casei* in the CTL cheese is likely to be as a result of environmental contamination. Furthermore, previous studies have indicated that some *L. casei* isolates show particular resistance to pasteurization temperatures (Beresford et al., 2001). Although not significantly so, *L. casei* cell numbers were observed to be consistently higher in the SPC cheeses compared to all other cheeses, possibly due to the presence of higher levels of lactose and galactose encountered in those cheeses at the early stages of ripening.

Mean NSLAB counts were similar in CTL, SPC, SLC, and SLPC cheeses at d 1 of ripening (5.8 log cfu/g; Figure 2B). Nonstarter lactic acid bacteria counts were higher than observed in similar studies (Kocaoglu-Vurma et al., 2008), which most likely reflects post-pasteurization contamination (i.e., from equipment or environment) or a result of failure of pasteurization to fully inactivate lactobacilli populations (Martley and Crow, 1993; Beresford et al., 2001; Ortakci et al., 2015). A significant ($P < 0.0001$) increase in viable counts was

evident throughout the ripening process and particularly when the cheeses were transferred to the hot-room. This effect was most obvious in cheeses with added *L. casei*. As NSLAB numbers are heavily influenced by temperature, significant increases in cell numbers would be expected to occur during hot room ripening, as previously described (Gilles et al., 1983; Fox et al., 1993). As expected, mean viable counts were consistently lower in the control throughout ripening than in cheeses to which *L. casei* was intentionally added. The highest viable counts were noted in the SPC cheeses, particularly at d 45 and 95 (8.8 log cfu/g at d 95), although they were not significantly different to those in other cheeses. Total lactobacilli counts were higher (~6 log cfu/g immediately after production) than encountered in similar studies (Swiss and semihard cheeses manufactured using thermophilic starters and PAB; Thierry et al., 1998; Sheehan et al., 2007a, 2008). Final viable cell counts in the control were similar to those encountered in the aforementioned studies. Plating was also carried out to determine coliform numbers present in the cheeses; however, no viable cells were recovered.

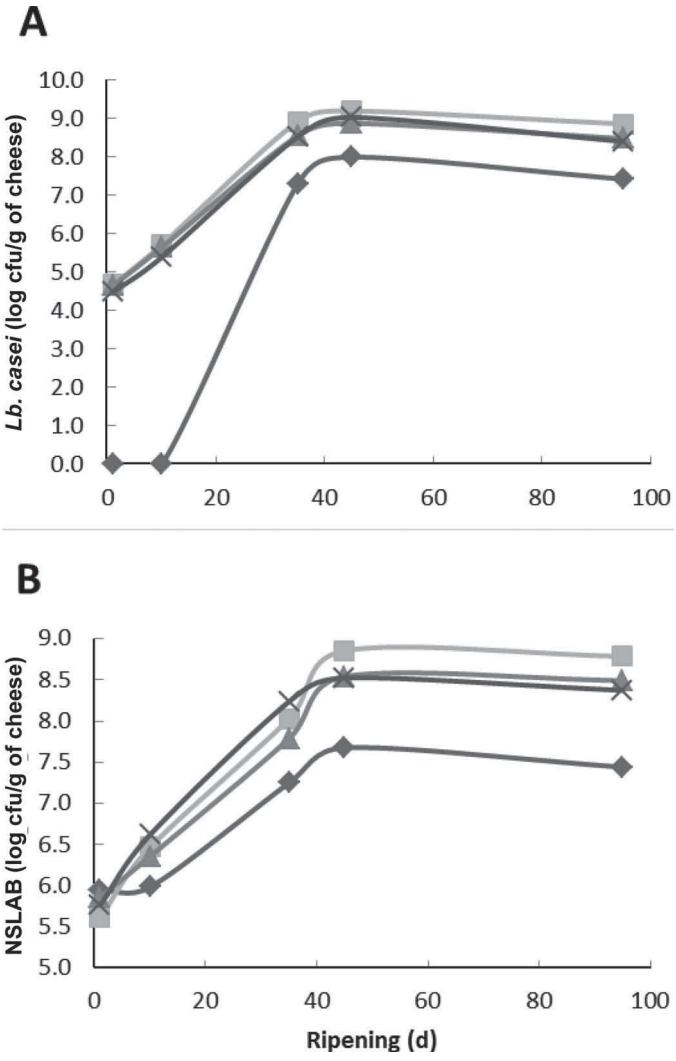

Figure 2. Effect of the respective treatments on mean viable counts of (A) *Lactobacillus casei* and (B) total lactobacilli, enumerated on de Man, Rogosa, Sharpe agar (BD, Oxford, UK) supplemented with vancomycin (Sigma, Arklow, Ireland) and LBS agar (BD), respectively. NSLAB = nonstarter lactic acid bacteria. Cheeses included control (CTL, ◆; containing *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii*), cheese without *L. helveticus* (SPC, ■), cheese without *P. freudenreichii* (SLC, ▲), and cheese with *S. thermophilus*, *L. helveticus*, *P. freudenreichii*, and *Lactobacillus casei* (SLPC, ×). Values presented are means of 3 replicate trials.

Changes in pH

In Swiss-type cheese, pH decreases in the initial stages of ripening due to the metabolism of residual sugars (lactose and galactose) before increasing in the later stages of ripening due to proteolytic liberation of short peptides and AA (McSweeney, 2004). In our study, a significant ($P < 0.01$) effect of ripening time on pH was noted (Figure 3). pH was higher than observed in similar studies during initial stages of ripening, but was similar to that of Emmental (pH 5.5–5.7) toward the end of ripening (Fröhlich-Wyder and Bachmann, 2007). This reflects the continual metabolism of residual lactose and galactose present during the early stages of ripening by *L. helveticus* or *L. casei* and NSLAB populations. Furthermore, the higher average pH levels in the SPC cheeses 1 d postproduction (although not significant) likely reflect the absence of the *L. helveticus* starter.

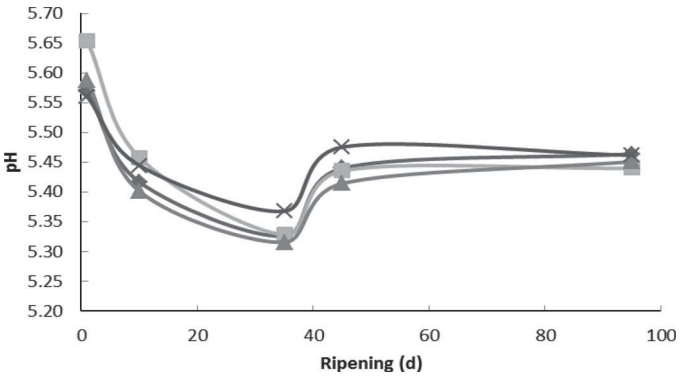

Figure 3. pH values throughout ripening for all cheeses. Cheeses included control (CTL, ◆; containing *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii*), cheese without *L. helveticus* (SPC, ■), cheese without *P. freudenreichii* (SLC, ▲), and cheese with *S. thermophilus*, *L. helveticus*, *P. freudenreichii*, and *Lactobacillus casei* (SLPC, ×). Values presented are means of 3 replicate trials.

Table 3. Cheese composition (protein, moisture, salt, calcium, and salt in moisture), 1 d after manufacture, and pH at 10 d after manufacture[1]

Compositional indices	CTL	SPC	SLC	SLPC
Protein (%)	25.78[a]	25.13[a]	25.02[a]	25.81[a]
Moisture (%)	39.63[a]	40.37[ab]	40.7[b]	39.91[a]
Salt (%)	1.19[a]	1.12[a]	1.24[a]	1.04[a]
Calcium (mg/100 g)	895[a]	886[a]	880[a]	887[a]
pH at d 10	5.42[a]	5.46[a]	5.40[a]	5.45[a]
Salt in moisture, (%)	3.0[a]	2.77[a]	3.04[a]	2.63[a]

[a,b]Means sharing a common letter are not significantly different ($P < 0.05$).

[1]Values presented are means of 3 replicate trials. CTL cheese: control cheese containing *Streptococcus thermophilus*, *Lactobacillus helveticus,* and *Propionibacterium freudenreichii*; SPC cheese: contains *S. thermophilus, P. freudenreichii*, *Lactobacillus casei*, and no *L. helveticus*; SLC cheese: contains no *P. freudenreichii* populations; SLPC cheese: contains *S. thermophilus*, *L. helveticus*, *P. freudenreichii*, and *L. casei*.

Cheese Composition

Moisture, Protein, Salt, Calcium, and pH Levels. The addition of *L. casei*, as well as the omission of *L. helveticus* (SPC) and *P. freudenreichii* (SLC), had no significant effect on mean levels of protein (%), salt, calcium, and pH (at 10 d; Table 3). Differences ($P < 0.05$), however, were observed with respect to moisture, as the SLC cheeses were significantly higher than that of the CTL and SPLC cheeses, likely due to reduced acidification during cheese manufacture. This is surprising, as PAB are not considered to affect rates of acidification during cheese manufacture. It was, however, notable that, although significantly different, the magnitude of the difference was not large (~1%) and may therefore have little biological significance. Compositional indices were similar to those encountered in similar studies (Sheehan et al., 2008). No significant difference in salt in moisture levels was observed.

Lactose and Galactose. A significant ($P < 0.0001$) reduction in lactose levels was observed in the CTL, SPC, SLC, and SLPC cheeses throughout ripening (Figure 4A). This effect was expected, as lactose is rapidly metabolized by *S. thermophilus* in the first few hours of ripening, with residual lactose being metabolized by starter and nonstarter lactobacilli (McSweeney and Fox, 2004). A significant ($P < 0.05$) effect of treatment was noted, as lactose levels were observably higher in the SPC than in the CTL or SPLC cheeses. This effect is attributed to the absence of *L. helveticus* in the SPC cheeses. Lactose levels were not significantly different in the SLC cheeses compared with the CTL or SPLC cheeses. A significant ($P < 0.01$) positive correlation was also observed between the CTL and SPC cheeses at 1 d postproduction. This is, again, likely due to the absence of *L. helveticus* populations. Similarly, a significant ($P < 0.01$) correlation was observed between SPC and SLPC cheeses at 1 d postproduction. In this case, the presence of both *L. helveticus* and *L. casei* in the SLPC cheeses likely resulted in a significant and rapid reduction in lactose levels. Low residual levels of lactose (<0.0005 g/100 g) were present in control and SLPC cheeses at 10 d of ripening, whereas lactose was undetectable in all cheeses by 35 d postproduction.

Galactose is metabolized primarily by lactobacilli (starter lactobacilli). Therefore, absence or failure of

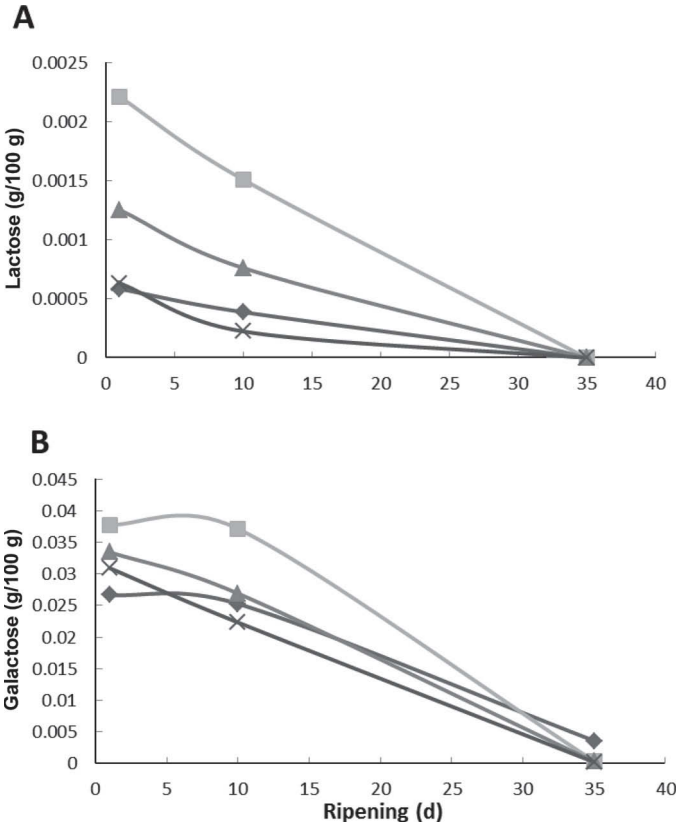

Figure 4. Levels of (A) lactose and (B) galactose expressed in grams per 100 g of cheese. Cheeses included control (CTL, ◆; containing *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii*), cheese without *L. helveticus* (SPC, ■), cheese without *P. freudenreichii* (SLC, ▲), and cheese with *S. thermophilus*, *L. helveticus*, *P. freudenreichii*, and *Lactobacillus casei* (SLPC, ×). Values presented are means of 3 replicate trials.

a galactose fermenting starter, such as *L. helveticus*, can allow for galactose accumulation, leading to undesirable bacterial growth or fermentation (Turner et al., 1983; Fröhlich-Wyder and Bachmann, 2004). In our study, galactose levels declined significantly ($P < 0.0001$), as expected, throughout ripening (Figure 4B). A significant ($P < 0.05$) positive correlation was observed with respect to galactose levels at 10 d postproduction in the SPC cheeses when compared with the control. This effect is likely due to the absence of *L. helveticus* populations. Additionally, a significant ($P < 0.01$) positive correlation was also observed between the SPC and SLPC cheese at 10 d postproduction. Galactose levels were lowest in the control cheeses at 1 d postproduction. Upon entering the hot room ripening phase, galactose was rapidly metabolized in all cases and was not detected by d 35 in all cheeses with added *L. casei* (SPC, SLC, and SLPC cheeses). It is feasible that the additional galactose present in the SPC cheeses provides a suitable substrate for *L. casei* populations, particularly upon transfer to the hot room, resulting in the production of gas before propionic acid fermentation.

D-, L-, and Total Lactate. Starter bacteria, including *S. thermophilus* and *L. helveticus,* produce L-lactate and a mixture of D- and L-lactate, respectively, during Swiss-cheese production (McSweeney and Fox, 2004). Levels of both D-, L-, and total lactate were monitored throughout the course of ripening (Figure 5 A–C). Significant effects of time ($P < 0.05$) and treatment ($P < 0.05$) were observed throughout ripening on levels of total lactate. Due to the absence of PAB, which metabolize lactate to propionate, acetate, and CO_2, total lactate levels were highest in the SLC cheeses. Differences were observed between the control and SLC cheeses from d 35 until the end of ripening and were significant ($P < 0.05$) at d 45 and 95. Total lactate levels were similar in SPC and SLPC cheeses, both of which contained PAB and *L. casei*. This effect has also been noted in previous studies where lactate levels were higher in cheeses produced with FHLb, which may be due to the competition or inhibition of PAB by FHLb (Fröhlich-Wyder and Bachmann, 2004; Weinrichter et al., 2004; Bouton et al., 2009). Total lactate levels were similar in our control cheeses to those reported to levels encountered in similar Swiss-type cheeses (1,200–1,500 mg/100 g; Sheehan et al., 2007a).

A significant effect of both time ($P < 0.0001$) and treatment ($P < 0.05$) was noted on levels of D-lactate throughout ripening (Figure 5B). D-Lactate levels were low in the early stages of ripening due to the slower metabolism of lactose by *L. helveticus* in comparison to that of *S. thermophilus*. As *L. helveticus* was not present in the SPC cheeses, no D-lactate was detected 1 d postproduction, and only slight increases were observed 10 d postproduction, possibly due to metabolism of residual lactose by FHLb. Levels of D-lactate increased significantly ($P < 0.0001$) across all treatments once the cheeses entered the hot room ripening phase, as previously described (McSweeney and Fox, 2004). Levels then decreased due to metabolism by PAB. No

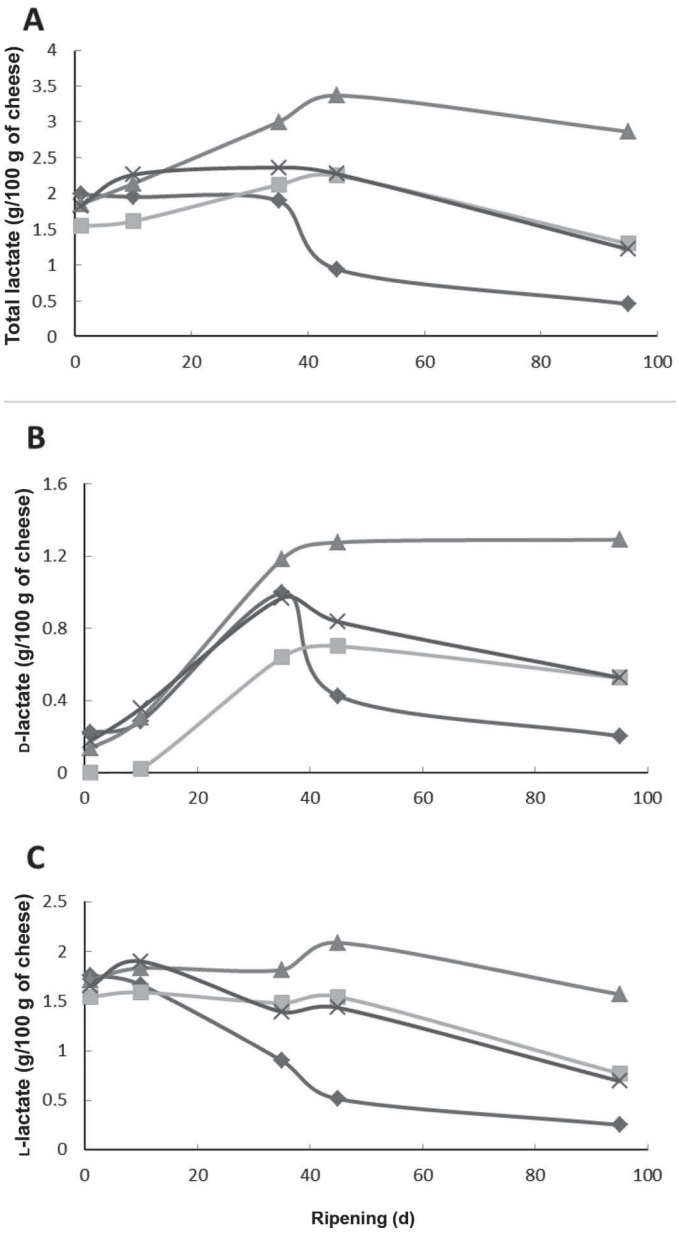

Figure 5. Levels of (A) total lactate, (B) D-lactate, and (C) L-lactate (g/100 g) present in the control and treatments 1 to 3. Cheeses included control (CTL, ◆; containing *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii*), cheese without *L. helveticus* (SPC, ■), cheese without *P. freudenreichii* (SLC, ▲), and cheese with *S. thermophilus*, *L. helveticus*, *P. freudenreichii*, and *Lactobacillus casei* (SLPC, ×). Values presented are means of 3 replicate trials.

consequent reduction of D-lactate was observed in SLC cheese due to the absence of PAB. A significant ($P < 0.05$) treatment by time interactive difference was observed in D-lactate levels between the control and SLC cheese at d 45 and 95 of ripening. Low levels of D-lactate were observed in the control cheeses (~0.2 g/100 g of cheese) at the end of ripening, whereas cheese containing PAB and *L. casei* displayed similar D-lactate levels, again likely due to the inhibitory action of FHLb on PAB activity.

Levels of L-lactate were similar across all cheeses and are considerably higher than that of D-lactate at 1 d postproduction due to the presence of *S. thermophilus*, which produces L-lactate from lactose. Thereafter a significant ($P < 0.01$) reduction was observed in levels of L-lactate throughout ripening (Figure 5C). Similar to total and D-lactate levels, L-lactate was highest in the SLC cheeses due to the absence of PAB, which preferentially metabolize L-lactate. L-Lactate levels were significantly ($P < 0.05$) lower in the control cheese at d 45 of ripening than the SLC cheeses on that day. L-Lactate considerably decreased in the control throughout ripening, whereas similar levels of L-lactate were again observed in both the SPC and SLPC cheeses. A noticeable reduction in L-lactate levels, together with a corresponding increase in D-lactate, at d 35 of ripening may be due in part to racemization of L-lactate to D-lactate by NSLAB or *L. casei* present, numbers of which increase considerably during hot room ripening (McSweeney and Fox, 2004).

Citrate Levels. Citrate metabolism is responsible for eye formation in Dutch-type cheeses (e.g., Edam and Gouda, which are made without added PAB; McSweeney and Fox, 2004), and acts as a potential substrate for gas formation by FHLb in both Cheddar and Swiss-type cheeses. (Porcellato et al., 2015). Initially, citrate levels averaged 0.13 mg/kg at 1 d postproduction across all cheeses, and decreased significantly ($P < 0.0001$) thereafter throughout the ripening process (Figure 6). Once the cheeses entered the hot room, a significant ($P < 0.0001$) reduction in citrate levels occurred in all cheeses. A significant ($P < 0.0001$) interactive effect (treatment by time) was observed between the CTL and all other cheeses from d 35 until the end of ripening (d 95). The SPC, SLC, and SLPC cheeses containing *L. casei* displayed lower levels of citrate (0.01 mg/kg at the end of ripening) than were observed in the CTL cheese (0.06 mg/kg at the end of ripening). As NSLAB such as *L. casei* are capable of metabolizing citrate (Mullan, 2000) to produce CO_2, it is feasible that the addition of this adjunct resulted in the differences in levels observed between the control and experimental cheeses. Furthermore, significantly reduced levels of citrate have been observed in cheeses manufactured with FHLb, such as *L. paracasei* and *L. rhamnosus*, as has previously been reported (Bouton et al., 2009).

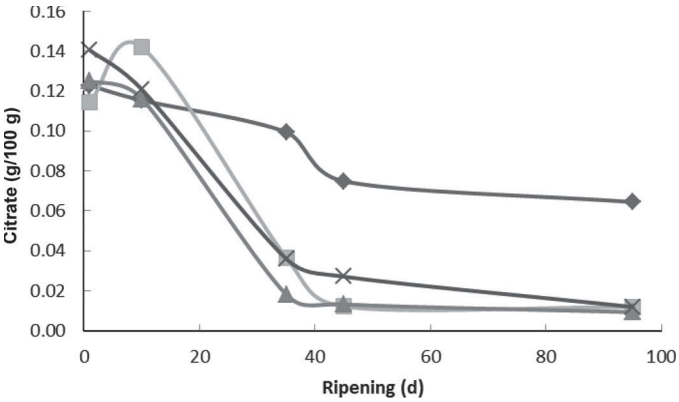

Figure 6. Citrate levels (g/100 g) present in the control and treatments 1 to 3 throughout ripening. Cheeses included control (CTL, ◆; containing *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii*), cheese without *L. helveticus* (SPC, ■), cheese without *P. freudenreichii* (SLC, ▲), and cheese with *S. thermophilus*, *L. helveticus*, *P. freudenreichii*, and *Lactobacillus casei* (SLPC, ×). Values presented are means of 3 replicate trials.

Short-Chain Volatile Carboxylic Acids. Acetic acid (acetate) is produced by propionic acid fermentation by PAB as well as metabolism of citrate by members of the LAB (Sheehan et al., 2008). Initial levels of acetate were low in all cheeses (215 mg/kg) and increased significantly ($P < 0.01$) upon transfer to the hot room (Figure 7A). This is likely due to the metabolism of citrate by *L. casei* as well as the metabolism of lactate by PAB. No significant differences were observed with respect to treatment. As shown previously, viable numbers of *L. casei*, NSLAB, and PAB all increased significantly when the cheeses were transferred to the hot room, likely resulting in the observed increase in levels of acetate produced. As hot room ripening progressed into cold storage, acetate levels were similar in CTL, SPC, and SPLC cheeses, whereas levels were noticeably lower in SLC cheeses. The latter effect is most likely due to the absence of PAB. Therefore, acetate levels present were likely as a result of NSLAB and *L. casei* populations. The levels of acetate produced were similar to those in similar Swiss-type cheese studies (Sheehan et al., 2008).

Propionic acid (propionate) is produced via the metabolism of lactate by PAB, primarily during the hot room phase of ripening (20–24°C; Fröhlich-Wyder and Bachmann, 2004). As no PAB were present in the SLC cheeses, no propionate was detected. A significant effect of time ($P < 0.01$) was observed throughout the ripening process in all other cheeses (Figure 7B). No significant effect of treatment was observed. A sig-

nificant increase in viable cell counts of PAB occurred once the cheeses were transferred to the hot room, and this correlated with an increase in levels of propionate detected. By the end of ripening (d 95) the highest levels of propionate were observed in the control cheeses. Propionate levels were similar in the SPC and SLPC cheeses, providing further evidence for an inhibitory effect of FHLb on PAB activity. This effect may be due to the production of acetate, which inhibits PAB growth. Similarly, the presence of complexed copper, released during metabolism of citrate also has an inhibitory effect on PAB growth (Fröhlich-Wyder et al., 2002). Propionate levels in the control cheeses at the end of ripening were similar to those encountered by Fröhlich-Wyder and Bachmann (2004; 5,000 mg/kg); however, levels encountered in SPC and SPLC cheeses were considerably lower.

The stoichiometric equation of PAB lactate metabolism describes 2 molecules of propionate produced for every 1 molecule of acetate (Piveteau, 1999). As NSLAB populations can produce acetate rather than propionate, the contribution of both PAB and NSLAB to acetate and propionate production can be roughly ascertained by deducing the ratio of propionate to acetate. In this case, ratios of propionate to acetate averaged 1.59 in the control, 1.05 in the SPC cheeses, 0 in the SLC, and 0.81 in the SPLC cheeses (Table 4). This indicated that the SPC and SPLC cheeses (i.e., those containing PAB and *L. casei*) displayed considerably lower ratios than that of the control, likely due to acetate production by NSLAB populations. As SLC cheese contained no PAB, no propionate was produced. The highest ratios were observed in the control cheeses due to the absence of added *L. casei*.

In our study, butyrate levels were low in the control, SLC, and SLPC cheeses and in line with levels previously reported in Swiss-type cheese (150 mg/kg; Figure 7C; Fröhlich-Wyder et al., 2002; Lawlor et al., 2002). A significant ($P < 0.01$) effect of time, particularly between 35 and 45 d postproduction, was observed. Additionally, a significant ($P < 0.01$) treatment by time interactive effect was observed in butyrate levels between the SPC and all other cheeses at d 45 and 95 of ripening. The reason for the accumulation of butyrate in the SPC cheeses is unknown at this stage.

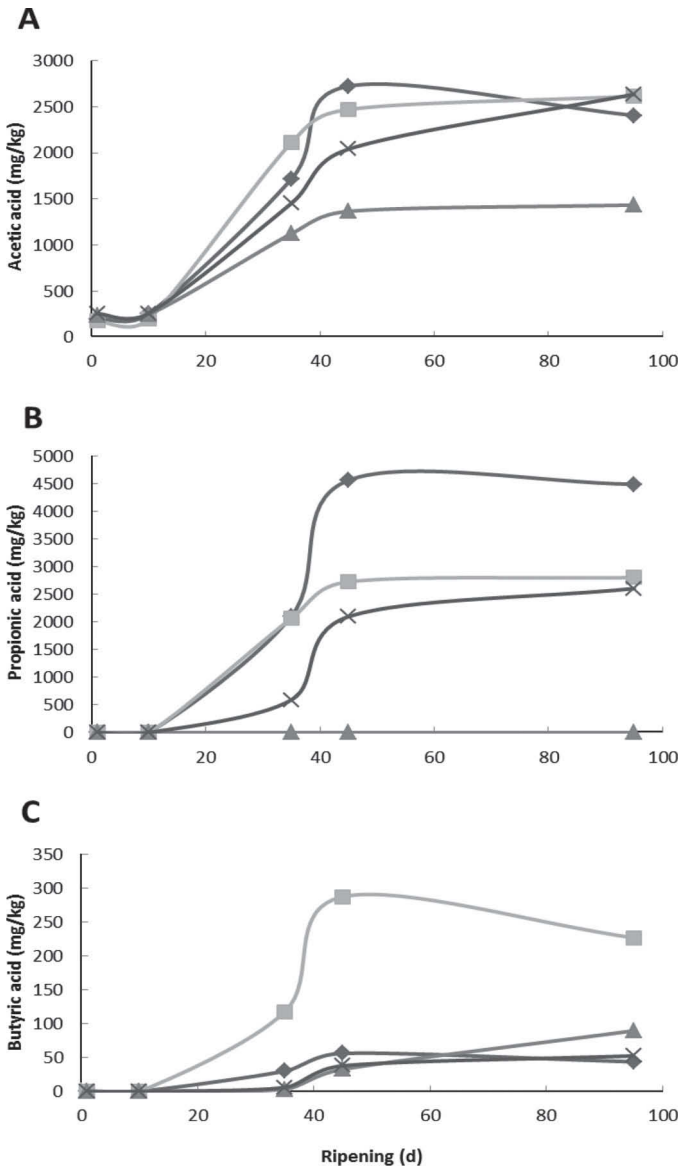

Figure 7. Short-chain volatile carboxylic acids, including (A) acetic acid, (B) propionic acid, and (C) butyric acid, presented in milligrams per kilogram of cheese. Cheeses included control (CTL, ◆; containing *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii*), cheese without *L. helveticus* (SPC, ■), cheese without *P. freudenreichii* (SLC, ▲), and cheese with *S. thermophilus*, *L. helveticus*, *P. freudenreichii*, and *Lactobacillus casei* (SLPC, ×). Values presented are means of 3 replicate trials.

Table 4. Ratio of propionate to acetate during the later stages of ripening (d 35–95) in the control and 3 treatment cheeses[1]

Ripening day	CTL	SPC	SLC	SLPC
35	1.23	0.98	0.00	0.41
45	1.68	1.10	0.00	1.03
95	1.87	1.07	0.00	0.99

[1]Ratios displayed are an average of mean propionate and acetate production cross replicate trials. Ratios are not included before d 35, as no propionate was produced. CTL cheese: control cheese containing *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii*; SPC cheese: contains *S. thermophilus, P. freudenreichii*, *Lactobacillus casei,* and no *L. helveticus*; SLC cheese: contains no *P. freudenreichii* populations; SLPC cheese: contains *S. thermophilus*, *L. helveticus*, *P. freudenreichii*, and *L. casei*.

However, it may be due to bacterial lipases, such as those from PAB, or AA catabolism (Christensen et al., 1999). Propionic acid bacteria are among the major contributors to lipolysis in Swiss-type cheeses; however, thermophilic bacteria, including *S. thermophilus* and *L. helveticus*, have previously been shown to exhibit lipolytic and esterolytic capabilities (Chamba and Perreard, 2002). Butyrate can also be formed by clostridia, and is responsible for blowing of Swiss-type cheeses (Fröhlich-Wyder and Bachmann, 2007); however, no evidence of blowing was detected in our study.

Proteolysis

Soluble N at pH 4.6. Levels of soluble N at pH 4.6 as a percentage of total N increased significantly ($P < 0.0001$) throughout ripening (data not shown), with a marked increase occurring when cheeses were transferred to the hot room. No effect of treatment was observed. The increase in pH 4.6-soluble N observed was expected and similar to trends seen in studies on Swiss-type cheeses (Sheehan et al., 2008). Levels of soluble N as a percentage of total N (11.3, 11.4, 12.4, and 12.3% in the CTL, SPC, SLC, and SLPC cheeses, respectively, at d 95) were similar to those described in the literature (Upadhyay et al., 2004; Sheehan et al., 2007b).

Total and Individual FAA. Levels of total FAA increased significantly ($P < 0.0001$) throughout the ripening process (Figure 8A), particularly when the cheeses entered the hot-room ripening phase. A significant ($P < 0.05$) treatment by time interactive effect was also observed at d 95, where SPC cheeses had significantly lower levels of total FAA in comparison to all other cheeses. This significant difference between SPC and the other cheeses is likely due to the absence of highly proteolytic *L. helveticus* populations in the SPC cheeses (Beresford et al., 2001). Highest levels of total FAA were encountered at d 95 in the control cheeses (9,063 mg/kg), whereas the lowest levels were observed in the SPC cheeses (3,168 mg/kg).

Levels of individual FAA at 95 d postproduction are shown in Figure 8B. A significant ($P < 0.05$) effect of treatment was observed, as individual FAA levels were lower in SPC cheeses than in all other cheeses. The FAA detected at highest concentrations at d 95 included glutamate, leucine, valine, lysine, and proline with proportions similar to those commonly observed in Swiss-type cheeses, such as Emmental (Lawlor et al., 2002; Upadhyay et al., 2004; Sheehan et al., 2008). Levels of glutamate, leucine, lysine, and proline were significantly ($P < 0.01$) higher at d 95 in the CTL than in the SPC cheeses.

Eye Formation in Swiss-Type Cheeses as Determined by X-ray CT

Swiss-type cheeses were investigated, using nondestructive X-ray CT, to allow for examination of the 3-dimensional spatial distribution of eyes produced by the various treatments as well as the size of the eyes present (Figure 9). With respect to the physical appearance (shape, distribution, size, and number) of eyes formed during the ripening process, the control cheese resembled most closely a standard Swiss-type cheese. As the control cheese was manufactured at pilot scale and not in an industrial setting, eye formation would still be regarded as somewhat irregular. However, marked physical differences were observed in the control compared with the other cheeses. In the SPC cheeses a large number of small eyes were distributed throughout the cheese wheel. This observation is consistent with prior studies, which describe the presence of FHLb (such as *L. casei*) providing conditions conducive to the production of a large number of small eyes, likely due to citrate and carbohydrate metabolism (Weinrichter et al., 2004; Law and Tamime, 2011; Guggisberg et al., 2015). Several eyes with a very large volume were also present. In the SLC cheeses, normal eye formation did not occur due to the absence of PAB. However, a large number of minute eyes were distributed throughout the cheese wheel. It is likely that these small eyes were produced as a result of CO_2 production by FHL present in the cheese but were not enlarged due to the absence of a PAB fermentation. In the SLPC cheeses, a large number of eyes with varying volumes were observed. These eyes are distributed throughout the cheese wheel and are observably larger than those present in the SPC cheeses.

With respect to void percentage, at 95 d post production the greatest ($P < 0.05$) void volume occurred in the SPC cheeses (22.6%; Table 5). Following this, SLPC and control cheeses (14.6 and 12.6%, respectively) displayed similar void percentages. The SLC cheeses displayed the lowest void percentage at 1.5%. Defect volume is represented (in mm^3) by the coloration of the void spaces (B images in Figure 9).

CONCLUSIONS

The results of our study demonstrate that the failure of starter bacteria (*L. helveticus*) coupled with the presence of a faculatively heterofermentative lactobacilli (*L. casei*) led to a greater propensity for excessive eye formation in Swiss-type cheeses during ripening. The availability of residual amounts of lactose, galactose, and citrate, present during the initial stages of ripening

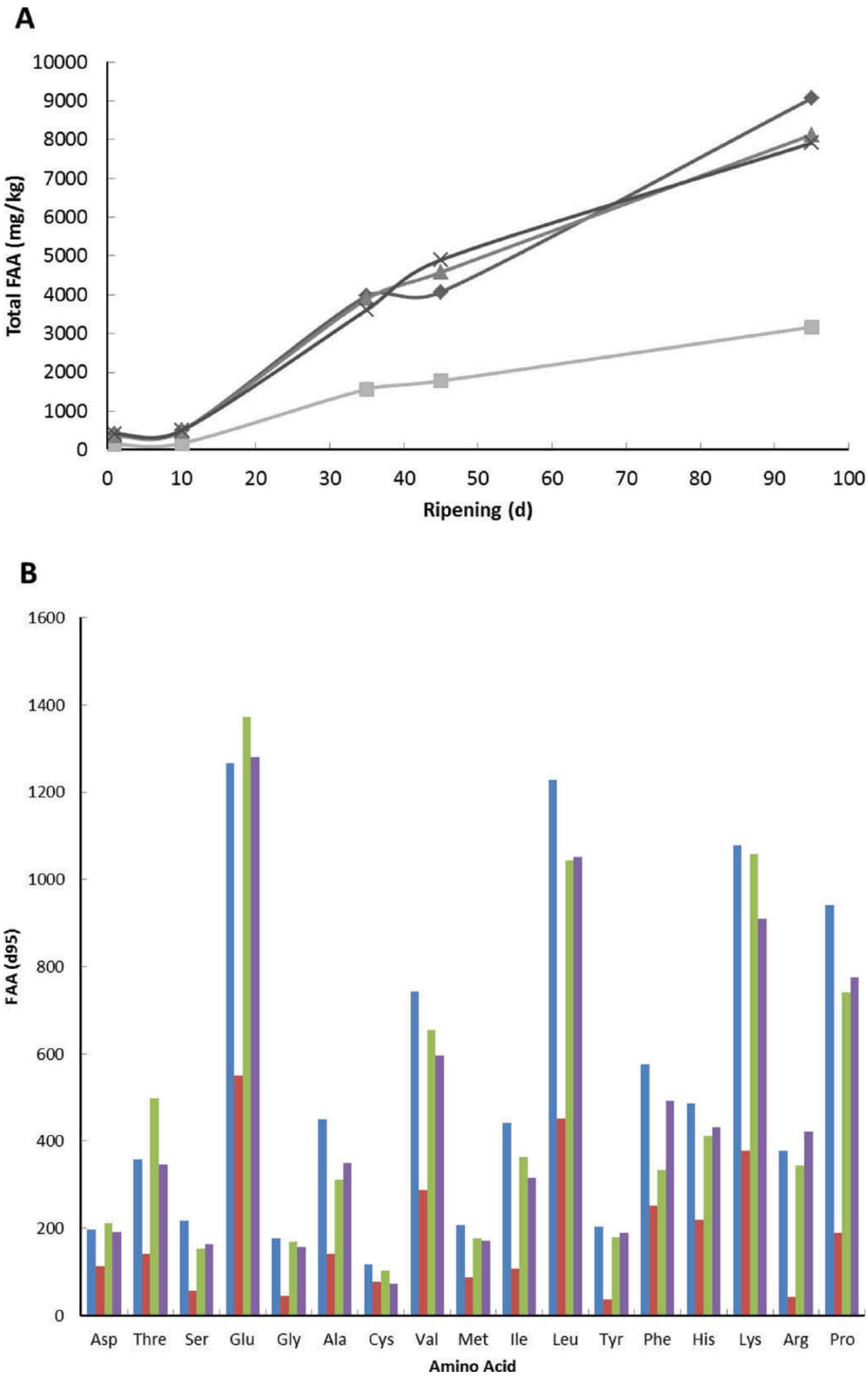

Figure 8. Total free amino acids (FAA) expressed in milligrams per kilogram of cheese (A) and individual FAA at 95 d (B) after production, in the control and treatments 1 to 3. Cheeses included control [CTL, ◆, dark gray (blue) bar; containing *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii*], cheese without *L. helveticus* [SPC, ■, white (red) bar], cheese without *P. freudenreichii* [SLC, ▲, light gray (green) bar], and cheese with *S. thermophilus*, *L. helveticus*, *P. freudenreichii*, and *Lactobacillus casei* [SLPC, ×, black (purple) bar]. Values presented are means of 3 replicate trials. Color version available online.

Table 5. Void percentage summary for each treatment, at 95 d of ripening; 3 sections were analyzed per treatment group[1]

Computed tomography section	CTL	SPC	SLC	SLPC
Section 1 (%)	16.6	25.48	1.76	17.49
Section 2 (%)	12.59	25.64	1.1	20.31
Section 3 (%)	8.54	16.62	1.55	5.97
Average (%)	12.6[ab]	22.6[a]	1.5[b]	14.6[ab]

[a,b]Means with the same letter are not significantly different ($P < 0.05$).

[1]CTL cheese: control cheese containing *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii*; SPC cheese: contains *S. thermophilus, P. freudenreichii*, *Lactobacillus casei*, and no *L. helveticus*; SLC cheese: contains no *P. freudenreichii* populations; SLPC cheese: contains *S. thermophilus*, *L. helveticus*, *P. freudenreichii*, and *L. casei*.

due to the absence of *L. helveticus*, likely provided the heterofermentative *L. casei* with sufficient substrates for gas formation. The accrual of these fermentable substrates was notable in cheeses lacking the *L. helveticus* starter population (SPC cheeses), and consequently excessive eye formation occurred. The presence of citrate likely provided a further substrate for CO_2 accumulation. As the cheese body can only accommodate a certain amount of gas, it is conceivable that increased amounts of fermentable substrates, coupled with the presence of heterofermentative microbial populations, resulted in build-up of CO_2 within the cheese before propionic acid fermentation. Once propionic acid fermentation occurred, toward the end of hot room ripening, an additional accumulation of gas resulted in the excessive eye formation observed. A stimulatory effect of LAB on PAB was not evident in our study, but rather, contrastingly, indicators of PAB activity, such as propionic acid production, were lower in cheeses containing both *L. casei* and *P. freudenreichii*. This suggested an inhibitory effect of *L. casei* metabolism on PAB activity. Heterofermentative adjuncts, such as *L. casei*, are often intentionally added to artisanal Swiss-type cheeses to control and reduce the occurrence of secondary fermentation defects. Whereas *L. casei* addition has proved a successful method for controlling excessive gas formation, our study has shown that the addition of FHLb, such as *L. casei*, can promote gas defects particularly in situations where starter cultures fail. X-ray CT analysis of the various cheese treatments

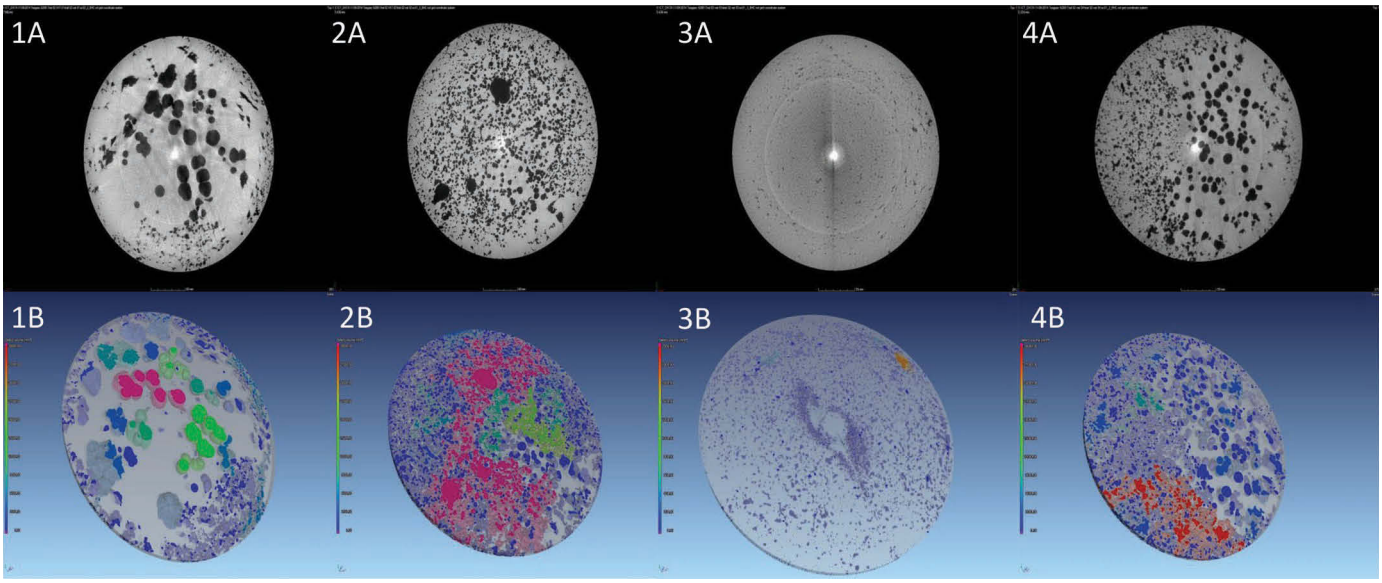

Figure 9. Eye formation in Swiss-type cheeses as determined by x-ray computed tomography (CT). The CT images are represented, in the particular sections of the cheeses, with an A whereas a void overview is represented by a B. Control cheese (1A and B; containing *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Propionibacterium freudenreichii*), SPC cheese (2A and B; cheese without *L. helveticus*), SLC cheeses (3A and B; cheese without *P. freudenreichii*), SLPC cheese (4A and B; cheese with *S. thermophilus*, *L. helveticus*, *P. freudenreichii*, and *Lactobacillus casei*). Images were taken from trial 2 at 95 d postproduction and are representative of trials 1 and 3. Colors in the blue spectrum represent voids of 0 to 6,000 mm^3, those in green represent 9,000 to 21,000 mm^3, and those in red represent 24,000 to 30,000 mm^3.

provided an accurate, noninvasive, overall image, not only of eye formation but of eye size, distribution, and overall void percentage. This method also allows for the establishment of relationships between the biochemical characteristics of the cheese and the physical manifestation of eyes.

ACKNOWLEDGMENTS

This work was funded by the Department of Agriculture, Food and the Marine (Ireland) under the Food Institutional Research Measure through the Cheeseboard 2015 project. Daniel J. O'Sullivan is in receipt of a Teagasc Walsh Fellowship (Grant Number: 2012205). The authors acknowledge Colin Grimes and Juliet Wiley for technical assistance with cheese trials and compositional analysis, Paula O'Connor for amino acid analysis and Helen Slattery for carbohydrate analysis (all from Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland). The authors also acknowledge Brendan Phelan at South Eastern Applied Materials (SEAM; Waterford Institute of Technology, Waterford, Ireland) for CT scanning and image analysis.

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