

## Accepted Manuscript

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C.D. Hickey, B.W.K. Diehl, M. Nuzzo, A. Millqvist-Feurby, M.G. Wilkinson, J.J. Sheehan

PII: S0963-9969(17)30653-1  
DOI: [doi:10.1016/j.foodres.2017.09.067](https://doi.org/10.1016/j.foodres.2017.09.067)  
Reference: FRIN 7016

To appear in: *Food Research International*

Received date: 19 July 2017  
Revised date: 31 August 2017  
Accepted date: 24 September 2017

Please cite this article as: C.D. Hickey, B.W.K. Diehl, M. Nuzzo, A. Millqvist-Feurby, M.G. Wilkinson, J.J. Sheehan, Influence of buttermilk powder or buttermilk addition on phospholipid content, chemical and bio-chemical composition and bacterial viability in Cheddar style-cheese. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. *Food Research International* (2017), doi:[10.1016/j.foodres.2017.09.067](https://doi.org/10.1016/j.foodres.2017.09.067)

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**Influence of Buttermilk Powder or Buttermilk addition on Phospholipid content, chemical and bio-chemical composition and bacterial viability in Cheddar style-cheese**

**C. D. Hickey,<sup>a,b,\*</sup> B. W. K. Diehl,<sup>c</sup> M. Nuzzo,<sup>d</sup> A. Millqvist-Feurby,<sup>d</sup> M. G. Wilkinson,<sup>b</sup>  
and J.J. Sheehan<sup>a,\*</sup>**

<sup>a</sup> *Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland*

*(Tel.: +353(0) 25 42232; e-mail: [Cian.Hickey@teagasc.ie](mailto:Cian.Hickey@teagasc.ie); [Diarmuid.Sheehan@teagasc.ie](mailto:Diarmuid.Sheehan@teagasc.ie))*

<sup>b</sup> *University of Limerick, Castletroy, Limerick, Ireland*

*(Tel.: +353 (0) 61 213440; e-mail: [Martin.Wilkinson@ul.ie](mailto:Martin.Wilkinson@ul.ie))*

<sup>c</sup> *Spectral service AG, Emil-Hoffmann-Straße 33, 50996 Köln, Germany*

*([Diehl@spectralservice.de](mailto:Diehl@spectralservice.de))*

<sup>d</sup> *RISE-Research institutes of Sweden, Stockholm, Sweden*

*([Anna.Feurby@ri.se](mailto:Anna.Feurby@ri.se))*

*\*Corresponding author*

**Abstract**

The effect of buttermilk powder addition post curd formation or buttermilk addition to cheese milk on total and individual phospholipid content, chemical composition, enzyme activity, microbial populations and microstructure within Cheddar-style cheese was investigated. Buttermilk or buttermilk powder addition resulted in significant increases in total phospholipid content and their distribution throughout the cheese matrix. Addition of 10 % buttermilk powder resulted in higher phospholipid content, moisture, pH and salt in moisture levels, and lower fat, fat in dry matter, *L. helveticus* and non-starter bacteria levels in cheeses. Buttermilk powder inclusion resulted in lower pH4.6/Soluble Nitrogen (SN) levels and significantly lower free amino acid levels in 10 % buttermilk powder cheeses. Buttermilk addition provided a more porous cheese microstructure with greater fat globule coalescence and increased free fat pools, while also increasing moisture and decreasing protein, fat and

pH levels. Addition of buttermilk in liquid or powdered form offers potential for new cheeses with associated health benefits.

## 1. Introduction

The potential health benefits of phospholipids and glycoproteins have been well documented in recent years (Spitsberg 2005, Vanderghem *et al.* 2010, Küllenberg *et al.* 2012, Verardo *et al.* 2017). The link between these components and benefits such as improved cognitive development (Naor *et al.* 1998, McDaniel *et al.* 2003, Radlowski *et al.* 2015), reduced risk of infection and toxicity (Anand *et al.* 1999, Fuller *et al.* 2013), competitive antigen binding (Sprong *et al.* 2002) and anti-adhesion properties (Ward *et al.* 2006, Dewettinck *et al.* 2008) have led to increased interest in their use in food. Phospholipids in milk are predominantly (60-70 %) located in the milk fat globule membrane (MFGM) and make up 0.6-1% of the fat in bovine milk (MacGibbon and Taylor 2006). The MFGM is a tri-layer structure rich in proteins, phospholipids, glycoproteins, neutral lipids, enzymes and various smaller components (Danthine *et al.* 2000, Dewettinck *et al.* 2008, Vanderghem *et al.* 2010). The tri-layer structure consists of an inner monolayer of polar lipids with their hydrophobic tails in direct contact with the triglyceride (TAG) rich core. The outer bi-layer, which originates from the secretory cell apical plasma membrane, consists of polar lipids arranged with their hydrophilic head groups in contact with the aqueous phase of milk (Keenan and Mather 2006, Vanderghem *et al.* 2010).

Destruction of the MFGM via heating, centrifugation, churning or chemical processing results in the release of phospholipid rich material into the aqueous fraction of milk or cream (Gallier *et al.* 2010). Buttermilk (BM) results from the churning or exposure of a high fat cream fraction to high shear rates, resulting in destabilisation of the o/w emulsion and

subsequent phase separation. The exposed TAG components coalesce due to their hydrophobic nature forming butter grains which are further combined to produce butter (> 80% total fat content). The aqueous side stream of butter making, known as buttermilk, is naturally rich in proteins, polar lipids, glyco-proteins and other MFGM components (Govindasamy-Lucey *et al.* 2006). Buttermilk is viewed as a low-value side-stream which is still not fully valorised. The concentration of buttermilk and subsequent spray drying results in a stable, PL rich buttermilk powder (BMP).

The possibility of increasing the phospholipid content in everyday foods including various dairy products may have potential benefits for the end consumer (Spitsberg 2005, Ward *et al.* 2006). Addition of condensed sweet buttermilk to cheese milk prior to manufacture of pizza cheese has been investigated previously by Govindasamy-Lucey, *et al.* (2006) (2007), who found increased rennet coagulation time (RCT) and moisture content, decreased curd strength, decreased protein and fat levels and reduced phospholipid recovery (PL) despite the addition of the PL rich ingredient. Turcot, *et al.* (2001) investigated the effect of buttermilk and subsequent phospholipid addition on low-fat Cheddar cheese manufacture. Moisture levels increased significantly as PL content increased. Increased PL content in the cheese milk increased fat losses to the resulting cheese whey and increases in gross cheese yields were solely due to moisture retention in the cheese. Similar results were also obtained by Morin *et al.* (2008), who investigated the effect of heat treatment of cream prior to buttermilk generation for gel/curd strength, moisture content, protein and fat levels. However, they reported significant differences in PL levels between control and experimental model cheese systems with 6 times more phospholipids on average in cheeses made with BM addition compared to their skim milk counterparts, contradicting the findings of the previous studies. The variations observed in previous studies for PL retention in cheese upon

buttermilk addition to cheese milk, highlights the need for alternative methods to increase PL content in cheese.

Increased moisture levels observed in cheese made using sweet cream buttermilks is likely to be partly due to the amphipolar nature of phospholipids, which possess a hydrophilic head and two hydrophobic tails, allowing PL to act as microscopic emulsifiers in oil-in-water emulsions. The presence of denatured whey proteins in the BM, due to high heat treatments of cream prior to butter formation may also result in increased moisture due to the formation of casein- whey complexes. The denaturation of  $\beta$ - lactoglobulin can result in interactions with casein (CN) micelles and  $\kappa$ - CN, forming complexes, which can increase rennet coagulation time (RCT) and impair syneresis, due to reduced accessibility of the coagulant to  $\kappa$ - CN (Govindasamy-Lucey *et al.* 2006). Romeih *et al.* (2012) investigated the effect of MFGM material, originating from BMP or skim milk powder, on the microstructure of low-fat Cheddar cheese and found BMP addition to cheese milk, resulted in decreased cheese hardness compared to control cheese. BMP addition resulted in a denser and smoother protein matrix with uniform globular fat dispersion, improving the overall texture in comparison to control low- fat Cheddar cheese.

Consideration is also necessary of the potential for MFGM material partitioned inhomogeneously within the cheese matrix to create variability in cheese moisture, S/M, protein and fat levels possibly resulting in ripening hotspots within the cheese matrix with potential for localised differences in lipolysis and/ or proteolysis, resulting in possible variations in cheese texture and flavour which may have a positive or negative effect on the overall resulting cheese. The effect of MFGM material, glycoprotein and significant carbohydrate addition due to the use of BMP/BM on bacterial viability is also of importance, as previous studies suggest starter and non-starter bacteria may utilise these materials as a

possible energy source, potentially influencing ripening of the resulting cheese (Moe, et al. 2012, 2013).

The use of confocal microscopy for the localisation of bacteria, fat and protein material in food systems has been well established in recent years (Ong *et al.* 2011, Huc *et al.* 2013, C. D. Hickey *et al.* 2015, Cian D. Hickey *et al.* 2015).. Previous applications of CRM in cheese research are limited but it has gained some attention in recent years. Roeffaers *et al.* (2011) identified Raman microscopy as a tool capable of identifying the distribution of fat, protein and water in Swiss- type cheese. However, the study focused on Raman microscopy techniques and methodology specifically. Burdikova *et al.* (2015) utilised the technique to distinguish differing components within a defect 'pink' section of cheddar cheese including fat, protein and  $\beta$ -carotenoids, which was identified as a potential cause of the pinking defect in Cheddar style cheese. Smith *et al.* (2017) utilised Raman microscopy to identify tri-sodium citrate, corn starch and paprika in processed cheese. This current study utilised both confocal laser scanning microscopy (CLSM) and confocal Raman microscopy (CRM) to determine the location of phospholipids within the cheese matrix of experimental cheeses.. The technique has not previously been used to identify the distribution of phospholipids within a cheese matrix.

The objective of this study was to investigate the effect of BMP addition, after whey separation, i.e. at the point of salting in Cheddar style cheese manufacture, on PL content, composition and distribution, and on cheese chemical composition, microbial populations, enzyme activity and protein breakdown in comparison to Cheddar made with cheese milk fortified with buttermilk, as well as to a control Cheddar cheese.

## 2. Materials and methods

## 2.1 Starter cultures

A starter culture blend consisting of strains of *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *Streptococcus thermophilus* (A2055) and an adjunct culture *Lactobacillus helveticus* (LH-B02) were sourced (Chr. Hansen Ltd, Little Island, Co. Cork, Ireland) as frozen DVS concentrates and stored at -80 °C until cheese manufacture.

## 2.2 Cheese manufacture

Three replicate cheesemaking trials, each consisting of four vats of 454 kg cheese milk were undertaken over a 2 month period. Raw milk and buttermilk were obtained from a local dairy company. Cheese milk was standardised to a protein: fat ratio of 0.95:1 by blending of appropriate quantities of skim milk and cream or buttermilk and ultra-filtration (UF) retentate obtained from skim milk. The UF retentate was prepared by concentration to > 5 % protein of skim milk via ultra-filtration (Single module ultra-filtration unit, GEA Process Engineering A/S, Skanderborg, Denmark, 14 m<sup>2</sup> spiral wound membrane, 10 kDa molecular weight cut-off, Koch membrane systems, Massachusetts, USA). The cheese trials are summarized as follows:

- Control: Cheddar cheese
- BM cheese: cheese milk comprising 70 % milk + 30 % buttermilk
- 5 % BMP cheese: 5 % buttermilk powder (BMP) added to curd (w/w) during salting
- 10 % BMP cheese: 10 % BMP added to curd (w/w) during salting

Cheese milks were held overnight at < 10 °C, pasteurized at 72 °C for 15 s, and pumped at 32 °C into cylindrical, jacketed, stainless steel vats (500L) with automated variable speed cutting and stirring equipment (APV) Schweiz AG, Worb, Switzerland). Cheese milk was inoculated with A2055 (0.005 % w/v) and LH-B02 (0.004% w/v). After a 60 min ripening period, chymosin (Chymax plus, Chr. Hansens Ltd., 200 IMCU.mL<sup>-1</sup>), diluted ~1:6 with de-





Phospholipid analysis was conducted on standardized cheese milk, buttermilk, cream and buttermilk powder, cheese whey and experimental cheeses. Analysis of cheese samples was conducted at 7 and 60 d ripening using a  $^{31}\text{P}$ - NMR method according to SAA-MET002-03 (McGill 2012) using a Bruker Avance III 600 MHz equipped with an automatic sample changer and cQNP probe. An internal standard of Triphenyl phosphate was used (Spectral services AG., Germany). Liquid samples were freeze dried followed by a subsequent soxhlet extraction, while cheese samples were subjected to a soxhlet extraction only.

## 2.5 Enumeration of starter and non-starter bacteria

During cheese manufacture, curd samples were removed aseptically prior to salting. Further samples were acquired aseptically at 7, 14, 21, 28, 60, 90, 120, 150 and 180 d of ripening. Samples were placed in a sterile stomacher bag, diluted 1:10 with sterile 2 % (w/v) trisodium citrate and homogenized in a stomacher (Stomacher, Lab-Blender 400, Seward, Thetford, Norfolk, UK) for 10 min. Further serial dilutions were prepared as required using maximum recovery diluent (MRD).

Viable mesophilic bacterial cells (*L. lactis*, *L. cremoris*) were enumerated on lactose-M17 agar after aerobic incubation at 37 °C for 3 d (Terzaghi and Sandine 1975), mean viable counts of the adjunct *L. helveticus* were enumerated on MRS agar pH 5.4 after anaerobic incubation for 3 d at 42 °C (IDF 1988b), Enterococci cells were enumerated on kanamycin aesculin azide (KAA) agar after aerobic incubation at 37 °C for 24 hrs, Coliform bacterial levels were enumerated on violet red bile agar (VRBA) after aerobic incubation at 37 °C for 24 hrs and non-starter lactic acid bacteria (NSLAB) were enumerated on LBS agar with an LBS agar overlay after incubation at 30 °C for 5 d (Rogosa *et al.* 1951). Thermophilic streptococci bacteria were not enumerated during the study.

## 2.6 Lactate dehydrogenase analysis

Autolysis of starter cultures in cheese during ripening was monitored in triplicate by assaying for the release of the intracellular enzyme, lactate dehydrogenase (LDH) as described by Cogan *et al.* (1981). Sampling time points correspond to those for starter bacteria enumeration. Both lactobacilli and lactococci contain the enzyme LDH. However, lactococcal LDH requires a co-factor, fructose-1,6 bis-phosphate (FBP), for activation (Hannon *et al.* 2003, Yarlagadda *et al.* 2014). Activity was expressed as units per mL of extract, where one unit was defined as the amount of enzyme that catalyses the oxidation of 1 mM NADH per min.

## 2.7 Proteolysis

### 2.7.1 Primary and secondary proteolysis

Levels of primary proteolysis, expressed as % pH 4.6 soluble N/TN (% pH 4.6 SN/TN), were measured by the methods described by Kuchroo and Fox (1982) and (Sheehan and Guinee 2004). Nitrogen was determined by the macro-kjeldahl method (IDF 1993). Secondary proteolysis was determined via the quantification of free amino acids present in a pH 4.6 SN extract at 7, 28, 60, 120 and 180 days of ripening. Analysis was carried out as described by Fenelon *et al.* (2000). Samples were deproteinised by mixing equal volumes of % pH 4.6 SN extract and trichloroacetic acid ( $240 \text{ g kg}^{-1}$ ). Free amino acids were separated using ion-exchange chromatography with post-column ninhydrin derivatisation and visible colorimetric detection.

### 2.7.2 Polyacrylamide gel electrophoresis

Urea-polyacrylamide gel electrophoresis was performed on cheese samples after 28, 120, 180 d of ripening, using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, Herts., UK) and the stacking gel system described by Andrews (1983). The gels

were stained directly by the method of Blakesley and Boezi (1977) using Coomassie Brilliant Blue G250.

## 2.8 Confocal microscopy

### 2.8.1 Confocal laser scanning microscopy

Phospholipids were identified using LipidTOX™ Red phospholipid detection reagent (excitation/emission maxima of 595/615 nm; HCS LipidTOX™ Phospholipidosis staining kit, Invitrogen molecular probes, Bio-sciences, Dublin, Ireland) and neutral lipids were identified using LipidTOX™ Green neutral lipid stain (excitation/emission maxima of 495/505 nm; HCS LipidTOX™ Phospholipidosis staining kit, Invitrogen molecular probes, Bio-sciences, Dublin, Ireland). Cheese samples were sectioned using a scalpel into 10 mm × 10 mm × 2 mm sections and incubated at room temperature with 20 µl of the aforementioned labelling dyes, mixed 1:1, applied to the surface. Images were acquired using the Leica TCS SP5 CLSM (Heidelberg GmbH, Mannheim, Germany), 63× (oil, Na 1.4) objective of a Leica TCS SP3 CLSM (Leica Microsystems, Heidelberg GmbH). Image analysis was carried out using ImageJ software (National Institutes of Health, Bethesda, Maryland) to compare relative fluorescent intensities for the various dyes.

### 2.8.2 Confocal Raman microscopy

Confocal Raman microscopy (CRM) analysis was performed with a WITec alpha300 (Ulm, Germany) system which has a lateral resolution of 250 nm and a vertical resolution of 500 nm. In this study we used a 532 nm laser for excitation. The sample spectra were fitted by a linear combination with the reference spectra (Lecithin) in each pixel to obtain the

relative occurrence of each component per pixel, from which an image was constructed based on the relative intensity of each component. The measured spectrum ( $s$ ) is described as:

$$S = B \times h + e$$

where  $B$  is the matrix of the reference spectra of the components,  $h$  is the vector providing the fractions of the components and  $e$  is the error spectrum.

The mixing values were fitted by the method of least squares minimizing the error:

$$(e)^2 = \text{Minimum}$$

As long as there are intensity differences in the peaks for different components, it is possible to generate an image describing the localization of the components.

Samples were analysed from Control and 5 % BMP cheeses by confocal Raman microscopy. The surface scan XY is a horizontal optical section. The area of analysis was 25  $\mu\text{m}$  x 25  $\mu\text{m}$ . Two samples were measured from the experimental cheeses. Each sample was measured in duplicate.

## 2.9 Statistical analysis

Analysis of variance (ANOVA) was performed using the general linear model (GLM) procedure (SAS 1995) where the effects of treatments (BMP addition or use of liquid buttermilk in cheese milk) and replicates were estimated for response variables relating to cheese composition, individual free amino acid profiles and fluorescence intensity of confocal images relating to phospholipid distribution. Duncan's multiple comparison tests was used as a guide for paired comparisons of the treatment means. The level of significance was determined at  $P < 0.05$ .

A split plot design was used to determine the effects of BMP addition at time of salting or the use of liquid buttermilk in cheese milk, ripening time and their interaction on microbial (mesophilic *Lactococci* cultures, *L. helveticus* and NSLAB viability) and biochemical (LDH enzyme activity, pH, total and individual free amino acids) parameters measured at regular intervals during manufacture and/or ripening.

ANOVA for the split plot design was carried out using a GLM procedure (SAS 1995). Statistically significant differences ( $P < 0.05$ ) between means were determined by Fisher's least significant difference. Application of a split plot design to determine the effects of the aforementioned treatments, ripening time and their interaction on the various parameters were performed as reported by Sheehan *et al.* (2007a).

### 3. Results and discussion

#### 3.1 Chemical composition

##### 3.1.1 Raw materials and cheese milks

The composition of cream, buttermilk, standardised cheese milks and BMP prior to cheese manufacture are shown in Table. 1. The standardised BM cheese milk had a significantly higher PL content ( $P < 0.05$ ) compared to the standardised cheese milk used for manufacture of the control, 5 % BMP and 10 % BMP cheeses. The levels of PL (g PL/ 100 g of fat) were significantly higher in the buttermilk ( $P < 0.05$ ) compared to the cream. The concentration of PL in the BMP is similar to levels reported by Sodini *et al.* (2006) for BMP of a similar fat content (1.29 g PL/ 100 g powder).

##### 3.1.2 Experimental cheeses

Levels of protein (26.91 %), moisture (36.15 %), fat (31 %), FDM (48.55 %) and salt (1.74 %) in the Control cheeses were similar to those reported previously for Cheddar- style

cheeses (Guinee *et al.* 2000, Ong *et al.* 2006). The addition of BMP had no significant effect on cheese MNFS compared to the control cheese; however the addition of liquid BM resulted in significantly ( $P < 0.05$ ) higher MNFS levels compared to all other cheeses (Table 2).

Cheeses with 10% BMP added at salting had significantly higher levels of moisture, salt and S/M levels compared to the control cheese, most likely due to the retention, by the BMP, of whey produced during curd syneresis and pressing, leading to greater moisture retention in the pressed curd and increasing salt retention as it is mixed with the powdered ingredient. The 5 % BMP cheese did not display the same level of moisture (despite similar MNFS values) or salt retention, possibly indicating that powder hydration level is a determining factor for salt retention in this case.

The results obtained suggest that it may be possible to significantly reduce the quantities of NaCl addition during cheese manufacture, made possible by the retention of  $> 85$  % of added NaCl on addition with 10 % BMP compared to  $< 65$  % retention observed in standard Cheddar cheese manufacture (Sutherland 1974). Addition of BMP could significantly reduce the quantity of salty whey produced during Cheddar cheese manufacture with associated economic cost reductions. As less salt is lost to the whey, there is a reduction in sodium content requiring demineralisation during subsequent isolation and production of high economic value whey products.

The addition of 30 % liquid BM to cheese milk prior to manufacture resulted in a cheese with significantly higher moisture content (39.45 %) in comparison to the control (equating to a 9 % increase in cheese moisture). This can be attributed to the presence of denatured whey proteins, resulting from high heat treatment of both the cream and subsequent buttermilk, forming casein-whey complexes reducing curd syneresis and also the presence of MFGM material including phospholipids which are capable of binding excess moisture (Corredig & Dalgleish, 1996; Govindasamy-Lucey, *et al.* 2006; Morin, *et al.* 2008; Pesic, *et al.* 2014)and.

Despite a significant increase in moisture levels there was no significant increase in salt content in comparison to the control cheese, similar to results reported by Govindasamy-Lucey *et al.* (2006) where buttermilk addition to cheesemilk had no significant effect on salt levels in pizza cheese. Standardisation of milk with buttermilk or the addition of BMP at salting resulted in a significant reduction ( $P < 0.05$ ) in levels of protein. The reduction in protein observed in the BM cheeses is most likely due to the significant increase in MNFS levels compared to all other cheeses, combined with a reduction in fat retention in comparison to the control cheeses, resulting in the loss of protein as indicated previously by (Govindasamy-Lucey *et al.* 2006, Guinee and McSweeney 2006, Hickey *et al.* 2017).

There was a significant ( $P < 0.05$ ) reduction in cheese fat in dry matter (FDM) levels with the addition of 5 % and 10 % BMP compared to the control cheese, while the BM cheese did not differ significantly from the control. This reduction in FDM levels is attributed to the addition of BMP, resulting in a dilution of the cheese fat content as indicated by increased protein: fat ratio in these cheeses (Table 2). Total fat levels were significantly lower ( $P < 0.05$ ) in the 10% BMP and BM cheeses. The cheese with 10 % BMP addition had a significantly higher pH at 14 d of ripening compared to all other cheeses, most likely due to significant levels ( $P < 0.01$ ) of un-metabolized and possibly un-hydrated lactose ( $2.03 \text{ g} / 100 \text{ g}^{-1}$ ) detected at 7 d of ripening compared to  $0.26$  and  $0.24 \text{ g} / 100 \text{ g}^{-1}$  in BM and 5 % BMP cheeses respectively (supplementary Fig. 1), in combination with high S/M levels which have been shown to result in higher pH levels (Upreti and Metzger 2007). The pH of the BMP (pH 6.68) may have also influenced cheese pH on addition to the cheese curd. Un-metabolized lactose was still detected at low levels ( $0.07 \text{ g} / 100 \text{ g}^{-1}$ ) after 60 d of ripening. The presence of unmetabolised lactose in the early stages of ripening may have been due to incomplete hydration of all lactose present due to the limited availability of moisture (whey) within the cheese matrix at that stage of ripening.

There was a significant interaction between treatment and time ( $P > 0.01$ ) for pH levels over 180 d of ripening (Fig. 1). There was a significant decrease ( $P > 0.05$ ) in pH for the 5 % BMP cheese over the 180 d ripening period with significantly lower ( $P > 0.01$ ) pH evident from 60 (Compared to control cheese) and 28 d (Compared to 10 % BMP cheese) of ripening onwards. A possible reason for difference in pH observed between the 5 and 10 % BMP cheeses could be the continued acidification of the 5 % BMP cheese due to addition of excess lactose which was sufficiently hydrated and hydrolysed, providing an energy source for both starter and NSLAB populations. The pH of the 10 % BMP cheese increased due to the addition of a larger quantity of a neutral pH powder (pH 6.6) and which, upon hydration of lactose and mineral components (calcium, phosphorous etc.), could cause an increase in cheese pH, while also increasing the buffering capacity of the cheese resulting in a difficulty in reducing the pH as ripening progressed (Fox *et al.* 1990, Salaün *et al.* 2005, Upreti and Metzger 2007, Boivin-Piché *et al.* 2016). BM cheese had a significantly lower pH compared to the control cheese ( $P > 0.05$ ) at 180 d and 10 % BMP cheese from 35 d of ripening onwards ( $P > 0.01$ ). Govindasamy-Lucey *et al.* (2007) indicated that BM addition (without modification of the cheese make procedure) resulted in lower pH compared to control cheese in pizza style cheese. No hypothesis was proposed for this lower pH but as the cheese making procedure was subsequently modified to standardize the moisture content across all cheeses and no difference in pH was observed. This suggests that it may have been due to the higher moisture and possible increase in microbial activity in that particular study.

### 3.2 Phospholipid content and composition

Cheeses with added BMP or made from milk supplemented with buttermilk had a significantly higher ( $P < 0.05$ ) concentration of phospholipids in comparison to the control cheese at 7 and 60 d of ripening, (The latter results are shown in Fig. 2). The most significant increases were in the 10 % BMP cheeses (47 % increase), followed by the BM cheeses (37 %



increase) and the 5 % BMP cheeses (23.5 % increase) in total phospholipid content compared to the control cheeses.

Significant differences ( $P < 0.05$ ) in individual phospholipids levels were also apparent between the control and experimental cheeses. Levels of phosphatidylethanolamine (PE) and sphingomyelin (SPH) were increased significantly ( $P < 0.05$ ) by the addition of BMP or liquid BM. Phosphatidylcholine (PC) levels were significantly higher ( $P < 0.05$ ) in the 10 % BMP cheese and in the liquid BM cheese compared to the control cheese. Phosphatidylinositol (PI) was not detected in any of the control cheeses at 0 or 60 d of ripening; testing of the cheese whey indicated that the limited quantity present in the standardised cheese milk (0.002 g/100g milk) was totally lost to the cheese whey during cheese manufacture (results not shown). This was not observed in the cheeses where BM was added to the cheese milk indicating the composition of the buttermilk may influence individual PL retention in the resulting cheese matrix. Phosphatidic Acid (PA) was not observed in the raw cream, buttermilk or in the various standardised milks used for cheese manufacture but was subsequently observed in all cheeses from 7 d of ripening and represented  $< 10\%$  of the total phospholipid in the control, BM and 10 % BMP cheese at 60 d of ripening. The formation of PA during cheesemaking may be due to the degradation of PC by phospholipase D to form PA and choline (Stace and Ktistakis 2006).

The results obtained in this study indicate the addition of BMP at the time of salting, as opposed to the cheese milk prior to manufacture, resulted in a greater increase in both total and individual phospholipid levels in the resultant cheeses. Furthermore, the increase in phospholipid content coincided with a 12 % reduction ( $P < 0.05$ ) in total fat levels in cheeses containing 10% BMP compared to the control cheeses. This offers potential for the development of reduced fat cheeses but with increased levels of bioactive ingredients such as phospholipids.

### 3.3 Viability of starter and NSLAB populations

#### 3.3.1 Mesophilic cultures

Levels of *Lactococcus* bacteria reached  $> 10^9$  cfu/g<sup>-1</sup> in all cheeses prior to cheddaring of the cheese curd (Fig. 3 A). The addition of BMP or the supplementation of cheese milk with BM did not significantly affect ( $P = 0.902$ ) the viability of mesophilic *Lactococcus* bacteria, with no significant difference observed between treatments at any point during the first 60 d of ripening.

#### 3.3.2 *Lactobacillus helveticus*

Viable counts of *L. helveticus* reached  $> 10^{7.5}$  cfu/g<sup>-1</sup> prior to cheddaring in all cheeses (Fig. 3 B), similar to levels reported by Hannon *et al.* (2003). Cell viability decreased significantly from 7 to 60 days of ripening in all cheeses ( $P < 0.001$ ). There was significant interaction between treatment and ripening time ( $P < 0.01$ ) with significantly lower viable counts in the 10 % BMP cheese compared to all other cheeses at 60 d. This may be due to the higher S/M levels attributed to salt retention as discussed in section 3.1.

#### 3.3.3 Non-starter lactic acid bacteria

NSLAB are adventitious microflora, consisting predominantly of mesophilic lactobacilli and pediococci, which can result from post pasteurisation contamination or due to incomplete inactivation of NSLAB during pasteurisation. There was a significant increase ( $P < 0.05$ ) in NSLAB populations over 180 d of ripening (Fig. 3 C) achieving counts of  $10^8$  cfu/ g<sup>-1</sup> (BM cheese),  $10^7$  cfu/ g<sup>-1</sup> (control) and  $10^6$  cfu/ g<sup>-1</sup> (5 % BMP cheese) at 180 d respectively. Counts observed in the control cheese were similar to those observed by McCarthy *et al.* (2015) for a similar style cheese. NSLAB levels increased steadily in these cheeses from 35 d of ripening onwards, with significant increases evident from 90 d onwards. Recent studies by

Moe *et al.* (2012), (2013) investigated the survival of *Lactococci* and NSLAB respectively in media containing MFGM material as the only energy source. Both studies found that *Lactococci* and common NSLAB bacteria could survive for an extended period of time on energy sources resulting from MFGM material, as seen in the BM cheese.

There was a significant interaction between treatment and time ( $P < 0.0001$ ) with the 10 % BMP cheeses displaying significantly lower levels ( $P < 0.01$ ) of NSLAB viability from 120 d onwards compared to all other cheeses. Lower NSLAB levels observed in the 10 % BMP cheese were most likely due to the high S/M levels ( $> 6\%$ ), which have been shown to retard NSLAB growth especially in the early stages of ripening (Jordan and Cogan 1993, Guinee and Fox 2004) and possibly be due to lower levels of free amino acids in this cheese throughout ripening. NSLAB are capable of utilising various amino acids and peptides as an energy source (Williams *et al.* 2001, Ur Rehman *et al.* 2003, Moe *et al.* 2013).

Enterococci and Coliform bacteria were also monitored throughout ripening and both were present at very low levels in the early stages of ripening ( $> 10^3$  cfu/ g<sup>-1</sup> and  $> 10^2$  cfu/ g<sup>-1</sup> respectively), but no viable counts for Enterococci or Coliform bacteria were detected after 35 d of ripening in any cheese (results not shown).

### 3.4 Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) is an intercellular enzyme found in the majority of bacterial species; especially those used in the food fermentation industry and is often used as a marker for cell lysis in cheese (Bunthof *et al.* 2001). The use of the co-factor FBP allowed for the monitoring of LDH activity in both *L. helveticus* (no FBP required) and *L. lactis* bacteria (FBP required). There was a significant increase in LDH activity for all treatments over 180 d of ripening for both *Lactococci* (FBP) and *Lactobacilli* (no FBP) bacteria (Fig. 4 A & B). The high level of salt in the 10 % BMP cheese could explain why LDH levels were not

significantly higher despite significantly lower viable cell counts, as high salt and S/M levels may result in the inactivation of LDH enzymes or potentially affect the bacterial cell directly preventing the release of intracellular enzymes, due to cell membrane rigidity, despite a loss in viability (Vafopoulou-Mastrojiannaki 1999, Guerzoni *et al.* 2001, Palomino *et al.* 2013, Hickey *et al.* In press). There was a significant increase ( $P < 0.05$ ) in *Lactococci* LDH activity (Fig. 4 B) from 90 to 120 d. Enzyme activity levels were significantly higher ( $P > 0.05$ ) in *Lactococci* bacteria in comparison to *Lactobacilli* bacteria throughout ripening, despite no significant decrease in *Lactococci* viability over the first 60 d of ripening. This may be due to differences in LDH enzymes between the 2 classes of bacteria or competition between the various strains of *Lactococci* and the adjunct *L. helveticus* bacteria. Although not significantly different, the 10 % BMP cheese recorded the lowest levels for both *Lactobacilli* and *Lactococci* LDH activity; which may be due to the high S/M levels observed in this cheese.

### 3.5 Proteolysis

#### 3.5.1 Primary proteolysis

Primary proteolysis is an indication of the initial breakdown of  $\alpha_{s1}$ - casein and  $\beta$ - casein molecules during cheese manufacture and ripening (Upadhyay *et al.* 2004, Sheehan *et al.* 2008). Breakdown is closely linked to activity of plasmin/ plasminogen, found naturally in milk, and the retention of residual coagulant such as chymosin as used in this study. There was a significant increase ( $P < 0.05$ ) in levels of primary proteolysis for all cheeses over the 180 d of ripening (Fig. 5) and levels of % pH 4.6 SN/ TN for the control and BM cheeses were similar throughout ripening and corresponded to those observed by McCarthy *et al.* (2016) for Cheddar cheese. There were significantly lower levels ( $P > 0.05$ ) of % pH 4.6 SN/TN observed in the 10 % BMP compared to the BM and control cheeses from 28 and 60

d of ripening respectively. The addition of 5 % BMP resulted in significantly lower levels ( $P > 0.05$ ) of % pH4.6 SN/TN compared to both the control and BM cheeses from 120 d of ripening onwards.

Similar trends were observed within a Urea-PAGE gel performed on samples at 7, 60 and 180 d of ripening (Fig. 6). There was evidence of  $\alpha_{s1}$ - casein breakdown in all cheeses as ripening progressed, with higher breakdown evident in the control and BM cheeses compared to the 5 % BMP and the 10 % BMP cheese, the latter of which showed limited breakdown of casein throughout ripening. There was limited breakdown of  $\beta$ - casein evident throughout ripening with a small reduction in the intensity of the  $\beta$ -casein band in the control and BM cheeses but no reduction evident in the 5 or 10 % BMP cheeses. The breakdown of  $\beta$ -casein is linked to the activity of plasmin and low levels of  $\gamma$ - CN formation indicates limited activity of the enzyme plasmin/ plasminogen, suggesting that residual chymosin is the main agent producing the levels of % pH 4.6 SN/TN observed.

The significantly lower levels of primary proteolysis observed in the BMP cheeses may be due to a number of possible factors, such as interactions between denatured whey proteins, caseins present in the cheese curd as discussed previously and the composition of the BMP. These factors may have also influenced the levels of proteolysis, as protein found in the BMP may not be readily available to residual coagulant enzymes such as chymosin or plasmin due to incomplete rehydration of the BMP and the casein micelles present within. Increased lactose concentration has previously been associated with slower casein hydration in milk powders (Sodini *et al.* 2004). The higher pH observed in the 10 % BMP cheese could result in decreased chymosin activity compared to cheeses at a lower pH (Sheehan *et al.* 2007b, Hickey *et al.* 2017). To the author's knowledge, no study has been previously conducted relating to the addition of powdered dairy ingredients to cheese curd and the subsequent effect on proteolysis.

### 3.5.2 Secondary proteolysis

#### 3.5.2.1 Total free amino acids

There was a significant influence of ripening time ( $P < 0.0001$ ) as total free amino acid (TFAA) levels increased significantly in all cheeses during ripening (Fig. 7). There was a significant interaction between treatment and ripening time with significantly lower levels ( $P < 0.001$ ) of TFAA in the 10 % BMP cheese compared to all other cheeses from 120 d onwards. Release of FAA's is closely linked to the activity of intracellular enzymes released by the starter and NSLAB populations within the cheese matrix, as observed for both the control and BM cheeses, as LDH levels were highest for these cheeses indicating higher levels of cell lysis and release of intracellular enzymes. Reduced FAA release in the 10 % BMP may be due to a combination of high S/M content, reduced levels of protein breakdown and lower enzymatic activity. The results indicate an apparent relationship between the level of BMP addition and rate of both enzyme activity and FAA release.

#### 3.5.2.2 Individual free amino acids

The addition of 10 % BMP resulted in a significant reduction ( $P > 0.05$ ) in all 15 individual free amino acids in comparison to both the control and BM cheeses at 186 d of ripening (Table 3). There were also significantly lower levels ( $P > 0.05$ ) of valine, methionine, phenylalanine, histidine, lysine and arginine in the 10 % BMP compared to the 5 % BMP cheese. The addition of 5 % BMP resulted in significantly lower levels of leucine and phenylalanine compared to the control and BM cheeses. The predominant FAA's in all cheeses at 186 d of ripening were glutamate, valine, leucine, lysine and proline, which are similar to the principle FAA's observed previously for Cheddar style cheese with the exception of proline (Wallace and Fox 1997, Fenelon *et al.* 2000). The use of *L. helveticus* as

an adjunct culture has been shown previously to increase the production of glutamic acid and proline (Gagnaire *et al.* 2001, Sheehan *et al.* 2008).

### 3.6 Confocal microscopy analysis

#### 3.6.1 CLSM

The use of modified fluorescent dyes allowed for the visualization of phospholipids within the cheese matrix. Image analysis indicated a significant increase ( $P > 0.05$ ) in fluorescence intensity of the phospholipid detection dye associated with the 5 % and 10 % BMP cheeses in comparison to the control cheese (Data not shown). A difference in phospholipid distribution is evident between the control cheese and all other cheeses, with phospholipids (red) located around the fat globules in the control cheese as expected, while there is evidence of phospholipids located throughout the protein matrix as well as in the surrounds of the milk fat globules particularly in the 5 and 10 % BMP cheeses and, to a lesser extent, in the BM cheeses (Fig. 8 A-D). There is evidence of large coalesced fat pools and areas of free fat in the BM cheeses compared to the control, 5 % and 10 % cheeses which show various sized fat globules with small areas of coalescence and limited evidence of free fat pools within the cheese matrix (Fig. 8 A-D).

#### 3.6.2 Confocal Raman microscopy

Confocal Raman microscopy was utilised to identify component distribution within the cheese matrix. Phospholipid distribution was clearly visible via CRM. Phospholipids (blue) in the control cheese appeared to be located within the lipid phase (green) of the cheese only (Fig. 9 C). A continuous protein phase was evident as an intense red colour of the protein phase (Fig. 9 C). Phospholipids in the 5 % BMP cheese appeared to be evenly distributed almost throughout the entire cheese sample (Fig. 9 B) indicating that PL's were present in

both the protein and lipid phases of the cheese samples (Fig. 92 D). This would indicate that the PL from the added BMP did not solely associate with the fat globules as they were also observed within the protein phase as indicated by the pink colour in image 1D, indicating that addition of BMP had a major influence on the distribution and location of phospholipids compared to the control cheese. The use of confocal Raman proved to be a useful tool in determining the location and distribution of phospholipids in cheese samples and would offer a sensitive analytical tool in future studies for observing specific cheese components within the matrix throughout ripening.

#### 4. Conclusion

This study aimed to investigate increasing the phospholipid content of Cheddar style cheese via the addition of BMP in comparison to the use of BM and also to control cheeses. The addition of BM or BMP (at the point of salting) resulted in a significant increase in total and individual PL and a significant reduction in total fat levels for BM and 10 % BMP cheeses compared to control cheese. The addition of BM and 10 % BMP resulted in significant increase in moisture content compared to the control cheese, with no difference in MNFS levels in the latter. The addition of BMP resulted in decreased levels of primary proteolysis possibly due to slow rehydration of casein in the buttermilk powder and possible reduction in chymosin activity due to the significantly higher pH on addition of 10 % BMP. Increased salt retention, significantly higher S/M and pH levels were observed in the 10 % BMP cheeses compared to all other cheeses. Significantly lower levels of total FAA's were detected for the 10 % BMP, possibly due to high S/M levels and lower levels of primary proteolysis products as substrates. Intracellular enzyme activity appeared unaffected by the addition of BM or BMP despite differences in *L. helveticus* viability. In comparison to the



BM cheeses, BMP addition resulted in moisture and MNFS levels closer to that of the control cheese. Overall the addition of BMP had a positive effect on cheese phospholipid content thus increasing levels of potentially health benefitting bioactive components and lowering fat content. This may offer new opportunities in relation to the emerging healthy cheese market for a cheese product promoting potential health benefits. It also allows for the possible valorization of a low value commodity such as buttermilk powder and creates areas for further research into the use of milk powders post curd formation as a method of improving the benefits of cheese within the diet.

#### Acknowledgment

This research was funded by the Dairy Levy Trust, Ireland.

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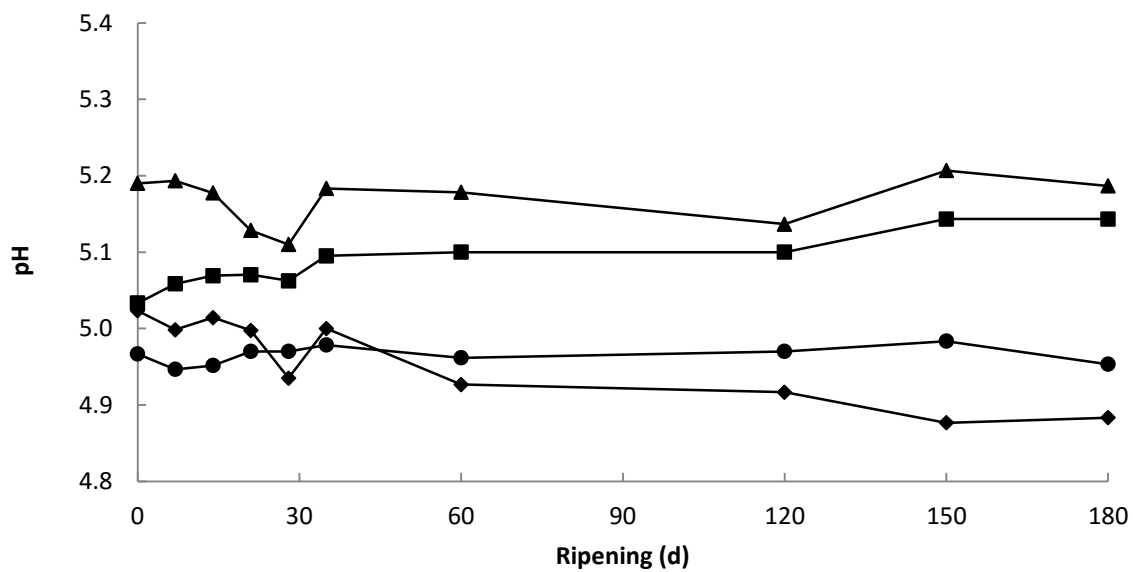
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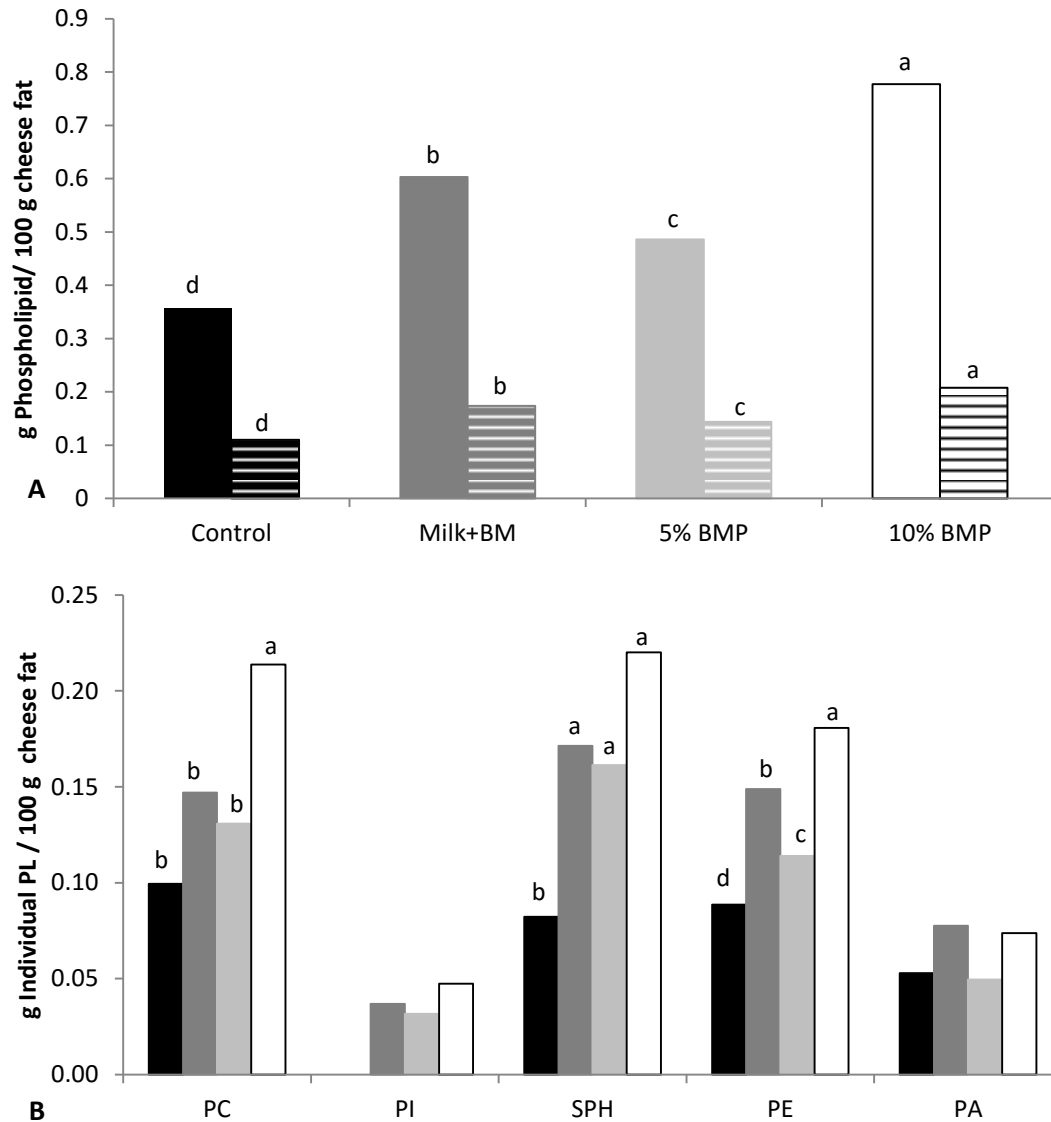
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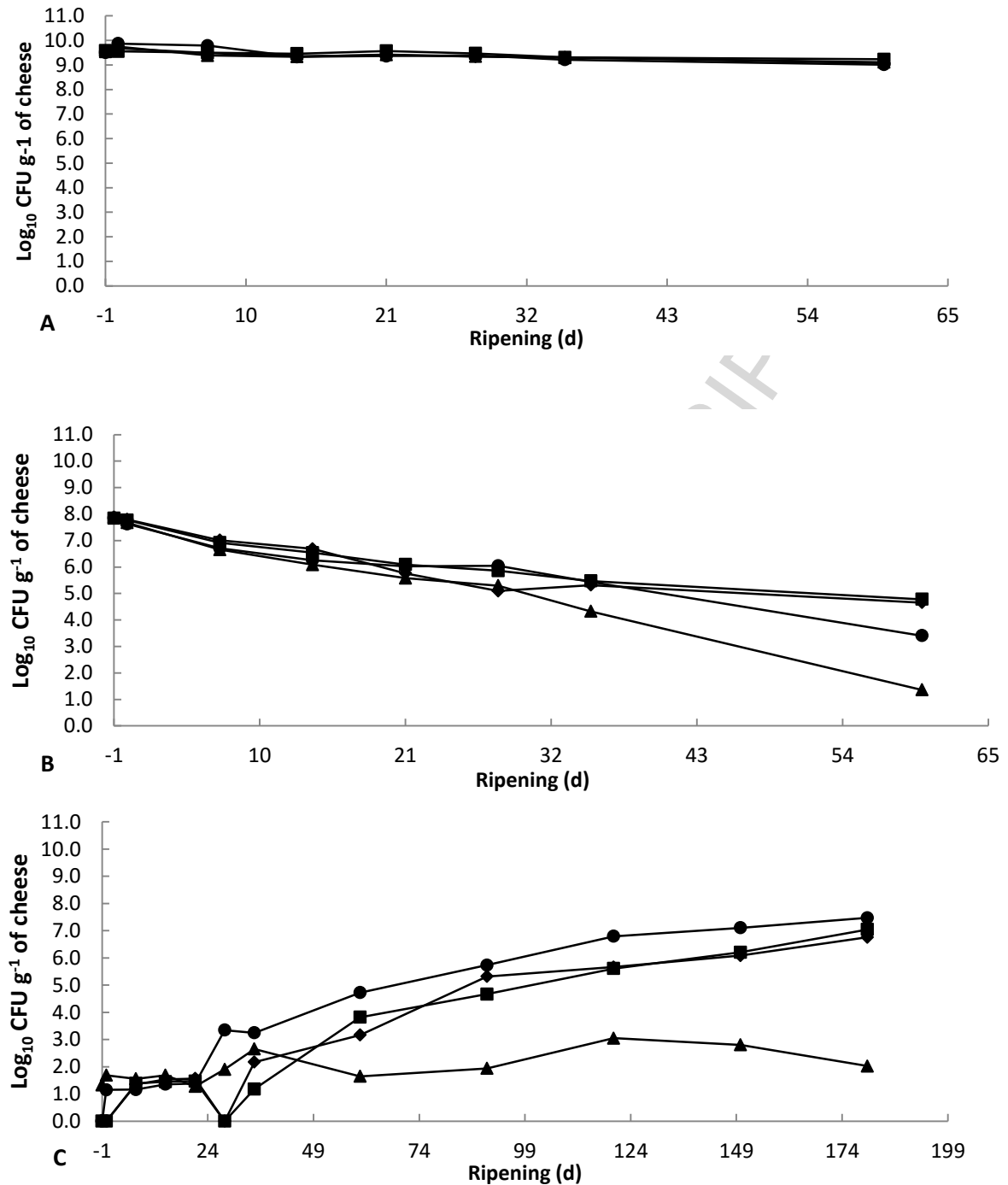
## Figures



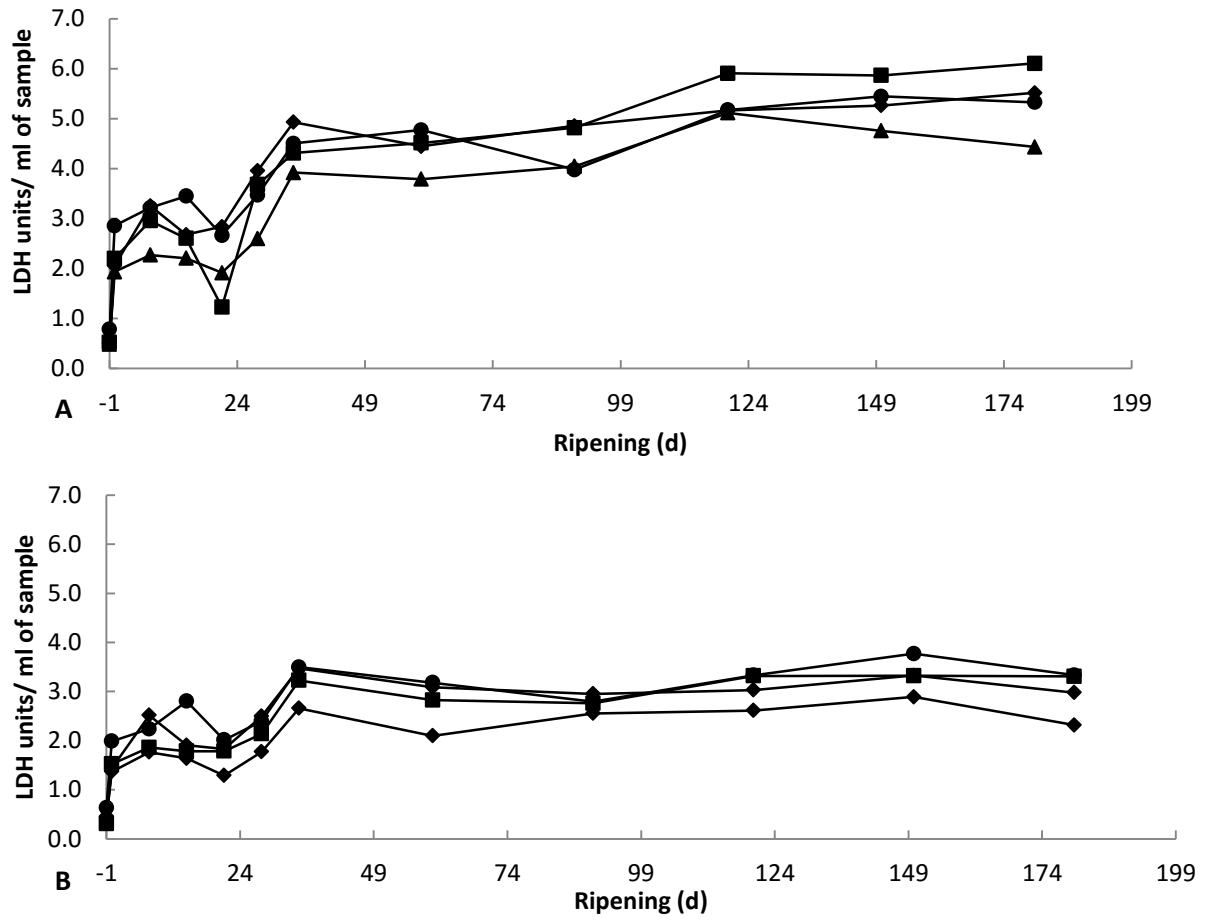
**Fig. 1** pH of control (■), BM (●), 5 % BMP (◆) and 10 % BMP (▲) cheeses over 180 d of ripening. Values presented are the means of three replicate trials.



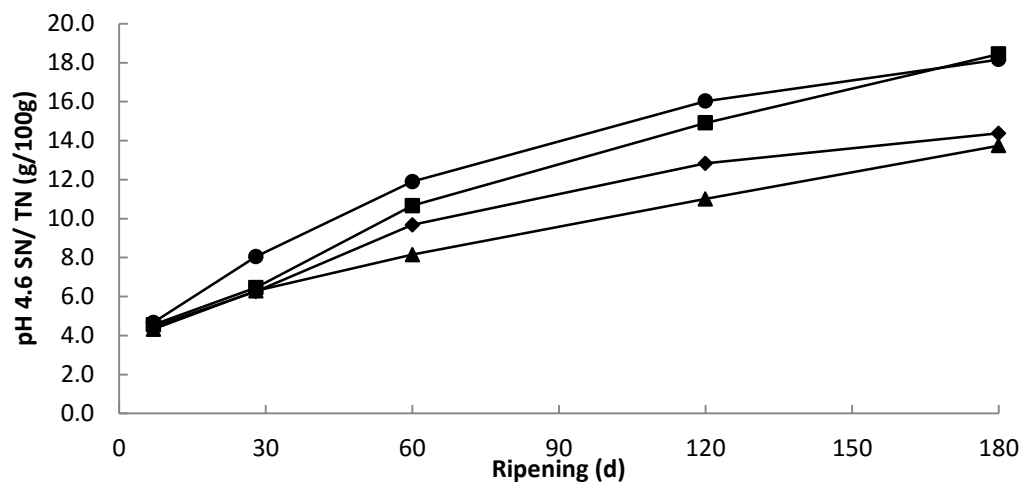
**Fig. 2** Levels of total Phospholipids expressed as (A) g/ 100 g cheese fat (■) and as g/ 100 g of cheese (▨) and individual phospholipids (B) as g/ 100g of cheese fat in Control (■), BM (■), 5 % BMP (■), 10 % BMP (□) cheeses at 60 d of ripening. Values presented are the means of three replicate trials. Letters (a-d) indicate significant ( $P > 0.05$ ) differences within series. PC = Phosphatidylcholine, PI = Phosphatidylinositol, SPH = Sphingmyelin, PE = Phosphatidylethanolamine, PA = Phosphatidic Acid.



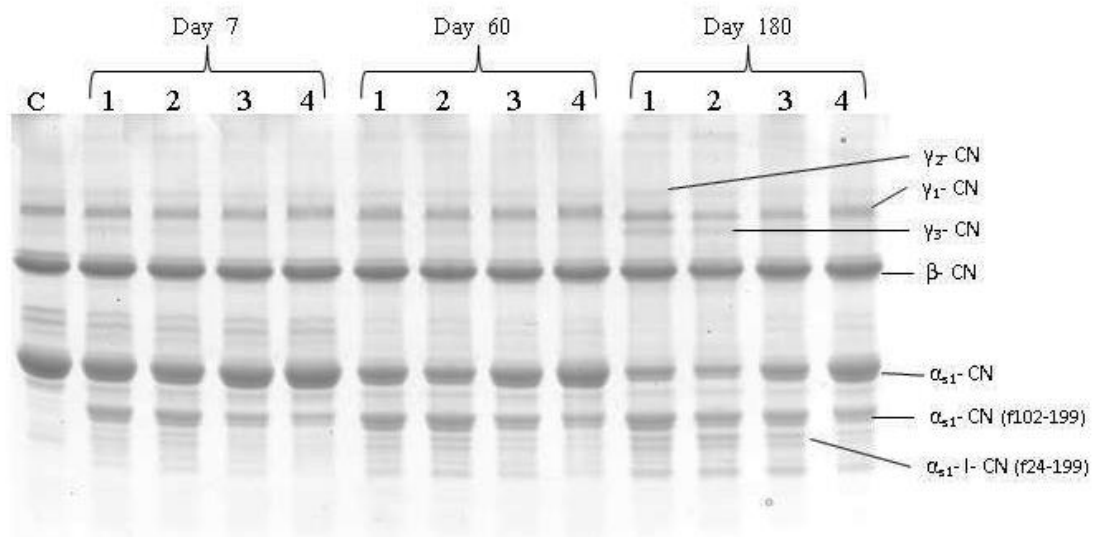
**Fig. 3** Mean viable counts of (A) Mesophilic *lactococcus lactis* species (B) *L. helveticus* and (C) NSLAB in control (■), BM (●) 5 % BMP (◆), 10 % BMP (▲) cheeses over 60 and 180 d of ripening respectively. Values presented are the means of three replicate trials.



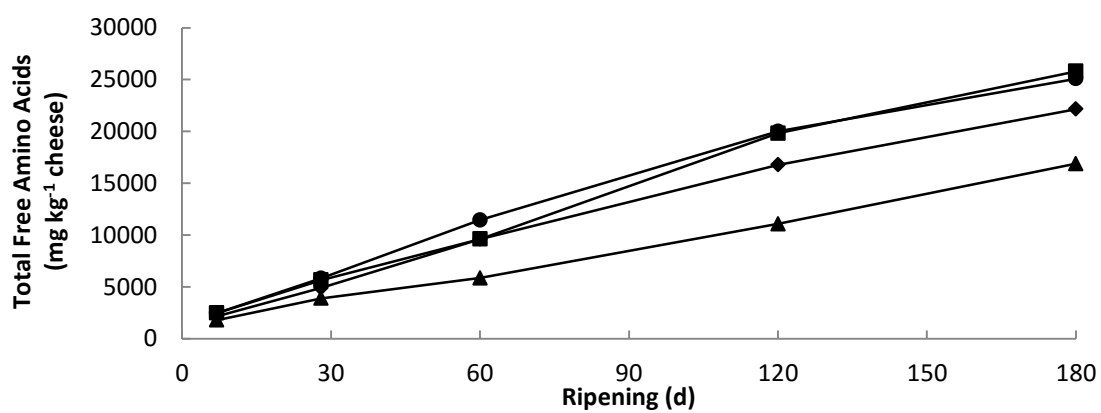
**Fig. 4.** Release of lactate dehydrogenase (LDH) from (A) mesophilic *Lactococci* (co-factor FBP) and (B) *Lactobacillus helveticus* bacteria in control (■), BM (●), 5% BMP (◆), 10% BMP (▲) cheeses over the 180 d ripening period. Values presented are the means of three replicate trials.



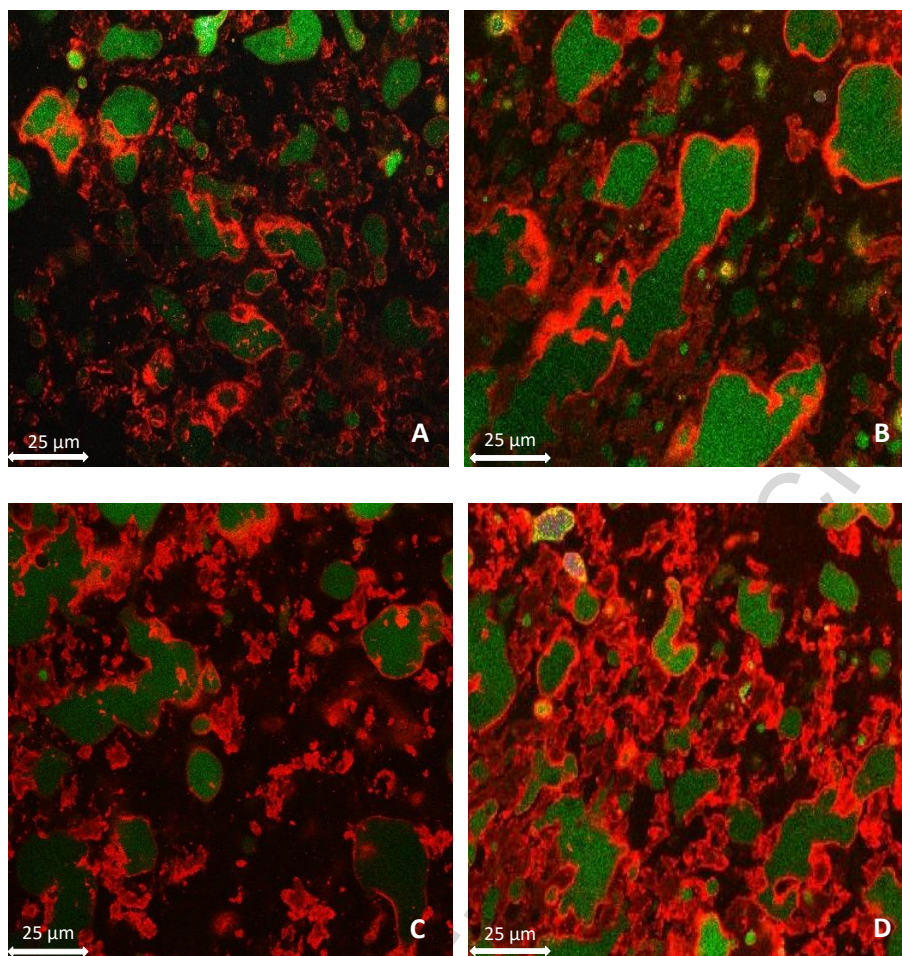
**Fig. 5.** Levels of % pH 4.6 SN/ TN in control (■), BM (●), 5 % BMP (◆) and 10 % BMP (▲) cheeses over 180 d of ripening. Values presented are the means of three replicate trials.



**Fig. 6.** Urea-PAGE electrophoretograms of primary protein breakdown in Control (lane 1), BM (lane 2), 5 % BMP (lane 3), 10 % BMP (lane 4) cheeses at 7, 60 and 180 d of ripening. Lane C = Sodium caseinate.

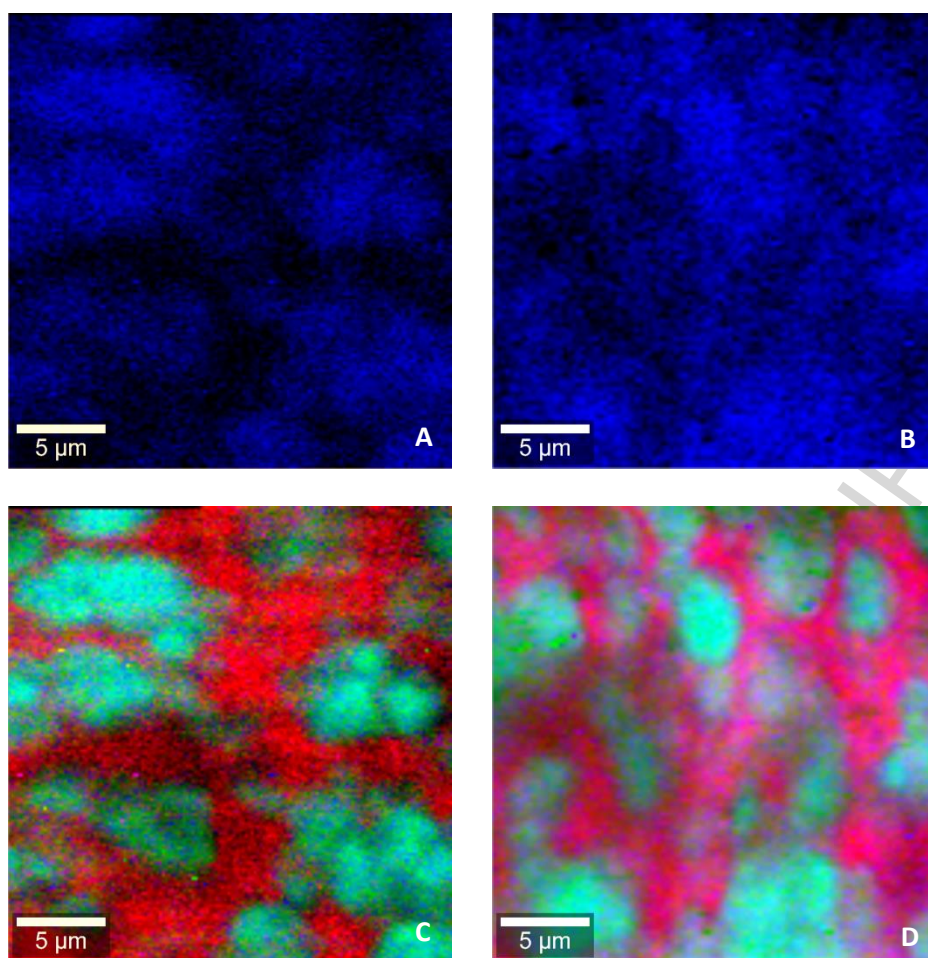


**Fig. 7.** Levels of total FAA's in control (■), BM (●), 5 % BMP (◆) and 10 % BMP (▲) cheeses over 180 d of ripening. Values presented are the means of three replicate trials.



**Fig. 8** CLSM images of cheese matrix containing Phospholipids (red), protein (dark) and neutral lipids (green) in control (A), BM (B), 5 % BMP (C) and 10 % BMP (D) cheeses after 7 d of ripening.





**Fig. 9** Confocal raman images indicating the location and distribution of Phospholipids (blue), Fat (green) and Protein (red) in control (A & C) and 5 % BMP ( B & D) cheeses after 60 d of ripening. Resolution: 4 pixels/ $\mu\text{m}$ .

**Table 1.** Composition of raw materials and standardised cheese milks

Components	Cream	Buttermilk	Buttermilk Powder	Standardised Cheese milk	Standardised milk + Buttermilk
<sup>1</sup> Total solids %	47.40	8.80	96.40	-	-
Protein %	1.9	2.8	31.4	3.65	3.65
Fat %	42	0.6	6.8	3.83	3.83
Lactose + Ash %	3.5	5.4	58.2	-	-
Phospholipid g / 100g sample	0.14	0.14	1.26	0.03	0.06
Phospholipid g / 100g fat	0.33	23.33	18.53	0.78	1.57
<sup>2</sup> Phosphatidylcholine %	22.86	25.74	27.24	25.81	25.45
<sup>2</sup> Phosphatidylinositol %	4.29	8.09	9.26	6.45	9.09
<sup>2</sup> Sphingomyelin%	35.71	20.59	23.75	29.03	23.64
<sup>2</sup> Phosphatidylethanolamine %	13.57	29.41	14.57	22.58	27.27
<sup>2</sup> Phosphatidic Acid %	N.D	N.D	0.63	N.D	N.D

<sup>1</sup>Total solids = Protein+ fat + Lactose + ash.

<sup>2</sup> Individual PL's expressed as % of the overall PL in sample

**Table 2.** Chemical composition and pH (14 d of ripening) of experimental cheeses

Components	Treatments			
	Control	BM	5 % BMP	10 % BMP
Protein (% w/w)	26.92 <sup>a</sup>	25.46 <sup>c</sup>	26.12 <sup>b</sup>	25.68 <sup>c</sup>
Moisture (% w/w)	36.15 <sup>c</sup>	39.45 <sup>a</sup>	36.70 <sup>b,c</sup>	37.74 <sup>b</sup>
<sup>1</sup> MNFS (% w/w)	52.38 <sup>b</sup>	56.07 <sup>a</sup>	52.12 <sup>b</sup>	51.85 <sup>b</sup>
Fat (% w/w)	31.0 <sup>a</sup>	28.87 <sup>b</sup>	29.59 <sup>a,b</sup>	27.23 <sup>c</sup>
<sup>1</sup> FDM (% w/w)	48.55 <sup>a</sup>	47.67 <sup>a,b</sup>	46.75 <sup>b</sup>	43.73 <sup>c</sup>
Protein: Fat ratio	0.86 <sup>c</sup>	0.88 <sup>b</sup>	0.88 <sup>b</sup>	0.94 <sup>a</sup>
Salt (% w/w)	1.74 <sup>b</sup>	1.85 <sup>b</sup>	1.79 <sup>b</sup>	2.38 <sup>a</sup>
<sup>1</sup> S/M (% w/w)	4.82 <sup>b</sup>	4.72 <sup>b</sup>	4.88 <sup>b</sup>	6.31 <sup>a</sup>
pH	5.07 <sup>b</sup>	4.95 <sup>c</sup>	5.01 <sup>b,c</sup>	5.18 <sup>a</sup>
Phospholipid g / 100g sample	0.11 <sup>d</sup>	0.17 <sup>b</sup>	0.14 <sup>c</sup>	0.21 <sup>a</sup>
Phospholipid g / 100g fat	0.36 <sup>d</sup>	0.60 <sup>b</sup>	0.49 <sup>c</sup>	0.78 <sup>a</sup>

<sup>a-d</sup> Means within a column with different superscripts differ significantly ( $P < 0.05$ ). Values presented are the means of three replicates.

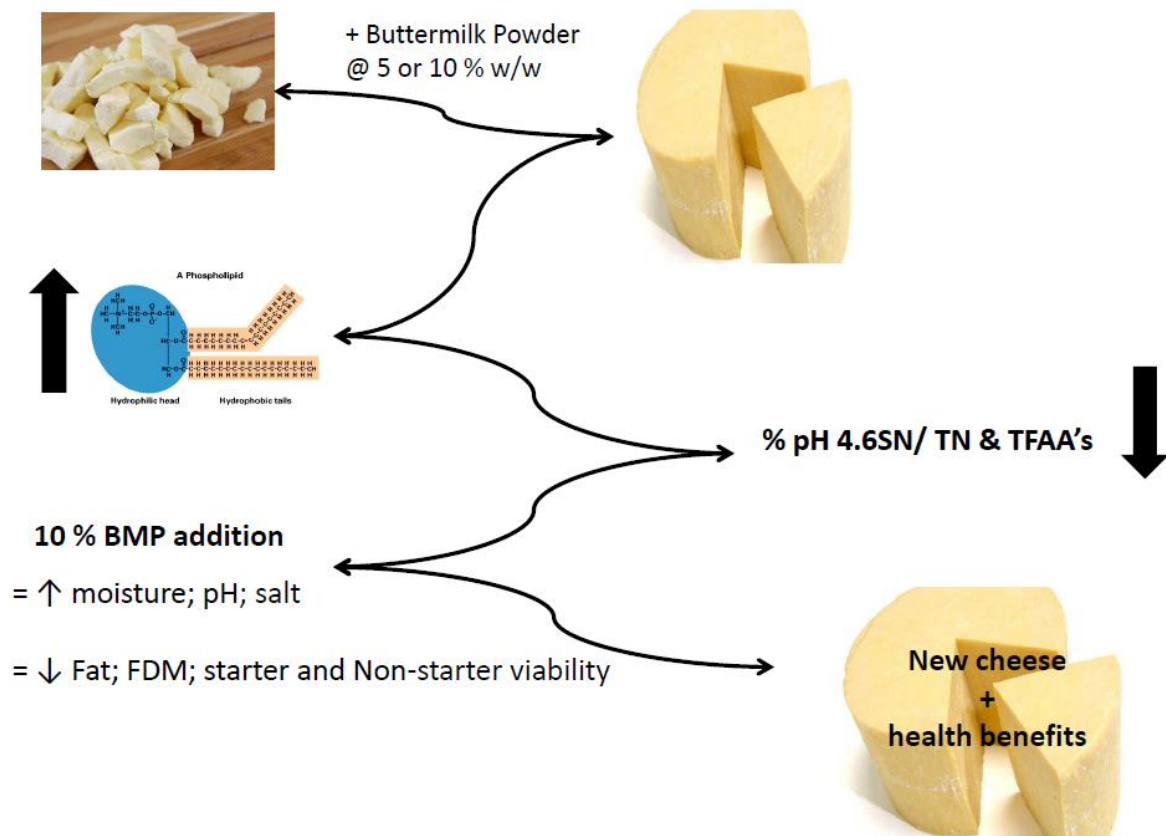
<sup>1</sup>Abbreviations: MNFS, moisture in the nonfat substance; FDM, fat in dry matter; S/M, salt in moisture

**Table 3.** Concentration (mg/kg of cheese) of Free Amino Acids at 180 d of ripening

	Asp	Thr	Glu	Gly	Ala	Val	Met	Iso	Leu	Tyr	Phe	His	Lys	Arg	Pro
<b>Control</b>	697 <sup>a,b</sup>	1587 <sup>a</sup>	5191 <sup>a</sup>	578 <sup>a</sup>	712 <sup>a</sup>	1808 <sup>a</sup>	600 <sup>a</sup>	1496 <sup>a</sup>	3025 <sup>a</sup>	774 <sup>a</sup>	1481 <sup>a</sup>	1227 <sup>a</sup>	3086 <sup>a</sup>	177 <sup>c</sup>	2147 <sup>a</sup>
<b>BM</b>	817 <sup>a</sup>	1400 <sup>a</sup>	4870 <sup>a</sup>	565 <sup>a</sup>	722 <sup>a</sup>	1711 <sup>a</sup>	584 <sup>a</sup>	1406 <sup>a</sup>	2974 <sup>a</sup>	803 <sup>a</sup>	1486 <sup>a</sup>	1171 <sup>a</sup>	2940 <sup>a</sup>	484 <sup>b</sup>	2057 <sup>a</sup>
<b>5 % BMP</b>	648 <sup>a,b</sup>	1317 <sup>a,b</sup>	4205 <sup>a</sup>	487 <sup>a,b</sup>	619 <sup>a,b</sup>	1524 <sup>a</sup>	529 <sup>a</sup>	1288 <sup>a,b</sup>	2361 <sup>b</sup>	683 <sup>a</sup>	1270 <sup>b</sup>	1071 <sup>a</sup>	2562 <sup>a</sup>	798 <sup>a</sup>	1811 <sup>a,b</sup>
<b>10 % BMP</b>	474 <sup>b</sup>	1065 <sup>b</sup>	3400 <sup>b</sup>	384 <sup>b</sup>	525 <sup>b</sup>	1161 <sup>b</sup>	372 <sup>b</sup>	946 <sup>b</sup>	1871 <sup>c</sup>	465 <sup>b</sup>	903 <sup>c</sup>	805 <sup>b</sup>	1921 <sup>b</sup>	379 <sup>b,c</sup>	1458 <sup>b</sup>

<sup>a-d</sup> Means within a column with different superscripts differ significantly ( $P < 0.05$ ). Values presented are the means of three replicates.

## Graphical abstract



## Highlights

- Buttermilk powder addition to curds significantly increases phospholipid levels
- Addition of 10 % BMP results in lower *L. helveticus* and NSLAB viability.
- BMP addition results in decreased levels of primary and secondary proteolysis.
- Confocal raman and confocal laser scanning microscopy utilised to identify more homogenous phospholipid distribution in BMP cheeses.
- BMP addition to cheese curd can influence ripening patterns and allow for new cheese types with possible health benefits.