LWT - Food Science and Technology 169 (2022) 113998

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Effect of negative air ionization technology on microbial reduction of food-related microorganisms

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ARTICLE INFO

Keywords:
Negative air ions
Ozone
Antimicrobial effect
Food-related microorganisms

ABSTRACT

Negative air ions (NAI) have been shown to have bactericidal effects on various microbial species.

Different concentrations of NAI and ozone (10^6-7x10^6 NAI cm^{-3} s^{-1}, 2.20 mg h-1 of ozone or 8 × 10^6-2x10^7 NAI cm^{-3} s^{-1}, 0.02 mg h^{-1} of ozone, respectively) were studied to determine whether the effect of these treatments on six food-related microorganisms was due to NAI or also to the ozone that forms.

Bacillus subtilis, *Escherichia coli*, and *Listeria monocytogenes* showed a significantly microbial reduction after the treatments of NAI with a low concentration of ozone, while *Pseudomonas fluorescens*, *Penicillium roqueforti*, and *Saccharomyces cerevisiae* were affected mainly by NAI and higher ozone concentration. It should be noticed that the reduction of the pathogen *L. monocytogenes* has been 2.17 Log after 72 h treatment to NAI and low ozone concentration.

The results proved that ions (NAI) contribute to microbial killing. However, the sensitivity of microbial species is different and depends on individual parameters of the different microorganisms.

NAI technology with low ozone production can be rapid, green, and low-cost and it can represent an alternative to the use of chemicals for the sanitation of air and surfaces in the food sector in order to reduce microbial contamination.

1. Introduction

Negative air ions (NAI), being airborne charged molecules naturally present in nature, form when neutral air molecules are broken down by energy taken from sunlight, radiation, atomization of water, and air movement (Tyagi et al., 2012). The generation of ions is also possible by photon, nuclear or electronic processes (Daniels, 2002). The most common among these is the corona discharge method, which is used to ionize air through appropriate high voltage between the ground and discharge electrodes. The result is an electric field that interacts with the molecules producing ions of the same polarity as the applied voltage (Roth, 1995). Commercial ion generators produce negative ions, and the main ones are O^-, O^{2-} and OH^- generated in a corona discharge (Nagato et al., 2006). These ions react with components in the air to generate the secondary negative ions, giving rise to a continuous creation of ions by ion-ion and ion-electron recombination reactions (Kim et al., 2017; Skalny et al., 2004). The concentration of ions produced depends on the surrounding operating conditions (relative humidity, temperature, and distance from the source) and it follows a logarithmic linear tendency within a specific distance depending on the relative humidity (Wu et al., 2006).

In recent years, ionization systems have been proposed for the cleaning treatment of air and surfaces. Ions react and destroy air pollutants, such as volatile organic compounds, dust, tobacco smoke, and other odors, improving air quality (Kim et al., 2017; Kim et al., 2011). This technology has been demonstrated to be effective in the treatment of biological contaminants as well as microorganisms. The antimicrobial capacity of NAI has been evaluated against different microorganisms related to the food industry, like *Salmonella enteritidis*, *Pseudomonas fluorescens*, *Erwinia carotovora* pv. *carotovora*, *Escherichia coli*, bacterial spores of *Bacillus stearothermophilus*, *Campylobacter jejuni*, *Listeria monocytogenes*, and *Staphylococcus aureus* (Arnold et al., 2004; Fan et al., 2002; Seo et al., 2001; Tyagi et al., 2008).

These studies showed that the reduction of microbial viability by NAI depended on the microbial strain, the environmental conditions, and the characteristics of the device used for the NAI generation. Furthermore, three mechanisms are involved in causing microbial death: electrodynamic, electrostatic, and electrochemical effects. The chemical species involved are ions, electrons, and other ionizing radiations (electrodynamic effect), electric charge and electric field (electrostatic effect), and ozone (electrochemical effect) (Fletcher et al., 2007). Results show that

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https://doi.org/10.1016/j.lwt.2022.113998
Received 8 July 2021; Received in revised form 11 September 2022; Accepted 17 September 2022
Available online 19 September 2022

Abbreviations

room T	room temperature
ref T	refrigeration temperature

negative energization and ozone are the main causes of death for the tested species (*S. aureus*, *Mycobacterium parafortuitum*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, *Burkholderia cenocepacia*, *B. subtilis,* and *Serratia marcescens*). Researchers have found that NAI and ozone released during the ionization process have a synergistic effect on microbial cells and that the efficacy is not the same if the microorganism is individually treated with ozone or ions (Becker et al., 2004; Fan et al., 2002; Kampmann et al., 2009; Tanimura, 1997). The effects of ions and ozone on microbial cells can be attributed to oxidative processes. Ozone reacts with fatty acids, glycoproteins, and glycolipids of the microbial cell membrane (Khadre et al., 2001). Ions use their electrostatic force to interact with charged groups in the cell wall causing physiological and biochemical changes leading to the formation of intracellular reactive oxygen species that generate oxidative reactions (Park et al., 2016). Since ozone can be toxic at certain levels (Pascual et al., 2007), new devices are currently being developed to improve NAI generation and to reduce, at the same time, the release of ozone (Jiang et al., 2018). Mitchell and Stone (2000) developed the electrostatic space charge system (ESCS), which generates a very high negative ion concentration ($>10^6$ NAI cm^{-3}) minimizing the production of ozone (<0.01 ppm). Kim et al. (2011) submitted *E. coli* and *S. epidermidis* to ionization treatment with a concentration of ozone lower than 0.01 ppm; they found that there is an antimicrobial effect mainly due to electroporation. Park et al. (2016) showed that ions generated during the process under a low concentration of ozone are responsible for the reduction in viability of *E. coli*, *Enterococcus faecalis*, *B. subtilis,* and *S. aureus*. Moreover, the concentrations of ozone used to obtain a microbial reduction may often cause oxidation phenomena in food components (Baggio et al., 2020) limiting the possibility of using this technology for food preservation.

From these observations and considering the synergism between NAI and low levels of ozone, the ionization technology could be applied in the food industry to reduce the viable microbial load present in equipment for food storage, and on the surfaces for food processing (Hildebrand et al., 2001; Kampmann et al., 2009; Seo et al., 2001; Tuffi et al., 2012). Moreover, the use of NAI may be considered a green technology since it does not leave residual chemicals and it may be applied as alternative to chemicals in the sanitization procedures.

However, few studies have been carried out on the antimicrobial activity of NAI in the food sector.

The aim of this study has been to investigate the antimicrobial activity of NAI technology on different microbial species of the food sector considering different concentrations of NAI and ozone. The microorganisms were subjected to treatments with NAI at different temperatures and exposure time to mimic the real conditions at which food is processed or stored.

2. Materials and methods

2.1. Microbial cultures

Six microorganisms were used in this study: *Bacillus subtilis* DSMZ 4181, *Escherichia coli* ATCC 8048, *Listeria monocytogenes* 284, and *Pseudomonas fluorescens* L22 (wild-type strains belonging to the collection of DI4A, University of Udine), *Penicillium roqueforti* PR N (used for the preparation of blue cheese), and *Saccharomyces cerevisiae* SCHLB (used for the preparation of fermented milk). Stock cultures of the strains were stored at −80 °C in their medium and glycerol at 30% (v:v) as cryoprotectant was added until use. For the experiments, an aliquot of each stock culture was transferred into appropriate broth to prepare the overnight suspension.

2.2. Culture media

Brain Heart Infusion (BHI, Oxoid, Milan, Italy) was used to prepare both the suspensions of *B. subtilis*, *E. coli*, *L. monocytogenes*, and *Ps. fluorescens,* and the agar plates for the spread of *L. monocytogenes*. Plate Count Agar (PCA, Oxoid, Milan, Italy) was used to prepare the solid medium for the spread of *B. subtilis*, *E. coli*, and *Ps. fluorescens*. Malt Extract (ME, Oxoid, Milan, Italy) broth and agar were used to cultivate *P. roqueforti*. Yeast extract Peptone Dextrose (YPD, Sigma Aldrich, Milan, Itlay) broth and agar were used for *S. cerevisiae*.

2.3. Sample preparation

The suspensions were incubated overnight at 37 °C for *E. coli* and *L. monocytogenes*, at 30 °C for *B. subtilis, Ps. fluorescens,* and *S. cerevisiae,* and at 22 °C for *P. roqueforti* reaching a final concentration of 10^8–10^9 CFU mL^{-1}. From the overnight suspensions of each microorganism, serial decimal dilutions were made in Maximum Recovery Diluent (MRD, Oxoid, Milan, Italy); the dilutions of each strain were plated on the respective agar medium according to the drop plate method (Herigstad et al., 2001). For each microorganism, two agar plates containing the decimal serial dilutions of the overnight suspension were prepared. The inoculated plates were used both for exposure to the ionization treatment and for control (without ionization treatment).

2.4. Operative conditions

Two negative air ion generators were characterized to verify the statements reported in the technical data sheet (Table 1) concerning particularly the amount of NAI and ozone generated. The technical characteristics of the devices were provided by the manufacturers. Conventionally, the devices were named A and B (to avoid any type of conflict of interest).

Ions were generated by corona discharge method, which has been identified as the common technology for the generation of NAI. The ionization generators used for the tests were small sized, in order to be easily used in a treatment chamber having reduced volumes (Kampmann et al., 2009; Seo et al., 2001).

The number of NAI was measured by Air Ion Counter Model AIC2 (AlphaLab Inc., Salt Lake City, UT, USA). The amount of ozone was estimated by Ozone Analyzer Model UV-100 Serial (Eco Sensors, Santa

Table 1
The main characteristic of devices A and B reported in the technical datasheets provided by the sellers.

	device A	device B
Description	Electronic housing linked with the load part -made by grid and needles- through high voltage silicon cables	Compact generator made by a ground electrode and a discharge needle electrode
Generation principle of NAI and ozone	Corona discharge	Corona discharge
Input voltage	230 V AC	12 V DC
Output voltage	−3.7 to −4.7 kV DC	−2.5 to −3.5 kV DC
Generation of NAI (at 30 cm from the source)	$\geq 6.0 \times 10^6$ NAI cm^{-3}	7.2×10^6 NAI cm^{-3}
Generation of ozone	0.10 mg m^{-3}*	0.04 mg h^{-1}
Usage temperature	−10 – +60 °C	NA**
Usage relative humidity	<90%RH	NA

* expressed as active oxygen concentration (volume of measurement not reported in the datasheet).
** NA = data not available in the technical datasheet.

Fe, NM, USA), which used a filtered mercury lamp for absorption at 254 nm. The evaluation of NAI and ozone production was carried out inside the air-tight chamber used for the treatments (Fig. 1) with and without the presence of inoculated plates. Two different conditions resulted: NAI + O_3 and NAI + lowO_3 (discussed in 3.1).

2.5. Preparation of the treatment chamber

An air-tight chamber made up of plastic material was used (size 30 cm × 50 cm x 30 cm; H x L x W). The total volume of the chamber was 0.045 m^3. Before the exposure to the ionization treatment, the chamber was cleaned with ethanol 70% (v:v). The device was placed on the base in the center inside the chamber. A fan (NMB 3610RL-04W–B10, Minebea Co., Ltd), having a maximum airflow of 1.13–1.84 m^3 min^{-1} and a rating voltage of 12 V DC, was placed on the base near the device to guarantee air circulation. A sensor (AMS CCS811) was fixed for continuous monitoring of the values of temperature and relative humidity (RH) during the treatments. Humidity in the chamber was not controlled. The inoculated plates (samples prepared as reported in section 2.3) were placed on the base of the chamber and removed from their lid (Fig. 1).

2.6. Ionization treatment

The samples were exposed to the treatment with NAI + O_3 for 1, 2, 3, 4, 5, 6 h at room T and ref T, and with NAI + lowO_3 for 4, 16, 24, and 72 h at ref T (discussed in 3.1). Negative control samples were put into an identical air-tight chamber under the same treating time conditions as the treated samples, without any ionization treatment, to assess the influence of flowing air (desiccating effect). When the tests were carried out at ref T, additional controls at room T were carried out to assess the effect of the exposure to refrigeration temperature. After the treatment, the plates were removed from the chamber, closed with the lid, and incubated under specific conditions (*E. coli* and *L. monocytogenes* at 37 °C for 24 h, *B. subtilis*, *Ps. fluorescens,* and *S. cerevisiae* at 30 °C for 24 h, and *P. roqueforti* at 22 °C for 72 h). Finally, the count of viable cells was evaluated.

2.7. Statistical analysis

Two technical repetitions were done for each experiment and the tests were carried out in two biological replicates. The results were expressed as Log (N/N_0), where N = viable count after treatment and N_0 = viable count of negative controls. The means obtained from replicate tests were subjected to one-way analysis of variance ($p < 0.05$), preceded by Levene test to verify the homogeneity of variance between means using Statistics 8.0 (Statsoft software, Tulsa, Oklahoma, USA). Differences between the means were assessed using Tukey's HSD post-hoc test.

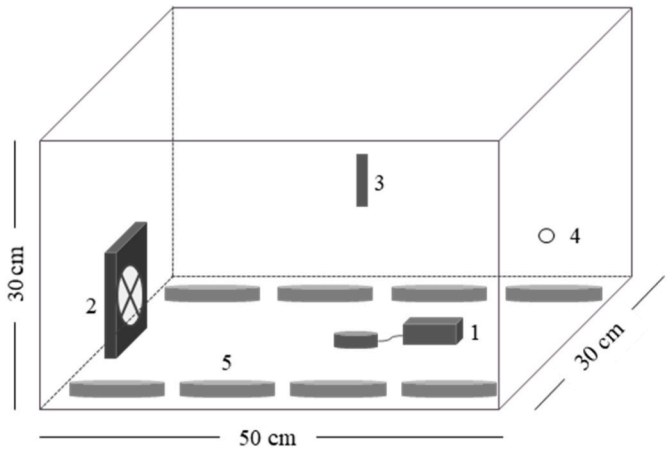

Fig. 1. Setup realized for the treatment of microorganisms. 1 = device, 2 = fan, 3 = T-RH sensor, 4 = hole for monitoring ozone, 5 = inoculated plates. The same setup was used for the characterization of the NAI generators.

3. Results and discussion

3.1. Device characterization

Table 1 reported the main characteristics of the two devices used. The characterization was carried out inside the 0.045 m^3 air-tight chamber. The volume selected represented an intermediate value usually observed in other studies using NAI technology (Chauhan et al., 2015; Fan et al., 2007; Kampmann et al., 2009; Park et al., 2016; Tyagi & Malik, 2010, 2012). The characterization was carried out considering two different setups. For the first setup (setup 1) the device and the fan were placed inside the chamber to estimate the amount of NAI and ozone generated. The aim of this test was to exclude adverse conditions, such as the presence of organic matter that could interact with the species generated: this allowed the effective potentiality of the devices to be checked. The second setup (setup 2) was arranged by adding the inoculated agar plates, to simulate the conditions in which the tests would be later conducted. This setup aimed to study the tendency of NAI and ozone to react with microorganisms and organic and inorganic substances contained in the agar medium composition, which are known for reacting to ozone (Güzel-Seydim et al., 2004; Marino et al., 2015; Restaino et al., 1995) and NAI (Fletcher et al., 2007; Noyce & Hughes, 2002, 2003). The characterization of the devices was performed at room T and ref T to evaluate how this parameter influenced the devices while working. The values of temperature measured in the air-tight chamber were 22.8 °C ± 1.1 °C at room T and 5.4 °C ± 0.7 °C at ref T. The RH detected inside the chamber was 75% ± 3% in setup 1 and 89% ± 1% in setup 2, the latter was probably higher due to the presence of agar medium. According to the results found by Wu et al. (2006) and considering the dimensions and the volume of the chamber, the concentration of NAI remained almost stable regardless of the RH. The detected values were within the ranges reported in the datasheet of device A (Table 1); thus, the same ranges were considered valid also for device B.

The characterization of the devices was carried out by evaluating the amounts of generated NAI and ozone (Table 1), which are responsible for giving an antimicrobial effect.

NAI. Device B generated ions in the range of $8 \times 10^6 - 2 \times 10^7$ NAI cm^{-3} s^{-1}, which was slightly higher than the range of device A ($1 \times 10^6 - 7 \times 10^6$ NAI cm^{-3} s^{-1}), confirming the values of the technical datasheets. The amount of NAI was almost constant during the overall period of 6 h for each setup and at the different temperatures taken into consideration. This was due to the short lifespan of NAI (100–1000s in clean air), which allowed an average concentration of NAI to be maintained unchanged over time (Gaunt et al., 2005). This result was very important since it demonstrated that a constant production of more than 10^6 ions cm^{-3} is needed to produce an antimicrobial effect (Fan et al., 2002; Tyagi et al., 2008).

Ozone. By the study of setup 1, whose aim was to yield the amount of ozone generated, device A production was 2.20 mg h^{-1} ozone, while device B production was 0.02 mg h^{-1}, with an accumulation of the gas inside the chamber during the 6 h testing time for both devices. The expression in mg h^{-1} is calculated from the concentration of the ozone after 1 h in the 0.045 m^3 chamber; this unit is correct when the aim is to express the production capacity and not a concentration value. The assessment confirmed the value reported in the technical specification of device B. Regarding device A, it emerged that the generation of ozone was higher than expected. The active oxygen concentration (referring to the totality of the oxygen active species, i.e., singlet oxygen, oxygen atoms, and ozone) was reported in the technical datasheet (Table 1); as expected, considering the volume of the chamber, the values of ozone

were no greater than 0.0045 mg h^{-1}. The trend of the amount of ozone detected inside the chamber with setup 2 is reported in Table 2. Considering the production rate (mg h^{-1}) of the devices stated with setup 1, the concentration (mg m^{-3}) of ozone was lower. This outcome confirmed what had already been reported by Khadre et al. (2001): the presence of microorganisms and organic and inorganic matter interacts with ozone and reduces its amount in the air. The values increased over time for device A, especially at ref T: this could be explained because the solubility of ozone in water (i.e. molecules of water present in the air) increases as the temperature decreases (Khadre et al., 2001). For device B, during the study of setup 2, the concentration of ozone was lower than the detection limit of the instrument (0.02 mg m^{-3}) throughout the whole test; tests/experiments led to similar results to those obtained by Arnold et al. (2004) and Seo et al. (2001). In conclusion, two different conditions of production of NAI and ozone were identified:

1. NAI + O_3 (NAI and high concentration of ozone);
2. NAI + lowO_3 (NAI and low concentration of ozone).

3.2. Explorative assessment of NAI + O_3 and NAI + lowO_3 at room and refrigeration temperatures

This part of the study was considered to understand the potential antimicrobial effect given by the two conditions identified (NAI + O_3 and NAI + lowO_3) on the microorganisms *L. monocytogenes* and *Ps. fluorescens*. They were chosen among all those used for the present experimentation since they are considered peculiar species of interest in the food industry. *L. monocytogenes* is a Gram-positive pathogen, cause of listeriosis in humans, psychrotrophic, can grow at refrigeration temperature (−0.5 – 9.3 °C), and is ubiquitous as it is able to grow and survive in different environments (Ferreira et al., 2014; Walker et al., 1990). For this microorganism, European mandatory limits for food (Reg. CE 2073/2005) are stated. *Ps. fluorescens* is Gram-negative, psychrotrophic, ubiquitous, and typical spoilage bacteria involved in a lot of food-quality decays (fresh vegetables, meat and poultry, eggs, fish, and dairy products). Moreover, *L. monocytogenes* and *Ps. fluorescens* are known to be able to form biofilms in food processing environments (Marino et al., 2018) representing an important challenge for the hygienic conditions of foods, food working surfaces, and consumer health (Møretrø & Langsrud, 2017).

The exposure to ionization treatment continued for 1, 2, 3, 4, 5, and 6 h at room T (22.8 °C ± 1.1 °C–97% ± 3% RH) and ref T (5.4 °C ± 0.7 °C–98% ± 1% RH) (Table 3). For none of all the times considered, a desiccating effect was observed, and the viable count of the control samples always remained 10^8–10^9 CFU mL^{-1}. Moreover, the controls exposed to ref T were not significantly affected by the cold temperature compared to the same controls at room T This highlighted the psychrotrophic nature and adaptation of the two microorganisms to low temperature (Collins & Margesin, 2019). Generally, both devices perform an antimicrobial activity in the tested conditions, except for NAI + lowO_3 in the case of *Ps. fluorescens*. The efficacy of the treatment was observed to be higher using NAI + O_3, confirming what had been previously found by other studies (Fan et al., 2002; Fletcher et al., 2007). Focusing on L. *monocytogenes* the antimicrobial effect after 6 h was higher at room T (reductions by 3.03 ± 0.14 Log with NAI + O_3 and 2.42 ± 0.11 Log with NAI + lowO_3) than at ref T (reductions by 1.63 ± 0.32 Log with NAI + O_3 and 1.34 ± 0.72 Log with NAI + lowO_3) independently of the amount of NAI or ozone applied. This behavior could find a possible explanation in the psychrotrophic nature of this microorganism, for which the ability to adapt to cold stress conditions through the production of cold shock proteins is demonstrated (Santos et al., 2019). Indeed, in both conditions at ref T, after the reduction during the first hour of exposure, no significant reduction was observed and the trend remained almost stable throughout the 6-h treatment. Similar behavior was previously found by Marino et al. (2018) after the exposure of microbial biofilms to ozonated water and gaseous ozone, suggesting that the antimicrobial effect of these oxidative technologies (i.e. NAI and ozone) can be achieved in the first stages of the exposition. Another observation was that the exposure for 6 h at ref T produced a similar result for both conditions identified (reductions by 1.63 ± 0.03 Log with NAI + O_3 and 1.34 ± 0.11 Log with NAI + lowO_3), suggesting that during the exposures for prolonged periods, NAI and low concentration of ozone can achieve similar results to those obtained in the presence of NAI and to a high concentration of ozone. A study by Boumail et al. (2016) demonstrated the antimicrobial effect of negative air ionization with a minimal amount of ozone on ready-to-eat cauliflower florets inoculated with *L. innocua*, showing a reduction by 2 Log CFU g^{-1} after one week exposure to NAI treatment.

These findings suggested the opportunity to increase the ionization treatment time to avoid dangerous levels of ozone, which, under certain exposure conditions, is toxic for humans (Karaca & Velioglu, 2007). Nonetheless, prolonged time of ionization treatment could be used for food preservation at ref T in the food sector.

The results for *Ps. fluorescens* showed a different trend of microbial reduction compared to that of *L. monocytogenes*. The different efficacy related to the presence of varying amounts of ozone was very clear, with NAI + lowO_3 not being able to generate any logarithmic reduction in the test conditions adopted in this study. It may be hypothesized that the presence of NAI and a low concentration of ozone (ozone was not detected inside the chamber in the presence of inoculated plates as previously detailed in paragraph 3.1) exerted a form of sub-lethal response by this bacteria, which can adapt its metabolism to survive oxidative stress (Mailloux et al., 2011) deploying cellular defences in enzymes (superoxide dismutase, catalase) and DNA repair enzymes. At a genomic level, most bacteria possess genes that regulate response to environmental stresses such as the OxyR system and soxRS system. Moreover, this behavior may be related to the different structures and composition of the cell wall between Gram-negative and Gram-positive bacteria. According to Pascual et al. (2007), Gram-positive bacteria are more sensitive than Gram-negatives to ozone exposure, and therefore their very low presence using the NAI + lowO_3 condition may explain the sensitivity of *L. monocytogenes* but the non-effect on *Ps. fluorescens*. However, the sensitivity to ozone can be different for each microorganism (Marino et al., 2018), and also two Gram-negative bacteria could have a different behavior in the presence of this molecule. On the contrary, NAI + O_3 caused a reduction in viability at both exposure temperatures, revealing that the presence of ozone at high concentrations was essential for achieving the microbial death of this microorganism. This observation was in agreement with the results by Fan et al. (2002), who found that exposure to NAI alone did not affect the viability of *Ps. fluorescens*, but the simultaneous exposure to ozone and NAI caused an

Table 2
Values of ozone (mg m^{-3}) and NAI (ions cm^{-3} s^{-1}) detected with setup 2 inside the air-tight chamber during the characterization of devices A and B at room T and ref T (mean ± SD; n = 3).

		Ozone (mg m^{-3})		NAI (ions cm^{-3} s^{-1})
	hours	room T	ref T	room T & ref T
device A	1	1.50 ± 0.36 [a]*	3.64 ± 0.06 [a]	$1 \times 10^6 - 7 \times 10^6$
	2	1.38 ± 0.14 [a]	4.90 ± 0.12 [b]	
	3	2.08 ± 0.24 [a]	5.02 ± 0.08 [b]	
	4	2.24 ± 0.50 [a]	5.54 ± 0.08 [bc]	
	5	2.62 ± 0.80 [a]	6.08 ± 0.24 [cd]	
	6	2.74 ± 1.06 [a]	6.88 ± 0.30 [d]	
device B	1	**ND	ND	$8 \times 10^6 - 2 \times 10^7$
	2	ND	ND	
	3	ND	ND	
	4	ND	ND	
	5	ND	ND	
	6	ND	ND	

*Means with different letters within a column are significantly different ($p < 0.05$).
**ND: not detected (<0.02 mg m^{-3}, the detection limit of the ozone analyzer).

Table 3
Effect of the treatments with NAI + O_3 and NAI + lowO_3 on *L. monocytogenes* and *Ps. fluorescens* at room T and ref T. Data are expressed as Log reduction (mean ± SD; n = 3).

	L. monocytogenes				*Ps. fluorescens*			
Exposure times (h)	NAI + O_3		NAI + lowO_3		NAI + O_3		NAI + lowO_3	
	room T	ref T	room T	ref T	room T	ref T	room T	ref T
1	$1.85 \pm 0.32^{a\ B}$*	$1.49 \pm 0.48^{a\ A}$	$0.32 \pm 0.52^{a\ A}$	$0.41 \pm 0.45^{a\ A}$	$1.13 \pm 0.87^{a\ A}$	$0.14 \pm 0.02^{a\ A}$	$0.03 \pm 0.04^{a\ A}$	$0.03 \pm 0.03^{a\ A}$
2	$2.54 \pm 0.28^{bc\ A}$	$1.21 \pm 0.19^{a\ A}$	$1.23 \pm 0.53^{a\ A}$	$1.21 \pm 0.49^{a\ A}$	$1.84 \pm 0.47^{ab\ A}$	$0.47 \pm 0.11^{a\ A}$	$0.08 \pm 0.08^{a\ A}$	$0.02 \pm 0.19^{a\ A}$
3	$2.86 \pm 0.04^{cd\ B}$	$1.35 \pm 0.09^{a\ A}$	$1.14 \pm 0.21^{a\ A}$	$0.98 \pm 0.06^{a\ A}$	$2.87 \pm 0.64^{ab\ A}$	$2.14 \pm 0.87^{b\ A}$	$0.26 \pm 0.22^{a\ A}$	$0.16 \pm 0.24^{a\ A}$
4	$2.88 \pm 0.17^{cd\ B}$	$1.50 \pm 0.53^{a\ A}$	$1.65 \pm 0.56^{a\ A}$	$1.57 \pm 0.78^{a\ A}$	$3.21 \pm 0.36^{ab\ A}$	$2.46 \pm 0.72^{b\ A}$	$0.40 \pm 0.71^{a\ A}$	$0.45 \pm 0.54^{a\ A}$
5	$2.85 \pm 0.24^{b\ B}$	$1.28 \pm 0.16^{a\ A}$	$1.28 \pm 0.73^{a\ A}$	$1.18 \pm 0.76^{a\ A}$	$3.72 \pm 0.17^{b\ A}$	$3.38 \pm 0.70^{bc\ A}$	$0.00 \pm 0.05^{a\ A}$	$0.05 \pm 0.03^{a\ A}$
6	$3.03 \pm 0.14^{d\ B}$	$1.63 \pm 0.32^{a\ A}$	$2.42 \pm 0.11^{a\ A}$	$1.34 \pm 0.72^{a\ A}$	$4.04 \pm 0.56^{b\ A}$	$4.52 \pm 0.07^{c\ A}$	$0.06 \pm 0.17^{a\ A}$	$0.04 \pm 0.19^{a\ A}$

*For each microorganism and then ionizer, means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

important rate of cell death. Considering the exposure of *Ps. fluorescens* to NAI and ozone at 2.20 mg h^{-1}, the antimicrobial effect appeared to be slower at ref T than at room T in the first 3 h of the treatment, probably due to the psychrotrophic nature of this microorganism. The effect of the exposure became significantly greater ($p < 0.05$) after 6 h of exposure time, generating reductions by 4.04 ± 0.56 Log at room T and by 4.52 ± 0.07 Log at ref T.

3.3. Antimicrobial assessment of ionization technology on six food-related microorganisms

The outcomes of the explorative assessment of the ionization technology on *L. monocytogenes* and *Ps. fluorescens* showed a different behavior of the microorganisms when undergoing the exposure to various ionization treatments. Therefore, interest arose to extend the study to other food-related microorganisms having different specific characteristics. Four additional microorganisms were selected: *Bacillus subtilis*, *Escherichia coli*, *Saccharomyces cerevisiae*, and *Penicillium roqueforti*. *B. subtilis*, the latter is a spore-forming Gram-positive frequently reported as an important spoiler of heat-treated foods, dairy and bakery products, and fresh vegetables (Møretrø & Langsrud, 2017). *E. coli* is a Gram-negative pathogen commonly considered as an indicator of fecal contamination and a cause of foodborne illnesses related to the consumption of fresh fruits and vegetables (Fan et al., 2007). *S. cerevisiae* and *P. roqueforti* are eukaryotic microorganisms: yeast and mold, respectively. Even if the bacteria species are most frequently related to the quality and safety of foods, they are also involved in the spoilage of refrigerated foods, having the ability to grow at low temperatures (Rico-Munoz et al., 2019; Tuffi et al., 2012).

Only the ref T was considered in the following steps of the study since it is the temperature commonly used in the food sector for the storage of food, especially for perishable foods that need maintenance of the cold chain (Kampmann et al., 2009; Panou et al., 2021). Indeed, the aim was to investigate whether ionization technology could be applied during cold storage while avoiding high levels of ozone for consumer safety and food quality (Cárdenas et al., 2011; Muhlisin et al., 2016; Sheelamary & Muthukumar, 2011). Considering the results obtained with the characterization of the devices regarding the production of ozone and the results obtained in the explorative assessment (section 2.2), the following part of the experimentation was divided into two sections:

1. Antimicrobial assessment of NAI + O_3 ($1 \times 10^6 - 7 \times 10^6$ NAI cm^{-3} s^{-1} and 2.20 mg h^{-1} of ozone) for a total exposure time of 6 h;
2. Antimicrobial assessment of NAI + lowO_3 ($8 \times 10^6 - 2 \times 10^7$ NAI cm^{-3} s^{-1} and 0.02 mg h^{-1} of ozone) for a total exposure time of 72 h.

Referring to L. *monocytogenes* and *Ps. fluorescens*, the presence of a high ozone concentration determined an improvement in the antimicrobial effect of NAI in the short treatment times.

Therefore, the exposure time of the low amount ozone treatments was prolonged to investigate whether the action of NAI against microorganisms could be enhanced by a continuous and protracted exposure to NAI ions.

Moreover, for L. *monocytogenes* the reduction after 6 h was lower at ref T than at room T: being an important psychrotrophic pathogen, the interest to investigate its behavior at ref T for an extended period was significant.

3.3.1. Antimicrobial assessment of NAI + O_3 at refrigeration temperature

B. subtilis, *E. coli*, *P. roqueforti*, and *S. cerevisiae* were exposed to ionization treatment using NAI + O3 ($1 \times 10^6 - 7 \times 10^6$ NAI cm^{-3} s^{-1} and 2.20 mg h^{-1} of ozone). The test conditions were the same as the assessment previously conducted on *L. monocytogenes* and *Ps. fluorescens*: 1, 2, 3, 4, 5, and 6 h at ref T. The cold temperature represented a real condition; it simulated the cold chain as a food preservation method. At this operational condition, in the presence of inoculated plates, no prolonged exposure times were considered here since the concentration of ozone inside the chamber after 6 h at ref T reached a concentration of about 6.88 mg m^{-3} (Table 2), a value considered high in the presence of personnel. It is known that ozone toxicity is an important parameter to consider both during the treatments and the work of operators.

The Federal Occupational Safety and Health Administration (OSHA, 1994) in the USA and the Health and Safety Executive (Health & Executive, 2014) in the UK specify 0.2 mg m^{-3} as the currently allowed exposure limit for continuous exposure during 8 h day/40 h week period or 0.6 mg m^{-3} for a 15 min period not to be exceeded four times per day. Although the value measured (6.88 ± 0.30 mg m^{-3}) exceeded the allowed one, many recent works have been carried out using much higher concentrations of ozone (Chen et al., 2020; Miller et al., 2021; Panou et al., 2021).

Fig. 2 illustrates the results of the treatment with NAI + O_3. To

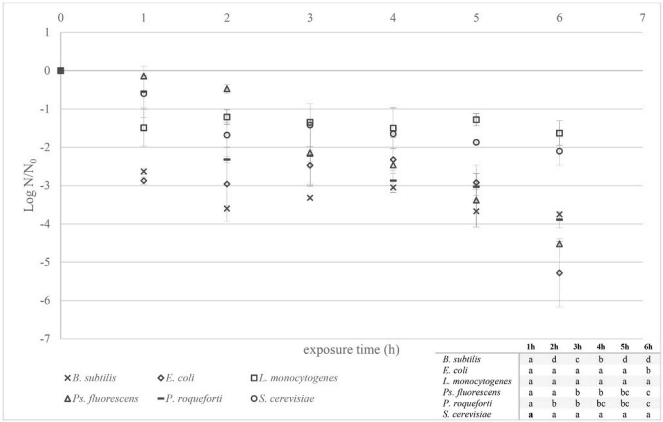

Fig. 2. Log reduction (mean ± SD; n = 2) of *B. subtilis*, *E. coli*, *L. monocytogenes*, *Ps. fluorescens*, *S. cerevisiae*, and *P. roqueforti* after the exposition to NAI + O_3 at ref T. In the table, the different letters refer to means significantly different ($p < 0.05$).

facilitate comparison, the results of *L. monocytogenes* and *Ps. fluorescens* at ref T, previously reported in Table 3, are also shown in the graph. The effect of desiccation was never observed, and the viable count of the control samples always remained 10^8–10^9 CFU mL^{-1}. The controls exposed to ref T were not significantly affected by the cold temperature compared to controls at room T. In general, for the additional four species involved, after 6 h the values of Log reduction ranged between 2.10 ± 0.37 Log for *S. cerevisiae* and 5.28 ± 0.89 Log for *E. coli*; for *B. subtilis* and *P. roqueforti* intermediate values of Log reduction were reached (3.75 ± 0.07 and 3.89 ± 0.21 Log, respectively). The trend of microbial reduction was different between bacteria and the eukaryote species. *B. subtilis* and *E. coli* proved to be quite sensitive to the NAI + O_3 ozone exposure after their first hour of treatment, and prolonging the exposure time to 6 h, the cells decreased significantly ($p < 0.05$). *P. roqueforti* and *S. cerevisiae* showed to be affected by NAI and ozone more gradually during the exposure time, demonstrating a different behavior towards an antimicrobial treatment for bacteria, as highlighted by the results obtained with other types of technologies (Erkmen & Özcan, 2004; Tawema et al., 2016).

3.3.2. Antimicrobial assessment of NAI + lowO_3 at refrigeration temperature

B. subtilis, *E. coli*, *L. monocytogenes*, *Ps. fluorescens*, *P. roqueforti*, and *S. cerevisiae* were exposed to ionization treatment using NAI + lowO_3 for 4, 16, 24, and 72 h at ref T (Fig. 3). The exposure times were extended to 72 h, since the amount of ozone was very low compared to the NAI + O_3 condition and the effect of the synergism between NAI and ozone could be appreciated by prolonging the treatment time. Similar, to the previous assessment, a desiccating effect was not observed, and the viable count of the control samples always remained 10^8–10^9 CFU mL^{-1}; the controls exposed to ref T were not significantly affected by the cold temperature compared to the same controls at room T. For L. *monocytogenes,* the antimicrobial effect of the exposition to NAI with a low amount of ozone was confirmed: even though an effect of the treatment after 4 h was not observed, after 16 h of exposure the decrease in viability was significant ($p < 0.05$), reaching 1.59 ± 0.01 Log reductions. By increasing the treatment time to 24 h, an almost unchanged effect was observed, and, by extending the ionization treatment to 72 h, a further effect on microbial viability was obtained, leading to a final reduction by 2.17 ± 0.12 Log. A similar result was obtained by Arnold et al. (2004), who reached 3.70 Log reductions after 3 h of biofilm treatment with ESCS (Electrostatic Space Charge System), a technology designed to produce NAI while minimizing the generation of ozone. After 72 h of exposure, *B. subtilis* and *E. coli* proved to be microorganisms having the highest sensitivity to ionization treatment reaching a reduction of nearly 4 Log. These results are in agreement with Arnold et al. (2004) and Park et al. (2016) confirming the antimicrobial ability of NAI. Particularly, by using an ion capture system (Park et al., 2016), it was verified that the ozone generated had very little or no bactericidal effect: this demonstrated the great contribution of NAI in the killing of bacteria. Moreover, L. Fan et al. (2002), despite using a low concentration of ozone (100 nL L^{-1}), found that the synergism between ozone and NAI was indispensable for achieving the death of the bacterial cells of *E. coli* and *Ps. fluorescens* with the degree of the effect depending on the microorganism. Nonetheless also in this part of the experimentation no effect was observed for *Ps. fluorescens*, with an exposure time of 72 h. This confirmed that the contribution of high levels of ozone for this microorganism is crucial to exert an antimicrobial effect. Also, for the yeast and the mold, no significant effect was observed after 72 h of exposure, despite a fungistatic effect previously verified for *Botrytis cinerea* and *Penicillium expansum* (Chauhan et al., 2015).

4. Conclusions

Ionization technology is considered a rapid, green, and low-cost strategy for possible use in the food industry to improve microbiological safety during food preservation. The antimicrobial effect was evaluated on six food-related microorganisms having different behavior for their intrinsic characteristics and impact on the food sector. Generally, this technology proved to affect microbial viability: the antimicrobial effects were related to several factors, i.e. the amount of generated NAI and ozone and their synergism, specific microorganism, temperature, and treating time exposure. The synergism between NAI and ozone was evident when low ozone levels were present. Therefore, the correct application of operational conditions (i.e., increasing the treatment period when low levels of ozone are generated) was essential to achieving a significant reduction in microbial viability, especially in the case of a pathogen microorganism such as *L. monocytogenes*. However, further studies should be carried out involving other food-related microbial species to confirm the antimicrobial efficacy of the ionization. This outcome was significant especially considering the possibility of using this technology in a safe context of an industrial or domestic environment.

The characteristics of NAI, such as low cost and user-friendly devices, offer the opportunity of application in food preservation at refrigeration or room temperatures.

Moreover, at room temperature, NAI guarantees food safety through the decontamination of air and surfaces of food processing areas. This technology may be applied to establish sanification protocols for the food industry, as well as medical and health-care areas.

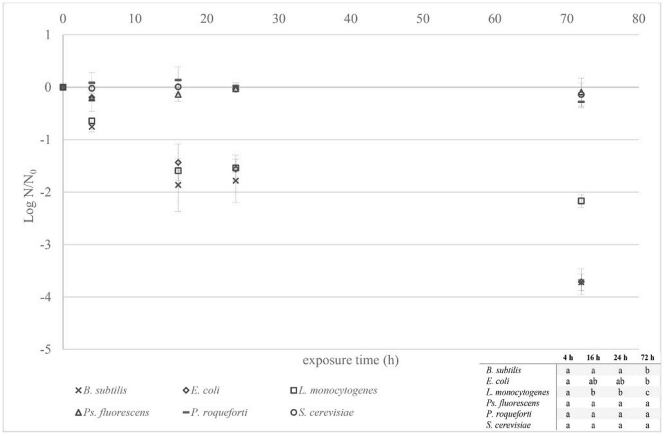

Fig. 3. Log reduction (mean ± SD; n = 2) of *B. subtilis*, *E. coli*, *L. monocytogenes*, *Ps. fluorescens*, *P. roqueforti*, and *S. cerevisiae* after the exposition to NAI + lowO_3 at ref T. In the table, the different letters refer to means significantly different ($p < 0.05$).

CRediT authorship contribution statement

Anna Baggio: Investigation, Writing, Writing – original draft. **Marilena Marino:** Methodology, Formal analysis, Writing – review & editing. **Michela Maifreni:** Conceptualization, Methodology, Writing, Writing – original draft, Funding acquisition.

Data availability

Data will be made available on request.

Acknowledgments

The authors are grateful to Monica Celotto, PhD, from Group Technology Organization by Electrolux Italia for the technological support for the experimentation.

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