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ORIGINAL ARTICLE

Antibacterial isoeugenol coating on stainless steel and polyethylene surfaces prevents biofilm growth

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Abstract

Aims: Pathogenic bacteria can spread between individuals or between food items via the surfaces they share. Limiting the survival of pathogens on surfaces, therefore, presents an opportunity to limit at least one route of how pathogens spread. In this study, we propose that a simple coating with the essential oil isoeugenol can be used to circumvent the problem of bacterial transfer via surfaces.

Methods and Results: Two commonly used materials, stainless steel and polyethylene, were coated by physical adsorption, and the coatings were characterized by Raman spectroscopy, atomic force microscopy and water contact angle measurements. We quantified and visualized the colonization of coated and uncoated surfaces by three bacteria: *Staphylococcus aureus, Listeria monocytogenes* and *Pseudomonas fluorescens*. No viable cells were detected on surfaces coated with isoeugenol.

Conclusions: The isoeugenol coating prepared with simple adsorption proved effective in preventing biofilm formation on stainless steel and polyethylene surfaces. The result was caused by the antibacterial effect of isoeugenol, as the coating did not diminish the adhesive properties of the surface.

Significance and Impact of the Study: Our study demonstrates that a simple isoeugenol coating can prevent biofilm formation of *S. aureus, L. monocytogenes* and *P. fluorescens* on two commonly used surfaces.

Introduction

Staphylococcus aureus and *Listeria monocytogenes* are common human pathogens. They can be transferred to humans via several routes, and one of these routes is via surfaces where they attach and form biofilm that are robust and highly tolerant to biocides. Such biofilms can, therefore, become persistent sources of contamination or spread of infections. Antibacterial surfaces are designed to eliminate or reduce bacterial growth and can be classified in two categories: antibiofouling surfaces that prevent bacterial attachment and bactericidal surfaces that kill the bacteria upon contact (Hasan *et al.* 2013). The approaches to modifying surfaces include, among others, chemical surface modification via adsorption or covalent binding, and tailoring the topography of the surface (Hasan *et al.* 2013). Researchers have used a plethora of materials, such as antimicrobial peptides (Etienne *et al.* 2004), enzymes (Yuan *et al.* 2011), polymers (Lin *et al.* 2003) and essentials oils (Suppakul *et al.* 2011), to tailor the surface properties.

Essential oils are secondary metabolites extracted from plants. Some of them work in defence of the plant and can, therefore, be used as antibacterial compounds (Burt 2004). Isoeugenol is an essential oil compound that occurs naturally in, for instance, cinnamon, clove and nutmeg (Janssens *et al.* 1990). It has been shown to be active against a range of Gram-positive and Gram-negative

bacteria (Laekeman *et al.* 1990; Hyldgaard *et al.* 2015b), and it is more effective than the isomer eugenol (Zemek *et al.* 1979, 1987). In this study, we coated two commonly used surfaces, stainless steel and polyethylene, with isoeugenol via physical adsorption and show how this coating affects the attachment and growth of two pathogens and one type of spoilage bacteria: *S. aureus, L. monocytogenes* and *Pseudomonas fluorescens*. This is the first study to exploit isoeugenol as a means to prevent biofilm formation on stainless steel and polyethylene.

Materials and methods

Oil coating and characterization

Coupons of stainless steel (316, 10 mm × 10 mm × 1 mm) or polyethylene (Isoplast, 10 mm × 10 mm × 2 mm) were sterilized by immersion in 70% ethanol for 30 min. The coupons were dried in a laminar flow bench for 15 min before addition of coating material. Fifteen microlitres of oil was dropped onto the surface of stainless steel or polyethylene and incubated at 37°C for 15 min. Excess oil was removed by tilting the coupon with a tweezer. Pure isoeugenol (Sigma Aldrich, Brøndby, Denmark) was compared with rapeseed oil (Nordic Food Partner, bought from local supermarket) or dilutions of isoeugenol in rapeseed oil (1 : 1 or 1 : 3 mixture). Rapeseed oil was included for comparison because of its low antibacterial properties. Possible physical effects of an oil coating with low antibacterial properties could, thereby, be investigated. Uncoated control surfaces were treated similarly but without addition of oil.

The oil coating was characterized by contact angle measurements, atomic force microscopy (AFM) and Raman spectroscopy. Contact angle measurements were performed using KRUSS DSA 100 (KRUSS, Hamburg, Germany) under ambient conditions. Surface coatings were produced as described above, and measurements were done with deionized water. Contact angles were calculated from the images using the Drop Snake plug-in of ImageJ (National Institutes of Health, Bethesda, MD). Each value was obtained by averaging four droplets on one sample. Three samples were measured for each coating.

A Nanowizard II AFM (JPK Instruments, Berlin, Germany) mounted on an inverted microscope (Zeiss Axiovert 200 M; Carl Zeiss, Jena, Germany) was used for AFM measurements. AFM imaging of coating on stainless steel surfaces was performed under ambient conditions by quantitative imaging (QI) mode using OMCL-AC160TS (Olympus, Hamburg, Germany) cantilevers with nominal spring constant of 26 N m^{-1}. The sensitivity of cantilevers was calibrated on glass, and the spring constant was obtained by thermal tuning. Imaging was performed at least three locations per sample.

Raman spectroscopy measurements were performed with a Renishaw InVia Raman Microscope (Renishaw, Wotton-under-Edge, United Kingdom) using 514 nm laser (five acquisition and exposure time of 10 s). The lateral resolution using a 50× objective was 1·2 μm. The software WIRE 4.1 from Renishaw was used for data analysis.

Culture preparations and biofilm growth

Overnight cultures of *S. aureus* (DSM20231), *P. fluorescens* AH2 (Gram *et al.* 1990) and *L. monocytogenes* (NCTC 12426) were prepared by transferring a single colony from an agar plate to 15 ml TSB ((3·0% (w/v), pH adjusted to 6 with HCl; Sigma Aldrich) in Erlenmeyer flask with a cellulose stopper. The suspension was incubated overnight at 25°C with orbital shaking 180 rev min^{-1}, and the $OD_{600\ nm}$ was adjusted to 0·5 with fresh TSB before transfer into 24-well plates (1 ml per well).

Coatings were prepared as previously described, and the coated and uncoated stainless steel or polyethylene coupons were individually placed in bacterial suspensions in a 24-well plate and incubated at 25°C for 1 h to allow bacterial attachment. After this inoculation step, each coupon was gently washed by two consecutive transfers into new wells with fresh media, and then incubated for additional 24 h at 25°C to allow biofilm growth. Finally, loosely attached or planktonic bacteria were gently rinsed off by immersion in 1 ml phosphate buffered saline (PBS) twice before further analysis by microscopy or colony forming unit (CFU) enumeration.

Quantification of viable cells in biofilms by CFU enumeration

In order to quantify the number of viable cells in the biofilms, CFUs were measured. Coupons were sonicated for 20 min at 45 kHz in 1 ml PBS. From the sonicated suspension, 10-fold dilutions were performed in PBS and 3 × 15 μl from each dilution step was transferred to an agar plate (detection limit 33 CFU per cm^2). The plates were incubated at 37°C and CFUs were counted. Three biological replicas were included: one per replicate sample.

To test the stability of the coating, stainless steel coupons were coated and incubated in TSB or air for 10 days at room temperature without shaking, followed by inoculation, biofilm growth, and enumeration of CFU as described above.

Visualization of biofilm by confocal laser scanning microscopy and scanning electron microscopy

A fluorescence staining solution that allows distinction between living and dead bacteria was prepared with

2·4 μmol l^{-1} of the membrane-impermeable DNA-binding stain TOTO-1® and 12·2 μmol l^{-1} of the membrane-permeable DNA-binding stain SYTO-60 in 0·85% (w/v) NaCl in demineralized water. Unfixed biofilms on stainless steel or polyethylene coupon were stained by placing the coupon face-down in a drop (90 μl) of staining solution on either a coverslip or a μ-Dish with glass bottom (Ibidi, uncoated) and incubating for at least 15 min in the dark before confocal laser scanning microscopy (CLSM) imaging (LSM700; Zeiss) using lasers 488 and 639 nm for excitation of the dyes. The "fast option" built in settings for imaging with TOTO-1 and Alexa647 were applied. The two dyes were excited simultaneously, and the emitted light was split at 630 nm and detected by two photomultipliers to allow simultaneous detection of fluorescence from both dyes.

Scanning electron microscopy (SEM) imaging of the biofilms required fixation with 2·5% glutaraldehyde for 2 h at 4°C followed by dehydration using ethanol series (35, 50, 70, 95, 100%) incubated for 3 min in each. Surfaces were air dried and sputter coated with Au. Secondary electron imaging was done using a Nova 600 Nano SEM (FEI, Eindhoven, The Netherlands) at 5 kV.

Measurement of *S. aureus* adhesion forces

Overnight cultures of *S. aureus* were prepared by transferring a single colony from an agar plate to 15 ml TSB ((3·0% (w/v), pH adjusted to 6 with HCl; Sigma Aldrich) in Erlenmeyer flask with a cellulose stopper. The suspension was incubated overnight at 25°C with orbital shaking 180 rev min^{-1}. Bacterial cells were harvested by centrifugation at 1677 ***g*** for 5 min, washed three times with PBS, and diluted in PBS to an optical density at 600 nm (OD_{600}) of 0·1.

To immobilize bacterial cells, 50 μl drop was placed on a glass coverslip for 5 min, gently washed with PBS to remove unattached cells and mounted on AFM. Cells were covered with a drop of PBS to protect bacteria from drying out.

Tipless cantilevers with nominal spring constant of 0·03 N m^{-1} (CSC12/NoAl; MikroMasch, Wetzlar, Germany) were submerged in a droplet of 2 μl of isoeugenol. The cantilever was allowed to incubate for 20 min, washed with Milli-Q water, and mounted on the AFM microscope. The sensitivity of tipless cantilevers was calibrated on glass before immobilization of *S. aureus,* and the spring constant was obtained by thermal tuning. To measure bacterial adhesion, a coated cantilever was approached on a single bacterial cell with the aid of the optical microscope. The contact force of the cantilever was kept at 1 nN for a specific period of time (0–30 s), and the cantilever was then retracted to record a complete force curve. At least 10 force curves were recorded on one cell and around 10 cells from the same culture were measured. The force curves were analysed by data processing software from JPK Instruments.

Isoeugenol coating release

Isoeugenol-coated SS were placed in demineralized water in a 24-well plate and samples from aqueous phase were taken out at specific time points for absorbance measurement at 330 nm using UV-visible spectrophotometer (VarioSkan Flash; Thermo Fisher Scientific, Hvidovre, Denmark). A standard curve with pure Isoeugenol in demineralized water was made. For concentrations below 24 mg ml^{-1}, we observed a linear relationship (r^2 = 0·8155) between absorbance at 330 nm and concentration. The release concentration of Isoeugenol was calculated at different time points.

Statistics

Statistics for wettability studies were performed via ANOVA analysis followed by a Tukey's HSD post hoc test and $P < 0·01$ was considered significant. Three replicas for each coating were analysed. Significant differences between CFU quantification were tested with the Wilcoxon rank sum test where $P < 0·05$ was considered significant. Three biological replicas were analysed for each coating.

Results

Characterization of coatings

Coatings with isoeugenol, rapeseed oil or 1 : 1 and 1 : 3 mixtures of these on stainless or polyethylene were achieved through physical adsorption, and the coated and uncoated surfaces were characterized in terms of differences in their wettability, chemistry, topography and adhesiveness.

Raman spectroscopy confirmed the presence of coatings on stainless steel (Fig. 1). The isoeugenol coating (blue curve, Fig. 1) showed three characteristic bands; one at 3068 cm^{-1} corresponding to aromatic carbon and two around 1700 cm^{-1} corresponding to the benzene ring. The rapeseed sample (red curve, Fig. 1) showed one broad peak at around 2900 cm^{-1} corresponding to aliphatic carbon characteristic of hydrocarbon oil. The mixtures of isoeugenol and rapeseed oil (green and pink curves, Fig. 1) resulted in mixtures of the bands characteristic of the pure samples.

Isoeugenol coatings increased the wettability of both stainless steel and polyethylene, as seen from the lower water contact angles of coated surfaces (Table 1).

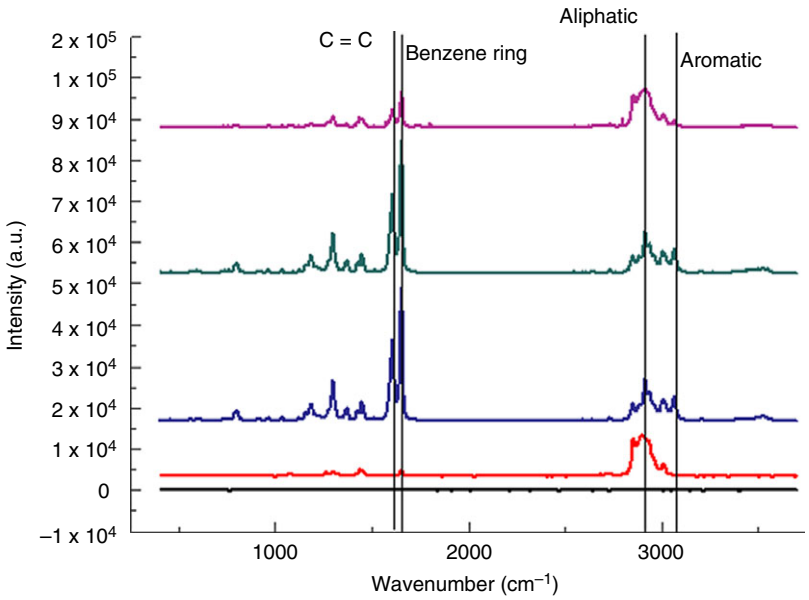

Figure 1 Raman Spectroscopy of the following coatings on stainless steel: (blue curve) isoeugenol (IE), (red curve) rapeseed oil (RS), (green curve) 1 : 1 and (pink curve) 1 : 3 mixtures of isoeugenol and rapeseed oil. A (black curve) control sample of pure stainless steel (blank) is included. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1 Wettability measurements of coatings on stainless steel and polyethylene. Standard deviations are shown in parenthesis (n = 9)

	Control	Isoeugenol	1 : 1	1 : 3	Rapeseed oil
Stainless steel	66·5 (4·9)	39·1 (3·0)*	47·6 (2·1)*,†	48·4 (3·2)*,†	50·5 (5·2)*,†
Polyethylene	86·5 (1·8)	45·9 (2·2)*	68·2 (1·6)*,†,‡	69·8 (1·9)*,†,‡	77·1 (1·5)*,†

*Significant difference to the control.
†Significant difference to the isoeugenol coating.
‡Significant difference to the rapeseed oil coating. 1 : 1 and 1 : 3 refers to 1 : 1 and 1 : 3 mixtures of isoeugenol : rapeseed oil, respectively.

Rapeseed and mixtures of the oils showed the same effect, although to a lesser extent.

Isoeugenol coating prevented biofilm formation on stainless steel and polyethylene due to its antimicrobial properties

Colonization and subsequent growth of bacteria on stainless steel and polyethylene were substantially impaired by the isoeugenol coating. After 1 h bacterial attachment followed by 24 h incubation of the samples in pure TSB, we found no viable cells on isoeugenol-coated surfaces for any of the bacterial strains tested (Fig. 2a,b). We included coatings comprised a mixture of isoeugenol and rapeseed oil to assess if isoeugenol was equally effective, even when diluted in another oil that had no antimicrobial properties. This was not the case, and the impairment of biofilm formation appeared to be concentration dependent.

In order to assess the stability of coatings, we prepared the coatings and incubated them in media or air for 10 days before inoculation and growth of *S. aureus*. The coatings lost some activity after 10 days, albeit the pure isoeugenol coating still caused a reduction in the number of viable bacteria by two log units (Fig. 2c).

We visualized biofilms formed by *P. fluorescens*, *S. aureus* and *L. monocytogenes* on coated and uncoated stainless steel by CLSM (Fig. 3). The images confirmed that biofilms of *P. fluorescens*, *S. aureus* and *L. monocytogenes* formed on uncoated and rapeseed oil-coated surfaces, while no bacteria were visible on surfaces coated with isoeugenol. Bacteria were present on surfaces coated with the isoeugenol/rapeseed oil mixture, but many of these were dead as indicated by TOTO-1 staining. The visualization of the biofilms thus supported the results from CFU enumeration.

In the CLSM images, the contours of the stainless steel grain boundaries were visible in the background, as reflection of the laser allowed us to capture a dim image of the stainless steel surface. Isoeugenol is slightly autofluorescent in the red spectrum, and amorphous structures of oil droplets were visible on some samples (Fig. 3d,g,m,n). However, oil and living bacteria were easily distinguishable by morphology and size.

The absence of both viable and dead cells in the CLSM images of isoeugenol-coated surfaces could indicate that the coatings were not only antibacterial but also antiadhesive. SEM images were taken of *S. aureus* biofilms on coated and uncoated stainless steel surfaces to complement the CLSM images (Fig. 3p–t), and they showed that

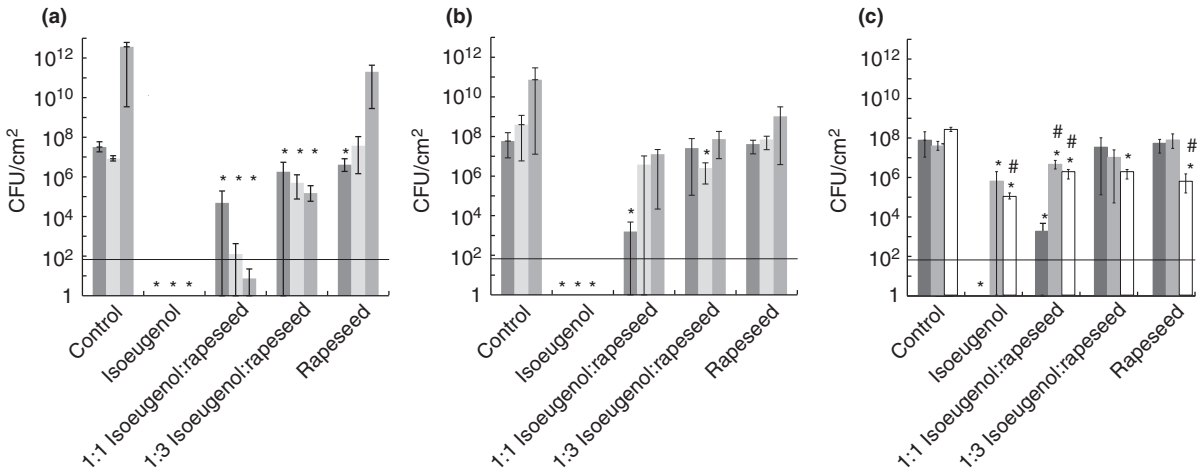

Figure 2 Enumeration of CFU from (■) *Staphylococcus aureus,* (■) *Listeria monocytogenes* and (■) *Pseudomonas fluorescens* biofilms after 24 h of growth on freshly prepared coatings of (a) polyethylene or (b) stainless steel. (c) Enumeration of *S. aureus* after growth for 24 h on stainless steel coated with (■) freshly prepared coatings and with coatings prepared 10 days prior and stored in (■) TSB or (□) air. The horizontal line indicates the detection limit. Values are averages (error bars = range). *Statistical significance (Wilcoxon rank sum test, $P < 0{\cdot}05$) compared with the uncoated control. #Statistical significance compared with freshly prepared coatings among the same coatings with different storage time.

the same overall trends were as CLSM images. However, some features on the isoeugenol-coated surfaces indicated the presence of cell debris rather than intact *S. aureus* cells (Fig. 3q), indicating that bacterial cells may have adhered to the isoeugenol-coated stainless steel.

The adhesive properties of isoeugenol-coated surfaces were then explored further by AFM, using the QI imaging mode which provides simultaneous information about surface contours and adhesiveness. Isoeugenol completely covered the grain boundaries on stainless steel surfaces, and QI imaging showed that the adhesiveness of isoeugenol-coated stainless steel (range 70–350 nN) was substantially higher than the bare stainless steel (range 10–50 nN) (Fig. 4). These forces resulted from the interaction of the AFM cantilever with the surface, and to better understand how isoeugenol coatings affected the interaction between bacteria and a surface, we quantified the adhesion force of single *S. aureus* cells and isoeugenol-coated cantilevers, using single-cell force spectroscopy. The adhesion force measured was in the range of 0·21–1·19 nN for isoeugenol-coated cantilevers and 0·38–3·18 nN for uncoated cantilevers. Statistical analysis (paired *T* test, $n = 10$) showed no significant difference; hence, isoeugenol coating did not appear to impair the adhesion of *S. aureus*.

Discussion

Increasing consumer interest in natural products has spurred investigations into the use of antimicrobial essential oils as replacement for synthetic disinfectants. Isoeugenol has previously been investigated for its use as an antimicrobial compound (Hyldgaard *et al.* 2015a), but has to our knowledge not previously been used as an antibacterial surface coating. In this study, we show that coating of stainless steel and polyethylene surfaces by physical adsorption of isoeugenol resulted in no detection of live bacteria of *P. fluorescens, S. aureus* and *L. monocytogenes* on the surfaces, even after 24 h of incubation in nutrient media to allow bacterial growth (Fig. 2).

It is known that isoeugenol has antimicrobial properties, and its antibacterial properties has been linked to its free hydroxyl group, the position of double bonds in α and β positions of the side chain, and a methyl group in the γ position (Laekeman *et al.* 1990). Hyldgaard *et al.* (2015b) suggested that isoeugenol has intracellular targets, but also targets the cell membrane through a nondestructive detergent-like mechanism that leads to increased permeability (Hyldgaard *et al.* 2015b). The apparent absence of bacteria on the isoeugenol-coated surfaces could potentially be caused by a combination of antimicrobial and antiadhesive properties of the isoeugenol coating. Other essential oils have previously been shown to inhibit bacterial adhesion at concentrations below the minimal inhibitory concentration for growth (Dal Sasso *et al.* 2006). Dal Sasso *et al.* (2006) showed that this effect was caused by aromatic compounds, such as thymol, which interfere with bacterial surface appendages. We, therefore, investigated if isoeugenol prevented biofilm formation through a combination of antibacterial and antiadhesive properties. However, adhesion mapping (Fig. 4) and single-cell force spectroscopy analysis did

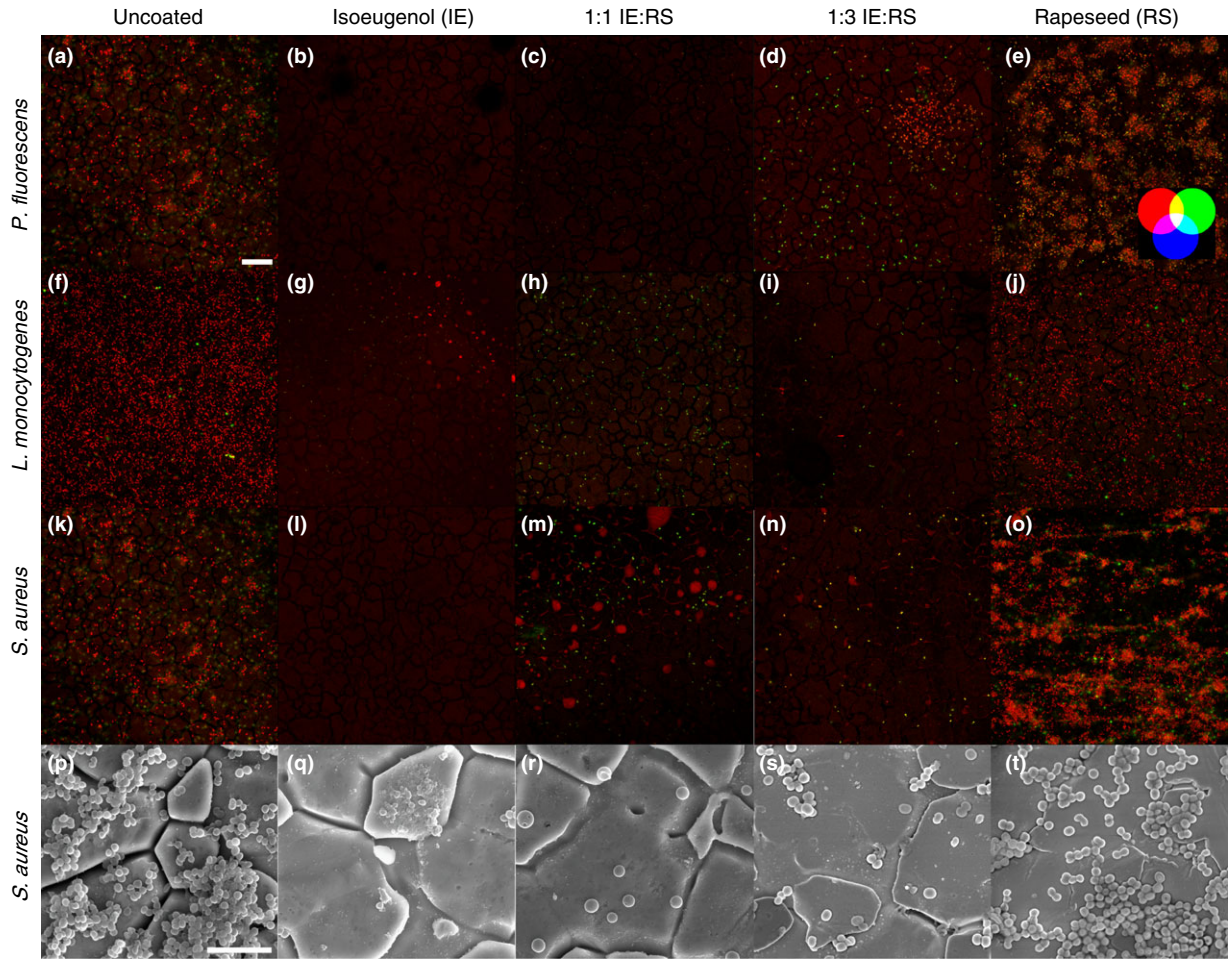

Figure 3 Biofilm growth of *Pseudomonas fluorescens* (a–e), *Listeria monocytogenes* (f–j) and *Staphylococcus aureus* (k–o) on stainless steel with different surface coatings imaged by CLSM (a–o) and SEM (p–t): Uncoated controls (a, f, k, p), Isoeugenol (b, g, l, q), 1 : 1 isoeugenol : rapeseed oil (c, h, m, r), 1 : 3 isoeugenol : rapeseed oil (d, i, n, s) and rapeseed oil (e, j, o, t). The isoeugenol coating has red autofluorescence and is recognized by a weak red signal in the background of the entire image, or as amorphous shapes of oil that does not cover the entire surface. Viable bacteria are red (SYTO 60). Dead bacteria and extracellular DNA are green (TOTO-1). Cells that appear orange are viable cells surrounded by extracellular DNA. Scale bar = 20 μm in CLSM images and 5 μm in SEM images. [Colour figure can be viewed at wileyonlinelibrary.com]

not suggest that bacterial adhesion was impaired to isoeugenol-coated surfaces. Single-cell force spectroscopy was only performed for *S. aureus,* and the results do, therefore, not exclude that antiadhesive effects may exist for other bacteria.

As the isoeugenol coating did not appear to be antiadhesive, one would expect to observe dead cells adsorbed to the coated surfaces. However, this was not the case according to CLSM images (Fig. 3b,g,l). SEM images did show the presence of cell debris (Fig 3q), indicating that adhesion does occur, and the absence of cells in CLSM images could, therefore, be caused by the absence of DNA in the cell debris, leaving the dead cells undetectable by the DNA-binding stains used for visualization.

Other researchers have demonstrated the use of essential oils as antimicrobials, but few investigated their use in surface coatings. For example, Suppakul *et al.* (2011) incorporated linalool and methylchavicol in polyethylene by an extrusion film blowing process, resulting in films that maintained antibacterial properties against *Escherichia coli*, even after storage for a year. Guarda *et al.* (2011) demonstrated the antimicrobial properties of polypropylene film with a coating of microcapsules containing thymol and carvacrol. These coatings showed significant antimicrobial activity against *S. aureus* ATCC 25923, *Listeria innocua* ATCC 33090 and *E. coli* O157:H7. Higueras *et al.* (2015) covalently attached cinnamaldehyde to chitosan films. Cinnamon essential oil has also been incorporated into a paper packaging, resulting in growth inhibition of the bread

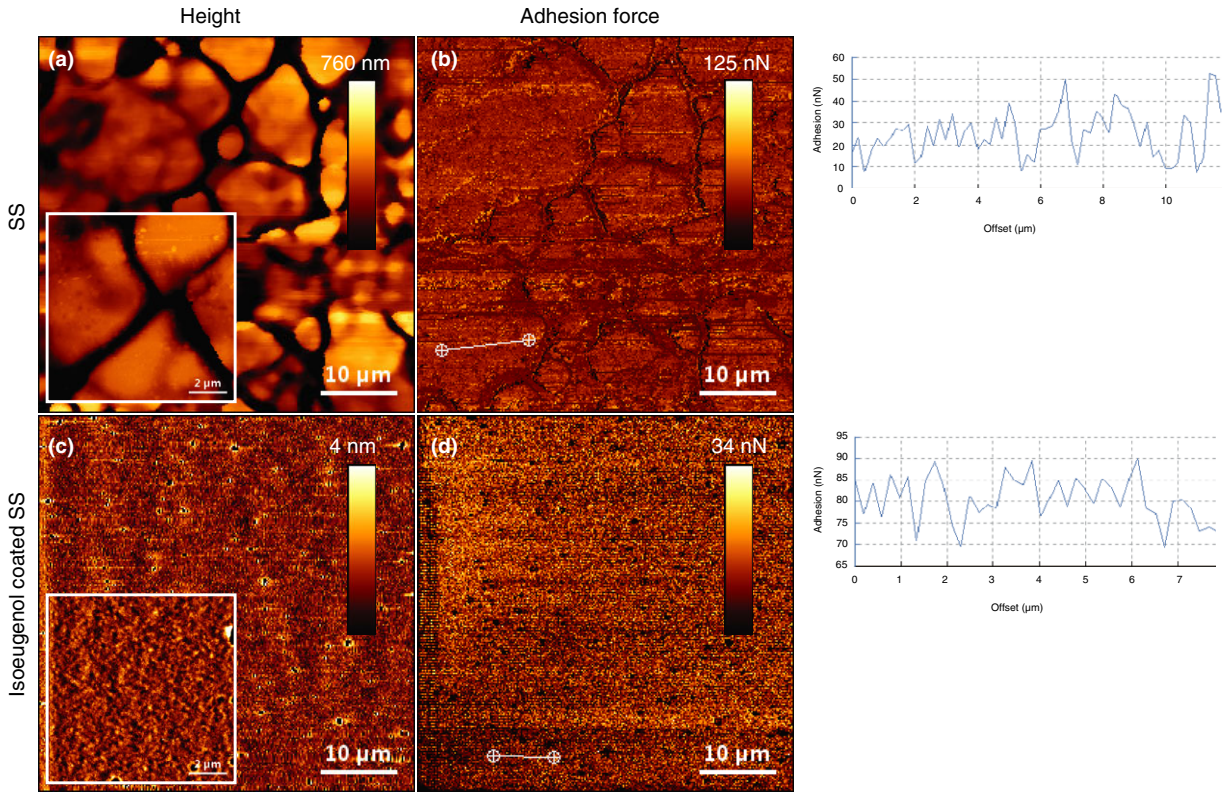

Figure 4 AFM images of uncoated and isoeugenol-coated stainless steel surfaces, showing the height (left) and adhesion force (right) images obtained by quantitative imaging (QI) mode. All images are 50 × 50 μm^2 with inset image are of size 20 × 20 μm^2. From three replicate images the range in adhesion force was from 10 to 50 nN for SS and 70–350 nN for isoeugenol-coated SS surfaces. [Colour figure can be viewed at wileyonlinelibrary.com]

mould *Rhizopusstolonifer* after 3 days of storage with 6% (w/w) of cinnamon oil (Rodriguez *et al.* 2008).

In the context of using essential oils directly as surface coatings, Magetsari *et al.* (2014) studied the effect of coating stainless steel surfaces via physical adsorption with a mixture of essential oil (cinnamon oil, 3%) and chitosan (2%, serial concentration of cinnamon oil from 0·125% to 2%) to prevent biofilm formation by *Staphylococcus epidermidis*. The study showed that a mixture of cinnamon oil (2%) and chitosan (1%) was most effective. In another study, Rajaraman *et al.* (2015) coated poly(methyl methacrylate) and polystyrene surfaces with a *Ocimum sanctum* oil extract, using a physical adsorption process similar to our study. They showed that the coating increased the surface wettability and significantly reduced (by 90%) the number of adhered bacteria of *S. aureus, P. aeruginosa* and *E. coli* after 3 h of incubation. The authors also suggested that the antimicrobial activity of *O. sanctum* oil can be attributed to the presence of more than 50% of eugenol in extract. Collectively, these and our findings suggest that essential oils, and isoeugenol in particular, can provide highly effective antimicrobial coatings.

Assessment of coating stability showed that coatings lost some activity after 10 days, but the pure isoeugenol coating still caused a reduction in the number of viable bacteria by two log units (Fig. 2c). The cause for reduction in activity after 10 days could be due to its high volatility and low stability. Isoeugenol release from the surface was evaluated, and almost 93% of the coating was released after 24 h of sample immersion in water (Fig. 5). The stability of isoeugenol may be improved by encapsulation, for example, in liposomes, which could provide good stability and sustained release of isoeugenol.

The isoeugenol coating employed in this study adds to the field by its effectiveness and simplicity. Application of this coating could, for example, be on tables, sinks, toilet seats or other hotspots for bacterial transfer between individuals in hospitals, or it could be used on surfaces in production facilities where microbial contamination is an issue. Another application could be in private homes to prevent growth of bacteria on, e.g. chopping boards where the long-lasting antimicrobial effect could eradicate bacteria in crevices where

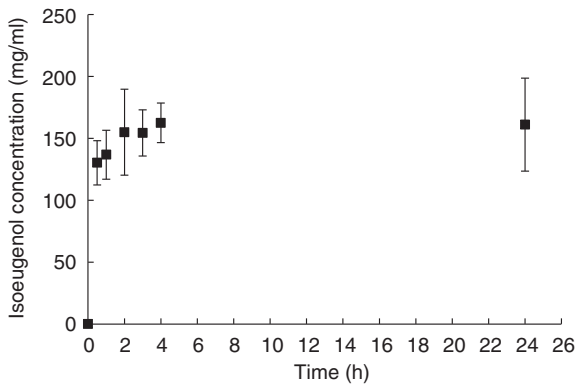

Figure 5 Isoeugenol concentration release from SS surface when immersed in demineralized water for 24 h. Values are averages (N = 3, error bars = SD).

mechanical cleaning is inefficient. Finally, coating with isoeugenol might also have potential applications on medical implants. However, for this application, the effect of the coating on human cells and immune factors should be investigated.

In conclusion, we have shown that a simple isoeugenol coating obtained by physical adsorption to stainless steel and polyethylene eliminated bacteria adsorbing to the surface and thereby prevented biofilm formation.

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Conflict of Interest

No conflict of interest declared.

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