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1 **Minimizing bacterial adhesion on membrane: multiscale**
2 **characterization of surface modifications**

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16 **Highlights**

- 17 • Filtration at low frontal flux allows to optimize vanillin coating on PES membranes
- 18 • Homogeneous vanillin coating was confirmed by macroscopic and microscopic methods
- 19 • Single Cell Force Spectroscopy validates the reduction of bacterial adhesion forces
- 20 • AFM and EFM assess vanillin coating effect at different stages of biofilm growth
- 21 • PES-VAN membrane is efficient to reduce bacterial adhesion and EPS production
- 22

23 **Abstract**

24 The control of biofouling is required to improve membranes performances for water filtration. In this
25 context, developing bioinspired approaches to modify membranes and give them antifouling properties is
26 an interesting alternative. In this study, we elaborated new anti-biofouling membranes by coating them
27 with vanillin, a natural bioactive molecule acting as a Quorum Sensing Inhibitor (QSI). First, the
28 adsorption of vanillin by filtration was optimized to obtain homogeneous surface modifications. The
29 modified membranes were then characterized in terms of physical-chemical properties: the results showed
30 that they were more hydrophobic and exhibited a 14% decrease in pure water flux. Then, their anti-fouling
31 properties were evaluated using complementary multiscale characterization methodologies; AFM single-
32 cell experiments showed that vanillin adsorption had an effect on both the probability of bacterial cells to
33 adhere to the surface, and on the force of adhesion. Direct EFM and SEM observation and adhesion assays
34 further confirmed the effect of vanillin-modified membranes at the population-scale which showed a
35 decrease in the bacterial coverage rate up to 50 %. Altogether, it confirms the multifunctionality of
36 vanillin-coated membranes against biofouling: reduction of bacterial initial adhesion by changing the
37 membranes surface properties and reduction of biofilm formation by quorum sensing inhibition.

38

39

40 **Keywords**

41 Microfiltration, anti-biofouling, surface modification, interfacial characterization, atomic force
42 microscopy

43

1. Introduction

45 Because of their high efficiency for water pollution remediation and the high quality of the produced
46 water, membrane processes are nowadays considered as improved technologies for sustainable
47 management of water resources [1, 2]. However, some issues remain, such as membrane biofouling,
48 which significantly hinders the development of these processes. Membrane biofouling occurs through the
49 deposition and accumulation of microorganisms on membrane surfaces. Approaching the surface, various
50 sequential mechanisms are identified: initial reversible adhesion controlled by van der Waals, electrostatic
51 and hydrophobic interactions, production of exopolymeric substances (EPS) leading to the irreversible
52 attachment of the bacteria and implying steric interaction, then the development of the biofilm to a mature
53 state at the surface and inside the pores of the membrane [3-5]. It results in a significant loss of membrane
54 performance (increase in the pressure drop, decrease in membrane permeability, and decline in product
55 quality) [6]. For these reasons, biofouling is responsible for a large part of the operating costs associated
56 with membrane processes, which include high-frequency cleaning cycles (use of chemicals, water, and
57 energy for back-flush and cleaning in place) and, consequently, replacement of filtration units after
58 premature ageing. PolyEtherSulfone (PES) membranes are widely used for microfiltration, ultrafiltration,
59 or nanofiltration in wastewater treatment, water disinfection processes, and pretreatment prior to
60 desalination mainly because of their resistance to cleaning solutions [7]. But even in this case, to control
61 biofouling development on these membranes, extensive chemical cleaning steps are needed, which
62 generate all the disadvantages previously mentioned and considerably shorten the lifetime of membrane
63 modules [8, 9]. In this context, controlling bioadhesion and biofouling would stabilize the water flux and
64 help improve cleaning protocols, thereby meeting industrial challenges to increase membranes' lifetime
65 and develop more efficient filtration processes for water treatment.

66 Different strategies to control biofouling and improve membrane cleaning have been explored. While
67 optimization of hydrodynamic conditions represents an important part of these strategies, membrane
68 surface modifications are also investigated [5, 10]. These recent years, bioinspired membranes have
69 attracted increasing interest to improve the performances of the separation processes they are involved in
70 [11]. These membranes reproduce the properties (forms, functions, etc.) of biological systems to solve
71 technological problems, and in this sense, they represent the next generation of filtration membranes for
72 water treatment [11-15]. Among these biological functions, those that control material fouling represent an
73 important target as they could significantly contribute to improving the performance of membranes and of
74 water treatment plants in which they are involved [16]. For that, some membrane modification strategies
75 considered are those that: (i) mimic biological structures, such as grafting of polymers containing
76 zwitterionic moieties [17], coating of thin film polymer brushes [18, 19], and creating Sharklet patterns on
77 the membrane surface [20-22] to prevent physical-chemical interactions, and/or (ii) mimic biological
78 functions, such as grafting to or adding in the bulk material of membranes antibacterial or enzymatic
79 molecules to inhibit and/or kill microorganisms [23]. However, while for antibacterial there is a high risk
80 to develop bacterial resistance, enzymes are costly and unstable [14]. The contact-type antibiofouling
81 effects are promising to limit the adhesion of bacteria to the surface but is limited to micro-organisms that
82 come into contact with the surface [24] with a risk for the cell contents to be released into the treated
83 water. Thus, membrane surface modifications combining a reduction of the bacterial adhesion and in the
84 biofilm development in a single step, easy to process and using non-dangerous chemicals are required.

85 An alternative and promising approach is to inhibit Quorum Sensing (QS), which is a form of intercellular
86 communication used by bacteria to coordinate biofilm formation [25]. Inhibiting QS is a way to reduce the
87 production of bacterial exopolysaccharides (EPS) needed for biofilm formation and responsible for
88 biofouling growth, without the issues associated with antibacterial or enzymes. These recent years,
89 numerous QS inhibitors (QSI) have shown promising results [26-29] towards different bacterial species.
90 In nature, plants produce phytochemicals, such as phenolic aldehydes, which act as antimicrobial agents
91 against a wide range of microbial species [30]. In particular, vanillin, a phenolic aldehyde from vanilla
92 bean extract and a known QSI, reduced the biofilm of *Aeromonas hydrophila* isolated from a biologically

93 fouled reverse osmosis (RO) membrane on polystyrene surface by up to 46.3% [31, 32]. It was also
94 demonstrated that a solution of 2 mM of vanillin could interfere with QS by binding to the active site of
95 PqsR which acts as a response regulator in the QS signaling molecule, reducing *Pseudomonas*
96 *aeruginosa*'s twitching motility and virulence [33, 34]. In addition to these demonstrated effects, vanillin
97 presents major advantages compared to other phenolic compounds used for their bactericidal effects: (i) it
98 is recognized as safe by the FDA (Generally Recognized As Safe, GRAS status), (ii) it has a disruption
99 effect on bacteria cell wall probably due to its low intrinsic hydrophobicity and homeostatic effects [35],
100 and (iii) it exhibits a bacteriostatic effect rather than a bactericidal one at measured inhibitory
101 concentrations (MIC), preventing the establishment of bioresistance mechanisms [36]. Moreover, vanillin
102 has recently been shown to inhibit biofilm formation in different bacterial species [37], while the use of
103 other QSI may be species-dependent [38]. Lastly, it was also demonstrated that vanillin could cause
104 changes in the amount and composition of EPS produced by bacteria, which has consequences on the
105 formation of biofilms and their structure [31, 37, 39-41]. Because of the multifunctional effects of vanillin,
106 it is a promising candidate to be incorporated into a membrane material or grafted at a membrane surface
107 to improve biofouling control. However, these multifunctional effects should be investigated further.

108 There are already studies exploring the effects of vanillin-modified membranes against different species.
109 However, these studies are mainly focused on bacterial growth inhibition or on the changes it caused on
110 the hydrophilicity or roughness of the materials, and there seems to be contradicting results when it comes
111 to vanillin quantity, hydrophilicity, and membrane performance [42-45]. For instance, Esmaeili *et al.*
112 showed an increase in polyethylene glycol (PEG) solution permeability after vanillin adsorption [42], but
113 the correlation of permeability with hydrophilicity and vanillin adsorption on the membrane surface at
114 high concentration of vanillin ($>1.8 \text{ g.L}^{-1}$) was not clearly demonstrated. The authors thus suggested that
115 the physical changes of the membrane surface could be responsible for the modification of the filtration
116 performances. In another study, Shin *et al.* found a decrease of the water contact angle (WCA) on thin-
117 film composite (TFC) RO membrane coated with vanillin (28°) compared to pristine membranes (40°),
118 and there was minimal decrease in the surface roughness [43]. Despite the improvement in hydrophilicity,
119 a 10% decrease in pure water permeability after the modification was reported. Nonetheless, they
120 confirmed the inhibition of bacterial growth on the vanillin-coated membrane for *Escherichia coli* and
121 *Bacillus subtilis* after 24 h of cultivation. However, no investigation was performed to observe the initial
122 stage of bacterial adhesion. Lastly, Kumar *et al.* worked on the incorporation of vanillin by blending it
123 into PES-based polymers [45]. When compared to virgin PES membranes, vanillin-modified membranes
124 showed a higher permeability caused by an increase in the hydrophilicity and higher pore radius and
125 volume due to thermodynamic instability. It also resulted in a bacteriostatic effect towards *E. coli* and *P.*
126 *aeruginosa*. Although these studies illustrate new possibilities that the incorporation of vanillin, as a
127 bioinspired approach, may offer to control membrane biofouling, further studies are needed to correlate
128 the change of surface properties caused by vanillin and membrane performance. There are several
129 hypotheses that could explain the anti-biofilm effect of vanillin membranes. It may be due to (i) the
130 modification of the physical-chemical properties of the membrane by vanillin, or (ii) the biological
131 properties of QS inhibition of vanillin, or (iii) a combined effect (additive or synergistic) of both. These
132 could affect biofilm development at different stages, from initial adhesion to biofilm maturation (EPS
133 production). While existing studies have focused on bacterial growth on modified membranes, so far, no
134 work has investigated the effect of vanillin incorporation in membranes at different stages from initial
135 bacterial adhesion/attachment to biofilm development. Moreover, the biofouling assessment techniques
136 used for vanillin-modified membranes were indirect methods. Given the high sensitivity of biofilm
137 formation to its environment, it is more appropriate to use direct observation of the biofouling evolution
138 without introducing multiple environmental changes.

139 In this study, we modified with vanillin the surface of microfiltration membranes used for water treatment
140 in reuse applications. For that, vanillin was adsorbed on PES membrane surfaces by filtration in optimized
141 condition to increase the load rate and homogeneity of the surface coverage. Using these membrane
142 samples, we then used, for the first time, a combination of direct and indirect techniques for surface
143 characterization and imaging techniques at different size scales from macro- (contact angle measurement,
144 FTIR mapping) to nanoscale (atomic force, epifluorescence, and electron microscopy) to further
145 investigate and correlate the effects of vanillin on the membranes' physical-chemical properties, pure
146 water flux, and initial bacterial attachment and biofilm maturation. Particularly interested in the first stage
147 of adhesion, a new protocol was developed and assessed to directly evaluate bacterial adhesion at the
148 population scale and coupled with measurements at single-cell level using AFM. This original approach
149 provides a better understanding of the mechanisms responsible for the control of biofouling from a
150 bioinspired approach using a natural biochemical molecule.

151 **2. Experimental**

152 **2.1. Chemicals and Materials**

153 **Chemicals**

154 Vanillin (99% reagent grade) was purchased from Alfa Aesar (UK). Phosphate-buffered saline solution
155 (PBS 1X) was obtained from the dilution of a 10X stock solution provided by ThermoFisher. Culture
156 media were prepared from powders: Tryptic Soy Broth (Corning), and Tryptic Soy Agar (VWR
157 chemicals).

158 Fluorescent probes, SYBR™ Green I nucleic acid gel stain (10000X in DMSO), and Concanavalin A
159 tetramethyl rhodamine conjugate, were provided by Invitrogen ThermoFisher Scientific.

160 The solvents used in this work, methanol (HPLC grade), ethanol absolute (analytical grade), formaldehyde
161 solution (38% in water), and hexamethyldisilazane (reagent grade, ≥99%), were purchased from Sigma-
162 Aldrich Merck (FR). Hydrochloric acid 37% was from Acros Organics.

163 The commercial membrane used as support for this study was polyethersulfone (PES) membrane with a
164 pore size of 0.04 μm, MicroPES® 1F PH. It was provided by Membrana GmbH (3M, Germany,
165 Wuppertal).

166 **Bacterial strains and growth conditions**

167 *Pseudomonas aeruginosa* (PAO1, CIP104116) was purchased from the Centre de Ressources Biologiques
168 de l'Institut Pasteur (France) as a freeze-dried sample. A single colony from Tryptic Soy Agar (TSA) was
169 inoculated in 50 mL of Tryptic Soy Broth (TSB). After cultivation in TSB medium for 24h at 37°C,
170 glycerol 20% final concentration was added to the culture. The mixture was divided into 1 mL aliquots
171 that were stored at -20°C until their preparation for the experiments. Before the experimentation, 100 μL
172 of *P. aeruginosa* cells from aliquot were cultured in TSB, 37°C, 150 rpm for 15h. Cells were then
173 harvested by centrifugation (4100 g, 10 min, 6°C, JOUAN-G4-11) and rinsed in PBS 1X twice.

174 **2.2. PES membranes coating with vanillin and evaluation of membrane filtration** 175 **performances**

176 **Surface modification of PES membrane with vanillin via coating**

177 PES membrane modification was obtained by filtration of vanillin through the PES membrane; the
178 resulting membranes samples were called PES-VAN. As a control, membranes were also conditioned
179 with only the solvent, PBS 1X pH=3.8, and were called PES-PBS. For that, PES membranes were cut into
180 44 mm diameter pieces and rinsed multiple times in hot MilliQ water (<60°C) to remove the preservative

181 agents. Membrane pieces were placed in a dead-end Amicon® filtration cell (Series 8050, Merck
182 Millipore) with a backing fibrous layer with high porosity to avoid alteration of the membrane surface
183 during filtration. The effective membrane surface is 13.4 cm² according to commercial specifications.

184 Pure water permeability was measured after a membrane compaction step at 0.6 bar for one hour
185 (necessary duration to reach a stable permeate flux) and served as the ultimate washing of the membrane
186 before the coating step. Filtration methods will be elaborated further in a section below.

187 Vanillin solution of 2000 mL at 3 g.L⁻¹ in PBS 1X, pH adjusted to 3.8, was filtered in this system at
188 different transmembrane pressures, from 0.05 to 0.4 bar. The pH adjustment allows the vanillin to be in its
189 protonated form (pKa 7.4) to avoid repulsive interaction with the negatively charged PES membrane [46].
190 After coating, PES-VAN membranes were rinsed with PBS 1X pH 3.8 to remove non-adsorbed vanillin
191 from the surface and were then cut into several small pieces for the different characterization experiments.

192 Vanillin stability on the membrane was evaluated in two ways. The first evaluation was desorption under
193 stirring wherein the PES-VAN membrane samples were immersed in ultrapure water and agitated at 150
194 rpm. The second assessment was through dead-end filtration at 0.1 bar wherein ultrapure water was
195 filtered through the membranes. The desorption tests were done in triplicates for similar time (three hours)
196 and water volume (2000 mL).

197 **Coating quantity**

198 The coating quantity of vanillin was obtained after extraction of the adsorbed vanillin from two or three
199 pieces of the same membrane (n≥3) in an ultrasonic methanol bath. Based on a protocol described in the
200 literature [47], this step was repeated three times. UV spectrophotometer (Lambda 365 Perkin Elmer)
201 quantified the vanillin concentration in methanol according to a specific calibration curve. The coating
202 quantity was calculated as the mean of membrane samples after summing the concentration of vanillin in
203 methanol after three runs of extraction for each membrane sample.

204 **Filtration experiments**

205 Pure water flux, J, was assessed before and after the surface modification of the compacted PES
206 membrane using equation 1 and the same filtration set-up as the one used for the PES membrane coating
207 with vanillin.

$$208 \quad J = \frac{\text{Permeate volume (L)}}{\text{Effective membrane area (m}^2\text{)} \times \text{Time (h)}} \quad (1)$$

209 To assess the change in membrane filtration performance, pure water permeability, L_p, (equation 2) and
210 flux decline ratio (FDR) (equation 3) were then calculated from the fluxes. J_o and J_f are the pure water
211 fluxes before and after the membrane coating, respectively. FDR represents the flux decline after the
212 modification step.

$$213 \quad L_p = \frac{J}{\Delta P} \quad (2)$$

$$214 \quad FDR = \frac{J_f}{J_o} \quad (3)$$

215 **2.3. Characterization**

216 **Water contact angle measurements**

217 Water contact angles (WCA) of membranes modified at different coating conditions were measured using
218 the sessile drop method (Drop Shape Analyzer DSA30, KRÜSS, Germany). The results showed how the
219 hydrophilicity changed upon modification of the membrane surface. 10 to 15 measurements were
220 performed for each sample at different locations to reduce the experimental error. Average values were
221 reported.

222 **FTIR and IR mapping**

223 Fourier transform infrared (FTIR) mapping of virgin and modified membranes was performed to
224 determine the minimum quantity necessary to obtain a homogeneous coverage of the vanillin on the
225 membrane surface, as described elsewhere [48]. Briefly, a Micro-FTIR spectrometer (iN10MX Thermo
226 Fisher Scientific Nicolet) equipped with a liquid nitrogen-cooled MCT detector was used in ATR mode to
227 scan 1.25 x 1.25 mm for each sample, meaning 6.25 mm² membrane surface for 4 samples in a row. One
228 spectrum was registered for each 50x50 μm point (one point was measured every 50 or 100 μm) with a
229 spectral resolution of 8 cm⁻¹ for 16 scans. To generate the chemical maps, first, the absorbance peak
230 unique to vanillin was identified, which is 1511 cm⁻¹, corresponding to the aromatic ring [49]. Then, the
231 peak height was measured by taking the baseline limit. The chemical maps generated were color-coded
232 according to the absorption peak height intensity with a rainbow color scale from blue (lowest intensity) to
233 red (highest intensity). A higher peak intensity indicates more vanillin is present on the surface. The
234 generated FTIR maps were not processed further except for the atmospheric correction. This technique
235 visualizes the coating coverage on the surface but does not provide quantitative data. Thus, a python code
236 was developed, as described elsewhere [50], to convert the colors on the chemical maps to peak height
237 intensity average and standard deviation values. To summarize, first, the code transforms the image
238 having a rainbow scale to a grey scale, from black (lowest intensity) to white (highest intensity). Then,
239 using the grey values obtained from the image, it calculates the peak height intensity average value and
240 standard deviation.

241 Attenuated total reflectance FTIR spectroscopy (ATR-FTIR) was used to validate the presence and
242 relative quantity of vanillin on the PES membrane surface modified at different coating conditions.
243 Average ATR-FTIR spectra were obtained from 3 independent measurements using ThermoNicolet 6700
244 spectrometer with Diamond ATR equipped with a DTGS detector. The spectral resolution was 4 cm⁻¹, and
245 16 scans were taken between 400 and 4000 cm⁻¹. The ratio of the absorbance of vanillin's specific peak at
246 1510 cm⁻¹ (aromatic ring) to the PES's specific peak at 1485 cm⁻¹ (aromatic band) was calculated to
247 estimate the relative quantity of vanillin at different coating conditions.
248

249 **AFM imaging, surface roughness, and apparent pore size estimation**

250 Surface topography images of PES-PBS or PES-VAN membranes were recorded using atomic force
251 microscope, AFM Nanowizard III (Bruker, USA), in contact mode in PBS 1X pH 7.4 at room
252 temperature, using MLCT cantilevers (Bruker, USA, nominal spring constant of ~0.01 N/m, determined
253 using the thermal noise method [51]) with a scanning rate of 4 Hz and a resolution of 512 x 512 lines.
254 Roughness measurements (Ra) were performed using JPK Data Processing software (Bruker, USA) on 5
255 areas of 0.5 x 0.5 μm at the surface of the membranes. The apparent pore size was calculated using two
256 10x10 μm AFM images of each sample. For each image, 15 pore size measurements were performed with
257 standard image analysis procedures using ImageJ. Lastly, the mean and standard deviation were
258 calculated.

259 **2.4. Bacterial adhesion and antifouling properties evaluation**

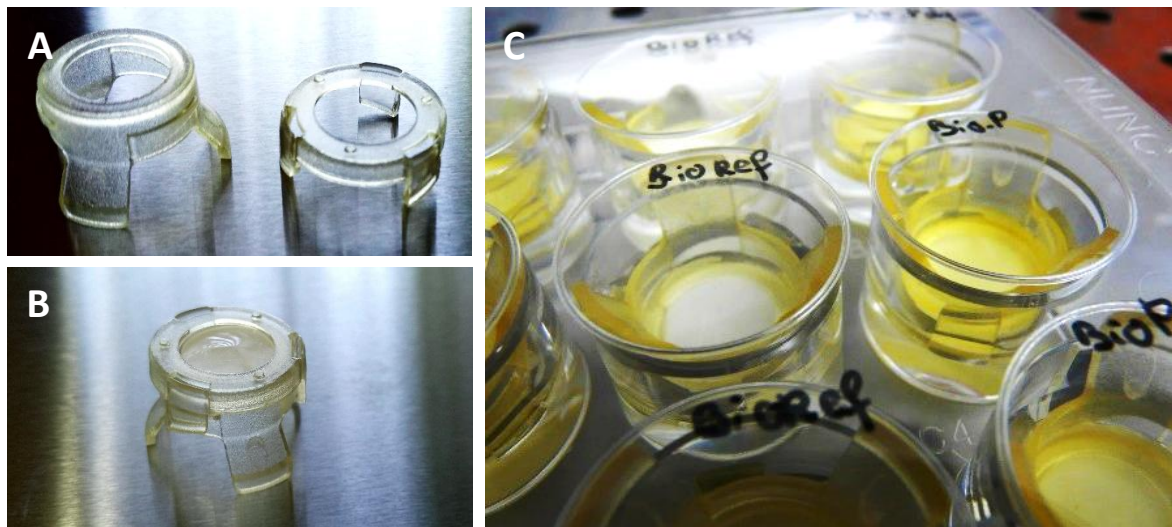
260 **Adhesion test and microscopic observations**

261 The effect of vanillin on the adherence of *P. aeruginosa* (*Pa*) to the PES membrane surface was studied by
262 performing culture tests in microplates. Several tests with the control membranes, PES-PBS, were
263 performed to validate the conditions under which the adhesion tests will be carried out, with the objective
264 to reduce the disturbances that may result from agitation and sedimentation conditions. Indeed, agitation
265 of the medium can generate stress and stimulate the production of EPS which will hinder the counting and
266 estimation of the number of cells adhered to the surface of the membrane [52]. Furthermore, if the
267 membrane is placed at the bottom of the culture well, in the absence of agitation, the deposition of DNA
268 and cell fragments by sedimentation can lead to a bias in the estimation of the initial adhesion of cells to
269 the PES membrane surface [53]. To counteract the sedimentation phenomena, inserts (CellCrown,
270 Scaffoldex) were used to keep the membrane coupon immersed upside down in the medium on the top of

271 the culture well (Figure 1). Thus, only mass transport mechanisms governed by convection and diffusion
272 are responsible for bacterial colonization and adhesion in our experiments.

273 Bacteria adhesion tests (biotic condition) with and without inserts, under agitation at 150 rpm and without
274 agitation, were first performed on triplicate PES-PBS membrane samples (1.5 cm²) in a 12-well culture
275 plate. Once the optimum conditions were identified, bacterial adhesion tests were then performed on PES-
276 VAN membranes. As a control, triplicates of PES-PBS and PES-VAN membrane samples were also
277 introduced in sterile 12-well culture plates under abiotic conditions.

278 For abiotic condition tests, 2 mL of sterile PBS 1X at pH 7.4 was added to each well. This pH was chosen
279 as the physiological pH for bacterial assays. For the biotic condition, 2 mL of *Pa* resuspended in PBS 1X
280 (pH 7.4) at a cell concentration of about 10⁷ CFU.mL⁻¹ was added to the corresponding well. Lastly, the



281 plates were incubated at 37°C for 3h in the dark.

282 **Figure 1.** CellCrown inserts to support the membrane during adhesion experiments. (A) Opened insert,
283 (B) Insert with membrane, (C) Inserts in a 12-well culture plate.

284 **EFM imaging**

285 The bacteria adhered to the membrane surface were directly observed under an epifluorescence
286 microscope (EFM) after specific staining. The membrane samples were carefully rinsed three times with 1
287 mL of PBS 1X (pH = 7.4; ThermoFisher) to remove the bacteria not adhered to the surface. For the
288 labeling step, the samples were completely immersed for 15 min in a solution of PBS 1X and SYBR™
289 Green (Invitrogen, USA) with a final stain concentration of 10X. The staining solution was then gently
290 removed, and the samples were rinsed with PBS 1X to remove the excess stain before microscopic
291 observations.

292 The calculation of the coverage rate by *Pa* was carried out with the Zeiss Zen Blue software on three
293 independent membrane samples taking 40 to 50 images on each sample to obtain a satisfactory statistical
294 representation of the observed phenomenon. A Z-stack acquisition (10 in-depth scans) was performed at
295 20x magnification. The Extended Depth of Focus tool was used to reconstruct a planar image when
296 necessary. The bacteria were quantified by defining a circular area with a diameter of 216 μm to eliminate
297 the non-homogeneous fluorescence between the center and the edge of the image.

298 **SEM imaging**

299 To further verify the structuration of the biofilm on PES-PBS and PES-VAN membranes, images were
300 taken using a scanning electron microscope (SEM-FEG Jeol JSM 7100F-TTLS Tokyo, Japan) at 10 kV.
301 Before performing SEM observations on biological samples, it is essential to have a good sample
302 preparation protocol. For this purpose, the protocol described by Voegel *et al.* [54] was adapted to the
303 membrane samples. They were immersed in a solution of 4% paraformaldehyde (1:2), PBS 1X (1:4), and
304 ultrapure water (1:4) for 1h at room temperature, as a fixation step. Then, they were washed twice with
305 PBS 1X and 0.4 M sucrose (1:2) solution. Lastly, in consecutive order, the samples were gradually
306 dehydrated in ethanol-water solution (50%, 70%, 90% of ethanol for 5 min each), pure ethanol (30 min),
307 ethanol – hexamethyldisilazane (HMDS) solution (50%, 75% of HMDS for 15 min each), and pure
308 HMDS (until total evaporation). Afterwards, a 60 s metallization with gold was immediately performed.

309 **AFM Single-Cell Force Spectroscopy experiments**

310 Cell probes were prepared using the protocol described by Beaussart *et al.* [55]. Briefly, colloidal probes
311 were obtained by attaching a single silica microsphere (5 μm diameter, Bangs Laboratories) with a thin
312 layer of UV-curable glue (NOA 63, Norland Edmund Optics) on triangular tipless cantilevers (NP-O10,
313 Bruker, USA) and using a Nanowizard III AFM (Bruker, USA). The cantilevers were then immersed for 1
314 h in PBS 1X containing 4 mg/mL of dopamine hydrochloride (Sigma-Aldrich), rinsed in PBS 1X, and
315 used directly for cell probe preparation. The cantilever spring constant was determined prior to cell
316 immobilization using the thermal noise method [51]. To attach the bacterial cell, the colloidal probe was
317 then brought into contact with an isolated bacterium and retracted. Proper attachment of the cell on the
318 colloidal probe was checked using optical microscopy. These cell probes were used to measure the
319 interactions between cells and PES-PBS or PES-VAN membranes at room temperature, using a maximum
320 applied force of 0.25 nN, a constant approach retraction speed of 2.0 $\mu\text{m}/\text{s}$, and a contact time of 1 s, on
321 areas of 1 x 1 μm . Data were analyzed using the Data Processing software from JPK Instruments (Bruker,
322 USA). Adhesion forces histograms were obtained by measuring the height of the biggest peak in each
323 retract curves for each force curve. For each condition, experiments were repeated for 7 bacteria coming
324 from 3 independent cultures.

325 **AFM quantification of the membrane hydrophobicity**

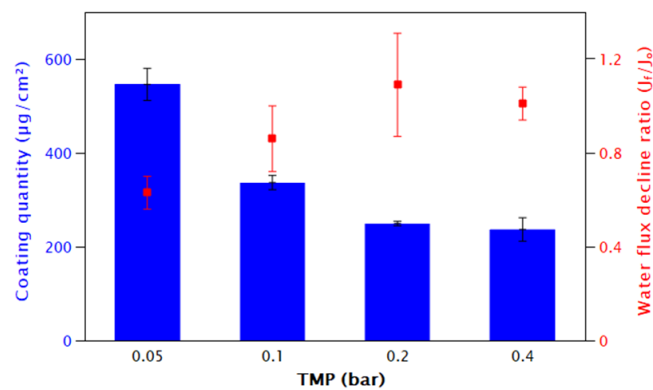
326 To measure the membrane's surface hydrophobicity, a recently developed method was used [56] which
327 consists in measuring, using Fluidic force microscopy (FluidFM), the interactions between a bubble
328 (hydrophobic interface in water) and the membranes. For that, FluidFM probes with an aperture of 8 μm
329 in diameter (Cytosurge AG, Switzerland) were hydrophobized by coating with self-assembled monolayers
330 (SAMs) of silanes *via* SAMs vapor deposition technique. FluidFM cantilevers were functionalized with
331 1H,1H,2H,2H-Perfluorodecyltrichlorosilane (FDTS) using Orbis-1000 equipment (Memsstar, Livingston,
332 UK) to make their external surface and inside microchannel hydrophobic. The deposition was realized
333 under vacuum at 0.05 bar and -40°C, for 5 min. Then, the microchannel of these silanized cantilevers was
334 filled with air and the probe was immersed in PBS 1X. To eliminate any particle or dust contamination or
335 to prevent clogging of the FluidFM cantilever, a slight overpressure of 20 mbar was applied. Then, to
336 produce a bubble at the aperture of the cantilever, a positive pressure of 200 mbar was applied inside the
337 microfluidic cantilever immersed in the buffer. The silanized probes were calibrated using the thermal
338 noise method before each measurement [51]. The interactions between the bubbles produced and PES-
339 PBS or PES-VAN membranes were then recorded in force spectroscopy mode using a maximum applied
340 force of 1.2 nN, a constant approach retraction speed of 3.0 $\mu\text{m}/\text{s}$, and a delay time of 1.0 s. For each
341 membrane, several areas of 4 x 4 μm were probed. Adhesion forces were obtained by calculating the
342 maximum adhesion force on the retract force curves.

343 **3. Results and discussion**

344 **3.1. Membrane coating with vanillin**

345 In the first step of the study, we optimized the conditions to provide membranes coated with vanillin. PES
346 membranes are negatively charged and hydrophilized to ensure low protein binding capacities
347 (specifications from the provider, <https://www.3m.com>). Their high permeability comes from a highly
348 asymmetric porous structure. Vanillin is a slightly hydrophobic molecule ($\log K_{ow} = 1.23$, OECD data)
349 depending on the pH of the solution. At pH 3.8, vanillin is mainly in its protonated form ensuring
350 maximum coating densities by reducing electrostatic repulsion with the sulfur oxygen groups of the PES
351 membrane. Moreover, in this condition, hydrophobic interactions are enhanced between the different
352 vanillin groups and PES phenyl rings and the OH groups of the vanillin can H-bond with the sulfur
353 oxygens of the PES [46]. For these reasons, pH 3.8 was chosen for the membrane coating step.

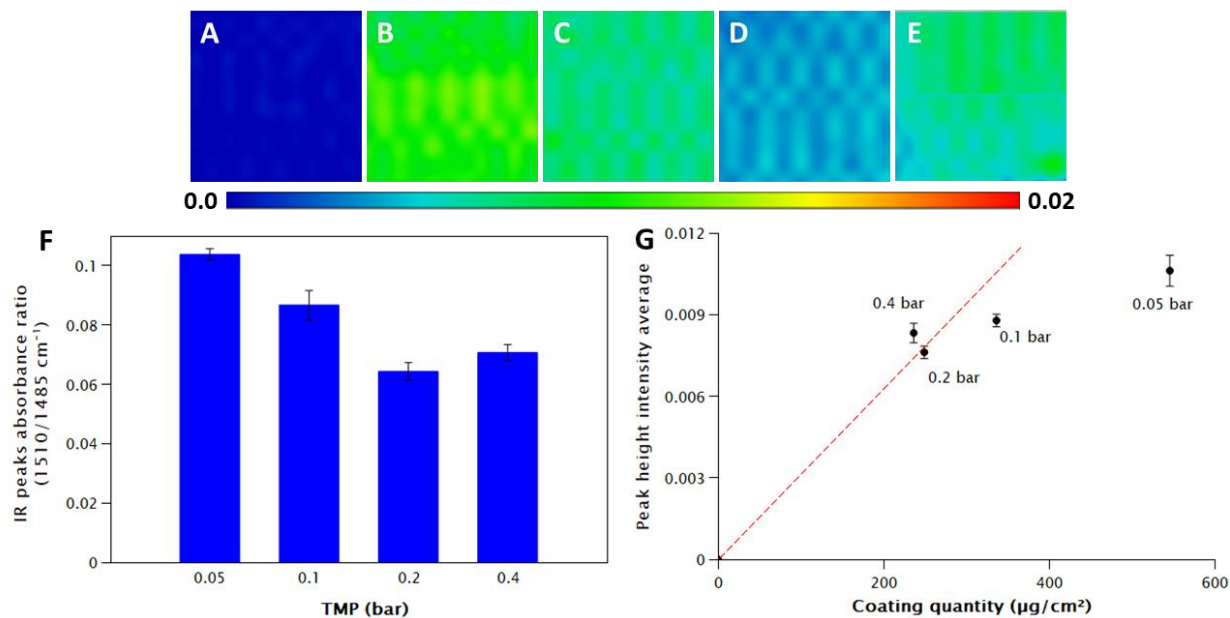
354 The first objective was to define the transmembrane pressure (TMP) that would result in a homogeneous
355 and high vanillin quantity on the membrane surface while minimizing the decline in transfer properties of
356 the membrane. The effect of varying TMP (0.05 bar, 0.1 bar, 0.2 bar, and 0.4 bar) on coating quantity and
357 membrane performance (pure water flux decline) before and after the membrane modification was
358 investigated. A previous study by Virtanen *et al.* (2018) demonstrated that PES-vanillin interaction
359 (graphical abstract) occurs in two timescales [46] (Figure S1). At a rapid timescale, the dominant
360 interaction is the hydrophobic ones between the aromatic rings of vanillin and of PES. At a slower
361 timescale, the OH group of vanillin H-bonds with the sulfur oxygens of PES and vanillin disperses from
362 each other which leads to an even coating coverage on the PES surface. In this study, the effect of pressure
363 resulted in two general trends in the obtained coating quantity and pure water flux (Figure 2A). Pure water
364 permeability results are presented in Figure S2. At higher TMP, 0.2 and 0.4 bar, the coating quantities
365 were lower and comparable with each other (249 ± 5 and $237 \pm 25 \mu\text{g}\cdot\text{cm}^{-2}$, respectively), while the pure
366 water flux decline ratios (FDR) were close to unity (1.09 and 1.01, respectively) indicating that the
367 membrane performance is retained even after surface modification. At these pressures, the hydraulic
368 conditions are stronger. Consequently, as per the previous study by Virtanen *et al.*, it could mean that
369 there is insufficient time to form stronger PES-vanillin interaction (H-bond) and the hydrophobic
370 interactions between the aromatic rings of PES and vanillin are more dominant. Vanillin, therefore, gets
371 flushed through the membrane's porous structure which explains the retention of membrane performance
372 as demonstrated by FDR values close to 1. While at lower TMP, 0.05 and 0.1 bar, the coating quantity
373 significantly increased to 546 ± 34 and $337 \pm 15 \mu\text{g}\cdot\text{cm}^{-2}$, respectively. When working at lower TMP,
374 vanillin accumulation in the porous structure is facilitated by lower hydraulics thereby increasing the
375 probability of adsorption of vanillin molecules on the membrane surface. This could also imply that there
376 is sufficient time for the vanillin to form both hydrophobic and H-bond interactions with the PES and
377 distribute evenly to the PES surface. This was evident in the decrease of pure water flux after modification
378 (lower FDR values). Although 0.05 bar TMP demonstrated the highest coating quantity, the membrane
379 performance was significantly reduced with the lowest FDR of 0.63 ± 0.07 compared to 0.1 bar with an
380 FDR of 0.86 ± 0.14 . For this reason, 0.1 bar TMP was chosen as a good compromise as it has a relatively
381 high coating quantity with a minor membrane performance loss of only 14%. From this result, we assume
382 that the coating will not have a significant impact on the pore size.



383

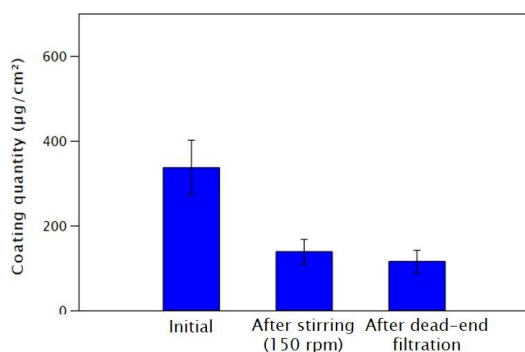
384 **Figure 2.** Coating quantity (histogram) and pure water flux decline ratio (FDR, dots) of PES-VAN
 385 membranes filtered with 2000 mL of $3\text{g}\cdot\text{L}^{-1}$ vanillin solution at varied transmembrane pressure (TMP)

386 The FTIR maps generated at 1511 cm^{-1} , which corresponds to the aromatic ring of vanillin, and IR peaks
 387 absorbance ratio confirmed the presence and coverage of vanillin on the PES surface at varied TMP
 388 (Figure 3A-F). The full IR spectra of the samples, highlighting the relevant peaks for their respective
 389 chemical groups are presented in supplementary data (Figure S1). Pristine PES membrane has blue color
 390 only, implying that there is no vanillin present on their surface. These results agree with the obtained
 391 coating quantity. In addition, the relationship between the average peak height intensity of the FTIR maps
 392 and the coating quantity is non-linear as shown in Figure 3G. A linear correlation, represented by the
 393 dashed line, was chosen assuming that at high pressure, vanillin was retained and adsorbed only at the
 394 surface. The points falling below the dashed line show that at lower pressure, 0.1 and 0.05 bar, we observe
 395 a deviation in the total quantity adsorbed compared to the coating coverage on the surface as observed
 396 through the FTIR mapping. This deviation indicates that a portion of vanillin penetrates through the
 397 membrane and adsorbs into the pores at low pressure. This agrees with the literature where Virtanen *et al.*
 398 [46] showed that vanillin penetrated a few nanometers deep into the PES membrane when it was modified
 399 via cross-flow filtration. Moreover, the depth of penetration of FTIR-ATR analysis is usually in a few
 400 micrometer range, approximately $3\text{-}5\text{ }\mu\text{m}$ using diamond crystal [9]. This implies that at lower TMP, the
 401 vanillin has more time to adsorb into the depth of the membrane for more than a few micrometers. This
 402 could also explain the flux decline under these conditions. Nevertheless, despite vanillin penetration
 403 through the membrane, FTIR maps show that at 0.1 bar, a homogeneous coating coverage on the
 404 membrane surface was still achieved. In conclusion, after consideration of vanillin coating quantity,
 405 coating coverage on the membrane surface, and membrane performance, a TMP of 0.1 bar will be used for
 406 the following experiments.



407
 408 **Figure 3.** FTIR maps generated at 1511 cm^{-1} of A) pristine PES membrane, PES-VAN at B) 0.05 bar, C)
 409 0.1 bar, D) 0.2 bar, and E) 0.4 bar TMP. Each map has an area of 0.25 mm^2 . The vanillin concentration
 410 used was $3\text{ g}\cdot\text{L}^{-1}$ and filtrate volume of 2000 mL. F) Mean absorbance ratio of the vanillin to the PES
 411 membrane peaks (1510 and 1485 cm^{-1} , respectively) as a function of the TMP applied during the filtration
 412 of 2000 mL of $3\text{ g}\cdot\text{L}^{-1}$ vanillin solution. G) Peak height intensity average from FTIR maps as a function of
 413 the coating quantity; the dashed line represents the hypothetical correlation for which vanillin is adsorbed
 414 only at the membrane surface.

415 The stability of PES membranes modified with vanillin at 0.1 bar was also evaluated. Results showed that
 416 41% ($138 \pm 29\text{ }\mu\text{g}\cdot\text{cm}^{-2}$) and 34% ($115 \pm 26\text{ }\mu\text{g}\cdot\text{cm}^{-2}$) of vanillin was retained after stirring and filtration
 417 desorption tests, respectively (Figure 4). Although a portion of vanillin was removed, the quantity left was
 418 sufficient to minimize biofouling in single-cell scale and population scale adhesion studies (sections 3.3
 419 and 3.4).



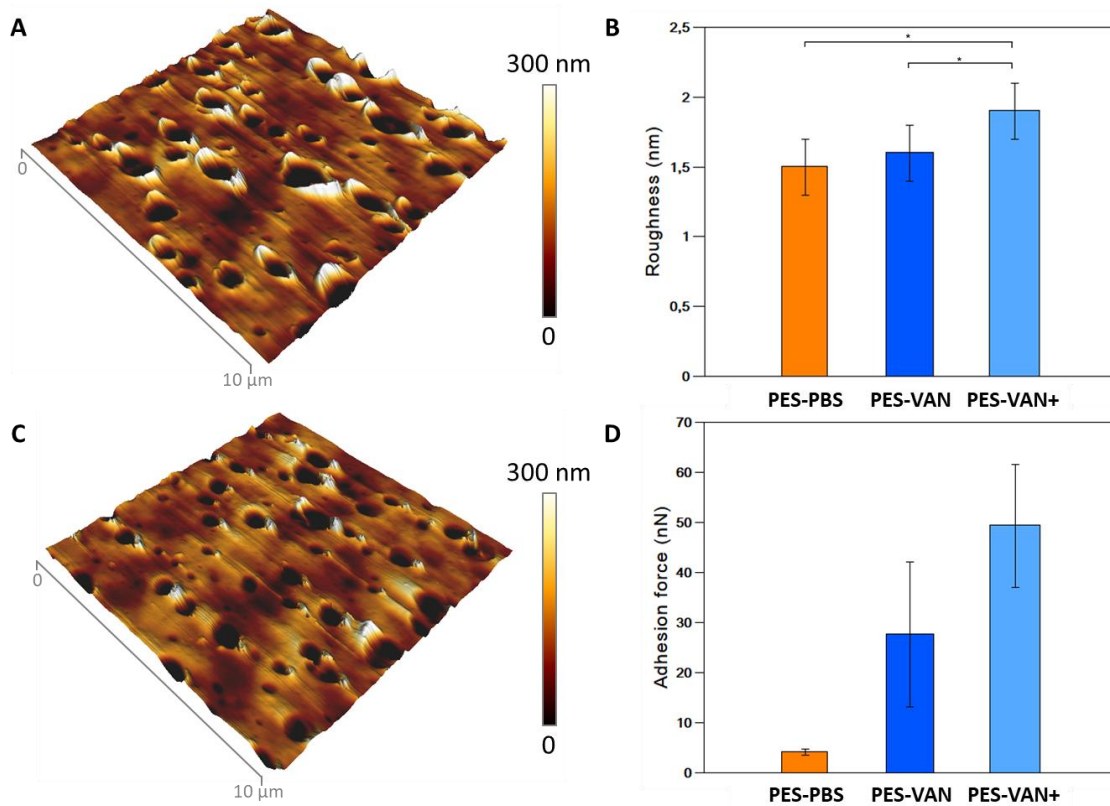
420
 421 **Figure 4.** Vanillin coating quantity on PES-VAN membranes before and after desorption assays under
 422 stirring or dead-end filtration mode (three hours and 2000 mL of ultrapure water).

423 3.2. Characterization of the surface modification

424 According to the literature, surface modification with vanillin renders the surface hydrophilic with static
 425 contact angles ranging from 30 to 55° [42-44]. These results are not coherent with the inherent

426 hydrophobic nature of vanillin and no significant gain in the pure water permeability was observed. Shin
427 *et al.* (2020) reported a 10% decrease in pure water permeability after the modification [43] and Katebian
428 *et al.* (2018) showed that there is no statistical difference in the water permeability before and after the
429 modification [44]. The change in the measured contact angles of the membrane surface can depend on
430 several factors: the new properties that the vanillin imparts and the rugosities created by the deposition or
431 accumulation of the molecule on the surface. Consequently, the combination of these may not be
432 measurable through macroscopic characterization such as water flux making it challenging to isolate the
433 nature and mechanisms of the membrane performance. In addition, parameters from filtration tests may
434 also be dependent on complex membrane internal structure. For these reasons, this section will be devoted
435 to the local characterization (in nano- and micrometer scale) of membrane properties after coating at 0.1
436 bar.

437 Both roughness and hydrophilicity have to be considered to assess the effect of surface modification on
438 bacterial adhesion. Atomic force microscopy was further used to characterize the PES-PBS and PES-VAN
439 membranes. AFM height images of the membrane surfaces are presented in Figure 5A and C. The
440 estimated apparent pore sizes measured from these images did not decrease after surface modification
441 (Figure S3), which was also verified on SEM images (Figure S4). This further confirms our previous
442 assumption at section 3.1 that the vanillin coating will not have a significant impact on the pore size.
443 Furthermore, although the pores of the membranes are clearly visible in the images, it is difficult to
444 conclude from these images whether vanillin (effective diameter in water is 6 Å [57]) is present or not on
445 the membrane surfaces. We thus performed roughness measurements on these membrane surfaces, on
446 areas of 500 x 500 nm located between the pores. Examples of the images used for roughness
447 measurements are presented as supplementary information (Figure S5). Filtration of vanillin increased the
448 surface roughness (Figure 5B, full data are in Supplementary table 1). From 1.5 ± 0.2 nm, indeed, at $71 \pm$
449 $12 \mu\text{g}\cdot\text{cm}^{-2}$ of vanillin, the roughness slightly increased to 1.6 ± 0.2 nm and at $105 \pm 28 \mu\text{g}\cdot\text{cm}^{-2}$ of
450 vanillin, the increase in roughness became significant (Mann and Whitney test, $p \leq 0.05$), to 1.9 ± 0.2 nm.
451 This increase in roughness is attributed to the deposition of vanillin on the membrane surface especially at
452 larger volumes of vanillin solution. Indeed, the presence of vanillin on the membrane surface was verified
453 by other characterization techniques such as FTIR mapping and FTIR-ATR (section 3.1).



455

456 **Figure 5.** AFM 3D height images of areas of $10 \times 10 \mu\text{m}^2$ on the surface of (A) PES-PBS membrane and
 457 (C) PES-VAN+ membrane containing $105 \pm 28 \mu\text{g}\cdot\text{cm}^{-2}$ of vanillin. Roughness measurements were
 458 performed on $500 \times 500 \text{ nm}^2$ areas between the pores of the membranes; the histogram in (B) shows the
 459 quantification of these measurements. Lastly, the interactions between membranes and a bubble probe
 460 were performed using FluidFM; the histogram in (D) shows the average adhesion forces recorded in each
 461 case.

462 In addition, AFM was used to characterize the hydrophobicity of the membranes used in this study. For
 463 that, their interactions with a bubble probe produced using FluidFM technology [56] were measured. As
 464 bubbles behave like hydrophobic surfaces, the forces recorded then directly reflect the hydrophobicity of
 465 the materials probed. The results showed that the adhesion force recorded increased with the amount of
 466 vanillin filtered on the membranes (Figure 5D). From approximately 5 nN for untreated or PES-PBS
 467 membranes, the adhesion force increased to $27.6 \pm 14.5 \text{ nN}$ for PES-VAN membrane with a coating
 468 quantity of $71 \pm 12 \mu\text{g}\cdot\text{cm}^{-2}$, and to $49.3 \pm 12.3 \text{ nN}$ for PES-VAN+ membrane with coating quantity of
 469 $105 \pm 28 \mu\text{g}\cdot\text{cm}^{-2}$. This increase in the adhesion force indicates that vanillin modifies the physical-chemical
 470 properties of the membranes and renders them hydrophobic, with more vanillin adsorbed on the
 471 membranes resulting in a higher hydrophobicity of the material. These results are in accordance with
 472 water contact angle measurements performed on these membranes. WCA was found to increase with
 473 vanillin coating quantity, from $65.2 \pm 4.4^\circ$ for the PES-PBS membrane to $75.7 \pm 2.8^\circ$ for the PES-VAN
 474 membrane (coating quantity of $142 \pm 25 \mu\text{g}\cdot\text{cm}^{-2}$). The increase in hydrophobicity could also explain the
 475 slight decrease in pure water flux upon increase of vanillin quantity on the membrane surface. Our results
 476 combining the water contact angle and AFM measurements provide an answer to the contradictory results
 477 found in literature. Indeed, Esmaili *et al.* [42] showed that contact angle increased at higher vanillin
 478 concentration while Kumar *et al.* [45] and Shin *et al.* [43] demonstrated the opposite. Considering that

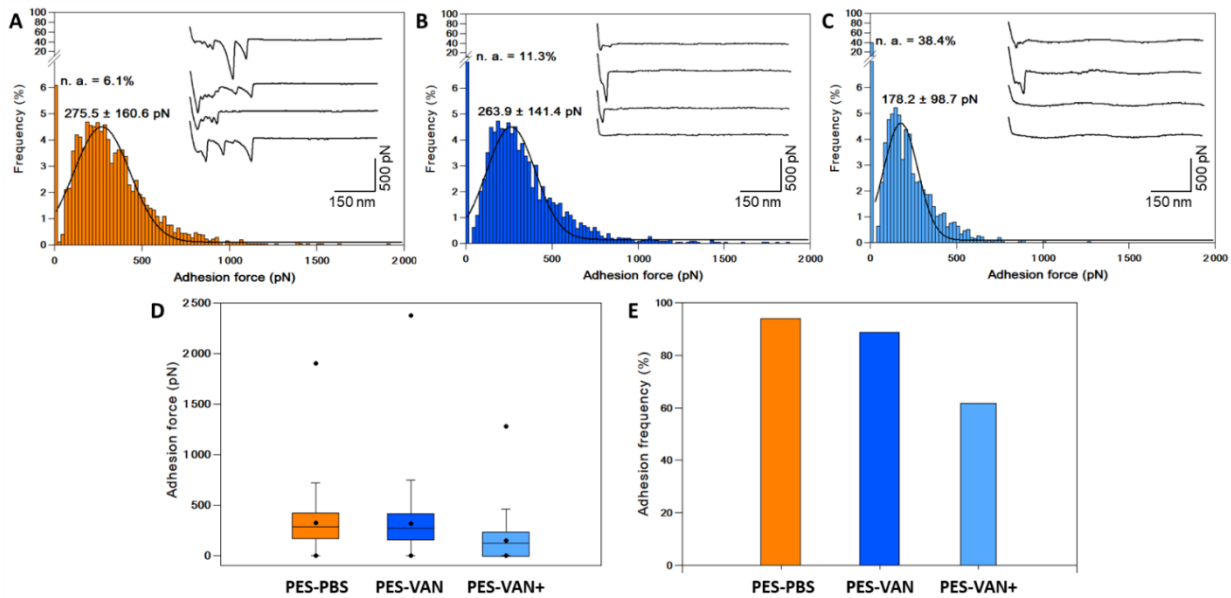
479 contact angle measurements are influenced not only by the material surface's intrinsic physical-chemical
480 properties but also by the surface topography, it is challenging to use this method in identifying the
481 surface's inherent property, thus, resulting in varying degrees of hydrophilicity. Using AFM for
482 hydrophobicity measurement offers an advantage to study the surface locally in the micrometer range in
483 comparison with contact angle measurements with bubble sizes in the millimeter range. This method
484 revealed the inherent hydrophobic nature of vanillin as a result of the surface modification strategy used
485 on the membrane and highlighted the fact that despite a subnanometer scale change in surface roughness,
486 it had a significant effect on hydrophobicity. In addition, a small-scale study like this could be more
487 advantageous in understanding bacterial interaction as they happen on a smaller scale.

488 **3.3. Initial adhesion of bacteria measured at the cell scale by AFM**

489 To answer the question of whether or not vanillin could have a potential effect on the initial attachment of
490 bacterial cells onto the surfaces, we decided to directly probe, using AFM, the interactions between single
491 cells and the different membranes produced in this study. *P. aeruginosa* was chosen as it rapidly produces
492 biofilm to attach over surfaces, and biofilm maturation was found to be interconnected with quorum
493 sensing activities [58]. Hence, it is expected to be sensitive to vanillin as a quorum sensing inhibitor and
494 represents a bacteria strain model for biofilm development control. In these experiments, we used the
495 single-cell force spectroscopy technique, which consists in attaching one bacterial cell to a colloid AFM
496 cantilever, making it then possible to probe the interactions between this bacterial cell and a surface in
497 force spectroscopy mode. This gives access to force vs distance curves, from which information can be
498 extracted. The adhesion force, which quantifies the force needed to break the interaction between the cell
499 and the surface, is a direct reflection of the possibility of the cell to adhere to the material or not. It can be
500 calculated from the force curves by measuring the height of the biggest retract adhesion peak visible on
501 the force curves. Such calculations are then realized for arrays of force curves (400 in our case) recorded
502 at the surface of membranes (Figure 6). In the case of PES-PBS membranes (Figure 6A), the force curves
503 recorded show multiple retract adhesions, meaning most likely that polymers from the bacterial cell
504 surface are unfolded upon retraction of the AFM cantilever. Indeed, physical-chemical forces such as
505 electrostatic or hydrophobic are usually materialized by a single peak taking place at the contact point
506 [59], whereas multiple peaks distributed over the retract curve on long distances are usually due to the
507 successive unfolding of polymer sub-units such as polysaccharide present at cell surfaces [60]. The
508 adhesion force recorded in this case was 275.5 ± 160.6 pN, and only 6.1 % of the force curves recorded
509 did not present adhesive events. On PES-VAN membranes with a vanillin coating quantity of 71 ± 12
510 $\mu\text{g}\cdot\text{cm}^{-2}$, the adhesion force recorded was similar (263.9 ± 141.4 pN); however, the force curves recorded
511 did not present multiple interactions unlike for PES-PBS membranes, meaning that probably fewer
512 molecules from the cell surface are involved in the interactions. Another important difference is in the
513 adhesion percentage, as the % of non-adhesive curves recorded increased to 11.3%. Such increase is even
514 more important for PES-VAN+ membranes with a vanillin coating quantity of 105 ± 28 $\mu\text{g}\cdot\text{cm}^{-2}$, as 38.4%
515 of the force curves did not present adhesive events anymore. In addition, in this case, the adhesion force
516 significantly decreased to 178.2 ± 98.7 pN (independent student test, $p \leq 0.001$). Adhesion forces obtained
517 and % of adhesion are summarized in Figure 6D and E, respectively. From these results, it is then clear
518 that the presence of vanillin on the surface of the membranes affects both the probability of cells to adhere
519 to the surface and the adhesion forces. This has important implications, as this decrease in the adhesion
520 force means that a small flux might be able to detach cells from the surface, thus alleviating the cleaning
521 procedures.

522 Biofilm formation on surfaces usually involves three steps. The first step consists of the reversible
523 adhesion of cells to surfaces, followed by irreversible microbial adhesion [61] where physical-chemical
524 bonds between cells and the surface are strengthened. Finally, after these adhesion steps, cells undergo
525 important changes (cell wall deformation, production of exopolysaccharides, gene expression, etc.) which

526 lead to the formation of a mature biofilm [62]. In this work, only the first step was assessed, the initial
 527 adhesion to the surface. This step can be influenced by different surface properties, such as surface charge,
 528 roughness, or hydrophobicity [63]. AFM measurements were performed on PES-PBS and PES-VAN
 529 membranes submerged in PBS at a pH 7.4. In these conditions, vanillin (pKa = 7.4) exists in both
 530 protonated and unprotonated forms and the PES membrane (pHi = 7) exhibits negative charges which is
 531 favorable to the electrostatic repulsion of bacteria that are negatively charged [64]. An increase in
 532 adhesion of microorganisms is generally observed when the surface roughness increases to the micrometer
 533 scale [3, 65]. In our case, the roughness changes induced by the incorporation of vanillin are in the
 534 subnanometer scale. Nevertheless, a decrease in the adhesion of *E. coli* cells in static condition was
 535 observed on surfaces with increased roughness at the subnanometer scale [66] which is more similar to
 536 our experimental conditions. In addition, previous studies have shown that when the sizes of surface
 537 patterns are smaller than the bacterium such as *P. aeruginosa*, the adhesion is significantly reduced [3]. A
 538 theoretical model, based on extended Derjaguin-Landau-Verwey-Overbeek (XDLVO) theory, showed that
 539 the contribution of electrostatic interactions to the total interfacial interaction was found to be minimal on
 540 smooth surfaces. However, when surface roughness in nanometer range was considered, the contribution
 541 of electrostatic repulsion increased [67] and could end up in an easier detachment of the bacteria from the
 542 surface [68]. This is in line with the results obtained during AFM force spectroscopy measurements,
 543 which showed a reduced adhesion force on vanillin-coated membranes, which could result in easier
 544 bacterial detachment. Regarding hydrophobicity, some studies have shown a stronger attachment of
 545 bacteria to hydrophobic surfaces [64, 69, 70]. Vanillin-modified membranes are more hydrophobic
 546 compared to the control membranes, and yet, the cell adhesion was reduced. First, this means that the anti-
 547 adhesion effect is stronger than a potential attractive effect caused by an increased hydrophobicity, and
 548 second that the anti-adhesion effect could be also due to the biochemical effect of vanillin which affects
 549 bacterial cell surface integrity via pH homeostasis loss as observed in *L. plantarum*, *L. innocua*, and *E.*
 550 *coli* [36] or even in *M. smegmatis* [35] which has a more complex cell wall core. A previous study also
 551 demonstrated that damage to the cell surface of *P. aeruginosa* could decrease its capacity to adhere to a
 552 hydrophobic surface [71, 72]. When disrupted, the bacteria cell wall itself undergoes a change in its
 553 surface properties, releasing lipid components from the cell, and cell wall stress also induces the inhibition
 554 of EPS production via cyclic adenosine monophosphate (cAMP) signaling during cell wall repair [73].



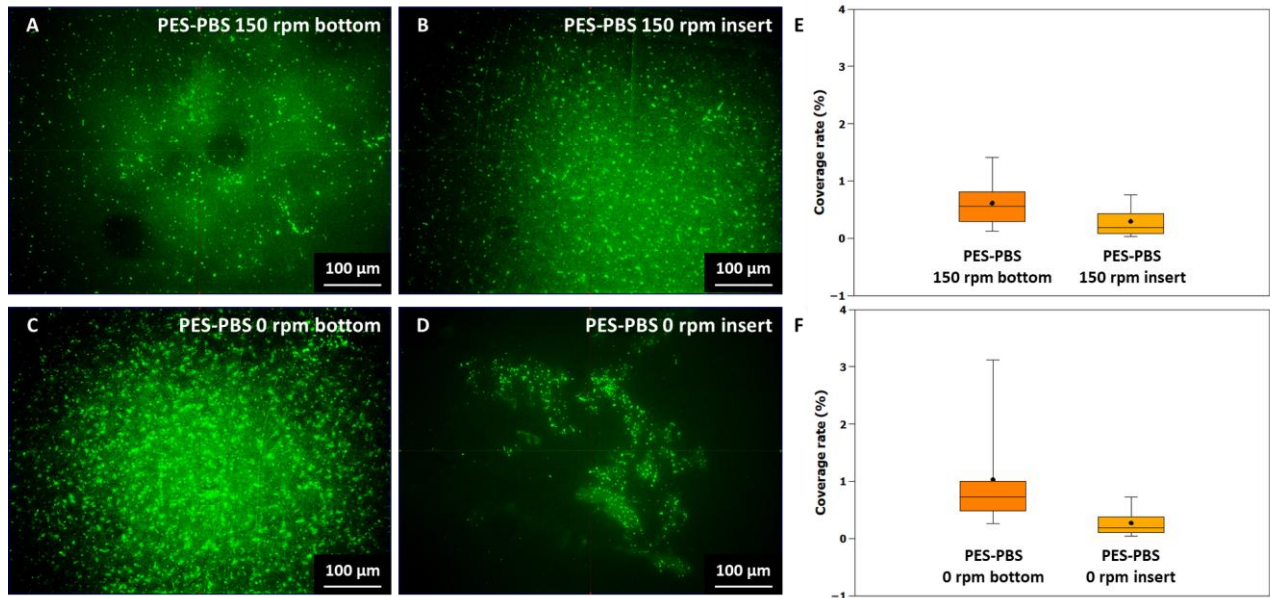
555

556 **Figure 6.** Interactions of *P. aeruginosa* cells with membrane surfaces. Histograms showing the
557 distribution of adhesion forces recorded between single *P. aeruginosa* cells and (A) PES-PBS membranes,
558 (B) PES-VAN membrane containing $71 \pm 12 \mu\text{g}\cdot\text{cm}^{-2}$ of vanillin, and (C) PES-VAN+ membrane
559 containing $105 \pm 28 \mu\text{g}\cdot\text{cm}^{-2}$ of vanillin. In each case, insets show representative force curves. The results
560 presented come, in each case, from 7 cells from 3 independent cultures. (D) Box-plot summarizing the
561 adhesion force results and (E) histogram summarizing the percentages of adhesion recorded in each case.

562 **3.4. Adhesion of bacteria at the population scale**

563 In the frame of our study, the bacterial adhesion at the initial stage of biofilm development is required to
564 understand the impact of the physical-chemical surface modifications. Thus, we investigated the effects of
565 vanillin at the cell population scale using adhesion assays of *Pa* in contact with the membranes for 3h.
566 This was performed with the objective to distinguish the effect of vanillin on the initial adhesion of
567 bacteria from the biofilm development wherein EPS are present. For this purpose, we developed a
568 different protocol than the one usually performed in the literature [74] which does not differentiate
569 bacterial adhesion from sedimentation. For that, we used inserts placed upside down in the suspension, as
570 described in the material and methods, section 2.4. The adhesion of bacteria was also evaluated under
571 agitation and static conditions on virgin PES membrane placed in a multi-well culture plate as classically
572 performed. Moreover, instead of detaching the adhered bacteria from the membrane surface by ultrasonic
573 treatment for enumeration, in our case, we quantified the adhered bacteria directly on membrane surfaces
574 using epifluorescence microscopy. This way we could determine the bacterial coverage rate using image
575 analysis [75].

576 First, we validated this new protocol by comparing the bacterial coverage rate (i) obtained under static
577 conditions (0 rpm) with the one obtained under agitation (150 rpm), and (ii) depending on the position of
578 the PES-PBS membrane in the culture plate well, allowing the sedimentation of bacteria (at the bottom) or
579 not (in the insert). Looking at the effect of agitation on PES-PBS membranes placed at the bottom of the
580 well (Figure 7E and F), under agitation of 150 rpm, the bacterial coverage rate for the membranes was on
581 average $0.61 \pm 0.41\%$, while in the absence of agitation, the mean coverage rate was $1.03 \pm 1.29\%$. The
582 absence of agitation led to a 69% increase in the mean coverage rate and high heterogeneity in the values
583 obtained for the membranes placed at the bottom of wells (Figure 7A, C, and F). Agitation had no
584 significant effect on the coverage rate of the membranes placed in the inserts; in fact, the mean coverage
585 rate was $0.29 \pm 0.29\%$ with 150 rpm of agitation and $0.27 \pm 0.24\%$ without agitation, highlighting the
586 importance of our approach to differentiate between bacterial adhesion and sedimentation. These results
587 imply that hydrodynamic conditions induced by plate agitation affect the bacterial colonization of the PES
588 membrane surface less when placed in the inserts. In addition, diffuse fluorescence around the cells was
589 observed in conditions under agitation, which suggests the presence of extracellular DNA. Therefore,
590 biofilm formed more rapidly in response to hydrodynamic stress [52, 76]. The use of inserts without
591 agitation allows clear visualization of the cells on the surface of the samples and a better estimation of the
592 initial adhesion before the production of EPS in the tested experimental conditions (Figure 7D). Thus,
593 adhesion assays were performed for 3h in PBS 1X (pH 7.4) as a holding medium, with the use of inserts
594 and without agitation. In this condition, the probability of adhesion is reduced to natural convection,
595 diffusion, and motility of the bacteria to the membrane surface. This means that after potential repulsion
596 of the bacteria from the membrane surface due to a change in surface properties, only irreversibly adhered
597 bacteria will stay attached after rinsing. In addition, these conditions do not promote biofilm formation
598 due to the absence of stress conditions.

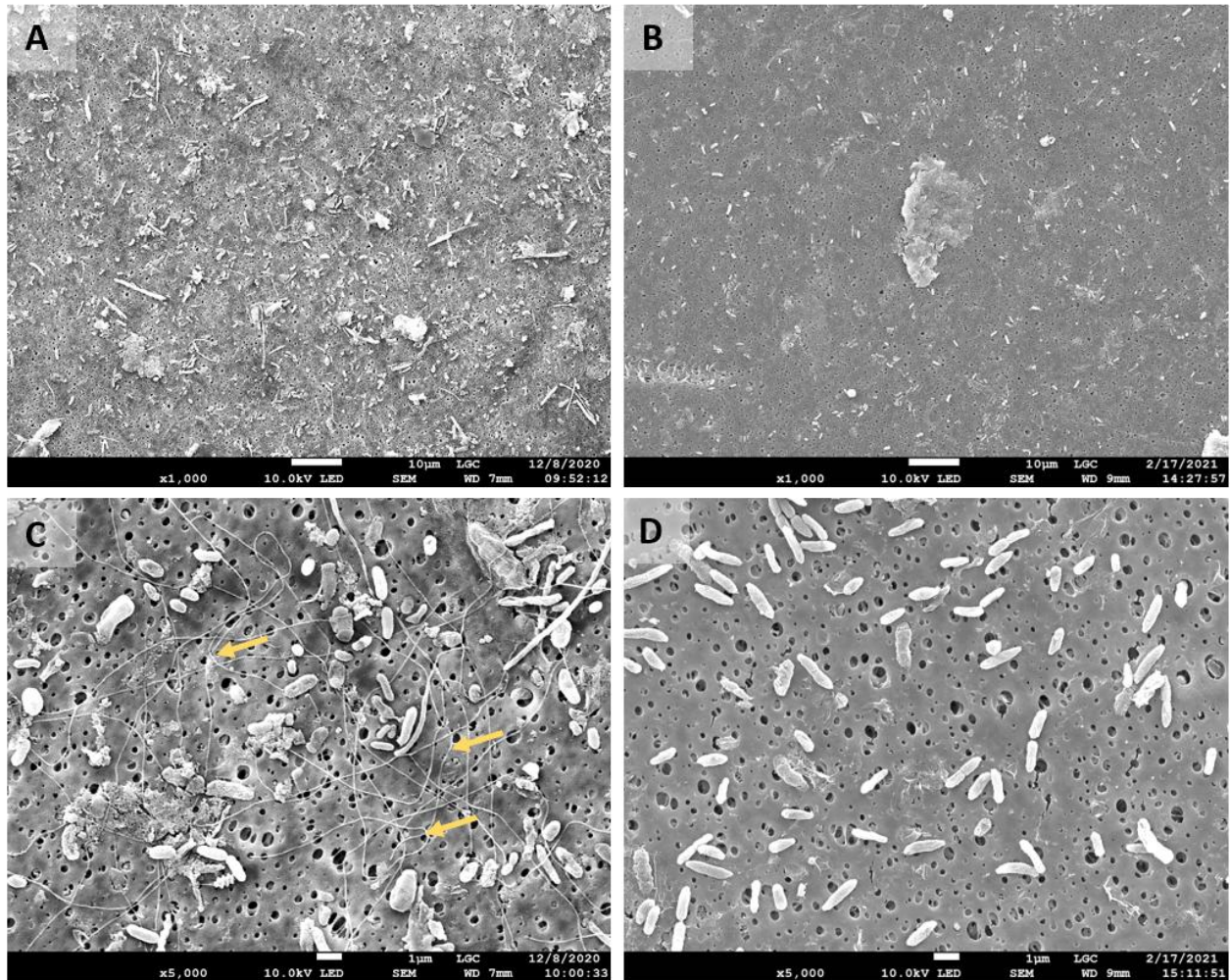


599
 600 **Figure 7.** Effect of agitation and sedimentation on the bacterial colonization of PES membranes.
 601 Epifluorescence microscopy (EFM) images of bacterial adhesion assays on PES-PBS membrane surface
 602 under different conditions stained with SYBR™ Green: (A) agitation at 150 rpm with the membrane at the
 603 bottom of the well, (B) agitation at 150 rpm with the use of insert, (C) static condition with the membrane
 604 at the bottom of the well, and (D) static condition with insert. (E, F) Box and whisker plots showing the
 605 coverage rates by *P. aeruginosa* on PES membranes and quantification in EFM with ZEN blue image
 606 analysis tools.

607 After these adhesion tests, the membrane coupons were prepared for scanning electron microscopy to
 608 observe the morphology of the bacteria present on the membranes. SEM observations showed similar
 609 morphologies between bacteria adhered to PES-PBS and PES-VAN membranes (Figure 8). However, a
 610 lower density of bacteria on the surface of PES-VAN membranes was observed (Figure 8B and D). The
 611 presence of filaments in the vicinity of *P. aeruginosa* cells was clearly observed on the PES-PBS
 612 membranes (Figure 8A and C) whereas these same filaments were almost invisible on the vanillin-
 613 modified membranes (Figure 8B and D), probably due to their thinness. These filaments could correspond
 614 to EPS filaments and the presence of vanillin seems to affect their quantity and/or structure.

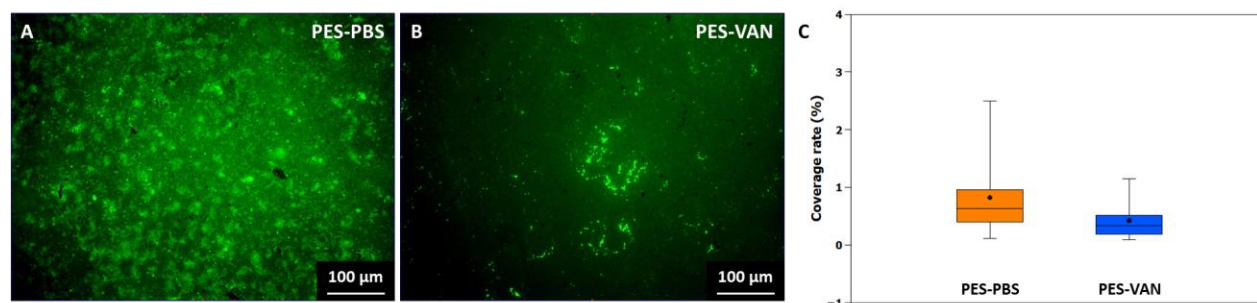
615 Observations were also carried out in epifluorescence microscopy with the highest coating quantity
 616 obtained in this study, 193 μg.cm⁻² (Figure 9). They showed that, from a qualitative point of view, the
 617 microbial density at the surface of the PES-VAN membranes is significantly lower than on the PES-PBS
 618 membranes, which is consistent with the observations made in SEM. It was further confirmed by the
 619 quantification of the bacterial coverage rate carried out by analyzing a total of 78 images. The coverage
 620 rate of *P. aeruginosa* on PES-PBS membrane was 0.82 ± 0.74%, while 0.42 ± 0.39% for the PES-VAN
 621 membrane. The bacterial coverage rate on PES-VAN membranes was reduced by 50% compared to the
 622 PES-PBS membranes (p-value < 0.05, Welch test, Figure 9C). Therefore, the presence of vanillin
 623 adsorbed on the membrane surface with a coating quantity of 193 μg.cm⁻² affects the initial adhesion by
 624 decreasing the coverage rate of adhered bacteria to the surface by half after 3h adhesion assay. Moreover,
 625 it seems that the quantity, quality, and/or structure of the EPS were also affected by the presence of
 626 vanillin, confirming the results from SEM. At pH 7.4, the vanillin is half protonated and could desorb
 627 slowly from the surface. In the worst case, if all the vanillin desorbs, the estimated concentration of
 628 vanillin in the solution would be less than the value required to produce a deleterious or QSI effect on

629 bacteria [32, 33, 36]. The results obtained then demonstrated that in our experiments the QSI effect can be
630 mainly attributed to the vanillin remaining adsorbed on the membrane surface.



631
632 **Figure 8.** SEM images of (A x1000, C x5000) PES-PBS and (B x1000, D x5000) PES-VAN membranes
633 (vanillin coating quantity of $142 \pm 25 \mu\text{g}\cdot\text{cm}^{-2}$) after 3h adhesion assay.

634 The 3h adhesion assay corresponds to the bacterial irreversible adhesion stage of biofilm formation at the
635 membrane surface. These results showed that even with such short contact time (3h) of *P. aeruginosa* with
636 the membrane in control conditions (PES-PBS membrane), EPS were produced and bacteria adhered to
637 the membrane surface. This results from a cascade of signaling molecules (proteins) expressed shortly
638 after the incubation (occurs in less than 2 hours) which are responsible for the rapid attachment kinetics of
639 the bacteria after its first contact with the surface [73, 77]. For QS mechanisms to take place, it is required
640 that numerous bacteria attach to the surface to induce EPS production and initiate biofilm formation [78-
641 80]. In the case of PES-VAN membranes, not only the adhesion of bacteria is reduced but also the
642 production of EPS is affected by the presence of vanillin at the membrane surface. Previous studies have
643 shown that vanillin leads to a modification of EPS components with a decrease in exopolysaccharides and
644 exoproteins [37, 41], via QSI [32, 33], which is in agreement with the present results. Also, it is not
645 surprising that no induction of the QS mechanisms responsible for EPS production and biofilm
646 development was observed when the number of bacteria adhered to the membrane surface decreased. It
647 thus confirms that the homeostatic and quorum sensing inhibitory effect of vanillin for the gram-negative
648 bacteria are effective already at the first stage of bacterial adhesion and biofilm development.



649
 650 **Figure 9.** Epifluorescence microscopy images of PES membrane surface labeled with SYBR™ Green: (A)
 651 PES-PBS and (B) PES-VAN (vanillin coating quantity of $193 \mu\text{g}\cdot\text{cm}^{-2}$) membranes after 3h adhesion
 652 assay, and (C) corresponding box and whisker plot showing the coverage rates of *P. aeruginosa* on PES-
 653 PBS and PES-VAN membranes and quantification in EFM with ZEN blue image analysis tools.

654 4. Conclusion

655 Modified PES membranes were obtained by dead-end filtration of vanillin solution. The vanillin-coated
 656 membranes, PES-VAN, were characterized in terms of filtration performances, physical-chemical surface
 657 properties, and adhesion of bacteria at the cell and population scales. For this, a new method for bacterial
 658 adhesion assay was developed and a combination of characterization techniques at different size scales,
 659 from macro- to nanoscale, was performed to validate the multifunctional effects of vanillin.

660 The results from this study conclude that:

- 661 • The coating of vanillin by filtration was optimized by decreasing the flux through the membrane at
 662 low transmembrane pressure.
- 663 • Small-scale localized measurements using AFM revealed the inherent hydrophobicity of PES-VAN
 664 membranes and that there is only subnanometer scale change in surface roughness. This was
 665 correlated to the membrane's macroscale property wherein a small reduction in membrane
 666 permeability was observed.
- 667 • The new method for bacterial adhesion assay by using inserts to support membranes avoids the
 668 sedimentation effect on bacterial attachment and promotes only the initial stage of biofilm
 669 development. Moreover, it reduces DNA fragment deposition and optimizes the observation and
 670 quantification of bacterial adhesion on the membrane surface.
- 671 • The combination of multiscale (from micro- to nanometer scale) characterization strategies using
 672 AFM, EFM, and SEM demonstrated that vanillin on the membrane's surface: (i) reduces the adhesion
 673 forces and the probability of the cells to adhere to the surface at the initial stage of biofilm
 674 development, and (ii) affects the structure and development of the biofilm.

675 Altogether, the combination of multiscale characterization methods demonstrated how vanillin minimizes
 676 biofouling on membranes, which intervenes at different levels of biofilm development from surface
 677 colonization and adhesion of bacteria to the early stages of biofilm formation. These results show that
 678 vanillin adsorption on the membrane surface will limit the bio-adhesion and biofouling during filtration
 679 processes and will facilitate and alleviate the cleaning procedures usually required. In addition, the
 680 proposed surface modification method in this study offers advantages. The use of dead-end filtration is
 681 fairly simple and adaptive to water treatment applications. Consequently, it can be easily scaled up and
 682 vanillin could be reloaded as needed making it suitable for an industrial setting.

683 **Supplementary Information (SI)**

684 The supplementary information provides examples of the images and cross-section profiles used for
685 roughness measurements in AFM.

686 **Declaration of competing interest**

687 The authors declare that they have no known competing financial interests or personal relationships that
688 could have appeared to influence the work reported in this paper.

689 **Author statement**

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