EFFICACY OF CHLORINE DIOXIDE GAS IN

INACTIVATING *SALMONELLA* SPP. ON MUNG BEANS SPROUTS

By

VARA PRODDUK

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ABSTRACT OF THE THESIS

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Rising popularity of fresh sprouted beans and grains as a source of good nutrition also brings increased challenges for maintaining food safety and quality. Internalization of various microorganisms apart from surface localization is a hurdle in ensuring safe produce of high quality. Microorganisms internalized in stomata and crevices may not be affected by the traditional chlorine sanitizing wash treatment. Sprouts, integral to many cuisines, have been the focus of media attention due to the frequency and severity of microbial outbreaks. Sprouts provide good matrices for microbial localization and growth due to optimal conditions of temperature and humidity while sprouting and lack of post-processing. In fact, the microbial populations on sprouts are some of the largest reported for fresh produce.

As conventional aqueous chlorine sanitizing wash treatment was unable to provide more than 2 $\log_{10}$ CFU/gram reduction in *Salmonella* spp. levels, this research explores the antimicrobial effectiveness of gaseous chlorine dioxide on mung bean sprouts. 3 – 4 log CFU/gram reduction was achieved in *Salmonella* serovar inoculated sprouts by gaseous chlorine dioxide application at various time combinations.

The difference in microbial reduction points to the important role of surface physiology, pore structure and bacterial internalization in sprouts. Consequently, the surface morphology of sprouts has been studied to understand the better efficacy of gaseous antimicrobial chlorine dioxide.

As the microbial loads and microorganism distribution on sprouts is variable, a three-dimensional printer was utilized to design and create consistent reliable silicone substrates which can simulate the surface physiology and pore structure of sprouts.

Sprouts have a complex farm-to-fork path with multiple microbial contamination scenarios. This research helps understand how gaseous antimicrobials and increase in surface area available for antimicrobial application can provide more effective microbial load reduction.

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Vara Prodduk

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1. INTRODUCTION

1.1 Sprouts

Sprouts are essentially young green plants germinated from the seeds of almost any living vegetation which may include, but are not limited to, nuts, seeds, grains, beans, legumes, as well as various grasses such as barley grass or wheat grass. Some of the most common sprout sources are alfalfa, mung, radish, clover, aduki, garbanzo (chickpea), lentil, soybean, sunflower, millet, quinoa, buckwheat, fenugreek, wheat, barley, soy, corn, oats, green peas and lima bean. Essentially any seed or bean equipped with the genetic fabric potential to reproduce the next generation of plant life is sproutable [48].

Sprouts are popular because they contain a high concentration of antioxidants and nutrients. Sprouts are also packed with vitamins, minerals, protein, enzymes and fiber [48].

1.2 Sprouting and its benefits

Sprouting is the process of soaking and germinating the grain or seed to produce edible live sprouts. Each sprouting seed is packed with the nutritional energy needed to create a full grown healthy plant.

Once the seed is soaked in water, a process necessary for sprouting, enzymes are released. Upon germination, the seed rapidly absorbs water (from soaking) and swells to at least twice its original size. Simultaneously, the nutrient content also increases. Finally, the germination process effectively pre-digests the seed, making digestion and assimilation of its nutrients easy [48].

Sprouting causes increased activities of hydrolytic enzymes, improvements in the contents of total proteins, fat, certain essential amino acids, total sugars, B-group vitamins, and a decrease in dry matter, starch and anti-nutrients. The increased contents of protein, fat, fiber and total ash are only apparent and attributable to the disappearance of starch. However, improvements in amino acid composition, B-group vitamins, sugars, protein and starch digestibilities, and decrease in phytates and protease inhibitors are the metabolic effects of the sprouting process [40]. Table 1 shows the percentage of crude protein and crude fiber change during a 7 day sprouting process of barley sprouts.

Table 1: Crude Protein and Crude Fiber changes in Barley Sprouted over a 7-day period [10, 42]

	Crude Protein (% of DM)	Crude Fiber (% of DM)
Original seed	12.7%	5.4%
Day 1	12.7%	5.6%
Day 2	13.0%	5.9%
Day 3	13.6%	5.8%
Day 4	13.4%	7.4%
Day 5	13.9%	9.7%
Day 6	14.0%	10.8%
Day 7	15.5%	14.1%

The consumption of sprouts is increasingly becoming popular in North America and some other parts of the world along with the growing consumption of fruits and vegetables. Besides having a lot of nutritional benefits, epidemiological studies suggest that sprouts may have a beneficial role in protecting against a number of chronic diseases and conditions such as cancer and osteoporosis. Studies conducted at Johns Hopkins University in 1997 concluded that raw broccoli sprouts may be rich in sulforaphane, a product that reduces the risk of cancer [54]. Figure 1shows the stages of sprouting which typically take 3 – 4 days.

Figure 1: Stages of growth from bean to fully formed sprout

1.3 Mung bean sprouts

The research documented in this thesis utilizes mung bean sprouts. Mung beans are a member of the legume family of plants and have the unique capacity to fix atmospheric nitrogen by the nodules on their roots which harbor nitrogen fixing bacteria. These bean sprouts are popularly consumed in the United States either fresh or stir-fried.

The fiber in mung beans has the capacity to combine with bile acids. The decrease in bile available for digestion may stimulate the body's conversion of blood cholesterol into bile resulting in lower levels of blood cholesterol. One phyto-nutrient unique to legumes is a group of molecules called saponins. Unlike most beans, mung beans contain very few oligosaccharides, the sugars responsible for flatulence. This unique biochemistry makes mung beans suitable for children and anyone suffering from delicate digestive systems [37]. This implies that the food safety of mung bean sprouts is even more critical.

The biochemical process of sprouting transforms this seed which already has a number of significant health benefits into a highly nutritious food. Consumers are aware of the mung bean sprouts, where the seed has germinated and is six fold bigger than its original size and the white radicle or root has emerged. It is often eaten in this form as a snack. At this stage the mung bean still resembles a pea in shape. The sprouting biochemistry has made minerals, enzymes and vitamins available in a form easily assimilated by the body [37].

Figure 2: Image of mung bean sprouts

1.4 Microbial risks related to sprouts

The production of sprouts has become a growth industry in response to consumer demand for a continuous supply of fresh produce. The U. S. market is valued at approximately $ 25 million with over 400 growers producing 300,000 tons of sprouts annually [28].

Microbiological analyses have shown that alfalfa seeds routinely contain high levels of microbial flora, including coliforms and fecal coliforms [38]. Of even greater public health significance however, is the fact that pathogens can exceed 1 X 10^7 CFU/gram in sprouts without affecting the appearance of the product [52]. With respect to bacterial growth and proliferation, sprouts present a special risk compared to other fresh produce. The conditions under which they are produced – sprouting time, temperature, moisture, and nutrients –are ideal for bacterial proliferation. The microbial

flora of sprouts was, therefore, often 2 to 3 $\log_{10}$ higher than that observed in seeds [14, 46].

Microbiological surveys have shown the presence of a variety of foodborne pathogens in sprouts. *Salmonella* species, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Aeromonas hydrophila* have been isolated from sprout seeds including alfalfa, mung, cress, and mustard. *Klebsiella pneumoniae* was also isolated from sprouts and seeds. Sprouts follow a complex path from farm-to-fork that includes growing, harvesting, processing, and shipping of seeds, followed by sprouting and distribution of the finished product [41].

There are several opportunities for the contamination of seeds or sprouts at any one of growing, harvesting, processing, and shipping points in production and distribution. Such contamination can be introduced in sprouts via a number of pathways including seeds, irrigation water used for sprouting, unsanitary production practices, improper packaging or mishandling by consumers [52].

1.5 Outbreaks of *Salmonella* in sprouts

Over the past decade, the consumption of seed sprouts has been linked to multiple outbreaks of foodborne illness throughout the world, affecting thousands of people. Between 1990 and 2010, over 2,500 Americans were sickened due to consumption of contaminated sprouts in at least 46 outbreaks, as reported to the Center for Disease Control and Prevention (CDC). *Salmonella* was identified in 37 out of 46 outbreaks, with *E. coli* associated with eight outbreaks, and *Listeria* with one outbreak [9].

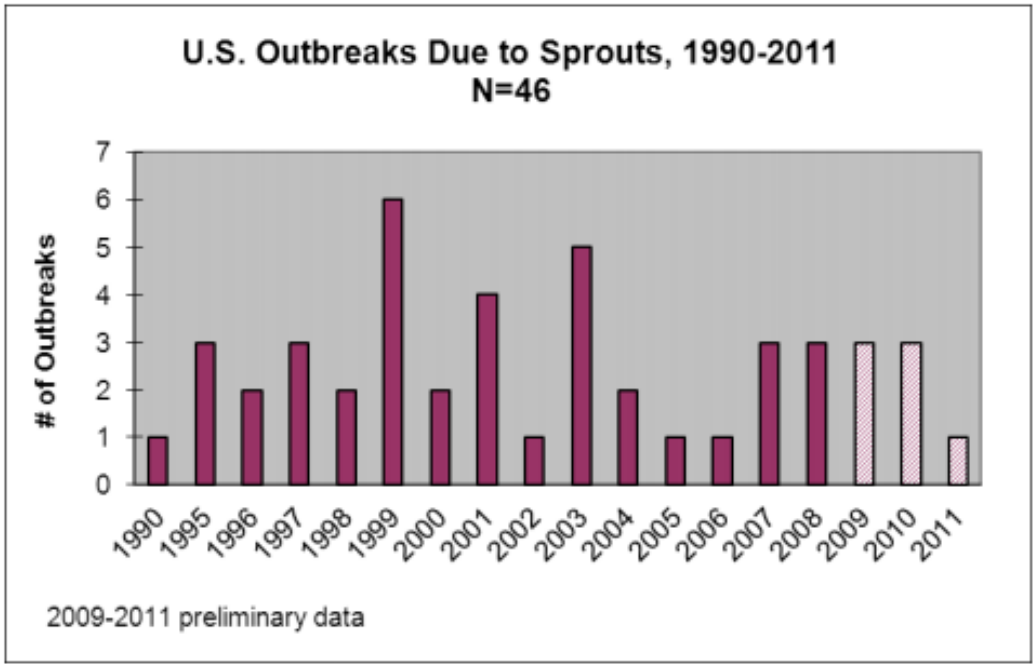


Figure 3: Number of outbreaks due to contaminated sprouts in the U.S. from 1990-2011 [9]

Some recent prominent sprout outbreaks include [9]:

- In June 2011, alfalfa sprouts contaminated with *Salmonella* Enteritidis sickened 21 people in five states, three of whom required hospitalization.
- In May-June 2011, fenugreek sprouts sickened more than 4,300 people and killed 50 in Europe, United States and Canada.
- In April-July 2011, alfalfa sprouts and spicy sprout mix were linked to *Salmonella* Enteritidis that affected 25 people in five states.
- In November 2010-February 2011, alfalfa sprouts and spicy sprouts sickened 140 people with *Salmonella* in 26 states; 24% are hospitalized.
- In 2010, sprouts contaminated with *Salmonella* Newport infected 44 people in 11 states, at least seven of whom required hospitalization. The same year, sprouts

from an Illinois sprout grower sickened 140 individuals from 26 states. FDA investigators observed numerous production practices that were unsanitary and provided ample opportunity for cross-contamination.

- In 2009, nearly 250 sprout eaters became ill from two outbreaks traced back to sprouts.
- More outbreaks from contaminated sprouts are included in Table 2

Table 2: Outbreaks in the U.S. from contaminated sprouts in from 2000-2011 [9]

Year	States	Vehicle	Cases	Etiology	Cause
2011	Multi-state	Sprouts, alfalfa	21	*S*.Enteritidis	Unknown
2010	Multi-state	Sprouts, alfalfa, radish, clover	140	*Salmonella* spp.	Cross-contamination at growing facility
2010	Multi-state	Sprouts, clover, others	7	*S*.Newport	Unknown
2010	Multi-state	Sprouts, alfalfa	44	*S*.Newport	Unknown
2009	MI	Sprouts, alfalfa	12	*S*.Typhimurium	Unknown
2009	Multi-state	Sprouts, alfalfa	229	*S*.Saintpaul	Contaminated seeds
2009	MN	Sprouts, alfalfa	16	*S*.Cubana	Unknown
2008	Multi-state	Sprouts, alfalfa	24	*S*.Typhimurium	Unknown
2008	NY	Sprouts, alfalfa	6	*L.monocytogenes*	Unknown
2008	CO	Sprouts, alfalfa	21	*E.coli* O157:H7	Infected restaurant workers
2007	CA	Sprouts, bean	24	*S*.Montevideo	Unknown
2007	CA	Sprouts, bean	20	*S*.Mbandaka	Unknown
2007	Multi-state	Sprouts, alfalfa	15	*S*.Mbandaka	Unknown
2006	OR	Sprouts, bean	4	*S*.Braenderup	Unknown
2005	MA	Sprouts, mung bean	2	*S*.Braenderup	Unknown
2004	Multi-state	Sprouts, alfalfa	35	*S*.Bovismorbificans	Contaminated seeds
2004	GA	Sprouts, alfalfa	2	*E.coli* O157:H7	Unknown
2003	Multi-state	Sprouts, alfalfa	16	*S*.Saintpaul	Unknown
2003	Multi-state	Sprouts, alfalfa	26	*S*.Chester	Unknown
2003	CO	Sprouts, alfalfa	13	*E.coli* O157:H7	Contaminated seeds suspected
2003	Multi-state	Sprouts, alfalfa	20	*E.coli* O157:H7	Unknown
2003	MN	Sprouts, alfalfa	7	*E.coli* O157:H7	Contaminated seeds suspected
2002	CA	Sprouts, alfalfa	5	*E.coli* O157:H7	Irrigation water at growing facility
2001	CA	Sprouts, alfalfa	22	*Salmonella* spp.	Unknown
2001	Multi-state	Sprouts, alfalfa	32	*S*.Kottbus	Contaminated seeds
2001	FL	Sprouts, mung bean	35	*S*.Enteritidis	Contaminated seeds
2001	HI	Sprouts, mung bean	21	*S*.Enteritidis	Unknown
2000	FL	Sprouts, alfalfa	3	*Salmonella* spp.	Unknown
2000	Multi-state	Sprouts, mung bean	75	*S*.Enteritidis	Contaminated seeds

Aware of the high risk associated with consumption of raw sprouts, the FDA recommends [9]:

- Children, the elderly, pregnant women, and persons with weakened immune systems should avoid eating raw sprouts of any kind (including alfalfa, clover, radish, and mung bean sprouts).
- Sprouts should be cooked thoroughly to reduce the risk of illness.
- Raw sprouts should not be mixed with cooked and processed food.

1.6 *Salmonella* spp.

Salmonella spp. is a genus of rod-shaped, gram-negative, non-spore-forming predominantly motile bacteria with peritrichous flagella belonging to the family *Enterobacteriaceae*. *Salmonellae* are facultative anaerobes and contain two species *Salmonella enterica* and *Salmonella bongori* which currently include 2,443 and 20 serovars respectively [35].

Salmonella species are associated with a number of foodborne and waterborne illnesses worldwide. Salmonellosis is the type of food poisoning that is caused by *Salmonella*. Most persons infected with *Salmonella* develop diarrhea, fever, and abdominal cramps within 12 to 72 hours after ingestion. The illness usually lasts 4 to 7 days, and most victims recover without treatment. However, in some people, the diarrhea may be so severe that the individual needs to be hospitalized. In that case the *Salmonella* infection may spread from the intestines to the blood stream, and then to other body sites and can cause death unless the person is treated with antibiotics. Every

year, approximately 40,000 cases of salmonellosis are reported in the United States [8]. Because many milder cases are not diagnosed or reported, the actual number of infections may be thirty or more times greater.

Salmonella serotype Typhimurium and *Salmonella* serotype Enteritidis are the most common in United States [8]. Sources of infection include infected food, poor kitchen hygiene, and fluids from sick or infected people or animals, polluted surfaces or standing water and unhygienically thawed meat. *Salmonella* is unique as it can survive several weeks in a low-moisture environment as well as several weeks in water [8].

There have been several outbreaks of *Salmonella* in the recent past in various disparate food forms such as cantaloupe, turkey burgers, sprouts, ground beef, pine nuts, tomatoes, peppers, shell eggs, pistachios, frozen entrée meals [8] etc., which has led to growing awareness and extensive research on *Salmonella* survival and growth in foods as well as technologies to eliminate their survival in foods during manufacture.

1.7 Seed disinfection and effectiveness

In 1999, FDA released guidelines, based largely on the work of National Advisory Committee on Microbiological Criteria for Foods (NACMCF), which included 5 basic recommendations [12]:

(1) Growing seed for sprouting using good agricultural practices,

(2) Conditioning and storing seed under sanitary conditions,

(3) Following good manufacturing practices (GMPs), as appropriate, at sprouting facilities,

(4) Applying a disinfection treatment to seed immediately before sprouting, and

(5) In-process testing of spent sprout irrigation water for pathogens of concern before finished product enters market channels

As an example, FDA cited 20000 ppm calcium hypochlorite for seed disinfection treatment [12]. Unfortunately, there does not seem to be a clear treatment procedure for post-sprouting.

Current treatment methods utilized for seed disinfection are:

1.7.1 Chemical treatment

Chemical treatment of seeds to kill pathogenic bacteria that may be present has been investigated by a number of researchers. In several studies, treatment of seeds with 20,000 ppm calcium hypochlorite reduced the population of the target pathogen to undetectable levels on culture media, but the pathogen could be detected by enrichment [19].

Various postharvest aqueous antimicrobial wash treatments such as chlorine, hydrogen peroxide, ozone, trisodium phosphate and peroxyacetic acid have been ineffective in eliminating inoculated bacteria. The effectiveness of chemical treatments may vary as the concentration of antimicrobial drops to sub-lethal levels due to reaction with cell wall materials, exudates and exposed plant tissue [2]. Microbial cells attached to inaccessible sites of sprout surfaces such as stomata and crevices may not be affected by the sanitizer treatment [6].

Table 3: Chemical interventions for reducing pathogens on inoculated sprouting seeds [15]

Treatment	Conditions	Time	Seed type	Bacterium	Log reduction (CFU/g)	Seed germination
Acetic acid, vapor	242 µl/l air, 45°C	12 h	Mung bean	*Salmonella*	>5, no survivors	No effect
Acetic acid, vapor	242 µl/l air, 45°C	12 h	Mung bean	*E.coli* O157:H7	>6, no survivors	No effect
Acetic acid, vapor	242 µl/l air, 45°C	12 h	Mung bean	*L. monocytogenes*	4.0	No effect
Acetic acid, vapor	300 mg/l air, 50°C	24 h	Alfalfa	*Salmonella*	0.8	No effect
Acidic EO water	1081 mV, 84 ppm chlorine	10 min	Alfalfa	*Salmonella*	1.5	No effect
Acidic EO water	1150 mV, 50 ppm chlorine	64 min	Alfalfa	*E.coli* O157:H7	1.6	Significant reduction
Acidic EO water	1079 mV, 70 ppm chlorine	15 min	Alfalfa	*Salmonella*	2.0	No effect
Allyl isothiocyanate	50 µl/950cm^3 jar, 47°C	24 h	Alfalfa	*E.coli* O157:H7	>2.0, survivors present	Slight reduction
Ammonia, gas	300 mg/l	22 h	Alfalfa	*Salmonella*	2.0	No effect
Ammonia, gas	300 mg/l	22 h	Mung bean	*Salmonella*	5.0	No effect
Ammonia, gas	300 mg/l	22 h	Alfalfa	*E.coli* O157:H7	3.0	No effect
Ammonia, gas	300 mg/l	22 h	Mung bean	*E.coli* O157:H7	6.0	No effect
$Ca(OH)_2$	1%	10 min	Alfalfa	*E.coli* O157:H7	3.2	No effect
$Ca(OH)_2$	1%	10 min	Alfalfa	*Salmonella*	2.8-3.8	No effect
$Ca(OCl)_2$	20,000 ppm	3 min	Alfalfa	*E.coli* O157:H7	>2.3, survivors present	Reduced rate
$Ca(OCl)_2$	20,000 ppm	10 min	Alfalfa	*Salmonella*	2.0	Slight reduction
$Ca(OCl)_2$	18,000 ppm	10 min	Alfalfa	*Salmonella*	3.9	No effect
$Ca(OCl)_2$	18,000 ppm	10 min	Alfalfa	*E.coli* O157:H7	4.5	No effect
$Ca(OCl)_2$	16,000 ppm	10 min	Mung bean	*Salmonella*	5.0	No effect
$Ca(OCl)_2$	16,000 ppm	10 min	Mung bean	*E.coli* O157:H7	3.9	No effect
Chlorine dioxide, acidified	500 ppm	10 min	Alfalfa	*E.coli* O157:H7	>2.4, survivors present	Significant reduction
H_2O_2	8%	3 min	Alfalfa	*E.coli O157:H7*	>2.9, survivors present	No effect
H_2O_2	8%	10 min	Alfalfa	*Salmonella*	3.2	No effect
Lactic acid	5%, 42°C	10 min	Alfalfa	*E.coli* O157:H7	3.0	No effect
Supercritical CO_2	4000 psi, 50°C	60 min	Alfalfa	*E.coli,* generic	1.0	No effect
Ozone, aqueous	21 ppm with sparging	64 min	Alfalfa	*E.coli* O157:H7	2.2	No effect
Ozone, aqueous	21.3 ppm with sparging	20 min	Alfalfa	*L. monocytogenes*	1.5	No effect

1.7.2 Heat treatment

Application of heat to kill pathogens on alfalfa seeds has been investigated [28] in a study that found treatment at 57°C or 60°C for 5 minutes appeared to be effective in killing *Salmonella* Stanley without substantially decreasing germinability of seeds. However, heat treatment has limited appeal because there is such a fine threshold at which bacteria can be killed and germination not destroyed. Heat treatment may impact the fresh flavor and quality attributes of sprouts.

1.7.3 Irradiation

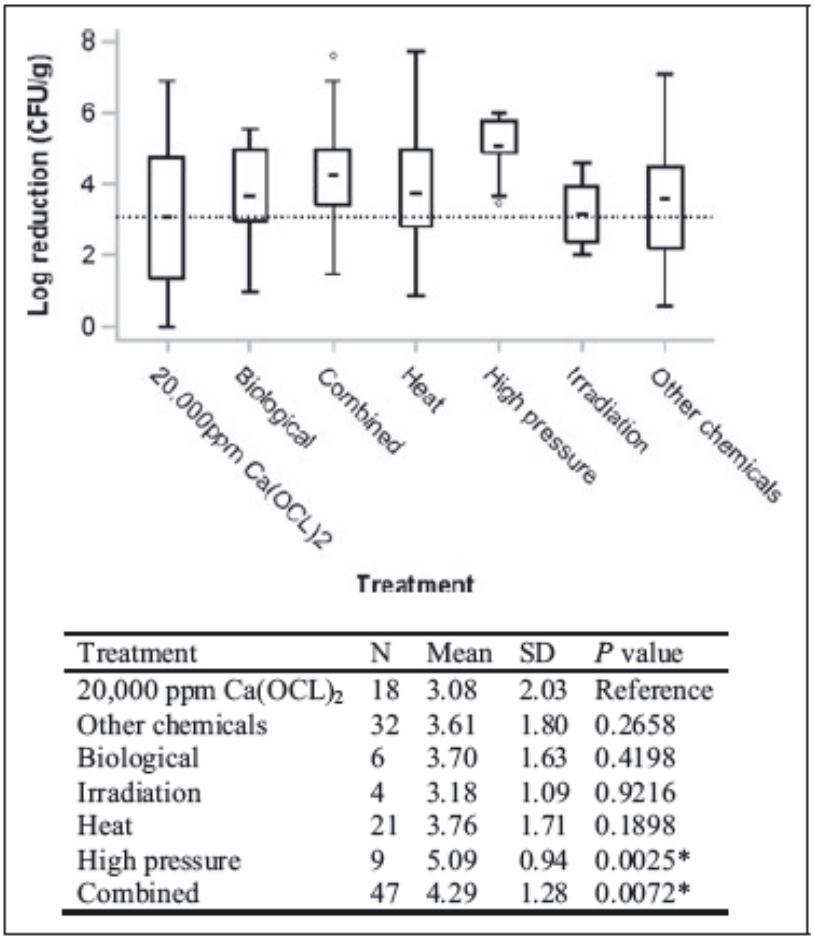
On October 30, 2000, the Food and Drug Administration (FDA) announced that it had approved the use of ionizing radiation on seeds used for producing sprouts. The goal is to reduce pathogens in and on the seeds. While this process cannot ensure elimination of every organism, it can achieve a considerable reduction. The likelihood of viable pathogens remaining will depend both on the original microbial load and on the irradiation dose applied. The new regulation allows a dose of up to 8 kilograys (kGy). However, the actual dose depends on the ability of various seeds to tolerate irradiation and remain able to sprout [17].

Table 4 shows various physical interventions and the log reductions achieved on pathogen inoculated sprouts.

Table 4: Physical interventions for reducing pathogens on inoculated sprouting seeds [15]

Treatment	Conditions	Time	Seed type	Bacterium	Log reduction (CFU/g)	Seed germination
Dry heat	50°C	60 min	Alfalfa	*E.coli* O157:H7	1.7	No effect
Dry heat	70°C	3 h	Alfalfa	*Salmonella*	3.0	Slight reduction
Hydrostatic pressure	300 mPa	15 min	Garden cress	*Salmonella*	5.8	Reduced rate
Hydrostatic pressure	300 mPa	15 min	Garden cress	*Shigella flexneri*	4.5	Reduced rate
Radiation, gamma	Various		Alfalfa	*Salmonella*	D-value of 0.97 kGy	Dosage dependent
Radiation, gamma	Various		Alfalfa	*E.coli* O157:H7	D-value of 0.60 kGy	Dosage dependent
Radiation, gamma	Various		Broccoli	*Salmonella*	D-value of 1.10 kGy	Dosage dependent
Radiation, gamma	Various		Broccoli	*E.coli* O157:H7	D-value of 1.11 kGy	Dosage dependent
Pulsed UV light	5.6 J/cm^2, 270 pulses	90 sec	Alfalfa	*E.coli* O157:H7	4.9	Significant reduction
Dielectric heating, radio frequency	39 MHz, 1.6 kV/cm	26 sec	Alfalfa	*Salmonella*	1.7	No effect

Figure 4 shows that the highest log reduction was achieved by high hydrostatic pressure processing (HHPP), however, HHPP reduces rate of germination in the seeds.



Treatment	N	Mean	SD	*P* value
20,000 ppm $Ca(OCL)_2$	18	3.08	2.03	Reference
Other chemicals	32	3.61	1.80	0.2658
Biological	6	3.70	1.63	0.4198
Irradiation	4	3.18	1.09	0.9216
Heat	21	3.76	1.71	0.1898
High pressure	9	5.09	0.94	0.0025*
Combined	47	4.29	1.28	0.0072*

Figure 4: Comparison of seed disinfection efficacies by various treatments. Bar in the middle of the box is mean and the dashed line represents the mean reduction of reference treatment, 20000 ppm $Ca(OCl)_2$. Statistical analyses are summarized in the figure [12].

1.8 FDA and Food Safety Modernization Act (FSMA) focus on sprouts

In accordance with the risk associated with sprout consumption, FDA has issued guidance documents to reduce the risk of future outbreaks. Commercial sprout growers need to follow good manufacturing practices (GMPs) and have written standard sanitation operating procedures (SSOPs) and have a hazard analysis and critical control point (HACCP) plan in place. Growers should follow detailed methods for testing of spent irrigation water for *Salmonella* and *E. coli* O157:H7. Seed should be of high quality

and all bags of seed should be inspected for evidence of rodent activity. Thorough testing of all lots of sprout seed for bacterial pathogens is desirable and should reduce the risk of sprout-related outbreaks of foodborne disease. A sampling and testing protocol for use with sprout seed for human pathogens has been proposed. However, due to the sporadic and low level of contamination with human pathogens often encountered, a negative sample test cannot guarantee that the entire lot is pathogen free. This is another reason why the present food safety measures for sprout safety may not be sufficient. Thus, an effective, approved seed-sanitizing step should be applied by the grower, and the spent irrigation water or sprouts should be tested for the presence of pathogens. Irrigation water needs to be of high quality and the use of well water also requires regular testing for adequate levels of residual chlorine. Postharvest contamination of sprouts can occur during transit, storage, display, and by cross contamination in restaurant or home kitchens and adequate precautions need to be taken. [12]

As part of the FSMA requirements to address sprout safety, the FDA, in cooperation with the Illinois Institute of Technology's Institute for Food Safety and Health (IIT IFSH), has created the Sprouts Safety Alliance (SSA) to help sprout producers in identifying and implementing best practices in the safe production of sprouts. The Alliance will develop a core curriculum and training and outreach programs for stakeholders in the sprout production community. Sprouts present a unique food safety risk because the warm, moist and nutrient-rich conditions required to produce sprouts are the same conditions that are also ideal for the growth of pathogens. The Alliance is composed of members from the FDA, local and state food protection agencies, the food industry, and academia. It is funded by a one-year, $100,000 grant to

the Illinois Institute of Technology's Institute for Food Safety and Health (IIT IFSH), a nationally-recognized leader in food safety [18].

The current goals for this Alliance are to [18]:

- Develop training materials to assist sprouters in adopting best practices for the safe production of sprouts based on available FDA guidance documents
- Provide tools to assist growers in conducting self-audits of their sprouting operations and production practices to minimize microbial hazards associated with sprouts
- Develop training materials that facilitate industry understanding and implementation of relevant requirements in produce safety regulation
- Serve as a network hub and resource for the sprout industry, and federal and state regulatory agencies
- Develop a technical assistance network for the sprout industry
- Collaborate with USDA, states, trade associations, and land-grant university extension services to provide classroom and distance training and workshops for stakeholders across the U.S

1.9 Need for further research

There is relatively little scientific literature available pertaining to the reduction or elimination of pathogenic bacteria on sprouts during growth. A substantial amount of research however, has been focused on prevention and intervention technologies to eliminate pathogens from seeds prior to sprouting as described above. However, the conditions in which sprouts are grown are conducive for the growth of many types of bacteria such as *E. coli, Salmonella* and other enteric pathogens [19]. Thus, if seed disinfection does not completely eliminate the target pathogen, then, the organism may

grow during germination and sprout growth to levels capable of causing human illness [19].

Despite considerable research efforts towards the development of sprout seed sanitizing methods there is still a need for highly effective, low-cost, easily implemented, and environmentally benign strategies that can be used by organic and conventional sprout growers. It has been shown that the use of 20,000 ppm $Ca(OCl)_2$ creates worker and environmental safety concerns, may not always be effective in eliminating human pathogens from contaminated seed lots under commercial practice, and can be highly detrimental to the germination capacity of some seed types [16]. The potential for internalization of bacterial human pathogens into sprouts during germination and growth from contaminated sprouting seed has been demonstrated, but the location of pathogens on naturally contaminated seed is still not known. The optimization and commercialization of biological control agents for use on sprouting seed and sprouts as an alternative to chemical sanitizers is highly desirable [16].

The ecology of human pathogens on sprouts is not well defined and several questions remain unanswered. Further research in the areas mentioned above should assist in the development of improved strategies for reducing the risk of future foodborne outbreaks allowing for greater consumer confidence in the microbiological safety of sprouts and ensuring the survival of a strong sprout industry worldwide. Intervention strategies developed for seeds and sprouts may also be applicable to ensuring the microbiological safety of other types of produce [16].

Warriner et al. (2003) showed that *E. coli* or *Salmonella* present on seeds become internalized with subsequent sprouts and cannot be removed by post-harvest biocidal

washing. *E. coli* or *Salmonella* Montevideo introduced on mung beans became established both internally and externally on sprouts after the initial 24 hour germinating period. In both cases the inoculated bacterium formed the predominant microflora on the sprouted beans throughout. From the bioluminescent profile of inoculated sprouting beans, bacterial growth was found to be in close proximity to the roots but not on the hypocotyls. Biofilms of cells with low viability were observed within the grooves between epidermal cells on hypocotyls. Treatment with 20,000 ppm sodium hypochlorite removed the majority of bacteria from the surface of hypocotyls although nonviable single cells were occasionally observed. However, viable bacteria were recovered from the apoplastic fluid, and extracts of surface-sterilized sprouts indicating that the internal bacterial populations had been protected [60].

Pathogens that reach elevated numbers during germination and sprouting may be practically impossible to eliminate from the mature sprout. Itoh et al. (1998) reported that *E. coli* O157:H7 was found in the inner tissue of experimentally contaminated sprouts during growth. Treatment of the outer surface of sprouts with mercury chloride did not kill the *E. coli* O157:H7 located at subsurface locations [23]. Using laser scanning confocal microscopy, Gandhi et al (2001) showed that *Salmonella* can localize at subsurface locations in mature sprouts produced from contaminated seeds. Exposure of sprouts for 5 min to concentrations of 2000 ppm chlorine resulted in >5 log reduction in numbers of *Salmonella*, but did not eliminate the pathogen. The internal location of the *Salmonella* likely protected the pathogen from the action of the surface sanitizing compound [20]. Fett (2002) reported that the addition of antimicrobial compounds to

irrigation water did not reduce the levels of native microflora associated with alfalfa sprouts by more than 1 log [16].

Chlorine dioxide (ClO_2) gas has been suggested as an effective alternative to chlorine [26]. Chlorine dioxide has a much higher oxidation capacity than chlorine and does not generate undesirable chemicals because its antimicrobial effectiveness is due to oxidation and not chlorination. Chlorine dioxide is highly soluble in water; works over a broad pH range (2-10), has a wide spectrum of antimicrobial capabilities and has low dosage requirements. It can be used in both aqueous and gaseous forms [26].

Table 5 below shows the effect of gaseous chlorine dioxide treatment on various microorganisms in various fruits and vegetables [32].

Table 5: Effect of gaseous chlorine dioxide treatment on various microorganisms in various fruits and vegetables [32]

Organism	Surface	Concentration	Time	Log reduction (CFU/g)
Lactobacillus spp. *Penicillium spp.* *S.cervasia*	Epoxy tank surfaces	10 mg/l	30 min	6.0
Bacillus spores	Plastic, paper, wood	15 mg/l	30 min	5.0
E.coli O157:H7	Green peppers	0.6 mg/l	30 min	7.3
L monocytogenes	Strawberries	0.6 mg/l	15 min	5.6
E.coli O157:H7 *L monocytogenes*	Apples	4.0 mg/l	10 min	5.5
Salmonella spp.	Oranges	0.3 mg/l	13 min	5.0
L.monocytogenes	Lettuce	0.2 mg/l	30 min	1.3
E.coli O157:H7 and *Salmonella* spp.	Cantaloupes	2.8 mg/l 8.6 mg/l	10 min 10 min	2.0 3.0

Singh et al (2002) showed that rinsing of inoculated alfalfa seed growing in plastic jars with aqueous ClO_2 (25 mg/l) or ozonated water (9.27 mg/l) after 48 or 72 hours of sprouting was ineffective in reducing populations of *E. coli* O157:H7 [49].

Kim et al (2009) studied the combined effect of aqueous chlorine dioxide (ClO_2) and fumaric acid as a chemical treatment to inactivate pre-existing microorganisms was evaluated on broccoli sprouts. Treatment with 50 ppm ClO_2 and 0.5% fumaric acid reduced the initial populations of total aerobic bacteria, yeasts and molds, and coliforms in broccoli sprouts by 2.70, 2.46, and 1.71 $\log_{10}$ CFU/gram, respectively. In addition, the combined treatment of 50 ppm ClO_2 and 0.5% fumaric acid reduced the initial populations of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* inoculated on broccoli sprouts by 2.39, 2.74, and 2.65 $\log_{10}$ CFU/gram, respectively, compared to the control. These results suggest that the combination of aqueous ClO_2 and fumaric acid can be useful as a hurdle for extending the shelf life of broccoli sprouts during storage [26].

Still, there is very limited research exploring the effectiveness of chlorine dioxide gas in ensuring the microbiological safety of sprouts. There exists a lot of research investigating the use of chemical and sanitizing treatments of seeds and sprouts but it has also been concluded that these methods are not effective to completely eliminate internalized bacteria. My research aims to explore the potential of gaseous chlorine dioxide on *Salmonella* inactivation in mung bean sprouts.

2. OBJECTIVES

2.1 Rationale of Research Work

Eliminating pathogens on sprout seeds prior to germination and sprouting has not been completely successful. On a similar basis, methods to eliminate pathogenic bacteria post-sprouting have resulted in only limited decrease in microbial populations and were carried out using aqueous chlorine compounds. The complexity of sprout physiology is a probable reason for the localization of microbes and limited effectiveness of antimicrobial treatments. This research explores the potential of gaseous chlorine dioxide as an antimicrobial and its ability to inactivate internalized microorganisms. Since *Salmonella* spp. was responsible for 80% (37 out of 46) of the outbreaks in sprouts over the past two decades, they were chosen as the test microorganisms [9].

Since the cultivation and processing conditions of sprouts are conducive to microbial contamination and growth, the primary objective of this research is to reduce the microbiological (in this case *Salmonella*) populations on sprouts with gaseous chlorine dioxide application. This broad objective is divided into the following sub-objectives:

1. Compare the effectiveness of gaseous chlorine dioxide against a control of conventional aqueous chlorine produce wash treatment.

   Testing antimicrobials with different systems of delivery (gaseous versus aqueous) will provide information on whether gaseous antimicrobials having higher permeability can reach bacteria internalized in sprouts.

2. Conduct surface electron microscopy imaging to assess the *Salmonella* distribution on the sprouts as well as to validate the reduction due to the antimicrobials
3. Design and create a methodology to print silicone substrates using a three-dimensional printer that can simulate the surface physiology of sprouts to reduce the experimental variability due the varying microorganism distribution on fresh produce

The impact and uniqueness of this research is:

1. Finding a more effective treatment than conventional aqueous treatments that do not have the ability to inactivate internalized bacteria
2. Investigating three-dimensional printers as a medium to create bio-mimicking substrates

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Mung bean seeds and sprouting conditions

Raw mung bean (*Vigna radiata*) seeds, unprocessed and not subjected to any additional cleaning procedure, were obtained from The Sprout House (Lake Katrine, NY, USA).

3.1.2 *Salmonella* spp. serovars

Salmonella enterica serovars Infantis F4319, Muenchen HERV2C, and Newport H1275, sprout related isolates were obtained from Dr. Bassam Annous (USDA – ARS – ERRC, Wyndmoor – PA) for this study.

3.1.3 Media for culturing and enumeration of *Salmonella*

1. Brain Heart Infusion (BHI): BHI broth was prepared by suspending BHI broth powder (BBL, Sparks, MD, USA) in 1 liter of distilled water. This was heated with agitation and boiled for 1 minute to completely dissolve the powder. This broth was sterilized in the autoclave at 121°C (250˚F) for 15 minutes.
2. 0.1% Peptone Water: 1.5 grams of peptone powder (Difco™, Benkitson and Dickson, MD, USA) was dissolved in 1 liter of distilled water. 9 ml aliquots and 225 ml glass bottles were created with the peptone water. This media was sterilized in the autoclave at 121˚C (250˚F) for 15 minutes.

3. Tryptic Soy Agar (TSA): Tryptic Soy Agar was prepared by suspending 40.0 grams of the tryptic soy agar (Soybean – Casein Digest Agar) powder (Difco™, Benkitson and Dickson, MD, USA) in 1 liter of distilled water. This media was sterilized in the autoclave at 121˚C (250˚F) for 15 minutes.
4. Tryptic Soy Broth (TSB): Tryptic soy broth was prepared by suspending 30.0 grams of the tryptic soy broth (Soybean – Casein Digest Medium) powder (Difco™, Benkitson and Dickson, MD, USA) in 1 liter of distilled water. This media was sterilized in the autoclave at 121˚C (250˚F) for 15 minutes.
5. Xylose Lysine Tergitol 4 Agar (XLT-4): XLT-4 media was prepared by suspending 59.0 grams of the XLT-4 Agar Base (Difco™, Benkitson and Dickson, MD, USA) in 1 liter of distilled water. 4.6 ml of XLT-4 Agar Supplement was added. A stir-bar was added and the bottle was heated on a hot-plate and media boiled.
6. XLT-4 Agar Supplement: 4.6 ml of this supplement (Difco™, Benkitson and Dickson, MD, USA) was added during the preparation of the XLT-4 Agar.

3.1.4 Salad Spinner

A 5 quart plastic salad spinner (OXO products, New York, NY, USA) was used to gently spin off excess inoculum suspension from the sprouts.

Figure 5: OXO Salad Spinner

3.1.5 Chlorine dioxide generation and exposure system

A ClorDiSys Minidox chlorine dioxide generation system (ClorDiSys, Lebanon, NJ, USA) was used to generate gaseous chlorine dioxide at the required concentration. The generated chlorine dioxide gas was pumped into a custom fabricated glass-walled exposure chamber.

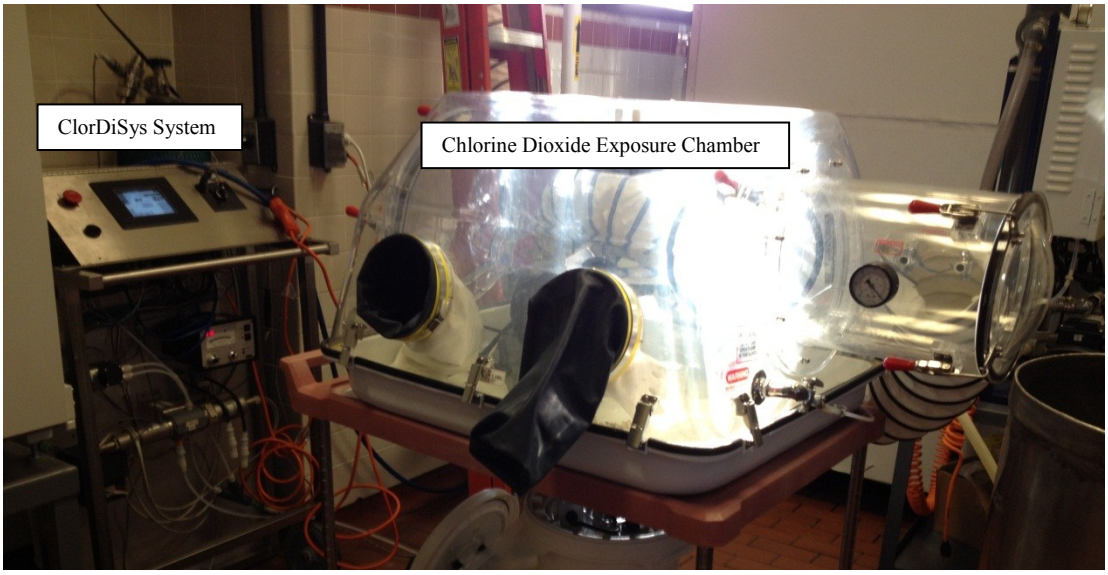


Figure 6: ClorDiSys chlorine dioxide generation system

3.1.6 Three – dimensional (3D) printer

A Fab@Home Model 1 material depositor which is more commonly known as a three-dimensional (3D) printer was used to deposit and create silicone substrates which can bio-mimic sprout physiology. This printer was sourced from NextFab Studio, (Philadelphia, PA, USA) who developed turn-key 3D printing software solutions for this application. The software Fab@Home V0.23 was used to create the three-dimensional substrate design and also functioned as the graphical user interface (GUI) between the operator and the Fab@Home Model 1 printer. The software program can accept 3D models saved with a .stf file extension. Many common 3D modeling programs such as SolidWorks™ CAD or Rhino® CAD can create 3D models with a .stf file extension and these models can be printed on the 3D printer.

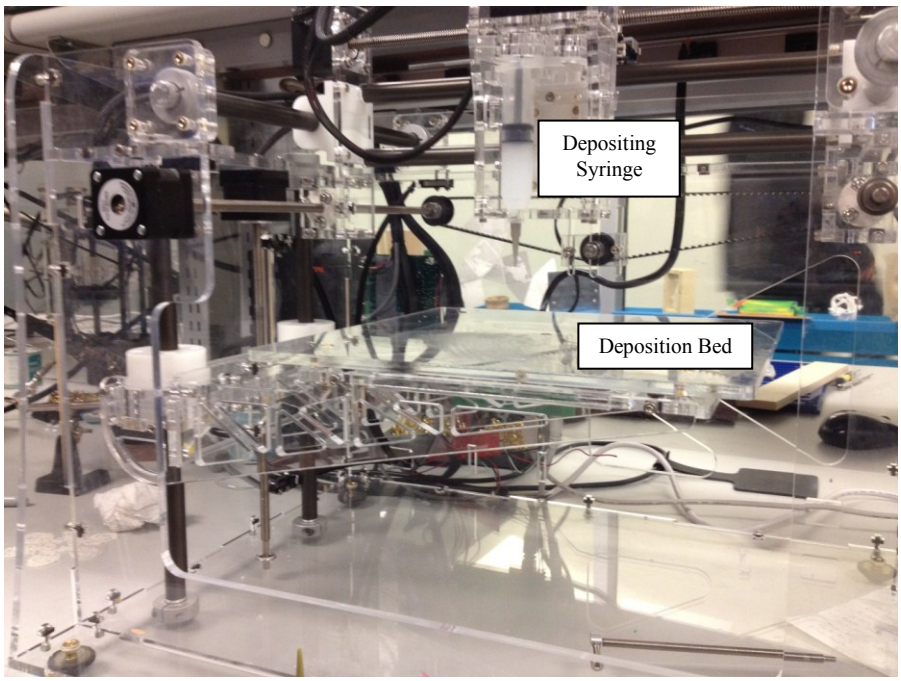


Figure 7: Fab@Home Model 1 printer

The 3D printer has two cables, a power cable which plugs into an 110V – 120V power supply and a USB connection to connect to the computer.

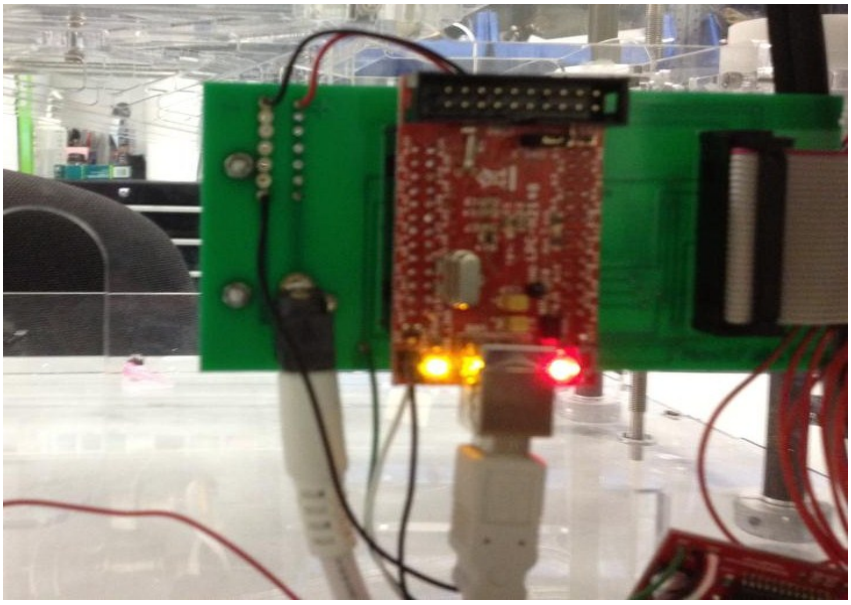


Figure 8: Power cables of the 3D printer

The components of the 3D printer are detailed in Figure 9.

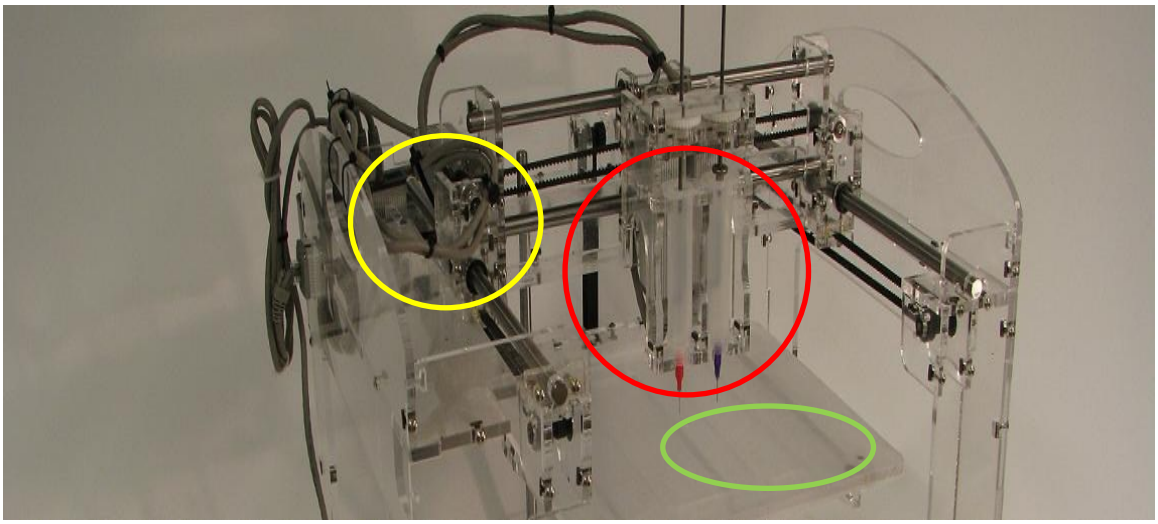


Figure 9: Components of 3D printer

- Red circle: Carriage containing two syringes and respective nozzles.
- Yellow circle: Motors which control movement of carriage along the X and Y axes.
- Green circle: Bed of the printer which can move up and down along the Z-axis.

3.2 Methods

3.2.1 Sprouting of mung bean seeds

Mung bean seeds were rinsed with deionized double distilled water till the water ran clear to remove surface debris. After draining and rinsing, sprouting was done in suitably sized food grade high-density polyethylene sprouting containers at a temperature of 30°C as mung beans can increase six-fold in size during sprouting. Spray irrigation was carried with double distilled deionized water every day so that the seeds were moist but not water soaked. Sprouting was carried out to a sprout length of 8 – 12 cm for 4 days. The sprouting containers were placed in dark conditions to avoid excessive greening of sprouts. The sprouts were refrigerated at 4°C for 24 hours following germination to slow down their respiration rate.

3.2.2 Initial estimation of microbial load on mung bean sprouts

In order to determine the base microbial loads typically present on mung bean sprouts sprouted from irrigated seeds, aerobic plate count were done in triplicate to calculate the base microbial content. This data was important to show the levels of competitive micro flora present in the sprouts consequent from the bean seeds themselves.

3.2.3 Bacterial cultures and inoculum preparation

Stock cultures of Infantis F4319, Muenchen HERV2C, and Newport H1275 *Salmonella enterica* serovars were maintained in tryptic soy broth (TSB; BBL, Sparks,

MD) containing 30% glycerol at -80°C. Working cultures were maintained on tryptic soy agar (TSA; BBL) slants containing 0.6% yeast extract (BBL) stored at 4°C for 2-4 weeks.

Infantis F4319, Muenchen HERV2C, and Newport H1275 serovars were revived by introducing a loopful of culture to Brain Heart Infusion (BHI; BBL, Sparks, MD) broth tubes. Each serovar of *Salmonella* was grown in 100 mL BHI tubes twice over a period of 48 hours. After 48 hours incubation at 30°C, the individual serovar tubes were spread plated in duplicate on the selective media for *Salmonella*, Xylose Lysine Tergitol-4 agar (XLT-4 BBL). After incubation for 24 hours at 30°C, the colonies were counted to determine the level of *Salmonella* to be in the order of 1 X 10^9 CFU/gram. The serovars were re-plated on XLT-4 media, incubated for 48 hours and inoculated on XLT-4 agar slants which served as working cultures. Fresh working cultures were prepared every week and stored at 4°C. The cultures were spun down and the pellet was re-suspended in fresh BHI medium and mixed again. Each of these tubes was then added to a master tube to create a 1:1:1 ratio of the three serovars and used for inoculation. This procedure was followed for each control experiment, aqueous and gaseous antimicrobial experiment.

3.2.4 Inoculation of mung bean sprouts

As this research focusses on the ease of contamination of sprouts during sprouting, 200 grams of mung bean sprouts were dipped in a 500mL 1:10 dilution of the cocktail with distilled water (this was determined to contain *Salmonella* level 1 X 10^8 CFU/mL). Immediately, sprouts were gently spun in a salad spinner for 2 minutes to remove excess inoculant and the sprouts were placed under a laminar flow hood and

allowed to dry for 4 hours. Figure 10 shows the inoculation procedure visually. The sprouts were sampled in triplicate for microbial assays 24 hours post-inoculation.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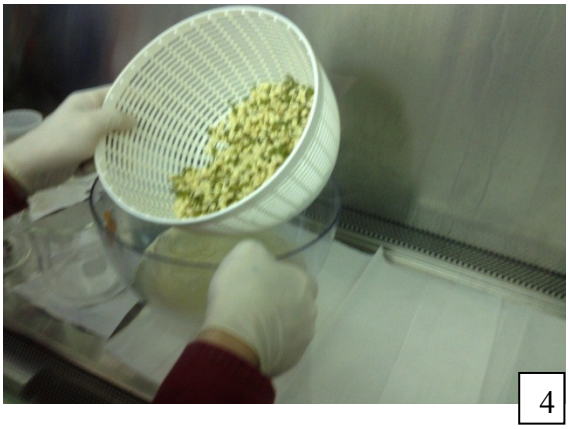
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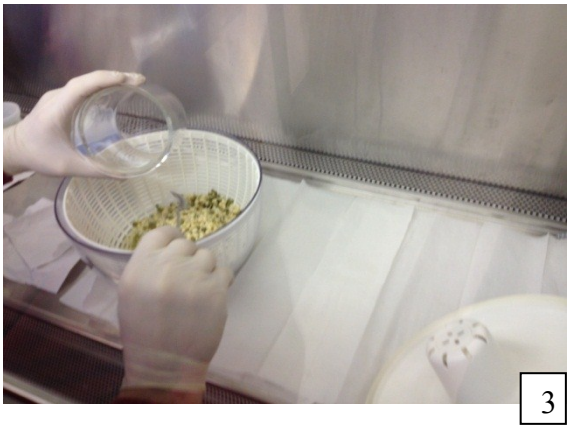
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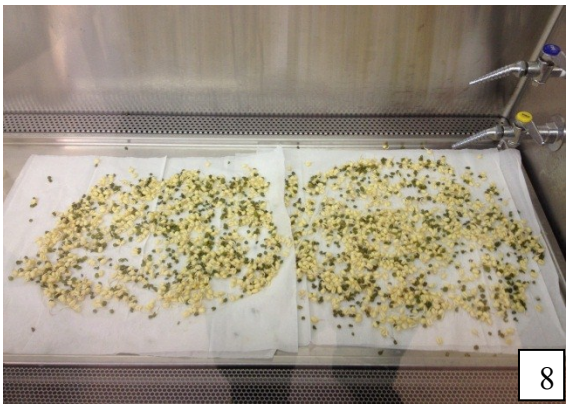
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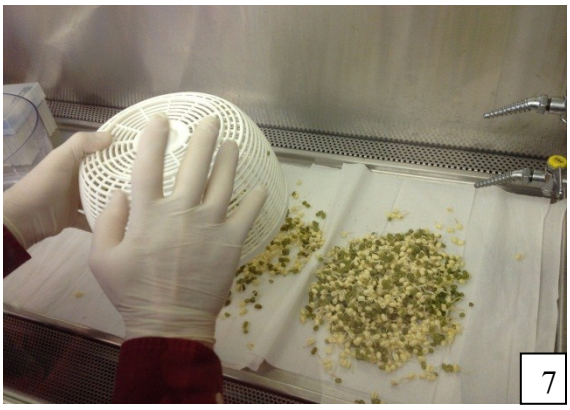
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Figure 10: Inoculation of mung bean sprouts

3.2.5 Aqueous chlorine treatment

In order to simulate a commercial cleaning procedure, the inoculated sprouts were gently rinsed for 30 minutes in a high-density polyethylene container with 200ppm (volume of chlorine /volume of water basis) chlorine (added as 15mL/gallon of water of household bleach containing 5.25% sodium hypochlorite). The chlorine treatment was carried out 6 hours after inoculation to allow time for bacterial internalization. The sprouts were sampled in triplicate.

3.2.6 Gaseous chlorine dioxide treatment

The advantage of chlorine dioxide gas (ClO_2) is that its efficacy is less affected by pH and organic matter and it is does not react with ammonia to form chloramines, as do liquid chlorine.

The inoculated sprouts were placed in a closed container inside a double walled glass chamber. Chlorine dioxide gas was generated using the ClorDiSys generation system. This gas was allowed to continuously flow into a double glass-walled enclosure till the concentration inside the chamber attained 0.5mg/L (or 185ppm volume of gaseous chlorine dioxide/volume of air basis) of chlorine dioxide at a temperature of 22.4°C and a relative humidity of 50-51%. The closed container was opened and the sprouts were spread in a single layer on the bed of the chamber and exposed to the chlorine dioxide gas for a period of 15, 30 and 60 minutes respectively.

3.2.7 Gaseous chlorine dioxide treatment with tumbling

The effect of increasing the available surface area of exposure of the sprouts was also tested by placing a tumbling machine in the enclosed glass chamber. This was sterilized under constant flow of chlorine dioxide. The inoculated sprouts were introduced into the tumbler and exposed to the chlorine dioxide gas while tumbling continuously for a period of 15, 30 and 60 minutes respectively as shown in Figure 11.

Figure 11: Gaseous chlorine dioxide treatment along with tumbling of inoculated sprouts

3.2.8 Surface Electron Microscopy of sprouts to study the influence of surface geography on antimicrobial efficacy

Scanning electron microscopy (SEM) has been used successfully to examine the attachment of spoilage or pathogenic micro-organisms to food and food contact surfaces. 5mm X 5mm longitudinal sections of bean sprout were cut using a sterile blade. The samples were fixed on clear plastic plates and subjected to dehydration in gradually increasing ethanol concentrations (10%, 20%, 50%, and 100%) for on a shaker table. Fixed samples were dried using a critical point dryer with liquid CO_2 as a transition gas and sputter-coated with a thin layer of gold coating. The samples were then observed under SEM for the distribution of *Salmonella* spp. Observation of the interior surfaces was carried out by fracturing the critical point dried samples with light impact [60].

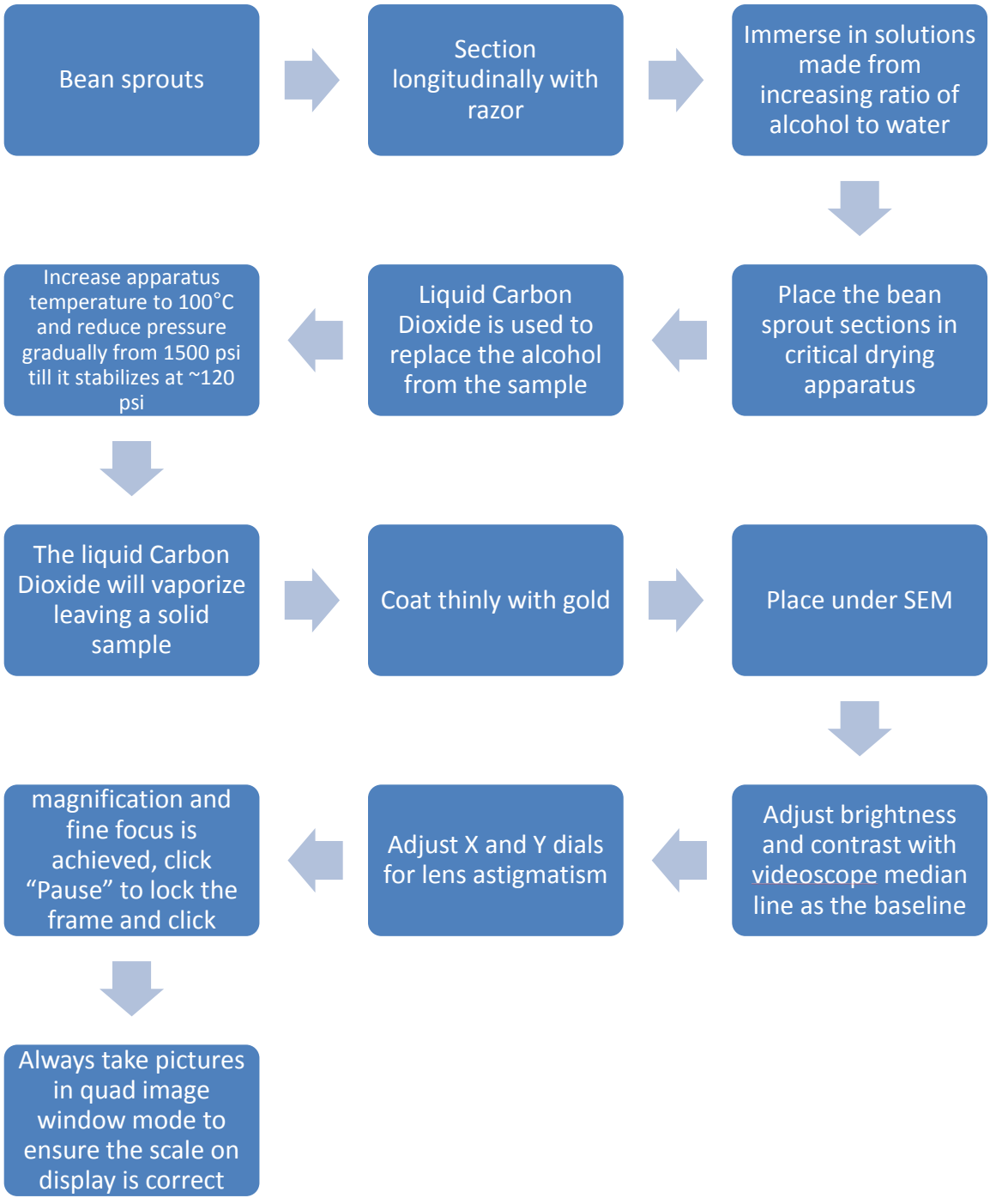


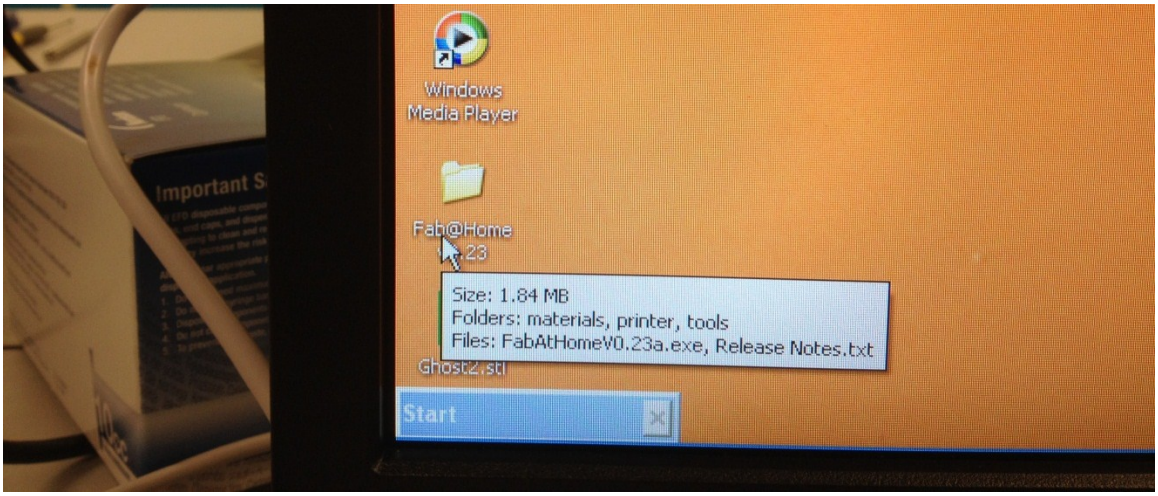
Figure 12: Surface Electron Microscopy of sprouts to study the influence of surface geography on antimicrobial efficacy

3.2.9 Fab@Home Model 1 printer setup and methodology

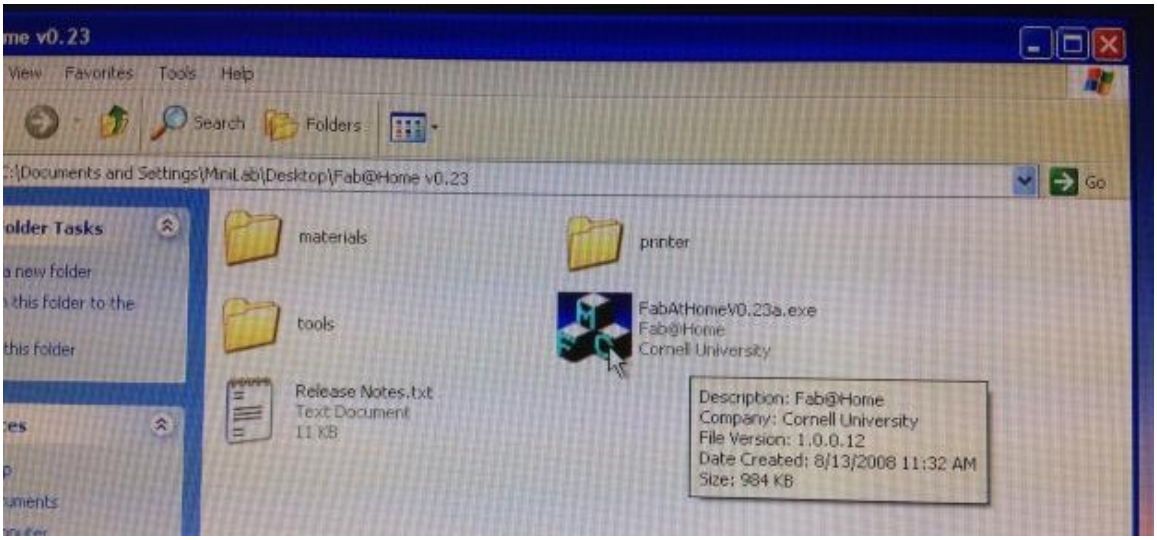
There was no previous literature present in the utilization of 3D printers for printing bio-mimicking substrates. The methodology to setup the Fab@Home Model 1 printer for printing 3D sprouts was designed after much trial and error regarding software calibration, substrate material selection, flow parameters of the flow material, degree of accuracy of printing.

After repeated experimentation and refining, the following method was developed to create bio-mimicking substrates:

- After making sure all the cables of the printer are correctly connected, double click to open the Fab@Home V0.23 folder to access the software .exe file.



- Double click to run the Fab@Home V0.23a.exe software program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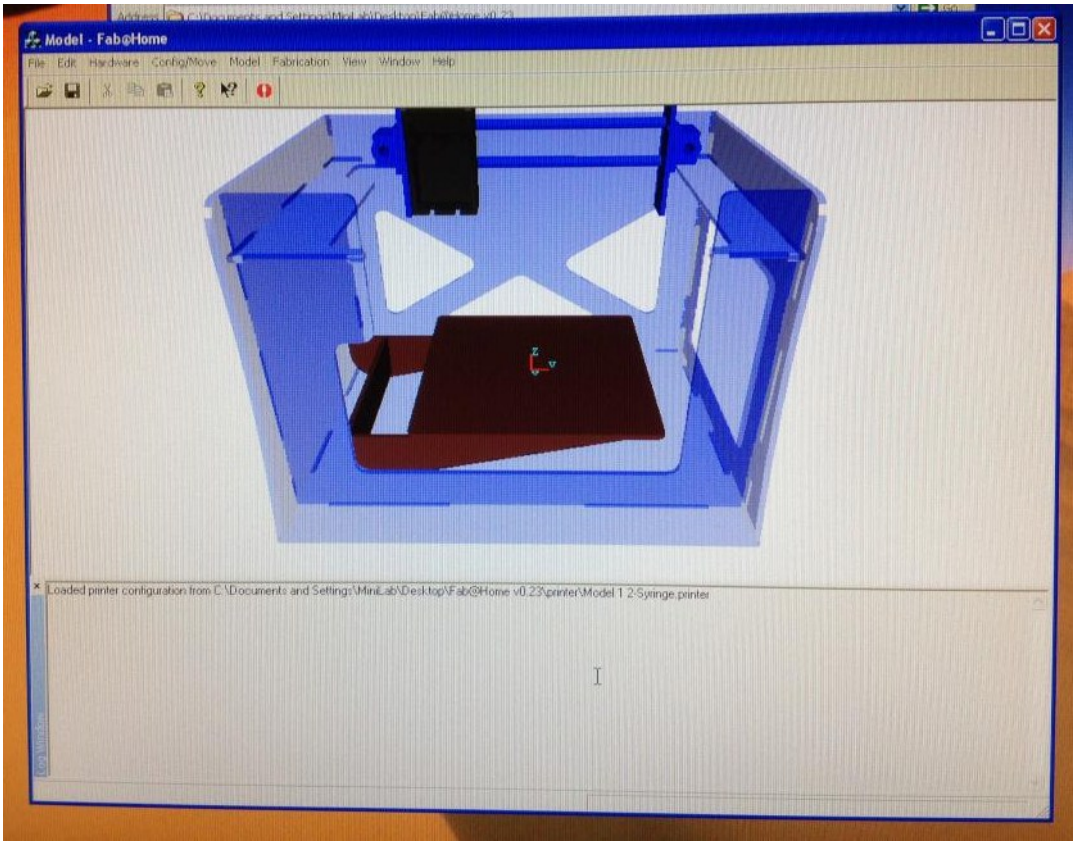
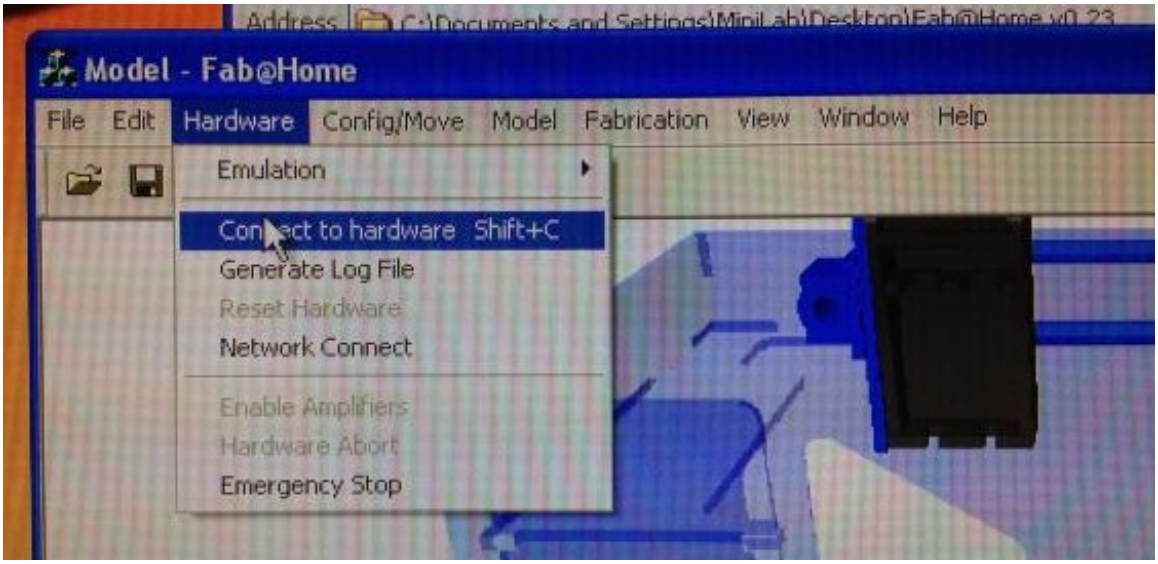
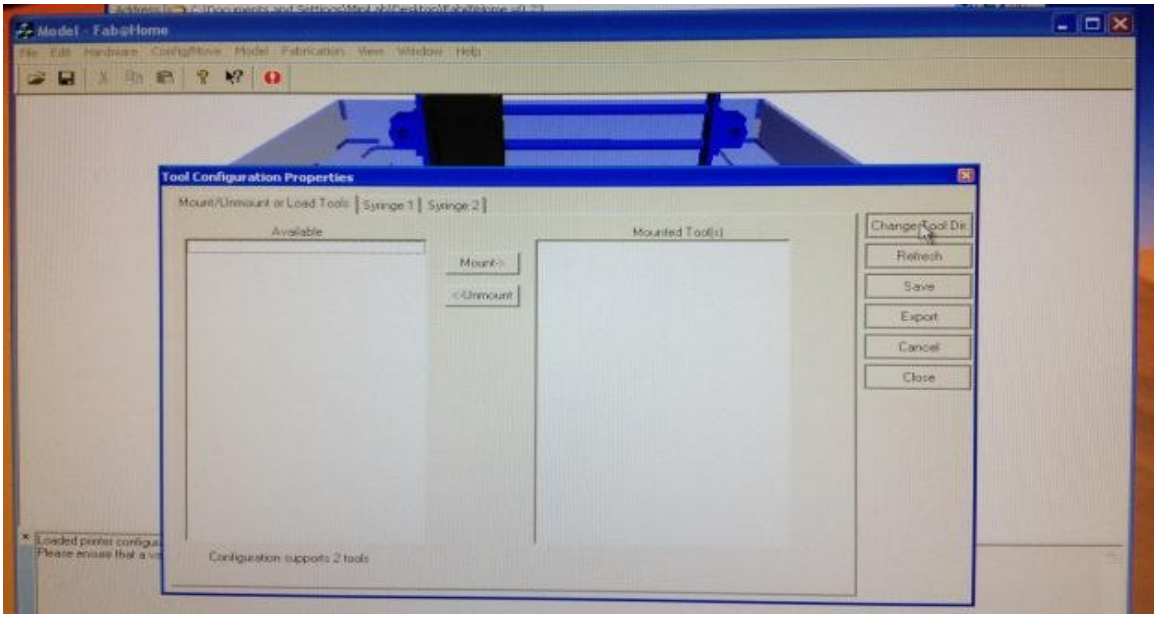
- Successful running of the program will cause the following screen will load.



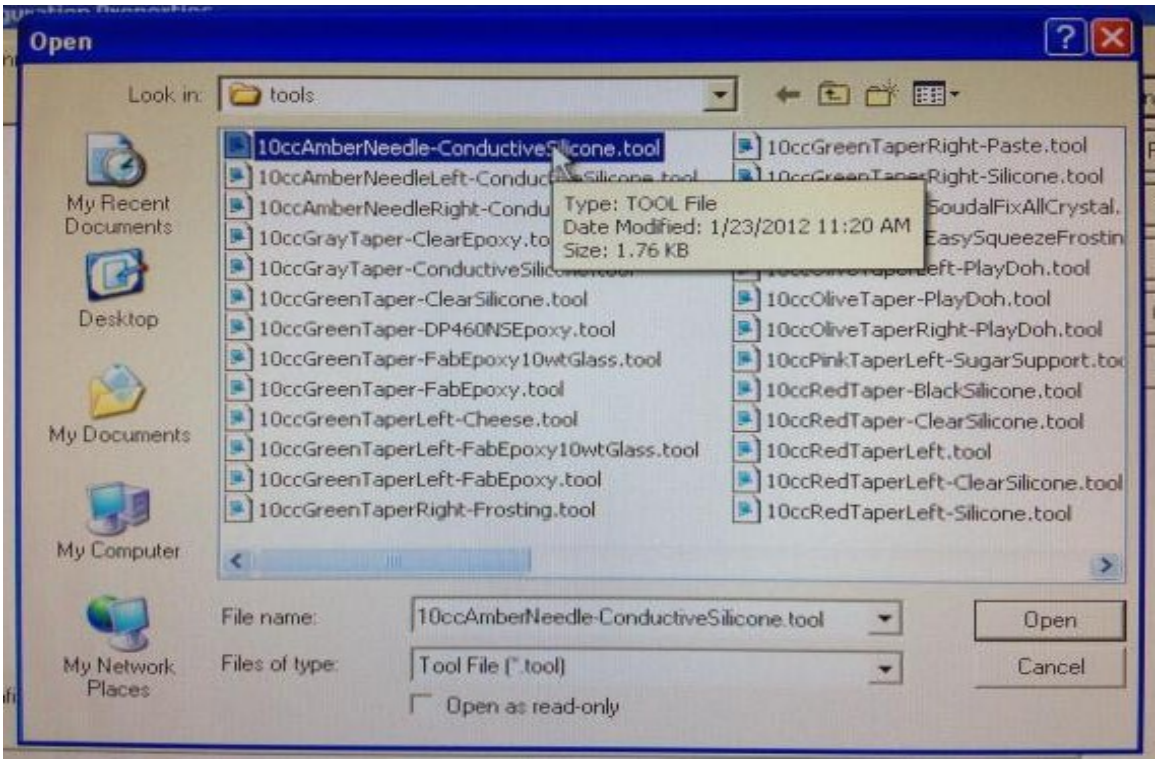
- Select **Hardware** from the top row of tabs and then click **Connect to Hardware**. This instructs the software to connect to the printer.



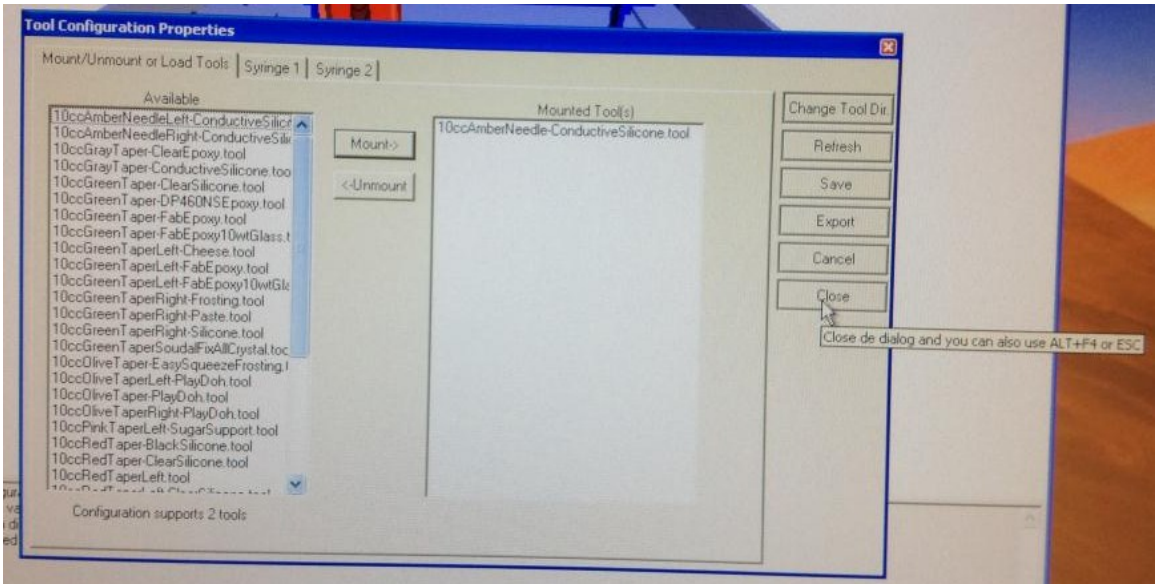
- Select **Config/Move** from the first row of tabs and click **Tool Configuration**. On the following window (**Tool Configuration Properties**), select **Change Tool Directory**. This step opens a folder of preset "tools" which are text files with instructions regarding the properties of the printer elements. The preset tools can be tweaked appropriately and new tools can be programmed as per requirements in a text editor.



- Select any tool and click **Open**

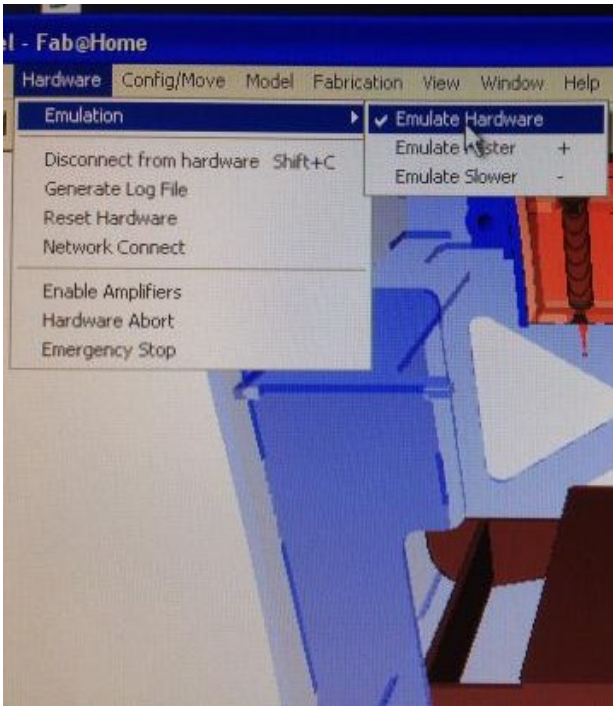


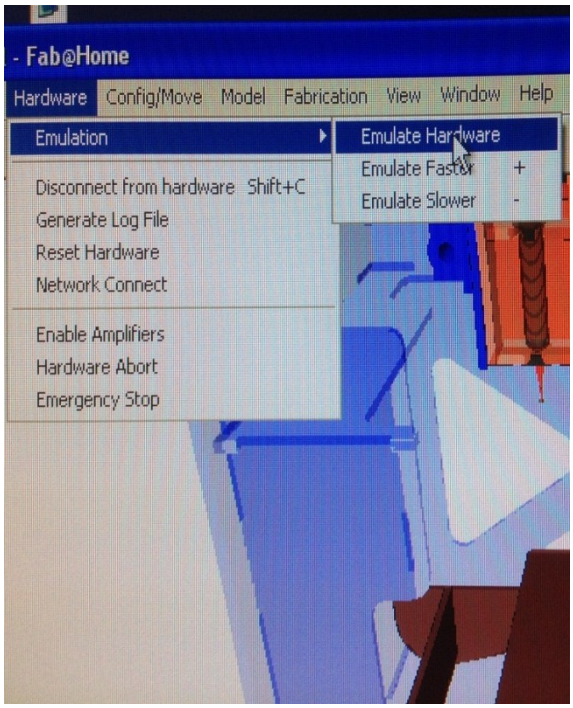
- This populates the **Tool Configuration Properties** window with ALL the available tools. **Mount** the appropriate tool for the printing task from here and click **close**.



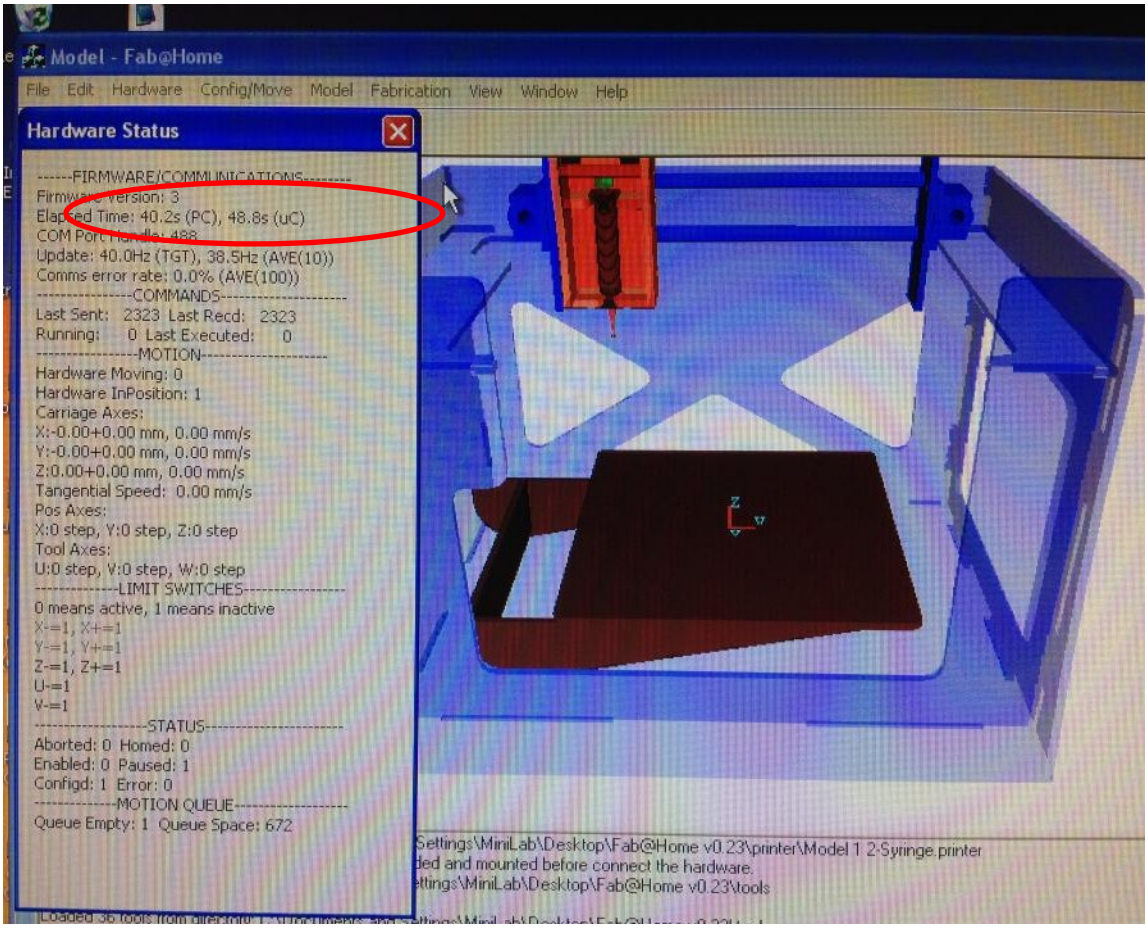
- Once again, select **Hardware** from the top row of tabs and then click **Connect to Hardware**.

- Select **Hardware** and select **Emulation**. Disable the **Emulate Hardware** so that the check mark is removed.

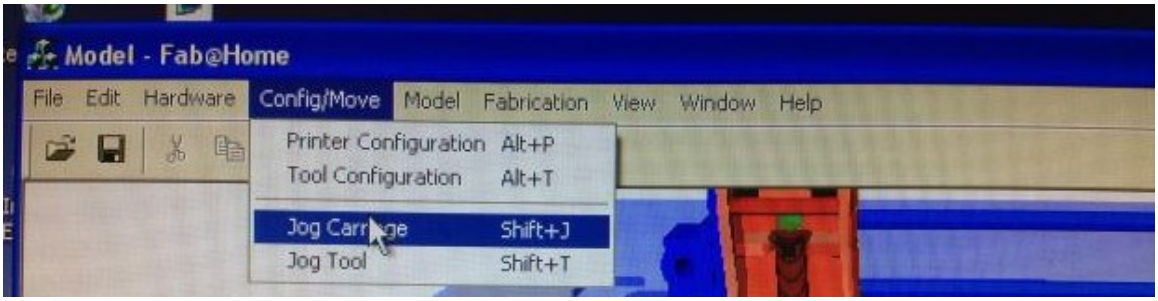




- Select **View** and then click **Show Hardware Status**. The following window should show up. If **Elapsed Time** is incrementing, then, the tools have been loaded correctly and the printer is connected to the computer.



- Close the **Hardware Status** window and select **Config/Move**. Then, select **Jog Carriage** to bring up the carriage movement control window.



- The Carriage holds the printing head and can move in X and Y axes. The base/bed of the printer moves up and down along the Z axis.
- On the **Jog Carriage** window, clicking **Find Home** will move the Carriage to the **Home** position which is usually a corner of the bed.

- Set “Commanded” values of 100 and 100 in the X-axis and Y-axis and hit Enter on your keyboard. Wait for the carriage to move to approximately the center of the printer.
- **The movement of the carriage is relative to the current position of the carriage. E.g., entering a X-axis value of 50 and Y-axis value of 50 will move the carriage 50 units on the X and Y axes from the current position of the carriage.**
- The **Set Safe** button allows the carriage to return to a safe height after finishing printing so that the printer head does not disturb the printed model. Enter an appropriate positive value in the Z-axis “Commanded” button to move the bed vertically up from the base position and click **Set Safe**. Now click the **Find Safe** button to move the bed to the set safe position whenever required.
- Tape wax paper on the printer bed on an appropriate area so that it is easy to collect the model and clean the bed.
- Slowly move the bed higher up such that the distance between the bed and the syringe tip is approximately **half the diameter of the syringe tip** used.
- Once the previous steps have been carried out and the carriage is an appropriate place over the printer bed, click **Set Origin** to indicate to the software the current position of the carriage as the printing origin point.
- Click **Go to Origin** for the carriage to adjust its position and now the printer is ready to print.

4. RESULTS AND DISCUSSION

4.1 Initial estimation of microbial load on mung bean sprouts

The Aerobic plate count estimation was in the order of 1 X 10^9 CFU/gram. This is in accordance with Viswanathan (2001) who found that the average aerobic plate counts for salad vegetables, fruits and sprouts were greater than 10^{10} CFU/gram and 10^9 CFU/gram respectively [58].

4.2 Efficacy of gaseous application of chlorine dioxide on *Salmonella* spp. inoculated mung bean sprouts

Figure 13 shows the effect of 0.5mg/L (185ppm volume of gaseous chlorine dioxide/volume of air basis) gaseous chlorine dioxide on *Salmonella* spp. inoculated mung bean sprouts at different exposure times.

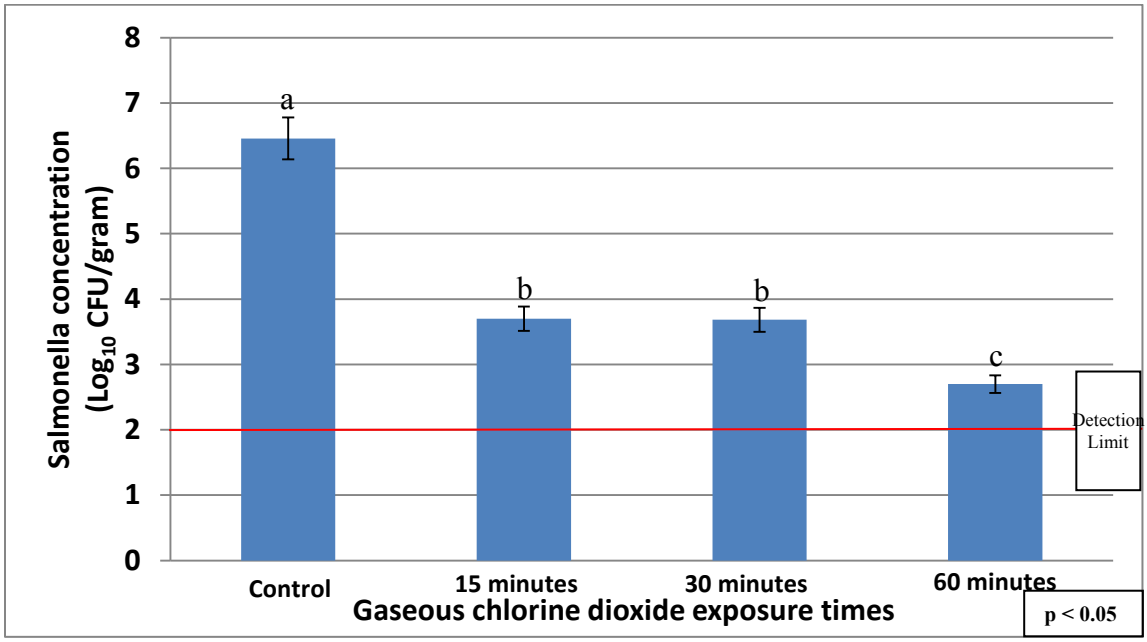


Figure 13: Efficacy of gaseous application of chlorine dioxide on Salmonella spp. inoculated mung bean sprouts

The control samples which were inoculated sprouts with no gaseous chlorine dioxide treatment had *Salmonella* levels of 6.5 $\log_{10}$ CFU/gram. The 15 and 30 minute exposure times resulted in log reduction of 3 $\log_{10}$ CFU/gram. This is in accordance with Kostrzynska et al's work (2001) where irrigation of mung bean sprouts with 0.2ppm gaseous ozone resulted in 4 $\log_{10}$ reduction in coliforms [27]. The difference in *Salmonella* concentrations between the 15 and 30 minute exposure times was not significant. Exposure to gaseous chlorine dioxide at the same concentration (0.5mg/L) for 60 minutes however reduced the *Salmonella* populations to 2.5 $\log_{10}$ CFU/gram which is a 4 $\log_{10}$ reduction. This experiment proved effective compared to Gandhi's work (2003) where daily spraying of alfalfa sprouts with aqueous chlorine (100 mg/L) led to reduction of less than 2 $\log_{10}$ in the population of salmonella at day 4 of sprouting [19]. Kim et al (2009) observed that the combined treatment of 50 ppm ClO_2 and 0.5% fumaric acid reduced the initial populations of *Escherichia coli* O157:H7, *Salmonella Typhimurium*, and *Listeria monocytogenes* inoculated on broccoli sprouts by 2.39, 2.74, and 2.65 $\log_{10}$ CFU/gram, respectively, compared to the control [26].

The results show that gaseous chlorine dioxide is effective in reducing *Salmonella* concentrations by 2 $\log_{10}$ CFU/gram more than conventional washing with chlorine solution. This can be explained by the higher diffusion capability of a gaseous antimicrobial. A useful corollary of the increased efficacy can be that less antimicrobial needs to be used and exposed to the sprouts in a gaseous versus aqueous scenario and this can reduce the risk of chlorine exposure to industry personnel.

4.3 Comparison of aqueous chlorine and gaseous chlorine dioxide treatment

200ppm of aqueous chlorine was applied to the inoculated mung bean sprouts using a gentle rinse process for 30 minutes. As Figure 14 shows, this simulation of a commercial process showed a reduction of 2 $\log_{10}$ CFU/gram *Salmonella* concentration versus a reduction of 4 $\log_{10}$ CFU/gram *Salmonella* concentration for the 60 minute gaseous chlorine dioxide treatment. This indicates that the gaseous treatment may be more effective in reaching internalized *Salmonella* microorganisms. Beuchat et al (1998) observed a reduction of 1.9 $\log_{10}$ CFU/gram upon treatment of *Salmonella* inoculated seeds with a 200ppm chlorine wash for 30 minutes [7]. Taormina (1999) reported that free chlorine in a 200ppm solution reduced by 90% to about 20ppm within 15 minutes [52].

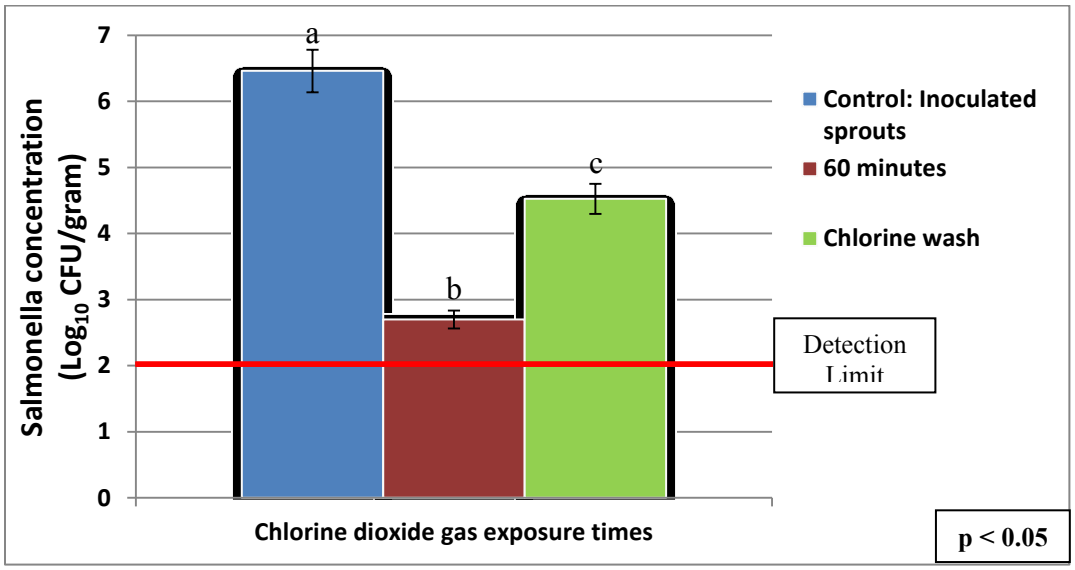


Figure 14: Comparison of aqueous chlorine and gaseous chlorine dioxide treatment

4.4 Tumbling and gaseous chlorine dioxide treatment

Tumbling the sprouts while exposing them to gaseous chlorine dioxide resulted in increased efficacy and *Salmonella* reduction. Tumbling the inoculated mung beans sprouts during the gaseous chlorine dioxide application for 15, 30 and 60 minutes resulted in the reduction of 3.0, 4.0 and 5.5 $\log_{10}$ CFU/gram *Salmonella* compared to the 4.5 $\log_{10}$ reduction seen in gaseous application alone. The control sample in both cases had 6.5± 0.2 $\log_{10}$ CFU/gram levels of *Salmonella* spp. The chlorine dioxide in both cases was applied at a concentration of 0.5mg/L. Consequently, there seems to be a correlation with increasing the sprout surface area with decreasing numbers of *Salmonella* spp. This shows that gaseous treatment of ClO_2 along with tumbling can achieve below detection limit (BDL) but the commercial feasibility of this process still needs to be explored.

The increased efficacy may be attributed to the increased surface area available for antimicrobial activity due to the tumbling. The increased efficacy of gaseous antimicrobial combined with the tumbling treatment can be visualized in the Surface Electron Microscopy images.

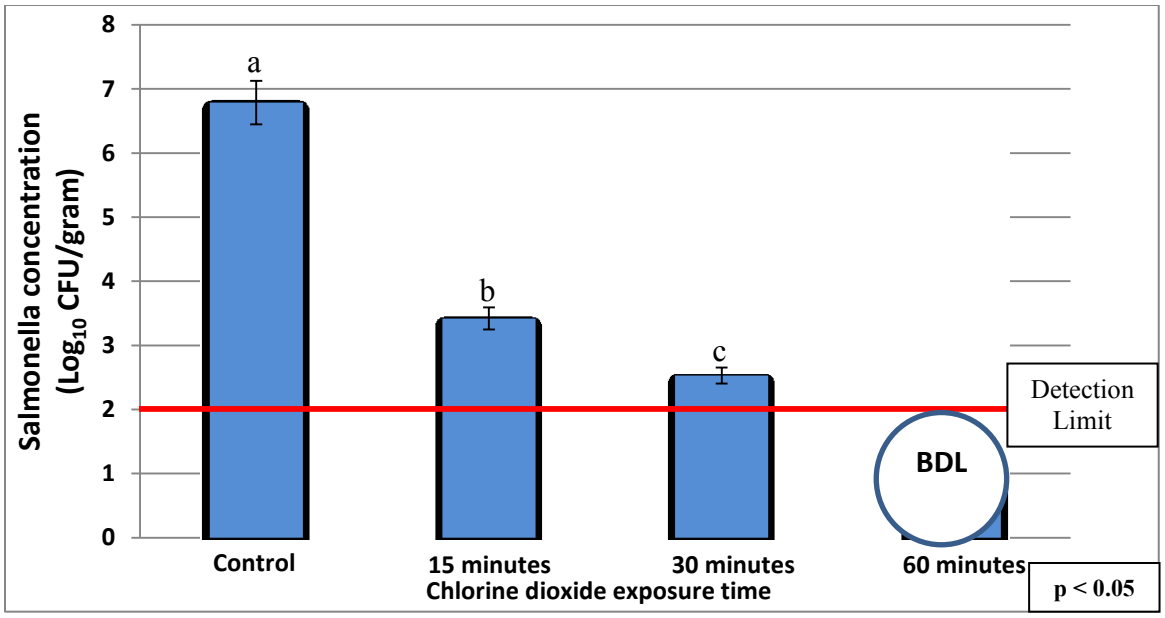


Figure 15: Gaseous chlorine dioxide exposure by tumbling

Table 6 is a summary of the log reductions obtained from all the three variables. From this table, it is clear to see that the gaseous application combined with tumbling has the largest log reduction.

Table 6: Log reductions from different variables compared to control (no treatment) (All values in log_{10} CFU/gram)

Treatment	Aqueous	Gaseous	Gaseous + Tumbling
15 minutes	NA	3	3
30 minutes	2	3	4
60 minutes	NA	4	5.5

4.5 Surface electron microscopy of bean sprouts

The surfaces of bean sprouts are covered by wrinkles and pores. Green leafy produce have trichomes and hairs apart from stomata which can increase the risk of

internalization of microorganisms. Studies have shown that internalized microorganisms in cracks and crevices were unaffected by chlorine washing [7]. The cuticle on surfaces of fruits and vegetables being hydrophobic also can protect microbial cells from antimicrobial exposure. The efficacy of vapor antimicrobial compared to liquid antimicrobial is related to the surface morphology of bean sprout. This matrix provides a reliable repeatable medium for inoculating microbes and measuring antimicrobial efficacy.

As microbial assays destroy the sample, it is unable to be determined if the *Salmonella* levels are from the surface or the interior. This is where SEM is extremely useful in determining the efficacy of antimicrobial on internalized bacteria and to determine whether surface geography of the sprouts hinders the efficacy of chlorine compounds in microbial inactivation [44].

The following images show *Salmonella* localized on surface and interior of bean sprouts and their consequent reduction after chlorine dioxide gas treatment.

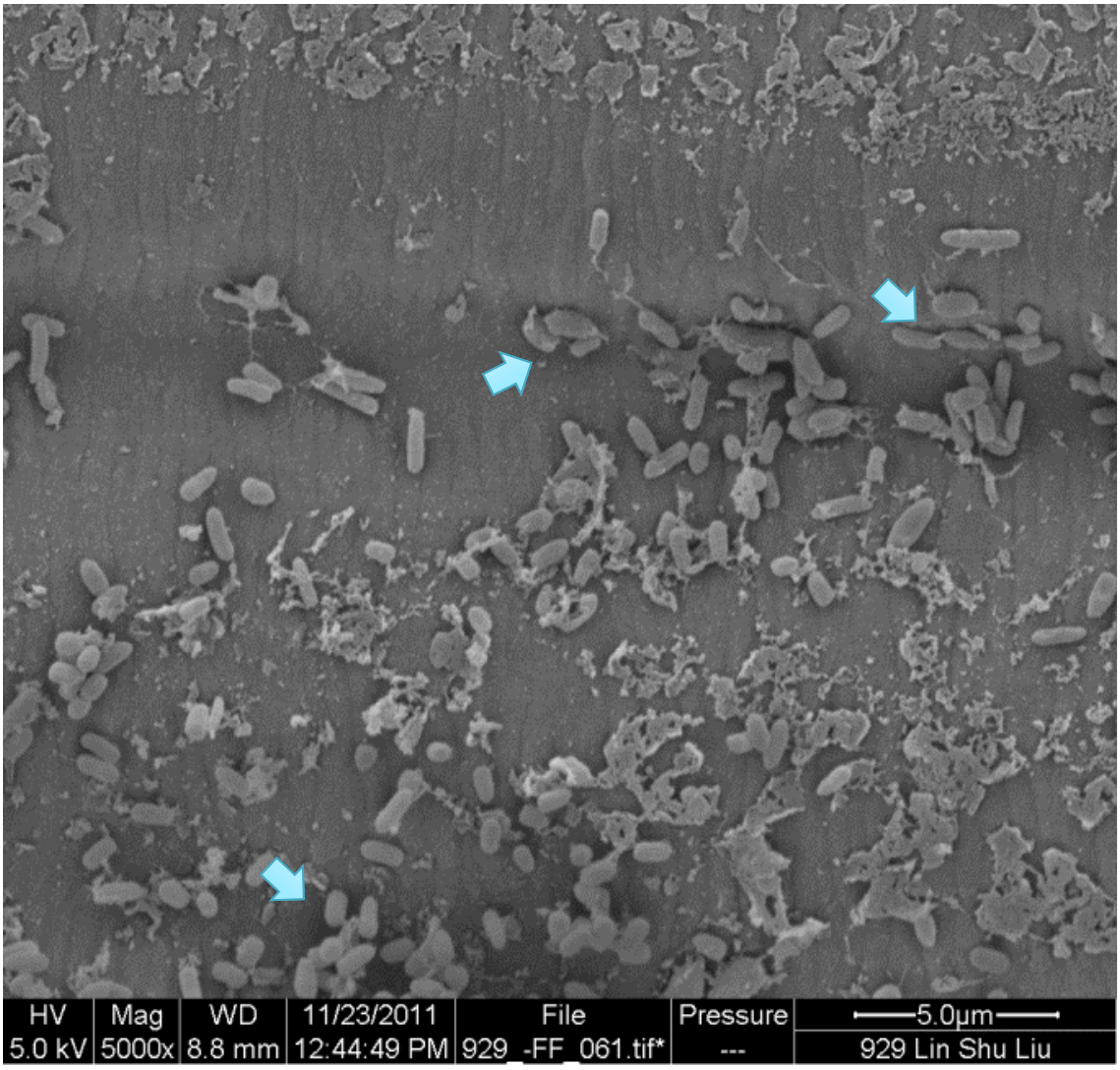


Figure 16: 5000X magnification of *Salmonella* inoculated sprouts showing bacteria on surface of sprout (arrows show bacteria)

The rod shaped structures are the *Salmonella* species.

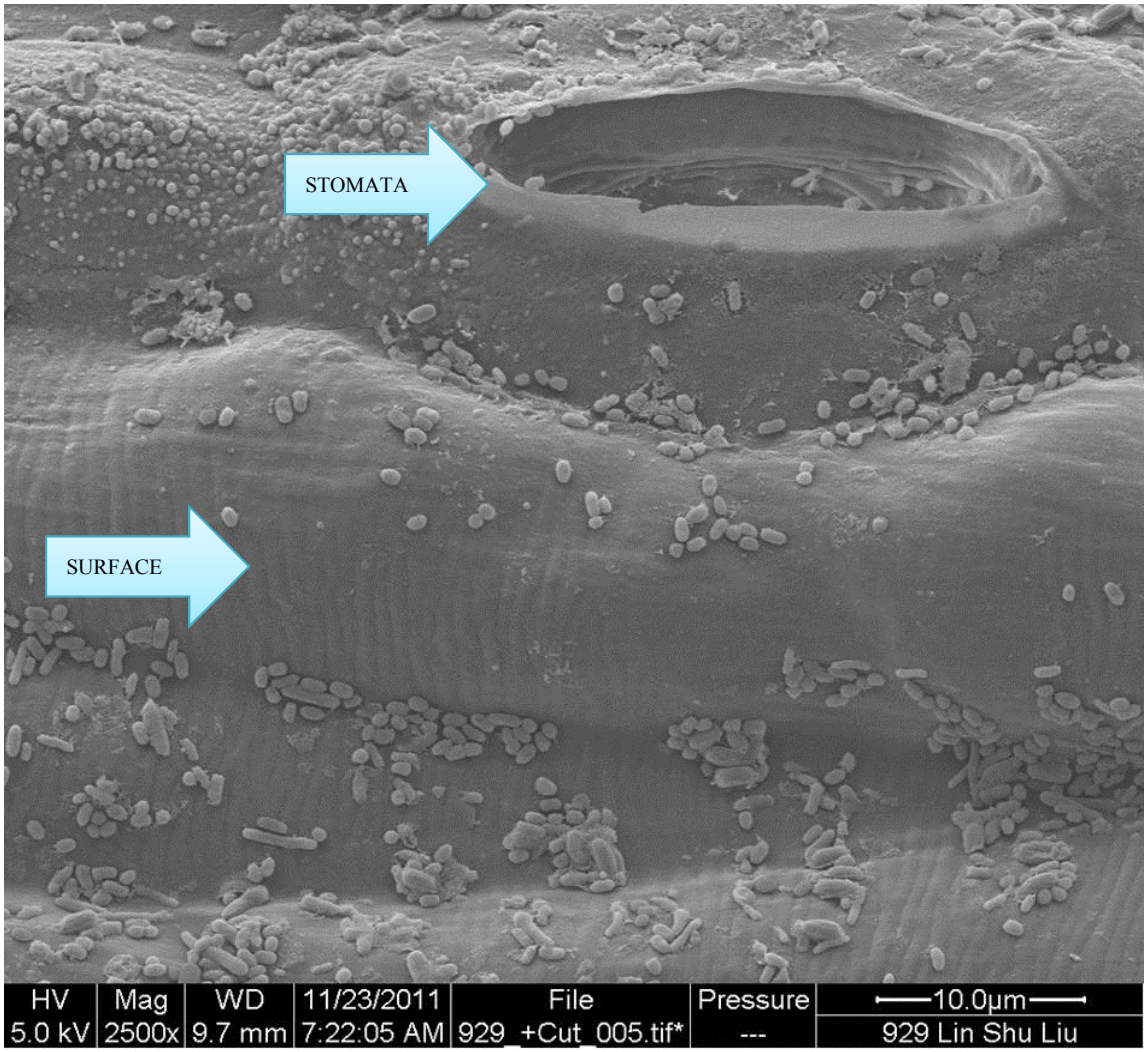


Figure 17: 2500X magnification of *Salmonella* inoculated sprouts showing bacteria on surface and interior of sprout

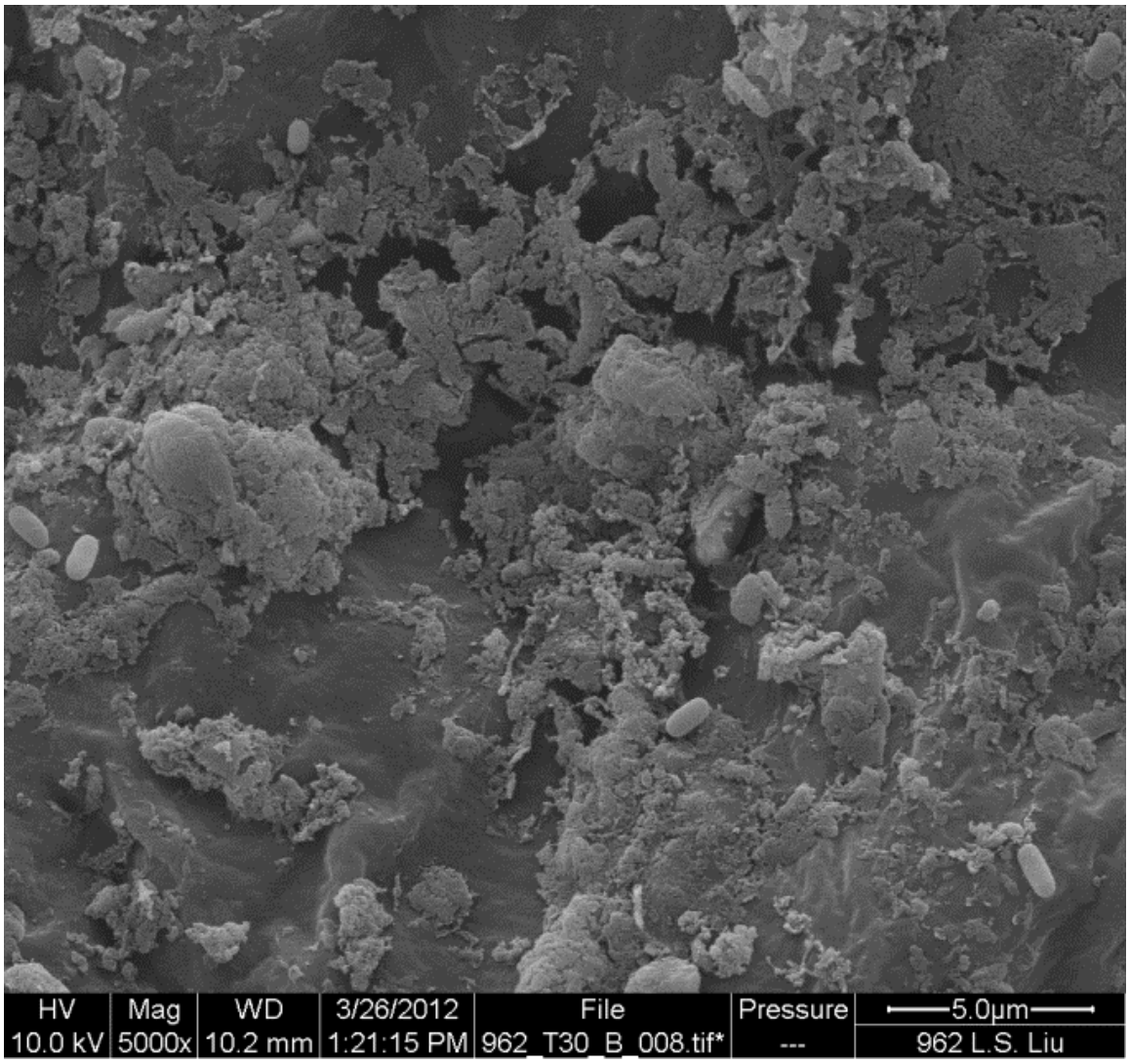


Figure 18: 5000X magnification of gaseous chlorine dioxide treated sprouts showing bacteria reduction on surface of sprout

Reduction in concentrations of *Salmonella* cells was observed on exterior surfaces of bean sprout stalks and roots. Biofilm formation was also noticed in many places. This may be due to the dehydration process in SEM preparation which leads to the mucilage getting stripped down to a web like structure. Some researchers have noted that bacteria often exuded substances that corroded the plant tissues and made themselves internalized

into the sprout tissue. This could explain the higher concentrations of bacteria in the root regions as the sprout releases nutritious exudates during growth.

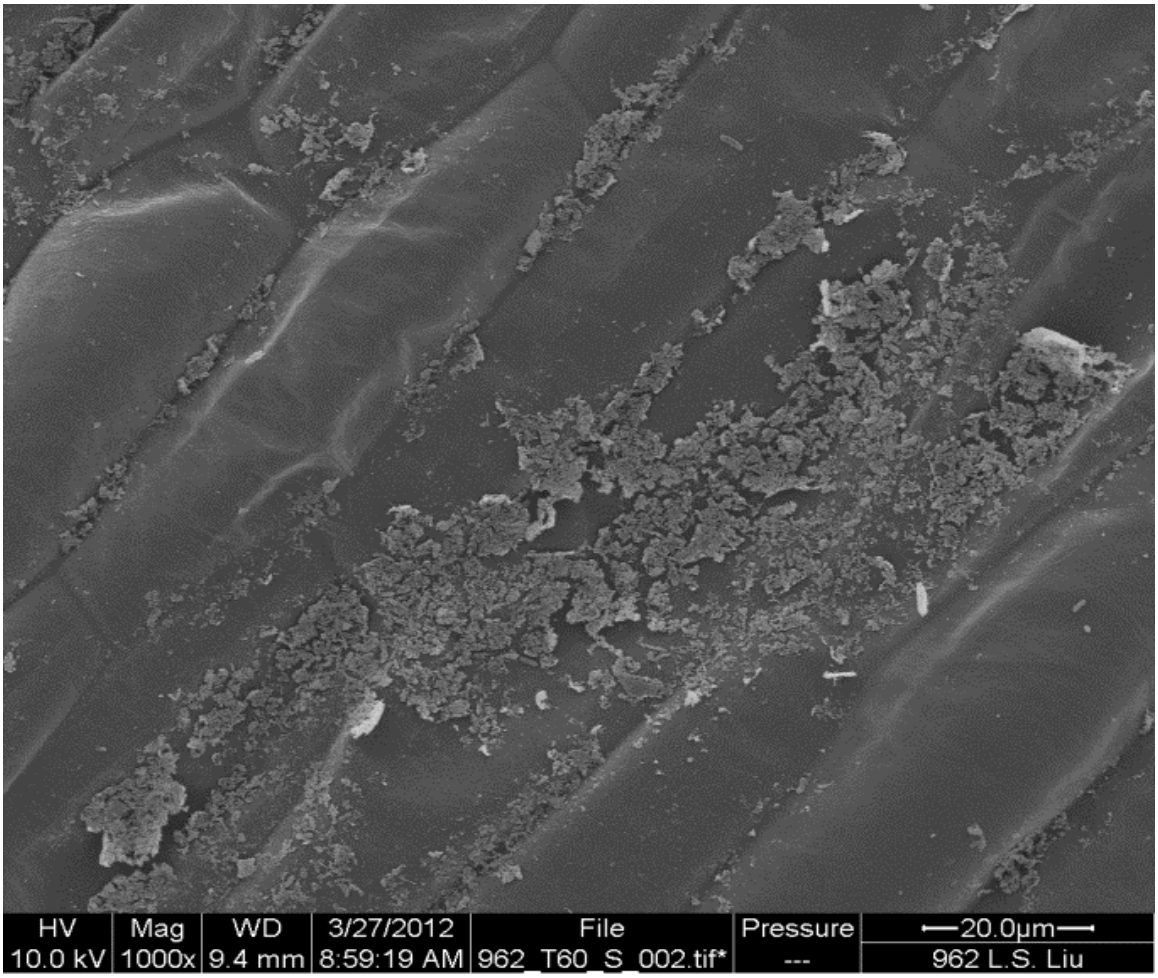


Figure 19: 1000X magnification of gaseous chlorine dioxide treated sprouts showing bacteria reduction on interior of sprouts

4.6 Designing and 3D printing synthetic matrices

A new methodology was successfully developed to setup the Fab@Home Model 1 printer for printing 3D sprouts. This was designed after much trial and error regarding software calibration, substrate material selection, flow parameters of the flow material, degree of accuracy of printing.

SolidWorks™ CAD software was used to create three-dimensional STL (STereoLithography) files. The surface physiology of sprouts was simulated to reduce the experimental variability due the varying microorganism distribution on fresh produce.

Due to the early pipeline nature of this work and to reduce the number of variables and consequent complexity, the assumptions made during the creation of the models were:

- Always open stomatal apertures, no chloroplasts and absence of cuticle in sprouts
- Average stomatal pore length: ~10μ
- Average stomatal pore width: ~5μ

With this set of assumptions, three-dimensional STL files were created. Silicone was determined as the substrate material suitable for three-dimensional printing of sprout surfaces based on the flow viscosity of common three-dimensional printing materials and also the ease of setting of the substrate. The STL files were loaded into the Fab@Home Model 1 printer software and the printer was prepared for printing as per the validated methodology. Figure 20 shows the deposition of the silicone material onto the printer bed and the gradual layer-by-layer development of the sprout surface.

Due to the limitations of the syringe nozzle size and flow parameters of silicone, the surface physiology attained was relatively simplistic.

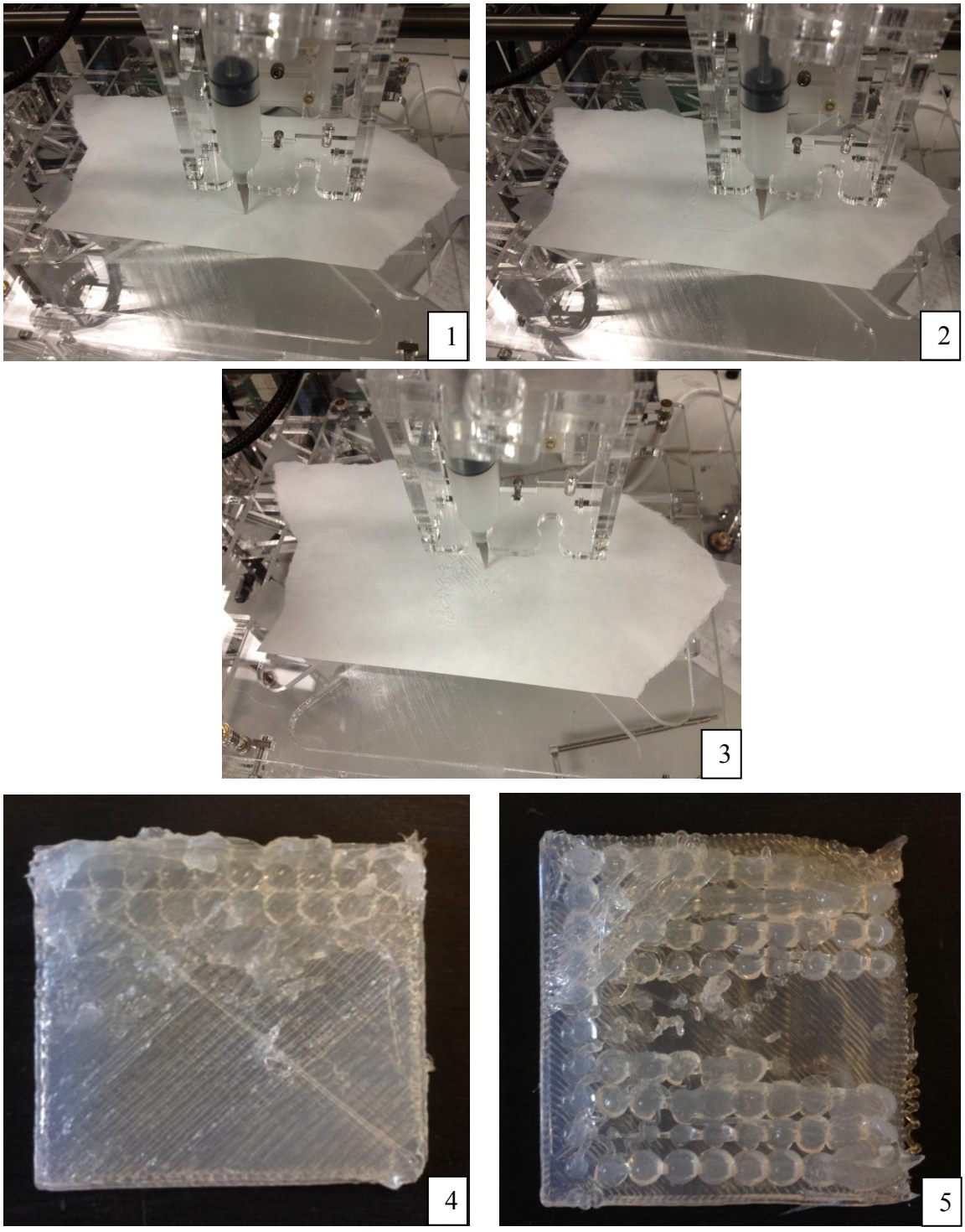


Figure 20: Deposition of silicone material to form substrates

5. CONCLUSIONS

This research has contributed to understanding the importance of the physical phase of the antimicrobial treatment and available surface area of the sprouted mung beans for antimicrobial effectiveness. This is correlated to the research rationale of understanding the complexity of sprout physiology as a probable reason for the localization of microbes and limited effectiveness of antimicrobial treatments.

- Rinsing of inoculated sprouts in 200ppm of aqueous chlorine can produce up to 2 $\log_{10}$ reduction from a control level of 6.5 $\log_{10}$ CFU/gram *Salmonella* serovar cocktail of Infantis F4319, Muenchen HERV2C, and Newport H1275
- Gaseous exposure of 0.5mg/L concentration chlorine dioxide resulted in a log reduction ranging from 3 $\log_{10}$ CFU/gram for the 15 and 30 minute exposure times to 4 $\log_{10}$ CFU/gram *Salmonella* concentrations for the 60 minute gaseous chlorine dioxide treatment.
- Tumbling the inoculated mung beans sprouts during the gaseous chlorine dioxide application for 15, 30 and 60 minutes resulted in the reduction of 3.0 , 4.0 and 5.5 $\log_{10}$ CFU/gram *Salmonella* compared to the 3.0, 3.0 and 4.5 $\log_{10}$ reduction seen in gaseous application alone. As time of exposure increases, the log reduction also increases.
- Greater reduction in *Salmonella* concentrations was observed going from an aqueous based antimicrobial to a gaseous antimicrobial to a gaseous antimicrobial paired with a tumbling treatment. Increasing the surface area available to action by the antimicrobial had a clear benefit. The log reduction increased as the available surface area increased. These points to the important role that sprout

physiology may play in harboring and sustaining the growth of microbial populations.

- As sprout physiology is complex and facilitates multiple internalization scenarios, this heightens the importance of gaseous antimicrobial because the gaseous antimicrobial can permeate to the internalized microorganisms due to the greater diffusivity.
- Tumbling provides avenues for increasing the surface area available for antimicrobial coverage.
- Surface electron microscopic imaging helped corroborate the microbial assay data that antimicrobial treatments were able to reduce the *Salmonella* spp. populations not just on the surface of the sprouts, but also, the internal regions and stomata of the sprouts.
- A new methodology was successfully developed to setup the Fab@Home Model 1 printer for printing 3D sprouts
- The substrate material suitable for three-dimensional printing of sprout surfaces was determined based on the flow viscosity of common three-dimensional printing materials and also the ease of setting of the substrate.
- Three-dimensional models were created for sprout surface physiology and the deposition method was designed and evaluated for the specific model and the specific substrate.

6. FUTURE WORK

- Chlorine dioxide efficacy in affecting internalized microorganisms should be tested in a larger exposure chambers which can simulate commercial chambers. These can include using larger scale chlorine dioxide generators such as Cloridox-GMP.
- Similarly, down-scaling this antimicrobial technology into in-package generation and delivery systems can allow for sustained antimicrobial impact and creation of Modified Atmospheres. These technologies may provide economies of scale for smaller fresh produce manufacturers who cannot invest in the larger chlorine dioxide generation and dispersion systems.
- The molecular mechanism behind the survival of internalized *Salmonella* in the complex sprout physiology needs to be researched and understood. Fluorescent microscopy and bioluminescent serovars can help better track the effectiveness of gaseous antimicrobials against internalized microbes.
- High Hydrostatic Pressure Processing (HHPP) can be investigated as a combination method with gaseous antimicrobial.
- Optimizing some of the present limitations of 3D printing (nozzle size, substrate flow parameters) and validating the created models
- Additional variables such as cuticle height, contact angle, surface tension and ionic composition of exudates need to studied and added to the 3D printer created substrates to for a better correlation between real-life behavior and the physical models.

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