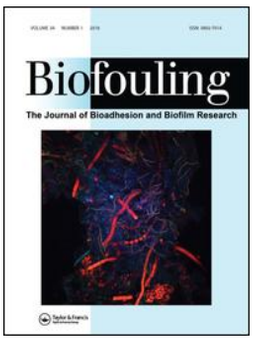

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The synergistic effect of enzymatic detergents on biofilm cleaning from different surfaces

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ABSTRACT

Biofilm growth is a significant source of contamination in the food industry. Enzymes are considered green countermeasures against biofilm formation in the food industry owing to their biodegradability and low toxicity. In this study, the synergistic effect of enzymes was studied against biofilm cleaning from hard surfaces. A mixed-microbial sample was sourced from a meat packaging line and biofilms were grown under high shear conditions on stainless steel and polyethylene surfaces. A model cleaning-in-place (CIP) parallel-plate flow chamber was used for firstly, the enzymatic cleaning and secondly, a disinfection step. The cleaning effectiveness was evaluated in response to different formulations containing non-foaming commercial surfactants among with amylase, protease and lipase at neutral pH. The formulation combining all three enzymes was the most effective, showing a synergy essential for the deformation of biofilm structure and consequently better disinfection of both material surfaces.

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Biofilm; cleaning; enzymes; CIP; biofouling; surfaces

Introduction

Microorganisms can live and proliferate as planktonic cells or they can attach to surfaces, where they grow as highly organized multicellular communities, known as biofilms (Otto 2008). Biofilms are considered the major type of microbial life in nature and exist as microorganism associations embedded in self-produced matrix of extracellular polymeric substances (EPS), which gives them consistency and resistance to antibiotics and disinfectants (Katsikogianni and Missirlis 2004; Flemming et al. 2016). Biofilm formation and proliferation affects many aspects of public health and industrial processes including being a source of contamination in the food and beverage industries (Johansen et al. 1997; Palmer et al. 2007; Goode et al. 2013). Some biofilm-forming species in food factory environments are human pathogens that are able to grow and proliferate on substrata such as stainless steel, polyethylene, wood, glass, polypropylene, rubber, etc. (Kohila et al. 2013; Abdallah et al. 2014; Colagiorgi et al. 2017).

In order to control biofilm formation, food industries use cleaning processes, such as cleaning-in-place (CIP) or cleaning-out-place (COP) systems which utilise shear stress together with chemical agents (Keener 2005; Miles et al. 2017). To understand the mechanisms that enhance biofilm removal, several studies have tried to model detachment kinetics of biofilms from artificial surfaces. The most important outcome was the observation that it is not a straightforward process and that adequate elimination requires the combination of hydrodynamic forces coupled with chemical agents (Lelièvre et al. 2002, 2007; Lécrigny-Nolf et al. 2009; Faille et al. 2013; Bénézech and Faille 2018). While routine CIP regimes in food-processing lines use strong alkaline or acidic agents (Antoniou and Frank 2005), this strategy is not always enough for biofilm removal. Also, their safe handling requires the utmost care, and hence milder, safer and more efficient biofilm CIP cleaning agents are desirable (Serena et al. 2018).

Research has shown that bacterial cells within biofilms are physiologically distinct from their planktonic counterparts and, for this reason, are more resistant to cleaning (Lindsay and Holy 2006). An interesting alternative to the use of cleaning agents is the application of enzymes. Since enzymes are biodegradable and show low toxicity, they are considered to be

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green countermeasures against biofilm formation (Serena et al. 2018) and they have been used in detergents designed for biofilm removal in the food industry (Furukawa et al. 2010). In order to understand the mechanism of enzymatic cleaning against biofilms, Molobela et al. (2010) studied commercial proteases and amylases for their effectiveness in the deformation and detachment of the EPS matrix produced by *Pseudomonas fluorescens*. In that study, all of the enzymes except the protease polarzyme degraded EPS, with savinase and everlase being the most effective. Earlier studies (Vickery et al. 2004; Walker et al. 2007) investigated enyzme cleaning effectiveness against biofilms and it was found that mixtures of enzymes were more effective in removing biofilms than single enzyme cleaning treatments.

In order to eliminate biofilm, a key component to control is the EPS matrix, which is mostly comprised of water (up to 97%) and contains the structural and functional components of the matrix: soluble, gel-forming polysaccharides, proteins and eDNA, as well as insoluble components such as amyloids, cellulose, fimbriae, pili and flagella (Flemming et al. 2016). Although little is known about the interactions between enzymes and EPS, it has been reported that polysaccharases, polysaccharide lyases, and to some extent proteases, disrupt the EPS structure (Sutherland 2001; Vickery et al. 2004). From a physico-chemical point of view, the removal of biofilms can be achieved by using substances that induce detachment by reducing the cohesiveness of the EPS matrix (Xavier et al. 2005), and enzymes have been proved to do this (Augustin et al. 2004; Vickery et al. 2004; Xavier et al. 2005). Along with chemical agents, enzymes are categorised as detachment-promoting agents (DPAs) *via* a range of mechanisms that have not yet been fully characterized (Vickery et al. 2004; Xavier et al. 2005; Palmer et al. 2007). It has been postulated that enzymes reduce the physical integrity of the EPS by weakening the structural bonds of the proteins, carbohydrates and lipids that form its structure (Melo and Bott 1997; Furukawa et al. 2010; Lequette et al. 2010; Molobela et al. 2010).

Nonetheless, it is essential to consider that a key factor for biofilm cleaning is the initial biofilm adhesion force of the biofilm to the solid surface it is on (Donlan 2002). The physico-chemical properties of both the substratum and the bacteria can impact on how cells attach and hence the response of the biofilm to cleaning. Microorganisms have been reported to attach more rapidly to hydrophobic, nonpolar surfaces such as Teflon and other plastics than to hydrophilic materials such as glass or metals (Fletcher and Loeb 1976; Boonaert et al. 2003; Bayoudh et al. 2005; Guillemot et al. 2006; Detry et al. 2010). However, Flint et al. (2000) reported that bacteria would adhere more easily to hydrophilic substrata, while Parkar et al. (2001) reported that there was no correlation between bacterial adhesion and surface energy for different 316-stainless steel substrata although surface energy may have influenced the adhesive strength. Consequently, interfacial phenomena between the biofilm and the substratum to which it is attached might be important in the choice of appropriate cleaning agents (Detry et al. 2010), although any relationship made between bacterial adhesion and surface energy must be considered with caution (Boonaert et al. 2003).

In the current study, the activity of enzymes in different formulations against a mixed-microbial biofilm sourced from a meat packaging line was evaluated using a model CIP system containing stainless steel and polyethylene surfaces. As these two material surfaces are widely used in the food industry, the first objective was to examine whether there would be a difference in their cleanability, specifically with regard to the removal of biofilms. Moreover, the synergistic effect of three enzymes, amylase, protease and lipase, was investigated in combination with non-foaming surfactants at a neutral pH. The hypothesis was that the combination of the enzymes would be more effective against biofilm but the goal was to explore whether there were significant differences between their cleaning efficacies.

Materials and methods

Biofilm growth

Biofilms were developed on the surfaces using cylindrical glass beakers of 2 l capacity and 18 cm in diameter, named microcosms. Specifically, stainless steel (316 L) (SS) and polyethylene terephthalate (PET: Polyester, Dacron) coupons (diameter 12.7 mm; BioSurface Technologies Corporation) were placed at the bottom of the microcosms filled with 500 ml of Ringers solution (Oxoid) and 50 ml of Tryptone Soy Broth (TSB; PanReac AppliChe). A pump (Eden 105) was placed inside the microcosms to create shear as in a dynamic environment of an industrial process previously line. Next, 2 ml of microbial inoculum were transferred in the microcosms to a final concentration of 10^7 CFU ml^{-1}. The inoculum was prepared from swab samples collected from a meat packaging process line (Vic, Catalonia, Spain) that were

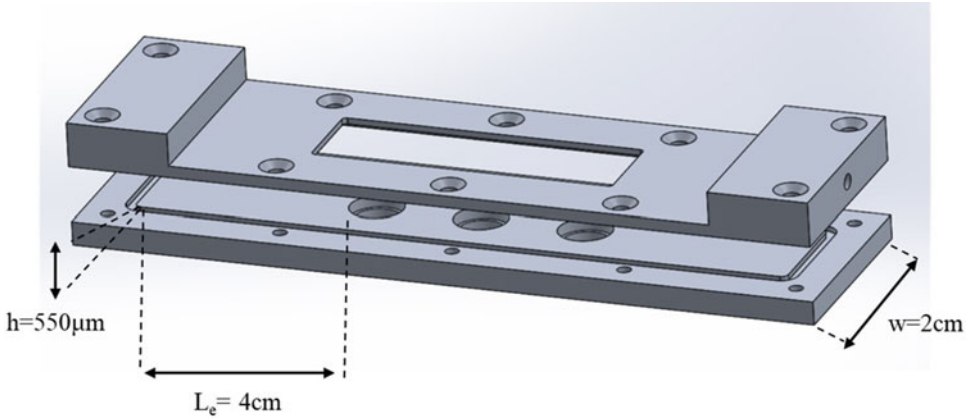

Figure 1. Schematic representation of the parallel-plate flow chamber.

incubated overnight at 30 °C in 500 ml of TSB to a final concentration of 2×10^{12} CFU ml^{-1}. The microcosms were covered with 5 layers of aluminium foil and wrapped with plastic tape, and incubated at 30 °C for 25 days. The volume of the solution became reduced due to evaporation and, in order to maintain a constant volume and to avoid modification of the physical-chemical environment due to microbial activity (i.e. pH, oxygen), 250 ml of the medium were removed from each microcosm every 3 days and replaced with 500 ml of fresh Ringers solution. The biofilm colonized SS and PET coupons, were removed aseptically, rinsed with sterile PBS water and subsequently used for the CIP enzymatic cleaning experiment.

Design of a parallel-plate chamber

A cleaning-in-place protocol was applied in a parallel-plate flow chamber, designed to apply a constant and predefined shear stress to the coupons. The parallel-plate flow chamber was composed of two SS slides that were considered to act as two infinite parallel plates, further assumptions were that the fluid was incompressible and Newtonian and that the flow was steady. Three parameters were calculated for parallel-plate flow chamber (Martines et al. 2004): (1) The Reynolds number, *Re*; (2) the entrance length, l_e and (3) the wall shear stress, τ_w.

Reynolds number, Re

The calculation of the dimensionless number Re, was selected as a fast and easy way to predict the profile of the flow in the chamber. The flow profile is generally described as the ratio between the inertial forces and the viscous forces that are present due to the flow. If *Re* is smaller than 2,300, the viscous forces are predominant, so the flow is considered laminar

$$Re = \frac{Dv\rho}{\mu} \quad (1)$$

Where ρ is the fluid density, v is the mean velocity of the flow and D is the hydraulic diameter for rectangular pipes, calculated from the equation

$$D = \frac{4wh}{2(w+h)} \quad (2)$$

where w is the width of the chamber and h the height between the two parallel slides. In order to achieve a well-developed laminar flow, the width of the chamber was 23 times greater than its height (Martines et al. 2004).

Entrance length

When the flow enters the chamber, a certain length is needed (so-called entrance length) before it becomes fully developed, i.e. before the velocity profile becomes parabolic. For the calculation of the entrance length of the chamber Equation (3) shown below was used, where D is the hydraulic diameter (Martines et al. 2004)

$$l_e = 0.065ReD \quad (3)$$

Wall shear stress

The wall shear stress was calculated based on the equation of the flow rate inside the chamber (Mark et al. 2007).

$$Q = \frac{wh^2\tau_w}{6\mu} \quad (4)$$

where Q represents the flow rate, τ_w the wall shear stress, w the width of the chamber, h the height of the flow chamber, and μ the viscosity of the flow medium. The parallel plate flow chamber was designed using fluid water at 40 °C as a reference. Thus, the physico-chemical properties used in all the above equations were the following (ThermExcell 2003–2019):

$$\text{Density, } \rho = \ 992.25\,\text{kg m}^{-3}$$
$$\text{Viscosity, } \mu = 0.653 * 10^{-3}\text{kg (m s}^{-1})$$

Hence, after the relevant calculations, the design dimensions of the parallel-plate flow chamber were as follows: width, $w = 2$ cm and height, $h = 550$ µm (Figure 1). For the flow to be fully developed according to these dimensions the entrance length should be $L_e > 0.78$ cm and thus it was chosen to be 4 cm for a fully developed laminar flow (Figure 1).

Cleaning-in-place protocol

In order to evaluate the effectiveness of different enzymes in biofilm removal, CIP comprising different steps was performed by using the parallel-plate flow chamber containing triplicate SS or PETcoupons colonized by biofilm as described above. During the first cleaning step, enzymatic formulations were applied for 30 min. The initial temperature of the formulation was 55 °C and after the 30 min of cleaning the temperature was 45 °C. In the second step, a commercial disinfectant based on peracetic acid (1% w/w) was applied for 10 min at room temperature. The temperature and the exposure time used for the detergents and the disinfectant were as recommended by their manufacturer. The parallel-plate flow chamber operated under stable flow in all the experiments ($Q = 1{\cdot}10^{-6}$ m^3 s^{-1}). The wall shear stress was calculated at 0.129 (N m^{-2}) for 25 °C and at 0.647 (N m^{-2}) for 50 °C. The wall shear stress conditions were chosen based on previous work cited in the literature (Lelièvre et al. 2007; Bénézech and Faille 2018). The different enzymatic products that were tested were prepared by the authors using standard enzymatic formulations from the Itram Higiene S.L. company that contained (1) amylase-protease-lipase (Formulation A), (2) amylase-protease (Formulation B), and (3) amylase-lipase (Formulation C), in combination with a non-foaming Itram enzymatic solution. The total concentration of enzymes in each formulation was kept constant in all the experiments. The formulations provided by Itram that were used for the preparation of the formulations tested in the experiments had standard concentrations of enzymes and surfactants. Each time the formulation tested (A, B or C) was prepared fresh with the same concentration of the enzyme to be tested. The efficacy of these products was compared to a non-foaming Itram solution without enzymes, which was used as a control.

In order to evaluate the effectiveness of biofilm reduction the parameters investigated were (1) bacterial viability, (2) the polysaccharide content in EPS and (3) the percentage (%) of the area covered by biofilm, biovolume and average biofilm thickness from confocal laser scanning microscopy (CLSM) images. Complementarily, scanning electron microscopy (SEM) images were obtained to study the microbial biofilm structure. All measurements were performed (1) before cleaning, (2) after cleaning with the different enzymatic formulations and (3) after disinfection. In each experiment a different formulation (control, A, B or C) was tested in combination with a disinfection process on freshly grown biofilms. Three replicates of biofilm without any treatment were initially tested. The enzymatic detergent treatments were performed in six different replicates and three of them were tested. Finally, the disinfection process was performed on the three remaining replicates that had been treated with the enzymatic formulations and they were all subjected to the same analysis techniques.

For all of the parameters analysed, the effectiveness of biofilm reduction, $E_{f,}$ was calculated as:

$$E_f(\%) = \frac{|Q_{BC} - Q_{AC}|}{Q_{BC}} * 100 \qquad (5)$$

where, Q_{BC} and Q_{AC} are the parameters measured before and after each cleaning step of the process.

Bacterial viability

The number of live and dead bacteria was obtained and the viability of biofilm bacteria was evaluated. The biofilm was scraped off the coupons using a cell-scraper (sterile silicone scraper, Nunc, Wiesbaden, Germany) and 2 ml of Ringer solution. The resulting cell suspension was vortexed and 1 ml was added to 4 ml of sodium pyrophosphate decahydrate, 99% A.C.S. reagent (50 mM, sterilised using a 0.2 µm pore size filter) which was incubated for 15 min and then sonicated for 10 s to avoid disruption of the cells. Next, 1 ml of the cell suspension was diluted in 9 ml of Ringer solution and the final samples were homogenized by vortexing. For flow cytometry analysis, 400 µl from the above solutions were transferred into the sample tubes. Next, 3 µl of Backlight stain (SYTO/PI, in 1:1) were added to the sample tubes, which were vortexed and incubated for 15-30min in the dark at room temperature. To normalise fluorescence data a bead solution (10 μl of 10^6 beads ml^{-1}, Fisher 1.0 μm) was added and the number of live, dead and damaged cells were counted by flow cytometry (FACSCalibur, Becton Dickinson) and the LIVE/DEAD bacterial cells were evaluated. This was done in all cleaning steps (before cleaning, cleaning with enzymatic detergent and after disinfection) and the effectiveness of biofilm reduction was studied as described in Equation (5).

Polysaccharide content of biofilm EPS

The biofilm EPS was extracted by cation exchange resin (CER) and the content of the polysaccharides was measured as glucose equivalents after digestion (Romani et al. 2008). The cation exchange resin (CER, Dowex® Marathon™ C sodium form, Sigma-Aldrich, 91973-250 G-F) was conditioned prior to application for EPS-extraction, following the manufacturer's instructions. The biofilm was scraped off the coupons into 1 ml of phosphate buffer (4.49 g $Na_2HPO_4 \cdot 12\,H_2O$, 1.7 g KH_2PO_4, adjust to 1 l, pH 7) and pipetted into 2 ml microtubes (Eppendorf). Then, 0.5 g of CER were added to the scraped biofilm and the microtubes incubated in ice for 1 h at 250 rpm agitation. After incubation the particulates of the biofilm were removed from the extract by centrifugation at 12,000 x g for 15 min at 4 °C (Sorvall RC 5B Plus). From the total volume of the supernatant 500 µl of each microtube were pipetted into 10 ml glass tubes. The polysaccharide content of the EPS was determined with the phenol/H_2SO_4 determination assay of Dubois et al. (1956) as phenol can be used for the colorimetric quantification of polysaccharides in the presence of sulphuric acid, as glucose equivalents after digestion. To the 500 µl EPS-extract in a glass-tube, a volume of 12.5 µl of phenol solution (80%) were added and the solution was gently mixed, prior to the addition of 1.25 ml of concentrated 95.5% reagent grade H_2SO_4 and the glass-tubes was sealed with caps. The samples were left to stand for 10 min and then they were gently shaken and incubated in a water bath for 20 min at 30 °C. The colour developed was measured at 485 nm against a reagent blank (U-2000 Spectrophotometer, Hitachi). To calculate the polysaccharide content a glucose calibration curve was prepared with standard glucose concentrations and 500 µl of the prepared glucose standard solutions were used for the phenol-H_2SO_4 assay as a comparison to the samples. The results were calculated as glucose equivalents per square centimeter of biofilm surface area. The EPS-polysaccharide content was evaluated in all cleaning steps (before cleaning, cleaning with enzymatic detergent and after disinfection) and the effectiveness of biofilm reduction was studied as described in Equation (5).

Confocal laser scanning microscopy

Multichannel confocal and transmission imaging of the biofilm samples was performed using a multispectral Confocal Leica TCS SP8 microscope. A high-speed module and three light channels and laser lines were used: 405 nm, 488 nm and white laser for 470-670 nm excitation at 592 nm. The samples were marked with three different fluorophores, SYTO/PI (LIVE/DEAD™ BacLight™ Bacterial Viability Kit) for the live and dead bacterial cells respectively and HCS CellMask (H32720) lectin for the EPS bioorganic compounds. For the sample preparation, 15 µl of SYTO and 15 µl of PI were diluted in 1 ml of phosphate buffer saline (PBS) solution and 30 µl of the lectin were added. The samples were stained using 60 µl of the above solution and were incubated in dark, at room temperature for 10 min. The stain was washed with PBS solution and the samples were studied using a 63 ×-magnification oil-lens. For the image acquisition, LAS X Leica software was used with 1024 × 1024-image resolution, speed 600 and zoom factor 1. The 3-D biomass information was obtained using a z-stack step of 1 µm and the image processing and quantification was performed using Fiji (ImageJ). For all samples, the biovolume, the percentage of the surface area covered with biofilm and the average biomass thickness were calculated. The biovolume (μm^3) represented the total volume of the biofilm, hence the EPS, the dead and the live bacteria. The percentage of the surface area covered with biofilm was calculated by dividing the values with the surface area studied and the number of the stacks measured in each case. Finally the average biomass thickness represented the number of the z-stacks that were studied in each case. Finally, in all experiments the biovolume, the surface coverage and the average biomass thickness were measured before applying any cleaning process, and, in order to compare the results for the different enzymatic formulations the effectiveness of the cleaning was also calculated for all cases, as the percentage of the reduction of biovolume and surface area coverage respectively.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to study biofilm structure during the different cleaning steps. Samples were fixed with 2.5% (wt./vol) glutaraldehyde EM grade for 4 h and stored in 0.1 M cacodylate buffer, pH 7.4 at 4 °C until measurement. Next, they were washed and dehydrated successively in ethanol, dried at the critical point CO_2 (Emitech, Germany, model K 850 CPD), and evaporated carbon (Emitech, Germany, model K950 turbo evaporator). Examinations were carried out with a FE-SEM

(Hitachi, Japan, S-4100) scanning electron microscope. Digital images were collected and processed by Quartz PCI (version 5.1) digital software.

Water contact angle measurement – surface energy

A theta optical tensiometer – contact angle meter was used for the measurement of the water contact angle with the surfaces and hence the calculation of the average surface energy or the work of adhesion. According to the Young- Laplace equation of surface tension, the higher the contact angle of water on the surface, the more hydrophobic is the surface. The work of adhesion was calculated according to the Young-Laplace equation of surface tension

$$\mathrm{Wa} = \gamma \cdot (1 + \cos\theta) \quad (6)$$

where γ, is the surface tension of water at 25 °C and θ is the contact angle of the water and the surface.

Table 1. Effects of enzymatic cleaning and disinfection treatments on bacterial viability and EPS-polysaccharide content.

Surface	Parameter	Contrast	Enzymatic cleaning	Disinfection
SS	Bacterial viability	A vs B	**<0.0001**	**0.000**
		B vs C	**<0.0001**	**0.001**
		C vs A	0.268	0.079
PET		A vs B	**0.039**	**0.001**
		B vs C	**0.047**	**0.004**
		C vs A	0.987	0.205
SS	EPS-polysaccharide content	A vs B	0.103	0.795
		B vs C	0.147	0.952
		C vs A	0.957	0.932
PET		A vs B	**0.011**	**0.032**
		B vs C	0.308	**0.001**
		C vs A	0.071	**0.001**

Significance (probability, p) after one-way ANOVA analyses is indicated. All contrasts of the A, B, and C treatments with the Control were significant ($p < 0.0001$, not shown).

Data analysis

The data analysis was performed using analysis of variance (ANOVA) in combination with Tukey's Honestly Significant Differences data comparison system. The one-way ANOVA was used in all analyses to test whether there were significant differences in the data between each treatment and at each cleaning step (enzymatic cleaning and disinfection step) for both surfaces.

Results

Bacterial viability

Bacterial viability before cleaning showed no significant differences between experimental groups ($p > 0.05$) but values were higher for PET (2.89 in average) than for SS grown biofilms (0.68 in average). After the enzymatic cleaning and the disinfection step, bacterial viability was significantly reduced ($p < 0.05$) for all the enzymatic formulations, A, B and C when compared with the control, although this depended on the enzyme formulation and the substratum for biofilm growth (Table 1). For biofilms grown on SS, bacterial viability was significantly reduced in A and C formulations (82.9% and 73.5% reduction effectiveness in average, for A and C respectively) while formulation B caused only a 14.4% reduction. For biofilms grown on PET, cleaning with formulations B and C (containing two enzymes) significantly reduced 25% of bacterial viability while formulation A (containing three enzymes) significantly reduced 82.6% of bacterial viability (Table 2, Figure 2). Differences between treatments after disinfection were the same as those observed after enzymatic cleaning (Table 2, Figure 2). The formulation that contained all three enzymes evaluated in this study, amylase-protease-lipase (A), was the most effective for both surfaces, as there was a clear reduction in

Table 2. Effects of enzymatic cleaning and disinfection treatments on bacterial viability and on the EPS-polysaccharide content.

Surface	Parameter	Formulation	Enzymatic cleaning (%)	Disinfection (%)
SS	Bacterial viability	Control	10.6	17.4
		A	82.9	92.1
		B	14.4	27.9
		C	73.5	82.0
PET		Control	8.6	16.9
		A	82.6	90.8
		B	26.8	36.1
		C	34.6	63.7
SS	EPS-polysaccharide content	Control	4.4	8.7
		A	45.0	55.9
		B	43.6	51.2
		C	41.0	43.9
PET		Control	4.4	8.8
		A	81.2	96.0
		B	7.2	10.7
		C	31.8	61.4

The effectiveness of biofilm reduction (E_f, %) is indicated.

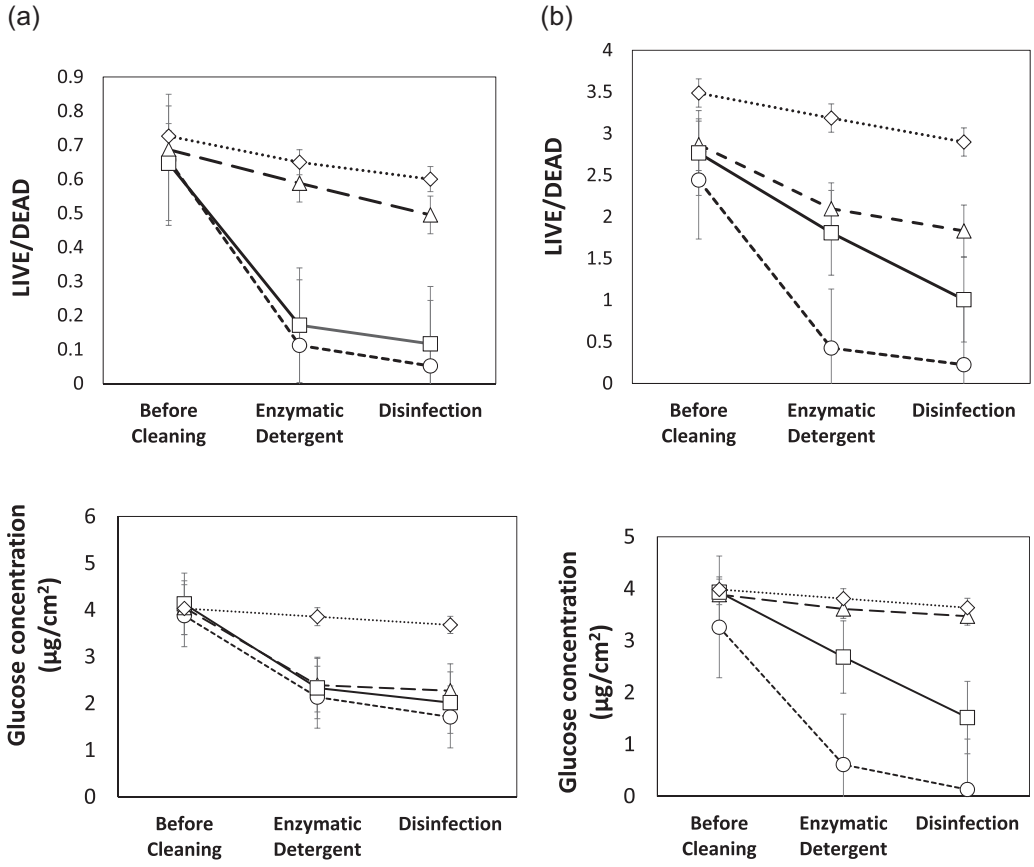

Figure 2. Bacterial viability (as the ratio of live over dead bacteria cm^{-2}) and EPS polysaccharide content (as μg cm^{-2} of glucose-equivalents) during cleaning on (a) SS and (b) polyethylene (PET).

bacterial viability after each cleaning step. In the case of polyethylene surfaces, amylase-lipase (C) and amylase-protease (B) combinations had a similar effect on the bacterial viability over the first step of enzymatic cleaning, while formulation A was more effective. After the disinfection step according to the marginal error all formulation showed similar effect, though again formulation A seemed to be the most effective. In contrast, on SS surfaces, the presence of protease (B) and lipase (C) showed a more distinct cleaning behaviour, since between these two the latter was clearly more effective (Table 2, Figure 2).

Table 3. Effects of enzymatic cleaning and disinfection treatments on the reduction in biofilm average thickness.

	Formulation	Before cleaning (μm)	Enzymatic cleaning (μm)	Disinfection (μm)
SS	Control	35.8	30.9	27.3
	A	27.3	20.1	16.4
	B	30.9	28.6	20.3
	C	35.8	24.4	15.1
	Average	**32.5**	**26**	**19.8**
PET	Control	49.8	40.8	35.0
	A	49.8	25.9	24.2
	B	40.8	36.0	31.2
	C	45.0	33.6	25.3
	Average	**46.4**	**34.0**	**28.9**

Polysaccharide content in biofilm EPS

EPS-polysaccharide content before cleaning showed no significant differences between experimental groups ($p > 0.05$) and biofilms grown on both surfaces had similar contents (4.02 μg cm^{-2} and 3.81 μg cm^{-2} on average for SS and PET, respectively). As would be expected based on the bacterial viability of biofilms, the EPS reduction expressed in polysaccharide content was more effective when the surfaces were cleaned using the formulation that contained amylase-protease-lipase (formulation A, Table 2, Figure 2). For biofilms grown on SS, the three formulations had a similar effect on EPS-polysaccharide content, reducing around 45% and 50% after enzymatic cleaning and disinfection, respectively (Table 2, Figure 2). For both cleaning steps, in SS there were no significant differences between enzymatic treatments A, B and C (Tukey's test, $p > 0.05$) but all three were significantly different from the control ($p < 0.05$) (Table 1). For biofilms grown on PET, the EPS-polysaccharide content was significantly reduced (81%) after enzymatic cleaning with formulation A, the EPS content being significantly different in A to that in

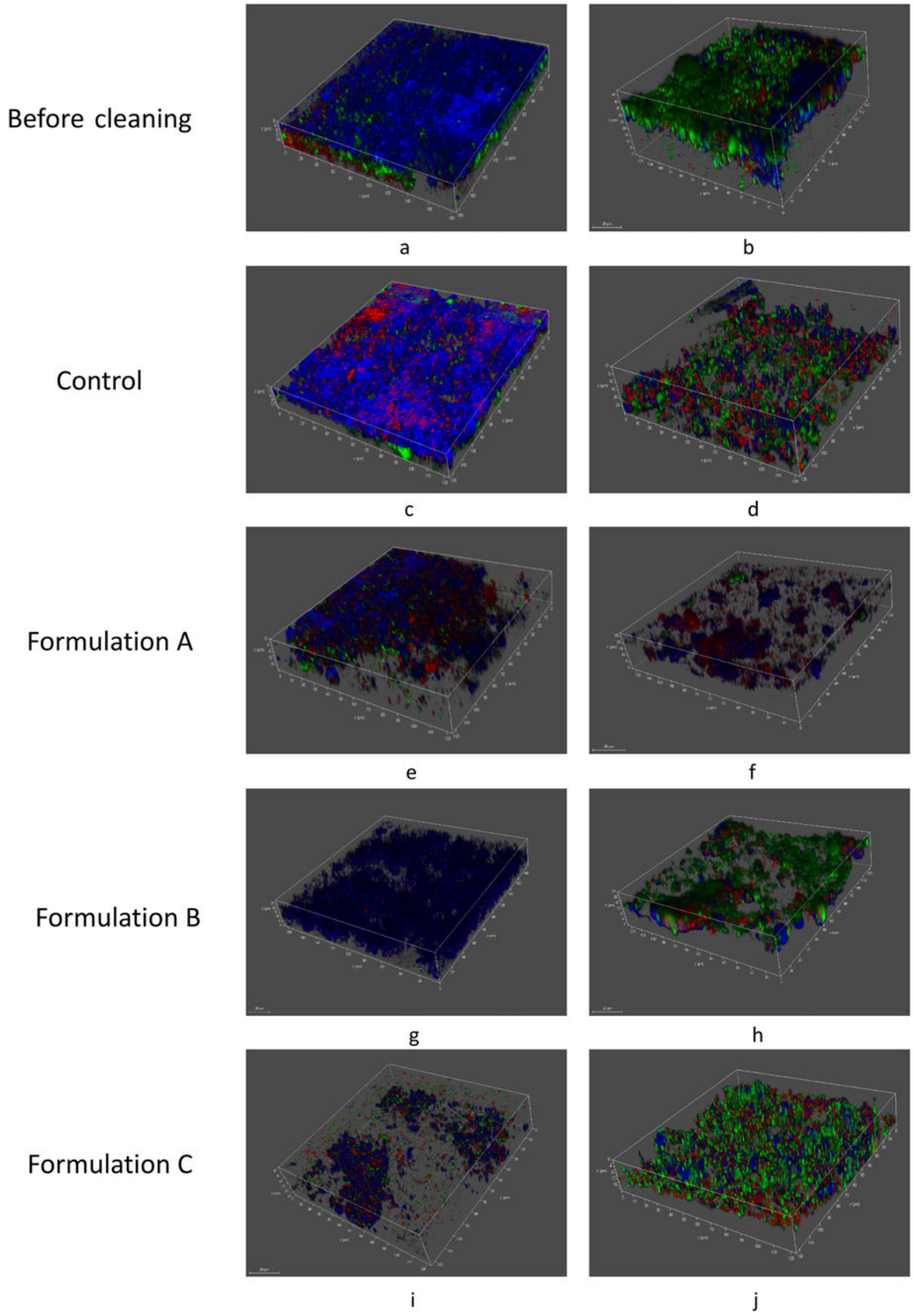

Figure 3. CLSM images of the effect of different enzymatic formulations on 25-day-old biofilms grown on SS and polyethylene (PET) surface coupons. (a) SS before cleaning, (b) PET before cleaning, (c) control on SS, (d) control on PET, (e) formulation A on SS, (f) formulation A on PET, (g) formulation B on SS, (h) formulation B on PET, (i) formulation C on SS and (j) formulation C on PET. Blue represents EPS-polymers, green live bacteria and red dead bacteria.

Table 4. Average work of adhesion for the surfaces of interest.

Surfaces	Average work of adhesion (mN m^{-1})
Stainless steel	119.8
PET	8.7

B and control treatments (ANOVA, $p < 0.05$, Table 1, Figure 2). After disinfection, EPS reduction slightly increased in A (up to 96%) and also occurred in C (61.4%) and the EPS content was significantly different between the three enzymatic treatments ($p < 0.05$) and also different form the control treatment.

Biovolume, biofilm surface area and biofilm thickness

Prior to any cleaning application, CLSM images showed that both surfaces were covered with biofilm and that the biofilm grown on PET tended to be thicker (46.4 µm on average) compared to SS (32.5 µm on average) (Table 3, Figure 3). It is believed that this phenomenon can be explained as the two substrata show significantly different values of average work of adhesion (Table 4). In the case of PET surfaces, it is thought that the bacteria would adhere more easily to already formed aggregates than to the clean surface, creating biofilm structures that would grow more in height than in surface area. On the other hand, on SS surfaces the higher work of adhesion (Table 4) facilitates the initial attachment of microbes throughout the surface rather than creating biofilms with increased thickness. In the presence of all three enzymes amylase-protease-lipase (formulation A) a distinct deformation of the biofilm structure was observed on both substrata (biofilm thickness, 20.1 µm for SS and 25.9 µm for PET), while the formulation containing amylase-protease (B) was not that effective and the formulation containing amylase-lipase (C), had a better effect on biofilm removal from SS than from PET (Table 3, Figure 3). Furthermore, it was observed that the synergy of all three enzymes (formulation A) had a significant effect on the viability of bacteria on both surfaces (Figure 3), a result that is in agreement with the observations from the flow cytometry data (Table 2, Figure 2).

After the disinfection step (Figure 4) the biofilm that was treated with the control formulation remained intact with dead bacteria (red colour) only occurring in the outer regions of the EPS matrix. In contrast, for the surfaces that had been cleaned using the combination of the three enzymes (formulation A) a significant reduction in biofilm was observed after the disinfection step on both surfaces and especially for SS, the majority of the biomass assemblies had been removed from the substratum (Figure 4). Interestingly, the enzymatic formulation that contained amylase-lipase (C) also proved to be effective, as although the biomass was not completely removed the majority of the bacteria appeared to be dead (Figure 4). Finally, the formulation containing amylase-protease (B) was the least effective as only a very small portion of the biofilm was either removed or deactivated (Figure 4).

Biovolume and the biofilm surface area were considerably reduced during the cleaning but the reduction differed depending on the enzymatic treatments and surface (Figure 5). For biofilms on the SS, the biovolume was lowest on those cleaned by enzymatic formulations A and B and also decreased in C treatment after disinfection (Figure 5). This resulted in 87.6, 71.9 and 86% biovolume reduction effectiveness on average, for A, B and C respectively after disinfection (Figure 6). For biofilms grown on SS, the biovolume was significantly reduced in formulations A and C, while for biofilms grown on PET, cleaning with formulations B and C (containing two enzymes) significantly reduced the biovolume by 21.8% and 49.5% respectively, while formulation A (containing three enzymes) significantly reduced the biovolume by 80.2% (Figure 6). Differences between treatments after disinfection were the same as those observed after enzymatic cleaning in all cases (Table 3, Figure 4). Similarly, biofilm surface coverage was significantly reduced in formulations A and C, and especially on the SS surfaces (39.2% and 58.0% effectiveness on average for A and C respectively), reaching an effectiveness in cleaning of 87.5% and 80.5% respectively for A and C after the disinfection step (Figure 6).

The biofilm surface coverage data depended on the average biomass thickness and, although in some cases the total biovolume was clearly reduced during the cleaning process, the covered surface area did not change significantly. For example, PET surfaces that were cleaned using amylase-protease (B) showed a lower percentage of covered surface area than those cleaned with amylase-lipase (C); however, the biovolume values were higher (Figure 5). Thus, as expected, they also had higher average biomass thicknesses than those that were cleaned with formulation C (Table 3). Consequently, the effectiveness of cleaning for the same surface would differ for the biovolume and the covered surface area data. A similar case was observed for SS surfaces cleaned with amylase-protease (B), where the effectiveness of cleaning from the biovolume data was 3 times larger than that from the covered surface area (Figure 6).

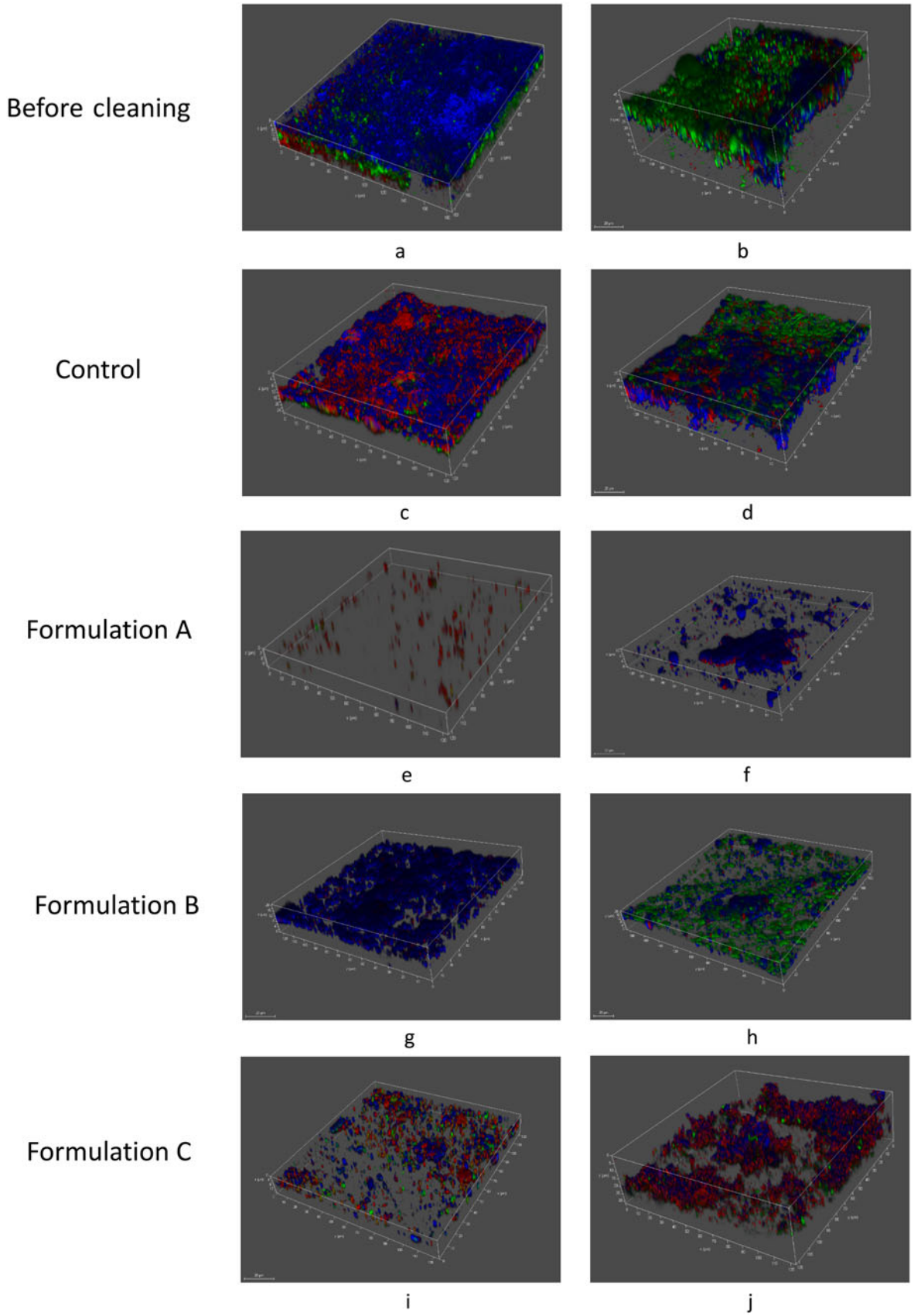

Figure 4. CLSM images of the effect the disinfection step after cleaning with different enzymatic formulations on 25-day-old biofilms grown on SS and polyethylene (PET) surface coupons. (a) SS before cleaning, (b) PET before cleaning, (c) control on SS, (d) control on PET, (e) formulation A on SS, (f) formulation A on PET, (g) formulation B on SS, (h) formulation B on PET (i) formulation C on SS and (j) formulation C on PET. Blue represents EPS-polymers, green live bacteria and red dead bacteria.

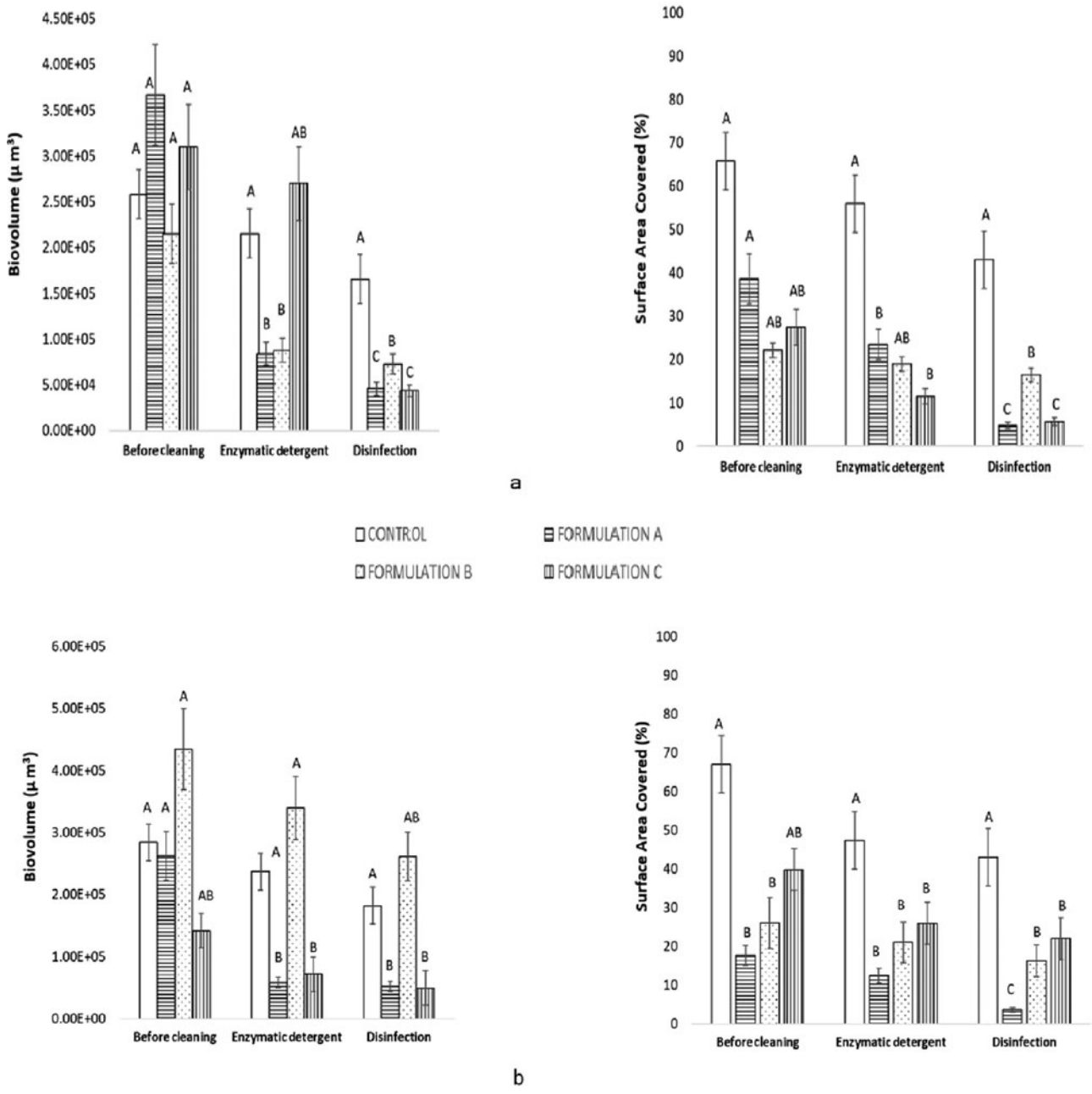

Figure 5. Biofilm biovolume (µm^3) and the percentage of surface area covered by biofilm during the enzymatic cleaning with the different enzymatic formulations and after the disinfection step for (a) SS and (b) polyethylene (PET) surfaces. Capital letters indicate statistically significant different groups at each cleaning step (Tukey's test, $p < 0.05$).

Biofilm structure on different artificial substrata

The biofilm structure was investigated using SEM and a distinct difference was observed in geometry between the SS and the PET substrata (Figure 7). On SS the biofilm formed ring-shaped structures with a diameter of 6-7µm, whilst on PET the EPS created an arbitrary network of organic compounds where bacteria were attached. In addition, the microbial biofilm was composed of bacteria of different shapes and sizes that along with the EPS components formed small and large aggregates on both surfaces. In this study the aim was to evaluate the cleaning efficacy of the enzymes on a real case mixed-microbial biofilm and therefore the analysis was limited to SEM analysis. Further characterisation by gene sequencing would add to future investigations.

Similar to the previous analyses, the effect of different enzymatic formulations, A, B and C on SS and PET coupons was studied (Figure 8). In the first step of the cleaning with amylase-protease-lipase (A), a distinct disruption of the biofilm structure was

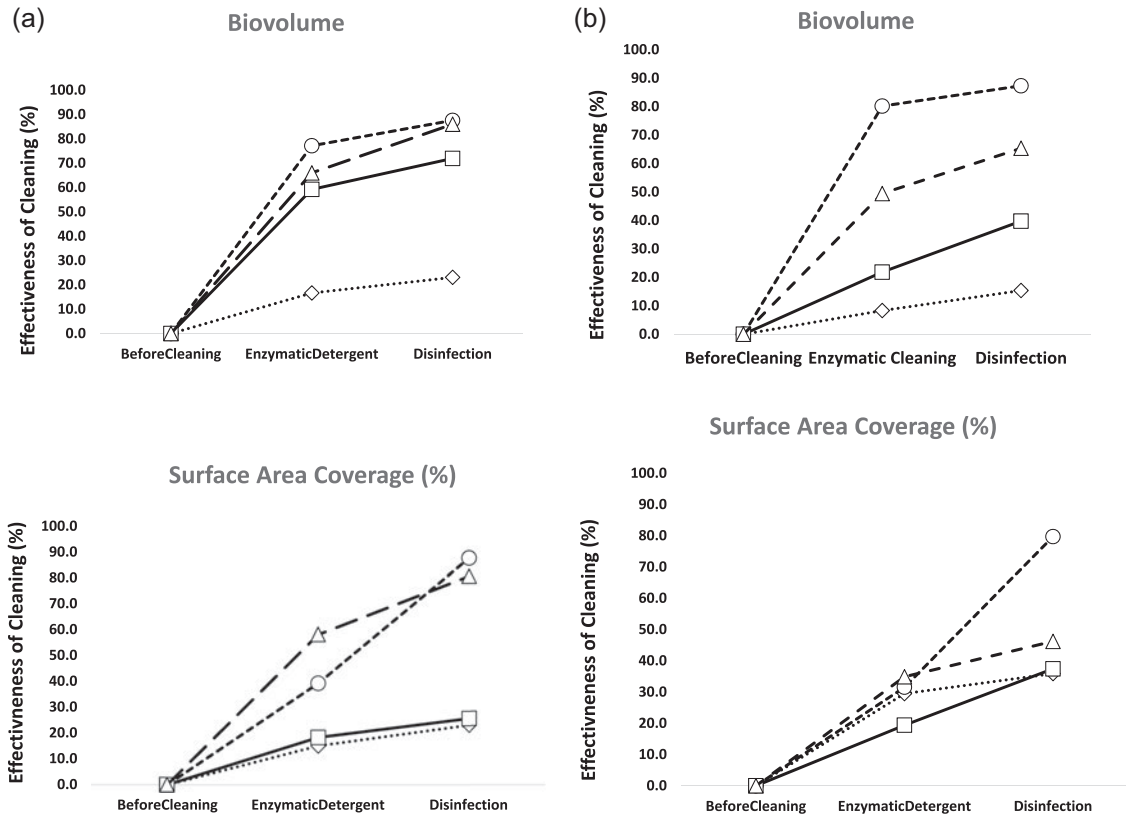

Figure 6. Effectiveness of cleaning (%) calculated from the data obtained for the biovolume and the percentage of surface area covered by biofilm during the enzymatic cleaning with the different enzymatic formulations and after the disinfection step for (a) SS surfaces and (b) polyethylene (PET) surfaces.

observed on both surfaces. It was evident that the combination of the three enzymes and the surfactants caused the decomposition of the EPS components and spherical assemblies of biomass were formed (Figure 8e and f). Formulations containing amylase-protease (B) and amylase-lipase (C) did not change the biofilm structure in the same way as formulation A, as there was no sign of spherical assemblies (Figure 8g–j). After the disinfection step (Figure 9), a significant reduction in organic compounds was observed on both surfaces, although on SS it seemed that most of the biofilm had been removed (Figure 9e and f). On the contrary, the formulations containing amylase-protease (B) and amylase-lipase (C) respectively appeared to be less effective than formulation A, as even after the disinfection step a substantial amount of biomass remained on both surfaces (Figure 9g–j). It could be concluded that formulation A, containing amylase-protease-lipase was the most effective in biofilm cleaning.

Discussion

Biofilms are communities of living microorganisms that can adapt and change their structure according to the environmental conditions, a feature that make them unpredictable and challenging to study. Further, biofilm growth is of great concern for various sectors, like the food industry, where day-to-day cleaning with common chemicals is not always efficient for biofilm control. Thus, enzymes like protease and α-amylase, have gained attention as alternative agents that could destroy the EPS matrix and attack bacterial cells (Vickery et al. 2004; Xavier et al. 2005; Lindsay and Holy 2006; Walker et al. 2007; Galié et al. 2018). Moreover, in a study by Kiran et al. (2014) it was suggested that lipases were between 90% and 95% effective in biofilm destruction and, hence, protease, amylase and lipase were chosen in this study as the enzymes of interest against a mixed-microbial biofilm obtained from a meat packaging process line.

In all, it was observed that enzymes were highly effective in biofilm cleaning and that the synergistic effect of all enzymes was essential for the most complete deformation of the biofilm structure and consequently a better disinfection of the surface. Formulation A, containing all three enzymes, caused the biggest reduction in all the parameters studied, ie biovolume, surface coverage, bacterial viability

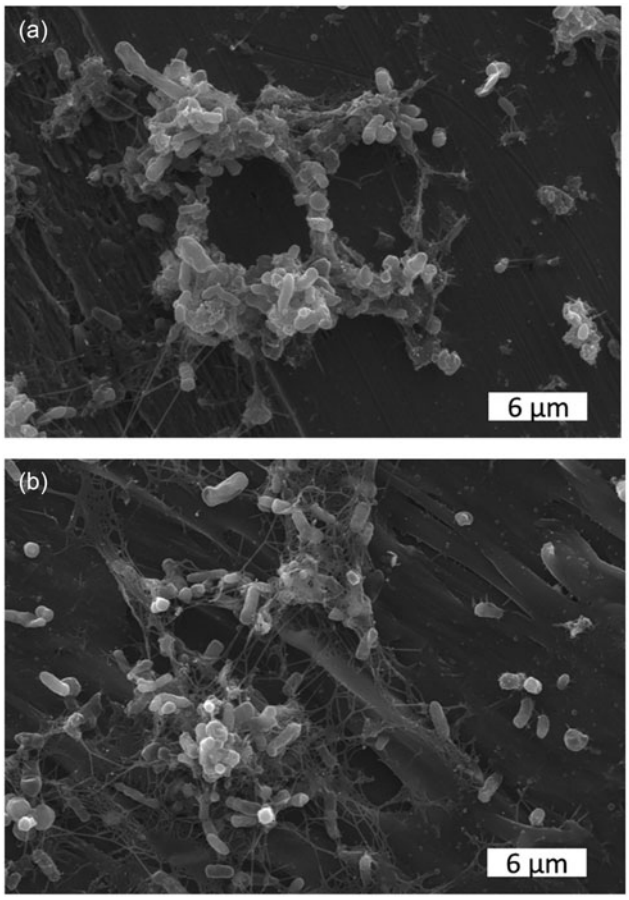

Figure 7. SEM images of 25-day-old biofilms grown on (a) SS and (b) and polyethylene (PET) surface coupons before cleaning.

and the polysaccharide content in the biofilm. It is believed that the combination of the three enzymes weakened the adhesive and cohesive forces of the organic compounds of the EPS and removed the biofilm from the surfaces more effectively than the other two formulations that were missing the enzymes lipase (formulation B) and protease (formulation C) respectively. As reported by Flemming et al. (2007), an increasing number of components have been identified in EPS, but many are yet to be identified and information about their localization and stability is not well known. For proteins, enzymatic degradation causes hydrolysis of the protein molecules and the resulting smaller molecules can be transported through the cell membranes and be metabolized (Molobela et al. 2010). Furthermore, it was suggested by Lequette at al. (2010) that a combination of enzymes targeting several components of the EPS, surfactants, dispersing and chelating agents would be an efficient alternative to chemical cleaning agents. It is thus believed that the efficiency of formulation A that contained all three enzymes, amylase-protease-lipase, may be due to the broad-spectrum activity in degrading a variety of EPS compounds.

The fact that the least efficient agent was formulation B, containing amylase and protease, is in contradiction with the literature where protease has been widely studied and proved many times to be the most effective enzyme in biofilm cleaning (Vickery et al. 2004; Walker et al. 2007; Lequette et al. 2010; Molobela et al. 2010). However, these studies focused on single species bacterial biofilms, whereas in this work the effectiveness of enzymatic cleaning was studied against a mixed-microbial biofilm. Additionally, Donlan (2002) indicated that biofilm EPS may be hydrophilic or hydrophobic depending on its structural components, which are mostly polysaccharides and proteins, followed by lipids, and DNA (Sutherland 2001; Czaczyk and Myszka 2007; Flemming et al. 2007; 2016). Consequently, it was expected that amylase, which degrades polysaccharides, and protease, which degrades proteins (Molobela et al. 2010), would be the most effective enzymes against biofilm EPS. In this case, it was shown that the presence of lipase in the enzymatic formulations A and C was decisive in biofilm deformation and subsequently better disinfection as shown by the reduction in biovolume and bacterial viability. It is thus believed that the stereochemistry of the polysaccharides, proteins and lipids in the EPS biomass, play a crucial role in the cohesive strength of their structure and, consequently, their decomposition by the appropriate enzymes. The results suggest that the complex chemistry and the 3-D structure of the matrix may need for their removal a combination of enzymes that will target selectively the different EPS compounds. It is also postulated that despite lipids being present in a much lower amount than polysaccharides and proteins within the matrix, they may play a key structural role.

When comparing the two material surfaces, it was initially observed that the biofilm structure was different. On SS, the biofilm was thinner whilst the biofilm on PET showed greater bacterial viability and increased biovolume. However, both biofilms had a similar surface area and EPS-polysaccharide content. Moreover, in terms of different cleaning performance, it was shown that the SS was cleaned more successfully than PET. It is thought that the increased biovolume and average biomass thickness on PET surfaces compared to SS played an important role in the difference in their cleaning efficacy. Moreover, the higher bacterial viability that was observed on PET

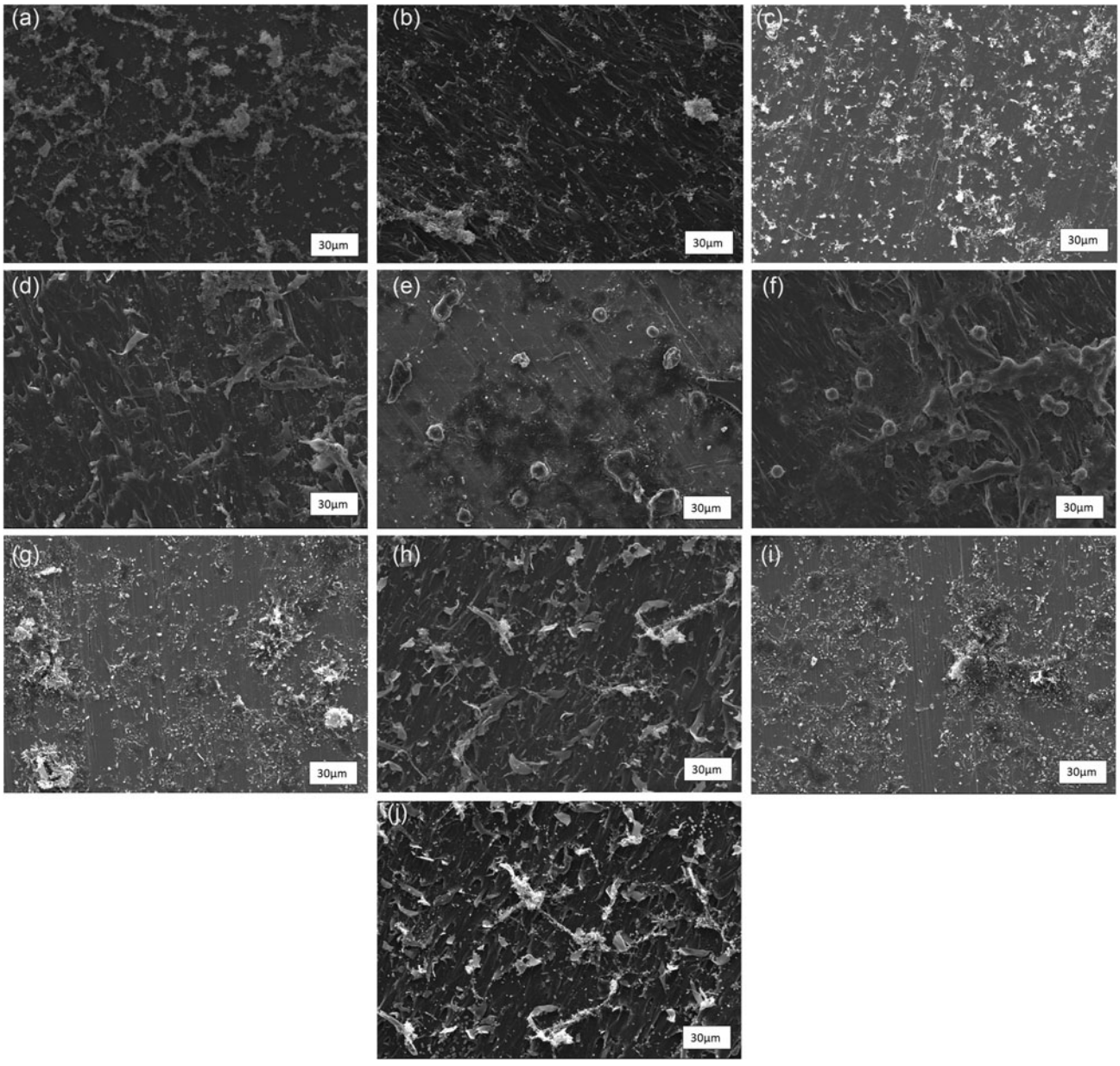

Figure 8. SEM images of the effect of different enzymatic formulations on 25-day-old biofilms grown on SS and polyethylene (PET) surface coupons. (a) SS before cleaning, (b) PET before cleaning, (c) control on SS, (d) control on PET, (e) formulation A on SS, (f) formulation A on PET, (g) formulation B on SS, (h) formulation B on PET, (i) formulation C on SS and (j) formulation C on PET.

might have aided the stronger attachment of the bacteria to the surfaces, which lead to the reduced cleanability of the surface. As mentioned in the literature, it is believed that this distinct contrast between the two surfaces is caused by the variation in their physico-chemical properties, such as their roughness, chemical composition and surface energy which affect the initial biofilm structure and hence, determines their cleanability (Fletcher and Loeb 1976; Detry et al. 2007, 2010). Surface energy, otherwise known as work of adhesion, is one of the parameters believed to play a key role in biofilm adhesion (Katsikogianni and Missirlis 2004), as bacteria have a tendency to grow on substrata that have similar surface energy (Detry et al. 2010). Furthermore, the different cleaning performance of the three formulations on PET surfaces against polysaccharide reduction was not expected, as all the formulations contained the same concentration of amylase and surfactants. The results suggest that the physical chemistry of the interfaces between the surface, the surfactants and the enzymes in the formulations might have played a significant role in biofilm cleaning. In this study, the hydrophobicity/hydrophilicity of the biofilm, the enzymatic detergent

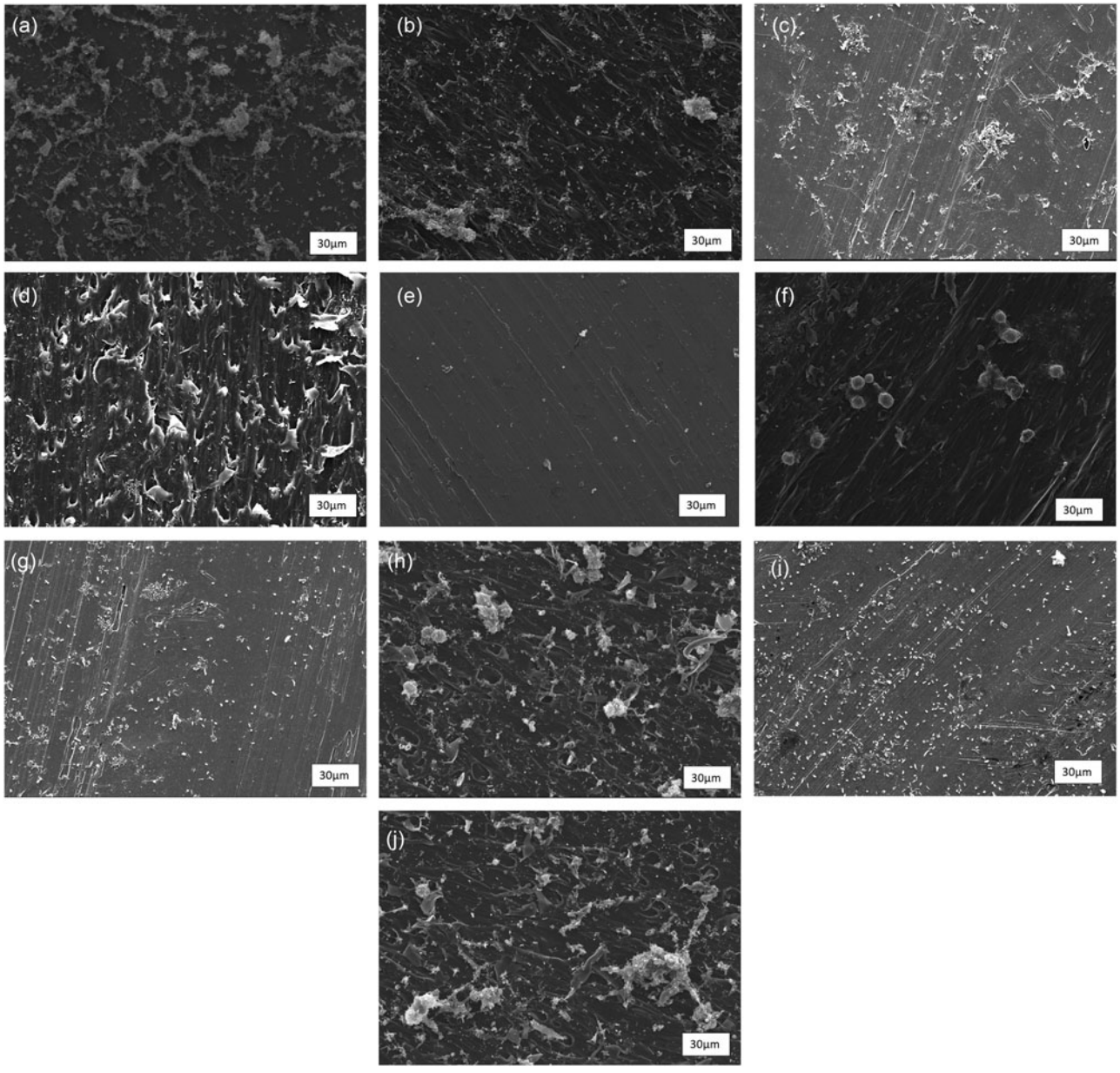

Figure 9. SEM images of the effect of the disinfection step after cleaning with different enzymatic formulations on 25-day-old biofilms grown on SS and polyethylene (PET) surface coupons. (a) SS before cleaning, (b) PET before cleaning, (c) control – surfactants without enzymes on SS, (d) control – surfactants without enzymes on PET, (e) amylase/lipase/protease on SS, (f) amylase/lipase/protease on PET, (g) amylase/protease on SS, (h) amylase/protease on PET, (i) amylase/lipase on SS and (j) amylase/lipase on PET.

and the material surface, was not specifically analysed. However, measuring the hydrophobicity/hydrophilicity of the interfaces involved might work as a useful tool in improving the biofilm cleaning process, as it is important to understand the phenomena that occur on the interfaces between the biofilm, the cleaning agent and the material surface.

Moreover, it was confirmed that the disinfection process was relevant to the final cleaning of the surfaces, as, although most reduction occurred due to the enzymatic cleaning, there was further biofilm elimination between the enzymatic cleaning and the disinfection step as clearly shown by the biovolume and surface coverage results. Finally, from the biovolume and biofilm surface area results, it was observed that the disinfection process was not effective without the initial enzymatic cleaning (control treatment), which is another indication that the presence of the enzymes was responsible for disrupting the structure of the EPS, which made the bacteria more vulnerable to the disinfectant used in the second step of cleaning.

Conclusions

The microscopic observation of changes in biofilm structure using SEM and confocal analyses indicated that enzymes were very effective in biofilm removal, especially on SS surfaces. It was observed that the combination of enzymes was more efficient than formulations based in a single enzyme regardless of surfaces (SS and PET). The treatment with a formulation combining amylase, protease and lipase, effectively decreased the total biofilm mass, the bacterial viability and the polysaccharide content of the biofilm. Moreover, it was observed that the surfaces differed in initial biofilm growth and this needs to be considered in consequent different cleaning patterns.

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Disclosure statement

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