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Improving Microbiological Safety and Quality Characteristics of Wheat and Barley by High Voltage Atmospheric Cold Plasma Closed Processing

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Improving microbiological safety and quality characteristics of wheat and barley by high voltage atmospheric cold plasma closed processing

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Abstract

Contamination of cereal grains as a key global food resource with insects or microorganisms is a persistent concern for the grain industry due to irreversible damage to quality and safety characteristics and economic losses. Atmospheric cold plasma presents an alternative to conventional grain decontamination methods owing to the high antimicrobial potential of reactive species generated during the treatment, but effects against product specific microflora are required to understand how to optimally develop this approach for grains. This work investigated the influence of ACP processing parameters for both cereal grain decontamination and grain quality as important criteria for grain or seed use. A high voltage (HV) (80 kV) dielectric barrier discharge (DBD) closed system was used to assess the potential for control of native microflora and pathogenic bacterial and fungal challenge microorganisms, in tandem with effects on grain functional properties. Response surface modelling of experimental data probed the key factors in relation to microbial control and seed germination promotion.The maximal reductions of barley background microbiota were 2.4 and 2.1 $\log_{10}$ CFU/g and of wheat - 1.5 and 2.5 $\log_{10}$ CFU/g for bacteria and fungi, respectively, which required direct treatment for 20 min followed by a 24 h sealed post-treatment retention time. In the case of challenge organisms inoculated on barley grains, the highest resistance was observed for *Bacillus atrophaeus* endospores, which, regardless of retention time, were maximally reduced by 2.4 $\log_{10}$ CFU/g after 20 min of direct treatment. The efficacy of the plasma treatment against selected microorganisms decreased in the following order: *E. coli* > *P. verrucosum* (spores) > *B. atrophaeus* (vegetative cells) > *B. atrophaeus* (endospores). The challenge microorganisms were more susceptible to ACP treatment than naturally present background microbiota. No major effect of short term plasma treatment on the retention of quality parameters was observed. Germination percentage measured after 7 days cultivation was similar for samples treated for up to 5 minutes, but this

was decreased after 20 min of direct treatment. Overall, ACP proved effective for cereal grain decontamination, but it is noted that the diverse native micro-flora may pose greater resistance to the closed, surface decontamination approach than the individual fungal or bacterial challenges, which warrants investigation of grain microbiome responses to ACP.

Key words: plasma, wheat, barley, germination, *E. coli*, *B. atrophaeus*, *P. verrucosum* spores.

1. Introduction

Microbial contamination of cereal grains derives from several sources, such as air, dust, water, soil, insects, birds and animal faeces, and can occur during crop growth, harvesting, post-harvest drying and storage (Laca, Mousia, Díaz, Webb, & Pandiella, 2006; Magan, Sanchis, & Aldred, 2003). Bacteria commonly found on cereals belong to the families *Pseudomonadaceae*, *Micrococcaceae*, *Lactobacillaceae* and *Bacillaceae* (Laca et al., 2006). Grains can also be contaminated by pathogenic bacteria, including *Salmonella, Escherichia coli* and *Bacillus* spp., as well as a range of moulds - associated with grains in the field ('field fungi'), e.g. *Alternaria* and *Fusarium* species, and 'storage fungi', e.g. *Aspergillus* and *Penicillium*, that are known to form mycotoxins in stored cereals (Hocking, 2003). These secondary metabolites are toxic and harmful in varying degrees, posing a serious health risk for both human and animals. Moreover, mycotoxins are resistant to current food-processing methods and may contaminate finished processed foods (Bullerman & Bianchini, 2009). Mitigating the presence of mycotoxin producing microorganisms or mycotoxins themselves on cereal grains is an important issue for addressing sustainability and nutritional impact of diet in regions where nutrition is principally reliant on grains.

Conventional techniques for controlling spoilage of cereal grains include thermal and chemical sterilization methods, however, these methods can negatively affect the quality and functional properties of cereals and cereal products; moreover, complete elimination of mycotoxins from food product by processing can rarely be achieved (Karlovsky et al., 2016; Oghbaei, Prakash, & Yildiz, 2016). Hence, the demand for novel methods that overcome these limitations. Alternative methods for cereal grain decontamination include irradiation (a full review can be found in Lorenz & Miller (1975)), ozone treatment (Allen, Wu, & Doan, 2003; Tiwari et al., 2010; Wu, Doan, & CUenca, 2006), microwave (MW) treatment (Reddy et al.,1998; Vadivambal et al., 2007), pulsed ultraviolet (UV) light treatment

(Maftei et al., 2013) and atmospheric cold plasma (ACP) treatment. ACP is generated at atmospheric pressure and consists of UV photons, neutral or excited atoms and molecules, negative and positive ions, free radicals and free electrons. The plasma treatment efficacy for microbial inactivation is under investigation in a number of food systems. Strong antimicrobial effects of ACP are due to chemical and bioactive radicals generated during electrical discharge, e.g. reactive oxygen species (ROS) and reactive nitrogen species (RNS), which cause damage to proteins and nucleic acids, as well as lesions in cellular membranes (Laroussi & Leipold, 2004; Scholtz, Pazlarova, Souskova, Khun, & Julak, 2015). As a non-thermal process, ACP causes minimal or no thermal damage to the food product treated (Niemira, 2012).

Application of atmospheric and low pressure cold plasma for decontamination of cereal grains has been reported both for inactivation of indigenous microbial communities of grains (Brasoveanu, Nemtanu, Surdu-Bob, Karaca, & Erper, 2015; Filatova et al., 2013; Kordas, Pusz, Czapka, & Kacprzyk, 2015; Selcuk, Oksuz, & Basaran, 2008; Zahoranova et al., 2015) and for artificially contaminated cereal grains and seeds (Butscher, Zimmermann, Schuppler, & Rudolf von Rohr, 2016; Butscher, Loon, et al., 2016; Schnabel et al., 2012; Zahoranova et al., 2015). There are a number of processing stages involved with key grain commodities, therefore the flexibility of ACP in terms of delivery in either contained or open, or dry or liquid forms provides a rich resource to develop risk appropriate cold plasma based interventions. This study examines the effects of closed delivery of ACP against a range of microbiological challenges, presenting an alternative approach to the surface only or fluidised bed approaches previously reported. Our previous studies demonstrated that an extended exposure of microorganisms to antimicrobial reactive species, achieved through generation of HV DBD plasma in a contained environment, enhanced antimicrobial effects of plasma treatment (Misra, Keener, Bourke, Mosnier, & Cullen, 2014; D. Ziuzina, Patil, Cullen,

Keener, & Bourke, 2014; Dana Ziuzina, Han, Cullen, & Bourke, 2015). Thus, the aim of this work was to study the potential of HVDBD ACP treatment generated in contained environment to improve both the microbiological safety whilst maintaining grain quality. The influence of plasma critical control parameters on antimicrobial efficacy of ACP against background microbiota of wheat and barley and against challenge pathogens; *E. coli*, *B. atrophaeus* (vegetative cells and endospores) and *P. verrucosum* (spores) inoculated on barley was studied. To investigate the mechanism responsible for the potential enhancement of early wheat growth, the influence of plasma treatment on wheat quality parameters, such as germination rate and surface hydrophobicity, was also examined. To our knowledge, the presented research is the first one directly comparing ACP inactivation of background microflora of cereals versus a range of artificially inoculated microorganisms including both bacteria and fungi.

2. Materials and methods

2.1. Bacterial and fungal strains

Two bacterial and one fungal strain were used in this study. *E. coli* NCTC 12900 was 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. *P. verrucosum* DSM 12639 was obtained from Leibniz Institute, German collection of microorganisms and cell cultures (DSMZ) and resuscitated according to manufacturer instructions. All strains were maintained at -70°C in the form of protective beads.

2.2. Inocula preparation

One protective bead of *E. coli* and *B. atrophaeus* was streaked onto separate tryptic soy agar (TSA, Biokar Diagnostics, France) plate and incubated at 37°C for 24 h. One protective bead of *P. verrucosum* was placed in the centre of potato dextrose agar (PDA, Biokar Diagnostics, France) plate which was further incubated at 30°C for 5 – 7 days or until an adequate growth occurred. The plates of the selected microorganisms were further maintained at 4°C.

A single isolated colony of either *E. coli* or *B. atrophaeus* was inoculated into tryptic soy broth without glucose (TSB-G, Biokar) and incubated overnight (18 h) at 37°C. Cells were harvested by centrifugation at 10,000 rpm for 10 min, the pellet was washed twice with sterile phosphate buffered saline (PBS, Sigma Aldrich) and re-suspended in PBS. 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 the average 8.0 $\log_{10}$ CFU/ml was prepared in PBS. The concentration of inoculum for *E. coli* and *B. atrophaeus* was confirmed by plating appropriate dilutions on TSA and incubation at 37°C for 24 h.

B. atrophaeus endospore suspension was prepared according to the procedure described by Zhao et al. (2008) with minor modifications. Briefly, *B. atrophaeus* was incubated at 30°C for 10 days on TSA supplemented with 3.0 mg/L of manganese sulphate. Spores were collected by flooding the agar plate with sterile PBS (10 ml). The obtained suspension was washed two times in PBS by centrifugation at 10,000 rpm for 10 min. To ensure inactivation of vegetative cells, bacterial suspension was heat-shocked for 20 min at 80°C using a water bath, washed two times in PBS at 4°C and finally re-suspended in sterile ice-cold PBS. The purity of spore suspensions was examined by using spore staining method (Hamouda et al., 2002) and optical microscopy. Spores were stained using malachite green stain solution, steaming for 3 min and counterstaining with safranin for 30 seconds. The concentration of spores was adjusted to 8-9 $\log_{10}$ CFU/ml and enumerated after plating aliquots of the appropriate dilutions on TSA and incubation at 37°C for 24 h. Spore suspension was stored at 4°C before use.

For fungal spore suspension preparation, PDA was inoculated with *P. verrucosum* by transferring small quantities of conidia with inoculation needle onto a three separate locations of the plate. Inoculated PDA was incubated at 30°C until colony size expanded with good sporulation obtained. The spores were harvested by flooding the agar surface with 10 ml of sterile PBS containing Tween 20 (1%) and scraping the spores from mycelia with a sterile spreader. The suspension was washed twice in sterile PBS, adjusted to 8-9 $\log_{10}$ CFU/ml and stored at 4°C until use. The concentration of spores was confirmed by plating appropriate dilutions on PDA and incubating at 30°C for 5-7 days.

2.3. Preparation of cereal grains

Organic wheat (origin: Ireland) and barley (origin: United Kingdom) grains were purchased from a local retailer. Barley grains were sterilized by autoclaving at 121°C for 15 min and used to study the effect of ACP on inactivation of challenge pathogens. In order to assess ACP treatment efficacy for the reduction of background microbiota, unsterilized wheat and barley grains were used. Germination studies and contact angle measurements were performed using unsterilized wheat grains.

In order to confirm the absence of background microbiota, sterilized grains (1 g) were aseptically transferred into a sterile Stomacher bag (Seward LTD, UK) containing 5 ml of maximum recovery diluent (MRD, ScharlauChemie, Spain) and stomached for 10 min. The resulting suspension (1 ml) was plated on either TSA or PDA with further incubation of plates for 48 h or 5 days, respectively. In addition, grains were enriched in either TSB or MRS broth for 72 h. These tests confirmed complete inactivation of grains microbiota as there was no growth observed on either TSA or PDA or after grain enrichment. The moisture content of barley used for inoculation and plasma inactivation experiments corresponded to 11.9% calculated on wet weight basis.

2.4. Barley grains inoculation procedure

For inoculation, sterilized barley grains were aseptically transferred into sterile Petri dishes (10 g per dish) and sprayed with a suspension of selected microorganism (0.5 ml). The grains were vigorously mixed by shaking for approximately 30 s to ensure even distribution of microorganisms. Inoculated grains were dried for 1 h at room temperature in a laminar flow

214 safety cabinet to allow the attachment of microorganisms on the grain surface. To evaluate
215 the effect of ACP treatment on grain background microbiota, unsterilized and uninoculated
216 wheat and barley grains were used.

217

218 **2.5. Experimental design**

219 The ACP system used in this study was a high voltage (HV) dielectric barrier discharge
220 (DBD) system with a maximum voltage output in the range 0-120 kV_{RMS} at 50 Hz, described
221 previously by Ziuzina et al., (2013) (Fig. 1). The distance between the two round aluminium
222 electrodes (diameter 15 mm) was equal to the height of the polypropylene container (310 x
223 230 x 22 mm) used to provide a contained environment during and post treatment. All
224 samples were subjected to ACP treatment at 80 kV under atmospheric pressure and
225 atmospheric air as a working gas. Sample holders containing either inoculated or un-
226 inoculated wheat or barley grains (2 g) were placed inside the polypropylene container. For
227 direct mode of plasma exposure, the sample was placed directly between the electrodes or
228 within the plasma discharge with 10 mm distance between the sample and top electrode,
229 whereas for indirect plasma treatment the sample was placed outside plasma discharge with
230 23 cm distance between the centres of the sample holder and the top electrode. Each
231 container was sealed with a high barrier polypropylene bag (B2630; Cryovac Sealed Air Ltd,
232 Dunkan, SC, USA) and placed between the aluminium electrodes of the transformer for
233 treatment. Inoculated barley or wheat samples were exposed to 80 kV_{RMS} ACP treatment for
234 5 or 20 min and analysed immediately or stored unopened for either 2 or 24 h at 15°C post
235 treatment to monitor effects of retention time. The same treatment durations and retention
236 times were assessed for effects against grain background microbiota using unsterilized and
237 uninoculated wheat and barley samples. To investigate the effect of ACP on grain functional

properties and physical quality parameters, unsterilized wheat grains were exposed to 5 min of treatment followed by 0, 2 or 24 h of post treatment retention time at 15°C. Untreated controls were stored under identical conditions as treated controls. 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 g ($\log_{10}$ CFU/g).

2.6. Microbiological analysis

ACP-treated and the corresponding control grains (1 g) were transferred into separate sterile Stomacher bags containing 10 ml of sterile MRD and stomached for 10 min. Samples inoculated with bacterial endospores were heat-shocked for 20 min at 80°C and cooled in ice for 10 min to inactivate the vegetative cells prior to microanalysis. Samples were serially diluted in MRD. Aliquots (0.1 ml and 1 ml) of appropriate dilutions were plated on corresponding media: TSA was used for grain indigenous mesophilic bacteria and for artificially inoculated *E. coli* and *B. atrophaeus*, while PDA was used for grain indigenous yeasts and moulds and for artificially inoculated *P. verrucosum*, which were incubated at 37°C for 24-48 h or at 30°C for 5-7 days, respectively.

2.7. Germination studies

ACP treated and control wheat grains (10 grains) were transferred into sterile Petri dishes containing Whatman paper moistened with 2 ml of sterile deionised water. Samples were incubated at room temperature in the dark for 7 days. In order to maintain sufficient moisture for germination, 1 ml and 2 ml of sterile deionised water were added during the experiment on days 1 and 3, respectively. The germination percentage was recorded on day 2, 3 and 7.

Germination was considered complete once the radicle protruded ~2mm in length, i.e. half of the grain length (Ling et al., 2014). Estimated values were germination percentage: $G\% = (n \times 100\%) / Nt$, and mean germination time: $MGT = \sum (n \times d) / N$, where n is number of grains germinated on each day, Nt – *total number of grains*, N is total number of grains germinated at the termination of the experiment and d is the number of days from the beginning of the test (Ellis & Roberts, 1981). All germination experiments were performed at least three times.

2.8. Contact angle measurement

The surface hydrophobicity of untreated and ACP-treated wheat grains was examined. The apparent contact angles of deionised water, ethylene glycol and diiodomethane (Sigma Aldrich, Ireland) were measured by sessile drop technique using contact angle meter (Theta Lite Optical Tensiometer, Biolin Scientific, UK). Analysis was performed immediately after deposition of a single droplet of one of three tested liquids on the grain surface. The images were recorded at 15 frames per second for 10 seconds and analyzed using the OneAttension software.

2.9. Statistical analysis

Statistical analysis was performed using IBM SPSS statistics 21 Software (SPSS Inc., Chicago, USA). The surviving populations of bacteria and fungi, contact angle values and values of germination parameters following ACP treatment were subjected to 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.

2.10. Response surface modelling

The datasets of the microbial inactivation and germination studies were modelled using polynomial response surface models. These mathematical models were fitted to the datasets using the *lsqnonlin* routine of the Optimization Toolbox of Matlab version 7.14 (The Mathworks Inc.). The mathematical model for the effect of treatment time (*tt*) and retention time (*rt*) on the inactivation of microorganisms on barley and wheat was formulized as: $\delta\,(tt, rt) = a_1 * tt + a_2 * rt + a_2 * tt * rt$, where δ is the decimal reduction of the microbial population as calculated by comparing the microbial quantities with the average of the controls. The parameters a_1 to a_4 are regression coefficients. The response surface model for the effect of treatment time, retention time and incubation time (*it*) on the germination rate γ was described as: $\gamma\,(tt,rt,it) = a_1 + a_2 * tt + a_3 * rt + a_4 * it + a_5 * tt * rt + a_6 * tt * it + a_7 * rt * it$.

The 95% confidence bounds on the model parameter estimates were determined using the *nlparci* Matlab function. A more detailed description of the calculation of confidence bounds on the parameter estimates is available in Walter & Pronzato (1997).

3. Results

3.1. Inactivation of barley background microbiota

The efficacy of ACP treatment against microorganisms naturally occurring on barley is presented in Fig. 2. Initial counts of mesophilic bacteria and fungi of barley grains were 4.6 and 4.4 $\log_{10}$ CFU/g, respectively, which were unaffected by the sample retention for either 0, 2 or 24 h at 15°C. Combining either 5 or 20 min of treatment with no post-treatment retention time or 5 min of treatment with 2 h of retention time resulted in no significant differences between microbial populations of treated samples and untreated controls. Significant reductions in barley background microbiota were only achieved after longer treatment for 20 min in combination with 2 h retention time or after either 5 or 20 min treatment time in combination with an extended retention time of 24 h ($p<0.05$), with no statistical difference between surviving populations of the treated groups recorded. After 20 min of treatment and a subsequent retention time of 24 h, microbial levels were reduced by 2.4 and 1.7 $\log_{10}$ CFU/g for bacteria treated directly (Fig. 2a) and indirectly (Fig. 2b), and 2.1 and 1.5 $\log_{10}$ CFU/g for fungi treated directly (Fig. 2c) and indirectly (Fig. 2d), respectively. Although extending the retention time of grains in contact with longer lived reactive species from 2 h to 24 h did not significantly enhance the inactivation effect of 20 min of treatment, it did improve the efficacy of shorter treatment (5 min) against both mesophilic bacteria and yeasts/moulds, with reductions by 1.9 and 1.7 $\log_{10}$ CFU/g achieved for bacteria treated directly (Fig. 2a) and indirectly (Fig. 2b), and 1.8 and 1.2 $\log_{10}$ CFU/g for fungi treated directly (Fig. 2c) and indirectly (Fig. 2d), respectively. In terms of the effects of mode of plasma exposure, direct treatment always resulted in slightly higher reductions of both mesophilic bacteria and fungi on grains. The modelling results with respect to the inactivation of naturally present fungi and mesophilic bacteria in barley are presented in Table 2. The models are visualised in Fig. 3e and Fig. 3f for the direct treatment and Fig. 4e and Fig. 4f for

the indirect treatment, respectively for fungi and mesophilic bacteria. The relatively low values of all coefficients confirm that these microorganisms have a high resistance against the applied ACP treatments.

3.2. Inactivation of wheat background microbiota

The reductions of wheat background microbiota by ACP treatment are shown in Fig. 5. Initial microbial populations of wheat grains were 4.0 and 3.8 CFU/g for the aerobic mesophilic counts and yeasts and moulds, respectively, which were slightly lower than that of barley. Microbial populations of the controls deceased significantly, by ~0.6 $\log_{10}$ CFU/g when wheat grains were stored for 24 h at 15°C ($p<0.05$). As compared to the inactivation of barley background microbiota, no clear trends with regards to the influence of treatment critical controls parameters, such as mode of plasma exposure, treatment time and post-treatment retention time were noted. Regardless of the mode of exposure, populations of bacteria and fungi were significantly reduced after either 5 or 20 min of treatment only for samples with a 24 h retention time at 15°C when microbial levels were compared with corresponding untreated and stored controls ($p<0.05$). Generally, higher inactivation levels were achieved for direct treatment as compared to indirect for most of the samples. Again, an enhanced antimicrobial effect of 20 min of treatment was noted only in combination with 2 h of retention. Overall, the maximal reductions were achieved after 20 min of direct plasma treatment - 1.5 $\log_{10}$ CFU/g for bacteria (when assessed without or combined with 2 h of post-treatment retention time – Fig. 5a) and 2.5 $\log_{10}$ CFU/g for fungi (with post-treatment retention time extended to 24 h – Fig. 5c). Maximal reductions due to indirect treatment constituted 1.2 $\log_{10}$ CFU/g for bacteria (Fig. 5b) and 1.7 $\log_{10}$ CFU/g for fungi (Fig. 5d) when 20 min of treatment was combined with either 2 or 24 h of post-treatment retention time, respectively. The polynomial coefficients for the effect of treatment time on the decimal reduction of fungi and mesophilic bacteria on wheat (Table 2) are generally much higher than

352 those of barley, except for the indirect treatment of mesophilic bacteria. In case of the direct
353 treatment of mesophilic bacteria, the overall effect of is reduced due to a negative interaction
354 between treatment time and retention time. These results are also illustrated in Fig. 3g, Fig.
355 3h, Fig. 4g and Fig. 4h. As such, the ACP treatment appears to be more effective against the
356 background microbiota on wheat than on barley.

357

358 **3.3. Inactivation of microorganisms inoculated on barley grains**

359 Reductions of microorganisms inoculated on barley grains are presented in Fig. 6.
360 The average initial populations of *E. coli*, *B. atrophaeus* vegetative cells, *B. atrophaeus*
361 endospores and *P. verrucosum* spores were 4.8, 4.8, 7.4 and 6.8 $\log_{10}$ CFU/g, respectively.

362 It should be noted that after 24 h retention, the levels of control *E. coli* and *P. verrucosum*
363 decreased by 1.2 and 0.4 $\log_{10}$ CFU/g, respectively, and increased by 0.4 $\log_{10}$ CFU/g for
364 *B. atrophaeus* endospores. The levels of attached vegetative cells of *B. atrophaeus* were not
365 affected by 24 h of retention time.

366 ACP treatment efficacy was strongly affected by the type of microorganism studied.
367 The highest resistance was observed for *B. atrophaeus* endospores, which were reduced by
368 a maximum of 2.4 $\log_{10}$ CFU/g after direct and 1.3 $\log_{10}$ CFU/g after indirect plasma
369 treatment for 20 min combined with 2 h of post-treatment retention time (Fig. 6c). For other
370 microorganisms tested, 20 min of treatment with 24 h retention time was the most efficient
371 combination. The levels of *E. coli* (Fig. 6a), *B. atrophaeus* vegetative cells (Fig. 6b) and
372 *P. verrucosum* spores (Fig. 6d) were reduced significantly ($p<0.05$) by 3.5 (undetectable
373 levels), 3.2 and 3.6 $\log_{10}$ CFU/g after direct and by 3.3, 2.7 and 2.7 $\log_{10}$ CFU/g after indirect
374 treatment, respectively. The efficacy of the plasma treatment of barley grains inoculated with
375 microorganisms decreased in the following order: *E. coli* > *P. verrucosum* (spores) >

B. atrophaeus (vegetative cells) > *B. atrophaeus* (endospores). Comparing Fig. 3a and Fig. 3b on the one hand and Fig. 4a and Fig. 4b on the other (in combined with the results of Table 2) confirm that the endospores of *B. atrophaeus* were considerably more resistant against ACP treatment than the vegetative cells. These results also demonstrate that the effect of treatment time itself is comparable between *E. coli* and *P. verrucosum* spores. The modelling analysis also indicates that the challenge microorganisms used were much more susceptible to the ACP treatments than the background microbiota. Comparing the Fig. 3a-d with Fig. 4a-d indicates that, even though the effect of treatment time on itself was always lower for the indirect treatment, the combined effect of treatment time and retention time can still be similar (this was not the case for *B. atrophaeus* spores).

3.4. Effect of plasma treatment on wheat grain germination

The effect of 5 and 20 min of plasma treatment on wheat grain germination was investigated. In general, 5 min of treatment had minimal effect on the grain germination rate, regardless of mode of exposure or retention time used, whereas 20 min of direct treatment significantly decreased germination for most samples ($p<0.05$) (Fig. 7). By Day 7, no significant differences were observed between the samples subjected to 5 min of plasma treatment, with either 2 or 24 h retention time, and the control samples ($p<0.05$). A maximum germination rate (80%) was recorded for samples treated indirectly for 5 min with a 2 h retention time at 15°C. Extending treatment time from 5 to 20 min significantly increased the germination times for all directly plasma-treated samples and indirectly treated samples with 24 h retention time ($p<0.05$) (Fig. 8). The parameter estimates and 95% confidence bounds of the response surface model for the effect of treatment, retention and incubation time on the germination rate is presented in Table 3. Response surface models are compared with average

measured germination rates in Fig. 9 for both the (a) direct and (b) indirect treatment. The effect of the treatment time itself is much higher with the direct treatment than with the indirect treatment (more than a factor of 2 higher). Also the retention time has a larger effect on the germination rate for the direct treatment. In case of the indirect treatment, it is mostly the interaction between the treatment time and retention time that has an effect on the germination rate (as illustrated clearly in Fig. 9b). The standard deviation of the difference between the response surface model and the measurements, as approximated by the RMSE, is relatively high for both models. In this case, the high RMSE points to high variability of the germination rate, given the same experimental conditions.

3.5. Effect of plasma treatment on wheat surface hydrophobicity

Contact angle measurements were used to determine whether plasma treatment affects the surface characteristics of wheat grains. Apparent contact angles and free surface energy of deionised water, ethylene glycol and diiodomethane are presented in Table 1. The values were dependent on mode of plasma exposure with similar values obtained for the control samples and samples treated indirectly. Direct plasma treatment dramatically decreased the apparent contact angles of all the tested liquids deposited on wheat grains. Extending treatment time from 5 to 20 min resulted in a further decline (Fig 7). No effect of extending retention time to 24 h on the surface hydrophobicity of grains was recorded.

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4. Discussion

ACP treatment inactivation efficacy against microorganisms naturally present on the surface of barley and wheat was evaluated in this study. Similar reduction levels of mesophilic bacteria and fungi were achieved for both types of cereal grains, which could be explained by the fact that the components of wheat and barley micro-floras bear a strong resemblance and similar microbial species are likely to be found on these cereals (Flannigan, 1996). The maximal reductions achieved for barley background microbiota were 2.4 and 2.1 $\log_{10}$ CFU/g for bacteria and fungi, respectively, while for wheat - 1.5 and 2.5 $\log_{10}$ CFU/g for bacteria and fungi, respectively. As ACP microbial inactivation efficacy depends on many different factors, such as type of source used to generate plasma, process parameters, food produce type, investigated microorganism and its physiological state, it is difficult to compare results obtained by different plasma applications other than in terms of overall efficacy and treatment durations needed. With regard to the inactivation of background microbiota of cereal grains, previous studies have shown the reduction of microbial challenges on wheat using a packed-bed reactor - treatment time of 10 seconds was found to be the most effective in the reduction of fungal contaminants up to around 10% of the initial load (Kordas et al., 2015). Brasoveanu et al., (2015) reported that the number of fungi was decreased up to 25% after 20 min of plasma treatment of barley seeds and up to 40% after 10 min of treatment of corn seeds. Zahoranova et al. (2016) investigated ACP microbial inactivation on wheat, reaching 1 log CFU/g reduction of bacteria after 10 min and complete inactivation of yeasts and filamentous fungi after 2 min.

A strong influence of microorganism type and its physiological state on ACP inactivation efficacy was observed in this work. Plasma treatment was conducted against bacterial vegetative cells (*E. coli* and *B. atrophaeus*) and endospores (*B. atrophaeus*) in addition to fungal spores (*P. verrucosum*) artificially inoculated on barley grains. Among the bacteria

studied in their vegetative form, *E. coli* was the most susceptible. There are no clear differences in inactivation levels in relation to bacterial cell wall structure. Various studies showed that Gram-positive bacteria are more resistant to plasma treatment (Lee, Paek, Ju, & Lee, 2006; D. Ziuzina et al., 2014), while other reported higher resistance for Gram-negative bacteria (Fan, Sokoral, Engemann, Gutler, & Liu, 2012; Han, Patil, Keener, Cullen, & Bourke, 2014) or similar susceptibility between the two groups of bacteria (Klämpfl et al., 2012). Han et al. (2016) observed two different mechanisms of inactivation for Gram-positive and Gram-negative bacteria by ACP – it was found that *E. coli* was inactivated mainly by cell leakage and low-level DNA damage, while *S. aureus* was eliminated by intracellular damage, with significantly higher levels of intracellular ROS observed and little envelope damage. Mai-Prochnow, Clauson, Hong & Murphy (2016) investigated shows a correlation of ACP inactivation of bacteria and the thickness of the cell wall – the results presented in the study showed that biofilms of Gram-negative species with a thinner cell wall are inactivated more rapidly than biofilms of Gram-positive bacteria with a thicker cell wall. However, the effect of bacterial cell wall on plasma inactivation efficacy needs further investigation.

The influence of bacterial physiological state on plasma decontamination efficacy was evident in this study. In comparison to the endospores, vegetative cells of *B. atrophaeus* do not have such a complex cell structure, and are more sensitive to physical and chemical environmental influences (Muranyi et al., 2010). The complex spore coat structure of *B. atrophaeus* has been identified as a resistance mechanism against various chemicals, particularly oxidizing agents, such as hydrogen peroxide, ozone, chlorine dioxide and hypochlorite (Sella et al., 2014). In the current work, *B. atrophaeus* endospores were reduced by a maximum of 2.4 $\log_{10}$ CFU/g, while the reduction achieved for vegetative cells was 3.2 $\log_{10}$ CFU/g. Muranyi et al. (2010) investigated the inactivation of *B. atrophaeus* vegetative cells and spores subjected to a cascaded dielectric barrier discharge (CDBD)

treatment and in the case of the vegetative cells, complete inactivation of the sample was achieved within 1 second of treatment time, while for spores a decrease of about 6 log cycles was achieved in 7 s. Investigating ACP interactions with *P. verrucosum*, this study reports that fungal spores were more resistant to high voltage ACP treatment than Gram-negative *E. coli* and less resistant than both vegetative cells and spores of Gram-positive *B. atrophaeus*. Eissa et al. (2014), studying the efficacy of sporicidal agent based on a mixture of peroxyacetic acid and hydrogen peroxide, also demonstrated higher resistance of bacterial spores: elimination time for *B. subtilis* spores was 15 min, which was about double the time required for inactivation of *Aspergillus brasiliensis* spores inoculated on vinyl surface material. Other studies that focus on ACP treatment of artificially contaminated grains reported 2-log reduction of *Bacillus amyloliquefaciens* endospores on wheat grains within 30 s of treatment (Butscher et al. 2015), while 5 min treatment reduced populations of *Geobacillus stearothermophilus* endospores by 0.8-log and by 3-log after 60 min of plasma treatment (Butscher et al., 2016). Selcuk et al. (2008) studied the effect of low pressure cold plasma inactivation of *Aspergillus* spp. and *Penicillum* spp. artificially inoculated on surface of wheat, barley, rye and corn and demonstrated reduction in the fungal attachment by 4-log after 20 min of sulfur hexafluoride (SF_6) plasma treatment.

When comparing the effect of the mode of treatment on the microbial inactivation, the observed differences were limited. In case of applying the indirect treatment, all treatments that resulted in a significant reduction of the microbial load also showed antagonistic interactions between the effects of treatment and retention time. As such, combining both a long treatment time and retention time may not be efficient. Thus, it is advised to combine shorter treatment times with longer retention times to achieve the required inactivation efficacy.

The complexity of the surface structure is a major challenge in the plasma decontamination of cereal grains. Both wheat and barley grain surfaces can be characterized as rough, porous and uneven. Wrinkles or crevices observed on grains allow better attachment of microorganisms to the surface and are another factor decreasing plasma treatment efficacy. Brasoveanu et al. (2015) observed higher plasma inactivation levels of fungal load for smoother corn seeds than for barley seeds. Butscher et al., (2016) noted that bacterial endospore reduction on wheat grains is considerably less efficient than on flat and granular polypropylene substrates.

A closed process ACP treatment combined with retention time of up to 24 h allowed the extended interaction of the plasma generated reactive species with the sample. This facilitated antimicrobial action of long lived reactive species and it explains why in our study extending post-treatment retention time was generally more efficient for microbial inactivation, which is important for translation to process design. When barley background microbiota were assessed immediately after treatment, there was no significant reduction achieved for either 5 or 20 min of treatment (Fig. 2), therefore for challenge studies treatment combined with either 2 or 24 h of post-treatment retention time were performed (Fig. 6). Considering that grain contact area with reactive species generated during plasma treatment is crucial for an efficient microbial inactivation, ACP treatment could be optimized to ensure a uniform exposure of the whole grains to surface, e.g. by agitating or rotating the samples during treatment.

In recent years, cold plasma technology has received increased attention as an alternative approach for enhancement of seed germination and promoting the growth process of the plants. Although insights have been provided by several authors, the mechanism of action is still not fully understood. Many studies have suggested that enhancement of seed germination and seedling growth rates might be associated with the water uptake of seeds. It was found that after plasma treatment the wetting properties of the seeds surfaces are altered.

A dramatic decrease in the apparent contact angle of treated wheat was noted by Bormashenko et al. (2012). Improvement of seed surface wettability can be partially attributed to the oxidation of seed surface by reactive species formed during plasma formation and may eventually increase germination speed. Šerá et al. (2010) observed that penetration of active species from plasma through the porous seed coat into the seed leads to their reaction with cellular components and caused changes in the metabolism of oats and wheat. In the same paper, the authors noted that other stimulation effect of cold plasma might be due to slight erosion of the surface after the treatment. Although plasma treatment inhibited the germinating acceleration of wheat and did not affect germination of oat seeds in the first days, the enhancement of footstalk and rootlet generation was observed in plants grown from treated wheat and oats, respectively. Nevertheless, various authors noted that extended plasma treatment times seem to be unfavourable for seed growth; therefore, the reports on cold plasma effect of seed germination are not consistent. The results obtained by Zahoranova et al. (2016) indicate that the germination rate, dry weight and vigour of plasma-treated wheat seeds significantly increased after 20 to 50 s of treatment. Similarly, treatment of 80 W significantly increased germination potential and germination rate of wheat in a study performed by Jiang et al. (2014). Also, Filatova et al. (2013) reported that ACP increased wheat germination, however, it was accomplished only for lower treatment times (up to 10 min). Moreover, the authors reported that when plasma treatment time of spring wheat, maize and lupine seeds was extended up to 20 min, their was an impact on microbiological quality, where the percentage of infected seeds increased, which could be due to the damage of the seed coat caused by longer treatments. In contrast, Dobrin et al. (2015) found that plasma treatment had little effect on wheat germination rate (95% and 98% for the untreated and plasma-treated seeds, respectively), but positively influenced other early growth parameters. Similarly, Selcuk et al. (2008) reported no influence of plasma on

the germination rate of wheat. In the present study it was demonstrated that germination percentage of wheat seeds measured after 7 days of start of cultivation remained unaffected after 5 min of treatment, regardless of mode of plasma exposure or retention time used (0, 2 or 24 h), but the germination rate decreased significantly after 20 min of direct treatment. The germination rate of plasma-treated seeds varies between individual plant species and higher doses of ACP can significantly inhibit seed germination, and also retard the seedling growth (Volin, et al., 2000; Zahoranova et al., 2016).

The values of apparent contact angles and free surface energy of deionised water, ethylene glycol and diiodomethane deposited on wheat grains, were heavily dependent on the mode of plasma exposure. Direct plasma treatment dramatically decreased the apparent contact angles of all the tested liquids (Table 1. and Fig. 10), while there were no significant differences between the control and samples treated indirectly. Similarly, Bormashenko et al. (2012) reported that radio-frequency plasma treatment of lentil and wheat seeds decreased the apparent contact angle of water. Dobrin et al. (2015) reported a decrease in contact angle of water due to plasma treatment of wheat grains from 92 ± 0.73° to 53 ± 0.85°, which was then accompanied by a 10–15% rise in water absorption. Therefore, the mode of exposure can be exploited to modulate the impact of reactive species on the functional properties of grains and seeds, with the potential to modulate quality while enhancing or maintaining microbiological safety.

5. Conclusion

In summary, ACP treatment was effective against microorganisms on the surface of cereal grains. Plasma treatment combined with a retention time up to 24 h significantly reduced the number of microbial counts on grains. The efficacy was dependent on processing parameters (treatment and retention time, mode of plasma exposure) and the type and physiological state of microorganisms tested. The maximal reductions achieved for barley background microbiota were 2.4 and 2.1 $\log_{10}$ CFU/g for bacteria and fungi, respectively, and wheat - 1.5 and 2.5 $\log_{10}$ CFU/g for bacteria and fungi, respectively. Among microorganisms artificially deposited onto barley grains, the highest resistance to plasma treatment was observed for *B. atrophaeus* endospores, which was maximally reduced by 2.4 $\log_{10}$ CFU/g. Generally, the native microflora of grains was more resistant to ACP treatments than inoculated microorganisms. In our study, short plasma treatment had minimal influence on the germination rate of wheat, however, extending treatment time up to 20 min negatively affected this quality parameter. Surface hydrophobicity of wheat grains was decreased as a result of direct ACP treatment and remained unaffected in case of indirect treatment. Therefore ACP technology may be a promising tool for effective cereal grain decontamination and modulation of functional properties.

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752 **Figure legends**

753 Fig. 1. Schematic of the experimental set-up for dielectric barrier discharge plasma system
754 (adapted from: Ziuzina et al., 2013).

755 Fig. 2. Effect of 5 min and 20 min of ACP treatment with different post-treatment retention
756 times at 15°C on barley background microbiota: mesophilic bacteria – direct (a) and indirect
757 (b) treatment, yeasts and moulds – direct (c) and indirect (d) treatment; —●— - control, - - ●- - 5
758 min treatment, ···●··· - 20 min treatment. Vertical bars represent standard deviation.

759 Fig. 5. Effect of 5 min and 20 min of ACP treatment with different post-treatment retention
760 times at 15°C on wheat background microbiota: mesophilic bacteria – direct (a) and indirect
761 (b) treatment, yeasts and moulds – direct (c) and indirect (d) treatment; —●— - control, - - ●- - 5
762 min treatment, ···●··· - 20 min treatment. Vertical bars represent standard deviation.

763 Fig. 6. Effect of 5 min and 20 min of ACP treatment with different post-treatment retention
764 times at 15°C on microorganisms inoculated on barley grains: (a) *E. coli*, (b) *B. atrophaeus* –
765 vegetative cells, (c) *B. atrophaeus* – endospores and (d) *P. verrucosum* – spores; post-
766 treatment retention time: 2 h - —○— - direct treatment and - -○- - - indirect treatment, 24 h -
767 —●— - direct treatment and - -●- - - indirect treatment. Vertical bars represent standard
768 deviation.

769 Fig. 7. Effect of 5 min of ACP treatment with different post-treatment retention times at 15°C
770 on wheat germination rate (%) measured on: (a) day 2, (b) day 3 and (c) day 7 from the start
771 of experiment; ■ - untreated control, ▮ - 5 and ▯ - 20 min of treatment. Experiments were
772 performed three times. Different letters indicate significant difference between the control
773 and ACP treated samples within each post-treatment retention time and day of experiment
774 ($p<0.05$). Vertical bars represent standard deviation.

775 Fig. 8. Effect of 5 min of ACP treatment with different post-treatment retention times at 15°C
776 on wheat mean germination time (days); ■ - untreated control, ▯ - 5 and ▯ - 20 min of
777 treatment. Experiments were performed three times. Different letters indicate significant
778 difference between the control and ACP treated samples within each post-treatment retention
779 time ($p<0.05$). Vertical bars represent standard deviation.

780 Fig. 10. Water droplet deposited on wheat grains: (a) untreated grains and treated directly for
781 (b) 5 and (c) 20 min in combination with 24 h of post-treatment retention time at 15°C.

782

783 Fig. 3. Response surface models for the effect of treatment time and retention time on the
784 decimal reduction for the direct treatment. The model is compared with the average of the
785 measurements (**x**) for (a) *B. atrophaeus* spores, (b) *B. atrophaeus* cells, (c) *E. coli*, (d) *P.*
786 *verrucosum*, (e) fungi and (f) mesophilic bacteria on barley and (g) fungi and (h) mesophilic
787 bacteria on wheat.

788

789 Fig. 4. Response surface models for the effect of treatment time and retention time on the
790 decimal reduction for the indirect treatment. The model is compared with the average of the
791 measurements (**x**) for (a) *B. atrophaeus* spores, (b) *B. atrophaeus* cells, (c) *E. coli*, (d) *P.*
792 *verrucosum*, (e) fungi and (f) mesophilic bacteria on barley and (g) fungi and (h) mesophilic
793 bacteria on wheat.

794

795 Fig. 9. Response surface models for the effect of treatment time and retention time on the
796 germination rate compared with the average of the measurements (**x**) for the (a) direct and (b)
797 indirect treatment.

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801 **Figure 1**

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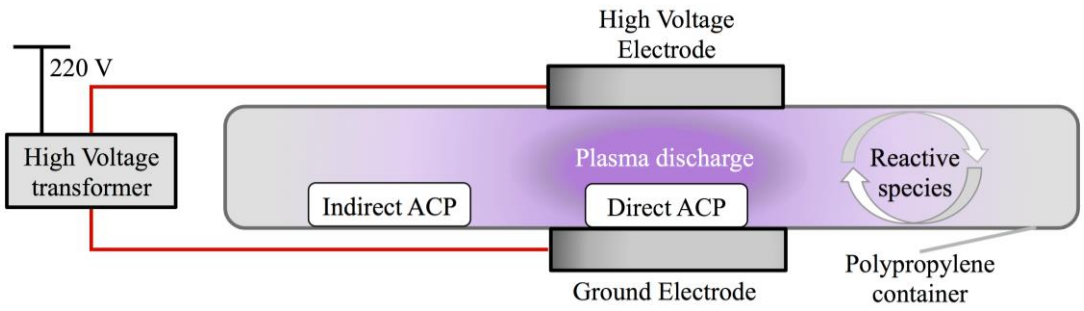

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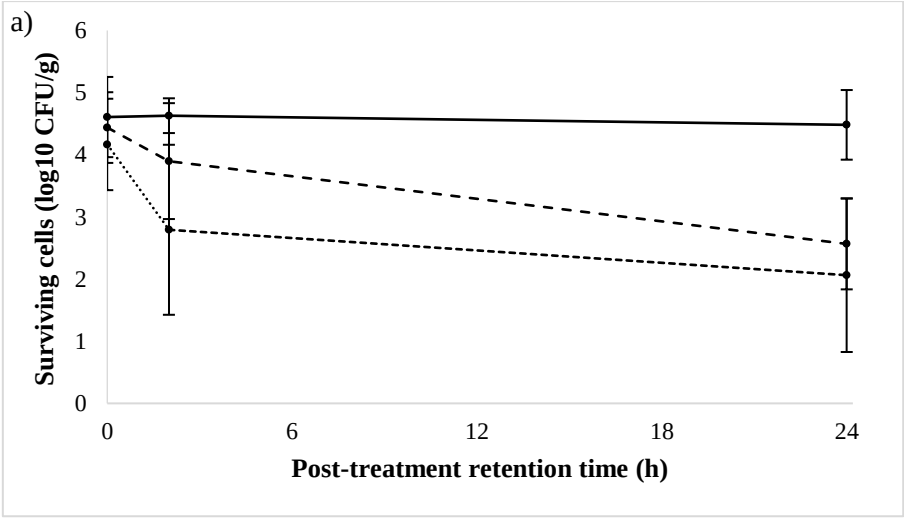
808 **Figure 2**



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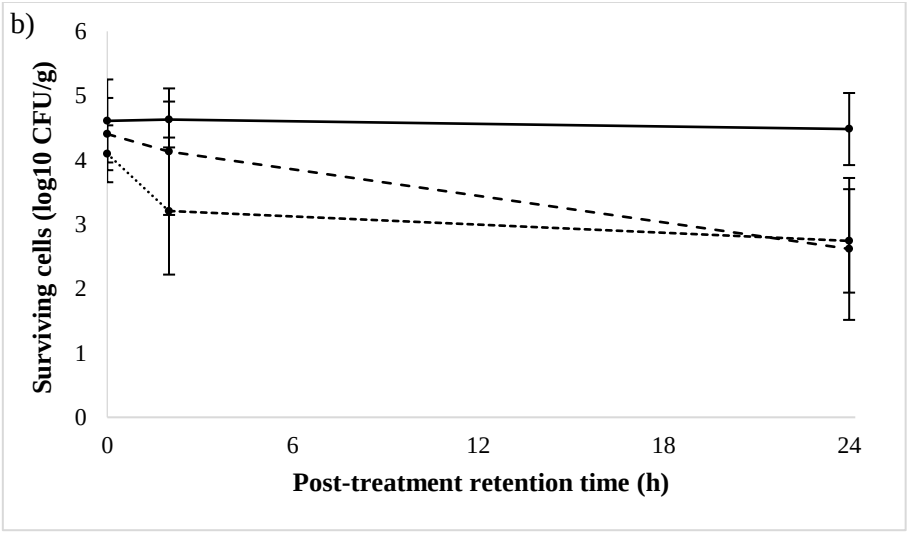

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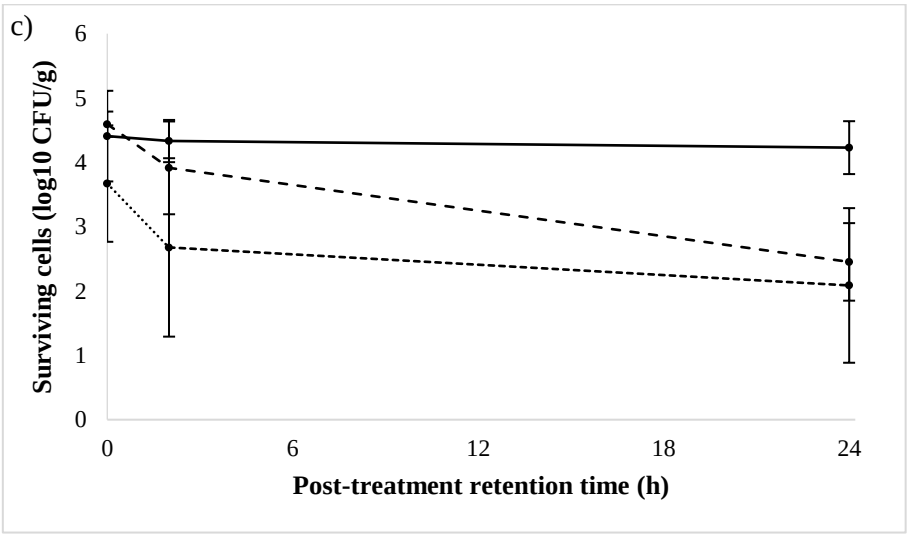

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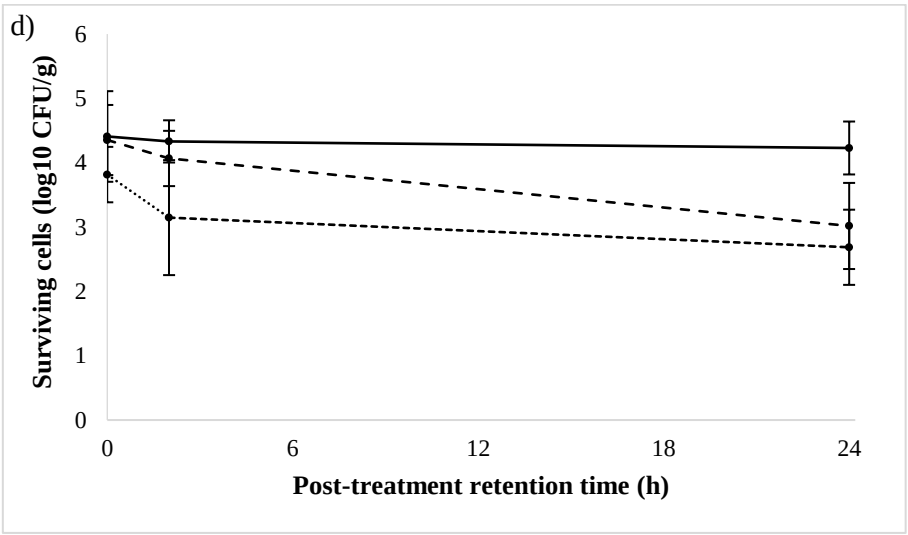

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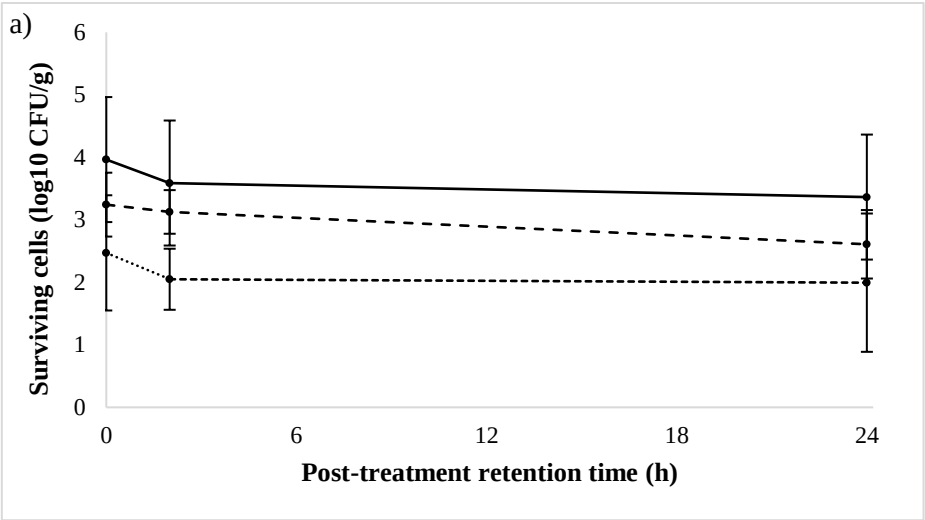
814 **Figure 5**



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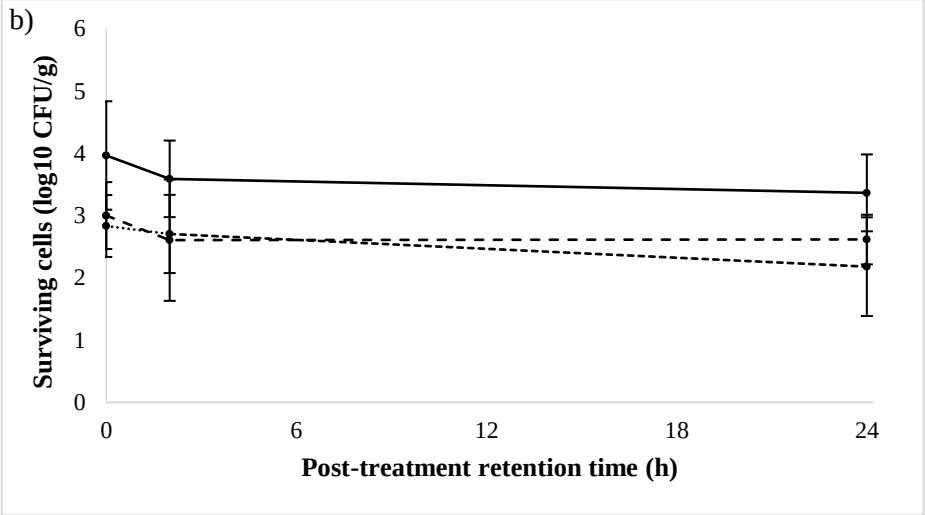

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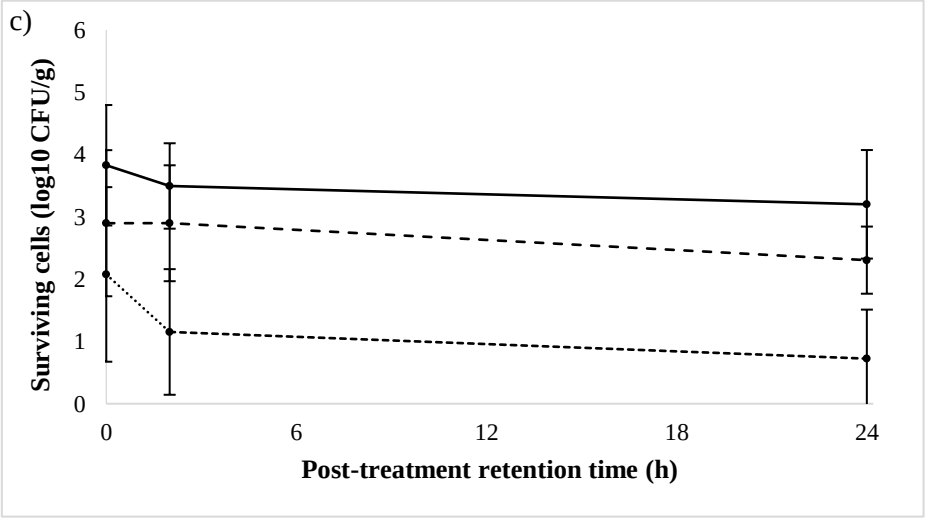

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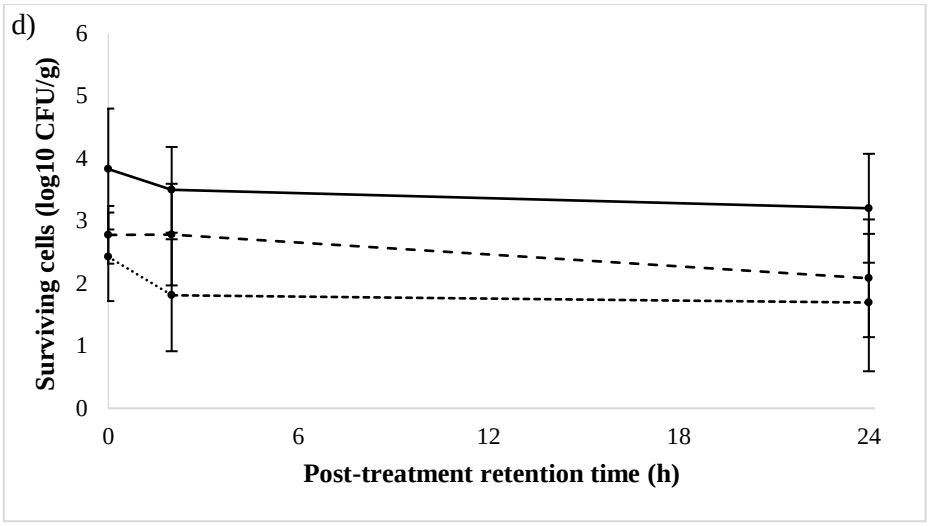

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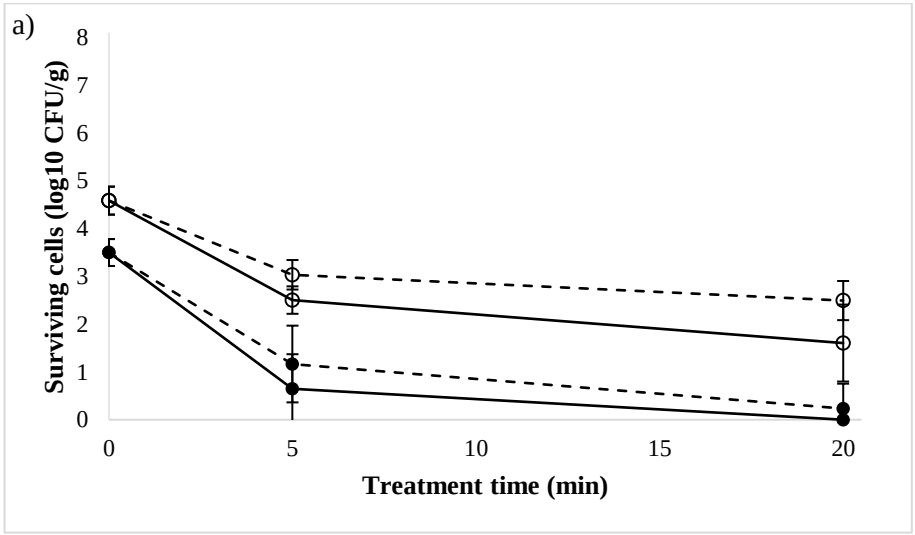
819 **Figure 6**



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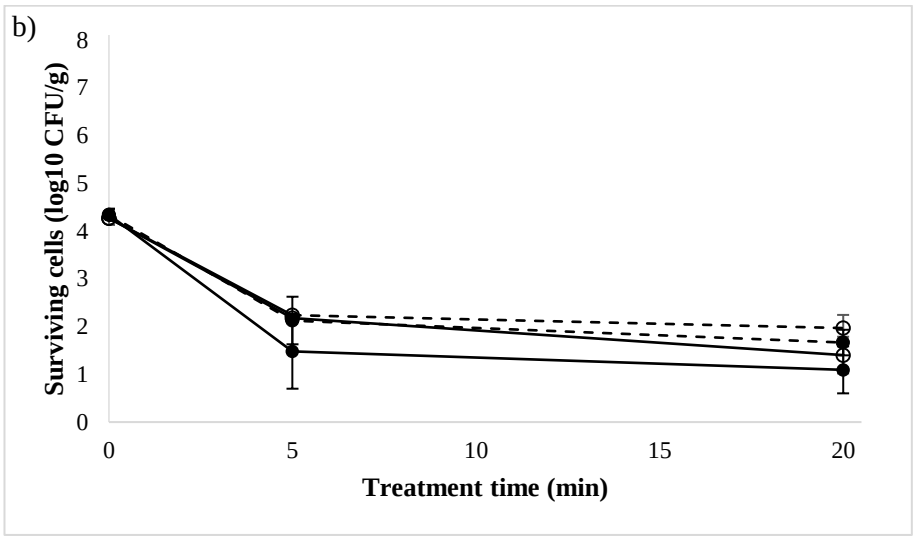

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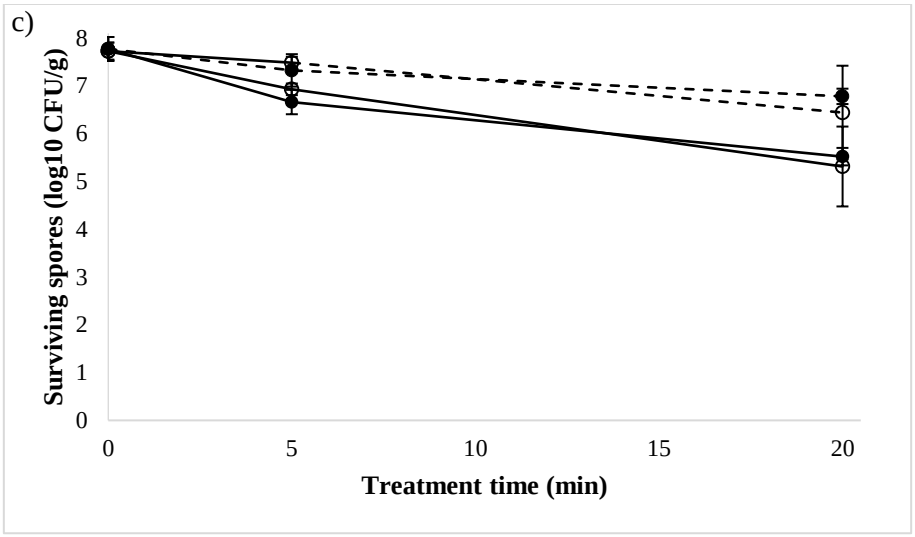

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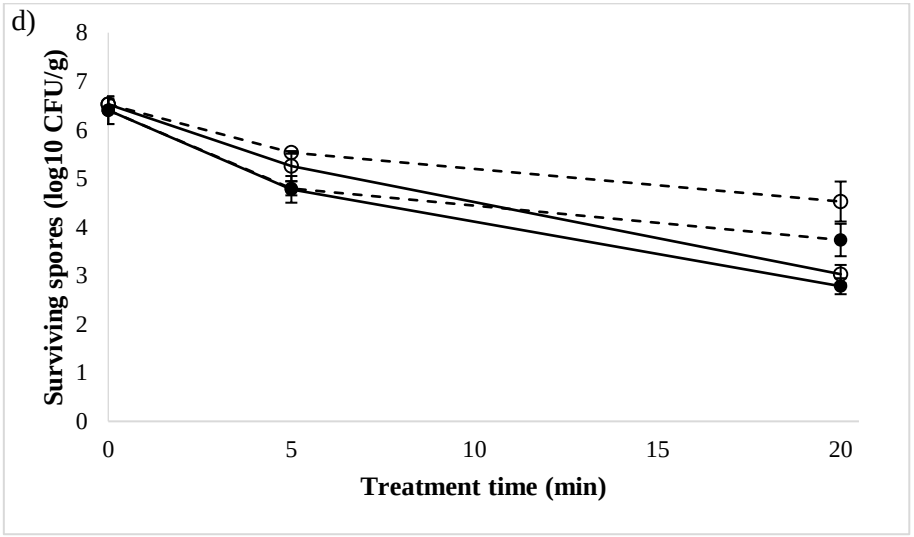

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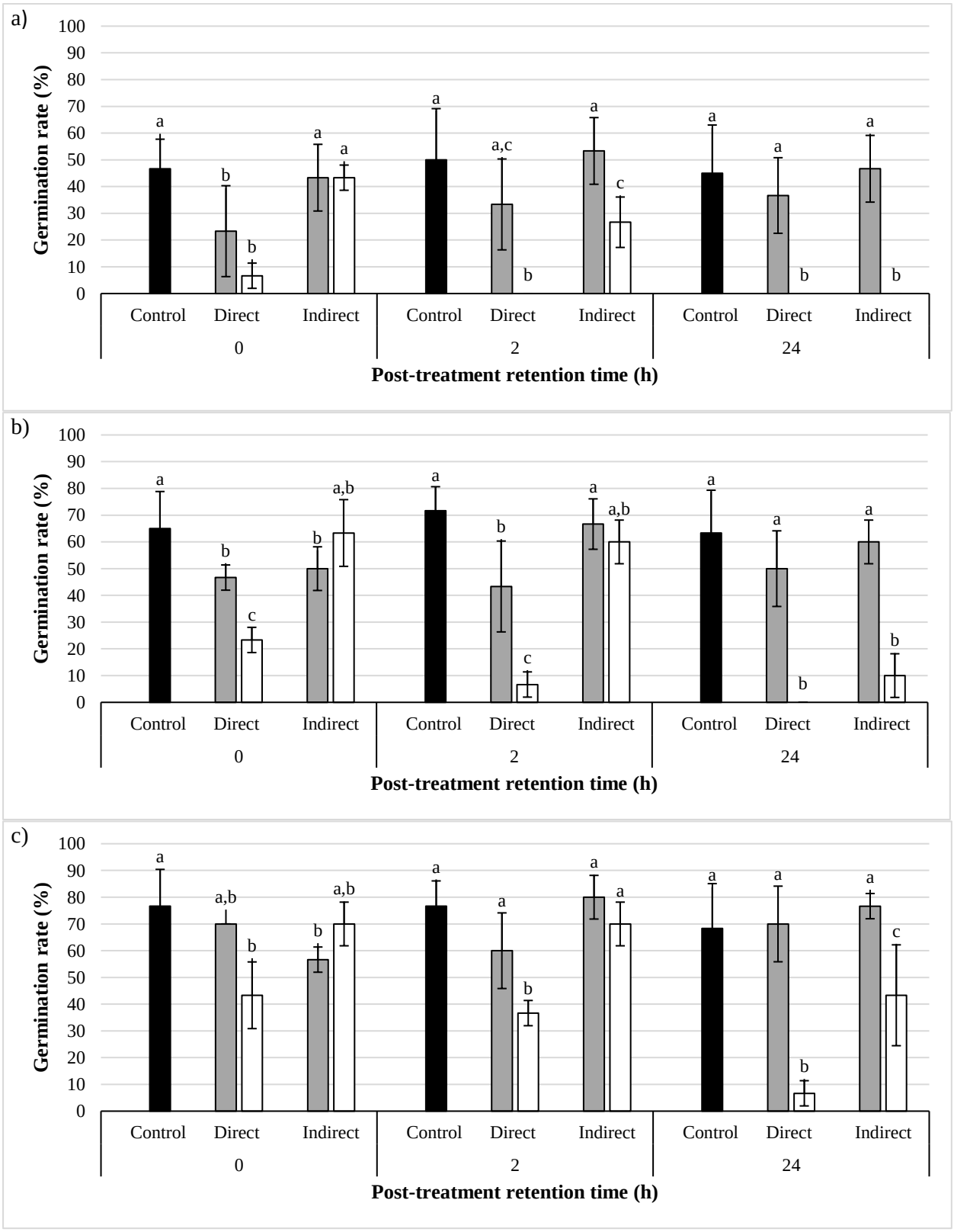
831 **Figure 7**



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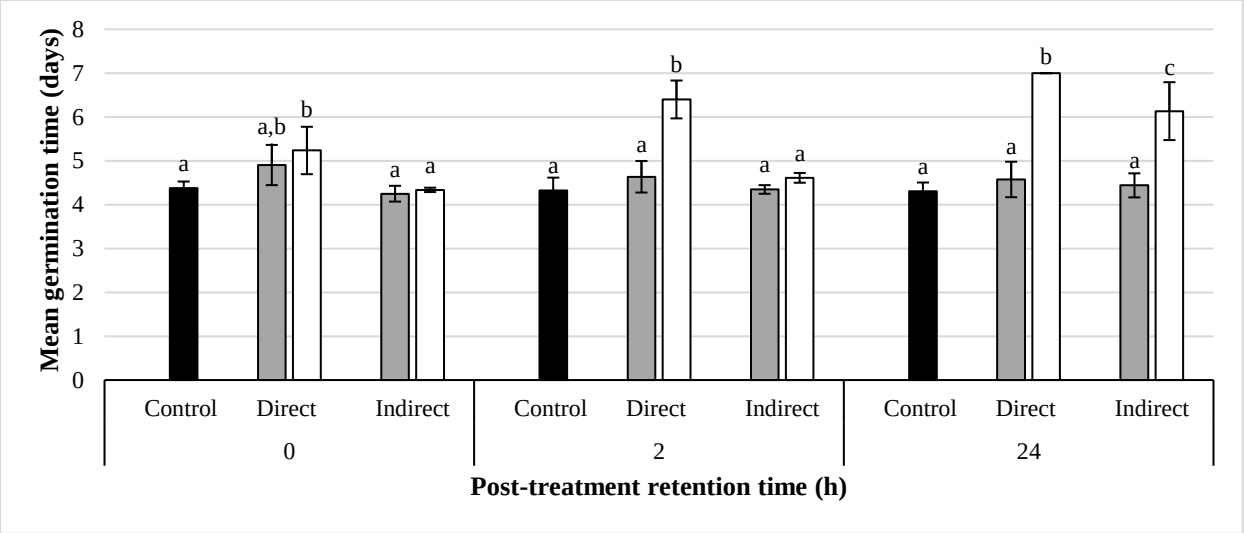
836 **Figure 8**



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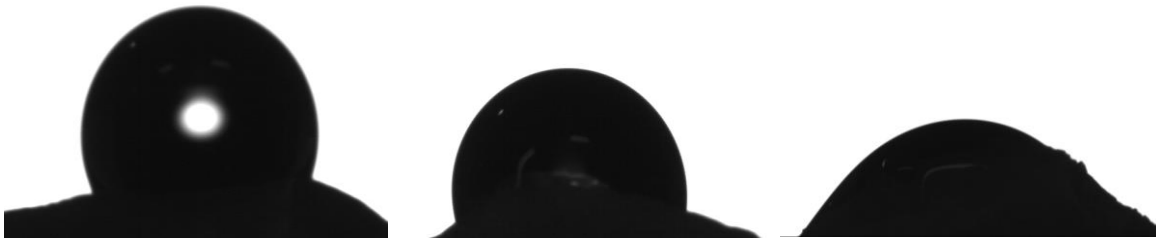
840 **Figure 10**

841 (a) (b) (c)



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Tables

Table 1. Effect of 5 and 20 min of ACP treatment with different post-treatment retention time on contact angle (CA) and surface free energy (γtot) of wheat grain surface. Different letters indicate significant difference between the control and ACP treated samples within each post-treatment retention time ($p<0.05$).

Table 2. Response surface model coefficients for the treatment time, retention time and interaction term. The magnitude of each coefficient indicates the influence of the related term on the decimal reduction. The 95% confidence bounds on the parameter estimates were omitted as all parameters had a high accuracy.

Table 3. Parameter estimates and 95% confidence bounds for the response surface model relating treatment, retention and incubation time with the germination percentage. The Root Mean Squared Error (RMSE) of each model is also presented. This error is an estimate of the standard deviation of error between the model and the measurements.

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870 **Table 1**

Post-treatment retention time (h)	Mode of plasma exposure	Treatment time (min)	Water		Ethylene glycol		Diiodomethane	
			CA (°)	γtot [mN/m]	CA (°)	γtot [mN/m]	CA (°)	γtot [mN/m]
0	Control	0	$104.6^{a} \pm 5.5$	$20.3^{a} \pm 3.3$	$78.1^{a} \pm 2.7$	$21.0^{a} \pm 1.2$	$61.1^{a} \pm 11.0$	$30.8^{a} \pm 5.3$
	Direct	5	$84.7^{b} \pm 1.7$	$32.5^{b} \pm 1.1$	$41.7^{b} \pm 1.9$	$37.6^{b} \pm 0.8$	$38.8^{b.c.d} \pm 4.5$	$37.6^{b} \pm 5.5$
		20	$45.8^{c} \pm 6.0$	$56.1^{c} \pm 3.3$	$41.0^{b} \pm 4.5$	$37.7^{b} \pm 1.6$	$32.0^{b} \pm 2.6$	$43.9^{c} \pm 1.0$
	Indirect	5	$102.7^{a} \pm 4.1$	$21.5^{a} \pm 2.5$	$75.9^{a} \pm 4.4$	$22.0^{a} \pm 2.0$	$49.0^{c} \pm 4.3$	$36.6^{b} \pm 2.0$
		20	$105.8^{a} \pm 5.1$	$19.6^{a} \pm 3.0$	$74.4^{a} \pm 3.9$	$22.7^{a} \pm 1.8$	$41.3^{c.d} \pm 3.6$	$40.1^{b.c} \pm 1.5$
2	Control	0	$102.8^{a} \pm 4.2$	$21.4^{a} \pm 2.6$	$77.2^{a} \pm 2.3$	$21.3^{a} \pm 1.1$	$56.8^{a} \pm 4.3$	$32.9^{a} \pm 2.1$
	Direct	5	$83.5^{b} \pm 0.7$	$33.3^{b} \pm 0.5$	$36.5^{b} \pm 1.8$	$39.7^{b} \pm 0.7$	$35.6^{b} \pm 1.2$	$42.4^{b.d} \pm 0.5$
		20	$45.3^{c} \pm 5.5$	$56.5^{c} \pm 3.2$	$38.5^{b} \pm 5.0$	$38.3^{b} \pm 1.8$	$30.8^{b} \pm 1.5$	$44.4^{b} \pm 0.6$
	Indirect	5	$94.8^{d} \pm 4.7$	$26.3^{a} \pm 2.9$	$74.0^{a} \pm 4.5$	$22.9^{a} \pm 2.1$	$54.1^{a.c} \pm 1.7$	$34.2^{a.c} \pm 0.8$
		20	$100.3^{a.d} \pm 5.1$	$22.9^{a} \pm 3.1$	$69.9^{a} \pm 2.5$	$24.8^{a} \pm 1.2$	$44.4^{b.c} \pm 2.1$	$38.7^{c.d} \pm 0.9$
24	Control	0	$106.4^{a} \pm 5.9$	$19.2^{a} \pm 3.5$	$77.9^{a} \pm 6.2$	$21.1^{a} \pm 2.8$	$55.8^{a} \pm 6.4$	$33.4^{a} \pm 3.0$
	Direct	5	$86.2^{b} \pm 2.7$	$31.6^{b} \pm 1.7$	$41.9^{b} \pm 5.5$	$37.5^{b} \pm 2.3$	$41.7^{b.c} \pm 4.2$	$39.9^{b} \pm 1.9$
		20	$39.7^{c} \pm 1.6$	$59.5^{c} \pm 0.8$	$40.4^{b} \pm 3.6$	$38.1^{b} \pm 1.5$	$33.0^{c} \pm 4.7$	$43.4^{b} \pm 1.8$
	Indirect	5	$99.8^{a} \pm 3.2$	$23.2^{a} \pm 2.0$	$79.3^{a.c} \pm 2.0$	$20.4^{a} \pm 0.9$	$52.9^{a.b} \pm 2.9$	$34.8^{a.b} \pm 1.3$
		20	$105.8^{a} \pm 1.3$	$19.5^{a} \pm 0.8$	$72.7^{a} \pm 1.0$	$23.5^{a} \pm 0.5$	$45.5^{b} \pm 3.2$	$38.2^{b} \pm 1.4$

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873 **Table 2**

Grain type	Microorganism	Mode of plasma exposure					
		Direct			Indirect		
		tt	rt	tt*rt	tt	rt	tt*rt
Barley	*B. atrophaeus* spores	0.077	-0.004	1.39E-03	0.019	0.001	7.56E-04
	B. atrophaeus cells	0.181	0.044	-1.29E-03	0.154	0.109	-5.44E-03
	E. coli	0.176	0.083	-4.31E-04	0.124	0.145	-2.86E-03
	P. verrucosum	0.193	0.023	-6.56E-04	0.114	0.070	-1.94E-03
	Fungi	0.056	0.018	2.05E-03	0.043	0.081	-2.18E-03
	Mesophilic bacteria	0.039	0.020	3.30E-03	0.045	0.085	-2.16E-03
Wheat	Fungi	0.114	0.031	3.82E-04	0.094	0.069	-2.90E-03
	Mesophilic bacteria	0.088	0.032	-8.97E-04	0.007	0.056	-4.67E-04

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875 **Table 3**

Model term	Direct	Indirect
Constant	39.851 ± 41.716	47.864 ± 44.076
tt	-2.304 ± 0.309	-0.808 ± 0.326
rt	0.302 ± 0.174	-0.158 ± 0.183
it	5.651 ± 1.945	3.837 ± 2.055
tt*rt	-0.030 ± 0.001	-0.075 ± 0.001
tt*it	0.032 ± 0.013	0.116 ± 0.014
rt*it	-0.089 ± 0.007	0.034 ± 0.008
RMSE	14.94	15.35

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