PLASMA-AIDED SURFACE DISINFECTION

FINAL REPORT

by

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List of Abbreviations

AC	Alternating current
AMR	Antimicrobial resistance
BOPP	Biaxially oriented polypropylene
CFU	Colony forming unit
DBD	Dielectric barrier discharge
DC	Direct current
DPI	Drexel Plasma Institute
HBSS	Hanks' balanced salt solution
LDPE	Low-density polyethylene
NTP	Nonthermal plasma
ROS	Reactive oxygen species
TSA	Tryptic soy agar

1 Introduction

The vast majority of people will experience some kind of foodborne illness throughout their lives. Due to the immense variety of food groups as well as production/preparation methods available, food safety is a highly complex issue. As food can possibly be contaminated at any given point during production, processing and transport, the sources of contamination are numerous. Most commonly the quality of food is negatively influenced by the unwanted introduction of potentially harmful bacteria, fungi, viruses, chemicals or toxins. [1]

Therefore, food safety is a topic of incredible importance. The absence of undesired and harmful contamination is of highest priority along the entire food production-, processing-, and distribution chain. As sub-qualitative food products would result in a large financial loss, companies are constantly on the lookout for novel strategies to guarantee the safety of their products.

The Drexel Plasma Institute (DPI) is conducting research on developing a potential method to ensure the disinfection of various food products using plasma. That way, potential health hazards can be inactivated without the use of any antibacterial- and/or antifungal agents or other aggressive chemicals.

1.1 Foodborne Diseases

In the U.S., a staggering 48 million people fall ill each year as a direct result of consuming contaminated food products, and more than 3000 die. Individuals who are at a particular high risk to develop a foodborne disease are the elderly, infants, pregnant women and immunodeficient people as their bodies are less efficient in clearing or fighting hazardous agents or contaminants introduced via food from their system.

Although those numbers are alarming, the main advantage of foodborne illnesses is the preventability thereof. By using stringently tested methods and applying

them without exceptions, these – sometimes fatal – diseases can be avoided completely.

While a lot of the most commonly encountered foodborne diseases are short-term in nature, food can also cause severe long-term health problems. Toxins, chemicals or heavy metals ingested with the food products can lead to an accumulation if the exposure lasts for an extended period of time. As a consequence, threshold limits for these substances may be exceeded in the body and serious reactions might follow.

Although food safety has always been a critical feature of the food production chain, it is steadily gaining importance as well as complexity. With the ongoing globalization and the more and more interconnected exchange of food-related products, food safety has to be guaranteed throughout several steps along the production chain. These days it is the absolute exception for the on-farm production, harvesting/slaughtering, processing steps, transport and distribution to happen under the supervision of one single. It follows that systems have to be developed to reliably set and meet quality standards within the interlinked processes. Another aspect that is influenced by the longer and more segregated food chain is the response time in case of any recall event. Should any complications arise, the contaminated food products are harder to recall and a possible disease outbreak investigation is much more difficult to conduct comprehensively.

Additionally, the endpoints of this production chain – the consumers – play a vital role as well. The wrong storage conditions, preparation methods and the consumption of expired or spoilt food is one of the most common causes for developing a foodborne illness. [1, 2]

1.1.1 Common Diseases and Sources of Contamination

To date, more than 250 different foodborne diseases have been identified. While the symptoms vary a lot from disease to disease, the common path is the ingestion of a contaminant and the entering of a microbe, virus, toxin or heavy metal

through the gastrointestinal tract. Frequently, the first signs of illness are therefore found in the digestive tract and may include nausea, vomiting, abdominal cramps and diarrhea. Table 1 includes a list of illness-causing microorganisms that frequently cause foodborne diseases in the United States of America. It becomes obvious that the sources of potentially hazardous contaminants are very diverse. Also, the experienced symptoms cover a wide spectrum, ranging from mild discomfort to life-threatening conditions. As mentioned before, the very young/old as well as sick individuals are at the highest risk of developing these diseases. Additionally, the symptoms experienced tend to be a lot more severe in these population groups. However, some of the listed organisms pose serious threats to otherwise perfectly healthy people also. [2]

Table 1. List of the most common foodborne illnesses, the organism, onset time after ingestion of a contaminated product, signs and symptoms, usual duration of illness as well as foo sources [3]

Organism	Common Name of Illness	Onset Time After Ingesting	Signs & Symptoms	Duration	Food Sources
Bacillus cereus	*B. cereus* food poisoning	10-16 hours	Abdominal cramps, watery diarrhea, nausea	24-48 hours	Meats, stews, gravies, vanilla sauce
Campylobacter jejuni	Campylobacteriosis	2-5 days	Diarrhea, cramps, fever, and vomiting; diarrhea may be bloody	2-10 days	Raw and undercooked poultry, unpasteurized milk, contaminated water
Clostridium botulinum	Botulism	12-72 hours	Vomiting, diarrhea, blurred vision, difficulty in swallowing, muscle weakness. Can result in respiratory failure and death	Variable	Improperly canned foods, especially homecanned vegetables, fermented fish, baked potatoes in aluminum foil
Clostridium perfringens	Perfringens food poisoning	8-16 hours	Intense abdominal cramps, watery diarrhea	Usually 24 hours	Mears, poultry, gravy, dried or precooked foods, time and/or temperature-abused foods
Cryptospoidium	Intestinal cryptosporidi-	2-10 days	Diarrhea (usually watery), stomach cramps, upset	May be remitting	Uncooked food or food contaminated

	osis		stomach, slight fever	and relapsing over weeks to months	by an ill food handler after cooking, contaminated drinking water
Cyclospora cayetanensis	Cyclosporiasis	1-14 days, usually at least 1 week	Diarrhea (usually watery), loss of appetite, substantial loss of weight, stomach cramps, nausea, vomiting, fatigue	May be remitting and relapsing over weeks to months	Various types of fresh produce (imported berries, lettuce, basil)
E. coli (Escherichia coli) producing toxin	*E.coli* infection (common cause of "travelers' diarrhea")	1-3 days	Watery diarrhea, abdominal cramps, some vomiting	3-7 or more days	Water or food contaminated with human feces
E. coli O157:H7	Hemorrhagic colitis or *E. coli* O157:H7 infection	1-8 days	Severe (often bloody) diarrhea, abdominal pain and vomiting. Usually, little or no fever is present. More common in children 4 years or younger. Can lead to kidney failure.	5-10 days	Undercooked beef (especially hamburger), unpasteurized milk and juice, raw fruits and vegetables (e.g. sprouts), and contaminated water
Hepatitis A	Hepatitis	28 days average (15-50 days)	Diarrhea, dark urine, jaundice, and flu-like symptoms, i.e., fever headache, nausea, and abdominal pain	Variable, 2 weeks - 3 months	Raw produce, contaminated drinking water, uncooked foods and cooked foods that are not reheated after contact with an infected food handler; shellfish from contaminated waters
Listeria monocytogenes	Listeriosis	9-48 hours for gastrointestinal symptoms, 2-6 weeks for invasive disease	Fever, muscle aches, and nausea or diarrhea. Pregnant women may have mild flu-like illness, and infection can lead to premature delivery or stillbirth. The elderly or immunocompromised patients may develop bacteremia or meningitis	Variable	Unpasteurized milk, soft cheeses made with unpasteurized milk, ready-to-eat deli meats
Noroviruses	Variously called viral gastroenteritis, winter diar-	12-48 hours	Nausea, vomiting, abdominal cramping, diarrhea, fever, headache. diarrhea is more prevalent	12-6- hours	Raw produce, contaminated drinking water, uncooked foods

	rhea, acute non-bacterial gastroenteritis, food poisoning, and food infection		in adults, vomiting more common in children		and cooked foods that are not reheated after contact with an infected food handler; shellfish from contaminated waters
Salmonella	Salmonellosis	6-48 hours	Diarrhea, fever, abdominal cramps, vomiting	4-7 days	Eggs, poultry, meat, unpasteurized milk or juice, cheese, contaminated raw fruits and vegetables
Shigella	Shigellosis or Bacillary dysentery	4-7 days	Abdominal cramps, fever, and diarrhea. Stools may contain blood and mucus	24-48 hours	Raw produce, contaminated drinking water, uncooked foods and cooked foods that are not reheated after contact with an infected food handler
Staphylococcus aureus	Staphylococcal food poisoning	1-6 hours	Sudden onset of severe nausea and vomiting. Abdominal cramps. Diarrhea and fever may be present.	24-48 hours	Unrefrigerated or improperly refrigerated meats, potato and egg salads, cream pastires
Vibrio parahaemolyticus	*V. parahaemolyticus* infection	4-96 hours	Watery (occasionally bloody) diarrhea, abdominal cramps, nausea, vomiting, fever	2-5 days	Undercooked or raw seafood, such as shellfish
Vibrio vulnificus	*V. vulificus* infection	1-7 days	Vomiting, diarrhea, abdominal pain, bloodborne infection. Fever, bleeding within the skin, ulcers requiring surgical removal. Can be fatal to persons with liver disease or weakened immune systems.	2-8 days	Undercooked or raw seafood, such as shellfish (especially oysters)

An additional aspect to be considered is the growing threat of antimicrobial resistance (AMR). Often times, antimicrobials are overused and misused in agriculture and especially in animal husbandry. The intensive use of antimicrobials is a standard strategy to minimize the financial loss attributed to diseased or dead live-

stock. These conditions facilitate the emergence of multi-resistant bacteria that are then transmitted to humans via food. As a consequence, previously effective treatments become less and less effective, acquired infections persist and might spread to others. [4-6]

1.1.1.1 *Penicillium* genera

Most famously known as the first producers of antibiotics, *Penicillium* spp. are mesophilic fungi, which means their growth optimum lies between 20-30°C at a pH of 3-4.5. More than 200 species of *Penicillium* have been identified, 20 of which are regularly found in the indoor environment. Most commonly, *Penicillium* growth can be observed in soil, on decaying vegetation, fresh produce as well as on dry food products, such as bread.

The colonies are typically fast growing, flat in shape and mostly consist of a dense felt. Initially they growing mold colonies may appear white, whereas the color changes to shades of blue and green at maturity. The mycelium of *Penicillium* spp. typically consists of a large amount of multinucleate, septate hyphae that form a highly branched network. Conidiophores sprout from the mycelia, which bear individually constricted conidiospores. The spores represent the main dispersal route of the fungi, are often blue/green in color and are responsible for the characteristic appearance of the mold (see Fig. 1). [7-9]

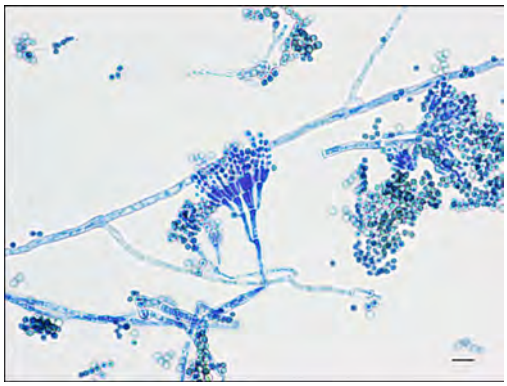


Fig. 1. Microscopic picture of Penicillium *spp. showing the highly branched network of hyphae, canidiophores and conidiospores [10]*

Penicillium species are among the main causes for the spoilage of food products. Due to the mold's ability to thrive in low humidity conditions and their rapid colonization by aerial dispersion, the control and inactivation of *Penicillium* is a major issue in food safety. [11]

1.2 Common Methods to Ensure Food Safety

Stemming from the diverse sources of contaminant commonly found in food products, numerous different approaches to minimize contamination have been developed. These measures have to be implemented at each and every step along the food production chain to ensure the safety of the end product.

Cleaning and disinfecting play an essential role in achieving this goal. Cleaning is defined as the "complete removal of residues and soil from surfaces, leaving them visually clean so that subsequent disinfection will be effective". [12]
Per usual, detergents are used to remove soil (a mixture of food waste and bacteria) from the surface of all processing equipment, floors or walls. The use of detergents separates the soil from the surface in order to allow the residues to be rinsed off or removed in any other way appropriate.

Disinfection on the other hand is the "process by which microorganisms are killed so that their numbers are reduced to a level which is neither harmful to health nor to the quality of perishable goods". [12]

Disinfection takes place after cleaning, where any soil has already been removed, but microorganisms remain. The mode of action with which a disinfectant inactivates microorganisms depend on the active ingredient of the disinfecting agent and has to be chosen in consideration of various parameters (e.g. compatibility with materials to be treated, required concentration, toxicity, required temperature).
There must not be any remaining residues of the cleaning- and disinfection agents used after the entire cleaning process is completed to prevent the transmission of undesired chemicals onto food products. [12]

Another main concern in food processing facilities is the formation of biofilms. Biofilms are communities of bacterial cells that adhere to both surfaces and each other and they produce glue-like materials (polysaccharides) to hold together and for additional protection. What makes biofilm a major issue is the fact that their presence severely impairs the adequacy of cleaning and disinfection efforts and in turn leads to even more contamination. [13, 14]

Apart from cleaning and disinfection product contact surfaces, the products themselves often require similar treatment as well. Again, the method has to be selected with respect to the product to be cleaned/disinfected. The goal is to achieve sufficient results (microorganism counts low enough as to not pose a health threat), while keeping any potential negative impact on the quality of a product to a minimum.

Leafy greens, such as spinach or lettuce, are commonly washed extensively before being packaged and distributed to vendors. These washing steps, however, often include bleach rinses or irradiation. Despite those aggressive measures taken by the food processing facilities, the effectiveness is comparably low. A vast majority of outbreaks involving leafy greens can be traced back to issues in the disinfection process. [15]

Other food products do not require the use of chemicals to meet the safety criteria. Milk, for example, is treated by pasteurization. Heat is applied in order to destroy pathogens for a defined amount of time to ensure effective inactivation. The most commonly method of pasteurization used in the U.S. is High Temperature Short Time pasteurization, during which the milk is heated to at least 161°F/72°C for no less than 15 seconds, before being cooled down rapidly. [16]

1.3 Plasma

Plasma describes one of the four fundamental states of matter, the other three being solid, liquid and gaseous. The most basic distinction among solids, liquids and gases is the difference between the strength of the bonds that hold their particles together. These forces are relatively strong in a solid, weak in a liquid and essentially non-existing in a gas. Whether a substance if present in one of the mentioned states of matter depends on the random kinetic energy (thermal energy) of the atoms or molecules making up the substance. By applying heat to a substance in solid or liquid phase, the atoms/molecules of the substance acquire more and more thermal kinetic energy. At one point, they are able to overcome the binding energy, which leads to a phase transition. A gas is formed.

If a sufficient amount of energy is provided, those particles – whose thermal kinetic energy now exceeds the molecular binding energy – start to collide at an increasing frequency until eventually the molecular gas gradually dissociated into an atomic gas. Now, the outermost orbital electrons are able to overcome their binding energy to the rest of the atom by collisions. The result is an ionized gas, or plasma. Simply put, plasmas are a gas-like mixture of negatively charged electrons and highly charged positive ions, whose overall charge is roughly zero (see Fig. 2).

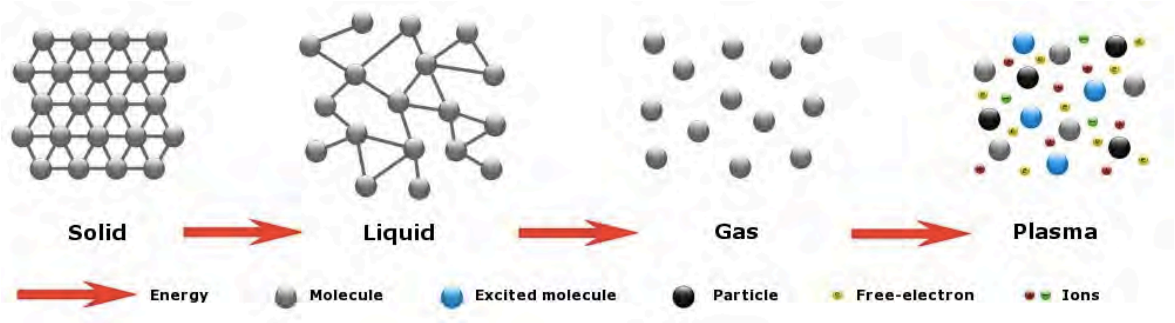


Fig. 2. Transition through the four states of matter by addition of energy [17]

Plasmas can be generated by raising the temperature of a given substance until a high fractional ionization is achieved. They can also be produced by several ionization processes that raise the degree of ionization highly above the thermal equilibrium value of the substance. Depending on the method of plasma generation, the resulting ionized gas can possess a large variety of characteristics, such as high/low temperature, density or stability. They have properties unique to plasmas, including the response to magnetic fields and high electrical conductivity. [18, 19]

1.3.1 Degree of Ionization

As the term 'ionized gas' indicates, some extent of ionization is necessary for plasma to exist. The degree of ionization describes the proportion of atoms that have lost or gained electrons and is mainly controlled by the temperature. A gas does not have to be fully ionized to qualify as plasma – a gas in which as few as 1 in 100 particles are ionized, already displays the characteristics of plasma. The degree of ionization of a plasma α is described as follows:

$$\alpha = \frac{n_i}{n_i + n_n}$$

where n_i is the number density of ions and n_n is the number density of neutral atoms.

A plasma is commonly referred to as "hot" if it is nearly fully ionized, or "cold" if the fraction of the ionized molecules is very small. [18]

1.3.2 Thermal and Nonthermal Plasmas

The classification of plasma to be thermal or nonthermal solely depends on the relative temperatures of the electrons, ions and neutrals. When talking about thermal plasmas, the electrons and heavy particles of an (partially) ionized gas are at the same temperature, meaning they are in thermal equilibrium with each other.

On the other hand, nonthermal plasmas have the neutrals and ions at a much lower temperature than the electrons, i.e. they are not in thermal equilibrium with each other.

Typically, plasma temperature is measured in electronvolts or kelvins and is informally used as a measure of the thermal kinetic energy per particle of the (partially) ionized gas. [18, 20]

1.3.3 Artificial Plasmas In Laboratory Use

Technological plasmas that are used in technology are usually nonthermal plasmas (NTPs) and only a small part of the gas molecules is in an ionized state. Most of the artificially generated plasmas used in a laboratory setting are produced by the application of an electric and/or magnetic field to a gas.

The characteristics of the resulting plasma can be influenced by a number of variables, such as the type of power supply, the pressure under which it is generated or the degree of ionization.

These nonthermal or “cold” plasmas are able to exist and be sustained at room temperature and atmospheric pressure, which allows for potential applications in the medical field or food processing. [21, 22]

1.3.3.1 Examples of Artificial Plasma

Due to the fact that plasmas can be controlled and influenced in various ways, they display a rather large range in temperature and density. This enables a broad field of potential applications in research as well as in industry. Some examples of plasmas and their applications follow:

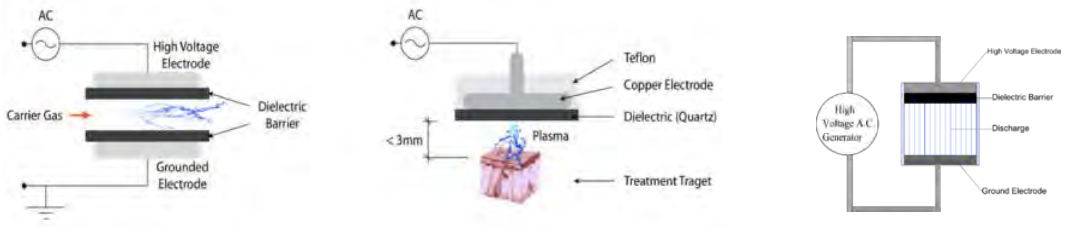
- Glow Discharge Plasma: A NTP generated by the application of a direct current (DC) power supply to a gap between two metal electrodes. This type of plasma is produced in fluorescent light tubes.

- Arc Discharge: A very high temperature, high power discharge (about 10.000 K) which is commonly used in metallurgical processes.
- Corona Discharge: A nonthermal discharge produced by the application of a high voltage to sharp electrode tips. It is commonly used in ozone generators or particle precipitators. [23-25]

1.3.3.2 Dielectric Barrier Discharge

Dielectric barrier discharge describes the electrical discharge between two electrodes separated by a dielectric barrier. They are a combination of the advantages of nonthermal plasmas with the relatively simple handling of atmospheric pressure operations. DBDs can be generated using several power supplies, such as AC-, micro- or nano-pulsed ones.

Devices producing dielectric barrier discharge can be set up in various configurations, the most common one being planar. Two parallel plated are separated by a dielectric barrier. With the application of a power supply, a discharge is then produced, which can bridge a few millimeters. Planar set-ups can also be modified in a way to omit one of the electrodes and use a target surface as a grounded element instead (Floating Electrode DBD). Another possibility of using DBD is by constructing a tubular system. A carrier gas is supplied with constant flow, sent through the discharges and the plasma-treated gas is then applied onto a target. These configurations are illustrated in Fig. 3. [21, 26]



a b c

Fig. 3. Different possible configurations of DBD systems. a) Tubular system supplied with carrier gas, which is treated by plasma; b) Floating Electrode DBD system modified to use a treatment target as a grounded element; c) Basic DBD set-up using two metal electrodes and a dielectric barrier [27, 28]

The generation of dielectric barrier discharge results in the generation of a multitude of reactive species, charged particles and UV photons. These components are the major players involved in the sterilization capabilities of plasma applications. Using plasma can inactivate a wide range of gram-positive- and negative bacteria, spores, biofilms, viruses and fungi. While a lot of the produced components have an influence on the sterilization powers, reactive oxygen species (ROS) seem to play the most important role. In an experiment comparing the D-values (time required to kill 90% of microorganisms) after plasma treatment with and without oxygen supply, the latter resulted in a higher value and therefore longer treatment time required to achieve the desired inactivation.

Considering these properties of dielectric barrier discharge systems, plasma-aided disinfection presents a potential solution to contamination issues in the food industry. The disinfecting potency and adjustability in parameters as well as application configurations of DBD set-ups are a highly interesting research field as a plasma-based device could potentially replace the use of antibacterial- and antifungal agents in the preparation of food products. In this thesis, research on replacing the adding of these chemicals in the production of bread with plasma-aided disinfection systems is described. [22, 29-31]

2 Materials and Methods

The overall goal of the conducted research project was to develop and evaluate plasma-based disinfection systems in order to eventually be able to replace the use of antibacterial and antifungal agents to bread. This way, the finished bread product can be disinfected, thereby prolonging the shelf life, and the consumers will not be ingesting any added chemicals.

To prevent any source of contamination along the production line, the plasma disinfection is intended to take place after the bread has already been placed inside the plastic wrapping and sealed shut.

The path leading to the desired outcome was organized into four project phases and research questions, wherein a project phase has to be completed in order to move on to the next phase.

Throughout the entire research work, the laboratory groups from the Drexel Plasma Institute and the company producing the bread (Campbell's Soup/Pepperidge Farm) were working together closely.

2.1 Phase I: Can plasma kill *Penicillium* spp.?

The first issue to be addressed in tackling the challenge of in-package bread disinfection was identifying the main contaminant during bread production. Personnel working at the company's main production site of bread were able to isolate *Penicillium* spp. spores. According to them, this is the strain that is responsible for most cases of spoilage of the bread produced. The Drexel Plasma Institute was supplied with a dilution of those spores, in order to be able to conduct experiments in house as well as at a site of Campbell's Soup.

As a first experiment to serve as a proof of concept, Rifampicin resistant *E. coli* was used as a model organism. Tryptic soy agar (TSA) plates were inoculated with approximately 10^5 colony forming units (CFUs) and subsequently treated with direct dielectric barrier discharge. The power supplies used in these experiments

varied in order to see any differences in the inactivation potency of the differently pulsed plasmas. The treatments times were in a range from 3s to 60s. After applying direct DBD treatment to *E. coli*, the dishes were incubated at 37°C over night. The next day, the now visible colonies were photographed and the area of inactivation visually observed and compared between the different power supplies.

As a next step, petri dishes were inoculated with mold spores. The dishes either contained tryptic soy agar or were left unfilled, i.e. the mold spores were applied directly to the 'dry' plastic. The two different concentrations of *Penicillium* spp. spores that were used were 10^2 and 10^4 per dish. An untreated control was included as well and the experiments were performed in duplicates. It should be noted that these concentration are much higher than any amount that might be found in a real-life scenario on bread.

The experiments were set up in way that the inoculated dishes were treated by directly exposing them to plasma and therefor to the generated reactive species. The treatment times were varied between 3s, 8s, 15s, 30s and 60s. A control was included that remained untreated and all experiments were performed in duplicates. The power supply used to treat the mold spores was micro-pulsed. The experimental set-up is shown in Fig. 4.

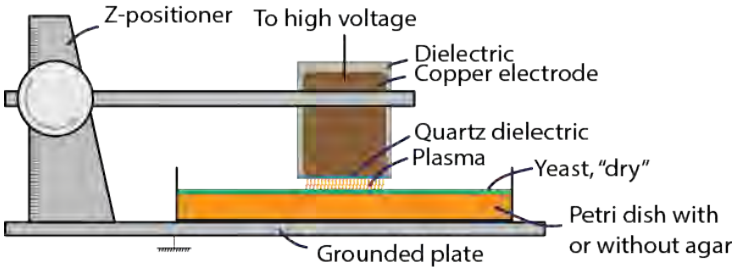


Fig. 4. Schematic of the experimental set-up used to treat both E.coli *and* Penicillium *spp. on either TSA plates or on 'dry' inoculated dishes*

The electrode that was used to generate plasma has a solid copper core with a diameter of 25mm, which is surrounded by an insulating layer made of Teflon. Serving as a dielectric barrier a thin quartz disc is used.

After subjecting the mold spore inoculated plates to direct DBD treatment, the dishes already containing TSA were incubated at 28°C (growth optimum for *Penicillium* spp.). The treated mold spores on the 'dry' inoculation plates were first transferred to TSA plates and then placed into the incubator as well. After about 3-5 days, visible mold growth was typically starting and the plates were photographed. The pictures were processed using ImageJ and the area of inactivation was plotted comparing the various treatment times.

2.2 Phase II: Does plasma treatment damage the bread?

One of the requirements from the bread producing company was not to change the bread in any form. Most importantly, the color of the crust of the bread was not to be altered. To assess any potential color changes plasma treatment causes on the bread, DPI was supplied with bread samples to conduct experiments on.

To compare the effect of different power supplies, AC-, micro- and nano-pulsed power supplies were tested. The loaf of bread remained unsliced and was treated using direct DBD (see Fig. 5). The time of treatment was varied from 10s up to 4min. Pictures were taken before and after every treatment. Using a digital gray card, the photos were color corrected in the program Lightroom and subsequently a square of 31x31 pixels was selected to derive the average *Lab* values in Photoshop. The *Lab* color space describes any color in three dimensions, *L* being lightness and *a* and *b* being the color opponents green-red and blue-yellow. The measured colors could then be digitally recreated and plotted.

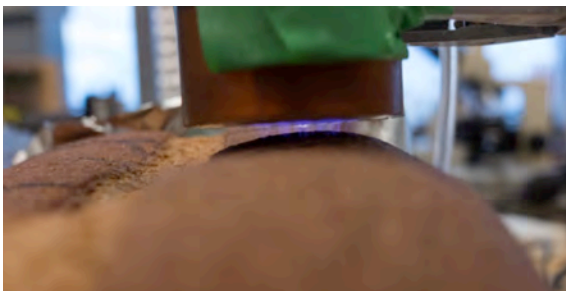


Fig. 5. Picture taken during the direct DBD treatment of an unsliced loaf of bread to assess any possible color change of the crust

2.3 Phase III: Can plasma be generated inside the packaging?

Bread produced by Campbell's Soup/Pepperidge Farm is packaged in a two-step procedure. First, the finished loaf of bread is wrapped in a completely clear plastic layer made from biaxially oriented polypropylene (BOPP). After this, the wrapped loaf is placed into an additional plastic bag that has the labels and nutritional information printed on. This outer bag is made from low-density polyethylene (LDPE). For better visualization, a completely packaged loaf of bread can be seen in Fig. 6.

Fig. 6. Loaf of bread that is completely packaged; wrapped in an inner layer of BOPP and placed into an outer bag of LDPE

Due to the fact that the plasma-disinfection step is intended to take place after the packaging of the loaves of bread, it has to be assessed if plasma can be generated within these packaging materials. Another important aspect is if the plasma treatment has any possible effect on the plastic layers. Since the inner and outer layer of packaging consist of two different types of polymers, it was important to check if the application of direct DBD has any negative impact on either layer. For this, DPI was supplied with the exact packaging materials that are used in production to conduct in house experiments. The two kinds of plastic were layered and subjected to micro-pulsed plasma treatment for a duration of 30s. Afterwards, any potential damage was visually assessed.

To confirm that plasma can indeed be generated within those two layers of plastic wrapping, an indicator for the presence of plasma – or the reactive species produced – was needed. The decision was made to use a dye named Indigo Carmine (see Fig. 7). The central double bond is oxidized upon the presence of plasma, which results in the rotation of the molecule. The formerly blue dye turns colorless. This reaction was used to confirm the presence of plasma inside of the packaging.

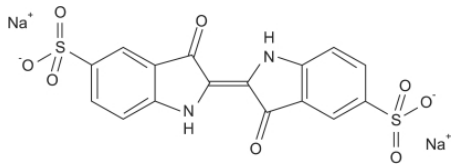


Fig. 7. Chemical structure of Indigo Carmine [32]

The powdered dye was diluted using distilled water to make a dark blue liquid. Small squares of white paper were dyed in a 2g/L Indigo Carmine solution and dried. Then, one of these dyed paper squared was used as a model for the bread, and layered with a square of the inner and outer packaging material each. To simulate possible air gaps, microscope slide with a thickness of 1mm were used. The electrode was placed on top of these layers and plasma was generated for 1.5 minutes. A schematic of the experimental set-up is shown in Fig. 8.

The discoloration of the blue paper square was assessed by taking pictures every 5s of plasma treatment. After color correcting the pictures using digital gray cards in Lightroom, the *Lab* values could be obtained in Photoshop and eventually recreated digitally.

The power supply used to conduct these color change experiments as well as the experiments testing for possible damage after the plasma treatment mentioned above was micro-pulsed in both cases.

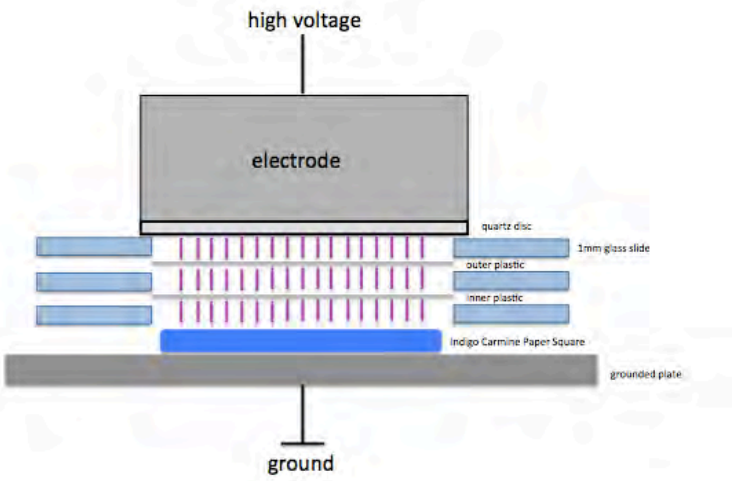


Fig. 8. Schematic of the experimental set-up used to confirm the presence of plasma within two different layers of packaging material

The treatment times used in these experiments are a lot longer than the expected time of plasma application in the bread production line. The intended duration of DBD treatment is under three seconds.

These long plasma treatment times were decided on to achieve a high degree of certainty in meeting the desired targets of not causing any damage to both of the different kinds of polymers (BOPP and LDPE), while still being able to generate plasma and the produced reactive species within the packaging.

2.4 Phase IV: Can plasma kill *Penicillium* spp. on bread?

To reach the final goal of having a plasma-based system capable of disinfecting packaged bread within a few seconds without altering the wrapping materials or the bread, the ability to kill *Penicillium* spp. on the bread itself has to be tested.

As in previous experiments *E. coli* exhibited an almost identical response to plasma treatments, it was decided to be used as a model organism for the first experiments. Small pieces of the crust of the bread – not containing any antibacterial or antifungal agents – were inoculated with a 10µl drop of a 10^4/mL *E. coli* solution. They were then treated with direct DBD using a micro-pulsed power supply. The treatment times ranged between 15s and 10min. Untreated controls and controls containing no bacteria were included. All experiments were performed in duplicates.

After the direct DBD treatment, the small pieces of crust were washed in 1mL of buffer (HBSS) and subsequently plated onto TSA plates. The plates were incubated over night at 37°C. The next day, pictures were taken and the colonies counted in order to plot the results.

Additionally, this experiment was repeated following the same protocol, but using a 10^3 *Penicillium* spore dilution. Instead of using HBSS as a washing solution, distilled water was used.

3 Results

3.1 Phase I: Can plasma kill *Penicillium* spp.?

As a first experiment to serve as a proof of concept, *E. coli* was used as a model organism. Approximately 10^5 CFUs were plated onto TSA plates and subsequently treated with direct DBD generated by three different power supplies (AC-, micro- and nano-pulsed). The treatment times were 3s, 7s, 20s and 60s. Untreated controls were included and the experiments were conducted in duplicates. After plasma treatment the plates were incubated at 37°C over night and the following day pictures were taken, which can be seen in Fig. 9.

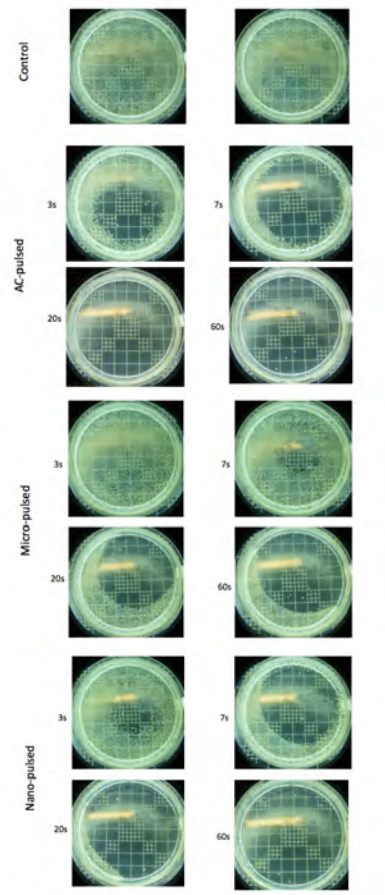


Fig. 9. Pictures of 10^5 E.coli *inoculated TSA plates following direct DBD treatment and incubation; AC-, micro-, and nano-pulsed power supply were used; treatment times were 3s, 7s, 20s, 60s, untreated control is included; n=2*

To investigate the inactivation capabilities of plasma in *Penicillium* spp. plates were inoculated under two different conditions (directly on agar and 'dry' on plastic) and in two different concentrations (10^2 and 10^4). Following direct DBD treatment for 3s, 8s, 15s, 30s and 60s the treated plates were incubated at 28°C until mold growth was visible. A micro-pulsed power supply was used. Untreated controls were included and the experiments were conducted in duplicates. The pictures of these plates can be seen in Fig. 10.

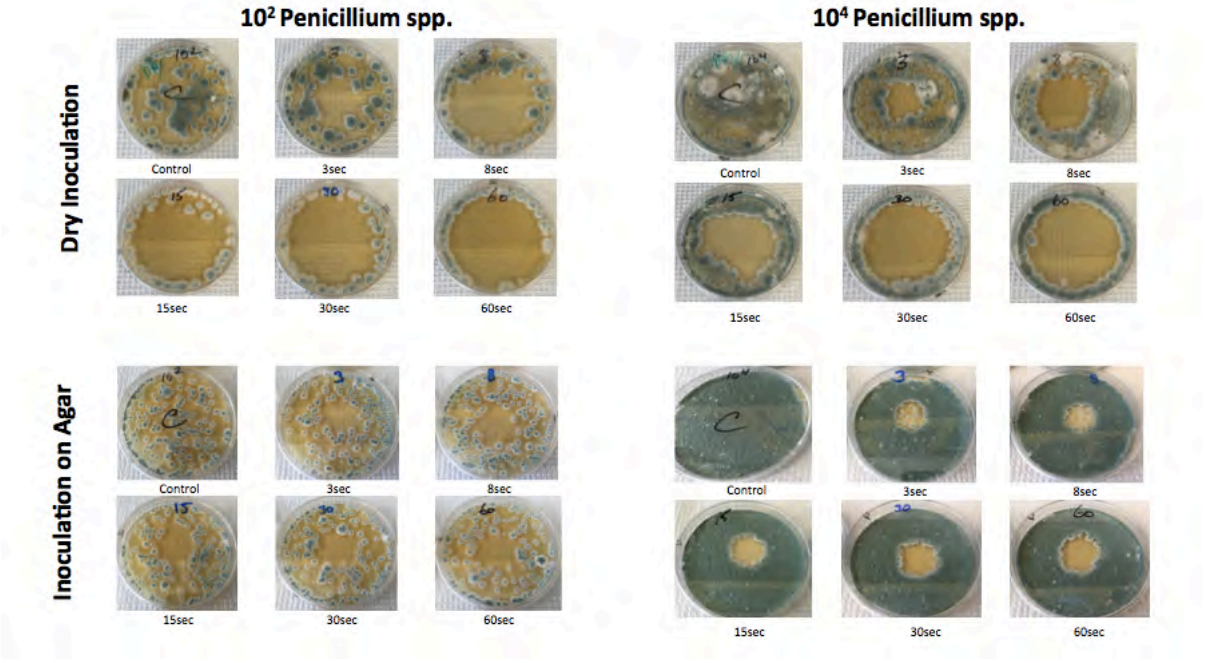


Fig. 10. Pictures of Penicillium *spp. inoculated plates following plasma treatment and incubation; two different inoculation procedures were used (inoculation on agar, dry inoculation); micro-pulsed power supply was used to generate direct DBD; treatment times were 3s, 8s, 15s, 30s and 60s as well as an untreated control; n=2*

To analyze the results obtained from these pictures, the observable area of inactivation, i.e. the area in which no mold growth is visible, was measured using ImageJ. The results were plotted comparing the different inoculation conditions as well as the used spore concentration. Additionally, the diameter of the electrode that was used to treat is indicated in order to determine if the area of inactivation is able to surpass the area of the electrode. The obtained graph is shown in .

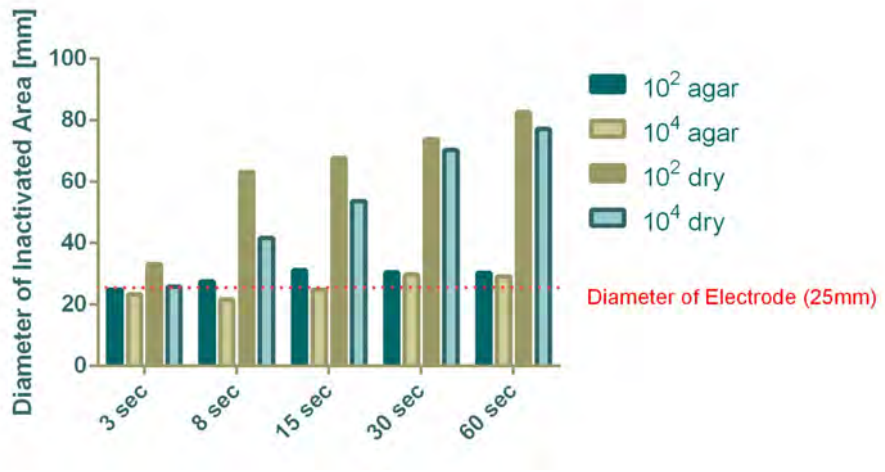


Fig. 11. The diameter of the inactivated area is plotted per treatment time, spore concentration used and inoculation method; the diameter of the used electrode is indicated as reference; a micro-pulsed power supply was used; n=2

After testing for ability of direct dielectric barrier discharge to inactivate both *E. coli* using three different power supplies and confirming these result in using the target microorganism *Penicillium* spp., the next phase of the research project was started. Knowing that the main contaminant of bread can be killed using a plasma-based disinfection system, the potential impact of the treatment on the bread itself had to be evaluated.

3.2 Phase II: Does plasma treatment damage the bread?

In order to determine if the plasma treatment of the bread has any negative effect on the bread itself, color analysis tests were conducted. An unsliced loaf of bread was treated with direct DBD for up to 4 minutes and pictures were taken before and after the treatments. With the help of digital gray cards the pictures were color corrected before the *Lab* values of a 31x31 pixel area were obtained and re-created digitally (see Fig. 12).

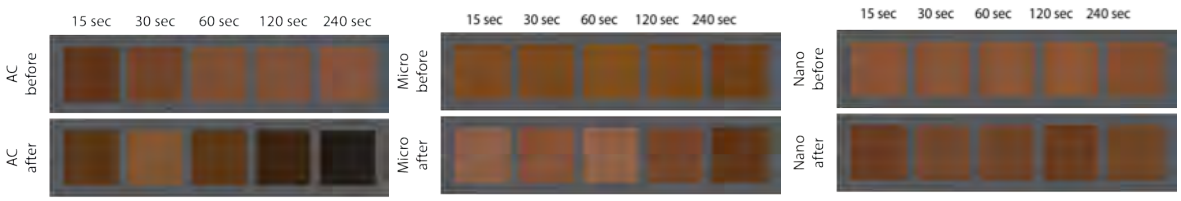


Fig. 12. Digitally re-created colors measured from the bread crust throughout direct DBD treatment; AC-, micro- and nano-pulsed power supplies were used; treatment times were 15s, 30s, 60s, 120s and 240s

Similarly to the experiments in Phase I, three different power supplies were used once again to generate direct DBD (AC-, micro- and nano-pulsed). The distance between the surface of the crust of the bread and the electrode was manually controlled due to the uneven surface inherent to bread.

3.3 Phase III: Can plasma be generated inside the packaging?

To confirm the generation of plasma and therefore the presence of the produced reactive species inside of the packaging materials used to wrap the loaves of bread, a detection system using the dye Indigo Carmine was used. The powdered dye was made into a 2g/L solution in which small squares of paper were dyed blue. Upon contact with plasma, the blue dye on the dried paper squares is chemically changed and turns colorless, i.e. the paper appears white again.

To confirm this theoretical assumption, dyed paper squares were treated with direct dielectric barrier discharge generated by a micro-pulsed power supply. Throughout the experiment pictures were taken in s 5 second interval for 1.5 minutes. With the help of digital gray cards the pictures were then color corrected using Lightroom and the Lab values measured in Photoshop afterwards. The exact color of the treated paper squared was then re-created digitally (see Fig. 13).

Fig. 13. Digitally re-created colors measured from the Indigo Carmine dyed paper squares throughout direct DBD treatment; pictures were taken every 5s up to 90s; micro-pulsed power supply was used

After assuring that Indigo Carmine is a suitable indicator for the presence of plasma, further experiments were conducted. The two types of plastic used by Campbell's Soup/Pepperidge Farm (BOPP and LDPE) were used to simulate the conditions under which the eventual plasma disinfection step would operate as a part of the production line. Squares from both kinds of wrapping materials were cut and then layered on top of the dyed paper square. Microscope glass slides were used to achieve an air gap of approximately 1mm between the different layers. The blue paper square was then treated with direct DBD through the two layers of plastic and the air gaps for 30 seconds. A micro-pulsed power supply was used. Various combinations and orders of the layering were tested, varying the plastics used and

the presence of air gaps. After the plasma treatment the two different polymers were visually inspected for any damage or change in the plastics and the paper square was inspected for a change of color (whitening). The results can be seen in Table 2.

Table 2. Impact of plasma treatment regarding decolorization of a dyed paper square and potential damage to BOPP and LDPE (inner and outer packaging material); micro-pulsed power supply was used to generate direct DBD; treatment time of 30s

Experiment Number	Layers (bottom/ground plate to top/electrode)	Decolorization (of dyed paper square)	Damage BOPP (inner layer of plastic)	Damage LDPE (outer layer of plastic)
1	paper – air – BOPP – air	✔	yes	-
2	paper – BOPP – air	✔	yes	-
3	paper – air – BOPP – air – LDPE – air	✔	no	no
4	paper – BOPP – air – LDPE – air	✔	no	no
5	paper – BOPP – air – LDPE	✔	no	no
6	paper – BOPP – LDPE – air	✔	no	no
7	paper – BOPP - LDPE	✔	no	no

3.4 Phase IV: Can plasma kill *Penicillium* spp. on bread?

As previous experiments showed an almost identical behavior of *E. coli* and *Penicillium* spp., the decision was made to start the experiments testing the plasma-aided inactivation of contaminants on bread with *E. coli*.

The Drexel Plasma Institute was supplied with unsliced loaves of bread that did not contain any added antifungal or antibacterial agents. From these loaves, small pieces of crust were cut off and inoculated with 10µl of a 10^4 *E. coli* dilution. The pieces of crust were then treated with direct DBD generated by a micro-pulsed power supply. The treatment times were chosen to be in a range between 15 seconds and 10 minutes. Following the plasma treatment, the crust pieces were washed in 1mL of HBSS and subsequently plated onto TSA plates. After an over night incubation at 37°C pictures were taken of the plates (see Fig. 14) and the colonies counted and plotted. Untreated controls as well as controls that were not inoculated with bacteria were included. All the experiments were conducted in duplicates.

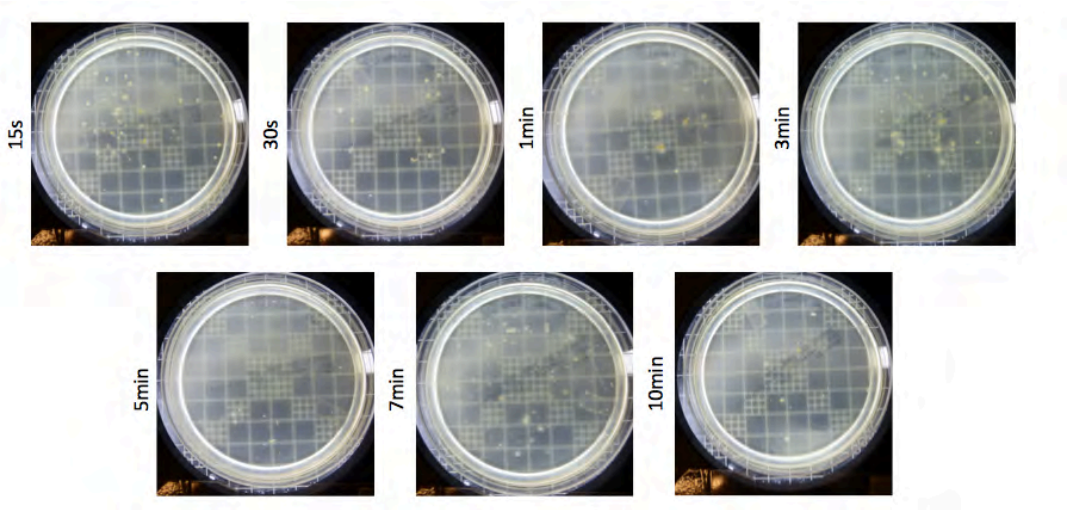


Fig. 14. Pictures of the transferred bacteria following direct DBD treatment on small pieces of crust; samples were inoculated with 10µl of a 10^4 E.coli *dilution; treatment times were 15s, 30s, 1min, 3min, 5min, 7min and 10min; micro-pulsed power supply was used*

It was noticed that the treated pieces of crust were starting to disintegrate during the washing step. Because of this it caused some difficulty telling colonies and bread residues apart on the photographs that were taken. For a better visualization of the inactivation following plasma treatment, the colonies were counted additionally and plotted (see Fig. 15).

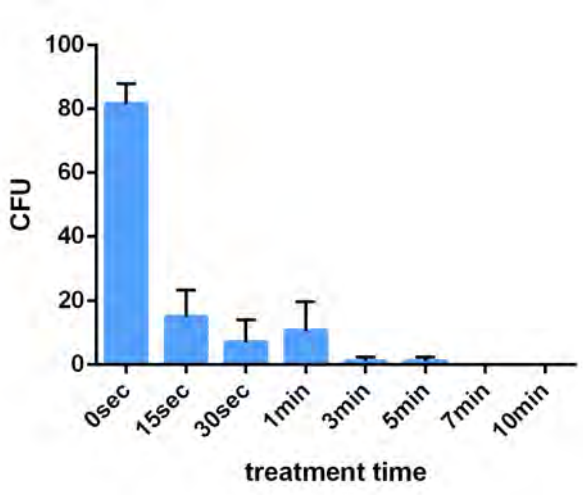


Fig. 15. CFUs observed after direct DBD treatment on crust and subsequent over night incubation; micro-pulsed power supply was used; treatment times were 15s, 30s, 1min, 3min, 5min, 7min and 10min; untreated control is plotted for reference; 10µL of a 10^4 E.coli *dilution were used for inoculation; n=2*

Following the experiments using E. coli as a model organism and confirming the suitability of the experimental protocol, the tests were repeated using *Penicillium* spp.

Some modifications were made, such as lowering the concentration of the microorganism dilution to 10^3. Also, the pieces of crust were washed in distilled water instead of buffer and the incubation was at 28°C and for about 3-5 days. The pictures taken after this incubation period can be seen in Fig. 16.

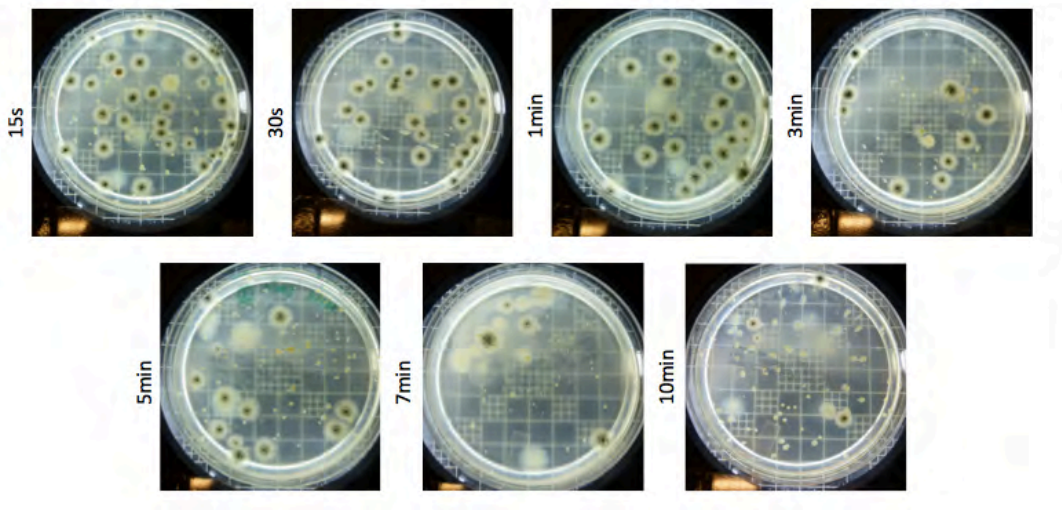


Fig. 16. Pictures of the transferred mold spores following direct DBD treatment on small pieces of crust; samples were inoculated with 10µl of a 10^3 Penicillium *spp. dilution; treatment times were 15s, 30s, 1min, 3min, 5min, 7min and 10min; micro-pulsed power supply was used*

To facilitate the visualization of the inactivation of the mold spores achieved with direct DBD treatment, the colonies were manually counted and plotted (see).

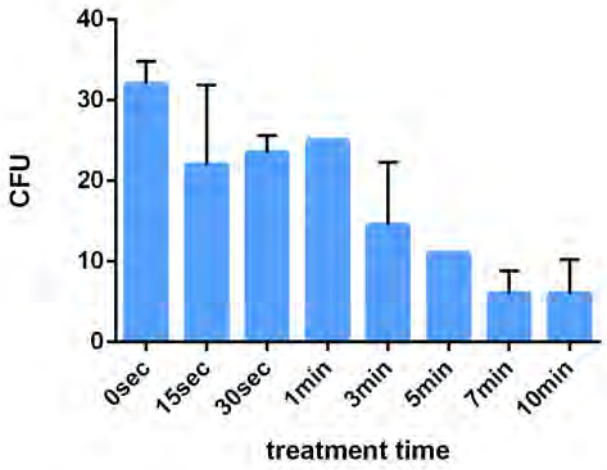


Fig. 17. CFUs observed after direct DBD treatment on crust and subsequent incubation; micro-pulsed power supply was used; treatment times were 15s, 30s, 1min, 3min, 5min, 7min and 10min; untreated control is plotted for reference; 10µL of a 10^3 Penicillium *spp. dilution were used for inoculation; n=2*

4 Discussion

4.1 Phase I: Can plasma kill *Penicillium* spp.?

The first experiment using *E. coli* as a model organism to conduct preliminary proof on concept tests were successful. Three different power supplies were used to generate plasma in order to investigate any potential differences between the results regarding the inactivation ability. As can be seen in Fig. 9, the AC-pulsed power supply was able to achieve the highest level of inactivation. Comparable levels of inactivation were achieved using the micro- and nano-pulsed power supplies. There was no inherent advantage or disadvantage observable in regards to the ability to kill bacteria on TSA plates between those two.

After taking the end goal and the feasibility of each of the three power supplies to be incorporated in an industrial scale into consideration, the decision was made to move forward using the micro-pulsed power supplies. The main factors in favor of a micro-pulsed system are the medium range price (nano-pulsed being the most expensive one, AC-pulsed the cheapest) as well as the filament production compared to the other power supplied. While the AC-pulsed power supply resulted in the largest area of inactivation, the plasma produced is highly filamentous. This presents no issue when treating contaminant located on an agar dish, however, when working with an uneven surface as a bread crust, it can lead to problems. The more filamentous plasma is, the more individual streamers are formed. These streamers tend to form on the spot that presents the easiest path for them to discharge. Applied to a loaf of bread, this would mean that the “highest” spots on the surface of the bread would be receiving the major part of the generated plasma, which in turn may lead to a drastic increase in the local temperature and might cause some damage.

Moving on, the experiments were repeated using *Penicillium* spp. spores on either an agar dish or using “dry” inoculated dishes at two different concentrations. Fig. 10 shows the comparison of those different inoculation conditions as well as the

varying concentrations used. In all the chosen experimental set-ups, inactivation of the mold spores was already achieved after 3 seconds.

For a better visualization of these results, the area of inactivation is plotted in Fig. 11. It can be observed that the dry inoculation (i.e. no agar at point of plasma treatment) resulted in a larger diameter lacking mold growth. The agar inoculated spores show an area of inactivation that is roughly the size of the electrode used to generate plasma and does not surpass it. On the other hand, with an increasing treatment time, the dry inoculated plates show an increasing area of inactivation that becomes much larger than the area of the electrode. This means that the created reactive species were able to propagate across the plastic surface better than across the agar surface, which has a rather high moisture content that seems to impair the propagation of the reactive species.

4.2 Phase II: Does plasma treatment damage the bread?

The color analysis that was conducted in order to assess if the treatment of bread with direct dielectric barrier discharge has any impact on the surface of the bread is shown in Fig. 12. Three loaves of bread were treated using the three different power supplies.

As expected, the most powerful but also most filamentous plasma (generated by the AC-pulsed power supply) had the biggest impact on the crust of the bread. After only a few seconds of plasma application, the surface of the bread began to show first signs of damage. Throughout the treatment, which lasted for up to 4 minutes, the surface of the bread started to char heavily. As mentioned earlier, this is mainly due to the very few streamers that form and concentrate on the highest spots on the bread that represent the shortest distance to form a discharge.

The micro- and nano-pulsed power supplies delivered comparable results. There was no visible damage caused on the surface of the bread throughout the entire experiment.

It should be noted that the color of a loaf of bread varies tremendously, which is why the digitally re-created squares seem not uniform in the shades of brown.

4.3 Phase III: Can plasma be generated inside the packaging?

To test if plasma can be generated inside two layers of packaging in order to actually achieve disinfection of the bread itself, several experiments using the exact materials used in the production line were conducted. Table 2 shows the different arrangements of an Indigo Carmine dyed paper square, the inner plastic wrapping (BOPP) and the outer plastic bag material (LDPE) that were tested. In order to simulate real life conditions, air gaps were introduced or removed to cover all possibilities. After 30 seconds of direct DBD treatment using a micro-pulsed power supply, the blue square was visually inspected for discoloration and potential damage was assessed on the two kinds of plastic.

As can be seen from the results, the outer layer of plastic (LDPE) seems to have a higher robustness against the plasma treatment and acted as protection to BOPP (inner wrapping). Without adding the outer plastic, the plasma treatment caused the inner plastic to develop small punctures. This damage is of course inacceptable to Campbell's Soup/Pepperidge Farm, but due to the fact that the outer layer of plastic is not only able to withstand 30 seconds of plasma treatment, but also completely protects the inner layer from being damaged in any way, this presented no problem.

The air gaps that were introduced did not have any influence on the outcome of the conducted experiments.

Discoloration of the Indigo Carmine dyed paper square was achieved in all of the tests, which proved the presence of plasma and the generated reactive species throughout all the layers and air gaps, regardless of the arrangement.

4.4 Phase IV: Can plasma kill *Penicillium* spp. on bread?

Similarly to the experiments in Phase I, *E. coli* was once again used as a model organism to start inoculation and plasma inactivation on the bread itself. Small pieces of crust were inoculated with approximately 100 CFUs, treated for different amount of times, washed and plated. Following incubation, pictures of the plates were taken, the colonies counted and plotted. As can be seen in Fig. 14 and Fig. 15, there was a significant reduction in the amount of colonies following the treatment with direct DBD. The recovery after the washing step was at around 85%. With increasing treatment times the amount of bacteria was reduced drastically until eventually a 100% inactivation was achieved.

Following the same protocol, the experiments were repeated using *Penicillium* spp. spores on bread. Fig. 16 and Fig. 17 show the pictures of the plates as well as the plotted amount of colonies in respect to the treatment time. Again, the amount of colonies is reduced upon plasma treatment. However, no complete inactivation could be achieved within the parameters of this experiment.

5 Conclusion

Overall, plasma-aided disinfection systems proved to be a viable option for bread disinfection. Direct dielectric barrier discharge is able to inactivate *Penicillium* spp. very effectively within a short amount of time. All three power supplies that were tested were able to achieve significant areas of inactivation.

However, AC-pulsed power supplies are not a suitable option to use on bread. The plasma generated is very powerful as well as filamentous which led to heavy charring of the surface of the bread.

Micro- and nano-pulsed power supplies resulted in similar areas of inactivation achieved in roughly the same amount of time. Also, the use of these two power supplied did not lead to any damage on the surface of the bread. Due to the high price of nano-pulsed systems, micro-pulsed power supplied represent the most feasible option if the plasma-based disinfection systems are to be implemented at an industrial scale.

Plasma was generated inside of the two layers of packaging material without causing any damage or visible changes to the two different polymers. This confirms that in package disinfection of bread is possible, which would make the adding of antifungal or antibacterial agents to the bread unnecessary.

It was proven that the application of plasma onto inoculated pieces of bread crust results in a decrease of the bacterial/spore load. No damage to the bread was caused.

5.1 Outlook

Future challenges following this research work include the development of a system that can be implemented in a production line. It has to sufficiently achieve inactivation within a few seconds and treat all sides of a loaf of bread evenly.

Also, the decision on what power supply is most suitable for an industrial setting has to be made.

Finally, the developed system has to be tested on a completely packaged and unsliced loaf of bread that has previously been inoculated with a known amount of contaminant and the inactivation has to be evaluated.

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