ENZYME SUPPLEMENTATION AS A STRATEGY TO IMPROVE NUTRIENT UTILIZATION, PRODUCTION PERFORMANCE AND MITIGATION OF NECROTIC ENTERITIS IN POULTRY

A thesis Submitted to the Faculty of Graduate Studies, University of Manitoba

By

WEI JIA

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Department of Animal Science

DEDICATION

To my husband Shunjie Lau,

my parents, Hou Jia and Yufang Liu,

my grandparents, Tianzeng Liu and Chunying Yu,

whom I love very much.

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FOREWORD

This thesis was prepared following a manuscript format including four manuscripts. Manuscript I has been submitted to Poultry Science. Manuscripts II and III have been published in Poultry Science. Manuscript IV has been accepted for publication by Canadian Journal of Animal Science. All manuscripts are formatted to meet the guidelines of Poultry Science.

ABSTRACT

Incorporation of full-fat flaxseed, and to a lesser extent, canola seed in diets to produce n-3-enriched products has attracted interest in the poultry industry. However, high amounts of nonstarch polysaccharides (NSP) in oilseeds compromise their nutritive value. The objectives of the current research were to develop enzyme supplements effective in cell wall depolymerization and viscosity reduction, particularly in flaxseed; to evaluate the effects of enzyme addition and feed processing on oil utilization and egg n-3 fatty acid deposition in broiler chickens and laying hens fed oilseed-containing diets; to characterize the NSP hydrolysis products and to investigate the effects of diet type and enzyme addition on growth performance and the incidence of necrotic enteritis (NE) in broiler chickens challenged with *Clostridium perfringens*. Results showed that diets containing high levels of flaxseed reduced egg production and shell quality in laying hens, and impaired final body weight and feed conversion ratio (FCR) in broiler chickens. Reducing flaxseed particle size via grinding did not improve the growth performance of broiler chickens, whereas diet pelleting showed more pronounced and beneficial effects in improving the nutritive value of flaxseed, particularly when intact seeds were used. Multicarbohydrase supplementation resulted in a significant depolymerization of cell wall polysaccharides in soybean, canola and flaxseed meals, which was followed by the production of water-soluble NSP hydrolysis products, and the reduction of flax mucilage viscosity in vitro was also evident. Enzyme addition to flaxseed-containing diets improved FCR of broiler chickens and egg production performance of laying hens, and facilitated egg n-3 fatty acid deposition. The *C. perfringens* challenge caused intestinal

NE lesions and increased the mortality of broiler chickens with the highest NE mortality and intestinal *C. perfringens* counts observed in those fed flaxseed-containing diets. Enzyme supplementation to diets containing high levels of water-soluble NSP (wheat/barley- or wheat/barley/flaxseed-based) facilitated post-disease compensatory growth in pathogen challenged birds. This was accompanied by a numerical reduction of intestinal *C. perfringens* by 1.4 $\log_{10}$ cfu/g in birds fed the flaxseed-containing diets. Such findings indicated that enzyme addition may be used as a nutritional strategy to reduce the risk of NE development in broiler chickens.

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LIST OF ABBREVIATIONS

AIA – Acid insoluble ash

ALA – α-linolenic acid

AME – Apparent metabolizable energy

AME_n – Nitrogen corrected apparent metabolizable energy

ANF – Antinutritional factors

BW – Body weight

CM – Canola meal

DHA – Docosahexaenoic acid

DM – Dry matter

EPA – Eicosapentaenoic acid

ETEC – Enterotoxigenic *Escherichia coli*

FCR – Feed conversion ratio

FM – Flaxseed meal

FOS – Fructooligosaccharides

GOS – Glucooligosaccharides

MOS – Mannanoligosaccharides

MUFA – Monounsaturated fatty acids

NDF – Neutral detergent fiber

NE – Necrotic enteritis

NSP – Nonstarch polysaccharides

PUFA – Polyunsaturated fatty acid

SBM – Soybean meal

SFA – Saturated fatty acids

SNE – Subclinical form of necrotic enteritis

TIA – Trypsin inhibitor activity

TME – True metabolizable energy

TME_n – Nitrogen corrected true metabolizable energy

1. GENERAL INTRODUCTION

Exogenous enzymes have been used for nearly 30 years in diets for monogastric animals. Conventional enzyme supplements developed for wheat/barley-based diets are mainly composed of xylanase and glucanase, enzymes that have been found effective in improving growth performance of broiler chickens via elimination of the high intestinal viscosity caused by water-soluble arabinoxylans and β-glucans (Burnett, 1966; Choct and Annison, 1992; Cowieson et al., 2006). The success of enzyme supplementation to viscous cereal-based diets has stimulated the interest in developing enzyme products for other feed ingredients, including oilseeds.

Over the last few years, full-fat flaxseed, and to a lesser extent canola seed, have been used in laying hen diets for the production of n-3-enriched eggs due to their high-quality oil (41-44%) rich in α-linolenic acid (**ALA**, 8-12% and 48-58% in canola and flax oil, respectively). Such an approach offers the consumers an alternative to enhance their daily intake of n-3 fatty acids. However, impaired egg production and growth performance have been reported in laying hens and broiler chickens fed flaxseed-containing diets (Leeson et al., 2000; Alzueta et al., 2003). A careful examination of the literature indicated that animal performance and nutrient utilization is more affected by cell wall/nonstarch polysaccharides (**NSP**) than by any other antinutritional factors present in oilseeds.

Oil is the main energy source in oilseeds with oil droplets being located in the cotyledon cells that are surrounded by the thick walls composed of polysaccharides. Due to the small seed size and the high oil content of canola seeds and flaxseed, an effective

rupture of the cell wall matrix can not be achieved under commercial grinding conditions. Consequently, they form a physical barrier which prevents oil exposure to digestive secretions (Lee et al., 1995). In addition, this nutrient encapsulating effect can not be overcome by poultry because they lack endogenous enzymes to digest NSP. A significant negative correlation between seed particle size and AME_n content has been reported for canola seeds (Danicke et al., 1998), and to our knowledge, no similar study has been done with flaxseed yet. In an attempt to reduce lipid oxidation during storage and to avoid handling problems associated with grinding (sieve plugging), feeding whole and intact seeds would be advantageous. However, the potential drawback would be a less-than-optimum deposition of n-3 fatty acids in poultry products since the ability of broiler chickens and laying hens to utilize oil from the whole seeds is questionable. In contrast to canola seed, flaxseed contains significant amounts of water-soluble NSP, including mucilage, which was found to markedly increase the viscosity of intestinal contents (Alzueta et al., 2003). It is known that high intestinal viscosity caused by water-soluble NSP of cereal grains has a negative effect on nutrient utilization, results in an increased nutrient supply for bacterial growth in the small intestine, and thus may facilitate *Clostridium perfringens* proliferation (Smits et al., 1998; Langhout et al., 1999; Collier et al., 2003). Feeding rye, wheat and barley has been found to be responsible for outbreaks of necrotic enteritis (**NE**) in broiler chickens, a disease caused by the overgrowth of *C. perfringens* which is becoming increasingly prevalent with the removal of feed antibiotics (Kaldhusdal and Skjerve, 1996; McDevitt et al., 2006). Whether or not feeding flaxseeds affects the susceptibility of broiler chickens to NE has not yet been investigated.

Due to a more complex composition of cell wall polysaccharides in oilseeds than that of cereal grains (Bacic et al., 1988), conventional enzyme products lack the activities necessary for targeting the NSP of oilseeds. A new generation of enzyme supplements is now being developed in our laboratory, which includes a blend of multicarbohydrase activities that has been demonstrated to be effective in depolymerizing cell wall polysaccharides thereby improving oil utilization from canola seed and flaxseed by broiler chickens (Meng et al., 2005; Meng et al., 2006; Slominski et al., 2006). However, the enzyme supplements failed to reduce the high intestinal viscosity caused by flax mucilage (Slominski et al., 2006). Therefore, fine tuning of the enzyme activities and their inclusion rate is needed to further improve the effectiveness of enzyme supplementation to flaxseed-containing diets.

In addition to canola seed and flaxseed, feedstuffs commonly used in Canada such as corn, wheat, barley, soybean meal, peas, and wheat by-products contain significant amounts of NSP including arabinoxylans, β-glucans, galactans, galactomannans, rhamnogalacturonans, arabinogalactans, mannans, and arabinans (Selvendran et al., 1987). Moreover, these feedstuffs are rich in certain galactooligosaccharides, which, along with resistant starch and glycoproteins, represent components poorly metabolized by poultry (Slominski, 1991). In the process of depolymerizing various dietary polysaccharides, enzymes may produce galacto-, gluco-, manno-, or xylo-oligomers which, similarly to prebiotics, may facilitate the proliferation of bacteria beneficial for gut health such as *Bifidobacterium* and *Lactobaccillus* spp. (Silva et al., 1983; Monsan and Paul, 1995; Vardakou et al., 2008), thereby decreasing the growth of certain pathogenic species (Gibson and Roberfroid, 1995). The use of lactic acid bacterial

cultures, *Lactobacillus acidophilus* and *Streptococcus faecalis*, has shown promising results in suppressing *C. perfringens* proliferation (Fukata et al., 1991) and reducing NE mortality (Hofacre et al., 2003) in broiler chickens. In this context, we hypothesized that NSP hydrolysis products resulting from dietary enzyme addition may serve as prebiotics and facilitate the growth of intestinal lactic acid bacteria, thereby indirectly reducing the proliferation of *C. perfringens* and NE outbreaks in broiler chickens.

Therefore, the objectives of this research were:

1. To further improve the multicarbohydrase supplements so they become more effective in both cell wall polysaccharide depolymerization and viscosity reduction when used in flaxseed-containing diets.
2. To explore dietary means of minimizing the antinutritive effects of flaxseed polysaccharides on the growth performance of broiler chickens, including enzyme addition, particle size reduction via grinding, feed pelleting and bile salt addition.
3. To examine the effects of enzyme supplementation on the production performance and egg n-3 fatty acid deposition in laying hens fed diets containing canola seeds and flaxseed.
4. To validate an in-feed *C. perfringens* challenge model to experimentally induce a mild form of NE in broiler chickens under commercial flock conditions, and to investigate the effects of diet type (corn- vs. wheat-based) and enzyme addition on growth performance and gut health of broiler chickens.
5. To characterize the NSP hydrolysis products derived from enzyme addition to soybean, canola and flaxseed meals, and to investigate the effects of flaxseed and

enzyme addition on growth performance and NE incidence in broiler chickens challenged with *C. perfringens*.

2. LITERATURE REVIEW

2.1 The content and structure of cell wall/nonstarch polysaccharides of feed ingredients with emphasis on oilseeds

2.1.1 Classification of plant carbohydrates

Plant carbohydrates are predominant components of poultry diets. In general, plant carbohydrate can be classified into 3 distinct categories: 1) simple sugars; 2) storage reserve compounds including sucrose, oligosaccharides, fructans and starch; and 3) structural polysaccharides that are mainly associated with cell walls (Slominski, 1991).

From the nutrition point of view, carbohydrates fall into 2 categories: 1) available substances that can be absorbed directly or digested by endogenous digestive enzymes of animals (e.g. glucose, fructose, sucrose and starch); and 2) unavailable substances including oligosaccharides, cell wall polysaccharides and carbohydrate-protein complexes that are only partly degraded by the intestinal microflora (Asp, 1996).

Based on molecular weight, carbohydrate can be classified into 5 categories (Slominski, 1991; Lehninger et al., 2000): 1) monosaccharides, e.g. glucose and fructose; 2) disaccharides, e.g. sucrose; 3) oligosaccharides, consisting of short chains of monosaccharide units or residues joined by characteristic linkages called glycosidic bonds with the degree of polymerization from 3 to 10 (i.e., galactooligosacchardies: raffinose, stachyose, verbascose; and fructosans: kestose, neokestose); 4) polysaccharides, containing more than 10 monosaccharide units, with some such as starch or fructans serving as storage compounds in plants, whereas others (e.g. cellulose and pectic

polysaccharides) being structural polysaccharides; and 5) carbohydrate-protein complexes, e.g. proteoglycans and glycoproteins (Fincher et al., 1983).

2.1.2 Plant cell wall structure and nonstarch polysaccharides (NSP) of cereal grains

Plant cell walls consist of a series of cell wall/nonstarch polysaccharides (**NSP**) often associated and/or substituted with proteins and phenolic compounds, and in some cells with the lignin polymers. The major NSP present in poultry diets are cellulose, arabinoxylans, mixed linked β-glucans, xyloglucans, xylans, rhamnogalacturonans, or arabinogalactans (Selvendran et al., 1987; Theander et al., 1989). The total content of NSP in commonly used feed ingredients is summarized in Table 2.1 with the structure of the major NSP being listed in Figure 2.1.

Cellulose fibres make up an important part of the framework of cell walls. Cellulose is a linear β-1,4-linked polymer of 10,000 units of glucose. This great regularity permits the formation of strong hydrogen bonds between cellulose molecules, resulting in the formation of water-insoluble crystalline microfibrils (Lehninger et al., 2000). Not being soluble in water, cellulose is also insoluble in alkali or dilute acids, and concentrated acids (i.e. 12 M sulphuric acid) are required for cellulose hydrolysis (Carré, 2002). About 100 cellulose molecules form cellulose fibrils that are bound together by a matrix of other polymeric materials such as hemicellulose, pectic polysaccharides, glycoprotein and lignin (Selvendran et al., 1987). The term hemicellulose was originally applied to polysaccharides which were structurally related and associated with cellulose and were preferentially solubilised by aqueous alkali after removal of water-soluble polysaccharides (Theander et al., 1989). The term hemicellulose gives little indication of

the chemical structures of component polysaccharides which may include β-glucans, arabinoxylans, xyloglucans, arabinans, arabinogalactans or mannans. Beta-glucans are linear polysaccharides with β-1,4- and β-1,3-linked glucose and are predominant polysaccharides of barley (Bacic et al., 1988; Bach Knudsen, 1997). Arabinoxylans predominate in endosperm cell walls of wheat and corn (Henry, 1987), and consist of a backbone of β-1,4-linked xylose residues with terminal 1,2 and 1,3 arabinose substitutions.

Table 2.1 Total content and predominant nonstarch polysaccharides (NSP) in commonly used feed ingredients

Ingredient	Total NSP (%, DM)	Reference	Predominant NSP
Corn	9.7	Bach Knudsen (1997)	Arabinoxylan
	8.1	Choct (2002)	
Wheat	11.9	Bach Knudsen (1997)	Arabinoxylan
	11.4	Choct (2002)	
	10.0	Slominski et al. (2004)	
	8.8[1]	Meng et al. (2005)	
Rye	15.2	Bach Knudsen (1997)	Arabinoxylan
	13.2	Choct (2002)	
Barley	18.6	Bach Knudsen (1997)	β-glucan, arabinoxylan
	16.7	Choct (2002)	
	16.8	Slominski (1991)	
Soybean meal	16.4-22.2	Irish and Balnave (1993)	Pectic polysaccharides (arabinan, arabinogalactan, galactomannan)
	19.2	Choct (2002)	
	14.8[1]	Meng et al. (2005)	
Canola meal	22.0	Bach Knudsen (1997)	Pectic polysaccharides (arabinan, arabinogalactan, galactomannan)
	17.8-21.3	Slominski and Campbell (1990)	
	17.1[1]	Meng et al. (2005)	
Flaxseed meal	30.3	Bach Knudsen (1997)	Pectic polysaccharides, mucilage (arabinoxylan)
	27.1[1]	Slominski et al. (2006)	

[1] %, as is basis.

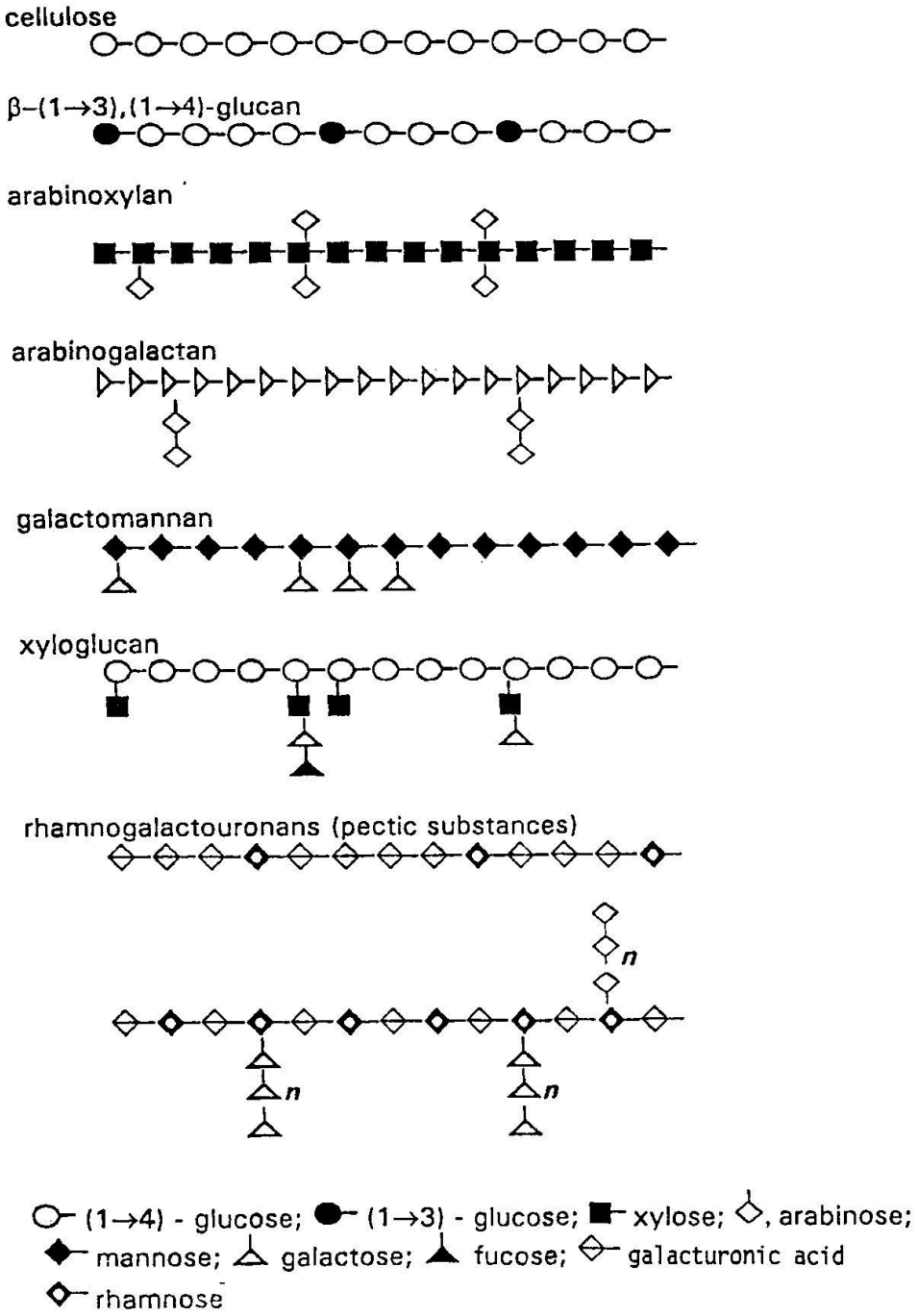

Figure 2.1 Structure of NSP commonly found in poultry diets.

(Smits and Annison, 1996, used with permission of World's Poultry Science Association, May 22, 2009)

2.1.3 Nonstarch polysaccharides of oilseeds

In oilseeds, the cell wall polysaccharides are diverse, and their specific structures are not very well understood. The main cell wall polymers of parenchymatous tissues of dicotyledons are pectic polysaccharides, hemicellulose, and cellulose, whereas those of lignified tissues include lignin, hemicelluloses, and cellulose. Different types of hemicellulosic polysaccharides occur in the cell walls of parenchymatous and lignified tissues, e.g. xyloglucans in the former and glucuronoxylans in the latter (Selvendran et al., 1987). Whereas very few or no pectic polysaccharides can be found in cereal grains, oilseeds such as soybeans, canola seeds or flaxseed are rich sources of pectic polysaccharides. Pectic polysaccharides, probably the most complex class of cell wall polysaccharides, consist of a family of acidic polysaccharides (rhamnogalacturonans) and several neutral oligosaccharides and polysaccharides (arabinans, galactans, and arabinogalactans) which are believed to be covalently attached to the rhamnogalacturonan backbone primarily through the rhamnopyranosyl residues. The α-1,4-linked galacturonic acid residues in the backbone are interspersed with α-1,2 rhamnose residues (Bacic et al. 1988). Pectic polysaccharides are partially soluble in water, and soluble in neutral detergents. Therefore, the values from neutral detergent fibre analysis (**NDF**) are significantly lower than those from the NSP analysis (Chesson, 2001; Choct, 2002).

2.1.3.1 Soybean meal

The total NSP content of soybean meal (**SBM**) ranges from 16 to 22% DM (Irish and Balnave, 1993; Bach Knudsen, 1997; Choct, 2002; Meng et al., 2005), with 90.2%

being water-insoluble (Meng and Slominski, 2005). The amounts of total NSP reported in literature vary markedly, presumably due to differences in analytical techniques used, growing conditions, variety, or the degree of hull removed.

Aspinall and Cottrell (1971) suggested that defatted soybean cotyledon fraction contained arabinan and arabinogalactan consisting of arabinose and galactose residues in the proportions of 1:2.8, and an acidic polysaccharide complex. The pectic polysaccharides from purified cotyledon cell walls were reported to be made up of arabinose and galactose residues in a molar ratio of 1:1.5 (Brillouet and Carré, 1983).

Soybean hulls contain 2.3% acid detergent lignin, 17.7% insoluble hemicelluloses (the difference between neutral detergent fibre and acid detergent fibre), 47.0% cellulose, and 83.3% of total dietary fibre with an insoluble to soluble fibre ratio of 5:1 (Dust et al., 2004). Aspinall et al. (1966) showed that major NSP in soybean hulls include pectic polysaccharides, galactomannan, xylan, and cellulose.

2.1.3.2 Canola meal

Limited information exists in the literature regarding the structural analysis of canola meal (**CM**) polysaccharides. The total NSP content of CM has been reported to range from 17 to 22% (Slominski and Campbell, 1990; Bach Knudsen, 1997; Meng et al., 2005). Slominski et al. (1994) found that NSP of yellow- and brown-seeded CM are for the most part water-insoluble (16.4-19.4%) with a small amount of water-soluble NSP (1.5-2.0%).

In addition to high concentration of pectic polysaccharides, non-cellulosic polysaccharides such as xylan, xyloglucan, arabinan, arabinogalactan and galactomannan

are present in CM (Siddiqui and Wood 1977; Slominski and Campbell 1990). Eriksson et al. (1996) isolated the water-soluble polysaccharides from dehulled rapeseed meal, which yielded 2 major fractions containing arabinose and galactose residues. Further analyses showed that one of the fractions with arabinose to galactose ratio of 5.4:1 mainly contained the arabinan fragments.

2.1.3.3 Flaxseed

Flaxseed is made up of 55% cotyledon, 36% hull and 4% embryo. The hull consists of 4 layers with the outermost layer containing a water-soluble mucilaginous carbohydrate material, known as mucilage (Mazza and Biliaderis, 1989; Bhatty, 1995).

Although the cellulose content in flaxseed meal (**FM**) is similar to that of SBM and CM (5.3 vs. 6.3 and 4.6-6.0%, respectively), the total NSP of FM is much greater and averaged 30% (Slominski and Campbell 1990; Bach Knudsen et al. 1997; Slominski et al., 2006). Oomah et al. (1995) analyzed 109 samples of flaxseed and reported that the content of water-soluble polysaccharides ranged from 3.6% to 8.0% with an overall mean of 6.2%.

There is a paucity of information regarding the composition of cell wall components in flaxseed except for mucilage. Mucilage consists of 2 fractions, a neutral fraction composed of arabinoxylans, and an acidic pectin-like fraction consisted of polysaccharides containing rhamnose, galactose and galacturonic acid residues. The presence of a β-1.4-xylan backbone of the highly branched arabinoxylan component is mainly responsible for the viscous property of mucilage (Wannerberger et al., 1991; Cui et al. 1994).

2.2 Nutritive value of flaxseed for poultry

Oilseed meals such as SBM and CM have been widely used in diets of monogastric animals. Due to their high nutritive value, there has been growing interest in feeding full-fat oilseeds to poultry. The nutrient contents of Canadian soybeans, canola seeds and flaxseed harvested in 2007 and 2008 are presented in Table 2.2. Canola seed and flaxseed are not only valuable sources of protein but also provide considerable amounts of energy (Lee et al., 1991; DeClercq, 2009 a, b). In addition, the lipids of these seeds are an excellent source of α-linolenic acid (**ALA**, $C_{18:3n-3}$) with canola oil containing 8-12% whereas flax oil 48-58% of this fatty acid (Ajuyah et al., 1991; DeClercq, 2009 a, b). Omega-3 polyunsaturated fatty acids (**PUFA**) such as ALA, eicosapentaenoic acid (**EPA**, $C_{20:5n-3}$) and docosahexaenoic acid (**DHA**, $C_{22:6n-3}$) have been indicated to be beneficial for human health (Leskanich and Noble, 1997), and an average of 1.0-1.6 g daily intake of ALA has been recommended by Health Canada (DRIs, 2006). However, research has shown that the consumption of n-3 PUFA in most of the Western countries is less than the recommended values (Gonzalez-Esquerra and Leeson, 2001). Enrichment of the poultry products with n-3 fatty acids by dietary inclusion of flaxseed, the richest terrestrial source of ALA, will offer consumers an alternative to enhance their n-3 fatty acids daily intake.

Table 2.2 Protein, oil and fatty acid composition of oilseeds (%, DM)

Oilseed	Protein	Oil	Fatty acid composition (% in oil)				
			Palmitic ($C_{16:0}$)	Stearic ($C_{18:0}$)	Oleic ($C_{18:1}$)	Linoleic ($C_{18:2n6}$)	Linolenic ($C_{18:3n3}$)
Flaxseed	23	46	4.8	3.5	18.9	15.2	56.0
Canola seed	23	48	3.9	1.9	63.2	18.4	9.1
Soybean seed	40	22	9.9	4.2	21.7	53.7	9.1

(DeClercq, 2008; 2009 a, b)

2.2.1 Digestion and absorption of dietary fat in poultry

Oil is mainly found as triacylglycerols in flaxseed with ALA present in all 3 positions on the glycerol molecule in nearly equivalent amounts (Daun et al., 2003). Dietary fats enter the duodenum and become emulsified upon contact with the conjugated bile salts. Emulsification is required for fat digestion because lipases act at the oil-water interface. Pancreatic lipase breaks down the emulsified fats into fatty acids, monoacylglycerols, and glycerol. The products of lipolysis are removed from the water-oil interface by incorporation into bile salt micelles. Mixed micelles are formed spontaneously by the interaction of bile salts and monoacylglycerols, medium-chain fatty acids (6-12 carbons), long-chain unsaturated fatty acids (> 12 carbons) and phospholipids. Long-chain saturated fatty acids such as palmitic and stearic acids, however, are only slightly soluble in bile salts in emulsion form, but are markedly solubilized in the presence of a mixed micelle. The micelles facilitate fat absorption by providing a high concentration of lipids in the unstirred water layer adjacent to the mucosal cells. Transport of lipids through the brush border membrane is passive. The absorption rates vary with chain length and degree of saturation and seem to be influenced by a fatty acid-binding protein (Garrett and Young, 1975; Krogdahl, 1985; Leeson and Summers, 2001).

Bile acids are a family of acidic sterols synthesized in the liver from cholesterol. Following synthesis and conjugation with glycine or taurine, they are secreted into bile. Bile salts are continuously re-utilized for micelle formation and eventually absorbed in the small intestine (Krogdahl, 1985).

The ability to utilize fat is not fully developed in the very young broilers and it increases with age (Carew et al., 1972; Polin and Hussein, 1982). Lipase activity has been reported to be low and secretion of bile acids seemed to be inadequate in the very young birds (Krogdahl, 1985; Nir et al., 1993). However, Meng et al. (2004) demonstrated that lipase addition had no effects on nutrient utilization and growth performance of broiler chickens. The addition of bile salts markedly increased fat digestibility with a more pronounced effect observed for saturated fatty acids when compared with unsaturated ones (Garrett and Young, 1975; Kussaibati et al., 1982b; Polin and Hussein, 1982; Campbell et al., 1983).

2.2.2 Deposition of n-3 fatty acids into poultry products

Once inside the mucosal cells, fatty acids and monoacylglycerol are re-esterified into triacylglycerols and then assembled into chylomicrons and transported via the portal vein to the liver. Enlongation and desaturation of ALA into longer chain fatty acids such as EPA and DHA occur in the liver. The efficiency of this conversion seems to be low. The liver forms a special yolk-targeted very low density lipoprotein which is transported to the ovary and deposited in the developing yolk primarily as lipoprotein (Brenner, 1971; Leeson and Summers, 2001; Scheideler, 2003). Yolk lipid storage has been shown to reflect the composition of the dietary fat (Leskanich and Noble, 1997). Feeding flaxseed to laying hens resulted in direct incorporation of ALA into eggs and it is suggested that every 1% of flaxseed addition to poultry diets can increase total n-3 fatty acids by around 40 mg per egg (Leeson and Summers, 2005). Research data on the effects of flaxseed or fish oil addition on egg fatty acid profile are summarized in the Table 2.3. A proportional

increase in yolk ALA was observed with increased dietary inclusion rate of flaxseed, whereas the increase of DHA and particularly that of EPA was less pronounced (Caston and Leeson, 1990; Cherian and Sim, 1991; Jiang et al., 1991; Aymond and Van Elswyk, 1995; Scheideler and Froning, 1996). In an effort to increase the oxidative stability of flaxseed, whole seeds instead of ground seeds were used in a few studies. The efficiency of yolk ALA deposition seemed to be reduced when whole seeds were used, and the addition of grit or coarse oystershell has been suggested to facilitate seed rupture in the gizzard (Aymond and Van Elswyk, 1995; Scheideler and Froning, 1996).

Similarly, the fatty acid composition of the muscle and adipose tissue in broiler chickens can also be influenced by dietary inclusion of n-3 fatty acids. In comparison with eggs, lipid fractions are unevenly distributed in different marketable meat portions with white meat (breast) being rich in phospholipids while dark meat (thigh) and skin prevalent in triacylglycerols (Leskanich and Noble, 1997). The adipose tissue has been shown to be influenced by dietary lipid composition to a greater extent than the breast muscle, presumably due to the lipid storage function of the adipose tissue (Yau et al., 1991). However, most of the fat in poultry meat is subcutaneous and many consumers discard it before consuming the meat. Gonzalez-Esquerra and Leeson (2000a) examined the fatty acid contents in the carcases of broiler chickens fed 10% of flaxseed when they were given flaxseed-containing diets 7 or 14 days before slaughter. The results showed that flaxseed addition significantly increased the total n-3 fatty acids in the editable carcases, which was mainly due to the increase of ALA. The n-3 fatty acid content was greatly influenced by the period of feeding with increased deposition occurring over time.

Table 2.3 Effects of dietary addition of flaxseed or menhaden oil on the deposition of n-3 fatty acids in eggs (mg/60 g egg[1])

	Ground flaxseed								Whole flaxseed			Menhaden oil
	0%	5%	8%	10%	15%	16%	20%	30%	5%	10%	15%	1.5%
Caston and Leeson (1990)												
ALA	22.8			276.0			534.0	690.0				
EPA	0.0			3.6			7.2	3.0				
DHA	2.4			6.0			13.2	12.0				
Cherian and Sim (1991)												
ALA	35.0		337.0			512.5						
EPA	0.0		7.0			8.8						
DHA	57.5		82.6			90.1						
Jiang et al. (1991)												
ALA	54.0				348.0							
EPA	0.0				18.0							
DHA	90.0				162.0							
Aymond and Van Elswyk (1995)												
ALA	17.1	145.4			289.4				119.2		216.5	23.4
EPA	33.8	7.0			27.0				4.3		10.1	20.2
DHA	37.6	89.6			123.1				90.4		97.2	110.7
Scheideler and Froning (1996)												
ALA	15.6	156.6		247.8	395.4				120.6	253.8	424.2	22.8
EPA	-[2]	-		-	-				-	-	-	-
DHA	31.2	86.4		106.2	108.6				109.8	102.0	106.8	172.2

[1]Assumed 6 g fat and 18 g yolk in a 60 g egg if not indicated by original paper. [2]Not reported by the original paper.

In cooked skinless meat, the ALA showed preferential deposition in thigh and wing whereas EPA and DHA in breast.

Deposition of n-3 fatty acids into eggs by dietary inclusion of flaxseed appears to be a more feasible source of n-3 fatty acids for the consumers than through chicken meat. The production of 350-400 mg n-3 PUFA per egg can be achieved by 10 to 20% of flaxseed in laying hen diets (Scheideler, 2003). More research is needed to further clarify the optimum inclusion rate and feeding time of flaxseed to broiler chickens in order to achieve a steady production of n-3-enriched meat products, as well as to develop strategies to ameliorate the antinutritional effects of flaxseed for young birds.

2.2.3 Effects of flaxseed on nutrient utilization and production performance in poultry

Lee et al. (1995) reported that although canola seed and flaxseed have similar contents of energy-contributing components, the TME_n of flaxseed was significantly lower than that of canola seed (3,750 vs. 4,560 kcal/kg). Ortiz et al. (2001) observed that increasing dietary flaxseed content in broiler chicken diets from 4 to 24% decreased the AME_n of diet, from 2816 to 2091 kcal/kg DM, and AME_n of flaxseed from 3260 to -100 kcal/kg DM. The negative values clearly indicated that flaxseed interfered with other dietary ingredients and impaired their energy utilization. The study by Alzueta et al. (2003) showed that the inclusion of 16% of flaxseed decreased the digestibilities of total fatty acids from 89 to 60% and ALA from 74 to 43%. Conflicting data exist regarding the effects of flaxseed on protein digestibility. Lee et al. (1991) reported that the inclusion of 10 or 20% flaxseed in the diet did not adversely affect protein retention in broiler chickens. However, in another broiler study, apparent retention of nitrogen and apparent

digestibility of amino acids (except for methionine) decreased as the concentration of flaxseed increased (Rodriguez et al., 2001). As a result of depressed nutrient utilization, reduced body weight (**BW**) gain and poor feed conversion ratio (**FCR**) have been observed in broiler chickens when the dietary inclusion rate of flaxseed was in excess of 8% (Ajuyah et al., 1991; Lee et al., 1991; Alzueta et al., 2003).

When birds were fed diets containing 10% ground flaxseed, the flaxseed AME_n values obtained with broiler chickens were 2,055 - 2,118 kcal/kg, whereas 3,560 - 3,654 kcal/kg values were determined with adult roosters indicating that a certain degree of tolerance develops as the birds age (Gonzalez-Esquerra and Leeson, 2000b). Although there is some evidence within the feed industry that egg production could be significantly reduced in layer flocks when flaxseed inclusion rate is greater than 8% (Leeson et al., 2000), published research data on the effect of flaxseed on laying hen performance are inconsistent. Jiang et al. (1991) and Caston et al. (1994) reported no effect of flaxseed on egg production, whereas Aymond and Van Elswyk (1995) suggested that flaxseed addition may have negative effects. Bean and Leeson (2003) suggested that duration of the trial could also affect performance. Among the long-term studies, increased feed consumption and reduced hen weight were reported by Caston et al. (1994), Leeson et al. (2000), and Novak and Scheideler (2001), whereas a decrease in feed consumption was reported by Bean and Leeson (2003) for flaxseed-containing diets. Decreased egg production was observed in the study by Leeson et al. (2000), which was in disagreement with findings by Caston et al. (1994), Novak and Scheideler (2001), and Bean and Leeson (2003), who reported no adverse effect of flaxseed addition (up to 20% of the diet)

on egg production. Differences in diet composition and/or strain of hens could be partially responsible for such conflicting results.

2.2.4 The antinutritional factors present in flaxseed

The negative effects associated with feeding flaxseed have been attributed to various antinutritional factors (**ANF**) present in flaxseed including mucilage, linatine, cyanogenic glycosides, and trypsin inhibitors (Bhatty, 1995).

Bhatty (1993) analyzed the trypsin inhibitor activity (**TIA**) of flaxseed samples and compared with samples of SBM and rapeseed meal. Their data showed that flaxseed contained only about one-half of TIA present in rapeseed meal and much less than that of SBM. Therefore, the author concluded that the low TIA present in flaxseed may not be of significance in livestock and human nutrition. Linatine, a dipeptide formed by the condensation of 1-amino-D-proline and glutamic acid is an antagonist of pyridoxal phosphate (vitamin B_6). Acute doses of linatine may induce the characteristic vitamin B_6 deficiency and hydrazine-poisoning symptoms, which can be alleviated by administration of pyridoxine. Cyanogenic glycosides such as linustatin, neolinustatin, and linamarin are present in flaxseed. They are not toxic per se, but can yield hydrogen cyanide following glycoside hydrolysis by specific enzymes i.e., β-glucosidase and hydroxynitrile-lyase (Bhatty, 1995; Leeson and Summers, 2001). However, careful examination of the literature concerned with feeding flaxseed indicates that bird performance and nutrient utilization is affected more so by high amount of cell wall polysaccharides than other ANF. Mucilage, a water-soluble polysaccharide, has been found to markedly increase the viscosity of intestinal contents in broilers (Rodriguez et al., 2001; Alzueta et al., 2003). In

addition, the decreased energy utilization from flaxseed-containing diets has been reported to result from incomplete rupture of the seed and nutrient encapsulation by the cotyledon cells (Slominski et al., 2006).

2.3 Antinutritive effects of dietary NSP in poultry

Poultry is not capable of cleaving and digesting dietary NSP such as cellulose, arabinoxylans, β-glucans, or pectic polysaccahrides. Because only glycosidic bonds of α-1,4 and α-1,6 in starch, α-1,2 between glucose and fructose in sucrose, and the β-1,4 between glucose and galactose in lactose can be cleaved by endogenous avian enzymes. All other glycosidic bonds are resistant to poultry digestive enzymes, but may be cleaved by enzymes of microbial origin (Smits and Annison, 1996). However, in general the digestion of NSP remains poor in poultry.

In addition, cell wall polysaccharides have been demonstrated to increase endogenous losses and to decrease digestibilities of dietary nutrients in monogastric animals, which become the basis for NSP being considered an ANF for poultry (Annison and Choct, 1991; Montagne et al., 2003). Three mechanisms have been proposed for their antinutritive effects, namely intestinal viscosity increase, dietary nutrients encapsulation, and interaction with intestinal microflora.

2.3.1 Intestinal viscosity increase

Some cell wall polysaccharides, such as arabinoxylans of wheat and rye, flax mucilage, as well as β-glucans of barley, form viscous solutions when dissolved in water. Viscosity caused by dietary NSP refers to the ability of some polysaccharides to thicken or form gels when mixed with fluids resulting from physical entanglements among the

polysaccharide constituents within the fluid or solution (Dikeman and Fahey, 2006). Many factors determine the viscosity of NSP including their water solubility, concentration, molecule weight, whether they are branched or linear, the presence of charged groups, and the surrounding structures (Smits and Annison, 1996; Choct, 2002). Wannerberger et al. (1991) reported that mucilage extracted from different flax cultivars exhibited different rheological properties. The viscosity varied over a wide range from 0.02 to 0.28 Pa·s for 1% mucilage solution, and it increased with increasing proportion of xylose residues, and with decreasing proportion of uronic acids in the mucilage polysaccharides.

Extensive studies with viscous cereal grains have demonstrated that arabinoxylans and β-glucans produce high viscosity in the small intestine of poultry, particularly of broiler chickens. The viscous intestinal environment causes a general inhibition of digestion and absorption of dietary nutrients including starch, protein and fat. As a consequence, a depressed growth performance has been reported in broiler chickens fed rye, barley or wheat (Misir and Marquardt, 1978; Fengler and Marquardt, 1988; Campbell et al., 1989; Bedford and Classen, 1992; Choct and Annison, 1992a,b).

A few mechanisms have been suggested to be responsible for such a response. Digestion is a dynamic process which depends on diffusion of enzymes, substrates and products, therefore any interference with free molecule movement will reduce the efficiency of the entire process (Bedford, 2002). In vitro study by Fengler and Marquardt (1988) demonstrated that viscous solutions reduced the diffusional rates of salts and glucose. The effect of intestinal contraction on the absorption of glucose was simulated in an in vitro model, and the presence of viscous polysaccharides (guar gum) led to the

reduced absorption by resisting the convective effects of intestinal contractions (Edwards et al., 1988). Johnson and Gee (1981) suggested that viscous polysaccharides may inhibit absorption by increasing apparent thickness of the intestinal unstirred water layer, across which nutrients must diffuse before reaching the epithelium. In addition, viscous polysaccharides cause physiological and morphological changes in the digestive system. Increasing dietary content of NSP has been demonstrated to stimulate secretions from salivary glands, stomach, liver, exocrine pancreas and the intestinal wall in pigs, rats and humans. Increased endogenous secretions included water, protein, electrolytes and lipids indicating an increased metabolic cost (Low, 1989). Enlarged digestive organs and increased secretion of pancreatic-bile secretion was found as a compensation for reduced nutrient utilization when viscous polysaccharides were fed to rats (Ikegami et al., 1990). Viscous polysaccharides such as barley β-glucans were also reported to prolong the rate of feed passage in rats (Gohl and Gohl, 1977) and in poultry (Salih et al., 1991).

Although viscous digesta causes an inhibition of utilization of all dietary nutrients, fat digestibility suffers the most among the macronutrients (Smits et al., 1997). This is because high intestinal viscosity may further impair fat emulsification and micelle formation by reducing the efficacy of bile salts. Feeding psyllium (a gell-forming polymer that does not bind bile acids in vitro) to rats increased the excretion of fecal bile acids and total steroids as well as up-regulated bile acid biosynthesis (Buhman et al., 1998). Smits et al. (1998) demonstrated that the addition of a nonfermentable gelling fiber (carboxymethylcellulose) decreased apparent lipid digestibility by reducing the concentration of bile acids in the chyme of broiler chickens. In addition, intestinal

microflora may proliferate under high intestinal viscosity and lead to an increased deconjugation of bile salts (Edwards et al., 1988; Feighner and Dashkevicz, 1988).

2.3.2 Nutrient encapsulation

In wheat (Figure 2.2, a), the endosperm contains starch, protein and cell wall materials and the thickness of the cell wall varies through different wheat samples. The whole endosperm is enclosed by a thin layer of cells with very thick cell walls, known as the aleurone layer containing enzymes required for release of nutrients from the endosperm during germination. Within the aleurone cell walls, protein bodies are tightly packed. The aleurone layer is surrounded by the pericarp and seed coat which consist of several layers of cells protecting the grain. Thus, in order for the digestive enzymes of poultry to access the nutrients of a grain, they must be able to penetrate the pericarp, aleurone layer and the final barrier, endosperm cell walls (Bedford, 1995; Tervilä-Wilo et al., 1996). Feed processing such as grinding and pelleting can facilitate the grain rupture and would result in a significant damage to the endosperm cell walls. However, processing alone does not break open all endosperm cells, and some may remain intact. Thus the encapsulated nutrients will escape digestion, reach the hindgut, and undergo fermentation with a relatively low energy yield (Józefiak et al., 2004). Microscopic examination confirmed that there were considerable amounts of starch and protein surrounded by intact cell walls in the intestinal contents of broilers fed wheat-based diets (Bedford and Autio, 1996; Tervilä-Wilo et al., 1996).

As shown in Figure 2.2 (b), oilseeds such as canola seeds and flaxseed contain significant amounts of oil that is located in the cotyledon cells which are surrounded by

thick walls of indigestible polysaccharides (Bhatty, 1995). Due to the high oil content, high-diameter sieves (i.e. 4 mm) in the hammer mill are used under commercial conditions for grinding to avoid sieve plugging. However, such grinding is insufficient for effective disruption of the cell wall structure due to the small size of seeds. Consequently, NSP associated with the cell wall matrix form a physical barrier and thus encapsulate oil, and impair dietary energy utilization. This rationale was substantiated in a rooster study with the TME_n values for full-fat canola seed and flaxseed being lower than their corresponding meal and oil mixture (canola seed: 4.6 vs. 5.6; flaxseed: 3.7 vs. 5.1 kcal/g, Lee et al., 1995).

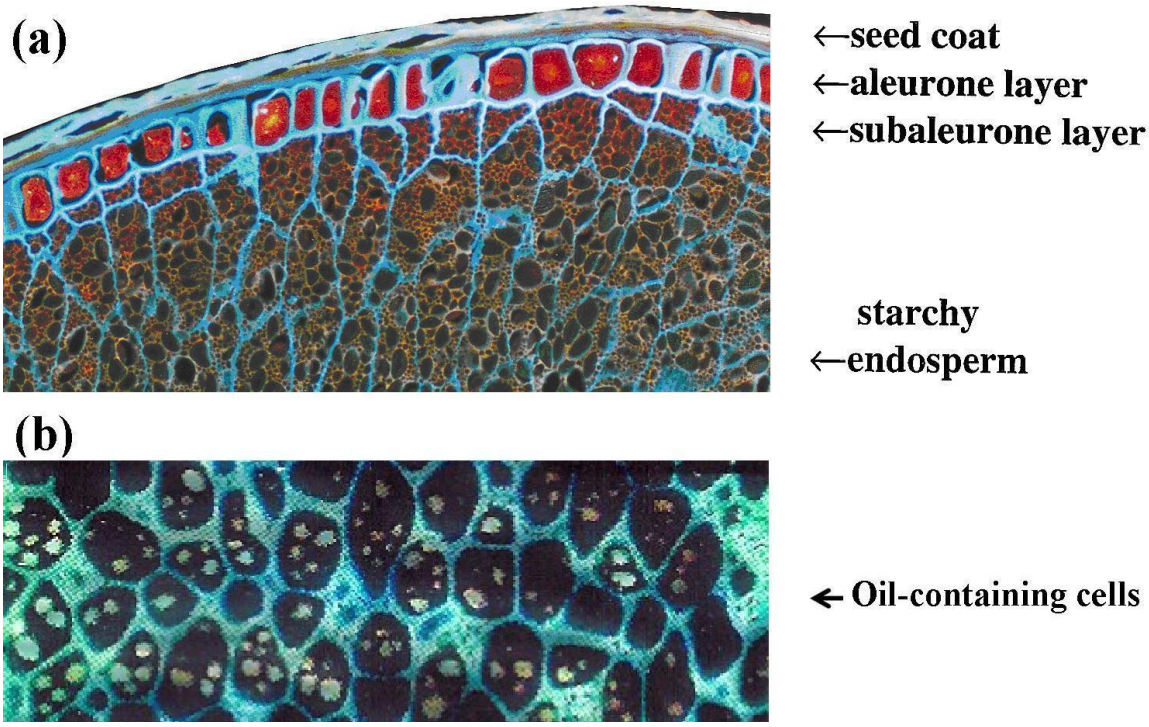

Figure 2.2 Microstructure of wheat grain and canola seed.

(a) wheat grain (Tervilä-Wilo et al., 1996, used with permission of Elsevier Limited, June 10, 2009) and (b) the cotyledon of canola seed.

2.3.3 Interaction with intestinal microflora

Addition of penicillin was found to improve the growth performance of broiler chickens fed viscous cereal-based diets, especially those containing rye. Such a finding demonstrates another potential antinutritive activity of viscous water-soluble NSP as it relates to microflora proliferation in the digestive tract (Misir and Marquardt, 1978; Wagner and Thomas, 1978).

2.3.3.1 General features of the intestinal microflora of the chicken

The intestinal microflora is an integral part of the digestive system of all animals. Among all the microorganisms which inhabit the poultry gut, bacteria are predominant, and the main bacterial activities are found in the crop and the ceca, and, to a lesser extent, in the small intestine (Gabriel et al., 2006). The bacterial population density tends to increase from the proximal to distal digestive tract. In the duodenum, environmental conditions are not favourable for bacterial growth because of high concentrations of digestive enzymes and antimicrobial compounds including pepsin, bile salts, lecithin or lysozyme, the high oxygen tension, and reflux movements from the jejunum to the gizzard resulting in a rapid change in pH values (5.7-6.1 in duodenum and jejunum, and 2.5-2.8 in gizzard). Further along the small intestine, the environment becomes more favourable for bacterial growth due to the reduction in concentrations of enzymes and bile salts, and in oxygen tension. In the ceca, the slow turnover of contents (1 to 2 times a day) facilitates bacterial growth and leads to an increase in their number and diversity (Farner, 1942; Bedford, 2000; Gabriel et al., 2006). Apajalahti et al. (2004) reported that 1 day after hatching, bacterial densities in the ileum and ceca of broiler chickens reached

10^8 and 10^{10} per gram of digesta, respectively, as measured by a culture-independent flow cytometric method. The numbers of microbes reached 10^9 / g of ileal digesta and 10^{11} / g of cecal digesta during the first 3 days post hatching, and remained relatively stable for the following 30 days. In total, they found 640 different species and 140 different bacterial genera in the chicken gastrointestinal tract.

A large proportion of bacteria is Gram+ and mainly includes facultative anaerobes from the crop to the terminal ileum, whereas obligate anaerobes are dominant in the ceca with less numbers of facultative anaerobes (Gong et al., 2002; Lu et al., 2003; Gabriel et al., 2006). Most researchers agree that lactobacilli predominate in the ileum, whereas ceca harbour a more diverse microbial community (Gong et al., 2002, Zhu et al., 2002; Lu et al., 2003). Lu et al. (2003) analyzed 16S rRNA gene sequences from chickens fed corn-soybean diet free of animal protein, antibiotics and coccidiostats. They reported that nearly 70% of gene sequences from the ileum were related to those of *Lactobacillus*, with the majority of the rest being related to *Clostridiaceae* (11%), *Streptococcus* (6.5%), and *Enterococcus* (6.5%). In contrast, *Clostridiaceae*-related sequences (65%) were the most abundant group detected in the cecum, with the other most abundant sequences being related to *Fusobacterium* (14%), *Lactobacillus* (8%), and *Bacteroides* (5%).

It is of importance to note that large variation exists in published reports regarding the composition of intestinal microbial community because of its diversity and complexity as well as the enumeration methods used. Moreover, the bacterial community can be affected by various factors from animal strain, gender, age, and immunologic status, diet (feed ingredients and additives including viscous cereal grains, antibiotics, prebiotics, probiotics etc.), to environment (housing conditions, thermal stress, disease

infections, etc.) (Suzuki et al., 1989; Knarreborg et al., 2002b; Lu et al., 2003; Apajalahti et al. 2004; Bjerrum et al., 2006; Gabriel et al., 2006; Gong et al., 2008). Research by Wielen et al. (2002) demonstrated that every chicken as well as every compartment of the intestinal tract within one chicken has its own specific dominant bacterial community, except for the left and right ceca, which are similar.

2.3.3.2 Impact of dietary NSP on intestinal microflora

Intestinal bacteria obtain most of their nutrients for reproduction and growth from dietary compounds which are either undigested or absorbed slowly by the host animals. Because different bacterial species have different substrate preferences and growth requirements, changes to the diet composition will lead to the shift in the structure of intestinal bacterial community (Apajalahti et al. 2004). Water-insoluble NSP e.g. cellulose are considered practically undigested by poultry, and they are not degraded extensively by bacterial fermentation either, which makes their influence on the intestinal microflora relatively insignificant (Hetland et al., 2004; Gabriel et al., 2006). Viscous water-soluble NSP, on the contrary, has been found to affect the intestinal microflora considerably.

Viscous cereal-based diets are known to increase intestinal viscosity which results in a prolonged feed passage rate and reduced nutrient utilization in broiler chickens. Increased substrate supply and digesta retention time under such conditions facilitate bacterial growth and colonization in the digestive tract (Choct et al., 1999; Bedford, 2000; Yegani and Korver, 2008). Increased microbial activity in the small intestine due to dietary supplementation of viscous water-soluble NSP has been demonstrated in a few

studies with broiler chickens (Choct et al., 1996; Smits et al., 1998; Langhout et al., 1999).

Not only the population density, but the species dominance may also change. Wagner and Thomas (1978) found that ileal anaerobe counts in broiler chickens fed diets containing rye or pectin were higher than those when corn-based diets were fed. Proliferation of detrimental bacteria belonging to the genus *Clostridium* within the intestine was suggested to be responsible for the depressed growth of birds fed rye or pectin. Study by Langhout et al. (1999) demonstrated that numbers of *Clostridia*, *Enterococci*, *Bacteroidaceae* and *Escherichia coli* were significantly increased in the ileum of broiler chickens with increased intestinal viscosity caused by dietary addition of high-methylated citrus pectin. However, another study by Smits et al. (1998) showed that feeding the high-viscosity nonfermentable carboxymethylcellulose to broiler chickens did not affect microbial counts in the ileum, but significantly increased the numbers of *Clostridia*, *Lactobacillus*, *Bacteroides*, and yeasts and molds in the digesta from duodenum and jejunum. Microbial numbers in both studies were determined using a conventional culture-based approach.

2.3.3.3 Adverse effects of intestinal bacteria on nutrient utilization

Intestinal bacteria, on one hand, may provide some benefits to the host animal by producing vitamins, digestive enzymes, and short-chain fatty acids (Ewing and Cole, 1994; Lan et al., 2005). On the other hand, bacterial activities may negatively affect the utilization of dietary nutrients by the host. Early studies demonstrated that germ-free chickens grew faster than the conventional birds without significant difference in feed

intake (Coates et al., 1963; Kussaibati et al., 1982a; Muramatsu et al., 1987). Addition of penicillin improved the weight gain of conventional but not the germ-free birds (Coates et al., 1963).

The presence of intestinal bacteria affects the morphology of the small intestine. When comparing with the germ-free with conventional animals, the former have a more regular and thinner villus structure with narrower lamina propria (Ewing and Cole, 1994). The relative weight of small intestine is higher in conventional animals due to the increased relative length and a thicker wall, mainly associated to the connective tissues, particularly the lamina propria, but also to the lymphoid tissue (Gabriel et al., 2006). Intestinal bacteria also stimulate the renewal of epithelium and increase nitrogen loss from the gut (Ewing and Cole, 1994). Protein synthesis was found to be higher in jejunum and ileum of conventional chickens than the germ-free birds (Muramatsu et al., 1987). In general, morphological changes in conventional animals indicate a reduced absorptive capacity and an increased maintenance requirement of the small intestine.

In addition, intestinal bacteria are in competition with the host for the use of dietary nutrients in the digestive tract. The energy and nutrients consumed by them would otherwise be available for the growth of host animals (Lan et al., 2005; Gabriel et al., 2006).

Regarding the utilization of dietary macronutrients, the total-tract apparent digestibility of corn starch was not different between germ-free and conventional birds (Kussaibati et al., 1982a). Conflicting results of protein digestibility exist in literature indicating that diet composition may have some influence. When corn/SBM-based diets were fed, conventional chickens had a decreased protein digestibility comparing with

their germ-free counterparts. An increased endogenous nitrogen loss found in conventional chickens may have originated from mucus, cellular debris and bacterial biomass (Kussaibati et al., 1982a; Gabriel et al., 2006). Intestinal bacteria reduced the total-tract fat digestibility by 2% and 10% in chickens fed diets containing corn oil and beef tallow, respectively (Boyd and Edwards, 1967). The reduction in fat digestibility of conventional birds is mainly due to the deconjugation of bile salts by intestinal bacteria (Kussaibati et al., 1982b).

Many indigenous intestinal bacteria including *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Bifidobacterium*, *Clostridium*, and *Bacteroides* are capable of hydrolyzing the amide bond of conjugated bile salts, liberating free bile salts (Hayakawa, 1973; Cole and Fuller, 1984). Deconjugation markedly reduces the detergent properties of bile salts in the emulsification of fat, and thus results in an impaired utilization of dietary lipids, particularly the long-chain saturated fatty acids (Krogdahl, 1985). Knarreborg et al. (2002a) isolated bacteria from the small intestine of chickens and found that *Enterococcus faecium* and *Clostridium perfringens* had the highest levels of bile salt hydrolase activity.

2.3.3.4 Proliferation of Clostridium perfringens and necrotic enteritis in poultry

From the viewpoint of gut health, intestinal bacteria can be categorized as potentially pathogenic or health-promoting groups. In general, lactobacilli and bifidobacteria may offer health-promoting benefits to the host such as stimulation of the immune system through non-pathogenic means, and inhibition of the growth and colonization of harmful microbial species. Some pathogenic species are associated with

localized or systemic infections, intestinal putrefaction, and toxin production (Jeurissen et al., 2002). A good example is the development of necrotic enteritis (**NE**) in poultry caused by the proliferation of *Clostridium perfringens* in the digestive tract.

Necrotic enteritis is an enteric disease affecting a variety of avian species including broiler chickens, laying hens, turkeys, and quails (McDevitt et al., 2006). Necrotic enteritis, especially in broilers, has long been controlled effectively by in-feed antimicrobial growth promoters. Concerns about increased antibiotic resistance in human pathogens have led to the removal or reduction of the use of these compounds in animal production. Consequently, NE, as a re-emerging disease, becomes increasingly prevalent (Boulianne, 1999; Van Immerseel et al., 2004).

2.3.3.4.1 Clinical and subclinical forms of NE in broiler chickens

The causative microorganism of NE is *Clostridium perfringens*, a Gram+, spore-forming, ubiquitous anaerobe. *C. perfringens* is a normal inhabitant of the intestine usually at low numbers (< 10^4 cfu/g). However, under certain circumstances, it may proliferate rapidly to 10^7-10^9 cfu/g which would lead to the onset of NE (McDevitt et al., 2006). Majority of outbreaks in broiler chickens typically occur at 2-5 weeks of age, and clinical signs include marked to severe depression, decreased appetite, reluctance to move, diarrhea, and ruffled feather (Ficken and Wages, 1997). Clinical illness is very short and birds are often found acutely dead. Mortality rate of the affected flocks may reach 1% per day, and overall 10% to 40% (Boulianne, 1999; McDevitt et al., 2006). At necropsy, large necrotic foci are found in the small intestinal mucosa, and in severe cases, the whole mucosal surface of gut is affected, with extensive necrosis of the lumen surface

(Figure 2.3). Intestines are often friable, thin walled, and distended with gas. Lesions generally occur in the small intestine, particularly in the jejunum and ileum and, less commonly, the caeca (Wilson et al., 2005).

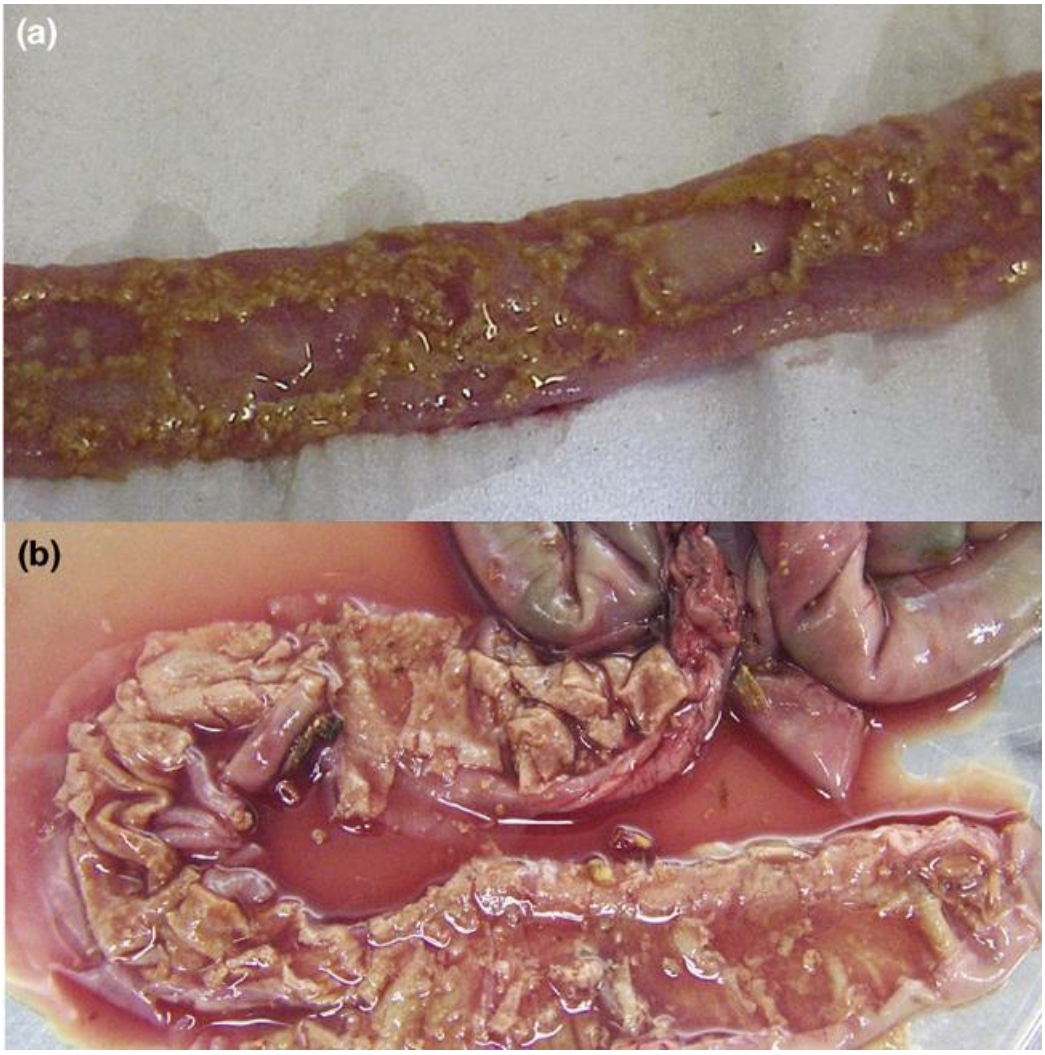

Figure 2.3 Typical gut lesions in severe NE in broiler chickens.

Patches of necrosis (a), and in extreme cases, extensive necrosis (b), are found in the small intestinal mucosa surface (Van Immerseel et al., 2009, used with permission of Elsevier Limited, June 10, 2009).

In the past few years, a subclinical form of NE (**SNE**) has been reported. Infection is associated with impaired growth performance without causing any clinical signs (sticky droppings may be observed). The diagnosis of SNE is based on the detection of macroscopically visible, focal necrotic lesions in the small intestinal mucosa (Kaldhusdal and Hofshagen, 1992; Wilson et al., 2005). Therefore, SNE is often not detected until the carcasses are inspected at the processing plants, when carcasses are rejected, and birds remain untreated (McDevitt et al., 2006). The damage to the intestinal mucosa results in decreased nutrient digestion and absorption, and impaired growth performance of broiler chickens. When compared with the clinical form of NE, SNE may have a greater impact on the welfare of birds and result in greater economic losses to the poultry industry (McDevitt et al., 2006; Van Immerseel et al., 2009).

2.3.3.4.2 C. perfringens virulence factors

As a species, *C. perfringens* can produce various toxins and enzymes responsible for the associated lesions and symptoms, but individual strains produce only a subset of these toxins (Table 2.4). *C. perfringens* strains are classified into 5 toxin types A, B, C, D, and E based on the production of 4 major toxins α-, β-, ε-, and ι-toxin (Hatheway, 1990). In poultry, NE is typically caused by *C. perfringens* type A, and to a lesser extent type C. The α-toxin, produced by all 5 toxin types, is the main virulence determinant in gas gangrene and mutated strains incapable of producing α-toxin are unable to cause disease in mice (Titball et al., 1999). The α-toxin is a phospholipase C sphingomyelinase that hydrolyzes phospholipids and promotes membrane disorganization. Hydrolysis of cell membrane phospholipids yields diacylglycerol and ceramide, resulting in activation of

protein kinase C, and subsequent stimulation of the arachidonic acid cascade. This induces the synthesis of inflammatory mediators including leukotrienes, thromboxane, platelet-agglutinating factor and prostacyclin, which cause blood vessel contraction, platelet aggregation and myocardial dysfunction, leading to acute death (Van Immerseel et al., 2004; McDevitt et al., 2006).

Although substantial evidence supports the role of α-toxin in the pathogenesis of NE (Ficken and Wages, 1997; Titball et al., 1999; Si et al., 2007), some recent studies have questioned it and indicated new NE causing toxins β-2 and NetB. NetB toxin, identified in *C. perfringens* type A strains isolated from NE broilers, has been demonstrated to be a key virulence factor in the pathogenesis of NE (Keyburn et al., 2008). The role of β-2 toxin in the pathogenesis of NE has been inconclusive (Van Immerseel et al., 2009).

2.3.3.4.3 Dietary factors affecting the incidence of NE

C. perfringens type A is ubiquitous in the environment, thus it is generally accepted that predisposing factors are required for these bacteria to cause clinical signs and gut lesions. Suggested predisposing factors are numerous including management practices, environmental conditions, climate, coccidiosis, and diets (Kaldhusdal and Skjerve, 1996; Dahiya et al., 2006; McDevitt et al., 2006; Hermans and Morgan, 2007). The best-known predisposing factor is the prior mucosal damage caused by coccidiosis. Among others, many are ill-defined and experimental results have been contradictory (Kaldhusdal et al., 1999). In the current review, emphasis is placed on the dietary effects.

Table 2.4 *C. perfringens* types and major toxins they produce

Major toxins	Biological activity	*C. perfringens* type				
		A	B	C	D	E
α	A phospholipase C sphingomyelinase that hydrolyzes phospholipids and promotes membrane disorganization.	+[1]	+	+	+	+
β	Induces hemorrhagic necrosis of the intestinal mucosa. Trypsin labile.	-[2]	+	+	-	-
ε	Permease. Trypsin activatable	-	+	-	+	-
ι	Has dermonecrotic activity. A binary toxin consisting of an enzymatic and binding component. Trypsin activatable.	-	-	-	-	+

[1]Toxin is produced by most strains. [2]No strain of the indicated toxin type has been shown to produce the toxin. (Hatheway, 1990; Van Immerseel et al., 2004)

Diet composition has been shown to have a great influence on the incidence of NE. Diets rich in viscous water-soluble NSP (e.g. wheat-, barley- and rye-based diets) were found to be responsible of NE outbreaks in broiler chickens (Branton et al., 1987; Kaldhusdal and Hofshagen, 1992; Riddell and Kong 1992; Kaldhusdal and Skjerve, 1996). Kaldhusdal and Skjerve (1996) concluded that the ratio of wheat plus barley to corn was positively correlated with the incidence of NE. Despite considerable research efforts, the actual mechanisms underlying this cereal effect are still not fully understood. It has been suggested that under the high intestinal viscosity, the substrate supply for microbial growth increases as a result of reduced nutrient utilization by host animals. In addition, high intestinal viscosity may prolong the rate of feed passage (Gohl and Gohl, 1977; Salih et al. 1991), and enhance mucus production (Larsen et al. 1993; Langhout et al. 1999; Piel et al. 2005), which could facilitate the overgrowth of anaerobic bacteria in the small intestine, particularly of *Clostridia* (Wagner and Thomas, 1978; Choct et al. 1996; Smits et al. 1998; Langhout et al. 1999; Deplancke et al. 2002; Collier et al. 2003). A study by Deplancke et al. (2002) showed that *C. perfringens* had significant acidomucolytic potential and grew rapidly on mucin-containing medium. Therefore, Collier et al. (2003) suggested that *C. perfringens* may be particularly mucolytic and its growth would be favored by the increased host mucus production associated with coccidiosis or a viscous intestinal environment.

High protein diets have been shown to be another predisposing factor to clostridial overgrowth and thus to NE, particularly when protein ingredients of animal origin such as fish meal are used (Kaldhusdal and Skjerve, 1996; Truscott and Al-Sheikhly, 1977; Drew et al., 2004; Wilkie et al., 2005; Dahiya et al., 2005 and 2007).

Drew et al. (2004) reported that a greater population of *C. perfringens* was found in the intestinal tract of broiler chickens fed fish meal-containing diets compared with those fed soy protein. Dietary amino acid analysis showed that the content of glycine and methionine in fishmeal diets were higher than that of soy protein diets. Dahiya et al. (2007) documented that dietary glycine concentration was an important determinant of *C. perfringens* growth in the intestinal tract of broiler chickens. Very little information is available in peer-reviewed literature regarding the mechanisms responsible for the increased incidence of NE due to high dietary protein, or glycine *per se*. The amino acid requirements of *C. perfringens* were studied by Fuchs and Bonde (1957), and the authors reported that the requirements varied between the strains. Eleven amino acids were found to be essential for all strains, including arginine, aspartic acid, cystine, glutamic acid, histidine, leucine, phenylalanine, threonine, tryptophan, tyrosine and valine. Omission of methionine, isoleucine, or alanine had no effect on some strains but depressed the growth of others. Although not essential, the presence of lysine, glycine and serine were necessary for maximal growth with glycine having the greatest effect and lysine the smallest, and glycine + lysine having a synergistic effect. Another study by Nakamura et al. (1968) reported that the in vitro α-toxin production required the presence of glycine-containing peptides in defined media. Alternatively, Dahiya et al. (2007) postulated that the effect of glycine on intestinal *C. perfringens* growth maybe such that the high-glycine diets stimulate the mucin production by the host. However, the exact mechanism requires further clarification.

Allen et al. (1996) reported that the addition of n-3 fatty acids (menhaden and flaxseed oil) reduced cecal lesions and maintained BW gains in broiler chickens infected

with *Eimeria tenella*. If true, n-3 fatty acids may provide some protection against NE given the fact that coccidiosis is an important risk factor in NE development. However, the subsequent study from the same group showed that diets containing 5 or 10% ground flaxseed exacerbated lesions in chickens infected with a high dose of *E. maxima* oocysts, indicating that n-3 fatty acids may affect the development of *Eimeria* spp. at different manner (Allen et al., 1997). Only one report can be found on the effects of dietary fat source (mixture of lard and tallow vs. soybean oil, at 10% inclusion rate) on *C. perfringens* growth (Knarreborg et al., 2002b). The results showed that numbers of ileal *C. perfringens* were higher in broiler chickens fed diets containing animal fat. However, too little work has been done to draw any sound conclusions.

The influence of feed processing method on NE incidence or *C. perfringens* proliferation was examined in two studies. Branton et al. (1987) reported that the NE associated mortality was increased in birds fed the finely-ground wheat-based diet (by a hammer-mill) compared with the coarsely-ground diet (by a roller-mill). In contrast, Engberg et al. (2002) found that particle size had no effects on intestinal *C. perfringens* counts. However, *C. perfringens* and lactobacilli counts were numerically higher in the small intestine of mash-fed birds and significantly higher in the ceca and rectum when compared with those fed pelleted diets. Such a response was most likely due to the increased nutrient utilization in birds fed pelleted diets.

2.3.3.4.4 Dietary strategies to protect against NE in broiler chickens

Alternative strategies have been explored to protect against the outbreaks of NE in broiler chickens in the absence of growth-promoting antibiotics. Detailed reviews

regarding NE vaccination and coccidiosis control have been published by Williams (2005) and Van Immerseel et al. (2004; 2009). Nutritional strategies including diet formulation, ingredient selection, and certain feed additives (competitive exclusion products, probiotics and prebiotics) are part of the current review. Other possible strategies such as organic acids, plant extracts or essential oils, hen egg antibodies and bacteriophages (Van Immerseel et al., 2004; Dahiya et al., 2006; McDevitt et al., 2006; Van Immerseel et al., 2009) will not be discussed.

Dietary components such as wheat, barley, rye, and fish meal have been widely accepted as predisposing factors of NE. Therefore, ingredient selection for diet formulation should take this into consideration. Similarly, diets formulated with high protein level or imbalanced amino acid profile should be avoided, as this may lead to increased amino acid supply for microbial growth. Therefore, any means of improving nutrient utilization in the upper gut would be beneficial for maintaining healthy environment of the gastrointestinal tract. In this context, enzyme supplements have potential to become another means of protecting broiler chickens against NE (see Section 2.4.2.1.3).

The concept of competitive exclusion was originally developed by Nurmi and Rantala (1973). They found that by oral administration of suspension of intestinal contents from healthy adult birds to newly hatched chickens, an adult-type intestinal microflora would establish and thus would protect young birds against *Salmonella* infection. A probiotic is defined as a live microbial feed supplement which beneficially affects host animals by improving its intestinal microbial balance (Fuller, 1989). The most typical active components of probiotics are lactic acid bacteria, including

Bidifobacterium, *Lactobacillus* and *Enterococcus* spp. (Holzapfel and Schillinger, 2002). Competitive exclusion products including probiotics have been demonstrated to be capable of reducing colonization of *Salmonella* and *Campylobacter jejuni* (Morishita et al., 1997; Chaveerach et al. 2004; Yang et al., 2009). The inhibition of pathogenic bacteria by probiotic bacteria can be achieved through several different mechanisms such as occupation of attachment sites on the gut wall, competition for nutrients, production of acid and lowering the gut pH, production of antibacterial substances, or immune system stimulation (Gibson and Roberfroid, 1995; Dugas et al., 1999; Lan et al., 2005). However, only few studies in this area have been carried out with *C. perfringens*. One in vitro study demonstrated that the selected lactic acid bacteria strains such as *Lactobacillus rhamnosus*, *L. pentosus*, *Bifidobacterium lactis*, and *Enterococcus faecium* reduced the adhesion of *C. perfringens* to immobilized canine mucus (Rinkinen et al., 2003). Reduced NE incidence and mortality, as well as decreased intestinal numbers of *C. perfringens* were found in broiler chickens fed the competitive exclusion products (Elwinger et al., 1992; Craven et al., 1999). However, the products have not been identified. Fukata et al. (1991) reported that when germ-free, monoflora (inoculated with only *Lactobacillus acidophilus* or *Streptococcus faecalis*) and conventional broiler chickens were challenged with *C. perfringens*, mortality rate of conventional and monoflora birds was much lower than that of germ-free birds (0 and 7-9 vs. 44-53%). Duodenal *C. perfringens* numbers were either decreased or not detected in monoflora and conventional birds, indicating that the proliferation of *C. perfringens* was suppressed by the administration of probiotics or normal intestinal microflora. Another probiotic candidate is *Bacillus subtilis*. La Ragione and Woodward (2003) documented that when broiler chickens were challenged with 10^9

spores of *Bacillus subtilis* and infected with 10^5 cfu of *C. perfringens* 24 h later, lower *C. perfringens* counts and reduced shedding were found in birds treated with *B. subtilis*.

Prebiotics are defined as nondigestible food and feed ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health (Gibson and Roberfroid, 1995). Prebiotic products are predominantly oligosaccharides, especially those containing fructose, xylose, galactose, glucose and mannose. Products including fructooligosaccharides (**FOS,** oligofructose, inulin), glucooligosaccharides (**GOS**), xylooligosaccharides or soybean galactooligosaccharides have been investigated in humans and animals (Gibson, 1998; Holzapfel and Schillinger, 2002; Rehman et al., 2009). Wang and Gibson (1993) demonstrated that FOS exerted a preferential stimulatory effect on most strains of bifidobacteria from human large intestine in an in vitro fermentation study, whilst maintaining populations of *Escherichia coli* and *Clostridia* at relatively low levels. Batch culture experiments showed that growth of *Bifidobacterium infantis* had an inhibitory effect towards *E. coli* and *C. perfringens*. Similarly, GOS are metabolized by bifidobacteria, lactobacilli, and *Bacteroides* spp., whereas they are poorly used as substrates by *Clostridia*, *Eubacteria*, *Enterobacteria* and *Coliforms* (Monsan and Paul, 1995). Another type of prebiotics, mannanoligosaccharides (**MOS**), have been reported to have a different mode of action. MOS has been suggested to prevent the pathogenic bacteria possessing type-1 fimbriae such as *E. coli* from attaching to gut wall as well as to stimulate the immune system (Spring et al., 2000; Rehman et al., 2009). Conflicting results exist in literature regarding the effectiveness of MOS in inhibiting the growth of *C. perfringens*. Hofacre et al. (2003) reported that a lactic acid-producing

bacterial culture alone or in combination with MOS was effective in reducing *C. perfringens*-associated mortality and the subclinical effects on feed utilization. Addition of either MOS or FOS alone had no positive effects. Yang et al. (2007) also found that counts of total anaerobic bacteria, lactic acid bacteria, and *C. perfringens* in digestive tract of broiler chickens were not affected by MOS supplementation. In contrast, Sims et al. (2004) observed reduced *C. perfringens* numbers in the large intestine of turkey of 6 wk of age when MOS was included in the diets.

Although some promising results have been demonstrated, much work remains to be done to confirm the effectiveness of dietary strategies in controlling *C. perfringens*. To date, no single strategy has been identified to be effective in replacing antibiotic growth promoters. The combined action of antibiotic alternative addition, consideration of diet composition, and good hygiene management (cleaning and disinfection), may be effective, to certain extent, in maintaining performance and controlling NE (Dahiya et al., 2006).

2.3.3.4.5 Experimental models for NE studies

In order to explore new strategies of controlling NE, it is necessary to be able to experimentally induce NE in a controlled environment. However, NE is difficult to reproduce. To date, there is no standardized model, and published models vary in *C. perfringens* strains, challenge procedures (challenge does; in-feed or gavage inoculation), number of challenge days, and whether or not having a prior mucosal damage (coccidial infection). As a consequence, there is much variation in severity of the NE induced by

different research groups (Riddell and Kong, 1992; Kaldhusdal et al., 1999; Brennan et al., 2001 a, b; Drew et al., 2004; Olkowski et al., 2006; Gholamiandehkordi et al., 2007).

A challenge model developed by Netreco Canada Agresearch in Burford, Ontario (originally ShurGain Agresearch) has been used in the current study to induce NE in broiler chickens. *C. perfringens* type A was originally isolated from a field case of NE in Ontario, and was known to produce lesions typical of NE with mild suppression of growth rate and minimal mortality (Brennan et al. 2001 a, b; Chalmers et al., 2007). This model has been documented to be reliable in identifying the potential effects of dietary treatments on subclinical or clinical form of NE under typical Canadian commercial broiler flock conditions. The specific intestinal lesions of randomly selected birds have been indicated to be a more sensitive disease indicator than the NE-specific mortality (Kaldhusdal et al., 1999).

2.4 Dietary means to improve the nutritive value of oilseeds for poultry

2.4.1 Feed processing

Various processing techniques have been employed in preparation of the oilseed-containing diets to improve their nutritive value for poultry. Grinding is the most commonly used method to facilitate seed rupture. Due to the high oil content, high-diameter sieves (i.e., 4 mm) are often used for seed processing to avoid plugging of the sieves. However, an effective seed rupture and thus oil exposure is difficult to achieve under such conditions. When full-fat canola seeds were fed to broiler chickens, a marked increase in the AME_n content was observed when the average particle size of seeds was decreased to ≤0.56 mm. This effect was much more pronounced for broiler chickens than

for laying hens (Danicke et al., 1998). To our knowledge, no similar study has been done with flaxseed yet.

Theoretically, seed grinding before diet preparation would accelerate lipid oxidation and would result in a short shelf life of diets. Thus, feeding whole seed would be advantageous, because it will eliminate the grinding cost and may reduce the potential for lipid oxidation during storage. However, Nam et al. (1998) documented that TME of whole flaxseed was much lower than that of ground seed (3786 vs. 4386 kcal/kg), and increased significantly to 5404 kcal/kg following the addition of builder's sand. The effects of mechanical dehulling were investigated by Leeson and Caston (2004). An increased AME_n of flaxseed and a higher content of ALA were found in eggs from laying hens fed dehulled flaxseed compared with those fed the regular ground seeds. Gonzalez-Esquerra and Leeson (2000b) reported that feeding flaxseed to mature roosters in pelleted or crumbled form significantly increased its AME_n from 3654 kcal/kg to 4578 and 4277 kcal/kg respectively. Similar results have been reported by Shen et al. (2004). They applied various processing methods including pelleting, autoclaving, or microwave roasting, and the TME_n values of flaxseed increased by 22-25% when compared with that of untreated seeds. Beneficial effects of the extruding process were reported by Thacker et al. (2005). Broiler chickens were fed diets containing 25% of an extruded or non-extruded flaxseed product (i.e. Linpro, flaxseed:peas, 1:1 wt/wt). The energy and nitrogen digestibility as well as BW gain and FCR were significantly improved in birds fed an extruded Linpro product. It would appear that the shear force, heat and pressure imposed during pelleting and extruding may facilitate an effective rupture of the seeds and thus better exposure of oil to digestive enzymes. In addition, the heat-labile ANF present in

flaxseed (i.e., trypsin inhibitors and enzymes responsible for cyanogenic glycosides conversion to toxic end products) could be inactivated (Feng et al., 2003). However, the mucilage and the high amount of cell wall polysaccharide in flaxseed may still pose a problem.

2.4.2 Addition of NSP-degrading enzymes

NSP-degrading enzymes (carbohydrase or glycosidase) are a class of enzymes that are capable of depolymerizing NSP in feed ingredients, and they have been used for nearly 30 years in diets for monogastric animals. Numerous studies have shown that the addition of xylanase and β-glucanase can counteract the adverse effects of arabinoxylans in wheat and rye, and β-glucans in barley, thereby improving their nutritive value and growth performance of monogastric animals. The success of enzyme addition to viscous cereal-based diets stimulated interest in developing enzyme preparations for other feed ingredients, including oilseeds.

2.4.2.1 Mode of action of NSP-degrading enzymes

2.4.2.1.1 Intestinal viscosity reduction

The mode of action of NSP-degrading enzymes is to cleave the large molecules of plant NSP into smaller poly- and oligo-mers. Viscosity is partially determined by the chain lengthen of the water-soluble polysaccharides. Thus, the viscosity can be substantially reduced by enzyme preparations (i.e. endo-hydrolases rather than exo-hydrolases) able to cleave a few linkages in the backbone of the polymer (Chesson, 1993; Bedford, 2002). In an in vitro study (Silva et al., 1983), incubation of barley with β-

glucanase resulted in reduced viscosity and β-glucan contents in endosperm cell walls, and was associated with the production of low molecular weight glucooligosaccharides. Barley β-glucans have a consistent linear structure, and the lack of branching points makes them relatively sensitive to the hydrolysis by β-glucanase (Carré, 2002). As a result, consistently beneficial responses have been observed in most broiler chicken studies with barley-based diets showing the reduction in viscosity of intestinal contents and improved BW gain and feed utilization after β-glucanase supplementation (Burnett, 1966; Hesselman and Åman, 1986; Classen et al., 1988; Campbell et al., 1989; Salih et al., 1991).

In contrast, water-soluble arabinoxylans present in rye or wheat, particularly those from endosperms and aleurones, are highly branched. As shown in Figure 2.4, a complete hydrolysis of arabinoxylans requires not only the enzymes responsible for the primary attack on the backbone but also another set of glycosidases. Although an effective viscosity reduction does not demand the complete hydrolysis of arabinoxylans, it may require the presence of debranching activities in the form of α-arabinanase/ arabinofurnosidases (Chesson, 2001). Similarly to barley, xylanase addition to rye-based diets reduced intestinal viscosity, and improved growth performance and feed utilization of broiler chickens (Bedford et al., 1991; Bedford and Classen, 1992). However, literature data concerned with the xylanse supplementation to wheat-based diets shows more variable results which seem to depend on the characteristics of wheat varieties used. Positive effects of enzyme addition on viscosity reduction and growth performance were observed when broilers were fed low-AME Australian wheats that caused a high intestinal viscosity of 10 - 40 mPa·s (Choct et al., 1995; Dusel et al., 1998). However,

feeding Canadian wheats would not result in such a high intestinal viscosity, and values ranging from 3.3 to 5.0 mPa·s have been observed in the intestinal contents of broiler chickens (Meng et al., 2004; 2005). However, enzyme addition to such diets also resulted in an improved nutrient utilization and growth performance. Such findings indicate that multiple mechanisms may be involved in the beneficial response to enzyme supplementation.

Although monosaccharides may be released following enzyme supplementation, the improved growth performance can not be attributed to simple sugar utilization and the subsequent energy yield. It appears that some sugars (e.g. xylose, arabinose or uronic acids) are poorly absorbed and/or utilized that it may be advantageous for them to pass into the hind gut as oligosaccharides, and be fermented by the microflora (Longstaff et al., 1988; Chesson, 1993).

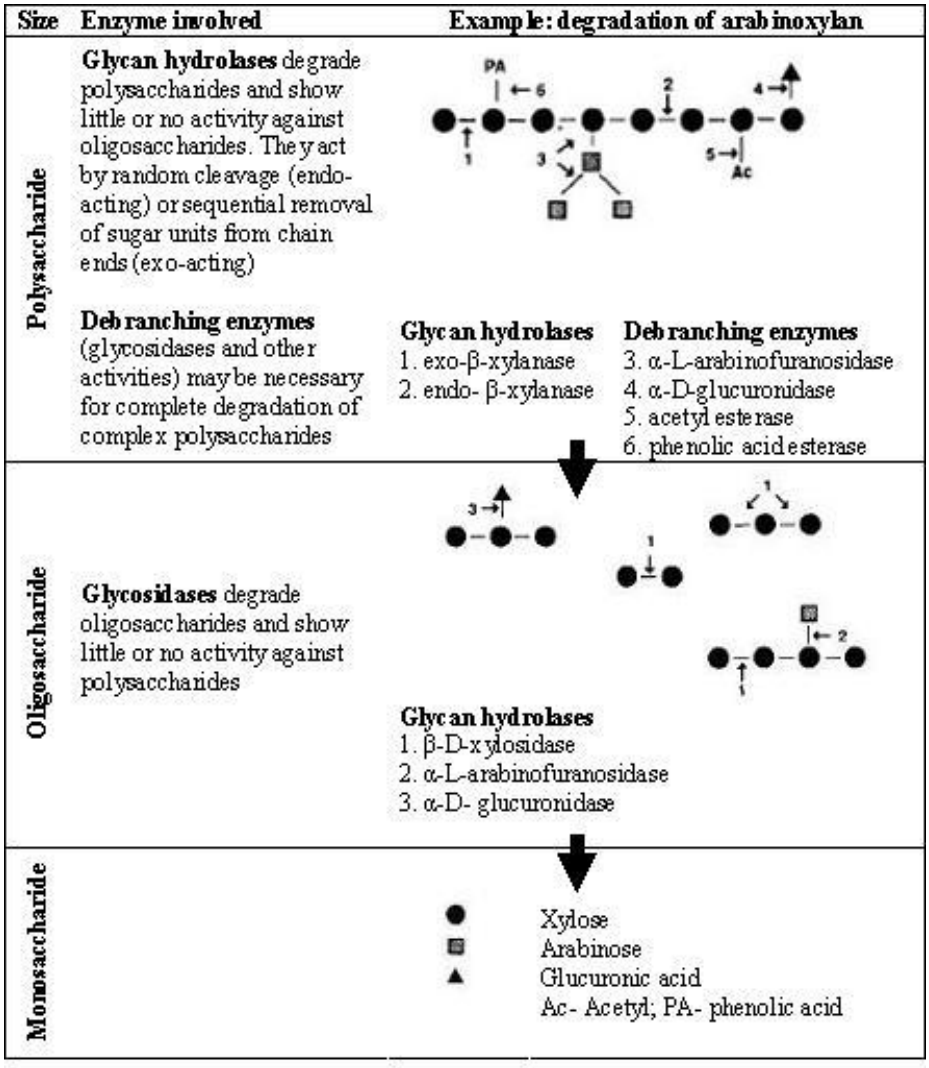

Figure 2.4 The enzyme-catalysed hydrolysis of arabinoxylan to its constituent monosaccharides.

A small portion of an arabinoxylan molecule is used to demonstrate the variety of enzymes required for the initial disruption of the polysaccharide and, subsequently for the complete hydrolysis of released oligosaccharides (Chesson, 1993, used with permission of Elsevier Limited, June 10, 2009).

2.4.2.1.2 Elimination of nutrient encapsulating effect of cell walls

Because β-glucans and arabinoxylans comprise the major components of endosperm cell walls, it seems very likely that enzymes capable of depolymerizing these polymers would also solubilize, to certain extent, the cell walls. Pettersson and Åman (1989) demonstrated that the addition of a feed enzyme preparation containing xylanse and β-glucanase to rye/wheat-based diets resulted in improved growth performance and nutrient digestibility in broiler chickens. However, no significant decrease could be observed in the content of water-soluble pentosans and relative viscosity of an extract from the enzyme-supplemented diet during in vitro experimentation. The researchers suggested that although enzyme addition destroyed the viscous property of water-soluble arabinoxylans, it simultaneously solubilised additional and, otherwise water-insoluble, arabinoxylans from the cell walls. In this context, disruption of the intact cell walls and release of entrapped nutrients would be one possible explanation of improved growth performance of birds fed enzyme-supplemented diets, particularly in situations when the viscosity of intestinal contents is low (Meng, 2005). A microscopic study by Bedford and Autio (1996) supports this rationale. They examined the intestinal contents from birds fed wheat-based diets without or with a xylanase-based enzyme supplement. The results showed that digesta from enzyme-treated group had less undamaged endosperm cells, indicating potential cell wall degradation. The authours concluded that the disruption of intact cell walls would facilitate an easier access of digestive enzymes to their substrates within the short feed transit time which would lead to an increased nutrient utilization.

Plant cell walls have a structural function and they are intrinsically more resistant to degradation. Unlike viscosity reduction, cell walls require not only large amount but also different types of enzymes in order to disrupt them to a significant extent within a relatively short period of time (Chesson, 1993; Bedford, 2002). Earlier research from our laboratory has demonstrated that a more pronounced NSP depolymerization in wheat, peas, SBM, canola meal and flaxseed was achieved only when several enzyme preparations were used in combination (Meng et al., 2005; Slominski et al., 2006).

2.4.2.1.3 Modification of intestinal microflora and the potential prebiotic effect of enzyme hydrolysis products

Enzymatic depolymerization of cell wall polysaccharides removes the constraints on nutrient digestion and absorption and thus increases the rate of utilization of dietary starch and protein in the small intestine. Such effects may reduce microbial activity as a result of substrate limitation in the small intestine. Bedford and Apajalahti (2001) demonstrated that in birds fed wheat-based diets, the addition of a xylanase-based enzyme resulted in a 60% reduction in microbial numbers. Study by Choct et al. (1999) supported these findings since a decreased ileal volatile fatty acid production was observed after xylanase addition to a wheat-based diet.

In the process of depolymerizing various polysaccharides in the diet, enzymes may produce a wide array of NSP hydrolysis products differing in sugar component and molecular sizes. Such products may include low molecular weight polysaccharides, oligosaccharides and simple sugars. Marsman et al. (1997a) studied the effects of several commercial enzyme supplements on cell wall depolymerization of SBM in vitro. The

degradation of SBM polysaccharides to small oligomers and monomers was confirmed by using high-performance anion-exchange chromatography. Although several standards were used, the authors could not identify the various oligomers, because of a high number of different products and peeks overlapping each other on the chromatogram. The incubation of wheat arabinoxylans with an endo-xylanase resulted in a mixture of up to 12 oligosaccharides (Austin et al., 1999), whereas the production of gluco-oligomers after β-glucanase addition to barley was demonstrated by Silva et al. (1983). Among the enzyme hydrolysis products that would be obtained from poultry feed, many of them i.e. galacto-, gluco- or xylo-oligomers are somewhat similar in nature to the prebiotics. Thus they may have the same property of indirectly prohibiting the growth of certain pathogenic species by stimulating the growth of lactic acid bacteria in the lower gut (Gibson and Roberfroid, 1995). In addition, certain hydrolysis products such as mannan-oligomers may attract microbes away from the intestinal binding sites by means of competitive exclusion, thereby reducing colonization and disease development as well as releasing the mucosa to perform its function of secretion as well as digestion and absorption of nutrients (Iji and Tivey, 1998; Spring et al., 2000).

Although not always consistent, there is some evidence in the literature supporting the beneficial effects of enzyme addition on the growth of lactic acid bacteria. Vahjen et al. (1998) documented that xylanase addition to a wheat-based diet increased *Lactobacillus* spp. counts in mucosal tissue samples from ileum of broiler chickens, but did not affect lactobacilli counts in digesta samples. Engberg et al. (2004) reported that xylanase addition slightly but significantly increased the counts of lactic acid bacteria in the small intestine (9.1 vs. 8.9 $\log_{10}$ cfu/g digesta, P=0.02), which was accompanied by a

higher lactic acid concentration (67.2 vs. 55.7 µmol/g). Similarly, higher ileal lactobacilli counts and lactate content were observed in piglets following the addition of a multicarbohydrase supplement to a starter diet containing wheat, barley, SBM, CM and flaxseed (Kiarie et al., 2007).

The effects of competitive exclusion products on the proliferation of *C. perfringens* have been discussed earlier in the current review (see section 2.3.3.4.4). In this context, it could be hypothesized that cell wall hydrolysis products resulting from enzyme addition may serve as prebiotics and facilitate the growth of lactic acid bacteria, thereby indirectly reducing *C. perfringens* proliferation. Very recently, Kiarie (2008) reported that enzyme hydrolysis products obtained from wheat, flaxseed, SBM and CM enhanced net absorption of fluid and solutes in enterotoxigenic *Escherichia coli* (**ETEC**) infected jejunal segments of piglets, suggesting for a potential of these products in controlling enteric infections such as ETEC-secretory diarrhea. Studies on the effects of enzymes on growth of *C. perfringens* are scarce, and the available data in the literature have been contradictory and very difficult to compare because of the use of different disease challenge models, diet types, and enzyme supplements. Riddell and Kong (1992), who used an in-feed *C. perfringens* challenge model, found that the addition of pentosanase to a wheat-based diet did not affect NE mortality. However, no *C. perfringens* enumeration was performed in their study. In contrast, Choct et al. (2006) reported that xylanase supplementation reduced *C. perfringens* numbers in the caeca of healthy broiler chickens fed wheat-based diets. In another study, Jackson et al. (2003) showed that the addition of β-mannanase to corn-based diets improved performance and reduced lesion scores in birds challenged with both *Eimeria* and *C. perfringens*. However,

the authors postulated that the benefits of enzyme addition were due to the depolymerization of β-mannans, which may exacerbate the disease symptoms via a stimulatory effect on the immune system. In another study, broiler chickens were fed wheat/SBM-based or flaxseed-containing diets without or with a multicarbohydrase enzyme (Wang, 2008). Intestinal segments from birds were excised, ligated and inoculated with a *C. perfringens* spore cocktail. The results showed that enzyme addition significantly reduced the in vitro growth of *C. perfringens* in digesta from both dietary groups by 50 and 67%, respectively, with a more pronounced effect observed for the flaxseed group. This was followed by only a slight and not statistically significant increase in the growth of lactic acid bacteria.

It must be emphasized that enzyme supplements are not drugs and their effects would have to be investigated from the prospective of gut health, reduction of pathogenic bacteria population as well as growth performance. It will be too optimistic to consider that the enzyme addition will be used solely to control intestinal bacteria. However, the effects and mechanisms of NSP-degrading enzymes should be more clearly established, and such findings would offer tremendous marketing advantages in the form of value-added products. At the very least, a reduction in the use of antibiotic growth promoters via employment of specific substrate producing enzymes would be welcomed in the feed industry which is currently under heavy pressure to initiate change.

2.4.2.2 Enzyme addition to oilseed meals or full-fat oilseeds

The success of enzyme supplementation to viscous cereal-based diets has stimulated the interest in the application of enzymes to target cell wall components in meals or full-fat oilseeds such as soybean, canola seeds, and more recently flaxseed.

In addition to high concentration of pectic polysaccharides, various cell wall polysaccharides presented in SBM and CM including cellulose, xylan, xyloglucan, arabinan, arabinogalactan and galactomannan (Aspinall and Cottrell, 1971; Siddiqui and Wood 1977; Slominski and Campbell 1990; Bach Knudsen et al. 1997). Due to such a complex structure, it is difficult to establish a clear enzyme-substrate relationship. However, because of more than 90% of total NSP in SBM and CM being water-insoluble (Meng and Slominski, 2005), the impervious nature of the cell walls should be the target for enzyme supplementation.

When SBM and CM were incubated with various carbohydrase preparations in vitro (i.e. cellulase, pecitnase, xylanase, glucanase, galactanase, and mannanase), consistent degradation of cell wall polysaccharides was observed in a few studies. Study by Marsman et al. (1997a) demonstrated that enzyme addition solubilised cell wall polysaccharides of SBM, and released oligosaccharides and monosaccharides. Incubation of CM with a commercial enzyme supplement revealed that in vitro hydrolysis pattern may be explained by a relatively rapid hydrolysis of the small water-soluble fraction followed by relatively slow release of component sugars from the larger water-insoluble and hence inaccessible cell wall fraction (Slominski and Campbell, 1990). Meng et al. (2005) documented that the highest degree of total NSP degradation following enzyme addition to SBM and CM in vitro was 26 and 36%, respectively.

Addition of a multicarbohydrase preparation to a semipurified diet containing high concentration (38%) of SBM increased apparent ileal digestibilities of protein and NSP in broiler chickens by 1.5% and 6.1%, respectively (Marsman et al., 1997b). Such an improvement most likely resulted from the disruption of the cell wall matrix leading to the release of entrapped protein. When enzyme supplement containing xylanase, glucanase and cellulase fortified with pectinase and galactanase activities was used in corn/SBM-based diets broiler chickens, increased apparent protein digestibility and dietary AME_n were observed and were accompanied by an increased NSP digestibility (Kocher et al., 2002; Meng and Slominski, 2005). However, such improvements are not always translated into a better growth performance of broiler chickens (Marsman et al., 1997b; Douglas et al., 2000; Kocher et al., 2002; Meng and Slominski, 2005). When using a blend of NSP-degrading enzymes in a laying hen diet containing 40% CM, Slominski and Campbell (1990) documented an increase in total NSP digestibility by 34%. However, growth performance was not affected when similar enzyme products were added to broiler diets with high level of CM (Simbaya et al., 1996). Kocher et al. (2001) investigated the effects of two commercial enzyme products on the replacement value of CM for SBM in broiler chickens. Their findings showed that when high levels of CM were included in broiler diets, carcass yield and quality was reduced. Enzyme addition to CM-based diet increased the production of breast meat as well as thigh and drumstick portion without affecting BW and FCR. Although the authors made no reference to any improvement in nutrient digestibility, it is possible that enzyme addition increased dietary protein utilization.

Over the last few years there has been interest in feeding full-fat canola seeds and flaxseed (ground or whole seeds) to poultry because they provide a convenient package for high-quality protein and oil rich in α-linolenic acid. However, the potential drawback would be less-than-optimum energy utilization from the seeds, since substantial amounts of oil and protein may be encapsulated in the cotyledon cells even after conventional grinding. Application of NSP-degrading enzymes has been proven beneficial in facilitating the extraction of canola oil in the aqueous extraction process (Sosulski and Sosulski, 1993). Enzyme addition to canola seed-containing diet (15%) resulted in an improved FCR, total tract fat and NSP digestibilities and AME_n content in young broiler chickens (Meng et al., 2006). Using enzymes to target full-fat flaxseed is a relatively new initiative and reports on this topic are rare. Recent research in our laboratory investigated the efficacy of a multicarbohydrase blend containing xylanase, glucanase, cellulase, and pectinase activities in improving energy utilization from full-fat flaxseed (Slominski et al., 2006). Enzyme addition increased the TME_n content of flaxseed from 2,717 to 3,751 kcal/kg, which was associated with increased digestibilities of fat and NSP by adult roosters used in the TME assay. Effects of enzyme were further investigated in a study with broiler chickens fed a corn/SBM-based diet containing 15% of flaxseed without or with enzyme addition at 0.002, 0.01, and 0.05%. Improved FCR and total tract digestibilities of fat and NSP were only observed when enzyme was used at the highest level. Such findings indicated that despite of the complex nature of flaxseed cell wall structure, enzymatic degradation of cell wall polysaccharides is possible and enzyme supplementation may be used as a means to improve its nutritive value for poultry. Unlike soybean and canola seeds, flaxseed contain significant amount of water-soluble

NSP. The intestinal viscosity in broilers fed flaxseed-containing diets appeared to be high. Despite the benefits on energy utilization and growth performance, no effect of enzyme addition on viscosity reduction was observed in this study. Therefore, further research is needed to screen for enzyme blends more effective in not only cell wall degradation but also viscosity reduction.

2.5 Research hypothesis

The hypothesis of the current research program was two fold: 1) Addition of specific carbohydrase supplements would depolymerize cell wall polysaccharides in oilseeds, thereby improving oil utilization and growth or production performance in poultry. 2) NSP hydrolysis products may facilitate proliferation of beneficial bacteria, thereby indirectly reducing the proliferation of *C. perfringens* and NE outbreaks in broiler chickens.

3. MANUSCRIPT I

Means to Improve the Nutritive Value of Flaxseed for Broiler Chickens: The Effect of Particle Size, Enzyme Addition and Feed Pelleting

W. Jia and B. A. Slominski

Department of Animal Science, University of Manitoba, Winnipeg, Canada R3T 2N2

(Submitted to Poultry Science)

3.1 Abstract

Three experiments were conducted to explore dietary means (particle size, enzyme addition, bile salts addition and feed pelleting) of minimizing the antinutritive effects of cell wall/nonstarch polysaccharides (NSP) of flaxseed. Broiler chickens were fed corn/soybean meal-based diets containing 15% full-fat flaxseed from 5 to 18 d. The effects of 2 enzyme preparations containing viscosity-reducing or cell wall-degrading activities on growth performance and nutrient digestibility were evaluated in Experiment 1 at 2 inclusion rates (0.02 vs. 0.05%). Enzyme addition had beneficial effects ($P < 0.05$) in increasing NSP digestibility and reducing intestinal viscosity when used at 0.05%. However, no differences in growth performance or fat digestibility were observed between the enzyme types or inclusion rates. Therefore, the enzyme supplement containing both viscosity-reducing and cell wall-degrading activities was used in subsequent studies. A 2 × 2 × 2 factorial arrangement was used in Experiment 2 to investigate the effects of particle size (coarse vs. fine), enzyme supplementation, and bile salt addition on the nutritive value of flaxseed for broiler chickens. In Experiment 3, a 4 × 2 factorial arrangement was used to further investigate the effects of feed processing (whole seed, coarsely ground seed, and finely ground seed in pelleted diets; or finely ground seed in mash diets) and enzyme addition on growth performance and fat utilization. Bile salt addition did not improve fat digestibility. Particle size reduction via grinding had no significant effect on growth performance no matter if present in the mash or pelleted diets. When compared with grinding, feed pelleting showed more pronounced and beneficial effects on growth performance particularly when whole, intact seeds were

used, indicating a potential for using whole flaxseed in the pelleted diets. Enzyme addition resulted in an increase in total tract fat digestibility by 3 to 6%, which was reflected in an improved FCR by 1 to 3%, regardless of the processing method used ($P < 0.05$). In conclusion, enzyme addition and feed pelleting offer practical solutions to improve the nutritive value of flaxseed for broiler chickens.

Key words: Flaxseed, enzyme, feed processing, broiler chicken

3.2 Introduction

Flaxseed is a rich source of protein (22%), oil (34%) and α-linolenic acid (ALA, 50% of oil), and its use in poultry diets to produce n-3-enriched eggs or meat products has attracted interest in the poultry industry (Leeson and Summers, 2005). However, low energy utilization and poor growth performance have been observed in broiler chickens fed flaxseed-containing diets (Ajuyah et al., 1991; Lee et al., 1991; Ortiz et al., 2001; Alzueta et al., 2003). Such adverse effects have been attributed to the various anti-nutritional factors (**ANF**) present in flaxseed (Bhatty, 1995), including high levels of cell wall/nonstarch polysaccharides (**NSP**). Oil is the main energy source in flaxseed with oil droplets located in the cotyledon cells that are surrounded by the thick walls of polysaccharides. When compared with other ingredients of poultry diets, flaxseed contains relatively high amounts of NSP (i.e., 165.2 mg/g, full fat basis), with 46% of those being water-soluble (Manuscript IV).

Poultry do not possess endogenous enzymes capable of cleaving and digesting cell wall components, which form a physical barrier and thus reduce oil exposure to digestive enzymes and less-than-optimum energy utilization from flaxseed (Lee et al.

1995). A significant negative correlation between the degree of seed rupture on grinding and AME_n content has been reported for canola seeds (Danicke et al., 1998).

Mucilage is an important water-soluble polysaccharide of flaxseed and has been reported to increase the viscosity of intestinal contents in broiler chickens (Rodriguez et al., 2001; Alzueta et al., 2003) with the viscous environment causing a significant inhibition of digestion and absorption of dietary nutrients, with fat digestibility suffering the most among the macronutrients (Smits et al., 1997). Possible mechanisms involved include a reduced diffusion rate between digestive secretions (i.e., lipases and bile salts) and their substrates (Ikegami et al., 1990), less mixing of chyme (Edwards et al., 1988), and an increased apparent thickness of intestinal unstirred layer (Johnson and Gee, 1981). As a result, emulsification and hydrolysis of dietary lipids, micelle formation and their transport to the epithelial surface may be impaired by high intestinal viscosity. Under such conditions, small intestine microflora may proliferate and lead to a subsequent excessive deconjugation of bile salts (Feighner and Dashkevicz, 1988; Choct et al., 1996; Smits et al., 1997; Langhout et al., 1999), which may further reduce their efficacy in lipid emulsification and micelle formation (Krogdahl, 1985). Addition of NSP-degrading enzymes has been demonstrated to be effective in reducing viscosity caused by arabinoxylans and β-glucans of cereal grains (Burnett, 1966; Choct and Annison, 1992). The use of dietary enzymes to target NSP of flaxseed is a relatively new initiative with some reports from this laboratory demonstrating their potential beneficial effects in poultry nutrition (Slominski et al., 2006; Manuscript II). Despite the fact, however, that the enzyme cocktails were effective in cell wall polysaccharide depolymerization, they were not effective in reducing the viscosity of flax mucilage (Slominski et al., 2006).

Therefore, three experiments were conducted to further explore dietary means to overcome antinutritive effects of flax polysaccharides and to increase the nutritive value of this new ingredient for poultry. The effects of multicarbohydrase supplementation, particle size reduction on grinding, feed pelleting, and bile salt addition on growth performance and fat digestibility were investigated with young broiler chickens fed corn/soybean meal-based diets containing 15% of full-fat flaxseed. The viscosity-reducing enzyme preparations used in the study were screened in a preliminary in vitro experiment, whereas the cell wall-degrading enzyme was selected based on earlier research from this laboratory (Slominski et al., 2006).

3.3 Materials and Methods

In vitro Viscosity-reducing Enzyme Evaluation

An incubation study was conducted to screen for viscosity-reducing enzymes using a mucilage isolate as a substrate. For mucilage isolation, whole flaxseed samples (50 g each) were extracted with distilled water (1:8, wt/v) by stirring the seed in the environmentally controlled shaker for 1 h at 40˚C. The samples were then centrifugated at 1,990 × *g*. The extraction was repeated twice. The supernatants were frozen, freeze-dried, and the mucilage collected. For enzyme evaluation, 7 mL mucilage solution (50 mg mucilage in 0.1 M sodium acetate buffer, pH 5.2) was incubated without or with enzyme addition at 20:1 wt/wt substrate to enzyme ratio for 1 h at 40 ºC. Following incubation, the viscosity of the solution was determined at 40 ˚C using the Brookfield digital viscometer (model DV-II+LV, Brookfield Engineering Laboratories, Stoughton, MA). Enzyme preparations **C** (cellulase, 600 U/g, mannanase, 7,900 U/g), **P** (pectinase,

10,000 U/g), and **XG** (xylanase, 13,900 U/g; glucanase, 19,600 U/g), alone and in combination (C + P, C + XG, P + XG, and C + P + XG) were evaluated.

Broiler Chicken Studies

Experiment 1

An experiment was conducted to evaluate the efficacy of two NSP-depolymerizing enzymes on fat utilization and growth performance of broiler chicken. Enzyme A, which was found to be the most effective in the in vitro study, was the viscosity-reducing enzyme and contained 300 U cellulase, 5,000 U pectinase, 3,950 U mannanase and some xylanase and glucanase side activities per g of premix. Enzyme B was used as a cell wall-degrading activity with some viscosity-reducing capability and contained 200 U cellulase, 3,000 U pectinase, 3,300 U xylanase, 2,500 U glucanase, 2,700 U mannanase per g of premix. The experimental treatments included a corn/soybean meal-based control diet containing 15% full-fat flaxseed, and the control diet supplemented with Enzyme A or B at 2 levels: 0.02% or 0.05%, giving a total of 5 treatments. The flaxseed used in the study was a food grade product and was provided by the Flax Council of Canada, Winnipeg, MB. The seed was hammer-milled to pass through a 3.5-mm sieve using a Wiley mill standard model No. 3 grinder (Arthur H. Thomas Company, Philadelphia, PA).

Experiment 2

A 2 × 2 × 2 factorial arrangement of 8 dietary treatments was used to evaluate the effects of flaxseed particle size (coarse vs. fine; 15% of diet), and an addition of a multicarbohydrase enzyme (none vs. 0.05%) or bile salts (none vs. 0.05% of Bile Extract containing glycine and taurine conjugates of hyodeoxycholic acid; Sigma B8631) or both

on nutrient utilization and growth performance of broiler chickens. Ground, feed grade flaxseed (i.e., “coarse”) was used in this experiment and was obtained from a local feed mill. To produce a finely ground seed, the sample was re-ground twice in a hammer-mill using a 3.5-mm sieve. Enzymes A and B used in Experiment 1 were combined to generate a new enzyme preparation with equal effects on viscosity reduction and cell wall degradation. The enzyme product provided 1,400 U pectinase, 160 U cellulase, 2400 U, xylanase, 1200 U glucanase, 1500 U mannanase, 50 U galactanase and other minor enzyme activities per kg of diet.

Experiment 3

A 4 × 2 factorial arrangement of 8 dietary treatments was used to further investigate the effects of particle size, enzyme addition and feed pelleting on the nutritive value of full-fat flaxseed for broiler chickens. The pelleted diets contained 15% of whole (intact) flaxseed, coarsely ground flaxseed, and finely ground flaxseed. The latter product was also used in mash diets (15% of diet) without and with enzyme supplementation. To produce the “coarsely” ground sample, the seeds were premixed with corn (1:1, wt/wt) to facilitate their flowability during grinding. The flaxseed-corn mixture was hammer-milled to pass through a 3.5-mm sieve using a Wiley mill standard model No. 3 grinder. To produce the finely ground sample, the mixture was ground to pass through a 2-mm sieve. The enzyme preparation used in this study was the same as that used in Experiment 2. To minimize enzyme inactivation, the pelleting temperature did not exceed 75˚C.

To determine particle size distribution, the samples of ground flaxseeds or flaxseed-corn mixtures (30 g) were sieved using a set of Endecotts (London, England) sieves of 2.0, 1.0 and 0.5 mm. The samples were shaken with an aid of Endecotts Test

Sieve Shaker for 20 minutes. The sample mass recovered from each sieve was expressed as percent of the total sample used. Average particle size distribution of "coarse" and "fine" seed samples used in Experiments 2 and 3 are presented in Table 3.1.

Animals, Management, Sample Collection and Chemical Analysis

One-day-old male Ross-308 broiler chickens were obtained from a local commercial hatchery. The birds were held in electrically heated Jamesway battery brooders (James Mfg. Co., Mount Joy, PA) for a 4 d pre-experimental period, and were fed commercial chick starter crumbles (21% CP). On day 5, birds were fasted for 4 h, individually weighed, and randomly distributed among treatments. There were 5 birds per pen, and 16 (in Experiment 1) or 10 (in Experiments 2 and 3) replicate pens per treatment giving a total of 400 birds per each experiment. Experimental diets were fed for 14 days (5 to 18 d of age), and all diets were formulated to contain 3000 kcal/kg ME and 21% CP (Table 3.2). Birds had free access to water and feed, and were provided with continuous light. Body weight (**BW**) and feed intake were recorded on day 18 with pen as the experimental unit. Mean BW gain, feed intake, and feed conversion ratio (**FCR**) were calculated to determine growth performance. All animal procedures were conducted according to the guidelines of the Canadian Council on Animal Care (1993).

At the termination of Experiments 1 and 2, excreta samples from each pen were collected over a 3 h period and immediately frozen at -20ºC. Samples were then freeze-dried, finely ground and pooled to produce 4 replicates per treatment in Experiment 1 and 5 replicate samples in Experiment 2. The pooled excreta samples were then subjected to analyses of fat (AOAC, 920.39, 1990), NSP (only in Experiment 1, Slominski and Campbell, 1990) and chromic oxide contents (Williams et al., 1962). On Day 19, 16 birds

(Experiment 1) or 12 birds (Experiment 2) were randomly selected from each treatment, and killed by cervical dislocation. The contents of jejunum were collected, and were pooled to yield 8 replicate samples per treatment in Experiment 1 and 6 samples in Experiment 2. In addition, fresh digesta samples (1.5 g) were collected, centrifuged at 3,600 × *g* for 10 minutes, filtered through a filter cartridge containing Whatman 541 filter paper and viscosity of the filtrate was determined at 40˚C using the Brookfield digital viscometer (model DV-II+LV, Brookfield Engineering Laboratories, Stoughton, MA). In Experiment 2, the contents of ileum were also collected, freeze-dried, finely ground, and pooled to yield 2 replicates per treatment with each replicate representing digesta from 6 birds. The pooled ileal samples were then subjected to fat, NSP and chromic oxide analysis. In Experiment 3, 12 birds were randomly selected from each treatment on Day 19 and killed by cervical dislocation. Intestinal contents from the ileum were collected, freeze-dried, finely ground, and pooled to yield 3 replicate samples per treatment. The pooled samples were then subjected to analyses of fat and titanium dioxide contents (Lomer et al., 2000).

Statistical Analysis

All studies were set up as completely randomized designs, and data were tested by the GLM procedure of SAS program (version 9.1, SAS Institute Inc., Gary, NC). Results were presented as means ± SEM. Means were separated by Tukey's Honestly Significant Difference. All statements of significance are based on $P \leq 0.05$.

Table 3.1 Particle size distribution of flaxseed and flaxseed-corn mixture used in Experiments 2 and 3 (%)

Item	Particle size[1]			
	> 2.0 mm	2.0 - 1.0 mm	1.0 - 0.5 mm	< 0.5 mm
Flaxseed (Experiment 2)				
Coarsely ground	4.8	40.7	26.9	27.6
Finely ground	0.2	19.1	51.9	28.7
SEM	0.1	0.9	1.4	1.4
P	<0.001	<0.001	<0.001	0.592
Flaxseed/corn (Experiment 3)				
Coarsely ground	-[2]	14.6	46.1	39.3
Finely ground	-	1.7	33.5	64.9
SEM		0.3	2.1	2.0
P		<0.001	0.012	<0.001

[1]Quadruplicate samples were tested for particle size in Experiment 2 and triplicate samples in Experiment 3. No intact seeds were found in finely ground flaxseed samples used in both experiments, whereas 6.7% of intact seeds was found in coarsely ground sample used in Experiment 2, and 0.6% was found in coarsely ground flaxseed-corn sample used in Experiment 3. [2]Not present.

Table 3.2 Composition and calculated analysis of basal diets

Item	Experiment 1	Experiment 2	Experiment 3
Ingredient, % of diet			
Corn	44.9	43.9	47.4
Soybean meal	31.2	32.2	30.9
Flaxseed	15.0	15.0	15.0
Canola oil	1.9	1.7	0.9
Limestone	1.3	1.3	1.3
Dicalcium phosphate	1.5	1.5	1.5
Methionine	0.17	0.17	0.16
Wheat middlings/corn[1]	2.25	2.38	1.00
Mineral and vitamin premix[2]	1.50	1.50	1.50
Chromic oxide/Titanium dioxide[3]	0.30	0.30	0.30
Total	100.0	100.0	100.0
Calculated analysis			
CP, %	21.0	21.0	21.0
ME, kcal/kg	3000.0	3000.0	3000.0
Calcium, %	0.95	0.95	0.95
Nonphytate phosphorus, %	0.43	0.43	0.43
Methionine, %	0.51	0.51	0.51
Methionine + cystine, %	0.86	0.85	0.85
Lysine, %	1.16	1.16	1.14
Total NSP, mg/g[4]	109.1	95.2	107.3

[1]Wheat middlings were used as a carrier for enzyme supplement in Experiments 1 and 2, and corn in the Experiment 3. [2]Provided per kilogram of diet: Mn, 70 mg; Cu, 10 mg; Fe, 80 mg; Zn, 80 mg; Se, 0.3 mg; I, 0.5 mg; Na, 1.7 g; vitamin A, 8,255 IU; vitamin D_3, 3,000 IU; vitamin E, 30.0 IU; vitamin B_{12}, 0.013 mg; vitamin K, 2.0 mg; niacin, 24.5 mg; choline, 1,081 mg; folic acid, 4.0 mg; biotin, 0.25 mg; riboflavin, 6.0 mg. [3]Chromic oxide was used as an external marker in Experiments 1 and 2, and titanium dioxide in Experiment 3. [4]Determined values.

3.4 Results

The in vitro study (Table 3.3) demonstrated that enzyme addition resulted in a significant reduction of mucilage viscosity. The three enzyme combinations containing pectinase (C + P, P + XG, and C + P + XG) but not pectinase alone were found to be the most effective with C + P showing a viscosity reduction from 10.0 to 2.6 mPa·s. Therefore, this combination was used as a viscosity-reducing enzyme (Enzyme A) in a subsequent study (Experiment 1).

In Experiment 1 (Table 3.4), enzyme addition significantly improved FCR and increased total tract digestibilites of fat and NSP by an average of 2.8, 5.7 and 9.3%, respectively, in broiler chickens fed corn/SBM-based diets containing 15% flaxseed. When compared with 0.02% of enzyme addition, no further improvement in growth performance was noted for the 0.05% enzyme-containing diets, as well no difference in growth performance was observed between the two types of enzyme supplements used. Enzyme addition resulted in decreased digesta viscosity which was significant only at the 0.05% enzyme inclusion rate.

In Experiment 2, 41% of material in flaxseed samples was between 2.0 to 1.0 mm for the coarsely ground seeds, while 52% of material was between 1.0 to 0.5 mm for the finely ground seeds (Table 3.1). However, the main effect of particle size was not significant for all the parameters examined except that an increased ileal fat digestibility was noticed with birds fed diets containing finely ground flaxseed (Table 3.5). Enzyme addition significantly reduced FCR regardless of particle size or bile salt addition. This was accompanied by a decreased digesta viscosity as well as increased digestibilities of

NSP (by 6%) and fat (by 4.6% and 3.4% at the ileal and total tract levels, respectively). Bile salts addition had no effect on all the parameters studied except that it interacted with the particle size for feed intake. Regardless of enzyme addition, feed intake of birds fed diets containing the coarsely ground flaxseed increased following bile salt addition (coarse, no bile salt vs. coarse, bile salt: 674.0 vs. 698.7 g, P = 0.03), whereas no significant difference was found among birds fed the finely ground seed (680.3 vs. 664.1, P = 0.15). Feed intake and BW gain were not affected by either particle size or enzyme addition.

Using corn as a carrier facilitated the grinding process as both "coarsely" and "finely" ground seeds used in Experiment 3 were finer than their counterparts in Experiment 2 (Table 3.1). Among the pelleted diets, birds consuming diets originally containing whole, intact seeds showed a lower ileal fat digestibility than those fed the ground seeds (Table 3.6). However, no difference in growth performance was observed due to particle size among birds fed pelleted diets. When mash diets were fed, birds consumed less feed and gained less weight comparing with those fed the pelleted diets regardless of enzyme addition. The FCR value in birds fed mash diets was higher than that in pelleted diets containing finely ground flaxseeds, but were not significantly different from those fed diets containing whole or coarsely ground seeds. Enzyme addition decreased the feed intake without affecting BW gain. Consequently, FCR decreased significantly by 2.5% and ileal fat digestibility increased by 5.3% following enzyme addition, regardless of the processing method used.

Table 3.3 Viscosity reduction following incubation of the mucilage solution with different enzyme preparations

Treatment	Viscosity (mPa·s)
Control (no enzyme)	10.0[1a]
Cellulase (C)	4.0[c]
Pectinase (P)	4.4[bc]
Xylanase/glucanase (XG)	5.1[b]
C + P	2.6[d]
C + XG	4.1[c]
P + XG	2.7[d]
C + P + XG	2.7[d]
SEM	0.1
P	<0.001

[a,b]Means within a column with no common superscript differ significantly ($P < 0.05$).

[1]Mean of triplicate determination.

Table 3.4 Growth performance, digesta viscosity, and apparent total tract digestibilities of fat and NSP in broiler chickens fed diets containing different enzymes and their concentrations (5 to 18 d, Experiment 1)

Treatment[1]	Feed intake (g/bird)	BW gain (g/bird)	FCR (g of feed/ g of gain)	Viscosity (mPa·s)	Total tract digestibility (%)	
					Fat	NSP
Control (no enzyme)	686.9	459.4	1.496[a]	17.1[a]	53.9[b]	4.3[b]
Control + Enzyme A[2], 0.02%	690.9	478.3	1.446[b]	13.8[ab]	58.7[a]	14.5[a]
Control + Enzyme A, 0.05%	699.3	480.3	1.458[b]	10.0[b]	59.7[a]	12.1[a]
Control + Enzyme B[3], 0.02%	692.1	475.3	1.456[b]	12.4[ab]	61.0[a]	14.6[a]
Control + Enzyme B, 0.05%	687.2	471.3	1.458[b]	9.7[b]	59.1[a]	13.3[a]
SEM	9.1	7.1	0.009	1.5[4]	0.7	1.9
P	0.862	0.225	0.002	0.013	<0.001	0.003

[a,b]Means within a column with no common superscript differ significantly ($P < 0.05$). [1]Four hundred broiler chickens were randomly assigned to 5 treatments with 16 replicate pens per treatment and 5 birds per pen. On Day 18, excreta samples were collected from each pen and pooled to yield 4 replicates per treatment for digestibility determination. On Day 19, 16 birds per treatment were killed and jejunum digesta were collected and pooled to yield 8 replicate samples for viscosity determination. [2]Contained 300 U cellulase, 5,000 U pectinase, 3,950 U mannanase and some xylanase and glucanase side activities per g of premix. [3]Contained 200 U cellulase, 3,000 U pectinase, 3,300 U xylanase, 2,500 U glucanase, 2,700 U mannanase per g of premix. [4]One value in "Control + Enzyme A, 0.02%", "Control + Enzyme A, 0.05%" and "Control + Enzyme B, 0.05%" was missing and thus SEM=1.6 for these 3 treatments.

Table 3.5 Effects of seed particle size, enzyme and bile salts addition on growth performance, digesta viscosity, apparent ileal and total tract fat digestibilities and ileal NSP digestibility in broiler chickens (5 to 18 d, Experiment 2)

Effect[1]	Feed intake (g/bird)	BW gain (g/bird)	FCR (g of feed/ g of gain)	Viscosity (mPa·s)	Fat digestibility (%)		Ileal NSP digestibility (%)
					Total tract	Ileal	
Particle size × enzyme × bile salt							
Coarse (control)	676.7	480.0	1.411	9.2	83.8	78.5	12.0
Coarse + enzyme	671.2	486.6	1.381	7.3	85.4	83.9	13.6
Coarse + bile salt	697.9	491.6	1.421	12.5	80.7	73.6	5.0
Coarse + enzyme + bile salt	699.5	498.5	1.404	8.1	85.7	82.8	14.3
Fine (control)	669.7	473.5	1.414	9.3	82.6	83.8	10.7
Fine + enzyme[2]	690.9[u]	495.6[w]	1.397[y]	5.9	86.4	87.9	17.7
Fine + bile salt	668.8	475.9	1.405	8.8	82.7	86.5	6.8
Fine + enzyme + bile salt	659.4	470.1	1.404	7.8	85.8	86.1	13.0
SEM	10.9	9.7	0.011	1.2	1.3	2.0	3.7
Main effects							
Coarse	686.3	489.2	1.404	9.3	83.9	79.7	11.2
Fine	672.2[v]	478.8[x]	1.405[z]	8.0	84.3	86.1	12.0
No enzyme	678.3	480.3	1.413	9.9	82.4	80.6	8.6
Enzyme	680.3[v]	487.7[x]	1.396[z]	7.3	85.8	85.2	14.6
No bile salt	677.1[v]	483.9[x]	1.401[z]	7.9	84.5	83.5	13.5
Bile salt	681.4	484.1	1.409	9.3	83.7	82.2	9.7
SEM	5.4	4.8	0.005	0.6	0.6	1.0	1.9
Particle size	0.073	0.137	0.942	0.123	0.636	0.002	0.759
Enzyme	0.795	0.285	0.033	0.002	0.001	0.011	0.052
Bile salt	0.584	0.986	0.312	0.107	0.368	0.384	0.194
Particle size × enzyme	0.613	0.920	0.383	0.570	0.914	0.085	0.837
Particle size × bile salt	0.010	0.095	0.263	0.413	0.549	0.249	0.829

Enzyme × bile salt	0.450	0.322	0.354	0.996	0.452	0.875	0.529
Particle size × enzyme × bile salt	0.229	0.308	0.904	0.145	0.247	0.173	0.447

[1]Four hundred broiler chickens were randomly assigned to 8 treatments with 10 replicate pens per treatment and 5 birds per pen. On Day 18, excreta samples were collected from each pen and pooled to yield 5 replicates per treatment for fat digestibility determination. On Day 19, 12 birds per treatment were killed. The jejunum digesta were collected and pooled to yield 6 replicate samples for viscosity determination, whereas ileal digesta were collected and pooled to yield 2 replicate samples per treatment for fat and NSP analyses. [2]Growth performance result from 1 pen in this group was missing, and thus the SEM for [u], [v], [w], [x], [y], and [z] was 11.5, 5.5, 10.2, 4.9, 0.012, and 0.006, respectively.

Table 3.6 Effects of flaxseed particle size, enzyme addition and feed pelleting on growth performance and apparent ileal fat digestibility in broiler chickens (5 to 18 d, Experiment 3)

Effect[1]	Feed intake (g/bird)	BW gain (g/bird)	FCR (g feed/g gain)	Ileal fat digestibility (%)
Process × enzyme				
Whole seed, pelleted diet	732.4	500.6	1.464	69.6
Whole seed + Enzyme, pelleted diet	692.0	473.6	1.462	69.6
Coarsely ground seed, pelleted diet	706.0	471.6	1.498	85.3
Coarsely ground seed + Enzyme, pelleted diet	689.9	480.0	1.439	83.6
Finely ground seed, pelleted diet	703.4	480.3	1.465	76.9
Finely ground seed + Enzyme, pelleted diet	693.0	479.0	1.449	87.5
Finely ground seed, mash diet	600.9	391.4	1.537	76.6
Finely ground seed + Enzyme, mash diet	593.6	405.8	1.464	88.9
SEM	10.1	8.1	0.016	3.2
Main effects				
Whole seed, pelleted diet	712.2[a]	487.1[a]	1.463[ab]	69.6[b]
Coarsely ground seed, pelleted diet	697.9[a]	475.8[a]	1.468[ab]	84.4[a]
Finely ground seed, pelleted diet	698.2[a]	479.7[a]	1.457[b]	82.2[a]
Finely ground seed, mash diet	597.2[b]	398.6[b]	1.500[a]	82.7[a]
SEM	7.2	5.7	0.011	2.3
No enzyme	685.7	461.0	1.491	77.1
Enzyme	667.1	459.6	1.453	82.4
SEM	5.1	4.1	0.008	1.6
Process	<0.001	<0.001	0.036	0.001
Enzyme	0.012	0.810	0.001	0.035
Process × enzyme	0.355	0.064	0.082	0.106

[1]Four hundred broiler chickens were randomly assigned to 8 treatments with 10 replicate pens per treatment and 5 birds per pen. On Day 19, 12 birds per treatment were killed and ileal digesta were collected and pooled to yield 3 replicate samples for fat analysis.

[a,b]Means within a column and within a source with no common superscript differ significantly ($P < 0.05$).

3.5 Discussion

Earlier research from this laboratory has demonstrated that flaxseed contains a considerable amount of NSP with similar proportion of water-insoluble to water-soluble fractions (54 vs. 46%) (Manuscript IV). Water-solubility is an important characteristic which determines the antinutritive activities of NSP in broiler diets (Smits and Annison, 1996). In addition, the water-insoluble NSP components act as a physical barrier and encapsulate nutrients within the cotyledon cells. This has been substantiated in a TME_n assay with full-fat flax seeds showing a lower energy value than their corresponding meal and oil mixture (3.7 vs. 5.1 kcal/g) (Lee et al., 1995). On the other hand, the water-soluble fraction, including mucilage, was found to increase viscosity of intestinal contents of broiler chickens (Rodriguez et al., 2001; Alzueta et al., 2003). The results of the current study confirmed the literature data as the viscosity determined in the jejunum was high and ranged from 8.8 (Table 3.5) to 17.1 mPa·s (Table 3.4) in broiler chickens fed diets containing 15% flaxseed with no enzyme added. The variation in viscosity values observed in the current study may be due to the origin of flaxseed used. In this regard, the samples used in Experiment 1 and 3 were of food grade and most likely contained more NSP due to minimal contamination with foreign material than the feed grade sample used in Experiment 2. This seemed to be reflected in the higher NSP content of diets used in Experiments 1 and 3 (Table 3.2). The geographical regions of flaxseed may also contribute to this variation. Oomah et al. (1995) reported that the content of water-soluble polysaccharides ranged from 3.6% to 8.0% in 109 samples of flaxseed analyzed. In addition, mucilage isolated from different flax cultivars exhibited

different rheological properties with viscosity varying over a wide range from 0.02 to 0.28 Pa·s when using a 1% solution (Wannerberger et al.,1991). Ortiz et al. (2001) observed that increasing dietary flaxseed content in broiler chicken diets from 4 to 24% decreased the AME_n value from 3260 to -100 kcal/kg, which clearly indicated that flaxseed interfered with the utilization of energy from other dietary ingredients as well. Alzueta et al. (2003) reported that the inclusion of 16% flaxseed decreased digestibilities of total fatty acids from 89 to 60% and ALA from 74 to 43% in broiler chickens. The digestibility values were restored to 80 and 73% for total fatty acids and ALA, respectively, following removal of the mucilage fraction from flaxseed. The authors concluded that the presence of mucilage was responsible for impaired energy utilization from the flaxseed-containing diets.

The success of applying xylanase and β-glucanase to depolymerize the indigestible cell wall polysaccharides such as arabinoxylans in wheat or β-glucans in barley has stimulated interests in developing the NSP-degrading enzymes for oilseeds. In this context, understanding the substrate structure is critical for the development of an effective product. Flaxseed is known to contain pectic polysaccharides, which are probably the most complex class of cell wall polysaccharides. Pectic polysaccharides consist of a family of acidic rhamnogalacturonans and several neutral polysaccharides including arabinans, galactans, and arabinogalactans which are believed to be covalently attached to the rhamnogalacturonan backbone (Bacic et al. 1988). Pectic polysaccharides do not exist alone and they interconnect with cellulose and other polysaccharides within the cell wall matrix (Darvill et al., 1980). The information on the structure of the cell wall components of flaxseed, however, is limited except for mucilage. Mucilage is present in

the outermost layer of the hull and consists of two fractions, a neutral fraction composed of arabinoxylans and an acidic pectin-like fraction consisting of rhamnose, galactose and galacturonic acid residues (Cui et al., 1994). In agreement with our previous research (Slominski et al., 2006), the current in vitro study demonstrated that the reduction of viscosity was more pronounced when enzyme products were used in combination (Table 3.3), which is not surprising considering the complexity and heterogeneity of the flax cell wall polysaccharides. Although mucilage arabinoxylans have been suggested to be responsible for the viscous property of mucilage (Cui et al., 1994), Carré (2002) documented that a commercial xylanase preparation was much more effective in arabinoxylan depolymerization of wheat than flaxseed. It is of interest to note that in the current study enzyme combinations containing pectinase but not pectinase alone were most effective in viscosity reduction (Table 3.3). This would indicate that in addition to pectinase, other NSP-degrading enzymes are needed to facilitate the hydrolysis of mucilage.

Enzyme A composed of C + P decreased the viscosity of mucilage solution in vitro by 74%. The results of Experiment 1 (Table 3.4) demonstrated, however, that a relatively high inclusion rate of enzyme (i.e. 0.05%) was needed to reduce the viscosity of intestinal contents in vivo. Even though Enzyme A was used at 0.05%, the viscosity values of jejunum digesta still remained relatively high (10.0 mPa·s, Table 3.4) and much higher than the intestinal viscosity of 3.0 to 5.0 mPa·s observed in broiler chickens fed diets based on Canadian wheats (Meng et al., 2005). The reason for such a discrepancy in enzyme efficacy in vitro and in vivo is not clear. In the last few years, xylanase inhibitors in wheat have been found responsible for compromising the efficacy of exogenous

xylanase added to poultry diets (Ponte et al., 2004). However, no similar inhibitors have been reported for flaxseed. Nevertheless, such a response indicates that certain feed compounds or conditions in the gut may interfere with the activity of viscosity-reducing enzymes. Therefore, although fat digestibility increased following enzyme addition in all three experiments, the inhibition of fat utilization caused by digesta viscosity was not totally eliminated.

Enzyme B used in Experiment 1 has been previously demonstrated to be effective in cell wall polysaccharides depolymerization with a minimal effect on viscosity reduction (Slominski et al., 2006). However, no difference was observed between the two types of enzymes used in the current study (Table 3.4). Lack of difference was probably due to the fact that feed enzymes are crude preparations and would contain some side activities, including xylanase and glucanase, which were present in Enzyme A as well. Therefore, it is difficult to differentiate between the effects of enzymes on cell wall degradation or viscosity reduction. In subsequent experiments, the two enzyme preparations were used in concert and contained activities towards both viscosity reduction and disruption of the cell wall structure. This enzyme preparation was similar to that used in our earlier research (Slominski et al., 2006). The results showed that in addition to the decrease in viscosity, cell wall polysaccharide depolymerization by this enzyme combination was reflected in increased ileal digestibility of NSP (from 8.6 to 14.6%) (Table 3.5). Overall, the benefits of enzyme supplementation were reflected in the increased total tract fat digestibility by 3 to 6% and improved FCR by 1-3%. Such beneficial effects most likely resulted from the disruption of the cell wall structure allowing for a better access of digestive enzymes to their corresponding substrates (i.e.,

oil), and to certain degree, elimination of the constraints associated with intestinal viscosity and nutrient digestion and absorption.

Low concentration of bile salt in the intestinal contents is considered to be the most limiting factor in lipids utilization by young birds (Krogdahl, 1985). The efficacy of bile salts in lipid digestion and absorption may be further reduced under the conditions of high intestinal viscosity due to a reduced convection and diffusion or an increased microbial bile salts deconjugation (Edwards et al., 1988; Feighner and Dashkevicz, 1988) or both. Feeding psyllium (a gell-forming polymer that does not bind bile acids in vitro) to rats increased the excretion of fecal bile acids and total steroids as well as up-regulated bile acid biosynthesis (Buhman et al., 1998). Smits et al. (1998) also demonstrated that the addition of a nonfermentable gelling fiber (carboxymethylcellulose) decreased apparent lipid digestibility by reducing the concentration of bile acids in the chyme of broiler chickens. However, addition of 0.05% bile extract in the current study had no effects on fat digestibility (Table 3.5). This is in agreement with reports by Kussaibati et al. (1982) indicating that the addition of bile salts markedly improved the digestibility of long-chain saturated fatty acids in conventional birds, whereas its effect was marginal for long-chain unsaturated fatty acids. Such a response may be due to the fact that unsaturated fatty acids (e.g. ALA in flaxseed) are more effectively absorbed than the saturated ones. Garrett and Young (1975) found that although bile salts were important for optimum absorption, considerable amounts of long-chain unsaturated fatty acids can be absorbed even in the absence of bile salts (oleic acid, 86 to 94% vs. 35 to 46%, with or without bile salts). In the current study, addition of bile salts appeared to interact with the particle size but only for feed intake, which is difficult to explain.

Studies by Danicke et al. (1998) suggested that when full-fat canola seeds were fed to broiler chickens, appropriate grinding of the seed was critical for oil utilization. A marked increase in the fat digestibility and dietary AME_n was observed when the average particle size of seeds was decreased to ≤0.56 mm and the effect was more pronounced for broiler chickens than for laying hens. In addition, the whole and intact seeds may pass through the digestive tract undigested due to their small size. Results of the current study showed that particle size reduction via grinding increased ileal fat digestibility, but did not affect the growth performance of broiler chickens no matter if present in the mash (Table 3.5) or pelleted diets (Table 3.6). This would indicate that seed coat rupture is of primary importance more so than any further particle size reduction. However, it is of interest to note that processing tended to increase the susceptibility of diets to NSP-degrading enzymes (Table 3.6). Enzyme addition had no effect on FCR in birds fed pelleted diets containing whole flaxseed, whereas an improvement from 1 to 4% was observed in those fed pelleted diets containing ground flaxseed and the highest improvement of 5% was observed in those consuming the mash diets containing finely ground flaxseed (Process×Enzyme, P=0.08). In comparison with grinding, feed pelleting showed a pronounced effect on growth performance. It would appear that the pressure forcing feed ingredients leaving the conditioner through the holes in a pellet die and the steam imposed during the pelleting process may facilitate an effective rupture of the seed, disruption of the cell wall structure and thus better exposure of oil to digestive enzymes. In addition, some ANF present in flaxseed (i.e., trypsin inhibitors and enzymes responsible for cyanogenic glycosides conversion to toxic end products) could be inactivated by heat employed during the pelleting process (Feng et al., 2003).

In conclusion, high levels of NSP in flaxseed would have some antinutritional effects by causing a high intestinal viscosity and encapsulating dietary lipids. Particle size reduction via grinding had no significant effects on growth performance of broiler chickens no matter if present in the mash or pelleted diets. Therefore, fine grinding of the seed would not be recommended. When compared with grinding, pelleting had more pronounced and beneficial effects. Pelleting of diets containing intact seeds was found to be very effective in achieving an optimum growth performance indicating that the use of whole seeds in pelleted diets would be feasible. Enzyme addition may further improve fat digestibility and FCR of birds, particularly when used in mash diets. In conclusion, physical rupture of the seed and disruption of cell wall structure via diet pelleting and the addition of NSP-degrading enzyme offer practical solutions to improve the nutritive value of flaxseed.

3.6 Acknowledgements

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4. MANUSCRIPT II

The Effect of Enzyme Supplementation on Egg Production Parameters and Omega-3 Fatty Acid Deposition in Laying Hens Fed Flaxseed and Canola Seed

W. Jia*, B. A. Slominski*, W. Guenter*, A. Humphreys† and O. Jones‡

* Department of Animal Science, University of Manitoba, Winnipeg, Canada R3T 2N2;

† Nutreco Canada Inc., Winnipeg, Canada R3P 2P2;

‡ Canadian Bio-Systems Inc., Calgary, Canada T2C 0J7

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4.1 Abstract

An experiment was conducted to investigate the effects of a multicarbohydrase enzyme on egg production parameters, nutrient digestibility and egg fatty acids composition in Hy-Line CV-20 laying hens (39 to 63 wk of age) fed diets containing 150 g/kg of diet of canola seed, flaxseed, or Linpro (flaxseed : peas, 1:1 wt/wt). The diet effect on each parameter was also evaluated. Hens consuming the canola seed and Linpro diets had greater egg production, lower feed consumption, and therefore better feed conversion than those fed the flaxseed diets. Enzyme supplementation significantly increased ($P < 0.01$) egg production (from 78.0 to 80.9%) and improved ($P < 0.001$) feed conversion ratio (from 2.15 to 2.03) in hens fed flaxseed. Hens fed the canola seed and Linpro diets produced eggs with greater egg specific gravity than those from birds consuming flaxseed. Enzyme supplementation significantly increased egg specific gravity in hens fed flaxseed (from 1.0773 to 1.0800, $P < 0.01$) in phase I of the experiment. There was no effect of diet on fat digestibility, and similar fat digestibility values with enzyme supplementation were observed for canola seed (92.1 vs. 96.7%) and flaxseed (87.4 vs. 92.4%). Eggs produced by hens fed flaxseed had the highest n-3 fatty acids content (562 mg/60 g of egg) when compared with those from hens consuming canola seed (207 mg/60 g of egg) or Linpro (427 mg/60 g of egg). Enzyme supplementation increased the egg n-3 content for the flaxseed diet (from 546 to 578 mg/60 g of egg; $P = 0.01$) and for the Linpro diet (from 415 to 438 mg/60 g of egg; $P = 0.05$). In addition, enzyme addition increased the egg docosahexaenoic acid content from 91.8 to 101.9 mg/60 g of egg ($P < 0.01$) and from 89.4 to 96.8 mg/60 g of egg ($P = 0.01$)

for the flaxseed and Linpro diets, respectively. When compared with canola seed, long-term feeding of flaxseed to laying hens resulted in reduced egg production and eggshell quality. Enzyme supplementation had positive effects on feed utilization, egg shell quality, and n-3 fatty acids deposition in the egg.

Key words: laying hen, flaxseed, canola seed, Linpro, omega-3 egg

4.2 Introduction

Flaxseed and canola seed provide a convenient package for the high-quality protein, vitamins, available phosphorus, and, more importantly, the high-quality oil rich in α-linolenic acid (**ALA**). Flaxseed is being widely used in laying hen nutrition for n-3-enriched egg production, whereas canola seed and off-grades of canola seed are financially attractive and are becoming an interesting alternative to animal fats in poultry and swine feeding programs. Flax oil contains 48-58% of n-3 fatty acids, and it has been indicated that an increase of flaxseed in laying hen diets by 1% would result in an increase in n-3 fatty acids deposition by 40 mg per egg (Leeson and Summers, 2005). However, published research data on the effect of flaxseed on egg production parameters has been inconsistent. Jiang et al. (1991) and Caston et al. (1994) reported no effect of flaxseed on egg production, whereas Aymond and Van Elswyk (1995) suggested that flaxseed addition may have negative effects. There is also some evidence, and belief within the feed industry, that egg production could be significantly reduced in layer flocks when flaxseed inclusion rate is greater than 8% (Leeson et al., 2000). This has been attributed to low nutrient utilization or the response of birds to antinutritional factors (**ANF**) such as mucilage, cyanogenic glycosides or trypsin inhibitors (Bhatty, 1995).

Mucilage, a water-soluble nonstarch polysaccharide (**NSP**), significantly increases the intestinal viscosity, which has been shown to have a negative effect on nutrient digestion and absorption (Choct and Annison, 1992; Rodriguez et al., 2001). In addition, the decreased energy utilization from flaxseed-containing diets has been reported to result from incomplete rupture of the seed and nutrient encapsulation by the cell wall structure (Slominski et al., 2006). Careful examination of the literature concerned with the feeding of full-fat flaxseed and canola seeds clearly demonstrates that bird performance and nutrient utilization is affected more so by insufficient seed processing and ineffective cell opening for optimal nutrient utilization than by any negative effects associated with ANF.

A common characteristic of flax and canola is the small seed size, which limits nutrient utilization, because there is no satisfactory practical grinding-processing technology available for an effective rupture of the tissue structure. Furthermore, the nutrient encapsulating effect of the cell walls can not be overcome by monogastric animals, because they lack enzymes to digest the polysaccharides of the cell wall. Research studies, including our own data (Jiang, 1999), support this concept. As reported by Lee et al. (1995), availability of oil from canola seed has been questioned, because metabolizable energy values were lower than those for corresponding canola meal plus canola oil mixtures. This was further substantiated in the broiler chicken study in which poorer feed conversion, lower AME_n content (2,963 vs. 3200 kcal/kg) and lower ileal fat (65.6 vs. 85.6%) and protein (75.6 vs. 81.2%) digestibilities were observed for the canola seed diet compared with the canola meal plus canola oil diet (Meng et al., 2006). A highly significant effect of particle size on apparent digestibility of nutrients by broiler chickens and laying hens fed full-fat canola seed has also been reported (Danicke et al.,

1998). In a similar study with full-fat flaxseed, it was demonstrated that various seed rupture processes such as pelleting, autoclaving, or microwave roasting had a significant effect on fat and energy utilization, with TME_n values of flaxseed increasing by 22-25% (Shen et al., 2004). In addition to seed rupture, the advantage of using heat treatment would be that some heat-labile ANF (i.e., trypsin inhibitors, cyanogenic glycosides) can be inactivated during feed processing (Feng et al., 2003). The mucilage and the intact NSP associated with the cell wall structure, however, may still pose a problem.

Oilseeds are often added to poultry diets after grinding. Under the commercial conditions, high-diameter sieves (i.e., 4 mm) are used for seed processing to avoid sieve plugging due to high oil content. However, when the seeds are premixed with cereal grains to overcome this problem, the grinding may still be insufficient for an effective rupture of the seed structure. In addition, seed grinding before diet preparation would accelerate lipid oxidation and would result in a short shelf life of diets. Therefore, it would be advantageous to feed whole seed in form of a pelleted diet, because this would eliminate the grinding cost and would reduce the potential for lipid oxidation during storage. The potential drawback could be a low deposition of n-3 fatty acids due to the cell wall physical barrier for effective nutrient utilization (Aymond and Van Elswyk, 1995).

Earlier research from this laboratory has demonstrated that the use of a multicarbohydrase enzyme containing cell wall-degrading and viscosity-reducing activities can improve oil utilization from full-fat oilseeds in broiler chickens and adult roosters (Meng et al., 2006; Slominski et al., 2006). However, the effectiveness of

enzyme addition on egg production and n-3 fatty acids deposition in hens fed oilseeds has not yet been investigated.

Therefore, the objective of this study was to evaluate the effects of a multicarbohydrase enzyme cocktail on performance parameters, egg fatty acids deposition and nutrient digestibility in hens fed diets containing canola seed, flaxseed, and a Linpro product (Werner Agra Ltd., Regina, Saskatchewan, Canada; flaxseed:peas, 1:1 wt/wt; ground-extruded). Pelleting and crumbling of the diets was chosen as a means of rupturing the seeds for effective nutrient utilization.

4.3 Materials and Methods

Birds and Housing

Six hundred and forty-eight Hyline CV-20 laying hens were kept in confinement housing under semicontrolled environmental conditions and were exposed to a 16-h photoperiod. Feed and water were available ad libitum. Each dietary treatment was replicated six times and each replicate consisted of 18 adjacently caged birds (6 cages of 3 birds each) fed as a group with a total of 108 hens per treatment. The cage dimensions were 30.5 × 40.6 cm, providing 413 cm^2 per bird. The birds were fed experimental diets through the production peak (39 to 63 wk of age) consisting of 2 phases and three 28-d periods in each phase. All birds were weighed individually at the start and the end of the experiment. Egg production was recorded daily and calculated as hen-day production. Feed consumption was measured on a 28-d basis and calculated as a mean for each 18-bird replicate. Eggs were weighed for 3 consecutive days in the middle of each period. All animal procedures were conducted according to the guidelines of the Canadian

Council on Animal Care (1993). The protocol for this study was approved by the Animal Care Protocol Review Committee of the University of Manitoba.

Diets

Birds were randomly assigned to 6 test diets, containing 150 g/kg diet of canola seed, flaxseed, or Linpro (flaxseed:peas, 1:1 wt/wt; ground-extruded) each without and with a multicarbohydrase enzyme addition (Superzyme OM; Canadian Bio-systems Inc., Calgary, Alberta, Canada; Table 4.1). The enzyme preparation supplied 1,100 U pectinase, 50 U cellulase, 1000 U xylanase, 600 U glucanase, 400 U mannanase, and 50 U galactanase per kilogram of diet. Intact flax and canola seeds were added to the diets, and all diets were pelleted and crumbled. The diets were examined in the laboratory and no intact seeds were found.

Sample Collection and Chemical Analysis

At 43 and 63 wks, eggs were collected for specific gravity measurements using the saline flotation method (Holder and Bradford, 1979). Five eggs per cage unit and 30 eggs per treatment were collected at 51 wk, and yolks from 10 eggs were pooled to yield 3 replicates per treatment. The yolk samples were frozen at -20ºC, freeze-dried, and finely ground before fatty acid analysis by the Lipid Analytical Laboratories, University of Guelph. The weights of fresh eggs as well as the weights of yolks before and after freeze-drying were recorded. At 59 wk, excreta samples from 6 cage units per treatment (108 birds) were collected over a 3-h period and immediately frozen at -20ºC. The samples were freeze-dried, finely ground and pooled to produce 3 replicates per treatment. The pooled samples were then subjected to analysis of acid insoluble ash (**AIA**), which served as an internal marker (McCarthy et al., 1974), fat (AOAC, 1990; 920.39) and NSP

content. Total NSP were determined by gas-liquid chromatography (component neutral sugars) and by colorimetry (uronic acids) using the procedure described by Englyst and Cummings (1988) with some modifications (Slominski and Campbell, 1990). At the end of the study, 12 hens (2 hens per replicate) were randomly selected from each treatment group and killed by cervical dislocation. The contents of jejunum (from the end of the duodenum to Meckel's diverticulum) were collected and pooled from 2 birds to yield 6 replicate samples per treatment. Fresh digesta were centrifuged at 3,600 × *g* for 10 min, and viscosity of the supernatant was determined at 40ºC using the Brookfield digital viscometer (model DV-II+LV, Brookfield Engineering Laboratories, Stoughton, MA).

Calculations and Statistical Analysis

The following equation was used for calculation of the digestibility of fat and NSP (using fat calculation as an example):

Fat digestibility (%) = $\{1- [(AIA_{\%diet} / AIA_{\%excreta}) \times (Fat_{\%excreta} / Fat_{\%diet})]\} \times 100$

A 3 × 2 factorial arrangement of a completely randomized design was used for statistical analysis. Experimental unit was a replicate, previously defined as 18 adjacently caged birds fed as 1 group. All of the statistical analysis was conducted by the SAS program (version 9.1, SAS Institute Inc., Cary, NC). Digestibility of nutrients, digesta viscosity, and fatty acid profile parameters were analyzed by the GLM procedure. Main effects of diet and enzyme and the interaction between diet and enzyme were tested. Performance parameters (feed consumption, egg production, feed conversion ratio, egg weight, hen weight, and specific gravity) from each phase were analyzed as repeated measures by the MIXED procedure. This model included main effects (diet, enzyme, and phase) and the associated 2- and 3-way interactions (diet × enzyme, diet × phase, enzyme

× phase, diet × enzyme × phase). Means were separated by Tukey's honestly significant difference. Contrasts of enzyme effect (i.e., without vs. with enzyme addition) within each diet type and phase were made. All statements of significance are based on $P < 0.05$.

Table 4.1 Composition and calculated analysis of experimental diets

	Canola seed diet		Flaxseed diet		Linpro diet	
Item	Phase I	Phase II	Phase I	Phase II	Phase I	Phase II
Ingredient, % of diet						
Wheat	42.4	42.2	42.2	42.8	42.2	44.6
Barley	10.0	10.0	10.0	10.0	10.0	10.0
Soybean meal	4.8	5.0	4.2	4.5	4.3	5.0
Canola seed	15.0	15.0	-	-	-	-
Flaxseed	-	-	15.0	15.0	-	-
Linpro	-	-	-	-	15.0	15.0
Wheat millrun	8.1	10.0	8.7	10.0	8.4	7.6
Porcine meat meal	7.5	7.5	7.5	7.5	7.5	7.5
Limestone	7.2	7.6	7.3	7.6	7.3	7.7
Shell and bone meal	1.3	1.3	1.3	1.3	1.3	1.3
Dicalcium phosphate	0.5	0.2	0.5	0.2	0.5	0.2
Mineral and vitamin premix[1]	0.25	0.25	0.25	0.25	0.25	0.25
Salt	0.25	0.26	0.25	0.27	0.25	0.28
$_{DL}$-Methionine	0.04	0.05	0.06	0.06	0.06	0.08
Choline (70%)	0.03	0.03	0.03	0.03	0.03	0.03
Calculated analysis						
CP, %	19.0	18.0	19.0	18.0	19.0	18.0
ME, kcal/kg	2800.0	2800.0	2800.0	2800.0	2800.0	2800.0
Calcium, %	4.00	4.10	4.00	4.10	4.00	4.10
Nonphytate phosphorus, %	0.44	0.38	0.44	0.38	0.44	0.38
Methionine, %	0.36	0.33	0.37	0.34	0.38	0.35
Methionine + cystine, %	0.70	0.66	0.70	0.66	0.70	0.66
Lysine, %	0.79	0.79	0.73	0.73	0.78	0.78
Sodium, %	0.17	0.17	0.17	0.17	0.17	0.17

[1]Provided the following per kilogram of diet: Mn, 137.9 mg; Cu, 18.3 mg; Fe, 212.8 mg; Zn, 123.5 mg; Se, 0.3ppm; I, 0.6 mg; vitamin A, 9,000 IU; vitamin D_3, 3,000 IU; vitamin E, 27.0 IU; riboflavin 6.3 mg; pantothenic acid, 17.1 mg; niacin, 93.3 mg; folic acid, 1.4 mg; vitamin B_{12}, 0.02 mg; menadione, 2.2 mg.

4.4 Results

Egg Production Parameters

Diet had a significant effect on all the production parameters measured (Table 4.2). Hens fed the canola seed diet consumed less feed than those fed flaxseed and Linpro (94.2, 100.1, and 98.5 g/hen per day, respectively) with a similar effect observed for each phase. There was no significant difference in feed consumption between the flaxseed and Linpro diets, except for phase II, in which greater consumption of the flaxseed diet was observed. Enzyme supplementation of canola seed and flaxseed diets resulted in lower feed consumption in phase II and over the entire trial, with no significant effect in phase I. No significant effects of diet on egg production were observed until phase II, at which time hens consuming flaxseed had lower egg production compared with those consuming canola seed or Linpro. Enzyme supplementation had a more pronounced effect on egg production in hens fed flaxseed than the other diets, with a significant increase in egg production during phase II and over the entire trial. There was no difference in egg weight between any of the dietary treatments in phase I of the experiment. In phase II, eggs from the flaxseed group were heavier than those from the canola seed group but not different from those of the Linpro treatment. Enzyme had no significant effect on egg size except for hens fed the flaxseed diet, who laid slightly smaller eggs after enzyme addition in phase II of the experiment. As a result, hens consuming canola seed diets had better feed conversion than those consuming flaxseed during each phase. Hens consuming Linpro diets had superior feed conversion to those consuming flaxseed diets in phase II, and no significant difference was noted in phase I. The use of enzyme

significantly improved feed conversion in the flaxseed diet during each phase and over the entire trial but had no effects on canola seed and Linpro diets.

Overall, feeding flaxseed (150g/kg of diet) negatively affected egg production parameters. During each phase, hens consuming the Linpro diets achieved similar egg production and feed conversion as those consuming canola seed diets. The effect of enzyme supplementation was more pronounced in hens fed flaxseed compared with those fed the canola seed or Linpro diets. The overall egg production increased from 78% to 81%, and feed conversion ratio improved from 2.15 to 2.03 after enzyme addition.

Hen Weight and Eggshell Quality

As shown in Table 4.3, flaxseed-fed hens were significantly lighter at the end of the trial than those from the other treatments. Enzyme supplementation had no effect on hen weight.

Eggs produced from hens fed flaxseed had consistently the lowest shell quality (Table 4.3). Adding enzyme to the flaxseed diet significantly increased the specific gravity measurements in phase I, but not in phase II. Eggs from Linpro-fed hens had an intermediate shell quality between those from canola and flaxseed-fed hens only in phase I of the experiment, whereas in phase II, egg specific gravity from Linpro-fed hens was similar to that from canola diet, which was probably due to the adaptation of the birds to the flaxseed component of the Linpro diet.

Table 4.2 The effect of diet and enzyme supplementation on egg production parameters

Effect	Feed consumption (g/hen per day)			Egg production (%)			Egg weight (g)			Feed conversion ratio (g of feed/g of egg)		
	Phase I	Phase II	Overall	Phase I	Phase II	Overall	Phase I	Phase II	Overall	Phase I	Phase II	Overall
Canola seed	96.2	94.6[a]	95.4[a]	84.3	82.7	83.5	59.0	60.9	59.9	1.940	1.880	1.910
Canola seed + enzyme	94.6	91.7[b]	93.1[b]	85.2	81.9	83.5	58.9	60.5	59.7	1.891	1.851	1.871
Flaxseed	100.6	101.7[a]	101.1[a]	82.8	73.2[b]	78.0[b]	59.1	62.4[a]	60.8	2.060[a]	2.231[a]	2.146[a]
Flaxseed + enzyme	99.4	98.8[b]	99.1[b]	83.9	77.9[a]	80.9[a]	59.7	61.3[b]	60.5	1.989[b]	2.073[b]	2.031[b]
Linpro	100.0	98.1	99.1	85.7	83.7	84.7	58.4	60.9	59.6	2.005	1.929	1.967
Linpro + enzyme	99.0	96.9	98.0	85.6	83.7	84.6	59.2	61.1	60.2	1.957	1.896	1.927
Pooled SEM	0.7		0.7	0.8		0.7	0.3		0.3	0.023		0.020
Diet												
Canola seed	95.4[b]	93.1[c]	94.2[b]	84.8	82.3[a]	83.5[a]	58.9	60.7[b]	59.8[b]	1.915[b]	1.866[b]	1.890[c]
Flaxseed	100.0[a]	100.2[a]	100.1[a]	83.3	75.5[b]	79.4[b]	59.4	61.8[a]	60.6[a]	2.025[a]	2.152[a]	2.089[a]
Linpro	99.5[a]	97.5[b]	98.5[a]	85.6	83.7[a]	84.7[a]	58.8	61.0[ab]	59.9[ab]	1.981[ab]	1.913[b]	1.947[b]
Pooled SEM	0.5		0.5	0.6		0.4	0.2		0.2	0.016		0.014
Diet	<0.0001			<0.0001			0.0355			<0.0001		
Enzyme	0.0026			0.1120			0.9238			0.0004		
Diet × enzyme	0.6702			0.0721			0.3861			0.1128		
Phase	<0.0001			<0.0001			<0.0001			0.7317		
Diet × phase	0.0001			<0.0001			0.0277			<0.0001		
Enzyme × phase	0.0289			0.3649			0.0001			0.3312		
Diet × enzyme × phase	0.3753			0.0172			0.0081			0.0302		

[a-c]Means within a column and within a source with no common superscript differ significantly ($P < 0.05$).

Table 4.3 The effect of diet and enzyme supplementation on hen weight and egg shell quality

Effect	Hen weight (kg)		Egg specific gravity	
	Start	Finish	Phase I	Phase II
Canola seed	1.55	1.61	1.0832	1.0763
Canola seed + enzyme	1.58	1.64	1.0828	1.0773
Flaxseed	1.57	1.56	1.0773[b]	1.0723
Flaxseed + enzyme	1.61	1.56	1.0800[a]	1.0725
Linpro	1.58	1.63	1.0802	1.0782
Linpro + enzyme	1.57	1.63	1.0812	1.0778
Pooled SEM	0.02		0.0006	
Diet				
Canola seed	1.56	1.63[a]	1.0830[a]	1.0768[a]
Flaxseed	1.59	1.56[b]	1.0787[c]	1.0724[b]
Linpro	1.57	1.63[a]	1.0807[b]	1.0780[a]
Pooled SEM	0.01		0.0004	
Diet	0.1792		<0.0001	
Enzyme	0.1431		0.0777	
Diet × enzyme	0.4735		0.4174	
Phase	0.0003		<0.0001	
Diet × phase	<0.0001		<0.0001	
Enzyme × phase	0.3092		0.1002	
Diet × enzyme × phase	0.4570		0.0104	

[a-c]Means within a column and within a source with no common superscript differ significantly ($P < 0.05$).

Digesta Viscosity and Total Tract Fat and NSP Digestiblities

Hens consuming flaxseed diets had the greatest jejunal digesta viscosity, whereas those fed canola seed had the lowest (Table 4.4). Diet had no significant effect on either NSP or fat digestibilities (Table 4.4). The NSP digestibility in hens fed flaxseed increased from 12 to 24% after enzyme supplementation, whereas NSP digestibility in hens fed canola seed or Linpro was not affected by enzyme addition. No statistically significant effect of enzyme addition on fat digestibility was noted.

Egg Fatty Acid Profile

There was no difference in total fatty acid content among the treatments, although the fatty acid profile changed significantly (Tables 4.5 and 4.6). When compared with canola seed, incorporation of flaxseed and Linpro into the diets increased the contents of saturated fatty acids (**SFA**) and total n-3 fatty acids and decreased monounsaturated fatty acids (**MUFA**). The content of n-6 fatty acids was not affected by dietary treatments; hence, n-6:n-3 fatty acid ratio was significantly lower in eggs produced by hens fed the flaxseed and Linpro. Eggs laid by the flaxseed group contained the greatest total n-3 fatty acids and the lowest MUFA. The decrease in egg MUFA content was mainly attributed to the decrease in oleic acid ($C_{18:1n-9}$), although the palmitoleic acid ($C_{16:1n-7}$) content increased when compared with that of the canola seed diets. Deposition of ALA ($C_{18:3n-3}$), eicosapentaenoic acid (**EPA**, $C_{20:5n-3}$), and docosahexaenoic acid (**DHA**, $C_{22:6n-3}$) was significantly greater for the flaxseed-containing diets than that of the canola seed diet. As a result, the n-6:n-3 ratio differed significantly (Table 4.6).

When compared with the flaxseed diet, eggs produced by hens fed Linpro contained similar amounts of SFA and n-6 fatty acids, greater MUFA, and lower n-3 fatty

acid content. The DHA content in eggs from Linpro-fed hens was similar to that of the flaxseed diet, but the ALA and EPA contents were lower, which contributed to a much greater n-6:n-3 ratio (1.71).

Enzyme had no effect on fatty acid deposition in eggs produced by hens fed canola seed. For the flaxseed diet, enzyme addition increased the egg DHA content from 91.8 to 101.9 mg/60 g of egg and tended to increase the contents of ALA ($P = 0.06$) and EPA ($P = 0.09$), hence significantly increasing the total n-3 fatty acid content from 546 to 578 mg/60 g of egg. For the Linpro group, EPA significantly increased from 6.5 to 7.2, DHA from 89.4 to 96.8, and total n-3 fatty acids from 415 to 438 mg/60 g of egg after enzyme supplementation. As well, the total fatty acids and stearic and oleic acids increased after enzyme supplementation.

Table 4.4 The effect of diet and enzyme supplementation on digesta viscosity and total tract nonstarch polysaccharide (NSP) and fat digestibilities

Effect	Viscosity (mPa·s)	Digestibility (%)	
		Fat	NSP
Canola seed	5.05	92.1	16.2
Canola seed + enzyme	4.96	96.7	21.6
Flaxseed	18.59	87.4	12.1[b]
Flaxseed + enzyme	19.09	92.4	24.2[a]
Linpro	10.79	97.0	22.4
Linpro + enzyme	9.00	94.2	24.9
Pooled SEM	0.86	2.4	2.8
Diet			
Canola	5.01[c]	94.4	18.9
Flaxseed	18.84[a]	89.9	18.1
Linpro	9.89[b]	95.6	23.7
Pooled SEM	0.61	1.7	2.0
Diet	<0.0001	0.0759	0.1519
Enzyme	0.5456	0.2668	0.0134
Diet × enzyme	0.4387	0.2171	0.2627

[a-c]Means within a column and within a source with no common superscript differ significantly ($P < 0.05$).

Table 4.5 The effect of diet and enzyme supplementation on egg fatty acid composition (mg/60 g of egg)

Effect	Palmitic ($C_{16:0}$)	Palmitoleic ($C_{16:1}$)	Stearic ($C_{18:0}$)	Oleic ($C_{18:1}$)	Linoleic ($C_{18:2n6}$)	Linolenic ($C_{18:3n3}$)	Arachidonic ($C_{20:4n6}$)	EPA[1] ($C_{20:5n3}$)	DHA[2] ($C_{22:6n3}$)	TFA[3]
Canola seed	1043.11	89.15	318.41	2085.96	674.83	115.76	68.97	2.17	81.87	4549.32
Canola seed + enzyme	1028.78	89.34	331.92	2142.91	662.77	105.85	70.85	1.90	84.00	4588.34
Flaxseed	1046.53	112.71	391.86	1742.29	667.14	419.88	44.20	8.46	91.82^{b}	4599.32
Flaxseed + enzyme	1068.46	112.16	405.08	1729.81	672.56	438.06	45.97	8.87	101.89^{a}	4661.91
Linpro	1080.91	125.36	381.02^{b}	1792.36^{b}	656.93	297.72	48.97	6.51^{a}	89.43^{b}	4550.85^{b}
Linpro + enzyme	1117.72	124.26	409.07^{a}	1875.72^{a}	668.00	311.36	50.96	7.20^{b}	96.80^{a}	4734.56^{a}
Pooled SEM	14.95	2.72	5.67	20.06	14.18	6.08	0.95	0.16	1.82	47.41
Diet										
Canola seed	1035.95^{b}	89.25^{c}	325.17^{b}	2114.43^{a}	668.80	110.81^{c}	69.91^{a}	2.03^{c}	82.94^{b}	4568.83
Flaxseed	1057.50^{b}	112.44^{b}	398.47^{a}	1736.05^{c}	669.85	428.97^{a}	45.08^{c}	8.67^{a}	96.85^{a}	4630.62
Linpro	1099.32^{a}	124.81^{a}	395.05^{a}	1834.04^{b}	662.47	304.54^{b}	49.97^{b}	6.85^{b}	93.12^{a}	4642.71
Pooled SEM	10.57	1.92	4.00	14.19	10.03	4.30	0.67	0.11	1.29	33.52
Diet	0.0036	<0.0001	<0.0001	<0.0001	0.8551	<0.0001	<0.0001	<0.0001	<0.0001	0.2848
Enzyme	0.2485	0.8294	0.0019	0.0232	0.9005	0.1673	0.0328	0.0568	0.0009	0.0302
Diet × enzyme	0.2523	0.9718	0.3580	0.0852	0.7041	0.0837	0.9930	0.0306	0.1279	0.2982

[a-c]Means within a column and within a source with no common superscript differ significantly ($P < 0.05$). [1]EPA = eicosapentaenoic acid. [2]DHA = docosahexaenoic acid. [3]TFA = total fatty acids.

Table 4.6 The effect of diet and enzyme supplementation on egg saturated, monounsaturated, n-3, n-6 and n-6:n-3 ratio (mg/60 g of egg)

Effect	SFA[1]	MUFA[2]	n-3[3]	n-6[4]	n-6:n-3
Canola seed	1379.06	2197.26	211.49	761.51	3.60
Canola seed + enzyme	1379.58	2254.14	203.12	751.50	3.70
Flaxseed	1457.13	1870.53	545.73^{b}	725.93	1.33
Flaxseed + enzyme	1493.35	1857.21	578.04^{a}	733.32	1.27
Linpro	1482.16^{b}	1932.28^{b}	415.44^{b}	720.97	1.74
Linpro+ enzyme	1547.82^{a}	2013.78^{a}	438.48^{a}	734.49	1.67
Pooled SEM	18.21	21.78	6.69	15.00	0.03
Diet					
Canola seed	1379.32^{b}	2225.70^{a}	207.31^{c}	756.51	3.65^{a}
Flaxseed	1475.24^{a}	1863.87^{c}	561.88^{a}	729.63	1.30^{c}
Linpro	1514.99^{a}	1973.03^{b}	426.96^{b}	727.73	1.71^{b}
Pooled SEM	12.88	15.40	4.73	10.61	0.02
Diet	<0.0001	<0.0001	<0.0001	0.1425	<0.0001
Enzyme	0.0405	0.0371	0.0142	0.7717	0.7427
Diet × enzyme	0.2412	0.1193	0.0252	0.7246	0.0472

[a-c]Means within a column and within a source with no common superscript differ significantly ($P < 0.05$). [1]SFA = saturated fatty acids; include $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{18:0}$, $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$. [2]MUFA = monounsaturated fatty acids; include $C_{14:1}$, $C_{16:1}$, $C_{18:1}$, $C_{20:1}$, $C_{22:1}$ and $C_{24:1}$. [3]n-3 fatty acids; include $C_{18:3n3}$, $C_{18:4n3}$, $C_{20:3n3}$, $C_{20:4n3}$, $C_{20:5n3}$, $C_{22:5n3}$, and $C_{22:6n3}$. [4]n-6 fatty acids; include $C_{18:2n6}$, $C_{18:3n6}$, $C_{20:2n6}$, $C_{20:3n6}$, $C_{20:4n6}$, $C_{22:2n6}$, $C_{22:4n6}$, and $C_{22:5n6}$.

4.5 Discussion

The utilization of flaxseed in laying hen diets to produce n-3-enriched eggs has become a common practice in the table egg industry (Gonzalez-Esquerra and Leeson, 2001). A potential drawback of feeding flaxseed could be the negative affects of its various components on egg production. In fact, the present study demonstrated that feeding flaxseed at 150 g/kg of diet resulted in increased feed consumption and reduced egg production, feed conversion, and hen weight when compared with canola seed (Table 4.2). Lee et al. (1995) reported that although canola seed and flaxseed have similar contents of energy-contributing components, the AME_n and TME_n of flaxseed (3.75 and 3.75 kcal/g) were, respectively, lower ($P < 0.05$) than those of canola seed (4.46 and 4.56 kcal/g). They suggested that the seed size, hull coat thickness, high fiber, and other ANF may affect energy utilization. It is a well-known fact that energy utilization could be reduced due to the nutrient-encapsulating effect of the cell wall-NSP (Bedford, 2002). In this context, the total NSP content of flaxseed meal has been reported to average 271 g/kg (Slominski et al., 2006) and was much greater than that of canola meal (171 g/kg; Meng et al., 2005). In addition, water-soluble mucilage present in the hull fraction of flaxseed is known to significantly increase the intestinal viscosity (Rodriguez et al., 2001; Alzueta et al., 2003). Mucilage consists of 2 fractions, a neutral fraction composed of arabinoxylans and an acidic pectic-like fraction consisting of polysaccharides containing rhamnose, galactose and galacturonic acid residues. The arabinoxylans are the major components responsible for the viscous properties of mucilage (Cui et al., 1994), which, similarly to viscous polysaccharides of cereal grains, could decrease nutrient availability by

increasing the rate of feed passage and reducing the rates of diffusion of endogenous enzymes and nutrients (Classen and Bedford, 1991). Ortiz et al. (2001) concluded that deleterious components of flaxseed interact with dietary nutrients of flax-based diets thereby decreasing energy (AME_n) utilization. This could explain an increase in feed consumption observed in the current study to compensate for low nutrient availability. In addition, the depressed egg production and body weight have been indicated to result from inadequate AME_n content of the flaxseed-containing diets (Leeson et al., 2000). In general, published research data on the effect of flaxseed on laying hens performance is inconsistent. Bean and Leeson (2003) suggested that duration of the trial could affect performance. Among the long-term studies, similar to our data, responses in feed consumption and hen weight were reported by Caston et al. (1994), Leeson et al. (2000), and Novak and Scheideler (2001), whereas a decrease in feed consumption was reported by Bean and Leeson (2003) for flaxseed-containing diets. Lower egg production observed in the current study is in agreement with the research data by Leeson et al. (2000) but in disagreement with findings by Caston et al. (1994), Novak and Scheideler (2001), and Bean and Leeson (2003), who reported no adverse effect of flaxseed addition, up to 20% of the diet, on egg production. Differences in diet composition or strain of hens could be partially responsible for such conflicting results. In the current study, eggs laid by hens consuming flaxseed were slightly but significantly heavier ($P = 0.047$) than those from birds fed canola seed (60.6 vs. 59.8 g). It has been demonstrated that there is a relationship between increased egg size and increased protein-amino acid intake (Waldroup and Hellwig, 1995). As discussed earlier, hens fed the flaxseed diets increased feed consumption to compensate for low energy utilization compared with those fed the

canola seed diets. As a result, protein-amino acid intake increased as well, resulting in a bigger egg size.

Egg specific gravity has been used extensively as a measure of shell strength (Holder and Bradford, 1979). Because the egg contents (yolk and albumen) maintain a constant specific gravity, any difference in egg specific gravity relates to the amount of calcium deposition (shell thickness). In the current study, eggs from hens fed flaxseed had consistently the lowest specific gravity (Table 4.3). Therefore, it is possible that calcium absorption was impaired due to increased digesta viscosity associated with the flax mucilage.

Earlier research from this laboratory has demonstrated that multicarbohydrase enzymes can improve energy (lipids) utilization from ground full-fat flaxseed or extruded flaxseed products in adult roosters and broiler chickens (Slominski et al., 2006). In the present study, the addition of a multicarbohydrase enzyme (Superzyme OM) significantly increased egg production and improved feed conversion in hens consuming flaxseed (Table 4.2). The improved egg production parameters would suggest better energy utilization from the flaxseed diet, although only a trend in improved fat digestibility with enzyme supplementation was noted (Table 4.4). A significant increase in NSP digestibility indicates that the enzyme (Superzyme OM) was effective in flaxseed cell wall polysaccharide depolymerization and elimination, at least in part, of the nutrient encapsulating effect of the cell wall structure. The use of flaxseed markedly increased the viscosity of jejunum digesta compared with that of canola seed (18.8 vs. 5.0 mPa·s; Table 4.4). Enzyme addition failed to reduce the intestinal viscosity, although in our earlier research, a significant viscosity-reducing activity was demonstrated in vitro (Manuscript

I). It would appear that flaxseed mucilage is much more difficult to degrade in vivo, and more effective enzyme combinations are needed to target this component. Egg specific gravity significantly increased with enzyme supplementation (Table 4.3), which could be indicative of improved calcium utilization.

Linpro is an extruded product consisting of full-fat flaxseed and peas (1:1 wt/wt). The flaxseed in Linpro is used as a whole seed and is ground with peas before extruding. In addition to providing nutrients, peas serve as a carrier to increase the flow of flaxseed during grinding. Hens fed the Linpro diets had overall similar feed consumption and egg size but greater egg production and greater feed conversion than those fed flaxseed (Table 4.2). This was not surprising, because the level of flaxseed in the Linpro diets was much lower than that present in the flaxseed diets (75 vs. 150 g/kg of diet). Hence, the Linpro diets contained lower levels of ANF, including mucilage, which as shown in Table 4.4 resulted in lower intestinal viscosity. It could be speculated that in addition to grinding, the pressure and heat used during the extrusion process could contribute to the effective rupture of the seed structure and elimination of the negative effect of some heat-labile ANF (i.e., trypsin inhibitors, cyanogenic glycosides). This, in turn, would increase the overall nutrient utilization (Thacker et al., 2005) and explain why in the current study enzyme addition showed no effect on most of the egg production and nutrient digestibility parameters in hens fed the Linpro diet.

In the present study, hens consuming flaxseed deposited significantly more n-3 fatty acids in the egg (562 mg/60 g of egg, equal to 11.6% of total yolk fat; the average of total fat per 60 g of egg was 4.85 g) than those fed canola seed (207 mg/egg, equal to 4.3% of total yolk fat). This is in agreement with research by Cherian and Sim (1991),

who reported the total n-3 fatty acids content in the egg yolk fat to average 10.75 and 4.15%, respectively, in hens fed flaxseed or canola seeds both at 16% of the diet. In the current study, the increase in total n-3 fatty acids in eggs produced by hens consuming flaxseed resulted from the increase of not only ALA but also both EPA and DHA (Table 4.5). The EPA and DHA deposition in the egg is a consequence of in vivo metabolism, with ALA serving as a precursor for their synthesis through the desaturation-chain elongation pathway within the liver (Brenner 1971). Conversion of ALA to EPA parallels that of linoleic acid ($C_{18:2n-6}$) to arachidonic acid ($C_{20:4n-6}$). Both fatty acids compete for the same enzyme Δ^6-desaturase in the first step of their respective conversions to form polyunsaturated 20-carbon derivatives (Brenner 1971; Nettleton, 1991). The decrease in arachidonic acid deposition in eggs produced by birds fed flaxseed (Table 4.5) supports this well-known competitive inhibition between n-3 and n-6 fatty acids. The increase in SFA observed in the current study was most likely due to much greater SFA content of flax oil than canola oil (i.e., 10.2 vs. 6.0%; Vaisey-Genser, 1994). The lower level of MUFA in eggs produced by hens fed flaxseed resulted from either lower concentration of oleic acid in this ingredient or the inhibitory effects of polyunsaturated fatty acids, or both. Mahfouz et al. (1984) and Garg et al. (1988) have reported that polyunsaturated fatty acids inhibit the activity of Δ^9-desaturase, which is involved in MUFA synthesis.

It is of interest to note that the egg enrichment with DHA, an important fatty acid for human health, was very effective in hens consuming canola seed and was only lower by 12 mg/60 g of egg (i.e., 82.9 vs. 95.0 mg/60 g of egg) from that of flaxseed and Linpro (Table 4.5). This could indicate that DHA deposition reaches the plateau at much lower concentration of dietary n-3 fatty acids than that corresponding to the 15% inclusion rate

of flaxseed. This is further substantiated by the fact that no difference in DHA deposition was observed between the flaxseed and the Linpro diets, both products included in the diets at 15% but Linpro contributing only 7.5% of the flaxseed. This finding is consistent with earlier studies indicating that the increase in EPA and DHA was not proportional to the level of ALA, which increased significantly in the eggs from hens fed flaxseed (Caston and Leeson, 1990; Cherian and Sim, 1991; Aymond and Van Elswyk, 1995). The poor conversion rate of ALA to DHA probably relates to the complexity of DHA biosynthesis. Recent studies have shown that the biosynthesis of polyunsaturated fatty acids is more complex than previously recognized, because enzymes from more than one intracellular compartment are required for the synthesis of 22-carbon polyunsaturated fatty acid with their first double bond at position 4 in a partial degradation-resynthesis cycle (Sprecher, 2000).

Enzyme addition significantly increased n-3 fatty acids deposition, particularly DHA, in eggs produced by hens fed both flaxseed and Linpro diets. This may due to the depolymerisation of the cell wall polysaccharides after enzyme supplementation, which resulted in more oil release and thus more oil exposure to digestive enzymes.

In conclusion, high levels of dietary flaxseed had adverse effects on hen production performance. Such negative effects were minimized by enzyme supplementation, which had a positive effect on feed utilization and n-3-enriched egg production in hens fed pelleted and crumbled diets containing flaxseed.

4.6 Acknowledgements

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5. MANUSCRIPT III

Effects of diet type and enzyme addition on growth performance and gut health of broiler chickens during subclinical *Clostridium perfringens* challenge

W. Jia*, B. A. Slominski*, H. L. Bruce†, G. Blank‡, G. Crow* and O. Jones§

* Department of Animal Science, University of Manitoba, Winnipeg, Canada R3T 2N2;

† Nutreco Canada Agresearch, Burford, Ontario, Canada N0E 1A0;

‡ Department of Food Science, University of Manitoba, Winnipeg, Canada R3T 2N2;

§ Canadian Bio-Systems Inc., Calgary, Canada T2C 0J7

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5.1 Abstract

The effects of diet type (corn- vs. wheat-based) and multicarbohydrase addition on growth performance, digesta pH and viscosity, intestinal populations of *Clostridium perfringens* and lactic acid bacteria, and gut lesion score (from 0 to 4, where 0 = no gross lesions, 4 = severe extensive necrosis) of broiler chickens during oral challenge with *C. perfringens* (none or 10^8 cfu/bird on d 13) were studied in a 39-d experiment. A total of 1,216 male Ross-308 chickens was assigned to 8 dietary treatments in a randomized complete block design providing 8 replicate pens per treatment. Diets were formulated to meet the NRC protein requirement but were suboptimal in energy level. When compared with birds fed corn-based diets, chickens fed wheat-based diets had inferior ($P < 0.01$) final BW (2.49 vs. 2.59 kg) and feed conversion ratio (FCR; 1.83 vs. 1.78). Pathogen challenge significantly ($P < 0.05$) impaired growth performance and increased *C. perfringens* numbers and average lesion score. Increased ($P < 0.01$) *C. perfringens* counts (2.4 vs. 1.5 $\log_{10}$ cfu/g of digesta) and intestinal lesion score (0.9 vs. 0.4) were observed for challenged birds fed wheat-based diets. No difference in digesta pH and lactic acid bacteria numbers were found among the treatments. Enzyme addition to both the corn- and wheat-based diets increased bird final BW (2.57 vs. 2.51 kg; $P < 0.01$), decreased overall FCR (1.78 vs. 1.83; $P < 0.01$), and, in those consuming wheat-based diets, reduced digesta viscosity (from 4.1 to 2.7 mPa·s; $P < 0.01$). Enzyme supplementation assisted the challenged birds in maintaining their optimal growth performance by improving ($P < 0.05$) average daily gain (59.5 vs. 56.9 g) in those consuming corn-based diets and FCR (1.83 vs. 1.90) in those consuming wheat-based diets to values similar to

those observed in control birds (59.7 g/d and 1.84, respectively). In conclusion, enzyme addition improved growth performance and mitigated the negative effects of *C. perfringens* challenge.

Key words: Enzyme, *Clostridium perfringens*, broiler chicken

5.2 Introduction

Necrotic enteritis (**NE**) is a disease observed in most poultry-growing areas of the world, and it becomes increasingly prevalent with the removal of feed antibiotics (Mcdevitt et al., 2006). The causative agent, *Clostridium perfringens*, is a spore-forming anaerobic bacterium found in the intestinal tract of healthy birds, usually in low numbers (Barnes et al., 1972). However, its rapid growth under certain conditions can lead to an outbreak of NE. Several dietary factors are known to predispose broiler chickens to NE, including great amounts of wheat, barley or rye (Branton et al., 1987; Kaldhusdal and Hofshagen, 1992; Riddell and Kong, 1992). It is well documented that increased intestinal viscosity caused by water-soluble cell wall or nonstarch polysaccharides (**NSP**) of cereal grains has a negative effect on nutrient absorption (Gohl and Gohl, 1977; Johnson and Gee, 1981; Edwards et al., 1988; Fengler and Marquardt, 1988; Ikegami et al., 1990; Choct and Annison, 1992a) and influences the gut microflora (Wagner and Thomas, 1978; Choct et al., 1996). There is also evidence that a high dietary amount of water-soluble NSP may be associated with *C. perfringens* proliferation (Wagner and Thomas, 1978; Langhout et al., 1999; Annett et al., 2002). Among other dietary factors predisposing broiler chickens to NE is a high dietary protein content (Kaldhusdal and Skjerve, 1996; Drew et al., 2004). This could also relate to low protein digestibility,

resulting in a significant supply of this nutrient for bacterial growth. In this context, *C. perfringens* is highly proteolytic and could multiply.

Carbohydrase enzymes have a direct, positive effect on animal performance by improving nutrient digestion and absorption, thereby reducing substrate availability for microbial growth in the ileum (Choct et al., 1999; Bedford and Apajalahti, 2001); therefore, these enzymes may be beneficial for maintaining a healthy environment in the gastrointestinal tract of growing broiler chickens. Conventional carbohydrase supplements are mainly composed of xylanase and glucanase, enzymes that have been found effective in improving growth performance because of a reduction in digesta viscosity. However, a new generation of enzyme supplements is now being developed for specific use in the feed industry. This includes a multicarbohydrase blend of activities that, in our laboratory, has been proven to be effective in cell wall polysaccharide depolymerization (Meng et al., 2005), with the hydrolysis products potentially having a direct effect on animal health by manipulating the growth of pathogenic and nonpathogenic gastrointestinal microorganisms. Such effects mainly originate from the chemical nature of the substrates produced via enzymatic action on feed components. Among the feedstuffs commonly used in Canada, wheat, barley, corn, soybean meal, peas, canola meal, and wheat by-products contain significant amounts of NSP such as arabinoxylans, β-glucans, galactans, galactomannans, rhamnogalacturonans, arabinogalactans, mannans, and arabinans (Theander et al., 1989). In addition, these feedstuffs are rich in certain galactooligosaccharides, which, along with resistant starch and glycoproteins, represent components poorly metabolized by poultry (Slominski, 1991). In the process of depolymerizing various polysaccharides in the diet, exogenous

enzymes may produce galacto-, gluco-, manno- or xylooligomers, which are similar to prebiotics and which may facilitate the proliferation of health-promoting bacteria such as *Bifidobacterium* and *Lactobacillus* (Monsan and Paul, 1995). In this context, the enzyme hydrolysis products may indirectly prohibit the growth of certain pathogenic species by stimulating the growth of lactic acid bacteria in the lower gut (Gibson and Roberfroid, 1995). Monsan and Paul (1995) demonstrated that glucooligosaccharides could be assimilated well by *Bifidobacterium* spp. In contrast, pathogenic species, including *Clostridium* and *Salmonella*, showed poor assimilation. In addition, certain enzyme hydrolysis products may attract microbes away from the intestinal binding sites by means of competitive exclusion, thereby reducing colonization and disease and releasing the mucosa to perform its function of secretion, digestion, and nutrient absorption (Iji and Tivey, 1998). For example, a reduced colonization of *Salmonella* was shown for diets after the addition of mannan-oligosaccharides (**MOS**; Spring et al., 2000). Therefore, we hypothesized that the NSP hydrolysis products may serve as prebiotics and indirectly prohibit the growth of certain pathogenic species.

Reports on the effects of enzyme supplementation on the growth of *C. perfringens* and the incidence of NE are scarce. Therefore, the objective of this study was to investigate the effects of diet and a multicarbohydrase enzyme supplement on growth performance, gut health, and the incidence of NE in broiler chickens during *C. perfringens* challenge.

5.3 Materials and Methods

Housing and Management

A total of 1,216 male Ross-308 broiler chickens, vaccinated for Marek's disease and infectious bronchitis, were purchased from a commercial hatchery. The broiler chicken research facility at Nutreco Canada Agresearch in Burford, Ontario, Canada, was used to conduct this study. Sixty-four pens were randomly assigned to treatment groups, with 19 birds per pen. Each pen provided 0.75 m^2 of floor space, with a concrete floor and new wood shavings for bedding. A solid 12-inch-high (31 cm) plastic barrier separated adjacent pens. Precautions such as changing gloves and foot coverings between treatment pens were taken to avoid accidental contamination of unchallenged pens with the challenge organism. Lighting program, heating, ventilation, and other management procedures were typical of broiler chicken producers in the local geographic area of Ontario, Canada. Water was provided by nipple-type drinker and feed was provided by trough-type feeders ad libitum. All animal procedures were conducted according to the guidelines of the Canadian Council on Animal Care (1993).

Experimental Design and Diets

A 2 × 2 × 2 factorial arrangement of treatments was used in a randomized complete block design to study the effects of diet type (corn- or wheat-based), enzyme addition (none or multicarbohydrase enzyme supplement), pathogen challenge, and their interactions. There were 8 blocks of 8 pens per block, with 1 pen of each treatment in each block, and each replicate pen consisted of 19 birds, for a total of 152 birds per treatment. The carbohydrase enzyme supplement supplied 60 U of cellulase, 1,400 U of pectinase, 1,200 U of xylanase, 800 U of glucanase, 500 U of mannanase, 30 U of galactanase, and other minor enzyme activities per kilogram of diet. Antibiotic- and coccidiostat-free diets were formulated to contain 3,000 kcal/kg of ME and 23% CP in

the starter phase and 3,000 kcal/kg of ME and 20% CP in the grower phase (Table 5.1). All diets were pelleted and crumbled, and the pelleting temperature did not exceed 75 ˚C.

Experimental Procedures, Sample Collection and Chemical Analysis

The experiment lasted for 39 d and consisted of 2 phases (0 to 21 d, starter; 21 to 39 d, grower-finisher). The *C. perfringens* challenge model used in the study was originally developed based on the method of Prescott et al. (1978) and has been described in several publications (Brennan et al. 2001a,b). The *C. perfringens* strain used was originally isolated from a field case of NE in Ontario and was known to produce lesions typical of NE, with mild suppression of growth rate and minimal mortality. Feed was withdrawn from all birds approximately 8 h before challenge. Inoculum was mixed with feed, and the feed was offered on the afternoon of d 13. Inoculation lasted for 16 h, and the remaining inoculum-containing feed was weighed and discarded on the morning of d 14. The calculated inoculation dose ranged from 6.7×10^8 to 8.9×10^8 cfu/bird. During inoculation, the control birds received their regular feed.

Feed consumption and BW were measured on a pen basis on d 0, 13, 21, and 40, whereas mortality was recorded daily. Average daily feed intake, average daily gain, and the feed conversion ratio (**FCR**) were calculated for each period (d 0 to 13, d 13 to 21, d 21 to 39, and d 0 to 39). On d 17, 40 birds per treatment (5 birds per pen) were randomly selected and killed by asphyxiation with carbon dioxide. Following euthanasia, the small intestine from each bird was removed, opened, and subjected to scoring by the same poultry pathologist for NE and coccidiosis lesions using the following scale: 0, no gross lesions; 1, thin, friable small intestine; 2, focal necrosis, ulceration or both; 3, patchy necrosis; 4, severe, extensive mucosal necrosis (Johnson and Reid, 1970; Prescott et al.,

1978). The intestinal contents were collected, and samples from 10 birds were pooled to yield 4 replicates per treatment for enumeration of bacteria. Subsamples (1.5 g from each pooled sample) were frozen in liquid nitrogen and stored at -20˚C until needed for viscosity and pH measurements. The thawed samples were centrifuged at 3,600 × *g* at room temperature for 10 minutes, and viscosity of the supernatant was determined at 40˚C using a Brookfield digital viscometer (model DV-II+LV, Brookfield Engineering Laboratories, Stoughton, MA). The pH was determined using a conventional pH meter.

Enumeration of Bacteria

Pooled digesta (10 g) were transferred into 90 mL of sterile peptone containing 0.5% cysteine hydrochloride and serially diluted. For *C. perfringens* enumeration, dilutions were plated on *Perfringens* agar base (OPSP, Oxoid Inc., Nepean, ON, Canada) containing supplements SR 76 and SR 7 (Oxoid Inc.) and were incubated at 38˚C for 48 h in jars containing gas generation kits (BBL GasPak Plus, Becton Dickinson, Sparks, MD). Lactic acid bacteria were enumerated by using de Man, Rogosa, Sharpe agar (Difco, Detroit, MI) after incubation at 37˚C for 48 h. Each sample was plated in duplicate.

Statistical Analysis

Statistical analysis was conducted by the SAS program (SAS Institute, 2003). Bacterial enumeration data were converted to $\log_{10}$ cfu/g before analysis. Because of the presence of a random effect (block), the performance parameters and lesion score data were analyzed by the MIXED procedure. The fixed effects in the model for performance parameters included diet, enzyme, challenge, and the associated 2- and 3-way interactions. Because no lesions were observed in the unchallenged birds, the model simply included diet, enzyme, and the 2-way interaction. Analyses based on pooled samples (bacterial

numbers, pH, and viscosity) were tested by the GLM procedure, and the model included diet, enzyme, challenge, and the associated 2- and 3-way interactions. Mortality was tested by the same model but was subjected to FREQ and GENMOD procedures. The results are presented with actual frequencies with SE. The GENMOD analysis was performed by using the binomial distribution and the logit function. Contrasts of enzyme effects (i.e., without vs. with enzyme addition) within each diet type and pathogen treatment (none or challenge) were made. All statements of significance were based on $P < 0.05$.

Table 5.1 Composition and calculated analysis of experimental diets

Item	Corn-based diets		Wheat-based diets	
	Starter	Grower	Starter	Grower
Ingredient (% of diet)				
Corn	49.3	58.6	-	-
Wheat	-	-	40.4	48.0
Barley	-	-	13.0	16.0
Soybean meal	28.5	19.9	22.4	12.0
Canola meal	4.0	5.0	4.0	5.0
Wheat middlings	8.0	8.0	8.0	8.0
Porcine meat meal	5.0	5.0	5.0	5.0
Vegetable oil	2.9	1.7	4.9	3.9
Calcium carbonate	1.0	1.1	1.0	1.2
Dicalcium phosphate	0.4	0.1	0.3	0.1
Mineral & vitamin premix[1]	0.25	0.20	0.25	0.20
Salt	0.36	0.25	0.36	0.22
DL-Methionine	0.15	0.05	0.17	0.10
L-Lysine	-	-	0.03	0.20
L-Threonine	-	-	-	0.06
Choline	0.07	0.06	-	-
Calculated analysis				
CP (%)	23.0	20.0	23.0	20.0
ME (kcal/kg)	2,997.0	3,012.0	2,985.0	2,991.0
Calcium (%)	0.95	0.93	0.95	0.93
Available phosphorus (%)	0.44	0.37	0.44	0.38
Methionine (%)	0.52	0.38	0.50	0.39
Methionine + cystine (%)	0.88	0.71	0.87	0.72
Lysine (%)	1.24	1.03	1.17	1.06
Sodium (%)	0.20	0.15	0.20	0.15

[1] Mineral and vitamin premix provided the following: Mn, 89 mg; Zn, 88 mg; Fe, 34 mg; Cu, 63 mg; Se, 0.3 mg; I, 1.8 mg; vitamin A, 6,238 IU; vitamin D_3, 2,275 IU; vitamin E, 20 IU; vitamin B_{12}, 0.013 mg; vitamin K, 2.9 mg; niacin, 75 mg; folic acid, 0.86 mg; biotin, 0.1 mg; riboflavin, 5.5 mg per kilogram of the starter diet; and Mn, 71 mg; Zn, 71 mg; Fe, 27 mg; Cu, 50 mg; Se, 0.24 mg; I, 1.4 mg; vitamin A, 4,990 IU; vitamin D_3, 1,820 IU; vitamin E, 16 IU; vitamin B_{12}, 0.011 mg; vitamin K, 2.3 mg; niacin, 60 mg; folic acid, 0.69 mg; biotin, 0.08 mg; riboflavin, 4.4 mg per kilogram of the grower diet.

5.4 Results

Growth Performance

Feed intake was not affected by dietary treatments in the starter phase, except that from d 0 to 13, birds consumed slightly but significantly more corn-based diets than the wheat-based diets, regardless of enzyme addition or disease challenge (Table 5.2). In the grower phase, a significant interaction between enzyme addition and diet type was observed. Enzyme addition resulted in an increase in feed consumption in birds fed corn-based diets (187.8 vs. 183.0 g; $P = 0.01$), whereas a reduction in feed intake was observed among birds fed wheat-based diets from 21 to 40 d (178.3 vs. 183.8 g; $P < 0.01$), regardless of disease challenge. Over the entire trial (0 to 40 d), birds consumed less wheat-based diets when enzyme was added (101.8 vs. 104.8 g; $P = 0.04$), but no difference was found in those consuming corn-based diets (106.7 vs. 104.2 g; $P = 0.09$), irrespective of pathogen challenge. *Clostridium perfringens* challenge significantly suppressed BW gain during the postchallenge period (13 to 21 d) in both the corn (55.3 vs. 60.1 g; $P < 0.01$) and the wheat group (47.2 vs. 55.3 g; $P < 0.01$), regardless of enzyme addition. An increased BW gain was observed in challenged birds consuming enzyme-supplemented corn-based diets (57.6 vs. 53.0 g), and this positive effect of enzyme addition was maintained throughout the grower phase. As a result, the final BW at the end of the trial was similar to that of the unchallenged birds (corn + enzyme, challenged vs. corn, unchallenged; 2.62 vs. 2.60 kg; $P > 0.05$). Over the entire trial, birds fed corn-based diets had a greater average daily gain than those fed the wheat-based diets. *Clostridium perfringens* challenge caused significant growth inhibition, and enzyme

addition resulted in increased average BW gain (58.5 vs. 57.2 g) as well as increased final BW (2.57 vs. 2.51 kg). Feed conversion ratio was affected by the pathogen challenge from d 13 to 21 of the experiment, with a more pronounced effect observed for the wheat-based diets (none vs. challenge; 1.53 vs. 1.79; $P < 0.0001$) than for the corn-based diets (1.44 vs. 1.53; $P = 0.11$), regardless of enzyme addition. The effect of enzyme on feed utilization was significant and interacted with the diet type during each period, except from d 13 to 21. Enzyme addition significantly decreased FCR from d 0 to 13 of the experiment (1.36 vs. 1.44; $P < 0.01$) in birds consuming corn-based diets, irrespective of the disease challenge group. For those consuming wheat-based diets, FCR was decreased by 5.1 and 4.5% from d 21 to 40, and by 4.9 and 3.7% for the entire trial in unchallenged and challenged birds, respectively.

Mortality, Lesion Score, Intestinal Bacteria Numbers, Viscosity and pH of the Intestinal Contents

Total mortality increased from 0.8% to 3.8% because of the pathogen challenge (Table 5.3). Neither diet type nor enzyme addition affected the mortality rate. The number of *C. perfringens* was below the detection limit among the unchallenged birds (Table 5.3). Among the challenged birds, those consuming wheat-based diets had greater *C. perfringens* counts than those consuming corn-based diets, regardless of enzyme addition. No intestinal lesions were observed in birds from the unchallenged groups (Table 5.3). For those exhibiting intestinal lesions, all were scored as focal necrosis, except that one bird consuming the wheat-based diet was scored as patchy necrosis. The average lesion score was greater in birds consuming wheat-based diets than in birds consuming corn-based diets. Addition of enzyme did not affect the *C. perfringens*

numbers or lesion score. The number of lactic acid bacteria was similar among the treatment groups, except that a reduction in bacteria number was found because of enzyme addition in the unchallenged birds consuming wheat-based diets. When compared with corn-based diets, digesta viscosity was greater in birds consuming wheat-based diets (Table 5.3) and was reduced significantly after enzyme addition (from 4.1 to 2.7 mPa·s; $P < 0.01$), regardless of pathogen challenge. No differences in pH values were observed among the treatments.

Table 5.2 The effect of diet, enzyme supplementation, and *Clostridium perfringens* challenge on the growth performance of broiler chickens

Item	Average feed intake (g/bird per day)				Average daily gain (g/bird per day)				BW[1] (kg)	Feed conversion ratio (g of feed/g of gain)			
	0-13 d	13-21 d	21-40 d	0-40 d	0-13 d	13-21 d	21-40 d	0-40 d		0-13 d	13-21 d	21-40 d	0-40 d
Treatment[2,3]													
Corn, unchallenged	33.4	86.5	183.6[b]	106.1	22.5[b]	60.0	94.3	59.7	2.60	1.48[a]	1.45	1.95	1.78
Corn + enzyme, unchallenged	32.5	86.0	189.9[a]	107.8	24.1[a]	60.2	95.4	60.7	2.64	1.35[b]	1.43	1.99	1.78
Corn, challenged	31.5	81.3	182.4	102.5	22.7	53.0[b]	92.2[b]	56.9[b]	2.51[b]	1.39	1.54	1.98	1.80
Corn + enzyme, challenged	32.0	88.2	185.8	105.6	23.6	57.6[a]	95.3[a]	59.5[a]	2.62[a]	1.36	1.53	1.95	1.78
Wheat, unchallenged	32.0	85.6	184.2[a]	105.4	20.5	54.6	93.0	57.3	2.50	1.56	1.57	1.98[a]	1.84[a]
Wheat + enzyme, unchallenged	30.6	82.9	178.2[b]	101.9	20.3	56.0	95.1	58.3	2.56	1.51	1.48	1.88[b]	1.75[b]
Wheat, challenged	31.0	87.3	183.4	104.2	20.6	47.7	91.7	54.9	2.45	1.51	1.83	2.00[a]	1.90[a]
Wheat + enzyme, challenged	31.7	81.3	178.5	101.7	20.4	46.7	93.4	55.6	2.46	1.55	1.75	1.91[b]	1.83[b]
SEM	0.7	2.7	2.0	1.5	0.6	0.8	1.3	0.7	0.03	0.02	0.06	0.02	0.02
Least squares means for main effects													
Corn-based diets	32.3	85.5	185.4	105.5	23.2	57.7	94.3	59.2	2.59	1.40	1.49	1.97	1.78
Wheat-based diets	31.3	84.2	181.1	103.3	20.5	51.3	93.3	56.5	2.49	1.53	1.66	1.94	1.83
No challenge	32.1	85.3	184.0	105.3	21.9	57.7	94.5	59.0	2.57	1.48	1.48	1.95	1.79
Challenge	31.5	84.5	182.5	103.5	21.8	51.2	93.2	56.7	2.51	1.45	1.66	1.96	1.83
No enzyme	32.0	85.2	183.4	104.5	21.6	53.8	92.8	57.2	2.51	1.49	1.59	1.98	1.83
Enzyme	31.7	84.6	183.1	104.3	22.1	55.1	94.8	58.5	2.57	1.44	1.55	1.93	1.78
SEM	0.5	1.4	1.1	0.8	0.3	0.5	0.8	0.4	0.02	0.02	0.06	0.02	0.02
Factors and significance[4]													
Diet	0.025	0.523	0.002	0.039	<0.001	<0.001	0.192	<0.001	<0.001	<0.001	<0.001	0.190	0.004
Enzyme	0.567	0.779	0.831	0.795	0.173	0.020	0.013	0.003	0.003	0.016	0.273	0.011	0.004

Challenge	0.191	0.701	0.279	0.079	0.896	<0.001	0.102	<0.001	0.001	0.193	<0.001	0.433	0.012
Diet × enzyme	0.807	0.058	<0.001	0.009	0.076	0.038	0.908	0.293	0.304	0.051	0.390	0.004	0.030
Diet × challenge	0.160	0.682	0.388	0.280	0.719	0.003	0.795	0.499	0.491	0.274	0.049	0.337	0.067
Enzyme × challenge	0.051	0.592	0.740	0.562	0.706	0.355	0.607	0.481	0.730	0.006	0.910	0.433	1.000
Diet × enzyme × challenge	0.709	0.169	0.466	0.946	0.658	0.003	0.471	0.227	0.089	0.972	0.970	0.241	0.375

[a,b]Means within a column and within a diet type with no common superscript differ significantly ($P < 0.05$). [1]Final BW at d 40. [2]Birds in the challenged groups received an in-feed *C. perfringens* inoculation on d 13 that lasted for 16 h, and the calculated dose ranged from 6.7×10^8 to 8.9×10^8 cfu/bird. [3]The carbohydrase enzyme supplement supplied 60 U of cellulase, 1,400 U of pectinase, 1,200 U of xylanase, 800 U of glucanase, 500 U of mannanase, 30 U of galactanase, and other minor enzyme activities per kilogram of diet. [4]Based on the MIXED analysis.

Table 5.3 The effect of diet, enzyme supplementation, and *Clostridium perfringens* challenge on total mortality, average intestinal lesion score, bacterial numbers, viscosity, and pH values of intestinal contents

Item	Mortality (%)	Average intestinal lesion score[1]	*C. perfringens* (log_{10} cfu/g)	Lactic acid bacteria (log_{10} cfu/g)	Viscosity (mPa·s)	pH
Treatments[2,3]						
Corn, unchallenged	0.66 ± 0.66	-[4]	-	7.3 ± 0.2	2.5 ± 0.2	6.1 ± 0.1
Corn + enzyme, unchallenged	1.32 ± 0.93	-	-	6.9 ± 0.2	2.5 ± 0.2	6.1 ± 0.1
Corn, challenged[4]	3.95 ± 1.58	0.35 ± 0.11	1.6 ± 0.2	7.2 ± 0.2	2.8 ± 0.2	6.1 ± 0.1
Corn + enzyme, challenged	5.26 ± 1.81	0.43 ± 0.11	1.4 ± 0.2	7.0 ± 0.2	2.4 ± 0.2	6.1 ± 0.1
Wheat, unchallenged	0.66 ± 0.66	-	-	7.2 ± 0.2[a]	3.8 ± 0.2[a]	6.3 ± 0.1
Wheat + enzyme, unchallenged	0.66 ± 0.66	-	-	6.2 ± 0.2[b]	2.6 ± 0.2[b]	6.3 ± 0.1
Wheat, challenged	3.95 ± 1.58	1.03 ± 0.11	2.5 ± 0.2	7.0 ± 0.2	4.3 ± 0.2[a]	6.1 ± 0.1
Wheat + enzyme, challenged	1.97 ± 1.13	0.80 ± 0.11	2.3 ± 0.2	7.3 ± 0.2	2.8 ± 0.2[b]	6.1 ± 0.1
Least squares means for main effects						
Corn-based diets	2.80 ± 0.67	0.39 ± 0.11	1.5 ± 0.2	7.1 ± 0.1	2.5 ± 0.1	6.1 ± 0.1
Wheat-based diets	1.81 ± 0.54	0.91 ± 0.11	2.4 ± 0.2	6.9 ± 0.1	3.4 ± 0.1	6.2 ± 0.1
No challenge	0.82 ± 0.37	-	-	6.9 ± 0.1	2.8 ± 0.1	6.2 ± 0.1
Challenge	3.78 ± 0.77	-	-	7.1 ± 0.1	3.1 ± 0.1	6.1 ± 0.1
No enzyme	2.30 ± 0.86	0.69 ± 0.11	2.1 ± 0.2	7.2 ± 0.1	3.4 ± 0.1	6.1 ± 0.1
Enzyme	2.30 ± 0.86	0.61 ± 0.11	1.9 ± 0.2	6.8 ± 0.1	2.6 ± 0.1	6.2 ± 0.1
Significance of factors and interactions[5]						
Diet	0.409	0.002	0.023	0.255	<0.001	0.107
Enzyme	0.890	0.615	0.504	0.043	<0.001	0.526
Challenge	<0.001	-	-	0.196	0.101	0.118
Diet × enzyme	0.409	0.319	0.940	0.728	0.003	0.661
Diet × challenge	0.880	-	-	0.117	0.311	0.168
Enzyme × challenge	0.592	-	-	0.022	0.242	0.781
Diet × enzyme × challenge	0.880	-	-	0.098	0.616	0.893

[a,b]Means within a column and within a diet type with no common superscript differ significantly ($P < 0.05$). [1]The following scale was used: 0 = no gross lesions; 1 = thin, friable small intestine; 2 = focal necrosis, ulceration, or both; 3 = patchy necrosis; 4 = severe, extensive mucosal necrosis. [2]The carbohydrase enzyme supplement supplied 60 U of cellulase, 1,400 U of pectinase, 1,200 U of xylanase, 800 U of glucanase, 500 U of mannanase, 30 U of galactanase, and other minor enzyme activities per kilogram of diet. [3]Birds in the challenged groups received an in-feed *C. perfringens* inoculation on d 13 that lasted for 16 h, and the calculated dose ranged from 6.7×10^8 to 8.9×10^8 cfu/bird. [4]No intestinal lesions were observed or the numbers of *C. perfringens* were not detectable in unchallenged birds. [5]From GENMOD analysis (mortality), MIXED (lesion score), and GLM (bacteria numbers, viscosity and pH) analyses.

5.5 Discussion

The results of the current study showed that birds consuming the corn-based diets had overall superior BW gain and FCR compared with those consuming the wheat-based diet. In general, wheat and barley as dietary ingredients are of lower quality than corn because of high amounts of water-soluble and viscous NSP (Leeson and Summers, 2005). Arabinoxylans and β-glucans are known to adversely affect nutrient utilization and growth performance in broiler chickens by increasing the viscosity of intestinal contents (Choct and Annison, 1992a,b; Burnett, 1966), which can be overcome by adding NSP-degrading enzymes (Choct et al., 1995; Classen et al., 1988). Earlier research from our laboratory demonstrated that the multicarbohydrase enzyme preparation used in the current study was effective in both viscosity reduction and degradation of the cell wall structure, which resulted in increased digestibilities of fat, starch, nitrogen, and NSP in the small intestine of young broiler chickens fed wheat-based diets (Meng et al., 2004). The results of this study are in agreement with these findings because, because when enzyme was added to the wheat-based diets, a reduction in intestinal viscosity was observed on d 17 (Table 5.3). However, the decrease in FCR reached the level of significance only in birds fed the wheat-based diets in the grower phase, despite the fact that birds at this phase of production are known to be able to tolerate high intestinal viscosity (Salih et al., 1991). In addition, enzyme supplementation resulted in a decreased FCR in birds consuming corn-based diets from d 0 to 13, given that minimal amounts of water-soluble NSP are present in this type of diet. The results of the current study

indicated that the cell wall-degrading activities are at least of equal importance as the viscosity-reducing properties.

In the current study, *C. perfringens* challenge caused an inhibition of BW gain and inferior FCR over the entire trial, regardless of diet type. No overt behavioral signs or death caused by NE were observed, although macroscopically visible focal or patchy necrosis in the small intestinal mucosa and increased numbers of intestinal *C. perfringens* were noted in birds from the challenge group. Such responses would reflect a subclinical form of NE (Kaldhusdal and Hofshagen, 1992). The presence of typical intestinal lesions was used to diagnose whether the mortality was due to NE. In the current study, *C. perfringens* challenge significantly increased mortality even though no lesions were observed in dead birds, indicating that nonspecific, subtle histological changes may exist (Wilson et al., 2005; Olkowski et al., 2008). There are some concerns within the poultry industry that the subclinical form of NE may contribute to poor flock health (J. Wilson, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada, personal communication). Clinical outbreaks of NE may cause high mortality; however, subclinical NE may also lead to severe economic losses. Because NE is not usually detected in broiler chickens unless there are associated mortalities, it is often untreated; thus, performance and bird welfare could be greatly affected and associated with high condemnation rates at slaughter (Mcdevitt et al., 2006).

Numerous studies have shown that birds consuming diets with high amounts of wheat and barley, which are known to increase the viscosity of the intestinal contents, have a greater incidence of NE than those fed corn-based diets (Branton et al., 1987; Kaldhusdal and Hofshagen, 1992). Kaldhusdal and Skjerve (1996) concluded that the

ratio of wheat plus barley to corn was positively correlated with the incidence of NE. The results from the current study support these findings, because greater *C. perfringens* numbers and average lesion scores were observed in birds fed the wheat-based diets. Despite considerable research efforts, the actual mechanisms underlying this cereal effect are still not fully understood (Dahiya et al., 2006). It has been suggested that high intestinal viscosity reduces nutrient absorption by the host animal, increases the rate of feed passage (Gohl and Gohl, 1977; Johnson and Gee, 1981; Edwards et al., 1988; Fengler and Marquardt, 1988; Ikegami et al., 1990), and may enhance mucus production (Larsen et al., 1993; Langhout et al., 1999; Piel et al., 2005), which could lead to increased numbers of anaerobic bacteria in the small intestine, particularly *C. perfringens* (Wagner and Thomas, 1978; Langhout et al., 1999). A study by Deplancke et al. (2002) showed that *C. perfringens* had significant acidomucolytic potential and grew rapidly on mucin-containing medium. Therefore, Collier et al. (2003) suggested that *C. perfringens* may be particularly mucolytic and its growth would be favoured by the increased host mucus production associated with coccidiosis or a viscous intestinal environment.

Carbohydrase enzyme supplementation is known to accelerate dietary nutrient utilization by the host, which may reduce microbial activity as a result of substrate limitation in the ileum (Choct et al., 1999). Bedford and Apajalahti (2001) demonstrated that in birds fed wheat-based diets, addition of a xylanase-based enzyme preparation resulted in a 60% reduction in bacterial numbers, and research by Hubener et al. (2002) supported this finding. Any potential reduction in ileal fermentation could, in fact, be beneficial, because the substrates being fermented in this region are mainly undigested or encapsulated starch and protein, which would otherwise be available to the bird (Bedford,

2000). Furthermore, in the process of depolymerizing various NSP in the diet, the enzyme may produce galacto-, gluco-, manno- or xylooligomers (Silva et al., 1983), which could serve as prebiotics because of their ability to selectively stimulate the growth, activity or both of beneficial lactic acid bacteria (Gibson and Roberfroid, 1995; Monsan and Paul, 1995). The use of both undefined competitive exclusion products (Elwinger et al., 1992; Craven et al., 1999) and defined bacterial cultures, including *Lactobacillus acidophilus*, *Streptococcus faecalis* and *Bacillus subtilis* (Fukata et al., 1991; La Ragione and Woodward, 2003), have shown promising results in suppressing the proliferation of *C. perfringens*. Hofacre et al. (2003) reported that a lactic acid-producing bacterial culture alone or in combination with the mannan-oligosaccharides (**MOS**) was effective at reducing *C. perfringens*-associated mortality and the subclinical effects on feed utilization, but addition of neither MOS nor fructooligosaccharides alone had any positive effects. In contrast, Sims et al. (2004) observed reduced *C. perfringens* numbers in the large intestine of turkey of 6 wk of age when MOS was included in the diets. In another study, Monsan and Paul (1995) demonstrated that glucooligosaccharides were assimilated well by beneficial *Bifidobacterium* spp., whereas *Clostridium* and *Salmonella* showed poor assimilation. In this context, we hypothesized that enzyme hydrolysis products may facilitate proliferation of lactic acid bacteria, thereby reducing *C. perfringens* growth. The results of the current study, however, do not fully support this hypothesis, because the parameters such as *C. perfringens* numbers, digesta pH, lesion score or mortality in birds consuming enzyme-supplemented diets did not differ significantly from those consuming diets without the enzyme. Only a few studies exist in the literature on the effects of enzyme supplementation on NE incidence, and the results have been contradictory and

very difficult to compare because of the use of different disease challenge models, diet types, and enzyme supplements. Riddell and Kong (1992), who used a similar in-feed *C. perfringens* challenge model as that in the present study, found that the addition of pentosanase to a wheat-based diet did not affect NE mortality. However, no *C. perfringens* enumeration was performed in their study. In contrast, Choct et al. (2006) reported that xylanase supplementation reduced *C. perfringens* numbers in the caeca of healthy broiler chickens fed wheat-based diets. In another study, Jackson et al. (2003) showed that the addition of β-mannanase to corn-based diets improved performance and reduced lesion scores in birds challenged with both *Eimeria* and *C. perfringens*. They postulated that the benefits of enzyme addition were due to the depolymerisation of β-mannans, which may exacerbate the disease symptoms via a stimulatory effect on the immune system. In the current study, enzyme supplementation did not impact the growth of *C. perfringens*. However, it ameliorated the growth inhibition of disease challenge by increasing the BW gain in birds consuming the corn-based diets and improving the FCR in those consuming the wheat-based diets. The different responses of birds to enzyme supplementation may be due to the different natures of the diet (i.e., wheat vs. corn) and the content of water-soluble NSP.

Some earlier studies demonstrated that xylanase addition stimulates the growth of lactic acid bacteria in the small intestine (Vahjen et al., 1998; Engberg et al., 2004). However, the results of the current study demonstrated that the number of lactic acid bacteria was slightly but significantly reduced by enzyme addition in the nonchallenged birds consuming the wheat-based diets. Whether this is the true microbial response is

difficult to determine because it was observed only in one enzyme-supplemented group, and further research may be needed.

Overall, enzyme supplementation minimized the growth suppression associated with the *C. perfringens* challenge, with the most pronounced effect observed in birds fed the wheat-based diet. The beneficial effect of enzyme addition was likely due to enhanced nutrient utilization as a consequence of reduced intestinal viscosity and elimination of the nutrient-encapsulating effect of the cell wall polysaccharides. This finding may assist in the development of nutritional strategies to maintain performance in broiler chickens without using antibiotic growth promoters.

5.6 Acknowledgements

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6. MANUSCRIPT IV

Enzyme addition facilitates the post-disease compensatory growth of broiler chickens challenged with *Clostridium perfringens*

W. Jia*, B. A. Slominski*, H. L. Bruce†, C. M. Nyachoti* and O. Jones‡

* Department of Animal Science, University of Manitoba, Winnipeg, Canada R3T 2N2;

† Nutreco Canada Agresearch, Burford, Ontario, Canada N0E 1A0;

‡ Canadian Bio-Systems Inc., Calgary, Canada T2C 0J7

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6.1 Abstract

In vitro incubation studies using a multicarbohydrase enzyme showed a significant depolymerization of nonstarch polysaccharides (NSP) of soybean meal (SBM), canola meal and flaxseed meal, which was associated with the production of water-soluble NSP hydrolysis products. Effects of diet type and enzyme addition on growth performance of broiler chickens were investigated in a *Clostridium perfringens* challenge (10^9 cfu/bird on d14) study. A total of 2,640 male chickens were assigned to 6 treatments (wheat/SBM, unchallenged; wheat/SBM challenged; wheat/SBM + enzyme, challenged; wheat/flaxseed, unchallenged; wheat/flaxseed, challenged; wheat/flaxseed + enzyme, challenged). When compared with the wheat/SBM-based diets, birds fed flaxseed-containing diets had a decreased final body weight, an inferior overall feed conversion ratio (FCR), and an increased intestinal digesta viscosity ($P < 0.01$). Pathogen challenge caused mucosal lesions and increased ($P < 0.05$) the incidence of necrotic enteritis mortality, with the highest mortality rate observed in birds fed the flaxseed diet without enzyme. Enzyme addition decreased ($P < 0.05$) the overall FCR from 1.88 to 1.77 and 1.96 to 1.86 in challenged birds fed wheat/SBM and flaxseed-containing diets, respectively. In conclusion, feeding flaxseed-containing diets had adverse effects on growth performance, and enzyme addition improved the nutritive value of flaxseed-containing diets, and facilitated the post-disease compensatory growth of chickens after *C. perfringens* challenge.

Key words: Enzyme, flaxseed, *Clostridium perfringens*, broiler chicken

6.2 Introduction

Necrotic enteritis (**NE**) is a disease observed in most poultry-growing areas of the world and it becomes increasingly prevalent with the removal of feed antibiotics (McDevitt et al., 2006). The causative agent, *Clostridium perfringens*, is a spore-forming anaerobic bacterium found in the intestinal tract of healthy birds, usually in low numbers (Barnes et al., 1972). Its rapid growth under certain conditions can lead to an outbreak of NE. Several dietary factors are known to predispose broiler chickens to NE including high levels of cereal grains rich in viscous polysaccharides (e.g. wheat, barley, rye) or animal protein supplements, including fish meal (Branton et al., 1987; Kaldhusdal and Hofshagen, 1992; Riddell and Kong, 1992; Truscott and Al-Sheikhly, 1997). High intestinal viscosity caused by water-soluble cell wall/nonstarch polysaccharides (NSP) of cereal grains may impair dietary nutrient utilization (Gohl and Gohl, 1977; Johnson and Gee, 1981; Edwards et al., 1988; Fengler and Marquardt, 1988; Ikegami et al., 1990; Choct and Annison, 1992), and may result in a significant supply of nutrients for bacterial growth (Wagner and Thomas, 1978; Choct et al., 1996), and, therefore, facilitate *C. perfringens* proliferation (Wagner and Thomas, 1978; Smits et al., 1998; Langhout et al., 1999).

Carbohydrase enzymes have a direct, positive effect on animal performance by improving nutrient digestion and absorption, thereby reducing substrate availability for microbial growth in the ileum (Choct et al., 1999; Bedford and Apajalahti, 2001). In the process of depolymerizing various polysaccharides in the diet, carbohydrase enzymes may produce galacto-, gluco-, manno-, or xylo-oligomers (Silva et al., 1983) which, similarly to prebiotics, may facilitate proliferation of bacteria beneficial for gut health

such as *Bifidobacterium* and *Lactobaccillus* (Monsan and Paul, 1995), thereby decreasing the growth of certain pathogenic species (Gibson and Roberfroid, 1995). The use of lactic acid bacterial cultures *Lactobacillus acidophilus* and *Streptococcus faecalis*, has showed promising results in suppressing *C. perfringens* proliferation (Fukata et al., 1991) and reducing *C. perfringens*-associated mortality (Hofacre et al., 2003). Kiarie et al. (2008) reported that the NSP hydrolysis products obtained from soybean and canola meals had positive effects against infection of enterotoxigenic *Escherichia coli* in piglets. In addition, higher ileal lactobacilli counts and lactate content were found in piglets fed diets supplemented with a multicarbohydrase enzyme (Kiarie et al., 2007).

Incorporating flaxseed in poultry diets to produce n-3-enriched eggs or meat products has attracted interest of the poultry industry. However, depressed energy utilization and growth performance have been observed in broiler chickens fed increased amounts of flaxseed (Ajuyah et al., 1991; Lee et al., 1991; Ortiz et al., 2001; Alzueta et al., 2003). This is associated with the presence of various anti-nutritional factors (**ANF**), including mucilage which is a water-soluble polysaccharide and can markedly increase the intestinal viscosity in broiler chickens (Alzueta et al., 2003). However, whether or not feeding flaxseed affects the susceptibility of broiler chickens to NE has not yet been investigated.

Earlier research from this laboratory has demonstrated that a multicarbohydrase enzyme was effective in depolymerizing NSP of flaxseed and canola seed thereby improving oil utilization (Slominski et al., 2006; Manuscript II). Therefore, we hypothesize that the addition of a multicarbohydrase enzyme to broiler chickens diets would facilitate the production NSP hydrolysis products which may exert some beneficial

effects against NE outbreaks by promoting the growth of lactic acid bacteria and thus reducing *C. perfringens* proliferation.

The current study was undertaken to evaluate the effectiveness of enzyme addition on NSP depolymerization of flaxseed meal (**FM**), soybean meal (**SBM**) and canola meal (**CM**) and to investigate the effects of flaxseed and enzyme addition on growth performance and NE incidence in broiler chickens consuming practical diets and challenged with *C. perfringens*.

6.3 Materials and Methods

In Vitro Study

NSP Depolymerization by a Multicarbohydrase Enzyme

A multicarbohydrase supplement used in the study contained cellulase, pectinase, xylanase, glucanase, mannanase and galactanase and was similar to that used in our earlier research on NSP depolymerization of SBM and CM (Meng et al., 2005; Meng and Slominski, 2005) and flaxseed (Slominski et al., 2006). Soybean meal, CM, and FM ground to pass through a 1 mm sieve were used in the study. The incubation procedure applied in this study was similar to that described by Slominski and Campbell (1990) while NSP were determined by gas-liquid chromatography (component neutral sugars) and by colorimetry (uronic acids) using the procedure described by Englyst and Cummings (1988) with some modifications (Slominski and Campbell, 1990). Briefly, 100 mg samples were boiled with 7 mL of 0.1 M sodium acetate buffer (pH 5.2) for 5 min and then incubated at 40˚C in an environmentally controlled shaker with the enzyme supplement added at 4 different levels: 0.5, 1.0, 2.5 and 5.0% along with a buffer solution

containing starch-degrading enzymes (amylase, pullulanase, and amyloglucosidase). Following incubation, ethanol was added to a final alcohol concentration of 80% and the mixture was left for 1 h at room temperature before being centrifuged. The supernatant was discarded and the dried residue was dissolved in 1 mL of 12 *M* sulfuric acid and incubated for 1 h at 35˚C. Six milliliters of water and 5 mL of myo-inositol (internal standard) solution were then added and the mixture was boiled for 2 h. One milliliter of the hydrolysate was taken and neutralized with 12 *M* ammonium hydroxide, reduced with sodium borohydride, and acetylated with acetate anhydride in the presence of 1-methylimidazole. Component sugars were separated using a SP-2340 column and a Varian CP 3380 Gas Chromatograph. Uronic acids were determined using the procedure described by Scott (1979).

The degree of NSP depolymerization was indicated by a reduced recovery of total NSP compared with the control treatment (without enzyme).

Characterization of NSP Hydrolysis Product

Soybean meal, CM and FM samples (30 g) were further subjected to a vigorous mixing with 400 mL of 80% ethanol to extract simple sugars, sucrose and oligosaccharides. The extraction was performed overnight in an environmentally controlled shaker and was repeated 4 times. After each extraction, the samples were subjected to centrifugation at 1,990 × *g* and the supernatant (ethanol) was discarded. After last extraction, the samples were dried under a fume hood for 2 days.

Ethanol-extracted (sugar-free) meals were used to determine the effect of enzyme addition on NSP depolymerization and the production of simple sugars, oligosaccharides and low molecular weight polysaccharides. To identify these products, any changes in the

content of total NSP, water-insoluble NSP, water-soluble NSP, and monosaccharides following enzyme addition were monitored.

For simple sugar (monosaccharide) analysis, the ethanol-extracted meals (0.1 g) were incubated without or with enzyme addition (2.5 and 5.0%) at 40ºC for 5 h in a 0.1 M sodium acetate buffer (pH 5.2) containing starch-degrading enzymes. Ethanol was then added to a final alcohol concentration of 80% and the mixtures were left for 1 h at room temperature. Following centrifugation, the supernatants were transferred to separate test tubes for simple sugar (monosaccharide) analysis, which involved ethanol evaporation, residue solubilization and sugar reduction, acetylation and GLC analysis as described above.

For water-insoluble NSP analysis, the ethanol-extracted meals (0.1 g) were incubated without or with enzyme addition (2.5 and 5.0%) at 40ºC for 5 h in a 0.1 M sodium acetate buffer (pH 5.2) containing starch-degrading enzymes. Following centrifugation, the supernatants were discarded. To ensure a complete removal of water-soluble NSP, the pellets were washed with buffer, centrifuged and the supernatants discarded. The dried residues were subjected to hydrolysis with 12 *M* sulfuric acid for 1 h at 35˚C and the hydrolysis in 1 *M* sulfuric acid at 100ºC for 2 h. The hydrolyzates were then neutralized with 12 *M* ammonium hydroxide, reduced with sodium borohydride, acetylated and the component sugars analyzed using the GLC methodology described above.

Water-soluble NSP were calculated by difference between the total and the water-insoluble NSP.

Broiler Chicken Study

Housing and Management

A total of 2,640 male Ross-308 broiler chickens, vaccinated for Marek's disease and infectious bronchitis, were purchased from a commercial hatchery. The broiler chicken research facility at Nutreco Canada Agresearch in Burford, Ontario, Canada was used to conduct the study. Forty-eight pens were randomly assigned to treatment groups, with 55 birds per pen. Each pen provided 13.7 square meters of floor space with a concrete floor and new chopped straw for bedding. A solid 31 cm high plastic barrier separated adjacent pens. Precautions such as changing gloves and foot coverings between treatment pens were taken to avoid accidental contamination of unchallenged pens with the challenge organism. Lighting program, heating, ventilation and other management procedures were typical of broiler chicken producers in the local geographic area of Ontario, Canada. Water was provided by nipple-type drinkers and feed by trough-type feeders *ad libitum*. All animal procedures were conducted according to the guidelines of the Canadian Council on Animal Care (1993).

Experimental Design and Diets

A 2 × 3 factorial arrangement of treatments was used in a randomized complete block design to study the effects of diet type (wheat/SBM-based or wheat/flaxseed-based diet, 120 g/kg of diet), enzyme addition and pathogen challenge (control, unchallenged; challenged; challenged with dietary enzyme addition). There were 8 blocks of 6 pens per block, with 1 pen of each treatment in each block, and each replicate pen consisted of 55 birds for a total of 440 birds per treatment. The carbohydrase enzyme supplement was added at the rate of 1 kg per 1 tonne of feed and supplied 60 U cellulase, 1400 U pectinase, 1200 U xylanase, 800 U glucanase, 500 U mannanase, 30 U galactanase and

other minor enzyme activities per kg of diet. Antibiotic- and coccidiostat-free diets were formulated to contain 3100 kcal/kg ME and 23% CP in the starter phase and 3100 kcal/kg ME and 20% CP in the grower phase (Table 6.1). All diets were pelleted and crumbled and pelleting temperature did not exceed 75˚C.

Experimental Procedure, Sample Collection and Chemical Analysis

The experiment lasted for 37 days, consisting of two phases (0 to 21d, starter; 21 to 37d, grower-finisher). The *C. perfringens* challenge model used in this study was originally developed based on Prescott et al. (1978) and has been described in a number of publications (Brennan et al., 2001 a, b). The *C. perfringens* strain used was originally isolated from a field case of NE in Ontario, and was known to produce lesions typical of NE with mild suppression of growth rate and minimal mortality. Feed was withdrawn from all birds for approximately 8 h before challenge. Inoculum was mixed with feed and the feed offered to birds on the afternoon of Day 14. Inoculation lasted for 16 h and the remaining inoculum-containing feed was weighed and discarded on the morning of Day 15. Calculated inoculation dose ranged from 1.6×10^9 to 2.4×10^9 cfu per bird. During inoculation, control birds received their regular feed.

Feed consumption and body weight (**BW**) were measured on a pen basis on days 0, 14, 17, 21 and 37; whereas mortality was recorded daily. The dead birds were subjected to necropsy and the presence of intestinal lesions was used to diagnose whether the mortality was due to NE. Average daily feed intake, average daily gain and feed conversion ratio (FCR) were calculated for each period (days 0-14, 14-17, 17-21, 21-37, 0-37). On day 17, 16 birds per treatment (2 birds per pen) were randomly selected and euthanized by asphyxiation with carbon dioxide. The small intestine from each bird was

removed, opened and subjected to scoring for NE lesions by the same poultry pathologist using the following scale: 0, no gross lesions; 1, thin, friable small intestine; 2, focal necrosis and/or ulceration; 3, patchy necrosis; 4, severe, extensive mucosal necrosis (Johnson and Reid, 1970; Prescott et al., 1978). The intestinal contents from jejunum and ileum were collected and samples from 4 birds were pooled to yield 4 replicates per treatment for bacteria enumeration. Sub-samples (1.5 g from each pooled sample) were frozen in liquid nitrogen and stored at -20ºC until needed for viscosity measurement. The thawed samples were centrifuged at 3,600 × *g* for 10 min, and viscosity of the supernatant was determined at 40°C using the Brookfield digital viscometer (model DV-II+LV, Brookfield Engineering Laboratories, Stoughton, MA).

Bacteria Enumeration

Pooled digesta (10 g) were transferred into 90 mL sterile peptone and serially diluted. For *C. perfringens* enumeration, dilutions were plated on Perfringens agar base (OPSP, Oxoid Inc., Nepean, ON, Canada) containing supplements SR 76 and SR 7 (Oxoid Inc.) and were incubated at 38°C for 48 h in jars containing gas generation kits (BBL GasPak Plus™, Becton Dickinson). Lactic acid bacteria were enumerated using MRS (de Man, Rogosa, Sharpe) agar (Difco, Detroit, MI, USA) following incubation at 37°C for 48 h. For Coliform enumeration, dilutions were plated on Petrifilm™ Coliform and *Escherichia coli* plates (3M Canada, Inc., London, ON, Canada). Typical colonies were counted following incubation at 35°C for 24 h. Each sample was plated in duplicate.

Statistical Analysis

All of the statistical analysis was conducted by the SAS program (version 9.1, SAS Institute Inc., Cary, NC). *In vitro* data were tested by GLM procedure and means

were separated by Tukey's Honestly Significant Difference (Steel et al., 1997). In the broiler chicken study, bacterial enumeration data were converted to $\log_{10}$ cfu/g before analysis. Performance parameters and lesion scores were analyzed by the MIXED procedure due to the presence of a random effect (Block), whereas analyses based on pooled samples (bacterial numbers and viscosity) were tested by the GLM procedure (McLean et al., 1991). The fixed effects in the model included diet (wheat/SBM or wheat/flaxseed), treatment (control; challenge; challenge + enzyme), and the 2-way interaction. Means were separated by Tukey's Honestly Significant Difference except for mortality data. Mortality was tested by the same model but subjected to the FREQ and GENMOD procedures (Steel et al., 1997). The results were presented with actual frequencies with standard errors. The GENMOD analysis was performed using the binomial distribution and the logit function. All statements of significance are based on $P < 0.05$.

Table 6.1 Composition and calculated analysis of experimental diets

	Wheat/SBM-based diets		Wheat/flaxseed-based diets	
Item	Starter	Grower	Starter	Grower
Ingredient (% of diet)				
Wheat	40.4	48.0	37.0	46.7
Barley	13.0	16.0	12.5	13.4
Soybean meal	22.4	12.0	18.5	8.0
Flaxseed	-	-	12.0	12.0
Canola meal	4.0	5.0	3.0	4.0
Porcine meat meal	5.0	5.0	5.0	5.0
Wheat middlings	8.0	8.0	8.0	8.0
Canola oil	4.90	3.90	1.86	0.83
Calcium carbonate	1.05	1.16	1.05	1.12
Dicalcium phosphate	0.33	0.08	0.28	0.07
Mineral & vitamin premix[z]	0.25	0.20	0.25	0.20
Salt	0.36	0.22	0.36	0.22
DL-Methionine	0.17	0.10	0.16	0.08
L-Lysine	0.03	0.20	0.04	0.25
Choline	0.08	0.06	0.08	0.06
Threonine	-	0.06	-	0.06
Calculated analysis				
CP (%)	23.1	19.9	22.8	19.7
ME (kcal/kg)	3100.0	3100.0	3100.0	3100.0
Calcium (%)	1.02	1.00	1.03	1.00
Nonphytate Phosphorus (%)	0.43	0.37	0.43	0.38
Sodium (%)	0.19	0.13	0.19	0.13
Methionine (%)	0.55	0.42	0.53	0.39
Methionine + Cystine (%)	0.92	0.75	0.89	0.71
Lysine (%)	1.19	1.06	1.17	1.06
Threonine (%)	0.77	0.69	0.80	0.71

[z]Mineral and vitamin premix provided: Mn, 89 mg; Zn, 88 mg; Fe, 34 mg; Cu, 63 mg; Se, 0.3 mg; I, 1.8 mg; vitamin A, 6238 IU; vitamin D_3, 2275 IU; vitamin E, 20 IU; vitamin B12, 0.013 mg; vitamin K, 2.9 mg; niacin, 75 mg; folic acid, 0.86 mg; biotin, 0.1 mg; riboflavin, 5.5 mg per kilogram of the starter diet and Mn, 71 mg; Zn, 71 mg; Fe, 27 mg; Cu, 50 mg; Se, 0.24 mg; I, 1.4 mg; vitamin A, 4990 IU; vitamin D_3, 1820 IU; vitamin E, 16 IU; vitamin B12, 0.011mg; vitamin K, 2.3 mg; niacin, 60 mg; folic acid, 0.69 mg; biotin, 0.08 mg; riboflavin, 4.4 mg per kilogram of the grower diet.

6.4 Results

In Vitro Study

The total NSP contents of SBM, CM and FM averaged 142.6, 177.2 and 250.3 mg/g, respectively, with a significant degree of NSP degradation observed following incubation of the meals with an enzyme supplement (Table 6.2). When compared with the control samples (without enzyme), NSP degradation averaged 21%, 30% and 20% for SBM, CM and FM, respectively, when 5.0% enzyme was used. The highest enzyme concentration (5.0%) resulted in the highest cell wall polysaccharide depolymerization in SBM. However, there was no significant difference between 1.0% and 5.0% enzyme concentrations in flaxseed NSP degradation, and the depolymerization of canola NSP was similar for 2.5% and 5.0% enzyme levels.

The characterization of NSP and NSP hydrolysis products following incubation with a multicarbohydrase enzyme is presented in Figure 6.1. The total NSP content of ethanol-extracted SBM averaged 179.7 mg/g, and consisted of 136.1 mg/g water-insoluble and 43.6 mg/g water-soluble NSP. Ethanol-extracted CM contained 227.2 mg/g total NSP, including 185.4 mg/g water-insoluble and 41.8 mg/g water-soluble NSP, whereas ethanol-extracted FM contained 188.7 mg/g water-insoluble and 157.9 mg/g water-soluble NSP that resulted in a total of 346.6 mg/g NSP. After 5 h-incubation with a multicarbohydrase enzyme, the amount of water-insoluble NSP in SBM numerically decreased ($P > 0.05$), whereas the content of water-soluble NSP and NSP hydrolysis products increased with some monosaccharides being released in the enzyme-treated samples. Among the two enzyme-treated SBM samples, no significant difference was observed in water-insoluble NSP or NSP hydrolysis products content, however, 5.0%

enzyme concentration resulted in a higher release of monosaccharides. A similar response was found for CM and FM samples with enzyme addition resulting in reduced amounts of water-insoluble NSP and increased amounts of water-soluble NSP and NSP hydrolysis products. When compared with the 2.5% level, the use of 5.0% enzyme did not increase the degradation of water-insoluble NSP further, but produced more NSP hydrolysis products in CM. Larger amounts of monosaccharides were released in both samples at the 5% than at the 2.5% enzyme concentration level. Among the monosaccharides released, glucose, galactose and uronic acid were predominant in all meal samples (Table 6.3). The increase in total sugars associated with 5.0% enzyme concentration was due to an increase in glucose, galactose and mannose in SBM; glucose, galactose, arabinose and mannose in CM; and glucose, galactose and xylose in FM.

Table 6.2 Recovery of total nonstarch polysaccharides (NSP) from soybean meal (SBM), canola meal (CM) and flaxseed meal (FM) following incubation with a multicarbohydrase enzyme (mg/g)

Enzyme dose	SBM	CM	FM
None (control)[z]	142.6 ± 2.2[a]	177.2 ± 2.9[a]	250.3 ± 1.6[a]
0.5%	135.3 ± 1.8[ab]	153.9 ± 3.5[b]	211.1 ± 1.6[b]
1.0%	129.0 ± 1.8[b]	144.2 ± 3.5[bc]	202.3 ± 1.6[bc]
2.5%	119.5 ± 1.8[c]	127.4 ± 3.5[cd]	200.5 ± 1.6[c]
5.0%	112.9 ± 1.8[d]	124.0 ± 3.5[d]	200.1 ± 1.6[c]
P	<0.001	<0.001	<0.001

[z]Values are means ± SEM, n = 2 except that n = 3 for enzyme treated SBM and CM control samples. [a-d]Means within a column with no common superscript differ significantly ($P < 0.05$).

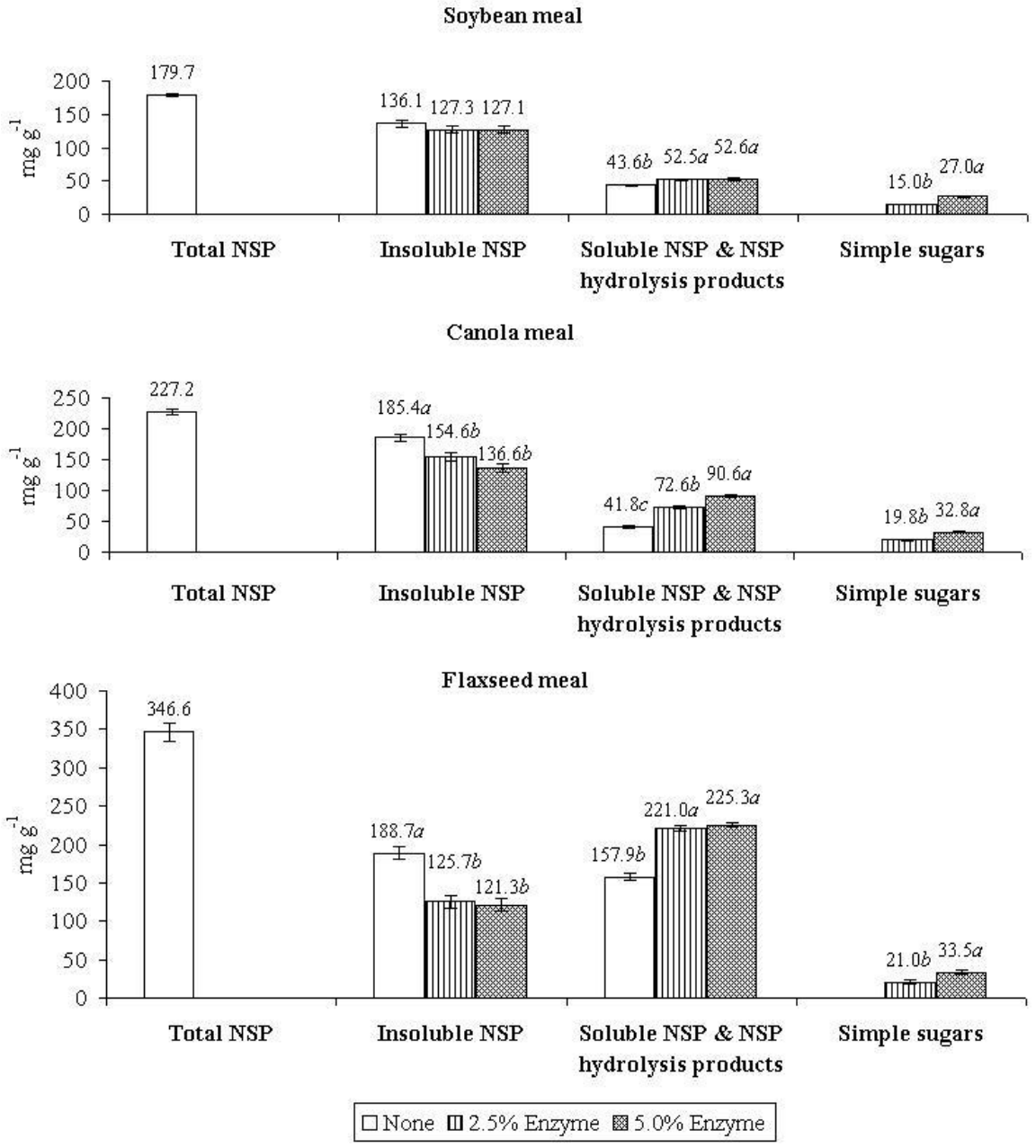

Figure 6.1 Nonstarch polysaccharides (NSP) and NSP hydrolysis product balance after incubation of ethanol-extracted soybean meal, canola meal and flaxseed meal with a multicarbohydrase enzyme at 2.5% and 5.0% for 5 h.

Soluble NSP & NSP hydrolysis products for the control samples represent water-soluble NSP only. Values are means ± SEM. n = 3, 3, 3, 2, 3, 5, 3, 5, and 3, respectively, for each

bar of soybean meal, n = 2, 4, 4, 4, 2, 6, 5, 6, and 5, respectively, for each bar of canola meal, and n = 2, 5, 5, 5, 2, 6, 5, 6, and 5, respectively, for each bar of flaxseed meal. [a,b]Means within a source with no common superscript differ significantly ($P < 0.05$).

Table 6.3 Profile of monosaccharides released from ethanol-extracted soybean meal (SBM), canola meal (CM) and flaxseed meal (FM) following incubation with a multicarbohydrase enzyme (mg/g)

Treatment	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acids	Total
SBM + 2.5% enzyme[z]	0.4 ± 0.2	-[w]	1.1 ± 0.2	3.6 ± 0.1	7.3 ± 0.3	2.5 ± 0.4	15.0 ± 0.6
SBM + 5.0% enzyme[y]	1.0 ± 0.3	-	2.2 ± 0.3	6.3 ± 0.2	14.0 ± 0.3	3.5 ± 0.5	27.0 ± 0.7
P	0.156	-	0.033	<0.001	<0.001	0.158	<0.001
CM + 2.5% enzyme[x]	2.7 ± 0.4	-	0.2 ± 0.1	1.2 ± 0.1	11.3 ± 0.5	4.3 ± 0.4	19.8 ± 1.1
CM + 5.0% enzyme[z]	4.9 ± 0.4	-	0.7 ± 0.1	2.1 ± 0.1	19.5 ± 0.5	5.6 ± 0.4	32.8 ± 1.2
P	0.004	-	0.007	<0.001	<0.001	0.053	<0.001
FM + 2.5% enzyme[x]	1.5 ± 0.2	0.0±0.1	0.0 ± 0.1	1.9 ± 0.2	10.5 ± 1.0	7.1 ± 0.8	21.0 ± 2.2
FM + 5.0% enzyme[z]	2.1 ± 0.2	0.3±0.1	0.1 ± 0.1	3.4 ± 0.3	18.7 ± 1.1	8.9 ± 0.9	33.5 ± 2.4
P	0.073	0.027	0.104	0.003	<0.001	0.156	0.004

[z]Values are means ± SEM. n=5. [y]n=3. [x]n=6. [w] Not detected.

Broiler Chicken Study

Growth Performance

Before *C. perfringens* challenge (0-14 d), birds consuming wheat/SBM-based diets had greater feed intake and BW gain than those consuming flaxseed-containing diets, and thus an improved FCR was observed regardless of experimental treatment (Table 6.4). After *C. perfringens* challenge (14-21 d), impaired BW gain and FCR were observed in challenged birds consuming the wheat/SBM-based diet, whereas feed intake was not impacted. Among those fed flaxseed diets, feed intake (14-17 d) and BW gain (14-21 d) decreased due to *C. perfringens* challenge, and an impaired FCR was observed in challenged birds consuming the flaxseed-containing diet during periods of 14-17 d and 17-21 d. In the grower phase (21-37 d), regardless of diet type, pathogen challenge no longer affected the feed consumption, and an increased BW gain was observed in the challenged groups when compared with control birds. In the absence of enzyme, birds consuming wheat/SBM-based diets had similar FCR irrespective of pathogen challenge (wheat/SBM, unchallenged vs. challenged, 21-37 d). However, a decreased FCR was noted in challenged birds fed flaxseed-containing diet compared with the unchallenged birds during this period. Enzyme addition reduced the feed intake of challenged birds regardless of diet type in the grower phase, whereas the average daily gain was not affected, therefore FCR of both dietary groups decreased significantly following enzyme addition during this period (21-37 d). Over the entire trial (0-37 d), pathogen challenge resulted in an impaired FCR among birds consuming wheat/SBM diets (1.88 vs. 1.82), whereas an improved FCR was observed in those fed flaxseed-containing diets (1.96 vs. 2.03). Enzyme addition significantly improved the FCR of *C. perfringens* challenged

birds in both dietary groups. With the inclusion of enzyme, the FCR of the challenged birds consuming the wheat/SBM diet was decreased to a level similar to that of unchallenged birds (1.77 vs.1.82, $P = 0.273$), with the FCR of birds fed the flaxseed-containing diet being even better than that of unchallenged birds (1.86 vs. 2.03, $P < 0.05$). In the absence of enzyme supplement, feeding flaxseed negatively affected the growth of birds, which was reflected by an increased FCR (0-37 d) and a decreased average final BW (Table 6.5). Neither pathogen challenge nor enzyme addition influenced the final BW.

Average Lesion Score and NE Mortality

No intestinal lesions were observed in unchallenged birds (Table 6.5). For those exhibiting lesions, they were scored as either focal or patchy necrosis. Neither diet type nor enzyme addition affected the average lesion score. *C. perfringens* challenge increased NE mortality from 1.1% to 3.2% in the wheat/SBM group and up to 7.7% in the flaxseed group (Table 6.5).

Bacterial Population and Viscosity of Intestinal Contents

Pathogen challenge and enzyme addition did not affect the intestinal *C. perfringens* numbers in birds consuming wheat/SBM-based diets (Table 6.6). Although the multiple comparison was not significantly different, contrast of mean *C. perfringens* numbers only in flaxseed groups showed that pathogen challenge significantly increased intestinal *C. perfringens* population from 2.3 in unchallenged birds to 4.3 $\log_{10}$ cfu/g in challenged birds ($P = 0.025$; not included in Table 6.6) in the absence of enzyme supplement. Similar *C. perfringens* counts of ileal digesta (i.e., 4.1 $\log_{10}$ cfu/g) were

observed in broiler chickens 4 days after the challenge and were lower than the number of 7.3 $\log_{10}$ cfu/g observed on day 1 post-challenge (Si et al., 2007).

Enzyme addition to the flaxseed-containing diet was accompanied by a 1.3 log reduction in *C. perfringens* counts (from 4.3 to 3.0 $\log_{10}$ cfu/g), however, this was not statistically significant. The numbers of total coliform and lactic acid bacteria were not influenced by any of the experimental treatments. A higher digesta viscosity was found in birds fed flaxseed diets than in those fed the wheat/SBM diets regardless of experimental treatment. Only a trend (P = 0.06) in viscosity reduction following enzyme supplementation was observed in birds fed flaxseed-containing diets.

Table 6.4 The effects of diet, enzyme addition and *C. perfringens* challenge on growth performance of broiler chickens[z]

	Average daily feed intake (g/bird per day)				Average daily gain (g/bird per day)				Feed conversion ratio (g of feed/g of gain)				
	0-14 d	14-17 d	17-21 d	21-37 d	0-14 d	14-17 d	17-21 d	21-37 d	0-14 d	14-17 d	17-21 d	21-37 d	0-37 d
Least squares means for diet × treatment													
Wheat/SBM, unchallenged	35.3^{ab}	73.7^{a}	93.6^{a}	153.1	25.0^{a}	45.7^{a}	59.3^{a}	75.8	1.42	1.41	1.71	2.02^{bc}	1.82^{d}
Wheat/SBM, challenged	37.2^{a}	72.8^{a}	97.3^{a}	154.9	25.3^{a}	39.6^{b}	56.6^{b}	76.2	1.47	1.59	1.96	2.04^{bc}	1.88^{c}
Wheat/SBM+Enzyme,challenged	36.1^{a}	71.1^{a}	90.4^{a}	148.3	25.5^{a}	38.4^{b}	55.9^{b}	78.6	1.41	1.59	1.91	1.90^{d}	1.77^{d}
Wheat/Flaxseed, unchallenged	34.1^{b}	72.7^{a}	97.1^{a}	152.2	22.2^{b}	45.*0*^{a}	57.2^{a}	66.9	1.54	1.42	1.86	2.33^{a}	2.03^{a}
Wheat/Flaxseed, challenged	34.1^{b}	64.9^{b}	87.0^{ab}	151.6	22.5^{b}	31.5^{c}	48.5^{c}	72.7	1.52	1.75	2.25	2.10^{b}	1.96^{b}
Wheat/Flaxseed+Enzyme, challenged	32.1^{b}	57.7^{c}	78.6^{b}	142.9	21.4^{b}	30.4^{c}	47.4^{c}	71.6	1.50	1.63	1.97	1.99^{cd}	1.86^{cd}
SEM	0.5	1.5	2.5	2.2	0.2	1.2	1.0	1.1	0.02	0.04	0.10	0.03	0.02
Least squares means for main effects													
Wheat/SBM	36.2	72.5	93.8	152.1	25.3	41.2	57.3	76.9	1.43	1.53	1.86	1.99	1.83
Wheat/Flaxseed	33.4	65.1	87.6	148.9	22.0	35.7	51.0	70.4	1.52	1.60	2.03	2.14	1.95
SEM	0.3	0.9	1.5	1.4	0.2	0.7	0.7	0.7	0.01	0.03	0.07	0.02	0.01
Control	34.7^{ab}	73.2^{a}	95.4^{a}	152.7^{a}	23.6	45.4^{a}	58.2^{a}	71.3^{b}	1.48	1.41^{b}	1.79^{b}	2.18^{a}	1.92^{a}
Challenge	35.6^{a}	68.9^{b}	92.1^{b}	153.3^{a}	23.9	35.5^{b}	52.6^{b}	74.5^{a}	1.50	1.67^{a}	2.11^{a}	2.07^{b}	1.92^{a}
Challenge + Enzyme	34.1^{b}	64.4^{c}	84.5^{c}	145.6^{b}	23.5	34.4^{b}	51.6^{b}	75.1^{a}	1.46	1.61^{a}	1.94^{ab}	1.94^{c}	1.82^{b}
SEM	0.3	1.0	1.8	1.6	0.2	0.9	0.8	0.8	0.01	0.03	0.08	0.02	0.01
Factors and their significance based on MIXED analysis													
Diet	<0.001	<0.001	0.004	0.063	<0.001	<0.001	<0.001	<0.001	<0.001	0.019	0.020	<0.001	<0.001
Treatment	0.007	<0.001	<0.001	0.001	0.210	<0.001	<0.001	0.003	0.061	<0.001	0.002	<0.001	<0.001
Diet × treatment	0.020	0.001	0.007	0.539	0.004	0.002	0.004	0.061	0.087	0.057	0.335	<0.001	<0.001

[z]Two thousand six hundred and forty birds were assigned to 6 treatments in a randomized complete block design with 8 blocks in total, 6 pens per block, and 55 birds per pen. Birds in challenged groups received an in-feed *C. perfringens* inoculation on day 14 that lasted for 16 h and the calculated dose ranged from 1.6 to 2.4×10^{9} cfu/bird. [a-d]Means within a column and within a source with no common superscript differ significantly ($P < 0.05$).

Table 6.5 The effects of diet, enzyme addition and *C. perfringens* challenge on final BW, average lesion score, and necrotic enteritis (NE) mortality in broiler chickens[z]

	Final BW (kg, day 37)	Average lesion score	NE mortality (%)
Least squares means for diet × treatment			
Wheat/SBM, unchallenged	1.98 ± 0.02	-[y]	1.1 ± 0.5
Wheat/SBM, challenged	1.96 ± 0.02	0.6 ± 0.3	3.2 ± 0.8
Wheat/SBM + Enzyme, challenged	2.00 ± 0.02	0.7 ± 0.3	5.4 ± 1.2
Wheat/Flaxseed, unchallenged	1.79 ± 0.02	-	1.1 ± 0.5
Wheat/Flaxseed, challenged	1.81 ± 0.02	0.9 ± 0.3	7.7 ± 1.3
Wheat/Flaxseed + Enzyme, challenged	1.77 ±0.02	0.6 ± 0.3	5.2 ± 1.1
Least squares means for main effects			
Wheat/SBM	1.98 ± 0.01	0.6 ± 0.2	3.3 ± 0.6
Wheat/Flaxseed	1.79 ± 0.01	0.7 ± 0.2	4.7 ± 0.5
Control	1.88 ± 0.01	-	1.1 ± 0.4[b]
Challenge	1.89 ± 0.01	0.7 ± 0.2	5.5 ± 0.8[a]
Challenge + enzyme	1.88 ± 0.01	0.6 ± 0.2	5.3 ± 0.8[a]
Significance of factors and their interaction from MIXED analysis (final BW and lesion score) and Genmod analysis (mortality)			
Diet	<0.001	0.732	0.252
Treatment	0.983	0.732	<0.001
Diet × treatment	0.098	0.425	0.065

[z]Two thousand six hundred and forty birds were assigned to 6 treatments in a randomized complete block design with 8 blocks in total, 6 pens per block, and 55 birds per pen. Birds in challenged groups received an in-feed *C. perfringens* inoculation on day 14 that lasted for 16 h and the calculated dose ranged from 1.6 to 2.4×10^9 cfu/bird. On day 17, 2 birds per pen were randomly selected and subjected to scoring for NE lesions using the following scale: 0, no gross lesions; 1, thin, friable small intestine; 2, focal

necrosis and/or ulceration; 3, patchy necrosis; 4, severe, extensive mucosal necrosis. Values are means ± SEM. [y]No intestinal lesions were observed in unchallenged birds. [a-b]Means within a column and within a source with no common superscript differ significantly ($P < 0.05$).

Table 6.6 The effects of diet, enzyme supplementation and *C. perfringens* challenge on bacteria numbers and viscosity of the intestinal contents[z]

	C. perfringens (log_{10} cfu/g)	Coliforms (log_{10} cfu/g)	Lactic acid bacteria (log_{10} cfu/g)	Viscosity (mPa·s)
Least squares means for diet × treatment				
Wheat/SBM, unchallenged	2.15 ± 0.54	6.40 ± 0.34	7.45 ± 0.16	2.6 ± 1.5
Wheat/SBM, challenged	2.96 ± 0.54	6.58 ± 0.34	7.59 ± 0.16	2.9 ± 1.5
Wheat/SBM + enzyme, challenged	2.61 ± 0.54	5.56 ± 0.34	7.39 ± 0.16	2.1 ± 1.5
Wheat/Flaxseed, unchallenged	2.29 ± 0.54	5.50 ± 0.34	7.42 ± 0.16	11.0 ± 1.5
Wheat/Flaxseed, challenged	4.32 ± 0.63[y]	6.09 ± 0.34	7.63 ± 0.16	9.0 ± 1.5
Wheat/Flaxseed + enzyme, challenged	2.96 ± 0.54	6.20 ± 0.34	7.55 ± 0.16	4.8 ± 1.5
Least squares means for main effects				
Wheat/SBM	2.57 ± 0.31	6.18 ± 0.20	7.48 ± 0.09	2.5 ± 0.9
Wheat/Flaxseed	3.19 ± 0.33	5.93 ± 0.20	7.53 ± 0.09	8.3 ± 0.9
Control	2.22 ± 0.39	5.95 ± 0.24	7.44 ± 0.11	6.8 ± 1.1
Challenge	3.64 ± 0.42	6.33 ± 0.24	7.61 ± 0.11	5.9 ± 1.1
Challenge + enzyme	2.79 ± 0.39	5.88 ± 0.24	7.47 ± 0.11	3.5 ± 1.1
Factors and their significance based on GLM analysis				
Diet	0.193	0.387	0.674	<0.001
Treatment	0.068	0.380	0.504	0.091
Diet × treatment	0.532	0.093	0.827	0.194

[z]Two thousand six hundred and forty birds were assigned to 6 treatments in a randomized complete block design with 8 blocks in total, 6 pens per block, and 55 birds per pen. Birds in challenged groups received an in-feed *C. perfringens* inoculation on day 14 that lasted for 16 h and the calculated dose ranged from 1.6 to 2.4×10^9 cfu/bird. On day 17, 2 birds per pen were randomly selected,

and intestinal contents from jejunum and ileum were collected and samples from 4 birds were pooled to yield 4 replicates per treatment for bacteria enumeration and viscosity measurement. Values are means ± SEM. [y]n=3.

6.5 Discussion

The use of carbohydrases to improve the nutritive value of viscous cereal-based diets has become a common practice in the feed industry (Cowieson et al., 2006). However, using enzymes to target flaxseed is a relatively new initiative and would appear to be more challenging (Slominski et al., 2006) because the composition of the cell wall polysaccharides in oilseeds is more complex than that of cereal grains (Chesson, 2001). Pectic polysaccharides are predominant in dicotyledons, and they consist of a family of acidic polysaccharides (rhamnogalacturonans) and several neutral oligosaccharides and polysaccharides (arabinans, galactans, and arabinogalactans) which are believed to be covalently attached to the rhamnogalacturonan backbone (Bacic et al., 1988). It has been indicated that arabinogalactan and pectins are primary polysaccharides in the cotyledons of soybean, whereas the hulls contain cellulose, pectins, and hemicellulose polysaccharides, including galactomannan and xylan (Karr-Lilienthal et al., 2005). In addition to a high concentration of pectins, non-cellulosic polysaccharides such as xylan, xyloglucan, arabinan, arabinogalactan and galactomannan are present in CM (Siddiqui and Wood, 1977; Slominski and Campbell, 1990). Although the cellulose content of FM is similar to that of SBM and CM (5.3% vs. 6.3% and 5.3%, respectively; Slominski and Campbell, 1990; Bach Knudsen, 1997), the total amount of cell wall polysaccharides in FM is much greater (Table 6.2), which is mainly due to the high content of water-soluble NSP (Figure 6.1). There is a paucity of information regarding the composition of cell wall components in flaxseed except for mucilage. Mucilage is present in the outermost layer of the hull, and consists of 2 fractions, a neutral fraction composed of arabinoxylans

and an acidic pectin-like fraction consisting of polysaccharides containing rhamnose, galactose and galacturonic acid residues (Cui et al., 1994). The arabinoxylans are the major components responsible for the viscous property of mucilage (Cui et al., 1994). Poultry do not possess endogenous enzymes capable of cleaving and digesting cell wall polysaccharides such as cellulose, arabinoxylan, or pectic polysaccharides, therefore the nutritive value of flaxseed could be compromised. Results of the current study are in agreement with the literature data indicating that feeding flaxseed (12%) causes growth depression in broiler chickens (Ajuyah et al., 1991; Lee et al., 1991; Alzueta et al., 2003). When comparing the 2 unchallenged groups, birds fed the flaxseed-containing diet had inferior final BW (Table 6.5) and overall FCR (Table 6.4) with no significant difference in feed intake when compared with those fed the wheat/SBM diet. This would indicate that the observed growth depression caused by flaxseed was related to poorer nutrient utilization.

Various ANF present in flaxseed, including mucilage, linatine, cyanogenic glycosides, and trypsin inhibitors, may contribute to the depressed nutrient utilization (Bhatty, 1995). The process of feed pelleting used in this study could inactivate some heat-labile components (i.e., trypsin inhibitor, enzymes responsible for cyanogenic glycosides conversion to toxic end products), however, the mucilage and the NSP associated with the cell wall structure could still pose a problem and be responsible for growth depression. Oil is the main energy source in oilseeds, and is located in the cotyledon cells which are surrounded by thick walls composed of polysaccharides. Because poultry can not digest such polysaccharides, they create a physical barrier preventing oil from its full utilization. This rationale was substantiated in a rooster study

in which the TME_n value of full-fat flaxseed was lower than the corresponding meal and oil mixture (3.7 vs. 5.1 kcal/g) (Lee et al., 1995). Furthermore, Ortiz et al. (2001) observed that increasing dietary flaxseed content in broiler chicken diets from 0 to 24% decreased the AME_n of the diet from 2799 to 2091 kcal/kg, which clearly indicate that, in addition to the oil encapsulating effect of cell walls, some flaxseed components may interfere with dietary nutrient utilization. Mucilage has been suggested to be responsible for the depressed growth rate and fat digestibility in broiler chickens by causing increased digesta viscosity (Alzueta et al., 2003). Although wheat is high in water-soluble arabinoxylans, the intestinal viscosity caused by mucilage present in the flaxseed-containing diets was much higher than that of wheat/SBM (Table 6.6).

Under high intestinal viscosity, substrate supply for microbial growth increases as a result of reduced nutrient utilization by host animals. Under such conditions, the rate of feed passage may increase (Salih et al., 1991), and mucus production may be enhanced (Larsen et al., 1993; Langhout et al., 1999; Piel et al., 2005). Such conditions may facilitate the overgrowth of ileal anaerobic bacteria, particularly *Clostridia* (Choct et al., 1996; Smits et al., 1998; Langhout et al., 1999; Deplancke et al., 2002; Collier et al., 2003). Feeding viscous cereal-based diets (e.g., wheat and barley) has been indicated to be responsible of NE outbreaks in broiler chickens (Branton et al., 1987; Kaldhusdal and Hofshagen, 1992; Kaldhusdal and Skjerve, 1996; Annett et al., 2002; Manuscript III). In the present study, the greatest values of NE mortality, intestinal *C. perfringens* number, and gut lesion score were observed in challenged birds fed the flaxseed-containing diet (Tables 6.5 and 6.6), which suggests that the viscous property of flax mucilage may have played some role. On the other hand, Allen et al. (1996) reported that the addition of n-3

fatty acids reduced cecal lesions and maintained BW gains in broiler chickens infected with *Eimeria tenella*. If true, then α-linolenic acid in flaxseed may provide some protection against NE given that coccidiosis is an important risk factor in NE development (McDevitt et al., 2006). The current results do not conflict with this postulate, because no mucosal lesions could be found in randomly selected unchallenged birds consuming the flaxseed-containing diet, and the mortality and *C. perfringens* numbers were also minimal in this group despite the high intestinal viscosity. In this context, it could be speculated that when birds are exposed to high numbers of *C. perfringens*, the high intestinal viscosity caused by flaxseed may overwhelm its potential protection and to facilitate NE outbreaks. Therefore, the hygienic management of poultry farms, particularly those using flaxseed to produce n-3-enriched egg or meat products, is critical. Pathogen challenge did not affect the growth performance of birds during the grower phase (21-37 d), and superior BW gain and FCR were noted in challenged birds fed the flaxseed-containing diet compared with the control birds (flaxseed, challenged vs. flaxseed, unchallenged). Considering the high mortality rate of this group, the better growth performance was due to the compensatory growth achieved by birds survived from the disease challenge.

The mode of action of carbohydrase enzymes is plant cell wall polysaccharide depolymerisation resulting in the production of water-soluble NSP and NSP hydrolysis products, including low molecular weight polysaccharides, oligosaccharides, disaccharides and monosaccharides (Silva et al., 1983; Pettersson and Aman, 1989; Marsman et al., 1997). In the current study, incubation of the meals with a multicarbohydrase enzyme *in vitro* (Figure 6.1) resulted in a significant decrease in

water-insoluble NSP in CM and FM, which was associated with an increase in water-soluble NSP and NSP hydrolysis products including simple sugars. The relatively high concentrations of glucose and uronic acid residues (Table 6.3) produced by enzyme addition indicated, at least to some extent, that the hydrolysis of cellulose and pectic polysaccharides took place (Slominski and Campbell 1990), resulting in a disruption of intact cell walls. However, as demonstrated in Table 6.2 and Figure 6.1, enzyme specific activities rather than the enzyme concentration would appear to be a limiting factor in achieving an effective NSP depolymerization. The in vitro study also demonstrated that the high production of water-soluble carbohydrate (soluble NSP and NSP hydrolysis products) due to enzyme addition to FM was followed by their minimal conversion, at least when compared to other meals, to simple sugars. This may explain why only a trend toward a reduction in digesta viscosity with enzyme supplementation was observed for birds fed the flaxseed-containing diets (from 9.0 to 4.8 mPa·s, $P = 0.06$, Table 6.6).

Among the NSP hydrolysis products, galacto-, gluco-, manno- or xylo-oligomers could act as prebiotics and selectively stimulate proliferation of the gut health-promoting bacteria such as *Bifidobacterium* and *Lactobaccillus* (Monsan and Paul, 1995), thereby decreasing growth of certain pathogenic species including *C. perfringens* (Gibson and Roberfroid, 1995; Fukata et al., 1991; La Ragione and Woodward, 2003). Kiarie et al. (2008) reported that carbohydrase hydrolysis products obtained from SBM and CM had positive effects against infection of enterotoxigenic *Escherichia coli* in piglets. Higher ileal lactobacilli counts and lactate content were found in piglets fed enzyme-supplemented diets (Kiarie et al., 2007). In another study from this research group, broiler chickens were fed similar wheat/SBM-based and flaxseed-containing diets

without or with a multicarbohydrase enzyme. Intestinal segments from birds were excised, ligated and inoculated with a *C. perfringens* spore cocktail. The results showed that enzyme addition significantly reduced the *in vitro* growth of *C. perfringens* in digesta from both dietary groups by 50 and 67%, respectively, with a more pronounced effect observed for the flaxseed group. This was followed by only a slight, and not statistically significant, increase in the growth of lactic acid bacteria (Wang, 2008). However, only a numerical difference was observed in the current study with enzyme addition to the flaxseed-containing diet resulting in a 1.3 log reduction in *C. perfringens* numbers ($P > 0.05$, Table 6.6). A study from Australia (Choct et al., 2006) reported that xylanase supplementation reduced the *C. perfringens* numbers in the ileum and ceca of healthy broiler chickens fed low-ME (high viscosity) wheat. Because Canadian wheat is usually of lower viscosity, the better quality wheat used in the current study may have accounted for the lack of response from enzyme addition in terms of pathogen growth. Some literature reports demonstrated that enzyme addition stimulated growth of lactic acid bacteria in the small intestine (Vahjen et al., 1998; Engberg et al., 2004; Kiarie et al., 2007). However, numbers of lactic acid bacteria and coliform were unaffected by experimental treatments in the current study, which did not fully support our original hypothesis. Although not statistically significant, the trend toward numerical reduction of intestinal viscosity and *C. perfringens* number after enzyme addition to the flaxseed diet should not be ignored. It may, to certain extent, reduce the risk of clinical outbreaks or development of subclinical NE in broiler chickens under extensive rearing conditions. Further research in this area is needed.

Effects of enzyme on growth performance were most pronounced during post-disease recovery period (21-37 d) in that enzyme addition significantly reduced FCR of challenged birds fed both diet types during the grower phase and over the entire experiment. Feeding flaxseed was associated with an inferior FCR, whereas after enzyme addition there was no significant difference in FCR between the 2 dietary treatments during the grower phase and over the entire trial (wheat/flaxseed + enzyme, challenged vs. wheat/SBM + enzyme, challenged) (Table 6.4), reflecting an enhanced dietary nutrient utilization and an improved nutritive value of flaxseed for poultry. This may lead to a reduced substrate supply for bacterial growth in the ileum (Choct et al., 1999; Bedford and Apajalahti, 2001; Hubener et al., 2002), which, in turn, may potentially contribute to the control of *C. perfringens* growth and NE development in birds fed flaxseed-containing diets.

In conclusion, feeding flaxseed had adverse effects on growth performance of broiler chickens, and enzyme addition improved the nutritive value of flaxseed-containing diets, and facilitated the post-disease compensatory growth of birds after *C. perfringens* challenge. Although no significant changes in the incidence of NE were observed following enzyme addition, the numerical reduction of intestinal viscosity and *C. perfringens* numbers in birds fed high dietary level of flaxseed may, to some extent, lead to a reduced risk of clinical outbreaks or subclinical NE development without using antibiotic growth promoters.

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7. GENERAL DISCUSSION

Using enzymes to target full-fat oilseeds is a relatively new initiative, and one of the objectives of the current research was to develop carbohydrase combinations with better efficacy than the existing enzyme products, particularly when used for flaxseed-containing diets. When compared with canola seed and soybeans, flaxseed contains much greater amounts of total and water-soluble NSP. Reduced egg production and shell quality, as well as inferior BW and overall FCR were observed in laying hens and broiler chickens fed diets containing high levels (12-15%) of flaxseed (Manuscripts II, IV), the responses mainly due to the reduced dietary nutrient utilization caused by the oil encapsulating effects of cell wall polysaccharides and the high intestinal viscosity associated with flax mucilage. Enzyme preparation used in the previous research from this laboratory was demonstrated to be effective in degrading flaxseed cell wall structure, but was ineffective in reducing the mucilage viscosity (Slominski et al., 2006). Therefore, an in vitro study was conducted to screen for the viscosity-reducing enzymes (Manuscript I), and the new enzyme combination was found to be capable of decreasing the viscosity of the mucilage solution by 74%. Unfortunately, it did not show its superiority in the subsequent broiler trial, suggesting that certain feed components or gut conditions may interfere with the enzyme activity. In fact, no difference was observed between these two types of enzyme preparations (Table 3.4), which was probably due to the complexity and heterogeneity of pectic polysaccharides present in flaxseed (Bacic et al., 1988) as well as the side activities associated with the commercial enzyme products. Thus, both preparations were used in concert in the subsequent studies with flaxseed.

The effectiveness of enzyme supplement on cell wall depolymerization was substantiated using an in vitro incubation study with 20-30% of total NSP in soybean, canola, and flaxseed meals being degraded following enzyme addition (Manuscript IV). Enzyme addition to diets containing 15% flaxseed increased total tract NSP digestibility by 9% in broiler chickens and by 12% in layers, and significantly reduced intestinal viscosity in broiler chickens, although it was not as pronounced as in the in vitro experiment (Manuscripts I, II). Research findings demonstrated that the adverse effects of flaxseed cell wall polysaccharides on dietary energy utilization were ameliorated by enzyme addition. As a consequence, total tract fat digestibility was increased by 3-6% in broiler chickens, which was reflected in an improved FCR by 1-3% (Manuscripts I); whereas the deposition of egg n-3 fatty acids and in particular that of DHA significantly increased by 28 and 9 mg, respectively, following enzyme addition to flaxseed-containing diets (Manuscript II).

Characterization of hydrolysis products from oilseed polysaccharides (Figure 6.1) illustrated that enzyme addition resulted in a significant depolymerization and solubilization of water-insoluble fraction of canola seed and flaxseed, which was accompanied by a considerable increase in water-soluble fraction with some conversion to simple sugars. Considering that in vitro enzymatic hydrolysis pattern of canola meal was revealed as a rapid hydrolysis of the water-soluble NSP followed by a slow degradation of the water-insoluble NSP (Slominski and Campbell, 1990), and flax mucilage can be rapidly hydrolyzed within 1 h (Manuscript I), water-soluble fraction in Figure 6.1 would mainly include various oligomers differing in sugar components and molecular sizes. Therefore, enzyme supplementation to practical diets would generate an

even wider array of hydrolysis products including galacto-, gluco-, manno-, or xylo-oligomers, which may be similar in nature to prebiotics (Monsan and Paul, 1995; Gibson and Roberfroid, 1995; Vardakou et al., 2008).

In order to investigate the potential prebiotic effects of enzyme hydrolysis products on growth of *Clostridium perfringens* in vivo, an in-feed challenge model was examined in the preliminary study (Manuscript III). The subclinical form of NE was induced after pathogen challenge, and birds fed the wheat/barley-based diets had greater intestinal *C. perfringens* numbers and gut lesion score when compared with those fed the corn-based diets. Focal or patchy necrosis observed in mucosal tissue would be expected to limit nutrient absorption (Wilson, 2004), and, in turn, would contribute to growth depression observed in the current study for the challenged birds. Such results support the literature data indicating that feeding viscous cereal-based diets (e.g. wheat and barley) would predispose broiler chickens to NE outbreaks (Kaldhusdal and Skjerve, 1996). Although the actual mechanism is not fully understood, it has been postulated that viscous intestinal contents caused by water-soluble arabinoxylans and β-glucans may play some role. In this context, the growth of *C. perfringens* under viscous intestinal conditions would be favoured by the increased substrate supply, prolonged feed passage rate, and enhanced mucus production (Salih et al., 1991; Langhout et al., 1999; Collier et al., 2003). If true, then feeding flaxseed may also facilitate NE development in broiler chickens.

Following the establishment of the challenge model, the effects of flaxseed and enzyme addition on NE incidence under commercial broiler flock conditions were investigated (Manuscript IV). Although as in the preliminary study, the same *C.*

perfringens strain was used, a mild form of NE was induced in challenged birds with those fed flaxseed having a greater intestinal *C. perfringens* count and relatively more severe gut lesions and NE mortality (Manuscript IV). Enzyme addition to the flaxseed-containing diets was associated with a numerical reduction in *C. perfringens* counts (by 1.4 $\log_{10}$ cfu). Although not statistically significant, this may be indicative of the potential for the reduced risk of clinical outbreaks or development of subclinical NE in broilers under extensive rearing conditions. The numbers of lactic acid bacteria in the intestinal contents as determined using a conventional culture-based approach were not significantly different following enzyme addition, which does not fully support our original hypothesis. Due to gut lesion scoring, the population of lactic acid bacteria attached to the intestinal mucosa was not enumerated in the current study. However, an increase in lactobacilli counts has been documented for pigs fed diets containing NSP hydrolysis products (Kiarie et al., 2009).

The results of the current research would also suggest that enzyme supplementation to diets containing a high level of water-soluble NSP (wheat/barley- or wheat/barley/flaxseed-based diets, Manuscripts III, IV) supported the optimal growth performance of broiler chickens after *C. perfringens* challenge as documented by FCR improvement (by 5%) during the post-disease recovery period (21-37 or 40 d). The beneficial effect of enzyme supplementation was most likely due to the enhanced dietary nutrient utilization, which, in turn, potentially contributed to the numerical decrease in *C. perfringens* counts in birds fed flaxseed-containing diets (Manuscript IV), possibly due to the reduced substrate supply for bacterial growth (Choct et al., 1999; Bedford and Apajalahti, 2001). Necrotic enteritis is a disease having a high animal welfare and

economic cost which is estimated as $0.05 per broiler chicken in the US (McDevitt et al., 2006). Findings from the current research indicate that enzyme supplements may be used as a nutritional strategy to reduce the risk of NE development in broiler chickens, and at the very least, their use could reduce the economic cost of the disease to the poultry industry.

Another means of facilitating the access of digestive secretions to flax or canola oil encapsulated within the cotyledon cells is feed processing. Results from the current research (Manuscript I) suggest that the rupture of flaxseed is of primary importance more so than any further particle size reduction. Diet pelleting could be effective in not only rupturing the seed, but also in inactivating some heat-sensitive ANF (Feng et al., 2003). With enzyme supplementation, it is feasible to feed intact oilseeds in the form of pelleted diets in both broiler chickens and laying hens without compromising growth performance and n-3 fatty acid deposition in the egg (Manuscripts II and IV). An average of 562 mg of n-3 fatty acids including 97 mg DHA per egg can be provided to consumers by incorporating 15% flaxseed into the laying hen diets, whereas 207 mg and 83 mg of egg n-3 fatty acids and DHA, respectively, can be provided by feeding canola seed (Manuscript II). The increase in egg ALA content was almost proportional to the inclusion rate of flaxseed. However, the conversion of ALA to PUFA was relatively low with DHA deposition reaching the plateau at 7.5% of flaxseed inclusion rate (provided as Linpro), which may relate to the complexity of DHA biosynthesis in vivo (Sprecher, 2000).

8. CONCLUSIONS

1. Feeding diets containing high levels of flaxseed had negative effects on egg production parameters of laying hens and growth performance of broiler chickens.
2. Reduction of particle size of flaxseed via grinding did not improve growth performance of broiler chickens, whereas diet pelleting showed more pronounced and beneficial effects in improving the nutritive value of flaxseed, particularly when intact seeds were used.
3. Multicarbohydrase supplementation resulted in a significant depolymerization of cell wall polysaccharides of oilseeds, which was followed by the production of water-soluble NSP hydrolysis products. Reduction of mucilage viscosity in vitro was also evident.
4. Enzyme supplementation to flaxseed-containing diets increased the total tract fat digestibility in broiler chickens, which was reflected in an improved FCR, as well as increased egg production performance of laying hens and n-3 fatty acid deposition in the egg.
5. *C. perfringens* challenge caused typical NE lesions in the intestinal mucosa and increased the mortality with the highest NE mortality and intestinal *C. perfringens* counts found in broiler chickens consuming flaxseed-containing diets.
6. Enzyme supplementation to diets containing high level of water-soluble NSP facilitated the post-disease compensatory growth in broiler chickens challenged with *C. perfringens*.

9. FUTURE STUDIES

The present research demonstrated that enzyme addition ameliorated the adverse effects of flaxseed cell wall polysaccharides on nutrient utilization in poultry, and assisted in maintaining the growth performance of broiler chickens particularly when they were challenged with *C. perfringens*, with some trends in limiting the intestinal growth of this pathogen. A better understanding of the structure of flax polysaccharides and intestinal conditions of birds is needed to further improve the efficacy of enzyme products in viscosity reduction.

Enzyme supplementation to practical poultry diets produces a wide array of NSP hydrolysis products differing in sugar component and molecular sizes. Identification of enzyme hydrolysis products may be noteworthy in investigating the impact of enzyme addition on the composition of intestinal bacterial community.

A recent study analyzed the genetic diversity among *C. perfringens* isolates from broiler chickens using pulsed-field gel electrophoresis (PFGE) and the results suggested that single NE-producing *C. perfringens* strain displaced the genetically heterogeneous enteric population of *C. perfringens* in broiler chickens with natural outbreaks of NE (Barbara et al., 2008). The *C. perfringens* counts reported in the current study were determined using the conventional culture-based technique, which is incapable of detecting any changes in the diversity of *C. perfringens* population. Thus, future studies using culture-independent molecular techniques may provide supplementary information regarding the composition of bacterial community in the gastrointestinal tract of broiler chickens following enzyme addition.

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