Inactivation of foodborne pathogens on leek and alfalfa seeds with supercritical carbon dioxide

Siméon Bourdoux[a], Alessandro Zambon[b*], Inge Van der Linden[a], Sara Spilimbergo[b], Frank Devlieghere[a], Andreja Rajkovic[a]

[a]Department of Food Safety, Quality and Health; Faculty of Bioscience Engineering; Ghent University; Belgium

[b]Department of Industrial Engineering: University of Padova; Italy

*Corresponding author:

alessandro.zambon@unipd.it

ABSTRACT

This study aimed to evaluate the effect of supercritical CO_2 process for the inactivation of artificially contaminated seeds of leek and alfalfa. The seeds were inoculated with *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* Thompson and *S.* Typhimurium and treated at 80 and 120 bar and at 35 and 45°C for 20 min. The process did not influence the germination rate of the seeds. The inactivation was dependent from the type of seed and pressure and temperature. At 120 bar and 45°C *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* spp. were reduced by 2.92 ± 0.27, 1.14 ± 0.63, and 1.74 ± 0.55 log CFU/g, respectively, on alfalfa and by 4.96 ± 0.37, 2.93 ± 0.27, and 3.18 ± 0.27, respectively on leek. Overall, these results indicated that supercritical CO_2 can be used to improve the microbial safety of sprouts, especially for leek.

Key words: supercritical CO_2, microbial inactivation, seeds, alfalfa, leek, sprouting

1. Introduction

Sprouted seeds are commonly consumed raw or minimally processed and have been associated to outbreaks of illnesses caused by several pathogens[1,2]. According to European Food Safety Authority [3], the majority of sprouted seed-associated outbreaks have been reported in the United States, but reported outbreaks have also occurred in in Germany, Sweden, Denmark, France and the United Kingdom. In USA the majority of outbreaks were attributed to alfalfa sprouts, and *Salmonella* was the most common pathogen identified followed by pathogenic *E. coli* and *Listeria* [4]. The process of sprouting consists in allowing the seeds to germinate in a moist and warm (25 – 35°C) environment [5]. These conditions are ideal for the growth of many foodborne pathogens if they were initially present on the raw materials [6]. Seed disinfection treatment has been recommended as a preventative treatment to reduce the risk of illness associated with contaminated sprouts[7]. Nevertheless, when decontamination is applied after the germination process a risk of quality impairment of the final product occurs [8]. Moreover, microorganisms that are internalized in the sprouts can be difficult to remove by washing procedures or other (surface) decontamination procedures [5]. FDA recommend application of a high concentration (20,000ppm) of calcium hypochlorite solution to seeds before germination [9]. However chlorine-based treatments are associated with negative health [10] and environmental issues [11] and more and more attention is given to alternative residue-free decontamination technologies. However, the innovative decontamination strategy should not impede the germination capability of the seeds as observed for example with high hydrostatic pressure treatments [12]. Among alternatives, supercritical CO_2 ($scCO_2$) has been shown~~ed~~ to be effective against *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* inoculated on alfalfa seeds [13] and background ~~microflora~~ bacteria [14], without relevant impairment of the seeds germination. However, the inactivation capacity of $ScCO_2$ is well known to be product dependent [15] and scientific evidence of its effects is needed to promote

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the development of the technology at industrial level. To the best of our knowledge currently available, publications do not provide sufficient elaboration and evidence of the $scCO_2$ decontamination efficacy on different seeds meant for human consumption.

The aim of this study was to investigate the efficiency of $scCO_2$ to inactivate foodborne pathogens on two different seed types: leek and alfalfa. Experiments were focused on artificially inoculated *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes.* The process was optimized to preserve the germination rate and sprouting of the untreated seeds.

2. Materials and methods

2.1. Bacterial strains

Two strains of *E. coli* O157:H7 (ATCC 700728, LFMFP 846), *L. monocytogenes* (LMG 23192, LMG 23194) and *Salmonella* enterica (serovars Thompson RM1987 and Typhimurium SL1344) were used in this study. LFMFP strains are part of the Laboratory of Food Microbiology & Food Preservation culture collection at the Faculty of Bio-Science Engineering, Ghent University. LMG strains are part of the Belgian Coordinated Collection of Micro-organisms (BCCM) situated at the Laboratory of Microbiology at the Faculty of Sciences, Ghent University. The *E. coli* O157:H7 strains are stx-negative strains. *E. coli* O157:H7 LFMFP 846 is a nalidixic acid resistant variant (50 µg/mL) of *E. coli* O157:H7 MB3885 (obtained from Prof. Piérard, EHEC Reference Laboratory at the University hospital in Brussels). *E. coli* O157:H7 ATCC 700728 originated from a verocytotoxigenic strain which lost its ability to produce toxin (as confirmed by absence of Shiga toxin genes). The strains, stored in glycerol stock at -80°C were revived in 10 mL of Brain Heart Infusion (BHI, Sacco, Italy) at 37°C for 24 h and then isolated on Tryptic Soy Agar (TSA, Oxoid, UK) and on an appropriate selective medium (see 2.6. Microbiological Analyses). Working cultures were prepared on TSA slants inoculated with one isolated colony from the selective media and incubated at 37°C for 24 h. These working cultures were stored at 4°C for maximum 2 weeks.

2.2. Inoculation cocktail

The two strains of each microorganism were cultured separately in BHI at 37°C for 24 h, after which the cells were harvested by centrifugation at 2900 g for 10 min (Megafuge 16 R, Heraeus). The supernatant was discarded and the pellet was washed twice with Peptone Buffered Saline (PBS, Oxoid). The two final suspensions were mixed together in a 1:1 ratio to obtain the inoculation cocktail. The cell concentration in the inoculation cocktail was 9.03 ± 0.03, 9.15 ± 0.19, and 8.72 ± 0.25 log CFU/mL for *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella*, respectively, as verified by plate counts.

2.3. Seeds and inoculation

Organic alfalfa seeds for sprouting (lot RO3350/165017, origin Italy, REM Sprout, Italy) and conventional leek seeds (lot RE3200/160064, origin Italy, REM Sprout, Italy) were bought from a local sprouting company. Alfalfa and leek seeds were packed in July 2017 and August 2017, respectively. For each experiment with inoculated seeds, 3 g of either alfalfa or leek were mixed with 300 µL of inoculation cocktail to reach a final inoculation level of 6 log CFU/g. After inoculation, the seeds were left to dry overnight (18 h) under a laminar flow at room temperature. Counts for *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* on the dried seeds were 6.32 ± 0.13, 6.64 ± 0.34, and 5.82 ± 0.34 log CFU/g, respectively, on alfalfa and 6.34 ± 0.30, 6.92 ± 0.28, and 5.64 ± 0.28 log CFU/g, respectively, on leek. The final water activity of the seeds after drying and before the $scCO_2$-treatment was 0.642 ± 0.006 for alfalfa and 0.659 ± 0.004 for leek. During preliminary experiments, *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* were not detected on the non-inoculated seeds.

2.4. Supercritical CO_2 treatment

Seeds were treated with $scCO_2$ in a high pressure system as previously described [16] with minor modifications. Briefly, the seeds were inserted into a 15 mL falcon tube without cap; the tube was then placed inside the reactor and pressurized in less than 2 min using the maximum (23 mL/min) flow rate of the pump (Gilson 25SC; Gilson S.A.S., France) to reach the pressure set point. Depressurization took place in less than 2 min. For each experiment, experimental control seeds were prepared in the identical manner, but without $scCO_2$ treatment.

First, experiments with non-inoculated seeds were performed to determine the potential impact of $scCO_2$ on the germination capability. Tubes containing 5 g of alfalfa and leek seeds were treated following a 2^3 factorial design (single trial) varying temperature (35-45°C), pressure (80-120 bar) and holding time (0-20 min). Double replication was performed for the central point (40°C, 100 bar, 10 min). The variable domain was chosen to guarantee supercritical conditions (35°C and 80bar as

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minimal temperature and pressure, respectively) and to keep mild temperature and pressure (45°C and 120 bar as maximum)[17]. Inactivation experiments were performed at two temperature (35°C and 40°C) two pressure (80 and 120 bar) for a holding time of 20 min.

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2.5. Seeds germination assessment

After treatment, 100 non-inoculated seeds were randomly picked and individually placed in folds of a 50-folded paper (Grade 3014 seed testing paper, GE Healthcare Whatman) in sterile aluminum tray (Product reference 0838610799, AVA papier, Belgium). The trays were then filled with 45 mL of sterile water, covered by a lid, and incubated at 22°C for 4 and 6 days for alfalfa and leek, respectively. After incubation, the germinated seeds were retrieved and placed on a dark background and a picture was taken (see an example in ~~Figure 1~~Figure ~~figure 1~~). To measure the length of the seeds on the pictures, a homemade algorithm, consisting of an OTSU binarization and a SOBEL pattern detection. The algorithm was developed *in-house* in Python using Anaconda distribution (v5.1.0). ~~, was applied using Python.~~ Each processed picture was then manually verified to check for mistakes made by the algorithm. The percentage of germination is indicated as the number of germinated seeds divided by the total number of seeds and the average lengths are given in cm.

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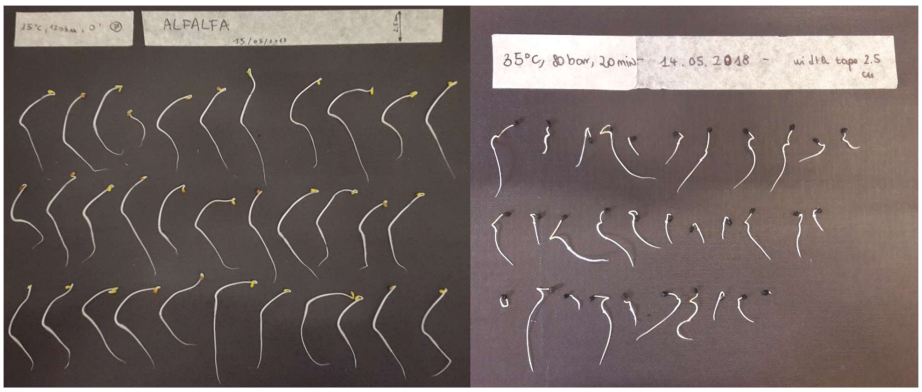

Figure 1 *– Representative picture of the seeds after the treatment: alfalfa germinated seeds (left) and leek germinated seeds (right)*

2.6. Microbiological analyses

Treated and control seeds were retrieved in 50-mL tubes and diluted in 27 mL of Buffered Peptone Water (BPW, Oxoid) to obtain a 1:10 dilution. The tubes were left 5 min at room temperature to rehydrate the seeds and then vortexed at 60 Hz for 30 s. The resulting homogenates were then 10-fold diluted in BPW and appropriate dilutions were spread-plated on selective media. Samples containing *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* spp. cocktails were plated on Sorbitol-MacConkey Agar supplement (Oxoid) with cefixime-tellurite (Oxoid), AL (Bio-Rad, France), and XLD Agar (Oxoid), respectively, and were incubated at 37°C for 24, 48, and 24 h, respectively.

2.7. Data processing

Experiments with inoculated seeds (single purchase) were done in three independent experiments comprising three independent inoculation, i.e. cultures of each microorganism were made at three different times ~~made in three time-separated occasions~~ and inoculated on seeds. Experiments to determine the germination rate and the length of the seeds were repeated in two independent experiments. Data was analyzed using SPSS 25 (IBM).

To measure the length of the seeds on the pictures, a homemade algorithm, consisting of an OTSU binarization and a SOBEL pattern detection. The algorithm was developed in-house in Python, using the Anaconda distribution (v5.1.0).

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Assumption of equal variances was tested with Levene's test. Comparison of average reductions was performed using t-tests. Two-way ANOVA was performed to evaluate the impact of the independent variables on inactivation. For the tests, the parameters were pressure/temperature, temperature/type of seeds, and pressure/type of seeds. All statistical tests were performed at a $p < 0.05$ significance level. ~~and effects of pressure, temperature and type of seeds on the inactivation was assessed by two-way ANOVA. All statistical tests were performed at a $p < 0.05$ significance level.~~

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3. Results and discussion

3.1. Germination of the seeds

Data presented in table 1 and 2 show the effect of $scCO_2$ treatment on the germination of alfalfa and leek seeds, respectively. Overall, alfalfa had a better germination ratio than leek. ~~After treatment,~~ 92.0 ± 1.4 % and 27.5 ± 0.7 % of the alfalfa and leek seeds, respectively, were able to germinate in the control (untreated) samples. For both alfalfa and leek, treatments did not influence the germination rate. Because the holding time did not seem to effect the germination capability of the seeds for any given temperature/pressure combination, the inactivation of inoculated pathogens was further performed with the longest treatment time (20 min). Indeed holding time was proven to influence the microbial inactivation rate of ScCO2 processes [13,14], therefore higher treatment times are expected to improve the inactivation capacity. The germination rate of alfalfa is similar to what observed by Hall *et al.* [18] from seeds subjected to freeze-thaw scarification. The germination of leek seeds was lower but comparable between treated and untreated samples. The germination achieved is similar with previous works at 35°C [19]. In their study, Hanci *et al.* found 21°C as optimal temperature for the germination of leek, while lower and higher temperature resulted in a lower germination rate. In our study, the average temperature was not controlled and measured during the experiments, but it was reasonable closer to 18°C. ~~Indeed, considering data from meteorology archive in Padua (Italy) in May 2018, the average maximum and minimum temperature was 21.3 and 11.3°C, respectively.~~ Although the germination rate of untreated and treated seeds is comparable, which is sufficient to support the further inactivation results.

Table 1 *– Germination percentage and length of alfalfa seeds after supercritical CO_2 treatment. Germinated fraction and lengths are expressed in % and cm, respectively, as mean ± standard deviation (n = 2).*

Treatment conditions	Control	35°C				40°C	45°C			
		80 bar		120 bar		100 bar	80 bar		120 bar	
		0 min	20 min	0 min	20 min	10 min	0 min	20 min	0 min	20 min
Germinated fraction (%)	92.0 ± 1.4	91.0 ± 5.7	93.5 ± 6.4	89.0 ± 7.1	89.5 ± 2.1	86.5 ± 6.4	91.5 ± 4.9	92.0 ± 2.8	92.5 ± 0.7	93.5 ± 0.7
Average length (cm)	6.97 ± 2.96	6..11 ± 3.04	7.27 ± 3.28	7.51 ± 3.21	8.02 ± 2.78	6.79 ± 2.69	6.18 ± 3.18	6.96 ± 2.99	6.89 ± 3.62	7.47 ± 2.73

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Table 2 – Germination percentage and length of leek seeds after supercritical CO_2 treatment. Lengths are expressed in cm as mean ± standard deviation (n = 2).

Treatment conditions	Control	35°C				40°C	45°C			
		80 bar		120 bar		100 bar	80 bar		120 bar	
		0 min	20 min	0 min	20 min	10 min	0 min	20 min	0 min	20 min
Germinated fraction (%)	27.5 ± 0.7	32.5 ± 6.4	30.5 ± 2.1	21.0 ± 8.5	20.0 ± 5.7	22.3 ± 5.9	23.5 ± 0.7	19.5 ± 5.0	25.0 ± 5.7	26.0 ± 5.7
Average length (cm)	2.54 ± 1.47	2.30 ± 1.47	2.32 ± 1.35	2.35 ± 1.62	1.72 ± 1.42	2.05 ± 1.34	3.16 ± 1.62	2.63 ± 1.76	2.35 ± 1.63	1.75 ± 1.41

3.2. Microbial decontamination of the seeds

The effect of the $scCO_2$ treatments on *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* spp. for leek and alfalfa is displayed in Figure 2. Overall, reduction on alfalfa was lower than on leek. With the harshest conditions (45°C/120 bar/20 min) 4.96 ± 0.37 log reduction of *E. coli* O157:H7 was achieved on leek while only 2.92 ± 0.27 log reduction was achieved in alfalfa. The type of seed, temperature, and pressure had a significant effect on the inactivation of pathogens ($p < 0.05$). The type of seed had the strongest effect on inactivation for each microorganism, followed by temperature. Pressure also had a significant effect on *L. monocytogenes*, however out of the three tested pathogens, *L. monocytogenes* exhibited the strongest resistance to the process, especially in alfalfa. According to Garcia-Gonzalez *et al.* [20], the effectiveness of $scCO_2$ treatments is strongly dependent on process variable like pressure and temperature. High pressure enhances the solubilization of CO_2 in the food matrix, facilitating contact with the cells and pH reduction of the external medium. Higher temperature increases the diffusivity of CO_2, allowing it to penetrate into a complex matrix easily. Moreover, higher temperatures can also fluidize the membranes of microorganisms, favoring penetration of CO_2 into the cell. In this study, both pressure and temperature had an effect on the inactivation of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* inoculated on alfalfa and leek seeds and the best reductions were obtained with the highest temperature/pressure combinations.

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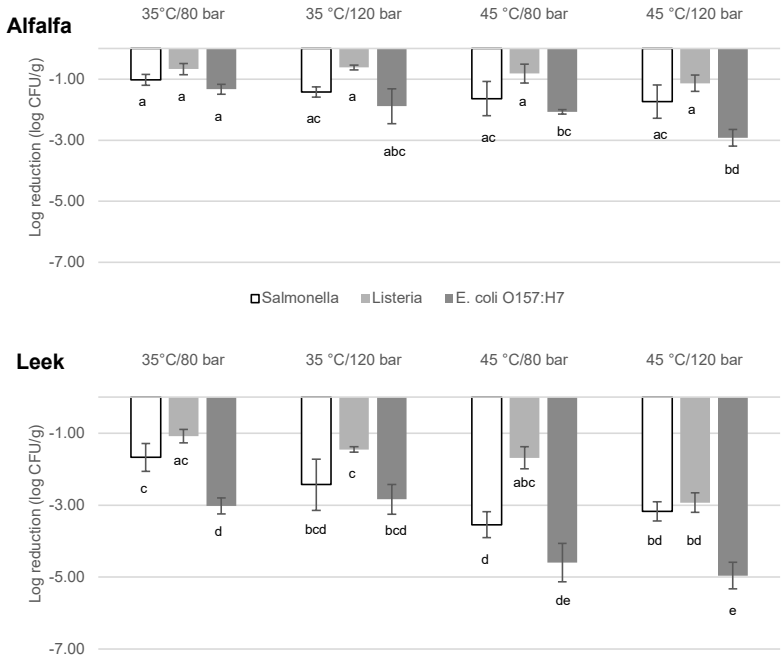

Figure 2 *– Average log reduction of* Salmonella *(white),* L. monocytogenes *(light grey), and* E. coli *O157:H7 (dark grey), inoculated on alfalfa and leek seeds after treatment with supercritical CO_2 for 20 min. Bar indicates standard deviations (n = 3). Different letters indicate significant differences ($p < 0.05$).*

Most studies on the decontamination of dry seeds focused on the use of chemical treatments, which involved chlorine, ozone, and/or organic acids and their combination alone [21] or together with other non-chemical techniques such as dry heat and ultrasounds [1,22]. According to a scientific opinion of EFSA, results from various studies using 10 000 to 20 000 mg/L of chlorine on dry seeds achieved between 2 and 5 log reduction of artificially inoculated *Salmonella* and *E. coli* O157:H7 [3]. In our study, the decontamination with $scCO_2$ at 120 bar and 45°C resulted in 5 log reduction of *E. coli* O157:H7 and 3 log reduction of *Salmonella* and *L. monocytogenes* which is higher or comparable to what was reported with chemical surface decontaminations.

Our finding for alfalfa is not comparable with the previous study by Juan *et al.*[13]. Specifically a lower reduction was obtained in our investigation for all the pathogenic microorganisms. Jung *et al.*

reported more than 5 log reduction of *L. monocytogenes* on alfalfa seeds after treatment at 100 bar and 45°C for 15 min while less than 1.5 log was here found after treatment at 100 bar, 45°C for 20 min. The discrepancy could be explained by a different water activity of the seeds before the treatment, as well as by possible inter-strain variability. Indeed in the work by Juan *et al.* there is no specific indication about the final water activity of the seeds after the inoculation procedure, which was performed by immersing the seeds in the inoculation cocktails, thereby adding more water to the seeds. On the contrary, we achieved an average water activity of 0.640 ± 0.01 and 0.66 ± 0.01 for alfalfa and leek, respectively, before the $ScCO_2$ treatment which was similar to the one measured in the commercial seeds (data not shown). Dehghani *et al.* demonstrated that low quantity of water faster and increase $ScCO_2$ microbial inactivation on ginger powder [23]. Similarly Calvo and Torres [24] reported that $ScCO_2$ inactivation is depended on the initial water content in paprika powder. Chen *et al.* demonstrated that dry *E.coli* were resistant to liquid and $ScCO_2$ at any temperature tested [25]. Recently, Fang et al. reported a further evidence about the importance of the water activity over the $ScCO_2$ inactivation capacity investigating cereal grains and beans[26]. In their funding the optimal antimicrobial effect was achieved by equilibration of the water activity to 0.75, following by soaking in water and treatment with water-saturated CO_2.

The most significant effect on the inactivation of microorganisms was observed when we tested a different type of seed. Indeed, although little to no reduction was observed for *L. monocytogenes* on alfalfa, with only 1 log reduction at 45°C/120 bar, up to 3 log reduction could be achieved on leek under the same treatment conditions. The increased resistance on alfalfa could be explained by the presence of antimicrobial compounds in the seeds of leek. Leek is part of the genus *Allium*, and many antimicrobial compounds have been isolated from other species of this genus (such as garlic, and onion) [27–29]. A fraction of these antimicrobial compounds could be dissolved in the $scCO_2$ during the treatment and act as co-solvents, effectively increasing the inactivation of the pathogens. Indeed supercritical CO_2 has been extensively investigated for the extraction of essential oil in many

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seeds[30], like coriander[31], clove[32] and parsley[33] seeds. Gonzalez-Alonso *et al.* reported a synergistic effect between $ScCO_2$ with coriander essential oil for the microbial inactivation of chicken breast [34]. Huang *et al.* reported a possible extraction of antimicrobial molecules from rosemary during the inactivation of ground pork [35]. Further research is needed to determine the cause of the increased reductions on leek and the potential antimicrobial compounds from these seeds or other seeds of the genus *Allium* which could be added as natural co-solvents during $scCO_2$ decontamination for other types of matrices. It is worth to notice that uncertain level of inactivation has to be taken into account since the enumeration of bacterial cells after lethal treatments on selective media might not account for sub-lethally injured cells and thus likely over-estimates the treatment lethality[36,37]. Further studies should be also investigate the effect of the treatment over the potential or likely survival of sub-lethally injured cells.

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4 Conclusion

The present work investigated the effects of $scCO_2$ on three relevant pathogens inoculated on alfalfa and leek seeds. The treatment did not substantially impact the germination capability of the seeds nor the length of the sprouts after 4 and 6 days, for alfalfa and leek, respectively. The type of seeds was found to be the most important parameter to take into account when assessing decontamination with $scCO_2$. Pathogens inoculated on alfalfa seeds were less prone to $scCO_2$ inactivation in comparison to those inoculated on leek seeds. A treatment at 120 bar, 45°C for 20 min resulted in 5 log reduction of *E. coli* O157:H7 and 3 log reduction of *Salmonella* spp. and *L. monocytogenes* in leek. Overall results obtained indicate relevant potential to reduce the level of contamination and therefore also the risk of outbreaks of foodborne illnesses caused by sprouts contaminated with bacterial pathogens. Further study should be performed to demonstrate the potential of the techniques on other seeds, as well as to investigate impact on the characteristics of surviving microbial fractions (e.g. virulence, cross resistance, growth characteristics). Further research could also investigate the e growth rate in the

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early days after germination or even at a later stage in life including also qualitative aspects to the study.

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References

[1] D. Millan-Sango, E. Sammut, J.F. Van Impe, V.P. Valdramidis, Decontamination of alfalfa and mung bean sprouts by ultrasound and aqueous chlorine dioxide, LWT - Food Sci. Technol. 78 (2017) 90–96. https://doi.org/10.1016/j.lwt.2016.12.015.

[2] J.M. Soon, P. Seaman, R.N. Baines, Escherichia coli O104: H4 outbreak from sprouted seeds, Int. J. Hyg. Environ. Health. (2013). https://doi.org/10.1016/j.ijheh.2012.07.005.

[3] E. Panel, H. Biohaz, Scientific Opinion on the risk posed by Shiga toxin-producing Escherichia coli (STEC) and other pathogenic bacteria in seeds and sprouted seeds, EFSA J. 9 (2011) 1–101. https://doi.org/10.2903/j.efsa.2011.2424.

[4] K. Gensheimer, D. Gubernot, 20 Years of Sprout-Related Outbreaks: FDA's Investigative Efforts, Open Forum Infect. Dis. (2016). https://doi.org/10.1093/ofid/ofw172.1140.

[5] K. Warriner, S. Spaniolas, M. Dickinson, C. Wright, W.M. Waites, Internalization of bioluminescent Escherichia coli and Salmonella Montevideo in growing bean sprouts, J. Appl. Microbiol. (2003). https://doi.org/10.1046/j.1365-2672.2003.02037.x.

[6] W.N. Wade, A.J. Scouten, K.H. McWatters, R.L. Wick, A. Demirci, W.F. Fett, L.R. Beuchat, Efficacy of ozone in killing Listeria monocytogenes on alfalfa seeds and sprouts and effects on sensory quality of sprouts, J. Food Prot. (2003). https://doi.org/10.4315/0362-028X-66.1.44.

[7] H. Ding, T.J. Fu, M.A. Smith, Microbial Contamination in Sprouts: How Effective Is Seed Disinfection Treatment?, J. Food Sci. (2013). https://doi.org/10.1111/1750-3841.12064.

[8] S.A. Mir, S. Farooq, M.A. Shah, S.A. Sofi, B.N. Dar, A.M. Hamdani, A.M. Khaneghah, An overview of sprouts nutritional properties, pathogens and decontamination technologies, LWT. (2021) 110900. https://doi.org/10.1016/j.lwt.2021.110900.

[9] A.M.D. Sikin, C. Zoellner, S.S.H. Rizvi, Current intervention strategies for the microbial safety of sprouts, J. Food Prot. (2013). https://doi.org/10.4315/0362-028X.JFP-12-437.

[10] H. Komulainen, Experimental cancer studies of chlorinated by-products, in: Toxicology, 2004.

https://doi.org/10.1016/j.tox.2004.01.031.

[11] S.S. Liu, H.M. Qu, D. Yang, H. Hu, W.L. Liu, Z.G. Qiu, A.M. Hou, J. Guo, J.W. Li, Z.Q. Shen, M. Jin, Chlorine disinfection increases both intracellular and extracellular antibiotic resistance genes in a full-scale wastewater treatment plant, Water Res. (2018). https://doi.org/10.1016/j.watres.2018.02.036.

[12] E.Y. Wuytack, A.M.J. Diels, K. Meersseman, C.W. Michiels, Decontamination of seeds for seed sprout production by high hydrostatic pressure, J. Food Prot. (2003). https://doi.org/10.4315/0362-028X-66.6.918.

[13] W.Y. Jung, Y.M. Choi, M.S. Rhee, Potential use of supercritical carbon dioxide to decontaminate Escherichia coli O157:H7, Listeria monocytogenes, and Salmonella typhimurium in alfalfa sprouted seeds, Int. J. Food Microbiol. (2009). https://doi.org/10.1016/j.ijfoodmicro.2009.08.014.

[14] A.M. Mazzoni, R.R. Sharma, A. Demirci, G.R. Ziegler, Supercritical carbon dioxide treatment to inactivate aerobic microorganisms on alfalfa seeds, J. Food Saf. (2001). https://doi.org/10.1111/j.1745-4565.2001.tb00320.x.

[15] G. Ferrentino, S. Spilimbergo, High pressure carbon dioxide pasteurization of solid foods: Current knowledge and future outlooks, Trends Food Sci. Technol. (2011). https://doi.org/10.1016/j.tifs.2011.04.009.

[16] A. Zambon, M. Vetralla, L. Urbani, M.F. Pantano, G. Ferrentino, M. Pozzobon, N.M. Pugno, P. De Coppi, N. Elvassore, S. Spilimbergo, Dry acellular oesophageal matrix prepared by supercritical carbon dioxide, J. Supercrit. Fluids. 115 (2016) 33–41. https://doi.org/10.1016/j.supflu.2016.04.003.

[17] S. Spilimbergo, D. Komes, A. Vojvodic, B. Levaj, G. Ferrentino, High pressure carbon dioxide pasteurization of fresh-cut carrot, J. Supercrit. Fluids. 79 (2013) 92–100.

[18] J.W. Hall, D.G. Stout, B.M. Brooke, Hard seed and field establishment of irrigated alfalfa, Crop Sci. (1993). https://doi.org/10.2135/cropsci1993.0011183X003300050030x.

[19] F. HANCI, M. ÇINGI, H. AKINCI, Influence of L-Tryptophan and Melatonin on Germination of Onion and Leek Seeds at Different Temperatures, Türkiye Tarımsal Araştırmalar Derg. (2019). https://doi.org/10.19159/tutad.559617.

[20] L. Garcia-Gonzalez, A.H. Geeraerd, S. Spilimbergo, K. Elst, L. Van Ginneken, J. Debevere, J.F. Van Impe, F. Devlieghere, High pressure carbon dioxide inactivation of microorganisms in foods: The past, the present and the future, Int. J. Food Microbiol. (2007). https://doi.org/10.1016/j.ijfoodmicro.2007.02.018.

[21] M. Trząskowska, Y. Dai, P. Delaquis, S. Wang, Pathogen reduction on mung bean reduction of Escherichia coli O157:H7, Salmonella enterica and Listeria monocytogenes on mung bean using combined thermal and chemical treatments with acetic acid and hydrogen peroxide, Food Microbiol. (2018). https://doi.org/10.1016/j.fm.2018.04.008.

[22] A.J. Scouten, L.R. Beuchat, Combined effects of chemical, heat and ultrasound treatments to kill Salmonella and Escherichia coli O157:H7 on alfalfa seeds, J. Appl. Microbiol. (2002). https://doi.org/10.1046/j.1365-2672.2002.01571.x.

[23] F. Dehghani, N. Annabi, M. Titus, P. Valtchev, A. Tumilar, Sterilization of Ginseng using a high pressure CO2 at moderate temperatures, Biotechnol. Bioeng. (2009). https://doi.org/10.1002/bit.22059.

[24] L. Calvo, E. Torres, Microbial inactivation of paprika using high-pressure CO2, J. Supercrit. Fluids. 52 (2010) 134–141. https://doi.org/10.1016/j.supflu.2009.11.002.

[25] Y.Y. Chen, F. Temelli, M.G. Gänzle, Mechanisms of Inactivation of Dry Escherichia coli by High-Pressure Carbon, Appl. Environ. Microbiol. 83 (2017) 1–10. https://doi.org/10.1128/AEM.00062-17.

[26] Y. Fang, C. Franke, A. Manthei, L. McMullen, F. Temelli, M.G. Gänzle, Effects of high-pressure carbon dioxide on microbial quality and germination of cereal grains and beans, J. Supercrit. Fluids. (2021). https://doi.org/10.1016/j.supflu.2021.105272.

[27] G. Goncagul, E. Ayaz, Antimicrobial effect of garlic (Allium sativum) and traditional

medicine, J. Anim. Vet. Adv. (2010). https://doi.org/10.3923/javaa.2010.1.4.

[28] B. Phillippe, B.P.A. Cammue, K. Thevissen, M. Hendriks, K. Eggermont, I.J. Goderis, P. Proost, J. Vandamme, R.W. Osborn, F. Guerbette, J.C. Kader, W.F. Broekaert, A POTENT ANTIMICROBIAL PROTEIN FROM ONION SEEDS SHOWING SEQUENCE HOMOLOGY TO PLANT LIPID TRANSFER PROTEINS, Plant Physiol. (1995).

[29] C.L. Ye, D.H. Dai, W.L. Hu, Antimicrobial and antioxidant activities of the essential oil from onion (Allium cepa L.), Food Control. (2013). https://doi.org/10.1016/j.foodcont.2012.07.033.

[30] M. Yousefi, M. Rahimi-Nasrabadi, S.M. Pourmortazavi, M. Wysokowski, T. Jesionowski, H. Ehrlich, S. Mirsadeghi, Supercritical fluid extraction of essential oils, TrAC - Trends Anal. Chem. (2019). https://doi.org/10.1016/j.trac.2019.05.038.

[31] Z. Zeković, O. Bera, S. Đurović, B. Pavlić, Supercritical fluid extraction of coriander seeds: Kinetics modelling and ANN optimization, J. Supercrit. Fluids. (2017). https://doi.org/10.1016/j.supflu.2017.02.006.

[32] E. Reverchon, C. Marrone, Supercritical extraction of clove bud essential oil: Isolation and mathematical modeling, Chem. Eng. Sci. (1997). https://doi.org/10.1016/S0009-2509(97)00172-3.

[33] A. Piras, S. Porcedda, D. Falconieri, A. Fais, B. Era, G. Carta, A. Rosa, Supercritical extraction of volatile and fixed oils from Petroselinum crispum L. seeds: chemical composition and biological activity, Nat. Prod. Res. (2020). https://doi.org/10.1080/14786419.2020.1810031.

[34] V. González-Alonso, M. Cappelletti, F.M. Bertolini, G. Lomolino, A. Zambon, S. Spilimbergo, Research Note: Microbial inactivation of raw chicken meat by supercritical carbon dioxide treatment alone and in combination with fresh culinary herbs, Poult. Sci. (2020). https://doi.org/10.3382/ps/pez563.

[35] S. Huang, B. Liu, D. Ge, J. Dai, Effect of combined treatment with supercritical CO2 and rosemary on microbiological and physicochemical properties of ground pork stored at 4 °C, Meat Sci. (2017). https://doi.org/10.1016/j.meatsci.2016.11.022.

[36] F. Zhao, X. Bi, Y. Hao, X. Liao, Induction of Viable but Nonculturable Escherichia coli O157:H7 by High Pressure CO2 and Its Characteristics, PLoS One. (2013). https://doi.org/10.1371/journal.pone.0062388.

[37] L. Garcia-Gonzalez, A.H. Geeraerd, K. Elst, L. Van Ginneken, J.F. Van Impe, F. Devlieghere, Influence of type of microorganism, food ingredients and food properties on high-pressure carbon dioxide inactivation of microorganisms, Int. J. Food Microbiol. (2009). https://doi.org/10.1016/j.ijfoodmicro.2008.12.005.