

Antimicrobial Activity Testing Methods for Hydrophobic Patterned Surfaces

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Research

Keywords: Antimicrobial testing methods, hydrophobic patterned surfaces, touch-transfer method, swab-inoculation method, active surfaces, antimicrobial surfaces

Posted Date: June 30th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-38112/v1>

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Abstract

Background: One strategy to decrease the incidence of hospital-acquired infections is to avoid the survival of pathogens in the environment by the development of surfaces with antimicrobial activity. To study the antimicrobial behaviour of active surfaces, different approaches have been developed of which ISO 22916 is the standard.

Aim: To assess the performance of different testing methodologies to analyse the antimicrobial activity of hydrophobic surface patterned plastics as part of a Horizon 2020 European research project (FLEXPOL: Antimicrobial FLEXible POLymers for its use in hospital environments No. 721062).

Methods: Four different testing methods were used to study the antimicrobial activity of a patterned film, including the ISO 22916 standard, the immersion method, the touch-transfer inoculation method, and the swab inoculation method, this latter developed specifically for this project.

Findings: The non-realistic test conditions of the ISO 22916 standard showed this method to be non-appropriate in the study of hydrophobic antimicrobial patterned surfaces. The immersion method also showed no differences between patterned films and smooth controls due to the lack of attachment of testing bacteria on both surfaces. The antimicrobial activity of films could be demonstrated by the touch-transfer and the swab inoculation methods that more precisely mimicked the way of high-touch surfaces contamination.

Conclusion: The touch-transfer and the swab inoculation methods proved to be the best methodologies to test the antimicrobial activity of patterned hydrophobic surfaces. In our opinion, a new ISO standard should be developed on the basis of these tests to study the antimicrobial behaviour of patterned surfaces.

Background

Hospital-acquired infections (HAI) can be defined as the infections acquired by a patient who was admitted to a hospital or other healthcare facility that were not present (or incubating) at the moment of admission. HAI represent a significant healthcare problem with an estimated total of 8.9 million cases occurring in the EU during 2016–2017 in acute care hospitals and long-term care facilities ¹. The prevalence of HAI has been estimated to vary between 4.4% in primary care hospitals to 7.1% in tertiary care hospitals, reaching 19.2% in intensive care units ¹. In the EU in 2015, around 25% of HAI were caused by antibiotic-resistant bacteria with an estimated annual mortality of 33,000 patients ².

HAI can be caused by pathogens coming from other patients, the staff or the hospital environment. The different high-touch surfaces such as door handles, tables, nurse-call buttons or bed rails can be contaminated by potential pathogens which are able to form biofilms and survive on the surfaces for a long time ³. Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Enterococcus* (VRE) have been shown to survive on surfaces for weeks and in some cases for months ⁴. Recent

evidence confirmed that patients admitted to rooms previously occupied by patients infected or colonized with MRSA, VRE or other antibiotic-resistant pathogens were at increased risk of acquiring those pathogens⁵. Consequently, appropriate protocols to clean and disinfect hospital surfaces are crucial to prevent HAI of which manual cleaning with disinfectants is the most commonly used. However, chemical cleaners used to disinfect surfaces are not exempt of certain degree of toxicity, can be incorrectly applied and have to be used at their effective concentration. Moreover, once applied on surfaces, disinfectant activity disappears and the objects could contaminate again within minutes⁶.

Modified surfaces can lead to a decrease in the bacterial attachment and biofilm production, which has raised high interest in the research on polymeric films with structured surfaces with intrinsic antimicrobial properties or on films with embedded antimicrobial agents. There is evidence that different polymers with specific micro- and nano-topographies can inhibit the attachment, growth and spread of microorganisms⁷⁻⁹. Furthermore, different polymeric materials treated with silver, copper, polycations, triclosan, bacteriophages or light activated biotoxic radicals have been developed¹⁰.

To assess the activity of films with antimicrobial behaviour, appropriate evaluation tests are needed. The ISO 22196 standard (Japanese test method JIS Z 2801) is used for the measurement of antibacterial activity on plastic surfaces¹¹. However, some studies have described this test as inappropriate, since the temperature of incubation (35 ± 1 °C) and the relative humidity (higher than 90%) do not reflect real conditions¹². Thus, alternative and more realistic methods for the *in vitro* study of antimicrobial activity of plastic surfaces have been described^{7,13}.

The present study was carried out as part of a European project called “Antimicrobial FLEXible POLymers for its use in hospital environments” (FLEXPOL grant agreement No. 721062) funded by Horizon 2020 Framework Programme for Research and Innovation (2014–2020). The objective of the FLEXPOL project was to develop a pilot line for the production of a cost effective antimicrobial adhesive film for its use in hospitals.

In this study, the main objective was to compare the performance of different described tests to measure the antimicrobial activity of plastic surfaces. To achieve this goal, the antimicrobial activity of a three-layer polypropylene (PP) polymer matrix with two different nano- and micro-structures was evaluated with previously described tests (the ISO 22916 standard¹¹, the immersion method⁷, and the touch-transfer inoculation method⁷). Moreover, a new methodology based on the protocols described by *Mann et al.*⁷ was designed for this study, called the “swab inoculation method”.

Methods

Preparation of film specimens

The test film was composed by a smooth three-layer film made of two 25 µm external PP layers (Sabic grade, PP-2 Sabic® PP 520L), and a 50 µm central layer of an olefin based thermoplastic elastomer

(Zelas™ 7025) produced at Propagroup (Torino, Italy). The inner layer was ready to function as a reservoir for the progressive release of encapsulated essential oils or other compounds with antimicrobial activity. In this work, only structured films without antimicrobial compounds were tested to exclusively check the antimicrobial activity of patterned surfaces.

A topography containing ordered micro- and nano-patterns was imprinted on the surface of the PP film by means of nanoimprint lithography. A nickel stamp was used as a stamp, which was copied from an original silicon master¹⁴. Non-structured smooth films were used in the different tests as control.

Bacterial strains and growth conditions

The bacteria used in this study were the Gram-negative *Escherichia coli* (ATCC 25922) and the Gram-positive *Staphylococcus aureus* (ATCC 25923). Bacteria from the frozen stock culture were transferred to Tryptic Soy Agar (TSA) supplemented with 5% sheep blood plates (TSA, bioMérieux, France) and incubated at 37 °C for 18–24 h. Bacteria were transferred to 50 mL of sterile Tryptic Soy Broth (TSB) medium and grown at 37 °C, 80 rpm, 18–24 h. Prior to inoculation, strains were subcultured into fresh TSB at 1:50 and incubated for 2 h at 37 °C, 80 rpm. Bacteria were collected by centrifugation (2000 rpm, 10 min) and resuspended in phosphate buffered saline (PBS). The number of bacteria was spectrophotometrically adjusted to $O.D._{600nm} = 0.5$ (equivalent to 5×10^8 CFUs/mL).

The patterned films evaluated in this study were highly hydrophobic so it was difficult to homogeneously spread the aqueous inoculum over the surface (Figs. 1a and 1b). In order to resolve this problem, the bacterial inocula were applied using glycerol to reduce the surface tension of the suspension. Three different final concentrations of glycerol were tested (5%, 10% and 40%). For all experiments it was decided to use the lowest concentration of glycerol (5%) as the distribution of the inoculum was as homogenous as with higher concentrations of glycerol (data not shown).

Antibacterial activity tests

a) ISO 22196 standard method

The ISO 22196 was performed with slight modifications¹¹. Pieces of 50 mm x 50 mm of the film and of non-structured smooth control films were placed in sterile 90 mm Petri dishes. Serial dilutions of the bacteria inoculum were made to reach a final bacterial concentration of 6×10^5 CFUs/mL of which 400 µL were pipetted onto the test surfaces. Bacterial inoculum was then covered with 40 mm x 40 mm piece of Stomacher® bag in order to homogeneously spread the inoculum over the surface. The specimens were incubated for 24 h at 35 °C and a relative humidity > 90%. To recover the bacteria from the surfaces of the plastic samples, 10 mL of Soybean Casein Lecithin Polysorbate (SCDLP) broth was added to the Petri dish. The SCDLP was collected and released at least four times to ensure that the specimens were completely washed. Then, 10-fold serial dilutions of the SCDLP were made in PBS and

50 μL of 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} dilutions were cultured on TSA with 5% sheep blood plates. Plates were incubated at 35 °C for 24 h. The number of colonies per plate was recorded and used to determine the number of viable bacteria per cm^2 in accordance with the equation $N = (100 \times C \times D \times V)/A$, where C is the average of CFU count, D is the dilution factor, V is the volume in mL of SCDLP and A is the surface (cm^2) of the film.

b) Immersion inoculation assay

Immersion inoculation assay⁷ evaluated the attachment and survival of the bacteria directly from the surface of the film. Serial dilutions of the bacteria inoculum were made in PBS to reach a bacterial concentration of 6×10^4 CFUs/mL. Film samples (50 mm x 50 mm) were placed in a 90 mm sterile Petri dish and were completely covered with 20 mL of bacterial inoculum for 1 h at room temperature without shaking. The films were then washed 3 times with 20 mL of PBS for 10 s while rotating at 80 rpm and allowed to dry under ambient conditions for 1 h before being sampled.

c) Touch-transfer inoculation assay

The Touch-transfer inoculation assay was described by *Mann et al.*⁷ and assessed the attachment (transference) and survival of the bacteria (persistence) directly on the surface of the film. In this study, the protocol described by *Mann et al.*⁷ was used with minor changes. Briefly, serial dilutions of the bacteria inoculum were made in PBS to reach a bacterial concentration of 6×10^4 CFUs/mL. This inoculum was experimentally established on 50 mm x 50 mm smooth control films, because it provided an adequate number of CFUs (around 50 well defined CFUs per plate using the agar contact method described in the *Sampling and colony counting* section) to have statistical difference between surfaces with or without antimicrobial activity. As described above, one of the modifications made was the addition of glycerol 5% to the bacterial suspension to reduce the surface tension of the PBS on the film, which allows a more homogeneous distribution of the bacterial suspension on the surface. A sterile velveteen cloth was placed on a replica plating tool (Bel-Art Products, Wayne, NJ) and flooded into 10 mL of the bacterial suspension by direct contact during 1 min. The excess of bacterial suspension was eliminated by placing the inoculated cloth for 10 s onto another dry sterile velveteen cloth. To inoculate the 50 mm x 50 mm film specimens, the inoculated velveteen cloth was pressed for 10 s contact time against the surface of the films. The surfaces were allowed to dry under ambient conditions (± 24 °C and humidity of $\pm 30\%$) for 0 min (transference) or 90 min (persistence). Films were sampled and colonies counted as described in the *Sampling and colony counting* section.

d) Swab inoculation assay

The Swab inoculation assay or Swab assay was designed for this study based in the Touch-transfer inoculation assay in order to obtain more reproducible results. This assay also assessed the attachment

and survival of the bacteria on the surface of the film. Serial dilutions of the bacteria inoculum were made in PBS supplemented with glycerol 5% to reach a bacterial concentration of 6×10^4 CFUs/mL. Fifty microliters of the bacterial suspension were pipetted on the films (50 mm x 50 mm) and spread homogeneously by rolling a cotton swab over their surface. Films were allowed to dry under ambient conditions (± 24 °C and humidity of $\pm 35\%$) for 0 min (transference) or 90 min (persistence) before being sampled.

Sampling and colony counting

To quantify the bacteria on the film surface, the agar contact method was used¹⁴ to directly transfer the bacteria of the surface of the films to the culture agar plates (Count-Tact® plates, bioMérieux, France). Contact plates were pressed for 10 s onto the surfaces of the films with the help of a Count-Tact® applicator (bioMérieux, France) that applies a uniform pressure of 0.5 kg on the surface of the contact agar plate. After inoculation, plates were incubated for 18–24 h at 35 °C. Colony counting was performed manually.

Data reporting and statistical analysis

For each assay, 3 samples of each plastic film with micro- and nano-structures and 3 smooth control samples were analysed. Mean colony counts and standard deviation (SD) were calculated for each film and controls. Results were expressed as logarithm of CFUs/cm². A single student's *t*-test was used to compare the bacterial counts between the patterned and the smooth surfaces. A *p*-value lower than 0.05 was considered statistically significant. Statistical analyses were performed using Prism GraphPad version 8 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

The antibacterial activity of the micro- and nano-patterned film against *E. coli* and *S. aureus* was assessed by the four described methodologies. In the ISO 22196 assay (Fig. 2), the inoculum had to be spread over the surface of the film with the help of a piece of Stomacher bag. During the performance of the assay, it was observed that due to the hydrophobicity of the film it was difficult to spread the inoculum homogeneously over the surface leaving some parts of the film not in contact with the bacterial inoculum. As shown on Fig. 2, no reduction in the bacterial count was observed on the patterned film compared to the smooth control film after 24 h of incubation in any of the assays.

The bacterial attachment to the film was also tested by the immersion assay (Fig. 3). The bacterial counts in the patterned film were not statistically lower than the counts on the control film ($p > 0.05$ for all assays). However, close to zero attachment of bacteria was observed in the film with this method (Fig. 3: 23 CFU/cm²) as compared to the touch-transfer and swab-transfer inoculation assays (Figs. 4 and 5: 10^5 CFU/cm²).

The Touch-transfer inoculation assay (Fig. 4) and the Swab inoculation assay (Fig. 5) were performed to test the attachment of bacteria (after 0 min of incubation) to the surface and the antibacterial activity (after 90 min of incubation) of the film against *S. aureus* and *E. coli*. The bacteria inoculum was prepared with and without glycerol 5% in order to spread the inoculum more homogeneously over the film. The addition of glycerol 5% showed a better spread of the inoculum over the surface without altering the growth of the bacteria as observed in the control film (data not shown).

As depicted on Figs. 4 and 5, at time-point 0, on the patterned film, a lower bacterial attachment for *S. aureus* was observed by both methods ($p < 0.05$), while for *E. coli* a lower bacterial attachment was only demonstrated by the swab assay ($p < 0.01$). The antibacterial activity of the film was also studied after 90 min of contact between the bacteria and the surface of the film, either the inoculum was transferred with the help of a velveteen cloth (Fig. 4) or with a swab (Fig. 5). Although a reduction in bacterial counts was observed in both bacterial species with both methods, a statistical significance was only achieved against *S. aureus* when the antimicrobial activity was tested by the swab assay ($p < 0.01$; Fig. 5).

Discussion

Frequently-touched environmental surfaces play an important role as reservoirs and sources of microorganism transmission¹⁰. Microbial pathogens are able to survive on the surfaces for long time, and could be transferred and cause HAI in patients admitted to hospitals¹². There are different current strategies to prevent the contamination of surfaces and therefore, reduce the possibility of patients' infection. Manual cleaning with antibacterial products is the disinfection method most commonly used in healthcare settings. Automated cleaning using UV light is another method that can be employed to provide not only surface but also environmental disinfection in critical areas of the hospitals⁶. More recently, the use of antimicrobial surfaces to permanently prevent their role as reservoir for potential pathogens has emerged as a potential solution^{7,8}. Among them, antiadhesive surfaces, contact-active surfaces, biocide-releasing surfaces or modified topographies have been developed¹⁴. It has been demonstrated that the micro-patterned surface of films reduces bacterial contamination⁷. To evaluate the antimicrobial activity of patterned films different tests can be used and depending on the methodology used, the antimicrobial activity results could vary^{7,17,18}.

One of the most commonly applied tests for the measurement of antimicrobial activity on surfaces is the ISO 22196 standard, which evaluates the antimicrobial activity of plastic surfaces after the incubation of a known bacterial inoculum for 24 h¹¹. However, the usefulness of the ISO 22196 assay for the evaluation of new antimicrobial surfaces is unclear as it showed discrepancies with other methods mainly due to the unrealistic way of transferring bacteria to the plastic surfaces, the high inoculum used and the amount of culture medium left in the liquid layer for 24 h for the diffusion of chemical antimicrobial products present in plastics^{13,18}. In a study carried out in 8 different research facilities, differences between laboratories were found using the ISO 22196 standard method, mainly for materials with intermediate antimicrobial activity¹⁷. Four critical factors that influence the outcome of antibacterial

testing in a series of experiments were described: (1) incubation time, (2) bacteria starting concentration, (3) physiological state of bacteria (stationary or exponential phase of growth), and (4) nutrient concentration. It has been demonstrated that the incubation conditions used in the ISO 22196 can influence the antibacterial activity of some materials^{12,19}. Differences in the activity of silver-ion containing materials were observed when they were tested using the ISO 22196 conditions (high activity at > 90% relative humidity) instead of more realistic conditions of 22% relative humidity (nearly no activity)¹⁹. However, humidity was not critical for the activity of copper and copper alloys.

In our study, no antimicrobial activity of the film was shown by the ISO 22196 method. The conditions used in the ISO 22196 (high inoculum, bacterial culture media and incubation temperature of 35 °C, and relative humidity of 90%) were far away from the conditions observed in the real setting. By using the more realistic touch-transfer and swab inoculation assays the antimicrobial activity of patterned film could be demonstrated. The activity of the film was more related to the anti-attachment activity, probably due to its high hydrophobicity, than to a direct killing of the bacteria. It was also demonstrated that the film was more effective against *S. aureus* than to *E. coli* probably in relation with the shape and composition of each bacteria. The ISO 22196 test is adequate for testing films releasing antimicrobial active compounds but not for films whose antimicrobial activity is based in their surface structure.

The touch-transfer and the swab inoculation assays, both studying transference and persistence events, were highly reproducible. The swab assay was designed in our laboratory taking the protocol of *Mann et al.*⁷ as a reference but trying to better standardize the inoculum applied to the film by controlling the amount of bacteria placed on it. Another modification was the addition of glycerol 5% to the inoculum to obtain a better distribution of the bacterial suspension all over the surface of the highly hydrophobic film. Glycerol was innocuous for the bacteria and reduced the surface tension of the bacterial suspension thus achieving a homogeneous distribution of the bacteria on the film. After inoculating the film surfaces, bacteria were left for 90 min at room temperature (24 °C) and humidity (31%) before taking samples with the contact plates better mimicking the conditions found in a hospital ward. In fact, the experiment was performed in the laboratory of a hospital which better represented the environment of the patients' rooms. Finally, the immersion assay revealed not to be as accurate as both touch-transfer and the swab inoculation methods since a much lower attachment of bacteria was observed. This might be explained by the high hydrophobicity of our film, indicating that this methodology should not be used with hydrophobic films.

Comparing all approaches tested, we concluded that the touch-transfer and swab inoculation methods were the most reproducible and the best at mimicking the various aspects of a real-world surface bacterial contamination. These procedures evaluated the antimicrobial activity of hydrophobic films under real temperature and humidity conditions. These tests were suitable for the analysis of the antimicrobial film under study and should be applied instead of the ISO 22196, which assesses a non-realistic antimicrobial activity. Therefore, taking these results into account, a new ISO method should be developed to study the antimicrobial activity of patterned plastic surfaces.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests in this work

Funding

This work was in part financed by the European Union Horizon 2020 program grant agreement FLEXPOL (grant agreement No. 721062).

Authors' contributions

APG, JVC, AA, NN, JMM: prepare the draft and were major contributors in the writing of the manuscript.

SM, AR, ASA, AF, NK, CST: contributed with the pictures, checked the technical and methodologic parts of the article and reviewed the manuscript.

DC, RM, SC, CP, MCG, AJ, BG, MCA, DD: contributed with the analysis of figures, statistics and other data and with the management of databases and review of the article.

All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

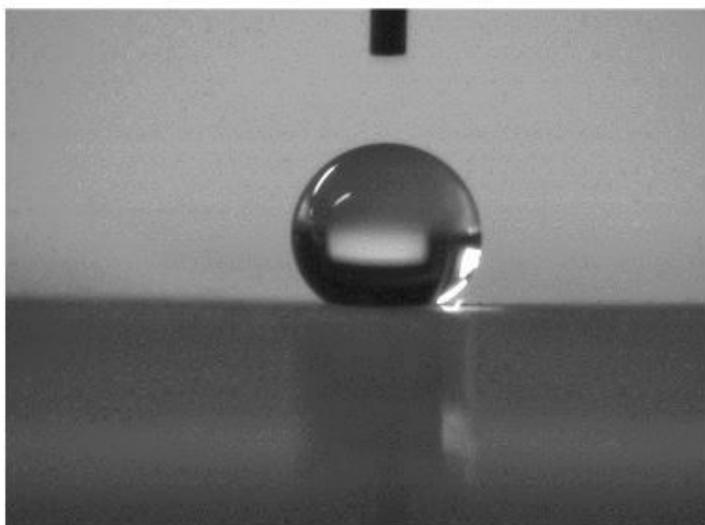


Figure 1

- a) Hydrophobic behavior of a microstructured PP film when it is placed into contact with drops of water.
b) Hydrophobic behaviour of the PP film showing a high contact angle (138°) of a drop of water with the patterned surface.

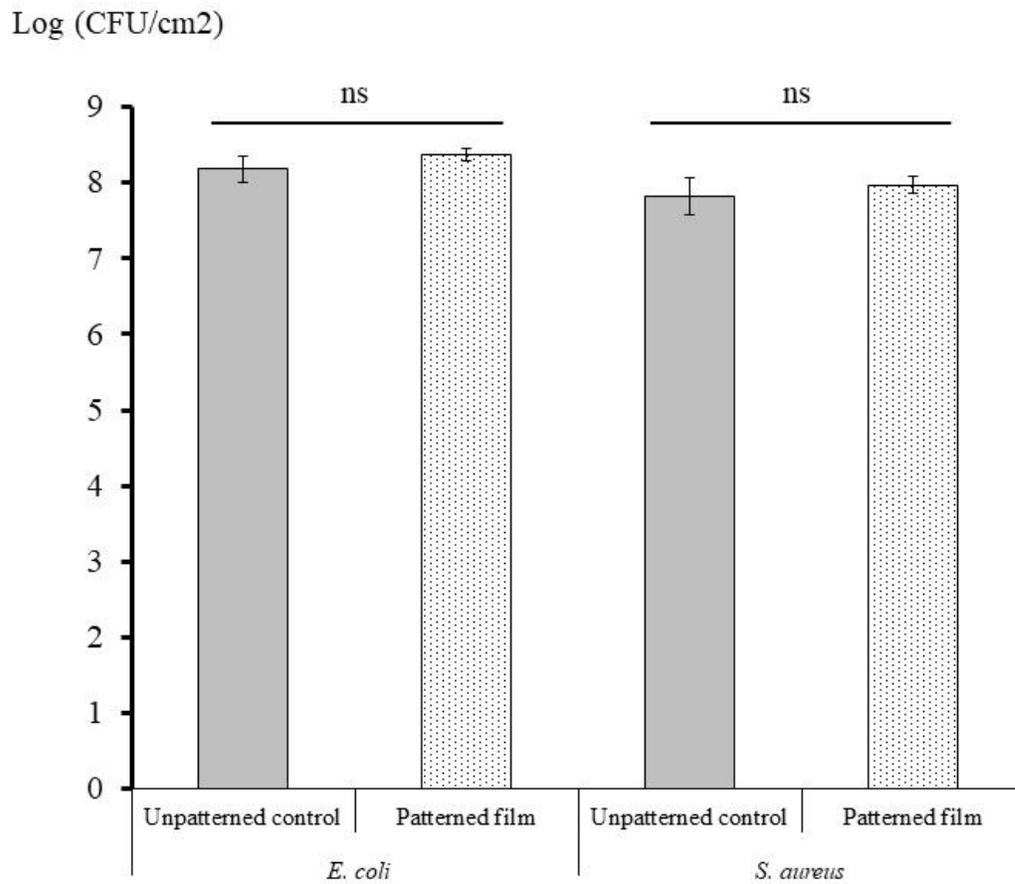


Figure 2

ISO 22196 standard. Antibacterial activity of a patterned film on *E. coli* and *S. aureus* according to the ISO 22196 standard. Bars represent average bacterial counts expressed as Log(CFU/cm²) and vertical lines the standard deviation. ns: no significant.

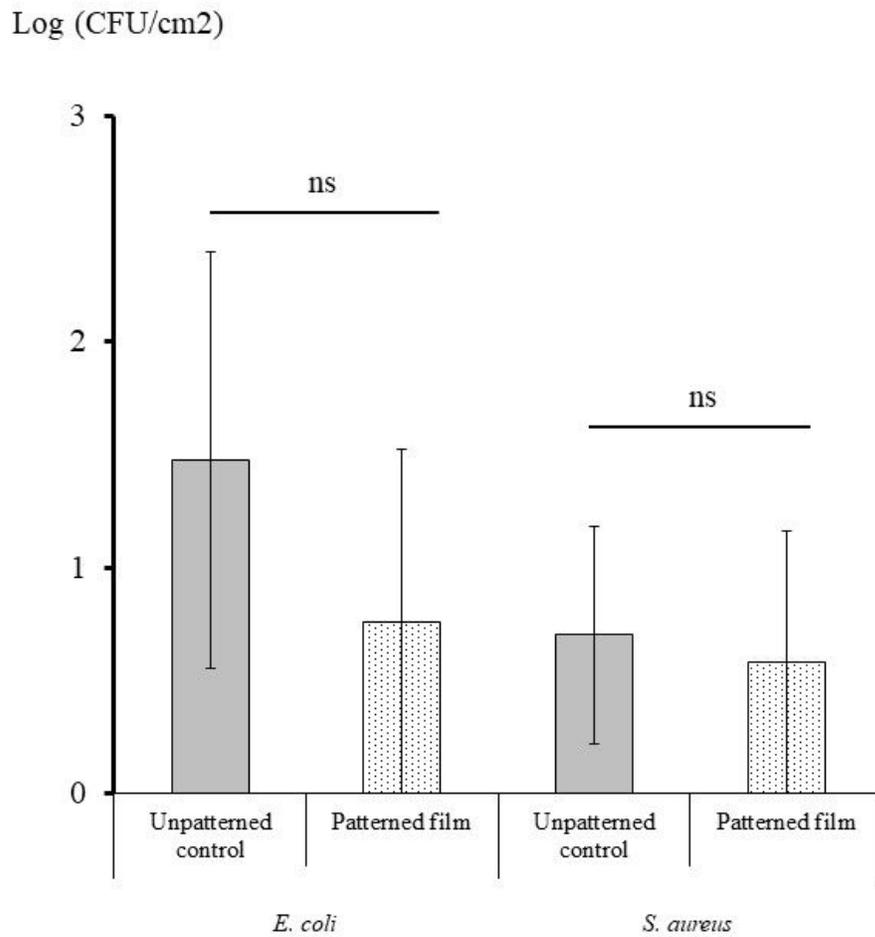


Figure 3

Immersion assay. Anti-attachment activity of a PP patterned film on *E. coli* and *S. aureus* studied by the immersion method. Bars represent average bacterial counts expressed as log (CFU/cm²) and vertical lines the standard deviation. ns: no significant.

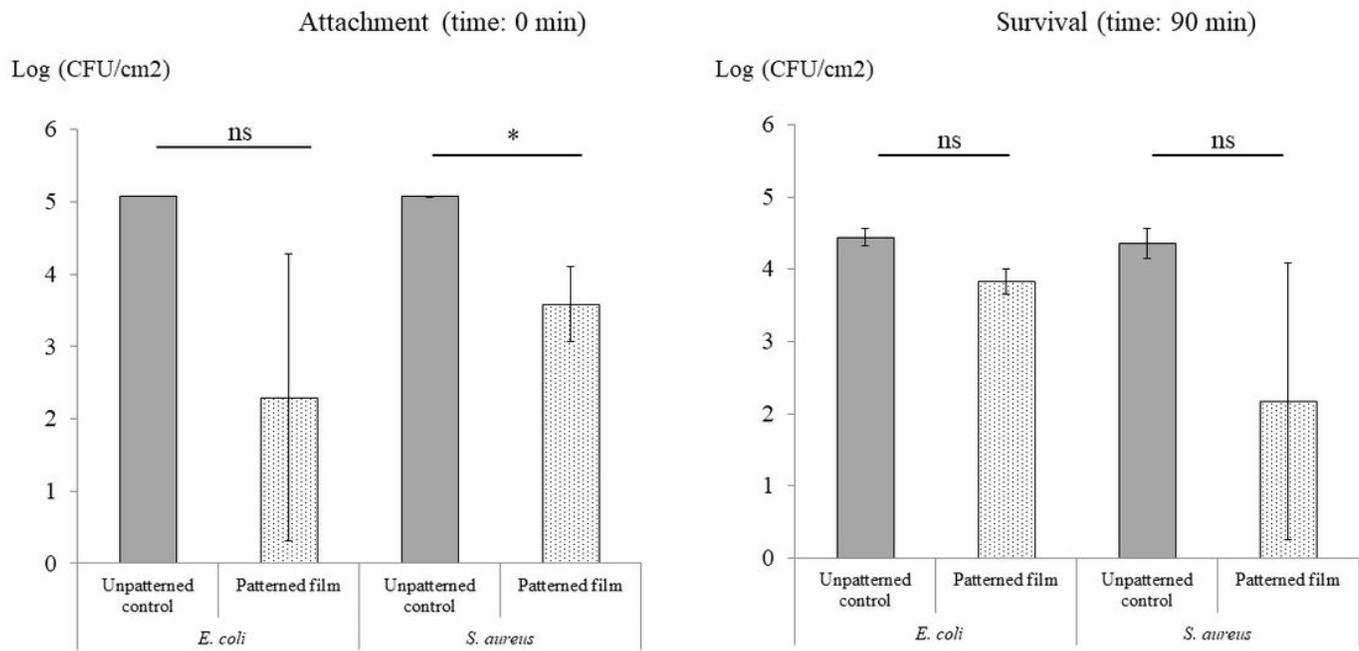


Figure 4

Touch-transfer assay. Attachment and survival of *E. coli* and *S. aureus* on a PP patterned film after 0 and 90 min of incubation, respectively, according to the Touch-transfer assay. Bars represent average bacterial counts expressed as log (CFU/cm²) and vertical lines the standard deviation. ns: no significant. *: $p < 0.05$.

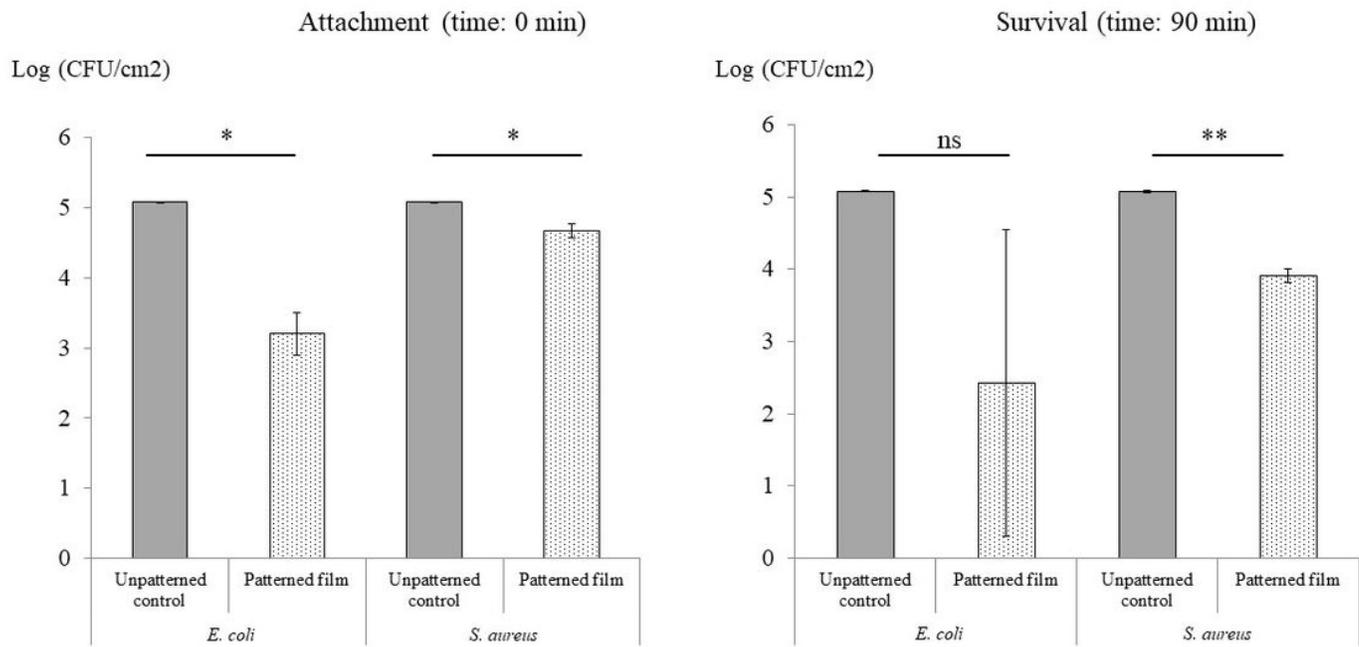


Figure 5

Swab assay. Attachment and survival of *E. coli* and *S. aureus* on a PP patterned film after 0 and 90 min of incubation, respectively, according to the swab assay. Bars represent average bacterial counts expressed as log (CFU/cm²) and vertical lines the standard deviation. ns: no significant. *: $p < 0.05$. **: $p < 0.01$.

Supplementary Files

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