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Non-chlorine detergent formulations as an alternative for unpasteurised milk removal from stainless steel surfaces

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ABSTRACT

Hygiene is a major concern in the dairy industry, and detergents based on hypochlorite have commonly been utilised for cleaning-in-place (CIP) regimes. However, due to concerns about chlorate residues entering the milk processing chain, new detergent alternatives that are free of chlorate sources are required. Two new formulations were developed based on ethylenediaminetetraacetic acid (EDTA) and wetting agents. Stainless steel surfaces were fouled with milk and cleaned once or 10 times using water, a caustic-EDTA product, a sodium hypochlorite product, an acid, or new cleaning formulations (Product A and Product B). The results demonstrated that the use of acid did not result in successful cleaning. The two new products performed well, with Product B performing equivalently during cleaning compared with the established hypochlorite or caustic-EDTA products. Product A exhibited better cleanliness than the other detergents tested. When allergen removal was considered, residual material was found to be retained on the surfaces, regardless of the cleaning type used. This study suggests that the new product formulations may be used to replace hypochlorite-based detergents to increase the hygienic status of a surface.

1. Introduction

The dairy industry is a huge business worth an estimated 674 billion USD in 2019 globally, and it is projected to grow to 1033 billion USD by 2024. The UK dairy industry was worth 4.5 billion GBP in 2018 and the Irish dairy industry had a value of 4.4 billion Euros in 2019 (Bia, 2019; Uberoi, 2020). For both the UK and Ireland, dairy is a major industry with dairy exports accounting for 16.9% of agricultural exports in the UK and 9.8% of Ireland's merchandise exports coming from the agri-food sector (Bia, 2019; Teagasc, 2017a; Uberoi, 2020).

Food hygiene is a vital component of the dairy industry. Previously, many dairy operations were cleaned using a hypochlorite-based detergent because of its high efficiency in washing, sanitation of food contact surfaces and relatively low price (Siobhan et al., 2012). The use of a hypochlorite-based detergent provides alkalinity for saponifying fats, with the ability to break up proteins via oxidation reactions, whereas the use of a caustic-EDTA detergent provides alkalinity to saponify fats with the ability to chelate minerals (Bylund, 2015). However, owing to changes in legislation related to residual chlorate levels in milk, hypochlorite-based detergents are being reduced, and alternative formulations are being recommended (Gleeson et al., 2022; Teagasc, 2017b).

One suggestion to replace the use of hypochlorite has been to use acid-based detergents (e.g. phosphoric acid) (Teagasc, 2017b). However, this remains controversial since the primary function of acid solutions in dairy cleaning is to remove mineral fouling, and their role in the removal of organic (e.g. protein) fouling is minimal. Therefore, an alternative product that is effective in removing surface milk fouling

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while posing minimal or no risk of contamination to subsequent production cycles would be beneficial.

The development of a new product requires consideration of the major components of milk and the most effective way to remove these components from the surface during the cleaning process. Unpasteurised milk is mostly water, with carbohydrates in the form of lactose (around 38.10%), fats (29.36%), casein (22.22%) and whey proteins (4.76%), and a variety of vitamins and minerals, including calcium and magnesium (Mesilati-Sthay et al., 2019). However, the levels of fat and protein can differ considerably depending on the breed of cow or animal from which the milk is obtained (Dominguez-Salas et al., 2019).

A combination of surfactants, ethylenediaminetetraacetic acid (EDTA), and wetting agents can be used to remove milk from production surfaces and maintain hygiene at the correct concentrations and formulations. Surfactants reduce the surface tension of a liquid, thereby enhancing its wetting properties. EDTA is used to collate minerals, which can cause significant fouling issues in the dairy industry. One issue with using hypochlorite-based products to clean the equipment used in the dairy industry is that it is becoming increasingly difficult to achieve chlorate residues within the required specifications (Teagasc, 2017b). The EU maximum residue limit (MRL) for perchlorate in foods ranges from 0.01 mg kg^{-1} in infant foods to 0.75 mg kg^{-1} in tea (European Commission, 2020). An MRL of 0.10 mg kg^{-1} has been applied to milk in its 'ready to use state' with no further specificity regarding different types of dairy products (Twomey et al., 2023). The dairy industry in the Republic of Ireland has taken action to mitigate chlorate contamination in foods and has primarily focused on the prohibition of chlorine-based chemicals for cleaning and disinfection on farms and in processing plants (Twomey et al., 2023). The prohibition on using chlorine-based chemicals came into effect on January 1, 2021 (Phelan, 2019). The removal of hypochlorite from cleaning agents would reduce these residues, which is particularly important in the production of foods such as infant milk formula and sports nutrition foods since these markets are particularly aware of and sensitive to chemical contaminants, including chlorates (O'Brien and Gleeson, 2023). In the work of Twomey et al. (2023), with the exception of milk, chlorate levels in Irish dairy products were higher on a mg kg^{-1} basis relative to previously conducted research (EFSA, 2015; Kettlitz et al., 2016; Twomey et al., 2023). The removal of hypochlorite from cleaning agents would reduce these residues, which is particularly important in the production of foods such as milk powder.

A further issue when considering the cleaning of milk-contaminated surfaces is the removal of potential allergens. Such considerations may be particularly important when new products are being developed, or when dairy products such as milk are used in only some of the food products being produced in the factory. Two important milk allergens are casein and β-lactoglobulin (β-LG) (Docena et al., 1996). One way to determine the levels of allergens retained on a surface is to consider the eliciting doses as determined by the VITAL Scientific Expert Panel Recommendations (Bureau, 2019), which summarises individual challenge studies to determine the dose at which an allergenic protein can trigger a reaction. The recommendations state that a dosage of 0.2 mg of milk protein would trigger a reaction in 1% of the allergenic population whilst a dosage of 2.3 mg would trigger a reaction in 5% of the allergenic population. Undeclared allergens can be inadvertently introduced into food via cross-contact during manufacturing. However, the information on the effectiveness of cleaning procedures for removing allergenic materials from surfaces is limited (Jackson et al., 2008).

This study was designed to determine the efficacy of novel cleaning formulation designs for the removal of unpasteurised milk from stainless steel surfaces, and to quantify the residual allergenic proteins remaining on the surfaces following cleaning, so that such formulations may be considered for application in processing industries that use milk in their food products.

2. Materials and methods

2.1. Coupon preparation

2.1.1. Stainless steel coupons

Type 304, 2B finish stainless steel (SS) (Aalco, UK) was cut into 20 mm × 20 mm square coupons using a guillotine and cleaned by submersion sequentially into acetone (BDH, UK), methanol (BDH, UK), and ethanol (BDH, UK), each for 10 min (with coupons being rinsed by submersion into sterile distilled (DI) water between each solvent), with a final rinse in DI water before being air-dried.

2.1.2. Coupon fouling and cleaning

Stainless steel coupons were placed into 30 mL of unpasteurised milk (Milk Maids, UK) at room temperature with gentle agitation (~150 rpm) for 30 min. This method was used to simulate a surface where milk is stored or held for short periods of time. The coupons were removed from the milk, allowing the excess to run from the coupon. The fouled surfaces were placed into either deionised (DI) water, or 2% (v/v) detergent solutions made up of DI water. The detergents included a commercially available single-stage disinfectant acid based on nitric acid (Airdale Group, UK), a commercially available caustic (INEOS, UK)-EDTA (BASF, UK) detergent, a commercially available chlorinated-alkaline detergent (Holchem, UK), and two new detergents, Product A and Product B. Product A and Product B were composed of a solution of caustic (INEOS, UK), EDTA (BASF, UK), and a blend of surfactants (BASF, UK). Product B contained 50% EDTA of Product A. The coupons were left in the solutions with gentle agitation (~150 rpm) for 30 min. The coupons were removed from the wash solution and submerged into water to rinse off the excess cleaning solution. The coupons were either dried following a single wash, or after a single wash, excess rinse water was tapped off the coupons, and the surfaces were re-fouled and cleaned as above 10 times.

2.1.3. Coupon staining and imaging

Each coupon was stained by covering the surfaces with 500 μL of 0.03% (w/v) acridine orange (Sigma Aldrich, UK; CAS 494-38-2) for 2 min, followed by submersion into water to rinse off excess stain. In this study, acridine orange was used to stain surfaces soiled with organic material (Whitehead et al., 2010). The coupons were then dried in the dark and imaged using an epifluorescence microscope (Nikon Eclipse E600; Nikon, UK). For each surface, 10 images were taken at 100 × magnification under epifluorescent immersion oil (Leica, UK), and the percentage coverage of the surface for each image was determined using the Cell F software (Olympus, UK) to generate an appropriate intensity profile and phase analysis, as performed by Whitehead et al. (2010). The percentage coverage was averaged for each set of 10 images taken from three surface replicates ($n = 30$) for each treatment.

2.2. Coupon analysis

2.2.1. Scanning electron microscopy (SEM)

The stainless steel coupons that had been fouled and cleaned were left in 4% (v/v) glutaraldehyde (Agar Scientific Ltd, UK) (made up of phosphate-buffered saline (Oxoid, UK)) for 24 h. The coupons were removed from glutaraldehyde using tweezers and rinsed with sterile distilled water to remove any residual solvent. The coupons were dried in a fume hood for 1 h before an ethanol gradient (BDH, UK) of 30%, 50%, 70%, 90%, and 100% (v/v) (composed of absolute ethanol diluted with distilled water) was used to remove moisture from the samples. Starting at 30% (v/v), each coupon was left at each ethanol concentration for 10 min before moving to the next highest concentration. Once removed from the 100% ethanol solution, the coupons were placed in a desiccator and when dried, attached to SEM stubs with carbon tabs (Agar Scientific Ltd., UK) prior to being sputter coated with a gold and palladium coating (Model SC7640, Polaron, Au/Pd target, deposition time: 1.5 min). The prepared coupons were stored in a desiccator until

imaging was performed. Analysis was carried out using a Supra 40VP scanning electron microscope (Carl Zeiss Ltd., UK).

2.2.2. Multifractal analysis

Multifractal analysis can be used to measure density, dispersion, and clustering of objects on a surface using mathematical packages (Lynch, 2023). Fig. 1 shows examples of surface multifractals generated with certain motifs. To obtain multifractal surfaces, one simply assigns weights (or probabilities) to the subdivided areas of a square. In Fig. 1A and D, 2 × 2 motifs were used, whereas in Fig. 1G–a 3× 3 motif with holes was used. The motifs were applied to smaller and smaller squares; thus, at stage one, for a 2 × 2 motif (array), a 4 × 4 array would be generated, and at stage eight, a 512 × 512 array is formed. For the 3 × 3 motif, stage five gives a 729 × 729 array. The pixels in this array were assigned to the weights, and then an image was generated by adjusting the contrast of the image. Without the adjustment, the figures would be black as the numerical values are all very close to zero. A white pixel would have a numerical value of one, and a black pixel would have a value of zero. Values between zero and one are on a gray scale. Note that many of the pixels in Fig. 1H are black and represent holes in the

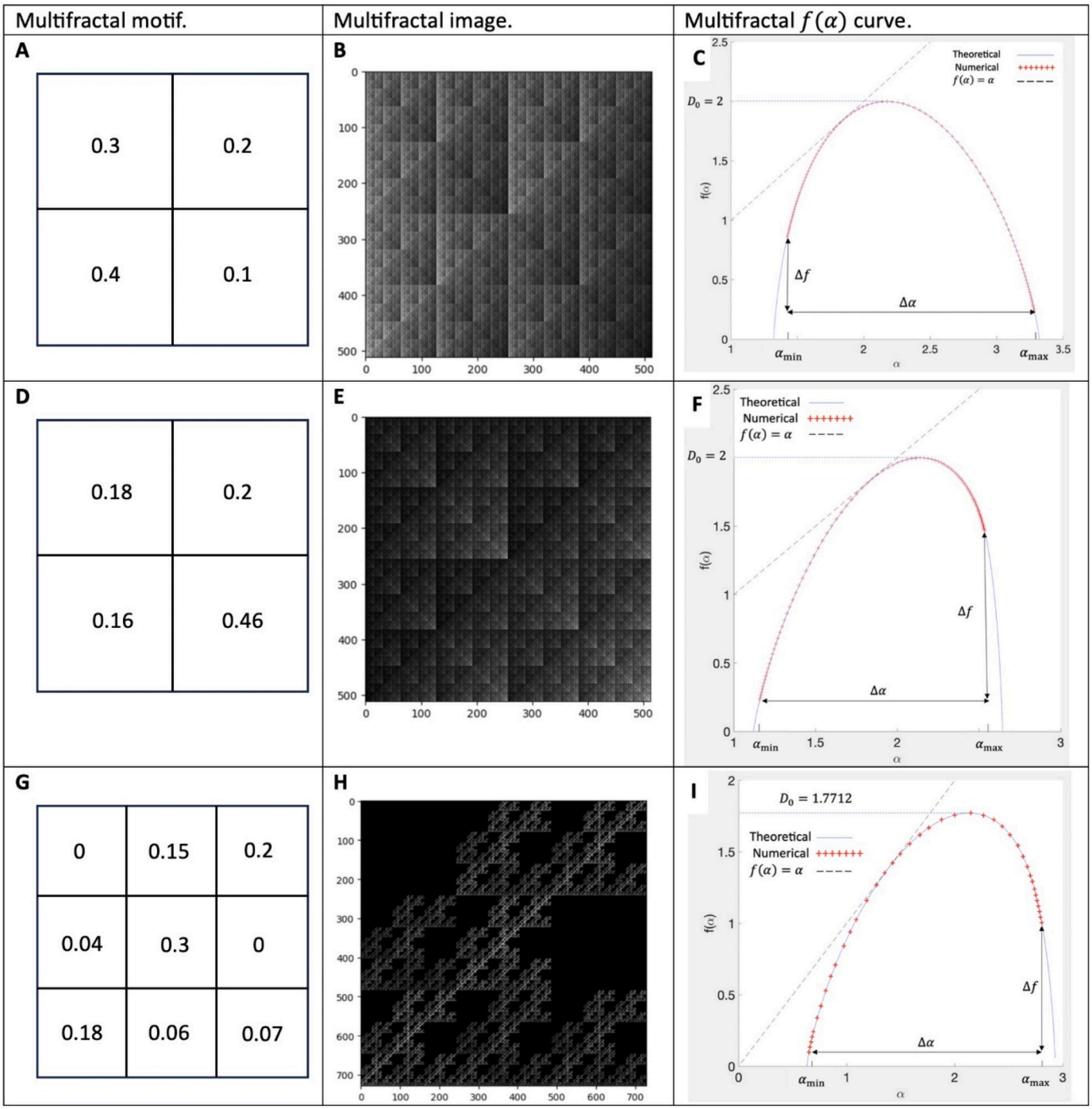

Fig. 1. Multifractal motifs, images and $f(\alpha)$ curves. (A) 2 x 2 motif; (B) 512 x 512 image; (C) $f(\alpha)$ curve, asymmetry shows clustering of brighter pixels; (D) 2 x 2 motif; (E) 512 x 512 image; (F) $f(\alpha)$ curve, asymmetry shows clustering of darker pixels; (G) 3 x 3 motif; (H) 712 x 712 image with holes; (I) $f(\alpha)$ curve, asymmetry indicates clustering of gaps. Notice that $D_0 = 1.7712$ is the fractal dimension.

multifractal object. The q'th moment (or partition function) Z_q is defined by Equation (1):

$$Z_q = \sum_{i=1}^{n} p_i^q(\ell) \quad [1]$$

where p_i are weights, which sum to one, ℓ is a length scale, and q is a parameter. A scaling function, labelled $\tau(q)$, satisfies Equation (2):

$$\sum_{i=1}^{n} p_i^q r_i^{\tau(q)} = 1, \quad [2]$$

with r_i being the fragmentation ratios, which is defined by Equation (3):

$$\tau(q) = \frac{ln\left(Z_q(\ell)\right)}{-ln\,(\ell)}\ . \quad [3]$$

The $f(\alpha)$ spectrum of dimensions is then determined using the parametric equations:

$$f(\alpha(q)) = q\alpha(q) + \tau(q), \alpha = -\frac{\partial \tau}{\partial q} \quad [4]$$

Fig. 1B–E, and H show the corresponding multifractal images, which can be generated using mathematical packages such as Python, MATLAB, Mathematica, and Maple. Fig. 1C–F, and I display the corresponding $f(\alpha)$ curves.

2.3. Density

The fractal dimension, D_0, (Equation (5)):

$$D_0 = f(\alpha_0) \quad [5]$$

gives the fractal dimension and is a measure of how much of the object covers the surface. In Fig. 1C and F, the dimension is two as the entire surface is covered. In Fig. 1I, two of the weights are zero, and hence, the multifractal has holes and does not cover the entire surface. In this case, the fractal dimension, $D_0 = 1.7712$, means that the multifractal object takes up more room than a straight line (which has dimension one) but less room than a plane (which has dimension two).

2.4. Dispersion

The width of the $f(\alpha)$ curve, $\Delta\alpha$, defined by Equation (6):

$$\Delta\alpha = \alpha_{max} - \alpha_{min} \quad [6]$$

and gives a measure of dispersion, that is, how evenly or unevenly the points (grayscale values) are dispersed over the surface.

2.5. Clustering

The difference in the height of the numerical $f(\alpha)$ curve is given by Equation (7):

$$\Delta f = f(\alpha_{min}) - f(\alpha_{max}) \quad [7]$$

and gives a measure of clustering. In Fig. 1C, Δf is positive, indicating clustering of brighter pixels, as indicated in the corresponding motif and multifractal images A and B, respectively. In Fig. 1F, Δf is negative, indicating clustering of darker pixels, as indicated in the corresponding motif and multifractal images D and E, respectively.

Readers should note that binary images have been used in this work, but the same arguments hold for binary images as for grayscale images. The only difference is that pixels have values of either zero (black) or one (white).

2.5.1. Energy Dispersive X-Ray spectroscopy (EDX)

The surfaces were prepared in the same manner as the SEM samples but were not sputter-coated. Samples were imaged using SEM at 20 kV with a 15 mm working distance before EDX analysis was carried out on the samples.

2.5.2. Attenuated Total Reflection – Fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR was used to determine the molecular structures and chemical bonds present on the surface of each sample (Spectrum 65 FTIR with an ATR attachment; PerkinElmer, UK). Background spectra were captured prior to each measurement with no sample present to remove the background noise from the obtained spectra. Spectra were acquired at room temperature using the Spectrum IR software (PerkinElmer, UK), with each run consisting of eight scans and a resolution of 4 cm^{-1}. The analysis was performed in triplicate and the average spectra were recorded.

2.5.3. Enzyme-Linked Immunosorbent Assay (ELISA) for surface allergen detection

The surfaces were prepared by fouling and cleaning as previously described, until they were fully dry. After drying, each surface was thoroughly swabbed using swabs provided in the Surface-Check Swabbing Kit (Bio-Check Ltd., UK) in three directions, while the swab was rotated to ensure maximum recovery from the surface. The swabs were stored in tubes containing storage media as part of the Surface-Check Swabbing Kit, and vortexed for 10 s to disperse the collected material into the solution. Samples were processed using either a Milk-check (Casein) or Milk-check (β-LG) ELISA kit (Bio-Check Ltd., UK), according to the manufacturer's instructions.

2.6. Statistical analysis

The average of the data was plotted, and the standard errors were calculated and are denoted as error bars in the graphs. The statistical differences between the surfaces were evaluated using one-way ANOVA followed by Bonferroni's multiple comparison tests. The statistical analysis was performed for confidence levels of 95% (differences are reported as significant for p values < 0.05) using IBM SPSS Statistics version 24.0 for Windows (IBM SPSS, Inc., USA).

3. Results

3.1. Visual determination of residual fouling using scanning electron microscopy (SEM)

The SEM images demonstrated that the control (pristine) stainless steel (Fig. 2A) had no visual fouling, and the grain boundaries of the stainless steel were visible. When the stainless steel was conditioned with milk (Fig. 2B), the underlying surface was not visible since it was covered with a film of foulant. When the surfaces were cleaned with water (Fig. 2C and D) or acid (Fig. 2E and F), there was some visible fouling remaining on the surfaces. The fouled stainless steel surfaces that were cleaned using an acid detergent (Fig. 2E and F) demonstrated significant fouling. Cleaning with either one or 10 cleans using caustic EDTA, hypochlorite, Product A or Product B did not show any residual fouling left on the surface using this method.

3.2. Epifluorescence microscopy of retained biofouling

The epifluorescence images of the control (pristine) stainless steel surface (Fig. 3A) showed little fouling of the surface, whereas the milk-fouled stainless steel surface (Fig. 3B) demonstrated heterogeneous fouling across the surface. The surfaces cleaned using only water (Fig. 3C and D) appeared to have globules on the surfaces. In addition, soil became retained within the grooves of the stainless steel surface. When an acid detergent was used to clean the surfaces (Fig. 3E, F), a layer of fouling was present on the surface, which was too thick to discern

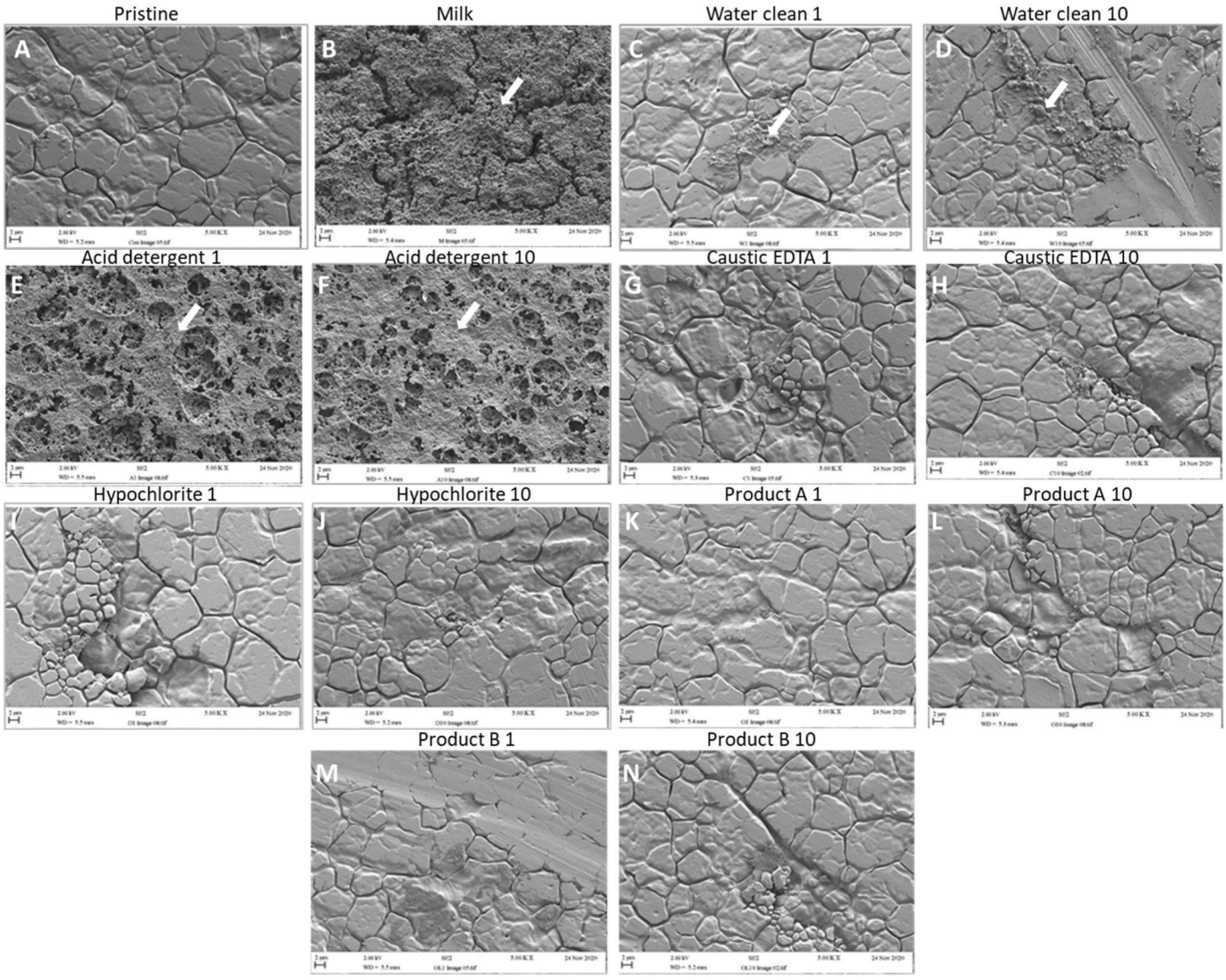

Fig. 2. SEM images of (A) unfouled stainless steel surface (pristine) and (B) stainless steel surfaces fouled with milk, along with images of stainless steel surfaces fouled with milk and cleaned using (C) water for 1 cleaning cycle, (D) water for 10 cleaning cycles, (E) acid for 1 cleaning cycle, (F) acid for 10 cleaning cycles, (G) caustic-EDTA for 1 cleaning cycle, (H) caustic-EDTA for 10 cleaning cycles, (I) hypochlorite for 1 cleaning cycle, (J) hypochlorite for 10 cleaning cycles, (K) Product A for 1 cleaning cycle, (L) Product A for 10 cleaning cycles, (M) Product B for 1 cleaning cycle, and (N) Product B for 10 cleaning cycles. White arrows indicate fouling regions. Scale bars of 2 μm.

globules or soil within the stainless steel grain boundaries. The retained soil within the crevices observed when cleaning using only water (Fig. 3C and D) was also present when either caustic-EDTA (Fig. 3G and H) or hypochlorite (Fig. 3I and J) was used after a single repetition; however, the soil was less visible for both cleaning agents after 10 repetitions (Fig. 3H–J). When the surfaces were cleaned using Product A (Fig. 3K and L), little fouling was observed, particularly after 10 repetitions (Fig. 3L). Fouling was more clearly visible after one and 10 cleaning repetitions with Product B (Fig. 3M and N).

3.3. Percentage coverage measurements of retained biofouling on surfaces

A pristine stainless steel surface was tested with no fouling as a control, and the percentage coverage results (Fig. 4) demonstrated minimal soil presence (1.8%). When milk was dried onto the stainless steel surfaces, the surface coverage significantly increased when compared to the pristine surface to 63% ($p < 0.05$). When the surfaces were fouled with milk and cleaned once with water, significant soil coverage of 24% was observed, whereas after 10 repeated fouling and cleaning steps, the surface demonstrated 19% fouling ($p < 0.05$). The use of an acid detergent to clean the milk-fouled stainless steel demonstrated a significant increase in the amount of material present when compared to the control, with one fouling and cleaning repetition demonstrating 75% and after 10 fouling/cleaning cycles 89% fouling ($p < 0.05$). The standard products that are typically used to clean milking equipment (caustic-EDTA or a hypochlorite product) showed no significant difference ($p > 0.05$) in the levels of fouling after both one fouling and cleaning (9.3% and 8.6%, respectively) and 10 repetitions (6.6% and 6.8%, respectively). The lowest percentage coverage for a cleaning solution was observed when Product A was used to clean the surfaces (a statistically different result was observed when compared to all the other surfaces ($p < 0.05$)), with only 2.2% and 0.2% coverage after one and 10 repetitions, respectively. Product B performed similarly to the hypochlorite product whereby after one and 10 cleaning and fouling repetitions, 6.3% and 6.4% coverages were determined, respectively.

3.4. Multifractal analysis of fouling Distribution across the surfaces

Multifractal analysis of the surfaces demonstrated that the density of the fouling across the surfaces was greatest on the one and 10 acid-

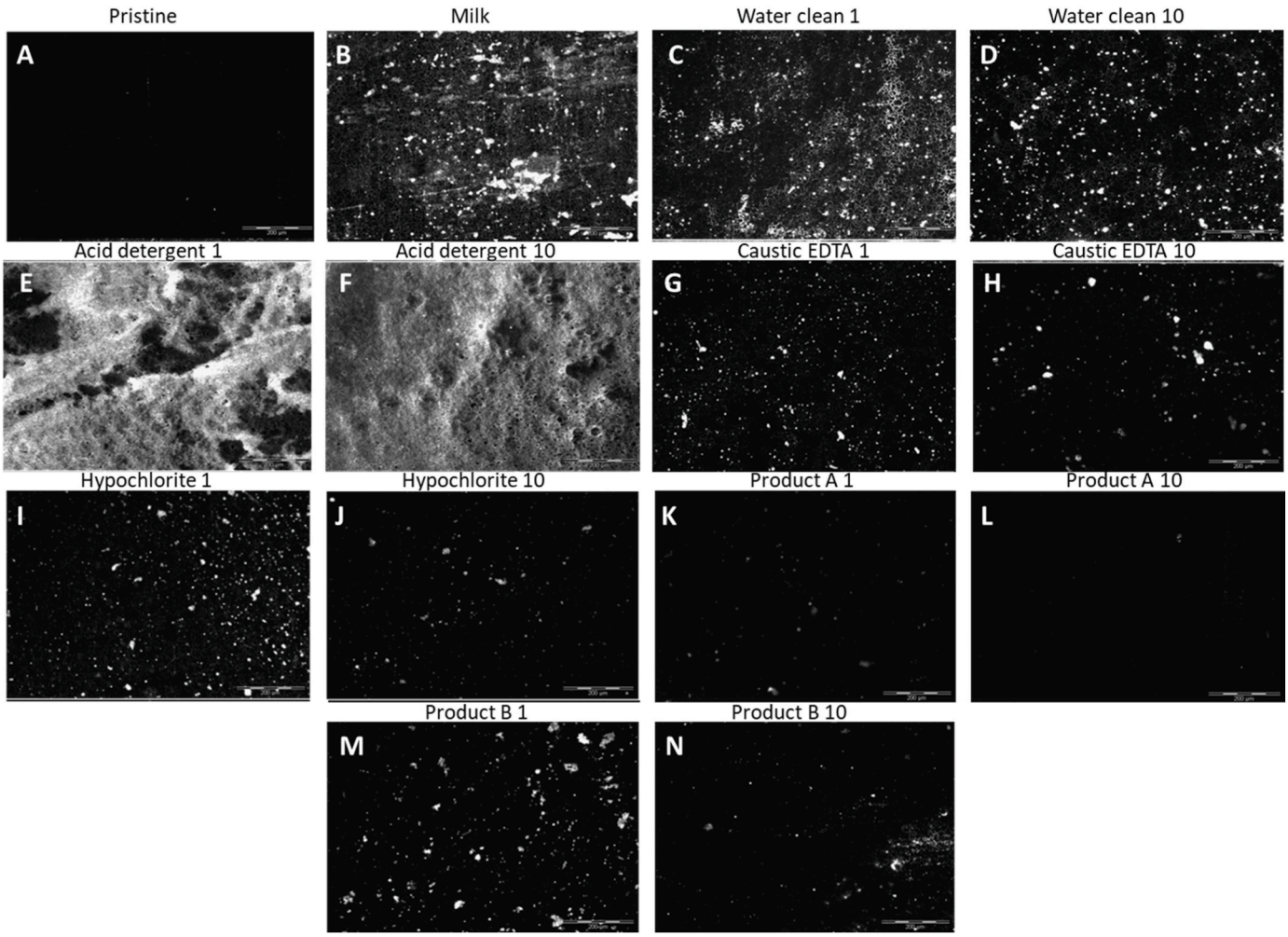

Fig. 3. Epifluorescence images of (A) unfouled stainless steel surface (pristine) and (B) stainless steel surfaces fouled with milk, along with images of stainless steel surfaces fouled with milk and cleaned using (C) water for 1 cleaning cycle, (D) water for 10 cleaning cycles, (E) acid for 1 cleaning cycle, (F) acid for 10 cleaning cycles, (G) caustic-EDTA for 1 cleaning cycle, (H) caustic-EDTA for 10 cleaning cycles, (I) hypochlorite for 1 cleaning cycle, (J) hypochlorite for 10 cleaning cycles, (K) Product A for 1 cleaning cycle, (L) Product A for 10 cleaning cycles, (M) Product B for 1 cleaning cycle, and (N) Product B for 10 cleaning cycles. Scale bars of 200 μm.

cleaned surfaces (D_0 values of 2) since these surfaces were completely covered with material. In contrast, the control stainless steel and stainless steel cleaned 10 times with Product A demonstrated no surface coverage of the residual foulant (Fig. 5A). Generally, the pattern of density of fouling retained on the surface was more dense for the fouled control ($D_0 = 1.7$), water ($D_0 \approx 1.7$), caustic-EDTA after 10 cleans ($D_0 = 1.7$), acid-treated surfaces ($D_0 = 2$), or Product B ($D_0 = 1.6$), and less dense on the surfaces treated with caustic-EDTA after one clean ($D_0 = 1.4$), hypochlorite after 10 cleans ($D_0 = 1.5$), or Product A after one clean ($D_0 = 1.1$).

The dispersion of the retained fouling across the surfaces (Fig. 5B) showed an opposite trend to that of the density. The surfaces cleaned for one or 10 repetitions with caustic-EDTA (1.1, 1.2), hypochlorite (0.9, 1.3), Product A (0.0, 1.2) or Product B (1.2, 1.2) presented a wider pattern of dispersion between the retained fouling, whereas the control surfaces (0.0, 1.0) or those cleaned with water (1.0, 1.0) or acid (0.0, 0.0) demonstrated that the retained foulant was closer together (Fig. 5B).

Analysis of the clustering of the fouling across the surfaces (Fig. 5C) demonstrated that the surface cleaned by the acid solution after both one and 10 repetitions was completely covered, and thus a value could not be obtained because there were no clusters of foulant to measure. After one cleaning cycle, Product A provided the least number of retained foulant clusters (−0.96). However, after 10 cycles of cleaning with the same product, no visible fouling was detected, and thus the clustering pattern could not be measured using this technique.

3.5. *Elemental analysis of residual fouling on the surfaces using Energy Dispersive X-Ray spectroscopy (EDX)*

When EDX was performed on the surfaces (Table 1), the chemical composition of the stainless steel was removed and the carbon (C), oxygen (O), and phosphate (P) elements were compared. Only trace amounts of calcium (<0.1%) were detected on all surfaces (data not shown), and nitrogen and magnesium were below the level of detection for the instrument. When a layer of milk was applied to the stainless steel surface, significant amounts of carbon (47.5%), oxygen (6.3%), and phosphate (0.3%) were detected. The lowest amounts of carbon and oxygen were detected following one wash using Product A (1.3% and 0.0%, respectively), whilst after 10 washes, carbon and oxygen were lowest following the use of Product B (1.0% and 0.5%, respectively). Phosphate levels were the lowest following a number of cleans, including one clean with Product A or Product B, and 10 cleans with caustic-EDTA, hypochlorite, and Product B (0.2%).

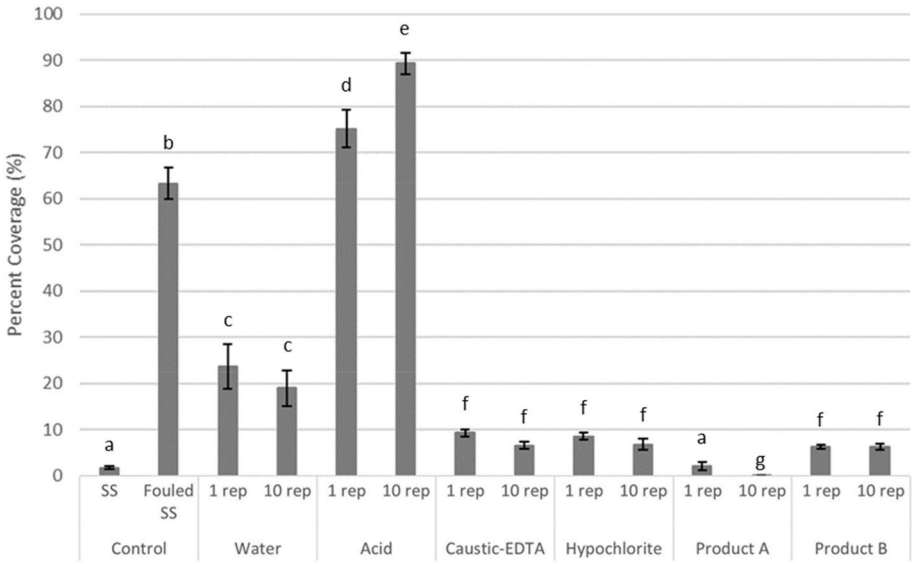

Fig. 4. Percentage coverage of stainless steel surfaces after one repeat (1 rep) or 10 repeats (10 rep) of fouling with unpasteurised milk followed by cleaning using either water or 2% (v/v) solutions of an acidic, caustic-EDTA, hypochlorite, Product A, or Product B detergent. Results are presented as average ± standard error. Letters were assigned (from a to g) as long as statistically significant differences exist between surfaces (for a confidence level greater than 95%, $p < 0.05$). This was evaluated by one-way analysis of variance (one-way ANOVA, Bonferroni's *post-hoc* test).

3.6. Determination of the Biochemistry on the surfaces using Attenuated Total Reflection – Fourier transform infrared spectroscopy (ATR-FTIR)

When ATR-FTIR was performed on the cleaned surfaces, no significant differences were observed between the spectra obtained from a single cleaning cycle (Fig. 6A) and those obtained after 10 repeated fouling and cleaning cycles (Fig. 6B). The main difference was in the spectra obtained for the surfaces cleaned with acid compared to those cleaned with different detergents. When water, caustic-EDTA, or hypochlorite was used, there was a small peak at ~1744 cm^{-1} representing a stretching vibration of the carbonyl functional group (C═O), which may have been indicative of the remaining fatty acids on the surface. This peak was not present when either of the novel compilations, Product A or Product B, were used. However, the use of an acid detergent caused a significantly different spectrum, resulting in increased and reduced absorption intensities at different points. Little differences were detected between the spectra obtained after one and 10 cycles. Compared to the control, the broad peak at approximately 3265 cm^{-1} was reduced after cleaning with acid, which could be due to a reduction in the water content (O–H stretching), changes in the lactose content (also O–H stretching), part of the O–H stretching in fatty acids, or N–H changes in proteins. However, the other major water peak, 1165 cm^{-1} (H–*O*–H scissoring), increased, whereas other peaks related to lactose were reduced, including those at ~1032 cm^{-1} (C–O/C–C/C–O–O/C–H functional groups) and ~882 cm^{-1} (a functional group with a vibration ring of lactose). On the milk alone and the hypochlorite-cleaned surfaces, many of the peaks that potentially indicated components of fatty acids demonstrated no significant differences in their biochemical structures. This included peaks at ~2922 cm^{-1} and 2852 cm^{-1} representing CH_3 stretching both asymmetrically and symmetrically, respectively, a peak at ~3004 cm^{-1} due to the cis olefinic stretching of a C═C bond, which may be indicative of molecular vibrations in the middle of the unsaturated fat chain, and a peak at ~1744 cm^{-1} representing a stretching vibration of carbonyl functional group (C═O) possibly at the end of the fatty acid chain. However, there was an increase in the intensity of the peak at ~1157 cm^{-1}, indicating either H–C–H wagging or a C–O ester group, whereas the presence of a new peak at approximately 1709 cm^{-1} supports the possible presence of a fatty acid ester. Peaks found in the regions of 1450 cm^{-1} (C–H bending), 1525 cm^{-1} (N–O stretching), and 1650 cm^{-1} (C–H bending) were also found on the milk and acid-cleaned surfaces, which were indicative of retained lipid and protein moieties.

3.7. Enzyme-Linked Immunosorbent Assay (ELISA) to detect residual allergens

The casein ELISA results (Fig. 7) demonstrated that the control stainless steel surface had no recoverable casein present on the surfaces. The surfaces fouled with milk demonstrated 8.5 ppm of casein recovered, which was around the maximum detectable level. When the surfaces were washed using water, there were no significant differences between one repetition (4.1 ppm) and 10 repetitions (4.0 ppm), but the result was significantly reduced compared to the fouled control ($p < 0.05$). Likewise, there was no significant difference between one repetition (8.7 ppm) and 10 repetitions (8.8 ppm) for the acid-washed surfaces, but both values were around the upper limit of detection ($p > 0.05$). When the other products were tested, there were no significant differences in the levels of recovered casein after one repetition between Product A and water, caustic EDTA or hypochlorite cleans ($p > 0.05$). After 10 repetitions, the recovered casein levels were at 1.6 ppm for the caustic-EDTA product, 1.1 ppm for the hypochlorite product, 1.8 ppm for Product A, and 2.7 ppm for Product B. Product B demonstrated significant differences to the results from cleaning using Products A, hypochlorite or caustic EDTA ($p < 0.05$).

Similar to the casein results, the ELISA test for β-LG (Fig. 8) demonstrated almost no recovered allergen from the control stainless steel (1.3 ppb). The surface fouled with milk demonstrated a recovery level of 579 ppb, which was the limit of detection of the instrument. The recovered β-LG after washing with water was 230 ppb after one repetition and significantly reduced after 10 repetitions (80.6 ppb) ($p < 0.05$). When acid was used, the results were similar to casein, with β-LG recovery after both one and 10 repetitions (529 and 497 ppb, respectively) being significantly greater than that of all other detergents used. After a single repetition, the results for the caustic-EDTA (237 ppb), hypochlorite (278 ppb), Product A (373 ppb), and Product B (267 ppb) detergents all demonstrated recovery levels greater than that of the water control. However, after 10 repetitions, the caustic-EDTA (9.2 ppb), hypochlorite (11.9 ppb), and Product A (58.4 ppb) demonstrated reduced recovery compared to the water control. After one clean, all the surfaces cleaned with caustic EDTA and Product A were found to recover significantly different amounts of β-LG ($p < 0.05$). Following 10 cleans,

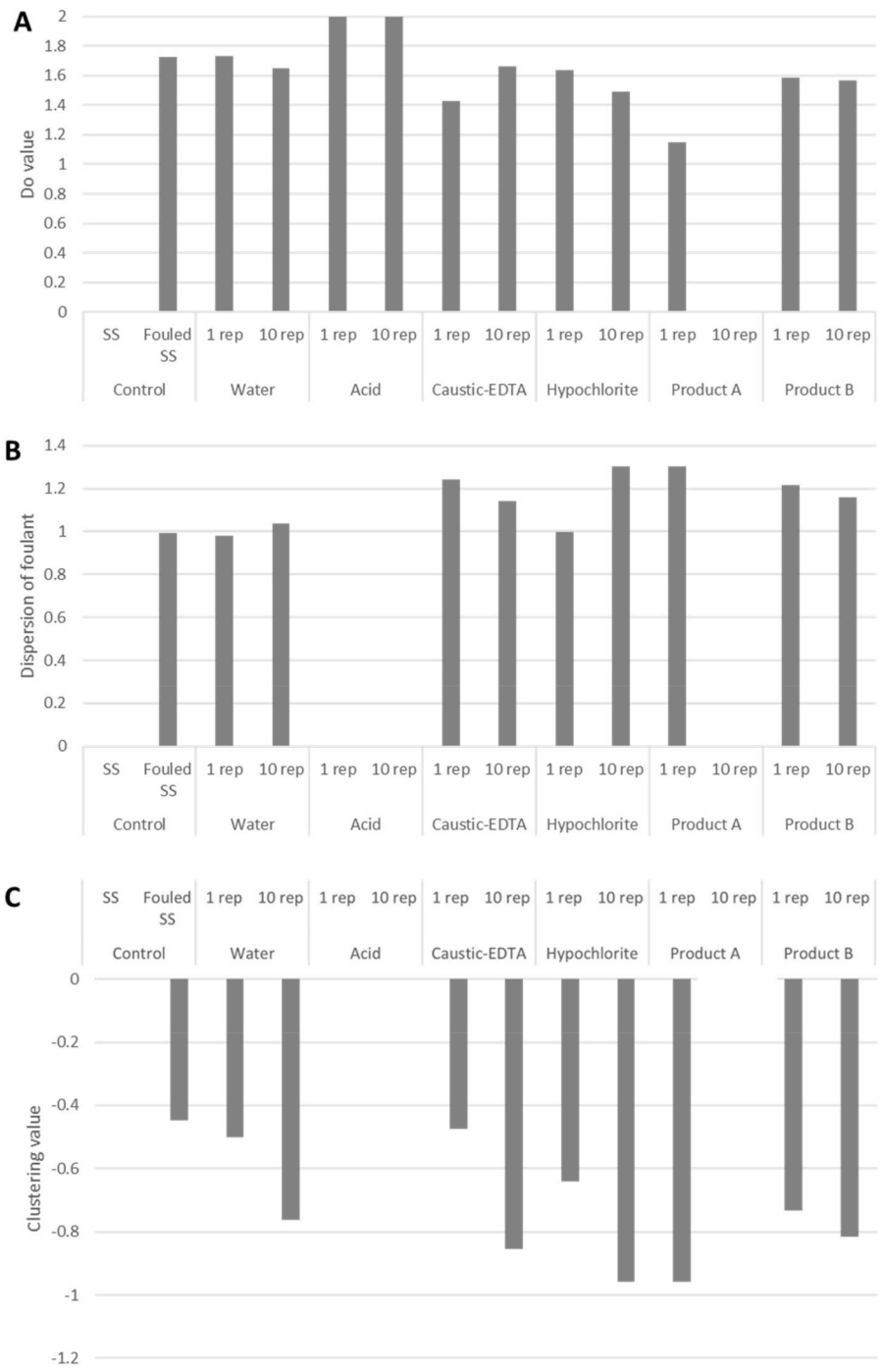

Fig. 5. Multifractal analysis of the surfaces demonstrating the (A) density, (B) dispersion, and (C) clustering of the soil retained on the unfouled stainless steel surface and stainless steel surface fouled with milk and cleaned using either water or 2% (v/v) solutions of caustic-EDTA, hypochlorite, Product A, or Product B after one (1rep) or 10 repetitions (10 rep).

all the surfaces cleaned using caustic EDTA, hypochlorite, Product A or Product B recovered significantly different amounts of β-LG ($p < 0.05$).

4. Discussion

While the results for the hypochlorite and caustic-EDTA cleaners demonstrated that the fouling on the surfaces was reduced by cleaning, the results obtained when using the acid-based detergent demonstrated the formation of a film when in contact with milk. Product A and Product B demonstrated the best removal of milk from the surfaces. The main chemical properties of the novel formulations were a high pH (prepared from a mixture of NaOH and KOH), high EDTA, and multiple surfactants to reduce surface tension. Product A demonstrated a greater cleaning efficacy than the currently used products, whereas Product B demonstrated a cleaning efficacy equivalent to that of the currently used products, considering the three commercial cleaners in the range of the conditions tested in this study.

The chemistry of the fouling retained on the surfaces was determined and it was found that when EDX was performed on the surfaces, the chemical composition of the stainless steel was removed and the carbon (C), oxygen (O), and phosphate (P) elements were compared. The results demonstrated that after one wash, Product A performed well, resulting

Table 1
Element weight (%) obtained from Energy Dispersive X-Ray Spectroscopy for all fouled and cleaned surfaces after one or 10 repeated fouling and cleaning cycles, as well as control stainless steel (SS) surface and stainless steel surface fouled with milk. Results are presented as average ± standard error.

	SS	Milk	Water		Acid		Caustic-EDTA		Hypochlorite		Product A		Product B	
			1	10	1	10	1	10	1	10	1	10	1	10
C	0.0 ± 0.0	47.5 ± 1.6	6.2 ± 4.8	13.5 ± 9.7	24.8 ± 0.3	10.9 ± 14.5	1.4 ± 0.3	1.7 ± 0.2	1.7 ± 0.2	13.8 ± 7.0	1.3 ± 0.1	6.3 ± 4.4	4.7 ± 3.4	1.0 ± 0.1
O	0.0 ± 0.0	6.3 ± 0.4	0.4 ± 1.8	4.9 ± 3.9	2.1 ± 0.3	6.7 ± 4.4	1.2 ± 6.0	1.0 ± 1.0	0.8 ± 0.7	0.8 ± 0.8	0.0 ± 0.0	1.3 ± 0.3	0.6 ± 0.5	0.5 ± 0.5
P	0.0 ± 0.0	0.3 ± 0.2	0.3 ± 0.1	0.3±0.1	0.3 ± 0.1	1.0 ± 0.6	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ±0.1

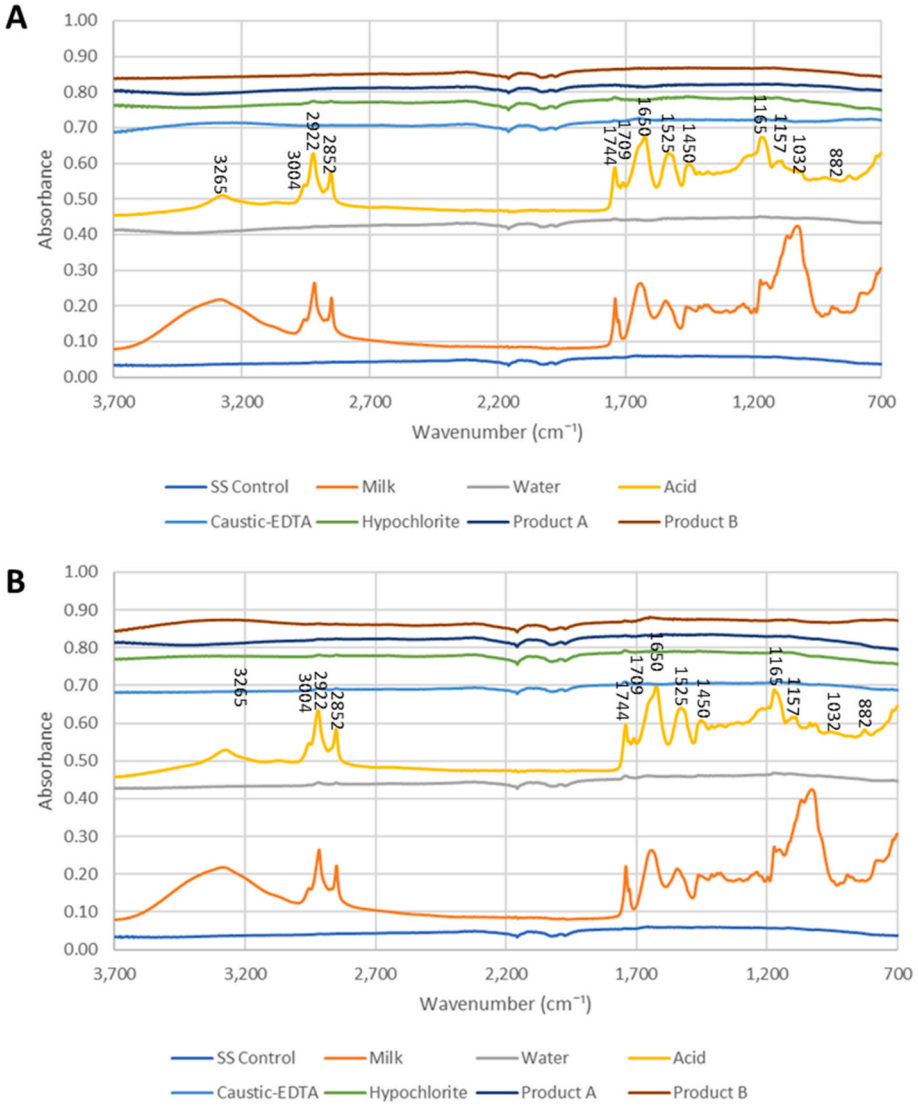

Fig. 6. FTIR of all fouled and cleaned surfaces after (A) one or (B) 10 repeated fouling and cleaning cycles, as well as control stainless steel surface and stainless steel surface fouled with milk.

in low amounts of residual C, O, and P. After 10 washes, Product B was the best at removing the three elements. The difference between Product A and Product B was the amount of EDTA in the product, and it is unclear why a lower amount of EDTA improved the hygienic status of the surfaces at lower concentrations. However, it may be hypothesised that there is an optimum concentration of EDTA that results in hygienic cleaning after one and 10 washes, but this requires further investigation. FTIR was useful to determine the biochemical moieties that had been removed from the surfaces following cleaning but this method could not discriminate between the type of foulant retained.

The patterns of density, dispersion, and clustering of fouling were different across the surfaces. The density of the fouling was greatest for the one and 10 times acid-cleaned surfaces, but this was due to the film that was left across the surface.

The ELISA results demonstrated that all fouled surfaces had recoverable levels of either casein or whey (β-LG) protein in varying amounts.

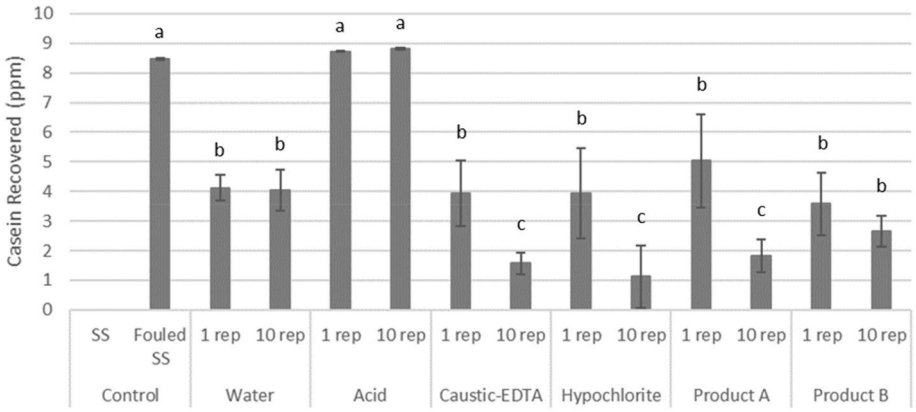

Fig. 7. Amount of casein recovered from unfouled stainless steel surfaces, milk-fouled stainless steel surfaces, and stainless steel surfaces fouled with milk and cleaned using water, acid, caustic-EDTA, hypochlorite, Product A, and Product B after one (1 rep) or 10 repetitions (10 rep). This was determined by ELISA after surface swabbing. Results are presented as average ± standard error. Letters were assigned (from a to c) as long as statistically significant differences exist between surfaces (for a confidence level greater than 95%, $p < 0.05$). This was evaluated by one-way analysis of variance (one-way ANOVA, Bonferroni's *post-hoc* test).

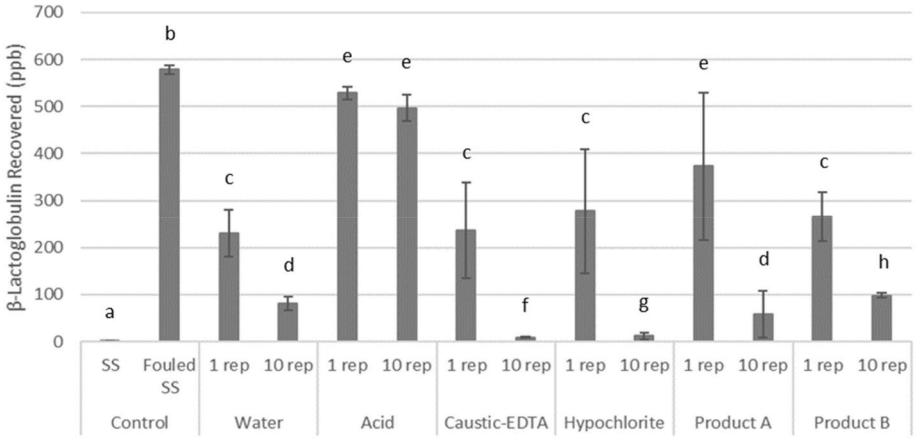

Fig. 8. Amount of β-lactoglobulin recovered from unfouled stainless steel surfaces, milk-fouled stainless steel surfaces, and stainless steel surfaces fouled with milk and cleaned using water, acid, caustic-EDTA, hypochlorite, Product A, and Product B after one (1 rep) or 10 repetitions (10 rep). This was determined by ELISA after surface swabbing. Results are presented as average ± standard error. Letters were assigned (from a to h) as long as statistically significant differences exist between surfaces (for a confidence level greater than 95%, $p < 0.05$). This was evaluated by one-way analysis of variance (one-way ANOVA, Bonferroni's *post-hoc* test).

However, the results demonstrated that there were different patterns of allergen removal which were dependent not just on the cleaner, but also on the number of cleans used and on the type of allergen being detected, which should be considered by the food industry.

The two cleaners typically used for cleaning milk from surfaces, either a caustic-EDTA detergent or a sodium hypochlorite detergent, were both effective. These two detergents use different chemical processes to remove milk from the surfaces. The alkalinity of the caustic-EDTA could have caused saponification of the fats present within milk due to the high pH, whereas the presence of EDTA would have helped remove calcium from the milk and prevent the deposition of calcium onto the surface (Marriott et al., 2018; Oude, 1992), including calcium native to the dilution water in the form of hardness salts. The hypochlorite detergent also saponifies the fats present within the milk owing to its high alkalinity, and this cleaner would also aid in soil breakdown/modification by peptising proteins present in the milk (Marriott et al., 2018). Both detergents demonstrated similar levels of overall soil removal and remaining recoverable allergenic protein. Interestingly, for the chlorinated detergent, the soil was clearly visible by surface staining after one washing cycle. However, after ten cleaning and fouling cycles, the soiling appeared to be more globular, with a more organised deposition. This may have occurred since the globules of fat in milk are surrounded by a membrane consisting of a trilayer of polar lipids, proteins, and cholesterol (Lopez, 2011; Obeid et al., 2019) and other compounds (van Der Berg, 1988). Hypochlorite is capable of peptising proteins, and this mode of action may have damage the globular membrane of milk fats, resulting in the ability of the globules to unite and form larger masses.

Regarding the acid detergent, the film soil was present on the surface. One of the issues in cleaning milk with acid is the casein content of milk, which can coagulate when the pH becomes acidic (Lucey, 2016). The coagulation of casein is driven by its micellar structure (composed of casein molecules, calcium, inorganic phosphate, and citrate ions), which is highly stable at a pH of 6.6, the typical pH value of milk (Sarode et al., 2016). However, the micellar structure of casein is sensitive to environmental pH and becomes coagulated and precipitated from milk when the pH becomes less than its isoelectric point of 4.6 at 20 °C, causing insoluble flocculation of the protein to occur (Ali et al., 2019; Marchal and Waters, 2010; Sarode et al., 2016). This may indicate that the instability of the micellar structure caused a loss of the calcium component. In contrast to casein, the whey component of milk proteins typically does not become insoluble during the acidification of milk, like casein at pH 4.6 (Sindayikengera and Xia, 2006). Since the ELISA results demonstrated that the film formed by treating milk with an acid-based detergent contained significant amounts of both casein and whey (β-LG), it is likely that the formation of the film on the surface was

partially caused by the deposition of casein onto the surface and the whey protein interacting with casein to build a thicker layer of material. This was confirmed by FTIR peaks, indicating the presence of protein Amides I, II and III, all of which were more apparent compared to the control milk or stainless steel spectra (Conceição et al., 2018; Nicolaou et al., 2010). Furthermore, the film development on the acid-cleaned surfaces from multiple fouling and cleaning attempts, supports that after the initial film was formed on the surface, the addition of more milk and acid-enabled interactions between the film and the foulantl further increased the amount of material retained. This mechanism of action may have occurred because the fat or lipids present in mammalian milk are typically present as milk fat globules, colloidal lipid assemblies that contain bioactive molecules (Lee et al., 2018). A study on milk fat globules has previously demonstrated that upon acidification of casein into a gel, the milk fat globules that arose from casein acidification were instead trapped in pockets of the protein network (Obeid et al., 2019). This could indicate that the network prevented the fats from being removed from the surface, thus increasing the layer that was apparent on the acid-treated milk-fouled surface. Furthermore, the peaks that potentially represented the lipids in the FTIR spectra remained unchanged compared to the control milk spectra, indicating that these molecular species remained present (Conceição et al., 2018; Nainggolan et al., 2018; Nicolaou et al., 2010).

In Products A and B, the combination of the three components (high pH, high EDTA, and multiple surfactants) should enable the detergent to saponify fat, solubilise minerals, and retain fat and other components of milk in suspension to facilitate the removal of milk fouling from the surfaces. The combination of caustic soda with caustic potassium carbonate mixtures provided a means of saponifying fats, with potassium carbonate derivatives generally being more soluble than those of caustic soda and thus more easily held in suspension. While the EDTA level in the new products was lower than that in the caustic-EDTA detergent, it may be speculated that it acted in the same manner to chelate minerals. Such a mechanism of action prevents the deposition of saponified fats and denatured proteins/amino acids from sticking together via calcium bridges (Joyce et al., 2017). It is interesting to note that, while the new products had similar or lower levels of EDTA than the caustic-EDTA product, they appeared to work as well or better. This may have been due to the presence of multiple wetting agents, likely enabling better initial contact of caustic and EDTA with milk. Furthermore, in the case of denatured soils, the presence of wetting agents may have enabled EDTA to gain better penetration of the milk and hence dissolve the calcium bridges.

When the ELISA results were compared with the allergenic eliciting thresholds after combining casein and whey (β-LG) data, the results demonstrated that every fouled surface exceeded the 5% eliciting dose threshold (2.4 mg protein) after a single fouling and cleaning cycle (Bureau, 2019). After 10 fouling and cleaning cycles, each fouled and cleaned surface was still above the 1% eliciting dose threshold (0.2 mg protein), despite the caustic-EDTA and hypochlorite cleaned surfaces dropping below the 5% eliciting threshold (Bureau, 2019). Hence, none of the cleaners successfully removed allergens from the surfaces.

To summarise the efficacy of the different methods used to determine residual biofouling left on the surfaces, SEM only demonstrated macro fouling on the surfaces, whilst epifluorescence microscopy with staining enabled areas with less fouling to be determined. In addition, the use of epifluorescence microscopy enabled the percentage coverage of the retained material to be quantitatively enumerated. Multifractal analysis of the surfaces allowed the quantification of the pattern of biofouling across the surfaces in terms of density, dispersion, and clustering which added further information on the binding pattern of the soil to the surface. EDX enabled the elemental analysis of the carbon, oxygen, and phosphate on the surface, but the amount of calcium retained was at the limit of detection for the instrument, and in future work more sensitive surface science analysis should be used. FTIR was also useful to demonstrate the biochemical moieties that had been removed from the surface following cleaning. However, this method was not sensitive enough to discriminate between the type of soil left on the surfaces following different cleaning types.

5. Conclusions

In this study, two new formulations were produced based on the selection of specific surfactants, EDTA, and wetting agents. This work demonstrated that the new products were highly effective at removing non-thermal milk fouling, with Products A and B being better or equivalent to the currently used cleaning solutions. Hence, the results showed that the use of either Product A or Product B would still provide effective cleaning and may potentially be used as an alternative to traditional cleaning products. However, when allergens were considered, the new products left a similar amount of allergenic protein behind on stainless steel compared to the currently used products. This is an important consideration for dairies that may produce dairy products where residual allergens are of concern, such as in the production of baby milk formulations. This study suggests the use of new product formulations to replace hypochlorite-based detergents and increase the hygienic status of a surface.

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CRediT authorship contribution statement

Joels Wilson-Nieuwenhuis: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Jim Taylour:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Luciana C. Gomes:** Writing – review & editing, Validation, Formal analysis. **Stephen Lynch:** Writing – review & editing, Software, Resources, Investigation, Formal analysis, Data curation. **David Whitehead:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Kathryn A. Whitehead:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that this study received funding from Holchem Laboratories Ltd.

Data availability

Data will be made available on request.

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