ASSESSMENT OF VACUUM STEAM PASTEURIZATION TO IMPROVE SAFETY AND QUALITY OF LOW MOISTURE FOODS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Low moisture foods such as grains, seeds, spices and flour are part of our daily diet. While they are rich in bioactive compounds, they can be minimally processed or often consumed raw. Several outbreaks due to *Salmonella* and *E. coli* O157:H7 have been attributed to low moisture foods. The efficacy of vacuum steam pasteurization in inactivation of *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium* on whole flaxseed, quinoa, sunflower kernels, milled flaxseed and whole black peppercorns was determined. The impact of pasteurization on microbial shelf life of whole and milled flaxseed was also monitored along with their water activity. Vacuum steam pasteurization was effective at inactivation of each bacteria, providing >5.0 log CFU/g reduction at temperatures as low as 75 and 85°C. Also, there was no negative impact on microbial shelf life or water activity on pasteurized flaxseed as compared to unpasteurized products.

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DEDICATION

My sincerest dedication is to my late Father, Lal Babu Sah, who was an inspiration to me for seeking the best education, and also to all my family members who were always supportive of my desire for higher education.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX TABLES

LIST OF APPENDIX FIGURES

INTRODUCTION

Food products are vulnerable to contamination by pathogens that can cause foodborne illnesses, hospitalizations or death. *Salmonella, Listeria monocytogenes* and *Escherichia coli* O157:H7 are three major pathogens of concern in ready to eat food products(1). *Salmonella* is the most frequently occurring pathogen in contamination of low moisture foods that are involved in outbreaks and recalls. A few foodborne outbreaks have also been attributed to *E. coli* O157:H7 contamination in low moisture foods (2).

Salmonella has been shown to be heat resistant in low moisture foods (3-5). Several inactivation methods have been explored to eliminate pathogens in such foods (6-10) with dry heat used mainly for treatment of various nuts. However, inactivation processes such as dry heat, oil roasting, high pressure processing, irradiation, and other methods have their own advantages and disadvantages when used as a treatment method for low moisture foods. While one technology might be best for one product type, it may not be feasible for another. In addition, it is necessary to maintain the chemical and physical properties of the foods and their quality during the treatment process. Spoilage organisms can also be naturally present in low moisture foods and an increase in their numbers to $10^7 – 10^9$ CFU/g can lead to spoilage (11). While their water activity is low, treatment processes can increase the water activity (a_w) of these foods affecting growth of microorganisms, and other chemical and sensory properties. Hence, it is also important to investigate if the processing method impacts the naturally present microbiota and the moisture content.

In this study, I have explored the use of vacuum steam pasteurization to inactive pathogens in low moisture products such as whole flaxseed, milled flaxseed, quinoa, sunflower kernels and whole black peppercorns. Most of the seeds for my experimental selection are

usually consumed raw or if processed, have the post processing potential for contamination. Inactivation data for bacterial pathogens on these seeds are minimal or non-existent. On the other hand, there are hundreds of studies conducted on the health benefits of usage of these foods leading to increased consumption of these products over the recent years. My goal was to fill in the gaps of knowledge for inactivation of *Salmonella* and *E. coli* O157:H7 on such foods with the use of vacuum steam pasteurization.

Vacuum steam pasteurization is a technology that utilizes steam under vacuum to treat low moisture food products. The efficiency of vacuum steam pasteurization to inactivate *Salmonella* phage type 30 and *Escherichia coli* O157:H7 from low moisture products such as whole flaxseed, milled flaxseed, quinoa, sunflower kernels and whole black peppercorns was evaluated in this thesis research. *Enterococcus faecium*was investigated as a surrogate for these pathogens when using vacuum steam pasteurization. In addition, the data obtained from the inactivation study was used to generate survival curves using the modified Weibull and Geeraerd-tail models (12, 13). Moreover, microbial shelf life and water activity of vacuum steam pasteurized whole and milled flaxseed was evaluated for periods of 6 and 9 months, respectively. The overall outcomes of this study will help determine the efficiency of vacuum steam pasteurization to improve quality and safety of low moisture foods. The industry should be able to successfully utilize vacuum steam pasteurization to treat low moisture foods for safe consumption.

LITERATURE REVIEW

Epidemiology of *Salmonella* and *E. coli* O157:H7

It is estimated that each year in the United States 31 pathogens cause 37.2 million illnesses of which 3.6 million (39%) are due to bacterial infection. It is estimated that *Salmonella* causes the second most foodborne illnesses (1.0 million) and is the leading cause of hospitalizations (19,336 cases, 35%) and deaths (378 cases, 28%) associated with foodborne illnesses per year (14). *E. coli* O157:H7 causes an estimated 63,153 illnesses, 2138 hospitalizations and 20 deaths per year (14). Of total *Salmonella enterica* illnesses from 1998-2008, 13.0 % were attributed to fruits and nuts and 2.9 % were attributed to grains and beans. Of total illnesses due to Shiga toxin producing *E. coli* O157, 21.1% and 0.7% were attributed to fruits and nuts and grains and beans, respectively (1).

From 2010-2015 there were 5 outbreaks due to *Salmonella* in low moisture foods (nut butter, peanut butter, tahini sesame sauce, Turkish pine nuts, and black and red pepper salami), causing 310 illnesses, 65 hospitalizations, and 1 death (15). In addition, the number of recalls due to *Salmonella* is alarmingly high for low moisture products. There were 46 instances of recalls in 2015 alone due to possible *Salmonella* contamination in various products such as flax powder, whole flaxseeds, sunflower kernels, organic coriander powder, macadamia nuts, and cashew splits. Also, the total number of recalls in low moisture products due to possible *Salmonella* contamination were 22, 9, 34 and 10 for the years 2014, 2013, 2012, and 2011, respectively (16). In the years 2003-2011, 238 and 104 recalls due to *Salmonella* were observed in nuts and edible seeds and spices, respectively. Of the total outbreaks due to *E. coli* O157:H7 from the years 2003-2012, 65% were due to contaminated foods; beef and leafy vegetables together were the source for >25% of all the reported outbreaks (17). Other food sources linked

to these outbreaks were cheese, fruits, sprouts, and low moisture foods (flour and nuts) (17). Also, one recall each due to *E. coli* O157:H7 was observed in nuts and edible seeds and bakery goods from 2003-2011 (18).

Salmonella and its pathogenesis

Salmonella is a Gram-negative, facultative anaerobic, motile, non-lactose fermenting bacteria that belongs to the family *Enterobacteriaceae* (19). *Salmonella* was first isolated by Salmon and Smith in 1886 from pigs and was considered to exist as a secondary pathogen during viral swine fever (20). Several animals are found to be asymptomatic carriers of *Salmonella*. However, *Salmonella* is shown to cause infections both in human beings and some animals. This pathogen is usually found in soil and water exposed to fecal contamination. Most human infections result from the ingestion of foods of animal origin that are contaminated with *Salmonella.* Non-animal vehicles include food of plant origins such as vegetables, fruits, nuts, water.(21). Non-typhoidal *Salmonella* infections result in gastroenteritis and enteric fever. Acute gastroenteritis is self-limiting and may not require antimicrobial therapy (22). However, in approximately 5% of *Salmonella* infections patients may develop bacteremia that leads to complications such as endovascular infections, osteomyelitis and abscesses(22). The risks of increased bacteremia are observed in children and immuno-compromised patients, and 21% of the bacteremic patients subsequently develop focal infections, including septic arthritis, pneumonia, and cutaneous abscess (22).

As few as 10-100 *Salmonella* cells can cause an infection (23). Samples of suspected chocolate from the outbreak in 1986 showed as little as 4.3 to 24.0 MPN of *S. nima* per 100 g of product (23). Similarly, other outbreaks due to peanut dressing, bean sprouts, and spinach with peanut dressing showed cell counts of 4.3 MPN/g, 40 CFU/g and 1.4 MPN/g, respectively, in the

outbreak samples with infectious dose of 344 cells, 880 cells and 49 cells, respectively (24). A dose-response model incorporating data from *Salmonella* outbreaks in Japan showed that ID50 of 7 CFU/g and 36 CFU/g was enough to cause an infection and illness in 50% of mice, respectively (25).

E. coli O157:H7 and its pathogenesis

*E. coli*O157:H7 is a gram negative, rod-shaped, facultative anaerobic bacteria. An outbreak investigation of hemorrhagic colitis in 1982 in Oregon and Michigan was linked to the first outbreak of *E. coli* O157:H7 that infected 47 people after eating at a fast-food restaurant chain (26). Prior to that, the only other known isolation of *E. coli* O157:H7 was from a sporadic case of hemorrhagic colitis in 1975 (26). In 1993, undercooked ground beef patties were implicated in a large outbreak causing 501 infections, 151 hospitalizations, 45 cases of Hemolytic Uremic syndrome (HUS), and three deaths (27). Since then, *E. coli* O157 has been attributed to several foodborne outbreaks and has emerged as a major foodborne pathogen.

E. coli O157:H7 causes severe stomach cramps, bloody diarrhea, vomiting and fever. While most infections are mild, 5 to 10% of infections can cause complications such as HUS, leading to permanent kidney damage or death (28). Fecal contaminated water and soil are the major sources of this pathogen. Cattle and wild animals are also found to serve as reservoirs, which in turn contaminate the irrigation water sources and produce fields. Hence, foods may be directly contaminated in the fields due to the usage of contaminated irrigation water, run off from cattle farms, and wildlife activities (29-31). Beyond the cultivation fields, several food processing stages are vulnerable to contamination, such as harvesting, processing, packaging, storage, shipment, and food preparation through contaminated equipment, improper sanitation measures and ill trained unhygienic employees (28, 32). The food processing facilities may be

contaminated from these processing steps and harbor pathogens in their environment leading to future cross-contaminations.

The infectious dose of *E. coli* O157:H7 is shown to be less than 100 CFU/g or MPN (33). In an *E. coli* O157:H7 outbreak associated with raw beef liver with infection rate of 100%, the contamination level and ingested dose was found to be 0.04- 0.18 CFU/g and 2 - 9 CFU, respectively. Similarly, in an outbreak due to *E. coli* O157:H7 in cooked hamburger patties, the contamination level and ingested dose was found to be 1.45 CFU/g and 108-216 CFU, respectively (34).

Economic burden of foodborne illnesses

Foodborne illnesses are expensive. There are economic losses due to medical costs, productivity losses and illness-related mortality. Costs for hospital services, physician care and laboratory services, pharmaceuticals, work-absence, and tradeoff for fatality risks are included in economic losses (35). In addition, losses occur due to pain, suffering and functional disability, all of which are measured as lost quality of life (QALY) (35). Enhanced costs are estimates of both economic costs and QALY. The average estimated cost per foodborne illness was estimated to be $1626 and the total estimated annual cost of illnesses was $77.7 billion. Total cost due to non-typhoidal *Salmonella* and *E. coli* O157:H7 (Shiga toxin-producing *E. coli*) related foodborne illnesses accounted for $11,391 million and $635 million per year, respectively (35). *Salmonella* and *E. coli* O157:H7 rank as the 1st and 8th most costly pathogens (14, 35).

Besides the costs of foodborne illnesses, food recalls also add to the economic burden for the industries and consumers. Food recalls occur when a food is likely contaminated with an organism of concern. As mentioned earlier, there were several recalls due to possible *Salmonella* contamination and a few due to *E. coli* O157:H7 in low moisture foods. There are several costs

associated with a food recall, primarily is warranted. First of all, there is loss of the value of the recalled product. Other costs include product recovery and disposal of the recalled products. In addition, product recalls may result in loss of consumer confidence.

Low moisture foods

Food products with water activity (a_w) less than 0.7 are considered low moisture foods. Water activity is the ratio of vapor pressure of water in the food to that of distilled water at 25°C. Nuts, dried fruits, edible beans, flour, baking mixes, spices, lentils, seeds and grains are a few examples of low water activity foods. Water availability is an important factor for microbial growth. Bacterial growth is impaired at lower water activity levels. *Salmonella* and *E. coli* O157:H7 require a minimum of 0.92 and 0.95 water activity levels, respectively, for growth (36). While low water activity does not support the growth of bacteria, it has been shown that these pathogens can survive for long periods of time in low moisture foods (36).

Survival of *Salmonella* in low moisture food is also dependent upon several factors, such as storage temperature and product type (37). Increased survival is observed at decreasing water activity levels and lower storage temperatures (38, 39). *Salmonella* inoculated at 4.0 and 5.0 log CFU/g on almonds and pistachios, respectively, showed survival for up to a year when stored at 24°C. Similarly, *E. coli* O157:H7 survived for 203 and 365 days on almonds and pistachios, respectively, when stored at 24°C. However, no decline was observed for both *Salmonella* and *E. coli* O157:H7 on almonds or pistachios when stored at 4°C and -19°C (40).

Several other studies show survival of different strains of *Salmonella* in matrices such as pasta, peanut butter, paprika powder for ≤ 12 months, ≤ 24 weeks and > 8 months, respectively (41, 42). Death of *Salmonella* and *E. coli* O157:H7 increases with increase in a_w, as observed on

alfalfa seeds (43). In addition to all these, *Salmonella* is also shown to survive for long periods of time in processing environments and on equipment surfaces (44).

Heat resistance of *Salmonella* in low moisture foods

The health and economical costs due to *Salmonella* and *E. coli* O157:H7 are substantial and it is necessary to understand the behavior of these pathogens in low moisture foods in order to eliminate them successfully. *Salmonella* in low moisture foods are found to be heat resistant and may be influenced by matrix type, a_w of the product, storage conditions, and type of inactivation method used. After storage at 4°C, almonds were exposed to hot oil treatment at 121°C for 0.72±0.09 and 1.3±0.17 minutes that provided 4.0 to 5.0 CFU/g log reduction for *Salmonella*, respectively (45). However, when almonds were subjected to hot oil roasting at 121°C after storage at 23°C, 4.0 or 5.0 CFU/g log reduction was achieved after 1.2±0.31 and 2.0±0.50 minutes of treatment, respectively (45). Longer time was required for inactivation of *Salmonella* on walnuts as compared to almonds. Treatment of almonds and walnuts provided different D_{10} values of 0.226±0.039 and 0.474±0.062, respectively when treated with low-energy X-ray inactivation methods at same water activity levels of 0.2. Changes in water activity did not impact D value when exposed to X-ray inactivation (10).

However, usually decreasing the a_w of a low moisture food increases the resistance of pathogens when using most inactivation methods. When alfalfa seeds were treated at 70°C at varied a_w of 0.25, 0.42 and 0.59, the highest and lowest log reduction was observed at a_w levels of 0.59 and 0.25, respectively (43). Heat treatment of peanut butter at 90°C for 50 minutes reduced *Salmonella* by 3.2 log CFU/g (46). Similarly, heating wheat flour at 75 -77°C for 2.5 minutes (5) and pecan nutmeats at a_w of 0.52 at 120°C reduced *Salmonella* only by 1.0 log CFU/g (4). While it is difficult to remove *Salmonella* from low water activity foods, their low

infectious doses and high infection rates increase the risks of infections. Studies which show that there may be increased risks when *Salmonella* are present particularly in low moisture foods. The fatty nature of some of the low moisture foods, mainly nuts and seeds, also helps in evading the acidic condition in the stomach (23). In addition, some low moisture products such as nuts, flour, and dried fruits might be minimally processed or consumed raw. One example of an outbreak due to *E. coli* O157:H7 was due to consumption of raw cookie dough tainted with *E. coli* O157:H7 contaminated raw flour (2).

Selection of strains for inactivation study

Salmonella enterica serovar Enteritidis phage type 30 was isolated from patients infected with *Salmonella* after having consumed raw almonds in 2000-2001 during an outbreak in Canada and the United States. The source of *Salmonella* PT 30 was linked to three farms growing the almonds. Previously, *Salmonella* PT 30 had been isolated from human cases in the United Kingdom (48, 49), broiler carcasses, pig feces, and humans in Korea (50). Since then, *Salmonella* PT 30 has been widely used in inactivation studies involving several technologies such as high hydrostatic pressure, moist air convection heating, gas catalytic infrared heat, X-ray Irradiation, hot air treatment, oil roasting and radio frequency energy (4, 9, 51-53).

Similarly, *E. coli* O157:H7 RMID 0509952 was implicated in a huge outbreak in Sakai, Japan in 1996 which resulted in 9,451 cases and caused 12 deaths. *E. coli* O157:H7 was linked to consumption of uncooked white radish sprouts (54).

Selection of low moisture foods

An objective of this thesis research was to investigate the efficacy of vacuum steam pasteurization to inactivate *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium* on low moisture foods. Among several inactivation methods, dry roasting and blanching are commonly used for

nuts such as almonds, pistachios, pecans and walnuts. However, these processes involve heat treatment at high temperatures for long period of pasteurization times. Blanching uses hot water which may not be appropriate for all foods such as for flaxseed, quinoa or milled flaxseed. High pressure processing and radio frequency has not been greatly explored in treatment of low moisture foods. Irradiation has shown effective treatment of low moisture products without changes in sensory attributes of walnuts and almonds but consumer concerns may exist due to use of irradiation.

Vacuum steam pasteurization

Vacuum steam pasteurization uses steam under vacuum that can operate at pasteurization temperatures above and below 100°C. The pasteurization system may include pre-heat treatment and cooling steps before and after vacuum steam pasteurization, respectively. Vacuum steam treatment process is the inactivation process and the pre-heat treatment is only to facilitate homogenous distribution of steam during steam treatment, whereas the cooling step lowers the temperature of treated product post pasteurization if needed. Products to be treated are placed in a sterilizer metal bin and are subjected to the pasteurization process. The vacuum steam treatment process works in four steps: initial vacuum, pre-vacuum, pasteurization, and post vacuum. The time and pressure can be adjusted as needed at each cycle dependent upon matrix type except at the pasteurization cycle, where the pressure is dependent upon the set temperature. The pasteurization cycles can only be conducted in batches and a cycle lasts approximately 20 to 25 minutes, which is dependent upon the set time for initial and pre-vacuums, pasteurization and post vacuum steps. No research has been extensively conducted using vacuum steam pasteurization that shows detailed time, temperature, and pressure parameters. In this study, the

parameters used during the pasteurization and the microbial shelf life study has been well explained with intricate details for various food matrices.

Enterococcus faecium as a surrogate organism

While I want to quantify the inactivation of *Salmonella* and *E. coli* O157:H7 from low moisture foods, it is also important to establish a surrogate organism for these pathogens when using vacuum steam pasteurization. The industry can use non-pathogenic bacteria to validate their pasteurization system without worries of contaminating their facility with pathogenic bacteria. *E. faecium* is a Gram-positive commensal bacteria in human and animal intestines and some strains of these bacteria can cause nosocomial infections (55). In addition, more than 90% of *E. faecium* strains that cause diseases are multidrug resistant (56). However, *Enterococcus faecium* NRRL B-2354 (ATCC 8459) has been shown to contain fewer mobile elements, antibiotic resistance, and virulence genes(57). Some well-known virulence genes may be present but they have been found to remain as nonfunctional copies (57). Hence, *E. faecium* ATCC 8459 has been widely used as a surrogate microorganism for *Salmonella* in validation of thermal processing technologies. The parameters of thermal inactivation such as D values or number of log reductions of *E. faecium* are found to be similar to that of *Salmonella*. However, *E. faecium* may behave differently in different treatment systems. Hence, it is necessary to measure the behavior of *E. faecium* as compared to *Salmonella* and *E. coli* O157:H7 when using vacuum steam pasteurization. Food industries can utilize such findings to validate their inactivation systems using a non-pathogenic strain.

Model fitting for microbial survival curves

Predictive microbiology provides the ability to mathematically describe the phenomenon of bacterial survival or decline. Survival curves are generated with observed counts after

exposure of bacteria under certain stress conditions. These curves can be fitted to a mathematical model with desired parameters that provide relevant information about thermal death time (D values and Z values), inactivation rates, and shape of inactivation (58). These values obtained after experimentation over myriad ranges of treatment conditions such as temperature, and time provide the ability to predict the nature of bacterial inactivation without the need of further experimental data. These abilities to predict the behavior of microorganisms are often helpful in determining the safety of food processing, such as cooking and storage. Hence, predictive microbiology aids in safe implementation of Hazard Analysis Critical Control Point (HACCP) providing increased understanding of process behavior to achieve desired inactivation of microorganisms (59, 60). Moreover, these survival curves can also be used as an essential tool in microbial food safety risk assessment with information on levels of inactivation obtained after pasteurization (61, 62). Survival curves are often dependent upon intrinsic factors that are the properties of food being treated, such as aw, pH, matrix shape/size, bulk density, and extrinsic factors such as temperature and pressure of treatment conditions.

While survival curves can be of first order (log linear), recently it has been shown that in several instances the microorganisms, such as *Salmonella*, show non-log linear survival (13, 39). In this study, the observed survival curves were non-log linear with a shoulder and a tailing effect. Hence, the modified Weibull and Geeraerd-tail models, which are, used to best-fit survival curves of non-linear nature or curves with a shoulder and a tailing effect was used in my experiment (12, 13).

Shelf life study

It is important to determine if there is any effect on the naturally occurring microbial flora on the low moisture foods after processing. Shelf life is the amount of time during which a

food can be consumed safely without any noticeable change to color, flavor, and texture. Raw products usually have higher amounts of natural microbes present on them. The measurement of total aerobic plate count (APC), yeast and mold, and *Bacillus cereus* provides an indication of spoilage. In a study by Postollec et al (63)found that 86% of raw products (dried vegetables, spices, egg mix powder, texturing agents) were contaminated with *Bacillus* species. *Bacillus* species causes rope formation in bread, shortening their shelf life (64). Similarly, yeast and mold growth on bread is a major concern for bread spoilage. Molds and fungus growth can be visually observed on bread, which not only causes discolorations of the bread, but can also be toxic if consumed.

Pasteurization of low moisture products using vacuum steam pasteurization may reduce the number of spoilage organisms. Decreasing the natural microbes would reduce the food spoilage due to these bacteria and enhance the microbial shelf life of these pasteurized products. However, steam treatment may increase the amount of moisture in the pasteurized products. The increased moisture content in low moisture products may negatively impact the shelf life of these products by helping the growth of surviving microbiota or by affecting other chemical properties such as, peroxide values and fatty acid contents. Hence, it is necessary to evaluate if the vacuum steam pasteurization not only inactivates pathogens but also decreases the natural microbial flora without significant effect on a_w, which in turn would help enhance the shelf life of the treated low moisture products.

EFFICACY OF VACUUM STEAM PASTEURIZATION FOR INACTIVATION OF *SALMONELLA* PT 30, *ESCHERICHIA COLI* O157:H7 AND *ENTEROCOCCUS FAECIUM* ON LOW MOISTURE FOODS

Abstract

With increasing demand for foods with bioactive compounds, low moisture foods such as flaxseeds, sunflower kernels, peppercorns, and quinoa have been studied a great deal for their health benefits and disease prevention. Such foods may be minimally processed and consumed raw, and several outbreaks of *Salmonella*, and *E. coli* O157:H7 have been attributed to contaminated spices, seeds and grains. Previous studies show that these pathogens are more resistant to dry heat in low moisture foods and processes such as chemical treatments and blanching may have negative effects on product quality and functionality (65, 66). It is important to investigate inactivation methods to minimize risks due to pathogens in such foods with minimum impact on product quality and functionality. Vacuum steam pasteurization uses steam under vacuum, which can be operated at temperatures below 100°C. The objective of this study was to determine the efficacy of vacuum steam pasteurization in inactivation of pathogens on whole flaxseed, quinoa, sunflower kernels, milled flaxseed and whole black peppercorns. The use of *E. faecium* as a potential surrogate for *Salmonella* and *E. coli* O157:H7 in vacuum steam pasteurization was also investigated. In addition, survival curves were generated using the modified Weibull and Geeraerd-tail models. Pasteurization for 1 minute at 75°C yielded average log reductions of 5.48±1.22, 5.71±0.40 and 5.23±0.61 on flaxseed, 4.29±0.92, 5.89±0.26 and 2.39±0.83 on quinoa, and 4.01±0.74, 5.40±0.83 and 2.99±0.92 on sunflower kernels for *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium*, respectively. Similarly, on milled flaxseed and black peppercorns average log reductions of 3.02±0.79 and 6.10±0.64 CFU/g were observed

for *Salmonella* PT 30 after 1 minute of treatment at 75°C. On average, >6.0 log reductions were observed after pasteurization at 85°C. The predicted δ and *Kmax* values from modelling shows similar findings to the log reductions observed in the inactivation studies. Vacuum steam pasteurization can be effectively used to reduce pathogens in these low moisture foods at temperature as low as 75 and 85°C and *E. faecium* may be used as a potential surrogate for *Salmonella* PT 30 and *E. coli* O157:H7 with careful considerations of treatment time and temperatures.

Introduction

Low moisture foods such as seeds, grains and spices have been demonstrated to have beneficial nutrients such as antioxidants, dietary fiber, proteins, lignans, omega acids, and many other bioactive compounds (67-69). While foods such as flaxseed, quinoa, sunflower kernels and peppercorns have been consumed since ancient times, their consumption may be increasing with increasing awareness of their health benefits. These foods may be assumed to be safe for consumption in their raw form, though recent outbreaks of *Salmonella* and *E. coli* O157:H7 associated with low moisture foods would suggest otherwise.

Salmonella is a major pathogen of concern in low moisture foods with increased outbreaks and recalls in recent years. In the U.S., *E. coli* O157:H7 has also been attributed to a few outbreaks in low moisture foods. Of total *Salmonella* enterica illnesses from 1998-2008, 13.0 % were attributed to fruits and nuts and 2.9 % were attributed to grains and beans (1). Similarly, 21.1% of total illnesses due to *E. coli* O157 was attributed to fruits and nuts, and and 0.7% were attributed to grains and beans (4). In the years from 2010-2015, there have been five outbreaks due to *Salmonella* in low moisture foods (nut butter, tahini sesame sauce, Turkish pine nuts, and red black pepper salami) causing 310 illnesses, 65 hospitalizations, and one death (15).

In addition, the number of recalls due to *Salmonella* was alarmingly high for low moisture products in 2015. There were 46 instances of recalls in 2015 due to possible *Salmonella* contamination in various products such as milled flaxseed, whole flaxseeds, sunflower kernels, organic milled coriander, macadamia nuts, and cashew splits. Also, the total number of recalls in low moisture products due to *Salmonella* contamination were 10, 34, 9, 22 for the years 2011, 2012, 2013, and 2014, respectively (16). Similarly, in the years 2003-2011, 238 recalls due to *Salmonella* were observed in nuts and edible seeds, and 104 recalls were seen in spices. One recall each, due to *E. coli* O157:H7, was also observed in nuts and seeds, and bakery goods (18).

The practices of cultivation, processing, and packaging increases risks of contamination by pathogen in foods (3, 31). The estimated annual risks due to pathogen contamination in nuts and dried fruits rank anywhere from moderate to high for number of illnesses, hospitalizations and deaths(1). Thermal processes such as dry heat are widely used to inactivate pathogens on nuts and edible seeds (4, 45, 70). However, they are effective at inactivation of pathogens only at high treatment temperatures after long pasteurization times. High treatment temperatures may also have negative impact on the chemical and physical properties, and loss of nutrition (71). Besides heat treatment, there are several other inactivation methods such as irradiation, fumigation, and high pressure processing explored for inactivation of pathogens in low moisture foods. However, most of these technologies are evaluated for inactivation of pathogens on almonds and walnuts. Implementation of the Food Safety Modernization Act (FSMA) to encompass preventative control measures would require the food industry to show adequate reduction of pathogens of concern in foods of all types (72).

Vacuum steam pasteurization uses steam under vacuum to operate at temperatures above or below 100°C for microbial inactivation, and then removes the steam with post vacuum cycles.

There were three major objectives of this study. The first objective of this study was to determine the efficacy of vacuum steam pasteurization in inactivation of pathogens on whole flaxseed, quinoa, sunflower kernels, milled flaxseed and whole black peppercorns. Next, the use of *E. faecium* as a potential surrogate for *Salmonella* and *E. coli* O157:H7 in vacuum steam pasteurization was also investigated. Lastly, survival curves were generated for *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium* after pasteurization at 75 and 85°C using the modified Weibull and Geeraerd-tail models to predict survival behavior of these bacteria in the tested low moisture products.

Materials and Methods

Inoculation of foods

Raw whole and milled flaxseed (*Linnum usitatissimum*), quinoa (*Chenopodium quinoa*), sunflower kernels (*Helianthus annuus*), and whole black peppercorn (*Piper nigrum*) were obtained from local commodity suppliers. Upon receipt, these products were tested negative for *Salmonella* and *E. coli*. Then, each product was separately mixed and then packaged into Mylar bags (Uline Inc., Pleasant Prairie, WI) and sealed. These bags were stored at room temperature until inoculation for inactivation studies.

*Salmonella enterica*subsp. *enterica* serovar Enteritidis PT 30 (ATCC BAA-1045) and *Enterococcus faecium* NRRL B-2354 (ATCC 8459) were obtained from the American Type Culture Collection (Manassas, VA) and *Escherichia coli* O157:H7 (RMID 0509952) was obtained from the Thomas S. Whittam STEC Center (Michigan State University, East Lansing, MI). The cultures were stored at -80°C in Brain Heart Infusion Broth (BHI) (Difco, Becton Dickinson, Sparks, MD) with 10% glycerol. The cultures were streaked onto BHI agar plates and were incubated overnight at 35°C. An isolated colony was transferred to 5ml BHI broth and was

incubated at 35°C static for 20 hours. Cultures were spread plated in the amount of 250 µl onto BHI agar plates (100 mm x 15 mm) and were incubated at 35°C for 24 hours. The resulting lawn of culture from a single BHI plate was sufficient to provide a target inoculum level of ~7-8 log CFU/g when added to 100g of product.

2.2kg of each product were required for one complete replicate of the inactivation study. Hence, 22 BHI agar plates were used for bacterial lawn growth for each replicate. Lawn culture from 11 BHI agar plates was collected with a sterile hockey stick (Fisher Scientific, Waltham, MA) into a beaker containing 2.5 ml of sterile water and was stirred well using an inoculating loop (Fisher Scientific, Waltham, MA). The 2.5 ml bacterial suspension was poured into 1.1kg product in a whirl pak bag (Nasco, Fort Atkinson, WI). The culture in the product was mixed and massaged for 5 minutes. The same procedure was followed for the remaining 1.1kg of the product. For milled flaxseed, 2.2kg was divided into three bags and each bag was inoculated with 2.5ml of inoculum. After mixing, the bags were transferred to a sterile stainless steel tray (12" X 9") for water activity (a_w) equilibration. *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium* were inoculated separately onto the food products following the same procedure to obtain ~7-8 log CFU/g count. All the inoculation procedures were conducted in class II biosafety cabinet.

a_w equilibration

Water activity (a_w) was measured using an Aqualab 4TE a_w meter (Aqualab, Pullman, WA). The a_w of the raw products increased upon inoculation. Following inoculation, 2.2 kg of the inoculated product in the stainless steel tray was placed in a closed chamber (Coleman cooler 24" X 16", Coleman Company, Inc., Kingfisher, OK). Approximately 25-50 g of Magnesium chloride 6-Hydrate crystal (Avantor Performance materials, Inc., Center valley, PA) or Lithium chloride anhydrous 99% -20 Mesh (Alfa Aesar, Ward Hill, MA) were weighed in

plastic weigh boats (Fisher Scientific, Waltham, MA) and was saturated with water. These boats were placed in the closed chamber (Coleman cooler 24" X 16", Coleman Company, Inc., Kingfisher, OK) with the inoculated product to equilibrate the a_w to the initial level (10). The water activity was equilibrated to the original level within 24hours. Inactivation experiments were conducted at the equilibrated a_w.

Assessing homogeneity of inoculation

Before conducting the inactivation studies, multiple samples of the inoculated and a_w equilibrated foods were plated to enumerate inoculated bacteria to ensure homogenous distribution of inoculum in the food products. Immediately after inoculation, two 25g samples of food with each bacteria type was plated in duplicates on Luria Bertani agar plates (Difco, Becton Dickinson, Sparks, MD). Then, eight 25g samples were plated in duplicates 24, 48, and 72 hours post inoculation for each product type.

Heat resistance test

To verify appropriate heat resistance of the inoculum in each product, guidelines provided by the Almond Board of California for using *E. faecium* NRRL B-2354 as a surrogate microorganism in almond process validation were used(73). After a_w equilibration, 100g of each inoculated product with each inoculum type were held at 280°F for 15 minutes in an Isotemp oven (Fisher Scientific, Waltham, MA, model no. 6903). Three 25g samples of each inoculum were plated in duplicates on LB agar to quantify bacteria survival.

Vacuum steam pasteurization

The vacuum steam pasteurization system at the pilot plant facility of Napasol North America (Fargo, ND) was used to conduct the pasteurization studies. Vacuum steam pasteurization (SMC, Statisol 50, model no. NA 07) uses steam under vacuum to obtain

treatment temperatures below 100°C (Figure A1). The pasteurization system works in three steps: pre-heat treatment, steam treatment and cooling (Figure 1). The steam treatment is the inactivation step and the pre-heat treatment is mainly to alleviate the temperature of raw product to a pre-determined set level in order to help facilitate homogenous distribution of steam during the steam treatment. Products to be treated are filled in a sterilizer metal bin (Statisol) and are subjected to the pasteurization process. The steam treatment process works in four steps: initial vacuum, pre-vacuum, pasteurization, and post vacuum (Figure 1). The amount of time and pressure can be adjusted as needed at each cycle dependent upon matrix type except at the pasteurization cycle, where the pressure is dependent upon the set temperature.

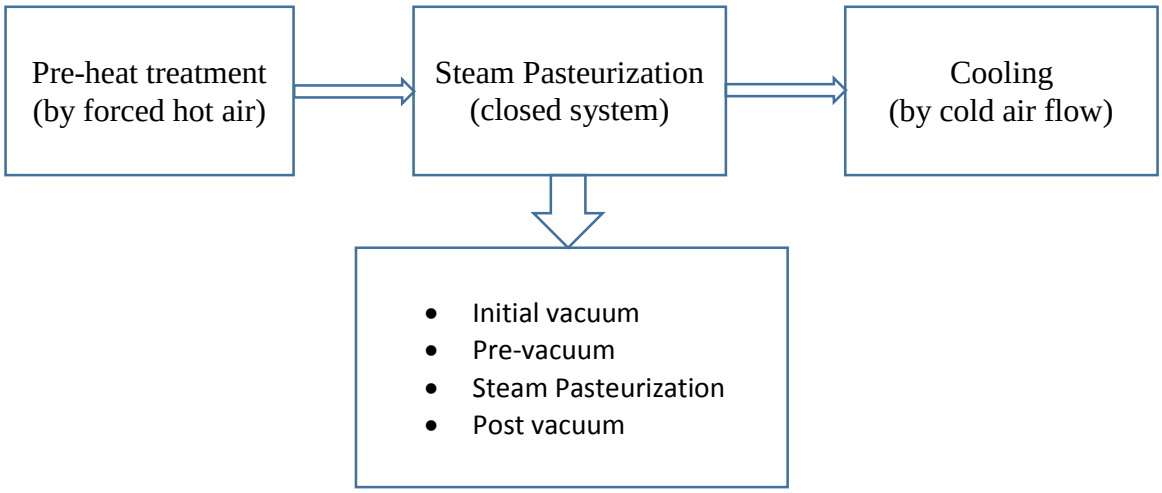

Figure 1. Vacuum steam pasteurization process diagram

Pasteurization conditions

After a_w equilibrations the inoculated seeds were divided into 25g portions and were placed into cotton bags (Uline, Pleasant Prairie, WI). Three bags of each bacterial strain were treated at each pasteurization condition and represent one biological replicate for that bacteria and product combination. The sterilizer bin was filled with product specific to the inoculated samples (45.0 kg for whole flaxseed, quinoa, and sunflower kernels, and 22.5kg for milled flaxseed and black peppercorns) and was pre-heated as needed using forced dry heat (Table A3). In this experiment, whole flaxseed, quinoa, and whole black peppercorns were pre-heated to 40±3°C, and sunflower seeds and milled flaxseed were pre-heated to 60±3°C, and 50±3°C, respectively, using forced dry heat (Table A4). After pre-heating, if needed, the nine inoculated bags and three temperature data loggers were sandwiched halfway in the product in the sterilizer bin. Then, the sterilizer bin was loaded for vacuum steam treatment. Pasteurization temperatures of 75, 85, 95and 105°C were chosen to conduct the inactivation experiments. Data loggers (Madge Tech, Inc., Warner, NH) were used to measure the temperature readings during the processing cycles (Table A4). However, during processing the observed temperature fluctuated from the set points. The treatment condition, which includes the measured pre-pasteurization, pasteurization and post pasteurization temperatures, will be referred to as the set pasteurization temperature (Table A4). At 75 and 85°C, pasteurization was conducted for 0.5, 1, 2, 3, 4, and 5 minutes and at the higher treatment temperatures of 95 and 105°C, pasteurization was conducted for 1, 2, 3, and 4 minutes. After vacuum steam treatment, the inoculated bags were retrieved and left to cool down for ~15 minutes at room temperature. The product used in the sterilizer bin was cooled to below 45°C if no pre-heat treatment was required before starting inactivation study at another condition. Three inoculated samples that were taken to the pilot plant facility but

untreated were used as controls. The pasteurization cycles can only be conducted in batches and hence, to obtain one replication of the experiment at all temperatures and pasteurization times for a product, 20 pasteurization cycles were conducted. Approximately, a cycle lasted 20-25 minutes dependent upon the set pasteurization parameters.

Enumeration of surviving bacteria

The samples were weighed in a sterile plastic bag (Whirl-pak, Nasco, Fort Atkinson, WI) and Butterfield dilution buffer was added in appropriate amounts. These bags were homogenized or massaged for 90 seconds and appropriate serial dilutions were spread plated in duplicate on non-selective agar manually or using Spiral plater Autoplate 4000 (Spiral Biotech, Norwood, MA). Luria Bertani agar plates were used to enumerate *Salmonella* PT 30, *E. coli* O157:H7 and *Enterococcus faecium* after pasteurization of whole flaxseed, quinoa, and sunflower kernels whereas modified Tryptic soy yeast extract agar (TSAYE) supplemented with ammonium ferric citrate and sodium thiosulfate (Difco, Becton Dickinson, Sparks, MD) was used to enumerate *Salmonella* PT 30on milled flaxseed and whole black peppercorns (74). The respective agar plates were incubated at 37±2°C for 48 hours. Following the incubation, the colonies were counted using a Q-Count reader model 350 (Advanced Instruments Inc., Norwood, MA).

Model fitting for microbial survival curves

Two models, the modified Weibull (12) and Gerraerd-tail (12) models, were used to generate survival curves for each bacteria at 75 and 85°C using GInaFiT Version 1.6. The equations with their parameters are listed and defined below.

Albert and Mafart modified Weibull model:

$$N= (No-N\_res)10^{**}(-((t/\delta)^{**}p))+ N\_res \quad \text{(Eq. 1)}$$

Where N is the population at time t (CFU/g), $N_o$is the population at time 0 (CFU/g), N_res is the heat resistant population, t is the time in minute, delta is the time of the first decimal reduction concentration, and p defines the shape of the curve (concavity or convexity).

Gerraerd-tail model:

$$N= (N_o- N\_res) * exp(-kmax * t) + N\_res \quad \text{(Eq. 2)}$$

Where N, N_o, N_res, and t are defined as above, and K_{max} is the maximum specific inactivation rate (min^{-1})

Statistical Analysis

Pasteurization of each inoculated product at each set temperature was repeated three times providing nine data points at each pasteurization condition for each bacterial strain. The duplicate counts obtained in CFU/g were averaged and log transformed using Microsoft Excel. The limit of detection (LOD) was used as the count for samples with no colony detected. Log reductions were calculated by subtraction of the log CFU/g from the observed unpasteurized control CFU/g. Analysis of Co-variance (ANCOVA) was conducted using Proc GLM in SAS V.9.4 (SAS Institute, Cary, NC). For ANCOVA, log reduction was considered as the dependent variable. Temperature and time measurements during pre-pasteurization, pasteurization, and post-pasteurization were included as dependent variables. Based on LS means adjusted for the significant interactions, adjusted p values were used. Survival curves were generated in Microsoft Excel 2013 using GinaFiT Version 1.6 add-in freeware (75).

Results

Inoculation and a_w equilibration

The inoculum levels of each bacteria on each product were confirmed and their heat resistance was verified prior to conducting the pasteurization experiments. Inoculation of *Salmonella* PT 30, *Escherichia coli* and *Enterococcus faecium* resulted in ~7 to 8 log CFU/g for each product (Table A1). When stored for three days after inoculation, <0.5 log CFU/g decline in CFU/g were observed (Table A1). Heat resistance tests performed after a_w equilibration on the tested foods following the Almond Board of California (ABC) protocol showed on average <2.5 log CFU/g reduction (Table A2). Inactivation studies were conducted on these foods at equilibrated a_w of approximately 0.35, 0.54, 0.50, 0.45 and 0.42 for whole flaxseed, milled flaxseed, quinoa, sunflower kernels and whole black peppercorns, respectively (Table 1). As inoculations were conducted on each product separately for each bacteria, the a_w was equilibrated separately (Table 1).

Table 1. Water activity of inoculated foods with control counts

Product	Before Inoculation	Bacteria	a_w After Inoculation[a]	a_w After Equilibration[a]	Unpasteurized (Controls) Log CFU/g at 75/85°C[a]	Unpasteurized (Controls) Log CFU/g at 95/105°C[a]
whole flaxseed	0.325 ± 0.05	*Salmonella* PT 30	0.438 ± 0.02	0.349 ± 0.02	8.08 ±0.17	8.11 ± 0.10
		E. coli O157:H7	0.421 ± 0.02	0.358 ± 0.02	7.90 ± 0.29	7.94 ± 0.11
		E. faecium	0.432 ± 0.01	0.361 ± 0.02	7.41 ± 0.22	7.44 ± 0.14
quinoa	0.513 ± 0.16	*Salmonella* PT 30	0.550 ± 0.17	0.494 ± 0.12	8.22 ± 0.18	8.01 ± 0.29
		E. coli O157:H7	0.554 ± 0.02	0.489 ± 0.07	7.92 ± 0.31	7.85 ± 0.29
		E. faecium	0.551 ± 0.02	0.488 ± 0.02	7.39 ± 0.17	8.02 ± 0.30
sunflower kernels	0.445 ± 0.03	*Salmonella* PT 30	0.505 ± 0.03	0.436 ± 0.04	8.17 ± 0.22	8.31± 0.11
		E. coli O157:H7	0.501 ± 0.04	0.438 ± 0.05	8.03 ± 0.18	7.89 ± 0.44
		E. faecium	0.505 ± 0.05	0.446 ± 0.03	7.85 ± 0.18	7.91 ± 0.23
milled flaxseed	0.567 ± 0.05	*Salmonella* PT 30	0.605 ± 0.01	0.543 ± 0.05	7.81 ± 0.15	7.92 ± 0.42
black peppercorns	0.417 ± 0.04	*Salmonella* PT 30	0.553 ± 0.06	0.419 ± 0.04	7.80 ± 0.16	7.76 ± 0.08

[a]averages and standard deviations for 3 replicates are reported

Pasteurization temperature, time and vacuum settings

Pasteurization was conducted for 0.5, 1, 2, 3, 4 and 5 minutes at 75 and 85°C, and for 1, 2, 3 and 4 minutes at 95 and 105°C for all the tested foods. The pre-pasteurization temperatures were selected based on preliminary experiments to provide homogenous distribution of steam and temperature in the chosen food matrix. However, once the pasteurization cycles started, the time required to reach the set pasteurization temperature from the pre-heat temperature lasted a few seconds to a maximum of 3 minutes (Table A4). Similarly, after pasteurization was completed, the time required to reduce the temperature to the pre-heat temperature ranged from 10-15 minutes (Table A4).Usually, these pre-pasteurization and post-pasteurization times with their corresponding temperatures increased with higher set pasteurization temperatures (Table

A4). ANCOVA showed that pre-and post-pasteurization temperatures and times had significant effect along with pasteurization time and temperature on bacterial reductions. Hence, LS means were adjusted for the significant interactions and the Tukey adjusted p values were used.

Pasteurization of whole flaxseed

Inoculated *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium* on whole flaxseed ranged from 7.41±0.22 to 8.11±0.10 log CFU/g (Table 1). Pasteurization for 30 seconds at 75°C yielded an average log reduction of 2.24±1.17, 2.50±1.36 and 1.81±0.58 for *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium*, respectively, (Figure 2), which were significantly less than average log reductions of 5.48±1.22, 5.71±0.40 and 5.23±0.61 for each strain after 1 minute of pasteurization at 75°C (Figure 2), (adj.p<0.05). On average, greater than 6.0 log CFU/g reduction was observed after pasteurization for longer than 1 minute at 75°C for *Salmonella* and *E. coli* O157:H7. For each bacteria, pasteurization at 75°C resulted in similar log reduction during pasteurization times of 1 to 5 minutes (adj.p>0.05). At each pasteurization time, the average log reduction for all bacteria were not significantly different from each other (adj.p>0.05) except for *Salmonella* PT 30 and *E. faecium* after 4 minutes of pasteurization at 75°C (adj.p<0.05)(Figure 2).

Pasteurization at 85°C for 30 seconds significantly increased the average log reduction to 5.59±0.14 and 5.25±0.02 and 4.97±0.21, respectively, for *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium* when compared to the log reduction observed after 30 seconds of pasteurization at 75°C (adj.p<0.05) (Figure 2). There were no significant differences in log reduction for each bacteria after 30 seconds of pasteurization at 85°C (adj.p>0.05). Increasing the pasteurization time to 1 and 2 minutes yielded an increase in log reduction on average by 0.5 CFU/g and by 1.0 CFU/g at 3 and 5 minutes, respectively at 85°C, but these were not significantly different from log reduction after 0.5 minutes (adj.p>0.05). The maximum log reduction was achieved at 3

minutes of pasteurization at 85°C as no further significant increases in inactivation was observed with increase in pasteurization time for each bacteria (adj.p>0.05). Pasteurization at 95 and 105°C for all bacteria resulted in ≥6 CFU/g log reductions irrespective of treatment times (adj.p>0.05) (Figure 2). Also, similar average log reductions were achieved by pasteurization at 75°C for 1 minute, 85°C for 0.5 minutes, 95°C for 1 minute, and 105°C for 1 minute for all bacteria (adj.p>0.05).

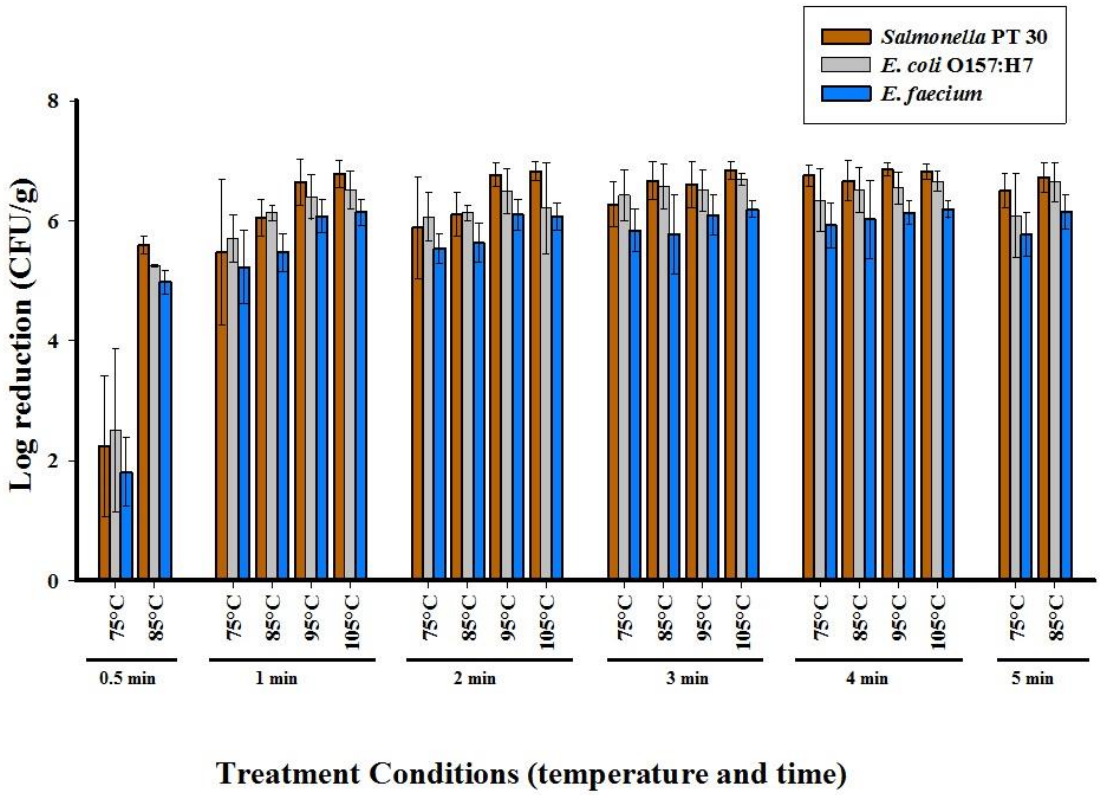

Figure 2. Average log reduction in CFU/g as observed after vacuum steam pasteurization on whole flaxseed for *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium* at treatment temperatures of 75, 85, 95 and 105°C. Log reductions are average of 9 data points and error bars denote standard deviation.

Pasteurization of quinoa

Inoculated *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium* on quinoa ranged from 7.39±0.17 to 8.22±0.18 log CFU/g (Table 1). Pasteurization at 75°C for 30 seconds yielded on average 4.09±0.67, 5.83±0.31, and 2.45±0.74 log CFU/g reduction for *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium*, respectively (adj.p<0.05) (Figure 3). Interestingly, increasing pasteurization times to 3 and 4 minutes at 75°C increased the log reduction by approximately 1.0 log CFU/g for all bacteria, but a decrease in log reduction after pasteurization for 5 minutes was observed. For each bacteria, pasteurization at 75°C resulted in similar log reduction during pasteurization times of 1 to 5 minutes (adj.p>0.05).

Pasteurization at 85°C for 30 seconds resulted in greater than 6.0 log CFU/g reduction for *Salmonella* PT 30 and *E. coli* O157:H7 (Figure 3). However, for *E. faecium* 6.0 log CFU/g reductions were observed only after pasteurization for 3 and 4 minutes. Increase in pasteurization time at 85°C did not yield significantly different log reductions for each bacteria (adj.p>0.05). The log reductions were similar for *Salmonella* PT 30 and *E. coli* O157:H7 at all treatment times at 85°C, but were only similar to *E. faecium* after pasteurization for 3-5 minutes (adj.p>0.05). Similar average log reductions were observed for *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium*, respectively, at pasteurization temperatures of 95°C, which were not different from those observed at 105°C (adj.p>0.05). Also, log reductions observed after pasteurization for 0.5 minutes at 85°C for each strain were not significantly different from their respective log reductions obtained after 1 minute of pasteurization at 95 and 105°C (adj.p>0.05) (Figure 3).

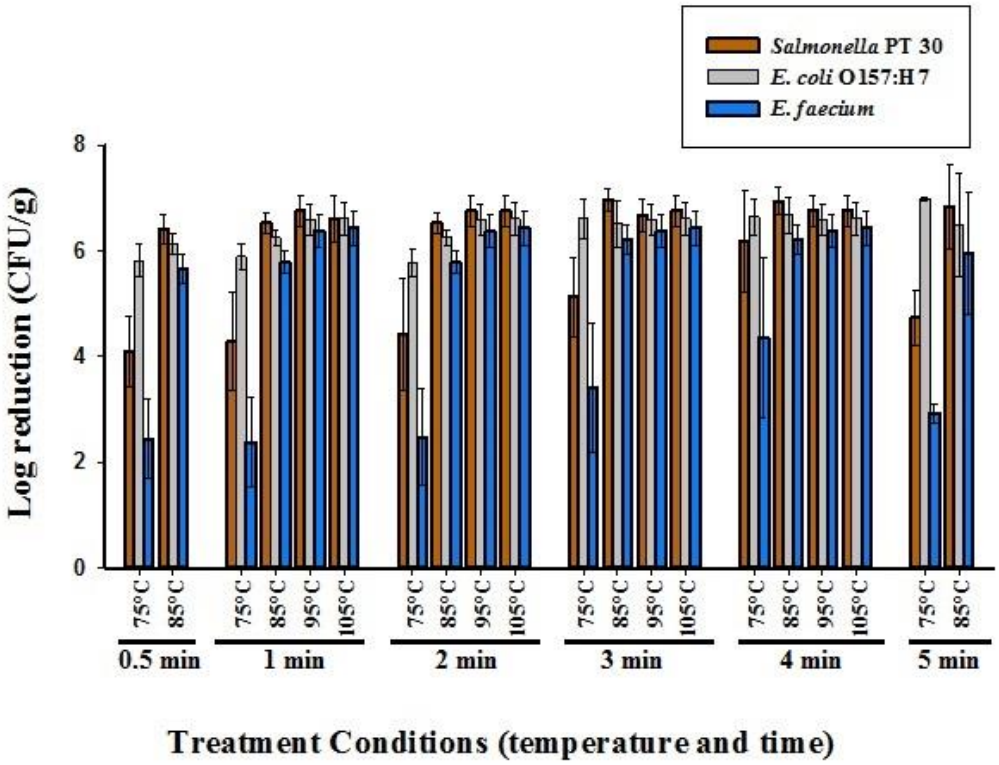

Figure 3. Average log reduction in CFU/g as observed after vacuum steam pasteurization on quinoa for *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium* at treatment temperatures of 75, 85, 95 and 105°C. Log reductions are average of 9 data points and error bars denote standard deviation**.**

Pasteurization of sunflower kernels

For sunflower kernels, the initial log CFU/g ranged from 7.85±0.18 to 8.31±0.11 for *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium* (Table 1). Pasteurization for 30 seconds at 75°C resulted in similar average log CFU/g reduction of 3.92±1.18, 4.85±1.33 and 3.14±0.58 for *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium*, respectively (adj.p>0.05) (Figure 4). For *E. coli* O157:H7, pasteurization at 75°C yielded similar log reduction of ~5.0 (adj.p>0.05). However, >5.0 log reduction for *Salmonella* PT 30 was achieved after 4 and 5 minutes of pasteurization. For *E. faecium*, a maximum of only 3.89±0.72 log CFU/g reduction was observed

at 75°C after pasteurization for 4 minutes, which was not significantly different from log reductions at 1,2,3, and 5 minutes of pasteurization (adj.p>0.05) (Figure 4).

Pasteurization at 85°C resulted in similar log reductions of >6.4 log CFU/g for *E. coli* O157:H7 (adj.p>0.05) at all pasteurization times. For *Salmonella* PT 30, log reduction of 6.75±0.28 CFU/g was observed after pasteurization for 4 minutes, which was similar to log reductions after pasteurization at all times at 85°C (adj.p>0.05) (Figure 4). On average, log reduction of 6.38±0.41 CFU/g was observed after pasteurization for 3 minutes at 85°C for *E. faecium*, which was not significantly different from log reductions obtained at any other pasteurization times (adj.p>0.05). Log reductions observed for *E. faecium* at 85°C after pasteurization for 3,4 and 5 minutes were similar to log reductions of *Salmonell*a PT 30 and *E. coli* O157:H7 at these conditions (adj.p>0.05) (Figure 4). Pasteurization at 95 and 105°C yielded on average log reductions of greater than 6.24 CFU/g at all treatment time for all bacteria with the highest reduction of 7.05±0.25 observed for *Salmonella* PT 30 at 95°C after pasteurization for 2 and 3 minutes (Figure 4). The log reductions observed at 95 and 105°C for *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium* were not significantly different from each other at any pasteurization times (adj.p>0.05) (Figure 4).

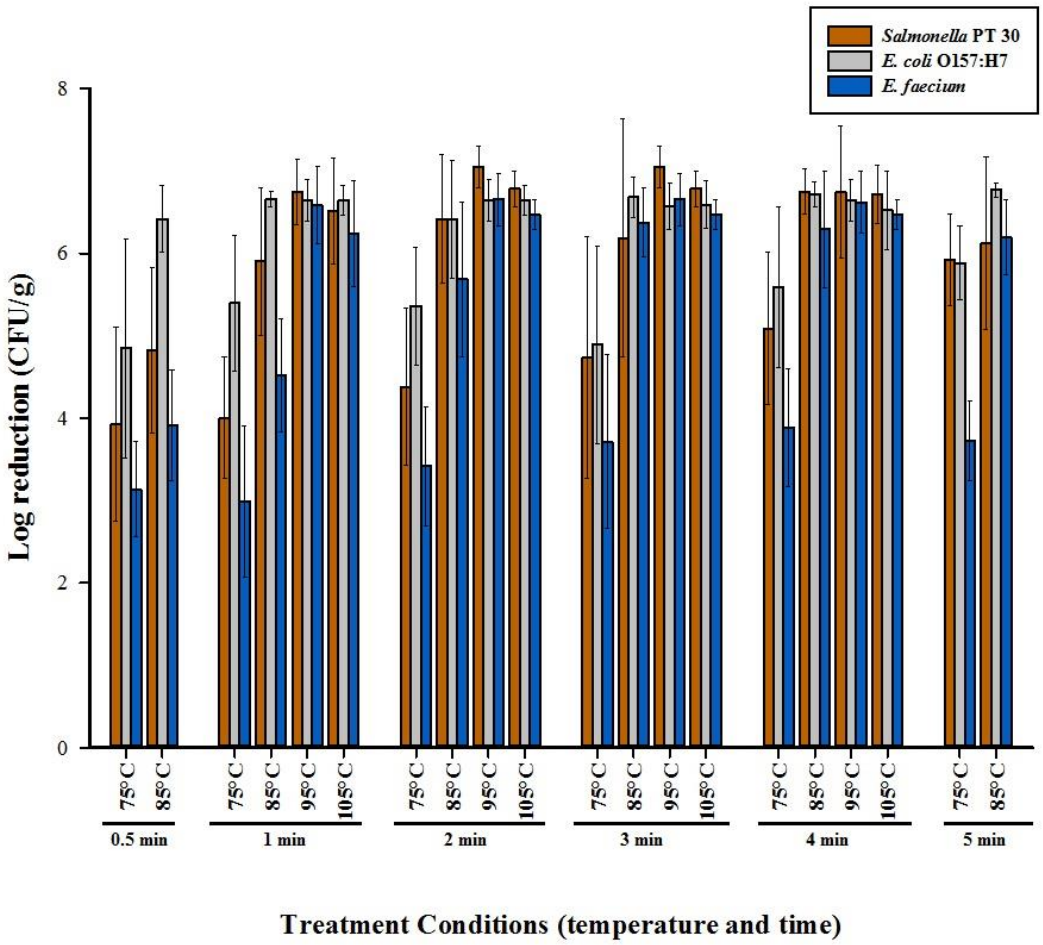

Figure 4. Average log reduction in CFU/g as observed after vacuum steam pasteurization on sunflower kernels for *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium* at treatment temperatures of 75, 85, 95 and 105°C. Log reductions are average of 9 data points and error bars denote standard deviation.

Pasteurization of milled flaxseed

Inoculation of *Salmonella* PT 30 on milled flaxseed resulted in 7.81±0.15 log CFU/g for inactivation at 75 and 85°C and 7.92±0.42 log CFU/g for inactivation at 95 and 105°C (Table 1). At 75°C, the lowest average log reduction of 2.21±1.06 CFU/g and the highest average log reduction of 4.01±0.79 CFU/g was observed after pasteurization for 0.5 and 4 minutes, respectively (Figure 5). There were no significant differences in log reductions for *Salmonella* PT 30 after pasteurization at 1, 2, 3, 4 and 5 minutes at 75°C (adj.p>0.05). Vacuum steam

pasteurization at 75°C did not result in a log reduction of 5.0 log CFU/g on milled flaxseed. Pasteurization for 30 seconds at 85°C yielded an average log reduction of 5.56±1.31 CFU/g whereas, an average log reduction of 6.77±0.47 CFU/g was observed after 4 minutes (adj.p<0.05). Pasteurization at 85°C resulted in similar log reductions at 1, 2, 3, 4 and 5 minutes (adj.p>0.05). Log reductions observed after 1 to 5 minutes of pasteurization at 85°C were not significantly different from log reductions at 1 to 5 minutes of pasteurization at 75°C (adj.p>0.05). At 95 and 105°C no colonies were detected (LOD 1.0 log CFU/g) at any pasteurization conditions providing same log reduction of 6.92±0.42 CFU/g (adj.p>0.05) (Figure 5). The log reduction observed at 75°C after 1 minute of pasteurization was similar to log reductions observed at 95 and 105°C (adj.p>0.05) (Figure 5).

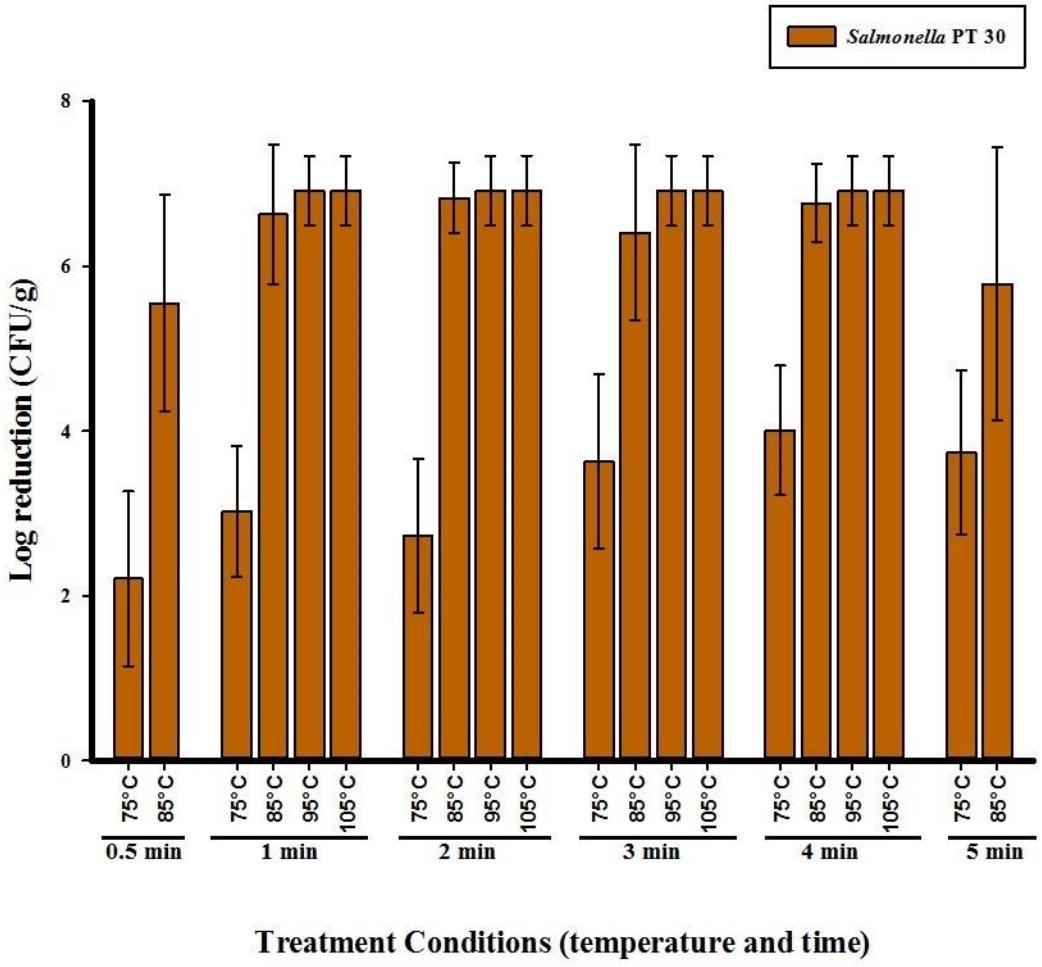

Figure 5. Average log reduction in CFU/g as observed after vacuum steam pasteurization on milled flaxseed for *Salmonella* PT 30 at treatment temperatures of 75, 85, 95 and 105°C. Log reductions are average of 9 data points and error bars denote standard deviation.

Pasteurization of whole black peppercorns

Inoculation of *Salmonella* PT 30 on black peppercorns resulted in 7.80±0.15 log CFU/g for inactivation studies at 75 and 85°C and 7.76±0.08 log CFU/g for inactivation studies at 95 and 105°C (Table 1). Pasteurization on peppercorns at 75°C for *Salmonella* PT 30 resulted in similar log reductions of 5.64±0.97, 6.10±0.64, 6.38±0.58, 6.67±0.26, 6.38±0.59, and 6.56±0.36 CFU/g after pasteurization at 0.5, 1, 2, 3, 4 and 5 minutes, respectively (adj.p>0.05) (Figure 6). Similarly, average log reductions of 5.40±2.45, 6.60±0.30, 6.67±0.23, 6.62±0.35, 6.43±0.51 and

6.67±0.23 CFU/g were observed after treatment at 85°C for 0.5 to 5 minutes, respectively (adj.p>0.05). Pasteurization at 95°C for 4 minutes yielded an average log reduction of 6.60±0.30 CFU/g, which were similar to log reductions at all pasteurization times at 95 and 105°C (adj.p>0.05). Interestingly, there were no significant differences in log reduction observed after 0.5 minute of pasteurization at 75°C and any other pasteurization times at 85, 95 and 105°C (adj.p>0.05) (Figure 6).

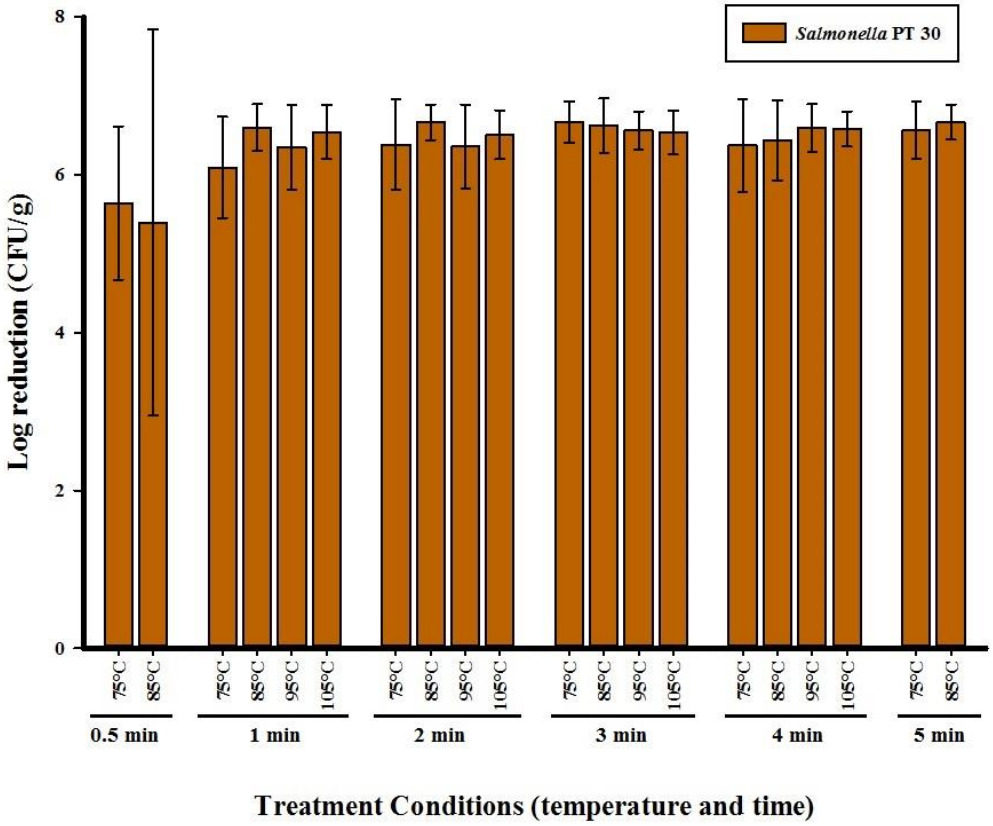

Figure 6. Average log reduction in CFU/g as observed after vacuum steam pasteurization on whole black peppercorns for *Salmonella* PT 30 at treatment temperatures of 75, 85, 95 and 105°C. Log reductions are average of 9 data points and error bars denote standard deviation.

Survival curves

As the survival rates were non-log linear, the modified Weibull and Geeraerd-tail models were used to generate survival curves (Table 2). The goodness of fit of data generated through use of both of these models was tested by comparing the *f* value against F table value at 95% Confidence (76). The *f* values were observed to be smaller than the F table value (F DF_{model}/DF_{data}). Given the nature of the curves as non-log linear, the modified Weibull model was expected to have lower Mean-sum of squared error (MSSE) values compared to those generated by Geeraerd-tail model for log-linear models. However, lower MSSE values were observed with Geeraerd-tail model on 17/ 22 occasions as shown in bold (Table 2). The non-log linear curves showed a shoulder and a tailing effect and hence, Geeraerd-tail model provided the best fit of data.

The *Kmax* is the maximum specific inactivation rate (min^{-1}) obtained through the Geeraerd-tail model where the highest *Kmax* value indicates more rapid inactivation. At all instances, *E. faecium* had the lowest *Kmax* values showing lower inactivation rates than *Salmonella* PT 30 and *E. coli* O157:H7 as suggested by the lower log reductions of *E. faecium* (Table 2). Also, *Kmax* values were smallest for *E. faecium* and largest for *E. coli* O157:H7 on quinoa and sunflower kernels at 75°C pasteurization, which agrees with inactivation results that showed lowest log reduction for *E. faecium* and highest log reductions for *E. coli* O157:H7 on quinoa and sunflower kernels (Table 2).

The δ value obtained through the modified Weibull model is the time in minutes required to obtain the first log reduction in bacteria. Similarly, the *p* parameter explains the shape of the curve and shows if there is a shoulder effect. As expected, the δ values were low for these products at both of these treatment temperatures. The highest δ value for *Salmonella* PT 30 was

observed to be 0.35 minutes on quinoa when treated at 75°C and the lowest δ value of 0.02 minutes was observed at 75°C for *Salmonella* PT 30 on milled flaxseed (Table 2). Interestingly, δ values for *Salmonella* PT 30 at 75°C were lower than that at 85°C for milled flaxseed and equal at both temperatures for black peppercorns. The highest δ value observed for *E. coli* O157:H7 was 0.31 on whole flaxseed at pasteurization temperature of 75°C and the lowest (0.23 minute) on quinoa (Table 2). Similar to the findings on milled flaxseed, δ values for treatment on quinoa at 75°C was found to be lower than the treatment at 85°C for *E. coli* O157:H7 and *E. faecium*. The highest δ value for *E. faecium* was observed to be 0.31 after treatment at 85°C on sunflower kernels. The δ values on whole flaxseed for *E. faecium* were the same irrespective of pasteurization temperatures. Also, the δ values were greater after treatment at 85°C than after treatment at 75°C on sunflower kernels for *E. faecium*. For all instances where the δ values were observed to be greater at 85°C than at 75°C for the same product type, the *p* values were also observed to be greater showing higher upward concavity (Table 2).

Table 2. Parameters estimated through use of modified Weibull and Geeraerd-tail model

			Modified Weibull				Geeraerd-tail		
Product	Temp	Bacteria	δ	p	RMSE[a]	4D	k_{max}	RMSE[a]	4D
whole flaxseed	75°C	*Salmonella* PT 30	0.22	1.12	0.7632	±0.75	12.71	**0.7598**	±0.75
		E. coli O157:H7	0.31	2.42	0.6665	±0.6	13.62	**0.637**	±0.7
		E. faecium	0.28	1.33	**0.3673**	±0.85	12.16	0.3974	±0.8
	85°C	*Salmonella* PT 30	0	2.36	0.4135	±0.05	27.71	**0.3584**	±0.35
		E. coli O157:H7	0	2.4	0.3375	±0.05	27.48	**0.2679**	±0.35
		E. faecium	0.28	2.93	0.4034	±0.5	25.03	**0.4**	±0.4
quinoa	75°C	*Salmonella* PT 30	0.35	4.15	0.9452	±0.5	19.17	**0.9372**	±0.5
		E. coli O157:H7	0.23	1.9	**0.6993**	±0.5	-	-	-
		E. faecium	0.02	0.24	**1.0042**	-	11.73	1.0452	-
	85°C	*Salmonella* PT 30	0	2.47	0.4329	±0.05	30.82	**0.4146**	±0.3
		E. coli O157:H7	0.26	2.79	0.4911	±0.45	29.87	**0.487**	±0.35
		E. faecium	0.28	3.04	0.5155	±0.45	27.58	**0.5112**	±0.35
sunflower kernels	75°C	*Salmonella* PT 30	0.04	0.49	**1.0684**	±0.7	18.34	1.0731	±0.55
		E. coli O157:H7	0.26	1.9	1.1977	±0.55	22.94	**0.9088**	±0.45
		E. faecium	0	1.41	0.7664	-	15.43	**0.746**	-
	85°C	*Salmonella* PT 30	0	2.36	1.0348	±0.05	22.3	**0.8889**	±0.45
		E. coli O157:H7	0	2.35	0.3509	±0.05	31.37	**0.3386**	±0.3
milled flaxseed	75°C	*E. faecium*	0.31	2.77	0.8645	±0.55	10.63	**0.8285**	±0.9
		Salmonella PT 30	0.02	0.24	**0.9252**	-	9.42	0.9689	-
	85°C	*Salmonella* PT 30	0.27	2.81	0.9354	±0.45	25.84	**0.9276**	±0.4
black peppercorn	75°C	*Salmonella* PT 30	0.25	2.57	0.557	±0.45	26.36	**0.5523**	±0.35
	85°C	*Salmonella* PT 30	0.25	2.5	0.9706	±0.45	24.98	**0.9625**	±0.4

[a]bolded RMSE value highlights the model that was best fit for each product/bacteria/temperature

Discussion

Inoculation of low moisture foods

To assess inactivation of pathogens by a specific processing technique, low moisture foods are typically inoculated with a high level of pathogen (7-8 log CFU/g) in order to detect a 5 log reduction. In addition, the inoculum should show sufficient thermal resistance prior to conducting inactivation studies. At the same time, inoculation tends to increase the moisture content that needs to be addressed before the pasteurization study (77, 78). However, a single inoculation protocol may not be the best for all types of food matrices. In order to inoculate whole flaxseed, quinoa, sunflower kernels, milled flaxseed and whole black peppercorns, modifications to existing inoculation methods were investigated and used in this study. The inoculation method resulted in ~7-8 log CFU/g for all three bacteria *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium* on 2.2 kg of food matrices. In this study, BHIA grown bacteria were dislodged into 5-10 ml of water then transferred to the food matrices. The bacteria were spread over these matrices from seed to seed by massaging and shaking which mimics a scenario where contamination may occur due to contaminated water and spread within a food matrix. Using the inoculation protocol described in this study, the counts on each product for each bacteria did not appear to decrease by greater than 0.5 log CFU/g during storage for three days. Also, use of Magnesium chloride and Lithium chloride salts were efficient at equilibrating a_w to the initial level as described by Jeong et al.(10).

Approximately 8.0 log CFU/g was achieved by Danyluk et al., using 25 ml of TSB grown *Salmonella* culture to inoculate 400g of almonds (79). Given the nature of foods tested in my experiment, it was in my best interest to use minimal amount of inoculum suspended in water in the ratio of 1 ml to 440 g to minimize increase in a_w, which helped to equilibrate a_w within 24

hours. A study by Enache et al.(80, 81),used talc inoculated with *Salmonella* and *E. faecium* to transfer to peanut paste but preparation of inoculated talc required lengthy drying and sieving procedure before transfer to peanut paste. A similar study by Blessington et al.(81), used sand and chalk to inoculate *Salmonella* on walnuts and almonds. In their study, 35ml of culture were used to inoculate 200g of sand and 62.5 ml of culture were used to inoculate 200 g of chalk, and after drying and sieving they were mixed with almond or walnut kernels at sand-to-nut ratios of 5, 25, or 50 g per 200 g. The inoculation procedure yielded inoculation levels of approximately 4.0 log CFU/g of *Salmonella* PT 30 which is not high enough to achieve 5-6 log reduction during an inactivation study but rather helpful for survival studies.

Bowman et al.(82) inoculated whole black peppercorns and cumin seeds following both dry and wet inoculation method which yielded 5.52 log CFU/g for dry transfer and 8.15 CFU/g for TSB grown culture on peppercorns, and 6.79 CFU/g for dry transfer and 6.89 CFU/g for TSB grown culture on cumin seeds. The dry inoculum showed to be more resistant over 28 days of storage but again the initial level of inoculation did not reach as high at 7-8 log CFU/g. However, when following the dry inoculation method, the ratio of inoculated sand to seed was very high (i.e. 25 g to 50 g) and the amount of seeds inoculated were only 100 g for each seed type. To inoculate a product as much as 2.2 kg would require a lot of inoculum. Also, it has been shown that agar plate grown bacteria shows greater resistance than broth grown culture (83). Hence, culture grown on 22 BHIA plates (100 X 15mm) was selected to inoculate sufficient amount of food matrices in the ratio of 1 BHIA plate culture to 100 g of each product. In addition, the thermal resistance test conducted after inoculation and a_w equilibration in this study showed, on average, less than 2.5 CFU/g log reduction following Almond Board of California protocol (73).

Vacuum steam pasteurization of low moisture foods

An inactivation technique for low moisture food products is recommended to achieve a minimum of 4.0-5.0 log CFU/g reduction for a pathogen (77, 78). Pathogens in low moisture foods are shown to be resistant to thermal heat treatments, which typically yield low reductions. Hence, alternative methods have been studied and new methods are being explored for use in inactivation of pathogens in low moisture foods. Treatment of *Salmonella* on immersion-inoculated pecan nutmeat pieces and halves by hot air treatment at 120°C for 20 minutes yielded a log reduction of 1.26 and 1.18 log CFU/g, respectively (4). In the same study, dry-inoculated pecan nutmeats pieces and halves after treatment at 120°C for 20 minutes yielded log reduction of 2.36 and 2.43, respectively. Only at temperatures of 160 or 170°C for 15 minutes were able to achieve reductions >6 and >7 CFU/g for dry and immersion-inoculated nutmeats, respectively. The high treatment temperatures may not affect sensory and chemical properties of nuts but can impact the quality and functionality of other seeds, grains and flour.

The results in my study show that vacuum steam pasteurization is effective at >5.0 log CFU/g reduction of *Salmonella* PT 30 and *E. coli* O157:H7 at treatment temperatures of 75 and 85°C just after a few minutes of pasteurization for various kinds of low moisture foods. However, it is necessary to note that there was variability in death rates between replicates, mainly at 75°C, as observed in treatment of quinoa for *Salmonella* PT 30 and *E. faecium*, with lower reductions observed at 5 minutes. Previously, inactivation studies done on almonds with steam showed that ~ 4.6 and ~8.5 log CFU/g reductions was observed for *Salmonella* PT 30 after treatment for 25 and 65 seconds, respectively at 95°C (84). These results appear similar to the results obtained in this study, but for different types of food matrices. A study by Lee et al., on nonpareil and mission almonds when subjected to steam produced by boiling water showed log

reductions of 3.73±0.48 and 2.05±0.12 CFU/g after 25 sec of treatment at 93±1°C (85). In the same study, 5.76±0.38 and 4.10±0.62 log CFU/g reduction was obtained after 65 sec of treatment for *Salmonella* Enteritidis for each product type at 93±1°C. Ban et al.(6), obtained log reductions of 3.8 and 2.4 CFU/g for *E. coli* O157:H7 and *Salmonella* Enteritidis PT 30 respectively, with the use of saturated steam at 100°C for 15sec on almonds. Also, log reductions of 3.0 and 3.3 CFU/g was obtained after saturated steam treatment at 100°C for 30sec for *E. coli* O157:H7 and *S.* Enteritidis PT 30 on pistachios (6). However, combination of superheated steam for 70sec followed by catalytic infrared heat treatment for 70sec yielded log reduction of *Salmonella* by 5.73 log CFU/g (52). Higher log reductions, as in my studies, may be due to the fact that the steam was applied in a closed system with initial pre-vacuum providing homogenous distribution of steam and causing higher log reductions as compared to an open system. Also, the types of food matrices tested here could have an effect in the differences observed for the different amount of log reductions observed.

Several other methods have been investigated for inactivation of pathogens in low moisture foods. High hydrostatic pressure (HHP) was investigated for inactivation of *Salmonella* Enteritidis on almonds, which resulted in a log reduction of 0.51 CFU/g after pressurizing the almonds at 70,000 psi and 55°C for 10 minutes (51). Also, irradiation with X-ray treatment of walnuts and almonds at varied a_w of 0.23 and 0.64 was able to obtain >5 log reduction for both *Salmonella* Enteritidis and *Salmonella* Tennessee without any negative impact on sensory of almonds (10). Inactivation of *Salmonella* enterica serovars Agona, Enteritidis and Typhimurium in peanut butter subjected to 90°C in water bath yielded a log reduction of 3.2 CFU/g (46). An experiment set up similar to vacuum steam pasteurization termed as vacuum/steam/vacuum was operated in a closed system with settings of initial vacuum time of 0.1 min and final vacuum

time of 0.3 min was conducted at two treatment temperatures of 138 and 143°C for maximum of 0.3 min on fruits and vegetables (grapefruit, papaya, mangoes, avocadoes, kiwis, and peaches) for the treatment of *Listeria innocua*. During this experiment, log reduction ranged from 3.1-4.7 CFU/g for various fruits treated (86). Although similar principle has been applied in this study, the tests were performed on fruits and vegetables.

An inactivation study conducted on black peppercorns with atmospheric pressure plasma treatment resulted in a log reduction of ~1.5 CFU/g and ~5 CFU/g after 20 and 80 sec of treatment (8). The temperature measurements during 20 and 80 sec of treatments were recorded to be ~70 and ~125°C, respectively. In this experiment, greatest log reductions were observed for peppercorns, where counts reaching below the limit of detection, after pasteurization for 1 minute at 75°C. This effect may be due to the volatile compounds such as phenolic amides that act as an antimicrobial agent, providing synergistic effect for inactivation of *Salmonella* (87, 88).

Sensory properties may be affected by steam treatment as observed in steamed black peppercorns that appeared darker, and had a considerable decrease in the piperine content after treatment and storage (71). Also, increase in moisture content was noticed after steam treatments (84). Similarly, it was also observed that during pressurizing the surface of almonds became visibly oily, presumably due to high pressure that forced oil out of the interior of the almonds (51). In current experiment, no such analysis was performed for the inactivation part, but no visual changes in color were observed. However, a study conducted using the same technology on almonds yielded a 4 log CFU/g reduction at 88°C after 4 minutes of pasteurization. Also, lower peroxide values were observed for pasteurized samples during a shelf life study conducted over 13 months period (89).

E. faecium as a surrogate

E. faecium has been widely used as a surrogate for its similar thermal sensitivity as *Salmonella* (74, 90, 91),and its safety has also been evaluated (57). It is important to investigate the potential of *E. faecium* as a surrogate for different product types and inactivation methods. *E. faecium* has not been studied as a potential surrogate for vacuum steam pasteurization. It has also not been directly investigated as a surrogate for *E. coli* O157:H7. Studies show that the log reduction in *E. faecium* is often lower than that of *Salmonella* at the same pasteurization conditions (74). My study shows similar results. Also, it is shown that *E. coli* O157:H7 has higher log reductions than *E. faecium* asserting that *E. faecium* is a safe conservative surrogate for both *Salmonella* PT 30 and *E. coli* O157:H7 (74, 90).

In this experiment, *E faecium* had the greatest survival on quinoa and sunflower kernels at a pasteurization temperature of 75°C. At most pasteurization conditions tested here, the log reduction in *E. coli* O157:H7 was observed to be greater than that of *Salmonella* PT 30 and *E. faecium*, with *E. faecium* being the most resistant bacteria. Although at higher pasteurization temperatures and longer times, similar log reductions were observed for all bacteria. With a conservative approach, it can be said that *E. faecium* can be used as a surrogate when using vacuum steam pasteurization. However, on whole flaxseed, pasteurization at 75°C for 1 minute yielded a log reduction of 5.23 CFU/g for *E. faecium* similar to log reductions observed for *E. coli* O157:H7 and *Salmonella* PT 30. This supports that behavior of *E. faecium* is dependent upon the food treated and that they may show different inactivation pattern, mainly at lower pasteurization temperatures.

Microbial survival curves after vacuum steam pasteurization

Survival curves are generated from results of an inactivation study obtained after a stress exposure to bacteria, which explain survival or decline in the amount of bacteria under certain defined parameters leading to prediction of its inactivation or survival without the need of an experiment. Such models provide parameter estimates such as δ (first decimal reduction), *p*-value (shape of the curve) and *kmax* (inactivation rates), which can be used for risk estimation or for development of HACCP plans and evaluation of food safety processes during intervention steps (60, 92). The survival curves in this study were not of first order kinetics rather they had a shoulder and a tailing effect. We found that the Geeraerd-tail model provided better goodness of fit with lower RMSE values when compared to modified Weibull-model (12, 13). When $p = 1$, the curves are log linear in nature and when $N\_res \neq 0$, it shows a tailing effect. In this experiment, *p* and *N_res* values generated through use of modified Weibull model were observed to be greater than 1, showing that the curves were upward concave with a shoulder and a tailing effect (12, 93).

In my experiment, the predicted survival curves generated at 75 and 85°C yielded 4D values that were supportive of the log reductions observed at most inactivation conditions. δ value, which is the first decimal reduction, had a maximum value of 0.35 minutes among all survival curves generated in this study. Inactivation of *Salmonella* PT 30 on almonds yielded a log-linear reduction where the calculated D values were 2.6, 1.2, 0.75 and 0.39 minutes for temperatures of 60, 70, 80 and 88°C, respectively (94). D values of 0.2 and 0.3 minutes at 93°C for Salmonella on almonds after exposure to steam were determined (85). The δ values observed in my study were similar to the findings of Harris et al.(94), and Lee et al.(85), but after pasteurization at lower temperatures. D values obtained using Weibull model with peanut butter

at 71°C ranged from 6.44 to 965 minutes to achieve 1 to 7 log reductions, respectively (95), which is higher than those observed in this study. However, their inactivation study was conducted at a lower temperature and *Salmonella* in peanut butter has been shown to be highly resistant to thermal treatments. Applied cold atmospheric pressure plasma to inactivate *Salmonella* on whole black peppercorns yielded a log reduction of 3.2 CFU/g after 15 minutes (7). In their study, the Weibull model provided δ value of 0.13 minute and a *p* value of 0.25 for *Salmonella* enterica for an upward concave survival curve (7). The survival curves obtained in my experiment were upward concave with observed *p* values always greater than 0. It is also important to note that the tailing effect observed in this experiment may have been impacted due to the fact that lowest limit of detection was used when no colonies were detected mostly at higher treatment parameters. The fact that tailing occurs due to more resistant subpopulation *N-_res* might not be true in our case since counts below the limit of detection was often observed during longer pasteurization time of 3 to 5 minutes which were also used for generation of survival curves (12, 13).

Conclusions

Vacuum steam pasteurization, under the settings at which this experiment was conducted, was effective in obtaining greater than 5.0 log CFU/g reduction at low pasteurization temperatures of 75 and 85°C for *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium*. Hence, vacuum steam pasteurization can be used for inactivation of pathogens in low moisture foods. In addition, log reduction for *E. faecium* was lower than that of *Salmonella* PT 30 and *E. coli* O157:H7 and thus *E. faecium* can be used as a surrogate when using vacuum steam pasteurization. The survival curves with the predicted parameters from the Geeraerd-tail and

modified Weibull models obtained through this study can be used for quantitative risk assessments.

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EFFECT OF VACUUM STEAM PASTEURIZATION ON MICROBIAL SHELF LIFE AND WATER ACTIVITY OF WHOLE AND MILLED FLAXSEED

Abstract

Low moisture foods have a long shelf life because of their low water activity, which does not permit growth of bacteria, yeast or molds. However, bacteria, yeast and mold can survive on low moisture foods. The native microbiota can grow at favorable conditions such as when the low moisture foods are used as an ingredient in other foods. In addition, thermal processing of low moisture foods may change the water activity of the foods or lead to activation of spores. These effects can cause microbial growth leading to untimely spoilage of foods. Vacuum steam pasteurization uses steam under vacuum for inactivation of microorganisms. In this experiment, the objective was to determine if the pasteurization process increased or decreased bacteria, yeast and mold on pasteurized whole and milled flaxseed. Inactivation of *B. cereus* was also observed on milled flaxseed along with the effect of pasteurization on a_w of the products. The shelf life of pasteurized products was conducted for a period of 6 months for milled flaxseed and 9 months for whole flaxseed. It was observed that pasteurization yielded lower counts for total aerobes, yeast and mold for both of the products at all pasteurization conditions. Similarly, *B. cereus* was only observed over the period of a month on milled flaxseed. However, after pasteurization there was an increase in a_w, but the increased a_w was still below 0.65. Moreover, a_w of unpasteurized products was observed to be similar or greater than the pasteurized products after 2 months of storage for whole flaxseed and 1 month of storage for milled flaxseed. Hence, vacuum steam pasteurization was effective in reducing native microbiota without any negative effect on a_w.

Introduction

Consumption of seeds and grains are on the rise among consumers due to awareness and willingness to eat healthy. Flaxseed is one such commodity which has been shown to be beneficial to human health because its high omega-3 fatty acids, α-linolenic acid, high dietary fiber, anti-carcinogenic lignans, and high protein compounds (67, 96-98). Flaxseed has also been used as feed for cattle to enhance milk production with increased omega-3 fatty acid content (99). Similarly, when hens were fed flaxseed, an increase in quantity of omega-3 fatty acids was observed in their eggs and meat (99, 100). Flaxseed is consumed in its natural form or is used as an ingredient in other foods such as salads, soups, muffins, bread, macaroni, spaghetti, and energy snack bars (101-104).

Flaxseed is either consumed raw or is minimally processed. Flaxseed in its raw form may pose a risk to human health due to possible microbial contamination. There have been several outbreaks of *Salmonella* and *E. coli* O157:H7 in such low moisture foods (15-17). In the years 2014-2015, there were 3 recalls due to *Salmonella* contamination in flaxseed and 1 recall was also seen in February 2016 in flaxseed powder due to *Salmonella* (16). Cultivation, harvesting and processing conditions are prone to contamination due to microorganisms (3, 29, 105, 106). Hence, it is important to reduce these microorganisms in such foods before consumption. However, it has also been shown that pathogens in low moisture foods are more resistant to dry heat treatment. Therefore, it is important to eliminate such harmful pathogens from low moisture foods with alternative technologies. At the same time, it is important to investigate whether these treatment procedures have any impact on naturally occurring microbiota, which is responsible for spoilage of such low moisture foods, influencing their shelf life.

The shelf life of food products is dependent upon background microbiota in addition to other chemical properties. To monitor the microbial shelf life of low moisture seeds and grains, the total aerobic plate count (APC), yeast and mold, and *Bacillus cereus* are measured. In a study by Postollec et al, 86% of raw products (dried vegetables, spices, egg mix powder, texturing agents) were contaminated with *Bacillus* species (63).A higher number of these indicator organisms can shorten the shelf life of a product due to changes in its physical properties such as flavor and texture. *Bacillus cereus* produces rope like slimes in food products such as bread made with flour (107, 108). In addition, *B. cereus* forms endospores, which remain inactive in a nutrient deprived condition, but mild heat treatment and temperature abuse during storage of cooked food can result in spore germination leading to production of toxin and growth of *B. cereus* cells to a high level causing food poisoning (109, 110). The low water activity of foods such as whole and milled flaxseed limits the growth of vegetative bacteria, yeast and molds. However, an increase in moisture content can allow growth of vegetative bacteria, yeast and mold over its storage period (111, 112). Yeast and mold cause visible growth and contamination, making these foods unfit for consumption. In addition, an increase in moisture can cause rancidity with increase in peroxide values and free fatty acid contents (113, 114). This usually occurs when ingredients such as flour are formulated to be used in ready to eat food products like bread and snack bars. Hence, it is equally important to monitor the water activity of low moisture foods in order to monitor their shelf life.

Vacuum steam pasteurization as explained in previous pages utilizes steam under vacuum for inactivation of bacteria in low moisture foods. Here, the objective of this study was to determine if vacuum steam pasteurization increased shelf life of whole and milled flaxseed by

inactivation of naturally occurring microbes. Also, it was determined if the water activity of the product was influenced by pasteurization.

Materials and methods

Sources of flaxseed

Raw whole and milled flaxseed were received from three different local suppliers from Fargo area, and they were homogenized to obtain well mixed samples.

Pasteurization parameters and shelf life study period

For each flaxseed type, pasteurization experiments were conducted at 75, 90 and 105°C for 3 minutes and for 9 minutes at 90°C (Tables A5 and A6). The experiment was repeated three times at each pasteurization condition. Samples for the control were packaged and handled similarly as the treated samples except for the pasteurization step. Water activity (a_w) measurements and microbiological shelf life were conducted at week 0 (the day of pasteurization), week 1, week 2, week 4, week 6, week 8, and every month until 9 months. However, the microbial analyses and a_w measurements for milled flaxseed were limited to 6 months.

Vacuum steam pasteurization

Vacuum steam pasteurization on whole and milled flaxseed was conducted separately. Flaxseed was packed in portions of 100 g and 250 g into cotton bags (Uline Inc., Pleasant Prairie, WI). Fifteen small (100 g) and 2 large (250 g) bags were pasteurized at each selected temperature and time combinations. These bags were sandwiched halfway between fill volume of 45 and 22.5 kg in the sterilizer bin for whole and milled flaxseed, respectively (Table A6). Three data loggers (Madge Tech, Inc., Warner, NH) were used to measure the temperature readings during the pasteurization process. These data loggers were placed in three different

locations along with sample bags halfway in the sterilizer bin. Prior to placing samples for pasteurization, the fill volume was pre-heated to a temperature of 40 and 50°C for whole and milled flaxseed, respectively (Table A5). The pre-pasteurization temperature, initial vacuum, and post vacuum parameters were selected based on preliminary experiments to provide homogenous distribution of steam and temperature in the food matrix. However, once the pasteurization cycles started, the time required to reach the set pasteurization temperature from the pre-heat temperature lasted a few seconds to a maximum of 3 minutes (Table A6). Similarly, after pasteurization was completed, the time for reducing the temperature to the pre-heat temperature ranged from 10-15 minutes (Table A6). Usually, these pre-pasteurization and post-pasteurization times with their corresponding temperatures increased with higher set pasteurization temperatures (Table A6). The details of the vacuum steam pasteurization system mechanism were explained in previous pages for my first experiment. After pasteurization, the samples were left to cool to room temperature. Then the individual bags were emptied into Kraft bags (Uline Inc., Pleasant Prairie, WI) providing 15 small and 2 large bags for each week of data collection for each replicate. These bags were stored at room temperature in the lab for microbial enumeration and chemical shelf life determination (not presented here).

a_w measurement

Three bags each for unpasteurized and treated samples were opened at each week of the experiment. A_w was measured for 3 samples from each bag using a water activity meter (Aqua lab, Decagon devices Inc., Model series 3TE). Before measurement of a_w, the water activity meter was verified with two standards of a_w 0.760 and 0.250.

Enumeration of total aerobic bacteria, B. cereus, yeast, and mold

Three bags each for unpasteurized and pasteurized conditions were opened at each week of the experiment. Three samples from each bag were plated in duplicates onto Plate Count Agar-PCA (Difco, Becton Dickinson, Sparks, MD), Dichloran-Glycerol Agar –DG-18 (Oxoid LTD., Basingstoke, Hampshire, England) with Glycerol (Fisher chemical, New Jersey), and Mannitol Egg Yolk Polymyxin Agar MYP (Difco, Becton Dickinson, Sparks, MD) to count total aerobic bacteria (APC), yeast and mold and *B. cereus*, respectively. Samples (25 g) were weighed in sterile whirl Pak bags (Nasco, Fort Atkinson, WI) for each replicate and were homogenized with 225 g of Butterfield's dilution buffer for 90 seconds using a Masticator (IUL instruments, Spain). After homogenization, appropriate serial dilutions were spread on their respective agar plates. After spread plating, the PCA, DG-18, and MYP plates were incubated at 37°C for 48±2 hours, 25°C for 7 days and 30°C for 48±2 hours, respectively. After incubation, the colonies on these plates were counted, and the counts for *B. cereus* were considered presumptive counts since they were not confirmed using further tests.

Statistical Analyses

Pasteurization of each flaxseed type at each selected condition was conducted to obtain three biological replicates each with three technical replicates, providing nine data points for each condition including the unpasteurized control samples. Microbial counts were obtained in duplicates in CFU/g which were averaged and log transformed using Microsoft Excel. The limit of detection (1 log CFU/g) was used as the count for samples with no colony detected. Analysis of variance (ANOVA) was conducted using Proc GLM in SAS V.9.4 (SAS Institute, Cary, NC). For ANOVA, log CFU/g was considered as the dependent variable and pasteurization condition

and week were used as the independent variables. Based on LS-means adjusted for the significant interactions, adjusted p values were used.

Results

Shelf life of whole flaxseed

Total aerobic plate counts after pasteurization of whole flaxseed

Total APC for unpasteurized whole flaxseed averaged 5.37±0.17 log CFU/g which was reduced to average counts of 3.77±1.07, 3.04±1.17, 4.17±0.48 and 2.68±1.79 log CFU/g after pasteurization for 3 minutes at 75°C, 3 minutes at 90°C, 9 minutes at 90°C and 3 minutes at 105°C, respectively (Figure 7). The greatest and the least log reduction in APC were observed after pasteurization for 3 minutes at 105°C and 9 minutes at 90°C on week 0. The average APC after 3 minutes of pasteurization at 90 and 105°C were significantly lower than the APC for unpasteurized whole flaxseed (adj.p<0.05). During storage, the log counts for unpasteurized flaxseed remained similar over the period of 36 weeks (adj.p>0.05). The APC after pasteurization at 75°C for 3 minutes increased from week 0 to 5.29±0.25 log CFU/g by week 2, but steadily decreased to 3.79±1.23 log CFU/g at 36 weeks of storage, which was not significantly different from week 0 (adj.p>0.05) (Figure 7).

APC after pasteurization for 3 minutes at 90°C was observed to be greater than 3.0 CFU/g on average until week 6 but after that, APC decreased by an average count of 2.23±1.03 log CFU/g at week 36, not significantly different from week 0 (adj.p>0.05) (Figure 7). The APC after pasteurization for 9 minutes at 90°C was reduced by ~1.0 log CFU/g during week 8 to 36, but were not significantly different from week 0 (adj.p>0.05). Pasteurization for 3 minutes at 105°C observed decreases in APC over the storage periods with counts below limit of detection at week 20 and 24. However, APC on weeks 28, 32 and 36 were observed to increase providing

counts similar to the first two weeks (Figure 7). At week 36, only APC observed after pasteurization for 3 minutes at 90°C was significantly lower than APC for unpasteurized whole flaxseed (adj.p<0.05) (Figure 7).

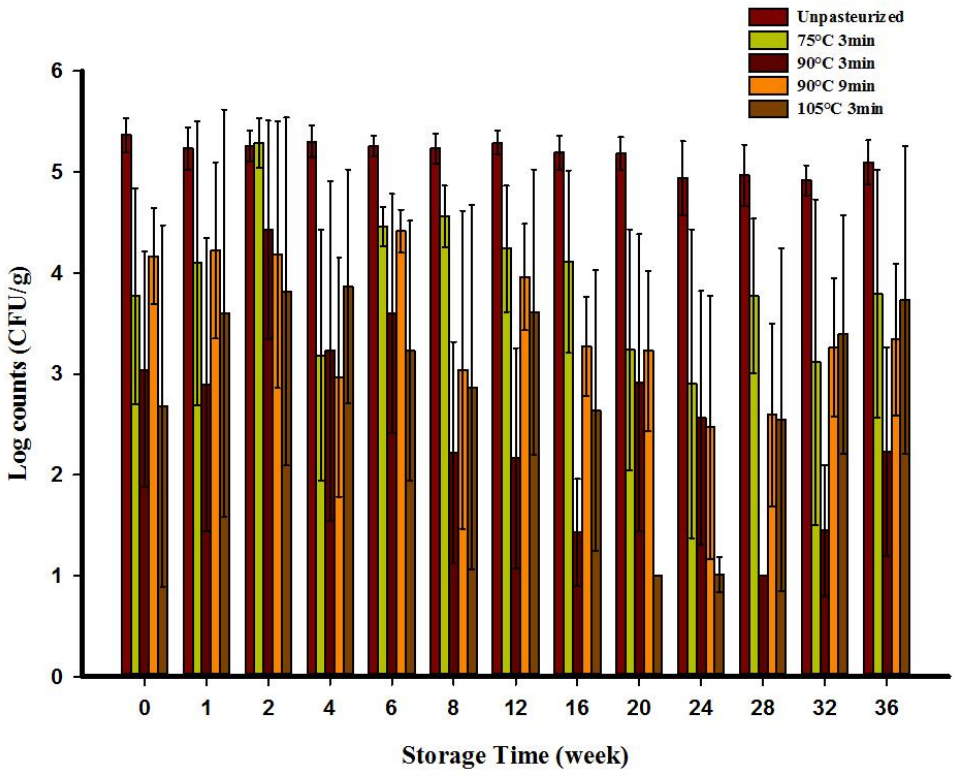

Figure 7. Total aerobic plate count on whole flaxseed during 36 weeks of storage for unpasteurized and pasteurized conditions. The counts are average of three samples at each replicate with error bars as standard deviation.

Total yeast counts after pasteurization of whole flaxseed

Average yeasts for unpasteurized whole flaxseed were 2.62±0.61 log CFU/g, which was similar to total yeasts after storage at week 36 (adj.p>0.05) (Figure 8). However, the number of yeast cells were observed to be as low as 1.48±0.35 CFU/g during week 24 for unpasteurized whole flaxseed. After pasteurization, total yeast cells of 1.73±0.92, 1.10±0.30, 1.00±0.00 and 1.41±0.68 log CFU/g was observed after 3 minutes at 75°C, 3 minutes at 90°C, 9 minutes at 90°C and 105°C, respectively. Pasteurization yielded significant decrease in total yeasts at

pasteurization temperatures of 90 and 105°C (adj.p<0.05) (Figure 8). However, total yeasts observed after pasteurization at all conditions were observed to be similar on week 0 (adj.p>0.05).

Total yeast cells after pasteurization at 75°C for 3 minutes increased over 2 week period but steadily decreased over time yielding counts that were not significantly different from week 0 (adj.p>0.05) (Figure 8). Similarly, after pasteurization for 3 minutes at 90°C, total yeast cells increased to ≥1.50 log CFU/g at 2, 4 and 6 weeks. However, total yeast cells steadily decreased over the remaining weeks of storage with counts observed below limit of detection during several weeks. Although, pasteurization after 9 minutes at 90°C and 3 minutes at 105°C yielded ~1.0 log CFU/g on week 0, total yeast cells as high as 1.64±0.98 log CFU/g was observed during the first month of storage, but from week 16-36 counts below limit of detection were often observed. At week 36, total yeast cells were enumerated to be 2.21±0.30, 1.21±0.35, 1.00±0.00, 1.09±0.18 and 1.00±0.00 log CFU/g for unpasteurized whole flaxseed, and pasteurization after 3 minutes at 75°C, 3 minutes at 90°C, 9 minutes at 90°C and 3 minutes at 105°C, respectively (Figure 8). The average total yeasts at week 36 was observed to be significantly greater for unpasteurized whole flaxseed than at any pasteurized conditions (adj.p<0.05) (Figure 8).

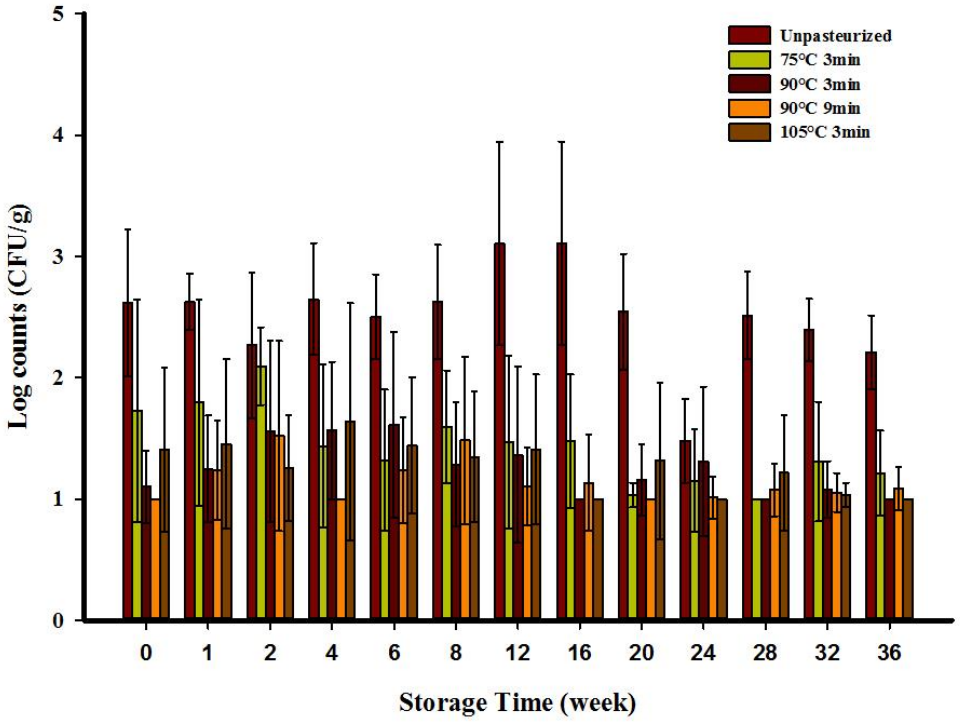

Figure 8. Total yeast on whole flaxseed during 36 weeks of storage for unpasteurized and pasteurized conditions. The counts are average of three samples at each replicate with error bars as standard deviation.

Total mold counts after pasteurization of whole flaxseed

Unpasteurized whole flaxseed had average initial mold levels of 2.09±0.64 log CFU/g, which increased to a maximum count of 2.50±0.32 log CFU/g at week 4 with the lowest count of 1.34±0.55 log CFU/g observed at week 24 (Figure 9). The observed levels of mold were similar at all weeks (adj.p>0.05) except at week 24 for unpasteurized whole flaxseed (adj.p<0.05). After pasteurization, similar total mold levels of 1.57±0.69 and 1.30±0.49 log CFU/g were observed after 3 minutes at 75°C and 9 minutes at 90°C, respectively on week 0 (adj.p>0.05) (Figure 9). Pasteurization for 3 minutes at 90 and 105°C provided similar total mold levels of 1.00±0.00 and 1.17±0.30, respectively (adj.p>0.05) (Figure 9). Total molds observed at all pasteurization

conditions except 3 minutes at 75°C were found to be significantly lower than the unpasteurized whole flaxseed (adj.p<0.05). The highest reduction of 1.09±0.64 log CFU/g was observed after pasteurization for 3 minutes at 90°C on week 0 (Figure 9).

After pasteurization for 3 minutes at 75°C, total number of molds increased to 2.23±0.31 on week 2 with gradual, but not significant, decline observed through 36 weeks of storage (adj.p>0.05). Pasteurization at 90°C irrespective of treatment time yielded total mold levels of ~1.0 log CFU/g during several weeks of the shelf life period. However, pasteurization at 105°C for 3 minutes provided mold counts as high as 1.60 log CFU/g during week 1, 4, 6, and 12, which were similar to the count of 1.47±0.39 CFU/g observed at week 36 (adj.p>0.05) (Figure 9). At week 36, mold counts were observed to be significantly lower after pasteurization at 75 and 90°C than unpasteurized whole flaxseed and pasteurization after 3 minutes at 105°C (adj.p<0.05) (Figure 9).

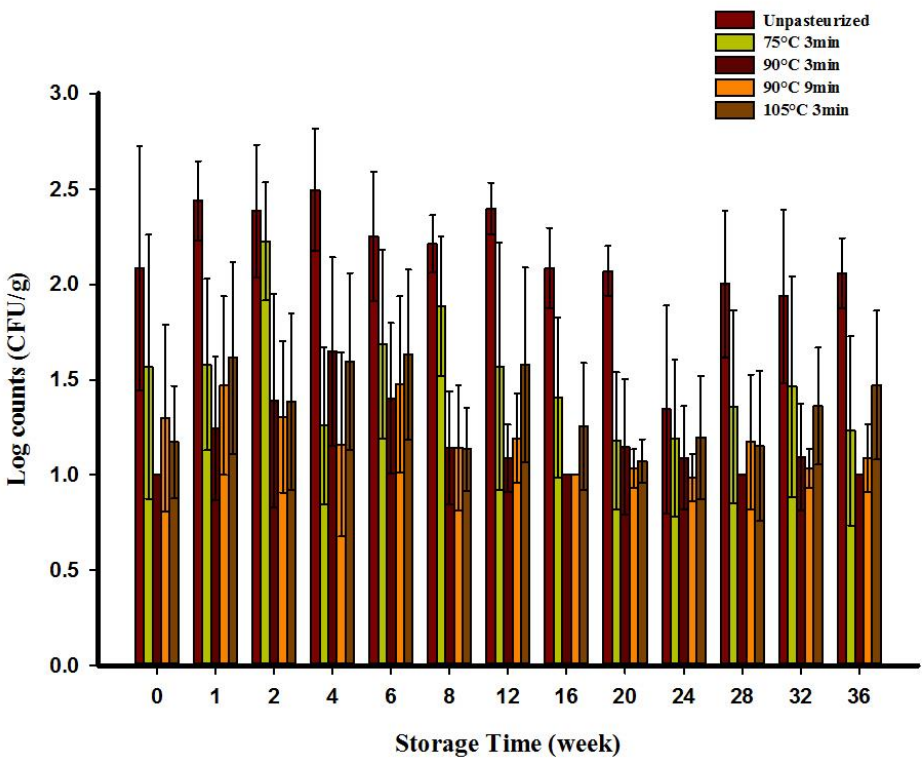

Figure 9. Total mold on whole flaxseed during 36 weeks of storage for unpasteurized and pasteurized conditions. The counts are average of three samples at each replicate with error bars as standard deviation.

a_w after pasteurization of whole flaxseed

The average a_w for unpasteurized whole flaxseed was measured to be 0.488±0.005, which increased to 0.544±0.028, 0.628±0.017, 0.625±0.021 and 0.634±0.028 after pasteurization for 3 minutes at 75°C, 3 minutes at 90°C, 9 minutes at 90°C and 3 minutes at 105°C, respectively (Figure 10). A_w of pasteurized flaxseed at all conditions except pasteurization for 3 minutes at 75°C were significantly greater than a_w of unpasteurized samples on week 0 (adj.p<0.05). Pasteurization for 3 minutes at 105°C yielded the highest increase in a_w, which was similar to a_w observed after pasteurization for 3 and 9 minutes at 90°C (adj.p>0.05).

a_w for unpasteurized flaxseed was measured to be similar until 8 weeks of storage (adj.p>0.05) and decreased significantly with increase in storage time (adj.p<0.05) (Figure 10).

However, a_w after pasteurization at all conditions significantly decreased on week 1 from week 0 (adj.p<0.05) and were found to be similar to a_w for unpasteurized whole flaxseed (adj.p>0.05) (Figure 10). A_w after pasteurization at 75°C for 3 minutes remained similar until week 6, except at week 1 (adj.p>0.05) and decreased significantly with increase in storage time (adj.p<0.05). At 36 weeks of storage, the a_w were measured to be 0.252±0.014, 0.228±0.006, 0.226±0.008, 0.231±0.016 and 0.231±0.008 for unpasteurized flaxseed, and after pasteurization for 3 minutes at 75°C, 3 minutes at 90°C, 9 minutes at 90°C and 3 minutes at 105°C, respectively, which were found to be similar (adj.p>0.05), but were significantly lower than the first week of measurements (adj.p<0.05) (Figure 10).

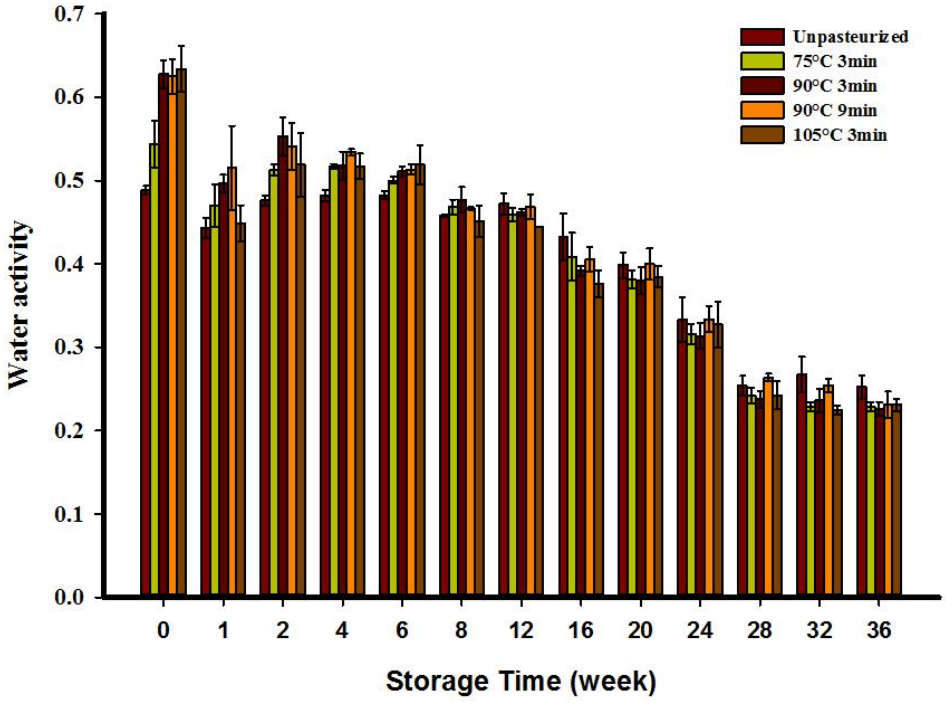

Figure 10. Measured water activity (a_w) for whole flaxseed during 36 weeks of storage for unpasteurized and pasteurized conditions. Measurements are average of one sample for each replicate with error bars as standard deviation.

Shelf life of milled flaxseed

Total aerobic plate counts after pasteurization of milled flaxseed

The average APC for unpasteurized milled flaxseed was observed to be 4.35±0.18 CFU/g (Figure 11). After pasteurization, average APC in milled flaxseed after 3 minutes at 75, 90 and 105°C were 4.38±0.30, 3.70±0.24 and 1.34±0.60 log CFU/g, respectively. Similarly, milled flaxseed pasteurization for 9 minutes at 90°C had an average APC of 2.75 ± 0.58 CFU/g (Figure 11). There was no reduction in APC after pasteurization at 75°C for 3 minutes and were similar to unpasteurized milled flaxseed (adj.p>0.05). However, pasteurization at 90°C for 3 and 9 minutes and at 105°C for 3 minutes yielded significantly lower APC than the unpasteurized milled flaxseed (adj.p<0.05).

Unpasteurized milled flaxseed had similar APC counts on week 0 and 1 but increased by ~1.0 log CFU/g at week 3. For unpasteurized flaxseed, the observed APC at week 2 was similar at all other weeks during the shelf life study (adj.p>0.05). APC for flaxseed after 3 minutes at 75°Cwas observed to be similar through week 24 (adj.p>0.05). After pasteurization for 3 minutes at 90°C, significant decrease in APC was observed at week 1 and 2 (adj.p<0.05) with similar counts observed through week 24 (adj.p>0.05) (Figure 11). Pasteurization for 9 minutes at 90°C resulted in gradual decrease of APC over time, with the lowest count of 1.86±0.40 CFU/g observed at week 6. However, pasteurization at 105°C for 3 minutes resulted in counts similar to ~1.0 log CFU/g from week 2 to 24 (adj.p>0.05) (Figure 11). At week 24, average APC of 5.49±0.27, 4.27±0.66, 3.03±0.41, 2.17±0.31 and 1.00±0.00 log CFU/g was observed for unpasteurized milled flaxseed, and after pasteurization for 3 minutes at 75°C, 3 minutes at 90°C, 9 minutes at 90°C and 3 minutes at 105°C, respectively (Figure 11). At week 24, APC for

unpasteurized milled flaxseed were observed to be significantly higher than at all pasteurized conditions (adj.p<0.05).

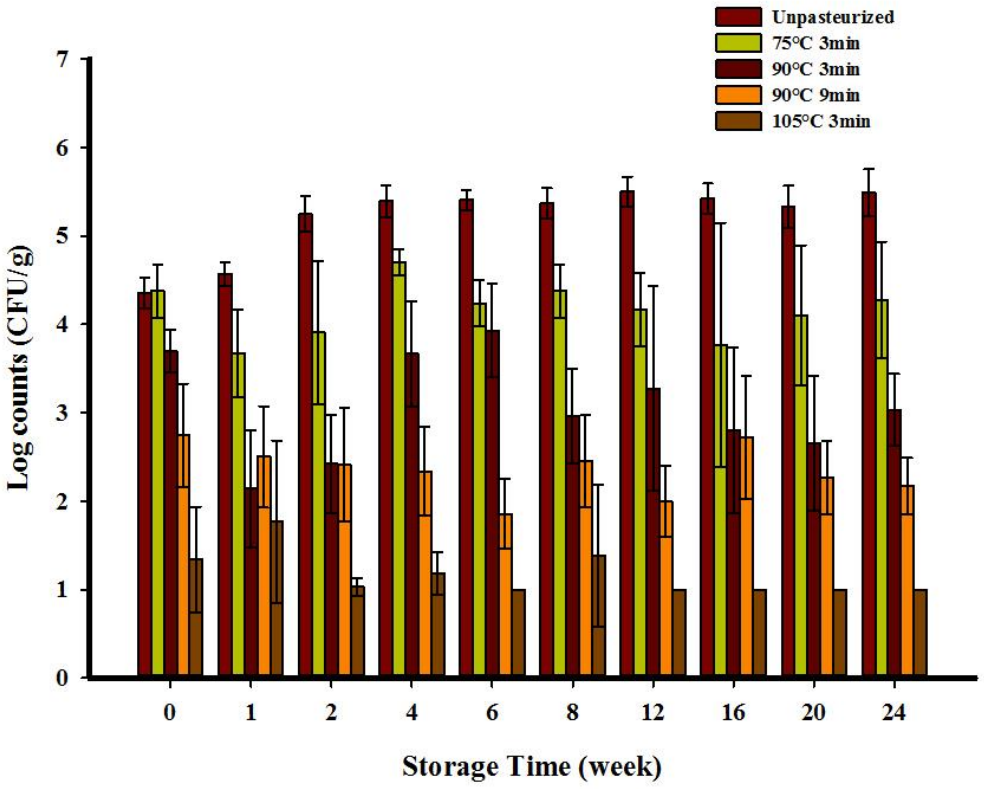

Figure 11. Total aerobic plate count on milled flaxseed during 24 weeks of storage for unpasteurized and pasteurized conditions. The counts are average of three samples at each replicate with error bars as standard deviation.

Total yeast counts after pasteurization of milled flaxseed

Total average yeast cells for unpasteurized milled flaxseed were 1.18±0.30 log CFU/g at week 0, which was similar to counts observed on weeks 1, 6, 8, 12, 16, 20 and 24 (adj.p>0.05) with the highest average count of 3.08 ± 0.43 log CFU/g observed on week 2 (adj.p<0.05) (Figure 12). No yeast colonies were observed on week 16, 20 and 24 for unpasteurized milled flaxseed. After pasteurization, no yeast colonies were detected on milled flaxseed at a limit of

detection of 1.0 log CFU/g throughout the entire shelf life study period except at week 6 for the pasteurization condition of 105°C, where a sample resulted in count of 3.05 log CFU/g (Figure 12).

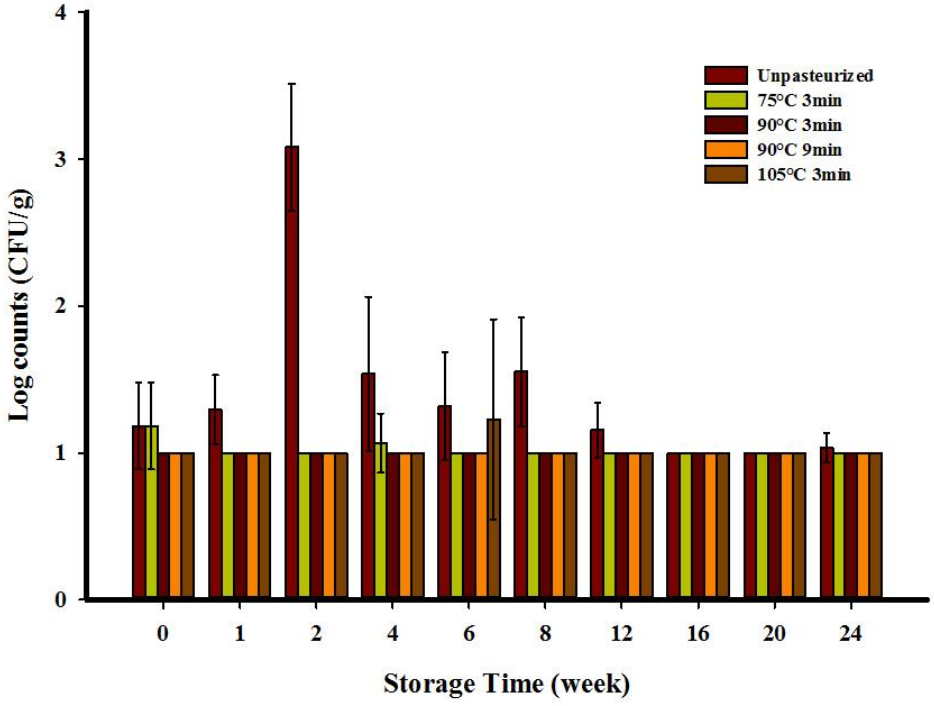

Figure 12. Total yeast count on milled flaxseed during 24 weeks of storage for unpasteurized and pasteurized conditions. The counts are average of three samples at each replicate with error bars as standard deviation.

Total mold counts after pasteurization of milled flaxseed

Total average mold count for unpasteurized flaxseed was 1.54±0.43 log CFU/g and remained similar over the entire storage period except at week 2 where the highest count of 2.15±0.44 log CFU/g was observed (Figure 13). After pasteurization for 3 minutes at 75°C, 3 minutes at 90°C, 9 minutes at 90°C and 3 minutes at 105°C, average mold count of 1.31±0.47, 1.10±0.31, 1.00± 0.00 and 1.03± 0.10 log CFU/g was observed, respectively (Figure 13). Mold levels observed after pasteurization for 9 minutes at 90°C and 3 minutes at 105°C were

significantly lower than unpasteurized milled flaxseed (adj.p<0.05). Total average mold counts after pasteurization for 3 minutes at 75°C were observed to be similar through week 24 (adj.p>0.05). Counts similar to limit of detection at all weeks through week 24 was observed after milled flaxseed pasteurization at 90 and 105°C (adj.p>0.05) (Figure 13).

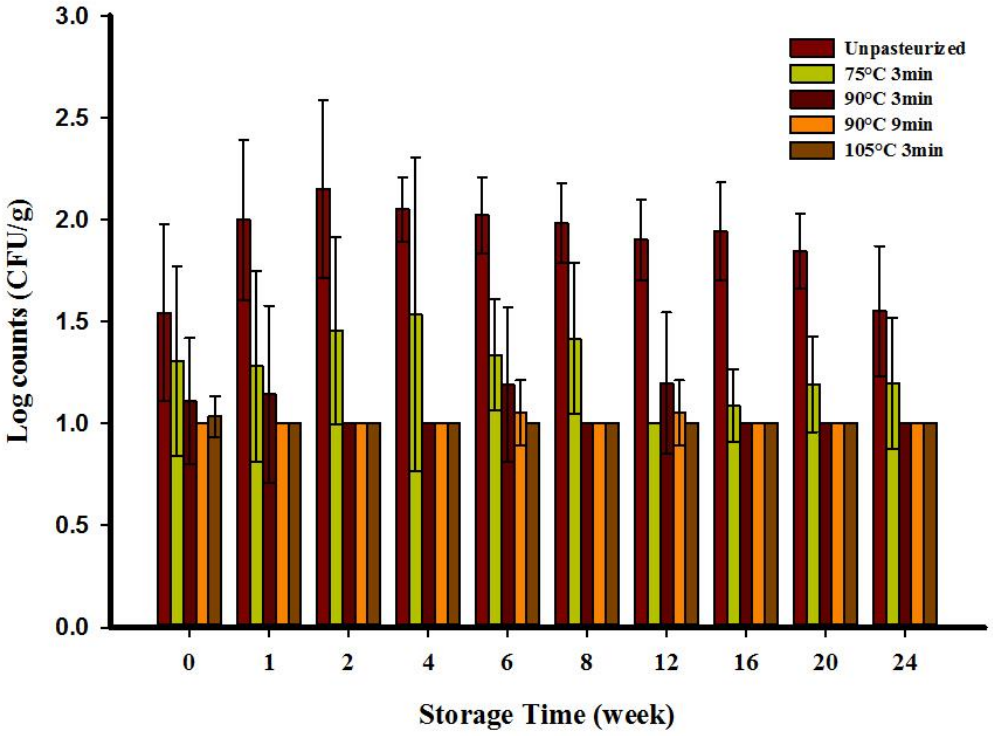

Figure 13. Total mold count on milled flaxseed during 24 weeks of storage for unpasteurized and pasteurized conditions. The counts are average of three samples at each replicate with error bars as standard deviation.

Total *B. cereus* counts after pasteurization of milled flaxseed

Presumptive *B. cereus* for unpasteurized milled flaxseed was observed to be 1.52±0.63 log CFU/g on average and increased to 1.95±0.37 CFU/g on week 1 (adj.p>0.05) (Figure 14). *B. cereus* for unpasteurized milled flaxseed on week 2 and 4 was similar to week 0 (adj.p>0.05), but were below the limit of detection on week 6 and 8. Pasteurization for 3 minutes at 75 and 90°C

yielded an average log count of 1.53±0.57 and 1.08±0.23 CFU/g on week 0. No *B. cereus* colonies were observed after pasteurization for 9 minutes at 90°C and 3 minutes at 105°C at the detection limit of 1.0 log CFU/g (Figure 14). However, *B. cereus* counts of 1.54±0.35, 1.46±0.31 and 1.40±0.53 log CFU/g were observed at week 1, 2 and 4 after pasteurization for 9 minutes at 90°C (Figure 14).

Over the next 2 weeks during storage, *B. cereus* counts of 1.75±0.42 and 1.45±0.44 log CFU/g were observed after pasteurization for 3 minutes at 75°C (adj.p<0.05) (Figure 14). Similarly, *B. cereus* counts of 1.97±0.31 and 1.79±0.60 log CFU/g were observed after pasteurization for 3 minutes at 90°C on week 1 and 2, respectively (adj.p<0.05). At week 0 and 4, *B. cereus* counts were not observed to be significantly different between unpasteurized and pasteurized milled flaxseed at any conditions (adj.p>0.05). No *B. cereus* colonies were observed below limit of detection at weeks 6 and 8 for unpasteurized and pasteurized milled flaxseed at all conditions (Figure 14).

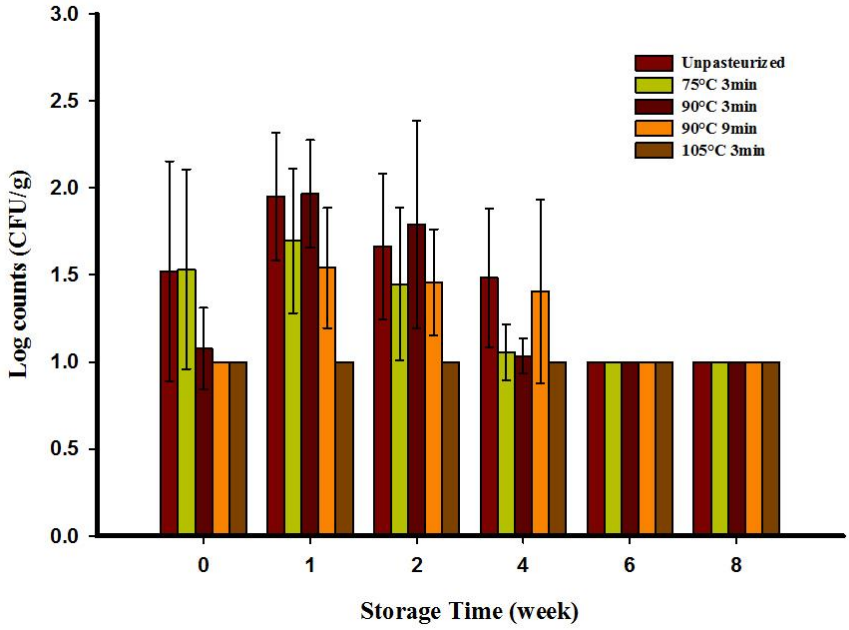

Figure 14. Total presumptive *Bacillus cereus* count on milled flaxseed during 8 weeks of storage for unpasteurized and pasteurized conditions. The counts are average of three samples at each replicate with error bars as standard deviation.

a_w after pasteurization of milled flaxseed

a_w for unpasteurized milled flaxseed was measured to be 0.512±0.002, and after pasteurization for 3 minutes at 75°C, 3 minutes at 90°C, 9 minutes at 90°C and 3 minutes at 105°C, a_w measurements of 0.497±0.029, 0.512±0.004, 557±0.007 and 0.578±0.017were observed, respectively (Figure 15). Significant increase in a_w was observed only after pasteurization at 105°C for 3 minutes (adj.p<0.05). However, no significant differences in a_w was observed after pasteurization for 3 minutes at 75 and 90°C (adj.p>0.05). Similarly, no significant difference in a_w was observed after 9 minutes at 90°C and 3 minutes at 105°C (adj.p>0.05).

a_w for unpasteurized milled flaxseed were similar during the first four weeks of storage (adj.p>0.05), but decreased steadily to a significantly low a_w of 0.252±0.014 at week

24(adj.p<0.05). Similarly, a_w after pasteurization at 75°C for 3 minutes were measured to be similar to unpasteurized milled flaxseed atall weeks (adj.p>0.05) except week 16where it was significantly lower (adj.p<0.05) (Figure 15). Pasteurization for 3 minutes at 90°C yielded similar a_w measurements to unpasteurized samples atall weeks (adj.p>0.05) except at weeks 16 and 20, where a_w were measured to be significantly lower than unpasteurized samples (adj.p<0.05) (Figure 15).

Pasteurization for 9 minutes at 90°C yielded similar a_w to unpasteurized milled flaxseed through week 20 (adj.p>0.05) with significantly lower $a_w$observed on week 24 (adj.p<0.05) (Figure 15). Similarly, pasteurization for 3 minutes at 105°C provided similar a_w measurements as unpasteurized milled flaxseed during weeks 1, 2, 4, 6, 8, 12, and 24 (adj.p>0.05) with significantly lower a_w measured during weeks 16 and 20 (adj.p<0.05). At week 24, a_w for unpasteurized milled flaxseed were not significantly different from a_w measured at any pasteurized conditions (adj.p>0.05) (Figure 15).

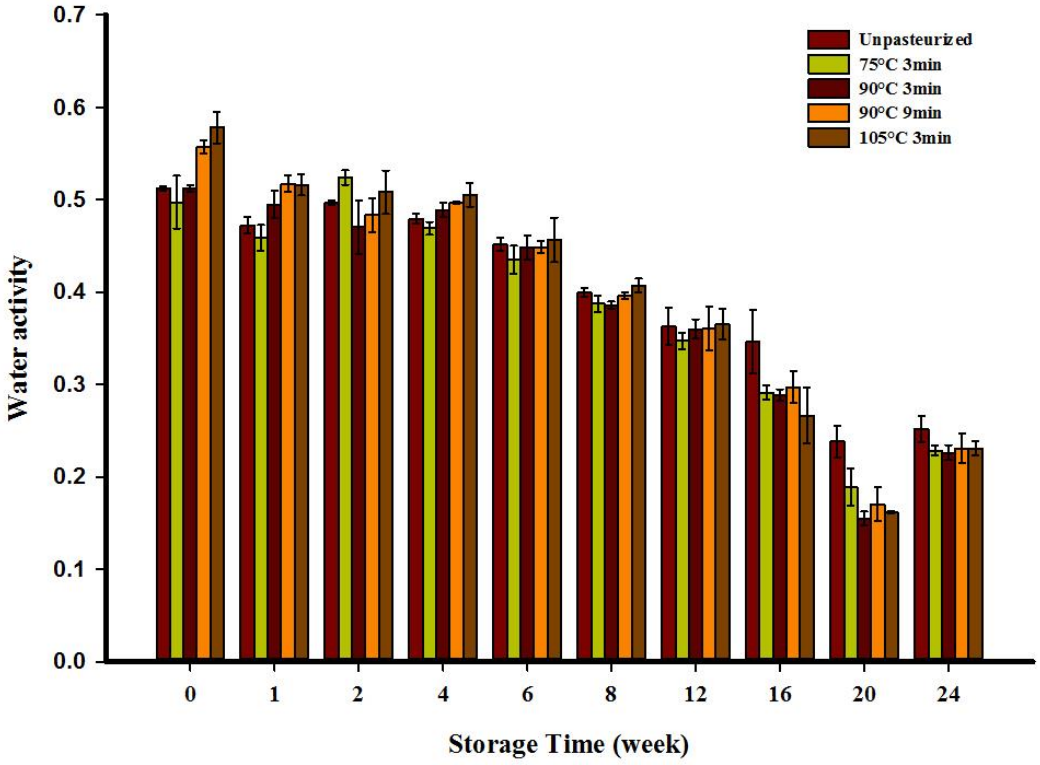

Figure 15. Water activity (a_w) for milled flaxseed during 24 weeks of storage for unpasteurized and pasteurized conditions. Measurements are average of one sample for each replicate with error bars as standard deviation.

Discussion

Factors affecting shelf life

The shelf life of a product is dependent upon biochemical and microbiological deterioration (115). However, microbial growth is limited by low a_w, so spoilage rates due to microbial activity is low in low moisture food products. Also, spoilage due to microorganisms can be reduced by inactivation methods such as heat treatment or irradiation. In cereal grains and flour, heat treatments at higher temperatures may induce enzyme susceptibility and decrease swelling power and paste consistency (65, 66). While irradiation is effective, consumers' perception regarding usage of irradiation has been negative. Therefore, an alternative

pasteurization process needs to be investigated to inactivate native microorganisms at lower treatment temperatures. However, any pasteurization techniques may affect the intrinsic factors that can lead to food spoilage. Intrinsic factors are the physical, chemical and structural properties of food which are defined by a_w, pH, redox potential, and available nutrients and antimicrobial substances present in them. The extrinsic factors are storage environment conditions, which include temperature and humidity.

In this experiment, microbial shelf life was monitored as affected by a_w and room temperature storage conditions after vacuum steam pasteurization at four different time and temperature conditions. Microbiological spoilage occurs in a food matrix usually when growth occurs leading to high numbers in the ranges of $10^7 – 10^9$CFU/g (11). In this experiment, the total aerobic counts observed in non-pasteurized whole and milled flaxseed were in the ranges of 10^4-10^5CFU/g, and yeast, mold and *B. cereus* were observed to be less than 1,000 CFU/g. A study conducted in 1989 profiled total aerobes, yeast and molds in milled wheat flour. It was observed that the average total aerobes, yeasts and molds were 4.17±0.52, 2.12±0.40 and 2.90±0.52 log CFU/g, respectively, similar to the counts observed for unpasteurized whole and milled flaxseed in this study. A survey conducted from 2003-2005 on whole wheat flour found similar counts of 4.41±1.15, 1.79±0.93 and 2.58±0.81 CFU/g for total aerobes, yeasts and molds, respectively (116). Also, a_w of the unpasteurized products were measured to be ~0.4-0.6. The low microbial counts may not cause spoilage at such a low a_w over a long duration of storage. However, when such products are used to prepare other foods such as bread, cake mixes, and spaghetti, their intrinsic factors such as moisture content and nutrient availability, and extrinsic factors, such as storage temperature, may lead to an increase in a_w and subsequent growth of the microbiota, shortening the shelf life of these products. After vacuum steam pasteurization,

decrease in total aerobes by 1-3 log CFU/g were observed at pasteurization temperatures of 90 and 105°C.

Effect of yeast and mold on shelf life

Fungi is most responsible for bread spoilage, and mycobiota belonging to the genera *Penicillium, Aspergillus, Monilia, Mucor, Endomyces, Cladosporium, Fusarium* and*Rhizopus* are studied as common spoilage fungi (117, 118). Fungi not only cause spoilage but also produce mycotoxins, which can cause health issues (119). In this study, vacuum steam pasteurization was implemented to investigate if the pasteurization led to increase or decrease in native mycobiota present in these two foods (120, 121). Vacuum steam pasteurization yielded ~1.0 log CFU/g reduction for yeasts and molds with counts observed below the limit of detection after pasteurization.

Several other inactivation and packaging methods have been implemented to prevent mold growth in bread. Dababneh et al.(122), reported that 2450 MHz microwave oven at 900 Watt inactivated fungi and thermophilic spore former on several spices. In their study, the fungi and thermophilic spore former counts for black pepper were approximately 4.0 and 3.0 log CFU/g and after inactivation over the storage periods of 60 days there was 1.0 log reduction in fungi reaching below limit of detection for thermophilic spore former. Similarly, for oats fungi and thermophilic spore former counts were approximately 4.0 and 3.0 log CFU/g, and after inactivation over the storage period of 60 days, there was 1.0 log reduction in fungi reaching below limit of detection for thermophilic spore former. Ozonization with 0.33 mg of ozone per gram of wheat after 1 minute of treatment reduced 96.9% of fungal spores (123). Similarly, 0.16 mg of ozone applied per gram of barley per minute was able to inactivate 96% of fungal inactivation (124). Modified atmosphere packaging, and irradiation have also been studied. In

addition, natural antimicrobial agents have also been investigated to a great deal for the purpose of usage in bread to enhance their shelf life. Propionic acid is one major chemical used to inhibit fungal growth.

Effect of B. cereus on shelf life

Endospores are often present in soil in the numbers of 10^4CFU/g and contamination can lead to their presence in foods (125). During storage or after pasteurization, such spores may be activated, provided that favorable conditions exist, causing increase in their numbers. *B. cereus* is an opportunistic pathogen and its major toxins are cytotoxins haemolysin BL (Hbl), nonhaemolytic enterotoxin (Nhe) and cytotoxin K (CytK) (126-128). The infectious dose of *B. cereus* has been determined as $10^5 – 10^8$ cells or spores. However, doses as low as 200 CFU/g have been associated in a foodborne outbreak due to *B. cereus*. After vacuum steam pasteurization of whole and milled flaxseed, decreases in *B. cereus* cells by ~0.5-1 log CFU/g were observed at all treated conditions when compared to unpasteurized whole and milled flaxseed. Interestingly, *B. cereus* cells were below limit of detection just after a month of storage. Another study for inactivation of *B. subtillis* spores by infrared radiation provided D values of 26 and 1.1 minutes at 120 and 140°C, respectively (129). A study by Molin et al., on ground pistachios yielded a 1.5 log CFU/g reduction in *B. cereus* after treatment at 0.1 and 0.5 ppm ozone for 360 minutes (130). In this study, vacuum steam pasteurization was able to achieve similar log reductions at temperatures as low at 75 and 90°C.

Effect of a_w on shelf life

Pasteurization processes often change the intrinsic factors of the pasteurized foods because of an increase in moisture content. Increase in a_w may cause negative impact on the shelf life of the pasteurized product. Increase in moisture content and change in sensory properties has

been reported in many studies (9, 71, 84). Here, a_w was observed to increase after vacuum steam pasteurization of whole and milled flaxseed after pasteurization at higher temperatures, but decreased over time with no significant differences between unpasteurized and pasteurized flaxseed. However, the increased a_w were still below 0.65 which inhibits microbial growth, including most yeasts and molds. It was also shown that, for milled wheat flour, a_w decreases during storage (116); similar observations were made in this study for whole and milled flaxseed.

Conclusions

Vacuum steam pasteurization is effective in reducing naturally occurring microbiota in low moisture foods, such as whole and milled flaxseed. In addition, vacuum steam pasteurization does not lead to activation of spores as observed by decreased microbal counts at all pasteurization temperatures. Although water activity increased after pasteurization, it was observed to be below 0.65. Moreover, a_w decreased after a week of storage, and were found to be similar to unpasteurized flaxseed after a month. In conclusion, vacuum steam pasteurization was not found to have any negative impact on microbial shelf life and water activity.

Acknowledgements

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CONCLUSIONS

Vacuum steam pasteurization is an effective method to reduce harmful pathogens in low moisture foods, making them safer for consumption. Vacuum steam pasteurization achieved a 5.0 log reduction for both pathogens *Salmonella* PT 30 and *Escherichia coli* O157:H7 at pasteurization temperatures as low as 75 and 85°C on all the tested food matrices, whole flaxseed, quinoa, sunflower kernels, milled flaxseed and whole black peppercorns, after treatment for as little as 1 minute. During pasteurization at lower treatment temperatures, *E. coli* O157:H7 and *E. faecium* yielded the most and the least amount of log reductions, respectively. At higher treatment temperatures, there were no significant differences in log reductions observed between *Salmonella* PT 30, *E. coli* O157:H7 and *E. faecium*. Therefore, *E. faecium* can be used as a surrogate for both pathogens when using vacuum steam pasteurization. However, there were variability in log reductions observed between replicates of experiments on quinoa at lower treatment temperatures. Therefore, inactivation of pathogens and usage of *E. faecium* as a surrogate needs to be considered carefully at lower treatment temperature, which was found to be dependent upon food matrices pasteurized. Moreover, the predicted survival curves that provided δ and *Kmax* values can be used as guidance for quantitative microbial risk assessment when utilizing vacuum steam pasteurization.

Vacuum steam pasteurization of raw whole and milled flaxseed yielded lower counts of native microbiota, such as total aerobes, yeasts, molds and *Bacillus cereus*. Higher log reductions were observed with increase in pasteurization temperatures, but not necessarily in pasteurization time. In addition, the increased a_w after pasteurization was observed to be below 0.65, which limits the growth of most bacteria and fungi. Also, similar a_w measurements were observed after

storage for a week. Hence, it can be seen that vacuum steam pasteurization had a positive effect on shelf life of the pasteurized products.

In conclusion, vacuum steam pasteurization is effective at reduction of pathogens on low moisture foods while maintaining product quality. Hence, this technology can be efficiently used by the food industry to pasteurize low moisture products. *Enterococcus faecium* can also be used as a surrogate for *Salmonella* and *E. coli* O157:H7 when validating their vacuum steam pasteurization system. In addition, the parameters obtained through modelling can be used for predictive microbiology. Overall, the results of this study establish vacuum steam pasteurization as an effective processing tool.

FUTURE STUDIES

This research study showed that vacuum steam pasteurization is effective at inactivation of *Salmonella* and *E. coli* O157:H7 on low moisture foods without any negative impact on microbial shelf life and water activity. Therefore, vacuum steam pasteurization can be considered as an alternative pasteurization method to other methods such as heat and irradiation. Further investigation on inactivation of pathogens on nuts and other seeds will showcase its inactivation potential to a greater extent. In addition, scale up for pasteurization of large quantities of low moisture food commodities using vacuum steam pasteurization needs to be investigated. The food industry can use the results obtained through this study as a guidance to select appropriate pasteurization parameters for treatment of low moisture foods. The industry should also be able to use *E. faecium* as a surrogate for *Salmonella* and *E. coli* O157:H7. Studies have shown that reduction in pathogens is dependent upon several factors such as initial inoculum levels, a_w of products, and strains of inoculated pathogens. Therefore, more studies are needed to understand the inactivation of pathogens as affected by inoculum levels, a_w levels and different strains of pathogens. In addition, it would be beneficial to understand the underlying mechanisms for the survival of the pathogens during vacuum steam pasteurization. Overall, these studies would provide enhanced knowledge on inactivation of pathogens when using vacuum steam pasteurization.

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APPENDIX. TABLES AND FIGURES

Table A1. Observed bacterial counts after inoculation and storage for three days

Product Type	Bacteria	Day 0 (CFU/g)[a]	Day 1 (CFU/g)[a]	Day2 (CFU/g)[a]	Day 3 (CFU/g)[a]
flaxseed whole	*Salmonella*PT 30	8.20 ± 0.2	8.04 ± 0.1	7.69 ± 0.3	7.77 ± 0.2
	E. coli O157:H7	7.36 ± 0.1	6.64 ± 0.1	6.93 ± 0.1	6.73 ± 0.0
	E. faecium	6.60 ± 0.1	6.32 ± 0.1	6.41 ± 0.2	6.50 ± 0.1
quinoa	*Salmonella*PT 30	8.39 ± 0.1	8.05 ± 0.1	8.04 ± 0.1	7.98 ± 0.2
	E. coli O157:H7	8.06 ± 0.1	7.98 ± 0.1	7.86 ± 0.1	7.88 ± 0.1
	E. faecium	6.96± 0.0	6.83 ± 0.1	7.11 ± 0.3	6.80 ± 0.1
sunflower kernel	*Salmonella*PT 30	8.40 ± 0.0	8.33 ± 0.0	8.36 ± 0.1	8.40 ± 0.0
	E. coli O157:H7	8.26 ± 0.2	7.89 ± 0.1	7.96 ± 0.1	7.83 ± 0.1
	E. faecium	7.96 ± 0.0	7.80 ± 0.2	7.91 ± 0.1	7.90 ± 0.1
milled flaxseed	*Salmonella*PT 30	7.56 ± 0.0	7.62 ± 0.1	7.60 ± 0.1	7.48 ± 0.1
black peppercorns	*Salmonella*PT 30	7.71 ± 0.1	7.43± 0.1	7.39 ± 0.1	7.42 ± 0.1

[a]denotes average counts with standard deviation for each bacteria at each day

Table A2. Heat resistance test on food matrices after a_w equilibration provided <2.5 log CFU/g reduction after treatment at 280°F for 15 minutes

Product Type	Bacteria	Log reduction (CFU/g)[a]
whole flaxseed	*Salmonella*PT 30	0.93 ± 0.34
	E. coli O157:H7	2.49 ± 0.40
	E. faecium	0.89 ± 0.20
quinoa	*Salmonella*PT 30	1.22 ± 0.22
	E. coli O157:H7	2.21 ± 0.12
	E. faecium	1.02 ± 0.04
sunflower kernels	*Salmonella*PT 30	0.85 ± 0.09
	E. coli O157:H7	1.79 ± 0.17
	E. faecium	0.59 ± 0.13
milled flaxseed	*Salmonella*PT 30	1.33 ± 0.20
black peppercorns	*Salmonella*PT 30	2.0 ± 0.35

[a] denotes average log reduction with standard deviation for each bacteria for each type of food

Table A3. Vacuum steam pasteurization parameters set during the inactivation experiments

Product	Set parameters											
	Fill volume (kg)	Pre-heat temp (°C).	Initial Vacuum	Initial Vacuum Time (min)	No. of Pre-vacuums	Pre-vacuum Pressure-1 Step 1(bar)	Pre-vacuum Pressure-1 Step 2(bar)	Pre-vacuum Pressure -2 Step 1(bar)	Pre-vacuum Pressure -2 Step 2(bar)	Pasteurization temp (°C)	Post-vacuum Time (min)	Post-vacuum Pressure (bar)
whole flaxseed	45	40	-0.9		0	-	-	-	-	75		-0.8
										85		-0.8
										95		-0.8
										105		-0.8
quinoa	45	40	-0.9		0	-	-	-	-	75		-0.8
										85		-0.8
										95		-0.8
										105		-0.8
sunflower kernel	45	60	-0.91	2	1	-0.62	-0.8	-	-	75	10	-0.85
						-0.4	-0.8			85		-0.85
						-0.15	-0.8			95		-0.85
						0.2	-0.8			105		-0.85
milled flaxseed	22.5	50	-0.91		2	-0.62	-0.9	-0.62	-0.9	75		-0.85
						-0.4	-0.9	-0.4	-0.9	85		-0.85
						-0.15	-0.8	-0.15	-0.8	95		-0.85
						0.2	-0.8	0.2	-0.8	105		-0.85
black peppercorn	22.5	40	-0.89		1	-0.62	-0.88	-	-	75		-0.8
						-0.45	-0.8			85		-0.8
						-0.15	-0.6			95		-0.85
						0.2	-0.4			105		-0.85

Table A4. Vacuum steam pasteurization temperatures and times measured during the inactivation experiments

Product	Set Past-eurizationtemp(°C)[a]	Measured parameters				
		Pre-Past-eurization time (min)[b]	Pre-Past-eurization temp °C[c]	Past-eurization temperature °C[d]	Post-Past-eurization time (min)[e]	Post-Past-eurization temperature °C[f]
Whole flaxseed	75	0.40 ± 0.3	63.13 ± 4.8	76.68 ± 1.8	11.71 ± 4.5	50.31 ± 4.7
	85	0.78 ± 0.5	70.55 ± 6.1	86.21 ± 1.7	12.03 ± 3.1	53.04 ± 6.4
	95	0.64 ± 0.5	76.01 ± 9.5	96.66 ± 2.4	14.86 ± 1.6	49.82 ± 2.2
	105	0.55 ± 0.3	85.22 ± 6.5	105.47 ± 2.2	13.93 ± 2.3	52.25 ± 1.1
quinoa	75	1.04 ± 1.2	58.77 ± 8.9	75.39 ± 2.6	12.55 ± 1.1	49.46 ± 1.5
	85	2.20 ± 2.9	56.36 ± 12.8	85.68 ± 1.0	14.96 ± 5.9	51.49 ± 2.1
	95	1.16 ± 1.0	66.54 ± 8.9	97.57 ± 2.1	14.04 ± 1.2	55.55 ± 1.0
	105	0.83 ± 0.4	89.07 ± 6.5	106.64 ± 1.4	15.16 ± 2.2	58.90 ± 4.7
sunflower kernel	75	0.11 ± 0.0	68.71 ± 1.2	75.10 ± 2.1	11.29 ± 2.7	64.65 ± 1.1
	85	0.76 ± 0.4	73.28 ± 3.0	85.32 ± 1.7	12.91 ± 3.0	69.40 ± 3.0
	95	1.29 ± 0.7	79.59 ± 3.9	95.21 ± 0.6	13.88 ± 1.0	72.97 ± 1.4
	105	1.68 ± 0.3	85.97 ± 3.9	106.84 ± 1.3	13.15 ± 3.0	75.86 ± 2.7
milled flaxseed	75	2.10 ± 1.7	62.03 ± 6.6	81.67 ± 5.5	10.80 ± 5.0	59.98 ± 2.3
	85	2.97 ± 3.4	64.57 ± 6.0	86.47 ± 2.6	13.76 ± 1.1	61.09 ± 2.5
	95	1.85 ± 1.0	73.14 ± 8.3	97.15 ± 1.3	13.34 ± 1.8	62.02 ± 4.6
	105	2.60 ± 1.0	74.61 ± 9.6	107.88 ± 0.9	12.94 ± 3.8	65.16 ± 3.1
black peppercorn	75	2.61 ± 2.3	61.38 ± 4.3	73.30 ± 1.3	12.67 ± 0.9	54.29 ± 2.6
	85	1.93 ± 1.4	71.70 ± 6.3	83.68 ± 3.3	12.85 ± 0.6	56.55 ± 2.3
	95	1.57 ± 1.1	78.52 ± 8.9	95.00 ± 1.4	13.38 ± 0.4	60.46 ± 3.0
	105	1.74 ± 0.3	83.61 ± 1.3	108.67 ± 1.7	12.99 ± 2.6	64.60 ± 1.6

a. Set-pasteurization temperature is the selected temperature for pasteurization experiments
b. Pre-pasteurization time is the average time with standard deviation taken to reach from pre-heat temperature to set pasteurization temperature
c. Pre-pasteurization temperature is average temperature with standard deviation during pre-pasteurization time
d. Pasteurization temperature is average temperature with standard deviation that was observed after reaching set pasteurization temperature for the amount of set pasteurization time.
e. Post-pasteurization time is average time with standard deviation required for pasteurization temperature to lower to pre-pasteurization temperature
f. Post-pasteurization temperature is average temperature with standard deviation during post-pasteurization time

Table A5. Vacuum steam pasteurization conditions set during pasteurization of whole and milled flaxseed

	Set parameters											
Product	Fill volume (Kg)	Pre-heat temp (°C)	Initial Vacuum (bar)	Initial Vacuum Time (min)	No. of Pre-vacuums	Pre-vacuum Pressure 1 Step 1 (bar)	Pre-vacuum Pressure 1 Step 2 (bar)	Pre-vacuum Pressure 2 Step 1 (bar)	Pre-vacuum Pressure 2 Step 2 (bar)	Past-eurization temp (°C)	Post-vacuum Time (min)	Post-vacuum Pressure (bar)
Whole flaxseed	45	40	-0.9	2	0	-	-	-	-	75	10	-0.8
										90		-0.8
										105		-0.8
Milled flaxseed	22.5	50	-0.91		2	-0.62	-0.9	-0.62	-0.9	75		-0.85
						-0.4	-0.9	-0.3	-0.8	90		-0.85
						0.2	-0.8	0.2	-0.8	105		-0.85

Table A6. Parameters observed during pasteurization for the shelf life study

Product	Set pasteurization condition (temp/time)[a]	Measured parameters				
		Pre-pasteurization time (min)[b]	Pre-pasteurization temperature (°C)[c]	Pasteurization temperature (°C)[d]	Post-pasteurization time (min)[e]	Post-pasteurization temperature (°C)[f]
Whole flaxseed	75°C/3min	0.26 ± 0.3	59.14 ± 26.6	74.36 ± 3.0	15.58±	48.88 ± 12.0
	90°C/3min	1.62 ± 2.2	63.31 ± 26.4	90.56 ± 2.2	14.36 ± 0.9	56.41 ± 19.2
	90°C/9min	2.09 ± 1.7	56.47 ± 18.4	89.61 ± 7.3	14.03 ± 1.1	55.09 ± 17.9
	105°C/3min	0.63 ± 0.5	80.21 ± 40.2	101.95 ± 3.0	14.84 ± 0.7	58.04 ± 24.2
Milled flaxseed	75°C/3min	0.86 ± 0.1	58.60 ± 13.0	75.85 ± 3.4	13.21 ± 1.0	59.62 ± 12.1
	90°C/3min	0.66 ± 0.2	75.85 ± 17.3	92.88 ± 2.5	13.52 ±0.7	59.91 ± 15.8
	90°C/9min	0.54 ± 0.0	75.76 ± 16.9	91.00 ± 4.0	13.92 ± 0.7	58.71 ± 16.1
	105°C/3min	2.34 ± 0.9	82.20 ± 26.0	108.51 ± 1.8	13.81 ± 0.3	64.47 ± 21.6

a. Set-pasteurization temperature is the selected temperature for pasteurization experiments
b. Pre-pasteurization time is the average time with standard deviation taken to reach from pre-heat temperature to set pasteurization temperature
c. Pre-pasteurization temperature is average temperature with standard deviation during pre-pasteurization time
d. Pasteurization temperature is average temperature with standard deviation that was observed after reaching set pasteurization temperature for the amount of set pasteurization time.
e. Post-pasteurization time is average time with standard deviation required for pasteurization temperature to lower to pre-pasteurization temperature
f. Post-pasteurization temperature is average temperature with standard deviation during post-pasteurization time

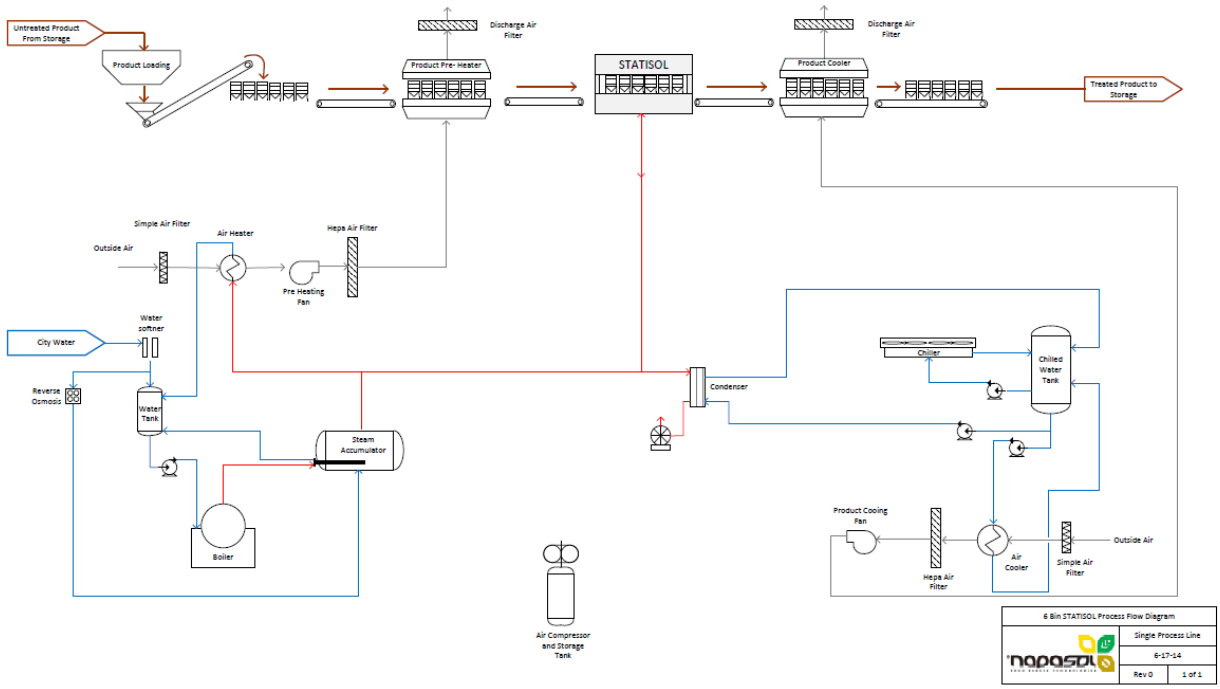

Figure A1. Schematic diagram showing details of vacuum steam pasteurization system used for the inactivation and shelf life study (source: Napasol North America, Fargo, ND)