Digestive enzyme physiology and fibre nutrition in layer strain chickens

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Summary

Growth of layer pullets before point of lay is a key factor in their later performance and health and this is influenced by the feeding and managing of the pullets before the onset of egg production. Improving the early development of digestive function in growing pullets will enable them to utilise nutrients and grow efficiently. Inclusion of fibre to the diet of chickens (both broilers and layer strain poultry) has been shown to induce positive changes on transit time of ingested food and can also stimulate organ development such as the gizzard. However, most of the research on the effects of fibre on growth and development of the digestive tract has been conducted in rapidly growing young broiler strain poultry before they reach maturity.

The experiments described in this thesis were designed to gain information on the effects of fibre supplements on gastrointestinal tract (GIT) development and function in layer pullets at different stages of growth.

Because there is little information on rates of development of organs of the GIT and the functional activity of enzymes involved in digestion of protein, in the first experiment measurements of GIT organs and activities of proventricular pepsin, pancreatic general proteolytic (GP) enzymes, chymotrypsin and trypsin, and small intestinal dipeptidase and aminopeptidase were carried out from 1 to 18 weeks of age in Hy-Line Brown strain of poultry. Results showed that the weights of supply organs of the GIT tended to reach their maximum weight at 8 to 12 weeks of age whereas live weight had not reached a maximum by the end of the experiment when pullets were 18 weeks of age. Also, activities of digestive enzymes tended to peak before live weight gain.

In a second experiment, 0.8% insoluble fibre in the form of the commercial product, Arbocel® RC, was added to the diet of point of lay hens (19 weeks of age) kept under commercial conditions. After 12 weeks, the gizzards were significantly heavier than in controls and pepsin activity, expressed per organ, was significantly greater. Pancreatic GP and trypsin activities were significantly increased when expressed /g tissue or /organ.

Based on the positive response of mature hens and knowledge of GIT growth and function from the first experiment it appeared that there could be a link between the

growth of internal organs and the addition of fibre which might result in improved weight gain of young pullets. The third experiment determined the effects of feeding 1% insoluble fibre (Arbocel) at different ages and for different lengths of time to pullets between 8 weeks of age (when growth rates of GIT organs and increases in rates of digestive enzyme activities were decreasing) and 18 weeks of age when pullets are at the point of lay. Results showed significant increases in live weights and weights of supply organs, liver, proventriculus and gizzard and in activities of pepsin, GP, trypsin and chymotrypsin. Gene expression for pepsinogens A and C in proventricular tissue of pullets given the 1% IF diet for 10 weeks were also significantly increased compared to controls. A shorter period of feeding (5 weeks) and removing IF from the diet after 5 weeks did not result in equivalent beneficial effects as in pullets supplemented for 10 weeks with IF.

When two different forms of fibre, mixed (soluble/insoluble) and insoluble, were compared in young (4 to 8 weeks) or growing (8 to 16 weeks) pullets the results showed that in the younger pullets IF (Arbocel) had a greater effect on increasing the enzyme activities than the mixed fibre (MF) supplement (Opticell^{C5}) however, both treatments resulted in increased mRNA expression for pepsinogens A and C though pepsin activity in the MF pullets was not increased. When IF was added to rations of the 8 to 16 week old pullets, it resulted in increased body weight and that of liver and gizzard: pepsin and GP activities were also increased. On the other hand, MF supplementation did not cause increases in organ weights or increases in activities of pepsin or pancreatic enzymes, however dipeptidase and aminopeptidase activities were significantly increased. Thus different responses to supplementation were observed with the different fibre types and at different ages. Efficient digestion of protein in the upper digestive tract may be more important for protein utilization and growth than that in the small intestine. The beneficial effects in IF pullets could have contributed to the weight gain of the pullets.

The findings reported in this thesis show that the age at which fibre supplementation is started, the length of time it is added to diets and the type of fibre supplement used, can all affect responses. It is therefore necessary to carefully match feed supplementation with growth of pullets in order to improve weight gains before maturity in order to improve health and productivity of commercial layer hens.

Statement of Authorship

Except where reference is made in the text of the thesis, this thesis contains no materials published elsewhere or extracted in whole or in part from a thesis submitted for the award of any other degree or diploma.

No other person's work has been used without due acknowledgement in the main text of the thesis.

This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

All research procedures reported in the thesis were approved by the relevant Ethics or Safety Committee or authorised officer as appropriate.

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Dedication

I dedicate this thesis to Margreet and Shumuel Yokhana my parents in Iraq who have been amazingly supportive through my entire life. I appreciate your eternal love, prayers and care for me and my family. Thank you.

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Chapter 1
Introduction

Growth and development in layer pullets during rearing and early lay is important to the commercial poultry industry because overall uniformity in a flock promotes production levels and feed efficiency (Summers and Leeson, 1994). Rates of growth and development can be modified by multiple means and certain classes of fibres when used as feed additives in poultry diets have been shown to enhance physiological activity and the health of the gastrointestinal tract in chickens (Svihus and Hetland, 2001; Hetland *et al.*, 2003; Jimenez-Moreno *et al.*, 2009a).

However, soluble, non-starch polysaccharides (NSPs) have been found to impair nutrient digestion and hence cause poor performance of poultry (Annison and Choct, 1991; Choct *et al.*, 1996; Smits and Annison, 1996). Soluble NSPs increase the viscosity of intestinal contents, which in turn decreases the rate of diffusion of digestive enzymes into digesta and hinders their effective interaction at the mucosal surface (Iji *et al.*, 2001b).Viscosity also increases the proliferation rate of enterocytes and causes a change in morphology of villi and microvilli resulting in poor absorption (Smits and Annison, 1996).

However, research into chickens has shown that insoluble fibre sources are beneficial to gut structure and can stimulate digestive secretions (Hetland *et al.*, 2003; Gonzalez-Alvarado *et al.*, 2007; Hetland and Svihus, 2007; Jimenez-Moreno *et al.*, 2009b; Svihus, 2011). Insoluble NSPs have a non viscous nature, are not fermentable and can lead to beneficial effects on digestive processes (Souffrant, 2001; Hetland *et al.*, 2005). In young broiler chickens, Sarikhan *et al.* (2010) observed that low concentrations of insoluble raw fibre concentrate (IRFC) (less than 1% of the diet) resulted in an increased intestinal villus height and body weight and a decrease in feed conversion ratio (FCR). Insoluble fibre supplements have also been shown to affect the development and also the pH of various segments of the gastrointestinal tract (Jimenez-Moreno *et al.*, 2009a).

Studies of Szymeczko (2000), Boguslawska-Tryk (2005) and Boguslawska-Tryk *et al.* (2012) showed that levels of 0.25 or 0.95% insoluble fibre (Arbocel BWW-40) in

broiler diets had a positive effect on growth including muscle protein synthesis, pancreatic enzyme activity and calcium absorption as compared to those birds on a diet without added fibre.

An experiment by Cao *et al.* (2003) showed that a higher concentration of insoluble fibre, 100g pure cellulose / kg (or 10%) in the diet of Leghorn chickens has a detrimental effect on bird performance while lower levels, 35g pure cellulose / kg diet (3.5%) could enhance performance. In their study the reduction in performance at the higher cellulose level was due to a considerable increase in the ingesta volume and intestinal feed passage time which decrease the time of nutrient exposure to digestive enzymes. Although the beneficial effects of different insoluble fibre supplements or feeds containing different types of insoluble fibres have been demonstrated in many experiments on broiler chickens (Hetalnd *et al.,* 2003; Svihus, 2011) very little work has been done on the effects of insoluble fibre in diets of layer pullets. Investigating the effects of fibre supplements on digestive function in growing layer pullets could be important because, if there are similar beneficial effects to those seen in broilers, then improvement in growth of layer pullets would provide a good foundation for the optimum performance and health of mature egg laying hens.

In this thesis the effects of fibre supplements on layers of different ages is described. A review of the literature of fibre types and development of enzymes function is described in Chapter 2. Pullets grow very rapidly during the first few weeks after hatching and require high levels of protein and energy for development of supply organs of the gastrointestinal tract (GIT). However, little is documented on the development of GIT and function of enzymes involved in protein digestion of layer pullets to point of lay. Chapter 3 contains data obtained from Hy-Line Brown layer pullets from 1 to 18 weeks of age on growth of organs of the GIT and the activities of enzymes of the proventriculus, pancreas and small intestine that are involved in protein digestion.

The results described in Chapter 3 showed that growth of digestive tract supply organs and digestive enzyme activities decreased relative to body weight. Therefore, to determine whether it would be possible to enhance digestive function in hens after point of lay the diet of 19 to 31 week old hens was supplemented with 0.8% of the insoluble fibre product Arbocel and effects on GIT and enzyme function were compared to layers without added fibre (Chapter 4).

In Chapter 5 is described an experiment in which Arbocel is added to the diet of growing pullets at different ages and for different lengths of time in order to determine an optimum time for adding fibre that could improve growth and proteolytic digestive enzyme function. In order to understand the effects of fibre on enzyme function messenger RNA expression of pepsinogens A and C was also determined. Chapter 6 contains a description of an experiment on the effects of different fibre sources on young pullets. In the first experiment (Experiment A) the effects of an insoluble fibre supplement (Arbocel) is compared with the effects of a soluble/insoluble fibre mixture (Opticell) on young pullets from 4 to 8 weeks of age. In the second experiment (Experiment B) a similar experiment was carried out on older pullets (8 to 16 weeks of age) fed the two fibre supplements used in the first part. In addition to GIT organ weights and enzyme activities, the expression of pepsinogens A and C in proventriculus and mucin gene (MUC2) in the jejunum were also measured.

The overall significance of the results of the experiment described in this thesis and their significance for improving the productivity of laying hens is described in Chapter 7 and suggestions and recommendations for further work are made.

Chapter 2

Literature review

2.0 Introduction

The nutrition of young layer strain pullets has not been as well researched as that of growing broilers or of laying hens (Mateos *et al.,* 2014) however, such information is necessary for developing methods of management that can produce optimal growth in modern strains of pullets before point of lay (Balnave and Brake, 2005). The productivity and health of hens during egg laying is affected by growth and development of pullets before they reach point of lay (Summers, 1993). Mateos *et al.* (2002) pointed to the possibility of enhancing health in growing birds by diet manipulation: for example, stimulation of gizzard development and its motility may influence the gut health, absorption of nutrients and the performance of growing birds. The presence of relatively inert components of feeds, such as fibre components, not only affect the anatomical development of the GIT of poultry (Iji, 1998, 1999; Teirlynck *et al.,* 2009) but also the physiological development and function of digestive enzymes produced by the GIT and organs associated with the digestive process (Hetland *et al.*, 2004). Such effects can therefore influence the growth and development of the whole bird.

2.1 Growth and development of chicken digestive tract

The GIT in chickens starts to develop as the embryo grows in the incubating egg and increases rapidly in size especially close to hatching and after the chick hatches (Uni *et al.,* 2003). Although the GIT at hatching is anatomically developed (Lim and Low, 1977) it undergoes dramatic changes in morphology and function with age and access to feed. Access to feed after hatching and the nutritional composition and physical characteristics of feed are considered as the most important factors for rapid development of the GIT (Noy and Sklan, 1997).

Studies carried out by Nitsan *et al.* (1991a,b), Sell *et al*. (1991), Obst and Diamond, (1992) and Jin *et al.* (1998) clearly showed that early growth of the digestive tract in broiler chicks and poults is very rapid and exceeds that of body weight gain for

the first seven days of age. Growth is also not linear, for instance the pancreas in broilers has been reported to have two different growth phases, a rapid growth phase during the first three days after hatching, and a slow growth phase from day 4 to day 8 (Nir *et al.*, 1993).

Besides changes to the weight or size of different parts of the GIT, changes can also occur in the size of the villi in the small intestine. In both broiler and White Leghorn layer strains the size of the intestinal villi increased during the first 10 days after hatching with the villi in the duodenum being longest followed by those in the jejunum and then ileum (Yamauchi and Isshiki, 1991). In Leghorn chicks the height and diameter of villi in duodenum, jejunum and ileum increased by 34 to 100 percent between 4 to 10 days after hatching (Uni *et al.,* 1996). In addition, crypt depth and the number of enterocytes per longitudinal section of villi also increased with age. Uni *et al.* (1998) showed that early access to feed can improve the mucosal structure of the small intestine and hence increase the absorption of ingested nutrients that promote early growth of chicks.

The majority of research work on the GIT post hatching and the effects of different feeds or feeding regimes on growth of the different parts (e.g., proventriculus, gizzard, small intestine and pancreas) of the GIT, has been carried out in broiler (meat strain poultry). There has been very little systematic work on the development of the GIT of layer strain poultry to point of lay.

In recent years the addition of different types of fibre products has been used to improve growth and functional development of the GIT of poultry (Mateos *et al.,* 2012).

2.2 Fibre Composition, Definition and Classification

Carbohydrates (CHOs) are chemically very diverse and they represent a major part of poultry diets with content ranging from 40 to 70%. Digestible CHOs supply energy to poultry. However, variations in the chemical and physical structure of the indigestible types of CHOs in feed can affect the GIT of poultry resulting in for example, altered digesta transit time and microflora modulation (Smits and Annison, 1996). Non-digestible carbohydrates (NDC) were originally described as the skeletal remains of plant cells in the diet that are resistant to degradation (in mammals) by the endogenous

digestive enzymes (Trowel, 1976) and for more than three decades Theander and Amman's (1979) definition has been accepted: ie that dietary fibre (DF) is a group of polysaccharides and other polymers in plant materials that are neither digested by normal secretions nor absorbed from the gastrointestinal tract.

Dietary fibre also can be further defined as the components of plants, which are resistant to, or non-digestible by, endogenous enzymes and that it consists of cellulose and hemicelluloses, resistant starches, guar-gums, pectic-substances, glycoproteins and lignin (McNab and Boorman, 2002; Fischer, 2003). The non-starch polysaccharides (NSPs) of dietary fibre can nutritionally, chemically and physically be described as heterogeneous materials that can be divided into two major categories (1) soluble viscous, fermentable fibre (e.g., pectic polysaccharides or pectin) and (2) insoluble non-viscous, and non-fermentable fibre (e.g., cellulose), (Choct and Annison, 1990; Bedford *et al.*, 1991; Smits and Annison, 1996; Sarikhan *et al.*, 2010).

The fibre components of feed have been of concern due to their potential anti-nutritive effects and because the hygroscopic and viscose nature of NSPs has an effect on metabolism of dietary nutrients in poultry (McNab and Boorman, 2002). The group of non-digestible carbohydrates (NDC) includes cellulose (which occurs in all plant cell walls) representing 5 to 95% of glucose polymers with β (1-4) linkages and hemicelluloses, also found in the cell walls of plant derived feedstuffs used for poultry and are composed of mixed linkage (1-3, 1-4) β-D-glucans, xyloglucans and mannans (Bach Knudsen, 1997 and Table 2.1).

Pectic substances are a complex group of polysaccharides in which D-galacturonic acid is a principal component with residues of, rhamanose, xylose, galactose and fructose along with some uronic acids present as methyl esters (Fischer, 2003). Another component of the cell wall of plants is lignin, a high molecular weight aromatic polymer composed of phenyl propane (Selvendran, 1984) and this non-carbohydrate compound cannot be digested by enzymes secreted either in the small or large intestine of poultry.

Table. 2.1 Chemical classification of dietary fibre*

Fibre source	Main chain	Side chain	Description
Non-starch Polysaccharides			
Cellulose	Glucose	None	• Main structural component of plant cell wall. • Insoluble in concentrated alkali; soluble in concentrated acid.
Non-cellulose			
Hemicelluloses	Xylose Mannos Galactose Glucose	Arabinose Galactose Glucoronic acid Glucoronic acid	• Cell wall polysaccharides containing backbone of 1-4 linked pyranoside sugars. • Varying degree of branching and uronic acid content. • Soluble in dilute alkali
Pectic substances	Galacturonic acid	Rhamanose, Arabinose, Xylose, Fucose (hexose deoxy sugar)	• Components of primary cell wall and middle lamella. Vary in methyl ester content. • Generally, water soluble and gel-forming.
Lignin	Sinapyl alcohol Coniferyl alcohol P-Coumaryl alcohol	3D structure	• Non-carbohydrate cell wall component. Insoluble in 72% sulphuric acid. • Resist microbial degradation

***** Source: partial adaption from McPherson (1985). McPherson R., Classification of the fibre types. In: The Clinical Role of Dietary Fibre. MES Canada Inc., Mississauga, 1985, pp. 13-22

2.2.1 Effect of fibre on organ weights

The structure of the feed of chickens is considered as one of the main factors that may interact with the morphology of the gastrointestinal tract and alter it. Studies carried out by Hetland *et al.* (2001, 2002, 2003) showed that feeding ground oats and whole wheat increased gizzard weight in growing broilers. Also, coarse dietary fibres such as oat hulls caused changes in the weight of the gizzard and its development and also altered feed passage rate in the chicken digestive tract (Hetland *et al.,* 2004). Oats consists of 20 to 25% NSP with the highest percentage, 85 to 90 %, being insoluble fibre (Fincher and Stone 1986). Pullets fed 10% oat hulls during the pre-lay period up to 17 weeks of age had increased gizzard weight, however, body weight and feed conversion ratio were negatively affected by this level of oats (Scheideler *et al.,* 1998).

Fibre source can also affect the physio-chemical development of gastrointestinal tract in chickens (Jamroz *et al.,* 2001; Amerah *et al.,* 2009). The weight and length of GIT parts e.g. caeca and large intestine, can be increased by different sources and/or amounts of fibre in the diet of broiler chicks (Jorgenson *et al.,* 1996). Insoluble fibre in the form of wood shavings and cellulose (Avicel microcrystalline) was found by Amerah *et al.* (2009) to decrease small intestine length however, the wood shavings increased the relative gizzard weight and ileal starch digestion.

The mechanism by which dietary fibre alters the size of the GIT organs might be through the action of the physical form of the undigested part of the fibre that accumulates in the digestive tract, especially in the gizzard. In the study of Elsenhans and Caspary (2000) increasing the concentration of hydroxymethylcellulose from 8 to 32% in rat diets increased weight of small intestine and that of the caecum.

Gastrointestinal mucosa is the first tissue that comes in contact with dietary constituents and Chinery *et al.* (1992) found that a fibre free diet in rats caused a significant decrease across the GIT in intestinal cell proliferation, organ tissue weight and intestinal brush border enzymes. According to Iji (1998, 1999) the presence of fibre-NSPs in chicken diets may lead to increased intestinal tissue weight and the depth of the crypts in jejunum and ileum.

2.2.2 Effect of fibre on digestion and gut function

Choct *et al.* (1996) showed a negative effect of soluble fibre on bird performance because of modulation and interaction within gut function and enzymatic secretions. The addition of the equivalent of 40 g/kg soluble NSPs (4%) to a commercial-type broiler diet decreased apparent metabolizable energy (AME), of the diet and reduced feed conversion efficiency (FCE) and weight gain in 21-day old broilers given the diet for 8 days.

In young non-ruminant animals such as pigs and poultry, fibre can alter physiological processes such as, digestion and absorption (Iij, 2001a; Metzler and Mosenthin, 2008). According to Smits and Annison, (1996) some fibre fractions may inhibit nutrients metabolism and thereby increase the viscosity in the GIT: a high proportion of soluble NSPs negatively affect digestion and absorption of chickens and can also affect the physical form of the droppings.

The anti-nutritive effects of NSPs in broiler and layer diets may lead to reduced bird performance and increased health problems on commercial chicken farms (Choct and Annison, 1992; Smits *et al.*, 1997; Iji, 1999). Several studies have been carried out on adult animals to determine the physiological effects of the presence of fibre in feeds; in such studies, digestion and metabolism of nutrients have been affected negatively.

In studies carried out on broilers (Choct and Annison, 1990; Annison, 1991; Annison and Choct, 1991) the soluble fibre fraction may alter the digesta transit time and digestive secretions, which in turn reduce digestibility of protein and starch. Furthermore, as NSPs can alter digesta viscosity, this can affect nutrient transport across mucosal cell membranes as a result of the higher density of the unstirred water layer covering the intestinal cells (Van der Klis *et al.*, 1993a,b).

Adding carboxyl methylcellulose (CMC) to broiler diets from 21 to 35 days of age decreases nutrient digestion and absorption because of increased luminal surface viscosity (Smits *et al.*, 1996). The increased viscosity caused decreased macronutrient digestibility as well as increased microbial fermentation.

Earlier studies indicated that microbial degradation of dietary fibre in the GIT might benefit the host animals, by providing an extra amount of metabolizable energy (ME) from short chain volatile fatty acid (SCVFA) (Moss, 1989; Muramatsu *et al.,* 1991) and SCVFA may inhibit the growth of pathogenic bacteria (Józefiak *et al.*, 2004).

It has also been shown that fibre may slow nutrient digestion, through encapsulating chyme particles and this may interfere with secretion of digestive enzymes (Hetland *et al.*, 2004).

In studies on chickens and turkeys, fibre enhanced performance and promoted GIT development (Jørgensen *et al.,* 1996; Sklan *et al.,* 2003). In recent studies it has been shown that diluting broiler diets with up to 20% rice hulls had no adverse effect on bird performance (Rezaei and Hajati, 2010), and α-cellulose at 30 – 80 g/kg (3 – 8%) had no adverse influence on amino acid structure and endogenous protein composition (Kluth and Rodehutscord, 2009).

Other studies have shown that using indigestible polysaccharides, oat hulls and whole grain as a fibre source in broiler feeds leads to increased performance and digestive functions (Rogel *et al.,* 1987b; Hetland and Svihus, 2001; Svihus and Hetland, 2001), Furthermore, adding 10% insoluble cellulose to chicken diets improved the efficiency of dietary nutrient utilization (Svihus and Hetland, 2001) and oat hulls and wood shavings in broiler and layer diets increased ileal starch digestibility (Hetland *et al.,* 2003).

Jiménez-Morenzo *et al.* (2009b) showed that inclusion of insoluble fibre as oat hulls, rice hulls, and sunflower hulls at levels of 2.5 to 5% had no negative effect on broiler performance, suggesting that broiler chicks might have a requirement for minimum levels of fibre in their diets.

The fibre fraction in a diet causes bulkiness to the digesta and this speeds up feed passage in the GIT (Smith and Annison, 1996). Coarse and insoluble fibre provided as oat hulls may change the rate of feed passage especially in the gizzard (Hetland *et al.,* 2004). The mechanism by which insoluble fibre enhances nutrient digestibility may also be related to effects on pancreatic function and microbial activity (Choct *et al.,* 1996; Preston *et al.,* 2000; Svihus and Hetland, 2001; Hetland *et al.,* 2004) or by effects that reduce pH, enhance gizzard development and stimulate gut motility (Nir *et al.,* 1994; Hetland *et al.,* 2002, 2003, 2004; Conzalez-Alvarado *et al.,* 2008; Jimenez-Moreno *et al.,* 2009a,b,c).

Inclusion of raw fibre concentrates (RFC) in broiler diets has been reported (Sarikhan *et al.,* 2010) to enhance bird performance through alterations in physiological function and health. During the past decade, researchers have shown that a small amount of RFC was indispensable in broiler diets in order to ensure the proper growth of birds and for correct functioning of their digestive tracts (Smulikowska, 2002).

Recent research has confirmed that the inclusion of insoluble fibre such as micronized cellulose at levels of 0.25 to 0.74% may increase villi height to crypt depth ratio in the ileum of 42 day-old broilers (Sarikhan *et al.,* 2010; Rezaei *et al.,* 2011).

Fibre may also affect the mucus layer that is involved in protection, lubrication and nutrient transport (Montagne *et al.,* 2004). Table 2.2 shows information on current application of cellulose and fibre concentrates on the digestive function of chickens and is mainly focused on the broiler chickens because of the limited information on their use in layer pullets'.

Table 2.2 Fibre sources and their use in experimental studies

Age	Source	Poultry	Level	Main effects	Author and year
19 to 35 wks	IRFC[1]	Layers	0.8%	Improved egg production	Lim *et al.* (2013)
42 d	IRFC[2]	Broilers	0.25 to 0.95%	Improved Ca absorption	Boguslawska-Tryk *et al.* (2012)
42 d	MIF[3]	Broilers	0.5%	Improve performance, intestinal morphology and litter quality	Rezaei *et al.* (2011)
21 d	Cellulose[4], Oat hulls and Sugar beet	Broilers	3%	Improved performance and organ function	Jiménez-Moreno *et al.* (2010)
21 d	Cellulose (α-cellulose)[5]	Broilers	30 to 80g	Increased amino acid at terminal ileum	Kluth and Rodehutscord (2009)
18 to 26 wks	Low and high NSPs[6]	Pullets/layers	10%	Increased feed intake	van Krimpen *et al.* (2007)
42 d	IRFC[2]	Broilers	0.25 to 0.95%	Increased growth and enzyme activity	Boguslawska-Tryk (2005)
10 to 21 d	Cellulose powder[7]	Broilers	100g/ kg	Improved organ and nutrient digestibility	Svihus and Hetland (2001)
7 to 18 d	Carboxymethylcellulose	Broilers	1%	Lower lipid digestibility	Smits *et al.* (1998)
3 to 5 wks	Carboxymethylcellulose	Broilers	5, 10 g / kg	Poor digestion and absorption	Van Der Klis *et al.* (1993a,b)
3 wks	Cellulose, pectin, alginic acid, rice straw	Leghorn chicks	5 to 20%	No health problem	Siri *et al.* (1992)

[1]IRFC (insoluble raw fibre concentrates as lignocelluloses of woody plant with a crude fiber content of 65%).[2]IRFC (Arbocel BWW-40 from J. Rettenmaier and Söhne GMBH + Co).[3]MIF= micronized insoluble fibre, 97% fibre known as Vitacel (JRS Co. Inc., Rosenberg, Germany).[4]Microcrystalline cellulose. [5]Purified α-cellulose (Jeluxyl WEHO 500 S, Jelu, Rosenberg, Germany; crude fiber: 746 g/kg).[6]Dietary energy (11.8, 11.2 and 10.6 MJ/kg), NSPs (oat hulls, beet pulp, Arbocel (pure alpha cellulose), soy hulls and straw) soluble NSP, particle size (fine and coarse) and feed form (mash and crumble).[7]Cellulose powder-product C 8002 from Sigma Aldrich.

2.3 Protein digestion in chicken gastrointestinal tract

Protein digestion occurs by breaking down the three dimensional tertiary structures of protein into protein and polypeptide chains (secondary and primary structures) until individual amino acids are released (Delia, 2008). The crop plays an important role in preparing the food for digestion in the chicken; however, little protein digestion occurs in this organ although Hinton *et al.* (1990) showed that acidogenic bacteria in the crop can cause a decrease in the pH which contributes to maintain a low pH in the proventriculus thus helping the initial stages of protein digestion to occur.

The biochemical secretions e.g. mucus and hydrochloric acid (HCl) in the proventriculus carry out the first stages of chemical digestion of protein molecules and this is continued by pepsin. After protein is denatured by HCl, pepsin acts on internal bonds within peptide chains and breaks protein molecules into poly peptides of varying lengths. Pepsin is released as the inactive pepsinogen molecule and is converted in the low pH of the proventriculus to its active form (discussed further in Section 2.3.1). A pH around 2.5 as a result of HCl secretion from the cells of the proventriculus stimulates the conversion of pepsinogen into its active form pepsin (Levchuk and Orekhovich., 1963; Esumi *et al.*, 1980). Parietal cells of the proventriculus are stimulated to secrete HCl on stimulation by hormones e.g gastrin (Li and Owyang, 1993). The pH in the proventriculus and gizzard can vary between 2 and 4 depending upon the buffering capacity of feed present (Bohak, 1973; Khan and James, 1998). At this pH range (which is considered as the optimum range), pepsinogen can be activated to pepsin.

Breakdown of proteins begins in the lumen of the proventriculus but the process is continued in the muscular gizzard where mixing of food and enzymes and other secretions (chyme) occurs (Hill, 1971). The gizzard grinds food and allows mixing of the food with pepsin and this continues until the chyme is in small enough particles to be moved to the small intestine which forms the lower part of the digestive tract.

Protein digestion continues as digested food pass from the gizzard to the duodenum (Susbilla, 1996) but when pancreatic secretions increase the pH, peptic digestion ceases. In the small intestine, the peptide molecules can be further hydrolyzed by the action of both pancreatic and intestinal proteases. According to Moran (1982) as the digested particles move closer to the duodenal-jejunal junction mixing occurs with

pancreatic enzymes and bile acid salts. When the chyme reaches the jejunal lumen, pancreatic endogenous enzymes and intestinal brush border peptidases (Coring, 1980) hydrolyse non-degraded proteins and polypeptides released from proventriculus and gizzard digestion.

In the jejunum and ileum, digestion occurs in the lumen and on the surface of the intestinal cells. A number of different enzymes are required to break-down peptides to simple amino acids that can be absorbed effectively (Cavides-Vidal *et al.,* 2000). McClelland, (1979) showed that in the intestinal tract the enzymes involved in extracellular digestion of simple and complex protein are derived from the pancreas and small intestinal cells. The pancreatic enzymes include trypsin, chymotrypsin, endopeptidase (elastase) and exopeptidases (carboxypeptidase A and B) (Alpers, 1994; Karasov and Hum, 1997). The exopeptidases and aminopeptidases are the main enzymes that hydrolyse molecules in the small intestine (Tarvid, 1995). These enzymes will be further discussed in Section 2.3.2.

2.3.1 Pepsinogen precursor of pepsin

Pepsinogens are inactive precursors or zymogens of pepsins, gastric (or proventricular) proteinases that belong to the family of aspartic proteinases (Kageyama, 2002). The gastric proteinases are synthesized as inactive precursors so as to protect gastric or proventricular tissue from self-autolysis (Richter *et al.*, 1998). Pepsinogens are rapidly converted to pepsin in an acidic environment with the removal of 42-47 amino acid residues from the N-terminal end of the zymogens (Taylor and Tyler, 1986; Foltmann, 1988).

In mammals, five types of pepsinogens have been found, pepsinogens A, B, C (progastricsin), F and Y (prochymosin) (Kageyama, 2002). These pepsinogens are mainly synthesized and stored in the chief cells of the gastric mucosa and make up more than half of the proteins synthesized in the stomach. In poultry, unlike mammals, the pepsinogens are secreted from the same cells (oxynticopeptic cells) as produce acid (Hirschowitz, 1991; Gawlicka *et al.*, 2001).

In ostriches, the largest living species of birds, only two pepsinogens have been reported and they share identical N-terminal sequences with the pepsinogen A group of aspartyl proteases (Pletschke *et al.*, 1995). In the chicken, three major pepsinogens were

detected; embryonic pepsinogen (ECPg), pepsinogen A (cPgA) and pepsinogen C (cPgC). The embryonic chicken pepsinogen (ECPg), also known as prochymosin, has been reported to be produced only in the embryonic stage (Hayashi *et al.*, 1988). From day 7 of incubation, the proventricular epithelium cells begin to express ECPg and the amount increases until day 15 of incubation (Hayashi *et al.*, 1988).

Pepsinogen A (Donta and Van Vunakis, 1970) is secreted in highest amounts in adult chickens while chicken pepsinogen C is a minor component. Sakamoto *et al.* (1998) studied expression patterns of cPgA and cPgC genes from embryonic day 12 to post hatching day 7. Their results show that transcripts of both genes were detected in embryonic and adult proventriculi. However, mRNA expression of cPgA was seen from day 16 to day 19, and disappeared temporarily just before hatching while its transcription started and increased rapidly within one day after hatching. Pepsinogen C gene had similar pattern but the rate of expression was lower than that of pepsinogen A (Sakamoto *et al.*, 1998).

2.3.2 Pancreatic and intestinal enzymes

The pancreas is a major organ for synthesizing and secreting different kinds of enzymes produced by the pancreatic acinar cells. Secretions from the pancreas are physiologically regulated by the vagal nerve, acetylcholine released from postganglionic neurons and by the gastrointestinal hormone, cholecystokinin (CCK) (Owyang and Logsdon, 2004). Several investigators have described the roles of gastrointestinal hormones, particularly CCK and secretin on the growth of the pancreas and its tissue activity (Jorpes, 1968; Louie *et al.*, 1986; Wang and Cui, 2007). Pancreatic enlargement and increased protein synthesis have been reported in rats treated with CCK (Rothman and Wells, 1967) and stimulation of pancreatic DNA was observed by Mainz *et al.* (1973) after CCK administration. Compared to other animals, it is possible that there is an additional factor involved in the regulation of pancreatic function in chickens.

Watanabe and Yasuda (1977) showed that in contrast to other simple stomached animals, the nerve endings in the pancreas of the domestic chicken terminate in direct contact with the acinar cells. This shows greater involvement of neuro-control in synthesizing and secreting pancreatic enzymes in the domestic fowl than in mammals.

The pancreatic juice secreted into the duodenum consists of a number of different proteolytic enzymes that act on different parts of peptide chains. Protease enzymes are synthesised in an inactive form (zymogens) and are stored in the cells of the pancreas and secretion of enterokinase or enteropeptidase (Figure 2.1) from intestinal cells initiates proteolytic enzyme activation by first converting zymogens to their active forms (Boorman, 1976). The enzymes are capable of digesting the cells lining the GIT but are prevented from doing so by being stored in an inactive form. Activation of trypsin by enterokinase dramatically stimulates the activation of other pancreatic enzymes such as chymotrypsin from chymotrypsinogen, carboxypeptidase A and B from paracarboxypeptidase A and B, and elastase from proelastase (Figure 2.1).

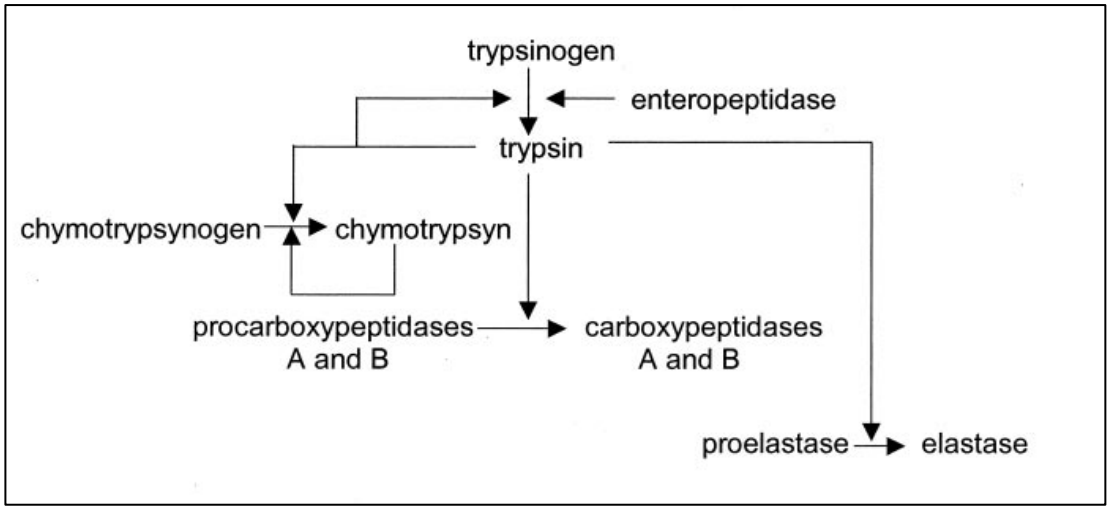

Figure 2.1 Schematic representation for the activation process of pancreatic proteolytic enzymes triggered by enteropeptidase from Orten *et al*. (1982)

The digestion of protein in the small intestine requires the action of several pancreatic enzymes. Each enzyme is specific for the cleavage of bonds between specific amino acids sequences.

Pancreatic enzymes for the hydrolysis of protein and peptide molecules are endopeptidases such as trypsin, chymotryspin, elastase and the exopeptidases, such as carboxypeptidase A and B. Both endopeptidases and exopeptidases catalyze the hydrolysis of peptide bonds at the initial and terminal polypeptide chain-linkages within the protein molecules (Vonk and Western, 1984) and carboxypeptidases hydrolyse peptide bonds at the carboxyl end of the protein molecule and remove amino acid residues sequentially (Tarvid, 1995). In younger poultry (broiler chicks and turkey poults)

the activity of pancreatic trypsin and chymotrypsin (Nitsan *et al.*, 1991a,b; Krogdahl and Sell, 1989) is well documented. Nitsan *et al.* (1991b) showed that heritability of birds may influence both the allomorphic changes and the activity of digestive enzymes such as trypsin, chymotrypsin and amylase. In a similar study carried out by Nir *et al.* (1993) the rates of activity and development and secretion of pancreatic enzymes varied between broiler and layer stains of poultry in the first two weeks after hatching. The rate of digestive enzyme development can affect the efficiency of digestion and subsequent growth of young birds.

Nitsan *et al.* (1974) showed that mechanical and humoral stimulation also causes increased enzyme activity along the small intestine. Feeding systems, according to some studies, may influence the response of the gastrointestinal organs in young birds either positively or negatively (Deaton, 1992; Barash *et al.*, 1993). Pinchasov *et al.* (1990) and Barash *et al.* (1993) both showed that enzyme development in the pancreas is a result of synthesis and secretion activities.

The two major groups of exopeptidases synthesised by intestinal cells and secreted into the lumen of small intestine are aminopeptidases and dipeptidases (Tarvid, 1995). These enzymes may be found within the intestinal cells and on the brush border membranes of proximal and also distal parts of the luminal intestinal cells. According to Desnuelle (1979) aminopeptidases, which hydrolyze peptide bonds between amino acids such as glycine and valine or glycine and leucine at the amino-end of polypeptide chain, are mainly associated with cell membranes: dipeptidases may found both in cell membranes and in intracellular parts of the small intestine.

2.3.3 Effect of age on enzyme function

Pancreatic enzymes have been detected during the incubation period in both chickens and turkeys before and after hatching (Moran, 1985; Sell *et al.*, 1991). Krogdahl and Sell, (1989) showed that there was a rapid increase in pancreatic amylase, trypsin and total protease activities in male turkeys during the first 14 to 21 days after hatch and slower increases or variable activities thereafter. Trypsin activity of the pancreas in poults changed dramatically in the first four days of age, but there was an increase between four and six days of age (Sell *et al.,* 1991). On the other hand, Pubols (1991) showed that the ratio of digestive enzymes could change with age and the diet of the birds. In posthatch broiler chicks, the amount of trypsin produced per gram of feed eaten decreased from 4 to 8 days of age but the actual amount of trypsin produced was increased (Noy and Sklan, 1997).

In chicks, chymotrypsin activity was reported by Nitsan *et al.* (1991b) to reach a maximum level at 11 days of age and then decrease. Trypsin development after hatching has been reported to not be active during the first two weeks of age, after that it started to increase significantly reaching higher levels after 21 days of age (Krogdahl and Sell 1989). In addition, in growing poults the activity of trypsin remained constant until eight weeks of age. In a study by Shih and Hsu (2006) on White Roman goslings, the activities of pancreatic trypsin showed a gradual increase from 7 to 11 days and 11 to 14 days of age while for chymotrypsin the activity increased from 3 to 14 d ages and remained high throughout the experimental period (up to 28 days after hatching). Nitsan *et al.* (1991b) showed that chymotrypsin activity in broiler chicks was highest at 10 to 11 days of age then it decreased significantly. In Krogdahl and Holm's (1982) study on pancreatic tissues they reported that the activity of proteolytic enzymes in White Leghorn chicks increased about 30% between 5 and 20 weeks of age and that it had probably reached mature levels before the first 20 weeks of age.

Changes in the activity of digestive enzymes with increase in age could be a result of stage of progressive development or maturity of a bird but could also be in response to dietary substrate levels or to amount of feed eaten (Karasov and Hume., 1997). Activity of aminopeptidase-N in birds and mammals is usually more dependent on diet content than age especially compared to other digestive enzymes (Sabat *et al.,* 1998; Sabat *et al.,* 1999). Although, diet affected the activity of some digestive enzymes in an experiment

carried by Brzek *et al.* (2009) on house sparrows no significant effect of diet on activity of aminopeptidase-N was found.

Finally, levels of digestive enzymes in the organ tissue and gastrointestinal tract may also vary according to the genetic stock of the poultry (O'Sullivan *et al.,* 1992; Nitsan *et al.,* 1991b).

2.3.4 Effect of diet on enzyme function

Nutrient digestion is an extremely complex process and it requires concurrent digestive enzyme activation. However, the rate of passage of food in the GIT may be altered by the amount of food consumed (Nitsan *et al.,* 1984) and this could alter digestive function. Diet manipulation may cause alterations in the synthesis and secretion of enzymes (Nir and Nitsan, 1979). In other studies, the presence of substrates in the small intestine can lead to the stimulation of pancreatic trypsin and chymotrypsin secretion (Niederau *et al.,* 1986; Valette *et al.,* 1992). Increasing the amounts of substrates such as carbohydrates and proteins resulted in increased activities of the relevant brush border enzymes (Siddons, 1972; Biviano *et al.,* 1993; Caviedes-Vidal *et al.,* 1994). In chickens fed a diet containing 50% carbohydrate there was almost twice the level of sucrase and maltase activity compared with chickens on a carbohydrate-free diet (Biviano *et al.,* 1993).

Presence of feed in the digestive tract (Pinchasov and Nitsan, 1990) and diet composition can alter synthesis or activity of pancreatic enzymes (Brannon, 1990). Supplementing a basal broiler chicken diet with 0.24% DL-methionine (Met) and 0.36% DL-methionine hydroxyl analogue (MHA) from hatch to three weeks of age, changed pepsin activity significantly. At 21 days of age pepsin activity in the proventriculus had increased with the Met supplementation, whereas for chicks fed MHA, the pepsin activity increased between 7 to 14 days of age (Lu *et al.,* 2003). Pancreatic trypsin activity increased at seven days of age with the addition of the MHA however, pancreatic chymotrypsin and intestinal dipeptidase activities did not change when birds where fed with either the Met or MHA supplements (Lue *et al.,* 2003).

Changes in pancreatic enzymes as a function of diet in the chick were also reported in an early study of Salman *et al.* (1967) who showed that changing the diet from one containing unheated to one with heated soybean meal caused an increase in the specific activities of pancreatic trypsin, chymotrypsin and amylase. Feeding regimes can also alter enzyme activity. In broiler chicks, a quantitative feed restriction (QFR) feeding regime, where the chicks were restricted to 40% of their intake from 5 - 10 days of age, and a meal-feeding (MF) regime where the chicks had access to their feed for two periods of three hours and one period of five hours per day from 5 – 17 days of age (Susbilla *et al.*, 2003), pancreatic general proteolytic activities (GPA) and carboxypeptidase-A activities were altered whereas the proteolytic activity in the proventriculus was unchanged by MF and reduced by QFR. On the other hand the QFR regime increased the amino and dipeptidase activities, whereas the dipeptidase activity was unaffected when the birds were on MF regime (Susbilla *et al.*, 2003).

Martin *et al.* (1998) showed that feeding growing ducklings a diet containing rice bran (600g/ kg) resulted in higher intestinal trypsin activity and increased pancreatic size. Activity of intestinal enzymes also can be affected by the concentration of protein in the diet. For example, increased aminopeptidase-N activity was seen in house sparrows (*Passer domesticus*) fed a diet high in protein, 60.3%, compared to when the birds were fed a diet low in protein, 12.8% (Caviedes-Vidal *et al.*, 2000).

2.4 Effect of fibre on enzyme function

Fibre added to the diet has been shown to enhance physiological activity and health of the GIT and, thus, increase nutrient digestion and absorption (Svihus and Hetland, 2001; Hetland *et al.*, 2003). However, Ikeda and Kusano, (1983) have also shown that NSPs can bind *in vitro* to digestive enzymes to reduce enzyme activities and digestion. Reductions in the activities of intestinal enzymes in the presence of soluble NSPs may however, be compensated for by increased cell division and size as has been shown in rats (Ikegami *et al.*, 1990).

In a review conducted by Dikeman and Fahey, (2006) the importance of assessing the physical properties of fibrous substrates and their effect on digestive secretions was highlighted. A number of experiments in other species have shown different effects of fibre types on enzyme activities. Early studies in rats also have suggested that short-term

intake of diets containing a 10% level of guar gum (Poskay and Schneeman, 1983) or a 5% level the soluble fibre pectin, did not affect the activities of pancreatic amylase, lipase, trypsin and chymotrypsin in rats (Forman and Schneeman, 1980). Also, the results of dietary supplements such as fructo-oligosaccharides (FOS) on the digestive enzyme activity in the pancreas of growing pigs, showed that supplementation with FOS has no significant effect on the activities of total protease, trypsin and amylase (Xu *et al.,* 2002). It has also been shown that feeding rats 50g/ kg dietary carboxymethycellulose (CMC) of varying viscosity rates had no significant effects on intestinal enzyme activities (Larsen *et al.,* 1994).

Feeding insoluble fibre to monogastric animals such as pigs alters the secretion of saliva, gastric juice, pancreatic juice, and bile salts suggesting that these effects mainly depend on the type of fibre used (Zebrowska *et al.,* 1983; Low, 1989; Dongowski *et al.,* 2002; Lallès *et al.,* 2007; Piel *et al.,* 2007; Wilfart *et al.,* 2007).

Lin *et al.,* (2010) showed that feeding young goslings to 21 days of age with a diet that included insoluble NSP such as barley, rice bran and wheat bran did not affect performance and pancreatic enzymes while those given soluble fibre from a pectin source tended to have reduced growth and to show a decreased activity of pancreatic enzymes. Isaksson *et al.* (1982) showed in *in vitro* studies that dietary fibre could inhibit pancreatic enzyme activity in human duodenal juice, and that this effect was mainly due to the viscosity, pH, and absorbability of fibre. When rats were fed a diet supplemented with 20% cellulose it did not cause any effect on pancreatic enzyme activity (Schneeman and Gallaher, 1980).

Sheard and Schneeman, (1980) added 5% wheat bran (WB) to the control diet of rats for up to 10 days and showed that there were no differences in the activity of pancreatic lipase, amylase, trypsin and chymotrypsin expressed per mg protein of pancreas. It has been reported that addition of 10% insoluble fibre as cellulose and alfalfa or 5% soluble fibre as pectin, guar gum and metamucil to a fibre free diet did not change the activity of pancreatic enzymes in the pancreatic gland of rats (Calvert *et al.,* 1985). Whole grains can stimulate gizzard development and promote gastroduodenal reflux leading to enhanced digestive enzymes activity on chyme particles (Hetland *et al.,* 2003; Taylor and Jones, 2003; Svihus *et al.,* 2011).

Changing feed form (Svihus *et al.*, 2004) from whole wheat to ground wheat stimulated both pancreas and liver secretions. Sarikhan *et al.* (2009) showed that the ratio of insoluble to soluble dietary fibre is important in formulating poultry diets in order to increase productivity.

It has been shown that adding insoluble dietary fibre as oat hulls and whole grain can increase pancreatic enzymes as well as bile salts levels in gizzard through promoting chyme reflux between gizzard and duodenum. A beneficial effect of fibre in the form of pure cellulose was noted by Boguslawska-Tryk (2005) on broiler chicken causing an increased body weight and a higher content of protein in the pancreas and a higher activity of proteolytic enzymes of the pancreas gland in those birds fed a diet containing the commercial product Arbocel.

Dunaif and Schneeman (1981) using an *in vitro* method, found that insoluble fibre decreased the activity of human pancreatic enzymes such as amylase, lipase, trypsin and chymotrypsin. An *in vitro* study carried out by Leng-Peschlow (1989) showed that the effect of fibre on the various enzymes varied individually and was not predictable. For example, wheat bran increased lipase, maltase and lactase activity and inhibited α-amylase activity while pectin and xylan decreased lipase and pepsin activity and increased chymotrypsin activity.

It is assumed that the inhibitory effects of fibre on enzymes are related to effects on viscosity, pH, and adsorption, and further that gastric acidification of fibre and conditions lowering intestinal pH may enhance these effects (Isaksson *et al.*, 1982). Schneeman *et al*., (1982) studied the response to dietary fibre in the pancreas and intestine of rats. In that study the addition of 20% insoluble fibre such as wheat bran indicated almost two-fold significant increases in the activity of trypsin and non-significant increases in the activity of chymotrypsin.

Poultry feed constituents can lead to changes in intestinal viscosity and mucin composition (Sharma *et al.*, 1997). Mucins are glycoproteins synthesized and secreted by goblet cells of the intestine (Sharma and Schumacher, 1995) and have protective properties in the gastrointestinal tract of animals. MUC2 is fundamental in maintaining the the gel layer on the intestinal surface and preventing microorganisms from attacking the host (Johansson *et al.*, 2008).

The study of Sharma *et al.* (1997) indicated that the consumption of either a maize-based diet or wheat-based diet or wheat based diet supplemented with 0.1% xylanase enzyme lead to changes in intestinal viscosity and mucin composition and these were associated with alterations in the goblet cell glycoconjucates of the chick intestinal tract. Montagne *et al.* (2004) observed that dietary factors such as fibre protein and anti-nutritional factors can influence the secretion and recovery of mucins in endogenous GIT losses.

Despite there being a considerable amount of information suggesting beneficial effects of fibre on digestive enzyme activity there has been very little systematic analysis of the effects of fibre on the activities of digestive proteases. There has also been very little work on the growth of GIT organs of layer, rather than broiler chicks, pullets to point of lay or in the possible changes with age in activities of proteolytic enzymes in different organs of the GIT of layer pullets.

Table 2.3 Enzyme activity in relation to the purpose of study

Age	Type of bird	Aim of study	Enzyme type	Organ	Activity	Author and year
120 d	RJF* + Broilers	Comparison	Trypsin and Chymotrypsin	Pancreas	60 to 150 40 to 80 U/g tissue	Kadhim *et al.* (2011)
21 d	Goslings	NSP sources	Trypsin and Chymotrypsin	Pancreas	1.2 to 1.4 15 to 20 U/mg protein	Lin *et al.* (2010)
42 d	Broilers	Cellulose	Trypsin and Chymotrypsin	Pancreas	29 30 U/mg protein	Boguslawska-Tryk (2005)
42 d	Broilers	Probiotics and Energy	Trypsin	Pancreas	5.8 U/ g tissue	Lima *et al.* (2003)
15 d	Broilers and Layers	Comparison	Trypsin and Chymotrypsin	Pancreas	200 to 250 80 to 90 U/g pancreas	Nir *et al.* (1993)
23 d	Broilers	Growth and development	Trypsin and Chymotrypsin	Pancreas	210 95 U/g pancreas	Nitsan *et al.* (1991a)
56 d	Turkey poults	Age	Proteas and Trypsin	Pancreas	50 to 100 70 to 100	Krogdahl and Sell (1989)

* Red Jungle Fowl

Chapter 3
Organ growth and activity of proteolytic enzymes in layer pullets of varying ages

3.0 Introduction

Early growth of the digestive tract in broiler chicks and poults is very rapid and the rate exceeds that of body weight gain for the first seven days of age (Nitsan *et al.*, 1991a,b; Sell *et al*., 1991; Obst and Diamond, 1992). After hatching, the gastrointestinal tract in chickens undergoes growth and maturation. The pancreas and small intestine in particular have critical impacts on nutrient digestibility and growth of chickens during the early post-hatching periods (Katanbaf *et al.,* 1988; Nitsan *et al.,* 1991a; Nir *et al.,* 1993). The pancreas of young broilers has been shown to have two different growth phases, a rapid growth phase during the first three days after hatching, and a slow growth phase from day 4 to day 8 (Nitsan *et al.*, 1991a,b; Sell *et al*., 1991; Obst and Diamond, 1992). However, Nir *et al.* (1993) showed an almost linear growth for pancreas, small intestine and liver from hatch to 15 days of age in broilers but in egg strain chicks those organs tended to plateau at about 5 to 8 days after. Relative growth tended to be similar in the two strains but that of the pancreas peaked later in the egg strain chicks (Nir *et al.,* 1993).

The growth rate in chickens can be altered via genetic selection (Barbato, 1991), however, feed composition can also influence growth. Several studies have reported that dietary composition and status may cause changes to the digestive enzyme system of the birds (Nir and Nitsan, 1979; Poort and Poort, 1981; Niederau *et al.,* 1986; Valette *et al.,* 1992; Brannon, 1990; Pinchasov *et al.,* 1990; Pubols, 1991). Improving the early development of digestive function in posthatch chicks will enable them to better utilise nutrients, grow efficiently and achieve their genetic potential (Ravindran, 2003). Noy and Sklan (1995) showed that during the first 4-7 days after hatching, digestibility of protein was 78% whereas by three weeks of age protein digestibility had increased to 90%. Changes in enzymes activity either in the pancreatic tissue or within the pancreatic juice could also be related to the diet and especially the concentration of dietary protein (Corring, 1980).

Layers are characterised by a slow growth rate relative to the modern meat type chickens and most of work on organ development and digestive enzyme function has been done on meat type chickens at early ages rather than in layer strain poultry (Chapter 2 Tables 2.2 and 2.3). Although the maintenance of growth and body weight is important during lay for successful egg production, the functional development of digestive enzymes in different parts of the gastrointestinal tract (GIT) and over the full growth period up to point of lay has not been as well documented in layers. Efficient digestive enzyme function is important for the supply of protein and other nutrients to laying pullets, however, little work has been done on proteolytic digestive enzyme activities beyond the first few weeks of life.

Therefore, the present study was designed to examine the development of the digestive organs of layer strain pullets from 1 to 18 weeks of age and to evaluate the activity of a number of enzymes essential for adequate digestion of dietary protein. The enzymes selected for study function at different stages of the breakdown of protein in the GIT and these were pepsin in the proventriculus, general proteolytic activity (GPA), trypsin and chymotrypsin in the pancreas and small intestinal dipeptidase and aminopeptidase. From such data on functional development it may be possible to develop feeding practices that optimise feed utilisation or stimulate protein digestive function.

3.1 Aims

To determine in normal layer pullets reared under commercial conditions,

- gross development of the digestive system and the organs that provide digestive secretions,
- rates of activity of enzymes in the proventriculus, pancreas and intestines that function to digest proteins and peptides.

3.2 Materials and Methods

3.2.1 Experimental animals, diets and design

Hy-Line Brown pullets were obtained at different ages from the same commercial pullet rearing farm in Yarrambat, Victoria. All pullets were from the same batch reared in the same shed on a free range floor system. They were fed according to the guidelines of Hy-Line Australia. The diets (Table 3.1) used followed the Hy-Line recommendations and covered the three growth periods; starter (1 to 8 weeks), grower (8 to 14 weeks) and developer (14 weeks to point of lay). The environmental conditions were carefully controlled and changes made gradually so that sudden changes would not affect the physiological status of the growing birds.

Eight pullets per age were selected randomly from the shed (7500 pullets max.) at weeks 1, 2, 4, 6, 8, 10, 12, 14, 16 and 18 after hatch. Only healthy pullets without injury were selected. The pullets were transported to the University by road and were killed 3 to 4 hours after being removed from feed in the growing shed. Pullets were weighed and euthanized by an intravenous overdose of 325 mg/ml of sodium pentobarbital (Valabarb Pitman-Moore Limited, Inc. USA).

The experiment was conducted in accordance with the principles and specific guidelines of La Trobe University Animal Ethics Committee, project number AEC10-53.

Table 3.1: Composition of commercial diets used to rear the layer pullets

Ingredients	Starter	Grower	Developer
Wheat Fine	57.86	57.97	56.04
Oats Fine	3.23	10.57	11.93
Peas Fine	15.00	12.00	5.87
Meat Meal	5.73	3.83	2.97
Blood Meal	2.00	2.00	0.00
Tallow Coater	0.50	0.00	0.00
Canola Meal	3.84	5.00	5.00
Soybean Meal	6.83	2.90	3.93
Millrun	0.00	3.27	12.00
Canola Seed	2.17	0.00	0.00
Limestone Fine	0.45	1.00	1.15
Supplements	1.94	1.46	1.11
Total (%)	100	100	100
Chemical composition (%)			
Dry mater	89.74	89.39	89.24
Moisture	10.26	10.61	10.76
Protein	20.52	18.01	15.97
Fat	5.02	3.04	2.90
Fibre	3.69	4.36	4.95
ME kcal/kg	2942.5	2796.6	2750.0
Calcium	1.00	1.00	1.00
Phosphorus	0.64	0.56	0.62
Av. P	0.49	0.40	0.45

Starter diet supplements (as percentage of total): RAM dried fine no. 2 salt 0.15; Sodium bicarbonate 0.15; Choline chloride (70%) 0.08; Dl-methionine 0.2; Avizyme 1210 Liquid 0.02; Ronozyme NP CT broiler 0.02; Biofix select 0.1; RAP-200 chick pullet PMX 0.2; L-threonine 0.03; L-Lysine plus 0.09; Acid oil mixer 1.20 0.33; Fysal FIT-4 0.15. (Hy-Line Brown-Ridley AgriProducts Pty Ltd;http://www.agriproducts.com.au).

Grower diet supplements (as percentage of total): RAM dried fine no. 2 salt 0.13; Sodium bicarbonate 0.25; Choline chloride (70%) 0.09; Dl-methionine 0.12; Avizyme 1210 Liquid 0.02; Ronozyme NP CT broiler 0.02; Biofix select 0.1; RAP-200 chick pullet PMX 0.2; L-Lysine plus 0.18; Acid oil mixer1.20 0.33; Potassium carbonate 0.02. (Hy-Line Brown-Ridley AgriProducts Pty Ltd;http://www.agriproducts.com.au).

Developer diet supplements (as percentage of total): RAM dried fine no. 2 salt 0.19; Sodium bicarbonate 0.17; Choline chloride (70%) 0.05; Dl-methionine 0.05; Avizyme 1210 Liquid 0.02; Ronozyme NP CT broiler 0.01; Biofix select 0.1; RAP-200 chick pullet PMX 0.2; Fysal FIT-4 0.15. (Hy-Line Brown-Ridley AgriProducts Pty Ltd;http://www.agriproducts.com.au).

3.2.2 Collection of samples

Immediately after birds were killed, the abdominal cavity was opened and the entire gastrointestinal tract was separated from the body cavity and placed on a glass sheet resting on ice. The proventriculus, gizzard, pancreas and small intestine were washed in cold normal saline, 0.9% w/v, dried with paper towel and then weighed. The small intestine included duodenum (from gizzard to pancreo-biliar ducts), jejunum (from pancreo-bilary ducts to Merkel's (vitelline) diverticulum, and ileum (from Mekel's diverticulum to ileo-cecal junction). The whole proventriculus and pancreas were stored at -80ºC. The jejunum and ileum were divided into proximal, medial and distal sections and 1 cm sections taken from the centre of each were weighed and stored at -80ºC (Appendix Photo 3.1).

3.2.3 Tissue preparation for enzyme assays and protein analysis

3.2.3.1 Proventriculus

The whole proventriculus was thawed and cut into small pieces and then placed into a glass centrifuge tube on ice. After thawing 0.01 M sodium phosphate buffer pH 7.0 was added at a ratio of 3ml buffer to 1g tissue and was homogenised for 55 seconds with a 12 mm Polytron homogeniser probe (Kinematica AG, Switzerland (Susbilla *et al*., 2003; Delia, 2008). The glass test tube that contained the tissue sample and phosphate buffer was kept on ice while it was homogenised. Each sample after homogenising was diluted 20 times with the buffer, 10.5 ml of buffer (containing about 0.5 ml of sample homogenate) was removed and placed into a plastic centrifuge tube kept on ice. Samples were centrifuged in a Multi-Purpose Refrigerated Centrifuge at 4ºC for 60 minutes at 12000 x g (ScanSpeed 1580R, Edwards Instrument Co. Code 7647518201 Denmark). The supernatant was kept at -80^{o}C until analysed for pepsin activity (Tarvid, 1992).

3.2.3.2 Pancreas

Whole pancreas was thawed and cut into small pieces and homogenised in a glass centrifuge tube kept on ice. A cold Ringer's solution, pH 7.4 at a ratio of 1 ml Ringer's to 60 mg of pancreatic tissue (Susbilla *et al,* 2003) was added to each sample before homogenisation using the 12 mm Polytron homogeniser at speed 6 for about 55 seconds. A 0.1ml aliquot of enterokinase solution (5 mg enterokinase from porcine intestine (Sigma E-0632)/ 10 ml Tris- HCl buffer (3.025 g Trizma base Tris (hydroxymethyl) amino-methane hydrochloride, Sigma T3253 + 20.95 ml 1 M HCl / 500 ml deionised water, pH 7.4) was added to 1ml of pancreatic homogenate. For general proteolytic activity the homogenate plus enterokinase was incubated on ice for to 2 hours, so that activation of zymogen granules would occur (Susbilla *et al.* 2003). For trypsin and chymotrypsin assays, the prepared homogenate plus enterokinase solution was incubated for one hour in a shaking water bath (Julabo SW, West Germany) at 25^{o}C (Erlanger *et al.*, 1961; Caviedes-Vidal and Karasov, 2001).

3.2.3.3 Small intestine

Proximal, medial and distal samples were taken from each small intestine as mentioned before; each segment part was placed at a glass test tube on ice. A ratio of 60 mg of tissue sample: 1 ml of ice cold Ringer's solution was added to each glass centrifuge tube. Each sample was homogenised by using the 12 mm Polytron homogeniser at speed 6 for 20 second. After that, each sample was kept on ice for the analysis of dipeptidase and aminopeptidase N activities (Susbilla *et al.,* 2003).

3.2.4 Measurement of enzyme activity

3.2.4.1 Pepsin activity in the proventriculus

The analytical procedure for the assay of pepsin activity of the proventriculus was based on the method of Anson (1938) modification by Tarvid (1992), Susbilla *et al.* (2003) and Delia (2008). Bovine haemoglobin (Hb) (Sigma H-2625) was used as the substrate (2.5 g Hb / 100 ml deionised water) after acidifying it with 25 ml of 0.2 M HCl solution to obtain pH 2.5. An aliquot of 1 ml of acidified haemoglobin was added to a glass test tube and was incubated for 5 minutes at 41^{o}C in a shaking water bath at 160 occultations per minute.

An aliquot of 10µl extracted supernatant was added to each glass test tube containing acidified haemoglobin solution and was incubated for a further 10 minutes. The enzyme reaction was stopped with the addition of 1 ml of 5% (w/v) trichloroacetic acid (TCA) solution. The sample was allowed to stand for 5 minutes at room temperature, and then filtered through Whatman No. 1 filter paper. From the filtrate 0.4 ml was added to a glass test tube containing 2 ml of 0.5 M sodium bicarbonate solution, and then the sample was mixed again and left to stand at room temperature for another 5 minutes.

A Folin-Ciocalteu's phenol reagent (Sigma F-9252) solution was prepared by mixing it 1: 1 v/v with deionised water, and 0.4 ml of this solution was added to each sample. After 20 minutes incubation at room temperature, free tyrosine released through hydrolysis of haemoglobin was measured at 750 nm in a spectrophotometer (Model U-1100 Spectrophotometer, Hitachi, Japan).

The amount of tyrosine released was determined from a tyrosine standard curve (Sigma T-3754) prepared from standard solutions ranging in concentration from 0 to 1mM. Standards were made up in a 1:1 v/v 0.01 M sodium phosphate buffer, pH 7.0: 5% TCA (w/v) solution. A 0.4 ml aliquot of each standard was added to a glass test tube containing 2 ml of 0.5 M Na_2CO_3 solution and each sample was left to stand at room temperature for 5 minutes. An aliquot of 0.4 ml of Folin-Ciocalteu's phenol reagent was added to each standard. The standards were incubated at 20 minutes at room temperature (Susbilla, 1996; Delia, 2008) and absorbance was measured at 750nm.

One unit (U) of general proteolytic activity was defined as 1µmole of tyrosine released per min under the assay conditions.

To convert units of proteolytic activity to pepsin units, porcine stomach mucosal pepsin (pepsin A, EC 3.4.23.1, Sigma P-7012) was diluted in 0.01 M sodium phosphate buffer, pH 7.0 and standards at concentrations ranging from 0 to 350 µg/ml were prepared. Aliquots of 0.1 ml of each pepsin standard were added to 1 ml of acidified haemoglobin, pH 2.5, which was incubated in a water bath at 41ºC for 5 minutes. Trichloroacetic acid (TCA), 5% (1ml, w/v) solution was used to stop the reaction of standards which were then filtered and absorbance was measured at 750 nm and activity expressed as 1µmole of tyrosine released either as units of pepsin per gram tissue or units of pepsin per total organ (Susbilla, 1996; Delia, 2008).

3.2.4.2 Pancreatic enzyme activities

a. General proteolytic activity (GPA)

The methods of Tarvid (1992) and Susbilla *et al.* (2003) with slight modification by Delia, (2008) were used to determine the general proteolytic activity of the pancreas. An aliquot of 2 ml of 2% casein solution (2 g casein Sigma C-8654 / 100 0.2 M sodium phosphate buffer pH 8.0) was added to a glass test tube as substrate and a 20µl aliquot of tissue extract after activation with enterokinase was added (Section 3.2.3.2). The solution was incubated in a shaking water bath at 41^{o}C for 20 minutes. Trichloroacetic acid 5% (1ml, w/v) was added to terminate the enzyme reaction.

The mixture was filtered through Whatman No.1 filter paper, a 0.4 ml aliquot was transfered to a test tube containing 2 ml 0.5 M Na_2CO_3 and allowed to stand for 5 minutes at room temperature. An aliquot of 0.4 ml Folin-Ciocalteu's solution (Section 3.2.4.1) was then added to each sample and they were allowed to stand for 90 minutes at room temperature. The absorbance was read at 720 nm. A tyrosine standard curve (Delia, 2008) was made in 0.2 M sodium phosphate buffer pH 8.0 and was kept at room temperature for 90 minutes after the addition of 0.4 ml Folin-Ciocalteu's solution. The GPA of the pancreas in growing layer pullets was determined as µmol of free tyrosine released from casein either per gram of tissue or per organ per minute.

b.Trypsin and chymotrypsin activities

The activity of pancreatic trypsin and chymotrypsin in the tissue homogenate was determined by applying the method of Erlanger *et al.* (1961) and modified by Caviedes-Vidal and Karasov (2001). Trypsin activity was determined by adding 16 µl tissue homogenate to 0.8 ml of 1 mM Nα-Benzoyl-DL-arginine-*p*-nitroanilide (DL-BAPNA, Sigma B4875) solution.

The DL-BAPNA solution contained 43.49 mg DL-BAPNA dissolved in a dimethylsulfoxide solution (1 ml dimethylsulfoxide (Sigma D2650) / 100 ml 0.05 M Tris-HCl buffer). The Tris-HCl buffer, pH 8.2 contained 3.54 g Trizma HCl (Sigma T3253), 3.34 g Tris (hydroxymethyl) aminomethane (Boehringer Mannheim Cat. No. 604205) and 2.94 g calcium chloride (Ajax Chemicals) / 1000 ml deionised water.

Chymotrypsin activity was determined by adding 16 µl of tissue homogenate to 0.8 ml of 1 mM N–glutaryl-L-phenylalanine-p-nitroanilide (GPNA, Sigma G2505) solution. The GPNA substrate solution contained 39.90 mg GPNA dissolved in 1 ml methanol / 100 ml 0.05 M Tris-HCl buffer, pH 7.6. The Tris-HCl buffer, pH 7.6 contained 6.06 g Trizma HCl, 1.39 g Tris (hydroxymethyl) aminomethane (Boehringer Mannheim Cat. No. 604205) and 2.94 g calcium chloride (Ajax Chemicals) / 1000 ml deionised water.

Samples for trypsin and chymotrypsin determinations were incubated in a shaking water bath at 40^{o}C for 20 minutes. For each sample an aliquot of 0.16 ml of 30% acetic acid was added to stop the enzyme reaction and then the absorbance was read at 410 nm. The concentration of *p*-nitroaniline was obtained from a *p*-nitroaniline (Sigma N2128) standard in 0.05 M Tris-HCl buffer pH 8.2 for trypsin activity and pH 7.6 for measuring chymotrypsin activity at concentrations ranging from 0 to 0.3 mM. A 0.8 ml aliquot of each standard was added to 16 µl of 0.05 M Tris-HCl buffer and 0.16 ml of 30% acetic acid. The absorbance of the sample was measured at 410nm.

A trypsin standard curve was prepared with trypsin (EC 3.4.21.4) from bovine pancreas, (Sigma T-1426) in 1 mM HCl. Concentrations ranged from 0 to 450 µg/ml. An aliquot of 5 ml of 1 mM DL-BAPNA solution, pH 8.2 was added to 0.9 ml deionised water. The solutions were incubated for 5 minutes in a water bath at 25^{o}C.

Trypsin standards (0.1 ml) were added to the incubated solutions and each standard was further incubated for 10 minutes at 25^{o}C. The enzyme reaction was stopped by adding 1 ml of 30% acetic acid solution to the incubated standards.

For chymotrypsin activity, standards (α-chymotrypsin, EC 3.4.21.1, Type II from bovine pancreas, Sigma C-4129) were prepared in a 1 mM HCl solution at concentrations ranging from 0 – 400 µg/ml. An aliquot of 5 ml of 1 mM GPNA solution, pH 7.6 was incubated in a water bath for 5 minutes at 25^{o}C for each standard. Chymotrypsin standards (1 ml) were added to the substrate solutions and further incubated for 10 minutes at 25^{o}C. With the addition of 1 ml of 30% acetic acid solution, the enzyme reaction was stopped and the absorbance was read at 410 nm.

The trypsin and chymotrypsin activities in layer pullets were expressed according to the method used by Delia (2008). For trypsin and chymotrypsin activities, results were expressed either as trypsin or chymotrypsin units per gram of tissue or per organ per minute.

3.2.4.3 Intestinal dipeptidase and aminopeptidase activities

Dipeptidase activity in the intestinal segments (proximal, medial and distal) was measured according to the method described by Tarvid (1992) and Susbilla *et al.* (2003) and modified by Delia (2008). Glycyl-L-leucine (Sigma EC 212.785.9) at concentration of 10 mM was prepared (188.23 mg / 100 ml Ringer's solution, pH 7.4). Two ml of this solution was added to a glass test tube containing 30 µl of tissue homogenate. After mixing gently, 100 µl was removed immediately (blank sample) and added to a glass test tube containing 2 ml absolute ethanol. The remaining sample was placed in a shaking water bath for 10 minutes at 41^{o}C. Another 100 µl was removed and mixed with 2 ml ethanol in a glass test tube, and after that the absorbance was measured at 220 nm.

The amount of glycyl-L-leucine hydrolysed by the activity of dipeptidase in the small intestine was determined by calculating the decrease in absorbance.

The activity of dipeptidase was expressed as nmoles of glycyl-L-leucine hydrolysed per gram of tissue by calculating the mean acivity/ g tissue from the sum of the proximal, medial and distal activities/ g.

Total activity for the small intestine (jejunum and ileum) was calculated by multiplying the means for the three parts (proximal, medial and distal)/ g by the total weight of the small intestine weight after subtracting the weight of duodenum.

The method of Caviedes-Vidal and Karasov, (2001) for the determination of aminopeptidase N activity was carried out by taking 1 ml of a 2 mM L-alanine-*p*-nitroanilide (Sigma A9325) solution (49.14 mg L-alanine-*p*-nitroanilide / 100 ml 0.2 M sodium phosphate buffer pH 7.0) and incubating for 5 minutes in a shaking water bath. A 10 µl aliquot of tissue homogenate was added to the substrate solution and was incubated at 40^{o}C for 10 minutes. To stop the reaction 3 ml of an ice-cold 2M acetic acid solution was added to the samples.

The absorbance was read at 384 nm. The concentration of *p*-nitroaniline formed was calculated from a *p*-nitroaniline standard curve in 0.2 M sodium phosphate buffer pH 7.0, at concentrations ranging from 0 to 0.3 mM.

The activity of aminopeptidase in the small intestine homogenate was expressed as µmoles of free *p*-nitroaniline released from L-alanine-*p*-nitroaniline per gram tissue or per total small intestinal parts (proximal, medial and distal) and was calculated as for dipeptidase activity.

3.2.4.4 Protein measurements

The protein concentrations in the homogenate extracts of the proventriculus, pancreas and small intestine were measured using the method of Lowry *et al*. (1951). Lowry mixture A was prepared from 0.5 % $CuSO_4.5H_2O$ MW 249.68 and 1 % Na-citrate MW 294.1 dissolved in distilled water.

Lowry mixture B was prepared from 2% Na_2CO_3 MW 105.99 and 0.4% NaOH dissolved in distilled water. Lowry mixture C was prepared immediately before use by mixing 1 ml of Lowry A and 50 ml of Lowry B. For the proventriculus, 100 µl of the homogenates was added to a test tube contains 10 ml of 0.01 M sodium phosphate buffer, pH 7.

Bovine serum albumen (Sigma Cat. No. A2153) was used as standard and for proventriculus this standard was prepared in 0.01M phosphate buffer pH 7.0 while for pancreas and small intestine it was prepared in Ringer's solution, pH 7.4 at concentrations ranging from 0 - 500 µg / ml. An aliquot of 0.4 ml of the diluted tissue samples was added to a glass test tube containing 2 ml Lowry mixture C.

Each sample (tissue sample, blank and standard) was mixed and left to stand at room temperature for 5 minutes. An aliquot of 0.4 ml of Folin-Ciocalteu's solution was added to each tissue sample and standard. The samples were mixed and left to stand at room temperature for another 40 minutes. The absorbance was read at 750 nm.

3.2.5 Statistical analysis

Data were checked for normality and were analysed using the General Linear Model procedure (SPSS 19.0 for Windows, USA). The differences between ages were determined by Turkey's test. Significance was at $\leq 5\%$. The histogram and the Q-Q plots for the Shapiro-Wilk's test (Shapiro and Wilk, 1965) showed that experimental data were normally distributed for all independent variables with skewness and kurtosis values in accordance to Doane and Sward (2011).

3.3 Results

3.3.1 Live body and organ weights

The live body weights and organ weights (Table 3.2) increased with age. Rates at which organs increased in weight tended to decrease after 8 to 10 weeks of age whereas gains in body weight tended to remain constant up to 18 weeks. Relative weights (RW) of proventriculus, gizzard, liver, pancreas and small intestine as percentage of the live body weight (g/100g body weight) are also presented (Table 3.3). Highest RW was observed in the first week and there was a steady decline in the RW of most internal organs with age. The RW of all organs at 18 weeks was about a third of that at 1 week.

Table 3.2: Live body weight and the weights of internal organs (g) of layer pullets at different ages (Mean ± SD, n = 8)

Age/ weeks	Live body	Liver	Gizzard	Proventriculus	Pancreas	Small intestine*
1	64.4 ± 4.38^{a}	2.50 ± 0.31^{a}	4.23 ± 0.39^{a}	0.70 ± 0.06^{a}	0.40 ± 0.05^{a}	5.44 ± 0.24^{a}
2	168.9 ± 6.79^{b}	5.09 ± 0.45^{b}	7.42 ± 0.46^{b}	1.22 ± 0.10^{b}	0.74 ± 0.08^{b}	9.73 ± 0.38^{b}
4	271.4 ± 17.78^{c}	7.56 ± 0.81^{c}	9.83 ± 1.00^{c}	1.57 ± 0.23^{b}	1.04 ± 0.13^{c}	14.61 ± 0.54^{c}
6	469.0 ± 26.36^{d}	12.94 ± 2.04^{d}	15.87 ± 1.84^{d}	2.44 ± 0.23^{c}	1.63 ± 0.10^{d}	20.73 ± 0.67^{d}
8	594.9 ± 67.54^{e}	18.63 ± 2.11^{e}	24.94 ± 3.66^{e}	3.49 ± 0.49^{d}	2.28 ± 0.21^{e}	30.88 ± 1.32^{e}
10	773.3 ± 97.10^{f}	18.90 ± 1.47^{e}	31.46 ± 6.48^{ef}	4.18 ± 0.42^{de}	2.64 ± 0.23^{ef}	35.39 ± 0.98^{e}
12	1008.5 ± 49.29^{g}	20.90 ± 2.09^{e}	30.41 ± 5.16^{ef}	3.98 ± 0.39^{de}	2.70 ± 0.35^{ef}	33.91 ± 1.56^{e}
14	1147.2 ± 50.44^{h}	20.65 ± 2.26^{e}	36.76 ± 4.49^{f}	4.32 ± 0.58^{de}	2.66 ± 0.44^{ef}	33.37 ± 1.11^{e}
16	1256.8 ± 81.16^{hi}	22.88 ± 2.46^{e}	37.39 ± 3.67^{f}	4.79 ± 0.71^{e}	2.79 ± 0.37^{ef}	33.64 ± 1.40^{e}
18	1407.0 ± 112.83^{i}	24.11 ± 4.05^{e}	34.12 ± 4.09^{f}	4.78 ± 0.71^{e}	2.92 ± 0.35^{f}	39.72 ± 1.77^{e}

Means within the same columns with different superscripts differ significantly ($P < 0.05$).*Small intestine (duodenum, jejunum and ileum).

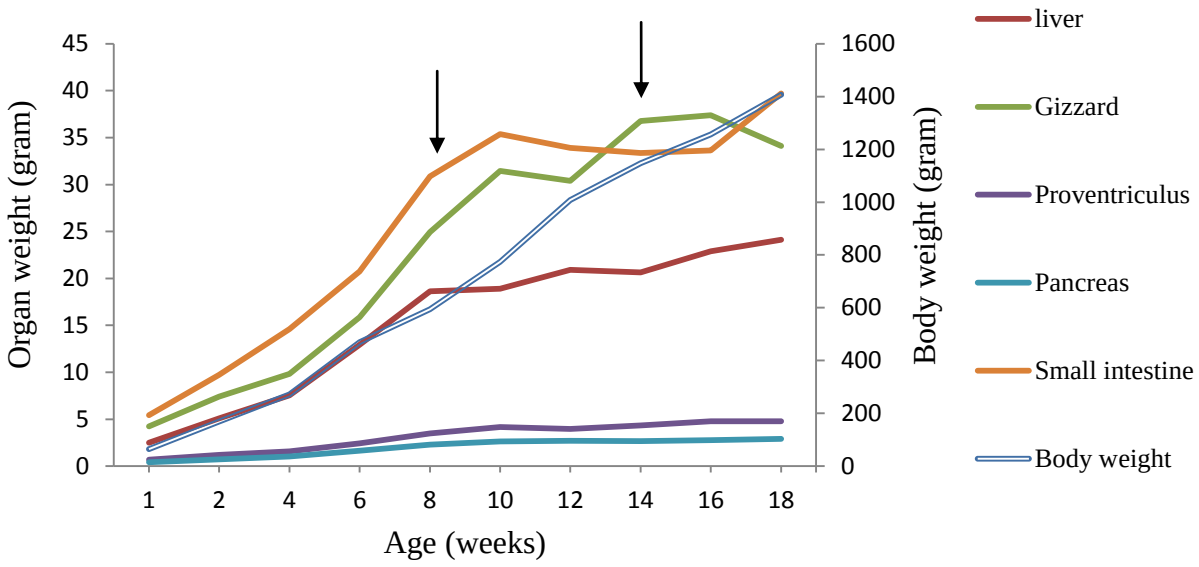

Figure 3.1 Live body weight and the weights of internal organs (g) of laying pullets at different ages (Mean ± SD, n= 8). Arrows indicate when feeds were changed from starter to grower (8 weeks) and grower to developer (14 weeks) feeds.

Table 3.3 The relative organ weights (RW, % body weight) of layer pullets at different ages (Mean ± SD, n = 8)

Age (weeks)	Liver	Gizzard	Proventriculus	Pancreas	Small intestine
1	3.90 ± 0.57^{a}	6.59 ± 0.71^{a}	1.09 ± 0.08^{a}	0.63 ± 0.08^{a}	8.46 ± 1.03^{a}
2	2.98 ± 0.27^{bc}	4.36 ± 0.42^{b}	0.71 ± 0.06^{b}	0.43 ± 0.05^{b}	5.77 ± 0.66^{b}
4	2.89 ± 0.26^{bc}	3.77 ± 0.46^{bd}	0.60 ± 0.06^{bc}	0.40 ± 0.07^{bc}	5.39 ± 0.54^{bc}
6	2.77 ± 0.37^{bc}	3.43 ± 0.53^{bd}	0.52 ± 0.04^{c}	0.35 ± 0.03^{c}	4.42 ± 0.35^{c}
8	3.09 ± 0.25^{ac}	4.14 ± 0.56^{bc}	0.58 ± 0.10^{bc}	0.38 ± 0.04^{bc}	5.25 ± 0.83^{bc}
10	2.52 ± 0.34^{bcd}	4.23 ± 1.23^{bc}	0.56 ± 0.13^{bcd}	0.35 ± 0.08^{bcd}	4.65 ± 0.77^{bc}
12	2.11 ± 0.16^{d}	3.08 ± 0.59^{cde}	0.40 ± 0.04^{de}	0.27 ± 0.03^{d}	3.36 ± 0.33^{d}
14	1.83 ± 0.13^{de}	3.25 ± 0.33^{cd}	0.38 ± 0.04^{e}	0.24 ± 0.03^{de}	2.91 ± 0.23^{de}
16	1.83 ± 0.14^{de}	3.01 ± 0.41^{de}	0.39 ± 0.07^{de}	0.22 ± 0.03^{de}	2.68 ± 0.26^{e}
18	1.69 ± 0.25^{de}	2.40 ± 0.24^{e}	0.34 ± 0.04^{e}	0.21 ± 0.02^{e}	2.82 ± 0.25^{de}

Means within the same columns with different superscript letters differ significantly ($P < 0.05$).

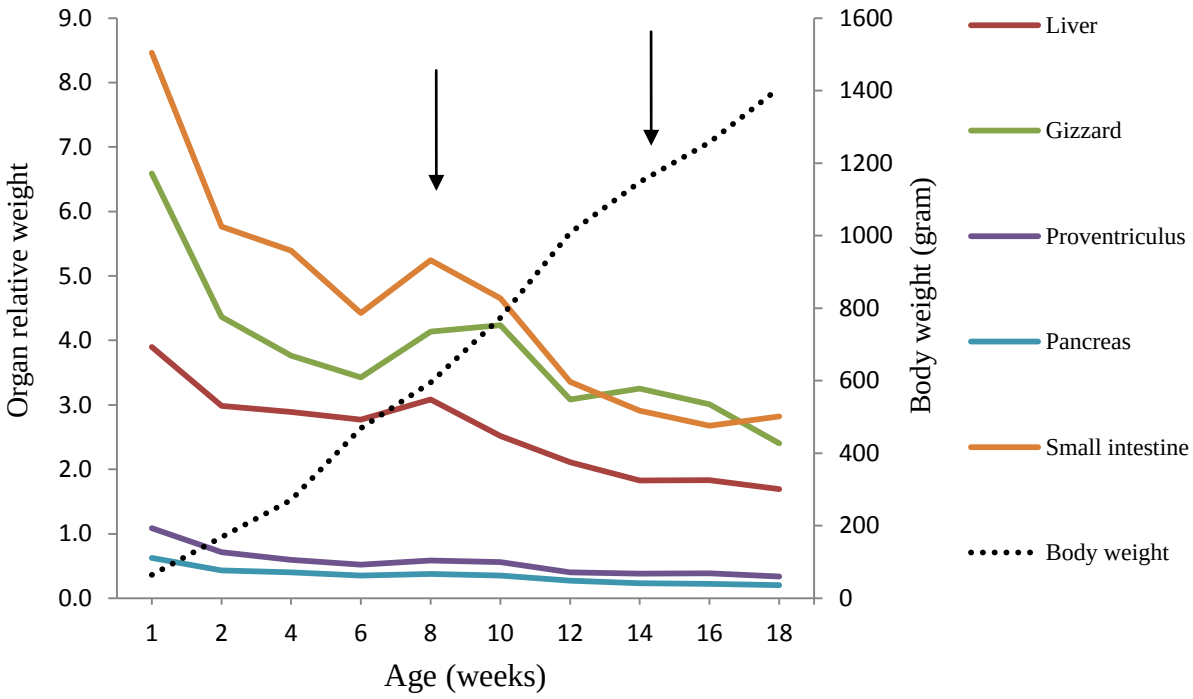

Figure 3.2 Live body weight and the relative weights of internal organs (g) of laying pullets at different ages (Mean ± SD, n= 8).). Arrows indicate when feeds were changed from starter to grower (8 weeks) and grower to developer (14 weeks) feeds. 3.3.2 Enzyme activities

3.3.2 Digestive enzyme activities

a. Pepsin activity in proventriculus

Pepsin activity in the proventriculus of layer pullets per gram tissue (Table 3.4) increased significantly ($P < 0.05$) to 4 weeks posthatch and thereafter remained at a plateau until week 18 except for a significant increase at week 12 compared with all other ages. The rates at 12 weeks of age showed a maximum of 98.50 porcine pepsin units per gram tissue / min. Overall pepsin activity per organ increased about 10 times from 1 to 18 weeks of age with the greatest increase (about 7.5 times) occurring between 1 and 8 weeks of age. Pepsin activity per organ showed a significant increase ($P < 0.05$) between weeks 1 and 4 and while there were no differences between activities at 4 and 6 weeks of age, significant differences were seen between activity per organ at 6 weeks and those in pullets aged 8 to 18 weeks.

Table 3.4 Pepsin activity in proventriculus of layer pullets at different ages (Means ± SD, n = 8)

Age	Porcine pepsin units per min per	
weeks	g tissue	organ (g)
1	58.19 ± 1.84^{a}	40.72 ± 3.99^{a}
2	74.77 ± 3.43^{b}	91.22 ± 10.53^{ab}
4	85.42 ± 6.09^{cd}	133.55 ± 20.31^{bc}
6	81.59 ± 2.40^{c}	198.90 ± 19.53^{c}
8	84.93 ± 2.46^{cd}	296.20 ± 44.23^{d}
10	86.70 ± 2.39^{cd}	361.26 ± 29.67^{de}
12	98.50 ± 2.85^{e}	392.31 ± 43.77^{e}
14	87.74 ± 3.56^{d}	378.42 ± 44.26^{e}
16	88.79 ± 4.54^{d}	427.08 ± 79.67^{e}
18	86.35 ± 2.64^{cd}	411.97 ± 56.52^{e}

Means within the same columns with different superscripts differ significantly ($P < 0.05$).

b. Pancreatic enzyme activities

1. General proteolytic activity

The general proteolytic activity (GPA) of the pancreas in the layer pullets increased ($P < 0.05$) with age (Table 3.5) with the highest rate of activity being observed at 12 weeks of age. Overall there was an 8-fold increase in activity from 1 to 18 weeks of age. There was a rapid increase from week 1 to 6 followed by significant decline at week 8 and then another increase to 12 weeks of age. Rate tended to plateau between 10 and 14 weeks of age. Although activity per g tissue at 8 weeks of age was significantly lower than activities at 6 weeks of age, when calculated on an organ basis there was no significant difference. When the activity was expressed as µmol free tyrosine released per organ / min. higher activities were observed between 10 and 18 weeks of age than those at 8 weeks of age or less.

Table 3.5: General proteolytic activity in pancreas of layer pullets at different ages (Means ± SD, n = 8)

Age	µmol free tyrosine released per min per	
weeks	g tissue	organ (g)
1	15.85 ± 1.33^{a}	6.37 ± 1.06^{a}
2	31.77 ± 1.78^{b}	23.51 ± 3.38^{a}
4	50.04 ± 4.79^{c}	52.11 ± 8.60^{a}
6	89.02 ± 2.93^{e}	145.04 ± 9.77^{b}
8	57.34 ± 5.99^{c}	130.23 ± 11.08^{b}
10	152.18 ± 2.96^{e}	400.31 ± 31.00^{c}
12	174.70 ± 2.65^{f}	470.87 ± 60.45^{d}
14	140.07 ± 3.93^{g}	372.74 ± 61.56^{c}
16	131.35 ± 3.23^{h}	366.63 ± 55.11^{c}
18	142.96 ± 2.40^{g}	417.01 ± 53.09^{cd}

Means within the columns with different superscript letters differ significantly ($P < 0.05$).

2. Trypsin

Although trypsin activity per gram pancreatic tissue of pullets increased significantly between 1 and 18 weeks of age the overall increase was only about 0.75 times (Table 3.6). Activity at 2 weeks of age was significantly ($P < 0.05$) higher than in the week before but there was no significant increases between weeks 2 and 8 weeks of age and differences among older pullets were only small. When the activity was expressed per organ (Table 3.6) there was an 8.5 fold increase between 1 and 18 weeks of age with the greatest relative increase (6.5 times) occurring between 1 and 10 weeks of age.

Table 3.6: Trypsin activity in pancreas of layer pullets at different ages (Means ± SD, n = 8)

Age	DL-BAPNA * trypsin units per min per	
weeks	g tissue	organ (g)
1	242.83 ± 8.23^{a}	97.59 ± 13.68^{a}
2	272.77 ± 6.61^{b}	201.49 ± 23.99^{ab}
4	280.64 ± 5.72^{b}	292.02 ± 37.21^{b}
6	280.11 ± 4.54^{bc}	456.58 ± 31.67^{c}
8	285.61 ± 3.58^{bc}	652.56 ± 66.14^{d}
10	275.07 ± 5.54^{c}	724.27 ± 65.17^{de}
12	285.76 ± 6.86^{c}	769.54 ± 93.08^{def}
14	309.48 ± 3.83^{d}	823.96 ± 138.15^{ef}
16	309.81 ± 5.31^{d}	864.22 ± 124.33^{f}
18	303.01 ± 6.24^{d}	884.14 ± 116.03^{f}

Means within the same columns with different superscript letters differ significantly ($P < 0.05$).

3. Chymotrypsin

The chymotrypsin activity per gram of pancreatic tissue of the layer pullets significantly increased in the first 4 weeks after hatching (Table 3.7). The maximum activity for chymotrypsin was at 6 weeks of age; however, no significant difference in activity was observed between week 4 and week 6.

Rate tended to be relatively constant from 4 to 18 weeks of age. The pattern of the activity per organ showed significant increases to 8 weeks of age and thereafter tended to be constant to 18 weeks of age.

Table 3.7: Chymotrypsin activity in pancreas of layer pullets at different ages (Means ± SD, n = 8)

Age	GPNA*chymotrypsin units per min per	
weeks	g tissue	organ weight (g)
1	5.07 ± 0.09^{a}	2.04 ± 0.25^{a}
2	6.43 ± 0.15^{b}	4.74 ± 0.51^{a}
4	7.38 ± 0.19^{c}	7.67 ± 0.93^{b}
6	7.41 ± 0.12^{c}	12.06 ± 0.62^{c}
8	6.70 ± 0.07^{de}	15.29 ± 1.34^{d}
10	6.45 ± 0.20^{b}	16.98 ± 1.41^{de}
12	6.78 ± 0.12^{e}	18.28 ± 2.39^{e}
14	6.64 ± 0.11^{bde}	17.64 ± 2.80^{de}
16	6.77 ± 0.13^{e}	18.87 ± 2.56^{e}
18	6.49 ± 0.14^{bd}	18.90 ± 2.11^{e}

Means within the same columns with different superscript letters differ significantly ($P < 0.05$).

c. Intestinal dipeptidase and aminopeptidase activities

Dipeptidase enzyme activity per g tissue of the small intestine of layer pullets did not increase significantly between 1 and 10 weeks of age after which there was a slight but significant increase to 18 weeks of age (Table 3.8). When expressed as activity per total small intestine per minute there were significant increases in activities between each age from 1 to 18 weeks (Table 3.8). Overall, activity increased 10 fold between 1 and 18 weeks of age with the greatest relative increase (7.5 times) between 1 and 10 weeks of age. Rate of amniopeptidase-N activity per gram small intestinal tissue did not change between 1 and 18 weeks of age (Table 3.8), however, when expressed per total small intestine (Table 3.9), activity increased significantly at each sampling time between 1 and 18 weeks of age.

Overall, activity increased more than 10 fold between 1 and 18 weeks of age with the greatest relative increase (10 times) between 1 and 10 weeks of age.

Table 3.8: Activities of dipeptidase and aminopeptidase-N per g tissue in small intestine (SI[1]) of layer pullets at different ages (Mean ± SD, n = 6)

Age weeks	Dipeptidase µmoles glycyl-L-leucine hydrolysed / g tissue/ min	Aminopeptidase-N nmoles *p*-nitroanilide released/ g tissue/ min
1	16.23 ± 0.48^{ab}	88.11 ± 10.46^{ab}
2	16.10 ± 1.06^{a}	91.38 ± 7.63^{ab}
4	17.09 ± 0.91^{a}	90.06 ± 11.42^{ab}
6	17.94 ± 0.89^{a}	94.74 ± 10.94^{ab}
8	17.78 ± 0.88^{ab}	76.80 ± 11.33^{b}
10	17.33 ± 0.38^{a}	101.23 ± 8.29^{ac}
12	18.12 ± 1.03^{cde}	99.34 ± 7.07^{ac}
14	19.71 ± 0.76^{e}	100.33 ± 6.40^{ac}
16	18.84 ± 1.30^{de}	101.64 ± 3.91^{ac}
18	18.83 ± 0.33^{de}	94.81 ± 4.42^{ac}

Means within the Columns with different superscript letters differ significantly (P < 0.05). [1]Small intestine [1]SI only (jejunum and ileum).

Table 3.9: Activities of dipeptidase and aminopeptidase-N in the whole small intestine[1] of layer pullets at different ages (Mean ± SD, n = 6)

Age weeks	Dipeptidase µmoles glycyl-L-leucine hydrolysed / small intestine/ min	Aminopeptidase-N nmoles *p*-nitroanilide released/ small intestine/ min
1	51.51 ± 5.09^{a}	242.21 ± 21.19^{a}
2	85.31 ± 3.43^{b}	420.56 ± 23.35^{b}
4	140.33 ± 4.10^{c}	614.82 ± 21.83^{c}
6	214.77 ± 2.36^{d}	1197.44 ± 21.00^{d}
8	313.57 ± 2.33^{e}	1465.42 ± 16.05^{e}
10	380.13 ± 4.52^{f}	2337.43 ± 23.77^{f}
12	330.13 ± 2.95^{g}	1680.16 ± 27.54^{g}
14	355.16 ± 4.60^{h}	1772.23 ± 35.49^{h}
16	371.32 ± 3.43^{i}	1975.89 ± 62.63^{i}
18	536.23 ± 7.76^{j}	2723.78 ± 69.76^{j}

Means within the columns with different superscript letters differ significantly (P < 0.05). [1]Small intestine (SI) only (jejunum and ileum).

3.3.3. Protein concentration of internal organs

a. Protein in pancreas and proventriculus

The protein concentration in both proventriculus and pancreas (Table 3.10) of the layer pullets increased with age. Highest protein concentration in proventriculus was (127.83 mg / g tissue) at 6 weeks while the maximum protein concentration in pancreas (442.69 mg / g tissue) was seen at 16 weeks. For the total amount of protein per organ (Table 3.9) both increased with age, with an 8 fold increase in proventriculus and 15 fold increase in protein in the pancreas between 1 and 18 weeks of age.

b. Protein in small intestine

Protein content in mg / g intestinal tissue increased significantly ($P < 0.05$) with age and maximum content of protein was recorded after 10 weeks (Table 3.11). The same patterns were obtained for the expression of protein content per organ and the highest rates for the amount of protein per organ was obtained at 10 weeks of age: overall there was a 10 fold increase in protein content per intestine (Table 3.10).

Table 3.10: Protein content (mg/g tissue) and (g/ organ) in proventriculus and pancreas of layer pullets at different ages (Means ± SD, n = 8)

Age	Proventriculus		Pancreas	
weeks	mg /g tissue	mg/ organ (g)	mg/ g tissue	mg/ organ (g)
1	102.56 ± 2.12^{a}	71.78 ± 7.03^{a}	217.34 ± 8.40^{a}	84.46 ± 10.38^{a}
2	115.40 ± 8.26^{ab}	134.85 ± 16.84^{ab}	271.39 ± 7.32^{b}	196.53 ± 24.20^{a}
4	115.15 ± 8.93^{ab}	180.83 ± 33.95^{b}	260.66 ± 16.43^{b}	256.17 ± 34.80^{a}
6	127.83 ± 7.08^{b}	312.13 ± 39.22^{c}	395.34 ± 6.64^{c}	658.06 ± 43.07^{b}
8	125.61 ± 5.15^{b}	437.30 ± 60.06^{d}	398.02 ± 9.32^{c}	932.25 ± 89.54^{c}
10	125.63 ± 9.50^{b}	523.78 ± 60.51^{def}	424.58 ± 13.14^{d}	1111.42 ± 95.55^{cd}
12	122.38 ± 8.35^{b}	486.56 ± 54.65^{de}	438.67 ± 8.58^{de}	1172.28 ± 150.86^{d}
14	125.36 ± 7.67^{b}	542.70 ± 86.72^{ef}	423.91 ± 8.19^{d}	1124.46 ± 195.38^{d}
16	121.88 ± 9.78^{b}	581.85 ± 82.26^{ef}	442.69 ± 13.68^{e}	1239.79 ± 178.31^{d}
18	123.23 ± 10.42^{b}	590.42 ± 107.74^{f}	438.39 ± 9.35^{de}	1277.20 ± 145.91^{d}

Means within the columns with different superscript letters differ significantly ($P < 0.05$).

Table 3.11: Protein content in the small intestine (mg/ g tissue) and (g/ organ) of pullets at different ages (Mean ± SD, n = 6)

Age	Protein content in small intestine*	
weeks	mg / g tissue	g / organ
1	143.63 ± 9.86[a]	0.77 ± 0.10[a]
2	166.35 ± 11.59[ab]	1.59 ± 0.22[ab]
4	163.60 ± 7.22[ab]	2.33 ± 0.28[bc]
6	168.85 ± 10.88[ab]	3.52 ± 0.39[c]
8	174.63 ± 19.19[b]	5.52 ± 0.66[d]
10	239.86 ± 17.13[d]	8.60 ± 0.54[g]
12	205.99 ± 18.38[c]	6.96 ± 0.40[ef]
14	178.99 ± 16.17[bc]	6.07 ± 0.89[de]
16	190.02 ± 16.70[bc]	6.39 ± 1.30[de]
18	187.99 ± 15.31[bc]	7.72 ± 0.91[efg]

Means within the columns with different superscript letters differ significantly ($P < 0.05$). *Small intestine (jejunum and ileum).

3.4 Discussion

3.4.1 Live body and organ weights

The live weights of the pullets obtained at different ages from the same commercial farm were within the growth weight guidelines recommended in the Hy-Line Brown Management Guide (Hy-Line International, 2014): from 1 to 18 weeks of age they were in the linear phase of weight gain and had not yet reached the plateau which normally occurs at about 22 – 25 weeks for layers of this strain.

The weights of specific organs; proventriculus, gizzard and other supply organs; small intestine, pancreas and liver all continued to increase to 18 weeks but the rates of gain tended to slow at about 8 – 10 weeks. Relative to body weight the weights of all the organs measured declined from 1 week of age. Nitsan *et al.* (1991b) showed a positive and significant correlation between age and the development of the gastrointestinal tract in chickens, and data presented here show a similar pattern of growth and development of the gastrointestinal tract.

The relative growth decreased from week 1 to week 2 as has been shown by Nir *et al.* (1993) for egg strain chicks. In the chicks used here, relative growth rate continued to decrease rapidly in all organs measured to about 8 weeks when rate of decrease slowed.

The rapid gastrointestinal growth rate in young chickens after hatching demonstrates the developmental changes in all internal organs (Katanabaf *et al.,* 1988; Jin *et al.,* 1998) and the need for adequate feed assimilation to support growth requirements. The relative pancreatic weight in commercial broiler chicks peaked at day 5 (Nir *et al.,* 1993) and continued until it decreased after day 11. Uni *et al.* (1999) showed that the relative weight of gizzard was almost constant from hatching to day 12, however, in the current experiment it decreased after week 3, in agreement with data of Ravindran *et al.* (2006). The small intestine in the present experiment had a maximal RW at one week of age, and then it declined rapidly to plateaued and previous studies had reported similar increases and decreases of RW of organs with ages (Sell *et al.,* 1991; Sell, 1996 and Sklan, 2001). The relative weight of the small intestine decreased with age, which is consistent with the findings of Santos *et al.* (2006) and Ravindran *et al.* (2006).

3.4.2 Pepsin activity of the proventriculus

No available published data was obtained to compare pepsin activity over different ages to 18 weeks in layer pullets. Pepsin activity in the proventriculus was measured at pH 2.5 the value that was also used in the study of Crévieu-Gabriel *et al.* (1999). The same authors measured chicken pepsins for protein hydrolysis with haemoglobin substrate at pH ranging from 1 to 5. (Crévieu-Gabriel *et al.,* 1999) and showed that pepsin activity in 25-d-old broiler chickens was optimal between pH 2.5 to 3.0. Pepsin activity also has been studied by Péron *et al.* (2007) in two different broilers genetic lines (D^+ selected for increased feed digestibility and D^- selected for lower feed digestibility) fed two wheat cultivars (Baltimor and Scipion). Results of that study showed that the pH level for optimum activity was around 3.0. The same authors recorded that proventriculus pepsin activities for the two broiler genetic lines at 27 days of age were respectively 16.5 and 19.2 (U/mg tissue/ min.) and however when the activity was expressed as (U/g BW) the values were higher in the D^+ (134.6) than D^- strain (84.8).

Overall, there was an increase in the rate of pepsin activity with age in the layer pullets used here. However, some changes could be related to the diet changes. Pepsin activity in proventricular tissue relative to body weight decreased in 29-day old broiler chicks fed a whole wheat protein concentrate diet as compared to the birds fed the complete more finely ground diet and the authors suggest that this may have been due to dilation of submucosal glands in the proventriculus and a resulting decreased stimulation of gastric juice secretion (Gabriel *et al.,* 2003). In the current experiment the diet of pullets was changed at 8 weeks from starter to grower and from grower to developer at week 14. This resulted in protein levels changing from 20% in starter diet to 18% and 15% in grower and developer diets, respectively: there was also an increase in fibre from 3.69 to 4.36 and 4.95% respectively. The change in fibre content from starter to grower diets did not appear to cause a significant effect on pepsin activity per gram tissue but may have contributed to the significant decrease in activity at 14 weeks.

3.4.3 General proteolytic activity of the pancreas

The GPA per g tissue tended to plateau at 8 weeks then rise between 8 and 10 weeks. The GPA in pancreatic tissue homogenates of layer pullets aged 2 and 4 weeks were respectively 31.77 and 50.04 µmol free tyrosine released /g tissue/ min.

In Susbilla *et al.'s* (2003) study the GPA activities of the pancreas of 12, 19 and 26 day old broiler chickens (37.0, 50.6 and 78.3, respectively) were similar to the activities of the 2 to 6 week old pullets measured here. In 21 day old layer chickens activity was about twice (112.7µmol free tyrosine released /g tissue/ minute) that measured here (Delia and Frankel, 2006). This difference could be related to the changes in feeding system and the feeding staus of the birds before collection of samples. Pancreatic proteolytic activity has also been measured in 27 day old broilers (Péron *et al*., 2007) in the two genetic lines (D^+ and D^-) described previously and fed two wheat cultivars (Baltimor and Scipion). In this study data indicated that the GP activities for both broiler genetic lines were 24.5 and 21.8 µmol free tyrosine released /g tissue repectively when fed the Baltimore strain of wheat and and 19.3 and 24.1 respectively when fed the Scipion strain. This suggests that source of feed as well as strain of poultry can affect the activity of enzymes.

Susbilla *et al.* (2003) showed an increase in the rates of GPA between 19 and 26 day old broiler chickens (50.6 and 78.3 µmol free tyrosine released/ g tissue respectively) and a similar result was seen in the present study at similar ages, 2 and 4 weeks, the GPA rates increased with age from 31.77 to 50.04 µmol free tyrosine/ g tissue. The change in the rate of GPA could be related to synthesis and secretion activities in pancreatic tissues and also to sampling conditions. Enzyme activities in chickens (Pubols, 1990) can be changed according to the diets as well as to the ages. The activity in the pancreas is a result of synthesis and secretion (Pinchasov *et al.,* 1990; Barash *et al.,* 1993).

3.4.4 Trypsin

The rates of trypsin activity in layer pullets measured here in 2 and 4 week old pullets old (272.7 and 280.64 DL-BAPNA trypsin units /g tissue/minute respectively) were similar to those in three week-old layer chicks (252.1 DL-BAPNA trypsin units /g tissue/minute) measured by Delia and Frankel, (2006). Activity slowly increased about 20% from 1 to 18 weeks of age: a different pattern from that reported by Kadhim *et al.* (2011) in broiler chicks from day old to 17 weeks old where trypsin activity increased about 2.5 times from 1 to 10 days (1.5 weeks) and then tended to remain constant to 17 weeks. In red jungle fowl on the other hand, activity increased from day old to 17 weeks old (Kadhim *et al.,* 2011). Nir *et al.* (1993) showed that between hatching and 14 days of age trypsin activity increased steadily to 11 days and then decreased to 14 days old and showed that activities in broiler and layers was similar.

Nitsan *et al*. (1991b) and O'Sullivan *et al.* (1992) showed that genetic background is an important factor that can affect the development of the intestinal tract and consequently digestive enzyme activity in chickens. Diet or feeding regime may influence rates of activities, however, Pinchasov *et al*. (1990) showed that the activity of the digestive enzymes in the pancreas of broilers under two feeding systems were not affected consistently by the feeding regime, but were more age related. Nitsan *et al.* (1991b) showed that selection for growth rate of Plymouth Rock dual purpose poultry for fast or slow growth rate affected the relative rates of activitry of pacreatic trypsin activity between 3 and 23 days of age and that there was a difference in activities between those strains and broiler chicks. For example in the broiler chicks trypsin activity increased slightly from 3 to 9 days and then decreased at 23 days: in the line selected for fast

growth pancreatc trypsin activity decreased from 3 to 23 days and in the slow growing birds the pattern was similar to the broilers but decline to 23 days was not as great.

Nir and Nitsan (1979) speculated that synthesis and release of pancreatic enzymes might depend on the amount of feed that had been delivered to the gastrointestinal tract and Niederau *et al.* (1986) and Valette *et al.* (1992) showed that in rats the secretion of trypsin and chymotrypsin in particular depends on the rate of substrate available at the small intestine. However, in this experiment there was not a consitent effect at the ages (8 and 14 weeks) when diets were changed.

3.4.5 Chymotrypsin

The chymotrypsin activity in the pancreas of pullets aged 2 and 4 weeks were 6.4 and 7.4 GPNA chymotrypsin units /g tissue/minute which is similar to the activity of 10.8 GPNA chymotrypsin units /g tissue/ minute obtained by Delia and Frankel, (2006) on a layer strain at three weeks of age.

In layer and broiler strain chicks, chymotrypsin activity decreased slightly from hatching to 8 days of age and then increased more than two-fold at 14 days (Nir *et al*., 1993). Kahdim *et al.* (2011) also showed differences between breeds of poultry: pancreatic chymotrypsin activity increased from about 40 units/g at day old to about 80 units/g at 10 days in broiler chicks but then remained constant to 120 days of age (17.5 weeks). In red jungle fowl activity slowly increased from about 20 units/g at day-old to about 40 at 120 days. Previous reports correlate pancreatic enzymes activity according to the body weight and the intestinal development (Nitsan *et al.*, 1991a,b; Nir *et al.*, 1993; Sklan and Noy, 2000).

The chymotrypsin activity in young avian species has been previously documented (Nitsan *et al.*, 1974; Nitsan *et al.*, 1991a,b; Nir *et al.*, 1993; Publos, 1991; Cavides-Vidal and Karasov, 2001). The activity of digestive enzyme from hatching to 14 days of age in broiler and layer chicks was also clearly discussed by Nir *et al.*, (1993) and the patterns of activities were similar in both types except the activities in the small intestinal contents were lower in broilers. For example, enzyme secretions to the duodenum per gram feed intake (Uni *et al.*, 1995) was higher in heavy-strain Arbor Acres birds than in light-strain Lohmann chicks on day 4 after hatching but thereafter no

differences were apparent. Also, because of the limitations of research data available in adult birds, the ensuring discussion were focused on young birds, however, data may partially agree to previous studies that correlated the enzymes activities to age and diet factors. A recent study carried by Brzęk *et al.* (2013) showed that the activity of pancreatic enzymes in birds is under strong genetic control, which enables evolutionary adjustment to typical diet composition but is less adapted for short term, diet-related flexibility.

The content of enzymes in the pancreas is highly dependent on the maturity of the animal, the feeding state, the chemical composition of the feed and the feed quality (Poort and Poort, 1981). Activation of digestive enzymes could be related to the physiology of the birds to modulating specfic enzymes according to substrate levels, rather than maintaing high enzyme activity constantly (Karasov and Hume, 1997). Pinchasov *et al.* (1990) showed that when broilers were under *ad libitum* or alternate day feeding from 14 to 83 days of age, the activity of the digestive enzymes, such as amylase, lipase, trypsin and chymotrypsin was not affected by the feeding regime. In addition, when the activity was expressed as U/g tissue for amylase it increased and that of chymotrypsin decreased with age.

3.4.6 Dipeptidase and Aminopeptidase activities

Intestinal enzymes are responsible for the terminal digestion of peptides and play a vital role in regulating the amount of amino acids available for absorption (Tarvid, 1995). Dipeptidase activity measured in the homogenates increased significantly for the first 10 weeks of age. Both enzymes, aminopeptidase and dipeptidase, have been studied in chicken small intestines and were found to be expressed early posthatch (Tarvid, 1992). After hatch, the profile of enzymatic activity in the small intestine adapts to the amount of exogenous substrate delivered to the intestinal tract. Different regions of the small intestine have their own distinctive patterns for the development of enzyme activity. In the present study increase in the rates of activities has been recorded with ages. Part of these changes in peptide activity could be related to a functional adaptation of the small intestine of growing birds which occurred as a result of dietary manipulation (Susbilla *et al.*, 2003).

Intestinal enzymes have been studied extensively in domestic birds (Nir *et al.,* 1978; Sell *et al.,* 1989; Biviano *et al.,* 1993; Jackson and Diamond, 1995), however, dipeptidase enzyme has been reported only in very few studies (Tarvid, 1991, 1992; Lu *et al.*, 2003; Susbilla *et al.,* 2003; Delia, 2008). Also, most previous studies focused on chickens at early ages. In the study of Susbilla *et al.* (2003) dipeptidase activity in intestinal segments (proximal, medial, and distal) of broiler chickens at day 5, 12, 19 and 26 were differed in the different segments. Yasumoto and Sugiyama, (1981) showed that the distal part of the small intestine was more sensitive to the changes in the feeding regime. In Susbilla *et al.* (2003), the rates of activities (µmoles boc-glycyl-L-leucine/g tissue/ min) in the small intestine jejunum and ileum of broilers at day 5, 12, 19 and 26 of age, were 200, 460, 380 and 380 respectively. In present study, the rates of dipeptidase activities in the total small intestine of growing pullets measured with a different substrate at 1, 2, 4 and 6 weeks of ages were slightly lower 51.5, 85.3, 140.3 and 214.8 µmoles glycyl-L-leucine released/ organ/ min. It has been observed that the genetic background of the breed has a profound effect on the maturation of the GIT and consequently on the development patterns of the digestive enzymes (Tarvid, 1995). It is possible therefore that the differences between enzyme activities in the broiler and layer strains may be the results of the variation in strain.

Aminopeptidase-N activity increased linearly with age until 10 weeks. A previous study showed that activity of 19 day old broiler chickens was 2.47 µmoles free leucine released from L-leucine-β-naphthylamide/g tissue/ minute (Susbilla *et al.,* 2003). The present study showed that the activity of aminopeptidase-N at weeks 2 and 4 were 0.42 and 0.62 µmoles L-alanine-*p*-nitroanilide/ small intestine/ minute. In the study of Jang *et al.* (2007) activity of leucine aminopeptidase in 35 day-old broiler chickens in the proximal intestine, was 489 µmoles *p*-nitroanilide /g proximal mucosa (surface)/ minute. It was also found that the medial segment of the small intestine showed maximum aminopeptidase-N activity at different incubation periods (Delia, 2008). Tarvid (1991, 1992) showed that different regions of the small intestine have their own distinctive patterns for the development of enzyme activity. Vonk and Western (1984) showed that body mass and dietary feed are believed to be considered as the main factors affecting the activity of intestinal enzymes. Moreover, aminopeptidase-N is considered as the most important exopeptidase in the small intestine of birds, often used as index for protein digestion capacity (Tarvid, 1991).

In conclusion, the data reported here supply additional evidence regarding the rates of aminopeptidase activity in the intestinal segments at different ages. Additional factors such as feeding system, breed of chickens and bird status may affect and alter the activity in different patterns. However, the present data indicated significant increases in the rates of aminopeptidase-N with age in layer pullets up to 18 weeks of age.

3.4.7 Protein concentrations (proventriculus, pancreas and small intestine)

The protein concentration in both proventriculus and pancreas of the layer pullets was measured at different ages. Data for proventriculus showed that levels of protein started to increase significantly up to 2 weeks of age. Susbilla *et al.* (2003) found that two weeks old broiler chickens had protein concentration of 61.7 mg/g tissue while the protein concentration in two weeks growing pullets was 115.4 mg/ g tissue. Delia (2008) found a similar protein concentration in three week old layer chickens (122.2 mg/g tissue) to the present study.

The reason for some discrepancy in difference between data from the current study and Susbilla *et al.* (2003) could be due to differences in strain of chickens studied and assay conditions. In the present experiment, the protein concentration in the pancreas of 21 day old layer chicks was 271.4 mg/g tissue and was similar to 237.3 mg/tissue found by Susbilla *et al.* (2003) in 19-d-old-broiler chickens while the layer chickens in Delia's (2008) study had protein concentrations of 508.4 mg/g tissue. Pancreatic protein level was shown to increase with the age of chickens (Jamroz *et al.*, 2002), but values mainly depend on poultry species.

In broiler breeder pullets, Iji *et al.* (2002) found that the pancreas of 10 weeks old pullets contained 200 mg / g tissue protein while at 14 weeks of age the protein concentration was almost 100 mg/ g tissue. Diet composition and structure also can affect the protein concentration in the pancreas. For example, Schneeman and Richter (1993), noted a significant increase in the concentrations of protein in the pancreas in rats fed with a diet enriched with dietary fibre such as oat bran. Increased pancreas weights and increased concentration of protein in the gland were also reported by Isaksson *et al.* (1983) feeding wheat bran to rats for 10 days. The protein concentration in small intestine also increased with age.

3.4.8 Conclusion

Internal organ development in growing laying pullets showed increases with ages of birds. Rapid increases were recorded in particular for the supply organs (e.g., pancreas and small intestine) that are essential to achieve maximal growth at an early age. Internal organs such as liver and gizzard both are important for the chemical and physical food digestion and rapid growth in these organs as well as the development in proventriculus will help to support body requirements. Therefore, the functional maturation of these organs is important in the assimilation of feed to reach optimum weights.

The findings of this study suggest that the activity of enzymes for protein digestion could each be regulated by different mechanisms, and there were positive increases in the rates of activity as pullets increased in age. The greatest changes in digestive organ growth and enzyme function were seen between the ages of 6 to 12 weeks however some intestinal peptidases showed increases up to 18 weeks of age that is to the point of egg production. However, in some instances, diet can be considered as one of the main factors that regulate the synthesis and secretions of digestive enzymes from specific organs of the gastrointestinal tract. From the pattern of expression of digestive enzymes activities and organ development in the present results, further studies could be developed to draw a clear conclusion concerning the possibility of using dietary feeds to improve feed formulation and optimize growth and development in layer hens.

Chapter 4
Effect of insoluble fibre on digestive organs and enzymes of egg laying hens

4.0 Introduction

Inclusion of fibre in the diet of growing or productive chickens has been shown to induce positive changes in digestive physiology. For instance, both insoluble and soluble fibres influence transit time (retention time) of ingested food from the crop to the gizzard and can also stimulate gizzard activity (Mateos *et al.,* 2012) which improves mixing of feed particles with digestive secretions and hence improve digestion and absorption of nutrients.

The potential benefits of fibre depend to a great extent on the physicochemical characteristics of the fibre source. Soluble fibres can increase viscosity in the small intestine and thereby inhibit digestion and absorption (Choct and Annison, 1990; Smits and Annison, 1996). Most of the research work that has been done on insoluble fibre indicates that this fraction is considered as a nutrient diluent or bulking material in monogastric animal diets (Mateos *et al.,* 2012).

However, more recently the inclusion of insoluble NSPs in chicken diets has been proposed as an important means for modifying gut development and digestive functions (Hetland *et al.,* 2004). For example, the inclusion in the diet of insoluble fibre, from oat hulls, at levels between 2 to 3% usually improves the growth performance of broilers fed low-fibre diets (Mateos *et al.,* 2012) and diluting broiler and layer diets with insoluble fibres in the form of oat hulls and wood shavings, can benefit nutrient digestion (Hetland *et al.,* 2003; Hetland *et al.,* 2005). The increase in starch digestibility they described may have been the result of an increased amylase concentration in the chyme of the jejunum (Hetland *et al.,* 2003).

A study by Roberts *et al.* (2007b) showed that there is no negative effect of including dietary fibre in the form of 10% dried distillers grains, 7% wheat middlings, or 5% soybean hulls on the productivity and feed consumption of laying hens at point of lay.

It has also been shown that inclusion in the diet of 3% inulin or cellulose (Arbocel FD00) improved egg production and reduced feed intake and body weight gain of broiler breeder hens from 43 to 55 weeks of age (Mohiti-Asli *et al.*, 2012). Inclusion of fibre in the diets of laying hens may also influence ammonia emission from poultry manure in laying-hen facilities (Roberts *et al.*, 2007a).

In the previous chapter (Chapter 3 Section 3.3.1) weights of supply organs tended to reach their maximum weight at 8 to 10 weeks of age whereas live weight had not reached a maximum by the end of the experiment when pullets were 18 weeks of age. Activities of digestive enzymes also tended to reach maximum activities before live weight had reached mature weight.

The experiment described in this chapter was designed to determine whether it was possible to increase the weights of supply organs of laying hens after point of lay by supplementing their feed with a commercially available insoluble fibre product. In addition, the effect of the insoluble fibre supplement on proteolytic enzymes was determined in order to understand possible mechanisms by which insoluble fibre may influence productivity and body growth.

4.1 Aim

To determine the effect of the 0.8% insoluble fibre concentrate, Arbocel (Arbocel® RC, J. Rettenmaier and Söhne GmbH and Co., Rosenberg, Germany) on the growth of the gastrointestinal organs and on digestive enzyme function of layer hens kept under commercial conditions from 19 to 31 weeks of age.

4.2 Materials and Methods

4.2.1 Experimental Birds

The experiment was carried out using hens obtained from a commercial hatchery, Country Lane Poultry Farm, Tynong North, Victoria Australia. The company was testing, for their own information, whether addition of 0.8% Arbocel (Table 4.2) to the ration they normally used, would increase egg productivity, decrease canabilism and decrease water

content of the manure. The company agreed to let us collect hens before the start of their feeding trial and at 3, 6, 9, and 12 weeks after the start.

Two diets were formulated for the company by Ridley AgriProducts Pty Ltd, the Control diet (Table 4.1) was fed to half the hens in the shed and the other diet (IF) containing 0.8% Arbocel (Table 4.2) was fed to hens in the other half of the shed. The shed contained 50,000 Hy-Line Brown hens which were placed there at 18-19 weeks of age. The concentrations of fibre in the two treatments were analysed by ***FEEDTEST*** (Agrifood Technology Pty Ltd) and were as follows for Control 3.3%, and for the IF treatment diet, fibre content was 4.0%.

The shed housing the hens was fully automated temperature and humidity controlled with, temperature sensors throughout the shed to monitor conditions and adjust ventilation accordingly. In order to be able to obtain hens that were kept under as similar environmental conditions as possible, hens for this experiment were sampled from the middle tiers of the two central rows in the shed. Thus hens in the row on one side of the center most passage way were fed the control ration, (Group C), and the hens in the adjacent row facing them were fed the ration containing 0.8% Arbocel (Group IF). There were 208 cages per row; the size of each cage was 500mm x 500mm or 500 cm^2 per bird floor space. Hens were housed five per cage.

At time 0, soon after hens were placed in the shed and before the Arbocel ration was fed, eight hens were randomly collected and at 3, 6, 9 and 12 weeks after the start of the feeding trial, eight hens from Group C and eight from Group IF were collected. Hens were transported by road from the farm in Tynong North to La Trobe University.

On each sampling day eight cages per treatment were randomly picked and one hen removed. The hens were weighted and killed with an overdose of barbiturate and samples of the GIT and organs were weighed and tissue samples of proventriculus and pancreas were collected and stored at -80^{o}C until analysed for enzyme activity as described in Chapter 3. The experiment was conducted in accordance with the principles and specific guidelines of LTU-AEC the project number AEC11-42 Section 3.2.3.

Table 4.1 Ingredient composition and calculated nutrient content of the control diet (as-fed basis)

Raw materials	%
Wheat (11.5%)	53.73
Soya (47.5%)	16.1
Limestone (38%)	5.57
Canola Exp (36/9)	5
Peas	5
Meat M (50%)	4.83
Oats (7%)	3.33
Lime Grit (38-Ca)	3.33
Acid Oil-Coater	1.33
Acid Oil	0.90
Farm Pride Layer supplement **	0.83
Rovabio Excel (75%)	0.03
Choline CHL (75%)	0.02
Chemical composition	**Analysis**
ME Kcal/kg	2800
Crude protein	19.57
Lysine	0.944
Methionine	0.469
Crude Fat	4.626
Crude Fibre	3.450
Ash	13.277
Calcium	4.099
Av. Phos.	0.450

**Farm Pride Layer supplement (premix) (http://www.agriproducts.com.au)

Table 4.2 Arbocel properties (Arbocel® RC, J. Rettenmaier and Söhne GmbH and Co., Rosenberg, Germany)

Properties	Arbocel® RC Fine Lignocelluloses
Colour	Yellowish
Structure	Granules
Crude fibre content	65%
Acid detergent lignin	20%
pH	4.5-6.5
Water holding capacity	500% - 700%

4.2.2 Statistical analysis

Data were analysed using the General Linear Models procedures of SPSS statistics (SPSS 19.0 for Windows, USA). All data were tested for normality. The significance between time after feeding means were examined by Turkey's test. Significant was at 5%. A t-test was used to compare live weights and organ weights of Control hens at the start of the experiment with those at the end.

4.3 Results

Information on the laying performance up to 40 weeks of age (May 2011 till February 2012) provided by the company showed that the hens in Group IF had 2.1% higher rate than those hens in Group C. They also reported an improvement of litter/ manure quality.

4.3.1 Body weight and internal organs weight

The live weights of control hens increased significantly ($P < 0.05$) from the initial weights at Time 0 (age 19 weeks) to those at Time 12 (age 31 weeks) (Table 4.3): all organs except for the gizzard and spleen increased significantly with age. The live weights of hens in Group IF were not significantly different from control hens (Table 4.3). Although only liver, gizzard and spleen of Group IF hens were significantly heavier than those of Group C hens at a few time points; the general trend was that organ weights were higher in Group IF hens (Table 4.3). Only the gizzard at 12 weeks after the start of the trial and liver and spleen at 9 weeks showed significantly higher relative weights (Table 4.4).

Table. 4.3 Live body weights and the weights of internal organs (g) of laying hens fed a commercial diet (C) or with a commercial diet plus insoluble fibre (IF) from 19 to 31 weeks of age (Mean ± SD, n = 8)

Time on diet	0	3		6		9		12	
Age (weeks)	19	22		25		28		31	
Treatments	Initial weight	C	IF	C	IF	C	IF	C	IF
Body weight	1519.3 ± 96.3	1773.9 ± 111.3	1743.9 ± 101.4	1793.1 ± 165.9	1822.1 ± 90.3	1888.3 ± 105.7	1882.7 ± 156.7	1902.1 ± 114.7	1919.4 ± 104.9
Liver	21.32 ± 3.65	33.91 ± 4.34	38.50 ± 4.73	36.82 ± 4.64	42.58 ± 3.32	39.97 ± 1.52*	45.13 ± 2.63*	40.01 ± 7.29	43.57 ± 5.22
Gizzard	20.85 ± 1.63	21.11 ± 1.23	21.76 ± 1.97	21.19 ± 2.52	22.01 ± 2.79	21.36 ± 1.96	22.97 ± 2.42	20.93 ± 2.35*	25.19 ± 1.57*
Proventriculus	4.45 ± 1.03	4.50 ± 0.53	4.71 ± 0.50	4.82 ± 0.81	5.27 ± 0.50	5.30 ± 0.59	5.55 ± 0.42	5.58 ± 0.50	5.97 ± 0.74
Pancreas	2.97 ± 0.49	2.98 ± 0.51	2.95 ± 0.42	3.24 ± 0.48	3.24 ± 0.43	3.47 ± 0.21	3.29 ± 0.39	3.66 ± 0.48	3.90 ± 0.59
Duodenum	10.25 ± 1.95	13.02 ± 1.90	13.29 ± 1.36	14.81 ± 2.17	15.52 ± 2.72	14.80 ± 0.84	14.93 ± 2.06	14.61 ± 1.77	15.56 ± 1.64
Small intestine	28.55 ± 2.78	35.03 ± 2.29	34.24 ± 3.52	40.33 ± 4.84	42.30 ± 5.81	39.23 ± 4.14	43.07 ± 2.78	36.96 ± 3.76	41.19 ± 3.80
Spleen	3.04 ± 0.55	1.87 ± 0.69	2.29 ± 0.64	2.16 ± 0.27	2.62 ± 0.50	2.24 ± 0.26*	3.19 ± 0.45*	2.68 ± 0.72	2.90 ± 0.31

* Means within the same row at same time point differ significantly ($P < 0.05$).

Table. 4.4 Relative weights (g/ 100g body weight) of internal organs of laying hens fed a commercial diet (C) or with a commercial diet plus insoluble fibre (IF) (Mean ± SD, n = 8)

Time on diet	0	3		6		9		12	
Age (weeks)	19	22		25		28		31	
Treatments	Initial weight	C	IF	C	IF	C	IF	C	IF
Proventriculus	0.29 ± 0.06	0.25 ± 0.04	0.27 ± 0.03	0.27 ± 0.03	0.29 ± 0.03	0.28 ± 0.04	0.30 ± 0.02	0.29 ± 0.02	0.31 ± 0.04
Gizzard	1.38 ± 0.12	1.19 ± 0.07	1.25 ± 0.11	1.18 ± 0.10	1.21 ± 0.13	1.14 ± 0.14	1.23 ± 0.19	1.10 ± 0.11*	1.32 ± 0.08*
Pancreas	0.19 ± 0.03	0.17 ± 0.03	0.17 ± 0.02	0.18 ± 0.02	0.18 ± 0.02	0.18 ± 0.01	0.18 ± 0.02	0.19 ± 0.02	0.21 ± 0.03
Liver	1.40 ± 0.16	1.91 ± 0.19	2.21 ± 0.25	2.10 ± 0.17	2.34 ± 0.14	2.23 ± 0.14*	2.40 ± 0.14*	2.20 ± 0.34	2.27 ± 0.24
Duodenum	0.67 ± 0.09	0.74 ± 0.13	0.77 ± 0.09	0.83 ± 0.14	0.86 ± 0.18	0.79 ± 0.08	0.79 ± 0.09	0.77 ± 0.12	0.81 ± 0.07
Small intestine	1.88 ± 0.09	1.98 ± 0.20	1.96 ± 0.15	2.25 ± 0.16	2.33 ± 0.37	2.09 ± 0.30	2.30 ± 0.17	1.95 ± 0.23	2.15 ± 0.24
Spleen	0.20 ± 0.04	0.11 ± 0.04	0.13 ± 0.03	0.12 ± 0.02	0.14± 0.02	0.12 ± 0.01*	0.17 ± 0.02*	0.14 ± 0.04	0.15 ± 0.02

*Means within the same row at same time point differ significantly ($P < 0.05$).

4.3.2 Pepsin activity in proventriculus

In both treatments pepsin activities per gram tissue per minute and per organ per minute increased gradually with age of the hens from initial values at 19 weeks to 31 weeks and at that age were significantly greater ($P < 0.05$) than at 19 weeks (Table 4.5). Pepsin activities per gram tissue were significantly ($P < 0.05$) greater at 3 and 9 weeks in Group IF hens (Table 4.5) compared with those in the Control Group.

When calculated per organ, significantly greater activity was only seen at 3 and 12 weeks after the start of the feeding trial. The protein content in the proventriculus per gram tissue increased significantly ($P < 0.05$) in both treatment groups from the initial concentration at 19 weeks to that at 31 weeks of age. There was no effect of treatment on protein content.

4.3.3 Pancreatic enzyme activities

Compared with the activity at the start of the experiment there was a significant decrease in general proteolytic (GP) activity in Group C hens at 22, 25, and 28 and 31 weeks of age while in Group IF significantly lower values were only seen at 25 and 28 weeks of age (Table 4.6). The GP activities (Table 4.6) were significantly ($P < 0.05$) greater in Group IF hens after 3, 6, 9 and 12 weeks than those of hens in Group C but in Group IF there was no significant increase with age.

When activities were expressed per organ, activities were higher in Group IF at 9 and 12 weeks after the start of the experiment. Activities from 19 weeks of age tended to decrease until 28 weeks of age but at 31 weeks of age there was no significant difference when compared with initial activities.

Chymotrypsin activities (Table 4.6) expressed per gram pancreatic tissue was significantly higher in Group IF ($P < 0.05$) compared with those of the control group at 9 and 12 weeks after the start of the feeding trial. When expressed per organ, activities of chymotrypsin in Group IF hens at 12 weeks were significantly higher ($P < 0.05$) than those in Group C.

For trypsin activities (Table 4.6) the only significant difference between treatments was seen after 12 weeks on the diets, with Group IF having higher activities

when expressed per gram pancreatic tissue ($P < 0.05$). Over time activities tended to decrease in both treatment groups.

Protein content of Group IF was significantly higher ($P < 0.05$) only after 3 weeks after the start of the treatments: after that no significant differences between treatments were seen. Protein content of the pancreas tended to increase in both treatment groups with age from 19 to 31 weeks After 3 weeks on the experimental diets significant differences ($P < 0.05$) were detected in Group IF compared to Group C.

Table 4.5. Enzyme activity and protein content in proventriculus of laying hens fed a commercial diet (C) or with a commercial diet plus insoluble fibre (IF) (Mean ± SD, n = 8)

Time on diet	0	3		6		9		12	
Age (weeks)	19	22		25		28		31	
Treatments	Initial value	C	IF	C	IF	C	IF	C	IF
Pepsin activity per g tissue	71.04 ± 1.69	80.01 ± 3.03*	89.08 ± 5.41*	86.99 ± 4.84	89.18 ± 4.52	89.96 ± 4.45*	95.52 ± 6.06*	87.89 ± 6.84	93.09 ± 8.63
per organ	315.09 ± 69.46	359.58 ± 38.77*	418.48 ± 41.43*	418.82 ± 73.50	469.65 ± 49.49	476.52 ± 57.19	529.64 ± 46.73	490.29 ± 55.22*	557.54 ± 102.45*
Proventriculus Protein mg/ g tissue	143.81 ± 11.55	171.73 ± 9.16	165.76 ± 6.81	175.04 ± 10.74	179.28 ± 19.40	179.10 ± 17.59	182.60 ± 14.67	174.56 ± 13.03	178.01 ± 16.84

*Means within the same row at same time point differ significantly ($P < 0.05$).
Pepsin activity in proventriculus was defined as the pepsin hydrolysis of 1µmol of substrate (haemoglobin) in 1 min per g tissue or per organ weight.

Table 4.6. Enzyme activity and protein content in pancreas of laying hens fed a commercial diet (C) or with a commercial diet plus insoluble fibre (IF) (Mean ± SD, n = 8)

Time on diet	0	3		6		9		12	
Age (weeks)	19	22		25		28		31	
Treatments	Initial value	C	IF	C	IF	C	IF	C	IF
GP									
per g tissue	136.42 ± 8.51	116.36 ± 8.92*	139.01 ± 9.57*	93.84 ± 9.53*	107.81 ± 6.95*	59.24 ± 13.43*	81.58 ± 9.31*	96.95 ± 9.44*	122.18 ± 7.01*
per organ	405.62 ± 80.16	344.56 ± 50.72*	406.27 ± 70.96*	303.73 ± 47.66	349.39 ± 53.38	203.57 ± 36.65*	269.21 ± 48.90*	356.40 ± 66.75*	475.01 ± 65.68*
Chymotrypsin									
per g tissue	5.27 ± 0.35	5.22 ± 0.33	5.15 ± 0.17	5.14 ± 0.12	5.27 ± 0.33	5.82 ± 0.20*	6.12 ± 0.34*	5.04 ± 0.35*	5.46 ± 0.31*
per organ	15.75 ± 3.50	15.55 ± 2.89	15.01 ± 2.18	16.64 ± 2.27	16.98 ± 1.64	20.18 ± 1.57	20.18 ± 2.86	18.42 ± 2.39*	21.27 ± 3.40*
Trypsin									
per g tissue	257.51 ± 12.47	229.20 ± 18.01	217.30 ± 8.07	266.92 ± 29.73	278.72 ± 20.35	210.78 ± 16.25*	237.84 ± 18.21*	208.76 ± 15.10	219.99 ± 23.72
per organ	760.40 ± 108.86	688.83 ± 162.47	632.07 ± 83.08	867.35 ± 170.15	900.38 ± 120.89	732.17 ± 87.90	780.52 ± 89.38	767.69 ± 136.70	865.86 ± 198.45
Protein									
per g tissue	378.73 ± 8.73	352.04 ± 9.40*	399.53 ± 9.65*	468.88 ± 21.73	493.83 ± 16.87	423.46 ± 21.17	445.21 ± 19.09	397.38 ± 14.74	412.40 ± 14.19

*Means within the same row with at same time point differ significantly ($P < 0.05$).

GP = General proteolytic activity, µmoles free tyrosine released from casein substrate/ min/ g tissue or pancreas.

Chymotrypsin = chymotrypsin units of *p*-nitroaniline released from N –glutaryl-L- Phenylalanine-p-nitroanilide (GPNA)/ min / g tissue or pancreas.

Trypsin = trypsin units of *p*-nitroaniline released from Nα-Benzoyl-DL-arginine-p-nitroanilide (DL-BAPNA) / min/ g tissue or pancreas.

4.4 Discussion

4.4.1 Live body and organ weights

The live weights at the start and end of the experiment were within the normal ranges for the Hy-Line Brown strain (Hy-Line Brown Management Guide, 2014). From 19 to 31 weeks of age there is a cessation of weight gain and increasing reproductive tract and fat cell development (Hy-Line Brown Management Guide, 2014). Comparing organ weights at the start of this experiment with those of the same strain at 18 weeks of age (Chapter 3), shows the liver and gizzard weights of the hens used here to about 16% and 42% less than those used previously. By 31 weeks of age the liver weights in both treatment groups were greater than those in the previous experiment but gizzard weights had not increased above those seen in birds in Chapter 3. On the other hand relative weights of the liver were only slightly lower at 19 weeks of age in the birds used here than in the 18 week old birds described in Chapter 3 and by 31 weeks of age relative weights were higher in these birds. Relative weights of the gizzard used in this experiment were about 43% less than those described in the previous experiment and in control hens the relative weight decreased by 31 weeks of but not in hens in the IF Group.

The poultry farm was unable to provide any information as to the rearing and feeding of the pullets prior to their being placed in the layer shed that could account for these differences in liver and gizzard weights.

The inclusion of the insoluble fibre, Arbocel, into the diet of pullets at the point of lay was able to influence the weights of some internal organs and the activities of digestive enzymes involved in protein digestion. The relative weights of gizzard and pancreas in 29 week old Leghorn hens (Hetland *et al.,* 2003) were similar to those of the Hy-Line Brown hens in this experiment and they reported a similar increase in relative gizzard weight with increased insoluble fibre intake: Hetland *et al.* (2003) showed a significant increase in the relative weight of the gizzard in 29 week old Leghorn pullets supplemented with wood shavings (insoluble fibre) for 14 weeks when compared with unsupplemented controls. As was found in this experiment, Hetland *et al.* (2003) also reported no effect of the fibre supplement on pancreas weight.

Feeding 19 week old Dekalb strain layer hens a diet supplemented with 0.8% insoluble raw fibre concentrate (IRFC) for 16 weeks improved egg production and improved efficiency of feed utilization (Lim *et al.*, 2013) however there was no significant effect on relative organ weights although the relative weights of the organs reported by Lim *et al.* (2013) are similar to those in the Hy-Line Brown hens used here. Lim *et al.* (2013) suggest that the improvement in feed utilisation could have been due to changes in the physical properties of the digesta allowing more rapid penetration of digestive enzymes and increased retention time in the gut however, it is possible that increased pancreatic enzyme activity indicate of increased production seen here (Table 4.6) could also have contributed to the improved feed utilisation.

Short term feeding of a high fibre diet (21.5% NDF + ADF) to adult hens (approximate live weight 2kg) for only 2 weeks did not result in differences in digestive organ weights when compared with hens given a low fibre diet (11.5% NDF + ADF) (Courtney Jones *et al.*, 2013). This suggests length of time for feeding insoluble fibre is important for achieving positive responses. The liver (38.4 – 41.0g), proventriculus (8.1 – 9.2g) and gizzard (26.5 – 29.6g) weights in the hens used by Courtney Jones *et al.* (2013) were similar to the weights of the hens in this experiment at 31 weeks of age.

Several studies showed the positive action of dietary fibre source on organ growth and development while little is known of the mechanism by which insoluble fibre can change the digestive secretions. However, recent work (Hetland and Svihus, 2001; Svihus *et al.*, 2002) indicates that the passage time of the feed particles decreases when coarse fibre or whole cereals are fed to chickens. Insoluble fibres have been shown to enhance physiological activity of the gastrointestinal tract and, thus, increase nutrient digestion and absorption (Hetland and Svihus, 2001; Gabriel *et al.*, 2003; Hetland *et al.*, 2003; Engberg *et al.*, 2004) and its positive effects may be related to the formation of a fibre network in the GIT which changes the transit time of digesta thus allowing better penetration of digestive enzymes (Choct, 2001; Lim *et al.*, 2013).

4.4.2 Enzyme activities

The activity of pepsin in the 19 week old hens in this experiment were about 17% lower the 18 week old birds (86.4 ± 2.64) in the previous experiment (Chapter 3) but per organ the activity was about 25% lower even though there was very little difference in the organ weights of the two groups of birds. By 31 weeks of age activity per g tissue in the control and IF groups were greater than that of 18 week old birds in Chapter 3 but on a whole organ basis that of the control birds was about 16% and IF Group birds about 26% higher than that of the 18 week old birds in Chapter 3. This suggests that activity can be improved with age and (on and organ basis) with fibre supplementation.

The inclusion of fibre in poultry diets has been shown to increase the retention time of the digesta in the upper part of the digestive tract from crop to gizzard and to stimulated gizzard function (Rogel *et al.*, 1987b; Hetland *et al.*, 2005) and the production of HCl in the proventriculus (Duke, 1986). The inclusion of coarse and insoluble fibre can reduce the pH of the gizzard contents and promote its development as well as stimulating gut motility (Hetland *et al.*, 2002, 2003, 2004; Gonzalez-Alvarado *et al.*, 2008; Jimenez-Moreno *et al.*, 2009a,c). A low pH less than 2 in the upper GIT improved pepsin activity (Guinotte *et al.*, 1995). The increase in pepsin activity in the hens given the insoluble fibre supplement could have been due to changes in gizzard function as well as to increased production of the proenzymes of pepsinogen.

Activities of the pancreatic enzymes expressed either per g tissue or per organ were slightly lower in the 19 week old hens than the 18 week old birds in Chapter 3 (Tables 3.5 to 3.7). Although the general proteolytic activity of the pancreas decreased with age total activity of the whole organ increased by 31 weeks of age and that of the IF group was significantly greater. A similar effect was seen for chymotrypsin but not trypsin suggesting the mechanisms of control on the different pancreatic enzymes could be different. The rates of synthesis and secretion of pancreatic enzymes can influence enzyme activity measured in pancreatic tissue (Barash *et al.*, 1993) and by the presence of feed in the digestive tract. In goslings Lin *et al.* (2010) showed that different types of fibre in the diet could influence pancreatic enzyme functions with soluble NSP causing a decrease in trypsin and chymotrypsin activities and rice bran causing an increase in

trypsin, but not chymotrypsin, activity relative to barley hulls but not the other insoluble fibre sources they used.

The effect of insoluble fibre on the pancreas and on enzyme activities could have been due to the release of hormones such as cholecystokinin (CCK) and secretin that can control growth and function of the pancreas (Watanabe and Yasuda, 1977). However, adding insoluble dietary fibre in the form of oat hulls and whole grain has been shown to increase pancreatic enzymes as well as bile salt levels in the gizzard through promoting chyme reflux between gizzard and duodenum (Hetland *et al.*, 2003, 2004) and it is possible that effects on pancreatic enzymes is due to mechanisms involving reflux from the duodenum or gizzard.

5.4.3 Conclusion

In conclusion, this study shows that laying hens fed a diet containing 0.8% insoluble raw fibre concentrate (Arbocel) had a significant increase in gizzard weight compared to the hens fed a control diet but there was no significant effect of the insoluble fibre on live weight or weights of other organs even though they were higher than weights in control hens. The increases in pepsin and pancreatic enzymes possibly contributed to the improvement in egg production reported by the poultry farm but did not affect growth rate.

An interesting observation has been that though the hens in the previous study described in Chapter 3 were lighter at 18 weeks than the 19 week old hens at the start of this experiment the gizzard weight and relative gizzard weights were very much lower. Without the insoluble fibre supplement gizzard weights did not increase over the 12 weeks of the experiment whereas other supply organs did. The observation of the wide variation in gizzard weights amongst poultry flocks within Victoria and the effects of a small addition of insoluble fibre could be of importance to the commercial poultry industry and to the development of feeds that improve productivity.

Chapter 5

Effect of insoluble fibre supplementation applied at different ages on digestive organs and enzyme function of growing pullets

5.0 Introduction

The role of fibre in the nutrition of chickens and its possible beneficial effects has attracted increasing attention (Choct, 1997). Growth and development in layer pullets during rearing and early lay is important to the commercial poultry industry because overall uniformity in a flock promotes improved production levels and feed efficiency (Summers and Leeson, 1994). Growth and development can be modified by multiple means and dietary fibre is being recommended as a feed additive by many commercial companies worldwide. The results of Incharoen and Maneechote, (2013a,b) indicate that dietary insoluble fibre such as whole rice hulls (WRH) at 60 g/ kg diet can be used to enhance growth and uniformity of pullets and to improve egg production of laying hens. The the same studies also indicated that WRH at 100 g/ kg diet can be used to enhance broiler performance without having any advers effect on GIT development.

Digestive enzymes that are secreted by the specific organs and sections of the digestive system such as pancreas and small intestine are responsible for the hydrolysis of dietary nutrients and play vital roles in regulating absorption of products of digestion.

In the experiment described in Chapter 3, it was shown that growth rate of the digestive organs and activities of digestive enzymes varied with age in pullets from 1 to 18 weeks and that the greatest changes in digestive organ growth and enzyme function were found before 10 - 12 weeks of age. In addition, the results from the previous chapter (Chapter 4) showed that proventricular pepsin and pancreatic general proteolytic and chymotrypsin activities in 19 week old laying hens can be increased by the inclusion of an insoluble fibre (0.8% Arbocel) for 12 weeks.

The experiments described in this chapter were therefore designed to determine whether the addition of insoluble fibre to the diets of younger pullets and at different ages and for different lengths of time would be able to increase GIT development and the activity of enzymes involved in digestion of protein beyond the maxima observed in Chapter 3. Such effects might be of value for improved growth of pullets as improved digestibility could lead to improved supply of amino acids for body growth which continues after growth of gastrointestinal tract organs.

5.1 Aims

To determine the effects of the insoluble fibre product Arbocel on the GIT organ weight and proventricular and pancreatic enzymes of immature layer pullets when:

1. added to their diet at different ages
2. added to their diet for different lengths of time, and
3. removed from the diet after supplementation for five weeks.

5.2 Materials and Methods

5.2.1 Birds, housing and management

Fifty-six 8 week old Hy-Line Brown pullets were obtained from the same commercial farm in Victoria from which chickens were obtained for the experiment described in Chapter 3. On arrival all pullets were weighed and leg bands attached. Feed and water were provided *at libitum.* All pullets were kept in the same shed containing pens (1 x 2 x 2 m, w x l x h), 4 pullets per pen. Two experimental diets used: a control diet (Ridley AgriProducts Pty Ltd., http://www.agriproducts.com.au) without additive and the IF diet consisting of the control diet plus 1% insoluble fibre Arbocel (JRS Co. Inc., Rosenberg, Germany). The concentrations of fibre in the two diets were analysed by ***FEEDTEST*** (Agrifood Technology Pty Ltd) and were 4.3%, and 5.0% in the control and IF, respectively.

5.2.2 Design of the experiment

On the day of arrival, eight pullets were chosen randomly weighed and killed (Fig 5.1). This group was considered as the baseline or initial weight. The remaining 48 pullets were allocated randomly to 12 pens, four birds per pen. Pullets in six pens were fed the control diet (Group C) for the first 5 weeks. The pullets in the remaining six pens were fed the control diet to which 1% Arbocel was added (Group IF). After five weeks, when the pullets were 13 weeks of age, eight pullets were randomly selected from the Group C and eight from Group IF. The pullets were weighed and killed with an overdose of pentobarbital sodium and liver, gizzard, pancreas, duodenum, small intestine (jejunum and ileum), caeca and spleen were weighed. Samples of the proventriculus, pancreas and small intestine were rinsed with cold normal saline and were stored at –80^{o}C for later analysis of enzyme activities (described in Chapter 3 Section 3.2.3).

The remaining 16 pullets in the Group C were divided so that eight pullets continued on the control diet (Group C) and eight were given the Arbocel diet (Group C-IF). Eight of the pullets in the Group IF continued to be fed the Arbocel diet (Group IF) and eight were given the control diet (Group IF-C) (Figure 5.1). After a further 5 weeks (10 weeks after the start of this experiment when the pullets were 18 weeks of age) the pullets were killed and samples collected as before

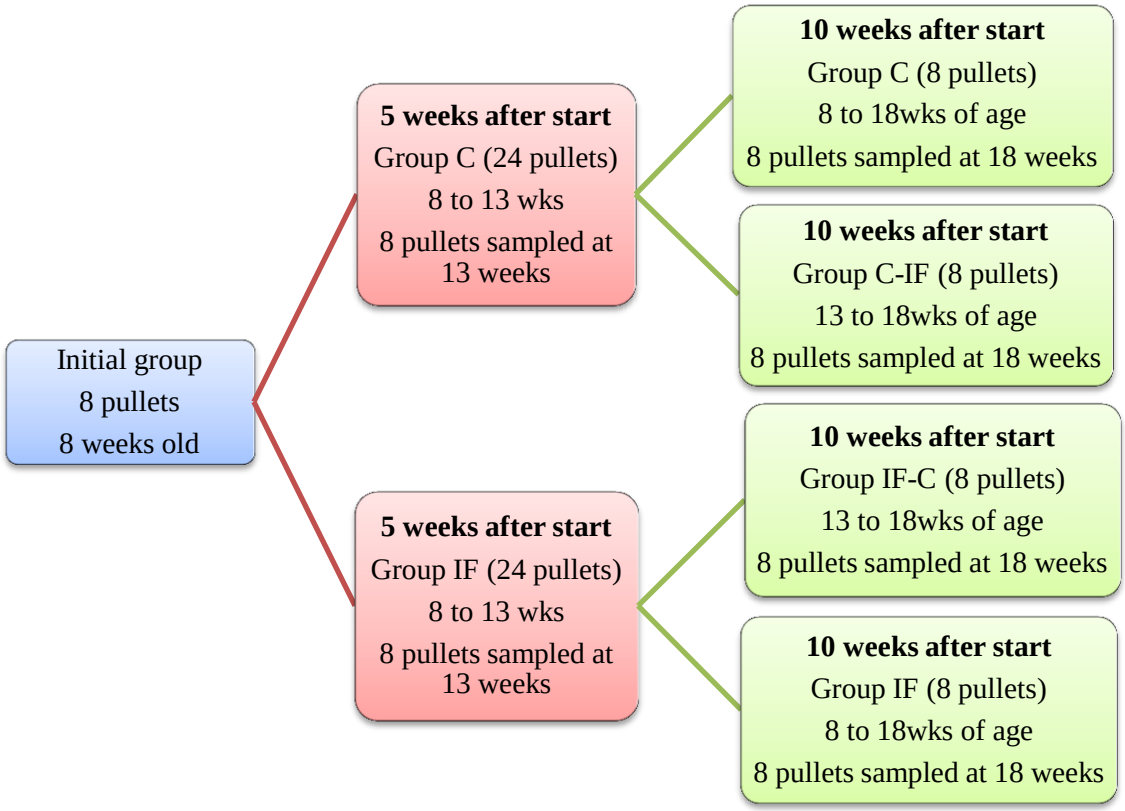

Figure 5.1 Diagram of the design of the experiment. The total number of the birds at the start was 56 pullets. For definition of treatment groups see text of Section 5.2.2.

5.2.3 Sample collection and enzyme analyses

All pullets were killed, organ samples collected and enzyme assays carried out as described in Sections 3.2.2 to 3.2.3. In addition, at 18 weeks of age, tissues samples from the proventriculi of pullets in Groups C and IF were taken and stored at -20ºC in RNA*later*® (Cat. No. R0901, Sigma) for later analysis of pepsinogen gene expression.

The experiment was conducted in accordance with the principles and specific guidelines of LTU-AEC as the project number AEC12-12.

Table 5.1 Composition of the control diet – manufacture's information (Ridley AgriProducts†)

Components	%
Wheat Fine	57.97
Oats Fine	10.57
Peas Fine	12.0
Meat Meal	3.83
Blood Meal	2.0
Canola Meal	5.0
Soybean Meal	2.9
Limestone Fine	1.0
Supplements	4.73
Total	100
Chemical composition	(%)
Dry mater	89.39
Moisture	10.61
Protein	18.01
Fat	3.04
Fibre (not including Arbocel)	Max. 4.36
ME	2796.7 kcal/kg
Calcium	1.00
Phosphorus	0.56
Available Phosphorus	0.40

Supplements in % Millrun 3.27, RAM dried fine NO_2 salt 0.13, Sodium bicarbonate 0.25, choline chloride (70%) 0.09, Potassium Carbonate 0.02, Dl-methionine 0.12, L-Lysine plus 0.18, Avizyme 1210 Liquid 0.02, Acid oil mixer 0.33, Ronozyme NP CT broiler 0.02, Biofix select 0.10, RAP-200 chick pullet PMX 0.20. Ridley †AgriProducts Pty Ltd http://www.agriproducts.com.au.

5.2.4 mRNA expression of pepsinogens A and C

a. Isolation of total RNA

Samples of proventriculus stored in RNA*later* were allowed to thaw at room temperature before extracting RNA. Approximately 100 mg of tissue were homogenized in 1 ml of TRIzol reagent (Invitrogen, Life Technologies, Australia). Homogenized samples were incubated at room temperature for 5 minutes and centrifuged at 12,000 x g (Heraeus Fresco 17, Thermo Scientific, USA) for 10 minutes at 4ºC.

The clear homogenate solution was transferred to a new sterile 1.5 ml microcentrifuge tube containing 200 µl of chloroform. Each sample was incubated at room temperature for 3 minutes and then all samples were vortexed briefly and centrifuged at 12,000 × g for 15 minutes at 4°C. Centrifugation resulted in separation of the lower red phenol-chloroform phase and upper colourless, RNA containing aqueous phase. The upper, colourless layer of the supernatant was transferred into a new sterile 1.5 ml microcentrifuge tube. Precipitation of RNA was achieved by the addition of 500 µl of isopropanol to each tube followed by incubation at room temperature for 10 minutes. Each sample was again centrifuged at 12,000 x g for 10 minutes at 4ºC. The supernatant was discarded and the RNA pellet was washed with 1 ml 75% ethanol and was centrifuged at 7500 x g for 5 minutes. The pellet was left to dry at room temperature for a few seconds and then dissolved in 30µl TE Buffer (Tris EDTA buffer). A Nano Drop ND-1000 spectrophotometer was used to quantify the RNA. Samples with an absorbance A260/280 ratio of 1.9 to 2.0 were accepted and the RNA solution was stored at -80ºC.

Before conversion of RNA to cDNA contaminating DNA was removed. To remove contaminating DNA, a 0.1 volume of Ambion 10x TURBO DNase Buffer (Life Technologies, USA) and 1µL TURBO DNase was added to the extracted samples of RNA, and mixed gently. The sample was incubated at 37ºC for 20–30 min, DNase Inactivation Reagent (0.1 times of the total volume) was added at 5.6µl addition of resuspended and was mixed by flicking the tube well. The samples was incubated for 2 minutes at room temperature followed by centrifugation at 10,000 × g for 1.5 min, and then the RNA was transferred to a new sterile 0.5 ml micro centrifuge tube and stored at -80ºC.

The synthesis of cDNA from extracted RNA was carried out with a DyNAmo™ cDNA synthesis kit (ThermoFisher Scientific, Australia). The reagents and volumes used for the conversion were as recommended for the kit (see Appendix Table 5.2). A thermal cycler (Stratagene Mx3000P QPCR Systems, Agilent Technologies, USA), was used to convert the RNA into cDNA following the cycle program shown below in Table 5.2.

Table 5.2 Reverse-Transcription PCR thermal cycler condition for synthesising cDNA from extracted RNA

Cycles	Time (minutes)	Temperature (°C)
1	10	25
1	30	37
	5	85
	1	25

Real time qPCR (RT-qPCR) was conducted using (Stratagene Mx3000P QPCR Systems, Agilent Technologies, USA). Primers for target and reference genes are given below in Table 5.3.

Table 5.3 Forward and reverse primer sequences of target and housekeeping genes for RT-qPCR

Gene	NCBI Accession Number	Forward primer	Reverse primer
Pepsinogen A	AB025283	GGGTGCCCTCTATCTATTGC	CAGTGTCATAGCCCAGGATG
Pepsinogen C	AB025284	GGTGTCCTACTGTGCCTGTG	CCTGGTTTGTGATGGAGATG
β-Actin	NM_205518	ATGGCTCCGGTATGTGCAA	TGTCTTTCTGGCCCATACCAA
GAPDH	n/a	GCCATCACAGCCACACAGA	TTTCCCCACAGCCTTAGCA
Mucin2	n/a	TCACCCTGCATGGATACTTGCTCA	TGTCCATCTGCCTGAATGACAGGT

Sources: Pepsinogen A and C were sequenced from National Centre for Biotechnology Information (NCBI).
β-actin (Grommen *et al.*, 2008).
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Mucin2 (Azzam *et al.*, 2011). Mucin 2 (MUC2) is presented and discussed in Chapter 6.

b. PCR Thermocycle and agarose gel electrophoresis

The PCR protocol for *Taq* DNA polymerase with Standard *Taq* Buffer contains 15 mM $MgCl_2$ in 25µl reaction volume as recommended in the kit instructions (see Appendix 5.4). The thermocycle conditions are given in Table 5.4. All the reagents were mixed according to the kit instructions and placed in a PCR machine (Takara Scintifix Pty. Ltd).

Table 5.4 Thermocycling conditions for PCR reaction

Cycles No.	TEMP	TIME
1	95ºC	30 seconds
40	95ºC 55ºC 68ºC	15-30 seconds 15-60 seconds 1 minute/kb
1	68ºC	5 minutes
Hold	4-10ºC	

Agarose gel electrophoresis (Figure 5.2) was carried out under the same conditions to confirm the purity and size of the bands of the PCR products resulting from amplification of the mRNA samples for target genes pepsinogens A and C and also for housekeeping genes β-actin and GAPDH.

1. Preparing the agarose gel

Two grams of agarose powder (Cat. No. BIO-41025) were added to 100 ml of Tris-acetate-EDTA (TAE) buffer. The agarose was melted for 1-2 min in a microwave oven, left to cool to 50-55ºC and then 5µl nucleotide staining dye (SensiMix SYBR Low-ROX Kit Cat.No. QT625-02) was added. The melted agarose solution was poured into the casting tray and left to cool until it was solid.

2. Loading the gel

A 12µl sample from the PCR reaction (see Appendix 5.4) was added to 2µl of sample loading buffer (6X DNA Loading Dye, R0611 Thermo Fisher Scientific Australia Pty. Ltd.) and 12µl of the mixture was pipetted to each well in the gel comb. This step was repeated for each sample. An 8µl aliquot of the 100 bp DNA ladder (New England Biolabs, Cat # N3231L (https://www.neb.com/products/n3231-100-bp-dna-ladder) was added into the first well (line 1). Then the gel was run at 100 volts for 30 min and the gel image was recorded in a gel doc instrument (Gel Doc™ EZ System #170-8270, *BIO-RAD*, Australia).

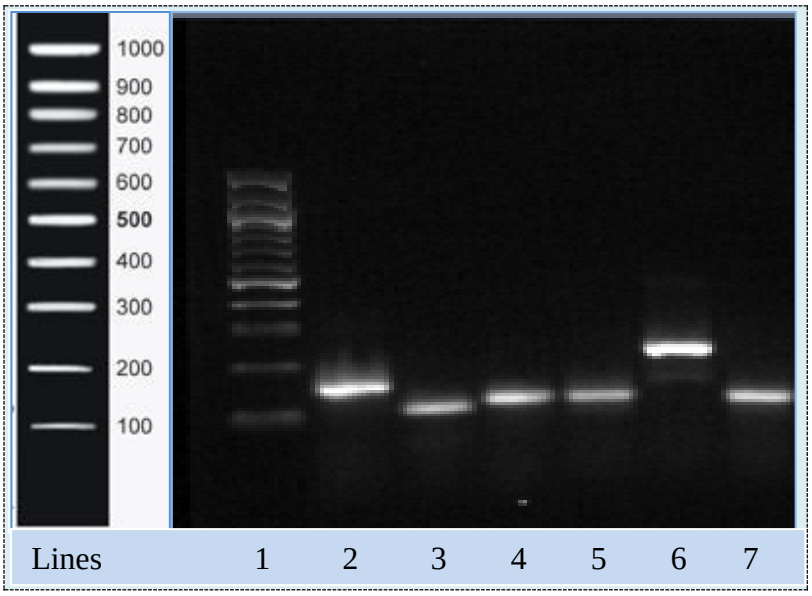

Figure 5.2 Agarose gel of the target and housekeeping genes. Line 1- ladder, L2 pepsinogen A, L3 pepsinogen C, L4 β-actin, L5 and L7 GAPDH, L6 Mucin2 (see Chapter 6 for details).

c. Real-time qPCR (Single-Plex protocol)

For singleplex real-time qPCR, each well of a 96 well plate contained the same volume of both forward and reverse primer of either a target or a housekeeping gene along with template cDNA (or DNase-free water for no template controls) and SensiMix SYBR Low-ROX (Bioline Australia, Pty Ltd) according to manufacturer's instructions (as shown in Appendix Table 5.5). The thermocycle conditions are shown in Table 5.5.

For each sample a mixture of all reagents was added to a sterile 1.5ml microcentrifuge tube and mixed well.

The first two wells of a 96 well plate contained buffer instead of the cDNA template and were used as a control. From each 1.5 ml microcentrifuge tube that contained a 50µl reaction mixture a 25µl was aliquot added to each well (2 replicates per sample).

Table 5.5 Thermocycling conditions for qPCR conditions:

No. Of cycles	TEMP	TIME
1	95ºC	10 min
40	95ºC 55ºC 72ºC	15 seconds 15 seconds 15 seconds
1	95ºC 55ºC 95ºC	1min 30 seconds 30 seconds

d. Calculation of gene expression

Standard curve

Primer efficiency of the target genes was tested using five dilutions of cDNA in duplicate. Complementary deoxyribonucleic acid (cDNA) was used and diluted as Neat (no dilution), 1:5, 1:50, 1:500 and 1:5000 in nuclease free water (NFW) and was made up to a final reaction volume of 50µL. The reaction for each dilution was performed by using 25µl SensiMix^{TM} SYBR Low-ROX (Bioline Australia, Pty Ltd), 1µl forward primer (50µM), 1µl reverse primer (50µM), 18µl nuclease free water plus 5µl diluted cDNA. The PCR cycling conditions were 95^{o}C for 10 minutes, 40 cycles as shown in Table 5.5. The integrity of amplification indicated by a single melt peak (Appendix Figures 5.1 to 5.5) for each product was verified by a melt standard curve analysis; purity and size of the amplicon were previously confirmed by agarose gel electrophoresis (Section b).

e. qRT-PCR data analysis

Efficiency of PCR was determined for both the target and endogenous control genes from the standard curves generated with different dilutions of the cDNA synthesized from the chicken proventriculus using the formula PCR Efficiency = 10 (−1/slope). The slope values were calculated from standard curves and used in the following formula (Harrison *et al.,* 2007; Gopinath *et al.,* 2011) to obtain the corrected Ct:

$$\textbf{Corrected Ct} = \textbf{Ct} + (\textbf{Nt} - \textbf{Ct'}) \times (\textbf{S}/\textbf{Ś})$$

where Ct is the mean target gene Ct,

Nt is the mean Ct of housekeeping genes of the entire experimental samples,

Ct' is the mean Ct of housekeeping gene of that particular sample,

S is the target gene slope, and

Ś is the endogenous control slope

5.2.5 Statistical analysis

Data were tested for normality and were statistically analysed using the General Linear Models procedures of SPSS statistics (SPSS 19.0 for Windows, USA). The significances of differences between age means were examined by Turkey's test. Significance was set at $P \leq 0.05$.

5.3 Results

5.3.1 Growth and development

5.3.1.1 Live body and organ weights

After 5 weeks on the treatments there was no effect on body weights of pullets in the IF Group given the insoluble fibre, Arbocel compared with controls (Group C) without added Arbocel (Table 5.6). However, after 10 weeks Group IF body weights were significantly heavier ($P < 0.05$) than those fed the control diet, Group C.

Although groups fed Arbocel over different time periods (Groups IF-C and C-IF) were heavier than controls at the end of the experiment, the differences were not significant.

There was no effect of treatment on liver weight of Group IF after 5 weeks compared with Group C. However, with the longer period of feeding, liver weights after 10 weeks in Group IF were significantly heavier ($P < 0.05$) than those in Group C. For both groups fed IF over different time periods (Groups IF-C and C-IF), liver weights were lower than controls but the differences were not significant.

Relative to body weights (Table 5.7) the weights of the liver decreased with age in the control Group C but no significant differences were seen between Group IF and control pullets without IF supplement. The relative weights of the liver in Groups C, C-IF and IF-C decreased significantly ($P < 0.05$) with age from 13 to 18 weeks as compared to a significant increase in that of pullets in Group IF.

The mean weight of the gizzards increased with age of the pullets with a significant increase from the initial weight at 8 weeks to that at 13 weeks of age in the controls (Group C) and a further significant increase in weight from 13 to 18 weeks of age (Table 5.6). In Group IF, after 5 weeks on the Arbocel supplement, the gizzard was heavier than Group C but the difference was not significant. However, after a further 5 weeks on the treatments the gizzards of the 18 week old Group IF pullets were significantly heavier than those of Group C. No significant differences were found between gizzards of pullets at 18 weeks of age compared with those at 13 weeks or between treatments at 18 weeks of age.

Relative to body weights (Table 5.7), the weights of the gizzards decreased with age: except those of pullets in Group IF supplemented for 10 weeks. For all treatments, relative gizzard weights were lower than those of the initial group of pullets at 8 weeks of age. The relative decrease in gizzard weight with age was not observed in the 18 week old Group IF pullets and their relative weights were not significantly different from that at 8 weeks of age.

Table 5.6 Live body weight (g) and the weight of internal organs (g) of laying pullets given a control diet (C) and the control diet supplemented with insoluble fibre 1%, Arbocel (IF) at different ages and for different length of time (Mean ± SD, n = 8)

Time on diet	0	5		10			
Age (weeks)	8	13		18			
Treatments	Initial weight	C	IF	C	C-IF	IF-C	IF
Live body	796.7 ± 44.77^{a}	1346.2 ± 27.31^{b}	1362.7 ± 28.60^{b}	1573.48 ± 50.06^{c}	1613.4 ± 52.13^{cd}	1631.1 ± 19.44^{cd}	1672.5 ± 47.68^{d}
Liver	22.43 ± 2.67^{a}	30.66 ± 3.09^{b}	33.38 ± 2.51^{b}	36.93 ± 5.02^{b}	33.12 ± 4.91^{b}	35.47 ± 5.22^{b}	46.95 ± 5.04^{c}
Gizzard	27.26 ± 4.79^{a}	36.77 ± 3.69^{b}	38.72 ± 3.58^{b}	35.95 ± 3.85^{b}	35.22 ± 4.96^{b}	38.29 ± 4.15^{b}	49.54 ± 7.96^{c}
Proventriculus	3.88 ± 0.53^{a}	4.79 ± 0.31^{ab}	5.43 ± 0.59^{b}	5.31± 0.38^{b}	5.53 ± 0.71^{b}	5.70 ± 0.63^{b}	6.88 ± 1.09^{c}
Pancreas	2.54 ± 0.41^{a}	3.29 ± 0.46^{b}	3.42 ± 0.34^{bc}	3.24 ± 0.41^{b}	3.01 ± 0.37^{ab}	3.23 ± 0.35^{b}	4.03 ± 0.64^{c}
Spleen	1.96 ± 0.18^{a}	2.21 ± 0.51^{ab}	2.58 ± 0.44^{abc}	1.98 ± 0.54^{a}	2.35 ± 0.37^{abc}	2.74 ± 0.21^{bc}	2.92 ± 0.53^{c}
Small Intestine	27.60 ± 3.64^{a}	27.19 ± 1.05^{a}	28.78 ± 2.80^{a}	30.17 ± 2.03^{a}	27.13 ± 1.93^{a}	30.88 ± 2.35^{a}	35.48 ± 2.83^{b}
Caeca	4.57 ± 0.59^{a}	8.62 ± 0.82^{b}	9.71 ± 1.31b^{bc}	10.55 ± 1.15^{cd}	11.09 ± 0.93^{cd}	11.68 ± 1.17^{de}	13.09 ± 0.66^{e}

Means within the same row with different superscript letters differ significantly ($P < 0.05$). C = control group (no Arbocel added), IF= Arbocel group (1% to control C diet), C-IF = no Arbocel from 8 to 13 wks of age, after 13 wks Arbocel was added, IF-CO = Arbocel from 8 to 13 weeks, and no Arbocel from 13 to 18 wks of age, IF = Arbocel from 8 to 18 wks.

After 5 weeks of feeding Arbocel supplement, weights of the proventriculus were significantly heavier in Group IF than those at the start of the experiment: this was not due solely to an increase with age as the weight of the proventriculus in the control, Group C, pullets was not significantly different from that of pullets at the start of the experiment (Table 5.6). At 18 weeks of age the weights of the proventriculus in Group C pullets were significantly heavier than those of the pullets at 8 weeks of age. Although supplementing with Arbocel in Groups C-IF and IF-C resulted in an increase in weights of the proventiculus, the increases were not significantly different from those of Group C. However, supplementing the pullets with Arbocel for 10 weeks (Group IF) resulted in a significant ($P < 0.05$) increase in the weight of the proventriculus (Table 5.6). The relative weights of the proventriculus decreased significantly ($P < 0.05$) with age from 8 to 13 weeks but thereafter there was no significant change in relative weights (Table 5.7).

Weights of the pancreas in both Group IF and Group C at 13 weeks of age were heavier than those in the initial group at 8 weeks old but differences were not significant (Table 5.6). After 10 weeks on Arbocel supplement, weights of pancreas in Group IF pullets were significantly greater than those of Groups C, C-IF and IF-C. The relative weights of the pancreas decreased with age from 8 to 13 weeks of age but thereafter there was no significant change in relative weights (Table 5.7).

There was no effect of age or treatment on weight of small intestine. The only significant effect was seen after 10 weeks of supplementing diets with Arbocel (Group IF) when the small intestine was significantly heavier than at all other times or treatments (Table 5.6). Relative weights decreased with age but in Group IF pullets after 10 weeks supplementation with Arbocel, relative weights of the small intestine were significantly higher than those in pullets in Group IF-C (Table 5.7). The weight of the caeca increased with age from 8 to 13 weeks and 13 to 18 weeks but there was no effect of feeding Arbocel for 5 weeks on the caecal weights. However, supplementation with Arbocel for 10 weeks caused a significant increase ($P < 0.05$) in weight of caeca of Group IF pullets compared with those of pullets in Groups C and C-IF but not with group IF-C pullets (Table 5.6). Relative weights of caeca increased from 13 to 18 weeks of age but there was no effect of Arbocel supplementation on the relative weights (Table 5.7).

Table 5.7 Relative weights of internal organs (% of body weight) of laying pullets given a control diet (C) and the control diet supplemented with insoluble fibre 1%, Arbocel (IF) at different ages and for different length of time (Mean ± SD, n = 8)

Time on diet	0	5		10			
Age (weeks)	8	13		18			
Treatments	Initial RW*	C	IF	C	C-IF	IF-C	IF
Liver	2.81 ± 0.24^{a}	2.27 ± 0.21^{b}	2.45 ± 0.19^{ab}	2.35 ± 0.32^{b}	2.06 ± 0.31^{b}	2.18 ± 0.32^{b}	2.80 ± 0.25^{a}
Gizzard	3.41 ± 0.50^{a}	2.73 ± 0.25^{bcd}	2.84 ± 0.25^{bc}	2.28 ± 0.23^{de}	2.18 ± 0.28^{e}	2.35 ± 0.25^{cde}	2.96 ± 0.47^{ab}
Proventriculus	0.49 ± 0.06^{a}	0.36 ± 0.03^{bc}	0.40 ± 0.04^{bc}	0.34 ± 0.02^{c}	0.34 ± 0.04^{c}	0.35 ± 0.04^{bc}	0.41 ± 0.07^{b}
Pancreas	0.32 ± 0.04	0.25 ± 0.04	0.25 ± 0.02	0.20 ± 0.02	0.19 ± 0.02	0.20 ± 0.02	0.23 ± 0.04
Spleen	0.25 ± 0.02^{a}	0.17 ± 0.04^{bc}	0.19 ± 0.03^{b}	0.13 ± 0.03^{c}	0.15 ± 0.02^{bc}	0.17 ± 0.01^{bc}	0.17 ± 0.04^{bc}
Small intestine	3.47 ± 0.43^{a}	2.02 ± 0.11^{b}	2.11 ± 0.18^{b}	1.92 ± 0.14^{bc}	1.68 ± 0.14^{c}	1.89 ± 0.15^{bc}	2.13 ± 0.20^{b}
Caeca	0.57 ± 0.08^{a}	0.64 ± 0.06^{a}	0.71 ± 0.08^{a}	0.67 ± 0.07^{b}	0.69 ± 0.07^{b}	0.72 ± 0.08^{b}	0.78 ± 0.04^{b}

Means within the same row with different superscript letters differ significantly ($P < 0.05$).* Initial relative weight. For abbreviations of treatments see Table 5.6.

5.3.2 Digestive enzyme activities

5.3.2.1 Pepsin activity in proventriculus

Pepsin activity in proventricular tissue homogenates showed significant ($P < 0.05$) differences with higher activities for pullets in Group IF (Table 5.8) compared to those in Groups C, C-IF and IF-C. Short term supplementation (5 weeks) from 8 to 13 weeks with Arbocel did not produce significant differences ($P < 0.05$) between Groups C and IF pullets, while the long term feeding (10 weeks) showed that pepsin activity was significantly greater for pullets in Group IF compared to those in Groups C, C-IF and IF-C. There were significant differences between Groups IF-C and C-IF with higher pepsin activity in IF-C while there was no difference between Groups C and C-IF. The pepsin activity per organ was significantly greater in IF group at 18 weeks (10 weeks on treatment) compared to those pullets in Groups, CC, C-IF and IF-C. The activity expressed per organ was also significantly greater for pullets in Group IF after 10 weeks on treatments (18 weeks of age) compared to all the other treatments.

5.3.2.2 Pancreatic enzyme activities

Table 5.8 shows the effects of different periods of feeding Arbocel on the activities of general proteolytic activity (GPA) and chymotrypsin and trypsin activities in pancreatic homogenates. The results showed a higher GPA for Group IF ($P < 0.05$) when expressed as µmol of free tyrosine released from the casein substrate per gram of tissue per minute at, both 5 and 10 weeks compared to the other treatment Groups. The rate of GPA increased by 22% compared to Group C in pullets in Group IF fed Arbocel fibre from 8 to 13 weeks of age, while the rates for the IF group on long term feeding from 8 to 18 weeks of age were 23.5, 19.4 and 14.5% greater than those pullets in Groups C, C-IF, and IF-C respectively. The GPA expressed per organ was significantly greater in all groups at 13 and 18 weeks of age than that at 8 weeks of age (Table 5.9). The activity expressed per organ was also significantly greater for pullets in Group IF after 10 weeks on treatments (18 weeks of age) compared to all the other treatments after 5 weeks, except for that of Group IF.

For chymotrypsin activity per g tissue there was a significant decrease at 13 and 18 weeks of age compared to the initial value at 8 weeks of age in all treatment groups

except Group IF fed Arbocel for 10 weeks (Table 5.8). The activity expressed per organ was also significantly greater ($P < 0.05$) for pullets in Group IF after 10 weeks on treatments (18 weeks of age) compared to all the other treatments.

Trypsin activity per gram tissue at 13 weeks of age decreased significantly ($P < 0.05$) from the initial rate at 8 weeks of age. By 18 weeks of age activities in Groups C and C-IF were still lower than at 8 weeks of age but the difference was not significant. Activity of trypsin per g tissue (Table 5.8) at 18 weeks of age (10 weeks on treatments) were significantly greater ($P < 0.05$) in pullets in Groups IF-C and IF when compared with activities of pullets in other treatments or at other ages. The trypsin activity expressed per organ was also significantly greater ($P < 0.05$) for pullets in Group IF after 10 weeks on treatments (18 weeks of age) compared to all the other treatments. However, there were significant differences ($P < 0.05$) between group IF-C and those in Groups C and C-IF.

5.3.2.3 Dipeptidase and aminopeptidase activities in small intestine

Activity of dipeptidase per g tissue at 13 weeks of age (5 weeks on treatments) decreased significantly ($P < 0.05$) from the initial rate at 8 weeks of age. By 18 weeks the differences were not significant among the treatment groups. Aminopeptidase activity per gram tissue at 13 weeks of age increased significantly ($P < 0.05$) from the initial rate at 8 weeks of age. By 18 weeks of age activities decreased significantly ($P < 0.05$) in all treatment groups compared to those in pullets at 13 weeks of age. For activity per organ (combined jejunum and ileum) a similar pattern was observed as that for the activity per g tissue.

Table 5.8. Enzyme activities (g tissue/ min) in proventriculus and pancreas of laying pullets given a control diet (C) and the control diet supplemented with insoluble fibre 1%, Arbocel (IF) at different ages and for different length of time (Mean ± SD, n = 8)

Time on diet	0	5		10			
Age (weeks)	8	13		18			
Treatments	Initial value	C	IF	C	C-IF	IF-C	IF
Pepsin	71.04 ± 2.95^{a}	70.78 ± 3.49^{a}	75.44 ± 2.66^{ab}	86.06 ± 5.15^{cd}	83.23 ± 6.28^{bc}	92.76 ± 8.39^{d}	105.26 ± 5.14^{e}
GPA-pancreas	42.50 ± 4.83^{a}	95.90 ± 10.11^{b}	123.13 ± 4.45^{d}	96.46 ± 6.18b^{c}	101.63 ± 10.93 ^{bc}	107.85 ± 7.22^{c}	126.13 ± 7.25^{d}
Chymotrypsin	4.69 ± 0.40^{a}	3.99 ± 0.15^{c}	4.36 ± 0.20^{ab}	3.80 ± 0.18^{c}	3.94 ± 0.25^{c}	4.06 ± 0.23^{bc}	4.63 ± 0.16^{a}
Trypsin	270.42 ± 35.89^{a}	196.62 ± 10.80^{b}	207.23 ± 13.47^{b}	257.02 ± 24.82^{c}	247.43 ± 18.25^{c}	369.26 ± 23.13^{d}	401.26 ± 15.26^{d}
Dipeptidase	21.87 ± 3.48^{a}	14.14 ± 0.68^{b}	14.68 ± 1.43^{bc}	12.74 ± 1.49^{bc}	12.38 ± 1.15^{bc}	10.94 ± 2.22^{c}	12.56 ± 1.36^{bc}
Aminopeptidase	34.64 ± 3.11^{a}	79.22 ± 6.31^{b}	87.24 ± 5.99^{b}	55.72 ± 10.03^{c}	59.95 ± 2.94^{c}	58.61 ± 8.64^{c}	59.53 ± 11.63^{c}

Means within the same row with different superscript letters differ significantly ($P < 0.05$). For abbreviations of treatments see Table 5.6. Pepsin activity in proventriculus was defined as the pepsin hydrolysis of 1µmol of substrate (haemoglobin) in 1 min per g tissue or per organ. General proteolytic activities in pancreas is defined as µmoles free tyrosine released from casein substrate in 1 min per g tissue and per organ. Chymotrypsin activity in pancreas was expressed as chymotrypsin units of *p*-nitroaniline released from N –glutaryl-L- Phenylalanine-p-nitroanilide in 1 min per g tissue or per organ. Trypsin activity in pancreas was expressed as trypsin units of *p*-nitroaniline released from Nα-Benzoyl-DL- arginine-p-nitroanilide in 1 min per g tissue or per organ weight. Dipeptidase activity µmoles glycyl-L-leucine in 1 min per g tissue or per small intestine. Aminopeptidase activity nmoles*p*-nitroanilide in 1 min per g tissue or per small intestine. For aminopeptidase and dipeptidase (n) is 6 replicates.

Table 5.9. Enzyme activities (per organ/ min) in proventriculus, pancreas and small intestine of laying pullets given a control diet (C) and the control diet supplemented with insoluble fibre 1%, Arbocel (IF) at different ages and for different length of time (Mean ± SD, n = 8)

Time on diet	0	5		10			
Age (weeks)	8	13		18			
Treatments	Initial value	C	IF	C	C-IF	IF-C	IF
Pepsin	276.63 ± 45.59^{a}	339.13 ± 31.31^{ab}	408.66 ± 39.41^{bc}	456.67 ± 38.78^{cd}	463.03 ± 88.74^{cd}	532.01 ± 96.99^{d}	699.77 ± 105.70^{e}
GPA-pancreas	107.76 ± 20.51^{a}	316.47 ± 58.58^{b}	421.15 ± 48.03^{cd}	313.45 ± 54.90^{b}	331.79 ± 64.67^{bc}	350.14 ± 54.63^{bc}	478.27 ± 101.19^{d}
Chymotrypsin	12.01 ± 2.90^{a}	13.15 ± 1.94^{a}	14.90 ± 1.52^{ab}	12.34 ± 1.97^{a}	12.81 ± 1.97^{a}	13.17 ± 1.96^{a}	17.57 ± 3.74^{b}
Trypsin	695.99 ± 207.46^{a}	650.62 ± 111.96^{ab}	710.23 ± 102.14^{ab}	839.33 ± 178.63^{ab}	805.34 ± 134.47^{ab}	1196.81 ± 170.83^{c}	1610.32 ± 211.84^{d}
Aminopeptidase	894.69 ± 98.59^{a}	1957.57 ± 95.99^{b}	2128.54 ± 79.67^{b}	1657.54 ± 102.39^{c}	1610.77 ± 116.89^{c}	1673.99 ± 158.34^{c}	1792.49 ± 101.76^{c}
Dipeptidase	505.30 ± 45.30^{a}	362.28 ± 18.37^{b}	369.84 ± 16.52^{b}	382.52 ± 78.56^{ab}	334.97 ± 43.36^{b}	309.88 ± 46.59^{b}	391.49 ± 54.91^{b}

Means within the same row with different superscript letters differ significantly ($P < 0.05$). For abbreviations see Table 5.8.

5.3.3 mRNA expression of pepsinogens A and C

The mRNA expression in proventricular tissues at 18 weeks of age (10 weeks on treatments) was greater for pepsinogen A than for pepsinogen C (Figure 5.3) in both treatment groups. Pullets in Group IF had significantly greater ($P < 0.05$) expression of both pepsinogen A and C than those in Group C.

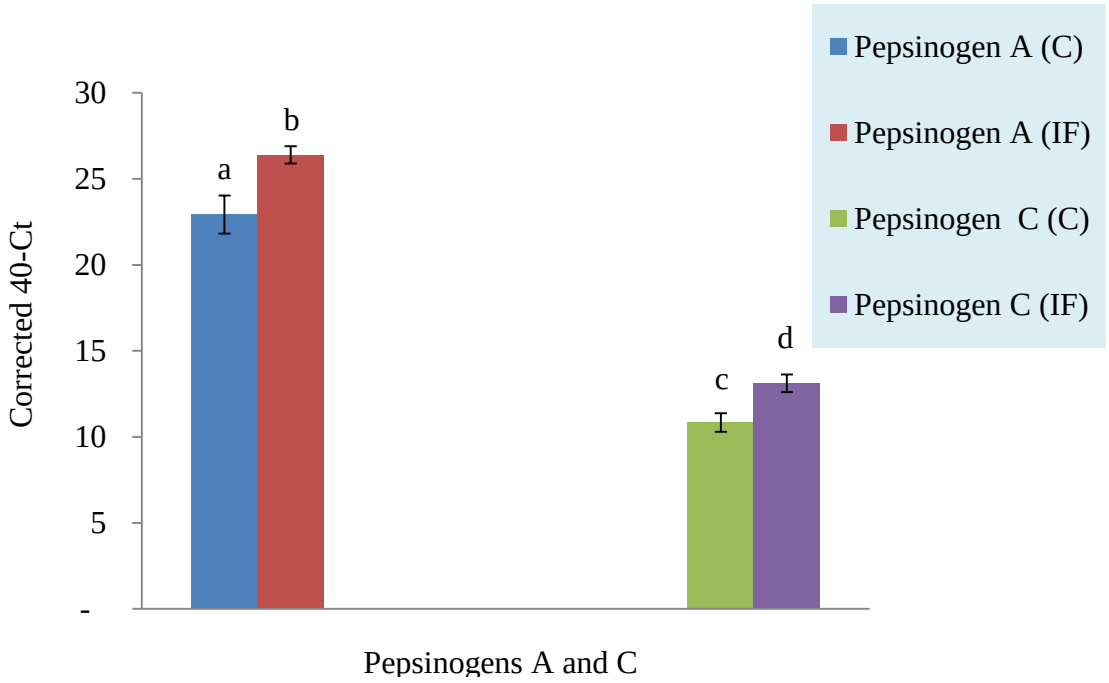

Figure 5.3 mRNA expression of pepsinogens A and C in proventricular tissue of 18 week old layer strain pullets given a control diet (Group C) and the control diet supplemented with insoluble fibre (1% Arbocel, Group IF) from 8 to 18 weeks of age (Means ± SE, n= 8).

5.4 Discussion

5.4.1 Live body and organ weights

The results presented here show that the insoluble fibre Arbocel provided as a supplement of 1% for 10 weeks in the diet of layer pullets improved growth of all digestive organs when compared to controls. Supplementing the diets with Arbocel for 5 weeks (Groups IF and IF-C) had no significant effect on any of the weights measured and supplementing the diets for 5 weeks after 5 weeks on the control diets (Group C-IF) also had no significant effect on weights. However, the although the live body weights of the pullets in Group IF-C were not significantly different from controls at 10 weeks they were also not significantly different from the pullets in Group IF suggesting that there may have been some residual effect of IF after it was removed from the diet: however the pullets in Group C-IF 10 weeks after the start of the experiment were also heavier than controls but not significantly. This could indicate that in older pullets IF may have a different effect than seen in younger birds (Group IF at 5 weeks after the start of the experiment). Similar responses across treatments were seen in the organs of Group IF and IF-C after 10 weeks but not in Group C-If suggesting that for organs, age at which supplementation occurs and length of time on the supplement may be important.

Although pullets of 8 weeks old had been ordered it is possible, based on the initial body weights, that the age at which they were provided was older than that and implications of this will be discussed later. Current results agree with those of Bogusławska-Tryk (2005) who showed that feeding broiler chickens Arbocel BWW-40 at three different levels from day-old to 42 days of age increased the body weight significantly at the lowest and highest levels when compared with controls without the insoluble fibre supplement: however, unlike in this experiment there was no effect on pancreas weight. In studies on rats results showed a significantly lower body weight in those fed with a feed containing 15% cellulose compared with rats fed with a standard laboratory diet (Bragado *et al*., 2001). In Szymeczko's (2000) study Arbocel BWW-40 at 0.25 to 0.95% was added to the diet of broiler chickens and it enhanced the final body weight and the digestion of dietary nutrients.

The results obtained here on the effects of IF on gizzard and proventricular weights is supported by those from an experiment by González-Alvarado *et al.* (2007) who showed that inclusion of insoluble fibre either as 3% oat hulls or 3% soy hulls in low-fibre diets (crude fibre 2.5%) for broiler chicks at early ages, 1 to 21 days, caused a significant increase in gizzard weight but proventricular weight was only significantly increased with soy hulls. The results also showed improved total tract apparent retention of feed, body weight gain and feed conversion and the pH in the gizzard was significantly decreased by both supplements. The results suggested that inclusion of moderate amounts of fibre in pre-starter feeds might be beneficial for poultry (González-Alvarado *et al.,* 2007). Addition of pea fibre, wheat bran and oat bran either at low or high levels (187, 375) g/ kg in broiler diets was shown by Jørgensen *et al.* (1996) to cause a significant increase in the GIT tract and caecum which supports the results obtained here. The results of an experiment by Freitas *et al*. (2014) in which Hy-Line Brown strain of 7 week old pullets were given diets containing different levels of neutral detergent fibre (NDF) for 5 weeks showed that the liver, and intestines increased significantly in weight with the highest NDF concentration but gizzard weight, unlike in the experiment described here, was reduced.

A fibre concentration in broiler diets of 15g of either barley or 15g oats hulls (Sacranie *et al.*, 2012) increased gizzard size and activity and decreased pH. This is in agreement with previous studies highlighting the stimulatory effect of crude fibre on the gizzard activity (Preston *et al.*, 2000; Hetland *et al.*, 2002; Plavnik *et al.*, 2002; Svihus *et al.*, 2004b; Ravindran *et al.*, 2006; Jiménez-Moreno *et al.*, 2009b,c) and as discussed in a recent review (Svihus, 2011). A larger gizzard improves the grinding action, gastric reflux, and surface area of contact between nutrients and digestive enzymes (Gabriel *et al.*, 2003).

It has also been shown that long term feeding on a fibre rich diet was more effective compared with a low fibre diet at enhancing the enlargement of gizzard size (Rogel *et al.*, 1987a). Adding fibre to poultry feeds has been shown to change the weight and length of the gastrointestinal tract (Siri *et al.*, 1992; Viverose *et al.*, 1994; Jorgenson *et al.*, 1996; Smits *et al.*, 1997) and support the result obtained here. However, the results in the present study disagree with the study of Rogel *et al.* (1987a) who showed that the lengths and weights of intestine segments in broilers decreased in birds fed 10% fibre. A study by Taylor and Jones, (2004) suggested that the increase in gizzard size could be the

reason for reduction of the relative weight of the small intestine, which in turn might reflect an adaptation of the gut to an increased availability of nutrients. On the other hand, Lin *et al*. (2010) showed that young goslings raised on a diet supplemented with high amount of insoluble NSPs from maize, barley hulls, rice bran, wheat bran, had significantly better performance than those given highly soluble non-starch polysaccharides (NSP) ingredients like pectin.

A well developed gizzard can positively enhance digestion since food is retained for a long time in the upper digestive tract (proventriculus and gizzard) and this may activate digestive secretions from specific organs and result in more efficient nutrient assimilation (Jones and Taylor 2001).

Comparing the effects of IF on organ weights in this experiment with those in Chapter 4 on older hens at point of lay, it can be seen that that the younger pullets here responded with significant increases in liver, gizzard, proventriculus, pancreas and small intestine weights whereas the older hens had significantly increased weights of only the gizzard after 12 weeks. This effect was most likely owing to the differences in age of the birds in the two experiments but the difference in levels of fibre in the diets (0.8% in the older hens and 1% in this experiment) could also have contributed to the differences in effects on organ weights.

5.4.2 Enzyme activities

The effects of IF treatments on activities of pepsin and the pancreatic enzymes (expressed per g tissue) at 10 weeks showed a similar pattern to that seen on organ weights with the greatest effect of treatment being in the IF Group compared to the other three groups. However, there was no significant effect on intestinal enzymes. Again when enzyme activities were expressed per organ, significant effects were only observed in the IF Group but there were no significant effects on the intestinal enzymes compared to controls.

The results obtained here on increased trypsin and chymotrypsin activities in the Group IF pullets after 10 weeks on the supplemented diet confirm those of Boguslawska-Tryk (2005) who showed that in 21 day old broilers fed the IF product Arbocel BWW-40

from day old, trypsin and chymotrypsin activities were increased relative to controls. Regulation of pancreatic enzymes is through hormones such as cholecystokinin, secretin and gastrin, produced in the digestive tract (Sommer and Kasper, 1981; Li and Owyang, 1993; Owyang and Logsdon, 2004; Wang and Cui, 2007). Soluble and insoluble fibres can influence the structure of the villi of the small intestine and the turnover of mucosal cells (Langhout, 1998; Langhout *et al*, 1999; Iji *et al.*, 2001b). Therefore, it is probable that hormone production in intestinal mucosa of pullets fed insoluble fibre supplement can be increased and lead to increased production and/or activity of the pancreatic enzymes. In people, Mössner *et al.* (1992) and Bourdon *et al*. (2001) have shown that dietary fibre can stimulate CCK release and in pigs, intravenous infusions of CCK and secretin caused an increase in pancreatic juice and increased trypsin (Jakob *et al.*, 2000).

The increase in pepsin activities of these pullets given IF was similar to the effects observed in the older hens described in Chapter 4, however, the differences in the older hens was not always significantly different from the controls. For pancreatic enzymes significant increases in GP and chymotrypsin activities were seen in both experiments in birds given IF compared to controls but trypsin activity was only significantly increased in the younger birds. This could have been due to the stage of maturity of the birds or to the difference in fibre concentration of the diets.

Results on the activity of digestive enzymes in the literature are conflicting. A possible explanation could come from the different experimental conditions used. In some studies the effect of fibre was negatively correlated to enzyme activity. For example, Isaksson *et al.* (1982) observed in their *in vitro* studies that dietary soluble fibre inhibited pancreatic enzyme activity and this was mainly due to the viscosity, pH and adsorption of enzymes by fibre. Hesselman and Aman, (1986) showed that insoluble fibre NSP can surround endosperm of cells and inhibit the catalytic action of digestive enzymes.

There was no significant effect of the insoluble fibre Arbocel at 1% level on the activities of intestinal enzymes; however, activities of dipeptidase and aminopeptidase were higher numerically in IF group and this could be related to level of fibre used in this study.

5.4.3 mRNA expression of pesinogens A and C

The significantly greater mRNA expression of pepsinogens A and C in the pullets given IF compared with the control pullets reflects the higher pepsin activities in proventricular homogenates and probably reflects an increase in production of the zymogens of pepsin enzymes. The similarity in increase between mRNA of pepsinogen A and that of pepsinogen C suggests that the mechanism of stimulation was similar.

Gene expression for pepsinogen could have been induced through increased production of CCK, increased reflux of bile from the small intestine and gizzard or though decreased pH (Konturek, 1976; Sommer and Kasper, 1981; Duke, 1982 and 1986; Hasik and Bartnikowska, 1987; Li and Owyang, 1993; Jiménez-Moreno *et al.,* 2009b,c; Sacranie *et al.,* 2005; Svihus, 2011; Sacranie *et al.,* 2012).

5.4.4 Conclusion

In conclusion, this study shows that growing pullets fed a diet containing 1% insoluble fibre Arbocel for 10 weeks had a significant increase in body weight and the weights of liver, gizzard, proventriculus and pancreas as compared to pullets fed the control diet.

The increase in total enzyme activities in the proventriculus and pancreas of IF supplemented pullets was probably caused partly by an increase in enzyme function and partly by the increase in organ weight. Intestinal enzymes dipeptidase and aminopeptidase activities did not change because of the feed supplement but there were changes over ages.

A greater mRNA expression of pepsinogens A and C in the pullets given insoluble Arbocel fibre compared with to the control pullets reflects the higher pepsin activities in proventricular homogenates and probably reflects an increase in production of the zymogens of pepsin enzymes

Improvement in growth and in enzyme activities in the growing pullets fed Arbocel fibre for 10 weeks may improve digestion of food and increase support for growth

Chapter 6

Effect of two different fibre sources on growth and enzyme function in layer pullets at different ages

6.0 Introduction

Chickens have a requirement for dietary fibre in order to optimise nutrient digestibility and growth performance. Responses produced from fibre depend on the physico-chemical characteristics of the fibre source used (Jiménez-Moreno *et al.,* 2013a). Inclusion of 5% of either oat hulls or sugar beet pulp in the diet of broiler chickens from 1 to 18 days of age improved nutrient digestibility and feed conversion ratio (FCR), but when both sources were increased to 7.5% it had detrimental effects on growth and intestinal mucosa morphology (Jiménez-Moreno *et al.,* 2013a,b). The authors suggested that the inclusion of oat hulls, as an additional insoluble fibre source (oat hulls contains 706 g/kg insoluble fibre) was more useful in improving nutrient digestibility than the inclusion of sugar beet pulp because an increase in nitrogen excretion was observed with the sugar beet pulp diet. Unlike the insoluble fibre in oat hulls, sugar beet pulp contains soluble and fermentable pectin (Bach Knudsen, 2001; Serena and Knudsen, 2007) which could have contributed to increase nitrogen excretion. Pectin has also been shown to increase digesta viscosity and decrease nutrient digestion and absorption in the GIT (Langhout and Schutte, 1996; Langhout *et al.,* 1999).

Ikegami *et al.* (1990) showed that viscous indigestible polysaccharides present in a variety of sources (e.g. apple pectin, λ-carrageenan, locust bean gum, gum xanthan, guar gum and sodium alginate) inhibited pancreatic-biliary function of rats and depressed the process of digestion and absorption. In addition, digestibility of protein and fat has been observed in rats fed 5% viscous (soluble) fibres and this suggests that the fibre may have a direct inhibitory effect on the pancreatic enzymes (Ikegami *et al.,* 1990).

It is well documented that broilers (Jørgensen *et al.,* 1996) and turkeys adapt to insoluble non-starch polysaccharides (NSPs) by increasing the gastrointestinal tract (GIT) size (Sklan *et al.,* 2003). Commercial diets for young chickens usually contain less than 2.5 to 3.0% crude fibre (CF) per kg (Swennen *et al.,* 2010) and the inclusion of moderate

amounts of dietary fibre sources in the diet (e.g. between 2 and 10% oat hulls per kg) can increase production of HCl (Jiménez-Moreno *et al.,* 2010), bile acids and some digestive enzymes (Hetland *et al.,* 2003). A reduction in the pH as a result of higher HCl concentrations, in the upper part of the gastrointestinal tract might be necessary to activate peptic digestion (Sklan *et al.,* 1978).

Increased body size of pullets during the growing stage has a positive effect on hens' subsequent performance during the laying cycle (Summers *et al.,* 1987; Leeson and Summers, 1987; Leeson *et al.,* 1991). Pullets on or above target body weight at 6 to 8 weeks of age have greater performance during the laying cycles than those below target weight (Leeson and Summers., 1987). The activities of gut enzymes in posthatch broiler chicks have been shown to be correlated with body weight and intestinal development (Sklan and Noy, 2000) and may also have an influence on body weight gains in layer strain poultry.

In the previous experiments described in Chapters 4 and 5, adding an insoluble fibre preparation (Arbocel) into laying hens and pullet diets at 0.8% and 1%, respectively, resulted in significant increases in the digestive organ weights, including the gizzard and also increases in some digestive enzyme activities. Organ weights of those of pullets (Chapter 5) on long term (10 weeks) feeding were significantly heavier than those pullets that had no added fibre. Also, digestive enzyme activity increased in the organs of those birds fed Arbocel for 10 weeks (Chapter 5 Section 5.3).

There has been very little work on the effects of different sources of fibre on digestive tract development and proteolytic enzyme activities in growing layer pullets. Therefore two related experiments were carried out at different times to examine the effects of insoluble and soluble fibre preparations on enzyme activities in the digestive tracts of young pullets. In this chapter the first experiment (Experiment A) compared the effects of two different types of fibre supplements (added at 1% of the diet) in pullets aged 4 to 8 weeks which corresponds to a period during which there is maximal increase in rate of growth and enzyme function (Chapter 3).

In Experiment B a similar experiment compared the effects of the same two fibre supplements at a higher concentration (1.5% of the diet) on older pullets between 8 and 16 weeks of age when the growth phase of supply organs is slower but rate of body

weight gain continues to be high (Chapter 3). The design and results of the two parts are described separately and then discussed together.

6.1 Aim:

To determine the effects of two different fibre sources on growth and enzyme function in pullets of different ages.

6.2 Experiment A - Materials and Methods

6.2.1 Birds and experimental design

Forty-four Hy-Line Brown pullets were obtained from a commercial grower farm at 4 weeks of age. Eight pullets were randomly selected, weighed and killed on day of arrival and the internal organs weighed and samples were taken for enzyme function as described in Chapter 3 section 3.2.3. The remaining 36 pullets were weighed and randomly placed, four per pen in slatted floor pens (1.8 x 0.9 m), three pens per treatment. Plastic slatted flooring (1208 x 500 mm, sourced from Australasian Agricultural Services Qld., Australia) rather than sawdust was used to keep the birds clean.

The three treatments were Control pullets (Group C) given a commercial starter feed (from 4 to 6 weeks) and a commercial grower feed (from 6 to 8 weeks) with no additives, a second group (Group MF) was given the same diet as the controls but it was supplemented with 1% of a mixed fibre preparation of insoluble and soluble fibre (Opticell^{C5} Agromed Austria GmbH), the third group (Group IF) was given the same diet as the controls but it was supplemented at 1% with an insoluble fibre preparation (Arbocel® RC, J. Rettenmaier and Söhne GmbH and Co., Rosenberg, Germany). The commercial feed used was as described in Chapter 3, Section 3.2.1. The concentrations of fibre in the three treatments were analysed by ***FEEDTEST*** (Agrifood Technology Pty Ltd., Victoria, Australia) and were Control, 3%; MF, 3.8%, and IF 3.6%.

At 8 weeks age (after 4 weeks on the experimental diets) all 12 birds from each group were weighed, killed and samples were taken for measuring organ weights and enzyme function as described in Section (Chapter 3 section 3). Tissue samples from the

jejunum of Groups C, MF and IF were taken and stored at -20^{o}C in RNA*later*® (Cat. No. R0901 SIGMA) for the analysis of Mucin 2 (MUC2) gene expression as described in Chapter 5, Section 5.2.3. The experiment was conducted in accordance with the principles and specific guidelines of LTU-AEC the project number AEC12-68.

6.2.2 Statistical analysis

Data were checked for normality and were analyzed using one-way analysis of variance (SPSS 19.0 for Windows, USA). Statistical significance between means of different treatment groups was compared by Turkey's test at $P \leq 0.05$.

6.3 Experiment B - Materials and Methods

6.3.1 Birds and experimental design

This part of experiment was conducted in the same shed as used for Part A. Sixty-eight Hy-Line Brown pullets aged 8 weeks were obtained from a commercial chicken supplier in Victoria. The experiment consisted of three treatments. At the start of the experiment, 8 pullets were killed to provide initial information on weight of organs and enzyme activities. The remaining 60 were randomly placed into 12 pens, 5 pullets / pen with 20 pullets / treatment (Appendix Photo 6.1). The pens with plastic slatted floors were the same as those described in Section 6.2.1. All pullets were fed according to amounts recommended by Hy-Line Company (Chapter 3 Section 3.3).

The three treatments were Control pullets (Group C) given an un-supplemented commercial grower feed (from 8 to 12 weeks) and commercial finisher from 12 to 16 weeks, a second group (Group MF) was given the same diet as the Controls but it was supplemented with 1.5% of a mixed fibre preparation of insoluble and soluble fibre (Opticell^{C5} Agromed Austria GmbH), the third group (Group IF) was given the same diet as the Controls but it was supplemented at 1.5% with an insoluble fibre preparation (Arbocel® RC, J. Rettenmaier and Söhne GmbH and Co., Rosenberg, Germany).

The commercial feed used was as described in Chapter 3, Section 3.2.1. The concentrations of the fibre supplements were higher (1.5%) than in the experiment described in Section 6.2.1 (Experiment A). The concentrations of fibre in the three

treatments (commercial grower and finisher) were analysed by ***FEEDTEST*** (Agrifood Technology Pty Ltd) and were Control, 4%; MF, 6%, and IF, 5.5%.

Four and 8 weeks after the start of the experiment, 10 pullets from each treatment were killed for collection of samples as described in Chapter 3, Section 3.2.2. In addition, tissues samples from the proventriculus of Groups C, MF and IF were taken and stored at -20^{o}C in RNA*later*® (Cat. No. R0901 SIGMA) for the analysis of pepsinogen gene expression as described in Chapter 5 Section 5.2.3.

The experiment was conducted in accordance with the principles and specific guidelines of LTU-AEC the project number AEC12-68.

6.3.2 Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA, SPSS 19.0 for Windows, USA). All data were checked for mormality. Statistical significance between means of different treatment groups was compared by Turkey's test at $P \leq 0.05$.

6.4 Results Experiment A

6.4.1 Live body and organ weights

Pullets in Groups MF and IF were significantly heavier in live weight than those in the Control group after 4 weeks on the treatments (8 weeks of age) (Table 6.1). The weights of gizzards of pullets in Group MF and IF were significantly ($P < 0.05$) heavier than those of Group C: although weights were heavier in Group MF than IF the difference was not significant. The weights of proventriculus, pancreas and liver did not differ significantly among the treatments at 8 weeks of age but all had increased significantly from the initial weights at 4 weeks of age.

The relative weight (RW) of the gizzard expressed as a percentage of body weight (Table 6.1) in the MF Group was significantly ($P < 0.05$) higher than that of the Control Group while the RW of proventriculus, pancreas, and the liver was not significantly different among the experimental groups.

Table 6.1 Live body (g) and organ weights (g) and the relative weights of organs of 8 week old pullets fed control diet (C) without additives, diet MF (1% mixture of insoluble + soluble fibre) and diet IF (1% mixture of insoluble for 4 weeks (Mean ± SD, n = 12)

Time on diet	0	4		
Age (weeks)	4	8		
Treatments	Initial weight	C	MF	IF
Initial body weight	-	350.5 ± 14.23	357.8 ± 12.65	351.9 ± 16.43
Live body weight	369.6 ± 27.48^{a}	647.8 ± 42.82^{b}	696.4 ± 34.32^{c}	717.5 ± 50.50^{c}
Liver	13.19 ± 2.49^{a}	25.67 ± 4.62^{b}	26.54 ± 2.57^{b}	29.02 ± 3.87^{b}
Gizzard	11.93 ± 1.29^{a}	23.56 ± 3.46^{b}	30.66 ± 4.01^{c}	27.92 ± 3.16^{c}
Proventriculus	2.31 ± 0.14^{a}	3.97 ± 0.59^{b}	4.08 ± 0.59^{b}	4.06 ± 0.43^{b}
Pancreas	1.43 ± 0.19^{a}	2.61 ± 0.55^{b}	2.67 ± 0.24^{b}	3.05 ± 0.49^{b}
Relative weight (% of body weight)				
Liver	3.56 ± 0.57	4.05 ± 0.45	4.09 ± 0.28	4.07 ± 0.65
Gizzard	3.22 ± 0.18^{a}	3.77 ± 0.45^{ab}	4.41 ± 0.62^{c}	3.91 ± 0.53^{bc}
Proventriculus	0.63 ± 0.07	0.62 ± 0.05	0.59 ± 0.09	0.57 ± 0.06
Pancreas	0.39 ± 0.03	0.42 ± 0.07	0.41 ± 0.02	0.43 ± 0.08

Means with different subscript letters in the same row indicates significant ($P < 0.05$) difference between mean treatments, n for age 4 weeks is 8 samples.

6.4.2 Enzyme activities in proventriculus and pancreas

There was a significant ($P < 0.05$) decrease in pepsin activity per g tissue/ min in Groups C and MF, 4 weeks after the start of the treatments and, for both groups activity in Group IF did not decrease significantly from that at 4 weeks of age and was also significantly greater than that of Groups C and MF (Table 6.2). When the pepsin activity was expressed per whole organ/ min it increased significantly with age (Group C vs Initial). But only pullets in Group IF had higher rates of activity than Controls 4 weeks after the start of the experiment (Table 6.2).

The general proteolytic activity (GPA) per gram tissue increased significantly with age (Group C vs Initial) and treatment: activities in pullets in Groups MF and IF were significantly greater than those of Control but there was no significant difference between activities of Groups MF and IF. The GPA per pancreas had a similar effect with both supplements resulting in a significant increase in activity compared to the Control group.

Chymotrypsin activity per g tissue/ min and per organ decreased significantly after 4 weeks in all treatment groups: per organ there were no significant effects of the treatments. Trypsin activity per g tissue/ min increased significantly from 4 to 8 weeks of age (Control vs Initial) and the increase in activity in Group IF was significantly greater than the increases in Groups C and MF (Table 6.2) after 4 weeks on the treatments. Trypsin activity per organ increased in all groups after 4 weeks on the experimental diet but there was no significant difference between treatment groups.

Table 6.2 Enzyme activities per gram tissue and per organ of 8 week old pullets fed control diet (C) without additives, diet MF (1% mixture of insoluble + soluble fibre) and diet IF (1% mixture of insoluble) for 4 weeks (Mean ± SD, n = 12)

Time on diet	0	4		
Age (weeks)	4	8		
Treatments	Initial value	C	MF	IF
Pepsin				
per g tissue	87.92 ± 6.07[a]	74.79 ± 1.89[b]	75.34 ± 3.66[b]	85.13 ± 6.01[a]
per organ	203.21 ± 19.45[a]	295.92 ± 17.25 [b]	306.79 ± 36.90[b]	345.23 ± 25.03[c]
GPA				
per g tissue	25.76 ± 3.36[a]	96.80 ± 6.95[b]	111.61 ± 7.35[c]	112.71 ± 5.36[c]
per organ	36.98 ± 7.57[a]	271.18 ± 31.92[b]	321.54 ± 29.19[c]	324.03 ± 42.50[c]
Chymotrypsin				
per g tissue	4.84 ± 0.19[a]	4.17 ± 0.11[b]	4.23 ± 0.10[b]	4.33 ± 0.12[b]
per organ	6.92 ± 0.95[a]	11.33 ± 1.84[b]	12.19 ± 0.87[b]	12.44 ± 1.55[b]
Trypsin				
per g tissue	209.63 ± 5.31[a]	231.69 ± 9.33[b]	230.93 ± 8.69[b]	243.87 ± 8.67[c]
per organ	300.38 ± 44.44[a]	629.36 ± 101.37[b]	666.15 ± 60.98[b]	701.68 ± 93.63[b]

Means with different superscript letters in the same row indicates significant ($P < 0.05$) differences between treatments, n at 4 weeks of age is 8.

Units: Pepsin - pepsin hydrolysis of 1µmol of haemoglobin per g tissue or per organ per min. GPA - General proteolytic activity - µmoles free tyrosine released from casein substrate per g tissue or per organ per min. Chymotrypsin activity - *p*-nitroaniline released from N–glutaryl-L-phenylalanine-p-nitroanilide per g tissue or organ per min. Trypsin activity - trypsin units of *p*-nitroaniline released from Nα-benzoyl-DL- arginine-p-nitroanilide per g tissue or organ per min.

6.4.3 mRNA expression of mucin 2 (MUC2)

Expression of mRNA for jejunal mucin 2 (MUC2) gene was increased significantly ($P < 0.05$) in Group IF compared to Groups C and MF and mRNA expression in the MF treatment was not significantly different from that of Group C (Figure 6.1).

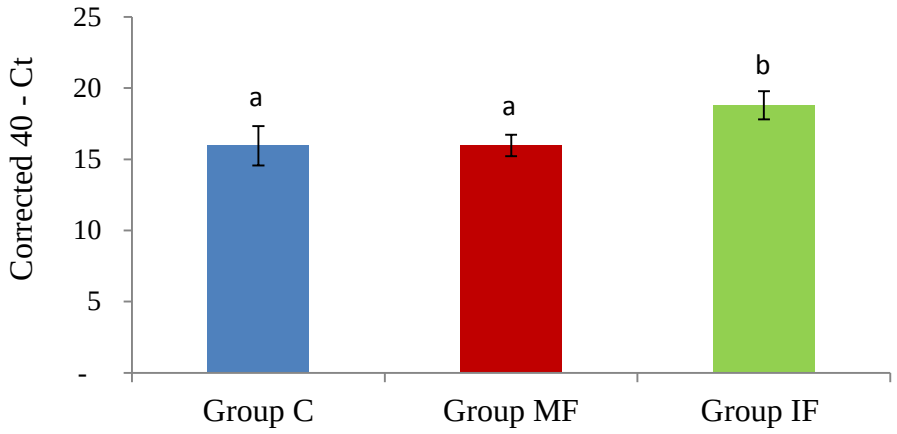

Figure 6.1 Mucin 2 (MUC2) mRNA expression of growing pullets fed control diet (C) without additives, diet MF (1% mixture of insoluble + soluble fibre) and diet IF (1% mixture of insoluble) (Mean ± SE, n = 5). Changes in MUC2 mRNA expression are normalized to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) mRNA.

6.5 Results Experiment B

6.5.1 Live body and organ weights

Body weights (Table 6.3) of pullets in all treatment groups after 4 and 8 weeks had increased significantly ($P < 0.05$) compared to the initial weights at the start of the experiment. There was no significant effect of fibre on weight of pullets after 4 weeks on the treatments although both Groups (MF and IF) fed fibre were heavier than Controls. After 8 weeks however, body weights of pullets in Group IF (with 1.5% Arbocel) were significantly ($P < 0.05$) heavier than pullets fed the control diet (Group C). Although live weight of pullets in Group IF was greater than those on the mixed soluble and insoluble fibre (Group MF) diet, the difference was not significant and there was no significant difference between Group MF and Group C pullets.

At 8 weeks after the start of the treatments the weights of liver, gizzard and small intestine in Group IF were significantly heavier ($P < 0.05$) than those of Groups C and MF. There was no other effect of treatment on organ weights (Table 6.3).

6.5.2 Enzyme activities

a. Enzyme activities in proventriculus and pancreas

Pepsin activity per gram tissue after 4 weeks on the experimental diets was not significantly different from initial activity at the start of the experiment or among treatments (Table 6.4). Pepsin activity per gram tissue /min decreased in both Control and MF Groups after 8 weeks on the diets compared with initial activity and activity was significantly greater in Group IF compared to the Control group (Table 6.4). After 8 weeks on the diets, activity of pepsin in Control fed pullets was significantly lower than activities at 4 weeks in pullets in Groups C and IF.

Table 6.3 Body and organ weights (g) of pullets fed a control diet (C) without additives, diet MF (1.5% mixture of insoluble + soluble fibre) and diet IF (1.5% mixture of insoluble fibre) from 8 to 16 weeks of age (Mean ± SD, n = 12)

Time on diet	0	4			8		
Age (weeks)	8	12			16		
Treatments	Initial weight	C	MF	IF	C	MF	IF
Initial body weight	-	576.4	575.5	580.5	585.4	580.9	578.3
Live body weight	572.33 ± 49.09^{a}	1030.9 ± 63.86^{b}	1113.7 ± 79.6^{b}	1130.0 ± 101.90^{b}	1419.3 ± 75.39^{c}	1485.9 ± 47.04^{cd}	1580.0 ± 60.56^{d}
Liver	18.69 ± 2.17^{a}	24.96 ± 3.32^{b}	27.67 ± 3.20^{b}	26.40 ± 1.36^{b}	30.45 ± 3.03^{b}	29.84 ± 2.21^{b}	37.07 ± 2.83^{c}
Gizzard	16.06 ± 2.91^{a}	24.92 ± 3.35^{b}	27.45 ± 3.01^{b}	30.30 ± 3.30^{b}	30.20 ± 3.77^{b}	32.20 ± 4.66^{bc}	39.44 ± 4.40^{c}
Proventriculus	3.43 ± 0.50^{a}	4.03 ± 0.28^{ab}	4.26 ± 0.46^{ab}	4.67 ± 0.58^{b}	4.18 ± 0.37^{ab}	4.46 ± 0.46^{ab}	4.66 ± 0.59^{b}
Pancreas	1.99 ± 0.37^{a}	2.4 ± 0.19^{ab}	2.71 ± 0.22^{bc}	2.65 ± 0.19^{bc}	2.71 ± 0.30^{bc}	2.80 ± 0.18^{bc}	3.09 ± 0.24^{c}
Small intestine	22.76 ± 2.80^{a}	21.96 ± 1.75^{a}	24.20 ± 2.40^{ab}	24.6 ± 2.09^{ab}	23.75 ± 1.78^{a}	23.44 ± 1.65^{a}	28.25 ± 1.82^{b}

Means within the same row with different superscript letters differ significantly ($P < 0.05$), n for initial weight (age 8 weeks) was 8 pullets.

When the activity was expressed as pepsin units per whole organ (Table 6.4) it was significantly higher ($P < 0.05$) in Group IF after 4 weeks than in pullets in Groups C and MF: compared with initial values, activities in Groups MF and IF were significantly greater after 4 weeks on the treatments. Activity in the IF Group after 8 weeks was also significantly higher ($P < 0.05$) than activity in Group C but not different from Group MF. After 8 weeks, activity in Group MF was not significantly different from Controls.

General proteolytic activities in the pancreas expressed per gram tissue/ min were significantly greater in all treatments at 4 and 8 weeks after feeding (Table 6.4) compared to the initial value at the start of the experiment when pullets were 8 weeks old. After 8 weeks the GPA activities per gram tissue were significantly higher in the IF group compared to Group C. The activity in the MF Group pullets was significantly lower 4 weeks after the start of the treatments than in those birds fed insoluble fibre (Group IF) but, although lower than Group IF after 8 weeks on treatments, it was not significantly different. General proteolytic activity per organ increased significantly ($P < 0.05$) in Group IF fed pullets compared to those in Groups C and MF. In addition, activity after 4 weeks on the diets did not change significantly between pullets on the control (Group C) and Group MF diets.

Chymotrypsin activity (per g tissue or per pancreas) did not show any significant differences between diet treatments. Activity of trypsin (per g tissue) decreased after 8 weeks (Table 6.4) for all treatment groups. There was a decrease in activity over time but the activity in Group IF pullets was significantly higher compared to those pullets fed control or MF diets.

There was a decrease in trypsin activity g tissue over time but the activity in Group IF pullets was significantly higher compared to those pullets fed control or MF diets. For activity of trypsin per pancreas, at 4 weeks no differences were seen between experimental groups however, there was a slight increase in those in Group IF.

Table 6.4 Enzyme activities in proventriculus and pancreas of pullets fed control diet (C) without additives, diet MF (1.5% mixture of insoluble + soluble fibre) and diet IF (1.5% mixture of insoluble Fibre) from 8 to 16 weeks of age (Means ± SD, n = 10)

Time on diet	0	4			8		
Age (weeks)	8	12			16		
Treatments	Initial value	C	MF	IF	C	MF	IF
Pepsin							
per g tissue	62.55 ± 5.42^{ab}	65.16 ± 6.83^{a}	65.10 ± 9.90^{ab}	68.63 ± 8.14^{a}	53.50 ± 5.64^{b}	60.61 ± 6.82^{ab}	70.66 ± 6.17^{a}
per organ	214.41 ± 34.32^{a}	261.58 ± 25.81^{ab}	273.70 ± 18.74^{b}	314.87 ± 22.27^{c}	224.01 ± 29.78^{ad}	268.95 ± 27.35^{abc}	326.98 ± 32.22^{c}
GPA							
per g tissue	36.02 ± 12.82^{a}	60.91 ± 11.36^{bc}	59.51 ± 10.06^{b}	80.47 ± 10.19^{cd}	72.84 ± 12.99^{bc}	74.89 ± 13.73^{bd}	94.10 ± 6.70^{d}
per organ	69.88 ± 19.06^{a}	150.65 ± 27.85^{b}	160.67 ± 26.89^{b}	211.67 ± 16.34^{c}	195.92 ± 92^{bc}	207.49 ± 25.29^{bc}	338.93 ± 32.02^{d}
Chymotrypsin							
per g tissue	3.77 ± 0.18^{a}	3.99 ± 0.40^{ab}	3.98 ± 0.30^{ab}	4.28 ± 0.21^{b}	3.84 ± 0.20b^{c}	3.80 ± 0.11^{ac}	4.02 ± 0.42^{bc}
per organ	7.52 ± 1.61^{a}	9.91 ± 1.15^{ab}	10.78 ± 1.10^{b}	11.34 ± 1.05^{b}	10.37 ± 1.04^{b}	10.62 ± 0.67^{b}	12.46 ± 1.95^{b}
Trypsin							
per g tissue	254.06 ± 27.62^{ab}	260.91 ± 24.17^{ac}	231.70 ± 21.45^{ab}	271.58 ± 26.76^{a}	219.53 ± 15.70^{bc}	204.87 ± 4.73^{b}	252.01 ± 10.16^{ad}
per organ	501.69 ± 95.20^{a}	646.74 ± 65.41^{ab}	625.28 ± 84.36^{ab}	716.38 ± 56.57^{bc}	594.47 ± 79.53^{abd}	572.63 ± 36.50^{ad}	778.492 ± 58.71^{c}
Dipeptidase							
per g tissue	15.11 ± 1.62^{a}	18.92 ± 1.28^{b}	20.19 ± 1.07^{b}	19.73 ± 2.75^{b}	14.23 ± 0.92^{a}	19.96 ± 2.98^{b}	14.29 ± 1.51^{a}
Aminopeptidase							
per g tissue	57.31 ± 5.39^{a}	84.25 ± 11.51^{bc}	81.62 ± 9.12^{bc}	80.40 ± 7.59^{bc}	63.40 ± 7.05^{a}	82.86 ± 3.63^{b}	69.18 ± 5.89^{ac}

Means with different superscript letters in the same row indicates significant ($P < 0.05$) differences between treatments, n at 4weeks of age is 8.

Units: :Pepsin - pepsin hydrolysis of 1µmol of haemoglobin per g tissue or per organ per min. GPA - General proteolytic activity - µmoles free tyrosine released from casein substrate per g tissue or per organ per min. Chymotrypsin activity - *p*-nitroaniline released from N–glutaryl-L-phenylalanine-p-nitroanilide per g tissue or organ per min. Trypsin activity - trypsin units of *p*-nitroaniline released from Nα-benzoyl-DL- arginine-p-nitroanilide per g tissue or organ per min. Dipeptidase activity - µmoles glycyl-L-leucine hydrolysed per g tissue per min. Aminopeptidase activity - nmoles *p*-nitroanilide released per g tissue per min.

b. Enzyme activities in small intestine (combined jejunum and ileum)

Dipeptidase activities per g tissue increased significantly in all groups after 4 weeks on the treatments compared with initial values (Table 6.4). Eight weeks after the start of the experiment, activity was significantly greater in Group MF than activities in Groups C and IF: these had decreased significantly from the values at 4 weeks after the start of the experiment and were not significantly different from those of the 8 week old pullets at the start of the experiment. Total activity of dipeptidase of Group MF pullets per small intestine /min (Figure 6.1) after 4 weeks on the treatments (12 weeks of age) was significantly greater ($P < 0.05$) than the activity at the start of the experiment and greater than that of Groups C and IF. After 8 weeks on the treatments activity in Group MF pullets was significantly greater ($P < 0.05$) than that of Control pullets but not those in Group IF.

Aminopeptidase activity per gram tissue was increased in all treatments 4 weeks after the start of the experiment compared with the initial activities at 8 weeks of age (Table 6.4) however there was no significant difference among activities of the three treatments. After 8 weeks on the treatments the activity in Group MF pullets had not changed from that after 4 weeks but was significantly greater than activities in Groups C and IF: activity in Group IF was lower but not significantly than that at 4 weeks but activity in Controls had decreased significantly from that at 4 weeks and was not significantly different from intitial activities at the start of the experiment.

Small intestinal aminopeptidase activity expressed per organ (Figure 6.2) was significantly greater ($P < 0.05$) in all treatment groups after 4 weeks on the treatments than initial activity at the start of the experiment: there was no significant difference between treatments. After 8 weeks on the treatments, activities in Groups MF anf IF were significantly greater than in the Control Group, the activity of which had decreased significantly below that at 4 weeks but was still significantly greater than that at the start of the experiment. The activity in Group IF pullets was not significantly different from that of Group MF or activities in all groups after 4 weeks on the treatments.

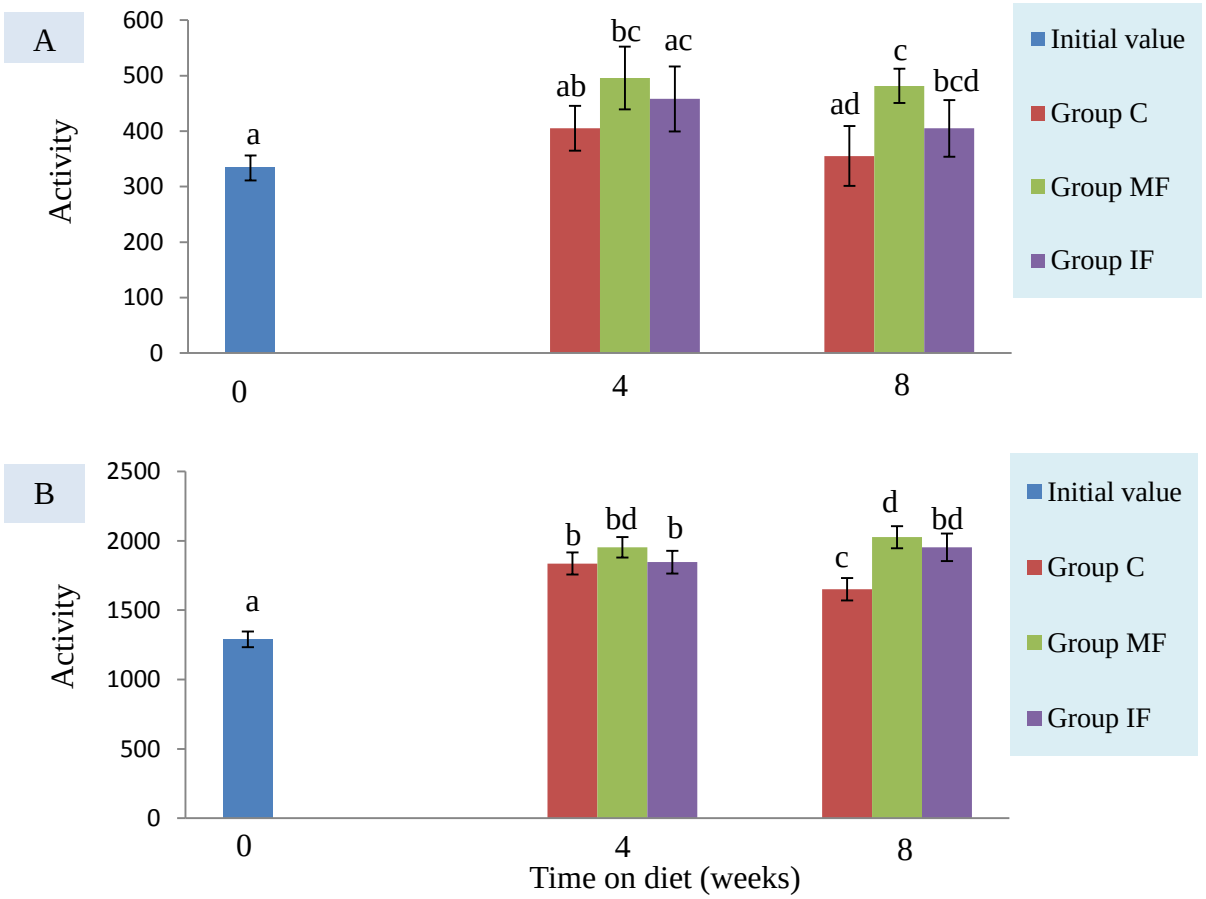

Figure 6.2 Activities of Dipeptidase (A) (µmoles glycyl-L-leucine) and aminopeptidase (B) (nmoles *p*-nitroanilide) hydrolysed or released per small intestine per min of layer pullets at different ages fed control diet (C), mixed (MF) and insoluble fibre (IF) (Means ± SD, n = 8). Different letters above bar for each enzyme indicates a significant difference ($P < 0.05$).

6.5.3 Gene expression for pepsinogens A and C

After 8 weeks on the experimental diets mRNA expression of pepsinogen A in the proventriculus of pullets in Groups MF and IF was significantly greater ($P < 0.05$) than in control pullets (Figure 6.3): although mRNA expression was greater in Group IF than MF there was no significant difference. Expression of pepsinogen C mRNA in pullets 8 weeks from the start of the experiment was significantly ($P < 0.05$) greater in Groups IF and MF compared to those in the Group C: there was no significant difference between Group IF and MF even though mRNA expression was greater in Group IF than in Group MF tissue.

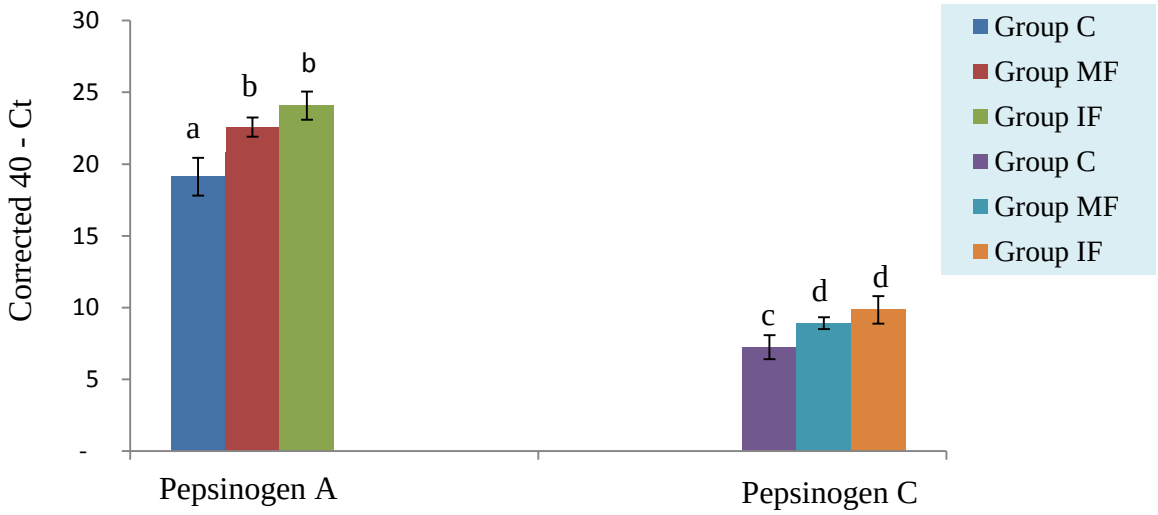

Figure 6.3 Pepsinogens A and C gene expression of layer pullets at different ages fed control diet (C), mixed (MF) and insoluble fibre (IF) (Means ± SE, n = 8).
a,b indicates significant differences for pepsinogens A
c,d indicates significant differences for Ppesinogen C.
($P < 0.05$)

6.6 Discussion

The present results show that giving supplements of both fibre products, MF and IF, from 4 to 8 weeks of age increased the growth of the pullets (Experiment A). However, in the older pullets (Experiment B), supplementing the diet from 8 weeks old for 4 weeks did not result in a significant increase in body weights compared to controls. Only after 8 weeks on the two supplements, MF and IF, were increases observed in body weight but only that of IF pullets was significantly heavier than controls. Body weight is the most frequently used indicator for growth and production and pullets must grow in weight in order to commence egg production (Summers *et al.,* 1987). An increase in the body weight of meat type chickens fed Arbocel BWW-40 has been reported by Szymeczko (2000) and Boguslawska-Tryk (2005). The increase in the broilers' body weight at 6 weeks of age in Boguslawska-Tryk's (2005) study was 7.5% as compared to the control treatment while in this experiment (Experiment A) pullets body weight at 8 weeks of age increased almost 9.7% as compared to the control group.

A similar effect to that seen here of fibre type on weight gain, was reported by Jimenez-Moreno *et al*. (2009b) in broilers fed different fibre sources from 1 to 21 days of age. In their experiments weight gain was significantly greater in the chicks fed oat hulls (insoluble fibre) compared with controls but not affected in those given sugar beet pulp (mixed soluble and insoluble fibres) (Jimenez-Moreno *et al*., 2009b). They also showed improvements in digestibility (retention) of dry matter, energy, ash and fat (ether extract) with addition of both sources of fibre but that of organic matter and nitrogen was only significantly improved by inclusion of the insoluble fibre source (oat hulls). The results obtained here also support those of Sarikhan *et al.* (2010) who showed that insoluble fibre incorporated in broilers diets at 0.25, .5 or 0.75% improves growth rate compared with unsupplemented controls.

The effect of dietary cellulose (insoluble fibre) on growth and nitrogen utilization was examined in 7 to 15 day old broiler chicks fed 65% crude protein (CP) and 80% metabolizable energy (ME) of Japanese feeding standard (Cao *et al.,* 1998). It was shown that body weight gain and nitrogen utilisation in chicks fed 1.5% and 3.5% cellulose were increased significantly while weight gain and retention rate of absorbed N were decreased when the inclusion levels were greater than 3.5% (Cao *et al.*, 1998; Cao, 2001).

In older, 8 week-old Single Comb White Leghorn male chickens fed 3 purified diets that contained 0, 3.5 and 10g cellulose /100g for 7 days (Cao *et al.*, 2003) weight gain was reduced by the 10g cellulose diet as was nitrogen retention and digestibility but no differences from controls were observed in group given 3.5 g cellulose / 100g diet. These changes in fibre effects could have been due to cellulose causing reduced passage of digesta through the intestinal tract and decreased nitrogen utilization as has been shown in rats (Shah *et al.*, 1982). A significant increase in growth and in nitrogen retention compared with controls has also been shown in broiler chicks fed different fibre sources (pea fibre, wheat bran or oat bran) from 12 to 28 or 35 days of age (Jørgensen *et al.*, 1996). Sklan *et al*. (2003) observed that growth rate and feed efficiency in turkeys decreased when the diet contained 8 to 9% crude fibre (CF) in the form of sunflower meal and soybean hulls. However, growth remained unaffected when 6% crude fibre was fed between 1 and 4 weeks or between 6 and 8 weeks of age, however it was increased between 11 and 14 weeks of age. As poultry lack enzymes to digest cellulose, part of the improved performance observed may have been due to digestion of some non-cellulose fraction of the CF, or, alternatively to intestinal microbial fermentation (Green, 1988).

Besides causing improvements in growth rate, supplementing the diets of poultry has also been shown to affect organ weights and the activity of digestive enzymes in those organs, however results across different experiments, do not always show similar responses. It has been shown that meat type chicks, up to 22 days may require an addition of fibre to their diets to stimulate the development of the upper gastrointestinal tract (González-Alvarado *et al.*, 2008). In their study González-Alvarado *et al.* (2008) showed that the relative weight of proventriculus and gizzard increased by adding 3% CF such as oat hulls and soy hulls to the starter diet of broiler chicks.

The weight of proventriculus in the 8 week old pullets in the experiments described here (Experiment A), was not significantly different from the controls or pullets fed the MF diet however in the older pullets (Experiment B) after 8 weeks on the 1.5% Arbocel diet (but not after 4 weeks) weight of the proventriculus was significantly greater than controls without added fibre which suggests that there could be an interaction between age and length of time fibre is included in the diet.

Jiménez-Moreno *et al*. (2009b) showed a significant increase in weight of the proventriculus in broilers given sugar beet pulp (SBP, soluble and insoluble fibre) but not

in those given oat hulls (OH, insoluble fibre) for 15 days. Although weight of the proventriculus was not increased in the IF pullets at 8 weeks old, pepsin activity was significantly increased by adding 1% insoluble fibre Arbocel but not with MF: this suggests that the weight of the organ may not reflect activity of the enzyme. Although pH in the gizzard of broilers is significantly reduced by both OH and SBP the pH in the proventriculus is not affected (Jiménez-Moreno *et al.*, 2009b, c; Jiménez-Moreno *et al.*, 2013b). However, the reduction of gizzard pH probably results from higher HCl secretion from proventriculus and this is considered to influence pepsin activity (Jiménez-Moreno *et al.*, 2009b, c, Sacranie *et al.*, 2012, Jiménez-Moreno *et al.*, 2013b**)**.

It is probable, however, that the increase in pepsin activity was due to an increase in mRNA expression for pepsinogens A and C which was observed in both treatments in the 16 week old pullets after 8 weeks on the diets. The effects of the fibre sources pectin, guar gum and lignin at levels of 10, 10 and 5 g/100g respectively reduced potential pepsin activity in GIT contents of the rat (Shah *et al.*, 1986) which suggests that the effect of fibre on overall enzyme function and efficacy is affected by many factors. However, improved nutrient digestibility reported by many authors (Rogel *et al.*, 1987; Petterson and Razdan, 1993; Jørgensen *et al.*, 1996; Sklan *et al*., 2003, Svihus *et al.*, 2003; Svihus, 2011; Mateos *et al.*, 2012 and 2014) suggests that overall inclusion of both mixed soluble and insoluble or insoluble fibre would be advantageous for growth and productivity of pullets.

The current experiments showed that the weight of the gizzard in MF and IF groups was significantly higher as compared to the control group at 8 weeks old after being on the diets for 4 weeks, however, in the older birds after 8 weeks on the diets only gizzards from the IF group were significantly heavier than controls. A well developed gizzard can increase the motility of the digestive tract and refluxing of feed from the duodenum to the gizzard, facilitate the mixing of digesta with enzymes and hence improve nutrient utilization (Guinotte *et al.*, 1995; Svihus and Hetland, 2001; Gabriel *et al.*, 2003a; Hetland *et al.*, 2005; Mateos *et al*., 2012; Mateos *et al.*, 2014).

The weight of the pancreas slightly increased in response to fibre treatments. Also the reports by Longstaff and McNab (1991) indicated no effect of the addition of fibre from faba bean hulls on the chicken pancreas weight a similar effect to that seen with the two fibre sources in these experiments.

The effects of the diet containing insoluble fibre on the pancreas and pancreatic function in the experiments reported here are similar to those reported in Chapters 4 and 5 in which pullets and hens given diets with insoluble fibre had increased general proteolytic, trypsin and chymotrypsin activities although the increases observed here were not always significant. The length of time on the diets, the concentration of insoluble fibre and also the ages of the pullets could have contributed to the differences observed. The mixed, soluble and insoluble diet (MF diet) induced a different response to that of the IF diet with no significant increase in the pancreatic enzymes after 4 and 8 weeks on the diets in the pullets given the diet from 8 weeks of age. However, in the younger pullets given diet MF from 4 to 8 weeks of age there was a significant increase in general proteolytic activity compared to controls but not in the two other enzymes, chymotrypsin and trypsin. The lack of effect of the MF diet on trypsin activity is also different from the results of an experiment in 8 week old piglets fed a diet containing potato fibre which consists of a mixture of soluble and insoluble fibre (Jakob *et al*., 2000). In that experiment there was a significant increase in trypsin activity in pancreatic secretions of the 8 week old piglets compared with the activity before and after being given the diets with potato flour.

Hormonal stimulation of pancreatic enzyme secretion is dependent on the entry of chyme into the intestine (Harper *et al.,* 1979), thus fibre could influence enzyme levels by affecting the passage rate of digesta in the digestive tract. Changes in pancreas exocrine activity, enzyme secretion and synthesis could also be related to the effect of fibre in the diet on release of hormones of the digestive tract (Hasik and Bartnikowska, 1987). Hormones such as CCK, secretin, gastrin, VIP and glucagon together with acetylcholine and noradrenaline act as intermediaries in the mechanism of activation of pancreatic secretion (Konturek, 1976; Sommer and Kasper, 1981). The relationship between the presence of fibre in the diet and release of CCK has been confirmed by research carried out in humans (Mössner *et al.,* 1992; Bourdon *et al.,* 2001).

The synthesis of proteolytic enzymes in pancreatic tissue is dependent on the maturity of the animal, the feeding system and the structure of the diet (Poort and Poort, 1981). Long term feeding (10 weeks) on Arbocel increased the activity of pancreatic enzymes such as GPA and trypsin in 8 to 18 weeks old pullets (Chapter 5), however, the effects of fibre in the present findings may disagree with other studies that showed a reduction in pancreatic enzymes when fibre was incorporated in the diet (Isaksson *et al.*, 1982; Dutta and Hlasko, 1985).

Trypsin activity in the IF Group increased in the present study in agreement with effects seen in the study of Boguslawska-Tryk (2005) who showed increased trypsin activities in broilers give the insoluble fibre Arbocel BWW-40 at different levels in the diets. The activity of trypsin was significantly greater than that of control broilers but chymotrypsin activity was reduced when Arbocel was included at the highest level (0.75, 0.85 and 0.95% of the starter, grower and finisher diets). It is therefore possible that the difference between the effects of IF on trypsin activity in the 8 week old pullets in Experiment A compared with the older pullets in Experiment B was due to a concentration effect.

A higher content of protein in the pancreas of birds fed Arbocel perhaps indicates increased enzymatic protein synthesis in the pancreas gland. In this study, pullets in Groups IF and MF had higher growth rate compared with the control group, which shows the benefits of the dietary supplements at this period of age 4 to 8 weeks.

The synthesis of proteolytic enzymes in pancreatic tissue is dependent on the maturity of the animal, the feeding system and the structure of the diet (Poort and Poort, 1981). Long term feeding (10 weeks) on Arbocel increased the activity of pancreatic enzymes such as GPA and trypsin in 8 to 18 weeks old pullets (Chapter 5), however, the effects of fibres in the present findings may disagree with other studies that showed a reduction in pancreatic enzymes when fibre was incorporated in the diet (Isaksson *et al.*, 1982; Dutta and Hlasko, 1985).

The present findings are similar to early studies that showed that the activity of pancreatic enzymes amylase, lipase, trypsin and chymotrypsin did not change when 10 % insoluble fibre in the form of cellulose or alfalfa and 5.0% soluble fibre in the form of pectin, guar gum, or metamucil were added to a fibre-free diet of rats (Calvert *et al*., 1985). Also, 20% additive of pure cellulose to a rat diet resulted in a slight decrease in the activity of pancreatic trypsin and a slight increase in the activity of chymotrypsin, as well as a significant decrease in the activity of both enzymes in the ileal digesta (Schneeman and Gallaher, 1980).

In Experiment B, the weight of the small intestine in pullets on the IF diet was increased and this finding is in conflict with other researchers who have shown that cellulose usage decreases small intestine weight in younger broilers (Noy and Sklan, 2002).

The activity of the peptidases, aminopeptidase N and dipeptidylpeptidase IV, increase when feeding pectin and barley hulls to monogastric animals in high fibre diets (Hedemann *et al*., 2006). In this experiment increases in activity were seen only in the pullets fed the MF diet. Jimenez-Moreno *et al*. (2009c) has shown that sugar beet pulp can increase digesta pH and this may have contributed to improve the activity of intestinal enzymes in the MF Group of pullets. It has also been shown that SBP but not OH can cause reductions in villus height and affect development of the intestinal tract (Jimenez-Moreno *et al*., 2013b), however, in this experiment the MF diet did not appear to affect enzyme function.

Feeding a diet containing carboxymethylcellulose (CMC) increases the intestinal viscosity reduces the villous height and increases the crypt depth (McDonald *et al.*, 2001). However, feeding a low viscosity carboxymethycellulose supplement resulted in longer villi (McDonald *et al*., 2001). Furthermore, type of fibre could partly be a means for changing the intestinal enzymes activity when diet MF was given to the pullets.

The effect of MF diet on the intestinal enzymes is unlikely to be a result of changes to mucin levels as expression of MUC2 mRNA was significantly increased in both treatment groups in Experiment A.

Insoluble fibre has an abrasive action, scraping mucins from intestinal mucosa as it passes through the GIT (Leterme *et al.*, 1998). The effect of insoluble fibre as cellulose or wheat bran at 10% levels of both was also tested in rats, and results indicated that the addition of fibre stimulated the activity of goblet cells and the small intestinal cytokinetics (Cassidy *et al.*, 1981). In another study on rats, Satchithanandam *et al.* (1990) showed that supplementation of 5% citrus fibre in a purified diet lead to a significant increase in intestinal mucin secretion. Their explanation to the effect of fibre was related to the cellulose and lignin of the citrus fibre. In another study carried by Satchithanandam *et al.* (1989) supplementing the diet of rats with 10% wheat bran stimulated intestinal mucin while 20% rice bran or cellulose did not.

Enzyme activities in the intestinal lumen can be changed according to the type of fibre being ingested by the animal (Schneeman *et al.*, 1982). In their study feeding rats wheat bran lead to changes in the exocrine pancreas and this may also have contributed to changes in the mucus production in the intestine. The mucus layer provided an area in

which enzymes act close to the intestinal epithelium surface for active hydrolysis and absorption of ingested nutrient (Montagne *et al.*, 2004).

6.6.1 Conclusion

The experiments carried out so far showed that the inclusion of Arbocel as a source of insoluble fibre increased body weight, organ weights and the expression and activities of digestive enzymes in the upper part of the digestive tract but had little effect on the development of the distal part. However, Opticell as a mixture of both soluble and insoluble fibre positively increased the activity of intestinal enzymes such as dipeptidases and aminopeptidase. In conclusion, selecting an appropriate fibre source at a level that enhances gain in weight and the expression of mRNA of proteolytic enzymes such as pepsin for better protein digestion could help to support the body requirements of protein for growth, maintenance and development. Finally, a level of 1 to 1.5 g IF/100g is recommended as a dietary additive for improving organ development and hence enhancing nutrient digestibility through active enzyme function.

Chapter 7
General Conclusions

The experiments described in this thesis show that at all ages tested enzyme activity and growth in layer pullets can be increased by fibre supplementation. New information is presented on the effects of fibre supplementation on supply organ development and of the activities of digestive enzymes in different organs that are involved in breakdown of dietary proteins to amino acids. Such information has not been well documented in layer strain poultry but could be of considerable value in formulating rations that allow layer hens to reach their optimal potential for egg production and maintenance of good health.

Although the rates of weight gain by supply organs and the rates of increase in enzyme activities declined from about 10 to 12 weeks of age in the layer pullets (Chapter 3), insoluble fibre was able to induce a positive effect on digestive organ growth and enzymes activities in hens over 18 weeks of age. In hens supplemented from the point of lay for 9 to 12 weeks (Chapter 4), increases in live body weights, organ weights (liver and gizzard) and activities of some of the digestive enzymes (pepsin and general pancreatic (GP), chymotrypsin and trypsin enzymes) were observed and indicate that there is plasticity in supply organ development that could be useful for improving live weight gains in pullets before and after point of lay.

The length of time pullets were given supplements was shown to affect responses (Chapter 5). In 8 week old pullets supplemented for 5 weeks with a higher concentration of insoluble fibre than used previously (1% vs 0.8% in hens), significant increases in organ weights were not seen and only increases in pancreatic GP and chymotrypsin activities were observed (Chapter 5). Supplementation for a longer period of 10 weeks resulted in significant increases in weights of all the supply organs measured and in pepsin and pancreatic, but not intestinal enzymes. Removing IF from the diet for 5 weeks after 5 weeks supplementation from 8 to 13 weeks of age did not result higher rates of enzyme activities at 13 weeks of age: starting the 1% IF supplement at the later age of 13 weeks and continuing it for 5 weeks until 18 weeks of age also had no significant effect on organ weights or enzyme activities. These results suggest that age of development of

pullets when supplementation is begun and the duration of supplantation are important if beneficial effects of IF supplementation is to be achieved.

Two further experiments described in Chapter 6 again showed that the timing of IF supplementation was important if significant improvements in organ growth or enzyme function were to be achieved: at the same time, comparison of the effects of IF supplementation with those of supplementation with a mixed, soluble/insoluble fibre (MF) preparation, Opticell, showed differences in effects on the functioning of the supply organs. The first experiment (Experiment A) was carried out in young pullets from 4 weeks of age and diets were supplemented with 1% IF or 1% MF for the relatively short period of 4 weeks. The period of supplementation coincided with the period when the most rapid rates of growth of supply organs and increases in enzyme activities occurred (Chapter 3). Results showed significant increases in weight gain, and gizzard weights and GP activities of the pancreas with supplementation of both fibre types: pepsin and trypsin activities were increased only in the IF supplemented pullets, the results show that, unlike the older pullets (8 to 18 weeks of age) fibre supplementation for a short period is beneficial.

In the second experiment (Experiment B) where pullets of 8 weeks old were given diets supplemented with a higher concentration of fibre, 1.5% compared with similar aged birds in the experiment described in Chapter 5, the IF caused increases in supply organ weights compared with controls after 8 weeks but still had no significant effect after a short period of 4 weeks on the supplement: significant effects of MF were not observed after 4 or 8 weeks on the supplement. Significant effects on enzyme activities were not seen at 4 weeks and, at 8 weeks, pepsin and pancreatic GP and trypsin activities were only greater in the IF supplemented group: on the other hand intestinal dipeptidase and aminopeptidase activities were only greater in the MF supplemented pullets after 8 weeks.

The experiments carried out here indicated that the inclusion of Arbocel as a source of insoluble fibre increased body weight, organ weights and the expression and activities of digestive enzymes in the upper part of the digestive tract but had little effect on the development of the distal part of the GIT. However, Opticell as a mixture of both soluble and insoluble fibre increased the activity of intestinal dipeptidases and aminopeptidase.

Because of limited facilities for rearing pullets from hatching until the age required for an experiment, it was necessary to purchase pullets from commercial growers

and there was no control of their management prior to their being received and the information on age of pullets came from the growers. This in some respects makes the positive results of fibre on the gastrointestinal tract more robust. However it is worthwhile comparing live weights (Appendix 7.1) at the time the pullets were received with the standards for the Hy-Line Brown strain of poultry (Hy-Line International, 2014). The pullets at all ages obtained for the experiment in Chapter 3 were close to the standard weights for age. The 8 week old pullets for the experiment in Chapter 5 were heavier than the standard weights for 8 week old pullets and could have been 9 weeks old, however, at 13 weeks and 18 weeks the control pullets were heavier than the standards for those ages. Pullets obtained for the experiment described in Chapter 6 (Experiment A) may have been about 5 rather than 4 weeks old but after 4 weeks their weights were similar to the standard for 8 week old pullets and in the final experiment (Chapter 6 Experiment B) the 8 week pullets were a little lighter than the 8 week standard weight but after 8 weeks they had exceeded the 16 week standard weights.

These results across the experiments suggest that there is probably some flexibility availably when applying dietary fibre treatments to layer pullets. However, it is possible that a better understanding of the effects controlling liver and other supply organ growth may help to contribute to improve uniformity of poultry in egg laying flocks and thus improve productivity.

Because the variation in weights for age were probably less than 7 days for all experiments it is probable that this did not affect substantially the outcomes of the treatments. However, it is also worth noting that there were also differences in weights of the supply organs in control pullets across the different experiments (Appendix 7.2 to 7.6). Although poultry breeders emphasise the importance of uniform live weight in flock management there is not much information on variations in organ weights for age in different strains of layer poultry. The data available from the experiments describe in this thesis is not comprehensive enough but does suggest that as supply organs play an important role in growth of pullets it may be important to assess variability (in particular that of liver) as a means of contributing to improved management strategies.

The findings in Chapter 3 of this experiment suggest that the activity of digestive enzymes for protein digestion in layer pullets changes according to the age of bird. However the experiments described here in subsequent chapters, also show that inclusion

of fibre in the diet can influence enzyme activity and thus availability of nutrients for absorption.

Suggestions for future research

Results in this thesis showed that purified fibre can increase rate of growth of supply organs and digestive enzyme activities in layer pullets before and after their periods of linear growth. Suggested further studies to clarify the mechanisms by which fibre influence digestive function and by which optimal economic benefits to the poultry industry can be achieved are by:

- determining the effects of dietary supplementation of purified forms of insoluble (eg cellulose, hemicellulose, lignin) and soluble (eg pectin) fibres and of different ratios of insoluble and soluble fibres on growth of supply organs and digestive enzyme gene expression and function,
- determining how the changes to supply organ development and digestive enzyme activities at different ages relate to improved production,
- identifying the effect of different types and concentrations of fibre given at different ages on endocrine hormones eg CCK, gastrin and secretin through measurement of mRNA expression of the hormones or of their concentrations in blood,
- determining optimal age(s) for supplementation that will result in long term, life-time benefits to pullet health and to producers,
- determining the short and long term production benefits of fibres through their effects on feed conversion efficiency of different supplement ratios and types of fibres.

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Appendix Tables and Figures

Appendix Table 5.2 Reverse-Transcription PCR reagents and volumes used with the DyNAmo™ cDNA synthesis kit (ThermoFisher Scientific, Australia)

Reagents	Stock	Reaction volume 20µl
RT Buffer	2x	10µl
Random hexamer primer set	300 ng/µl	1µl
Template RNA		5µl
M-MuLV RNase H^+ reverse transcriptase		2µl
RNase-Free Water		2µl
Total volume		20µl

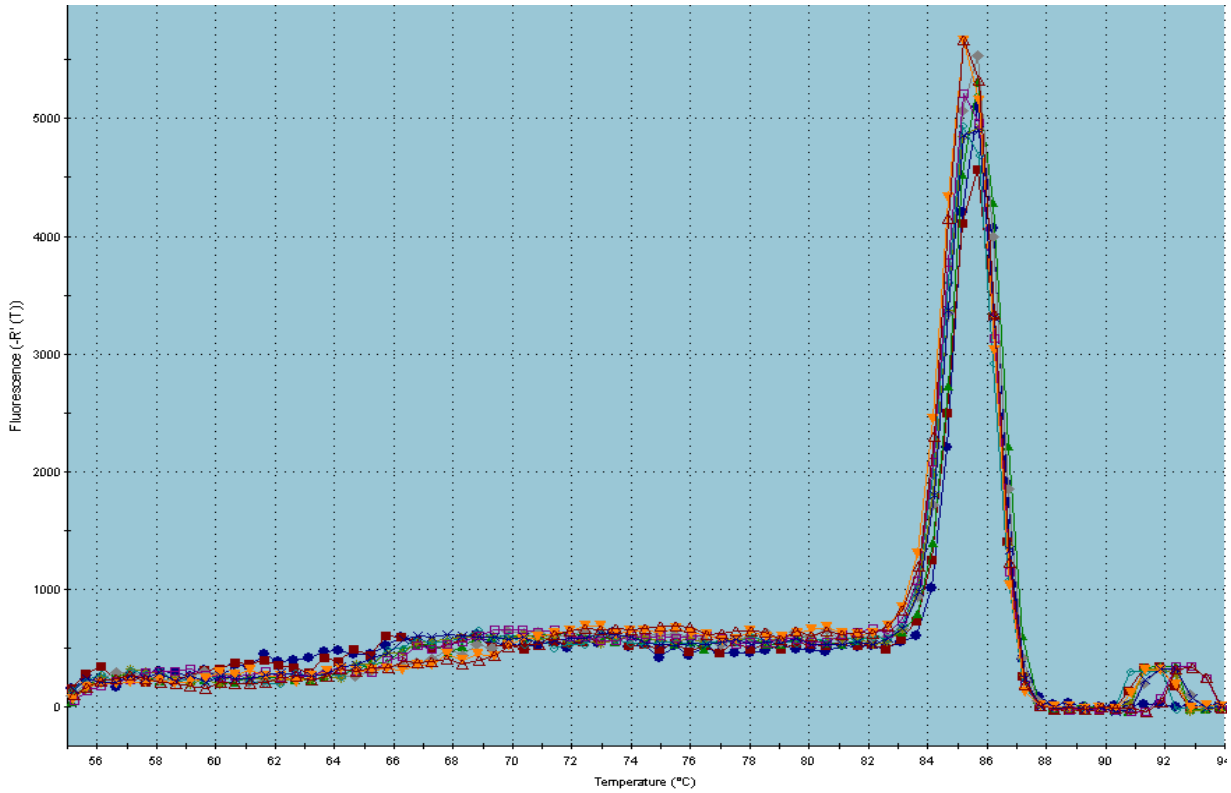
Appendix Table 5.4 Protocol for PCR reaction (QIAGEN – Australia)

Reagent	25µl reaction	Final concentration
10X Standard *Taq* Reaction buffer*	2.5µl	1x
10 mM dNTPs	0.5 µl	200 µM
10 µM Forward Primer	1 µl	0.5 µM
10 µM Reverse Primer	1 µl	0.5 µM
Template DNA (cDNA)	5 µl	<1,000ng
Taq DNA Polymerase	0.125 µl	1.25 units
Nuclease-free water	14.875 µl	n/a

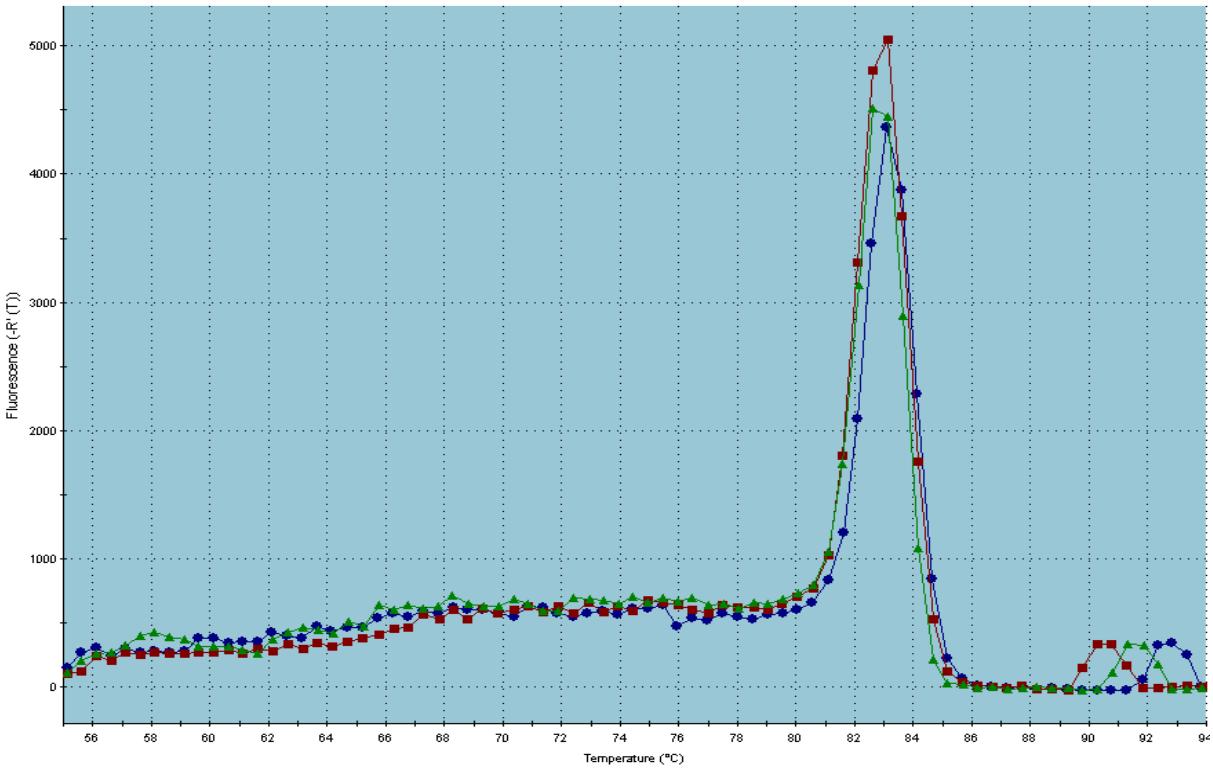
*Contains 15 mM MgCl2 (QIAGEN)

Appendix Table 5.5 Reagents and volumes for RT-qPCR singleplex

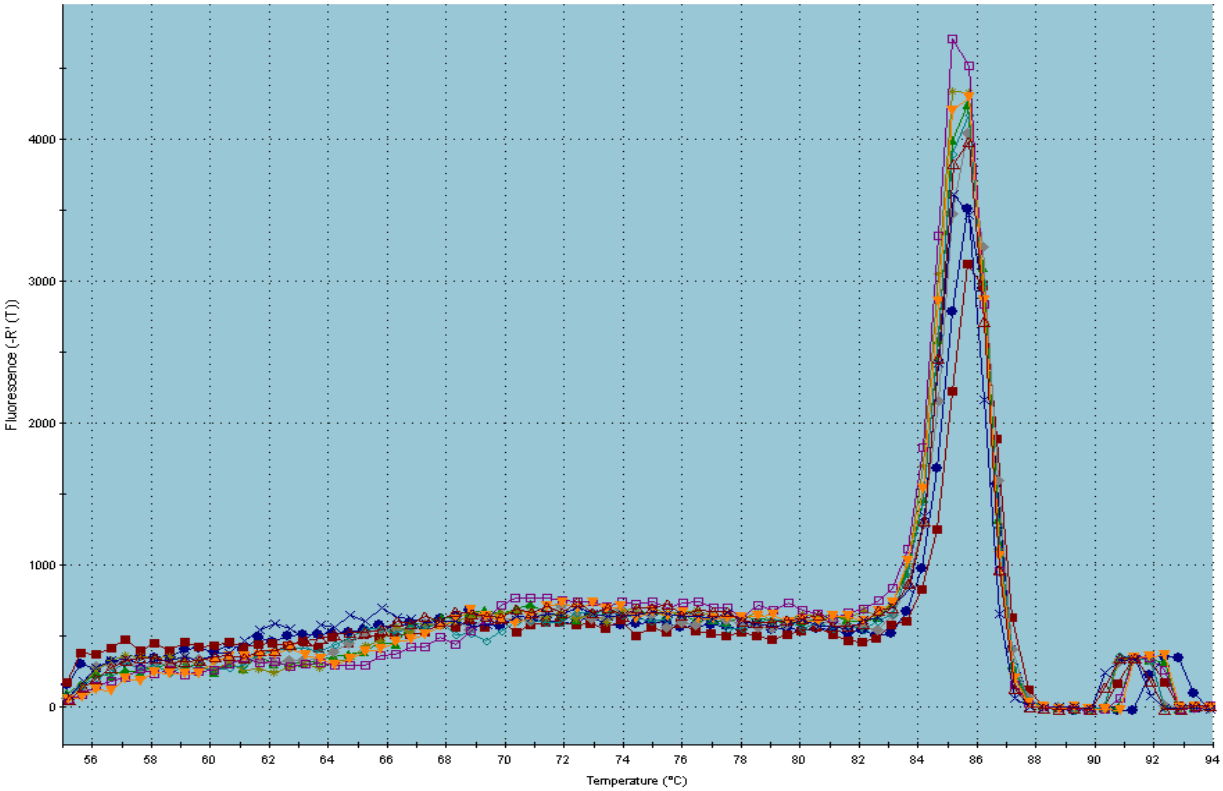
Reagent	50µl reaction	Final concentration
2x SensiMix SYBR Low-ROX	25µl	1x
25 µM Forward Primer	1µl	250 nM
25 µM Reverse Primer	1µl	250 µM
Template DNA (cDNA)	5µl	
Nuclease-free water	18µl	



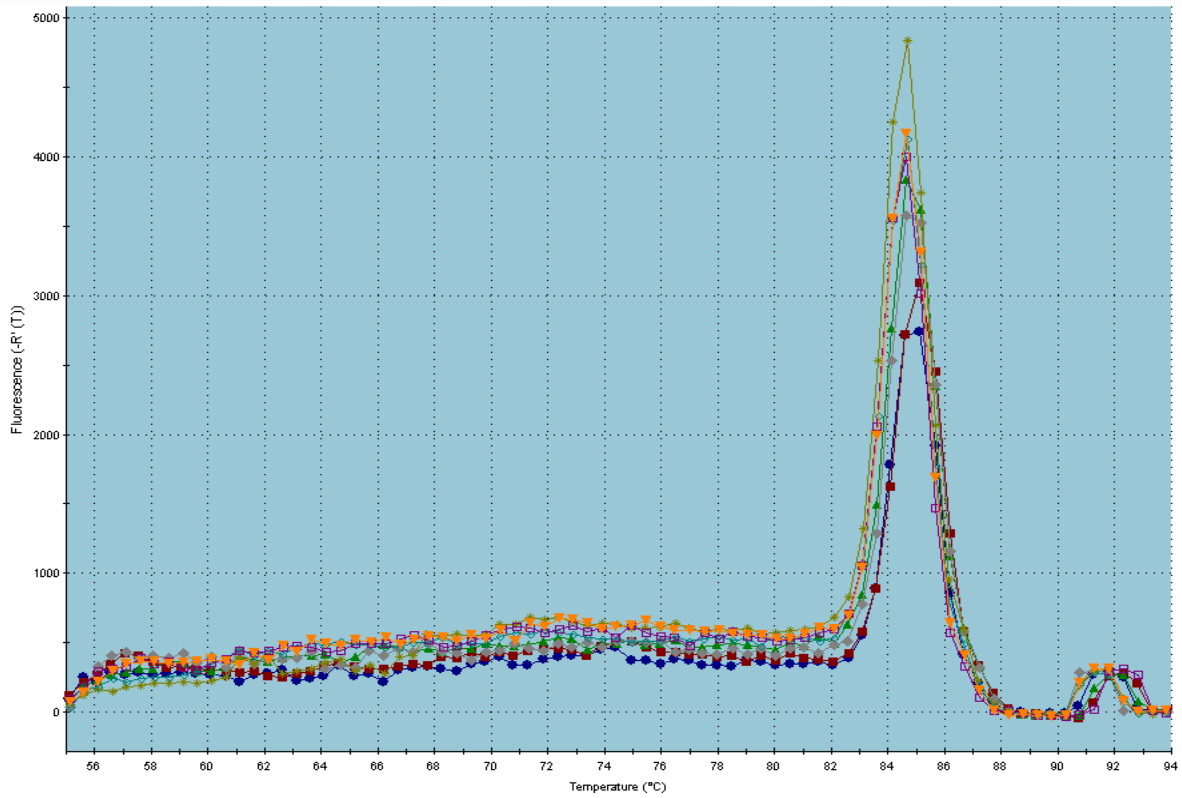
Appendix Figure 5.1. Melt curve plot for Pepsinogen A in proventriculus of layer pullets age 16 weeks (n = 6)



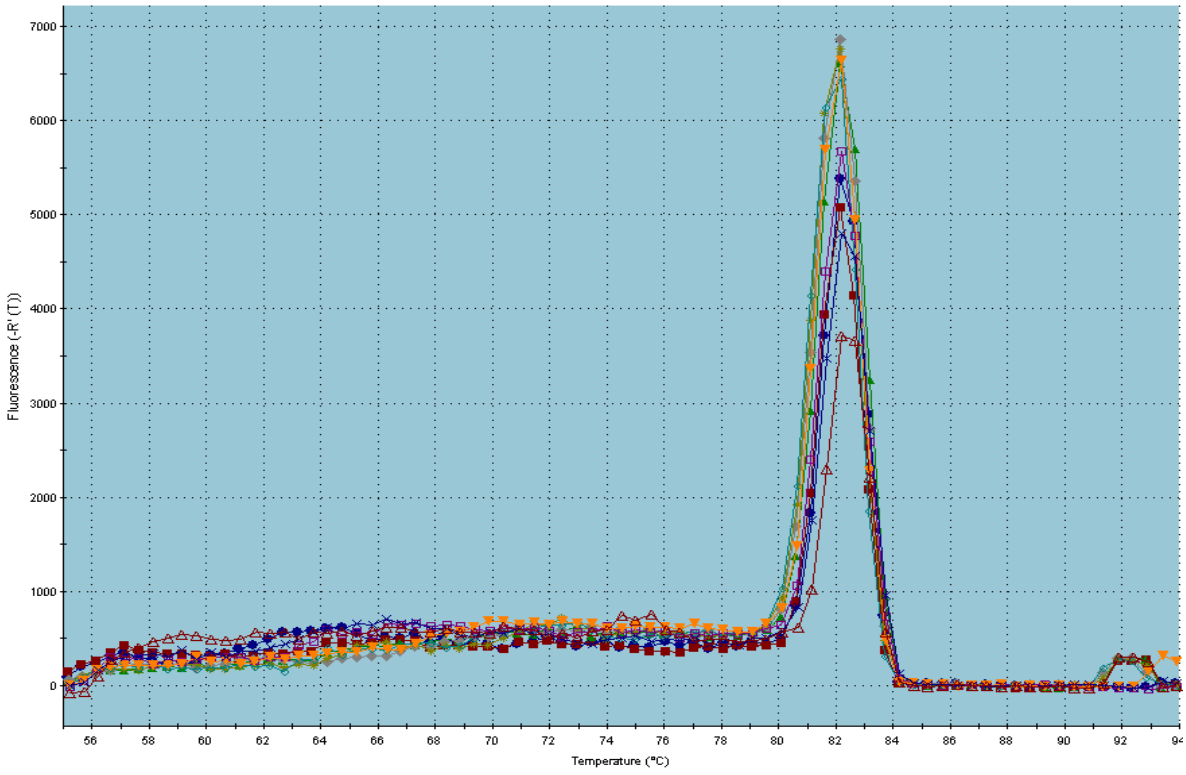
Appendix Figure 5.2. Melt curve plot for Pepsinogen C in proventriculus of layer pullets age 16 weeks (n = 3)



Appendix Figure 5.3. Melt curve plot for β actin in proventriculus of layer pullets age 16 weeks (n = 6).



Appendix Figure 5.4. Melt curve plot for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in proventriculus of layer pullets age 16 weeks (n = 6)



Appendix Figure 5.5. Melt curve plot for mucin 2 in small intestine (jejunum) of layer pullets age 16 weeks (n = 6)

Appendix 7.1 Live body weight of Hy-Line Brown chickens across all experiments (Mean ± SD)

Age/weeks	Chapter 3	Chapter 4	Chapter 5	Chapter 6 (A)	Chapter 6 (B)
2	168.88 ± 6.79				
4	271.38 ± 17.78			369.6 ± 27.48	
6	469.01 ± 26.36				
8	594.88 ± 67.54		796.7 ± 44.77	647.8 ± 42.82	572.3 ± 49.09
10	773.33 ± 97.10				
12	1008.48 ± 49.29				1030.9 ± 63.86
13			1346.2 ± 27.31		
14	1147.20 ± 50.44				
16	1256.78 ± 81.16				1419.3 ± 75.39
18	1407.00 ± 112.83		1573.48 ± 50.06		
19		1519.3 ± 96.30			
22		1773.86 ± 111.35			
25		1793.13 ± 165.99			
28		1888.34 ± 105.70			
31		1902.01 ± 114.69			

Appendix 7.2 Liver weight of Hy-Line Brown chickens across all experiments (Mean ± SD)

Age/weeks	Chapter 3	Chapter 4	Chapter 5	Chapter 6 (A)	Chapter 6 (B)
2	5.09 ± 0.45				
4	7.56 ± 0.81			13.19 ± 2.49	
6	12.94 ± 2.04				
8	18.63 ± 2.11		22.43 ± 2.67	25.67 ± 4.62	18.69 ± 2.17
10	18.90 ± 1.47				
12	20.90 ± 2.09				24.96 ± 3.32
13			30.66 ± 3.09		
14	20.65 ± 2.26				
16	22.88 ± 2.46				30.45 ± 3.03
18	24.11 ± 4.05		36.93 ± 5.02		
19		21.32 ± 3.65			
22		33.91 ± 4.34			
25		36.82 ± 4.64			
28		39.97 ± 1.52			
31		40.01 ± 7.29			

Appendix 7.3 Gizzard weight of Hy-Line Brown chickens across all experiments (Mean ± SD)

Age/weeks	Chapter 3	Chapter 4	Chapter 5	Chapter 6 (A)	Chapter 6 (B)
2	7.42 ± 0.46				
4	9.83 ± 1.00			11.93 ± 1.29	
6	15.87 ± 1.84				
8	24.94 ± 3.66		27.26 ± 4.79	23.56 ± 3.46	16.06 ± 2.91
10	31.46 ± 6.48				
12	30.41 ± 5.16				24.92 ± 3.35
13			36.77 ± 3.69		
14	36.76 ± 4.49				
16	37.39 ± 3.67				30.20 ± 3.77
18	34.12 ± 4.09		35.95 ± 3.85		
19		20.85 ± 1.63			
22		21.11 ± 1.23			
25		21.19 ± 2.52			
28		21.36 ± 1.96			
31		20.93 ± 2.35			

Appendix 7.4 Proventriculus weight of Hy-Line Brown chickens across all experiments (Mean ± SD)

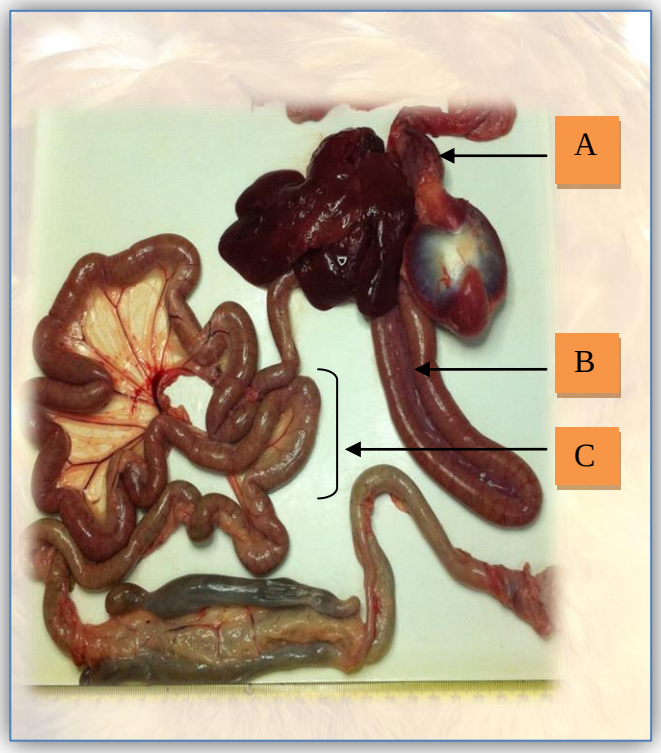
Age/weeks	Chapter 3	Chapter 4	Chapter 5	Chapter 6 (A)	Chapter 6 (B)
2	1.22 ± 0.10				
4	1.57 ± 0.23			2.31 ± 0.14	
6	2.44 ± 0.23				
8	3.49 ± 0.49		3.88 ± 0.53	3.97 ± 0.59	3.43 ± 0.50
10	4.18 ± 0.42				
12	3.98 ± 0.39				4.03 ± 0.28
13			4.79 ± 0.31		
14	4.32 ± 0.58				
16	4.79 ± 0.71				4.18 ± 0.37
18	4.78 ± 0.71		5.31± 0.38		
19		4.45 ± 1.03			
22		4.50 ± 0.53			
25		4.82 ± 0.81			
28		5.30 ± 0.59			
31		5.58 ± 0.50			

Appendix 7.5 Pancreas weight of Hy-Line Brown chickens across all experiments (Mean ± SD)

Age/weeks	Chapter 3	Chapter 4	Chapter 5	Chapter 6 (A)	Chapter 6 (B)
2	0.74 ± 0.08				
4	1.04 ± 0.13			1.43 ± 0.19	
6	1.63 ± 0.10				
8	2.28 ± 0.21		2.54 ± 0.41	2.61 ± 0.55	1.99 ± 0.37
10	2.64 ± 0.23				
12	2.70 ± 0.35				2.48 ± 0.19
13			3.29 ± 0.46		
14	2.66 ± 0.44				
16	2.79 ± 0.37				2.71 ± 0.30
18	2.92 ± 0.35		3.24 ± 0.41		
19		2.97 ± 0.49			
22		2.98 ± 0.51			
25		3.24 ± 0.48			
28		3.47 ± 0.21			
31		3.66 ± 0.48			

Appendix 7.6 Small Intestine weight of Hy-Line Brown chickens across all experiments (Mean ± SD)

Age/weeks	Chapter 3	Chapter 4	Chapter 5	Chapter 6 (A)	Chapter 6 (B)
2	9.73 ± 0.38				
4	14.61 ± 0.54			N/A	
6	20.73 ± 0.67				
8	30.88 ± 1.32		27.60 ± 3.64	N/A	22.76 ± 2.80
10	35.39 ± 0.98				
12	33.91 ± 1.56				21.96 ± 1.75
13			27.19 ± 1.05		
14	33.37 ± 1.11				
16	33.64 ± 1.40				23.75 ± 1.78
18	39.72 ± 1.77		30.17 ± 2.03		
19		28.55 ± 2.78			
22		35.03 ± 2.29			
25		40.33 ± 4.84			
28		39.23 ± 4.14			
31		36.96 ± 3.76			



Appendix Photo 3.1 The digestive system in immature pullets. Samples were taken from proventriculus (A) pancreas (B) and small intestine (C) for enzyme analysis

Appendix Photo 6.1 Hens reared on slatted floor

Publications

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P196 Effect of two different fibre sources on growth and digestive enzyme function in layer pullets

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In order to assess the effects of fibres of different chemical structures on growth and digestive enzyme functions, 68 week old pullets were randomly allocated to 3 dietary groups 20/group: Group CO was fed un-supplemented layer pellets; Group MF was fed the CO diet plus 1.5% of a commercial mixture of soluble (SF) and insoluble fibre (IF); Group IF was fed the CO diet plus 1.5% of a commercial insoluble fibre (IF) product. After 4 and 8 weeks the pullets were weighed, killed and samples of proventriculus, pancreas and small intestine stored for measurement of proventricular pepsin, general proteolytic (GP), chyomtrypsin and trypsin in the pancreas and intestinal amino- and dipeptidase activities. For body weights, only pullets fed the IF diet for 8 weeks were heavier (1.58±0.06 kg, $P<0.05$) than CO pullets (1.42±0.08) although IF and MF pullets were heavier than CO pullets at 4 weeks. After 8 weeks, pepsin (327±32 U/organ/min) and pancreatic GP (339±32 U/organ/min) activities were greater ($P<0.05$) in IF pullets than in CO (224±30; 196±92) and MF (269±27; 207±25) pullets. Both IF (1952±98; 405±51 U/organ/min) and MF (2026±80; 481±31) pullets had higher ($P<0.05$) intestinal aminopeptidase and dipeptidase activities than the CO pullets (1650±81; 355±54). The increase in upper digestive tract enzymes, pepsin, GP and trypsin caused by IF may have had a greater effect on improving digestion of protein and increasing body weight than MF treatment.

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Effect of two different fibre sources on growth and immune function in layer pullets

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Implications Supplementing pullet grower diets with fibre may be a useful replacement for antibiotics in order to enhance growth and disease resistance and thus improve sustainable egg production.

Introduction Restrictions or complete bans to the use of antibiotics as feed additives in rations of poultry have resulted in negative impacts on their health and productivity. Knowledge of alternatives to antibiotics for enhancement of immune function is important if sustainable and economic production is to be achieved. Different types of dietary fibres may be effective alternatives to antibiotics for enhancement of immunity and performance of layers. In broilers and ducks increasing fibre to the diet has been shown to increase T and B lymphocyte proliferation as well as improve growth of their lymphoid organs (Dong *et al.* 2007; Shi-bin and Hong 2012). The aim of this study was to determine whether dietary fibre would enhance layer pullets' innate immune function and growth of lymphoid organs.

Material and methods Thirty-six 4 week old Hy-line brown pullets were weighed and randomly placed, four/ pen, in slatted floor pens (1.8 x 0.9 m), three pens/ treatment. The three dietary treatments were Control, a commercial starter and grower feed with no additive; Group MF, given control diet with 1g of a commercial mixed soluble/insoluble fibre supplement (~59% crude fibre, ~85% mixed soluble and insoluble fibres, ~30% lignin, manufacturer's data) per 100g diet; Group IF, given control diet with 1g of a commercial insoluble fibre supplement (65 – 70% crude fibre high in insoluble cellulose and >20% lignin, manufacturer's data) per 100g diet. At 8 weeks of age blood samples were taken over 4 consecutive days from the brachial vein of 8 pullets/ treatment: pullets were taken randomly from the pens and not all pullets in a treatment came from the same pen. Heterophils were isolated using Ficoll-Hypaque discontinuous gradients (Andreasen and Latimer 1989) and heterophil oxidative burst measured (Wan *et al.* 1993). All pullets were then weighed and killed on the same day with intravenous pentobarbitone sodium under La Trobe University Animal Ethics Committee approval (No. AEC12-68) and guidelines. From each pullet the spleen, bursa of Fabricius, and combined left and right thymus glands were collected and weighed. Data were analysed using one-way analysis of variance (ANOVA, SPSS, 2012). Statistical significance between means of different treatment groups was compared by Turkey's test at $P<0.05$.

Results Heterophil oxidative burst in both MF and IF pullets was significantly increased at 8 weeks of age (Table). Live body weight and relative weights of bursa of Fabricius and thymus glands of pullets in Groups MF and IF were significantly ($P<0.05$) higher than the Control group, while those in Group IF group had significantly ($P<0.05$) higher relative weights of the spleen compared to those in the Control group (Table).

Table Mean live weight (lwt) and relative weights (% of lwt) of immune organs (Mean ± SE, N = 12) and heterophil oxidative burst (Mean ± SE, N = 8, ΔRFU = relative fluorescence units) of pullets fed different diets.

Group	Live weight (g)	Spleen (% of lwt)	bursa of Fabricius (% of lwt)	Thymus (% of lwt)	Heterophil oxidative burst (ΔRFU)
Control	647.9±12.3^{a}	0.40±0.02^{a}	0.50±0.01^{a}	0.50±0.01^{a}	3509±207^{a}
MF*	696.3±9.9^{b}	0.42±0.02^{ab}	0.57±0.01^{b}	0.77±0.02^{b}	4330±183^{b}
IF*	717.5±14.7^{b}	0.47±0.02^{b}	0.57±0.02^{b}	0.78±0.01^{b}	5264±199^{c}

* See methods for definition. a-c Values with different superscripts in the same column are significantly different ($P<0.05$).

Conclusion Compared to controls, live weight and innate immune function were increased in pullets fed a mixture of soluble and insoluble fibre or insoluble fibre alone. The benefits may have been the results of improved immune function in the gut or decreased gut pathogens but further work would be needed to confirm this and to identify whether insoluble fibre contributed more to improvement than soluble fibre.

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