

This document is confidential and is proprietary to the American Chemical Society and its authors. Do not copy or disclose without written permission. If you have received this item in error, notify the sender and delete all copies.

Colonization of electrospun polycaprolactone fibers by relevant pathogenic bacterial strains.

Journal:	<i>ACS Applied Materials & Interfaces</i>
Manuscript ID	am-2017-19440d.R1
Manuscript Type:	Article
Date Submitted by the Author:	08-Mar-2018
Complete List of Authors:	Rumbo, Carlos; Universidad de Burgos, Tamayo-Ramos, Juan Antonio; Universidad de Burgos - Campus San Amaro, ICCRAM Caso, M. Federica; Nanofaber srl. Rinaldi, Antonio; ENEA and University of L'Aquila , UTT-MAT and MEMOCS Romero-Santacreu, Lorena; International Research Centre in Critical Raw Materials-ICCRAM, Universidad de Burgos Quesada, Roberto; Universidad de Burgos, Química Cuesta-López, Santiago; International Research Centre in Critical Raw Materials-ICCRAM, Universidad de Burgos

SCHOLARONE™
Manuscripts

Colonization of electrospun polycaprolactone fibers by relevant pathogenic bacterial strains

Carlos Rumbo^{1, 2, ‡, *}, Juan Antonio Tamayo-Ramos^{1, ‡, *}, M. Federica Caso³, Antonio Rinaldi⁴, Lorena Romero-Santacreu¹, Roberto Quesada², and Santiago Cuesta-López^{1, *}

1 International Research Centre in Critical Raw Materials-ICCRAM, Universidad de Burgos, Plaza Misael Bañuelos s/n, 09001 Burgos, Spain.

2 Departamento de Química, Facultad of Ciencias, Universidad de Burgos, 09001, Burgos, Spain.

3 Nanofaber srl. Via Anguillarese 301, 00123 Rome, Italy.

4 Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), Casaccia Research Centre, Via Anguillarese 301, 00123 Rome, Italy.

ABSTRACT

Electrospun biodegradable polymers have emerged as promising materials for their applications in several fields, including biomedicine and food industry. For this reason, the susceptibility of these materials to be colonized by different pathogens is a critical issue for public health, and their study can provide future knowledge to develop new strategies against bacterial infections. In this work, the ability of three pathogenic bacterial species (*Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Listeria monocytogenes*) to adhere and form biofilm in electrospun polycaprolactone (PCL) microfibrinous meshes was investigated. Bacterial attachment was analyzed in meshes

1
2
3 with different microstructure, and comparisons with other materials (borosilicate glass
4 and electrospun polylactic acid (PLA)) fibers were assessed. Analysis included colony
5 forming unit (CFU) counts, scanning electron microscopy (SEM), and crystal violet
6 (CV) staining. All the obtained data suggest that PCL meshes, regardless of their
7 microstructure, are highly susceptible to be colonized by the pathogenic relevant
8 bacteria used in this study, so a pretreatment or a functionalization with compounds that
9 present some antimicrobial activity or antibiofilm properties is highly recommended
10 before their application. Moreover, an experiment designed to simulate a chronic wound
11 environment was used to demonstrate the ability of these meshes to detach biofilms
12 from the substratum where they have developed, thus making them promising
13 candidates to be used in wound cleaning and disinfection.
14
15
16
17
18
19
20
21
22
23
24
25

26
27 **KEYWORDS:** Electrospun polycaprolactone, microfibers, nosocomial pathogens,
28 foodborne pathogens, bacterial attachment, biofilm.
29
30

31 32 **INTRODUCTION**

33
34
35 The use of biodegradable polymers as biomaterials in medical equipment and hospital
36 settings set a major advance in modern medicine, being the main advantage their
37 capacity to be degraded, excreted or reabsorbed in the organism, thus avoiding surgical
38 removal. A wide range of natural and synthetic biodegradable polymers are being used
39 in biomedicine, with a great variety of medical applications.¹ Tissue engineering is one
40 of the main fields where these polymers are implemented. The production of three-
41 dimensional scaffolds provides a space with an structure similar to the extracellular
42 matrix, which is appropriate for cell adhesion and proliferation, playing a crucial role in
43 new tissue regeneration.² Many advanced procedures have been developed to obtain
44 these scaffolds.³ Among them, electrospinning is one of the most successful techniques.
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 Through this method, fibers in the nanoscale or microscale range are obtained, with the
4 possibility of controlling morphology, diameter and patterning of electrospun nanofibers
5 over a wide range value.⁴ Polycaprolactone (PCL), a synthetic biodegradable and
6 semicrystalline polyester, is one of the most suitable materials for use in tissue
7 engineering. Several studies have demonstrated that electrospun PCL scaffolds can be
8 efficiently applied in different fields, such as bone regeneration,^{5, 6} cardio-vascular
9 tissue engineering^{7, 8} or skin tissue engineering.^{9, 10} Moreover, the use of PCL in
10 combination with other compounds, such as nanoparticles or hyaluronic acid, has been
11 shown to be a safe and effective wound dressing.^{11, 12}
12
13
14
15
16
17
18
19
20
21
22

23 Hospital-acquired infections caused by multidrug-resistant bacteria are one of the most
24 important problem that modern medicine is facing nowadays, presenting incidence rates
25 of 5% and 7.1% in the United States and the European Union respectively in 2013.¹³
26 Bacteria can attach to biomaterials and proliferate forming biofilms, which can cause
27 implant related infections.¹⁴ Biofilms are microorganism associations, formed by one or
28 more species, attached to a substratum and embedded in a complex self-produced
29 matrix of polymeric substances, being the main components exopolysaccharides,
30 proteins and nucleic acids.^{15, 16} This structure confers protection to the microorganisms
31 contained in it. Compared to their planktonic states, biofilm-embedded bacteria are
32 significantly more resistant to desiccation, environmental stress, and ultraviolet (UV)
33 light exposure, as well as to ordinary antibiotics and disinfectants.¹⁷ All of this turns
34 biofilms into a serious risk in medicine. Biofilm formation is frequent in clinical strains
35 and plays an important role in chronic colonization of human tissues and their
36 persistence in implanted medical devices.^{18, 19} For all these reasons, investigating
37 biofilm formation on biomaterials is a crucial issue for modern medicine.
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 Beyond the biomedical field, there is a growing interest in the utilization of electrospun
4 biodegradable polymers in the food industry, due to the many possible applications,
5 such as encapsulation of food materials, and their use in packaging or as processing
6 aids.^{20, 21} However, most of the foodborne pathogens, such as *Listeria monocytogenes*,
7 *Salmonella enterica* or *Escherichia coli*, can manage to colonize different materials and
8 surfaces present in food processing plants through the development of biofilm²², being
9 this an important public health issue. In fact, as with the biomedical field, microbial
10 biofilms are a problematic and persistent issue in the food industry, where they have
11 been involved in many foodborne outbreaks.²³

12
13 Bacterial attachment and biofilm formation in abiotic surfaces is a complex process that,
14 besides bacterial surface characteristics, is critically affected by surface topography.
15 Substratum properties such as morphology, surface chemistry, roughness, material
16 surface energy, wettability, or zeta potential influence the colonization of a
17 biomaterial.^{24, 25} Despite the critical importance of biofilms for public health, and the
18 growing use of electrospun microfibers in several fields, to our knowledge, there are
19 only a few studies focusing on microbial adhesion, colonization and biofilm formation
20 on these structures. The analysis of interactions between bacteria and electrospun
21 micro/nanofibers is relevant to develop new strategies to prevent the occurrence of
22 associated infections, and to determine new potential applications of these novel
23 materials. Kargar *et al.* investigated the interactions of bacteria with fibrous substrates,
24 finding that the adhesion of *Pseudomonas aeruginosa* on polystyrene (PS) fibers is
25 dependent on fiber diameter and spacing.²⁶ Abrigo *et al.* observed that the diameter of
26 the fibers influences the ability of *E. coli*, *P. aeruginosa* and *Staphylococcus aureus* to
27 proliferate and colonize electrospun PS fibers.²⁷ By the same token, these authors

1
2
3 described the effect of surface chemistry in the attachment and proliferation onto
4
5 electrospun PS fibers.²⁸
6
7

8 In this study, the ability of three pathogenic species belonging to different environments
9
10 to adhere to electrospun PCL microfiber meshes and form biofilms was analyzed and
11
12 compared using different assays. Two of the most important Gram-negative nosocomial
13
14 pathogens, *P. aeruginosa* and *Acinetobacter baumannii*, with the capacity to acquire
15
16 and develop multiple resistance mechanisms^{29, 30} were used in this study. In addition,
17
18 the ability to form biofilm in these microfibers was studied in *L. monocytogenes*, one of
19
20 the most important foodborne pathogens.³¹ The attachment, the structure and the
21
22 viability of the biofilms formed on these meshes were studied using crystal violet (CV)
23
24 staining, scanning electron microscope (SEM) and colony forming unit (CFU)
25
26 quantification. Also, since *P. aeruginosa* and *A. baumannii* are frequently involved in
27
28 wound infections,³² bacterial transfer experiments using PCL meshes in direct contact
29
30 with bacteria previously grown in agar plates were performed to simulate a wound
31
32 environment. Furthermore, CFU count assays were carried out to study the influence of
33
34 the PCL mesh microstructure on bacterial attachment, and to perform comparative
35
36 experiments with borosilicate glass and electrospun polylactic acid (PLA) fibers,
37
38 materials commonly used in hospital environments and biomedicine applications
39
40 respectively.
41
42
43
44

45 **MATERIALS AND METHODS**

46 **Materials and specimen preparation**

47
48
49 All PCL microfiber meshes used in this study (NBARE, Pro3 and Pro4) were supplied
50
51 by Nanofaber S.R.L. All of them were synthesized using the linear thermoplastic PCL
52
53 diol polymer CAPA® 6800 (80,000 MW) (Prestor), chloroform (99.2% purity,
54
55
56
57
58
59
60

stabilized with 0.6% ethanol; VWR) and dimethylformamide (DMF) (100% purity; VWR). PCL was dissolved to obtain 12% (w/w) solutions in 40 mL DMF/chloroform 35:65 (NBARE), 40 mL DMF/chloroform 20:80 (Pro3) or 40 mL chloroform (Pro4). Table 1 shows the values of the electrospinning parameters that were used for each fiber type. The electrospun material was deposited on a flat aluminium collector. NBARE meshes are formed by 2 kinds of microfiber with different size, while Pro3 and Pro4 meshes present a uniform microfiber size (Table 1). PLA fiber meshes were supplied by Nanopharma a. s. Round dishes of all biomaterials were obtained with a round punch with a diameter of 12 mm. Borosilicate glass coverslips of 12 mm in diameter (Thermo Scientific Menzel) were purchased from Fisher Scientific. Before use, all materials were sterilized by immersion in 70% ethanol (v/v) for 30 minutes, and UV irradiation for 30 minutes.

Table 1. Parameters used for the electrospinning of PCL solutions and microfiber diameter in the different fiber types.

fiber type	flow rate (μL/h)	applied voltage (kV)	Moving needle diameter (mm)	X axis speed (mm/s)	Y axis speed (mm/s)	spinning distance (cm)	deposition time (min)	microfiber diameter (μm)
NBARE	6000	23	1.7	60	6	24	60	2.19±0.13
								0.80±0.007
Pro3	4000	29	1.7	60	6	24	60	2.21±0.48
Pro4	4000	23	1.7	60	6	24	60	6.43±1

Bacterial strains and culture conditions

The bacterial strains *A. baumannii* ATCC 19606, *P. aeruginosa* PAO1 and *L. monocytogenes* NCTC 11994 were purchased from Spanish Type Culture Collection. *A. baumannii* and *P. aeruginosa* were maintained at 30°C in Luria-Bertani (LB) broth or agar. *L. monocytogenes* was maintained in Tryptic Soy broth (TSB) or agar at 30°C.

Bacterial count determination

To study the number of bacteria attached to the different materials, one colony of each strain grown on agar media was inoculated into 5 mL of liquid media and incubated overnight. Then, a dilution 1:100 of the overnight culture was dispensed into each well of a 24 well plate containing materials, and incubated at 30°C for 48 hours under static conditions. The materials were then rinsed three times with Dulbecco's Phosphate-Buffered Saline (DPBS) to remove any unbound bacteria, and transferred into sterile conical tubes containing 5 mL of DPBS. The tubes were vortexed at full speed for 1 minute and then placed in an ultrasonic bath and sonicated for 15 minutes at low power intensity, to release the attached bacteria from the material. After an additional vortex step, suspensions were serially diluted with DPBS and cultured overnight. The CFUs were counted to determine the number of viable adherent bacteria, and the Log base 10 of the CFUs/cm² (Log CFUs/cm²) was calculated. Each assay included at least three independent replicates.

Scanning electron microscopy

Biofilms of each strain were grown under the same conditions as those explained above. Once microfiber meshes were washed 3 times, they were fixed with 2% paraformaldehyde, 2% glutaraldehyde, 3 mM CaCl₂ in DPBS, and sequentially dehydrated for 10 min in 50, 70, 80, 90, 95 and 100% (x2) (v/v) ethanol. Finally, the meshes were coated with a layer of gold and examined with environmental scanning electron microscope (FEI-Quanta 200F).

Electrospun meshes NBARE, Pro3 and Pro4 were previously coated with gold (15-30 nm) using the Edwards Sputter/Coater S150B deposition chamber and observed and analyzed with scanning electron microscope (Leo 1530, ZEISS, Germany).

Crystal Violet assay

Biofilms of each strain were grown under the same conditions as those explained above. After the washing step with DPBS, meshes were transferred into clean wells, and stained with a 1:2 dilution of 1% (w/v) crystal violet solution (Sigma-Aldrich) for 30 minutes at room temperature. The excess stain was washed 3 times with DPBS, and membranes were transferred again to clean wells and photographed. All experiments were carried out in triplicate.

Agar-mesh bacterial transfer experiments

Bacterial transfer experiments were performed according to a method described previously by Abrigo *et al.*²⁷ In brief, one colony of each strain grown on agar media was inoculated into 5 mL of liquid media and incubated overnight. The obtained culture was diluted up to an optical density at 600 nm (OD₆₀₀) of 0.3 in clean media, and 100 μ L of the suspension was spread onto agar plates, which were incubated 18 h at 30°C to obtain a confluent biofilm. Subsequently, microfiber meshes were placed on the agar in contact with the biofilm, and the plates were incubated 1 h at 30°C. After incubation, meshes were removed from the plate, and the culture was checked for the presence of spots.

Statistical analysis

Statistical analysis data are presented as means \pm SD. Differences between the attachment in PCL microfiber meshes and other materials were established using a Student's t-test. The one-way analysis of variance (ANOVA) was used for multiple comparisons, followed by Tukey *post hoc* test. Statistical tests were carried out using

Prism 6.0 (GraphPad Prism, GraphPad Software, Inc.). Differences were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Determination of biofilm formation in PCL microfiber meshes by different pathogenic strains.

Microbial biofilms are formed by a matrix of extracellular polymeric substances that allow bacteria to resist chemical and physical stress.³³ Biofilms have a high impact in industrial and clinical settings, representing a significant concern for human health. In this study, the susceptibility of commercial electrospun PCL microfiber meshes specially designed for use in biomedicine, named as NBARE, to be colonized by different bacterial pathogens has been analyzed. For this purpose, different methodologies were applied. Figure 1 shows the structure of NBARE fibers. This mesh type consists in two kinds of fiber with different size, one with an average fiber thickness of 2.19 μm , and the other with an average fiber thickness of 0.80 μm .

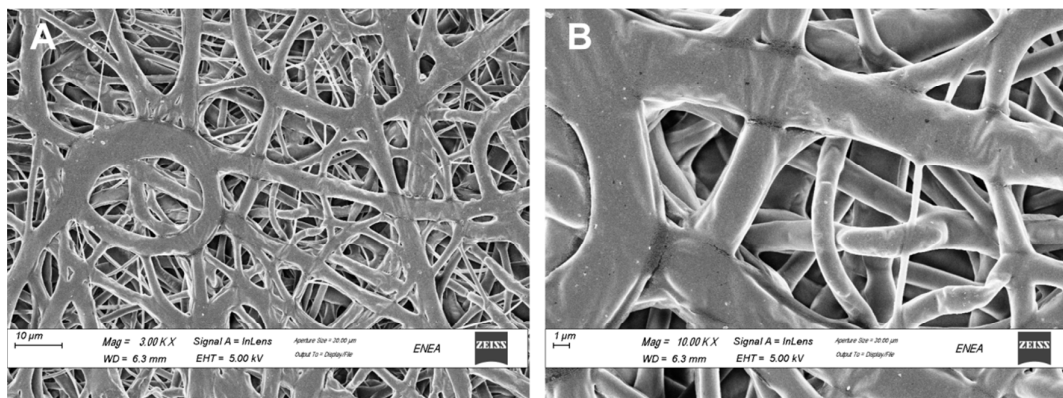


Figure 1: SEM image showing the microstructure of NBARE fibers. Overview (A) and detail (B). Image A: Original magnification $\times 3000$ (Scale bar = 10 μm); Image B: Original magnification $\times 10000$ (Scale bar = 1 μm)

To investigate the ability of the different pathogenic species to colonize PCL microfibers, meshes were exposed to liquid cultures of the strains under study during 48 h. After this incubation period, meshes were washed and adhered bacteria were detached by vortexing and sonication. CFU counts were used to analyze the number of viable bacteria attached. The results obtained are represented in Figure 2. *P. aeruginosa* and *L. monocytogenes* had similar Log CFUs/cm² attached to PCL microfibers, while *A. baumannii* presented a statistically significant reduction of attached bacteria in comparison with the two other strains ($P \leq 0.001$), presenting a bacterial load of around 1 log unit lower than the others.

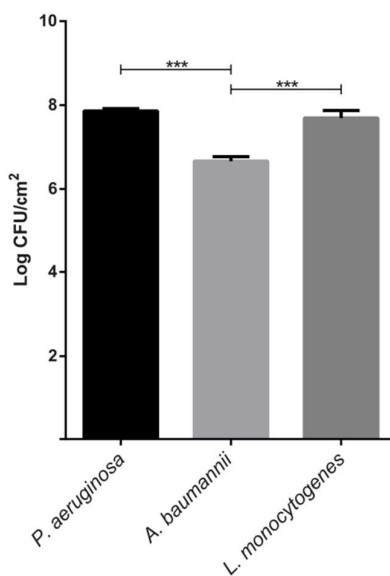
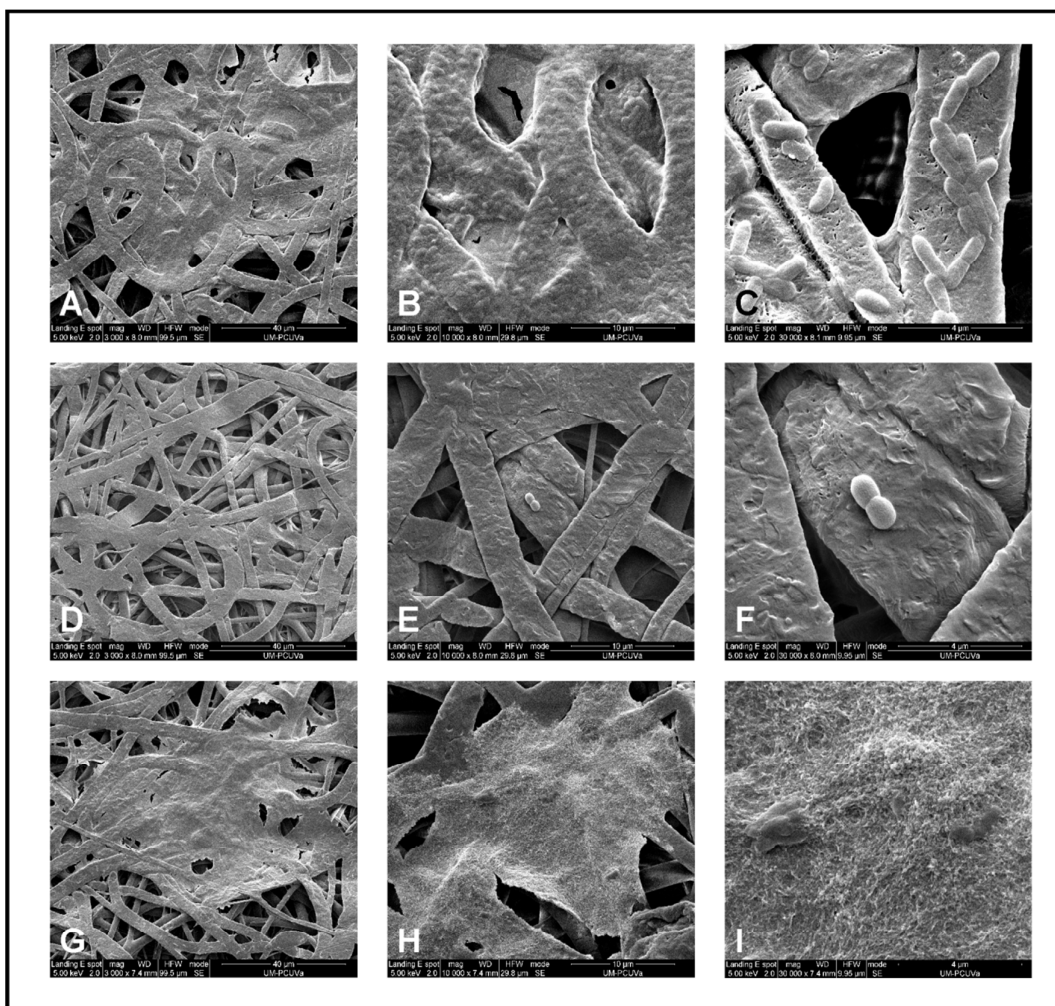


Figure 2: Ability of different bacterial strains to attach to PCL fibers expressed as Log (CFU/cm²). Data represent the mean (\pm standard deviation, SD) of three independent replicates. Differences were established using a One-way ANOVA, and considered significant at $P \leq 0.05$. *** $P \leq 0.001$.

Biofilms were also analyzed using SEM (Figure 3). We observed that *P. aeruginosa* (Figure 3A, B, C) and *L. monocytogenes* (Figure 3G, H, I) showed high ability to form biofilms in PCL microfibers. In both cases, the microbial biofilms spread across the

1
2
3 mesh, being able to form continuous layers over the microfiber nets. These results are
4
5 consistent with those obtained in CFU counts, which showed very high values of
6
7 bacteria/cm². However, the images obtained for *A. baumannii* are intriguing (Figure 3D,
8
9 E, F), since we could not find a clear evidence of the attachment of this bacteria to PCL
10
11 microfibers through the SEM analysis. CFU data corresponding to the attachment
12
13 analysis of this strain suggested that it has the ability to form biofilms into electrospun
14
15 surface. Therefore, it could be possible that the sample preparation protocol for SEM
16
17 analysis alters the weakly formed biofilm, causing a massive detachment.
18
19
20



53
54
55
56
57
58
59
60

Figure 3: SEM images showing bacterial biofilms of different pathogenic strains formed on PCL fibers. Biofilms formed by *P. aeruginosa* (A, B, C); *A. baumannii* (D,

1
2
3 E, F) and *L. monocytogenes* (G, H, I). Images A, D and G: Original magnification
4 $\times 3000$ (Scale bar =40 μm); Images B, E and F: Original magnification $\times 10000$ (Scale
5 bar =10 μm); Images G, H and I: Original magnification $\times 30000$ (Scale bar =4 μm).
6
7
8
9

10 A qualitative analysis of the bacterial attachment to the PCL meshes was also performed
11 using crystal violet staining (Figure 4). Crystal violet stains the whole biofilm biomass,
12 including cells and extracellular polymeric matrix.³⁴ As expected after the results
13 obtained in the CFU determination and SEM experiments, all meshes appeared
14 completely stained. Depending on the bacterial culture put in contact with the
15 electrospun polymeric surface, different intensities of the stain could be distinguished,
16 suggesting differences in the biomass content of the biofilms attached to them. Meshes
17 incubated with *P. aeruginosa* (Figure 4B) presented the most intense color, followed by
18 the meshes incubated with *A. baumannii* and *L. monocytogenes* (Figure 4C and E
19 respectively), indicating that biofilms formed by this strain presented the higher amount
20 of biomass. Control meshes incubated with culture media without bacteria were not
21 stained by CV (Figure 4A and D). Remarkably, the PCL microfiber meshes incubated
22 with *A. baumannii* showed to be well stained by the crystal violet reagent, indicating the
23 formation of a biofilm by this bacterial specie. This result is coherent with the CFU
24 quantification results obtained for this strain, but not with our observation through SEM
25 analysis. In this regard, as suggested above, it may well be that sample preparation
26 protocol for SEM analysis caused complete bacterial detachment.
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

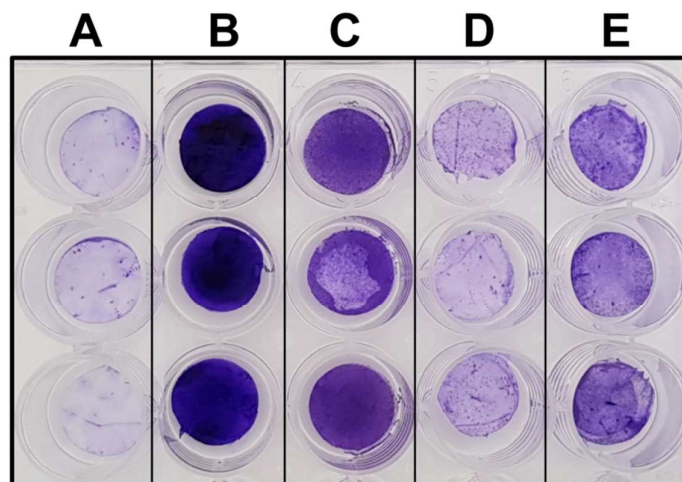
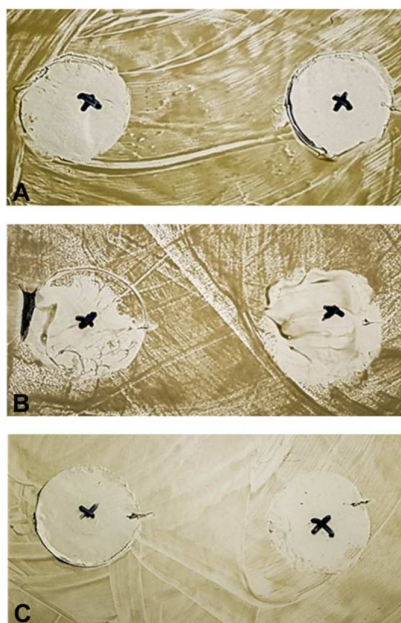


Figure 4: CV staining of biofilms onto PCL microfiber meshes. A: Control meshes incubated with LB; B: Meshes incubated with *P. aeruginosa*; C: Meshes incubated with *A. baumannii*; D: Control meshes incubated with TSB; E: Meshes incubated with *L. monocytogenes*.

Ability of electrospun PCL microfiber meshes to detach preformed biofilms from the substrate.

PCL presents good characteristics such as biocompatibility, biodegradability, and great strength properties, which make this material appropriate to be used as wound dressing.¹² For this reason, we found interesting to examine the ability of the selected bacterial strains to attach to the PCL microfibers in a wound dressing experiment following the method described by Abrigo *et al.*²⁷ Through this method, by putting PCL meshes in direct contact with confluent biofilms grown in agar plates, a realistic model that simulates a wound bed contaminated by bacteria is obtained, and the capacity of the meshes to attract and remove the bacterial cells can be studied. *P. aeruginosa* and *A. baumannii* are frequently involved in wound infections.³² *L. monocytogenes* was also included in this experiment in spite of the fact that wound infections caused by this

1
2
3 pathogen are uncommon. Figure 5 shows agar cultures where the mesh was in contact
4 with biofilms for 1 hour and removed afterwards. As shown in this figure, it can be
5 clearly distinguished where the meshes were placed, as the bacterial biomass present
6 under them was almost completely removed in all cases. In a deep examination of the
7 plates, it was observed that the spots left by *P. aeruginosa* and *L. monocytogenes*
8 (Figure 5A, C) were almost clean and free of remains of culture, which suggests that
9 these strains attached very efficiently to the meshes. In case of *A. baumannii* (Figure
10 5B), remnants of the biofilm could be observed in the spots left once the PCL meshes
11 were removed, which indicates that this pathogen has a lower ability to attach to this
12 material. The obtained results are in concordance with those observed in CFU
13 determination experiments, where *A. baumannii* was the strain that presented the lowest
14 number of attached bacteria to PCL meshes. Nevertheless, the obtained results suggest
15 that electrospun PCL microfibers could be used to attach and significantly remove the
16 bacteria present in the surface of a wound.

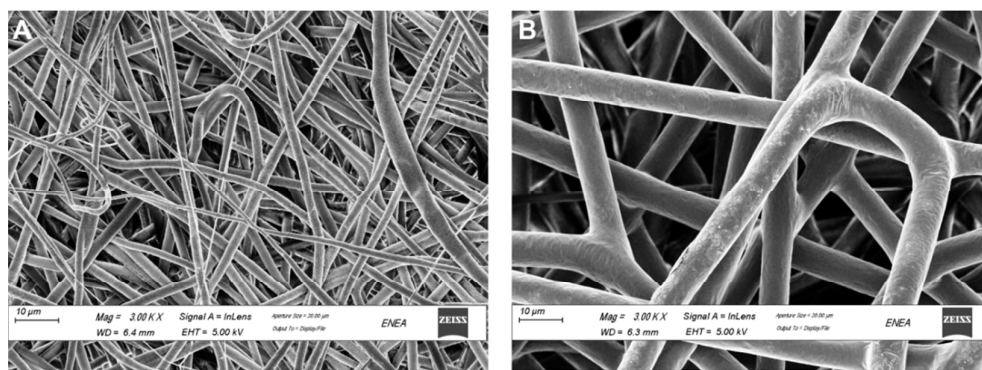


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 **Figure 5:** Agar culture after mesh removal: A: *P. aeruginosa*; B: *A. baumannii*; C: *L.*
4
5 *monocytogenes*.

6
7
8 **Impact of different electrospun PCL mesh microstructure on bacterial attachment.**

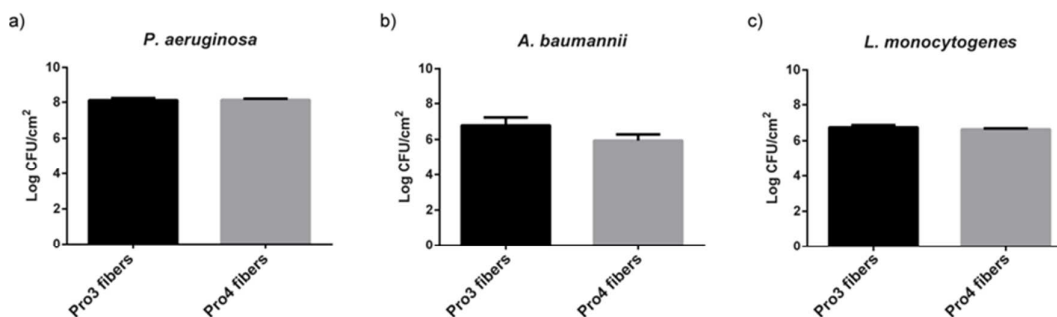
9
10
11 In order to study if the ability of the selected bacterial strains to adhere to the PCL
12 meshes is affected by the microstructure, two types of meshes with clear microstructural
13 differences and different microfiber diameter, named as Pro3 and Pro4, were analyzed
14 using CFU counts and SEM. In Figure 6 the structure of both types of these fibers is
15 represented. Pro3 microfiber meshes (Figure 6A) were designed with an average fiber
16 thickness of 2.21 μm , while Pro4 fiber meshes (Figure 6B) were designed with an
17 average fiber thickness of 6.43 μm .
18
19
20
21
22
23
24
25
26
27
28
29



43 **Figure 6:** SEM images showing the microstructure of Pro3 (A) and Pro4 PCL fibers
44 (B). Differences between both types are clearly distinguishable. Original magnification
45 $\times 3000$ (Scale bar = 10 μm).
46
47
48
49

50
51 Figure 7 displays the results obtained in CFU determinations corresponding to the
52 biofilm formation of *P. aeruginosa*, *A. baumannii* and *L. monocytogenes* on the
53 microfibers Pro3 and Pro4. In case of *A. baumannii*, bacterial counts obtained showed
54
55
56
57
58
59
60

1
2
3 that a higher number of bacteria were attached to the Pro3 material when compared to
4 the Pro4, but the differences obtained were not statistically significant (Figure 7b). *P.*
5 *aeruginosa* and *L. monocytogenes* presented a similar number of bacterial attachment to
6 both fiber types, suggesting that their ability to form biofilms on PCL microfibers is not
7 easily altered by the material microstructure (Figures 7a, c).
8
9
10
11
12
13



14
15
16
17
18
19
20
21
22
23
24
25
26 **Figure 7:** Bacterial attachment (Log CFUs/cm²) to Pro3 fibers (black bars) and Pro4
27 fibers (Grey bars. a): *P. aeruginosa* ; b): *A. baumannii*; c): *L. monocytogenes*. Data
28 represent the mean of three independent replicates (\pm standard deviation, SD).
29
30
31
32

33 To illustrate biofilm formation in both kinds of meshes, *P. aeruginosa*, the pathogen
34 that showed the highest ability to colonize PCL microfibers, was selected to perform
35 SEM experiments (Figure 8). As expected, and in line with the CFU counts, both Pro3
36 and Pro4 presented biofilms spread across the mesh, appearing areas completely
37 covered.
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

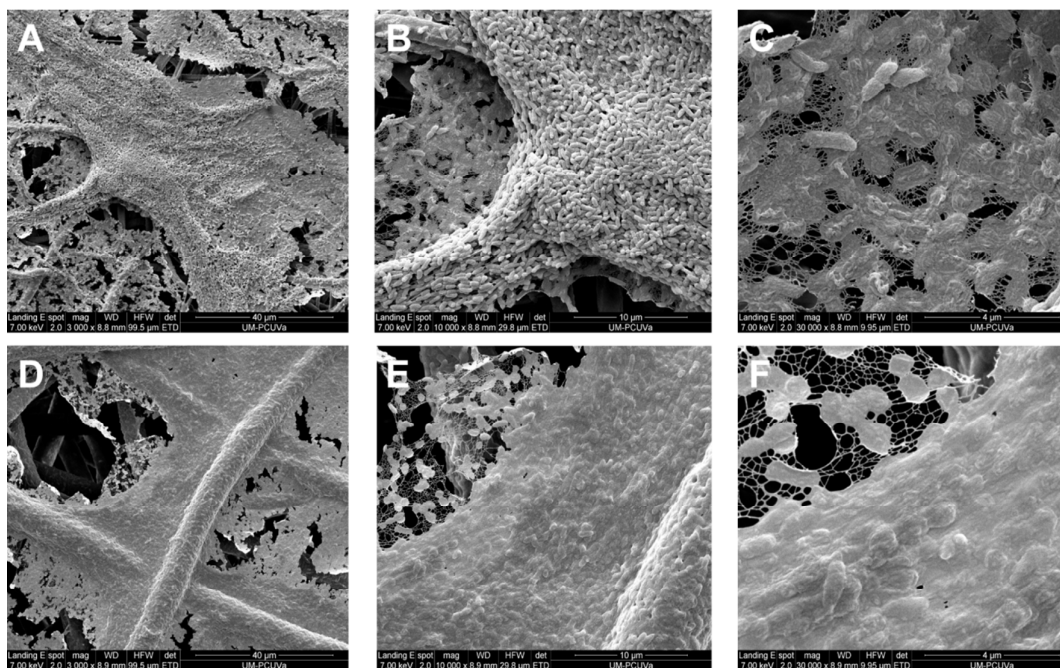


Figure 8: SEM images showing bacterial biofilms of *P. aeruginosa* formed on Pro3 (A, B, C) and Pro4 fibers (D, E, F). A and D: Original magnification $\times 3000$ (Scale bar =40 μm); Images B and E: Original magnification $\times 10000$ (Scale bar =10 μm); Images C and F: Original magnification $\times 30000$ (Scale bar =4 μm).

Previous studies have shown that fiber diameter and spacing influence the attachment to electrospun PS of some pathogenic species.^{26, 27} Abrigo *et al.* concluded that meshes with an average fiber diameter close to bacterial size were the best support for bacterial adhesion.²⁷ In our study, changes in the bacterial load between meshes with different microstructure and microfiber diameter were not observed. In the present study, the microfibers had, in all cases, a diameter higher than that of the selected bacteria, and incubation time of the liquid cultures was much longer (48 hours) than that used by Abrigo *et al.* (1 hour). Therefore, both studies are not clearly comparable, as they focus on different stages of bacterial colonization.

Comparative analysis of bacterial colonization on different materials applied in the food and biomedical fields

The pathogenic strains selected have shown good ability to colonize different electrospun PCL surfaces under the specific experimental conditions used. At this point, we decided to study their colonizing ability in other materials used in medical and food applications as well. Borosilicate glass and electrospun PLA microfiber meshes were selected. Borosilicate glass can be found in hospital environments, being part of medical devices, and coverslips made of this material are commonly used in research studies that involve the development of microbial biofilms. PLA is a thermoplastic and biodegradable aliphatic polyester derived from lactic acid that presents an excellent biocompatibility and mechanical properties, which makes it highly suitable for its use in a wide spectrum of applications in the medical field and in food packaging. The same experimental approach was used. Therefore, CFU counts were carried out on PCL (NBARE meshes), PLA and glass surfaces, and the obtained results were compared. The number of viable bacteria attached to borosilicate glass in comparison to PCL (Log CFUs/cm²) is shown in Figure 9. Clear differences could be observed between both surfaces. In all cases a statistically significant reduction in the number of bacteria attached to glass coverslips was observed. The colonization of PCL fibers was found between 1 and 3 log units higher than that of borosilicate glass coverslips in *P. aeruginosa*, *A. baumannii* and *L. monocytogenes*.

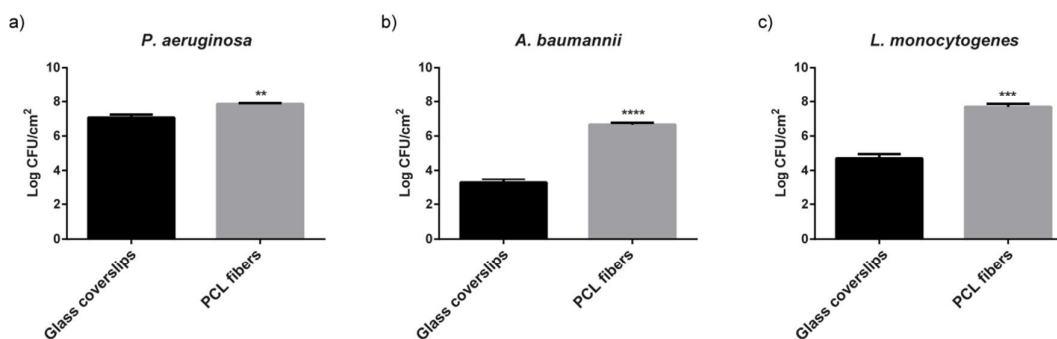


Figure 9: Bacterial attachment (Log CFUs/cm²) to borosilicate glass coverslips (black bars) and electrospun PCL meshes (Grey bars). a): *P. aeruginosa*; b): *A. baumannii*; c): *L. monocytogenes*. Data represent the mean of three independent replicates (\pm standard deviation, SD). Differences were established using a Student's t-test, and considered significant at $P \leq 0.05$. ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

In order to study whether the attachment of the pathogenic strains to electrospun materials is influenced by their composition, the bacterial attachment in commercial PLA meshes was studied and compared with the PCL meshes. The viable bacterial counts corresponding to the biofilm formation in both fiber types is represented in Figure 10. PCL fibers seem to be more likely to be colonized by *A. baumannii* than PLA fibers (Figure 10b), but the observed differences were not statistically significant. In the case of *L. monocytogenes* and *P. aeruginosa*, very similar levels of colonization were observed in both types of fibers (Figure 10a, c). The results obtained in these comparative studies revealed that the scaffold provided by electrospun microfibers facilitates the attachment and proliferation of bacteria, being these structures more suitable to be colonized than borosilicate glass, whose characteristics such as its smooth surface and its hydrophilicity make this material surface more difficult to be colonized by pathogens.³⁵

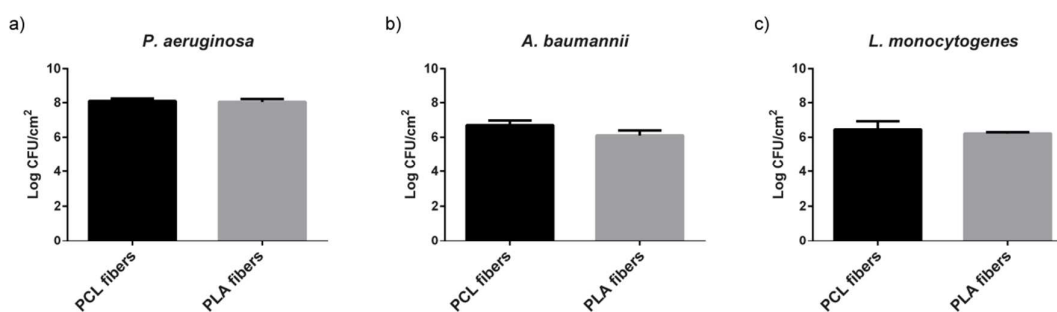


Figure 10: Bacterial attachment (Log CFUs/cm²) to electrospun PCL meshes (black bars) and electrospun PLA meshes (Grey bars) a): *P. aeruginosa*; b): *A. baumannii*; c): *L. monocytogenes*. Data represent three independent replicates (\pm standard deviation, SD).

CONCLUSION

Electrospun PCL microfiber meshes showed to be highly susceptible to be colonized by different pathogenic bacteria, such as *P. aeruginosa*, *A. baumannii* and *L. monocytogenes*, important pathogens involved in nosocomial infections and foodborne outbreaks respectively. SEM and CV experiments indicated that biofilms spread across the mesh, and the microbial biomass is uniformly dispersed along them. PCL and PLA microfiber meshes are better substrata for bacterial adhesion than other materials such as borosilicate glass, suggesting that the 3D structure of electrospun microfibers constitutes a an adequate support for bacterial attachment and proliferation. In this regard, we have also observed that the microfiber microstructure of the electrospun PCL meshes do not seem to influence the bacterial attachment in the species studied after long incubation times. Furthermore, the ability of the PCL meshes to detach biofilms from the substrate, as it was observed in the bacterial transfer experiments, opens the possibility of using this material as an efficient tool for cleaning and disinfecting wounds. The presented results suggest the relevance of functionalizing electrospun

1
2
3 microfibers with antimicrobial or antibiofilm agents prior their use in biomedicine or
4
5 food industry applications to prevent their colonization by undesired pathogenic species.
6
7

8 **AUTHOR INFORMATION**

9

10 **Corresponding Authors**

11
12
13
14 *Carlos Rumbo: E-mail: crumbo@ubu.es
15

16
17 *Juan Antonio Tamayo-Ramos. E-mail: ja.tamayoramos@gmail.com
18

19
20 *Santiago Cuesta López: E-mail: scuestalopez@gmail.com
21
22

23 **Author Contributions**

24
25
26 ‡These authors contributed equally.
27
28

29 **Notes:**

30
31
32
33 The authors A. Rinaldi and F. Caso are affiliated with the company NANOFABER srl
34
35 (www.nanofaber.com).
36
37

38 **ACKNOWLEDGEMENTS**

39

40
41 This research work has been developed at the Consolidated Research Unit UIC-154.
42
43 The Project has received funding from the European Union's H2020 research and
44
45 innovation programme under the Marie Skłodowska-Curie grant agreement N° 691095
46
47 and Junta de Castilla y Leon-FEDER (projects BU079U16 and BU092U16). JATR and
48
49 CRL thank Junta de Castilla y León and FEDER for a postdoctoral contract.
50
51
52
53
54
55
56
57
58
59
60

REFERENCES

- (1) Ulery, B. D.; Nair, L. S.; Laurencin, C. T., Biomedical Applications of Biodegradable Polymers. *J. Polym. Sci. B. Polym. Phys.* **2011**, *49*, 832-864.
- (2) Wei, G.; Ma, P. X., Nanostructured Biomaterials for Regeneration. *Adv. Funct. Mater.* **2008**, *18*, 3566-3582.
- (3) Lu, T.; Li, Y.; Chen, T., Techniques for fabrication and construction of three-dimensional scaffolds for tissue engineering. *Int. J. Nanomedicine.* **2013**, *8*, 337-350.
- (4) Ribba, L.; Parisi, M.; D'Accorso, N. B.; Goyanes, S., Electrospun nanofibrous mats: from vascular repair to osteointegration. *J. Biomed. Nanotechnol.* **2014**, *10*, 3508-3535.
- (5) Son, S. R.; Linh, N. B.; Yang, H. M.; Lee, B. T., In vitro and in vivo evaluation of electrospun PCL/PMMA fibrous scaffolds for bone regeneration. *Sci. Technol. Adv. Mater.* **2013**, *14*, 015009.
- (6) Heydari, Z.; Mohebbi-Kalhari, D.; Afarani, M. S., Engineered electrospun polycaprolactone (PCL)/octacalcium phosphate (OCP) scaffold for bone tissue engineering. *Mater. Sci. Eng. C. Mater. Biol. Appl.* **2017**, *81*, 127-132.
- (7) Reddy, C. S.; Venugopal, J. R.; Ramakrishna, S.; Zussman, E., Polycaprolactone/oligomer compound scaffolds for cardiac tissue engineering. *J. Biomed. Mater. Res. A.* **2014**, *102*, 3713-3725.
- (8) Fu, W.; Liu, Z.; Feng, B.; Hu, R.; He, X.; Wang, H.; Yin, M.; Huang, H.; Zhang, H.; Wang, W., Electrospun gelatin/PCL and collagen/PLCL scaffolds for vascular tissue engineering. *Int. J. Nanomedicine.* **2014**, *9*, 2335-2344.
- (9) Lou, T.; Leung, M.; Wang, X.; Chang, J. Y.; Tsao, C. T.; Sham, J. G.; Edmondson, D.; Zhang, M., Bi-layer scaffold of chitosan/PCL-nanofibrous mat and PLLA-microporous disc for skin tissue engineering. *J. Biomed. Nanotechnol.* **2014**, *10*, 1105-1113.
- (10) Sharif, S.; Ai, J.; Azami, M.; Verdi, J.; Atlasi, M. A.; Shirian, S.; Samadikuchaksaraei, A., Collagen-coated nano-electrospun PCL seeded with human endometrial stem cells for skin tissue engineering applications. *J. Biomed. Mater. Res. B. Appl. Biomater.* **2017**, DOI: 10.1002/jbm.b.33966.
- (11) Cai, E. Z.; Teo, E. Y.; Jing, L.; Koh, Y. P.; Qian, T. S.; Wen, F.; Lee, J. W.; Hing, E. C.; Yap, Y. L.; Lee, H.; Lee, C. N.; Teoh, S. H.; Lim, J.; Lim, T. C., Bio-conjugated polycaprolactone membranes: a novel wound dressing. *Arch. Plast. Surg.* **2014**, *41*, 638-646.
- (12) Nhi, T. T.; Khon, H. C.; Hoai, N. T.; Bao, B. C.; Quyen, T. N.; Van Toi, V.; Hiep, N. T., Fabrication of electrospun polycaprolactone coated with chitosan-silver nanoparticles membranes for wound dressing applications. *J. Mater. Sci. Mater. Med.* **2016**, *27*, 156.
- (13) Fair, R. J.; Tor, Y., Antibiotics and bacterial resistance in the 21st century. *Perspect. Medicin. Chem.* **2014**, *6*, 25-64.
- (14) Zimmerli, W., Clinical presentation and treatment of orthopaedic implant-associated infection. *J. Intern. Med.* **2014**, *276*, 111-119.
- (15) Sutherland, I., Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology.* **2001**, *147*, 3-9.
- (16) Payne, D. E.; Boles, B. R., Emerging interactions between matrix components during biofilm development. *Curr. Genet.* **2016**, *62*, 137-141.
- (17) Davey, M. E.; O'Toole, G. A., Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev* **2000**, *64*, 847-867.

- 1
2
3 (18) Donlan, R. M.; Costerton, J. W., Biofilms: survival mechanisms of clinically
4 relevant microorganisms. *Clin. Microbiol. Rev.* **2002**, *15*, 167-193.
- 5 (19) Donlan, R. M., Biofilms on central venous catheters: is eradication possible?
6 *Curr. Top. Microbiol. Immunol.* **2008**, *322*, 133-161.
- 7 (20) Kriegel, C.; Arecchi, A.; Kit, K.; McClements, D. J.; Weiss, J., Fabrication,
8 functionalization, and application of electrospun biopolymer nanofibers. *Crit.*
9 *Rev. Food Sci. Nutr.* **2008**, *48*, 775-797.
- 10 (21) Noruzi, M., Electrospun nanofibres in agriculture and the food industry: a
11 review. *J. Sci. Food Agric.* **2016**, *96*, 4663-4678.
- 12 (22) Bridier, A.; Sanchez-Vizueté, P.; Guilbaud, M.; Piard, J. C.; Naitali, M.;
13 Briandet, R., Biofilm-associated persistence of food-borne pathogens. *Food*
14 *Microbiol.* **2015**, *45*, 167-178.
- 15 (23) Srey, S.; Jahid, I. K.; Ha, S.-D., Biofilm formation in food industries: A food
16 safety concern. *Food Control.* **2013**, *31*, 572-585.
- 17 (24) Mitik-Dineva, N.; Wang, J.; Truong, V. K.; Stoddart, P.; Malherbe, F.;
18 Crawford, R. J.; Ivanova, E. P., *Escherichia coli*, *Pseudomonas aeruginosa*,
19 and *Staphylococcus aureus* attachment patterns on glass surfaces with
20 nanoscale roughness. *Curr. Microbiol.* **2009**, *58*, 268-273.
- 21 (25) Yuan, Y.; Hays, M. P.; Hardwidge, P. R.; Kim, J., Surface characteristics
22 influencing bacterial adhesion to polymeric substrates. *RSC Adv.* **2017**, *7*,
23 14254-14261.
- 24 (26) Kargar, M.; Ji, W.; Amrinder, N. S.; Bahareh, B., Controlling bacterial
25 adhesion to surfaces using topographical cues: a study of the interaction of
26 *Pseudomonas aeruginosa* with nanofiber-textured surfaces. *Soft Matter.* **2012**,
27 *40*, 10254-10259.
- 28 (27) Abrigo, M.; Kingshott, P.; McArthur, S. L., Electrospun polystyrene fiber
29 diameter influencing bacterial attachment, proliferation, and growth. *ACS Appl.*
30 *Mater. Interfaces.* **2015**, *7*, 7644-7652.
- 31 (28) Abrigo, M.; Kingshott, P.; McArthur, S. L., Bacterial response to different
32 surface chemistries fabricated by plasma polymerization on electrospun
33 nanofibers. *Biointerphases.* **2015**, *10*, 04a301.
- 34 (29) Peleg, A. Y.; Seifert, H.; Paterson, D. L., *Acinetobacter baumannii*: emergence
35 of a successful pathogen. *Clin. Microbiol. Rev.* **2008**, *21*, 538-582.
- 36 (30) Lister, P. D.; Wolter, D. J.; Hanson, N. D., Antibacterial-resistant
37 *Pseudomonas aeruginosa*: clinical impact and complex regulation of
38 chromosomally encoded resistance mechanisms. *Clin Microbiol Rev* **2009**, *22*,
39 582-610.
- 40 (31) NicAogain, K.; O'Byrne, C. P., The Role of Stress and Stress Adaptations in
41 Determining the Fate of the Bacterial Pathogen *Listeria monocytogenes* in the
42 Food Chain. *Front. Microbiol.* **2016**, *7*, 1865.
- 43 (32) Di Domenico, E. G.; Farulla, I.; Prignano, G.; Gallo, M. T.; Vespaziani, M.;
44 Cavallo, I.; Sperduti, I.; Pontone, M.; Bordignon, V.; Cilli, L.; De Santis, A.;
45 Di Salvo, F.; Pimpinelli, F.; Lesnoni La Parola, I.; Toma, L.; Ensoli, F.,
46 Biofilm is a Major Virulence Determinant in Bacterial Colonization of Chronic
47 Skin Ulcers Independently from the Multidrug Resistant Phenotype. *Int. J.*
48 *Mol. Sci.* **2017**, *18*. DOI: 10.3390/ijms18051077.
- 49 (33) Branda, S. S.; Vik, S.; Friedman, L.; Kolter, R., Biofilms: the matrix revisited.
50 *Trends Microbiol.* **2005**, *13*, 20-26.
- 51 (34) Merritt, J. H.; Kadouri, D. E.; O'Toole, G. A., Growing and analyzing static
52 biofilms. *Curr. Protoc. Microbiol.* **2005**, *Chapter 1*, Unit 1B.1.
- 53
54
55
56
57
58
59
60

- 1
2
3 (35) Donlan, R. M., Biofilms: microbial life on surfaces. *Emerg. Infect. Dis.* **2002**,
4 8, 881-890.
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Graphical abstract

