Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Inactivation of pathogenic bacteria inoculated onto a Bacto™ agar model surface using TiO_2-UVC photocatalysis, UVC and chlorine treatments

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Keywords
agar model surface, cell morphology, pathogenic bacteria, TiO_2-UV photocatalysis, UVC treatment.

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2014/2646: received 23 December 2014, revised 28 May 2015 and accepted 14 June 2015

doi:10.1111/jam.12877

Abstract

Aims: The aim of this study was to study inactivation of different pathogenic bacteria on agar model surface using TiO_2-UV photocatalysis (TUVP).
Methods and Results: A unified food surface model was simulated using Bacto™ agar, a routinely used microbial medium. The foodborne pathogenic bacteria *Escherichia coli* K12 (as a surrogate for *E. coli* O157:H7), *Salmonella Typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes* were inoculated onto the agar surface, followed by investigation of TUVP-assisted inactivation and morphological changes in bacterial cells. The TUVP process showed higher bacterial inactivation, particularly for Gram-negative bacteria, than UVC alone and a control (dark reaction). A TUVP treatment of 17·2 mW cm^{-2} (30% lower than the UVC light intensity) reduced the microbial load on the agar surface by 4·5–6·0 log CFU cm^{-2}. UVC treatment of 23·7 mW cm^{-2} caused 3·0–5·3 log CFU cm^{-2} reduction.
Conclusions: The use of agar model surface is effective for investigation of bacterial disinfection and TUVP is a promising nonthermal technique.
Significance and Impact of the Study: The results showing effects of photocatalysis and other treatments for inactivation of bacterial pathogens on model surface can be useful for applying such processes for disinfection of fruit, vegetables and other similar surfaces.

Introduction

Consumer interest in minimally processed foods (MPF) is increasing. However, minimally processed products deteriorate during storage and can present health hazards. Chlorinated water (sodium hypochlorite mixed in wash water) is applied to disinfect fruit and vegetables surfaces either before processing or during pre- and postcutting operations (Soliva-Fortuny and Martín-Belloso 2003). The use of chlorinated water that may be insufficient for inactivation of all bacterial pathogens can cause health hazards due to generation of trihalomethanes (Kim *et al.* 2008, 2009) and can also result in undesirable by-products and, at the same time, create disposal problems. Treated products may also require water rinsing before use to remove residual chlorine. As the use of thermal treatments is discouraged for MPF, novel processes need to be developed to ensure microbial safety of MPF products.

TiO_2 in the anatase crystal form is a semiconductor with a band gap of 3·2 eV or more. When illuminated using light of wavelength <385 nm, an electron hole pair on the TiO_2 surface is generated due to photon energy. The hole in the valence band reacts with H_2O or hydroxide ions on the surface of TiO_2 particles to produce hydroxyl radicals (OH•) and the electron in the conduction band reduces O_2 to produce superoxide ions (O_2^-). Both holes and OH• are extremely reactive against

organic compounds. Moreover, detection of other reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and singlet oxygen, has also been reported after illumination with TiO_2 (Maness *et al.* 1999). ROS generated in photocatalytic reactions are reactive against living cells and can cause cell death (Beyer Jr and Fridovich 1987; Maness *et al.* 1999). TiO_2-UV photocatalysis (TUVP) is effective for inactivation of vegetative microbes and bacterial endospores via DNA and cell membrane disruption (Setlow and Carrier 1966; Maness *et al.* 1999; Kim *et al.* 2013). Customized simple TUVP systems have shown outstanding effects for inactivation of micro-organisms on agricultural product surfaces (Cho *et al.* 2007; Kim *et al.* 2009; Chai *et al.* 2014). The TUVP process has advantages because it is nonthermal, simple, and leaves no residual chemicals on the food surface.

Disinfection effects of surface decontamination methods, including TUVP, are difficult to evaluate on fruit and vegetables due to an inconsistent degree of contamination and irregular surface characteristics. The physicochemical properties of fruit and vegetables and surface structures may affect light penetration and cause shading effects during the TUVP process (Alvaro *et al.* 2009). Hence, to develop a surface decontamination process, a food surface model needs to be developed for investigation of disinfection effects. In this study, an ellipsoidal model surface was developed to evaluate the disinfection effects of the TUVP process. An artificial food surface model was prepared using Bacto™ agar, which is a solidifying agent in which extraneous material, salts, and pigmented portions have been reduced to a minimum. The agar is then used for preparation of a microbiological culture medium (Waksman and Bavendamm 1931; Duckworth and Yaphe 1971).

The objectives of this study included, (i) development of an agar model surface to simulate a food surface, (ii) study of the disinfection effects of the TUVP process against the pathogenic bacteria *Escherichia coli* K12, *Salmonella Typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes* inoculated onto an agar model surface using different process times and conditions and (iii) evaluation of morphological changes in bacterial cells using scanning electron microscopy.

Materials and methods

TiO_2-UVC photocatalytic reactor

A lab-scale TiO_2-UVC photocatalytic (TUVP) reactor was fabricated (Fig. 1a) following a previous study (Kim *et al.* 2009). The reactor consisted of a stainless steel vessel (30 l), an air pump and six UVC lamps (254 nm, 35 W, 25 mW cm^{-2}, Sankyo Denki, Tokyo, Japan) in TiO_2-coated quartz tubes (38 cm length, 24·5 mm outer diameter, TiO_2 coating thickness 0·7–0·9 mm; Taekyeong UV Co., Namyangju, South Korea). An air pump at the bottom of the reactor was used to produce agitation in water during treatment. UVC lamps fitted in TiO_2-coated quartz tubes were placed in the TUVP reactor cap. UVC lamps were fitted in non-TiO_2 coated quartz tubes for use as a UVC treatment alone. These assemblies were submerged in water used inside the reactor. Within the same reactor water, a bubble treatment (Dark, Dark-TiO_2: lamp off with TiO_2 reactor cap) was carried out as a control based on disconnection of the UVC lamp/TiO_2 assembly. A chlorine solution (200 ppm) was prepared using a 12% hypochlorite solution (Yakuri Pure Chemicals Co., Kyoto, Japan) and, similar to the water bubble treatment, this solution was used for treatment of the Bacto™ agar model surface in the same reactor without photocatalysis. Inoculated agar was immersed in the vessel containing 28 l of tap water and TUVP, UVC, dark, dark-TiO_2 and sodium hypochlorite (NaOCl) treatments were applied. Samples were treated for 0, 10, 20, 30, 40, 50, 60, 80, 100 and 120 s and subsequently analysed for microbial counts. Treatment with NaOCl followed an immediate neutralizing step of washing in tap water for 1 min.

The small-sized reactor used for study of morphological changes was also customized (Fig. 1b) according to a previous study (Kim *et al.* 2013). The reactor consisted of a cap, a UVC lamp, and a quartz tube with an internal diameter of 25 mm and a height of 50 mm. $TiCl_4$ was used as a source of titanium for preparation of TiO_2 thin films. A TiO_2 solution was prepared using dissolution of $TiCl_4$, HCl, NH_4HCO_3 and H_2O_2 in distilled water. This solution was deposited on the surface of a quartz tube and completely dried at 250°C for 24 h (Cho *et al.* 2007). Control experiments were also performed using UVC irradiation alone without TiO_2.

Preparation of *Escherichia coli*, *Salmonella Typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes* cultures

The four foodborne bacteria *E. coli* K12 (ATCC 43895), *Salm. Typhimurium* (ATCC 14028), *Staph. aureus* (ATCC 29213), and *L. monocytogenes* (ATCC 15313) were supplied by the American Type Culture Collection (Rockville, MD) and the Korean Culture Center of Micro-organisms (Seoul, South Korea). *E. coli* K12 was used as surrogate bacterium of *E. coli* O157:H7. Difco™ culture media were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ). Stock cultures of *E. coli* K12, *Salm. Typhimurium*, *Staph. aureus*, and *L. monocytogenes* were prepared in nutrient broth (NB)

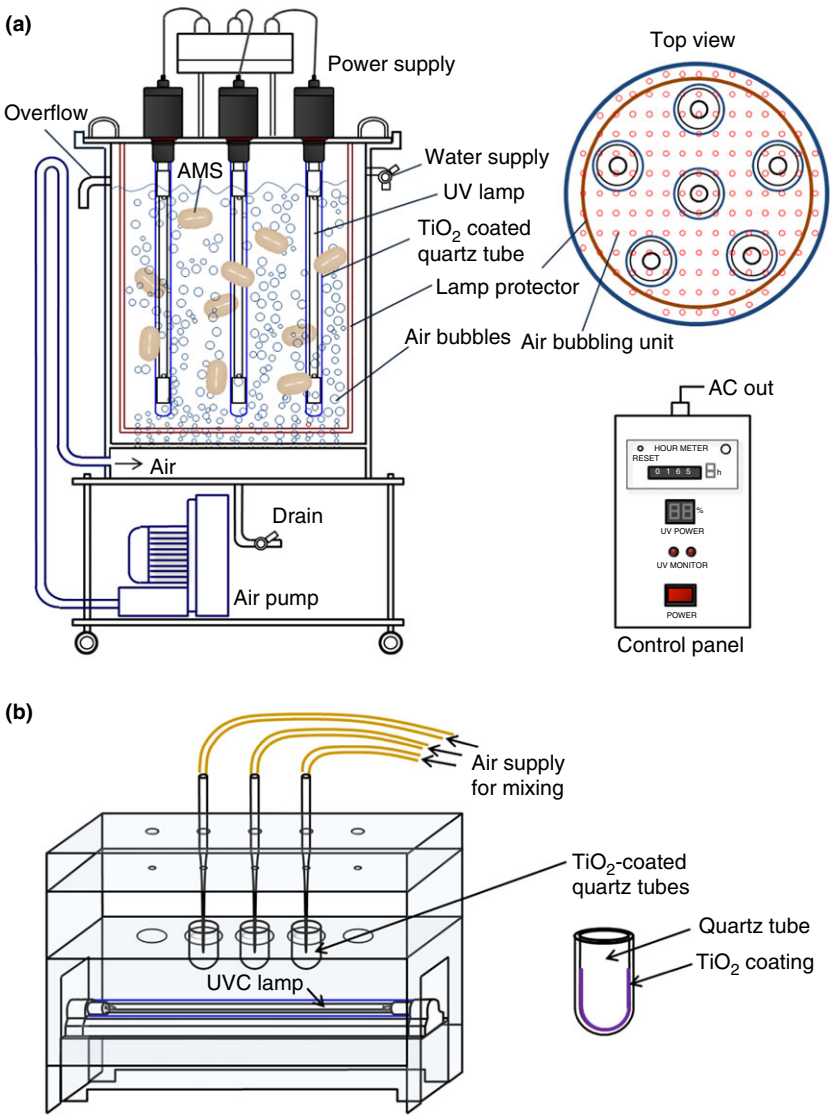

Figure 1 Schematic diagrams of a lab-scale TiO_2-UVC photocatalytic reactor (a) consisting of a 30 l stainless steel vessel, an air pump and 6 UV lamps (254 nm wavelengths, 35 W, 25 mW cm^{-2}) in TiO_2-coated quartz tubes (24·5 mm outer diameter, 0·7–0·9 mm TiO_2 coating thickness). AMS was free-floating with agitation due to air bubble provided by air pump at the bottom of the reactor during processing. A small-sized TUVP reactor (b) for morphological studies consisted of a UVC lamp and a quartz tube with an internal diameter of 25 mm and a height of 50 mm.

after incubation at 38°C for 24 h along with shaking at 200 rev min^{-1}. A 50 ml cell aliquot was transferred to 200 ml of fresh NB and incubated at 38°C for 24 h with shaking at 200 rev min^{-1}. Cell suspensions of each of the microbial strains were centrifuged at 2870 ***g*** for 10 min. The cell pellet of each strain was suspended in an equal volume (30 ml) of a 0·85% NaCl solution to be used as an inoculum. Colony counts for bacteria in the inoculum were obtained using the spread plate method. Nutrient agar was used after serial dilution using a 0·85% NaCl solution.

Preparation of a model surface

Bacto™ agar was cast in an ellipsoidal chocolate mould and solidified to simulate a fruit or vegetable surface. The agar model surface (AMS) was prepared by casting of an agar solution (2·5%) in a modified chocolate mould after sterilization at 121°C for 15 min. The shape of the surface model was ellipsoidal having a surface area of 20·54 cm^2 and calculated following a previously described method (Maritz and Douglas 1994). AMSs were rapidly cooled in a deep freezer (−78°C) for 30 min to harden the surface. AMSs were irradiated with UVC light on a clean bench (VS-1400LS; Vision Scientific, Daejeon, South Korea) for 30 min to ensure sterilization prior to inoculation. The morphology of surface models is shown in Fig. 2a-c. The surface was prepared to be porous and to exhibit stable cell adhesion properties with homogenous physicochemical characteristics. Inoculation of these models was achieved by immersion in a prepared inoculum (8–9 log CFU ml^{-1})

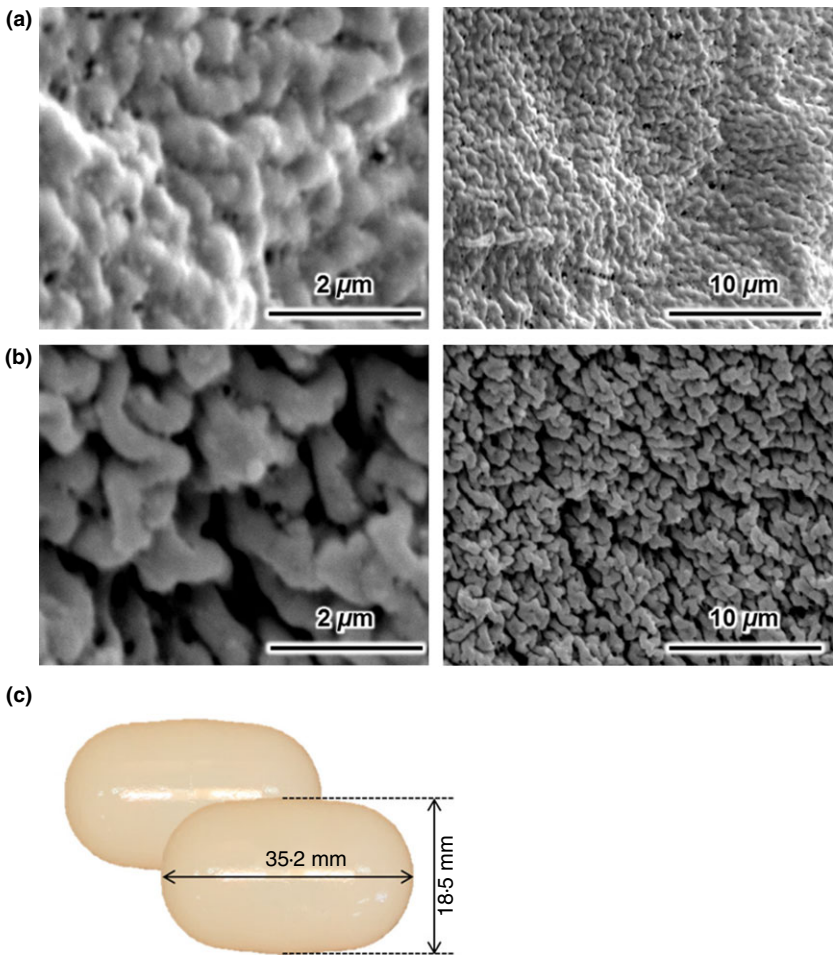

Figure 2 Scanning electron microscopic images of an agar model surface dried at room temperature for 40 min (a), a surface frozen at −78°C for 10 min (b), and a final Bacto™ agar model surface (c).

for 3 h at 38°C using a 200 rev min^{-1} shaking incubator, followed by 30 min air drying on a clean bench to ensure bacterial attachment to the model surface. In each experiment a total of 27 AMSs (three for each treatment consisting of nine different time intervals) were used.

Scanning electron microscopy

Samples were fixed in modified Karnovsky's fixative (2% paraformaldehyde, 2% glutaraldehyde, 0·5% $CaCl_2$ in 0·1 mol l^{-1} phosphate buffer) overnight, washed with a 0·1 mol l^{-1} cacodylate buffer, and postfixed with 1·0% OsO_4 in a 0·1 mol l^{-1} cacodylate buffer. Dehydration was achieved by passing cover slips through 50, 60, 70, 80, 90, 95, and 100% ethyl alcohol solutions for 5 min at each dilution, followed by dehydration with 100% ethyl alcohol for 10 min. Samples were dried in a critical-point drying apparatus (HCP-2; Hitachi Ltd., Tokyo, Japan), followed by mounting on SEM stubs. Samples were coated with approx. 300 Å of gold and observed under a field emission scanning electron microscopy (FE-SEM S-800; Hitachi Ltd.). Magnifications (3000×, 5000×, 20 000×), resolution, and the tilt angle were adjusted, then photo images were recorded.

Microbiological analysis

The total plate count method was used to enumerate viable microbial cells on model surfaces after inactivation treatments. Three AMSs (61·62 cm^2) from before and during inactivation treatments were homogenized on a stomacher (MIX 2, AES Laboratories, Combourg, France) in 61·62 ml of a sterile 0·85% NaCl solution for 2 min, then serially diluted using the saline solution, followed by inoculation of 1·0 ml of the solution onto duplicate plates containing the appropriate agar. Nutrient agar was used to detect culturable *E. coli* K12, *Salm. Typhimurium*, *Staph. aureus*, and *L. monocytogenes* cells after incubation at 37°C for 24 h. Colonies were counted in each case at the end of the incubation period. The detection limit was 1 CFU per plate which is equated to 1 CFU cm^{-2}. The log (N/N_0) value was calculated to determine the inactivation effect, where N_0 is the initial microbial count in

samples before treatment, and N is the viable microbial count after inactivation treatments.

Statistical analysis

Results from triplicate experiments were analysed using a one-way analysis of variance (ANOVA) with the Statistical Package for the Social Sciences (SPSS ver. 21; IBM Corporation, NY) and expressed as a mean values along with standard deviation (means ± SD). Statistical analysis was performed using the Student–Newman–Keuls test and the confidence level for statistical significance was set at a probability value (P) of 0·05.

Results

Obtaining an appropriate microbial load on simulated food model surfaces

In preliminary studies, a variety of solid materials were used to investigate attachment of bacterial cells and to simulate a food model. Stainless steel, polyvinyl chloride, glass and rubber were found not to be suitable as surface models due to erratic cell attachment and cell sensitivity towards the physical factors of shear stress of water flow in the TUVP reactor (data not shown). An ellipsoidal surface model made using Bacto™ agar was suitable due to a high efficacy of homogeneous cell attachment and reduced cell sensitivity to physical factors. The resulting solidified agar model surface (AMS) was porous and can effectively simulate a fruit or vegetable surface (Fig. 2). To obtain optimum microbial counts on AMS, a distinct inoculum cell population was obtained by changing the immersion time in the inoculum. As an example, 5·28 log CFU cm^{-2} of *E. coli* K12 was obtained after 1 h of AMS immersion in an 8 log CFU ml^{-1} inoculum. A similar AMS immersion time in a 9 log CFU ml^{-1} inoculum of *E. coli* K12 resulted in a bacterial count of 6·31 log CFU cm^{-2} on the agar surface. Increasing the immersion time to more than 1 h did not affect the initial count of *E. coli* K12 on AMS (Table 1). However, when AMS was immersed in an inoculum for a different time (1–3 h) and subsequently washed for 100 s in sterilized tap water using the air blower of the TUVP reactor without application of photocatalytic reaction or UV irradiation, *E. coli* K12 attached more strongly when AMS was immersed for a longer time (3 h) and the washing process removed fewer bacteria than for AMS immersed for 1–2 h (Table 1). Hence, the cell population of the inoculum affected the initial AMS count, whereas an increase in the immersion time improved adhesion of bacterial cells. Study of *E. coli*, *Salm. Typhimurium*, *Staph. aureus,* and *L. monocytogenes* inactivation on AMS using TUVP and other treatments was based on these results.

Table 1 The effect of immersion time on reduction in *Escherichia coli* K12 counts inoculated on an agar model surface

Immersion time	*E. coli* K12 counts (log CFU cm^{-2})		Loss during washing for 100 s (log CFU cm^{-2})
	Initial inoculated cells	After washing	
1 h	6·31 ± 0·09	4·34 ± 0·34	1·97 ± 0·43
2 h	6·29 ± 0·08	4·77 ± 0·11	1·53 ± 0·19
3 h	6·26 ± 0·04	5·11 ± 0·15	1·08 ± 0·10

Values are expressed as a mean ± SD ($n \geq 3$).

Inactivation of pathogenic bacteria on an agar model surface using TUVP and other treatments

The bactericidal effects of dark, dark-TiO_2 (without light), NaOCl, UVC, UVC-TiO_2 (TUVP) and treatments for inactivation of the pathogenic bacteria *E. coli* K12, *Salm. Typhimurium*, *Staph. aureus,* and *L. monocytogenes* on AMS were evaluated and results are shown in Fig. 3. The initial population of these individual pathogens on AMS was in the range of 5·28–5·93 log CFU cm^{-2} in each case. Dark reaction treatments produced no bactericidal effects. Slight reductions (<1·5 log) in bacterial counts during dark reactions were attributed to air bubbles produced during the reaction. For *E. coli* K12, *Salm. Typhimurium* and *Staph. aureus*, the TUVP process in a lab-scale reactor (Fig. 1a) showed higher inactivation effects than UVC and chlorine treatments and resulted in an earlier bactericidal phase. As shown in Fig. 3, a complete inactivation of microbes was achieved under UVC-TiO_2 treatment within short time of 30–50 s (*E. coli* K12 at 50 s, *Salm. Typhimurium* at 40 s, and *Staph. aureus* at 30 s). In contrast, UVC treatment comparatively took longer time for bacterial inactivation (*E. coli* K12 at 120 s, *Salm. Typhimurium* at 60 s, and *Staph. aureus* at 50 s). However, treatment with NaOCl proved ineffective to inactivate bacteria even after 5 min (data not shown). In contrast to other bacteria, *L. monocytogenes* showed higher resistance against UVC-TiO_2 treatment. UVC treatment successfully inactivated *L. monocytogenes* after 50 s, whereas TUVP treatment failed to do so even after 5 min.

Morphological changes in pathogenic bacteria after inactivation treatments

Pathogenic bacteria inoculated onto AMS were treated using TUVP (17·2 J cm^{-2}) in a small-sized reactor (Fig. 1b) and UVC (23·7 J cm^{-2}) for 40 s and SEM images

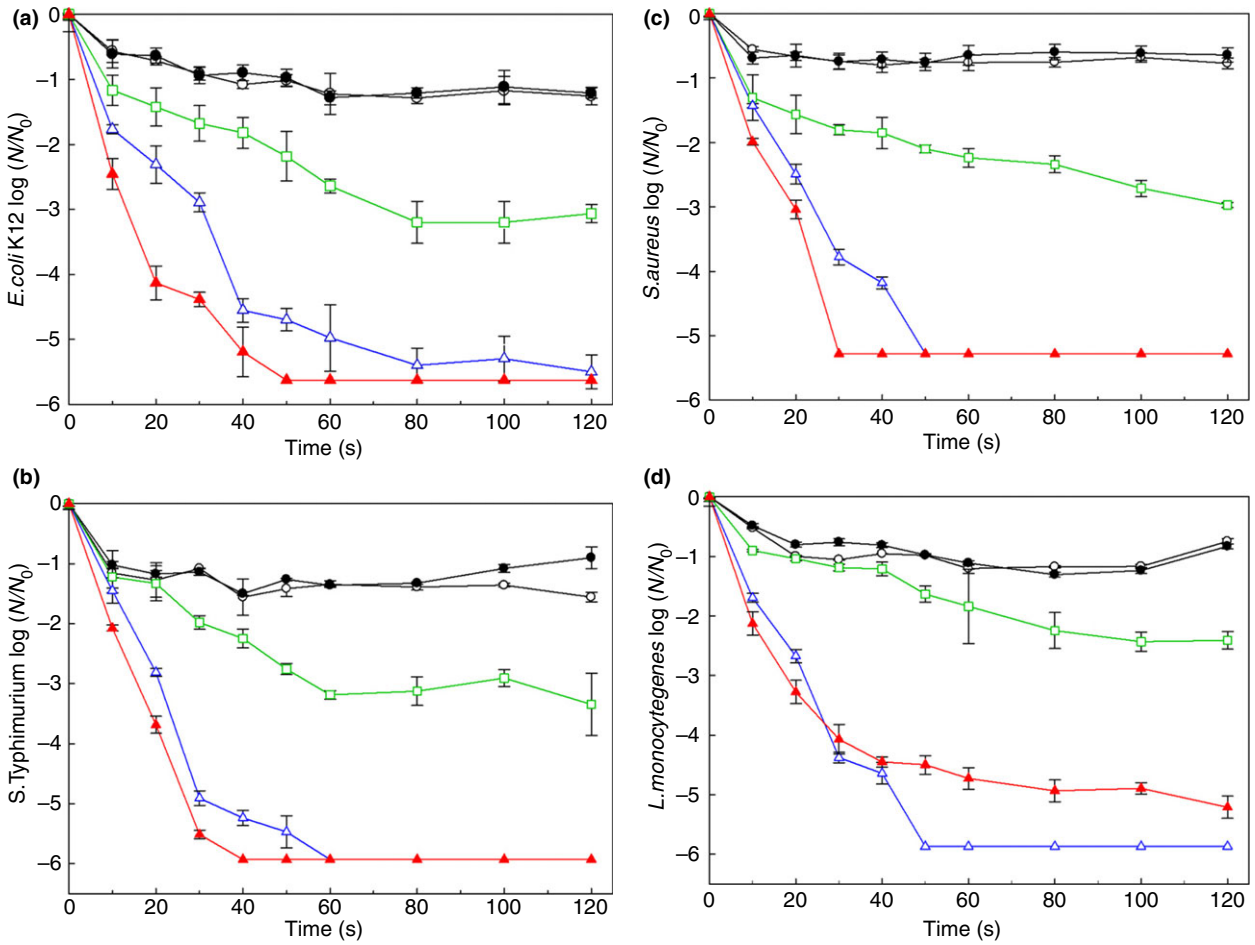

Figure 3 Bactericidal effects against *Escherichia coli* K12 (a), *Salmonella Typhimurium* (b), *Staphylococcus aureus* (c) and *Listeria monocytogenes* (d) for different non-thermal treatments. N/N_0 is the survival ratio with standard error of means (n = 3). (–○–) Dark; (–●–) Dark-TiO_2; (–□–) NaOCl; (–△–) UVC; (–▲–) UVC-TiO_2.

were obtained for study of morphological changes (Fig. 4). Control cells (untreated bacteria) showed uniform and smooth morphologies with an average size of 1·5–3·0 μm. Bactericidal treatments resulted in a deformed cell morphology of bacteria. Treated cells of *E. coli* K12, *Salm. Typhimurium*, *Staph. aureus*, and *L. monocytogenes* were integrated with each other and appeared like crumpled surfaces because the cell volume was reduced due to mass transfer from cells to surroundings. Both types of treatments (UVC-TiO_2 and UVC) at 40 s showed the most severe morphological disruptions in Gram-positive and Gram-negative bacteria, consistent with results shown in Fig. 3, providing support for bactericidal effects.

Discussion

The results obtained in relation to obtaining a desired level of microbial load were similar to previous studies when microbial inocula were applied to fruit and egg surfaces to obtain a desired level of attachment on a food surface (Castillo *et al.* 2006; Lasagabaster *et al.* 2011). It was observed that adhesion of a pathogen (*E. coli* K12) was stronger when AMS was immersed for longer time in inoculum.

Among different bactericidal treatments, TUVP showed considerable inactivation effects for three pathogenic bacteria (*E. coli* K12, *Salm. Typhimurium* and *Staph. aureus*), attributable mainly to photocatalysis as the TiO_2 coating on the quartz tube reduced the light (UVC) intensity by 70%, resulting in a shadowing effect against UVC emission. The intensity of a noncoated UV lamp is 23·7 mW cm^{-2}, whereas the intensity of a TiO_2 coated lamp is 17·2 mW cm^{-2}. Although the TUVP process allowed exposure at a lower UVC dose than the UVC process, the disinfection effects of TUVP were higher against *E. coli* K12, *Salm. Typhimurium* and *Staph. aureus*

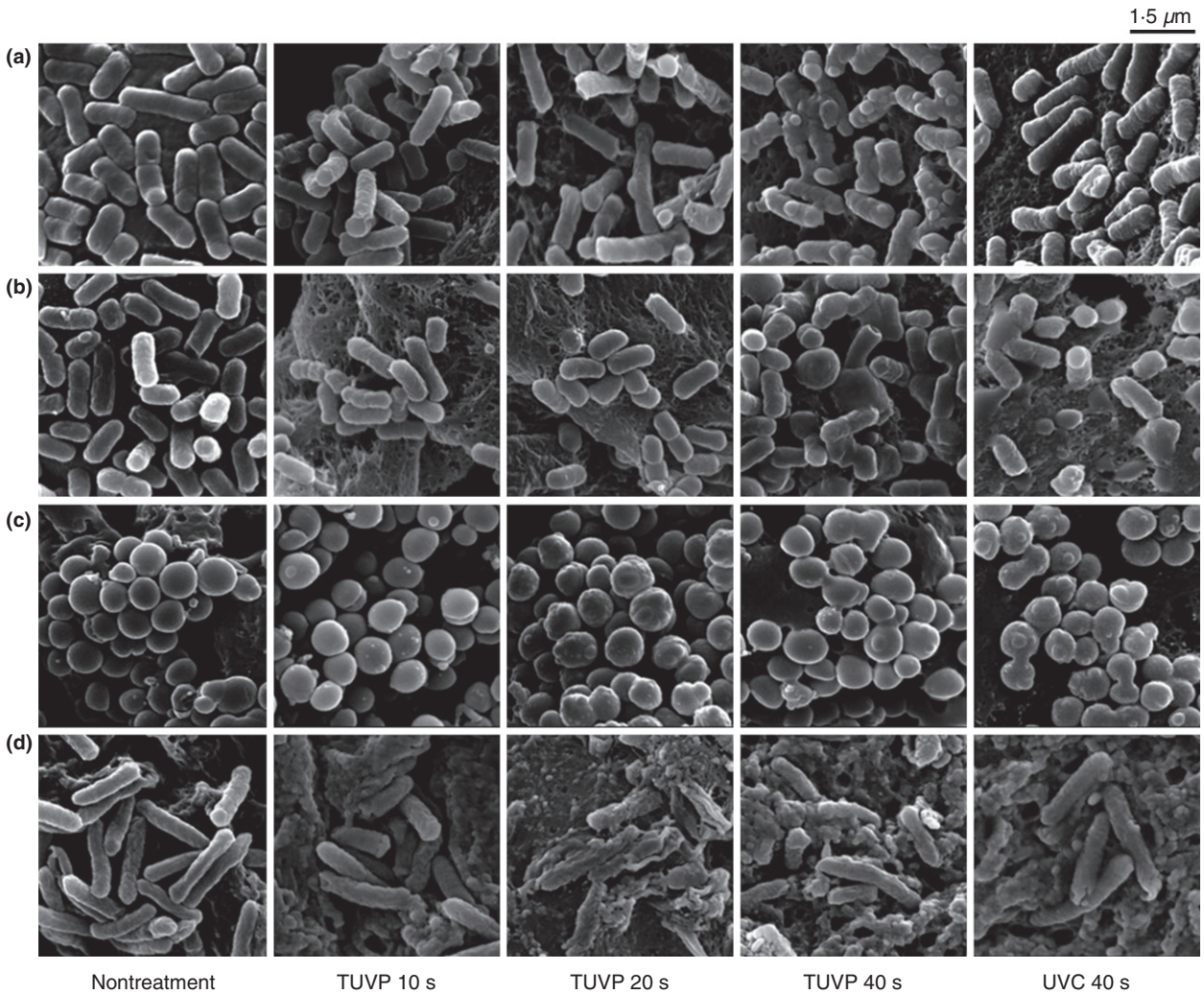

Figure 4 Scanning electron microscopic images of bacterial cell structure on an agar model surface after TUVP and UVC treatments. *E. coli* K12 (a), *Salm. Typhimurium* (b), *Staph. aureus* (c), and *L. monocytogenes* (d).

than UVC, attributable to generation of highly oxidizing reactive oxygen species (ROS) from the TiO_2 surface. Superoxide dismutases (SODs), a family of three metalloenzymes, are capable of disproportionating O_2^- (the first ROS formed in the metabolic reduction of oxygen) into H_2O_2. Subsequently, catalase activity decomposes H_2O_2, into O_2 and water. Previous reports of *L. monocytogenes* resistance to ROS indicated that this behaviour results from different properties of the cell wall, and from enzyme activities. Both SOD and catalase have been reported to protect certain pathogenic bacteria. SODs of *L. monocytogenes* have been reported to reduce the killing efficacy of ROS (Chun *et al.* 2009; Gabriel and Nakano 2009), in agreement with results reported herein as *L. monocytogenes* was more sensitive towards UVC than TUVP.

TiO_2 increased the inactivation effects of bacteria in association with UVC, particularly for *E. coli* K12 and *Salm. Typhimurium*. These two bacteria are Gram-negative and showed less resistance to TUVP than other strains. Previous studies (Li and Logan 2004; Hu *et al.* 2006; Cohen-Yaniv *et al.* 2008; Van Grieken *et al.* 2010) related this behaviour to distinct interactions of Gram-positive and Gram-negative bacteria with the TiO_2 catalyst. Hydrophobic Gram-negative bacteria probably show greater bacterial-TiO_2 adhesion than Gram-positive bacteria when contact is made with an immobilized TiO_2 catalyst. It was also proposed that distinctive wall structures in Gram-positive and Gram-negative bacteria are responsible for different sensitivities towards TUVP and UVC light (Kim *et al.* 2013). Gram-positive *L. monocytogenes* and *Staph. aureus* have thicker cell walls than Gram-negative

bacteria, such as *E. coli* K12 and *Salm. Typhimurium*, which may have obstructed penetration of free radicals generated from TiO_2 particles and, hence, have reduced the extent of damage to cell membranes and internal organelles in Gram-positive bacteria (Kuhn *et al.* 2003; Rincón and Pulgarin 2005; Malato *et al.* 2007; Chung *et al.* 2009; Gomes *et al.* 2009; Kim *et al.* 2013).

Scanning electron microscope images have been previously used to demonstrate effects of inactivation treatments on the morphology and structure of microbial cells (Marx *et al.* 2011; Kim *et al.* 2013). SEM observations also suggested the presence of birth and bud scars (Marx *et al.* 2011). TUVP treatment (10–40 s) caused cell shrinkage and fusion, resulting in a cell volume reduction as a result of mass transfer from cells to surroundings (Pilavtepe-Çelik *et al.* 2008). In previous reports it was observed that TUVP induces destruction of the cytoplasmic membrane, DNA, and internal organelles of bacterial cells. TUVP induced disruption of the internal organization of *E. coli*, *L. monocytogenes*, and *Salm. Typhimurium* cells in a liquid culture (Kim *et al.* 2013), leading to leakage of cytoplasmic contents and cell death (Krishnamurthy *et al.* 2010). The bacterial inactivation effects of TUVP can be attributed to free radicals generated from the TiO_2 surface as most of the generated UVC photons did not actually strike pathogenic bacteria on the AMS. Hence, TUVP treatment can be effectively used as a microbial inactivation treatment at a lower dose than a separate UVC treatment. TUVP can also induce multiple inactivation mechanisms in microbial cells and is a promising nonthermal treatment. Furthermore, use of a simulated food model developed using Bacto™ agar was effective for demonstration of the actual bactericidal effects of different treatments.

In brief, AMS can be used as a simulated food model to study effects of different disinfection methods against pathogenic bacteria. Similar approaches were followed previously for studying the efficiency of various disinfection treatments, including the use of atmospheric pressure plasma to inactivate *L. monocytogenes* (Lee *et al.* 2011) and photodynamic-assisted inactivation of *E. coli* (Caminos and Durantini 2006) on agar surfaces. The use of an artificial surface is important for the establishment of a new surface disinfection method and its comparison with existing techniques. Among different techniques, TUVP (13·72 J cm^{-2}) caused complete inactivation of *E. coli* K12 (ATCC 43895), *Salm. Typhimurium* (ATCC 14028), *Staph. aureus* (ATCC 29213), and *L. monocytogenes* (ATCC 15313). *E. coli*, *Salm. Typhimurium* and *Staph. aureus* showed similar and lower resistance values to TUVP treatment. However, *L. monocytogenes* resisted disinfection effects of photocatalysis and was more sensitive to UVC treatment. SEM images of treated microbial cells showed bacterial cell deformation that was consistent with observed inactivation patterns. TUVP treatment caused cell damage after 20 s and complete cell rupture and leakage was observed after 40 s. The TUVP process, with an ability to generate different bacterial inactivation mechanisms, can be used as an alternative to existing nonthermal food surface disinfection methods.

Acknowledgements

This research was supported by a grant (No. 114035-2) from High Value-added Food Technology Development Program, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea.

Conflict of Interest

Authors declare no conflict of interest.

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