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Effect of Ozone on *Enterobacteriaceae* Counts in Wheat Grain, Alfalfa, Radish, Broccoli Seeds and Sprouts

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Abstract. The sprouts of various seeds have garnered considerable regard amid consumers due to its verdure, crispiness, and germination potential, offering a cost-effective and straightforward process. The moist and warm conditions are very conducive to germination, fostering the growth of microorganisms and thereby raising food safety concerns. The aim of the study was to investigate the impact of ozone on *Enterobacteriaceae* counts by subjecting wheat (*Triticum aestivum*) grains, alfalfa (*Medicago sativa*), radish (*Raphanus sativus*) and broccoli (*Brassica oleracea*) seeds to ozone gas treatment. Additionally, the grain and seeds were rinsed with ozonated water during germination. Three different treatment methods were employed for seeds and grains. In the first method, samples were exposed to ozone gas at 20 ppm for 10 minutes, with subsequent rinsing every 12 hours during germination until the rinse water appeared visually clean, at a concentration of 2.0 mg L^{-1} of ozone. The second method involved rinsing with ozonated water at a concentration of 2.0 mg L^{-1} for durations of 20, 40, and 60 minutes. The third method - treated with ozone gas at 50 ppm and the exposure time of 1, 2, 3, 4 and 5 h. Untreated samples were used as controls. Evaluation of the samples revealed that *Enterobacteriaceae* was virtually undetectable in dry seeds. However, after 72 hours of germination in both the control and ozone-treated sprouts and following 7 days of storage at 4±2 °C, *Enterobacteriaceae* were found at an average of 7 to 8.1 log CFU g^{-1} in all samples, irrespective of the treatment method. Additionally, mould was observed only in wheat and radish sprouts during the assessment period. The study showed that there was no significant reduction in the number of mesophilic aerobic and facultative anaerobic microorganisms, moulds and *Enterobacteriaceae* then treated with ozone gas.

Keywords: grains, germination, seeds, *Enterobacteriaceae*, microorganisms.

Introduction

Sprouts from grains and vegetable seeds have been considered a healthy food for over 5000 years. Sprouts are usually produced from wheat grains, Chinese beans, alfalfa, broccoli and other *Brassica* spp., fenugreek, radishes, mustard, and hemp seeds. The rich nutritional value and low-calorie content of sprouts make them particularly appealing to modern consumers, especially those interested in healthy eating. However, the amount of nutrients in sprouts, coupled with the warm and humid conditions required for germination, also makes them an ideal environment for the growth of microorganisms, including pathogens, moulds, and yeasts (Tournas, 2005; Waje *et al*., 2009). Unfortunately, ready-to-eat sprouts have been implicated in major disease outbreaks, raising significant food safety concerns for the entire sprout industry. Contaminated dry seeds are often identified as the primary source of sprout-related outbreaks, although other contamination routes may occur during production due to inadequate manufacturing and hygiene practices (EFSA, 2011). Pathogenic and opportunistic bacteria can be transmitted by humans and animals, while seeds themselves may become contaminated from the environment, leading to potential contamination throughout the entire seed germination process. There are several risk factors associated with seed production, including cultivation, harvesting, storage, and distribution. Possible sources of contamination include manure, contaminated irrigation water, the presence of birds and rodents, soil particles, and storage (Thompson & Powell, 2000; Tournas, 2005; EFSA, 2011). The microorganism-friendly conditions during sprout

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production, characterised by warmth and humidity (Bergspica *et al*., 2020), promote the growth and spread of microorganisms, including pathogens, representing a significant risk factor. Studies have demonstrated that cereals and seeds harbour a variety of moulds, such as *Aspergillus, Penicillium* spp*., Fusarium* and *Alternaria* species, which can lead to spoilage during storage when moisture levels exceed 12% (Tournas & Niazi, 2018). Some of these microorganisms are capable of producing toxic secondary metabolites known as mycotoxins (Tournas, 2005). Seeds can acquire microorganism contamination from the surrounding environment, and there are numerous available methods for both physical and chemical treatment. Chemical methods for improving seed germination have significant limitations. These are harmful to the environment. Currently, ozone is one of the most popular ways of treating fruits, vegetables, cereals and seeds (Augspole *et al*., 2014; Brandão *et al*., 2016; Pandiselvam *et al*., 2020; Wang *et al*., 2022). Recently, the use of ozone in food production and processing has gained great importance. Ozone (O_3) is a gaseous substance, which rapidly breaks down into oxygen without any traces (Pandiselvam, Thirupathi, & Anandakumar, 2015b), a highly reactive and powerful oxidiser that has been recognized by the Food and Drug Administration (FDA) as GRAS (Generally Recognised as Safe) and commonly used for treating bottled drinking water in accordance with good hygienic practices (FDA, 1995). Ozone is environmentally friendly, and the company doesn`t necessitate significant investments (Pascual, Llorca, & Canut, 2007). Ozone is fairly soluble in water, so it can have both oxidising and purifying properties (Brodowska *et al*., 2014). Ozone has been shown to improve seeds germination and is utilised as a disinfectant in seed and sprout production, although comprehensive methods for decontaminating all types of seeds or germinated seeds are still under development (EFSA, 2011). The aim of the study was to investigate the impact of ozone on *Enterobacteriaceae* by subjecting wheat (*Triticum aestivum*) grains, alfalfa (*Medicago sativa*), radish (*Raphanus sativus*) and broccoli (*Brassica oleracea*) seeds to ozone gas treatment. Additionally, the grain and seeds were rinsed with ozonated water during germination.

Materials and Methods

Materials

The seeds were purchased in the local market in Latvia, intended for foods and sprouting. Broccoli (Brassica oleracea) expiration date April 2022 and radish (Raphanus sativus) August 2023 seeds originated in Italy. Wheat (Triticum aestivum) was grown in Latvia in 2021 and 2022, but alfalfa (*Medicago sativa*) seeds were grown in Latvia 2021, but alfalfa seeds with expiration date 2023 was originated in Italy. The research was conducted from 2021 to 2023 at the scientific laboratories of the Faculty of Agriculture and Food Technology, Latvia University of Life Sciences and Technologies.

Treatment with gaseous ozone and with ozonated water

Dry seeds/grains were treated with gaseous ozone and ozonated water generated using the BNP OZ-7G series water–air ozone generator (BNPOZONE, China). The ozone concentration was measured using a PSE – Priggen Special Electronic meter. Ozone was introduced into a 10.0 L plastic container with a lid containing 20±1 g of seeds/grains. The container had a lid equipped with connections for gas supply and release. The concentration of ozone in water (2.0 mg L^{-1}) was measured using a portable ozone meter DO3 (Eco Sensors Division of KWJ Engineering Inc., ASV). Untreated, edible seeds/grains were used as controls.

The seeds and grains were subjected to three different types of ozone treatment:

Type 1 – Considering ozone's strong oxidising properties, the following ozone gas concentrations were selected: 20 ppm (Pandiselvam *et al*., 2020; Sharaf – Eldin *et al*., 2022) at an afflux rate of 1.0 L min^{-1} (Rosa *et al*., 2022) for seed exposure of 10 minutes (Singh *et al*., 2002). After treatment with gaseous ozone, the seeds were allowed to germinate, and they were rinsed every 12 hours by immersion in ozonated water. During immersion of ozone the maximum concentration in the water is 2.0 mg L^{-1}, and rinsing continued until the water appeared visually clean. Germination and rinsing were conducted for 72 hours.

Type 2 – Ozone gas concentration were chosen: 20 ppm at a flow rate of 1.0 L min^{-1} for seed exposure of 15 minutes (Singh *et al*., 2002). The maximum ozone concentration in water is 2.0 mg L^{-1}, exposure time 20, 40, 60 minutes. Timing was started when the ozone concentration reached 2.0 mg L^{-1}. After the exposure time, excess ozonated water was drained from the test sample. Rinsing with ozonated water was performed every 12 h for the next 72 h.

Type 3 – Ozone gas concentration was chosen: 50 ppm at a flow rate of 1 L min^{-1} for seed exposure for 1, 2, 3, 4 and 5 hours. After treatment with ozone gas, seeds and grains were germinated for 72 hours.

Seeds germination

The washed seeds were germinated in a glass container in a climate chamber ICH110 (Memmert, Germany) at 23±2 °C in darkness with 80% humidity. After ozone treatment, the samples were washed in

potable water until the water used became visually clear, then soaked. Clean seeds/grains were soaked in potable water at a ratio of 2:1 (water:seed). Seed rinsing was performed every 12 hours using three different treatment methods as mentioned in the section – treatment with gaseous ozone and with ozonated water. Seed rinsing was carried out for 72 h. Untreated wheat grains, broccoli, radish, and alfalfa seeds were used as controls.

Samples storage

Sprouts were germinated for 3 days and then rinsed with potable water or ozonated water until the rinse water was visually clear. Subsequently, they were individually placed in sealed 300 mL polypropylene packaging. Wheat sprouts were packaged in 100 g portions, alfalfa sprouts in 50 g portions, and broccoli and radish sprouts in 75 g portions. The packaged sprouts were then stored for 7 days in a cold chamber at 4±2 ºC in darkness. For microbiological analysis, samples of 10 g and 25 g were taken from each type of sprout.

Microbiological analyses

Microbiological analyses were conducted on three types of dry and soaked seeds and wheat grains, as well as on sprouts. Repeat analyses were performed on the sprouts after 7 days of storage. Total plate count (TPC) was determined in accordance with standard LVS EN ISO 4833:2003A, while mould plate count was determined following standard LVS ISO 21527 – 2:2008. The mould colonies were transferred to a Nutrient agar (NA) plate and analysed by matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry using the MALDI Compass BioTyper ™ 3 database (Bruker Daltonics). *Enterobacteriaceae* determination was conducted according to the standard LVS EN ISO 21528-1:2017 using Violet Red Bile Glucose and to the standard LVS EN ISO 6887-1 decimal dilutions were prepared for the samples. The minimum dilution used in the experiments was 10^{-1} for dry seeds, 10^{-4} for sprouts and the maximum was 10^{-7} (Bernate & Sabovics, 2021). Untreated grains, seeds, and sprouts were tested as controls. Each sample was tested in triplicate, with 10±0.02 g of the sample per replicate placed in sterile filter bags and combined with 90 mL of physiological solution. The result was expressed as the decimal logarithm of colony-forming units (CFU) per gram of the product ($\log_{10}$CFU g^{-1}).

Mycotoxins analyses

Mycotoxins - aflatoxin, ochratoxin, and fumonisin were assessed in radish sprouts and wheat grain sprouts after 7 days of storage at the J.S. Hamilton Baltic laboratory. Aflatoxin was determined using the PN-EN ISO 16050:2011 method, ochratoxin- PB-456 ed. I of 15.10.2021 and fumonisin using PB-43/HPLC ed. III of 28.02.2009 method. The results showed no detection of mycotoxins in any sample.

Statistical analyses

The data were analysed using the Excel program, and the average and standard deviations ($\log_{10}$CFU per gram) were calculated for the listed bacterial populations. An analysis of variance (ANOVA) was employed to determine if there was a significant difference between the control and ozone-treated samples at a confidence level of 95% ($P < 0.05$). Correlation was determined between control and ozone-treated samples between seed types.

Results and Discussion

Assessment of microbiological contamination

Bacteria thrive in environments with sufficient moisture and nutrients (Gan *et al*., 2017), making the sprouts and their germination conditions ideal for bacterial growth.

However, the germination process cannot be considered as one of the main sources of sprout contamination. Sprouts can become contaminated at any stage of the food chain. Although washing and rinsing with clean water can remove dirt and some microorganisms from seeds and grains, a simple rinsing method is often insufficient to eliminate microorganisms, including pathogens. The hazards associated with sprouts emphasize the need for regular surveillance to identify the most suitable treatment methods that ensure the destruction of pathogenic bacteria and to provide the safety of sprouts (Ding, Fu, & Smith, 2013). To mitigate these risks, various treatments using ozone, including gaseous and ozonated water treatments, have been implemented to reduce the microorganism population as much as possible.

*Processing of Type 1***:** The ozone concentration in the water reached 2.0 mg L^{-1}, and the seeds were rinsed until the ozonated water appeared visually clean. Microbiological indicators were determined for both untreated and ozone-treated dry seeds, soaked after 12 hours, germinated after 72 h and storage after 7 days. Based on the previous results, appropriate dilutions were made to determine *Enterobacteriaceae* (Bernate & Sabovics, 2021). Overall, there was no significant difference observed among the analysed sprout samples wheat, alfalfa and radish ($P > 0.05$). After mathematical data processing, insignificant differences between the analysed samples were found, $P = 1.8$. The average number of $\log_{10}$CFU g^{-1} *Enterobacteriaceae* is summarized in Table 1.

Analysis of the samples revealed that no *Enterobacteriaceae* were found in dry broccoli and alfalfa seeds, as well as in soaked broccoli seeds. This shows that the surface of broccoli seeds

Table 1

***Enterobacteriaceae* log_{10}CFU g^{-1} in dry seeds and sprouts both treated with ozone and untreated**

Seeds type	Untreated dry seeds	Untreated soaked seeds	Untreated sprouts 72 h	Untreated sprouts stored for 7 days	Treated dry seeds	Treated soaked seeds	Treated sprouts 72 h	Treated sprouts stored for 7 days
Broccoli	n.d.*	n.d.	7.5±0.1	7.2±0.2	n.d.	n.d.	5.5±0.3	6.2±0.3
Wheat	0.8±0.2	4.8±0.2	7.8±0.1	7.8±0.1	0.2±0.1	5.2±0.2	7.8±0.2	7.6±0.2
Alfalfa	n.d.	2.3±0.2	8.1±0.1	7.6±0.3	0.0±0.0	4.3±0.3	7.2±0.3	7.4±0.3
Radish	0.2±0.1	5.4±0.2	6.8±0.1	7.2±0.2	2.5±0.2	4.9±0.4	7.9±0.3	7.1±0.1

*n.d. – not detected

is relatively free from microbial contamination with *Enterobacteriaceae.* However, during the germination process, under favourable conditions for microorganisms, the number of *Enterobacteriaceae* in untreated sprouts increased from 7.5 to 8.1 log_{10}CFU g^{-1}. From the results, it can be seen that rinsing with ozonated water has a 2 log_{10}CFU g^{-1} reduction in the number of *Enterobacteriaceae* in broccoli sprouts from 7.5 to 5.5 log_{10}CFU g^{-1}. However, after storage, the reduction was only 1 log_{10}CFU g^{-1}, from 7.2 to 6.2 log_{10}CFU g^{-1}. Although a small number of *Enterobacteriaceae* were already detected in dry wheat grains and radish seeds, the effect of treatment with ozonated water on wheat and radish sprouts between control and ozonated water treated samples was statistically insignificant (P=0.39). Both dry raw and treated radish seeds showed *Enterobacteriaceae* contamination of 2.0 – 2.5 log_{10}CFU g^{-1}, comparing to other seed types. Overall, *Enterobacteriaceae* increased dramatically in all seed samples during germination. Microorganism counts in sprouts were significantly higher than pre-germination and soaking rates, with P=0.003 between untreated dry seeds and sprouts and with P=0.01 between ozone-treated dry seeds and sprouts, increasing by 3 log_{10} CFU g^{-1} in wheat grain sprouts and by 4 log_{10}CFU g^{-1} in alfalfa sprouts sample. The correlation coefficient between untreated and treated seeds and sprouts was determined (r = 0.90), indicating a close correlation. Separate observations for each seed type revealed Pearson's correlation coefficients of r = 0.99 broccoli sprouts and wheat sprouts, r = 0.95 for alfalfa sprouts and r = 0.93 for radish seed sprouts. The findings suggest that decontamination of seeds and wheat grains infected during growing, harvesting, and storage with low doses of ozone and short exposure time may not be effective. High levels of *Enterobacteriaceae* populations in sprouts were also observed in other studies (Kim, Kim, & Rhee, 2013; Iversen, & Forsythe, 2004; Bergspica *et al.,* 2020; Abadias *et al.,* 2008; Jang *et al.,* 2021). *Enterobacteriaceae* were most dominant in alfalfa, wheat sprouts and radish sprouts. Regardless of the plant culture, similar trends were observed, with high numbers of *Enterobacteriaceae* and coliforms in the sprouts, posing potential risks to sprout quality and safety (Jang *et al*., 2021). Other studies have shown similar results on microbial contamination in alfalfa sprouts, with initial counts ranging from 3 to 4 log_{10}CFU g^{-1}, increasing to 7-8 log_{10}CFU g^{-1} in finished sprouts. (Kim, Kim, & Rhee, 2013). Singh *et al*. (2002); Wade *et al.* (2003) and Mir *et al.* (2021) studies also indicated that treatment of micro lettuce with ozonated water (5.2 mg L^{-1}) did not significantly reduce *E. coli* O157:H7 populations during 1, 5, 10 or 15 minutes of washing. Augspole *et al*. (2014) carrots were treated with ozonated water to 2 ppm for 60 min ± 1 min before packaging. Samples were analysed after 3.5, 8 and 10 days, the study proved that when treated with ozonated water it was proven that microbiological safety of chopped carrots was possible. According to the results of this study, a reduction was achieved in the total number of microorganisms of shredded carrots to 4.63 log_{10} CFU g^{-1} on the 10th day of storage, similar results for non-treated carrots were acquired on the 5th day of storage. In addition to *Enterobacteriaceae*, the count of facultative anaerobic and mesophilic aerobic microorganisms was determined in these seed and grain samples (Table 2). Evaluation of the results indicates that there is no significant difference in the quantity of microorganisms between the treated and control samples (P > 0.05) when treated with ozone. Both the treated samples and untreated samples had very similar results, P = 0.93. After 72 hours of germination, the number of microorganisms increased in all samples to an average of 8.3 log_{10}CFU g^{-1}. Nevertheless, ozone treatment did not result in a reduction of facultative anaerobic and mesophilic aerobic microorganisms in the sprouts. After 7 days of storage, a similar number of *Enterobacteriaceae* was found for all types of germinated seeds, as observed after 72 h, with an average of 8.3 log_{10}CFU g^{-1} without significant changes P=0.62, regardless of the

type of seeds. The obtained data demonstrate a close correlation between the ozone-treated samples and the control samples, where the Pearson's correlation coefficient is r = 0.99. In this study, the presence of moulds in seeds, grains, and sprouts from the investigated samples was determined (refer to Figure 1). Moulds were found in two samples: soaked wheat grains and sprouts, as well as in radish seeds and sprouts. *Aspergillus, Fusarium*, and *Penicillium* moulds were identified in wheat, but radish *Fusarium proliferatum*. Evaluating the results of mould reveals higher contamination levels in raw wheat and radish sprouts at 3.1 $\log_{10}$CFU g^{-1}. However, treated wheat sprouts exhibited a 48% reduction in mould, and a 37% reduction was observed after 7 days of storage. Despite these reductions, differences between samples were not statistically significant (P = 0.529).

In addition to mould detection, mycotoxin analysis was conducted on wheat grain sprouts and radish sprouts. Tournas (2005) indicated in his study that bean sprouts exhibited high mould contamination at 8.4 $\log_{10}$CFU g^{-1}, while alfalfa sprouts and broccoli sprouts respectively showed 6.3 $\log_{10}$CFU g^{-1} and 5.6 $\log_{10}$CFU g^{-1}. *Penicillium* spp. were predominant in most samples, while *Fusarium* spp. contamination remained low. The author suggests that these organisms may be contaminants carried over from the field, unable to thrive after harvest.

Processing of Type 2: The sprouts were treated with ozonated water, ensuring the flow of ozonated water with an ozone concentration in the water of 2.0 mg L^{-1}. The exposure times were 20, 40, and 60 minutes. No significant changes were found (P>0.05), P values ranging from 0.17 to 0.99 between control seed samples and for treated seeds samples. Dry broccoli seeds showed no presence of *Enterobacteriaceae*, but in soaked seeds, it reached up to 4.1 $\log_{10}$CFU g^{-1}. After 24 hours, both the control and ozone-treated seeds exhibited *Enterobacteriaceae* levels ranging from 6.3 to 6.6 $\log_{10}$CFU g^{-1}. No *Enterobacteriaceae* contamination was detected in dry alfalfa and radish seeds. However, in soaked alfalfa seeds, *Enterobacteriaceae* reached 3.4 $\log_{10}$CFU g^{-1} in the control sample and 5.5 $\log_{10}$CFU g^{-1} in the ozone-treated samples. Similarly, radish seeds exhibited 4.2 $\log_{10}$CFU g^{-1} in the control and 5.0 $\log_{10}$CFU g^{-1} in the ozone-treated samples. As the germination process continued, *Enterobacteriaceae* levels increased in both the control and ozone-treated alfalfa samples, reaching from 8.0 to 8.5 $\log_{10}$CFU g^{-1}. Radish sprouts exhibited *Enterobacteriaceae* contamination levels ranging from 7.0 to 7.6 $\log_{10}$CFU g^{-1}. Upon evaluating the samples, it was observed that after 7 days of storage *Enterobacteriaceae* decreased in wheat sprouts by an average of 1 $\log_{10}$CFU g^{-1} (Figure 2). The study indicates that the microorganisms remaining in seeds during cultivation, harvesting, and storage are present in small amounts.

Dry seed samples showed either no detection or very low levels of microorganisms, which do not pose a threat to consumer health. However, upon soaking and sprouting, bacterial contamination levels increase dramatically. Jang *et al*. (2021) also noted in their study that the number of *Enterobacteriaceae* increases from 3 to 7 and 8 $\log_{10}$CFU g^{-1} in alfalfa, rape and radish sprouts during germination. Wade et al. (2003) was report that ozone treatment does not significantly reduce the population of *Listeria monocytogenes* similar as to *Enterobacteriaceae,* regardless of treatment time (10 or 20 min) or seed type. On the contrary, seeds treatment with water for 20 minutes, where gaseous ozone was passed (0.34 m^3 h^{-1}) through the seed-water mixture, significantly increased the number of *L. monocytogenes*. The slight decrease (0.21 to 0.27 $\log_{10}$CFU g^{-1}) in the first 10 minutes of treatment is due to the partial destruction of *L. monocytogenes* on the surface of the seeds. Wade et al. (2003) mention that the ozone

Table 2

Number of mesophilic aerobic and facultatively anaerobic microorganisms (MAFAM) $\log_{10}$CFU g^{-1} in untreated, treated dry seeds and sprouts

Seeds type	Untreated dry seeds	Untreated soaked seeds	Untreated sprouts 72 h	Untreated sprouts stored for 7 days	Treated dry seeds	Treated soaked seeds	Treated sprouts 72 h	Treated sprouts stored for 7 days
Broccoli	2.4±0.20^{d}	2.4±0.10^{d}	8.3±0.20^{a}	8.3±0.05^{a}	2.4±0.05^{d}	2.9±0.15^{d}	8.3±0.10^{a}	8.3±0.05^{a}
Wheat	4.1±0.10^{b}	5.2±0.15^{b}	8.3±0.10^{a}	8.4±0.20^{a}	3.5±0.20^{d}	5.3±0.15^{b}	8.4±0.15^{a}	8.6±0.05^{a}
Alfalfa	1.2±0.15^{c}	3.6±0.15^{d}	8.3±0.10^{a}	8.3±0.15^{a}	1.3±0.10^{c}	4.6±0.10^{b}	8.3±0.05^{a}	8.3±0.01^{a}
Radish	3.5±0.05^{d}	5.5±0.25^{b}	8.2±0.10^{a}	8.1±0.05^{a}	4.2±0.20^{b}	5.4±0.10^{b}	8.2±0.10^{a}	8.2±0.10^{a}

Note. Data are shown as mean values (n = 3) ± standard error (SE). Different letters indicate significant differences at p < 0.05 one-way ANOVA

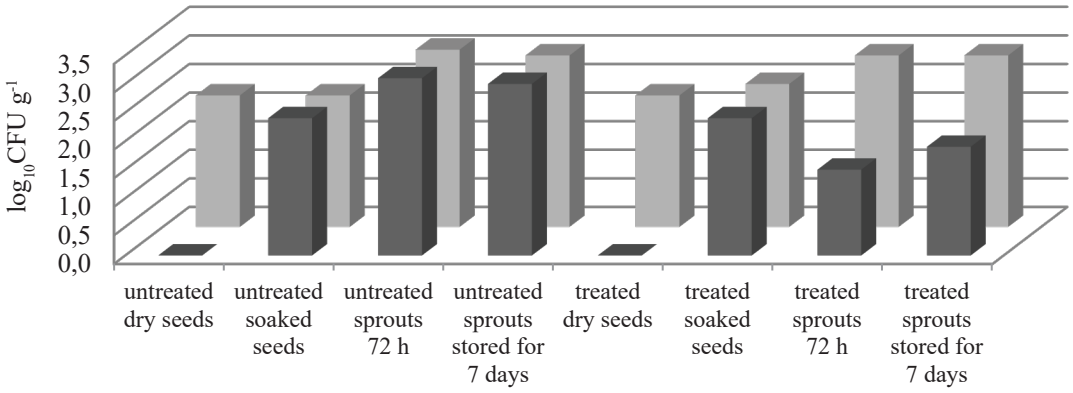

Figure 1. Mould count $\log_{10}$CFU g^{-1} in untreated dry seeds and sprouts.

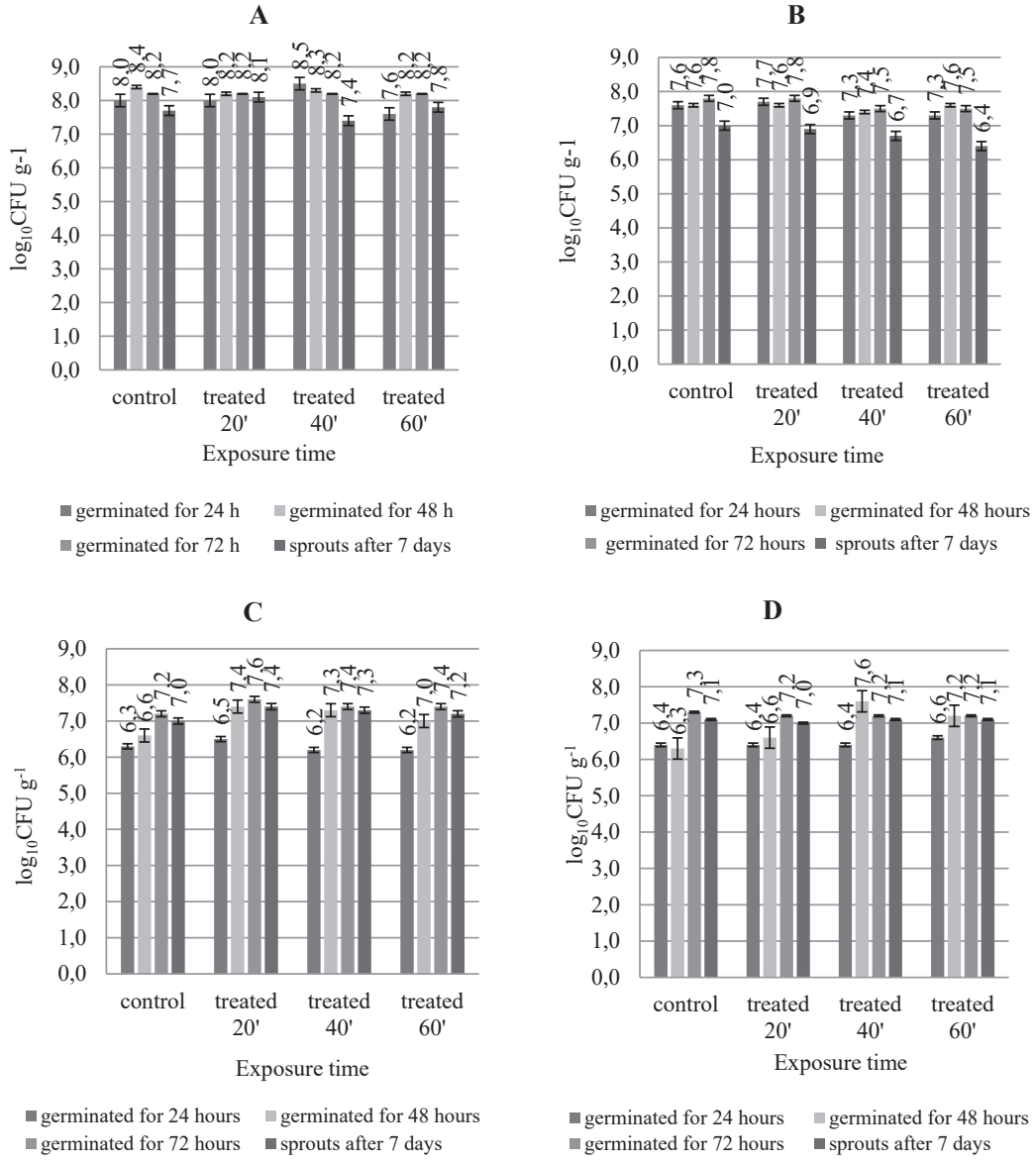

Figure 2. *Enterobacteriaceae* $\log_{10}$CFU g^{-1} in untreated and ozone-treated dry seeds and sprouts, alfalfa (A), wheat (B), radish (C), broccoli (D).

concentration decreases to an ineffective level at some point during the 20-minute treatment. Subsequent rinsing, where water may become oxygenated due to ozone decomposition, along with the moist and warm germination conditions, seem to facilitate the release of viable *L. monocytogenes* from the surface, particularly from areas under the shell. This results in a significant increase in *L. monocytogenes* after 20 minutes of treatment compared to 10 minutes (Wade *et al*., 2003). In their study, Kwack *et al*. (2014) also observed that treatment with ozonated water, where the maximum dissolved ozone concentration was 3.5 ppm for 5 minutes, resulted in an insignificant reduction of total microbial population, *E. coli* and *Salmonella spp*. Similarly, Sharma *et al*., 2002 reported that although alfalfa seed coat is smooth, when observed by microscopy, alfalfa seed surface had many cracks and gaps. These imperfections provide favourable environments for microorganisms, including pathogens.

Consequently, the reduction in microorganism populations was attributed to the lower concentration of dissolved ozone, which was unable to penetrate damaged areas on or beneath the surface of the alfalfa seed. Consistent with the findings of Kwack *et al*. (2014) and Wade *et al*. (2003), this study shows that the reduction in *Enterobacteriaceae* is nonsignificant ($P > 0.05$), irrespective of seed type or duration of treatment with ozonated water.

*Processing of Type 3***:** This type of processing exclusively utilised gaseous ozone at a concentration of 50 ppm, with a flow rate of 1.0 L min^{-1} for seed exposure ranging from 1 to 5 hours. Untreated edible seeds/grains served as the control. Upon evaluating the results, it is evident that the reduction in the number of microorganisms due to ozone gas treatment is generally insignificant $P = 0.74$. *Enterobacteriaceae* contamination remains high, and reduction was not achieved in alfalfa sprouts. *Enterobacteriaceae* was detected in all samples, ranging from an average of 6.3 $\log_{10}$CFU g^{-1} in broccoli and radish sprouts to 8.5 $\log_{10}$CFU g^{-1} in alfalfa sprouts (Table 3). In broccoli sprouts, *Enterobacteriaceae* decreased by 1 $\log_{10}$CFU g^{-1} after one hour of treatment, while in wheat grain sprouts, reduction occurred after 4 hours of treatment. Similarly, in radish seed sprouts, reduction was observed after 1, 4, and 5 hours of treatment collate to the control sample. After 7 days of storage in a cold chamber at a temperature of 4±2 ºC, a similar number of *Enterobacteriaceae* were found for all types of sprouts as observed after 72 hours. Despite the high number of microorganisms present, the sprouts showed no signs of spoilage after 7 days of storage, and there were no significant differences between seed types, as indicated by a P-value of 1.06. In fact, crops of various vegetable seeds and grains grown and harvested in a natural environment will not be free of microorganisms (Sagoo *et al*., 2003), and germination conditions further contribute to the proliferation of microorganisms. However, even with effective washing and treatment with ozone gas, decontamination cannot be achieved. Despite the high microbial counts, National Advisory Committee on Microbiological Criteria for Foods (1999) stated that high microbial levels in themselves do not necessarily pose a public health concern in sprouts; the presence and growth of pathogenic microorganisms is a concern, and opportunistic pathogens can cause health harm to at-risk groups of consumers.

Table 3

***Enterobacteriaceae* $\log_{10}$CFU g^{-1} in untreated and ozone-treated dry seeds and sprouts**

Seeds type	Untreated	Ozonated 1h	Ozonated 2h	Ozonated 3h	Ozonated 4h	Ozonated 5h
Broccoli sprouts	7.4±0.15	6.4±0.15	6.7±0.20	7.2±0.06	7.2±0.10	8.0±0.10
Broccoli sprouts after 7 days of storage	7.0±0.11	6.3±0.10	6.5±0.10	7.0±0.20	6.5±0.20	7.5±0.30
Wheat sprouts	7.3±0.05	7.6±0.05	7.7±0.06	7.3±0.10	6.4±0.20	7.0±0.10
Wheat sprouts after 7 days of storage	7.0±0.10	7.0±0.06	7.1±0.10	7.2±0.11	6.8±0.10	7.2±0.06
Alfalfa sprouts	7.8±0.05	8.5±0.10	8.3±0.20	8.1±0.10	7.3±0.06	7.4±0.10
Alfalfa sprouts after 7 days of storage	7.0±0.10	7.4±0.10	7.6±0.10	8.0±0.10	7.6±0.15	7.7±0.05
Radish sprouts	7.5±0.11	7.5±0.15	6.7±0.25	6.8±0.15	6.6±0.06	6.3±0.10
Radish sprouts after 7 days of storage	6.3±0.10	6.5±0.10	7.2±0.21	7.2±0.11	7.2±0.11	6.6±0.06

Conclusions

The study revealed that rinsing seeds and grains with ozonated water, regardless of the exposure time, or treating them with ozone gas at a concentration of 50 ppm for 1-5 hours, did not result in a significant reduction in the number of facultative anaerobic and mesophilic aerobic microorganisms, moulds, and *Enterobacteriaceae.* While there was a reduction of *Enterobacteriaceae* by 2 $\log_{10}$CFU g^{-1} in the first type of processing, and a reduction of mould in radishes from 3 to 1.5 $\log_{10}$CFU g^{-1}, these reductions were insufficient to ensure the production of a harmless final product. Despite the high microbiological contamination observed, no signs of sprout spoilage were detected. This study highlights that effective washing, rinsing with clean drinking water, and ozone treatment alone are not sufficient to completely decontaminate products to reduce microorganisms to levels that are safe for consumer health, especially for the at-risk groups. To achieve a significant reduction in the number of microorganisms, it will be necessary to implement combined processing methods.

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