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The potential of atmospheric air cold plasma for control of bacterial contaminants relevant to cereal grain production

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ABSTRACT

The aim of this work was to investigate the efficacy of dielectric barrier discharge atmospheric cold plasma (DBD ACP) against bacteria associated with grains quality and safety. ACP inactivation efficacy was tested against biofilms formed by different strains of *E. coli*, *Bacillus* and *Lactobacillus* in grain model media and against *B. atrophaeus* endospores either in grain media or attached on abiotic surfaces. Effects were dependent on bacterial strain, media composition and mode of ACP exposure. ACP treatment for 5 min reduced *E. coli* spp., *B. subtilis* and *Lactobacillus* spp. biofilms by > 3 $\log_{10}$, whereas insignificant reductions were achieved for *B. atrophaeus*. ACP treatment of 5–20 min reduced *B. atrophaeus* spores in liquids by > 5 $\log_{10}$. Treatment for 30 min reduced spores on hydrophobic surface by > 6 $\log_{10}$, whereas maximum of 4.4 log reductions were achieved with spores attached to hydrophilic surface. Microscopy demonstrated that ACP caused significant damage to spores. In package ACP treatment has potential to inactivate grain contaminants in the form of biofilms, as well as spores and vegetative cells.

Industrial relevance: This study demonstrates that ACP technology is a promising tool for effective bio-decontamination which offers a wide range of possible applications including inactivation of microorganisms on cereal grains. However, due to the nature of the microbial contamination of grains and complex grain structures it may be necessary to optimise the potential for surface inactivation at several stages of grain processing and storage to enhance ACP efficacy against bacterial endospores.

1. Introduction

Due to their worldwide significance and extensive use as human food and livestock feed, optimising the quality and safety of cereal grains and cereal products remains an important food safety issue. Microbial contamination of cereal grains derives from several sources, including air, dust, water, soil, insects, birds and rodents faeces. The main factors influencing microbial contamination and persistence on cereals are environmental conditions, such as drought, rainfall, temperature and sunlight, as well as unsanitary handling, harvesting and processing equipment, and poor storage conditions (Bullerman & Bianchini, 2009).

The microflora of cereal grains will depend on the stage of processing and consists of moulds and bacteria. Bacteria that commonly occur on cereal grains include *Pseudomonadaceae*, *Micrococcaceae*, *Lactobacillaceae* and *Bacillaceae* (Laca, Mousia, Díaz, Webb, & Pandiella, 2006) and they are generally non-pathogenic, however, pathogens such as *Salmonella*, *Escherichia coli* and *Bacillus cereus* also contaminate grains (Hocking, 2003). Although appropriate storage conditions do not promote microbial growth because of the decreased moisture content of the stored grains, a common problem of mould growth predominates on cereals, showing that these conditions are not always met (ICMSF, 1980). Some bacterial pathogens and spore-forming species can survive during storage and thus contaminate more processed products. For example, lactic acid bacteria present in the raw grain may persist through the processing and spoil doughs prepared from flour and cornmeal (Bullerman & Bianchini, 2009; Justé et al., 2011).

Laca et al. (2006) studied the distribution of microorganisms within wheat grains and found that most of bacteria and moulds are concentrated on the surface of the grain in the pericarp surrounding the endosperm and the germ. However, some species can invade the inner part of the grains and cause internal infections. Andersen and Thrane (2006) reported that grain surface disinfection with sodium hypochlorite removed only 10–15% of *Alternaria* and *Bipolaris*, which indicated that the grains were contaminated beneath the pericarp.

As microorganisms associated with cereals are strongly adhered to the grains surface, debranning (removing the outer layers of the cereal grains) is a conventional mechanical method used to decrease cereal

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microbial contamination (Laca et al., 2006), however, this destroys some grains. Chemical methods for sterilization may leave residues on the cereal products that may be toxic in themselves or negatively impact on subsequent grain uses. There is a need for a rapid, low energy and cost effective technology that minimises residual chemistry on the product and does not affect grain quality. An ideal method for cereals preservation should not adversely affect their quality characteristics in order to maintain high-quality processed foods with minimal changes in nutritional and sensory properties. Potential sterilization techniques include: microwave treatment (Reddy, Raghavan, Kushalappa, & Paulitz, 1998), low-energy electrons (Röder et al., 2009), pulsed UV light (Maftei, Ramos-Villarroel, Nicolau, Mart, & Soliva-Fortuny, 2014), organic acids (Sabillon, Stratton, Rose, & Flores, 2016) and cold plasma treatment.

Dielectric barrier discharge (DBD) atmospheric cold plasma (ACP) has recently found an extensive range of applications in biocontrol due to the reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated (Ziuzina, Han, Cullen, Keener, & Bourke, 2015). Cold plasmas can offer a uniform non-thermal treatment and mitigate the presence of chemical residues or environmental pollutants remaining after the treatment (Dhayal, Lee, & Park, 2006). A further advantage of cold plasma for microbial decontamination of grains is that is proven to degrade a range of pesticides that are commonly employed for cereal crops (Sarangapani et al., 2016).

The application of a new technology for food decontamination requires the evaluation of efficacy within the actual food products or in model systems that simulate food substrate composition. Therefore, to successfully apply ACP in complex food matrices such as cereal grains, primary studies in representative food model media can be employed to determine potential interactions between species generated during plasma treatment and food components. In our study, cereal-based model media were used to provide insight for the potential application of ACP for cereal grains decontamination.

The main objective of this work was to determine the efficacy of high voltage contained DBD-ACP for control of key bacteria posing grain quality and safety concerns. Different combinations of ACP treatment parameters such as mode of plasma exposure and treatment time were investigated. Thus, the efficacy against *Bacillus* spp., *Lactobacillus* spp. and *E. coli*, in the form of 72 h biofilms formed in cereal-based model media was evaluated in addition to vegetative cells and endospores suspended in liquid cereal-based media. The control of endospores attached on different types of abiotic surfaces was also examined. Contact angle measurements were conducted to characterize changes in abiotic surface properties used for bacterial inoculation as well as the surface properties of the spores attached on these surfaces post plasma treatment. The morphological changes in spores resulting from cold plasma treatment were characterized using both optical and scanning electron microscopy.

2. Materials and methods

2.1. Bacterial strains and inocula preparation

Six bacterial strains were used in this study. *B. subtilis* ATCC 6633, *Lactobacillus plantarum* ATCC 8014, *Lactobacillus brevis* ATCC 8287 and *E. coli* NCTC 12900, *E. coli* ATCC 25922, were obtained from the microbiology stock culture of the School of Food Science and Environmental Health of the Dublin Institute of Technology. *B. atrophaeus* var. *niger*, obtained in the form of spore strips (Sportrol®/Namsa®, VWR International, Radnor, PA, USA), was resuscitated and preserved in the form of protective beads (Technical Services Consultants Ltd., UK) in-house. All stock cultures in the form of protective beads were maintained at − 70 °C. One protective bead of culture of *E. coli* and *Bacillus* spp. was streaked onto separate tryptic soy agar (TSA, Biokar Diagnostics, France) plate and incubated at 37 °C for 24 h. Cultures of *Lactobacillus* spp. were streaked onto separate de Man, Rogosa and Sharpe agar (MRS agar, Biokar Diagnostics, France) plates and incubated at 37 °C for 48 h. The plates were further maintained at 4 °C. A single isolated colony of either *E. coli* or *Bacillus* spp. was inoculated into tryptic soy broth without glucose (TSB-G, ScharlauChemie, Spain) and incubated overnight (18 h) at 37 °C; *Lactobacillus* spp. was inoculated in MRS and incubated overnight at 37 °C. The bacterial density was determined by measuring absorbance at 550 nm using the McFarland standard (BioMerieux, Marcy-l'Etoile, France) and a working inoculum corresponding to a concentration of 7.0 $\log_{10}$ CFU/ml was prepared in either standard optimal media, model wheat or barley media, maximum recovery diluent (MRD, ScharlauChemie, Spain) or sterile deionised water. The concentration of inoculum was confirmed by plating appropriate dilutions on TSA and incubation at 37 °C for 24 h for *E. coli* and *Bacillus* spp.; dilutions of *Lactobacillus* spp. were plated on MRS and incubated at 37 °C for 48 h.

2.2. Preparation of spore suspension

Endospores of *B. atrophaeus* were isolated according to the procedure described by Zhao, Krishna, Moudgil, and Koopman (2008) with minor modifications. Briefly, *B. atrophaeus* was incubated on TSA supplemented with 3.0 mg/l of manganese sulphate for 10 days at 30 °C. Spores were collected by flooding the agar plate with sterile PBS (10 ml). The resulting suspension was washed twice in PBS by centrifugation at 8720*g* for 10 min. In order to inactivate and remove vegetative cells, bacterial suspension was heat shocked for 20 min at 80 °C using a water bath, washed twice at 4 °C and finally resuspended in sterile ice cold PBS. The purity of spore suspensions was examined by using spore stain method described in Hamouda, Shih, and Baker (2002) following by optical microscopy. Spore stain method involved application of malachite green stain solution, steaming for 3 min and counterstaining with safranin for 30 s. Concentration of spores was estimated by plating aliquots of the appropriate dilutions on TSA. Spore suspension was stored at − 20 °C before use.

2.3. Preparation of wheat and barley model media

Wheat and barley model media were prepared as for the method described by Charalampopoulos, Pandiella, and Webb (2002) with minor modifications. Either wheat or barley flour (50 g) was mixed with 450 ml of tap water and centrifuged at 6000*g* for 30 min at room temperature. The supernatant was sterilized at 121 °C for 45 min. The procedures of centrifugation and sterilization were repeated four times in order to avoid the presence of sediments caused by sterilization in the final media. Prepared wheat and barley model media was stored at 4 °C before use. The pH value of wheat and barley media corresponded to 5.85 ± 0.14 and 5.25 ± 0.12, respectively.

2.4. Biofilm formation

Bacterial monoculture biofilms were formed by adding 200 µl of prepared bacterial suspension of *E. coli*, *Bacillus* spp. and *Lactobacillus* spp. in either wheat or barley model medium into the wells of the 96-well flat-bottomed microtiter plate (Sarstedt, Nümbrecht, Germany) and each microtiter plate contained three independently grown cultures of each bacterium. The plates were incubated at 37 °C for 72 h without supernatant replacement. After incubation, the supernatant containing suspended bacterial cells was removed and the wells were washed twice with 200 µl of sterile PBS, leaving only bacterial biofilms for further investigations. Biofilms were air dried for 60 min prior to ACP treatment.

2.5. Inoculation procedure

Prepared wheat and barley media, MRD and sterile deionised water were inoculated with either vegetative cells or spores of *B. atrophaeus* to a final concentration of 7.0 $\log_{10}$ CFU/ml. The resulting bacterial

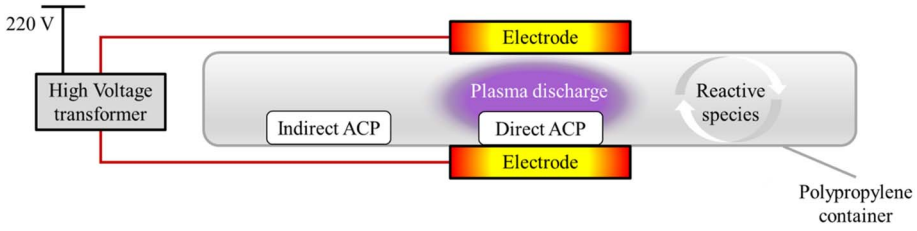

Fig. 1. Dielectric barrier discharge atmospheric cold plasma generator set-up.
(Adapted from: Ziuzina, Boehm, Patil, Cullen, & Bourke, 2015.)

suspensions were further dispensed into the 96 well plate (100 µl per well) with a total volume of each media in each 96-well plate of 1.5 ml.

For abiotic surface inoculation, either synthetic butyl rubber disks (diameter 27 mm), borosilicate glass slides (76 mm × 26 mm × 1 mm) (VWR, Ireland) or polyethylene (PE) coupons of medium porosity (75 mm × 25 mm × 1.6 mm, pore size 45–90 µm) (VWR, Fritware®) were used. The rubber disks were inoculated with *B. atrophaeus* either vegetative cell or spore suspension (100 µl per disk). The glass slides and PE coupons were inoculated only with *B. atrophaeus* spore suspension (100 µl per slide/coupon). Inoculated surfaces were left in the laminar flow cabinet until the inocula is completely dry.

For each ACP treatment two rubber disks inoculated with spore suspension and two rubber disks inoculated with vegetative cells were placed in sterile petri dish. In the case of other surfaces, either two glass slides or PE coupons were used for each ACP treatment.

2.6. Experimental design

The ACP system used (Fig. 1) in this study was a dielectric barrier discharge system with a maximum high voltage output of 120 kV at 50 Hz, described previously in Ziuzina, Han, et al. (2015). The distance between the two aluminium electrodes was equal to the height of the polypropylene container, used as a sample holder and a dielectric barrier, i.e. 2.2 cm. All samples were subjected to ACP treatment at 80 kV under atmospheric pressure. The plasma working gas was atmospheric air.

Either two microtiter plates (containing bacterial biofilms or inoculated liquids) or two petri dishes containing either rubber disks, glass slides or PE coupons were placed inside the polypropylene container. The direct and indirect plasma treatments were conducted simultaneously. For direct exposure, one microtiter plate or petri dish containing samples was placed directly between the electrodes, i.e. within the plasma discharge with 10 mm distance between the sample and top electrode. For indirect plasma treatment, the sample was placed in the corner of the container, the distance between the samples and the centre of the electrodes varied from 120 to 160 mm owing to sample distribution on the plate. Each container was sealed with a high barrier polypropylene bag (B2630; Cryovac Sealed Air Ltd., Dunkan, SC, USA) and placed between the aluminium electrodes of the transformer. The temperature increase inside the container and at the surface of the samples due to plasma treatment was < 5 °C.

Before treatment the temperature of the sample surface and the temperature inside the container was at room temperature. Bacterial biofilms were exposed to direct and indirect ACP treatment for 5 min. Bacterial vegetative cell suspensions were treated for 2, 5 and 10 min, while 5 and 20 min of treatment was used for spores. Bacterial vegetative cells and spores inoculated on butyl rubber disks were treated for 10, 20 and 30 min; bacterial spores attached on glass slides or PE coupons were treated for 30 min. Except for bacterial biofilms, which were assessed immediately after treatment, liquid and surface inoculated samples were stored at 15 °C for 2 h after treatment prior to microbiological analysis. Unless otherwise stated, all experiments were performed in duplicate and replicated at least three times. Results are expressed as logarithmic units of colony forming units per ml ($\log_{10}$ CFU/ml).

2.7. Microbiological analysis

Following ACP treatment, 200 µl of sterile PBS was added into the wells containing biofilms and plates were sonicated using a water table sonicator (Bransonic 5510E-MT, USA, Mexico) for 10 min. Suspensions from each well of corresponding culture were collected and vortexed in a sterile Eppendorf tube.

In order to determine the number of spores in biofilms for *Bacillus* spp., the suspensions were subjected to a heat-treatment (20 min at 80 °C) and cooled on ice (10 min) to inactivate the vegetative cells prior to enumeration.

To assess the effect of ACP treatment on bacterial cell suspensions in liquid media, corresponding samples were also collected into separate Eppendorf tubes.

To study the effect of ACP treatment on attached bacteria, the single rubber disk, glass slide or PE coupon was transferred into sterile stomacher bag containing 4 ml of MRD. Surfaces inoculated with bacterial endospores were heat shocked for 20 min at 80 °C and cooled in ice for 10 min. Rubber disks or PE were stomached for 5 min, while glass slides were rubbed for approximately 1 min.

Samples were serially diluted in MRD and aliquots of appropriate dilutions, e.g. 10^{-1}, 10^{-2}, and 10^{-3} (0.1 ml) and undiluted sample (0.1 and 1 ml) were plated on corresponding media (TSA for *E. coli* and *Bacillus* spp. and MRS agar for *Lactobacillus* spp.). Plates were incubated at 37 °C for either 24 h or 48 h, respectively. Results are presented as surviving bacterial population in $\log_{10}$ CFU/m1 units for biofilm and liquid studies and in $\log_{10}$ CFU/sample units for surface inoculation studies. The limit of detection for bacterial biofilm recovery was 2.0 $\log_{10}$ CFU/sample (plated volume 0.01 ml of either diluted or undiluted samples), for liquid samples was 1 CFU/ml (plated volume 1 ml of undiluted sample) and surface inoculation studies was 4 CFU/sample (plated volume 1 ml of undiluted sample, i.e. suspension of cells that was detached form solid surface by transferring the samples into 4 ml of diluent in the stomacher bag).

2.8. Optical microscopy

In a separate set of experiments, optical microscopy technique was used in order to visualise spore structural changes caused by either direct or indirect ACP treatment for 30 min and stored post treatment for 2 h at 15 °C. *B. atrophaeus* spores were inoculated on glass slides as described in Section 2.5. Either untreated or ACP treated glass slides were subjected to spore staining as described in Section 2.2. Spores were observed using light microscope (Optika, Italy) under 100 × objectives (oil immersion lens, 1000 × total magnification). Images were acquired with digital camera and analyzed using computer software (Optika Vision Pro).

2.9. Contact angle measurement

The static contact angles of deionised water (Sigma Aldrich, Ireland) were measured by sessile drop technique using contact angle meter (Theta Lite Optical Tensiometer, Biolin Scientific, UK). The surface hydrophobicity of uninoculated untreated glass slides, PE coupons and untreated glass slides was examined in addition to *B. atrophaeus* endospores inoculated on glass slides which were treated directly/

indirectly for 30 min with 2 h of post treatment storage at 15 °C was examined. Analysis was performed immediately after deposition of water droplet on the studied surfaces; the image was recorded at 15 frames per second for 10 s. Images were analyzed using the OneAttension software.

2.10. Scanning electron microscopy (SEM)

The effect of ACP treatment and subsequent storage for 2 h at 15 °C on *B. atrophaeus* endospores inoculated on PE coupons was analyzed by SEM. The spores attached on PE coupons were fixed in ice-cold 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) (SCB) for 2 h. The spores were washed with the same buffer three times and fixed in 1% osmium tetroxide for 2 h at 4 °C. After 2 h of fixation, the spores were washed with SCB followed by three washes with distilled water. The samples were dehydrated using increasing concentrations of ethanol (30%, 50%, 70%, 80%, 95% and 99.5%) following by dehydration with series of 33%, 50%, 66%, and 100% of hexamethyldisilazane (Sigma Aldrich, Ireland). In order to prevent surface charging by the electron beam, the samples were sputter-coated with gold particles using Emitech K575X Sputter Coating Unit resulting in a coating of 10 nm after 30 s. The samples were examined visually using a FEI Quanta 3D FEG Dual Beam SEM (FEI Ltd., Hillsboro, USA) at 5 kV.

2.11. Statistical analysis

Statistical analysis was performed using IBM SPSS statistics 21 Software (SPSS Inc., Chicago, USA). The surviving bacterial biofilm populations, bacteria suspended in liquids and inoculated on abiotic surfaces following ACP treatment were subjected to Mixed Design Analysis Of Variance (ANOVA). Means of ACP treated and untreated controls were compared according to the method of Fisher's Least Significant Difference-LSD at the 0.05 level.

3. Results

Results of this work demonstrated that intrinsic parameters such as bacterial type, strain, mode of existence, physiological state and the substrate properties significantly affected decontamination efficacy of ACP treatment.

3.1. Effect of ACP treatment on bacterial biofilms formed in cereal model media

The effect of the type of cereal grain model media, bacterial strain and mode of exposure on ACP treatment efficacy against bacterial biofilms was investigated (Fig. 2). Both direct and indirect ACP treatment for 5 min significantly reduced 72 h biofilms regardless of the media used for biofilm formation, for all the strains tested except *B. atrophaeus*. Bacterial growth within 24 h, assessed by OD measurement as well as colony count assay, was comparable in both wheat and barley model media (data not shown), suggesting that the cereal media selected supported microbial growth equally. Higher inactivation effect was achieved for direct mode of exposure as compared to indirect, reducing the number of bacterial cells in biofilms to undetectable levels or slightly above the detection limit (2.0 $\log_{10}$ CFU/ml) for all of the species examined except for *B. atrophaeus*. In wheat model medium, direct ACP treatment reductions achieved were up to 3.27, 3.67, 3.44, 2.38 and 3.03 $\log_{10}$ CFU/ml for *E. coli* NCTC 12900, *E. coli* ATCC 25920, *B. subtilis*, *L. plantarum* and *L. brevis*, respectively. In barley model medium, populations of the same strains were reduced up to 1.72, 4.01, 3.38, 1.99 and 3.07 $\log_{10}$ CFU/ml, respectively. In general, indirect treatment was the least efficient mode against bacterial biofilms, however, biofilms formed by *E. coli* ATCC 25920 and *L. brevis* were reduced to undetectable levels in both cereal-based media.

ACP treatment was strongly affected by phenotype and strain of bacteria studied. The highest resistance to treatment was observed for biofilms formed by *B. atrophaeus*, regardless of the media and mode of plasma exposure, with no significant reductions achieved for this strain. The percentage of spores in the total counts for *B. atrophaeus* biofilms before the ACP treatment constituted 92.9% ± 3.1 and 87.7% ± 4.0 in wheat and barley model medium, respectively, and for *B. subtilis* constituted 77.2% ± 12.4 and 84.7% ± 4.4 in wheat and barley model medium, respectively.

3.2. Effect of ACP on B. atrophaeus vegetative cells and spores suspended in liquid media or inoculated on the surface of rubber

The antibacterial efficacy of ACP treatment against bacterial vegetative cells and spores suspended in liquid media is presented in Figs. 3 and 4, respectively. Reduction achieved for *B. atrophaeus* vegetative cells for direct plasma treatment in wheat model media was 3.3, 3.4 and 5.1 $\log_{10}$ cycles after 2, 5 and 10 min of treatment, respectively. Similar reduction levels of 3.2, 3.3 and 5.2 $\log_{10}$ cycles were observed for barley model media. The highest inactivation levels were obtained for *B. atrophaeus* in distilled water. Overall, within 10 min of direct treatment vegetative cells of *B. atrophaeus* were reduced to undetectable levels in all media tested. In case of indirect mode of exposure, recorded plasma treatment efficacy was lower - only treatment for 10 min in MRD and water resulted in values below detection limits. *B. atrophaeus* spores showed higher resistance to plasma treatment. After 5 min of treatment *B. atrophaeus* spores were significantly reduced by 3.5 $\log_{10}$ cycles in MRD and 6.2 $\log_{10}$ cycles in distilled water for direct treatment and 2.8 $\log_{10}$ cycles in distilled water for indirect treatment. Extending treatment time up to 20 min was necessary to significantly reduce the number of remaining spores in wheat and barley model media. Inactivation to undetectable levels was only achieved for spores in MRD, treated directly with plasma and in distilled water in both modes of exposure tested after 20 min.

Reductions of *B. atrophaeus* vegetative cells and spores inoculated on the surface of synthetic butyl rubber discs are shown in Fig. 5. Populations of vegetative cells and spores of the control samples recovered after 2 h of storage at 15 °C were 4.4 and 6.4 $\log_{10}$ CFU/sample, respectively. After 10 min of ACP treatment, the number of vegetative cells was reduced by 2.8 and 2.4 $\log_{10}$ CFU/sample for direct and indirect mode of exposure, respectively. Vegetative cells were completely inactivated after 20 min, regardless of the mode of exposure. Higher resistance was observed for *B. atrophaeus* spores - 10 min of treatment resulted in reduction of 1.6 $\log_{10}$ CFU/sample spores regardless of the mode of exposure. Extending the treatment time from 20 min to 30 min did not lead to further reductions of spores in case of both direct and indirect plasma exposure (up to 3.1 $\log_{10}$ CFU/sample).

3.3. Effect of ACP on B. atrophaeus endospores inoculated on glass and porous polyethylene (PE) surfaces

Fig. 6 shows the effect of 30 min of direct and indirect ACP treatment with subsequent storage for 2 h at 15 °C on viability of *B. atrophaeus* spores inoculated on either glass slides or porous PE coupons. Regardless of the mode of exposure, higher reductions were achieved when spores were inoculated on porous PE surface, where the number of viable spores was reduced to undetectable levels (~6 $\log_{10}$ reduction). In contrast, spores inoculated on glass slides were reduced by 3.5 and 4.4 $\log_{10}$ cycles after exposure to direct and indirect treatment, respectively.

The hydrophobic property of glass and PE material used for inoculation was determined by measuring water contact angles, which corresponded to 7.71° ± 1.11 and 124.37° ± 9.05 (Table 1), indicating hydrophilic and hydrophobic characteristics of tested surfaces, respectively (Fig. 7).

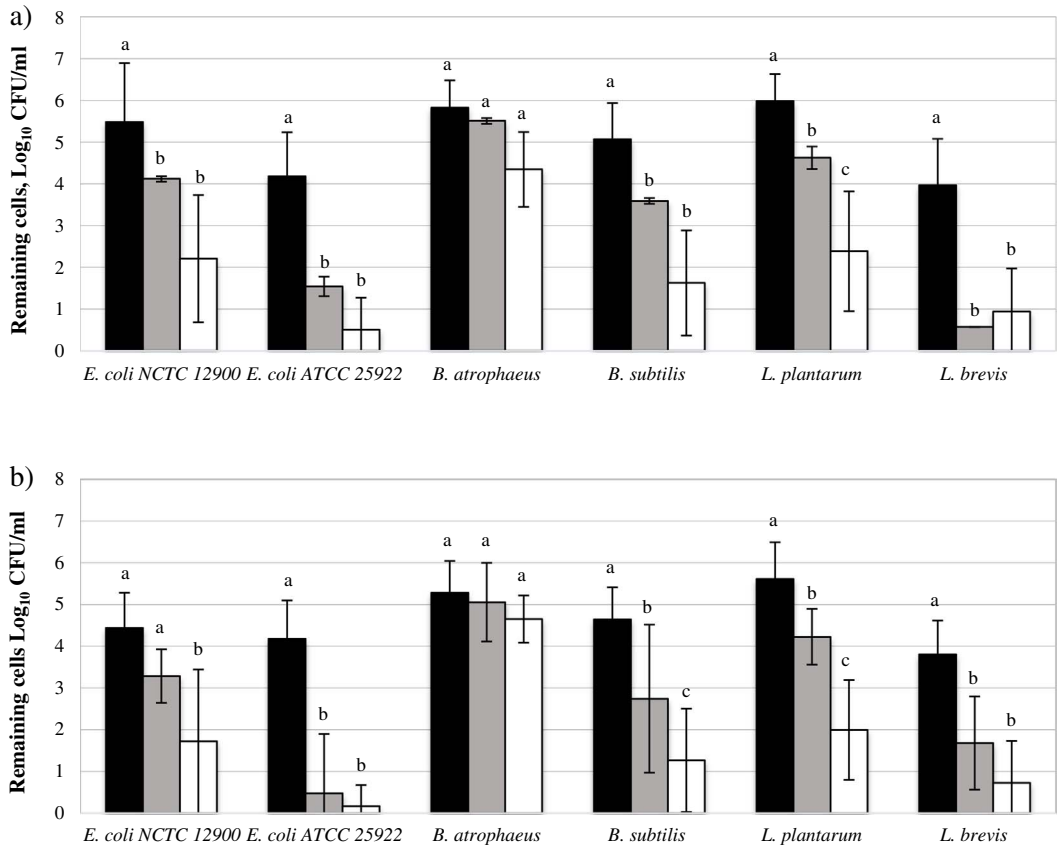

Fig. 2. Effect of 5 min of ACP treatment on 72 h bacterial biofilms formed in (a) wheat model medium and (b) barley model medium; untreated control (▮), direct (▯) and indirect (▯) treatment. Limit of detection 2.0 $\log_{10}$ CFU/ml. Experiments were performed in duplicate and replicated 3 times (n = 6). Different letters indicate significant difference between bacterial populations of the control and ACP treated samples within each type of bacteria ($p < 0.05$). Vertical bars represent standard deviation.

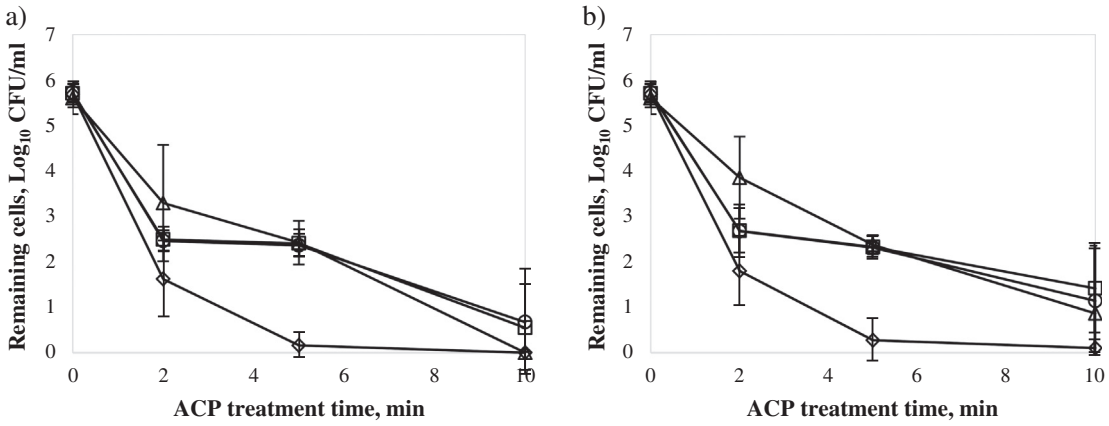

Fig. 3. Effect of direct (a) and indirect (b) ACP treatment and 2 h post treatment storage at 15 °C on *B. atrophaeus* vegetative cells suspended in wheat model medium (○), barley model medium (□), maximum recovery diluent (MRD) (Δ) and distilled water (◊). Limit of detection 1 CFU/ml. Experiments were duplicated and conducted 3 times (n = 6). Vertical bars represent standard deviation.

3.4. The effect of ACP on structural characteristics of *B. atrophaeus* spores

The effect of 30 min of direct and indirect ACP treatment with subsequent storage for 2 h at 15 °C on structural characteristics of *B. atrophaeus* spores inoculated on glass slides and porous PE surface was determined using optical microscopy (Fig. 8, top panel), water contact angles (Fig. 8, bottom panel) and SEM analysis (Fig. 9), respectively. Microscopic analysis of spores subjected to direct treatment demonstrated that most of the spores that retained structures similar to the spores of the untreated control sample (Fig. 8a) were stained green due to application of malachite green staining solution (Fig. 8b). However, spores subjected to indirect treatment were not able to retain the green dye and were counterstained in the following step involving safranin dye, thereby appearing red on the slide (Fig. 8c). Contact angle measurement also indicated the physical changes of spores following treatment (Fig. 8, bottom panel). If the water contact angle formed on the surface of spores before treatment was 7.05° ± 1.99, this value increased to 27.15° ± 3.88 and 39.52° ± 3.04 after direct and indirect treatment, respectively (Table 1). SEM analysis confirmed physical damage of bacterial endospores inoculated on porous PE surface following treatment (Fig. 9). Unlike the micrographs of the untreated control samples (Fig. 9a), spore debris was present on the images of

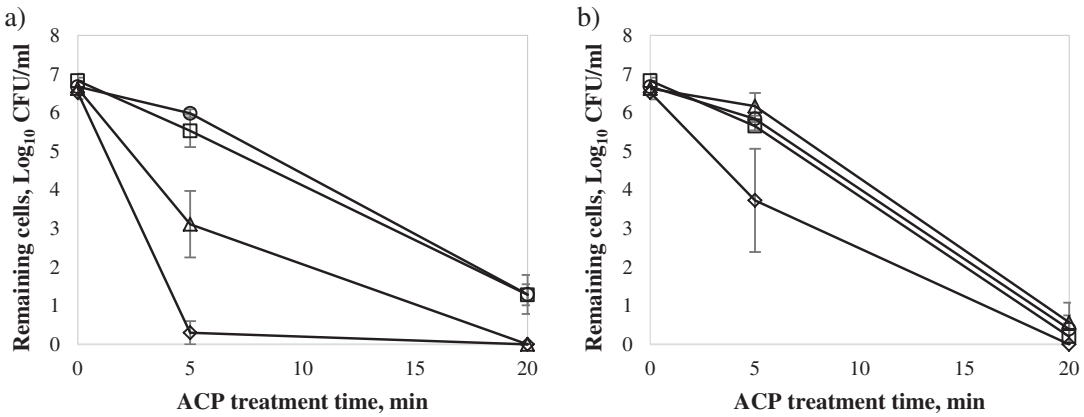

Fig. 4. Effect of direct (a) and indirect (b) ACP treatment and 2 h post treatment storage at 15 °C on *B. atrophaeus* endospores suspended in wheat model medium (○), barley model medium (□), maximum recovery diluent (MRD) (Δ) and distilled water (◊). Limit of detection 1 CFU/ml. Experiments were duplicated and conducted 3 times (n = 6). Vertical bars represent standard deviation.

either directly or indirectly treated samples. This indicates that ACP largely affected physical characteristics of spores through rupture and disintegration of spore structures (Fig. 9b or c, respectively).

4. Discussion

In summary, results of this study show that various factors including bacterial type, different physiological states of the cells and the phenotype, as well as the substrate properties play an important role in the decontamination efficacy of plasma treatment. The process parameters studied, such as treatment time and mode of plasma exposure, were also critical to achieve successful decontamination processing. Model conditions were employed to extend the findings to a range of grains.

Many human pathogens as well as spoilage microorganisms grow predominantly as biofilms, which are known to tolerate various environmental stresses and action of applied antimicrobial agents, thereby presenting a major challenge in food industry (Giaouris et al., 2013; Sharma et al., 2014). In this work high voltage DBD ACP treatment was challenged with 72 h biofilms of *E. coli*, *Lactobacillus* spp. and *Bacillus* spp. formed in 96 well plate model. Moreover, cereal-based liquid model media was used for biofilm formation in order to address the influence of substrate chemical composition in relation to grain industry on antimicrobial efficacy of treatment. Regardless of the type of the media selected for biofilm formation, plasma treatment for 5 min significantly reduced bacterial biofilms for most of the strains tested. However, the highest resistance to the treatment was observed with *B. atrophaeus* biofilm. A possible reason for the achievement of relatively low reduction levels for *B. atrophaeus* biofilms could be the induced cell sporulation during biofilm formation. Sporulation levels within biofilms depend on the incubation conditions, such as growth medium, incubation time and indeed the bacterial strain (Ramirez-Peralta et al., 2012). In this work it was experimentally determined by plate count method that the 72 h biofilms of *B. atrophaeus* developed in 96 well plates constituted on average of 90% of spores using either wheat or barley model media for biofilm formation, which is in accordance with previous reports. For example, Wijman, De Leeuw, Moezelaar, Zwietering, and Abee (2007) noted that percentages of spores in the total counts of 48 h *B. cereus* biofilm cells constituted up to 90%. Faille et al. (2014) also showed that *Bacillus* spp. biofilms may contain up to 90% spores after 48 h of incubation. Based on these results *B. atrophaeus* was a target microorganism for further investigations. Moreover, because previous studies reported that extending the post treatment storage time enhances bactericidal action of ACP treatment through the retention of plasma generated species in a sealed package (Ziuzina, Patil, Cullen, Keener, & Bourke, 2013), further experiments applied a 2 h post treatment containment at 15 °C. Thus, the influence of bacterial physiological state of *B. atrophaeus* on decontamination efficacy of ACP treatment was evident in the case of treatment of inoculated liquid media as well as for inoculated dry surfaces, where vegetative cells were readily inactivated as compared with inactivation of spores in general. These results are consistent with previous reports (Muranyi, Wunderlich, & Langowski, 2010). Moreover, interactive effects of media composition and mode of plasma exposure were also noted. For instance, *B. atrophaeus* vegetative cells suspended in more complex media, either cereal-based broth or MRD, were inactivated by an average of 5 $\log_{10}$ CFU/ml after 10 min of treatment despite the mode of action applied, whereas similar inactivation levels were achieved after 20 min of treatment in the case of *B. atrophaeus* spores. This

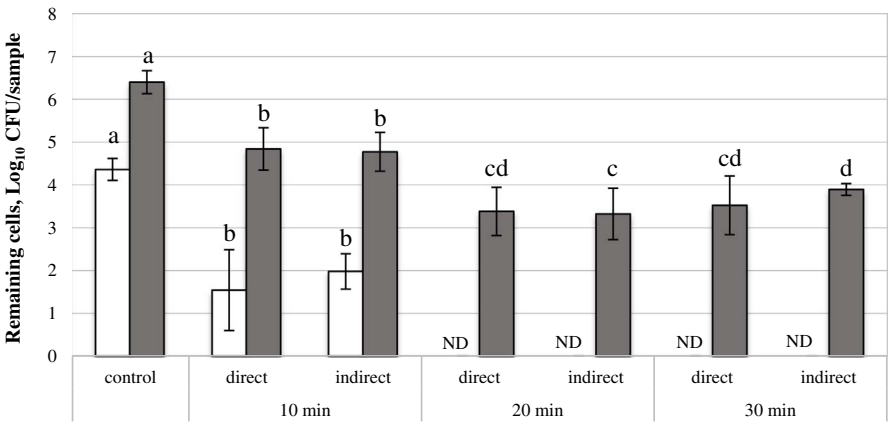

Fig. 5. Effect of direct/indirect ACP treatment and 2 h post treatment storage at 15 °C on *B. atrophaeus* vegetative cells (▯) and spores (▯) on synthetic butyl rubber disks. ND – not detected. Limit of detection 4 CFU/sample. Experiments were duplicated and conducted 4 times (n = 8). Different letters indicate significant difference between bacterial populations of the control and ACP treated samples within either vegetative or spore group ($p < 0.05$). Vertical bars represent standard deviation.

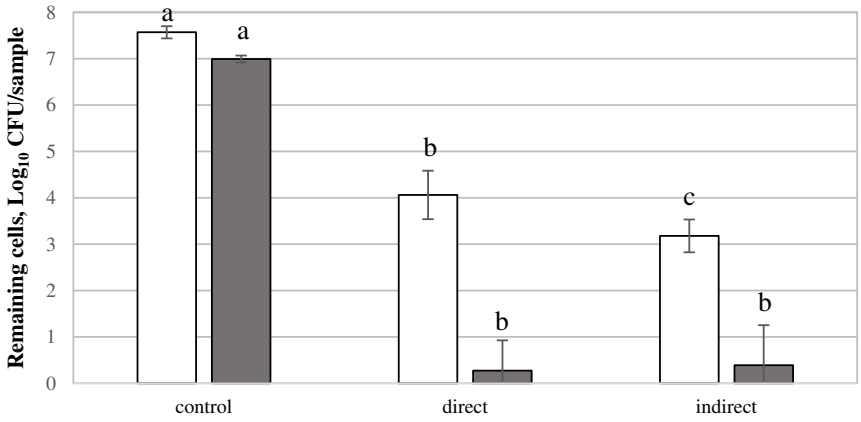

Fig. 6. Effect of direct and indirect ACP treatment of 30 min and 2 h post treatment storage at 15 °C on *B. atrophaeus* spores inoculated on glass slides (▯) and PE coupons (▮). Limit of detection 4 CFU/sample. Experiments were duplicated and conducted 4 times (n = 8). Different letters indicate significant difference between bacterial populations of the control and ACP treated samples within either Glass slide or PE group ($p < 0.05$). Vertical bars represent standard deviation.

Table 1
Water contact angle measurements on glass slide, porous polyethylene coupons and either untreated or ACP treated and stored for 2 h at 15 °C *B. atrophaeus* spores inoculated on glass slides (– untreated; ± standard deviation). Experiments were performed in duplicate and replicated 3 times.

Surface	ACP treatment	Contact angle °
Glass slide	–	7.71 ± 1.11
PE coupon	–	124.37 ± 9.05
B. atrophaeus	–	7.05 ± 1.99
B. atrophaeus	Direct	27.15 ± 3.88
B. atrophaeus	Indirect	39.52 ± 3.04

demonstrates that the complexity of nutritional environment of wheat and barley model media provided a protective effect against the action of plasma generated reactive species. Inactivation of *B. atrophaeus* vegetative cells and endospores was achieved in distilled water where 5 min of direct treatment led to undetectable levels, thus a complex medium minimises the effect of bacterial physiological state on decontamination efficacy of treatment.

Reduced inactivation efficacy of treatment was noted when either vegetative cells or spores of *Bacillus* were inoculated on rubber surface by comparison with liquids. Similarly, the effect of bacterial substratum on the effects of ACP was recorded in Ziuzina, Boehm, et al. (2015). Vegetative cells were not detected when an extended treatment time of 20 min was applied, whereas neither 20 nor 30 min of treatment reduced bacterial spores by > 3 $\log_{10}$ CFU/sample. A non-linear tailing effect for inactivation of spores could be due to the resistant nature of spores to treatment and/or limitation in the antimicrobial effects with the treatment time tested. Shintani, Sakudo, Burke, and McDonnell (2010) reports that tailing could be due to spore clumping. However, further research is needed to determine the reasons for such a response and what the optimal treatment parameters are that will be sporicidal taking factors, such as contamination levels, type of microorganisms, presence of multiculture species, cells/spores clumping, the presence of biofilms, as well as food physical and chemical characteristics to demonstrate the real potential of plasma in food processing into account. Previously we observed that relative humidity (RH) facilitated *B. atrophaeus* spores inactivation by atmospheric cold plasma (Patil et al., 2014), where a combination of 1 min of treatment and 70% RH resulted in > 6 $\log_{10}$ reduction cycles. However, in some stages of cereal grain processing, increase in RH is not recommended, therefore other aspects must be taken into consideration to enhance the plasma treatment efficacy for spore inactivation on grain surfaces and in the processing environment.

In general, bacterial endospores are characterized by increased resistance to environmental stresses, including heat, radiation and common industrial processes such as pasteurization. Besides the increased resistance, other spore characteristics that make them critical for food industry include elevated adhesiveness, ability to survive for extended time and germinate after sub-lethal stress conditions (Heyndrickx, 2011; Sella, Vandenberghe, & Soccol, 2014). Increased resistance of *Bacillus* endospores as compared to *Bacillus* vegetative cells is mainly associated with their structural complexity and chemical composition. *Bacillus* endospores are surrounded by a multilayered coat predominantly composed of proteins (up to 80% of total spore protein) and a minor fraction of carbohydrate components (6%). This complex spore outer structure provides initial barrier to large molecules and has been identified as a resistance mechanism against many chemicals, particularly oxidizing agents, such as hydrogen peroxide, ozone, chlorine dioxide and hypochlorite (Sella et al., 2014). Moreover, as compared to other *Bacillus* spp., *B. atrophaeus* spores are known to be particularly resistant to ozone and plasma treatment. Under dry gaseous ozone exposure, Mahfoudh et al. (2010) showed that the inactivation rate of *B. atrophaeus* in comparison to spores of *B. pumilus* and *G. stearothermophilus* spores, was the slowest. Similarly, Klämpfl et al. (2012) observed the differences between the strains after 1 min of plasma treatment on agar plates: *B. atrophaeus* spores were reduced by approximately 2.5 $\log_{10}$ cycles, while the same treatment time was sufficient for reducing the number of *B. subtilis* spores by 4.0 $\log_{10}$ cycles. Therefore, we examined ACP inactivation efficacy against *B. atrophaeus* spores associated with different types of contact surfaces. For this, PE and glass material with different physical properties were selected as model systems representing both industrially relevant equipment and food surfaces with different surface topography and physical characteristics. Glass surface possesses nonporous hydrophilic features and PE has porous hydrophobic surface characteristics representing cracked grain structures and environmental

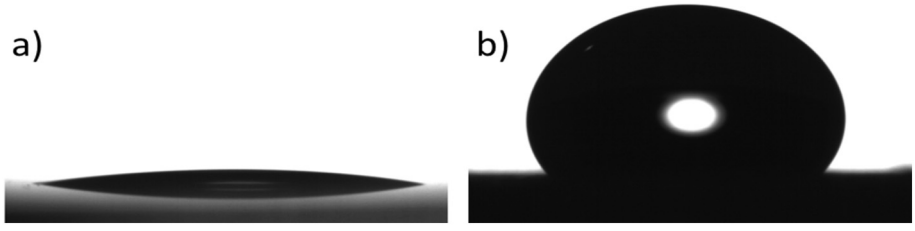

Fig. 7. Images of water droplet deposited on a) glass slide and b) polyethylene coupon.

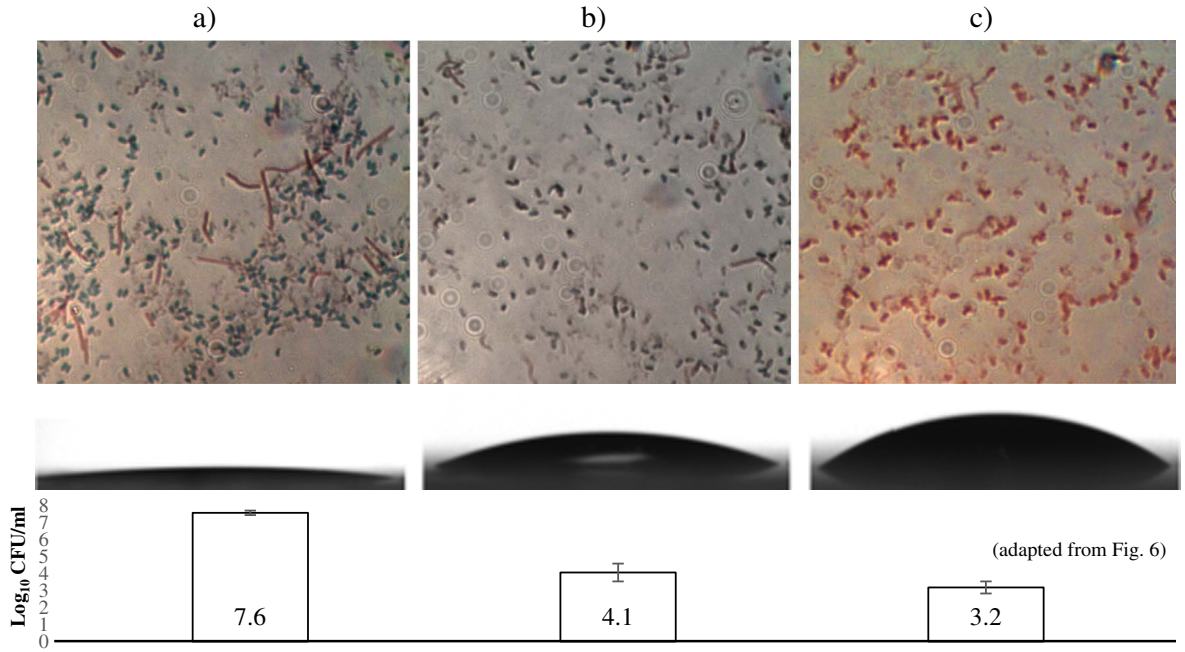

Fig. 8. Top panel – optical microscope images (100 × oil objective, total magnification 1000 ×); middle panel – images of water droplet; bottom panel – population of spores for *B. atrophaeus* inoculated on glass slides a) untreated control, b) treated directly and c) indirectly for 30 min and stored post treatment for 2 h at 15 °C.

contamination. It is widely accepted that surface roughness is one of the important factors involved in bacterial attachment and plays an important role in decontamination. Microorganisms readily attach to macroscopic crevices, pits and convolutions on inanimate and produce surface where they are protected from unfavorable environmental stresses and these features significantly alter plasma decontamination efficacy by protecting microbial cells from the action of ACP generated reactive species (Fernández, Noriega, & Thompson, 2013; Hsu et al., 2013; Jahid, Han, Zhang, & Ha, 2015; Ziuzina, Boehm, et al., 2015). For instance, the efficacy of atmospheric pressure DBD generated pulsed plasma treatment tested for inactivation of *Geobacillus stearothermophilus* was influenced by the substrate shape and surface properties. Butscher, Zimmermann, Schuppler, & Rudolf von Rohr (2016) achieved ~5 $\log_{10}$ reductions within 10 min on polypropylene granules, but found that spore inactivation on wheat grains was less efficient and reached a maximum reduction of approximately 3 $\log_{10}$ units after 60 min of treatment. In the current work, SEM micrographs of the inoculated PE coupons confirmed aggregation of spores within the macropores of the PE material (Fig. 9, right panel), indicating the preferential places for bacterial spore attachment. However, despite apparent irregularities and pores on the surface of PE, which could prevent direct contact of plasma generated species with spores, higher inactivation levels were obtained with porous PE substrate than with nonporous glass surface (> 3 $\log_{10}$ difference between inactivation levels). Hence, consideration of the surface porosity alone is not sufficient for the purpose of plasma processing optimization with this system. The substrate hydrophobicity was another important intrinsic parameter that played an important role in antimicrobial efficiency of ACP treatment, where enhanced plasma inactivation was associated with highly hydrophobic PE material with up to 7 $\log_{10}$ reduction achieved. In general, hydrophobic materials do not support bacterial cell adhesion at the initial stages (Myszka & Czaczyk, 2011). However, the strength of bacterial spores attachment on the surface of abiotic materials with different physical properties was not investigated in this work. Standard inoculation procedure provided only ~0.5 $\log_{10}$ CFU/sample difference in the initial populations of spores attached on either studied surface before treatment. Moreover, SEM micrographs of untreated surface indicated the presence of high levels of spores after inoculation (Fig. 9a, right panel). A similar concentration of spores in the form of both intact and completely disintegrated spore structures were found following direct and indirect ACP treatment (Fig. 9b, c, right panel). Therefore, intrinsic factors, such as mechanism and strength of bacterial attachment, require further research to characterize plasma-spore interactions, which in turn will help in establishing efficient target-oriented microbiological control. It is also important to note, that the presence of a mixture of intact spores and spore debris as a result of relatively long treatment time (30 min) demonstrates the range of inactivation mechanisms, where multiple chemical reactions and high concentrations of reactive oxygen and nitrogen species in conjunction with plasma physical disruptive ability play a role in plasma interaction with spores. Similarly, Veen et al. (2015) reported distinct morphological changes including etching effects and the appearance of rough *B. cereus* spore surfaces observed by SEM after 20 min of treatment with nitrogen plasma. In the current study the occurrence of intact spores after ACP treatment as observed on SEM and low numbers of recovered spores on TSA plates indicate that ACP reactive species could possibly penetrate the spores causing major disruption at DNA levels. Muranyi et al. (2010) reported that plasma treatment could cause significant damage to the bacterial endospores genome changing the fingerprint after a treatment time of 5 s as demonstrated by the appearance of additional PCR products.

Furthermore, in the current study with longer treatment duration, different inactivation patterns were observed for different modes of plasma exposure examined. In the case of hydrophilic glass surface where spores exhibited higher resistance to plasma, higher reduction levels were achieved with indirect treatment (4.4 $\log_{10}$) when compared to the reductions obtained after direct treatment (3.5 $\log_{10}$) (Fig. 8b, c, bottom panel). The observed reductions were correlated with physical changes of spores observed through optical microscopy and contact angle measurements to determine hydrophobic characteristics of *B. atrophaeus* spores before and after treatment. The principle of spore staining with malachite green, is that the dye is forced into the spore by mild heating, which cannot be washed with water from spore structures once the dye is inside the spore. This results in green appearance of spores under the light microscope. As demonstrated on Fig. 8(b, top panel), spores that were exposed to direct treatment had

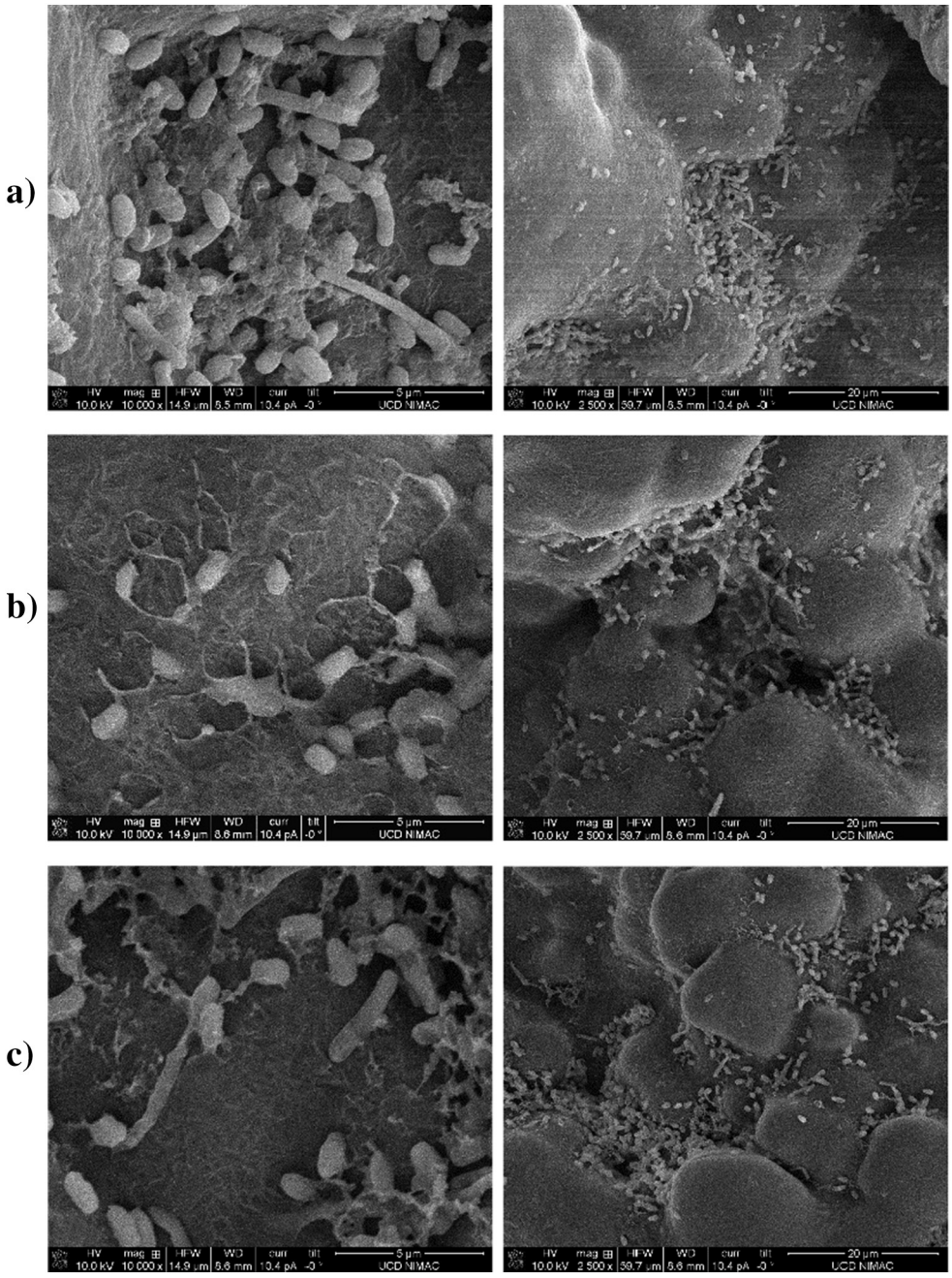

Fig. 9. SEM images of *B. atrophaeus* spores inoculated on polyethylene coupons: a) untreated control, b) treated directly and c) indirectly for 30 min and stored post treatment for 2 h at 15 °C. Magnification: left panel 10,000 × and right panel 2500 ×.

the ability to retain the dye. In contrast, the green dye was easily washed off from the spores after exposure to indirect treatment and the majority of spores were stained red as a result of the application counterstain safranin (Fig. 8c, top panel). These results indicated major alterations to the spore structures caused by plasma treatment, which could affect the staining process, but also lead to lower spore recovery levels as demonstrated by plate count assay. The loss of the spores ability to retain the stain could be associated with changes in the outer pericortex region of the spores (Kozuka & Tochikubo, 1991), however, to determine whether these changes influence the recovery of spores on TSA, a more detailed analysis of spore components responsible for the stain retention as well as for viability of spores is required. In addition, ACP treatment caused significant changes in water contact angles for bacterial spores changing hydrophilic surface of the untreated spores to more hydrophobic, which could be probably due to the local damage of proteins constituting the spore coat. Again, higher water contact angle of the spores was recorded after exposure to indirect treatment than after direct exposure. The protective mechanisms of spores in association with a range of surface and structural characteristics require elucidation for successful application of ACP technology to grain processing. Another important aspect to consider for successful application of ACP to real food systems concerns retention of physicochemical and physiological properties of grains and seeds as maximal microbial control treatment conditions may not always be associated with significant improvements in the quality. Therefore, further studies involving grain models are warranted in order to draw conclusions about possible inactivation effects and grain quality characteristics retention.

5. Conclusion

Overall, a significant reduction of microorganisms associated with cereal grains after ACP treatment was achieved. Results demonstrated that intrinsic parameters such as bacterial type, strain, mode of existence, substrate composition and surface hydrophobicity, as well as

process parameters such as treatment time and mode of plasma exposure, significantly impact decontamination efficacy. Plasma treatment of 5 min was required to effectively reduce bacterial biofilms formed in grain model media for all the strains tested except for *B. atrophaeus*. Reduced inactivation efficacy of ACP treatment both in liquid cereal-based media and inoculated on rubber surface was observed for bacterial endospores as compared to vegetative cells. Enhanced plasma inactivation of *B. atrophaeus* endospores was associated with higher hydrophobicity of inoculated abiotic surface: higher reduction was achieved for hydrophobic PE coupons as compared to hydrophilic glass slides, providing insights in terms of the effect of substrate surface properties to optimise ACP processes for decontamination of cereal grains.

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