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Control of Bacterial Proliferation and Formation of Biofilm in Membranes for Food Packaging Manufactured By Electrospinning

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The application of micro/nanotechnologies in the development of food contact materials is currently of great interest for the food technology sector. The materials for this purpose include various types of polymers, among which are PLGA (polylactic-co-glycolic acid) and PCL (polycaprolactone), biodegradable synthetic polymers. Electrospinning is a low-cost and easily reproducible technique for the production of polymeric micro/nanofiber membranes that can be used in the packaging of food. This work establishes the parameters for the manufacturing, by means of electrospinning, of micro/nanofiber membranes made of PLGA, PCL and a combination of both of these polymers. The resulting fibers were characterized via scanning electron microscopy. Given the importance, within the food industry, of knowing the effect that the package components may have on bacterial proliferation, the resulting fibers were incubated with three of the most frequent microorganisms that contaminate food: *Staphylococcus aureus*, *Enterococcus faecalis* and *Escherichia coli*. Both the viability and the bacterial adhesion were quantitatively determined in terms of the biofilm formation capacity. The formation of biofilms was greater in the PLGA membranes. When any of the microorganisms were exposed to the membranes made of the mixture of the two polymers, or with only PCL, a marked reduction in the formation of biofilm was observed. These results show that it is possible, by means of a proper selection of the materials, to reduce the possibility of contamination in both the packaging and the food, without the need to add products that might be inconvenient due to their possible side effects.

1. Introduction

The basic function of packaging is to protect its content, but in the case of food, the most important aspect is safety, which is why agencies such as the European Food Safety Authority (EFSA) and the Food and Drug Administration (FDA) have strict regulatory standards related to the materials that may come into contact with food (Karmaus, Osborn and Krishan, 2018). In the traditional concept, the packaging material is limited to containing and separating food from the environment, thus working as a passive and inert barrier that protects the content from the passage of light, heat, humidity, undesired chemical substances, inadequate handling and biological contamination. The concept of active packaging involves the idea that the packaging must generate a favorable response, such as extending the shelf life of the food product and its nutritional qualities, as well as prevent the growth of microorganisms, the latter being a growing area of research in the recent years that includes the addition of natural or synthetic antimicrobials, and metallic elements. Both micro and nanotechnology have produced a great impact at the level of development of active packaging. Nanomaterials can be incorporated into the packaging material, stay still on its surface, or be used as coating. Due to their capacity to inhibit different types of microorganisms, Ag, Au, Cu, ZnO and TiO_2 nanoparticles have been used, as well as carbon nanotubes, which are not biodegradable and have recently been attributed toxic effects (Hadrup and Lam, 2014). Given the risk of metallic nanoparticles transfer from the packaging to the food product, and thus to the consumer, the EFSA does not authorize their use in packaging. Based on the above, the use of biodegradable synthetic polymers, whose final products are ultimately excreted, such as PLGA and

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PCL (approved by the FDA), has generated substantial interest in the food packaging sector (Grumezescu, 2017).
Electrohydrodynamic techniques enable the manufacturing of different structures on a nanoscopic scale (films, particles, fibers, etc.), and they are based on the application of an electric field in the vicinity of a liquid conductor that flows through a needle, which originates an electric current in the liquid itself and the accumulation of surface charge (Malagon-Romero, Clavijo and Lopez, 2018). The electric field, together with the surface charge, generates an electrical effort. When said effort is increased enough to overcome the surface tension, the interface of the liquid is deformed with its external means in the direction of the applied field, adopting different geometries with a conical tendency (Clavijo-Grimaldo et al, 2018). Next to the vortex of the cone, the electric field is so intense that mass and charge emission is produce in the form of a stable stream, small in diameter, which is fragmented in a spray of charged drops (electrospray) or fibers (electrospinning). During the process, the solvent is completely evaporated (Clavijo-Grimaldo, 2015).
By means of electrospinning, membranes have been manufactured for the packaging of food products; said membranes are made of micro/nanofibers (Quirós, Boltes and Rosal, 2016). With this versatile, efficient and controllable technique it is possible the manufacturing of fibers with different diameters and porosity, controlling three groups of variables: variables dependent on the polymer and its solvent (molecular weight of the polymer, concentration, viscosity, electric conductivity, temperature and surface tension of the solution), variables dependent on the process (voltage, flow of the polymer, distance between the needle through which the polymer comes out and the collector where the fibers are collected, the diameter of the needle, type of collector and velocity of the movement of the collector) and variables dependent on the environmental conditions (temperature and humidity, for example).
Although both the PLGA and the PCL have been approved to be used, some research shows that the PGLA degradation products (glycolic and polylactic acids) can be metabolic substrates and increase bacterial adhesion and favor infection (Al-Ahmad et al., 2008). This effect not observed in the PCL, which is why the objective of this work is to qualitatively assess the proliferation, adhesion and biofilm formation capacity of three of the most frequent microorganisms that contaminate packaging and food products in micro/nanofiber membranes made of PLGA, PCL and a combination of both polymers.

2. Materials and methods

2.1 Manufacturing and characterization of membranes

For the manufacturing of the membranes by means of the electrospinning technique, 3 solutions were prepared:

- PLGA solution: Poly (D, L-lactide-co-glycolide) (50:50, CAS# 26780-50-7 and molecular weight 30,000 - 60,000 g/mol) was used in a solution with 20% W/V in Chloroform (99,5 %, CAS # 67-66-3)
- PLGA+PCL Solution: Poly(L-lactide-co-caprolactone-co-glycolide) (L-Lactide 70%, CAS # 134490-19-0 and molecular weight 100 kg/mol) was used in a solution with 10% W/V in Dichloromethane (99,8% CAS Number: 75-09-2)
- PCL Solution: Polycaprolactone (CAS # 134490-19-0 and molecular weight Mn=80.000) was used in a solution with 9% W/V in a 50:50 V/V mixture of chloroform and Isopropyl alcohol (99,7 % CAS # 67-66-3)

All the chemical agents used were supplied by Sigma-Aldrich. The resulting solutions were subjected to an ultrasound with a frequency of 50Hz per 60 minutes at 17°C. The collection of fibers was done on microscope slides (22mmx22mm) placed on the collector. The process variables were adjusted (solution flow, needle-collector distance, and voltage) until uniform, flawless fibers were obtained. The resulting membranes were characterized by means of Scanning Electron Microscopy (SEM).

2.2 Microbiological assays

Staphylococcus aureus ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922 reference strains were used, which were supplied by the Microbiology laboratory of Keralty. The manufactured membranes were sterilized by means of ultraviolet radiation in order to reduce the risk of contamination.
The bacterial strains were incubated for 24h in a concentration of 10^8 UFC/ml at 37°C so as to assess their viability as well as their biofilm formation capacity. Subsequently, they were washed 3 times with a saline solution, and the coloration was carried out with LIVE/DEAD™ BacLight™ Bacterial Viability Kit for Microscopy (Molecular Probes™), based on the use of a two-colorant mixture, the SYTO9, which turns the nucleic acids of the live bacteria fluorescent green, and the propidium iodide, which turns the bacteria with damaged membranes fluorescent red. To identify the presence of exopolysaccharides present in the biofilm, 0.02% of calcofluor stain (Sigma-Aldrich) was added for 20 minutes in darkness, and the observation was

done through a fluorescence microscope (Izquierdo-Barba et al., 2015). All the assays were carried out in triplicate.

3. Results

3.1 Parameters to obtain membranes and characterization

The Table 1 shows the definitive parameters used to manufacture membranes with each one of the polymer solutions.

Table 1: Parameters of the electrospinning process for manufacturing the membranes

Polímero	Flujo (ml/h)	Voltaje(kV)	Distancia aguja-colector (cm)
PLGA	0.8	22.5	15
PLGA+PCL	0.8	21	20
PCL	0.8	20	15

Figure 1 show the macroscopic aspect of the PCL membranes obtained at 5, 10 and 20 minutes of electrodeposition. The aspect of the PLGA and PLGA+PCL membranes is similar. The thickness of the membranes increases as the process time is prolonged. For the characterization to the SEM a deposition time of 5 minutes was standardized.

Figure 1: Macroscopic appearance of the PCL membranes at 5 (A), 10 (B) and 20 (C) minutes of electrodeposition

Figure 2 shows the morphology seen through SEM (3000x) of the fibers forming the manufactured membranes. The fibers are stable, flawless. The diameter of the PLGA fibers (A) is greater than that of the PCL fibers (B), which is evident in the membrane containing both polymers (C). When observed at 5000x, it is evident that, besides the differences in the diameter, the PLGA fibers have a porous surface not seen in the PCL fibers.

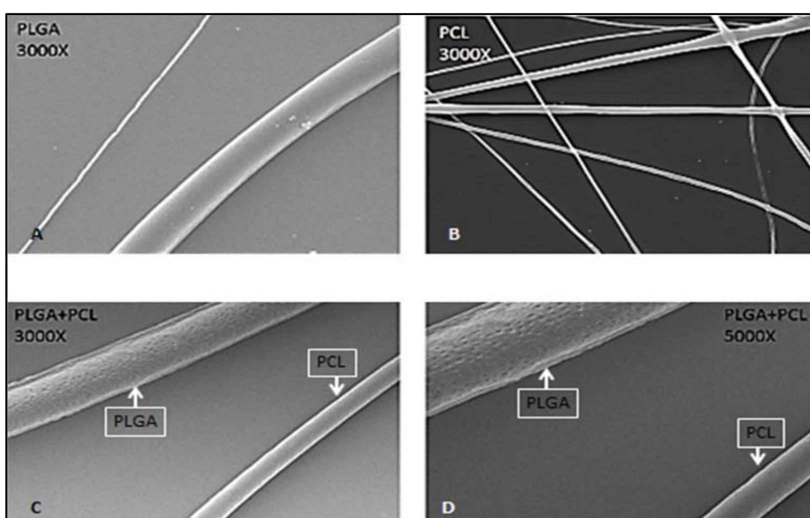

Figure 2: Microphotographs of the membranes of PLGA (A), PCL (B) at 3000x and PLGA + PCL at 3000x (C) and 5000X (D)

These photographs confirm that although PLGA and PCL are combined in a solution, the electrospinning process does not form fibers that combine the two polymers; on the contrary, fibers are formed that preserve the individual characteristics (Quirós, Boltes and Rosal , 2016)

3.2 Viability, adhesion and formation of bacterial biofilms

Figure 3 shows the merged optical fluorescence microscopy images obtained after the incubation of different types of membranes (PLGA, PLGA+PCL and PCL) with the *Staphylococcus aureus*, *Enterococcus faecalis* and *Escherichia coli* strains.

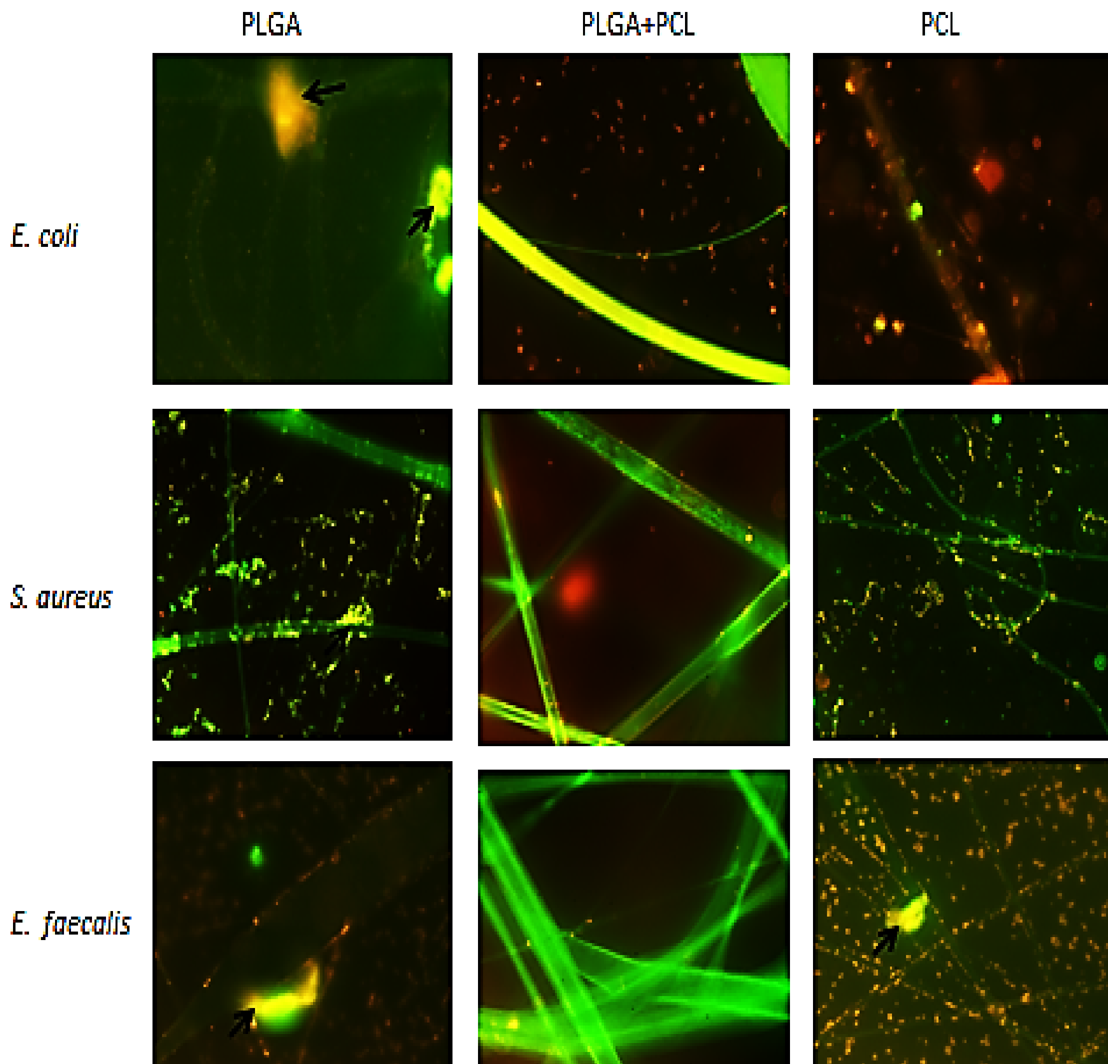

Figure 3: Merged optical fluorescence microscopy images (40X) after the incubation of the microorganisms with the different types of membranes (Green fluorescence: live bacteria and red fluorescence: dead bacteria. Arrows: exopolysaccharide)

Green fluorescence indicates the integrity of the nucleic acids of the live bacteria, while the red fluorescence indicates damage in the cellular wall typical of dead bacteria. When bacteria are bonded to one another, they form a biofilm characterized by a matrix of exopolysaccharides that are secreted so as to guarantee the survival and resistance of antimicrobial agents. When the three genera of bacteria were exposed to the PLGA, PLGA+PCL and PCL matrix, a greater proliferation and biofilm formation was observed, which can be recognized thanks to the fluorescence of the exopolysaccharide, in the PLGA membrane. The PLGA+PCL membranes did not display any biofilm formation, and a larger quantity of non-viable bacteria can be observed in the PCL membranes. Scarce exopolysaccharide fluorescence with the PCL membrane was only observed for *Enterococcus faecalis*, but the predominance of non-viable bacteria is evident.

Figure 4 shows other fields in which the formation of biofilm in the PLGA membranes incubated with the three genera of bacteria is evidenced.

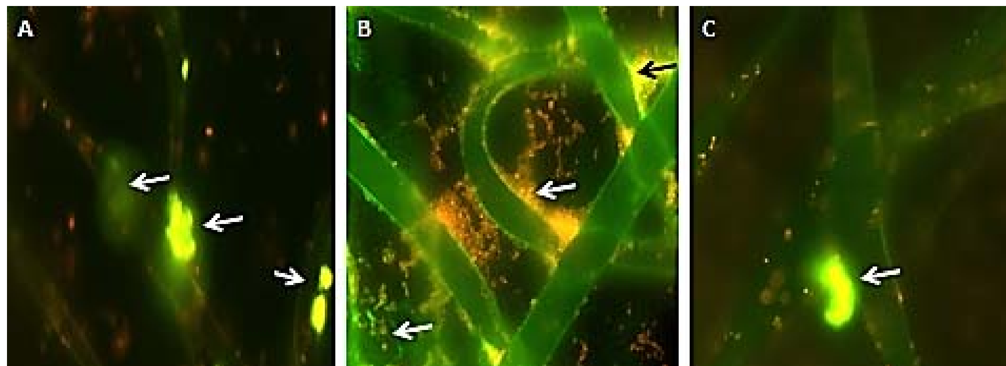

Figure 4: Fluorescence optical micrograph (40x) showing the results of the incubation of the PLGA membranes with Escherichia coli (A), Staphylococcus aureus (B) and Enterococcus faecalis (C). (Arrows: exopolysaccharide

The greatest concentration of exopolysaccharide of the biofilm matrix can be seen in the membrane incubated with *Staphylococcus aureus.* The metabolites released from the PLGA are likely to generate a microenvironment that allows the proliferation and adaptation of the microorganism enabling the segregation of exopolysaccharide to live in community, forming the biofilms. The topography observed in the surface of the PLGA fibers may also promote the formation of biofilms, given that it is known that the porosity and roughness of a material are factors that facilitate migration and adhesion in various cell types, among them bacteria. Unfortunately, the capacity to produce biofilm also generates greater resistance to eradication.
Studies conducted on devices for biomedical use and on matrixes manufactured by means of 3D-prototyping showed similar results. When the adhesion of various microorganisms to PLGA matrixes, tricalcium phosphate (TCP), and alginate (Na/Ca) was assessed, it was observed that the *Streptococcus mutans*, *Enterococcus faecalis*, *Prevotella nigrescens*, *Porphyromonas gingivalis*, *Streptococcuss anguis* and *Candida albican* strains showed greater adhesion to the PLGA matrixes.

4. Conclusions

Food intoxication is a public health problem worldwide. The microorganisms that are most frequently related to this pathology (*Staphylococcus aureus*, *Enterococcus faecalis* and *Escherichia coli*) have the ability to generate a variety of mechanisms to ensure their survival, for example, the formation of biofilm (microbial community surrounded of a polymeric matrix, generally of exopolysaccharides) that favors infection, resistance to antimicrobials and the maintenance of microorganisms on food surfaces and their packaging, which is why the materials used to manufacture the packaging also reduce bacterial proliferation they must inhibit the formation of biofilm (Kirk et al., 2015). Synthetic polymers such as PLGA and PCL are frequently used in the manufacture of food packaging. One of the limitations in the use of PLGA is that several microorganisms display good adhesion to matrixes, membranes and devices manufactured with this polymer. In this study, it was evidenced that there was a greater capacity for the formation of biofilms in the membranes made of PLGA micro/nanofibers versus those made of PLGA+PLC or exclusively PCL micro/nanofibers. On the biofilm, the oxygen and nutrient needs of the bacterial population change, as well as their resistance to antimicrobial agents; this increases the survival of microorganisms in different environments. Given the high adherence and formation of biofilm of microorganisms that cause food intoxication to PLGA membranes, it is suggested that the packaging made exclusively with this material be more susceptible to bacterial contamination and therefore, if its use is considered, it should be carried out modifications or, as in this investigation, combine with other polymers that reduce this risk
Given that the incorporation of metallic particles with antimicrobial properties into food packaging has not been approved by the regulatory agencies, an adequate combination of polymers becomes a practical and efficient alternative to reduce bacterial proliferation, as well as the risk of biofilm formation.

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