Inactivation of *Bacillus atrophaeus* endospores using cold plasma treatment

Juan Carlos Figueroa Sorto

Escuela Agrícola Panamericana, Zamorano
Honduras
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ZAMORANO
FOOD SCIENCE AND TECHNOLOGY MAJOR

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Presented by

Juan Carlos Figueroa Sorto

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Abstract. Cold plasma treatment is a novel technology that is being investigated for its use in the food industry due to its ability to inactivate microorganisms, and keep certain characteristics of freshness in food products. The efficiency of the cold plasma treatment using Argon over *Bacillus atrophaeus* endospores was evaluated at different exposure times (30 s, 2, 4 and 6 min). Four concentrations (10^4 to 10^7) of *B. atrophaeus* endospores were inoculated on polyethylene plastic strips and treated inside a sterile sealed sample box filled with Argon. The samples were treated using a Dielectric Barrier Discharge Cold Plasma equipment with 60 kV. Results were collected using plate count method 24 and 48 h after treatment. A 1.8 Log CFU/ mL reduction of *Bacillus atrophaeus* endospores was achieved after 6 min of exposure using 60 kV. A standard operating procedure was elaborated for *Bacillus atrophaeus* spore inactivation using cold plasma. More research is needed to understand the effect of different gases over endospores of *Bacillus atrophaeus*.

Keywords: Argon, dielectric barrier discharge, nonthermal technology, pathogen control.

Resumen. El tratamiento de plasma frío es una tecnología novedosa que se está promoviendo en la industria alimentaria debido a su capacidad para inactivar microorganismos y mantener ciertas características de frescura en los alimentos. La eficacia del tratamiento con plasma frío utilizando Argón sobre las endosporas de *Bacillus atrophaeus* se evaluó en diferentes momentos de exposición (30 s, 2, 4 y 6 min). Se inocularon cuatro concentraciones (10^4 a 10^7) de endosporas de *B. atrophaeus* en tiras de plástico de polietileno y se trataron dentro de una caja de muestras estéril y sellada llena de Argón. Estas muestras se trataron utilizando un equipo de plasma frío de descarga de barrera dieléctrica con 60 kV. Los resultados se obtuvieron utilizando el método de recuento en placa, 24 y 48 h después del tratamiento. Se demostró que se logró una reducción de 1.8 Log UFC/ mL en el conteo de endosporas de *Bacillus atrophaues* en 6 minutos usando 60 kV. Se elaboró un proceso operativo estandarizado del proceso de inactivación de esporas de *Bacillus atrophaeus* utilizando plasma frío. Se requiere más investigación para comprender el efecto de otros gases sobre las endosporas de *Bacillus atrophaeus*.

Palabras clave: Argon, control esporulados, descarga de barrera dieléctrica, tecnología no térmica.

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1. INTRODUCTION

Sterilization is a critical control point on a package surface that prevents food from contamination in aseptic processing and packaging system. Packaging material main purpose is to protect the food from recontamination and preserve food quality throughout its distribution chain. Improper sterilization of the food packaging can lead to serious consequences associated with health risks and economic losses (Pankaj *et al*. 2014). Sterilization may be achieved by the application of different type of treatments, such as chemical, thermal and non-thermal.

Cold plasma treatment is a method that was used in hospitals and other industries to sterilize equipment. Recently, this method is being used in the food industry in the fields of conservation and protection from microorganisms (Lee *et al*. 2005). This novel non-thermal technology has been viewed for years as an effective method to achieve microbial and endogenous enzymes inactivation. In addition, cold plasma sterilization is an eco-friendly process, which is used for food preservation as an alternative to conventional methods such as, heat application and hydrogen peroxide. This method has certain advantages that are important for the food industry including reduced water usage, cost effective, time efficient and lack of chemical residue (Bourke *et al.* 2017; Thirumdas *et al.* 2015; Morris *et al.* 2009). This treatment affects microorganisms present depending on the different conditions applied in the treatment chamber. The effect of this method over the microorganisms present depends on the type of microorganism, the inoculated medium, the exposure method (direct or indirect) and conditions during the exposure to cold plasma (Pignata *et al.* 2017).

The Dielectric Barrier Discharge (DBD) is one of the techniques used to apply cold plasma treatment, this includes normal atmospheric pressure and high voltage. DBD is mainly used in the food industry to achieve microbial decontamination of foodborne pathogens, specially spores (Min *et al.* 2016). Due to its non-thermal properties, this plasma treatment has the advantage of achieving microbial deactivation at moderate temperatures. This treatment is beneficial for sterilization of high temperature sensitive material (Butscher *et al.* 2015). The process uses high voltage using two parallel aluminum plates separated by a dielectric barrier. Atmospheric pressure causes high energy levels to be sustained by dielectric barrier; common barrier materials include glass, quartz, polymers and ceramics (Pankaj *et al*. 2014).

Spores, also known as endospores, are a major issue when sterilization takes place because these are able to resist extreme conditions. *Bacillus* and *Clostridium* species are microorganisms that develop these dormant structures allowing them to survive environments full of stress, such as acid, alkaline, heat, high pressure, ultra violet light and atmospheric cold plasma, for long periods of time (Bourke *et al*. 2017).

Bacillus atrophaeus var. *niger* is a mesophilic, Gram-positive, aerobic, non-pathogenic, and endospore forming microorganism that is used as a sterilization control strain (ATCC® 9372™, Manassas, United States of America). *B. atrophaeus* spores have played an important role in the food industry as a *B. anthracis* surrogate. The microorganism has been used in different studies to assess the effectiveness of spore inactivation methods (Sella *et al.* 2014).

There are many studies that demonstrate the inactivation of *B. atrophaeus* using cold plasma treatment. Dobrynin *et al.* (2010) demonstrated that Double Barrier Discharge Cold Plasma (DBD-CP) is able to effectively inactivate *Bacillus* spores up to 5 log in less than a minute of treatment using 30 kV. *Bacillus atrophaeus* inactivation was achieved by the application of a 70 kV DBD within 60 seconds of exposure (Patil *et al.* 2014a). Hertwig *et al.* (2015) demonstrated 2.4 and 2.8 log reduction for *B. subtilis and B. atrophaeus* spores that were inoculated on whole black pepper, using direct plasma application for 15-30 minutes.

This research focuses on the development of a standard operating procedure to realize further studies using the cold plasma treatment and demonstrate that the protocol can achieve microbial inactivation by exposing *B. atrophaeus* spore strips to cold plasma treatment.

For this research, the following objectives were established:

- Develop a standard operating procedure to treat *Bacillus atrophaeus* spores in cold plasma equipment.

- Determine the logarithmic reduction of *B. atrophaeus* by cold plasma treatment using Argon.

2. MATERIALS AND METHODS

Location.
This study required three phases in order to achieve the objectives: 1) spore population and sample preparation, 2) cold plasma treatment, and 3) spore recuperation. All phases were carried out in the Food Science Department of Purdue University, West Lafayette, Indiana, United States of America. More specifically in the process modeling and validation laboratory.

Phase 1.
This phase consisted in the preparation of the spores and the samples that will undergo cold plasma treatment.

Spore population preparation. The microorganism used in this study was *Bacillus atrophaeus.* This is a biosafety level 1, mesophilic, aerobic and Gram-positive microorganism and it is used as a sterilization control strain (ATCC® 9372™). The spore suspension of *B. atrophaeus* was prepared by inoculating 1 ml of microorganism seed suspension to glass test tubes containing 9 mL of Tryptic Soy Broth (TSB) (Acumedia, Neogen Corporation, Michigan, USA) and incubated the solution 35 °C for 24 h. One liter of sporulation medium (8.0 $g \cdot L^{-1}$ yeast extract, 4.0 $g \cdot L^{-1}$ nutrient broth, 0.05 $g \cdot L^{-1}$ $MnSO_4$, 0.05 $g \cdot L^{-1}$ $CaCl_2$, and 30.0 $g \cdot L^{-1}$ agar) (Sella *et al.* 2012) was prepared, autoclaved at 121 °C for 15 min and poured into 20 mL petri dishes (diameter: 150 mm, height 15 mm, Fisherbrand, Fisher Scientific, Massachusetts, USA).

Petri dishes containing sporulation media were inoculated with 75 µL of TSB containing vegetative cells of *B. atrophaeus*. The petri dishes with bacteria were incubated at 35 °C for 7 days. A petri plate with 6 days of incubation was used to perform spore staining to verify the presence of spores (Sella *et al.* 2012). The glass slides with the stained solutions were observed in a optical microscope at 100X, in which spores were green colored and vegetative cells were red colored as observed in figure 1. Spores were harvested from petri dishes using distilled water. A total of 50 mL of spore solution was collected and placed in sterile test tubes (Falcon, Corning Science México S.A., Tamaulipas, México).

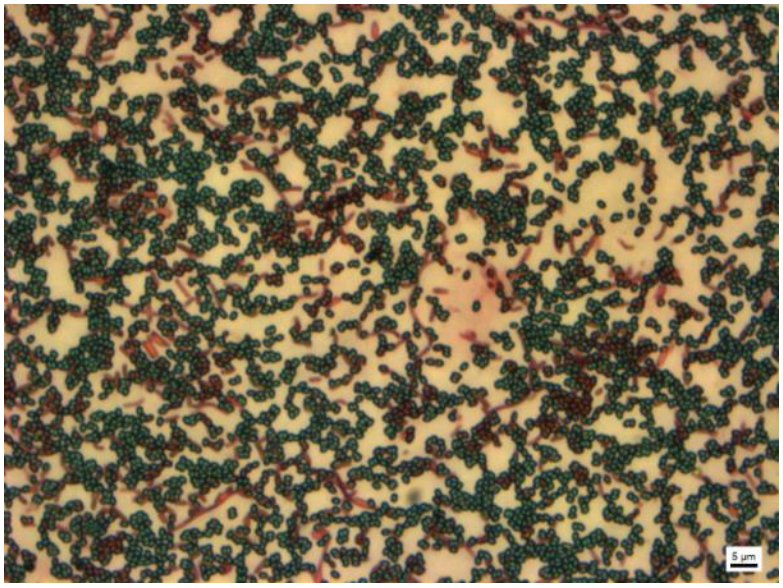

Figure 1. *Bacillus atrophaeus* spores viewed from digital microscope.

Viable spore quantification. Viable spores in the solutions were quantified by the plate count method. Serial decimal dilutions were made in phosphate buffered solution, and 75 µL of each dilution was surface inoculated on nutrient agar plate. Plates were incubated at 35 °C for 24 h.

Sample preparation. Viable spore quantification demonstrated that spore concentration on the spore solution was of 1×10^9 UFC/ mL, which was used to prepare spore strips. Plastic strips of polyethylene were cut in dimensions of 1 × 2 cm and autoclaved at 121 °C for 20 min. Serial dilutions (10^6 to 10^9) were made, 10 µL of each were placed in the center of the plastic strip and were dried in a sterile environment. These resulted in 10^4 to 10^7 dilutions. A strip of each concentration was placed separately inside a labeled sterile petri dish as shown in figure 2.

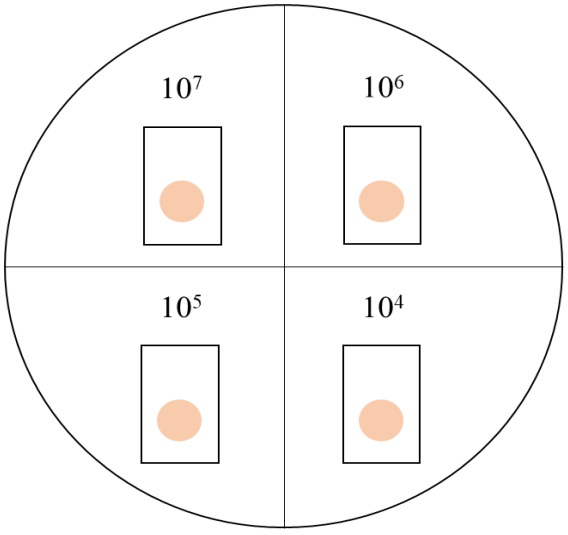

Figure 2. Plastic spore strip placement inside the Petri plates.

Phase 2.
This phase consisted on the treatment of *Bacillus atrophaeus* spores with cold plasma at 60 kV.

Cold plasma treatment. Dielectric barrier discharges were employed to inactivate *B. atrophaeus* spores using a high voltage transformer (Phenix BK130, Phenix Technologies, MD, USA) with an input voltage of 120 V, a voltage regulator (0-100%, output voltage controlled within 0-120 kV), and two aluminum electrodes (15 cm diameter) between which the plasma was generated. Dielectric barriers, made of low density polyethylene, were placed in between the aluminum electrodes and the sample box as shown in figure 3.

A petri dish containing plastic spore strips was placed in the center of a sample box (Low density polyethylene;16.8 × 26.9 × 4 cm) that was handled adequately to avoid movement of strips. Sample box was placed inside a bag (25 cm × 35 cm), which was sealed, purged and filled with Argon gas. This system was run at 60 kV and sample exposures time to cold plasma discharge were 30 s, 2 min, 4 min and 6 min. A triplicate of independent set of cultures were used in this experiment.

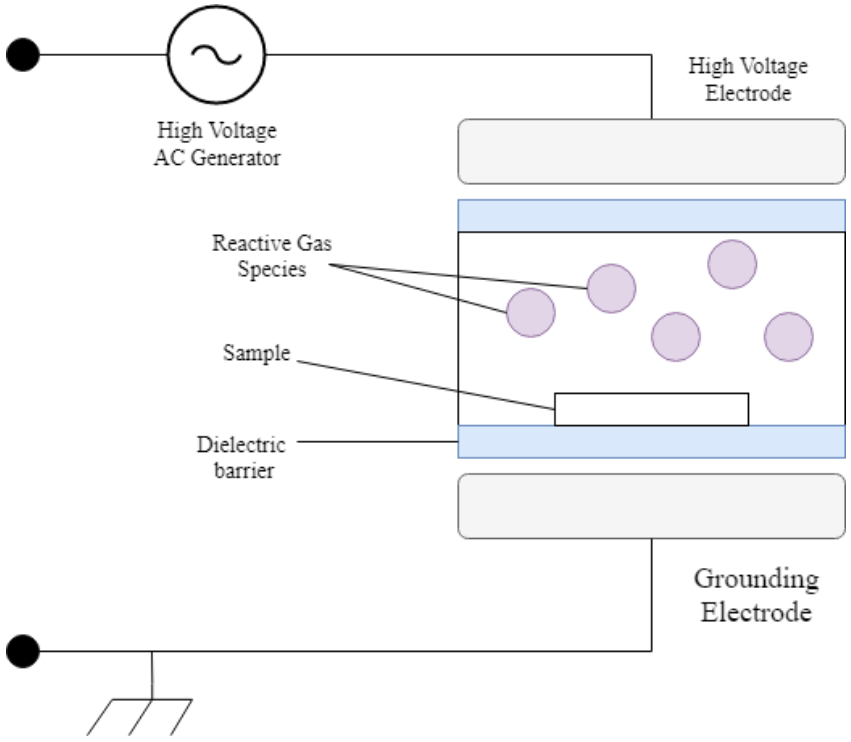

Figure 3. System diagram of the Dielectric Barrier Discharge (DBD) used to generate cold plasma using Argon as the reactive gas.

Phase 3.
This phase consisted in the recuperation of spores from plastic strips after the cold plasma treatment and the elaboration of a standard operating procedure.

Spore recuperation. This step was done in sterile conditions, tha sample box was taken off from the cold plasma equipment once it was turned off and disconnected. The bag was opened and the sample box was taken out. Petri dishes containing the four spore strips with different concentration were placed out of the box. Each strip was placed on a labeled test tube containing 1 mL of Phosphate Buffered Solution (PBS) (Fisherbrand, Fisher Scientific, Massachusetts, USA) and mixed in the vortex (Thermo Scientific, Thermo Fisher Scientific, Massachusets, USA) for 3 min.

Afterwards, the solution was transferred to a labeled 2 mL micro centrifuge tube (Eppendorf, Eppendorf Manufacturing Corp., Enfield, Conneticut, USA). Serial dilutions from 10^{-2} to 10^{-9} were performed by using a 100 µL pipette and 2 mL micro centrifuge tubes containing 900 µL of PBS. Petri dishes with nutrient agar (Difco, Difco Laboratories, Sparks, Maryland, USA), were prepared in order to inoculate the dilutions. Inoculation of 100 µL of each serial dilution took place once the nutrient agar solidified and incubated at 35 °C for 48 h.

Data were collected at 24 and 48 h of incubation and the Colony Forming Units (CFU) were counted on the serial dilutions made from each concentration of the different treatments. Morphology of *Bacillus atrophaeus* was taken into consideration during data collection. Realpe *et al.* (2002) stated that *Bacillus atrophaeus* colonies morphology had between 2 to 4 mm in diameter, with an appearance that could be smooth, mucoid or rough, and the edges were wavy. After the collection of information generated throughout the experiment, a Standard Operating Procedure (SOP) was elaborated explaining in detail the process for the cold plasma treatment of *Bacillus atrophaeus* endospores.

Experimental design and statistical analysis.
A Randomized Complete Block design with a factorial arrangement of 4 × 4, four concentrations of *B. atrophaeus* endospores and four times of exposure (30 s, 2, 4 and 6 min) was used to analyze the data obtained from phase 3. The experiment was carried out in triplicate of independent set of cultures. The data analyzed was based on serial dilutions performed in phase 3, obtaining a total of 48 experimental units. Data obtained was analyzed using the Statistical Analysis Software (SAS® 9.4) by performing an analysis of variance to determine the significance of the model. The Duncan test was used to determine statistical differences among the exposure times on the inactivation of the endospores. Also, this test was used to investigate significant differences in logarithmic reduction for each concentration after applying the cold plasma treatment. LS Means was used to analyze the interactions between the factors evaluated.

3. RESULTS AND DISCUSSION

Double Barrier Discharge Cold Plasma using Argon at 60 kV had a significant influence ($P < 0.0001$) in the inactivation of *Bacillus atrophaeus* endospores at different bacterial concentrations and exposure times (figures 4 and 5).

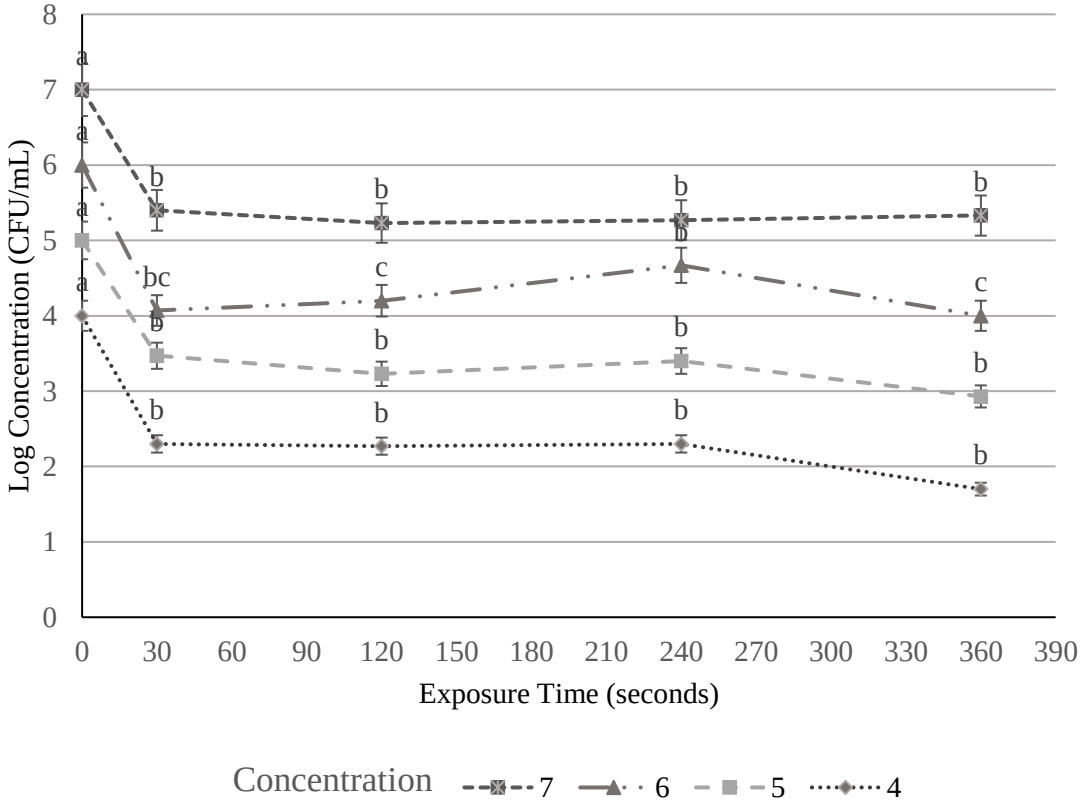

Figure 4. Inactivation using Double Barrier Discharge Cold Plasma at 60 kV using Argon on various concentrations of *Bacillus atrophaeus* endospores on plastic strips 24 h after treatment.

[abc]Means with different letters in the same concentration represent statistical differences ($P < 0.05$).

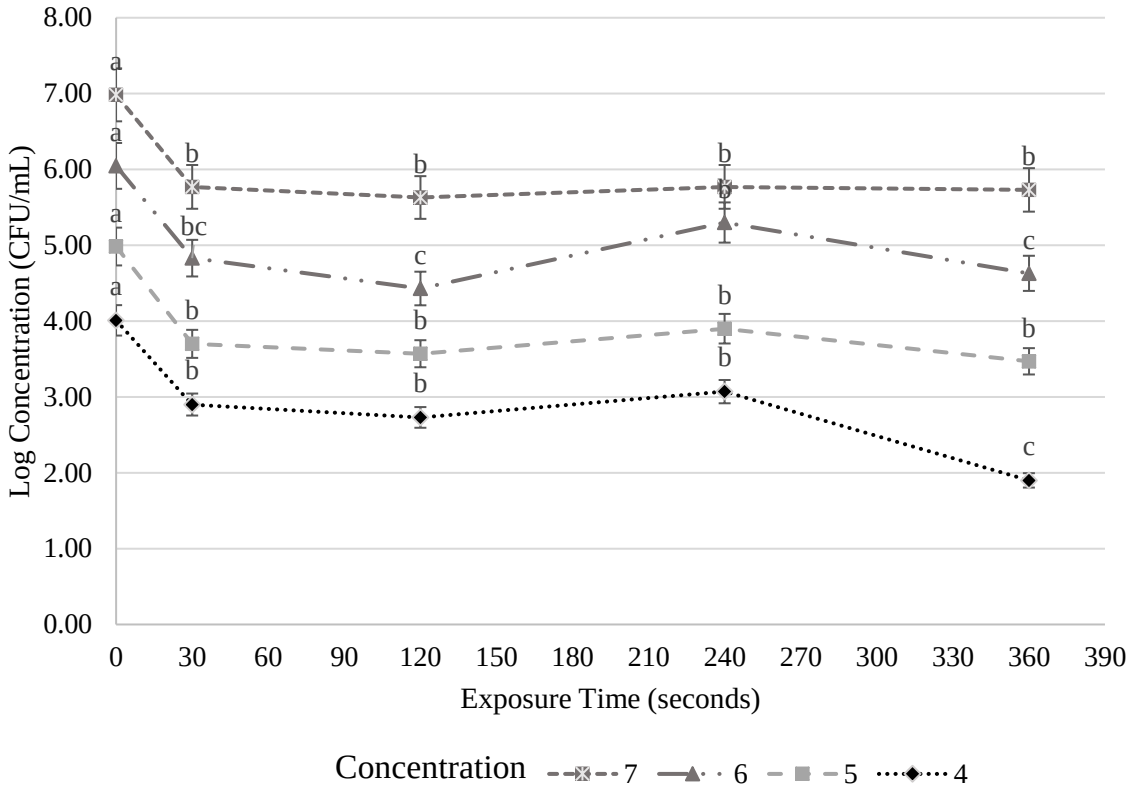

Figure 5. Inactivation using Double Barrier Discharge Cold Plasma at 60 kV using Argon on various concentrations of *Bacillus atrophaeus* endospores on plastic strips 48 h after treatment.
[abc]Means with different letters in the same concentration represent statistical differences ($P < 0.05$).

Results of exposure times studied (30 s, 2, 4 and 6 min) cold plasma nonthermal processing treatment reduced effectively ($P < 0.0001$) *B. atrophaeus* endospores. Within each exposure time there were statistical differences among concentrations, at 30 s and 4 min it can be observed that certain concentrations demonstrated no statistical difference among them.

A Duncan test was realized for the exposure time and concentration due to the results obtained on separation of means, which showed logarithmic reduction in both factors. The exposure times applied independently to each set of samples had a different effect on the logarithmic reduction of the microorganism. The results shown in table 1 demonstrate that more exposure time resulted in a lower spore concentration after treatment. In this case, 6 min of exposure to cold plasma treatment reduced from 5.5 Log (CFU/ mL) before treatment to 3.71 Log (CFU/ mL) after treatment. Statistical differences ($P < 0.05$) were observed among the results of each exposure time in which all concentrations were taken into consideration.

Table 1. Final logarithmic concentration of Bacillus atrophaeus endospores for each time of exposure to cold plasma treatment at 60 kV.

Time (s)	Log (CFU/ mL)
0	5.51 ± 1.17^{A}
30	4.05 ± 1.22^{BC}
120	3.92 ± 1.16^{C}
240	4.21 ± 1.21^{B}
360	3.71 ± 1.45^{D}
CV (%)	28.96%

^{ABCD}Means with different letters in the same column represent statistical differences ($P < 0.05$).
CV (%)= Coefficient of variation in percentage.

In table 1, the final logarithmic concentration of the 4 min exposure is higher than the other exposure times. This high concentration can be due to a resistance of the *Bacillus atrophaeus* spores. The resistance of *Bacillus* endospores can be attributed to a different set of factors that can differ tremendously depending on spore strain and cold plasma method used (Pedraza-Reyes *et al.* 2012). Different parameteres during the process have an effect over the efficacy of cold plasma treatment on microorganisms spores and vegetative cells. In a study realized by Patil *et al.* (2014b), they demonstrated that relative humidity has a greater effect over the efficacy of the cold plasma treatment on microbial inactivation.

Another parameter that is crucial in order to achieve microbial inactivation is the type of gas used to generate plasma. van Bokhorst-van de Veen *et al.* (2015) suggested that cold atmospheric plasma using Nitrogen as gas feed was able to inactivate chemical and heat-resistant spores. This indicates the potential of cold plasma to target a diverse range of sporeformers microorganisms. Keener *et al.* (2011) achieved 6.23 log reduction of *Bacillus atrophaeus* spores in less than 15 seconds with 80 kV. This reduction was achieved using air and a modified atmosphere (65% O_2, 30% CO_2, 5% N_2). According to Hertwig *et al.* (2017), *Bacillus atrophaeus* endospores have lower resistance than *B. subitilis* wild endospores, however, they observed that in the presence of O_2 and CO_2 in plasma the *B. atrophaeus* spores had a higher resistance to the treatment and lower logarithmic reduction. In this study, cold atmospheric pressure plasma method was used to achieve an inactivation on *Bacillus* endospores.It was observed that higher ionization voltages lead to shorter sterilization times. As mentioned in a study realized by Pignata *et al.* (2017), Gram-negative bacteria showed a membrane rupture after cold plasma treatment and Gram-positive bacteria did not show any damage. This membrane rupture affects the viability of bacteria, it is suggested that Gram-positive bacteria are more resistant to cold plasma treatmen than Gram-negative bacteria.

This experiment started with different concentrations (7, 6, 5, and 4 Log CFU/ mL) of *Bacillus atrophaeus* endospores which were exposed to 60 kV of cold plasma treatment. The results showed that concentrations 6, 5 and 4 Log CFU/ mL were statistically different to 7 Log CFU/ mL (table 2). The plastic strip inoculated with 4 Log of *Bacillus atrophaeus* endospores and treated with cold plasma using Argon at 60 kV achieved a reduction of 2.21

Log in 6 min of exposure (table 2). Results from this logarithmic reduction differ from results obtained by Klämpfl *et al.* (2012), in which a 6 Log reduction was achieved in 3.4 min. This difference is due to the application of different methods of cold plasma, in this study a Surface Micro-Discharge plasma was applied to *Bacillus atrophaeus* endospores.

Table 2. Logarithmic reduction of four concentrations of Bacillus atrophaeus endospores applying cold plasma treatment using Argon at 60 kV in different exposure times.

Inoculum Level	**Logarithmic reduction (CFU/mL)**			
	30 s	120 s	240 s	360 s
10^7	1.39 ± 0.39^{ABx}	1.53 ± 0.28^{Ax}	1.45 ± 0.46^{Ax}	1.46 ± 0.30^{Ax}
10^6	1.61 ± 0.57^{Ax}	1.72 ± 0.21^{Ax}	1.07 ± 0.35^{Ax}	1.73 ± 0.43^{Bxy}
10^5	1.41 ± 0.22^{Ax}	1.59 ± 0.20^{Ax}	1.31 ± 0.30^{Ax}	1.76 ± 0.49^{Ay}
10^4	1.42 ± 0.67^{Bx}	1.50 ± 0.50^{Ax}	1.34 ± 0.54^{Bx}	2.21 ± 0.24^{Ay}

^{AB}Means with different letters in the columns represent statistical differences among esposure times ($P < 0.05$).
^{xy}Means with different letters in the rows represent statistical differences among inoculum levels ($P < 0.05$).

The results of this study had a similar behavior as the results found by Dobrynin *et al.* (2010) in which different concentrations of *Bacillus* endospores in liquid and in dry form were exposed to DBD cold plasma treatment. It was observed that lower concentration of spores achieved more logarithmic reduction than higher concentrations did in a certain amount of time. However, tis results differ from tose obtained by Keener *et al.* (2011)in which a 6.23 log reduction of *Bacillus atrophaeus* spores was achieved in less than 15 seconds with 80 kV.

Standard operating procedure

Is a document that was prepared based on the information collected in the laboratory logbook. It describes the objectives, responsibles, materials, procedures of sample preparation, cold plasma treatment and spore recuperation.

4. CONCLUSIONS

- A standard operating procedure to treat *Bacillus atrophaeus* endospores using cold plasma was developed.

- Cold plasma treatment at 60 kV during 6 min using Argon had a reduction of 1.8 Log UFC/ mL over *Bacillus atrophaeus* endospores.

5. RECOMMENDATIONS

- Evaluate the effect of different gases on *Bacillus atrophaeus* endospores and vegetative cells.

- Carry on studies using variables such as; relative humidity, temperature and voltage input used in cold plasma treatment of *Bacillus atrophaeus* endospores.

- Evaluate the effect of cold plasma treatment on different packaging materials.

6. REFERENCES

Bourke P, Ziuzina D, Han L, Cullen PJ, Gilmore B. 2017. Microbiological interactions with cold plasma. Journal of Applied Microbiology. 123: 308-324. eng. doi:10.1111/jam.13429.

Butscher D, Zimmermann D, Schuppler M, von Rohr PR. 2015. Plasma inactivation of bacterial endospores on wheat grains and polymeric model substrates in a dielectric barrier discharge. Food Control. Elsevier

Dobrynin D, Fridman G, Mukhin Y, Wynosky-Dolfi M, Rieger J, Rest R, Gutsol A, Fridman A. 2010. Cold plasma inactivation of *Bacillus cereus* and *Bacillus anthracis* (Anthrax) spores. IFEE Transactions on Plasma Science. 38: 1878-1884.

Hertwig C, Reineke K, Ehlbeck J, Knorr D, Schlüter O. 2015. Decontamination of whole black pepper using different cold atmospheric pressure plasma applications. Food Control. 55: 221–229.

Hertwig C, Reineke K, Rauh C, Schülter O. 2017. Factors involed in *Bacillus* spore´s resistance to cold atmospheric pressure plasma . Innovative Food Science and Emerging Technologies. 43: 173-181. eng. URL: http://dx.doi.org/10.1016/j.ifset.2017.07.031.

Klämpfl TG, Isbary G, Shimizu T, Li Y, Zimmermann JL, Stolz W, Schlegel J, Morfill GE, Schmidt H. 2012. Cold atmospheric air plasma sterilization against spores and other microorganisms of clinical interest. Appl. Environ. Microbiol. eng. doi: 10.1128/AEM.00583-12.

Keener KM, Jensen JL, Valdramidis VP, Byrne E, Connolly J, Mosnier JP, Cullen PJ. 2011. Decontamination of *Bacillus subtilis* spores in a sealed package using a non-thermal plasma system. Plasma for Bio-Decontamination, Medicine and Food Security. NATO Science for Peace and Security Series A: Chemistry and Biology. 445-455. URL: https://doi-org.ezproxy.lib.purdue.edu/10.1007/978-94-007-2852-3_34.

Lee K, Park B, Lee D, Lee I, Hyun S, Chung K, Park J. 2005. Sterilization of *Escherichia coli* and MRSA using microwave-induced argon plasma at atmospheric pressure. Surface & Coating Technology. 193: 35-38. eng. doi:10.1016/j.surfcoat.2004.07.034.

Min SC, Roh SH, Niemira BA, Sites JE, Boyd G, Lacombe A. 2016. Dielectric barrier discharge atmospheric cold plasma inhibits *Escherichia coli* O157:H7, *Salmonella*, *Listeria monocytogenes* and Tulane virus in romaine lettuce. International Journal of Food Microbiology. 237: 114-120. eng. URL: http://dx.doi.org/10.1016/j.ijfoodmicro.2016.08.025.

Morris AD, McCombs GB, Akan T, Hynes W, Laroussi M, Tolle SL. 2009. Bactericidal effect on *Geobacilus stereathermophilus* and *Bacillus cereus* microorganisms. The Journal of Dental Hygiene. 83: 55-62. eng.

Pankaj S, Bueno-Ferrer C, Misra N, Milosavjeviić, O´Donell C, Bourke P, Keener K, Cullen P. 2014. Applications of cold plasma technology in food packaging. Trends in Food Science & Technology. 35: 5-17. eng. URL: http://dx.doi.org/10.1016/j.tifs.2013.10.009.

Patil S, Keener K, Mosnier T, Misra NN Cullen PJ, Bourke P. 2014a. In package inactivation of *Bacillus atrophaeus* spores using high voltage atmospheric cold plasma [internet]. Society of Engineering Science 51st Annual Technical Meeting. URL: https://pdfs.semanticscholar.org/1a85/d16eacf33ee47d4cc0abe3c233fdad0e8945.pdf

Patil S, Moiseev T, Misra N, Cullen J, Mosnier P, Keener K, Bourke P. 2014b. Influence of high voltage atmospheric cold plasma process parameters and role of relative humidity on inactivation of *Bacillus atrophaeus* spores inside a sealed package. J Hosp Infect. 88:162–169.

Pedraza-Reyes M, Ramírez- Ramírez N, Vidales Rodríguez L, Roberto E. 2012. Mechanisms of bacterial spore survival. Caister Academic Press. In E. Abel-Santos (Ed.), Bacterial spores: Current research and applications (pp. 73–87).

Pignata C, D'Angelo D, Fea E, Gill G. 2017. A review on microbiological decontamination of fresh produce with nonthermal plasma. Journal of Applied Microbiology. 122: 1438-1455. eng. doi: 10.1111/jam.13412.

Realpe ME, Hernández CA, Agudelo CI. 2002. Especies del genero *Bacillus:*morfología macroscópica y microscópica [internet]. Colombia. Instituto Nacional de Salud. https://www.revistabiomedica.org/index.php/biomedica/article/download/1148/1263/0.

Sella SRBR, Guizelni BP, Gouvea PM, Figueiredo LFM, Ribeiro CAO, Vanderberghe LPS, Minozzo JC, Soccol CR. 2012. Relations between phenotypic changes of spores and biofilm production by *Bacillus atrophaeus* ATCC 9372 growing in solid-state fermentation. Arch Microbiol. 194:815-825. eng. doi:10.1007/s00203-012-0815-0 .

Sella SRBR, Vaderberghe LPS, Soccol CR. 2014. *Bacillus atrophaeus*: main characteristics and biotechnological applications. Critical Reviews in Biotechnology. 1-13.

Thirumdas R, Sarangapani C, Annapure US. 2015. Cold plasma: a novel non-thermal technology for food processing. Food biophysics. 10: 1-11. eng. doi:10.1007/s11483-014-9382-z.

van Bokhorst-van de Veen H, Xie H, Esveld E, Abee T, Mastwijk H, Groot MN. 2015. Inactivation of chemical and heat-resistant spores of *Bacillus* and *Geobacillus* by nitrogen cold atmospheric plasma evokes distinct changes in morphology and integrity of spores. Food Microbiology. 45: 26-33. eng. URL: http://dx.doi.org/10.1016/j.fm.2014.03.018.

7. APPENDICES

Appendix 1. Cold Plasma Treatment Standard Operating Procedure (SOP).

PURDUE UNIVERSITY

Purdue University
Food Science Department
Policy No:

Standard Operating Procedure

Cold Plasma Treatment for
***Bacillus atrophaeus* endospores**

Date when SOP was approved by laboratory supervisor:_____
Laboratory Manager/ Safety Coordinator:_____
Laboratory Phone:_____ Office Phone:_____
Emergency Contact:_____
_____
Location:_____

Objective:
Achieve a microbial inactivation applying double barrier discharge cold plasma (DBD-CP) treatment on a plastic surface with a set of controlled factors.

Responsibilities:
The person operating the equipment is responsible:

- To inform other people in the laboratory that the equipment has been turned on and the area where it is located is dangerous due to high voltage.
- For being at all times near the control panel of the equipment.

Materials:

- 15 mL glass test tubes
- 20 mL sterile petri plates
- Polyethylene strips
- Glass spreader
- Pipette tips
- 2 mL micro centrifuge tube
- Vortex
- Autoclave
- AC Dielectric Test Set
- Bunsen burner

1

Procedure:

Sample Preparation

1. Inoculate 1 mL of microorganism seed suspension on glass test tubes containing 9 mL of Tryptic Soy Broth (TSB).
2. Incubate inoculated test tubes at 35 °C for 24 h.
3. Prepare Sporulation medium (8.0 g·L-1 yeast extract, 4.0 g·L-1 nutrient broth, 0.05 g·L-1 MnSO4, 0.05 g·L-1 CaCL2, and 30.0 g·L-1 agar) (Sella et al. 2012).
4. Pour into 20 mL petri plates.
5. Inoculate petri plates with 75 µL of TSB containing vegetative cells of B. atrophaeus.
6. Incubate at 35 °C for 7 days.
7. Perform spore staining on a petri plate on the sixth day of incubation.
    a. Sterilize working area with 70 % alcohol and light up a Bunsen burner.
    b. Label glass slides as the petri plate (Initials of person responsible, date and bacteria)
    c. Place 100 µL of sterile distilled water in the glass slide.
    d. Sterilize inoculation loop in Bunsen burner until red.
    e. Scrape carefully the surface of petri plate and dissolve scraped out bacteria on distilled water in glass slides.
    f. Pass glass slides through the Bunsen burner flame to fix bacteria to glass.
    g. Place a beaker containing 500 mL of water on a hotplate at 110 °C.
    h. Place the glass slides over the beaker and put a piece of paper towel over glass slides.
    i. Place seven drops of green malachite on glass slide and let it sit for 5 min.
    j. Wash out excess of green malachite with distilled water and let it dry for 15 min.
    k. Take glass slides to beaker containing boiling water, apply three drops of saphranine and let it sit for 2 min.
    l. Take glass slides to a microscope and use immersion oil to view spores at 100×.
8. Perform viable spore quantification by plate count method.
9. *B. atrophaeus* spores were collected from petri dishes using distilled water and scraping out slowly the surface with a sterile spreader.
10. Prepare plastic spore strips by cutting down strips of polyethylene in 1 × 2 cm dimensions.
11. Sterilize plastic strips in autoclave at 121 °C for 20 min.
12. Prepare serial dilutions according to the concentration obtained from plate count method.
13. Place 10 µL of each serial dilution on a plastic strip and let dry on a sterile area.
14. Place concentration separately on a labeled sterile petri dish.
15. Place petri dish in the center of sample box.
16. Introduce slowly the sample box into a plastic bag and seal 75 % of the bag.
17. Fill and purge Argon gas into the sample box 5 times and seal bag completely.
18. Place the sample in the dielectric setup.

Cold Plasma Treatment

1. Place sample box between dielectric barriers (3 in each side).
2. Electrodes must be exactly one above the other ad the treated sample in between them.
3. Cover wires and metal with dielectric barriers in the chamber.
4. Turn On the AC Dielectric Test Set.
    a. Plug in.
    b. Turn on Main Power switch.
    c. Turn on Transformer Power switch.
    d. Push High Voltage On button.
5. Manage voltage slowly until reaching the desired power on the voltmeter panel.
6. With a stopwatch measure exposure time of the sample done by cold plasma treatment.
7. Once the exposure time has finished, proceed to turn off the AC Dielectric Test Set.
    a. Lower voltage to zero.
    b. Turn off Transformer Power.
    c. Turn Off Main Power.
    d. Unplug.

Spore Recuperation

1. After treatment take out the sample and place on sterile work area.
2. Open the bag using sterile scissors and take out the box.
3. Place the box between two Bunsen burners and proceed to open.
4. Using sterile tweezers take the strips out of sample box and place in test tubes containing 1 mL of Phosphate Buffered Solution.
5. Mix in the vortex for 3 minutes.
6. Make serial dilution for each concentration (discard pipette tip every time and use sterile glass spreaders).
7. Mix each serial dilution during 30 s, take out 100 µL and place in a petri dish containing Nutrient Agar.
8. Spread 100 µL in nutrient agar using sterile glass spreader.
9. Label each petri dish with concentration, serial dilution, exposure time, medium and microorganism.
10. Incubate at 35 °C for 48 h.

Appendix 2. Phenix Technologies High Voltage Transformer.

Appendix 3. Control panel of Double Barrier Cold Plasma Dielctric Set.

Appendix 4. Results of analysis of variance applied to evaluate the logarithmic reduction of *Bacillus atrophaeus* spores at different exposure times using cold plasma treatment. Statistical analysis SAS Program Version 9.4® $P < 0.05$ indicate the existence of statistical difference.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
CONCEN	3	0.30510312	0.10170104	0.60	0.6140
TIME	3	3.20695313	1.06898438	6.35	0.0006
TIME*CONCEN	9	2.23616771	0.24846308	1.48	0.1708

Appendix 5. Results of analysis of variance applied to evaluate the influence of different variables of cold plasma treatment over the inactivation of *Bacillus atrophaeus* spores. Statistical analysis SAS Program Version 9.4® $P < 0.05$ indicate the existence of statistical difference.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
CONCEN	3	145.7120815	48.5706938	471.27	<.0001
TIME	4	28.2411949	7.0602987	68.50	<.0001
INCUB_TIME	1	5.3534260	5.3534260	51.94	<.0001
TIME*CONCEN*INCUB_TI	27	2.9809716	0.1104064	1.07	0.3959