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Characterization of antimicrobial efficacy of photocatalytic polymers against food-borne biofilms

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ABSTRACT

Biofouling of food industry equipment and other surfaces that food products contact during processing is a threat to food safety, which results in infectious outbreaks and economic losses due to corrosion, equipment impairment, and reduced heat transfer efficiency. Once firmly attached to a surface, biofilms can be almost impossible to remove using current sanitation procedures. Self-cleaning surfaces with TiO_2 coatings that are activated with ultraviolet (UV) light may be effective in preventing bacterial growth or killing or removing adherent organisms but require studies to demonstrate their efficacy and determine optimum conditions for use. Therefore, we examined the efficacy of TiO_2-based polymer coatings against key food-borne pathogens namely, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*. Upon photo-catalytic activation of the coatings, the viability of early stage biofilms formed on each coated surface and the relative contribution of reactive oxygen species was evaluated. Results show that the relative antimicrobial activity strength was dependent on the length of UV irradiation; 5–10 min exposure was sufficient to inhibit/kill biofilms of each pathogenic species tested. The results of this study render contact surfaces less attractive for pathogenic biofilms while doubling as an effective mitigation strategy to remove biofilms that form despite coating.

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1. Introduction

Within the food manufacturing and processing industry, microbial biofilms constitute a difficult challenge to overcome because they cause spoilage and threaten public health by contaminating food products (Shi & Zhu, 2009). Regulations of increasing strictness are being placed on food processing plants, and the widespread and sensational publication of contamination events and foodborne illness outbreaks pressures the food industry to take every possible precaution to prevent food contamination. Biofilms can form on almost any surface (dependent on the organisms involved) including silver, aluminum (Al), stainless steel, and glass, which are commonly used surfaces in the food processing industry (Araújo et al., 2010). Once a biofilm forms, it is notoriously difficult to remove as the formation of complex three-dimensional biofilm structures protects inhabitants from chemical disinfectants and mechanical cleaning processes (Kordmahaleh & Shalke, 2013; Simões, Simoes, & Vieira, 2010). The frequent and excessive use of disinfectant agents to remove biofilms has resulted in a sharp and dangerous increase in the number of microbial strains due to the development of resistance (Dorotkiewicz-Jach, Augustyniak, Olszak, & Drulis-Kawa, 2015). The implementation of self-cleaning surfaces can enhance current cleaning and sanitation protocols by providing sustained surface cleanliness and minimizing the incidence of insufficient cleaning practices that are unable to completely remove adherent organisms (Araújo et al., 2010). Advances in surface and cleaning technology will likely help to prevent and control biofilm contamination without the overuse of disinfectants that give rise to resistance. Such approaches may be more effective mitigation strategies for improving food safety and reducing the risk of contamination.

In recent years, the interest in the antimicrobial and self-cleaning coatings has grown rapidly, mainly because these applications may be a valuable weapon in the fight against microbial biofilm contamination, which may fall under more than one antifouling technology category - those that prevent attachment and those that facilitate detachment (Araújo et al., 2010). The photocatalysis of titanium dioxide (TiO_2) is one strategy that has been widely investigated and successfully applied to disinfection and the

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development of self-cleaning surfaces (Bonetta1 et al., 2013; Cai, Strømme, & Welch, 2014a; Robertson, Robertson, & Bahnemann, 2012; Sánchez et al., 2012); TiO_2 has been largely exploited for the ability to kill microorganisms upon photoactivation, which signals radical release (Matsunaga, Tomoda, Nakajima, & Wake, 1985). TiO_2 is considered an excellent photocatalyst because of its high efficiency, low cost, general lack of toxicity and its photostability (Uboncholakate, Sikong, & Saito, 2012). The mechanism of TiO_2 induced photocatalysis involves the formation of reactive oxygen species (ROS), including superoxide radicals (O), hydroxyl radicals (•OH), and hydrogen peroxide (H_2O_2), which can be induced by the absorption of light at a wavelength of 385 nm or less, depending on the size of the band gap. ROS are very strong oxidants, which cause the oxidation of organic materials, deterioration of cellular membranes, growth inhibition, and microbial cell death, which may consequently prevent or destabilize biofilm growth (Dalrymple, Stefanakos, Trotz, & Goswami, 2010).

In this study, we used the durable coatings of polyurethane and TiO_2 as food contact surfaces to test against food-borne pathogenic bacterial biofilms. The novelty and key advantage of such photocatalytic polymer coatings is that they increase the stabilization and dispersion of TiO_2 particles throughout the polymer matrix by chemically and permanently attaching the TiO_2 particles directly to the polymer backbone as it is formed. Linking TiO_2 to the polymer backbone enhances its necessary optical properties; light is required to penetrate the coating to in turn activate the photocatalytic sites within the polymer (Faure et al., 2013). This process also allows for the catalytic properties to be fully transferred to the bulk material, for example in event that the surface is damaged. This method also offers a solution to safety concerns related to TiO_2 dust formation, because the nanoparticles are chemically attached to the polymer backbone and unable to detach and form dust particles.

The overall aim of this study was to test the antimicrobial efficacy of an innovative antibacterial polymer coating using TiO_2, which was applied to steel and aluminum surfaces. We selected to evaluate its efficacy against common and relevant food-borne pathogens *Escherichia coli, Listeria monocytogenes*, *Pseudomonas aeruginosa,* and *Salmonella typhimurium.*

2. Material and methods

2.1. Bacterial cultures and material

E. coli (ATCC 25992) was purchased from the American Tissue Culture Collection (ATCC). All other bacterial stains (*P. aeruginosa* BK-76, *L. monocytogenes* and *S. typhimurium*) were isolated from clinical samples and received as a gift from the Canadian Research Institute for Food Safety (CRIFS) and from the Centre for Public Health and Zoonoses (CPHAZ) of the Ontario Veterinary College of University of Guelph. All other reagents were purchased from Sigma-Aldrich (Oakville, Canada). TiO_2 polyurethane photocatalytic film coatings (SunWash EverPur PA401) were obtained as a gift from Sun-Wash Technologies Inc., (London, ON, Canada). SunWash EverPur is a 2-part polyaspartic photocatalytic coating. Steel and Al substrates (grades Q-Panel QD36 and A36) without coating (control), coated with polyurethane alone, and coated with 1% TiO_2 and polyurethane were cut into 20 mm diameter discs.

2.2. Material characterization

The surface morphologies of the bare surface, polyurethane, and dual-coated (polyurethane and titanium oxide) films on steel and aluminum surfaces were observed using a scanning electron microscope (JCM-5000 NeoScope™, JEOL, QC, Canada) and a portable Raman system (Sierra, SnRI, WY, USA) with a laser of 785 nm at 70 mW with 5 s exposure time and 5 accumulations.

2.3. Photocatalytic characterization of coatings

Methylene blue (MB) degradation assays were performed under UV irradiation to evaluate the photocatalytic activities of TiO_2-based polymers coatings. Briefly, MB solution was prepared to a concentration of 0.05 mM (Navabpour et al., 2014). Four-hundred μL of MB solution was placed on these substrates and irradiated by an integrated power flux of 950 $\mu W/cm^2$ at a height of 5 cm with a 6 W UV handheld lamp (UVGL-55 handheld lamp- 254/365 nm, CA, USA) for the following intervals: 0, 10, 30, 60, and 90 min. Spectra of the MB solution were taken by a portable compact VIS-NIR spectrometer (CCS175, ThorsLab, QC, Canada). Viability tests were performed on glass slides using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Life Technologies Inc., Burlington, ON, Canada). Fluorescence images were acquired using a Confocal Laser Scanning Microscope (Leica TCS SP2, Leica Microsystems, ON, Canada). Live bacteria were imaged using an excitation wavelength (Ex) of 488 nm and emitted light in the "green" channel was collected in a 514–535-nm window. Dead bacteria were imaged at Ex543 and collected in the "red" channel at 633 nm.

2.4. Photocatalytic disinfection against bacteria

L. monocytogenes, *E. coli*, *Salmonella typhymurium*, and *P. aeruginosa* were used to test the antibacterial efficacy of TiO_2-based photocatalytic polymer coatings. *L. monocytogenes*, *E. coli*, *S. typhymurium*, and *P. aeruginosa* were grown overnight in 5 mL of brain heart infusion broth (BHI) and tryptic soy broth (TSB) at 37 C. The bacterial cultures were adjusted to 0.5 McFarland Standard cell densities (optical density (OD) at 600 nm between 0.08 and 0.1 before a test. Then, 400 μL of diluted culture (of each species) was pipetted individually onto the steel and aluminum discs and glass slides. After incubation at room temperature for 4 h, the solution was removed and the unbound bacteria were gently washed away with 500 μL deionized water. Antibacterial activity tests were performed under UV irradiation (6 W, 1200 $\mu W/cm^2$, UVGL-55 handheld lamp- 254/365 nm, CA, USA) kept at a height of 5.5 cm from discs for 0, 5, 10 and 30 min. Bacteria were swabbed off from the substrates both with and without coating (control) were performed the swab test (Path-Check, Microbiology International, MD, USA), and the CFU counting test. For the colony counting, swabbed-off bacteria were inoculated into the corresponding broth medium for overnight incubation at 37 °C. Ten-fold dilution series of the overnight bacterial culture (broth) were prepared (10^{-4} to 10^{-6} final dilutions). 100 μL of each dilution in triplicate were spread-plated onto Mueller Hinton agar and incubated at 37 °C for 24 h. The numbers of bacterial colonies appeared between 30 and 300 on each plate were counted and recorded as estimates of the viable bacteria counts. To directly demonstrate the sanitizing effects of the coated substrates with photocatalytic treatment, the disinfection efficiency was estimated by the following equation:

$$D = \frac{N_0 - N}{N_0} \times 100\%$$

Where N_0 is the average number of colonies on the control plates, and N is the average number of colonies counted on plates for treated bacteria samples.

2.5. Metabolic activity assays for reactive oxygen species (ROS)

TiO_2-based photocatalytic polymers can produce ROS, free

hydroxyl radicals (•OH), and hydrogen peroxide (H_2O_2), by receiving energy from UV irradiation. This metabolic activity assay based on resazurin is a method used to evaluate the contribution of ROS to the antibacterial effects of TiO_2-induced photocatalysis by measuring the accumulation of resorufin (pink) from resazurin (blue), which is produced due to bacterial metabolism (Cai, Strømme, & Welch, 2014a, 2014b). For a typical test, overnight bacterial cultures were first collected by centrifugation (850 × g, 5 min) and re-suspended in PBS containing the ROS scavengers D-mannitol and catalase, which bind and block •OH and H_2O_2, respectively. An optimized concentration of scavengers (1 mg/mL) based on Cai's (2014a) study was used. Sixty μL of the sample suspension was spread evenly over the surface of the discs and then irradiated (6 W, −254/365 nm) for 0, 5, 10, and 20 min. After irradiation, the discs were transferred into sterile centrifuge tubes containing 1 mL of PBS and were mixed at 300 rpm for 5 min (IncuShaker Mini, Southwest Science, NJ, USA) to re-suspend the bacteria from the surface. One-hundred μL of the bacteria suspension was transferred to a test tube containing 900 μL of BHI broth and resazurin (2.5 μg/mL), which was incubated at 37 °C for 20 min. A series of untreated bacterial dilutions of known OD values were made for the preparation of the standard solutions. Visual color changes were compared to the standard solutions to investigate the bacterial metabolism by reading the absorbance values.

2.6. Statistical analysis

Biological experiments were performed independently twice with 3 biological replicates. Characterization experiments were performed twice with triplicate repeated measures for each time point. Statistical significance was calculated by Student's t-test in each group with significant differences defined as $p < 0.05$ (R Open Source Statistical Programming, Auckland, New Zealand).

3. Results and discussion

3.1. Titanium dioxide coating forms

The coatings use a combination of rutile and anatase. From a formulation point of view SunWash polymers use 2% TiO_2 - where 1% is pure rutile and 1% is Evonik P25 where the anatase to rutile ratio is 80/20. So a final ratio of Anatase to Rutile is 2:3. The novelty and key advantage of the nTiO2-PU technology is that it increases the stabilization and dispersion of TiO_2 nanoparticles throughout the polymer matrix by chemically attaching the TiO_2 nanoparticles directly to the polymer backbone as it is being formed. From a chemistry point of view, this is accomplished by coordinating the TiO_2 to the polymer through carboxyl group coordination. Better dispersion of TiO_2 in the polymer matrix has several key advantages. It not only improves the photocatalytic activity or "self-cleaning" but it also decreases the tendency of TiO_2 agglomeration. It also allows for the catalytic properties to be fully transferred to bulk material i.e. if the surface is damaged the newly exposed surface retains its catalytic properties.

3.2. Characterization and methylene blue (MB) degradation

Fig. 1A–D shows SEM images and RAMAN spectra of the bare surfaces, polyurethane only, and dual-coating (polyurethane and titanium oxide) films on both steel and Al surfaces. Results show that the dual coatings were not uniform on both substrates. This feature in coating may increase the roughness of the surface, possibly leading to the differences in the disinfection efficacy due to the non-uniform photocatalytic reaction. The major characteristic peaks of titanium oxide (Hardcastle, 2011), 394 cm^{-1}, 448 cm^{-1},516 cm^{-1}, 613 cm^{-1} and 640 cm^{-1}, were observed in the RAMAN spectra of the dual-coated (polyurethane and titanium oxide) film. The spectra show similarities and are indicative of the film coating on both surfaces. The presence and the appropriate concentration of titanium dioxide are being confirmed by the obtained RAMAN spectral data. Fig. 1E shows photocatalytic activity of the coating by the degradation of MB with respect to the length of UV irradiation. There was a significant increase in MB degradation from 0 to 10 min, the rate of which slowed after 30 min for both coating on aluminum and on steel. However, we determined that steel exhibited enhanced photocatalytic activity, evidenced by its more rapid and complete degradation of MB, as compared to the curve for aluminum. These findings suggest that while both surfaces may be suitable substrates for TiO_2 coating, steel may be a superior substrate in terms of its photocatalytic activity.

3.3. Viability tests by confocal imaging

The confocal laser scanning microscopy aided in the visual confirmation of a reduced live bacteria count indicating the increase in the dead bacterial count on the polyurethane-titanium dioxide coated surfaces. *L. monocytogenes* biofilm was uniformly grown across both the polyurethane-TiO_2 coating and the uncoated glass substrate. Seven different sections of the samples were imaged for each biological replicate, and the live-dead cells were distinguishable at the magnification used for imaging. Bacterial viability was first assessed by microscopy. The numbers of viable cells on coated glass slides after photocatalytic treatment were determined and used as a measure of disinfection strength. Fig. 2A and B show the uncoated samples upon irradiation with UV. Fig. 2C and D show the photocatalytically-treated *L. monocytogenes* cells on coated substrates. Fig. 2C and D indicates that a considerable amount of bacteria were killed after the photocatalytic treatment for TiO_2 polyurethane photocatalytic film coated surfaces, where the red fluorescence signal is dominant as observed from the image. In contrast, the green fluorescence signal appears to be dominant in Fig. 2A and B which shows that only fewer cells were killed after photocatalytic treatment of uncoated surfaces. Our results indicate that the coating is effective in killing cells that attach to the surface. Exposure of TiO_2 coated substrates to the UV radiation resulted in a significant ($P < 0.05$, R Open Source Statistical Programming, Auckland, New Zealand) reduction in the viability in comparison to the uncoated sample surfaces. Our results confirm that the presence of titanium dioxide coating is significant in inactivating the bacterial cell.

3.4. Swab and colony counting

Tables 1 and 2 show the antibacterial efficiency of TiO_2 polyurethane photocatalytic film coatings with varying lengths of UV irradiation (in minutes). On the steel surfaces, UV irradiation (1200 $\mu W/cm^2$) for 5 min was sufficient to inhibit *E. coli.* and *S. typhymurium*, while 10 min was necessary to exhibit the same effect on *L. monocytogenes*. Similar results were obtained for coated aluminum surfaces (Table 2). *L. monocytogenes* required a longer UV irradiation time to kill cells as compared to *E. coli* and *S. typhymurium*. The reason for this can be at least partially attributed to the difference in cell wall structure between Gram-negative and Gram-positive bacteria (Foster, Ditta, Varghese, & Steele, 2011; Kim, Kim, Cho, & Cho, 2003; Ramani, Ponnusamy, & Muthamizhchelvan, 2012; Skorb et al. 2008). The matrix of these biofilms and the difference in the virulence properties and the metabolism of these organisms could also be the reasons for the differences in the antibacterial effect of the studied species.

CFU counting was used to evaluate the number of viable bacteria

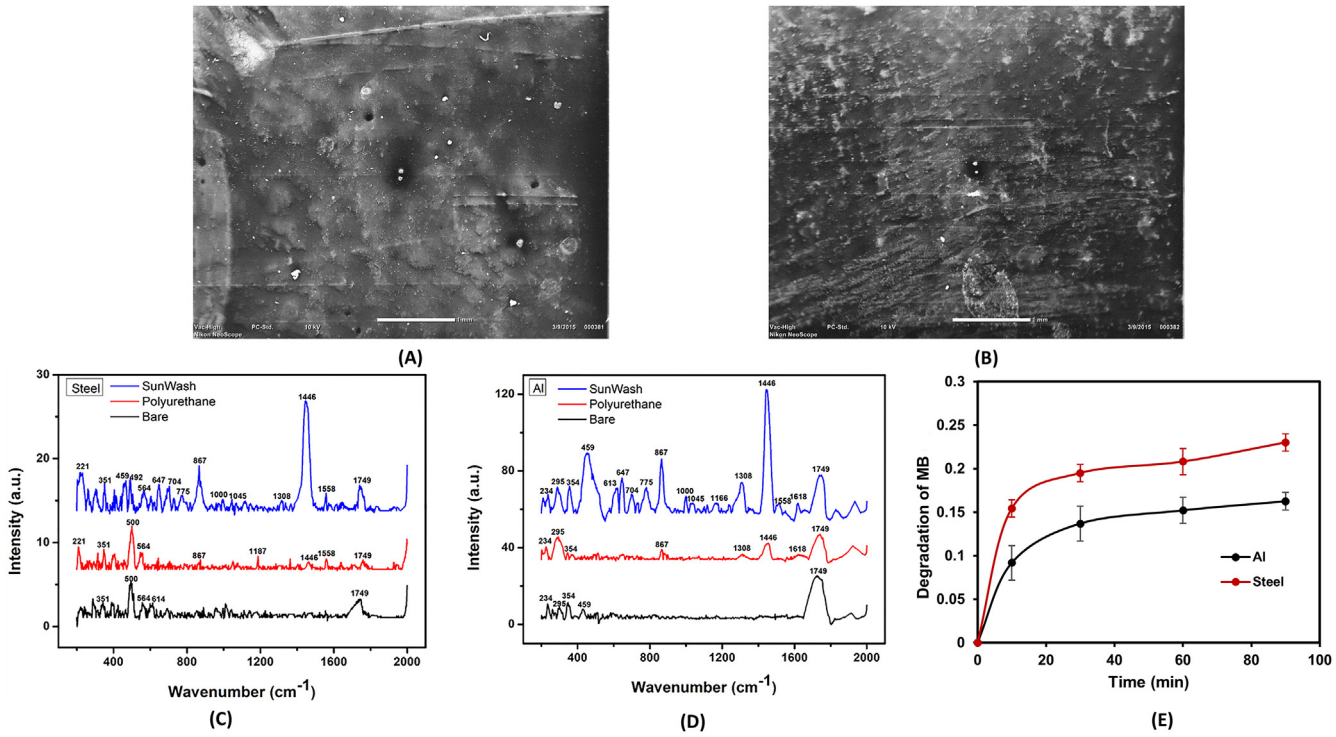

Fig. 1. SEM images of (A) Steel surface with duplex coating of polyurethane and TiO_2; (B) Al surface with duplex coating of polyurethane and TiO_2; Raman spectra of (C) bare steel substrate, steel substrate coated with polyurethane film, and duplex coating (SunWash) of polyurethane and TiO_2; (D) bare Al substrate, Al substrate coated with polyurethane film, and duplex coating (SunWash) of polyurethane and TiO_2; (E) Effect of UV irradiation on the degradation of MB on coated steel and aluminum substrates. Error bars are expressed as mean ± SE (standard error) with $n \geq 3$ ($p < 0.05$).

that form colonies on agar plates after UV irradiation and collection of cells. Although a proven method, CFU counting can sometimes underestimate the viability of the bacterial cells due to aggregate formation (each multicellular aggregate forms a single colony) (Cai, Strømme, & Welch, 2014b). Fig. 3 shows the antibacterial efficiency of TiO_2 polyurethane photocatalytic film coated steel and aluminum surfaces first seeded with *E. coli*, *L. monocytogenes*, *P. aeruginosa*, and *S. typhymurium* and then treated by UV irradiation. We found that the disinfection efficacy is strongly dependent on the bacterial species colonizing the surface, which is unsurprising as each strain has unique virulence and attachment characteristics that influence survival on a surface; these results also agree with a previous study by Francolini, Donelli, Crisante, Taresco, and Piozzi (2015). Coating was most effective against Gram-negative bacterial strains *E. coli*, *P. aeruginosa,* and *S. typhymurium*, while the Gram-positive bacteria *L. monocytogenes* was more resistant, which may be a product of the differences in cell membrane structure; Gram-positive organisms are typically more recalcitrant to lysis due to a thick peptidoglycan layer surrounding outside of the cell (Kongsong, Sikong, Niyomwas, & Rachpech, 2014). Furthermore, the surface used for coating influences the degree of disinfection. We found that we could kill 75–92% of the adherent cells on steel, while we only killed 61–77% the cells attached to aluminum after 10 min of UV irradiation. Therefore, steel, which is commonly used in the food industry, may be a superior substrate for self-cleaning coatings. It should be pointed out that the surface roughness of the substrate may contribute to the overall disinfection efficacy. It should be emphasized that Class B UV systems allow upto (16 mJ/cm^2 or 16,000 μW-sec/cm^2) for supplemental bactericidal treatment of treated and disinfected public drinking water. Class A systems (40,000μw-sec/cm2) are designed to disinfect and remove microorganisms and are approved by regulatory agencies such as EPA, NSF and ANSI for point of use and point of entry systems as well as in the food processing industries. In our study, the energy of the UV irradiation is only 1200 μw/cm^2. It is expected that few seconds of exposure of Class A UV irradiation on the TiO_2 polymer coating will provide almost 100% antibacterial effect.

3.5. ROS testing

Fig. 4 shows the results of ROS inhibition on the antibacterial properties of steel and aluminum surfaces coated with TiO_2 polyurethane photocatalytic films. Viability of the bacteria was evaluated via the absorbance determined by correction to the standard curve of known bacterial concentrations. The blue (in web version) line shows the viability of *L. monocytogenes* as a function of UV irradiation time on the uncoated surfaces (control) while the red (in web version) and black lines show the polymer coated surfaces with scavenger treatment. A relative higher value and a slight drop upon time were observed from the blue line, which indicated that with the same UV dose, the antibacterial effect of the UV light alone on the uncoated surfaces resulted in less viability reduction than that on the TiO_2-coated surfaces from the photocatalytic effect. As seen in the figure, after 5 min of UV irradiation on steel surfaces, the disinfection rate was similar between surfaces treated with enzymes to block •OH and H_2O_2 production. However, when the length of UV irradiation was increased from 10 to 20 min, the disinfection rate was significantly decreased in samples treated with the H_2O_2 scavenger. On the aluminum surface, the disinfection rate was consistently lower for surfaces treated with the H_2O_2 scavenger (over the entire 0–20 min). Altogether, reducing the amount of H_2O_2 on both surfaces resulted in a decrease in disinfection efficiency, indicating that the generation of H_2O_2 is a

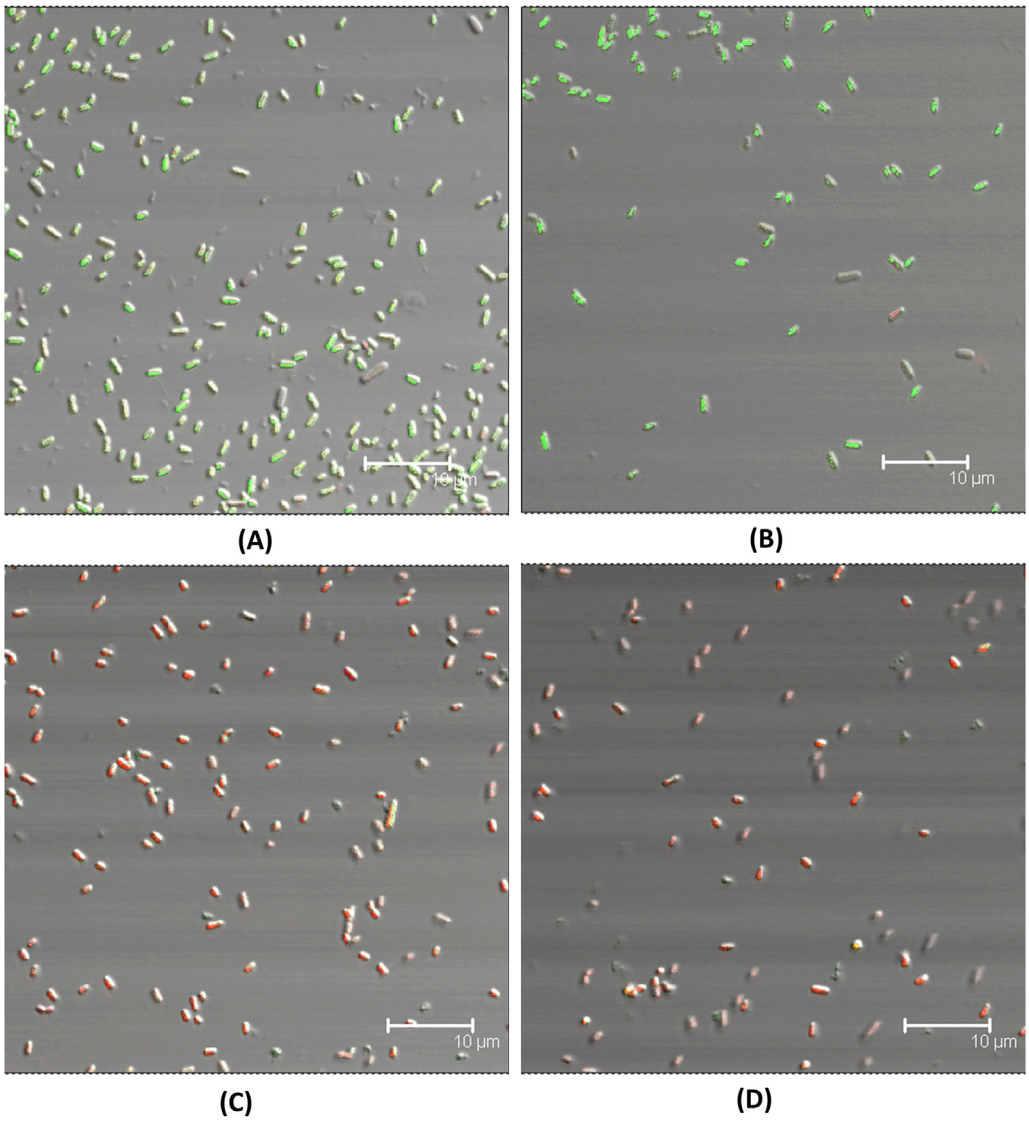

Fig. 2. Confocal Laser Scanning Microscopy images of live/dead stained *Listeria Monocytogenes* cells within a biofilm on the glass surfaces. The green and red fluorescence is generated by dye SYTO®9 and propidium iodide indicating viable and dead bacteria, respectively. (A–B) photocatalytically LM on uncoated surfaces (control); (C–D) photocatalytically treated LM on coated surfaces. The fluorescence images appear to show a substantially increased number of dead (red) bacteria than live (green) bacteria on the polyurethane-titanium dioxide coatings. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Antibacterial effects of TiO_2 polyurethane photocatalytic film coated stainless steel against *E. coli*, *L. monocytogenes*, and *S. typhimurium* under UV irradiation.

Stainless steel	*E. coli*			*L. monocytogenes*			*S. typhymurium*		
UV irradiation time [min]	30	10	5	30	10	5	30	10	5
Control	−	+	+	−	+	+	−	−	+
Polyurethane	−	+	−	−	−	−	−	−	+
TiO_2 polyurethane photocatalytic film	−	−	−	−	−	+	−	−	−

+, − indicate the positive and negative results, respectively.

Table 2
Antibacterial effects of TiO_2 polyurethane photocatalytic film coated aluminum against *E. coli*, *L. monocytogenes*, and *S. typhymurium* under UV irradiation.

Al	*E. coli*			*L. monocytogenes*			*S. typhymurium*		
UV irradiation time [min]	30	10	5	30	10	5	30	10	5
Control	−	+	+	−	+	+	−	+	+
Polyurethane	−	+	−	−	+	+	−	−	−
TiO_2 polyurethane photocatalytic film	−	−	−	−	−	+	−	−	−

+, − indicate the positive and negative results, respectively.

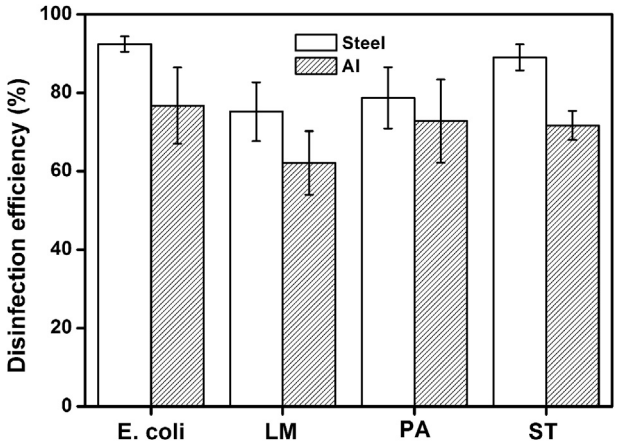

Fig. 3. Antibacterial effects of photocatalytic TiO_2 polymer film coated steel and Al surfaces against *E. coli*, LM, PA, and ST after UV irradiation for 10 min. Error bars are expressed as mean ± SE (standard error) with $n \geq 3$ ($p < 0.05$).

the overall sensitivity to H_2O_2. Altogether our results demonstrate that TiO_2 photocatalytic polymer coatings may be ideal for the development self-cleaning surfaces, especially for food safe coatings, by virtue of its profound and broad antimicrobial effects as well as its compatibility with existing sanitizing procedures, low toxicity, reduced chemical usage, and reduced labor associated with cleaning. Future studies are warranted to examine the integrity of the polyurethane or the TiO_2 on the steel or aluminum surfaces. Ultimately, we determined that our disinfection strategy effectively reduces the number of adherent viable microbial cells, while the degree of effectiveness is influenced by the surface coated and the bacterium tested. These results hold promise for the application of technologies that will appreciably improve disinfection in the food industry.

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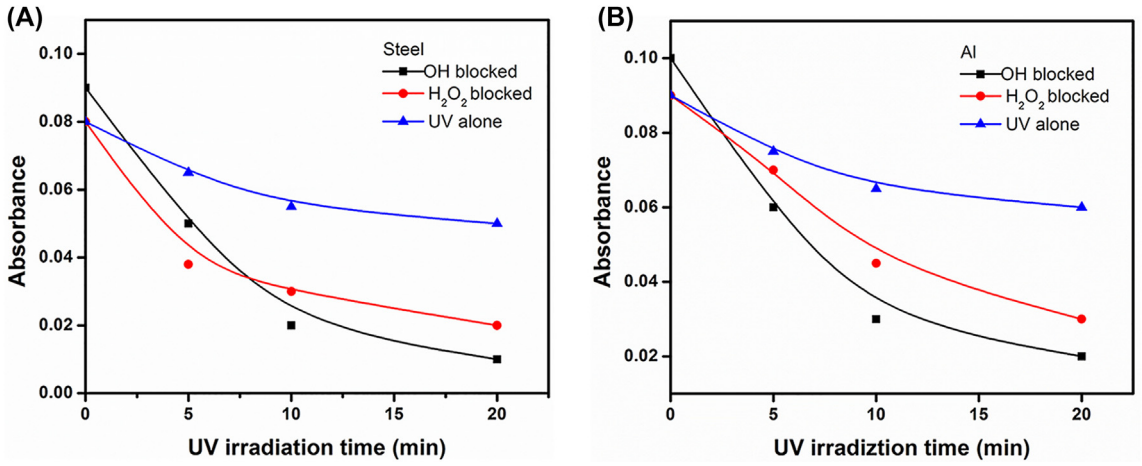

Fig. 4. Viability of *L. monocytogenes* as a function of UV irradiation time. Contributions of •OH and H_2O_2, to the antimicrobial effect on coated steel (A) and Al (B) by metabolic activity test in the presence of oxidative radical scavengers ($n = 5$).

significant contributor to the antibacterial effects of our disinfection strategy (TiO_2 coatings treated with UV).

4. Conclusions

Our work demonstrates the antimicrobial efficacy of the TiO_2-based polymers on the steel and aluminum surfaces against *E. coli*, *L. monocytogenes*, *P. aeruginosa*, and *S. typhimurium*. Coatings on both steel and aluminum reduced the total number of adherent viable cells after brief UV treatment (20 min or less), while steel, a common material surface used in the food industry, may be a more ideal substrate for coating as disinfection rates were enhanced on steel. The coating is effective against a broad range of organisms tested, which commonly contaminate foods and pose a serious threat to human health. The coating may be slightly more effective against specific strains, as some strains are more or less sensitive to oxidative radical production. Most bacterial strains possess mechanisms for reducing harmful reactive oxygen and nitrogen species, although some bacteria have redundant or overlapping mechanisms for coping with oxidative stress and/or are more efficient at reducing and tolerating oxidative stress. Our results suggest that of the radicals generated by our coating, H_2O_2 has the greatest impact on disinfection and that relative bacterial sensitivity is explained by

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