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Validation of Extrusion Processing for the Safety of Low-Moisture Foods

Tushar Verma
University of Nebraska-Lincoln, Tushar.verma@huskers.unl.edu

Validation of Extrusion Processing for the Safety of Low-Moisture Foods

by

Tushar Verma

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Tushar Verma, M.S.

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Advisors: Jeyamkondan Subbiah & Andreia Bianchini

Salmonella in low-moisture foods is an emerging challenge due to numerous food product recalls and foodborne illness outbreaks. The new Food Safety Modernization Act requires the food processors to validate their process controls that indeed kills the desired number of bacteria. This research had two major objectives: 1) Develop a response surface model for *Salmonella* inactivation during the extrusion of low-moisture food 2) Evaluate the use of *Enterococcus faecium* NRRL B-2354 as an adequate surrogate for *Salmonella* during the extrusion of low-moisture food. Oat flour was selected as a low-moisture food model. For inoculation, cocktail of five different strains of *Salmonella* was used and *Enterococcus faecium* NRRL B-2354 was used as a potential surrogate for *Salmonella*. The inoculated samples were adjusted to different moisture (14 to 26%) and fat levels (5 to 15%) that were then extruded in a single-screw extruder running at different temperatures (65 to 85˚C for *Salmonella*; 75 to 95˚C for *E. faecium*) and screw speeds (75 to 225 rpm). A split-plot central composite 2^{nd} order response surface design was used, with central points replicated six times. Temperature showed the highest significant effect on *Salmonella* ($p < 0.0001$) and *E. faecium* ($p < 0.0001$) reduction. Moisture content showed a significant quadratic effect on *Salmonella* ($p = 0.0005$)

reduction and linear and quadratic effect on *E. faecium* ($p = 0.0002$) reduction. Fat content showed a significant protective effect on *Salmonella* ($p < 0.0001$) reduction, whereas fat content had a significant interactive effect with moisture on *E. faecium* ($p < 0.0001$) reduction. The screw speed had a significant interactive effect with the temperature on *Salmonella* ($p = 0.0004$) reduction, whereas it had a linear effect on *E. faecium* ($p < 0.0001$) reduction. The results showed that both microorganisms showed a different response depending upon fat, moisture content, and screw speed. However, temperature showed a similar effect on both the microorganisms when thermal inactivation was considered. The reduction of *E. faecium* was always lower than the one obtained for *Salmonella* under similar conditions. Therefore, *E. faecium* may be an acceptable surrogate for *Salmonella* due to its higher thermal resistance.

Keywords: extrusion; low-moisture food; *Salmonella*; *E. faecium*; thermal inactivation

To my family & friends

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Chapter I: Introduction

1.1 Introduction

Food safety continues to be a major challenge for both consumers and manufacturers. Foodborne illness is an issue of great concern not only in United States but also all over the world which affect millions of people, killing thousands of them each year. The Centre for Disease Control and Prevention (CDC) estimates that each year roughly 1 in 6 Americans (ca. 48 million) get sick, 128,000 get hospitalized, and 3,000 die of foodborne diseases (CDC 2008). Historically, these illnesses have been only associated with high-moisture foods, however recently low-moisture foods have also been implicated. *Salmonella* is an emerging issue in low-moisture food as found in different product recalls and foodborne illness outbreaks (GMA 2009).

Low-moisture foods can no longer be considered safe simply because they do not support the growth of *Salmonella* (GMA 2009). Low-moisture foods inherently have low-water activity which prevents the growth of pathogenic bacteria like *Salmonella*. While growth no longer happens, the low-water activity does not affect *Salmonella* that may already be present in the food. The infectious dose of *Salmonella* has been estimated to be as low as one cell so its presence in foods at any level is a risk to consumers. Indeed, infection has occurred from consuming low-moisture products contaminated with less than 1 CFU/g (GMA 2009) like paprika potato chips (Lehmacher et al. 1995), peanut butter (Zink 2008) and chocolate (ACMSF 2006).

The increase in the number of outbreaks and recalls due to *Salmonella* in low-moisture foods has resulted in a greater need for development and validation of process controls to assure the microbiological safety of the final product. The new Food Safety Modernization Act of 2011, requires food processors to validate their

processes to ensure food safety. Following validation of the microbial control measure, there is also a need for verification and monitoring that can be used to assure that the control measure is implemented as planned (Covance 2013).

Extrusion is one of the most commonly used process in the food industry and has been shown to be effective in reducing *Salmonella* in high-moisture foods at temperatures above 85˚C (Anderson et al. 2017; Li et al. 1993; Likimani et al. 1990; Okelo et al. 2006; Quéguiner et al. 1989; Walsh and Funke 1975). While most studies focused on extrusion of high-moisture foods, studies on extrusion of low-moisture food is limited. One of the challenges related to validation of processes for inactivation of pathogens in low-moisture foods is the identification of an adequate surrogate for the target microorganism, for the specific product, and for the specific process being validated.

A surrogate is a non-pathogenic microorganism that has equal or greater resistance to inactivation when compared to the pathogen of concern (Bianchini et al. 2012; Gurtler et al. 2014). According to Gurtler et al. (2014), there is a need for the identification of appropriate surrogates to assist with the large-scale validation studies for low-water activity foods. The need for surrogates is related to the fact that initial validation studies conducted in laboratory-scale equipment may not scale up very well to large-scale equipment. Therefore, the industry must validate their processes in their own facility using a suitable surrogate since introducing pathogens into the processing plant would not be an option. *Enterococcus faecium* NRRL B-2354 is a recognized surrogate for validating the thermal processing of almonds (ABC 2014) and in the extrusion of carbohydrate-protein meal (Bianchini et al. 2014). While these studies show promise for use of *E. faecium* as a surrogate, a more comprehensive research is

necessary to validate the use of this organism as a *Salmonella* surrogate during the extrusion of low-moisture food across a broad range of process parameters and food composition.

1.2 Objectives

The main goal of this research is to fulfil a gap related to the lack of research regarding *Salmonella* inactivation in low-moisture foods. The major objectives of this study are:

- To develop a response surface model to predict *Salmonella* spp. inactivation in oat flour, as affected by moisture, fat content, screw speed, and temperature.
- To evaluate the use of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* spp. in extrusion of oat flour.

1.3 Thesis Organization

Chapter II includes a literature review that provides background regarding the microorganism of interest in low-moisture food, the survivability of *Salmonella* in low-moisture foods, the previous research conducted in this area and the identification of any research gaps related to the reduction of this microorganism.

Chapter III describes the development of a response surface model to estimate the inactivation of *Salmonella* spp. during the extrusion process in oat flour. Parameters considered during the development of the model include screw speed, temperature, moisture content, and fat content. Also, it contributes to expansion of the use of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* in low-moisture foods.

Chapter IV evaluates the use of *Enterococcus faecium* NRRL B-2354 as a potential surrogate for the extrusion process in low-moisture foods.

Chapter V summarizes the results of chapter III and IV and presents the suggestions for future work.

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Chapter II: Literature Review

2.1 *Salmonella* spp.

Salmonella is a bacterium which is known to cause one of the most common intestinal infections called salmonellosis. *Salmonella* is one of the bacteria that has been most frequently reported cause of foodborne illness (USDA-FSIS 2016). The CDC estimates that ca. 1.2 million illnesses and ca. 450 deaths occur due to *Salmonella* every year in the United States (CDC 2016). This microorganism is present in every aspect, present in all levels of food chain, from production to consumption.

2.1.1 History

The genus *Salmonella* was originally isolated in 1885 by Dr. Daniel E. Salmon, a pioneer American veterinary pathologist after whom this pathogen was named (Pegues, Ohl, and Miller 2004). In 1886, Salmon, along with Theobald Smith discovered the organism that causes hog cholera (*Salmonella enterica* serotype Choleraesuis), when they were studying the causes of hog cholera (Durecko et al. 2004; Kass 1987).

2.1.2 General Characteristics

Table 2.1 describes the taxonomic hierarchy of *Salmonella* as described by the Integrated Information System (ITIS).

The genus *Salmonella* is facultatively anaerobic, gram-negative rod-shaped, non-spore forming, oxidase-negative, catalase-positive bacteria. Most strains are motile due to peritrichous flagella and ferment glucose with the production of both acid and gas. *Salmonella* spp. are ubiquitous in nature, being found just about anywhere in the general environment. Specifically related to food safety is the fact

that this organism may be found on many raw foods, including those of oilseed and animal origin, on animals and humans and in their gastrointestinal tract. Three species have been established in the genus *Salmonella*, which include *S. bongori, S. enterica* and *S. subterranae* (ITIS 2012). Table 2.2 describes some *Salmonella* species, subspecies and the number of serotypes associated with those.

2.2 Low-Moisture Foods

Low-moisture foods or low water activity foods (a_w<0.70) (Blessington, Theofel, and Harris 2013) are those foods naturally low in moisture or those with originally high moisture that undergo a drying or dehydration process (GMA 2009). Traditionally, low-moisture foods, until recently, have always been considered to be microbiologically safe and were not the target of any discussion in terms of food safety mainly because these products do not offer a desirable environment for the growth of microorganisms. Some food products that are classified as low-moisture foods include: flours, cereals, chocolates, grains, dried fruits and vegetable, spices and herbs, and other nut based products. Because consumers perceive these products as safe for consumption, usually additional precautions such as cooking are not performed at consumer level. Indeed, some of these foods are considered ready-to-eat (RTE) and their safety should be ensured.

2.2.1 Low-Moisture Foods Are Safe: A Misconception

In recent years, the association of low-moisture foods with pathogens has gained the attention of the scientific community and regulations. According to Reportable Food Registry maintained by FDA, the majority of entries associated with *Salmonella* were animal food and feed (18/58 entries), followed by nut products (11/58 entries) and spices and seasonings (10/58 entries) in the year of 2012-2013.

Other reports have associated *Salmonella* with the contamination include infant formula, chocolates, spices, toasted oat breakfast cereals, and peanut butter (GMA 2009). In different microbial challenge studies, *Salmonella* was observed to survive in chocolate after a storage period of 1-9 months (Tamminga et al. 1976); while in peanut butter products it survived a 6-month storage at both room temperature and refrigerated temperature (Burnett et al. 2000).

The infectious dose of different serovars of *Salmonella* has been established at $>10^5$ organisms (Kothary and Babu 2001). Indeed, the outbreak associated with peanut butter in the year 2006-2007 was reported to have been caused due to low numbers of *Salmonella* in the product. Contamination levels were around 1.5 MPN/g in an unopened jar (Zink 2008).

Salmonella contamination in chocolates, candies, and cocoa powder has been studied since the 1970s. *Salmonella* Oranienburg, ranging from 1.1-2.8 cells per gram, was found in an international outbreak that was associated with chocolates produced in Germany (Werber et al. 2005). In another outbreak, the level of *Salmonella* contamination was reported as low as 0.04 cells per gram in chocolate coins made in Belgium that caused illnesses in Canada (Hockin et al. 1989). Surveys have reported the incidence of *Salmonella* in wheat flour ranged from 0.14% to 1.3% (Sabillón and Bianchini 2016).

A report related to product recalls revealed that between 1970 and 2003 (GMA 2009), there were 21 recalls that included spices and herbs contaminated by *Salmonella* (Vij et al. 2006). The spices recalled, were imported from different parts of the world such as India, Egypt, Spain, Mexico, Taiwan, and Jamaica, to name a

few. The spices included ground black pepper, ground oregano, paprika, ground cumin seeds, herb basil leaves, among others (Vij et al. 2006).

In 2005, 25 people in the United States developed salmonellosis due to the consumption of ice cream manufactured with contaminated cake batter. Upon investigation, the Food and Drug Administration (FDA) indicated that the source of contamination with *Salmonella* was the ingredients that were listed as part of dry cake mix. The FDA has issued a warning that dry cake mix batter should not be considered as a RTE food if it has not been processed to ensure it is safe for the consumers to ingest without further processing (Food and Drug Administration 2005).

An outbreak of *Salmonella* Typhimurium in flour took place in New Zealand in the year 2008-2009, with the source of contamination being determined as raw wheat flour. The contamination affected 67 people, out of which 12 were hospitalized with cases of bloody diarrhea being reported (McCallum et al. 2013). Grocery Manufacturers Association (GMA) compiled 23 *Salmonella* outbreaks in low-moisture foods between 1970 and 2009 (GMA 2009). Out of those, 6 outbreaks were associated with chocolates, 5 with infant foods, 3 with peanut butter and its products, 5 with nuts and nut products and 2 with spices and seasonings.

Based on the FDA-Reportable Food Registry (RFR), in the United States alone, between 2009 and 2013, 54 recalls were associated with spices and seasonings and 42 with nuts and nut products because of their contamination with *Salmonella*. In 2009, three major recalls in the United States involving pistachio nuts, peanut butter and non-fat dry milk, were examples of serious health and economic burden due to the pathogen contamination of low-water activity foods (Gurtler et al. 2014).

Based on all the information available regarding *Salmonella* and low-moisture foods, it becomes clear the need for the industry to validate processing parameters in order to ensure the safety of foods and consumers.

2.3 Extrusion

As the world population reaches an estimate of seven billion by 2017, and with further increase, the demand for food products, or rather safer and more attractive ones also increases. The food industry is always innovating by creating solutions and new processing methods to address emerging issues. There are several unit operations in the food industry to ensure the food safety. One of the most common unit operation used in the low-moisture food industry is extrusion.

2.3.1 Principle of Extrusion

Extrusion processing is very popular in the food as well as in the feed industry. This is a process that combines various operations including cooking, mixing, kneading, shaping, and forming (Riaz 2000).

A typical food extrusion process involves the addition of raw materials into the extruder barrel where screws are placed to convey the food through the length of the equipment. As the material moves slower, down the barrel, its resistance to movement increases due to the small clearance between the screw flight and the barrel. Along the barrel, material gets kneaded into a semi-solid mass by the help of the extrusion screws (Riaz 2000). Generally, the food is heated at a temperature above 75ºC, which is known as hot extrusion or extrusion cooking. The heating provided along with the frictional forces leads to a rapid increase in the temperature inside the barrel. As the food moves towards the end of the barrel the shearing and pressure becomes more intense. Finally, the food exits the barrel through an opening called die.

Because the food inside of the barrel is under pressure, it expands to its final shape as it emerges, cooling down immediately. The most common extruded products are RTE puffed cereals and low density foods.

The purpose of the extrusion is to cook, to change or to create texture, to reduce microbial hazards and to inactivate enzymes (Riaz 2000).

2.3.2 Development of Extruder

The term extrusion typically refers to the process by which a liquid or semi-liquid product gets forced through a desired die opening. The basic science behind extrusion has not undergone a drastic change in over 60 years. However, new designs and application have been developed. The last two centuries have witnessed a steady progress of the extrusion method. Joseph Bramah was the first man to obtain the extrusion patent in the year 1797. His process was used to produce pipes of specific diameter and length without joints by forcing liquid metal (i.e. lead) out of a die in a continuous profile (Riaz 2000).

The single-screw extruder became popular in the year 1930. In late 1930s, General Mills first used single-screw extruder in manufacturing of RTE cereal (Riaz 2000). Its application became preferred over the hot shaping and rolling in a hydraulic press equipment. The single-screw design was then followed by the development of the twin-screw extruders in the mid 1930's, which were both co-rotating and counter-rotating (Riaz 2000). Additionally, there have also been advancements in the design of the screw and die parts. Currently the process is carried out under better temperature control, with the possibility of temperature control for different zones of the barrel. Advances have also been made in regards to the average production that has increased from a few hundred kilograms to several metric tons per hour.

2.3.3 Role of Extrusion in Inactivation of Microorganisms

Even though several food preservation methods exist, the most widely used method is thermal processing. The use of heat to eliminate microbial hazards, is usually more efficient in products that have high-water activity (Puupponen-Pimiä et al. 2003; Nieto, Castro, and Alzamora 2001). Moist heat processes, that include controlled condensation and blanching, have been known to be an effective method for microbial reduction due to the increased moisture that helps in reducing the thermal resistance of microorganism (Gurtler et al. 2014). According to Gurtler et al. (2014), the increased moisture can reduce the shelf life of food, therefore the product is re-dried to remove the excess moisture which adds up the energy cost. Furthermore, other methods such as drying, hot air and baking, dry and oil roasting have also been tested for their ability to reduce microbial contamination (Gurtler et al. 2014). These processes however required extended heating time and higher temperatures to achieve the desired microbial reduction. Because hot or thermal extrusion is a complex process that involves temperature, pressure and shear forces, it may provide the desired effect on microbial load while allowing for the use of reduced temperature and moisture.

2.3.3.1 Cold Extrusion

Cold extrusion is a processing technique typically used in the single-screw extruder in which the temperature of the food products remains at ambient and is used to produce products such as pasta and various meat products (Riaz 2000). These cold extruded products (e.g. pasta and macaroni) have been recalled from the market due to the presence of microorganisms. Because these products are made with raw ingredients such as flour and semolina that may be contaminated with pathogens and

the product is not heated during the commercial processing which may lead to presence of some microorganism in the product. Viable microorganism remains unaffected by the cold extrusion processing technique.

The interest in research related to the safety of extruded products led to the study of the effect of temperature and screw speed on the survival of *Staphylococcus aureus* and *Salmonella* Typhimurium during the production of spaghetti. Walsh et al. (1974) determined that *S*. Typhimurium population survived the extrusion process at temperatures from 35 to 55˚C and 12 rpm. Faster screw speeds (12-30 rpm) led to the greater reduction of *S*. Typhimurium at 35˚C due to the mechanical destruction (Walsh, Funke, and Graalum 1974). Walsh et al. (1975) also determined that a 1-log reduction of *S. aureus* was obtained at 35-55˚C and 20-50 rpm (Walsh and Funke 1975). To ensure the food safety of pasta products, food components used for products with intermediate moisture content need to be pasteurized before the mixing process to the lower water activity (a_w) (Hsieh et al. 1976).

2.3.3.2 Hot or Thermal Extrusion

When the food is heated typically above 75˚C, the process is known as hot extrusion, also known as extrusion cooking or thermal extrusion. This high-temperature relatively short-time process has proved to be effective in reducing the load of microbial contamination. Studies of extrusion on microbial inactivation are usually of limited scope often targeting one product matrix and characterizing a limited set of processing parameters (Anderson et al. 2017; Li et al. 1993; Likimani et al. 1990; Okelo et al. 2006; Quéguiner et al. 1989; Walsh and Funke 1975).

In 1989, a study was done using a twin-screw extruder on reduction of *Streptococcus thermophilus* in whey protein isolate with low moisture content (4-5 %

w/w), operating temperature of 80-204˚C, and a constant screw speed of 50 rpm (Quéguiner et al. 1989). Results indicated a reduction of 4.2 log of *Streptococcus thermophilus* at 143˚C. This study demonstrated the efficacy of hot extrusion in assuring the safety of food and feed products.

Likimani et al. (1990) studied the inactivation of *Bacillus globigii* during the extrusion of corn/soybean mixture (70/30%, w/w) at a moisture of 18% running at different screw speeds (80-160 rpm). Results indicated a reduction of 1-7 log when the product was extruded at high temperatures (110-130˚C), while the increased screw speed resulted in lesser lethality.

In another study, thermal inactivation and injury of *Clostridium sporogenes* spores were studied in a mixture of mechanically deboned turkey and white corn flour. Thermal inactivation was observed at 93.3˚C with a 2-log reduction and at 115.6˚C with a 4-5 log reduction (Li et al. 1993).

Bianchini et al. (2012) used a response surface methodology to study the effect of moisture and temperature on the inactivation of *Enterococcus faecium* NRRL B-2354 in a carbohydrate-protein mix. The greatest reduction of 5-log occurred at the temperature of 81.1˚C and at the highest moisture content of 28.1% evaluated in their study.

Crane et al. (1973), determined that thermal extrusion completely eradicated *Salmonella* when the processing temperatures were above 93.3˚C. Anderson et al. (2017) studied the destruction of *Salmonella* Agona during the extrusion of oat flour with indigenous fat content of 8% when extruded at different temperatures (65-100˚C) and water activity (0.72-0.96). Pathogen reduction was observed and ranged from 1-7 log CFU/g with the greatest reduction obtained at higher temperature and high water

activity levels. Processing conditions above 82˚C and 0.89 a_w achieved on average greater than a 5-log reduction of *Salmonella.*

Based on the available research, it seems that the bacterial destruction is greatest at higher temperatures with the mixture to be extruded having higher moisture levels. Little or no destruction was observed at low moisture and low temperature.

2.3.4 Research Gap in Extrusion

A recent increase in the number of outbreaks and recalls due to *Salmonella* in low-moisture foods has resulted in greater need for the development and improvement of process controls that ensure the microbiological safety of these products (Gurtler et al. 2014).

At high a_w and temperatures above 85˚C, extrusion has been reported to be effective in reducing microbial contamination in the final product (Anderson et al. 2017; Bianchini et al. 2012; Li et al. 1993; Okelo et al. 2006; Quéguiner et al. 1989; Crane et al. 1973; Likimani et al. 1990). However, most of these studies targeted only one product matrix and characterized a limited set of processing parameters. Thus, there is a critical need to study different extruder parameters (screw speed, screw type) and a broader matrix composition (fat content, low moisture levels).

2.4 Kill-Step Validation for Food Safety

Many food products undergo a kill step during processing, which is designed to eliminate pathogens and reduce spoilage microorganism. The new Food Safety Modernization Act (FSMA) signed into law on 4 January 2011, requires the food industry to implement and validate controls to prevent or significantly minimize

identified hazards (Bianchini et al. 2012). This has created an industry-wide need for science based validation procedures that can ensure product safety.

When designing a kill-step validation experiment, the conditions tested should include any potential "worst-case scenario" to best assess how the process performs. Therefore, in the case of extrusion validations it should include lower temperatures, slower screw speed, high fat content (which may have protective effect), lower moisture content etc. (Gurtler et al. 2014; Anderson et al. 2017).

2.5 Surrogate Microorganism: Why Are They Important?

Surrogates are non-pathogenic microorganisms that when used for validation studies behave similarly to the pathogen of interest. In the case of thermal process, a surrogate should have heat resistance comparable to or greater than the target pathogenic microorganism (GMA 2009). There is a need for the identification of surrogate microorganisms and standard protocols to help the food industry companies in the validation of processes that are necessary for the control of pathogenic microorganisms (Gurtler et al. 2014). Some validation studies can be accomplished using a laboratory-scale equipment using the specific pathogen of concern, but sometimes the scale-up does not reflect well the results obtained in the laboratory. Hence, it becomes difficult to extrapolate laboratory results to be used in a large-scale processing plant, so the validation study must be repeated in the processing plant. When this is required, the use of a surrogate organism is essential since introducing pathogen into the food facility would not be an option.

Enterococcus faecium NRRL B-2354 is a non-pathogenic microorganism that has been used as a surrogate for *Salmonella* spp. in the validation of thermal processes. One of such processes is almond roasting and *E. faecium* has been

identified as an adequate surrogate (ABC 2014). Bianchini et al. (2014) studied the use of *E. faecium* as surrogate for *Salmonella enterica* during extrusion. Results showed that the minimum temperature needed to achieve a 5-log reduction of *E. faecium* was 73.7˚C, while *Salmonella* was reduced by 5-log at 60.6˚C. This study indicated that *E. faecium* could be used as a surrogate for *Salmonella* under the parameters evaluated (Bianchini et al. 2014). However, further evaluation of this microorganism as *Salmonella* surrogate during extrusion is necessary to consider other parameters such as screw speed or lower moisture content.

2.6 References

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Table 2.1. Taxonomic Hierarchy of *Salmonella* (ITIS 2012).

Rank	**Taxon**
Kingdom	Bacteria
Subkingdom	Negibacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Salmonella*

Table 2.2. *Salmonella* species and subspecies (Brenner et al. 2000)

***Salmonella* species and subspecies**	**Number of serotypes**
S. enterica subsp. *enterica*	1,454
S. enterica subsp. *salamae*	489
S. enterica subsp. *arizonae*	94
S. enterica subsp. *diarizonae*	324
S. enterica subsp. *houtenae*	70
S. enterica subsp. *indica*	12
S. bongori	20
Total	2,463

Chapter III: Develop a response surface model for *Salmonella* inactivation during the extrusion of low-moisture food, oat flour

Abstract

A recent increase in the number of foodborne illness outbreaks and recalls due to *Salmonella* in low-moisture foods has resulted in the need for the development and validation of process controls to assure their microbiological safety. Further, the Food Safety Modernization Act rule for Preventive Control for Human Food requires food processors to validate their process controls to ensure food safety. The objective of this study was to develop a response surface model to predict *Salmonella* inactivation in oat flour, as affected by product composition and extrusion process parameters. Oat flour was adjusted to different moisture (14 to 26% wb) and fat (5 to 15%) contents and was then inoculated with a 5-strain cocktail of *Salmonella* to attain ca. 8.0 log CFU/g. Inoculated material was extruded through a single-screw extruder running at different screw speeds (75 to 225 rpm) and temperatures (65 to 85ºC), without a die. Once steady state conditions were attained, extruded samples were collected, cooled, stored under refrigeration and *Salmonella* survivors were enumerated. A split-plot central composite 2nd order response surface design was used, with the central point replicated six times. Temperature showed the highest significant ($p < 0.0001$) effect on microbial reduction. Moisture content showed a significant linear ($p = 0.0014$) and quadratic ($p = 0.0005$) effects whereas higher fat content showed a significant ($p < 0.0001$) protective effect on *Salmonella* destruction. The screw speed did not play a major role in inactivating *Salmonella*, but it had a significant ($p = 0.0004$) interactive effect with temperature. Results indicated that >5 log reduction was achieved in oat flour extruded at temperature above 85˚C at all moisture and fat content evaluated at

screw speed of 150 rpm. The developed response surface model can be used to identify the extrusion process conditions to achieve a desired reduction of *Salmonella* based on the moisture and fat content of the product.

3.1 Introduction

Low-moisture foods are those with a water activity (a_w) typically below 0.70 (GMA 2009). Historically, low-moisture foods had been considered microbiologically safe because they do not favor the growth of bacteria. However, between 2006-2009, 10 foodborne illness associated with *Salmonella* were reported to be linked to low-moisture foods like puffed cereals, peanut butter, nuts, spices and pet foods (GMA 2009). Many of these *Salmonella* outbreaks have been associated with the consumption of extruded products like cereals and snacks or due to consumer handling of extruded pet food products (Bianchini et al. 2012; GMA 2009).

Extrusion is a thermal process that combines several unit operations like mixing, kneading, shaping, and forming (Riaz 2000). Typical extruded products include a variety of low-density, puffed and snack foods which are often further processed by baking, frying or drying. Extrusion is commonly assumed to be a process that significantly minimizes or eliminates pathogens in food products due to the high temperature, high shear, and high pressure experienced by the product inside the extruder barrel. At temperature above 85˚C, thermal extrusion was demonstrated to be an effective tool to inactivate *Salmonella* in high-moisture foods (Crane et al. 1973; Li et al. 1993; Likimani et al. 1990; Ukuku et al. 2012). However, studies on validation of extrusion for low-moisture foods are lacking. Validation of the microbiological safety of extrusion process is necessary (21 CFR 117) as the thermal destruction parameters (*D*- and *z*-values) of microorganisms are very high in low-

moisture foods (Archer et al. 1998; Goepfert et al. 1968). Additionally, published literature primarily focused moisture content and temperature as factors affecting microbial inactivation during extrusion; while the effects of fat content and screw speed have not yet been studied. While higher screw speed results in shorter residence time, it also creates more friction inside the barrel. Therefore, the effect of this variable must be systematically evaluated. The fat content of pork burger patties, ground chicken, turkey and beef has been shown to have a protective effect on *Salmonella* inactivation during thermal processing (Gurman et al. 2016; Juneja et al. 2000; Juneja et al. 2001; Smith et al. 2001). It is unknown whether fat would provide the same protective effect in foods processed by extrusion.

Further, the Food Safety Modernization Act (FSMA) rule for Preventive Controls for Human Food requires food processors to validate their process controls to ensure food safety. Because the food industry uses a broad set of processing parameters and product formulations, a response surface model would serve as an excellent tool for process validation. Such a model would allow processors to adjust extrusion process parameters for different foods to achieve the desired level of microbial inactivation, ensuring food safety.

The complex nature of the extrusion along with its wide variety of process parameters (screw speed, temperature) and food properties (moisture content, fat content) demands a comprehensive study to understand the effect of food composition and extrusion process parameters on microbial inactivation during the extrusion of low-moisture foods. Therefore, the objective of this research was to develop a response surface model to predict *Salmonella* inactivation in oat flour, as affected by

product composition (moisture content and fat content) and process parameters (temperature and screw speed).

3.2 Materials and Methods

3.2.1 Low moisture food: Oat flour

Whole grain oat flour was obtained from Bob's Red Mill (Milwaukie, OR, USA) and was stored under refrigerated conditions (4˚C) until it use. Microbiological testing was performed to determine the background microflora and the presence of any potential *Salmonella* in the flour before conducting the experiments. Twenty-five grams of flour was taken from each bag, diluted in 225 mL of 0.1% buffered peptone water (BPW; Becton, Dickinson and Company, Sparks, MD), plated on tryptic soy agar with 0.6% yeast extract (TSAYE; Becton, Dickinson and Company, Sparks, MD) and xylose lysine deoxycholate (XLD; Becton, Dickinson and Company, Sparks, MD) agar plates and incubated for 24 h at 37˚C. The total microbial population and typical *Salmonella* populations were below the detectable limit (<10 CFU/g).

3.2.2 Bacterial Strains

A cocktail of five different serovars of *Salmonella enterica* was used to conduct the microbiological studies. The cocktail included *Salmonella* Agona 447967 associated with a puffed rice cereal recall in Minnesota (CDC 2008); *Salmonella* Enteritidis PT30 associated with a raw almonds recall (CDC 2004); *Salmonella* Tennessee K4643 associated with a 2006 peanut butter outbreak (CDC 2007); *Salmonella* Montevideo 488275 associated with a 2009-2010 black pepper outbreak (CDC 2010); and *Salmonella* Mbandaka 698538 associated with a sesame tahini recall in Turkey (CDC 2013). These strains were selected because of their thermal

resistance and frequency of occurrence in low-moisture food. *Salmonella* Agona 447967, *Salmonella* Montevideo 488275 and *Salmonella* Mbandaka 698538 were obtained from FDA, ORA Regional Lab in Jefferson, Arkansas. *Salmonella* Enteritidis PT 30 was obtained from ATCC BAA-1045 and *Salmonella* Tennessee K4643 from Dr. Larry Beuchat, University of Georgia, Griffin (GA). These bacterial strains were stored at -80˚C as frozen stocks in 80% glycerol.

3.2.3 Inoculum Preparation

The frozen stocks of 5 strains of *Salmonella* were individually streaked for reactivation on TSAYE plates and incubated at 37˚C for 24 h. After 24 h, the different strains were then transferred to 10 mL tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) and incubated at 37˚C for 24 h. Bacterial lawns were created by spreading 100 µL of cultured TSB onto TSAYE plates and incubating at 37˚C for 24 h. Finally, the cells were harvested by adding 3 mL of 0.1% BPW to each plate and loosening the lawns with sterile L-shaped spreaders. To prepare the *Salmonella* cocktail, equal volume of cell suspensions of each strain was combined in a sterile container and vortexed for 30 s to ensure uniform distribution of cells.

3.2.4 Sample Inoculation

For inoculation, moisture, and fat adjustments on the oat flour, a commercial mixer (Kitchen aid, S. No. W53294842) capable of handling 1 kg of product was used. The mixer was kept inside a biosafety hood throughout the sample preparation process and the mixer bowl was covered with a lid to contain dust generated during mixing. The moisture content of the sample was adjusted to 14.00, 16.35, 20.00, 23.65, and 26.00% and the fat content was adjusted to 5.00, 6.96, 10.00, 13.04, and 15.00% as determined by the experimental design (Table 3.1).

To prepare each of the inoculated samples, oat flour (1 kg) was aseptically transferred to a sterile mixing bowl, followed by double deionized water, oil (Great Value, Wal-Mart Stores, Inc., Bentonville, AR) and inoculum were added to the flour. The amount of water and oil required to achieve the moisture content and fat level desired was calculated based on the initial moisture content and fat level of the flour. The amount of inoculum (10 mL) to be added was then subtracted from the amount of water. After adding the desired amount of water, oil and inoculum, the mixer was set to run at the lowest speed for 5 min. After 5 min, the flour was scraped off from the walls of bowl and paddle using a sterile spatula. The mixer was then again set to run for another 5 min at the same speed. After mixing, the inoculated flour was carefully transferred to a sterile bag and stored at room temperature (25˚C) for 5 days for moisture distribution within the sample and adaptation of bacterial cells to the low water activity. The period of five days was selected based on the results reported by Anderson et al. (2017) on the number of days required for achieving homogeneity in oat flour.

3.2.5 Extrusion

The prepared inoculated samples were extruded in a laboratory-scale GR-8 single-screw extruder (C.W. Brabender Instruments, South Hackensack, NJ). To feed the extruder continuously, an external volumetric feeder (Brabender Technologie, Ontario, CA) was used in which the speed was controlled by a frequency controller. The feeder had a screw size of 18 mm and pitch of 19 mm. The screw used in the extruder barrel had a compression ratio of 3:1 with length/diameter ratio of 20. The die face was equipped with a thermocouple (CW Brabender Instruments, South

Hackensack, NJ) that constantly measured the product temperature throughout the extrusion process.

In an industrial setting, the extrusion process is not performed with the barrel filled at 100% of its capacity. To simulate such conditions, extrusion was performed with the barrel filled at 75% of its capacity. A preliminary experiment was conducted to identify different feed rates to achieve 75% capacity for the different screw speeds tested in the study. As the moisture content and fat content also affect the movement of the material in the barrel, an appropriate feeding rate was identified for each trial.

The extruder barrel had three temperature zones with the temperature of the first two zones maintained at 50˚C, while the last zone was adjusted to achieve the desired product temperature as determined by the experimental design. No die was used at the barrel end, which resulted in minimal back-pressure and shear, when compared to industrial extrusion. Thus, this study represented the worst-case scenario.

Residence time was measured at different screw speeds by adding Food, Drug, and Cosmetic Act (FD&C) Red #40 into the inlet of the extruder barrel and measuring the time required for the color to appear at the die end. After achieving the desired product temperature at the die, the extruder was run for another 5 min to achieve steady state conditions before the samples were collected. Extruded samples were collected for 5 min in a sterile whirl pack bags and cooled immediately in an ice-water bath.

3.2.6 Extruder Cleaning

To avoid bacterial carryover, proper cleaning of the extruder barrel between runs was essential. Uninoculated oat flour was run in-between the trials to clean the barrel as a push-through sanitation. Preliminary microbiological analysis was done

with samples collected at 5, 15, and 20 min after processing inoculated oat flour to establish the minimum push-through period necessary to prevent cross-contamination. Microbiological analysis of the samples was performed to identify the time required for push-through sanitation of the barrel.

3.2.7 Moisture Determination and Sorption Isotherm

The moisture content of the inoculated material was determined before and after the extrusion. A Halogen Moisture Analyzer HR73 (Mettler Toledo Laboratory and Weighing Technologies, Greifensee, Switzerland) was used to measure the moisture content.

The adsorption isotherm was generated using an AquaLab Vapor Sorption Isotherm (Decagon Devices, Inc., Pullman, WA) for oat flour (5, 10, and 15% fat) at 25˚C.

3.2.8 Microbial Analysis

Non-extruded and extruded inoculated samples were enumerated to determine the destruction of *Salmonella* during the extrusion process. During extrusion, extruded samples were collected in a sterile bag and cooled immediately in ice-water bath for further microbial analysis. From each bulk sample, two subsamples were taken for microbial analysis. Bacterial counts were determined by the serial dilution method, where 25 g of sample was diluted in 0.1% BPW to achieve a 1:10 dilution. Subsequent dilutions (1:10) were done to provide plates with 25-250 colonies.

Bacterial enumerations were done using TSAYE and TSAYE overlaid with XLD, with incubation at 35±2˚C for 24 h. The non-selective media (TSAYE) allowed the growth of any heat injured *Salmonella* cells and other bacteria present in the sample capable of growing under the incubation conditions. The selective media

(XLD) was used as an overlay on TSAYE. In this case, the TSAYE allowed the growth of any heat injured cells, while the XLD as an overlay selected and differentiated for *Salmonella*.

3.2.9 Experimental Design and Statistical Analysis

The experiment was conducted as a split-plot second order central composite design with temperature as the whole-plot factor and moisture content, fat content, and screw speed as the split-plot factors. In general, split-plot experiments are special blocked experiments where the blocks are the whole-plot units to which levels of the whole-plot factor are applied and the split-plot treatment factors are applied to the smaller split-plot units within blocks (Chukwu et al. 2012; Mugabi et al. 2017). In this experiment, moisture, fat, and screw speed are applied to 1 kg samples of product which are the split-plot experimental unit for these three factors. However, the extruder die exit temperature takes a prohibitively long time to adjust and stabilize which greatly limits the number of treatments that can be conducted on a day. Therefore, temperature was applied to blocks of either 6 or 8, one-kg samples at a time where all the product extruded at a given temperature was a whole-plot unit (Table 3.2). In this experiment, a blocked central composite design was used for the split-plot factors and replicated 3 times for a total of 60 runs, where temperatures were applied to blocks as described in Table 3.2.

A split-plot analysis of variance model with temperature as the whole-plot factor and terms for the second-order response surface for the split-plot factors including their interactions with temperature was fit for log reduction of *Salmonella* inactivation using least-squares. A final model was fit following the procedures of testing the highest order terms first and keeping significant ($p < 0.05$) higher order

terms and all their lower order components. SAS version 9.4 (SAS Institute, Cary, NC) was used to fit the response surface models and MATLAB R2016b (MathWorks, Natick, MA) was used to create contour plots.

3.3 Results

3.3.1 Extrusion of Inoculated Flour

Bacterial counts on the 5-strain *Salmonella* cocktail inoculum indicated a level of ca. 9.5 log CFU/mL. This prepared inoculum was used to inoculate the oat flour at a level of ca. 8.0 log CFU/g. The inoculated flour was left at room temperature (25˚C) for 5 days for homogeneity and stability. Anderson et al. (2017) demonstrated that storing the inoculated sample at 25˚C for five days resulted in homogeneous distribution of *Salmonella* and stable population of *Salmonella* in the oat flour.

The inoculated oat flour was extruded at different conditions based on the experimental design. However, before starting the extrusion of inoculated sample for each set of variables tested, steady state conditions were achieved by running the uninoculated sample first through the extruder barrel. The product temperature at the exit of the barrel was measured continuously by a thermocouple placed at the die face. This temperature was then used to determine when the extruder reached the desired temperature. Once the equipment was running consistently, the inoculated samples were processed.

The residence time inside the barrel was measured as 55, 40, 34, 26, and 21 s at 75, 100, 150, 200, and 225 rpm, respectively. While developing a push-through sanitation method for the trials, data shown in Figure 3.1 were collected. Results showed that 20 min were sufficient, at different screw speeds, to provide non-detectable levels of *Salmonella* (<10 CFU/g) in the uninoculated extruded samples

that followed the extrusion of an inoculated sample. Therefore, uninoculated oat flour was fed for 20 min for the push-through sanitation between extrusion trials.

The moisture content of the inoculated sample was measured before and after the extrusion process. A reduction in moisture content by approximately 1.5 to 2.5% was observed in the product subsequent to extrusion. Bianchini et al. (2012) observed a moisture loss of 0.5 to 2.0% during extrusion at different temperatures (ranging from 67.5 to 85˚C). Similarly, Riaz (2000) reported that 4 to 7% reduction of moisture is obtained by flash-off as hot products expand at the die during industrial extrusion. The lower levels of moisture reduction observed in this study and the one by Bianchini et al. (2012) may be related to the fact that temperatures tested were lower than those normally used by the industry. These lower temperatures may have resulted in lower flash-off of product moisture at the die (Bianchini et al. 2012).

The target water activity of the oat flour (5, 10, and 15% fat) was estimated based on the moisture adsorption isotherm (Figure 3.2). Results indicated that the moisture content of the oat flour decreased as the fat content increased from 5 to 15% at a certain water activity.

After extrusion, samples were collected and enumerated for *Salmonella*. Samples were plated on TSAYE, which allowed the growth of any heat injured cells and other bacteria surviving the extrusion process, and plated on TSAYE overlaid with XLD which is a selective media for *Salmonella*. Bacterial counts on TSAYE was always higher than TSAYE-XLD, since the non-selective media allowed the growth of any bacteria that survived the extrusion process.

3.3.2 Response Surface Model for *Salmonella* Inactivation

The split-plot analysis of variance model with terms for second-order response surface was fit for log reduction (log N_o/N_t) for *Salmonella* in oat flour when extruded at different temperature, screw speed, moisture content, and fat content. The final model was developed as described above and fit well with a R^2 of 0.83. The final intercept value was calculated by taking the average of estimate of all the blocks and was added to the intercept value given in Table 3.3. The response surface equation for the inactivation of *Salmonella* during extrusion of oat flour under the studied conditions based on enumeration conditions in selective media is given in Equation 3.1.

$$\text{Log } (N_o/N_t) = -7.9212 + 0.3419*T - 0.2676*F - 1.0076*M + 0.07615*S - 0.00118*T*S + 0.0277*M^2 \quad (3.1)$$

Where:

N_o = Initial bacterial population before extrusion (CFU/g)

N_t = Final bacterial population after extrusion (CFU/g)

T = Extruder die exit temperature (˚C)

M = Moisture content of sample before extrusion (% wb)

F = Fat content (%)

S = Screw speed (rpm)

The model showed a significant linear effect of temperature, fat content, and screw speed. Moisture content had both significant linear and quadratic effect, while temperature and screw speed showed significant interaction with each other. Temperature and fat content were the most significant factors (Table 3.3). Fat had a negative coefficient indicating that it had a protective effect on *Salmonella*, while

temperature and screw speed had a positive coefficient indicating their positive effect on *Salmonella* inactivation (Table 3.3).

3.3.2.1 Effect of Extruder Die Exit Temperature

The inoculated samples were extruded at die exit temperatures of 65, 75, and 85˚C. It was anticipated that the higher the temperature, the higher would be the log reduction of *Salmonella* irrespective of screw speeds, fat content, and moisture content. As seen in the contour plots of Figure 3.3, as the temperature increases from 65˚C (Figure 3.3A) to 85˚C (Figure 3.3B) the log reduction increases. For instance, the minimum log reduction achieved at 65˚C was 2.0 log CFU/g; while at 85˚C it was 5.5 log CFU/g. At 85˚C, >5 log reduction was achieved at all moisture and fat contents. In general Figure 3.3 shows that an increase in extruder die exit temperature from 65 to 85˚C for the same fat and moisture contents, yielded a difference of ca. 3.5 log CFU/g in *Salmonella* inactivation.

3.3.2.2 Effect of Fat Content

Several studies have shown that fat has a protective effect on *Salmonella* inactivation (Gurman et al. 2016; Juneja et al. 2000; Juneja et al. 2001; Smith et al. 2001). One of the objectives of this research was to evaluate the effect of fat content on *Salmonella* inactivation during the extrusion of oat flour. According to the results obtained, fat content showed a pronounced protective effect on *Salmonella* inactivation. At 150 rpm, oat flour formulated with lower fat content (5%) resulted in higher bacterial log reduction (Figure 3.4A) than those containing higher fat content (15%) (Figure 3.4B). To achieve >5 log reduction, higher temperatures were required as the fat content increased. For example, a 5 log reduction was achieved at 65˚C in formulations containing 5% fat (Figure 3.4A) and 80˚C when the fat content was 15%

(Figure 3.4B). A similar trend was observed for other screw speeds as well. To evaluate the exclusive effect of fat content in the formulation, contour plots were built with the moisture level fixed at 14%, 20% and 26% (Figure 3.5). When the moisture content was 14% (Figure 3.5A), as the fat content increased, a higher temperature was needed to achieve a desired log reduction. Similar trends were also seen at 20% (Figure 3.5B) and 26% (Figure 3.5C) moisture content.

3.3.2.3 Effect of Moisture Content

The effect of moisture on *Salmonella* inactivation was studied by extruding oat flour at different moisture levels (14, 20, 26%). According to the results, the moisture content showed both a significant linear and a quadratic effect on microbial inactivation. To understand the effect of moisture on *Salmonella* inactivation, contour surface plots were created at the screw speed of 150 rpm (Figure 3.5). As the moisture content increased from 14 to 20% (Figure 3.5A to 3.5B), surprisingly the log reduction achieved in general was slightly decreased. For example, at 65˚C and 5% fat content, the log reduction went from 5 log CFU/g to 4.5 log CFU/g as the moisture went from 14 to 20%. However, further increasing the moisture content to 26% (Figure 3.5C) resulted in higher log reductions (i.e. 6.0 log CFU/g at 65˚C and 5% fat content). This may be due to the quadratic effect observed for moisture content in the response surface model (Equation 3.1). In general, for moisture levels 14, 20, and 26%, a bacterial reduction of 2.5 to 8.0 log CFU/g, 2.5 to 7.5 log CFU/g, and 4.0 to 8.5 log CFU/g was observed, respectively. Similar trends were also seen at other screw speeds.

3.3.2.4 Effect of Screw Speed

The residence time inside the extruder barrel affects the inactivation of microorganisms (Okelo et al. 2006). To understand the effect of screw speed, contour plots were built at different screw speeds (100, 150, and 200 rpm) for oat flour extruded at the highest fat content (15%). The statistical analysis of the results showed that screw speed had a significant linear effect. In moving gradually from 100 (Figure 3.6A) to 200 rpm (Figure 3.6B), *Salmonella* inactivation was reduced for any combination of moisture and temperature (Figure 3.6). These results are in agreement with the fact that higher screw speeds mean lower residence time inside the extruder barrel. For example, at 14% moisture content and 85˚C, a screw speed of 100 rpm (40 s of residence time) resulted in 6.5 log reduction (Figure 3.6A), while at 200 rpm (26 s of residence time) only a 4.5 log reduction (Figure 3.6B) was achieved. Similar trends were also seen at other fat contents.

Results also showed a significant interaction effect of temperature and screw speed on *Salmonella* inactivation. Figure 3.6 clearly shows that as the screw speed increases from 100 to 200 rpm, the curvature of the contour plot changes (Figure 3.6A to 3.6B), showing the interactive effect of screw speed with temperature on *Salmonella* inactivation. To better understand this interaction a contour plot (Figure 3.7) was built with temperature and screw speed as variables at a fixed moisture and fat content. This contour plot shows that at lower temperatures (< 70˚C), the contour lines have a slightly negative slope. As a result, as the screw speed increases, the temperature required to achieve a certain log reduction decreases. However, as the temperature increases (> 70˚C), the slope of the contour lines change to positive indicating that as the screw speed increases, the residence time decreases, therefore

higher temperatures are now required to achieve the same log reduction. Similar trends were seen at other moisture and fat contents as well.

3.4 Discussion

3.4.1 Effect of Extruder Die Exit Temperature

In this study, the temperature of the product had a significant effect on the *Salmonella* survival, with increasing temperature leading to greater microbial reduction. At temperatures, above 85˚C at 150 rpm, >5 log reduction was achieved at different moisture (14 to 26%) and fat (5 to 15%) contents. These results were consistent with previous studies in this area. For instance, Crane et al. (1973) reported that thermal extrusion completely eradicated *Salmonella* from feed when the processing temperatures were above 93.3˚C at 25 to 35% moisture content. Ukuku et al. (2012) determined that corn meal products extruded at 55˚C or above and whey protein isolate extruded at 75˚C inactivated *Escherichia coli*, resulting in population reductions greater than 5 logs. Queguiner et al. (1989) reported a reduction of more than 4 log CFU/g of *Streptococcus thermophilus* in a whey protein isolate with low moisture content (4-5%) when extruded in a twin-screw extruder operating at temperatures greater than 130˚C. Bianchini et al. (2012) used a response surface methodology to study the effect of moisture content and temperature on the inactivation of *Enterococcus faecium* in a carbohydrate-protein mix. Results indicated a 5 log CFU/g reduction at the temperature of 81.1˚C and moisture content of 28.1%. Results obtained in this research also concur with Anderson et al. (2017) who reported an average of 5 log reduction when extruding oat flour (8.5% fat) at conditions above 82˚C and a_w of 0.89 (19% moisture) on a pilot-scale extruder. Using the response surface equation (Equation 3.1) obtained in this research, it was confirmed that this

study was also consistent with Bianchini et al. (2014) who reported that *Salmonella* was reduced by 5 log CFU/g at 60.6˚C at 28% moisture content during the extrusion of a carbohydrate-protein meal.

3.4.2 Effect of Fat Content

Results indicated that fat content had a significant effect on *Salmonella* reduction, with increasing fat content correlating with longer *Salmonella* persistence during the extrusion cooking. Therefore, for a 5 log reduction to be achieved, higher temperatures were needed as the fat content increased. For example, as the fat content increased from 5 to 10 to 15%, a minimum temperature of 65, 72, and 80˚C, respectively, was required to achieve a 5 log reduction for all moisture contents evaluated at the screw speed of 150 rpm. These observations regarding the effect of fat content on microbial survival were consistent with those of Smith et al. (2001) who found that *Salmonella* in beef mince containing 19% fat was more heat resistant than in the beef mince containing 4.8% fat. Bover et al. (2015) also reported a protective effect of fat content (10 to 50%) on *Listeria monocytogenes* in dry-cured ham during high-pressure processing (HPP) where treatments above 700 MPa were applied. Juneja et al. (2000) reported the protective effect of fat content (4 to 28%) on microbial survival in ground beef and pork, at temperatures ranging from 58 to 68˚C. Their results showed an increase in D-value for *Salmonella* in fresh ground beef and pork with increasing fat levels. Gurman et al. (2016) reported that the fat content (5% and 17%) in pork burger patties influenced the rate of *Salmonella* inactivation, with the higher fat levels being more protective.

Several studies have showed the protective effect of fat content on microbial inactivation during various industrial process, and this study confirms that a similar protective effect is found during extrusion as well.

3.4.3 Effect of Moisture Content

In this study, a significant linear and quadratic effect of moisture was observed on *Salmonella* inactivation. As the moisture content increased from 14 to 20%, *Salmonella* reduction decreased slightly, and then increased as the moisture content increased from 20 to 26%. These results are consistent with those reported by Anderson et al. (2017), where a quadratic effect of water activity on inactivation of *Salmonella* Agona was also reported for a pilot-scale extruder. Their study showed that microbial log reduction initially decreased (a_w from 0.74 to 0.84) and then increased (a_w from 0.84 to 0.96).

3.4.4 Effect of Screw Speed

A significant linear effect of screw speed and a significant interaction of screw speed and temperature were observed in this study. As the screw speed increased, *Salmonella* reduction decreased except at lower temperatures (< 70˚C). These results are in general consistent with those reported by Likimani et al. (1990) while studying the inactivation of *Bacillus globigii* during the extrusion of corn/soybean blend (70/30%, w/w). In their study moisture was maintained constant at 18% while temperature (110-130˚C) and screw speed (80-160 rpm) varied during processing. The study showed that increasing temperature resulted in greater reduction while increasing screw speed, which reduced the residence time, resulted in lower reduction. However, a different tendency was observed in another study by Walsh et al. (1974) where the effect of temperature (35-55˚C) and screw speed (12-30 rpm) on the

inactivation of *Salmonella* Typhimurium was evaluated during the extrusion of spaghetti. In this later study, results indicated that faster screw speed (12-30 rpm) resulted in greater reduction of *S.* Typhimurium at 35˚C due to mechanical shear.

3.5 Conclusion

This research investigated the inactivation of *Salmonella* over a broad range of factors like matrix moisture content (14, 20, 26%) and fat content (5, 10, 15%) and processing screw speed (75, 150, 225 rpm) and temperature (65, 75, 85˚C). A response surface model was developed to predict the effect of extrusion process variables and product characteristics on *Salmonella* inactivation. The extruder die exit temperature significantly affected the inactivation of *Salmonella* with higher temperatures leading to greater microbial reduction. Temperature also showed a significant interactive effect with the screw speed on *Salmonella* reduction. Regarding the fat content of the material to be extruded, it was shown to provide a protective effect on the inactivation of *Salmonella* where the higher the fat content resulting in lower *Salmonella* reduction. The moisture content had a significant linear and quadratic effect on microbial reduction. As the moisture content increased from 14 to 20 to 26%, the microbial log reduction first decreased and then increased. Screw speed by itself had a slight, but significant effect. As the screw speed increased, the microbial reduction decreased. However, the effect of screw speed was much smaller compared to the effect of the temperature.

In general, results indicated that extrusion is an effective method to reduce *Salmonella* in oat flour. A >5 log reduction was achieved for *Salmonella* when the processing temperature was above 85˚C at different moisture (14 to 26%) and fat (5 to 15%) contents during the extrusion of oat flour at screw speed of 150 rpm. The

response surface model developed in this research is a great tool for the food and feed industry in identifying the possible combinations of extrusion process conditions required to achieve a desired microbial reduction for *Salmonella*.

3.6 References

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Table 3.1. Coded levels of extrusion variables for *Salmonella* inactivation.

Variable	Coded Levels*	-1.633	-1	0	1	1.633
M, % wb		14	16.35	20	23.65	26
S, rpm		75	104	150	196	225
F, %		5	6.96	10	13.04	15

M= Moisture content of the product, S= Screw speed of the extruder, F= Fat content in the product

*Coded values used in experimental design in Table 3.2.

Table 3.2. Blocked 2nd order central composite design (Cox and Cochran 1953).

Block 1*			**Block 2**			**Block 3**		
x_1	**x_2**	**x_3**	**x_1**	**x_2**	**x_3**	**x_1**	**x_2**	**x_3**
-1	-1	1	-1	-1	-1	-1.633	0	0
1	-1	-1	1	-1	1	1.633	0	0
-1	1	-1	-1	1	1	0	-1.633	0
1	1	1	1	1	-1	0	1.633	0
0	0	0	0	0	0	0	0	-1.633
0	0	0	0	0	0	0	0	1.633
						0	0	0
						0	0	0

x_1 = Moisture content**;** **x_2** = Fat Content**;** **x_3** = Screw Speed

*Temperatures were applied to separate blocks so as each temperature was applied to each different type of block for a total of 60 runs.

Table 3.3. Final response surface model parameters for *Salmonella* inactivation in single-screw extruder (R^2=0.83).

Parameter	Estimates	SE	*P* value
Intercept	-8.5807	4.894	0.0862
T	0.3419	0.0505	<.0001
block (T) 65	1.8574	0.5581	0.0017
block (T) 65	1.8201	0.5581	0.0021
block (T) 65	1.1125	0.5432	0.0463
block (T) 75	0.7842	0.338	0.0251
block (T) 75	0.7314	0.3387	0.0361
block (T) 75	0	0	0
block (T) 85	-0.3242	0.3387	0.3435
block (T) 85	-0.0425	0.3387	0.9006
block (T) 85	0	0	0
F	-0.2676	0.0325	<.0001
M	-1.0076	0.2958	0.0014
S	0.07615	0.0199	0.0004
T*S	-0.0011	0.0002	0.0001
M*M	0.0277	0.0073	0.0005

SE = Standard error, T = extruder die exit temperature (˚C), M = moisture content (%,wb), F = Fat content (%), S = screw speed (rpm)

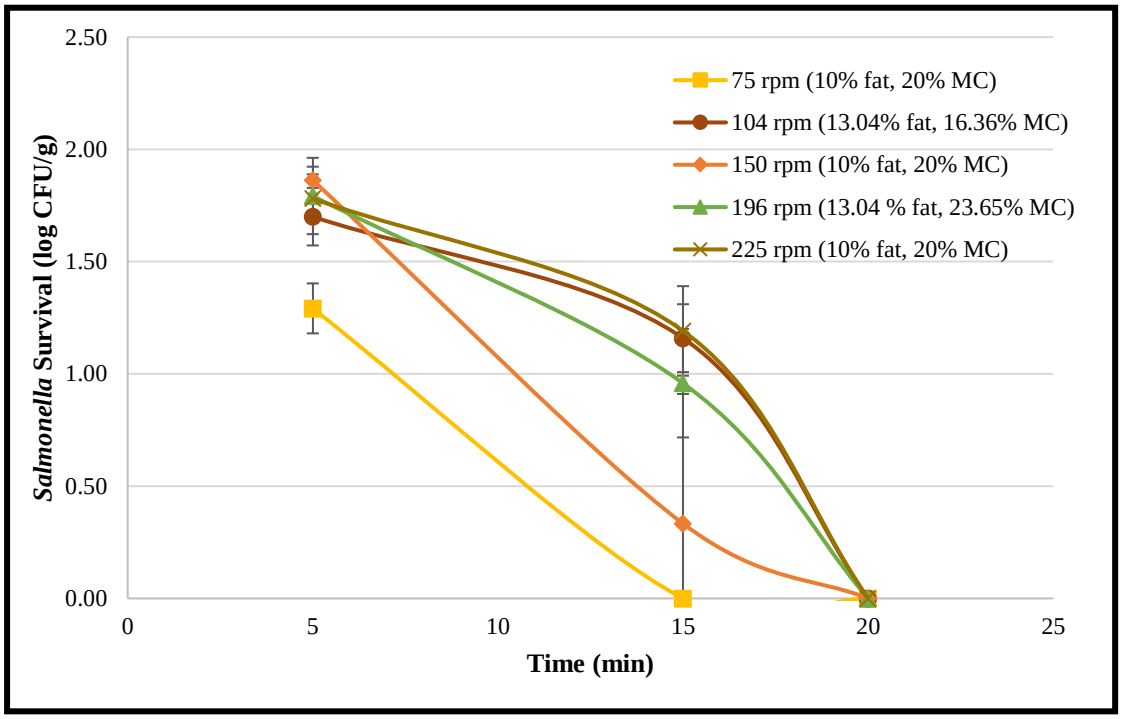


Figure 3.1. Uninoculated samples collected at 5, 15 and 20 min to establish time required to clean the extruder barrel between runs.

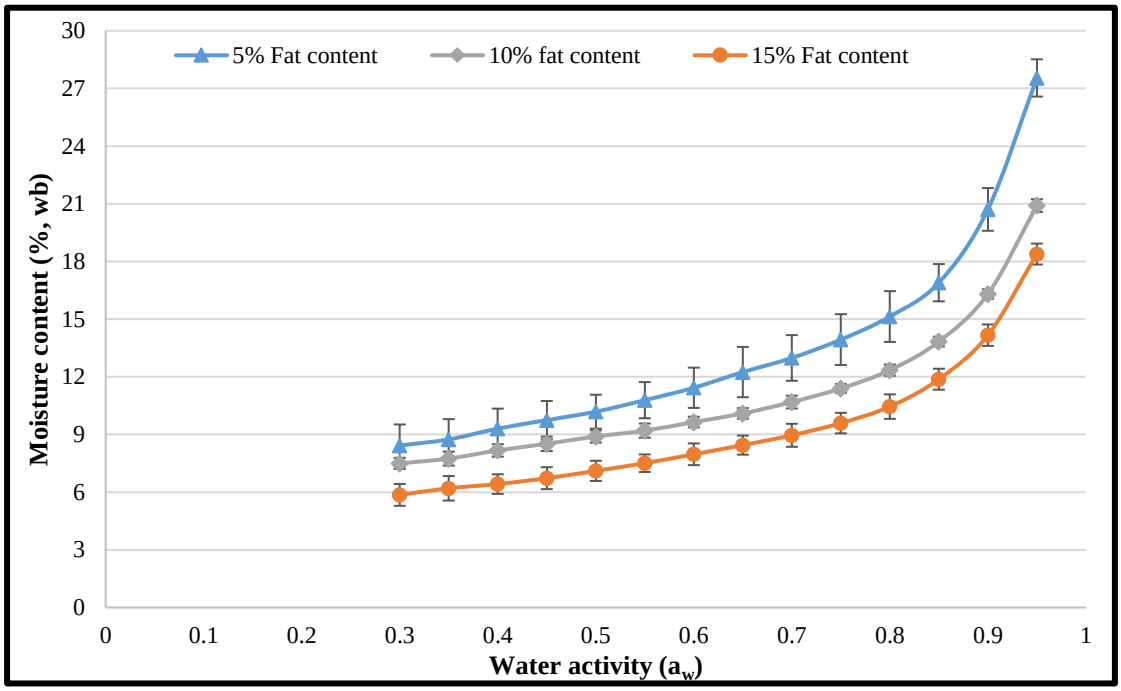
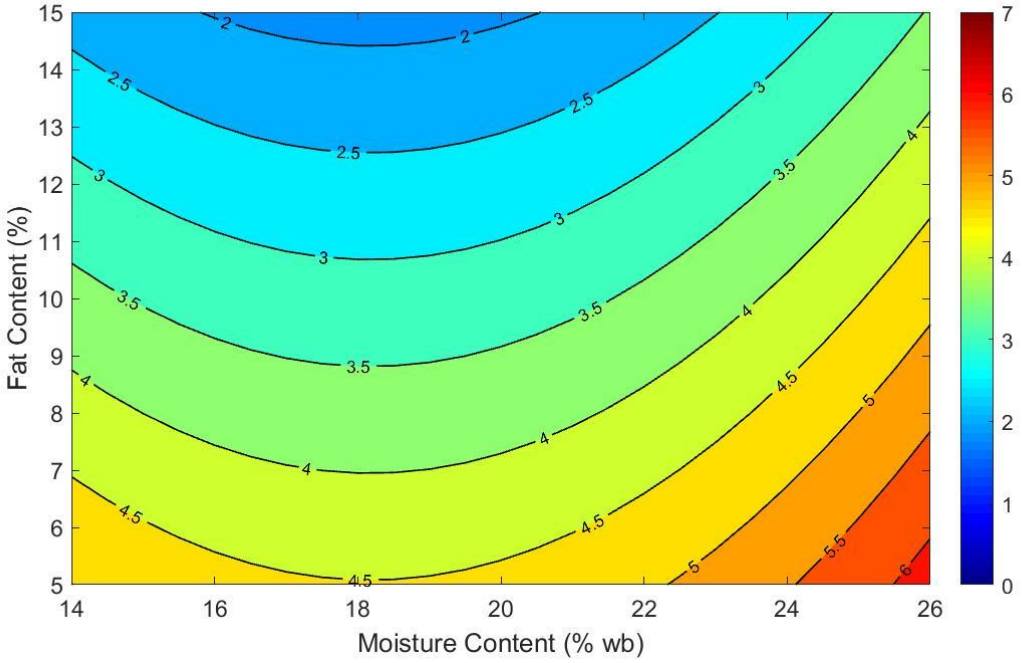
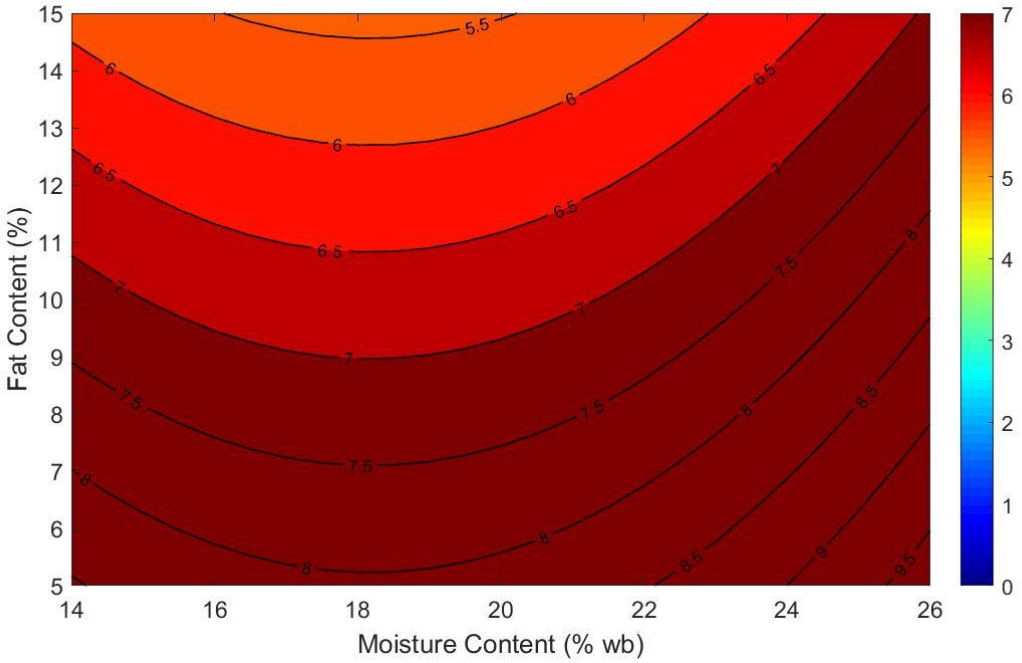


Figure 3.2. Moisture adsorption isotherm of oat flour (5, 10, and 15% fat) at 25˚C.

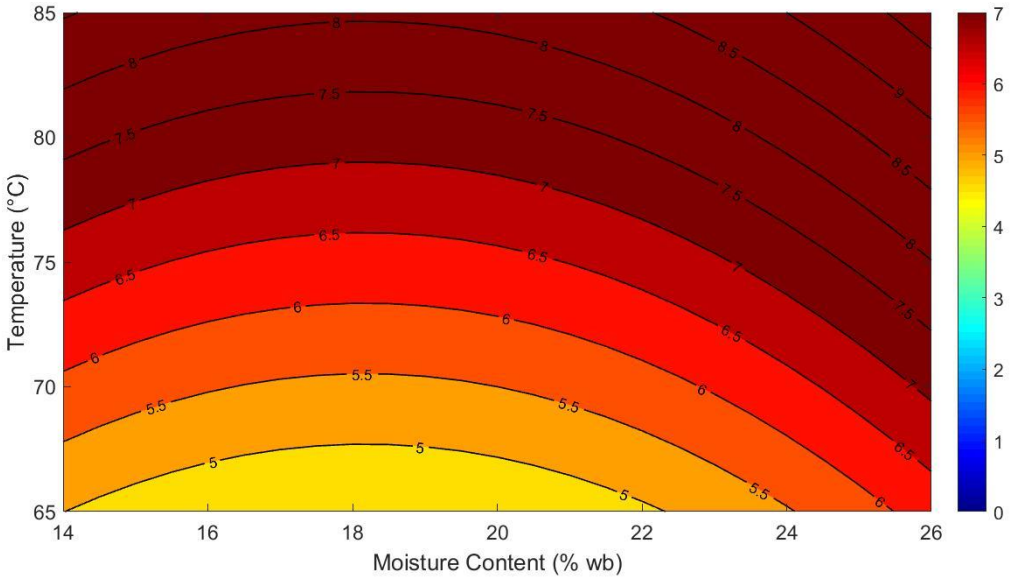


(A)

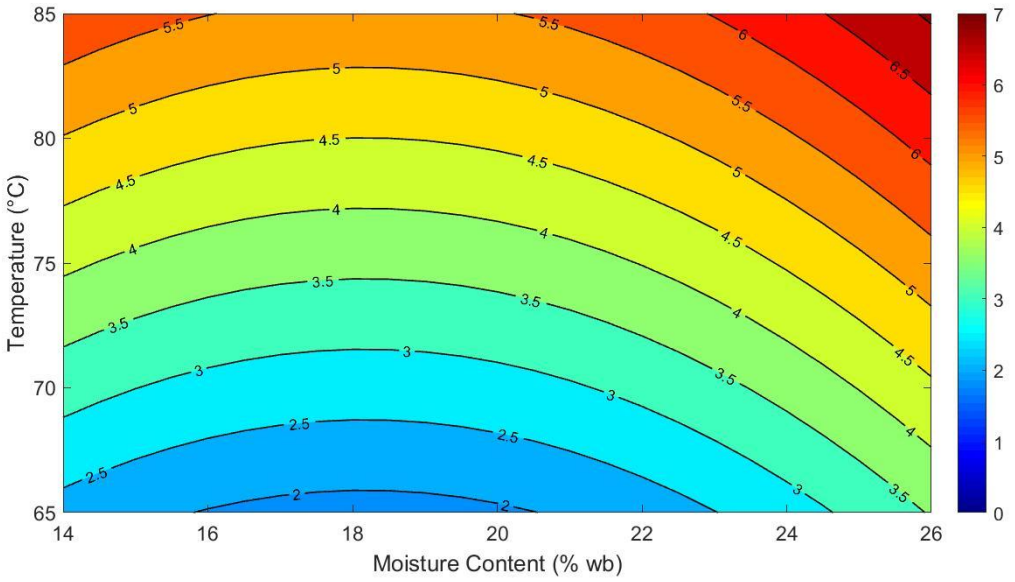


(B)

Figure 3.3. Contour surface plots showing *Salmonella* inactivation at temperatures of (A) 65˚C and (B) 85˚C when oat flour was extruded at a constant screw speed of 150 rpm at varying levels of moisture and fat content. The color bar indicates the log reduction (log N_o/N_t) of *Salmonella* corresponding to the range of colors.

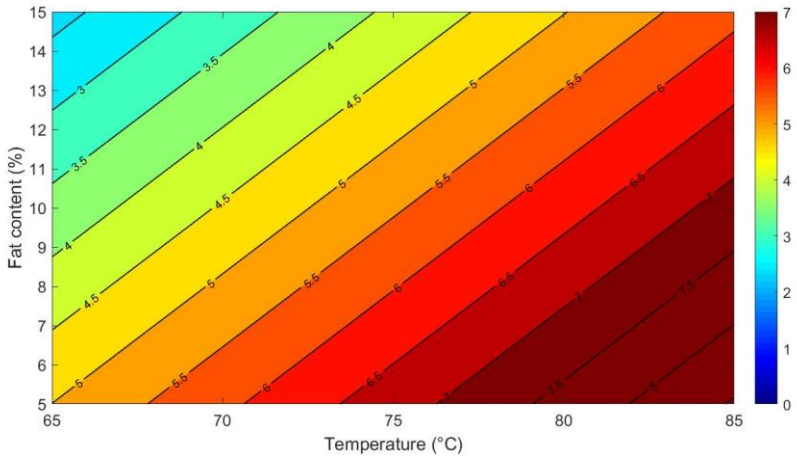


(A)

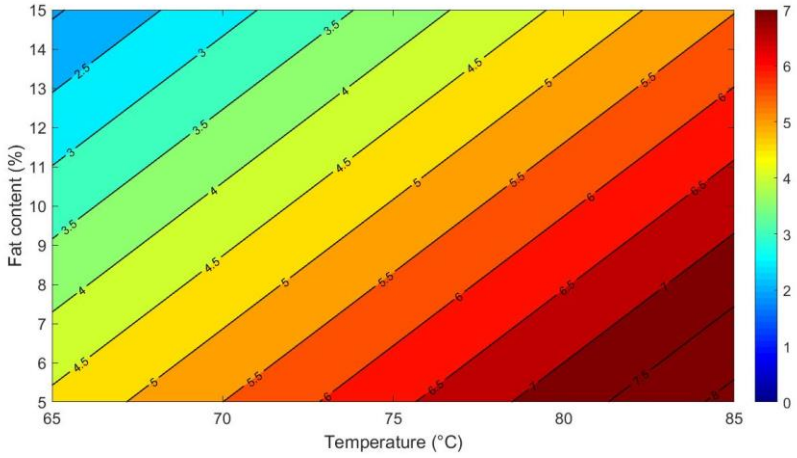


(B)

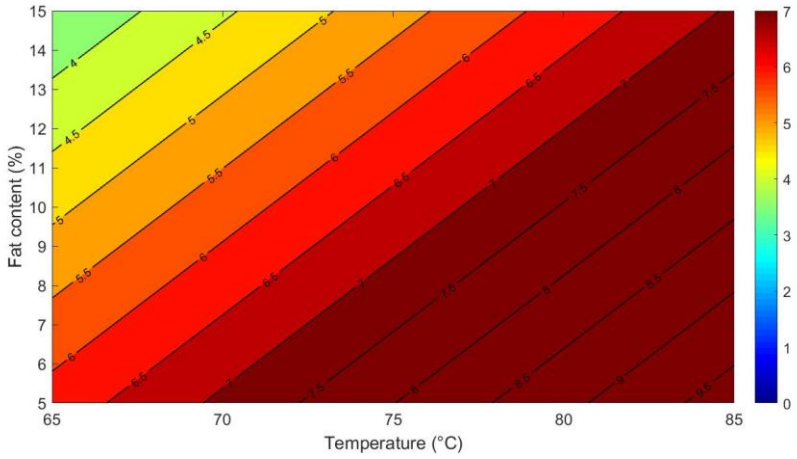
Figure 3.4. Contour surface plots showing *Salmonella* inactivation in formulations with fat contents of (A) 5% and (B) 15% when the oat flour was extruded at a screw speed of 150 rpm at varying levels of moisture content and temperature. The color bar indicates the log reduction (log N_o/N_t) of *Salmonella* corresponding to the range of colors.



(A)

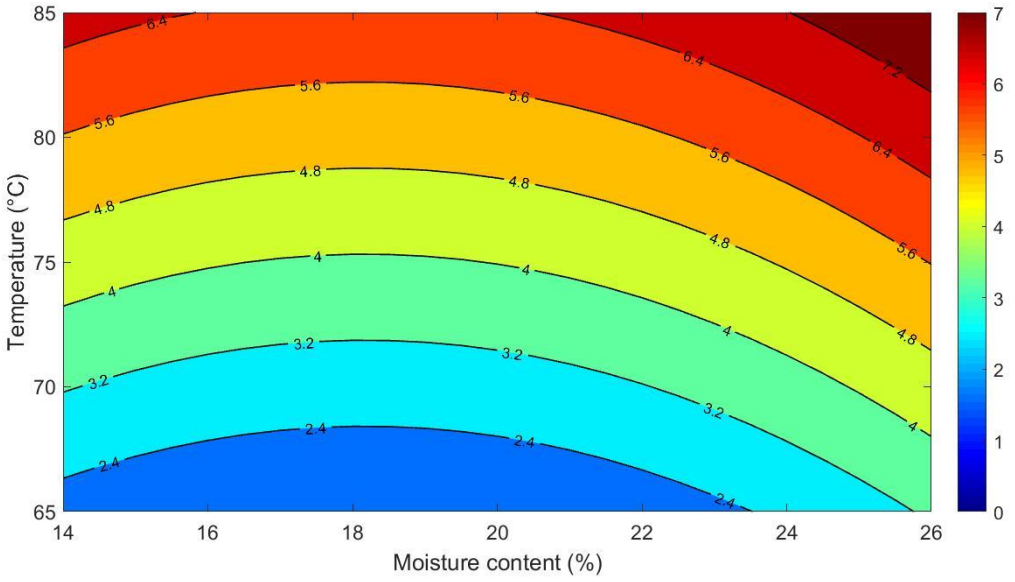


(B)

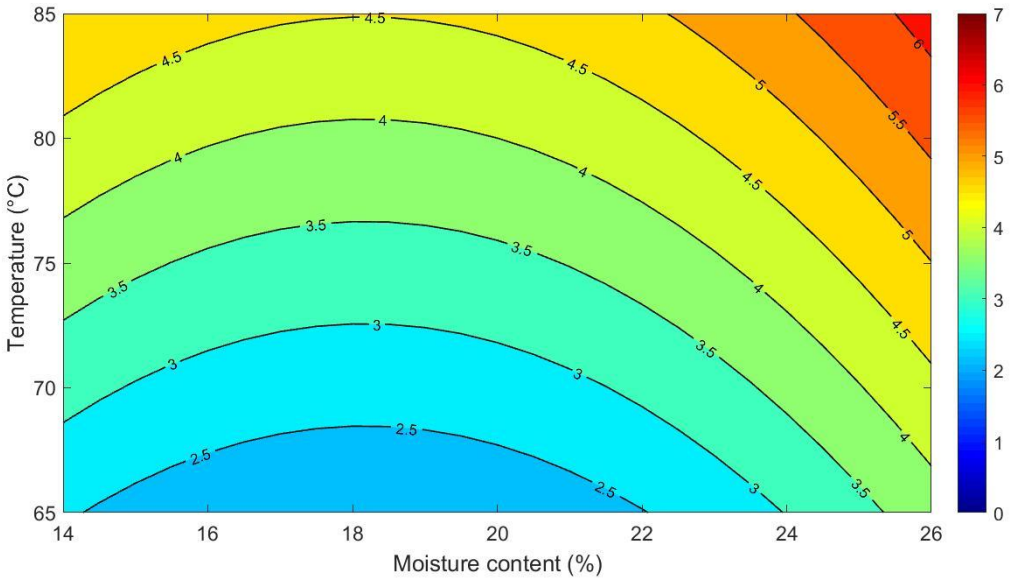


(C)

Figure 3.5. Contour surface plots showing *Salmonella* inactivation at moisture content of (A) 14%, (B) 20%, and (C) 26% when oat flour was extruded at a screw speed of 150 rpm at varying levels of temperature and fat content. The color bar indicates the log reduction (log N_o/N_t) of *Salmonella* corresponding to the range of colors.



(A)



(B)

Figure 3.6. Contour surface plot showing *Salmonella* inactivation at screw speeds of (A) 100 rpm and (B) 200 rpm when oat flour was extruded at a fat content of 15% at varying levels of moisture content and temperature. The color bar indicates the log reduction (log N_o/N_t) of *Salmonella* corresponding to the range of colors.

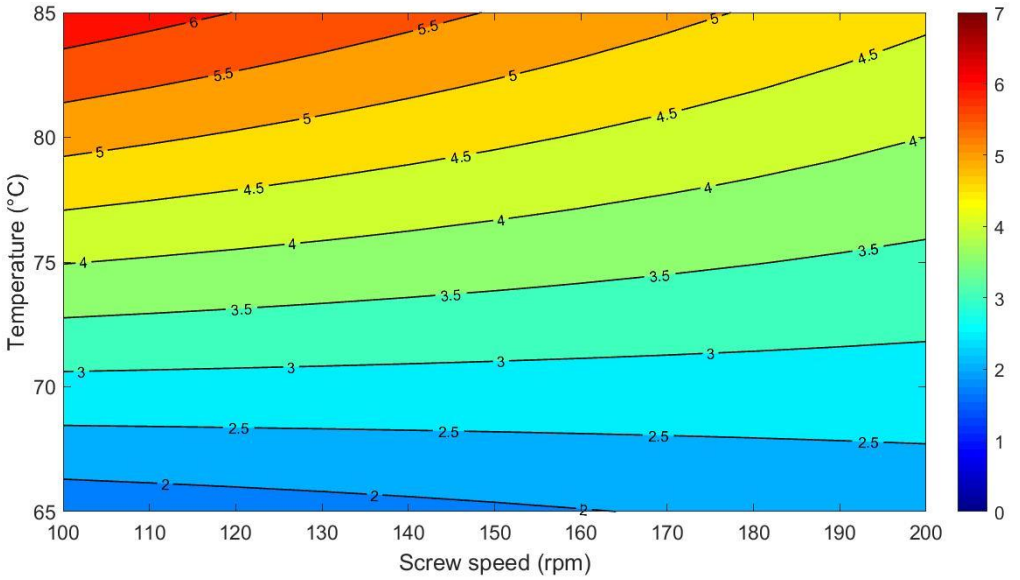


Figure 3.7. Contour surface plot showing the interaction between temperature and screw speed for *Salmonella* inactivation when oat flour was extruded at 20% moisture and 15% fat. The color bar indicates the log reduction (log N_o/N_t) of *Salmonella* corresponding to the range of colors.

Chapter IV: Evaluate the use of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* during extrusion of oat flour

Abstract

Salmonella in low-moisture foods is an emerging challenge due to numerous food product recalls and foodborne illness outbreaks. This has resulted in the need for development and validation of process controls to assure the microbiological safety of the final product. Additionally, the new Food Safety Modernization Act of 2011 requires food processors to validate preventive controls to ensure food safety. One of the challenges related to validation of processes for the inactivation of pathogens in low-moisture foods at the industrial level is the identification of an adequate surrogate for the target microorganism, for the specific product, and for the process being validated. The objective of this study was to evaluate *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* during the extrusion of low-moisture food. In this study, oat flour, a low-moisture food, was adjusted to different moisture (14 to 26% wb) and fat (5 to 15%) contents and was inoculated with *E. faecium*. Inoculated material was then extruded in a lab-scale single-screw extruder running at different screw speeds (75 to 225 rpm) and different temperatures (75 to 95ºC). A split-plot central composite 2nd order response surface design was used, with the central point replicated six times. Results indicated that *E. faecium* always had higher heat resistance compared to *Salmonella* at all conditions evaluated in this study. However, the patterns of contour plots showing the effect of various product and process parameters on inactivation of *E. faecium* was different from that of *Salmonella*. While *E. faecium* may be an acceptable surrogate for extrusion of low-moisture

products due to higher resistance than *Salmonella*, another surrogate with similar inactivation behavior may be preferred and needs to be identified.

4.1 Introduction

Outbreaks and recalls continue to be an issue not only in high-moisture food but also in low-moisture foods. Recently, several outbreaks of salmonellosis have been linked with the consumption of low-moisture foods. Therefore, low-moisture foods can no longer be considered safe simply because they do not support the growth of *Salmonella* (GMA 2009). Low-moisture foods are those with low-water activity which prevents the growth of pathogenic bacteria like *Salmonella*. However, even though growth does not occur, the low-water activity does not affect *Salmonella* that may already be present in the food. Therefore, *Salmonella* may persist for prolonged time in dry environments, being recognized now as a biological hazard in low-moisture foods and ingredients. In this context, the Food Safety Modernization Act (FSMA) signed a law on 4 January 2011, requires the food industry to implement and validate processing interventions to prevent and control identified hazards, including pathogens in low-moisture foods.

Usually, microbial challenge studies are conducted at laboratory-scale equipment using the pathogen of concern. However, sometimes it is hard to translate the results from laboratory-scale to industrial-scale processing systems especially process like extrusion. For those, validation at industrial-scale would be preferred but introduction of pathogen into the process would be unacceptable. That is when a surrogate organism needs to be identified. An ideal surrogate is a non-pathogenic microorganism that has equal or greater resistance to inactivation when compared to the pathogen of concern. According to Gurtler et al. (2014), there is a need for the

identification of appropriate surrogates to assist with large-scale validation studies for low-water activity foods.

One surrogate microorganism that has been commonly used in thermal processing of low-moisture food is *Enterococcus faecium* NRRL B-2354 (ABC 2014; Jeong et al. 2011a; Kornacki 2012). Also, the Almond Board of California found *Pantoea agglomerans* as an appropriate surrogate for *Salmonella* Enteritidis PT30 under dry heat almond processing (ABC 2007a). In another study, *Enterococcus faecium* NRRL B-2354 was used as a surrogate for *Salmonella* Enteritidis PT30 in almonds that was infrared heated at 120˚C (Bingol et al. 2011). Bianchini et al. (2014) determined that *Enterococcus faecium* NRRL B-2354 is an appropriate surrogate for *Salmonella* in a balanced carbohydrate-protein meal extruded at 28% moisture over a temperature range of 55-100˚C.

Enterococcus faecium NRRL B-2354 has been recognized as a surrogate for *Salmonella* in various validation studies. Bianchini et al. (2014) demonstrated the adequacy of the surrogate organism in extrusion process for one moisture content (28%) at one screw speed and one fat content. Therefore, a comprehensive evaluation of *E. faecium* as a surrogate in low-moisture food across a range of moisture content, fat content, and screw speed in addition to different temperatures is required. The objective of this study was then to validate the use of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* during the extrusion of a low-moisture model food over a range of moisture content, fat content, screw speed, and temperature.

4.2 Materials and Methods

4.2.1 Oat flour

Whole grain oat flour was used as a low-moisture food model and it was purchased from Bob's Red Mill (Milwaukie, OR, USA). The oat flour had an indigenous fat content of 5% and moisture content of 8.73% wb. Microbiological testing was performed to determine the background microflora and the presence of *Salmonella* in the flour. Twenty-five grams of flour was taken from each bag, diluted in 225 mL of 0.1% buffered peptone water (BPW; Becton, Dickinson and Company, Sparks, MD) and was plated on tryptic soy agar with 0.6% yeast extract (TSAYE; Becton, Dickinson and Company, Sparks, MD) and xylose lysine deoxycholate (XLD; Becton, Dickinson and Company, Sparks, MD) agar plates and incubated at 37˚C for 24 h. The results showed that the presence of *Salmonella* or any other microorganism was at non-detectable limit (<10CFU/g). Oat flour was stored under refrigerated conditions (4˚C) until its use.

4.2.2 Bacterial strain

For this study, *Enterococcus faecium* NRRL B-2354 was used as a potential surrogate for *Salmonella*. This organism was obtained from Agriculture Research Service at the U.S. Department of Agriculture. A cocktail of five different strains of *Salmonella enterica* was used in this study. The cocktail included *Salmonella* Agona 447967, *Salmonella* Enteritidis PT30, *Salmonella* Tennessee K4643, *Salmonella* Montevideo 488275 and *Salmonella* Mbandaka 698538. All the strains were stored at -80˚C by creating a frozen stock in 80% glycerol.

4.2.3 Experimental design

A split-plot second order central composite design was used with temperature as a whole-plot factor and moisture content, fat content, and screw speed as the split-plot factors. The split-plot experiments are special blocked experiments in which blocks are the whole-plot units to which the levels of the whole-plot factor are assigned and split-plot treatment factors are assigned to the split-plot units within each block (Chukwu et al. 2012; Mugabi et al. 2017). Here moisture, fat, and screw speed are applied to 1 kg samples of product which are the split-plot experimental unit for these three factors. Since extruder die exit temperature is hard to modify and stabilize during the experiment. Therefore, the temperature was used as whole-plot factor and was applied to blocks of either 6 or 8, one-kg samples at a time (Table 4.1).

Blocking provides the opportunity for obtaining increased precision (Cox and Cochran 1953). The experiment was replicated 3 times for a total of 60 runs, where temperatures were applied to blocks as described in Table 4.1.

4.2.4 Preparation of inoculum and sample inoculation

For preparing the inoculum, a frozen stock of *E. faecium* was streaked on TSAYE for resuscitation of culture and incubated for 48 h at 37˚C. One isolated colony from TSAYE was also streaked on m-Enterococcus Agar (m-EA; Becton, Dickinson and Company, Sparks, MD) for confirmation of no contamination. The bacterium was then transferred to 10 mL tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) and incubated for 24 h at 37˚C. The TSB was later used to create lawns by spreading 100 µL on TSAYE plate. After 24 h, cells were harvested by adding 3 mL of 0.1% BPW to each plate, loosening the lawns with

sterile spreader. The method used to prepare the *Salmonella* cocktail has been described in Chapter 3.

The prepared inoculum was used to inoculate the flour at a level of ca. 7.5 log CFU/g. A commercial mixer (Kitchen aid, S. No. W53294842) was used which was capable to handle 1 kg of flour. The oat flour (1 kg) was aseptically transferred in a sterile mixer bowl. The amount of water and oil required was calculated based on the initial moisture content and fat content of the flour. The amount of inoculum (10 mL) to be added was subtracted from the amount of water. After adding desired amount of water, inoculum, and oil to the flour, the mixer was set to run on lowest speed for 5 min. After 5 min, the flour was scraped off well from the paddle and the sides of mixer bowl, was then mixed again at lowest speed for another 5 min. The prepared inoculated sample was transferred to the sterile bag and stored at room temperature for five days for homogenization of moisture and adaption of bacterial cells to the new environment. The moisture content was adjusted to 14.00, 16.35, 20.00, 23.65 and 26.00% and fat content was adjusted to 5.00, 6.96, 10.00, 13.04, and 15.00%, respectively (Table 4.2).

4.2.5 Extrusion of inoculated samples

The prepared inoculated samples were then extruded in a laboratory-scale GR-8 single-screw extruder (C.W. Brabender Instruments, South Hackensack, NJ). The extruder screw had a compression ratio of 3:1 with length/diameter ratio of 20:1. The flour was fed into the extruder using an external volumetric feeder whose speed was controlled by a frequency controller (Brabender Technologie, Ontario, CA). The feeder had a screw size of 18 mm and a pitch of 19 mm. The die face was equipped

with the thermocouple (CW Brabender Instruments, South Hackensack, NJ) that constantly measured the product temperature throughout the extrusion process.

At industrial level, extrusion is usually not performed with barrel filled at 100% of its fill capacity. To simulate such conditions, extrusion was then performed with the barrel filled at 75% fill capacity. Information from a preliminary experiment conducted in Chapter 3 was used to identify the feed rate to achieve 75% capacity for different screw speeds in the lab-scale extruder.

The extruder barrel had three temperature zones with temperature of the first two zones maintained at 50˚C, while the last zone was adjusted to achieve the desired product temperature as determined by the experimental design. No die was used at the die face, which resulted in minimal back-pressure. When compared to the industrial extrusion, this study would then represent the worst-case scenario.

After achieving the desired product temperature at the die, steady state operation was achieved by running the extruder for another 5 min before collecting the samples. Samples were then collected for 5 min in a sterile whirl pack and cooled immediately in an ice-water bath.

4.2.6 Moisture Determination

The samples were also tested for moisture content before and after the extrusion. A Halogen Moisture Analyzer HR73 (Mettler Toledo Laboratory and Weighing Technologies, Greifensee, Switzerland) was used to measure the moisture content.

4.2.7 Microbial Enumeration

To enumerate the population of *E. faecium*, samples were plated before and after the extrusion process. From the bulk samples, two subsamples were taken for

microbial analysis. Bacterial counts were determined by using serial dilution method, where 25 g of sample was diluted in 0.1% BPW to achieve a 1:10 dilution. Subsequent dilutions were prepared to allow the proper growth of bacteria with counts ranging from 25-250 colonies.

For enumeration of *E. faecium*, samples were plated on TSAYE and m-EA and incubated at 37˚C for 48 h. The non-selective medium (TSAYE) allowed the growth of heat injured *E. faecium* cells or any other bacteria capable of growing under the incubation conditions. The selective and indicator medium (m-EA), contains sodium azide as a selective agent that helps in suppressing the growth of gram-negative organisms. Additionally, in the m-EA medium, triphenyl tetrazolium chloride is the indicator dye, which is reduced to the insoluble formazan inside the bacterial cell, resulting in the production of red colonies. The enumeration method used for *Salmonella* has been described in Chapter 3.

4.2.8 Statistical Analysis

A split-plot analysis of variance (ANOVA) was used to determine the effect of moisture content, fat content, temperature, and screw speed on inactivation of *E. faecium*. SAS version 9.4 (SAS Institute, Cary, NC) was used to fit the final response surface model following the procedures of testing the highest order terms. Only factors with significant effects ($p < 0.05$) were considered in the response surface model. MATLAB R2016b (MathWorks, Natick, MA) was used to create the contour plots using the response surface equation and Microsoft Excel v.2013 was used to compare the heat resistance of *Salmonella* and *E. faecium* by creating a scatter plot.

4.3 Results and Discussion

4.3.1 Extrusion of Flour Inoculated with *E. faecium*

The oat flour was inoculated with *E. faecium* at a level of ca. 7.5 log CFU/g. This flour was extruded at different conditions based on the experimental design. A steady state condition with desired temperature and flow rate was achieved by running the uninoculated oat flour through the extruder. Temperature is usually considered as the most important factor during the thermal inactivation of microorganisms (Bianchini et al. 2014). Therefore, the die exit temperature was continuously monitored by a thermocouple equipped at the die face during the complete extrusion run. Once the desired temperature was achieved at the die, inoculated sample was introduced. In between the trials, a push-through sanitation was performed using the uninoculated oat flour for the proper cleaning of the barrel. Chapter 3 demonstrated that 20 min was sufficient for push-through sanitation at all different screw speeds.

Samples were analyzed for moisture content before and after the extrusion process. An approximate moisture loss of 1.0 to 3.0% was seen in all the samples. Bianchini et al. (2012) reported a loss of 0.5 to 2.0% during the extrusion of a carbohydrate-protein meal at temperatures ranging from 67.5 to 85˚C. The study done here had a slightly higher temperature range (75-95˚C) which resulted in a slightly higher moisture loss (1.0-3.0%). These results are also in agreement with the study done in Chapter 3, which reported a loss of 1.5 to 2.5% moisture during extrusion at similar conditions to those reported here. In industrial applications moisture losses obtained by flash-off as hot products expand at the die during the extrusion may be as high as 4.0 to 7.0%.

Extruded samples were plated on TSAYE and m-EA for enumeration of *E. faecium*. TSAYE is a non-selective media which allowed the growth of any heat injured cells and other bacteria surviving the extrusion process, whereas m-EA is a selective media which provided specific information about survivors of *E. faecium*. Results indicated that bacterial counts on TSAYE was always higher than m-EA, which was expected since the non-selective media allowed the growth of all bacteria surviving the extrusion process. Therefore, the data from TSAYE was not used for the statistical analysis. Instead the data from the selective media (m-EA) was used for all statistical analysis and to build the response surface model for inactivation of *E. faecium*.

4.3.2 Response Surface Model for *E. faecium* Inactivation

A split-plot analysis of variance model with terms for second-order response surface fit for *E. faecium* reduction when extruded at different temperatures, screw speed, moisture content, and fat content. The final intercept in the equation was calculated by taking the average of estimate of all blocks and was added to the intercept value given in Table 4.3. After removing the non-significant terms ($p \geq 0.05$), the model fit well with $R^2 = 0.84$. The final response surface model for *E. faecium* inactivation is presented in Equation 4.1.

$$\text{Log}\,(N_o/N_t) = 4.4588 + 0.243*T - 1.06*F - 1.099*M - 0.0085*S + 0.0473*F*M + 0.0349*F^2 + 0.0141*M^2 - 0.0093*T*F \quad (4.1)$$

Where:

N_o = Initial bacterial population before extrusion (CFU/g)

N_t = Final bacterial population after extrusion (CFU/g)

T = Extruder die exit temperature (˚C)

M = Moisture content of sample before extrusion (% wb)

F = Fat content (%)

S = Screw speed (rpm)

The model showed a significant linear effect of temperature and screw speed. Also, fat content and moisture content showed both a significant linear and quadratic effects. Among all variables, fat content showed a significant interaction with temperature and moisture content (Table 4.3).

4.3.2.1 Effect of Temperature

Inoculated samples were extruded at different temperatures (75, 85, and 95˚C). Based on results, temperature showed a significant positive effect on the inactivation of *E. faecium*. Figure 4.1 shows that within each contour plot, as the temperature increases, the log reduction increases. At 5% fat and 14% moisture content, >5 log reduction was achieved at temperature above 80˚C. However, as the fat and moisture content increased, a higher temperature would be required to achieve the same log reduction.

Results obtained here are in agreement with other studies. Crane et al. (1973) reported that thermal extrusion completely eradicated *Salmonella* from feed when the temperatures were above 93.3˚C at 25 to 35% moisture content. Anderson et al. (2017) studied the effect of temperature (65-100˚C) and water activity (0.72-0.96) on inactivation of *Salmonella* during extrusion of oat flour. Their results indicated a reduction of 5 log CFU/g when the temperature was above 85˚C. However, it seems that the matrix under consideration may have an effect as Bianchini et al. (2012) reported that the minimum temperature needed to achieve 5 log reduction of *E. faecium* in a carbohydrate-protein meal was 81.1˚C at 28.1% moisture whereas, under

similar conditions, using Equation 4.1, the reduction achieved in oat flour would be 1.7 log CFU/g. This difference in reduction might be due to the use of different product matrix or the use of die in the study done by Bianchini et al. (2012) which adds back-pressure whereas no die was used in this study.

4.3.2.2 Effect of Fat Content

Fat content showed both a significant linear and quadratic effect on the inactivation of *E. faecium*. In general Figure 4.1 shows that as the fat content increased from 5 to 15%, the log reduction obtained at the same moisture and temperature decreased. For example, at 14% moisture content, 5 log reduction was achieved at temperatures above 77˚C at the lowest fat content (5%), whereas, only 2.4 log reduction was achieved at the highest fat content (15%) even at the highest temperature (95˚C). However, when the effect of fat content is further explored, it shows an interaction with moisture content. Figure 4.2 helps to further explain this interactive effect when samples were extruded at 75˚C and 150 rpm. A considerable quadratic effect is displayed by the fat content at the highest moisture content (26%). However, as the moisture content decreased, the quadratic effect of fat content also reduced. A similar effect was seen at other temperatures and screw speeds. Figure 4.2 also shows that at the lowest moisture content (14%), as the fat content increased, bacterial reduction decreased, as expected. Surprisingly, the trend changed at the highest moisture content (26%) where the bacterial reduction increased with the increase in fat content. This shows that the protective effect of the fat present in the samples was more pronounced at lower moisture contents.

Very few studies have evaluated the effect of fat content on microbial reduction in different foods. Li et al. (2009) reported that peanut butter with a fat level

of 50% enhanced *Salmonella* survival compared to product with 33.33% fat content. Smith et al. (2001) found that *Salmonella* in beef containing 19% fat was more heat resistant than in beef containing 4.8% fat. Juneja et al. (2000) reported the effect of fat content (4 to 28%) in ground beef and pork, at different temperatures that ranged from 58 to 68˚C. Their results showed an increase in D-value of *Salmonella* with increasing fat levels. The protective effect of fat was also shown in another study by Gurman et al. (2016) who reported that increasing fat content was correlated with longer *Salmonella* persistence during cooking of pork patties. However, the effect of this variable was reduced at higher cooking temperatures (Gurman et al. 2016).

4.3.2.3 Effect of Moisture Content

The effect of different moisture content (14, 20, and 26%) on *E. faecium* inactivation was studied. Based on the results obtained, moisture content showed a significant linear and quadratic effect on the inactivation of *E. faecium*. In high-moisture foods, microbial inactivation increases, as the moisture content or water activity increases. The same trend was observed at the highest fat content (15%). Figure 4.1 shows that as the moisture content increased from 14 to 26%, the log reduction of *E. faecium* increased in those samples with 15% fat. However, as the fat content decreased to 5%, the trend reversed. This can also be seen on the response surface equation (Equation 4.1), where there is a significant interaction term between moisture content and fat content.

Previous research has demonstrated a quadratic effect of moisture content on the reduction of different microorganisms (Anderson et al. 2017; Bianchini et al. 2012). Anderson et al. (2017) reported a quadratic effect of water activity on *Salmonella* reduction. Their results indicated that as the water activity increased from

0.72 (14%) to 0.96 (28%), the log reduction decreased first and then increased. Bianchini et al. (2012) also observed a significant quadratic effect of moisture content on the inactivation of *E. faecium*. However, in their research higher log reduction for *E. faecium* was achieved when compared to those reported here. According to Bianchini et al. (2012) a 5 log reduction of *E. faecium* was achieved at 81.1˚C and 28.1% moisture during extrusion of a carbohydrate-protein meal. The higher values reported by Bianchini et al. (2012) may be the result of a product matrix effect or extruder set up.

4.3.2.4 Effect of Screw Speed

To understand the effect of screw speed on the inactivation of *E. faecium*, contour plots were built with screw speeds as a variable (100 to 200 rpm) (Figure 4.1). According to the results, screw speed showed a significant linear effect on *E. faecium* inactivation. As the screw speed increases from 100 to 200 rpm, *E. faecium* inactivation decreases. The higher the screw speed the lower the residence time inside the extruder barrel. Okelo et al. (2006) demonstrated that the residence time inside the barrel affects the inactivation of microorganisms. Here at 14% moisture and 5% fat content, as the screw speed increased, higher temperatures were required for a desired log reduction. Similar trends were also seen at other moisture and fat contents. These results are consistent with the study conducted by Likimani et al. (1990) who studied the inactivation of *Bacillus globigii* during the extrusion of corn/soybean blend (70/30%, w/w) at a moisture of 18% running at different temperatures (110-130˚C) and screw speeds (80-160 rpm). Their study showed that increasing screw speed, which reduced the residence time, resulted in lesser bacterial reduction.

4.3.3 Comparison of Microbial Reduction between *E. faecium* and *Salmonella*

Figure 4.3 represents the bacterial reduction data for *Salmonella* and *Enterococcus faecium* during extrusion of oat flour at 75˚C and 85˚C irrespective of fat content, moisture content, or screw speed. The purpose of this scatter plot was to compare the heat resistance of *E. faecium* with *Salmonella*. The correlation coefficient between reduction of *Salmonella* and *E. faecium* during the extrusion processing of oat flour was $R^2 = 0.33$. The data presented in Figure 3 shows that reduction of *E. faecium* was always lower than those observed for *Salmonella*. A 95% lower confidence interval was also generated for the reduction of *E. faecium*. The results showed that even under the worst-case situations, reduction of *E. faecium* was always lower than *Salmonella*. This indicates that *E. faecium* was always more heat resistant than *Salmonella* when processed under similar conditions. Additionally, there was a margin of ca. 4.0 log CFU/g between the microbial reduction observed for *Salmonella* and *E. faecium*, indicating the suitability of this organism as a surrogate for *Salmonella* during the extrusion process.

To further evaluate the use of *E. faecium* as a surrogate for *Salmonella*, the effect of moisture content, fat content, temperature, and screw speed on their inactivation was compared. Due to their individual response to the process of extrusion, data for *E. faecium* inactivation was collected in the temperature range of 75 to 95˚C, while *Salmonella* inactivation data was collected in the range of 65 to 85˚C (Chapter 3). To compare the behavior of both organism during extrusion, the models were extrapolated to the same temperature range of 65 to 95˚C (Figure 4.1 and 4.4).

According to the inactivation data, temperature showed a pronounced significant effect on the reduction of both *Salmonella* and *E. faecium*. As the temperature increased from 65 to 95˚C, log reductions increased. Figure 4.4 shows that a 5 log reduction of *Salmonella* was achieved at temperatures as low as 65˚C depending upon the conditions fixed, whereas for *E. faecium*, a 5 log reduction was only achieved when the temperature was above 80˚C at the lowest fat (5%) and moisture content (14%) (Figure 4.1). Additionally, for *E. faecium* as the moisture and fat content increased, a higher temperature was required to achieve a desired log reduction.

The fat content had a significant protective effect on inactivation of *Salmonella*, with increasing fat content correlated with longer persistence of this pathogen during the extrusion of oat flour. As the fat content increased from 5 to 10, and to 15%, the minimum temperature required for a 5 log reduction among all moisture content and screw speed tested was 68, 72, and 83˚C (Figure 4.4). For *E. faecium*, the protective effect of the fat content was dependent on the moisture content. This was due to the significant interaction between these two variables (Equation 4.1). In summary, the fat content showed an interactive effect with moisture on *E. faecium* inactivation; whereas fat content just had a simple protective effect on the inactivation of *Salmonella*.

The moisture content showed a significant effect on the inactivation of both *Salmonella* and *E. faecium* during extrusion. Figure 4.4 shows that as the moisture content increased from 14 to 20%, *Salmonella* reduction decreased slightly when all other variables were maintained, and then increased from 20 to 26%. For *E. faecium*, as the moisture content increased from 14 to 26%, the bacterial log reduction

decreased at 5% fat (Figure 4.1). However, the trend reversed as the fat content was increased to 15%. This can be explained by the significant interaction effect between moisture and fat contents which was not observed while evaluating *Salmonella*. Results showed that the effect of the moisture content on bacterial inactivation was different for *Salmonella* and *E. faecium*.

A significant linear effect of screw speed was seen for both *Salmonella* and *E. faecium*. Specifically, for *Salmonella*, as the screw speed increased from 100 to 200 rpm, microbial log reduction decreased slightly (Figure 4.4). There was also a significant interaction effect between temperature and screw speed on the inactivation of *Salmonella*. So, at lower temperature (<70˚C), as the screw speed increased, the temperature required to achieve a certain log reduction decreased. However, at temperatures >70˚C, as the screw speed increased, higher temperature was required to achieve the same log reduction. For *E. faecium*, as the screw speed increased, the log reduction decreased (Figure 4.1) and no interaction between temperature and screw speed was observed.

Results obtained from our study indicated that the effect of moisture content, fat content, and screw speed on inactivation of *E. faecium* was different from that of *Salmonella*. However, for a microorganism to be described as a surrogate, it is important that it responds in a similar pattern as the target pathogen. In fact, according to the FDA states that a surrogate must be "a non-pathogenic species and strain responding to a particular treatment in a manner equivalent to a pathogenic species and strain" (FDA 2000). If this requirement alone is taken into consideration perhaps *E. faecium* would not be the ideal surrogate for *Salmonella* during extrusion of low-moisture foods. However, the criteria for electing a surrogate is broader and must

consider: non-pathogenic, similar behavior, easy to culture, and more resistant than the pathogen of concern. When these factors are considered it is plausible to consider *E. faecium* as a suitable surrogate for *Salmonella* in extrusion of low-moisture foods.

In fact, *E. faecium* NRRL B-2354 is a non-pathogenic microorganism that has been used as an appropriate surrogate for *Salmonella* during thermal inactivation studies in different products (ABC 2014; Jeong et al. 2011a; Kornacki 2012). In a study done by Bingol et al. (2011) it was reported the use *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* Enteritidis PT30 in almonds that were treated with infrared heat at 120˚C. Bianchini et al. (2014) determined that *Enterococcus faecium* NRRL B-2354 is an appropriate surrogate for *Salmonella* during the extrusion of a balanced carbohydrate-protein meal at 28% moisture over a temperature range of 55-100˚C. Villa-Rojas et al. (2017) also reported that *E. faecium* is a an adequate surrogate for *Salmonella* during RF treatment of wheat flour.

Other surrogates for *Salmonella* have also been proposed. The Almond Board of California found *Pantoea agglomerans* to be an appropriate surrogate for *Salmonella* Enteritidis PT30 when almonds were processed by dry heat (ABC 2007a). Ceylan et al. (2015) reported that *Pediococcus acidilactici* ATCC 8042 can be utilized as a surrogate for *Salmonella* in dry pet food that are thermally processed at temperature below 90˚C. Jeong et al. (2011b) demonstrated that *Pediococcus* sp. NRRL B-2354 can be used as a surrogate for *Salmonella* Enteritidis PT30 during moist-air heating. Liu et al. (2015) demonstrated that *E. faecium* NRRL B-2354 is a suitable surrogate for *Salmonella* during thermal treatments of wheat flour. In all these instances, results indicated that *Salmonella* suffered a greater reduction than *E. faecium* when both organism were subjected to similar treatments.

The results reported here corroborate with findings in the literature since they indicate that *E. faecium* had a greater thermal resistance than *Salmonella* during the extrusion of oat flour and could therefore serve as a surrogate to validate extrusion processes. However, that effect of fat content, moisture content, and screw speed may affect the inactivation of *E. faecium* differently than *Salmonella*. While based on thermal resistance alone *E. faecium* may be an acceptable surrogate for *Salmonella*, other surrogates with more similar inactivation behaviors may be preferred and would need to be identified.

4.4 Conclusion

The current study evaluated the use of *E. faecium* NRRL B-2354 as a surrogate for *Salmonella* during the extrusion of low-moisture food. The inactivation of *E. faecium* was studied over a broad range of moisture content, fat content, screw speed, and temperature. Temperature showed a significant effect on *E. faecium* inactivation with microbial reduction increasing with an increase in temperature. The protective effect of fat content was dependent on the moisture content due to a significant interactive effect between these two variables. At 15% fat content, as the moisture content increased, the log reduction increased. However, at 5% fat content, the trend reversed. Moisture by itself also showed a significant quadratic effect on inactivation of *E. faecium*. Screw speed had a significant linear effect on *E. faecium* inactivation.

To evaluate the use of *E. faecium* as a surrogate for *Salmonella*, the effect of temperature, moisture content, fat content, and screw speed on the inactivation of both microorganisms were compared. The results indicated that the two microorganisms tested showed a different response to the processing depending upon fat content,

moisture content, and screw speed. However, temperature showed a similar effect on both the microorganisms when thermal inactivation was considered. The reduction of *E. faecium* by extrusion was always lower than the one obtained for *Salmonella* under similar conditions. Therefore, *E. faecium* may be an acceptable surrogate for *Salmonella* due to its higher thermal resistance.

4.5 References

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Table 4.1. Blocked 2nd order split-plot central composite design (Cox and Cochran 1953).

Block 1*			**Block 2**			**Block 3**		
x_1	**x_2**	**x_3**	**x_1**	**x_2**	**x_3**	**x_1**	**x_2**	**x_3**
-1	-1	1	-1	-1	-1	-1.633	0	0
1	-1	-1	1	-1	1	1.633	0	0
-1	1	-1	-1	1	1	0	-1.633	0
1	1	1	1	1	-1	0	1.633	0
0	0	0	0	0	0	0	0	-1.633
0	0	0	0	0	0	0	0	1.633
						0	0	0
						0	0	0

x_1 = Moisture content**; x_2** = Fat Content**; x_3** = Screw Speed

*Temperatures were applied to separate blocks so as each temperature was applied to each different type of block for a total of 60 runs.

Table 4.2. Coded levels of extrusion variables for *E. faecium* inactivation.

Variable	Coded Variable Levels*				
	-1.633	**-1**	**0**	**1**	**1.633**
Fat Content (%)	5	6.96	10	13.04	15
Moisture Content (% wb)	14	16.35	20	23.65	26
Screw Speed (rpm)	75	104	150	196	225

*Coded values used in experimental design in Table 4.1.

Table 4.3. Statistical analysis of the parameters (temperature, screw speed, fat content, and moisture content) for *E. faecium* inactivation during extrusion of oat flour (R^2=0.84).

Parameter	Estimates	SE	*P* value
Intercept	4.2469	4.943	0.3949
T	0.2429	0.0423	<.0001
block (T) 75	0.5972	0.4660	0.2067
block (T) 75	0.6789	0.4660	0.1523
block (T) 75	0.1175	0.4536	0.7968
block (T) 85	0.2876	0.2828	0.3147
block (T) 85	0.3193	0.2828	0.2650
block (T) 85	0	0	0
block (T) 95	0.03478	0.2828	0.9027
block (T) 95	-0.1285	0.2828	0.6517
block (T) 95	0	0	0
F	-1.0656	0.3865	0.0085
M	-1.0997	0.2657	0.0002
S	-0.0085	0.0018	<.0001
F*M	0.04735	0.00963	<.0001
F*F	0.0349	0.0088	0.0003
M*M	0.0141	0.00616	0.0267
T*F	-0.00928	0.0033	0.0078

SE = Standard error, T = extruder die exit temperature (˚C), M = moisture content (%, wb), F = Fat content (%), S = screw speed (rpm)

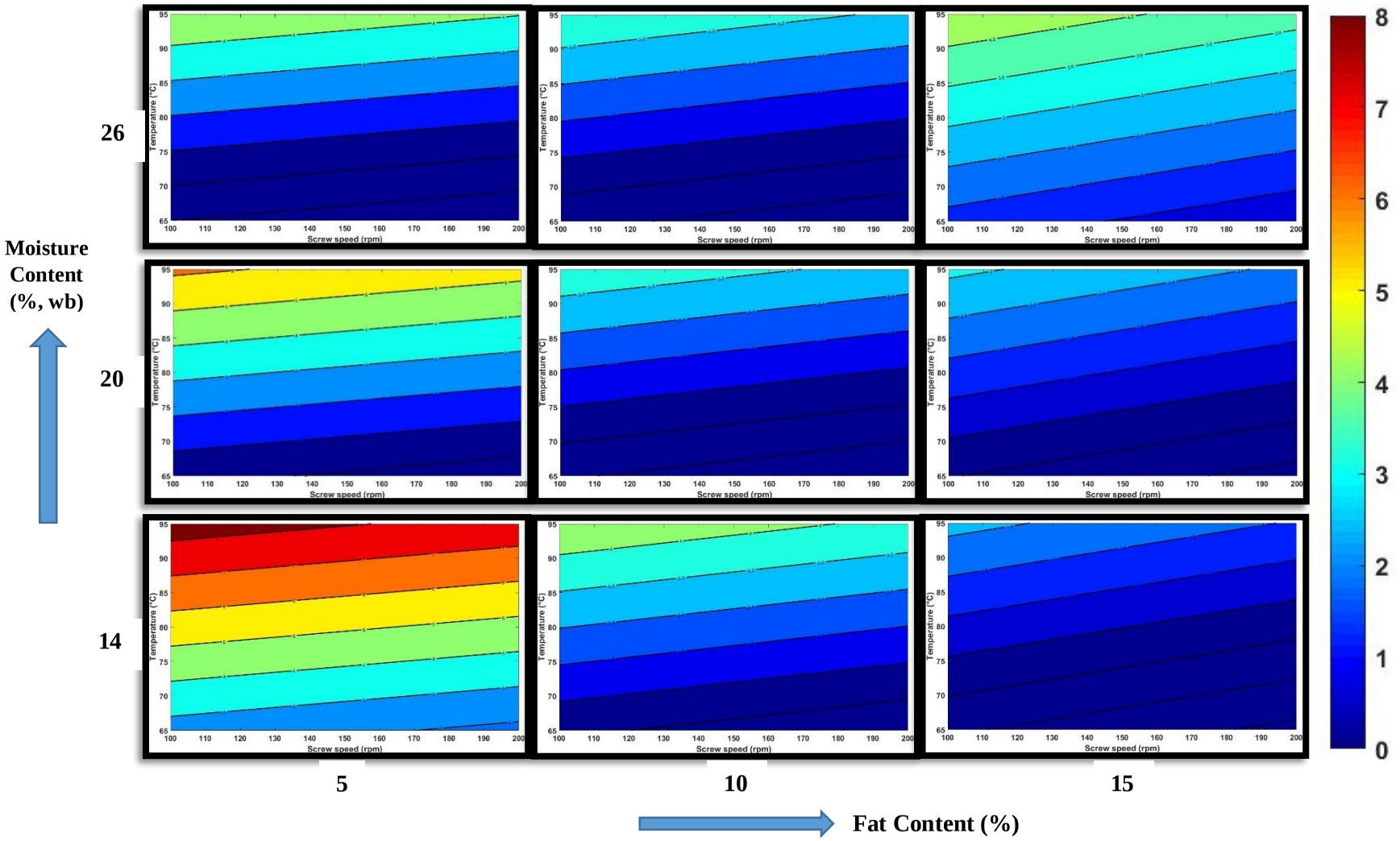

Figure 4.1. Contour plots showing *E. faecium* inactivation at different fat (5, 10, and 15%) and moisture (14, 20, and 26%) content. The color bar indicates the log reduction (log N_o/N_t) of *E. faecium* corresponding to the range of colors.

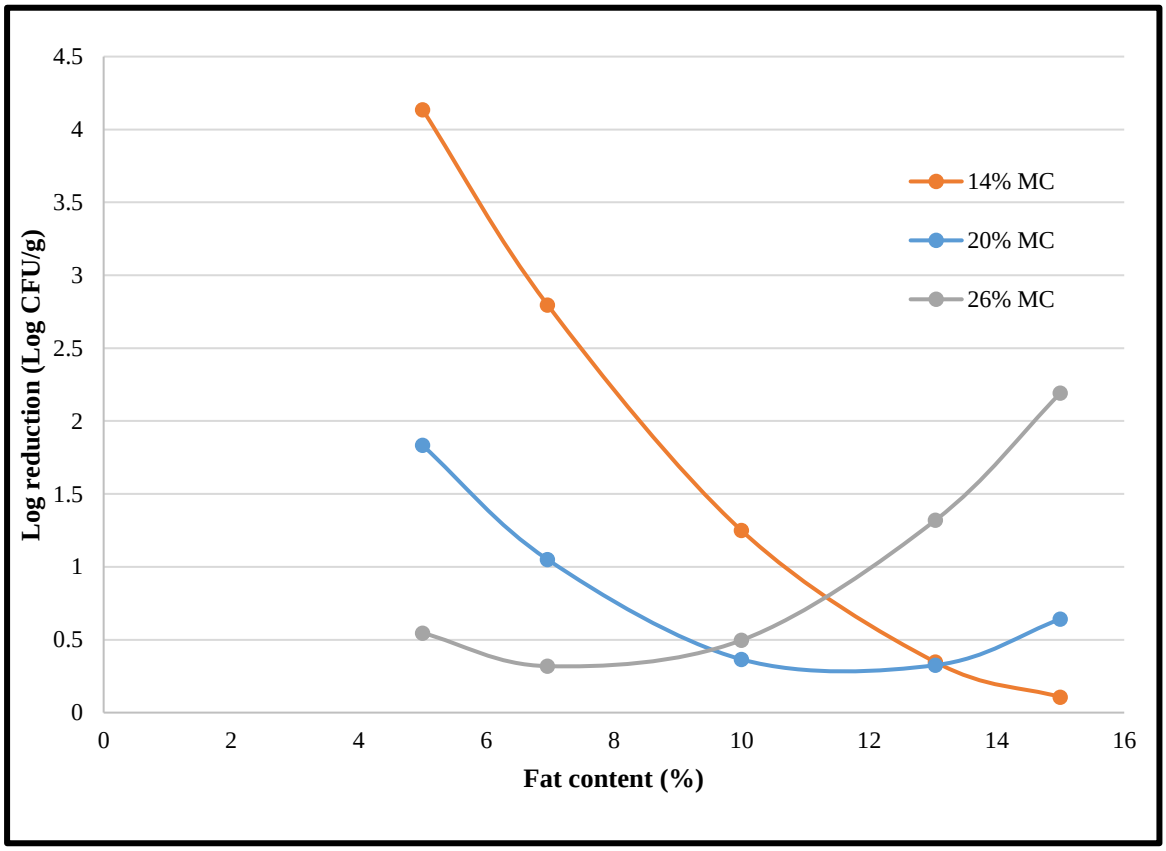

Figure 4.2. Interactive effect between fat and moisture content of oat flour extruded at 75˚C and 150 rpm on the log reduction of *E. faecium*.

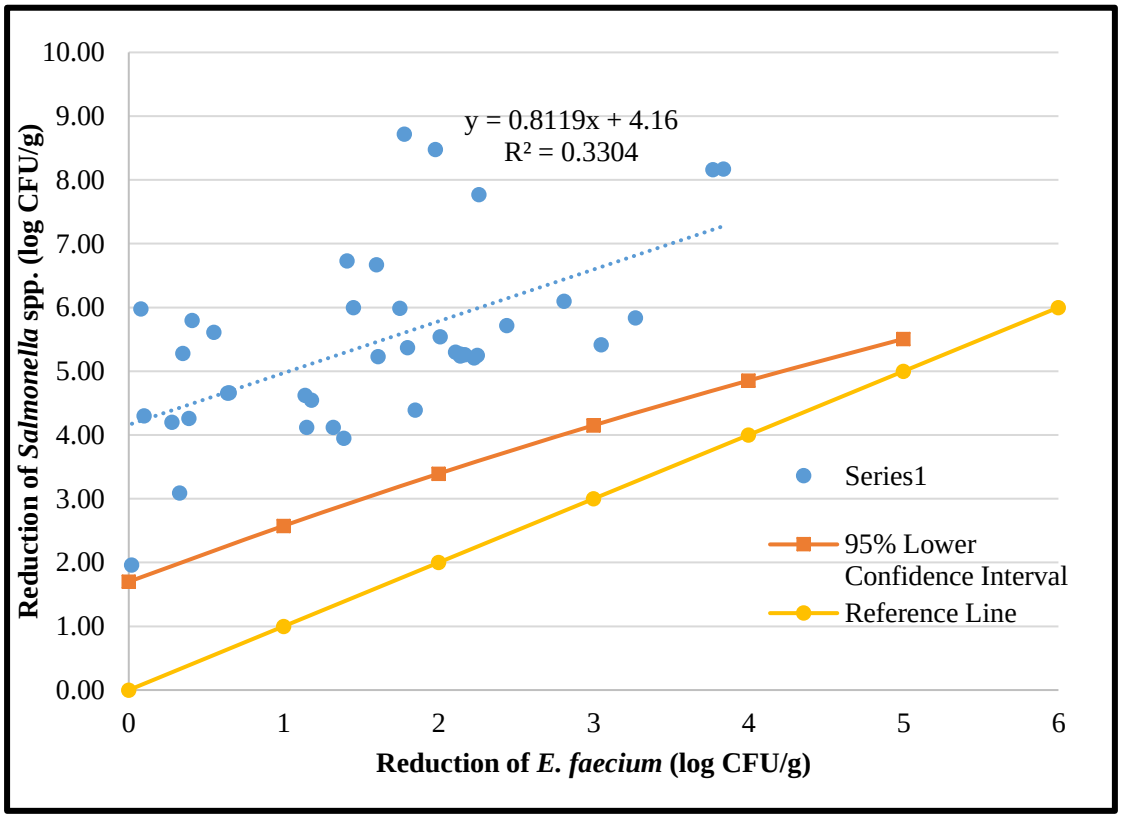

Figure 4.3. Bacterial reduction (log CFU/g) for *Salmonella* and *E. faecium* during the extrusion of oat flour at 75 and 85˚C.

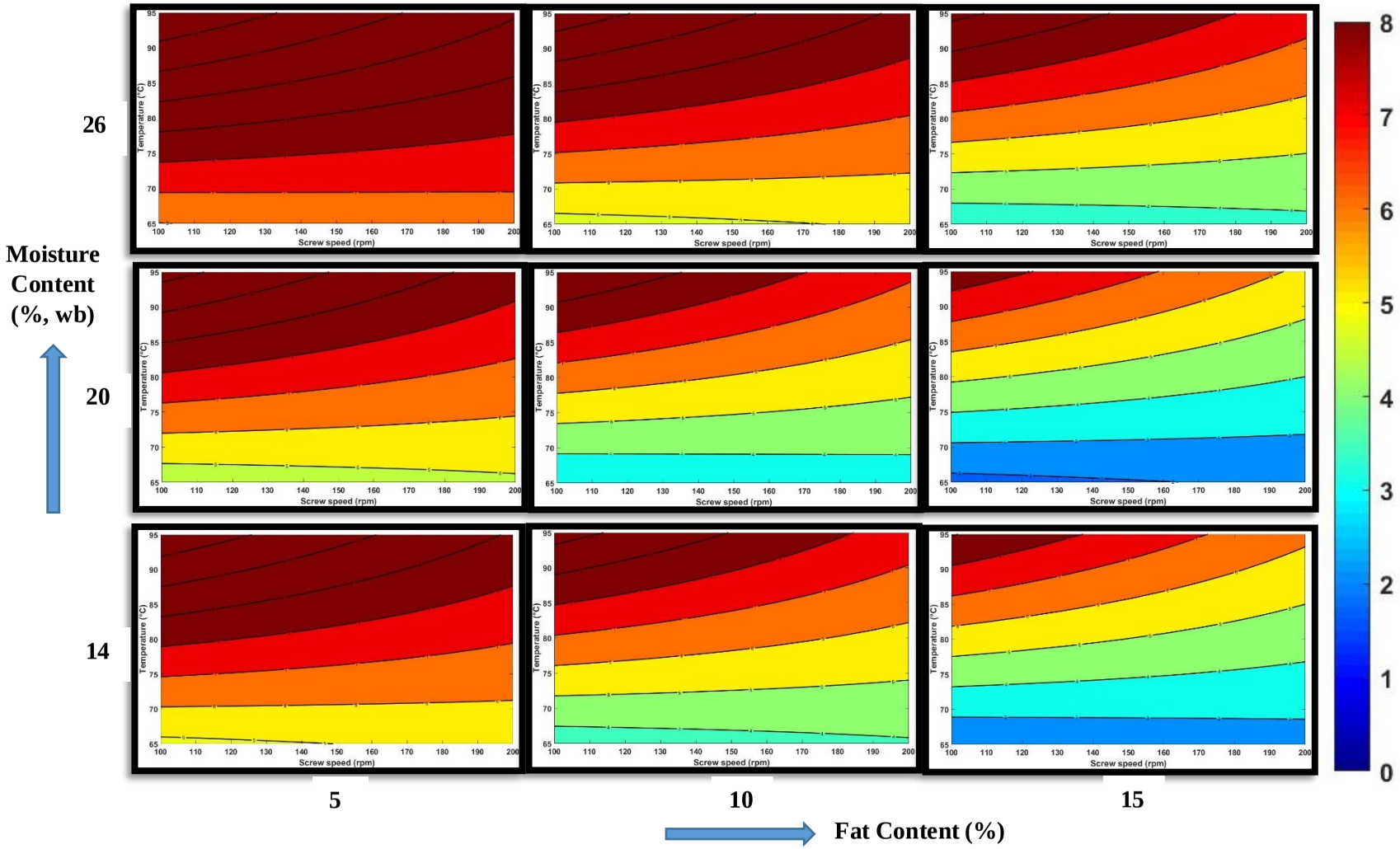

Figure 4.4. Contour plots showing *Salmonella* inactivation at different fat (5, 10, and 15%) and moisture (14, 20, and 26%) content. The color bar indicates the log reduction (log N_o/N_t) of *Salmonella* corresponding to the range of colors.

Chapter V: Conclusion and Suggestions for Future Research

5.1 Conclusions

This research was conducted to evaluate the microbial inactivation efficacy of extrusion in low-moisture food. The main objectives of this research were: 1) Develop a response surface model for *Salmonella* inactivation during the extrusion of low-moisture food 2) Evaluate the use of *Enterococcus faecium* NRRL B-2354 as a good surrogate for *Salmonella* during the extrusion of low-moisture food. Oat flour was selected as a food model due to its low-moisture content (8.73% wb) and high fat content (5%). Inoculated samples were adjusted to different moisture (14 to 26%) and fat levels (5 to 15%) and were extruded in a lab-scale single-screw extruder at different temperatures (65 to 95ºC) and screw speeds (75 to 225 rpm). To carry out the experiment, a central composite 2nd order design in incomplete blocks was used. The major findings of this research are summarized as follows:

	Salmonella	***Enterococcus faecium***
Temperature	Highest significant effect. ($p < 0.0001$)	Highest significant effect. ($p < 0.0001$)
Moisture content	Significant quadratic effect. ($p = 0.0005$)	Significant interactive effect with fat content. ($p < 0.0001$)
Fat content	Significant protective effect. ($p < 0.0001$)	Protective effect of fat content was only seen at lowest fat content (14%). However, the protective effect reduced as the moisture content increased. This was due to the significant interaction effect of moisture with fat content. ($p < 0.0001$)

Screw speed	Significant interaction effect with temperature. ($p = 0.0001$)	Significant linear effect. ($p < 0.0001$)

5.2 Suggestions for Future Research

While this thesis demonstrated the efficacy of extrusion process in reducing the microbial load in low-moisture food, there are several interesting research opportunities arising from this work which should be pursued.

- In this study, the moisture content, fat content, and screw speed gave a different response for *Salmonella* and *E. faecium*. Therefore, it is important to identify a new surrogate for *Salmonella* during the extrusion of oat flour that shows a similar behavior over a broad range of moisture content, fat content, and screw speed.
- This study used screw speed as one of the process parameters. It would be interesting to expand the study of *Salmonella* inactivation using the residence time distribution as affected by moisture content, fat content, and temperature in a single-screw extruder.
- The use of single-screw extruder in this study represented one of the worst-case scenario. Future experimental work should study the effect of twin-screw extruder on *Salmonella* inactivation and compare its effect with single-screw on inactivation of *Salmonella* by developing a response surface model.
- Many researchers have used water activity as one of the important factor on microbial inactivation. The use of water activity (as affected by temperature,

moisture content, and fat content) in the response surface model should be investigated further.

- This study focussed on the use of a single unit operation i.e. extrusion on microbial inactivation in low-moisture food. It would be interesting to evaluate how effective the other process technologies, like gas treatments (ClO_2, H_2O_2, Ethylene dioxide) are on reducing microbial load in low-moisture foods.

APPENDICES

Table A.1. Feed rates at 75% capacity at different screw speeds, fat content, and moisture content.

S. No.	Fat %	%MC	Screw Speed	100% Feed Rate (g/min)	Calculated 75% Feed Rate (g/min)	Observed 75% Feed rate (g/min)
1	5	20	150	82.8	62.1	64.5
2	15	20	150	120.5	90.4	87.9
3	10	14	150	88.7	66.5	64.9
4	10	26	150	129.2	96.9	93.4
5	10	20	150	113.6	85.2	86.7
6	10	20	75	69.4	52.1	47.1
7	10	20	150	113.6	85.2	86.7
8	10	20	225	125.8	94.4	91.6
9	6.96	16.35	196	81.3	61.0	58.2
10	13.04	16.35	196	88.7	66.5	70.2
11	6.96	23.65	196	97.5	73.1	71.9
12	10	20	150	113.6	85.2	86.7
13	10	20	150	113.6	85.2	86.7
14	13.04	23.65	196	110.8	83.1	79.6
15	13.04	16.35	104	56.6	42.5	39.1
16	6.96	23.65	104	48.3	36.2	35.6
17	6.96	16.35	104	58.1	43.6	44.2
18	13.04	23.65	104	64.9	48.7	45.6
19	10	20	150	113.6	85.2	86.7
20	10	20	150	113.6	85.2	86.7

Table A.2. *Salmonella* reduction on the selective media (TSAYE-XLD) at different temperatures, screw speed, moisture content, and fat content.

Sample #	% fat	% MC	Temp.	Screw speed	Log reduction
1	6.96	16.35	65	196	5.16
2	13.04	16.35	65	104	3.87
3	6.96	23.65	65	104	5.23
4	13.04	23.65	65	196	4.21
5	10	20	65	150	4.31
6	10	20	65	150	4.36
7	6.96	16.35	65	104	5.21
8	13.04	16.35	65	196	4.19
9	6.96	23.65	65	196	5.31
10	13.04	23.65	65	104	3.13
11	10	20	65	150	4.67
12	10	20	65	150	4.42
13	5	20	65	150	4.65
14	15	20	65	150	2.70
15	10	14	65	150	3.61
16	10	26	65	150	4.63
17	10	20	65	75	3.16
18	10	20	65	225	3.84
19	10	20	65	150	3.81
20	10	20	65	150	3.86
21	6.96	16.35	75	196	5.72
22	13.04	16.35	75	104	4.12
23	6.96	23.65	75	104	6.73
24	13.04	23.65	75	196	5.28
25	10	20	75	150	4.66
26	10	20	75	150	4.66
27	6.96	16.35	75	104	5.41
28	13.04	16.35	75	196	4.30
29	6.96	23.65	75	196	5.98
30	13.04	23.65	75	104	5.99
31	10	20	75	150	4.55
32	10	20	75	150	4.62
33	5	20	75	150	6
34	15	20	75	150	1.96
35	10	14	75	150	4.39
36	10	26	75	150	5.61
37	10	20	75	75	5.8

38	10	20	75	225	3.09
39	10	20	75	150	4.2
40	10	20	75	150	4.26
41	6.96	16.35	85	196	5.84
42	13.04	16.35	85	104	5.27
43	6.96	23.65	85	104	7.77
44	13.04	23.65	85	196	5.54
45	10	20	85	150	5.26
46	10	20	85	150	5.30
47	6.96	16.35	85	104	8.17
48	13.04	16.35	85	196	5.23
49	6.96	23.65	85	196	6.67
50	13.04	23.65	85	104	6.10
51	10	20	85	150	5.24
52	10	20	85	150	5.26
53	5	20	85	150	8.16
54	15	20	85	150	3.95
55	10	14	85	150	5.37
56	10	26	85	150	8.72
57	10	20	85	75	8.48
58	10	20	85	225	4.12
59	10	20	85	150	5.21
60	10	20	85	150	5.25

Table A.3. *E. faecium* reduction on the selective media (m-EA) at different temperatures, screw speed, moisture content, and fat content.

Sample #	% fat	%MC	Screw speed	Temp.	Log Reduction
1	6.96	16.35	196	75	2.44
2	13.04	16.35	104	75	1.32
3	6.96	23.65	104	75	1.41
4	13.04	23.65	196	75	0.35
5	10	20	150	75	0.64
6	10	20	150	75	0.65
7	6.96	16.35	104	75	3.05
8	13.04	16.35	196	75	0.1
9	6.96	23.65	196	75	0.08
10	13.04	23.65	104	75	1.75
11	10	20	150	75	1.18
12	10	20	150	75	1.14
13	5	20	150	75	1.45
14	15	20	150	75	0.02
15	10	14	150	75	1.85
16	10	26	150	75	0.55
17	10	20	75	75	0.41
18	10	20	225	75	0.33
19	10	20	150	75	0.28
20	10	20	150	75	0.39
21	6.96	16.35	196	85	3.27
22	13.04	16.35	104	85	2.14
23	6.96	23.65	104	85	2.26
24	13.04	23.65	196	85	2.01
25	10	20	150	85	2.17
26	10	20	150	85	2.11
27	6.96	16.35	104	85	3.84
28	13.04	16.35	196	85	1.61
29	6.96	23.65	196	85	1.6
30	13.04	23.65	104	85	2.81
31	10	20	150	85	2.14
32	10	20	150	85	2.15
33	5	20	150	85	3.77
34	15	20	150	85	1.39
35	10	14	150	85	1.8
36	10	26	150	85	1.78

37	10	20	75	85	1.98
38	10	20	225	85	1.15
39	10	20	150	85	2.23
40	10	20	150	85	2.25
41	6.96	16.35	196	95	4.01
42	13.04	16.35	104	95	2.76
43	6.96	23.65	104	95	5.08
44	13.04	23.65	196	95	3.76
45	10	20	150	95	2.9
46	10	20	150	95	2.94
47	6.96	16.35	104	95	5.26
48	13.04	16.35	196	95	2.11
49	6.96	23.65	196	95	3.34
50	13.04	23.65	104	95	3.87
51	10	20	150	95	2.98
52	10	20	150	95	2.91
53	5	20	150	95	7
54	15	20	150	95	2.02
55	10	14	150	95	4.7
56	10	26	150	95	2.78
57	10	20	75	95	3.9
58	10	20	225	95	1.87
59	10	20	150	95	3.04
60	10	20	150	95	3.05