



Strategic Management of Inactivation of Food borne Pathogens on Nuts by Non-Thermal Plasma

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Abstract: Nuts a great source of vital vitamins, minerals and bioactive substances, and their consumption is increasing globally. In the present study, Walnut, Cashew and Almond samples were collected from local market of District Islamabad to optimize bacterial isolation protocol and use the cold plasma technique to reduces these pathogens from the surface of nuts. Bacteria were characterized by colony morphology/shape, biochemical tests and Gram. Six bacterial species were identified through gram staining and by chemical testing. All the nut (walnut, cashew and almond) samples were treated with Surfaguide microwave (MW) plasma device for 10, 15, 30 second and for 1 to 2 minutes. The gas flow rate range was (1.5-1.7 SCCM), plasma generating power (0.38,0.7,1.21 kW) and treatment time was 15s-2min. Among all of these three samples, after the treatment shown the reduction of bacterial colonies. After 10 and 15s treatment time, minimal bacterial reduction rate was observed in the three tested nut samples. At 30s treatment time walnut sample, almond samples, and cashew samples showed 4.36 log reduction, 1.70 log reduction, and 1.81 log reduction respectively. Scanning electron microscopy (SEM) was used to check the surface morphology of nuts. Thirty seconds treatment of non-thermal plasma (NTP) did not affect nuts morphology, and one-minute NTP treatment affected the nut samples morphology. This study proved that Surfaguide Cold Plasma efficiently reduces surface bacterial count on walnut, almond and cashew at 30 seconds NTP treatment time without effecting nuts physical morphology.

Keywords: MALDI-TOF MS, Gram Staining, Biochemical Tests, Colony Morphology, Scanning Electron Microscopy (SEM), Log Reduction, SCCM (Standard Cubic Centimeters per Minute), Plasma Power (kW).

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Introduction

Almonds, cashews, and walnuts are the three tree nuts that are most frequently farmed around the world. Foodborne microorganisms are the source of many diseases, which has a significant effect on both the economy and human health. Since the traditional hazard-based approach to food safety management systems has been shown to be insufficient, prominent academics and organisations now advocate for a risk-based approach to food safety. Accordingly, a food safety management system should be designed to identify and implement mitigation actions to control and reduce the health risks associated with food consumption after analysing those risks (Gama et al., 2018).

On the other hand, less research has been done on preventing food-borne illnesses without sacrificing food quality. It

was commonly believed that both raw and processed edible tree nuts contained foodborne pathogens, such as moulds, aerobic bacteria, and yeasts. *Bacillus cereus* and *Escherichia coli* are the two primary bacteria that infect nuts and are known to cause diseases that are spread through them (Akbas & Ozdemir, 2006). *Escherichia coli*, which can persist for long periods of time in unfavourable settings, is responsible for almost 90% of food poisoning cases (Khalili et al., 2018). *Salmonella* and *Escherichia coli* O157:H7 are the most harmful pathogenic bacteria found in nuts, although less harmful pathogens include *Clostridium* spp., *Pseudomonas*, and *Xanthomonas* (Atungulu & Pan, 2012). Numerous post-harvest contamination pathways have been proposed. In California, motorised tree shaking is mostly utilised for the collection of almonds and walnuts between mid-August and October. Additionally, during the drying process, they are



mechanically lifted from the ground, increasing the risk of contamination in the unlikely case that the soil contains *Salmonella* (Janse, 2010). Based on all of the information shown thus far, NTP appears to be a potent and useful decontamination and disinfection technique.

Given the aforementioned characteristics, which have been thoroughly described in a number of publications, NTP undoubtedly has a great potential for use in biotechnology (Scholtz et al., 2015). Non-thermal technology for antibacterial food and nut treatment is effective. These methods have low financial and environmental costs, and cold plasma can reduce *Salmonella*, *E. coli*, and *Aspergillus* without affecting the quality of the fruit being treated. It also extends the shelf life of food goods (Sánchez-Bravo et al., 2022). There haven't been many published studies on the safety of non-thermal plasma technology up to this point. Reports state that following prolonged exposure to plasma, a surge in peroxidation produced the bitter flavours of nuts. Long-term exposure can damage plasma's lipid content and cause it to oxidise into aldehydes, keto acids, and shorter-chain fatty acids. Long-term plasma exposure may also be significantly influenced by antioxidant molecules. A reduction in fruit firmness and browning effects are also observed in some cases (Hati et al., 2018).

NTP is primarily used for surface sterilizing in the food processing industry, food packaging, seed germination, changing the biochemical characteristics of grains and flour, eliminating pathogens, improving taste and smell and modifying packaging materials to extend shelf life (Misra et al., 2011). The objective of this study is to use standardised procedures and parameter exploration to create and refine a pilot-scale non-thermal plasma treatment method for drying and microbial decontamination of nuts, guaranteeing significant bacterial reduction while maintaining their nutritional, physical, and sensory attributes.

Literature Review

Over the past few decades, there has been a steady rise in the number of research indicating a connection between nut consumption and a decreased risk of serious chronic disorders. Dietary fibre, found in nuts, has been associated with a decreased risk of cardiovascular disease and obesity (Gonçalves et al., 2023). Consumption of raw agricultural goods has been connected to outbreaks of disease. Bacterial infections are believed to have a significant impact on food safety. Most reporting countries identify *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* O157:H7 as the primary bacteria of concern (Palareti et al., 2016). According to Mañas and Pagán (2005), a food must be treated as much as necessary to ensure its safety against germs while minimising alterations to its sensory, nutritional, and functional properties. Fruits, spices, nuts, and other goods are typically surface disinfected using sterilisation techniques including heat and chemical solutions, which are often time-consuming, damaging, or leave dangerous residues. Cold plasma's pathogen-inactivating capabilities might offer a step in the fresh produce processing process that reduces the microorganism burden without sacrificing nutritional content or other crucial characteristics (Muranyi et al., 2007). The first bio-decontamination by non-thermal plasma tests were carried out in the beginning of the 1990s with the aim of identifying alternative methods for the disinfection of heat-sensitive compounds. When contrasted with risky methods such as

the use of noxious gas, the results appeared to be quite promising (Moreau et al., 2008).

According to rules established by the US Food and Drug Administration (FDA), processors must cut the amount of the most resistant bacteria in their final goods by five logs. Fascinatingly, research conducted at the Commonwealth Scientific and Industrial Research Organization's (CSIRO) Australian Food and Nutritional Sciences division has revealed that bacteria can be decreased by as much as five logs after just a few seconds of coming into touch with cold plasma. Similar results have also been obtained from numerous additional investigations (Misra et al., 2011). The inactivation rates of three foodborne pathogens—*Salmonella* Typhimurium, *Escherichia coli* O157:H7, and *Listeria monocytogenes*—were examined in sets of fresh fruits, vegetables, nuts, and powdered goods in response to cold plasma treatment. For each pathogen, several strains were evaluated. Following infection with the food-borne pathogens, samples were exposed to cold atmospheric plasma for a maximum of 20 minutes. As the length of treatment increased, there were differences in the amounts of microbial decline (Manuscript, 2018). Many studies have been conducted on the effects of cold plasma as a microbial inactivator in almonds. Deng et al. reduced *E. coli* by 5 log cfu/g using 30-s treatments at a voltage level of 30 kV and 2 kHz (Sánchez-Bravo et al., 2022).

Bacillus subtilis and *Clostridium* spores were exposed to oxygen gas plasma in order to render them inactive. These bacteria were injected into strips, which were then exposed to gas plasmas at two different power levels (50 and 200 watts) for three exposure intervals (five, thirty and sixty minutes). For both bacteria, spore logarithmic reductions (SLR) in a 50-watt plasma were more than 3.5 for a 60-min exposure and greater than 3.4 for a 30-min exposure. According to this study, bacteria resistant to conventional disinfection methods can be rendered inactive by employing oxygen gas plasma (Nelson & Berger, 1989). Niemira investigated how well three isolates of *Salmonella* and *E. coli* that had been injected on almonds were eliminated using pulsed cold plasma. A 20-second treatment at 6-cm intervals resulted in a significant 1.34 log CFU decrease in the *E. coli* O157:H7 C9490 strain. Nitrogen as a source gas has less antibacterial action than dry air, and longer treatment times did not always result in larger decreases (Prakash, 2013). Shirani et al. investigated the effects of plasma on peeled and sliced almonds using a 17 V and 2.26 A current for 5, 10, 15, and 20 minutes. Following a 20-minute argon cold plasma jet treatment, the total number of almond pathogens was reduced by 2.95 log cfu/g, mould and yeast by 1.81 log cfu/g, and *Staphylococcus aureus* by 2.72 log cfu/g (Sánchez-Bravo et al., 2022). According to Hati et al. (2018), *Bacillus subtilis* counts were shown to drop by two logs when exposed to O₂ and by five logs when subjected to a combination of O₂ and CF₄ in a plasma therapy.

SEM, or scanning electron microscopy, was used to study the physical change. The almond's morphology following gliding arc treatment was investigated using electron microscopy. They discovered that for up to four minutes following processing, there were no discernible changes to the surface colour or structure (Khalili et al., 2018).

The strategic control of microbial inactivation from the surface of nuts is a crucial area of study in the context of food

safety and public health. Few reports of outbreaks caused by contaminated nuts are recorded, and the microbial relationship with nuts is not as well explored in terms of food safety protocols. The absence of cutting-edge technology is Pakistan's largest issue. Successful and economical results can be achieved with early diagnosis and efficient treatment techniques. Numerous time-consuming techniques, such as thermal, chemical, roasting, boiling, UV, and heating procedures, affect the morphology and nutritional value of nuts. The main issue these days is the growing demand for high-quality, safe food.

Materials and Method

Adequate number of nuts samples (Almond, cashew and walnut) were collected from local market (commercial) of Rawalpindi in sterilized bags. Date and area were noted in the paper. After this half of walnut, cashew and almond sample were placed in safety cabinet to check the naturally occurring microbial contamination on them and half of walnut, cashew and almond sample were placed in sterile petri plates to decontaminate them by NTP treatment. Take caution when handling the samples to prevent contamination. Put on gloves to stop any contamination from spreading by our hands. To preserve the samples' integrity, seal them inside sterile bag or autoclaved petri plates.

Luria-Bertani (LB) broth and LB agar media were used for culturing bacteria. Media preparation was done by using earlier enhanced procedures. For bacterial isolation from nuts the samples were dipped into the LB broth in test tubes that were covered with aluminum foil. After that they were placed in a shaking incubator at 110 rpm and 30 to 37 °C for 24 hours.

S.No.	Ingredients	Concentration
1.	NaCl	1 g
2.	Peptone	1 g
3.	Yeast	0.5 g
4.	Distilled water	100 ml
5.	Ph	7

Table 1. Concentration of ingredients for 100ml of LB broth media

In order to prepare LB agar media, ingredients used were yeast, peptone, agar, sodium chloride and distilled water, also the pH was adjusted to 7. At that time media was autoclaved for about 60 minutes at 121 °C and 15 psi pressure after its preparation. After this media was allowed to cool down at normal room temperature and then poured it into the autoclaved petri plates inside the laminar flow to avoid the contamination.

S.No.	Ingredients	Concentrations
1	Yeast	0.5 g
2	Peptone	1 g
3	LB agar	1.5 g
4	NaCl	1 g
5	Distilled water	100 ml
6	pH	7

Table 1. Concentration of ingredients for 100 ml of LB agar media preparation

Serial dilutions method was used for the isolation of bacteria from nuts. 1 g of nut was combined with 9 ml of LB broth and vortexed for 10–20 minutes in order to separate bacteria from nut samples. In order to perform serial dilutions, 1 ml of LB broth was transferred from one test tube to the next, repeating the process for three to four tubes. To observe bacterial colonies, 100 µl aliquots from each dilution were plated on agar and incubated for 24 hours at 30–37 °C. To ensure pure culture growth, distinct bacterial colonies were chosen, streaked on LB agar plates, and then incubated for 24 hours. Bacterial preservation involved inoculating isolated bacteria into sterile LB broth, shaking the mixture at 37°C, and then preserving it in autoclaved Eppendorf tubes at 4°C by combining the bacterial suspension with 50% glycerol (1:1). The following formula was used to count colonies on plates containing 25–300 CFUs: $\text{Microbes/ml} = (\text{colonies} \times \text{dilution factor}) / \text{volume plated}$

Gramme staining was performed on a single colony that had been smeared on a slide and heat-fixed. A method of differential staining that helps identify bacteria by differentiating between gram-positive (purple) and gram-negative (red) bacteria using crystal violet, iodine, ethanol, and safranin.

The bacterial isolates were verified using biochemical tests. By forming bubbles, the catalase test determines whether the enzyme catalase is present. Catalase breaks down hydrogen peroxide (H₂O₂) into oxygen and water. One millilitre of H₂O₂ is diluted with fifteen millilitres of distilled water, and the volume is adjusted to twenty millilitres to create a 5% H₂O₂ solution. Using a sterile loop, a drop of 5% H₂O₂ is added to a dried slide along with a tiny amount of fresh bacterial culture. A catalase-positive organism is indicated by the immediate formation of bubbles. The Kovács reagent, which changes colour in 20 seconds if cytochrome oxidase is present, is used in the oxidase test to identify the enzyme in bacteria.

By creating distinct mass spectral fingerprints, MALDI-TOF MS is a quick, precise, and economical method of microorganism identification. Bacterial isolates are applied to target slides using VITEK MS, followed by matrix overlay, drying, and analysis. A confidence score is determined by comparing the resultant spectra with a database.

In present study Surfaguide microwave system (WR-340) was used to kill the foodborne pathogens from the surface of walnut, cashew and almond nuts. This equipment consists of following parts; power supply, Isolator, Stub Tuners, Surfaguide, Sliding short circuit and Flow meter and gas cylinder.

The experimental setup for bacterial deactivation on nut surfaces includes a magnetron powered by a source to generate microwaves, which are directed through a waveguide to a quartz reactor tube (20 mm thick). Plasma is generated in the tube using a mixture of argon and oxygen gases at flow rates between 1.5–1.7 SCCM and power levels from 0.38–1.21 kW. Nut samples in petri plates are placed beneath the plasma plume for surface decontamination.

The Surfaguide MW plasma device was used to treat almond, cashew and walnut samples for a range of times (10 seconds to 2 minutes) while maintaining controlled gas flow rates (1.5–1.7 SCCM) and power settings (0.38–1.21 kW). In order to track bacterial regrowth, samples were kept in sterile petri plates

for a month after treatment. Colony-forming units (CFUs) were counted before and after plasma exposure, and log reduction was used as a metric to evaluate the treatment's efficacy. Furthermore, surface morphology changes were examined using a Scanning Electron Microscope (SEM), which offers high-resolution structural insights.

Results was expressed as mean score standard deviation after analytical findings. And results were recorded triplicates to five times of every sample. Analysis of variance (ANOVA) was used to determine the statistical significance of the observed differences between their means.

Results and Discussion

Culturing was done on LB broth and LB agar medium. Plates were incubated at 37° for 24 hours. Isolated purified bacterial colonies from walnut sample round, small and white in color. The appearance of colonies was round, smooth and white color. Isolated purified bacterial colonies from the almond sample. The appearance of colonies was yellow smooth and rough white, while the size varied. Isolated colonies of bacteria from cashew sample was white and yellow color colonies that appeared after the purification of isolates of bacteria.

Each isolate of bacteria from nuts was grown on LB broth and then smear was made on autoclaved filter paper, then 1 to 2 drops of oxidase reagent was put on the smear. Walnut sample 1 isolate was negative for the oxidase test, and no color change was observed. Walnut sample 2 isolate was negative for the oxidase test, and no color change was observed. Cashew sample 1 isolate was positive for the oxidase test, and color change was observed. Cashew sample 2 isolate was positive for the oxidase test, and color change was observed. Almond sample 1 isolate was positive for the oxidase test, and color change was observed. Almond sample 2 isolate was negative for oxidase test and no color change was observed.

1 to 2 drops of 3 percent hydrogen peroxide were added to an isolated colony of bacteria from nuts sample on a clean glass slide and formation of bubbles was observed for a minute. Wa1 and Wa2 shows bacteria isolates from walnut were catalase negative, no bubble formation was observed. Ca 1 shows bacteria isolate from cashew sample was catalase positive, bubble formation was observed. Ca 2 shows bacteria isolate from cashew was catalase negative, no bubble formation was observed. Al 1 shows bacteria isolate from almond sample was catalase negative, no bubbles formation was observed. Al 2 show bacteria isolate from almond sample was catalase positive, bubbles formation was observed.

In the present study almost, similar results were found. Walnut, Cashew and Almond samples were treated with Surfaguide MW plasma device for 10, 15, 30 second and for 1 to 2 minutes. The argon and oxygen gas flow rate (1.5-1.7 SCCM), plasma generating power was (0.38,0.7,1.21 kW) and treatment time (15s-2min) were ranging. The reduction of bacterial colonies was observed in all three of these samples following the treatment. None of the three nut samples showed a significant decrease rate after the 10- and 15-second treatment periods except walnut. Without changing their morphology, walnut samples showed a 4.36 log reduction after 30 seconds of treatment, almond samples showed a 1.70 log reduction, and cashew samples showed a 1.81 log reduction. All three samples showed morphological changes

but no growth after a one-minute NTP treatment. According to this study, Surfaguide cold plasma was effective in lowering the number of bacteria after a short treatment period without affecting the physical morphology of the nuts, and longer treatment times had an impact on the bacterial growth and very small effect on the morphology of the sample. Statistical analysis of five time repeatedly experiments showed the results that bacterial population on nuts sample significantly reduced after 30s and 60s treatment time.

Conclusion

The present study concludes that 30 seconds NTP treatment on nuts (walnut, almond and cashew) for surface decontamination reduced bacterial growth efficiently 99.99% (4.36log reduction) from walnut surface and 90% (1.70log reduction and 1.81) from almond and cashew surface. The study results found that 10s and 15s treatment on nuts showed a significant reduced bacterial growth from walnut while, cashew and almond sample showed non-significant reduction. And one minute treatment completely inhibited bacterial growth on the studied nuts but affected nuts surface morphology/damage the surface as well. The 30 sec, treatment was most effective for dried nuts surface bacterial growth inhibition without affecting their morphology. It was the best scale setup for nuts drying and removal of microbes. Nuts can be preserved for long time through 30 sec NTP treatment without affecting their texture.

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