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Bactericidal efficacy of UV activated TiO_2 nanoparticles against Gram-positive and Gram-negative bacteria on suspension

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ABSTRACT

Two different TiO_2 nanoparticles, NM101 and NM105, were evaluated against a range of Gram-positive (*Staphylococcus aureus, Bacillus cereus, Lactobacillus casei, Lactobacillus bulgaricus, Lactobacillus acidophilus* and *Lactobacillus lactis*) and Gram-negative (*Salmonella enterica* var. Enteridis and *Escherichia coli*) bacteria. Both NM101 and NM105 TiO_2 nanoparticles (UV-exposed or none) had a significant antibacterial activity when the concentration of TiO_2 suspension was 100 μg mL^{-1}. The activation of the TiO_2 NPs led, in all cases, to a shift in the growth curve, revealing lower counts as the concentration increased. *E. coli* was the most significantly affected pathogen by both TiO_2 nanoparticles reaching among 2–3 log $CFU.mL^{-1}$ reduction. In addition, in the case of the probiotic bacteria, NM105 TiO_2 nanoparticles had similar effects as the bacterial density was reduced by 2–3 log $CFU.mL^{-1}$. These results may be applied as a potent technology to be included in the formulation of new disinfectants.

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Eficacia bactericida de nanopartículas de TiO_2 activadas por luz UV contra bacterias Gram-positivas y Gram-negativas en suspensión

RESUMEN

Se evaluaron dos nanopartículas diferentes de TiO_2, denominadas NM101 y NM105, contra una batería de bacterias Gram-positivas (*Staphylococcus aureus, Bacillus cereus, Lactobacillus casei, Lactobacillus bulgaricus, Lactobacillus acidophilus and Lactobacillus lactis*) y Gram-negativas (*Salmonella enterica* var. Enteridis and *Escherichia coli*). Ambas nanopartículas de TiO_2, NM101 y NM105 (expuestas o no a luz UV) tuvieron una actividad antibacteriana significativa cuando la concentración de suspensión de TiO_2 alcanzó los 100 μg mL^{-1}. La activación de las nanopartículas de TiO_2 condujo, en todos los casos, a un desplazamiento en la curva de crecimiento microbiano, revelando recuentos más bajos a medida que concentración de TiO_2 aumentaba. *E. coli* fue el patógeno más afectado, alcanzando entre 2-3 log $UFC.mL^{-1}$ de reducción. Además, en el caso de las bacterias probióticas, las nanopartículas NM105 TiO_2 tuvieron efectos similares, ya que la densidad bacteriana se redujo en 2-3 log $UFC.mL^{-1}$. Estos resultados podrían aplicarse como una potente tecnología a incluir en la formulación de nuevos desinfectantes

PALABRAS CLAVE
TiO_2; nanopartículas; desinfección fotocatalítica; UV; higiene alimentaria

1. Introduction

Food safety is an important topic of food industrial development (Wang et al., 2016). This is mainly related to foodborne diseases which have been a widespread health problem in the contemporary world and an important cause of reduced economic productivity (WHO, 2015).

Even though disinfection methods commonly employed in the food industry can control microbial pathogens, researchers have shown that there is an increase of bacteria resistant to conventional treatments (González-Rivas, Ripolles-Avila, Fontecha-Umaña, Ríos-Castillo, & Rodríguez-Jerez, 2018; Zhao, Zhao, Wang, & Zhong, 2017). Consequently, there is a necessity to reevaluate traditional methods of disinfection, as well as, to consider new approaches oriented to improve the reliability and robustness of disinfection whilst eluding the resistance of bacteria (Li et al., 2008; Özkalelí & Erdem, 2017; Ripolles-Avila, Ríos-Castillo, & Rodríguez-Jerez, 2018). In this sense, a technology that has stimulated a significant interest in finding new possibilities in this area is nanotechnology. In fact, it has been shown that many nanoparticles (NPs) such as copper oxide, silver and zinc oxide possess antimicrobial properties and could be a possible promising solution (Bondarenko et al., 2013; Duffy, Osmond-McLeod, Judy, & King, 2018; Sirelkhatim et al., 2015). However, there are some concerns about the residues liberation of these nanomaterials to the environment, and how the different organisms present in the environment may be affected (Bondarenko et al., 2013). With this respect, Kahru and Dubourguier (2010) demonstrated that over seven nanoparticles analyzed (ZnO, Ag, CuO, TiO_2, C60-fullerenes, SWCNTs and MWCNTs), TiO_2 NPs were the least environmentally harmful. Furthermore, International Agency for Research on Cancer (IARC) classified TiO_2 as a human carcinogen group 2B although this category is used for chemicals for which less than sufficient evidence of carcinogenicity in experimental animals and also there is limited evidence of carcinogenicity in humans (Jovanović, 2015a). Furthermore, something to have into consideration is the lack

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of consistent toxicity data of TiO_2 NPs due to the diversity of materials and test conditions followed by the different researchers, which makes a real challenge the fact of conducting risk assessment (Jovanović, 2015b).

TiO_2 is a semiconductor which upon UV light excitation, it can react with H_2O or hydroxide ions adsorbed on the surface to produce hydroxyl radicals (·OH), or reduce O_2 to produce superoxide ions (Banerjee, Muraleedharan, Tyagi, & Raj, 2006). In addition, it has been shown that there is a production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and singlet oxygen, which are closely related to cell damage, when TiO_2 is activated by UV light (Carp, Huisman, & Reller, 2004; Foster, Ditta, Varghese, & Steele, 2011). All of those ROS can cause damage to living cells. Three principal polymorphs of TiO_2 exist: anatase, rutile and brookite (Landmann, Rauls, & Schmidt, 2012). In fact, some studies have revealed that anatase form is the most active photocatalyst and, rutile normally less effective (Foster et al., 2011). Nevertheless, Miyagi, Kamei, Mitsuhashi, Ishigaki, and Yamazaki (2004) have shown that rutile and anatase mixtures have a higher photocatalytic effect than only anatase and also are more efficient to generate ROS.

Matsunaga (1985) reported the effect of photocatalytic inactivation of several microorganisms such as *E. coli* and *L. acidophilus* using nano-TiO_2. Since then, research on nano-TiO_2 photocatalytic effect has been intensively conducted on a wide range of microorganisms to evaluate photocatalytic disinfection activities. Furthermore, it would be also worth to evaluate the effects of TiO_2 embedded in surfaces. As it has been proposed by other authors, surfaces made up of light-guiding materials, coated with specific semiconductors (i.e. TiO_2 NPs) and stimulated by UV light could provide a new way of disinfection (Barthomeuf et al., 2017; Jalvo, Faraldos, Bahamonde, & Rosal, 2017; Yemmireddy & Hung, 2017). In fact, the ability of TiO_2 for microbial elimination on photocatalytic self-cleaning/self-disinfecting surfaces may contribute an additional mechanism to the control of disease transmission. Thus, photooxidation mediated by TiO_2 appears to be promising for microbial elimination in areas where the employment of biocides or chemical cleaning agents is not effective or is restricted by food industry regulations (Yemmireddy & Hung, 2015). However, the bactericidal effect of TiO_2 NPs seems to be higher when applied in suspension rather than embedded in surfaces (Kim, Kim, Cho, & Cho, 2003), although comparative effects need to be investigated. Foster et al. (2011) have shown that a closer contact within the TiO_2 and the organism enhances the amount of oxidative damage. On this sense, it has been demonstrated that on water suspended TiO_2 has a greater effect than TiO_2 immobilized on surfaces like thin films (Alhaji et al., 2017; Sun, Tay, & Tan, 2003). This is presumably because of extended contact between the microbial cells in suspension and the TiO_2 NPs, augmenting the surface area for ROS production. A number of studies have demonstrated the significance of such contact (Caballero, Whitehead, Allen, & Verran, 2009; Cheng et al., 2009; Horie, David, Taya, & Tone, 1996).

This study aims to consider the potential of TiO_2 for the development of new surface disinfection alternatives to be employed in the food industry. The main purpose was to compare the bactericidal effect of photoactivated and non-photoactivated TiO_2 NPs on a range of bacterial indicators of contamination in food, both pathogenic and spoilage microorganisms, tested on suspension, as a way to reduce the use of other disinfectants and contemplate it as an alternative. Two different forms of TiO_2 NPs were used in this study, one containing purified anatase form and the other containing a mixture of anatase/rutile phase. The ability of all the microorganisms to survive photo-inactivation with the UV light was assessed by varying the periodicity of irradiation. Furthermore, the turbidity of the two NPs in the different media used in the experiment and the photoactivation of the TiO_2 NPs was also evaluated.

2. Materials and methods

2.1. Nanoparticles

Two different types of TiO_2 nanoparticles named NM101 and NM105 were employed in this study. The NM101 NPs were obtained from the nanomaterials repository at the Institute for Health and Consumer Protection at the European Commission Joint Research (Ispra, Italy) and NM105 NPs were purchased from Degussa (Frankfurt, Germany). Sample NM101 consisted of anatase crystal phase with 7 ηm size. Besides, sample NM105 was a combination of anatase-rutile phase (80:20 wt/wt) with 21 ηm size. The main characteristics of the nanoparticles provided by the individual supplier are listed in Table S1.

Suspensions of TiO_2 nanoparticles at concentration of 1 mg mL^{-1} using Nutrient Broth (NB; Oxoid, Hampshire, United Kingdom) and MRS (Oxoid, Hampshire, United Kingdom) were prepared and then vortexed (SLS, Southampton, United Kingdom) to mix the preparations in order to have a homogeneous mixture. Fresh suspensions were prepared prior to each experiment.

2.2. Bacterial cultures

Salmonella enterica var. Enteridis (ATCC 13076), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29247) and *Bacillus cereus* (ATCC 29247) were purchased as a Culti-Loops (Remel Europe, Dartford, United Kingdom). The Culti-Loops were then streaked onto sterile plate count agar (PCA; Oxoid, Hampshire, United Kingdom) to obtain pure cultures on plates. After streaking, the tips of the Culti-Loops which contained the microorganisms were separated and deposited in sterile nutrient broth. Both the inoculated plates and broths were incubated at 37 ± 2 ºC during 24 h. After that, the inoculated plates were sealed with Parafilm and kept as stock cultures at 4 ± 2 ºC for a maximum period of 1 month. The broth cultures were subcultured into new sterile nutrient broth every day by transferring 200 µL from the initial broth culture and incubated at 37 ± 2 ºC for 24 h.

Probiotic strains *Lactobacillus casei* (ATCC 393), *Lactobacillus delbrueckii subsp. bulgaricus* (ATCC 11842), *Lactobacillus lactis subsp. lactis* (ATCC 19435) and *Lactobacillus acidophilus* (ATCC 4356) were provided by Nutraceutech, Montreal, Canada. Individual strains were cultivated in MRS liquid medium (Oxoid, Hampshire, United Kingdom) and incubated at 37 ± 2 ºC as static cultures for 48 h. Then, broths were subcultured into 20 mL of new sterile MRS liquid medium every 48 h by transferring 200 µL from the initial broth culture and then were kept at 37 ± 2 ºC for 48 h more.

2.3. Inoculum preparation

Seed cultures of the four pathogens were inoculated into 100 mL nutrient broth and the four probiotic seed cultures were inoculated into 20 mL MRS. Cell concentration was adjusted at approximately 10^7 colony forming units (CFU). mL^{-1} by measuring the absorbance of bacterial suspension at 650 ηm using a UV/Vis spectrophotometer (Helios Epsilon, Thermo Scientific, Loughborough, United Kingdom). This cell adjustment was done using the calibration curves obtained by each microorganism.

2.4. Impact of the NPs on the turbidity of the media

A turbidity testing of NM101 and NM105 TiO_2 was done to investigate whether nano-TiO_2 would change the turbidity of the media through time. A range of 8 different concentrations from 0.78 to 100 µg mL^{-1} of both NPs were prepared and measured in nutrient broth and MRS. Figure 1 shows representative results for NM101 TiO_2 in NB; which are consistent with the results observed for the NM105 TiO_2 nanoparticle in NB media (supplementary materials). TiO_2 showed a different turbidity between concentrations but it did not change the turbidity of the media over the time. In concordance, non-significant differences ($P > 0.05$) were found between the turbidity achieved by the diverse concentrations of the nanoparticles tested.

2.5. Photocatalytic experiments

The photocatalytic bactericidal tests were performed in sterile microtitre plates. Twenty microliters of the aqueous suspensions of TiO_2 nanoparticles (both NM101 and NM105) were added to each well in triplicate along with 180 µL of bacterial cultures to give final nanoparticle concentration of 0.78–100 µg mL^{-1}. In all the tests, four different conditions were examined: (i) presence of TiO_2 and UV light, (ii) no TiO_2 and presence of UV light, (iii) presence of TiO_2 under dark conditions, (iv) no TiO_2 under dark conditions.

The microtitre plates with bacteria-nanoparticles suspension were located in a noncommercial chamber which was fitted with a UVSPOT 1000 UV lamp (UV Technologies, Germany), emitting radiation over 315–400 ηm. The distance within the samples and the lamp was set up to acquire 6.9 mW cm^{-2} of UV light incident on the samples. As *S. aureus* was more sensitive to UV light than the rest of microorganisms (see section 3.2), the time of UV light exposure was set up at 6 min, which was the time period that did not compromise the growth of the microorganism. Furthermore, UV intensities higher than 1 mW cm^{-2} were always applied, which has been shown to be sufficient to begin the photocatalytic reaction at the established exposure time (Oka et al., 2008). After the UV exposure, samples were shaken to resuspend the cultures and the optical density was measured at 650 ηm wavelength on a microplate reader (Spectra MAX M5, Molecular Devices, California, United States). The microtitre plates were incubated at 37 ± 2 ºC during 24 h. After the incubation, samples were shaken again and the optical density was measured at 650 ηm wavelength on the same microplate reader.

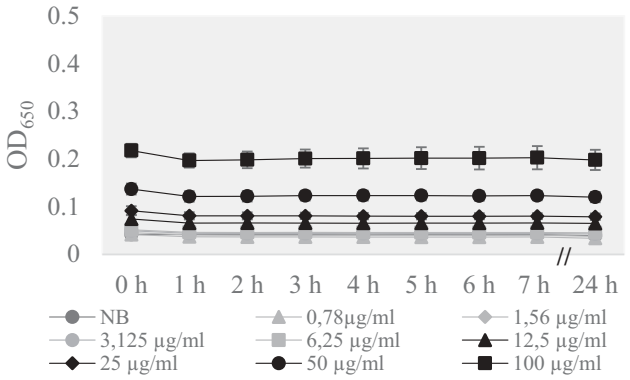

Figure 1. Optical density measurements of different concentrations of NM101 TiO_2 in nutrient broth over time. Each value corresponds to the mean of three replicates performed on three separated days (n = 9). The error bars represent standard deviation.

Figura 1. Densidad óptica obtenida a partir de diferentes concentraciones de NM101 TiO_2 en caldo nutritivo a lo largo del tiempo. Cada valor corresponde a la media de tres repeticiones realizadas en tres días separados (n = 9). Las barras de error representan la desviación estándar.

2.6. Correlation between optical density (OD) values and CFU.mL^{-1}

Serial dilutions of all microorganisms (ranging from 10^{-1} until 10^{-9}) were prepared in 200 µL of nutrient broth and MRS broth using sterile microtitre plates and the optical density was measured at 650 ηm wavelength on a microplate reader. From the dilution 10^{-3}–10^{-9}, 100 µL of aliquots were withdrawn and plated in sterile petri dishes with plate count agar (PCA and MRS, respectively, for pathogens and probiotic bacteria). The plates were incubated at 37 ± 2 ºC for 24 h (*S. enterica* var. Enteridis, *E. coli, S. aureus* and *B. cereus*) and 48 h (*L. casei, L. bulgaricus, L. acidophilus* and *L. lactis*). All microbial counts were reported as CFU.mL^{-1} values.

2.7. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay

The level of ROS generated by TiO_2 NPs was detected by carboxy-2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA) staining. DCFH-DA is a fluorescent probe that can be deacetylated to form DCFH. DCFH is non-fluorescent unless oxidized by ROS to transform DCF. The fluorescence intensity of DCF can indicate the quantity of ROS produced (Sun et al., 2011). Ten microliters of DCFH-DA probe (Sigma-Aldrich, Poole, UK) and 40 µL of sodium NaOH (Sigma-Aldrich, Poole, UK) solution were added to the wells of a microtitre plate in triplicate and incubated for 30 min at room temperature. After this period, 68 µL of sterile phosphate buffered saline (PBS, Sigma-Aldrich, Poole, UK) solution was added to the mixture. In parallel, suspensions of TiO_2 NPs (both NM101 and NM105) in nutrient broth and MRS broth were exposed to UV light during 6, 12 and 24 min. After the UV exposure, 118 µL of the TiO_2 nanoparticle suspension was added to the DCFH-DA+NaOH+PBS mixture and incubated during 10 min at room temperature. The investigated range of nanoparticle concentrations was between 0.78 and 100 µg mL^{-1}. After the incubation, fluorescence was measured every 1 min until 15 min and then at 30, 45 and 60 min at 485 ηm (excitation) and 535 ηm (emission) wavelengths on a microplate reader.

2.8. Statistical analysis

All experiments were performed three times on three different days (n = 9). Data from all experiments were analyzed using the Minitab statistical package using ANOVA general linear model with subsequent analysis of variance and Tukey's test. A P value of <0.05 was considered to be significant. The global differences were examined followed by an analysis of the individual data sets from the two TiO_2 nanoparticles.

3. Results

3.1. Effect of UV light over microorganisms

A preliminary study was conducted to select the time of exposure of the samples (bacteria + NPs) and to confirm that the time of UV exposure would not have impact on the growth of the bacteria. Suspensions of *S. enterica* var. Enteridis, *E. coli, B. cereus* and *S. aureus* in nutrient broth and suspensions of *L. casei, L. bulgaricus, L. acidophilus* and *L. lactis* in MRS broth were exposed to 3, 6, 12, 24, 36 and 48 min of UV light with an intensity of 6.9 mW cm^{-2} incident on the samples. The microorganism that showed to be more sensitive to UV light was *S. aureus*, increasing its sensitivity when the time of exposure was raised (Figure 2). Only an exposure period of 3 and 6 min did not have an impact on the growth of *S. aureus*. Because of that, 6 min of UV exposure was selected in order to maximize the potential for photoactivation of nano-TiO_2. The microorganism less affected on its growth curve by UV light was *S. enterica* var. Enteridis (supplementary materials). In addition, it is also important to highlight that UV light did not have any impact on the turbidity of the culture broths employed in the study (i.e. NB and MRS). As can be observed in Figure 2, the increase of the exposure time to UV let to no change in the obtained OD_{650} values on NB and MRS broths.

3.2. Photoactivation test with a fluorescent probe

Results showed that DCF fluorescence intensity slightly increased through the time, increasing in parallel the ROS level with the increase of the concentration of TiO_2 NPs from 0.78 to 100 μg mL^{-1} although non-significantly ($P > 0.05$) (Figure 3). Moreover, no significant differences ($P > 0.05$) were found between the ROS level produced after exposure of the UV samples with respect to those subjected to dark conditions, suggesting that there is no need to expose the samples with UV so that the TiO_2 NPs could play an antimicrobial role.

3.3. Effect of photocatalytic TiOtio$_2$ NPs on different microorganisms

The inhibitory effects of both TiO_2 NPs, NM101 and NM105, on pathogenic bacteria is shown in Figure 4. All the pathogens in nutrient broth decreased their growth in a dose-dependent manner following exposure to NM101 and NM105 TiO_2. The bacterial culture of *S. enterica* var. Enteridis showed a significant decrease ($P< 0.01$) in the OD_{650} value at concentrations of 50 and 100 μg mL^{-1} of NM101 and NM105 TiO_2, compared to the control suspension. The pathogen *E.coli* showed a significant growth decrease ($P< 0.01$ and $P < 0.001$) in the OD_{650} at concentrations of 12.5 μg mL^{-1} when compared to the control. Using the standard curves obtained from the relationship between absorbance and CFU.mL^{-1}, an estimated 2 log CFU.mL^{-1} reduction of the initial concentration of *E. coli* was obtained when NM101 and NM105 TiO_2 was activated by 6 min UV light exposure. In contrast, when both TiO_2 NPs were not UV activated (TiO_2+ UV-), *E. coli* was 2.3–3 log CFU.mL^{-1} reduced, respectively, for NM101 and NM105. In the case of *S. aureus* and *B. cereus*, a significant decrease in the OD_{650} value at concentrations of 50 and 100 μg mL^{-1} of non-photoactivated ($P< 0.001$) and photoactivated ($P< 0.05$) NM101 TiO_2 was observed when compared with the control. However, when *S. aureus* and *B. cereus* were exposed to NM105 TiO_2 showed a significant ($P< 0.01$ for *S. aureus* and $P< 0.05$ for *B. cereus*) decrease in the OD_{650} value just at the highest concentration (i.e. 100 μg mL^{-1}). Again, estimating the reduction in terms of CFU.mL^{-1} using the standard curves for each microorganism, in the presence of TiO_2 and dark conditions, NM101 at 100 μg mL^{-1} reduced 2 log CFU.mL^{-1} the initial microbial load of *S. aureus* and *B. cereus*, and NM105 reduced the load of them by 2.69 log CFU.mL^{-1}. With all these results, growth curves of the different pathogens (including Gram-positive and Gram-negative) have been observed to be significantly affected in a directly proportional way as applied TiO_2 concentration increased.

From the data of the impact of photoactivated and non-photoactivated TiO_2 NPs on probiotic bacteria, it was evident that there was a dose-dependent decrease in the OD_{650} values after 48 h of incubation (Figure 5). There was no significant difference between photoactivated and non-photoactivated TiO_2, showing in all cases, the same effects on probiotic bacteria ($P> 0.05$). Furthermore, the OD_{650} values of the probiotic cultures showed no significant changes during the tests under 0.79 to 25 μg mL^{-1} NM101 and NM105 TiO_2 ($P > 0.05$). However, using the standard curves to obtain an estimated result of CFU.mL^{-1}, it was apparent that NM105 TiO_2 UV activated and non-activated (i.e. dark conditions) induced a very significant 3 log CFU.mL^{-1} reduction in the cases of *L. casei, L. bulgaricus* and *L. lactis*, and 2 log reduction in the case of *L. acidophillus*. Significant, but less pronounced, decreases on the OD_{650} values were also detected in the presence of photoactivated and non-photoactivated NM101 TiO_2 (around 1 log CFU.mL^{-1} reduction in all the cases at the highest concentration). NM101 and NM105 TiO_2 had a significant difference on decreasing the number of probiotic cultures between them, being NM105 TiO_2 the one that had the strongest effect ($P< 0.01$).

4. Discussion

This study was aimed to evaluate the antibacterial effect of photoactivated and non-photoactivated TiO_2 nanoparticles on a range of Gram-negative (*S. enterica* var. Enteridis and *E. coli*) and Gram-positive (*S. aureus, B. cerus, L. casei, L. bulgaricus, L. acidophilus* and *L. lactis*) bacteria.

Preliminary tests for the correlation between OD_{650} values and CFU.mL^{-1} (data not shown) were performed in nutrient broth and MRS, the growth media used for the cultivation of the pathogens and probiotic bacteria, respectively. This was performed to set up a correlation curve as a basis to

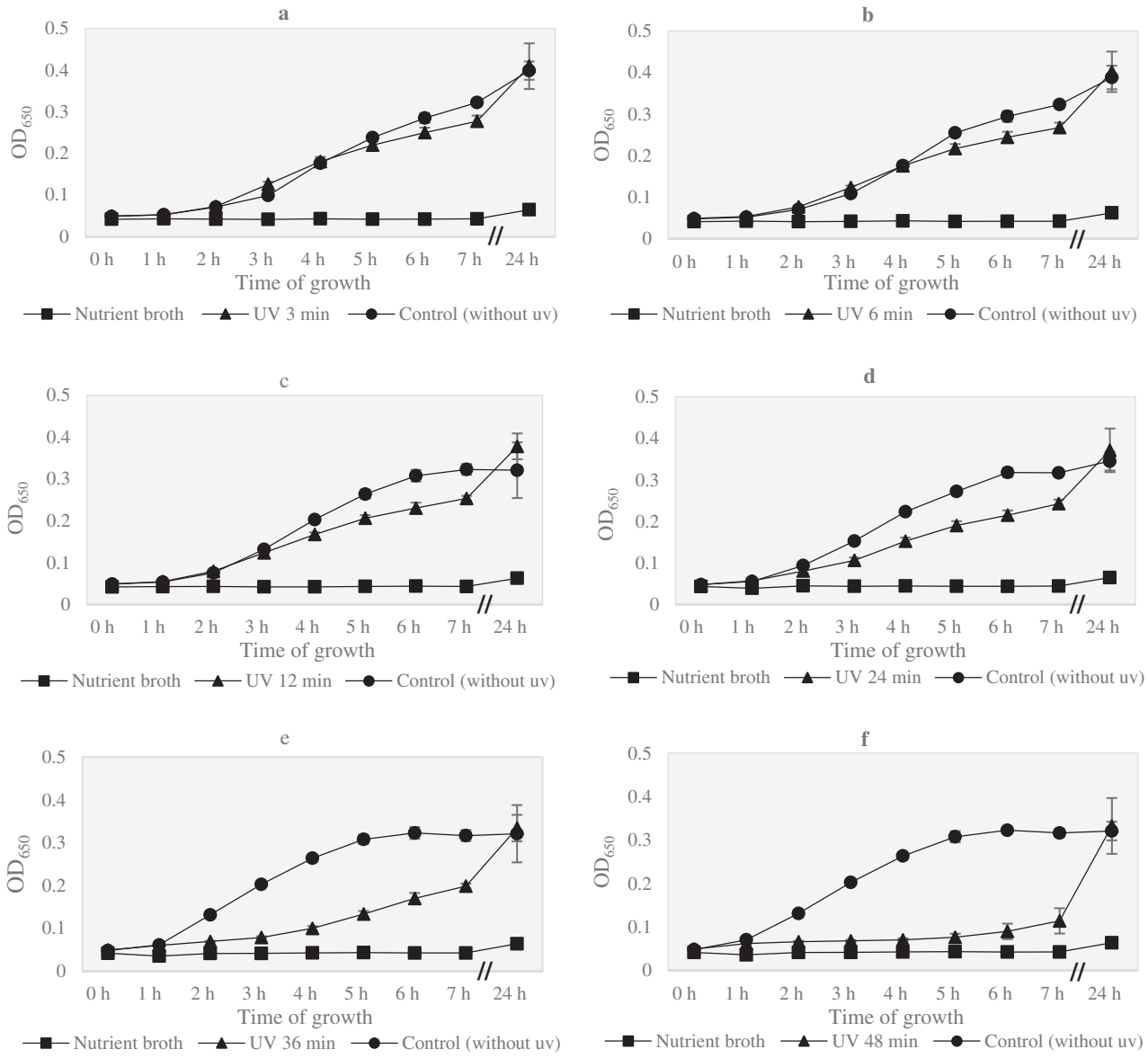

Figure 2. Effect of UV light at different exposure times on the growth curve of *S. aureus*. The graphs represent: (a) 3 min; (b) 6 min; (c) 12 min; (d) 24 min; (e) 36 min; and (f) 48 min of UV exposure. An every hour measurement was taken until 7 h of incubation and after that, another reading at 24 h of incubation was measured. Each value corresponds to the mean of 3 replicates performed on three separated days (n = 9). The error bars represent standard deviation.

Figura 2. Efecto de la luz UV, a distintos tiempos de exposición, sobre la curva de crecimiento de *S. aureus*. Los gráficos representan: (A) 3 minutos; (B) 6 minutos; (C) 12 minutos; (D) 24 minutos; (E) 36 minutos; y (F) 48 minutos de exposición a luz UV. Se tomó una medición cada hora hasta las 7 horas de incubación y, posteriormente, se realizó otra lectura a las 24 horas de incubación. Cada valor corresponde a la media de 3 repeticiones realizadas en tres días separados (n = 9). Las barras de error representan la desviación estándar.

compare the OD values from the experiments with estimates of the numbers of bacteria that were represented by these OD values, as other studies did (Kim, 2012; Ripolles-Avila, Ríos-Castillo, Guerrero-Navarro, & Rodríguez-Jerez, 2018; Yemmireddy & Hung, 2015). Similarly, we described a high correlation was observed between OD_{650} values and CFU. mL^{-1} for the different bacteria employed for the study.

Investigation of the effect of UV light on the different microorganisms used in this study was performed in the second instance to identify whether the exposure time of UV light on the bacteria had an impact on their growth. By establishing a UV light exposure time for the activation of the NPs that did not affect the growth of none of the bacteria, the bactericidal effects were able to be attributed to the effects of TiO_2 photocatalytic activation. Under the tested conditions, *S. aureus* was shown to be the most sensitive bacteria. When the time of UV light exposure increased, the growth curve of *S. aureus* was affected. UV light can cause DNA damage (Hijnen, Beerendonk, & Medema, 2006). So the use of UV irradiation alone can have an impact on the growth of the bacteria but is incapable of bacterial disinfection; only strong UV irradiation (UV-B and UV-C) can have a disinfecting effect on bacteria (Pham & Lee, 2015; Yu, Lee, Lin, & Huang, 2008). The UV irradiation employed in the study was between the range 315–400 nm, which has also been used by other authors (Adhikari, Thind, Chen, Schraft, & Chen, 2018). The microorganism that we observed that had greater resistance to UV light was *S. enterica* var. Enteridis, which can be due to the range of UV light used was considered to be UV-A, and as it is not strong it has not had a bactericidal effect against this pathogen. Nevertheless, the TiO_2 NPs are photoactive and produce reactive oxygen species under natural sunlight (UV-A and UV-B) (Jovanović, 2015b).

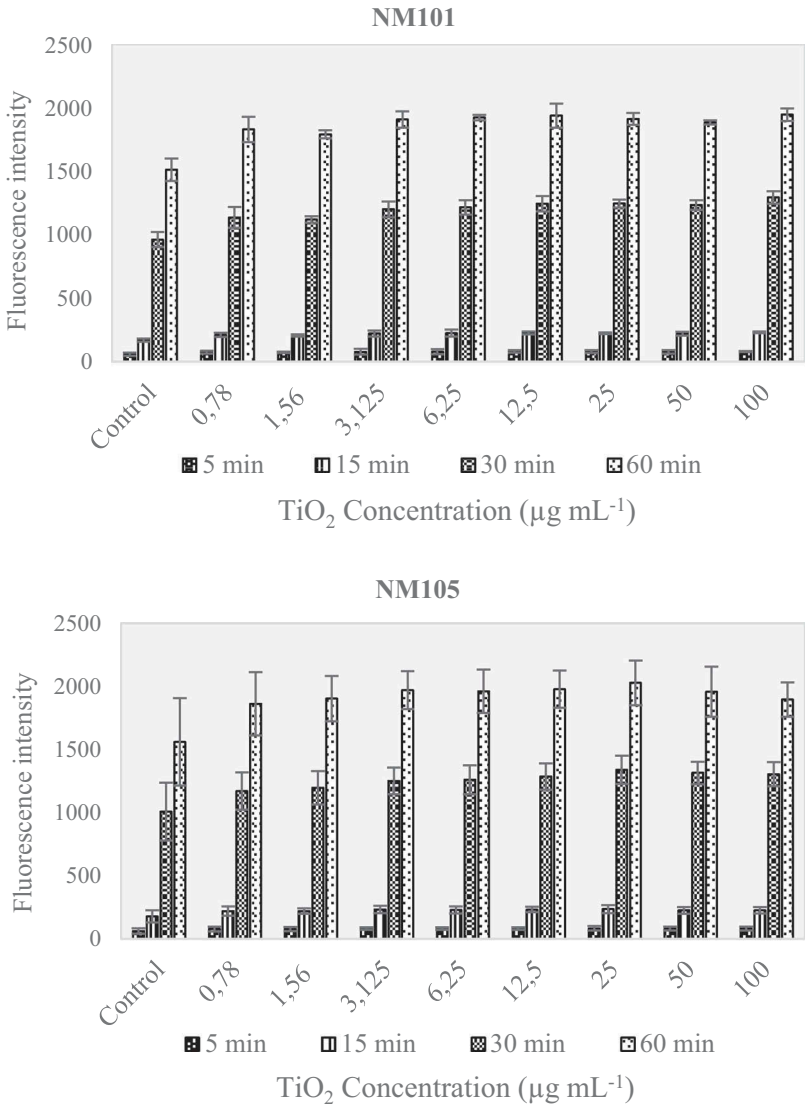

Figure 3. DCF fluorescence intensity produced by TiO_2 NPs at different concentrations ranging from 0.78 to 100 µg mL^{-1}. The UV exposure time was 6 min, but the results are representative for 12 and 24 min of exposure. The control sample did not contain TiO_2. Each value corresponds to the mean of 3 replicates performed on three separated days (n = 9). The error bars represent standard deviation.

Figura 3. Intensidad de fluorescencia de DCF producida por nanopartículas de TiO_2 a diferentes concentraciones que varían de 0.78 a 100 µg mL^{-1}. El tiempo de exposición a luz UV fue de 6 minutos, pero los resultados son representativos de 12 y 24 minutos de exposición. La muestra de control no contenía TiO_2. Cada valor corresponde a la media de 3 repeticiones realizadas en tres días separados (n = 9). Las barras de error representan la desviación estándar.

The ability of nano-TiO_2 to be photoactivated in the bacterial cultures was investigated using a DCFH-DA fluorescent emission assay. This fluorometric probe is primarily utilized for oxidative stress measurements. Our assay was based on the ability of the photoactivated TiO_2 NPs to create ROS on the surface of the NPs. Aranda et al. (2013) and Griffiths et al. (2011) have already demonstrated the interference of different types of NPs with DCFH-DA fluorescence emission. A modification of the DCFH-DA test from the original protocol was done in order to adapt the assay to the experimental conditions, for example when the NPs were exposed to UV light the media used in the assay was culture medium rather than water or PBS. Although increasing the period of UV light exposure increased the fluorescence emission observed in the assay, non-significant enhancement with increasing concentrations of either TiO_2 NPs was observed. Therefore, as the fluorescence of the control was not different to the fluorescence produced by the different concentration of NPs, the increased fluorescence observed in the assay might be associated to NPs-culture media interactions rather than ROS production. For example, the enhancement of fluorescence emission could be caused due to agglomerate formation of the particles in the cell culture medium. Some studies have had similar problems (Aranda et al., 2013; Qi et al., 2012; Sabatini, Pereira, & Gehlen, 2007). Consequently, this photoactivation test using DCFH-DA as a fluorometric probe did not provide any evidence of TiO_2 being photoactivated. Although some studies have not used any test to prove photoactivation of TiO_2 (e.g. Long et al., 2014; Yemmireddy & Hung, 2015), some others have shown that a degradation test on an organic dye, rhodamine B, was very efficient to show if the NP was being photoactivated by UV light (e.g. Koizumi & Taya, 2002; Li et al., 2008; Polo et al., 2011).

TiO_2 NPs are a highly effective disinfectant once they are activated by UV light (Kermanizadeh et al., 2013; Kim et al., 2003). In water and air systems, TiO_2 absorbs photons, resulting in the formation of active hydroxyl (·OH) and other ROS (O_2^-, H_2O_2, among others) in the presence of O_2 and H_2

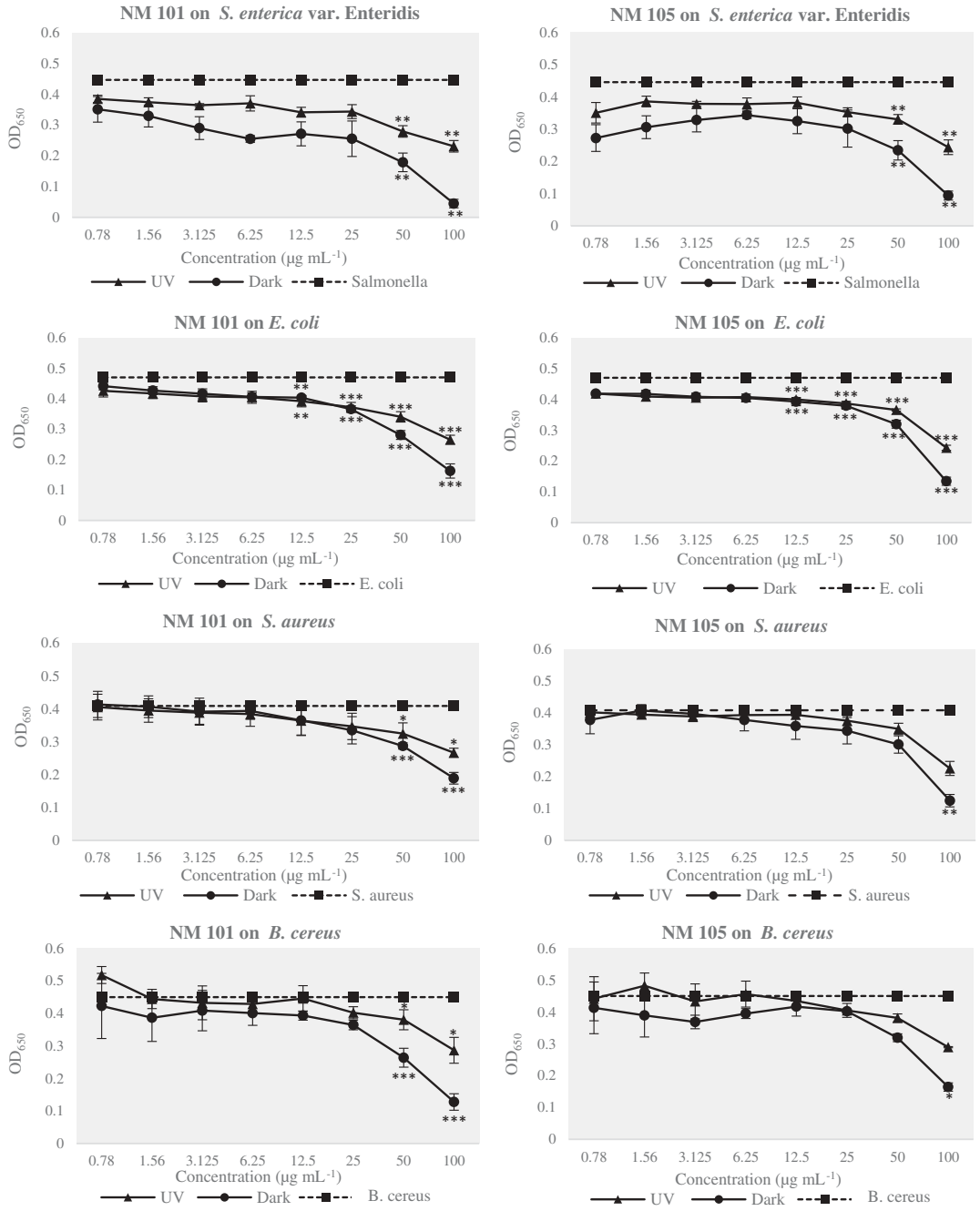

Figure 4. OD_{650} values obtained in the tests of *S. enterica* var. Enteridis, *E. coli, S. aureus* and *B. cereus* with photocatalytic TiO_2 NPs in nutrient broth. In the graphs, y-axis shows the OD_{650} values for each test condition and x-axis shows the different concentrations ranging from 0.78 to 100 µg mL^{-1} of TiO_2 tested (NM101 and NM105). Samples were subjected to UV activation (UV) or non-UV activation (Dark). Each value corresponds to the mean of three replicates (n = 9). The error bars represent standard error of the mean. The graphs provide the *P*-values obtained by ANOVA analysis. The statistical analysis was performed separately for each experimental condition. Significance indicated by: * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$.

Figura 4. Valores OD_{650} obtenidos para *S. enterica* var. Enteridis, *E. coli, S. aureus* y *B. cereus* después de aplicar un tratamiento con nanopartículas de TiO_2 en caldo nutritivo. En los gráficos, el eje y muestra los valores de OD_{650} para cada condición y el eje x muestra las diferentes concentraciones que van desde 0.78 a 100 µg mL^{-1} de TiO_2 (NM101 y NM105). Las muestras se sometieron a activación por UV (UV) o no se activaron (Oscuridad). Cada valor corresponde a la media de tres repeticiones realizadas en tres días separados (n = 9). Las barras de error representan el error estándar de la media. Los gráficos proporcionan los valores de *P* obtenidos por análisis ANOVA. El análisis estadístico se realizó por separado para cada condición experimental. Significancia indicada por: * = $P < 0.05$, ** = $P < 0.01$, y *** = $P < 0.001$.

O. Those ROS produced, particularly H_2O_2, cannot only transverse the cell membranes, but also produce oxidative active hydroxyl through a reaction inside bacterial cells that finally leads to the disinfection (Fujishima, Rao, & Tryk, 2000; Kikuchi, Sunada, Iyoda, Hashimoto, & Fujishima, 1997). There is overwhelming evidence that the mechanism of killing bacteria is due to membrane and cell wall damage (Foster et al., 2011). Some studies have demonstrated that once TiO_2 NPs are mixed with the bacterial cultures, TiO_2 is attached to the bacterial cells and damages the cell wall under UV light (Dalrymple, Stefanakos, Trotz, & Goswami, 2010; Demidova & Hamblin, 2005; Kubacka, Ferrer, & Fernández-García, 2012). The damaged cell wall allows TiO_2 NPs to enter into the cells and releases the cell contents from the cells, resulting in cell death (Long et al., 2014). In this sense, we described TiO_2 NPs significantly reduced the populations of all tested Gram-positive and Gram-negative bacteria in the suspension solutions, being more effective NM105 than NM101. Although TiO_2 demonstrated antibacterial activity for each of the evaluated bacterial strains in

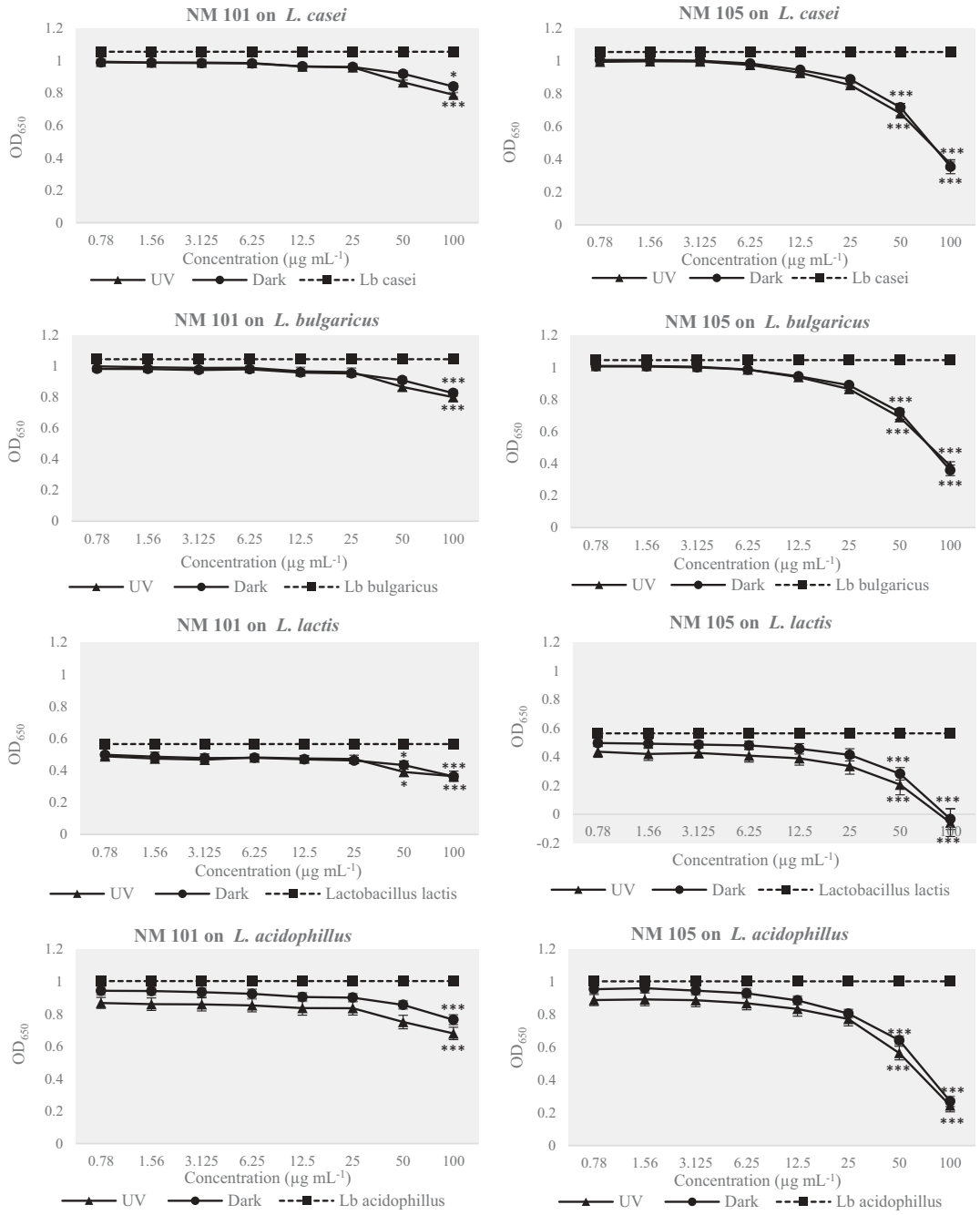

Figure 5. OD values obtained in the tests of *L. casei, L. bulgaricus, L. lactis* and *L. acidophillus* with photocatalytic TiO_2 NPs in MRS. In the graphs, y-axis shows the OD_{650} values for each test condition and x-axis shows the different concentrations ranging from 0.78 to 100 µg mL^{-1} of TiO_2 tested (NM101 and NM105). Samples were subjected to UV activation (UV) or non-UV activation (Dark). Each value corresponds to the mean of three replicates (n = 9). The error bars represent standard error of the mean. The graphs provide the *P*-values obtained by ANOVA analysis. The statistical analysis was performed separately for each experimental condition. Significance indicated by: * = $P < 0.05$, ** = $P< 0.01$, and *** = $P < 0.001$.

Figura 5. Valores OD_{650} obtenidos para *L. casei, L. bulgaricus, L. lactis* and *L. acidophillus* después de aplicar un tratamiento con nanopartículas de TiO_2 en caldo MRS. En los gráficos, el eje y muestra los valores de OD_{650} para cada condición y el eje x muestra las diferentes concentraciones que van desde 0.78 a 100 µg mL^{-1} de TiO_2 (NM101 y NM105). Las muestras se sometieron a activación por UV (UV) o no se activaron (Oscuridad). Cada valor corresponde a la media de tres repeticiones realizadas en tres días separados (n = 9). Las barras de error representan el error estándar de la media. Los gráficos proporcionan los valores de *P* obtenidos por análisis ANOVA. El análisis estadístico se realizó por separado para cada condición experimental. Significancia indicada por: * = $P < 0.05$, ** = $P < 0.01$, y *** = $P < 0.001$.

a dose-dependent way, there were no significant differences ($P > 0.05$) between the activity of the UV-exposed and non-UV-exposed TiO_2. After 24 h of incubation, the biocidal effect of TiO_2 NPs was evident in the microorganisms tested. These results are in agreement with studies reported by Kim et al. (2003), Rincón and Pulgarin (2003), Rizzo (2009) and Swetha, Santhosh, and Geetha Balakrishna (2010), where, in similar conditions, *S. aureus, E. coli, Salmonella* spp.,*Clostridium perfringens* spores, *Pseudomonas stutzeri, Pseudomonas aeruginosa, Listeria monocytogenes, Serratia marcescens, Vibrio parahaemolyticus, Actinomyces viscosus, L. acidophilus, Streptococcus* spp., and coliform bacteria were killed by photoactivated TiO_2 NPs.

However, according to our results, UV-exposed and non-UV-exposed TiO_2 did not show significant differences in their bactericidal activity. As an inhibition of the bacteria was produced with the presence of TiO_2 NPs in dark conditions, this suggests that the NPs were effective and showing a dose-dependent response even when TiO_2 was not exposed to UV-A light. The decrease in CFU.mL^{-1} associated

with the non-photoactivated TiO_2 has been shown in other studies (Lalucat, Bennasar, Bosch, García-Valdés, & Palleroni, 2006; Polo et al., 2011; Rincón & Pulgarin, 2004) where they suggested that the reduction of the bacterial cells can be put down to a fall in pH as a result of the addition of the photocatalyst. This fall in pH would be associated to both conditions (UV activated TiO_2 and non-UV activated TiO_2) but with the difference that UV-exposed TiO_2 would have both inhibitory effects, the reduction of the pH and the photocatalytic activity. Nevertheless, this finding cannot be justified by the drop in the pH because of UV-exposed and non-UV-exposed TiO_2 did not show significant differences in their bactericidal activity. A possible explanation might be that TiO_2 nanoparticles are sensitive to photo-oxidation and when oxidized they lose their photocatalytic activity (Hu, Song, Jiang, & Wei, 2015; Lin et al., 2014). The fact that the TiO_2 NPs had been in use during some time suggests that light could have damaged the NPs. However, the results obtained demonstrated that although NPs were slightly activated by UV light, they presented an important role as antimicrobial for both Gram-positive and Gram-negative bacteria used in the study, as growth curves were compromised revealing a bacteriostatic effect, which is something interesting to continue investigating. In posterior studies, it would be interesting to include microbial viability tests to observe the real impact of TiO_2 NPs as potential disinfectants. On this sense, the applications of international standards such as UNE-EN 1040 or UNE-EN 1276 could be relevant. Furthermore, it is also interesting to evaluate the effectivity of these nanoparticles once they are embedded on surfaces in order to know the impact on biofilms, employing *in vitro* models to form mature structures and have results similar to real conditions (Ripolles-Avila, Hascoët, Guerrero-Navarro, & Rodríguez-Jerez, 2018).

Additionally, NM105 TiO_2 was more effective than NM101TiO_2. Similar findings have been reported by Miyagi et al. (2004) who stated that anatase and rutile mixtures have a greater photocatalytic effect than 100% anatase. The increased activity of NM105 TiO_2 is commonly associated to interactions between the two forms, reducing the recombination between electrons and holes (Foster et al., 2011). In this regard, Yemmireddy and Hung (2015) reported that among the three TiO_2 tested (NM105 and two purified anatase crystal phase with different size measurements), NM105 was found to be the most efficient photocatalyst.

Furthermore, no significant differences were observed between Gram-negative (*Salmonella* spp. and *E. coli*) and Gram-positive (*S. aureus, B. cereus* and the probiotic bacteria) in their resistance to nano-TiO_2 treatment. Some studies have suggested that Gram-negative bacteria are more sensitive to photocatalytic disinfection than Gram-positive bacteria (Backhaus, Marugán, Van Grieken, & Sordo, 2010; B. Kim et al., 2003; Pal, Pehkonen, Yu, & Ray, 2007). This difference on sensitivity is normally associated to the differences in the cell surface structure such as cell wall and cell membrane, between Gram-negative and Gram-positive bacteria. However, several researchers have also stated that they found no difference within the bactericidal effect of photoactivated TiO_2 on Gram-negative and Gram-positive bacteria. For example, Wong et al. (2006) showed that the sensitivity of some human pathogens (*S. aureus, V. parahaemolyticus* and *L. monocytogenes*) to TiO_2 was not affected whether the target was Gram-negative or Gram-positive bacteria. Another investigation reported analogous survival ratios when testing different bacteria (*E. coli, S. aureus* and *P. aeruginosa*) with a TiO_2 coated implant (Tsuang et al., 2008).

Regarding the impact of these NPs to human health, it is considered that toxicity and exposure data on TiO_2 NPs is not well defined until now in emerging nanomaterials, which makes the risk assessment more challenging (Tsang et al., 2017). However, TiO_2 is a food additive approved by different agencies such as the FDA and can be used in foods up to a concentration of 1% (Food and Drug Agency [FDA], 2015). In this case, the development of integrative technologies to respond to the food industry needs for the cleaning and disinfection protocols would include concentrations of these NPs lower than this established percentage for food matrices.

Considering the results obtained, the potential field of application of TiO_2 NPs can be related to the elimination of pathogenic bacteria that cause food poisoning or bacteria that cause food spoilage such as *Lactobacilli* spp. in meat and brewing industries. For example, Matsunaga (1985) demonstrate that *Lactobacillus* spp. was very sensitive to a Pt-doped TiO_2 catalyst and this could be exploited as an alternative to eliminate these microorganisms. In this time, Joost et al., (2015) suggested that nanosized TiO_2 based novel surface coating materials might be very effective for the elimination of pathogenic bacteria such as *E. coli*. In addition, (Long et al., 2014) suggested that TiO_2 NPs may be used as an effective antimicrobial treatment to improve food safety of meat products. Recently, UV-TiO_2 photocatalysis has been studied as an alternative for disinfection of fruits with a delicate texture, demonstrating highly effectivity against *E. coli* K12 on blueberry fruit surface (Lee et al., 2018).

5. Conclusions

All together, our results obtained in this study indicate that both UV-exposed and non-UV-exposed TiO_2 NPs (both NM101 and NM105) show, in a dose-dependent manner, an antibacterial activity against a range of suspended Gram-negative (*S. enterica* var. Enteridis, *E. coli*) and Gram-positive bacteria (*S. aureus, B. cereus, L. casei, L. bulgaricus, L. acidophilus*and*L. lactis*) as growth curves are compromised. The data demonstrate that the optimal content of TiO_2 NPs in a suspension for the disinfection of the bacterial cultures was 100 μg mL^{-1} which was able to reduce an estimated amount of 2–3 log the bacterial population after a 24 h incubation. We suggest that further research into the comparative effects between TiO_2 embedded in surfaces and suspended TiO_2 need investigating. Additionally, the toxicological influence of TiO_2 NPs should be evaluated to determine the consequences of using these NPs in food safety. On the foundation of the results acquired and the literature survey, it can be concluded that the technology has the potential to provide, particularly in view of the development of visible light-activated catalysts, a forceful weapon to combat both, the transmission of food-borne diseases and the spoilage of food originated in, for example, meat and brewing industries by *Lactobacillus* spp.

Disclosure statement

No potential conflict of interest was reported by the authors.

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