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2018

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

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### Recommended Citation

Los, A., Ziuzina, D. & Akkermans, S. (2018). Improving microbiological safety and quality characteristics of wheat and barley by high voltage atmospheric cold plasma closed processing. *Food Research International*, vol. 106, pg. 509-521. doi.org/10.1016/j.foodres.2018.01.009

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4 This document contains the **post-print pdf-version** of the referred paper:

5  
6 **“Improving microbiological safety and quality characteristics of wheat and barley by**  
7 **high voltage atmospheric cold plasma closed processing”**

8  
9 by **Agata Los, Dana Ziuzina, Simen Akkermans, Daniela Boehm, Patrick J. Cullen,**  
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18 When referring to this work, please cite the full bibliographic info:

19  
20 Los, A., Ziuzina, D., Akkermans, S., Boehm, D., Cullen, P.J., Van Impe, J., Bourke, P., 2018.  
21 Improving microbiological safety and quality characteristics of wheat and barley by high  
22 voltage atmospheric cold plasma closed processing. *Food Research International*. (In Press)

23  
24 The journal and the original published paper can be found at:

25 <https://doi.org/10.1016/j.foodres.2018.01.009>

26  
27 The corresponding author can be contacted for additional info.

28  
29 Conditions for open access are available at: <http://www.sherpa.ac.uk/romeo/>

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

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46

47 **Abstract**

48 Contamination of cereal grains as a key global food resource with insects or microorganisms  
49 is a persistent concern for the grain industry due to irreversible damage to quality and safety  
50 characteristics and economic losses. Atmospheric cold plasma presents an alternative to  
51 conventional grain decontamination methods owing to the high antimicrobial potential of  
52 reactive species generated during the treatment, but effects against product specific  
53 microflora are required to understand how to optimally develop this approach for grains.  
54 This work investigated the influence of ACP processing parameters for both cereal grain  
55 decontamination and grain quality as important criteria for grain or seed use. A high voltage  
56 (HV) (80 kV) dielectric barrier discharge (DBD) closed system was used to assess the  
57 potential for control of native microflora and pathogenic bacterial and fungal challenge  
58 microorganisms, in tandem with effects on grain functional properties. Response surface  
59 modelling of experimental data probed the key factors in relation to microbial control and  
60 seed germination promotion. The maximal reductions of barley background microbiota were  
61 2.4 and 2.1 log<sub>10</sub> CFU/g and of wheat - 1.5 and 2.5 log<sub>10</sub> CFU/g for bacteria and fungi,  
62 respectively, which required direct treatment for 20 min followed by a 24 h sealed post-  
63 treatment retention time. In the case of challenge organisms inoculated on barley grains,  
64 the highest resistance was observed for *Bacillus atrophaeus* endospores, which, regardless of  
65 retention time, were maximally reduced by 2.4 log<sub>10</sub> CFU/g after 20 min of direct treatment.  
66 The efficacy of the plasma treatment against selected microorganisms decreased in  
67 the following order: *E. coli* > *P. verrucosum* (spores) > *B. atrophaeus* (vegetative cells) >  
68 *B. atrophaeus* (endospores). The challenge microorganisms were more susceptible to ACP  
69 treatment than naturally present background microbiota. No major effect of short term plasma  
70 treatment on the retention of quality parameters was observed. Germination percentage  
71 measured after 7 days cultivation was similar for samples treated for up to 5 minutes, but this

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

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

78

79 **1. Introduction**

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

95 Conventional techniques for controlling spoilage of cereal grains include thermal and  
96 chemical sterilization methods, however, these methods can negatively affect the quality and  
97 functional properties of cereals and cereal products; moreover, complete elimination of  
98 mycotoxins from food product by processing can rarely be achieved (Karlovsky et al., 2016;  
99 Oghbaei, Prakash, & Yildiz, 2016). Hence, the demand for novel methods that overcome  
100 these limitations. Alternative methods for cereal grain decontamination include irradiation  
101 (a full review can be found in Lorenz & Miller (1975)), ozone treatment (Allen, Wu, & Doan,  
102 2003; Tiwari et al., 2010; Wu, Doan, & CUenca, 2006), microwave (MW) treatment  
103 (Reddy et al., 1998; Vadivambal et al., 2007), pulsed ultraviolet (UV) light treatment

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

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



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

141

142

143 **2. Materials and methods**

144 **2.1. Bacterial and fungal strains**

145 Two bacterial and one fungal strain were used in this study. *E. coli* NCTC 12900 was  
146 obtained from the microbiology stock culture of the School of Food Science and  
147 Environmental Health of the Dublin Institute of Technology. *B. atrophaeus* var. *niger*,  
148 obtained in the form of spore strips (Sportrol®/Namsa®, VWR International, Radnor, PA,  
149 USA), was resuscitated and preserved in the form of protective beads (Technical Services  
150 Consultants Ltd, UK) in-house. *P. verrucosum* DSM 12639 was obtained from Leibniz  
151 Institute, German collection of microorganisms and cell cultures (DSMZ) and resuscitated  
152 according to manufacturer instructions. All strains were maintained at -70°C in the form of  
153 protective beads.

154

155 **2.2. Inocula preparation**

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

161 A single isolated colony of either *E. coli* or *B. atrophaeus* was inoculated into tryptic soy  
162 broth without glucose (TSB-G, Biokar) and incubated overnight (18 h) at 37°C. Cells were  
163 harvested by centrifugation at 10,000 rpm for 10 min, the pellet was washed twice with  
164 sterile phosphate buffered saline (PBS, Sigma Aldrich) and re-suspended in PBS.

165 The bacterial density was determined by measuring absorbance at 550 nm using  
166 the McFarland standard (BioMerieux, Marcy-l'Etoile, France) and a working inoculum

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167 corresponding to a concentration of the average  $8.0 \log_{10}$  CFU/ml was prepared in PBS.

168 The concentration of inoculum for *E. coli* and *B. atrophaeus* was confirmed by plating

169 appropriate dilutions on TSA and incubation at  $37^{\circ}\text{C}$  for 24 h.

170 *B. atrophaeus* endospore suspension was prepared according to the procedure described by

171 Zhao et al. (2008) with minor modifications. Briefly, *B. atrophaeus* was incubated at  $30^{\circ}\text{C}$

172 for 10 days on TSA supplemented with 3.0 mg/L of manganese sulphate. Spores were

173 collected by flooding the agar plate with sterile PBS (10 ml). The obtained suspension was

174 washed two times in PBS by centrifugation at 10,000 rpm for 10 min. To ensure inactivation

175 of vegetative cells, bacterial suspension was heat-shocked for 20 min at  $80^{\circ}\text{C}$  using a water

176 bath, washed two times in PBS at  $4^{\circ}\text{C}$  and finally re-suspended in sterile ice-cold PBS.

177 The purity of spore suspensions was examined by using spore staining method

178 (Hamouda et al., 2002) and optical microscopy. Spores were stained using malachite green

179 stain solution, steaming for 3 min and counterstaining with safranin for 30 seconds.

180 The concentration of spores was adjusted to  $8-9 \log_{10}$  CFU/ml and enumerated after plating

181 aliquots of the appropriate dilutions on TSA and incubation at  $37^{\circ}\text{C}$  for 24 h. Spore

182 suspension was stored at  $4^{\circ}\text{C}$  before use.

183 For fungal spore suspension preparation, PDA was inoculated with *P. verrucosum* by

184 transferring small quantities of conidia with inoculation needle onto a three separate locations

185 of the plate. Inoculated PDA was incubated at  $30^{\circ}\text{C}$  until colony size expanded with good

186 sporulation obtained. The spores were harvested by flooding the agar surface with 10 ml of

187 sterile PBS containing Tween 20 (1%) and scraping the spores from mycelia with a sterile

188 spreader. The suspension was washed twice in sterile PBS, adjusted to  $8-9 \log_{10}$  CFU/ml and

189 stored at  $4^{\circ}\text{C}$  until use. The concentration of spores was confirmed by plating appropriate

190 dilutions on PDA and incubating at  $30^{\circ}\text{C}$  for 5-7 days.

191

### 192 **2.3. Preparation of cereal grains**

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

199 In order to confirm the absence of background microbiota, sterilized grains (1 g) were  
200 aseptically transferred into a sterile Stomacher bag (Seward LTD, UK) containing 5 ml of  
201 maximum recovery diluent (MRD, ScharlauChemie, Spain) and stomached for 10 min.  
202 The resulting suspension (1 ml) was plated on either TSA or PDA with further incubation of  
203 plates for 48 h or 5 days, respectively. In addition, grains were enriched in either TSB or  
204 MRS broth for 72 h. These tests confirmed complete inactivation of grains microbiota as  
205 there was no growth observed on either TSA or PDA or after grain enrichment. The moisture  
206 content of barley used for inoculation and plasma inactivation experiments corresponded to  
207 11.9% calculated on wet weight basis.

208

### 209 **2.4. Barley grains inoculation procedure**

210 For inoculation, sterilized barley grains were aseptically transferred into sterile Petri dishes  
211 (10 g per dish) and sprayed with a suspension of selected microorganism (0.5 ml). The grains  
212 were vigorously mixed by shaking for approximately 30 s to ensure even distribution of  
213 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<sub>RMS</sub> 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<sub>RMS</sub> 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

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238 properties and physical quality parameters, unsterilized wheat grains were exposed to 5 min  
239 of treatment followed by 0, 2 or 24 h of post treatment retention time at 15°C. Untreated  
240 controls were stored under identical conditions as treated controls. Unless otherwise stated,  
241 all experiments were performed in duplicate and replicated at least three times. Results are  
242 expressed as logarithmic units of colony forming units per g ( $\log_{10}$  CFU/g).

243

## 244 **2.6. Microbiological analysis**

245 ACP-treated and the corresponding control grains (1 g) were transferred into separate sterile  
246 Stomacher bags containing 10 ml of sterile MRD and stomached for 10 min. Samples  
247 inoculated with bacterial endospores were heat-shocked for 20 min at 80°C and cooled in ice  
248 for 10 min to inactivate the vegetative cells prior to microanalysis. Samples were serially  
249 diluted in MRD. Aliquots (0.1 ml and 1 ml) of appropriate dilutions were plated on  
250 corresponding media: TSA was used for grain indigenous mesophilic bacteria and for  
251 artificially inoculated *E. coli* and *B. atrophaeus*, while PDA was used for grain indigenous  
252 yeasts and moulds and for artificially inoculated *P. verrucosum*, which were incubated at  
253 37°C for 24-48 h or at 30°C for 5-7 days, respectively.

254

## 255 **2.7. Germination studies**

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

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

268

## 269 **2.8. Contact angle measurement**

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

277

## 278 **2.9. Statistical analysis**

279 Statistical analysis was performed using IBM SPSS statistics 21 Software (SPSS Inc.,  
280 Chicago, USA). The surviving populations of bacteria and fungi, contact angle values and  
281 values of germination parameters following ACP treatment were subjected to analysis of  
282 variance (ANOVA). Means of ACP-treated and untreated controls were compared according  
283 to the method of Fisher's Least Significant Difference-LSD at the 0.05 level.

284

## 285 **2.10. Response surface modelling**

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

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

300

301



### 302 3. Results

#### 303 3.1. Inactivation of barley background microbiota

304 The efficacy of ACP treatment against microorganisms naturally occurring on barley is  
305 presented in Fig. 2. Initial counts of mesophilic bacteria and fungi of barley grains were 4.6  
306 and 4.4 log<sub>10</sub> CFU/g, respectively, which were unaffected by the sample retention for either 0,  
307 2 or 24 h at 15°C. Combining either 5 or 20 min of treatment with no post-treatment retention  
308 time or 5 min of treatment with 2 h of retention time resulted in no significant differences  
309 between microbial populations of treated samples and untreated controls. Significant  
310 reductions in barley background microbiota were only achieved after longer treatment for  
311 20 min in combination with 2 h retention time or after either 5 or 20 min treatment time in  
312 combination with an extended retention time of 24 h (p<0.05), with no statistical difference  
313 between surviving populations of the treated groups recorded. After 20 min of treatment and  
314 a subsequent retention time of 24 h, microbial levels were reduced by 2.4 and  
315 1.7 log<sub>10</sub> CFU/g for bacteria treated directly (Fig. 2a) and indirectly (Fig. 2b), and 2.1 and  
316 1.5 log<sub>10</sub> CFU/g for fungi treated directly (Fig. 2c) and indirectly (Fig. 2d), respectively.  
317 Although extending the retention time of grains in contact with longer lived reactive species  
318 from 2 h to 24 h did not significantly enhance the inactivation effect of 20 min of treatment, it  
319 did improve the efficacy of shorter treatment (5 min) against both mesophilic bacteria and  
320 yeasts/moulds, with reductions by 1.9 and 1.7 log<sub>10</sub> CFU/g achieved for bacteria treated  
321 directly (Fig. 2a) and indirectly (Fig. 2b), and 1.8 and 1.2 log<sub>10</sub> CFU/g for fungi treated  
322 directly (Fig. 2c) and indirectly (Fig. 2d), respectively. In terms of the effects of mode of  
323 plasma exposure, direct treatment always resulted in slightly higher reductions of both  
324 mesophilic bacteria and fungi on grains. The modelling results with respect to the inactivation  
325 of naturally present fungi and mesophilic bacteria in barley are presented in Table 2. The  
326 models are visualised in Fig. 3e and Fig. 3f for the direct treatment and Fig. 4e and Fig. 4f for

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

### 330 **3.2. Inactivation of wheat background microbiota**

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

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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<sub>10</sub> 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<sub>10</sub> CFU/g, respectively, and increased by 0.4 log<sub>10</sub> 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<sub>10</sub> CFU/g after direct and 1.3 log<sub>10</sub> 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<sub>10</sub> CFU/g after direct and by 3.3, 2.7 and 2.7 log<sub>10</sub> 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) >

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376 *B. atrophaeus* (vegetative cells) > *B. atrophaeus* (endospores). Comparing Fig. 3a and Fig.  
377 3b on the one hand and Fig. 4a and Fig. 4b on the other (in combined with the results of  
378 Table 2) confirm that the endospores of *B. atrophaeus* were considerably more resistant  
379 against ACP treatment than the vegetative cells. These results also demonstrate that the effect  
380 of treatment time itself is comparable between *E. coli* and *P. verrucosum* spores. The  
381 modelling analysis also indicates that the challenge microorganisms used were much more  
382 susceptible to the ACP treatments than the background microbiota. Comparing the Fig. 3a-d  
383 with Fig. 4a-d indicates that, even though the effect of treatment time on itself was always  
384 lower for the indirect treatment, the combined effect of treatment time and retention time can  
385 still be similar (this was not the case for *B. atrophaeus* spores).

386

### 387 **3.4. Effect of plasma treatment on wheat grain germination**

388 The effect of 5 and 20 min of plasma treatment on wheat grain germination was investigated.  
389 In general, 5 min of treatment had minimal effect on the grain germination rate, regardless of  
390 mode of exposure or retention time used, whereas 20 min of direct treatment significantly  
391 decreased germination for most samples ( $p < 0.05$ ) (Fig. 7). By Day 7, no significant  
392 differences were observed between the samples subjected to 5 min of plasma treatment, with  
393 either 2 or 24 h retention time, and the control samples ( $p < 0.05$ ). A maximum germination  
394 rate (80%) was recorded for samples treated indirectly for 5 min with a 2 h retention time at  
395 15°C. Extending treatment time from 5 to 20 min significantly increased the germination  
396 times for all directly plasma-treated samples and indirectly treated samples with 24 h  
397 retention time ( $p < 0.05$ ) (Fig. 8). The parameter estimates and 95% confidence bounds of the  
398 response surface model for the effect of treatment, retention and incubation time on the  
399 germination rate is presented in Table 3. Response surface models are compared with average

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

409

### 410 **3.5. Effect of plasma treatment on wheat surface hydrophobicity**

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

419

420

421 **4. Discussion**

422 ACP treatment inactivation efficacy against microorganisms naturally present on the surface  
423 of barley and wheat was evaluated in this study. Similar reduction levels of mesophilic  
424 bacteria and fungi were achieved for both types of cereal grains, which could be explained by  
425 the fact that the components of wheat and barley micro-floras bear a strong resemblance and  
426 similar microbial species are likely to be found on these cereals (Flannigan, 1996).

427 The maximal reductions achieved for barley background microbiota were 2.4 and  
428 2.1 log<sub>10</sub> CFU/g for bacteria and fungi, respectively, while for wheat - 1.5 and  
429 2.5 log<sub>10</sub> CFU/g for bacteria and fungi, respectively. As ACP microbial inactivation efficacy  
430 depends on many different factors, such as type of source used to generate plasma, process  
431 parameters, food produce type, investigated microorganism and its physiological state, it is  
432 difficult to compare results obtained by different plasma applications other than in terms of  
433 overall efficacy and treatment durations needed. With regard to the inactivation of  
434 background microbiota of cereal grains, previous studies have shown the reduction of  
435 microbial challenges on wheat using a packed-bed reactor - treatment time of 10 seconds was  
436 found to be the most effective in the reduction of fungal contaminants up to around 10% of  
437 the initial load (Kordas et al., 2015). Brasoveanu et al., (2015) reported that the number of  
438 fungi was decreased up to 25% after 20 min of plasma treatment of barley seeds and up to  
439 40% after 10 min of treatment of corn seeds. Zahoranova et al. (2016) investigated ACP  
440 microbial inactivation on wheat, reaching 1 log CFU/g reduction of bacteria after 10 min and  
441 complete inactivation of yeasts and filamentous fungi after 2 min.

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

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446 studied in their vegetative form, *E. coli* was the most susceptible. There are no clear  
447 differences in inactivation levels in relation to bacterial cell wall structure. Various studies  
448 showed that Gram-positive bacteria are more resistant to plasma treatment (Lee, Paek, Ju, &  
449 Lee, 2006; D. Ziuzina et al., 2014), while other reported higher resistance for Gram-negative  
450 bacteria (Fan, Sokoral, Engemann, Gutler, & Liu, 2012; Han, Patil, Keener, Cullen, &  
451 Bourke, 2014) or similar susceptibility between the two groups of bacteria (Klämpfl et al.,  
452 2012). Han et al. (2016) observed two different mechanisms of inactivation for Gram-positive  
453 and Gram-negative bacteria by ACP – it was found that *E. coli* was inactivated mainly by cell  
454 leakage and low-level DNA damage, while *S. aureus* was eliminated by intracellular damage,  
455 with significantly higher levels of intracellular ROS observed and little envelope damage.  
456 Mai-Prochnow, Clauson, Hong & Murphy (2016) investigated shows a correlation of ACP  
457 inactivation of bacteria and the thickness of the cell wall – the results presented in the study  
458 showed that biofilms of Gram-negative species with a thinner cell wall are inactivated more  
459 rapidly than biofilms of Gram-positive bacteria with a thicker cell wall. However, the effect  
460 of bacterial cell wall on plasma inactivation efficacy needs further investigation.

461 The influence of bacterial physiological state on plasma decontamination efficacy was  
462 evident in this study. In comparison to the endospores, vegetative cells of *B. atrophaeus* do  
463 not have such a complex cell structure, and are more sensitive to physical and chemical  
464 environmental influences (Muranyi et al., 2010). The complex spore coat structure of  
465 *B. atrophaeus* has been identified as a resistance mechanism against various chemicals,  
466 particularly oxidizing agents, such as hydrogen peroxide, ozone, chlorine dioxide and  
467 hypochlorite (Sella et al., 2014). In the current work, *B. atrophaeus* endospores were reduced  
468 by a maximum of 2.4 log<sub>10</sub> CFU/g, while the reduction achieved for vegetative cells was  
469 3.2 log<sub>10</sub> CFU/g. Muranyi et al. (2010) investigated the inactivation of *B. atrophaeus*  
470 vegetative cells and spores subjected to a cascaded dielectric barrier discharge (CDBD)

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

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



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495 The complexity of the surface structure is a major challenge in the plasma decontamination of  
496 cereal grains. Both wheat and barley grain surfaces can be characterized as rough, porous and  
497 uneven. Wrinkles or crevices observed on grains allow better attachment of microorganisms  
498 to the surface and are another factor decreasing plasma treatment efficacy. Brasoveanu et al.  
499 (2015) observed higher plasma inactivation levels of fungal load for smoother corn seeds  
500 than for barley seeds. Butscher et al., (2016) noted that bacterial endospore reduction on  
501 wheat grains is considerably less efficient than on flat and granular polypropylene substrates.

502 A closed process ACP treatment combined with retention time of up to 24 h allowed  
503 the extended interaction of the plasma generated reactive species with the sample. This  
504 facilitated antimicrobial action of long lived reactive species and it explains why in our study  
505 extending post-treatment retention time was generally more efficient for microbial  
506 inactivation, which is important for translation to process design. When barley background  
507 microbiota were assessed immediately after treatment, there was no significant reduction  
508 achieved for either 5 or 20 min of treatment (Fig. 2), therefore for challenge studies treatment  
509 combined with either 2 or 24 h of post-treatment retention time were performed (Fig. 6).

510 Considering that grain contact area with reactive species generated during plasma treatment is  
511 crucial for an efficient microbial inactivation, ACP treatment could be optimized to ensure  
512 a uniform exposure of the whole grains to surface, e.g. by agitating or rotating the samples  
513 during treatment.

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

520 A dramatic decrease in the apparent contact angle of treated wheat was noted by  
521 Bormashenko et al. (2012). Improvement of seed surface wettability can be partially  
522 attributed to the oxidation of seed surface by reactive species formed during plasma  
523 formation and may eventually increase germination speed. Šerá et al. (2010) observed that  
524 penetration of active species from plasma through the porous seed coat into the seed leads to  
525 their reaction with cellular components and caused changes in the metabolism of oats and  
526 wheat. In the same paper, the authors noted that other stimulation effect of cold plasma might  
527 be due to slight erosion of the surface after the treatment. Although plasma treatment  
528 inhibited the germinating acceleration of wheat and did not affect germination of oat seeds in  
529 the first days, the enhancement of footstalk and rootlet generation was observed in plants  
530 grown from treated wheat and oats, respectively. Nevertheless, various authors noted that  
531 extended plasma treatment times seem to be unfavourable for seed growth; therefore,  
532 the reports on cold plasma effect of seed germination are not consistent. The results obtained  
533 by Zahoranova et al. (2016) indicate that the germination rate, dry weight and vigour of  
534 plasma-treated wheat seeds significantly increased after 20 to 50 s of treatment. Similarly,  
535 treatment of 80 W significantly increased germination potential and germination rate of  
536 wheat in a study performed by Jiang et al. (2014). Also, Filatova et al. (2013) reported that  
537 ACP increased wheat germination, however, it was accomplished only for lower treatment  
538 times (up to 10 min). Moreover, the authors reported that when plasma treatment time of  
539 spring wheat, maize and lupine seeds was extended up to 20 min, there was an impact on  
540 microbiological quality, where the percentage of infected seeds increased, which could be  
541 due to the damage of the seed coat caused by longer treatments. In contrast, Dobrin et al.  
542 (2015) found that plasma treatment had little effect on wheat germination rate (95% and 98%  
543 for the untreated and plasma-treated seeds, respectively), but positively influenced other early  
544 growth parameters. Similarly, Selcuk et al. (2008) reported no influence of plasma on

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

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

564

565 **5. Conclusion**

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

582

583 **Acknowledgements**

584 This work was funded through Science Foundation Ireland (SFI) under Grant Number  
585 14/IA/2626 and the project PFV/10/002 (Center of Excellence OPTEC-Optimization in  
586 Engineering) of the KU Leuven Research Council, projects G093013N of the Fund for  
587 Scientific Research-Flanders and the Belgian Program on Interuniversity Poles of Attraction,  
588 through the Belgian Federal Science Policy Office.

589

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751

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.

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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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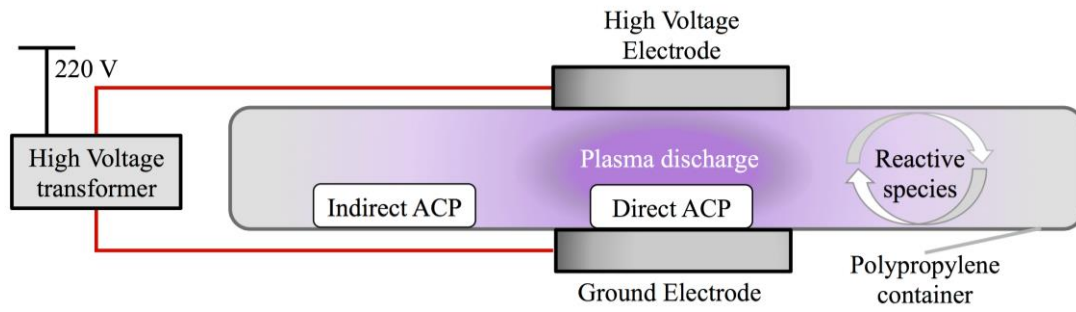
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801 **Figure 1**

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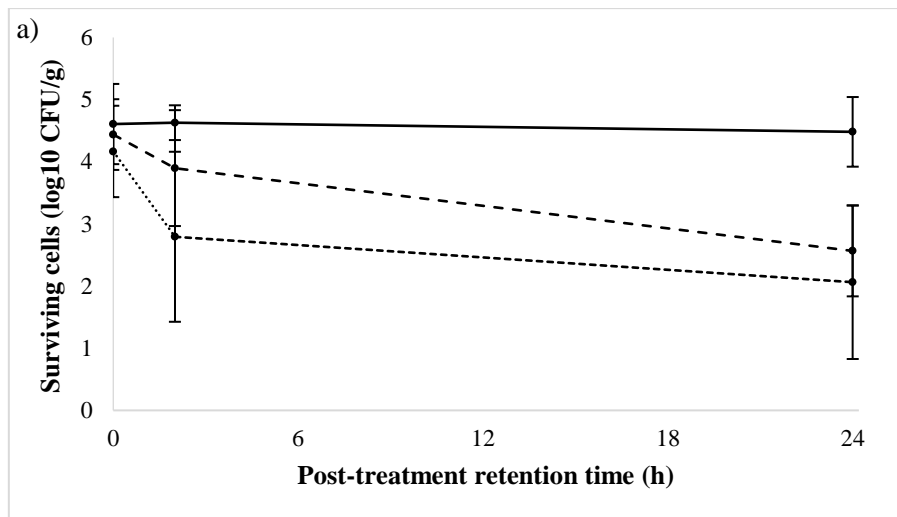
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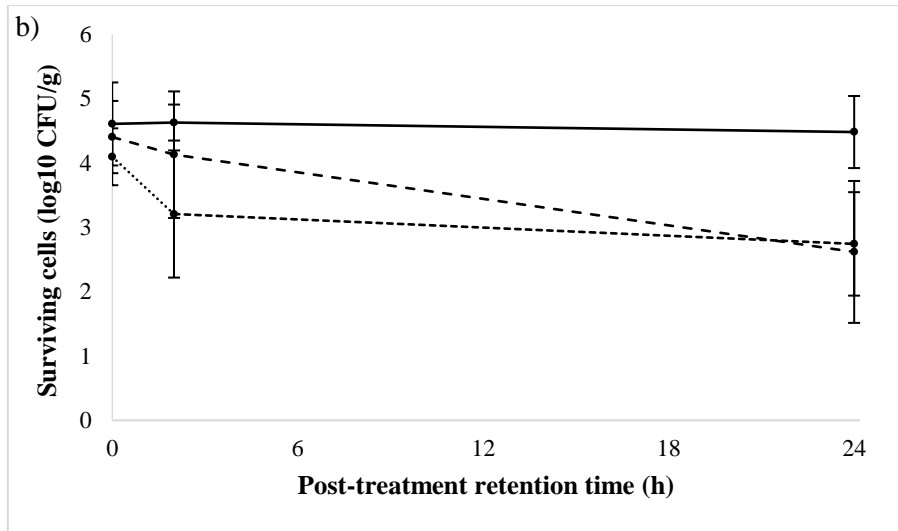
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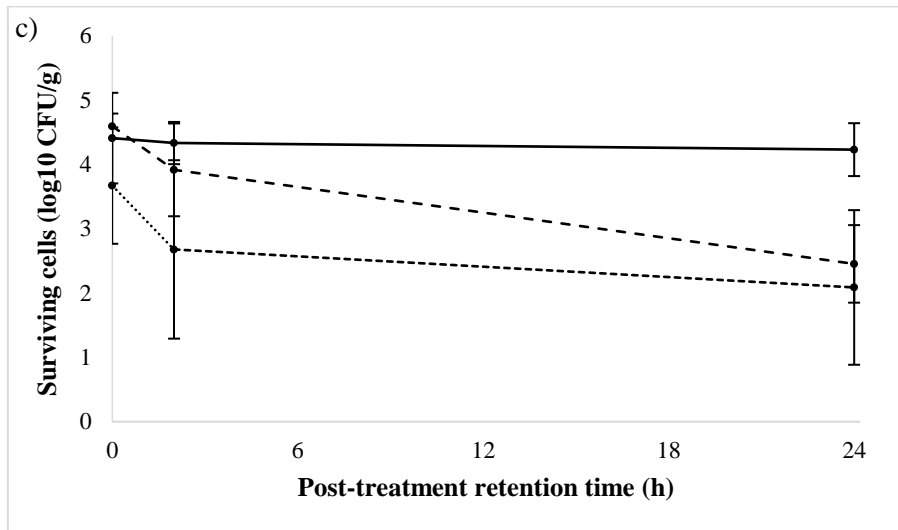
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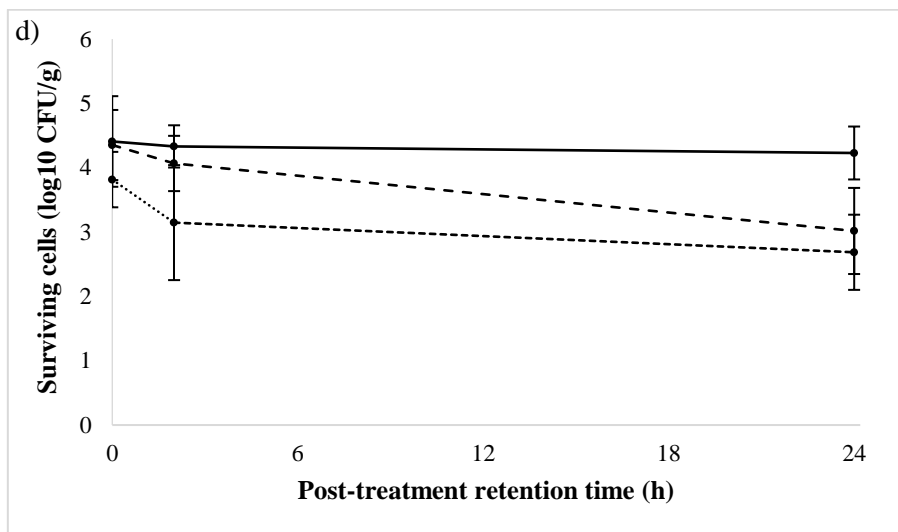
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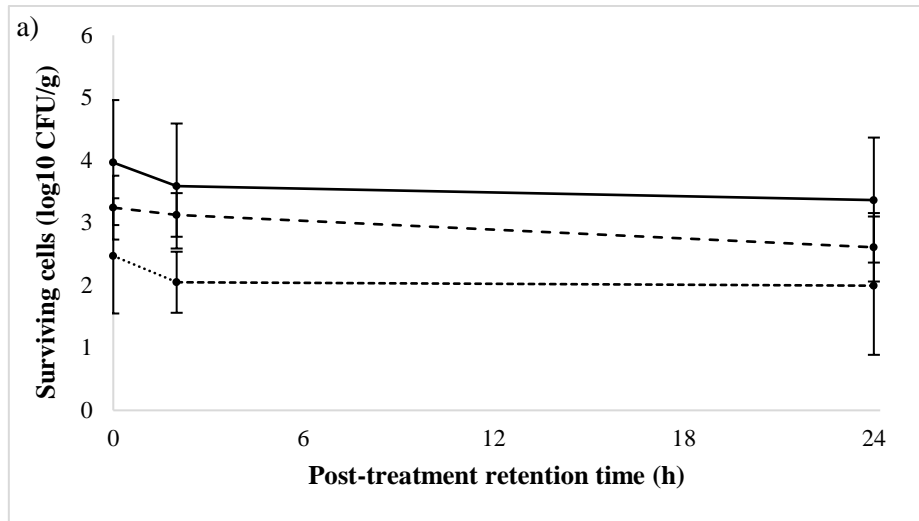


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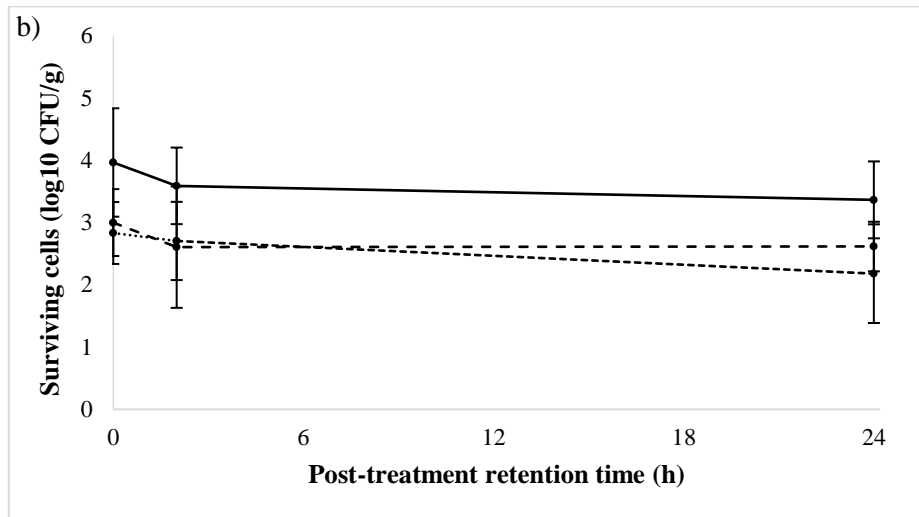
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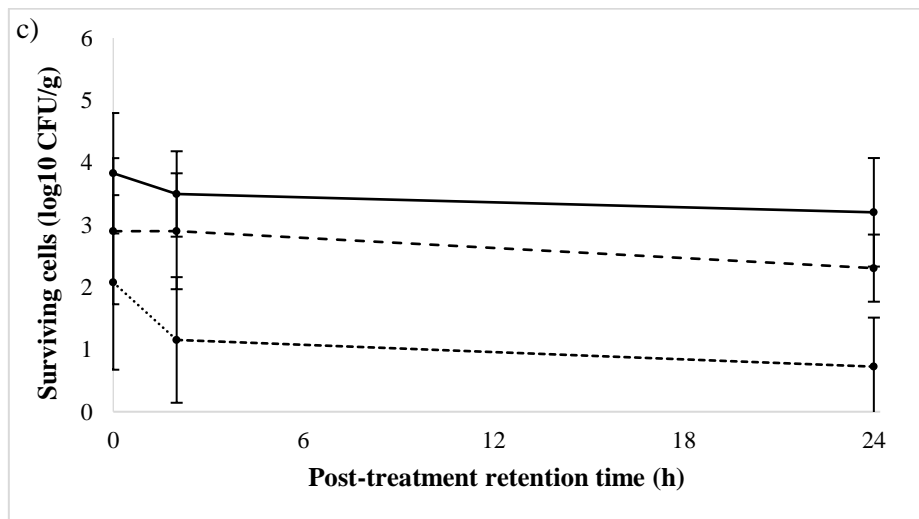
814 **Figure 5**



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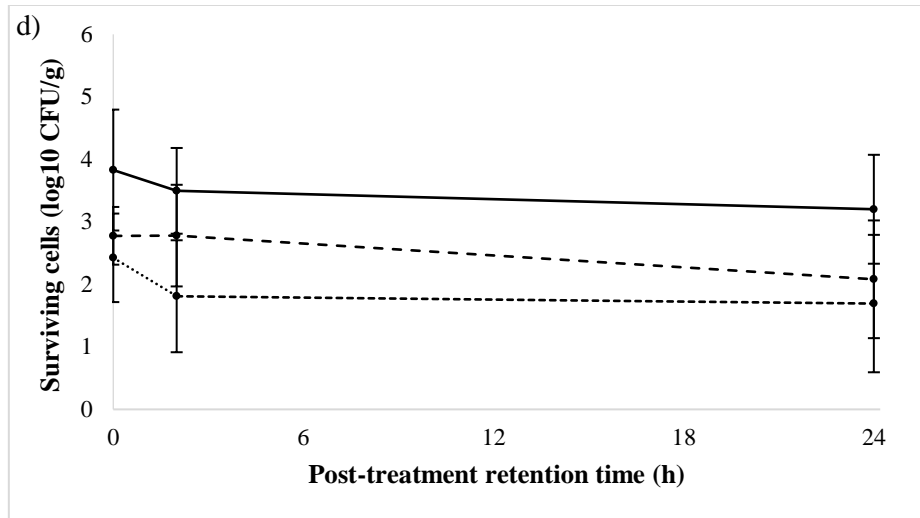


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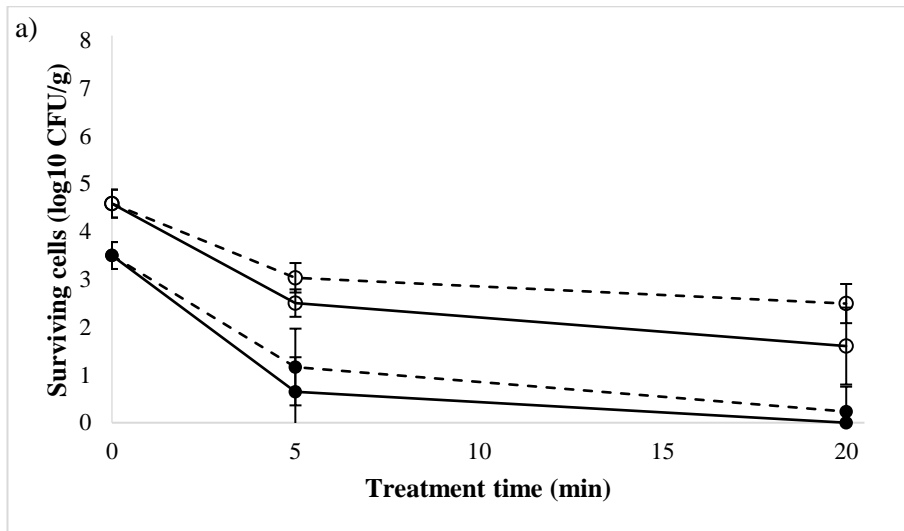
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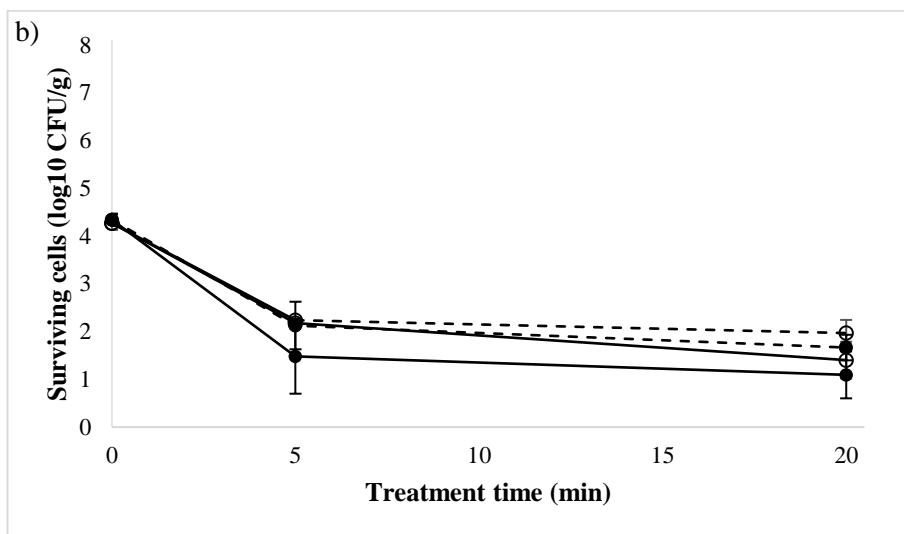


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819 **Figure 6**

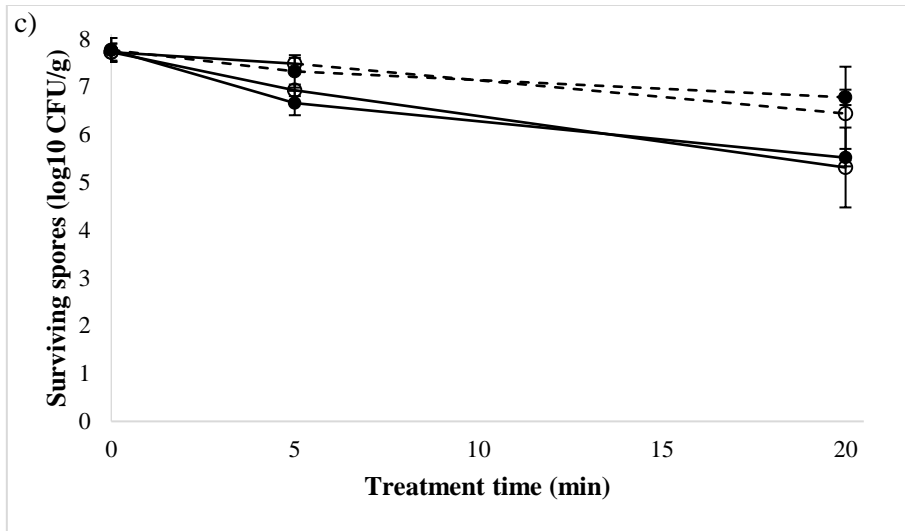


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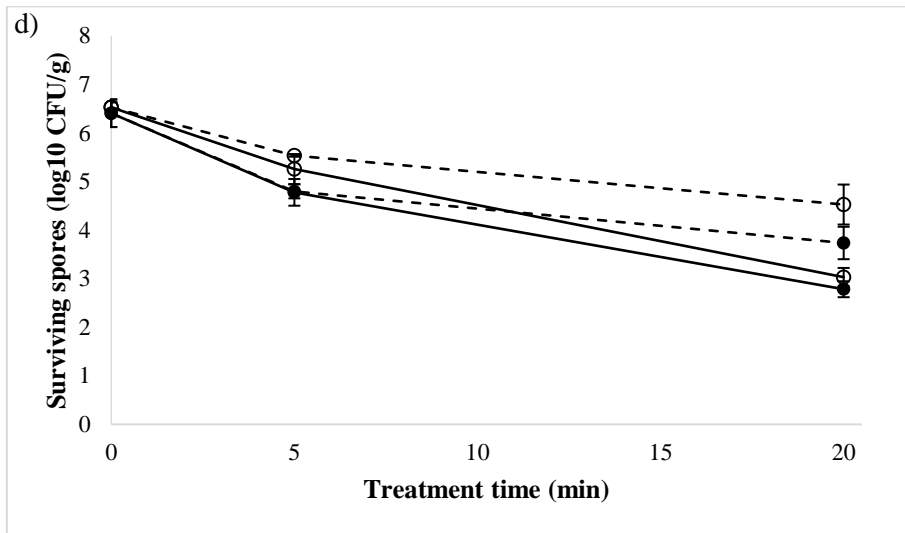


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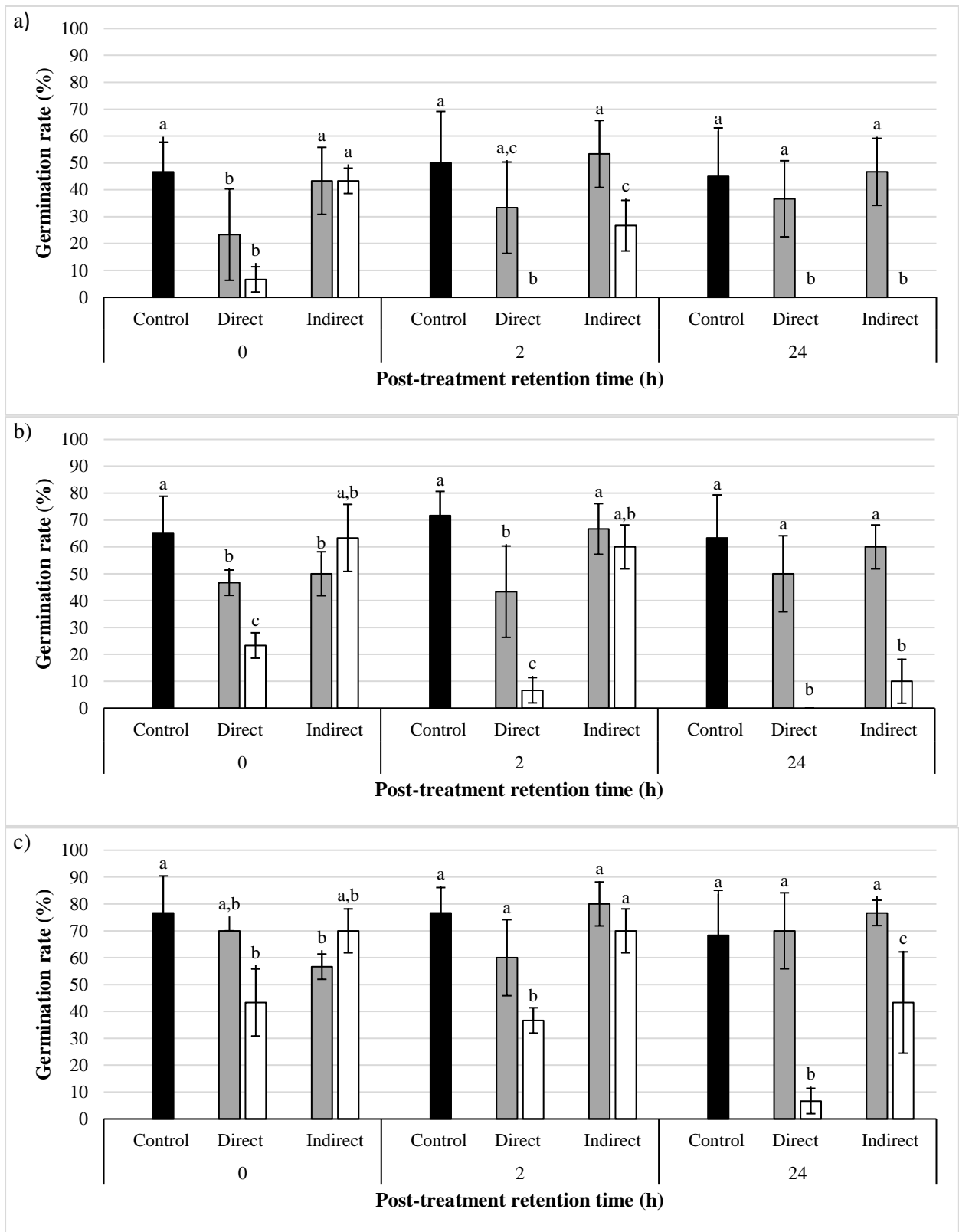
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831 **Figure 7**



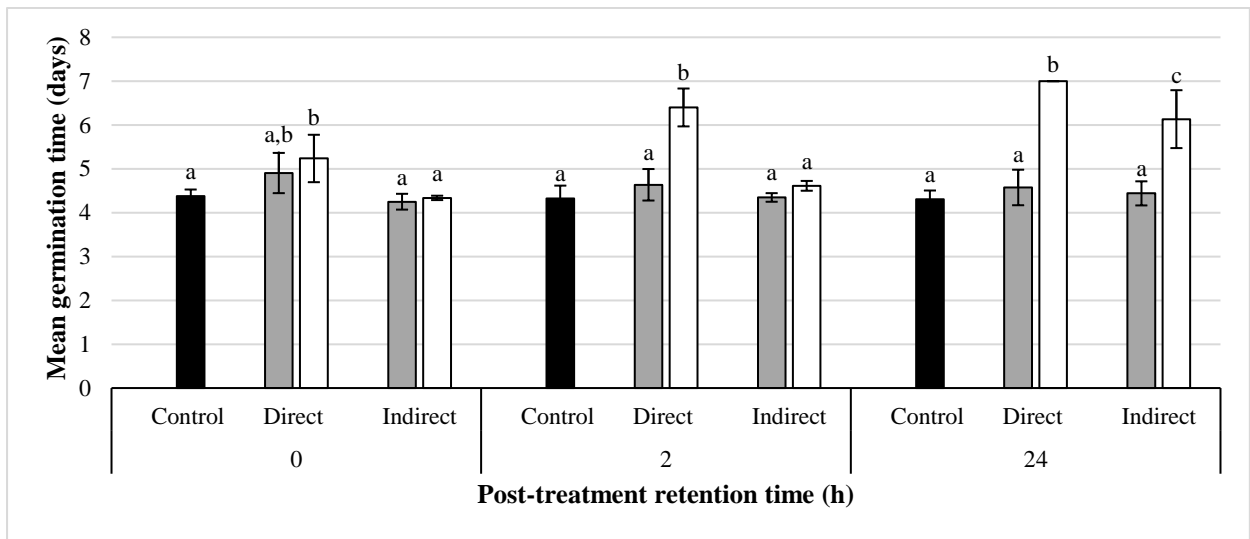
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836 **Figure 8**



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840 **Figure 10**

841 (a)

(b)

(c)

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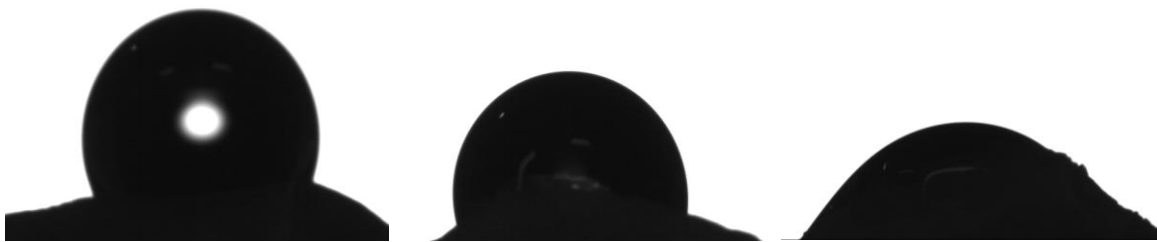
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848 **Tables**

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

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

857 Table 3. Parameter estimates and 95% confidence bounds for the response surface model  
858 relating treatment, retention and incubation time with the germination percentage. The Root  
859 Mean Squared Error (RMSE) of each model is also presented. This error is an estimate of the  
860 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 (°)	$\gamma_{tot}$ [mN/m]	CA (°)	$\gamma_{tot}$ [mN/m]	CA (°)	$\gamma_{tot}$ [mN/m]
0	Control	0	104.6 <sup>a</sup> ± 5.5	20.3 <sup>a</sup> ± 3.3	78.1 <sup>a</sup> ± 2.7	21.0 <sup>a</sup> ± 1.2	61.1 <sup>a</sup> ± 11.0	30.8 <sup>a</sup> ± 5.3
	Direct	5	84.7 <sup>b</sup> ± 1.7	32.5 <sup>b</sup> ± 1.1	41.7 <sup>b</sup> ± 1.9	37.6 <sup>b</sup> ± 0.8	38.8 <sup>b,c,d</sup> ± 4.5	37.6 <sup>b</sup> ± 5.5
		20	45.8 <sup>c</sup> ± 6.0	56.1 <sup>c</sup> ± 3.3	41.0 <sup>b</sup> ± 4.5	37.7 <sup>b</sup> ± 1.6	32.0 <sup>b</sup> ± 2.6	43.9 <sup>c</sup> ± 1.0
	Indirect	5	102.7 <sup>a</sup> ± 4.1	21.5 <sup>a</sup> ± 2.5	75.9 <sup>a</sup> ± 4.4	22.0 <sup>a</sup> ± 2.0	49.0 <sup>c</sup> ± 4.3	36.6 <sup>b</sup> ± 2.0
		20	105.8 <sup>a</sup> ± 5.1	19.6 <sup>a</sup> ± 3.0	74.4 <sup>a</sup> ± 3.9	22.7 <sup>a</sup> ± 1.8	41.3 <sup>c,d</sup> ± 3.6	40.1 <sup>b,c</sup> ± 1.5
	2	Control	0	102.8 <sup>a</sup> ± 4.2	21.4 <sup>a</sup> ± 2.6	77.2 <sup>a</sup> ± 2.3	21.3 <sup>a</sup> ± 1.1	56.8 <sup>a</sup> ± 4.3
Direct		5	83.5 <sup>b</sup> ± 0.7	33.3 <sup>b</sup> ± 0.5	36.5 <sup>b</sup> ± 1.8	39.7 <sup>b</sup> ± 0.7	35.6 <sup>b</sup> ± 1.2	42.4 <sup>b,d</sup> ± 0.5
		20	45.3 <sup>c</sup> ± 5.5	56.5 <sup>c</sup> ± 3.2	38.5 <sup>b</sup> ± 5.0	38.3 <sup>b</sup> ± 1.8	30.8 <sup>b</sup> ± 1.5	44.4 <sup>b</sup> ± 0.6
Indirect		5	94.8 <sup>d</sup> ± 4.7	26.3 <sup>a</sup> ± 2.9	74.0 <sup>a</sup> ± 4.5	22.9 <sup>a</sup> ± 2.1	54.1 <sup>a,c</sup> ± 1.7	34.2 <sup>a,c</sup> ± 0.8
		20	100.3 <sup>a,d</sup> ± 5.1	22.9 <sup>a</sup> ± 3.1	69.9 <sup>a</sup> ± 2.5	24.8 <sup>a</sup> ± 1.2	44.4 <sup>b,c</sup> ± 2.1	38.7 <sup>c,d</sup> ± 0.9
24		Control	0	106.4 <sup>a</sup> ± 5.9	19.2 <sup>a</sup> ± 3.5	77.9 <sup>a</sup> ± 6.2	21.1 <sup>a</sup> ± 2.8	55.8 <sup>a</sup> ± 6.4
	Direct	5	86.2 <sup>b</sup> ± 2.7	31.6 <sup>b</sup> ± 1.7	41.9 <sup>b</sup> ± 5.5	37.5 <sup>b</sup> ± 2.3	41.7 <sup>b,c</sup> ± 4.2	39.9 <sup>b</sup> ± 1.9
		20	39.7 <sup>c</sup> ± 1.6	59.5 <sup>c</sup> ± 0.8	40.4 <sup>b</sup> ± 3.6	38.1 <sup>b</sup> ± 1.5	33.0 <sup>c</sup> ± 4.7	43.4 <sup>b</sup> ± 1.8
	Indirect	5	99.8 <sup>a</sup> ± 3.2	23.2 <sup>a</sup> ± 2.0	79.3 <sup>a,c</sup> ± 2.0	20.4 <sup>a</sup> ± 0.9	52.9 <sup>a,b</sup> ± 2.9	34.8 <sup>a,b</sup> ± 1.3
		20	105.8 <sup>a</sup> ± 1.3	19.5 <sup>a</sup> ± 0.8	72.7 <sup>a</sup> ± 1.0	23.5 <sup>a</sup> ± 0.5	45.5 <sup>b</sup> ± 3.2	38.2 <sup>b</sup> ± 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	<i>B. atrophaeus</i> spores	0.077	-0.004	1.39E-03	0.019	0.001	7.56E-04
	<i>B. atrophaeus</i> cells	0.181	0.044	-1.29E-03	0.154	0.109	-5.44E-03
	<i>E. coli</i>	0.176	0.083	-4.31E-04	0.124	0.145	-2.86E-03
	<i>P. verrucosum</i>	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

876