RESIDENCE TIME AND SURVIVAL STUDIES FOR *ENTEROCOCCUS FAECIUM* AS A SURROGATE FOR *SALMONELLA* DURING PRECONDITIONING AND EXTRUSION PROCESSING OF DRY EXPANDED PET FOOD

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Abstract

Validation studies on process equipment are an important step for effective pathogenic control during dry expanded pet food manufacturing. The preconditioner is used to hydrate, mix and pre-cook raw materials before extrusion of pet food. The High-Intensity-Preconditioner (HIP) was designed with two independently driven shafts, thus offering control of both shaft speed and rotational direction with potential for improving residence time and thus pathogen inactivation. Residence time distribution (RTD) of raw dog food mix was impacted by the HIP process parameters (average residence time varying between 104-178 s for dry experiment and 65-177 s with steam addition) depending on shaft speed and direction. In general, increase in shaft speed resulted in shorter residence time with the larger shaft having a greater impact than the smaller shaft. Rotational direction of shafts also had an effect on average residence time (a maximum difference of 37 s was noticed between treatments with different shaft directions and the same speed). The uniformity of residence time distribution (difference of 97-132 s between 15 and 85 percentiles of the cumulative RTD) also varied considerably with process conditions, with uniformity increasing with shaft speed.

Enterococcus faecium (ATCC® 8459™) was chosen as a surrogate for *Salmonella* for microbial inactivation studies on the HIP. Both HIP shaft speed (200 and 300 rpm) and process temperature (67-70^{o}C and 89-91^{o}C) impacted *E.faecium* survival. Lower shaft speed (corresponding to longer residence time) or higher temperature led to greater *E.faecium* inactivation. A 5 log CFU/g of *E.faecium* was reduced using selective agar (m-Enterococcus or mE agar) after treatment with high temperature, but approximately 3.5 log CFU/g of *E.faecium* reduced on non-selective agar (Brain Heart Infusion or BHI agar). Uneven heat distribution, inadequate residence time and system instability might have negatively affected the inactivation.

Microbial inactivation, with *E.faecium* as surrogate, was also studied for the complete dry expanded pet food process using a pilot-scale single-screw extruder with a regular double shaft preconditioner. Meal was inoculated with *E.faecium* at 6 log CFU/g and processed. Preconditioner downspout temperature ranged from 89-94^{o}C and extrusion die temperature was between 120-140^{o}C. Complete inactivation was observed after extrusion.

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Chapter 1 - Introduction

1.1 Introduction

Salmonella is commonly found in dry pet food products (PetRecall, 2015). Therefore, *Salmonella* control is an important task as contaminated pet food not only affects pets but also pet owners who share the same environment (Murray et al, 2007). To comply with the Food Safety Modernization Act (FSMA), pet food manufacturers are required to develop a food safety plan to minimize or prevent hazards identified in the hazard analysis (Silliker, 2014).

Verification that equipment(s) such as preconditioners and extruders are effective critical control points (CCP) during pet food manufacturing is necessary to ensure pathogen control. Raw pet food materials are important sources of *Salmonella* (Buchanan et al, 2011), including animal-origin meal (Mehlenbacher et al, 2012) and grains (Grocery Manufacturers Association, 2004). Though dry pet food products are commonly considered to be a low water activity food and do not support the growth of *Salmonella*, *Salmonella* could potentially to survive manufacturing processes and contaminate dry pet food.

Most dry pet foods are manufactured using extrusion systems. The preconditioner is an essential part of the extrusion system. It can hydrate, mix and pre-cook the raw materials in a barrel prior to the extrusion process. Additional retention time can be achieved as well, which is critical to all types of reactions (Riaz, 2007). Preconditioned materials undergo a high-temperature, high-pressure and high-shear environment in the extruder when both thermal energy and mechanical energy input are generated. A die is placed at the end of the extruder for back pressure pushing and forming purposes, and a knife cuts the final products into the desirable size.

The extruder is recommended as a potential and logical critical control point (CCP) (Lambertini et al, 2016) in pet food manufacturing; however, few publications regarding

validation on *Salmonella* control using the extruder could be found. Thus, the ability of extruder for pathogen destruction must be validated. Since the preconditioner itself provides a heated, moist environment as well as the extruder, its effectiveness on *Salmonella* deactivation also needs to be investigated. A newly designed preconditioner, the High-Intensity-Preconditioner (HIP), was created to improve food safety and increase productivity. Compared to traditional double-shaft preconditioners, the HIP is capable of providing wider ranges of residence time and mixing capacity because the 2 shafts in the HIP are independently-driven, meaning they can offer control of both speed and rotational direction.

Enterococcus faecium (American Type Culture Collection, ATCC 8495) evaluated as a surrogate organism for *Salmonella* in this project. *E. faecium* is a non-pathogenic organism and is classified as a Biosafety level – 1 agent, which presents minimal risk to personnel and the environment. Most importantly, *E. faecium* shares similar or greater thermal lethality characteristics with *Salmonella* and has been used as a surrogate for *Salmonella* in several other studies (Jeong et al., 2011; Bianchini et al., 2014).

Moisture level and temperature in the preconditioner and the extruder could be easily controlled by the moisture injection rate in the form of steam or water (Riaz, 2007). The more complicated issue here is to accurately determine the duration of materials through the preconditioner system, which could also be used as heat exposure time of pet food materials. Residence time accounts for the time that materials required going through the preconditioner or the extruder barrel and it highly depends on the shaft/screw speed and/or the material throughput (Bouvier, 1996; Choudhury & Gautam, 1998). Properly manipulating the equipment(s) would result in ideal residence time. Residence time distribution (RTD) could be obtained at the same

time, which provides information such as mixing intensity and degree of cooking (Lee & McCarthy, 1996).

1.2 Objectives

The main goal of this project was to verify if the HIP (Model 150, Wenger® Mfg., Sabetha, KS) and the single-screw extruder (X-20, Wenger® Mfg., Sabetha, KS) could be considered as a valid CCP under certain circumstances (temperature, time, moisture level, etc.) during the dry expanded pet food manufacturing.

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Chapter 2 - Residence time distribution study in the High-Intensity-Preconditioner

Abstract

The High-Intensity-Preconditioner (HIP) was designed with 2 independently driven shafts, thus offering control of both shaft speed and shaft rotational direction with potential for providing wide ranges of residence time. Residence time distribution (RTD) was impacted by the HIP process parameters (average residence time varied between 104-178 s for dry experiment and 65-176 s with steam addition) depending on shaft speed and shaft direction. When shaft speed stayed consistently, shaft direction (4 combinations) had a huge impact on residence time: a difference of 37 s was noticed. Slight change (3%) on steam injection rate did not change residence time much while 10% change reduced 90 s of residence time. The mixing uniformity (value between 97-132) also varied considerably with process conditions: uniformity increasing with shaft speed. Correctly manipulating the High-Intensity-Precondition could generate a broad range of residence time, which would be helpful to improve food safety.

Keywords: residence time distribution, High-Inensity-Preconditioner, mixing uniformity

2.1 Introduction

Extrusion processing is widely used in the food industry for various applications, such as pet food (Gibson and Alavi, 2013; Koppel et al., 2015; Tran et al., 2008), animal feed (Lundblad et al., 2012; Rojas, et al., 2016), aquatic feed (Adedeji et al., 2015), instant porridge (Chanadang et al., 2016; Mayachiew et al., 2016) and expanded snack (Devi et al., 2013; Liu et al., 2011). During extrusion, raw materials are cooked using a combination of thermal and mechanical energy, shaped by forcing through a die, and often expanded to obtain a porous structure (Alavi and Ambrose, 2015; Riaz, 2007). Pet food, especially the dry expanded type of product, is commonly produced by extrusion as it is an energy efficient, high throughput, economical process that allows flexibility in product shapes, colors and other characteristics such as expansion (Rokey and Weier, 2015).

Preconditioning is an essential part of many extrusion-based process applications, during which raw materials are hydrated, mixed and pre-cooked with introduction of water and thermal energy in the form of steam (Riaz, 2007). Preconditioning serves to - reduce the wear of extruder barrel and screw components by softening the raw materials that are typically abrasive in natural crystalline or glassy state (Alavi and Ambrose, 2015; Rokey et al., 2010; Riaz, 2007); increase throughput (Riaz, 2000); improve final product quality (Rokey et al., 2010; Karkle et al., 2012; Mathew et al., 1999); and also facilitate chemical reactions among ingredients (Riaz, 2007; Clark, 1969). The thermal energy input and temperature during preconditioning is controlled by the amount of steam injected. For pet food extrusion, preconditioning is even more important as it provides a substantial proportion of required energy in the absence of adequate mechanical energy due to high process moisture. It can also potentially enhance safety of the pet food

products (Rokey and Weier, 2015), although there is a lack of published studies and data in this regard.

Traditional preconditioners have a single shaft or twin shaft configuration, with the latter having a higher residence time although the shaft speed and rotational direction are coupled leading to limited efficiency and operational flexibility. The latest development in preconditioner design for increased productivity and food safety is the High Intensity Preconditioner (HIP, Wenger Manufacturing, Inc., Sabetha, KS). This equipment is an improvement over traditional double shaft precoditioners as its two shafts are driven by separate motors, allowing control of both shaft speed and rotational direction independent of each other. This aspect of the design provides a wider range of capacity, mixing uniformity and residence time, making it a significant innovation in the extrusion cooking process.

Residence time is an important parameter during the extrusion process, and it is defined as the average amount of time that the raw materials spend in the extruder barrel and preconditioner (Choudhury & Gautam, 1998; Levine et al., 2002). However, all particles do not have the same residence time, and it is best to characterize not just the average time but also the residence time distribution (RTD). RTD can be used to characterize the process and also determine the optimum conditions for mixing, cooking, etc. (Lee & McCarthy, 1996). Characterization of RTD involves introduction of a tracer, such as $NaNO_3$, KCl and erythrosine dye, into the system in order to track the footprint of the materials. There are several studies that describe RTD in an extruder (Todd, 1975; Choudhury and Gautam, 1998; Lee and McCarthy, 1996; Altomare and Ghossi, 1986; Gogoi and Yam, 1994; Unlu and Faller, 2000; Davidson et al., 1983), but only few have investigated residence time in traditional preconditioners as described below (Levine et al., 2002; Bouvier, 1996; Morales et al., 1996). Levine et al. (2002)

used a dimensionless analysis to predict the RTD in the preconditioner. Bouvier (1996) used RTD to determine mixing efficiency in the preconditioner. Morales and coworkers (1996) compared the RTD under different feed rate, mixing speed, and moisture content.

However, no data has been published for RTD performance of HIP-type preconditioners, which was the primary objective of the current study. Besides investigating the impact of shaft speed, rotational direction and moisture content on residence time and RTD, the effectiveness of two different tracers/ methods for RTD determination were also investigated. As the preconditioner is often a critical control point for avoiding microbial contamination and RTD is an important factor for the pathogen inactivation process, its characterization in the HIP would be particularly useful for evaluating the potential for food safety. Residence time data obtained from this study was used in further studies focusing on *Salmonella* control during extruded pet food manufacturing as described elsewhere.

2.2 Materials and Methods

2.2.1 Materials.

A generic dry expanded dog food formulation (KSU Mix), obtained from Lortscher Animal Nutrition Inc. (Bern, KS), was used for the HIP RTD studies. The KSU Mix is described in Table 2-1. Two different types of materials were used as tracer, viz. plastic beads (5mm x 3mm x 2mm) and sodium chloride (NaCl, 7647-14-5, Fisher Scientific Co, L.L.C., Pittsburgh, PA).

2.2.2 High-Intensity-Preconditioner (HIP).

The HIP design is described in Figure 2-1. Two independently driven shafts offer both speed and rotational control. If viewed from the discharge end, the large shaft is on the left and the small shaft is on the right (Figure 2-1). The paddles on the large shaft were 15.2-cm long and

the ones on the small shaft were 7.6-cm long. The differential size shaft arrangement allows better mixing uniformity, while ensuring adequate residence time at the same time. Steam is injected from the bottom of the preconditioner chamber (Figure 2-2). A temperature probe measuring the material exit temperature was located at the downspout of the HIP.

2.2.3 RTD determination.

RTD was measured using the pulse stimulus response technique as described by Levenspiel (1972). This involves introduction of a pulse stimulus in the form of a tracer at the inlet or 'feed end' and tracking the evolution of the tracer or its concentration at the outlet or 'discharge end' using a suitable measurement method. Tracer was prepared by mixing 300 g of plastic beads or NaCl with 500 g of KSU mix. The preconditioner was operated at the desired throughput of the pet food formulation, and discharge rate was constantly monitored using a weighing scale and a timer. When steady state operation was reached, the pulse stimulus or tracer was introduced into the HIP inlet and simultaneously a timer was started to monitor the time. For the first 7 min, samples were collected from the outlet at 20 s time intervals for the whole duration of each interval. For the next 3 min, samples were collected at 1 min time intervals but only for the first 20 s of each interval. Sampling was stopped at 10 min after the tracer was introduced. A total of 24 response samples were thus collected. In the case of plastic beads, the tracer concentration in the response samples was measured by separating the beads from pet food formulation with a 2-mm sieve, and dividing the mass of the beads with the total sample mass. In the case of NaCl, a 10 g sub-sample was collected from each response sample and NaCl concentration was measured using a Quantab® test kit (30-600 mg/L, Hach, Loveland, CO) according to the method descried by Boyer (2004) as follows. Ten gram of the pet food sample was mixed into 90 g of hot water (to make a total of 100 g) in a cup. The mixture was

stirred for 1 min the dissolve the NaCl and allowed to settle for a short period so the heavy particles dropped to the bottom of the cup. A piece of filter paper was folded into quarters and placed in the cup along with the lower end of a Quantab strip (Figure 2-3) that works as a titrator. The wick of the strip was completely saturated by the solution and when the reaction was complete the indicator turned dark (Figure 2-4). A color change in the wick from yellow to white marks the Quantab value, which can be read from a graduated scale. NaCl concentration was then obtained from the Quantab value using a standard conversion table.

Microsoft® Excel 15.17 (Microsoft Corporation, Redmond, WA) was used to process the data. The average residence time ($\bar{t}$) and the the exit age (differential) distribution function, *E(t)*, and the cumulative distribution function, *F(t)* were calculated using the following equations adapted from Levenspiel (1972):

$$\bar{t} = \frac{\Sigma_0^\infty t*C_t}{\Sigma_0^\infty Ct} \quad (2\text{-}1)$$

where t = time (mid-point of each time interval for response sample collection) and C_t = tracer concentration at t.

$$E(t) = \frac{C_t}{\Sigma_0^\infty C_t} \quad (2\text{-}2)$$

$$F(t) = \frac{\Sigma_0^t C_t}{\Sigma_0^\infty C_t} \quad (2\text{-}3)$$

The average residence time can also be calculated as the ratio of degree of fill of material to the total mass flow rate (Mathew et al., 1999). For this measurement, after completion of the response sample collection, the preconditioner was brought to a “dead-stop” and all the material inside the chamber was scooped out and weighed. The average residence time was then calculated as shown in equation (4).

$$\bar{t} = \frac{M}{\dot{m}} \quad (2\text{-}4)$$

where $\dot{m}$ = feed rate or throughput and M = mass of material inside the preconditioning chamber or degree of fill.

The uniformity of RTD was proportional to the slope of the cumulative distribution function. The corresponding readings on the X-axis of the *F(t)* curve when $y_1 = 0.15$ and $y_2 = 0.85$ were determined as x_1 and x_2, respectively (Figure 2-5). The difference between the two ($x_2 - x_1$) represents the inverse (m) of the slope. The smaller the m value, the steeper would be the slope and better is the uniformity.

2.2.4 Experimental design.

The effects of shaft speed, rotational direction and steam injection level on average residence time and RTD were studied. The HIP was operated at a throughput of 453.6 kg/hr (1000 lbs/hr) for all experiments. In the first experiment (Table 2-2), effect of shaft speed was studied while keeping rotational directional constant (both shafts clockwise). The small shaft speed was varied at three levels (200, 300 and 400 rpm) and the large shaft at two levels (200 and 300 rpm) in a fractional factorial design with 4 treatments. In the second experiment (Table 2-3), effect of shaft rotational direction was studied in a full factorial design with 4 treatments, while keeping shaft speed constant (both shafts 200 rpm). Both experiments 1 and 2 were done with dry formulation. The third experiment was conducted with hydrated formulation to mirror actual processing of pet food, and different levels of steam injection were studied while keeping the shaft rotational direction constant (Table 2-3). The pet food formulation was pre-hydrated to 14% (w/w) moisture level (10.55 kg of water mixed with 226.8 kg of dry formulation) using a double paddle mixer (H.C. Davis Sons Mfg Co., Inc., Bonner Springs, KS) before feeding into the HIP. Steam injection rates varied at three levels, which were controlled through the steam

valve opening of 0%, 7% and 9% resulting in preconditioner downspout temperatures of 25, 68±2 and 90±1^{o}C, respectively.

2.3 Results and Discussion

2.3.1 Dead stop method versus pulse stimulus response method using tracers.

The dead-stop method was the method that estimate the most accurate residence time in the HIP since introduction of external materials would bring compatibility challenges in the HIP processing. Residence time obtained from stimulus response method were 13.6 to 39.3% less while using beads and 7.3 to 27.5% higher while using salt compared to the residence time obtained from dead stop method. Plastic beads were heavier than pet food materials and it was easier to convey material with a higher bulk density in the barrel as lighter materials tended to be more dispersed in the barrel and not stay in a tight, compact way. Salt, on the other hand, was lighter than the pet food materials and tended to be more dispersed during transportation resulted in a longer residence time. When shaft speed increased from 200 to 300 rpm, residence time obtained from 3 methods all decreased. However, introduction of tracers either overestimated or underestimated residence time.

Although when being used as tracer, both of them could provide a bell-shaped curve describing RTDs (Figure 2-6), the reason why plastic beads were chosen as the tracer over salt was because that plastic beads could be all collected for analysis during sample collection and under such circumstance, data was more representative while only 10 g of samples containing salt was obtained out of 2.5 kg was used to calculate tracer concentration.

2.3.2 Effect of shaft speed on residence time, RTD, and uniformity.

Although only few studies (Bouvier, 1996) investigated the RTD in the preconditioner, extrusion RTD analysis could be used as references in this paper as they have similar principles.

An increase of speed for either the large or small shaft led to shorter residence time (Figures 2-7 & 2-9). Similar results were reported by Gogoi & Yam (1994) from their twin-screw-extruder-based studies. When the small shaft speed increased from 200 to 400 rpm, residence time decreased about 74 s (Figure 2-7). Residence time decreased more (81 s) when the large shaft speed increased from 200 to 300 rpm (Figure 2-9). Residence time had a bigger drop when the same shaft speed change was applied on the large shaft rather than the small shaft. This difference implied that the large shaft is the main conveying shaft, thus, the large shaft had a greater effect on residence time.

Residence time distribution curves present the relationship between the tracer concentration and time: at a particular time, the quantity of tracers collected at the end of the HIP was noted. In Figures 2-8 & 2-10, a more left shifted the curve indicating a shorter residence time. The materials were transported through the HIP barrel faster because more beads were collected during the early stage of sample collection. This pattern was also described by Unlu & Faller (2002) and Gogoi & Yam (1994). Moreover, a higher peak in the RTD curves also indicated shorter residence time because more tracers were brought out of the HIP barrel within a specific 20 s collection interval.

Cumulative distribution curves describe the relationship between time and cumulative tracer concentration (the sum of tracer concentration obtained from all tracers before certain time point). A more left-shifted the curves standing for a shorter residence time. For instance, in Figure 2-11, at 90 s, more than 60% of the tracer was collected at the end of the HIP when the small shaft was rotating at 400 rpm, while only around 5% was coming out of the system when the small shaft speed was 200 rpm. A more left-shifted curves also suggested better uniformity, as the slope of the curve was steeper.

In Figure 2-11, the slopes (*m*) of the curves were 132, 107 and 97, respectively with increasing small shaft speed (Table 2-6). In Figure 2-12, *m* equaled to 132 and 99, respectively (Table 2-7), with increasing large shaft speed. Results obtained showed that the faster the shaft rotated, better uniformity was created because with an increase in screw speed, more mixing was generated within the HIP barrel. Unlu and Fallller's (2002) and Altomare and Ghossi (1986) measured the uniformity using normalized RTD or dimensionless RTD. Normalized RTD could be applied to compare the flow patterns of different processing conditions relative to each other (Unlu and Faller, 2002). A wider spread was observed under the normalized RTD corresponding to a better mixing uniformity.

Better uniformity (99 vs. 107) was achieved when the large shaft was rotating faster than the small shaft (300 rpm on the large shaft and 200 rpm on the small shaft vs. 200 rpm on the large shaft and 300 rpm on the small shaft). This phenomenon indicated that better mixing was created if the large shaft speed increased when compared to increase in the small shaft speed; thus, the large shaft was the main mixing shaft.

However, to achieve better uniformity by increasing the large shaft speed would compromise the residence time as the large shaft is also the main conveying shaft. Thus, increasing the small shaft speed while maintaining the large shaft speed at a lower level is a better way to create better uniformity without sacrificing the residence time.

2.3.3 Effect of shaft rotational direction on residence time, RTD, and uniformity.

Even though the 2 shafts in the HIP had the same rotational speed, residence time varied when shaft directions were different (Figure 2-13). The longest residence time generated from the HIP was 144 s when running at a 200 rpm shaft speed while the shortest was 106 s (approximate 38 s of difference was noted). The ability to provide different rotational speeds on

both shafts was one of the main reasons why the HIP was capable of providing a wider range of residence times and flexibility compared to traditional preconditioners. A possible reason why shaft rotational direction played a huge role in residence time was because the large shaft was the main conveying shaft and when both shafts had counterclockwise rotation, the materials were brought to the large shaft side of the HIP barrel so that they were carried out of the system faster. Similarly, when the materials were sent to the small shaft side of the barrel (when both shafts had clockwise rotations), the longest residence time was detected.

In Figure 2-14, the highest peak was observed when both shafts had counter-clockwise rotation indicating that the shortest residence time was generated. However, the most left-shifted curve was not the one achieving the shortest residence time in this case. The RTD curves (Figures 2-14 & 2-15) also differed somewhat from each other. The *m* value (Figure 2-15) of each curve was: 99, 41, 99 and 119, respectively (Table 2-8). The worst uniformity was observed when the big shaft had counter-clockwise rotation while the small shaft had clockwise rotation. Under such circumstance, the materials were pushed to the two sides of the preconditioner barrel and only part of the materials could make contact with either shaft instead of both; thus, uniformity was reduced.

The uniformity could not only be influenced by the shaft speed, but also the paddle arrangement in the HIP. There were 5 forward paddles at the beginning of the HIP barrel, and the rest were neutral. The 45-degree angle on the forward paddles played the most important role in adjusting residence time in the HIP barrel and improving the uniformity while the neutral paddles had no such contributions. What the neutral paddles did was to accumulate the materials in the barrel creating longer residence time. There was no backward paddle in this HIP.

2.3.4 Effect of steam injection rate on residence time, RTD, and uniformity.

A slight change (steam valve opening increased 2%) on the moisture level did not impact residence time, while a significant increase (steam valve opening from 0% to 9%) in the moisture level mainly influenced the residence time (see Figure 2-16 & 2-17). When the moisture level increased due to 9% increase on steam valve opening, the residence time had a 22 s decrease when the shaft speed was 200 rpm (Figure 2-16). An even more noticeable difference was observed when the shafts were rotating at 300 rpm (Figure 2-17): the residence time went down 87 s with an increase in the moisture level generated by 9% steam valve opening. However, when the valve opening only slightly changed (from 7% to 9%), no significant variation (65 s vs. 66 s) was found. Altomare and Ghossi (1986) concluded from their rice dough extrusion study that the moisture content hardly affected the residence time as the dough moisture content increased from 10.0% to 28.4%, while the mean residence time only increased 3.5 s: from 21 s to 24.5 s. This result was also agreed by Gogoi and Yam (1994). However, in some cases in Gogoi and Yam's study (1994), the residence time increased 47% when the moisture content increased from 20% to 30%, which also agreed with the data of Lin and Armstrong (1988) for their extruded cereal-mix study. Hydration made the pet food materials heavier and more cohesive and could stay in the bottom of the HIP instead of floating in the barrel. Thus, hydrated materials could be more easily conveyed in the HIP barrel.

The uniformity of the materials did not vary much between dry and wet treatments (m = 151 and 142, Table 2-9) when the HIP was running at 200 rpm-shaft-speed (Figure 2-18). However, when the shaft speed increased to 300 rpm, the uniformity exhibited a large difference between dry and wet treatments (Figure 2-19): the m values were very close under hydrated conditions: 52 for the low temperature/hydration condition and 51 for the high

temperature/hydration condition, while the uniformity value was 144 for the dry treatment (Table 2-10).

2.4. Conclusion

A decrease of shaft speeds resulted in longer residence time and worse uniformity; the shaft rotational direction had a significant impact on residence time, RTD and uniformity. The large shaft was the major conveying/mixing shaft as changes made on the large shafts had larger impact on both residence time and uniformity. To achieve better uniformity, an increase in the small shaft speed should be chosen over a change in the large shaft speed as in this way, residence time would have a relatively smaller reduction. This set of data will useful for the subsequent microbial destruction study: residence time might be helpful to indicate the average time duration that contaminated materials are exposed to the moist and heated environment; RTD and uniformity would inform about if the thermal energy could be evenly distributed on the contaminated materials.

Tables

Table 2-1. KSU mix formulation

Ingredients	Level (w/w)
Corn flour	48%
Poultry by-product meal	20%
Corn gluten meal	21%
Rice meal	10%
Vitamin premix	0.5%
Mineral premix	0.5%
Total	100%

Table 2-2. Investigation of the effect of shaft speed on residence time, residence time distribution and uniformity in the High-Intensity-Preconditioner

Shaft speed (rpm; big-small)	Shaft direction (big-small)
300-200	
200-200	
200-300	
200-400	

Table 2-3. Investigation of the effect of shaft rotational direction on residence time, residence time distribution and uniformity in the High-Intensity-Preconditioner

Shaft speed (rpm; big-small)	Shaft direction (big-small)
200-200	
200-200	
200-200	
200-200	

Table 2-4. Investigation the effect of steam injection level (discharge temperature as dependencies) on residence time, residence time distribution and uniformity in the High-Intensity-Preconditioner

Shaft speed (big-small, rpm)	Shaft direction	% valve opening / discharge temperature
200-200	↻↺	0% / room temp
300-300	↻↺	0% / room temp
200-200	↻↺	7% / 68±2°C
300-300	↻↺	7% / 68±2°C
300-300	↻↺	9% / 89±1°C

Table 2-5. Comparison of the residence time between using beads as the tracer and the dead-stop method in the High-Intensity-Preconditioner when both shafts had clock-wise rotation

Treatments	200 – 200 rpm (big-small)	300 – 200 rpm (big-small)
Beads	178 s	97 s
Dead-stop	206 s	160 s
Salt	221 s	204 s

Table 2-6. Uniformity value obtained when the shaft speed on the small shaft from 200 to 400 rpm while 200 rpm on the large shaft and both shafts had clockwise rotational direction in the High-Intensity-Preconditioner

Treatments (big shaft – small shaft)	Uniformity
200-200, C-C	132
200-300, C-C	107
200-400, C-C	97

Table 2-7. Uniformity value obtained when the shaft speed on the large shaft from 200 to 300 rpm while 200 rpm on the small shaft and both shafts had clockwise rotational direction in the High-Intensity-Preconditioner

Treatments (big shaft – small shaft)	Uniformity
200-200, C-C	132
300-200, C-C	99

Table 2-8. Uniformity value obtained when both shafts were rotating at 200 rpm with different shaft rotational directions in the High-Intensity-Preconditioner

Treatments (big shaft – small shaft)	Uniformity
200-200, C-C	99
200-200, CC-CC	41
200-200, C-CC	99
200-200, CC-C	119

Table 2-9. Uniformity value obtained when both shafts rotating at 200 rpm in the same rotational direction under room temperature and 68±2°C in the High-Intensity-Preconditioner

Treatments (big shaft – small shaft)	Uniformity
200-200, CC-C, room temp	151
200-200, CC-C, 68±2 °C	142

Table 2-10. Uniformity value obtained when both shafts rotating at 300 rpm in the same rotational direction under room temperature, 68±2°C and 90±1 °C in the High-Intensity-Preconditioner

Treatments (big shaft – small shaft)	Uniformity
300-300, CC-C, room temp	144
300-300, CC-C, 68±2 °C	51
300-300 CC-C, 90±1 °C	52

Figures

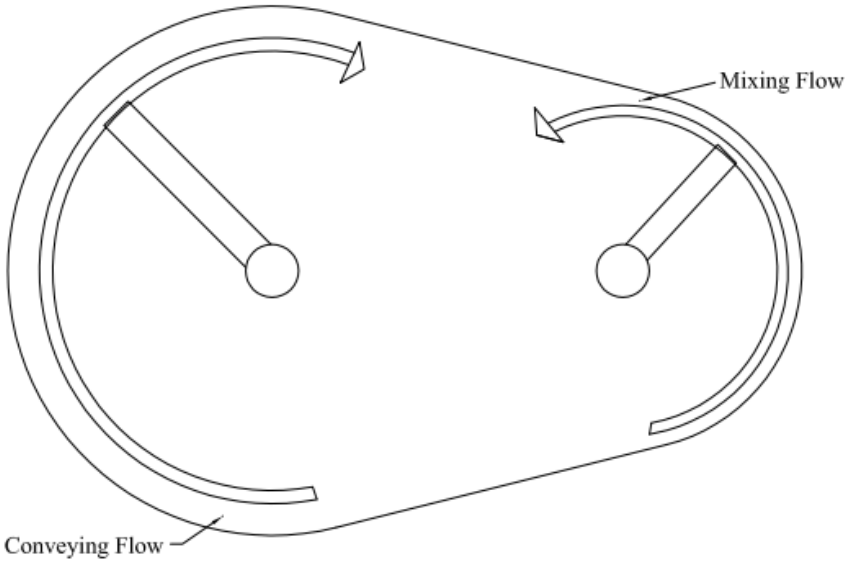

Figure 2-1. The two shafts in the High-Intensity-Preconditioner (HIP)

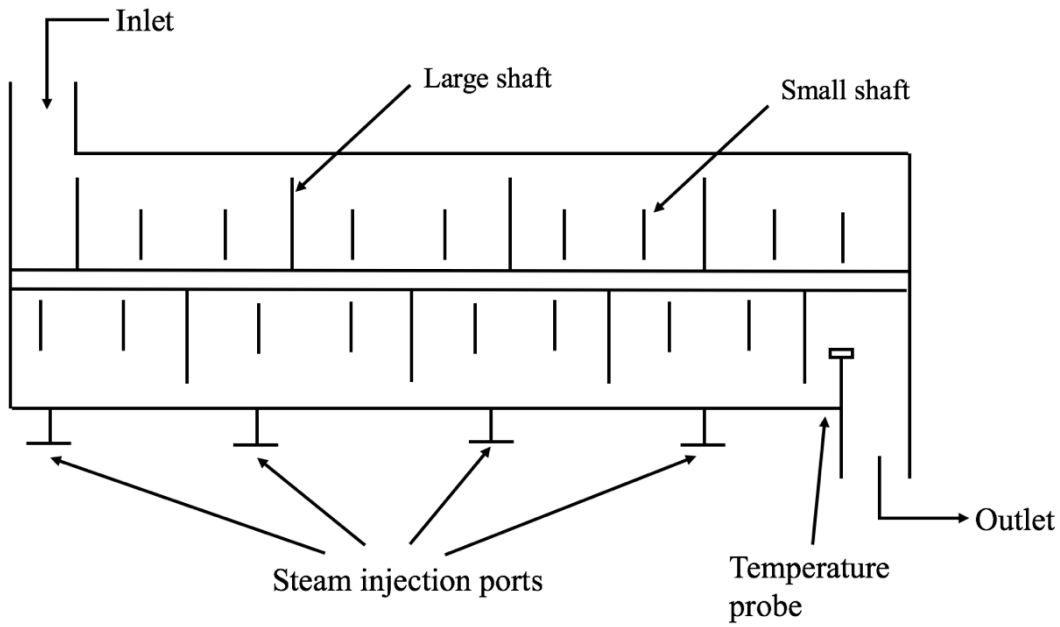

Figure 2-2. HIP system

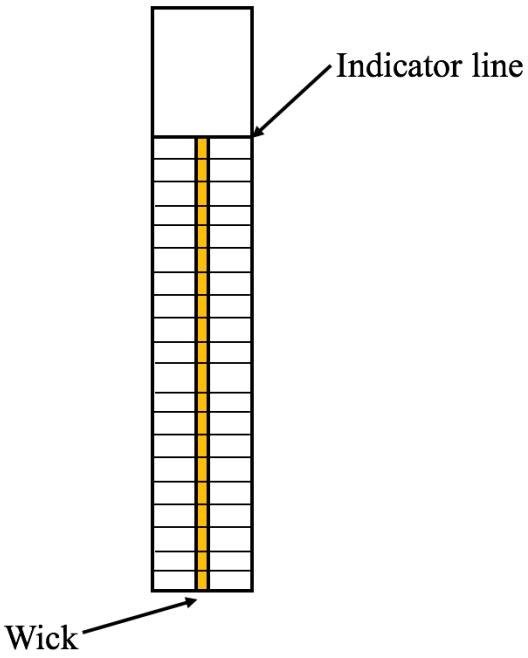

Figure 2-3. Quantab test strip

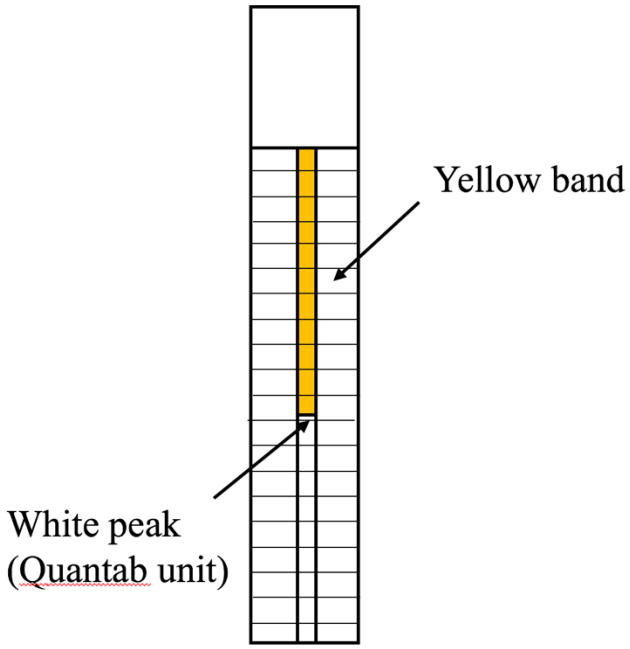

Figure 2-4. Used Qutantab® test strip

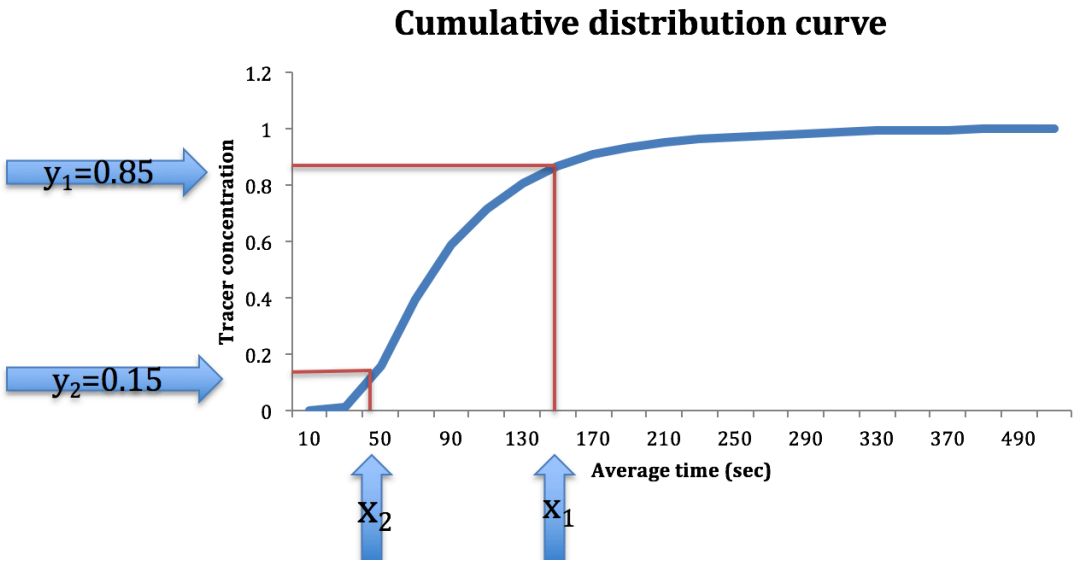

Figure 2-5. Using cumulative distribution curve to calculate uniformity

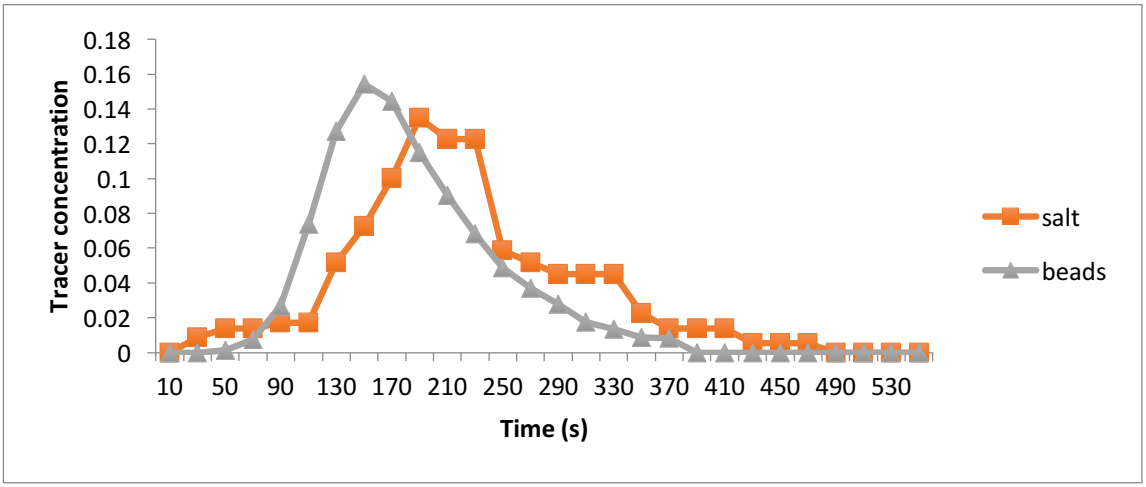

Figure 2-6. Comparison of residence time distribution curves between using beads and salt as the tracer when both shafts were at 200 rpm and had clockwise rotation in the High-Intensity-Preconditioner

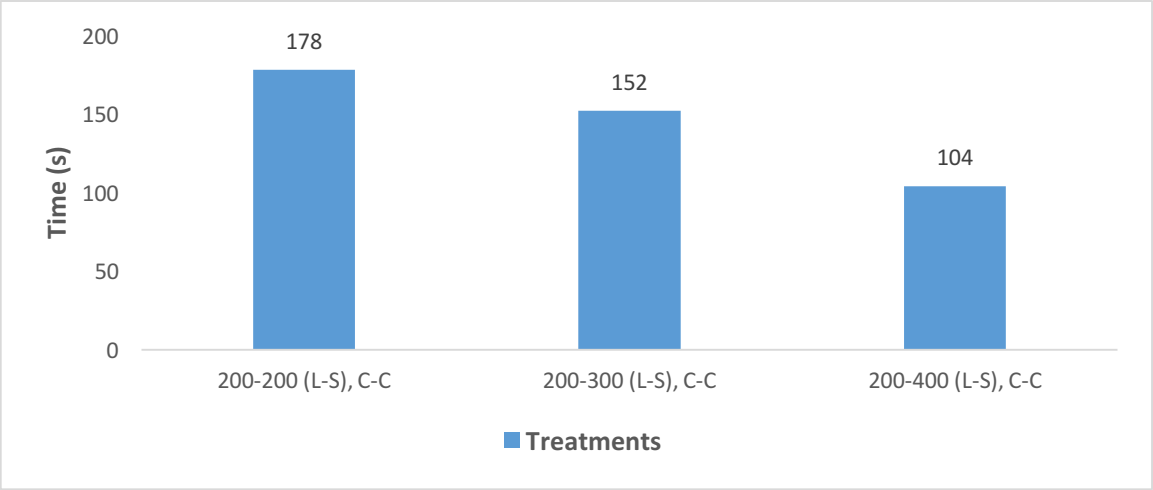

Figure 2-7. Residence time obtained when the shaft speed on the small shaft from 200 to 400 rpm while 200 rpm on the large shaft and both shafts had clockwise rotational direction in the High-Intensity-Preconditioner

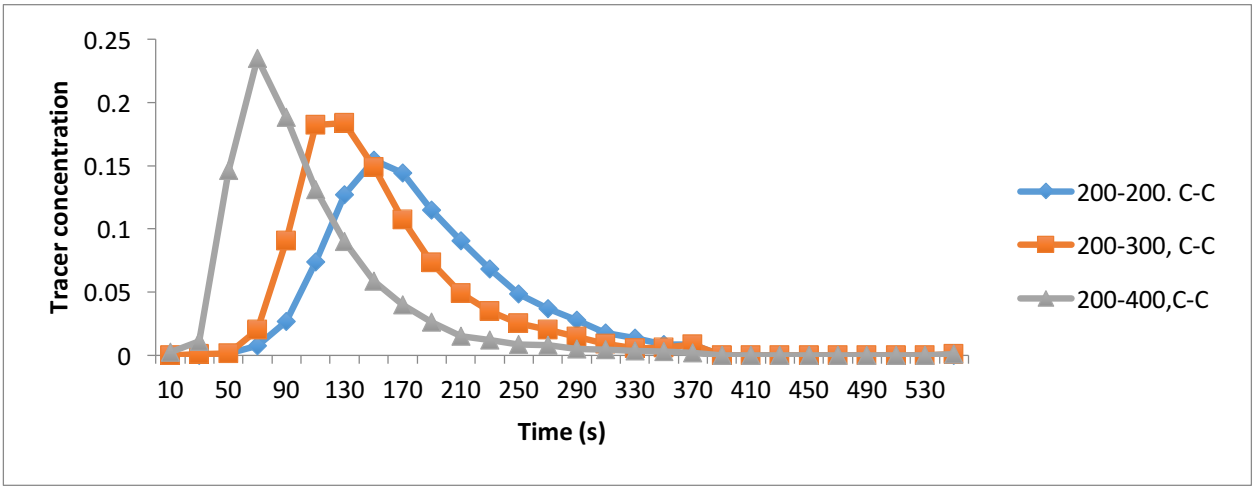

Figure 2-8. Residence time distribution curves obtained when the small shaft speed increased from 200 to 400 rpm while 200 rpm on the large shaft and both shafts had clockwise rotational direction in the High-Intensity-Preconditioner

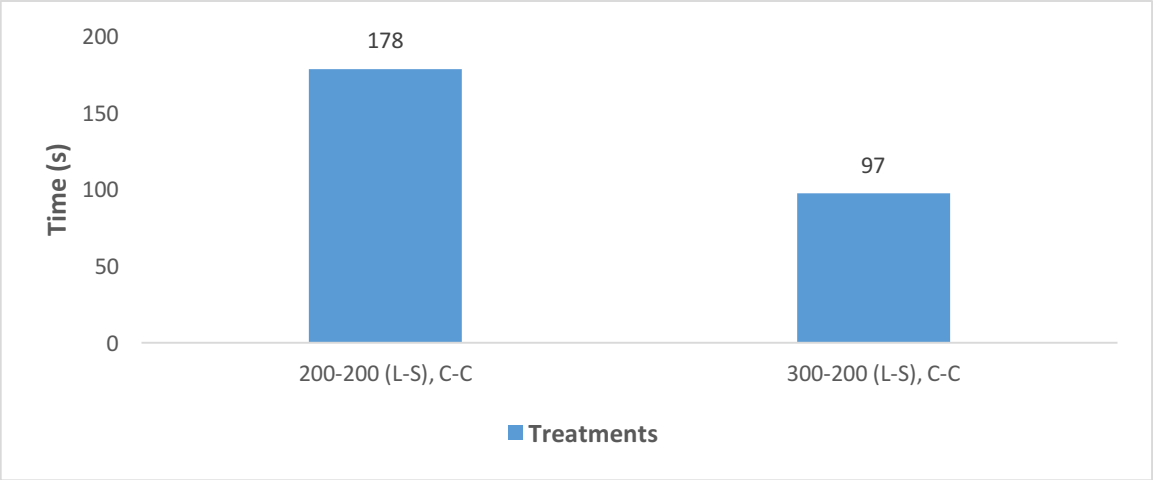

Figure 2-9. Residence time obtained when the shaft speed on the large shaft from 200 to 300 rpm while 200 rpm on the small shaft and both shafts had clockwise rotational direction in the High-Intensity-Preconditioner

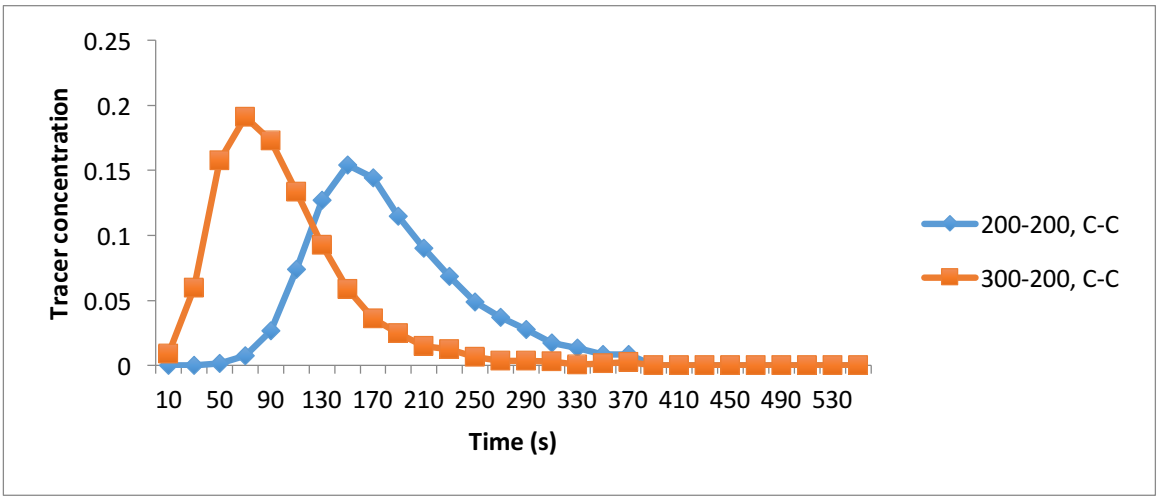

Figure 2-10. Residence time distribution curves obtained when the large shaft speed increased from 200 to 300 rpm while 200 rpm on the small shaft and both shafts had clockwise rotational direction in the High-Intensity-Preconditioner

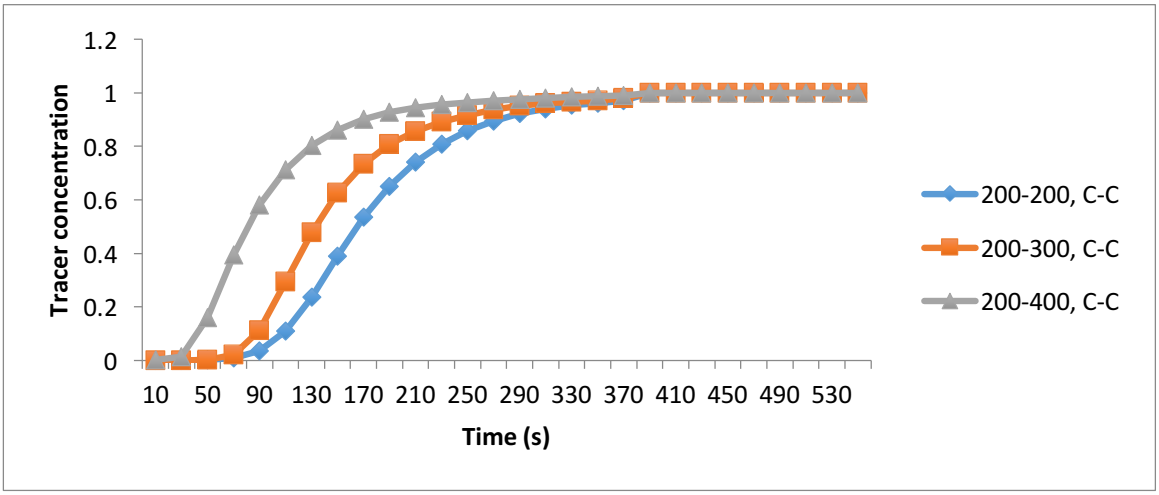

Figure 2-11. Cumulative distribution curves obtained when the small shaft speed increased from 200 to 400 rpm while 200 rpm on the large shaft and both shafts had clockwise rotational direction in the High-Intensity-Preconditioner

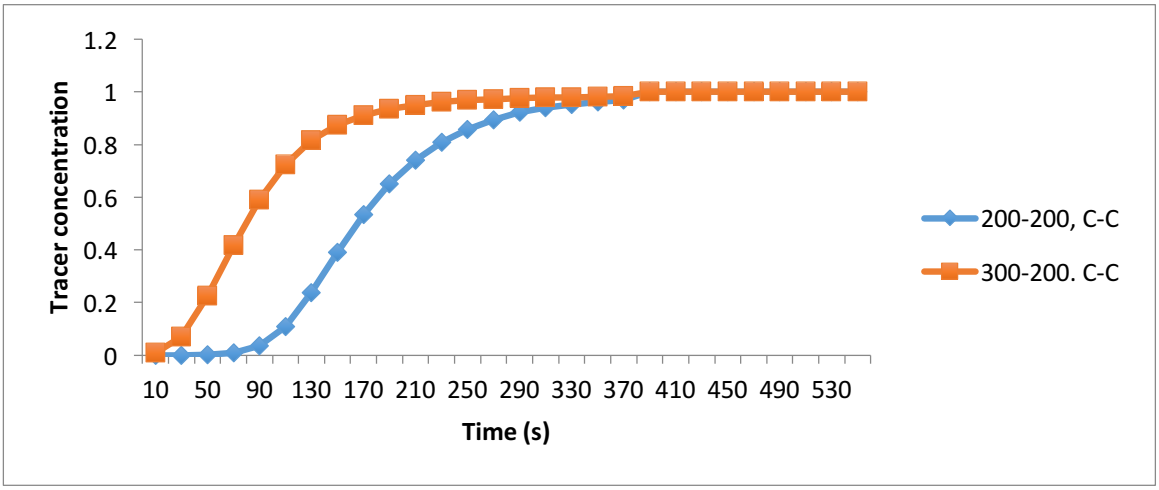

Figure 2-12. Cumulative distribution curves obtained when the large shaft speed increased from 200 to 300 rpm while 200 rpm on the small shaft and both shafts had clockwise rotational direction in the High-Intensity-Preconditioner

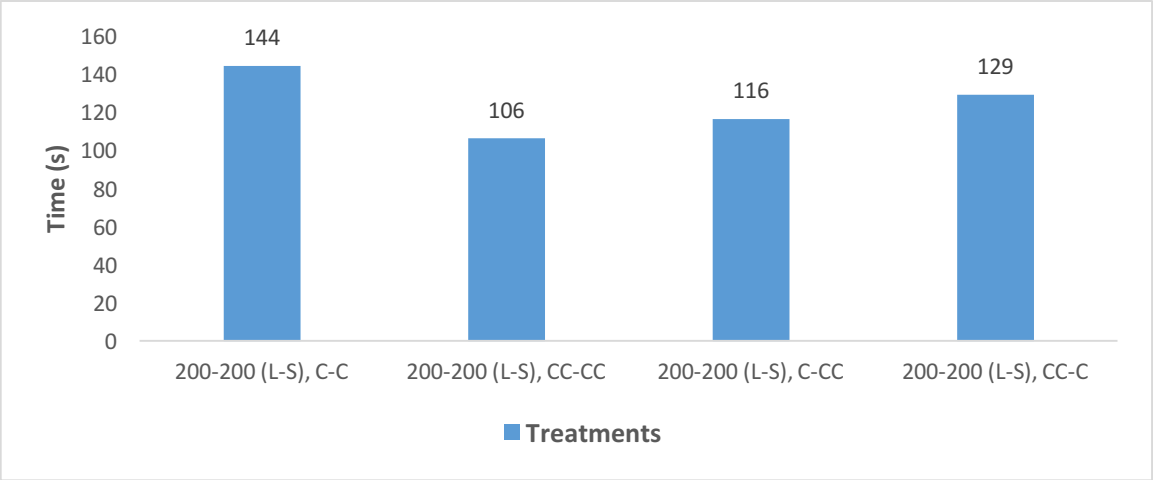

Figure 2-13. Average residence time obtained when both shafts were rotating at 200 rpm with four different combinations of shaft rotational direction in the High-Intensity-Preconditioner

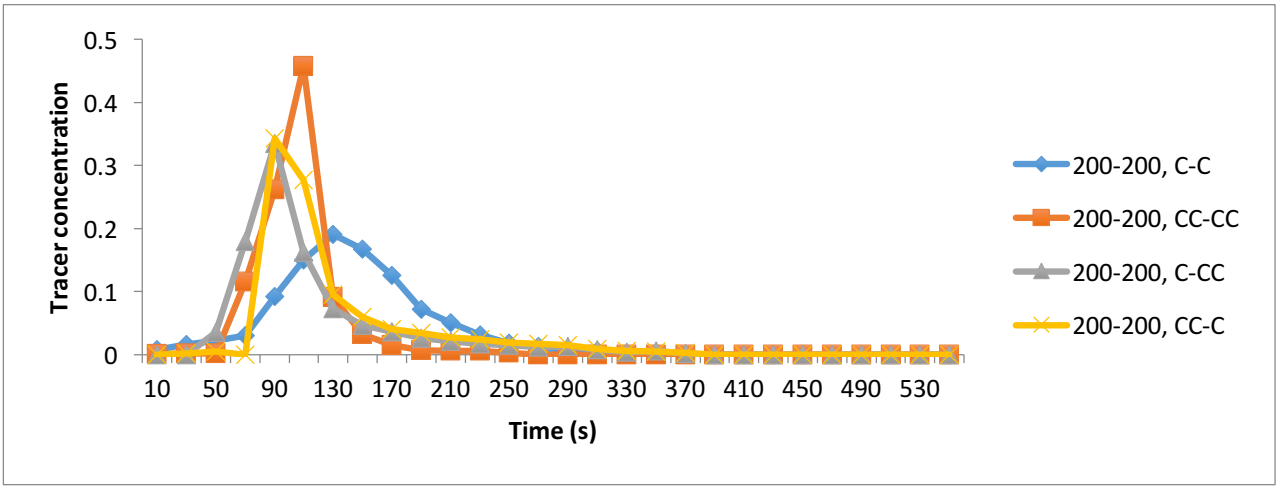

Figure 2-14. Residence time distribution curves obtained when both shafts were rotating at 200 rpm with different shaft rotational directions in the High-Intensity-Preconditioner

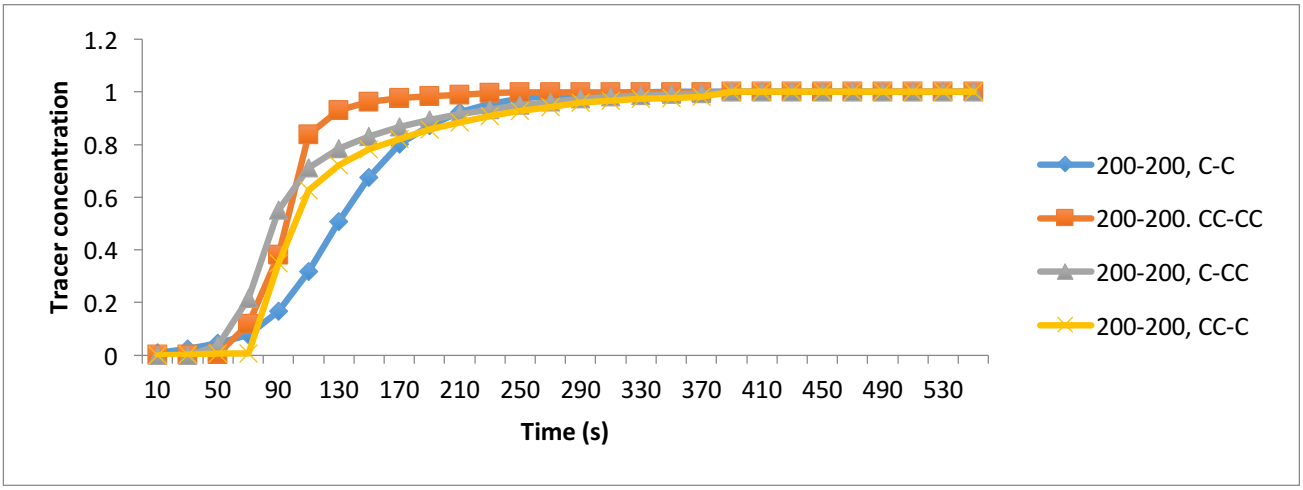

Figure 2-15. Cumulative distribution curves obtained when the shafts were rotating at 200 rpm with different shaft rotational directions in the High-Intensity-Preconditioner

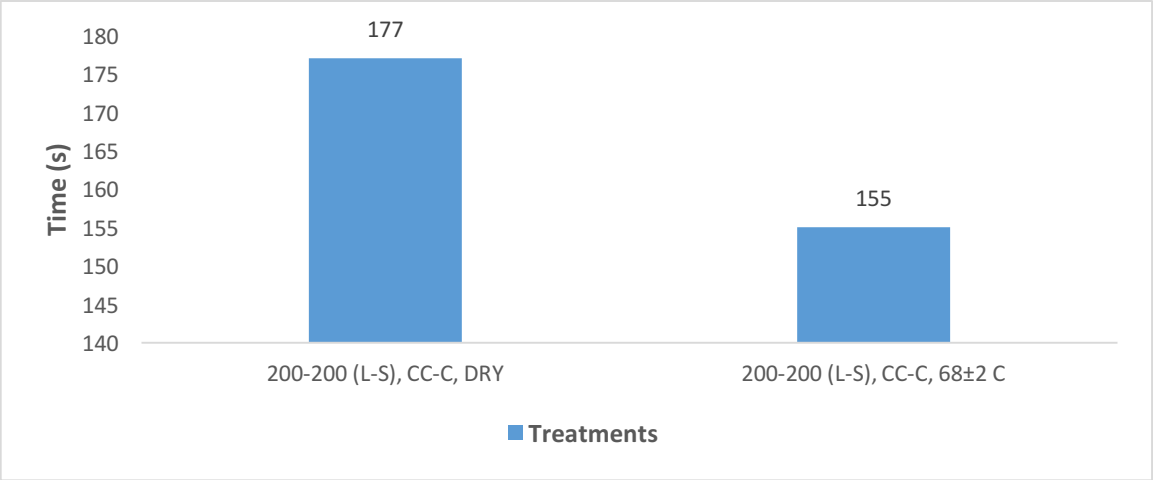

Figure 2-16. Residence time obtained when both shafts rotating at 200 rpm in the same rotational direction under room temperature and 68±2^{o}C in the High-Intensity-Preconditioner

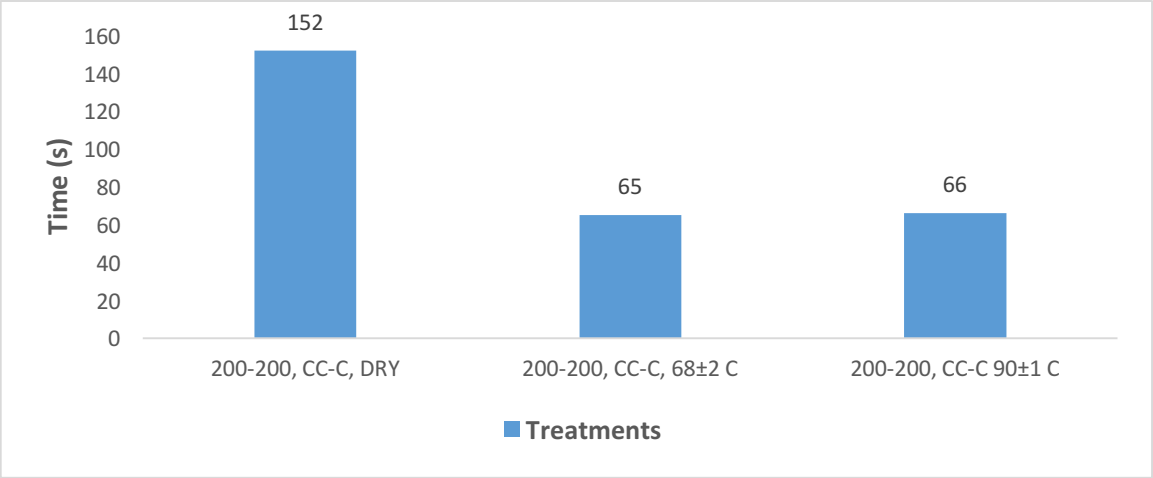

Figure 2-17. Residence time obtained when both shafts were rotating at 300 rpm under room temperature, 68±2 ^{o}C and 90±1 ^{o}C in the High-Intensity-Preconditioner

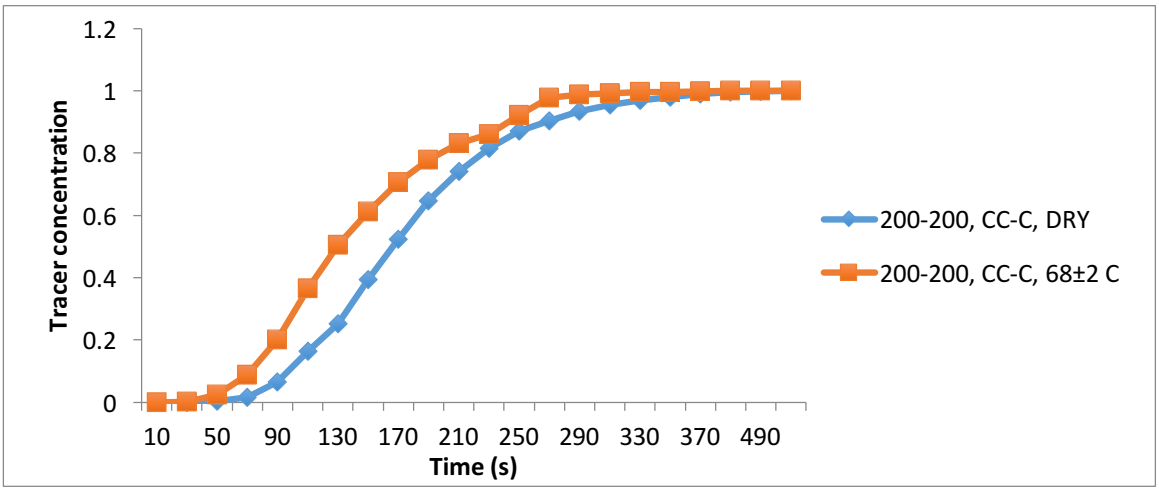

Figure 2-18. Cumulative distribution curves obtained when both shafts were rotating at 200 rpm under room temperature and 68±2 ^{o}C in the High-Intensity-Preconditioner

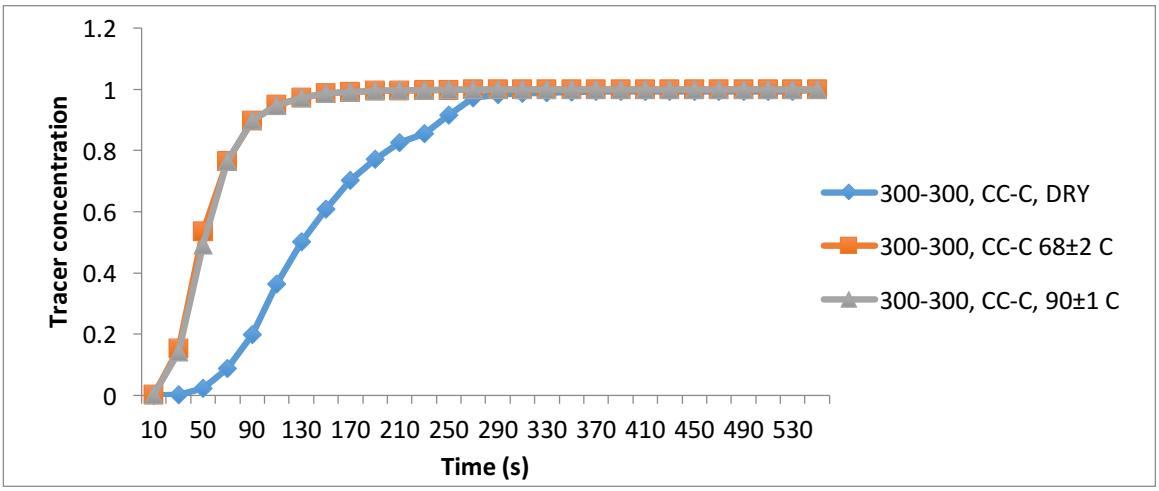

Figure 2-19. Cumulative distribution curves obtained when both shafts were rotating at 300 rpm under room temperature, 68±2 ^{o}C and 90±1 ^{o}C in the High-Intensity-Preconditioner

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Chapter 3 - Role of High-Intensity-Preconditioner in dry pet food safety – *Enterococcus faecium* as a surrogate for *Salmonella*

Abstract

An increase of *Salmonella* related dry pet food outbreaks hasd raised concerns regarding pet food safety. The efficacy of a High-Intensity-Preconditioner (HIP) was validated in this study to determine its ability to function as a critical control point (CCP) against *Salmonella* during dry expanded pet food processing. The preconditioner barrel provides a heated and moist condition, and is theoretically ideal for microbial destruction. *Enterococcus faeiucm* (American Type Culture Collection, ATCC® 8459™) was chosen as a surrogate organism for *Salmonella* because *E. faecium* possesses a higher heat-resistance than *Salmonella,* yet shares similar characteristics and behaviors with *Salmonella*. The impact of precondition temperature (68±2 ^{o}C and 90±1^{o}C) and preconditioner shaft speed (200 and 300 rpm, corresponding to 154 and 65 s of residence time, respectively) on *E. faecium* deactivation was analyzed. An increase in temperature and/or time resulted in a better microbial destruction. The HIP temperature range of 68±2 ^{o}C was not effective in eliminating *E. faecium*, while 90±1 ^{o}C for 65 s was sufficient to provide a 5 log CFU/g reduction in *E. faecium* as determined by plating on selective agar (mEnterococcus agar). However, only a 3.5 log CFU/g of *E. faecium* was reduced on the non-selective agar (Brain-Heart Infusion agar) after treatment at high temperature (90±1 ^{o}C) for 65 s, indicating the presence of sublethally injured cells in the pet food materials. The throughput in the HIP was 453.6 kg/hr and under such a large throughput, heat distribution might not be even, which could be the possible reason leading to incomplete destruction.

Keywords*: Salmonella, Enterococcus faecium,* pet food safety, preconditioner

3.1 Introduction

Salmonella spp. has been related to foodborne illness outbreaks associated with low-moisture food including powdered milk, beef jerky, peanut butter and cereal (Ceylan & Bautista, 2015). During the last five years, numerous pet food recalls have been found related to *Salmonella enterica* contamination. In 2014, 8 out of 12 pet food recalls were *Salmonella* related (2014 Pet Food Recall List, 2014). From 2009 to 2013, there were a total of 102 pet food recalls, 61% of which were *Salmonella* related (Mehlenbacher et al., 2012). On May 16, 2008, the U.S. Centers for Disease Control and Prevention (CDC) reported a 2006–2007 multi- state outbreak of human infections with *Salmonella enterica* serotype Schwarzengrund, which was associated with dry dog food (CDC, 2008). In the report (CDC, 2008), 79 cases from 21 states had been announced. The report stated "This outbreak is the first documented outbreak to associate human *Salmonella* infections with contaminated dry dog food and to trace human illness to a contaminated pet food plant" (CDC, 2008). Dry dog foods, like many low moisture food products, typically do not support the growth of *Salmonella*. However, *Salmonella* can endure for prolonged periods of time under dry conditions due to its innate ability to become resistant to desiccated conditions (Montville et al., 2012).

Pet food safety becomes a substantial challenge as the hazards can directly impact pets and indirectly affect owners who share the same environment. The pet owners can be infected with *Salmonella* from contacting with the feces of contaminated animals (Murray et al., 2007). Under the implemented Food Safety Modernization Act (FSMA), pet food facilities are required to establish food safety plans, which could be internationally recognized as modern preventive controls. To serve the purpose of minimizing or preventing the hazards identified in the hazard analysis, the preventive controls may comprise sanitation procedures for food contact surfaces

and utensils; employee hygiene training; recall plan; cGMPs; supplier verification activities related to food safety and microbiological environmental monitoring program (Silliker, 2014). During the extrusion process, expanded kibble-style pet food materials go through 2 different barrels individually providing thermal energy: the preconditioner and the extruder. Extrusion was recommended as a critical control point for *Salmonella* during dry pet food processing (American Feed Industry Association, 2010). However, few publications validating the preconditioning or extrusion steps during dry pet food manufacturing for this purpose have been published. Since most of the cooking (thermal energy input) takes place in the preconditioner, the preconditioner's capability of *Salmonella* inactivation should be first verified.

A High-Intensity-Preconditioner (Model 150, Wenger® Mfg., Sabetha, KS) was used in this study. The HIP is an innovation in the extrusion industry and compared to traditional preconditioners, the HIP is capable of providing a wider range of residence times – the time that pet food materials spend going through the preconditioner barrel where they receive a thermal treatment. In our previous study, residence time and residence time distribution (RTD) in the HIP barrel under the same condition was investigated and the data collected will be used in this study.

Enterococcus faecium has been introduced as a surrogate organism of *Salmonella* in several thermal lethality studies (Jeong et al., 2011; Bianchini et al., 2013). It presents a higher thermal resistance than *Salmonella*, yet shares similar thermal lethality characteristics with *Salmonella.* Besides, *E. faecium* (American Type Culture Collection, ATCC 8495) is a strain classified as biosafety level 1 (BSL-1) bringing minimal potential hazard to laboratory personnel and the food processing environment. These characteristics make *E.faecium* ATCC 8495 an adequate surrogate for *Salmonella* during the extrusion process.

The objective of this chapter was to determine if the HIP could serve as an effective critical control point (CCP) for *Salmonella* control by using *E. faecium* as a surrogate under two temperature levels and two residence time levels. The residence time data previously obtained was used as the time reference for materials being transported through the HIP.

3.2 Materials and Methods

3.2.1 Raw materials.

A generic dry dog food mix (KSU mix) obtained from Lortscher Animal Nutrition Inc. (Bern, KS) was used. The KSU mix was formulated with ingredients listed below: corn flour (48%), poultry by-product meal (20%), corn gluten meal (21%), rice meal (10%), vitamin premix (0.5%), mineral premix (0.5%). Ingredient specification of vitamin premix and mineral premix were listed, respectively in Tables 3-1 & 3-2. Protein content in the raw materials provided by Lortscher Anmial Nutrition Inc. was 30.94%, which was obtained using Dumas Combustion Method. An NIRS™ DA1650 Feed Analyzer (Hillerod, Denmark) was used to determine levels of protein, ash, moisture, fat and fiber in the KSU mix. Carbohydrate level of KSU mix could be calculated by the percent remaining after all the other components have been measured: % carbohydrates = 100 - %moisture - %protein - %fat - %ash (BeMiller, 2004).

3.2.2 Inoculum and cultures preparation.

Enterococcus faecium (ATCC® 8459™) was obtained from the American Type Culture Collection (Manassas, VA) and was maintained by Kansas State University Food Safety Defense Lab (FSDL) at -70 ^{o}C. To activate cultures for this study, frozen beads with *E.faecium* culture were transferred to a test tube combining 10 mL BBL™ Brain Heart Infusion (BHI) broth (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated at 35 ^{o}C for 24 h. After incubation, *E.faecium* broth from the test tube was streaked for isolation on Tryptic Soy Agar

(TSA) plates (Difco, Becton, Dickinson Co., Sparks, MD) agar. The TSA plates were incubated at 35 ^{o}C for 24 h. One isolated colony was picked for strain confirmation which was completed using an API® 20 Strep kit (Biomerieux, Marcy l'Etoile, France). Once confirmed as *E. faecium*, a single colony was picked and transferred to 10 mL of BHI broth and incubated at 35 ^{o}C for 24 h. Ten milliliters of culture were inoculated into each of 1-L jug of BHI broth, and all jugs were incubated for 48 h at 35 ^{o}C. The *E.faecium* concentration of this master inoculum achieved 7.4 log CFU/mL. This was done in the FSDL.

3.2.3 Pre-hydration and inoculation of pet food.

Although *E.faecium* is an approved non-pathogenic surrogate and can be used in commercial feed manufacturing, facilities, biosafety protocols established by the K-state Feed Safety Research Center (FSRC) and approved by the University's Institutional Biosafety Committee (IBC#1038.3) were followed during these studies. Water addition was necessary during pet food preconditioning. However, no water but only steam was injected into the HIP; thus, pre-hydration of raw material to 14% (w/w) moisture level was completed before raw materials were fed to the HIP. Four kilograms of inoculum and 6.54 kg of water were mixed with 226.8 kg (500 lbs) of pet food mix in a double paddle mixer (H.C. Davis Sons Mfg Co., Inc. Bonner Springs, KS) with a sealing lid and capable of handling 272 kg of materials. The inoculum was homogeneously mixed into the pet food materials. The mixing time was 15 min allowing *E.faecium* to absorb and attach to the dog food mix. *E. faecium* level in the inoculated feed reached an average of 5.6 log CFU/g. Inoculated samples without the thermal treatment were used as the control. After mixing, the inoculated dog food mix was collected into 50-pounds feed bags and transported to the HIP, where it was utilized to feed the HIP.

3.2.4 High-Intensity-Preconditioner (HIP) treatments.

The inoculated pet food materials were fed into the HIP at a throughput of 453.6 kg/hr. The HIP equipment was run for 10 min to establish a consistent throughput of materials and a stable temperature prior to sample collection. The shaft rotational direction was the constant all the treatments: the small shaft had counter-clockwise rotation while the big shaft had clockwise direction. The post-treatment samples were taken at the end of the HIP, as soon as the system reached equilibrium. The impact of residence time was tested first. The HIP was set at the temperature was approximate 68±2 ^{o}C, and both shafts were rotating at 200 rpm generating a relatively longer residence time treatment at first. Shorter residence time treatments were accomplished by increasing shaft speed to 300 rpm. Effect of temperature was analyzed when the shaft speed was maintained at 300 rpm and increased the temperature to 90±1 ^{o}C (Table 3-3).

Temperature ranges was controlled by the steam injection level: Time was manipulated by different shaft speeds: 200 rpm vs. 300 rpm in this case and according to the residence time study, 153 s of residence time was generated with a 7% steam valve opening at 200 rpm, while approximately 66 s was generated with a 7% and 9% steam valve opening at 300 rpm.

3.2.5 Sampling for microbial testing.

Samples of dog food mix were taken before inoculation, after inoculation, and after preconditioning. A probe was used to collect samples at the exit of the HIP and twenty-five grams of sample were weighed and instantly put in the 225 mL of chilled peptone water (1%) prepared earlier. For pre- and post-inoculation samples, duplicate samples were obtained and analyzed. For preconditioned samples, three samples were collected for each inoculated treatment at 1, 5, and 9 min after the system reached operational equilibrium. After the HIP reached an operational steady state, all the materials inside the barrel were treated the same

within one treatment and sampling at three different time points were considered to be as a triplicate sample. The peptone water is a buffered solution that protects microbial cells from breakage due to the difference in osmotic pressure and keeping peptone water chilled helped to stop the thermal destruction immediately after the samples were taken out of the HIP. The sample bags containing both chilled peptone water and preconditioned pet food were put in an ice chest and transported immediately to the FSDL for microbiological analysis.

3.2.6 Enumeration.

The 25-g treated samples contained in filtered stomacher bags in 225 mL of buffer (1:10 dilution) were mixed by stomacher (Smasher, AES Laboratorie, Bruz, France) for 1 min and serial dilutions (1:10) were subsequently prepared using 0.1% peptone water. The sample dilutions were spread-plated on modified Enterococcus (mE) agar (Becton, Dickinson, Sparks, MD) and BBL^{TM} BHI agar (Oxoid Ltd., Basingstoke, Hampshire, England). mE agar is selective while BHI agaris non-selective. mE agar plates were incubated at 35 ^{o}C for 24 h. BHI agar plates were sent to the incubator at 35 ^{o}C and after 6 h of incubation, a thin overlay of mE agar was added to the top of the BHI agar and incubated for another 18 h. If there was no presumptive *E. faecium* colony seen on the plates, an enrichment procedure was applied. There were two reasons why colony might not be found on the plates: 1. there was no microbial growth on the plates indicating the microbial destruction was complete; 2. if the inactivation was incomplete, viable *E.faecium* population level was below the detection limit for the direct plating procedure that was used (0.7 log CFU/g). Enrichment is a qualitative (+ or -) procedure applied to determine whether the destruction was complete or not. To perform enrichment, 25 mL of original sample (a mixture of peptone water and pet food) was taken and added to 250 mL of BHI broth for incubation at 37 ^{o}C for 24 h. The enriched sample was then spread-plated on mE agar and

incubated at 35 ^{o}C for 24 h. The presence of any presumptive colonies on these plate was confirmed as described in the next section.

3.2.7 Colony confirmation.

E.faecium is a gram-positive bacteria and *E.faecium* colonies appear as purple on both mE agar and BHI agar according to Public Health Agency of Canada (2011). However, some other bacterial species such as *Streptococcus bovis* might be able to grow on the media, presenting results similar to those of *Enterococcus* spp. (Manero and Blanch, 1999). Therefore, representation colonies on the agar, purple-coccus, was picked for further confirmation. A gram stain test kit (S25344, Fisher Science Co. L.L.C., Nazareth, PA) was first used to distinguish the colony into the gram-positive or negative group (Cornell University, n.d). Then an API® 20 Strep kit (Biomerieux, Marcy l'Etoile, France) was used to biochemically identify the colony as being *E.faecium*.

3.2.8 Water activity determination.

Water activity (a_w) samples were obtained before inoculation, after inoculation and after preconditioning and put in individual disposable cups. Testing cup containing 7.5mL of sample was placed in the water activity meter (AquaLab Series 4TEV, Decagon Devices, Inc., Pullman, WA) for analysis.

3.2.9 Statistical analysis.

The microbial data was expressed as the mean values of reduction rate and standard deviations after log transformation. Data analysis was following the model “Random Complete Block Design”. Significant differences in average values were established by the Tukey-Kramer multiple comparison methods at a 5% level of significance using SAS (version 9.4, 2012, SAS Institute, Cary, NC). Differences were considered statistically significant at $P<0.05$.

3.3 Results

Table 3-4 presented the approximate carbohydrate, protein, fat, ash, fiber and moisture content of the raw materials. The KSU mix had a 29.78% of protein level, which was the same as with the manufacturer's specification sheet.

The concentration of master inoculum reached 8.1 (±0.25) log CFU/mL. The *E.faecium* population in inoculated pet food had an average value of 5.9 (±0.47) log CFU/mL.

Since different *E.faecium* concentrations in inoculated pet food were observed from each treatment, the microbial data in Table 3-5 was presented in reduction rate instead of recovery rate. Higher reduction rate was noticed with increasing residence time or higher temperature. A 5 log reduction was observed after STHT treatment on mE plates while only 3.5 log reduction was found on BHI plates. The reduction rate in STLT treatment was significant different from the other 2 treatments: LTLT and STHT.

Water activity (a_w) value of raw, inoculated preconditioned pet food materials were listed in Table 3-6. Water activity of preconditioned pet food range from 0.83 to 0.85 under each treatment.

3.4 Discussion

A recent study focused on the use of *Pediococcus acidilactici* ATCC 8042 as a surrogate for *Salmonella* (Ceylan and Bautista, 2015) was published. The experiment was conducted under three different moisture levels (9.1%, 17.9%, and 27.0%) which were mimicking moisture levels for raw, preconditioned and extruded pet food. Samples were heated from 60 ^{o}C to 88 ^{o}C. At temperatures below 90 ^{o}C, *P. acidilactici* was found to have lower heat resistance than *E. faecium,* yet still had higher heat resistance than *Salmonella*. At 16.9% moisture level, $D_{82.2}$ at was 0.42, 0.91 and 3.99 respectively for *Salmonella, P. acidilactici* and *E.faecium*; $D_{87.8}$ was only

recored for *E.faecium*, which was 1.08. Therefore, Ceylan & Baustista (2015) concluded that *P. acidilatici* was a better surrogate for *Salmonella* than *E. faecium*. However, *P. acidilactici* was not chosen in this study because the heat resistance of *Salmonella* could be affected by many factors (Doyle and Mazzotta, 2000). *Salmonella* could easily develop thermal resistance due to several factors including growth medium composition, growth phase, growth temperature, intrinsic parameters (water activity, fat content, salt content for instance), and pathogen strains used (Harris, 2008; Tuntivanich et al., 2008). This was one of the reasons why *E.faecium* was chosen to act as a surrogate in our study: with the characteristic of being more heat-resistant that both *P. acidilatici* and *Salmonella*, if a 5 log reduction of *E.faecium* was observed, a better inactivation of *Salmonella* under the same processing condition could be inferred, even for *Salmonella* strains that potentially developed a degree of thermal resistance. Additionally, *E.faccium* (ATCC® 8459™) was easily obtained, cultivated and enumerated and would not lead to spoilage issues (Ceylan and Bautista, 2015) as this research facility is also utilized to manufacture consumable animal feeds. Consequently, *E.faccium* (ATCC® 8459™) was used as a surrogate for *Salmonella* spp. in our study.

Longer residence time (153 s vs. 66 s) or higher preconditioner temperature (90±1 ^{o}C vs. 68±2 ^{o}C) positively affected microbial inactivation (Table 3-5), which agreed with our expectation. Similar results were also collected by Tunivanich et al. (2008) in their turkey breast study and Villa-Rojas et al. (2013) in almond kernel study. The reduction rate of samples from STLT treatment obtained in replication two was at least 1 log CFU/g higher than it was in replication one on both mE agar and BHI agar. The reason for this difference might be the HIP itself: the system may not have been stable when samples were taken, therefore fluctuations might appear in preconditioner temperature which would lead to variation in microbiological

results. This inconstancy might have happened during experiment at random, so the only way to achieve valid results was repetition.

On mE plates, at least a 5 log of *E.faecium* was reduced after the preconditioning (Table 5). Even at the high-temperature range (90±1 ^{o}C), elimination of *E.faecium* was not complete: 3.5 log CFU/g of *E.feacium* was reduced on BHI plates (compared to *E.faecium* population in control sample: 5.6 log). At 68±2 ^{o}C, longer residence time (~87 s) significantly reduced *E.faecium* population (Table 3-5). On BHI plates, similar reduction rates were observed from both LTLT and STHT treatments: higher temperature might be more capable of damaging the cells but was not effective enough to remove them from pet food. There are a couple of possibilities leading to incomplete destruction in the HIP: the steam was injected into the bottom of the HIP barrel constantly and after the system reached the equilibrium, steam injection theoretically would be able to provide a stable-heated environment. However as discussed above, instability might happen at times. Moreover, the processing was running under a high throughput (485.3 – 494.4 kg/hr) and heat distribution might not be even. Pet food material would have hit any part of the barrel when the shafts were rotating, and the top part of the HIP barrel might have failed to reach the target temperature.

Insufficient residence time might also have resulted in incomplete microbial destruction. The residence time data presented here is the average residence time, meaning that some portion of the materials might have spent less time in the HIP compared to the rest. Based on the previous study regarding RTD, the treated pet food material could be seen at the end of the HIP as early as 10 s. Thus, some of the inoculated pet food only took 10-20 s traveling in the HIP, while some of the material may have likely accumulated in the barrel and received a longer

thermal treatment. Therefore, there is a real likelihood that a portion of the material will be inadequately exposed to the thermal treatment.

The reduction rate of *E.faecium* on the mE plates was higher than the reduction rate on the BHI-overlay plates indicating a portion of cells were injured during the HIP process (Table 3-5). *E.faecium* cells that were sublethally injured by the heat in the HIP barrel required more sensitive methods for recovery such as enrichment broth (Podolak et al., 2010). The temperature range of 68±2 ^{o}C was not sufficient to destroy *E.faecium* and even longer exposure time did not contribute much to lethality. According to the Public Health Agency of Canada (2011), *Enterococcus* should be destroyed by temperatures in excess of 80 ^{o}C and the range of 68±2 ^{o}C in this study was below this target. However, this value (80 ^{o}C) was the average of data obtained from various types of food products containing different ingredients, and this value cannot be applied to all foods because a pathogen surviving in a specific ingredient or product matrix may be more resistant to processing treatments than in other products (Li et al., 2014).

When bacteria were subject to multiple types of physical or chemical stresses, such as sub-lethal heat, their immediate growth was precluded (McKillip, 2001). Bacteria then entered a phase named viable-but-not-culturable (VBNC) and the bacteria in VBNC phase could not be cultured on selective-agar. This injury was characterized by decreased resistance to selective agents or by increased nutritional requirements (Doyle et al., 2001). If one wants to avoid an underestimation of the true counts of live target pathogens, a non-selective plating medium should be used to regain metabolic activity. Chemical component(s) in the selective medium such as antibiotics (in this case, Sodium Azide in mE 7544 agar), could inhibit growth of injured cells because cells are less antibiotics-resistant when they are injured. To enumerate sublethaly injured pathogens, a common approach was to employ a specialized agar overlay method during

plating of the serially diluted sample (Speck et al., 1975). The inoculum is first spread-plated on a non-selective solid nutritive medium and during a short non-selective incubation period, mixed bacterial populations have an opportunity to physically repair and begin to grow. Then a second thin layer of the selective medium is poured on top of the original layer to provide an environment that inhibits all or most non-target cells from growing. Injured cells pose a serious food safety problem: if injured cells are mistakenly categorized as dead cells, manufacturers may overestimate the effectiveness of their process for controlling target pathogens (Mafart, 2000). Further, these injured pathogen cells can potentially be infectious and pose a health risk to those who consume the product.

Based on the Food and Drug Administrations (FDA) Hazard Analysis and Critical Control Point (HACCP) guidance, a 5 log reduction standard must be implemented to verify the effectiveness of CCP(s) (Demirci and Ngdai, 2012). Though the 5-log-reduction rule is only mandatory for several types of food – juice, vegetable, seafood, for example – it is widely applied to other categories of foods. In our study, only the STHT samples (90±1 ^{o}C) on mE plates reached a reduction of 5-log. Even at high-temperature range (90±1^{o}C), only a 3.8 log reduction was noted when injured cells were taken into account. To receive better inactivation, significantly higher temperatures and/or residence time should be required. Such higher temperatures or long holding times may compromise the quality of treated products (Villa-Rojas et al., 2013) and would need to be evaluated appropriately.

3.5 Conclusion

Longer residence time or higher HIP preconditioning temperature would contribute to a decrease of microbial population. The temperature range from 89 to 91 ^{o}C was sufficient to provide a 5 log reduction of *E.faecium* when plated on mE agar but it was not an adequate

condition to completely inactivate *E.faecium* as only 3.5 log of population was reduced when plated on BHI agar. *E.faecium* cells were injured during the HIP processing yet still possessed food safety risks. Uneven heat distribution or unsatisfactory heat exposure time are the main possibilities resulting in incomplete microbial destruction. The HIP processing in our study did significantly reduced the *E.faecium* load, however complete inactivation was not observed while using direct plating method with BHI agar. To ensure safety, another CCP that should be validated in pet food manufacturing is the extrusion process other than the preconditioner downspout temperature.

Tables

Table 3-1. Vitamin premix specification in the KSU mix

Parameters	Specification	Parameters	Specifications
Calcium (%)	20.2	Copper (%)	1.1
Iodine (ppm)	1,320	Iron (ppm)	35,200
Selenium (ppm)	72.6	Manganese (ppm)	5,610
Zinc (%)	10.5		

Table 3-2. Mineral premix specification in the KSU mix

Parameters	Specification	Parameters	Specifications
Vitamin A (IU/lb)	5,278,000	Vitamin D3 (IU/lb)	347,000
Vitamin E (IU/lb)	33,113	Vitamin B12 (mg/lb)	7.2
Riboflavin (mg/lb)	1,635	D-Pantothenic Acid (mg/lb)	3,856
Niacin (mg/lb)	24,495	Folic Acid (mg/lb)	273
Vitamin B6 (mg/lb)	1,928	Thiamine (mg/lb)	2,268
Biotin (mh/lb)	28		

Table 3-3. Experimental design of thermal treatments on Enterococcus faecium in the High-Intensity-Preconditioner

Treatments	LTLT	STLT	STHT
Temperature range (^{o}C)	68±2	68±2	90±1
Shaft speed (big-small, rpm)	200-200	300-300	300-300
Shaft direction (big-small)	↻↺	↻↺	↻↺
Steam valve opening (%)	7%	9%	9%

Table 3-4. Proximate analyses of KSU dry dog food mix

Components	Content	Components	Content	Components	Content
Carbohydrate	46.3%±0.39	Protein	29.78%±0.26	Fat	6.66%±0.00
Ash	3.66%±0.00	Fiber	3.17%±0.03	Moisture	10.45%±0.01

Table 3-5. The Enterococcus faecium population reduced after treatment in the High-Intensity-Preconditioner on pet food mix under different temperatures (67-70 ^{o}C & 89-91 ^{o}C) and different residence time (153 s & 66 s)

	E.faecium population recovered (log CFU/mL)[a]	
	mE plate	BHI-overlay plate
153s, 68±2 ^{o}C (LTLT)	4.0±0.24[a]	3.4±0.29[a]
66s, 68±2 ^{o}C (STLT)	1.6±0.23[b]	1.5±0.29[b]
66s, 90±1 ^{o}C (STHT)	5.1±0.23[a]	3.5±0.29[a]

[a] The reduction rate data is represented by the mean of 3 samples at 3 sampling time from 1 treatment and standard deviations within 2 replications (n=6). Within individual columns, the values followed by different letters are significantly different (p<0.05). Reduction rate of each sample was obtained by using the population control samples to minus the recovery rate of each sample, respectively. The control samples were the inoculated dog food mix prior to thermal treatment.

Table 3-6. Water activity of pet food raw material, inoculated/hydrated material and preconditioned material

Water activity (%)[a]	Raw material	Post-inoculation/hydration	Post-HIP
153s, 68±2 ^{o}C (LTLT)	0.52±0.00	0.73±0.01	0.85±0.01
66s, 68±2 ^{o}C (STLT)	0.49±0.03	0.74±0.02	0.83±0.00
66s, 90±1 ^{o}C (STHT)	0.51±0.04	0.73±0.02	0.85±0.01

[a] The water activity data is represented by the mean of 4 samples and standard deviation (n=4)

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Chapter 4 - Microbial safety study of a dry expanded pet food process using extrusion – *Enterococcus faecium* as a surrogate for *Salmonella*

Abstract

Minimizing *Salmonella* contamination in dry pet food products is a substantial challenge in the pet food industry. The extruder provides a high-temperature, high-pressure and high-shear environment during the dry pet food processing and may serve as a potentially effective critical control point (CCP). *Enterococcus faecium* (American Type Culture Collection, ATCC® 8459™) was used as a surrogate organism for *Salmonella* in this project. The capability of a pilot-scale single-screw extruder equipped with a Differential Diameter Cylinder (DDC) preconditioner for reducing *E. faecium* population was investigated using an inoculated generic dog food formulation. Samples were collected at the end of the DDC preconditioner and the extruder while the extruder was operated at the feeder screw speed of 12.0 and 18.2 rpm, respectively and an extruder screw speed of 394 rpm; the downspout temperature ranged from 89 to 94 ^{o}C and the die temperature was between 120 and 140 ^{o}C. Preconditioned samples had a reduction rate of 3.1 and 2.9 log using BHI agar at 18.2 and 12.0 rpm feeder screw speed, respectively. Higher reduction rates were reported after preconditioning using mE agar: 6.0 log for 18.2 rpm feeder screw speed and 4.5 log for 12.0 rpm). *E. faecium* was completely removed from the pet food after both extrusion treatments, with no surviving cells detected by enrichment protocol. Titanium dioxide was introduced into the extruder to explore the impact of throughput and screw speed on residence time distribution (RTD).

Keywords: Extrusion, *Salmonella* control, *Enterococcus faecium*, dry pet food, validation

4.1 Introduction

Contaminated pet food poses health risks for both pets and pet owners. When pet owners improperly handle dry pet food (e.g., touch pet food; used food containers by bare hand and then touched their mouths; pet food placed in an open area where toddlers can easily reach), they may expose themselves to hazards. If their hands are not washed correctly, *Salmonella* might be transferred to human food or food contact surfaces easily. This explained why 79 people from 21 states were hospitalized from a dry pet food related *Salmonella* outbreak in 2007 (CDC, 2008).

Why do dry pet food products present a high risk of *Salmonella* contamination? Traditionally, dry pet food kibbles, as they are low-water-activity foods, were considered as low-risk for allowing *Salmonella* to survive. However, recent outbreaks have shown that *Salmonella* can survive in dry pet foods (Buchanan et al., 2011). Disease outbreaks product recalls were usually linked to products contaminated with *Salmonella* or manufactured with ingredients contaminated with pathogens (Buchanan et al, 2011). Pet food ingredients are a major source of *Salmonella*. Animal-origin products such as chicken meal or chicken by-product meal are usually at high risk of contamination with *Salmonella* (Mehlenbacher et al., 2012). Grain ingredients are also susceptible (Grocery Manufacturers Association, 2004). According to KuKanich (2011), commercial raw food diets that contained chicken are 5 times as likely to be contaminated with *Salmonella* organisms as are raw food diets that did not contain chicken.

Dry pet foods are commonly manufactured by extrusion systems. The raw materials are usually made with a mixture of ingredients containing grains, soy rendered protein meal and minor ingredients such as vitamins and minerals (Lambertini et al., 2016). The ingredients are first mixing in a mixer based on specific recipe, and further mixed is completed in the preconditioner. Other than mixing, the preconditioner hydrates the materials by injecting water

and steam into the barrel, and most of the cooking process is finished in the preconditioner (Riaz, 2007). The temperature and moisture level in the preconditioner barrel are brought to the levels needed for the following extrusion process. The extruder barrel provides a high-temperature, high-pressure and high-shear environment while coating, shaping and produces more mixing and cooking to the preconditioner (Riaz, 2007). Products are sent to the dryer after extrusion and at this time point, no recontamination is usually assumed (Lambertini et al., 2016). According to *Salmonella* control guidelines written by American Feed Industry Association (2010), extrusion is recommended as a critical control point (CCP) during dry pet food processing, but no validation study supporting its effectiveness on pet food had been published yet. As preconditioning is also a process that provides thermal energy in the form of moist heat, downspout temperature (temperature at the exit of the preconditioner) should also be verified as a CCP as well as the die temperature (temperature at the exit of the extruder).

Residence time stands for the duration that pet food materials spend in the extruder barrel. Longer residence time positively impacts *Salmonella* reduction, but might compromise pet food quality.

The objectives of this study were to 1) verify whether the preconditioner downspout temperature and the extruder die temperature could serve as an effective CCP for decreasing *Salmonella* contamination associated with raw ingredients to a negligible level; and 2) explore the residence time distribution in the single screw extruder under different throughputs or screw speeds.

4.2 Materials and Methods

4.2.1 Raw materials.

A generic dry dog food mix (KSU mix) obtained from Lortscher Animal Nutrition Inc. (Bern, KS) was used. The KSU mix was formulated with ingredients listed in Table 4-1. Ingredient specification of vitamin premix and mineral premix were listed in Tables 4-2 & 4-3. Protein content in the raw materials provided by Lortscher Anmial Nutrition Inc. was 30.94%, which was obtained using Dumas Combustion Method. An NIRS™ DA1650 Feed Analyzer (Hillerod, Denmark) was used to determine levels of protein, ash, moisture, fat and fiber in the KSU mix. Carbohydrate level of KSU mix could be calculated by the percent remaining after all the other components have been measured: % carbohydrates = 100 - %moisture - %protein - %fat - %ash (BeMiller, 2004).

4.2.2 Inoculum and culture preparation.

Three replicates were carried out and each replicate was prepared by obtaining the organisms from 3 different *E. faecium* (American Type Culture Collection, ATCC® 8459™) culture broth beads. The organism was obtained from the American Type Culture Collection (Manassas, VA) and was maintained by Kansas State University Food Safety Defense Lab (FSDL) at -70 ^{o}C. To activate stock cultures for each replication of this study, three frozen beads of *E.faecium* culture were transferred to three separate test tubes containing 10 mL BBL™ Brain Heart Infusion (BHI) broth (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated at 35 ^{o}C for 24 h. After incubation, *E.faecium* broth from each tube was streaked for isolation on Tryptic Soy Agar (TSA) plates (Difco, Becton Dickinson, Sparks, MD). The plates were incubated at 35 ^{o}C for 24 h. Colonies from each plate were picked for biochemical strain confirmation, which was completed using an API® 20 Strep kit (Biomerieux, Marcy l'Etoile,

France). Once the colonies were confirmed as *E. faecium*, a single colony from each plate was picked and transferred to 10 mL of BHI broth and incubated at 35 ^{o}C for 24 h. Ten milliliters of culture were inoculated into each of 1-L-jugs of BHI broth, and all jugs were incubated for 48 h. these jugs of culture were utilized to inoculate the dog food mix. This was done in the FSRC.

4.2.3 Inoculation of pet food.

Although *E.faecium* is an approved non-pathogenice surrogate and can be used in commercial feed manufacturing, facilities, biosafety protocols established by the K-state Feed Safety Research Center (FSRC) and approved by the University's Institutional Biosafety Committee (IBC#1038.3) were followed during these studies. Four liters of inoculum were mixed with one batch of KSU mix (226.8 kg) in a double paddle mixer (D.C. Davis Sons Mfg Co., Inc., Bonner Spring, KS) with a sealable lid and capable of handling 272.2 kg of materials. The mixing time was 15 min allowing *E.faecium* to absorb and attach to the materials in the mix. Inoculated dog food mix prior to thermal treatments were used as the control samples.

4.2.4 Throughput calibration.

The actual throughput might be influenced by the preconditioner bin level: if a fuller bin was observed, raw materials would be transported faster in the extrusion system due to the pressure generated by larger amount of the feed. The preconditioenr feed screw speed was set at 18.2 and 12.0 rpm respectively in a Differential Diameter Cylinder (DDC) preconditioner (Wenger Manufacturing, Sabetha, KS), which was attached on a pilot-scale single screw extruder (X20, Wenger Manufacturing, Sabetha, KS). No water or steam was injected into the preconditioner. Materials were collected at the end of the extruder for 1 min while the preconditioner was kept under operation. The feeder bin was kept at three different levels: full, half-full and empty and the throughput values were recorded under each level.

4.2.5 Extruder conditions.

The extrusion process was conducted on a pilot-scale single-screw extruder equipped with a DDC preconditioner and a 7.8- mm diameter die. Two levels of feed screw speed were chosen: 12.0 rpm and 18.2 rpm, corresponding to different levels of throughput and residence time. The shaft speed in the preconditioner cylinder was set at 400 rpm and the extruder screw speed ranged from 394 to 400 rpm. To maintain the same in-barrel moisture level and downspout temperature range, cylinder steam and water injection rate and extruder water injection rate were adjusted based on different raw material throughput (Table 4-4). The preconditioner downspout temperature was between 88 ^{o}C to 91 ^{o}C and the extruder die temperature fluctuated from 120 ^{o}C to 140 ^{o}C. The screw configuration and temperature profile are shown in Figure 4-1.

Specific mechanical energy (SME) for each treatment presented the mechanical energy going into the extrusion system per unit mass and was calculated by the equation below:

$$SME(kJ/kg) = \frac{\frac{(\tau-\tau 0)}{100} * Prated * \frac{N}{Nrated}}{\dot{m}} \quad (4\text{-}1)$$

Where τ = % torque, τ_0 = % torque at no-load (34%), N = screw speed (rpm), N_{rated} = rated screw speed (508 rpm), P_{rated} = rated motor power (37.3 kW) and $\dot{m}$= mass flow rate (kg/s).

In-barrel moisture (IBM) was the value that combined all moisture sources in the extruder and preconditioner. IBM could be calculated using the equation below:

$$IBM\ (\%) = \frac{RM + PS + PW + ES + EW}{\dot{m} + PS + P\dot{W} + ES + EW} * 100\% \quad (4\text{-}2)$$

Where RM = moisture level in raw material; PS = steam rate in preconditioner (kg/hr); PW = water rate in preconditioner (kg/hr); ES = steam rate in extruder (kg/hr); EW = water rate in extruder (kg/hr); $\dot{m}$ = throughput (kg/hr).

4.2.6 Sampling for microbial testing

Samples were collected before and after inoculation, at the exit of the preconditioner and at the exit of the extruder. After reaching a steady state, a timer started counting and samples were taken at 1, 5 and 9 mintues. Twenty-five grams of sample was weighed into 225 mL of chilled peptone water (1%) prepared earlier to instantly stop the thermal destruction. The peptone water is a buffer solution that protects microbial cells from breakage due to the difference in osmotic pressure and keeping peptone water chilled helps to stop the thermal destruction immediately after the samples being taken out of the system. Filtered sample bags containing both peptone water and treated pet food were kept in an ice chest and immediately sent for microbial analysis at the K-state Food Safety and Defense Laboratory.

4.2.7 Enumeration.

At the laboratory, the treated samples were further mixed by stomacher (Smasher, AES Laboratorie, Bruz, France) for 1 min. Serial dilutions in peptone water were subsequently prepared and were spread-plated on mE agar and BHI agar. The mE agar plates were incubated at 35 ^{o}C for 24 h. The BHI agar plates were overlayed with mE agar after 6 h of incubation at 35 ^{o}C and returned to the incubation for another 18 h of incubation. If there was no colony seen on the plates for each individual sample, enrichment was applied to determine whether the *E.faecium* destruction was complete or not (i.e. no survivors present below the detection level of our direct plating method). To perform enrichment, 25 mL of the original sample (a mixture of peptone water and pet food) was taken and added to 250 mL of BHI broth for incubation at 37 ^{o}C for 24 h. The enriched sample was streaked on agar and incubated at 35 ^{o}C for 24 h for qualitative (+ or -) detection.

4.2.8 Colony confirmation.

According to Public Health Agency of Canada (2011), *E. faecium* is a gram-positive coccus. *E.faecium* colonies appear as a purple color on both mE agar and BHI agar overlaid with mE agar. *Enterococcus* was able to grow on selective mE agar, and this characteristic is used for detection and enumeration. However, some other bacterial species such as *Streptococcus bovis* might be able to grow on the media, presenting results similar to those of *Enterococcus* spp. (Manero and Blanch, 1999). Therefore, representation colonies on the agar, purple-coccus, was picked for further confirmation. A gram stain test kit (S25344, Fisher Science Co. L.L.C., Nazareth, PA) was first used to distinguish the colony into the gram-positive or negative group (Cornell University, n.d). Then an API® 20 Strep kit (Biomerieux, Marcy l'Etoile, France) was used to biochemically identify the colony as being *E.faecium*.

4.2.9 Water activity determination.

Water activity (a_w) samples were obtained before inoculation, after inoculation and after preconditioning and put in individual disposable cups. Testing cup containing 7.5mL of sample was placed in the water activity meter (AquaLab Series 4TEV, Decagon Devices, Inc., Pullman, WA) for analysis.

4.2.10 Statistical analysis.

The microbial data were expressed as the mean values and standard deviations after log transformation. Data analysis was following the model “Random Complete Block Design with 2-way treatment structure”. Significant differences in average values were established by the Bonferroni multiple comparison methods at a 5% level of significance using SAS (version 9.4, 2012, SAS Institute, Cary, NC). Differences were considered statistically significant at $P<0.05$.

4.2.11 Residence time distribution measurement.

Residence time is the average time that materials spend in the extruder barrel (Todd, 1975; Choudhury & Gautam, 1998). The residence time distribution (RTD) is an important parameter in extrusion processing because it is critical for all reactions and can provide information such as cooking intensity and mixing intensity. The RTD strongly depends on variables associated with the system such as screw speed, feed rate, and moisture (Choudhury & Gautam, 1998). Since there are 3 critical parameters within the microbial destruction process: time, temperature and moisture level, the RTD data was the preliminary data for microbial deactivation analysis. Titanium dioxide, a white colored dye, was used as the tracer in the RTD determination. The impact of extruder screw speed and feeder screw speed (contribute to throughput) was analyzed in the extruder (Table 4-6). The usual way to assume throughput was to use the feeder screw speed times 10. Twenty grams of dye was introduced at the beginning of the extruder barrel and samples were collected at the exit of the extruder: the die. After reaching operational equilibrium, titanium dioxide was added into the system at time zero (t=0) and sample collection began in the thereafter. In the first 90 s, the samples were collected at the end of the extruder every 10 s and in the next 210 s, the samples were collected every 30 s. The whole sample collection process lasted for 5 min. The samples were visually inspected and a score was assigned to color intensity (where a score of 0 signifies no dye is visible in the sample and 10 signifies that the sample is extremely white in color). The average time, t, could be calculated by dividing the sum of starting time and ending time into two. For instance, if the starting time equaled to 0 s and the ending time was 20 s, the average time should be 10 s. From here, residence time could be calculated, and RTD could be measured using the following equations:

$$\bar{t} = \frac{\sum_0^\infty t*C_t}{\sum_0^\infty C_t} \quad (4\text{-}3)$$

$$E(t) = \frac{C_t}{\sum_0^\infty Ct} \quad (4\text{-}4)$$

4.3 Results

4.3.1 Raw materials.

Table 4-5 presented the proximate carbohydrate, protein, fat, ash, fiber and moisture content of the raw materials. The KSU mix had a 29.78% of protein level, which agreed with the manufacturer's specification sheet.

4.3.2 Microbial sample analysis.

Since different *E.faecium* concentrations in inoculated pet food were observed from each treatment, the microbial data in Tables 4-7 & 4-8 was presented in reduction rate instead of recovery rate. In treatment with 18.2 rpm feeder screw speed, 6.1 log of *E. faecium* was reduced after both the preconditioning and the extrusion using direct plating method on mE agar and the reduction rates from the preconditioning and the extrusion were not significantly different (P = 0.76). In treatment with 12.0 rpm feeder screw speed, reduction rate after the preconditioning (4.5 log) was significantly different from it after the extrusion (5.2 log, P=0.002). Reduction rates observed using direct plating method on BHI agar after the preconditioning and the extrusion were significantly different (P<0.0001) within treatments with 18.2 rpm and 12.0 rpm feeder screw speed, respectively. A 5 log reduction was achieved after the extrusion process using direct plating method on both mE and BHI agars.

4.3.3 Throughput calibration.

The lowest throughput was recorded when the feeder bin was empty for treatments with both feeder screw speeds. Fuller the feeder bin, higher the throughput that was observed (Table 4-9).

4.3.4 Extrusion conditions.

The SME decreased while the IBM increased with decreasing feeder screw speed (Table 4-10). The SME ranged from 75.44 to 103.56 kJ/kg, while the IBM varied between 21.59% and 21.77% at 18.2 rpm feeder screw speed; the SME ranged from 42.01 to 54.39 kJ/kg while the IBM varied between 29.4% and 31.6% at 12.0 feeder screw speed.

4.3.5 Extrudates physical characterization.

At 18.2 rpm feeder screw speed, moisture content of the pet food material reached 20.95% after the preconditioning and 22.59% after extrusion (Table 4-11). The bulk density of pet food reached an average of 436 g/L (Table 4-12). At 12.0 rpm feeder screw speed, moisture content of preconditioned and extruded pet food was 20.8% and 24.31%, respectively (Table 4-11). The bulk density of extruded pet food ranged from 381.7 g/L to 433.3 g/L (Table 4-12).

4.3.6 Water activity determination.

Water activity reached 0.63~0.64 after inoculation in both treatments (Table 4-13). After treatment with 18.2 rpm feeder screw speed, preconditioned and extruded pet food both had an a_w value of 0.93. After treatment with 12.0 rpm feeder screw speed, water activity value of extruded sample also reached 0.93 while preconditioned sample had an a_w value of 0.81.

4.3.7 Residence time distribution measurement.

Residence time increased with a decrease on both screw speed and throughput (Figure 4-2). Eight seconds of difference was noted when the screw speed increased from 250 rpm to 550

rpm. A bigger difference of 34 s was noticed when throughput lowered from 180 kg/hr to 120 kg/hr. The most left-shifted curve and the curve with the highest peak in Figure 4-3 was the one under the highest throughput (180 kg/hr) and highest screw speed (550 rpm) while shortest residence time was generated. The curve with lowest peak corresponded to the lowest throughput: 120 kr/hr. The curve with the lowest screw speed: 250 rpm, was the most right-shifted one.

4.4 Discussion

An increase of feeder screw speed and extruder screw speed resulted in shorter residence time (Figure 4-2). A similar result was obtained by Gogoi & Yam (1994). Change on feeder screw speed had a larger impact on the residence time than change on extruder screw speed (Figure 4-2). In Figure4-3, the curve with the highest peak as well as the left shifted one (under feeder screw speed of 18 rpm and extruder screw speed at 550 rpm) represented the treatment with the shortest residence time (71s). This phenomenon was also observed by Unlu & Faller (2002) and Gogoi & Yam (1994). To achieve longer residence time during the extrusion process, the better option is to decrease the feeder screw speed and increase the extruder screw speed a bit. Extruder screw speed does not have a significant impact on residence time as well as feeder screw speed yet increase on extruder screw speed brings higher mechanical energy into the system, which benefits both degrees of cooking and pet food safety. In our study, residence time increased 34 s when the feeder screw speed decreased from 18.2 rpm to 12.0 rpm.

How to obtain residence time in the extruder barrel requires more researches and explorations. Different dyes were commonly used as tracers and tracer concentration could be analyzed in several ways, such as using a UV spectrophotometer (Chen & Pan, 1990;), a spectrophotometer (Chuang & Yeh, 2004; Nwabueze & Iwe, 2010) or a Color-Eye colorimeter (Singh & Rizvi, 1998). In our RTD studies, color intensities were visually measured by different people and the mean value was obtained. The drawback of this method was too subjective and slight changes in color might not be able to be recognized by naked eyes, thus leading to inaccurate estimation. Therefore, a baseline and an upper-line was created to make sure value sets recorded by different participants within one treatment had the same range.

Choosing the most appropriate surrogate for *Salmonella* is critical for this project. Several types of microorganisms had been chosen as a surrogate for *Salmonella* in other studies, such as *Bacillus stearothermophilus* 12980 in animal feed (Okelo et al, 2006), *Enterococcus faecium* ATCC® 8459™ in Almonds (ABC, 2007b), and *Pediococcus* spp. in whole-muscle turkey jerky (Williams et al, 2010). Ceylan & Bautista (2015) pointed out that *Pediococcus acidilactici* ATCC® 8042™ might be an ideal surrogate for *Salmonella* in pet food based studies because *P.acidilactici* has a lower heat resistance than *E. faecium,* yet still poses a higher thermal resistance than *Salmonella*: at 16.9% moisture level, $D_{82.2}$ was 0.42, 0.91 and 3.99 respectively for *Salmonella, P.acidilactici* and *E.faecium;* even under 87.8 ^{o}C, D value for *E. faecium* was 1.08 while no data could be recorded for *Salmonella* and *P.acidilactici*. Therefore, compare to *E.faecium, P. acidilactici* shares a similar lethality characteristics with *Salmonella*. However, *Salmonella* might easily develop a higher thermal resistance due to many factors (Doyle and Mazzotta, 2000), including including growth medium composition, growth phase, growth temperature, intrinsic parameters (water activity, fat content, salt content for instance), pathogen strains used (Harris, 2008; Tuntivanich et al., 2008). Thus, the organism with higher heat resistance, *E.faecium,* was chosen in our study. Also, *E.faecium* ATCC® 8459™ is easily obtained, cultivated and enumerated and would not lead to spoilage issues (Ceylan and Bautista, 2015).

Extrusion temperature has been chosen as a potential CCP, yet few publications regarding validation of extrusion cooking to effectively reduce microbial counts in pet food processing were found (Rokey & Baldwin, 2013). Okelo (2006) pointed out that extrusion thermal processing was adequate to eliminate mesophilic organisms but not thermophilic organisms. *Salmonella* in animal feed was inactivated under an extrusion process at 28.5%

moisture level, 83 ^{o}C and 7 s of retention time (Okelo et al, 2006). For most of the conventional extruded products, microbiological quality could be assured if the extruder temperature reached 130 ^{o}C (Guy, 2001). A 5-log-reduction of *E.faecium* was observed in a carbohydrate-protein meal at the temperature of 81.1 ^{o}C and moisture level at 28.1% (Bianchini et al, 2012).

Theoretically, longer residence time resulting from lower feeder screw speed would positively impact microbial inactivation. However, 2 feeder screw speeds in our study, 18.2 rpm vs. 12.0 rpm, did not have a significant impact on *E.faecium* recovery rate on mE plates (p=0.44) while a significant difference were observed on BHI plates ($p \leq 0.0003$).

Higher reduction rate on *E.faecium* was observed after the extrusion and the more obvious difference was noted on BHI agars. At both 18.2 and 12.0 rpm feeder screw speed, minimum of a 5-log reduction rate on *E. faecium* after extrusion (Tables 4-7 & 4-8) while only the reduction rate after treatment with 18.2 rpm feeder screw speed on mE agar reached at least 5 log. The downspout temperature ranged from 89-94 ^{o}C, which should be adequate to deactivate *E.faecium*. According to Public Health Agency of Canada (2011), *E. faecium* should be destroyed at temperatures in excess of 80 ^{o}C. However, under lethal heat condition, *E.faecium* got injured and its antibiotics-resistance decreased. The antibiotics components in the selective agar prevent *E.faecium* from growing on the mE agar. After incubation on the BHI agar plates, injured *E.faecium* cells recovered and its resistance grow back and started to populate on the non-selective agar plates. Sublethally injured cells can potentially lead to a serious food safety problem because the organism's sensitivity or the production process' lethality overestimated if the injured cells are mistakenly classified as dead cells. The temperature in the preconditioner barrel was controlled by the injection rate of steam, and after the system reached operational equilibrium, the temperature would not fluctuate much, thus a relatively stable thermal

processing condition was created. The feeder bin level was kept from half-full to full throughout the experiment, and the dry throughput ranged from 165.9 kg/hr to 171.0 kg/hr (Table 4-11) at 18 rpm feeder screw speed and from 104.5 kg/hr to 115.2 kg/hr at 12 rpm feeder screw speed, which could be considered as a slight fluctuation. Heat distribution in the preconditioner barrel to the pet food materials might not be even under large and unstable throughput. That might be the main possibility resulting in incomplete *E.faecium* deactivation.

Complete destruction was obtained at the exit of the extruder. No population recovered either on mE agar or BHI agar (Tables 4-7 & 4-8). The set temperature in the extruder barrel was 50 ^{o}C, 70 ^{o}C and 90 ^{o}C in 3 temperature zones in the extruder (Figure 4-1) and the die temperature ranged from 120 ^{o}C to 140 ^{o}C. Thermal energy in the extruder was generated by several ways (Guy, 2001): 1. by means of direct hot water injection into the barrel; 2. by the process of conveying, pressurizing, mixing, kneading and grinding and energy converted from mechanical energy; 3. by the heat generated by the screw and due to conductive energy, heat transmits to the rest part of the barrel. The moisture contents of pet food at the exit of the extruder was 22.59% ± 0.73 at 18.2 rpm feeder screw speed and 24.31% ± 0.00 at 12.0 rpm feeder screw speed (Table 4-13). These values were lower than the moisture level of materials before the extruder die due to the steam loss at the die. When materials got pushed out of the extruder through the die, materials expanded after going through a high-pressure environment, and steam flashed off the second pet foods expanded.

Mechanical energy generated by the friction also contributed to microbial destruction because a high-shear environment was provided. Thermal energy was the primary cause leading to microbial inactivation in the extruder barrel, but shear force could enhance the effect (Guy, 2001). According to Fraiha et al. (2011), shear stress might be involved in the reduction of the

microbial population during the extrusion process and mechanical forces might cause cell rupture. The microbial reduction was observed even during low-temperature extrusion indicating that shear force did contribute to microbial elimination. The SME input in the extruder barrel ranged from 75.44 – 103.56 kJ/kg at 18.2 rpm feeder screw speed and 42.01 – 54.39 kJ/kg at 12.0 rpm feeder screw speed (Table 4-12). The SME for the traditional dog food extrusion in a single-screw extruder fluctuated from 79.37 to 111.11 kJ/kg (Streit, n.d.). The bulk density of the extruded pet food samples was 436 ± 9.9 g/L at 18.2 rpm feeder screw speed and 408 ± 26.3 at 12.0 rpm feeder screw speed, which was higher than the ideal range (dried pet food): 288.3 – 400.5 g/L (Coffee et al., 1980). The bulk density would increase after drying as most of the moisture would have been evaporated. Therefore, the actual bulk density of dried pet food samples in our study would be even higher compared to extruded pet food. The bulk density value was closely related to SME: high bulk density value corresponded to lower SME. The bulk density of pet food from 2 treatments did not vary too much: 436 g/L vs 408 g/L but both of them were higher than the ideal range for different reasons. At 18.2 rpm feeder screw speed, the SME input value fell within the optimal range (79.37 to 111.11 kJ/kg), therefore high bulk density of products might result from lack of thermal energy input: the IBM was 21.68% ± 0.09, lower than commonly accepted level (≥ 27%) (Koppel et al., 2014). Thermal energy input in the extrusion was controlled by moisture injection level and therefore the IBM value could be a nice indicator of thermal energy input. Insufficient thermal cooking could be one of the main reasons why the pet food products were poorly expanded. At 12.0 rpm feeder screw speed, the SME values was much lower than the ideal range while the IBM values were higher than the common accepted value. Lack of mechanical energy input would theoretically lead to higher bulk density of pet food products.

An increase of moisture level would have a positive impact on reducing pathogenic population. It required a lower temperature to eliminate pathogens in a higher moisture content condition, as heat would be easier to penetrate in moist heat than dry heat. The in-barrel moisture in our project reached 21.68% at 18.2 rpm feeder screw speed and 30.5% at 12 rpm feeder screw speed (Table 4-12) and it was relative low than other pet food processing (~ 27%) (Koppel et al., 2014).

4.5 Conclusion

A thorough *E. faecium* inactivation in pet food was accomplished after extrusion: 5-log reduction goal was achieved for using both mE agar and BHI agar after treatments with 2 different feeder screw speed. Feeder screw speed controlled the raw material throughput and it had a larger impact on residence time than extruder screw speed. This study was carried out under the longest and shortest residence time the extruder could achieve, and the extruder fulfilled the goal of full removal of *E.faecium* under both circumstances. The X-20 single-screw extruder could be served as an effective CCP during pet food manufacturing. What needed to be improved was to adjust mechanical and thermal energy input to get better expanded / quality products.

Tables

Table 4-1. KSU mix formulation

Ingredients	Level (w/w)
Corn flour	48%
Poultry by-product meal	29%
Corn gluten meal	21%
Rice meal	10%
Vitamin premix	0.5%
Mineral premix	0.5%

Table 4-2. Vitamin premix specification in the KSU mix

Parameters	Specification
Calcium (%)	20.2
Iodine (ppm)	1,320
Selenium (ppm)	72.6
Copper (%)	1.1
Iron (ppm)	35,200
Manganese (ppm)	5,610
Zinc (%)	10.5

Table 4-3. Mineral premix specification in the KSU mix

Parameters	Specification
Vitamin A (IU/lb)	5,278,000
Vitamin D3 (IU/lb)	347,000
Vitamin E (IU/lb)	33,113
Vitamin B12 (mg/lb)	7.2
Riboflavin (mg/lb)	1,635
D-Pantothenic Acid (mg/lb)	3,856
Niacin (mg/lb)	24,495
Folic Acid (mg/lb)	273
Vitamin B6 (mg/lb)	1,928
Thiamine (mg/lb)	2,268
Biotin (mh/lb)	28

Table 4-4. Moisture injection rate into the extrusion system under different feed screw speeds

Feed screw speed (rpm)	12.0	18.2
Cylinder steam flow (kg/hr)	21.9	16.9
Cylinder water flow (kg/hr)	0	12.1
Extruder water flow (kg/hr)	6-10	6

Table 4-5. Proximate analyses of KSU dry dog food mix

Components	Content	Components	Content	Components	Content
Carbohydrate	46.3%±0.39	Protein	29.78%±0.26	Fat	6.66%±0.00
Ash	3.66%±0.00	Fiber	3.17%±0.03	Moisture	10.45%±0.01

Table 4-6. Experimental design exploring the impact of throughput and screw speed on the residence time distribution in the extruder

	Feeder screw speed (rpm)	Extruder screw speed (rpm)
Treatment 1	18	250
Treatment 2	18	394
Treatment 3	18	550
Treatment 4	15	394
Treatment 5	12	394

Table 4-7. The Enterococcus faecium population in pet food reduced on mE plates after preconditioning and extrusion at 12.0 and 18.2 rpm feeder screw speed

	E. faecium population recovered on mE (log CFU/g)[a]	
	18.2 rpm feeder screw speed	12.0 rpm feeder screw speed
Preconditioned	6.0 ± 0.22[a]	4.5 ± 0.22[a]
Extruded	6.1 ± 0.22[a]	5.2 ± 0.22[b]

[a] The reduction rate data is represented by the mean of 3 samples at 3 sampling time from 1 treatment and standard deviations within 3 replications (n=9). Within individual columns, the values followed by different letters are significantly different (p<0.05). Reduction rate of each sample was obtained by using the population control samples to minus the recovery rate of each sample, respectively. The control samples were the inoculated dog food mix prior to thermal treatment.

Table 4-8. The Enterococcus faecium population in pet food reduced on BHI plates after preconditioning and extrusion at 12.0 and 18.2 rpm feeder screw speed

E. faecium population recovered on BHI (log CFU/g)[a]		
	18.2 rpm feeder screw speed	12.0 rpm feeder screw speed
Preconditioned	3.1 ± 0.22^{a}	2.9 ± 0.22^{a}
Extruded	6.1 ± 0.22^{b}	5.6 ± 0.22^{b}

[a] The reduction rate data is represented by the mean of 3 samples at 3 sampling time from 1 treatment and standard deviations within 3 replications (n=9). Within individual columns, the values followed by different letters are significantly different (p<0.05). Reduction rate of each sample was obtained by using the population control samples to minus the recovery rate of each sample, respectively. The control samples were the inoculated dog food mix prior to thermal treatment.

Table 4-9. Throughput calibration in the preconditioner while the bin feeder level was at full, half-full and empty

Feeder screw speed		18.2 rpm	12.0 rpm
Feeder bin level	Full	169.8 ± 1.20	115.2 ± 0.04
	Half-full	168.6 ± 2.75	107.7 ± 3.16
	Empty	160.4 ± 6.41	89.2 ± 0.92

[a] The calibration data is represented by the mean of three weight of materials collected at the end of the preconditioner and standard deviation (n=3).

Table 4-10. Specific mechanical energy and in-barrel moisture level in the extrusion system

Feeder screw speed (rpm)	18.2	12.0
Specific mechanical energy (SME, kJ/kg)	89.5 ± 14.06	48.2 ± 6.19
In-Barrel Moisture (IBM, %)	21.68 ± 0.09	30.5 ± 1.1

[a] The data is represented by the mean of four sets of extrusion parameters and standard deviation (n=4).

Table 4-11. Moisture content of preconditioned and extruded samples

Feeder screw speed (rpm)		18.2	12.0
Moisture content (%) [a]	Preconditioned	20.95 ± 0.61	20.8 ± 0.00
	Extruded	22.59 ± 0.73	24.31 ± 0.00

[a] The moisture content data is represented by the mean of 2 independent moisture samples and standard deviation (n=2)

Table 4-12. Bulk density of extruded and dried samples

Feeder screw speed (rpm)		18.2	12.0
Bulk density (g/L)[a]	Extruded	436 ± 9.9	408 ± 26.3

[a] The bulk density data is represented by the mean of 2 data obtained using a bulk density cup and standard deviation (n=2)

Table 4-13. Water activity of raw material, inoculated, preconditioned, and extruded samples

Feeder screw speed (rpm)		18.2	12.0
Water activity [a]	Post-inoculation	0.64 ± 0.00	0.63 ± 0.00
	Post-preconditioning	0.93 ± 0.00	0.81 ± 0.01
	Post-extrusion	0.93 ± 0.00	0.93 ± 0.02

[a] The water activity data is represented by the mean of 3 water activity values and standard deviation (n=3)

Figures

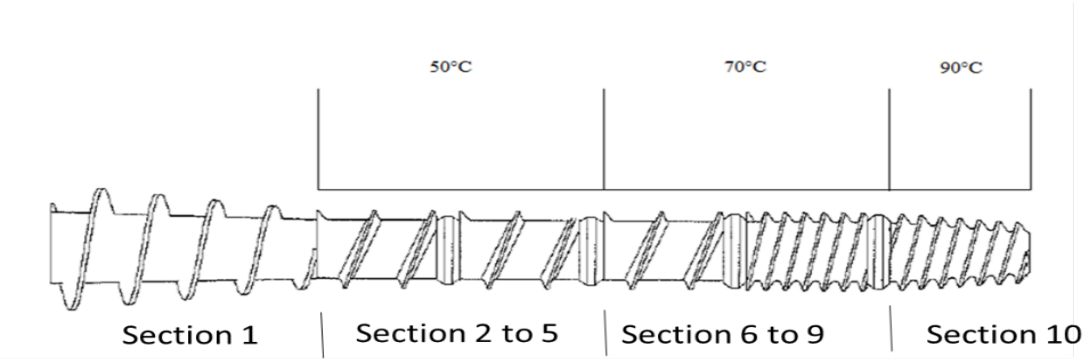

Figure 4-1. Extruder-based temperature set points and screw profile 1. Inlet; 2. Single flight inlet screw; 3. Single flight screw; 4. Small steam lock; 5. Single flight screw; 6. Single flight screw; 7. Medium steam lock; 8. Double flight screw; 9. Large steam lo lock; 10. Double flight cone.

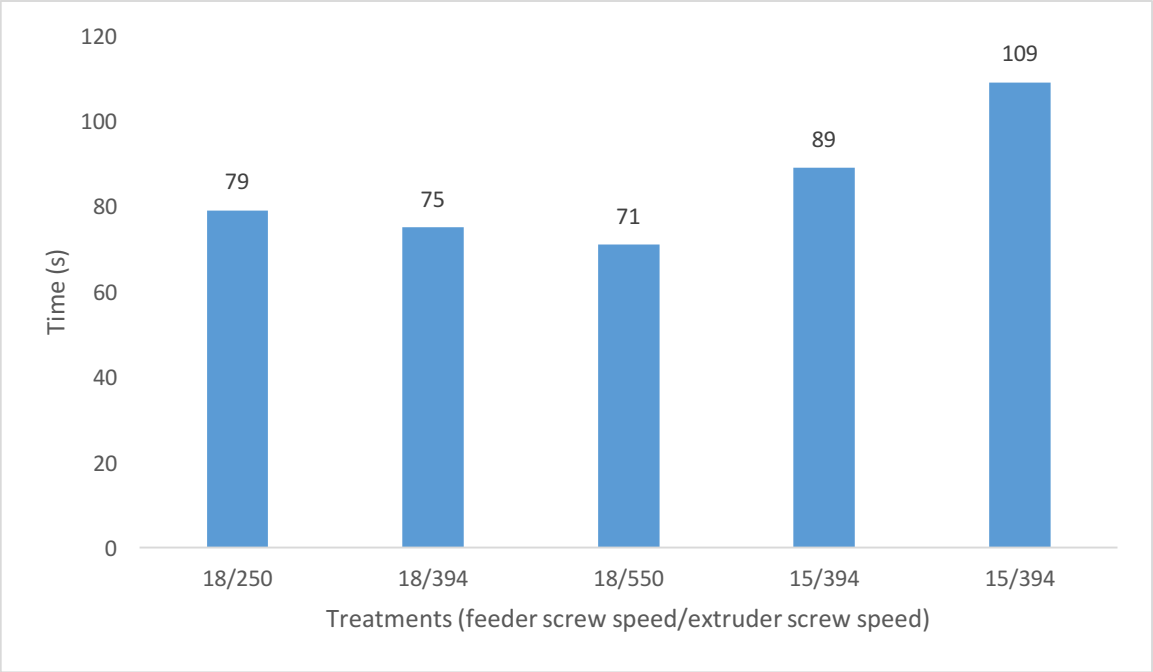

Figure 4-2. Residence time in the extruder under different feeder screw speed (12, 15 and 18 rpm) and screw speeds (250, 394 and 550 rpm)

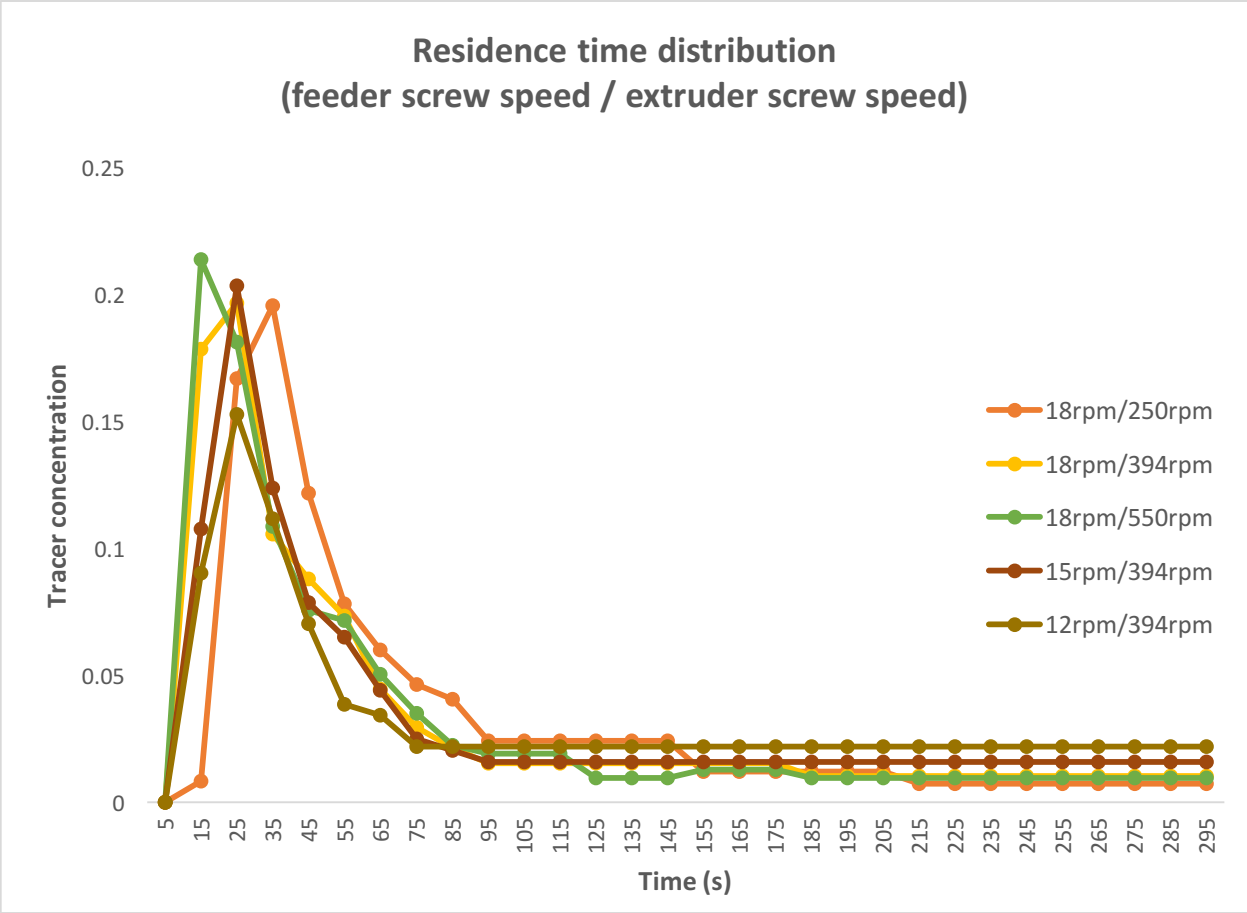

Figure 4-3. Residence time distribution curves when the extruder was running under different feeder screw speeds (12, 15 and 18 rpm) and screw speeds (250, 394 and 550 rpm)

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