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Effect of varying the salt and fat content in Cheddar cheese on aspects of the performance of a commercial starter culture preparation during ripening

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ABSTRACT

Production of healthier reduced-fat and reduced-salt cheeses requires careful selection of starter bacteria, as any substantial alterations to cheese composition may prompt changes in the overall performance of starters during cheese ripening. Therefore, it is important to assess the effect of compositional alterations on the individual strain response during cheese ripening for each optimised cheese matrix. In the current study, the effect of varying fat and salt levels in Cheddar cheese on the performance of a commercial *Lactococcus lactis* culture preparation, containing one *L. lactis* subsp. *lactis* strain and one *L. lactis* subsp. *cremoris* strain was investigated. Compositional variations in fat or salt levels did not affect overall starter viability, yet reduction of fat by 50% significantly delayed non-starter lactic acid bacteria (NSLAB) populations at the initial ripening period. In comparison to starter viability, starter autolysis, as measured by release of intracellular lactate dehydrogenase (LDH) or post-proline dipeptidyl aminopeptidase (Pep X) into cheese juices, decreased significantly with lower salt addition levels in full-fat Cheddar. Conversely, reducing fat content of cheese resulted in a significantly higher release of intracellular Pep X, and to a lesser extent intracellular LDH, into juices over ripening. Flow cytometry (FCM) indicated that the permeabilised and dead cell sub-populations were generally lower in juices from cheeses with reduced salt content, however no significant differences were observed between different salt and fat treatments. Interestingly, fat reductions by 30 and 50% in cheeses with reduced or half added salt contents appeared to balance out the effect of salt, and enhanced cell permeabilisation, cell death, and also cell autolysis in these variants. Overall, this study has highlighted that alterations in both salt and fat levels in cheese influence certain aspects of starter performance during ripening, including autolysis, permeabilisation, and intracellular enzyme release. However, it may be possible to reduce the fat and salt content of Cheddar cheese by 30 or 50%, respectively, without largely altering permeabilised and dead cell sub-populations and, in turn, the amount of released intracellular Pep X activity, such that these performance parameters are similar to those observed for control full-fat, full-salt Cheddar cheese.

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1. Introduction

Development of flavour during cheese ripening is significantly influenced by the type of starter lactic acid bacteria (LAB) used for cheese manufacturing and subsequently the various enzyme activities originating from them (Fox et al., 1993; Fox and Wallace, 1997; Smit et al., 2005). LAB serve as principal flavour development mediators during cheese ripening, as they provide an array of extracellular and intracellular enzymes that act on various substrates within the cheese matrix to generate important volatile flavour compounds (Lopez et al., 2006; McSweeney, 2004; McSweeney and Sousa, 2000; Wilkinson et al., 1995).

LAB in the form of *Lactococcus lactis* subsp. *cremoris* or *L. lactis* subsp. *lactis* strains are the main starter types added during cheese manufacturing. They possess an extracellular cell envelope proteinase (CEP I or CEP III) and a range of intracellular proteolytic/peptidolytic enzymes, which, during ripening can hydrolyse chymosin-derived casein fragments to generate lower molecular mass peptides and free amino acids (FAA), which directly or indirectly contribute to development of the characteristic flavour and aroma of mature Cheddar cheese (Børsting et al., 2015; Fox et al., 1993; Kunji et al., 1996; McSweeney, 2004; O'Reilly et al., 2002).

Contact between intracellular peptidases of LAB and various substrates within the cheese matrix, depends on the extent of cell permeabilisation and/or cell autolysis (Doolan and Wilkinson, 2009; Sheehan et al., 2005), which correlates with elevated levels of intracellular enzyme release with a significant impact on proteolysis and flavour development in cheese (Wilkinson et al., 1994b). However,

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starter enzyme release profiles are strain dependent and can vary as a result of microenvironmental changes in cheese during the maturation period (O'Donovan et al., 1996; Sheehan et al., 2005; Wilkinson et al., 1994b). Some of the important strain dependent aspects of starter performance relevant to cheese ripening include: starter viability, permeabilisation potential, autolytic response and ability to release intracellular enzymes (Chapot-Chartier et al., 1994; O'Donovan et al., 1996; Wilkinson et al., 1994a). These properties appear to be affected by cheese compositional parameters including moisture level, fat and also salt contents, which subsequently impact the final pH of cheese and water activity levels (Beresford et al., 2001; Feirtag and McKay, 1987; Lortal and Chapot-Chartier, 2005; O'Donovan et al., 1996; Steele et al., 2013; Wilkinson et al., 1994b).

Fat and salt are both fundamental components of cheese, which directly and indirectly control starter activity and various enzymes associated with cheese ripening (Fenelon et al., 2000; Hickey et al., 2013; Thomas and Pearce, 1981). Fat content has been shown to influence starter bacteria retention during whey drainage (Laloy et al., 1996), while the action of starter esterases can hydrolyse fat to free fatty acids (Collins et al., 2003; Hickey et al., 2006; Smittle et al., 1972), which can be converted to other volatile and non-volatile flavour compounds (Hickey et al., 2006; McSweeney and Sousa, 2000; Singh et al., 2003; Smit et al., 2005). Salt addition, on the other hand, promotes a higher rate of starter permeabilisation and cell autolysis during early stages of ripening (Wilkinson et al., 1994a, b), which can allow intracellular enzymes of LAB access to substrates within the cheese matrix (Bunthof et al., 2001; Doolan and Wilkinson, 2009; Guinee, 2004; Sheehan et al., 2006, 2005).

Individual studies of cheeses with either reduced-salt or reduced-fat contents have shown that both compositional alterations (e.g. salt and fat) significantly influence moisture and salt in moisture (S/M) levels in such cheese varieties (Banks, 2004; Banks et al., 1989; Fenelon et al., 2000; Rulikowska et al., 2013; Schroeder et al., 1988). A substantial increase in moisture with a significant decrease in salt content results in cheeses having very low S/M levels, which is a primary factor controlling microbial growth, cell permeabilisation, cell autolysis, and subsequently intracellular enzyme release during cheese ripening (Rulikowska et al., 2013; Wilkinson et al., 1994b). As a result, bitterness is the most common flavour defect in aged reduced-fat and reduced-salt Cheddar (Mistry, 2001). The latter parameters influence starter activity through higher cell densities and a prolonged viability during ripening with an accumulation of excessive levels of hydrophobic bitter peptides (Wilkinson et al., 1994a). Furthermore, significantly lower S/M levels result in lower cell autolysis and decreased intracellular enzyme release from starter cells, in particular Pep X, which assists de-bittering by cleaving proline residues from hydrophobic bitter peptides originating from proline rich caseins (Wilkinson and Kilcawley, 2005).

Substantial alterations in fat and salt levels may have a more significant impact on overall starter culture performance and, in turn, the flavour characteristics of such cheese varieties. However, very little is known regarding the extent of composition-related microbiological changes when both the salt and the fat content of cheese are lowered, and how this influences intracellular enzyme release, especially from defined strain starters in cheese.

Therefore this study was carried out in order to assess the impact of reducing salt and fat levels in Cheddar cheese by 30 and 50% on aspects of the performance of a commercial starter culture in relation to its viability, permeabilisation and autolytic response during the early ripening phase.

2. Material and methods

2.1. Cheddar cheese manufacture

Cheddar cheese was manufactured in triplicate in 500 L vats using a frozen DVS preparation of a mixed starter culture R604Y containing one *L. lactis* subsp. *lactis* and one *L. lactis* subsp. *cremoris* strain (Chr. Hansen's Ireland Ltd., Little Island, Co. Cork, Ireland). Starter culture was added to cheese milk at the rate of 0.012% (*w*/*v*), the curd was cooked to 38.5 °C, pitched at pH 6.15, and milled at pH 5.35. Salt was added at the rate of 0.9, 1.22 and 1.8% (*w*/w) to milled curd after which cheeses were pressed overnight at 264.6 kPa. Subsequently all cheeses were vacuum packed and ripened at 8 °C for 60 days. Resultant cheeses were denoted as FFFS, FFRS, FFHS, RFFS, RFRS, RFHS, HFFS, HFRS and HFHS based on target compositions (Fig. 4). Grated cheese samples (10 g) were taken at 1, 14, 28 and 56 days of ripening to assess starter and NSLAB populations. Cheese juice extracted using hydraulic pressure was used to assess the levels of released intracellular enzyme activities—LDH and Pep X. Flow cytometry (FCM) was used to evaluate the physiological status of starter bacteria present in juices during Cheddar cheese ripening.

2.2. Extraction of cheese juice

Cheese juices were expressed from all cheeses on each sampling day using a hydraulic cheese press (Wilkinson et al., 1994a). Grated cheese (300 g) was mixed with general purpose grade sand (600 g; Fisher Scientific, UK) and subjected to a gradual pressure increase to 32 MPa over one hour. The expressed aqueous phase was collected over three hours at room temperature following incubation at 4 °C for 30 min. Subsequently, the solidified fat layer was removed and cheese juice was centrifuged at 10,000 $\times g$ for 10 min. The centrifuged juice was used to determine released LDH and Pep X activities. Addition of a filtration step through a 10 μm pore size Partec filter (CellTrics®, Partec, Germany) generated the filtrate used for FCM analysis.

2.3. Determination of water activity (a_w) in cheese juice during ripening

Water activity (a_w) was measured in disposable sample cups each containing 3 mL of a freshly expressed cheese juice at 21 °C with an Aqualab Series 3TE (Decagon Devices, Inc.,WA, USA) dewpoint electronic water activity meter. Data is presented as the mean of three replicate measurements of three replicate trials.

2.4. Starter and NSLAB viability in cheese during ripening

Cheese extracts were prepared by diluting 10 g of cheese 1:10 (*w*/*v*) with sterilised tri-sodium citrate (2% *w*/*v*) in a sterile stomacher bag. The mixture was homogenised for 5 min using a stomacher (Seward Stomacher® 400 Circulator, Seward Ltd., UK). The resultant homogenate was used for microbiological analysis. L-M17 agar was used to enumerate starter LAB counts (Terzaghi and Sandine, 1975). NSLAB populations were enumerated on LBS selective agar (Rogosa et al., 1951). L-M17 and LBS agar plates were incubated at 30 °C for 3 and 5 days, respectively, after which plates were counted and the cfu/g of cheese were calculated. Data is presented as the mean of three replicate measurements of three replicate trials.

2.5. Starter autolysis in cheese

Starter autolysis was monitored by the release of the intracellular marker enzymes LDH and Pep X into cheese juice during ripening (Hickey et al., 2006; Sheehan et al., 2005; Wilkinson et al., 1994a). LDH activity was assayed by modification of the method of Wittenberger and Angelo (Wittenberger and Angelo, 1970) by measuring the decrease in absorbance at 340 nm (UV–vis spectrophotometer, Shimadzu, Japan) resulting from pyruvate-dependent oxidation of NADH. Activity was expressed as units/mL/min of cheese juice, where one unit is defined as the amount of LDH that catalyses the oxidation of 1 μmol NADH per min. Pep X activity was determined in cheese juice samples by a modification of the method of Booth (Booth et al., 1990) based on the ability of Pep X enzyme to hydrolyse Gly-Pro-AMC substrate (Bachem Feinchemikalien, Bubendorf, Switzerland). The release of AMC from the synthetic substrates was determined spectrofluorimetrically using

excitation and emission wavelengths of 370 ± 40 nm and 440 ± 40 nm, respectively (Synergy HT Microplate Reader, Winooski, VT, USA). The extent of release of AMC was calculated by reference to a standard curve relating AMC to fluorescence emission under the assay conditions. Activity was expressed as μmol AMC/mL/min of sample. Data is presented as the mean of three replicate measurements of three replicate trials.

2.6. Flow cytometry

FCM was used to monitor the physiological profile of cells as the percentage of sub-populations of live, permeabilised/damaged, and dead bacterial cells present in cheese juices over ripening (Sheehan et al., 2005). Reference control populations of live, permeabilised/damaged and dead starter cells were created using two approaches (see Fig. 3). Firstly, using cells harvested from cheese juice samples expressed at each sampling point and secondly using the R604Y starter culture grown in filter-sterilised cheese juice expressed on each day of sampling. The use of these controls was to account for any potential morphological or physiological differences between cells extracted from cheeses and those in the DVS preparation, which may affect FCM analytical parameters. Starter culture controls in cheese juices were created by sterilising expressed cheese juice by passage through a 0.2 μm filter; subsequently sterility was verified by the absence of colonies on L-M17 agar media after overnight incubation. Sterile juices were inoculated with the R604Y culture and grown overnight at 30 °C, thereafter (100 μL) of the overnight culture was used to inoculate a fresh sterile juice and allowed to grow to mid-log phase (OD ~0.8). A 1 mL sample of mid-log phase culture in juice was subsequently centrifuged at 10,000 ×*g* for 10 min, to recover bacterial cells. Cells pellets re-suspended in 1 mL of potassium phosphate buffer (50 mM pH 7.0) were used as unstained control cells to set voltages on a BD LSR II cytometer (BD Biosciences, Shannon, Ireland); these cells were also used as a live cell control population. Dead cell population controls of R604Y were generated by re-suspending a cell pellet in 100% IPA for 30 min. Permeabilised cell population controls were generated by treating cell pellets with 2 mM hexadecyltrimethyl ammonium bromide (CTAB) for 15 min. Both dead cells (IPA-treated) and permeabilised cells (CTAB-treated) were recovered by centrifugation at 10,000 ×*g* for 10 min and washed once in potassium phosphate buffer (pH 7.0) before being re-suspended in 1 mL of this buffer. Equal volumes (100 μL) of live/intact (Untreated), dead (IPA-treated) and permeabilised/damaged (CTAB-treated) cells were mixed with LIVE/DEAD® BacLight™ staining reagent (Molecular Probes, Leiden, Netherlands). This kit consists of two fluorescent stains SYTO9 and PI, where SYTO9 is a green fluorescent dye and penetrates all bacterial cells while PI displays red fluorescence and is only able to enter cells with compromised cell membranes (e.g. dead or permeabilised cells). As per manufacturing instructions, after labelling, cells were left to stand at room temperature in the dark for 15 min, after which they were transferred to BD TruCount™ tubes (Becton Dickinson, Oxford, UK) containing a known amount of fluorescent beads to enable actual population counts per sample. The fluorescence-labelled cells from each of the cheese juice samples and control panels were analysed in triplicate using a Becton Dickinson LSR II flow cytometer equipped with an Argon-ion laser at 488 nm. A total of 10,000 events were recorded for each sample. Forward Side Scatter (FSC) and Side Scatter (SSC), green fluorescence (515–545 nm) and red fluorescence (>670 nm) were also used to assess cell viability for each sample. Data acquisition and analysis were controlled using CellQuest software (Becton Dickinson, Oxford, UK). Control populations of live, permeabilised/damaged or dead cells were gated as reference regions for estimation of the presence of live/intact, dead and permeabilised/damaged cells in cheese juices during ripening. Cells falling within each region were presented as percentage of a total profile for each cheese at each sampling point during ripening. Data is presented as the mean of three replicate measurements from three replicate Cheddar cheese trials.

2.7. Statistical analysis

Analysis of variance (ANOVA) was carried out using a general linear model (GLM) procedure of SPSS (version 19, SPSS Inc., Chicago, IL, USA). In this study, the effect of treatment and replicates was estimated for each response variable at each sampling point.

Tukey's multiple comparison test was used as a guide for pair comparisons of the treatment means. Differences among treatments described subsequently as being significant were determined at $P < 0.05$. Description of data was based on salt reduction and fat reduction for each salt and fat series of cheeses, respectively.

3. Results

3.1. Water activity (a_w) in cheese juices from Cheddar cheeses

Water activity (a_w) values throughout 56 day ripening period were in the range of 0.962–0.999 (Fig. 1). Analysis of variance (ANOVA) indicated that both fat and salt reduction in cheese had a significant impact on a_w levels at different times during ripening with reductions in salt content generally exerting the greater effect.

3.1.1. Effect of fat reduction

In the FS cheese series, a significant impact of fat reductions on the a_w was observed on day 28 of ripening. Cheeses with FF and RF content displayed similar a_w values, yet those had significantly higher a_w (0.965) compared with HF cheese (0.959). In contrast, results from the *posthoc* (ANOVA) analysis indicated that a_w values in RS cheese series were significantly lower in FF and RF cheeses compared with their corresponding HF counterpart on day 1 and day 56 of ripening (Fig. 1). Furthermore, an effect of reducing fat contents on a_w levels in HS cheese series was noted only at day 14 of ripening, where a_w significantly increased with reducing fat content of cheese. During this period (at day 14) a_w values were recorded at 0.976, 0.980 and 0.987 for FF, HF, and RF cheeses, respectively.

3.1.2. Effect of salt reduction

In terms of FF and RF cheese series, significant differences ($P < 0.05$) were observed between the mean a_w values on day 1 of ripening and these reflect the percentage salt reduction made (i.e., 0.962, 0.974 and 0.977 were noted for FS, RS, and HS cheese, respectively). Despite these findings, *posthoc* (ANOVA) analysis showed that a_w in the RF cheese series was similar among RS and HS cheeses, but was significantly higher than in the corresponding FS cheese from day 14 to day 56 of ripening (Fig. 1). In contrast, the initial a_w values in the HF cheese series significantly differed between FS (0.966) cheese and its RS (0.978) and

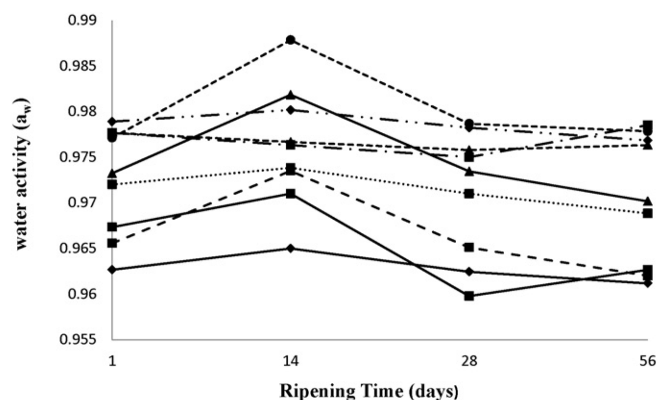

Fig. 1. Water activity (a_w) in cheese juices from Cheddar cheeses with differing fat contents and NaCl addition levels throughout ripening: —◆— FF — 1.8% NaCl; ···· ■ ···· FF — 1.2% NaCl; – –▲– – FF — 0.9% NaCl; – – ■ – – RF — 1.8% NaCl; —▲— RF — 1.2% NaCl; – – ● – – RF — 0.9% NaCl; —■— HF — 1.8% NaCl; —■ · HF — 1.2% NaCl; —◆ · · HF — 0.9% NaCl.

HS (0.980) counterparts. However, during the period of day 14 to day 56 of ripening, a_w levels were only significantly ($P < 0.05$) different between cheeses with HS (0.985) and FS (0.970) contents.

3.2. Viability of starter lactic acid bacteria and non-starter lactic acid bacteria

Starter populations enumerated on L-M17 agar on day 1 of ripening reached log 9.0 to 9.5 cfu/g of cheese and remained relatively constant throughout the ripening period (Fig. 2a). NSLAB populations, on the other hand, increased significantly from log 2.0–4.0 cfu/g on day 1 to log 4.5–6.0 cfu/g by day 56 of ripening (Fig. 2b).

3.2.1. Effect of fat reduction on starter LAB viability

Reducing the fat content of Cheddar cheese significantly affected the mean starter populations in the FS cheese series at day 56 and in RS and HS cheese series at day 14 of ripening. Mean starter counts in the FS cheese series were significantly higher in FF cheese (log 9.6 cfu/g) compared with those in the equivalent HF cheese (log 8.9 cfu/g) at day 56. Similarly, starter populations in RS and HS cheese series were generally higher in FF and RF cheeses when compared with starter counts in the HF cheese variant on day 14 of ripening.

3.2.2. Effect of salt reduction on starter LAB viability

Lowering cheese salt addition rates by 30 and 50% exerted no statistically significant effect on starter viability, yet some considerable numerical differences were observed. Generally, starter counts were highest in cheeses with RS and HS contents compared with starter populations in the FS equivalent (Fig. 1a). On day 1 of ripening starter populations among all cheeses in the FF series were similar (~log 9.6 cfu/g). Slightly higher starter populations were observed on day 14 of ripening in cheeses whose salt content was lowered by 30 or 50% (Fig. 1a). For both RF and HF cheese series, reduction in the salt level resulted in higher starter counts between day 1 and 14 of ripening, yet from day 28 to day 56 of ripening, all cheeses had similar starter populations (from 9.0 to 9.4 cfu/g). Overall, ripening time had no significant ($P > 0.05$) influence on the mean starter LAB populations throughout 56 days of ripening.

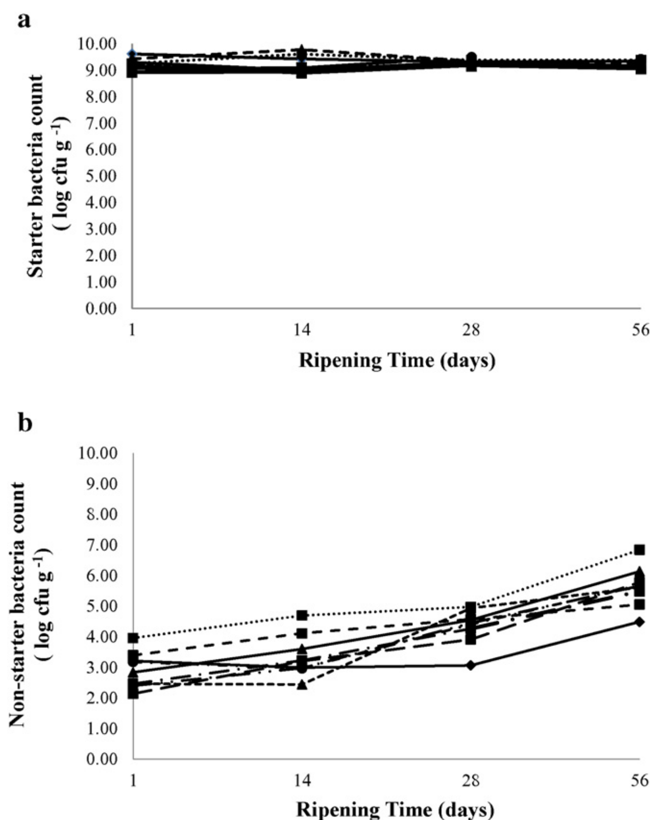

Fig. 2. (a) Starter lactic acid bacteria count and (b) Non-starter lactic acid bacteria count in Cheddar cheeses with different fat contents and NaCl addition levels throughout ripening: —◆— FF — 1.8% NaCl; ···· ■ ···· FF — 1.2% NaCl; – –▲– – FF — 0.9% NaCl; – – ■ – – RF — 1.8% NaCl; —▲— RF — 1.2% NaCl; – – ● – – RF — 0.9% NaCl; —■— HF — 1.8% NaCl; —■ · HF — 1.2% NaCl; —◆ · · HF — 0.9% NaCl.

3.2.3. Effect of fat reduction on NSLAB viability

Fat reduction had a significant ($P < 0.05$) impact on NSLAB populations at the initial stages of ripening (at day 1 and day 14), where NSLAB populations were significantly higher in FF and RF cheeses (~log 3.5 cfu/g) compared with HF cheese (log 2.5 cfu/g). Despite the differences noted at the outset of ripening, fat reduction did not exert a significant effect on NSLAB population dynamics from day 28 to day 56.

3.2.4. Effect of salt reduction on NSLAB viability

Reducing salt content of cheese had a significant effect on NSLAB populations in FF cheese series. Up to day 14 of ripening NSLAB counts were similar for FS, RS and HS cheeses (~log 3.4 cfu/g). A change in the growth dynamics of NSLAB was noted from day 28 to day 56 of ripening, where counts were significantly higher in RS and HS cheeses (~log 4.9 cfu/g) compared with those in the corresponding FS cheeses (log 3.06 cfu/g). In contrast to the FF cheese series, salt reduction had no significant impact on overall NSLAB populations in RF and HF cheese series, where mean NSLAB populations increased from log 2.4 cfu/g at day 1 to log 6.0 cfu/g by day 56 of ripening. Mean NSLAB populations increased significantly ($P < 0.05$) throughout 56 days of ripening.

3.3. Flow cytometric analysis of starter lactic acid bacteria in cheese juice during ripening

FCM identified the presence of three sub-populations of physiologically different cells in juices extracted at each point during ripening, corresponding to live/intact cells ($SYTO9^+$), permeabilised/damaged cells ($SYTO9/PI^+$), and also dead cells (PI^+) (Table 1). The intact/live cell sub-population generally comprised the predominant sub-population in the FCM profiles throughout the entire 56 days ripening period (Table 1). Alterations in cheese fat and salt addition rates, made in the present study, had no statistically significant effect on the percentages of various cell sub-populations, yet some considerable differences were noted when salt addition rates were lowered by 30 and 50%, respectively.

3.3.1. Effect of fat reduction on intact/live, permeabilised, and dead cells

Reductions in fat content of cheeses had no significant impact on the percentage of intact/live cells, permeabilised and dead cell fractions except in FS and HS cheese series on day 1 of ripening. Significant differences were observed in percentages of live/intact cells among all fat levels (Table 1). Similar to intact/live cell sub-populations, the percentage of permeabilised cells in the FS cheese series on day 1 of ripening was significantly different between FF and RF cheeses (~9 or 14%) to that recorded for the HF cheese variant (~23%). However, under HS conditions, permeabilised and dead cell fractions were significantly different among all fat levels and comprised 16.05%, 3.36% and 10.23% of the total cell population in cheeses with FF, RF and HF contents, respectively. Correspondingly, fat reduction had a significant impact on dead cell sub-populations in FS and HS cheese series, where FF, RF, and HF cheeses significantly differed from each other on day 1 of ripening. Fat reduction appeared to have no significant impact on percentage of various cell sub-populations from day 14 to day 56 of ripening.

3.3.2. Effect of salt reduction on intact/live, permeabilised and dead cells

Lowering salt addition rates had no significant impact on the percentage of intact/live, permeabilised and dead cell sub-populations, however some numerical differences were observed between various sub-populations of cells depending on the salt content of cheese. Generally the percentage of intact/live cells in FF, RF, and HF cheese series increased

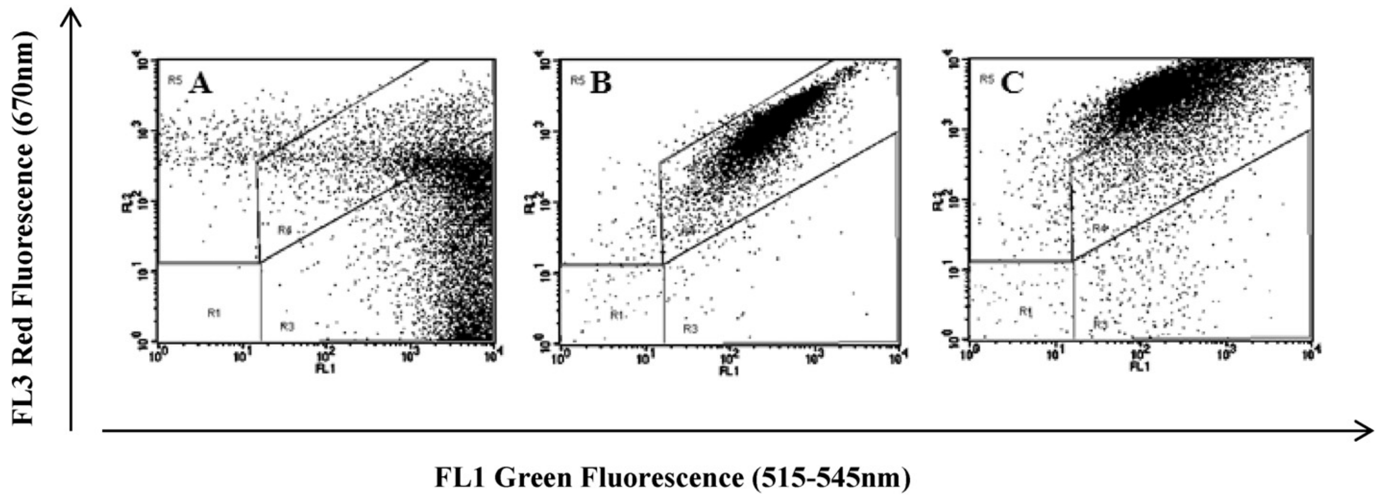

Fig. 3. Flow cytometry multiparameter dot plots of SYTO9 fluorescence (FL1) versus Propidium Iodide (PI) fluorescence (FL3) of *L. lactis* subs. *lactis* strain R604Y in cheese juice stained with the Live/Dead® BacLight™ kit. Plots represent following: (A) untreated mid-log phase cells (R3 region, Live), (B) cells treated with 100% IPA (R4 region, Dead) and (C) cells treated with 2 mmol L^{-1} CTAB (R5 region, Permeabilised).

with reduced salt content of cheese on days 1, 14, and 56 of ripening (Table 1). Consequently the percentage of permeabilised cells decreased in the FF cheese series with reductions in salt addition levels on days 14, 28, and day 56 (e.g. 12.85%, 8.71% and 5.32% for FS, RS, and HS cheese on day 14 of ripening, respectively). However, these trends were noted only at days 1 and 14 for RF cheese and at day 1 for HF cheese. The dead cell sub-population, on the other hand, followed similar trends but only on day 1 and 14 in the FF cheese series, while in HF cheese, the percentage of dead cells decreased as a result of salt reduction only on day 1 of ripening (47.83%, 22.41%, and 16.37% of dead cells noted for FS, RS, and HS cheeses, respectively). No such effect was observed among various salt treatments in the RF cheese series.

3.4. Starter autolysis in cheese

Starter autolysis was monitored by the release of the intracellular marker enzymes Pep X and LDH into cheese juice over 56 days of ripening (Tables 2, 3).

3.4.1. Effect of fat reduction on released Pep X activity

Fat reduction had a significant impact on released Pep X activity in FS and HS cheeses (at days 1 and 56 for FS cheese and at day 14 for HS cheese). In the FS series, Pep X activity increased with decreasing fat content of Cheddar on day 1, with 8.20, 18.77 and 21.74 µmol AMC/mL/min detected in FF, RF, and HF cheeses, respectively. From day 14 to day 28 of ripening fat content appeared to have no significant ($P > 0.05$) influence on cell autolysis with similar levels of Pep X activity (20.9 µmol AMC/mL min) released into juices of all FF, RF, and HF cheeses. However, by day 56 of ripening, Pep X activity had increased in tandem with lower fat contents. In contrast to FS series, fat reduction had no significant effect on starter autolysis in RS Cheddar cheeses, with comparable Pep X activity found in all juices (10–14 µmol AMC/mL/min on day 1 to 18.6–25 µmol AMC/mL/min on day 56). Similarly, in HS cheeses, no noticeable effect of fat reduction on overall Pep X activity was observed, apart from day 14 of ripening, where significantly higher Pep X activities (11.78 and 15.91 µmol AMC/mL min) were detected in RS and HS cheeses, respectively, compared with 5.89 µmol AMC/mL/min Pep X activity in their FS equivalent.

3.4.2. Effect of salt reduction on released Pep X activity

Reducing the amount of salt added to cheese appeared to have a more pronounced effect on intracellular Pep X release into cheese juice from FF cheeses. In the FF cheese series, significantly higher Pep X activity was observed in cheeses with highest added salt levels. At day 14 of

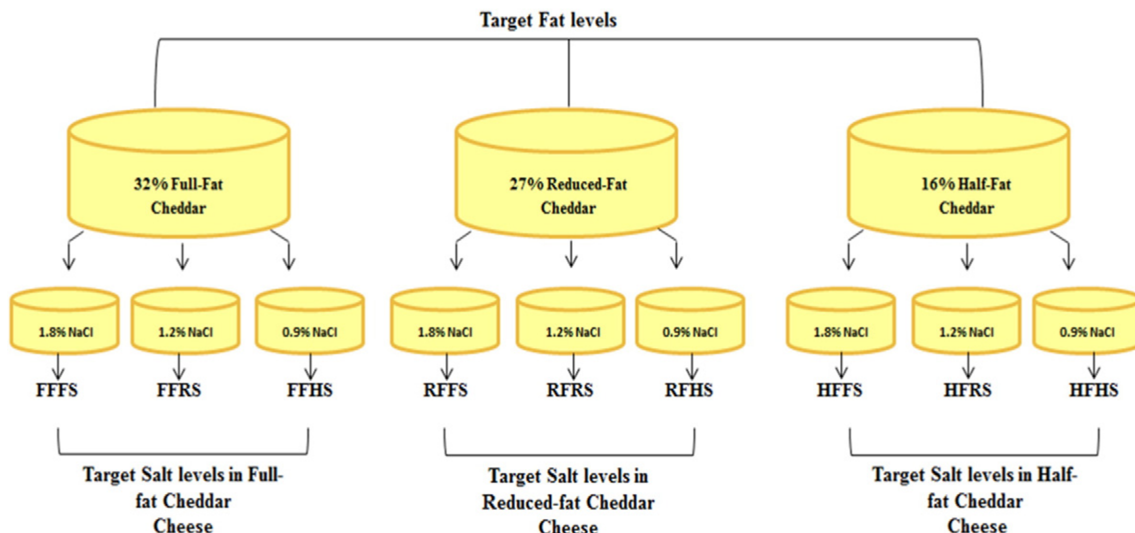

Fig. 4. Coded representation of target fat and target salt levels in Cheddar cheese.

Table 1
Flow cytometry profiles showing the percentage of live/intact, permeabilised and dead cells in cheese juices from Cheddar cheeses with differing Fat contents and NaCl addition levels throughout ripening.

Cheese (% fat and % NaCl)	Profile/regions	Ripening time (days)[a]			
		1	14	28	56
FFFS	Live/intact	56.44%	61.77%	72.92%	57.46%
FFRS	Live/intact	65.32%	70.91%	66.78%	54.77%
FFHS	Live/intact	57.62%	74.61%	66.92%	65.93%
RFFS	Live/intact	80.32%	66.71%	66.03%	58.48%
RFRS	Live/intact	69.94%	64.45%	70.08%	60.53%
RFHS	Live/intact	95.23%	73.27%	67.27%	67.40%
HFFS	Live/intact	27.57%	69.20%	65.28%	57.19%
HFRS	Live/intact	67.81%	77.54%	64.89%	64.88%
HFHS	Live/intact	72.78%	72.46%	68.24%	63.96%
FFFS	Permeabilised	14.01%	12.51%	10.52%	16.67%
FFRS	Permeabilised	13.79%	8.71%	14.85%	13.32%
FFHS	Permeabilised	16.05%	5.32%	17.06%	8.65%
RFFS	Permeabilised	9.07%	10.99%	17.32%	12.81%
RFRS	Permeabilised	11.10%	12.55%	14.32%	10.33%
RFHS	Permeabilised	3.36%	9.60%	12.93%	8.59%
HFFS	Permeabilised	22.78%	11.29%	14.33%	12.34%
HFRS	Permeabilised	9.47%	8.79%	14.65%	9.69%
HFHS	Permeabilised	10.23%	11.64%	11.27%	8.89%
FFFS	Dead	29.29%	26.63%	14.70%	25.51%
FFRS	Dead	18.61%	16.30%	17.79%	31.27%
FFHS	Dead	23.83%	16.26%	19.77%	25.24%
RFFS	Dead	7.10%	21.81%	16.01%	28.19%
RFRS	Dead	17.55%	22.39%	12.61%	28.85%
RFHS	Dead	1.08%	16.78%	19.38%	23.82%
HFFS	Dead	47.83%	9.44%	14.01%	29.66%
HFRS	Dead	22.41%	12.50%	20.20%	24.94%
HFHS	Dead	16.37%	14.40%	19.79%	26.96%

[a] Values presented are the means of each duplicate samples for triplicate trials.

ripening, activities of 19.25, 9.08 and 5.89 μmol AMC/mL/min were detected for cheese with FS, RS, and HS contents, respectively. However, by day 56 of ripening, Pep X activity was similar in all cheeses (11.7 to 15.6 μmol AMC/mL/min). In both RF and HF cheese series, Pep X activity on day 1 of ripening was highest in cheeses with FS level (Table 2). However, no significant differences were observed for released Pep X activity irrespective of salt addition rate from day 14 to day 56 of ripening.

3.4.3. Effect of fat reduction on released LDH activity

Similar to Pep X activity, LDH release into cheese juices was enhanced as a result of fat reduction, however, this trend was only noted in HS cheeses. For both FS and RS cheeses, fat reduction had no significant impact ($P > 0.05$) on released LDH activity, and all cheeses had similar levels of activity (1.7–4 and 0.27–2.41 units/mL/min in FS and RS, respectively). In contrast, fat reduction resulted in significantly ($P < 0.05$) higher levels of LDH release in the HS cheese series (Table 3). From day 1 to day 14 of ripening LDH activity was similar (0.97 units/mL/min) in RF and HF cheeses. However, released LDH activity in FF cheeses (0.66 units/mL/min) was significantly lower than the observed activities in RF and HF cheeses. From day 28 to day 56 of ripening, FF and RF had similar levels of LDH activity (0.62 units/mL/min), which were significantly lower than that found in HF cheeses (1.56 units/mL/min).

3.4.4. Effect of salt reduction on released LDH activity

Salt reduction had differing impacts on LDH release in the various series of cheeses. In the FF cheese series, LDH activity was significantly lower in cheeses with lowest added salt level (HS) on days 1, 14, and day 56 of ripening (Table 3). At day 28 of ripening, both FS and RS cheeses had similar LDH activities (1.5–2.5 units/mL/min), which were significantly higher than those noted in HS cheese (0.42 units/mL/min). In the RF cheese series, no significant difference was observed for LDH activity irrespective of added salt content on days 1 and 56 of ripening. From day 14 to day 28 of ripening, significantly lower (0.63 units/mL/min) LDH activity was found in HS cheeses compared with 1.44 and 0.82 units/mL/min of LDH activity detected in FS and RS cheeses, respectively. In the case of half-fat cheeses (HF) with varying salt levels, significantly lower LDH activity was released in cheeses with HS salt content on days 1, 14 and 28 of ripening.

3.5. Recommended RF and RS ratios for the optimal performance of a commercial R604Y mixed starter

Recommended combinations of fat and salt reduction levels, which result in similar culture performance, in terms of cell permeabilisation, cell death and cell autolysis, between FFFS control cheese and compositionally altered experimental cheeses are shown in Table 4. Those combinations include RFRS, RFHS, HFRS and also HFHS cheese equivalent and indicate that a 30% fat reduction and a 50% reduction in added salt are possible without adversely impacting on the performance of this particular commercial mixed strain starter.

4. Discussion

The focus of the current study was to assess the performance of a commercial mixed *L. lactis* subsp. *lactis* and subsp. *cremoris* starter following reductions in cheese salt and fat content by 30 and 50%. This study also provides recommended combinations of fat and salt levels, which potentially can result in similar starter response in lower-salt and lower-fat cheese variants to the FFFS control.

Table 2
Post-proline dipeptidyl aminopeptidase activity (μmol AMC/mL/min) in cheese juices from Cheddar cheeses with differing Fat contents and NaCl addition levels throughout ripening.

Cheese	Ripening time (days)	Salt content					
		Full-salt (1.8%)[a,b,c]	(SD)[d]	Reduced-salt (1.2%)[a,b,c]	(SD)[d]	Half-salt (0.9%)[a,b,c]	(SD)[d]
Full-fat (32%)	1	8.20[aA]	4.25	10.60[aA]	5.92	10.90[aA]	4.93
	14	19.25[aA]	3.72	9.08[bA]	4.78	5.89[bA]	3.81
	28	20.84[aA]	4.97	15.80[abA]	5.23	11.48[bA]	7.40
	56	11.77[aA]	6.96	18.64[aA]	3.07	15.60[aA]	6.40
Reduced-fat (27%)	1	18.77[aB]	6.22	12.67[abA]	6.27	10.10[bA]	2.71
	14	16.50[aA]	9.56	10.01[aA]	9.54	11.78[aAB]	11.13
	28	20.96[aA]	11.24	15.44[aA]	10.20	22.43[aA]	14.21
	56	23.90[aAB]	7.52	25.70[aA]	9.95	23.12[aA]	12.81
Half-fat (16%)	1	21.74[aB]	5.60	14.12[abA]	5.60	13.35[bA]	4.21
	14	14.87[aA]	9.09	12.67[aA]	2.07	15.91[aB]	3.63
	28	16.30[aA]	3.38	13.30[aA]	5.11	16.80[aA]	4.78
	56	27.39[aB]	10.90	19.43[aA]	4.61	17.84[aA]	7.81

[a] The results shown are the average of three replicate trials.
[b] Values within a row relating to full-salt (FS), reduced-salt (RS) and half-salt (HS) cheese at each sampling point, not followed by the same lower-case letter significantly differ ($P < 0.05$).
[c] Values within a column relating to full-fat (FF), reduced-fat (RF) and half-fat (HF) cheese at each sampling point, not followed by the same upper-case letter significantly differ ($P < 0.05$).
[d] SD—standard deviation between three replicate trials.

Table 3
Lactate dehydrogenase activity (units/mL/min) in cheese juices from Cheddar cheeses with differing Fat contents and NaCl addition levels throughout ripening.

Cheese	Ripening time (days)	Salt content					
		Full-salt (1.8%)[a,b,c]	(SD)[d]	Reduced-salt (1.2%)[a,b,c]	(SD)[d]	Half-salt (0.9%)[a,b,c]	(SD)[d]
Full-fat (32%)	1	1.78^{aA}	0.68	0.77^{bA}	0.36	0.59^{bA}	0.17
	14	2.84^{aA}	1.03	0.94^{bA}	0.33	0.66^{bA}	0.18
	28	2.56^{aA}	1.53	1.48^{aA}	0.92	0.42^{bA}	0.15
	56	3.44^{aA}	0.58	1.52^{bA}	0.36	1.34^{bA}	0.66
Reduced-fat (27%)	1	3.01^{aA}	1.65	1.89^{aA}	1.22	1.26^{aB}	0.39
	14	4.05^{aA}	1.47	1.79^{bA}	0.71	1.95^{bB}	1.12
	28	1.44^{aA}	0.27	0.82^{bA}	0.13	0.63^{bA}	0.14
	56	4.02^{aA}	2.41	2.98^{aA}	1.21	3.68^{aB}	2.26
Half-fat (16%)	1	2.67^{aA}	1.16	1.00^{bA}	0.37	0.97^{bAB}	0.48
	14	2.52^{aA}	0.49	1.61^{bA}	0.52	1.45^{bAB}	0.37
	28	2.78^{aA}	0.88	1.53^{bA}	0.64	1.56^{bB}	0.58
	56	2.69^{aA}	0.82	2.03^{aA}	1.08	2.24^{aAB}	1.53

[a] The results shown are the average of three replicate trials.
[b] Values within a row relating to full-salt (FS), reduce-salt (RS) and half-salt (HS) cheese at each sampling point, not followed by the same lower-case letter significantly differ ($P < 0.05$).
[c] Values within a column relating to full-fat (FF), reduced-fat (RF) and half-fat (HF) cheese at each sampling point, not followed by the same upper-case letter significantly differ ($P < 0.05$).
[d] SD—standard deviation between three replicate trials.

Water activity (a_w) was the main compositional factor assessed in this work (Hickey et al., 2013; Møller et al., 2013). It has been previously shown to directly control microbial growth, starter permeabilisation and also influence the activity of various enzymes involved in flavour and texture development (O'Donovan et al., 1996; Sheehan et al., 2005; Wilkinson et al., 1994b). Generally, a_w was shown to increase significantly as a result of fat and salt reductions in Cheddar cheeses. This may be explained by significantly higher moisture levels found in such cheese variants (Broadbent et al., 2003; Fenelon et al., 2000; Mistry, 2001; Mistry and Kasperson, 1998). Furthermore, additional decrease in S/M levels, which is the key determinant of a_w in young Cheddar cheese (Guinee, 2004; Lopez et al., 2006; Schroeder et al., 1988), can also account for significantly higher a_w levels in reduced-fat and reduced-salt cheeses. A similar relationship between a_w and the salt content of Cheddar cheese has been reported in the most recent studies by Møller et al. (2013) and Rulikowska et al. (2013), where it was noted that cheeses with the lowest [0.5–0.9% (*w*/*v*)] NaCl addition levels had higher a_w (~0.975) than the corresponding cheeses with 3–3.7% (*w*/*v*) added NaCl (a_w ~0.950).

The growth dynamics of starter LAB were not significantly affected by either of the compositional alterations. Initially higher starter counts observed for the FF cheese variant in FS and HS cheese series could potentially reflect a greater degree of starter LAB retention in full-fat cheese curd, or the possibility of more starter cells being associated with the milk fat globule membrane in FF cheese. Those findings concur with the results previously reported by Laloy et al. (1996) in 50 and 100% reduced fat cheese and by Fenelon et al. (2000) in full-fat (33%) and low-fat (6%) Cheddar cheese, where the authors directly linked starter LAB viability to the fat content of Cheddar cheese. Lowering the salt content of cheese by 30 and 50% numerically increased starter populations in RS and HS cheeses, with lower counts detected in the FS equivalent. These results are in agreement with the findings by Rulikowska et al. (2013); Schroeder et al. (1988), and Mistry and Kasperson (1998), and could be attributed to the lower degree of starter culture inhibition as a result of increased a_w levels and lower S/M levels in such cheeses. Overall, salt and fat reduction, together with the cheese ripening time, exerted no significant impact on the growth dynamics of this particular mixed strain commercial starter among the FFFS control and RF, HF, RS and HS experimental cheeses. Similar observations have been recently made by Møller et al. (2012) in reduced-salt Cheddar cheeses with added salt levels of 0.9, 1.3, 1.8 and 2.4% (*w*/w). Authors reported high viability, up to day 90 of ripening, for a commercial starter preparation (F-DVS R604, Chr Hansen's) and also showed that cheeses with a higher [4.8% (*w*/w)] salt addition level had similar viability to lower salt cheese. Therefore, these findings along with our data suggest a degree of tolerance by this mixed strain DVS preparation in response to compositional alteration in fat and salt levels, and also cheese ripening conditions.

Previous work on the factors affecting NSLAB populations during cheese ripening has shown that levels of moisture, salt and pH of the final cheese product have relatively little effect on NSLAB growth dynamics, providing that compositional factors are in the range for a good quality Cheddar cheese (Lane et al., 1997; Lawrence et al., 1999). However, in the present study, fat reduction by 50% had shown to significantly delay initial NSLAB propagation. This delay in the initial NSLAB growth may reflect the lack of sufficient energy sources (e.g. liberated glycoprotein molecules) available following fat reduction in cheese (Banks et al., 1989; Kelly et al., 1996; Williams et al., 2000). Furthermore, the substantial increase in free amino acid concentration and the higher fraction of cell lysate components, both used as energy sources by NSLAB, could potentially explain why the fat content of cheese had no significant impact on NSLAB growth at the later stages of ripening (Thomas, 1987). Salt reduction, on the other hand, had no significant impact on NSLAB viability in cheeses with RF and HF content, potentially reflecting the increase in a_w levels and subsequent further decrease in S/M levels (Banks, 2004; Fenelon et al., 2000; Mistry and Kasperson, 1998). Higher NSLAB counts in FF cheese with RS and HS content could indicate the presence of more salt sensitive species of

Table 4
Recommended fat and salt combinations for optimal performance of commercial R604Y mixed strain starter used in this study.

Starter performance criteria:	Cheese code	Starter permeabilisation and cell death		Starter autolysis	
		Permeabilised cells	Dead cells	Pep X activity	LDH activity
Control cheese	FFFS	10–16%	14–30%	8–21 μmol AMC/mL/min	1.78–3.44 units/mL min
Experimental cheeses	RFRS	10–14%	12–29%	10–25 μmol AMC/mL/min	0.82–2.98 units/mL/min
	RFHS	3–13%	1–24%	10–23 μmol AMC/mL/min	0.63–3.68 units/mL/min
	HFRS	8–15%	12–25%	12–19 μmol AMC/mL/min	1.00–2.03 units/mL/min
	HFHS	9–12%	14–27%	13–18 μmol AMC/mL/min	0.97–2.24 units/mL/min

NSLAB in these cheeses, also supporting the findings of Rulikowska et al. (2013) and Møller et al. (2013). In contrast to starter LAB growth patterns, NSLAB populations increased significantly over the ripening period among all cheese variants and those trends coincide with the previously reported NSLAB growth dynamics (Hickey et al., 2006; Rulikowska et al., 2013; Sheehan et al., 2005; Wilkinson et al., 1994a).

Various studies in the field of cheese microbiology have recorded the presence (Bunthof et al., 2001; Sheehan et al., 2005) and potential importance of metabolically active but yet un-culturable cells in cheese during ripening (Bunthof et al., 2001; Doolan and Wilkinson, 2009; Jacques and Hunt, 1980; O'Leary and Wilkinson, 1988; Sheehan et al., 2005). In this study, FCM analysis revealed the predominance of a live/intact cell sub-population throughout the whole ripening period, which may reflect the sustained viability of one particular strain in this DVS multi-strain starter culture preparation. Similar findings have been previously reported by Wilkinson et al. (1994a), who noted that a defined mixed-strain starter culture comprising two strains of *L. lactis* subsp. *cremoris* G11 and *L. lactis* subsp. *cremoris* C25 retained high viability throughout a 63 day ripening period. However, increasing levels of the autolytic marker enzyme LDH, were released indicating differences in viability and autolytic properties between the strains. Generally, FCM analysis has shown no significant impact of fat and salt reductions by 30 and 50% on overall fractions of live/intact, permeabilised or dead cells between control and experimental cheeses, which strongly supports the results of the starter viable count data carried out by standard plate count method. Reducing the salt content of cheese did not significantly affect starter cell permeablisation or cell death, yet a numerically lower percentage of intact/live cells with a higher percentage of permeabilised and dead cells were found in cheeses with higher salt contents. This trend, similar to that reported by Rulikowska et al. (2013) in full-fat Cheddar cheeses with 0.5%, 1.25% and 1.8% salt addition, was consistent with starter growth inhibition at higher salt addition rates as a result of increased starter cell autolysis in cheese and in this case autolysis of one particular strain within the culture blend (Wilkinson et al., 1994a). Based on previous studies by O'Donovan et al. (1996) and Rulikowska et al. (2013) it would be expected that starter cell permeabilisation and cell death should be reduced on lowering of salt content of cheese. However, it was noted that the impact of salt reductions on permeabilised and dead cell fractions was less pronounced following reductions in fat content of Cheddar cheese by 30 and 50%, respectively. These results suggest the potential of fat to act as a counter agent to salt, which in RS and HS cheeses may result in similar fractions of intact/live, permeabilised and dead cells in certain experimental cheeses and FFFS control. It is possible that one of the strains is autolysing considerably faster in the reduced-fat environment and, in turn, increasing the total percentage of permeabilised and dead cells in RS and HS cheeses to levels comparable to those of the FFFS control. Two scenarios can be proposed as to why this culture may autolyse quicker in a lower fat environment. Firstly, starter cells may lose the ability to form protectant molecules (e.g. cyclopropane fatty acids) derived from fat (Jacques and Hunt, 1980; O'Leary and Wilkinson, 1988; Smittle et al., 1972) that can act as protective agents for LAB against various environmental stresses (Johnsson et al., 1995). Secondly, the compact and high density protein cheese structure itself, as a result of fat reductions, can cause autolysis of one or both strains in this culture blend (Banks, 2004; Guinee et al., 2000).

Intracellular Pep X and LDH activities have been widely used as an indicator of cell autolysis in Cheddar cheese (Doolan and Wilkinson, 2009; Fenelon et al., 2000; O'Leary and Wilkinson, 1988; Sheehan et al., 2005) and Pep X activity in particular contributes to a multi-enzyme debittering mechanism. It is generally recognised that high levels of released Pep X, either from permeabilised or autolytic cells, are a desirable characteristic for a commercial starter preparation having potentially beneficial impacts on overall cheese quality (Fox and Wallace, 1997; McSweeney, 2004; O'Donovan et al., 1996; Wilkinson et al., 1994b). Fat reductions, in this study, were shown to increase intracellular Pep X release into the matrix of FS cheese series. No particular explanation can be given for this data, only that the conditions for permeabilisation or starter cell autolysis may be more favourable once fat content in FS cheese is lowered. In terms of salt reductions, a significant decrease in Pep X activity was observed in the FF cheese series. The impact of various salt addition rates on trends noted for Pep X activity, especially in the FF cheese series, supports data from Wilkinson et al. (1994b) and also Rulikowska et al. (2013), indicating the importance of salt content as a factor in contributing to starter autolysis. No differences, however, were observed for RF and HF cheeses irrespective of salt levels added. These results indicate that reduction in fat content of cheese may be an essential step for obtaining starter autolysis levels comparable to those in FFFS control cheese. Similarly, LDH activity was also enhanced as a result of fat reduction in the HS cheese series. This data, however, is not in agreement with the findings by Fenelon et al. (2000) in full-salt Cheddar cheese with variable fat levels. These authors found the highest LDH activity throughout ripening in cheeses with FF (33%) content compared with lower activity in cheese juices with RF (22%) and HF (17%) contents. Differences between both studies may be explained by the use of different starter systems, and most importantly, variation in cheese composition, as both fat and salt levels were altered in the present study. Lower LDH activity reported in HS cheese throughout ripening of FF cheese is in agreement with previous studies by Wilkinson et al. (1994a); Rulikowska et al. (2013), and Møller et al. (2013), reflecting the higher viability, and lower cell permeabilisation and cell death as the salt content of cheese is lowered. However, similar to Pep X activity, those trends were less pronounced in RF and HF cheese series. This is most likely due to enhanced cell permeabilisation as a result of fat reduction, which may ultimately lead to enhanced intracellular LDH release into the cheese matrix of RS, and HS cheeses.

5. Conclusions

Overall, this study has highlighted the necessity of evaluating cheese starter performance in compositionally altered cheese matrixes, as salt and fat reduction in Cheddar cheese was shown to significantly impact various aspects of starter performance, namely starter cell permeabilisation, cell death, and subsequent cell autolysis. Generally, reductions in fat and salt content significantly increased a_w levels in various cheeses. Those subsequently further decreased S/M levels, which was reflected in slightly higher starter populations in cheese with lower salt concentrations. However, fat and salt reductions by 30 or 50% had no significant impact on overall commercial mixed strain starter viability. Reducing the fat content of cheese, on the other hand, significantly delayed NSLAB populations in HF cheeses, while lowering salt addition rates only had a slight impact on NSLAB viability. Lowering salt addition rates was shown to marginally impact on cell permeabilisation and cell death with a significant decrease in overall cell autolysis in FF Cheddar cheese. Reduction in fat levels by 30 and 50% in RS and HS cheese series appeared to counteract the effect of salt reduction, where lower cell permeabilisation, cell death and cell autolysis was to be expected, and provided an environment to enable a comparable performance of this commercial mixed starter in terms of permeabilisation potential, cell death and cell autolysis between control FFFS cheese and the experimental cheeses with lower-salt and fat levels. Based on those findings, we propose that combined fat and salt ratios of RFRS, RFHS, HFRS, and HFHS could enable optimal performance of R604Y starter in reduced fat and reduced salt cheese.

This study also indicates that it may be possible to reduce fat and salt content of Cheddar cheese by 30 and 50%, without largely altering permeabilised and dead cell fractions and in turn the amount of released intracellular Pep X activity, such that those starter performance parameters are similar to those found for good quality standard fat and salt Cheddar cheese.

Additionally, FCM analysis, in this study, has emphasised the limitations of this technique when using a multi-strain starter, as current FCM staining techniques do not yet allow for individual strain-specific staining to be performed. Additionally, autolytic behaviour by an individual starter strain within a mixed culture is not yet amenable to FCM analysis. In fact complete cell autolysis is not possible to be measured by FCM due to extensive cell disruption/disintegration. This is well illustrated in the case of released intracellular Pep X and LDH where either numerical or statistically significant differences could be seen in terms of salt reduction in FF, RF, and HF cheeses indicating a degree of autolysis by one or other of the strains in the DVS-R604Y culture.

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