Microscopic analysis and microstructural characterization of the organic and inorganic components of dairy fouling during the cleaning process

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Interpretive Summary

Microscopic analysis and microstructural characterization of the organic and inorganic components of dairy fouling during the cleaning process.

The thermal treatment of milk produces fouling, which can accumulate on contact surfaces. The elimination of fouling is expensive, which is a serious problem in the dairy industry. Therefore, studying the composition and microscopic structure of fouling could be of great help to improve cleaning processes in this industry. This paper presents the composition and structure of dairy fouling in different setups. A new improved enzymatic formula for eliminating fouling is also tested as a real alternative for use in the dairy industry.

ABSTRACT

It is essential to have a thorough knowledge of the composition of the fouling and residues in facilities to ensure a correct and efficient cleaning process. There are many ways to determine the content of the organic and inorganic fraction in a fouling sample, ranging from classic chemistry techniques to advanced technological devices. In this study, the organic residues of milk fouling were evaluated using fluorescent microscopy. The inorganic content was analyzed with Energy-dispersive X-ray spectroscopy and complemented with Inductively Coupled Plasma Optical Emission Spectrometry. These techniques were also applied to the residues from two different cleaning procedures: (1) a commercial alkaline cleaner and (2) a formula of enzymes and surfactants. The results indicate a fouling removal efficiency of 86.9% for the alkaline product and 87.01% for the enzymatic formula. When the two products were compared, similar activities were found to remove the fouling. Knowing the average content of the fouling in the industry helps to formulate better strategies to save water, energy and time during the cleaning process. Additionally, studying the cleaning residues helps to avoid problems of cross-contamination between batches or subsequent microbial growths (biofilms) on surfaces with residues.

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

INTRODUCTION

There are two types of dairy fouling depending on the temperature of the process that promotes its formation. In Type A, the temperature range is from 75 ºC to 110 ºC and the composition is 50% - 70% proteins, 30% - 40% minerals, and 4% - 8% fat. Type B is formed at temperatures above 110 °C and the content is 70% - 80% minerals, 15% - 20% proteins, and 4% - 8% fat (Visser and Jeurnink, 1997). This study used fouling Type A, mainly organic residues produced at 90 ºC, similar to a pasteurization process (Bansal and Chen, 2006). However, the morphology and spatial composition is very heterogeneous in both types.

During heat treatments, there is a deposition of materials from the food to the surfaces of the facilities (Fryer and Asteriadou, 2009). The problems produced by fouling could be summarized into three different

categories: food security, shelf life, and operating problems (Barish and Goddard, 2013). This is important to avoid cross-contamination between different batches from the preexisting fouling in the facilities (Ayala *et al*., 2016; Hagsten *et al.*, 2016). While the cleaning process is costly and time consuming, it is essential to the food industry (Liu *et al*., 2006; Fryer *et al,* 2011). However, it is still poorly understood and cleaning protocols are designed in a semi-empirical manner. In the food industry, 25% of the operating time is spent on the cleaning process (Fryer *et al.*, 2011). In this industry, it is impossible to know the extent and composition of fouling throughout the plant, so the use of models to represent the fouling must be taken into account, perhaps through a stochastic rather than a purely deterministic approach (Fryer and Asteriadou, 2009). Fouling is formed by adherence to the surface and cohesion between elements of the layer on the surface. Cleaning can result from either or both adhesive and cohesive detachment (Liu *et al*., 2006]. Identifying the composition and its structure is a crucial step in studying the residues from industrial treatments to prevent their production and to know how to clean the facilities. There are a wide range of options for identifying waste elements. The choice depends on the kind of target identified: (1) whether the waste is attached to other items, (2) the budget, (3) the time available, and (4) access to new techniques or special requirements such as specialized personnel and additional safety features. In this case, the dairy fouling was a residue firmly attached to the metal surface of the facilities. For this type of residue, various analyses are required to identify the components of the organic and inorganic fraction of the dairy fouling.

Confocal Laser Scanning Microscopy (CLSM) technology is widely used to study food structure and composition because this method enables the use of a variety of selective stains, as well as post processes (Auty *et al.*, 2001). There are many studies on food products using CLSM, e.g. the distribution of fat and proteins in mozzarella cheese and chocolate (Auty *et al.*, 2001) and the fat globules in milk and milk products (Evers *et al.*, 2008). In other studies, this technology has been used to study the structure and composition of different residues such as fouling on membrane filtration units (Doumèche. *et al.*, 2007; Stoica *et al.*, 2018). Other authors have studied dairy fouling Type A (Boyce *et al*., 2010) and Type B by CLSM (Hagsten, *et al*., 2016).

The aim of our study was to improve knowledge about fouling Type A, especially its composition and microstructure, and to identify the composition of the residues from cleaning procedures. Residues were

produced to compare the effectiveness of two different cleaning products (a caustic one-pass commercial product and a novel enzymatic formula) using a laboratory-fouling model as a target for the cleaners.

MATERIALS AND METHODS

Milk and Test Surfaces

The fouling formed during the assays was generated from bovine whole raw milk refrigerated and supplied by a dairy farm (Granja Can Bordoi, Sant Antoni de Vilamajor, Catalonia, Spain) and stored at 5 ºC until the assays. The test surfaces were made of stainless steel (Type AISI 316 with a grade 2B finish), 2.0 cm in diameter and 1.2 mm thick (Fig. 1A). This kind of surface was selected as a sample for the studies on fouling growth and to develop cleaning formulations because it is one of the main materials for plate heat exchangers (PHE). Heat resistant aluminum foil tape (Ceys, L'Hospitalet de Llobregat, Spain) was used to make the walls of the test surfaces. Each side of the wall was approximately 6 mm high and 70 µm thick (Fig. 1B). Before using the stainless steel surfaces to produce the milk-fouling laboratory models (MFS), they were cleaned and disinfected in accordance with the standard EN 13697 (Anonymous, 2015). They were then kept in sealed bags to avoid contamination.

Fouling Production

The milk fouling models (MFM) were formed according to the modified procedure referenced by Guerrero-Navarro *et al*. (2019). First, the containers were weighed using an analytical balance (Mettler AE 100, Mettler-Toledo S.A.E., Hospitalet del Llobregat, Spain) and then pre-heated at 90 °C in a fan-assisted oven (IDL-FI-80, Labolan, S.L. 31191, Esparzar de Galar, Spain). When the containers reached the working temperature (Fig. 1B), 0.4 mL of whole raw milk was added and they were placed back in the oven for 30 minutes. The milk was then dried and attached to the surface. Thereafter, another 0.4 mL of milk was added. The addition of milk and the drying process were repeated 5 times, after which the washing process was performed by introducing each MFM into plastic flasks with 4 mL of distilled water at 50 ºC and shaking them at 2,500 rpm for 50 seconds using an agitator (REAX Top, Heidolph Instruments, Schwabach, Germany). The coupons were then rinsed with deionized water to remove the unattached milk on the surfaces and then pre-heated again at 90 ºC for 5 minutes. Once the working temperature was reached, five

additional steps of adding milk and drying were performed (10 cycles in total), after which the washing step was repeated using water at 50 ºC (Fig 1C).

Cleaning Solutions (CS)

Two kinds of cleaning products were selected to remove the dairy-fouling: an enzyme formulation and a commercial product. The enzymatic formulation was composed of protease (Savinase®, Novozymes, Bagsværd, Denmark), amylase (Termamyl Ultra®, Novozymes, Bagsværd, Denmark) and a non-foam commercial surfactant (Tensio CIP®, ITRAM Higiene, Vic, Spain). The commercial product used as the control was a one-pass alkaline formulation (BRIO COMPLEX®, ITRAM Higiene, Vic, Spain) currently used in the industry to remove fouling and was chosen to represent the classical strategy of chemical cleaning.

Products used as solutions:

- Cleaning Solution 1 (CS1): composed of 6% v/v alkaline cleaner in deionizate water. Applied at 70 °C for 45 minutes with a pH of 10 to 12, according to manufacturer's instructions.
- Cleaning Solution 2 (CS2): composed of 0.12% v/v of Savinase®, 0.10% v/v of Termamyl Ultra®, and 25% v/v of Tensio CIP®, diluted in buffer solution (pH 8.5) and used at 55 °C for 30 minutes.

Improving the Enzymatic Formulation

The enzymatic formulation used in this study was established based on the fouling components in previous experiments (Guerrero-Navarro *et al*., 2019). Moreover, the activity curves of the enzymes used in the formulation provided by the manufacturer were analyzed before the assays. Thereafter, the analysis was performed using a starch solution and bovine serum albumin (BSA) for amylase and protease, respectively. The pH was adjusted from 9.5 to 8.5 and the temperature from 50 °C to 55 °C to maintain the effectiveness of the enzymatic cleaning solution during the assays at >80%.

A buffer solution was used to avoid pH variations and thereby improve the activity of the enzymatic formulation, since a low pH close to neutrality would inactivate the enzymes (Argüello *et al*., 2003). Three kinds of solutions were evaluated to select the buffer solutions:

1. Borate Buffered Saline (BBS) (Sigma-Aldrich, Madrid, Spain) in ready-to-use tablets. One tablet was added to 500 mL of deionized water, homogenized, and adjusted to pH 8.2.
2. Carbonate-Bicarbonate Buffer (CBB) (Sigma-Aldrich, Madrid, Spain) in ready-to-use capsules. One capsule was added and homogenized in 100 mL of deionized water with a pH of 9.6. The solution was then diluted at a proportion of 1/16 until a pH of 8.45 was reached.
3. Borate Buffer Hydrochloric Acid (BB-HCl), following the protocol in the Handbook of Analytical Chemistry (Lurie, 1975). Borate buffer consists of a mixture of two solutions, and the pH required depends on the proportion of the solutions. It can be adjusted from 7.8 to 9.2 to achieve a pH of 8.5. The final solution was prepared with 65.25 mL of Solution 1, placed in a 100 mL volumetric flask and filled to 100 mL with Solution 2.
    - Solution 1: sodium tetraborate ($Na_2B_4O_7$) (0.05 mol/L) (Sigma-Aldrich, Madrid, Spain) in deionized water.
    - Solution 2: hydrochloric acid (HCl) 0.1 N (Panreac, Castellar del Vallès, Spain).

Testing the Buffer Solutions

The three buffer solutions were tested under similar conditions and the pH was recorded at different concentrations, times and temperatures. The initial pH of each buffer solution was measured with a pH meter (micro pH 2000, Crison Instruments, Barcelona, Spain) at room temperature (20 ºC). The solutions in closed containers were then immersed in a water bath at 55 ºC (Unitronic 6320100, J.P Selecta S.A, Abrera, Spain) and the pH was measured at 30 minutes and 1 hour. The solutions were tempered at room temperature and two and half hours after starting the experiments, the pH was measured again. Additionally, tap water was used (Aigües de Barcelona, Barcelona, Cerdanyola del Vallès, Spain) to evaluate the maximum dilution of activity of the enzymatic solution and to establish its real-life economic cost. All the buffer solutions were repeatedly diluted in 1/2 the volume of water until reaching 1/64, while the pH measurements were made at room temperature.

Cleaning Protocol

The procedure of the cleaning protocol used to test the enzymatic formulation and the commercial product was based on the previous research carried out by Guerrero-Navarro *et al.,* (2019), with modifications in the rescaling of volumes of the milk fouling adhered to surfaces.

The enzymatic cleaning solutions were concentrated ten-fold, stored in sterile tubes and frozen at -18ºC until use. Prior to the cleaning protocol, 5 mL of enzymatic cleaning solution was thawed at room temperature and diluted in 30 mL of buffer solution to obtain a test volume of 35 mL. The cleaning solution was placed in a 50 mL plastic flask with the MFM. The plastic flask was then placed in a water bath at maximum agitation (Unitronic 320 OR, J.P Selecta S.A, Abrera, Spain) at 55 °C and 70 °C for the enzymatic and chemical protocols, respectively.

The enzymatic cleaning protocol lasted 30 minutes and was split into two steps. First, the plastic flasks were placed at 55 °C in the stirred water bath for 15 minutes. Then, the MFMs were removed from the cleaning solution and placed in new plastic flasks with 5 mL of deionized water at 50 °C. Using a vortex at 2,500 rpm for 50 seconds, the detachment elements were removed to simulate the flow of liquid in the pipes of the facilities. The coupons were again placed in the cleaning solution, which was stirred in a water bath for an additional 15 minutes at 55 °C. Last, the coupons were washed with hot water and stirred for 50 seconds to complete the enzymatic cleaning.

The procedure for the chemical cleaning protocol was carried out in the same way, but lasted for 45 minutes in three 15-minute steps at 70 °C in a water bath at maximum agitation. After each 15 minute step in the water bath, a washing step was performed in the same manner as for the enzymatic cleaning protocol. The cleaned models were then left at room temperature and the fouling of the remaining milk products in the model was weighed once it had completely dried. The following techniques were applied to characterize the residues from the different cleaning protocols in the same way as for the untreated MFM.

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

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

Fouling Morphology. Scanning electron microscopy (SEM) EVO MA 10 (Carl Zeiss, Oberkochen, Germany) with no metallic coating, variable pressure and a maximum magnification of 1000x was used to study the morphology of the surface of the MFM.

Fouling Topography. The fouling topography was analyzed with the optical confocal microscope Leica, DCM 3D (Leica Microsystems, Bensheim, Germany). The roughness of the sample surfaces of the MFM was evaluated using the software Leica Map Premium (Version 6.2.6409).

Organic Milk Fraction. The organic components of whole raw milk were determined using Near-infrared Spectroscopy (NIRS) model NIR 5000 (1100-2500 nm) (FOSS-NIRSystems, Silver Springs, MD, USA), for a rapid, non-destructive analysis of the different components of the bovine milk. The results were compared with an internal calibration obtained from comparing the sample results and a standard of bovine whole milk. This calibration was previously obtained by classical analysis methods (Kjeldahl method, Gerber method and ashes by muffle).

All the samples were analyzed three times to determine the total solids, crude protein and crude fat, and the whole system was calibrated using internal and commercial calibration tools.

Epifluorescent Microscopy. The different components of the whole milk samples were determined using a mechanized fluorescence microscope (BX51, Olympus Optical, Tokyo, Japan) equipped with a mercury lamp. A combination of three different dyes (Tab. 1) were selected to dye the MFM at the same time. The stained samples remained in dark conditions for 25 minutes. For each sample, the dyes where mixed in 60 µL of sodium bicarbonate ($NaHCO_3$) 0.1M (Panreac, Castellar del Vallès, Spain) to avoid the dimerization of Concanavalin A, according to the manufacturer's instructions. Epifluorescent microscopy images were then taken and processed using the software CellSense (Olympus, Hospitalet del Llobregat, Spain). The selected microscopy filters were blue (ConA 350) and green-red (FITC-NR).

Confocal Laser Spectrometric Microscopy (CLSM). The CLSM used was a Confocal Laser Spectral Multiphoton Leica TCS SP5 **(**Leica Microsystems, Germany) to study the spatial composition of the milk fouling model microstructure. This technology determines the composition of the inner layers of fouling and residues using the 3D software (IMARIS, Bitplane, Zurich, Switzerland). Different representations of the samples can thereby be created with the corresponding microphotographs and their information. The microstructures were analyzed with an inverted 10x objective using the same fluorescent dyes as in the epifluorescent microscopy (Tab. 1). The samples were previously incubated in a humidity chamber for 15 hours and were then placed in microscopy cell culture capsules (µ-Dishes IBIDI, GmbH, Planegg, Austria), to be analyzed by CLSM. The software for processing microscopy images (Metamorph, Molecular Devices,

Sunnyvale, CA, USA) was used to quantify the different components of contamination of the dairy products and the post-cleaning residues.

To ensure the selective action of Concanavalin A-Alexa Fluor 350 to dye lactose, it was tested with a model consisting of lactose caramel monohydrate (Sigma, Madrid, Spain) and deionized water boiled until the solution acquired a brown appearance. The lactose in the form of caramel was poured onto the stainless steel discs and allowed to harden at 4 °C for 24 hours. They were then tested by epifluorescent microscopy and CLSM to look for autofluorescence and dyed with ConA 350 to identify the difference in fluorescence from the sample, the dye and the background.

Energy-Dispersive X-Ray Spectroscopy (EDX). The stainless steel discs, used as supports for the milk fouling model, were analyzed by energy dispersion X-ray spectroscopy (EDX) (INCAx-act detector, Oxford Instruments plc., Tubney Woods, United Kingdom), coupled with a SEM. The EDX quantifies or qualitatively identifies the elements of the disc surfaces to evaluate their possible effect on the fouling formation.

Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). All the samples were subject to acidic digestion with nitric acid (HNO3) (Panreact, Castellar del Vallès, Spain) in a microwave digestor Ultrawave (Milestone Inc, Shelton, CT, USA) for whole milk. The solid samples were boiled in nitric acid for 30 minutes and the liquid fraction was filtered by Milli-Q with a pore size of 0.45 (Millipore GmbH, Schwalbach, Germany). The Optical Emission Spectrometry of Inductively Coupled Plasma (ICP-OES, Optima 4300DV, Perkin-Elmer, Waltham, MA, USA) was used to analyze the inorganic fraction of bovine whole milk, the laboratory model of dairy fouling and the residues of the cleaning protocols. The amounts of Ca-Calcium (Ca), Potassium (K), Sodium (Na), Magnesium (Mg) and Phosphorus (P) was measured through this technique.

Statistical Processing of Data

The efficiency of the enzymatic treatment and the chemical product was compared by means of the independent Student's t-test, using the software R version 3.1.0 (R Development Core Team, 2014). A value of $p < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

The NIRS analysis of whole raw milk was carried out for 10 different batches used in fouling production (Tab 2). The predicted composition of the milk used for this study agreed with the data found by Bylund (1995) about the average composition of this type of food product.

Ten different batches of whole milk were used to follow the adapted protocols for creating dairy fouling (Fig. 1D). The average weight of the fouling obtained was 44.56 mg /cm^2, higher than that previously obtained in other studies. For example, Liu *et al.,* (2017) obtained 19.21 mg/cm^2 by using whole raw milk. Further, Zouaghi *et al*., (2018) obtained 30.8 mg/cm^2 with a solution of whey protein. From this stock of fouling models, 20 batches were cleaned using the chemical product and a further 20 were cleaned using the enzymatic formula.

Subgroups of fouling models were used for digestion and processed with ICP-OES: (1) ten models were processed using the chemical cleaner, (2) ten models using the enzymatic cleaning, and (3) ten of the original milk fouling models were used with no modifications. This allowed us to determine the quantity of five different elements of the mineral fraction of fouling. A second, identical subgroup of fouling models was analyzed using CLSM microscopy with fluorescent dyes, including a post-process to semi-quantify the components of the organic fraction from fouling.

Buffers

The results of the buffer solution test showed that the solution was quite stable over time, temperature and concentration variations. In addition, it stabilized the pH range, even when using potable water (Fig. 2 and 3). After testing the different buffer solutions for the enzymatic cleaning, hydrochloric acid (BB-HCl) was selected as the most balanced buffer. This was because the borate buffer stabilizes the pH during the different steps. Once the borate buffer was selected, all the following cleaning tests with enzymes were carried out with undiluted BB-HCl and deionized water.

Cleaning Efficacy

According to the results, the efficiency of the chemical and enzymatic products was 86.9% and 87.1%, respectively. These results show that there were no statistically significant differences between the two treatments ($p > 0.05$) (Fig. 4), while they were observed to be equally efficient in terms of the removal of scale (Fig. 1E). With an average efficiency value of 87% of dairy fouling, enzymatic cleaning procedures can be suggested as a feasible alternative to the use of chemical cleaners, which have the added benefits of reduced waste water and cleaning times and energy saving on thermal procedures (Takahasi *et al*., 1996; Fryer & Asteriadou, 2009; Boyce *et al*, 2010).

Morphology

The results of the SEM technique showed that two types of materials form the micro-morphology of the fouling model. These results are based on their density and their resistance to the passage through the electrons during this technique. Thus, one of them appears darker than the other (Fig. 5, B). This could be due to the presence of a higher concentration of mineral matter, which is denser than organic matter. In addition, there are fissures and pores in the surface (Fig. 5 A and B). These fissures in the morphology could be attributed to the desiccation of the sample during the vacuum step of the SEM. Other authors suggest that these pores could be gas bubbles formed within the fouling during the heat treatment (Boyce *et al*., 2010; Gandhi *et al*., 2017). Boyce *et al* (2010) suggest that these bubbles can increase the formation of the scale. Moreover, in the dairy industry, liquids under pressure reduce the formation of the air bubbles (Bansal and Chen, 2006).

Microstructure

The roughness analysis (Fig. 6) showed that the surface of the milk fouling model using a 3D topography measurement and SEM microphotography can produce cracks (Fig. 5). However, the pores are of a natural origin due to the absence of a drying process to carry out this topographic technique.

Composition Analysis

The dairy fouling was studied in two separate parts: the content of organic matter and the content of inorganic matter. Two different techniques, epifluorescent microscopy and CLSM, were used to evaluate the content of organic matter. Likewise, a fluorescence microscopy study was carried out to test the solid

components of the dairy fouling and the residues of the cleaning protocol. Epifluorescence microscopy using three dyes was an easy procedure to detect the presence of fouling components (Tab.1). However, it was impossible to detect the three compounds selected with the straining formula, probably because of the inherent auto-fluorescence of this type of residue (Fig. 7A and B). This was probably due to the compounds produced by the Maillard Reaction from the proteins and sugars in the milk. This was especially true for the ConA-350 dye, due to the amount of background auto-fluorescence in the blue channel. CLSM microscopy was also used because this technique is very sensitive to detecting fluorescent dyes, despite the samples being autofluorescent. Thus, the three dyes used were detected by this microscopy and the 2D models (Fig. 7C, E and F), and 3D models (Fig. 7D), which produced in milk fouling models and the residues of cleaning protocols. Data were also obtained on the average intensity of the fluorescence in each channel to semi-quantify the organic compounds and to trace the abundance of each compound in the depth of the sample.

CLSM microscopy can record each channel in different files, a feature that is very useful to obtain the spatial disposition of the different elements of the sample. In this example of a dairy fouling model (Fig 7D), different overlapping channels are visible where there are the different compounds. Figure 7C shows a green channel over a blue channel, which means that the proteins are on the surface and sugars are in the lowest part. On overlapping the three different channels, red dots appear over all the other compounds (Fig 7E), meaning that the fats are in the highest part of the fouling model.

By processing the image information with the Metamorph® software (Molecular Devices, Sunnyvale, CA, USA), the differences in abundance at different depths of the fouling can be observed (Fig. 8). At the top of the sample, there is a mixture of protein and fats (Fig 7E and 8), and sugars increase in intensity (Fig. 7C and 8) in the middle of the sample. The information offered by this technique as background to the samples can be inaccurate so it was removed from the graph due to its lack of informative content. The semi-quantification of the organic compounds in the dairy fouling models is based on an approximation taken from the average intensity of the fluorescence for each compound and the total fluorescence of the sample (Fig. 9).

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

The results of the EDX-SEM (Fig. 10) showed that there are three different peaks along the graph: Cr and Fe peaks, which correspond to stainless steel as the base material for the production of dairy model, C and O peaks that correspond to organic matter. The lower peaks represents the mineral salts of the milk. When the peaks are less than 2% - 4% they cannot be used for quantification, but they do indicate the presence of these elements qualitatively. Other authors suggest that the presence of C and O peaks obtained with EDX indicate the presence of lipids, the peaks of C and N indicate the presence of proteins, and the S peak indicates the presence of sulfur-containing amino acids (Gandhi *et al*., 2017). During the tests with SEM (Fig. 5), an elementary analysis was performed in parallel using EDX coupled with SEM (Fig. 10). This technique was used to identify the elements that would later be quantified by ICP (Fig. 11).

The highest mineral concentration in the whole milk was K (1.54 mg/g) followed by Ca (1.18 mg/g) and P (0.87 mg/g). However, the most important mineral on the fouling was Ca (14.83 mg/g) followed by P (9.79 mg/g) and K (7.39 mg/g). Therefore, a mineralization of the organic matter on the fouling was expected (Bansal and Chen, 2006). When an alkaline-acidic cleaning process was performed, Ca was reduced by 77.34%, which was similar to P (75.08%); K was solubilized completely, while Na increased more than three-fold (340.91%). Na increased due to the solubilization of the ion from the chemical used on the fouling. On the other hand, when the enzymatic treatment was performed, reductions in Ca and P concentrations of 71.61% and 74.67% were observed, respectively. The final mineral concentrations were 1.5x higher in the case of the chemical treatment compared to the enzymatic treatment, and they were more homogeneous in the case of the residues from the enzymatic treatment. The use of acid treatments after an alkaline treatment is useful for detaching the minerals by solubilization. However, if the adhesive element from the fouling, which initiates the accumulation of matter on the surface, is destroyed, an effective mineral elimination can be achieved without using chemicals that have a high environmental impact. This is one of the advantages of the enzymatic treatments; it is not necessary to focus on all the elements, only on those that start the adhesion process and promote the accumulation of food residues. In this study, the mixture of sugar and proteins in the form of caramel contributes to the fouling process.

CONCLUSIONS

Fluorescent microscopy with selective dyes in conjunction with micro-topographic measurement using SEM-EDX and ICP-OES can be a useful tool for understanding the composition, distribution and structure of the milk fouling matter adhered to surfaces. The use of these techniques enabled the representation of the compounds of fouling in a vertical profile, demonstrating our initial hypothesis of how important the role of the caramel component is in the entire process of the fouling adhesion to surfaces. The caramel component has a fundamental role in dairy fouling formation and its spatial location reinforces the notion that it acts as an adherent for the other residues during heat treatments. All this information is very useful when studying deposits from the thermic milk treatments and comparing the chemical and enzymatic cleaning formulas tested. The mixture of analytical techniques was used to obtain more information to support the argument for the advantages of using enzymes for cleaning this type of residue embedded in industrial surfaces. Epifluorescence microscopy with selective dyes is also very useful to assess formulas and methods before testing new cleaning products in the dairy processing plants, saving on the large amount of energy and water required during the rinse steps. Last, the use of the enzymatic cleaning product proposed in this study is more environmentally sustainable than the classic acid and alkaline products.

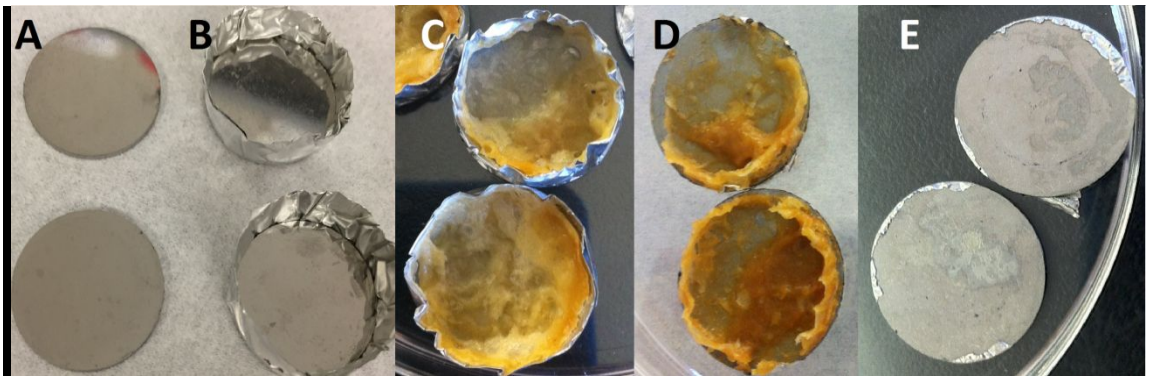

Figure 1. A) Stainless steel 316 2B discs 2 cm in diameter, B) Surfaces where the milk fouling models (MFM) were to be created, C) MFM once the production process was finished, D) MFM finished without aluminum walls, E) MFM once cleaned by the enzymes.

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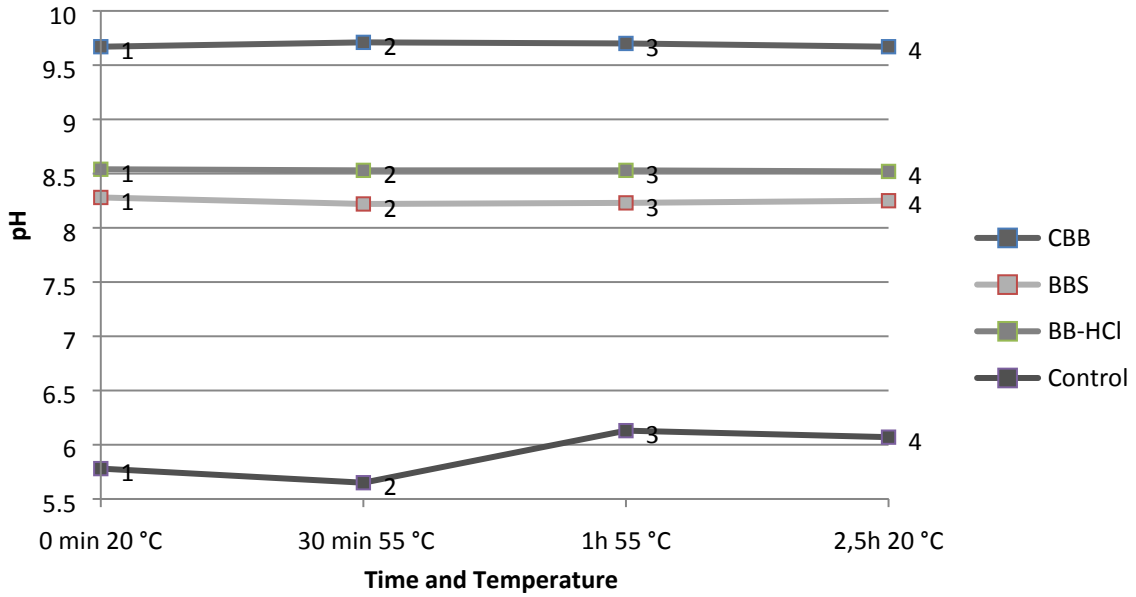

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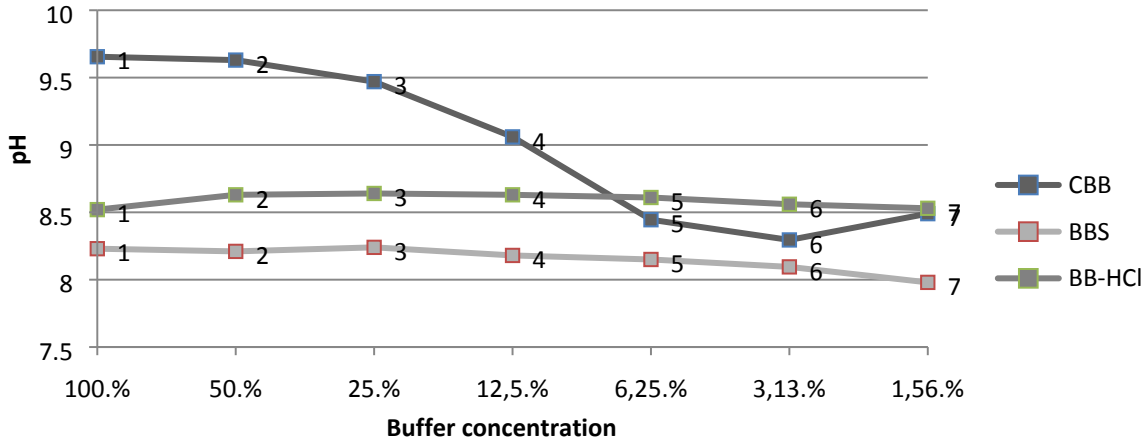
389 **Figure 2. Average pH of different buffer solutions exposed to heat over time, with tap water as a**

390 **control.**



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392 **Figure 3. pH of diluted buffer solutions using tap water at 20 ºC.**

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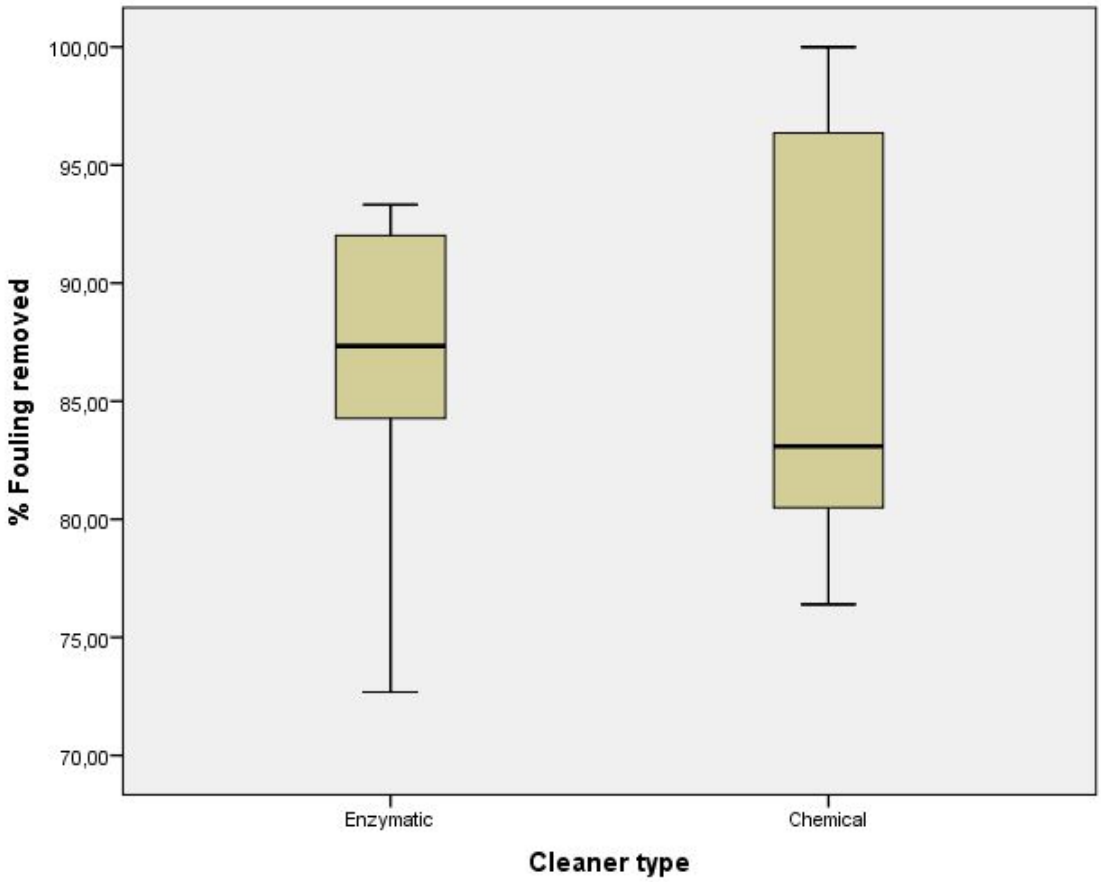

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396 **Figure 4. Boxplot of the efficiency of two different cleaners (n = 27).**

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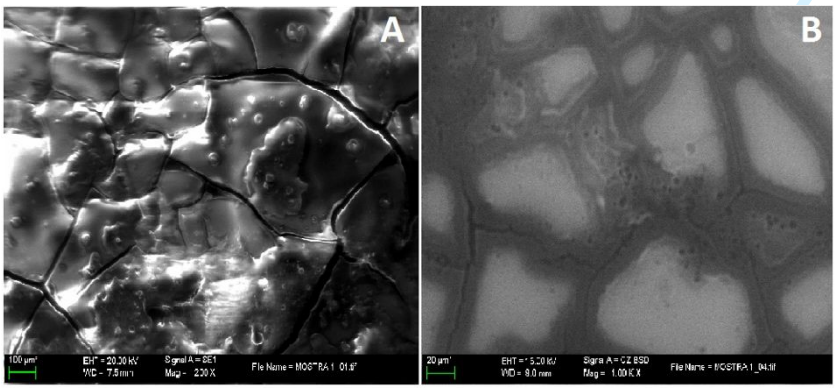

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400 **Figure 5. Microphotographs of milk fouling models using SEM. Amplifications A) 200x B) 1000x.**

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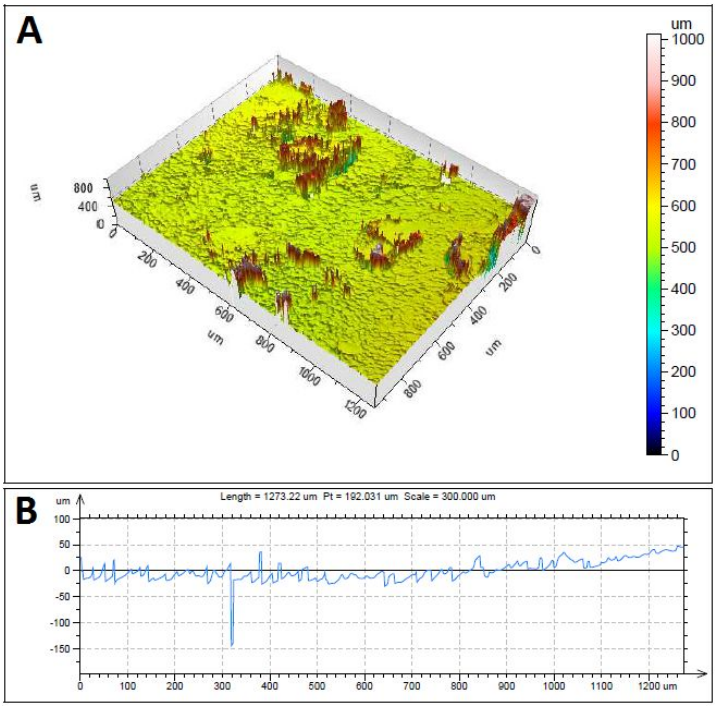

Figure 6. Topographic view of the milk fouling model. A) 3D view of the surface of the fouling model. Cold colors represent the depressions on the surface below the average level of the sample, and warm colors represent the elevations above the surface average B) Topographic profile of the milk-fouling 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.

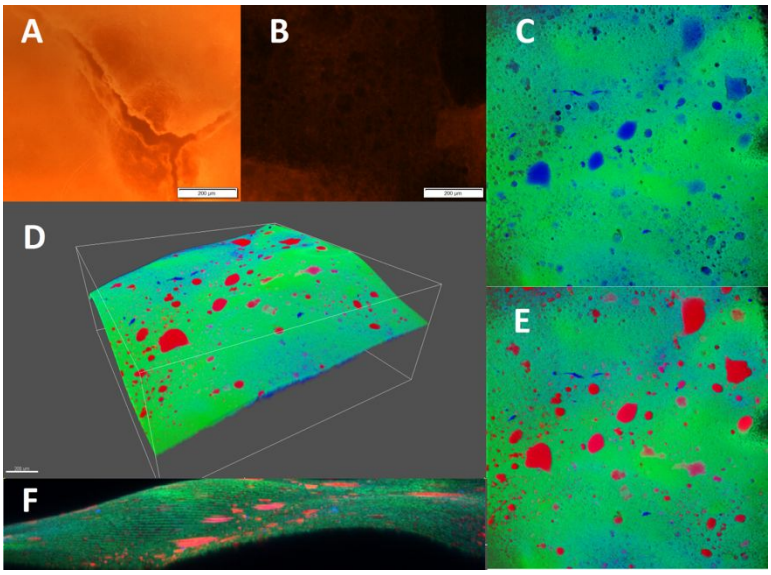

Figure 7. A) 4x Auto-fluorescence of dairy fouling from MFM, 4x red/green filter, captured using DEM. B) Auto-fluorescence after enzymatic cleaning. C) MFM dyed with selective fluorescence dyes, 10x channel blue (sugar/caramel) and green (protein) captured using CLSM. D) 3D reconstruction

using CLSM images of MFM, height of 618.3 µm. E) MFM dyed with selective fluorescence dyes, 10x channel blue, green and red (fats) captured using CLSM. F) Side view of the whole stack of microphotographs.

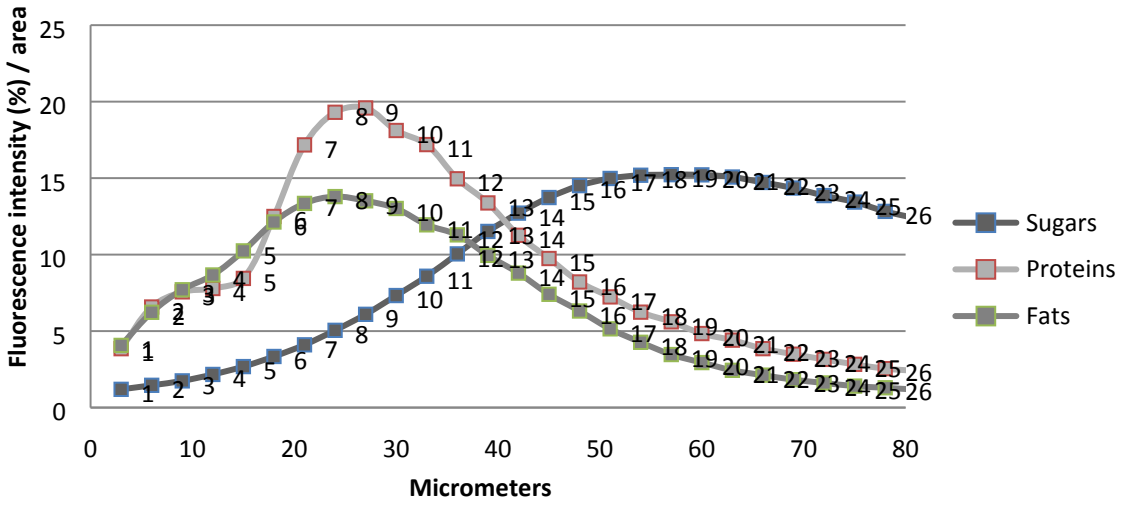

Figure 8. Representation of the intensity (%) of the blue, red and green CLSM channels from the depth of the milk fouling.

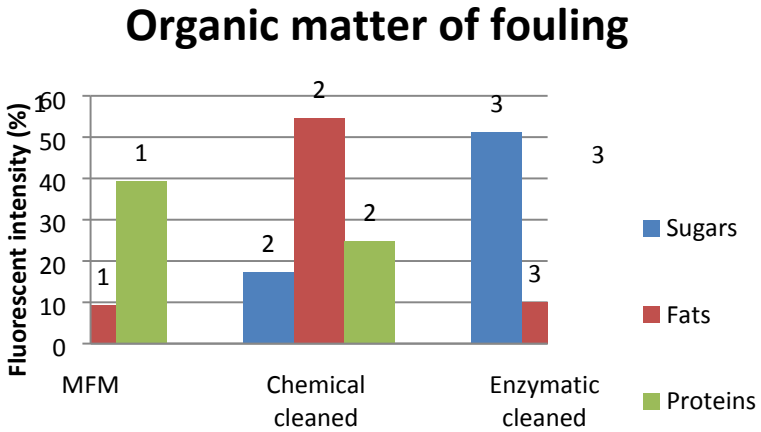

Fig 9. Average fluorescent intensity (%) in the organic matter fraction from MFM (n = 10).

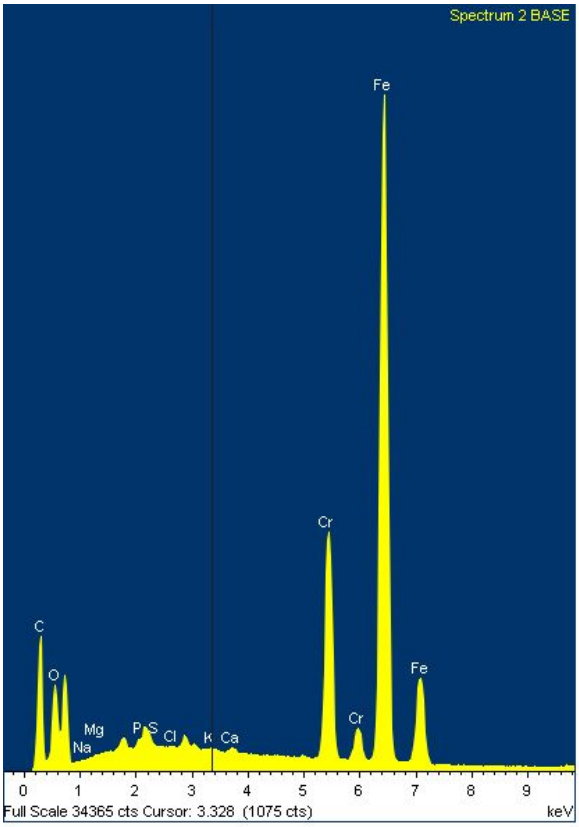

Figure 10. Results from EDX-SEM. The scale expressed in kilo-electron Volts (keV) shows the signals obtained by EDX using an internal standard and identifying each peak with the corresponding element of the periodic table. Lighter elements than carbon (C) are not shown by this technique.

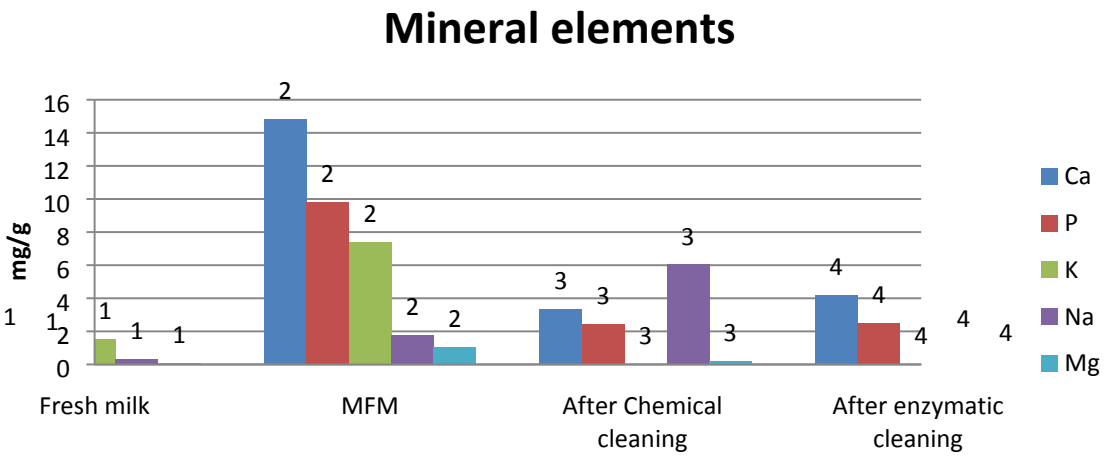

Figure 11. ICP-OES results from whole milk, milk fouling models and cleaning residues (n=10).

Table 1. Fluorescent dyes used in epifluorescent microscopy.

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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