EFFECT OF DRY HEAT TREATMENT ON INACTIVATION OF SALMONELLA AND GERMINATION OF ALFALFA, MUNG BEAN, RADISH, AND RED CLOVER SEEDS

by

Shunyang Yao

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the Master of Science in Food Science

Summer 2022

EFFECT OF DRY HEAT TREATMENT ON INACTIVATION OF SALMONELLA AND GERMINATION OF ALFALFA, MUNG BEAN, RADISH, AND RED CLOVER SEEDS

by

Shunyang Yao

Approved: ______________________________

Haiqiang Chen, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: ______________________________

Erin Connor, Ph.D.
Chair of the Department of Animal and Food Sciences

Approved: ______________________________

Calvin Keeler, Ph.D.
Interim Dean of the College of Agriculture and Natural Resources

Approved: ______________________________

Louis F. Rossi, Ph.D.
Dean of the Graduate College and Vice Provost for Graduate and Professional Education

ACKNOWLEDGMENTS

This project was supported by the Agriculture and Food Research Initiative Competitive Grants Program of the USDA National Institute of Food and Agriculture, NIFA Award No.: 2017-67017-26223.

I would like to thank my advisor, Dr. Haiqiang Chen. He is always optimistic, kind, supportive, and professional in my whole research program. He always supports and encourages me when I was down and uncertain about my research and my career. I cannot go this far without his guidance and encouragement. I also want to thank Dr. Rolf Joerger and Dr. Changqing Wu for being a part of my thesis committee and providing valuable advice for my research project.

I would like to express my appreciation of all my friends and colleagues during my research program as an ANFS student. Special thanks to my best friend Brianna LiBrizzi, who has always been in my corner. She helped me out a lot in the lab works and she assisted me to finish some parts in this project. She also lets me experience a lot of American culture when I feel diffident and nervous as an international student. I cannot finish this project without her help and her kindness to me. I am very glad and grateful to work with her as my best colleague and my best friend!

I would like to also thank my lab mates: Shiyun Yao, Corinna Darelius, Xuanjing Liu, Katya Anaya, Kassidy Raymond, Shiwei Xu. They are always kind and

helpful and provide the necessary assistance and training for my experimental skills and research project.

Finally, I would like to show my deep love for my parents. They are not only providing me the necessary financial support, but also the emotional support always in my life. Especially after the pandemic, they always talk to me and give me faith and hope during this uncertain era. When I was feeling confused and unsure about my future career, they are always giving me advice and guidance. Without their support and life advice, I cannot live my life with happiness and be certain about what I should do for my future career.

TABLE OF CONTENTS

LIST OF TABLES viii
LIST OF FIGURES x
ABSTRACT xi

Chapter

1 INTRODUCTION 1

1.1 Background and Issues 1
1.2 Significance and Value 4
1.3 Research Objective 6
1.4 Innovation and Application 9

REFERENCES 11

2 LITERATURE REVIEW 14

2.1 Origin of Artificial Sprouts Cultivation and Sprouts' Purpose 14
2.2 Structure and Germination of Seed and Sprouting 17
2.3 Nutrition and Health Benefits of Eating Sprouts 19
2.4 Industrial Sprouting Process and Sanitation Procedure 20
2.5 Sprout Production and Consumption in the US and the World 21

2.5.1 Alfalfa 21
2.5.2 Mung Bean 22
2.5.3 Radish 23
2.5.4 Red Clover 23

2.6 Microbiological Safety Issues of Sprouts 24
2.7 Contamination Sources and Pathways 25
2.8 Sprouts' Associated Foodborne Illnesses 27
2.9 *Salmonella* spp. 29

2.9.1 General Characteristics of *Salmonella* spp. 29
2.9.2 Pathogenesis of *Salmonella* spp. 30

2.10 Government Regulations and Guidelines 31
2.11 Decontamination Methods 34

2.11.1 Chemical Treatments 35
2.11.2 Biological Treatments 37
2.11.3 Physical Treatments 39

2.11.4 Hurdle Technology ... 41
2.11.5 Dry Heat and Effectiveness Factors ... 42

2.11.5.1 Water Activity and *Salmonella* Thermal Resistance ... 42

REFERENCES ... 44

3 MATERIALS AND METHODS ... 56

3.1 Alfalfa, Mung Bean, Radish, and Red Clover Seeds Preparation and Storage ... 56
3.2 Microbiological Analysis of Dry Heat Treatment on Seeds ... 56

3.2.1 Bacterial Strains and Culture Preparation ... 56
3.2.2 Seed Inoculations ... 57
3.2.3 Water Activity Control ... 58
3.2.4 Dry Heat Treatment ... 58
3.2.5 Microbiological Analysis ... 59

3.3 Germination Analysis of Dry Heat Treatment on Seeds ... 60

3.3.1 Water Activity Control and Dry Heat Treatment ... 60
3.3.2 Seed Cultivation and Yield Ratio Calculation ... 60

3.4 Combined Analysis of Microbiological and Germination Studies ... 61
3.5 Chemical Treatment ... 61
3.6 Temperature Profile Curve ... 62
3.7 Statistical Analysis ... 63

REFERENCES ... 64

4 RESULTS AND DISCUSSION ... 65

4.1 Temperature Curve ... 65
4.2 Alfalfa Seeds ... 66

4.2.1 Microbial Effect of Dry Heat Treatment ... 66

4.2.1.1 Results at 65.9°C ... 66
4.2.1.2 Results at 71.0°C ... 68
4.2.1.3 Results at 76.6°C ... 71

4.2.2 Germination Effect of Dry Heat Treatment ... 73

4.2.2.1 Results at 65.9°C ... 73

4.2.2.2 Results at 71.0°C 74
4.2.2.3 Results at 76.6°C 77

4.2.3 Chlorine Wash 78

4.3 Mung Bean 79

4.3.1 *Salmonella* Inactivation Results at 76.6°C 79
4.3.2 Combined Analysis of *Salmonella* Inactivation and Germination at 71.0°C 81
4.3.3 Chlorine Wash 82

4.4 Radish 82

4.4.1 Combined Analysis of *Salmonella* Inactivation and Germination at 71.0°C 82
4.4.2 Chlorine Wash 83

4.5 Red Clover 84

4.5.1 Combined Analysis of Salmonella Inactivation and Germination at 71.0°C 84
4.5.2 Chlorine Wash 85

REFERENCES 86

5 CONCLUSION 88

6 FUTURE AND ADDITIONAL RESEARCH 90

LIST OF TABLES

Table 4.1 Effects of dry heat treatment at 65.9°C on the log reduction of *Salmonella* and the germination yield ratio of alfalfa seeds at a_w of 0.2. Data here represents the average of 3 replicates ± standard deviation...................... 66

Table 4.2 Effects of dry heat treatment at 71.0°C on the log reduction of *Salmonella* and the germination yield ratio of alfalfa seeds. Data here represents the average of 3 replicates ± standard deviation 68

Table 4.3 Effects of dry heat treatment at 76.6°C on the log reduction of *Salmonella* and the germination yield ratio of alfalfa seeds. Data here represents the average of 3 replicates ± standard deviation 71

Table 4.4 Effects of 20,000 ppm sodium hypochlorite soak on log reduction of *Salmonella* and the germination yield ratio of alfalfa seeds. Data here represents the average of 3 replicates ± standard deviation 78

Table 4.5 Comparison of the optimal dry heat treatment parameters to chlorine wash. Data here represents the average of 3 replicates ± standard deviation... 79

Table 4.6 Initial comparison of 3-log reduction of *Salmonella* and germination results at 76.6°C.. 80

Table 4.7 Initial comparison of 5-log reduction of *Salmonella* and germination results at 76.6°C.. 80

Table 4.8 Effects of 20,000ppm sodium hypochlorite soak on the log reduction of *Salmonella* and the germination yield ratio of mung bean seeds. Data represents the average of 3 replicates ± standard deviation 82

Table 4.9 Combined effects of dry heat treatment at 71.0°C on the log reduction of *Salmonella* and the germination yield ratio of radish seeds. Data represent the average of 3 replicates ± standard deviation.. 82

Table 4.10 Effects of 20,000ppm sodium hypochlorite soak on the log reduction of *Salmonella* and the germination yield ratio of radish seeds. Data represents the average of 3 replicates ± standard deviation.. 83

Table 4.11 Combined effects of dry heat treatment at 71.0°C on the log reduction of *Salmonella* and the germination yield ratio of red clover seeds. Data represent the average of 3 replicates ± standard deviation 84

Table 4.12 Effects of 20,000ppm sodium hypochlorite soak on the log reduction of *Salmonella* and the germination yield ratio of red clover seeds. Data represents the average of 3 replicates ± standard deviation 85

LIST OF FIGURES

Figure 2.1 Alfalfa seed structure 18

Figure 2.2 Sprouting stages of alfalfa in different days 19

Figure 3.1 Lab-scale dry heat treatment system... 59

Figure 4.1 Temperature curve.. 65

Figure 4.2 *Salmonella* inactivation at 76.6°C treatment under different a_w levels....... 79

Figure 4.3 *Salmonella* inactivation & germination results at 71.0°C treatment with a_w 0.2 81

ABSTRACT

Alfalfa, mung bean, radish, and red clover are world's main sprout vegetables production categories and are consumed by a huge population both in the US and around the world. Due to their good taste and many health benefits, there is a growing trend to consume sprouts. However, sprouts are often associated with food-borne outbreaks. *Salmonella* is the major pathogenic bacteria associated with sprout outbreaks, due to their strong attachment to seeds and proliferation under drought environment. Seeds could be contaminated during growing, harvesting, transportation or distribution and the initial contamination load is usually low. During seeds' sprouting process, temperature, humidity, pH, and available nutrients in the sprouting environment provide an ideal warm environment for bacteria to multiply and the final pathogenic bacteria population could reach 6-7 log. Without cooking treatment, consumption of raw sprouts can easily make people sick and cause food safety issues. Therefore, effective decontamination methods are needed to treat seeds before sprouting to prevent pathogen contamination. Currently the Food and Drug Administration (FDA) no longer recommends using 20,000 hypochlorite chemical wash to decontaminate seeds because it was proven to be very inefficient; only achieve 2-3 log reduction on pathogens. The new 2017 FDA sprout production guidelines recommends using physical treatment or combined treatment methods to achieve 5 log reduction of pathogens. Dry heat treatment is considered as a physical treatment method and heat can penetrate the whole seed. Some previous research

already showed the high effectiveness of dry heat treatment on seed decontamination but not too many studies on seed germination after treatment.

In this research, different dry heat temperature, treatment time, and seeds' water activity (a_w) were precisely controlled to develop the optimal dry heat treatment conditions for alfalfa, mung bean, radish and red clover seeds. Both microbial log reduction and germination results were monitored during the treatment. The optimal conditions are considered to reach 5 log reduction of *Salmonella* population as well as maintain seeds germination after the dry heat treatment. Microbial and germination results after 20,000 ppm sodium hypochlorite wash for 15 or 20 minutes were also studied and compared to dry heat treatment results. Our results showed that when alfalfa seeds with a_w of 0.1 were dry heat treated at 71.0°C for 140 hours and 120 hours, *Salmonella* inactivation could reach 6.03 log and 4.53 log, respectively, while germination weight yield ratio could maintain 96.42% and 101.42%, respectively. When mung bean seeds with a_w of 0.2 were dry heat treated at 71.0°C for 50 hours, *Salmonella* inactivation could reach 5.14 log, while germination weight yield ratio could maintain 98.60%. When radish seeds with a_w of 0.2 were dry heat treated at 71.0°C for 50 hours, *Salmonella* inactivation could reach 3.80 log, while germination weight yield ratio could maintain 61.03%. When red clover seeds with a_w of 0.2 were dry heat treated at 71.0°C for 50 hours, *Salmonella* inactivation could reach 3.36 log, while germination weight yield ratio could maintain 66.73%.

Taking together, dry heat treatment has a very promising application on seeds decontamination. Our results showed that lower water activity level provided a better overall trade-off on each seed. Higher treatment temp could decrease seeds' viability, but could also shorten treatment time. Therefore, a proper treatment temperature is needed for each seed. Microbial results were similar among 4 seeds, while germination results varied per seeds. Germination results behaved well on alfalfa and mung bean, but not so well on radish and red clover. 71.0°C/a_w 0.1/140h is considered as the most optimal treatment condition for alfalfa, while 71.0°C/a_w 0.2/50h is considered as the most optimal treatment condition for mung bean, radish, red clover. Dry heat treatment showed better results for microbial/germination overall trade-off on alfalfa and mung bean, but not as ideal as for radish and red clover. However, even for radish and red clover, dry heat treatment method is still considered superior to the results from chlorine wash treatment groups.

From industry perspective, required processing equipment for dry heat treatment is very simple and low cost. Dry heat treatment technology developed in this study could be easily scaled up for large scale decontamination of seeds and easily applied to the sprout industry. Despite the cost of equipment investment, this new technology could also cut down the cost for sprouting yield loss and outbreak recall for sprout producers caused by using inefficient seeds decontamination methods. Overall, dry heat treatment is a very promising decontamination technology for sprouting seeds. It is recommended for sprout producers to use dry heat treatment technology for post-harvest treatment before sprout growth.

Chapter 1

INTRODUCTION

1.1 Background and Issues

Sprouts have been discovered and utilized to serve as human food for a very long time. Archaeological evidence indicates that soybean seeds were present in some bronze vessels in ancient China in 1,000 B.C. The sprouting of soybean seeds was also mentioned in Shennong Bencaojing (an ancient Chinese book on agriculture and medicinal plants), written between about 206 B.C. to 220 A.D. (Huang et al., 2008) With such a long history of planting, mung bean and soybean sprouts are widely consumed in East Asian countries. Nowadays, serval kinds of sprouts are consumed by humans. Mung bean, soybean, radish sprouts are mostly consumed by Asian countries. Alfalfa and red clover sprouts are mostly consumed by European countries and the US. It was estimated about 20 years ago that 80 million and 15-20 pounds of alfalfa and mung bean sprouts were produced each year, respectively (Russelle, 2001), forming a very large food market. Sprout production has been increasing since then and is becoming more popular in the United States.

Sprouts are popular due to their good taste and many health benefits. Sprouts have the following health benefits: 1) sprouting process can reduce the content of several anti-nutritional components like trypsin inhibitors and phytic acid which are often associated with digestion problems (Doblado, 2007), 2) sprouting process itself produces digestion-aiding enzymes (Ghani, 2016), 3) unlike eating seeds solely, sprouts contain an increased amount of bioactive compounds which can scavenge free radicals in the human body and protect cells (Hochstein and Atallah, 1988), and 4) macronutrient contents are broken down into amino acids, polyunsaturated fatty acids, dietary fiber and vitamins during the sprouting period (Hou et al., 2019). Due to all the above health benefits, sprouts are becoming more and more popular in many countries, especially developed countries where people pay more attention to the nutrition benefits of food. On the other hand, sprouts are higher value-added products than seeds and have a big economic benefit as well. In our study, 20 g of seeds can grow into 200-300 g of sprouts, which is a more than ten times the yield than seeds alone. Both nutritional and economic benefits make sprouts a commercial food product and a trend food in US.

However, sprouts are often associated with food-borne outbreaks. When seeds are harvested from the field, it is inevitable that bacteria can be introduced onto the seeds where they can survive for a considerable amount of time. The environment required for sprouts' growth is a very warm, humid, and high-nutrient environment with a proper pH, which is also ideal for bacteria to grow and thrive. If seeds are contaminated with bacterial pathogens, these pathogens could grow to a very high level during the growing process. When people eat these raw or under-cooked contaminated sprouts, the pathogens could cause gastrointestinal diseases. It is estimated that from 1996 to 2016, 48 food-borne outbreaks associated with sprouts occurred, resulting in 2499 cases, 179 hospitalizations, and 3 deaths (Gensheimer and Gubernot, 2016). Among these cases, alfalfa sprouts accounted for 30 cases, followed by 7 cases of clover, and 6 cases of mung bean sprouts. In terms of pathogenic bacteria causing these cases, *Salmonella* accounted for most of the outbreak cases (34 cases), followed by *Escherichia coli* (12 cases) and *Listeria* (2 cases) (Gensheimer and Gubernot, 2016). In 1996, an outbreak of *Escherichia coli* O157: H7 infection occurred among schoolchildren in Sakai City, Osaka, Japan. Radish sprouts from a local farm were contaminated with *E. coli* O157: H7 and raw sprouts were consumed by children, causing 9,000 sicknesses and 12 deaths (Michino et al., 1999). These outbreak cases caused a huge social and economic loss and it is urgent to find effective decontamination methods for sprouts.

Some food-borne bacteria, such as *Salmonella*, can be very persistent in a dry environment, because the drought can stimulate the formation of biofilms and filaments to protect the bacteria cells (Finn et al., 2013). Most of the seeds are stored in a relatively dry environment (usually in a RH 20%-40% environment), which is a very ideal *Salmonella* storage condition. Most of the *Salmonella* cells can survive this drought storage condition and thrive when seeds are sprouting in warm and humid conditions. Because of the drought-resistance property, *Salmonella* contributes to most of food-borne outbreaks in sprouts, and they are causing most of the food safety issues. In those 48 sprout-associated outbreak cases, almost 70% cases were related to *Salmonella*, and 63% cases were associated with alfalfa sprouts. Because *Salmonella* was causing most of the food safety issues on alfalfa sprouts in the US, alfalfa and *Salmonella* are two major and representative research objects in the research for the current thesis. Although other three types of seeds (mung bean, radish, red clover) were studied as well, alfalfa seeds were considered as the most important seed type in our study and the results for alfalfa seeds were the most detailed among the four seeds.

1.2 Significance and Value

The FDA used to recommend using 20,000 ppm of calcium hypochlorite to wash seeds for 15-20 minutes (Brooks et al., 2001; Ding et al., 2013). However, this method can only achieve about 1-2 log reduction of *Salmonella* and the surviving bacteria can still thrive to a very high concentration in the following sprouting process and cause food safety issues. In our research we also found that washing alfalfa seeds with 20,000 ppm of chlorine for 15 min resulted in a 1.9-log reduction of *Salmonella*. As mentioned above, biofilm and filament can form to protect *Salmonella* cells from being injured by chemical treatments. Secondly, the micro-structure of seeds is not as smooth as they are visually observed. Kwack et al. (2014) showed that under the electron microscope, many cracks and gaps on the surface of seeds are visible, which could possibly provide an ideal hiding place for bacteria. Fu et al. (2022) found out that the bacteria cells' distribution on the hilum part of alfalfa seeds was higher than that of other parts of the seed coat after the inoculation with *Listeria monocytogenes* or *Salmonella enterica* serovar Typhimurium on alfalfa seeds. It is mainly due to the hilum being more curve-shaped and could provide more hiding spots for bacteria on the seed's surface. Finally, compared to *E. coli*, *Salmonella enterica* Serovars experimentally showed a stronger attachment to alfalfa sprouts and were harder to be rinsed off (Barak, Whitehand, Charkowski, 2002). Based on these three major reasons, it is difficult to conduct decontamination process for sprouting seeds and sprouts are causing a lot of food-borne outbreaks and food-safety issues.

Chemical treatment is usually considered as a surface treatment method because the chemical compound can only reach the very top surface but not the inside of seeds. Due to the structure of sprouting seeds and properties of *Salmonella* cells, chemical treatment is not a very effective decontamination method. One outbreak case happened in 1999 in Wisconsin and six other states in US, where people consumed raw alfalfa sprouts and got sick. Although the alfalfa seeds were pre-washed by calcium hypochlorite before sprouting, *Salmonella* serovar Muenchen was not completely inactivated and multiplied during the sprouting process, causing at least 157 outbreak-related illnesses in multiple states (Proctor et al., 2001). The new FDA 2017 guideline for sprout decontamination no longer require hypochlorite wash treatment method. Instead, physical or combined treatment methods are recommended in this guideline to achieve a 5-log reduction on food pathogens (FDA, 2017). As a result, a new decontamination processing method is needed for the sprout industry to follow. An ideal method should be economical, more effective in bacteria inactivation, and less lethal to seeds' viability after the treatment.

Currently, some researchers are trying to develop a new treatment method for sprouting seeds rather than chemical wash method because of the drawbacks of chemical treatments. Besides calcium hypochlorite, acids are also used in seeds' decontamination study. Alfalfa seeds and radish seeds treated with 8.7% (v/v) acetic acid at 55°C for 2–3 h showed reductions in the populations of both *E. coli* O157:H7 and *Salmonella* on alfalfa and radish seeds by more than 5.0 log CFU/g, but the germination after treatment was be affected (Nei et al., 2011). Physical treatments are also studied as a potential treatment method for seeds, including wet heat, high hydrostatic pressure (HPP), UV, and irradiation. Neetoo et al. (2008) found out that when a 600 MPa high pressure was applied to alfalfa seeds at 20℃ for >6 min, a 5-log reduction of *E. coli* O157:H7 can be achieved. However, the problem with HPP is that the equipment cost is very high, usually greater than 1 million dollars. Biological treatment also resulted in a 5-log reduction of *Salmonella enterica*, without affecting yield, if *Pseudomonas fluorescens* 2-79 was applied to alfalfa seeds (Fett, 2006). Hurdle technology is another feasible method as well. It was proven that HHP processing together with adding antimicrobial compounds like hypochlorite or carvacrol can improve microbial inactivation results on both alfalfa and mung bean sprouts (Peñas et al., 2009)

Thermal processing method has been used to sterilize canned food for a very long time and remains as a popular food treatment method because of the convenience, long shelf life, and low cost of production (Al-Baali and Farid, 2006). The major difference between thermal treatment and UV treatment is that heat can penetrate the food sample, while UV light cannot.

1.3 Research Objective

Alfalfa is the most popular sprout product consumed in US. Approximately 80 million pounds of alfalfa seed are produced each year in the United States (Oregon Public Health Division, 2022). Mung bean sprouts are commonly consumed in most Asian countries and are also popular in US. About 15-20 million pounds of mung beans are consumed annually in the US, and most of them are consumed in the form of mung bean sprouts (Oregon Public Health Division, 2022). Radish and red clover are widely consumed all over the world. Based on this market share of sprouting seeds, alfalfa, mung bean, radish, and red clover seeds were chosen in our study.

We also chose *Salmonella* as our research bacteria model and indicator bacteria since most of the sprout contamination scenarios occurred because *Salmonella* is the most resistant food-borne pathogen in sprouts (Newell et al., 2010). *Salmonella* also caused most of the sprout associated food-borne outbreaks in the US. If *Salmonella* can be successfully inactivated, it is safe to say that other more vulnerable bacteria strains can be inactivated under the same treatment conditions.

Water activity is a very important parameter in our research as well. The definition of water activity of a food is the ratio between food's vapor pressure and the vapor pressure of pure water under the same temperature (Karel and Lund, 2003). In some previous research, water activity was associated with a higher *Salmonella* survival rate. Low water activity of food could preserve or even improve the survival of *Salmonella* Heidelberg and *Salmonella* Montevideo in dried milk and cocoa powder (Juven et al.,1984). It has been proven by serval researchers that when water activity is lower, the *Salmonella* is harder to inactivate. Beuchat and Scouten (2002) found out that the death rate of *Salmonella* on alfalfa seeds after heat treatment had a positive correlation with the level of water activity during the seeds' storage. Heat inactivation of *Salmonella* at 65.9°C was less effective in seeds with 0.3 or 0.5 a_w compared with seeds with an a_w of 0.6 (Neetoo and Chen, 2014). All these previous results are in accordance with our research results as well, as the lower a_w of seeds could result in a less efficient thermal inactivation on *Salmonella*. On the other hand, seeds' water activity is also associated with seeds' viability. Some pre-dry treatment for seeds of cucurbit crops before conducing dry heat treatment could slightly enhance the viability and germination rate of those seeds (Kubota et al., 2012). This is the same results for our research as well. When alfalfa, mung bean, radish, red clover seeds had a lower water activity, seeds were more thermally resistant and had a higher germination yield. The detailed results will be fully illustrated in Chapter 3. Because water activity can affect both *Salmonella* inactivation efficiency and germination yield rate, it is very important to precisely control the water activity to reach the balanced point. Since dry heat treatment method was also involved in this research, it was also very important to control the treatment temperature to a certain level. In our study, it was demonstrated that when water activity was kept in the range of 0.1-0.2 and treatment temperatures were kept at 71.0°C or 76.6°C, this optimal balanced point can be reached on alfalfa and mung bean seeds.

The goal of this research is to discover and develop a novel treatment method to successfully inactivate *Salmonella* inoculated on alfalfa, mung bean, radish, and red clover seeds, as well as maintain the seeds' viability and germination yield rate after treatment. The target we set for this research was to achieve >5 log reduction of *Salmonella* while minimizing the loss of sprout yield. In general, our research results showed that if water activity, treatment temperature, treatment time were properly controlled, this dry heat treatment method was very effective in *Salmonella* inactivation and maintaining seeds' viability. It is a very promising food processing technology and could be a potential superior alternative to the traditional chemical wash treatment method for the sprout industry.

1.4 Innovation and Application

Results from this study clearly demonstrated that alfalfa and mung bean seeds responded well to the dry heat treatment. Both 5-log reductions of *Salmonella* and over 90% of the yield ratio were achieved after the dry heat treatment with the proper control parameters. Although radish and red clover seeds showed a less ideal result than the other two types of seeds, the overall results are still considered superior to the results from chemical wash treatment.

The most innovative part in this project is that water activity parameter is taken into consideration while using the dry heat treatment to treat seeds before sprouting. Although there are many literatures studied on the correlations between water activity and *Salmonella* inactivation on seeds, there are not too many studies that determined the correlation between water activity and seed germination.

The dry heat processing technology has a very broad and promising application prospect and can be applied as a critical control point for microbial safety in sprouts production. It is also environmental-friendly and cost-effective. Compared with traditional chemical cleaning methods, dry heat processing can effectively inactivate food-borne pathogenic bacteria without a negative effect or with only a minor negative effect on germination and growth of heat-treated seeds. In addition, the technology does not leave any chemical residues on the surface of the seeds, thus has no safety or quality impact on food for human consumption. Compared to some non-thermal food processing technology, the required processing equipment for dry heat treatment is simple and low cost. The dry heat treatment processing method developed in this study could be easily scaled up for large scale decontamination of seeds.

REFERENCES

Al-Baali, A. G., & Farid, M. M. (2006). Thermal sterilization of food. *Sterilization Of Food In Retort Pouches* (pp. 1-16). Springer, Boston, MA.

Barak, J. D., Whitehand, L. C., & Charkowski, A. O. (2002). Differences in attachment of *Salmonella enterica* serovars and *Escherichia coli* O157: H7 to alfalfa sprouts. *Applied and Environmental Microbiology*, 68(10), 4758-4763.

Brooks, J. T., Rowe, S. Y., Shillam, P., Heltzel, D. M., Hunter, S. B., Slutsker, L., Hoekstra, R. M., & Luby, S. P. (2001). *Salmonella* Typhimurium infections transmitted by chlorine-pretreated clover sprout seeds. *American Journal of Epidemiology*. 154, 1020-1028.

Beuchat, L. R., & Scouten, A. J. (2002). Combined effects of water activity, temperature and chemical treatments on the survival of *Salmonella* and *Escherichia coli* O157: H7 on alfalfa seeds. *Journal of Applied Microbiology*, 92(3), 382-395.

Ding, H., Fu, T. J., & Smith, M. A. (2013). Microbial contamination in sprouts: how effective is seed disinfection treatment? *Journal of Food Science*, 78(4), R495-R501.

Doblado, R, Frías, J, and Vidal-Valverde, C (2007). Changes in vitamin C content and antioxidant capacity of raw and germinated cowpea (Vigna sinensis var. carilla) seeds induced by high pressure treatment. *Food Chemistry*. 101, 918-923.

Fett, W. F. (2006). Inhibition of *Salmonella enterica* by plant-associated pseudomonads in vitro and on sprouting alfalfa seed. *Journal of Food Protection*, 69(4), 719-728.

Finn, S., Condell, O., McClure, P., Amézquita, A., & Fanning, S. (2013). Mechanisms of survival, responses and sources of *Salmonella* in low-moisture environments. *Frontiers in Microbiology*, 4, 331.

Food and Drug Administration (FDA). (2017). Draft Guidance for Industry: Compliance with and Recommendations for Implementation of the Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption for Sprout Operations. Available at https://www.fda.gov/regulatory-information/search-fda-guidance-documents/draft-guidance-industry-compliance-and-recommendations-implementation-standards-growing-harvesting. Accessed on April 5, 2022.

Gensheimer, K., & Gubernot, D. (2016). 20 years of sprout-related outbreaks: FDA's investigative efforts. *In Open Forum Infectious Diseases* (Vol. 3, No. suppl_1, p. 1438). Oxford University Press.

Ghani, M., Kulkarni, K. P., Song, J. T., Shannon, J. G., & Lee, J. D. (2016). Soybean sprouts: A review of nutrient composition, health benefits and genetic variation. *Plant Breeding and Biotechnology*, 4(4), 398-412.

Huang, H. T., DuBois, C. M., Tan, C. B., & Mintz, S. W. (2008). Early uses of soybean in Chinese history. *The World of Soy*, 45-55.

Hochstein, P., & Atallah, A. S. (1988). The nature of oxidants and antioxidant systems in the inhibition of mutation and cancer. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 202(2), 363-375.

Hou D., Yousaf L., Xue Y., Hu J., Wu J., Hu X., Feng N., Shen Q., (2019) Mung Bean (Vigna radiata L.): Bioactive Polyphenols, Polysaccharides, Peptides, and Health Benefits. *Nutrients*. 11(6):1238. https://doi.org/10.3390/nu11061238

Juven, B. J., Cox, N. A., Bailey, J. S., Thomson, J. E., Charles, O. W., & Shutze, J. V. (1984). Survival of *Salmonella* in dry food and feed. *Journal of Food Protection*, 47(6), 445-448.

Karel, M., & Lund, D. B. (2003). *Physical Principles of Food Preservation: Revised and Expanded*. CRC Press. ISBN 9780824740634.

Kubota, M., Hagiwara, N., & Shirakawa, T. (2012). Disinfection of seeds of cucurbit crops infested with *Acidovorax citrulli* with dry heat treatment. *Journal of Phytopathology*, 160(7-8), 364-368.

Kwack, Y., Kim, K. K., Hwang, H., & Chun, C. (2014). An ozone micro-bubble technique for seed sterilization in Alfalfa sprouts. *Horticultural Science and Technology*. 901-905.

Michino, H., Araki, K., Minami, S., Takaya, S., Sakai, N., Miyazaki, M., Ono, A., & Yanagawa, H. (1999). Massive outbreak of *Escherichia coli* O157: H7 infection in schoolchildren in Sakai City, Japan, associated with consumption of white radish sprouts. *American Journal of Epidemiology*, 150(8), 787-796.

Neetoo, H., Ye, M., & Chen, H. (2008). Potential application of high hydrostatic pressure to eliminate *Escherichia coli* O157: H7 on alfalfa sprouted seeds. *International Journal of Food Microbiology*, 128(2), 348-353.

Neetoo, H., Ye, M., Chen, H. (2009). Factors affecting the efficacy of pressure inactivation of *Escherichia coli* O157:H7 on alfalfa seeds and seed viability. *International Journal of Food Microbiology*, 131, 218-223.

Neetoo, H., & Chen, H. (2014). Factors Influencing the Dry Heat Sensitivity of *Salmonella enterica* on Alfalfa Sprouting Seeds. *Journal of Food Safety*, 34(4), 312-320.

Nei, D., Latiful, B. M., Enomoto, K., Inatsu, Y., & Kawamoto, S. (2011). Disinfection of radish and alfalfa seeds inoculated with *Escherichia coli* O157: H7 and *Salmonella* by a gaseous acetic acid treatment. *Foodborne Pathogens and Disease*, 8(10), 1089-1094.

Newell, D. G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., Opsteegh, M., Langelaar, M., Threfall, J., Scheutz, F., van der Giessen, J., & Kruse, H. (2010). Food-borne diseases—the challenges of 20 years ago still persist while new ones continue to emerge. *International Journal of Food Microbiology*, 139, S3-S15.

Oregon Public Health Division, (2022). Sprouts. Available at: https://fsi.colostate.edu/sprouts/. Accessed on March 29, 2022.

Peñas, E., Gómez, R., Frías, J., & Vidal-Valverde, C. (2009). Efficacy of combinations of high pressure treatment, temperature and antimicrobial compounds to improve the microbiological quality of alfalfa seeds for sprout production. *Food Control*, 20(1), 31-39.

Proctor, M. E., Hamacher, M., Tortorello, M. L., Archer, J. R., & Davis, J. P. (2001). Multistate outbreak of *Salmonella* serovar Muenchen infections associated with alfalfa sprouts grown from seeds pretreated with calcium hypochlorite. *Journal of Clinical Microbiology*, 39(10), 3461-3465.

Russelle, M. P. (2001). Alfalfa: After an 8,000-year journey, the" Queen of Forages" stands poised to enjoy renewed popularity. *American Scientist*, 89(3), 252-261.

Chapter 2

LITERATURE REVIEW

2.1 Origin of Artificial Sprouts Cultivation and Sprouts' Purpose

Human consumption of sprouts has a very long history. Soybean (*Glycine max*) is believed to have one of the longest histories of human domestication. Soybean sprouts were recorded in ancient Chinese medical and agriculture book called "Shennong Bencaojing" (Huang et al., 2008). Soybean sprouts were first used for medical purpose and later ancient Chinese found their cuisine purpose and developed them into a very important food in China, and also spread them to other east Asian countries. The earliest document that records soybean sprouts as food appeared in Shanjia Qinggong [Basic Needs for Rustic Living], by Lin Hong in the Song Dynasty (year of 1127-1279) (Shurtleff and Aoyagi, 2016). It is clear from the document that soybean sprout was a very important daily food for the Chinese one thousand years ago. Soybean sprouts and other sprouts are believed to be brought into the United States by Chinese immigrants during 1930s. Some small firms in Chinatown in New York City were growing soybean into soybean sprouts and served local restaurants and markets (Trelease and Trelease, 1943). The first formal production of soybean sprouts in the United States was done by a Japanese company, Yuta Moyashi Seizo-sho (Utah Beans Sprout Co.) in Salt Lake City, Utah in 1952 (Shurtleff and Aoyagi, 2016). In the United States and Europe, soybean sprouts are growing in a rapid speed as a food type because Asian immigrant community cuisine habits influence the local cuisine habits as well.

Besides soybean sprouts, there are also other kinds of seed sprouts consumed widely around the world. Alfalfa (*Medicago sativa*) has the largest production among all sprouts in the United States nowadays. Alfalfa is believed to originate in south-central Asia and was first cultivated in ancient Iran (Oakley and Westover, 1922). Then throughout time, this type of seeds was introduced to Europe. According to Pliny, alfalfa was brought into Greece by Persian soldiers around 490 BC when the Persians invaded Greek territory (Stronk, 2019). Alfalfa sprouts were widely used for human food and horse fodder in Europe for two thousand years. In the 16th century, Spanish colonizers introduced alfalfa to the Americas as fodder for their horses (Bilello,2016). At first alfalfa was not produced in the United States, but only limited to the South America. Alfalfa seeds were imported to California from Chile in the 1850s, and that was the first time when alfalfa seeds were introduced and spread across the United States. Currently it is considered as the sprout type that is consumed the most in the United States. According to the data of the world total production of alfalfa in 2009, North America produced 41% (11.9 million hectares; 29 million acres), Europe 25% (7.12 million hectares; 17.6 million acres), and South America 23% (7 million hectares; 17 million acres) (Cash 2009). Both America and Europe share a big amount of alfalfa production, and North America has the most production according to the data.

Mung bean (*Vigna radiata*) sprouts have been consumed and planted for culinary purposes in China for over 2000 years. Mung bean is believed in Chinese culture to eliminate acne during hot and humid seasons. In the book Ben Cao Qiu Zhen, mung bean is documented to have the merit in regulation of gastrointestinal upset and moisturizing the skin (Min, 2001). Seeds and sprouts of mung beans are also widely consumed as fresh salad vegetable or common cuisine in India, Bangladesh, Southeast Asia, and western countries (Fery, 1990). Similar to soybean sprouts, mung bean sprouts became popular in the United States because of the Asian immigrants.

Radish (*Raphanus raphanistrum* subsp. sativus) is commonly distributed in the wild all over the world and is believed to have been firstly domesticated in Asia. However, no apparent archaeological evidence showed the exact place of origin for its artificial cultivation and early history (Zohary and Hopf, 2000). Some genetic evidence has located the initial origin of *Raphanus sativus* in Southeast Asia, where the truly wildtype was firstly formed. India, central China, and Central Asia seem to be the secondary centers where differing forms of radish were developed (Glaubrecht and Köhler, 2004). Radish was first recorded historically in the third century BC. Greek and Roman agriculturalists gave some definition of radish of different types according to their sizes and shapes. Radish is considered as the first type of European crop introduced into the Americas (Panetsos and Baker, 1967). Radish is commonly consumed and used for cuisine in China, Japan, and European countries.

Red clover (*Trifolium pratense*) is native to Europe, Western Asia, and northwest Africa, but it has been planted in other continents, like North and South America (Jones, 2020). The red clover was introduced and planted in Argentina and Chile over 100 years ago, although it is not yet known how exactly it was introduced (Rosso and Pagano, 2005). Red clover is widely used as fodder, ornamental plant, raw material for essential oil extract, and edible food. Because of red clover’s high economic value, it is a pillar crop in Chile (Arretz and Macfarlane,1986). Red clover has medical attributes as well. It is used in traditional Indian medicine as antispasmodic, expectorant, sedative, anti-inflammatory agent (Khare, 2008). Due to the medical and health benefits, red clover can be consumed as a functional food and some related food products are produced mainly in South America.

2.2 Structure and Germination of Seed and Sprouting

Sprouting is a process whereby seeds germinate and form a root from the inner parts of the seeds, then stem and leaves are formed. Because all sprouting seeds have a similar sprouting process, alfalfa seeds are used as an illustration. Alfalfa is a dicotyledonous plant. Usually, the outside coat of an alfalfa seed has a yellow to olive green color. Looking into the detailed structure of alfalfa seed, it is called a dicotyledonous plant because it contains mainly two parts: cotyledon and radicle (primary root). Cotyledon is a significant part of the embryo within the seed of a plant, and it stores the necessary nutrient for plant anabolism process during seeds' germination and growth (Kawashima and Goldberg, 2010). As shown Figure 2.1, the radicle part is in the lower part of the seed. The radicle develops into the root of alfalfa sprouts and absorbs essential nutrients and water for the seeds' growth needs. When the radicle part is big enough and forms an actual part of the root, it will penetrate the len (weak point) on the seed coat (Meyer, 1999). Hilum is a scar tissue left on a seed coat by the former attachment to the ovary wall of the seed pod, usually known as "eye" of seeds. (Undersander, et al., 1997) The hilum is usually less smooth than other parts on the seed coat and some studies have found that the hilum is the hiding site of most of the bacteria cells on seeds. Fu et al. (2022) found that after inoculation with *Listeria monocytogenes* or *Salmonella enterica* serovar Typhimurium, the bacteria load on the hilum of alfalfa seeds was higher than that of other parts of the seed coat. Micropyle is a remnant of the tiny opening, and this part grew during the process of fertilizing the female ovary (Meyer, 1999).

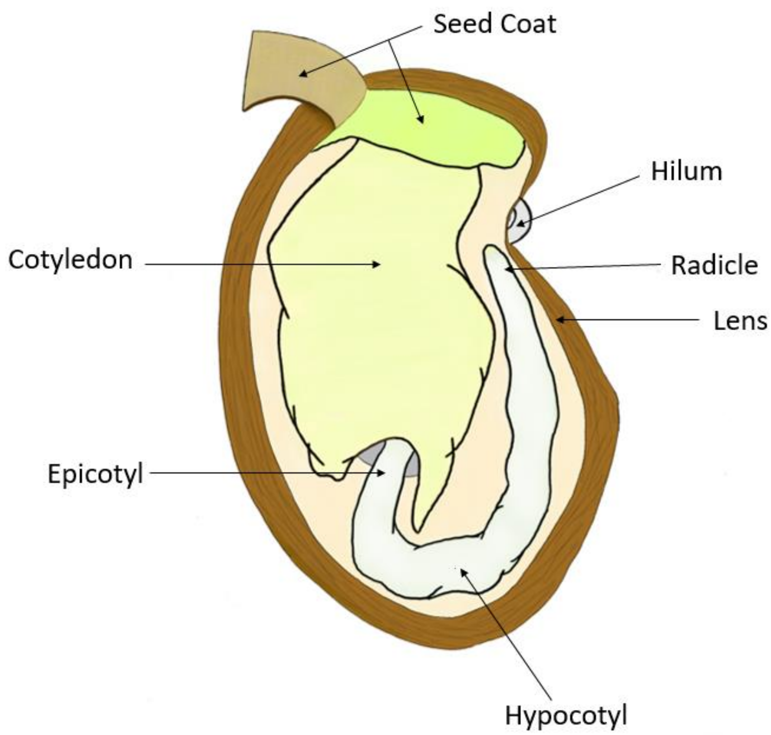

Figure 2.1 Alfalfa seed structure

During the germination stage, seeds show different forms in different stages. Seeds can either be planted in soil or water. Sprouts are usually developed into their immature form and then harvested for food purposes 3-8 days after seed gemination (DeEll, 2014). Figure 2.2 shows the time course of alfalfa seed sprouting from day 0 to day 8. The picture was taken using alfalfa seeds from room temperature storage and we usually harvested seeds at the eighth day after germination in our study. Usually during the first 1-3 days of sprouting, the radicle penetrates the seed coat and grows towards the water source. In our research, those seeds with heat treatment usually have a slower germination process than untreated seeds. It took longer for the radicle part to penetrate the seed coat, indicating that dry heat treatment can indeed hurt the seeds. After the seeds germinate, the stem of the cotyledons grows longer. Usually on days 5-8, leaves are formed, and the intact sprouts are formed and could be harvested.

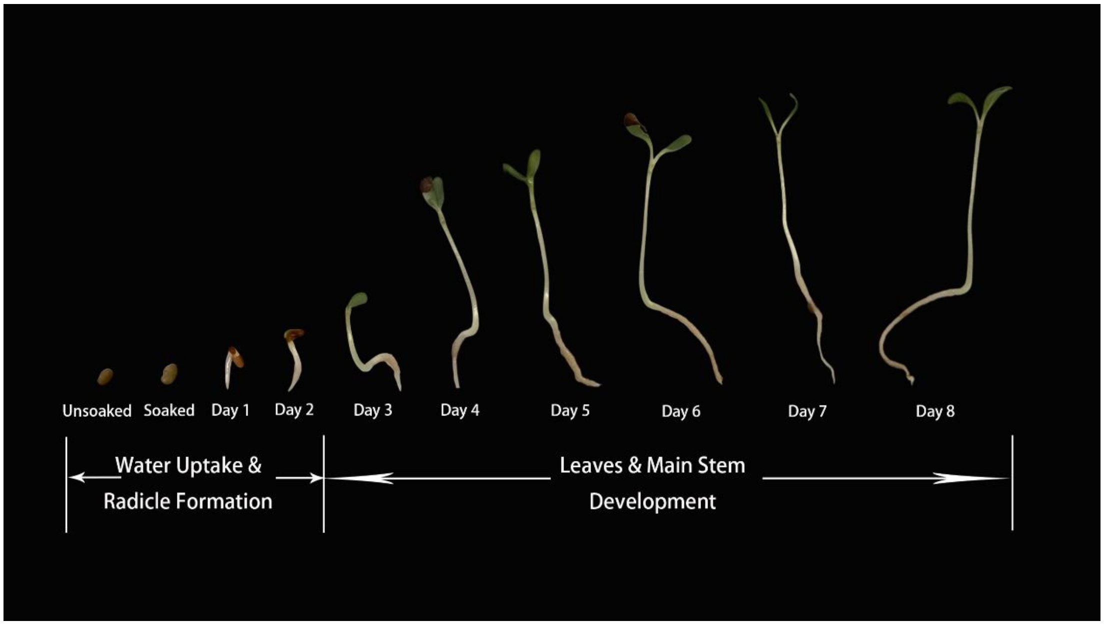

Figure 2.2 Sprouting stages of alfalfa in different days

2.3 Nutrition and Health Benefits of Eating Sprouts

Sprouts are considered as a very healthy food. During the sprouting process when seeds are transformed into sprouts, macronutrients are broken down into more digestible micronutrients (Peñas, 2008). Macronutrient contents are broken down into amino acids, polyunsaturated fatty acids, dietary fiber, and vitamins during sprouting period (Hou et al., 2019). During soaking and sprouting of seeds, cereal grains' stored protein content is broken down into albumins, globulins, and amino acid lysine during sprouting, which can increase the quality of cereal proteins

(Chavan et al., 1989). Alfalfa sprouts after freeze-drying contain 49.6% protein, 33.6% carbohydrate, 2.6% fats, 10.5% water, and 3.7% ash (Hong et al., 2009). This content analysis shows that sprouts contain a very high ratio of protein and carbohydrate content, which is very beneficial to human health.

On the other hand, the sprouting process can also produce other specific healthy organic compounds. It is proven that it can reduce the content of several anti-nutritional components such trypsin inhibitors and phytic acid which are originally present in seeds and often associated with digestion problems (Doblado, 2007). The sprouting process can also produce some digestion-aiding enzymes (Ghani, 2016). Sprouts contain a higher number of bioactive compounds than seeds, which can scavenge free radicals in human body (Hochstein and Atallah, 1988).

2.4 Industrial Sprouting Process and Sanitation Procedure

The usual industrial sprouting process includes several steps: harvesting seeds, packing into larger seed bags, seed storage, prewashing with clean water to remove dust, soaking with 20,000 ppm calcium hypochlorite to sanitize seeds for 15 minutes, rinsing seeds with water for serval times, growing seeds in trays, buckets or rotating drums, allowing some air circulation during sprouting, harvesting, weighing, and packaging (IIT, 2022). Some seed types like alfalfa, red clover, and radish are grown with light access to allow the chloroplasts to produce chlorophyll, while mung bean and soybean seeds are usually grown industrially in a dark environment (Fett, 2005). In the germination results of this research, when mung bean seeds were grown with light access, the seeds’ color usually turns into red and the leave color is green, which is different from mung bean sprouts consumed in the market.

To make sure the manufacture process is safe and clean, GMP is strictly followed during industrial sprouting. When seeds bags are received by sprouting factories, bags should be visually inspected to make sure the bags have no evidence of contamination such as insects, water stain or bird droppings. Before entering the facility, workers must wear proper personal protective equipment and wash hands before each operation. Sanitation and hygiene procedures are done on the working surfaces and especially on the equipment. The routine cleaning of sprouting equipment should be performed after each batch of sprouting, including physical rubbing and agitation, washing with soap or detergent, and followed by rinsing with flowing water (IIT, 2009).

2.5 Sprout Production and Consumption in the US and the World

Because of all the health and economic benefits and culinary usage of sprouts described above, sprout is a major food source in many countries in the world. Different kinds of sprouts are consumed in different amounts in different regions and countries. Alfalfa, mung bean, radish, and red clover are four types of sprouts that are commonly consumed by most of the countries. The detailed production amount by major countries in the world will be illustrated in this section.

2.5.1 Alfalfa

During the early 2000s, alfalfa was the most cultivated forage legume in the world, of which the total world annual production was 436 million tons in 2006 (Reddy et al., 2014). The data of the world total production of alfalfa in 2009 is that: North America produced 41% (11.9 million hectares), Europe 25% (7.12 million hectares), South America 23% (7 million hectares), Asia 8% (2.23 million hectares), and Africa & Oceania produced the remainder (Cash 2009). Among all the sprouts, alfalfa sprouts are the most consumed in the United States. Several other countries also have large farmlands to grow alfalfa. The top largest production area is found in Argentina (6.9 million hectares), Canada (2 million hectares), Russia (1.8 million hectares), Italy (1.3 million hectares), and China (1.3 million hectares) (Cash 2009).

In 2009, the United States was the largest alfalfa producer in the world by area, with 9 million hectares (22 million acres) (Cash 2009). It is estimated that 80 million pounds of alfalfa are produced annually in the United States, although most alfalfa is used for animal fodder (Mueller, 2008). In the United States in 2012, the top three alfalfa-growing states were California, Idaho, and Montana (USDA, 2013).

The most recent alfalfa production in the United States in 2021 was estimated at 49.2 million tons, a decrease by 7 percent from the 2020 total. Harvested area, at 15.2 million acres, was 6 percent lower than in 2020. Average yield was estimated at 3.23 tons per acre, decreasing 0.04 ton from 2020, and the top three production states were Idaho, California, Nebraska (USDA, 2022). The harvested area reduction from 2009 to 2021 is possibly due to the disruption of economy and supply chain after the COVID-19 pandemic.

2.5.2 Mung Bean

Mung bean sprouts are usually the most consumed sprouts around the world. The global mung bean planting area was about 7.3 million hectare and global output was about 5.3 million tons from 2015 to 2017, India and Myanmar each supplied about 30%, China 16%, and Indonesia 5% (World Vegetable Center, 2016). China produces 200,000 tons of mung bean and soybean sprouts each year (Kang, 2011). Japan produces 1,200-1,300 tons of bean sprouts per day (BSGAJ, 2011).

About 10% of the United States' population consume sprouts regularly (Sikin et al., 2013). Due to the Asian American community, mung bean sprouts are commonly served in Asian restaurants and Asian markets in the United States. It is reported that there are 15-20 million pounds of mung bean consumed in the United States each year and most of them are consumed in the form of sprouts, three fourth of which are imported from other countries (Small, 2009).

2.5.3 Radish

The domestication and human selection of preferred types of radishes have led to significant variations in their size, color and taste (Yang, 2022). Among those different varieties, small-rooted radishes are grown in temperate zones and harvested throughout the year (Crisp 1995), while large-rooted radishes such as Chinese radishes are predominant in East and Southeast Asia (Schippers 2004). Radish roots production is estimated to be 7 million tons per year worldwide, accounting for 2% of the total world production of vegetables (Schippers 2004). In China, Japan and Korea, and Yemen, radish is considered as a very importance vegetable category (Schippers 2004).

2.5.4 Red Clover

Red clover is an important forage crop in temperate zones of the world, especially in America and Europe (Steiner and Alderman, 2003). Red clover is grown throughout the northeastern United States for forage purposes and is mainly used for soil improvement (PSU, 1993). In Canada, the production of red clover seed averages about 3 million kg annually, with the farming land of 90,000 acres, although in 1970, output was more than 6 million kg. Most of the red clover produced in Canada are exported to the United States, where red clover is primarily used as a top dressing for winter wheat for plough-down purposes (PRFSA, 2022). In Chile, red clover is a very important resource for animal forage and the seed industry, representing almost 15% of the sown pastures and 60% of the forage seed exports (Ortega et al., 2014).

2.6 Microbiological Safety Issues of Sprouts

Sprouts used for commercial human consumption is often associated with food-borne pathogens, including *Salmonella* and toxic forms of *Escherichia coli* (Tournas, 2005), and such outbreaks caused by microbiological bacteria are so frequent in the United States that the investigators even call them "sproutbreaks" (Neuman, 2011). It is estimated that from 1996 to 2016 in the United States, 48 food-borne outbreaks associated with sprouts were reported, resulting in 2499 cases, 179 hospitalizations, and 3 deaths. Among these cases, alfalfa sprouts accounted for 30 cases, followed by 7 cases of clover, and 6 cases of mung bean. In terms of pathogenic bacteria causing these cases, *Salmonella* accounted for most of the outbreak cases (34 cases), followed by *Escherichia coli* (12 cases) and *Listeria* (2 cases) (Gensheimer and Gubernot, 2016).

Salmonella and *E. coli* can be introduced through irrigation system, harvesting process, or improper package operations. During the seeds' sprouting process, the warm, humid, and high nutrient environment creates an ideal environment for microbial growth. It is estimated that a single surviving bacterium in a kilogram of seeds is enough to contaminate a whole batch of sprouts (Neuman, 2011). Some researchers found that the microbial population of sprouts are often 2-3 log higher than that of seeds (Feng, 1997). When contaminated sprouts are packaged and sold in the market, people consume those contaminated sprouts with the high microbial load and easily get sick, especially when sprouts are eaten raw without heat treatment.

2.7 Contamination Sources and Pathways

Through seeds' growth on the farm and all stages of product handling from harvest to the point of sale, the food product may be contaminated with pathogens (Beuchat, 1996). In this case, seeds and sprouts are the contamination object for harmful pathogens. During seeds' growth, pathogenic bacteria could inhabit and contaminate seeds through contaminated soil and irrigation water, wild animals and birds, and dirty farming equipment (James, 2006). During harvest, farm tools, utensils, and packaging materials could possibly contaminate seeds. Worker' health and hygiene are also important. Unsanitary operation or seed contact surfaces could possibly cause contamination. In addition, transportation and distribution practices could affect product quality and safety (James, 2006). When it comes to seeds' sprouting, the initial microbial load is even more important for seeds' microbial safety, because sprouting is a perfect environment for microbial growth. If there are some initial contaminations on seeds, bacteria populations can increase to a very high-level during seeds' sprouting and cause food safety issues. Many epidemiological investigations showed that seed contamination is the main reason for sprout related outbreaks (Puohiniemi et al.,1997). As a result, it is very important and urgent to detect harmful bacteria initially on seeds and use effective seed decontamination methods.

However, the usual bacteria loads on seeds before sprouting are very low and unevenly distributed, which makes the early detection of pathogens very difficult (Cui et al., 2017). Seeds are usually produced in a very large quantity, therefore the detection and isolation of a small quantity of defect seeds cannot represent the whole batch of seeds and some contaminated seeds can still go into the sprouting process. During the sprouting process, high nutrition, warm temperature, and high humidity environment are providing the ideal growth condition for pathogenic bacteria. Bacteria population on the seeds will multiply quickly to a significant high level during sprouts' germination and growth. It is reported that a 24-hour sprout growth period could result in a 4-log increase of *Salmonella enterica* (Howard and Hutcheson, 2002), and a 48-hour sprout growth could result in the population of some strains of *Salmonella enterica* to reach as high as 10^6-10^7 CFU/g (Taormina et al., 1999). Fett (2005) found out that sprout growth under at 20-30°C could increase *E.coli* and *Salmonella* population by 4 log.

Besides fast bacteria multiplication, bacteria attachment to seeds and sprouts is another reason that causes food safety issues. Fu et al. (2022) found that *Listeria monocytogenes* or *Salmonella enterica* serovar Typhimurium tended to present on alfalfa seeds' hilum than on other parts of the seed coat. Both *Salmonella enterica* and *Escherichia coli* O157:H7 could adhere and grow on alfalfa sprouts, but the growth on and adherence to sprouts were different between *Salmonella enterica* and *Escherichia coli* O157:H7 (Barak, 2002). *Escherichia coli* O157:H7 tended to adhere to the radicle part of sprouts, while *Salmonella enterica* tended to attach to the broken edges of the seed coat, and large *Salmonella* aggregates were observed after 48 hours of sprouting (Charkowski et al., 2002). When the root part was submerged in *E. coli* O157:H7-contaminated water during sprouting, *E. coli* O157:H7 was found present in all edible parts of radish sprouts (Hara-Kudo, 1997). When radish seeds were inoculated with *E. coli* O157:H7 initially and then grown into sprouts, *E. coli* O157:H7 was detected on outer and in inner tissues of radish sprouts (Itoh, 1998). Fett (2000) found that *E. coli* and *Salmonella* were able to form biofilms during multiplication on sprouts and those biofilms were detected after 48-hour growth. Strong attachment, fast multiplication, and biofilm formation make seed decontamination processes very difficult and cause those food safety issues related to sprouts.

2.8 Sprouts' Associated Foodborne Illnesses

Bacteria contamination is often considered as the major cause of sprouts' associated food borne illnesses. The earliest sprout related illness documented was in 1973 from home sprouting kits with soybean, cress and mustard that were contaminated with *Bacillus cereus* (Taormina et al., 1999). There are two sprout related food-borne outbreak cases that cause the widest range of people to get sick, and they took place in Japan in 1996 and in Germany in 2011.

The case that happened in Osaka, Japan, was caused by elementary school students' lunch box containing white radish sprouts contaminated with *E. coli* O157:H7, resulting in a massive food poisoning outbreak with 6,000 people affected, 398 people hospitalized and 12 deaths (Michino et al., 1999). The following investigation suspected that white radish sprouts in lunch boxes supplied by a single local farm got contaminated with *E. coli* O157:H7 during sprout growth on the farm (Imamura et al., 2007). Radish sprouts were not cooked and were eaten raw and young school children didn't have a very strong immune system, which could cause this massive food-borne outbreak (Michino et al., 1999).

The case that initially happened in Germany in 2011 was caused by contaminated fenugreek sprouts (grown from seed from Egypt) and the bacteria strain was identified as *E. coli* O104:H4 (Beutin and Martin, 2012). The outbreak caused about 4,000 sicknesses, 852 of whom developed Hemolytic Uremic Syndrome (HUS) and 54 deaths (Soon et al., 2013). Many related cases were also reported in several countries including Switzerland, Poland, the Netherlands, Sweden, Denmark, the UK, Canada, and the USA (Bilinski et al., 2012). Most of the illness was characterized by bloody diarrhea and hemolytic-uremic syndrome (Soon et al., 2013). Hemolytic–uremic syndrome (HUS) is a group of blood disorders characterized by low red blood cells, acute kidney failure, and low platelets, and patients need urgent treatment when developing HUS (Cody and Dixon, 2019). *E. coli* O104:H4 was first identified and sequenced in this serious outbreak and this *E. coli* strain is classified as a virulent hybrid type of both enterohemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC) (Prager et al., 2014). *E. coli* O104:H4 strain can produce Shiga toxin 2 (stx2), which often shows aggregative adherence to intestinal epithelial cells (Bielaszewska et al., 2011).

While *E. coli* caused two outbreak cases which involved most of the sicknesses, *Salmonella*-related sprout food-borne outbreaks are more frequent in the

United States and the world. According to FDA investigations, from 1996 to 2016, 48 food-borne outbreaks associated with sprouts were reported in the US, resulting in 2499 cases, 179 hospitalizations, and 3 deaths. Among these cases, *Salmonella* accounted for most of the outbreak cases (34 cases), followed by *Escherichia coli* (12 cases) and *Listeria* (2 cases) (Gensheimer and Gubernot, 2016). This is a part of the reason in our research, we chose *Salmonella* as our research model. Because *Salmonella* is the major cause of sprout associated food-borne outbreaks.

Besides *Salmonella* and *E. coli* as the two major causes of sprout associated outbreaks, other pathogens such as *Listeria monocytogenes* and *Yersinia enterocolitica* also caused a portion of outbreak cases (Sivapalasingam et al., 2004). Other pathogens, including *Aeromonas hydrophila*, *Aeromonas caviae, Staphylococcus aureus*, and *Klebsiella pneumoniae*, were also isolated and detected from sprouts, although they were not considered as the microbial causes in outbreak cases (Callister and Agger, 1987; Robertson et al., 2002).

2.9 *Salmonella* spp.

2.9.1 General Characteristics of *Salmonella* spp.

Salmonella is non-spore forming, facultative anaerobic, motile, gram-negative, rod shape bacilli with 1–2 μm in length and 0.35–0.6 μm in width (Smith et al., 2021). *Salmonella enterica* and *Salmonella bongori* comprise 2 species in the genus *Salmonella*, and there are over 2,500 serovars in total (Fookes et al., 2011).

Salmonella serotypes that are commonly linked to food-borne diseases include Enteritidis, Newport, Stanley, and Typhimurium (Thung et al., 2018).

Salmonella is also notorious for its ability to survive in dry environments and can survive for years in dry environments or foods (Mandal and Kwon, 2017). *Salmonella* cannot be destroyed by freezing, but UV light and heat can inactivate them (Beuchat and Heaton, 1975). They will die after being heated at 55 °C (131 °F) for 90 min, or at 60 °C (140 °F) for 12 min (Goodfellow and Brown, 1978). Therefore, it is recommended by the USDA that heating food to an internal temperature of 76.6 °C (167 °F) is sufficient to inactivate *Salmonella* in food (USDA, 2022).

2.9.2 Pathogenesis of *Salmonella* spp.

Worldwide, it is estimated that the incidence rate for *Salmonella* infections was 1140 per 100,000 person-years in 2010 and 93.8 million cases of gastroenteritis were due to *Salmonella* infections The estimated number of deaths due to *Salmonella* infection was approximately 155,000 (Majowicz et al., 2010). In the United States, the Centers for Disease Control and Prevention (CDC) estimated that *Salmonella* causes about 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths every year. The more susceptible groups to salmonellosis are children, pregnant women, elderly people, and those with deficient immune systems (CDC, 2022).

The usual time for symptoms to show is 6 hours to 6 days after infection and symptoms will last 4–7 days. Most people with a *Salmonella* infection will have symptoms of diarrhea (that can be bloody), fever, stomach cramps, and some people can also have nausea, vomiting, or a headache (CDC, 2022).

Salmonella serotypes can be divided into two major groups: typhoidal and nontyphoidal. For non-typhoidal salmonellosis, the infectious dose is approximately

10^3 cells, while for enteric fever, the infectious dose is about 10^5 cells (Bronze and Greenfield, 2005). Due to the growth difference under different pHs, the infectious dose may also be dependent on a patient's stomach acidity (Bronze and Greenfield, 2005). When ingested, *Salmonella* can invade different cell types, including epithelial cells, M cells, macrophages, and dendritic cells (LaRock et al., 2015). Most of the *Salmonella* cells target the intestine epithelium cells. *Salmonella* pathogenicity islands (SPI) encode injecting effectors into the cytoplasm (Eng et al., 2015). SPI_1 is responsible for the initial infection, while SPI_2 accounts for the later spread to and infection of organs (Alghoribi et al., 2019). *Salmonella* can also attack macrophages and dendritic cells, which is usually associated with the death of host cells, causing chronic colonization, local inflammation, and fevers in patients (Fink and Cookson, 2007).

2.10 Government Regulations and Guidelines

Due to the large number of outbreak cases related to sprouts and the wide populations involved, government implemented some regulations and guidelines to improve food safety for sprouts. The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) released guidelines for microbial safety improvements, which include the education of seed growers and producers, good agricultural practices (GAPs), the use of one or more decontamination treatments, the development of food safety systems including a Hazard Analysis and Critical Control Point (HACCP) plan, and additional research in all categories of sprout production (NACMCF, 1999). In the same year, the Food and Drug Administration (FDA) also released two industrial guidance recommendations for sprouts: 1) sprout growth with treatment, storage/handling and pathogen testing; 2) pathogen detection from irrigation systems used for seeds (FDA, 1999a, b). In the first FDA document, FDA recommended decontaminating seeds with a 20,000 ppm hypochlorite wash, which is a frequently used treatment method by the sprouts industry. In the second document, *E. coli* and *Salmonella* were recommended to be tested for in both sprouts and irrigation water, because these two types of bacteria caused most of the sprout related food-borne outbreaks and needed a closer attention than other pathogens.

Initiated in September 2011, the U.S. Food and Drug Administration, in cooperation with the Illinois Institute of Technology's Institute for Food Safety and Health (IIT IFSH) created the Sprout Safety Alliance (SSA) to help sprout producers identify and implement best practices in the safe production of sprouts (FDA, 2018). All the sanitation procedures, good agricultural practices, and good manufacture practices during seed cultivation, harvesting, transportation, storage, pre-treatment, sprouting, packaging, and distribution were listed and described by the SSA, to help improve the microbial safety for sprouts during the whole process. There are also online and in-person safety training courses available from SSA for producers to have better safety practices during sprout productions (IIT, 2022). In 2017, FDA released a "Guidance for Industry: Sprouts" recommendation paper, that provides detailed guidelines from growing the seeds to the consumer's end. All the personnel training and equipment sanitation involved during the whole process are fully described in this guidance (FDA, 2017). This guidance withdrew the previous recommended 20,000 ppm hypochlorite wash, because this method is proven to be ineffective to decontaminate pathogens on seeds, which is also proven by our research as it also only reached a 1-2 log reduction of *Salmonella* on alfalfa, mung bean, radish, and red clover seeds. The new guidance paper also recommends using physical treatments or combination treatments to reach a higher log reduction on pathogens. It is recommended by FDA to use some new decontamination methods to reach a 5-log reduction in microbial inactivation.

In the United States, the Code of Federal Regulations (CFR) states that sprouts must be grown inside sealed buildings to prevent environment contamination sources (Food and Drugs, 2022a). Seed growers must establish sampling procedures to test spent water or sprouts for these two major pathogens, *E. coli* and *Salmonella*, as well as testing for the growth or survival of *Listeria* species, specifically *Listeria monocytogenes* (Food and Drugs, 2022b). Verifying documentation that the seeds have been treated in at least one FDA approved way, either from the supplier or grower is mandatory (Food and Drugs, 2022c). A written environmental monitoring plan, and written sampling plan are also mandatory for each batch, and sample frequency should be no less than once a month (FDA, 2022d, e). In addition, the FDA requires that one supervisor or responsible party who has received food safety training from the Sprout Safety Alliance or an equal level must be present during sprout production.

In the European Union, to prevent the sprout outbreaks similar to the 2011 Germany EHEC epidemic, the European Commission issued three new, tighter regulations: 1) Regulation (EU) No 208/2013 requires that the origins of seeds must always be traceable at all stages of processing, production, and distribution.

Therefore, a full description of the seeds or sprouts needs to be kept on record (European Commission, 2013a). 2) Regulation (EU) No 209/2013 amends Regulation (EC) No 2073/2005 in respect to microbiological criteria for sprouts and the sampling rules for poultry carcasses and fresh poultry meat (European Commission, 2013b). 3) Regulation (EU) No 211/2013 requires that imported sprouts and seeds intended to produce sprouts have a certificate, drawn up in accordance with the model certificate in the Annex of the regulation, that serves as proof that the production process complies with the general hygiene provisions in Part A of Annex I to Regulation (EC) No 852/2004 and the traceability requirements of Implementing Regulation (EU) No 208/2013 (European Commission, 2013c).

2.11 Decontamination Methods

Because there are so many food-borne outbreaks related to sprout, many researchers are seeking new decontamination methods to eliminate pathogens on sprouts. Chemical treatments were frequently used in the sprout industry for many years. FDA used to recommend the use of 20,000 ppm calcium hypochlorite to pre-wash seeds for 15 minutes before sprouting to reduce the contamination risks for bacteria on seeds and sprouts (Ding et al., 2013). But this method can only reach 1-2 log reductions on bacteria population and still cause food-borne outbreaks even from those chemically washed seeds. It is urgent to come up with some new decontamination methods for the sprout industry. The new method should be more effective in bacteria inactivation to reduce the microbiological risks in sprouts. On the other hand, the new treatment method should also maintain seeds' viability and germination. For economic reasons, the building cost of processing equipment should be reasonable for manufactures to use.

In this section, different treatment methods developed by different researchers for seeds and sprouts are listed and reviewed. Some of the methods achieved very good results on bacteria inactivation. However, when it comes to germination results, most of the researchers only studied seed germination rates after treatment. While germination rate is a very important factor in such studies, it is not a practical factor for the industry to follow because germination rate can only indicate the number of seeds germinated. This factor cannot indicate how healthy sprouts can grow after treatment. In our study, we tested dry heat treatment and, in some cases, although the germination rates were 100% compared to untreated control, seeds grew not as healthy as those from the untreated control and the actual height and yield weight of sprouts were much lower. That’s why in our study, we used germination yield ratio to examine the post treatment effects on seed germination and viability.

2.11.1 Chemical Treatments

Chemical treatments are frequently used in the sprout industry and this method is well studied by many researchers as well. FDA used to recommend use 20,000 ppm calcium hypochlorite to pre-wash seeds for 15-20 minutes before sprouting to reduce the contamination risks for bacteria on seeds and sprouts (Ding et al., 2013; Brooks et al., 2001). However, many studies showed that this treatment can only reach a 2-log reduction and some manufactures that used this method for treating seeds were still ending up causing outbreaks related to *E. coli* or *Salmonella* (Proctor et al., 2001). The reason for outbreaks after chemical treatment is that chemicals can only reach the surface of the seeds, and the bacterial cells hidden deeply inside the seeds' inner holes and caracks cannot be inactivated. In Wisconsin, in 1999, one food-borne outbreak related to alfalfa sprouts was caused by the fact that *Salmonella* cells were not completely decontaminated on seeds by hypochlorite wash, and the remaining bacteria cells proliferated in the subsequent growth of sprouts, causing food-borne diseases after being consumed (Proctor et al., 2001). Another reason for difficulties in decontamination with hypochlorite is due to the formation of biofilms and filaments when *Salmonella* is under the drought stress (Finn et al., 2013). Seeds are usually stored under dry conditions, which will stimulate *Salmonella* or other pathogens to form biofilms and filaments. Biofilms and filaments can make *Salmonella* more resilient to the outside environment and the matter that surrounds the bacteria can first interact with chlorine and eventually reduce the effectiveness of chemicals on the bacteria (Estrela et al., 2002).

Acids are also tested by researchers for seed decontamination. When alfalfa seeds were treated with 8.7% gaseous acetic acid for 1-3 hours, a 4.3-5-log reduction of *E. coli*, and a 2.5-5-log reduction of *Salmonella* was achieved, but the treatment resulted in a slight decrease in seed germination (Nei et al., 2011). Three percent of peroxyacetic acid wash for 15 minutes achieved a 1.34-log reduction in *Salmonella* Stanley populations, with a slightly negative effect on seed germination so this method is not commercially unacceptable (Buchholz and Matthews, 2010). Five percent lactic acid wash for 10 minutes at 42°C was shown to have a 3-log reduction in *E. coli* on alfalfa seeds (Lang et al., 2000).

Other chemicals were also applied to treat seeds and analyzed by different researchers. Treating alfalfa seeds with gaseous ozone at 25°C for 24 hours achieved a 1.5-log reduction of *Salmonella* (Rajkowski and Ashurst, 2009). Mung bean seeds that were treated with 100 ppm of Oxychloro-Based Sanitizer resulted in a 3.8 and 9.1 log-reduction of *Salmonella* and *E. coli*, respectively, with no effects on mung bean germination (Kumar et al., 2006).

Because of the seeds' structure and pathogens' characteristics mentioned above, it is very hard for chemical treatments to reach FDA recommended 5-log reduction of pathogens. Only two kinds of chemical treatment methods (gaseous acetic acid and oxychloro-based sanitizer) mentioned in this section can reach a 5-log reduction on either *E. coli* or *Salmonella* (Nei et al., 2011; Kumar et al., 2006). It was also shown that chemical compounds can possibly bind to produce after chemical treatment, which could potentially affect the quality of food product (Pradhan et al., 2007). In general, chemical wash is not recommended for seed decontamination treatment because of low efficacy in microbial inactivation and low product quality post-treatment. In 2017 FDA's guideline, physical treatment or hurdle technology are more recommended than chemical treatments as well.

2.11.2 Biological Treatments

In research, biological treatments are also explored to determine if they could be applied to the sprout industry. The basic principle of biological treatment is to use other nonpathogenic bacteria to compete with target pathogenic bacteria, so those pathogenic ones will die from lack of nutrient or bacteriocins produced by other bacteria strains. Unlike chemical treatment, the advantage of biological treatments is that these methods use other microorganism or organic substances, so they won't leave residues on sprouts after treatment.

Pseudomonas fluorescens 2-79 was shown to inhibit five serovars of *Salmonella enterica* and, when added to the soaking water for alfalfa seeds, a 5-log reduction was achieved without affecting alfalfa sprout yield or appearance (Fett, 2006). When *Pseudomonas jessenii* was added to *Salmonella* inoculated mung bean seeds, there was a 3-log reduction in *Salmonella* (Weiss et al., 2007). Mung beans inoculated with *P. jessenii* 24 hours prior *Salmonella* inoculation did not show any growth of *Salmonella* (Weiss et al., 2007).

Bacteriocins are defined as bacteria produced small poly peptides that have antimicrobial effects against other closely or non-closely related bacteria (Cleveland et al., 2001). Lactic acid bacteria (LAB) produce the majority of bacteriocins that contain a great variety of microorganisms defined as "generally recognized as safe (GRAS)" by the FDA. As some of the bacteriocins are commercially applied in food processing, their application was also explored to applied on sprouts (Güllüceet al., 2013; Ramu et al., 2015). A colicin produced by *E. coli* HU194 could reduce *E. coli* O157:H7 by 5 log CFU/g after one day of incubation on alfalfa seeds (Nandiwada et al., 2004). Mundticin synthesized by *Enterococcus mundtii*, could inactivate 2 log CFU/g of *L. monocytogenes* on mung bean sprouts when applied to washing water before sprouting (Bennik et al., 1999).

Although biological treatments showed good results on the inactivation of *Salmonella* and *E. coli*, the drawback of this treatment type is that the treatment itself could introduce new bacteria strains on food sample. Even if those stains are not considered as food-borne pathogens, other potential effects to the human body remain unknown and need to be investigated.

2.11.3 Physical Treatments

Physical treatments use different types of physical agents like heat, pressure, light, or irradiation to treat seeds or sprouts to inactivate bacteria and fulfill the decontamination requirement. The advantage of physical treatments is that the treatments themselves don't leave any foreign chemical or biological residues on food product. Without greatly affecting the product after treatment, physical treatments are more recommended by the FDA for the sprout industry, as long as the decontamination requirement are fulfilled.

Thermal treatment has a very long history of application in food processing, and the advantage of thermal treatment is that the required equipment is very simple and low cost. When it comes to thermal treatment, there are two categories: wet heat and dry heat. Wet heat involves the direct interaction of the heat medium such as heated water with seeds. Dry heat uses heated air to conduct heat to the seeds. Dry heat is milder compared to wet heat, so usually dry heat treatment takes longer than wet heat treatment. Wet heat treatment will be discussed here, while dry heat treatment will be discussed in Chapter 2.11.5. Mung bean seeds with a thicker coating can germinate better post wet heat treatment than those with thinner coats like alfalfa and radish seeds (Suzuki and Takizawa, 1997). This is also proven by our dry treatment method that mung bean seeds behaved better than alfalfa, radish, and red clover after treatment. Bari et al. (2008) found that when mung beans were dipped in 90°C hot water for 90 seconds and chilled for 30 seconds, a 6-log reduction and a 5.34-log reduction were achieved for *E. coli* and *Salmonella*, respectively. When treating alfalfa sprouts instead of seeds with a hot water dipping at 100°C for 3 seconds, a full elimination of *Salmonella* was achieved, though sprouts' quality after the treatment remains to be tested (Pao et al., 2008).

Nonthermal physical treatments, including high pressure processing (HPP), irradiation, supercritical carbon dioxide, and UV light, have also raised a lot of attentions by researchers. Neetoo et al. (2008) found that using HPP to treat alfalfa seeds for 2 minutes at 550 MPa/40°C could result in a 5-log reduction of *E. coli* O157:H7. Irradiation is another nonthermal physical treatment and treating with 0.5 kGy showed a 4-log reduction of *Salmonella* population (Rajkowski and Thayer, 2000). Supercritical carbon dioxide used on alfalfa seeds with 27.6 MPa at 50°C for 15 minutes reduced 81.3% of *E. coli* K12 (Mazzoni et al., 2001). Treating alfalfa seeds with pulsed ultraviolet light up to 90 s at 8-cm distance from the UV strobe reduced the population of *Escherichia coli* O157:H7 by more than 4 log CFU/g (Sharma and Demirci, 2003).

Although nonthermal treatment can usually achieve a very high microbial log reduction and also retain nutrients well after treatment, the drawback of these treatments is the high cost for building the whole production line, which is impracticable for most of the food producers. It is estimated that the initial investment of a whole HPP production line could cost $600,000-$4,000,000 and building a food irradiation plant could cost millions of dollars as well (FIPA, 2006; Yamamoto, 2017). If these new nonthermal treatment technologies are applied to sprouts decontamination, it will create a huge economic burden for sprout producers.

2.11.4 Hurdle Technology

Sometimes it is also a good approach to combine different treatment methods listed above into one combined method to increase the overall treatment effects, which is called hurdle technology in food processing. Hurdle technology is also widely researched in the seed and sprout processing area.

It is proven that HPP could be enhanced by using some antimicrobial compounds like hypochlorite and carvacrol on alfalfa or mung bean sprouts, but potentially could negatively affect the seeds' germination as well (Peñas et al., 2009). Millan-Sango et al. (2017) found out that when ultrasound and aqueous chlorine dioxide were combined to treat alfalfa and mung bean sprouts, the overall log reduction of *E. coli* and *Salmonella* increased than using these two methods alone. The combined treatment of soaking alfalfa seeds first in water for 60 min and then treating with HPP at 600 MPa for 2 min at 20 °C were successfully decontaminated to *E. coli* and afterwards showed a germination rate of 91% (Neetoo et al., 2009). Mung bean seeds dipped in hot water at 85°C for 40 seconds followed by dipping in cold water for 30 seconds then soaking into 2,000 ppm chlorine solution for 2 hours could decontaminate all inoculated *Escherichia coli* O157:H7 and *Salmonella* Enteritidis on mung bean seeds, and no pathogens were founded in the following sprouting process (Bari et al., 2010).

Different combinations of treatment methods could apply to seed decontamination and currently under research. There is a very high potential for hurdle technology to enhance the microbial inactivation than just the single treatment method.

2.11.5 Dry Heat and Effectiveness Factors

Dry heat treatment is a very good approach for inactivating pathogens, especially when it comes to seeds. Because seeds are usually oval shaped, some surface treatment method like UV light cannot reach the deep surface of seeds. Kwack et al. (2014) showed that under an electron microscope, there were many cracks and gaps on the surface of seeds, which could possibly provide an ideal hiding position for bacteria cells. Fu et al. (2022) found that bacteria cells' distribution on the hilum of alfalfa seeds was higher than that of other parts of the seed coat after the inoculation with *Listeria monocytogenes* or *Salmonella enterica* serovar Typhimurium on alfalfa seeds. With such seed structures and strong bacteria attachment to seeds, surface treatments cannot do very good work in pathogen inactivation. Dry heat will process the whole seed part because the heat can penetrate every part of the seed. In previous studies, dry heat treatment also showed a good inactivation efficiency of *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. With the 55°C dry heat treatment for 6 days, *Salmonella* and *Escherichia coli* O157:H7 were inactivated 2 log and 8 log, respectively, and no significant damage to seeds viability occurred (Feng et al., 2007). With the proper control of treatment temperature and time, dry heat can achieve higher log reductions on *Salmonella* and shows a promising application.

2.11.5.1 The Relationship between Water Activity and *Salmonella* Thermal Resistance

It is a heavily studied phenomenon that with a lower water activity, the heat-resistance of *Salmonella* can increase accordingly. Finn et al. (2013) stated that the formation of biofilm and filament in *Salmonella* could explain this heat-resistance increase due to lower water activity. Some other literatures have already proved this phenomenon as well, in dried food products such as dried milk powder (Dega et al.,1972; McDonough and Hargrove,1968; Wei et al., 2020). Wei et al. (2020) discovered that milk powder under 76.6°C heat treatment, the D-value of *Salmonella* can significantly increase as twice as much when a_w decreases from 0.3 to 0.2. In our study, at 71.0°C dry heat treatment, when a_w decreased from 0.2 to 0.1, the required treatment time for a 5-log reduction increased from 60 hours to 140 hours, which was more than 2 times. The same experimental phenomenon happened for 76.6°C treatment in our study as well. This is an indication that when seeds are heat treated under a lower a_w environment, *Salmonella* on seeds are usually more resistant and the treatment time should be extended to reach the same level of log reduction.

REFERENCES

Alghoribi, M. F., Doumith, M., Alrodayyan, M., Al Zayer, M., Köster, W. L., Muhanna, A., Aljohani, S. M., Balkhy, H. H., & Desin, T. S. (2019). *S.* Enteritidis and *S.* Typhimurium harboring SPI-1 and SPI-2 are the predominant serotypes associated with human salmonellosis in Saudi Arabia. *Frontiers in Cellular and Infection Microbiology*, 9, 187.

Arretz, P. V., & Macfarlane, R. P. (1986). The introduction of *Bombus ruderatus* to Chile for red clover pollination. *Bee World*, 67(1), 15-22.

Barak, J. D., Whitehand, L. C., & Charkowski, A. O. (2002). Differences in attachment of *Salmonella enterica* serovars and *Escherichia coli* O157: H7 to alfalfa sprouts. *Applied and Environmental Microbiology*, 68(10), 4758-4763.

Bari, M. L., Nazuka, E., Sabina, Y., Todoriki, S., & Isshiki, K. (2003). Chemical and irradiation treatments for killing *Escherichia coli* O157: H7 on alfalfa, radish, and mung bean seeds. *Journal of Food Protection*, 66(5), 767-774.

Bean Sprout Grower's Association of Japan (BSGAJ), (2011). The development of the Bean Sprout Market in Japan and the Activities of the Bean Sprout Grower's Association of Japan. [PPT]. Presented at *the 21st ISGA Convention*, Oct. 2011, Las Vegas, USA. Available at: http://www.isga-sprouts.org/wp-content/uploads/2013/05/JBSA_The_Bean_Sprout_Market_in_Japan_JBSC2011.pdf. Accessed on March 30, 2022.

Bennik, M. H. J., Van Overbeek, W., Smid, E. J., & Gorris, L. G. M. (1999). Biopreservation in modified atmosphere stored mungbean sprouts: the use of vegetable-associated bacteriocinogenic lactic acid bacteria to control the growth of *Listeria monocytogenes*. *Letters in Applied Microbiology*, 28(3), 226-232.

Beuchat, L. R., & Heaton, E. K. (1975). *Salmonella* survival on pecans as influenced by processing and storage conditions. *Applied Microbiology*, 29(6), 795-801.

Beuchat, L. R. (1996). Pathogenic microorganisms associated with fresh produce. *Journal of Food Protection*, 59(2), 204-216.

Beutin, L., & Martin, A. (2012). Outbreak of Shiga toxin–producing *Escherichia coli* (STEC) O104: H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. *Journal of Food Protection*, 75(2), 408-418.

Bielaszewska, M., Mellmann, A., Zhang, W., Köck, R., Fruth, A., Bauwens, A., Peters, G., & Karch, H. (2011). Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. *The Lancet Infectious Diseases*, 11(9), 671-676.

Bilello, Stanley (2016). 21st Century Homestead: Nitrogen-Fixing Crops. *Lulu.com*. ISBN 978-1-365-45290-1.

Bilinski, P., Kapka-Skrzypczak, L., Posobkiewicz, M., Bondaryk, M., Holownia, P., & Wojtyla, A. (2012). Public health hazards in Poland posed by foodstuffs contaminated with *E. coli* O104: H4 bacterium from the recent European out break. *Annals of Agricultural and Environmental Medicine*, 19(1).

Bronze, M. S., & Greenfield, R. A. (Eds.). (2005). *Biodefense: principles and pathogens*. Wymondham, Norfolk, England: Horizon Bioscience.

Brooks, J. T., Rowe, S. Y., Shillam, P., Heltzel, D. M., Hunter, S. B., Slutsker, L., Hoekstra, R. M., & Luby, S. P. (2001). *Salmonella* Typhimurium infections transmitted by chlorine-pretreated clover sprout seeds. *American Journal of Epidemiology*, 154(11), 1020-1028.

Buchholz, A., & Matthews, K. R. (2010). Reduction of *Salmonella* on alfalfa seeds using peroxyacetic acid and a commercial seed washer is as effective as treatment with 20 000 ppm of $Ca(OCl)_2$. *Letters in Applied Microbiology*, 51(4), 462-468.

Callister, S. M., & Agger, W. A. (1987). Enumeration and characterization of *Aeromonas hydrophila* and *Aeromonas caviae* isolated from grocery store produce. *Applied and Environmental Microbiology*, 53(2), 249-253.

Cash, D. (2009). "Chapter 1. Global Status and Development Trends of Alfalfa" (PDF). Alfalfa Management Guide for Ningxia (PDF). *United Nations Food and Agriculture Organization*. pp. 1–2. Archived from the original (PDF) on 24 September 2015. Retrieved 27 March 2022.

Centers for Disease Control and Prevention (CDC). (2022). *Salmonella*. Available at https://www.cdc.gov/salmonella/general/index.html Accessed on April 5, 2022.

Charkowski, A. O., Barak, J. D., Sarreal, C. Z., & Mandrell, R. E. (2002). Differences in growth of *Salmonella enterica* and *Escherichia coli* O157: H7 on alfalfa sprouts. *Applied and Environmental Microbiology*, 68(6), 3114-3120.

Chavan, J. K., Kadam, S. S., & Beuchat, L. R. (1989). Nutritional improvement of cereals by sprouting. *Critical Reviews in Food Science & Nutrition*, 28(5), 401-437.

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, 71(1), 1-20.

Cody, E. M., & Dixon, B. P. (2019). Hemolytic uremic syndrome. *Pediatric Clinics*, 66(1), 235-246.

Crisp P. (1995). Radish, *Raphanus sativus* (Cruciferae). In: Smartt J, Simmonds NW, editors. *Evolution of Crop Plants*. 2nd Edition, Longman Scientific & Technical, UK. pp. 86–89.

Cui, Y., Walcott, R., & Chen, J. (2017). Differential attachment of *Salmonella enterica* and enterohemorrhagic *Escherichia coli* to alfalfa, fenugreek, lettuce, and tomato seeds. *Applied and Environmental Microbiology*, 83(7), e03170-16.

DeEll, J.R. (2014). Sprouts. The commercial storage of fruits, vegetables, and florist and nursery stocks. Agriculture Handbook Number 66 (HB-66). Beltsville, MD: *United States Department of Agriculture*, Agricultural Research Service. Available from: www.ba.ars.usda.gov/hb66/sprouts.pdf. Accessed on March 30, 2022.

Dega, C. A., Goepfert, J. M., & Amundson, C. H. (1972). Heat resistance of salmonellae in concentrated milk. *Applied Microbiology*, 23(2), 415-420.

Ding, H., Fu, T. J., & Smith, M. A. (2013). Microbial contamination in sprouts: how effective is seed disinfection treatment? *Journal of Food Science*, 78(4), R495-R501.

Doblado, R., Frías, J., & Vidal-Valverde, C. (2007). Changes in vitamin C content and antioxidant capacity of raw and germinated cowpea (*Vigna sinensis* var. *carilla*) seeds induced by high pressure treatment. *Food Chemistry*, 101(3), 918-923.

Eng, S. K., Pusparajah, P., Ab Mutalib, N. S., Ser, H. L., Chan, K. G., & Lee, L. H. (2015). *Salmonella*: a review on pathogenesis, epidemiology and antibiotic resistance. *Frontiers in Life Science*, 8(3), 284-293.

Estrela, C., Estrela, C. R., Barbin, E. L., Spanó, J. C. E., Marchesan, M. A., & Pécora, J. D. (2002). Mechanism of action of sodium hypochlorite. *Brazilian Dental Journal*, 13, 113-117.

European Commission. (2013a). Commission Implementing Regulation (EU) No 208/2013. Accessed on April 6, 2022.

European Commission. (2013b). Commission Regulation (EU) No 209/2013. Accessed on April 6, 2022.

European Commission. (2013c). Commission Regulation (EU) No 211/2013. Accessed on April 6, 2022.

Feng, G., Churey, J. J., & Worobo, R. W. (2007). Thermal inactivation of *Salmonella* and *Escherichia coli* O157: H7 on alfalfa seeds. *Journal of Food Protection*, 70 (7), 1698-1703.

Feng, P. (1997). A summary of background information and foodborne illness associated with the consumption of sprouts. *Center for Food Safety and Applied Nutrition*. Available at: https://sproutnet.com/foodborne-illness-associated-with-the-consumption-of-sprouts/. Accessed on March 30, 2022.

Fery, R. L. (1990). The cowpea: production, utilization, and research in the United States. *Horticultural Reviews*, 12, 197-222.

Fett, W. F. (2000). Naturally occurring biofilms on alfalfa and other types of sprouts. *Journal of Food Protection*, 63(5), 625-632.

Fett, W. F. (2005). Interventions to ensure the microbial safety of sprouts. In *Microbiology of Fruits and Vegetables* (pp. 203-226). CRC Press. ISBN 9780367392215.

Fett, W. F. (2006). Inhibition of *Salmonella enterica* by plant-associated pseudomonads in vitro and on sprouting alfalfa seed. *Journal of Food Protection*, 69(4), 719-728.

Fink, S. L., & Cookson, B. T. (2007). Pyroptosis and host cell death responses during *Salmonella* infection. *Cellular Microbiology*, 9(11), 2562-2570.

Finn, S., Condell, O., McClure, P., Amézquita, A., & Fanning, S. (2013). Mechanisms of survival, responses and sources of *Salmonella* in low-moisture environments. *Frontiers in Microbiology*, 4, 331.

Food and Drug Administration (FDA). (1999a). Guidance for Industry: Reducing microbial food safety hazards in the production of seed for sprouting. Available at: https://www.ifsh.iit.edu/sites/ifsh/files/departments/ssa/pdfs/fda1999_sproutseedsguidance.pdf. Accessed on April 5, 2022.

Food and Drug Administration (FDA). (1999b). Guidance for Industry: Sampling and microbial testing of spent irrigation water during sprout production. Available at https://www.ifsh.iit.edu/sites/ifsh/files/departments/ssa/pdfs/fda1999_spent_irrigation_water.pdf. Accessed on April 5, 2022.

Food and Drug Administration (FDA). (2017). Draft Guidance for Industry: Compliance with and Recommendations for Implementation of the Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption for Sprout Operations. Available at https://www.fda.gov/regulatory-information/search-fda-guidance-documents/draft-guidance-industry-compliance-and-recommendations-implementation-standards-growing-harvesting. Accessed on April 5, 2022.

Food and Drug Administration (FDA). (2018). Sprout Safety Alliance. Available at https://www.fda.gov/food/food-safety-modernization-act-fsma/sprout-safety-alliance. Accessed on April 6, 2022.

Food and Drugs, 21 CFR §112.143, (2022a).

Food and Drugs, 21 CFR §112.44, (2022b).

Food and Drugs, 21 CFR §112.145, (2019c).

Food and Drugs, 21 CFR §112.147, (2019d)

Food and Drugs, 21 CFR §112.150, (2019e).

Food Irradiation Processing Alliance (FIPA). (2006). Food irradiation: Questions and Answers. *FoodIrradiation*. Available at: http://www.foodirradiation.org/PDF/FIPA%20QandA.pdf. Accessed on April. 7, 2022

Fookes, M., Schroeder, G. N., Langridge, G. C., Blondel, C. J., Mammina, C., Connor, T. R., Seth-Smith, H., Vernikos, G. S., Robinson, K. S., Sanders, M., Petty, N. K., Kingsley, R. A., Bäumler, A. J., Nuccio, S., Contreras, I., Santiviago, C. A., Maskell, D., Barrow, P., Humphrey, T., Nastasi, A., Roberts, M., Frankel, G., Parkhill, J., Dougan, G., & Thomson, N. R. (2011). *Salmonella bongori* provides insights into the evolution of the Salmonellae. *PLoS Pathogens*, 7(8), e1002191.

Fu, Y., Bhunia, A. K., & Yao, Y. (2022). Alginate-based antimicrobial coating reduces pathogens on alfalfa seeds and sprouts. *Food Microbiology*, 103, 103954.

Gensheimer, K., & Gubernot, D. (2016). 20 years of sprout-related outbreaks: FDA's investigative efforts. *In Open Forum Infectious Diseases* (Vol. 3, No. suppl_1, p. 1438). Oxford University Press.

Ghani, M., Kulkarni, K. P., Song, J. T., Shannon, J. G., & Lee, J. D. (2016). Soybean sprouts: A review of nutrient composition, health benefits and genetic variation. *Plant Breeding and Biotechnology*, 4(4), 398-412.

Glaubrecht, M., & Köhler, F. (2004). Radiating in a river: systematics, molecular genetics and morphological differentiation of viviparous freshwater gastropods endemic to the Kaek River, central Thailand (Cerithioidea, Pachychilidae). *Biological Journal of the Linnean Society*, 82(3), 275-311.

Goodfellow, S. J., & Brown, W. L. (1978). Fate of *Salmonella* inoculated into beef for cooking. *Journal of Food Protection*, 41(8), 598-605.

Güllüce, M., Karadayı, M., & Bariş, Ö. (2013). Bacteriocins: promising natural antimicrobials. *Local Environment*, 3, 6.

Hara-Kudo, Y., Konuma, H., Iwaki, M., Kasuga, F., Sugita-Konishi, Y., Ito, Y., & Kumagai, S. (1997). Potential hazard of radish sprouts as a vehicle of *Escherichia coli* O157: H7. *Journal of Food Protection*, 60(9), 1125-1127.

Hochstein, P., & Atallah, A. S. (1988). The nature of oxidants and antioxidant systems in the inhibition of mutation and cancer. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 202(2), 363-375.

Hong, Y. H., Chao, W. W., Chen, M. L., & Lin, B. F. (2009). Ethyl acetate extracts of alfalfa (*Medicago sativa* L.) sprouts inhibit lipopolysaccharide-induced inflammation in vitro and in vivo. *Journal of Biomedical Science*, 16(1), 1-12.

Hou D., Yousaf, L., Xue Y., Hu, J., Wu, J., Hu, X., Feng, N., & Shen, Q., (2019) Mung Bean (*Vigna radiata* L.): bioactive polyphenols, polysaccharides, peptides, and health benefits. *Nutrients*. 11(6):1238.

Howard, M. B., & Hutcheson, S. W. (2003). Growth dynamics of *Salmonella enterica* strains on alfalfa sprouts and in waste seed irrigation water. *Applied and Environmental Microbiology*, 69(1), 548-553.

Huang, H. T., DuBois, C. M., Tan, C. B., & Mintz, S. W. (2008). Early uses of soybean in Chinese history. *The World of Soy*, 45-55.

Illinois Institute of Technology. (2009). Safer Processing of Sprouts: Module 3E Cleaning and Sanitizing Equipment and Facilities. Available at: https://www.youtube.com/watch?v=esfI8LUyXSk. Accessed on March 29, 2022.

Illinois Institute of Technology. (2022). Sprout Safety Alliance. Available at: https://www.ifsh.iit.edu/ssa/resources. Accessed on March 29, 2022.

Imamura, T., Ide, H., & Yasunaga, H. (2007). History of public health crises in Japan. *Journal of Public Health Policy*, 28(2), 221-237.

Itoh, Y., Sugita-Konishi, Y., Kasuga, F., Iwaki, M., Hara-Kudo, Y., Saito, N., Noguchi, Y., Konuma, H., & Kumagai, S. (1998). Enterohemorrhagic *Escherichia coli* O157: H7 present in radish sprouts. *Applied and Environmental Microbiology*, 64(4), 1532-1535.

James, J. (2006). Overview of microbial hazards in fresh fruit and vegetables operations. *Microbial Hazard Identification in Fresh Fruit and Vegetables*, 1-36.

Jones, C., De Vega, J., Lloyd, D., Hegarty, M., Ayling, S., Powell, W., & Skøt, L. (2020). Population structure and genetic diversity in red clover (*Trifolium pratense* L.) germplasm. *Scientific Reports*, 10(1), 1-12.

Kang, Y. (2011). Current Situation of the Industrialization of Bean Sprouts Production, Processing, and Marketing in China. [PPT]. *China Agricultural University*. Presented at 21st ISGA Convention, Oct. 2011, Las Vegas USA. Available at: http://www.isga-sprouts.org/wp-content/uploads/2013/05/Roujun_Wang_KANG_YUFAN_Market_Demand_for_Mung_Bean_seeds_in_China_2011_10_19_ISGA.pdf. Accessed on March 30, 2022.

Kawashima, T., & Goldberg, R. B. (2010). The suspensor: not just suspending the embryo. *Trends in Plant Science*, 15(1), 23-30.

Khare, C. P. (2008). *Indian medicinal plants: an illustrated dictionary*. Springer Science & Business Media.

Kumar, M., Hora, R., Kostrzynska, M., Waites, W. M., & Warriner, K. (2006). Inactivation of *Escherichia coli* O157: H7 and *Salmonella* on mung beans, alfalfa, and other seed types destined for sprout production by using an oxychloro-based sanitizer. *Journal of Food Protection*, 69(7), 1571-1578.

Kwack, Y., Kim, K. K., Hwang, H., & Chun, C. (2014). An ozone micro-bubble technique for seed sterilization in Alfalfa sprouts. *Horticultural Science and Technology*. 901-905.

Lang, M. M., Ingham, B. H., & Ingham, S. C. (2000). Efficacy of novel organic acid and hypochlorite treatments for eliminating *Escherichia coli* O157: H7 from alfalfa seeds prior to sprouting. *International Journal of Food Microbiology*, 58(1-2), 73-82.

LaRock, D. L., Chaudhary, A., & Miller, S. I. (2015). Salmonellae interactions with host processes. *Nature Reviews Microbiology*, 13(4), 191-205.

Majowicz, S. E., Musto, J., Scallan, E., Angulo, F. J., Kirk, M., O'Brien, S. J., Jones, T. F., Fazil, A., Hoekstra, R. M. (2010). The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clinical Infectious Diseases*, 50(6), 882-889.

Mandal, R. K., & Kwon, Y. M. (2017). Global screening of *Salmonella enterica* serovar Typhimurium genes for desiccation survival. *Frontiers in Microbiology*, 8, 1723.

Mazzoni, A. M., Sharma, R. R., Demirci, A. L. I., & Ziegler, G. R. (2001). Supercritical carbon dioxide treatment to inactivate aerobic microorganisms on alfalfa seeds. *Journal of Food Safety*, 21(4), 215-223.

McDonough, F. E., & Hargrove, R. E. (1968). Heat resistance of *Salmonella* in dried milk. *Journal of Dairy Science*, 51(10), 1587-1591.

Meyer, D. W. (1999). Alfalfa: Seed Germination, Seedling Growth, Vegetative Development. Agricultural Experiment Station, *North Dakota State University*. Available at: https://library.ndsu.edu/ir/bitstream/handle/10365/9130/R648_1999.pdf?sequence=1&isAllowed=y. Accessed on April.2, 2022.

Michino, H., Araki, K., Minami, S., Takaya, S., Sakai, N., Miyazaki, M., Ono, A., & Yanagawa, H. (1999). Massive outbreak of *Escherichia coli* O157:H7 infection in schoolchildren in Sakai City, Japan, associated with consumption of white radish sprouts. *American Journal of Epidemiology*, 150(8), 787-96.

Millan-Sango, D., Sammut, E., Van Impe, J. F., & Valdramidis, V. P. (2017). Decontamination of alfalfa and mung bean sprouts by ultrasound and aqueous chlorine dioxide. *Lwt*, 78, 90-96.

Min, L. (2001). Research advance in chemical composion and pharmacological action of mung bean. *Shanghai J Trad Chin Med*, 5, 18.

Mueller, S. (2008). Alfalfa seed production in the western United States. *UC Cooperative Extension, University of California Davis*. Available at: http://www.alfalfaseed.ucdavis.edu/files/147738.html. Accessed on March 30, 2022.

Nandiwada, L. S., Schamberger, G. P., Schafer, H. W., & Diez-Gonzalez, F. (2004). Characterization of an E2-type colicin and its application to treat alfalfa seeds to reduce *Escherichia coli* O157: H7. *International Journal of Food Microbiology*, 93(3), 267-279.

National Advisory Committee on Microbiological Criteria for Foods. (1999). Microbiological safety evaluations and recommendations on sprouted seeds. *International Journal of Food Microbiology*, 52(3), 123-153.

Neetoo, H., Ye, M., & Chen, H. (2009). Factors affecting the efficacy of pressure inactivation of *Escherichia coli* O157: H7 on alfalfa seeds and seed viability. *International Journal of Food Microbiology*, 131(2-3), 218-223.

Nei, D., Latiful, B. M., Enomoto, K., Inatsu, Y., & Kawamoto, S. (2011). Disinfection of radish and alfalfa seeds inoculated with *Escherichia coli* O157: H7 and *Salmonella* by a gaseous acetic acid treatment. *Foodborne Pathogens and Disease*, 8(10), 1089-1094.

Neuman, W. (2011). The poster plant of health food can pack disease risks. *The New York Times*, 10.

Oakley, R. A., & Westover, H. L. (1922). How to Grow Alfalfa. *United States Department of Agriculture*. p. 3. OCLC 15432716.

Ortega, F., Parra, L., & Quiroz, A. (2014). Breeding red clover for improved persistence in Chile: a review. *Crop and Pasture Science*, 65(11), 1138-1146.

Panetsos, C. A., & Baker, H. G. (1967). The origin of variation in "wild" *Raphanus sativus* (*Cruciferae*) in California. *Genetica*, 38(1), 243-274.

Pao, S., Kalantari, A., & Khalid, M. F. (2008). Eliminating *Salmonella enterica* in alfalfa and mung bean sprouts by organic acid and hot water immersions. *Journal of Food Processing and Preservation*, 32(2), 335-342.

Peace Region Forage Seed Association (PRFSA). (2022). Red clover seed production. Available at: http://www.peaceforageseed.ca/pdf/publications_pamphlets/Red_clover_seed_production_guide.pdf. Accessed on March 30, 2022.

Peñas, E., Gómez, R., Frías, J., & Vidal-Valverde, C., (2008). Application of high pressure on alfalfa (*Medigo sativa*) and mung bean (*Vigna radiata*) seeds to enhance the microbiological safety of their sprouts. *Food Control*, 19, 698-705.

Peñas, E., Gómez, R., Frías, J., & Vidal-Valverde, C., (2009). Efficacy of combinations of high pressure treatment, temperature and antimicrobial compounds to improve the microbiological quality of alfalfa seeds for sprout production. *Food Control*, 20(1), 31-39.

Pennsylvania State University (PSU). (1993). Red clover. Available from: https://extension.psu.edu/red-clover. Accessed on March 30, 2022.

Pradhan, S. K., Nerg, A. M., Sjöblom, A., Holopainen, J. K., & Heinonen-Tanski, H. (2007). Use of human urine fertilizer in cultivation of cabbage (Brassica oleracea)—impacts on chemical, microbial, and flavor quality. *Journal of Agricultural and Food Chemistry*, 55(21), 8657-8663.

Prager, R., Lang, C., Aurass, P., Fruth, A., Tietze, E., & Flieger, A. (2014). Two novel EHEC/EAEC hybrid strains isolated from human infections. *PLoS One*, 9(4), e95379.

Proctor, M. E., Hamacher, M., Tortorello, M. L., Archer, J. R., & Davis, J. P. (2001). Multistate outbreak of *Salmonella* serovar Muenchen infections associated with alfalfa sprouts grown from seeds pretreated with calcium hypochlorite. *Journal of Clinical Microbiology*, 39(10), 3461-3465.

Puohiniemi, R., Heiskanen, T., and Siitonen, A. (1997). Molecular epidemiology of two international sprout-borne *Salmonella* outbreaks. *Journal of Clinical Microbiology*, 35, 2487–2491

Rajkowski, K. T., & Ashurst, K. (2009). Use of 1% peroxyacetic acid sanitizer in an air-mixing wash basin to remove bacterial pathogens from seeds. *Foodborne Pathogens and Disease,* 6(9), 1041-1046.

Rajkowski, K. T., & Thayer, D. W. (2000). Reduction of *Salmonella* spp. and strains of *Escherichia coli* O157: H7 by gamma radiation of inoculated sprouts. *Journal of Food Protection*, 63(7), 871-875.

Ramu, R., Shirahatti, P. S., Devi, A. T., & Prasad, A. (2015). Bacteriocins and their applications in food preservation. *Critical Reviews in Food Science and Nutrition*, 0-0.

Reddy, M. S., Ilao, R. I., & Faylon, P. S. (Eds.). (2014). Recent advances in biofertilizers and biofungicides (PGPR) for sustainable agriculture. *Cambridge Scholars Publishing*. p. 381. ISBN 978-1-4438-7105-1.

Robertson, L. J., Johannessen, G. S., Gjerde, B. K., & Loncarevic, S. (2002). Microbiological analysis of seed sprouts in Norway. *International Journal of Food Microbiology*, 75 (1-2), 119-126.

Rosso, B. S., & Pagano, E. M. (2005). Evaluation of introduced and naturalised populations of red clover (*Trifolium pratense* L.) at Pergamino EEA-INTA, Argentina. *Genetic Resources and Crop Evolution*, 52(5), 507-511.

Schippers R. R. (2004). *Raphanus sativus* L. [online] Record from Protabase. Grubben GJH, Denton OA, editors. *PROTA (Plant Resources of Tropical Africa / Ressources végétales de l'Afrique tropicale)*, Wageningen, Netherlands. Available from: http://database.prota.org/search.htm. Accessed on: March 30, 2022.

Sharma, R. R., & Demirci, A. (2003). Inactivation of *Escherichia coli* O157: H7 on inoculated alfalfa seeds with pulsed ultraviolet light and response surface modeling. *Journal of Food Science*, 68(4), 1448-1453.

Shurtleff, W., & Aoyagi, A. (2016). History of soy sprouts (100 CE to 2013). ISBN 9781928914549.

Sikin, A. M., Zoellner, C., & Rizvi, S. S. (2013). Current intervention strategies for the microbial safety of sprouts. *Journal of Food Protection*, 76(12), 2099-2123.

Smith, S. I., Seriki, A., & Ajayi, A. (2021). Molecular Epidemiology of *Salmonella*. *In Molecular Food Microbiology* (pp. 229-239). CRC Press.

Sivapalasingam, S., Friedman, C. R., Cohen, L., & Tauxe, R. V. (2004). Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *Journal of Food Protection*, 67(10), 2342-2353.

Small, E. (2009). Top 100 food plants. *NRC Research Press*.

Soon, J. M., Seaman, P., & Baines, R. N. (2013). *Escherichia coli* O104: H4 outbreak from sprouted seeds. *International Journal of Hygiene and Environmental Health*, 216(3), 346-354.

Steiner, J. J., & Alderman, S. C. (2003). Red clover seed production: VI. Effect and economics of soil pH adjusted by lime application. *Crop Science*, 43(2), 624-630.

Stronk, J. P. (2019). From Sardis to Marathon. Greco-Persian relations 499-490 BC: A Review, part two: The battle of Marathon and its implications. *Talanta*, 51, 77-226.

Suzuki, T., & Takizawa, T. (1997). *U.S. Patent No. 5,615,518*. Washington, DC: U.S. Patent and Trademark Office.

Taormina, P. J., Beuchat, L. R., & Slutsker, L. (1999). Infections associated with eating seed sprouts: an international concern. *Emerging Infectious Diseases*, 5(5), 626.

Thung, T. Y., Radu, S., Mahyudin, N. A., Rukayadi, Y., Zakaria, Z., Mazlan, N., Tan, B. H., Lee, E., Yeoh, S. L., Chin, Y. Z., Tan, C. W., Kuan, C. H., Basri, D. F., & Wan Mohamed Radzi, C. W. (2018). Prevalence, virulence genes and antimicrobial resistance profiles of *Salmonella* serovars from retail beef in Selangor, Malaysia. *Frontiers in Microbiology*, 8, 2697.

Tournas, V. H. (2005). Spoilage of vegetable crops by bacteria and fungi and related health hazards. *Critical Reviews in Microbiology*, 31(1), 33-44.

Trelease, Sam F., Trelease, Helen M. (1943). Sprouted soy and mungo beans. J. of the New York Botanical Garden 44(527):254-60. Nov.

Undersander, D. J., Vassalotti, P., & Cosgrove, D. (1997). Alfalfa germination & growth (Vol. 3681). *University of Wisconsin*--Extension, Cooperative Extension.

United States Department of Agriculture (USDA). (2013). Crop Production: 2012 Summary. Available at: https://usda.library.cornell.edu/concern/publications/k3569432s. Accessed on March 30, 2022.

United States Department of Agriculture (USDA). (2022). Crop Production: 2021 Summary. Available at:

https://usda.library.cornell.edu/concern/publications/k3569432s. Accessed on March 30, 2022.

United States Department of Agriculture (USDA). (2022). Safe Minimum Internal Temperature Chart. Available at: https://www.fsis.usda.gov/food-safety/safe-food-handling-and-preparation/food-safety-basics/safe-temperature-chart. Accessed on March 29, 2022.

Wei, X., Lau, S. K., Chaves, B. D., Danao, M. G. C., Agarwal, S., & Subbiah, J. (2020). Effect of water activity on the thermal inactivation kinetics of *Salmonella* in milk powders. *Journal of Dairy Science*, 103(8), 6904-6917.

Weiss, A., Hertel, C., Grothe, S., Ha, D., & Hammes, W. P. (2007). Characterization of the cultivable microbiota of sprouts and their potential for application as protective cultures. *Systematic and Applied Microbiology*, 30(6), 483-493.

World Vegetable Center. (2016). International mung bean improvement network. Available at: https://avrdc.org/intl-mungbean-network/. Accessed on March 30, 2022.

Yamamoto, K. (2017). Food processing by high hydrostatic pressure. *Bioscience, Biotechnology and Biochemistry*, 81(4),672-679.

Yang, Q. (2022). Radish genetic resources. *Crop Genebank Knowledge Base, Genebank Standards for Plant Genetic Resources for Food and Agriculture*. Accessed on March 30, 2022.

Zohary, D., & Hopf, M. (2000). *Domestication of plants in the Old World: The origin and spread of cultivated plants in West Asia, Europe and the Nile Valley* (No. Ed. 3). Oxford university press. ISBN: 0198503563.

Chapter 3

MATERIALS AND METHODS

3.1 Alfalfa, Mung Bean, Radish, and Red Clover Seeds Preparation and Storage

Alfalfa (*Medicago sativa*), mung bean (*Vigna radiata*), radish (*Raphanus raphanistrum subsp.* sativus), and red clover (*Trifolium pratense*) seeds were purchased from International Specialty Supply (Cookeville, TN, USA). Seeds were kept in nylon bags at room temperature (24°C±1) and natural relative humidity (RH) to mimic general industrial seed storage conditions. The water activity levels of seeds ranged from 0.3-0.5 (RH30%-50%), depending on different seasons and weather. Seeds were used for both microbial and germination studies.

3.2 Microbiological Analysis of Dry Heat Treatment on Seeds

3.2.1 Bacteria Strains and Culture Preparation

Four serotypes of *Salmonella* were used in this study: *Salmonella* Heidelberg 45955, *Salmonella* Typhimurium 14028, *Salmonella* Montevideo 51, *Salmonella* Newport H1073. Bacteria strains came from University of Delaware Culture Collections. All these bacteria strains contain the nalidixic acid resistance mutation, so they could grow in media containing nalidixic acid and media could select for the inoculated *Salmonella* only. One loopful of each of the four original *Salmonella* strains was inoculated into 10 ml of tryptic soy broth (Becton, Dickinson and Company, MD, USA) with 6% yeast extract (HiMedia Laboratories Pvt. Ltd., India) and 1mg/10ml nalidixic acid (Fisher Scientific, NH, USA) (TSBYE-N). Each of the TSBYE+N tubes was incubated at 35°C for 12 hours then streaked on XLT-4 Agar (Becton, Dickinson and Company, MD, USA) and incubated at 35°C for 24 hours to verify the inoculums as *Salmonella* spp. Each inoculum was also streaked on tryptic soy agar (Becton, Dickinson and Company, MD, USA) with 6% yeast extract (HiMedia Laboratories Pvt. Ltd., India) and 1mg/10ml nalidixic acid (Fisher Scientific, NH, USA) (TSAYE-N) and incubated at 35°C for 24 hours, then stored at 4°C for stock cultures (stock cultures could be used for one month, then repeat this culture preparation step to make new cultures).

3.2.2 Seed Inoculations

Each of the four stocks of *Salmonella* strains on TSAYE-N Petri dishes was inoculated into 10 ml of TSBYE-N. One colony was picked from each plate and transferred to four individual tubes. TSBYE-N tubes were incubated at 35°C for 12 hours, then 1 ml of culture from each TSBYE-N tube was transferred into 4 bottles with 300 ml TSBYE-N. The 4 bottles were shaken on an orbital shaker at 120 rpm and incubated at 35°C for 48 hours. The cultures were transferred into 4 centrifuge bottles. The 4 bottles were centrifuged at 20°C at 4000 RPM for 10 minutes. After the centrifugation, the bacteria were collected and resuspended in 300 ml of sterile 0.1% peptone water (Becton, Dickinson and Company, MD, USA) to obtain a cocktail of the strains. The cocktail was mixed with 400 g of alfalfa, mung bean, radish, or red clover seeds. The mixture was homogenized by constant stirring with a glass rod for 25 minutes to allow bacteria cells to attach to the seeds. After homogenization, seeds were placed on a double layer of cheesecloth and dried inside a biosafety hood at room temperature for 48 hours. The water activity range was usually 0.5-0.8 after 48-hour drying.

3.2.3 Water Activity Control

Inoculated seeds were dried and controlled to different water activity levels to meet the dry heat treatment water activity requirement. Water activity values were constantly measured by an AquaLab Water Activity Meter (METER Group, Inc., WA, USA). Self-build desiccators with fans were used to store the seeds. To reach different levels of water activity, different saturated salt solutions were used in each desiccator: potassium carbonate (a_w 0.432), magnesium chloride (a_w 0.328), and potassium acetate (a_w 0.225). Inoculated seeds were kept in different desiccators to reach the target a_w level. To reach a_w 0.2, a constant climate chamber (Memmert, Germany) was used to keep inoculated seeds and stabilized them at an a_w of 0.2. The lowest level the constant climate chamber could reach was 0.2, so other seeds were kept in sealed jars with silicon beads to continue to dry them to a lower a_w of 0.1 and 0.05. The seeds' water activity was constantly monitored and when the target a_w level was reached (a_w 0.1 or a_w 0.05), the silicon beads were removed from the jars and the jars were completely sealed to maintain the stabilized a_w level.

3.2.4 Dry Heat Treatment

Twenty grams of inoculated seeds were placed in a sealed glass tube. Figure 3.1 showed the actual set-up of lab-scale dry heat treatment system, using water bath as the heat source. Dry hot air was the heat conduction medium in the sealed jar. The tube was exposed to dry heat at temperatures of 65.9°C, 71.0°C, 76.6°C respectively.

Seeds were dry heat treated for at most 180 hours (65.9°C) and 160 hours (71.0°C, 76.6°C). Water activity levels of 0.45, 0.35, 0.25, 0.2, 0.1, 0.05 were used for dry heat treatment, with different treatment temperature and treatment time. Microbial population density was analyzed before and after the dry heat treatment.

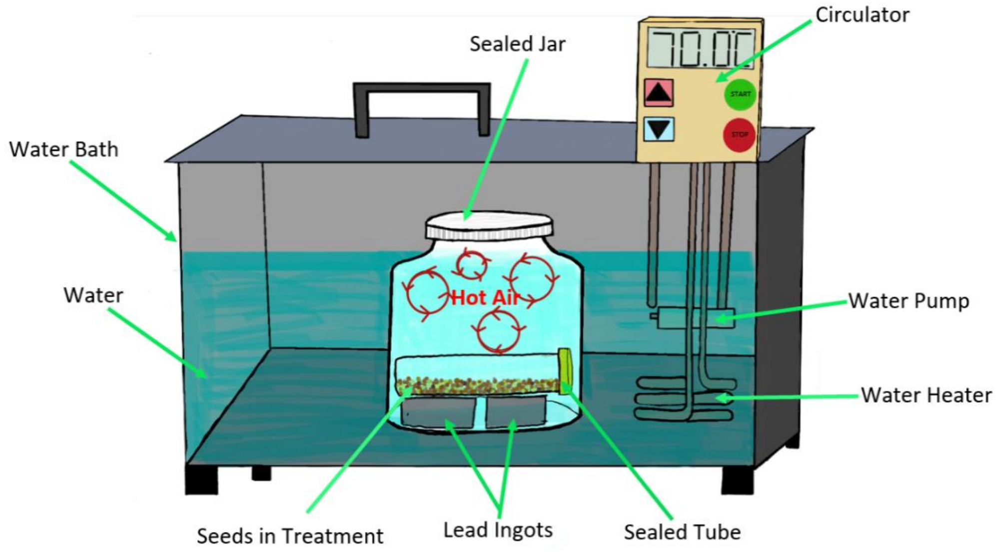

Figure 3.1 Lab-scale dry heat treatment system

3.2.5 Microbiological Analysis

Before and after the heat treatment, *Salmonella* inoculated seeds were collected and mixed with 0.1% peptone water, at a ratio of 1:5 or 1:10 (seeds: total weight of mixture). The mixture was homogenized in a stomacher for 2 min at 260 rpm (Seward Stomacher, UK). After homogenization, serial dilution was preformed to the appropriate microbial population load and plated on TSAYE-N plates. The plates for microbial population calculation before treatment were incubated at 35°C for 24 hours. The plates for microbial population calculation post treatment were incubated at 35°C for 48 hours, because *Salmonella* were likely injured after dry heat treatment and needed more time to form colonies. Colonies on plates were enumerated and CFU/g were calculated to estimate the log reduction of *Salmonella* population on alfalfa, mung bean, radish, and red clover seeds, before and after treatment.

3.3 Germination Analysis of Dry Heat Treatment on Seeds

3.3.1 Water Activity Control and Dry Heat Treatment

Procedures for water activity control and dry heat treatment in this part is very similar to the procedures used for part *3.2.3* and *3.2.4*, except in this germination study, no *Salmonella* inoculation was needed. Uninoculated seeds were directly taken from bags and placed separately in the desiccators (a_w 0.45, a_w 0.35, a_w 0.25), the constant climate chamber (a_w 0.2), and sealed jars with silicon beads (a_w 0.1 & a_w 0.05). Water activity level was precisely controlled to 0.45, 0.35, 0.25, 0.2, 0.1, 0.05. Twenty grams of a_w controlled seeds were placed in a sealed glass tube. The tubes were exposed to dry heat at temperatures of 65.9°C, 71.0°C, 76.6°C, respectively. Seeds were dry heat treated for at most 180 hours (65.9°C) and 160 hours (71.0°C, 76.6°C). Different treatment temperatures and different treatment times were used in accord to the parameters used in the microbiological analysis part. After the heat treatment, tubes were cooled for 1 minute.

3.3.2 Seed Cultivation and Yield Ratio Calculation

After dry heat treatment, seeds were soaked with DI water for 3 hours. Untreated seeds at the same a_w level were also soaked for 3 hours and served as the control group. Then heat treated and untreated seeds were placed as a single layer on seed sprouters. Daily watering was needed, and seeds were grown on the sprouters for 8 days. After 8 days, the seeds had grown into sprouts and the total weight of the sprouts was measured. Heat-treated seeds were visually examined to determine if they formed healthy sprouts. Yield ratios were determined by the following formula:

$$YR = \frac{Weight\ of\ heat\ treated\ sprouts}{Weight\ of\ untreated\ sprouts\ (control)} \times 100\%$$

3.4 Combined Analysis of Microbiological and Germination Studies

Both inactivation and germination studies were conducted separately, but with the same water activity level, treatment temperatures, and treatment time in each group. Therefore, under the same condition, both log reduction and yield ratio results could be compared and analyzed. With the analysis of all the results, the most optimal dry heat treatment was determined. *Salmonella* log reduction of 5-log reduction or higher and the best seeds yield ratio at the same time was considered as the optimal conditions for alfalfa, mung bean, radish, and red clover seed dry heat treatments. If only one aspect of both microbial and yield ratio standard could be fulfilled, then meeting the adequate yield ratio was the first priority.

3.5 Chemical Treatment

FDA used to recommend applying 20,000 ppm concentration of calcium hypochlorite to wash seeds for 15-20 minutes and this method is frequently used by the sprout industry (Brooks et al., 2001; Ding et al., 2013). This research project also conducted studies on *Salmonella* log reductions and on seed germination using the actual industry chemical decontamination method procedures. A 20,000-ppm chlorine solution was prepared by adding 104.13 g of bleach containing 6.05% (w/w) sodium hypochlorite into tap water for a final volume of 300 mL. Thirty grams of inoculated seeds were placed in chlorine solution for 15 or 20 minutes, with frequent agitation. After the time was over, the seeds were recovered using cheese cloth and rinsed serval times with tap water. The same viable plate count method described in section *3.2.5* was used to measure and analyze the population of *Salmonella* on the four different seeds before and after the chemical treatment. Then the log reduction of CFU/g of *Salmonella* during chlorine wash process was calculated.

Germination studies after the chlorine washes of the uninoculated seeds were done as well. Chlorine wash procedures were the same as described in section *3.5*. After chlorine wash, washed seeds and unwashed control seeds were soaked with tap water for 3 hours, then plant seeds on the sprouters. After 8 days' growth, seeds were harvested, and germination yield ratio was calculated with the formula in section *3.3.2*.

3.6 Temperature Profile Curve

To monitor the correct temperature during the dry heat treatment, temperature profiles were measured during the treatment. A 2-Channel Thermocouple Datalogger (Omega Engineering Inc., CT, USA) was used to monitor the temperature, and the probe was inserted into the seeds. The seeds were put into a glass tube and the tube was placed into a jar in a water bath. Temperature was monitored every 30 minutes during the treatment. Temperature profile curves were formed and used as reference to the 65.9°C, 71.0°C, 76.6°C dry heat treatment temperature groups.

3.7 Statistical Analysis

All the experimental results were repeated at least three times. Temperature profile curves were processed and presented in Origin (OriginLab, MA, USA). Averages and standard deviations were obtained in Excel (Microsoft, WA, USA) and JMP (SAS Institute, NC, USA).

REFERENCES

Brooks, J. T., Rowe, S. Y., Shillam, P., Heltzel, D. M., Hunter, S. B., Slutsker, L., Hoekstra, R. M., & Luby, S. P. (2001). *Salmonella* Typhimurium infections transmitted by chlorine-pretreated clover sprout seeds. *American Journal of Epidemiology*, 154(11), 1020-1028.

Ding, H., Fu, T. J., & Smith, M. A. (2013). Microbial contamination in sprouts: how effective is seed disinfection treatment? *Journal of Food Science,* 78(4), R495-R501.

Chapter 4

RESULTS AND DISCUSSION

4.1 Temperature Curve

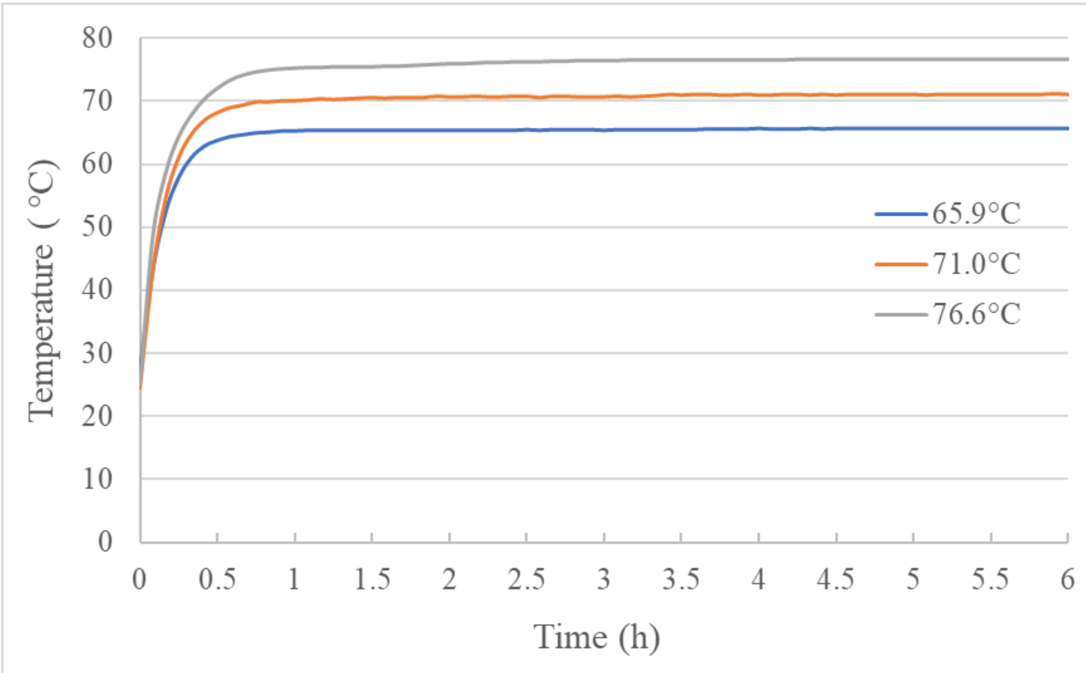

Figure 4.1 Temperature curve

Here is the temperature curve monitored for three treatment temperature groups used in this study: 65°C, 70°C, and 75°C. Temperatures was monitored every 5 minutes. Temperature was rapidly increasing in the first hour when seeds were initially put into the water bath, reaching to 65.3, 70.0, and 75.2°C, respectively. It took 2 hours for the temperature inside glass tubes to stabilize and stay consistent during the rest phase of heat treatment. The actual measured temperatures had some slight deviations from the target range. The actual measured stabilized temperature values were 65.9°C, 71.0°C, and 76.6°C, respectively.

4.2 Alfalfa Seeds

4.2.1 Microbial Effect of Dry Heat Treatment

4.2.1.1 Results at 65.9°C

Table 4.1
Effects of dry heat treatment at 65.9°C on the log reduction of *Salmonella* and the germination yield ratio of alfalfa seeds at a_w of 0.2. Data here represents the average of 3 replicates ± standard deviation

Treatment time (h)	*Salmonella* log reduction	Germination yield ratio (%)
180	5.76±0.33*	84.07±2.49
140	3.82±1.15	93.50±3.32
100	2.41±0.34	98.89±2.85

*: The detection limit of viable plate count was 1 log CFU/g. Data had no colony detection on plates and the final data was determined by initial log population – 1.

In this group, all the alfalfa seeds were treated by dry heat at 65.9°C and they were treated when the water activity had stabilized at 0.2±0.01. The treatment durations were 180, 140, and 100 hours, respectively. Table 4.1 shows the experimental results of *Salmonella* inactivation efficiency by dry heat treatment for different treatment times at 65.9°C. At 65.9°C and a_w 0.2, an 180-hour treatment was needed to reach a 5-log CFU/g reduction of *Salmonella* as an average of a 5.76 log reduction was observed. It is predictable that under the same treatment temperature and water activity, treating seeds with a longer treating time would cause more *Salmonella* cells to die and increase the average log reduction. Treating the inoculated seeds for 180, 140, and 100 hours reached log reductions of 5.76, 3.82, 2.41, respectively.

In the studies by Beuchat and Scouten (2002) and Feng et al. (2007), 5-log reduction could be achieved when seeds were dry heat treated at 55°C for 8 days and 6 days, respectively. In our study, we used 65.9°C as the lowest treatment temperature, and 180 h (7.5 days) was required to have a 5-log reduction. This result is similar to their research results, although the temperature difference was 10°C. It is possible that the different *Salmonella* serotypes used in the different studies differed in their susceptibility. The water activity differences of the alfalfa seeds used in the different studies are another possible cause of the differing results, since in our study, alfalfa seeds were treated with a lower water activity value (a_w 0.2). As described in Chapter 2, *Salmonella* resistance during dry heat treatment is always associated with water activity, because drought conditions can stimulate the formation of biofilms and filaments that protect the bacteria (Finn et al., 2013). Since we treated this group of seeds under very low water activity, it is possible that *Salmonella* cells gained more resistance to heat. Therefore, the treatment time was similar to those of the other studies that treated alfalfa seeds at 55°C, although the treatment temperature was 10°C higher in our study.

At a treatment time of 140 hours, the log reduction reached 3.8 and was reduced by 2 logs, compared to 180-hour treatment. When alfalfa seeds were treated for 100 hours, the final log reduction reached 2.4. From the 140-hour treatment to the 100-hour treatment, the log reduction decreased by 1.4. This indicated that 40 hours differences in treatment time could have a great impact on *Salmonella* log reduction.

In general, under a mild temperature of 65.9°C, dry heat requires 6-8 days to achieve a 5-log reduction of *Salmonella*. This is a very long treatment time as it will take more than a week to decontaminate seeds. Although 5-log reduction could still be fulfilled at 65.9°C, this temperature is not considered desirable in terms of energy consumption for sprouts manufactures.

4.2.1.2 Results at 71.0°C

Table 4.2

Effects of dry heat treatment at 71.0°C on the log reduction of *Salmonella* and the germination yield ratio of alfalfa seeds. Data here represents the average of 3 replicates ± standard deviation

a_w level	Treatment time (h)	*Salmonella* log reduction	Germination yield ratio (%)
0.2	60	5.25±0.16	71.18±2.25
0.2	50	4.21±0.03	83.13±4.78
0.2	40	3.75±0.07	94.22±2.30
0.1	160	6.40±0.23*	91.31±1.90
0.1	140	6.03±0.74*	96.42±0.21
0.1	120	4.53±0.19	101.42±0.58
0.1	100	4.18±0.08	101.62±1.80
0.1	80	3.17±0.10	102.76±2.18
0.1	60	2.21±0.06	103.90±2.11

*: The detection limit of viable plate count was 1 log CFU/g. Data had no colony detection on plates and the final data was determined by initial log population – 1.

According to Table 4.2, at 71.0°C and a_w 0.2, a 60-hour treatment time was needed for a 5-log reduction of *Salmonella*, and the average for this treatment time was a 5.25-log reduction. Under the same condition, 50-hour and 40-hour treatment achieved 4.21 and 3.75 log reductions, respectively.

Within the same treatment temperature, when the a_w decreased, the inactivation efficiency decreased as well, which means it took a longer time to treat alfalfa seeds in order to achieve the same log reduction. When seeds were treated at 71.0°C and a_w 0.1, at least 140 hours were required to reach a 5-log reduction as a

6.03 log CFU/g reduction was observed. The longest treatment time we used in this study for the same temperature and a_w condition was 160 hours, and it resulted in an average log reduction of 6.40. Under the same condition, 120, 100, 80, 60 hours treatment times achieved a 4.53, 4.18, 3.17, 2.21 log reduction, respectively.

With two groups of the same water activity treatments (a_w 0.2), when temperature increased from 65.9°C to 71.0°C, treatment time to reach 5-log reduction was greatly shortened from 180 to 60 hours. This 71.0°C treatment temperature is preferred over the 65.9°C treatment because the treatment time is greatly shortened.

The microstructure of the seeds is not as smooth as visually perceived. Under the scanning electron microscopy, a lot of cracks and folds on the surface of alfalfa seeds are observable, providing ideal spots for bacteria attachment (Kwack et al., 2014). Charkowski et al. (2002) found that under microscope, *Salmonella enterica* could quickly colonize the cracks on the surface of alfalfa seeds during the first 24 hours of germination. Fu et al. (2022) discovered that *Listeria monocytogenes* or *Salmonella enterica* serovar Typhimurium formed aggregates on the hilum part of alfalfa seeds and the bacteria counts of the hilum were higher than those of other parts of seeds coat after inoculation. The uneven surface of seed coats just provides a perfect spot for bacteria to colonize and attach, which make seed decontamination extremely hard. Barak et al. (2002) discovered that *Salmonella enterica* may endure more stress than *E. coli* O157:H7 on seeds because *Salmonella* usually shows a stronger attachment to seeds, sprouts, and plant tissues. Due to the special structure of alfalfa seeds and strong attachment of *Salmonella* to seeds, it is hard for most of the decontamination procedures to reach a high microbial inactivation efficiency. Even for dry heat treatment, it took 140 hours to reach a 5-log reduction of Salmonella at 71.0°C and a_w 0.1.

In another way, *Salmonella* can also be an ideal study model for seed decontamination research. If a 5-log reduction is reached on this most heat-resistant food-borne pathogenic bacterium, other more vulnerable pathogenic bacteria like *E. coli* or *Listeria* can be easier to inactivate under the same dry heat treatment condition applied on seeds. In addition, *Salmonella* is a major concern for food manufacturers especially for low-moisture food because of their high-resistance properties. *Salmonella* caused most cases of outbreaks related to sprouts because of their high survival rate in low moisture environment and higher heat resistance on low a_w seeds. All these reasons support using *Salmonella* as reference bacterium and study model in this research.

The advantage of heat treatment over other surface treatment methods is that it can penetrate food samples fully and provide a better microbial inactivation effect. Some of other kinds of treatment methods discussed in Chapter 2 could only achieve 2-3 log reduction of *Salmonella* on seeds, especially those surface treatment methods. While through dry heat treatment, 5-log reduction of *Salmonella* can easily be achieved at 65.9°C, 71.0°C, and 76.6°C. It took 140 hours of treatment to achieve 5-log reduction at 71.0°C with an a_w 0.1. Overall, *Salmonella* reduction can reach a higher level when seeds were treated with dry heat than other types of decontamination methods. It is a very effective decontamination method and can meet the FDA requirement for seeds to achieve a 5-log reduction of food pathogenic bacteria.

4.2.1.3 Results at 76.6°C

Table 4.3
Effects of dry heat treatment at 76.6°C on the log reduction of *Salmonella* and the germination yield ratio of alfalfa seeds. Data here represents the average of 3 replicates ± standard deviation

a_w level	Treatment time (h)	*Salmonella* log reduction	Germination yield ratio (%)
0.1	60	6.21±0.23*	75.90±4.38
0.1	50	4.27±0.02	81.53±1.74
0.1	40	3.66±0.46	89.26±2.30
0.1	30	2.92±0.27	101.22±0.72
0.05	120	3.81±0.03	61.62±2.21
0.05	100	2.99±0.09	77.10±1.79
0.05	80	2.94±0.11	85.45±2.55
0.05	60	2.75±0.04	98.78±3.07

*: The detection limit of viable plate count was 1 log CFU/g. Data here had no colony detection on plates and the final data was determined by initial log population – 1.

At 76.6°C and a_w 0.1, a 6.21-log reduction was observed with a 60-hour treatment and 4.27, 3.66, 2.92 log reductions were observed with a 50-, 40-, 30-hour treatment time, respectively. At the same treatment temperature with a lower a_w of 0.05, a longer treatment time was necessary to achieve the same log reduction. At 76.6°C and a_w 0.05, only a 3.81-log reduction was observed when seeds were treated for 120 hours, whereas this log reduction can be achieved by a 76.6°C/a_w0.1 treatment within only 50 hours. A 2.99-, 2.94-, 2.75-log reduction was observed with a shorter 100-, 80-, 60-hour treatment time, respectively.

Nissen et al. (1996) proved that low moisture food usually requires a higher treatment temperature and higher treatment time than high moisture food using heat treatment to decontaminate food. Alfalfa seeds are considered as low moisture food because the normal water activity level is 0.3-0.5 during seed storage. Although this storage environment is not considered as an ideal bacteria growing environment, bacteria can still survive for a long period of time in a low-moisture environment (Carrasco et al., 2012). Then surviving bacteria, especially *Salmonella*, can multiply during seed sprouting and cause food safety issues. The possible reason for this phenomenon is due to the formation of biofilms and filaments when *Salmonella* is under the dry environment stress (Finn et al., 2013). Biofilms and filaments can make *Salmonella* more resilient to the outside environment and gain a better resistance to stress like dry heat. Even a dry heat treatment took 140 hours to reach a 5-log reduction at 71.0°C. It took a 60-hour treatment to reach a 6-log reduction of *Salmonella*, even though the treatment temperature was as high as 76.6°C.

There are a lot of studies showing that with a lower water activity, the heat-resistance of *Salmonella* can increase accordingly. Some studies demonstrated this phenomenon in dried food products such as dried milk powder (Dega et al., 1972; McDonough and Hargrove, 1968; Wei et al., 2020). Wei et al. (2020) observed that treating milk powder at 75°C, the D-value of *Salmonella* increased twice as much when the a_w decreased from 0.3 to 0.2. In our study, at 71.0°C dry heat treatment, when a_w decreased from 0.2 to 0.1, the required treatment time for a 5-log reduction increased from 60 hours to 140 hours. The same experimental phenomenon happened for the 76.6°C treatment as well (120 hours/a_w0.05 & 50 hours/a_w0.1). This is an indication that, when seeds are heat treated under a lower a_w environment, *Salmonella* on seeds are usually more resistant and the treatment time should be increased to reach the same level of log reduction. At the same time, treatment time should not only fulfill the log reduction, but also fulfill the requirement for seeds germination, because the longer the treatment time is, the more harmful it will be for seed viability and germination. A “sweet spot” is needed to fulfill both requirements, which will be fully discussed in the next section.

4.2.2 Germination Effect of Dry Heat Treatment

In this section, germination effects results are listed and discussed. Most previous studies used the germination rate of seeds as their quantitative indicator. However, in our research, we found that this indicator cannot fully represent the actual height, weight, and health status of sprouts. Some heat-treated seeds had a 100% germination rate, but the sprouts looked shorter and weaker than untreated sprouts. Therefore, in our research we introduced the total weight of a whole tray of sprouts grown from 20 grams of seeds, compared to the total weight of untreated control seed sprouts. We call this quantitative indicator germination yield ratio to best describe the true germination status after treatment.

4.2.2.1 Results at 65.9°C

According to Table.1, under 65.9°C dry heat treatment with a_w 0.2 and treatment times of 180, 140, 100 hours, the germination yield ratio was 84.07%, 93.50%, and 98.89%, respectively. Combined this data with *Salmonella* inactivation data, this set of parameters did not fully achieve the goal of 5-log reduction of *Salmonella* and 90% of germination yield ratio. Although an 180-hour treatment reached the 5-log reduction goal (5.76 log), the yield weight ratio was only 84.07%

and not a very economic outcome for manufactures. The 140-hour treatment had a desirable yield rate of over 90% (93.50%), but the log reduction was only 3.82. Still, this log reduction was better than that of the chlorine wash, which only gave a 2-3 log reduction (Table 4.4). Compared to Table 4.4, with the similar yield rate of ~80%, chlorine wash could only give a 1.9-log reduction, while dry heat treatment at 65.9°C could give a 5.8-log reduction.

This 65.9°C/a_w 0.2/180h treatment condition is still considered to be much superior than that of the chlorine wash because the *Salmonella* inactivation efficiency was almost as three times higher than that of the chlorine wash (5.8 log to 1.9 log), with a similar germination yield outcome (84.1% to 82.9%). The drawback of this treatment parameter group is that the treatment time was too long, almost 8 days. But for the overall results, this is still an ideal group for the treatment, especially compared to the chlorine wash results.

4.2.2.2 Results at 71.0°C

When the treatment temperature was increased to 71.0°C, the treatment time to maintain the same germination yield ratio was shortened. At 71.0°C and a_w 0.2 (Table 4.2), when treating seeds for 60, 50, 40 hours, the germination yield ratio was 71.18%, 83.13%, and 94.22%, respectively. When comparing this group of data to that of the 65.9°C/a_w0.2 group, the increased temperature caused more harm to the seed viability. At 65.9°C, when extending the treatment temperature by 40 hours (100 h to 140 h), the yield ratio decreased by 5.39%. While at 71.0°C, extending the treatment temperature by only 10 hours (40h to 50h) decreased the yield ratio by 11.09%. Farooq (2004) stated that the dry heat effect on seed viability depended on the dry heat intensity (treatment temperature) and duration (treatment time), and our results for 65.9°C and 71.0°C showed accordance with this statement. With the treatment temperature increasing and water activity unchanged, the survival rate of seeds under dry heat decreased. When comparing this group of data to that of the 65.9°C and a_w 0.2 group, the increased temperature caused more harm to seed viability. At 65.9°C, when extending the treatment temperature by 40 hours (100 h to 140 h), the yield ratio decreased by 5.39%. While at 71.0°C, extending the treatment temperature by only 10 hours (40 h to 50 h) decreased the yield ratio by 11.09%. Thus, this was an indication that 71.0°C treatment can do more harm to the alfalfa seeds than 65.9°C. Seed viability decreased more, even when the treatment times were shorter in the 71.0°C group.

At 71.0°C, and when the water activity was lowered from 0.2 to 0.1, the seeds became more heat resistant and the treatment time to keep a 90% germination yield rate was much longer. When a_w was 0.2, ≤ 40 hours of dry heat treatment were needed to maintain the yield ratio at over 90%. When a_w was dropped to 0.1, ≤ 160 hours of dry heat treatment was needed to maintain the yield ratio at over 90%. However, lower water activity could also make *Salmonella* more heat resistant. And that's why we need to find a "sweet spot" to meet both microbial and germination requirement.

Another significant parameter that can influence alfalfa seeds' germination in our study (also applied to other three seeds as well) was water activity (a_w). All of our data showed that under the same treatment temperature, when a_w was lower, seeds tended to gain more heat resistance and the treatment time to maintain a good germination yield ratio increases more than those of the seed groups with a higher a_w. Some studies have shown that pre-drying seeds before dry heat treatments can make seeds more heat resistant and viable heat treatment time could increase because of this property (Nakamura et al., 1978). With a lower water content in seeds, it could increase the safety level to conduct dry heat treatment for seeds with minor damage to seed viability (Kubota et al., 2012). These research results were also proved by our experimental results. When a_w was 0.2, $\leq$ 40 h of dry heat treatment was needed to maintain the yield ratio at over 90%. When a_w was dropped to 0.1, $\leq$160 h of dry heat treatment was needed to maintain the yield ratio at over 90%. There was a four-fold difference in treatment time when a_w changed from 0.2 to 0.1. We speculate that the drying process before dry heat treatment could push seeds into a dormant phase and makes them more “stress tolerant”. The gene regulation and expression of seeds could have been altered as well. When the seeds were exposed to heat stress, dried seeds could have become more resistant to stress and thus survived better than those seeds with a higher a_w. However, this is only speculation and solid research results are still needed to verify the correctness of this speculation.

Combining this set of germination data with the microbial data, the 71.0°C treatment showed the best and most balanced results for both *Salmonella* inactivation and alfalfa seeds germination, especially treating seeds with 71.0°C/a_w0.1. Under this condition, when alfalfa seeds were treated for 140 hours, 6.0-log reduction of *Salmonella* and 96% germination yield ratio were obtained. Treating seeds for 120 hours gave a 4.5-log reduction but could even increase the yield ratio to 101%. This is also the most optimal treatment condition we recommend for alfalfa seed dry heat treatment.

4.2.2.3 Results at 76.6°C

When the treatment temperature jumped to 76.6°C, it became more lethal for seeds. With an a_w 0.1, and a treatment time of 60, 50, 40, 30 hours, the germination yield ratio was 75.90%, 81.53%, 89.26%, and 101.22% respectively. Because the a_w was lower than the a_w of the 71.0°C treatment group, seeds were more heat resistant and ≤30 hours were needed to keep seeds in a healthy state. With a_w dropping from 0.1 to 0.05, the seeds seemed to gain more heat resistance and the treatment time to keep a similar seed viability was even longer. With a_w 0.05 and treatment time of 120, 100, 80, and 60 hours, the germination yield ratio were 61.62%, 77.10%, 85.45%, and 98.78%, respectively.

Beuchat and Scouten (2002) found that seed germination rate was not so different from untreated seeds if seeds were dry heat treated at 60°C for 3 hours. But seeds' germination rate was greatly reduced if seeds were dry heat treated at 71.0°C for more than 3 hours or treated at 80°C for more than 1 hour. Neetoo and Chen's (2011) observed that alfalfa seeds could reach the germinate rate of above 90% when they were dry heat treated for 10 days at 55°C, 60°C, 65°C. However, if seeds were treated at 70°C, the germination rate was only 80%. Both results were corroborated by our study because dry heat treatment at 71.0°C was a turning point and all the treatment temperature above 71.0°C hurt seed viability greatly. In our research, seed viability could be well maintained at 65.9°C dry heat treatment for serval days, while 71.0°C and 76.6°C could maintain the seed viability only for hours. Martin et al.

(1975) found that plant seed germination rate could have a sudden drop after one temperature point during heat treatment. This temperature turning point in their study ranged from 70°C to 90°C among different species of legume seeds. Basavarajappa et al. (1991) explained this phenomenon by stating that a lethal treatment temperature could do damage to the structure and function of plant cells' membrane, plastids, and mitochondria. We speculate that a similar mechanism happened during the dry heat treatment of alfalfa seeds. When the temperature threshold is exceeded, which was 71.0°C, the seeds can be heavily damaged and cause seeds to lose viability.

Overall, combining the germination data with the microbial data at 76.6°C treatment, this group is not a very recommended treatment condition because seeds could get easily killed at this higher temperature, though the treatment time is greatly reduced. Although seeds' viability could be well maintained under a very low a_w 0.05, this water activity would make *Salmonella* extremely heat resistant and the overall *Salmonella* inactivation results were not as desired. Still, the results from this group were better than that for the chlorine wash.

4.2.3 Chlorine Wash

Table 4.4
Effects of 20,000 ppm sodium hypochlorite soak on log reduction of *Salmonella* and the germination yield ratio of alfalfa seeds. Data here represents the average of 3 replicates ± standard deviation

Soaking time (min)	*Salmonella* log reduction	Germination yield ratio (%)
15	1.85±0.11a	82.86±1.35a
20	1.96±0.19a	82.52±3.45a

Table 4.5
Comparison of the optimal dry heat treatment parameters to chlorine wash. Data here represents the average of 3 replicates ± standard deviation

a_w level	Treatment time (h)	*Salmonella* log reduction	Germination yield ratio (%)
0.1	140	6.03±0.74*	96.42±0.21
0.1	120	4.53±0.19	101.42±0.58

*: The detection limit of viable plate count was 1 log CFU/g. Data had no colony detection on plates and the final data was determined by initial log population – 1.

If we compare Table 4.4 and Table 4.5, the *Salmonella* log reduction under the optimal dry heat treatment for alfalfa seeds was significantly higher than that of the chlorine washed seeds. Even the germination results (101%/96%) were much better than those of the chlorine washed seeds (83%/82%). This is an almost three-fold log reduction and 20% promotion of germination, definitely showing the superiority of dry heat treatment method over chlorine wash method.

4.3 Mung Bean

4.3.1 *Salmonella* Inactivation and Germination Results at 76.6°C

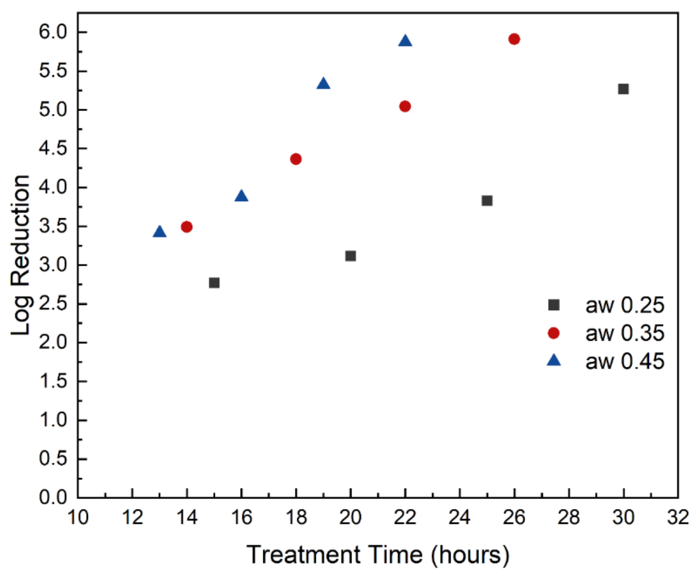

Figure 4.2 *Salmonella* inactivation at 76.6°C treatment under different a_w levels

Table 4.6
Initial comparison of 3-log reduction of *Salmonella* and germination results at 76.6°C

a_w level	Treatment time (h)	*Salmonella* log reduction	Germination yield ratio (%)
0.45	13	3.42±0.32	0.00
0.35	14	3.49±0.54	47.44
0.25	20	3.12±0.18	68.14

Note:
1. *Salmonella* data represent the average of 4 replicates ± standard deviation
2. Yield ratio data only represent one replicate.

Table 4.7

Initial comparison of 5-log reduction of *Salmonella* and germination results at 76.6°C

a_w level	Treatment time (h)	*Salmonella* log reduction	Germination yield ratio (%)
0.45	19	5.32±0.69	0.00
0.35	22	5.05±0.22	0.00
0.25	30	5.27±0.50	43.33

Note:
1. *Salmonella* data represent the average of 4 replicates ± standard deviation
2. Yield ratio data only represent one replicate.

Figure 4.1 showed that at 76.6°C treatment to reach the same level of 5-log or 3-log reduction, when water activity decreases, it took longer to inactivate *Salmonella*. This outcome confirmed results of previous studies. When seeds have lower water activity, *Salmonella* tended to become more heat-resistant. Combining the 3-log reduction (Table 4.6) and the 5-log reduction (Table 4.7) of the microbial results with the germination results, at 76.6°C, showed this treatment condition as not ideal or mung beans, since seed viability decreased greatly and even no germination was observed. However, this set of data provided the evidence for an experimental design path to go to relatively a lower water activity, because a lower water activity provides a better overall tradeoff for both microbial and germination results. This prediction is also proven by our experimental results.

4.3.2 Combined Analysis of *Salmonella* Inactivation and Germination at 71.0°C

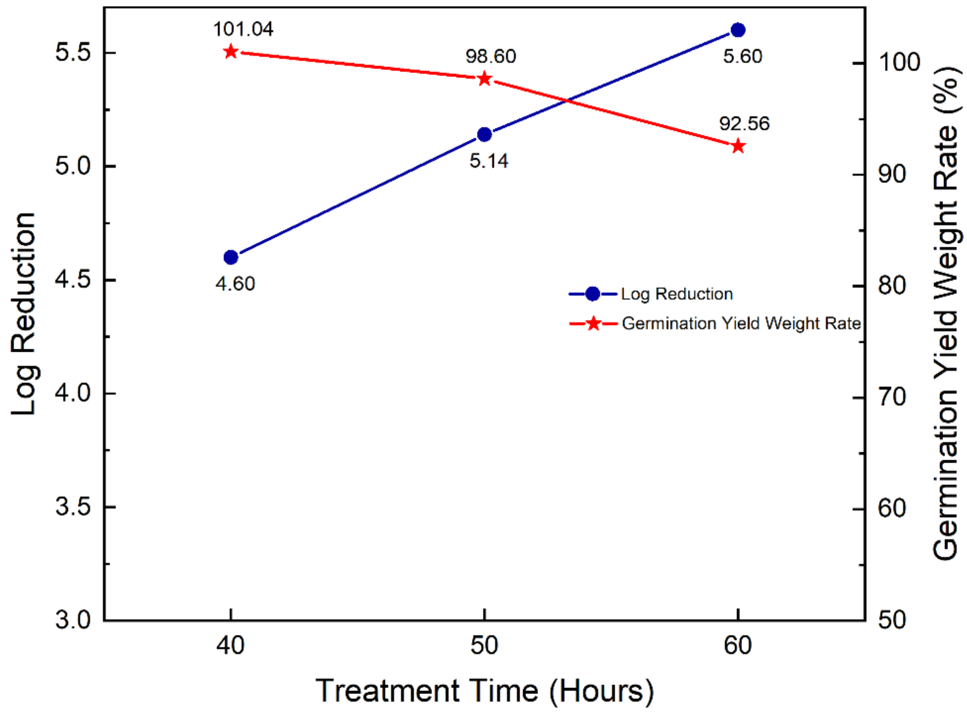

Figure 4.3 *Salmonella* inactivation & germination results at 71.0°C treatment with a_w 0.2

As Figure 4.3 showed, 71.0°C looked very promising with a very good balance between *Salmonella* inactivation and seed germination. At 71.0°C/a_w0.2 condition, dry heat treatment for 50 hours can achieve 5.14-log reduction and maintain 98.6% of germination yield ratio compared to untreated mung bean. Dry heat treatment at 71.0°C/a_w0.2 for 50 hours is considered as a “sweet spot” for mung bean dry heat treatment. This is the optimal treatment condition we decided for the mung bean sprout industry to reach the best balance of pathogen decontamination and seed viability. It is recommended that mung bean sprout producers can use dry heat treatment technology for post-harvest decontamination before sprout growth, because both microbial safety and germination yield requirement are fulfilled in this treatment condition.

4.3.3 Chlorine Wash

Table 4.8
Effects of 20,000ppm sodium hypochlorite soak on the log reduction of *Salmonella* and the germination yield ratio of mung bean seeds. Data represents the average of 3 replicates ± standard deviation

Soaking time (min)	*Salmonella* log reduction	Germination yield ratio (%)
15	1.30±0.16a	83.69±2.04a

If we compare Table 4.8 with Figure 4.3, *Salmonella* log reduction of optimal dry heat treatment (71.0°C/a_w0.2/50h) for mung bean seeds was significantly higher than that of chlorine-washed seeds (5.1 log to 1.3 log). Even the germination results (99%) were much better than those chlorine washed seeds (84%). This is even more than a 3-fold log reduction and 15% promotion of germination for mung bean seeds, definitely showing the superiority of dry heat treatment method over chlorine wash method.

4.4 Radish

4.4.1 Combined Analysis of *Salmonella* Inactivation and Germination at 71.0°C

Table 4.9
Combined effects of dry heat treatment at 71.0°C on the log reduction of *Salmonella* and the germination yield ratio of radish seeds. Data represent the average of 3 replicates ± standard deviation

a_w level	Treatment time (h)	*Salmonella* log reduction	Germination yield ratio (%)
0.2	60	4.22±0.18	50.37±4.27
0.2	50	3.80±0.67	61.03±1.57
0.2	40	2.71±0.37	67.46±2.97

As Table 4.9 showed, the overall germination results of three treatment times for radish seeds were not as good as those for alfalfa and mung bean seeds. Still, when radish seeds were treated for 60, 50, and 40 hours, germination yield ratio reached 50%, 61%, and 67%, respectively. Although the microbial results were similar among different seeds, germination results among different seeds were very different. Different seeds behaved differently when they were under heat stress and this may be due to their structure and genetic differences. Based on this result, we would recommend 71.0°C/a_w0.2/50h as optimal dry heat treatment parameter for radish seeds, because this parameter provided the best tradeoff.

4.4.2 Chlorine Wash

Table 4.10
Effects of 20,000ppm sodium hypochlorite soak on the log reduction of *Salmonella* and the germination yield ratio of radish seeds. Data represents the average of 3 replicates ± standard deviation

Soaking time (min)	*Salmonella* log reduction	Germination yield ratio (%)
15	1.38±0.08	64.70±2.29

If we compare Table 4.9 with Table 4.10, *Salmonella* log reduction of optimal dry heat treatment (71.0°C/a_w0.2/50h) for radish seeds was higher than that of the chlorine-washed seeds (3.8 log to 1.4 log). Although overall results were not as ideal as results for alfalfa and mung bean seeds, dry heat treatment still showed a better result than chlorine wash method. The germination results for dry heat-treated radish seeds (61%) were similar to those for chlorine-washed seeds (65%), and the better microbial results of the dry heat treatment method were better than those for the chlorine wash method for radish seeds.

4.5 Red Clover

4.5.1 Combined Analysis of *Salmonella* Inactivation and Germination at 71.0°C

Table 4.11
Combined effects of dry heat treatment at 71.0°C on the log reduction of *Salmonella* and the germination yield ratio of red clover seeds. Data represent the average of 3 replicates ± standard deviation

a_w level	Treatment time (h)	*Salmonella* log reduction	Germination yield ratio (%)
0.2	60	4.00±0.06	50.56±5.76
0.2	50	3.36±0.18	66.73±3.30
0.2	40	2.77±0.23	78.16±3.77

As Table 4.9 showed, the overall germination results of three treatment times for red clover seeds were not as good as those for alfalfa and mung bean seeds. Still, when red clover seeds were treated for 60, 50, and 40 hours, germination yield ratio reached 51%, 67%, and 78%, respectively. Although the microbial results were similar among different seeds, the germination results were different. Different seeds germinate differently when they are under the heat stress, and this may be due to their structure and genetic differences. Germination results of red clover were not as good as alfalfa and mung bean germination results. None of the treatment groups could reach more than 90% of germination yield ratio. Based on this result, we would recommend 71.0°C/a_w0.2/50h as optimal dry heat treatment parameter for red clover seeds, because this parameter provided the best tradeoff.

4.5.2 Chlorine Wash

Table 4.12
Effects of 20,000ppm sodium hypochlorite soak on the log reduction of *Salmonella* and the germination yield ratio of red clover seeds. Data represents the average of 3 replicates ± standard deviation

Soaking time (min)	*Salmonella* log reduction	Germination yield ratio (%)
15	1.48±0.08	69.96±1.44

If we compare Table 4.11 with Table 4.12, *Salmonella* log reduction of the optimal dry heat treatment (71.0°C/a_w0.2/50h) for red clover seeds were higher than those for chlorine-washed seeds (3.4 log to 1.5 log). The germination results for dry heat-treated red clover seeds (67%) were similar to chlorine washed seeds (70%), which showed a better microbial result of dry heat treatment method over the chlorine wash method for red clover seeds.

REFERENCES

Barak, J. D., Whitehand, L. C., & Charkowski, A. O. (2002). Differences in attachment of *Salmonella enterica* serovars and *Escherichia coli* O157: H7 to alfalfa sprouts. *Applied and Environmental Microbiology,* 68(10), 4758-4763.

Beuchat, L. R., & Scouten, A. J. (2002). Combined effects of water activity, temperature and chemical treatments on the survival of *Salmonella* and *Escherichia coli* O157: H7 on alfalfa seeds. *Journal of Applied Microbiology*, 92(3), 382-395.

Carrasco, E., Morales-Rueda, A., & García-Gimeno, R. M. (2012). Cross-contamination and recontamination by *Salmonella* in foods: a review. *Food Research International*, 45(2), 545-556.

Charkowski, A. O., Barak, J. D., Sarreal, C. Z., & Mandrell, R. E. (2002). Differences in growth of *Salmonella* enterica and *Escherichia coli* O157: H7 on alfalfa sprouts. *Applied and Environmental Microbiology*, 68(6), 3114-3120.

Dega, C. A., Goepfert, J. M., & Amundson, C. H. (1972). Heat resistance of salmonellae in concentrated milk. *Applied Microbiology*, 23(2), 415-420.

Farooq, M. (2004). Influence of high and low temperature treatments on seed germination and seedling vigor of coarse and fine rice. *International Rice Research.* Notes, 29, 69-71.

Feng, G., Churey, J. J., & Worobo, R. W. (2007). Thermal inactivation of *Salmonella* and *Escherichia coli* O157: H7 on alfalfa seeds. *Journal of Food Protection*, 70 (7), 1698-1703.

Finn, S., Condell, O., McClure, P., Amézquita, A., & Fanning, S. (2013). Mechanisms of survival, responses and sources of *Salmonella* in low-moisture environments. *Frontiers in Microbiology*, 4, 331.

Fu, Y., Bhunia, A. K., & Yao, Y. (2022). Alginate-based antimicrobial coating reduces pathogens on alfalfa seeds and sprouts. *Food Microbiology*, 103, 103954.

Kwack, Y., Kim, K. K., Hwang, H., & Chun, C. (2014). An ozone micro-bubble technique for seed sterilization in alfalfa sprouts. *Horticultural Science & Technology*, 32(6), 901-905.

McDonough, F. E., & Hargrove, R. E. (1968). Heat resistance of *Salmonella* in dried milk. *Journal of Dairy Science*, 51(10), 1587-1591.

Nissen, E., Koenig, P., Feinstone, S. M., & Pauli, G. (1996). Inactivation of hepatitis A and other enteroviruses during heat treatment (pasteurization). *Biologicals*, 24(4), 339-341.

Wei, X., Lau, S. K., Chaves, B. D., Danao, M. G. C., Agarwal, S., & Subbiah, J. (2020). Effect of water activity on the thermal inactivation kinetics of *Salmonella* in milk powders. *Journal of Dairy Science*, 103(8), 6904-6917.

Chapter 5

CONCLUSION

In conclusion, all the results showed that dry heat treatment is a decontamination method superior to that of hypochlorite washing seeds destined for sprouting. When treatment factors like treatment temperature, treatment time, and the seeds' water activity during treatment are properly controlled, a "sweet spot" can be found to fulfill a very high log reduction on *Salmonella* inactivation and maintain seed viability at the same time.

The optimal dry heat treatment parameter for alfalfa is to expose seeds to dry heat at 71.0°C, under a_w 0.1 for 140 or 160 hours. Under these conditions, both >5 log reduction and >96% germination yield ratio can be both achieved and is considered as the most optimal dry heat treatment condition for alfalfa seeds.

The optimal dry heat treatment parameter for mung bean is to treat the seeds with dry heat at 71.0°C, under a_w 0.2 for 50 hours. Under these conditions, both >5 log reduction and >98% germination yield ratio can be achieved and is considered as the most optimal dry heat treatment condition for mung bean seeds.

The optimal dry heat treatment parameter for radish is to expose the seeds to dry heat at 71.0°C, under a_w 0.2 for 50 hours. Under these conditions, both >3 log reduction and >60% germination yield ratio can be achieved and is considered as the most optimal dry heat treatment condition for radish seeds. Although this result was not as good as those for alfalfa and mung bean, it is still better than that for the chlorine wash treatment of radish (chemically washed only achieved 1.4-log reduction/64.7% yield).

The optimal dry heat treatment parameter for red clover is to treat the seeds with dry heat at 71.0°C, under a_w 0.2 for 50 hours. Under these conditions, both >3 log reduction and >60% germination yield ratio can be achieved and is considered as the most optimal dry heat treatment condition for radish seeds. Although this result was not as good as that for alfalfa and mung bean, it is still better than that of the chlorine wash treatment of red clover (chemically washed only got 1.5-log reduction/70.0% yield).

Dry heat treatment is very promising according to this research and can be applied to the critical control point for microbial safety in sprout production enterprises. Compared with traditional chemical cleaning methods, dry heat processing can effectively inactivate food-borne pathogenic bacteria without affecting the germination and growth of treated seeds too much. In addition, the technology does not leave any chemical residues on the surface of the seeds, thus has no safety or quality impact on human consumption. The required processing equipment is simple, easy-to-build, and low cost. If the equipment for industrial large-scale production can be determined later, this technology has a very promising future of application and promotion in the sprout industry.

Chapter 6

FUTURE AND ADDITIONAL RESEARCH

Most of this research project showed very ideal and promising results, especially the overall results for alfalfa and mung bean seeds. There are four major things I would like to refine and add to for this project.

First, although alfalfa and mung bean seeds could fulfill the required 5-log reduction and keep at least a 90% of germination yield ratio, the optimal treatment time was still a little bit long (alfalfa required 140/120 hours, mung bean 50 hours). In the future, we would like to test some higher treatment temperatures, for example 80°C or 85°C, to shorten the treatment time. But this needs a careful treatment because this treatment temperature could be detrimental to seed viability. Careful control is needed for treatment parameters.

Second, *E. coli* O157:H7 and *Listeria monocytogenes* are concerned pathogens that are often associated with sprout outbreaks as well. Although they are believed to be more vulnerable than *Salmonella*, we would like to test if the optimal dry heat treatment conditions for alfalfa and mung bean could still achieve 5-log reductions for these two pathogens on these two seeds.

Third, radish and red clover seed germination behaved not as good as that of alfalfa and mung bean seeds. Although the overall results were still better than those of the hypochlorite wash method, the germination ratios to reach at least 3-log reduction were low. Since we only tested these parameters for two seeds: 71.0°C/a_w 0.2, and these parameters were the same parameters used for optimal mung bean treatment, we would like to test radish and red clover with the same parameters as alfalfa's optimal treatment parameters, which is treating seeds at 71.0°C/a_w 0.1, to observe if we could achieve a better log-reduction and germination yield ratio.

Finally, we would like to look into the industry-scale processing equipment required for dry heat treatment of seeds and the actual cost for such equipment. Also, some humidity control equipment suitable for seed storage is worthwhile investigating.