The application of Near Infrared Transmittance (NIT) individual kernel sorting technology to improve grain quality from spring and durum wheat infected with *Fusarium* and the effects on broiler chicken performance and immune response.

A Thesis Submitted to the College of
Graduate Studies and Research
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the Toxicology Graduate Program
University of Saskatchewan
Saskatoon, Saskatchewan,
Canada

By

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ABSTRACT

This project investigated the use of new near infrared transmittance (NIT) technology for individual kernel sorting to reduce *Fusarium* damaged kernels (FDK) and mycotoxins in grain. There were two objectives: 1) determine the efficiency of sorting; and 2) assess how highly contaminated sorted grain fractions can be used in dietary challenges for broilers as a screen for methods to reduce mycotoxin exposure.

Fusarium damaged kernels are associated with lower crude protein (CP) caused by fungal infestation during kernel development, and may contain varying concentrations of mycotoxins (e.g. deoxynivalenol; DON). The BoMill TriQ measures the NIT of limited spectra to predict CP variation among individual kernels at ~2 - 3 MT/hour. Five sources of downgraded grain attained from grain producers in Western Canada in 2013 were sorted into ten calibration fractions, each analyzed for CP, FDK and 16 common mycotoxins. From these analyses, three wheat sources were individually sorted into three test fractions: outliers (10%); high FDK (low CP; 20% of source); and low FDK (high CP; 70% of source). Four diet recombinations were produced based on increasing inclusion of the high % FDK fraction [0% (M0), 20% (M20), 40% (M40) or 60% (M60)] of each wheat source, providing increasing mycotoxin concentrations in the test diets. Productions of these diets from re-combining the FDK fractions enabled a 3 wheat source x 4 FDK level (M0, M20, M40, M60) factorial design. The 12 test diets were included at 70 (starter, 0 - 21 d) and 75% (grow/finisher, 21 - 35 d) of a basal diet. Diets were formulated to meet or exceed NRC (1994) requirements for broilers.

Eight cages of five, one-day old male Ross 308 broilers were each randomly assigned to the 12 starter diets. The number of cages were reduced to three per diet at 21 d. Broiler performance were recorded for the 0 - 21 and 21 - 35 d. Apparent metabolizable energy (AME; kcal ME/kg diet) and nitrogen retention (NR; %) were determined using digestible markers and excreta collections. Five biomarkers of immune function were measured for starter and grower/finisher periods: 1) cell-mediated immune response to injection of the T cell mitogen phytohemagglutinin (PHA); 2) humoral response to immunization with bovine serum albumin (BSA) antigen; 3) relative weights of liver, spleen and bursa of Fabricius; 4) heterophil to lymphocyte (H:L) ratio; and 5) histopathology of primary and secondary immune organs. Analysis of sorting efficiency of this technology indicated that grain could be separated into 10% increments based on unique spectral ranges and their correlation to the chemical characteristics of CP.

Indications were that the lowest 20% CP kernels contained increased FDK (15.4%) and DON (10.2 ppm) compared to the unsorted kernels (2.4% and 1.7 ppm). The statistical correlations between FDK, DON and CP provided the capability to produce high and low mycotoxin fractions for use in the poultry feeding trial. Analysis of growth and performance endpoints of each exposure period indicated no significant difference ($P > 0.05$), however AME and NR were different ($P < 0.01$) among treatment groups at 21 and 35 d. Analysis of immune system endpoints indicated no significant differences ($P > 0.05$) among treatment groups in cell-mediated (PHA; 0.32 - 0.35 % change), humoral (BSA; 0.57 - 0.64 % change) or H:L ratio (0.03 - 0.13 % change) immune responses. However, histopathological examination of the spleen ($P < 0.05$) at 21 d and the liver ($P < 0.01$) at 35 d showed increases in lymphoid aggregates and/or granulopoisis in the diet containing 8 ppm DON suggesting potential adverse effects on the immune system.

Overall, the results of these studies indicate that the NIT technology has the potential to produce naturally contaminated diets with various levels of mycotoxins from a single source of grain. These naturally contaminated diets may improve our ability to evaluate models to examine the effects of mycotoxin exposures to poultry or livestock.

ACKNOWLEDGEMENTS

I would like to express a great amount of gratitude to many people in the Toxicology Centre and Department of Animal and Poultry Science in the College of Agriculture. My most sincere thank you goes to my co-supervisors Dr. Tom Scott and Dr. Mark Wickstrom. Without their leadership, guidance and support, I would not have been able to complete this project. I would also like to personally thank my advisory committee, Dr. Barry Blakley and Dr. Natacha Hogan for their assistance and my external examiner, Dr. Randy Kutcher.

I was lucky enough to have help from many people, far too many to be able to mention. From grain sorting and diet formulations with John Smillie and Scott Bishoff to experimental and analysis guidance from Dawn Abbott to statistical salvation from Drs. Karen Schwean-Lardner and Cheryl Waldner and everyone else I don't have the space to thank but were a large part of my success, a most sincere 'Thank you"! I am grateful for the assistance of Kaitlyn Brown, my research assistant for her battle with all immunological analysis.

To the staff, faculty and students in the Toxicology and Animal and Poultry Science I extend genuine appreciation for your support, especially during sample collections! Lastly, I want to especially thank my family for their love, encouragement and motivation – I am indebted to you all.

I would like to acknowledge BoMill for technical support with the TriQ and the Canadian Feed Research Centre. This project was funded by the Saskatchewan Ministry of Agriculture - Agriculture Development Fund, the Alberta Crop Development Fund, the Canadian International Grains Institute; and the Western Economic Diversification (AAFC). Additional funding for conference registrations and travel was provided as student travel awards from the University of Saskatchewan Graduate Student Association, the Cecil E. Doige Fund, the Poultry Science Association, the Miriam Green Ellis Scholarship, SETAC North America and the Agricultural Bioscience International Conference Foundation.

DEDICATION

This thesis is dedicated to my family;
Mom, Dad, Cheryl and Matthew.

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LIST OF ABBREVIATIONS

AA – Amino Acids
ADON – Acetyl Deoxynivalenol
AF – Aflatoxin
AME – Apparent Metabolizable Energy
BSA – Bovine Serum Albumin
BW – Body Weight
CMI – Cell mediated immunity
CP – Crude Protein
CWAD – Canadian Western Amber Durum
CWRS – Canadian Western Red Spring
CWSWS – Canadian Western Soft White Spring
DAS – Diacetoxyscirpenol
df – Degrees of Freedom
DM – Dry Matter
DON – Deoxynivalenol
FAO – Food and Agriculture Organization
FCR – Feed Conversion Ratio
FDK – *Fusarium* Damaged Kernels
FHB – *Fusarium* Head Blight
FI – Feed Intake
FUM – Fumonisins
FUS X – Fusarenon-X
GE – Gross Energy
H:L – Heterophil to Lymphocyte Ratio
Ig – Immunoglobulin
IgA – Immunoglobulin A
IgG/IgY – Immunoglobulin G (Y in poultry)
IgM – Immunoglobulin M
Kg/hL – Kilogram per Hectoliter
ME – Metabolizable Energy
MT – Metric Tonnes
mm - Millimeters
N – Nitrogen
n – Number of Observations
NEO – Neosolaniol
NIR – Near Infrared
NIT – Near Infrared Transmittance
NIV – Nivalenol
NR – Nitrogen Retention
NS – No Significance
OT-A – Ochratoxin A
P – Probability Value
PBS – Phosphate Buffered Saline
PHA – Phytohemagglutinin
ppm – Parts Per Million
RH – Relative Humidity
SEM – Standard Error of the Mean
SCIRP – Scirpentriol
SKNIR – Single Kernel Near Infrared
TKW – Thousand Kernel Weight
TW – Test Weight (formerly Bushel Weight)
ZEA – Zearalenone

PREFACE

This thesis has been organized as a series of manuscripts that will be submitted for publication in scientific journals. Some repetition of introductory and methodological material is unavoidable. Chapter 1 is a literature review describing the current state of knowledge relating to *Fusarium* mycotoxins including the various types and economic effects. Chapter 2 and 3 have been published in *Animal Nutrition* and *Poultry Science*, respectively (citations listed below). Chapter 4 has been submitted to *Mycotoxin Research*. Chapter 5 is a general discussion and concluding remarks.

Kautzman, M.E., Wickstrom, M.L., Scott, T.A., 2015. The use of near infrared transmittance kernel sorting technology to salvage high quality grain from grain downgraded due to *Fusarium* damage. Anim. Nutr. 1, 41 - 46.

Kautzman, M.E., Wickstrom, M.L., Hogan, N.S., Scott, T.A., 2015. Using near infrared transmittance to generate sorted fractions of *Fusarium* infected wheat and their impact on broiler performance. Poult. Sci. 94, 1619 - 1628.

1.0 LITERATURE REVIEW

1.1 General introduction

Mycotoxins are a large diverse group of naturally occurring secondary metabolites (CAST, 2003) produced in grain by fungal infectations pre and/or post-harvest. They have the potential to negatively impact animals and humans (Glenn, 2007). Mycotoxins occur particularly in regions or countries with high temperature and humidity and poor agronomy practices, such as inefficient crop rotations or poor grain storage conditions that encourage fungal growth. As environmental factors are the primary determinate in mycotoxin production, it is impossible to completely eliminate contamination of grain with mycotoxins (Choudhary and Priyanka, 2010).

The total number of mycotoxins characterized exceeds 400 (Lazicka and Orzechowski, 2010). They are classified based on fungal species, chemical structure and mechanism of action. A single species of fungi can produce more than one type of mycotoxin and a single mycotoxin can be produced by different fungal species (Zain, 2011; Table 1.1). The scientific literature indicates that detection of all mycotoxins is limited by plants "masking" mycotoxins by glycosylation, preventing detection using current methods of analysis (Fink-Gremmels, personal communication, 2013).

Table 1.1. Fungal species and associated mycotoxins.

Fungal species	**Mycotoxin**
Aspergillus flavus	Aflatoxins B1, B2
A. parasiticus	Aflatoxins B1, B2, G1, G2
A. ochraceus	Ochratoxin A
Penicillium verrucosum	Ochratoxin A
Fusarium sporotrichiodes	T-2 toxin, HT-2 toxin, Diacetoxyscirpenol
F. verticilloides	Fumonisin B1
F. graminearum	Deoxynivalenol, Nivalenol, Zearalenone

Numerous factors operate independently to affect fungal colonization and production of mycotoxins (Hussein and Brasel, 2001). These factors include: physical (environmental conditions such as temperature and relative humidity), chemical (the use of fungicides or

fertilizers), and biological (insect infestation and interactions between fungal species and substrate) conditions (D'Mello and Macdonald, 1997). Environmental conditions can make some plants more susceptible to fungal colonization, and may even increase the vulnerability of more resistant plants to fungal invasion. Studies have shown that the optimal conditions for fungal growth are not necessarily the same conditions needed for mycotoxin production (Moss, 1991). Consequently, a mycotoxin producing fungal species may be present, but this does not necessitate the presence of a mycotoxin(s) (Zain, 2011). Studies conducted in pre- and post-harvest grains have produced a better understanding of the potential impact of fungi on grains.

1.1.1 Pre-harvest vs. post-harvest mycotoxin formation

Generally, mycotoxins can be divided into two groups: those produced by plant fungal infestation in the field (pre-harvest), and those formed during improper handling and storage after harvest (post-harvest; Lazicka and Orzechowski, 2010). Although rare, mycotoxins may be produced during processing of grain into food and feed (Jouany, 2007).

1.1.1.1 Pre-harvest

The production of pre-harvest mycotoxins is influenced by environmental factors making the extent of contamination with a particular mycotoxin unpredictable. It can vary with geographical location, agricultural practices, drought and the susceptibility of the crop to fungal invasion (Wu et al., 2011; Murugesan et al., 2015). Plant growth and health, and the competitiveness of invasive mycotoxigenic fungi are greatly affected by temperature and moisture. Fungi can also grow on the vegetative part of plants, producing large amounts of mycelium on the stem, where it colonizes the vascular bundles, inhibiting the transfer of nutrients in the upper part of the plant. Members of the genus *Fusarium* including *graminearum*, *moniliforme*, and *roseus*, are ubiquitous soil organisms. These may infect grain directly in the field producing a variety of mycotoxins during growth, maturation and at harvest.

There have been strategies used to attempt to limit fungal infestations. These include crop rotation and soil tillage (Dill-Macky and Jones, 2000), breeding for genetic resistance (Anderson et al., 2001) and chemical or biological control (Parry et al., 1995). The efficacy of fungicides (chemical control) has been shown to vary with application timing, potential cultivar resistance, inadequate wheat head coverage and inefficient application technology (McMullen et al., 1997; Mesterhazy et al., 2003). McMullen et al. (2008) suggested that the best method for *Fusarium*

management was the use of multiple strategies and Wegulo et al. (2010) indicated that wheat cultivars also vary in resistance to fungal invasion.

1.1.1.2 Post-harvest

Fungal species producing post-harvest mycotoxins in stored grain include members of the genera *Aspergillus*, *Fusarium* and *Penicillium*. Most post-harvest mycotoxin production results from inadequate storage conditions (environmental and biological) and grain damage from handling (mechanical from harvest machinery and physical from insects; CAST, 2003). Mycotoxigenic fungi require specific humidity, moisture and temperature conditions for growth. Since these environmental conditions are (at least in part) controllable, mycotoxin production can be minimized by ensuring grain is stored and maintained dry, and treated with fungal inhibitors when necessary (Harris, 1998).

1.1.2 Mycotoxin combinations

Improved analytical techniques have led to the increased awareness of the potential significance of the co-occurrence of multiple mycotoxins (Placinta et al., 1999). The presence of multiple mycotoxins can lead to complex interactions with regard to clinical effects. These interactions can be classified as additive, antagonistic, synergistic (two mycotoxins each have an effect and when combined together, the effects are greater) or potentiative (one mycotoxin does not cause an effect alone, but enhances the effect of a mycotoxin that does cause an effect; Kubena et al., 1988; Kubena et al., 1997; Placinta et al., 1999). If multiple mycotoxins have similar mechanisms of action or target tissues, additive or synergistic effects appear to be the most common (Cheeke, 1998). A meta-analysis was conducted by Grenier and Oswald (2011) of over 100 experiments that evaluated the individual and combined effects of mycotoxins in animals. Results indicated that most of the studies demonstrated synergism or an additive interaction on animal performance, but other indicators of effects (i.e. organ weights, hematological, histological effects) showed a variety of interactions ranging from synergistic to antagonistic for the same combination of mycotoxins.

People and domestic animals in developed countries are at less risk of mycotoxin exposure than those in less developed countries. Regulatory controls deter the importation and sale of seriously contaminated material. Disease arising from ingestion of one or a combination of

mycotoxins may affect every organ resulting in clinical signs and lesions; which may vary markedly between animal species (Osweiler, 1985; Haschek et al., 2002).

1.1.3 Mycotoxin guidelines worldwide

International trade in wheat has risen from 100 million tonnes to 150 million tonnes from 1990 - 2011 (FAO, 2013a). *Fusarium* associated infestations (the most common fungal genus infecting grains) lead to significant mycotoxin contaminations and losses in yield (Desjardins and Proctor, 2007). The trade of food and feed worldwide is regulated in terms of mycotoxin contamination, particularly in more developed countries (Wu, 2004). As a consequence, developing countries tend to export their best quality crops while consuming the more highly contaminated grains, increasing the potential exposure to people and livestock. There is also the potential for lost value of some exported foodstuffs due to rejection by importing countries based on regulatory compliance. These regulations specify maximum limits for mycotoxins such as aflatoxins (AF), ochratoxin A (OT-A), patulin, fumonisins (FUM), zearalenone (ZEA) and deoxynivalenol (DON) for different foodstuffs (Kubo, 2012). A large worldwide survey from 2004 - 2013 indicated that AF, DON, FUM, OT-A and ZEA were the most economically important mycotoxins. The most prevalent mycotoxin in grain was DON (59%) and then FUM (57%) in finished feed. There were significant variations in prevalence and contamination levels year to year, with DON being most common in Asia, North America and central Europe (Murugasen et al., 2015). Indications are that regulatory limits, especially on DON, may become stricter, resulting in higher costs, decreased exports and expanded use of these grain as a source of feed (Bianchini et al., 2015). Fink-Gremmels (2013) estimated that the annual loss in North America alone due to mycotoxins was approximately 5 billion dollars.

From a Western Canadian perspective, three classes of *Fusarium* mycotoxins may be considered of particular importance in animal performance, immunity and productivity. These are fumonisins, zearalenone and trichothecenes. The most important and most commonly occurring representatives of the trichothecenes are DON, nivalenol (NIV), diacetoxyscirpenol (DAS), and T-2 toxin.

1.2 Aetiology of *Fusarium*: *Fusarium* head blight and *Fusarium* damaged kernels

1.2.1 *Fusarium* head blight

Fusarium head blight (FHB) occurs when wheat kernels are infected by *Fusarium* fungi (Parry et al., 1995). Outbreaks of FHB have been reported repeatedly in Canada (mainly Manitoba and Saskatchewan) and the USA (McMullen et al., 1997). Outbreaks are primarily a food safety concern because of the frequency and level of mycotoxins; but also an economic problem due to reductions in milling and processing quality. These effects on grain, including maize, are of great concern, since maize, wheat and barley constitute approximately 60% of the world grain production, and nearly 80% of the European grain production (Pronk et al., 2002).

Fusarium head blight was first described in 1884 in England, and was known as "scab" (Creelman, 1965), but later referred to as tombstone or blight disease because of the chalky, lifeless appearance of the infected kernels. The incidence of FHB is highest when the plant is exposed to moisture, mainly as rainfall, at the time of flowering (i.e., anthesis) or during the early kernel fill stages (Cheeke, 1998). The timing of this rainfall, rather than the amount, is the most critical factor (Canady, 2010). From 1991 – 1997, FHB was the major problem for wheat and barley growers in the Red River Valley region of Minnesota, North and South Dakota, with a reported economic loss of ~1 billion in 1993 alone. *Fusarium graminearum* has a sexual stage which is named *Gibberella zeae* that is the major causal agent in epidemics of FHB (CAST, 2003). *Fusarium* species overwinter on residue as mycelium (a mass of branching thread like hyphae), but then form perithecia (a flask shaped structure with an opening) in spring and early summer as wheat anthesis (flowering) occurs. Ascospores, the sexual spores of *G. zeae*, are considered the primary inoculum (Gilbert and Tekauz, 2000; Shaner, 2003) for FHB and are discharged from these perithecium. *Fusarium* species produce long, multicellular, canoe or banana shaped conidia which result in infection of host plants (Figure 1.1). Great potential exists for infection of plants with multiple *Fusarium* species resulting in contamination of grain with multiple *Fusarium* mycotoxins (Glenn, 2007). With increasing number of wet days following anthesis, the severity of *Fusarium* effects increases as well (Bianchini et al., 2015).

The four common *Fusarium* species known to cause FHB in Western Canada are *graminearum, avenaceaum, poae* and *sporotrichiodes* (Moss, 1991). *Fusarium graminearum* is the principle cause of FHB not only in Saskatchewan (Charmley and Trenholm, 2012), but also

Alberta and Manitoba (Gilbert and Tekauz, 2000). It is considered to be the most important *Fusarium* species because of its impact on grain yield and grain quality and its abundance in the Prairie region (Clear and Patrick, 2010).

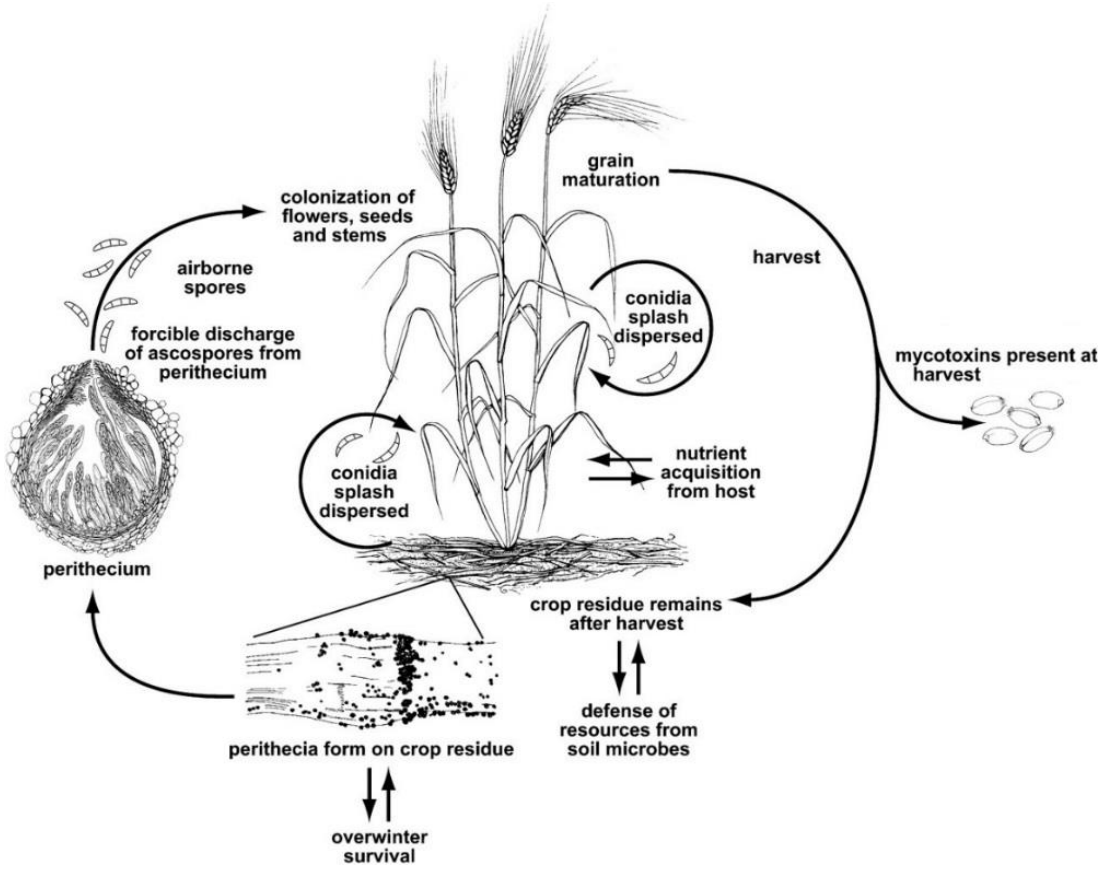

Figure 1.1. *Fusarium* head blight disease cycle. Adapted from Trail, 2009.

1.2.2 *Fusarium* damaged kernels

The physical change in the appearance of FHB infected kernels is collectively called *Fusarium* damaged kernels (FDK). The first sign of disease is premature discoloration or the bleaching of spikelets (Figure 1.2a), which become shriveled, indicating a disruption of kernel development (Figure 1.2b). This color change is an important sign, as it indicates that the infestation is contained to individual kernels, and not necessarily the entire grain head (CAST, 2003). Similarly, the mycotoxins associated with FDK are usually contained within kernels, minimizing more extensive contamination of the entire grain sample (Dr. Sheryl Tittlemier, personal communication, 2014). This indicates that it is possible to have individual kernels with

very high concentrations of mycotoxins but the overall contamination of the bulk grain is low (Streit et al., 2013).

Figure 1.2. Discoloration of kernels infected by *Fusarium* infections pre-harvest (a) and post-harvest (b). Adapted from the Canadian Grain Commission, 2013.

Grain are graded visually by many different criteria, including the percentage of FDK; the higher the percentage of FDK, the lower the yield, test weight and grade (Dexter et al., 1996; Dexter and Nowicki, 2003). Downgrading of grain due to FDK can greatly reduce the value and limit the use of grain (Table 1.2).

Table 1.2. The allowable limits as a percentage of *Fusarium* damaged kernels (%FDK) before grade reductions in Canadian Western Red Spring (CWRS), Canadian Western Soft White Spring (CWSWS) and Canadian Western Amber Durum (CWAD) wheat.

Wheat	CWRS	CWSWS	CWAD
Grade			
No. 1	0.25	1.50	0.50
No. 2	0.80	1.50	0.50
No. 3	1.50	1.50	2.00
Feed	4.00	4.00	2.00
AC Fusarium	<6.00	<6.00	<6.00
Salvage	>6.00	>6.00	>6.00

Adapted from the Canadian Grain Commission (2014a).

If these damaged kernels could be safely removed from grain, it would have a significant impact on the agricultural industry and the safety of feed and food. In addition, there would be

less potential for the spread of FHB when cleaned grain is used as seed. The Alberta Agricultural Pests Act indicates growers cannot sell, acquire, distribute or use *Fusarium* infected kernels for propagation purposes (Alberta Pests Act, 2012).

1.3 *Fusarium* mycotoxins and most common varieties reported in North America

1.3.1 *Fusarium* mycotoxins

Fusarium is the most prevalent mycotoxin producing fungal genus found on grain grown in the northern temperate regions (Murugesan et al., 2015). There is a relationship between *Fusarium* infection and mycotoxin contamination (D'Mello and Macdonald, 1998; Foroud et al., 2012). However, *Fusarium* species do not significantly contribute to mycotoxin production post-harvest. The concentrations of mycotoxins have been shown to decline from initial infection to harvest (Jouany, 2007) where it stabilizes even though the spread of *Fusarium* continues throughout maturation (Cowger and Arellano, 2013).

There are 24 toxigenic species of *Fusarium* which infect a wide range of agricultural crops and can have important effects on human and animal health (FAO, 2004). There is increasing evidence of global contamination of grain and animal feeds with *Fusarium* mycotoxins, and the grain trade may contribute to the worldwide dispersal of toxicogenic strains. These fungi infect wheat, barley, oats, rye, rice, sorghum, millet and maize, and can cause severe yield reductions (Pronk et al., 2002). *Fusarium graminearum* and *F. culmorum* are the most common species found worldwide. *Fusarium graminearum* grows optimally at a temperature of 25°C and above 88% relative humidity (RH). *Fusarium culmorum* grows optimally at 21°C and above 87% RH (Canady, 2010). The geographical distribution of the two species appears to be related to temperature. Forecasting systems such as the US *Fusarium* head blight risk assessment tool and Canada's DONcast® model are used to predict the risk of mycotoxin production, specifically DON to make fungicide application more efficient (Wegulo et al., 2013).

1.3.1.1 Trichothecenes

The trichothecenes are the most chemically diverse of the *Fusarium* mycotoxins, with approximately 200 varieties characterized as mycotoxins (Lazicka and Orzechowski, 2010). Trichothecenes are a family of chemically related organic compounds consisting of three isoprene molecular structures called sesquiterpenoids. They have a tetracyclic 12, 13-epoxytrichothecene skeleton which provides their stability and reactivity, and can be divided into four categories: Type

A (characterized by a functional group at C-8); Type B (characterized by a carbonyl at C-8); Type C (characterized by a second epoxide group at C-7, 8 or C-9, 10) and Type D (characterized by a macrocyclic ring system between C-4 and C-15 with two ester linkages) (WHO, 1990). Type A includes T-2 toxin, HT-2, neosolaniol (NEO) and DAS. Type B includes DON and its acetyl derivatives (3-ADON, 15-ADON), NIV and fusarenon X (FUS-X). Types C and D are not normally produced by *F. graminearum* (Placinta et al., 1999; Figure 1.3) and are less common in Canada.

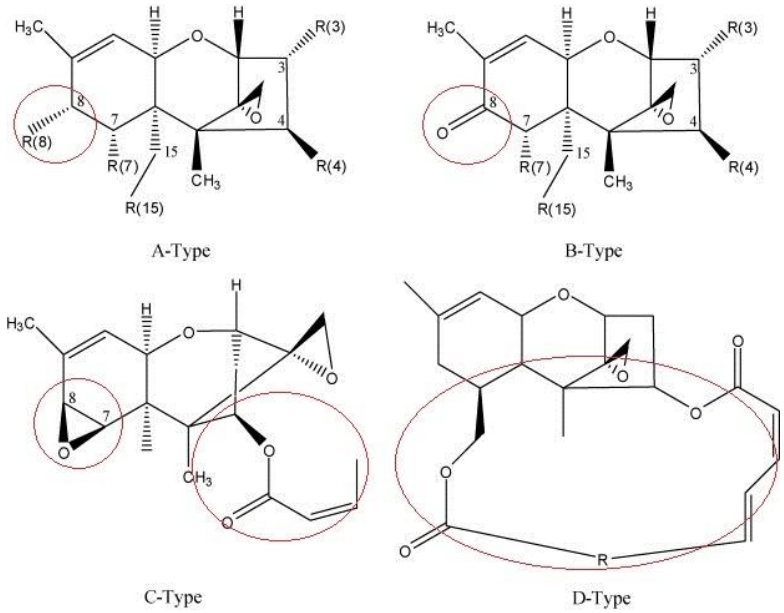

Figure 1.3. Differentiations in trichothecene skeletal and epoxide group types (A-D). Adapted from Turker and Gumus 2009.

Trichothecenes are generally considered to be potent inhibitors of protein synthesis, by binding to the ribosomal 60S subunit (Awad et al., 2008a). This causes inhibition of the initiation, elongation and/or termination stages for protein synthesis and mitochondrial function (Murugesan et al., 2015), with some suggestion of effects on lymphocytes due to up-regulation of cytokine production (Awad et al., 2008a). The structural diversity of toxic trichothecenes indicates that this binding occurs regardless of the variation in chemical structure as long as the skeletal and epoxide group integrity are preserved (Karlovsky, 2011). Differences in toxicity are difficult to differentiate (Pronk et al., 2002) but may be defined by the ability of individual trichothecenes to cross phospholipid membranes (Wu et al., 2013). Trichothecenes are produced under conditions of prolonged periods of high humidity and warm temperature during crop growth, and/or cool, wet

harvest conditions (McMullen et al., 2008). It is rare for only a single trichothecene to occur in contaminated feed. Usually there are multiple types of trichothecenes occurring together in feed at varying concentrations (Pronk et al., 2002).

Of the trichothecenes, DON, NIV, ZEA and FUM are of major concern due to their increase in global distribution and effects on overall animal health. However, concentrations of NIV and ZEA are usually lower in comparison to DON (Pronk et al., 2002). Incidence of mycotoxins in feed samples from Western Canada from 1982 – 1994 determined that DON and other type B trichothecenes were the most commonly observed, followed by HT-2 toxin, other type-A trichothecenes, and OT-A. The increase in DON in similar surveys of the 1970's and early 1980's reflected both the improvement in analytical methods and the spread of FHB in Western Canada. Co-occurrence of *Fusarium* mycotoxins in grain and animal feed has raised the question of interactions in the manifestation of toxicity. How do these co-occurring mycotoxins interact in their toxicity? Additionally, it is possible that fungi may be spread from one country to another with increased global grain trade, as there has been consistent, overwhelming evidence of global contamination of grain and animal feeds with *Fusarium* mycotoxins (Placinta et al., 1999). A number of trichothecenes are similar in structure and/or mechanism of action, and therefore are difficult to differentiate from one another (Pronk et al., 2002).

1.3.1.1.1 Deoxynivalenol

Deoxynivalenol is a trichothecene mycotoxin produced mainly by *F. culmorum* and *F. graminearum* including two chemotypes, one producing NIV and DON and the other a mixture of DON and the acetylated derivatives 3-ADON and 15-ADON (Perkowski et al., 1997). Deoxynivalenol is commonly referred to as vomitoxin (Vesonder et al., 1973; Cheeke, 1998) due to the feed rejection of pigs at low doses and emetic syndromes (i.e., vomiting) at high doses (Prelusky et al., 1994). Due to its prevalence, DON has been used as a marker for *Fusarium* infestation and it has been suggested that the concentration of DON is usually expected to be higher than that of other mycotoxins in feedstuffs naturally contaminated with *Fusarium* mycotoxins (Girish et al., 2010). The relative susceptibility of animals to DON varies with species. Pigs are the most susceptible domestic animal, followed by mice, poultry and ruminants (Prelusky et al., 1994). Differences in sensitivity are thought to result from differences in the absorption, distribution, metabolism and excretion characteristics (Pestka and Smolinski, 2005).

The reduced sensitivity of poultry compared with pigs has been shown to be due to their ability to detoxify the DON molecule to a less toxic form that mostly disappears between the crop and jejunum (Lun et al., 1988). These less toxic metabolites are excreted rapidly (Pestka, 2010) due to their water soluble nature (Bianchini et al., 2015). The protein synthesis effects of DON are related to a ribosomal stress response (impairment of translation of ribosomal RNA) and the phosphorylation of and activation of mitogen activated protein kinases (Grenier et al., 2011). Other toxic mechanisms include the activation of intracellular protein kinases, leading to apoptosis of all rapidly dividing cell types such as immune, gastrointestinal tract and liver cells (Pestka et al., 2004). Cases of acute poisoning with DON are relatively rare; however, chronic exposure of animals is a more serious concern (Rotter et al., 1996; Placinta et al., 1999; Canady, 2010). Since DON is considered the most important economic mycotoxin produced by *F. graminearum*, it is regulated in grain and food products in Canada, the US and most European countries (Bai et al., 2001). The DON mycotoxin is the most often reported next to aflatoxin, and is the most closely monitored in feed materials as a general representative trichothecene at the parts per million (ppm) level (Rotter et al., 1996).

The proportion of FHB and FDK to DON levels in grain has been positively correlated (Beyer et al., 2007; Gautam and Dill-Macky, 2012). Surveys from South America, Canada, China and Europe have showed DON contamination incidence in excess of 50% in oats, barley and wheat, with the mean concentration as high as 9 ppm (Moss, 2002). A joint study between Manitoba Agriculture and the Canadian Grain Commission indicated that DON is usually found in FDK. In most samples, negligible levels are found in non-damaged kernels and in the stem (Canadian Grain Commission, 2013). An overview of the DON concentrations found in Western Canada in 2011 is shown in Figure 1.4.

Reduced feed intake resulting from DON contamination is a serious issue pertaining to the overall health of animals. While this reduction in consumption decreases the mycotoxin intake, it constrains the nutrients and energy available for metabolic processes such as growth, which are more damaging than the direct toxic effect (Doll and Danicke, 2011).

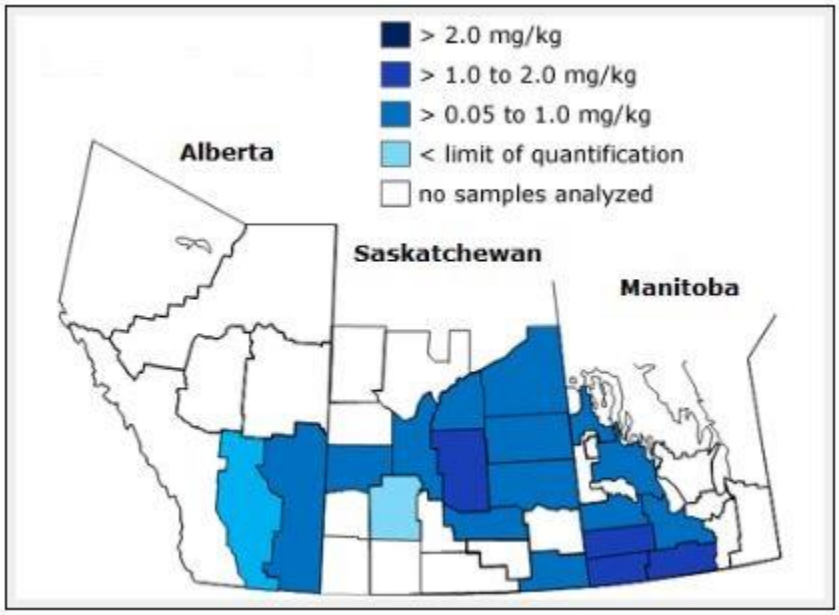

Figure 1.4. Average concentrations of DON (mg/kg; ppm) in Canadian wheat samples collected in Alberta, Saskatchewan and Manitoba during the 2011 year. Adapted from the Canadian Grain Commission, 2014b.

1.3.1.1.2 T-2 toxin

Type A trichothecenes are more toxic to poultry species than the Type B trichothecenes (Richard, 2007). Among the naturally occurring trichothecenes, T-2 toxin and DAS are the most toxic to animals (Lazicka and Orzechowski, 2010). T-2 toxin is produced by *F. sporotrichiodes* (Placinta et al., 1999), and is the most acutely toxic (Wu et al., 2010). The mechanism of action is similar to DON, involving the inhibition of protein synthesis, but T-2 binds peptidyltransferase, an integral part of the 60S ribosomal subunit (Rafai et al., 2000). T-2 may be associated with a fatal disease in humans called "Alimentary toxic aleukia" reported in Europe, but not North America (CAST, 2003). Visible evidence of the fungus may appear on corn as white mold growth, which in some instances, may appear pink to reddish, often beginning at the tip of the ear (Richard, 2007). The effects of T-2 toxicosis in young broiler chicks have been extensively studied (Jewers, 1990).

1.3.1.1.3 Diacetoxyscirpenol

Diacetoxyscirpenol is more likely associated with chronic toxicity of poultry as compared to T-2 (Jewers, 1990), and is rapidly and extensively absorbed and widely distributed in tissues

and organs. Relatively few studies have included repeated administration of DAS, some used only one dosage level. Although not fully understood, the mechanism of action of DAS is similar to T-2 (Rafai et al., 2000) and potentially other Type A trichothecenes (Pronk et al., 2002).

1.3.1.2 Zearalenone

Zearalenone is sometimes called F-2 toxin. The mechanism of action pertains to the competition with 17-beta estradiol (β-E2) for estrogen receptor binding sites, causing hyper-estrogenic effects and hormonal changes (Richard, 2007). Zearalenone has two metabolite forms: alpha (α) and beta (β) zearalenol, which have even higher binding affinities for the estrogen receptors, suggesting an increased potency compared to ZEA (Fink-Gremmels, 2008). However, with the exception of potential reproductive effects, ZEA is generally considered one of the least toxic trichothecenes in animals (Prelusky, 1993). It is distributed worldwide and is most commonly detected in corn (Cheeke, 1998), but is also detected in wheat, barley, sorghum and rye (CAST, 2003). Many *Fusarium* species including *graminearum, culmorum,* and *roseum* produce ZEA. Its co-occurrence with other trichothecenes provides potential issues regarding additivity and/or synergism mycotoxicoses in animals (Placinta et al., 1999). It sometimes co-exists with DON, but generally DON is detected in higher concentrations than ZEA (Bailly and Guerre, 2008). Grain infected with ZEA producing fungi may exhibit pink spore masses. In wheat, the conditions for the occurrence of ZEA are essentially the same as for DON. Swine are the most commonly affected and sensitive species to ZEA (Prelusky et al., 1994) while poultry are considered relatively resistant (Cheeke, 1998).

1.3.1.3 Fumonisins

The *Fusarium* species *moniliforme* and *proliferatum* produce the mycotoxins FUM, moniliformin and fusarin C (Placinta et al., 1999). The fumonisins are structurally related molecules consisting of long hydroxylated hydrocarbon chains (Heidtmann-Bemvenuti et al., 2011). The structural similarity between FUM and sphingosine is the basis for the toxic mechanism of action. This involves reducing sphingolipid formation through inhibition of sphingosine biosynthesis from sphinganine, causing an increase in free sphinganine that can impact nervous tissues (Richard, 2007). Six different variants have been isolated and characterized with FUM B1 the major metabolite (Norred, 1993). Corn is the major commodity affected by this group of mycotoxins, with sorghum and rice also occasionally contaminated (CAST, 2003).

Infected kernels or areas on an ear may exhibit “pink kernel rot” (sometimes appears white) with closely adhering fungal organisms on the kernels (Marasas et al., 1988).

1.3.1.4 Other *Fusarium* mycotoxins

Other lesser known *Fusarium* mycotoxins include NIV, FUS-X, NEO, Scirpentriol (SCIRP) and the DON metabolites, 3 and 15-ADON.

Nivalenol is produced by the fungal species *F. kyushuense* (Aoki and O’Donnell, 1998) and *F. poae* (Pettersson et al., 1995). It is the hydroxylated form of deoxynivalenol (IARC Monograph, 1997). Nivalenol is limited in distribution worldwide and is uncommon in Canada (Wu et al., 2012; Miller and Richardson, 2013), but is mostly found in grain and animal feed, often in co-contamination with DON (Konishi-Sugita and Nakjima, 2010). It is about ten times more toxic than DON, but due to its limited nature, it is not well studied (Mirocha et al., 1989; Kim et al,. 2003). Acute and chronic studies have been reported, but with doses that exceeded typical levels (Kawasaki et al., 1990).

Fusarenon-X is produced by the fungal species *F. crookwellense*. It is a precursor to nivalenol (Konishi-Sugita and Nakjima, 2010) which is the active metabolite. It rarely occurs in grain unless in co-occurrence with NIV or ZEA. Its toxicity is through the covalent binding of FUS-X molecule to sulphydryl groups on RNA, causing inhibition of protein synthesis (Pronk et al., 2002).

Neosolaniol (NEO) is produced by the fungal species *F. tricinctum*. It has been found in peanuts at low levels (Lansden et al., 1978). Given its structural similarity and toxicity, it is suggested that the mechanism of action of NEO is similar to DAS in terms of type and severity (Pronk et al., 2002).

Scirpentriol (SCIRP) is produced by the fungal species *F. equiseti* and *F. semitectum*. It has two main derivatives; 15-acetoxyscirpentriol and DAS. It is typically found in wheat, oats, soya beans and potatoes in low concentrations (Perkowski et al., 2003). The mechanism of action is inhibition of protein synthesis (Smith, 1992).

Both 3-ADON and 15-ADON are derivatives of DON (O’Donnell et al., 2000), and sometimes co-occur at low concentrations. They are considered equivalent to or less toxic than DON (Pestka, 2007). De-acetylation of 3-ADON to DON occurs in the liver (Ohta et al., 1978). Acute effects have only been reported with concentrations higher than normally expected to occur naturally (Kasali et al., 1985). Chronic exposure has been associated with minimal reductions in

feed consumption, weight gain and feed efficiency (Rotter et al., 1992). Most studies in the literature were conducted on mice. Very little data exist on the mechanism of toxicity of 3-ADON, other than reports of cytotoxicity to cultured cells and protein synthesis inhibiting activity. No data are available on the specific mechanism of toxicity of 15-ADON (Pronk et al., 2002).

1.4 Toxicological and economic effects of mycotoxins

1.4.1 Toxicological effects

The symptoms of mycotoxicosis depend on the type of mycotoxin, dose, length of exposure, and other factors such as species, age, health, and gender of exposed animals. The diagnosis of mycotoxicoses can be difficult due to possible co-occurrence and interaction of mycotoxins, formation of potentially toxic metabolites, and masked mycotoxins causing common clinical signs of disease (Richard, 2007). Most mycotoxins affect more than one target organ or system simultaneously, causing a multiplicity of biological responses (CAST, 2003). Understanding the mechanism of mycotoxin action at cellular and biochemical levels is important to the overall goal to treat or prevent mycotoxicosis.

Aflatoxin, ochratoxin, trichothecenes, zearalenone, fumonisins and ergot alkaloids represent the greatest public and animal health concerns (Hussein and Brasel, 2001). The clinical signs of mycotoxicosis are very diverse and include feed refusal and vomiting, impaired reproduction and reduced fertility, nephrotoxicosis, neurotoxicosis, hematopoietic effects, hepatotoxicosis, cancer, reduced immune function and reduced performance (growth rate and feed efficiency). Most of these effects occur after consumption of mycotoxin contaminated grain or products made from such grain, but other routes of exposure (litter, dermal and inhalation) exist (Richard, 2007). The widespread occurrence and potential potency of mycotoxins make this group of naturally occurring mycotoxins an ongoing animal health hazard, and a constant risk for contamination in the human food supply.

1.4.2 Economic impact

Worldwide contamination of feeds with mycotoxins is a growing problem (Hussein and Brasel, 2001). The Food and Agriculture Organization (FAO) has estimated that 25% of the world's crops are contaminated (at least at trace levels) with mycotoxins (Fink-Gremmels, 1999). Multiple criteria have been used to assess the severity of the economic impact of this global problem on humans and animal agriculture, including loss of life, human health care costs,

veterinary costs, decreased livestock productivity, loss or downgrading of feeds, and regulatory and research costs (Hussein and Brasel, 2001). Direct economic losses resulting from mycotoxin contaminated crops can be measured in reduced crop yields and lower quality, reduced animal performance (weight gain, feed efficiency) and productivity, interference with reproductive capabilities, and increased disease incidence due to immune suppression (Wu, 2004). These losses are much more significant than acute livestock death. Reduction in immune capacity also reduce efficacy of vaccines and may increase the need for antibiotics to treat secondary infections. More than 100 countries representing 90% of the world's population have specific regulatory limits for various mycotoxins in foods and feeds (van Egmond, 2004; van Egmond et al., 2007). Since FDK have been strongly correlated with mycotoxins, such as DON, their detection in grain sources is an issue of food safety (Beyer et al., 2010).

1.5 Grain sorting: Near infrared (NIR) spectroscopy and kernel sorting technology

Visual measurement of FDK, as used for grading grain, can be laborious and is subject to inconsistencies resulting in variability from intra-grader repeatability and/or inter-grader reliability. Chemical assessments (i.e. Kjeldahl and wet chemistry analysis for mycotoxins) require time, the destruction of the sample, and are expensive. Near infrared (NIR) spectroscopy is a technique that reduces the need for chemical analysis (after calibrations are validated) and does not require destruction of sample by relating the spectral information to specific characteristics (Pojie and Mastilovic, 2013). Wilbur Kaye demonstrated the theoretical principles of infrared spectroscopy and instrumentation analysis through the absorption of NIR wavelengths in the 1950's (Barton, 2002). There are four methods to detect the absorbed NIR wavelengths: interactance, transflectance, transmittance and reflectance, with the latter two being most common. Transmittance is the measurement of the wavelength as it passes through the samples, while reflectance is a measurement of the reflected wavelength. Interactance and transflectance use both transmittance and reflection analysis methods.

Karl Norris (1964) used multivariate statistical regression methods to correlate NIR spectral features with reference values, allowing the determination of moisture, protein and starch (Osborne and Fearn, 1986). These spectral features have also been used to evaluate basic intrinsic properties such as protein, oil and moisture (Williams and Sobering, 1993). Delwiche (1995; 1998) showed that hardness and protein, among other factors, produce accurate classifications by

NIR. The NIR spectroscopy analysis is based on the measurement of overtones and vibrations of molecules in a sample when exposed to light of different wavelengths. The energy peak in an absorption spectrum corresponds to the frequency of the vibrations (Stuart, 1997) producing a distinct calibration. Studies by Dowell et al. (1999), Pettersson and Aberg (2003) and Delwiche and Hareland (2004) indicated that the use of spectral data may allow for more precision in determining mycotoxin contents in grain sources compared to the visual approach. Pojie and Mastilovic (2013) suggested calibration models in combination with multivariate analysis such as multiple linear, principal component regression or partial least squares provide a relationship between the spectral data and the properties of the sample. The main functional groups producing these vibrations are; C-H, O-H, N-H and C=O (Murray and Williams, 2001). Near infrared spectroscopy is regarded as the legal method for determining crude protein (CP) content in grain (Pedersen et al., 2002) and could be used in the purchase and processing of grain for improved functionality and safety (Williams 2007; 2008).

A single kernel NIR (SKNIR) system was developed by Perten Instruments in Stockholm, Sweden to sort individual kernels for FDK. This system demonstrated a high level of accuracy (Dowell and Maghirang, 2007). A similar method was used by Peiris et al. (2010) to differentiate uninfected kernels from FDK. They found that the SKNIR system could distinguish between the two with ~99% accuracy. The FDK fraction could then be sorted into subsamples with low and high DON concentrations. The authors concluded that not all FDK contain DON. Results suggested that SKNIR could be used as a non-destructive, accurate, consistent and low cost method in FDK detection; however, the system could not sort bulk grain samples at a commercially viable rate. Beyer et al. (2010) evaluated the effectiveness using spectrometric reflectance data from grain sources to assess mycotoxins instead of the standard visual disease assessment. They concluded that the wavelengths of 1400 and 1900 nm showed different spectrometric signal intensities between healthy and damaged kernels with an accuracy of 100%. This reflectance data also showed a potential method of estimating mycotoxins, particularly DON content if the average FDK spectra range spanned 350 - 2500 nm. The largest differences in wavelength were at the 1408, 1904 and 1919 nm ranges when only DON was considered (Peiris et al., 2009). It was noted that the accuracy of spectral wavelengths predictive models is affected by environmental factors as indicated by Petterson and Aberg (2003).

1.6 BoMill TriQ assessment

The TriQ individual kernel sorter (BoMill, Sweden) has commercial capacity to analyze individual kernels using Near Infrared Transmittance (NIT) spectroscopy. This technology can sort individual kernels into calibration or commercial fractions based on chemical characteristics such as FDK, CP, hardness and vitreousness. The basic principles and design of the TriQ are shown in Figure 1.5. Presently, the technology is designed to sort barley, spring and durum wheat. Grain is fed into the center of a specifically designed drum or singulator (e.g. separate singulators for each grain type due to differences in kernel size) that has multiple rows of individual pockets for kernel placement. These pockets collect and hold individual kernels by the centrifugal force of the cylinder rotation. As the kernels are rotated past the NIT detector, it detects specific light spectra generated from the center of the cylinder through each kernel via a small opening in the bottom of each pocket. The transmittance spectral details of each kernel are then communicated to the ejection units that uses forced air to eject the appropriate kernels into one of three collection tubes. Kernels then slide through the collector tubes by gravitational force (BoMill, 2012). The TriQ system has the capacity to sort 2 - 3 metric tonnes (MT) of grain per hour into three discrete fractions, referred to as a commercial sort based on the user selected criteria (BoMill, personal communication, 2013).

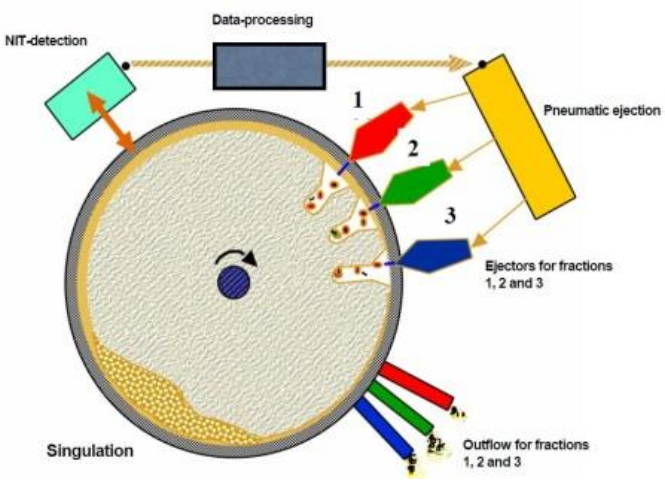

Figure 1.5. Basic principles and design of the TriQ. Adapted from BoMill, 2012.

The TriQ technology enables sorting for different chemical characteristics that would potentially improve grain quality and grade through reduction in variability and produce high quality food or feed from low quality ingredients, thereby maximizing safety and value (Agriculture and Agri-Food Canada, 2013). This technology may have the capability to improve the safety and functionality of contaminated bulk grain by removing the uncontaminated grain, increasing availability for human and animal consumption. With a growing emphasis on safe, high protein and quality grain for food production, the ability to remove FDK efficiently would have a significant impact on agricultural economics. Improving grain quality by the removal of FDK and mycotoxins by this kernel sorting would concentrate FDK and mycotoxins in the remaining grain, the disposition of which would represent an area for future research.

1.6.1 Physical variables: length, width, height and weight

Delwiche et al. (2005) showed that kernel morphology was a primary feature used for differentiating healthy and damaged grain. *Fusarium* infected grain has reduced thousand kernel weight (TKW; Schwarz et al., 2001) and yield (McMullen et al., 1997). It has been suggested that *Fusarium* infection impedes water flow and nutrient absorption to the upper spike at the infection point (Savard et al., 2000), reducing the nutrients available for normal kernel development. Grain shape, size, density and uniformity are key grading factors. Evers (2000) indicated that ideal kernels are large and spherical and these size characteristics are associated with protein content and are directly associated with milling performance and end use suitability. The weight of individual kernels tend to differ between healthy and damaged kernels but not between cultivars and not consistently. Studies have shown that separation based on physical characteristics was effective at removing *Fusarium* infected grain, however it was noted that many *Fusarium* infected kernels are physically indistinguishable from healthy kernels, and in the case of DON, removal of infected grain does not necessarily indicate mycotoxin removal (Scudamore and Patel, 2008).

1.6.2 Chemical variables: moisture, crude protein, starch, fibre

Wheat kernels infected with *Fusarium* differ in chemical composition from healthy kernels, resulting in lower grain quality (Jirsa and Polisenska, 2011) mainly through changes in starch and CP concentrations. The effect of *Fusarium* on CP is not consistent, as both reduced (Bechtel et al., 1985; Dexter et al., 1996) and increased (Trenholm et al., 1984; Mielke and Meyer, 1990; Matthaus et al., 2004) levels have been shown. Miller (2003) indicated that kernels in the

lower section of wheat heads contained higher CP than middle or top sections. Kernel hardness and protein content are important properties in bread making (Snape et al., 2005). Protein also affects dough properties and bread volume through gluten levels (Boz et al., 2011). Thousand kernel weight has been used as an indicator of potential flour quality by the milling industry (Mut et al., 2010). Nightingale et al. (1999) compared various analytical methods to determine differences in protein content. They found that total storage protein was lower with FDK than healthy kernels. Beyer and Aumann (2008) investigated the effects of *Fusarium* infection on the structural building blocks of proteins, the amino acids (AA). They concluded that *Fusarium* infection needed to be very severe for changes in AA composition to occur. These changes could result in either an increase or decrease in AA availability.

1.6.3 Masked mycotoxins

Masked mycotoxins are mycotoxin derivatives comprised of extractable conjugated and bound (non-extractable) metabolites that tend to produce an underestimation of the total mycotoxin concentration in a sample. Impaired extraction efficiency (or masking) is caused by changes in polarity, affecting the binding capability with carbohydrates or protein matrixes. Overestimation of risks may also occur when modification of the mycotoxin reduces or eliminates its toxicity. An example is deoxynivalenol-3-glucoside (D3G), which is the conjugation of DON to a glucose molecule during the malting process (Berthiller et al., 2013). This masked mycotoxin has reduced ability to inhibit protein synthesis compared to DON. Other *Fusarium* mycotoxins with masked mycotoxins include ZEA and FUM. There are limited data on some recent emerging *Fusarium* mycotoxins, (fusaproliferin, beauvericin, enniatins and moniliformin) but their occurrence has been found to be high in some food products (Escriva et al., 2015). Masked mycotoxins were usually undetectable by analytical methods due to chemical transformations in plants (Tran and Smith, 2011). These transformations are catalyzed by plant enzymes involved in the detoxification process. Recently developed methods have allowed the concentrations of these previously hidden metabolites to be determined (Murugesan et al., 2015).

1.7 Poultry as a model species

Many *Fusarium* mycotoxins have the potential to adversely affect poultry (D'Mello et al., 1999), however, differences in metabolism and detoxification capabilities between genetic lines cause some poultry species to be less sensitive (Awad et al., 2012) or even resistant (Swamy et al.,

2004a) to *Fusarium* mycotoxins with broiler chickens appearing to be the most tolerant. As a consequence, mycotoxins in contaminated feed are often diverted away from swine for use in poultry feed (Girgis and Smith, 2010). The co-occurrence of different *Fusarium* mycotoxins in grain (Abidin et al., 2011) may result in higher than expected concentrations to be found in these poultry diets. Although poultry may be relatively more resistant than pigs or cattle, poultry feeding trials can better provide essential information on the effects of mycotoxins due to lower feed and exposure time requirements. Examples of the effects of *Fusarium* mycotoxins on poultry performance and immune function are summarized in Table 1.3.

Table 1.3. Summary of previous exposure studies of important trichothecene mycotoxins, including test species, study duration, concentrations and effects.

Mycotoxin	Broiler / Age (d)	Toxin level (ppm)	Effect	Reference
DON	21	0; 9; 18	No effects on growth parameters but reduced liver mass and increased relative weights of heart, bursa of Fabricius and gizzard.	Kubena et al. (1985)
	35	0.1; 3.4	No effects seen on feed intake, weight gain or histological parameters.	Bergsjo and Kaldhusdal (1994)
	21	0; 1; 5; 10; 16	No effects seen on weight gain, FCR but decreased intestine weight; no effects on histological, hematological or serum parameters.	Awad et al. (2006b; 2011); Harvey et al. (1997)
	21	0; 16	No effects seen on feed intake, weight gain, hematological or serum parameters but increased relative weights of heart, bursa of Fabricius and gizzard.	Kubena et al. (1989)
	56	0; 5.9; 9.5	No effects on feed intake or weight gains in starter or finisher period. No effects on liver, kidney, spleen and bursa of Fabricius. Other parameters such as FCR, differential leukocyte count, serum parameters, skin thickness to DNCB and antibody responses were unaffected.	Swamy et al. (2004a)
	56	0.14; 4.7; 8.2; 9.7	No effects on feed intake, body weight, FCR, hematology, IgG or IgM. Quadratic responses were noted in serum concentrations of albumin and linear responses to IgA.	Swamy et al. (2002)

Table 1.3 continued

	42	0; 10	No effects on weight gain or FCR but decreased glucose absorption.	Awad et al. (2004)
	35	0; 10	Reduced feed intake and body weight, antibody titers. Increased FCR, heterophil to lymphocyte ratio. No effects on thymus, spleen or bursa of Fabricius.	Ghareeb et al. (2012)
	21	0.5; 3-4; 6-9; 17-18	Linear reduction of body weight and heterophil to lymphocyte ratio. Feed intake, crypt depth unaffected. Increased villi height.	Xu et al. (2011)
	21	0; 4	No effects on body weight, feed intake or FCR. Increased incidence and severity of oral lesions.	Kubena et al. (1989)
T-2	42	1; 2; 4	Reduced body weight and serum parameters but increased FCR. No effects on heterophil to lymphocyte ratio and hematological parameters.	Pande et al. (2006)
	35	0; 3	Reduced body weight and feed intake. No effects on liver and gizzard weight, serum levels or hematological parameters.	Raju and Devegowda (2000)
	21	0; 0.5; 1; 2; 4	Reduced body weight and feed intake but increases in mouth lesions	Ademoyero and Hamilton (1991)
DAS	35	0; 0.2; 0.5; 1	No effects on body weight, feed intake, histopathological or antibody parameters but increased oral lesions.	Sklan et al., 2001
	21	0; 10 – 800	No effects on body weight, feed intake, FCR, liver, heart, spleen or bursa of Fabricius.	Allen et al. (1981)
ZEA	21	0.3/kg BW	No effects seen after oral or intravenous administration.	Osselaere et al., 2013
	21	0; 300	Increased relative weights of liver and kidney, increased protein and albumin concentrations in serum. Reductions seen in body weight gains and higher FCR.	Kubena et al. (1997)
FUM	41	0; 50; 200	Decreased body weight, weight gain, and antibody titers. Increased relative heart weight and histological changes in the liver and kidney with cell proliferation in the liver.	Tessari et al. (2006)

Definitions: deoxynivalenol (DON), T-2 toxin (T-2), diacetoxyscirpenol (DAS), zearalenone (ZEA), fumonisins (FUM) and feed conversion ratio as feed to gain (FCR).

Many studies have demonstrated that mycotoxin exposure may impair nutrient utilisation. Bryden (2012) suggested this observation to be the result of mycotoxin-induced malabsorption. Discussions of mycotoxins often neglect the fact that fungal growth and metabolism may change the nutrient composition of infected feedstuff (Bryden, 1982). Synergistic and additive effects of mycotoxins have been reviewed in poultry (Pedrosa and Borutova, 2011) and indications are that weight gain is decreased more with certain combinations (i.e. DON and T-2 toxin) than with individual toxins. An examination of the influence of *Fusarium* infection and mycotoxin contamination on the nutrient value of grain demonstrated that some *Fusarium* mycotoxins increased CP, apparent metabolizable energy (AME) and AA availability in wheat (Trenholm et al., 1984).

1.8 Overview of poultry immunity

The poultry immune system has developed defenses against a wide range of pathogens. The two interactive categories of the immune system are the innate (non-specific) and acquired (specific). Innate immunity consists of phagocytic functions as the main defense to remove pathogens by various cell types including heterophils (Erf, 2004), which are highly efficient in phagocytosis and bacterial elimination, and are usually the first response to inflammation (Harmon, 1998; Fairbrother et al., 2004). As innate immunity lacks specificity, no antibodies are produced, allowing protection against multiple types of pathogens as the first line of defense (Fairbrother et al., 2004). When specific targeting is required for pathogen destruction, adaptive immunity is essential. This immunity is developed through exposure to pathogens (Grogan et al., 2008) with T helper cells which have a regulatory role through the stimulation and differentiation of lymphocytes (T and B cells), resulting in the elimination of pathogens and newly produced protection from a repeat exposure (Erf, 2004). A repeat exposure creates a "memory", which leads to a stronger, quicker response providing the basis for protection and vaccination protocols (Scott, 2004). Acquired immunity can be subdivided into a humoral response and cell-mediated response that consists mainly of B and T cell involvement, respectively (Fairbrother et al., 2004).

The immune system is considered to be the primary target of many trichothecenes (Bai et al., 2001). Most mycotoxicoses result from chronic, low level ingestion which can result in a measureable decline in immune function (D'Mello et al., 1999) through reductions in nutrient availability to support the immune response (Pestka, 2007). Tissues with higher protein turnover and rapidly dividing cells are especially affected by *Fusarium* mycotoxin exposure (Awad et al.,

2012). Numerous studies on resistance, antibody responses and cell-mediated immunity indicate that the structure of mycotoxins is a determinant in the immunostimulatory or immunosuppressive effect depending on dose, exposure time and age (Rotter et al., 1996; Bondy and Pestka, 2000; Danicke, 2002; Pestka et al., 2004). *Fusarium* mycotoxin induced immunosuppression manifests as depressed lymphocyte proliferation, decreases in resistance, immunoglobulin synthesis and antibody responses (Bondy and Pestka, 2000). The common mechanism of action for this immunosuppression is the inhibition of peptidyl transferase activity. Immune responses to different mycotoxins are highly variable (Pestka, 2008) and correlations between *Fusarium* mycotoxin mechanisms of action and immunotoxicity are still not fully understood (Murugesan et al., 2015).

Fusarium mycotoxin immune modulation might be considered as a spectrum of immune effects. High dose trichothecene exposure severely injures actively dividing tissues including bone marrow, lymph nodes, spleen, thymus, and intestinal mucosa that can result in immunosuppression (Ueno, 1984). Recent work by Grenier et al. (2011) showed low dose exposure impaired the stimulation of the humoral mediated lymphocytes that had been activated by an antigen, but had no effect on lymphocytes involved in the cell-mediated response. This would indicate that *Fusarium* mycotoxins do not cause immunotoxic effects, but instead they interfere with the signaling pathways of the immune system, affecting cell growth, apoptosis and overall immune responses, increasing susceptibility to infection. Evaluating immunological changes due to mycotoxin contaminants can provide information about the potential adverse effects of sub-lethal exposures (Fairbrother et al., 2004).

1.8.1 Immunotoxicity assays and assessment

The effects of mycotoxins on the immune system are an important consideration when determining consequences of mycotoxin exposure on poultry performance and health, including resistance to infections. These effects are often difficult to recognise because the signs of disease are associated with the infection rather than mycotoxin ingestion (Bondy and Pestka, 2000; Oswald et al., 2005). The immunosuppressant effect of mycotoxins occur at lower levels of intake than effects on performance such as growth rate. During mycotoxin induced immunosuppression leading to infections, nutrients are diverted away from growth and development to the immune system (Humphrey et al., 2004; Klasing, 2007) further contributing to reduced performance. Mycotoxins also reduce the effectiveness of vaccination programs (Oswald et al., 2005).

Commonly used *in vivo* tests for assessing immunotoxicity include measuring antibody titers in response to immunization with a foreign antigen, and delayed type hypersensitivity response to T cell mitogen challenge (Baecher-Steppan et al., 1989).

1.8.2 Cell-mediated response and assessment

The cell-mediated response is moderated by T cells produced in the thymus in conjunction with B cells. T cells are antigen specific (Grogan et al., 2008) and can enhance (via T helper cells) or suppress (via T suppressor cells) cell-mediated immunity responses (Chen et al., 1991; Cooper et al., 1991). In order for T cells to recognize an antigen, a fragment of that antigen must be bound to the T cell receptor on the surface of a cell (Erf, 2004) through the major histocompatibility complex. Once this binding occurs, the cell is called an antigen presenting cell (Fairbrother et al., 2004). The binding stimulates proliferation and differentiation of T cells into effector and memory cells. Effector cells can either enhance or suppress the immune response (Larsson and Carlander, 2002). Memory T cells function similar to memory B cells, but they can also differentiate into effector cells to eliminate the antigen (Fairbrother et al., 2004).

The most commonly used evaluation of cell-mediated immunity is the phytohemagglutinin (PHA) swelling test. This is a delayed type hypersensitivity response requiring the intradermal injection of PHA, a T cell mitogen into the interdigital web or wing web, stimulating T cell proliferation and an inflammatory response to this foreign challenge (Lochmiller et al., 1993). Phytohemagglutinin is usually dissolved in sterile phosphate buffered saline (PBS). To eliminate PBS as a possible confounding factor or potential cause of swelling, an injection of sterile PBS in the opposite web is used as a control. Relative changes in thickness (swelling) are measured at 24 hours.

Fusarium mycotoxins have also been shown to decrease lymphoid organ size, reducing lymphocyte production (Pestka and Smolinski, 2005). Dose dependent increases or decreases in B and T cell mitogen responses were observed in lymphocytes from poultry exposed to *Fusarium* mycotoxins (Pestka et al., 1994). Most *Fusarium* mycotoxins exhibit greater inhibitory effects on T cell proliferation and antibody dependent cytotoxicity (Awad et al., 2012).

1.8.3 Humoral mediated response and assessment

Humoral immunity is mediated by antibodies or immunoglobulins (Ig) produced by B cells originating in the bursa of Fabricius (Grogan et al., 2008). The humoral immune response is

categorized by Ig proteins with specific receptors for binding foreign antigens (Larsson and Carlander, 2002). There are three types of antibodies: IgM (associated with the primary response), IgG (mammalian) or IgY (avian; associated with the secondary response) and IgA (mucosal) antibodies (Benedict and Berestecky, 1987; Table 1.4). On initial exposure, a primary response occurs, causing B cells to be stimulated to proliferate and differentiate into plasma cells which secrete IgM antibodies specific to the antigen. A second, repeated exposure enhances this proliferation by triggering memory B cells, providing a more rapid, persistent and stronger response consisting of production of IgG or IgY antibodies (Fairbrother et al., 2004). Antibody titers are evaluated through enzyme linked immunosorbant assays (ELISA; Fairbrother et al., 2004).

One method of evaluating humoral immunity is through the injection of a foreign antigen with the intent to illicit a response (e.g. production of antibodies specific to the antigen). Bovine serum albumin (BSA) is a foreign protein that has been shown to illicit IgY and IgM responses in poultry (Mast and Goddeeris, 1999; Vandaveer et al., 2001), which is measured by increased serum immunoglobulin concentration. Injection of an antigen must be conducted after maternal antibodies have dissipated to prevent these non-self-antibodies from eliciting the response rather than those from the individual. It has been shown that maternal antibodies disappear within three weeks of hatch (Lee et al., 2009) indicating that determine effects on poultry immunity, exposure should occur after three weeks.

Table 1.4. Immunological characteristics of immunoglobulins IgM, IgG and IgA in poultry.

Immunoglobulin (Ig)	Secretion	Primary Location	Primary Functions
IgM	Primary Ig	Serum, B cell surfaces	Binds and activates complement.
IgG (IgY)	Secondary Ig	Serum, Egg yolk	Memory of previous exposure, binds antigen presenting cells.
IgA	Constant	Mucosal surfaces (i.e. saliva, tears)	Mucosal immunity, neutralizes virus and microbial toxins, prevents pathogen formation.

Adapted from Grogan et al., 2008.

1.8.4 Heterophil to lymphocyte ratio assessment

The ratio of heterophils to lymphocytes (H:L) is considered a reliable indicator of chronic stress reactions in poultry. This measure of immunotoxicity is nondestructive, compatible with other blood based assays such as ELISAs and is commonly used as an immune indicator (Fairbrother et al., 2004). Heterophils and lymphocytes are counted as a ratio in a blood smear to indicate the relative percent difference of these white blood cell types due to a stressor, in this case *Fusarium* mycotoxin contaminated diets. The number of lymphocytes have been shown to decrease while the number of heterophils increases in response to stress (Gross and Siegel, 1983; Davis et al., 2008). Heterophils possess an innate immune function while lymphocytes provide adaptive immune function. The relative proportions of these cell types are indicative of the overall immune cell profile (Davis et al., 2008).

1.8.5 Histopathological assessment

The modern, fast growing broiler requires healthy gastrointestinal tissues to absorb the required nutrients necessary for optimum performance and immunity. Any disruption or reduction in nutrient absorption can have significant effects on performance, productivity and immunity. A variety of tissues can be assessed histopathologically as an indicator of immunocompetence (Awad et al., 2006b), as *Fusarium* mycotoxins affect the functions of multiple organs in poultry. Specifically, they affect the thymus, liver, spleen, kidney, heart, bursa of Fabricius and intestinal tract (jejunum, ileum and caecum; Awad et al., 2006a). In addition, some mycotoxins have shown effects on the gizzard and proventriculus. The effects of feeding *Fusarium* mycotoxin contaminated grain on tissue weights of broiler chickens have been shown to be contradictory and highly variable, suggesting these tissues may not be relevant indicators (Awad et al., 2013). Tissue weights expressed relative to body weight are less sensitive than histopathological methods or cell counts (Fairbrother et al., 2004).

1.9 Research objectives and hypothesis

Many previous studies of the effects of mycotoxins in animal models used purified mycotoxins, fungal culture materials, or artificially inoculated feed, which do not account for the potential toxicological synergism and interactions arising from the feeding of combinations of mycotoxins (Smith, 2006). The use of naturally contaminated grain containing multiple *Fusarium* mycotoxins has been adopted in some recent experiments, as it is more representative of field

conditions (D'Mello and Macdonald, 1997; Girgis and Smith, 2010). Although these studies need naturally contaminated grain, they are confounded with the use of modified ingredients or a source of uncontaminated grain from a different location in combination with the contaminated source to produce the target levels. These adaptions may confound the results and their interpretation.

This project is the first step in the evaluation of potential applications of the BoMill TriQ individual kernel sorting technology using near infrared transmittance. Our focus was firstly to evaluate the capability of this technology as a method of separating FDK from healthy kernels to determine associations with mycotoxins. Secondly, to determine the capacity to sort bulk grain sources into low and high mycotoxin contaminated fractions, and finally to examine the effects of differing, controlled recombination diet ratios of low and high mycotoxin fractions on the performance and immunological competence of broiler chickens. Typical performance endpoints evaluated consisted of body weight (BW), feed intake (FI), feed conversion ratio (FCR), mortality, apparent metabolizable energy (AME) and nitrogen retention (NR). The physical response to diet treatments on gross gut segments was also measured. Cell-mediated and humoral immunity in addition to the H:L ratio, tissue weights relative to body weight and histopathological effects were evaluated as indicators of immune system effects.

1.9.1 Objectives

Specific objectives of this project were:

1) Evaluate the capability of the TriQ for salvaging high quality grain from bulk grain downgraded due to FDK, and reduce mycotoxin concentrations in salvaged grain.
2) Evaluate the sorted fractions of different grain as reconstituted ratios with controlled levels of mycotoxins on the performance and immune competence effects of broiler chickens.
3) Identify endpoints sensitive to increased mycotoxin exposure.

1.9.2 Hypothesis

1) The TriQ will show the capability to reduce levels of FDK and mycotoxins in salvaged grain at volumes useful for industry application.
2) Exposure of broiler chickens to sorted fractions of grain with controlled levels of mycotoxins will affect performance based on levels of natural sources of mycotoxins.

3) Exposure of broiler chickens to sorted fractions of grain with controlled levels of mycotoxins will impact the immune system as a dysfunction of cell-mediated and humoral response, and provide a more reliable endpoint for assessing mycotoxicosis.

2.0 THE USE OF NEAR INFRARED TRANSMITTANCE (NIT) KERNEL SORTING TECHNOLOGY TO SALVAGE HIGH QUALITY GRAIN FROM GRAIN DOWNGRADED DUE TO *FUSARIUM* DAMAGE.

Preface

This chapter was published in Animal Nutrition 1, 41 - 46 under joint authorship with Mark L. Wickstrom and Tom A. Scott (University of Saskatchewan). References cited in this chapter are listed in the reference section of this thesis.

The research described in this chapter was the preliminary investigation of new individual kernel sorting technology, the BoMill TriQ that uses Near Infrared Transmittance (NIT). The purpose of this study was to evaluate the capacity and capabilities of the sorter to remove *Fusarium* damaged kernels from bulk grain sources. My role for this chapter was to separate each procured wheat source into ten fractions representing 10% increments of the bulk grain based on crude protein using this technology and have these samples analyzed to confirm chemical (*Fusarium* levels, crude protein and associated mycotoxins) and physical (thousand kernel weight, test weight and grade) characteristics. Additionally, I was responsible for all data collection, the statistical analysis of that data and the authorship of this chapter in full.

2.1 Abstract

The mycotoxins associated with specific *Fusarium* fungal infections of grains are a threat to global food and feed security. These fungal infestations are referred to as *Fusarium* head blight (FHB) and lead to *Fusarium* damaged kernels (FDK). Incidence of FDK > 0.25% will lower the grade, with a tolerance of 5% FDK for export feed grain. During infestation, the fungi can produce a variety of mycotoxins, the most common being deoxynivalenol (DON). *Fusarium* damaged kernels have been associated with reduced crude protein (CP), lowering nutritional, functional and grade value. New technology has been developed using Near Infrared Transmittance (NIT) spectra that estimate CP of individual kernels of barley, spring and durum wheat. Our objective is to evaluate the technology's capability to reduce FDK and DON of downgraded wheat and its ability to salvage high quality safe kernels. In five FDK downgraded sources of wheat, the lowest 20% CP kernels had significantly increased FDK and DON with the high CP fractions having decreased FDK and DON, thousand kernel weights (TKW) and test weight (TW). Strong positive correlations were observed between FDK and DON (r = 0.90); FDK and grade (r = 0.62) and DON and grade (r = 0.62). Negative correlations were observed between FDK and DON with CP (r = -0.48 and -0.39); TKW (r = -0.49 and -0.50) and TW (r = -0.83 and -0.81). Results show improved quality and value of *Fusarium* downgraded grain using this technology.

2.2 Introduction

Wheat is the second most commonly grown grain next to maize and a major commodity worldwide for use in food and feed (FAO, 2013a). Wheat is susceptible to a fungal species, *Fusarium*, which is responsible for *Fusarium* head blight (FHB). *Fusarium* infection usually occurs at the plant flowering stage (i.e. anthesis) and incidence and severity is increasing worldwide (McMullen et al., 1997). *Fusarium* head blight causes physical damage to kernels and is referred to as *Fusarium* damaged kernels (FDK; Gilbert and Tekauz, 2000). The first sign of FHB is a change in kernel spikelet color from green to white in the grain head. These infected kernels are shriveled, which is associated with disruption of kernel development (CAST, 2003). Although FHB infestation is most common in temperate regions, its occurrence has been identified in all regions. *Fusarium graminearum* is the most common *Fusarium* species infecting grain (Parry et al., 1995), and is the principle cause of FHB in Saskatchewan (Charmley and Trenholm, 2012), affecting the grain industry (Windels, 2000). The impacts of infected kernels are widespread (McMullen et al., 1997; Bai et al., 2001) resulting in reductions in grain yield and quality, impacting the suitability for human and animal consumption (Dexter et al., 1996; Jin et al., 2014).

Although the level of FDK may not always be correlated to mycotoxin type or concentration (Liu et al., 1997), it does provide a rapid and economical means of reducing risk and grading grain (Tittlemier et al., 2013). Testing large numbers of samples for a variety of mycotoxin contaminants is expensive and time consuming (Beyer et al., 2007). Common *Fusarium* species (i.e. *graminearum, culmorum*, *crookwellense,* and *verticilloides*) have adapted to a wide range of habitats (Moss, 1991). During kernel infestation crude protein (CP), an important quality and nutritional trait (Tonning et al., 2009), can be negatively affected (Matthaus et al., 2004; Paul et al., 2005). Reduction in CP may be due to the destruction of the protein that surrounds the starch granules (Nightingale et al., 1999; Jackowiak et al., 2005), thereby lowering yield, milling and baking quality of flour (Dexter et al., 1996; Evers, 2000; Snape et al., 2005). It has been suggested that minimizing CP heterogeneity to obtain more uniform CP levels within a batch of grain could lead to improved processing capabilities and overall grain quality (Benseler, 2010). This would also suggest that removal of low CP grain may significantly reduce levels of mycotoxins (D'Mello and Macdonald, 1998).

There is increasing evidence of global contamination of grain by *Fusarium* mycotoxins (D'Mello and Macdonald, 1998) with deoxynivalenol (DON) considered the most prevalent (Pronk et al., 2002). It has been established that DON is associated with FDK (Symons et al., 2002) and indications are that removal of FDK would reduce DON, and thereby increasing grain quality and safety. Health Canada's current maximum allowable levels of DON in grain for human consumption is 2.0 ppm in non-staple foods and 1.0 ppm in baby food however these regulations are under review (Bianchini et al., 2015). Grain grading quality standards are used to subjectively evaluate grain quality for nutritional and functional value as food or feed (Alander et al., 2013). In Canada, key determinants to identify high quality grain (classified as No. 1 grade) from downgraded grain are the factors relating to level of CP and mycotoxins. Nearly 50 visual factors of grain quality are used in determining grade. If FDK is >4% the grain will be limited to feed usage and >6% FDK is graded as "Salvage". Estimates for the 2014 crop year are that 2 MMT (million metric tonnes) of wheat were graded as salvage primarily due to FDK and cannot be marketed legally (Dr. Rex Newkirk, Canadian International Grain Commission, personal communication, 2014). Typically grain graded as Feed has approximately 30% less value than grain graded as No. 1. Grain graded as salvage would normally be disposed of or diluted with higher quality grain to meet minimum standards for sale.

The relationship between visual grading for FDK and actual levels of DON are not consistent (Peiris et al., 2010) due to DON variation within kernels (Beyer et al., 2007, 2010; Berthiller et al., 2013) but it is generally accepted as the only rapid and economic means of grading grain for safety. Beyer et al. (2010) evaluated the effectiveness of Near Infrared (NIR) spectrometry to differentiate healthy, sound kernels from FDK. They concluded that the largest spectral changes between healthy and damaged kernels occurred in the 1400 and 1900 nm spectral range. Peiris et al. (2009) observed that absorption bands of DON were in a similar spectral range, indicating that the DON concentration could be estimated from specific NIR spectra. A single kernel NIR (SKNIR) system was developed by Perten Instruments (Stockholm, Sweden) to detect FDK accurately (Peiris et al., 2010), but was unable to sort large volumes of contaminated grain. BoMill (Lund, Sweden) has engineered a Near Infrared Transmittance (NIT) sorter, the TriQ (Figure 2.1) that has higher sorting capacity and uses a limited algorithm of spectra. The use of NIT spectroscopy allows a single kernel to be evaluated based on CP. Since FDK contains reduced

CP, the TriQ was evaluated to determine if it was capable of reducing FDK and DON by sorting individual kernels based on CP.

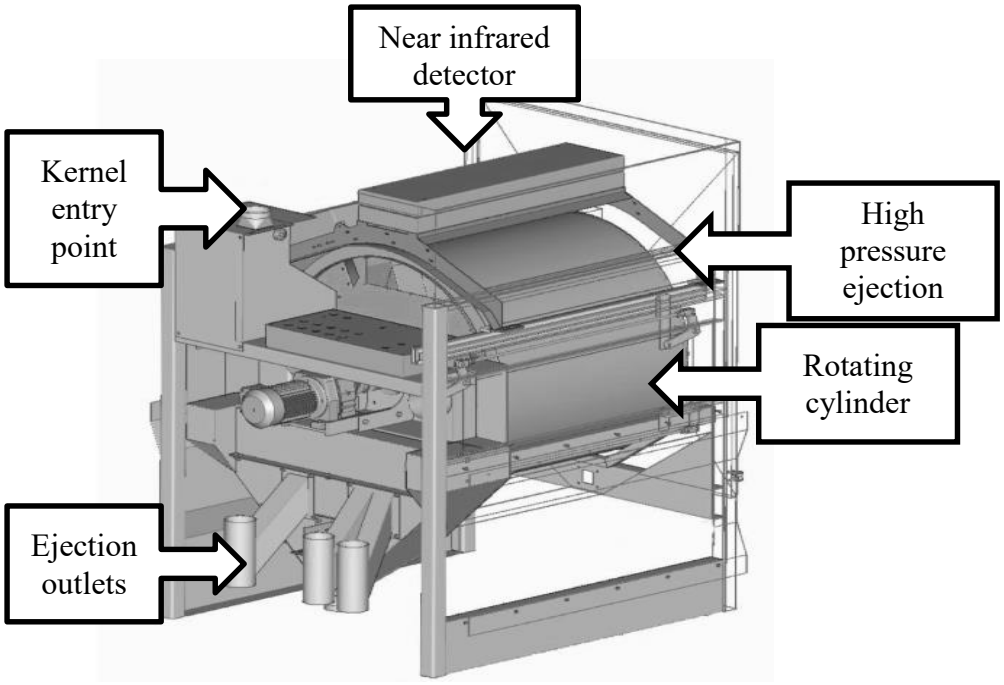

Figure 2.1. TriQ manufactured by BoMill. Note the location of the near infrared detectors at the top and the three ejection outlets at the bottom left. The TriQ dimensions (height x width x depth) are 1.8m x 1.2m x 1.75m.

Our objective was to evaluate this technology's capability for salvaging grain from multiple sources downgraded due to *Fusarium* infection (Canadian Grain Commission, 2014a). A capacity to reduce FDK and DON and salvage high quality grain would significantly reduce risks for humans and animals and increase food and feed security. The authors' note that such sorting technology would change the view of grain as a mass commodity to one based on individual kernels and has significant ramifications for food, malt and feed production.

2.3 Materials and methods

2.3.1 BoMill TriQ sorter

The development of the patented TriQ (BoMill, 2008; Patent #7417203) individual kernel sorter was based on application of NIT technology. This technology measures and sorts individual

kernels of barley, spring or durum wheat based on variability in the spectral range of 1100 – 1700 nm, which is used to estimate CP. The spectra of individual kernels with known CP were used to establish reference values for the TriQ to determine the CP of unknown kernels.

The TriQ uses a stainless steel rotating drum (i.e., a singulator) that has 256 rows of 88 laser etched singulator pockets designed to position individual kernels and allow near infrared wavelengths to pass through the kernels. Specifically designed drums are required for each grain type, and at present drums have been designed to sort barley, spring and durum wheat. The rotating drum uses centrifugal force to position individual kernels into the singulator pockets and carries the kernels past three detectors. These detectors measure NIT wavelengths passing through the kernels and obtain six to ten readings per kernel that are used to determine where the grain will be ejected. The processed information is then relayed to the compressed air ejection unit that ejects individual kernels into one of three user determined ejection outlets. A single unit can sort individual kernels at a rate of 2 - 3 tonnes/hour.

Prior to commercial sorting, the drums require calibration, which is done by scanning a sample (~100 - 200 kg) of grain within the TriQ using a specified number of near infrared spectra to determine the variability in kernel CP. Once the calibration curve has been established the TriQ software will produce a histogram indicating ten equal fractions of grain based on individual kernel CP. The ten calibration fractions are then produced for sampling in sequence from two of the three ejection outlets: one for the designated calibration fraction (~3 - 5 kg) and one for the remaining grain. These ten calibration samples can then be analyzed to verify the actual variability in CP (and indirectly FDK and DON) and grade; then the user is able to define three commercial fractions for sorting. The focus of this work is to discuss the ten calibration fractions and assess this variability from five different wheat sources. During detection some kernels are not properly identified and are classified as outliers. The outliers may represent kernels from other types of grain, improper positioning of kernels into pockets or two kernels in one pocket (BoMill, 2012). These outliers are included in the first and last of the ten calibration fractions. Typically outliers are reduced by pre-cleaning grain and removing excessively small kernels; this was not done in this study.

2.3.2 Wheat sources

Five sources of wheat (6 MT/source) downgraded based primarily on levels of FDK were purchased from grain producers in Western Canada. The characteristics of each grain source were

defined in Table 2.1. The five wheat samples were classified as: Canadian Western Red Spring (CWRS; n = 2); Canadian Western Soft White Spring (CWSWS; n = 2); and Canadian Western Amber Durum (CWAD; n = 1). Representative samples of 750 g of each grain were graded by a Canadian Grain Commission inspector. The grade information included: grade; bulk density as test weight (TW; kg/hL); and FDK (%) as defined by Official Grain Grading Guide (Canadian Grain Commission, 2014a). Samples were collected from each of the ten calibration fractions sorted (2 kg) and from each unsorted grain (1 kg) for characterization and mycotoxin analysis. For the calibration sort and subsequent analysis, outlier grain was included in the lowest 10% and highest 10% CP fractions.

Table 2.1. The estimations of *Fusarium* damaged kernels (FDK, %), deoxynivalenol (DON, ppm), grade, crude protein (CP, %), thousand kernel weight (TKW, g) and test weight (TW, kg/hL) of CWRS, CWSWS and CWAD.

	FDK, %	DON, ppm	Grade	CP, %	TKW, g	TW, kg/hL
Wheat types						
CWRS 01	1.4	1.1	3	19.7	38.5	73.7
CWRS 02	1.6	1	4	19.7	37.7	74.4
CWSWS 01	1.7	0.9	4	14.5	33.2	75.8
CWSWS 02	3.7	1.2	4	16.3	42.4	76.9
CWAD	7.6	8.4	6	18.1	43.7	74.1

CWRS = Canadian Western Red Spring, CWSWS = Canadian Western Soft White Spring, CWAD = Canadian Western Amber Durum, grade (based on Canadian grading classifications from 1 - 6 where 4 = Feed and 6 = Salvage).

2.3.3 Chemical assessments

All grain samples were ground through a 1 mm screen (SM 2000 High-Performance Cutting Mill, Retsch GmbH and Co., Haan, Germany) and thoroughly mixed prior to analysis. Ground samples (0.1 g) were analyzed in duplicate for CP (N x 6.25) with a Leco nitrogen analyzer (model FP528 601-500-100; Leco Corp., St. Joseph, MI, USA) using 0.1 g EDTA (ethylenediaminetetraacetic acid) as a standard (AOAC, 1995). Crude protein was evaluated on a dry matter (DM) basis using the formula CP (DM) = (CP % / (100 – moisture %)) / 100; AOAC, 1990).

2.3.4 Mycotoxin determinations

Mycotoxin analysis was conducted at North Dakota State University (Veterinary Diagnostic Laboratory, Fargo, North Dakota) based on their proprietary protocol. The suite of 16 mycotoxins measured included: DON (vomitoxin) and metabolites (3 and 15 acetyl DON); T-2 toxin and metabolites (T-2 triol, T-2 tetraol, Iso T-2 toxin and acetyl T-2); HT-2 toxin; FUS; DAS; SCIRP and metabolite (15 acetyl scirpentriol); NIV; NEO; zearalenol and ZEA. A standard curve using 0.2; 0.5; 1.0; 3.0 and 6.0 ppm *Fusarium* mycotoxins standards (Romer Labs Diagnostic GmbH, Tulln, Austria) mixed in a known wheat blank (0.0 ppm *Fusarium* mycotoxins) was prepared. A "wheat pool" sample produced by the lab was used as a positive control. Samples of 25 g were prepared in duplicate and analyzed through a solvent extraction solution of acetonitrile and water. Chemical derivatization was used to enhance the volatilization of potential mycotoxins for more accurate detection. The solution was filtered through a 1:1 mixture of C_{18} and alumina. Samples were analyzed using gas chromatography (model 689ON; Agilent Technology, Englewood, CO, USA) and mass spectrometer (model 5975B XL E1/C1; Agilent Technology, Englewood, CO, USA). Mirex (Absolute Standards Inc., Hamden, CT) was dissolved in iso-octane to establish a quantitation peak and all mycotoxin concentrations were determined using linear regression. Of all mycotoxins tested, only DON was found to be above the detection limit (0.5 ppm) in any of the grain samples tested.

2.3.5 Physical assessment – Grading

Samples of unsorted grain and ten calibration fractions were graded by inspectors of the Canadian Grain Commission and provided grade and estimates of %FDK. Thousand kernel weight (TKW, g) of each calibration fraction was determined in triplicate using an ESC-1 seed counter (model ESC120006; Agriculex Inc., Guelph, ON, CAN) and test weight (TW, kg/hL) was determined in triplicate with a UK imperial pint based on the formula: test weight = ((kg/pint) / (pint/hL)); where pint/hL = 0.00568.

2.3.6 Calculations

The data on grain quality was highly variable due to grain source and this interfered with our ability to correlate the differences between calibration fractions across all grain sources. Therefore, we expressed the values in fractions 2 - 10 relative to the first fraction and then calculated the correlations for the different measurements (i.e., CP, FDK, DON, TKW and TW).

2.3.7 Statistical design

To evaluate the sorters capacity to produce repeatable calibration fractions regardless of wheat type, data from the respective fractions for all sources of grain were pooled for statistical comparison. The variables described above for the ten calibration fractions were analyzed using a complete randomized design (PROC MIXED of SAS [Version 9.3]; SAS Institute, Cary, NC) statistical model ($y_{ij} = \mu + t_i + \varepsilon_{ij}$) where: y = measured parameter; μ = overall mean; t_i = fixed effects (individual calibration fractions) and ε_{ij} = error term for the fixed effects and associated variation between the five sources of wheat included. Multi-treatment means comparisons of the calibration fractions were separated using the Tukey test. Crude protein, TKW and TW analysis was based on the % difference compared to the first fraction. Linear associations among calibration fractions and all parameters were analyzed using Pearson (r) correlation coefficients (PROC CORR of SAS [Version 9.3]; SAS Institute, Cary, NC) statistical model. Further analysis using linear regression (PROC REG of SAS [Version 9.3]; SAS Institute, Cary, NC) model ($y_i = \alpha + \beta x_i + \varepsilon_i$; where α = y-intercept, β = slope of the line and ε_i = error term) was used to evaluate the relationship between the variables was conducted where appropriate. Data is presented as means with standard error of the mean (SEM). Means were compared using PDMIX800 (Saxton, 1998). Significance was declared at $P < 0.05$.

2.4 Results

2.4.1 Unsorted grain

Descriptive data for the original five unsorted sources of grain are provided (Table 2.1). Each unsorted source was analyzed for FDK, DON, CP, TKW and TW. The FDK of the five wheat sources ranged from 1.4 - 7.6%, with CWAD containing the highest level. For DON, the range was from 0.9 - 8.4 ppm, with CWAD containing the highest level. Crude protein on a dry matter basis ranged from 14.5 - 19.7%, thousand kernel weight from 33.2 (CWSWS 01) - 43.7 g (CWAD) and test weight from 73.9 (CWRS 01) - 76.9 kg/hL (CWSWS 02). Due to grading criteria being impacted by FDK, only the CWRS 01 sample graded #3. The CWRS 02 and both CWSWS wheat samples graded as Feed (#4) with CWAD graded as Salvage (#6) and not legal for sale. As Feed, AC Fusarium and commercial salvage grades are non-numerical, values of 4, 5 and 6, respectively, were dedicated to Feed, AC Fusarium and Salvage for statistical purposes.

2.4.2 Grain sorted into calibration fractions

There were significant differences among the ten calibrations fractions for all measurements. Calibration fractions one and two were the most significantly different from all other fractions after sorting. In comparison to the original total unsorted grain (Table 2.2); these two fractions contained the highest level of FDK and DON while remaining fractions (3 - 10) indicated an improvement in all measurements. Grade for the higher fractions (3 - 10) was improved to either No. 1 or No. 2 (with a grade of Feed being the original assessment) with the exception of those fractions from the sorted amber durum (CWAD) where a grade of No. 3 was obtained (original assessment was Salvage). Part of the explanation for the durum wheat was the higher contamination and damage due to FDK. The durum wheat was also downgraded based on other characteristics not related directly to FDK; however, improvements in grade were observed.

Table 2.2. The assessment of *Fusarium* damaged kernels (FDK, %), deoxynivalenol (DON, ppm), grade, crude protein (CP, %), thousand kernel weight (TKW, g) and test weight (TW, kg/hL) for the ten calibration fractions produced by the BoMill TriQ.

	n	FDK, %	DON, ppm	Grade	CP, %	TKW, g	TW, kg/hL
Combined unsorted		2.44	1.67	2.90	18.1	40.4	76.7
Fractions		**	**	**	**	**	**
1^{+}	10	9.79^{a}	7.38^{a}	5.60^{a}	17.7^{c}	38.3^{c}	72.8^{h}
2	10	5.65^{ab}	2.78^{b}	4.60^{ab}	17.7^{bc}	39.3^{bc}	74.9^{g}
3	10	2.51^{b}	1.62^{b}	3.60^{abc}	17.9^{abc}	41.0^{a}	75.8^{fg}
4	10	1.67^{b}	1.10^{b}	2.60^{bc}	18.1^{ab}	41.4^{a}	76.3^{ef}
5	10	2.09^{b}	0.88^{b}	2.40^{bc}	18.2^{ab}	40.8^{a}	76.8^{de}
6	10	0.61^{b}	0.80^{b}	2.00^{c}	18.2^{ab}	41.1^{a}	77.4^{cd}
7	10	1.03^{b}	0.64^{b}	2.20^{bc}	18.3^{a}	41.6^{a}	77.8^{bc}
8	10	0.48^{b}	0.54^{b}	2.00^{c}	18.3^{a}	41.2^{a}	78.7^{ab}
9	10	0.43^{b}	0.48^{b}	2.20^{bc}	18.1^{ab}	40.7^{ab}	79.3^{a}
10^{+}	10	0.13^{b}	0.52^{b}	1.80^{c}	18.0^{abc}	39.0c	77.1^{cde}
SEM	100	0.542	0.280	0.207	0.235	0.314	0.221

Means with different superscript letters in the same column are significantly different.
Significance indicated as: NS ($P \geq 0.1$); † ($P \leq 0.1$); * ($P \leq 0.05$); ** ($P \leq 0.01$) and *** ($P \leq 0.001$). $^{+}$ Outliers were not removed prior to analysis.

Pearson correlations (Table 2.3) based on the individual calibration fractions indicated that all correlations were significant ($P < 0.05$). There was a strong positive correlation (r = 0.90) between FDK and DON. Crude protein was negatively correlated with FDK (r = -0.48); DON (r

= -0.39) and grade (r = -0.36) but positively correlated with both TKW (r = 0.22) and TW (r = 0.54). It should be noted that the negative correlation of CP with grade is an increase in grain value indicating an improvement in grain quality. Grade was positively correlated with FDK (r = 0.62) and DON (r = 0.62) but negatively with both TKW (r = -0.21) and TW (r = -0.51). There were negative correlations between TKW and FDK (r = -0.49) and DON (r = -0.50); but a positive correlation with TW (r = 0.43). Finally TW was negatively correlated with FDK (r = -0.83) and DON (r = -0.81). Regression analysis of FDK (y) and DON (x) indicated that approximately 80% of DON levels can be explained by FDK % ($y = 1.665 + 0.993x$, $P < 0.01$; $r^2 = 0.803$).

Table 2.3. Pearson correlation coefficients between the measured variables (based on sorting by crude protein) of *Fusarium* damaged kernels (FDK, %), deoxynivalenol (DON, ppm), grade, crude protein (CP, %), thousand kernel weight (TKW, g) and test weight (TW, kg/hL) from all calibration fractions (n = 100) of all tested grain sources are compared statistically.

	FDK, %	DON, ppm	CP, %	Grade	TKW, g	TW, kg/hL
DON, ppm	0.90 ***	1.00				
CP, %	-(0.48) ***	-(0.39) ***	1.00			
Grade	0.62 ***	0.62 ***	-(0.36) ***	1.00		
TKW, g	-(0.49)***	-(0.50) ***	0.22 *	-(0.21) *	1.00	
TW, kg/hL	-(0.83) ***	-(0.81) ***	0.54 ***	-(0.51) ***	0.43 ***	1.00

Significance indicated as: NS ($P \geq 0.1$); † ($P \leq 0.1$); * ($P \leq 0.05$); ** ($P \leq 0.01$) and *** ($P \leq 0.001$). Correlations are calculated using the differences in numerical values in proportion to fraction 1 to account for variability of different wheat sources.

2.5 Discussion and conclusions

Previously, the ability to sort bulk grain based on individual kernel CP into user-defined fractions was unfeasible on a commercial scale. In the TriQ patent by Lofqvist and Nielsen (2003) the calibration and development of this technology was based on sorting using NIT spectra and comparing the results to the chemically determined CP through Kjedahl analysis. The use of NIT allows individual kernel CP to be evaluated based on spectral information converted into proprietary algorithms as an indication of kernel structural integrity and soundness. From the results of this study, the BoMill TriQ sorter has demonstrated capability to sort bulk sources of downgraded grain based on FDK indirectly by sorting directly on kernel CP. Reduction in FDK resulted in a reduction in DON and increased CP as indicated by the correlation results among FDK, DON and CP and past literature (reviewed by Paul et al., 2005; Beyer et al., 2007; Wegulo

et al., 2010). We acknowledge, however, that FDK has been reported without DON and vice versa (Mesterhazy, 2002). More sources of FDK downgraded grain are required to verify the consistency and capacity of the TriQ sorter.

As demonstrated previously by Tonning et al. (2009) and Benseler (2010), the TriQ is capable of differentiating individual kernels of grain based on CP, however neither researcher indicated the effect of sorting by CP on FDK and associated mycotoxins. In the present study, the two lowest CP fractions contained increased FDK and DON concentrations, while remaining fractions showed improved quality and value with the greatest improvements to CP in fractions 8 - 10. Visual grading by a Canadian Grain Commission inspector of each of the ten calibration fractions from all sources demonstrated that grade was significantly improved when FDK was removed. The decreased CP in fractions one and ten may be associated with the outlier kernels that were automatically assigned to these fractions in the calibration sort. It was suggested that pre-cleaning bulk grain would have reduced the number of outlier kernels and minimized the impact on fraction ten (Bo Lofqvist, 2014 personal communication).

It should be noted that the TriQ does not sort solely on CP. The selected spectral ranges utilized by the TriQ are also correlated to other indicators (i.e., hardness and vitreousness; Bo Lofqvist, 2014 personal communication). These correlations allow unknown kernel spectra to be compared against known kernel spectra for the determination of individual kernel CP. A potential reason for the lack of more pronounced CP variability within the ten calibration fractions could be the limited variability within the bulk grain obtained. Further work is also required to establish the sensitivity and accuracy of sorting grain with higher variability in individual kernel CP.

Downgrading of grain is typically in response to a small percentage of affected kernels that reduce safety or quality. This study has focused on the merits and capacity of removing these low quality kernels and salvaging the high quality kernels. With the increasing awareness of food security issues and one third of food lost or wasted (Gustavsson et al., 2011), salvage of the high quality kernels for use in food production is important (Benseler, 2010). Decreased grain grade can occur from as little as 0.25% FDK (Canadian Grain Commission, 2014a). Decreased concentrations of FDK and DON had a positive impact on the correlation to grade ($r = 0.62$).

The relationship between TKW and fraction observed in the present study was different than reported by Benseler (2010). While Benseler indicated that TKW decreased from calibration fraction 1 - 10, our results indicated a moderate increase and plateau. Analysis of TW was in

agreement showing a linear increase with increasing fraction. These measurements of TKW and TW weight can be influenced by a number of factors including kernel packing, size and density (Nielsen et al., 2003).

The relatively small number of FDK compared to uninfected kernels affects TKW and TW weight, as indicated by the negative correlations. This might suggest that the TriQ potentially sorts on kernel size. However, results by Tonning et al. (2009) indicated that the TriQ did not sort based on kernel size through observations of kernel mass, diameter and hardness measurements. Additionally, Benseler (2010) reported no significant differences in weight or size in barley when using three screen sizes to obtain four size fractions. Most kernels were located in a similar screen range indicating similar sizes except fractions 8 - 10 which had decreased size with the smallest kernels located in fraction ten and may be actually outliers that are assigned to fraction ten. *Fusarium* infected kernels tend to be smaller, lighter and shrunken leading to reduced weight (Matthaus et al., 2004; Jin et al., 2014).

This study indicated that the TriQ has the potential to sort bulk grain sources based on CP and reduce FDK and DON. Since FDK are strongly correlated with mycotoxins like DON, their detection in grain sources is an issue of food safety (Beyer et al., 2010). If these damaged kernels could be safely removed from grain, it would improve grain quality through reduction in variability; turn low quality ingredients into high quality food or feed; increase milling performance and baking with consistently better grain quality; decrease feed costs (which accounts for 60 - 70% of feed value); and reduce the spread of FHB by improving kernel characteristics (Gilbert and Tekauz, 2000).

By removing 20% of the low CP kernels, grain quality can be improved. Additional capabilities include the recombination of these ten calibration fractions into a commercial sort of three fractions: one to concentrate FDK and DON for removal; a second for outliers, preventing those kernels from impacting a third fraction consisting of kernels with improved safety and security. Upgrades to the TriQ software to improve the ability to sort bulk grain specifically on *Fusarium* damaged kernels or hard vitreous kernels will improve the sorter capabilities to remove healthy from infected kernels.

Many countries setting regulatory limits for mycotoxins in foods and feeds (van Egmond, 2004, 2007) and for the persistence and effects of mycotoxins, which continue to affect global food supply even after 30 years of research (Cardwell et al., 2001). Agricultural technologies such

as the BoMill TriQ may provide an effective solution to reduce or remove FDK and associated mycotoxins.

3.0 USING NEAR INFRARED TRANSMITTANCE (NIT) TO GENERATE SORTED FRACTIONS OF *FUSARIUM* INFECTED WHEAT AND THEIR IMPACT ON BROILER PERFORMANCE.

Preface

This chapter was published in Poultry Science 94, 1619 - 1628 under joint authorship with Mark L. Wickstrom, Natacha S. Hogan and Tom A. Scott (University of Saskatchewan). References cited in this chapter are listed in the reference section of this thesis.

The research described in this chapter is based on the conclusion in Chapter 2. With the capability to sort wheat sources into outlier (10%), high (highest 70%) and low (lowest 20%) crude protein fractions and subsequent determination of *Fusarium* damaged kernels and mycotoxin content, the purpose of this chapter was to evaluate the performance effects of feeding four diet ratios pertaining to the amount of high *Fusarium* damaged kernels and mycotoxin inclusion to Ross 308 male broiler chickens for 35 days. My roles consisted of reconstitution of the diet ratios and determining mycotoxin concentrations of the diet ratios, broiler chicken husbandry, all data and tissue collections, the statistical analysis of that data and the authorship of this chapter in full.

3.1 Abstract

The objective of this study was to investigate the effects of naturally contaminated *Fusarium* wheat containing deoxynivalenol (DON) on growth and performance of broiler chickens from 0 - 35 d of age. The BoMill TriQ individual kernel sorting technology uses Near Infrared Transmittance (NIT) spectra to separate *Fusarium* damaged kernels (FDK) from healthy kernels based on individual kernel crude protein (CP). Three *Fusarium* contaminated wheat sources were individually sorted into three test fractions consisting of: outlier (10% of the source); high mycotoxin (20% of the source); and low mycotoxin (70% of the source). These fractions were reconstituted into four ratios: M0; M20; M40; and M60 relating to the proportion of the high mycotoxin fraction in the reconstituted diets. These 12 reconstituted wheat sources with varying levels of DON were incorporated at 70% (starter) or 75% (grower/finisher) into diets. The fractions of wheat used had FDK ranging from 0.1 - 25.8% and DON from 0.0 - 14.3 ppm. A total of 480 newly hatched Ross 308 male broilers were randomly divided into 96 cages. Each test diet was assigned to eight replicates with five birds per replicate cage. At 21 d, 180 birds were transferred to 36 cages, allowing three replicates of five birds per diet until 35 d. A factorial arrangement analysis compared the three wheat sources and four ratios produced from each sorted wheat. Growth and performance was evaluated as: BW (g); feed intake (FI; g/bird/day); feed conversion ratio (FCR g:g); AME (kcal ME/kg diet); nitrogen retention (NR; %) and mortality (%) for 0 - 21 d and 21 - 35 d. Results indicate no significant difference ($P > 0.05$) in BW, FI and FCR. Significant differences ($P < 0.01$) were observed in AME and NR. This study demonstrated the potential of this novel sorting technology to produce naturally contaminated diets with a large range of mycotoxin concentrations from a single wheat source to enable future investigations of mycotoxin exposure in any species.

3.2 Introduction

Fusarium mycotoxin contamination of feed is a global concern (Schothorst and van Egmond, 2004; Streit et al., 2012) resulting in significant economic losses and adverse health effects in poultry (Awad et al., 2012). Previous estimations (CAST, 2003) have indicated that 25 % of the world's food crops are contaminated with mycotoxins resulting in losses to all aspects of the grain industry including food and feed production and animal productivity. It should be noted, that only a small percentage of contaminated kernels is required to legally lower the grade to feed or salvage depending on the grain type (Canadian Grain Commission, 2013). Cheeke (1998) showed that fungi in the genus *Fusarium* causes the plant disease *Fusarium* head blight (FHB) leading to the formation of *Fusarium* damaged kernels (FDK). These damaged kernels contain reduced crude protein (CP) (Jackowiak et al., 2005) and according to Pronk et al. (2002) usually contain mycotoxins, primarily deoxynivalenol (DON), although other mycotoxins are also associated with *Fusarium* infections. This contaminated wheat is often used as poultry feed (Awad et al., 2008a) and although poultry are considered relatively resistant to low DON exposures (Yunus et al., 2012a), these exposures may still lead to decreased performance, productivity and immune function (Awad et al., 2012).

A meta-analysis by Danicke et al. (2001) concluded that dietary concentrations of up to 5 ppm DON showed no effects on poultry performance. However, the impact of DON level on poultry performance is variable (Kubena et al 1997; Swamy et al 2002; Awad et al., 2008a; Ghareeb et al., 2012). Many previous studies were based on purified sources of DON that are considered to be less toxic than naturally contaminated diets (Canady et al., 2002). Naturally contaminated wheat increases the potential for synergistic or additive interactions from multiple mycotoxins (Smith et al., 1997) including DON metabolites and masked mycotoxins. Although acute DON mycotoxicosis is well-characterized (Devegowda et al., 2005; Smith et al., 2005); this level of exposure is not common in developed countries (Streit et al., 2013). However, chronic mycotoxicoses from lower dietary exposures are known to impact poultry performance, nutritional efficiency, and can increase the susceptibility of poultry to infections or reduce vaccine response (Swamy et al., 2004a; Pestka and Smolinski, 2005; Awad et al., 2008b). These effects are a factor of mycotoxin(s) concentration, duration of exposure, age and the type of mycotoxin(s) ingested (D'Mello et al., 1999; Pestka, 2008). Furthermore, poultry are increasingly challenged to meet

their ever increasing genetic potential for producing animal protein (meat or eggs). Even marginal losses in productivity due to mycotoxicosis would have serious economic ramifications.

Commercial methods to reduce FDK and associated mycotoxin contaminated kernels are not available (Awad et al., 2012). Beyer et al. (2010) evaluated the effectiveness of Near Infrared Reflectance (NIR) spectrometry to differentiate healthy and FDK. They concluded that the largest spectral changes between healthy and damaged kernels occurred in the 1400 and 1900 nm spectral range. Peiris et al. (2009) observed the absorption bands of DON in a similar spectral range, and indicated that DON levels could be estimated using specific NIR spectra. BoMill (Lund, Sweden) has engineered an individual kernel sorter using Near Infrared Transmittance (NIT), the TriQ (Figure 2.1), that has commercially interesting sorting capacity and uses only a specific spectral range. Compared to reflectance, transmittance measurements are less susceptible to surface properties and can more accurately detect internal differences in samples (Schaare and Fraser, 2000). Previous research (Tonning et al., 2009; Kautzman et al., 2015a) indicated that the TriQ could effectively separate FDK and DON contaminated kernels from healthy kernels. Estimated capacity of the TriQ is dependent on kernel density, but one machine is expected to sort ~2 - 3 metric tonnes (MT) of grain/hour into three user defined fractions. Economies of scale would involve 10 machines with an estimated cost of $10/MT for operation.

Our objectives were: 1) to sort three wheat sources with natural *Fusarium* contamination using the TriQ into three test fractions (outlier, high and low mycotoxin levels) and use these fractions to create four reconstituted diet ratios based on increasing amounts of the high FDK fraction (M0; M20; M40 and M60) to be used in a poultry feeding trial; and 2) to expose broiler chicks to these naturally contaminated diets and evaluate the effects of mycotoxin concentrations on broiler performance by measuring body weight (BW; g), feed intake (FI; g/bird/day), mortality corrected feed to gain ratio (FCR; g:g), apparent metabolizable energy (AME; kcal ME/kg diet), nitrogen retention (NR; %) and mortality (%).

3.3 Materials and methods

The experimental protocol and all procedures were approved (AUP# 20130047) by the University Committee on Animal Care and Supply, Animal Research Ethics Board, University of Saskatchewan, Saskatchewan, Canada. The care and handling used followed the principles established by the Canadian Council on Animal Care (1993).

3.3.1 BoMill TriQ sorter

The TriQ is based on the application of NIT technology for determination of CP within individual kernels. The TriQ consists of a rotating steel singulator drum (specific to grain type; presently only barley, spring and durum wheat) with approximately 22,000 laser etched pockets that facilitate positioning of individual kernels by centrifugal force. Each kernel is measured by three NIT detectors to detect transmittance of specific spectral wavelengths (BoMill, 2008; Patent #7417203) in the spectral range of 1100 – 1700 nm, to estimate CP. The absorptive spectra of individual kernels with known CP were used to provide reference values for the TriQ which were then compared to unknown kernel spectra to estimate CP (BoMill, 2012). The determined spectral data is then used to produce 10% increments as a histogram representing the natural variability of the sample which are analyzed chemically to verify CP. The user may then divide the 10% increments and subsequent CP level into three distinct commercial fractions of grain to be sorted into three ejection outlets. Recent modifications to the TriQ software have enabled additional capabilities to sort bulk grain on FDK or hard vitreous kernels. These will improve the capabilities of the TriQ to remove healthy from infected kernels, however these updates were not available at the time of this study. A more detailed description of this technology is available elsewhere (Kautzman et al., 2015a).

3.3.2 Diets

Three sources of contaminated wheat (approximately 6 MT/source) that had been downgraded based on % FDK were purchased from grain producers in Western Canada. The three wheat sources were classified as: Canadian Western Red Spring (CWRS); Canadian Western Soft White Spring (CWSWS); and Canadian Western Amber Durum (CWAD). Each source was initially sorted into outliers, high and low mycotoxin fractions based on CP concentration. Unsorted and sorted fractions were graded by a Canadian Grain Commission inspector for levels of FDK and assigned a grade. Samples from each source and fraction were analyzed for 16 common *Fusarium* mycotoxins (North Dakota State University, Veterinary Diagnostic Laboratory, Fargo, ND) via gas chromatography (model 689ON; Agilent Technology, Englewood, CO, USA) and mass spectrometer (model 5975B XL E1/C1; Agilent Technology, Englewood, CO, USA). Deoxynivalenol (DON) was the only toxin identified at levels above the detection limit of 0.5 ppm.

Re-constitutions of the outlier, high and low mycotoxin fractions into four ratios (Figure 3.1) consisted of: a control diet or M0 (low fraction only); M20 (10% outlier, 20% high fraction, 70% low fraction); M40 (10% outlier, 40% high fraction, 50% low fraction); and M60 (10% outlier, 60% high fraction, 30% low fraction). 'Outliers' represent kernels from other types of grain, improper positioning of kernels into pockets or two kernels in one pocket (BoMill, 2012). As the CP range varied from 16 - 20%, among wheat sources, diets were formulated with the average CP of 18% CP using Brill® (Formulation Optimization, [version 2.04.007]; Feed Management Systems Inc., Hopkins, MN). Starter and grower/finisher diets contained 70.7% and 75.7% wheat, respectively (Table 3.1). One starter and one grower/finisher basal diet were prepared with similar formulations. The grower/finisher was formulated with a reduced level of soybean meal to accommodate the increased inclusion of wheat. All wheat sources were ground using a Jacobson hammer mill (model 160-D; Jacobson Machining Works, Minneapolis, MN) equipped with a 4.2 mm screen. Ingredients were blended in a Marion 800 kg paddle mixer (model 2030; Rapids Machining Company, Marion, IA) for three minutes to produce the mash diets. Protein concentration and AME were within required ranges to meet requirements of broilers (Aviagen, 2012) and diets were formulated to meet or exceed the National Research Council (1994) requirements. Xylanase (0.05%) was included to aid in the digestion of the high wheat levels in the diets. There was also 0.8% Celite™ added as an acid insoluble ash marker for determining digestibility. The authors would like to note that due to the university's animal welfare concerns, diet formulation consisting solely of the high mycotoxin fraction was not approved.

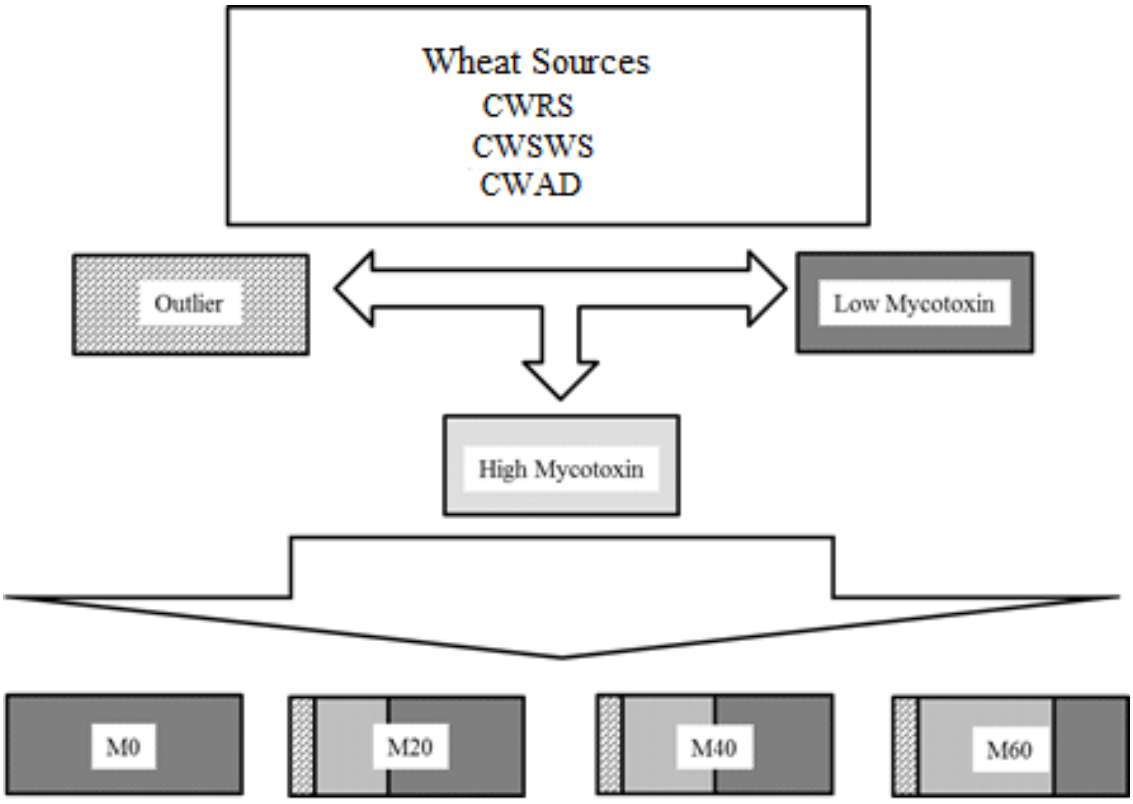

Figure 3.1. Diagram of diet formulation for each wheat source. Outlier fraction contains the top and bottom 5% of kernels, high mycotoxin fraction contains the lowest 20% CP kernels and the low mycotoxin fraction contains the remaining higher CP kernels. Formulated diets consist of mixed ratios of outlier, high and low mycotoxin fractions respectively. M0 contains no high fraction; M20 contains 20% high mycotoxin fraction; M40 contains 40% high mycotoxin fraction and M60 contains 60% high mycotoxin fraction.

Table 3.1. Calculated dietary composition of starter and grower/finisher test diets. Twelve sources of wheat based on three wheat types and reconstituted sorted fractions were used.

Ingredient	Starter (%)	Grower/Finisher (%)
Wheat	70.7	75.7
Soybean meal	20.0	14.0
Fish meal	1.00	1.00
Corn-gluten meal	2.00	3.00
Canola oil	2.00	2.00
DiCalcium Phosphate	0.50	0.50
Limestone	1.20	1.20
Salt (as NaCl)	0.25	0.25
Vitamin/mineral premix[1]	0.50	0.50
Choline chloride	0.10	0.10
DL-Methionine	0.50	0.50
L-Lysine	0.40	0.40
Celite	0.80	0.80
Xylanase	0.05	0.05
Total	**100%**	**100%**
Calculated Nutrient Composition[2]		
Protein, %	26.21	23.00
ME (kcal/kg)	3,212	3,260
Calcium, %	0.75	0.73
Phosphorus, %	0.41	0.38
Sodium, %	0.17	0.17
Lysine, %	1.17	1.02
Methionine, %	0.76	0.75
Cysteine, %	0.25	0.26

[1]Supplied per kilogram of diet: vitamin A (retinyl acetate + retinyl palmitate), 11000 IU; vitamin D, 2200 IU; vitamin E (dl-α-tocopheryl acetate), 30 IU; menadione, 2 mg; thiamine, 1.5 mg; riboflavin, 6 mg; niacin, 60 mg; pyridoxine, 4 mg; vitamin B_{12}, 0.02 mg; pantothenic acid, 10 mg; folic acid, 0.6 mg; and biotin, 0.15 mg. iron, 80 mg; zinc, 80 mg; manganese, 80 mg; copper, 10 mg; iodine, 0.8 mg; and selenium, 0.3 mg.
[2]Calculated on an as-fed basis with amino acids provided as digestible amino acids

3.3.3 Bird management and sample collection

This study was an assessment of the effects of mycotoxins on broiler performance and immune competence; however, only performance and digestibility data are presented here. A total of 480 1 d of age Ross 308 male broiler chicks were purchased from Lilydale Hatchery Inc. (Wynyard, Saskatchewan, S0A 4T0). Birds were individually weighed and randomly assigned to 96 battery cages (29 cm high; 48 cm wide; 83 cm long; providing 800 cm^2/bird floor space at 21 d) arranged into four levels from 1 to 21 d of age. The 12 starter test diets (described above) were

randomly assigned to eight replicate cages of five birds per cage with feed and water (two cup drinkers/cage) provided *ad libitum*. Ambient temperature was initially set at 32°C and was gradually reduced to 23°C by 21 d. The birds were provided with incandescent lighting with day length of 23 hours at 30 lux from 0 - 7 d then reduced to a day length of 20 hours at 10 lux for the remainder of the trial.

Individual BW, cage FI and FCR were recorded at 0, 7, 14 and 21 d. At 21 d of age two birds per cage were humanely killed by cervical dislocation for gut and organ measurements. A total of 180 of the remaining birds were then housed in 36 grow-out cages (38 cm high x 66 cm wide x 69 cm long; providing 900 cm^2/bird floor space) until 35 d of age, maintaining the same dietary treatments as fed from 0 - 21 d. The 12 test grower/finisher test diets (Table 3.1) were assigned to three cages of five broilers from 21 - 35 d based on the previously assigned starter diet. At 21 and 35 d organs relative to digestion (proventriculus, gizzard, jejunum, ileum and caecum) were measured and expressed as % relative to BW.

Apparent metabolizable energy and NR of the 12 starter and grower/finisher test diets were determined. A representative sample of each test diet was collected for the starter and grower/finisher diets. Clean (i.e., free of feathers and feed) excreta samples were collected from each cage twice daily at 19, 20, 33 and 34 d. The excreta samples were frozen, weighed, oven dried at 55°C for 72 hours, re-weighed and then pooled by cage based on sampling day (AOAC, 1995). The dried excreta and diet samples were ground through a 1 mm screen (SM 2000 High-Performance Cutting Mill; Retsch GmbH and Co., Haan, Germany) prior to analysis.

3.3.4 Chemical analysis

Wheat sources, ground diet and excreta samples were analyzed in duplicate. Dry matter was measured using a forced air drying oven (VWR Signature model 1330 GSM, VWR International, Radnor, Pennsylvania) at 135°C for 2 hours (AOAC, 1990). Crude protein (N*6.25) was measured with a Leco nitrogen analyzer (model FP-5281, Leco Corp, St. Joseph, Michigan) at 800°C using 0.1g EDTA as a standard (AOAC, 1995). Gross energy was measured by adiabatic oxygen bomb calorimeter (model 6400, Parr Instrument, Moline, Illinois) using benzoic acid as a standard. An acid insoluble ash digestible marker, Celite™ 585 (Fisher Scientific, Ottawa, Ontario, Canada) was analyzed in 1 g excreta and 2 g test diet samples in 125 mm glass tubes (VWR North America, West Chester, PA, USA). The tubes were ashed in glass beakers at 500°C for 24 hours. Ashed samples were mixed with 5 mL of 4 N HCl and heated for 1 hour at 120°C.

Samples were then centrifuged (model 5810R, 15 amp version, Eppendorf AG, Hamburg, Germany) at 2500 rpm for 10 mins and the supernatant removed using a vacuum siphon. Samples were washed twice with 5 mL of distilled water, centrifuged (as above), siphoned and dried overnight at 80°C. The samples were further ashed at 500°C overnight, cooled and measured (Vogtmann et al., 1975).

3.3.5 Calculations and statistical analysis

The following equations (Scott and Hall, 1998) were used to calculate the digestibility of diets as AME and NR:

$$AME\ (kcal\ ME/kg\ diet) = GE_{diet} - [GE_{excreta} * (Marker_{diet}/Marker_{excreta})]$$

$$NR = 100 - \left[100 * \left(\frac{\%Marker_{diet}}{\%Marker_{excreta}}\right) * \left(\frac{\%N_{excreta}}{\%N_{diet}}\right)\right]$$

where: GE = gross energy; Marker = Celite™ and N = nitrogen.

All data were subjected to statistical analysis using a complete randomized block design (PROC MIXED of SAS [Version 9.3]; SAS Institute, Cary, NC) with battery level as the blocking factor. Classes were wheat (CWRS, CWSWS, CWAD) and ratio (M0, M20, M40, M60), and cage was the experimental unit. Analysis was conducted using a 3 (wheat class) x 4 (ratio level) factorial arrangement. Multi-treatment means comparisons were separated using the Tukey test. Linear and quadratic effects of increasing ratio of mycotoxins were analyzed using regression (PROC REG and RSREG of SAS [Version 9.3]; SAS Institute, Cary, NC) model. Data was log transformed when required to achieve normality. Means were compared using PDMIX800 (Saxton, 1998). Significance was determined at $P < 0.05$.

3.4 Results

3.4.1 Wheat sources

Information on the physical, CP and DON levels of the unsorted wheat and the average for sorted fractions are presented in Table 3.2. Prior to sorting, all wheat sources were downgraded due to FDK. The DON levels were 1.1 and 1.2 ppm in the CWRS and CWSWS wheat, respectively, but 8.4 ppm in the CWAD. Similarly, CP for CWRS, CWSWS and CWAD were 19.7, 16.3 and 18.1% respectively. In regards to grade, the CWRS was graded as No. 3 due to an FDK of >1.0%, the CWSWS was graded as Feed (FDK = 3.7%) and the CWAD as Salvage (>5% FDK). The end result for salvage grain is either disposal or dilution with higher quality grain to

meet minimum standards for sale (Randy Dennis, Chief Grain Inspector for Canada, Canadian Grain Commission, personal communication, 2015).

Table 3.2. Measured characteristics of sorted wheat and fractions using the BoMill TriQ.

		FDK (%)	DON (ppm)[1]	CP (%)	TKW (g)	TW (kg/hL)	Grade
Unsorted		4.78	3.46	18.9	41.3	76.4	Feed
Sorted Wheat		**	***	***	***	***	
	CWRS	0.44^{b}	1.30^{c}	21.0^{a}	38.9^{b}	80.2^{a}	2
	CWSWS	1.92^{b}	1.83^{b}	16.1^{c}	42.1^{a}	76.8^{b}	4
	CWAD	12.1^{a}	7.17^{a}	19.5^{b}	42.7^{a}	72.9^{c}	4
Fractions		***	***	***	***	***	
	Outlier	2.40^{b}	3.16^{b}	18.9^{a}	41.2^{ab}	77.4^{b}	4
	High Mycotoxin	10.4^{a}	6.30^{a}	18.4^{b}	40.1^{b}	72.2^{c}	5
	Low Mycotoxin	1.73^{c}	0.80^{c}	19.3^{a}	42.5^{a}	80.3^{a}	3
SEM		6.366	0.997	0.510	0.468	1.157	

Means with differing letters in the same column are significantly different. Significance indicated as: NS $P \geq 0.1$; † $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$ and *** $P \leq 0.001$. Wheat types described include CWRS = Canadian Western Red Spring; CWSWS = Canadian Western Soft White Spring and CWAD = Canadian Western Amber Durum. Fractions described are the results of sorting wheat using the BoMill TriQ and were not sent for grading. *Fusarium* damaged kernels (FDK), deoxynivalenol (DON), Leco measured crude protein (CP), grade (based on Canadian grading classifications), thousand kernel (TKW) and test (TW) weight. [1]Other mycotoxins including: DON metabolites (3 and 15 acetyl DON), T-2 toxin and metabolites (tetrol; triol; iso T-2), fusarenon X, DAS, scripentriol and metabolite (15 acetyl SCIRP), nivalenol, neosolaniol, HT-2 toxin, zearalenol and zearalenone were analyzed and were below the detection limit of 0.5 ppm.

With respect to grade categories of the sorted fractions it was not possible to average across wheat as Feed and Salvage grades are non-numerical. TriQ sorting concentrated FDK and DON in the high mycotoxin fraction, reducing them in the low mycotoxin fraction. Increases in FDK corresponded to increases in DON concentrations and to decreases in CP, TKW and TW measurements. As mentioned, DON was the only mycotoxin of the 16 measured that was above the limit of detection (0.5 ppm). Grade was improved in all wheat types between high and low mycotoxin fractions after sorting.

To better illustrate the 12 starter and grower/finisher diets used, the DON levels for the respective diets are presented in Table 3.3. The concentration of DON measured in starter diets ranged from 0.5 - 8.3 ppm; in the grower/finisher diets DON ranged from 0.5 - 7.9 ppm.

Table 3.3. Concentrations of deoxynivalenol (ppm) inclusion in starter and grower/finisher ratio diets.

Diet		Ratio			
Starter		M0	M20	M40	M60
	CWRS	0.50	0.70	1.00	1.20
	CWSWS	0.50	1.40	1.60	2.20
	CWAD	1.50	3.70	6.90	8.30
Grower/Finisher					
	CWRS	0.50	0.70	0.90	1.00
	CWSWS	0.50	1.10	1.70	2.10
	CWAD	1.20	3.60	5.70	7.90

Wheat types described include CWRS = Canadian Western Red Spring; CWSWS = Canadian Western Soft White Spring and CWAD = Canadian Western Amber Durum. Formulated diets consist of mixed ratios of outlier, high and low mycotoxin fractions respectively. M0 - no high fraction; M20 - 20% high mycotoxin fraction; M40 - 40% high mycotoxin fraction and M60 - 60% high mycotoxin fraction.

3.4.2 Bird performance

Broiler performance was evaluated for the respective starter (0 - 21 d) and grower/finisher (21 - 35 d) phases based on individual wheat sources CWRS, CWSWS and CWAD. The results were based on starter diets for BW, FI, FCR, mortality, AME and NR based on excreta collected at 19 and 20 d are presented in Table 3.4. Overall mortality from 0 - 21 d was 2.3% and was not significantly different among treatments. With respect to BW, the expected BW for male broilers at 21 d is 945 g (Aviagen, 2012) and in the present study the average BW was 809 g. No significant differences in performance (BW or FI) was observed among wheat sources, the different ratios of high mycotoxin or interactions at 21 d. The FCR results among wheat sources were significantly ($P < 0.01$) higher in CWSWS (1.48) than CWRS (1.44) and CWAD (1.43).

Table 3.4. Broiler performance measurements for broiler starter phase (0 - 21 d) for three Canadian Western wheat types and respective ratios of mycotoxin-based diets.

Treatments			Broiler Starter Phase (0 - 21 d)					
			BW (g)	Feed Intake (g/b/d)	FCR (g/g)	AME (kcal ME /kg diet)	NR (%)	Mortality (%)
		n/df						
Total		96	809	52.0	1.45	3197	55.0	2.29
Wheat type		*2*	*NS*	*NS*	**	***	***	*NS*
	CWRS	32	817	52.0	1.44^{b}	3134^{b}	51.1^{c}	3.75
	CWSWS	32	801	52.9	1.48^{a}	3173^{b}	58.3^{a}	0.63
	CWAD	32	809	51.2	1.43^{b}	3283^{a}	55.5^{b}	2.50
Ratio of mycotoxin		*3*	*NS*	*NS*	*NS*	*	*	*NS*
	M0	24	806	52.5	1.46	3206^{ab}	55.5^{ab}	1.67
	M20	24	823	51.8	1.42	3231^{a}	56.2^{a}	2.50
	M40	24	814	52.2	1.45	3173^{b}	53.6^{b}	2.50
	M60	24	793	51.6	1.47	3175^{b}	54.7^{ab}	2.50
Wheat x Ratio		*6*	*NS*	*NS*	*NS*	***	***	*NS*
SEM			5.7	0.33	0.007	14.7	0.67	0.718
Regression (R^2)								
	Linear		0.00	0.01	0.01	0.00	0.00	
	Quadratic		$0.04^{\dagger}$	0.01	0.05*	0.02	0.01	
Intercept			804.89		1.46			
Ratio			0.0078		$-0.01e^{-3}$			
Ratio x Ratio			$-7.45e^{-8}$		$1.09e^{-10}$			

Means with differing letters in the same column are significantly different. Significance indicated as: NS $P \geq 0.1$; † $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$ and *** $P \leq 0.001$. Wheat types described include CWRS = Canadian Western Red Spring; CWSWS = Canadian Western Soft White Spring and CWAD = Canadian Western Amber Durum. BW= body weight; FCR = mortality corrected feed to gain ratio; AME = apparent metabolizable energy; NR = nitrogen retention. Formulated diets consisted of mixed ratios of outlier, high and low mycotoxin fractions respectively. M0 - no high fraction; M20 - 20% high mycotoxin fraction; M40 - 40% high mycotoxin fraction and M60 - 60% high mycotoxin fraction.

With respect to AME and NR for the starter phase, there were significant main effects and interactions (Table 3.4). The interactions were difficult to interpret, but were consistent between AME and NR, indicating this may be due to variability in sampling and subsequent variation in marker ratios measured. The interactions are not presented. There were significant effects of

wheat ratio on AME and NR with the highest levels measured for M60 or the highest level of DON contaminated wheat.

The performance and digestibility data for the grower/finish period are presented in Table 3.5. Overall mortality from 21 - 35 d was 4.4% and was not significantly different among wheat sources or ratio. With respect to 35 d BW there were numerical ($P < 0.10$) differences due to wheat source; the CWAD (2221 g) was higher than the CWRS (2191 g) and CWSWS (2145 g) wheat sources. Aviagen's performance guides (Aviagen, 2012) for 35 d of age male broilers is 2250 g. There were no significant treatment effects for FI. The FCR for 21 - 35 d was significant, birds fed CWAD had the lowest FCR (1.65) that was not significantly different from CWRS (1.70), but was significantly different than those birds fed CWSWS (1.74); there were no significant differences among birds FCR when fed CWRS or CWSWS. There were no significant interactions for BW, FI or FCR. Organ weights (proventriculus, gizzard, jejunum, ileum and caecum) relative to individual bird weight were measured and evaluated at 21 d and 35 d; there were no significant differences observed ($P > 0.10$) and data has not been presented.

There were significant interactions measured for AME and NR for the grower/finisher diets, and similar to the starter period there did not appear to be any consistent explanation for the interaction. Therefore, this data is not presented. There were no significant linear or quadratic estimations in wheat or diet ratio used in the study on performance or digestibility estimates during the grower/finisher period.

3.5 Discussion

Contamination of poultry feed with *Fusarium* fungi and mycotoxins have resulted in more than $1 billion in economic losses between 1992 and 1997 in Canada alone (Lombaert, 2002). The infection of grain due to FDK and mycotoxins cause reduced yield and quality and value of grain (Canadian Grain Commission, 2013). There are multiple factors contributing to the contamination of feed with *Fusarium* mycotoxins (Bryden, 2012). The most complicated of these are environmental conditions that fluctuate year to year. *Fusarium* infections continue to be a growing issue worldwide, as does our capacity to measure those infections (Alvarez et al., 2011).

Table 3.5. Broiler performance measurements for broiler grower/finisher phase (21 - 35 d) for three Canadian Western wheat types and respective ratios of mycotoxin-based diets.

Treatments			Broiler Grower/Finisher Phase (21 - 35 d)					
			BW (g)	Feed Intake (g/b/d)	FCR (g/g)	AME (kcal ME /kg diet)	NR (%)	Mortality (%)
		n/df						
Total		36	2186	165	1.70	3272	55.6	4.44
Wheat type		*2*	*NS*	*NS*	*	**	***	*NS*
	CWRS	12	2193	166	1.70^{ab}	3230^{b}	52.0^{b}	5.00
	CWSWS	12	2143	167	1.74^{a}	3277^{ab}	60.1^{a}	5.00
	CWAD	12	2221	162	1.65^{b}	3310^{a}	54.6^{b}	3.33
Ratio of mycotoxin		*3*	*NS*	*NS*	*NS*	***	***	*NS*
	M0	9	2201	165	1.70	3295^{a}	55.8^{a}	4.44
	M20	9	2181	162	1.70	3289^{a}	57.5^{a}	6.67
	M40	9	2168	164	1.72	3151^{b}	50.3^{b}	2.22
	M60	9	2193	168	1.68	3353^{a}	58.6^{a}	4.44
SEM			14.1	2.6	0.012	20.9	0.99	1.405
Wheat x Ratio		6	*NS*	*NS*	*NS*	***	***	*NS*
Regression (R^2)								
	Linear		0.01	0.01	0.00	0.01	0.00	
	Quadratic		0.01	0.08	0.01	0.05	0.01	

Means with differing letters in the same column are significantly different. Significance indicated as: NS $P \geq 0.1$; † $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$ and *** $P \leq 0.001$. Wheat types described include CWRS = Canadian Western Red Spring; CWSWS = Canadian Western Soft White Spring and CWAD = Canadian Western Amber Durum. BW= body weight; FCR = mortality corrected feed to gain ratio; AME = apparent metabolizable energy; NR = nitrogen retention. Formulated diets consist of mixed ratios of outlier, high and low mycotoxin fractions respectively. M0 - no high fraction; M20 - 20% high mycotoxin fraction; M40 - 40% high mycotoxin fraction and M60 - 60% high mycotoxin fraction. No linear or quadratic effects were detected.

The capacity to sort downgraded FDK wheat sources into high and low mycotoxin containing fractions was previously not commercially attainable. This new technology could provide an opportunity to reduce losses in broiler performance. In terms of economics, the use of at least a portion of this contaminated grain in feed should be allowed (Binder et al., 2007; Terzi et al., 2014), however this has raised the question of the impact on poultry performance (Binder et al., 2007). Variability in the quality among different wheat sources may impact broiler performance (Steenfeldt, 2001), therefore, reducing the variability through sorting may be a

method to minimize the impact on broiler performance. Impact on poultry performance and overall health are major concerns among producers as well as cost, and lower immune function or vaccine response, which may require higher antibiotic use (Shier et al., 2005).

Our results show that the test fractions sorted by the BoMill concentrated FDK and DON into the lowest 20% CP and were reduced in the remaining kernels. This indicates that reduced the amount of FDK in a grain source could reduce the amount of DON and improve feed quality. The reduction of both FDK and DON with improved CP level agrees with the literature that FDK and DON are positively correlated (Menniti et al, 2003; Paul et al., 2005; Wegulo et al., 2010; Kautzman et al., 2015a). Differences in TKW might be attributed to distinct differences in kernel composition (Matthaus et al., 2004). There are indications that both TKW and TW weight may be reduced by the shriveling effects of FDK on kernels (Dexter et al., 1996) and they would be smaller and lighter than healthy kernels (Symons et al., 2002).

Although DON was the only *Fusarium* mycotoxin present in measureable amounts in any fraction of all three grain sources, it is possible that unmeasured or "masked" mycotoxins were present. The differences in broiler response with similar DON concentrations found in the literature may be due to these unmeasured mycotoxins producing additive, synergistic or antagonistic effects (Fink-Gremmels, 2013). Detection and analytical limitation may have limited our ability to detect additional mycotoxins that may influence DON toxicity (Swamy et al., 2004a). However, based on our study, performance was not negatively impacted by mycotoxin enrichment through sorting and reassembling wheat with different levels of naturally contaminated kernels.

Poultry have been shown to have greater tolerance to DON in comparison to other species (Bohm, 2000; Razzazi-Fazeli et al., 2003). This tolerance enables poultry to be fed contaminated grain unfit for human consumption (Girgis and Smith, 2010). The natural contamination in feed is typically lower than concentrations tested in purified mycotoxin research studies (Dersjant-Li et al., 2003); however more recent studies are focusing on use of naturally contaminated grain (Girgis and Smith, 2010). Many of the studies that measured the effect of DON fed to poultry show variation in performances (Danicke et al., 2001). Awad et al. (2012) and Ghareeb et al. (2012) indicated that DON has negative influences on the performance of poultry including feed refusal and decreased weight gain at levels of 10 ppm or higher. Negative clinical effects were seen in diets containing less than 4 ppm (Bergsjo et al., 1993) and at higher concentrations (Leitgeb et al., 2000), yet other studies (Swamy et al., 2002; Awad et al., 2004, 2006a, 2011) reported no effects

at levels up to 15 ppm. In this study, DON measured in diets ranged from 1.2 and 8.3 ppm (starter) and from 1.0 - 7.9 ppm (grower/finisher). Similar to results reported by Awad et al. (2011), BW, FI and FCR were not affected by increasing levels of DON in diets. It appears that the broiler chickens were able to tolerate the levels of DON in the contaminated diets.

The lack of effects on bird performance measurements could be due to changing gut microbes as an adaptation to the diet (Rotter et al., 1992) or that broilers do not show feed refusal in the grower/finisher phases (Swamy et al., 2002). In regards to BW gain and FI, our results were similar to Swamy et al. (2004a). These authors reported that feed consumption and weight gains were not affected by DON inclusion in either starter (5.9 ppm) or grower/finisher (9.5 ppm) diets. Other studies have also shown a highly variable effect of DON on broiler performance (Kubena et al., 1997; Swamy et al., 2002; Danicke et al., 2003; Sypecka et al., 2004; Awad et al., 2004, 2006a). It appears that in order to obtain feed refusal and reduced weight gain, the DON concentrations in diets may need to reach levels of 16 - 20 ppm (Kubena et al., 1989; Harvey et al., 1991). There was no apparent effect of ratio on broiler performance based on any of the endpoints measured in this study. These are research studies, hence, it is often a cleaner and less challenging environment than may be found commercially. Whether these factors are important needs to be considered.

This study demonstrates the potential of individual kernel sorting technology to produce naturally contaminated diets with a range of mycotoxin concentrations from a single wheat source to enable future investigations of mycotoxicoses in any species. Previous to this, uncontaminated diets were spiked with pure mycotoxin to create a range of exposures or multiple wheat sources were used. This approach can alter mycotoxin bioavailability compared to natural contamination, and does not permit effective study of naturally-occurring mycotoxin mixtures (Danicke et al., 2000).

Our results provided evidence that the feeding of DON contaminated diets using realistic levels found in *Fusarium* contaminated wheat sources as a chronic exposure may not cause performance effects on broilers to 35 d of age. These results are of practical importance to poultry producers by providing evidence that wheat sources contaminated with *Fusarium* may be able to be fed at DON concentrations higher than 5 ppm. The results also indicate that further work is required to establish the upper limit of contaminated grain that could be fed safely as commercial NIT sorting may significantly increase the volumes of these grain and provide a means of salvaging them and converting them into high quality animal protein.

4.0 USING NEAR INFRARED TRANSMITTANCE (NIT) TO GENERATE SORTED FRACTIONS OF *FUSARIUM* INFECTED WHEAT AND THE IMMUNOLOGICAL IMPACT ON BROILER CHICKENS.

Preface

This chapter is to be submitted to Mycotoxin Research under joint authorship with Tom A. Scott, Natacha S. Hogan, Susantha M, Gomis, Kaitlyn S. Brown and Mark L. Wickstrom (University of Saskatchewan). References cited in this chapter are listed in the reference section of this thesis.

The research described in this chapter is a continuation of Chapter 3. The purpose of this chapter was to investigate the potential impacts on immune responses (cell mediated and humoral immune responses, the heterophil to lymphocyte ratio, relative tissue weights of immune organs and histopathological changes) of feeding broiler chickens four diet ratios pertaining to the amount of high *Fusarium* damaged kernels and mycotoxin fraction over 35 days. My roles consisted of random selection of birds to be placed in the trial, broiler chicken husbandry, injections of phytohemagglutinin, bovine serum albumin and saline, all data and tissue collections and preparations, the statistical analysis of that data and the authorship of this chapter in full.

4.1 Abstract

Fusarium mycotoxins, namely deoxynivalenol (DON), negatively impact the nutritional quality of grain. This study evaluated effects of feeding three naturally contaminated *Fusarium* downgraded wheat sources; Canadian Western Red Spring (CWRS), Canadian Western Soft White Spring (CWSWS) and Canadian Western Amber Durum (CWAD) on immunological parameters in broiler chickens. Sources were individually sorted into three test fractions: outlier (10% of the source); high mycotoxin (20% of the source); and low mycotoxin (70% of the source) then were reconstituted into four diet ratios: M0; M20; M40; and M60 relating to the proportion of the high mycotoxin fraction in the reconstituted diets, providing a 3 (wheat source) x 4 (diet ratio) factorial arrangement. Concentrations of DON ranged from 1 - 8 ppm. Immunological impacts of DON contaminated diets were assessed by evaluating: 1) the cell-mediated response to the mitogen phytohemagglutinin (PHA) through intermediate interdigital web swelling; 2) the humoral response to bovine serum albumin (BSA) antigen through induced antibody production, and 3) the heterophil to lymphocyte (H:L) ratio. Relative tissue weights and histopathology of selected immune tissues were also assessed. Results indicate that there were no significant differences ($P < 0.05$) in skin swelling response to PHA, secondary antibody response to BSA or the ratio of heterophils to lymphocytes with increasing inclusion of the high mycotoxin fraction. Significant differences in relative tissue weights were only observed in the spleen ($P < 0.05$) at 21 d and in the liver ($P < 0.01$) at 35 d. Histopathology showed increases in lesions as lymphoid aggregates or granulopoisis in more contaminated diets. These results indicate that broiler immunology was not impacted with increasing mycotoxin exposure.

4.2 Introduction

Fusarium mycotoxins are secondary metabolites produced from grain infected with *Fusarium* species (Doll and Danicke 2011). Ingestion has been shown to cause well-characterized toxicological symptoms including decreased immunity and resistance to infectious diseases (Bondy and Pestka 2000; CAST 2003). *Fusarium* contamination of grain may lead to the formation of *Fusarium* damaged kernels (FDK; Cheeke, 1998), which can decrease kernel crude protein (CP; Jackowiak et al. 2005) levels in addition to increasing levels of mycotoxins, most commonly deoxynivalenol (DON; Pronk et al. 2002; Girish et al. 2010). Overall, FDK results in poor quality feed. Ingestion of this feed may cause adverse health effects in production animals including decreased growth performance and require the increased use of antibiotics (Pronk et al. 2002; Binder 2007). It has been suggested that performance losses are due to the ability of *Fusarium* mycotoxins to alter the availability and quality of nutrients in animal feed potentially leading to mycotoxicosis *(*Bryden 2007; Doll and Danicke 2011*).*

Fusarium mycotoxins induce acute and chronic immune system alterations in poultry depending on the mycotoxin type, concentration, duration of exposure, species and age of the bird (D'Mello et al. 1999; Pestka 2007). The immune system and hematological alterations in poultry with chronic *Fusarium* mycotoxin consumption have not been well characterized (Awad et al. 2012) and are often contradictory (Awad et al. 2006). *Fusarium* mycotoxins target the rapidly proliferating and differentiating cells (monocytes, macrophages and lymphocytes) or organs (liver, spleen and bursa of Fabricius) of the immune system (Girish et al. 2010). This can reduce immune system function, leading to increased disease susceptibility (Pestka 2007) and reduced antibody production (Girish et al. 2010).

There is evidence that *Fusarium* mycotoxins can be immunosuppressant or immunostimulating (Rotter et al. 1996; Bondy and Pestka 2000; Danicke 2002; Awad et al. 2005) due to their highly variable and complex nature (Pestka 2008). Effects in animals have been observed on cellular responses, humoral factors and cytokine mediators of the immune system with consumption of *Fusarium* contaminated feed (Bondy and Pestka 2000). Effects of chronic, low level mycotoxin exposure are difficult to recognize since indications of disease tend to be diagnosed as secondary infections rather than a weakened immune system that predisposed the animal to the infection (Oswald et al. 2005; Antonissen et al. 2014) due to potential compensatory or adaptive mechanisms (Awad et al. 2008a). During infection the body will divert nutrients away

from growth and development to the immune system (Humphrey et al. 2004; Klasing 2007), which will further contribute to reduced performance following mycotoxin exposure.

Innate and acquired immunity collectively contributes to the immune system of poultry. Acquired immunity consists of cell-mediated and humoral immune systems. It has been suggested that T and B cells are highly sensitive to *Fusarium* mycotoxins (Awad et al. 2008). Cell-mediated immunity (CMI) acts through T cells and is responsible for delayed type hypersensitivity responses, which may be impaired or induced by DON exposure (Bondy and Pestka 2000; Pestka et al. 2004). The phytohemagglutinin (PHA) skin thickness swelling response after 24 hours is the most commonly used evaluation of cell-mediated immune system function (Fairbrother et al. 2004; Tella et al. 2008). Humoral immunity requires the production of B cells, which produce immunoglobulin (Ig) antibodies specific to the invading antigen. Primary response to an antigen produces IgM antibodies and subsequent exposure to the same antigen produces a secondary response of IgY antibodies (Fairbrother et al. 2004). The humoral immune competency of broiler chickens has been studied using injections of bovine serum albumin (BSA) antigen, a foreign protein that has been shown to cause an IgY response (Mast and Goddeeris 1999). One of the most dramatic effects of DON on antibody synthesis is the depression in IgY (Rotter et al. 1996; Pestka 2003). *Fusarium* mycotoxin immunosuppression or immunostimulation in poultry has been suggested to be dose dependent (Fairbrother et al. 2004). The ratio of heterophils to lymphocytes (H:L) is known to increase in broilers as a response to foreign challenge leading to decreased lymphocyte numbers (Gross and Siegel 1983). Organs with a higher protein turnover such as immune organs, liver, heart and small intestine are mainly affected by *Fusarium* exposures (Awad et al. 2012).

Several mitigation strategies have been investigated to combat the negative effects of DON. Ideally, contaminated grains would be avoided completely when formulating diets for livestock. Knowing the positive correlation between FDK and DON (Symons et al. 2002; Peiris et al. 2009), BoMill AB has engineered an individual kernel sorter, the TriQ, which uses Near Infrared Transmittance (NIT) technology to separate FDK contaminated kernels, based indirectly on CP from bulk grain (BoMill AB 2008; Patent 7417203). Previous research on the TriQ indicated it could effectively distinguish between healthy and damaged kernels contaminated with DON by reducing FDK based on CP (Kautzman et al. 2015a). Wheat varieties have inherent variability in grain characteristics, such as CP (Tonning et al. 2009) and FDK does not indicate DON presence

(Liu et al. 1997). Also, DON levels can vary within individual kernels (Berthiller et al. 2013). Previously, ranges of DON were obtained using purified sources mixed with grain or multiple sources of contaminated grain (Canady et al. 2002). The variability of CP, FDK and attained DON levels within diets makes it difficult to compare studies on the effects of mycotoxins on animal health. This technology would allow the establishment of a range of DON exposure levels using a single wheat source as opposed to using multiple sources thereby reducing much of the variability.

The purpose of this study was to evaluate the effects of 5 weeks of dietary exposure to ratios naturally contaminated with DON on cell mediated and humoral immune responses, the heterophil to lymphocyte ratio, changes in relative organ weights and histopathological differences in selected immune organs. We sorted three wheat sources containing natural *Fusarium* contamination using the TriQ into three test fractions (outlier, high and low mycotoxin levels) to formulate four reconstituted diet ratios (M0; M20; M40 and M60) with increasing amounts of the high mycotoxin fraction. We exposed broiler chicks to these naturally contaminated diet ratios in a 35 d feeding trial and evaluated the impact of DON on immune-relevant endpoints (CMI response, humoral response, H:L ratio, immune specific organ effects and histopathology of those organs). This allowed the assessment of potential differences among different wheat sources and among reconstituted diet ratios.

4.3 Materials and methods

The experimental protocol and all procedures were approved (AUP# 20130047) by the University Committee on Animal Care and Supply, Animal Research Ethics Board, University of Saskatchewan, Saskatchewan, Canada. The care and handling used followed the principles established by the Canadian Council on Animal Care (1993).

4.3.1 BoMill TriQ sorter

This technology is based on Near Infrared Transmittance (NIT) of individual kernels with the capacity to sort individual kernels of wheat based on CP at a rate of 2 - 3 tonnes/hour into three user-defined fractions. The TriQ consists of a rotating singulator drum (specific to grain type) with approximately 22,000 laser etched pockets that facilitate positioning of individual kernels by centrifugal force. Each kernel is measured by three NIT detectors to detect transmittance of specific spectral wavelengths to estimate the amount of CP in each kernel (BoMill 2008; Patent

#7417203). Once variability is known, the user can direct fractions of grain into three exit pipes. A more detailed description of this technology is available elsewhere (Kautzman et al. 2015a).

4.3.2 Diets

Three sources of contaminated wheat (approximately 6 MT/source) downgraded due to FDK were purchased from grain producers in Western Canada. The three wheat sources were classified as: Canadian Western Red Spring (CWRS); Canadian Western Soft White Spring (CWSWS); and Canadian Western Amber Durum (CWAD). Each source was initially sorted into outlier, high and low mycotoxin fractions based on CP concentration. Unsorted and sorted fractions were graded by a Canadian Grain Commission inspector for levels of FDK and assigned a grade. Samples from each source and fraction were analyzed for 16 common *Fusarium* mycotoxins (North Dakota State University, Veterinary Diagnostic Laboratory, Fargo, ND) via gas chromatography (model 689ON; Agilent Technology, Englewood, CO, USA) and mass spectrometer (model 5975B XL E1/C1; Agilent Technology, Englewood, CO, USA). Deoxynivalenol (DON) was the only mycotoxin identified at levels above the detection limit of 0.5 ppm.

Re-constitutions of the outlier, high and low mycotoxin fractions into four ratios consisted of: a control diet or M0 (low fraction only); M20 (10% outlier, 20% high fraction, 70% low fraction); M40 (10% outlier, 40% high fraction, 50% low fraction); and M60 (10% outlier, 60% high fraction, 30% low fraction). Levels of DON in the starter diets ranged from 0.5 - 8.3 ppm and 0.5 - 7.9 ppm in the grower diets (Table 3.3). 'Outliers' represented either kernels from other types of grain, improper positioning of kernels into pockets or two kernels in one pocket (BoMill 2012). Starter and grow/finisher diets contained 70.7% and 75.7% wheat, respectively. A more detailed description of these diets is available elsewhere (Kautzman et al. 2015b). The authors would like to note that due to the university's animal welfare concerns, diet formulation consisting solely of the high mycotoxin fraction was not approved.

4.3.3 Bird management and sample collection

This study was an assessment of mycotoxins on broiler performance and immune competence; however, only immune competence data is presented here. A total of 480 1 d of age Ross 308 male broilers were purchased from Lilydale Hatchery Inc. (Wynyard, Saskatchewan, S0A 4T0), individually weighed and randomly assigned to one of 12 starter diets. Each diet

consisted of eight replicate cages containing five birds with feed and water (two cup drinkers/cage) provided *ad libitum* for a total of 96 battery cages (29 cm high; 48 cm wide; 83 cm long; providing 800 cm^2/bird floor space) arranged into four levels from 1 - 21 d of age. At 21 d, a total of 180 birds previously assigned to the immunology trial were housed in 36 grow-out cages (38 cm high x 66 cm wide x 69 cm long; providing 900 cm^2/bird floor space) until 35 d of age. The grow-out cages maintained the same dietary treatments as fed 0 - 21 d. The 12 test grower/finisher test diets (Table 3.1) were assigned to three cages of five broilers from 21 - 35 d based on the previously assigned starter diet. Ambient temperature was set to 32^{o}C (1 d) and was gradually reduced to 23^{o}C (21 d), which was maintained until trial completion. The birds were provided with incandescent lighting and lighting program of 23 hours daylight at 30 lux from 0 - 7 d then reduced to 20 hours daylight at 10 lux for the remainder of the trial.

4.3.4 Lymphoproliferative response to phytohemagglutinin

The *in vivo* CMI response to PHA was evaluated as the change in thickness of the right and left intermediate interdigital webs of selected broilers. One bird from each cage (n = 96) was measured in triplicate using a digital caliper (model 54-101-150-2; Fowler Canada, Kitchener Ont.) to the nearest 0.01 mm to determine the average thickness. The site was swabbed with 70% ethanol and 100 ug PHA (Cat no. 151884; MP Biomedicals, Solon, OH) dissolved in 0.1 ml of 1x sterile phosphate buffered saline (PBS; Cat no. BP2438-4; Fisher BioReagents, Fair Lawn, NJ) was injected into the left intermediate interdigital web. At the same time, 0.1 ml of 1x sterile PBS was injected into the right intermediate interdigital web (Stoddart 2006). Skin thickness was then measured in triplicate 24 hours post-injection in millimeters (mm). The difference in thickness (swelling) response was calculated as: swelling index = (post injection thickness of intermediate interdigital web – pre injection thickness of intermediate interdigital web) / post injection thickness of intermediate interdigital web (Smits et al. 1999).

4.3.5 Antibody response to bovine serum albumin

Humoral effects of mycotoxin exposure were evaluated through the injection of BSA (Cat no. BP671-1; Fisher BioReagents, Fair Lawn, NJ) to elicit changes in 2^o IgY antibody titers (Bunn 2000). Two birds from each replicate (except for the eighth replicate where only one bird was used) from each diet ratio per wheat source (n = 180) were selected at 14 d and pre-exposure blood samples were collected from the left brachial vein. A total of seven birds per diet ratio and wheat

source (n = 168) were then injected with BSA. The left brachial vein was cleaned of feathers, swabbed with 70% ethanol and injected subcutaneously with 0.5 ml of 4 ppm BSA (Stoddart 2006). The remaining birds (n = 12) were similarly handled and received 0.5 ml of sterile PBS as a control. To prevent the collapse of the branchial vein in the 14 d birds, baseline blood samples were collected using heparinized 25G Monoject™ hypodermic needles with Monoject™ 1 mL syringes (Covidien, Mansfield, MA, USA). Samples were kept on ice for a maximum of 1 hour until processed. Challenged and control birds were administered a booster of 0.5 ml at 22 d to elicit the secondary response (Stoddart 2006). Final blood samples were collected at 35 d using heparinized 22G monoject™ hypodermic needles with 4 mL BD Vacutainers to evaluate 2^{o} antibody titer response and measured as ng/ml. The samples were placed on ice and immediately transported for processing.

Prior to the processing of 14 and 35 d blood samples, 0.5 ml subsamples were taken for the establishment of heterophil to lymphocyte (H:L) ratio. The remaining blood was centrifuged (Eppendorf 5810 R, Brinkmann Instruments Inc., Westbury, NY, USA) at 4000 rpm and 10^{o}C for 10 minutes. Plasma was pipetted off, transferred to low temperature cryovials and stored in aliquots at -80^{o}C. Analysis of the plasma samples was conducted using the Chicken IgG ELISA Quantification kit from Bethyl Laboratories (Cat. No. E30-104), following manufactures instructions, read on a microplate spectrophotometer reader (model SpectraMAX 190; Molecular Devices Corp., Sunnyvale, CA) to measure absorbance at 450 nm at room temperature (20 - 25^{o}C) and SOFTPRO Max software (Version 4.0; 2001) was used to calculate and interpret the absorbance readings. The difference in antibody titer response was calculated as: (35 d antibody titer response – 14 d antibody titer response) / 35 d antibody titer response (Embers et al. 2012).

4.3.6 Heterophil to lymphocyte ratio

Duplicate blood smears on microscope slides were made using 0.5 ml blood from pre- and post-challenged and control birds. Slides were sent to Prairie Diagnostic Services (College of Veterinary Medicine, University of Saskatchewan) for staining. A total of 100 leukocytes (either heterophils or lymphocytes) were counted per slide at 400X total magnification to establish H:L ratio. The H:L ratio was calculated and evaluated as: number of heterophils / number of lymphocytes (Gross and Siegel 1983). The difference between these H:L ratios were evaluated as (35 d H:L – 14 d H:L) / 35 d H:L (Maxwell and Robertson 1998).

4.3.7 Relative tissue weight

Lymphoid organ weights (liver, spleen, bursa of Fabricius) expressed as percentage body mass and can be assessed by histopathology as indicators of immunocompetence in poultry (Fairborther et al. 2004). The evaluation of potential differences in important immunological tissues based on the effects of the consumed diets can then be conducted. These tissues have primordial roles in poultry immunity (Toivanen et al. 1987) and their development may be affected by mycotoxins (Corrier 1991) through inhibitory effects on protein synthesis (Awad et al. 2013). At 21 d, 156 birds were weighed and euthanized by cervical dislocation. Selected tissues associated with immunological function (liver, spleen and bursa of Fabricius) and empty gut sections (jejunum, ileum and caecum) were collected and weighed to the nearest 0.01 g. At 35 d, 108 birds were selected, weighed and euthanized by cervical dislocation with similar tissues collected as 21 d. Relative tissue weight was calculated and expressed as % relative to body weight as (tissue weight / body weight)*100.

4.3.8 Histopathology

The heart, liver, spleen, kidney, bursa of Fabricius and gut sections from three birds per diet ratio at 14 and 35 d were removed carefully using a sharp scalpel and fixed in 10% neutral buffered formalin. The jejunum was defined as the proportion from the duodenal loop to Meckel's diverticulum; the ileum section included Meckel's diverticulum to the ileo-cecal junction and the caecum consisted of the two ceca. These gut sections were sampled as 1 cm samples according to the method indicated by Yunus et al. (2012a). These tissues were trimmed to fit into histopathological cassettes with a clean scalpel and placed in fresh 10% neutral buffered formalin. Tissues were paraffin embedded, sectioned at 5 μm thickness and stained with hematoxylin and eosin (Robertson and Maxwell 1990). The processed tissues were examined by a poultry pathologist (Dr. Susantha Gomis, Department of Veterinary Medicine, University of Saskatchewan) using 400X magnification. A histopathological score of 0 – 4: (0 = no visible lesions; 1 = mild, multifocal expansion of lymphoid aggregates or granulopoisis; 2 = moderate, multifocal expansion of lymphoid aggregates or granulopoisis; 3 = moderate to severe, multifocal expansion of lymphoid aggregates or granulopoisis or 4 = severe, multifocal expansion of lymphoid aggregates or granulopoisis) was assigned (Sharma et al., 1989).

4.3.9 Statistical analysis

All data were subject to statistical analysis using a complete randomized block design (PROC MIXED of SAS [Version 9.3]; SAS Institute, Cary, NC) with battery level as the blocking factor. Classes were wheat (CWRS, CWSWS, and CWAD) and ratio (M0, M20, M40, and M60). Analysis was conducted using a 3 (wheat class) x 4 (ratio level) factorial arrangement with cage as the experimental unit. Multi-treatment means comparisons were separated using the Tukey test. Linear and quadratic effects of increasing ratios of mycotoxins were analyzed using regression (PROC REG and RSREG of SAS [Version 9.3]; SAS Institute, Cary, NC) model. Data were log transformed when required to achieve normality. Histopathological lesion scores were analyzed using Chi-Square. Means were compared using PDMIX800 (Saxton 1998). Significance was determined at $P < 0.05$.

4.4 Results

4.4.1 Phytohemagglutinin

The lymphoproliferative response was evaluated for differences among wheat type (CWRS; CWSWS and CWAD), diet ratios (M0; M20; M40; M60) and potential interactions through intradermal administration of PHA into the intermediate interdigital webs. The DON exposure as presented in Table 4.1 indicated no significant difference in the pre-challenged birds in both the PHA and saline control injected birds. There was an expected swelling due to PHA mitogen post challenge, but no significant differences ($P > 0.05$) were observed in the change in percentage swelling in the intermediate interdigital webs among wheat types or diet ratios. No interactions were present.

4.4.2 Antibody production

The antibody titers against BSA are summarized in Table 4.2. The antibody response was evaluated for differences among wheat type (CWRS; CWSWS and CWAD), diet ratios (M0; M20; M40; M60) and potential interactions. Analysis of the baseline blood samples at 14 d indicated no significant differences in secondary response prior to BSA mitogen administration. Although levels of BSA antibody indicated a possible trend at 35 d ($P < 0.1$) associated with diet ratio, this was not determined to be significant ($P < 0.05$) and was not impacted by wheat type or diet ratio. No significant interactions were seen at either 14 or 35 d.

Table 4.1. A 24-hour cell-mediated immune response of broilers (n = 96) to phytohemagglutinin (PHA) injection of 1 ppm intradermally for three Canadian Western grain sources and four diet ratios.

		Day 20 Pre-Challenged (mm)		Day 21 Post Challenged (mm)		% Change in swelling	
Treatments	*n/df*	PHA	Saline	PHA	Saline	PHA	Saline
Wheat	*2*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
CWRS	32	1.64	1.61	2.49	1.73	0.33	0.08
CWSWS	32	1.67	1.68	2.52	1.76	0.33	0.05
CWAD	32	1.70	1.65	2.61	1.81	0.33	0.10
Ratio of mycotoxin	*3*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
M0	24	1.68	1.69	2.65	1.84	0.35	0.10
M20	24	1.72	1.66	2.57	1.85	0.32	0.12
M40	24	1.58	1.58	2.43	1.63	0.33	0.03
M60	24	1.69	1.65	2.51	1.74	0.32	0.05
SEM		0.023	0.020	0.042	0.033	0.012	0.017
Wheat x Ratio	*6*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
Regression (R^2)							
Linear			0.01	0.02	0.02	0.01	0.01
Quadratic			0.02	0.03	0.03	0.01	0.03

Means with differing letters in the same column are significantly different. Significance indicated as: NS $P \geq 0.1$; † $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$ and *** $P \leq 0.001$. Wheat types described include CWRS = Canadian Western Red Spring; CWSWS = Canadian Western Soft White Spring and CWAD = Canadian Western Amber Durum. Formulated diets consist of mixed ratios of outlier, high and low mycotoxin fractions respectively. M0 - no high fraction; M20 - 20% high mycotoxin fraction; M40 - 40% high mycotoxin fraction and M60 - 60% high mycotoxin fraction. No linear or quadratic effects were detected.

4.4.3 Heterophil to lymphocyte ratio

There were no significant effects of wheat source or diet ratio on the heterophil to lymphocyte (H:L) ratio (Table 4.3). The CWAD wheat was the most contaminated of all wheat sources. The change in the H:L ratio from 14 - 35 d indicated a larger increase in the amount of heterophils in birds fed the CWAD wheat type. Similarly to the diet ratio results, there was an increase in the H:L ratio for all diet ratios.

Table 4.2. Humoral immune response of broilers at 35 d to bovine serum albumin (BSA) injection of 4 ppm intramuscularly compared to 14 d baseline for three Canadian Western grain sources and four diet ratios.

Treatments		Day 14 *n/df*	Day 14 Antibody titers (ng/ml)	Day 35 *n/df*	Day 35 Antibody titers (ng/ml)	% Change
Wheat		*2*	*NS*	*2*	*NS*	*NS*
	CWRS	56	3.17	54	8.91	0.62
	CWSWS	56	3.18	53	8.21	0.58
	CWAD	56	3.20	54	9.27	0.62
Ratio of mycotoxin		*3*	*NS*		*NS*	†
	M0	42	3.29	40	8.68	0.58
	M20	42	3.14	40	8.96	0.63
	M40	42	3.07	41	9.34	0.64
	M60	42	3.24	39	8.17	0.57
SEM			0.055		0.215	0.010
Wheat x Ratio		*6*	*NS*		*NS*	*NS*
Regression (R^2)						
	Linear		0.01		0.00	0.00
	Quadratic		0.01		0.01	0.03†

Means with differing letters in the same column are significantly different. Significance indicated as: NS $P \geq 0.1$; † $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$ and *** $P \leq 0.001$. Wheat types described include CWRS = Canadian Western Red Spring; CWSWS = Canadian Western Soft White Spring and CWAD = Canadian Western Amber Durum. Formulated diets consist of mixed ratios of outlier, high and low mycotoxin fractions respectively. M0 - no high fraction; M20 - 20% high mycotoxin fraction; M40 - 40% high mycotoxin fraction and M60 - 60% high mycotoxin fraction. No linear or quadratic effects were detected.

4.4.4 Relative tissue weights

The relative tissues weights of the liver, spleen and bursa of Fabricius were summarized in Table 4.4. There were significant differences ($P < 0.01$) in relative liver weight among birds fed different wheat types at 35 d, with CWRS having different relative weight compared to CWSWS and CWAD. Liver weights at 35 d were lower than those observed at 21 d where there was no significant difference in weight seen. The heaviest liver weights were shown to be in the wheat type (CWAD) that contained the highest DON contamination.

Table 4.3. Heterophil to Lymphocyte (H:L) ratio of broilers pre and post exposure to bovine serum albumin (BSA) injection of 4 ppm intramuscularly for three Canadian Western grain sources and four diet ratios.

		H:L Ratio (cell count)		% Change
Treatments	*n/df*	Day 14	Day 35	
Wheat	*2*	*NS*	*NS*	*NS*
CWRS	56	0.90	0.95	0.05
CWSWS	56	0.94	0.98	0.04
CWAD	56	0.81	0.92	0.12
Ratio of mycotoxin	*3*	*NS*	*NS*	*NS*
M0	42	0.92	0.95	0.03
M20	42	0.98	1.06	0.08
M40	42	0.76	0.87	0.13
M60	42	0.87	0.91	0.04
SEM		0.036	0.040	0.055
Wheat x Ratio	*6*	*NS*	*NS*	*NS*
Regression (R^2)				
Linear		0.01	0.00	0.00
Quadratic		0.01	0.01	0.01

Means with differing letters in the same column are significantly different. Significance indicated as: NS $P \geq 0.1$; † $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$ and *** $P \leq 0.001$. Wheat types described include CWRS = Canadian Western Red Spring; CWSWS = Canadian Western Soft White Spring and CWAD = Canadian Western Amber Durum. Formulated diets consist of mixed ratios of outlier, high and low mycotoxin fractions respectively. M0 - no high fraction; M20 - 20% high mycotoxin fraction; M40 - 40% high mycotoxin fraction and M60 - 60% high mycotoxin fraction. No linear or quadratic effects were detected.

There was a significant difference ($P < 0.05$) in relative spleen weight at 21 d; however there was no significant difference in this response at 35 d. There was no significant difference in the relative weight of the bursa of Fabricius due to either the wheat type or the diet ratio level however relative bursa weight was lower at 35 d compared to 21 d.

4.4.5 Histopathology

Blind histopathological examination of liver, kidney, spleen, heart and bursa of Fabricius was conducted by a poultry pathologist and showed no mycotoxin induced alteration. Liver damage was observed, however among birds fed the M60 diet ratio (Table 4.5 and Figure 4.1).

Table 4.4. Relative tissue weights (%) for liver, spleen and bursa at 21 and 35 d for three Canadian Western grain sources and four diet ratios.

		Day 21				Day 35		
Treatments	*n/df*	Liver	Spleen	Bursa	*n/df*	Liver	Spleen	Bursa
Wheat	*2*	*NS*	*	*NS*	*2*	**	*NS*	*NS*
CWRS	52	2.94	0.09[a]	0.23	36	2.75[b]	0.12	0.19
CWSWS	52	3.07	0.07[b]	0.24	36	2.82[a]	0.11	0.18
CWAD	52	3.21	0.08[ab]	0.24	36	3.05[a]	0.13	0.19
Ratio of mycotoxin	*3*	*NS*	*NS*	*NS*	*3*	*NS*	*NS*	*NS*
M0	39	3.15	0.09	0.24	27	3.04	0.11	0.18
M20	39	3.08	0.08	0.23	27	2.84	0.13	0.19
M40	39	3.05	0.09	0.24	27	3.01	0.12	0.19
M60	39	3.03	0.08	0.23	27	2.85	0.12	0.19
SEM		0.033	0.003	0.005		0.081	0.006	0.004
Wheat x Ratio	*6*	*NS*	*NS*	*NS*	*6*	*NS*	*NS*	*NS*
Regression (R^2)								
Linear		0.01	0.00	0.00		0.01	0.01	0.00
Quadratic		0.01	0.00	0.00		0.01	0.01	0.01

Means with differing letters in the same column are significantly different. Significance indicated as: NS $P \geq 0.1$; † $P \leq 0.1$; * $P \leq 0.05$; ** $P \leq 0.01$ and *** $P \leq 0.001$. Wheat types described include CWRS = Canadian Western Red Spring; CWSWS = Canadian Western Soft White Spring and CWAD = Canadian Western Amber Durum. Formulated diets consist of mixed ratios of outlier, high and low mycotoxin fractions respectively. M0 - no high fraction; M20 - 20% high mycotoxin fraction; M40 - 40% high mycotoxin fraction and M60 - 60% high mycotoxin fraction. No linear or quadratic effects were detected.

Although there appeared to be no trend among wheat types, the appearance of the liver was similar between the M0 and M20 diet ratios, however birds fed the M60 diet ratio had higher lesion scores as lymphoid and granulopoisis damage when compared to the other diet ratios, although this was not significant ($P > 0.05$). Typically in poultry, some lymphoid damage is evident in healthy birds and was taken into account prior to visual lesion scoring (Susantha Gomis, personal communication).

Table 4.5. Histological lesion score from livers of three birds from selected diet ratios at 35 d.

Wheat type	Diet	Bird number	Lesion score	Histological Alteration
CWRS	M0	1	3	Lymphoidal and granulopoisis
	M0	2	0	Normal
	M0	3	0	Normal
	M20	1	1	Lymphoidal
	M20	2	1	Lymphoidal
	M20	3	3	Lymphoidal
	M60	1	2	Lymphoidal
	M60	2	2	Lymphoidal
	M60	3	3	Lymphoidal
CWSWS	M0	1	1	Lymphoidal and granulopoisis
	M0	2	1	Lymphoidal
	M0	3	3	Lymphoidal
	M20	1	0	Normal
	M20	2	3	Lymphoidal
	M20	3	3	Lymphoidal
	M60	1	3	Lymphoidal
	M60	2	2	Lymphoidal and granulopoisis
	M60	3	2	Lymphoidal
CWAD	M0	1	1	Lymphoidal and granulopoisis
	M0	2	1	Lymphoidal and granulopoisis
	M0	3	0	Normal
	M20	1	2	Lymphoidal
	M20	2	0	Normal
	M20	3	2	Lymphoidal and granulopoisis
	M60	1	1	Lymphoidal and granulopoisis
	M60	2	2	Lymphoidal
	M60	3	3	Lymphoidal and granulopoisis

Lymphoidal = damaged lymphoid cells were present. Granulopoisis = hematopoiesis of granulocytes. Scoring system = 0 - 3 (0 = no visible lesions; 1 = mild, multifocal expansion of lymphoid aggregates or granulopoisis; 2 = moderate, multifocal expansion of lymphoid aggregates or granulopoisis; or 3 = moderate to severe, multifocal expansion of lymphoid aggregates or granulopoisis) Wheat types described include CWRS = Canadian Western Red Spring; CWSWS = Canadian Western Soft White Spring and CWAD = Canadian Western Amber Durum. Formulated diets consist of mixed ratios of outlier, high and low mycotoxin fractions respectively. M0 - no high fraction; M20 - 20% high mycotoxin fraction and M60 - 60% high mycotoxin fraction.

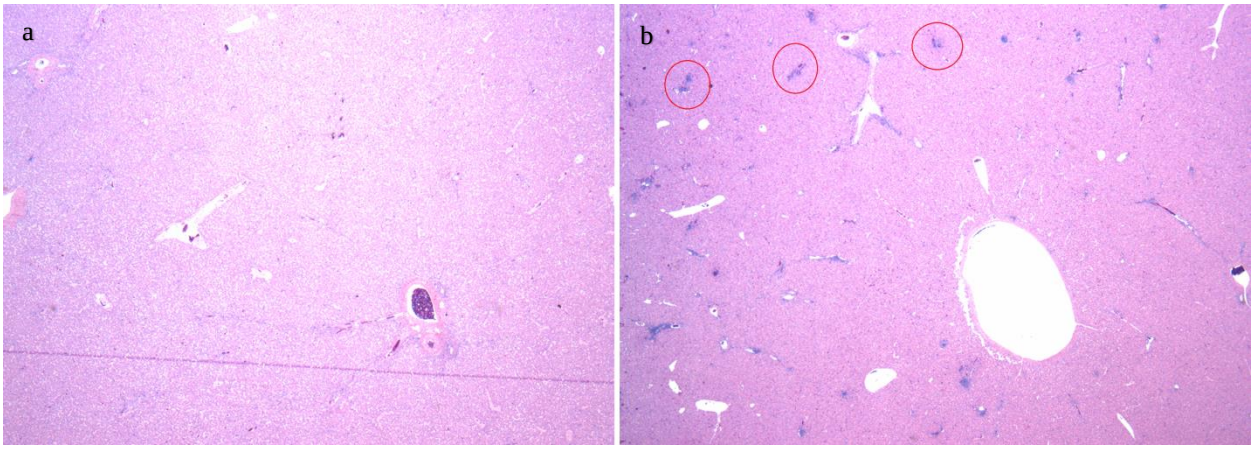

Figure 4.1. Histopathological comparison at 400X magnification of a healthy liver (a) to a damaged liver (b) of broilers fed the M60 diet ratio. Lymphoid aggregates can be seen in the damaged tissue while the healthy tissue is clear of these aggregates.

4.5 Discussion

In the present study we employed new NIT technology capable of sorting three wheat sources into three fractions and used these fractions to formulate four diets with increasing DON concentrations. Concentrations of DON up to 8.3 ppm indicated minimal effects on the immune system of broiler chickens. This is the first study to evaluate potential immune impacts on the CMI and humoral response, the H:L ratio, primary and secondary immune organ weights and histopathological lesion score using a single grain source to produce a range of DON containing diets through NIT sorting technology.

Animal feeding trials in studies on mycotoxicosis typically evaluate immune effects based on responses to diets spiked with purified mycotoxins (Girish et al. 2010). It has been argued that feeding diets formulated with naturally contaminated grain is more representative of commercial conditions than the feeding of purified or semi-purified form (Yegani et al. 2006). Feed diets formulated with naturally contaminated wheat account for potential variability between wheat sources and interactions among multiple mycotoxins in those sources (Smith et al. 1997), including the difficult to detect masked mycotoxins (Berthiller et al. 2013). With at least 25% of the world's wheat contaminated with mycotoxins (CAST 2003), contamination of feed has become a global concern (Streit et al. 2012). Adverse effects, including effects on immune function were reported in poultry feed diets containing DON (Awad et al. 2012). Additionally, there is limited information on the antibody titer response of poultry to DON (Awad et al. 2013).

Girish et al. (2008) suggested that the CMI response is more sensitive to the ingestion of mycotoxins than other immune responses. Phytohemagglutinin is known to inhibit T cell proliferation, the main immune cell in the CMI response (Swamy et al. 2004; Pestka 2007). Our results show that apart from the expected swelling response to the PHA mitogen, there was no difference in broiler response among different wheat sources or diet ratios. There was a negligible increase among the saline control birds; however, this may be attributed to the irritation from the injection. Our findings more closely agree with those of Chowdhury et al. (2005), who showed that the CMI response to PHA was not affected by the ingestion of DON up to 18.6 ppm over 6 weeks. However Swamy et al. (2004) showed that birds consuming diets containing 5.9 and 9.5 ppm DON had slightly reduced T cells after 28 d.

Antibody titer production in response to a foreign antigen can be a useful measure to evaluate the humoral immunotoxicity of DON (Danicke et al. 2003; Awad et al. 2006); however, the literature on DON effects on broiler humoral immunity is inconclusive (Yunus et al. 2012b). Our results from the use of BSA as a mitogen demonstrate a trend of secondary IgY antibody titer production among diet ratios. This suggests that immunostimulation occurred linearly as expected but the humoral response may have become overwhelmed at the highest dietary inclusion of DON (8 ppm), resulting in an inability to produce antibodies and causing immunosuppression to occur. Other studies have reported impairments in humoral immunity following consumption of DON contaminated grain (Ghareeb et al. 2012). Swamy et al. (2002) indicated that diets containing 4.7 - 8.2 ppm DON caused significant linear and quadratic declines in biliary IgA but not serum IgG and IgM. Interestingly, Yunus et al. (2012b) reported that a high DON diet resulted in not only a higher level of antibody titers, but a linear increase in those titers with increased dietary DON level.

The H:L ratio from a blood smear is a commonly used indicator of immune response (Gross and Siegel 1983; Banbura et al. 2013) and consumption of high levels of DON (12 ppm) has been shown to depress lymphocyte numbers (Girish and Smith 2008). Our study did not find any significant effect of wheat source or diet ratio on H:L ratio although the consumption of the most DON contaminated wheat source (CWAD) suggested a larger % change in the H:L ratio. Similar to the antibody titer response, there appeared to be a linear numerical trend of the H:L ratio when comparing diet ratios. There was an increase in the percent change from M0 - M40 before a drop in the H:L ratio at M60, the highest DON concentration. This suggested a reduced capacity of

lymphocyte proliferation. Swamy et al. (2004) observed decreased lymphocyte numbers but no alteration in immunoglobulin concentrations when feeding diets of *Fusarium* mycotoxins (DON, fusaric acid and zearalenone) to broiler chickens. Ghareeb et al. (2012) reported that broilers fed 10 ppm DON had increased the H:L ratio through decreased lymphocyte numbers. The combination of suppressed antibody production and reduced lymphocyte numbers are common characteristics of immunosuppressive effects (Corrier 1991).

Our results from the evaluation of the lymphoid organ weights at 21 and 35 d showed an effect of wheat sources but not diet ratios. At 21 d, the relative spleen weight from CWRS was the heaviest followed by CWAD and CWSWS respectively. This is interesting as the CWRS and CWSWS wheat types were similar in DON concentration with CWAD containing more DON, however this effect disappeared at 35 d suggesting this may have been due to variable development time among birds. Additionally this may be due to a direct mycotoxin effect or due to the slight differences in diet CP. At 35 d, the relative liver weight of CWSWS and CWAD were significantly different from CWRS. Additionally, these liver weights were lower than the 21 d relative weights, suggesting that liver damage may have occurred. Similar to the liver, there was a numerical, but not significant reduction in bursa weight, potentially an indication of bursal involution. It has been indicated that these decreases in organ weights relative to body weight is a part of normal development. As there was no significant difference among any diet, this would indicate that these reductions was consistent. Further research should look more closely at these potential effects to determine if they are consistent. Relative tissue weight is an endpoint that has provided contradictory results in studies where poultry were fed naturally contaminated *Fusarium* diets. Some reports of increasing specific tissue weights (Kubena et al. 1989; Li et al. 2012) while others reported no effect on tissue weights (Swamy et al. 2004b; Yunus et al. 2012a).

Histopathology results showed increases in lesions as lymphoid aggregates or granulopoisis in more contaminated diets. Histopathologically, our results were similar to Awad et al. (2006) who found a slight increase in liver lesions in birds fed 10 ppm DON for 42 days. Harvey et al. (1997) reported no histopathological lesions from boiler chicks fed a diet containing 16 ppm DON for 21 days while Bergsjo and Kaldhusdal (1994) observed no effect on heart weight and microscopic examination of organs did not reveal pathological changes following 35 day feeding trial with 3.4 ppm DON contaminated diet.

Deoxynivalenol is one of the most important *Fusarium* mycotoxins present in all grain sources (Dersjant-Li et al. 2003). Some studies indicate that dietary consumption by poultry of less than 5 ppm DON can cause suppression of immune response (Swamy et al. 2004; Pestka 2007) while others suggest the same concentration may cause immune stimulation with higher concentrations leading to suppressed immune responses (Pestka 2003; Pestka et al. 2004). The results of this study indicated that chronic exposure to upwards of 8 ppm DON did not cause significant effects on CMI or humoral responses, on the H:L ratio, nor on the relative tissue weights of primary or secondary immune tissues (except liver). There was no significant effect on histopathological tissues up to 35 d of age. Although performance parameters are used as the main indicators of poultry health, immunological effects have been shown to occur with consumption of feed with low level DON concentrations, predisposing poultry to infections through alteration of viability and proliferation of immune cells (Humphrey et al. 2004; Klasing 2007). Our work indicates that naturally contaminated *Fusarium* wheat and DON concentrations higher than the Canadian regulations of 5 ppm could potentially be fed to poultry. Additionally, with the capacity to reduce the variability of experimental results by sorting single sources of grain sorted by NIT technology, further studies are required to determine a concentration threshold for DON induced immunotoxicity in poultry.

5.0 GENERAL DISCUSSION

5.1 Past and present options for reducing impacts of *Fusarium*.

Fusarium species are considered some of the most devastating pathogens affecting the grain industry. The infection of wheat heads during anthesis and early kernel development results not only in FDK, but also contamination by mycotoxins, most commonly DON. The cost of mycotoxin contamination of food and feed to Canada and the USA alone ranges between 0.5 and 2 billion dollars a year (Dr. David Miller, Carlton University, personal communication). As indicated by the Canadian Grain Commission (2014a), as little as 0.25% FDK in bulk grain can significantly impact grain value and safety. The traditional methods of evaluating grain are visual evaluation, chemical and microbiological analysis (Huang et al., 2008). These assessments of grain are not only time consuming but destructive, expensive and lack consistency and reliability. Current methods in managing and handling infected grain are either physical removal or dilution/blending with higher quality grain to more acceptable levels (Charmley and Prelusky, 1994; Bianchini et al., 2015). Various decontamination or detoxification methods are used by industry, but none of them are sufficient or proven (Shetty and Jespersen, 2006; Canadian Grain Commission, 2013).

The variation in mycotoxin chemical structures provide the basis for the differences in their physical, chemical and biochemical properties. Since their biochemical nature explains mycotoxin toxicity, knowledge of the physical and chemical properties can therefore be used to detoxify or decontaminate (Murugasen et al., 2015). Mycotoxin decontamination requires potential methods to conform to six processes: 1) inactivate, destroy or remove the mycotoxin; 2) be non-toxic; 3) cause no change to the nutrient value; 4) not alter the technological properties; 5) destroy fungal spores and 6) be readily available and inexpensive (Galvano et al., 2001). They would also have to be regulatory compliant, demonstrating both safety and efficacy (EFSA, 2009). These requirements have limited the success of the physical, chemical and biological methods (Table 5.1) for detecting and contending with FDK and mycotoxins, as no single method has been found to be effective against the diversity and co-occurrences of mycotoxins.

One approach to detoxification has been the use of mycotoxin binding products. These binders are added to the diets and claim to sequester mycotoxins in the intestinal tract of animals, causing reduced bioavailability and mycotoxicosis (Milicevic et al., 2010). However, few are approved for commercial use. Several criteria are used to assess the functionality of a binding additive.

These include: low inclusion rate for effectiveness; stability over a range of pH; high capacity and affinity to absorb mycotoxins; proven chemical interaction between the binder and mycotoxins; low toxicity and environmental effects and proven scientific data with *in vivo* testing and efficacy of the active component (Kabak et al., 2006). Many potential compounds are found to be effective *in vitro* but do not attain similar results *in vivo* (Varga and Toth, 2005). A different approach uses adsorbents with biotransformation capabilities which alter the molecular structure of mycotoxins, converting them to non-toxic metabolites allowing their excretion. This strategy has shown potential to detoxify certain non-absorbable mycotoxins (Grenier et al., 2013).

5.1 TriQ technology and its impacts on the grain industry

Technology will change the way food and feed is produced and supplied; the most valuable and important advances may be in agricultural technologies. Technological inefficiencies result in one third of food produced going to waste, yet estimates are that food production will need to increase by 70% over the next 35 years to meet the global demand (FAO, 2002). The increased demand for animal based products will cause increased demand for higher quality feeds, which accounts for 50 - 75% of producer costs (Agriculture and Agri-Food Canada, 2013). According to the International Feed Industry Federation, 300 million tonnes of feed will be produced on farm where stringent regulations on feed production are not enforced, providing an opportunity for reduced nutrition in animal feeds due to inadvertent use of FDK and mycotoxin contaminated grain (EFSA, 2013). In addition, most feed manufacturers rely on producers to monitor potential effects such as mycotoxins. The use of NIR spectroscopy provides not only a potential screening but an analytical method to establish the overall quality, functionality and safety of grain for use in the food and feed industries. This will change the grain industry to one where bulk grain is evaluated on a kernel by kernel basis. Williams (2007) indicated that premiums are paid for as little as 0.1% increases in CP level of wheat, and our results indicated that the TriQ has the capacity to accurately reduce kernels that contain low CP such as FDK, on a consistently accurate basis. This technology, using NIT spectroscopy, is able to sort individual kernels based on their chemical profile allowing kernels to be evaluated individually. This kind of technology can sort more grain faster, eliminates kernel destruction by requiring no chemical analysis and has the potential to evaluate more than one chemical characteristic in a kernel's chemical profile (Pojie and Mastilovic, 2013).

Table 5.1. Physical, chemical and biological methods to reduce *Fusarium* infected kernels and associated mycotoxins

Physical Methods	Result	Author
Antimycotic agents	Prevent mold growth and disrupt mycotoxin production	Homdork et al., 2000; Gerez et al., 2009
Density segregation or dehulling	Sorting contaminated kernels or hulls which exhibit different properties than non-contaminated kernels by floatation	Jackson and Bullerman, 1999; Fandohan et al., 2005
Gravity table or color separation	Removal of kernels based on quality (i.e., scalpers, manual separation) since damaged kernels will vary in size, shape and color.	Tkachuk et al., 1991; Bullerman and Bianchini, 2007; Pearson, 2010.
Blending	Diluting contaminated grain with known concentrations to levels above salvage	Schaafsma, 2002
Washing	Using distilled water and sodium carbonate solution	Scott, 1998
Cleaning	Removing screenings and the outer layer of bran	Abbas et al., 1985; Lancova et al., 2008
Resistance breeding	Breeding plants to improve resistance to fungal invasion	Mesterhazy, 2002; Munkvold, 2003
Chemical Methods		
Ammoniation	Treatment with ammonia or ammonium hydroxide to cause structural changes at the 12,13-epoxy group	Bretz et al., 2005; 2006
Ozonization/Oxidation	Using oxidizing reagents to react with numerous functional groups (i.e. double bonds) causing molecular changes.	Young, Zhu and Zhou, 2006
Irradiation/Thermal activation	Exposure of contaminated kernels to UV or thermal heating to cause changes in the molecular structures of mycotoxins, reducing levels	Abramson et al., 2005; Stepanik et al., 2007; Beyer et al., 2009
Biological Methods		
Mycotoxin binding agents/adsorbents	Adhering of higher affinity polar absorbent particles to mycotoxin surfaces to reduce the bioavailability and are nutritionally inert.	Ramos et al., 1996; Huwig et al., 2001; Patil et al., 2005
Microbiological binding isolates	Bio-transformation of the epoxide group of trichothecenes using microbes isolated from nature	Binder et al., 2001; Fuchs et al., 2002; Diaz et al., 2005; Karlovsky, 2011
Mycofix®; MTx+; Toxfin; Cobind B	Encapsulated bacterium/enzyme designed for diets in commercial formulations for detoxification of mycotoxins	BIOMIN GmbH 2012; Olmix 2012; Kemin 2015; Provimi 2015
Detection Methods		
Micro columns; liquid and gas chromatography; and enzyme linked immunesorbent assays (ELISA)	Determination of mycotoxin concentrations and types	Asrani et al., 2013; Murugesan et al., 2015

With countries now enforcing different regulations for quality standards, the ability to consolidate these standards to a more uniform international equivalent would be beneficial for worldwide consistency. The NIR spectroscopy technology allows networks to be set up for the transfer and amalgamation of calibrations to ensure reliability, accuracy and uniformity in terms of grain quality regardless of operator or location (Pojie and Mastilovic, 2013).

5.2 TriQ technology and its impacts on the food industry

The consumption of poultry has increased at a greater rate than any other food animal species. Agriculture and Agri-Food Canada (2014) estimate that poultry consumption has increased 60% in the past two decades. There is expected to be a demand for increased poultry production in the future, as retail costs associated with animal protein continues to rise, especially in the pork and beef industries, making it more expensive to attain quality food products. With a growing consumer demand for poultry products, there is an increase demand for poultry feed. EFSA (2013) indicated that poultry feeds were found to be significantly ($P < 0.05$) more contaminated with DON than feed for other animal species. With poultry feed potentially containing increased mycotoxins and more and more feed becoming contaminated due to increased *Fusarium* infections, the ability to use low quality feed to produce high quality food is growing.

This project provided evidence that, there was no (or limited) adverse impacts on the performance and immune competence of broiler chickens fed diets containing FDK with DON concentrations up to 8 ppm. The exact dietary DON concentration that elicits performance and immunological responses in poultry is uncertain, with most literature indicating that exposure to greater than 16 ppm will impact performance, while other studies report that lower levels of 5 ppm have shown effects. Explanations for the reported variability in responses range from mycotoxicosis being a receptor mediated response on ribosomes, preventing peptide bond formation at the center of the 60S ribosomal subunit (McCormick et al., 2011) to a possible protective effect of poultry absorbing nutrients in the jejunum while DON affects the ileum absorptive capabilities (Awad et al., 2013). Previously none of these past studies had the capability to sort a single source of grain into distinct, controlled mycotoxin ratios providing a range of contamination as demonstrated in this project. This capability reduces the field to field variability of grain sources and limits the effect of non-detectable or masked mycotoxins caused by using more than one source of grain to attain the experimentally desired levels of mycotoxin contamination in test diets. These sources of variability may confound the observed effects in

animals. Since grain is not only a direct (cereals) but indirect (animal) source of food for human consumption, a reduction in grain quality limits the availability of food and feed (Smith et al., 2012).

5.3 Future research

The new TriQ NIT individual kernel sorting technology has been demonstrated effective in salvaging high quality grain from FDK contaminated materials. A consequence of this is the production of even more highly contaminated fractions and the consequent need to find safe means of use or disposal. Since the types of mycotoxin vary year-to-year, the focus should not solely be on those kernels contaminated with DON. The prevalence of fumonisins have steadily increased and in 2013, exceeded DON as the most commonly found *Fusarium* mycotoxin (Murugasen et al., 2015). Future research should focus on the need to develop a model for maximizing the use of highly contaminated grain regardless of associated mycotoxins. The question then becomes, what can be done?

Current methods are to burn highly contaminated grain, as they have little economic or industrial value. This leads to the possibility of combustion of the grain for heat to replace wood or gas. Grain produces approximately half the amount of heat produced by gasoline and is similar to wood. The amount of heat stored or energy density of grain is one third that of oil and three times that of wood, and has lower CO emissions (Teagasc Fact Sheet, 2007). This would suggest that, although it would take more grain to produce the same amount of heat as fuel or oil, it is similar, if not better than wood. One adverse outcome may be a reduction in air quality and secondly, the increased potassium content in grain may present a hazard due to the clumping of ash in the combustion chambers.

Research has been conducted on removing the bran portion of kernels to reduce mycotoxin levels, a process referred to as pearling. Dietary bran, while beneficial, contains high concentrations of mycotoxins, as *Fusarium* infection usually occurs near the surface of kernels (Vidal et al., 2014). Sovrani et al. (2012) evaluated pearling, which consisted of removing the outer bran layers of kernels as 5% fractions (0 - 5, 5 - 10, 10 - 15, 15 - 20 and 20 - 25%) and showed that removal of the 10 - 15% layer fraction provided the best compromise between maintaining kernel CP and removal of DON. However, both of these fractions typically are used in feed, so there would still be a problem for safe disposal of highly contaminated fractions.

A study by Kim and Dale (2004) evaluated the potential to use major crops, including wheat, in the production of bioethanol. Bioethanol is produced by fermenting renewable resources, such as wheat, to make ethanol. Ethanol is used to replace more hazardous compounds in gasoline and for octane enhancement. It was been documented that distillers dried grain with solubles (DDGS), a by-product of ethanol production may be enriched up to three times with regard to mycotoxin concentrations, but DDGS are still used in animal feeds (Bennett and Richard, 1996). A similar by-product from the malting process, brewers spent grain (BSG) is formed in a similar manner to DDGS. Some research has shown that both BSG and DDGS contain unmasked (Inoue et al., 2013) and masked (Berthiller et al., 2013) mycotoxins. Research by Low (2000); Russ et al. (2005) and Lu and Gibb (2008) have suggested three potential uses for BSG, which may allow the use of highly contaminated grain to be used in ethanol production if DDGS could be used in a similar manner as BSG.

One proposal is the use of these byproducts as building materials. Russ et al. (2005) suggested that BSG could be used in building materials such as bricks. These bricks had equal or greater strength, lower density and better thermal insulation properties than clay bricks. They also indicated that these effects were the result of replacement of the original sawdust with high amounts of fibrous material contained within the BSG. Proposals by Low et al. (2000) and Lu and Gibb (2008) suggested using BSG as a means of ion metal adsorption and immobilization of cadmium, lead and also copper from aqueous solutions. In addition, Low et al. (2008) suggested that treatment with sodium hydroxide could increase metal sorption. This application could benefit the mining and pulp and paper industries. Additionally, researchers have suggested uses including, paper manufacturing using DDGS (Ishiwaki et al., 2000), formation of plastics with properties similar to polypropylene and epoxies (Woerdman et al., 2004) and the formation of bioplastics to replace petroleum based plastics (Song et al., 2009).

Other suggestions for using contaminated whole grain include examination of the use of microbes in insect intestinal tracts to break down mycotoxins. Three companies, Enviroflight (Environflight, LLC, 2015), Enterra (Enterra Feed Corp., 2013) and Ynsect (Ynsect, 2015), have indicated that black soldier flies are capable of consuming DDGS and detoxifying mycotoxins. Hornung (1991) and Abado-Becognee (1998) examined yellow mealworms and found they were able to partly detoxify ZEA and FUM B1. Niu et al., 2008 suggested corn earworm may be able to detoxify AFB1. It has been suggested that insect detoxification is attained through the

cytochrome P450 monooxygenases (Feyereisen, 2005). A recent pilot project in Belarus evaluated mycotoxin detoxification by cockroaches (AllAboutFeed.net, 2015). These insects are then used as high protein replacements in animal feeds (FAO, 2013b). This would reduce the amount of plant-derived protein sources such as soybean meal which could then be used for human food.

5.4 Conclusion

Technology will change the way we produce, supply and consume food. Population growth, water scarcity and pollution increasingly threaten global food supplies. Advances in agricultural technology are quickly becoming the most important and valuable way to engage food and feed challenges. Inefficiencies result in one third of food produced (1.3 billion tonnes) being wasted, with 33% of the loss coming from agricultural production and 54% from production and post-harvest handling and storage (FAO, 2013c). Under ideal environmental conditions, yields can be maximized, but there appears to be an overall decline in the nutrition of grain due to *Fusarium* infection, which can be mitigated by modern agricultural technology. Reduced nutritional value of crops not only impacts food quality but also feed, resulting in synergistic decreases by reduced animal nutrition, decreased animal production, and reduced available food and food safety.

Decreasing these losses is possible with the use of NIR spectroscopy. The ability to detect structural differences between internally sound and damaged tissues has been used to expand NIR capabilities to other grain, fruits and even vegetables (Abbott, 1999). Near infrared spectroscopy could be used for any commodity that has chemical characteristics if a calibration model is developed and correlations can be found with characteristics such as maturity, sugar content, acidity or internal defects (Walsh et al., 2004). There are multiple examples of using NIR capabilities for more than just grain. Other uses in the food industry include: the quality, adulteration and botanical origin of honey (Davrieux et al., 2004); meat quality monitoring (Davies et al., 1998) and tenderness (Rodbotten et al., 2001); and even caffeine levels in coffee (Downey et al., 1994). Other, more unique possibilities have been examine such as the potential to determine blood glucose levels (Heise et al., 1998) and uses in the petroleum industry (Bohacs and Ovadi, 1998). One group of researchers even used NIR to determine the active ingredients in pharmaceuticals (Hopkins, 2003).

In conclusion, *Fusarium* mycotoxin contamination is on the rise, increasing the risks associated with the consumption of contaminated grain. Decreasing *Fusarium* effects in bulk grain can improve grading guidelines and regulatory limits by reducing variability. Visual inspection is insufficient in quantifying toxicity and feed mills cannot wait for the results from analytical testing prior to feed production. The ability to remove or reduce contamination through separation of FDK, that are known to be associated with *Fusarium* mycotoxins, such as DON can increase the feed value and safety of lower quality grain sources, thereby producing consistent, uniform, high quality feed. This will increase animal performance and efficiency, and improve overall health and well-being leading to better food quality (Alberta Agriculture and Rural Development, 2014).

The ability of the TriQ to sort on a 2 - 3 MT scale is a realistic, commercial application that would be of benefit to the agricultural economies of Saskatchewan and Canada. As the spread of *Fusarium* continues and becomes increasingly unpredictable, the levels and concentrations of mycotoxins will increase in kind. This technology has the capability and potential to sort infected grain on a kernel by kernel basis in a fast and efficient manner, providing safety, security and increase value to downgraded bulk grain sources for the use in food and feed. It is also expected that the capacity and complexity of the technology will evolve and become not only more adapted but efficient at supplying safe food for increased sustainability of the global supply of a worldwide major food source.

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