International Journal of Food Engineering and Technology
2025, Vol. 9, No. 1, pp. 20-33
https://doi.org/10.11648/j.ijfet.20250901.12

Research Article

Efficacy of Gliding Arc Plasma Discharge Remote Treatment on Microbial Decontamination of Black Pepper (*Piper nigrum*) Seeds

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Abstract

Cold plasma technology is experimentally investigated globally as a green approach to microbial decontamination. Gliding Arc Plasma Discharge is one cost-effective design among many cold plasma configurations. The present study aimed to investigate the efficacy of Gliding Arc Plasma Discharge treatment for decontaminating black pepper seeds. Samples of black pepper seeds were washed, dried, packed and drawn from a processing facility in Padukka, Sri Lanka, and selected for the study. The study isolated and genetically identified the microflora associated with black pepper seeds. Reductions occurring in the microflora after Gliding Arc Plasma Discharge treatment and respective changes in the black pepper seeds' physical, chemical, and structural parameters were studied. Three bacterial species, namely, *Bacillus safensis*, *Bacillus firmicutes*, and *Bacillus siamensis*, and seven fungal species, five of which were *Aspergillus spp.*, one of *Talaromyces spp*, and *Cladosporium spp.*, were isolated, and genetically identified. A few identified molds can cause the physical degradation of black pepper seeds. Results showed a reduction in aerobic bacteria by 51%, 69%, and 73%, and yeast and mold count by 58%, 92%, and 93% with Gliding Arc Plasma Discharge treatment time of 5,10, and 15 minutes respectively. When a known quantity of a reference strain, namely Bacillus cereus American Type Culture Collection 11778, was subjected to Gliding Arc Plasma Discharge treatment to analyze the performance of a single pathogenic microbe, a reduction of 99.9% was achieved after 15 minutes. Even though a significant decrease in moisture content and water activity levels was observed after Gliding Arc Plasma Discharge treatment, piperine content and volatile oils reduction were not significant at $p<0.05$ with treatment time. Scanning Electron Microscopic images showed surface changes in all treatments, while Attenuated Total Reflectance Fourier Transform Infrared spectra also confirmed chemical structural changes of piperine. The study concludes that Gliding Arc Plasma Discharge treatment is an effective method for decontaminating microbes present in black pepper seeds.

Keywords

Microbial Inactivation, Black Pepper Seeds, Gliding Arc Plasma Discharge, *Bacillus Cereus*, Physicochemical Properties

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Received: 15 February 2025; **Accepted:** 28 February 2025; **Published:** 26 March 2025

1. Introduction

Black pepper seeds of a tropical vine, "Piper nigrum," are used for culinary purposes as a flavoring agent in whole or powder form and consumed in both cooked and uncooked foods. The presence of alkaloid, piperine and volatile oils in black pepper seeds is known to possess anti-microbial properties [1]. Black pepper seeds in dried form with low water activity naturally inhibit the growth of bacteria and molds [2]. However, black pepper seeds are found to be associated with a range of microorganisms, including resilient spore-forming bacteria and molds that can tolerate low-humid environments. Notably, heat-resistant microorganisms and soil-born microorganisms, such as spore-forming *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, as well as molds such as *Aspergillus* spp, and *Penicillium* spp, have the potential to produce various toxins. *Bacillus cereus*, a human pathogen capable of producing toxins, is responsible for causing both food poisoning forms: diarrheal and emetic [3].

Within the last decades, research has demonstrated that thermal treatment methods, including conventional steam sterilization, could improve the microbiological stability of dried black pepper, minimizing its potential contaminations [2]. Among the non-thermal methods, gamma irradiation and fumigation are known to minimize the adverse effects of thermal methods; however, they show a loss of aroma and flavor. Nevertheless, both techniques are not widely applied due to low consumer acceptability and the presence of chemical residues [4]. Most black pepper processing industries in Sri Lanka do not employ sterilization methods. However, a few export-oriented enterprises utilize wet steam to decontaminate black pepper seeds, thus requiring an additional drying step after steam sterilization to reduce the moisture to acceptable levels. Combining steam sterilization and an extra drying process may attenuate aroma and odor. Hence, other sterilization methods for decontaminating the black pepper seeds are essential [5].

Cold plasma, an emerging technology for microbial decontamination, is being evaluated globally across different food types using a variety of configurations [6]. Cold plasma is a green approach [7-9]. Low-pressure and atmospheric pressure plasmas such as plasma jets, arc discharges, and Dielectric Barrier Discharge (DBD) plasmas are frequently used in research on food applications [10]. However, atmospheric pressure plasmas are more suitable for food industry applications than low-pressure plasma systems as the former does not present extreme conditions to the food materials [11].

Gliding Arc Plasma Discharge (GAPD), operated under atmospheric pressure, has demonstrated high plasma efficiency. This is attributed to its distinctive capability of operating with low gas flows at atmospheric pressure, coupled with low investment and operational costs and a high rate of microorganism inactivation [12]. GAPD can produce both hot (thermal) and cold (non-thermal) plasma [13] thus demonstrating exceptional productivity and excellent adaptability to plasma-assisted chemical processes [14]. In GAPD, an airflow between two metal knife-shaped electrodes converts gas discharge into plasma flame with a given supply voltage [15]. The principal characteristics of GAPD include elevated electron density, heightened electron temperatures, and relatively lower plasma temperatures [16]. Arc plasma has reported the highest plasma species density of 10^{16} - 10^{19} ionic matter per cm^3 when compared with the low-pressure, corona, and dielectric barrier discharges, as well as in plasma jets [10]. Similarly, the density of electrons was reported as 10^{17} - 10^{18} per cm^3 in arc plasma, which recorded the highest levels found in other non-thermal plasma configurations [14] when compared to low-pressure discharges, corona, DBDs, and plasma jets [10]. High plasma species density and electron density can bombard the surfaces, causing etching and death of microbes [17, 18].

The utilization of GAPD faces constraints as a non-thermal processing technique because of the heat produced during the arc discharge. Nonetheless, remote or indirect treatment methods are implemented to mitigate the adverse impacts of heating food materials. In the remote/indirect treatments, long-lived reactive species generated in GAPD should play a pivotal role in microbial decontamination. The effectiveness of microbial decontamination in GAPD hinges on how the plasma species (short or long-lived reactive species) interact with various bacteria and mold species found on the outer surface of black pepper seeds. These seeds naturally possess a heterogeneous surface with pits, grooves, and cracks at millimeter and micrometer scales. The plasma microbe interaction also depends on the type of microbial species present on the surface of black pepper seed, their cell numbers, and cell surface densities with stacking and aggregation of microbes [19]. Therefore, the hygienic levels maintained from cultivation fields to processing are paramount to implementing a decontamination technology with inherently low penetration power.

The objective of this study was to examine and explore the efficacy of GAPD treatment in reducing the microflora that survived in black pepper seeds after pre-processing and on the death kinetics of Bacillus cereus (ATCC 11778) a known pathogenic microorganism inoculated to black pepper seeds with predetermined concentrations. The changes in the physical, chemical, and structural properties of black pepper seeds as both positive and negative effects of the treatment were also quantified. The study further identified and characterized the microflora associated with black pepper found in the processing facilities.

2. Materials and Methodology

2.1. Sample Collection

Samples of black pepper seeds (commercial high-yielding variety MB12, recommended by the Department of Agriculture, Sri Lanka) grown in Matale District (Central Province, Sri Lanka) were selected for the study. Black pepper seeds were collected from a processing facility in Padukka (Western Province, Sri Lanka), which routinely collects dried black pepper seeds from registered suppliers in Matale District. The registered suppliers supply black pepper seeds after sun drying of fully mature green berries. The dried black pepper seeds are washed, dried, and packed at the processing center for retail sale.

Samples of black pepper seeds (n=6) were drawn aseptically at four different processing steps: storage, after washing, after drying, and after packaging. They were collected into sterile stomacher bags (Seward, UK) and sealed. The samples were transferred to the microbiology laboratory within 3 hours, stored at 4 ± 2 ℃ while on transport, and after that stored at a controlled temperature of 17 ℃ until use.

Samples drawn at each processing step were used to analyze the microbial contamination levels: aerobic bacteria, yeasts, and mold counts. The packed samples collected from the facility were used to isolate and identify bacteria and molds associated with black pepper seeds and to investigate the effect of GAPD.

2.2. Aerobic Plate Count (APC) and Yeasts and Molds (Y&M) Count of Black Pepper Seeds in Processing Steps

The representative portion of black pepper seeds (n=6) was aseptically weighed (10 g) and mixed in 90 ml of 0.1% peptone (Oxoid, UK) water and serially diluted up to 10^{-6} dilutions. Two dilutions, 10^{-5} and 10^{-6} were used for APC, and 10^{-2} and 10^{-3} were used for Y&M. Plate count agar and dichlro glycerol agar were used for APC (ISO 4883: 2013) and Y&M (ISO 21527:2008.) respectively. The APC was assessed using the pour plate method, while the spread plate method was employed for Y&M. For APC plates were incubated for 48 h at 30 ± 1 ℃ for a duration of 72 ± 3 h, and Y&M incubation took place at 25 ± 1 ℃ for 5 to 7 days. Enumeration of colonies on designated plates was conducted, and the results were utilized to determine the colony forming units (CFU) per gram of the sample's fresh weight.

2.3. Isolation and Identification of Microorganisms Associated with Processed Black Pepper Seeds

Packed samples drawn from the processing facility were used in the study. Each sample (n=6) underwent successive dilutions in sterilized saline (0.89% NaCl, w/v), reaching a dilution factor of 10^{-6}. Bacteria were isolated using Nutrient agar (Oxoid, UK) by following the pour plate technique, and the plates were placed in an incubator set at 30 ℃ for 48 hours [20]. Molds were isolated using Potato Dextrose Agar (Oxoid, UK) following the spread plate technique, and plates were incubated at 25 ± 1 ℃ for five days [21].

2.3.1. Phenotypical Characterization of Isolated Microorganisms from Black Pepper Seeds

As described in section 2.3, the isolated microorganisms were cultured on their respective growth media to obtain purified colonies. The plates were examined (ROCKER, Galaxy 230) to identify colonies displaying typical bacteria and mold colony morphology. Colony morphological characteristics were noted, including form, size, shape, surface, texture, color, elevation, and margin. Isolates were further characterized by performing gram staining [20] and Lactophenol cotton blue staining [22] for bacteria and molds, respectively.

2.3.2. Molecular Level Identification of Microorganisms

Isolates with distinct phenotypic characteristics (described in Section 2.3.1) were selected for molecular-level identification. The genomic DNA was extracted [20]. Out of the isolated molds, isolates with distinct phenotypic characteristics (09 Nos) were selected for molecular identification. The extraction of fungal genomic DNA followed the procedure outlined by Martin & Rygiewicz, 2005. DNA analysis for quantity and purity was conducted using a gel documentation system (BIO-RAD, UK). This involved combining 5 µl of gel loading dye, and the gel was run at 60 V for 15 minutes.

Bacteria genomic DNA was subjected to PCR amplification with universal primers 27F (5′GAGTTTGATCATGGCTCAG3′) and 1492R (5′GGTTACCTTGTTACGACTT3′) (Frank et al., 2008), while fungi DNA was subjected to PCR amplification using universal primers ITS4 (5′ TCCTCCGCTTATTGATATGC3 TAACAAGG3) and ITS5 (5′GGAAGTAAAAGTCGTAACAAGG3′) primers (Marttinen et al., 2020; Nurunnabi et al., 2020). The resulting PCR products of both bacterial and mold isolates were sequenced at Macrogen, Seoul, Republic of Korea. The Bioedit sequence alignment editor 7.2 software (Ibis Therapeutics, Carlsbad, CA) was utilized for sequence analysis. Homologous sequences were sought through a database search using the Basic Local Alignment Tool of the National Center for Biotechnology Information (NCBI), Republic of Moldova [22]. Sequences exhibiting an identity of 98 - 99% or higher than those in the databases were assigned to the corresponding species. The partial sequences of bacterial and mold isolates were deposited at GenBank NCBI, and accession numbers were obtained (Table 1).

2.4. GAPD Treatment for Black Pepper Seeds

GAPD, purchased from Eltech Pvt Ltd, Mumbai, India, was used in this study to decontaminate microflora. The step-up voltage was 15 kV with a domestic power supply of 50 Hz alternating current (AC). The carrier gas was ambient air at 26 ℃ and had a humidity of 65%. The airflow rate was fixed at 0.009 m^3/s until the plasma discharge came out as a flame. Black pepper seed samples (20 g) were packed in a petri dish as a single seed layer with a packing density set at 0.22 g/cm^2. Each sample was placed under the plasma discharge at a distance of 4 cm to carry out the experiments in remote mode. The Petri dishes were shaken manually twice per minute and kept horizontally. The samples underwent treatment for 5, 10, and 15 minutes, with each treatment replicated three times. Thermal images after each treatment were captured using IR thermography prime FLIR E60, Instrumart, USA.

Treated samples of black pepper seeds (10 g) were drawn before and after GAPD and were aseptically weighed and analyzed for total aerobic bacteria and molds as per the methods described in 2.2 following ISO 4883: 2013 & ISO 21527:2008.

2.4.1. Determining Inactivation Kinetics of *Bacillus cereus*

Based on the different types of bacteria and molds isolated in section 2.3, *Bacillus* species was selected as the potential organism to study further as a model organism to understand the efficacy of GAPD plasma when inoculated with known concentrations to black pepper seeds. *Bacillus cereus* was reported to have a relatively higher pathogenicity than the identified *Bacillus* species associated with black pepper seeds. Therefore, *Bacillus cereus* (ATCC 11778) was selected as the model organism to evaluate the inactivation kinetics with GAPD treatment.

(i). Culture Preparation

Based on the different types of microorganisms isolated in section 2.3, *Bacillus* species was selected as the potential organism to study further as a model organism to understand the efficacy of GAPD plasma when inoculated in known concentration to black pepper seeds. *Bacillus cereus* (ATCC 11778) was selected as the model organism. *Bacillus cereus* (ATCC 11778) was sub-cultured from the reference stock tier 2 of the culture maintenance system of the Microbiology Laboratory of the Food Technology Section of Industrial Technology Institute and incubated at 30 ± 1 ℃ for 18 hours. After completion of the incubation period, the cell concentration of the broth cultures was adjusted to 0.5 McFarland standard, which accounts for 1.5×10^8 CFU/ml, and absorbance was measured using a spectrophotometer (SPECTRA max plus 384, USA) at 600 nm. Following this, the culture was inoculated into Mannitol Egg Yolk Polymyxin (MYP) Agar (recommended culture medium by ATCC), and the number of colonies was enumerated.

(ii). Inoculation of Black Pepper Seeds, Subjecting to GAPD Treatment

Black pepper seed samples (n=3) were washed using potable water and dried in the open air for 2 hours until the water on the surface was removed. Ten grams of each sample was weighed and wrapped in Aluminum foils. Wrapped samples were sterilized in an autoclave (Sakura, ASV-3023, Japan) at 121 ℃ for 20 minutes. Each sample was transferred into sterile stomacher bags (Seward, UK) in a biological safety cabinet (BSL-2, Thermo Forma, Japan). *Bacillus cereus* (ATCC 11778) inoculum adjusted to 0.5 McFarland standard was uniformly inoculated into black pepper seeds samples and incubated for 1 h at 30 ± 1 ℃ [23]. Inoculated samples were brought to the GAPD treatment, and each sample was treated according to the method described in 2.4. Soon after the GAPD treatment, each sample was packed in a sterile stomacher bag (Seward, UK) and immediately transferred to the microbiology laboratory for enumeration studies.

(iii). Enumeration of *Bacillus cereus* After GAPD Treatment

To estimate the *Bacillus cereus* in black pepper seeds, it was enumerated using MYP agar, following the spread plate technique. Plates were incubated at 30 ± 1 ℃ for 24 hours; colonies were counted. Presumptive colonies were witnessed for distinctive phenotypic traits, characterized by large, pink-colored colonies encircled by a precipitate zone. Confirmation of presumptive *Bacillus cereus* was achieved through biochemical tests, including glucose agar, Voges-Proskauer (VP), and nitrate tests.

2.4.2. Determination of Inactivation Kinetics

Colony Forming Units were recorded for each treatment. Microbial inactivation kinetics, the time required to reduce the microbial cell count by one log cycle (D value) for the GAPD, were calculated. The inactivation effect of aerobic bacteria, molds, and *Bacillus cereus* (ATCC 11778) was analyzed using a first-order kinetic model according to Equation (1) [24, 25].

$$log_{10}\left[\frac{N}{N_o}\right] = -k\,t = -\frac{t}{D} \quad (1)$$

Where D (minutes) = 1/k is the decimal reduction time.

N - Microbial count after treatment

N_o - Microbial Count before treatment

2.5. Physical, Chemical, and Structural Properties After GAPD Treatment

After evaluating the microbial decontamination levels, black pepper seeds were evaluated for physical, chemical, and structural changes corresponding to the GAPD treatment.

2.5.1. Determination of Physical Properties of Black Pepper Seeds

The diameter and weight of black pepper seeds were measured using a 150 mm electronic digital Vernier caliper (IGaging IP54) and analytical balance (METTLER TOLEDO), respectively. The Bulk density was measured using the method given by [26].

The Moisture content was assessed using Dean and Stark method as described in AOAC 17th edition 2000, 986.21. Black pepper seeds (40 g), subjected to distillation, post-grinding a dry grinder (Singer, 650W). The resulting sample was distilled with Toluene (Sigma Aldrich) in a graduated distillation apparatus. Refluxing continued for 4 hours until two consecutive readings, spaced 15 minutes apart, showed no further alterations in the trap.

Water activity was analyzed using a Water Activity Meter (Aqualab, Washington, USA) [3].

Color measurements were taken from twelve points of each sample using a Chromometer (Konica Minolta) with a three-dimensional colorimetric system as per the International Commission of Illumination standards. In each treated and non-treated sample, three parameters, L, a, and b, were recorded [27].

2.5.2. Scanning Electron Microscopic (SEM) Images

Scanning electron microscopy (LEO 1420VP) examined black pepper seeds' morphology and surface features, maintaining an accelerated voltage within the 18-21 kV range. The examination includes the complete surface of pre-demarcated black pepper seeds, capturing representative images for analysis.

2.5.3. Extraction and Quantification of Piperine of Black Pepper Seeds

Piperine content was determined by a method described in ISO 5564-1982 (E). Ground black pepper seeds were accurately weighed (40 g), and hydro-distillation was carried out to remove volatile oils. The remaining residue after hydro-distillation was collected and dried to remove moisture. The oleoresin was extracted from the residue using Ethyl Alcohol (Sigma-Aldrich) and n-hexane (Sigma-Aldrich) (40:60 ratio). Then, oleoresin was dissolved in Ethyl Alcohol and n-hexane (40:60 ratio). The blending process occurred with a solvent-to-residue ratio of 3/5 (v/w). The resultant mixture was left to facilitate the crystallization of piperine, and it was subsequently stored in a refrigerator at 4 ℃ overnight. The crystallized piperine was filtered and washed with ethanol and n-hexane in a 40:60 ratio. The mixing process was executed with a solvent-to-residue ratio 3:5 (v/w). The resulting mixture was permitted to crystallize piperine and placed in a refrigerator at 4 ℃ overnight. The precipitated piperine crystals were filtered, followed by a thorough wash with a mixture of ethanol and n-hexane in a 40:60 ratio. This recovery of piperine was optimized using different ratios of Ethanol and Hexane. Dilutions of piperine were prepared, and absorbance was measured at 343 nm using a spectrophotometer (SPECTRA max plus 384, USA).

2.5.4. Extraction and Quantification of Volatile Oils of Black Pepper Seeds

Volatile oils were extracted as per the method (AOAC 17th edition 2000, 962.17). Crushed black pepper (50.00 g, n=3) was accurately weighed and quantitatively transferred to a 1000 ml round bottom flask. Then 500 ml of distilled water was added, and the flask was heated to boiling. The distillation was conducted for approximately 4 hours, persisting until two successive readings, obtained at one-hour intervals, revealed no alteration in the volume of oil collected in the trap. The oil was collected for glass vials, and anhydrous Na_2SO_4 was added and kept in the refrigerator at about 4 ℃ for further analysis.

2.5.5. Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy

Potential chemical structural changes in black pepper seeds were assessed using Attenuated Total Reflectance- Fourier Transform Infrared Spectroscopy (ATR-FTIR Bruker Tensor 27 IR spectrometer). Spectra were captured within the wavenumber range of 500 cm^{-1} to 4000 cm^{-1}, employing a resolution of 4 $cm^{-1,}$ exposing the outermost layers of black pepper seeds using diamond crystal, and the capturing was set at 16 scans [28].

2.5.6. Statistical Analysis

Each experiment was conducted in triplicate. The impact of the treatment was assessed for statistical significance at a threshold of $p<0.05$ through one-way analysis of variance (ANOVA). Differences between means were further elucidated using Tukey's test at a significance level of $p<0.05$.

3. Results and Discussion

3.1. Physical Properties of Black Pepper Seeds

Black pepper seeds, with a bulk density of 530 ±12 kg/m^3, a mean diameter of 4.52 ± 0.49 mm, and a mean weight of 0.056 ±0.015 g, were used throughout the experiments. Their water activity was 0.64.

3.2. Microbial Contamination of Black Pepper Seeds

Black pepper seed samples collected from the processing facility were subjected to microbiological analysis. The total aerobic plate and yeast and mold counts were quantified. The highest microbial contamination was observed in samples drawn from the initial storage location, with a bacterial count

of 9.9 log (CFU/g) and mold count of 4.9 log (CFU/g), as presented in Figure 1. When the black pepper seeds were subjected to washing with potable water, a significant loss at $p<0.05$ in the aerobic bacterial count was observed. Still, the reduction in the yeast mold count was not statistically significant at $p<0.05$. It may be postulated that microbial contamination of black pepper seeds occurs at the early post-harvest stage when green berries are sun-dried and stored repeatedly over several days, where the seeds are often exposed to both soil and the environment. The study also revealed that the technique used for washing the black pepper seeds with potable water at the processing facility was not effective. Contaminated black pepper could only pose the risk of causing foodborne diseases; nonetheless, it may also reduce the product's shelf life [5]. It is reported that both *Bacillus cereus* and *Clostridium botulinum* are recognized as two potentially hazardous microbes under Appendix 1 of the FDA's Draft Guidance for Industry (ASTA spice).

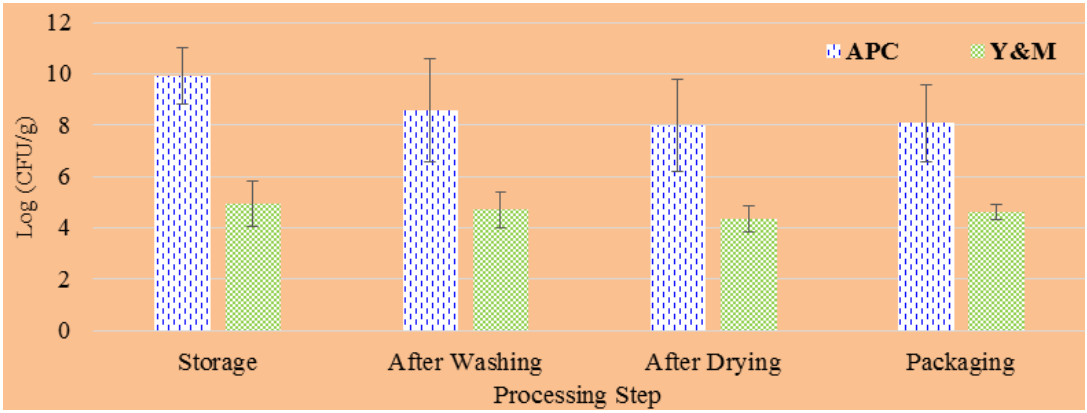

Figure 1. *Bacteria and molds contamination levels of black pepper seeds at the processing facility.*

3.3. Identification and Characterization of Bacteria and Molds in Black Pepper Seeds

An attempt was made to isolate and characterize microorganisms associated with black pepper seeds. Samples were drawn from the finished product (packaged) in the processing facility, and isolation and characterization were carried out using the methods described in Section 2.3. Three bacterial species and seven mold species were isolated from the black pepper seeds, presented in Table 1.

Table 1. *Microorganisms isolated from black pepper seeds and GenBank accession numbers.*

Type of microorganism	Code	Identity	Similarity to type strain%	Accession Number in NCBI submission
Bacteria	SLPB_110	Bacillus safensis	100.00	OR 418926
	SLPB_122	*Bacillus firmicutes*	99.87	OR 418431
	SLPB_198	*Bacillus siamensis*	98.80	OR 418489
Molds	SLBF_022	*Aspergillus penicilliodes*	100.00	OR 425146
	SLBF_025	*Aspergillus penicilliodes*	100.00	OR 425145
	SLBF_033	*Talaromyces pinophilus*	100.00	OR 425144
	SLBF_040	*Aspergillus sydowii*	100.00	OR 425142
	SLBF_045	*Aspergillus japonicus*	100.00	OR 425108
	SLBF_052	*Aspergillus sydowii*	100.00	OR 419503
	SLBF_061	*Cladosporium spp*	99.77	OR 418493

The letters S L P B refers to the Bacteria isolated from Sri Lankan Black Pepper and S L B F for the molds isolated from Sri Lankan Black Pepper.

All three bacterial species isolated from black pepper seeds, *Bacillus safensis*, *Bacillus siamensis*, and *Bacillus firmicutes*, were gram-positive. *Bacillus safensis* is an aerobic chemo-heterotrophic bacterium that forms spores and thrives in moderate temperatures. Its pathogenicity is not reported. This bacterium showcases behaviors that promote plant growth and holds promise in biotechnological realms by producing a range of industrial enzymes and secondary metabolites. It is considered a safe industrial microorganism as there is no evidence of its pathogenicity [29]. *Bacillus siamensis* is a gram-positive facultative anaerobic bacterium recorded as a non-pathogen [30] and reported to perform antifungal activities against *Aspergillus* spp [31]. *Bacillus firmicutes* is also a gram-positive bacteria in the gut microbiota [32] and demonstrates antimicrobial properties against pathogenic bacteria [33]. All three bacteria isolated in this study have not been reported previously to be associated with black pepper seeds.

Of the seven mold species isolated from the black pepper seeds, five were *Aspergillus* species, and the others were *Talaromyces spp.* and *Cladosporium spp*. The toxicity, allergy, and infection of *Aspergillus spp* can harm humans, which may adversely affect human health. Certain *Aspergillus* species can generate secondary metabolites or mycotoxin [34].

Among the molds identified, *Aspergillus penicillioides* can thrive in conditions of low water activity, exhibiting growth capabilities even at a water activity level as low as 0.68 and found in dried foods such as cereals [35]. *Talaromyces pinophilus* is also a species of mold that is extensively utilized for breaking down cellulose and decomposing waste [36]. Therefore, its presence can contribute to the degradation of the structure of black pepper seeds, reducing their shelf life. *Aspergillus sydowii* has been documented as a food contaminant and as a pathogen affecting immunocompromised individuals [37]. The presence of *Aspergillus sydowii* was also reported in Sri Lankan black peppers in previous research [38]. However, the presence of *Aspergillus japonicus* could damage the seeds during storage, as it can damage the chemical structure of the black pepper seeds by metabolizing lignin and many structure-related phenols [39] and aromatic compounds [40]. *Cladosporium spp*. ranks among the prevalent fungi in indoor and outdoor settings, thriving even in extreme ecological niches [41].

Both pathogenic and non-pathogenic microorganisms were associated with the surface of black pepper seeds. Notably, these microorganisms manage to survive despite acquiring high levels of antimicrobial properties inherent to black pepper seeds with their volatile oils and alkaloid content [1,44]. The molds isolated from this study can potentially affect the quality of the black pepper seeds as they can break down the lignocellulosic structure and modify odor compounds.

3.4. Effect of GAPD Treatment for Inactivation of Aerobic Bacteria, Molds, and *Bacillus cereus* Inoculum

The efficacy of the GAPD treatment in decontaminating the microflora on black pepper seeds was tested on naturally occurring microbial contaminants in black pepper seed surfaces and on the inactivation of *Bacillus cereus* at a known concentration. The results were evaluated by determining log reduction, percentage reduction, and D value.

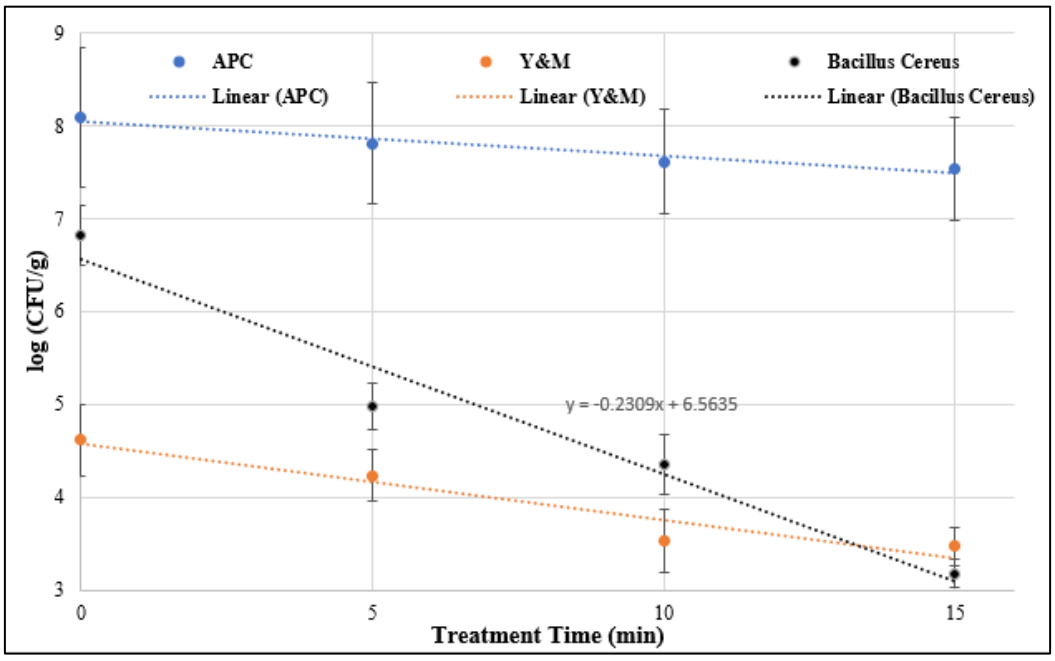

Figure 2. *Reduction of aerobic bacteria, molds, and Bacillus cereus inoculum in Black Pepper seeds after GAPD Treatment.*

Figure 2 presents the reduction of log (CFU/g) of aerobic bacteria, molds, and *Bacillus cereus* inoculum. The correlation between the log value of the viable microbial count and the treatment time was explored through regression analysis. This revealed that aerobic bacteria, yeasts and molds, and *Bacillus cereus* inactivation adhered to a linear, first-order kinetic model, with R^2 values of 0.9399, 0.9227, and 0.9620, respectively, as illustrated in Table 2.

Table 2. *Inactivation levels after 15 minutes of treatment and D Value (minutes) of bacteria, molds, and Bacillus cereus inoculum.*

Microorganism	Log Reduction (15 min)	Percentage Reduction (15 min)	D Value (min)	R^2
Aerobic Bacteria	0.55	73	-	0.9399
Molds	1.15	93	-	0.9227
Bacillus cereus (ATCC 11778)	3.64	99.9	4.35	0.9620

R^2 measures how the data fit the linear first-order kinetic model.

The microbial inactivation rate with the GAPD treatment differed between native microflora and the known inoculum. As given in Table 2, after 15 minutes of treatment time, the aerobic bacteria count was reduced by 73% (0.55 log (CFU/g)), and the yeasts and molds count were decreased by 93% (1.15 log (CFU/g)). The GAPD treatment on the known concentration of 6.81 x 10^6 (CFU/g) *Bacillus cereus* was reduced by 99.9% after 15 minutes of treatment. In line with the findings of the results obtained in this study, cold atmospheric pressure plasma driven with 1.2 kW microwave power reduced 2.0 $\log_{10}$ (CFU/g) of mesophilic aerobic bacteria after 30 min treatment time when air was used as the process gas. Similar to the higher inactivation levels obtained for *Bacillus cereus*, higher inactivation levels were obtained for inoculums used in treating black pepper seeds, reaching 2.4 $\log_{10}$ (CFU/g) for *B. subtilis* and 2.8 $\log_{10}$ (CFU/g) for *B. atrophaeus* [5].

The reduction of microbial counts depends on how far the plasma-microbe interaction occurred during the treatment. Corresponding to Table 1, native microflora in black pepper seeds are mixtures of bacteria, yeasts, and molds in different forms attached to the surface of black pepper seeds. As the literature reports, the attachments of microbes to different substrates follow adsorption, irreversible adsorption, micro-colonies, and natural biofilms [43]. The lowest inactivation was obtained with aerobic bacteria with 6.5 x 10^7 CFU/g before treatment. The inactivation of the multilayered microbial cell structure's outermost layer can establish a physical barrier. This barrier serves as a protective shield, keeping the reactive plasma species generated from reaching and deactivating the microorganisms in the bottom layers [44]. A higher number of initial counts of aerobic bacteria, which can create this shielding effect, may retard microbial inactivation levels.

According to the literature, microbial inactivation can occur through biological mechanisms, such as DNA damage from UV radiation, lipid peroxidation, protein modulation, and induction of apoptosis. Physical mechanisms such as electrostatic disruption and electroporation are also widely recognized [45]. Microbial inactivation mechanisms depend on the plasma species density, which continuously impacts the surface of the black pepper seeds. As per the literature, using a high-voltage power supply in GAPD plasma generates significant quantities of short-lived reactive plasma species [46]. Only the long-lived reactive species can reach the target microbe during a remote treatment, influencing the inactivation levels. The generated reactive species are mixed with the surrounding air by diluting the plasma density at the point of the target substrate, where the black pepper seeds' surface is. In addition to that, in the GAPD system, the electrodes employed in plasma generation in GAPD have a relatively limited effective area, potentially resulting in the conversion of a small volume of feeding gas into plasma species, thus [47], yielding low microbial inactivation levels.

However, the inactivation of *Bacillus cereus* with GAPD remote treatment was remarkable compared to native bacteria and molds. GAPD could remove 99.9% of the inoculated *Bacillus cereus* (ATCC 11778), yielding a decimal reduction time of 4.35 minutes as per Equation (1). This confirms the efficacy of GAPD for the pathogen inactivation. During the inoculation process, black pepper seed surfaces are evenly mixed with a known concentration of *Bacillus cereus* suspended in a liquid medium; thus, the exposure of microbial cells to GAPD is more straightforward than in the process of decontamination of native bacteria and molds. This can cause comparatively higher inactivation levels of *Bacillus cereus* than in the native bacteria and molds associated with black pepper seeds.

3.5. SEM Images

The interaction between plasma species and the surface of black pepper seeds extends beyond engaging with microbes, and prolonged interaction may result in the etching of the substrate surface. Figure 3 shows that the surface of untreated black pepper seeds initially has an irregular surface with fewer grooves, cracks, cavities, and pits, while the cavities on

the seed surface are wide and shallow. After 5 minutes of treatment, approximately 20 microns wide pits were created with the continuous bombardment of plasma species. The seed surface captured after 10 minutes revealed the deeper grooves and cracks in the epicarp, while after 15 minutes of treatment, the surface of the black pepper seeds was porous with excessive etching. The extent of etching depended on the plasma density and the energy levels of plasma species' impact on the surface during the treatment time [2, 30].

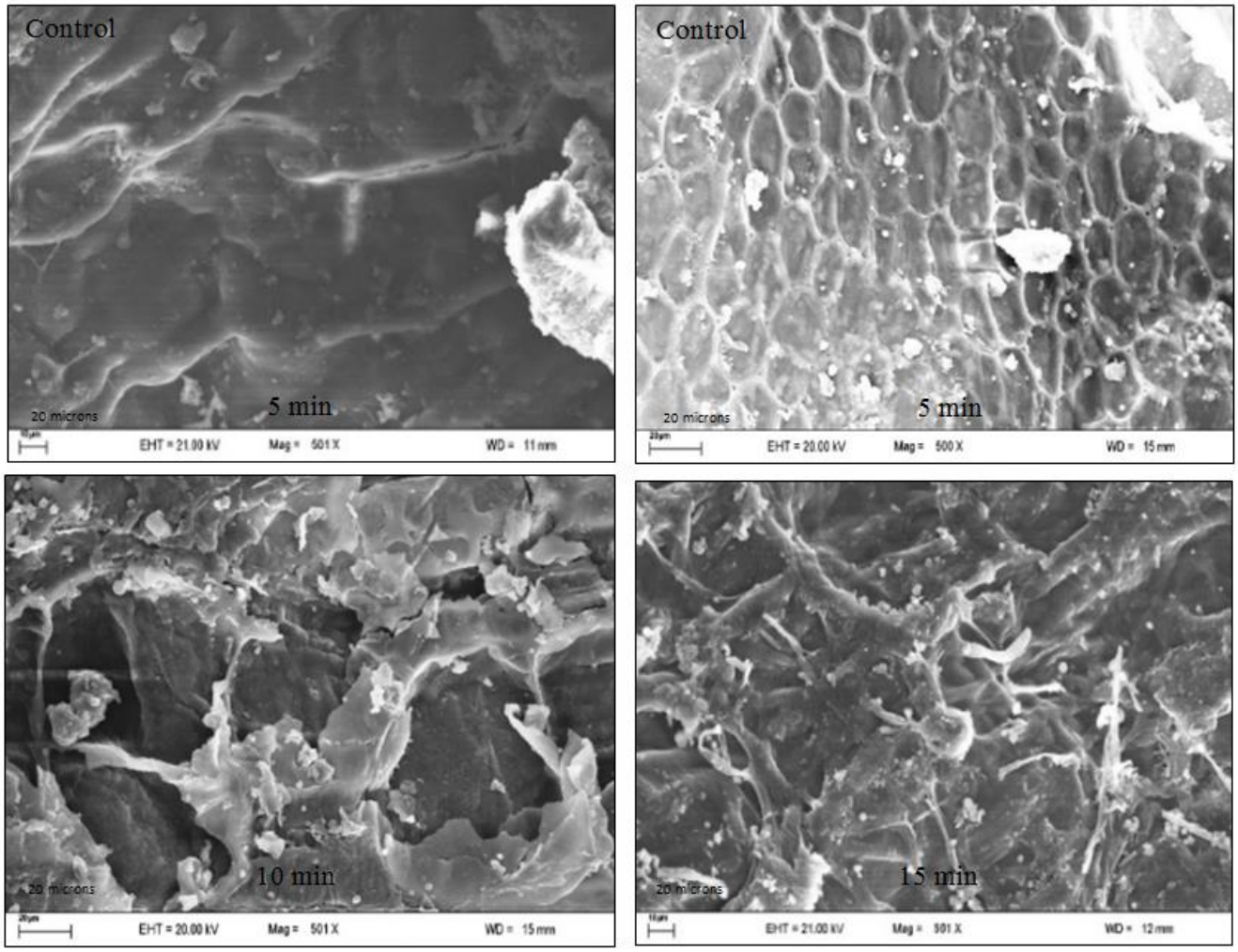

Figure 3. *SEM images of the surface of black pepper seeds before and after GAPD Treatment.*

The SEM micrographs obtained after 5 minutes of treatment of black pepper seeds using GAPD treatment have shown to be similar to the surface changes reported by [29], after 5 minutes of treatment using diffuse coplanar surface barrier discharge plasma treatment. A significant change on the surface affected the internal substances of the black pepper seeds, making micro and nanometer-level grooves. These surface changes can cause the interaction of the species generated in plasma with the internal compounds present in black pepper seeds. Supporting this, it was reported that plasma etching facilitates the movement of inner molecules of foodstuffs during processing [48].

3.6. Variation of Physicochemical Properties

Color, moisture content, water activity, and the mean surface temperature attained after each GAPD treatment are presented in Table 3. In color, a significant increase in L value after 5 minutes of treatment time was observed, with no changes in the “a” and “b” values. The color of black pepper seeds depends on the outer epicarp's chemical composition and bioactive components [49]. Changes in bioactive ingredients such as antioxidants and polyphenols in black pepper seeds during plasma treatment have been reported previously, with changes in the L value as the high heat-susceptible color component in paprika powder [5].

Table 3. Variation of physical properties of black pepper seeds with GAPD treatment.

Treatment	Color			Moisture (%)	Water Activity	MST (℃)
	L	a	b			
Control	10.29 ±0.25[a]	3.46 ±0.01[a]	3.79 ±0.51[a]	11.81 ±0.09[a]	0.640 ±0.003[a]	28.3 ±0.2[a]
5 min	13.59 ±0.47[b]	2.93 ±0.81[a]	3.94 ±0.51[a]	8.12 ±0.18[b]	0.344 ±0.018[b]	53.4 ±1.2[b]
10 min	13.38 ±1.61[b]	4.29 ±2.22[a]	3.79 ±0.61[a]	6.87 ±0.18[c]	0.286 ±0.002[b]	61.6 ±2.3[c]
15 min	13.01 ±2.14[b]	4.49 ±2.51[a]	3.87 ±0.72[a]	6.75 ±0.35[c]	0.231 ±0.001[c]	65.2 ±2.3[c]

Data is expressed as mean ±SEM, n = 3. Means followed by the different letters indicate a significant difference at $p<0.05$ between the data within the same columns, MST: Mean Surface Temperature

The moisture content and water activity of treated black pepper seed samples reduced significantly ($p<0.05$) with the treatment time. As shown in Figure 3, the surface changes occurred along with the heat generation and the flow of arc plasma discharge in the GAPD treatment, facilitating the evacuation of the moisture and active water (unbound water) presented in black pepper seeds. Eliminating moisture can cause shrinkage and contraction of the surface, which result in a color change.

Although it is generally accepted that water activities below 0.6 will not accommodate the growth of bacteria and molds, the results of the microbiological studies further confirmed that the bacteria and molds that were there on the surface of dry black pepper seeds were not eliminated with the reduced water activity along with GAPD treatment even at 0.231 water activity after 15 minutes treatment. From an economic point of view, excess removal of moisture and active water can cause significant reductions in weight, highlighting a negative effect in prolonged treatment times. As Table 2 shows, the mean surface temperature of the black pepper seeds increased to 65.2 ℃ after 15 minutes of GAPD treatment. This was identified as a limitation, evident even during the remote treatment.

As shown in Table 4, The piperine content after plasma treatment decreased gradually with the treatment time; however, it was not statistically significant (at $p<0.05$). Piperine is reported as heat stable around 50 ℃. Excessive surface temperature increments up to 65.2 ℃ in black pepper seeds during GAPD treatment might cause the degradation of piperine, which exists near the outermost layers of black pepper seeds [50]. Similar observations have also been made when black pepper seeds were treated with direct plasma jet treatment and microwave plasma remote treatment [5].

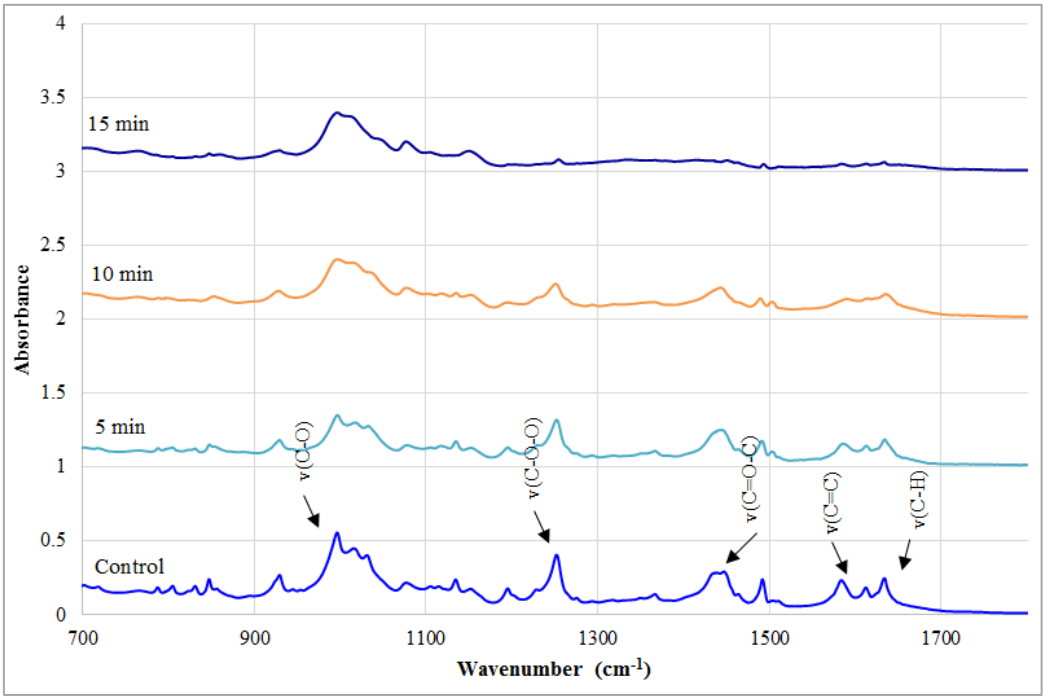

Figure 4. ATR-FTIR spectra of black pepper seeds after GAPD Treatment.

Table 4. *Variation of piperine and volatile oil content after GAPD treatment.*

Treatment	Piperine (w/w%)	Volatile Oil Content (ml/g)
Control	7.72 ±0.19[a]	0.029 ±0.001[a]
5 min	7.24 ±1.28[a]	0.032 ±0.003[a]
10 min	6.51 ±0.95[a]	0.037 ±0.001[a]
15 min	6.57 ±0.68[a]	0.032 ±0.001[a]

Data is expressed as mean ± SEM, n = 3. Means followed by the different letters indicate significant differences at $p<0.05$ between the data within the same columns

The changes in the volatile oil content were not significant, even though a decrease was shown after 10 minutes of GAPD treatment time. Microchannels created during the treatment may have facilitated the extraction of volatile oils. Thus, it is a favorable observation of the GAPD treatment. Similar findings have been obtained for lemon verbena and lemon peel, resulting in loss of volatile oils, using low-pressure cold plasma treatment and dielectric barrier discharge treatments respectively [51, 52].

Figure 4 shows ATR-FTIR spectra obtained before and after GAPD remote treatment. Within the alkaloids found in black pepper seeds, most infrared peaks align with distinctive peaks associated with piperine. In the mid-infrared spectrum, distinct bands of piperine can be observed at 1633 cm^{-1}, 1610 cm^{-1}, 1582 cm^{-1}, 1252 cm^{-1}, and 997 cm^{-1} [53]. Major peaks appeared in the fingerprint region of ATR-FTIR spectra from 500 cm^{-1} to 2000 cm^{-1} which changed after 15 minutes of treatment. Peaks specific to v(C-H), v(C=C), v(C=O-C), v(C-O-O), and v(C-O) were altered after 15 minutes of treatment confirming that prolonged treatment times could lead to chemical structural changes in the surface of black pepper seeds with respect to the IR peaks of piperine. These changes can be correlated with reduced piperine content with the treatment time (Table 4). Therefore, 10 minutes of GAPD can be recommended as a suitable treatment time to decontaminate the natural contaminations of the black pepper seeds.

Considering the microbial inactivation levels and changes in physical, chemical, and structural properties, 10 minutes of treatment can be selected as the best treatment time for microbial decontaminating black pepper seeds.

4. Conclusion

A few novel microflorae connected to black pepper seeds have been discovered, prompting intrigue regarding the non-pathogenic nature of the identified bacteria. Conversely, molds are susceptible to pathogenicity, posing contamination risks and physical degradation to the black pepper seeds. A 10-minute treatment time for decontaminating black pepper seeds using GAPD was adequate, with minimum changes in quality parameters (piperine content and volatile oil content). Ten minutes of treatment reduced aerobic bacteria by 69% and yeasts and molds by 92%, reducing the risk of potential hazards and the physical degradation of black pepper seeds during shelf life. The decimal reduction time of *Bacillus cereus* (ATCC 11778) inoculum was 4.35 minutes in GAPD remote treatment. After 10 minutes of GAPD treatment time, a statistically significant ($p<0.05$) reduction in moisture and water activity was observed while reporting no statistically significant ($p<0.05$) changes in both volatile oils and piperine content, emphasizing the minimal damage to significant quality attributes. It can be suggested that further research be conducted with low initial contamination levels, possibly complying with Good Agricultural Practices and Good Manufacturing Practices to achieve high microbial inactivation levels with GAPD. Additional research is required to analyze the potential variations in the microbiological quality during the shelf life. The results of this study would be helpful in scaling up research and development activities in either batch-mode GAPD reactors (e.g., Rotary arc discharges) or continuous-mode GAPD reactors (e.g., an array of arc discharges along with a conveyor) using GAPD-focusing industrial applications on microbial decontamination of black pepper seeds.

Abbreviations

DBD	Dielectric Barrier Discharge
GAPD	Gliding Arc Plasma Discharge
ATCC	American Type Culture Collection
NCBI	National Centre for Biotechnology Information
MYP	Mannitol Egg York Polymyxin
SEM	Scanning Electron Microscopy
AOAC	Association of Official Analytical Chemistry
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy
FDA	Food and Drugs Authority
ASTA	American Spice Trade Association
CFU	Colony Forming Units
DNA	Deoxyribonucleic Acid
UV	Ultraviolet

Acknowledgments

The collaborative research initiative spanning from 2017 to 2019, titled “Indo Sri Lanka Joint Research Project (FP/125),” brought together the Industrial Technology Institute (ITI) under the State Ministry of Technology, Government of Sri Lanka, and the Institute of Chemical Technology (ICT) in Mumbai, operating under the Department of Science, Government of India.

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Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Data Availability Statement

The data of the this study are available and can be provided through a request to the corresponding author.

Funding

Indo Sri Lanka Joint Research Project (FP/125)," brought together the Industrial Technology Institute (ITI) under the State Ministry of Technology, Government of Sri Lanka, and the Institute of Chemical Technology (ICT) in Mumbai, operating under the Department of Science, Government of India (2017 - 2019).

Authors would like to mention the financial support TG 21/222, the treasury, Sri Lanka granted project "Development of novel juice extraction technique for high-quality extracts from underutilized agro-based commodities, extract-based fermented/powder products" to the Food Technology Section of Industrial Technology Institute.

Conflicts of Interest

The authors declare no conflicts of interest.

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