Title: The influence of fat globule membrane components on the microstructure of low-fat Cheddar cheese

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Abstract: The microstructural characteristics of low-fat Cheddar cheese differing in the content of milk fat globule membrane (MFGM) components achieved by addition of either buttermilk powder (BMP) or skim-milk powder (SMP) to the cheese milk were investigated. Scanning electron microscopy (SEM) and confocal scanning laser microscopy (CSLM) were used to study the cheese structure and the distribution of the starter culture and fat globules.
Variations in the microstructure were observed relating to the MFGM content. The structure of the control cheese (SMP) was more irregular with inhomogeneous large voids. Whereas, cheese with BMP had a homogeneous protein network with small voids, showing a smoother, more compact and less coarse structure accompanied by more pronounced fat globules that were uniformly scattered throughout the protein matrix. The starter bacteria were located within the protein networks in clusters which were distributed homogeneously all over the cheese matrix regardless of treatment.

1   **The influence of fat globule membrane components on the microstructure of low-**

2   **fat Cheddar cheese**

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25   **Abstract**

26   The microstructural characteristics of low-fat Cheddar cheese differing in the content of milk

27   fat globule membrane (MFGM) components achieved by addition of either buttermilk powder (BMP)

28   or skim-milk powder (SMP) to the cheese milk were investigated. Scanning electron microscopy

29   (SEM) and confocal scanning laser microscopy (CSLM) were used to study the cheese structure and

30   the distribution of the starter culture and fat globules.

31   Variations in the microstructure were observed relating to the MFGM content. The structure of

32   the control cheese (SMP) was more irregular with inhomogeneous large voids. Whereas, cheese with

33   BMP had a homogeneous protein network with small voids, showing a smoother, more compact and

34   less coarse structure accompanied by more pronounced fat globules that were uniformly scattered

35   throughout the protein matrix. The starter bacteria were located within the protein networks in clusters

36   which were distributed homogeneously all over the cheese matrix regardless of treatment.

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1. Introduction

Consumers often regard cheese with reduced fat content to be of inferior quality (Banks, 2004). Nevertheless, novel technology in cheese manufacture and considerable advances in understanding the biochemical and physicochemical characteristics of low-fat cheese variants have led to potential improvements in terms of flavour, texture and functionality, with major achievements in the area of fresh and soft cheeses. However, there is still work to be done in the area of semi-hard and hard ripened cheeses. Besides, seeking new dairy products that possess health effects beyond the nutritional components has become a way of life for consumers during the last decade.

Buttermilk, a byproduct of butter making released during churning of cream, is very rich in milk fat globule membrane (MFGM) (Morin, Pouliot, & Britten, 2008). The MFGM is mainly composed of phospholipids, sphingolipids, glycoproteins and other minor compounds and Morin et al. (2008) found that the phospholipid content was 8.5 times higher in sweet pasteurized buttermilk than in skimmed milk (0.119 % and 0.014 %, respectively). The MFGM fragments have previously been shown to carry many beneficial health effects (Dewettinck et al., 2008; Spitsberg, 2005). It has been reported that MFGM fractions may inhibit colon cancer, suppress gastrointestinal pathogens and may be involved in stress responses (McDaniel, Maier, & Einstein, 2003; Parodi, 2001).

Buttermilk has been used as a functional ingredient in many food products, such as salad dressings, chocolate, cheese and yoghurt (Govindasamy-Lucey, Lin, Jaeggi, Johnson, & Lucey, 2006; Mistry, Metzger, & Maubois, 1996; Morin et al., 2008; Trachoo & Mistry, 1998). Many studies have used ultrafiltered or concentrated buttermilk in cheese manufacture (Govindasamy-Lucey et al., 2006; Poduval & Mistry, 1999; Raval & Mistry, 1999). Commercial buttermilk is often subjected to process conditions that are severe and variable (pasteurization temperatures of cream, fermentation, holding time through the evaporation and spray-drying process), which are likely to have major impact on buttermilk microstructure (Morin et al., 2008). Accordingly, most studies have not been able to fully explain the effects and changes in physical and structure characteristics resulting from adding

64 commercial buttermilk to cheese milk. Accordingly, in this present experiment, we produced the

65 buttermilk powder having control of the full processing history from the raw milk.

66   Cheese is stated to have a microstructure consisting mostly of the casein matrix in which the fat

67 globules are entrapped; water or serum is both bound to casein and fills interstices of the matrix that

68 forms a network (Hort & Grys, 2001). Thus knowledge and understanding of the way in which milk

69 components and functional ingredients affect cheese microstructure make it possible to direct the

70 industrial processes towards the production of high-quality low-fat cheeses. Electron microscopy is one

71 of the disciplines which may contribute to this objective.

72   Scanning electron microscopy technique (SEM) has been used extensively as a high resolution

73 analysis to elucidate the state of the casein micelles, type of aggregates and the formation of network

74 during cheese making and of the final cheese products, (Dabour, Kheadr, Benhamou, Fliss, &

75 LaPointe, 2006; Guinee, Auty, & Fenelon, 2000; Kalab, 1985; Kalab, Allan-Wojtas, & Miller, 1995;

76 Kaláb, Yang, & Chabot, 2008; Lopez, Camier, & Gassi, 2007; Poduval & Mistry, 1999; Morin et al.,

77 2008). Findings obtained by the SEM technique have made useful contributions to a better

78 understanding of the complex biochemical structure-function relationships of cheese components.

79 However, it is worthwhile to note that SEM operates under high vacuum where the cheese sample is

80 exposed to a high electron beam and, owing to the high moisture and fat content of the cheese, an

81 extensive sample preparation is required prior to analysis with such steps as sectioning, chemical

82 fixation and dehydration. Additionally, it has been reported that the well-defined structures, e.g. fat

83 globules, can be reliably seen by high resolution topographical images of SEM, and thereby ensure

84 their identification. However, less well-defined particles, e.g. starch or other food additives, were better

85 observed using specific staining microscopy and/or advanced examination techniques, e.g. CLSM

86 (Montesinos-Herrero, Cottell, O'Riordan, & O'Sullivan, 2006).

87   Since the early 1990s, confocal scanning laser microscopy (CSLM) has complemented SEM in

88 cheese microstructure studies (Everett, 2007). CSLM is a technique that has great potential as a tool to

89 improve our understanding of milk and cheese microstructure, and offers a number of advantages over

90 conventional techniques. One of the advantages of this technique is that it can both make visible and

91 chemically differentiate cheese components through the use of specific stains. The basis of staining

92 specimens relies on a number of different mechanisms, e.g. acidic dyes will bind to basic groups and

93 vice versa. In other cases, differential solubility will cause dyes to accumulate according to polarity

94 (Hassan, Frank, Farmer, Schmidt, & Shalabi, 1995; Ong, Dagastine, Kentish, & Gras, 2011). Also,

95 structural information can be obtained in a nondestructive manner and with minimal sample

96 preparation through this technique. In particular, CSLM has proved to be very useful for examination

97 of highly-hydrated and high-fat foods which are difficult in sample preparation using the conventional

98 microscopic techniques without the loss or migration of their components.

99   Defining the structural properties and their relative magnitude with respect to other similar

100 products will increasingly become a critical criterion for cheese manufacturers seeking to design new

101 products, to maintain the quality of current ones or understand the strengths and weaknesses of the new

102 relative to their competitors.

103   The objective of this study was to investigate the microstructural characteristics of low-fat

104 Cheddar cheese differing in the content of MFGM components. Scanning electron microscopy was

105 used to characterize cheese structure as well as the features of the pure starter culture in the cheese. A

106 further aim was to use CSLM to see and to differentiate the distribution of fat globules and bacterial

107 colonies within the protein matrices, providing complementary insights into the evaluation of cheese

108 microstructure.

109

110 *2. Material and methods*

111 *2.1 Experimental design*

112   The Cheddar cheese used in this experiment was made in a replicate block design with two

113 experimental factors. Factor 1. MFGM composition, achieved by adding either butter milk powder

114   (BMP) or skim milk powder (SMP) and cream to the cheese milk. SMP and cream were added to

115   standardize the fat and protein contents in the SMP cheese vats to that in BMP cheese vats; Factor 2.

116   Adjunct culture: addition of two different adjunct cultures *Lb. paracasei* INF448 and *Lb. paracasei*

117   INF456 (characterized by 16s rDNA sequence analysis); both added in the amount of 1 x 10^2 cfu mL^{-1}

118   milk. However this paper covers only the effect of factor 1, but the full factorial design is described as

119   factor 2 will influence the standard deviation of the mean of each level of factor 1.

120   Six cheese vats were made in three replicate blocks (i.e. cheesemaking days). The six cheese

121   vats were given sample codes according to their additions: SMP (cheese milk with SMP and without

122   adjunct culture), BMP (cheese milk with BMP and without adjunct culture), SMP+448 (cheese milk

123   with SMP and adjunct *Lb. paracasei* INF448), BMP+448 (cheese milk with BMP and adjunct *Lb.*

124   *paracasei* INF448), SMP+456 (cheese milk with SMP and adjunct *Lb. paracasei* INF456), BMP+456

125   (Cheese milk with BMP and adjunct *Lb. paracasei* INF456).

126

127   ***2.2. Production of skim milk powder (SMP) and butter milk powder (BMP)***

128   A quantity of 900 L whole milk from the University farm at the Norwegian University of Life

129   Sciences was separated, and the cream was standardized to 36 % fat by addition of skimmed milk. The

130   cream (~95 L) was pasteurized (73°C, 15 s), directly cooled to 7°C and stored at 4°C overnight. The

131   cream was churned to butter in 30 min, with a rise in temperature from 8.5°C to 13.8°C. The buttermilk

132   was sieved through a sterilized cloth bag. The churning yielded ~50 L buttermilk with 1.6 % fat. The

133   buttermilk was stored cold overnight, and then heated to 50°C, and separated to yield 45.8 kg

134   buttermilk with a fat content of 0.6 %. The buttermilk (0.6 % fat) was spray dried directly after

135   separation to yield 3.3 kg BMP (96.6 % dry matter (DM) and 9.8 % fat). From the original milk, 50 L

136   skimmed milk (0.5% fat) was pasteurised (73°C, 15 s) and spray-dried to yield 3.8 kg SMP (97.1 %

137   DM and 2.26 % fat). The SMP and BMP were produced by spray drying (Niro Atomizer, GEA NIRO,

138 Søborg, Denmark) with an inlet air temperature of 185-190°C, and a constant outlet air temperature of

139 85 °C. The spray drier rotary atomizer had a drying rate of 15 L liquid h^{-1}.

140

141 ### 2.3. Cheese milk

142 The raw milk was obtained from a local farm in Cork, Ireland. The raw milk was separated (45

143 °C) and standardized to 0.5 % fat using a table top milk separator (Claire, Milky; Althofen, Austria),

144 before pasteurization (72°C, 15 s). Before each cheese making session, three cheese making vats were

145 mixed containing 20 L of milk and 263 g BMP each. Further three vats contained 19.95 L of milk and

146 240 g SMP and 48 g cream (42 % fat; pasteurized at 80 °C) each, to standardise these to the increase in

147 dry matter and fat in vats with BMP. The vats of cheese milk were stored at 4 °C for 17 h and were

148 stirred every 30 min until the powders were fully dissolved to ensure complete hydration of the milk

149 proteins of the powders.

150

151 ### 2.4. Cheese making and ripening

152 The cheese milk was heated to 30°C, and 1 % of a single strain starter culture (*Lactococcus*

153 *lactis* ssp. *lactis* ML8), cultured for 24 h in 200 mL reconstituted skim milk (10 % w/v); was added to

154 each of the vats. The adjunct starters, cultured for 24 h in MRS broth, were added 15 min after starter

155 addition.

156 After 45 minutes, rennet (Chy-Max™ Plus; 190 mcu mL^{-1}; Chr. Hansen, Hørsholm, Denmark),

157 35 mL $100L^{-1}$ milk (7 mL diluted to 40 mL with pasteurized distilled water), and $CaCl_2$, 0.1 g $100L^{-1}$

158 (18 mL 0.1 M), was added to each of the vats.

159 The cheese milk was coagulated for 40-50 min, and the firmness of the gel was checked before

160 cutting. After cutting the curd was left undisturbed for 5 min before gentle stirring commenced, and

161 then stirred for 5 min followed by increasing the temperature to 39°C in the course of 10 min. The

162 whey was drained at pH 6.20, and the cheese was cut into blocks that were stacked at each side of the

7

163 cheese vat. The cheese blocks were inverted every 15 min during cheddaring, until pH 5.40 was

164 reached. The cheese blocks were milled (~2 x 2 x 15 cm) and 2.5 % salt (w/w) was added to the cheese

165 curd, and gently mixed in. The curd was transferred to cylindrical cheese moulds lined with

166 cheesecloth, and pressed at 1.0 bar for 30 min. The pressure was then increased to 2.5 bar, and the

167 pressing continued for another 18 h. The cheeses were vacuum packed, and ripened at 8°C over a

168 period of 24 weeks.

169

170 *2.5. Compositional and statistical analysis*

171 After 24 weeks of ripening the gross composition of the cheese was analysed. Sampling was

172 undertaken according to IDF Standard 50C (1995). Microbial counts, pH and dry matter were measured

173 immediately after sampling. The cheese for analysis of fat was wrapped in aluminum foil and packed in

174 plastic bags sealed under vacuum and then frozen (-20 °C) until analysis.

175 Dry matter was determined according to IDF Standard 4A (1982). pH was measured as

176 described by Skeie, Lindberg, and Narvhus (2001). Fat was analysed by the van Gulik method

177 according to IDF Standard 222 (ISO 3433) (2008). Total Nitrogen (TN) of cheese was determined by

178 the Kjeldahl method according to IDF standard 20 (1993). Total protein content was calculated by

179 multiplying the TN % by 6.38. Salt content was measured according to IDF Standard 88 (2004).

180 The microorganisms were enumerated on specific media giving the presumptive genera of

181 lactococci on M17 agar (Oxoid, Basingstoke, UK) after aerobic incubation at 30 °C for 4 days.

182 Cheese hardness was measured using the Texture Profile Analysis (TPA) technique on between

183 3 and 9 samples for each cheese (24 weeks of aging). The TPA was performed according to Romeih,

184 Michaelidou, Biliaderis, and Zerfiridis (2002) with a TA-XT2i Texture Analyser equipped with a flat

185 aluminum plunger 75mm in diameter, produced by Stable Micro Systems (Vienna Court, Surrey GU7

186 1YL, UK). Cylindrical samples, prepared using a cylindrical sharp hand cutter, were taken from at least

187 20 mm deep in the cheese blocks, and their dimensions were 35 mm in diameter and 20 mm in height.

188 Samples were compressed axially in two consecutive cycles without yield, with 35% deformation from

189 the initial sample's height at 120 mm/min rate of force application. The force required to attain a given

190 deformation or the maximum force during the first compression in TPA technique, is the TPA hardness

191 measured in Newton.

192 Analysis of variance (ANOVA) was performed using the SAS Enterprise guide 4.0 (SAS

193 Institute Inc., Cary, USA). The treatment factors replicate block and MFGM content formed the

194 statistical model. When analysing the TPA hardness, 7 outliers were removed from the data having

195 been defined as outliers by analysis of the normal distribution.

196

197 ***2.6. Microscopy***

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199 ***2.6.1 Scanning Electron Microscopy (SEM)***

200

201 ***Starter cultures***

202 The starter culture was activated in MRS broth media. After incubation for 24 h at 30 ℃, 1 mL

203 of the broth was spun down and the supernatant was decanted while the sediment was fixed by addition

204 of 1 mL of the fixation mixture consisting of 1.25 % (v/v) glutaraldehyde and 2 % (w/v) para-

205 formaldehyde in 0.1 M cacodylate buffer ($C_2H_6AsNaO_2.3H_2O$) for 2 h. A 8 mm glass slide of poly-l-

206 lysine was submerged in the fixed bacterial solution and held for 2 h to carry the bacterial cells on both

207 sides. The glassy film of bacteria was dehydrated in series of aqueous ethanol solutions (70%, 90%,

208 96% and 100%, 5 min in each), and then dried to critical point using CO_2 in a BAL-TEC CPD 030

209 Critical Point Dryer (BAL-TEC AG, FL-9496 Balzers, Germany), and mounted on aluminum SEM

210 stubs, followed by gold coating in a Sputter Coater Polaron SC 7640 (Quorum Technologies Ltd, East

211 Sussex, UK). A high vacuum Zeiss Scanning Electron Microscope EVO-50-EP (Carl Zeiss SMT Ltd.,

212 Cambridge CB1 3JS, UK) was used to view the strains at 10 kV and magnification of 5000x.

213

Cheese

Small cubic samples from the center of the Cheddar blocks (approximately 3 x 3 mm) were prepared using a surgical blade. The protein network of the cheese cubes was fixed overnight in 4% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 6.8. The samples were washed several times in 0.1 M sodium cacodylate buffer (pH 6.8) at 15 min intervals, and then the fat was fixed in 2% (w/v) osmium tetraoxide (OsO_4) in 0.1 M sodium cacodylate for 1-2 h. The cheese samples were re-washed several times in 0.1 M sodium cacodylate buffer at 15 min intervals, followed by dehydration in increasing concentrations of aqueous ethanol solutions (25%, 50%, 75%, 90% and 100%, 15 min in each). Samples were then dried to critical point using CO_2 in a BAL-TEC CPD 030 Critical Point Dryer (BAL-TEC AG, FL-9496 Balzers, Germany). Following the drying step, some of the dried cubic samples of cheese from each treatment were gently cut from the center into two pieces using a fine scalpel. This was done to explore the internal structure of the cheese cubes, which is free from the fat globules as a result of using series concentrations of ethanol in the dehydration step. This modified step may help in investigating the localization and incorporation of the bacterial cells into the cheese matrix without any interruption from overlapping fat globules. Finally, both samples of complete cubes and divided cubes were mounted individually on aluminum SEM stubs, followed by gold coating as described previously and examined at 5 kV and magnification of 5000x.

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2.6.2. Confocal Laser Scanning Microscopy (CLSM)

Cheese cubes (~ 4 x 4 x 10 mm) were prepared and fixed overnight in 4% (v/v) glutaraldehyde as described previously for the SEM method. Cryo-sections, 50 μm in thickness, were taken from the cheese cubes using a cryotome (Microm HM 560 MV, Microm International GmbH, D-69190 Walldorf, Germany). The sections were then incubated for 10 min in the dark at ambient temperature with a mix of three fluorescent dyes (50μl of each) consisting of 0.2% (w/v) Fast Green FCF (Sigma-

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238   Aldrich, UK), 0.01% (w/v) Nile Red (Sigma-Aldrich, USA) and 0.2% (w/v) Hoechst 33342 (AnaSpec

239   Inc., San Jose, California, USA) for labeling protein, fat and bacteria respectively. Each cheese section

240   was placed between a microscope slide and a cover slip. Samples were then examined at 25 ºC with a

241   63x oil objective lens and sequential scan using a Leica TCS SP5 confocal laser scanning microscope

242   (Leica Microsystems CMS GmbH, 68165 Mannheim, Germany), using a helium neon (HeNe) laser

243   with excitation wavelength of 633 nm for proteins (an excitation peak of 622-626 nm and the

244   maximum emission was at 640 nm) and an argon (Ar) laser with an excitation wavelength of 488 nm

245   for fat (an excitation peak of 515-530 nm and an emission of 525-605 nm), while employing a UV laser

246   with excitation wavelength of 405 nm for the bacterial cells (an excitation peak of ~ 350 nm and

247   maximum emission at 461 nm). In the CLSM micrographs, the protein network, fat and bacterial cells

248   were labeled in gray, green and blue, respectively, while the aqueous phase appears as black areas.

249

250   **3. Results and discussion**

251   Only the effects of the first experimental factor the milk fat globule membrane compounds are

252   covered by this paper. The effects of the adjuncts are described in another manuscript where the

253   microbial development of the cheeses is characterised.

254

255   *3.1. Cheese composition and texture*

256   Significant differences in the gross composition and the TPA hardness of the cheeses after 24

257   weeks of ripening are shown in Table 1. The butter milk powder considerably reduced the content of

258   dry matter by 1 % and the pH by 0.04 in the ripened cheese. Significant differences between the

259   replicate blocks and the adjuncts were found, explaining the large standard deviation found for some of

260   the parameters analysed. Additionally, no influence from the experimental factors was found on the

261   content of protein, fat in dry matter or the salt content which were on average 32.11 ± 0.64 %, $13.7 \pm$

262 0.6 % and 1.6 ± 0.2 %, respectively. The content of presumptive lactococci was on average log 6.9 ±

263 0.5 cfu g^{-1}.

264   The TPA hardness measurements (Table 1) clearly revealed a considerable reduction in the

265 textural hardness as a function of added BMP. The SMP cheese was the hardest, reflecting the potential

266 effect of BMP in softening the cheese texture. The TPA hardness is affected by cheese composition,

267 such as protein content, protein degradation and the interaction between casein and fat and/or other

268 cheese components (Heertje, 1993; Tunick, 2000). No significant difference between SMP and BMP

269 was found on the proteolysis as measured by the content of free amino acids (results not shown). The

270 effect of buttermilk in reducing cheese hardness appears to be linked to its MFGM fragments, which

271 were incorporated with the casein matrices, and played a lubricant role that provided a smoother and a

272 soft texture. This physical function of buttermilk has also been demonstrated by other studies; i.e.

273 Poduval and Mistry (1999) for reduced-fat Mozzarella cheese and Trachoo and Mistry (1998) for low-

274 fat yogurt. Also, Mistry et al. (1996) reported that reduced-fat Cheddar cheese made with 5% UF-sweet

275 buttermilk had lower hardness values after 4 weeks of ripening than its control counterpart cheese.

276

277 *3.2. Scanning Electron Microscopy (SEM)*

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279 *3.2.1 Conventional technique micrographs (surface scanning of cheese cube samples)*

280   The SEM micrographs of low-fat Cheddar cheese ripened for 6 months are shown in Fig. 1. The

281 protein matrix (gray area) formed a continuous phase permeated by amorphous voids (black areas), and

282 spherical fat globules of different sizes. As these micrographs show, an obvious variation in the cheese

283 microstructural properties was obtained between low-fat Cheddar cheeses made with either addition of

284 SMP (Fig. 1A) or BMP (Fig. 1B). An extremely porous, open and coarse structure was obtained in

285 low-fat Cheddar cheese with SMP addition, while cheese with added BMP was more dense and

286 homogeneous.

287   Despite the fact that a uniform protein content was achieved in both cheese treatments, cheese

288   with addition of SMP had irregularly aggregated protein folds and large matrix clusters interrupted by

289   large pores filled with serum which appeared as cavities embedded in the cheese matrix. The trend to

290   formation of apparent cavities was expected according to the age of the cheese. Earlier studies (El-

291   Zeini, El-Aasser, Anis, & Romeih, 2006; Poduval & Mistry, 1999; Tunick et al., 1993) have indicated

292   that an increase in the size of the cavities would occur during aging in different cheese varieties due to

293   the weakening of the paracasein matrix caused by proteolysis or CO_2 production by starter or non-

294   starter bacteria.

295   In contrast, the microstructure of the low-fat Cheddar cheese with BMP added, as shown in Fig.

296   1B, reveals a homogeneous systematic protein aggregate network. The protein matrix appeared as a

297   smooth continuous phase of aggregated micelles, characterized by a compact fusion and a dense

298   structure. The BMP cheese structure was more regular and had fewer voids compared to that of cheese

299   with SMP, and the effect of the BMP addition was most probably attributed to the high levels of

300   MFGM components in the BMP. The BMP used in this study was prepared from cream pasteurized at

301   73°C. This promotes a higher retention of MFGM components in the buttermilk than is obtained from

302   raw-cream as stated by Morin, Jimenez-Flores and Pouliot (2007). Furthermore, it has been reported

303   that MFGM fragments may physically be entrapped within the paracasein network (Morin et al., 2008).

304   It could induce direct interactions with casein (CN) by folding CN micelles inside reconstituted

305   aggregates reflecting the functional properties of buttermilk in dairy product structures (Morin et al.,

306   2008 and Ong et al., 2011). In this context, Lopez et al. (2007) have stated that cross-links can be

307   formed between MFGM components and the casein matrix, which in turn strongly affects the

308   rheological and microstructure properties of milk gels. In addition, BMP cheese tended to have a higher

309   moisture content compared to that of SMP cheese (Table 1), which is mainly attributed to the increased

310   hydration capacity of the buttermilk components, particularly its phospholipids. This result is in

311   agreement with those of Morin et al. (2008), Raval and Mistry (1999) and Turcot, Turgeon and St-

312 Gelais (2001) who reported that addition of buttermilk increased the moisture content of low-fat

313 cheese.

314   Although a uniform fat-in-dry-matter basis was achieved in all cheese treatments, the spherical

315 fat globules were more pronounced and more uniformly scattered throughout the protein matrices in the

316 BMP cheese compared to the SMP cheese structures (Fig. 1). Addition of BMP tended to cause

317 inclusion of a higher number of discrete fat globules differing in size within the protein matrix, whereas

318 fat globules were more often trapped and embedded within the protein matrix of the SMP cheeses. The

319 more hydrated the texture, the more systematically distributed were the fat globules and the presence of

320 MFGM components and these, taken all together, may contribute to a softer and less firm texture of the

321 BMP cheese compared to its SMP counterparts. This finding goes in parallel with the hardness values

322 (Table 1) for these treatments and is in agreement with the results of Mistry et al. (1996) and Turcot,

323 St-Gelais and Turgeon (2002) who concluded that addition of UF-buttermilk to cheese milk led to

324 softer texture properties of reduced and low fat Cheddar cheeses.

325   Surprisingly, the observation of starter within these cheese micrographs was infrequent and

326 difficult to clearly define (Fig 1). The starter culture (*Lc. lactis* subsp. *lactis* ML8) appeared attached

327 and embedded within the protein network and distributed all over the cheese matrix. This finding leads

328 us to investigate and develop other techniques to explore the distribution and localization of these

329 bacterial cells within the cheese matrix.

330

331 *3.2.2 Modified technique micrographs (entire matrix of cheese cube samples)*

332   The uneven clarity of the starter culture (*Lc. lactis* subsp. *lactis* ML8) in the cheese micrographs

333 (Fig. 1) stimulated a development and improvement in the microstructure examination in order to

334 explore the manner of distribution of the bacterial cells within the cheese matrix. By cutting the dried

335 cubic cheese samples from the center; the internal structure was exposed, and the fat globules were

336 removed by the ethanol series extraction during sample preparation (see section 2.6.1). The

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337 microstructure obtained with this technique as shown in Fig. 2 clearly revealed that the protein matrices

338 (gray area) appeared as a continuous phase of a smooth, flat consolidated surface permeated by

339 heterogeneous voids (black area), without the appearance of the network obtained in the previous

340 examination technique (Fig. 1). The appearance shown in Fig. 2 resulted from the fine scalpel cut step

341 of the fixed and dried cubic cheese samples (see section 2.6.1).

342 In this context, the distribution of milk fat globules can be clearly seen through the numerous

343 smooth surfaced concave areas, which are mostly spherical in shape and were originally occupied by

344 fat globules (white arrows) as described above.

345 These micrographs show that the cocci, most probably the starter culture (*Lc. lactis* subsp. *lactis*

346 ML8), appeared in clusters immersed and uniformly dispersed throughout the protein matrix (black

347 arrows), which is in parallel to the appearance of the pure strain (Fig 3). The starter cells appeared as

348 discrete globular cocci gathered in clusters randomly distributed. Besides in the cheese micrographs, a

349 collection of hollows appeared, which most probably are areas of cells removed during the sample

350 preparation process in particular with regard to the cutting of the dried cheese cube samples.

351 Overall, it can be observed from the microstructure properties revealed in Fig. 2 that the protein

352 matrix formed a continuous uniform phase in both SMP cheese (Fig. 2A) and BMP cheese (Fig. 2B).

353 However, the pronounced uneven size and shapes of voids were noticeably less marked and fewer in

354 number in the BMP cheese than in the SMP cheese, reflecting the higher fusion of casein aggregates

355 and the increased network formation obtained by addition of BMP. This effect of buttermilk was also

356 identified by Lopez et al. (2007) and Morin et al. (2007) as a vital factor influencing the microstructure

357 characteristics of the cheese. These structure characteristics are similar to those obtained by the

358 previous formal examination technique presented in this work.

359 By the conventional SEM technique it was difficult to see the distribution and localization of

360 bacterial colonies, most probably owing to the sample preparation. However, by the modified SEM

361   technique applied in the current study we were able to see and describe the bacteria with a degree of

362   high resolution.

363

364   *3.3. Confocal Laser Scanning Microscopy (CLSM)*

365   A CLSM technique was used to differentiate between the structural components of the cheese

366   and to make the preferential localization of bacterial colonies visible throughout this cheese matrix in

367   comparison with the findings of SEM.

368   The CLSM micrographs presented in Fig. 4 reveal a cheese structure with features that

369   resemble those observed by the SEM techniques (Fig. 1 and Fig. 2). Fig. 4 shows the protein matrix

370   (gray area) as a continuous uniform phase permeated by heterogeneous voids (black area) representing

371   the aqueous serum pores. The milk fat globules appear as discrete green spots differing in shape and

372   size. Previously, an observation using CLSM in Emmental cheese showed that fat was dispersed in the

373   cheese matrix in three phases; as individual fat globules, as coalesced fat globules resulting from the

374   fusion of individual fat globules, and finally as nonglobular fat (free fat) of larger size than the other

375   phases (Lopez et al., 2007). This may explain the different sizes and shapes of fat in Fig. 4. An obvious

376   finding was that the localization of fat noticed in CLSM images (Fig. 4) suggest that the globules are

377   not only entrapped in the protein network, but also protrude into the serum pores. According to Ong,

378   Dagastine, Kentish and Gras (2010) the native MFGM acts as a natural emulsifying agent that enables

379   the fat to remain in the aqueous phase.

380   Further, Fig. 4 shows that the clusters of starter cells were of uneven size and had different

381   shapes (blue spots) distributed randomly in the cheese matrix. This finding is compatible with Fig. 3 of

382   the pure starter strain image and Figs. 2A and 2B of the Cheddar cheese structure by the modified SEM

383   technique.

384   Consistent with the microstructural characteristics obtained by SEM, under CLSM the cheeses

385   with added BMP (Fig. 4B) appeared homogenous, with rather small and evenly distributed pores and a

16

386   network consisting of a relatively fused protein phase. Cheeses with added SMP (Fig. 4A), however,

387   had noticeably rather large pores which appeared as cavities embedded in the cheese matrix, reflecting

388   the porous and coarse structure of the SMP cheese.

389   Lopez (2005) has stated that milk caseins are able to associate with the fat globule membrane,

390   forming a protein layer which in turn enables the newly formed phase to behave as pseudo-protein

391   particles, becoming an integral part of the protein matrix during coagulation. Also, Ong et al. (2011)

392   have suggested that chemical bonds may exist between the fat globule membrane components and the

393   protein matrix. This might allow for more rearrangement to take place in the cheese matrix, again

394   favouring the formation of a more homogeneous and compact structure in the BMP cheeses.

395   In summary, CLSM provided structural information compatible with that from SEM.

396   Furthermore, CLSM allowed a visualization of the cheese chemical composition as well as the

397   distribution and localization of bacteria within the cheese matrix. Structure imaging was achieved by

398   the combination of specific stains, which effectively avoided any artifacts due to possible cross-

399   reactions of the multi-stains used.

400

401   **4. Conclusion**

402   Addition of BMP softens the texture of low fat Cheddar cheese as shown by decreased hardness

403   values, and the microstructure analysis supported these findings. The structural network of the BMP-

404   added cheeses was characterized by a smooth and dense protein matrix, in which spherical fat globules

405   exhibited a more uniform dispersion and were more pronounced compared to those of SMP cheeses.

406   The modified SEM technique used, provided a peerless tool over the conventional technique for

407   monitoring the genuine localization and distribution of bacterial colonies in the cheese matrix without

408   disturbance of other cheese structure compounds.

409   Qualitatively, the microstructure attributes revealed by CLSM were similar to the structure

410   observed using SEM, but CLSM also had the capacity to specifically distinguish the different

components of the cheese. The staining procedure we used has shown protein, fat and starter clusters and their manner of incorporation in the cheese matrix.

Together these techniques provide a complementary and more thorough assessment of the microstructure of cheese and of other more hydrated dairy products. The results of this study offer a better understanding of the functional impact of BMP on cheese structure, which may lead to a better comprehension of the functional properties and quality attributes of low-fat Cheddar cheese. Addition of BMP proved to be a promising option to direct the industrial processes to the production of high-quality low-fat cheeses with additional nutritional properties.

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520

Figure headings

522

523 **Fig. 1.** SEM micrographs (5000x) of low-fat Cheddar cheese (surface of cubic samples): **(A)** Cheese of

524   skim milk (SMP), **(B)** Cheese of butter milk (BMP). Scale bar is 2 µm.

525

526 **Fig. 2.** SEM micrographs (5000x) of low-fat Cheddar cheese (entire matrix of cubic samples): **(A)**

527   SMP and **(B)** BMP. Black arrows indicate the starter cluster cells and white arrows indicate

528   voids of removed fat globules. Scale bar is 2 µm.

529

530 **Fig. 3.** SEM micrographs (5000x) of *Lc. lactis* ssp. *lactis* ML8. Scale bar is 3 µm.

531

532 **Fig. 4.** CLSM micrographs (63x) of low-fat Cheddar cheese treatments: **(A)** Cheese of skim milk

533   (SMP), **(B)** Cheese of butter milk (BMP). Protein is labeled in gray, fat is in green and bacterial

534   cells are in blue. The aqueous phase appears in black. Scale bar is 10 µm

535

536

Table 1. Dry matter (DM %), pH and the texture properties as measured hardness on a Texture analyzer (TPA hardness) of the cheeses after 24 weeks of ripening (Values are means ± SD). The *p*-statistics of each experimental factor is shown in the last two rows of the table (n = 18).

	DM (%)	pH	Hardness (N)
SMP	52.97 (±0.95)	5.25 (±0.11)	148.2 (±12.7)
BMP	51.83 (±1.01)	5.22 (±0.11)	132.6 (±15.3)
Powder	0.0002	0.01	0.0024
Rep block	0.0002	0.0001	ns

[*] ns: not significant

Figure

A
2μm*
5A02.tif   EHT = 5.00 kV   I Probe =   3 pA   Free WD = 7.5 mm   Chamber = 6.91e-004 Pa   Signal A = SE1   Date :17 Mar 2009

B
2μm*
6B02.tif   EHT = 5.00 kV   I Probe =   3 pA   Free WD = 7.5 mm   Chamber = 2.26e-003 Pa   Signal A = SE1   Date :25 Mar 2009

Fig. 1.

Figure
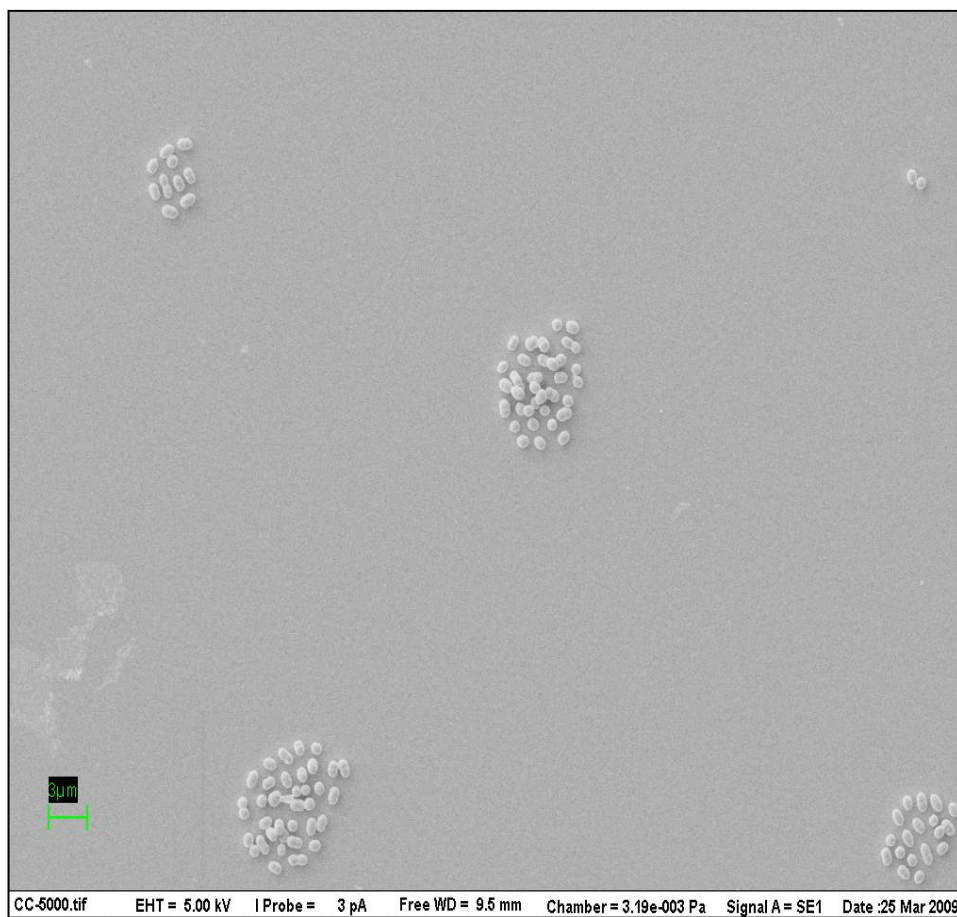

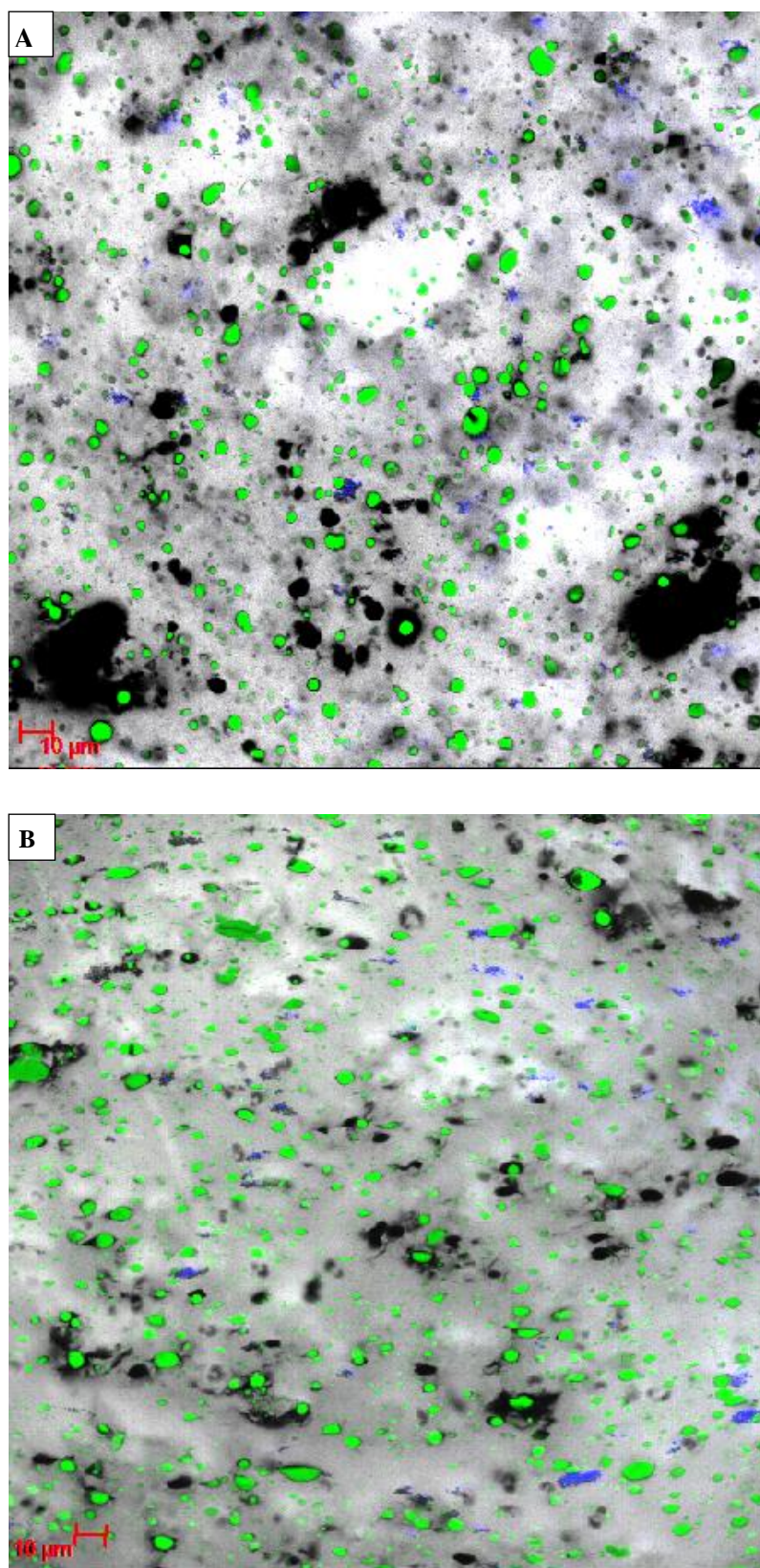**Fig. 2.**

Figure

Fig. 3.

Figure
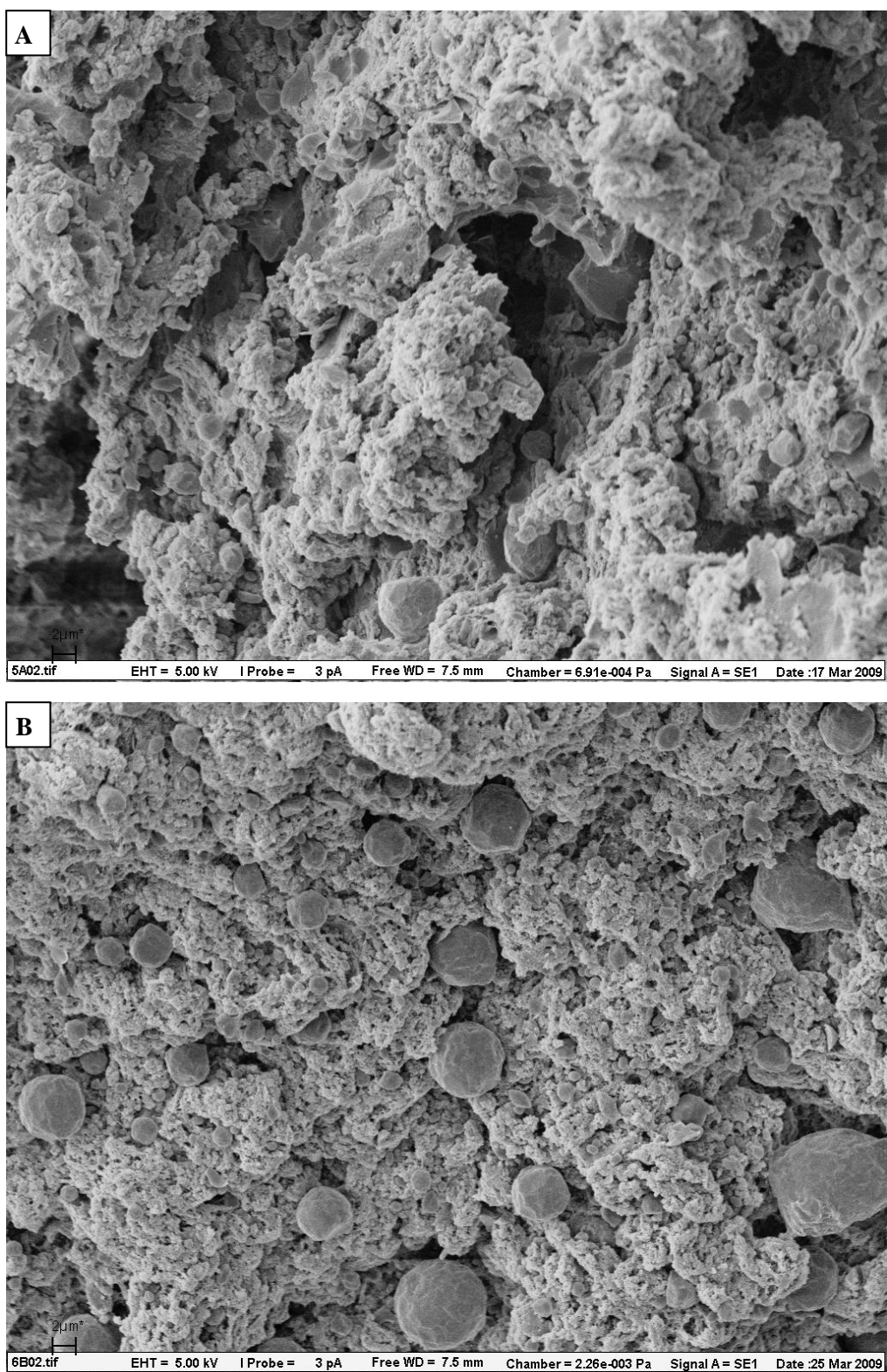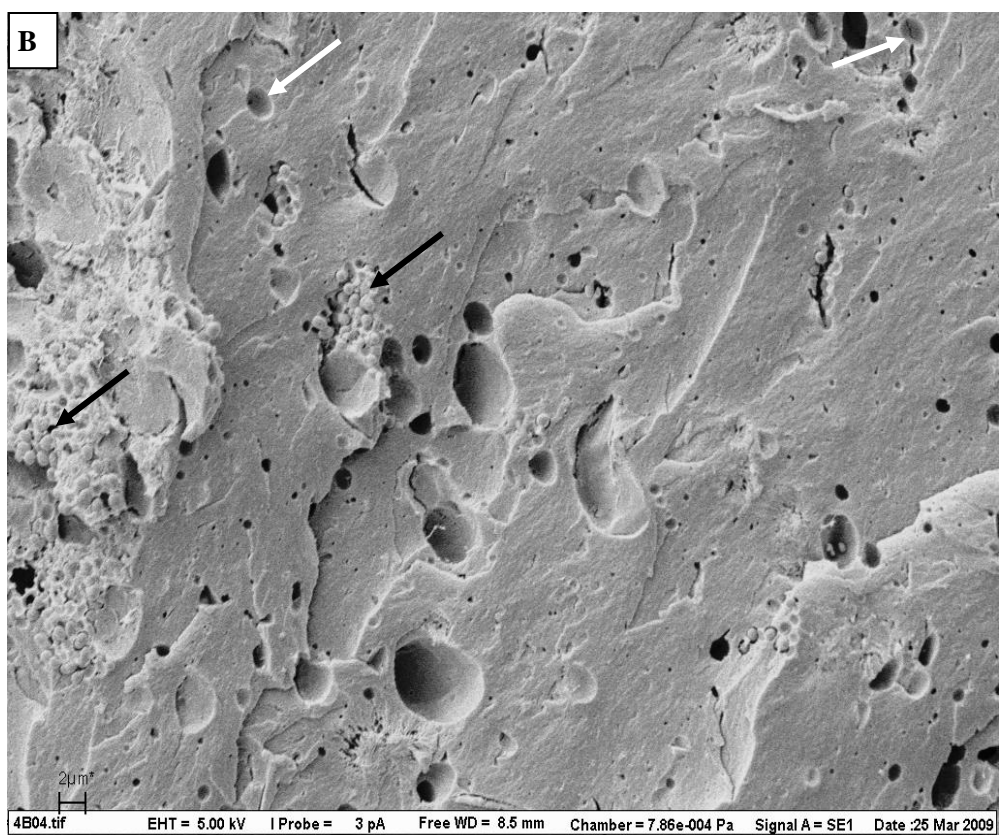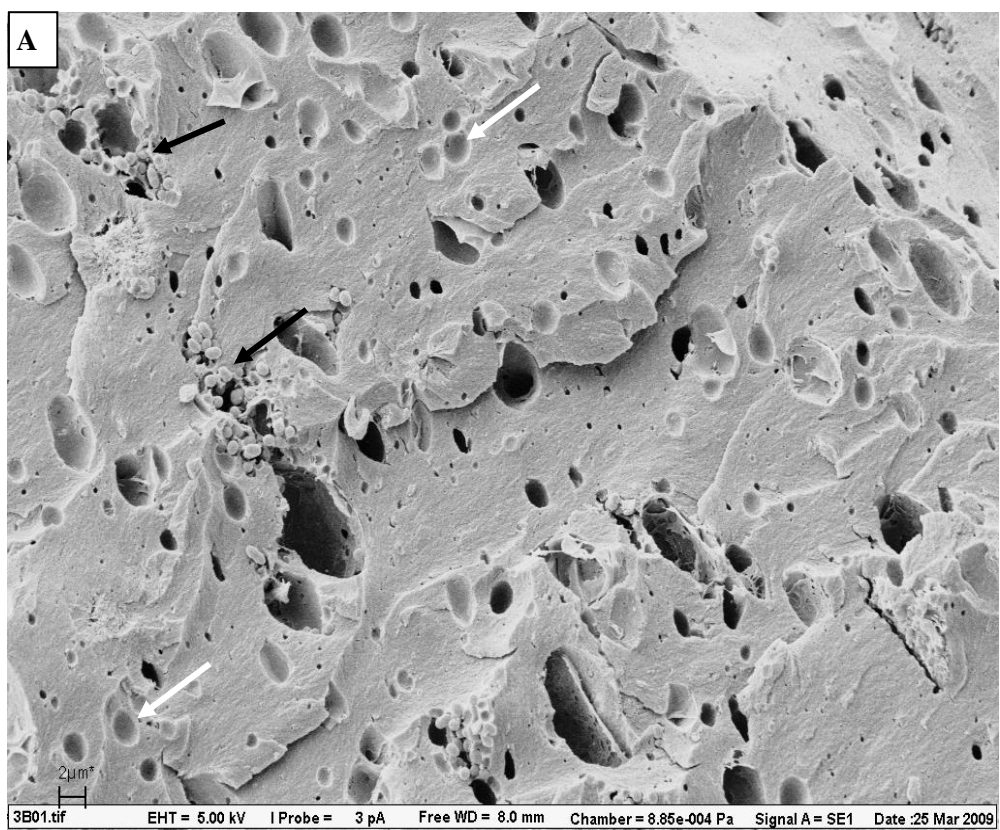
Fig. 4.

2.1 Experimental design

Cheddar cheeses were made in a replicate block design with two experimental factors; Factor 1. Replicate block, which was milk obtained at three different cheesemaking days; Factor 2. MFGM composition, achieved by adding either butter milk powder (BMP) or skim milk powder (SMP) and cream to the cheese milk. SMP and cream were added to standardize the fat and protein contents in the SMP cheese vats to that in BMP cheese vats. Six cheese vats were made in each of the three replicate blocks. Three vats where the milk was added SMP and three where the milk was added BMP.