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Effect of magnetic hyperthermia on the structure of biofilm and cellular viability of a food spoilage bacterium

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This work evaluated the effect of magnetic hyperthermia (MH) on planktonic cells and biofilms of a major food spoilage bacterium *Pseudomonas fluorescens* and its performance compared to a conventional direct heating (DH) technique. The results showed that MH had a greater and faster bactericidal effect, promoting a significant reduction in cell viability (≥3 Log CFU) in planktonic and biofilm cells, and leading to a complete eradication of planktonic cells at 55 °C (after only ~8 min). Accordingly, when comparing the same final temperatures, MH was more harmful to the integrity of cell membranes than DH, as observed in confocal laser scanning microscope images. Additionally, scanning electron microscope images revealed that exposure to MH had promoted modifications of the bacterial cell surface as well as of the structure of the biofilm. These results present the possibility of using MH out of the biomedical field as a potential disinfection method in food-related environments.

Keywords: magnetite nanoparticles; magnetic hyperthermia; biofilm; planktonic bacteria; *Pseudomonas fluorescens*

Introduction

The search for novel, safer and more effective disinfection methods remains a very relevant issue nowadays. Although extremely widespread and adopted by industry worldwide, traditional disinfection methods are chemically intensive, and have many associated disadvantages (SCENIHR 2008; SCHER/SCENIHR 2008). Moreover, it has been reported widely that most biocides are much less effective on the biofilms of bacteria than on planktonic cells (eg Gilbert et al. 2002). One of the several factors implicated in this phenomenon is the existence of different cell populations within biofilms, which present distinct metabolic states and, consequently, have different susceptibilities to biocides (Davey & O'Toole 2000; Vlamakis et al. 2008). All together, these facts highlight the great need to develop chemical-free disinfection methods with the ability to eradicate microbial cells regardless of their metabolic state.

A multitude of new possibilities to improve antimicrobial strategies have recently arisen, together with nanotechnology research. Within this vast investigation field, the synthesis and application of superparamagnetic nanoparticles in magnetic hyperthermia (MH) is of great interest. MH is based on the ability of magnetic nanoparticles (MNPs) to produce heat under an applied oscillating magnetic field through spin relaxation processes. The use of superparamagnetic nanoparticles in MH has been mostly studied for biomedical applications, specifically in cancer therapy (Fortin et al. 2007). Nevertheless, other applications of MNP-based hyperthermia have arisen, and reports have been published recently regarding the use of MH as a useful technique against the proliferation of undesirable bacterial cells in human cutaneous infections (Kim et al. 2013) and biofilms (Park et al. 2011). MH is a very promising method to inactivate planktonic bacterial cells and, thus, it is a viable alternative to traditional disinfection methods (Bañobre-López et al. 2013). Moreover, as a thermal approach, MH is able to overcome the limitation of biocides stated above by acting on microbial cells regardless of their metabolic state.

Despite the evidences of the potential of MH as an antimicrobial method, much remains unexplored. For example, fundamental studies comprising a direct comparison of its effect on planktonic and biofilm bacterial cells, or comparing the bactericidal effect of MNP-based hyperthermia with a conventional heating method are scarce. Moreover, since most reports are focused on control of clinical pathogens, there is a complete lack of information on the potential of this heating technique on food-related microorganisms, which also represent a highly important area concerning the food industry and public health. Thereby, the present work was aimed at evaluating the effect of MH on planktonic cells and biofilms of a major food spoilage bacterium *Pseudomonas fluorescens*, and comparing its efficiency with a direct heating (DH) technique.

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Materials and methods

PAA-coated Fe_3O_4 nanoparticles

Stable dispersions of magnetite nanoparticles of ≈10 nm were synthesized in aqueous solution by chemical co-precipitation of a stoichiometric mixture of Fe^{3+} ($FeCl_3 \cdot 6H_2O$, 97%) and Fe^{2+} ($FeSO_4 \cdot 7H_2O$, 99%) ions in the molar ratio Fe^{3+}/Fe^{2+} = 1.49 with ammonium hydroxide (NH_4OH, 28%). Poly-acrylic acid-coated magnetite nanoparticles, Fe_3O_4@PAA, were obtained by addition of poly-acrylic acid (PAA) immediately to the solution after precipitation of magnetite nanoparticles. PAA attaches covalently to the surface of nanoparticles providing them with a polymeric coating that improves their stability in aqueous solutions. The structural phase of magnetite was confirmed by X-ray powder diffraction to be a single phase of Fe_3O_4; no secondary phases or mixtures of other iron oxides were found as impurities. The average particle size and distribution were determined by transmission electron microscopy and found to be 10 ± 2 nm (data not shown).

Bacterial strains and culture conditions

For each assay, *P. fluorescens* ATCC 27663 (ATCC, American Type Collection Culture) was subcultured on tryptic soy agar (TSA, Merck, Germany) for 24–48 h at room temperature (20 ± 2 °C) and then grown in 30 ml of tryptic soy broth (TSB, Merck, Germany) for 18 ± 2 h at room temperature with agitation at 120 rpm. Cells were then harvested by centrifugation at 8,000 rpm at 4 °C for 5 min, and washed twice with 0.9% sterile saline. Finally, the cellular suspension was adjusted to a final concentration of ≈1×10^8 cells ml^{-1}, determined by the optical density at 640 nm. This suspension was directly used for the assays of planktonic cells, and to initiate the formation of biofilm.

Formation of biofilm

Biofilms were grown on silicone coupons (1 × 1 cm^2, 0.1 cm thick; Neves & Neves, Trofa, Portugal). All coupons were initially cleaned by immersion in a 0.2% solution of a commercial detergent (Sonazol Pril, Alverca, Portugal) followed by an immersion in ethanol. Each silicone coupon was then rinsed with ultrapure water and dried at 60 °C. Next, coupons were placed in a 24-well tissue culture plate (Orange Scientific, Braine-l'Alleud, Belgium) and UV irradiated for at least 15 min to ensure complete sterilization. Each coupon was finally submersed in 1.5 ml of TSB and 50 µl of cell suspension, and the plate was incubated for 3 days at room temperature with constant shaking at 120 rpm. The culture medium of each well was replaced daily, by removing the total volume in the well and adding 1.5 ml of fresh TSB.

MH assays

MH experiments were performed by applying an oscillating magnetic field of amplitude 100 Oe with a frequency of 873 kHz to each sample. All samples (planktonic cells and biofilms on silicone coupons) were transferred to specific glass tubes to be used in the hyperthermia equipment, to each of which the MNP solution was added. Each sample was then placed in the mid-point of a water-cooled refrigerated coil, and an optical fibre was inserted into the glass tube to register the generated temperature increase *vs* time under application of the oscillating magnetic field. A high vacuum was achieved in the chamber around the sample to avoid transfer of heat from the electronics to the sample and, thus, false temperature readings. To study cell viability as a function of temperature, magnetic heatings were performed at the same heating up rate by exposing the samples to the external magnetic field for different times. Whenever the desired temperature was reached, the glass tube containing the planktonic or biofilm sample was removed from the hyperthermia equipment and kept on ice until further analysis (determination of colony-forming units [CFU] or microscopy assays).

Concerning planktonic cells, the composition of the solution (Fe_3O_4@PAA nanoparticle and the bacterial concentrations) was selected taking into account the time scale needed to achieve the desired maximum temperature. Optimal results were obtained using a solution containing magnetite nanoparticle-based ferrofluid of 16 g l^{-1} and a bacterial cell suspension of 10^8 CFU ml^{-1} (prepared as described above) at a 1:1 ratio. For the MH assays with biofilms, after the incubation for 3 days all coupons were rinsed in 0.9% saline to remove unattached cells, and each silicone coupon was then inserted into a glass tube. In order to mimic the conditions used against planktonic cells, biofilms were submerged in a total volume of 1 ml composed by 500 µl of 0.9% saline and 500 µl of MNPs solution. Biofilms were subjected to MH as described for the planktonic cells.

For both planktonic and biofilm samples, controls corresponded to equally prepared samples that were not exposed to MH. All experiments were performed in triplicate, in at least three independent assays.

DH assays

In order to compare the antibacterial efficacy of MH with a conventional technique, DH assays were performed using a thermoblock (Thermomixer, Eppendorf Ibérica). Planktonic cells and biofilms were prepared as explained for MH assays. The same ratios of each suspension/solution were also adopted, and all samples were inserted into the thermoblock simultaneously. Temperature increase was measured directly inside the solution

by immersing a thermocouple, and the time needed to reach each preselected maximum temperature was also monitored. Whenever the desired temperature was reached, the corresponding samples were removed from the thermoblock and kept on ice until further analysis.

Controls corresponded to equally prepared samples that were not exposed to DH. All experiments were performed in triplicate, in at least three independent assays.

Enumeration of surviving cells

For the planktonic cells, the number of survivors was determined by the enumeration of CFU. In order to do this, the content of each glass tube was serially diluted in 0.9% saline and plated on TSA. Prior to the enumeration of colonies, the plates were incubated for at least 24 h at 37 °C. The same methodology was used to assess the number of viable biofilm cells, but an additional step was required in order to first remove the biofilm from the coupons. This was accomplished by transferring the coupons and all the associated suspension (1 ml) from the glass tubes to a well of a 24-well cell culture plate. The cells of biofilm were then scraped from each coupon using a sterile microbiological spatula and all the suspension was re-collected into the same sample tube. Prior to serial dilutions and platting in TSA, each resulting suspension following scraping of the biofilm was vigorously vortexed for 30 s to promote disaggregation of cells.

Confocal laser scanning microscopy

In order to assess the effect of both heating techniques on the integrity of bacterial cell membranes, planktonic cells and biofilms were stained with the Live/Dead (L/D) Backlight Kit (Molecular Probes, Invitrogen) and visualised through Confocal laser scanning microscopy (CLSM). For observing planktonic cells, 3 µl of a mixture of L/D fluorochromes (SYTO9 and Propidium Iodide [PI], prepared according to the instructions of the manufacturer and mixed in equal proportions) were added to each sample tube. After incubation for 10 min in the dark, the sample was concentrated by centrifugation at 8,000 rpm at 4 °C for 5 min. Twenty microliters of sterile water were added to the pellet, the suspension was sucked up into the pipette tip and dispensed back into the tube several times in order to disperse the biomass, and the suspension was then homogenised by vortexing. A drop of 5 µl was then transferred to a glass slide, covered with a coverslip and sealed with varnish. Confocal imaging was carried out with a 40× oil immersion objective of a Carl Zeiss inverted microscope attached to the LSM 780 confocal system (software; ZEN 2010) (Carl Zeiss Microimaging GmbH, Göttingen, Germany). Fluorescence images shown as sequential photographs were acquired at 512 × 512 pixel resolution in the same region but separately for each fluorophore and then mixed to prevent interfaces. Argon laser 488 nm was used to obtain the imaging from SYTO 9 in the green channel and diode-pumped solid-state 561 nm laser was used to obtain the imaging from PI in the red channel.

For observing biofilm, each coupon was removed from the sample tube and its surface was covered by 100 µl of L/D mixture. After incubation for 10 min in the dark, the excess of fluorochromes was removed by carefully washing each coupon twice in distilled water. The coupons were immobilised over 5 µl of mounting medium (1:1 mixture of water: glycerol) in between two coverslips. Microscopy observations were performed using the same settings as mentioned for the assays of planktonic cells. However, in this case, sequential acquisitions were taken all along the complete depth of the biofilms. The fluorescent images are shown as volume-rendered projections belonging to *Z*-stacks acquired at 0.5 or 1 µm intervals and 512 × 512 pixel resolution.

Scanning electron microscopy

Based on the most relevant results obtained concerning the bactericidal character of MH, SEM assays were performed in an attempt to get further information on the effects of this heating technique on the cell surface and on the structure of the biofilm. Therefore, planktonic cell samples were collected on a polycarbonate membrane with a pore size of 0.2 µm (Whatman Polycarbonate Nuclepore) and were then dehydrated by a immersion for 15 min in solutions with increasing concentrations of ethanol up to 100% (vol/vol). The same dehydration procedure was performed for the samples of biofilm, by directly submerging the silicone coupons in the ethanol solutions. All samples were then placed in a sealed desiccator until visualisation. Morphological analysis was performed in an Ultra-high resolution field emission gun scanning electron microscopy (FEG-SEM), NOVA 200 Nano SEM, FEI Company. Secondary electron images were performed with an acceleration voltage of 5 kV. Before morphological analyses, the samples were covered with a very thin film of Au–Pd (80–20 wt.%) of 15 nm thickness, using a high-resolution sputter coater, 208 HR Cressington Company, coupled to a MTM-20 high-resolution thickness controller.

Statistical analysis

The statistical analysis of the results of cell enumeration was performed using the statistical programme SPSS (Statistical Package for the Social Sciences). The results were compared using the non-parametric Mann–Whitney *U*-test at a 95% confidence level.

Results

Effect of DH and MH on survival of bacteria

Quantification of surviving cells after DH assays showed that eradication was not achieved for either planktonic cells or biofilms, even after exposure at 65 °C (Figure 1). Moreover, a significant reduction (3 Log) in the load of bacteria was only achieved for planktonic cells at that same maximum temperature tested. Also observed was the occurrence of a plateau concerning the viability of both planktonic and biofilm cells between 35 and 50 °C, values which were very similar to those found in the control (Figure 1).

On the other hand, exposure to MH promoted a continuous decrease in the load of bacteria as the temperature increased, in the case of both planktonic and biofilm cells (Figure 2). Even so, the effectiveness of MH was different for each bacterial life form, since a significant reduction (3 Log) in planktonic cells was achieved at 40 °C, while an equivalent reduction in biofilms required a temperature of 5 °C higher (Figure 2). Furthermore, it was observed that a temperature of 55 °C was enough to eradicate all planktonic cells, whereas biofilms could not be eliminated when exposed to MH up to 65 °C.

The temperature increase for MH was on average ~4 min faster than for DH, which consequently resulted in different heat exposure periods, shorter for the bacterial cells subjected to MNP-based hyperthermia, and longer for the bacteria inserted into the thermoblock.

Effect on cell membrane integrity and cell surface and biofilm structure

CLSM images of planktonic cells showed that both DH and MH had induced membrane damage, and revealed major differences between the two heating techniques based on the same temperature (Figure 3). While both controls hardly showed any injured/dead cells (red-stained), exposure to MH up to 40 °C inflicted membrane damage on a much larger quantity of cells (red stained) than exposure to DH up to the same temperature. Moreover, CLSM images following heating at 50 °C showed that almost all cells were injured/dead when heated by MH, while a substantial number of intact bacteria (green stained) were still observed when heated by DH (Figure 3). Focusing on samples exposed to MH, SEM images indicated that this heating technique had affected the surface of the bacteria, since controls presented a different appearance from the heated cells. Accordingly, these images showed that cells heated up to 40 °C looked thinner and rougher than the controls, while those heated up to 50 °C presented protuberances on their surface that were not found in the other tested conditions (Figure 3).

In general, CLSM images of biofilms showed similar results to those found for planktonic cells, given that both heating techniques affected the integrity of cell membranes, and MH was more deleterious than DH when based on the same temperature (Figure 4A). Additionally, these images of biofilms showed that red-stained cells were preferentially located in the upper layers, and that biofilms heated up to 65 °C were apparently thicker than the controls and those heated up to 45 °C. This finding was much more evident after MH treatment, as can be verified by analysing the *Z*-axis of the images presented. SEM images allowed comparison of the appearance of biofilms, and their structure before and after addition of MNPs and exposure to MH (Figure 4B). It was found that nanoparticles had completely covered the biofilms, such that individual cells became hard to distinguish when compared to biofilms without MNPs. Moreover, these images revealed that exposure to MH had influenced

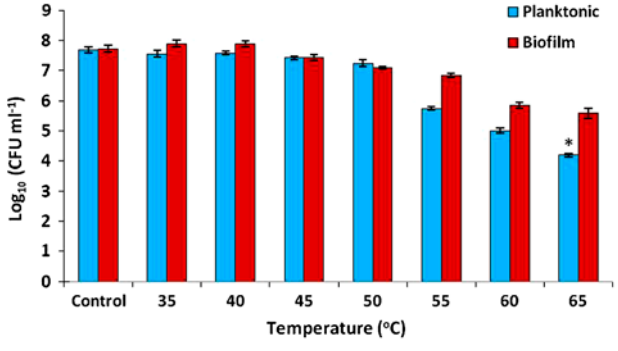

Figure 1. Survival of planktonic and biofilm cells after exposure to conventional DH in a thermoblock. *indicates values that are statistically different ($p < 0.05$) from the respective controls, and consistent with a survival reduction of at least 3 Log.

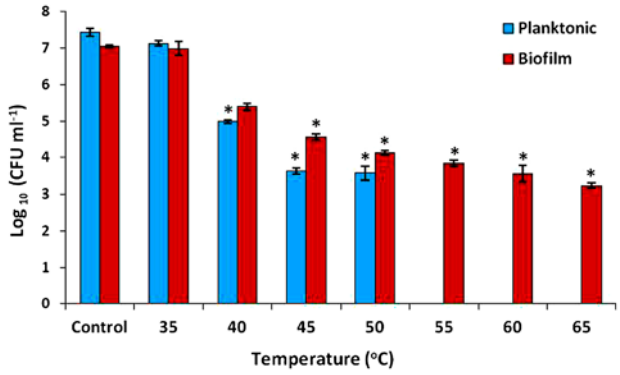

Figure 2. Survival of planktonic and biofilm cells after exposure to MH treatment. *indicates values that are statistically different ($p < 0.05$) from the respective controls, and consistent with a survival reduction of at least 3 Log.

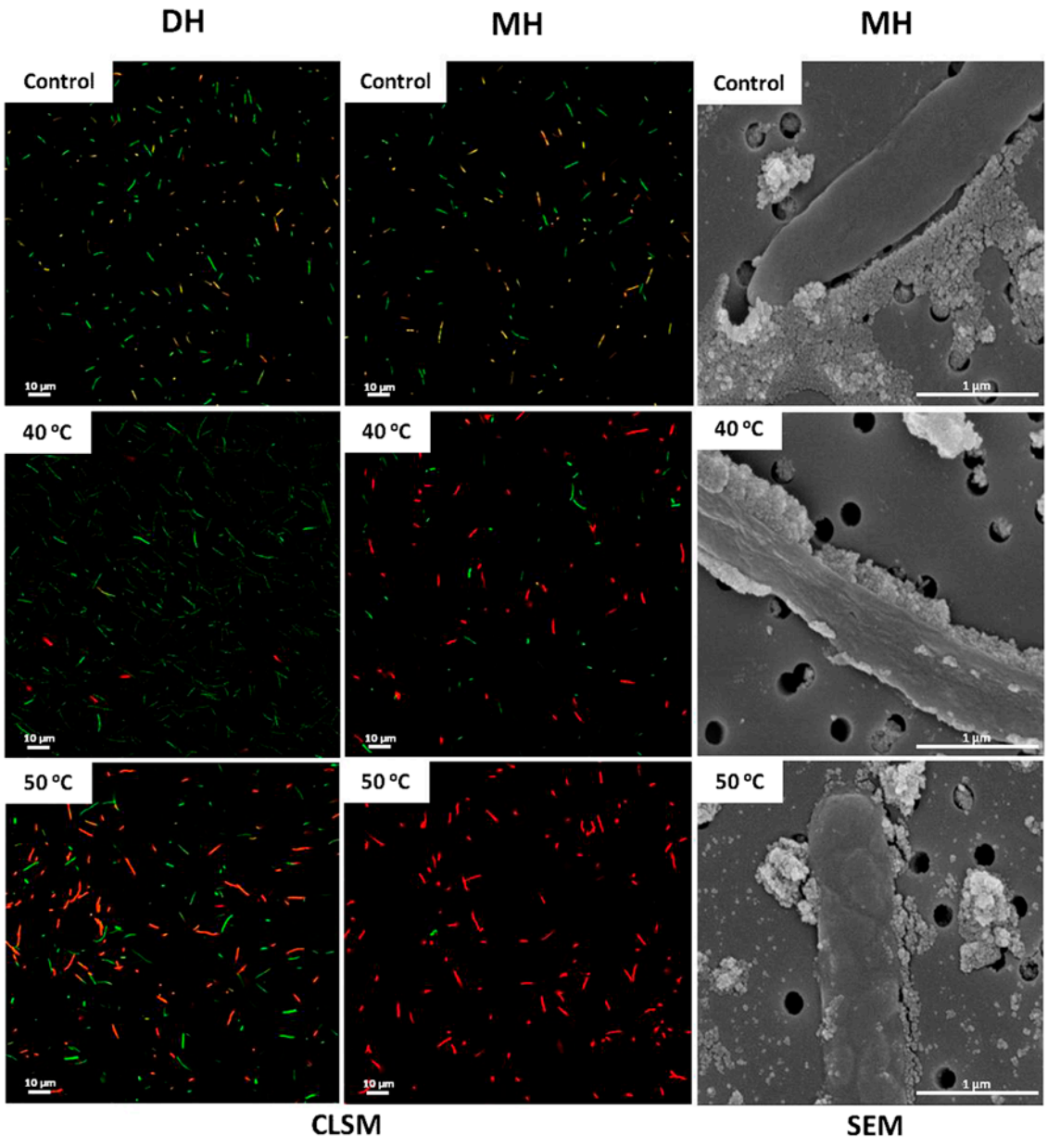

Figure 3. CLSM images of planktonic cells of *P. fluorescens* after DH and MH treatment, and SEM images after MH treatment. The controls correspond to samples of cells and MNPs that were not heated.

the structure of the biofilms and their cell surface, since biofilms that were heated up to 65 °C seemed more dispersed and presented more protuberances on the surfaces of the cells than the respective controls (Figure 4B).

Discussion

The current study confirmed that MH can inactivate an important spoilage microorganism and showed that the technique was more effective against both planktonic cells and cells within biofilms compared to conventional DH. Accordingly, while the only noteworthy effect of DH was a significant decrease in viable planktonic cells at the maximum final temperature tested (Figure 1), MNP-based hyperthermia accomplished a total eradication of these cells between 50 and 55 °C, as well as a significant decrease in surviving biofilm cells at 45 °C (Figure 2). The fact that these results were achieved over shorter heating periods compared to thermoblock heating (Table 1) further highlights the better performance of MH as a disinfection tool. These marked efficiency

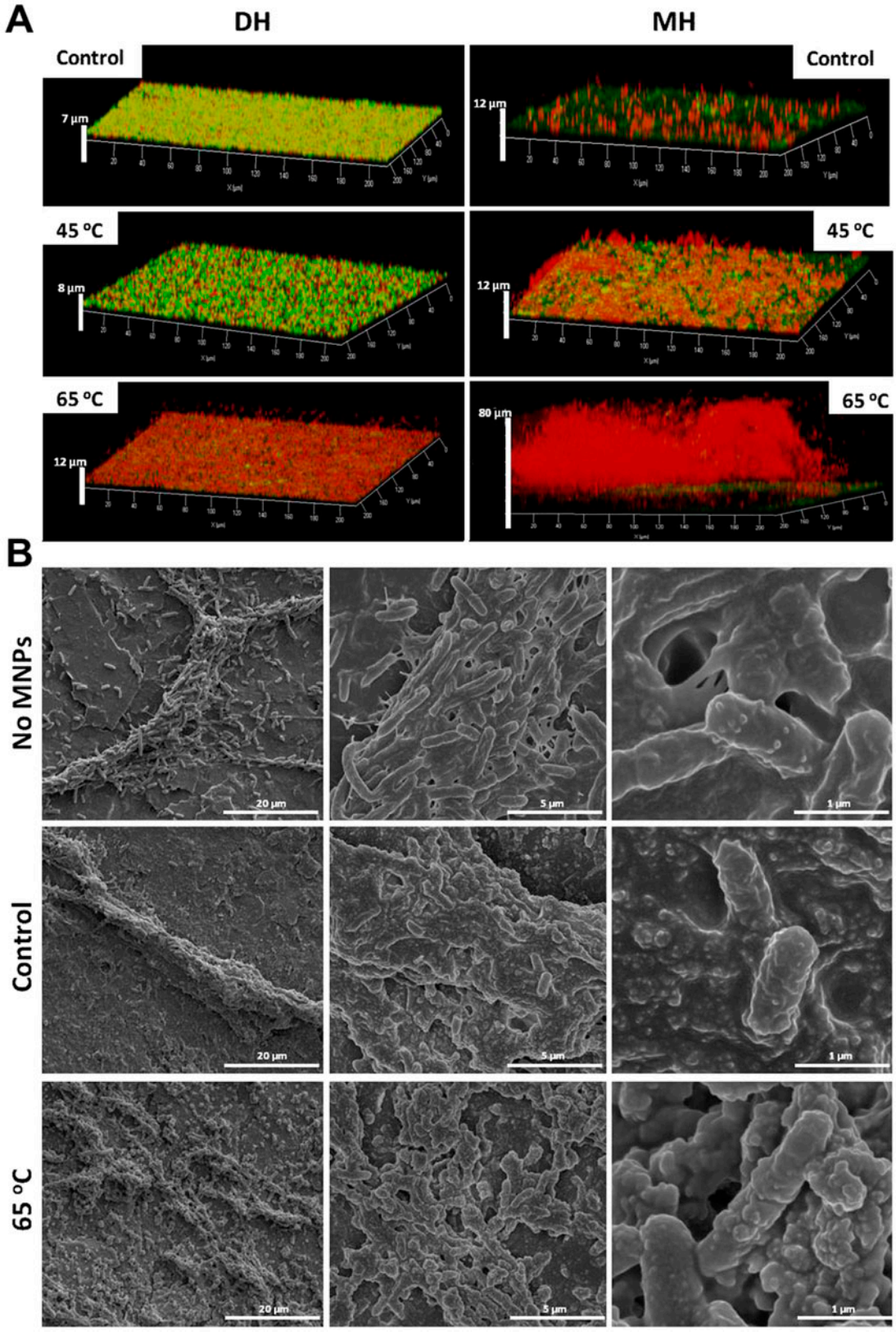

Figure 4. CLSM images of *P. fluorescens* biofilms after DH and MH treatment, and SEM images after MH treatment. The control samples correspond to cells and MNPs that were not heated. The samples designated 'No-MNPs' correspond only to biofilms, without MNPs and without heating.

differences between both heating techniques can be ascribed to the higher local temperature that MH can generate at the surface of the magnetite nanoparticles, which provokes a harmful heat transfer to the bacterial cells. Dana and Gannot (2012) have reported that alternating magnetic field-excited MNPs might produce a major temporary temperature increase within nanometers of each MNP surface, which presumably makes the local temperature reached around each nanoparticle a lot higher than the macroscopic value measured within all the suspension. In this way, even when the overall measured temperature is not life-threatening, the local heat emitted by nanoparticles can be sufficient to kill bacterial cells. In the present work, this effect must have been potentiated by the relatively high concentration of the MNP-based dispersion used (8 g l^{-1}), which led bacterial cells to be in direct physical contact with many magnetite nanoparticles, as was observed in SEM images (Figures 3 and 4). Nonetheless, MH was not able to eradicate biofilms of *P. fluorescens*, which is in agreement with previous reports that showed biofilms of bacteria to have a superior resistance to heat treatment compared to planktonic cells (Frank & Koffi 1990; Trachoo & Brooks 2005).

Based on the most relevant CFU results, CLSM and SEM assays were performed in order to gain further information on the effect of MH on cell membrane integrity, and cell surface and biofilm structure. As far as planktonic cells are concerned, a good correlation was found between CLSM images and the respective CFUs, since greater amounts of injured/dead (red-stained) cells were found as the temperature increased (Figure 3). Moreover, after exposure to temperatures superior to 50 °C, no viable planktonic cells were detected by CLSM, confirming the total eradication of these bacterial cells by MH (data not shown). Images also showed that, based on the same final temperature, more red-stained cells were found after MNP-based treatment, which corroborated the higher bactericidal effect of this heating technique towards DH. In addition, SEM images confirmed that a close interaction occurred between nanoparticles and planktonic cells, and clearly showed the emergence of modifications of the cell surface after MH heating (Figure 3). It can, then, be hypothesised that the higher bactericidal effect of MH is due to the proximity between MNPs and bacterial cells, which induces significant modifications in the cell surface by heat irradiation and greatly accelerates destruction of cells. Moreover, it is possible that the protuberances observed on cells heated up to 50 °C (Figure 3) are aggregates of MNP located somewhere in the cell wall, which would be in agreement with previous studies that showed the ability of nanoparticles to either adhere to the cell wall or get inside the bacteria (Feng et al. 2000; Morones et al. 2005; Raffi et al. 2008; Zhou et al. 2012). Although additional analysis would be required to confirm it, the existence of such agglomerates of nanoparticles would confirm the occurrence of a direct connection between MNPs and the bacterial wall, thus sustaining the suggested mechanism of action.

Table 1. Time required to reach each maximum temperature.

Temperature (°C)	Magnetic hyperthermia (min)*	DH (min)*
35	1.2	5.5
40	2.2	7.3
45	3.5	8.8
50	5.3	10.8
55	7.7	12.8
60	11.3	14.7
65	16.9	18.3

*Mean values of the performed assays.

Similar to planktonic cells, CLSM images of biofilms exposed to MH (Figure 4A) showed the emergence of greater amounts of injured/dead (red-stained) cells as the temperature increased, and also confirmed a greater bactericidal effect of this heating technique towards DH when the same final temperature was considered. It was also observed that biofilms heated up to 65 °C were thicker than all others, especially after MNP-based treatment, which may be due to the release of injured/dead cells from the biofilms. Red-stained cells seemed to be preferentially located in the upper layers of the biofilms (Figure 4A), indicating that both kinds of heating must have first affected the outer layers of these microbial communities. As far as MH is concerned, this hypothesis is in agreement with the fact that MNPs had completely covered the surface of the biofilms and established a direct connection with the biofilm cells (presumably responsible for the appearance of the large protuberances on the surface of the cells). In this way, the higher number of CLSM optical sections (larger Z-axis scale) found in biofilms heated up to 65 °C must be due to the gradual detachment of dead cells, and perhaps matrix material, from the upper layers of the biofilms.

Conclusions

MNPs were used as heating sources under an oscillating magnetic field, and allowed an efficient and fast heating of solutions containing nanoparticles and the important food spoilage microorganism *P. fluorescens*. MH revealed a better bactericidal performance compared to a conventional DH, since it led to a more efficient reduction in viable planktonic and biofilm cells at lower final solution temperatures. Even though MNP-based hyperthermia showed a more pronounced bactericidal effect against planktonic cells than on biofilms, the survival and the structure of such microbial communities were also affected by this treatment. Although further studies

are needed aimed at addressing other food-related microorganisms (in particular with foodborne pathogens), these preliminary results present the possibility of using MH out of the biomedical field as a potential method of disinfection in food-related environments, highlighting its promising character not only with respect to planktonic cells but also biofilms.

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