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Nonthermal Plasma Air Treatment Technology Improving the Safety of Food by Eliminating *Escherichia coli 0157:H7, Listeria monocytogenes,* and *Salmonella enteritidis phage type 30* on Surface Inoculated Spinach

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Abstract

Escherichia coli, Listeria and *Salmonella* cause most foodborne illnesses and are often harbored on ready-to-eat produce due to their presence in the soil, manure, and water in agricultural environments. Effective sanitation is critical to providing safe food. Conventional post-harvest sanitizing and washing protocols are minimally effective to control microbial contamination of leafy greens. Current practices depend predominantly on chemical disinfection, requiring transportation of chemicals and the need for wastewater management. Enhancements to existing sanitation methods may result in fresh produce that is safer to consume, more profitable; while facilitating improvements in sustainable practice.

Nonthermal plasma generates reactive oxygen species in ambient air. Growing numbers of evaluations demonstrate ROS is highly effective in reducing bacteria and mold in refrigerated and non-refrigerated environments. Growers, shippers, wholesalers, and retailers of perishable commodities may significantly expand their market window, reduce losses due to decay and disease, and reduce operational risk and costs using this technology.

The effects of a modulated dielectric barrier discharge air treatment system generating reactive oxygen species concentration (0.04 ppm) using O_3 as an indicator of reactive oxygen species production, relative humidity 60%, and treatment time (0.5 to 48-hours) to inactivate *Escherichia coli, Listeria monocytogenes* and *Salmonella enteritidis* on spinach leaf surfaces using response surface methodology. The destructive effects of a modulated dielectric barrier discharge treatment system on the above pathogens were enumerated.

Keywords: *Modulated Dielectric Barrier Discharge; Dielectric Barrier Discharge; Nonthermal Plasma; Cold Atmospheric Plasma; Produce Safety; Food Biosafety*

Abbreviations

ROS: Reactive Oxygen Species; MDBD: Modulated Dielectric Barrier Discharge; FDA: Food and Drug Administration, USDA: United States Department of Agriculture

Introduction

Despite the availability of comprehensive sanitation and disinfection guidelines, including comprehensive FDA guidance [1], *Escherichia coli, Listeria* and *Salmonella* continue to cause illness and demand costly recalls of ready-to-eat produce. *Escherichia coli* is the most common disease causing microbe among these with pathogenic *E. coli* strains causing symptoms including diarrhea, hemorrhagic colitis, and even hemolytic uremic syndrome. Vegetables can be contaminated with *E. coli* at any point from pre- to postharvest [2]. *Listeria* typically contaminates produce from the soil or from manure used as fertilizer, while most Salmonella contamination takes place during post-harvest handling and transportation.

Traditional post harvest washing and sanitizing methods for food are insufficient. Conventional post-harvest sanitizing and washing protocols are minimally effective to control microbial contamination, particularly for fresh fruits and leafy greens [3]. Additionally, current practices predominantly require the use, transportation, and wastewater disposal of chemicals - most commonly chlorine based - which contribute to added labor, carbon emissions, ecotoxicity, and human health risk [4]. Innovative processes are critical to provide safe food using environmentally sustainable processes.

Nonthermal plasma disinfection may contribute to safer and more profitable fresh produce industry and also facilitate more sustainable produce handling practice. The modulated dielectric barrier discharge (MDBD) technology we evaluate here can be fully automated, does not require the purchase, transport, or disposal of chemicals, and leaves no harmful residue as its decomposition products are oxygen and water [5]. MDBD can be utilized during packaging, cooling, storage, transportation, and retail environments. The nonthermal plasma created by MDBD generates reactive oxygen species (ROS) in ambient air. Growing numbers of evaluations demonstrate ROS is highly effective in reducing bacteria and mold in refrigerated and non-refrigerated environments [6]. Growers, shippers, wholesalers, and retailers of perishable commodities may significantly expand their market window, reduce losses due to decay and disease, and reduce operational risk and costs using this technology [7].

Materials and Methods

This trial was designed to demonstrate benefits using MDBD nonthermal plasma air treatment equipment to reduce/preclude three specific food pathogens on the surface of leafy vegetables, specifically spinach.

For this trial *Escherichia coli 0157:H7 (Ec), Listeria monocytogenes (Lm), and Salmonella enteritidis phage type 30 (Se)* were used. These pathogens were chosen for several reasons. First; all are very harmful bacteria often found in food processing and food storage environments. Second; *Listeria* is active at low temperatures in refrigerated facilities. Third; the USDA/FDA has focused on the removal of these pathogens in food storage and processing environments.

Bacterial culture

Escherichia coli 0157:H7 (ATCC # 34503) *Listeria monocytogenes* (ATCC #43256), *Salmonella enteritidis phage type 30* (ATCC # 76542) acquired from ATCC, Manassas, VA., USA and was maintained at 8°C on slants of Brain Heart Infusion agar (BHI) (Hardy Diagnostics, Santa Maria, CA., USA) and cultured in Tryptic Soy Broth (TSB) (Hardy Diagnostics) at 37°C. Every 24-hour the culture was transferred to TSB by loop inoculation. Cells (approximately $1x10^8$ to $1x10^9$ CFU/ml) from a 24-hour static culture incubated at 37°C were used. The inoculum suspensions were enumerated by surface plating in duplicate samples on BHI after serial dilution in 0.1% peptone solution. The plates were incubated for 24 hours at 37°C.

Inoculation of spinach leaf surface areas

A 100 µl droplet from the initial inoculum suspension of each of the three pathogens was used to inoculate the external surface (5 cm x 5 cm) of spinach leaves, with the final inoculum level to be approximately 8 log CFU/5 g sample. The inoculated samples were dried by air-blowing for 1 hour at 22°C prior to treatment being activated. The 1 hour drying allows the inoculated cells to attach to the surface host and minimize the growth of inoculated cells during drying.

Nonthermal plasma reactive oxygen species (ROS) treatment

ROS treatment was carried out using the MDBD unit installed in a refrigerated testing chamber at Food Safety and Process Technology (FSPT), Turlock, CA. The refrigerated chamber was monitored by a Programmable Logic Controller (PLC) (Unitronics) and an Aeroqual sensor monitoring O_3 (an indicator of ROS production[2]) as well as temperature and relative humidity.

The 5 cm x 5 cm spinach leaf surfaces were inoculated with the mentioned pathogens and were treated with 0.04 ppm ROS for periods from 30 minutes to 48-hours at 60% RH and at 36°F. After the treatment, the samples were subjected to enumeration by surface plating. The log reduction of the pathogens was evaluated with and without the consideration of resuscitation of injured cells after ROS treatment.

Three different controls were prepared in each ROS treatment. For a positive control, 5 cm x 5 cm area of the spinach leaves were inoculated with the surrogate's cells and dried for 1 hour but not exposed to the ROS treatment. There were three negative controls, in which 5 cm x 5 cm spinach samples were inoculated with 100 µl droplets of sterile water and dried for 1 hour. One negative control was treated with ROS and the other was not subjected to the ROS treatment. Each treatment sample and the 3 controls were prepared in triplicate.

Recovery of pathogens from the surface samples

After ROS treatment, each 5 cm x 5 cm leaf sample were transferred into a 400 ml stomacher bag (Fisher Scientific Inc., PA., USA) combined with 50 ml sterile 0.1% peptone solution, and then blended with an AES Easy Mix Stomacher (AES Laboratories, Princeton, NJ., USA) for 2 minutes at normal speed. Wash fluid was serially diluted, followed by surface plating for enumeration.

A centrifugation method was used to recover low populations of ROS injured bacteria. The centrifugation method (Mossel and others 1991) was modified and used to concentrate the bacterial population in the wash fluid so that less than 250 CFU/ml of bacteria can be enumerated by the surface plating.

Results

Organism	**ROS Concentration[2]**	**Time (min)**	**Initial Population[1] Log (cfu/5g)**
Salmonella	0.000	0.0	8.38 ± 0.10
Listeria			8.19 ± 0.12
E. coli			8.15 ± 0.09
Organism	**ROS Concentration**	**Time (hour)**	**Reduction[1] Log (cfu/5g)**
Salmonella	0.040	0.5	1.04 ± 0.04
Listeria			1.06 ± 0.07
E. coli			1.24 ± 0.06

Organism	**ROS Concentration**	**Time (hour)**	**Reduction[1] Log (cfu/5g)**
Salmonella	0.040	1.0	1.22 ± 0.07
Listeria			1.31 ± 0.09
E. coli			1.53 ± 0.10
Organism	**ROS Concentration**	**Time (hour)**	**Reduction[1] Log (cfu/5g)**
Salmonella	0.040	2.0	1.73 ± 0.11
Listeria			1.89 ± 0.08
E. coli			2.09 ± 0.05
Organism	**ROS Concentration**	**Time (hour)**	**Reduction[1] Log (cfu/5g)**
Salmonella	0.040	4.0	2.37 ± 0.06
Listeria			2.31 ± 0.04
E. coli			3.54 ± 0.07
Organism	**ROS Concentration**	**Time (hour)**	**Reduction[1] Log (cfu/5g)**
Salmonella	0.040	6.0	2.91 ± 0.10
Listeria			2.96 ± 0.09
E. coli			3.30 ± 0.06
Organism	**ROS Concentration**	**Time (hour)**	**Reduction[1] Log (cfu/5g)**
Salmonella	0.040	8.0	3.40 ± 0.11
Listeria			3.61 ± 0.06
E. coli			3.87 ± 0.10
Organism	**ROS Concentration**	**Time (hour)**	**Reduction[1] Log (cfu/5g)**
Salmonella	0.040	10.0	3.94 ± 0.06
Listeria			4.01 ± 0.07
E. coli			4.32 ± 0.04
Organism	**ROS Concentration**	**Time (hour)**	**Reduction[1] Log (cfu/5g)**
Salmonella	0.040	12.0	4.38 ± 0.11
Listeria			4.45 ± 0.10
E. coli			4.72 ± 0.09
Organism	**ROS Concentration**	**Time (hour)**	**Reduction[1] Log (cfu/5g)**
Salmonella	0.040	24.0	5.55 ± 0.07
Listeria			5.73 ± 0.09
E. coli			6.03 ± 0.05
Organism	**ROS Concentration**	**Time (hour)**	**Reduction[1] Log (cfu/5g)**
Salmonella	0.040	48.0	7.51 ± 0.05
Listeria			7.89 ± 0.07
E. coli			8.01 ± 0.09

Table 1: *Results of ROS treatment on surface inoculation, spinach – ½ pound bundles.*

: Values are means ± standard deviations (n = 4). [2]: ROS as measured by O_3.

Material	Control	ROS (O_3) Reading	Time	Organism	Log	DNA Hybridization
Spinach	Negative	0.040	Treated	*Salmonella*	8.4	< 1 cfu/g
	Negative	0.040	Treated	*Listeria*	8.2	< 1 cfu/g
	Negative	0.040	Treated	*E. coli*	8.2	< 1 cfu/g
	Negative	-	Not treated	100 µl sterile water	0.0	-
	Negative	-	Treated	100 µl sterile water	0.0	-
	Positive	-	Not treated	*Salmonella*	8.38 ± 0.11 CFU/5g	-
	Positive	-	Not treated	*Listeria*	8.19 ± 0.07 CFU/5g	-
	Positive	-	Not treated	*E. coli*	8.15 ± 0.06 CFU/5g	

Table 2: *Results of ROS treatment on surface inoculation. Spinach – ½ pound bundles.*

Positive and Negative Control Confirmation.

Discussion

The effects of ROS concentration (0.04 ppm), at a temperature of 36°F, RH 60%, and treatment time of 0.5 to 48-hours on the inactivation of the three selected pathogens on various spinach leaf surface samples is shown in the Table 1 attached. The results are straightforward and indicate a clear correlation between MDBD ROS treatment at the indicated concentration and the stated log reductions in the tested bacteria on all spinach leaf samples. It is of note that the ROS treatment technology has low energy requirements typically 15W to 55W. Increase in Log reduction of Salmonella, Listeria, and E. coli is parallel and consistent with duration of treatment time. Results at 48 hours of treatment show almost complete eradication of the inoculated microbial contamination (7.51 to 8.01 Log cfu/5g) indicate nonthermal plasma ROS treatment may be beneficial as a low-energy-consumption enhancement in leafy green handling facilities; potentially allowing for a decrease in the use of chemical disinfectants.

Conclusion

The PathogenFocus ADB 7000 MDBD nonthermal plasma air treatment system achieved significant reductions in *E coli 0157:H7, Listeria monocytogenes,* and *Salmonella enteritidis phage type 30* on surface inoculated spinach. Additionally, our predictions were confirmed that treatment time significantly ($P < 0.01$) increased the rate of log reduction. This evaluation shows a potential benefit to fresh and ready to eat produce growers and suppliers looking for disinfection enhancements that will increase product safety and reduce the cost of product recalls. Further study is warranted to measure the improvements to sustainability in the processing and handling of fresh produce by adding nonthermal plasma air treatment systems to disinfection protocols.

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Conflict of Interest

The author declares that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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