1 **Microscopic analysis and microstructural characterization of the organic and inorganic components**

2 **of dairy fouling during the cleaning process**

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16 **Interpretive Summary**

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18 **of dairy fouling during the cleaning process.**

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21 The thermal treatment of milk produces fouling, which can accumulate on contact surfaces. The

22 elimination of fouling is expensive, which is a serious problem in the dairy industry. Therefore, studying

23 the composition and microscopic structure of fouling could be of great help to improve cleaning processes

24 in this industry. This paper presents the composition and structure of dairy fouling in different setups. A

25 new improved enzymatic formula for eliminating fouling is also tested as a real alternative for use in the

26 dairy industry.

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ABSTRACT

31   It is essential to have a thorough knowledge of the composition of the fouling and residues in facilities to

32   ensure a correct and efficient cleaning process. There are many ways to determine the content of the organic

33   and inorganic fraction in a fouling sample, ranging from classic chemistry techniques to advanced

34   technological devices. In this study, the organic residues of milk fouling were evaluated using fluorescent

35   microscopy. The inorganic content was analyzed with Energy-dispersive X-ray spectroscopy and

36   complemented with Inductively Coupled Plasma Optical Emission Spectrometry. These techniques were

37   also applied to the residues from two different cleaning procedures: (1) a commercial alkaline cleaner and

38   (2) a formula of enzymes and surfactants. The results indicate a fouling removal efficiency of 86.9% for the

39   alkaline product and 87.01% for the enzymatic formula. When the two products were compared, similar

40   activities were found to remove the fouling. Knowing the average content of the fouling in the industry helps

41   to formulate better strategies to save water, energy and time during the cleaning process. Additionally,

42   studying the cleaning residues helps to avoid problems of cross-contamination between batches or

43   subsequent microbial growths (biofilms) on surfaces with residues.

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45   **Key words:** Milk fouling, microscopy, component, cleaning process, enzymatic product

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INTRODUCTION

48   There are two types of dairy fouling depending on the temperature of the process that promotes its

49   formation. In Type A, the temperature range is from 75 ºC to 110 ºC and the composition is 50% - 70%

50   proteins, 30% - 40% minerals, and 4% - 8% fat. Type B is formed at temperatures above 110 °C and the

51   content is 70% - 80% minerals, 15% - 20% proteins, and 4% - 8% fat (Visser and Jeurnink, 1997). This

52   study used fouling Type A, mainly organic residues produced at 90 ºC, similar to a pasteurization process

53   (Bansal and Chen, 2006). However, the morphology and spatial composition is very heterogeneous in both

54   types.

55   During heat treatments, there is a deposition of materials from the food to the surfaces of the facilities (Fryer

56   and Asteriadou, 2009). The problems produced by fouling could be summarized into three different

57 categories: food security, shelf life, and operating problems (Barish and Goddard, 2013). This is important

58 to avoid cross-contamination between different batches from the preexisting fouling in the facilities (Ayala

59 *et al.*, 2016; Hagsten *et al.*, 2016). While the cleaning process is costly and time consuming, it is essential to

60 the food industry (Liu *et al.*, 2006; Fryer *et al,* 2011). However, it is still poorly understood and cleaning

61 protocols are designed in a semi-empirical manner. In the food industry, 25% of the operating time is spent

62 on the cleaning process (Fryer *et al.*, 2011). In this industry, it is impossible to know the extent and

63 composition of fouling throughout the plant, so the use of models to represent the fouling must be taken into

64 account, perhaps through a stochastic rather than a purely deterministic approach (Fryer and Asteriadou,

65 2009). Fouling is formed by adherence to the surface and cohesion between elements of the layer on the

66 surface. Cleaning can result from either or both adhesive and cohesive detachment (Liu *et al.*, 2006].

67 Identifying the composition and its structure is a crucial step in studying the residues from industrial

68 treatments to prevent their production and to know how to clean the facilities. There are a wide range of

69 options for identifying waste elements. The choice depends on the kind of target identified: (1) whether the

70 waste is attached to other items, (2) the budget, (3) the time available, and (4) access to new techniques or

71 special requirements such as specialized personnel and additional safety features. In this case, the dairy

72 fouling was a residue firmly attached to the metal surface of the facilities. For this type of residue, various

73 analyses are required to identify the components of the organic and inorganic fraction of the dairy fouling.

74

75 Confocal Laser Scanning Microscopy (CLSM) technology is widely used to study food structure and

76 composition because this method enables the use of a variety of selective stains, as well as post processes

77 (Auty *et al.*, 2001). There are many studies on food products using CLSM, e.g. the distribution of fat and

78 proteins in mozzarella cheese and chocolate (Auty *et al.*, 2001) and the fat globules in milk and milk

79 products (Evers *et al.*, 2008). In other studies, this technology has been used to study the structure and

80 composition of different residues such as fouling on membrane filtration units (Doumèche. *et al.*, 2007;

81 Stoica *et al.*, 2018). Other authors have studied dairy fouling Type A (Boyce *et al.*, 2010) and Type B by

82 CLSM (Hagsten, *et al.*, 2016).

83 The aim of our study was to improve knowledge about fouling Type A, especially its composition and

84 microstructure, and to identify the composition of the residues from cleaning procedures. Residues were

85   produced to compare the effectiveness of two different cleaning products (a caustic one-pass commercial

86   product and a novel enzymatic formula) using a laboratory-fouling model as a target for the cleaners.

87

88   **MATERIALS AND METHODS**

89   *Milk and Test Surfaces*

90   The fouling formed during the assays was generated from bovine whole raw milk refrigerated and supplied

91   by a dairy farm (Granja Can Bordoi, Sant Antoni de Vilamajor, Catalonia, Spain) and stored at 5 ºC until the

92   assays. The test surfaces were made of stainless steel (Type AISI 316 with a grade 2B finish), 2.0 cm in

93   diameter and 1.2 mm thick (Fig. 1A). This kind of surface was selected as a sample for the studies on

94   fouling growth and to develop cleaning formulations because it is one of the main materials for plate heat

95   exchangers (PHE). Heat resistant aluminum foil tape (Ceys, L'Hospitalet de Llobregat, Spain) was used to

96   make the walls of the test surfaces. Each side of the wall was approximately 6 mm high and 70 μm thick

97   (Fig. 1B). Before using the stainless steel surfaces to produce the milk-fouling laboratory models (MFS),

98   they were cleaned and disinfected in accordance with the standard EN 13697 (Anonymous, 2015). They

99   were then kept in sealed bags to avoid contamination.

100

101   *Fouling Production*

102   The milk fouling models (MFM) were formed according to the modified procedure referenced by Guerrero-

103   Navarro *et al*. (2019). First, the containers were weighed using an analytical balance (Mettler AE 100,

104   Mettler-Toledo S.A.E., Hospitalet del Llobregat, Spain) and then pre-heated at 90 °C in a fan-assisted oven

105   (IDL-FI-80, Labolan, S.L. 31191, Esparzar de Galar, Spain). When the containers reached the working

106   temperature (Fig. 1B), 0.4 mL of whole raw milk was added and they were placed back in the oven for 30

107   minutes. The milk was then dried and attached to the surface. Thereafter, another 0.4 mL of milk was added.

108   The addition of milk and the drying process were repeated 5 times, after which the washing process was

109   performed by introducing each MFM into plastic flasks with 4 mL of distilled water at 50 ºC and shaking

110   them at 2,500 rpm for 50 seconds using an agitator (REAX Top, Heidolph Instruments, Schwabach,

111   Germany). The coupons were then rinsed with deionized water to remove the unattached milk on the

112   surfaces and then pre-heated again at 90 ºC for 5 minutes. Once the working temperature was reached, five

113   additional steps of adding milk and drying were performed (10 cycles in total), after which the washing step

114   was repeated using water at 50 °C (Fig 1C).

115

116   ***Cleaning Solutions (CS)***

117   Two kinds of cleaning products were selected to remove the dairy-fouling: an enzyme formulation and a

118   commercial product. The enzymatic formulation was composed of protease (Savinase®, Novozymes,

119   Bagsværd, Denmark), amylase (Termamyl Ultra®, Novozymes, Bagsværd, Denmark) and a non-foam

120   commercial surfactant (Tensio CIP®, ITRAM Higiene, Vic, Spain). The commercial product used as the

121   control was a one-pass alkaline formulation (BRIO COMPLEX®, ITRAM Higiene, Vic, Spain) currently

122   used in the industry to remove fouling and was chosen to represent the classical strategy of chemical

123   cleaning.

124   Products used as solutions:

125   • Cleaning Solution 1 (CS1): composed of 6% v/v alkaline cleaner in deionizate water. Applied at 70

126   °C for 45 minutes with a pH of 10 to 12, according to manufacturer's instructions.

127   • Cleaning Solution 2 (CS2): composed of 0.12% v/v of Savinase®, 0.10% v/v of Termamyl Ultra®,

128   and 25% v/v of Tensio CIP®, diluted in buffer solution (pH 8.5) and used at 55 °C for 30 minutes.

129

130   ***Improving the Enzymatic Formulation***

131   The enzymatic formulation used in this study was established based on the fouling components in previous

132   experiments (Guerrero-Navarro *et al*., 2019). Moreover, the activity curves of the enzymes used in the

133   formulation provided by the manufacturer were analyzed before the assays. Thereafter, the analysis was

134   performed using a starch solution and bovine serum albumin (BSA) for amylase and protease, respectively.

135   The pH was adjusted from 9.5 to 8.5 and the temperature from 50 °C to 55 °C to maintain the effectiveness

136   of the enzymatic cleaning solution during the assays at >80%.

137   A buffer solution was used to avoid pH variations and thereby improve the activity of the enzymatic

138   formulation, since a low pH close to neutrality would inactivate the enzymes (Argüello *et al*., 2003). Three

139   kinds of solutions were evaluated to select the buffer solutions:

140   1. Borate Buffered Saline (BBS) (Sigma-Aldrich, Madrid, Spain) in ready-to-use tablets. One tablet

141   was added to 500 mL of deionized water, homogenized, and adjusted to pH 8.2.

142   2. Carbonate-Bicarbonate Buffer (CBB) (Sigma-Aldrich, Madrid, Spain) in ready-to-use capsules. One

143   capsule was added and homogenized in 100 mL of deionized water with a pH of 9.6. The solution

144   was then diluted at a proportion of 1/16 until a pH of 8.45 was reached.

145   3. Borate Buffer Hydrochloric Acid (BB-HCl), following the protocol in the Handbook of Analytical

146   Chemistry (Lurie, 1975). Borate buffer consists of a mixture of two solutions, and the pH required

147   depends on the proportion of the solutions. It can be adjusted from 7.8 to 9.2 to achieve a pH of 8.5.

148   The final solution was prepared with 65.25 mL of Solution 1, placed in a 100 mL volumetric flask

149   and filled to 100 mL with Solution 2.

150   ○ Solution 1: sodium tetraborate ($Na_2B_4O_7$) (0.05 mol/L) (Sigma-Aldrich, Madrid, Spain) in

151   deionized water.

152   ○ Solution 2: hydrochloric acid (HCl) 0.1 N (Panreac, Castellar del Vallès, Spain).

153

154 ***Testing the Buffer Solutions***

155 The three buffer solutions were tested under similar conditions and the pH was recorded at different

156 concentrations, times and temperatures. The initial pH of each buffer solution was measured with a pH

157 meter (micro pH 2000, Crison Instruments, Barcelona, Spain) at room temperature (20 ℃). The solutions in

158 closed containers were then immersed in a water bath at 55 ℃ (Unitronic 6320100, J.P Selecta S.A, Abrera,

159 Spain) and the pH was measured at 30 minutes and 1 hour. The solutions were tempered at room

160 temperature and two and half hours after starting the experiments, the pH was measured again. Additionally,

161 tap water was used (Aigües de Barcelona, Barcelona, Cerdanyola del Vallès, Spain) to evaluate the

162 maximum dilution of activity of the enzymatic solution and to establish its real-life economic cost. All the

163 buffer solutions were repeatedly diluted in 1/2 the volume of water until reaching 1/64, while the pH

164 measurements were made at room temperature.

165

166 ***Cleaning Protocol***

167 The procedure of the cleaning protocol used to test the enzymatic formulation and the commercial product

168 was based on the previous research carried out by Guerrero-Navarro *et al.,* (2019), with modifications in the

169 rescaling of volumes of the milk fouling adhered to surfaces.

170 The enzymatic cleaning solutions were concentrated ten-fold, stored in sterile tubes and frozen at -18ºC until

171 use. Prior to the cleaning protocol, 5 mL of enzymatic cleaning solution was thawed at room temperature

172 and diluted in 30 mL of buffer solution to obtain a test volume of 35 mL. The cleaning solution was placed

173 in a 50 mL plastic flask with the MFM. The plastic flask was then placed in a water bath at maximum

174 agitation (Unitronic 320 OR, J.P Selecta S.A, Abrera, Spain) at 55 °C and 70 °C for the enzymatic and

175 chemical protocols, respectively.

176 The enzymatic cleaning protocol lasted 30 minutes and was split into two steps. First, the plastic flasks were

177 placed at 55 °C in the stirred water bath for 15 minutes. Then, the MFMs were removed from the cleaning

178 solution and placed in new plastic flasks with 5 mL of deionized water at 50 °C. Using a vortex at 2,500 rpm

179 for 50 seconds, the detachment elements were removed to simulate the flow of liquid in the pipes of the

180 facilities. The coupons were again placed in the cleaning solution, which was stirred in a water bath for an

181 additional 15 minutes at 55 °C. Last, the coupons were washed with hot water and stirred for 50 seconds to

182 complete the enzymatic cleaning.

183 The procedure for the chemical cleaning protocol was carried out in the same way, but lasted for 45 minutes

184 in three 15-minute steps at 70 °C in a water bath at maximum agitation. After each 15 minute step in the

185 water bath, a washing step was performed in the same manner as for the enzymatic cleaning protocol. The

186 cleaned models were then left at room temperature and the fouling of the remaining milk products in the

187 model was weighed once it had completely dried. The following techniques were applied to characterize the

188 residues from the different cleaning protocols in the same way as for the untreated MFM.

189

190 ***Methods to Study the Characterization of the Dairy Fouling After the Cleaning Protocols***

191 The methods used to evaluate the characterization of the dairy fouling were:

192 ***Fouling Morphology.*** Scanning electron microscopy (SEM) EVO MA 10 (Carl Zeiss, Oberkochen,

193 Germany) with no metallic coating, variable pressure and a maximum magnification of 1000x was used to

194 study the morphology of the surface of the MFM.

195 *Fouling Topography.* The fouling topography was analyzed with the optical confocal microscope Leica,

196 DCM 3D (Leica Microsystems, Bensheim, Germany). The roughness of the sample surfaces of the MFM

197 was evaluated using the software Leica Map Premium (Version 6.2.6409).

198 *Organic Milk Fraction.* The organic components of whole raw milk were determined using Near-infrared

199 Spectroscopy (NIRS) model NIR 5000 (1100-2500 nm) (FOSS-NIRSystems, Silver Springs, MD, USA), for

200 a rapid, non-destructive analysis of the different components of the bovine milk. The results were compared

201 with an internal calibration obtained from comparing the sample results and a standard of bovine whole

202 milk. This calibration was previously obtained by classical analysis methods (Kjeldahl method, Gerber

203 method and ashes by muffle).

204 All the samples were analyzed three times to determine the total solids, crude protein and crude fat, and the

205 whole system was calibrated using internal and commercial calibration tools.

206 *Epifluorescent Microscopy.* The different components of the whole milk samples were determined using a

207 mechanized fluorescence microscope (BX51, Olympus Optical, Tokyo, Japan) equipped with a mercury

208 lamp. A combination of three different dyes (Tab. 1) were selected to dye the MFM at the same time. The

209 stained samples remained in dark conditions for 25 minutes. For each sample, the dyes where mixed in 60

210 μL of sodium bicarbonate (NaHCO$_3$) 0.1M (Panreac, Castellar del Vallès, Spain) to avoid the dimerization

211 of Concanavalin A, according to the manufacturer's instructions. Epifluorescent microscopy images were

212 then taken and processed using the software CellSense (Olympus, Hospitalet del Llobregat, Spain). The

213 selected microscopy filters were blue (ConA 350) and green-red (FITC-NR).

214 *Confocal Laser Spectrometric Microscopy (CLSM).* The CLSM used was a Confocal Laser Spectral

215 Multiphoton Leica TCS SP5 (Leica Microsystems, Germany) to study the spatial composition of the milk

216 fouling model microstructure. This technology determines the composition of the inner layers of fouling and

217 residues using the 3D software (IMARIS, Bitplane, Zurich, Switzerland). Different representations of the

218 samples can thereby be created with the corresponding microphotographs and their information. The

219 microstructures were analyzed with an inverted 10x objective using the same fluorescent dyes as in the

220 epifluorescent microscopy (Tab. 1). The samples were previously incubated in a humidity chamber for 15

221 hours and were then placed in microscopy cell culture capsules (μ-Dishes IBIDI, GmbH, Planegg, Austria),

222 to be analyzed by CLSM. The software for processing microscopy images (Metamorph, Molecular Devices,

223   Sunnyvale, CA, USA) was used to quantify the different components of contamination of the dairy products

224   and the post-cleaning residues.

225   To ensure the selective action of Concanavalin A-Alexa Fluor 350 to dye lactose, it was tested with a model

226   consisting of lactose caramel monohydrate (Sigma, Madrid, Spain) and deionized water boiled until the

227   solution acquired a brown appearance. The lactose in the form of caramel was poured onto the stainless steel

228   discs and allowed to harden at 4 °C for 24 hours. They were then tested by epifluorescent microscopy and

229   CLSM to look for autofluorescence and dyed with ConA 350 to identify the difference in fluorescence from

230   the sample, the dye and the background.

231   ***Energy-Dispersive X-Ray Spectroscopy (EDX).*** The stainless steel discs, used as supports for the milk

232   fouling model, were analyzed by energy dispersion X-ray spectroscopy (EDX) (INCAx-act detector, Oxford

233   Instruments plc., Tubney Woods, United Kingdom), coupled with a SEM. The EDX quantifies or

234   qualitatively identifies the elements of the disc surfaces to evaluate their possible effect on the fouling

235   formation.

236   ***Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).*** All the samples were subject to

237   acidic digestion with nitric acid (HNO_3) (Panreact, Castellar del Vallès, Spain) in a microwave digestor

238   Ultrawave (Milestone Inc, Shelton, CT, USA) for whole milk. The solid samples were boiled in nitric acid

239   for 30 minutes and the liquid fraction was filtered by Milli-Q with a pore size of 0.45 (Millipore GmbH,

240   Schwalbach, Germany). The Optical Emission Spectrometry of Inductively Coupled Plasma (ICP-OES,

241   Optima 4300DV, Perkin-Elmer, Waltham, MA, USA) was used to analyze the inorganic fraction of bovine

242   whole milk, the laboratory model of dairy fouling and the residues of the cleaning protocols. The amounts of

243   Ca-Calcium (Ca), Potassium (K), Sodium (Na), Magnesium (Mg) and Phosphorus (P) was measured

244   through this technique.

245

246   ***Statistical Processing of Data***

247   The efficiency of the enzymatic treatment and the chemical product was compared by means of the

248   independent Student's t-test, using the software R version 3.1.0 (R Development Core Team, 2014). A value

249   of $p < 0.05$ was considered as statistically significant.

250

RESULTS AND DISCUSSION

251

252   The NIRS analysis of whole raw milk was carried out for 10 different batches used in fouling production

253   (Tab 2). The predicted composition of the milk used for this study agreed with the data found by Bylund

254   (1995) about the average composition of this type of food product.

255

256   Ten different batches of whole milk were used to follow the adapted protocols for creating dairy fouling

257   (Fig. 1D). The average weight of the fouling obtained was 44.56 mg /cm^2, higher than that previously

258   obtained in other studies. For example, Liu *et al.,* (2017) obtained 19.21 mg/cm^2 by using whole raw milk.

259   Further, Zouaghi *et al*., (2018) obtained 30.8 mg/cm^2 with a solution of whey protein. From this stock of

260   fouling models, 20 batches were cleaned using the chemical product and a further 20 were cleaned using the

261   enzymatic formula.

262

263   Subgroups of fouling models were used for digestion and processed with ICP-OES: (1) ten models were

264   processed using the chemical cleaner, (2) ten models using the enzymatic cleaning, and (3) ten of the

265   original milk fouling models were used with no modifications. This allowed us to determine the quantity of

266   five different elements of the mineral fraction of fouling. A second, identical subgroup of fouling models

267   was analyzed using CLSM microscopy with fluorescent dyes, including a post-process to semi-quantify the

268   components of the organic fraction from fouling.

269

270   ***Buffers***

271   The results of the buffer solution test showed that the solution was quite stable over time, temperature and

272   concentration variations. In addition, it stabilized the pH range, even when using potable water (Fig. 2 and

273   3). After testing the different buffer solutions for the enzymatic cleaning, hydrochloric acid (BB-HCl) was

274   selected as the most balanced buffer. This was because the borate buffer stabilizes the pH during the

275   different steps. Once the borate buffer was selected, all the following cleaning tests with enzymes were

276   carried out with undiluted BB-HCl and deionized water.

277

278   ***Cleaning Efficacy***

279 According to the results, the efficiency of the chemical and enzymatic products was 86.9% and 87.1%,

280 respectively. These results show that there were no statistically significant differences between the two

281 treatments ($p > 0.05$) (Fig. 4), while they were observed to be equally efficient in terms of the removal of

282 scale (Fig. 1E). With an average efficiency value of 87% of dairy fouling, enzymatic cleaning procedures

283 can be suggested as a feasible alternative to the use of chemical cleaners, which have the added benefits of

284 reduced waste water and cleaning times and energy saving on thermal procedures (Takahasi *et al*., 1996;

285 Fryer & Asteriadou, 2009; Boyce *et al*, 2010).

286 *Morphology*

287 The results of the SEM technique showed that two types of materials form the micro-morphology of the

288 fouling model. These results are based on their density and their resistance to the passage through the

289 electrons during this technique. Thus, one of them appears darker than the other (Fig. 5, B). This could be

290 due to the presence of a higher concentration of mineral matter, which is denser than organic matter. In

291 addition, there are fissures and pores in the surface (Fig. 5 A and B). These fissures in the morphology could

292 be attributed to the desiccation of the sample during the vacuum step of the SEM. Other authors suggest that

293 these pores could be gas bubbles formed within the fouling during the heat treatment (Boyce *et al*., 2010;

294 Gandhi *et al*., 2017). Boyce *et al* (2010) suggest that these bubbles can increase the formation of the scale.

295 Moreover, in the dairy industry, liquids under pressure reduce the formation of the air bubbles (Bansal and

296 Chen, 2006).

297

298 *Microstructure*

299 The roughness analysis (Fig. 6) showed that the surface of the milk fouling model using a 3D topography

300 measurement and SEM microphotography can produce cracks (Fig. 5). However, the pores are of a natural

301 origin due to the absence of a drying process to carry out this topographic technique.

302

303 *Composition Analysis*

304 The dairy fouling was studied in two separate parts: the content of organic matter and the content of

305 inorganic matter. Two different techniques, epifluorescent microscopy and CLSM, were used to evaluate the

306 content of organic matter. Likewise, a fluorescence microscopy study was carried out to test the solid

307 components of the dairy fouling and the residues of the cleaning protocol. Epifluorescence microscopy using

308 three dyes was an easy procedure to detect the presence of fouling components (Tab.1). However, it was

309 impossible to detect the three compounds selected with the straining formula, probably because of the

310 inherent auto-fluorescence of this type of residue (Fig. 7A and B). This was probably due to the compounds

311 produced by the Maillard Reaction from the proteins and sugars in the milk. This was especially true for the

312 ConA-350 dye, due to the amount of background auto-fluorescence in the blue channel. CLSM microscopy

313 was also used because this technique is very sensitive to detecting fluorescent dyes, despite the samples

314 being autofluorescent. Thus, the three dyes used were detected by this microscopy and the 2D models (Fig.

315 7C, E and F), and 3D models (Fig. 7D), which produced in milk fouling models and the residues of cleaning

316 protocols. Data were also obtained on the average intensity of the fluorescence in each channel to semi-

317 quantify the organic compounds and to trace the abundance of each compound in the depth of the sample.

318

319 CLSM microscopy can record each channel in different files, a feature that is very useful to obtain the

320 spatial disposition of the different elements of the sample. In this example of a dairy fouling model (Fig 7D),

321 different overlapping channels are visible where there are the different compounds. Figure 7C shows a green

322 channel over a blue channel, which means that the proteins are on the surface and sugars are in the lowest

323 part. On overlapping the three different channels, red dots appear over all the other compounds (Fig 7E),

324 meaning that the fats are in the highest part of the fouling model.

325

326 By processing the image information with the Metamorph® software (Molecular Devices, Sunnyvale, CA,

327 USA), the differences in abundance at different depths of the fouling can be observed (Fig. 8). At the top of

328 the sample, there is a mixture of protein and fats (Fig 7E and 8), and sugars increase in intensity (Fig. 7C

329 and 8) in the middle of the sample. The information offered by this technique as background to the samples

330 can be inaccurate so it was removed from the graph due to its lack of informative content. The semi-

331 quantification of the organic compounds in the dairy fouling models is based on an approximation taken

332 from the average intensity of the fluorescence for each compound and the total fluorescence of the sample

333 (Fig. 9).

334

335 ICP-OES (quantification) and EDX coupled with SEM (qualification) were used to study the inorganic

336 fraction.

337 The results of the EDX-SEM (Fig. 10) showed that there are three different peaks along the graph: Cr and Fe

338 peaks, which correspond to stainless steel as the base material for the production of dairy model, C and O

339 peaks that correspond to organic matter. The lower peaks represents the mineral salts of the milk. When the

340 peaks are less than 2% - 4% they cannot be used for quantification, but they do indicate the presence of

341 these elements qualitatively. Other authors suggest that the presence of C and O peaks obtained with EDX

342 indicate the presence of lipids, the peaks of C and N indicate the presence of proteins, and the S peak

343 indicates the presence of sulfur-containing amino acids (Gandhi *et al*., 2017). During the tests with SEM

344 (Fig. 5), an elementary analysis was performed in parallel using EDX coupled with SEM (Fig. 10). This

345 technique was used to identify the elements that would later be quantified by ICP (Fig. 11).

346

347 The highest mineral concentration in the whole milk was K (1.54 mg/g) followed by Ca (1.18 mg/g) and P

348 (0.87 mg/g). However, the most important mineral on the fouling was Ca (14.83 mg/g) followed by P (9.79

349 mg/g) and K (7.39 mg/g). Therefore, a mineralization of the organic matter on the fouling was expected

350 (Bansal and Chen, 2006). When an alkaline-acidic cleaning process was performed, Ca was reduced by

351 77.34%, which was similar to P (75.08%); K was solubilized completely, while Na increased more than

352 three-fold (340.91%). Na increased due to the solubilization of the ion from the chemical used on the

353 fouling. On the other hand, when the enzymatic treatment was performed, reductions in Ca and P

354 concentrations of 71.61% and 74.67% were observed, respectively. The final mineral concentrations were

355 1.5x higher in the case of the chemical treatment compared to the enzymatic treatment, and they were more

356 homogeneous in the case of the residues from the enzymatic treatment. The use of acid treatments after an

357 alkaline treatment is useful for detaching the minerals by solubilization. However, if the adhesive element

358 from the fouling, which initiates the accumulation of matter on the surface, is destroyed, an effective mineral

359 elimination can be achieved without using chemicals that have a high environmental impact. This is one of

360 the advantages of the enzymatic treatments; it is not necessary to focus on all the elements, only on those

361 that start the adhesion process and promote the accumulation of food residues. In this study, the mixture of

362 sugar and proteins in the form of caramel contributes to the fouling process.

363

CONCLUSIONS

364

365 Fluorescent microscopy with selective dyes in conjunction with micro-topographic measurement using

366 SEM-EDX and ICP-OES can be a useful tool for understanding the composition, distribution and structure

367 of the milk fouling matter adhered to surfaces. The use of these techniques enabled the representation of the

368 compounds of fouling in a vertical profile, demonstrating our initial hypothesis of how important the role of

369 the caramel component is in the entire process of the fouling adhesion to surfaces. The caramel component

370 has a fundamental role in dairy fouling formation and its spatial location reinforces the notion that it acts as

371 an adherent for the other residues during heat treatments. All this information is very useful when studying

372 deposits from the thermic milk treatments and comparing the chemical and enzymatic cleaning formulas

373 tested. The mixture of analytical techniques was used to obtain more information to support the argument

374 for the advantages of using enzymes for cleaning this type of residue embedded in industrial surfaces.

375 Epifluorescence microscopy with selective dyes is also very useful to assess formulas and methods before

376 testing new cleaning products in the dairy processing plants, saving on the large amount of energy and water

377 required during the rinse steps. Last, the use of the enzymatic cleaning product proposed in this study is

378 more environmentally sustainable than the classic acid and alkaline products.

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383 **Figure 1. A) Stainless steel 316 2B discs 2 cm in diameter, B) Surfaces where the milk fouling models**

384 **(MFM) were to be created, C) MFM once the production process was finished, D) MFM finished**

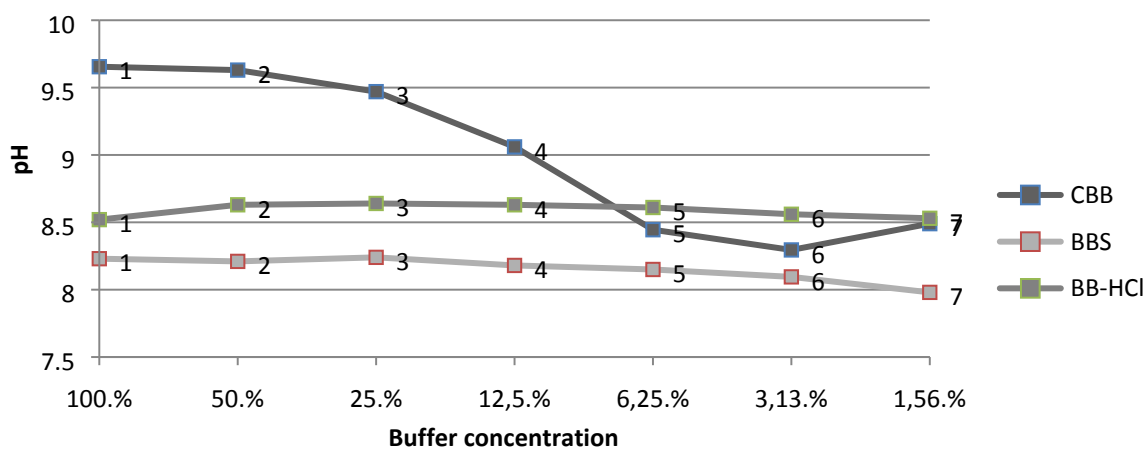385 **without aluminum walls, E) MFM once cleaned by the enzymes.**

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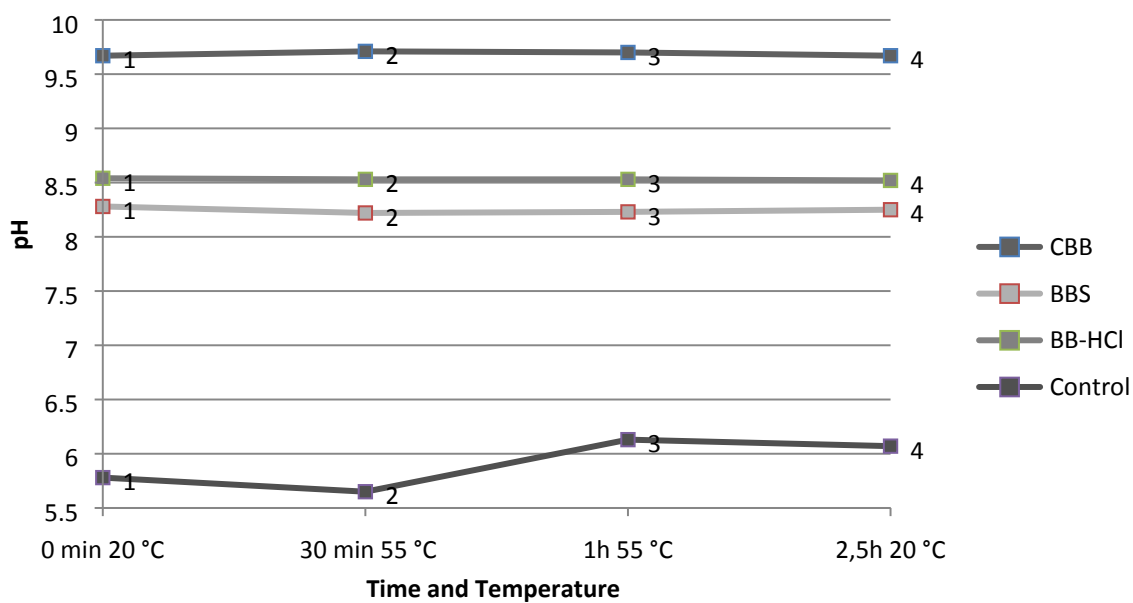
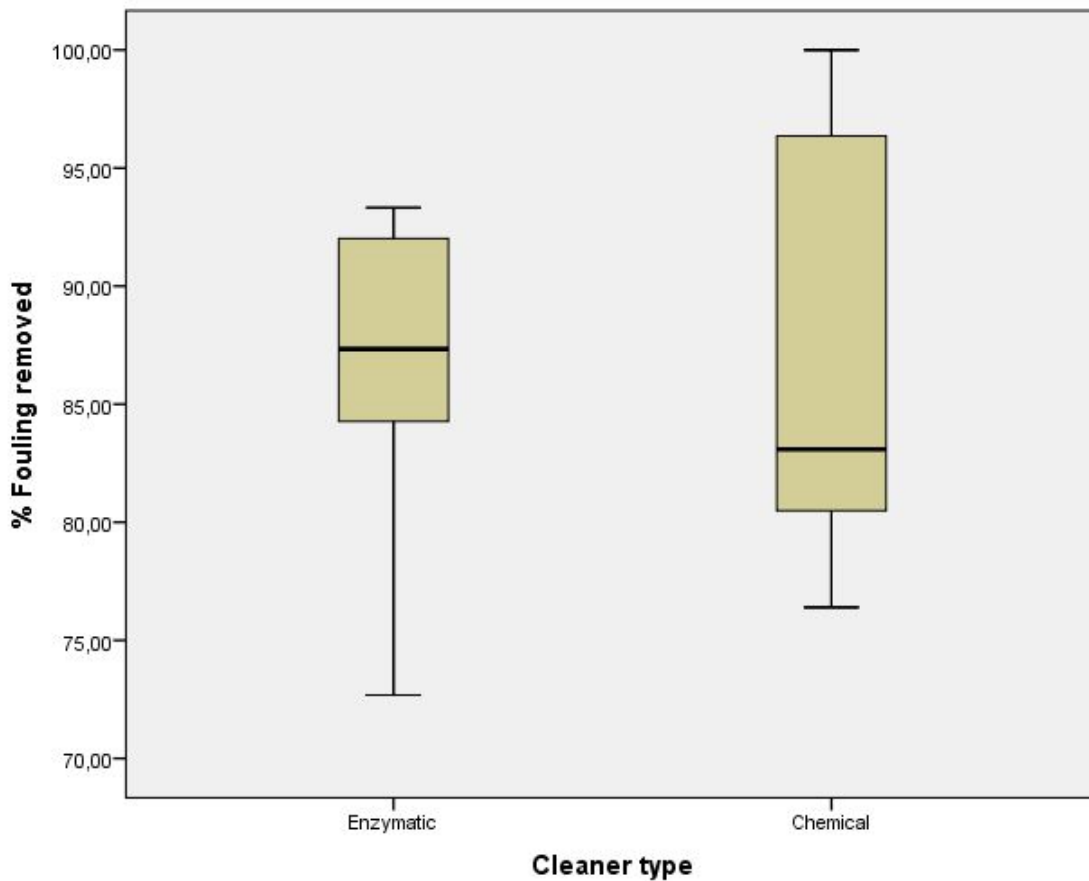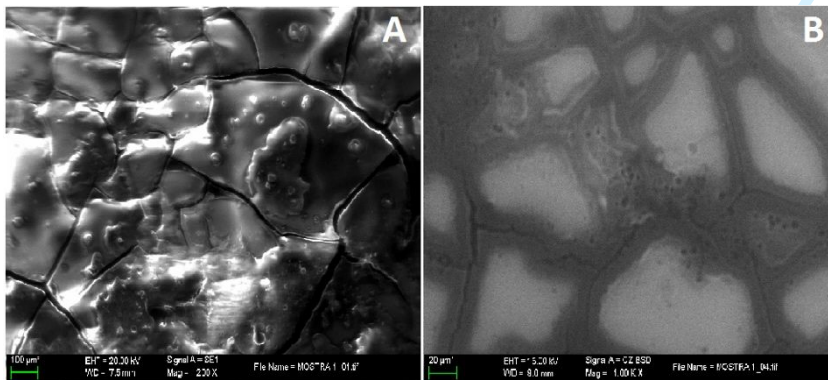**Figure 2. Average pH of different buffer solutions exposed to heat over time, with tap water as a control.**

Figure 3. pH of diluted buffer solutions using tap water at 20 ºC.
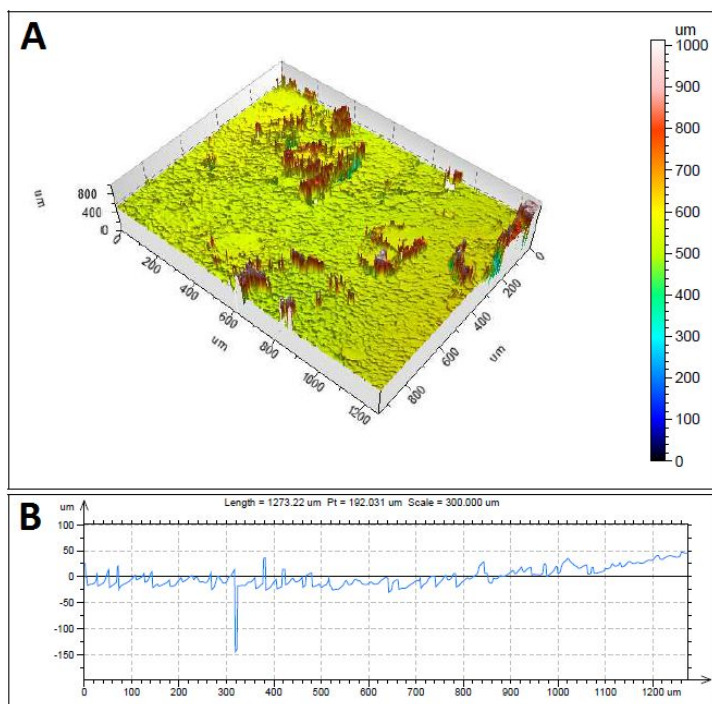
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Figure 4. Boxplot of the efficiency of two different cleaners (n = 27).

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Figure 5. Microphotographs of milk fouling models using SEM. Amplifications A) 200x B) 1000x.

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Figure 6. Topographic view of the milk fouling model. A) 3D view of the surface of the fouling model.

Cold colors represent the depressions on the surface below the average level of the sample, and warm

colors represent the elevations above the surface average B) Topographic profile of the milk-fouling

model. Level 0 represents the average height of the surface; negative values are cracks and

concavities, while positive values are undulations and accumulations above the average height.

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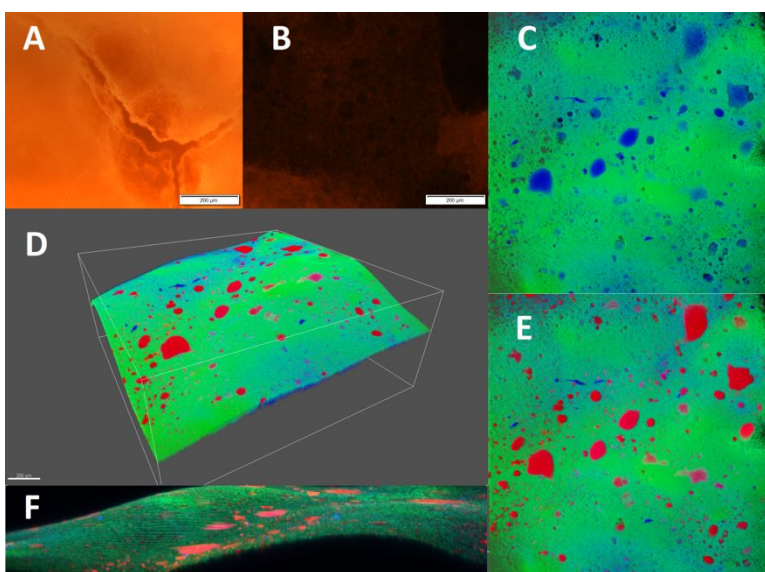
Figure 7. A) 4x Auto-fluorescence of dairy fouling from MFM, 4x red/green filter, captured using

DEM. B) Auto-fluorescence after enzymatic cleaning. C) MFM dyed with selective fluorescence dyes,

10x channel blue (sugar/caramel) and green (protein) captured using CLSM. D) 3D reconstruction

415 using CLSM images of MFM, height of 618.3 μm. E) MFM dyed with selective fluorescence dyes, 10x

416 channel blue, green and red (fats) captured using CLSM. F) Side view of the whole stack of
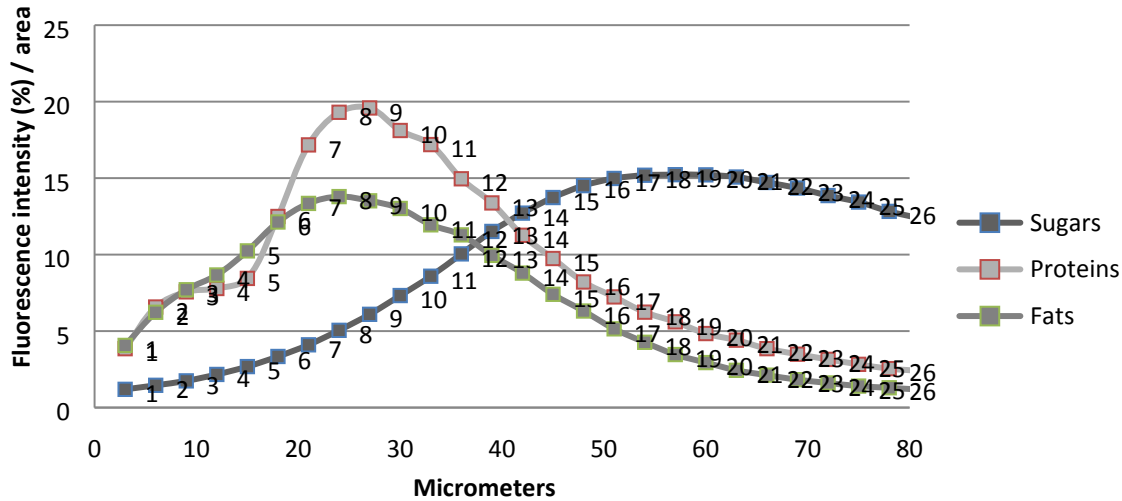
417 microphotographs.

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420 **Figure 8. Representation of the intensity (%) of the blue, red and green CLSM channels from the**

421 **depth of the milk fouling.**

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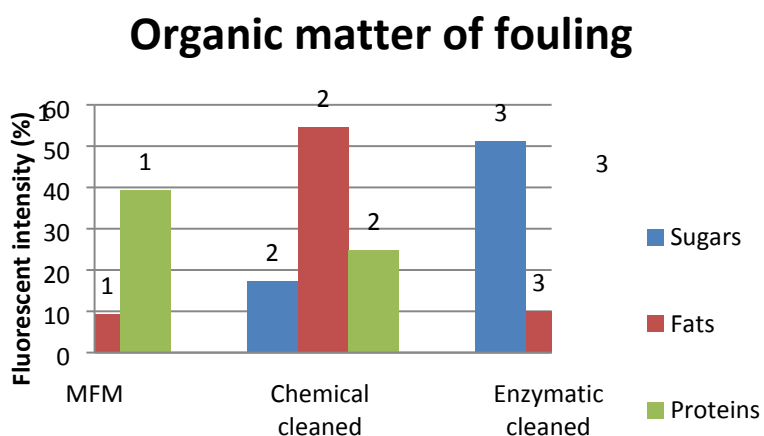
425 **Fig 9. Average fluorescent intensity (%) in the organic matter fraction from MFM (n = 10).**
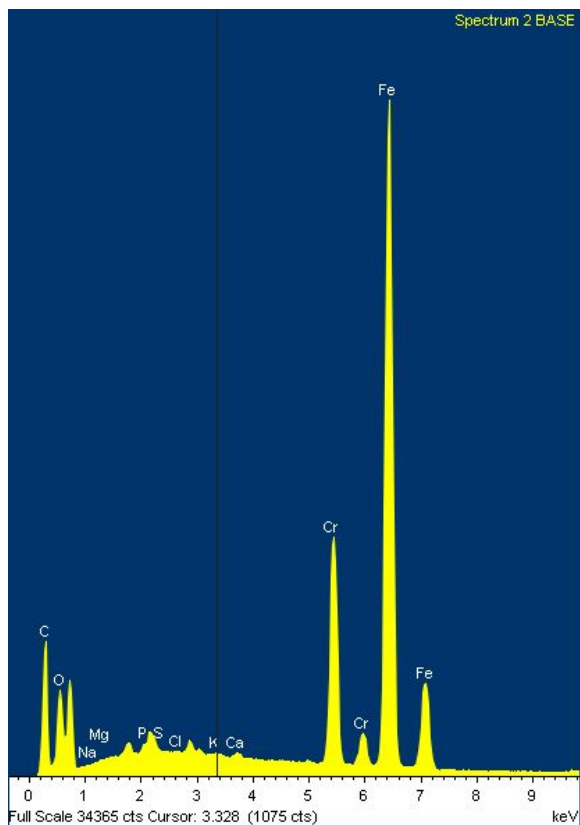
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429 **Figure 10. Results from EDX-SEM. The scale expressed in kilo-electron Volts (keV) shows the signals**

430 **obtained by EDX using an internal standard and identifying each peak with the corresponding**

431 **element of the periodic table. Lighter elements than carbon (C) are not shown by this technique.**
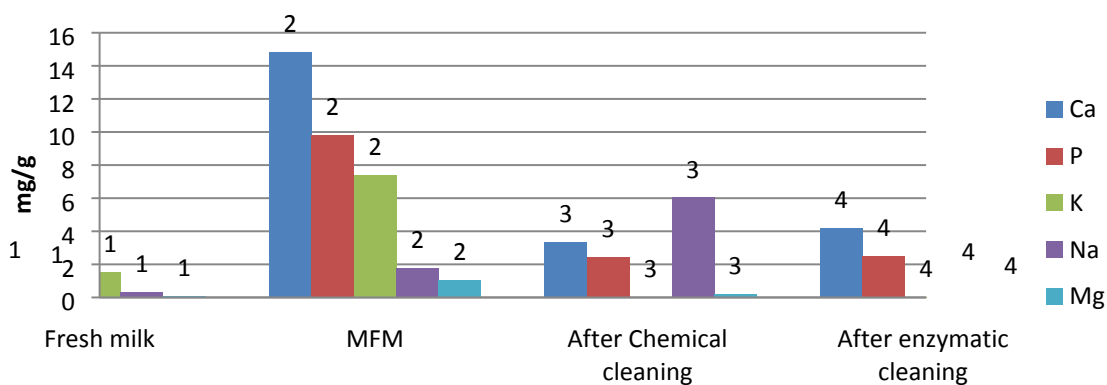
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435 **Figure 11. ICP-OES results from whole milk, milk fouling models and cleaning residues (n=10).**

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437 **Table 1. Fluorescent dyes used in epifluorescent microscopy.**

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Fluorescent Dye	Produced by	Concentration	Volum used	Target	Color
Fluorescein 5-isothiocyanate (FITC)	Sigma-Aldrich, St Louis, USA	1 mg/mL	10 μL	Proteins	Green
Nile Red (NR)	Sigma-Aldrich, St Louis, USA	0.1 mg/mL	10 μL	Lipids	Red
Concanavalin A-Alexa Fluor 350 (ConA 350)	Thermo Fisher/Molecular Proves, Eugene, USA	0.3 mg/mL	20 μL	Sugars	Blue

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441   **Table 2. Milk composition used for fouling generation (n = 30).**

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Component	Average %
Lipids	3.63 ± 0.14
Proteins	3.38 ± 0.10
Total solids	12.64 ± 0.19
Total sugars	5.63 ± 0.19

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