Effects of High-Pressure Carbon Dioxide on Microbial Quality and Germination of Cereal Grains and Beans

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Running title: CO_2 inactivation of low a_w foods

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Abstract

Decontamination of low a_w foods without impairing the food quality is challenging, particularly for sprouting seeds. This study aimed to explore the lethality of high pressure carbon dioxide (HPCD) to *Salmonella enterica*, *E. coli* AW1.7, *A. niger*, *P. roquforti*, and *Fusarium* on beans, cereal grains, and ground pepper. The optimal antimicrobial effect of HPCD was achieved by equilibration of the a_w to 0.75, followed by soaking in water and treatments with water-saturated CO_2. HPCD reduced the viable cell counts of *E. coli* and *Salmonella* by 3 to 10 log(CFU/g), and prevented fungal growth during 10 d of incubation. More than 90% of mung beans germinated but the germination of oats was impaired after fungicidal or bactericidal treatments with HPCD. Overall, HPCD can be a promising antimicrobial treatment but treatment parameters need to be optimized for each type of seed.

Key words: *E. coli*, *Fusarium*, High-pressure carbon dioxide, malting, *Salmonella*, spoilage fungi, sprouts

1. Introduction

Legumes and cereals are important staple foods worldwide. Microbiological contamination of legumes and cereal grains occurs during plant growth, harvest, and storage [1]. Microorganisms present on or in cereals and legumes are derived from plant microbiota, including seed endophytes [1–3] and phytopathogenic fungi [4] or represent post-harvest contaminants, including storage molds such as *Penicillium*, *Eurotium*, and *Aspergillus* [5] and low-infectious dose foodborne pathogens such as Shiga-toxin producing *Escherichia coli* (STEC) and *Salmonella* [6,7]. Seed endophytes include bacterial species with an alternate lifestyle as opportunistic pathogens or foodborne pathogens; examples include *Klebsiella pneumoniae*, *Cronobacter sakazakii* and organisms of the *Bacillus cereus* group [8–10]. Infection with *Fusarium* spp. and storage molds during plant growth or storage, respectively, results in a loss of grain yield and malt quality and may support mycotoxin formation during pre- or post-harvest stages [4,11]. *Salmonella enterica* and *Escherichia coli* are both able to colonize plants after pre-harvest contamination [12,13].

Dry storage of legume seeds and cereal grains prevents microbial activity and the development of filamentous fungi but does not eliminate contaminants. Most post-harvest disinfection treatments result in a low-level survival of microorganisms on or in seeds [14]. Additionally, bacteria, including pathogenic bacteria are more resistant to heat and other interventions in the dry state when compared to high moisture environments, making the decontamination of low water activity (a_W) foods including seeds and spices challenging [15]. As a consequence, the presence of pathogenic or toxin–producing microorganisms on seeds and grains poses a potential health risk. The health risk associated with microorganisms on legumes and cereal grains is enhanced by sprouting and malting; first, because environmental conditions for germination support microbial growth and toxin formation; second, because intervention

treatments of seeds intended for sprouting or malting must maintain the viability of the plant embryo. Sprouts frequently cause outbreaks of foodborne illness [14,16], including infections by *Salmonella* and STEC [17,18]. Seed decontamination with chlorine retains the germination capacity but only moderately reduces microbial counts [19,20]. Physical preservation methods, including thermal treatments and microwave heating, high hydrostatic pressure, or UV light reduce microbial contaminants but also impair the quality, viability and enzyme activity of seeds [21–23]. Thus, novel and cost-effective approaches to reduce bacterial and fungal contaminants on seeds are of major interest to the food industry.

High-pressure CO_2 (HPCD) treatment is a promising preservation technology for high moisture food products but only a few studies explored its use for dry foods [24–26]. The lethality of HPCD depends on the pressure and temperature, and whether liquid, gaseous or supercritical CO_2 is used [26,27]. The term HPCD is used to encompass CO_2 at pressures above ambient, regardless of its state. Bacteria in a low moisture environment are more resistant to HPCD when compared to high moisture environments [26,28]. Remarkably, supercritical CO_2 is more bactericidal than gaseous CO_2 in high moisture environments while the opposite was reported for inactivation of *E. coli* and *Salmonella* under low moisture conditions [24,27]. In addition, the water activity during treatment was identified as a key determinant of the lethality of HPCD [24]. The moisture content and the moisture sorption isotherm are specific for different foods [29], and HPCD treatment parameters must be optimized specifically for each food in question, particularly if treatments aim to reduce microbial contaminants while maintaining the viability of the plant embryo. This exploratory study therefore aimed to assess the potential application of HPCD to inactivate epiphytic, phytopathogenic and toxigenic microorganisms occurring on the surface of foods, and to evaluate the effects of HPCD on the seed germination. Six representative foods were

used in this study; these included coarse ground black peppercorns (ground pepper), and three types of plant seeds from legumes (soybeans and mung beans), oilseeds (flax seeds), and cereals (oat and barley grains). Plant seeds were selected to include seeds that are commonly used for sprouting and major Albertan crops. Target organisms used in this study included *E. coli* AW 1.7 and *Salmonella enterica*, the spoilage molds *Penicillium roqueforti* and *Aspergillus niger*, and phytopathogenic *Fusarium* spp.. *E. coli* AW1.7 is a non-pathogenic isolate from a meat processing plant, and its resistance to heat and HPCD is equal to or higher than that of STEC; thus, it is used as a surrogate organism to STEC [24,30,31]. *Salmonella enterica* was represented by a cocktail of five strains isolated from human, food and environment, and all of them exhibited high resistance to dry heat and HPCD [24,32].

2. Materials and methods

2.1. Bacterial and fungal strains and culture conditions

Bacterial and fungal strains used in this study are listed in Table 1. Strains were streaked out from 30% glycerol stock stored at -80 °C. *E. coli* and *Salmonella* were cultivated on Luria-Bertani (LB) agar (Fisher Scientific, Ottawa, ON, Canada) and incubated aerobically at 37 °C for over 18 h. A single colony was inoculated into 5 mL of LB broth and incubated at 37 °C with agitation at 200 rpm. To obtain a high cell density inoculum, liquid subcultures of 100 µL were plated onto the surface of LB agar and incubated at 37 °C for over 18 h. The bacterial lawn was removed with 1 mL of 0.1% peptone (BD Difico^{TM}, Mississauga, ON, Canada). Bacterial cells were collected by centrifugation and re-suspended in 1 mL of 0.1% peptone water (BD) to obtain inocula with a final concentration of 10-11 $\log_{10}$ CFU/mL. To create a cocktail of *Salmonella*, inocula of 5 strains of *Salmonella* were combined in equal volumes.

Fungal strains were cultivated on malt extract agar and incubated aerobically at 25 °C for 7 d. Spores were collected in 5 mL of physiological solution (0.85% NaCl, 0.01% Tween80), collected by filtration through filter paper (Whatman®, Fisher Scientific, Ottawa, ON, Canada), harvested by centrifugation, and re-suspended in 1 mL of physiological saline. The spore density was determined microscopically with a hemocytometer (Fein-Optik, Jena, Germany) and ranged from from10^3 to 10^6 spores/mL.

2.2. Sample preparation

Legume and flax seeds, and ground pepper (obtained from a local supermarket in Edmonton, AB, Canada) were decontaminated, and inoculated with bacterial cultures and/or fungal spores, as outlined below. Barley and oats were not decontaminated or inoculated with lab strains; therefore, the microorganisms derived from them represent the microbiota naturally present on these grains. Dehulled oat grains were kindly provided by Ceapro Inc. (Edmonton, AB, Canada), which were treated with a mild surface heat treatment by the supplier to inactivate surface enzymes. Hulled barley grains were obtained from an Albertan farm. Approximately 4% of the barley grains were naturally contaminated with *F. graminearum*. Barley grains that exhibited the visual characteristics of *Fusarium* head blight were selected manually and apparently healthy grains were discarded. An overview of the selection of plant material, decontamination treatments, and the treatments with HPCD is provided in Fig. S1 (bacterial contaminants or inocula) and Fig. S2 (fungal contaminants or inocula).

Sample decontamination and inoculations. To reduce microbial contaminants from flax seeds, soybeans and mung beans, 20 g of each plant seeds were treated with 0.2% chlorine for 15 min, followed by rinsing twice with sterilized H_2O. Then, plant seeds were air dried in a biosafety cabinet and stored in a desiccator at room temperature (23 °C) to keep the embryo dry for up to 4

months until use. Ground pepper was sterilized by autoclaving at 121 °C for 30 min. To ensure that the decontaminated samples were free of microorganisms, samples (0.1-0.15 g) were resuspended in 1 mL 0.1% peptone water, and the suspension was plated onto LB to determine the microbiological contamination. The microbial load was below the detection limit of 200 CFU/g.

Decontaminated foods were inoculated with 1 mL of bacterial culture or fungal spores separately. Bacterial cultures outlined in Section 2.1 were mixed with 1 g of ground pepper or flax seeds, or 5 g of soybeans or mung beans until the maximum absorption was reached. Another 5 g of soybeans or mung beans were inoculated with *A. niger*, or *P. roqueforti* with the same methods as above. After inoculation, samples were immediately air dried in a biosafety cabinet for 2 h and stored in a desiccator to keep the sample at dry conditions.

Preparation of samples for treatment. Inoculated foods were kept at dry conditions before treatments. To increase the water content that aims to achieve an optimal antimicrobial effect by HPCD, different foods were treated differently to increase the water content. Step 1, for foods inoculated with bacteria and air dried, 0.1 g of ground pepper or flax seeds, and two mung beans or one soybean (ca. 0.1-0.15 g) were transferred into a glass vial (Sigma-Aldrich, St Louis, MO, USA) and stored in an air-tight container with a saturated solution of NaCl for 3 d to equilibrate the sample to a_w of 0.75. The a_w of each food was measured by a a_w meter (AquaLab PRE, Decagon Devices, Pullman, WA, USA). Step 2, to increase the water content of plant seeds prior to treatment, 0.1-0.15 g of seeds were soaked in sterile H_2O for 20 min; whereas, ground pepper was treated without prior soaking. Initial bacterial cell counts on inoculated samples ranged from 8 to 10 log(CFU/g). All samples inoculated with bacteria (0.1-0.15 g) were treated with heat or water-saturated CO_2 or N_2 in the mobile CO_2 pasteurization apparatus described in Section 2.3.

To determine the antifungal effects of HPCD, legumes and cereal grains with fungal contaminants were soaked in water (Step 2) prior to treatment. At each experiment, 1 soybean and 2-3 mung beans inoculated with the same fungal strains were treated. Two barley grains and two oats grains were treated simultaneously.

2.3. Treatments with pressurized gas

HPCD treatments were carried out in a custom-built mobile CO_2 pasteurization apparatus described previously [24]. The system was equipped with a syringe pump (Model 260D, Teledyne ISCO Inc., Lincoln, NE, USA) and a high-pressure vessel (V=42.53 mL). The pressure vessel and the supply lines were heated with electric heating bands (Brisk Heat, Columbus, OH, USA). The temperature was monitored by thermocouples (CN7500, Spectris Canada, St-Eustache, QC, Canada) installed before, after and in the vessel, and attached to temperature controllers (Maxthermo, MC-2438, Taipei, Taiwan). The pressure was controlled by the pump and the depressurization rate was manually controlled by a micro-metering valve placed at the outlet of the vessel. A 0.2 µm filter was installed in line at the exit to prevent microbiological contamination. The system was preheated to 25, 35, or 65 °C and flushed with CO_2 or N_2 before pressurization. The system was kept at atmospheric pressure (1.013 bar) or 57 or 120 bar for 15 min, followed by de-pressurization at a rate of 60 bar/min. Sterile water was added onto a filter paper placed on the top shelf of the basket to ensure the CO_2 and N_2 in the system was saturated with water as described previously [24], which is referred to as water-saturated CO_2 or N_2. Dried samples not subjected to high-pressure CO_2 or N_2 treatments were untreated controls. Dried samples treated at 25, 35 and 65 °C without high-pressure CO_2 or N_2 in the same unit for 15 min serve as temperature controls.

2.4. Microbiological determination and seed germination

166 The lethality of treatments on bacteria and fungi were determined by bacterial enumeration
167 and visual observation, respectively. To enumerate viable bacterial cells, bacteria on treated food
168 samples (0.1-0.15 g) and their respective controls were washed off by vortexing in 1 mL of 0.1%
169 peptone water. The cell suspension was serial-diluted and plated onto LB for *E. coli* and
170 *Salmonella* inoculated samples. Microorganisms on oats and barley were determined by plating
171 on LB and Plate Count Agar (PCA). Colonies were counted after aerobic incubation at 37 °C for
172 18 h.

173 Seed germination and growth of fungi or molds on legumes and cereal grains were
174 observed for 10 d. Treated and untreated seeds were placed on a filter paper (Whatman®, Fisher
175 Scientific, Ottawa, ON, Canada) and transferred to a petri-dish. One-microliter of sterile H_2O was
176 added to the filter paper to provide humidity for germination. Then, the petri-dish was sealed with
177 wrapping film (Parafilm™ M, Fisher Scientific, Ottawa, ON, Canada) and incubated at 25 °C for
178 10 d. Development of mycelia was recorded as positive growth of fungi.

179 *2.5. Data analysis*

180 The antifungal effects of treatments were determined as the ratio of mold-free seeds to total
181 seeds from all treated seeds, corrected for the fraction of moldy seeds in untreated controls.
182 Individual seeds were considered as experimental units. The number of seeds from each
183 independent experiment using the same treatment parameters was added up for calculations. The
184 total number of treated beans is as follows: Twenty-five mung beans or soybeans treated at 65 °C
185 and 57 bar; three soybeans or mung beans for each fungal strain treated at 25 or 35 °C and 1.013
186 bar; nine mung beans or soybeans treated at 65 °C and 1.013 bar, 25 or 35 °C and 57 or 120 bar.
187 A total of six barley and oat grains were included in each HPCD treatment under different
188 conditions. The treatment effect was calculated using Eq. (1).

$$Treatment\ effect = \frac{\left(\frac{\#\ of\ treated\ and\ non\text{-}moldy\ seeds}{\#\ of\ treated\ seeds}\right)}{\left(\frac{\#\ of\ untreated\ and\ moldy\ seeds}{\#\ of\ untreated\ seeds}\right)} \quad (1)$$

All experiments were conducted with three or more independent replicates. The bacterial cell counts were log-transformed and the reduction of cell count was calculated as $\log_{10}(N_0/N)$ or $\log_{10}(N_1/N)$, with N_0 representing the cell counts of culture without any treatment, N_1 representing the cell counts after inoculation and drying (refer to Section 2.2), and N representing cell counts after treatments. Data were analyzed by two-way analysis of variance (ANOVA) using Tukey's test ($P<0.05$) to determine the statistical differences among means.

3. Results

3.1. Validation of food-specific pre-treatments prior to HPCD

The bactericidal effect of HPCD is mediated by dissolved CO_2 and dependent on the water activity of foods [24]; therefore, preliminary experiments assessed the effect of equilibration of the a_W of sample and soaking prior to HPCD treatment and water addition during HPCD treatment on the bactericidal effects by HPCD as described in Sections 2.2 and 2.3. Without equilibration of the a_W of samples and saturation of CO_2 with water, the lethality of HPCD to *E. coli* AW1.7 on ground pepper was 0.44±0.00 log(CFU/g) (Table 2). When CO_2 was saturated with water, the lethality to *E. coli* AW1.7 was ca. 1 log(CFU/g) on ground pepper and soybeans, and 2.3 – 3.4 log(CFU/g) on flax seeds and mung beans (Table 2). By testing different water addition strategies to enhance the antimicrobial effects of HPCD, food-specific methods were developed for each type of food. In the subsequent experiments, ground pepper was equilibrated to a_W 0.75; plant seeds for bacterial inactivation were equilibrated to a_W 0.75, followed by soaking in water for 20 min. All samples were treated with water-saturated high-pressure gas. Plants seeds contaminated with fungi were soaked in water prior to treatment, without the need for a_W adjustment.

3.2. Effect of HPCD on seed germination

The effect of HPCD on seed germination is critical for plant seeds that are used in malting or sprouting. The effects of treatment pressure and temperature on seed germination are shown in Table 3. The germination rate of control and treated samples was highly dependent on the type of seed. Soybean and barley germinated poorly at control conditions with only 8% and 11% germinated, respectively, which was further reduced by the treatments (Table 3). In contrast, germination was observed in 96% of mung beans without treatment. Treatment of mung beans with heat and HPCD did not affect the ability of more than 90% of mung beans to germinate. Germination was observed for untreated oats (58%) and germination was not affected by heat treatment, but HPCD treatment strongly reduced the proportion of germinated oat grains.

3.3 Antifungal effects of heat and high-pressure CO_2

The antifungal effects of treatments were calculated on the basis of the number of mold-free beans or cereal grains after treatment and incubation for 10 d (Table 4). Mung beans and soybeans were challenged by inoculation with *A. niger* or *P. roqueforti*. Treatments at 25 and 35 °C were not lethal to *P. roqueforti* and *A. niger,* but an increase in the treatment temperature and/or pressure enhanced the lethality towards both fungi. The treatment effect of heating to 65 °C alone was 0.5-0.7 for *A. niger* and 0.3-0.7 for *P. roqueforti*. At low temperatures, an increase in pressure to 57 bar, which maintains CO_2 in the gas phase, enhanced the lethality of HPCD to *A. niger* to 0.1-0.4 at 25 °C and 0.3-0.7 at 35 °C; the latter one was similar to the effect of heating at 65 °C alone. At 120 bar, CO_2 is supercritical at temperatures above 31 °C, and the antifungal effects were diminished to less than 0.5 at 35 °C. In addition, the effect of HPCD at 57 bar against fungi was generally more effective on mung beans than soybeans, and the lethality to both fungi on mung bean was greater by at least 0.2 than that on soybean. Maximum lethality (>0.9) to *A. niger* and *P.*

234 *roqueforti* was achieved by HPCD at 65 °C and pressures ≥57 bar. All mung beans treated with
235 HPCD at 65 °C remained mold-free.

236 The antifungal effects of HPCD treatment were validated on naturally contaminated
237 cereals; the barley grains selected for treatment showed visible signs of *Fusarium* head blight.
238 Similar to *A. niger* and *P. roqueforti*, temperatures of 25 or 35 °C had no or low lethality (<0.3)
239 against field molds on barley and oat grains. Different from the effect of HPCD against *A. niger*
240 and *P. roqueforti* on soybean and mung bean, gaseous CO_2 at 57 bar and all temperatures
241 eliminated fungi on oats and barley. Treatment with CO_2 at 120 bar and 35 °C was equally effective
242 as treatment with CO_2 at 57 and 35 °C (Table 4).

243 *3.4. Bactericidal effects of drying, heat, and high-pressure CO_2 or N_2 on E. coli and*
244 *Salmonella on seeds*

245 To determine whether the bactericidal effects of HPCD on dry foods are attributed to CO_2,
246 the lethality of drying and HPCD at 57 bar and 65 °C was compared to the lethality of heating
247 without compression, and to the lethality of high-pressure N_2 at the same temperature (Fig. 1).
248 Bacterial cell counts on seeds after drying and soaking were reduced by less than 1 log(CFU/g)
249 after inoculation with *E. coli* and *Salmonella*, except for *E. coli* AW1.7 on mung beans (>1 log).
250 The lethality of treatment at 65 °C varied among the samples tested and was less than 1 log(CFU/g)
251 for *E. coli* AW1.7 and *Salmonella* cocktail on ground pepper and flax seeds, less than 3 log(CFU/g)
252 on soybeans, and 3-6 log(CFU/g) on mung beans (Fig. 1). The lethality of treatments with high-
253 pressure N_2 at 65 °C was similar (P>0.05) to that of heating at 65 °C for all the samples tested.

254 Treatments with water-saturated CO_2 at 65 °C reduced cell counts of *E. coli* and *Salmonella*
255 by 9-10 log(CFU/g) on flax seeds. Cell counts of *E. coli* AW1.7 on ground pepper were reduced
256 by more than 8 log(CFU/g), while cell counts of the *Salmonella* cocktail were reduced only by 4

log(CFU/g) (Fig. 1). On the other foods, the viable cell counts of *E. coli* and the *Salmonella* cocktail were not different (P>0.05) after treatments with heat alone and high-pressure gases (Fig. 1). The treatment effects on mung beans had a very high experimental error; consequently, the survival of viable cells after treatments was not different (P>0.05) compared to the samples after drying. The lethality of HPCD to bacteria on mung bean and soybean was 5-6 log for *E. coli* and 3-3.5 log for *Salmonella* despite the high variation on mung beans. For soybean, the lethality of HPCD to *E. coli* AW1.7 was higher (P<0.05) than other treatments; however, the effect of HPCD on *Salmonella* cocktail was only significantly different from the lethality by drying.

3.5. Bactericidal effects of HPCD against natural microbiota of barley and oat

The bactericidal effects of HPCD were further validated on naturally contaminated cereal grains (Fig. 2). Bacteria on barley grains were more sensitive to high temperature when compared with *E. coli* AW1.7 and *Salmonella*. Treatments at 25 and 35 °C did not reduce the viable cell counts compared to other treatments. The lethality of treatments at 35 °C and CO_2 at 57 or 120 bar did not differ (P>0.05). The effects of treatments at 65 °C alone and in combination with gaseous CO_2 at 57 bar as well as the HPCD treatment at 35 °C were similar and significantly enhanced the reduction of total counts enumerated on LB and PCA.

The initial microbiological load on dehulled oat grains ranged from 3 to 7 log(CFU/g). After all the treatments, the average cell counts were below the detection limit of 2 log(CFU/g) (Table S1, Supplementary Material).

4. Discussion

This exploratory study provided proof of concept that HPCD can be used to reduce or eliminate microbial contaminants on some plant seeds, however, optimization of food-specific

pretreatments is crucial to achieve an antimicrobial effect of HPCD. In addition, further optimization is needed for those seeds targeted for germination since not all seeds germinated after the HPCD treatment. The treatment conditions that were lethal to bacteria and fungi achieved >90% germination of mung beans, which is comparable to the traditional NaOCl treatment but HPCD is more bactericidal to *E. coli* [25,33,34]. Barley grains with hulls are naturally contaminated with a heavy load of microorganisms [4,5]; using HPCD as a post-harvest intervention significantly reduced the microbiological load (this study) even though the germination was reduced after treatments. It is of value to use HPCD for cereal grains that are not targeted for malting.

Seed germination is not only affected by antimicrobial intervention treatments but also depends on the natural germination capacity of the specific seed type [25,35,36]. The water absorption of plant embryo is seed-specific due to the different surface topography and water absorption isotherm [19,37]. In the present study, the effect of HPCD on germination was evaluated with four types of seeds (soybean, mung bean, barley, oat). The water absorption capacity of soybeans was 2-5 times higher than that of mung beans [37,38], resulting in different levels of bean hydration. Germination of barley was completely inactivated when water was added to the grains during HPCD treatment [36]. Water around and in the seeds in contact with CO_2 generally lowers the pH in the environment by forming H_2CO_3 and forms bicarbonate complex with intracellular substances, which causes inactivation of enzymes required for seed germination, such as α-amylase and lipase [39,40]. Germination of mung beans was the least impacted by HPCD likely due to their waxy coat and low water absorption capacity, which might help alleviate any damage to the seed embryo due to the pressure and solubilized CO_2. Seed-dependent effect on germination was also demonstrated after high hydrostatic pressure treatment, which had limited

damage to seeds with low water absorption capacity [41]. Barley grains germinated poorly even without treatment in this study; however, Park et al. [36] found that germination of barley was 95% after HPCD at 100 bar and 50 °C. The weak germination level of barley used in the present study reflects the selection of *Fusarium* infected grains. Soybean germinated poorly for unknown reasons. Pressure and temperature (>60 °C) also reduced the germination of barley and alfalfa seeds [33,35,36].

Hydration levels of each food likely contributed to the differences in the bacterial reduction levels. Mucilage on the surface of flax seeds strongly absorbs water during soaking [42], and water uptake of cereal grains is impacted by the presence of hulls [43], suggesting the highest inactivation of *E. coli* in flax seeds and lowest on soybeans. Generally, HPCD is bactericidal only in the presence of water, regardless of increases in pressure and temperature [44–46]. Antimicrobial effects of HPCD have been facilitated by the membrane disruption, and intra- and extracellular acidification due to the high diffusion power and solubility of CO_2 [26,35]. Water plays an important role in the biochemical reaction mediated by dissolved CO_2; however, in a dry (food) matrix, such reactions are impeded due to the limited sorption of CO_2 in the lipid and cytoplasm membrane of microbial cells [26,47]. The use of water and/or ethanol as cosolvents in supercritical CO_2 increases the polarity of the solvent [48] and substantially increased the treatment lethality against conidia of *A. niger* [44] and *P. oxalicum* [36], *E. coli* and *Salmonella* [24,27, this study] in a dry environment. Inactivation of *A. niger* spores depended on the temperature and the amount of solubilized CO_2 [49]. The fungal and bacterial inactivation was consistently enhanced by adding water to the food matrix [24,36] or using water-saturated CO_2 [24, this study] during HPCD treatment. On the time scale typically used for HPCD treatments in this study, the moisture content

of microbial cells can be considered to be in equilibrium with the surrounding matrix and thus serves as a cosolvent dictating the amount of CO_2 absorbed by the cells [47].

Recognizing that control of the water in food matrix is critical for the antimicrobial efficacy of HPCD, seeds used in the present study were equilibrated to a_W 0.75, soaked with water prior to treatments, and then treated with water-saturated CO_2. This treatment protocol increased water availability only on the surface of the seeds, because full hydration of legume seeds and cereal grains during soaking requires several hours [43,50]. The seeds treated in this study were thus not in equilibrium with respect to the moisture content, with a hydrated exterior but a dry interior. This was sufficient for microbial inactivation since microbial contamination was on the surface and not in the interior, but protected the plant embryo in the interior of the seeds. It is suggested that equilibration of a_W can be replaced by soaking because equilibration of a_W alone was not as effective as the case involving the soaking step. In addition, a_W of foods will increase after soaking in water. Taken together, due to the difference in water absorption of seeds, application of HPCD requires careful validation of the water content of seeds to optimize the antimicrobial efficacy and germination rate.

The state of CO_2 is associated with different antimicrobial mechanisms owing to the different density, solubility in aqueous medium, and solvent power of gaseous, liquid and supercritical CO_2 [27]. Supercritical CO_2 is lethal to microorganisms in a high moisture environment [45–47,51,52]; but not in a dry environment [24,27]. The lethality of liquid and supercritical CO_2 on dry foods/matrix generally requires a treatment time of >60 min [25,26]; however treatment with gaseous CO_2 at 65 °C for 15 min achieved a higher bacterial reduction than supercritical CO_2 [24,27]. The present study adds further evidence that gaseous CO_2 at moderate temperature (65 °C) is bactericidal in dry foods by achieving >5 log reduction of *E. coli*

and *Salmonella*. Gaseous CO_2 has a higher diffusion rate than supercritical CO_2, which is further increased by elevated temperature, thus it diffuses and disrupts cell membranes [53]. Comparing the treatment lethality of CO_2 with high-pressure / high-temperature treatments or the use of high temperature only demonstrates that the effect of HPCD is not caused by the combination of pressure and temperature but depends on the specific properties of CO_2 [24,46,54, this study].

The present study extends prior observations in bacteria by demonstrating the inactivation of fungal conidiospores in dry foods using HPCD and documenting the inactivation of natural contamination with *Fusarium* spp. Because the contamination of grains with *Fusarium* is almost invariably related to pre-harvest infection of the plant [4], we employed *Fusarium* infected grains rather than artificial surface contamination in the laboratory. Few studies reported the lethality of supercritical CO_2 with conidiospores of *Aspergillus* and *Penicillium* spp. as targets [54–56]. Haas et al. [57] observed that gaseous CO_2 at 45 °C reduced conidiospores of *P. roqueforti* by 5 log (CFU/g) after 2 h. The present study showed that gaseous CO_2 at 65 °C was lethal to bacteria, fungi and molds after only 15 min. Treatment at 65 °C alone without pressure had <2.5 log reduction of conidia of *A.niger* [54], and moderate effects on fungal inhibition on food substrates, which was similar to the HPCD treatments at 35 °C and 57 bar (this study). When *A. niger* was treated with supercritical CO_2 at 65 °C and 350 bar, the lethality was equivalent to the treatment at 65 °C [54]. However, the lethality of HPCD to *A. niger* was enhanced by gaseous CO_2 at 65 °C and 57 bar (this study), which further indicates that the antimicrobial effect depends on the physical state of CO_2.

5. Conclusions

Although innovative decontamination approaches for seeds have been broadly investigated [23], achieving decontamination without compromising the viability of the seed embryo remains

a significant challenge for treatment of seeds intended for sprouting or malting [58]. Among the treatments investigated for this purpose, radiation is a promising technology to improve microbiological safety while retaining a sprouting rate acceptable to the industry [58]. Treatment with cold atmospheric plasma that maintained germination of lentil seeds reduced bacterial contaminants by less than 3 log(CFU/g) and was not effective against fungal conidia [59]. The present study adds HPCD to the toolset with potential use for decontamination of seeds, however, the antimicrobial efficacy of HPCD varied among the different seeds/grains tested in this study and also differently affected the ability of seeds to germinate after treatment. Successful seed decontamination treatment is dependent on the soaking parameters to hydrate the seed surface. Soaking parameters are likely crucial parameters on maintaining the viability of plant embryo during treatments. The present study also highlighted the importance of seed hydration on the antimicrobial effects of HPCD as well as the ability of seeds to germinate. Specifically, treatments with HPCD that are lethal to fungal spores and bacteria did not compromise the viability of the embryo of mung beans.

6. Acknowledgments

We acknowledge the funding support from Alberta Innovates Bio Solutions and Alberta Agriculture and Forestry (Grant No. 2016F052R); Michael Gänzle acknowledges support from the Canada Research Chairs program. We also thank Dr. Ricardo Couto for providing training, technical support and expertise for the HPCD apparatus, Ceapro Inc. (Edmonton, AB, Canada) for providing dehulled oat grains, and undisclosed Albertan farmers for providing *Fusarium* infected barley grains.

7. References

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Figure legends

Figure 1. Effect of treatments on the reduction of *E. coli* AW1.7 (black) and *Salmonella* cocktail (grey) inoculated onto flax seeds (panel A), ground pepper (panel B), mung beans (panel C), and soybeans (panel D). Dry foods inoculated with bacterial culture were air-dried. Dried samples were equilibrated to an a_W of 0.75, soaked in water for 20 min and then treated with HPCD at 57 bar and 65 °C, N_2 at 57 bar and 65 °C , or at ambient pressure at 65 °C for 15 min. Data are shown as means ± standard deviation (n = 3). The reduction of cell counts after drying alone was determined as $\log(N_0/N)$, where N_0 and N refer to the cell count before and after drying, respectively. The reduction of cell counts after treatments with heat alone or pressurized gas was determined as $\log(N_1/N)$, where N_1 and N refer to the cell counts of dried bacteria before and after treatments, respectively. Different letters denote significant differences between strains and among treatments in the same panel ($P<0.05$).

Figure 2. Reduction of bacterial cell counts on barley grains cultivated on LB media (panel A) and PCA media (panel B). Barley grains were treated with CO_2 at 1.013 (black), 57 (grey), and 120 bar (white) and temperatures at 25, 35, and 65 °C for 15 min. Supercritical CO_2 was evaluated at 120 bar and 35 °C only. Data are shown as means ± standard deviation (n = 3-5). Different letters denote significant differences among different treatments in each panel ($P<0.05$).

Table 1. List of bacterial and fungal strains and their source of isolation.

Strain	Source	Reference
Escherichia coli AW1.7	Beef-packing plant	[60]
Salmonella Typhimurium ATCC13311	Human	ATCC
Salmonella Senftenberg ATCC43845	Eggs	[61]
Salmonella Bareilly FUA1934	Waste water	[24]
Salmonella Enteritidis FUA1946	Waste water	[24]
Salmonella Thompson FUA1955	Waste water	[24]
Aspergillus niger FUA 5001	Spoiled bread	[62]
Penicillium roqueforti FUA 5004	Spoiled bread	[62]

Table 2. Effect of HPCD coupled with a_w equilibration of dry foods and/or saturation of CO_2 on the lethality to *E. coli* AW1.7. After equilibration in the saturated NaCl, the a_w of beans (0.1-0.15 g) was measured by an a_w meter. Samples were treated at 65 °C and 57 bar with CO_2. Water saturation of CO_2 during treatment was achieved by placing 30 µL of water onto a filter paper at the gas inlet of the treatment apparatus. Food samples were not soaked before HPCD treatment. Data from pepper and flax seeds are shown as means ± SD of triplicate experiments; only one replicate was performed with soy and mung beans.

Sample	**a_W of food**	**Vol of water added to the system**	**Reduction of cell counts [log(N_0/N)]**
Pepper	not equilibrated	not applied	0.44±0.001
Pepper	not equilibrated	30-180 µL	1.08±0.03
Flax seeds	0.60-0.63	30 µL	2.27±0.043
Soybean	0.67-0.692	30 µL	0.72
Mung bean	0.551-0.671	30 µL	3.36

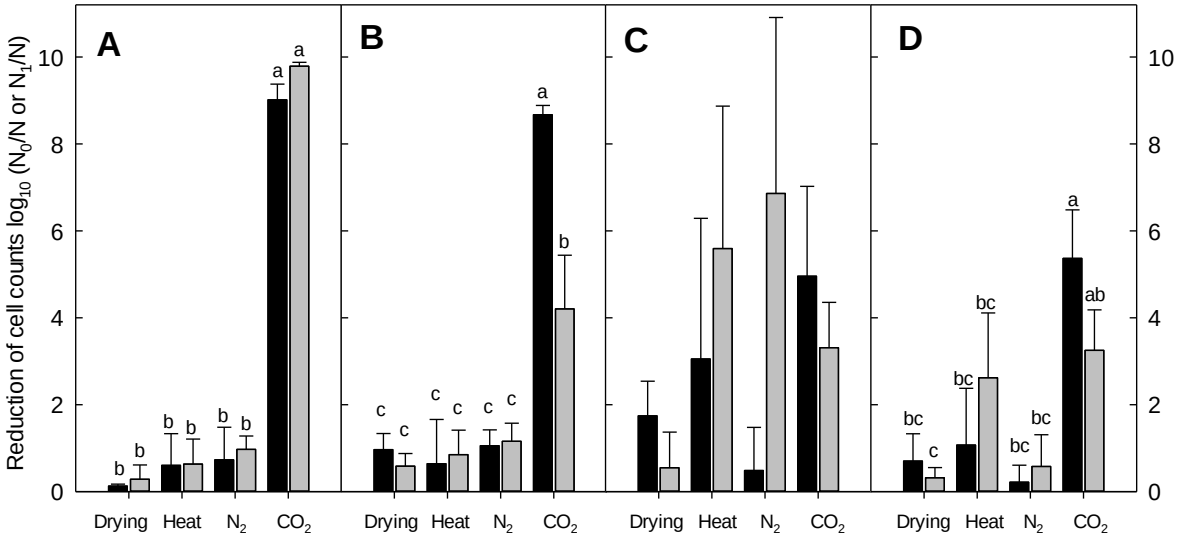
Table 3. Germination of seeds after treatments. The percentage of seeds that germinated within 10 d after treatments is reported; the total number of seeds is indicated in brackets.

	Percentage of germinated seeds (total number of seeds)			
	Soybean	**Mung bean**	**Barley**	**Oat**
Control	8 (146)	96 (146)	11 (27)	58 (26)
35 and 65°C	0 (24)	96 (24)	0 (26)	50 (26)
CO_2 at 57 bar; 35 and 65°C	2 (86)	90 (86)	0 (17)	6 (17)
CO_2 at 120 bar; 35 and 65°C	0 (34)	97 (34)	0 (6)	0 (6)

Table 4. Lethality of temperature and gaseous (57 bar) or supercritical (120 bar) CO_2 to fungal conidia on or in grains or beans. Mung beans and soybeans were surface-inoculated with *A. niger* or *P. roqueforti*. Barley and oat were naturally contaminated with *F.graminearum* and field molds without additional inoculations.

		Treatment effects (total number of seeds)							
	Temperature	25 °C		35 °C			65 °C		
	Pressure, bar	1.013	57	1.013	57	120	1.013	57	120
A. niger	Soybean	0 (3)	0.1 (9)	0 (3)	0.3 (9)	0.5 (9)	0.5 (9)	0.72 (25)	0.9 (9)
	Mung bean	0 (3)	0.4 (9)	0 (3)	0.7 (9)	0.4 (9)	0.7 (9)	1 (25)	1 (9)
P. roqueforti	Soybean	0 (3)	0.1 (9)	0 (3)	0.1 (9)	0.3 (9)	0.3 (9)	0.72 (25)	0.9 (9)
	Mung bean	0 (3)	0.6 (9)	0 (3)	0.3 (9)	0.5 (9)	0.7 (9)	1.0 (25)	1 (9)
F. graminearum	Barley	0 (6)	1 (6)	0 (6)	1 (6)	1 (6)	1(6)	1(6)	-
Field molds (unknown)	Oat	0.3 (6)	1 (6)	0.3 (6)	1 (6)	1 (6)	1(6)	1(6)	-

Grains or beans were treated with the given conditions for 15 min. Individual seeds were considered as an experimental unit. Treatment effects were calculated by Eq.1 where 1 indicates all seeds remained mold-free over 10 d of storage and 0 means that molds grew on all seeds.


A
B
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Reduction of cell counts log10 (N0/N or N1/N)
0
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4
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Drying
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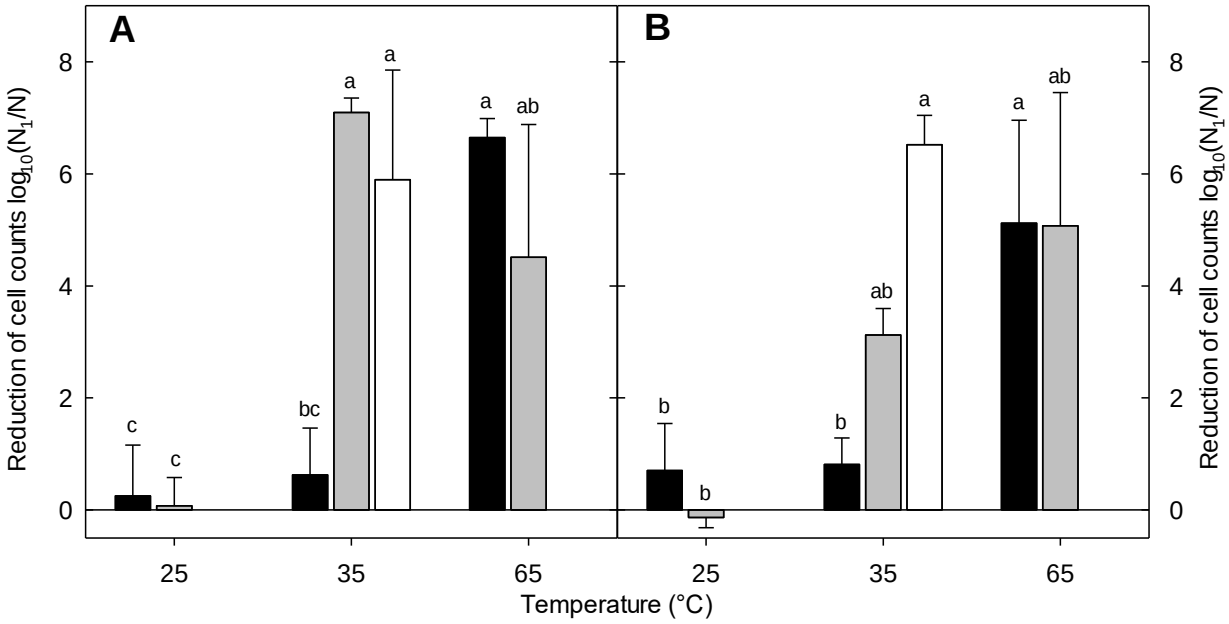
Figure 1.


A
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Reduction of cell counts log10(N1/N)
Temperature (°C)
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Figure 2.

Online Supplementary Material to

Effects of High-Pressure Carbon Dioxide on Microbial Quality and Germination of Cereal Grains and Beans

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Table S1. Viable cell counts of uninoculated dehulled oat determined on LB media and PCA media. Data are shown as means ± SD of 3-5 replicates.

Treatment parameters (gas, °C, bar)	Cell counts [log(CFU/g)]	
	LB	**PCA**
untreated	5.82±1.29	5.40±1.41
CO_2, 45, 1.013	1.8±2.5[a]	1.7±2.4[a]
CO_2, 45, 57	n.d.	1.2±1.7[a]
CO_2, 55, 1.013	n.d.	n.d.
CO_2, 55, 57	1.4±2.0[a]	1.5±2.1[a]
CO_2, 65, 1.013	1.5±1.8[a]	0.8±1.5[a]
CO_2, 65, 57	1.3±1.8[a]	1.2±1.8[a]
N_2, 65, 57	n.d.	n.d.

[a] For average values less than 2 [log(CFU/g)], the cell count of one or more of the replicates was below the detection limint of 200 cells / g; for calculation of the average, the cell count of these samples was assumed to be 0[log(CFU/g)].

n.d., cell counts in all replicates were below the detection limit of 200 cells / g

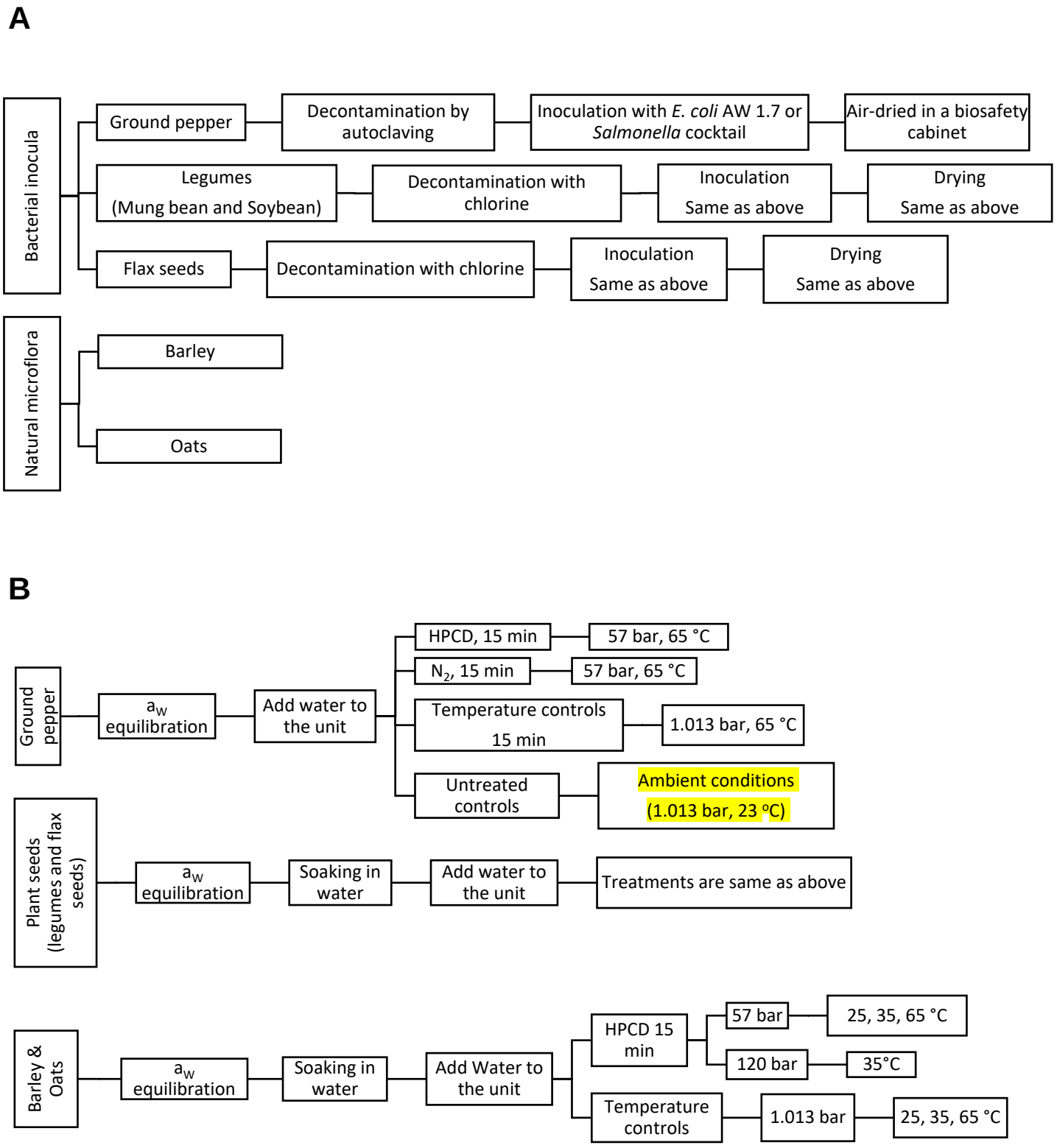

Figure S1. Flowchart depicting (A) the sample preparation for inoculation with bacterial cultures, and (B) treatments with HPCD.

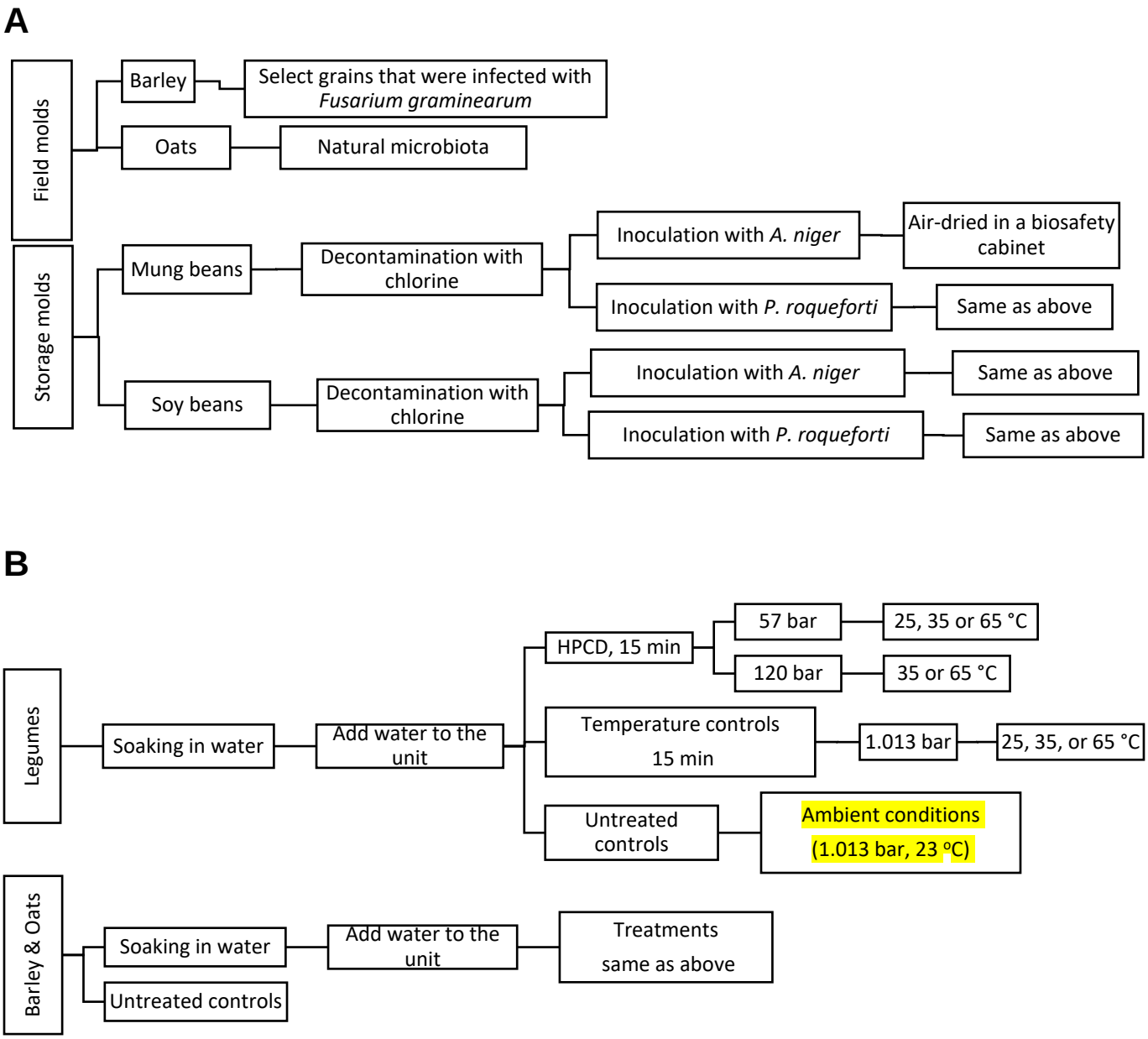

Figure S2. Flowchart depicting (A) the sample preparation for fungal inoculation, and (B) treatments with HPCD.