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Cold Plasma Technology:

Bactericidal Effects on *Geobacillus Stearothermophilus* and *Bacillus Cereus* Microorganisms

Angela D. Morris, RDH, MS; Gayle B. McCombs, RDH, MS; Tamer Akan, PhD; Wayne Hynes, PhD; Mounir Laroussi, PhD; and Susan L. Tolle, RDH, MS

Abstract

Introduction: Cold plasma, also known as Low Temperature Atmospheric Pressure Plasma (LTAPP) is a novel technology consisting of neutral and charged particles, including free radicals, which can be used to destroy or inactivate microorganisms. Research has been conducted regarding the effect of cold plasma on gram-positive bacteria; however, there is limited research regarding its ability to inactivate the spore-formers *Geobacillus stearothermophilus* and *Bacillus cereus*.

Purpose: The purpose of this study was to determine if cold plasma inactivates *G. stearothermophilus* and *B. cereus* vegetative cells and spores.

Methods: Nine hundred eighty-one samples were included in this study (762 experimental and 219 controls). Experimental samples were exposed indirectly or directly to cold plasma, before plating and incubating for 16 hours. Control samples were not exposed to cold plasma. The percentage-kill and cell number reductions were calculated from Colony Forming Units (CFU). Data were statistically analyzed at the .05 level using one-way ANOVA, Kruskal Wallis and Tukey's tests.

Results: There was a statistically significant difference in the inactivation of *G. stearothermophilus* vegetative cells receiving indirect and direct exposure (p=0.0001 and p=0.0013, respectively), as well as for *B. cereus* vegetative cells and spores (p=0.0001 for direct and indirect). There was no statistically significant difference in the inactivation of *G. stearothermophilus* spores receiving indirect exposure (p=0.7208) or direct exposure (p=0.0835).

Conclusion: Results demonstrate that cold plasma exposure effectively kills *G. stearothermophilus* vegetative cells and *B. cereus* vegetative cells and spores; however, *G. stearothermophilus* spores were not significantly inactivated.

Key Words: Cold plasma, Low Temperature Atmospheric Pressure Plasma (LTAPP), bacteria, spores, sterilization

Introduction

Low temperature atmospheric pressure plasma (LTAPP), also known as "cold" or nonthermal plasma, is an innovative technology that has the potential to destroy microorganisms.[1] Most of the visible universe is made up of plasma, referred to as the fourth state of matter, which is a partly ionized gas comprised of molecules, atoms, electrons and ions. The remaining 1% of the visible universe consists of three other states of matter: solids, liquids and gases. Most plasmas are very hot, with temperatures up to thousands of degrees centigrade; however, cold plasma denotes technology that operates at or near room temperature.

Plasma technology can be thought of as cold combustion producing highly reactive free radicals via electron-neutral collisions instead of using heat.[2] Researchers have investigated plasma technology for a wide spectrum of biomedical and commercial applications including decontamination of food and military equipment and sterilization of medical/dental instruments, as well as the killing of airborne and surface pathogens.[3] The development of an alternative to traditional sterilization methods that is safer, faster, and more cost effective would have far-reaching implications for the dental and medical professions. Moreover, cold plasma has the potential to impact the health care profession beyond sterilization purposes; in particular, inactivating microorganisms associated with oral diseases and wound infections.

Numerous studies have been conducted investigating the effectiveness of cold plasmas to inactivate strains of bacteria, such as *Bacillus atrophaeus* (previously called *Bacil-*

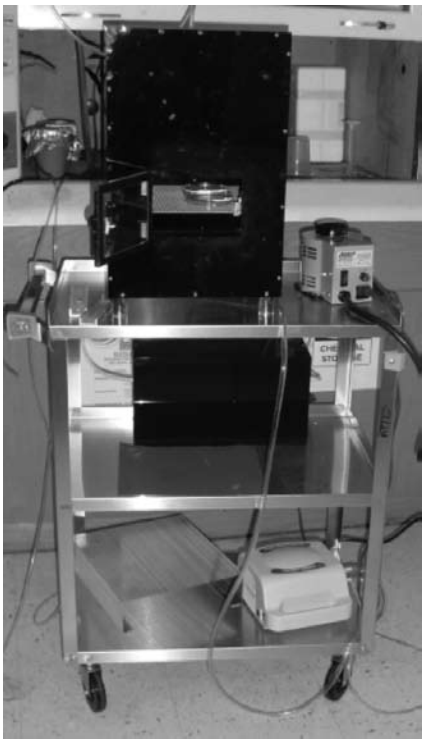

Figure 1. Indirect Cold Plasma Chamber

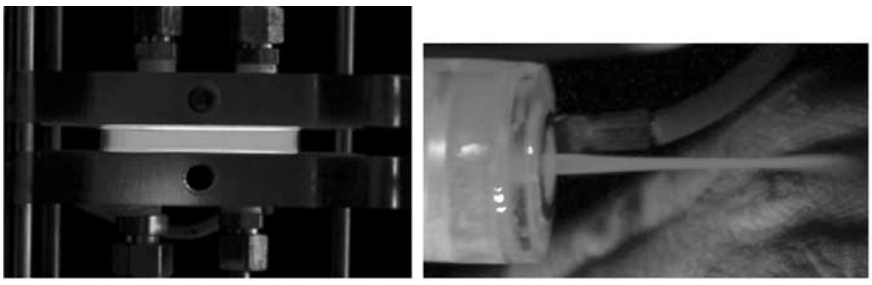

Figure 2. Direct Cold Plasma Plume

lus subtilis), *Escherichia coli*, and *Staphylococcus aureus*.[4-7] However, there is limited research regarding the inactivation of *Geobacillus stearothermophilus*, formerly called *Bacillus stearothermophilus*, and *Bacillus cereus*. The two microorganisms chosen for this study were selected because of their distinct differences. *G. stearothermophilus* is commonly found on biological indicator test strips to verify steam or chemical vapor sterilization of resistant microorganisms, whereas *B. cereus* is associated with food poisoning; both are extremely resistant to heat at the spore stage.[8,9]

Cold plasma may be utilized to inactivate microorganisms via indirect or direct methods. Indirect or "remote" cold plasma exposure requires the bacteria to be placed away from the plasma discharge; therefore, the samples are placed in an adjacent chamber (Figure 1). Conversely, direct cold plasma exposure occurs when the samples are placed directly under (within inches of) the plasma plume discharge (Figure 2).[4,10,11]

The purposes of this investigation were to (1) evaluate the effectiveness of cold plasma in destroying *G. stearothermophilus* and *B. cereus*, (2) determine which type of cold plasma exposure (indirect or direct) has the greatest kill and, (3) assess the minimum time needed to achieve a statistically significant reduction in the number of bacterial colonies.

Review of the Literature

Sterilization and decontamination are essential components of infection control within the dental and medical communities. Ensuring that sterilization techniques are effective is a major concern to health care professionals. Sterilization occurs when all microorganisms, spores and viruses are destroyed or inactivated.[12-14] Cold plasma technology has the potential to become a more cost effective and less time-consuming procedure, as well as produce less toxic waste when compared to the traditional types of sterilization, such as steam, dry heat, ethylene oxide, or chemical vapor methods.[4,12,14]

Cold plasma produces greater structurally damaging effects on gram-negative bacteria, such as *E. coli*, than to gram-positive bacteria.[5] Gram-negative bacteria experience structural damage to the outer membrane following exposure to cold plasma, whereas more resistant gram-positive bacteria do not show the same degree of morphological effects.[15] According to Laroussi and colleagues,[5] even though structural damage was not observed in gram-positive bacteria following exposure, the bacteria remaining were nonviable, suggesting that cold plasma inactivates the microorganisms without changing their structure.

G. stearothermophilus, a gram-positive, aerobic, spore-forming microorganism, is extremely resistant to high heat and pressure.[16] This microorganism is commonly associated with spoiling liquid foods in vending machines, such as coffee, and is incorporated on biological indicator strips as a way to monitor sterilization methods.[16]

B. cereus, a gram-positive, aerobic, spore-forming, microorganism, was chosen because it is an opportunistic pathogen which commonly causes food poisoning and has also been associated with periodontal disease and bacteremias.[17-19] Certain strains of *B. cereus* produce enterotoxins and emetic toxins, resulting in diarrhea and vomiting, the classic characteristics of food poisoning.[19] According to Marsili and colleagues, *B. cereus* microorganisms demonstrate susceptibility to plasma treatment by being inactivated within 10 to 30 seconds of exposure.[20] Moreover, when air was used as the gas for the plasma, a greater inactivation of *B. cereus* occurred after 50 seconds of treatment in comparison to using nitrogen or carbon dioxide gas mixtures. Researchers postulate that this interaction may be due to the ozone and free radicals that are produced in the breakdown of the air gas, causing inactivation of the *B. cereus*.[20]

Exposing bacteria inoculated on different types of media, such as liquid suspension, glass slab, or

Table 1. Sample Distribution and Cold Plasma Treatment Exposure Times

Dependent Variables		Independent Variables		Total
Bacteria and State	**Control (No Exposure)**	**Indirect Exposure**	**Direct Exposure**	**Sample Size**
G. stearothermophilus Vegetative	0 seconds	15, 20, 25 and 30 minutes	1, 2, 4, 5, 6, 8, 10, 15, 20 and 30 minutes	**440**
G. stearothermophilus Spores	0 seconds	15, 20, 25 and 30 minutes	10, 20 and 30 minutes	
Bacillus cereus Vegetative	0 seconds	1, 2, 3, 4, 5, 10, 15, 20, 25 and 30 minutes	10, 20, 30, 40 and 50 seconds and 1, 2, 4, 6, 8 and 10 minutes	**541**
Bacillus cereus Spores	0 seconds	1, 2, 3, 4, 5, 10, 15, 20, 25 and 30 minutes	30 seconds and 1, 2, 3, 4 and 5 minutes	
Total Sample Size	**219**	**344**	**418**	**981**

polypropylene, may also effect inactivation by cold plasma.[4] Laroussi demonstrated that the "survivor curves" of the microorganisms are related to the culture medium. For example, the D-value, or the time that was needed to destroy 90% of the original concentration of *B. atrophaeus* on a glass slab, was much shorter than the time required for inactivation of the same bacteria in a liquid suspension.[4]

The inactivation of resistant gram-positive bacteria, such as *B. atrophaeus*, without causing significant structural damage to the microorganism, suggests that cold plasma has the ability to kill without obvious morphological changes. Moreover, exposing cold plasma to a variety of materials used to manufacture dental and medical instruments may affect the amount of exposure time needed to effectively destroy resistant microorganisms such as *G. stearothermophilus* and *B. cereus*.

Methods and Materials

In the present study, the experimental group consisted of *G. stearothermophilus* and *B. cereus* vegetative cells (<18 hour culture) and spores (48-hour culture) on agar or glass slides, then exposed to either indirect or direct cold plasma. The control group, also referred to as "0 seconds exposure," utilized *G. stearothermophilus* and *B. cereus* vegetative cells and spores that did not receive cold plasma exposure.

Due to the exploratory nature of this study, various exposure times were evaluated for the indirect and direct plasma, resulting in a total sample size of 762 exposed and 219 unexposed control samples (N= 981). The indirect plasma chamber exposed 344 samples and the direct plasma device exposed 418 samples (n=762). The bacteria were purchased from the American Type Culture Collection (ATCC) (*G. stearothermophilus* ATCC 12980 and *B. cereus* ATCC 14579). Various exposure times were selected to evaluate the time points at which noticeable kill occurred as assessed by counting Colony Forming Units (CFU).

Laboratory Procedures

Indirect Plasma Exposure. Before exposure to cold plasma, the microorganisms were cultured in trypticase soy broth (TSB), diluted and plated onto trypticase soy agar (TSA) (Difco/Becton Dickinson Laboratories, Sparks, MD 21152) or Luria Bertani (LB) (Difco/Becton Dickinson Laboratories, Sparks, MD 21152) media for *G. stearothermophilus* and *B. cereus*, respectively. *G. stearothermophilus* vegetative cells and spores were exposed to indirect cold plasma for 15, 20, 25 and 30 minutes, whereas *B. cereus* vegetative cells and spores were exposed for 1, 2, 3, 4, 5, 10, 15, 20, 25 and 30 minutes (see Table 1). Following each exposure time, the cold plasma was turned off and the gases within the chamber were evacuated into a fume hood for 60 seconds. After treatment, the samples were incubated for up to 18 hours, and plates containing between 30 and 300 CFU were counted.

Direct Plasma Exposure. Prior to direct cold plasma exposure, the microorganisms were cultured overnight in TSB, diluted before 10 microliters of the culture was pipetted onto a sterile glass slide. The glass slides were placed directly under the plasma discharge. *G. stearothermophilus* vegetative cells were exposed for 1, 2, 4, 5, 6, 8, 10, 15, 20, and 30 minutes; *G. stearothermophilus* spores were exposed for 10, 20, and 30 minutes; *B. cereus* vegetative cells were exposed for 10, 20, 30, 40, and 50 seconds, as well as 1, 2, 4, 6, 8, and 10 minutes; and *B. cereus* spores were exposed for 30 seconds, 1, 2, 3, 4, and 5 minutes (see Table 1). After exposure, the glass slide was rinsed with sterile saline into a test tube, dilutions were made and the bacteria plated on either TSA or LB media prior to

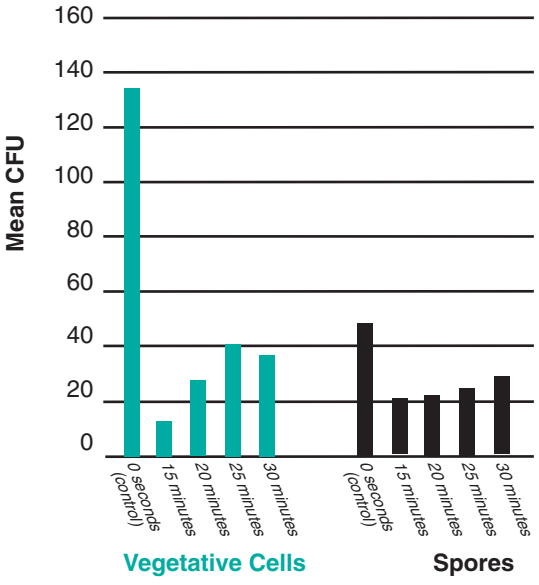

Figure 3. Mean CFU of* G. stearothermophilus *receiving indirect cold plasma

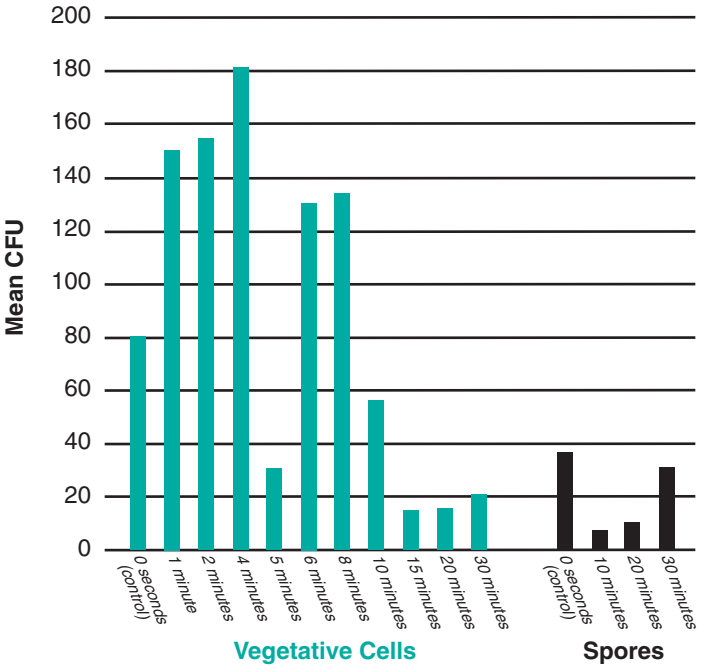

Figure 4. Mean CFU of* G. stearothermophilus *receiving direct cold plasma

incubation for 12-16 hours. Following incubation, CFU were counted.

Statistical Methods

Data were grouped for statistical analysis according to bacteria (*G. stearothermophilus* or *B. cereus*), bacteria state (vegetative or spores), cold plasma exposure (direct or indirect) and cold plasma exposure times (variable). Percentage kill was calculated by obtaining the total CFU for each bacteria, state, exposure type, and time and subtracting this number from each control group's CFU, dividing this total by the control CFU and then multiplying by 100 for the percentage value [i.e., (control CFU – experimental CFU / control CFU) x 100% = percentage kill]. Percentage kill is the proportion of colonies that were killed via cold plasma exposure (experimental group) compared to the number of colonies in the control group. The percentage kill provided the proportion of the bioburden of microorganisms that were effectively inactivated by cold plasma.[21] The concentration of cells (CFU/mL) was also calculated for each bacteria and state, exposure type, and time.

For data that were roughly normally distributed, the parametric test of one-way Analysis of Variance (ANOVA) was used to determine the means and standard deviations, whereas the Kruskal Wallis test was used to analyze data that were not roughly normally distributed. One-way ANOVA and the Kruskal Wallis test analyzed overall significance, and the Tukey's Studentized Range (HSD) test determined which cold plasma treatment times were statistically significant.

Results

Results demonstrate there was a statistically significant kill of *G. stearothermophilus* vegetative cells exposed in the indirect chamber at all time points, as well as direct exposure at 10 minutes (p-value of 0.0001 for indirect and 0.0013 for direct); however, there was not a statistically significant kill in *G. stearothermophilus* spores exposed to indirect or direct cold plasma (p-value of 0.7208 for indirect and 0.0835 for direct).

Data revealed a statistically significant kill of *B. cereus* vegetative cells at all time points for indirect exposure and starting at 50 seconds for direct exposure (p-value of 0.0001 direct and indirect). Statistically significant kill of *B. cereus* spores occurred at all time points for indirect exposure and beginning at 3 minutes for direct cold plasma exposure (p-value of 0.0001 for direct and indirect) (see Figures 3 - 6 for mean CFU and Table 2 for significance).

Discussion

This study was designed to evaluate the bactericidal effect of cold plasma on *G. stearothermophilus* and *B. cereus* vegetative cells and spores. The development of an alternative to traditional sterilization methods, such as cold plasma, would have a positive impact within the medical and dental communities. Furthermore, vegetative cells and spores were specifically tested to determine if differences occurred in the inactivation rates. Since spores

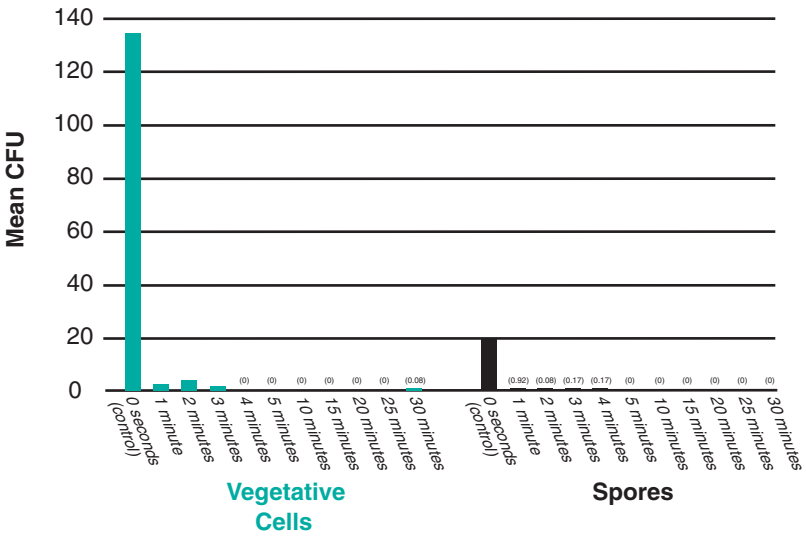

Figure 5. Mean CFU of* B. cereus *receiving indirect cold plasma

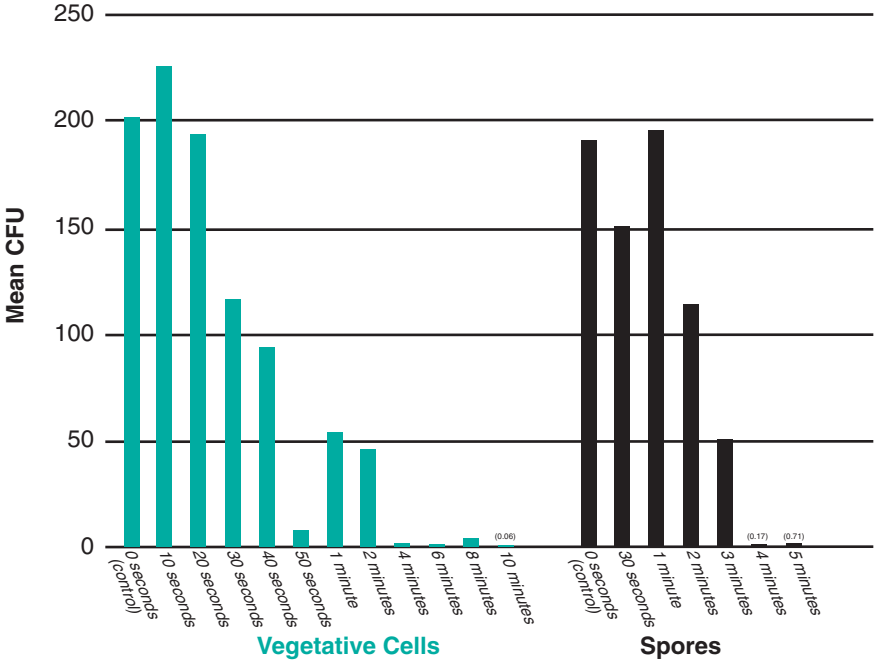

Figure 6. Mean CFU of* B. cereus *receiving direct cold plasma

are more resistant than actively dividing and growing vegetative cells, it was anticipated that vegetative cells would be inactivated at a faster rate than spores.[11,21,22] These findings suggest additional research is needed to determine how to best destroy spores.

G. stearothermophilus spores demonstrate extreme stability and require high heat and pressure for inactivation.[16] These factors may contribute to the difficulty experienced in killing *G. stearothermophilus* spores using cold plasma and may provide suggestions as to why there were no statistically significant reductions in CFU. Since *G. stearothermophilus* spores were more resistant to cold plasma than *B. cereus*, future studies are required to determine if modifications to the cold plasma device would increase its efficacy in killing *G. stearothermophilus* spores.

Exposing bacteria on various types of media, other than agar or glass slides, is recommended. Future studies are needed to compare the type of media and amount of time required for inactivation of *G. stearothermophilus* and *B. cereus*. It has been suggested that the type of media does affect cold plasma exposure times; however, this study did not address this aspect.[4]

The indirect chamber exposed 4 samples (Petri dishes) at one time. Additional research should evaluate variability of sample placement within the chamber. The researchers monitored plate location within the chamber (front left, front right, back left or back right); however, the results were not analyzed differentiating between the locations. Additionally, a distance of 0.25 inches from the direct plasma output to the glass side was utilized for each exposure. A recommendation for future studies would involve assessing the variability of direct exposure by using different distances between the cold plasma output and the culture.

This innovative technology holds commercial promise for a whole host of biomedical and industrial applications. Cold plasma, which could be thought of as room-temperature sterilization, has the potential to change the way we currently apply sterilization techniques. Potentially, cold plasma offers advantages over traditional methods, such as being more cost effective and time efficient, and producing less toxic byproducts than, for instance, ethylene oxide. Plasma technology has far-reaching implications for the development of an efficient and safer means of inactivating pathogenic microorganisms on hard surfaces and skin and in the air, as well as within the oral cavity. Researchers envision the implementation of a cold plasma device that can be used intraorally to inactivate cariogenic and periodontal pathogens, in addition to a device that can be

Table 2. Statistical Significance of Indirect and Direct Cold Plasma Exposure

Bacteria	State	Chamber	Significance
Geobacillus stearothermophilus	Vegetative	Direct	.0013*
		Indirect	.0001*
	Spore	Direct	.0835
		Indirect	.7208
Bacillus cereus	Vegetative	Direct	.0001*
		Indirect	.0001*
	Spore	Direct	.0001*
		Indirect	.0001*

*** Denotes statistical significance less than or equal to .05**

used for surface decontamination. The future of plasma technology is wide open and far reaching with tremendous potential for state-of-the-art biomedical and commercial applications.

Despite the limitations of this study, the data support that cold plasma is effective in killing *G. stearothermophilus* vegetative cells, as well as *B. cereus* vegetative cells and spores, for both direct and indirect exposure, at various time intervals. However, data revealed there was not a statistically significant kill in *G. stearothermophilus* spores.

Conclusion

The present study examined the bactericidal effects of direct and indirect cold plasma on *G. stearothermophilus* and *B. cereus* vegetative cells and spores. Results demonstrate that there is a statistically significant reduction in *G. stearothermophilus* vegetative cells and *B. cereus* vegetative cells and spores exposed to cold plasma; however, there is no statistically significant reduction in *G. stearothermophilus* spores. Spores are difficult to inactivate; therefore, further analysis is needed to determine how to penetrate the protective layers by modifications to the cold plasma devices.

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