**Atmospheric pressure cold plasma anti-biofilm coatings for 3D printed food tools**

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**Abstract**

Increasing adoption of 3D printing in the daily life and, specifically, the food field (kitchen tools and food contact containers) is associated with potential health risks when printed tools are made by specialized centers and designed for disabled patients, who cannot re-print them with the necessary periodicity. The characteristic pattern of 3D printing promotes bacterial adhesion and biofilm formation. In this work acrylic acid (AcAc) and tetraethyl orthosilicate (TEOS) coatings applied by plasma-polymerization have been developed to reduce biofilm formation by *Pseudomonas aeruginosa*, *Escherichia coli* and *Listeria monocytogenes* on 3D printed PLA materials. Chemical (generation of a hydration layer) and morphological (a decrease in distance between peaks) modifications provoked by plasma-polymerized treatments could explain the reduction in bacterial attachment and biofilm formation. AcAc coatings were more effective than TEOS coatings, and showed up to a 47.7% (*L. monocytogenes*), 50.4% (*P. aeruginosa*) and 64.1% (*E. coli*) relative biofilm production, when compared with untreated samples.

**Industrial relevance text**

Nowadays, products manufactured by 3D printing technologies are constantly growing; resulting in a greater flexibility and customization in the designs and a reduction of production costs. One of the main applications of 3D printed tools is the food industry, specifically, kitchen tools and containers for disabled people. There are great difficulties when it comes to disinfecting these types of parts. So, the health risks associated with the use of 3D printed tools for food contact applications should be attended. Our study demonstrated that acrylic acid plasma coatings are suitable for decreasing the amount of biofilm generated by different bacteria over 3D printed poly-lactic acid (PLA) substrates. Plasma coatings make it possible to address food-related health issues through the generation of safe tools manufactured by 3D printing. The mitigation of those issues will allow the food industry to safely employ a technology as versatile as 3D printing.

**Keywords:** 3D printing; Food industry; Atmospheric Pressure Plasma Jet; Plasma-polymerization; Antibiofilm coatings; Hydration layer

# INTRODUCTION

Additive manufacturing is capable of producing complex geometries that cannot be manufactured by other means, reducing times and production costs. Some entrepreneurs which have utilized 3D printing to create objects include aeronautic, architecture, automotive, dentistry, fashion, food, medicine, pharmaceutical and robotic industries (Gardan, 2016).

3D printing has increased over the last decade, with increases starting to be significant in 2004, when the industry grew from 10% in 2003 to 25% in 2004 (Wohlers Associates, 2017). This sharp improvement was driven by the RepRap Project, which allowed adopting an open source equipment and the development of cheap 3D printers (S. H. Chang, 2016; Domínguez-Robles et al., 2019). In 2014, another huge increase in the 3D printing field occurred due to the expiry of Stratasys patents, which caused a rise from 20% in 2013 to 33% in 2014 (Gardan, 2016; Wohlers Associates, 2017).

The decrease in prices along the years and the recent emergence of desktop 3D printers have provoked an interest in industrial and amateur 3D printing (Campbell, Williams, Ivanova, & Garrett, 2011; Chiulan, Frone, Brandabur, & Panaitescu, 2018).

The most widely used material in 3D printing is plastic. In particular, poly-lactic acid (PLA) is nowadays the most popular material, with annual growth rates of 19.5% (Baran, Erbil, Baran, & Erbil, 2019; Chiulan et al., 2018; Domínguez-Robles et al., 2019; Gardan, 2016; Zhang, Seong, Nguyen, & Byun, 2016). PLA is a thermoplastic obtained from corn and sugar cane and, therefore, it is a recyclable, biodegradable and biocompatible polymer. There are no complications during PLA impression due to its low thermal expansion coefficient and its non-adherent properties to 3D printed surfaces. By using PLA, high surface quality and precision pieces can be obtained. Besides, toxicity levels associated with PLA polymers during 3D printing processes are lower than those derived from fossil fuels (like acrylonitrile butadiene styrene -ABS) (Baran et al., 2019; Chiulan et al., 2018; Domínguez-Robles et al., 2019). Since the 1970s, PLA is approved by the U.S Food and Drug Administration (FDA) for food and pharmaceutical applications and, indeed, due to all of these properties PLA has ideal condition to be used in many biomedical and food-related applications (Baran et al., 2019; Chiulan et al., 2018; Guo, Jin, & Yang, 2014; Jin, 2010).

3D printing applications for food contact supply personalized tools and utensils with ergonomic improvements, tailored for instance to patients with hand mobility disabilities. People with burned hands or amputations can benefit from tools that are designed to their specific needs. There is also a necessity to manufacture specific utensils for people with Parkinson disease (e.g. with weighted handles to reduce tremors, curved handles to minimize wrist movement, pieces with hand straps or finger holes, or even deeper bowls on spoons to avoid spills) (Lipton, Witzleben, Green, Ryan, & Lipson, 2015). Thus, cutlery is specifically designed for each patient, impressed at hospitals and health centers and then delivered to the end users for daily applications.

However, in spite of these advantages, there are two important disadvantages to the use of 3D printed pieces in food contact applications. The first one is related to the chemical composition of PLA. Despite having a vegetal origin and the approbation of the FDA and the EU under “Commission Regulation (EU) No. 10/2011 on plastic materials and articles intended to come into contact with food”, as well as several commercial applications in the food field (e.g. Voltivo, 3DFilaPrint, Filaments.ca, Formfutura and Innofil3D), once the filament is casted, in the event of using Fused Filament Fabrication (FFF) technology, potential toxic transformations in its composition could occur (Oskui et al., 2016; Zhu, Friedrich, Nugegoda, Kaslin, & Wlodkowic, 2015). The main chemical pollutants generated in 3D printing using PLA are lactide, methyl methacrylate and aldehydes like formaldehyde and acetaldehyde (Azimi, Zhao, Pouzet, Crain, & Stephens, 2016; Y. Kim et al., 2015; Steinle, 2016). These modifications discourage the use of these pieces for food contact applications. For that reason, the FDA and EU No. 10/2011 approval only applies to natural PLA filament, before being impressed (3DFilaPrint, 2015; “DECLARATION OF CONFORMITY for Food Contact Products, PHILIPS,” 2011; Filaments.ca, 2020; Voltivo, 2020). In fact, suppliers like Ultimaker and NatureWorks inform that their filaments are not adequate for food contact applications (“Technical Data Sheet PLA Innofil3D,” 2017, *Technical Data Sheet PLA NatureWorks*, n.d., *Technical data sheet PLA Ultimaker*, 2018). Currently, some researches on 3D printed parts are being focused on the characterization of chemical toxicity and its mitigation (Alifui-Segbaya, Varma, Lieschke, & George, 2017; Rindelaub, Baird, Lindner, & Strantz, 2019). The second disadvantage is associated with the surface pattern of tools printed using the FFF technology, due to the deposition of material in successive rows. Once the piece is finished, some grooves are present on its surface, what could facilitate the colonization and proliferation of different microorganism (Formalabs, 2019; Lipton et al., 2015). Health risks linked to microbial contamination are greater when 3D printed products are recurrently in contact with food products or with raw foods, such as eggs, meat or fish. This problem is even more relevant in PLA 3D printed materials, due to their vegetal origin. In fact, bacteria can metabolize and degrade PLA objects. In addition to this, the fusion temperature of PLA is low (of around 60ºC) and as a consequence PLA 3D printed pieces are not suitable for being cleaned in wet and high temperature cleaning systems because they could deform and break the 3D printed object. In order to address this issue, 3D printed tools are sometimes commercialized coated to avoid bacterial multiplication. Nevertheless, the coatings might deteriorate and peel off a long time, exposing the original surface to new possible contaminations (Formalabs, 2019). In the report by Sandler et al. (2014), nitrofurantoin (NF) was added to PLA 3D printed pieces with the aim to reduce the adhesion of *Staphylococcus aureus*. A decrease of 24.6% in biofilm formation with regard to control samples was obtained with NF. (Domínguez-Robles et al., 2019) suggested the combination of lignin with PLA to form filaments which can be used for health applications. The antimicrobial capacities of the 3D printed materials were evaluated through the study of *S. aureus* adhesion. These authors showed that lignin did not provide antimicrobial activity to the printed materials, but the mechanical properties of the new material showed a lower mechanical resistance than pure PLA.

Atmospheric Pressure Plasma Jet (APPJ) non-thermal plasma technology is an innovative method of applying functional coatings with the capability of causing physical, chemical and morphological modifications on materials and surfaces without altering their mechanical properties. Plasma-polymerization coatings created with the APPJ technology offer some advantages over those applied with traditional methods: it is a clean and solvent-free process, it does not need to work under vacuum, it is cheap, with an easy scalability for industrial applications, it needs mild substrate temperatures and it is versatile, allowing the creation of different coatings by controlling different processing parameters (e.g. passes, power, speed…) (Bashir, Rees, & Zimmerman, 2013; Bismarck, Brostow, Chiu, Hagg Lobland, & Ho, 2008; Borcia & Brown, 2007; Merche, Vandencasteele, & Reniers, 2012). There are some studies that have used coatings applied with the APPJ technology with the aim of reducing biofilm formation by diverse bacteria. Sardella et al. (E. Sardella et al., 2005; E. Sardella, Gristina, Senesi, D’Agostino, & Favia, 2004) obtained cell-repulsive zones by applying cold plasma Polyethylene Oxide-like (PEO-like) coatings mixed with cell adhesive compounds derived from acrylic acid. Stallard et al. (Stallard, McDonnell, Onayemi, O’Gara, & Dowling, 2012) applied tetraethyl orthosilicate (TEOS) and other siloxane coatings using plasma-polymerization (APPJ system) over titanium coupons with the aim of analyzing their effects on protein adsorption and bacterial attachment. Other works have used this technology successfully in 3D printed objects since its temperature is not dangerous for the piece integrity (Baran et al., 2019).

At household level, and due to the low cost of printers, the most reasonable solution to control the microbial contamination of used 3D printed pieces would be their re-printing after a limited number of uses. Nonetheless, in 3D tools designed for disabled people, anti-biofilm coatings are promising solutions, since these users may not have access to printing technology and, consequently, they could not routinely re-print these utensils as necessary.

This research study intends to mitigate microbial contamination of PLA 3D printed tools by applying anti-biofilm plasma-polymerization coatings using an APPJ system. The effectivity of the coatings was assessed by measuring biofilm formation by *Pseudomonas aeruginosa*, *Escherichia coli* and *Listeria monocytogenes*, which all have a huge interest in the clinical and food fields. The influence of the process parameters such as, number of passes (1, 2, 6 and 12) and type of precursor (acrylic acid and TEOS) when depositing coatings on PLA Petri dishes was characterized from a morphological, physico-chemical and microbiological point of view. For these characterizations, atomic force microscopy (AFM), scanning electron microscopy (SEM), X-ray photoelectron spectroscopy (XPS), water contact angle (WCA) measurements and crystal violet biofilm formation assays for biofilm quantification were used.

# MATERIALS AND METHODS

## Materials

The filament used for the printing of PLA Petri dishes was White PLA RS PRO with 1.75 mm diameter (RS PRO, UK). Acrylic acid tech. 90% (AcAc; (C3H4O2)) (Alfa-Aesar, USA) and tetraethyl orthosilicate 98% (TEOS; Si(OC2H5)4) (Acros-Organics, USA) were used as plasma-polymerized coating precursors. To prevent from the environmental contamination of PLA Petri dishes, these were covered after plasma treatment with 35 mm diameter sterilized polystyrene (PS) lids (Thermo-Scientific, USA).

## 3D Printing

The 3D printer used was “Original Prusa i3 MK3” (Prusa3D, Czech Republic). The extruder temperature was 215ºC and the print bed temperature was 60ºC. PLA Petri dishes were printed with 0.2 mm thickness, using a 0.4 mm nozzle and 80% fill. With the view to facilitate the anti-biofilm capacity analysis of plasma-polymerized coatings, these were applied on 3D printed PLA Petri dishes. PLA Petri dishes showed a 31 mm inside diameter, 35 mm outside diameter and 15 mm high (Fig. 1[a]).

## Atmospheric Pressure Plasma Treatment

Once printed, different plasma-polymerization treatments were applied on the PLA Petri dishes (Table 1). The APPJ system used in the present study was PlasmaSpot500® (MPG, Luxemburg). It consists of two disk electrodes (one external connected to a high voltage source and the other one internal and grounded); between them there is an Al2O3 dielectric tube (Fig. 1[b-d]). The plasma flow gas was 80 slm and the precursor flow was 1.5 slm, both using nitrogen as a carrier gas (99.99%). Plasma power was 360 W. As shown in Table 1, three treatments were carried out using only plasma and eight treatments using coatings deposited with different passes (1, 2, 6 and 12) and precursor liquids (AcAc and TEOS). All treatments were applied employing a pattern scan in which the gun speed was 100 mm/s, the pitch was 2 mm and the gap between the plasma discharge and PLA Petri dishes was 10 mm. Each experiment was done in quadruplicate.

## Morphological and Chemical characterization

A Multimode AFM Bruker (Bruker Corporation, USA) with Nanoscope V Controller was used in order to analyze the surface topography of the samples. An area of 40 μm × 40 μm was studied with a frequency of 50 Hz. Three areas per sample were analyzed. The values of roughness (Ra) presented are the average ones and were determined by using NanoScope Analysis 1.4 (Bruker Corporation, USA) software. The surface morphology was examined by scanning electron microscopy (SEM) by using a microscope HITACHI S-2400 with 18 kV power. PLA samples were previously coated with gold and palladium to make them conductive.

For studying the surface profiles of the samples, as a first step, a smoothing technique was applied to the AFM profile in order to attenuate the signal distortion and to avoid false peak detections. Then, to detect true peaks and to neglect the peaks which were too small, each peak amplitude was compared to a threshold. Finally, the positions of the identified peaks were determined and the distance between peaks was averaged.

Chemical characterization of the coatings was achieved by X-ray photoelectron spectrometer (XPS) analyzes. X-ray photoelectron spectra were obtained using a Kratos AXIS Supra (Kratos Analytical, England) system with a hemispherical electrons analyzer and a Monochromatic AlKα X-ray source (120 W, 15 kV) operating at 1.33 x 10-7 Pa of residual pressure. Spectra were collected at 160 eV (general spectrums) and 20eV (high resolution spectrums). Binding energies were related to C1s signal for the adventitious carbon at 285 eV. Those results were deconvoluted by means of *PeakFit 4.12 (SPSS Inc.).* Each sample was analyzed in triplicate.

Due to the surface pattern of the printed samples (Fig. 1[e,f]) it was not possible to measure the water contact angle (WCA). For this reason, the same coatings were applied on a stainless steel surface and the sessile drop method (with 10 µL of distilled water) was used to measure the WCA.

## Bacterial strains and growth conditions

*Pseudomonas aeruginosa* PAO1, *Listeria monocytogenes* CECT911 and *Escherichia coli* CECT515 were used as reference strains to perform the microbiological and biofilm analysis. The strains were routinely grown onto Brain-Heart Infusion (BHI) agar (Pronadisa, Conda, Spain) incubated at 37ºC overnight. These agar plates were subsequently used for the regular culture and correct isolation of colonies for biofilm formation assays.

## Biofilm Determination

The crystal violet (CV) staining method (Peeters, Nelis, & Coenye, 2008) was followed to quantify the total biofilm biomass of each microorganism on PLA Petri dishes for each plasma treatment. Briefly, PLA Petri dishes were inoculated with an initial 106 CFU/mL bacterial inoculum prepared in 3 mL of Mueller Hinton (MH) Broth (Pronadisa, Conda, Spain) from cultures previously obtained after growing on BHI agar plates during 18 h. Then, the PLA Petri dishes were incubated at 37°C during 24 h. At the end of the incubation period, the medium was removed and the biofilm was washed with Phosphate Buffer Saline (PBS), and subsequently was fixed using methanol during 15 min at room temperature. After removing the methanol, PLA plates were dried during 20 minutes at room temperature. Then, 3 mL of CV solution (Sigma, final concentration 10% in PBS) were added and PLA plates were incubated during 10 minutes at room temperature. The excess of CV was removed under running water and PLA dishes were dried. Finally, the CV fixed to the biofilm biomass was resolubilized in 3 mL of 66% acetic acid, and incubated at room temperature during 1 h. Absorbance was measured at 570 nm using a plate reader (POLARstar Omega microplate reader, BMG Labtech). Four PLA Petri dishes were used per each treatment and microorganism, and three PLA Petri dishes without plasma coating were included as control plates in all assays.

# RESULTS AND DISCUSSION

## Morphological characterization (SEM and AFM)

Fig. 2 shows SEM and AFM images of PLA substrate without plasma treatment (Fig. 2[a]) and plasma treated substrates with 2, 6 and 12 passes (Fig. 2[b-d]). Untreated PLA (Fig. 2[a]) shows a lumpy surface with many grooves. During the first six passes (Fig. 2[b,c]), plasma treatments produce a slight superficial erosion that does not significantly modify the surface roughness (Ra: 408.8 nm, Fig. 3). However, plasma treatments with 12 passes (Fig. 2[d]) provoked a greater erosion and an increase in roughness (Ra: 494nm).

Fig. 4 illustrates SEM and AFM images of PLA coated with AcAc (Ac1p – Ac12p). During the first two passes (Fig. 4[a,b]) plasma-polymerized particles were deposited on PLA surface lumps and partially filled in its grooves. This double effect caused an increment in the surface roughness. Ac2p showed the highest roughness among the AcAc coated substrates (Ra: 531.5nm, Fig. 3). A further increase in the number of passes (Fig. 4[c,d]) caused a reduction in roughness, which showed values similar to those obtained for the original surface material. Indeed, Ac12p even showed lower roughness values than untreated PLA (Ra: 385.5nm, Fig. 3).

Fig. 5 shows SEM and AFM images of PLA substrates coated with TEOS (Te1p – Te12p). In the same way as with AcAc coatings, during the first two passes plasma-polymerized TEOS particles filled in the characteristic pits of the PLA surface. However, due to the different coating formation mechanisms, when 6 passes were applied (Fig. 5[c]) it was possible to identify the typical surface cracks due to the residual thermal stress produced on plasma-polymerized siloxane coatings (Sainz-García, Alba-Elías, Múgica-Vidal, & González-Marcos, 2017). In PLA coated with 12 passes (Fig. 5[d]) spherical particles coming from silicon dioxide aggrupation were observed (Sainz-García et al., 2017). Some of those particles were also visible on Te1p and Te2p (white circles in Fig. 5[a1,b1]). Under no circumstances, the different TEOS coating morphologies had an impact on the measured roughness (p>0.05) (Fig. 3).

2D AFM images and AFM profiles shown in Fig. 6 confirmed aspects previously discussed in relation to PLA substrates coated with AcAc. In AFM profiles illustrated in Fig. 6[a2,b2] it is possible to identify how particles deposited during the first pass changed the superficial pattern of untreated PLA, decreasing the distance between peaks. This distance decreased from 3.72±1.89 µm (untreated PLA) to 1.22±0.84 µm (Ac1p) (Fig. 6[a3,b3]). The distance between peaks on Ac2p is 1.70±1.22 µm; also fewer than untreated PLA (data not shown). When the number of passes was further increased, deposited particles filled in all pits and grooves and a smoother surface profile was obtained with a gradually increased distance between peaks, of up to 8.83±3.67 µm (Ac12p) (Fig. 6[c3,d3]).

In view of these results, it can be concluded that the morphology of coated PLA substrates (Fig. 4, Fig. 5) depends mainly on the coating applied and does not rely only on superficial modifications produced by the plasma flow (Fig. 2).

## Chemical characterization (XPS)

XPS analysis was used to determine the chemical composition of untreated and coated PLA samples. Table S1 (see Supplementary Material) indicates the functional groups and binding energies associated with peaks that correspond to the deconvolution of C1s high resolution spectra. Fig. 7 shows the atomic chemical composition of the samples according to the coating applied. Each treatment was analyzed in triplicate. Data shown in Fig. 7 are the average of these three measures. Fig. S1 (see Supplementary Material) illustrates deconvolutions of C1s spectra of untreated and coated PLA samples, with the objective of quantifying relative abundance of C-C/C-H, C-O y O-C=O groups and its possible relation to the coating anti-biofilm capacity. C-C/C-H, C-O and O-C=O relative abundances are also shown in Table S2 (see Supplementary Material). Both functional groups and binding energies were similar to those previously identified by other authors (Abourayana, Dobbyn, & Dowling, 2018; Jacobs et al., 2012; Wang et al., 2016; Zhao, Fina, Venturello, & Geobaldo, 2013).

In all coated samples, regardless of the number of passes and precursor used, an increase in the superficial oxidation degree, when compared with untreated samples, occurred (Fig. 7). This is due to effect of plasma energy, precursor employed and oxygen present in the atmosphere. The superficial oxidation degree of the samples depended on the type of precursor used. When AcAc was used, the chemical composition did not change, regardless of the number of passes applied (Fig. 7[a]). However, when TEOS was used (Fig. 7[b]), the oxidation degree increased with the number of passes. This different behavior can be due to the higher stability of the deposited molecules when AcAc is used. These molecules, mainly O-C=O groups, are more resistant to the successive passes of the plasma flow. It should be noted that Si abundance significantly increased when more than 2 passes of TEOS were applied. N was practically absent (<1%) for all coatings.

Fig. S1 (see Supplementary Material) illustrates C1s spectra deconvolution of the samples. These deconvolutions are formed by the three peaks previously defined in Table S1 (see Supplementary Material). An increment in oxygen functional groups (C-O, O-C=O) and decrease of carbon functional groups (C-C/C-H) occurred for Ac1p coatings. As previously reported, the oxidation degree significantly increased since the first pass and remained constant regardless of the number of passes applied. The same as for AcAc coatings, although to a lesser extent, in Te1p samples an increase in the abundance of oxygen functional groups (C-O, O-C=O) and a reduction in the amount of carbon functional groups (C-C/C-H) occurred. Nevertheless, a further increase in the number of TEOS coating passes provoked a reduction of both C-C/C-H, C-O and O-C=O functional groups, possibly due to an increment of Si-Si and Si-O functional groups. The specific atomic percentages of functional groups identified in C1s spectra deconvolution are shown in Table S2 (see Supplementary Material).

## Wettability

Wettability is a primordial aspect to evaluate the biological response such as the interaction of the bacteria and the substrate. The substrate wettability reflects the way of spreading of the liquid on its surface, which is associated with the intermolecular forces between the solid and liquid phases. This property is defined by the water contact angle (WCA); which is formed between the solid substrate and a tangent along the liquid-vapor interface where they meet. On hydrophilic surfaces (WCA<90º), the liquid molecules are strongly attracted to the solid surface; allowing the liquid drop to spread out on a greater area. Therefore, the water contact angle will be less on hydrophilic surfaces than on hydrophobic ones (Agrawal, Negi, Pradhan, Dash, & Samal, 2017). The morphological characteristics (Fig. 1[e,f]) of PLA printed dishes, typical from the FFF printing technology, make not possible the measurement of the WCA, because water drops are trapped in the grooves between adjacent filaments. Due to the impossibility of measuring the WCA in PLA dishes, other material was used as a template for the coatings deposition for wettability measurements. Many articles have analyzed the influence of surface roughness on substrate wettability (Wang & Zhang, 2020; Xia, Ni, & Xie, 2016). Although polystyrene (PS) is the material most commonly used to fabricate Petri dishes, stainless steel (SS) instead of PS was chosen to measure the WCA, due to the fact that SS has a roughness (Ra) of 106nm, closer than PS (Ra:4.9nm) to the PLA roughness (Ra:408.8nm). Besides, SS is the material most commonly used in the food industry (Dürr, 2007). Plasma-polymerized AcAc coatings generated a super-hydrophilic surface (<10º) on the SS substrate independently of the number of passes (data not shown). Fig. S2 (see Supplementary Material) shows WCA measurements of untreated and plasma-polymerized TEOS coated samples. SS wettability decreased when TEOS coatings were applied. Thus, the WCA of untreated SS was 89.5º, while for SS with TEOS it was <70º. When TEOS was applied with only one pass the WCA of the sample was 48.3º and then, the WCA slightly increased with the number of passes.

Results previously described on Section 3.2 could confirmed the hydrophilic character of all deposited coatings, and especially of AcAc coatings. Thus, an increase in the O/C ratio (Jordá-Vilaplana, Sánchez-Nácher, Fombuena, García-García, & Carbonell-Verdú, 2015; M. C. Kim & Masuoka, 2009; Lima Da Silva et al., 2018; Swilem et al., 2016) and specifically in the O-C=O functional group (Baran et al., 2019; Wang et al., 2016) were observed and support the wettability results.

## Biofilm Determination

The total biofilm biomass produced by each microorganism on coated PLA dishes was analyzed and compared with the biomass on control uncoated PLA plates without plasma treatment (used as a reference, with 100% relative biofilm production). Fig. 8 shows the relative biofilm production for each coated sample in comparison to the reference PLA plates. A relative biofilm production < 100% indicate a coating with potential anti-biofilm capacity, while relative biofilm production levels >100% indicate coatings with potential pro-biofilm capacity.

All coatings showed good anti-biofilm properties when they were applied with 1 pass (except Te1p for *P.aeruginosa* biofilms) and 2 passes. AcAc coatings were more effective than TEOS coatings and caused a greater decrease in biofilm production by the three pathogenic microorganisms. Generally, the anti-biofilm capacity of the coating increased as the number of passes decreased. Significant statistical differences (p<0.05) were found in biofilm formation by *P. aeruginosa* and *E. coli* according to the number of passes (2 and 6 passes). Finally, the Ac1p treatment can be considered the best coating, with a relative biofilm production of 47.7% for *L. monocytogenes*, 50.4% for *P. aeruginosa* and 64.1% for *E. coli*.

### Effect of coating chemistry

Several authors have shown a relationship between the reduction in bacterial adhesion (first step of biofilm formation) and the repulsion occurring as a consequence of the hydration layer created on hydrophilic surfaces. When a hydrophilic surface is immersed in a fluid, some attractive interactions between water molecules and functional groups from the surface of the substrate appear. As a result, water molecules tend to orientate to the surface and a hydration layer is generated. This layer causes a repulsive force on the sample surface resulting in a decrease in the bacterial adhesion and biofilm generation (Y. Chang, Cheng, Shih, Lee, & Lai, 2008; Díaz, Cortizo, Schilardi, de Saravia, & de Mele, 2007; Peng, Song, & Fort, 2006; Temmen, Ochedowski, Schleberger, Reichling, & Bollmann, 2014).

Taking into account the chemical characterization results above described under Section 3.2, it seems that the anti-biofilm character of most of the AcAc and TEOS coatings is result of the repulsion forces generated by its hydrophilic nature. As it is shown in Table S2 and Fig. S2 (see Supplementary Material), the increase in oxygen polar groups (C-O y O-C=O) induces an increase in surface hydrophilicity. As many authors have noted, O-C=O groups are associated with carboxyl groups (COOH) which have a strong hydrophilic character (Ba, Marmey, Anselme, Duncan, & Ponche, 2016; Ramkumar et al., 2017; Eloisa Sardella, Palumbo, Camporeale, & Favia, 2016; Shen et al., 2019; Zhao et al., 2013).

TEOS coatings have more O-C=O groups the lower the number of passes applied (see Supplementary Material, Table S2). This fact could explain, in most cases, the lower biofilm formation observed when 1 or 2 passes were applied. With regard to AcAc coatings, the number of passes did not provoke important chemical modifications in the surface (Fig. 7). For that reason, the different anti-biofilm capacity of these coatings (based on the number of passes) cannot be only explained by dissimilarities in chemical nature of surfaces. Therefore, it is necessary to consider the effect of the surface morphology on biofilm production.

### Effect of coating morphology

As indicated above, the biofilm reduction obtained with AcAc coatings depending on the number of passes used cannot be explained only by the generation of a hydration layer, since their chemical nature is the same whatever the number of passes applied. In this sense, the different biofilm production observed with different number of passes could be due to the distinct surface morphology of each AcAc coating.

Some authors have described how particular rough patterns are helpful to limit bacterial adhesion and, consequently, biofilm generation. In fact, in smooth surfaces bacteria tend to accumulate in large groups, which promotes biofilm formation. However, when the distance between peaks is lower than the bacterial dimensions, different situations occur which could reduce bacterial adhesion to the surface. Peaks separated distances smaller than bacterial dimensions may produce bacterial modifications (Díaz et al., 2007; Lorenzetti et al., 2015; Tripathy, Sen, Su, & Briscoe, 2017; Wu et al., 2018). They might even cause a rupture of the cell membrane and consequently the cell death (Elbourne, Crawford, & Ivanova, 2017; Li & Chen, 2016). Due to all the above, the shape and size of bacteria have to be taken into account to explain the possible bacterial adhesion reduction effects (Kargar, Chang, Khalili Hoseinabad, Pruden, & Ducker, 2016; Kelleher et al., 2016). The bacteria analyzed in the current study have a bacilli shape: *P. aeruginosa* (0.5-1 µm width and 2-4 µm length), *E. coli* (0.25-1 µm width and 2-3 µm length), *L. monocytogenes* (0.5-2 µm width and 0.5-4 µm length) (Jamshidi & Zeinali, 2019; Monteiro et al., 2015; Tripathy et al., 2017). Taking into consideration the dimension of the bacteria studied and the AcAc coatings morphology (Fig. 6), it is possible to justify the biofilm production level obtained for each coating according to the distance between the coated surface peaks.

With the aim of clarifying that point Fig. 9 illustrates a scheme of the possible interaction of bacteria with each different AcAc coating surface pattern shown in Fig. 6. Ac1p and Ac2p are the samples whose coatings produce the less biofilm. In Fig. 9[b] the possible reduction in bacterial adhesion is represented for Ac1p coatings. That decrease in biofilm formation occurred on coatings with 1.22±0.84 µm and 1.70±1.22 µm distance between peaks, respectively. These spaces between peaks are 2-3 times lower than the bacterial length and could reduce their adhesion for any of the reasons cited above. That phenomenon could be the cause why Ac1p and Ac2p coatings, despite having identical chemical nature than the rest of AcAc coatings, generate the lowest amount of biofilm for the bacteria studied.

# CONCLUSIONS

In the current study, different coatings were applied through plasma-polymerization with the aim of reducing the biofilm generated by three bacteria (*P. aeruginosa, L. monocytogenes and E. coli*) over the surface of 3D printed PLA pieces. With that purpose, two liquid precursors (AcAc and TEOS) were utilized with diverse number of passes.

The key findings of this research are the following:

* All coatings that were applied with 1 or 2 passes had anti-biofilm properties for all the bacteria studied, with the exception of Te1p in *P. aeruginosa*. It could be concluded that AcAc coatings are more effective than TEOS coatings. Generally speaking, the anti-biofilm capacity of the coatings was higher when they were applied with less passes.
* The Ac1p coating was the best one, giving rise to relative biofilm productions levels of 47.7% for *L. monocytogenes*, 50.4% for *P. aeruginosa* and 64.1% for *E. coli*.
* The hydrophilic properties of the coatings were key for the effectivity to these coatings, since hydrophilic coatings promote the generation of a hydration layer reducing bacterial adhesion and consequently biofilm formation.
* The increase in surface coatings hydrophilicity could be a result of the increase in oxygen polar groups (C-O y O-C=O). TEOS coatings have higher abundance of O-C=O groups when they are applied with a low number of passes. That fact may explain that, almost in all cases, lower biofilm formation was generated when less passes were applied.
* The anti-biofilm capacity of AcAc coatings cannot be explained only by the formation of a hydration layer, since their chemical nature did not change depending on the number of passes used. The different biofilm formation obtained could be justified by the morphology of AcAc coatings. When the distance between peaks was 2-3 times lower than the bacterial cellular length a reduction in adhesion occurred, maybe as a result of the smaller contact area between bacteria and coating and the potential rupture of the cellular membrane.

In future research, anti-biofilm capacity will be characterized for different clinical- and food- relevant bacteria, and other liquid precursors, such as polyethylene glycol methyl ether methacrylate (PEGMA, H2C=CCH3CO2(CH2CH2O)nCH3), oleic acid (C18H34O2), succinic acid (C4H6O4) and citric acid (C6H8O7), will be tested. The reasons for using these precursors are their similarity with acrylic acid and the possibility of generating hydrophilic surfaces thanks to the presence of hydroxy and carboxyl groups. Moreover, durability and toxicity tests will be performed in order to examine the shelf-life and safety of the coatings for their practical application.

Plasma-polymerization coatings can mitigate issues related to 3D printing food contact applications. Furthermore, atmospheric plasma technology can be easily implemented in industrial 3D printers, which facilitates its immediate integration into present production lines, thus enabling the food industry to safely employ a technology as versatile as 3D printing.

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LIST OF FIGURES



**Fig. 1.** Process sequence: [a] 3D Printed poly-lactic acid (PLA) Petri dishes, [b] Close view of the deposition process, [c] Plasma-polymerization (APPJ) equipment, [d] Scheme of the plasma-polymerization process, [e] Top view of a PLA Petri dish, and [f] SEM image with a magnification of x100 of an untreated PLA Petri dish.



**Fig. 2.**Atmospheric pressure plasma jet (APPJ) treatment: [1] Scanning electron microscopy (SEM) images (x2000), [2] Atomic force microscopy (AFM) images (40x40µm).



**Fig. 3.** Average roughness (Ra) of all samples. The red dashed line indicates the untreated sample roughness.



**Fig. 4.**AcAc coatings: [1] Scanning electron microscopy (SEM) images (x2000), [2] Atomic force microscopy (AFM) images (40x40µm).



**Fig. 5.**TEOS coatings: [1] Scanning electron microscopy (SEM) images (x2000), [2] Atomic force microscopy (AFM) images (40x40µm). White arrows indicate coating cracks and white circles indicate silicon dioxide particles.



**Fig. 6.**Profiles of untreated and AcAc coated samples: [1] 2D-atomic force microscopy (AFM) images, [2] Cross section along the dashed line and [3] deposited coating scheme. (d=distance between peaks, µm).



**Fig. 7.**Atomic percentages of C, O, Si and N: [a] AcAc coatings, [b] TEOS coatings.



**Fig. 8.**Relative biofilm production generated by *Pseudomonas aeruginosa*, *Escherichia coli* and *Listeria monocytogenes* on coated 3D printed PLA Petri dishes. The red dashed line indicates biofilm production on untreated sample.



**Fig. 9.**Bacterial behavior according to the surface morphology of the samples: [a] Untreated, [b] Ac1p, [c] Ac6p and [d] Ac12p.

SUPPLEMENTARY MATERIAL

|  |  |  |  |
| --- | --- | --- | --- |
| **Component** | **Functional group** | **Binding energy (eV)** | **Reference** |
| A | C-C/C-H | ~285 | (Abourayana et al., 2018; Jacobs et al., 2012; Zhao et al., 2013) |
| B | C-O | 286.5 – 287.2 | (Abourayana et al., 2018; Jacobs et al., 2012; Wang et al., 2016; Zhao et al., 2013) |
| C | O-C=O | 288.5 – 289.4 | (Jacobs et al., 2012; Wang et al., 2016; Zhao et al., 2013) |

**Table S1.** Functional groups and binding energies associated with peaks that correspond to the deconvolution of C1s spectra.

|  |  |  |  |
| --- | --- | --- | --- |
| **Samples** | **C-C/C-H** | **C-O** | **O-C=O** |
| Untreated | 41.15±1.98 | 16.04±0.59 | 13.89±1.09 |
| Ac1p | 22.95±0.18 | 19.38±0.17 | 19.35±0.06 |
| Ac2p | 23.02±0.18 | 19.10±0.09 | 19.11±0.11 |
| Ac6p | 22.71±0.05 | 19.46±0.09 | 19.66±0.09 |
| Ac12p | 22.64±0.10 | 19.11±0.20 | 19.72±0.20 |
| Te1p | 19.72±0.37 | 17.22±0.47 | 13.44±0.81 |
| Te2p | 26.68±3.47 | 21.33±1.50 | 10.76±2.27 |
| Te6p | 13.41±0.43 | 8.53±0.28 | 3.96±0.32 |
| Te12p | 14.07±1.09 | 9.87±0.38 | 4.75±0.56 |

**Table S2.** Relative percentages of groups found in the C1s signal of the analyzed samples



**Fig. S1.**Deconvolutions of C1s spectra: [a] Untreated, [b] Ac2p, [c] Ac12p, [d] Te1p, [e] Te2p, [f] Te12p



**Fig. S2.** Water contact angle (WCA) measurements for TEOS coatings over stainless steel surface. The red dashed line indicates WCA for untreated stainless steel (89.5±12.6º).