Isolation and Characterization of Thermal Resistance of *Salmonella* strains from Raw, Frozen Chicken Nuggets, Strips and Pelleted Broiler Feeds

BY

Oliver Bucher

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Food Science
University of Manitoba
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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of

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FOREWARD

This thesis has been prepared using a paper style format. It is composed of two papers prepared for journal publication (Chapters 3 and 4) as well as a literature review (Chapter 2) and consolidated introduction (Chapter 1), conclusion (Chapter 5) and references. Chapters 3 and 4 are the two papers presented as originally submitted for publication with formatting changes to reflect the paper style format of this thesis.

Chapter 3, "Occurrence and characterization of *Salmonella* from chicken nuggets, strips and pelleted broiler feed" was accepted for publication in the Journal of Food Protection, May 28 2007 with authorship by Bucher, O., Holley, R.A., Ahmed, R., Tabor, H., Nadon, C., Ng, L.K., and D'Aoust, J.-Y.

Chapter 4, "Thermal resistance of *Salmonella* serovars isolated from raw, frozen chicken nuggets/strips, nugget meat and pelleted broiler feed" was submitted for publication to the International Journal of Food Microbiology, August 17 2007 with authorship by Bucher, O. and Holley, R.A.

ABSTRACT

Raw, frozen chicken nuggets/strips have been identified as a significant risk factor in contracting food-borne salmonellosis. During processing, these products undergo partial frying to ensure their shape is maintained and give the batter and breaded coating a golden brown colour before they are frozen and packaged. This thermal treatment may mislead consumers to believe these products are fully-cooked, and that they require only re-heating before consumption. It is uncertain whether inadequate cooking (microwave oven) or the presence of *Salmonella* serovars with elevated thermal resistance is the more likely cause of salmonellosis associated with these products. Thermally resistant *Salmonella* strains may be selected by the heating step used in poultry feed pelleting and subsequently contaminate the animals, meat and enter the food distribution system.

This study was undertaken to determine the occurrence and characterize the strains of *Salmonella* contaminating raw, frozen chicken nuggets/strips and pelleted feeds in an attempt to demonstrate whether the same *Salmonella* strains present in broiler feed could be isolated from raw, frozen chicken nuggets/strips available for human consumption.

Salmonellae were isolated using the Health Canada MFHPB-20 method for the isolation and identification of *Salmonella* from foods. Strains were characterized through serotyping, phage typing, antimicrobial resistance typing (R-type) and pulsed-field gel electrophoresis pattern analysis (pulse type).

Thermal resistances (D- and z-value) were determined for cocktails of the same *Salmonella* serovar. Cocktails were challenged in chicken nugget meat heat challenged in a preheated waterbath and recovered on tryptic soya broth containing 1.5% agar and 0.6% yeast extract with 48 h incubation at 25°C.

Salmonellae were isolated from 27% of chicken nuggets/strips, 5% of pelleted feed and from 48% of meat blend samples. Of the different serovars, *S.* Enteritidis was the only organism isolated from feed, nuggets/strips and meat blend. *S.* Enteritidis strains with identical phage- pulse- and R-types were isolated from feed and nugget meat blend samples, while nugget isolates had different phage and pulse type combinations.

The thermal resistances found were lower than those reported for similar strains in the literature. D-values ranged from 6.93 to 0.12min at 55 and 62°C respectively, with z-values from 4.10 to 5.17°C. The two strains of *S.* Enteritidis separately isolated from pelleted feed and chicken nugget meat blend, with indistinguishable geno- and phenotypes, had lower (and probably identical) thermal resistances than the other isolates.

Results from this project indicate that indistinguishable *Salmonella* strains could be isolated from pelleted broiler feed and from meat blend used in the manufacture of raw, frozen chicken nuggets/strips. However results did not rule out the possibility that breeding stock or contamination during processing may have contributed to contamination by *Salmonella.* Thermal resistance data show that the strains did not exhibit unusually high thermal resistance, and that normal heating (71°C) prior to

consumption should eliminate these organisms from chicken nuggets/strips. Future development of this research should focus on investigating the potential for post-pelleting contamination of pelleted feed in the pellet mill via contaminated dust and the extent to which antibiotic-resistant strains of *Salmonella* persist in raw chicken meat products.

TABLE OF CONTENTS

PAGE

Acknowledgments ..... **iii**

Foreward ..... **iv**

Abstract ..... **v**

Table of Contents ..... **viii**

List of Figures ..... **xi**

List of Tables ..... **xii**

1. Introduction **1**

2. Literature Review **4**

2.1 *Salmonella* 4

2.1.1 Genus Description 4

2.1.2 Clinical Significance 5

2.2 Occurrence of *Salmonella* in feed and feed ingredients 10

2.3 Occurrence of *Salmonella* in raw, frozen chicken nuggets/strips 12

2.4 Pelleted Feed Processing 14

2.4.1 Ingredients and Formulations 14

2.4.2 Feed Pelleting 19

2.5 Chicken Nugget/Strip Processing 24

2.5.1 Ingredients and Formulations 24

2.5.2 Processing Steps and Conditions 28

2.6 Isolation, Identification and Subtyping of *Salmonella* 34

2.6.1 Serological Identification 38

2.6.2 Phage Typing 41

2.6.3 Pulsed-Field Gel Electrophoresis 42

2.7 *Salmonella* Thermal Resistance 44

3. Occurrence and characterization of *Salmonella* from chicken nuggets, strips and pelleted broiler feed 51

3.1 Abstract 51

3.2 Introduction 52

3.3 Materials and Methods 54

3.3.1 Sample Collection and Handling 54

3.3.2 Isolation from Pelleted Broiler Feed 55

3.3.3 Isolation from Nuggets/Strips, Batter Coating and Meat 57

3.4 Results 58

3.4.1 Occurrence of *Salmonella* in Pelleted Feed 58

3.4.2 Occurrence of *Salmonella* in Chicken Nuggets/Strips 58

3.4.3 Occurrence in Chicken Nugget Meat and Nugget Coating 60

3.5 Discussion 60

3.5.1 Occurrence of *Salmonella* 60

3.5.2 *Salmonella* Isolate Characterization 63

4. Thermal resistance of *Salmonella* serovars isolated from raw, frozen chicken nuggets/strips, nugget meat and pelleted broiler feed 75

4.1 Abstract 75

4.2 Introduction 76

4.3 Materials and Methods 78

4.3.1 Raw Material 78

4.3.2 Culture Storage Conditions and Inoculum Preparation 79

4.3.3 Thermal Resistance 80

4.3.4 D- and z-value Calculations and Statistical Analysis 81

4.4 Results 82

4.4.1 Raw Material Analysis 82

4.4.2 *Salmonella* Thermal Resistance 82

4.5 Discussion 83

5. Discussion 92

6. Conclusions 97

References 99

List of Figures

Figure 2.1: Ontario feed mill layout 20

Figure 2.2: Feed expander 22

Figure 2.3: Pelletizer 23

Figure 2.4: Chicken nugget shapes 29

Figure 2.5: Predust applicator and excess predust remover 30

Figure 2.6: Batter applicators 31

Figure 2.7: Chicken nugget/strip fryer 32

Figure 2.8: Gram negative outer membrane 39

Figure 3.1: Chicken nugget/strip process flow 67

List of Tables

Table 2.1: Feeding program duration 15

Table 3.1: Isolated *Salmonella* antigenic formula 68

Table 3.2: Inhibitory concentrations of antimicrobials 69

Table 3.3: *Salmonella* occurrence in pelleted feed 70

Table 3.4: *Salmonella* strains isolated from pelleted feed 71

Table 3.5: *Salmonella* occurrence in nuggets/strips, nugget meat and coating 72

Table 3.6: *Salmonella* strains isolated from nuggets/strips and nugget meat 73

Table 3.6 cont'd 74

Table 4.1: Description of *Salmonella* strains tested for thermal resistance 88

Table 4.2: Thermal resistance of isolated *Salmonella* strains 89

Table 4.3: D- and z-values of *Salmonella* cocktails from other investigations 90

Table 4.3 cont'd 91

1. INTRODUCTION

Investigations into a 1998 outbreak of *S.* Typhimurium phage type (PT) 12 in South Australia and in a 2003 family cluster of S. Heidelberg PT26 infections in British Columbia (Canada), as well as case control studies conducted in 2002 in Quebec (Canada) and nationwide (Canada) in 2003 have identified raw, frozen, chicken nuggets/strips bought at retail and prepared for consumption at home as significant risk factors in contracting foodborne salmonellosis (Kenny et al., 1999; MacDougall et al., 2004; Currie et al., 2005). With the exception of a few pre-cooked, microwavable luncheon meal products, most frozen chicken nuggets/strips sold in stores are raw and require consumers to fully cook these products before consumption. During processing these products undergo a partial frying (par-frying) step to maintain the product's shape and give the coating a golden brown colour (Barbut, 2001). Since the resulting nugget/strip appears cooked, confusion over the raw nature of the product may contribute to the increased risk of contracting salmonellosis. During their nationwide case control study, Currie et al. (2005) found that 34% of all laboratory confirmed cases of *S.* Heidelberg infection in Canada could be attributed to the consumption of raw, frozen chicken nuggets/strips. They also noted that 40% of respondents participating in the study believed these products to be pre-cooked and required re-heating only before consumption. In addition, 11% of respondents indicated using the microwave to heat these products, which may not provide a lethal heat treatment.

Use of feed contaminated with zoonotic pathogens, including *Salmonella* is not uncommon in the animal industry, but is a questionable practice (Rogers and Haines,

2005). It has been reported that during a study of primary breeders, close to 80% of *Salmonella* serovars isolated from feeds and feed ingredients were the same serotypes isolated weeks later in breeding flocks and their offspring (Jones and Richardson, 2004). Ingredients of animal origin have been shown to have the highest frequencies of contamination, though ingredients of vegetable origin have also been reported to harbour the organism (Isa et al., 1963; Hacking et al., 1978; Veldman et al., 1995; Zdragas et al., 2001). Recent work conducted in the southern United States (US) found that 8.8% of mash feed samples and 4.2% of pelleted feed samples were contaminated with *Salmonella*, suggesting that the pelleting process reduces *Salmonella* isolations from poultry feed but does not eliminate the organism (Jones and Richardson, 2004). Current European Union (EU) legislation requires that feed manufacturers adopt Hazard Analysis and Critical Control Point (HACCP) procedures to control *Salmonella* and other pathogens in feeds (Jones and Richardson, 2004). Currently, Canada is behind in its regulatory policies with no concise standard for feed pelleting time and temperature treatment. It is uncertain whether inadequate cooking (microwave oven) or the presence of *Salmonella* serovars with elevated thermal resistance is the more likely cause of salmonellosis associated with these products. Thermally resistant *Salmonella* strains may be selected by the heating step used in poultry feed pelleting and subsequently contaminate the animals, meat and enter the food distribution system.

This work was undertaken to determine whether the same strains of *Salmonella* could be isolated from pelleted broiler feed and raw, frozen chicken nuggets and strips. The objectives of this work were two-fold: 1) to determine the occurrence of *Salmonella* in pelleted feeds and raw, frozen, chicken nuggets/strips and, 2) characterize the strains

via serotyping, phage typing, antimicrobial resistance typing (R-Type), pulsed-field gel electrophoresis (PFGE) analysis and thermal resistance to establish whether the same strains could be found in both feed and in nuggets/strips. The thermal resistances were also assessed to determine whether this resistance was higher than normally would be expected.

2. LITERATURE REVIEW

2.1. *Salmonella*

Salmonella are enteric bacteria ubiquitous to animal gastrointestinal (GI) tracts, especially poultry, swine and reptiles that are frequently associated with human illness. They survive very well outside their animal hosts and can persist in the environment for years. Their persistence and proliferation in the environment coupled with large scale agricultural operations and global trade have made these organisms a global food challenge (Crump et al., 2002). Food of animal origin is especially of concern because of the organism's association with animal GI tracts, though their ability to persist in the environment has led to contaminated soil and water, implicating *Salmonella* in numerous outbreaks linked to the consumption of fruits and vegetables (Montville and Matthews, 2005).

2.1.1. Genus description

Salmonella comprise a genus of Gram negative, facultatively anaerobic, rod shaped, bacteria belonging to the family *Enterobacteriaceae.* They ferment glucose and can use citrate as their source of carbon (D'Aoust, 1989). They typically produce hydrogen sulfide, decarboxylate lysine and ornithine, and are urease negative (Bopp et al., 2003; D'Aoust, 1989), though a number of studies have shown that lactose-positive and hydrogen sulfide-negative variants exist (Gonzalez, 1966; Easterling et al., 1969; Falcão et al., 1975; MacDougall et al., 2004). The Kauffman-White classification scheme is the currently accepted taxonomic scheme for *Salmonella* (Popoff, 2001). This scheme splits the genus into two species: *S. enterica* and *S. bongori*, with *S. enterica*

being further subdivided into six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI). These subspecies are commonly subdivided into serotypes, of which there are over 2500 with approximately 1500 belonging to subspecies *enterica* (Bopp et al., 2003; Popoff, 2001). Most bacteria have their serotype denoted by an antigenic formula, but because there are so many *Salmonella* serovars whose names are already familiar among medical personnel and researchers it would be unrealistic to replace all the names with their antigenic formula. These names are denoted without italics and have their first letter capitalized. Serovar names for *S. enterica* ssp. *enterica* represent >99.5% of the strains isolated, so organisms belonging to this subspecies have kept their serovar name rather than their antigenic formula, while serovars from other *S. enterica* subspecies and those from *S. bongori* are denoted by their antigenic formula (Popoff, 2001). Subspecies *enterica* organisms are usually isolated from humans and other warm-blooded animals while the other remaining subspecies (II, IIIa, IIIb, IV and VI) and those belonging to *S. bongori* are usually associated with cold-blooded animals and the environment (Bopp et al., 2003).

2.1.2. Clinical significance

Disease caused by salmonellae can be classified into two categories: Typhoidal and non-Typhoidal. Typhoid Fever, sometimes referred to as Enteric Fever, is caused by *S. enterica* Typhi, though *S. enterica* Paratyphi A, B and C cause a similar but less severe set of symptoms (Bopp et al., 2003; Bhan et al., 2005). Typhoid fever is widespread across the globe with a high incidence of illness in south-central and south-east Asia (>100 cases of infection per 100 000 population per year), a medium incidence of illness (10-100 cases of infection per 100 000 population per year) in the rest of Asia,

Africa, Latin America and Oceania, except Australia and New Zealand, and a low (<10 cases of infection per 100 000 population per year) incidence of illness occurring in the rest of the world (Bhan et al., 2005). People are the only known natural host and reservoir for *S.* Typhi so improvements to hygiene have dramatically reduced its occurrence in these developed countries (Bhan et al., 2005). Infected persons may experience a prolonged high fever, headaches, abdominal pain, fatigue, diarrhea, rash, loss of appetite and death in rare cases (Bopp et al., 2003; Monteville and Matthews, 2005).

Non-typhoidal *Salmonella* infections are much more prevalent in developed countries than Typhoidal *Salmonella* infections, and have been estimated to cause close to 1.4 million foodborne infections in the United States with estimated annual costs due to medical care and lost productivity ranging from 0.5 to 2.3 billion dollars (Frenzen et al., 1999, Mead et al., 1999). Non-typhoidal strains of *Salmonella* normally cause a self-limiting gastrointestinal infection (termed Salmonellosis) that usually lasts over a week. Symptoms include: fever, diarrhea, abdominal cramps, nausea and occasional vomiting. More serious invasive infections can occur and lead to meningitis, reactive arthritis, myocarditis and death (Currie et al., 2005. MacDougall., 2004 Bopp et al., 2003). Successful treatment of uncomplicated salmonellosis is usually done with supportive therapy such as fluid and electrolyte replacement (Montville and Matthews, 2005), but in people with systemic infections, antibiotics (fluoroquinolones and extended-spectrum cephalosporins for adults and children, respectively) are needed for treatment (Fey et al., 2000; Cui et al. 2005).

Cases of salmonellosis as a result of consuming raw, frozen chicken nuggets/strips have commonly been associated with *S.* Heidelberg infection, though *S.* Typhimurium has also been identified as the etiological agent (MacDougall et al., 2004; Kenny et al., 1999). Disease symptoms were reported to last from 1 to 90 days with 47% of cases requiring hospitalization. Symptoms included: diarrhea (88%), fever (80%), abdominal cramps (65%), nausea (42%), vomiting (35%) and headache (29%) with 33% of cases experiencing bloody diarrhea (Currie et al., 2005). A couple of isolates have also been derived from blood samples, indicating the possibility of systemic infection (MacDougall et al., 2004).

To cause systemic infections and spread beyond the GI tract, *Salmonella* may have several virulence factors that allow the organisms to spread to the lymphoid tissue and spread via circulatory and lymphatic channels to major organs (Altier, 2005). Many of these virulence factors are found on *Salmonella* pathogenicity islands (SPIs) or islets. SPIs are distinct regions of DNA on the chromosomes of *Salmonella* that contain blocks of virulence-associated genes. In *Salmonella* there are 12 known SPIs. SPI1 and SPI2 encode structural components (*invE/A, orgA, ttrC, ssaQ*) for two different Type III secretion systems in *Salmonella* that deliver effector proteins to the host cell cytosol during host epithelial cell invasion (Soto et al., 2006; Hensel, 2004). Genes encoded on SPI3 (*mgtC, misL*) are necessary for survival within macrophages and growth in low Mg^{2+} environments, while genes present in SPI4 (*spi4R, spi4D*) are suspected to play a role in intramacrophage survival and toxin secretion (Soto et al., 2006; Hensel, 2004). Genes on SPI5 (*sopB, pipA*) encode effector proteins that are secreted by the Type III secretion systems (encoded by SPI1 and SPI2) which are involved in eliciting fluid

secretion that is typical of the diarrhoeal symptoms of salmonellosis (Soto et al., 2006; Hensel, 2004). SPI6 contains the *saf* gene cluster responsible for fimbriae, *pagN* which encodes an invasin and several other genes whose functions are still unknown (Hensel, 2004). SPI7 is a pathogenicity island specific to *S.* Typhi, *S.* Dublin and *S.* Paratyphi C. The Vi antigen, the *sopE* phage encoding effector protein and the *pil* gene cluster responsible for encoding the type IVb pilus are all present in SPI7 (Hensel, 2004). The virulence factors present in SPI8 have yet to be fully investigated, though bacteriocin genes as well as a gene encoding an integrase have been identified (Hensel, 2004). SPI9 encodes a Type I secretion system and large RTX-like exotoxin, while the known virulence factors of SPI10 encode the Sef fimbriae (Hensel, 2004). SPI11 or *Salmonella* genomic island 1 (SGI1) encodes the penta-resistance phenotype (resistance to tetracycline, ampicillin, chloramphenicol, streptomycin and sulfonamides) of *S.* Typhimurium DT104, *S.* Paratyphi B and *S.* Agona. The genes conferring this resistance are clustered in the multi-drug resistance region which is composed of two integrons. Other mobility genes such as transposase and excisionase genes have been detected in SGI1 which may have contributed to the appearance of variants of SGI1 in other serovars at the same chromosomal locations (Doublet et al., 2003). For example, a newly isolated strain of *S.* Albany with the streptomycin resistance gene was replaced with the integron that confers resistance to trimethoprim. The last SPI, SPI12, also known as the High Pathogenicity Island (HPI) encodes the biosynthesis pathway for a siderophore and iron uptake system (Hensel, 2004). This SPI was detected in several other Gram negative species and its presence appears to be correlated with the ability to cause septicaemia. Other genes that play a role in *Salmonella* virulence that are found unclustered on the bacterial chromosome are *slyA*, that encodes a transcriptional

regulator for genes required during survival in macrophages, *phoP/Q*, another transcriptional regulator for *Salmonella* virulence, *agfA*, that encodes for the thin aggregative fimbriae required for attachment to the host and *stn*, an enterotoxin (Soto et al., 2006). Many virulence factors are found on virulence (V) plasmids that vary in size and are serovar specific (Skyberg et al., 2006; Soto et al., 2006). All V plasmids in *Salmonella* share a highly conserved 8 kb region with 5 genes designated *spvRABCD* (*Salmonella* plasmid virulence) which has been shown to promote rapid growth and survival of *Salmonella* within the host cells (Soto et al., 2006).

The virulence genes, SPIs and Type III secretion systems with which they are associated seem to be widely distributed among many *Salmonella* poultry isolates regardless of whether their hosts of origin have salmonellosis or not. Skyberg et al. (2006) found all the genes localized in SPI1 and SPI2, along with the *pagC-msgA* pathogenicity islet in all the isolates tested. The *sopB* gene of SPI5 was also present in all isolates from healthy birds and in all but one of the isolates from birds diagnosed with salmonellosis. Nde and Logue (2008) made similar observations and found that all the genes of SPI2, the *pagC-msgA* pathogenicity islet and 5 of the 6 genes of SPI1 were present in all the isolates tested. In contrast, genes located on V plasmids are found less frequently. This may be because the V plasmids that carry the virulence genes are not always present in plasmid-bearing salmonellae and because these V plasmids can be serovar specific (Skyberg et al., 2006; Nde and Logue, 2008). Skyberg et al. (2006) found that, in contrast to the high prevalence (100%) of the virulence genes of SPI1, SP2 and the *pagC-msgA* pathogenicity islet in the isolates they tested, plus the genes *spvB* and *pefA* located on V plasmids, were found in <17% of the isolates tested. The lower

prevalence of the *spvB* and *pefA* genes was also observed by Nde and Logue (2008), who found them in 33.8% and 22.5%, respectively of the isolates examined.

2.2. Occurrence of *Salmonella* in feed and feed ingredients

In Canada the 10 most commonly isolated serovars associated with animal feed and feed ingredients in 2005 were: *S.* Thompson (16%), *S.* Schwarzengrund (8%), *S.* Cerro (7%), *S.* Anatum (6%), *S.* Heidelberg (6%), *S.* Montevideo (5%), *S.* London (5%), *S.* Infantis (4%), *S.* Orion (4%) and *S.* Alachua (3%) (Demczuk et al., 2007). Many of the larger feed companies in the U.S. and EU import raw materials like meat and bone meal, fish meal and cotton seed meal from South America, South Asia and Africa, respectively. These imported materials may potentially harbour exotic strains of *Salmonella* that have an increased resistance to heat and low humidity (Zdragas et al., 2001). A good example of this was indicated by Crump et al. (2002) who noted that the dramatic rise of *S.* Agona as a public health problem in the US (human isolations rose from 1 in 1967 and 1968 to 507 by 1972) was ultimately associated with poultry farms that incorporated Peruvian fish meal in their poultry feed. This fish meal was also blamed for the rises in *S.* Agona human infections in Isreal, the UK and the Netherlands. Increased awareness regarding the production and spread of *Salmonella* contaminated feeds has spurred legislation in the EU requiring that feed manufacturers adopt HACCP procedures to control *Salmonella* and other pathogens in feed. In Canada, no such legislation exists.

Contaminated feed ingredients and finished feeds have long been known to harbour *Salmonella*. In particular, ingredients of animal origin have been shown to have

the highest frequencies of contamination, though ingredients of vegetable origin have also been reported to harbour *Salmonella*. Isa et al. (1963) found *Salmonella* present in 31% of meat meal and 10% of soybean meal sampled. Hacking et al. (1978) reported that meat meal and broiler premix (containing trace amounts of vitamins and minerals, with protein filler from meat, fish, soybean and rapeseed meal) had the highest levels of contamination with rates of 81.0 and 84.2%, respectively, while the only vegetable/grain based feed ingredients contaminated with *Salmonella* were wheat screenings with 20% of the samples positive for *Salmonella*. Veldman et al. (1995) isolated *Salmonella* from 31% and 27% of fish meal and maize grits, respectively, in comparison with meat and bone meal and tapioca which were positive for *Salmonella* in 4% and 2% of the samples, respectively. Zdragas et al. (2001) reported isolating *Salmonella* from 24.2% of meat and bone meals while vegetable based meals that included grains and soya had 3.5 and 5.8% of samples positive, respectively.

A lower incidence of *Salmonella* in pelleted feeds compared to raw feed ingredients and raw mash feeds demonstrates that the pelleting process is capable of greatly reducing *Salmonella* in pelleted feed but is unable to eliminate the organism, possibly because of differences in feed mill management practices. Veldman et al. (1995) found that 21% of mash samples were positive for *Salmonella*, while only 1 of 162 samples of pelleted feed was contaminated. The temperatures used by the feed mills sampled in this study ranged from 70 to 75°C despite a suggested use of 80°C. Blank et al. (1995) evaluated the use of a Direct-Fired Steam Conditioner (DFSC) to control the microbial quality of mash and pelleted feeds. *Salmonella* were isolated from 8.3% of raw mash but only 1.7% of steam conditioned mash samples and from none of the

pelleted samples. Jones and Richardson (2004) collected feed (mash and pellet) and dust samples from three feed mills to investigate what effects the season, day of production and time of production had on *Salmonella* contamination. *Salmonella* was isolated from 8.8% of mash and 4.2% of pelleted samples. The season and time of production had no significant effects on *Salmonella* contamination, though the day of production did show significant effects ($P<0.05$) on *Salmonella* contamination with higher incidences of *Salmonella* observed on Fridays. Dust samples collected from feed mills in this study were found to be heavily contaminated with *Salmonella*, especially samples collected from the pellet coolers (ranging from 1.58% to 25.81%). It is thought that because pellet coolers take in large volumes of ambient air from within the feed mill, contaminated dust would have a greater likelihood to collect at the cooler rather than other areas, suggesting that post-pelleting contamination may negate the disinfecting effects of pelleting (Jones and Richardson 2004).

2.3. Occurrence of *Salmonella* in raw, frozen chicken nuggets/strips

During an outbreak investigation in Australia and examination of family-cluster cases of infection in British Columbia, both *S.* Typhimurium PT12 and *S.* Heidelberg PT26 were isolated from human cases and from samples of opened boxes of chicken nuggets/strips which corresponded to the brand and production lots of the index cases of illness (Kenny et al., 1999; MacDougall et al. 2004). In Canada the 10 most commonly isolated serovars associated with chicken and chicken meat in 2005 were: *S.* Heidelberg (43%), *S.* Kentucky (18%), *S.* Enteritidis (6%), *S.* Typhimurium (6%), *S.* Hadar (6%), *S.* Thompson (3%), *S.* Orion (2%), *S.* Brandenburg (2%), *S.* Agona (2%) and *S.* Infantis (1%) (Demczuk et al., 2007).

Chicken nuggets/strips are made using different forms of chicken meat depending on customer specifications with whole pieces or chunks of meat ground to improve protein extraction and facilitate binding between meat pieces (Owens, 2001). Thus, like ground beef with internalized *E. coli* O157:H7, chicken nuggets/strips are non-intact meats with the potential to have contaminating *Salmonella* distributed throughout the meat matrix. Since meat used in the manufacture of these products can come from a number of sources, the likelihood of obtaining meat contaminated with *Salmonella* is increased. To date, there have yet to be any studies that report the occurrence of *Salmonella* in these products, but because of the similarity of the processed meat to ground meats, comparisons between these products and ground poultry seem appropriate. Rose et al. (2002) collected random samples of ground chicken and broiler carcasses from large, small and very small federally inspected processing facilities in the United States over a two year period (1998 to 2000). Salmonellae were isolated from 14.4% of ground chicken samples and from 10.2% of broiler carcass samples. White et al. (2001) recovered *Salmonella* from 35% of the ground chicken sampled from three supermarket chains in the Washington D.C area (US). Smeltzer (1981) estimated most probable numbers of the organism in mechanically deboned meat (MDM) at 0.3 organisms per 100 g to greater than 550 organisms per 100 g. Other work in São Paulo, Brazil found that 15 of 60 MDM samples were *Salmonella* positive (Carvalho and Cortex, 2005). The extent of *Salmonella* contamination in these products does not seem unusual given their raw nature. In Edmonton (Canada), a survey of raw and ready-to-eat meat products sold at retail was done to examine these products for the presence of *Salmonella*. The organism was present on 30% of the raw chicken leg samples but absent from any of the ready-to-eat

products (Bohaychuck et al., 2006) suggesting that processing of products that are ready-to-eat will eliminate *Salmonella*. Since some chicken nuggets/strips sold in stores are fully cooked and only require re-heating before consumption perhaps all chicken nuggets/strips should be processed this way.

2.4. Pelleted feed processing

Poultry feed represents approximately 20-40% of the animal feed produced in most countries with North American production of the latter estimated at 127 million tonnes per year, 52 million tonnes of which represent broiler feed alone (Leeson and Summers, 2005). For the most part, broilers are fed pelleted feed except during their first two weeks of life when chicks are given crumbled pellets to facilitate feed consumption (Lacy, 2002).

2.4.1. Ingredients and formulations

Feeds are formulated based on the purpose of the chicken, the breed and the availability of feed ingredients. For example, birds destined for meat production such as broilers and roasters require feeds that maximize growth and muscle gain (Patrick and Schaible, 1980). The meat used in chicken nugget/strip manufacture will often come from broilers and other meat fowl. As the birds age, their nutritional requirements change and is reflected in their feed ration formulation. In general, starter, grower, finisher and withdrawal feeds are used at different animal ages to optimize the bird growth. Withdrawal feeds are feeds that do not contain any antibiotics and have their vitamin, amino acid and mineral content significantly reduced (Coon, 2002) and should not be confused with the feed withdrawal period where feed is withdrawn from birds

prior to shipment for slaughter so as reduce the intestinal content of the birds to decrease fecal contamination during transport and processing (Rigby and Pettit, 1981). Usually producers will try to use a minimal number of types of feed to help reduce costs associated with bin space and reduce mistakes associated with managing additional feed formulas, though for birds that are marketed at an older age and larger size (roaster), additional formulations are required (Table 2.1).

Table 2.1: Duration of starter, grower, finisher and withdrawal feed use for broilers. (Adapted from Coon, 2002)

Feed Type	Average Feeding Duration (days)	
	4-feed program[a]	5-feed program[b]
Starter	1-18	1-18
Grower	19-30	19-30
Finisher	31 to feed withdrawal	31-35
Initial Withdrawal	-[c]	36 to final feed withdrawal
Final Withdrawal	Last 5	Last 5

[a]Feeding program that uses 4 different types of feed
[b]Feeding program that uses 5 different types of feed
[c]There is no initial withdrawal feed for a 4-feed program

As birds get older, diets used are less nutrient rich because a greater proportion of nutrients are used for maintenance and less for growth (Leeson and Summers, 2005a). Starter and grower feeds will therefore have higher nutrient densities compared to finisher and withdrawal feeds to support the rapid growth rates early in the birds' life

(Leeson and Summers, 2005a). Finisher feeds may be used in place of withdrawal feeds if the antibiotic, growth promoter and/or anticoccidials are removed from the formulation (Coon, 2002). Often starter feeds will contain thermally labile enzyme supplements to support an underdeveloped digestive systems in broiler chicks (Zdragas et al., 2001). These enzymes are therefore usually added as post-pelleting additives, though others are more heat stabile and can be added to the mash prior to pelleting (Bedford, 2000; Leeson and Summers, 2005a).

Feed ingredients are chosen based on cost, with the relative value based on available energy, crude protein, and other essential nutrients. These ingredients are usually obtained locally to help keep the feed costs low (Patrick and Schaible, 1980). Major components of poultry feed are carbohydrates, lipids and proteins, plus major vitamins, minerals and feed additives such as antibiotics and other medications, antioxidants and pellet binders.

In terms of carbohydrate and energy content, corn is usually the main source because of its ready availability, low price and ease of digestibility. It contains carotenoid pigments called xanthophylls that impart a yellow colour to the fat and egg yolks in chickens and is a source of vitamin A. Sorghums such as kafir and milo are the two most widely used in poultry feed and can replace up to two-thirds of the portion of cereal grains. Whole wheat is analogous to corn and has a higher percentage of protein (10 to 17% compared to 8 to 11% in corn) but lacks vitamin A or other pigmenting properties (Coon, 2002a).

Lipids in the form of fat and/or oil are added to broiler feed because of their high energy content. They also reduce dust and improve palatability. Their use in broiler feed is usually based on the price relationship between the energy derived from the lipid and the energy derived from the carbohydrate source. When lipid energy is inexpensive it is more economical to use a higher proportion of fat or oil as part of the feed. Some typical sources of lipid include: hard fats like tallow and lard, soft fats that are semi-solid and hydrolyzed animal fats and vegetable oils like coconut, corn, soybean, palm and canola oil (Coon, 2002a).

Protein used for animal feeds can come from animal and vegetable sources. For the most part protein from animals sources like meat by-products, such as meat scrap (dry rendered animal flesh and tissue), meat and bone meal and fish meal are being replaced in favour of protein sources of vegetable origin because of concerns over *Salmonella* contamination (Coon, 2002a), though proteins from animal sources are still in use in Canada. Proteins from vegetable sources are the by-products of cereal grains that have had their oils extracted. Soybean meal is the most commonly used due to its availability, nutritional value and cost (Coon, 2002a), but should not be the only source of protein because of its low methionine and lysine content. Feeds are often built around a corn-soybean meal base with other ingredients added to make up for their deficiencies. Canola meal is another vegetable protein source often used as part of a feed ration. It contains a good balance of amino acids and newer varieties that have lower levels of glucosinolate and erucic acid have made it a suitable replacement for up to 75% of the soybean meal (Coon, 2002a).

There are 4 major macronutrients that make up poultry feed: calcium, phosphorous, sodium and chloride (Coon, 2002a), though zinc, manganese, iron, iodine, selenium, copper and molybdenum are micronutrients required in smaller quantities (Coon, 2002b). Steamed bone meal and rock phosphate contain considerable amounts of calcium (32 and 30%, respectively) and phosphorous (8 and 12.5%, respectively) though limestone and oyster shell are also excellent sources of calcium that contain up to 38% each. Salt provides a source of sodium and chloride necessary in small quantities. Typically 0.25% free salt is added to rations as large amounts increase water consumption and exhibits a laxative effect (Coon, 2002a).

Vitamins are another essential component of poultry feed. There are 14 listed as essential to poultry. Fat soluble vitamins include: vitamins A, D, E and K while water soluble vitamins include: vitamins B1, B2, B6, B12, C pantothenic acid, niacin, choline, biotin and folic acid. Most vitamins are present in feed ingredients, but their content varies substantially. Since adjusting the amount of feed ingredients to meet the required vitamin content is impractical, supplementation with commercially produced vitamins is done (Coon, 2002b).

Non-nutritive feed ingredients such as antioxidants, antibiotics and other medications, enzymes and pellet binders are added to poultry feed to increase meat yield, treat disease, improve feed digestion and increase pelleted feed quality. Many of the fat soluble ingredients are subject to oxidation so antioxidants like butylated hydroxytoluene and ethoxyquin are added in small quantities (0.0125%) (Coon, 2002b). Even though antibiotics are used to treat bacterial infections in chickens, several are added to feed because they have growth promoting effects when fed continuously at low

levels (Leeson and Summers, 2005a; Coon, 2002b). Due to concerns over the spread of antibiotic resistance, regulation governing their use has been implemented in many countries. The list of accepted antibiotics in Canada is available on the Canadian Food Inspection Agency website (Anon, 2004).

For a more complete list of feed ingredient descriptions, dietary supplements and sample feed diets, please refer to Leeson and Summers (2005a) and Coon (2002; 2002a; 2002b).

2.4.2. Feed pelleting

The pelleting process used during the manufacture of poultry feed can dramatically reduce the number of *Salmonella* and perhaps even eliminate the organism, barring any post-processing contamination. There don't exist any standard feed mill designs and several mixer-feeder operations (animal-producing farms large enough to produce their own feed) exist, but all mills have several key processes used in pelleting poultry feed: (1) formulation and mixing, (2) conditioning, (3) pellet formation and (4) cooling/drying. Figure 2.1 outlines the design of a typical poultry feed mill located in Ontario (Canada).

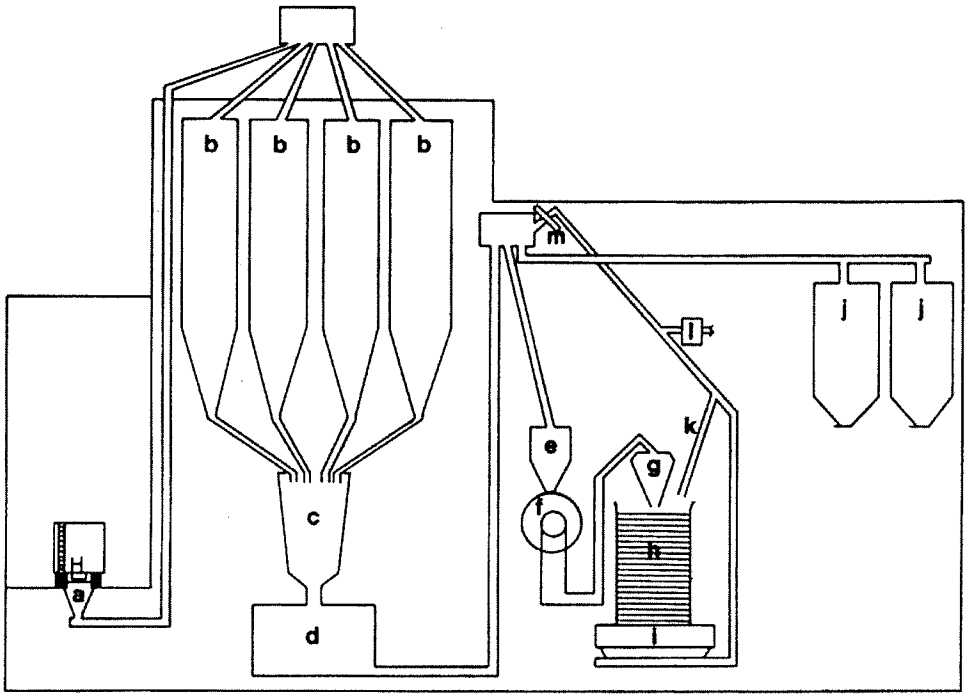


Figure 2.1: Basic schematic design of an Ontario feed mill. a –receiving chute; b – ingredient storage bins; c – blender scale; d – mixer; e – collecting bin; f – pellet mill; g – cone; h – cooler; i – crumbler; j – finished feed bins; k – return pipe; l – tallow meter; m – pellet sifter. (Adapted from Hacking et al., 1978)

Feed ingredients are delivered in bulk and deposited in a receiving chute (Fig. 2.1a) from which they are conveyed to bulk storage bins (Fig. 2.1b). Feed and feed ingredients can be conveyed or elevated along a production line using portable or vertical augers and cup elevators (Patrick and Schaible, 1980). From the storage bins ingredients are proportionately weighed in a blender scale according to the feed formulation (Fig. 2.1c) then sent to be mixed (Fig. 2.1d) below. Vertical or horizontal batch mixers are commonly used in today's industry. Vertical mixers are the more common of the two and use a large vertical screw to blend the ingredients. Horizontal mixers use two sets of

paddles or open spirals that move in opposite directions to move the ingredients forward and backwards (Patrick and Schaible, 1980). Once the raw feed ingredients are mixed, depending on the type of feed (mash, pellet or crumbled) the feed is conveyed to finished feed bins (Fig. 2.1j) for mash feeds, or are sent to a collecting bin before they are conveyed to the conditioner. Before raw mash is pelleted it passes through a conditioner where heat and moisture are added by injecting steam into the raw mash. As the mash moves through the conditioner paddles agitate the mash to evenly disperse the steam throughout the feed. The added heat and moisture increase gelatinization of the starches present in the raw mash which helps bind the wet mash into a pellet once it is pressed through the pellet mill die. Optimally, the steam will add about 15 to 18% moisture (Leeson and Summers, 2005a) to the dry mash and increase the temperature up to 75 to 80°C (Fancher et al., 1996). Depending on the feed ingredients mash can travel through the conditioner in 2 to 3 s or take as long as 22.5 s (Hacking et al., 1978; Zdragas et al., 2001). An annular gap expander is a type of conditioner, similar in design to a single screw extruder, sometimes used after the conditioner and before the pelletizer to pre-treat the feed before pelleting (Figure 2.2).

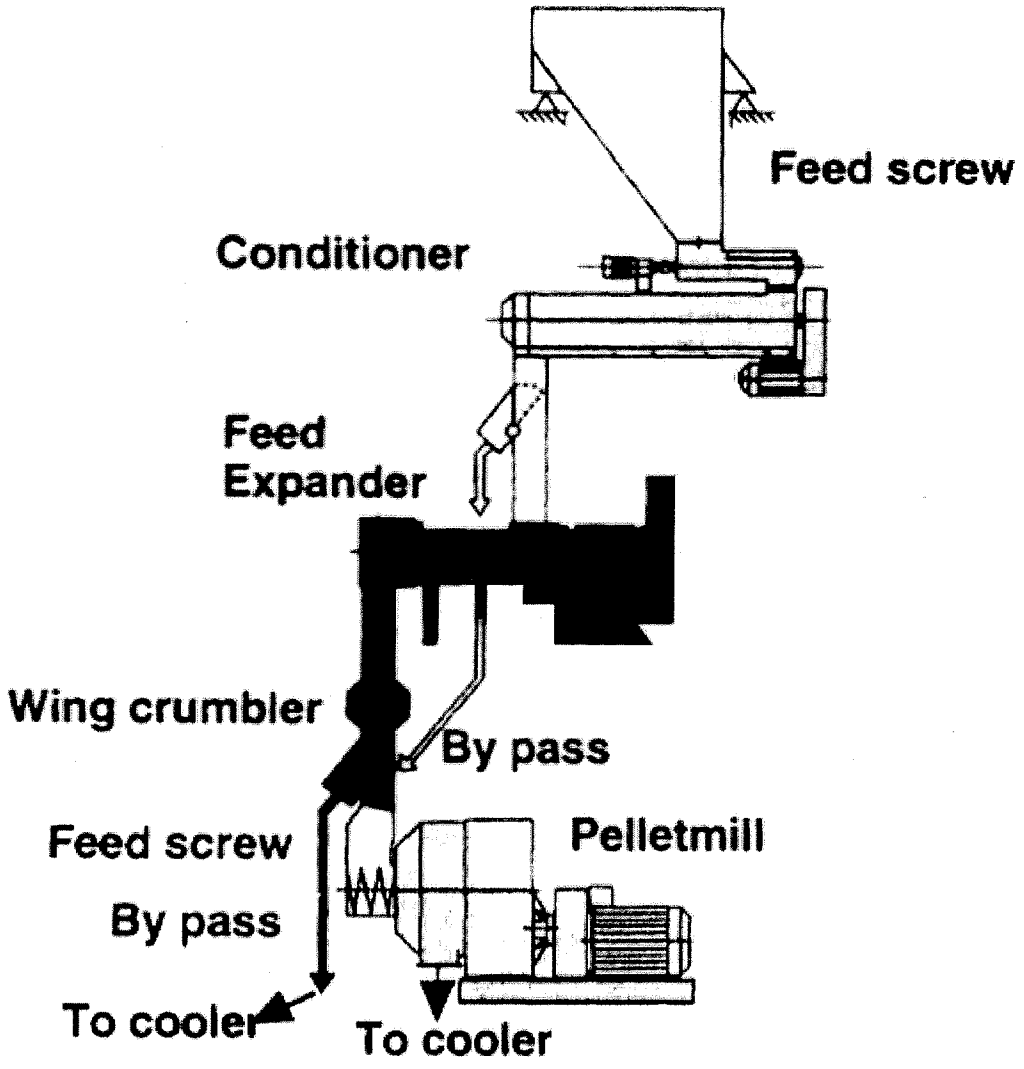


Figure 2.2: Process flow when a feed expander (highlighted in black) is integrated into the feed pelleting process after the conditioner and before the pelletizer. (Adapted from Fancher et al., 1996)

The annular gap expander uses a high temperature for a short time (HTST) to increase pellet hardness/durability when higher levels of liquid and by-product feed ingredients are part of the feed formulation (Fancher et al., 1996). Similarly to a conventional

conditioner, mash is metered into the expander where a heavy duty screw forces wet mash over the annular gap outlet. The transfer of mechanical energy to thermal energy can increase the mash temperature up to 127°C (Fancher et al., 1996) and can last approximately 5 s (Newcombe, 2007 personal communication). After conditioning and expanding the wet mash can be augered or simply fall into the pelletizer chamber. Once in the pelletizer, two or more rollers and feed ploughs push the material through the holes of the die plate (Figure 2.3).

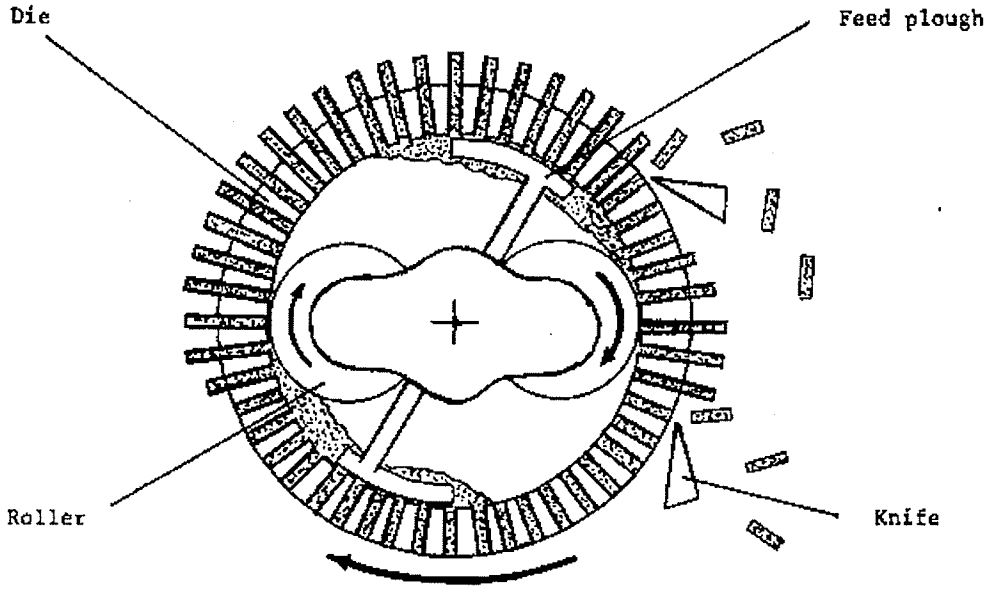


Figure 2.3: Pelletizer chamber with two rollers extruding pellets. (Taken from FAO, 1980)

After pellets are formed they must be cooled and dried (Fig. 2.1h) before being sent to the finished feed bins. Coolers use ambient air from within the feed mill to cool pellets with a counter flow system. In order for most coolers to work properly they need to be filled to capacity, so a return pipe (Fig. 2.1k) can return the first pellets to be processed

back to the cooler. The cooler has been a recent topic of discussion with regards to post-pelleting contamination of pellets with *Salmonella*. Jones and Richardson (2004) found high frequencies of *Salmonella* contamination around the coolers of three feed mills in the US. It is hypothesized that since the cooler pulls large amounts of air from within the feed mill, dust contaminated with *Salmonella* would have a greater likelihood of accumulating around the cooler and potentially contaminating finished pellets with *Salmonella* (Jones and Richardson, 2004). If crumbled pellets are desired, after cooling the pellets are crumbled using sharp, corrugated rollers to cut rather than crush the pellets into crumbles (Fig. 2.1i) before being augered to the finished feed bins. Often fat is added after the pelleting process (Fig. 2.1l) (rather than before) because pellet quality is improved (Patrick and Schaible, 1980).

2.5. Chicken nugget/strip processing

Since their initial introduction to North America by fast-food chains in the 1970's, chicken nuggets/strips have quickly become one of today's most popular food items available. Many poultry processors have added these products to their product line since they add convenience and variety for consumers, while at the same time increasing profits for processors.

2.5.1 Ingredients and formulations

Chicken nuggets/strips are coated poultry products that consist of two parts: (1) the coating, which includes a predust, batter and breading and (2) the meat product. The meat used in chicken nugget/strip production can come from a variety of sources, though for the most part broiler chickens are the main source of meat. Other sources can include

culled laying hens and other fowl. Processors very rarely manufacture whole breast muscle nuggets/strips. The meat used in manufacture varies depending on the preference of a particular locality. In North America white meat is preferred whereas in Pacific Asia dark meat is preferred. Due to their preference for white meat, a common formulation in North America is to use breast meat in combination with skin. The breast meat functions to provide a uniformly soft texture and colour whereas skin improves flavour because of its higher fat content (Owens 2001). Dark meat sources such as thigh, drumstick, rib as well as MDM have also been incorporated into North American nugget/strip formulations to reduce production cost and enhance flavour. A common ratio of white:dark meat is 70:30. Elevated levels of dark meat can compromise the overall texture of the nugget/strip because of its softer texture in comparison to white meat and result in a higher susceptibility to oxidative rancidity because of its higher fat content (Lai et al., 1991). Soy protein isolate has been found to firm-up the softer texture of dark meat as well as lighten its colour (Owens, 2001). Other non-meat ingredients such as salt and sodium tripolyphosphate (STP) are added to increase protein extraction, enhance flavour, increase water holding capacity and delay oxidative rancidity (Barbut, 2002). Salt is <1% of the overall nugget/strip formula and polyphosphates such as STP can only account for 0.5% of the finished product (O'Sullivan et al., 2004; Lai et al. 1991). Water is also added in the form of ice to solubilize the salt and STP as well as to reduce the meat temperature so that the meat maintains its shape after forming (Owens, 2001).

Generally the coating consists of the batter and breading layers, but can sometimes include an intermediate predust layer between the meat and batter. It is usually made from a fine coating of wheat flour or dry batter mix and seasonings if the

meat surface is too moist for the batter to properly adhere (Owens, 2001; Barbut, 2002). Adherence to the meat can be enhanced with the addition of non-meat proteins from soy, egg or whey to the predust mix, but can also be done with extracted actin and myosin meat proteins after tumbling with salt (Barbut, 2002). Volatile flavour compounds normally added in the outermost layer of the nugget/strip can also be incorporated in the predust since they would significantly be reduced or lost in the final cooking or par-frying step if applied in the outer layers of the nugget/strip (Barbut, 2002).

Following the predust, a batter is applied to cement the breading and meat as well as impart desirable flavours and textures to the final product. Several types of batters exist (leavened/tempura and unleavened) and consist of similar ingredients except for leavened batters which use leavening agents like sodium bicarbonate plus one or more acids in their formulations (Barbut, 2002). The main ingredients that make-up a batter are water, wheat flour, proteins, gums, spices and colouring agents as well as the leavening agents when appropriate. Wheat flour provides starch and protein. The starch absorbs water for good adhesion to the meat and influences batter drying time and oil pickup during frying, while the protein (gluten) acts as an emulsifier of the water and fat and forms a gel matrix that binds all the batter ingredients upon heating (Barbut, 2002). Xanthan, guar and modified cellulose gums can control the water holding capacity of the batter to increase viscosity for better adhesion to the meat source. Some gums, such as methyl cellulose increase batter viscosity as well as form a gel upon heating which helps reduce fat absorption during frying (Barbut, 2002). Flavouring and colouring agents such as white and black pepper, thyme, celery salt, marjoram, rosemary, paprika and reducing sugars with amino acids are added for flavour and colour development. On

average 3-5% of the dry batter with salt representing 10-15% of the batter mix make up the flavouring agents in batter. Artificial food colourings are usually not permitted (Barbut, 2002). These batters are used as the final coating for the nugget.

Since the breading is applied as the final layer, it contributes to product appearance and texture and also increases the final weight and volume of the nugget/strip. Breadings used yield a variety of sizes, shapes, textures, colours and flavours and can be used alone or in combination with spices and seasonings to provide extra flavour and colour to the final nugget/strip. Flour is one of the most economical breadings commonly used in conjunction with spices and seasonings to give the final product a fairly dense coating with relatively low browning and a flaky homemade appearance (Barbut, 2002; Owens, 2001). Cracker-type breadings have a range of fine to coarse granulation depending on final product specifications. Browning of this breading after frying is not extensive and so dextrose is often added if more browning is desired (Owens, 2001; Barbut, 2002). Homestyle or American bread crumbs resemble homemade bread crumbs with a two-tone appearance. This provides the nugget/strip with a highlighting effect with medium to high levels of browning upon frying. The higher granulation of this breading causes a more porous structure that results in a crispier texture compared to cracker and flour type breadings (Owens, 2001; Barbut, 2002). Unlike homestyle or American bread crumbs, Japanese bread crumbs are made from crustless bread giving them a similar porous texture but with a lighter colour. These crumbs have a very delicate elongated shape that requires special equipment to prevent the shape from breaking. They are commonly produced using electrical induction rather than conventional baking which allows for a very light crumb density

without crust formation. This type of breading is considered to have a coarse granulation that can adversely affect nugget/strip coating, but has a high degree of crispiness and highlighting upon frying (Owens, 2001; Barbut, 2002).

2.5.2 Processing Steps and Conditions

The steps in chicken nugget/strip processing are: (i) meat particle size reduction, (ii) nugget/strip forming, (iii) battering, and breading, (iv) cooking, and (v) freezing and packaging. The first step in production is to reduce the particle size of the meat ingredient. The whole or chunks of meat are either chopped or ground in a bowl chopper or grinder to the consistency of a paste. Excessive blending or chopping is avoided since it can denature these proteins (Barbut, 2002; Owens, 2001). At this stage of the process, ingredients like salt and STP can be tumbled in with the meat. Water, in the form of ice, is also added to solubilize the dry ingredients, but more importantly to reduce the meat temperature so that meat proteins are not denatured, and maintain enough moisture so that subsequent coating operations are more efficient as well as to ensure the nugget/strip keeps its shape after forming (Barbut, 2002; Owens, 2001). If the temperature is too low the formed product can break. Optimally, meat temperature should be between -3.3 to -2.2°C (Owens, 2001). To help reduce meat temperature, processors often use a combination of chilled and frozen meat pieces during the size-reduction stage of processing.

Following size-reduction and cooling, the meat is immediately sent along a conveyor belt to a hopper that augers the mixture to the forming machine. Dry-ice can be added to maintain the low temperature, but will increase production costs. The cool

meat is pressed into mold plates that later slide out and a knockout apparatus pushes the formed meat onto a conveyor belt. Figure 2.4 shows some of the many shapes of chicken nuggets that are made available to consumers.

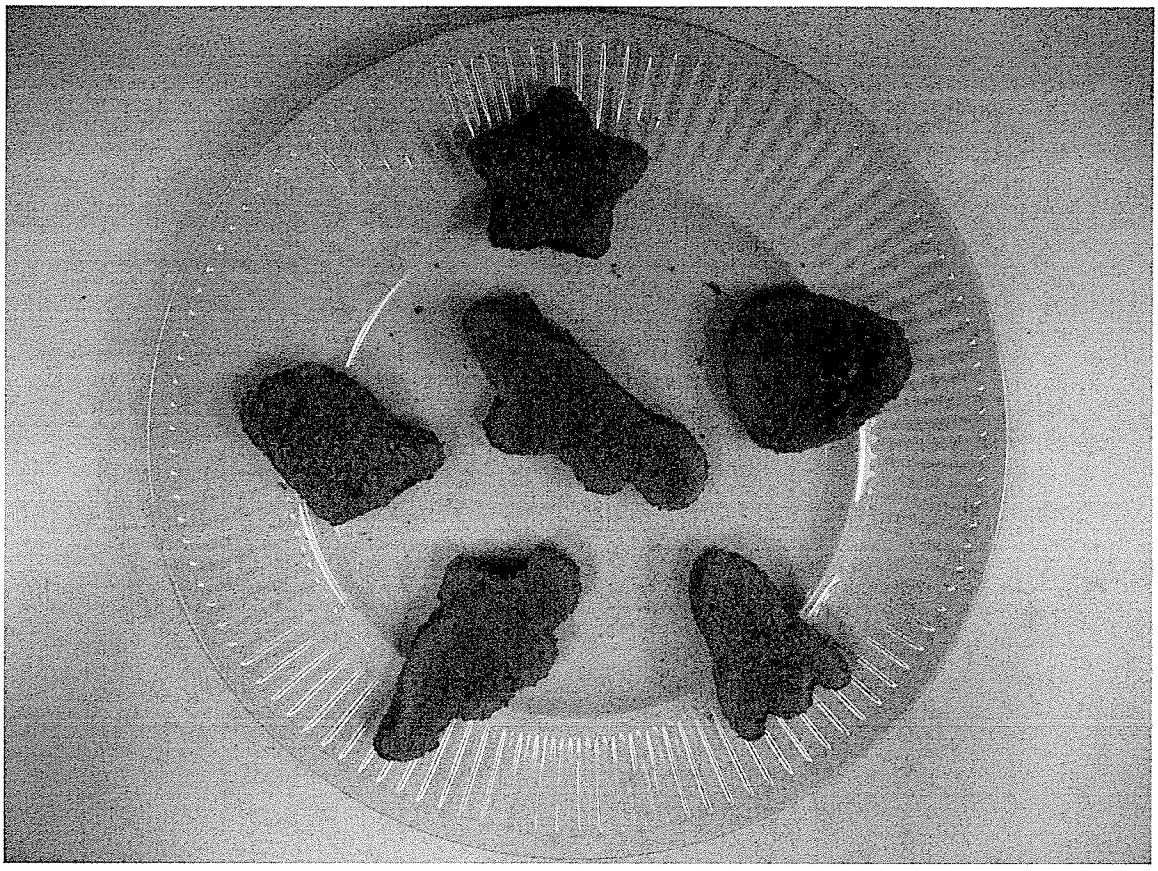


Figure 2.4: Dinosaur, space and generic shaped chicken nuggets.

After molding into the desired shape, the nugget/strip is coated. As mentioned in the above section, the coating system can be comprised of (1) a predust, (2) battering and (3) breading. Various combinations of the above steps can be incorporated into

nugget/strip production. Figure 2.5 shows a common method of applying predust to nugget/strips using a sprinkle/flatbed applicator.

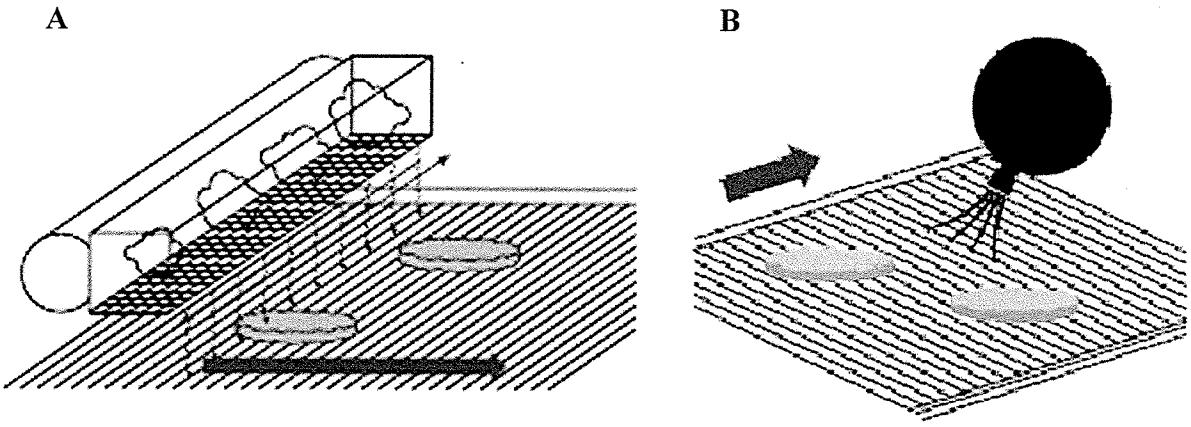


Figure 2.5: Sprinkle/flatbed predust applicator (a) and Air Knife blower (b). (Adapted from Anon, 1998)

With this type of application the nugget/strip travels along a mesh conveyor belt evenly layered with predust mix as more is sprinkled from the top before passing under pressure rollers that embed the predust in the nugget/strip. Excessive amounts of predust result in bare spots on the finished nugget/strip. The excess is therefore blown-off with air or shaken off with an agitating conveyor belt (Anon, 1998).

After predusting, the nuggets/strips are battered. Different equipment is required for leavened and unleavened batters since excessive pumping and movement can cause leavened batters to lose the entrapped gas. For this reason a Still or Submerging system is used to apply these batters. With this type of system a mesh conveyor belt carries the nuggets/strips along a path that submerges them in a pool of batter while the holes from

the mesh conveyor allow for batter to coat the bottom (Figure 2.6a) (Anon, 1998). For unleavened batters, an overflow system pumps batter into a trough that overflows and cascades over the side to coat the top and sides as the nuggets/strips are conveyed through a puddle of batter to coat the bottom (Figure 2.6b) (Anon, 1998).

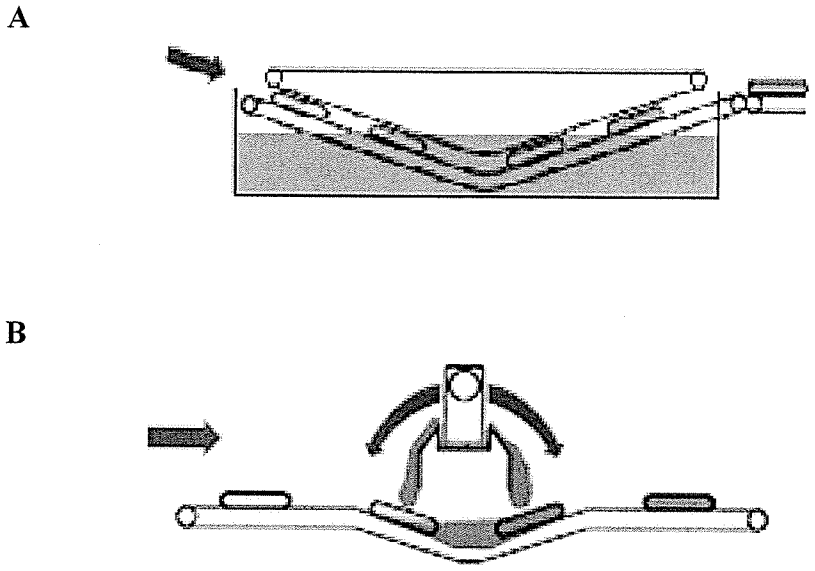


Figure 2.6: Batter application systems. (a) Top submerger system; (b) Overflow system (Adapted from Anon, 1998)

Most chicken nuggets/strips sold in North America, use an unleavened batter in conjunction with breading as a final coating for the nugget/strip. Equipment for breading is generally the same for all types of breading except for Japanese style bread crumbs which require gentler handling. The battered nuggets/strips are conveyed onto a wire conveyor that deposits them into a layer of breading to coat the bottom as they move underneath an applicator or sifter that sprinkles breading from the top. The nuggets/strips pass under pressure rollers that press the breading into the batter so that

bound breading remains embedded in the nugget/strip. Excess breading is then blown off or removed with vibrating belts to minimize loose breading from dropping into the fryer and reducing oil quality (Anon, 1998).

After breading the nuggets/strips are conveyed to the fryer or oven to be baked or fried. Frying is the most common means of cooking, although increasing consumer concern has created a demand for baked products as well (Barbut, 2002). In general, processors have three choices when cooking these products: par-frying, fully cooking or baking. Most nuggets/strips sold at retail are par-fried, which has become cause for concern since the raw nature of these products is not evident to consumers and has lead to cases of salmonellosis in Canada and Australia (MacDougall et al., 2004; Kenny et al., 1999). With this type of operation, the nuggets/strips are taken along a conveyor belt that submerges the nuggets into a pool of hot oil ranging from 179-200°C for anywhere from 20 to 45 s depending on the product specifications (Figure 2.7).

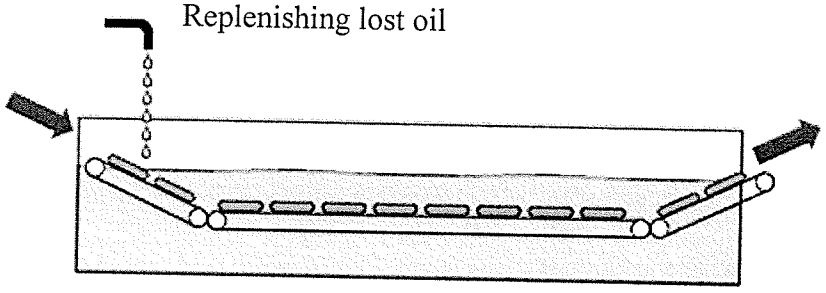


Figure 2.7: Chicken nugget/strip frying operation (Adapted from Anon, 1998).

Par-frying is designed to develop the characteristic golden brown colour commonly attributed to these products and solidifies the outer coating. The inner core of the

nugget/strip, however, remains raw leaving the final cooking step up to consumers. If processors choose to fully cook the nugget/strip they have the choice to proceed in one or two steps. When fully cooking nuggets/strips in a one step operation, there are fewer processes, which increases the production time and reduces the number of fryers and volume of oil required; however there is also the opportunity to increase the number of defective products. Nuggets/strips are more likely to stick together when fully cooked in one operation and voids in the breading can occur if the coating adhesion becomes compromised. Also, since frying evaporates moisture, product yield is reduced (Owens, 2001). If processors fully cook these products they usually use a two-step process and par-fry the nuggets first to solidify the coating before sending them to a second fryer where they are fully cooked at temperatures ranging from 166°C to 179°C (Owens, 2001). Challenges with keeping the same crunchy texture and golden brown colour for baked nugget/strips keep these products from being as popular as their fried counterparts (Owens, 2001).

When the nuggets/strips have been fried or baked, they are immediately sent to an individual quick freezer (IQF) for rapid freezing. These freezers typically use dips or sprays to expose the nugget/strip to liquid nitrogen (boiling point of -196°C) or carbon dioxide (boiling point of -78.5°C) as they move along a conveyor belt within an insulated tunnel. The temperature of the nugget/strip is allowed to equilibrate to the product's desired storage temperature (usually $\leq$-20°C) before leaving the freezer for weighing and packaging.

2.6 Isolation, Identification and Subtyping of *Salmonella*

The Health Canada MFHPB-20 method is the official method used for the isolation and identification of *Salmonella* from foods in Canada (D'Aoust and Purvis 1998). This method uses 6 steps to isolate the organism. The first step is a preenrichment to recover injured or stressed cells. In the second and third steps, selective media inhibit and delay the growth of other competing organisms present in the sample, and this is followed by plating onto agar plates that select and differentiate colonies of *Salmonella* from non-*Salmonella* colonies. The fourth step is done to purify suspected colonies so that they are well isolated and can be individually screened based on biochemical reactions in agar slants in a fifth step. After being screened on slants, cultures are confirmed as *Salmonella* through serological agglutination with polyvalent or somatic grouping antisera in the sixth step.

For sampling pelleted feed and raw, frozen chicken nuggets buffered peptone water (BPW) or nutrient broth (NB) can be used interchangeably as a preenrcihment. These media provide basic nutritional and growth requirements for most bacteria to recover and multiply. The peptone ingredients provide carbon, nitrogen and energy to cells while beef and yeast extracts provide sources of amino acids, peptides, nucleotides, organic acids, vitamins, minerals and nitrogen and carbon compounds (Prescott et al., 1990).

In the second step tetrathionate brilliant green (TBG) and selenite cysteine (SC) broths are used to select for the growth of *Salmonella* while discouraging the growth of non-salmonellae. The selectivity of the TBG broth comes from a combination of

incubation temperature (43°C) and the tetrathionate ion ($S_4O_6^{-2}$) that is formed when sodium thoisulfate in the broth medium reacts with an iodine-potassium iodide solution that is added to complete the medium (D'Aoust, 1989; Chapin and Lauerdale, 2003; Atlas, 1995). A membrane bound tetrathionate reductase enzyme in *Salmonella*, *Citrobacter*, *Serratia*, and *Proteus* genera provide these organisms resistance to the toxic effects of the tetrathionate ion (D'Aoust 1989; Palumbo and Alford, 1970). Bile salts and the addition of brilliant green dye also further enhance the selectivity of the medium and exclude Gram positive organisms, though brilliant green can also inhibit certain salmonellae, most notably *S.* Typhi (Chapin and Lauerdale, 2003; Atlas, 1995).

The SC broth used to isolate *Salmonella* is far less selective so is used in conjunction with TBG broth. The medium selects for salmonellae through the increased rate of uptake of selenite by non-salmonellae which becomes incorporated in cell proteins and other microbial constituents as a sulfur analog and delays the proliferation of these non-salmonellae organisms (D'Aoust 1989; Singleton and Sainsbury, 1987).

Subsequent to the selective enrichment, samples are plated in duplicate onto brilliant green sulfa (BGS) and bismuth sulfite (BS) agars to isolate suspected *Salmonella* colonies. These media selectively promote the growth of *Salmonella* and inhibit the growth of some non-salmonellae while differentiating between colonies of *Salmonella* and non-*Salmonella*. As with the TBG broth, brilliant green is added to BGS to inhibit Gram positive contaminants while the sodium sulfapyridine inhibits competitive Gram negative contaminants (Osborne and Stokes, 1955; Moats and Kinner, 1974). The differential ability of this media depends on the ability of an organism to

utilize lactose or sucrose. Phenol red acts as a pH indicator so when an organism utilizes lactose or sucrose, the pH of the medium drops and the indicator dye turns green/yellow. Salmonellae do not typically use lactose or sucrose and produce an alkaline reaction making the colonies pink/fuchsia (D'Aoust and Purvis, 1998). However, the appearance of lactose and sucrose positive biotypes (Gonzalez, 1966; Easterling et al., 1969; Falcão et al., 1975) has limited the ability of this medium to permit *Salmonella* identification, and has promoted the use of BS agar in conjunction with BGS to isolate *Salmonella.* BS agar does not rely on carbohydrate utilization to differentiate between *Salmonella* and other organisms but rather on the ability of *Salmonella* to produce hydrogen sulfide. Ferrous sulfate acts as an indicator in the media and reacts with metabolically produced hydrogen sulfide which creates black/green colonies with or without a metallic sheen and causes a gradual darkening of the media surrounding the colony (Chapin and Lauerdale, 2003). Bismuth sulfite and brilliant green dye are added to inhibit most Gram negative and Gram positive organisms. The isolation of hydrogen sulfide negative strains of *S.* Heidelberg from chicken nuggets/strips has called into question the efficacy of this and other media commonly used to isolate *Salmonella* (MacDougall et al., 2004). In addition, inconsistencies in the preparation and storage of this media have been noted and it was found in one study that newly prepared media can be inhibitory to several *Salmonella* serovars unless poured plates have been aged 24 h under refrigeration prior to streaking (D'Aoust, 1977).

After colonies suspected of being *Salmonella* have been isolated they are transferred to MacConkey (MacC) agar plates to purify the culture for well isolated colonies. This media has a very low selectivity and is used to isolate Gram negative

organisms, such as *Salmonella*, due to the inclusion of bile salts and crystal violet (Chapin and Lauerdale, 2003). Neutral red acts as an indicator that changes the colour of colonies to pink when the organisms use lactose and cause the pH of the medium to drop. The alkaline reaction that salmonellae typically produce yields colourless colonies (D'Aoust and Purvis, 1998).

After purifying the culture, colonies are screened using biochemical tests that distinguish salmonellae from non-salmonellae. This is done using triple sugar iron (TSI), lysine iron (LIA) and Christensen's Urea (Urea) agar slants. TSI slants distinguish among glucose, lactose and sucrose fermenting organisms as well as detect hydrogen sulfide and gas production (Bopp et al., 2003; Singleton and Sainsbury, 1978). Typically, salmonellae use glucose, and to distinguish this from other carbohydrate fermentation, one-tenth the amount of glucose compared to lactose or sucrose is present in the medium. Glucose fermentation occurs in the anaerobic butt of the slant and produces acid end products that change the phenol red to yellow. Once the glucose is used *Salmonella* are forced to deaminate the peptone in the media as a source of carbon. This is an aerobic process so it can only occur on the upper slanted portion of the agar and it produces an alkaline reaction because of the ammonia end products. The resulting slant has a yellow butt with a red slant. Ferrous sulfate is included in the medium to indicate whether hydrogen sulfide is produced. This process in anaerobic, so only the butt will turn black.

LIA was designed to isolate lactose fermenting *Salmonella* and so is best used in conjunction with other media when isolating *Salmonella* (Edwards and Fife, 1961). It

differentiates between enteric bacilli based on the ability of *Salmonella* to decarboxylate or deaminate lysine and produce hydrogen sulfide. Glucose is the only carbohydrate available for fermentation and its use changes the pH indicator bromcresol purple to yellow when the pH drops below 5.2. *Salmonella* decarboxylate lysine so the medium will change the initial acid reaction from yellow to purple (Edwards and Fife, 1961) and ferric ammonium citrate and sodium thiosulfate cause a blackening of the medium when hydrogen sulfide is produced.

Urea agar slants differentiate between enteric bacilli like *Salmonella* based on the presence of urease. Typical *Salmonella* are urease negative and consequently do not create alkaline end products (ammonia) that change the phenol red indicator colour red-pink (Atlas, 1995; Singleton and Sainsbury, 1978).

After test cultures are screened biochemically, they are then confirmed as belonging to the genus *Salmonella* using polyvalent antiserum or somatic grouping antisera and sent to a certified typing center for further identification (D'Aoust and Purvis, 1998).

2.6.1 Serological Identification

Salmonella serotyping is one of the most discriminatory methods of typing these bacteria and remains the standard method for identifying *Salmonella*, with other subtyping methods providing only supplemental information. Serological identification of *Salmonella* is based on the Kauffmann-White scheme which designates serotypes

based on the identification of somatic (polysaccharide O-antigens) and flagellar (protein H-antigens) antigens present in the cell wall (Figure 2.8) (Popoff, 2001).

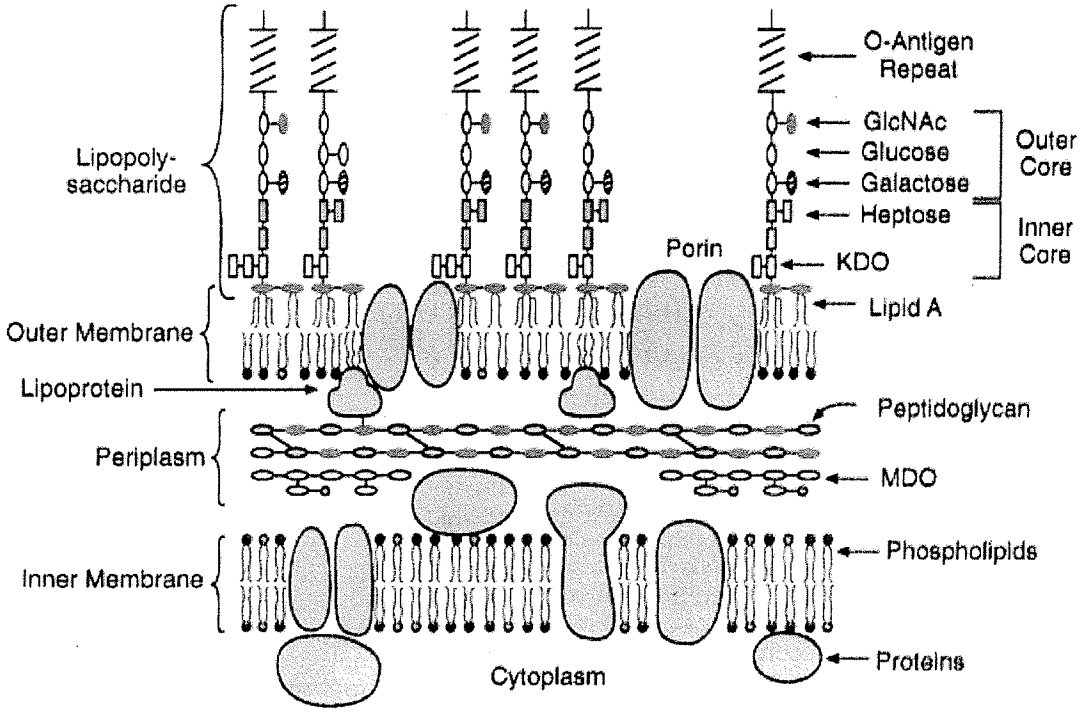


Figure 2.8: Section of a Gram negative bacterium inner and outer membrane depicting the location of the O-antigens. (Taken from Raetz, 1993)

In this scheme the O-antigens determine the grouping, while the H-antigens add more discriminatory criteria and complete the definition of the serotype. The notation used to denote the serotype is given by the major antigenic groups separated by colons:

O-antigens: phase1 H-antigens: phase2 H-antigens

With regards to serology, it is the outermost combination of rare polysaccharides within the lipopolysaccharide outer membrane that give rise to the O-antigens. The genes responsible for O-antigen synthesis are normally grouped together in a cluster called *rfb* (Herrera-León et al., 2007). These genes encode the nucleotide sugar synthesis pathways

as well as the transferase enzymes responsible for constructing the repeating polysaccharide unit in the outer membrane. The O-antigen is not necessary for cell viability and so mutants that do not express all the O factors produce rough (R) variants that lack some of the repeating polysaccharide units. Lysogenic conversion by bacteriophages can also produce lysogenic variants of certain serotypes and change the O grouping (Popoff, 2001). Flagellar antigens differ from the polysaccharide complexes of the somatic antigens in that they are made of proteins (flagellin) that make up the cell's flagella. Most *Salmonella* typically express two phases of H-antigens, H1 and H2 which are encoded by the *flicC* and *flicB* genes respectively, though variants that express none, only one or up to four different phases are known (D'Aoust, 1989; Herrera-León et al., 2007).

Antigens are identified using antiserum containing antibodies raised in an animal, usually rabbits. Testing for antigens is done through a slide agglutination reaction whereby the antigen and antibody bind at antigen binding sites on the antibody. There are usually multiple binding sites that allow for cross-linking to occur and this forms a clump visible to the naked eye. Autoagglutination can occur with rough variants which can create false positive reactions. To avoid this problem, rough cultures can be transferred and re-cultured until the smooth (S) colony state is recovered. In the case of flagellar antigens, all potential phases need to be screened for, so a phase inversion is done. After the first phase is identified, it is repressed by mixing a few drops of antiserum for that first phase in with melted semi-solid agar medium. This tethers any cells that express the first phase in place and forces other cells to express the other phase(s) in order to remain motile.

Although serotyping *Salmonella* is a very reliable and discriminatory method of identification, it does possess its drawbacks. Slide agglutinations are time consuming, use 167 different antisera and require well trained technicians to interpret the agglutination reactions. Despite the drawbacks to this method of *Salmonella* typing, no alternative methods are available which completely identify all the different serotypes. In a well equipped laboratory the method is reasonable and provides useful additional criteria to differentiate *Salmonella* strains.

2.6.2 Phage Typing

Phage typing is a method that exploits the highly specific, lytic reactions a bacteriophage (phage; a virus that attacks and lyses bacteria) has with its respective host bacterium to differentiate between related strains of *Salmonella*. Phages attach to specific antigenic markers on the surface and/or flagella of its host (Kutter et al., 2005). After a phage attaches to its host it penetrates the membrane and transfers its DNA to the host. There the DNA can take over the host bacterium's metabolism and begin replicating more phage particles that will eventually lyse the host cell (Guttman et al., 2005). When a sufficient number of bacteria are lysed, they can be visually observed as clear patches on the surface of agar plates called plaques (Summers, 2005). It is the formation and interpretation of the types of plaques that gives rise to the phage typing method.

The phage typing scheme used for *Salmonella* follows the protocols described by Anderson and Williams (1956). A series of phages selected on the basis of their stability, and host range, are spotted onto a lawn of *Salmonella* (Anderson and Williams, 1956).

The plaques formed by the phages are read and interpreted following incubation. There are a number of interpretations that can be made when reading phage plates. Lytic reactions of 100 plaques or more are considered positive lytic reactions. It is important to note that phages are not arbitrarily spotted onto plates, but that a routine test dilution, or the highest dilution of phages required to produce semi-confluent lysis of the propagating host is used (Demczuk et al., 2003). This concept arose as a result of the work done creating the Vi-typing scheme for *S.* Typhi (Anderson and Williams, 1956). When phages are spotted onto their host in high concentrations cell death is so great that the lytic reaction will appear as confluent lysis and mask the specific lysis caused by the phage. By using the highest dilution of phage necessary to yield semi-confluent lysis within the test spot on the host growth, the specific lytic reaction can be assessed.

Phage typing offers the advantages of very easily and rapidly obtained results with a minimum of personnel time required (results in 24 hr). Despite these advantages, the method does have drawbacks in that it relies on phenotypic markers that can change depending on the host bacterium's growth conditions. Also, typing schemes are not available for all isolates and maintenance of a large number of standardized stock cultures of host cells and bacteriophage is required.

2.6.3 Pulsed-Field Gel Electrophoresis

Historically, before the introduction of molecular methods, *Salmonella* isolates were characterized using phenotypic methods (serotyping, phage typing, biotyping) that distinguish isolates based on physiological and/or metabolic properties. Now with the introduction of molecular methods, isolates can be characterized beyond their

phenotypic properties to include a description of an isolate's chromosomal and/or extrachromosomal genetic material for increased discriminatory power. Molecular methods such as pulsed-field gel electrophoresis (PFGE) offer several advantages over traditional phenotypic typing such as: (i) increased reproducibility since genomic DNA is, in general, a stable component of a cell and its composition is independent of growth conditions; (ii) DNA is present in all living bacteria so all strains can be typed and; (iii) its better suited to automation and statistical analysis (Farber, 1996).

PFGE typing exploits the negative charge and molecular weight dependence of DNA molecules moving through an electrophoretic field to separate large DNA fragments (theoretically up to 12Mb) in an agarose gel. These fragments are subsequently dyed and analyzed to reveal a unique pattern that can be used to identify the *Salmonella* isolates (Wu and Della-Latta, 2006). PFGE differs from conventional electrophoresis methods in that (i) the electrophoresis chamber is modified to periodically change the orientation of the electric field applied across the agarose gel in different directions and (ii) it is capable of analyzing whole bacterial genomes (10 to 30 bands of large DNA fragments) (Wu and Della-Latta, 2006).

As a result of the establishment of PulseNet, a network of public health and food regulatory agency laboratories coordinated by the Centers for Disease Control and Prevention, the conditions, equipment and reagents used for PFGE are standardized and can thus be compared to worldwide data. Contour-clamped homogeneous electric field (CHEF) gel electrophoresis uses a hexagonal arrangement of 24 electrodes to create uniform electric fields oriented 120° to each other which keeps the DNA fragments

running along the gel in a straight line (Farber, 1996). As mentioned previously, the major advantage of using PFGE is that it is capable of separating very large fragments of DNA that could not previously be done. To create DNA fragments, restriction endonucleases with low cutting frequency are used to generate small numbers of large fragments. Unlike enzymes that recognize 4 bp sequences, low cutting frequency restriction enzymes recognize 6 to 8 bp groups (Farber, 1996). For *Salmonella*, the endonuclease *Xba*I is used.

PFGE is widely used for genotypic characterization of bacteria, and even though there is better reproducibility and the method can type all strains (and is very easy to perform), the laboratory techniques are tedious and time-consuming. Plus, the reagents, enzymes and equipment are very expensive.

2.7. *Salmonella* thermal resistance

In addition to increasing the palatability of certain foods, cooking also serves as a means of eliminating food-borne pathogenic bacteria like *Salmonella* from food. Over time the food industry has developed and optimized different cooking processes using experimental data from the destruction of *Salmonella* to design processes that eliminate this organism. Occasional survival of *Salmonella* may be the result of: changes in food composition; the solid or liquid nature of the food as well as its inherent properties, or stresses placed on the organisms that survive. In general *Salmonella* grow at temperatures of 5.5°C to 45°C (Doyle and Mazzotta, 2000), though many factors can extend this growth range. These include intrinsic and extrinsic factors such as bacterial strain, growth phase and prior exposure to sublethal heat stress (heat shock), as well as

the composition of the heating menstruum, pH and water activity (a_w) (Doyle and Mazzotta, 2000). The extent of heat resistance is described by calculation of D- and z-values. The D-value, or decimal reduction time, is the time (usually in min) at a specific temperature required to reduce the bacterial population by 1 logarithmic (log) unit, while the z-value expresses the temperature dependence of D-values as the increase in temperature required to decrease the D-value by 1 log unit (Geeraerd et al., 2000).

Salmonella thermal resistance differs among strains. Some are inherently more resistant than others, though the reasons for differences are not fully understood. A classic example is *S.* Senftenberg 775W. The heat resistance of this strain is several magnitudes higher than the average for most *Salmonella* strains. Ng et al. (1969) found the D-value at 57°C (D_{57}) in tryptic soy broth supplemented with 2% yeast extract of *S.* Typhimurium TM-1, *S.* Blockley 2004 and *S.* Senftenberg 775W to be 1.2, 5.8 and 31 min, respectively. They also found that in addition to the *S.* Blockley 2004 and *S.* Senftenberg 775W strains, 6 other *S.* Blockley and 19 additional *S.* Senftenberg strains did not demonstrate any difference in heat resistance in comparison to *S.* Typhimurium TM-1. This showed the unique resistance to heat possible at the strain level.

The growth phase of a *Salmonella* culture is another intrinsic factor that affects its thermal resistance. It has been documented that cultures of *Salmonella* in their stationary phase are more resistant to heat than cultures in their log phase of growth (Doyle and Maszzotta, 2000). Work done by Humphrey et al. (1990) found that log phase cells of *S.* Enteritidis PT4 had a D_{60} value of 5.2 s whereas stationary phase cells of the same strain a had a D_{60} value of 12.5 s. In addition, during a comparison of the

heat resistance of log and stationary phase cultures, Smith et al. (2001) found that an 8-strain *Salmonella* cocktail in log phase exhibited D-values ranging from 0.15 to 16.34 min at 63 and 55°C, respectively, and had a z-value of 3.90°C. The same 8-strain cocktail in stationary phase had D-values ranging from 0.20 to 18.6min at 63 and 55°C, respectively, with a z-value of 4.08°C.

Heat shock is a somewhat universal response observed in many cell types ranging from bacteria to mammalian cells (Xavier and Ingham, 1997). In bacteria, when a cell is subjected to a sublethal heat treatment, the cell responds by manufacturing proteins (heat shock proteins) to help repair damaged proteins and protect the cell from subsequent shifts to environments of increased temperature (Foster and Spector, 1995; Hurme and Rhen, 1998). This phenomenon has been demonstrated in several different bacteria including *Salmonella*. Mackey and Derrick (1987) observed increases in heat resistance of *S.* Thompson heated in tryptone soya broth and a variety of food systems (liquid whole egg, reconstituted dried milk and minced beef) at 48°C for 30 min before challenging the cultures at 54 and 60°C. Their results indicated that broth systems are not essential to elicit the heat shock response and suggested in that foods given a mild heat treatment prior to final cooking, thermally stressed pathogens with enhanced heat resistance may develop. Xavier and Ingham (1997) observed increases in D-values of 5.3 to 16.9 min at 52°C, 2.7 to 4.5 min at 54°C, 1.3 to 2.5 min at 56°C and 0.9 to 1.3 min at 58°C in heat shocked cultures of *S.* Enteritidis. The increase in thermal resistance was further enhanced when surviving cells were recovered using anaerobic incubation, though the increase was not statistically significant. Even though the heat shock response has clearly been shown to increase a culture's thermal resistance, the effects

are lost when the culture is not continuously heat shocked. Mackey and Derrick (1986) found that a heat-shocked culture of *S.* Typhimurium retained increased thermal resistance for 10 h.

The composition of the heating menstruum (culture media, food systems), pH/acidity and a_w are the main factors related to food that can influence *Salmonella* heat resistance. In terms of heating menstruum composition the type of medium (meat species, product formulation) and its physical form (ground, liquid or solid) influence the thermal resistance of *Salmonella.* In general, *Salmonella* thermal resistance is enhanced when challenged in solid food systems as opposed to ground or liquid foods and culture media modeled after food. When evaluating the thermal resistances of a 6 strain *Salmonella* cocktail in a peptone-agar (0.1%) liquid medium and ground chicken breast meat, Murphy et al. (2000) observed D-values in ground chicken breast meat ranging from 0.24 to 30.1 min at 70 and 55°C, respectively while D-values in the peptone-agar solution ranged from 0.15 to 22.8 min at 70 and 55°C, respectively. The z-values also showed a similar trend but were not statistically different. Orta-Ramirez et al. (2005) compared the thermal resistance of *Salmonella* in whole beef shoulder and ground beef and observed higher D-values in the whole muscle samples compared to the ground beef samples. Differences in thermal resistance due to heating in solid versus ground meat or liquid media could possibly be attributed to cells having greater opportunity to attach to surfaces within tissue structures of whole muscle resulting in different thermal resistances. Also homogeneous distribution of ingredients that have protective effects against heat might be diluted when ground product is used as opposed to segregated areas on whole muscle tissue that may concentrate ingredients with

protective effects (Orta-Ramirez et al., 2005). Humphrey et al. (1990) compared the effects of the suspending medium on the thermal resistance of *Salmonella* and found that *Salmonella* strains heat challenged in egg yolk had significantly higher thermal resistances than the same strains challenged in whole egg or egg albumen. Murphy et al. (2002) conducted a Type III analysis of covariance to determine if different meat product formulations (chicken patties, chicken tenders, franks, beef patties and blended beef and turkey patties) and heating temperatures would affect the thermal resistance of a 6 strain *Salmonella* inoculum. They found that heating temperatures and product formulations had statistically significant effects on *Salmonella* heat resistance possibly due to changes in the fat and moisture content and meat product type among the different products.

The pH and type of organic acid influence the thermal resistance of *Salmonella*. In general, as the pH of the heating menstruum moves away from the optimal pH for growth and/or survival, the thermal resistance of *Salmonella* decreases. (Doyle and Mazzotta, 2000). Blackburn et al. (1997) indicated that the optimal pH for *S.* Enteritidis was between 5.9 and 6.5. When temperature and NaCl concentration were maintained at 64.5°C and 3.5% (w/w), respectively but the pH was varied between 4.2 and 9.5 the highest D-value was observed at a pH of approximately 6.5. As the pH of the medium changed to more alkaline or acidic pH the D-value decreased. Similar observations were made by Kinner and Moats, (1981) who investigated the growth and/or survival of *Salmonella* and other bacteria commonly encountered on the surface of egg shells. As previously mentioned, the type of organic acid also influences the thermal resistance of *Salmonella*. The $D_{62.5}$ value of *S.* Enteritidis was lower when acetic acid was used as an

acidulant than when lactic, hydrochloric and citric acids were used (Blackburn et al., 1997). Investigation into the toxicity of organic acids has revealed that the ability of the undissociated acid to diffuse across cell membranes and dissociate in the cytoplasm, lowering the cytoplasmic pH, as well as the undissociated acid intercalating and disrupting the cell membrane lipid bilayer, plus the effects of anion accumulation contribute to the toxicity of these compounds (Roe et al., 2002). The ability of the acid to remain undissociated is the result of the acid's dissociation constant (pK_a), which is related to the pH of the medium (Rosso et al., 1997). The pK_a value represents the pH at which a weak acid liberates a proton. When pH is lowered, the further away we move from the pK_a value and so there exists more undissociated acid capable of entering microbial cells. This lowered pH therefore translates into greater inhibition of microorganisms (Ryu *et al.*, 1999).

The protective effects of fat on *Salmonella* from heat stress are well known. As fat levels increase, a concomitant rise in the D-value is observed (Juneja et al., 2001). The protective effects are thought to stem from lower heat conductivity due to lower water activity (a_w) (Murphy et al., 2003; Juneja and Eblen, 2000). Juneja et al. (2001) observed increases in D-values for an 8 strain *Salmonella* cocktail from 7.38 to 9.04 min at 58°C when fat was increased from 2 to 12% in ground chicken breast. Increases in D-value were also observed at 60, 62.5 and 65°C but were less dramatic. The thermal resistance of a cocktail of *S.* Typhimurium DT104 human and beef isolates in ground beef with varying fat levels of 7, 12, 18 and 24% also increased with increasing fat (Juneja and Eblen, 2000). Other researchers have observed an increased thermal resistance of *Salmonella* in other low a_w, high fat foods. Shachar and Yaron (2006)

observed only a 1.4 log reduction after 5 min for a three strain *Salmonella* cocktail at an initial cell density of 8 log CFU/g in peanut butter compared to a 7 log reduction in less than 5 min in saline.

3. Occurrence and characterization of *Salmonella* from chicken nuggets, strips and pelleted broiler feed.

3.1. Abstract

Raw, frozen chicken nuggets/strips have been identified as a significant risk factor in contracting food-borne salmonellosis. Cases of salmonellosis as a result of consuming partly cooked chicken nuggets may be due, in part, to *Salmonella* strains originating in broiler feed. This study was undertaken to determine the occurrence and characterize the strains of *Salmonella* contaminating chicken nuggets/strips and pelleted feeds in an attempt to demonstrate whether the same *Salmonella* strains present in broiler feed could be isolated from raw, frozen chicken nuggets/strips available for human consumption.

Salmonellae were recovered using Health Canada MFHPB-20 method for the isolation and identification of *Salmonella* from foods. Strains were characterized by serotyping, phage typing, antimicrobial resistance typing (R-type) and by pulsed-field gel electrophoresis (PFGE). Salmonellae were isolated from 25g samples in 25% (n=92) of nugget/strip samples, 95% (n=20) of chicken nugget meat samples and from 9% (n=111) of pelleted feed samples. *S.* Heidelberg, *S.* Enteritidis and *S.* Orion were the most commonly isolated serovars from chicken nuggets/strips, nugget/strip meat and pelleted broiler feeds, respectively. *S.* Enteritidis phage type (PT) 13a with PFGE pattern SENXAI.0006 and R-type Sensitive as well as *S.* Enteritidis PT 13a with PFGE pattern SENXAI.0068 and R-type Sensitive were isolated from pelleted feed and chicken nugget/strip meat in two separate instances.

Data showed that *Salmonella* strains isolated from broiler feed were indistinguishable from strains isolated from packaged raw, frozen chicken nuggets/strips. However results did not rule out the possibility that breeding stock or contamination during processing may have contributed to chicken meat contamination by *Salmonella*.

3.2. Introduction

Raw, frozen chicken nuggets and strips purchased at retail stores and prepared for consumption at home have been identified as a significant risk factor in contracting food-borne salmonellosis. A 1998 investigation into an outbreak of *Salmonella* Typhimurium phage type (PT) 12 infection in Australia, as well as a 2003 investigation into a family cluster of *S.* Heidelberg PT26 infections in British Columbia (Canada) and case-control studies conducted in 2002 in Québec (Canada) and in 2003 across Canada, have highlighted this problem (Kenny et al., 1999; MacDougall et al. 2004; Currie et al. 2005). With the exception of a few pre-cooked, microwave-ready products, most frozen chicken nuggets and strips sold in stores are not fully cooked. During processing, the product undergoes a partial frying (par-frying) step to ensure its shape is maintained and to provide the batter and breading coating a golden brown color before it is frozen and packaged (Fig. 3.1). Since the resulting nugget/strip neither looks nor smells raw, increased risk of contracting salmonellosis from these products has been attributed to uncertainty as to whether they are cooked (Kenny et al., 1999). A case-control investigation across Canada in 2003 found that consumption of home-prepared chicken nuggets and strips was a leading risk factor for *S.* Heidelberg infection, with 34% of all

laboratory confirmed cases of *S.* Heidelberg infection attributable to these products (Currie et al., 2005). In addition, one-third of the respondents participating in another 2003 case-controlled study in British Columbia believed the frozen nuggets were pre-cooked, requiring reheating only, with 27.3% indicating they either always or sometimes used a microwave oven to heat these products despite voluntary labeling advising against this method of cooking (MacDougall et al., 2004).

Pelleted and mash poultry feeds have long been recognized as vectors for *Salmonella* contamination of commercial poultry production systems. In particular, ingredients of animal origin have been shown to have the highest frequencies of contamination, though ingredients of vegetable origin have also been reported to harbor the organism (Erwin, 1955; Cox et al., 1983; Veldman et al., 1995; Blank et al., 1996; Ha et al., 1998; Zdragas et al., 2001). Recent work conducted in the southern United States (US) found that 8.8% of mash feed samples and 4.2% of pelleted feed samples were contaminated with *Salmonella*, suggesting that the pelleting process reduces *Salmonella* isolations from poultry feed but does not eliminate the organism (Jones and Richardson, 2004). In addition, it was also mentioned that contrary to other suggested temperatures (80°C), a temperature of 85°C is required to produce *Salmonella*-free feed. Current European Union (EU) legislation requires that feed manufacturers adopt Hazard Analysis and Critical Control Point (HACCP) procedures to control *Salmonella* and other pathogens in feeds (Jones and Richardson, 2004). Currently, Canada does not have a standard for feed pelleting time and temperature treatment. Even though poultry feeds have been suggested as significant vectors for *Salmonella* contamination of broiler flocks, the extent to which food-borne outbreaks have been linked back to the

consumption of poultry products manufactured from flocks fed contaminated feed is unknown (Blank et al., 1996). Nevertheless, it has been reported by primary breeders that close to 80% of *Salmonella* serovars isolated from feeds and feed ingredients were the same serotypes isolated weeks later in breeding flocks and their offspring (Jones and Richardson, 2004).

This work was undertaken to determine whether the same strains of *Salmonella* could be isolated from pelleted broiler feed and raw, frozen chicken nuggets and strips. The objectives of this work were two-fold: 1) to determine the frequency of *Salmonella* occurrence in pelleted feeds and chicken nuggets/strips and, 2) characterize the strains isolated by serotyping, phage typing, antimicrobial resistance typing (R-Type) and pulsed-field gel electrophoresis (PFGE) analysis to establish whether the same strains could be found in both feed and in nuggets/strips.

3.3. Materials and Methods

3.3.1. Sample Collection and Handling

A total of 92 raw, frozen, chicken nugget and strip samples representing 58 different lots prepared at 9 different establishments were collected. Chicken nuggets and strips used in this study were identical products, differing only in their shape. Of these, 57 (wholesale samples) were couriered to the university laboratory by the two major manufacturers of chicken nuggets and strips in Canada (from three different processing plants in Ontario). The remaining 35 were obtained from 4 local supermarkets (Winnipeg, MB, Canada). In addition to chicken nuggets and strips, 20 samples representing one lot of chicken nugget/strip meat (destined for nugget/strip manufacture)

was also provided by one of the major manufacturers. All samples were transported frozen and kept at -28°C upon arrival until analysis. Samples were typically analyzed upon receipt or within two weeks after arrival.

A total of 111 samples of pelleted broiler feed from different lots were couriered to the laboratory from the major manufacturer of pelleted poultry feed in Canada (southern Ontario). Of the samples provided, 13 contained ingredients of animal origin, while 98 were made from ingredients of vegetable origin only. Samples were kept under refrigeration (5°C) or briefly at room temperature (22°C) until analysis. Samples were analyzed within two weeks after arrival.

Although samples were collected from the major manufacturers of both pelleted feed and raw, frozen chicken nuggets/strips in Canada, it was not possible to determine whether the lots of feed analyzed were fed to broiler hens used in the production of the lots of chicken nuggets/strips sampled for *Salmonella* in this study.

3.3.2. Isolation from Pelleted Broiler Feed

Sampling was done in duplicate and followed the Health Canada MFHPB-20 method for the isolation and identification of *Salmonella* from foods (D'Aoust and Purvis, 1998). Isolation from pelleted feed was done by mixing the contents (about 500g) of the sample containers by hand for 30 s before aseptically weighing 25g into 225ml buffered peptone water (BPW) (Difco, Becton-Dickinson, Sparks, MD). Samples were soaked at 22°C for 1 h, treated in a stomacher (Bagmixer 400, Interscience, Bois Arpent, France) for 30 s and incubated for 24 h at 35°C. Following incubation, 1ml of

sample was pipetted into 9ml of selenite cystine (SC) (Fluka, BioChemika, Germany) and tetrathionate brilliant green (TBG) (Fluka, BioChemika, Switzerland) broth tubes. Tubes were vortex-mixed and incubated for 24 h at 35 and 43°C, respectively. Following incubation, a loopful of SC and TBG sample was streaked onto duplicate plates of brilliant green sulfa (BGS) (Difco) and bismuth sulfite (BS) (Difco) agar. The plates were incubated at 35°C for 24 and 48 h, respectively. Plates with suspect *Salmonella* colonies were counted and a number (the square-root) of those colonies was streaked on MacConkey (MacC) (Difco) agar plates and incubated for 24 h at 35°C. Single colonies from MacC plates were then stab/surface-inoculated into triple sugar iron (TSI) (Oxoid, Basingstoke, HAM), lysine iron (LIA) (Oxoid) and urea (UA) (BBL, Becton-Dickinson, Sparks, MD) agar slants. Slants were loosely-capped and incubated for 24 h at 35°C. Presumptive positive slants were confirmed as *Salmonella* using a *Salmonella* Rapid Latex Agglutination Test Kit (Oxoid) according to the manufacturer's instructions. Samples confirmed as *Salmonella* were stab-inoculated into nutrient agar (NA) (Oxoid) tubes and delivered to The National Microbiology Laboratory (Winnipeg, MB) for characterization by serotyping, phage typing (PT), antimicrobial resistance profiling (R-Type) and pulsed-field gel electrophoresis (PFGE). Serotypes were described by somatic (O) and flagellar (H) antigens according to the antigenic formulae (Table 3.1) outlined by Popoff (2001). Phage typing was done following the standard methods described by Anderson and Williams (1956). R-typing was performed according to CLSI (formerly NCCLS) guidelines (2003). Table 3.2 contains ranges and sensitivity/resistance breakpoint concentrations of the antimicrobials used for antimicrobial resistance profiling. PFGE analyses were performed by PulseNet Canada according to

internationally standardized procedures (Centers for Disease Control and Prevention, 2004).

3.3.3. Isolation from Nuggets/Strips, Batter Coating and Meat

Sampling of raw, frozen chicken nuggets and strips was done in triplicate to account for variance in contamination between individual chicken nugget and strip pieces. Samples in containers (ranging from 0.8-3.0kg containing between 20-50 individual nuggets/strips) were mixed for 30 s before aseptically weighing 25g of sample into 225ml BPW. Samples were thawed at 22°C for 1 h, treated for 30 s in a stomacher and incubated for 24 h at 35°C. Subsequent isolation and characterization steps were carried out as noted in the previous section.

Unbreaded, frozen chicken nugget meat received directly from the manufacturer was sampled as above by aseptically weighing 25g of partly-thawed meat into 225ml of BPW. Samples were held at 22°C to thaw for 1 h, pummeled for 30 s in the stomacher and incubated for 24 h at 35°C. Subsequent *Salmonella* isolation and characterization steps were carried out as noted previously.

Chicken nugget coating (batter and breading) samples were analyzed separately from a package of *Salmonella* positive chicken nuggets after partial thawing. The coating was removed, the surface previously exposed to the meat was treated with 95% ethanol and the excess alcohol burned away. Twenty, 25g samples of coating were aseptically weighed separately into 225ml of BPW, treated in the stomacher for 30 s and

incubated for 24 h at 35°C with subsequent isolation and characterization steps carried out as noted previously.

3.4. Results

3.4.1. Occurrence of *Salmonella* in Pelleted Feed

Salmonella were found in 9% of the 111 samples/lots tested (Table 3.3). Feed manufactured from ingredients of both animal and vegetable origin had a higher percentage of *Salmonella*–positive samples (15%) compared to feed manufactured from ingredients of only vegetable origin (8%). *S.* Enteritidis had somatic and flagellar antigen profiles typical for this organism (Table 3.1), while *S.* Orion had somatic antigens O15 and O34 indicating the culture had become lysogenized. *S.* Orion was the most common serovar isolated and was found in 8 of 10 positive samples (Table 3.4). All of the *S.* Orion isolates had PFGE pattern OrionXAI.0002 and were susceptible to all the antimicrobials tested. Two strains of *S.* Enteritidis were isolated (having different PFGE patterns), but were both PT13a and were sensitive to all the antimicrobials tested.

3.4.2. Occurrence of *Salmonella* in Chicken Nuggets/Strips

The results from analysis of 92 chicken nugget/strip samples are shown in Table 3.5. Sampling of chicken nuggets and strips from local retail stores revealed that 31% were contaminated with *Salmonella* while 25% of wholesale samples were found to be positive for *Salmonella*. Overall, 27% of samples were positive for *Salmonella*. Table 3.6 indicates the different strains of *Salmonella* isolated. Both retail and wholesale samples had similar diversity among the salmonellae isolated with 12 different strains isolated from retail sources and 13 different strains isolated from wholesale sources. All

12 of the retail isolates except for *S.* Heidelberg PT 29 were isolated from different lots. Salmonellae were absent from retail product from 3 establishments while one plant was a source of each of the different isolates except for *S.* Hadar and Infantis. The 13 isolates from wholesale samples were all found in different lots except for *S. enterica* subsp. *enterica* 6,8:-e,n,x. Another establishment served as a source of all serovars isolated except for *S. enterica* subsp. *enterica* 6,8:-e,n,x, *S.* Hadar and *S.* Mbandaka. All nugget/strip isolates had typical somatic and flagellar antigen profiles except serovars Heidelberg, Infantis and Kentucky. Two *S.* Heidelberg isolates from wholesale samples had an O1 antigen indicating the strains had become lysogenized. All strains of *S.* Kentucky had an O20 antigen while the single *S.* Infantis strain had an O14 antigen indicative of lysogenization. *S.* Heidelberg was the most frequently isolated serovar in both sets of samples and showed the most diversity among the strains isolated with 6 different strains isolated from both retail and wholesale samples. Five different PT's (19, 19b, 26, 29 and 54) and 4 different R-Types and PFGE patterns were identified in isolates from retail samples, while 4 PT's (4, 19, 29 and atypical), two R-Types (in common with retail isolates) and three PFGE patterns (two different from retail isolates) were found in isolates from wholesale samples. *S.* Kentucky was the second most frequently isolated serovar with two strains isolated from retail samples and 4 strains isolated from wholesale samples. These isolates from retail samples had the same R-Type (sensitive) but differed in their PFGE patterns, whereas isolates from wholesale samples had 4 different R-Types and PFGE patterns. *S.* Hadar was the only other serovar isolated from both retail and wholesale samples, though strains differed in PT, R-Type and PFGE patterns. *S.* Enteritidis was only isolated from retail samples, with the same strain (PT 13; R-Type sensitive; PFGE pattern SENXAI.0038) appearing in three

different samples. Single isolates of *S.* Indiana and *S.* Infantis were found in retail samples. Strains isolated only from wholesale samples were *S. enterica ssp. enterica var.* 6,8:-e,n,x (R-Type Te; PFGE SHAXAI.0037) and a single strain of *S.* Mbandaka.

3.4.3. Occurrence in Chicken Nugget Meat and Nugget Coating

Chicken nugget meat and chicken nugget coating were sampled to determine whether these components were possible sources of *Salmonella. Salmonella* were present in 95% of chicken nugget meat samples analyzed but were absent from the coating (Table 3.5). Three different *Salmonella* strains were isolated from chicken nugget meat: two were *S.* Enteritidis and one was *S.* Heidelberg (Table 3.6). All strains showed typical somatic and flagellar antigen profiles (Table 3.1). *S.* Enteritidis was the most common, and 19 strains were isolated from 18 of the 19 samples tested, whereas *S.* Heidelberg was isolated from only one of the samples. The *S.* Enteritidis strains were all PT13a with 11 of the strains having PFGE pattern SENXAI.0006 and the remaining 8 having pattern SENXAI.0068. Although PFGE can have limited discriminatory power for *S.* Enteritidis, SENXAI.0006 and SENXAI.0068 are relatively rare PFGE patterns for this serovar in Canada. The *S.* Heidelberg strain was found to be PT40 with PFGE pattern SHEXAI.0141. Strains isolated were found to be susceptible to all the antimicrobials tested.

3.5. Discussion

3.5.1. Occurrence of *Salmonella*

It should be noted that because feed analyzed was from one commercial mill and because of the restricted number of different lots of chicken nuggets/strips sampled, the

results presented may not truly represent the overall industry situation. Nonetheless, the samples were obtained from the largest commercial concerns active in the industry in Canada, and they demonstrated that *Salmonella* were present in these products. The observation here that *Salmonella* were present in 9% of pelleted feed samples is higher than the 0 to 6.6% incidence found in other reports (Cox et al., 1983; Blank et al., 1996; Zdragas et al., 2001). Hacking et al. (1978) investigated the presence of *Salmonella* from a single feed mill in Ontario (Canada) and found *Salmonella* in 3% of pelleted feed samples. Differences in results may be attributed to different sensitivities in isolation protocols used. Previous difficulty in isolating *Salmonella* from feed has been thought to stem from the non-uniform distribution of the organism among samples, as well as the effect of stress on the organisms from processing operations in feed mills which may injure the few cells (10-100CFU/g) that are present (Veldman et al., 1995; Zdragas et al., 2001; Jones and Richardson, 2004). Jones and Richardson (2004) found that feed mill management practices were related to *Salmonella* contamination and so differences in contamination seen here may reflect this influence. In addition to differences among different feed mills, differences in regulatory guidelines in different countries may also affect the frequency of reported *Salmonella* contamination. In Canada there are no strict guidelines for minimum time/temperature treatments when pelleting feed, and lower processing temperatures (57°C and 80°C are not uncommon) may be used when thermosensitive enzyme supplements are added (Zdragas et al., 2001). When feeds containing ingredients of animal origin were compared with those containing only vegetable ingredients, a higher incidence of *Salmonella* was found in the former. Though the number of feed samples containing ingredients of animal origin was small, the present results are consistent with others that show feeds with ingredients of animal

origin tend to have higher rates of *Salmonella* contamination (Erwin, 1955; Cox et al., 1983; Veldman et al., 1995; Blank et al., 1996; Ha et al., 1998; Zdragas et al., 2001).

Salmonellae were isolated from 27% of chicken nuggets/strips in the present work, and to our knowledge this is the first study to provide data on the occurrence of *Salmonella* in these products. During manufacture of nuggets whole breast muscle and chunks of white/dark meat, ground breast meat with and without MDM plus skin can be used, depending on customer specifications (Barbut, 2002). Whole pieces or chunks of meat must be size-reduced to improve protein extraction and facilitate binding of meat pieces to prevent development of loose texture (Owens, 2001). Thus, like ground beef with internalized *E. coli* O157:H7, chicken nuggets/strips are non-intact meats with the potential to have contaminating *Salmonella* distributed throughout the meat matrix (MacDougall et al., 2004). Since meat used in the manufacture of these products can come from a number of sources, the likelihood of obtaining meat contaminated with *Salmonella* is increased. Thus, comparisons between the present results and those found with ground or MDM chicken meat seem fitting. When random samples from large, small and very small Federally inspected processing facilities in the US were taken during 1998 to 2000, it was found that the overall prevalence of *Salmonella* in ground chicken meat was 14.4%. In comparison, *Salmonella* contamination on broiler carcasses was found to be 10.2% (Rose et al., 2002). The latter result is slightly lower than the results in the present study which may be a result of the implementation of the pathogen reduction HACCP rule since a general decline was observed in *Salmonella* prevalence over the course of the sampling period. Other work in São Paulo, Brazil found that of 60 MDM samples, 15 were *Salmonella* positive, whereas only 6 of 45 samples of broiler

carcass rinse water contained the organism (Carvalho and Cortez, 2005). The present results showed a similar prevalence of *Salmonella*.

3.5.2. *Salmonella* Isolate Characterization

In 2002 and 2003, laboratory surveillance data for enteric pathogens in Canada showed that *S.* Senftenberg was the most prevalent serovar isolated from animal feed and feed ingredients followed by *S.* Montevideo (Demczuk et al., 2005). In the present study three different strains of *Salmonella* from two different serovars (Enteritidis and Orion) with single PT and R-Types and three different PFGE patterns were found in pelleted chicken feed, but *S.* Senftenberg and *S.* Montevideo were absent. Previously, *S.* Orion and *S.* Enteritidis were isolated from raw materials used in feed manufacture as well as mash feeds, but to our knowledge these serovars have not been isolated from pelleted feeds (Hacking et al., 1978; Cox et al., 1983; Veldman et al., 1995; Blank et al., 1996; Zdragas et al., 2001). Although thermal treatment can drastically reduce *Salmonella* in feeds, the pelleting processes actually used in different pelleting mills can be highly variable in terms of time, temperature and feed moisture (Cox et al., 1983; Veldman et al., 1995; Blank et al., 1996).

Both chicken nugget meat and chicken nugget batter and breading were sampled for *Salmonella* to provide an indication of the source of chicken nugget/strip contamination. While three strains of *Salmonella* were isolated from chicken nugget meat, there were no *Salmonella* isolated from chicken nugget coating, suggesting that the source of *Salmonella* in chicken nuggets and strips was the meat. This result is not surprising since most of the chicken nuggets and strips purchased in stores are often par-

fried in oil for 20 to 30 s at temperatures of 179-200°C before freezing and packing (Owens, 2001; Barbut, 2002). Though it is established that chicken is a significant reservoir of *Salmonella* in food (Currie et al. 2005; Demczuk et al., 2005; MacDougall et al., 2004) there are other potential sources of contamination including spices and seasonings which are added to the meat after particle size reduction (Vij et al., 2006). The three contaminating salmonellae strains found in nugget meat were two strains of *S.* Enteritidis and a strain of *S.* Heidelberg. This result is somewhat surprising since preliminary surveillance results from three Canadian provinces in 2005 indicate that *S.* Heidelberg can make up anywhere from 24-46% of *Salmonella* isolates from retail chicken samples, whereas *S.* Enteritidis has been found in much lower percentages of 4-5% (Government of Canada, 2007). While it is recognized that the meat used in the present tests was from a single lot, it is important to note that the two strains of *S.* Enteritidis isolated from the nugget meat were indistinguishable, both phenotypically and genotypically from those isolated from pelleted feed. This finding is significant since the same strains of *S.* Enteritidis were found in both feed and chicken nuggets/strips, however, since it could not be determined if the feed contaminated with these two srains of *S.* Enteritidis was fed to broilers used to manufacture the sampled nuggets that were also contaminated with these same strains, there is no direct evidence to suggest their horizontal transfer.

S. Heidelberg has been identified as a significant source of food-borne illness associated with consuming under-cooked chicken nuggets and strips. The present findings support conclusions regarding the involvement of this serovar since just over half of the *Salmonella* positive samples contained *S.* Heidelberg. Even though two of

these *S.* Heidelberg isolates were the same PT (26) as isolates from a family cluster outbreak in British Columbia in 2003 from patients and uneaten nuggets, isolates in the present study were different because they all were able to produce H_2S (MacDougall et al., 2004). The second most commonly isolated serovar from the present study was *S.* Kentucky. This is in agreement with national findings indicating that after *S.* Heidelberg, *S.* Kentucky was the most frequently isolated serovar from chicken from 1999-2003 (Demczuk et al. 2005).

Serotyping isolates from the present study yielded lysogenic strains of *S.* Orion, *S.* Heidelberg, *S.* Infantis and *S.* Kentucky. Lysogenized host cells have often been shown to demonstrate an increased resistance to environmental stresses (Guttman and Kutter, 2005) and the presence of bacteriophages in foods has been demonstrated (Greer, 2005), so the presence of these strains in chicken nuggets/strips is not surprising. It is of interest that in the present study 60% the salmonellae isolated from nuggets/strips were resistant to at least one antimicrobial, and that 40% were resistant to multiple antimicrobials. Though previous investigations into food-borne illness associated with consuming chicken nuggets/strips (MacDougall et al. 2004; Currie et al., 2005) did not mention salmonellae with antimicrobial resistance, multi-drug resistant non-typhoidal salmonellae have been isolated from ground chicken meat sampled in the greater Washington D.C. area (White et al., 2001). In the present study, the isolation of ceftriaxone-resistant *S.* Heidelberg is notable. Antibiotics are not regularly used to treat *Salmonella* infections, though for people with an invasive infection they may be lifesaving. Ceftriaxone is a broad-spectrum cephalosporin commonly used to treat

Salmonella infections in children (Fey et al., 2000), and so the finding of ceftriaxone-resistant *S.* Heidelberg in chicken nuggets/strips is of great concern.

Despite its general acceptance that processing at 80°C for one min is satisfactory for killing salmonellae during poultry feed pelleting (Veldman, 1995; Zdragas et al., 2001) it is clear that *Salmonella* are still capable of contaminating pelleted broiler feed, perhaps by aerosolized dust in the pelleting mill. Recent work has found that pelleting at 85°C is more reliable for eliminating the organism (Jones and Richardson, 2004). It is possible that the *Salmonella* serovars isolated from pelleted poultry feed had enhanced thermal resistance, and this is being examined. Additional comparisons between strains isolated from human clinical sources and those isolated from poultry feed and chicken nuggets/strips should provide incentive to re-examine guidelines regarding poultry feed processing/pelleting and generate data on the extent to which food-borne illness can be traced back to contaminated animal feeds. The finding in the present study of two *S.* Enteritidis strains in pelleted feed, indistinguishable from two *S.* Enteritidis strains in chicken nugget meat provides some evidence that *Salmonella* contaminating pelleted feed may be passed along the production chain to food for human consumption. Since it is unknown whether *Salmonella* was present in the lots of poultry feed used for rearing animals from which sampled nugget/strip meat was obtained, evidence provided for this source of meat contamination is only circumstantial.

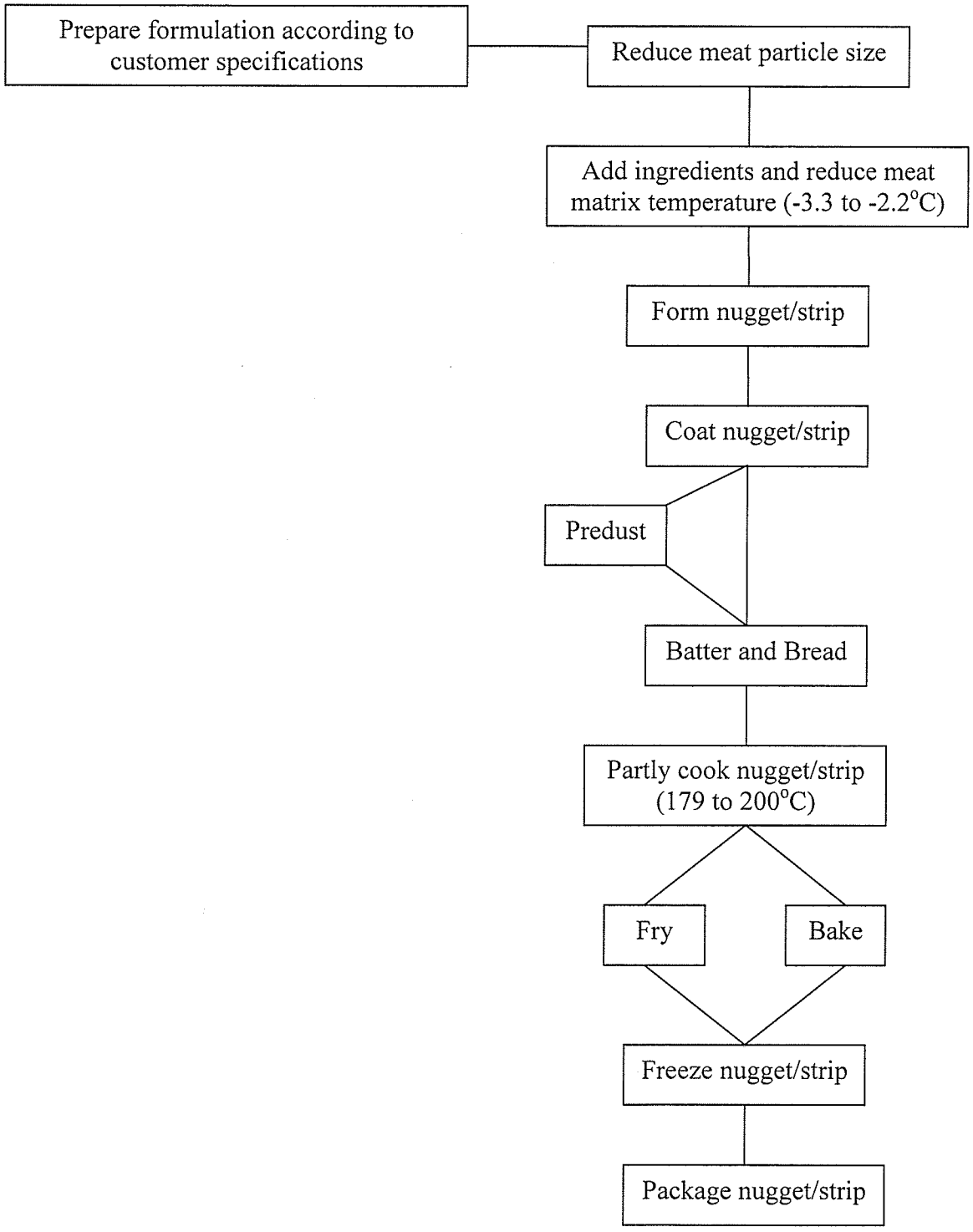


Figure 3.1 Process flow diagram of chicken nugget and strip manufacture. (Adapted from Owens, 2001).

Table 3.1: Serotype descriptions (antigenic formulae[a]) of the *Salmonella* serovars isolated from pelleted feed or raw, frozen chicken nuggets/strips and nugget meat.

Serovar	Somatic (O) Antigen	Flagellar (H) Antigen	
		Phase 1	Phase 2
Enteritidis	1,9,12	[f],g,m,[p]	[1,7]
Hadar	6,8	z_{10}	e,n,x
Heidelberg	1,4,[5],12	r	1,2
Indiana	1,4,12	z	1,7
Infantis	6,7,14	r	1,5
Kentucky	8,20	i	z_6
Mbandaka	6,7,14	z_{10}	e,n,z_{15}
Orion	3,10,[15],[15,34]	y	1,5

[a]Serotypes are classified by the Kauffman-White scheme outlined by Popoff (2001)
Underlined antigenic factors indicate bacteriophage conversion. These antigens are present only when the culture is lysogenized
[ ] indicates O or H antigens that may be present independent of bacteriophage conversion. H factors in [ ] denote antigens that are occasionally found in wild strains

Table 3.2: Antimicrobials and susceptible ranges used for resistance (R-) typing the *Salmonella* serovars isolated[a].

Antimicrobial	Susceptible Range (μg/ml)	Intermediate Range (μg/ml)	Resistant Range (μg/ml)
Amikacin-Ai	≤16	32	≥64
Amoxicillin/Clavulanic acid-Ac	≤8/4	16/8	≥32/16
Ampicillin-Am	≤8	16	≥32
Cefoxitin-Ce	≤8	16	≥32
Ceftiofur-Cf	≤2	4	≥8
Ceftriaxone-Cx	≤8	16-32	≥64
Chloramphenicol-Ch	≤8	16	≥32
Ciprofloxacin-Ci	≤1	2	≥4
Gentamicin-Ge	≤4	8	≥16
Kanamycin-Ka	≤16	32	≥64
Nalidixic Acid-Na	≤16	-	≥32
Streptomycin-St	≤32	-	≥64
Sulfizoxazole-Su	≤256	-	≥512
Tetracycline-Te	≤4	8	≥16
Trimethoprim/Sulfamethoxazole-Tm	≤2/38	-	≥4/76

[a]Antimicrobial susceptibility testing was performed according to CLSI (formerly NCCLS) guidelines (2003).

Table 3.3: Occurrence of *Salmonella* in pelleted broiler feed made from ingredients of animal plus vegetable origin (mixed) as well as from vegetable origin only.

Ingredient Origin	No. samples	No. lots	No. positive samples/lots (%)
Vegetable	98	98	8 (8)
Mixed	13	13	2 (15)
Total	**111**	**111**	**10 (9)**

Table 3.4: Serotype, PT, PFGE pattern and R-Type of the salmonellae isolated from pelleted broiler feed of animal plus vegetable origin (mixed) and from vegetable ingredients only.

Feed Type	Serovar	PT [a]	PFGE pattern[b]	R-Type[c]	No. contaminated samples (n=10)
Mixed	Enteritidis	13a	SENXAI.0068	Sensitive	1
	Orion	-	OrionXAI.0002	Sensitive	1
Vegetable	Enteritidis	13a	SENXAI.0006	Sensitive	1
	Orion	-	OrionXAI.0002	Sensitive	7

[a] There is no phage typing scheme available for *S.* Orion
[b] Pulsetypes (PFGE) were determined using *XbaI* nuclease
[c] Antimicrobial resistance (R-Type). Antimicrobials tested are listed in Table 3.2

Table 3.5: Occurrence of *Salmonella* in chicken nuggets/strips sampled from retail stores and manufacturing plants (wholesale), plus chicken nugget meat used in nugget/strip manufacture and chicken nugget coating.

Sample Type	No. samples	No. lots	No. positive samples (%)	No. positive lots (%)
Retail	35	20	11 (31)	9 (45)
Wholesale	57	38	14 (25)	13 (34)
Total	**92**	**58**	**25 (27)**	**22 (38)**
Nugget Meat	20	1	19 (95)	1 (100)
Nugget Coating	20	1	0 (0)	0 (0)

Table 3.6: Serotype, PT, PFGE patterns and R-Type of salmonellae isolated from chicken nuggets and strips sampled from retail and manufacturing plants (wholesale).

Sample Type	Serovar/Serotype	PT	PFGE pattern[a]	R-Type[b]	No. contaminated samples[c] (n=44)
Retail	Heidelberg	19	SHEXAI.0121	GeStSu	1
		19b	SHEXAI.0068	Am	1
		26	SHEXAI.0141	Sensitive	1
		26	SHEXAI.0001	Sensitive	1
		29	SHEXAI.0001	AcAmCeCf(Cx)	2
		54	SHEXAI.0001	Sensitive	1
	Kentucky	-[de]	KenXAI.0002	Sensitive	1
		-[de]	KenXAI.0004	Sensitive	1
	Hadar	23	SHAXAI.0003	StTe	1
	Indiana	-[d]	IndiXAI.0003	St	1
	Infantis	5	SINXAI.0028	AcAmCeCf(Cx)	1
	Enteritidis	13	SENXAI.0038	Sensitive	3
Wholesale	Heidelberg	4	SHEXAI.0007	AcAmCeCf(Cx)	2
		4	SHEXAI.0129	AcAmCeCf(Cx)	1
		19	SHEXAI.0001	Sensitive	1
		29[e]	SHEXAI.0001	Sensitive	3
		29	SHEXAI.0007	Sensitive	2
		atypical[e]	SHEXAI.0001	AcAmCeCf(Cx)	1

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Table 3.6 cont'd…

	Kentucky	-[de]	KenXAI.0019	AcAmCeCf(Cx)	1
		-[de]	KenXAI.0018	AcAmCeCf	1
		-[de]	KenXAI.0013	StTe	1
		-[de]	KenXAI.0021	Te	1
	Hadar	atypical	SHAXAI.0037	Te	1
	6,8:-e,n,x	-[d]	SHAXAI.0037	Te	2
	Mbandaka	-[d]	MbaXAI.0009	Sensitive	1
Nugget Meat	Enteritidis	13a	SENXAI.0006	Sensitive	11
	Enteritidis	13a	SENXAI.0068	Sensitive	8
	Heidelberg	40	SHEXAI.0141	Sensitive	1

[a]Pulsetypes were determined using *XbaI* nuclease

[b]Antimicrobial resistance (R-Type). Antimicrobials tested are listed in Table 3.2. Antimicrobials in parentheses showed intermediate resistance

[c]Three retail samples, 4 wholesale samples and a single sample of nugget meat contained two or more different *Salmonella* strains differing by serovar, PT or PFGE pattern

[d]No phage typing (PT) scheme is available to subtype these organisms

[e]Lysogenic *Salmonella* strains

4. Thermal resistance of *Salmonella* serovars isolated from raw, frozen chicken nuggets/strips, nugget meat and pelleted broiler feed.

4.1. Abstract

Raw, frozen chicken nuggets/strips available at retail and prepared at home before consumption have been identified as a significant risk factor in contracting food-borne salmonellosis. Cases of salmonellosis from consumption of these products may be due, in part, to *Salmonella* strains originating in broiler feed. In this study the thermal resistances of *Salmonella* strains isolated from chicken nuggets and strips, chicken nugget/strip meat and broiler feed were determined to assess whether they exhibited enhanced thermal resistance.

Thermal resistances (D- and z- values) of 7 cocktails (25 isolates, 4 serovars) were determined in commercially prepared irradiation-treated chicken nugget/strip meat blend, and heated in a constant temperature waterbath. The thermal resistances found were lower than those reported for similar strains in the literature. D-values ranged from 6.93 to 0.12min at 55 and 62°C respectively, with z-values from 4.10 to 5.17°C. Two strains of *S.* Enteritidis separately isolated from pelleted feed and chicken nugget meat blend, with indistinguishable geno- and phenotypes, had lower (and probably identical) thermal resistances than the other isolates.

Results from this study indicate that the strains of *Salmonella* isolated from raw, frozen chicken nuggets/strips and pelleted broiler feed did not exhibit unusually high

thermal resistance, and that normal heating (71°C) prior to consumption should eliminate these organisms from chicken nuggets/strips.

4.2. Introduction

A 1998 investigation into an outbreak of *Salmonella* Typhimurium phage type (PT) 12 infection in Australia (Kenny et al.,. 1999), as well as a 2003 investigation into a family cluster of *S.* Heidelberg PT26 infections in British Columbia (MacDougall et al. 2004) plus case-control studies conducted in 2002 in Québec (Canada) as well as a nation-wide (Canadian) study in 2003 (Currie et al.,. 2005), demonstrated that raw, frozen chicken nuggets/strips are a significant risk factor in contracting food-borne salmonellosis. During processing, these products undergo partial frying (par-frying) to ensure their shape is maintained and to give the batter and breaded coating a golden brown colour before they are frozen and packaged (Barbut, 2002). This external thermal treatment may mislead consumers to believe these products are fully-cooked, and that they require only re-heating before consumption. Of the respondents participating in the Canada-wide case study, 40% believed the nuggets/strips were fully pre-cooked and 11% reported heating them in a microwave oven (Currie et al.,. 2005), which may not evenly deliver lethal temperature.

It is uncertain whether inadequate cooking (microwave oven) or the presence of *Salmonella* serovars with elevated thermal resistance is the more likely cause of salmonellosis associated with these products. Thermally resistant *Salmonella* strains may be selected by the heating step used in poultry feed pelleting and subsequently contaminate the animals, meat and enter the food distribution system. Use of feed

contaminated with zoonotic pathogens, including *Salmonella* is not uncommon in the animal industry, but is a questionable practice (Rogers and Haines, 2005). Recent work conducted in the southern United States (US) found that 8.8% of mash feed samples and 4.2% of pelleted feed samples were contaminated with *Salmonella*, suggesting that the pelleting process reduces *Salmonella* isolations from poultry feed but does not eliminate the organism (Jones and Richardson, 2004). Current European Union (EU) legislation requires that feed manufacturers adopt Hazard Analysis and Critical Control Point (HACCP) procedures to control *Salmonella* and other pathogens in feeds (Jones and Richardson, 2004). Currently, Canada is behind in its regulatory policies with no concise standard for feed pelleting time and temperature treatment. Even though poultry feeds have been suggested as significant vectors for *Salmonella* contamination in broiler flocks, the extent to which food-borne outbreaks have been linked back to the consumption of poultry products manufactured from flocks fed contaminated feed is unknown (Blank et al., 1996). Nevertheless, it was reported during a study of primary breeders that close to 80% of *Salmonella* serovars isolated from feeds and feed ingredients were the same serotypes isolated weeks later in breeding flocks and their offspring (Jones and Richardson, 2004).

This work is the second of a two-part study undertaken to determine whether the same strains of *Salmonella* were present in both raw, frozen chicken nuggets/strips and pelleted broiler feed (Bucher et al., 2007). The objectives of the present study were to determine whether *Salmonella* strains isolated from the aforementioned sources had the same thermal resistance and to assess whether this resistance was higher than normally would be expected.

4.3. Materials and Methods

4.3.1. Raw Material

Chicken nugget/strip meat blend used in nugget manufacture was supplied frozen by one of the major manufacturers of nuggets and strips in Canada (southern Ontario) for use as a heating menstruum in thermal resistance experiments. The formulation and production of this meat blend was proprietary and included binder/filler to yield an almost homogenous blend of meat, fat, collagen and spices/seasonings. The meat blend was thawed and packaged in 53g portions in Deli*1 EVOH/Nylon/PE bags (17.5cm x 17.5cm) provided by WinPak Ltd. (Winnipeg, MB, Canada). Packaged samples were frozen at -30°C, irradiation-treated with an average electron beam dose of 4.75kGy at Acsion Industries Inc. (Pinawa, MB, Canada) and held at -30°C until analysis. Treated meat was analyzed for the presence of aerobic mesophilic bacterial contaminants by dilution plating on tryptic soya agar (tryptic soya broth, TSB; Becton-Dickinson, Sparks, MD, USA, supplemented with 0.6% yeast extract YE; Sigma, St. Louis, MO and 1.5% w/v granulated agar Difco, Becton-Dickinson). Triplicate bags of meat blend were analyzed for total moisture, protein, fat, carbohydrate and salt content along with pH and a_w. Procedures in the AOAC (2002) were used to determine protein and fat content by micro Kjeldahl and Soxhlet methods, respectively. Carbohydrate from binder/fillers, measured as dextrose equivalents, as well as total salt content were determined using Health Canada official methods FO-32 (Health Canada, 1981) and FO-38 (Health Canada, 1985), respectively. Total moisture was calculated as the percent water lost after freeze-drying (Genesis 25XL freeze-dryer; VirTis, Gardiner, NY, USA). The pH was determined with an with Accumet Basic pH meter (Denver Instrument Company, Denver, USA) after pummeling 1g of thawed meat blend in 9ml of distilled water for 2

min in a stomacher device (Bagmixer 400; Interscience, Bois Arpent, France). The a_w of the thawed meat was measured using an AW Sprint (Novasina, Switzerland).

4.3.2. Culture storage conditions and inoculum preparation

Salmonella strains tested for their thermal resistance were previously isolated from raw, frozen chicken nuggets/strips, pelleted broiler feed and from chicken nugget/strip meat blend (Bucher et al., 2007), and were grouped for analysis according to serovar and their origin (Table 4.1). Isolates were stored in TSB (Becton-Dickinson) supplemented with 50% (v/v) glycerol (Sigma, St. Louis, MO) at -80°C until thermal resistance analysis.

Before analysis, stock cultures were revived on nutrient agar (NA; Oxoid, Basingstoke, UK) using two consecutive overnight transfers. Inocula were prepared by transferring a single colony of each organism to 30ml TSB supplemented with 0.5% YE and incubated for 18 h at 37°C to yield late stationary phase cells (Bacon et al., 2003, Juneja et al., 2001). Cocktails of inocula were prepared with equal numbers of cells of several strains of the same serovar from the same source. Three strains of different serovars (Enteritidis, Orion, Heidelberg) having different traits (phage type, PT; pulsed-field gel electrophoresis pattern, PFGE), isolated from different sources were tested individually (Table 4.1). Cells were washed twice by collecting pelleted cells after centrifugation for 15 min at 5000x g (Juneja et al., 2001), discarding the supernatant and resuspending the pellet in buffered peptone water (BPW; Difco, Becton Dickinson). The absorbance at 650nm of the cell suspension was adjusted to 0.2 OD units (7 $\log_{10}$ CFU/ml) in 5ml. Mixtures or cocktails of strains were prepared by combining equal

portions of standardized cell suspensions to yield 6-7 $\log_{10}$CFU/ml of each strain in the mixture. Viable numbers were verified by spiral plating (Autoplate 4000, Spiral Biotech, Bethesda, MD) on TSB+0.6%YE agar plates.

4.3.3. Thermal Resistance

Thermal resistance protocols used an end-point procedure based on the methods of Peirson et al. (2003) with slight modification. Prior to inoculation, the 53g aliquots of irradiation-treated meat were thawed and 3g were aseptically transferred to another Deli*1 bag. Prepared inoculum (5ml) was added to the remaining 50g of meat and pummeled in a stomacher for 30 sec at high speed. Three grams of this inoculated meat were then aseptically weighed into each of 12 Deli*1 bags, vacuum-sealed with a model GM 2002 vacuum packer (Bizerba Canada Inc., Mississauga, ON, Canada) and flattened to a width of 0.3mm±0.01mm using a template. Bags were inserted into slots of a custom-made thermospacer (Peirson et al., 2003) and held with metal fold-back binder clips. The bag with 3g uninoculated meat was placed in the middle slot of the thermospacer and a K-type glass braid, insulated thermocouple (OMEGA Engineering, Laval, QC, Canada) connected to a data logger (OMEGA Engineering) was inserted to monitor the time required to reach the target temperature (come-up time). The thermospacer was fully immersed in a Magni-Whirl constant temperature waterbath (Blue M, Blue Island, IL, USA) set to one of four target temperatures in the meat (55, 58, 60 and 62°C). At time zero (when thermocouple temperature was 0.5°C below the target temperature) two inoculated bags were removed and immediately immersed in an ice water bath. The remaining bags were removed in pairs at pre-determined times,

based on the heating temperature, and immediately immersed in the ice bath for 10 min until analysis.

Bags of heat-treated meat were aseptically opened and 27ml of BPW were added before being pummeled with a stomacher for one min. Serial dilutions up to 10^{-3} in 9ml BPW were made and surface-plated manually or by spiral plater on TSB+0.6%YE and incubated at room temperature (22 to 25°C) for 48h. Colonies on plates surface-plated manually were counted visually while plates prepared by spiral plating were counted by CASBA-4 scanner with CIABEN software (Spiral Biotech, Bethesda, MD).

4.3.4. D- and z-value Calculations and Statistical Analysis

Microsoft® Excel® software was used to construct survival curves ($\log_{10}$CFU/g survivors vs. time) and D-values were calculated by fitting a regression line to the survival curve. From the regression analysis, the negative inverse from the slope of the regression equation was calculated giving the corresponding D-value for that temperature (Smith et al., 2001).

The z-value was calculated by constructing thermal death time curves (plotting $\log_{10}$D-values against the corresponding temperature), and using Microsoft® Excel® a regression line was fitted to the curve and the negative inverse from the slope of the regression equation was calculated which gave the corresponding z-value (Smith et al., 2001).

D- and z-values represented means from experiments carried out in triplicate. Differences were determined using Tukey's test (α=0.05) with SAS statistical software (SAS version 9.1, Institute Inc., Cary, N.C.).

4.4. Results

4.4.1. Raw Material Analysis

From proximate analysis after irradiation it was found that the chicken nugget/strip meat blend pH was 5.78 and the a_w was 0.96. The moisture content was 75.32% and the meat contained 1.09% carbohydrate, 13.93% fat, 70.07% protein and 5.27% salt on a dry weight basis. No contaminating organisms were recovered from the irradiated meat (<10 CFU/g).

4.4.2. *Salmonella* Thermal Resistance

Twenty-five different strains of *Salmonella* isolated from pelleted broiler feed, uncooked chicken nuggets/strips, and chicken nugget meat blend used for nugget manufacture were tested in 7 different groups for their thermal resistances (Table 4.1).

D-values of the *Salmonella* cocktails and individual strains tested ranged from 6.93 to 3.58 min at 55°C, 1.53 to 0.96 min at 58°C, 0.75 to 0.38 min at 60°C and from 0.29 to 0.12 min at 62°C (Table 4.2). As expected, all D-values decreased substantially with an increase in temperature and it was found that ≥20 min at 55°C, 7 min at 58°C, 5 min at 60°C but just over 1.5 min at 62°C were required to reduce *Salmonella* viability by 7 $\log_{10}$CFU/g chicken meat blend. The data indicated that as the temperature increased so did the differences among D-values of the different serovars (Table 4.2). Of

note were the significant differences observed in the D_{60} values of the *S.* Enteritidis cocktails isolated from feed and nugget meat blend. Each of these 2 cocktails of 2 strains contained one strain with identical phage type, pulsed-field gel electrophoresis pattern, and antimicrobial resistance profiles, and while they showed similar D-values at other temperatures tested, their D_{60} values (0.38 min for the feed and 0.75 min for the nugget/strip meat blend isolates) were the lowest and highest of D_{60} values determined for all other serovars including that of the *S.* Enteritidis strain isolated from nuggets.

The z-values of *Salmonella* strains tested ranged from 4.10°C to 5.17°C (Table 4.2). All values were approximately the same except for those of the two *S.* Enteritidis cocktails containing 4 strains either isolated from chicken nugget/strip blend or pelleted feed. Since these 4 of 5 strains of *S.* Enteritidis studied had the same z-values, it is possible that despite the differences in their D_{60} values, the overall thermal resistance of at least two of these 4 strains was the same and was slightly less than those of the other pair of test organisms.

4.5. Discussion

Despite the many advances made in food safety, cooking remains the primary means of destroying contaminating pathogens. Thus information on the thermal resistance of pathogens is still required for processors to adequately heat treat products and provide safe food preparation instructions for consumers. Results presented here show that the *Salmonella* strains isolated from raw, frozen chicken nuggets/strips, pelleted broiler feed and from chicken nugget/strip meat blend (Bucher et al., 2007) had lower heat resistances than reported for other *Salmonella* strains. Unfortunately, much of

the literature does not include thermal resistance data using chicken meat as a heating menstruum making comparison difficult, though some data exist for poultry and processed poultry products (Table 4.3).

Variations in bacterial thermal resistance are influenced by both intrinsic and extrinsic factors. In terms of extrinsic (environmental) factors, the effects of the heating menstruum composition which is influenced by meat species, pH, size, shape and type of muscle, plus the content of carbohydrate, fat and salt as well as the nature of the heating process used (continuous or batch), have dramatic effects on the thermal resistance of an organism. In terms of intrinsic factors, differences can been seen among strains as well as at different bacterial growth phases and in response to prior sublethal heat exposure (Doyle and Mazzotta 2000; Smith et al., 2001; Orta-Ramirez et al., 2005; Murphy et al., 1999, 2001, 2002).

The lower D- and z-values reported in this study compared to results in other studies may have been due to differences in composition of the heating menstruum. Of the factors in the heated medium that influence bacterial thermal resistance, the fat content has been reported to play a significant role (Juneja et al., 2001; Smith et al., 2001; Orta-Ramirez et al., 2005; Murphy et al., 2003). It has been speculated that the protective effects of fat may be due to its lower heat conductivity and water activity (Murphy et al., 2003). In general, the higher the fat content the higher the thermal resistance (Ahmed et al., 1995; Juneja et al., 2001; Veeramuthu et al., 1998) though others did not observe this effect (Fain et al., 1991; Young et al., 1991; Kotrola and Conner 1997), perhaps because the effects of fat may have been affected by unequal

distribution in the heated medium. Orta-Ramirez et al. (2005) observed lower heat inactivation rates in an 8 serovar cocktail of *Salmonella* in whole beef muscle compared to ground beef. It was suggested that bacteria attached to whole intramuscular fat may benefit more from the protective effects of fat than bacteria distributed in ground samples because the total fat present is diluted when homogenously distributed in ground beef.

Differences in the volume of the heating menstruum, physical nature and design of the thermal reaction containers (pouches versus tubes) affect the come-up time of the heating menstruum (Smith et al., 2001; Murphy et al., 1999). In the present study 3g samples of meat 0.3mm thick sealed in pouches were used, whereas Juneja et al. (2001a) used 5g samples 1 to 2mm thick in stomacher bags and Murphy et al., (2002) used 8.5g samples in thin-walled (0.3mm) metal containers (8.23mm diameter by 152.4mm length). Murphy et al. (1999) evaluated the effects of sample size on the come-up time and the reduction of *Salmonella* in ground chicken breast meat by using 8.5g, 15g or 30g samples packed in sealed brass containers 50.8, 25.4, or 8.2mm in diameter. They found that reducing the diameter of the thermal reaction container by half decreased the come-up time by about 27% and that *Salmonella* were reduced to <10CFU/g about 3 min faster. The larger sample sizes used in these other studies along with the greater thickness of meat medium may have resulted in longer come-up times leading to an overestimate of thermal resistance, possibly by lowering the initial microbial load at the target temperature, decreasing the slope of the regression line, and increasing the D-values determined.

It has also been documented that differences in thermal resistance can exist among different bacterial strains (Doyle and Mazzotta 2000; Murphy et al., 1999). Variations in D- and z-values seen in the present study may have this origin. Murphy et al. (1999, 2000, 2002) included *S.* Senftenberg (the most heat resistant *Salmonella* serovar) in their studies so that resulting D- and z-values would provide a margin of safety if processors used the data in designing thermal processes. Similarly, Juneja et al. (2001a) selected only the most heat resistant strains of *Salmonella* found during testing in chicken broth for use in ground breast meat tests. In the present study, the goal was to characterize the thermal resistances of all strains isolated from pelleted feed and chicken nuggets/strips, so strains were not pre-screened for thermal resistances prior to tests in the nugget/strip meat blend.

It was also of interest in the current study to gather additional information to substantiate whether the same strains of *Salmonella* had been isolated from pelleted broiler feed and raw, frozen chicken nuggets/strips. This was shown to be more probable as the 2 cocktails of *S.* Enteritidis strains (each containing one genotypically and phenotypically identical organism) isolated from feed and meat blend used for nugget/strip manufacture seemed to show the same thermal resistances. It should be noted that the thermal resistance of the fifth *S.* Enteritidis strain (with different PT and PFGE pattern) from frozen nuggets/strips was not significantly different from the other *Salmonella* serovars isolated.

Results from the present study showed that two cocktails of two strains of *S.* Enteritidis isolated from pelleted broiler feed or from chicken nugget meat blend had the

same thermal resistances. Combined with data from previously published work, results here are consistent with the hypothesis that one *S.* Enteritidis strain each from pelleted feed and nugget/strip meat blend were indistinguishable, though without nucleic acid sequencing some doubt remains. The lower thermal resistances of the strains tested in this study demonstrate that with adequate cooking (71°C), these organisms can be eliminated from raw, frozen chicken nuggets/strips, provided preparation instructions are followed.

Table 4.1: *Salmonella* isolate groups used in mixtures (cocktails) tested for thermal resistances.

Group	Serovar	Source[a]	No. Strains	Distinguishing traits[b]
1	Enteritidis	A	1	PT, PFGE
2	Enteritidis	B	2	PFGE (2)
3	Enteritidis	C	2	PFGE (2)
4	Heidelberg	A	12	PT (7), PFGE (7), AB resist. (5)
5	Heidelberg	C	1	PT
6	Kentucky	A	6	PFGE (6), AB resist. (4)
7	Orion	B	1	PFGE

[a]Nuggets/strips, A; Feed, B; chicken nugget/strip meat blend, C

[b]PT, phage type, PFGE, pulsed-field gel electrophoresis pattern; AB resist., antibiotic resistance profile to a panel of 16 clinical antibiotics. Numbers in parenthesis represent the number of different PT, PFGE or AB resist. profiles in each group of serovars. Where one strain was assigned to a group, the distinguishing trait is identified. Data from Bucher *et al.* (2007).

Table 4.2: D- and z-values of *Salmonella* serovars isolated from chicken nuggets/strips, pelleted feed and chicken nugget meat.

Source	Serovar	D-value (min ± std. dev.)				z-value (°C ± std. dev.)
		55	58	60	62	
Nuggets/Strips	Enteritidis	6.87±0.60^{a}	1.51±0.16^{c}	0.69±0.06^{e}	0.23±0.02^{h}	4.86±0.14^{l}
	Heidelberg	4.50±0.15^{b}	0.96±0.03^{d}	0.39±0.02^{g}	0.15±0.01^{ij}	4.73±0.08^{l}
	Kentucky	4.49±0.29^{b}	1.19±0.13^{cd}	0.39±0.02^{g}	0.17±0.03^{j}	4.81±0.16^{l}
Pelleted Feed	Enteritidis	6.93±0.58^{a}	1.53±0.17^{c}	0.38±0.02^{g}	0.15±0.01^{ij}	4.10±0.13^{m}
	Orion	3.58±0.03^{b}	1.45±0.22^{c}	0.38±0.03^{g}	0.18±0.03^{j}	5.17±0.25^{l}
Nugget Meat Blend	Enteritidis	6.54±0.53^{a}	1.40±0.15^{c}	0.75±0.03^{e}	0.12±0.02i	4.24±0.23^{m}
	Heidelberg	6.54±0.39^{a}	1.45±0.13^{c}	0.49±0.05^{f}	0.29±0.01^{k}	5.03±0.14^{l}

Values within columns with different superscripts are significantly different ($p<0.05$)

Table 4.3: D- and z-values of *Salmonella* serovar mixtures (cocktails) in various poultry products.

Serovar mixture	Product	Temperature (°C)	D-value (min)	z-value (°C)	Reference
6 serovar cocktail:	Chicken Strips	55	22.37	7.61	Murphy et al., 2002
Senftenberg ATCC 43845		57.5	9.92		
Typhimurium		60	8.50		
Heidelberg		62.5	4.55		
Mission		65	1.25		
Montevideo		67.5	0.38		
California		70	0.32		
6 serovar cocktail:	Chicken Patties	55	26.97	7.60	Murphy et al., 2002
Senftenberg ATCC 43845		57.5	14.55		
Typhimurium		60	8.09		
Heidelberg		62.5	3.98		
Mission		65	1.39		
Montevideo		67.5	0.61		
California		70	0.32		
8 serovar cocktail:	Ground Chicken Breast	58	7.08	6.11	Juneja et al., 2001a
Thompson FSIS 120		60	5.20		
Enteritidis H3527		62.5	1.36		
Thompson H2464		65	0.59		
Typhimurium H3380					
Hadar MF60404					
Copenhagen 8457					
Montevideo FS1S 051					
Heidelberg F5038BG1					

Table 4.3 cont'd...

Serovar mixture	Product	Temperature (°C)	D-value (min)	z-value (°C)	Reference
6 serovar cocktail:	Ground Chicken Breast	55	30.1	6.53	Murphy et al., 2000
Senftenberg ATCC 43845		57.5	12.9		
Typhimurium		60	5.88		
Heidelberg		62.5	2.51		
Mission		65	1.16		
Montevideo		67.5	0.358		
California		70	0.238		
6 serovar cocktail:	Fully Cooked Ground Chicken Breast	55	24.07	6.26	Murphy et al., 2003
Senftenberg ATCC 43845		57.5	9.60		
Typhimurium		60	3.83		
Heidelberg		62.5	1.53		
Mission		65	0.61		
Montevideo		67.5	0.24		
California		70	0.10		
7 serovar cocktail:	Ground Chicken Breast	56	3.2	5.7	Mazzotta, 2000
Typhimurium ATCC 13311		60	0.6		
Enteritidis ATCC 13076		62	0.31		
Enteritidis NFPA N-4016		63	0.18		
Montevideo					
Mbandaka					
Heidelberg					
Thompson					

5. Discussion

Observations made from the investigations in Chapters 3 and 4 into the occurrence and characterization of salmonellae strains contaminating raw, frozen chicken nuggets/strips and pelleted broiler feed demonstrate the persistence of this organism in these types of products despite the stresses placed on them during processing. Although the numbers of sampling locations of pelleted feed and lots of raw, frozen chicken nuggets/strips were limited the samples did represent the largest commercial concerns active in the industry in Canada, though this may not truly represent the entirety of the industry situation.

It should be noted that subsequent to the publication of Chapter 3, sampling of chicken nuggets/strips, pelleted feed and nugget meat and coating continued. Data from Chapter 3 combined with the additional unpublished data lowers the mean contamination rate of pelleted feed to 5% (13 of 267 samples/lots) from 9%. This updated result changes the previous observation that pelleted feed was more frequently contaminated with *Salmonella* in this study compared to other reports (Cox et al., 1983; Blank et al., 1996; Zdragas et al., 2001; Jones and Richardson, 2004), to being only slightly more contaminated and comparable to other reports (Zdragas et al., 2001; Jones and Richardson, 2004). Differences in contamination rates between results in the present study and those in other reports may be the result of the limited number of sampling locations in this study. Jones and Richardson (2004) found significant differences in the occurrence of *Salmonella* between feed mills, which is thought to stem from differences in feed mill management practices with respect to sanitation and the control of dust

contaminated with *Salmonella*. Dust present in and around the pellet mill was estimated to be 1.5, 0.8 and 6.4 mm in thickness from 3 separate feed mills, respectively. It was hypothesized that mechanical vibrations and air currents traveling through the feed mills could have resulted in dust contaminated with *Salmonella* falling into the pelleted feed, effectively negating the sanitizing effects of the pelleting process (Jones and Richardson, 2004).

The unpublished data regarding the contamination rates of chicken nuggets/strips combined with previous observations in Chapter 3 have not changed the overall frequency of *Salmonella* contamination in chicken nuggets/strips (54 of 203 samples from 166 lots were positive for *Salmonella*). The results presented in the present study are comparable to results presented on the incidence of *Salmonella* in ground chicken and MDM (White et al., 2001; Rose et al., 2002; Carvalho and Cortez, 2005). Rose et al. (2002) isolated *Salmonella* from 14.4% of ground chicken samples from large, small and very small federally inspected processing facilities in the US over a two year period. In contrast, White et al. (2001) found *Salmonella* from 35% of ground chicken samples from three supermarket chains in the Washington D.C. area. Differences in contamination between the latter and former results might be due to the implementation of the pathogen reduction HACCP rule since a general decline was observed in *Salmonella* prevalence over the course of the sampling period. Cavalho and Cortex (2005) observed similar rates of *Salmonella* contamination of MDM in São Paulo, Brazil with an incidence of 25% compared to the 27% incidence observed in the present study.

When combining the data presented in Chapter 3 with the additional unpublished data, the chicken nugget meat contamination rate becomes 48% (19 of 40 samples from 2 lots). Again *Salmonella* were absent from the nugget coating (0 of 31 samples from 12 lots positive for *Salmonella*), which is consistent with previous observations. These observations may suggest that the par-frying process is sufficient to eliminate *Salmonella* in the coating layer of the nugget/strip but is insufficient to eliminate the organism from the meat core. This provides additional support for the argument that uncertainty regarding the cooked state of these products seems to be the primary reason for cases of salmonellosis (Kenny et al., 1999; MacDougall et al., 2004).

Characterization of the *Salmonella* strains from pelleted feed and chicken nuggets/strips revealed that there is more diversity among the strains contaminating chicken nugget/strip strains than the strains contaminating feed. *S.* Orion, *S.* Heidelberg and *S.* Enteritidis were the most frequently isolated serovars from feed, nuggets/strips and nugget meat, respectively. The finding that *S.* Heidelberg is the most frequently isolated serovar from chicken nuggets/strips is consistent with other reports (MacDougall et al., 2004; Currie et al., 2005; Demczuk et al., 2007). Twelve different strains differing in PT, PFGE pattern and/or antimicrobial resistance were isolated. Of concern was the isolation of *S.* Heidelberg with resistance to multiple antimicrobials. Several strains were ceftriaxone-resistant. This antibiotic is a broad-spectrum cephalosporin commonly used to treat *Salmonella* infections in children (Fey et al., 2000), so the isolation of ceftriaxone-resistant *S.* Heidelberg strains in chicken nuggets/strips is of great concern. *S.* Orion was the most frequently isolated *Salmonella* strain from pelleted feed which is slightly surprising since *S.* Thompson and *S.*

Schwarzengrund were the most frequently isolated serovars in Canada in 2005 (Demczuk et al., 2007). Nevertheless, *S.* Orion had been isolated from animal feed and feed ingredients in Canada (Demczuk et al., 2007), so the finding in the present study that *S.* Orion was the most frequently isolated serovar from pelleted feed may be because only one feed mill was sampled which only represents the *Salmonella* contamination situation at the one feed mill. Of note is the finding of two strains of *S.* Enteritidis isolated from pelleted feed that had a phenotype and genotype indistinguishable from two strains of *S.* Enteritidis isolated from chicken nugget meat. This provides some evidence that *Salmonella* contaminating pelleted feed may be passed along the production chain to food for human consumption. However, since it could not be determined if the feed contaminated with these two strains of *S.* Enteritidis was fed to broilers used to manufacture the sampled nuggets that were also contaminated with these same strains, there is no direct evidence to suggest their horizontal transfer.

Chapter 4 investigated the thermal resistance of the *Salmonella* strains to determine whether strains isolated from raw, frozen chicken nuggets/strips and pelleted broiler feed had the same thermal resistance and to assess whether this resistance was higher than normally would be expected. To reduce the amount of time and materials needed to evaluate the thermal resistance of the *Salmonella* strains, cocktails, rather than individual strains of *Salmonella* strains were tested. Information on the thermal resistance of strains provide additional criteria by which the similarity of the *Salmonella* strains isolated from pelleted feed and chicken nuggets/strips could be compared. The two *S.* Enteritidis strains with indistinguishable pheno- and genotype from pelleted feed and chicken nugget meat blend were therefore combined into two different cocktails

consisting of *S.* Enteritidis from pelleted feed and chicken nugget meat blend. The two cocktails of *S.* Enteritidis had the same thermal resistance which further strengthens the belief that the same strains of *Salmonella* contaminating feed can potentially contaminate chicken meat used for human consumption. An assessment of the degree of thermal resistance of the strains isolated in this study indicated that all the strains tested did not have an increased thermal resistance. This suggests that the *Salmonella* isolated in this study were not likely selected for during the feed pelleting process. The heating step used during the pelleting of the samples tested in this study included conditioning at 80°C for approximately 5 s followed by expansion through an annular gap expander at 100 to 105°C for another 5 s before pelleting (Newcombe, 2007). This further enforces the theory that post-pelleting contamination of pelleted feed is a significant barrier towards producing *Salmonella*-free feed.

6. Conclusions

The investigations into the occurrence and the characterization of *Salmonella* strains in pelleted feed and raw, frozen chicken nuggets/strips presented in Chapters 3 and 4 were the first to report on the frequency and characterize the strains of *Salmonella* in raw, frozen chicken nuggets/strips in Canada. Results from the present work have also indicated the problems associated with tracing back the origin of *Salmonella* that contaminate raw, frozen chicken nuggets/strips to pelleted feed fed to chickens used in their manufacture. The finding in the present study of two *S.* Enteritidis strains in pelleted feed, indistinguishable from two *S.* Enteritidis strains in chicken nugget meat provides some evidence that *Salmonella* contaminating pelleted feed may be passed along the production chain to food for human consumption. However, since it is unknown whether *Salmonella* was present in the lots of poultry feed fed to the flocks of animals used in the manufacture of chicken nuggets/strips sampled in this study, evidence provided for this source of meat contamination is only circumstantial. Results however do suggest that it is the meat and not the nugget coating that is the source of *Salmonella* contamination. This provides a target for future work to investigate intervention strategies that can be used on farm and in chicken nugget/strip processing.

The thermal resistance of the strains tested indicates that the strains contaminating pelleted feed and raw, frozen chicken nuggets did not have an increased thermal resistance and that adequate cooking of chicken nuggets/strips (71°C) and pelleting (85°C) will eliminate these organisms from these products.

It should be noted that sampling of pelleted feed and raw, frozen chicken nuggets/strips lots is still ongoing and should provide a better representation of the overall industry situation. Work is therefore also continuing to characterize the thermal resistance of additional *Salmonella* strains as they become available. Also, additional comparisons between strains isolated from human clinical sources and those isolated from poultry feed and chicken nuggets/strips are being investigated and should provide incentive to re-examine guidelines regarding poultry feed processing/pelleting and generate data on the extent to which food-borne illness can be traced back to contaminated animal feeds.

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