CHAPTER 1 GENERAL INTRODUCTION

Over the past few decades there has been a dramatic increase in consumption of chicken meat world-wide due, in part, to concerns over diet and health as well as changes in everyday lifestyles. According to the Food and Agriculture Organisation statistics, the world chicken meat production in 2003 was 65.0 million tonnes, which was 27.3 % of the total meat production of the world (237.8 million tonnes) (FAO, 2004). Chicken meat production in Australia in 2003-04 was 0.68 million tonnes, which was 18.6 % of the total meat production in Australia (3.68 million tonnes) (Australian Bureau of Statistics, 2005). Chicken meat consumption in Australia has increased by 30 % during the decade 1988-1998 (Roenigk, 1999). Significant improvements in the performance of broilers have been achieved during the last half of the twentieth century through proper management of nutrition, genetics, health and welfare (Ferket, 2004). Feed additives constitute an important group of pre-harvest measures which can help to improve bird performance as well as meat quality.

Since the mid-1940s, many broad-spectrum antibiotics have been used intensively in the poultry industry as therapeutic and prophylactic agents to improve the health and well-being of birds; maximize efficiency of production and product quality, and to control diseases. However, in recent years, there has been growing concern that this use of antibiotics is leading to an increase of antibiotic-resistance in human and animal pathogenic bacteria (Aarestrup, 1999; Ferket, 2004). In addition, the use of antibiotics may negate many beneficial properties conferred by gastrointestinal bacteria of chickens (Poole *et al.*, 2004). Therefore, in the EU, the application of antibiotics as prophylactics has been banned, and countries such as Australia, Canada and the USA, are considering also banning the use of antibiotics in feed or setting up programs to reduce their overall use. The restriction on the use of in-feed antibiotics has resulted in an increase in enteric disorders such as NE and the widespread occurrence of ill-defined intestinal dysbacteriosis in poultry (Bager *et al.*, 2002; Grave *et al.*, 2004).

Currently, many countries are experimenting with alternative feed additives that may be used to alleviate the problems associated with the withdrawal of antibiotics from poultry feed. In this context, there has been increased interest in the use of biological products, including naturally occurring additives such as microbial enzymes, probiotics, prebiotics, synbiotics,

organic acids and plant extracts (phytobiotics) as alternatives to antibiotic feed additives in monogastric animal diets (Bedford, 2000; Wenk, 2003; Ferket, 2004).

Although the modes of action of antibiotic growth promoters (AGPs) are not fully understood, the main effects are thought to be mediated via the gut-associated bacteria (Gaskins *et al.*, 2002). Therefore, modification of the chicken gut microflora has currently become an important objective of the poultry industry, when introducing natural alternatives to conventional chemotherapeutic agents. The diverse microflora of the GIT plays a significant role in maintaining the health of the host. Supplementation of feeds that may improve the microbial balance in the GIT of broiler chickens by stimulating the growth of "healthier" endogenous bacterial populations has been proposed as an alternative to in-feed AGPs (Ferket, 2004). One such group of supplements, known as prebiotics, have been defined as 'non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the distal parts of the digestive tract, and thus improving the host health' (Gibson & Roberfroid, 1995). Members of the genera *Lactobacillus* and *Bifidobacterium* are examples of these supposedly beneficial bacteria that proliferate in broilers fed a diet supplemented with prebiotics (Patterson & Burkholder, 2003) and many of them have been identified as natural resistance factors against potentially pathogenic microorganisms, thereby reducing susceptibility to enteric pathogens and intestinal diseases (Flickinger *et al.*, 2003).

Many investigations have been made to explore the wealth of medicinal plants, which can be used for growth promotion as well as for therapeutic treatment in humans and animals (Guo *et al.*, 2003; Wenk, 2003; Cornelison *et al.*, 2006). Plant and fungal bioactive compounds such as oligosaccharides and polysaccharides have recently received increasing attention as potential AGP replacements in poultry feed (Iji & Tivey, 1998; Guo *et al.*, 2004b; Lan *et al.*, 2004). However, little research has been done to investigate the effects of prebiotics and bioactive compounds on nutrient digestibility and gut morphology of broilers. Among the vast sources of plant materials from which prebiotic and bioactive compounds may be extracted are Australian and New Zealand herbs, botanicals and seaweeds. These contain rich and largely untapped products which may have potential commercial uses. Furthermore, the health benefits of Australian and New Zealand plant extracts have been empirically known for thousands of years by Aboriginal and Maori populations (Cambie & Ferguson, 2003). However, these extracts have not been tested on poultry. The present study not only could open up new supply channels for basic raw materials for the poultry industry, but by

doing so it could generate possible alternatives to AGPs that have been used in the poultry feed industry. Furthermore, this research may lead to reduction in feed costs and improvements in broiler performance and uniformity, thus saving the industry significant amounts of money, and not at the expense of animal or human health.

It was hypothesized in the present study that dietary supplementation with plant extracts or commercially available prebiotic compounds with a similar chemical composition to the plant extracts would exert prebiotic effects and selectively stimulate growth and/or activity of favourable bacteria in the GIT, thereby improving the gut development, health and performance of broilers.

The objectives of this research were:

- To isolate and characterise prebiotic compounds from selected Australian and New Zealand plants;
- To investigate the effects of prebiotic plant extracts and commercially available prebiotic compounds on performance, organ development, gut morphology, microbial populations and microbial activity in broilers;
- To characterise and identify the ileal and caecal microflora stimulated by the prebiotic plant extracts: with emphasis on the populations of lactobacilli and bifidobacteria;
- To evaluate the effects of water-soluble prebiotic extracts on performance, gut morphology, gut microflora composition and humoral immune responses of broiler chickens subjected to a NE disease challenge model involving oral inoculation with *Clostridium perfringens*.

CHAPTER 2 LITERATURE REVIEW

2.1 GUT MICROFLORA OF POULTRY

The gastrointestinal tract (GIT) of poultry is a complex ecosystem divided into various well-defined anatomical regions. Each of these regions is colonised by a large variety of bacteria, protozoans and fungi, collectively referred to as the gut microflora (Mead, 1997; Koutsos & Arias, 2006). Each gut compartment can be further subdivided into microhabitats depending on the microflora present in the intestinal lumen, in the unstirred mucus layer that covers the epithelium of the entire tract and attached to the surface of mucosal epithelial cells (Berg, 1996; Gong *et al.*, 2002a; Gong *et al.*, 2002b). Many of the microorganisms that are present in the GIT of poultry, especially in the hindgut, are obligate anaerobes that are slow-growing on culture media and require specialized techniques for isolation and enumeration (Mead, 1997).

The metabolic capacity of microflora is extremely diverse and can produce both positive or negative effects on bird health (Apajalahti, 2005; Koutsos & Arias, 2006). Usually young chicks do not acquire a maternal microflora matched to the natural antibodies transferred with the yolk because in regular poultry houses chickens are raised under hygienic conditions in the absence of mother hens. Therefore, during early growth periods chicks are highly vulnerable to colonization by pathogenic bacteria. Thus, alterations to the intestinal microflora in a beneficial way with the aim of improving the health of poultry is most important (Apajalahti, 2005; Dibner & Richards, 2005).

2.1.1 Development of the microflora in young poultry

Although it was believed that the GIT of newly hatched chicks may be devoid of microflora or that the microflora may not be very diverse (van der Wielen *et al.*, 2002a), a recent study using denaturing gradient gel electrophoresis of 16S rDNA fragments indicated that day-old broiler chicks harbour a complex community of bacteria (48 different amplicons) in their intestine (Pedroso *et al.*, 2005). Furthermore, Pedroso *et al.* (2005) suggested that these amplicons, representing different genotypes, may have been acquired by the chicks during the incubation period. The intestinal bacterial population of chickens changes qualitatively and quantitatively, from one region to another over time, in response to nutritional and physiological interactions between different microflora and between the organisms and host

tissues (Mead, 1997; Knarreborg *et al.*, 2002b; Apajalahti, 2005). Diet composition directly influences the initial acquisition, developmental succession and eventual stability of microflora in the GIT of the chick (Rubio *et al.*, 1998; Knarreborg *et al.*, 2002b; Apajalahti, 2005). It has been estimated that the normal chicken intestine contains 10^8-10^{10} bacterial cells/g contents, belonging to as many as 640 different species (Apajalahti *et al.*, 2004).

The crop and gizzard contain relatively few types of bacteria in healthy chickens due to low pH in these regions (Mead, 1997). Culture-based experiments have shown that the microflora of the avian crop has a simple composition and is dominated by acid-tolerant *Lactobacillus* spp., coliforms and *Enterococcus/Streptococcus* spp. (Fuller & Brooker, 1974; Fuller, 1977). Recent work of Guan *et al.* (2003) found lactobacilli counts as high as 10^8 to 10^9 CFU per gram of crop contents and that *L. gallinarum*, *L. crispatus*, *L. reuteri*, *L. salivarius*, and *L. johnsonii* are present in the crop throughout the life cycle of broilers raised under commercial farming conditions. More recently, Smith and Berrang (2006) reported that crop contents harboured more bacteria than gizzard contents and were responsible for high incidences of *E. coli* and *Campylobacter* contamination in broiler carcasses.

The distal small intestine (ileum) has more diverse microflora and larger numbers of bacteria (10^9 to 10^{10} CFU/g) than the upper intestine (duodenum and jejunum) because of lower redox potential and lower enzyme and bile acid concentrations. Microorganisms of the genus *Lactobacillus* are present in the distal small intestine at concentrations of 10^8 to 10^9 CFU per gram of digesta (Vahjen *et al.*, 1998; Engberg *et al.*, 2000). *Lactobacillus johnsonii*, *L. crispatus*, *L. salivarius*, and *L. reuteri* were the species most commonly detected in ileal digesta of broilers (Knarreborg *et al.*, 2002b). Lu *et al.* (2003) used a 16S rRNA gene sequence analysis method to identify the bacterial composition and determine the community succession in the ileum of chickens. As shown in Table 2.1 the main organisms were low G+C (guanine plus cytosine) gram-positive, anaerobic bacteria and these organisms accounted for ~81% of the total bacterial population. The major bacteria groups were *Lactobacillus* (68 %), *Clostridiaceae* (10 %), *Streptococcus* (7 %), and *Enterococcus* (7 %). Furthermore, these authors also observed that the microbial community structure in the ileum varies with age (Lu *et al.*, 2003).

Table 2.1 Bacterial genera detected in ileal and caecal contents by 16S rRNA gene sequencing

Group (% total)	Genus	Percentage	
		Ileum	Caeca
Low G+C, Gram positive	*Lactobacillus*	67.6	7.8
(Ileum, 94.2; caeca, 76.9)	*Clostridium*	9.7	39.3
	Streptococcus	6.6	0.7
	Enterococcus	6.4	1.0
	Weisella	1.1	0.5
	Ruminococcus	0.4	16.5
	Eubacterium	0.7	9.9
	Bacillus	0.7	1.5
	Staphylococcus	0.9	0.0
High G+C, Gram positive	*Fusobacterium*	0.7	13.9
(ileum, 0.9; caeca, 13.9)	*Bifidobacterium*	0.2	0.0
Proteobacteria, Gram negative	*Ochrobacterium*	0.2	0.8
(ileum, 2.3; caeca, 2.8)	*Alcaligenes*	0.9	0.7
	Escherichia	0.4	1.3
	Campylobacter	0.9	0.0
Cytophaga/Flexibacter/Bacteroides	*Flavobacterium*	0.0	0.2
(ileum, 0.6; caeca, 5.2)	*Bacteroides*	0.6	5.0

Adapted from Lu *et al.* (2003).

The caeca of poultry contain far more diverse species of bacteria than does the ileum, with greater numbers of strictly anaerobic bacteria and fewer facultative bacteria (10^{10} to 10^{11} CFU/g) (Barnes *et al.*, 1972; Mead, 1997). Early culture-based studies identified at least 38 different strains of anaerobic bacteria isolated from chicken caeca with more than 200 bacteria strains being isolated in total (Barnes *et al.*, 1972; Barnes, 1979). The *Clostridiaceae*-related bacteria are the most abundant group that has been observed in the caeca of chickens (Lan *et al.*, 2002; Zhu *et al.*, 2002; Lu *et al.*, 2003). Recently, Zhu *et al.* (2002) identified 243 different 16S rRNA gene sequences from bacterial DNA extracted from the caecal content of broiler chickens; phylogenetically they were dominated by sequences related to those found in low-G+C Gram-positive bacteria, specifically from the *Clostridium coccoides* (27.1 %), *Clostridium leptum* (20.2 %), and *Sporomusa* spp. (21.2 %) groups. However, the strictly anaerobic culture-based methods, in conjunction with the 16S rDNA clone library analysis of caecal microflora in chickens, found that the *Clostridium* spp. (51 %), *Lactobacillus* spp. (24 %), and *Bacteroides* spp. (4 %) were the major bacterial constituents of the caecal content, with the *Clostridium* subcluster XIVa being the most phylogenetically diverse group (Lan *et al.*, 2002).

2.1.2 Colonization and colonization resistance

The GIT of poultry harbours a microflora formed immediately after the chick is hatched. A healthy microflora is an important barrier against colonization by potentially pathogenic microorganisms (Fukata *et al.*, 1991; Gaskins *et al.*, 2002); this phenomenon is known as colonization resistance. Moreover, prevention of colonization of pathogenic microorganisms in birds during growout may diminish the problem of poultry being infected by certain food-borne pathogens. Some bacteria which thrive in the gut and become members of the resident microbial community during early post-hatch period interact with and depend on other species in many ways (Apajalahti, 2005). It has long been known that dense bacterial colonization of the intestine plays an important role in health and performance through its effect on gut morphology, nutrition, and pathogenesis of intestinal disease, immune system development and inflammatory response of chickens (Lee *et al.*, 2002; Apajalahti *et al.*, 2004; Dibner & Richards, 2005). Although the specific mechanism by which protection occurs is not fully understood, many hypotheses have been proposed. One such hypothesis is that beneficial bacteria suppress the colonization of pathogenic species by secretion of bacteriocin-like compounds (Zhu *et al.*, 2000) and by production of organic acids (van der Wielen *et al.*, 2000; Apajalahti, 2005). Another suggested mechanism is that beneficial

bacteria positively affect humoral and cellular immune responses and thereby increase the intestinal host defense functions in chickens (Koenen *et al.*, 2004).

2.1.3 Adhesion of bacteria

Bacterial attachment to the epithelial wall of the GIT may be a defense mechanism against the colonization of the gut by pathogenic bacteria (Granato *et al.*, 1999; Edelman *et al.*, 2002). Several species of *Lactobacillus* are normal inhabitants of the GIT of healthy chickens (Guan *et al.*, 2003). *In vitro* studies have revealed that 13 strains of *Lactobacillus plantarum* can adhere strongly to Caco-2 epithelial cell layers (Pennacchia *et al.*, 2006). It is now known that surface-associated crystalline protein layers may mediate the adherence of the lactobacilli to the gut epithelium of chickens (Hagen *et al.*, 2005).

Certain pathogenic bacteria have surface glycoproteins (lectin/fimbriae) which can recognise and combine rapidly and selectively with the sugars on the surface of the gut wall (Ewing & Cole, 1994). However, if bacteria attach to a polysaccharide or oligosaccharide which is not part of the gut wall, but is an indigestible component of the feed, then they pass out with the digesta without causing any harmful effects (Newman, 2004). Carbohydrates that adhere to the cell surfaces of various bacteria are shown in Table 2.2. Lee & Puong (2002) found carbohydrates such as *N*-acetyl-glucoseamine, galactose, glucose, fructose, fucose and mannose inhibited the adhesion of *L. casei*, *E. coli* and *Salmonella* spp. to the Caco-2 intestinal cell lines with varying degrees. Mannan-oligosaccharides (MOS) are thought to act by binding and removing pathogens from the intestinal tract. Pathogens with mannose-specific Type-1 fimbriae adsorb to the MOS instead of attaching to intestinal epithelial cells and, therefore, move through the intestine without colonization (Spring, 1999; Spring *et al.*, 2000). Recently, Watarai & Tana (2005) found that activated charcoal made from the bark of an evergreen oak containing wood vinegar liquid could effectively be used as an adsorbent for eliminating the carriage of *S. enteritidis* in layer chickens.

2.2 TOOLS FOR ANALYSIS OF GUT MICROFLORA IN POULTRY

Tools for analysis of gut microflora in poultry include both culture-dependent and other approaches such as molecular techniques.

Table 2.2 Carbohydrate adhesions of various bacterial strains

Bacterial strain	Type of carbohydrate adhered to bacteria
Campylobacter coli	Glucose
Campylobacter jejuni	Glucose
Clostridium spp.	Galactose, glucose and lactose
Escherichia coli	Fucose, galactose and glucose
Fusobacterium spp.	Galactose, lactose, and raffinose
Haemophilus influenzae	Galactose and glucose
Klebsiella pneumoniae	Glucose
Salmonella spp.	Fucose and galactose
Shigella spp.	Fucose
Streptococcus bovis	Glucose
Streptococcus suis	Galactose

Adapted from Newman (2004).

2.2.1 Culturing techniques for identification of gut microflora

The enumeration of gut microflora of poultry has been investigated extensively in the past by plating on selective or non-selective media (Barnes *et al.*, 1972; Barnes, 1979) and species identification was based on phenotypic rather than genotypic characters (Vaughan *et al.*, 2000). However, it is now known that many bacteria in the GIT may not be cultivated by standard culture techniques (Vaughan *et al.*, 2000). Moreover, culture-based techniques are tedious and labour-intensive, especially for large numbers of samples (Vaughan *et al.*, 2000), and they all have inherent disadvantages of not being absolutely selective for specific species or genera (O'Sullivan, 2000). Furthermore, many of the strictly anaerobic GIT bacteria are difficult to grow on artificial media (Vaughan *et al.*, 2000). Lan *et al.* (2002) observed that approximately 90 % of the microscopically observable bacteria from chicken caeca could not be cultivated *in vitro*. Such drawbacks associated with culture-based techniques are exacerbated when enumerating bacteria from anaerobic habitats like the lower GIT (Vaughan *et al.*, 2000). Despite these limitations, culture-based techniques are very powerful and absolutely essential to depicting a complete picture of the diversity and role of the GIT microbial ecosystem (O'Sullivan, 2000; Zhu *et al.*, 2002).

2.2.2 Other techniques for identification of gut microflora

Direct microscopic analysis and monitoring of specific enzymes or metabolites of bacteria are two valuable techniques that have been widely used for characterization of gut microflora (O'Sullivan, 2000). Direct microscopic analysis gives reliable total counts of organisms present in the digesta or faecal matter and also identifies the morphological features of these bacteria. This technique is also a valuable aid for assessing the effectiveness of a culture based method for analysing gut microflora (O'Sullivan, 2000). Measurement of specific metabolites produced by certain species of bacteria can indirectly give more information on the presence of specific microflora, or on the metabolic activities of a specific group of bacteria (O'Sullivan, 2000). For example isolates belonging to the genus *Bifidobacterium* could be identified by the detection of fructose-6-phosphate phosphoketolase enzyme in cellular extracts as described by Scardovi (1986). Measurement of short-chain volatile fatty acids (acetate, propionate and butyrate) and organic acids such as lactate could also reveal information about the fermentation characteristics of the anaerobic gut microflora.

2.2.3 Molecular characterisations of gut microbial communities

One of the most widely used approaches in gut microflora studies has been the use of 16S rRNA and its encoding genes as target molecules. This technique involves amplifying the 16S rRNA gene using the polymerase chain reaction (PCR), employing primers which are targeted at universally conserved regions within this gene (O'Sullivan, 2000). After amplifying the 16S rRNA products, community analysis are carried out by strategies such as separation of individual rRNA products by denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), or terminal restriction fragment length polymorphisms (T-RFLP), sequence analysis of 16S rRNA genes and checkerboard hybridization with specific probes (O'Sullivan, 2000; Knarreborg *et al.*, 2002b; Zhu *et al.*, 2002; Lu *et al.*, 2006). The entire amplified product, which is ~1.5 kb can be directly sequenced and compared with those in the GeneBank database using the Basic Local Alignment Search Tool (BLAST) algorithm. These techniques have already been successfully applied to monitor bacterial populations in gut microflora in chickens (Zhu *et al.*, 2002; Zhu & Joerger, 2003; Amit-Romach *et al.*, 2004; Pedroso *et al.*, 2006).

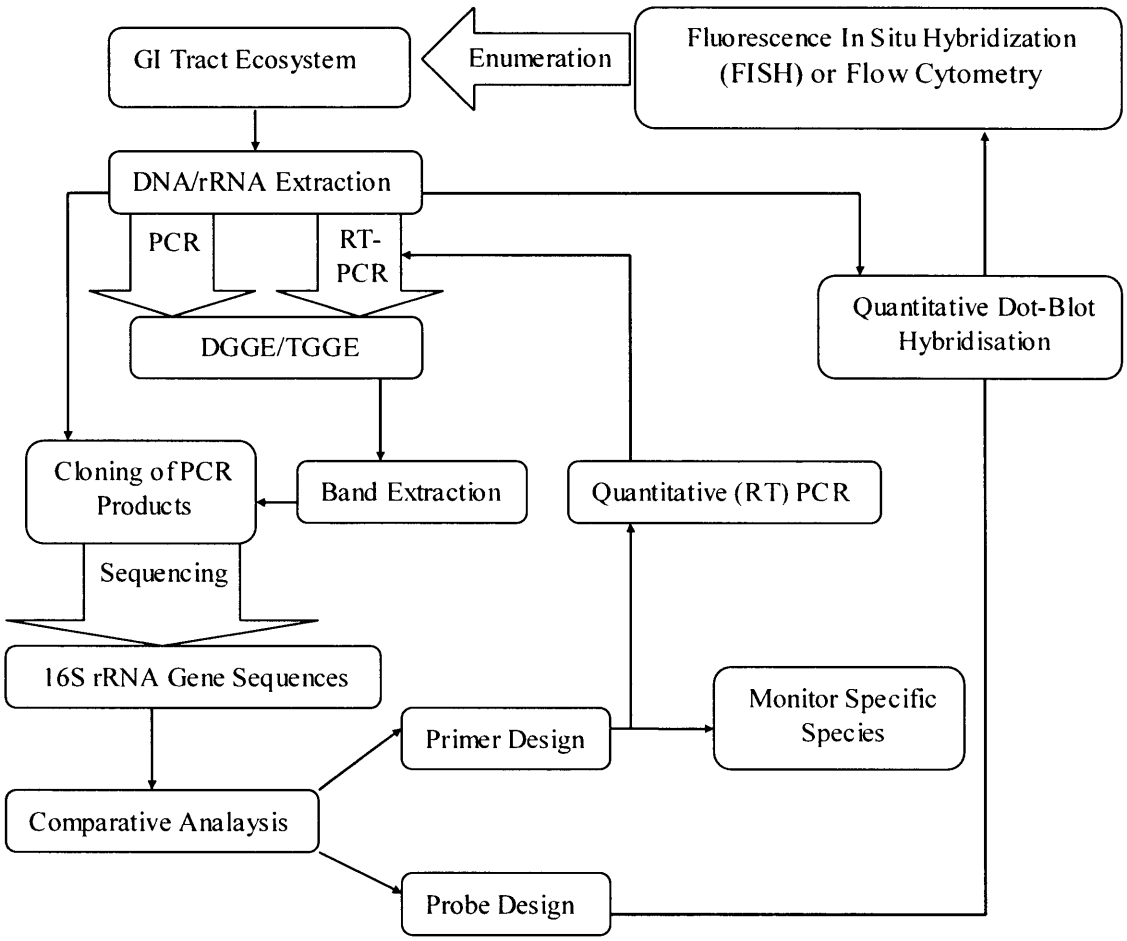
A major advantage of using a DNA-based molecular fingerprinting technique is its ability to rapidly analyse unknown bacteria (O'Sullivan, 2000). Apajalahti *et al.* (2004) recently

reported that, based on modern molecular techniques approximately 90 % of the gut microflora in chickens are previously unknown species. However, limitations like experimental biases and the sensitivity of the particular fingerprinting technique may cause errors (Vaughan *et al.*, 2000). For example, the universal 16S rRNA gene primers often used may not be identically homologous to all bacteria present in a sample and may not amplify all representative 16S rRNA gene products with the same efficiency: and could result in biased estimation of the numbers of those organisms (O'Sullivan, 2000). To overcome this limitation, different sets of primers targeting different universally conserved regions within the 16S rRNA gene can be used (O'Sullivan, 2000). Current molecular approaches to the phylogenetic identification and detection of microbial groups or species in the GIT is shown in Figure 2.1.

2.3 COMMONLY USED ANTIBIOTIC GROWTH PROMOTERS IN POULTRY FEED

The word "antibiotic", which is derived from the Greek words "against life", means a chemical substance derived from fungi, bacteria or other organisms, that kills or slows the growth of bacteria. The growth promoting effect of low levels of antibiotics in animal feed was first described in the late 1940s when chickens fed fermentation waste from tetracycline production grew more rapidly than controls (Dibner & Richards, 2005). Since 1950, a wide range of antibacterial growth promoters has been available to the broiler industry. In the 1950s, the bulk of the antibiotic agents reported in the pharmaceutical industry were derived from the *Streptomyces* spp. and in the two decades preceding the mid 1980s interest in higher plant antimicrobial agents was reawakened worldwide (Mitscher *et al.*, 1987). Table 2.3 summarizes the information on antibiotic usage in farm animals in different countries.

Many antibiotic substances have been shown to improve FCR and animal growth and reduce the morbidity and mortality due to clinical and subclinical diseases (Rosen, 1995; Butaye *et al.*, 2003). Ewing and Cole (1994) suggested that average growth improvement of antibiotic growth promoter (AGP) is between 4-8 %, and feed utilization can be improved by 2-5 %. Rosen (1995) reported that in 72 % of 12,153 studies from 55 countries, antibiotics improved growth and feed efficiency by 2-3 % over the past 50 years.



DGGE/TGGE = Denaturing or Temperature Gradient Gel Electrophoresis
RT-PCR = Reverse Transcription-Polymerase Chain Reaction

Figure 2.1 Flow chart of current molecular approaches to the phylogenetic identification and detection of microbial groups or species in the intestinal microbial community

Adapted from Vaughan *et al.* (2000).

Table 2.3 Comparison between countries in usage of antimicrobials for farm animals

Country	Antibiotics used (tonnes active ingredient)	Grams antibiotics/tonne of pig and poultry meat produced
Australia*	398	210
Denmark	103	53
Netherlands	394	209
New Zealand	36	206
Norway	6	38
Sweden	16	42
United Kingdom	384	191
United States	5260	220

All data for the year 2003, except Australia and New Zealand, where the data were obtained for 1999, and 2002, respectively.
Adapted from *JETACAR (1999) and Wheelock (2005).

Antibiotics suppress the growth of bacteria, and hence the diseases they cause, in two ways: by arresting growth and preventing the bacteria from dividing to produce new progeny (bacteriostatic) and by killing the bacteria (bactericidal) (JETACAR, 1999). The AGPs currently permitted for use in the livestock industry in Australia are shown in Table 2.4. Few of the commonly used AGPs in poultry are described in sections 2.3.1 to 2.3.5.

2.3.1 Bambermycin

Bambermycin, also known as flavomycin, moenomycin or flavophospholipol, is a phosphorus-containing glycolipid obtained mainly from *Streptomyces* spp. (Commission on Antimicrobial Feed Additives, 1997; Butaye *et al.*, 2003). Bambermycin exerts its effect by inhibiting cell wall synthesis, mainly in Gram-positive bacteria (Hudd, 1983). Concentrations used for growth promotion in poultry is in the range of 0.5-20 ppm (Commission on Antimicrobial Feed Additives, 1997). It has been observed that bambermycin improved weight gain and feed efficiency and significantly reduced the numbers of *C. perfringens* (*Cp*) counts in the ileum of broiler chickens (Stutz & Lawton, 1984).

Table 2.4 Antibiotics registered as growth promotants in Australia

Group	Antibiotic	Livestock species
Arsenicals	3-nitro-arsonic acid	Pigs, poultry
Glycopeptides	Avoparcin	Pigs, meat poultry, cattle
Marcolides	Kitasamycin	Pigs
	Oleandomycin	Cattle
	Tylosin	Pigs
Polyethers (ionophores)	Lasalocid	Cattle
	Monensin	Cattle
	Narasin	Cattle
	Salinomycin	Cattle
Polypeptides	Bacitracin	Meat poultry
Quinoxalines	Olaquindox	Pigs
Streptogramines	Virginiamycin	Pigs, meat poultry
Others	Flavophospholipol	Pigs, poultry, cattle

Adapted from JETACAR (1999).

2.3.2 Avilamycin

Avilamycin belongs to the family of orthosomycin group is a mixture of oligosaccharides that are produced by *Streptomyces virridochromogenes* and is mainly active against Gram-positive bacteria; it acts by interfering with bacterial polypeptide (protein) synthesis (Butaye *et al.*, 2003). Avilamycin has been used as an AGP in poultry, at dosages ranging from 2.5 to 10 ppm (Commission on Antimicrobial Feed Additives, 1997). The addition of 10 ppm of avilamycin to the feed resulted in significant reduction in *Cp* counts in broilers (Elwinger *et al.*, 1998).

2.3.3 Bacitracin

Bacitracin is a complex mixture of cyclic polypeptides produced by *Bacillus subtilis* and *Bacillus licheniformis* (Butaye *et al.*, 2003). It is mainly used in complex with zinc which seems to stabilize the antibiotic complex. The dosage permitted for growth promotion in poultry is in the range of 5-100 ppm (Commission on Antimicrobial Feed Additives, 1997). Zinc-bacitracin is active mainly against gram-positive bacteria, forming a complex with C_{55}-isoprenyl pyrophosphate, carrier for the *N*-acetylmuramyl pentapeptide intermediates for the

synthesis of the peptidoglycan in the bacterial cell wall (Butaye *et al.*, 2003). However. Stutz *et al.* (1983a) and Engberg *et al.* (2000) in addition to a reduction in the number of *Cp*, also observed a reduction in Gram-negative bacteria such as coliforms in the ileal contents of broiler chickens fed a Zinc-bacitracin supplemented diet. Stutz *et al.* (1983a) also observed that the antibiotic bacitracin significantly reduced the populations of *Cp* in the intestine and improved the growth and feed efficiency of chickens. Furthermore, Wicker *et al.* (1977) reported that additions of 55 mg/kg of Zn-bacitracin in broiler diets completely prevented mortality due to NE.

2.3.4 Streptogramins

The streptogramins are natural cyclic peptides such as virginiamycins and pristinamycins, mainly produced by *Streptomyces* spp. (Commission on Antimicrobial Feed Additives, 1997). They always consist of an "A" component and a "B" component which act synergistically on protein synthesis in bacteria (Butaye *et al.*, 2003). Virginiamycin is permitted for growth promotion in poultry at concentrations between 5-55 ppm. Miles *et al.* (2006) recently reported that feeding 15 ppm virginiamycin in starter diets and 10 ppm in grower and finisher diets in broilers resulted in increase in body weights and decrease in intestinal length and weight at various ages compared with control birds. In order to determine the effect of virginiamycin on the ileum microflora, Lee *at al.* (2002) sequenced the 16S rRNA genes of clone from libraries obtained from broiler chickens at 28 and 49 days of age. These authors found that *Lactobacillus* species were less abundance at both ages in birds fed virginiamycin compared with control birds.

2.3.5 Ionophores

The ionophore type antibiotics that have been used as therapeutic agents in livestock can be divided into three major classes on the basis of their transport modes: the neutral ionophores, the carboxylic ionophores, and the channel-forming quasi-ionophores (Butaye *et al.*, 2003). Most ionophore antibiotics are produced by *Streptomyces* spp. (Butaye *et al.*, 2003) and many of them are used as anticoccidial substances (coccidiostats) against *Eimeria* spp., including lasalocid, monensin, maduramycin, narasin and salinomycin (Williams, 2005). These substances interfere with the natural ion transport system of both prokaryotic and eukaryotic cells (Butaye *et al.*, 2003) and have been proven to have inhibitory effects on the growth of *Cp* because they also exhibit antibacterial activity (Elwinger *et al.*, 1998; Brennan

et al., 2001; Martel *et al.*, 2004). Thus, the continued use of ionophore antibiotics may be responsible for the fact that broiler producers did not notice an increase in clinical NE following the banning of antibiotic feed additives in Scandinavian countries (Bager *et al.*, 2002).

2.3.6 Modes of action of antibiotics

Considerable evidence suggests that growth-promoting antibiotics modify gut microbial populations or activities and four major theories have been proposed to explain their actions (Visek, 1978; Thomke & Elwinger, 1998; Gaskins *et al.*, 2002; Dibner & Richards, 2005). These are inhibition of sub-clinical infections, reduction of growth-depressing microbial metabolites, nutrient-sparing effects, and effects on intestinal ultrastructures (Hudd, 1983; Gaskins *et al.*, 2002; Apajalahti, 2005). Not all antibiotics accomplish these modifications by the same mechanism. Some of the growth promoting antibiotics are readily absorbed into the bloodstream of the host animal, whereas others are poorly absorbed and mainly act within the gut lumen (Hudd, 1983). Gram-positive members of the gut microflora are the main target for antibiotic growth promoters in poultry feed. Recently, Pedroso *et al.* (2006) suggested that changes in the composition, instead of reduction in the number, of bacterial genotypes in the intestinal bacterial community induced by antibiotics is responsible for improved growth performance in chickens.

2.3.6.1 Effects of antibiotics on microbial production of growth-depressing toxins

Certain toxic metabolites produced by bacterial fermentation can cause harmful effects to the host animal. Increased production of toxic phenolic and aromatic compounds such as phenol, 4-methylphenol (*p*-cresol), 4-ethylphenol, indole, and 3-methylindole (skatole) due to bacterial catabolism of aromatic amino acids could be a potential mechanism for gut microbial-induced growth depression (Anderson *et al.*, 1999; Anderson, 2002). It is also known that toxic amines such as histamine and cadaverine are produced by decarboxylation of amino acids by certain bacteria (protein-fermenting bacteria) including *Bacteroides*, *Clostridium*, *Enterobacterium*, *Lactobacillus* and *Streptococcus* (Apajalahti, 2005; Dibner & Richards, 2005). Bacterial enzymes, such as β–glucosidase (EC 3.2.1.21) and β-glucuronidase (EC 3.2.1.31), are the major microbial glycosidases which are responsible for production of these toxic compounds (Ling *et al.*, 1994; Jin *et al.*, 2000). These enzymes

release harmful metabolites from non-toxic glycosides and prolong the life of toxicants in the GIT of animals (Jin *et al.*, 2000).

Under certain conditions bacterial metabolic products are considered harmful for the well-being of the host, because these metabolic products may cause detrimental effects to cell membranes in the gut. Furthermore, these metabolic products can initiate the formation of free radicals that cause peroxidation of membrane lipids (Visek, 1978). Peroxidation can cause formation of aldehydes and different types of free radicals, resulting in widespread damage to DNA, enzymes, and structural proteins (Hoerr, 1998). Moreover, Visek (1978) and Anderson (2002) suggest that the Gram-positive facultative anaerobes, which are oxygen-tolerant and predominant in the small intestine, often produce toxic compounds such as urea; the end product is a high concentration of ammonia in the digestive tract, which adversely affects animal health and performance. It is also known that the gut-thinning effect following antibiotic usage results from a reduction in bacterial toxin production, thus permitting improved absorption of dietary nutrients (Hudd, 1983).

2.3.6.2 Efficiency of nutrient absorption

It has been hypothesised that feeding antibiotics reverses microbe-induced growth depression by increasing the utilization or availability of nutrients to or by the host animal (Anderson *et al.*, 1999). The gut microflora can capture up to 20 % of the total nutrients available for host animal (Apajalahti, 2005). Feighner and Dashkevicz (1987; 1988) proposed an important mechanism of growth-promoting antibiotics and suggested that antibiotics are important in the inhibition of microbial bile salt deconjugation in the intestine. These authors have shown an inverse relationship between the level of cholytaurine hydrolase activity in the upper part of the small intestine and growth rate in broiler chickens fed antibiotics. As conjugated bile salts are used for micelle formation, microbial deconjugation and decarboxylation of bile salts impairs lipid solubilisation and hence lipid absorption (Tannock *et al.*, 1989; Knarreborg *et al.*, 2002a). Many species of bacteria, such as *Bacteriodes*, *Bifidobacerium*, *Lactobacillus* and *Clostridium* spp. possess bile acid hydrolase activity (Mallory *et al.*, 1973; Srivastava & Hylemon, 1988; Tannock *et al.*, 1989; Mariam *et al.*, 2004). Bacterial utilization of nutrients such as glucose, fructose and galactose may also reduce the energy available to the host animal (Saunders & Sillery, 1982).

2.3.6.3 Effect of antibiotics on gut mucosal morphometry

The gut mucosal surface plays a key role in the exclusion and elimination of potentially harmful dietary antigens and enteric microorganisms. The epithelial lining of the GIT is characterized by a rapid cell turnover and the constant production of a protective mucus secreted by the mucin-secreting goblet cells (Deplancke & Gaskins, 2001). Gut tissues represent approximately 5 % of body weight but account for 15 to 30 % of whole body oxygen consumption and protein turnover because of the high rate of epithelial cell turnover and metabolism (Gaskins *et al.*, 2002).

Intestinal bacteria are mainly responsible for degrading the large amount of mucus produced by goblet cells in the intestinal epithelium (Meslin *et al.*, 1999). It has been estimated that 90% of the total protein synthesized by the GIT is lost due to mucus production and epithelial cell shedding (Gaskins *et al.*, 2002). Furthermore, mucus is also important for innate defence functions (Deplancke & Gaskins, 2001) which, however, are provided at the expense of animal growth efficiency (Anderson, 2002). As suggested by Anderson *et al.* (1999) and Miles *et al.* (2006), feeding antibiotics to chickens could spare energy from tissue maintenance that can instead be used for growth, or improving absorption of various nutrients, could result from gut morphometric parameters that would include decreased cell proliferation, thinner mucosa, less lamina propria, and an increase in absorptive surface area. Numerous reports have shown that antibiotics reduce the thickness of the intestinal wall and thereby improve the growth performance (Stutz *et al.*, 1983b; Stutz & Lawton, 1984; Miles *et al.*, 2006).

2.3.7 Antibiotic resistance

Extensive use of antibiotics for the prevention of infections and promotion of growth in livestock has led to an imbalance in the beneficial intestinal microflora and the appearance of resistant bacteria (Aarestrup, 1999; Dibner & Richards, 2005). Most classes of antibiotics used in animals have human analogues, and therefore, some antibiotics and coccidiostats are losing their effectiveness because of the acquisition of resistance among targeted organisms not only in poultry but also in humans (Aarestrup, 1999). Monitoring and identifying resistance mechanisms and their dissemination into the food chain were recently discussed by Khachatourians (1998) and Roe and Pillai (2003).

The EU banned the use of avoparcin in poultry diets in the late 1990s due to the risk that the use of avoparcin in feed may lead to development of enterococci resistance against vancomycin, an antibiotic used in human medicine (Dibner & Richards, 2005). According to Johansson *et al.* (2004), 76 %, 10 % and 29 % of the *Cp* strains isolated from intestine of broiler chickens in Sweden, Denmark and Norway, respectively carried the *tet*A(P) and *tet*B(P) resistance genes (tetracycline-resistant genes). Therefore, the EU has imposed a complete ban on prophylactic use of antibiotics from 2006. In Australia, the report of the Joint Expert Advisory Committee on Antibiotic Resistance (JETACAR) noted that new evidence has continued to emerge on the development of resistant bacteria in humans and animals and the transferring of antibiotic-resistance genes from animal bacteria to human pathogens following antibiotic use (JETACAR, 1999). Table 2.5 shows the negative effects of antibiotic resistance in humans and animals.

2.3.8 The effect of withdrawing growth promoting antibiotics from poultry feed

Restriction in the use of in-feed antibiotics in many European countries after the mid 1990s resulted in an increase in incidences of enteric disorders such as NE and the widespread occurrence of ill-defined intestinal dysbacteriosis (Kaldhusdal & Lovland, 2000; Bager *et al.*, 2002; Pattison, 2002). Birds affected with dysbacteriosis have substantially larger numbers of bacteria, including increased numbers of Clostridia in the small intestine (Panneman, 2000). Emborg *et al.* (2002) reported that although the productivity and mortality in the Danish broiler industry was not affected by the ban of AGPs in Denmark, FCR was increased by 0.016 unit from November 1995 to May 1999. It has also been observed that consumption of the ionophore type antibiotics increased steadily in Denmark since the ban on other AGPs (Bager *et al.*, 2002). In Norway, estimated annual percentages of broiler chickens treated against NE increased abruptly after the avoparcin ban in the mid-1990s but in 1996 this figure declined to the same level as before the ban and has remained at that low level since then (Grave *et al.*, 2004).

Table 2.5 Negative effects of antibiotic resistance in humans and animals

Humans	Animals
Short term effects	**Short term effects**
Patient suffering	Animal suffering
Cost of new antibiotics	Cost of new antibiotics
Cost of increased laboratory testing	Cost of increased laboratory testing
Longer course of diseases	Longer course of diseases
High cost of medical consultations	High cost of veterinary consultations
Long term effects	**Long term effects**
Irreversible damage to internal organs	Irreversible damage to internal organs
Costs for alternative measures and control and prevention	Production losses
	Costs for alternative measures and control and prevention
Shorter life span of new therapeutics	
Cost of increased monitoring	Shorter life span of new therapeutics
	Cost of increased monitoring
	Loss of consumer confidence

Adapted from Commission on Antimicrobial Feed Additives (1997).

2.3.8.1 Necrotic enteritis in poultry

Necrotic enteritis (NE) was first described by Parish (1961) and is a specific enterotoxaemic disease of chickens caused by the α-toxin of *Cp* type A and, to a lesser extent, by the β-toxin produced by *Cp* type C (van Immerseel *et al.*, 2004). *Clostridium perfringens* is a Gram-positive, obligate anaerobic, spore-forming bacterium that can be found in soil, litter, dust and at low levels in the intestine of healthy birds. In recent studies, Nauerby *et al.* (2003) and Engstrom *et al.* (2003) found considerable genetic diversity among *Cp* from healthy broilers. When the intestinal contents of boiler chickens were analysed for the presence of *Cp*, it was identified in approximately 75-90 % of birds (Miwa *et al.*, 1997; Craven *et al.*, 2001a; Craven *et al.*, 2001b). In flocks in which the median *Cp* count of the small intestine is above 10^6 CFU/g digesta, there is a higher risk that birds will suffer from some form of NE (Kaldhusdal *et al.*, 1999). *Clostridium perfringens* counts of 10^7-10^9 CFU/g of digesta can cause clinical NE (Drew *et al.*, 2004). Craven *et al.* (2001b) suggested that colonization of

poultry by *Cp* occurs very early in the life of chickens, and can be transmitted within the integrated broiler chicken operation, starting from the hatchery. The majority of outbreaks of NE have been reported in two-to-five-week-old broiler chickens raised on litter (Heier *et al.*, 2001; Hofacre, 2005).

2.3.8.2 Clinical signs of necrotic enteritis

Clostridium perfringens infections in poultry may present as acute clinical disease or subclinical disease, and is most common in broiler chickens (van Immerseel *et al.*, 2004). In the acute form of NE, birds often die without clinical signs. The commonly observed symptoms of the disease vary with the age of birds (van der Sluis, 2000b) and early signs of a necrotic NE outbreak include wet litter, depression, decreased appetite, diarrhoea and increased mortality, typically up to 1 % per day for up to seven consecutive days (Kaldhusdal & Lovland, 2000). It is known that death occurs because of the absorption of toxins produced in the intestine as well as intraluminal fluid loss and circulatory collapse (Al-Sheikhly & Truscott, 1977). The intestine of infected birds is friable, and distended with gas and gross lesions. Early studies of Al-Sheikly and Truscott (1977) found that histological lesions caused by *Cp* at 8 and 12 h post-inoculation are characterized by massive necrosis of the villi, with necrotic zones reaching the crypts in broiler chickens. These observations are consistent with the experimental findings of Fukata *et al.* (1988) who reported that oral inoculation of a broth culture of *Cp* resulted in detachment and disruption of the epithelial layer at the tips of the villi and sloughed epithelial cells in the intestine of broiler chickens. Moreover, large numbers of Gram-positive bacilli can be seen within the focal or diffuse mucosal coagulative necrotic areas (Wilson *et al.*, 2005).

In its subclinical form the disease is financially much more damaging to the producer because of impaired performance and increased condemnations at processing due to liver lesions (cholangiohepatitis) (Kaldhusdal & Lovland, 2000; Hofacre *et al.*, 2003). The subclinical form of NE has been identified as specific growth retardation around the twenty-third day of grow-out in broiler farms (van der Sluis, 2000a). The damage to the intestine and the subsequent reduction in digestion and absorption can reduce the weight gain of broilers by more than 200 g/bird (van der Sluis, 2000a) and increase the FCR at 35 days of age by up to 10 conversion points (Kaldhusdal & Lovland, 2000). It has been estimated that the total cost of clinical and subclinical NE can be as high as 5 US cents per bird (van der

Sluis, 2000b) or a staggering US 1084 million dollars based on the global production of chicken meat in 2003 (FAO, 2004).

2.3.8.3 Control of necrotic enteritis

Today, clinical NE outbreaks are effectively controlled and prevented with antibiotics such as virginiamycin, bacitracin, pencillin, tylosin, or flavomycin (Watkins *et al.*, 1997; Collier *et al.*, 2003). Included at sub-therapeutical dosage, AGPs reduce the colonization of *Cp* (Collier *et al.*, 2003). Several predisposing factors such as high percentages of proteins from animal sources in diets (Drew *et al.*, 2004), dietary amino acids such as glycine (Dahiya *et al.*, 2005; Wilkie *et al.*, 2005), dietary grains rich in non-starch polysaccharides (Craven, 2000), and damage of the intestinal mucosa, particularly sloughing of intestinal villi by various strains of *Eimeria* (Williams *et al.*, 2003) could enhance the development of the clinical signs and lesions of NE in poultry. Many researchers have hypothesized that increased NE susceptibility of chickens fed diets based on grains such as wheat, barley, oats and rye may be due to enhanced mucus production caused by water soluble non-starch polysaccharides that are present in these grains (Kaldhusdal & Skjerve, 1996; Craven, 2000; Annett *et al.*, 2002). However, this concept was not supported in a study by Riddell and Kong (1992) who observed that addition of pectin and guar gum to broiler diets eliminated NE induced by mixing *Cp* cultures in the feed. In line with this, recent work by Collier *et al.* (2003) showed that addition of highly methylated citrus pectin did not affect the occurrence of NE lesions in broiler chickens and Waldenstedt *et al.* (2000) observed no change in counts of caecal *Cp* when digesta viscosity was increased by dietary supplementation with carboxymethyl cellulose. Recently, Lovland *et al.* (2004) observed that vaccination of broiler breeder hens with a candidate vaccine based on *Cp* type A and type C (immunoprophylaxis) appeared to be an alternative to the use of antibiotics to control NE in broilers. Furthermore, Thompson *et al.* (2006) also found that it is possible to immunize broiler chickens successfully against NE and that immunogens other than α–toxin are important in protective immunity against oral infection. Williams (2005) proposed rational and integrated management strategies to control *Cp* associated NE in poultry, including improved hygiene, husbandry and nutrition.

It has been recognised that modulation of the natural bacterial population of the intestine in broilers through nutritional manipulation such as the selection of feed ingredients or the use of alternate feed supplements can be effective tools to control NE (Takeda *et al.*, 1995; van

Immerseel *et al.*, 2004). Engberg *et al.* (2004) found that feeding whole wheat reduced the intestinal counts of *Cp* in broilers. Few studies have been performed on the protective effects of prebiotics, probiotics and plant extracts against NE. Feeding oligosaccharides derived from the cell wall of the *Saccharomyces cerevisiae* (mannan-oligosaccharides) has been found to decrease the mortality due to NE in chickens (Hofacre *et al.*, 2003). These authors also found that an oral inoculation of lactobacilli at hatch could successfully reduce the NE-associated mortality. Takeda *et al.* (1995) found that lactose supplementation in feed at an inclusion rate higher than 10 % was effective in reducing *Cp* in the caecal contents of layer chickens. Growth of *Cp* was suppressed *in vitro* by inclusion of non-autoclaved or autoclaved water-soluble wheat extracts (Branton *et al.*, 1996). Cross *et al.* (2004) found that caecal counts of *Cp* were reduced when broiler diets were supplemented with thyme and yarrow extracts. Recently, Mitsch *et al.* (2004) observed that chickens on feed supplemented with a specific blend of essential oil components can control *Cp* colonization and proliferation in the gut of broilers and therefore may be of help in preventing NE. Although the exact reason/s for the above positive effects are not known, it is believed to be the result of both direct and indirect effects of probiotics, prebiotics and plant extracts on microbial populations in the digestive tract.

2.3.8.4 Avian coccidiosis

Coccidia are unicellular parasites which grow and multiply in the mucosa of poultry. Coccidiosis has the greatest economic impact of any parasitic disease on poultry production (Allen & Fetterer, 2002); the annual worldwide impact on poultry production is estimated at about 800 million US dollars (Williams, 1998). Most coccidia of poultry belong to the genus *Eimeria* and there are five main species of *Eimeria*; *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, and *E. tenella* have been isolated from the digestive tract of poultry (Allen & Fetterer, 2002). In chickens, invasion of the lamina propria by *E. maxima*, *E. necatrix* and *E. tenella* may result in less severe to severe haemorrhages (Hoerr, 1998). Young poultry are highly susceptible to coccidiosis due to lack of passively transferred maternal immunity. In adult birds losses are due to mortality, malabsorption, inefficient feed utilization and impaired growth rate (Allen & Fetterer, 2002).

There have been a number of studies that show a correlation between *Eimeria* and the development of NE (Shane *et al.*, 1985; Baba *et al.*, 1992). Colonization of the small intestine by *Eimeria* spp. leads to mucosal damage, which can provide a surface for *Cp* to

proliferate and produce toxins (Shane *et al.*, 1985). Coccidiosis has been controlled for many years using ionophores and coccidiostats in poultry feed. Vaccination with live oocysts may become an alternative to the use of coccidiostats in broilers (Williams, 2002; Dalloul & Lillehoj, 2005).

2.4 ALTERNATIVES TO SUBTHERAPEUTIC ANTIBIOTICS IN POULTRY FEED

Due to current consumer preferences for poultry products grown without antibiotics, many alternatives to antibiotics have been studied as performance enhancers or health promoters (Griggs & Jacob, 2005). It is very unlikely that even long-term withdrawal of antibiotics from feed will lead to a significant reduction in the pool of antibiotic-resistant bacteria. Hence, various alternative means for reducing the use of antibiotics should be considered (Collett & Dawson, 2001). Effectiveness, safety, acceptability to regulatory agencies, ease of use, and economics are some of the important characteristics of acceptable alternatives for subtherapeutic antibiotics (Collett & Dawson, 2001).

2.4.1 Organic acids

Organic acids (C_1-C_7) are primarily the saturated straight-chain carboxylic acids and their respective derivatives that are naturally present in many plant products (Gauthier, 2006). Most of the short-chain volatile fatty acids such as acetate, propionate and butyrate are also formed through microbial fermentation of carbohydrates mainly in the distal parts of the GIT in many animals (Ricke, 2003; Gauthier, 2006). Non-dissociated organic acids act on bacteria by penetrating the lipid membrane of the bacterial cell wall and disrupting the normal physiology of those bacteria that cannot tolerate a wide internal and external pH gradient, e.g. *E. coli*, *Salmonella* spp., *C. perfringens*, *Listeria momocytogenes* and *Campylobacter* spp. (Gauthier, 2006). It has been observed that upon passive diffusion of various organic acids into the cytoplasm of bacteria, where the pH is near or above neutrality, the acids will dissociate into anions and protons and lower the pH, leading to situations that will impair or stop the metabolic activity (Roe *et al.*, 1998; Gauthier, 2006). Furthermore, it has also been postulated that the anionic part of the organic acids which cannot exit the bacteria in its dissociated form, will accumulate within the bacterial cells and disrupt many cellular functions (Gauthier, 2006). Recent studies have indicated that certain individual or combinations of organic acids can have a significant beneficial impact on

poultry performance and health (Thompson & Hinton, 1997; Dibner & Buttin, 2002; Chaveerach *et al.*, 2004). Supplementation of butyric acid impregnated microbeads to the diets of chickens resulted in a decrease in colonization by *Salmonella* of the caecum (van Immerseel *et al.*, 2003).

2.4.2 Antibodies

In poultry, maternal antibodies, i.e. those that are passed to the offspring via the yolk of eggs, are recognized as an important factor in maintaining the chick's immunity. Recent evidence indicates that egg antibodies offer a new strategy in improving the efficiency of poultry performance, specifically through immune modulation (Cook, 2004; Berghman *et al.*, 2005). Based on the amount of IgY immunoglobulins present in an egg yolk (25 mg/mL of yolk) Berghman *et al.* (2005) estimated that around 1,000 kg of antibody per year can be produced from 10,000 layers. It is now known that cholecystokinin, a neuropeptide which is responsible for decreasing appetite during immune stimulation, binds to the egg antibodies and thereby improves animal performance (Cook, 2004). Research is beginning to explore the possibility of feeding the antibody-containing products to poultry. Kassaify & Mine (2004) demonstrated that inclusion of non-immunized egg yolk powder in layer feed is effective in controlling *Salmonella*, *Campylobacter jejuni* and *E. coli* colonization in faecal contents.

2.4.3 Bacteriocins

Bacteriocins are proteinaceous compounds of bacterial origin that are harmful to bacteria other than the strain producing the compound (Joerger, 2003). In contrast to the currently used antibiotics, bacteriocins are often considered as natural because these compounds are naturally present in many food products. Purified or partially purified bacteriocins could be used for suppression or elimination of certain pathogens. Intestinal strains of lactobacilli produce bacteriocin-like substances with inhibitory activity against Gram-positive and Gram-negative bacteria (Juven *et al.*, 1992; Zhu *et al.*, 2000). The best known bacteriocin produced by lactic acid bacteria, nisin, has been given the GRAS (generally recognised as safe) status from the United States department of Food and Drug Administration (FDA) (Joerger, 2003). An antimicrobial bacteriocin peptide, gassericin KT7, isolated from *L. gasseri* KT7, showed inhibitory activity against some pathogenic and food-spoilage species, including *Clostridium*, *Listeria* and *Enterococcus* (Zhu *et al.*, 2000). Recently, Teo and Tan

(2005) observed that bacteriocins produced by *Bacillus subtilis* isolated from the GIT of healthy chickens are broadly active against many pathogens, including *Cp*.

2.4.4 Bacteriophages

Another alternative to subtherapeutic antibiotics is the use of specific viruses that infect and multiply in certain pathogenic bacteria (Joerger, 2003). The concept of using bacteriophages to control common infections in animals is in its infancy, but it is gaining a great deal of attention. Lytic bacteriophages infect bacterial cells and multiply within the cytoplasm, a process that is completed when the bacterium lyses, releasing new infectious bacteriophage particles. One feature that makes bacteriophages attractive as an alternative to AGP is their highly discriminatory nature. Most of the known bacterriophages are specialists that interact only with a specific set of bacteria that express specific binding sites; bacteria without these receptors are not affected (Joerger, 2003). There are two general types of bacteriophages, virulent and temperate and their mode of action on bacterial lysis is different (Huff *et al.*, 2005). Recently, Toro *et al.* (2005) described the successful application of a bacteriophage in prevention of colonization by *Salmonella* in layer chickens. In a similar study, Fiorentin *et al.* (2005) also found that a mixture of bacteriophages was effective in reducing *Salmonella enterica* serovar Enteritidis in broiler caeca. Research demonstrating the efficacy of bacteriophages in preventing *E. coli*-associated colibacillosis on broiler chickens has been demonstrated by Huff *et al.* (2005).

2.4.5 Probiotics

Exclusion of pathogens from poultry began with the introduction of pathogen-free native adult gut microflora to day-old chicks in the early 1970s (Nurmi & Rantala, 1973). This concept is now known as "competitive exclusion" and many subsequent studies have shown that providing newly hatched chicks with intestinal microflora from adult chickens decreases the incidence of colonization by pathogenic bacteria such as *Cp* (Fukata *et al.*, 1991; Craven *et al.*, 1999; Kaldhusdal *et al.*, 2001) and *Salmonella enteritidis* (Pascual *et al.*, 1999). Probiotics are defined as live microbial cultures of a single bacterial strain or mixture of different strains that beneficially affect the host animal, either directly or indirectly, by improving its intestinal microbial balance (Fuller, 1989; Griggs & Jacob, 2005). They are also known as direct fed microbials (DFM).

The most commonly used probiotic strains belong to genera, *Lactobacillus, Bifidobacterim* or *Enterococci* (Klein *et al.*, 1998). For probiotic bacteria in feeds to be beneficial in the host, they should be able to survive gastric transit and reach the small intestine in sufficient numbers to be effective. Probiotics are able to exert their effects by one or more actions, e.g. creation of a restrictive physiological environment for potentially pathogenic microorganisms. These effects are achieved by lowering the pH through production of organic acids such as lactate and short chain volatile fatty acids due to break-down of complex carbohydrates (Ehrmann *et al.*, 2002; van der Sluis, 2003) or elaboration of antibiotic-like substances such as bacteriocin-like compounds (Chateau *et al.*, 1993; Zhu *et al.*, 2000). van der Wielen *et al.* (2002b) reported that undissociated forms of acetate and propionate produced in a mixed culture of *Lactobacillus crispatus* and *Clostridium lactifermentans* isolated from chicken caecal contents inhibited the *in vitro* growth of *Salmonella enterica* serovar Enteritidis. A genetically modified probiotic strain of *Lactobacillus reuteri* not only acquired the capacity to break down soluble complex carbohydrates such as carboxymethyl cellulose, β-glucans, and xylans but also showed high adhesion efficiency to mucin and mucus and resistance to bile salts and acids (Liu *et al.*, 2005). Certain probiotic bacterial species such as lactic acid bacteria can adhere to the intestinal epithelium and thereby prevent invasion by pathogenic bacteria such as *E. coli*, salmonella and *Cp* in the gut epithelium (Spencer & Chesson, 1994; Jin *et al.*, 1996). For example, many *Lactobacillus* spp. possess great ability to adhere to the epithelial cells of the GIT because of the high cell surface hydrophobicity (Gusils *et al.*, 1999; Ehrmann *et al.*, 2002).

Another possible mode of action of probiotic bacteria such as lactobacilli could be to increase the levels of digestive enzymes such as amylase (Jin *et al.*, 2000). This hypothesis was supported by these authors because they detected extracellular and intracellular amylase activity of *Lactobacillus* spp. in an *in vitro* experiment. Feeding probiotics may also help to modulate the cellular and humoral immune system thereby enhancing the host's resistance to enteric pathogens (Dalloul *et al.*, 2003; Koenen *et al.*, 2004). Recently, Dalloul *et al.* (2003) demonstrated an immunomodulatroy effect of dietary probiotic bacteria on the local immune system in broiler chickens which was characterized by altered intestinal intraepithelial lymphocyte subpopulations and which increased the birds' resistance to *E. acervulina* as reflected by reduced oocyst shedding. According to the model described by Clancy (2003), probiotic bacteria activate dendritic cells in Peyers' patches which, in turn, stimulate the mucosa-circulating pool of T-lymphocytes generated from within the Peyers' patch. Huang

et al. (2004) indicated that inactivated probiotics (*L. acidophilus* and *L. casei*) could have beneficial effects similar to those of live probiotics in enhancing humoral immune responses in broiler chickens.

Experimental results from the use of probiotics are contradictory; some authors have found them to improve broiler performance and health (Jin *et al.*, 1998b; Pascual *et al.*, 1999; Kalavathy *et al.*, 2003) while others have indicated that there are no beneficial effects on farm animals (Watkins & Kratzer, 1983). The strain of selected microorganisms, the dosage, method of preparation, susceptibility to low pH and/or bile, and ability to colonize the GIT could be partially responsible for such discrepancies (Fuller, 1989; Klein *et al.*, 1998). Studies with CE cultures suggest that early use (early nutrition) of these products can suppress colonization by enteric pathogens and can significantly decrease mortality in disease-challenged chickens. Growth studies without a disease challenge showed that the inclusion of *Bacillus coagulans* or *Lactobacillus* cultures in broiler diets significantly improved growth performance (Cavazzoni *et al.*, 1998; Jin *et al.*, 1998b), and in a challenge study with *Cp*, a defined lactic acid bacterial CE culture and mannan-oligosaccharides significantly reduced the severity of NE (Hofacre *et al.*, 2003). Hofacre *et al.* (1998) also observed that the commercially available natural microflora product Aviguard® is superior to virginiamycin and bacitracin for increasing BWG, and similar to them in controlling experimentally induced NE in broiler chickens. Fukata *et al.* (1991) found that the pathogenic effects of *Cp* could be reduced by feeding chicks a monoculture of *Lactobacillus acidophilus* or *Streptococcus faecalis*.

2.4.6 Prebiotic and bioactive compounds

A prebiotic was first defined as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the distal parts of the digestive tract, and thus improves the host health" (Gibson & Roberfroid, 1995). Recently, Gibson *et al.* (2004) indicated that three major criteria need to be satisfied for a food ingredient to be classified as a prebiotic: (1) resistance to gastric acidity, hydrolysis by host enzymes and gastrointestinal absorption; (2) fermentation by the intestinal microflora, and (3) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and wellbeing. Feed components that are resistant to enzymatic degradation, such as oligosaccharides, serve as substrates for bacterial activity in the intestinal lumen (Verdonk *et al.*, 2005). Like non-starch polysaccharides,

oligosaccharides cannot be degraded by the intestinal enzymes of chickens. Therefore, these compounds can only be degraded by intestinal bacteria.

The use of plants and their relevant bioactive compounds dates back thousands of years to the ancient Egyptians, Chinese, Indians and Greek (Gill, 1999; Kamel, 2000). Chinese herbal medicines have been used for many centuries for treating various human and animal diseases (Li, 2000). Phytobiotics are plant-derived natural bioactive compounds that can be incorporated into animal diets in order to enhance the performance and wellbeing of animals. Phytobiotics represent an enormous range of different compounds. The chemicals from plants which are bioactive are usually those commonly referred to as secondary metabolites. Wenk (2003) reported that herbs, spices and their extracts can stimulate appetite and endogenous secretions such as enzymes, or have antimicrobial, coccidiostatic or anthelmintic activities in monogastric animals. In recent years, there has been an increased awareness of the potential that natural plant compounds have in the prevention and treatment of poultry diseases (Chen *et al.*, 2003; Guo *et al.*, 2003; Cross *et al.*, 2004; Guo *et al.*, 2004b).

2.4.6.1 Essential oils and herbal extracts

Plants with leaves that contain oil glands provide essential oils and volatile oils which can be steam-distilled. These are mainly a mixture of fragrant, volatile compounds composed of two classes of compounds, terpenes and phenylpropenes (Lee, 2002). Many plants contain tannins, polyphenolic compounds whose name derives from their use in tanning hides due to bactericidal and fungicidal properties. The Australian flora is particularly rich in aromatic plants such as eucalyptus, tea-trees, boronias and mints and these have always been considered suitable for treating various diseases (Cribb & Cribb, 1981). Polyphenolic compounds possess bactericidal and fungistatic properties. The antibacterial effect of essential oil *in vitro* is well established and numerous reports exist of the antibacterial effects of essential oil components from various plant sources against *Cp* and other bacteria such as *E. coli* (Burt & Reinders, 2003; Mitsch *et al.*, 2004).

Hernández *et al.* (2004) observed that supplementation of broiler diets with 200 ppm essential oil extract from oregano, cinnamon and pepper and 5,000 ppm Labiatae extract from sage, thyme, and rosemary improved the apparent faecal digestibility of DM and CP of the finisher diet. Thus, supplementation of broiler diets with essential oil mixtures can create a healthier gut microflora, aiding optimum digestion and bird performance (Cruickshank,

2001). However, Lee *et al.* (2003) did not observe any positive effects on growth performance or macronutrient digestibilities in female broiler chickens when the diets were supplemented with thymol, cinnamaldehyde or commercially available essential oil product CRINA®. Similarly, Oviedo-Rondon *et al.* (2005) also could not find any significant beneficial effect or deleterious effect due to dietary supplementation with commercially available essential blends (CRINA®) given to broiler chickens that had been vaccinated against coccidiosis. In contrast, in a similar study Williams and Losa (2002) found that feeding of CRINA® to broiler birds significantly reduced the concentration of pathogenic microorganisms in the ileum, caeca and colon, which was accompanied by an increased weight gain of birds. According to Gill (1999) oregano essential oil also has anticoccidial activity against *E. tenella*, *E. acervulina*, *E. necartrix*, *E. mivati* and *E. bruneti*.

A solution of twelve soluble plant extracts has been shown to improve broiler performance and reduce ascites and NE (Cruickshank, 2001). An osmoprotectant extracted from sugar beet, betaine, can offset the negative effects of heat stress in poultry and improve feed efficiency by at least 6 % (Remus, 2002). A recent study by Youn and Noah (2001) tested extracts from 15 Asian herbs for their anticoccidial activity against *E. tenella* and found that amongst the species tested, extracts from *Sophora flavescens Aiton* was the most effective in reducing lesion scores and oocyst production, without affecting the growth performance of birds. In a similar study, Du and Hu (2004) found that a Chinese herbal complex could effectively control avian coccidiosis caused by *E. tenella.* Artemisinin is a Chinese herbal compound isolated from *Artemisia annua*; it is a naturally occurring endoperoxide with antimalarial properties. It has been found effective in reducing oocyst output from both *E. acervulina* and *E. tenella* infections when fed at levels of 8.5 and 17 ppm in broiler starter diets (Allen *et al.*, 1997).

2.4.6.2 Oligosaccharides and polysaccharides

Many plant extracts also contain compounds which are carbohydrates of various structures, being mostly heteroglycans composed not only of hexoses and pentoses, but also of methylated uronic acids (Delzenne & Roberfroid, 1994). Although most plants store insoluble starch as a reserve polysaccharide in the amyloplast, several plant families store soluble polymers of sugars such as fructose in the vacuole (Vergauwen *et al.*, 2003). Recent studies have shown that various oligosaccharides and polysaccharides may act as phytobiotics or prebiotics in poultry feed, exerting numerous growth promoting effects (Xu

et al., 2003; Lan *et al.*, 2004). Prebiotic oligosaccharides are essentially obtained by one of three processes: direct extraction of natural oligosaccharides from plants, controlled hydrolysis of natural plant polysaccharides, and enzymatic synthesis, using hydrolases and/or glycosyl transferases (Grizard & Barthomeuf, 1999).

Inulin and fructooligosaccharides

Fructans are oligomeric and polymeric carbohydrates composed of β–linked fructose monomers with one glucose unit at the reducing end (Figure 2.2). The majority of their polymeric structures are based on three trisaccharides (1-kestotriose, 6_G-kestotriose and 6-kestotriose) and two branched tetrasaccharides (1&6-kestotetraose and 6,1-kestotetraose). In higher plants three major classes of structurally different fructans can be distinguished: inulin, levan, and graminan (Vijn & Smeekens, 1999). Inulins and fructans extracted primarily from chicory root (*Cichorium intybus*) and Jerusalem artichoke (*Helianthus tuberosus*) tubers, are homologues of the GF_n type (where G and F represent glucose and fructose, respectively) consisting of β-(2→1) fructosyl residues with degree of polymerization (DP) of 2-60 with an average DP of 12 (Roberfroid, 2005). Partial enzymatic hydrolysis produces FOS, a mixture of small fructofuranose chains of the GF_n and F_m types with an average DP of 5 (Salminen *et al.*, 1998). Levan is a fructan which contains mostly or exclusively the (2→6) fructosyl-fructose linkage. The branched group has both (2→1) and (2→6) fructosyl-fructose linkages (graminan). Because of the β–configuration of the anomeric C_2 in its fructose monomers, inlulin-type fructans resist hydrolysis by intestinal digestive enzymes (Roberfroid, 2005). Thus, these compounds are available for fermentation to short chain volatile fatty acids and lactic acid by the intestinal bacteria in the distal parts of the intestine such as the colon and the caeca (Flickinger *et al.*, 2003). Indeed, low molecular weight fructooligosaccharides (GF_{2-4}) exhibit a variety of health benefits by stimulating the growth of favourable bacteria in the gut of the animals (Kleessen *et al.*, 2001; Biedrzycka & Bielecka, 2004). The use of inulin and oligofructose in livestock and companion animals was reviewed recently by Flickinger *et al.* (2003).

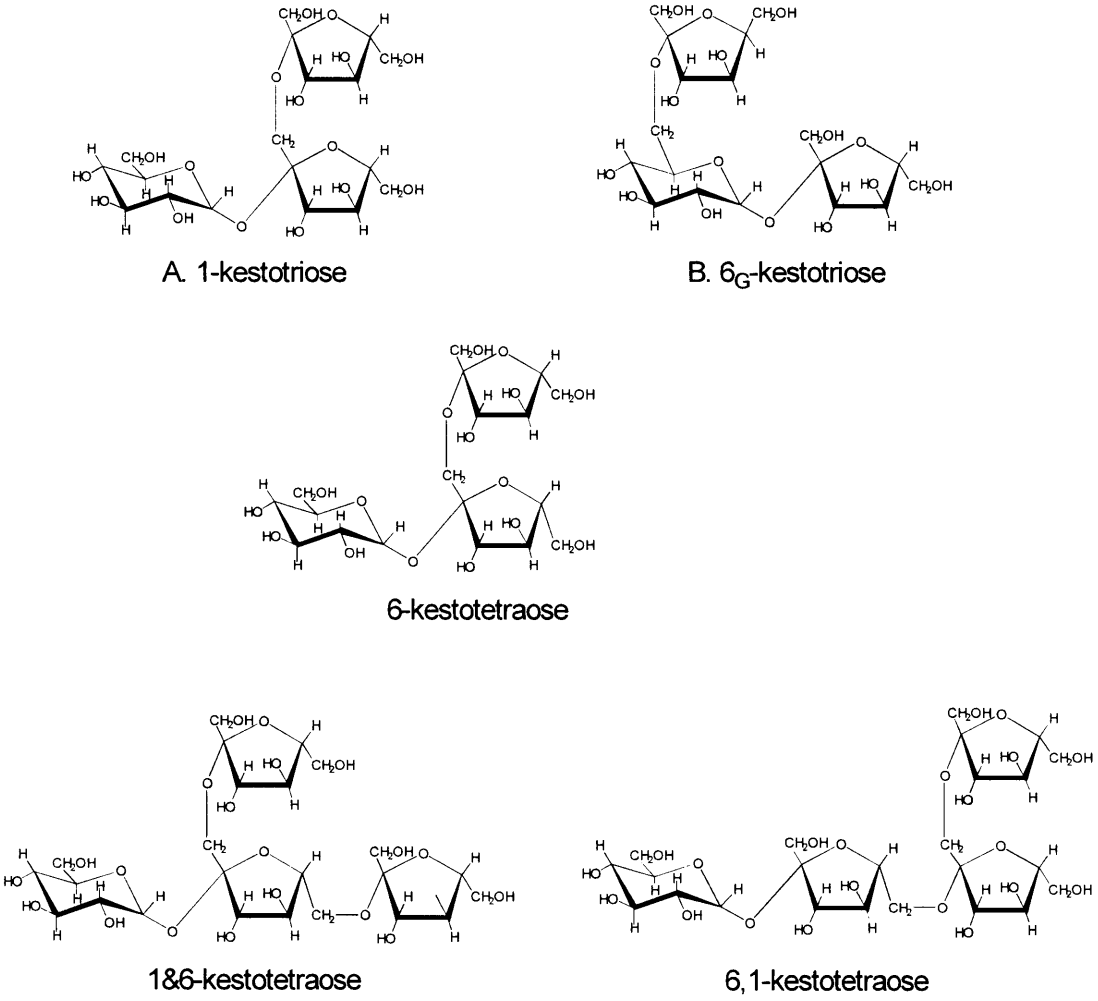


Figure 2.2 Structures of fructooligosaccharides

Adapted from Sims (2003).

2.4.6.3 Arabinogalactans

Arabinogalactans are found in many plants as they are essential for the strength and elasticity of plant cells (Paulsen, 2002). Based on the structural differences, arabinogalactans are divided into two groups, namely arabinogalactan type I and arabinogalactan type II. The major structural difference between these two types of arabinogalactans is that the galactose monomer units are (1→4)-linked in type I, and (1→3)- and (1→6)- linked in type II (Paulsen, 2002). High concentrations of arabinogalactans, with the type II being found to

possess most of the biological activities amongst the two types, have been found in the Western Larch (*Larix occidentalis*), Mongolian Larch trees (*Larix dahurica*) and resins exuded from stems and branches of Acacia trees (Fitzpatrick *et al.*, 2004; Meance, 2004). Gum exuding from branches is eaten as a soothing medication for gastrointestinal problems by Aboriginal people in Australia (Cribb & Cribb, 1981). Moreover, arabinogalactans and fucogalactoxylglucans are the major bioactive compounds of *Echinacea purpurea*, a plant that has a long tradition of use against common cold and influenza in humans (Wagner *et al.*, 1988; Paulsen, 2001). The arabinogalactan from *Acacia* has an average molecular weight of between 300-800 kDa and is a polysaccharide consisting of a highly branched arabinose and galactose which constitutes 95-99 % of dry weight, the remaining 1-5 % being protein (Meance, 2004) (Figure 2.3).

2.4.6.4 Seaweed sulphated fucans (fucoidans)

Among the vast sources of plant materials available for extracting bioactive compounds, seaweeds (macroalgae) represent a rich and largely untapped source (Tringali, 1997). Today, many countries, including Australia and New Zealand are looking at seaweeds more closely because they have a number of important uses. Southern Australia is one of the richest areas in the world for seaweeds and more than 1,000 species of macroalgae have been identified around Australia (Edgar, 1997). Seaweeds are an abundant source of natural polysaccharides, many of which have commercial uses, particularly in the food, cosmetic and medical industries (Renn, 1997).

Sulphated polysaccharides are abundant both in brown and red seaweeds (Paulsen, 2002). The terms fucan and fucoidan are generally applied to sulphated polymers with a high fucose content or where there is a fucosyl backbone (Falshaw *et al.*, 1999). The chemical structure of fucoidan from the brown seaweed *Fucus vesiculosus* is predominantly (1→2)-linked polymer of 4-sulphated fucopyranosyl residues with some (1→3)- and (1→4)-linkages (Figure 2.4). The wide range of biological properties exerted by sulphated seaweed polysaccharides is due to their structural conformation, close to that of endogenous sulphated glycosaminoglycans and other sulphated glucans found in mammalian cell membranes (Boisson-Vidal *et al.*, 1995; Berteau & Mulloy, 2003).

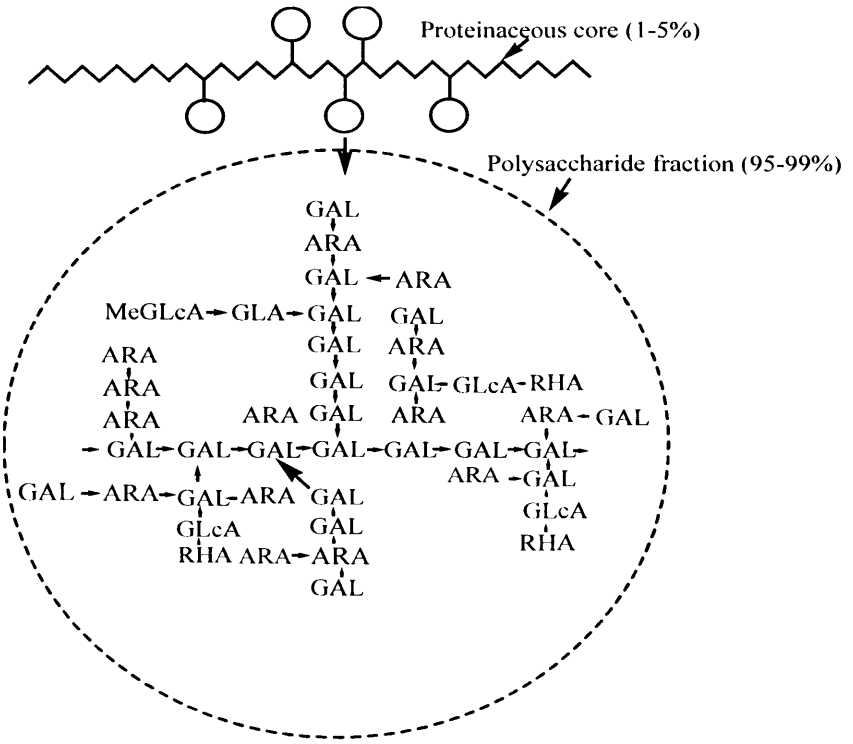


Figure 2.3 Schematic representation of the structure of acacia gum

Adapted from Meance (2004).

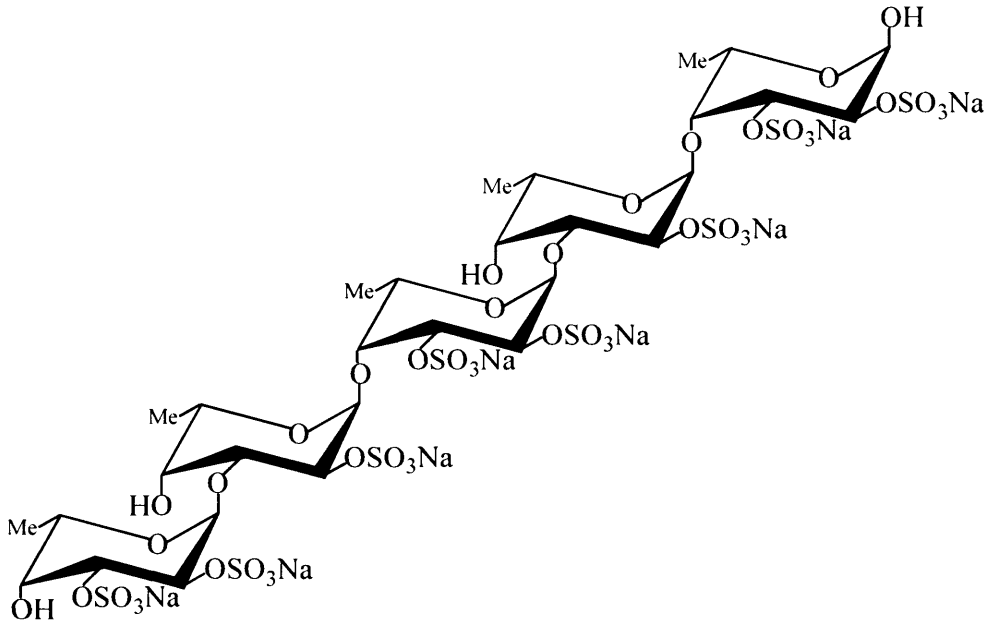


Figure 2.4 Structure of sulphated oligofucans extracted from brown algae *Ascophyllum nodosum*, shown as sodium salt

Adapted from Chevolot *et al.* (2001).

2.4.7 Modes of action of prebiotic and bioactive compounds

2.4.7.1 Direct antimicrobial effect

Certain bioactive substances from plants, like many antimicrobial agents, show their antimicrobial effects by modulating microbes at the cellular membrane (Kamel, 2000). Recently, antimicrobial activities of seaweed extracts have been reported (Vlachos *et al.*, 1999; Kumar & Rengasamy, 2000; Etahiri *et al.*, 2001). Kamel (2001) indicated that the minimum inhibitory concentration (MIC_{50}) and minimum bacteriocidal concentration (MBC_{50}) of various plant extracts tested in *in vitro* studies are clearly linked to the level of their active substances and purity of the plant extracts. Furthermore, a strong increase in hydrophobicity of the microbial species due to the presence of certain plant extracts may influence the surface characteristics of microbial cells and thereby affect the virulence of the microbes (Kamel, 2001). This could be one of the potentially important mechanisms by which certain plant extracts exert antimicrobial properties. This concept may have interesting implications in the animal intestine where the adhesion of microbes to intestinal mucosal cells is of vital importance in certain pathogenic microflora, a process that is influenced strongly by the hydrophobic surface properties of the microbial cells (Pusztai *et al.*, 1990; Gusils *et al.*, 1999; Kamel, 2001). Various essential oil mixtures, that contain natural polyphenolic compounds or flavonoids as major active ingredients, have been identified as excellent antimicrobial and antioxidant agents (Cruickshank, 2001; Friedman *et al.*, 2004). Recently, Cross *et al.* (2004) found that caecal counts of *Cp* were reduced when broiler diets were supplemented with thyme and yarrow extracts. Similarly, Jamroz *et al.* (2003) reported that dietary supplementation of plant extracts containing capsaicin (1.98 g/100g), carvacol (4.95 g/100g) and cinnamic aldehyde (2.97 g/100g), reduced *Cp* and *E. coli* counts in rectal contents to the same degree as an antibiotic (avilamycin) in treated birds.

2.4.7.2 Prebiotic effects

The effects of prebiotics and bioactive compounds are often indirect, created by metabolites, which are generated by gut microflora that use these compounds for their own metabolism (Kamel, 2000). It has been observed that prebiotic oligosaccharides and certain plant extracts can influence the growth of commensal gut microflora by providing a continuous supply of specific substrates for the protective intestinal flora, or by minimizing the risk of developing populations in which opportunistic pathogens can thrive (Mul & Perry, 1994; Lan *et al.*,

2005). Table 2.6 summarizes the effects of various prebiotic and bioactive compounds on gut-associated microflora.

The inclusion of substrates like FOS, transgalacto-oligosaccharides (TOS) or inulin can selectively stimulate the growth of beneficial microorganisms (bifidobacteria, *Lactobacillus* spp.) in the intestine (Ziggers, 2001; Bielecka *et al.*, 2002). Microorganisms like *E. coli* or *Cp* are unable to use FOS as an energy source and consequently the number of FOS-fermenters will increase (Rinkinen *et al.*, 2003). The increase in numbers of these bacteria not only reduces the amount of available substrates to potential pathogens but also decreases pH in the intestine due to increased fermentation and production of volatile fatty acids. Schematic presentation of characteristic pathways and potential health effects of saccharolytic and putrefactive fermentation by intestinal bacteria is shown in Figure 2.5.

In vitro fermentation and animal studies have indicated that inulin and oligofructose decrease the colonic and caecal pH and increase the size of the colonic and caecal pool of SCFAs (Flickinger *et al.*, 2003; Juskiewicz *et al.*, 2004). Recently, Tsukahara *et al.* (2003) reported that adding FOS to weaner pig ration caused a rapid increase in the concentration of total SCFAs and *n*-butyrate in the caecal contents. It is known that the undissociated SCFAs penetrate the bacterial cell walls and disrupt the normal physiology of certain types of bacteria (Alakomi *et al.*, 2000; Fons *et al.*, 2000). A recent study indicated that acetate, isovalerate, and succinate decreased the sporulation of *Cp* while isobutyrate decreased the vegetative cell numbers as well as sporulation of this species (Wrigley, 2004). An *in vitro* study by Gibson and Wang (1994) showed that inulin and FOS are preferentially fermented by bifidobacteria while populations of *E. coli* and *Cp* were maintained at relatively low levels. Moreover, a reduction in pH of the gut contents caused by the presence of SCFAs may stimulate the production of gut-associated protective agents such as mucus (Cummings & Macfarlane, 1991).

In addition, the inclusion of FOS in diets fed to broilers inoculated with *Salmonella typhimurium* caused a reduction in the intestinal colonization of *Salmonella* and also an improvement in the daily gain and feed efficiency (Choi *et al.*, 1994; Oyarzabal & Conner, 1996). In a similar study, Fukata *et al.* (1999) observed that adding low-level (0.1 %) of FOS to the diets of layers receiving a CE treatment resulted in reduced susceptibility to *Salmonella enteritidis* colonization in the caeca.

Table 2.6 Effects of various plant-derived bioactive compounds on gut-associated microflora

Prebiotic or bioactive compound	Study type	Evidence of prebiotic or bioactive effect/s	Reference
Thyme and yarrow extracts	Broiler chickens	Decrease in *Cp* in caecal content	Cross *et al.* (2004)
Capsaicin, carvacol, and cinnamic aldehyde mixture	Broiler chickens	Decrease in *Cp* and *E. coli* in rectal content	Jamroz *et al.* (2003)
Mushroom extracts	Broiler chickens	Increase in bifidobacteria and lactobacilli and decrease in *E.coli* in caecal content	Guo *et al.* (2004b)
Fructooligosaccharide	Broiler chickens	Increase lactobacilli, bifidobacteria and decrease *Cp*	Cao *et al.* (2005)
Oligosaccharides and water-soluble polysaccharides extracted from soybean meal	Broiler chickens	Increase in lactic acid bacteria and decrease in *E. tenella* in caecal content	Lan *et al.* (2004)
Chicory fructans	Broiler chickens	Increase in *Lactobacilli* in gizzard and small intestine, Decrease in *E. coli* and *Campylobacter* in large intestine	Yusrizal and Chen (2003b)
Jerusalem artichoke fructans	Broiler chicken	Decrease in *Cp* in caeca	Kleessen *et al.* (2003a)
Fructooligosaccharides	Broiler chickens	Increase in bifidobacteria and Lactobacilli and decrease in *E. coli* in ileal and caecal content	Xu *et al.* (2003)
Larch arabinogalactans	Dogs	Increase in bifidobacteria and Lactobacilli and decrease in *Cp* in faecal matter	Grieshop *et al.* (2002)
Larch arabinogalactans	Humans	Increase in Lactobacilli in stool	Robinson *et al.* (2001)
Acacia arabinogalactans	Humans	Increase in faecal lactic acid bacteria, Lactobacilli and bifidobacteria	Cherbut *et al.* (2003)

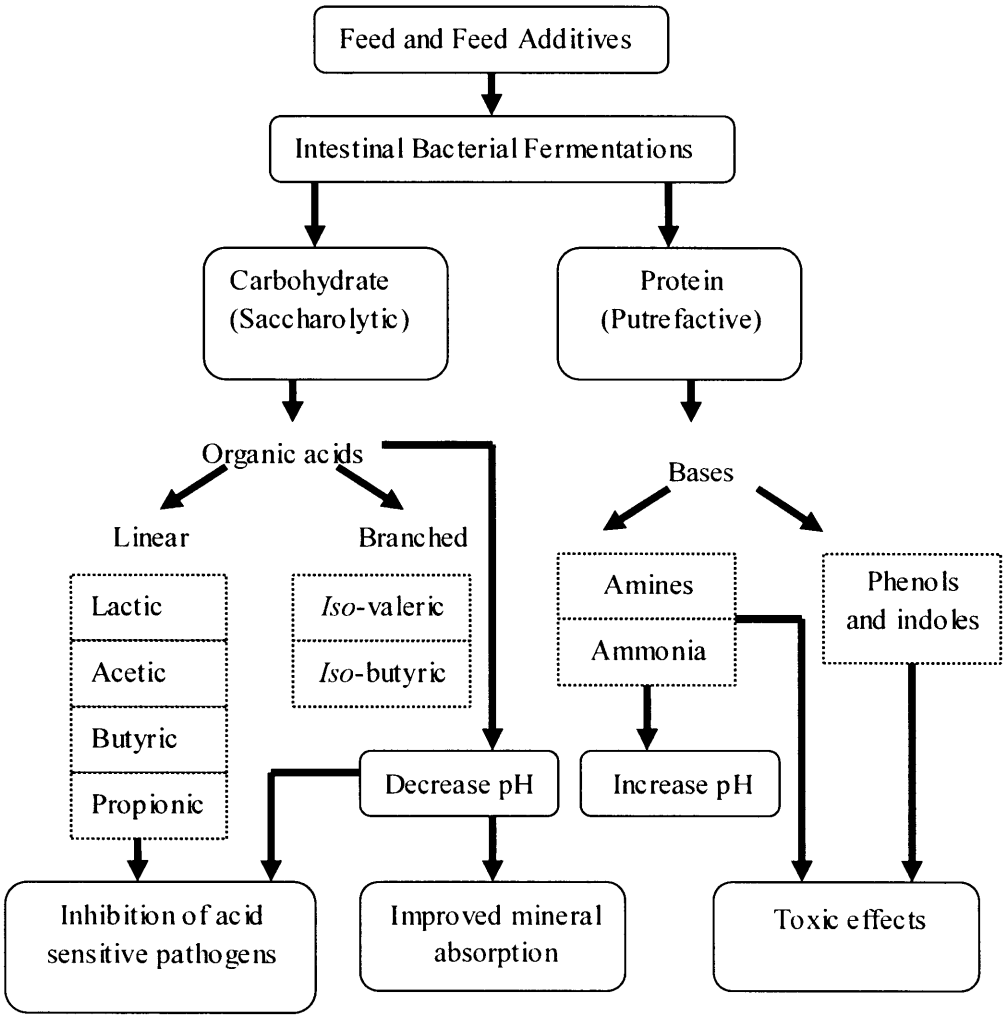


Figure 2.5 Schematic presentation of characteristic pathways and potential health effects of saccharolytic and putrefactive fermentations by intestinal bacteria

Adapted from Apajalahti (2005).

Furthermore, Bailey *et al.* (1991) observed that inclusion of 0.7 % (w/w) FOS in chicken diets led to a four-fold reduction in the counts of *Salmonella* in the caeca. Supplementing broiler diets with chicory FOS resulted in improved BWG, feed conversion, carcass weight, and a high density of ileal villi of female birds (Yusrizal & Chen, 2003a). Moreover, it increased the *Lactobacillus* counts in the gizzard and the small intestine, while reducing *Campylobacter* counts in the large intestine as well as faecal *E. coli* (Yusrizal & Chen, 2003b). In contrast, Juskiewicz *et al.* (2004) did not observe any improvement in growth parameters from supplementation of turkey diets with inulin.

Several bioactive compounds from mushrooms and plants have already been identified as compounds which differentially stimulate favourable bacteria such as *lactobacilli* and *bifidobacteria* without promoting the growth of pathogenic species (Jamroz *et al.*, 2003; Guo *et al.*, 2004b; Lan *et al.*, 2004). Stimulation of these beneficial bacteria could contribute to a balanced gut microflora, and may provide an optimal precondition for effective protection against pathogenic microorganisms and for an intact immune system (Piva & Rossi, 1998; Wenk, 2003). This, in turn, may be associated with a reduction of gut associated diseases such as coccidiosis and NE in chickens. Interestingly, Guo *et al.* (2004b) observed that dietary supplementation of mushroom and herb polysaccharide extracts in broiler chickens stimulated the growth of beneficial caecal bacteria such as *Bifidobacteria* and *Lactobacilli*, while reducing the number of potentially harmful bacteria such as *Bacteroides* spp. and *E. coli*. Recently, Lan *et al.* (2004) showed that dietary inclusion of oligosaccharides and water-soluble polysaccharides extracted from soybean meal promoted the growth of lactic acid bacteria in the caeca. They suggested that this approach can be used as a CE method in the caeca of *E. tenella*-challenged broilers.

Recent studies indicated that arabinogalactans can be used as bioactive compounds in humans and companion animals. Larch arabinogalactans are actively fermented by the intestinal microflora, thereby increasing the number of the beneficial anaerobic bacteria such as bifidobacteria and lactobacilli, while decreasing *Cp* in faecal material of dogs (Grieshop *et al.*, 2002). Moreover, Robinson *et al.* (2001) reported that supplementation with 15 or 30 g/day of Larch arabinogalactans in the healthy adult human diet resulted in increased total faecal anaerobic and *Lactobacillus* populations. These observations were similar to those of Michel *et al.* (1998) who showed a 6.75-fold increase in lactic acid bacteria counts and 1.5-1.8 log reduction in *Clostridium* spp. counts within 24 hours of inoculating chemostatic continuos-cultures of human faecal bacteria using two acacia gums (arabinogalactans)

(Fibregum standard and Fibregum AS®). Furthermore, an *in vivo* study by Cherbut *et al.* (2003) demonstrated that supplementation with *Acacia* gum (Fibergum®) in the diet of healthy adult humans increased the total lactic acid bacteria, lactobacilli and bifidobacteria counts in stools. Previous studies have shown that inclusion of arabinogalactans from *Acacia senegal* in the diet of a human subject increased the total counts of bacteroides and bifidobacteria in faeces (Wyatt *et al.*, 1986).

2.4.7.3 Competitive blocking of bacterial adhesion

Lectin-carbohydrate receptor interactions are the main mechanism for the adhesion of pathogens to the brush border of the gut mucosal epithelium. Many prebiotic and phytogenic bioactive substances may have the potential to block the adhesion of pathogens to the mucosal layer of the intestine through the 'lectin-receptor' mechanism (agglutination) (Pusztai *et al.*, 1990) or by steric hindrance (Figure 2.6). One such group of bioactive compounds that has been studied extensively is the mannan-oligosaccharide (MOS) from yeast cell walls (Spring *et al.*, 2000; Fernandez *et al.*, 2002). Mannose in the cell wall may cause the MOS to act as a decoy for the attachment of bacteria to the intestinal wall. Dietary MOS has been shown to decrease the prevalence of strains of *Salmonella* expressing Type-1 fimbriae in young chickens under laboratory conditions (Spring *et al.*, 2000). Recently, Piva and Rossi (1998) postulated that compounds like oligomannans and lectins could bind to the enterocyte receptors which are present on the cell walls of pathogenic bacteria, thus preventing them from colonizing the gut. Moreover, Bengmark (1998) suggested that certain prebiotic compounds such as pectin, guar gum and oat gum that are known to have a protective function in the mucosal layer of the intestine act by preventing colonization by pathogenic bacteria.

2.4.7.4 Stimulation of digestive enzymes

Another possible mode of action of phytogenic bioactive compounds on growth performance of farm animals could be through their effects on the activity of digestive enzymes. Xu *et al.* (2003) reported that dietary supplementation with fructooligosaccharides in male broiler chickens improved daily BWG by increasing the activities of amylase and protease. Furthermore, a study with broiler chickens indicated that feeding a diet containing a commercial blend of essential oils (CRINA®) in combination with lactic acid induced a significant increase in activities of digestive enzymes of the pancreas and intestinal mucosa

of birds, leading to a significant increase in growth (Jang *et al.*, 2004). Although Lee *et al.* (2003) observed significantly higher amylase activity in the intestinal digesta of broiler chickens fed the same commercial essential oil mixture, they did not observe any improvement in growth.

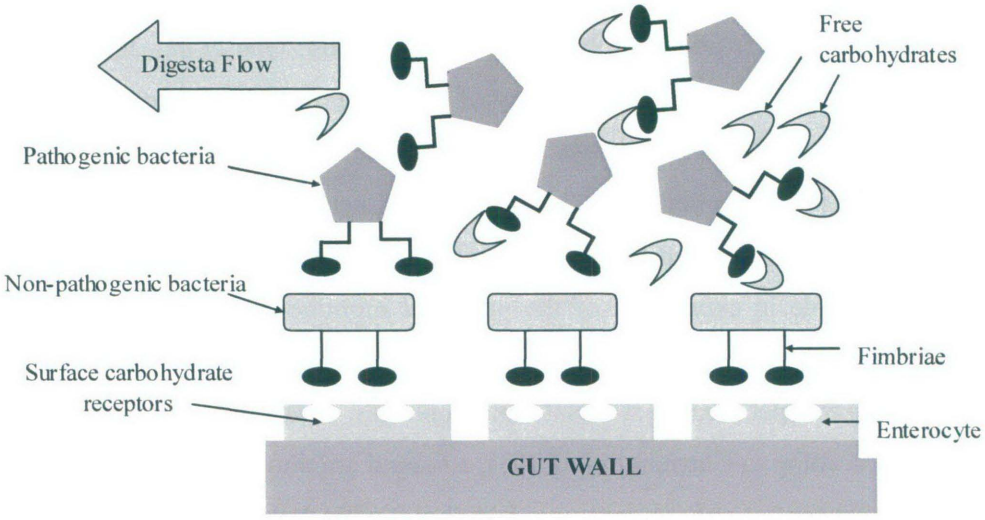


Figure 2.6 Schematic representation of protective function of compounds like lectins and oligomannans in the mucosal layer of the intestine and how they act by preventing the colonisation by pathogenic bacteria

Adapted from Ewing & Cole (1994)

2.4.7.5 Immunostimulatory effects

The gut-associated lymphoid tissue (GALT) plays a key role in immunomodulation in farm animals. Recent findings in many animal models clearly indicate that prebiotic and bioactive compounds can exert beneficial effects on gut health by enhancing GALT responses directly (systemic and local immunological effects) or indirectly by the mediation of bacteria which produce SCFAs such as butyrate and lactate (Gil & Rueda, 2002; Kaminogawa, 2002; Hosono *et al.*, 2003). Peyer's patches are unique islands of lymphoid tissue in GALT, and play an important role as an inductive site for IgA production. Since lymphocytes in Peyer's patches of GALT migrate through the thoracic duct to the systemic circulation to deliver into peripheral lymph nodes, spleen, and other mucosal sites, the intestinal immune system regulates the systemic immune system as well as the mucosal immune system (Schat & Myers, 1991). The regulatory molecules of the intestinal immune system have potential as

new immuno-modulators of both the mucosal and systemic immune systems. Wagner *et al.* (1988) have hypothesised that the immunological activity of a plant bioactive compound is dependent on a specific conformational feature and the presence of a certain number of anionic domains (Wagner *et al.*, 1988).

A number of plant glucans and glycans have been tested for their immunostimulatory properties (adjuvant effect) (Paulsen, 2002; Chen *et al.*, 2003; Guo *et al.*, 2003). One such glycan, an arabinogalactan from cell cultures of the plant *Echinacea purpurea*, has been shown to interact with mice macrophages and induce a cascade of cellular and biochemical events comparable to those elicited by β-1,3 glucans (Luettig *et al.*, 1989). Taguchi *et al.* (2004) have postulated that the β-D-(1→3)-galactan backbone of the arabinogalactans may play an important role in expression of the intestinal immune modulation activity against Peyer's patch cells. Moreover, Wagner *et al.* (1988) observed that arabinogalactans from *Echinacea purpurea* specifically stimulate macrophages to excrete the tumour necrosis factor (TNF). According to Currier *et al.* (2003), a neutral arabinogalactan from larch can act as an immunoenhancer not only on immune (lymphoid) cells, but also on natural killer (NK) lymphoid cells, as well as a variety of other haemopoietic cells in both the bone marrow and spleen of healthy, young adult mice.

Glucans have for a long time been known to have an effect on the immune system. A recent review by Guo *et al.* (2003) suggests that polysaccharides obtained from two mushroom species; *Lentinus endodes* and *Tremella fuciformis*, and a herb, *Astragalus membranaceus radix*, can be used as immune enhancers or modulators in chickens, to activate both innate and adaptive, or specific, immunity including cellular and humoral immunity. Recently, Huff *et al.* (2006) suggested that supplementation of broiler diets with β-1,3/1,6-glucan extracted from *Saccharomyces cervisiae* may be valuable for decreasing the production losses due to *E. coli* respiratory infection in broiler chickens.

The immunomodulatory effects of extracts of seaweeds are well documented and have been reported to include regulation of cytokine production and activation of macrophages in laboratory species such as rats and mice, but their influence on growth of farm animal species is less well-documented. Qureshi *et al.* (1996) observed that humoral and cell-mediated immune functions were enhanced in White Leghorn chickens fed with 10,000 ppm blue-green algae, *Spirulina platensis*. Hoshi *et al.* (1999) observed that saponins extracted

from Quillaja (*Quillaja saponaria*) are a useful mucosal adjuvant in chickens following oral immunization with a non-replicating antigen.

Oligosaccharides may act as immunomodulators at the intestinal level and recently, Kudoh *et al.* (1999) have observed that IgA secretion from caecal GALT is promoted by orally administered, highly fermentable, indigestible saccharides. These researchers also showed that the oral administration of indigestible saccharides increased the proportion of B lymphocytes in the small intestine and caecal mucosa, and suggested that lactic acid or lactic acid-producing bacteria in the caecum might be responsible (Kudoh *et al.*, 1998). Many pathogens establish contact with a potential host at mucosal surfaces. Mediation of adaptive immune defence at these sites is initiated by lymphocyte activation and local secretion of IgA (Muir *et al.*, 2000). Findings of Hosono *et al.* (2003) suggest that dietary supplementation of FOS changes the intestinal environment of microflora, and leads to up-regulation of immunoglobulin A (IgA) secretion in the murine Peyer's patch cells in the intestinal mucosa. These results were further confirmed by Roller *et al.* (2004), who showed that prebiotic inulin with oligofructose supplementation enhances the production of interlukin-10 in Peyer's patches of secretory immunoglobulin A (sIgA) in the caecum of rats. In a similar study, Kelly-Quagliana *et al.* (2003) indicated that dietary inclusion of oligofructose and inulin up-regulate the macrophage-dependent immune responses in a dose-dependent manner in rats. Moreover, Chen *et al.* (2003) found that the low-molecular weight Chinese herbal polysaccharide, achyranthan, was more effective than the high molecular weight polysaccharide, astragalan, in enhancing the immunity of broiler chickens. Verstegen and Williams (2002) pointed out that, in order to exert these immunomodulating properties, non-digestible oligosaccharides and polysaccharides need to act before fermentation and/or have to escape fermentation in the digestive tract of monogastric animals.

2.5 CONCLUSIONS

A stable and balanced gut ecosystem is of paramount importance for host animal health and for the protection of the gut environment, and is now known to be an important part of reducing disease challenge in animal production. During the past five decades, antibiotics have been used in the poultry industry as therapeutic and subtherapeutic agents in order to control diseases and to improve growth performance. Although the exact modes of action of antibiotic growth promoters are not fully understood, the main effects are thought to be mediated via the gut associated bacteria.

Many countries are moving towards a reduction in use of antibiotics in animal diets due to public concerns regarding development of antibiotic-resistant bacteria in humans. In Europe, the application of antibiotics as feed additives in order to enhance growth in production animals has been banned. However, initial restriction in the use of in-feed antibiotics in many European countries resulted in an increased incidence of enteric disorders such as NE and coccidiosis in poultry.

Use of alternatives to subtherapeutic antibiotics that improve gut health and bird performance may help to overcome the need for in-feed antibiotics. Thus, there has been increased interest in the use of biological products; this has included the use of a variety of naturally-occurring additives, including probiotics, prebiotics, synbiotics, organic acids, antibodies, bacteriocins, bacteriophages and plant extracts (phytobiotics) as alternatives to antibiotic feed additives in poultry diets.

Many alternatives to AGPs have been tested on various animal models under experimental and field conditions; some of them have been found effective in improving poultry health and performance. The exact modes of action whereby prebiotic and plant bioactive substances exert their positive effects *e.g.* antimicrobial, prebiotic, immunomodulator are not well understood, and further scientific research in this area is warranted. Many studies in poultry suggest that dietary supplementation with certain prebiotic and bioactive compounds are capable of inhibiting colonization of the gut by certain pathogenic bacteria; however, the effects are inconsistent in terms of growth performance of animals. Perhaps, this could be partly due to specific sanitary conditions of the experimental environment, and nutritional status of the animals. There is no doubt that many prebiotics and plant bioactive substances are capable of drastically modifying the gut microflora, which, in turn, can bring about a cascade of changes in the animal's responses to nutrients, disease challenge and physical environment. The question which remains unanswered is what type of extracts can be recommended and under what circumstances they can be used. The whole area of prebiotic and bioactive compounds is at an early stage of application in animal nutrition and will require much research.

CHAPTER 3 WATER-SOLUBLE PREBIOTIC COMPOUNDS FROM AUSTRALIAN AND NEW ZEALAND PLANTS: ISOLATION AND CHARACTERISATION

ABSTRACT

The water-soluble carbohydrates (WSCs) extracted from the underground parts (rhizome) of Arthropodium cirratum (Rengarenga lily extract); third order branches of Cordyline australis (Cabbage tree extract); a seaweed, Undaria pinnatifida (Undaria extract), and exudates from Acacia pycnantha (Acacia extract) were investigated. Extracts of Rengarenga lily, Cabbage tree, Undaria, and Acacia contained 576, 250, 275, and 794 g/kg DM WSCs, respectively. Constituent sugar analysis by gas-liquid chromatography (GLC) showed that extracts of Rengarenga lily and Cabbage tree contained predominantly fructose and glucose (82-95 %). The analysis also revealed that Acacia extract contained mainly galactose (78 %) and arabinose (22 %) while Undaria extract, contained fucose (55 %) and galactose (44 %). Thin-layer chromatography (TLC) showed that, on the basis of R_F values, fructan composition of Rengarenga lily extract and Cabbage tree extract was different. Cabbage tree extract contained 45% (w/w) fructans while Rengarenga lily extract contained 65 % (w/w) fructans. High performance size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS) showed that the extracts had varying weight average molecular weight due to differences in the average chain length of the major carbohydrates. Data for the amino acid compositions differed considerably depending on the type of extract. Water-soluble carbohydrate extracts prepared from the four plant sources gave a wide range of WSC (250-794 g/kg DM) due to the different proportions of structural material in different species.

3.1 INTRODUCTION

The use of plants and their relevant bioactive compounds dates back thousands of years to the ancient Egyptians, Chinese, Indians and Greeks (Gill, 1999; Kamel, 2000). Chinese herbal medicines have been used for many centuries for treating various human and animal diseases (Li, 2000). Since the late 1990s and the birth of the prebiotic concept, scientists

have begun to be interested in the health properties of prebiotic compounds so today there are many scientific papers describing them in relation to human health. In recent years there has been an increased awareness of the potential that various natural plant bioactive compounds such as oligosaccharides and polysaccharides may act as phytobiotics or prebiotics in poultry feed and to exert growth-promoting effects as well as health-improving effects (Guo *et al.*, 2004b; Lan *et al.*, 2004; Vidanarachchi *et al.*, 2005). Australia and New Zealand have rich reservoir of bioactive compounds, and the health benefits of plant extracts have been empirically known for thousands of years by Aboriginal and Maori populations. The incidence of non-infectious diseases during pre-modern times was low in these populations, perhaps in part due to the presence of protective chemical constituents within the plants that were eaten (Cambie & Ferguson, 2003).

Many flowering plant species store fructans, which are polymers of fructose, as reserve carbohydrates. *Arthropodium cirratum* (Rengarenga lily) is a slender herb which has starchy edible rhizomes rich in fructans as storage carbohydrate (Harris, 1996). The occurrence of large amounts of fructans in Rengarenga lily rhizomes and readily hydrolysable glucofructofuranan in *Cordyline australis* (Cabbage tree) shoots, explains why the early settlers in Australia and New Zealand not only used the tubers and cooked stems of these species as a food, but also used them in herbal medicine (Fankhauser & Brasch, 1985; Harris, 1996; Cambie & Ferguson, 2003).

Another highly abundant plant that has potential prebiotic properties is the wattle tree (*Acacia* spp.). Many *Acacia* species exude a complex arabinogalactan-type polysaccharide as sap. A report in the nineteenth century on Victorian Aborigines states that all their common ailments were effectively treated with wattle bark exudate (*Acacia* exudate), which may be apparent as a slimy exudate from a damaged portion of *Acacia* plants (Cribb & Cribb, 1981). Impressed with the "healing" powers of many wattle species, colonial doctors in Australia prescribed many of them for the treatment of dysentery and diarrhoea (Wickens & Pennacchio, 2002). American Indians also have traditionally consumed *Acacia* exudates for preventing and treating gastro-intestinal disorders. Today, *Acacia* gum is widely used for its nutritional and surface properties by the human food industry.

Seaweeds are an abundant source of natural polysaccharides, many of which have commercial uses, particularly in the food, cosmetic and medical industries (Renn, 1997). Southern Australia is one of the richest areas in the world for seaweeds and more than 1,000

species of macro-algae have been identified around Australia (Edgar, 1997). The health promoting effects of extracts of certain seaweeds in human and animal models are well known (Sakai *et al.*, 2002), but their influence on growth performance of farm animal species is less well-documented.

Prebiotic compounds from plants are obtained by one of three processes: direct extraction of natural carbohydrates (monosaccharides and oligosaccharides) from plants, controlled hydrolysis of natural plant polysaccharides and enzymatic synthesis, by using hydrolases and/or glycosyl transferases (Grizard & Barthomeuf, 1999). Of the several methods which can be used for extracting bioactive compounds from plant sources, ethanol-water (warm or hot) extraction of water-soluble compounds is the most popular. Only a few polysaccharides are soluble in ethanolic extract, while all mono- and oligosaccharides are soluble (Carre, 2002). Research on biologically active carbohydrates from higher plants and related species in broiler nutrition is relatively new and in this study, water-soluble carbohydrates from underground parts (rhizome) of Rengarenga lily, third order branches of Cabbage tree and exudates from *Acacia*, and a seaweed *Undaria* were extracted and their chemical composition analysed in order to study their bio-activities *in vivo*.

3.2 MATERIALS AND METHODS

3.2.1 Isolation of water-soluble carbohydrates

3.2.1.1 Plant materials

The underground parts (rhizome) of *Arthropodium cirratum* (Rengarenga lily) and third order branches (stems) of *Cordyline australis* (Cabbage tree) were collected in mid-winter of 2003 from a nursery in Wellington, New Zealand. Care was taken not to break the rhizomes, and they were thoroughly washed in order to remove soil. The exudates from *Acacia pycnantha* (Golden wattle) were obtained from trees grown in the Adelaide area of South Australia in December 2003. The *Undaria pinnatifida* seaweed samples were collected from Point Arthur, Wellington Harbour, New Zealand in June 2003 and the algae were washed thoroughly with seawater, followed by tap water, to remove soil particles and epiphytes. Several batches of plant extracts were prepared according to the following procedures in order to obtain sufficient materials for the broiler experiments. Materials to solvent ratios were changed accordingly during the large scale production of extracts.

3.2.1.2 Rengarenga lily and Cabbage tree extracts

The cleaned rhizome samples of Rengarenga lily and third order branches of Cabbage tree were air-dried, cut into small pieces and ground in a laboratory grinder (Mikro-Feinmuhle-Culatti MFC grinder, Janke & Kunkel GmbH & Co., Staufen, Germany). Powdered samples (1 g) from the two species were extracted in 50 mL of boiling 80 % (v/v) ethanol for 10 min and water-soluble carbohydrates were extracted twice with distilled water (50 mL/g, 70°C, 60 min). The ethanol and two water extracts from each species were combined and concentrated *in vacuo* at 40°C in a rotary evaporator (Buchi Rotavapor-R, Buchi Laboratories, Flawil, Switzerland). After freezing and thawing, the resulting insoluble materials were removed by centrifugation at 3500 x *g* for 20 min at 20°C using an MSE Mistral 2000R centrifuge (Sanyo Gallenkamp PLC, Leicestershire, UK), and the supernatants were passed through an ion exchange column consisting of both anion exchange resin (Amberlite IR 120, Na^+ form) and cation exchange resin (Amberlite IR 401, Cl^- form). The neutral elutes were immediately frozen in acetone cooled with dry-ice, and lyophilized for 72 h at -49°C and 62 x 10^{-3} mbar (Eyela Freeze Dryer FD-1, Rikakikai Co., Tokyo, Japan). The freeze-dried powders of Rengarenga lily extract, (off-white) and Cabbage tree extract (greenish-black) were incorporated into experimental diets (broilers) as supplements in later experiments.

3.2.1.3 Acacia extract

The Acacia exudate was dissolved in distilled water (50 g/L) and the solution was filtered through a 600 μm screen to remove particulate matter and then centrifuged at 12,000 x *g* for 10 min at 4°C in a Beckman model J2-21M Induction Drive centrifuge with a JA-21 rotor (Beckman Instruments Inc., Palo Alto, CA, USA) in order to remove insoluble tannins. The supernatant was collected and dialysed (MWCO 12-14 kDa, Medicell International Ltd., London, UK) exhaustively against distilled water (48 h). The dialysate was collected and then lyophilized to yield a fine off-white powder (*Acacia* extract) that was incorporated into the diets in experiments reported later in this thesis.

3.2.1.4 Seaweed extract

The seaweed samples (thalli) were air-dried and crushed into chips (less than 2.0 mm in diameter) using a laboratory grinder. The preparation of crude extracts of the *Undaria* seaweed samples were performed as described below. Unless indicated otherwise, all

extraction steps were performed at 25°C and all centrifugation steps were performed at 3500 x *g* for 20 min at 20°C using an MSE Mistral 2000R centrifuge. Extraction of fraction I was performed by constant mechanical stirring (Heidolph MR 3001K stirrer, Heidolph Instruments GmbH & Co., Schwabach, Germany) of the algal powder in 150 mL of 1 % (w/w) sulphuric acid, initially for 6 h, followed by overnight stirring with another 150 mL of 1 % (w/w) sulphuric acid. After each stirring step, samples were centrifuged and supernatants were saved and finally pooled together (fraction I). The pooled fraction was neutralized (1 % (w/v) sodium hydroxide) and dialysed exhaustively against distilled water (48 h). The dialysate was concentrated *in vacuo* at 40°C in a rotary evaporator and immediately frozen in acetone cooled with dry-ice, and lyophilized.

The insoluble matter was resuspended in 150 mL of 1 % (w/v) sodium carbonate and mechanically stirred (Heidolph Instruments GmbH & Co., Schwabach, Germany) three times (3 x 150 mL) for 4 h, 6 h, and 12 h, followed by centrifugation after each of the stirring steps. Supernatants were pooled together and neutralized with 10 % (w/w) sulphuric acid and the pH was adjusted to 1.6 with the same sulphuric acid solution. A creamy white flocculate (alginate) which appeared at about pH 3.0 was removed by centrifugation and the resultant supernatant (fraction II) was neutralized with 10 % (w/v) sodium hydroxide, followed by dialysis and lyophilization. The insoluble material was washed three times with distilled water (3x150 mL) and finally with 100 mL of acetone. Washed fractions were separated by centrifugation and pooled together (Fraction III), dialysed against distilled water (48 h) in tubing with a normal 10-12 kDa molecular weight cut-off, and then lyophilised. Fractions I, II and III were combined (*Undaria* extract) and incorporated in the experimental diets in later experiment.

3.2.2 Analytical techniques and measurements

3.2.2.1 Dry matter content

The DM content of extracts was determined gravimetrically according to the Association of the Official Analytical Chemists Official Method 934.01 (AOAC, 2002). Samples were accurately weighed (2-4 g) into preweighed silica crucibles and placed in a forced-air convection oven (Qualtex Universal Series 2000, Watson Victor Ltd., Perth, Australia) which was preheated to 105°C. Samples were held at this temperature overnight or until a

constant mass was obtained. The DM content was calculated as percent ratio of the weight difference of samples before and after drying to that of original material.

3.2.2.2 Crude Protein

The nitrogen content of the extracts was determined according to the DUMAS combustion technique following the method described by Sweeney (1989) using a LECO® FP-2000 automatic nitrogen analyser (Leco Corp., St Joseph, MI, USA). Nitrogen freed by combustion at high temperature in pure oxygen was measured by thermal conductivity detection and converted to equivalent protein by a numerical factor of 6.25. The furnace temperature was maintained at 1050°C for pyrolysis of sample in ultra high purity oxygen. To interpret detector response as percentage nitrogen (w/w) calibration was done using pure primary standard of ethylenediaminetetra-acetic acid (EDTA).

3.2.2.3 Analyses of water-soluble carbohydrates

Total water-soluble carbohydrates (WSC) were determined by the phenol-sulphuric acid method using glucose as a standard (Dubois *et al.*, 1956). An equal volume (400 µL) of 5 % (v/v) phenol was added to 400 µL plant extracts dissolved in distilled water (1 mg/mL) and gently mixed. Concentrated sulphuric acid (2 mL) was added and after 10 min incubation at room temperature the absorbance was read at 490 nm using a Hitachi 450-20 spectrophotometer (Hitachi Co., Japan). (Total carbohydrate in each extract was expressed as g/kg dry matter).

3.2.2.4 Constituent sugar analysis

The reductive hydrolysis and acetylation method of Stevenson and Furneaux (1991) was used to convert the constituent sugars in water-soluble carbohydrate fractions into alditol acetate derivatives. This method utilises *in situ* reduction with *N*-methylmorpholineborane (MMB) during hydrolysis to prevent degradation of 3,6-anhydrogalactose units.

A fresh stock solution of aqueous MMB (80 mg/mL, 0.2 mL per sample) was made beforehand. The water-soluble carbohydrate fractions (1 mg) were placed in Kimax screw-cap tubes (13 x 100 mm) equipped with Teflon-lined caps and 50 µL aqueous MMB and 200 µL 3M trifluoroacetic acid (TFA) which contained 2 mg/mL inositol (internal standard) were

added and heated at 80°C for 30 min. The tubes were cooled, a second portion of 50 µL MMB was added and reheated at 120°C for 1h. This step was repeated once more with the 100 µL aqueous MMB, and the solution was evaporated to dryness at 50°C. Residual water was removed by adding acetonitrile (CH_3CN) twice (2 x 0.4 mL) and concentrating to dryness again. The resulting dry solid was acetylated by adding 100 µL of acetic anhydride (Ac_2O) and 100 µL of neat TFA, followed by heating at 50°C for 30 min. Residual Ac_2O was decomposed by adding 1 mL of toluene and evaporating to dryness with a stream of dry air. The mixture was extracted with 2.5 mL of dichloromethane (CH_2Cl_2) and the organic phase was extracted first with 2.5 mL 0.5M aqueous-sodium carbonate and then with 2.5 mL distilled water, discarding the upper aqueous layer each time. The phases were separated by brief, low speed (1000 x *g*, 5 min, 20°C) centrifugation. The dichloromethane layer was then evaporated to near dryness and excess water was removed by adding 0.5 mL of CH_3CN and evaporating to dryness. The residue was dissolved in 350 µL of acetone and GLC was conducted on a Hewlett Packard 5890 Series II chromatograph (Global Medical Instrumentation Inc., Ramsey, MO, USA) fitted with a Supleco SP™ 2330 fused silica capillary column (15 m x 0.25 mm i.d., 0.25 µm film thickness). The sample was introduced by split injection using H_2 as the carrier gas with a split ratio of 65:1 (column flow, 1.5 mL/min). Injector and FID detector temperatures were 215°C. Relative response factors were determined using synthesised alditol acetate standards and identification of components was by comparison of retention times with authentic standards.

3.2.2.5 High performance size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS)

Ten milligrams (10 mg) of each of the water-soluble extracts were dissolved in 2 mL of 0.1M lithium nitrate ($LiNO_3$) and heated at 95°C for 15 min. Another 3 mL of 0.1M $LiNO_3$ was added and left overnight. The SEC-MALLS system consisted of a Waters® 2690 XE Alliance separations module, a Waters® 490 E programmable multi-wavelength detector set at 280 nm, a DAWN-EOS multi-angle laser light-scattering detector (Laser Photometer) with a laser at 690 nm (Wyatt Technology Corp., Santa Barbara, CA, USA), and a Waters® 2410 refractive index monitor. Samples (0.2 mg/mL) were filtered (0.45 µm) before injection (100 µL) and eluted with 0.1M $LiNO_3$ containing 0.02 % NaN_3 (0.7 mL/min) from two columns (TSK-Gel G5000PWXL and G4000PWXL, 300 x 7.8 mm, Tosoh Co., Tokyo, Japan) connected in series. Data for molecular weight determination and conformation were

analysed using ASTRA software (Wyatt Technology Corp., Santa Barbara, CA, USA) with a specific refractive index (*dn/dc*) of 0.145 mL/g (determined experimentally).

3.2.2.6 Determination of fructan contents and thin-layer chromatographic (TLC) analyses of fructans

Fructan content in Rengarenga lily extract and Cabbage-tree extract was determined using the full Megazyme fructan assay procedure (Megazyme, 2004). Structural composition of fructans was analysed by TLC using a method described by Sims (2003). Samples containing about 50 μg fructans were applied to the origin of a 20 x 20 cm silica gel TLC plate (Silica gel 60 F_{254}, Merck, Germany). Plates were developed three times in butan-1-ol/propan-2-ol/water (3:12:4, v/v/v) at room temperature. Compounds were visualised by spraying with the ketose-specific, urea-phosphoric stain.

3.2.2.7 Determination of amino acid composition of plant extracts

The amino acid composition of the plant extracts was determined using a gas chromatography-mass spectrometry (GC-MS) method described by Persson and Nasholm (2001). The extracts were hydrolysed with 6M HCl containing 0.1 % (w/v) phenol at 110°C overnight before derivitisation. The hydrolysed amino acids were derivitised to their *tert*-butyldimethylsilyl (tBDMS) form using N-methyl-N-*tert*-butyldimethylsilyl-trifluroacetamide (MTBSTFA). The derivitised amino acids were analysed by GC-MS using a Hewlett-Packard HP 6890 gas chromatograph (Global Medical Instrumentation Inc., Ramsey, MO, USA) on a CPSSil5 column (25 m x 0.3 mm, Chrompack, Bergen, The Netherlands) and detected by mass spectroscopy with a Hewlett-Packard 5973 mass selective detector. Quantifications were performed by the use of two internal standards, α-aminoisobutyric acid and hydroxyl-L-proline.

3.3 RESULTS

The four plant extracts showed considerable variation in their water-soluble carbohydrate (WSC) contents (Table 3.1). Rengarenga lily extract had 58 % (dry matter basis) WSC in rhizomatous roots. The results also showed that Cabbage tree extract and *Undaria* extract contained about one third (25-28 %) of the water-soluble carbohydrates of *Acacia* extract (79%). The DM contents of lily extract and *Acacia* extract were similar (Table 3.1).

Table 3.1 Yield and chemical analyses of the plant extracts

	Plant extract			
	Lily extract	Cabbage tree extract	*Undaria* extract	*Acacia* extract
Yield of water-soluble carbohydrates (g/kg DM)	576	250	275	794
Dry matter (g/kg)	938	880	885	930
Total sugar content (g/kg DM)	680	480	370	790
Crude protein (g/kg DM)	49	143	21	32
Molecular weight (Da)	~5,000[1]	N.D	511,000	30,000
Polydispersity index[2] (M_w/M_n)	1.10	-	1.46	1.07
Fructan content (%, w/w)	65	45	-	-

N.D = Molecular weight is too low for determination.
[1]approximate molecular weight only; close to limit of method.
[2]Polydispersity index (M_w/M_n) = M_w (Weight average molecular weight) / M_n (Number-average molecular weight).

The DM contents of Cabbage tree extract and *Undaria* extract were similar and lower than those of the lily extract and *Acacia* extract. Water-soluble carbohydrates from lily extract contained monosaccharides, fructose and glucose with similar retention times to those of a commercially available fructooligosaccharide (Frutafit) (Figure 3.1). The results of the sugar composition analysis (Table 3.2 and Figure 3.2) showed that *Acacia* extract contained mainly galactose and arabinose with a molar ratio of 7.8:2.2, while *Undaria* extract, contained fucose and galactose with a molar ratio of 5.5:4.4. Cabbage tree extract contained mainly fructose and glucose with a molar ratio of 3.2:4.9.

The Megazyme fructan assay results indicated that lily extract and Cabbage tree extract contained a high proportion of fructose and fructose-containing oligosaccharides. These sugars are highly acid-labile and are destroyed by the hydrolysing conditions in these analyses; as such values may have been underestimated in the composition analysis. Cabbage tree extract contained 45 % (w/w) fructans while lily extract contained 65 % (w/w) fructans (Table 3.1). Thin-layer chromatography (TLC) using ketose-specific urea-phosphoric acid stain showed that lily extract fructans did not migrate from the origin at DP>12, but that the Cabbage tree extract fructans all moved from the origin (Figure 3.3); therefore Cabbage tree extract fructans are much smaller fructans (oligofructans) than those of lily extract.

Table 3.2 Comparison of the constituent sugar analyses of the plant extracts[1]

Alditol acetate derivatives	Deduced sugar	Sugar composition (g/ 100 g))			
		Lily extract	Cabbage tree extract	*Undaria* extract	*Acacia* extract
Rhamnitol	Rhamnose	N.D	4.7	N.D	0.5
Fucitol	Fucose	N.D	1.2	55.2	N.D
Arabinitol	Arabinose	N.D	5.8	N.D	21.6
Xylitol	Xylose	N.D	1.4	N.D	N.D
Mannitol	Mannose/Fructose	34.4	32.1	1.0	N.D
Glucitol	Glucose/Fructose	61.0	49.6	N.D	N.D
Galactitol	Galactose	4.6	5.1	43.9	77.9

[1]Average of duplicate determinations.
N.D = Not detected.

Of the four extracts, Cabbage tree extract had the highest crude protein content (14.3 %), about 3, 4.5 and 7-fold those of lily extract, *Acacia* extract, and *Undaria* extract, respectively (Table 3.1). The amino acid analysis of plant extracts revealed that the lily extract and Cabbage tree extract contained high proportions of glutamic acid which is similar to the higher levels of glutamic acid found in commercially available fructooligosaccharide (Frutafit®) (Table 3.3). As expected, Hyp and Ser were the main amino acids in *Acacia* extract and accounted for 23 % (w/w) and 10 % (w/w) of the total amino acids, respectively. *Undaria* extract had a comparatively high proportion (20 % w/w) of Gly compared to other extracts. The concentrations of Met, Cys (sulphur-containing amino acids), and Tyr were lower in all plant extracts.

The analysis showed that the extracts had varying weight average molecular weights due to differences in the average chain length of major carbohydrates. The molecular weight of lily extract was ~5000 Da, meaning that the average chain length of the fructans in lily extract is about 30 (the number of sugar residues in the chain). *Undaria* extract and *Acacia* extract had weight-average molecular weights of 5.11×10^5 Da and 3.0×10^4 Da, respectively. The *Acacia* extract had a relatively low polydispersity index (M_w/M_n) of 1.07, whereas the same value for *Undaria* extract was relatively high (1.46).

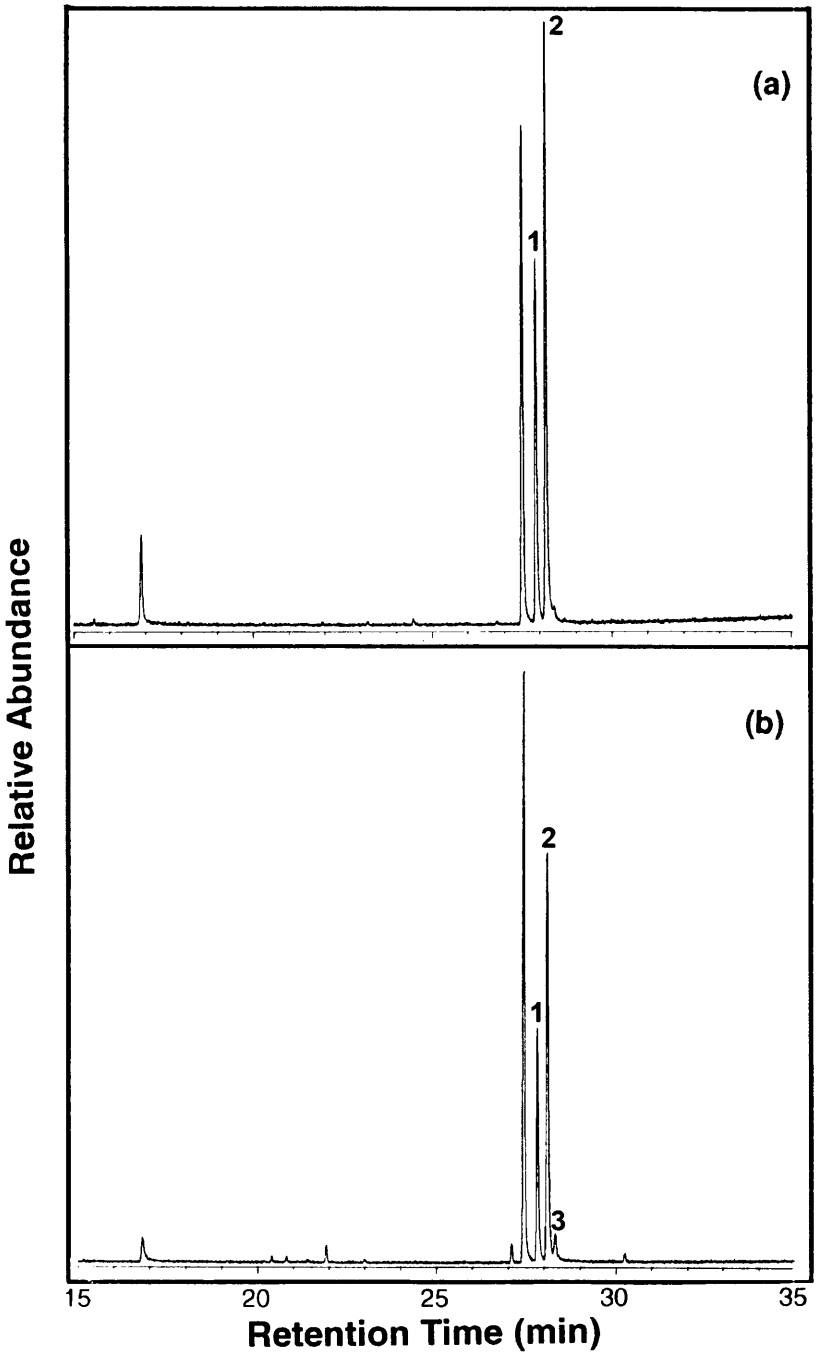


Figure 3.1 Gas-liquid chromatograms of the alditol acetates of sugars from (a) Fruatafit (fructooligosaccharide) and (b) Rengarenga lily extract obtained by reductive hydrolysis method

Peaks represent the sugars, mannose (1), fructose/glucose (2), and galactose (3).

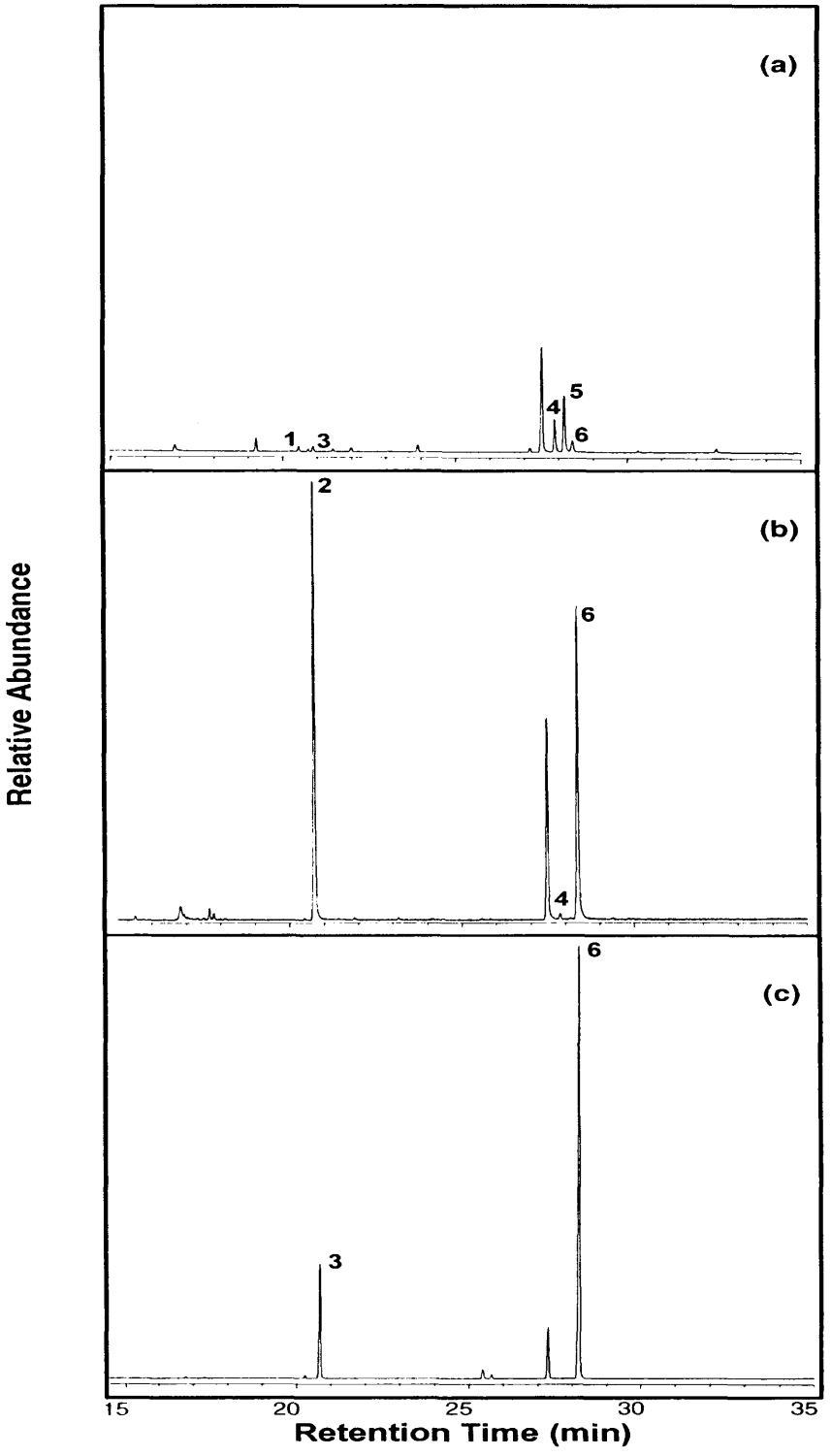


Figure 3.2 Gas-liquid chromatograms of the alditol acetates of sugars from Cabbage tree extract (a), *Undaria* extract (b), and *Acacia* extract (c) obtained by reductive hydrolysis method

Peaks represent the sugars, rhamnose (1), fucose (2), arabinose (3), mannose (4), fructose/glucose (5) and galactose (6).

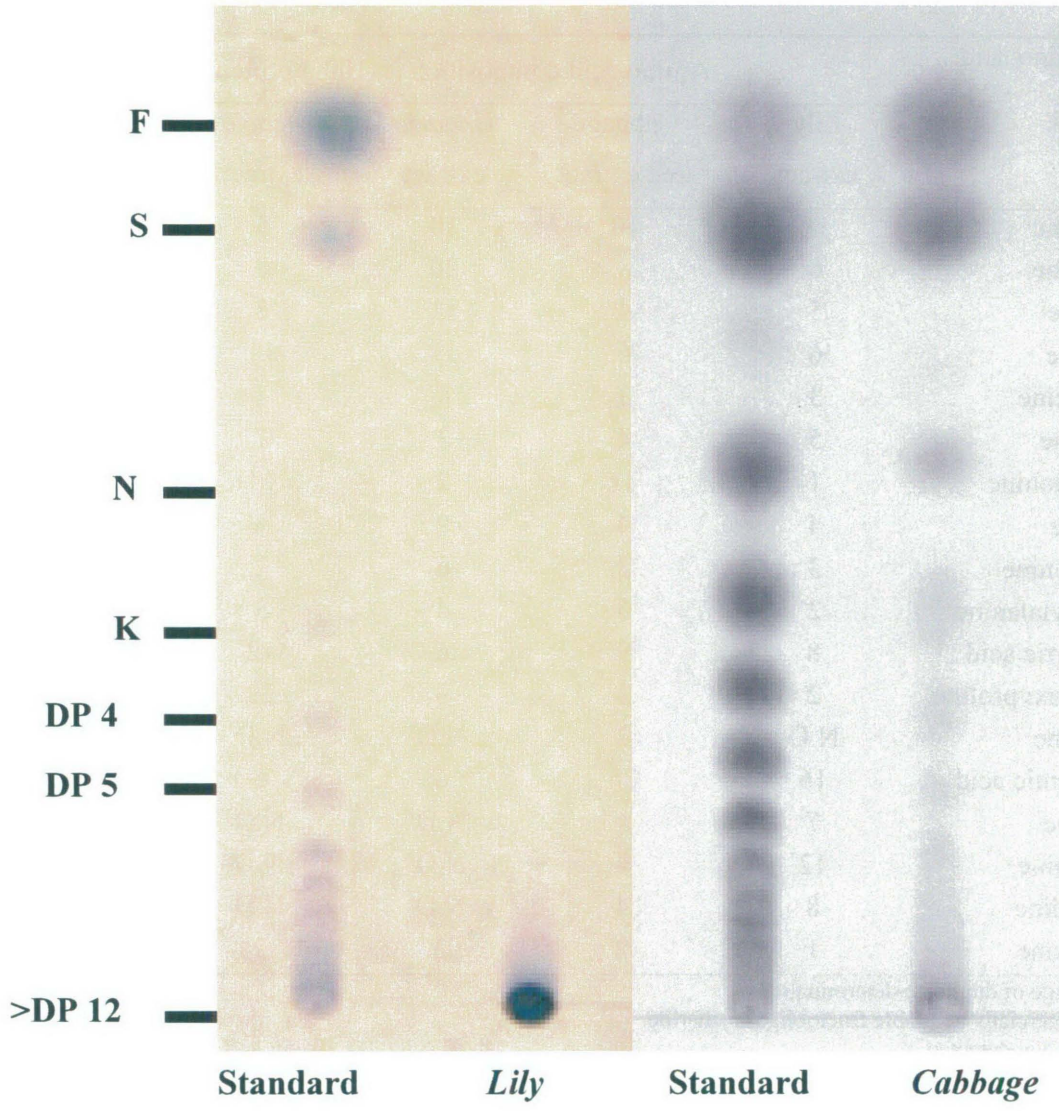


Figure 3.3 Thin-layer chromatogram of fructans from Rengarenga lily extract (*Arthropodium cirratum)* and *Cabbage tree extract* (*Cordyline australis*)

Markers represent the mobilities of fructose (F), sucrose (S), 6_G-kestotriose (N), 1-kestotriose (K), inulin tetrasaccharide (DP 4), and pentasaccharide (DP 5).

Table 3.3 The amino acid composition (weight percentage) of the plant extracts[1]

Amino acid	Amino acid composition (g/100 g protein)				
	Lily extract	Cabbage tree extract	*Undaria* extract	*Acacia* extract	Frutafit[2]
Alanine	11	10	10	5	6
Glycine	6	8	20	8	N.D
Valine	5	4	11	8	7
Lecine	6	6	11	11	7
Isolecine	3	3	4	4	7
Proline	5	4	3	7	6
Methionine	1	Tr	2	N.D	N.D
Serine	4	4	9	10	N.D
Threonine	3	3	6	2	1
Phenylalanine	2	3	4	3	N.D
Aspartic acid	8	9	6	12	30
Hydroxyproline	2	3	6	23	N.D
Cystine	N.D	2	N.D	N.D	N.D
Glutamic acid	16	26	6	6	36
Lysine	7	4	N.D	N.D	N.D
Arginine	12	4	N.D	N.D	N.D
Histidine	8	4	N.D	N.D	N.D
Tyrosine	1	2	2	2	N.D

[1]Average of duplicate determinations.
[2]Commercially available fructooligosaccharide.
N.D = Not detected.
Tr = Trace amount (<1 wt%).

3.4 DISCUSSION

The water-extractable compounds from plant materials are heterogeneous mixtures of compounds. The extracts obtained from the dry powders of Rengarenga lily, Cabbage tree, *Undaria* and exudate of *Acacia* were water-extractable carbohydrate compounds and part of the weight was likely to be water-extractable proteins and inorganic salts and other compounds such as phenolic compounds. The wide range of WSC (250-794 g/kg DM) is a reflection of the different proportions of structural material in different species.

The water-soluble carbohydrate (WSC) content of the rhizomes of Rengarenga lily was lower than the WSC contents reported for the underground parts of chicory (*Cichorium intybus*) and Jerusalem artichoke (*Helianthus tuberosus*) (van den Ende *et al.*, 1996; Schubert & Feuerle, 1997; Wilson *et al.*, 2004). Perhaps, the lower value of Rengarenga lily in this study was due to the limitations in the carbohydrate analysis as described below. Fructan contents can reach about 20 % of the fresh weight (roughly 80 % of the dry weight) in chicory taproots (Wilson *et al.*, 2004). The fructan composition (mainly long chain oligosaccharides; DP>12) of the lily extract in this study resembles that of the underground parts of chicory (Wilson *et al.*, 2004). In contrast, Monti *et al.* (2005) reported that chicory fructan contains a high proportions of short chain oligosaccharides (DP 2-10) instead of long chain fructans (DP>11) and DP varies widely from 2 to more than 100. These variations could be due to differences in soil conditions, climate, biosynthetic factors, season (harvest time), cultivar and geographical locations (Wilson *et al.*, 2004; Monti *et al.*, 2005). Fructans from the Cabbage tree extract contained greater amounts of monosaccharides (fructose and glucose) and sucrose, making them similar to fructans from adventitious root tubers of the *Caesia calliantha* (Incoll *et al.*, 1989). The amount of WSC extracted from Cabbage tree extract (250 g/kg DM) in this study is comparable to the values reported by previous studies on the same species (211-388 g/kg DM) (Fankhauser & Brasch, 1985). Furthermore, the same authors reported that the polysaccharide precipitated from the water extracts of Cabbage tree shoots is glucofructofuranan and contains more utilisable carbohydrate on a fresh weight basis than either sugar cane or sugar beet (Fankhauser & Brasch, 1985). The analytical data obtained for the sugar composition of *Acacia* extract are similar to those reported previously for *A. pycnantha* exudates (Annison *et al.*, 1995). *Acacaia* extract had very high galactose content (78 %) compared to similar types of arabinogalactans (18-49 % galactose) extracted from Vulgares and Gummiferae series *Acacia* species in Africa (Al-

Assaf *et al.*, 2005b). Although the uronic acid content of *Acacia* extract was not estimated in this study Annison *et al.* (1995) reported that *A. pycnantha* extract contains low level of uronic acid (3 %).

Fucose-containing carbohydrates (fucans) were extracted from *Undaria* seaweed with diluted sulphuric acid (1 % (w/w)) during which alginate was extracted with 1 % (w/v) Na_2CO_3 and discarded during the extraction process. Alginates form an insoluble precipitates at acidic pH (~3.0), but they are stable in solutions between pH 6 and 9. Total recovery corresponded to non-dialyzable compounds, as free minerals and low-molecular-weight substances were removed during exhaustive dialysis of the fractions. Sulphated fucans from brown algae may be classified into three main families: fucoidans (homofucans), fucoglucouronans (ascophyllans and sargassans), and glycuronofuco-glactans sulphate (Kloareg & Quatrano, 1988; Mabeau *et al.*, 1990). Homofucans (or fucoidans) are primarily composed of galactofucan-sulphate oligosaccharides with glucuronic acid and mannuronic acid as minor constituents (Mabeau *et al.*, 1990). Sulphate (and its counter-ions) present in the galactofucan sulphate account for a significant proportion of the weight of the water-soluble carbohydrates in the *Undaria* extract. For the total carbohydrate content of the *Undaria* extract if every sugar residue has a sulphate attached then it could easily double the weight as being due to galactofucan sulphate. This means there is not a lot of weight left for other organic and inorganic compounds to make up the 885 g/kg dry matter.

In general, total carbohydrates determined in the plant extracts may have been underestimated in this study. The different sugars give different responses in the phenol-sulpuric acid total sugar assay. The response of fructose in the phenol-sulphuric acid assay was lower than glucose and therefore, the total sugar estimate in lily extract and Cabbage tree extract was lower than expected. For instance, lily extract gave 680 g/kg carbohydrates using glucose as the standard. According to the Megazyme fructan assay lily extract contained 65 % (w/w) fructans and because of the lower response of fructose in the phenol-sulphuric acid assay, total carbohydrate was underestimated. Out of the 209 g unaccounted for in analysis (938-680-49 = 209 g) (938 = DM, 49 = CP) it would not be surprising if 150 g or more of this was because of underestimation in the carbohydrate assay, and similarly for the other extracts.

The constituent sugar analyses of lily extract and Cabbage tree extract indicated that these extracts contained 34 % (w/w) and 32 % (w/w) of mannose or manitol, respectively. This

observation is because of the procedure for analysis, which involves hydrolyzing the polymers into their individual monosaccharides. As shown in the section 3.3 both these extracts contained large amounts of fructose containing fructans and a small amount of glucose. Since fructose is a ketose sugar it reduces to both types of alditols such as glucitol and mannitol during the reductive hydrolysis process. Thus, the mannose detected in the analyses is almost certainly derived from the fructose released by hydrolysis of fructans and is not due to the presence of mannose sugar in these extracts. Harris and Mann (1994) reported that the fructose content of the *C. australis* stems harvested as an annual field crop is on average 50 % of their dry weight.

It has long been established that the proteinaceous content of complex plant extracts may comprise a mixture of proteins, whose relative proportions vary for different plant species. It seems that proteins are extracted with the water-soluble carbohydrates, possibly due to the presence of water-soluble proteins in the intact materials or may be proteins are present in a carbohydrate-protein complex. For example, it is known that the polysaccharide fraction from *Acacia* tree exudates constitutes 95 % of the dry weight of proteoglycans, which consist of highly-branched galactan polymers, with galactose and/or arabinose side chains and 1-5 % of covalently attached proteins rich in hydroxyproline and serine (Meance, 2004). This observation is typical for most of the type II arabinogalactans in many plant species which possess immunomodulatory properties (Paulsen, 2002; Inngjerdingen *et al.*, 2005). The Cabbage tree extract was shown to contain a large (14 % (w/w)) amount of protein compared to other extracts. This would also suggest that the considerable amount of water-soluble proteins in Cabbage tree shoot powder may solubilise and fractionate with the carbohydrates during the extraction process.

To complete the analysis of the primary structure of carbohydrates, it is important to determine the mean number of constituent sugars or repeating units, that is, their weight average molecular weight (M_w), and the distribution of the molecules (polydispersity). The molecular weight of the *Acacia* extract (3.0×10^4 Da) is lower than the molecular weight of the arabinogalactans extracted from *Acacia senegal* (Al-Assaf *et al.*, 2005a) and similar ($3.0 \times 10^4 - 3.9 \times 10^4$) to bioactive arabinogalactan polysaccharides extracted from *Glinus oppositifolius* (a medicinal plant) in Norway (Inngjerdingen *et al.*, 2005). The weight average molecular weight of *Acacia* extract is one sixteenth that of *A. senegal* reported by Al-Assaf *et al.* (2005a) and low molecular weight means that its ability to become viscous is limited. Exuded arabinogalactan-types from *Acacia* species show a wide range of molecular

weights (Anderson *et al.*, 1984; Anderson *et al.*, 1985). The molecular weight of the Cabbage tree extract was smaller than the lowest level that can be detected by the light-scattering system (SEC-MALLS). However, Brasch *et al.* (1988) reported that the number average molecular weight of the glucofructofuranan extracted from roots of the New Zealand Cabbage-tree is ~3000, which corresponds to a degree of polymerization of 19. The molecular weight of the seaweed fraction (5.11×10^5 Da) was similar to a value (6.8×10^5 Da) previously reported for high-molecular-weight fucans extracted from brown seaweeds (Patankar *et al.*, 1993; Nishino *et al.*, 1994). Differences could be due to the extraction method or to seasonal or geographical variations. The high polydispersity index of *Undaria* extract may indicate that some degradation might have occurred during extraction of galactose-containing fucans from *Undaria* seaweed. Because of the acid hydrolysis of sulphated fucans during the extraction process (*Undaria* seaweed), it may have resulted in sulphated oligosaccharides and their proportions not been estimated in this experiment.

3.5 CONCLUSIONS

It can be concluded that water-soluble carbohydrates extracted from Rengarenga lily and Cabbage tree contain mainly fructans and that their structures are different. It appears that the Cabbage tree extract contains mainly smaller fructans (oligofructans), whereas lily extract contains oligosaccharides with higher degree of polymerization (DP>12). The *Acacia* extract contained around 80 % (w/w) carbohydrate and only 3 % (w/w) protein with a high level of hydroxyproline. The carbohydrates extracted from *Undaria* seaweed were composed mainly of fucose and galactose. Further studies, such as the fractionation of water-soluble carbohydrate compounds using gel-permeation chromatography and glycosyl linkage analysis using gas chromatography-mass spectrometry (GC-MS), are needed to elucidate and confirm the chemical structures in these plant extracts.

CHAPTER 4 FRUCTANS FROM RENGARENGA LILY (*ARTHROPODIUM CIRRATUM*) EXTRACT AND FRUTAFIT AS PREBIOTICS FOR BROILERS: THEIR EFFECTS ON GROWTH PERFORMANCE, DIGESTIVE ORGAN SIZE, GUT MORPHOLOGY AND NUTRIENT DIGESTIBILITY

ABSTRACT

An experiment was conducted to evaluate the effect of dietary water-soluble carbohydrate extract from Rengarenga lily and Frutafit® (both fructans) on the performance, organ weights, ileal digestibility and gut morphology in male Cobb broilers. There were six treatment groups: a negative control with no supplements, a positive control supplemented with 45 ppm Zn-bacitracin, and four test diets each supplemented with Rengarenga lily extract or Frutafit at 5 or 10 g/kg diet. Supplementation with low levels of Rengarenga lily extract and Frutafit in the diet did not affect productive parameters, whereas the inclusion of a high level of Frutafit had a negative effect on BWG and FI compared with birds fed the negative control diet. The addition of an antibiotic to the diet significantly improved ($P < 0.05$) the BWG and FCR of broilers. Apparent ileal digestibility of dry matter, starch, protein and fat was not affected ($P > 0.05$) by supplementation with both levels of lily extract and low level of Frutafit. The apparent ileal digestibility of dry matter, protein and fat was decreased ($P < 0.05$) by the high level of Frutafit. The apparent metabolisable energy (AME) of broilers fed the high level of Frutafit was approximately 0.2 MJ/kg DM lower than that of the negative control group. The addition of Zn-bacitracin increased ($P < 0.05$) the apparent ileal digestibility of fat. The relative weight of the liver was higher ($P < 0.05$) in broilers supplemented with the high level of Frutafit than for negative control birds at 14 and 35 d of age. Feeding Rengarenga lily extract or Frutafit had no effect on the gut morphology of birds on d 14 and 35. It can be concluded that dietary inclusion of fructans from the two sources used in this study affected broiler performance differently and in a dose-dependent manner.

4.1 INTRODUCTION

The use of in-feed AGPs in the poultry industry has been under scrutiny for the past decade due to growing concerns about the development of microbial resistance and the potential harmful effects on human health. Hence, at present many countries are searching for suitable alternative(s) to AGPs. Prebiotics are feed ingredients that can potentially affect the host's nutrition and health by selectively stimulating the activity of a number of beneficial gut microflora (Gibson & Roberfroid, 1995). Prebiotics may also affect mucosal morphometry, probably because of their fermentation properties, which may strengthen mucosal protection and reduce the risk of gastrointestinal diseases (Kleessen *et al.*, 2003b; Koutsos & Arias, 2006). However, information about how prebiotic and bioactive compounds affect gut morphology, digestibility of nutrients and performance of broilers is far from complete.

Potentially, there are hundreds of different prebiotics which are naturally available or can be produced from plant parts. Inulins and oligofructans are linear β-2→1 fructans, widely distributed in nature as plant storage carbohydrates. Fructans are extracted primarily from chicory roots (*Cichorium intybus*) and Jerusalem artichoke (*Helianthus tuberosus*) tubers (Roberfroid, 2005). *Arthropodium cirratum* (Rengarenga lily) is a small perennial herbaceous plant commonly available in Australia and New Zealand, which has starchy edible rhizomes rich in fructans as storage carbohydrate (Harris, 1996). Rengarenga lily rhizomes contain about 65 % fructans. The early settlers in Australia and New Zealand used the tubers of Rengarenga lily as a food, as well as using them as a herbal medicine to treat boils and abscesses in humans (Harris, 1996; Cambie & Ferguson, 2003). The nutritional value of fructans in Rengarenga lily has not been studied in poultry.

The objective of the present study was to evaluate the effect of a water-soluble carbohydrate extract from Arthropodium cirratum (Rengarenga lily) and a commercially available prebiotic product Frutafit® on growth performance, ileal digestibility, organ development, and gut morphology of broiler chickens.

4.2 MATERIALS AND METHODS

4.2.1 Bird husbandry

Two hundred and eighty-eight (288) day-old male broilers (Cobb) vaccinated against Marek's disease, infectious bronchitis, and Newcastle disease were obtained from a local

hatchery (Baiada hatchery, Kootingal, NSW, Australia). At 1 d of age, chicks were randomly placed in 36 brooder cages (42 cm x 75 cm x 25 cm dimension) with wire floor and with a floor space of 0.32 m^2/cage. The cages were randomly assigned to one of six dietary treatments with six replicates per treatment. Each cage housed eight chicks. At 21 d of age, chickens from each replicate were placed into metabolic cages (60 cm x 45 cm x 38 cm dimension) with a 1 x 1-cm wire mesh bottom and housed in climate-controlled rooms. The temperature was set at 33-34°C during the first week and gradually decreased by 3°C per week until 24-25°C was reached by the third week. Relative humidity was between 65 and 70%. A photoperiod of 24 h from 1 to 21 d of age, and 18 h from 22 to 42 d of age was maintained. Each pen was equipped with a feeding and water trough placed outside and also an excreta collection tray. Water and feed were provided *ad libitum*. Weekly BWG and FI per cage were measured and FCR, adjusted for mortality, calculated on a cage basis. Birds were observed twice daily for general health. The experiment was approved by the Animal Ethics Committee of the University of New England (Approval No.: AEC04/111).

4.2.2 Experimental diets

The composition of the basal diets is shown in Table 4.1. The diets were formulated to meet the requirements set by the National Research Council (1994) for broilers using the "PRO-4 Standard Edition" software package (Version 1, Agri-Data Systems, Inc., Annapolis, Maryland, USA). Celite™(Food Chemicals Codex grade, Celite Corp., Lompar, CA, USA), a source of acid-insoluble ash, was added (5 g/kg) to finisher diets as an indigestible marker. The grains in the experimental diets were hammer-milled using a 5-mm screen, and all diets were mixed and cold-pelleted at the University of New England. Treatments were as follows: 1) basal diet with no additives (negative control); 2) basal diet with Zinc-bacitracin (45 mg/kg) (positive control); 3) basal diet with Rengarenga lily rhizome extract (5 g/kg); 4) basal diet with Rengarenga lily rhizome extract (10 g/kg); 5) basal diet with Frutafit (5 g/kg) and 6) basal diet with Frutafit (10 g/kg). Additions of Frutafit®-HD, consisting of fructooligosaccharide (FOS) (Sensus, Roosendaal, The Netherlands), and Rengarenga lily rhizome extracts consisting of 650 g fructans/kg were made in place of sorghum. The extraction of fructans from Rengarenga lily rhizomes is described in Section 3.2.1.2.

Table 4.1 Composition (g/kg as received) of experimental diets

Ingredient (g/kg)	Starter (d1-d21)	Finisher (d22-d35)
Sorghum	480	547
Corn	133	120
Soybean meal (48% CP)	274	230
Meat meal (50% CP)	80	56
Sunflower oil	7.5	10
Limestone (38% Ca)	5.0	5.8
Dicalcium phosphate	3.5	6.9
Lysine-HCl	1.2	1.7
DL-methionine	3.6	3.9
Salt	0.3	0.9
Sodium bicarbonate	5.0	5.0
Choline chloride	5.0	4.9
L-threonine	0.6	0.9
Celite	-	5.0
Premix[1]	2.0	2.0
Nutrient composition		
Metabolisable energy (MJ/kg)	12.3	13.1
Crude protein (g/kg)	230	205
Crude fat (g/kg)	39	46
Lysine (g/kg)	12	11
Methionine (g/kg)	7	7
Available phosphorous (g/kg)	5	4
Calcium (g/kg)	10	11

[1]Vitamin mineral premix (Ridley Agriproducts Pte Ltd., Tamworth, NSW) contained the following minerals in milligrams per kilogram of diet: Mn, 80; Zn, 60; Fe, 60; Cu, 8; I, 1.2; Co, 0.3; Se, 0.1; Mo, 1.0 and the following vitamins per kilogram of diet: Vitamin A, 12,000 IU from all *trans*-retinyl acetate; cholecalciferol D_3, 3.500 IU; vitamin E, 44.7 IU from DL-α-tocopherol; vitamin B_{12}, 12.75 μg; riboflavin, 6.0 mg; niacin, 50 mg; pantothenic acid, 12 mg; folic acid, 2 mg; biotin, 0.1 mg; thiamine, 2 mg; vitamin K, 2 mg and pyridoxine, 5 mg.

Frutafit®-HD contains mainly inulin (920 g/kg) (fructooligosaccharides) extracted from chicory root and some fructose and glucose (40 g/kg) and sucrose (40 g/kg). The average chain length of the inulin in Frutafit is about 9-11 and the content of fructooligosaccharides (with DP below 10) is about 25-30% with about 2-3% DP3 and DP4, and 3-4% of DP5.

4.2.3 Collection and processing of samples

Birds were killed for sampling on d 14 and 35. On d 35, three chickens were selected at random from each replicate and euthanized by cervical dislocation. To synchronise the feeding pattern of the birds, light was switched off for 2 h, followed by at least 1 h light before the chickens were sacrificed. Subsequently, the abdominal cavity was opened and the small intestine was ligated and removed. The contents of the ileum were collected into plastic containers. The ileal digesta samples were frozen immediately after collection, subsequently lyophilized (Martin Christ Gerfriertrocknungsanlagen, GmbH, Osterode am Harz, Germany), ground to pass through a 0.5 mm sieve (Cyclotec 1093 sample mill, Tecator, Höganes, Sweden), and stored at -20°C in airtight containers until chemical analyses were conducted. On d 14 and 35, approximately 2.5 cm of the middle portion of the ileum was excised from one bird per replicate, flushed with PBS buffer (pH 7.6) and fixed in 10 % (v/v) neutral buffered formalin for histomorphological analysis.

4.2.4 Organ weights

The weights of the proventriculus, gizzard, and small intestine without content, pancreas, bursa of Fabricius, caeca, spleen and liver without gall bladder were recorded on d 14 and d 35. The duodenum is the region from the outlet of the gizzard to the distal attachment of the pancreas, the jejunum, distally from the end of the pancreatic loop to Meckel's diverticulum, the ileum distally from Meckel's diverticulum to 1 cm above the ileo-caecal junction. The length and weight of each segment were recorded, as was the body weight of the bird they were excised from. The 'gut mass index' was subsequently calculated as an indication of mass per unit weight of body or length of the GIT region.

4.2.5 Apparent metabolisable energy (AME) bioassay

Apparent metabolisable energy (AME) evaluation was conducted over a period of 4 days during the fifth week of the experiment (36 to 39 d post-hatch). Clean excreta trays were

placed under each AME cage, droppings were collected daily, dried at 80°C to a constant weight in a forced-drought oven and collections from each pen were pooled for analysis. Care was taken to avoid contamination with feed, feathers, scales and debris. The moisture content of the excreta voided was measured. Diet and excreta were ground to pass through a 0.5 mm screen using a Cyclotec sample mill. Gross energy contents of diets and excreta were determined using an IKA bomb calorimeter system, C7000 with Cooler C7002 (IKA®-Werke GmbH & Co, Staufen, Germany) standardized with benzoic acid. Apparent metabolisable energy of diets was calculated using the equation below and values were corrected for zero nitrogen retention using a value of 34.4 MJ per g nitrogen retained (Hill & Anderson, 1958).

$$\text{AME diet (MJ/kg DM)} = GE_{diet} - [GE_{excreta} \times (AIA_{diet}/AIA_{excreta})]$$

where, GE_{diet} = gross energy content in diets

$GE_{excreta}$ = gross energy content in excreta

AIA_{diet} = Acid-insoluble ash content in diets

$AIA_{excreta}$ = Acid-insoluble ash content in excreta or ileal digesta.

4.2.6 Digestibility of nutrients

Apparent ileal digestibilities of protein, fat, starch and DM and the AME as a proportion of the gross energy of feed were estimated from the analyses of feeds, freeze-dried ileal digesta and excreta; an indigestible acid-insoluble marker was used to calculate digestibilities. Diets and ileal digesta were analysed for DM, protein, fat and starch as described below. The apparent ileal digestibility of protein, fat, starch and DM were calculated using the following formula. All values are expressed on a DM basis.

$$\text{Apparent nutrient digestibility} = \frac{(\text{Nutrient}/AIA)_{diet} - (\text{Nutrient}/AIA)_{ileum}}{(\text{Nutrient}/AIA)_{diet}}$$

where, $(\text{Nutrient}/AIA)_{diet}$ = ratio of nutrient and acid insoluble ash in diet, and

$(\text{Nutrient}/AIA)_{ileum}$ = ratio of nutrient and acid insoluble ash in ileal digesta.

4.2.7 Acid-insoluble ash

The concentration of acid-insoluble ash in the feed, freeze-dried ileal digesta and excreta was determined after ashing the samples and treating the ash with boiling 4M HCl, following the method described by Vogtmann *et al.* (1975) and Choct and Annison (1990). Samples (diet, 3 g; ileal digesta, 1 g) were weighed accurately into Pyrex® brand Gooch-type crucibles (porosity 4 μm) and dried (overnight, 105°C) in a forced-air convection oven (Qualtex Universal Series 2000, Watson Victor Ltd., Perth, Australia). After cooling and weighing, the samples were ashed (480°C, overnight) in a Carbolite CWF 1200 chamber furnace (Carbolite, Sheffield, UK). The crucibles were placed in a boiling 4M HCl acid bath so that the samples were wetted from underneath (just covered the ash). The samples were gently boiled twice in 4M HCl for 15 min and the acid was removed through suction, the residues (AIA) were washed with distilled water, and the crucibles were dried (105°C, overnight) and weighed. The acid insoluble ash content was calculated using the following equation:

$$\text{AIA (g/kg dry matter)} = \frac{\text{(Crucible + Ash weight) – (Crucible weight)}}{\text{(Crucible + Dry sample weight) – (Crucible weight)}} \times 1000$$

4.2.8 Dry matter content

The dry matter content was determined gravimetrically according to the Association of Official Analytical Chemists Official Method 934.01 (AOAC, 2002) as described in Section 3.2.2.1.

4.2.9 Total starch content

The total starch content of the diets and ileal digesta was determined using the Megazyme Total Starch Assay Kit (Megazyme Australia Pty. Ltd., Warriewood, NSW, Australia) based on the method developed by McCleary *et al.* (1994). Representative duplicate samples (20 mg of feed and 100 mg freeze-dried ileal digesta) were weighed into screw-capped reaction tubes (25 mL). To aid dispersion, 0.2 mL of 80 % (v/v) ethanol was stirred into the samples with a vortex mixer and 2.0 mL of dimethyl sulphoxide was added into each tube. Uncapped tubes were placed in a preheated stirring block at 100°C for 10 min. Three millilitres (3 mL) of thermostable α-amylase (*EC* 3.2.1.1, Megazyme Australia Pty. Ltd., Warriewood, NSW, Australia) in 50 mM MOPS buffer (3-[*N*-morpholino]propanesulfonic acid, pH 7.0, Sigma-Aldrich Co., St. Louis, MO, USA) were added and the capped tubes were incubated in a

preheated stirring block at 100°C for 30 min. The tubes were then placed in a 50°C water bath and 4 mL of 200 mM sodium acetate buffer (pH 4.5) was added, followed by 0.1 mL of amyloglucosidase (*EC* 3.2.1.3, Megazyme Australia Pty. Ltd., Warriewood, NSW, Australia). The tubes were stirred in a vortex mixer (every 15 min) and incubated for 1 h in water bath at 50°C. The tubes were weighed and reaction mixtures were centrifuged (3000 x *g* for 10 min). An aliquot (0.1 mL) of the supernatant was mixed with 3 mL of Glucose Determination Reagent (GOPOD), and incubated at 50°C for 20 min (glucose oxidase assay). The blank (0.1 mL of distilled water and 3 mL of GOPOD solution) and glucose standard were incubated concurrently using the following standards, 0.0; 0.05; 0.075; and 0.10 mg /mL. The absorbance was measured at 510 nm and 1 cm light path length using a Hitachi 150-20 spectrophotometer attached with Hitachi 150-20 Data Processor (Hitachi Science Systems Ltd., Ibaraki, Japan). When calculating starch digestibility it was assumed that the free glucose in ileal digesta derives from starch.

4.2.10 Crude Protein

Diets and ileal digesta were analysed for protein by the method of Sweeney (1989) using a LECO® FP-2000 automatic nitrogen analyser (Leco Corp., St. Joseph, MI, USA) as described in Section 3.2.2.2.

4.2.11 Crude Fat

The crude fat content of the ileal digesta samples was determined gravimetrically by the Soxhlet extraction procedure using Association of the Official Analytical Chemists Official Method 920.39 (AOAC, 2002). The fat content of the feed was determined gravimetrically using the modified Weibull acid hydrolysis method (4N HCl, 1 h, 100°C) as described by Wiseman *et al.* (1992). The feed samples (3 g) were weighed into paper thimbles, boiled for 1 h in 50 mL of 4M HCl solution and filtered through No. 541 Whatman paper following cooling and addition of 150 mL of distilled water. The residues were washed until acid-free, dried at 70°C overnight and then extracted with chloroform (overnight) in a Soxhlet extraction unit.

4.2.12 Gut histomorphology

Formalin-fixed tissue slices from the ileum, each 5 to 6 mm thick, were enclosed in tissue cassettes (Bayer Diagnostics Australia Pty Ltd., Pymble, NSW, Australia). The tissues were processed over 19 h in an automatic tissue processor (TOSCO, Thomas Optical & Scientific Co., Melbourne, Australia), and embedded in paraffin using a Histo Embedding Centre (Leica EG 1160, Leica Microsystems, Bensheim, Germany). Processing consisted of serial dehydration with ethanol, clearing with xylol and impregnation with paraplast (wax). Embedded samples were subsequently sectioned at a thickness of 5 μm with a Rotary Microtome (Leitz 1516, Leica Microsystems, Bensheim, Germany). The tissue sections on the slides were stained using Harris's hematoxylin (Gerorge Gurr Ltd., London, UK), and eosin (Gur Certistain, VWR International Ltd., Poole, UK), and mounted with DPX mountant (Distrene Polystyrene Xylene), for histology (BDH Laboratory Supplies, Poole, UK). Slides were viewed on a Leica DM LB microscope (Leica Microscope GmbH, Wetzlar, Germany) and morphometric indices were determined using computer-aided light microscope image processing analysis system (SPOT 3.1, Diagnostic Instruments, Inc., Sterlin Heigths, MI, USA) as described by Iji *et al.* (2001a). Villus height (tip of the villi to the villus crypt junction) and height from the villus tip to the muscularis mucosae (villus height + crypt depth) were measured in 10 vertically, well-oriented, intact villi and crypts. Crypt depth (depth of the invagination between adjacent villi) was then calculated as villus height plus crypt depth minus villus height. All measurements were calibrated using photomicrographs of a stage micrometer recorded at 5X magnification.

4.2.13 Statistical analysis

Each variable was analysed as a completely randomized design with a cage of broilers composing an experimental unit. Percentage data were arcsine transformed prior to analysis and data from all the response variables were analysed according to the General Linear Models procedure (GLM) for ANOVA (SAS Institute Inc., 2000). Variables having a significant F test were compared using Duncan's Multiple Range Test. Significant levels of $P \leq 0.05$ were considered significant, unless otherwise stated.

4.3 RESULTS

4.3.1 Bird performance

The growth performance of broilers is shown in Table 4.2. Mean BWG of the Rengarenga lily extract (10 g/kg) fed group during the first three-week period was similar to that of the negative control group and that of birds fed the antibiotic during the same period. Mean BWG in the group supplemented with high level of Frutafit was lower ($P < 0.05$) than that of the negative control group throughout the experimental period. The overall FI (1 to 42 d) and FI during the first three weeks (1 to 21 d) were lower ($P < 0.05$) in the same treatment group. Feed intake of all other treatment groups was not different from the negative control group. Feed efficiency was not affected by dietary supplementation with lily extract or Frutafit. Addition of 45 mg/kg Zn-bacitracin resulted in significant ($P < 0.05$) improvements in BWG and FCR of birds compared to those fed the negative control.

4.3.2 Organ weights

The relative weights of the major visceral organs and the relative lengths of the different sections of the intestinal tract are shown in Table 4.3 and Table 4.4. Dietary supplementation with Rengarenga lily extract or Frutafit had no effect on the relative weights of the proventriculus, gizzard or immune organs (bursa and spleen) on d 14 and 35. In birds fed Frutafit at high dosage, the relative weight of the liver was higher than that of the negative control group at d 14 and 35. In the same treatment group the relative weight of the pancreas was greater ($P < 0.05$) than that of positive control, but not significantly different from the negative control at d 14. The relative weight of the caeca was greater ($P < 0.05$) in birds fed the high level of Frutafit compared to both negative- and positive control groups at d 35. The relative weight of the jejunum of birds fed with Zn-bacitracin was lower than that of the negative control group on both sampling days (Table 4.4). Relative weight and length of duodenum were not altered by dietary supplements in either sampling period. Birds given antibiotics had numerically shorter relative length of jejunum and ileum at d 14 and 35.

Table 4.2 Mean body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) of broiler chickens fed diets supplemented with the Rengarenga lily extract and Frutafit[1]

Parameter	Period	Negative Control	Positive Control	Rengarenga lily extract		Frutafit		S.E.M.	Probability
				5 g/kg	10 g/kg	5 g/kg	10 g/kg		
BWG (g/bird)	1-21 d	823^{b}	853^{a}	832^{b}	838^{ab}	828^{b}	781^{c}	17.6	***
	22-42 d	1851^{b}	1926^{a}	1852^{b}	1858^{b}	1853^{b}	1831^{c}	15.7	***
	1-42 d	2668^{b}	2779^{a}	2678^{b}	2681^{b}	2664^{b}	2613^{c}	23.5	***
FI (g/bird)	1-21 d	1392^{a}	1407^{a}	1399^{a}	1407^{a}	1405^{a}	1336^{b}	15.2	***
	22-42 d	3716	3722	3721	3716	3713	3680	39.8	NS
	1-42 d	5116^{a}	5129^{a}	5120^{a}	5121^{a}	5096^{a}	5048^{b}	45.5	*
FCR	1-21 d	1.69^{ab}	1.65^{b}	1.68^{ab}	1.68^{ab}	1.70^{a}	1.71^{a}	0.04	*
	22-42 d	2.01^{a}	1.93^{b}	2.01^{a}	2.00^{a}	2.00^{a}	2.01^{a}	0.03	***
	1-42 d	1.92^{a}	1.85^{b}	1.91^{a}	1.91^{a}	1.91^{a}	1.93^{a}	0.02	***

[1]Least square means and pooled standard error of the mean (S.E.M.), $n = 6$.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, results not sharing the same superscripts within a row are significantly different ($P < 0.05$).

Table 4.3 Effect of Rengarenga lily extract and Frutafit on relative weight of organs (% body weight) of broilers at 14 and 35 days of age[1]

Treatment	Proventriculus		Gizzard		Liver		Pancreas		Spleen		Bursa		Caeca	
	14 d	35 d	14 d	35 d	14 d	35 d	14 d	35 d	14 d	35 d	14 d	35 d	14 d	35 d
	g/100 g body weight													
Negative control	0.82	0.46	2.53	1.20	3.90^{b}	2.56^{b}	0.44^{ab}	0.19	0.09	0.11	0.20	0.21	0.81	0.50^{b}
Positive control	0.82	0.37	2.57	1.26	3.92^{b}	2.48^{b}	0.38^{b}	0.20	0.11	0.11	0.17	0.23	0.78	0.48^{b}
Lily extract (5 g/kg)	0.76	0.41	2.37	1.27	4.10^{b}	2.63^{ab}	0.42^{ab}	0.21	0.11	0.12	0.19	0.21	0.64	0.51^{b}
Lily extract (10 g/kg)	0.73	0.45	2.51	1.09	4.07^{b}	2.51^{b}	0.43^{ab}	0.22	0.10	0.11	0.20	0.22	1.06	0.61^{ab}
Frutafit (5 g/kg)	0.82	0.46	2.40	1.20	3.84^{b}	2.66^{ab}	0.40^{ab}	0.21	0.11	0.11	0.18	0.26	0.67	0.56^{ab}
Frutafit (10 g/kg)	0.65	0.40	2.53	1.17	4.77^{a}	2.95^{a}	0.52^{a}	0.20	0.10	0.12	0.22	0.21	0.81	0.66^{a}
S.E.M.	0.16	0.14	0.51	0.26	0.53	0.31	0.11	0.05	0.03	0.03	0.07	0.05	0.41	0.12
Probability	NS	NS	NS	NS	**	*	*	NS	NS	NS	NS	NS	NS	*

[1]Least square means and pooled standard error of the mean (S.E.M.), $n = 6$.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, results not sharing the same superscripts within a column are significantly different ($P < 0.05$).

Table 4.4 Effect of Rengarenga lily extract and Frutafit on relative weight and length of the intestinal tract of broilers at 14 and 35 days of age[1]

Treatment	Relative weight (g/100 g body weight)						Relative length (cm/100 g body weight)					
	Duodenum		Jejunum		Ileum		Duodenum		Jejunum		Ileum	
	14 d	35 d	14 d	35 d	14 d	35 d	14 d	35 d	14 d	35 d	14 d	35 d
Negative control	1.72	0.71	2.62^{a}	1.13^{a}	1.58	0.90	6.44	1.59	14.26	3.36	13.60	3.46
Positive control	1.77	0.69	2.08^{b}	1.00^{b}	1.51	0.79	6.32	1.58	11.58	3.03	12.60	3.38
Lily extract (5 g/kg)	1.95	0.70	2.68^{a}	1.12^{a}	1.61	0.87	6.98	1.59	15.01	3.23	13.27	3.53
Lily extract (10 g/kg)	1.56	0.69	2.50^{ab}	1.11^{ab}	1.88	0.87	6.04	1.59	14.10	3.40	14.57	3.71
Frutafit (5 g/kg)	1.82	0.72	2.59^{a}	1.15^{a}	1.55	0.91	6.98	1.59	15.13	3.22	14.44	3.64
Frutafit (10 g/kg)	2.03	0.78	2.94^{a}	1.20^{a}	1.88	0.95	7.01	1.61	14.56	3.37	14.73	3.67
S.E.M.	0.50	0.10	0.43	0.11	0.44	0.15	2.09	0.20	3.99	0.44	5.10	0.31
Probability	NS	NS	**	*	NS	NS	NS	NS	NS	NS	NS	NS

[1]Least square means and pooled standard error of the mean (S.E.M.), $n = 6$.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, results not sharing the same superscripts within a column are significantly different ($P < 0.05$).

4.3.3 Ileal digestibility of nutrients

The apparent ileal digestibility of nutrients is shown in Table 4.5. Rengarenga lily extract supplementation at both levels had no effect on apparent ileal digestibility of nutrients. The apparent ileal digestibility of dry matter, protein and fat was decreased ($P < 0.05$) in birds fed Frutafit at the high level. The apparent ileal digestibility of fat was higher ($P < 0.05$) in the antibiotic fed group compared with the other treatment groups. High level of Frutafit decreased ($P < 0.05$) AME of birds between d 36 to 39, while the other dietary treatments showed no difference in AME compared to the negative control group (Figure 4.1).

Table 4.5 Apparent ileal digestibility of major nutrients in broilers fed the experimental diets[1]

Treatment	Apparent Ileal Digestibility			
	%			
	Dry matter	Starch	Protein	Fat
Negative control	72.59[a]	93.57	77.21[a]	74.32[b]
Positive control	72.62[a]	93.81	77.19[a]	75.29[a]
Lily extract (5 g/kg)	72.45[a]	93.71	77.27[a]	74.34[b]
Lily extract (10 g/kg)	72.44[a]	93.73	77.26[a]	74.32[b]
Frutafit (5 g/kg)	72.56[a]	93.71	77.27[a]	74.50[b]
Frutafit (10 g/kg)	71.02[b]	93.48	76.04[b]	70.76[c]
S.E.M.	0.73	0.32	0.60	0.73
Probability	***	NS	**	***

[1]Least square means and pooled standard error of the mean (S.E.M.), $n = 6$.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, results not sharing the same superscripts within a column are significantly different ($P < 0.05$).

4.3.4 Gut histomorphology

The effects of dietary treatments on the ileal morphological parameters are shown in Figures 4.2 and 4.3. There was no effect ($P > 0.05$) of any treatment on the villus height on either sampling day. Also, neither lily extract nor Frutafit had any significant ($P > 0.05$) effect on the crypt depth and villus height to crypt depth ratio, while Zn-bacitracin numerically decreased ($P < 0.10$) the crypt depth on d 35.

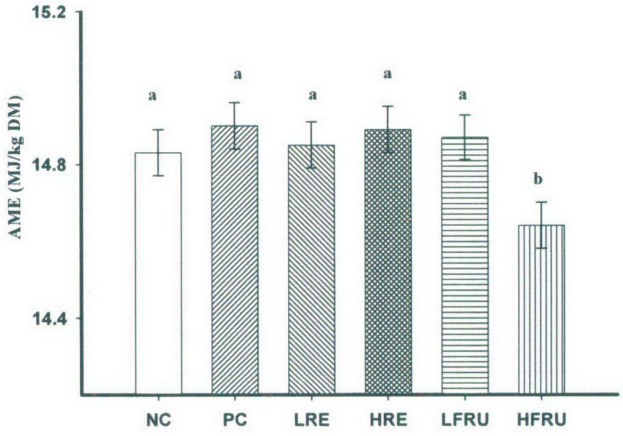


Figure 4.1 Effects of Rengarenga lily extract and Frutafit on AME of birds between 36 to 39 days of age

Negative control (NC), positive control (PC), low level of Rengarenga lily extract (LRE), high level of Rengarenga lily extract (HRE), low level of Frutafit (LFRU) and high level of Frutafit (HFRU). (Mean values, $n = 6$; error bars indicate pooled S.E.M.; bars with different superscripts are significantly different).

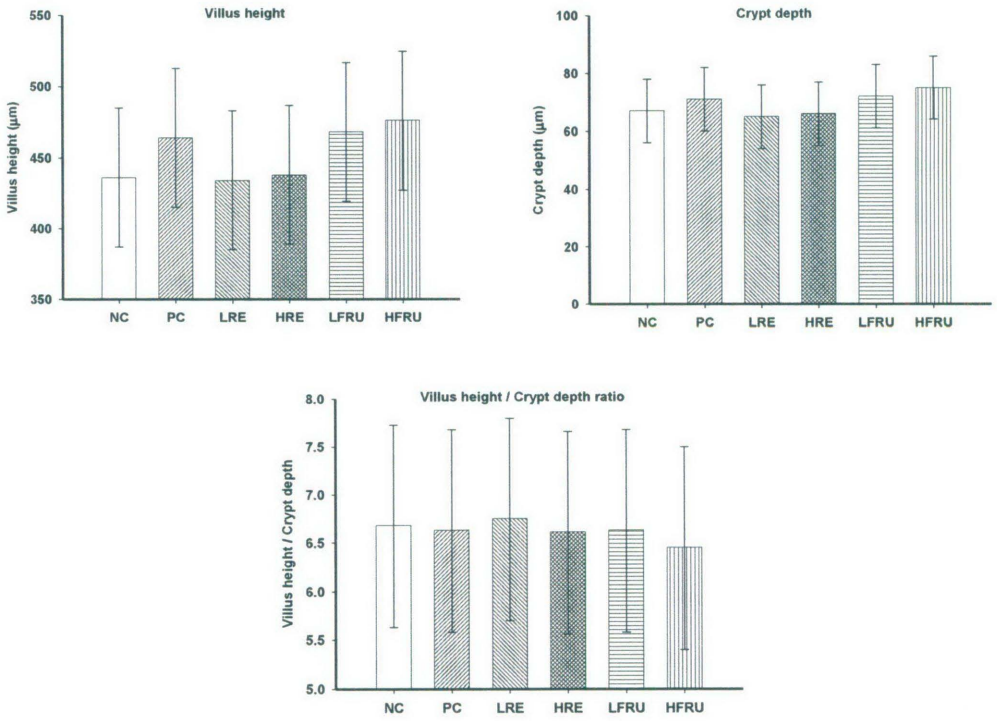


Figure 4.2 Effects of Rengarenga lily extract and Frutafit on the morphometric parameters of ileum at 14 days of age

Negative control (NC), positive control (PC), low level of Rengarenga lily extract (LRE), high level of Rengarenga lily extract (HRE), low level of Frutafit (LFRU) and high level of Frutafit (HFRU). (Mean values, $n = 6$; error bars indicate pooled S.E.M.).

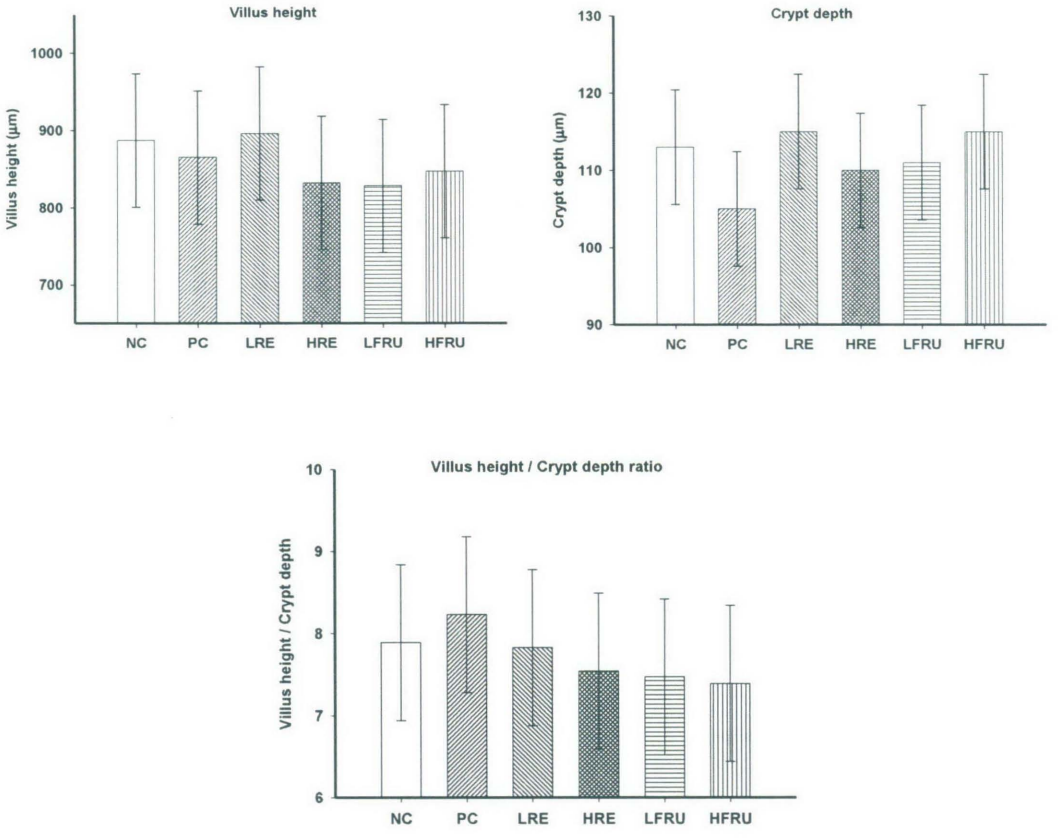


Figure 4.3 Effects of Rengarenga lily extract and Frutafit on the morphometric parameters of ileum at 35 days of age

Negative control (NC), positive control (PC), low level of Rengarenga lily extract (LRE), high level of Rengarenga lily extract (HRE), low level of Frutafit (LFRU) and high level of Frutafit (HFRU). (Mean values, $n = 6$; error bars indicate pooled S.E.M.).

4.4 DISCUSSION

4.4.1 Bird performance

Dietary supplementation with high level of Rengarenga lily extract resulted in numerically higher BWG compared to the negative control group and produced BWG equal to that of birds fed on antibiotic-containing diet during the first three 3-week period. This is in line with results by Yusrizal & Chen (2003a) who observed a significant improvement in BWG and FCR in female broilers when they were fed diets supplemented with chicory fructans (10 g/kg). In the current study, the high level of Frutafit, but not the low level, significantly depressed the weight gain of the birds. Similar results have been shown in chickens fed diets

supplemented with high levels of fructans (Wu *et al.*, 1999; Chen *et al.*, 2005). The reduction in BWG in the group supplemented with the higher dosage of Frutafit was due primarily to a reduction in FI. Possibly, digestion of large amounts of fructans is hindered in these birds, leading to an accumulation of indigestible material in the intestine, which then is degraded by microbes resulting in flatulence, a feeling of satiety, and thus reduction in FI (Iji & Tivey, 1998). As observed in the present study, Waldroup *et al.* (1993) reported that the inclusion of a low level of fructans (4 g/kg) in broiler diets did not have any positive effect on production parameters. The findings of this study agree with other observations that dietary Zn-bacitracin significantly improves the performance of broilers (Stutz & Lawton, 1984; Stanley *et al.*, 2004; Ravindran *et al.*, 2006).

4.4.2 Organ weights

The relative weights of the major digestive organs and immune organs were not affected by supplementation with Rengarenga lily extract or the lower level of Frutafit. The significant enlargement of the liver and pancreas of birds fed on the higher level of Frutafit may be attributed, to some extent, to the presence of a high concentration of indigestible oligosaccharides in the GIT. Perhaps the large amount of oligosaccharides in Frutafit may have acted as an anti-nutritional factor, inducing hypertrophy and hyperplasia in these organs. Similar results were observed by Lee *et al.* (2003), who found that addition of a plant extract containing the essential oil thymol to broiler diets caused an enlargement of the digestive organs such as the liver and pancreas. An accumulation of undigested oligosaccharides may also have caused an increase in the relative weight of the caeca in the high level of Frutafit fed group. In line with this, Jozefiak *et al.* (2004) reported that high dietary fibre components affected the fermentation in poultry caeca leading to caecal hypertrophy. A significant decrease in the relative weight of jejunum and numerically shorter relative lengths of jejunum and ileum following antibiotic supplementation are presumably related to a reduction in microbial populations and more efficient utilisation of nutrients. A similar effect of antibiotics on intestinal weight of broilers has been confirmed by earlier investigators (Stutz & Lawton, 1984; Dafwang *et al.*, 1985; Sarica *et al.*, 2005). By reducing populations of pathogenic bacteria in the intestine, antibiotics prevent the accumulation of lymphocytes in the epithelial layer and the underlying lamina propria, which, in turn, prevents the thickening of muscularis in intestine (Coates, 1980; Gunal *et al.*, 2006). Reduction in intestinal weight by thinning the intestinal mucosa may also enhance

nutrient absorption by the host (Visek, 1978; Henry *et al.*, 1987). The improved fat digestibility in birds fed with Zn-bacitracin in the current study also supports this hypothesis.

4.4.3 Ileal digestibility of nutrients

The apparent ileal digestibility of dry matter, fat, protein and AME was lowered in broilers fed with the high level of Frutafit. A high concentration of fructans in the GIT of broilers may lead to an increase in microbial fermentation in the lower GIT that could adversely affect the utilization and absorption of nutrients, such as protein and fat. Because of the lack of the enzyme galactosidase in the small intestine of birds, it can be assumed that break down of fructans in the distal intestine occurs by bacterial fermentation. The observed increase in the population of lactobacilli in the small intestine of broilers fed with prebiotic plant extracts and commercial prebiotic compounds supports this (Chapters 5 and 7). There is evidence that a low fat digestibility in broilers fed large amounts of soluble polysaccharides is due to bacterial overgrowth in the small intestine and reduction in the concentration of bile acids for the emulsification of fatty acids due to bacterial bile acid deconjugation (Danicke *et al.*, 1997). Apart from that, Chen *et al.* (2005) observed that pancreatic lipase activity was reduced in chickens fed with a high level (10 g/kg) of fructans and suggested that it could lead to lower lipid digestibility. Therefore, it is likely that similar effects may have caused the reduction (3.5 %) in apparent ileal digestibility of fat in birds fed the high level of Frutafit compared to the negative control group in the current study. It is likely that the increase in undigested fat in birds fed the high level of Frutafit caused the increase in the gross energy of the excreta and thereby reduced the AME. Similar to the findings in the present study, Leske *et al.* (1993) found that adding high levels of oligosaccharides to poultry diets reduced the true metabolisable energy in a dose-dependent manner. Furthermore, Coon *et al.* (1990) suggested that rapid intestinal transit of digesta caused by an increase in acidity resulting from the microbial fermentation of oligosaccharides, could cause reduction in metabolisable energy in chickens.

The reduction in the ileal digestibility of protein in birds fed the high level of Frutafit indicates that more protein was recovered in the ileal digesta, which in turn may be due to an increase in bacterial cell synthesis. Ravindran *et al.* (1999) suggested that the fermentation of carbohydrate components in the hindgut is likely to be responsible for the net microbial protein synthesis in broilers. The high concentration of fructans may have also increased the

rate of passage of digesta in the upper small intestine; this may prevent proteolytic enzymes from acting fully on proteins present in digesta.

Therefore, the reduction in performance of birds fed the high level of Frutafit may also be explained by the decrease in ileal nutrient digestibility, and AME, apart from the low FI observed with the same group.

The reason that the high level of Rengarenga lily extract had no effect on ileal digestibility of dry matter, fat and protein in this study may be because the concentration of fructans in lily extract is low (650 *vs.* 920 g/kg) compared to Frutafit. The low concentrations of fructans may slow down the microbial enzyme reactions because of the limitation of substrate. The other possibility is that the difference in average DP in fructans from two different sources can elicit varying responses. The average DP of fructans present in Frutafit is 9-11, whereas in Rengarenga lily extract it is >12 (Section 3.3). By measuring fructans disappearance, Roberfroid *et al.* (1998) indicated that the long-chain fructans fermented at least twice as slowly as their short-chain counterparts.

In the current study, apparent ileal digestibility of starch was not affected by dietary supplementation with fructans. Similarly, Trevino *et al.* (1990) found that dietary supplementation with oligosaccharides up to 56 g/kg in broiler diets did not affect the apparent ileal digestibility of starch. The results also agree with the findings of Twomey *et al.* (2003), who did not find any change in apparent starch digestibility in dogs fed with fructooligosaccharides.

The improvement in apparent ileal digestibility of fat in the antibiotic fed group in this study agrees with results reported by Knarreborg *et al.* (2004) and Chae *et al.* (2006). Knarreborg *et al.* (2004) found that ileal lipase activity and absorption of total and individual fatty acids were increased in broilers fed a diet supplemented with antibiotics. Furthermore, these authors reported that a decrease in deconjugation of bile salts which accompanied a decrease in the number of *C. perfringens* in the ileum is responsible for an improvement in lipid digestion in broilers fed with antibiotics. The same could apply to the current study, and therefore the improved performance observed in the antibiotic fed group could also be partly explained by the increased apparent ileal digestibility of fat.

4.4.4 Gut histomorphology

It has been observed that fructans in the diet can cause alterations in the intestinal morphology of farm animals (Pierce *et al.*, 2006). In the current study, neither the Rengarenga lily extract nor the Frutafit at either dose had any effect on the gut morphology of the ileum. Due to the large within-treatment variability it was difficult to observe significant differences between treatments in any of the estimated parameters. In contrast to the findings from the current study, Xu *et al.* (2003) observed that addition of a low level of fructooligosaccharides to broiler diets resulted in an increase in villus height and villus height to crypt depth ratio, and a decrease in crypt depth in the ileum. The antibiotic-fed birds tended to have shallower crypts compared to the negative control group at d 35. This is in agreement with results from Chapters 5 and 7.

4.5 CONCLUSIONS

Rengarenga lily extract at high level (10 g/kg) tended to increase BWG compared to the negative control group. Increasing the level of Frutafit in the diet from 5 g to 10 g/kg adversely affected the performance of broilers. The ileal digestibility of nutrients, organ weights and AME were not improved by dietary inclusion of Rengarenga lily extract at both levels or Frutafit at the lower rate. The reduced growth at the higher level of Frutafit may be due, in part, to depressed ileal digestibility of fat and protein and AME. Birds given the antibiotic showed clear improvement in performance, fat digestibility and intestinal development. Thus, high concentration of fructans in broiler diets may have no effect or negative effect on growth and nutrient utilisation.

CHAPTER 5 PREBIOTIC PLANT EXTRACTS FOR BROILERS: THEIR EFFECTS ON GROWTH PERFORMANCE, INTESTINAL MORPHOLOGY, MICROFLORA COMPOSITION AND ACTIVITY

ABSTRACT

An experiment was carried out using 384 broilers to study the effects of water-soluble carbohydrate extracts from Cabbage tree, Acacia, and Undaria seaweed on performance, gut morphology, microbial populations, and organic acid concentrations in the digestive tract of broilers. Each plant extract was supplemented at two levels (5 g/kg or 10 g/kg) to the diets. The plant extracts had no effect on BWG, but both levels of Acacia extract improved ($P < 0.05$) FCR during the first three weeks compared with the negative control group. The high level of Undaria extract suppressed growth throughout the experimental period. The positive control group fed on antibiotic supplemented diet showed improved ($P < 0.05$) BWG and FCR. Ileal digesta viscosity was increased ($P < 0.05$) and apparent ileal digestibility of fat was depressed ($P < 0.05$) in birds fed the high level of Undaria extract compared to the negative control. The plant extracts increased ($P < 0.05$) the numbers of lactobacilli in the ileum and caeca. The high levels of Acacia extract and Undaria extract significantly ($P < 0.05$) reduced the population of coliform bacteria in the ileum compared to the negative control group. The population of C. perfringens (Cp) was reduced ($P < 0.05$) in the caeca by plant extracts, but not in the ileum. The antibiotic reduced the population of Cp in both ileum and caeca compared with the negative control group. The number of bifidobacteria was below the detection limit in most samples and not affected by dietary supplementation of plant extracts. The organic acid analysis indicated that plant extract affected microbial fermentation patterns in the ileum and caeca. High level of Undaria extract reduced villus height in the ileum and the antibiotic diet resulted in higher ($P < 0.05$) villus height and villus height/crypt depth ratio compared with the negative control group. In conclusion, the results from this study demonstrate that prebiotic plant extracts had no or negative effect on performance of broilers, but beneficially modulated the composition of the microflora in the ileum and caeca by increasing the number of lactobacilli and reducing harmful bacteria, such as potential pathogenic E. coli and Cp.

5.1 INTRODUCTION

For the last five decades, AGPs have been used in poultry feeds in order to maximize efficiency of production and quality of product and to control diseases in chickens. Feeding antibiotics reverses microbe-induced growth depression by increasing the utilization of nutrients (Anderson *et al.*, 1999). Recently, Pedroso *et al.* (2006) found that changes in the composition, instead of reduction in the number of bacterial genotypes in the intestinal bacterial community induced by antibiotics, is responsible for improved growth performance in chickens. However, the extensive use of antibiotics in poultry has led to an imbalance in the beneficial intestinal microflora and the appearance of resistant bacteria (Aarestrup, 1999; Dibner & Richards, 2005). Due to current consumer preferences for poultry products grown without antibiotics, many alternatives to antibiotics have been studied as performance enhancers or health promoters (Griggs & Jacob, 2005; Verdonk *et al.*, 2005).

It has long been known that bacterial colonization of the intestine of chickens plays an important role in health and performance through its effect on gut morphology, nutrition, pathogenesis of intestinal disease and immune system development and response (Lee *et al.*, 2002; Apajalahti *et al.*, 2004; Dibner & Richards, 2005). Altering the intestinal microflora in a beneficial way, with the aim of improving the health of poultry, has become a subject of considerable interest. Hence, modulation of the natural bacterial population of the intestine in broilers through nutritional manipulation such as the selection of specific feed ingredients or the use of alternative feed supplements may be effective tools to control pathogens.

Oligosaccharides and polysaccharides, such as inulins (fructans), fructo-oligosaccharides (FOS) and arabinogalactans, extracted from plants, and sulphated fucans from seaweeds, are potential substitutes for currently used AGPs (Tringali, 1997; Iji & Tivey, 1998; Vidanarachchi *et al.*, 2005). Recent studies have shown that various oligosaccharides and polysaccharides may act as bioactive compounds or prebiotics in poultry feed, exerting growth-promoting effects (Lan *et al.*, 2004; Verdonk *et al.*, 2005). Several bioactive compounds from mushrooms and plants have already been identified as compounds which differentially stimulate favourable bacteria such as lactobacilli and bifidobacteria at the expense of pathogenic species in chickens (Gajewska *et al.*, 2002; Guo *et al.*, 2004b; Jamroz *et al.*, 2005). Stimulation of these beneficial bacteria could contribute to a balanced gut microflora, and may provide an optimal precondition for effective protection against pathogenic microorganisms, ultimately resulting in improved performance in animals (Flickinger *et al.*, 2003; Wenk, 2003; Verdonk *et al.*, 2005). Furthermore, it has been

observed that some non-digestible carbohydrates affect the development of the intestinal morphology and thereby improve performance in chickens (Iji *et al.*, 2001b; Santin *et al.*, 2001; Xu *et al.*, 2003). However, research on biologically active prebiotic compounds from higher plants and related species from Australian and New Zealand sources is scarce and their effects on broiler nutrition have not been investigated.

It was hypothesised in this study that dietary supplementation of water-soluble carbohydrate extracts from Cabbage tree, *Acacia*, and *Undaria* seaweed would exert prebiotic effects and selectively stimulate growth and/or activity of favourable intestinal bacteria, and furthermore influence gut morphology and hence improving the performance of broilers.

5.2 MATERIALS AND METHODS

5.2.1 Bird husbandry

A total of 384 Cobb male broilers were allocated to 8 treatments of 6 replicates (8 birds per replicate). Bird husbandry and performance measurements were as described in Section 4.2.1.

5.2.2 Experimental diets

The composition of the experimental basal diets is shown in Table 5.1. Formulation of diets and mixing of experimental diets were carried out as described in Section 4.2.2. Three different extracts were prepared from Cabbage tree, *Acacia* and *Undaria* seaweed, and were included at two levels (5 or 10 g/kg) in the diets. An antibiotic treatment group (45 mg Zn-bacitracin/kg) was used as a positive control and a non-supplemented diet was used as a negative control. Additions of plant extracts were made in place of sorghum. The extraction of water-soluble carbohydrates from the three plant sources was described previously (Section 3.2.1). The Cabbage tree extract contained mainly fructans (450 g/kg). The water-soluble carbohydrates extracted from *Undaria* seaweed (sulphated oligosaccharides) were composed mainly of fucose (55%, w/w) and galactose (44%, w/w). The *Acacia* extract was composed of naturally-occurring arabinogalactans (790 g/kg). Celite™(Food Chemicals Codex grade, Celite Corp., Lompar, CA, USA), a source of acid-insoluble ash, was added (5 g/kg) to the finisher diets as an indigestible marker. Chickens had *ad libitum* access to feed and water.

Table 5.1 Composition (g/kg as received) of experimental diets

Ingredient (g/kg)	Starter (d1-d21)	Finisher (d22-d35)
Sorghum	480	547
Corn	133	120
Soybean meal (48% CP)	274	230
Meat meal (50% CP)	80	56
Sunflower oil	7.5	10
Limestone (38% Ca)	5.0	5.8
Dicalcium phosphate	3.5	6.9
Lysine-HCl	1.2	1.7
DL-methionine	3.6	3.9
Salt	0.3	0.9
Sodium bicarbonate	5.0	5.0
Choline chloride	5.0	4.9
L-threonine	0.6	0.9
Celite	-	5.0
Premix[1]	2.0	2.0
Nutrient composition		
Metabolisable energy (MJ/kg)	12.3	13.1
Crude protein (g/kg)	230	205
Crude fat (g/kg)	39	46
Lysine (g/kg)	12	11
Methionine (g/kg)	7	7
Available phosphorous (g/kg)	5	4
Calcium (g/kg)	10	11

[1]Vitamin mineral premix (Ridley Agriproducts Pte Ltd., Tamworth, NSW) contained the following minerals in milligrams per kilogram of diet: Mn, 80; Zn, 60; Fe, 60; Cu, 8; I, 1.2; Co, 0.3; Se, 0.1; Mo, 1.0 and the following vitamins per kilogram of diet: Vitamin A, 12,000 IU from all *trans*-retinyl acetate; cholecalciferol D_3, 3.500 IU; vitamin E, 44.7 IU from DL-α-tocopherol; vitamin B_{12}, 12.75 μg; riboflavin, 6.0 mg; niacin, 50 mg; pantothenic acid, 12 mg; folic acid, 2 mg; biotin, 0.1 mg; thiamine, 2 mg; vitamin K, 2 mg and pyridoxine, 5 mg.

5.2.3 Collection and processing of samples

On d 35, two chickens were selected at random from each replicate and euthanized by cervical dislocation. Subsequently, each bird was dissected and the small intestine was ligated and removed from the bird. The contents of the ileum and caeca were pooled within a pen and collected into plastic containers by gently finger-stripping the respective intestinal segments. Around 0.2 g ileal and caecal contents were suspended in 0.8 mL of distilled water, and the pH was measured with a glass pH electrode (EcoScan 5/6 pH meter, Eutech Instruments Pte Ltd., Singapore). Intestinal contents (around 1 g) from ileum and caeca were aseptically transferred into McCartney bottles containing 10 mL of a pre-reduced salt medium (Holdeman *et al.*, 1977) and immediately transported to the laboratory for microbial analysis. Approximately 2 g fresh ileal digesta was centrifuged at 12,000 x *g* for 10 min at 20°C (Beckman model J2-21M, Beckman Instruments Inc., Palo Alto, CA, USA). After centrifugation, 0.5 mL supernatant was placed into a cone/plate Brookfield Model DV-III digital Rheometer (Brookfield Engineering Laboratories, Corp., Stoughton, MA, USA) with a CPE-40 spindle. The viscometer was set at 5-500 S^{-1} shear rates for determination of digesta viscosities at 25°C. The samples did not exhibit shear thinning at these shear rates. The results of the viscosity measurements were expressed in millipascal second (mPa.s; millipascal second = centipoise).

The remaining ileal and caecal digesta samples were frozen immediately after collection for further analysis of organic acid concentrations and apparent ileal digestibility of fat. For histomorphological analysis, approximately 2.5 cm of the middle portion of the ileum (one bird/replicate) was excised, flushed with PBS buffer (pH 7.6) and fixed in 10 % (v/v) neutral buffered formalin.

5.2.4 Apparent ileal digestibility of fat

The apparent ileal digestibility of fat was measured as described in Sections 4.2.6 and 4.2.11.

5.2.5 Gut histomorphology

The morphology of the ileal tissues was determined as described in Section 4.2.12.

5.2.6 Enumeration of intestinal bacteria

Samples in pre-reduced salt medium were homogenized for 2 min in CO_2-flushed plastic bags using a MiniMix® bag mixer (Interscience, St. Nom, France) and serially diluted in 10-fold increments in pre-reduced salt medium according to the technique of Miller and Wolin (1974). An aliquot (100 µL) was plated on the following agar media. Total anaerobic bacteria were enumerated on Wilkins-Chalgren anaerobic agar (Oxoid, CM0619) after incubation at 39°C for 7 days in an anaerobic cabinet (Model SJ-3, Kaltec Pty. Ltd., Edwardstown, SA, Australia). Lactobacilli were enumerated on Rogosa agar (Oxoid, CM0627) after anaerobic incubation at 39°C for 48 h in anaerobic jars (Oxoid Ltd, Hampshire, UK) with an anaerobic environment (<1% O_2 and 9-13% CO_2), generated using anaerobic AnaeroGen™ sachets (AN0025A, Oxoid Ltd, Hampshire, UK). Coliform bacteria and lactose-negative enterobacteria were counted on MacConkey agar (Oxoid, CM0115) after aerobic incubation at 39°C for 24 h. The population of *C. perfringens* (*Cp*) were counted on Tryptose-Sulfite-Cycloserine and Shahidi-Ferguson Perfringens agar base (TSC & SFP) (Oxoid, CM0587) mixed with egg yolk emulsion (Oxoid, SR0047) and Perfringens (TSC) selective supplement (Oxoid, SR0088E) according to the pour-plate technique, where plates were overlaid with the same agar after spreading the inoculum. Bacterial numbers were expressed as $\log_{10}$ CFU/g digesta.

For the enumeration of bifidobacteria, the diluted samples were spread on modified tryptone-neutralized soy peptone-yeast extract agar (MTPY) plates (Petr & Rada, 2001), and incubated at 39°C for 3 days in the anaerobic cabinet. The TPY agar was supplemented with mupirocin (100 mg/mL) and glacial acetic acid (1 ml/L). Mupirocin was extracted from antimicrobial discs (200 µg; Oxoid, A MUP 200) as described by Rada *et al.* (1999). In brief, 75 discs were placed in 15 mL TPY broth and stirred for 30 min; 10 mL of the broth was filter-sterilized and added to the medium (90 mL) after this had been autoclaved and cooled to 45°C. Bifidobacteria were enumerated in ileal and caecal digesta of the groups supplemented with 10 g/kg plant extracts and the negative control group. The corrected number of bifidobacteria was calculated for samples positive for bifidobacteria by correcting the counts on the MTPY agar plates with the proportion of bifidobacterial positive colonies determined by the fructose-6-phosphate phosphoketolase (F6PPK; EC 4.1.2.22) enzyme assay as described below.

5.2.7 Isolation and characterization of bacteria

All colonies from MTPY agar plates and twenty randomly selected colonies from Rogosa agar (lactobacilli) from the highest dilution of each sample were isolated and grown in TPY broth and MRS broth (Oxoid, CM0359), respectively. Isolates were grown at 39°C in the anaerobic cabinet for three days for bifidobacteria and two days for lactobacilli and stored at -20°C in 30% (v/v) sterilized glycerol. Further molecular and biochemical characterisation of the lactobacilli isolated from Rogosa agar are described in Chapter 6. Subcultures of isolates from MTPY agar were grown in 10 mL of TPY broth at 39°C overnight. Cells from isolates that did not produce gas during growth (99/402) were then harvested by centrifugation at 5,500 x *g* for 10 min and tested for the presence of F6PPK activity as described by Orban and Patterson (2000). Isolates recovered from MTPY agar were also subjected to Gram-staining and their cellular morphology was recorded. Furthermore, identified bifidobacteria were analysed by Amplified Ribosomal Restriction Analysis (ARDRA) and 16S rRNA gene sequencing as described by Mikkelsen *et al.* (2003). The sequences of the 16S rRNA genes determined in this study were deposited with the GeneBank nucleotide database under the accession number DQ676992.

5.2.8 Measurement of organic acids

For measurement of organic acid (SCFA, lactic and succinic acid) concentrations, about 2.0 g of thawed ileal and caecal sample was suspended in 1.0 mL of 0.02 M-*2*-ethylbutyric acid and thoroughly mixed by using a vortex mixer, followed by centrifugation at 25,700 x *g* at 4°C for 15 min in a Beckman model J2-21M Induction Drive centrifuge with a JA-21 rotor. To a sample of 1 mL supernatant fraction, 0.5 mL of concentrated HCl and 2 mL of diethyl ether were added and thoroughly mixed by using a vortex mixer, followed by centrifugation at 2060 x *g* at 4°C for 15 min. Aliquots (360 µL) from the ether phase were recovered and mixed with N-methyl-N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA; 40 µL) for derivatisation of organic acids. The concentration of derivatised organic acids was quantified using a Varian CP 3400 CX gas chromatograph (Varian Analytical Instruments, Palo Alto, CA, USA).

The chromatographic apparatus included a Varian CP3400 CX gas chromatograph fitted with a flame ionization detector (FID) and a Varian CP 8200 autosampler (Varian Analytical Instruments, Palo Alto, CA, USA) equipped with a capillary column (0.32 mm internal diameter, 30 m length and 0.25 µm film thickness) (Alltech ECONO-CAP^{TM}, Alltech

Associations Inc., Deerfield, IL, USA). The initial and final oven temperatures were 70°C and 240°C, respectively, and the injector and FID temperatures were 240°C and 280°C, respectively. Ultra high purity helium was used as the carrier gas (40 cm/sec). The organic acids were tentatively identified by comparing their retention times with those of authentic standard mixtures (formic acid: 20 μmol/mL, acetic acid: 20 μmol/mL, propionic acid: 10 μmol/mL, *iso*-butyric acid: 10 μmol/mL, butyric acid: 10 μmol/mL, *iso*-valeric acid: 5 μmol/mL, valeric acid: 5 μmol/mL, lactic acid: 15 μmol/mL, and succinic acid: 10 μmol/mL). The area under each peak was calculated on a weight percentage basis using *2*-ethylbutyric acid (Sigma-Aldrich Pty Ltd., Castel Hill, NSW, Australia) as an internal standard. Varian Star 5.52 chromatography workstation (integration system) software (Varian Analytical Instruments, Palo Alto, CA, USA) was used for data processing. Total organic acid concentration is the sum of the all organic acids observed in a sample, expressed as μmol/g digesta. Molar percentages of individual organic acids are expressed as mol/100 moles.

5.2.9 Animal ethics

All experimental procedures were approved by the University of New England Animal Care and Ethics Committee (Approval No.: AEC04/111), and throughout the experiments, health and husbandry practices complied with the *Code of Practice for the Care and Use of Animals for Scientific Purposes* (National Health and Medical Research Council, 2004), for the Commonwealth of Australia and the *Australian Model Code of Practice for the Welfare of Animals: Domestic Poultry* (Primary Industries Standing Committee, 2002).

5.2.10 Statistical analysis

Bacterial counts were transformed to $\log_{10}$ values, and apparent ileal fat digestibility coefficients and molar proportions of organic acids were subjected to arcsine transformation before statistical analysis; digestibility coefficients and organic acid data are presented as natural numbers (Steel & Torrie, 1981). Each variable was analysed according to a completely randomized design with a cage of broilers composing the experimental unit. The data were analysed according to the following model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij};$$

where,

Y_{ij} = observed dependent variable;

μ = overall mean;

α_i = fixed effect of treatment, i = 1,2,3,4,5,6,7,8;

β_j = random effect of replicate, j = 1,2,3,4,5,6;

ε_{ij} = residual error for treatment 'i' of replicate 'j' ~ N (0, σ_ε^2).

All possible interactions were tested for significance ($P \leq 0.05$) and were eliminated from the models because they were not significant. The errors were assumed to be independently and normally distributed with a mean of zero and variance of σ_ε^2. All data were analysed by using ANOVA option of PROC MIXED procedure (SAS Institute Inc., 2000). Treatment least-squares means were compared using predetermined contrasts and considered significant at $P \leq 0.05$. Results are reported as least-squares means (n = 6) and pooled standard error (SE).

5.3 RESULTS

5.3.1 Bird performance

The performances of chickens fed the experimental diets are shown in Table 5.2. In general, no differences in BWG or FI were observed in broilers fed diets supplemented with plant extracts, except the high level of *Undaria* extract supplemented-group, which had lower ($P < 0.05$) BWG throughout the experimental period compared to the negative control group. The same group also showed lower ($P < 0.05$) FI during the period d 21 to 35. Body weight gain of the high level of Cabbage tree extract fed group tended to be lower compared to the negative control group over the whole experimental period. Throughout the experimental period, broilers fed diets containing the antibiotic grew faster ($P < 0.05$) and had lower ($P < 0.05$) FCR than those fed the negative control or plant extract-supplemented diets. Over the first three week period, FCR of birds fed both levels of *Acacia* extract-supplemented diets was lower ($P < 0.05$) than that of the negative control group. However, the BWG or the FI were unaffected by *Acacia* extract supplementation. Birds supplemented with high level of Cabbage tree extract had a lower ($P < 0.05$) feed efficiency (increased FCR) during the period d 1 to 21 compared to the negative control group. This effect was not observed in the later stage of the growth.

Table 5.2 Mean body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) of broiler chickens fed diets supplemented with plant extracts[2]

Treatment	Body weight gain (BWG; g/bird)			Feed Intake (FI; g/bird)			Feed conversion ratio (FCR)		
	d 1 to 21	d 21 to 35	d 1 to 35	d 1 to 21	d 21 to 35	d 1 to 35	d 1 to 21	d 21 to 35	d 1 to 35
Negative-control (NC)	685	1497	1822	1172	2824	3340	1.71	1.89	1.83
Positive-control (PC)	716	1545	1886	1163	2830	3331	1.62	1.83	1.77
Cabbage tree (5 g/kg)	682	1515	1845	1176	2822	3351	1.72	1.86	1.82
Cabbage tree (10 g/kg)	671	1489	1801	1178	2816	3339	1.76	1.89	1.85
Undaria (5 g/kg)	686	1507	1831	1161	2825	3345	1.69	1.87	1.83
Undaria (10 g/kg)	667	1473	1786	1162	2790	3342	1.74	1.89	1.87
Acacia (5 g/kg)	690	1517	1847	1160	2825	3356	1.68	1.86	1.82
Acacia (10 g/kg)	693	1508	1844	1159	2819	3346	1.67	1.87	1.81
S.E.M.[1]	7.53	8.05	9.33	18.32	12.15	12.94	0.02	0.01	0.01
Orthogonal contrasts	Probability level of contrasts								
NC vs. Cabbage tree (5 g/kg)	NS	NS	NS	NS	NS	NS	NS	0.01	NS
NC vs. Cabbage tree (10 g/kg)	NS	NS	NS	NS	NS	NS	0.03	NS	NS
NC vs. *Undaria* (5 g/kg)	NS	NS	NS	NS	NS	NS	NS	NS	NS
NC vs. *Undaria* (10 g/kg)	0.04	0.03	0.006	NS	0.01	NS	NS	NS	0.01
NC vs. *Acacia* (5 g/kg)	NS	NS	NS	NS	NS	NS	0.04	0.01	NS
NC vs. *Acacia* (10 g/kg)	NS	NS	NS	NS	NS	NS	0.03	NS	0.04
NC vs. PC	0.0004	<0.0001	<0.0001	NS	NS	NS	<0.0001	0.0001	<0.0001

[1]S.E.M. = Pooled standard error of the least square means. [2]Results are given as least square means (n = 6).

5.3.2 Ileal and caecal microflora

The bacterial counts in ileal and caecal contents are shown in Table 5.3 and Table 5.4, respectively. The population of lactobacilli was higher ($P < 0.05$) in the ileum and caeca in chickens fed the plant extracts compared with the negative control group. The antibiotic diet decreased ($P < 0.05$) the population of total anaerobic bacteria both in the ileum and caeca, but did not reduce the number of lactobacilli compared to the negative control group (Table 5.3 and Table 5.4). Lower ($P < 0.05$) ileal coliform counts were observed in birds fed high levels of *Undaria* extract and *Acacia* extract-supplemented diets than in the negative control group. The caecal coliform counts in birds supplemented with *Undaria* extract (both levels), and high level of Cabbage tree extract were significantly lower than those of the negative control group. The numbers of *Cp* in the ileum tended to be reduced, although not significantly ($P > 0.05$), in plant extract-fed birds compared to non-supplemented birds. On the other hand, supplementation with plant extracts led to a reduction ($P < 0.05$) in caecal *Cp* counts. The numbers of lactose-negative enterobacteria in the ileum and caeca were unaffected by inclusion of the plant extracts in the diet.

The population of bifidobacteria enumerated on the MTPY medium from the ileal and caecal digesta is shown in Table 5.5. The bifidobacteria population was substantially low in both ileal and caecal digesta and in most of the replicates the numbers were below the detection limit. Many of the isolates (303/402) recovered from the MTPY agar medium were gas producers. Identification by fructose-6-phospahte phosphoketolase (F6PPK) activity revealed that only 27 out of 99 non gas producing isolates were bifidobacteria (formation of pink reddish colour) in this assay. Typical pleomorphic bifurcated, Gram-positive cells were observed with isolates that were positive for F6PPK activity. Amplified Ribosomal DNA Restriction Analysis (ARDRA) revealed that the isolates positive for F6PPK activity had similar restriction band patterns (Plate 5.1). The 16S rRNA gene sequencing of isolates confirmed as *Bifidobacterium* species had a very high sequence similarity (98 %) to *B. pseudolongum* (GeneBank accession number AB186304).

Table 5.3 Bacterial counts in ileal digesta of birds (35 d of age) fed diets supplemented with plant extracts[2]

Treatment	Total anaerobic bacteria	Lactobacilli	Lactose-negative enterobacteria	Coliform bacteria	*Clostridium perfringens*
	Log_{10} CFU/g digesta				
Negative-control (NC)	9.26	7.87	4.80	6.35	4.94
Positive-control (PC)	8.74	8.02	4.80	6.35	4.21
Cabbage tree (5 g/kg)	9.30	9.00	4.76	6.28	4.83
Cabbage tree (10 g/kg)	8.99	9.08	4.77	5.72	4.53
Undaria (5 g/kg)	9.32	8.97	4.76	6.08	4.55
Undaria (10 g/kg)	9.29	9.22	4.79	5.13	4.60
Acacia (5 g/kg)	9.38	8.67	4.76	6.30	4.68
Acacia (10 g/kg)	9.47	8.92	4.73	5.38	4.53
S.E.M.[1]	0.33	0.19	0.03	0.25	0.17
Orthogonal contrasts	Probability level of contrasts				
NC vs. Cabbage tree (5 g/kg)	NS	<0.001	NS	NS	NS
NC vs. Cabbage tree (10 g/kg)	NS	<0.001	NS	NS	NS
NC vs. *Undaria* (5 g/kg)	NS	<0.001	NS	NS	NS
NC vs. *Undaria* (10 g/kg)	NS	<0.001	NS	0.0009	NS
NC vs. *Acacia* (5 g/kg)	NS	0.002	NS	NS	NS
NC vs. *Acacia* (10 g/kg)	NS	<0.001	NS	0.005	NS
NC vs. PC	0.001	NS	NS	NS	NS

[1]S.E.M. = Pooled standard error of the least square means. [2]Results are given as least square means (n = 6).

Table 5.4 Bacterial counts in caecal digesta of birds (35 d of age) fed diets supplemented with plant extracts[2]

Treatment	Total anaerobic bacteria	Lactobacilli	Lactose-negative enterobacteria	Coliform bacteria	*Clostridium perfringens*
			Log_{10} CFU/g digesta		
Negative-control (NC)	9.54	8.76	5.84	8.40	6.11
Positive-control (PC)	9.22	8.65	5.10	7.85	5.43
Cabbage tree (5 g/kg)	9.75	9.49	5.18	8.22	5.25
Cabbage tree (10 g/kg)	9.52	9.50	5.34	7.70	5.13
Undaria (5 g/kg)	9.52	9.20	5.35	7.69	5.14
Undaria (10 g/kg)	9.79	9.35	5.62	7.94	5.14
Acacia (5 g/kg)	9.51	9.09	5.27	8.27	5.42
Acacia (10 g/kg)	9.54	9.53	5.07	8.34	5.38
S.E.M.[1]	0.18	0.16	0.21	0.16	0.19
Orthogonal contrasts			Probability level of contrasts		
NC vs. Cabbage tree (5 g/kg)	NS	0.0002	NS	NS	0.003
NC vs. Cabbage tree (10 g/kg)	NS	0.0002	NS	0.003	0.0009
NC vs. *Undaria* (5 g/kg)	NS	0.02	NS	0.003	0.001
NC vs. *Undaria* (10 g/kg)	NS	0.002	NS	0.04	0.0009
NC vs. *Acacia* (5 g/kg)	NS	NS	NS	NS	0.01
NC vs. *Acacia* (10 g/kg)	NS	0.0001	NS	NS	0.001
NC vs. PC	0.002	NS	NS	0.02	0.01

[1]S.E.M = Pooled standard error of the least square means. [2]Results are given as least square means (n = 6).

Table 5.5 Population levels of bifidobacteria in ileal and caecal digesta of broiler chickens fed the experimental diets

Segment	Treatment	No. of bifidobacteria (Log_{10} CFU/g digesta)	No. of replicates positive for bifidobacteria
Ileum	Negative control	<2.80 (-)[1]	0
	Cabbage tree (10 g/kg)	<3.18 (3.94)[2]	1
	Undaria (10 g/kg)	<2.98 (3.33)	1
	Acacia (10 g/kg)	<2.90 (-)	0
Caeca	Negative control	<3.15 (-)	0
	Cabbage tree (10 g/kg)	<3.13 (5.47)	1
	Undaria (10 g/kg)	<3.17 (3.50)	2
	Acacia (10 g/kg)	<3.05 (-)	0

[1]No bifidobacteria were detected.
[2]Corrected number of bifidobacteria in samples positive for bifidobacteria (see materials and methods for details).

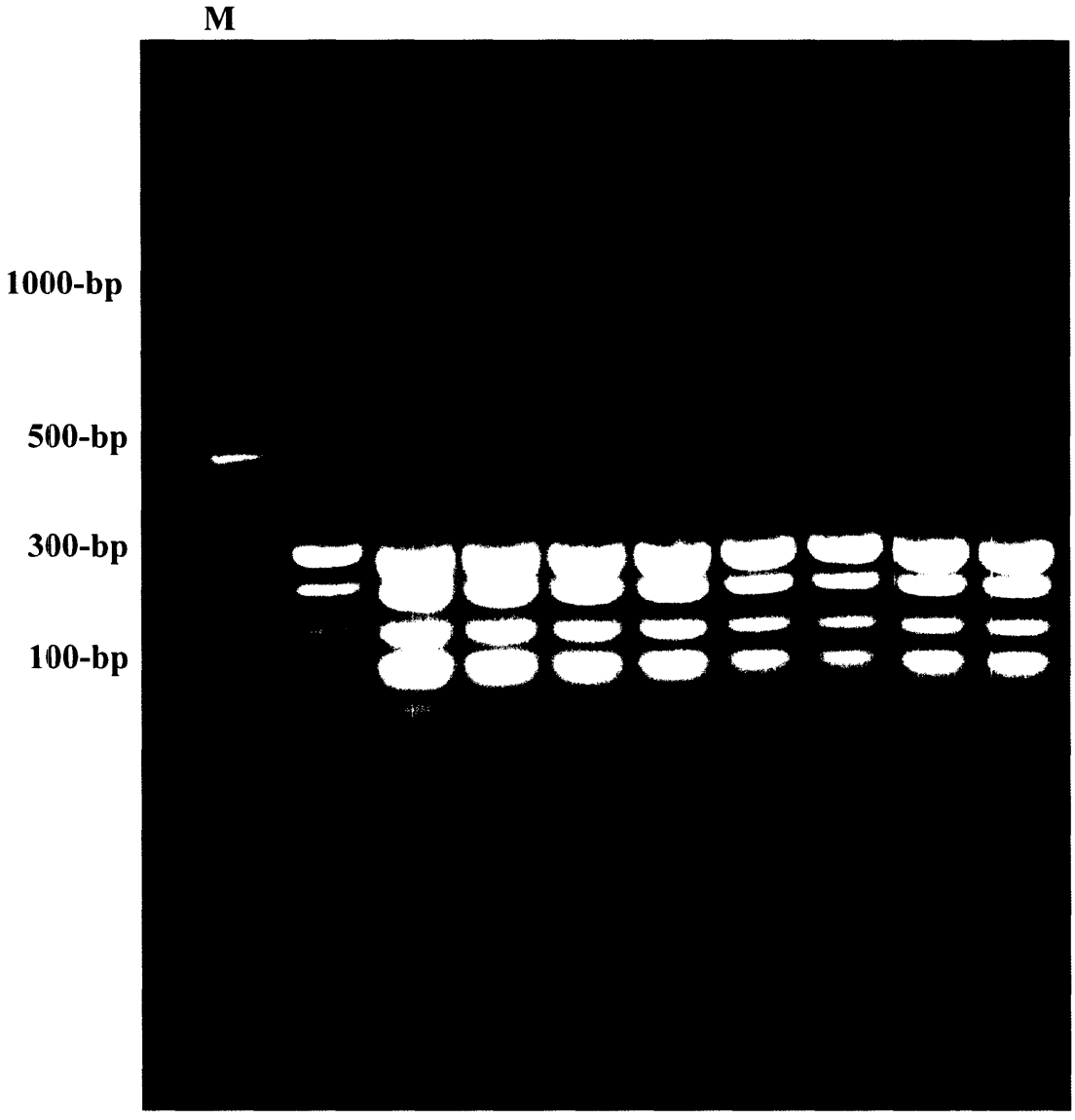


Plate 5.1 Amplified Ribosomal DNA Restriction Analysis (ARDRA) patterns of 16S rRNA genes from bifidobacterial isolates.

The amplified 16S rRNA gene fragments were digested with the restriction endonuclease *Hae*III enzyme and resolved by electrophoresis through a 2% agarose gel. Lane M, molecular weight marker (100-1000bp ladder).

5.3.3 Apparent ileal fat digestibility, ileal digesta viscosity and organic acids in ilea and caeca

Digesta viscosity, apparent fat digestibility, digesta pH and organic acid data in the ileum are shown in Table 5.6. High levels of *Undaria* extract and *Acacia* extract raised ($P < 0.05$) the viscosity of the digesta from the ileum compared with the negative control diet. The apparent ileal digestibility of fat was decreased ($P < 0.05$) in the *Undaria* extract (10 g/kg)-fed birds.

The pH of the ileal content was not affected by diet, although numerically lower pH values were observed for broilers supplemented with plant extracts compared with the negative control group. The results also demonstrated that ileal total organic acid concentrations were higher ($P < 0.05$) in high level of Cabbage tree extract-fed birds and values tended to be higher in birds supplemented with other plant extracts compared with the negative control group. The molar proportion of lactate in ileal digesta was increased ($P < 0.05$) in birds fed both levels of Cabbage tree extract and the high level of *Undaria* extract diets compared to the negative control.

Dietary supplementation with Cabbage tree extract (both levels) and high level of *Undaria* extract resulted in lower pH in the caecal digesta compared to the negative control group (Table 5.7). The concentration of total organic acids increased ($P < 0.05$) in the caecal digesta of broilers fed diets supplemented with *Undaria* extract (10 g/kg) and *Acacia* extract. The molar proportions of propionate in the caecal contents of birds fed with the diet containing the high level of *Acacia* extract were increased ($P < 0.05$), compared to the negative control group (Table 5.7). The caecal content of broilers fed all plant extracts, except the high level of *Acacia* extract had higher ($P < 0.05$) molar proportions of butyric acid than the negative control group (Table 5.7).

Table 5.6 Viscosity, apparent fat digestibility, pH and molar proportions of various organic acids in ileal digesta of broiler chickens fed the experimental diets at 35 days of age[2]

Treatment	Viscosity (mPa.s)	Apparent fat digestibility (%)	pH	Total organic acids (μmol/g digesta)	Molar proportions of organic acids	
					Acetate	Lactate
					mole/100 mole	
Negative-control (NC)	1.65	73.2	7.63	61.4	3.2	95.4
Positive-control (PC)	1.54	74.0	7.78	56.0	4.2	95.6
Cabbage tree (5 g/kg)	1.62	73.3	7.29	72.4	2.6	97.4
Cabbage tree (10 g/kg)	1.66	72.9	7.17	76.2	2.6	97.4
Undaria (5 g/kg)	1.77	73.2	7.29	70.3	1.7	96.7
Undaria (10 g/kg)	2.95	70.9	7.03	63.5	3.2	98.3
Acacia (5 g/kg)	1.70	73.1	7.39	66.7	2.8	95.4
Acacia (10 g/kg)	1.93	72.9	7.17	71.8	2.7	95.6
S.E.M.[1]	0.09	0.35	0.64	4.24	0.66	0.62
Orthogonal contrasts	Probability level of contrasts					
NC vs. Cabbage tree (5 g/kg)	NS	NS	NS	NS	NS	0.01
NC vs. Cabbage tree (10 g/kg)	NS	NS	NS	0.02	NS	0.01
NC vs. *Undaria* (5 g/kg)	NS	NS	NS	NS	NS	NS
NC vs. *Undaria* (10 g/kg)	0.0001	0.001	NS	NS	NS	0.0007
NC vs. *Acacia* (5 g/kg)	NS	NS	NS	NS	NS	NS
NC vs. *Acacia* (10 g/kg)	0.03	NS	NS	NS	NS	NS
NC vs. PC	NS	NS	NS	NS	NS	NS

[1]S.E.M. = Pooled standard error of the least square means. [2]Results are given as least square means (n = 6).

Table 5.7 pH and molar proportions of various organic acids in caecal digesta of broiler chickens fed the experimental diets at 35 days of age[2]

Treatment	pH	Total organic acids (μmol/g digesta)	Molar proportions of organic acids					
			Acetate	Propionate	*n*-Butyrate	*n*-Valerate	*iso*-But + *iso*-Vale	Succinate
			mole/100 mole					
Negative-control (NC)	7.13	91.2	71.2	11.4	10.3	2.0	1.9	3.2
Positive-control (PC)	7.12	79.9	72.3	9.8	11.1	2.0	1.8	3.1
Cabbage tree (5 g/kg)	6.78	101.0	66.1	10.7	14.1	2.2	2.1	3.5
Cabbage tree (10 g/kg)	6.66	102.0	69.7	10.3	14.0	1.9	1.4	2.7
Undaria (5 g/kg)	6.85	101.3	66.0	7.9	15.3	2.3	1.5	3.3
Undaria (10 g/kg)	6.61	106.7	68.3	10.6	15.9	2.4	1.8	4.7
Acacia (5 g/kg)	6.81	107.0	66.7	11.4	13.8	2.1	1.5	4.5
Acacia (10 g/kg)	6.84	112.1	68.4	12.0	13.2	1.8	1.2	4.7
S.E.M.[1]	0.12	4.15	1.87	1.08	1.13	0.19	0.27	0.85
Orthogonal contrasts	Probability level of contrasts							
NC vs. Cabbage tree (5 g/kg)	0.04	NS	NS	NS	0.03	NS	NS	NS
NC vs. Cabbage tree (10 g/kg)	0.009	NS	NS	NS	0.03	NS	NS	NS
NC vs. *Undaria* (5 g/kg)	NS	NS	NS	NS	0.004	NS	NS	NS
NC vs. *Undaria* (10 g/kg)	0.004	0.01	NS	NS	0.001	NS	NS	NS
NC vs. *Acacia* (5 g/kg)	NS	0.01	NS	NS	0.04	NS	NS	NS
NC vs. *Acacia* (10 g/kg)	NS	0.001	NS	0.03	NS	NS	NS	NS
NC vs. PC	NS	NS	NS	NS	NS	NS	NS	NS

[1]S.E.M. = Pooled standard error of the least square means. [2]Results are given as least square means (n = 6).

5.3.4 Gut histomorphology

The histology of ileal segments was studied at 35 days of age and the data are presented in Figure 5.1. No significant differences among plant extract-supplemented groups were noted in villus height in the ileum, except the high level of *Undaria* extract-fed group, in which shorter ($P < 0.05$) villi were observed when compared to the positive control group. The birds fed high level of *Undaria* extract had shallower ($P < 0.05$) crypts and lower ($P < 0.05$) villus/crypt ratio in the ileum compared to the positive control group. However, these differences were not significant compared with the negative control group. Birds fed with the positive control diet had marginally longer villi ($P < 0.05$) and higher ($P < 0.05$) ileal villus height/crypt depth ratio than the negative control group.

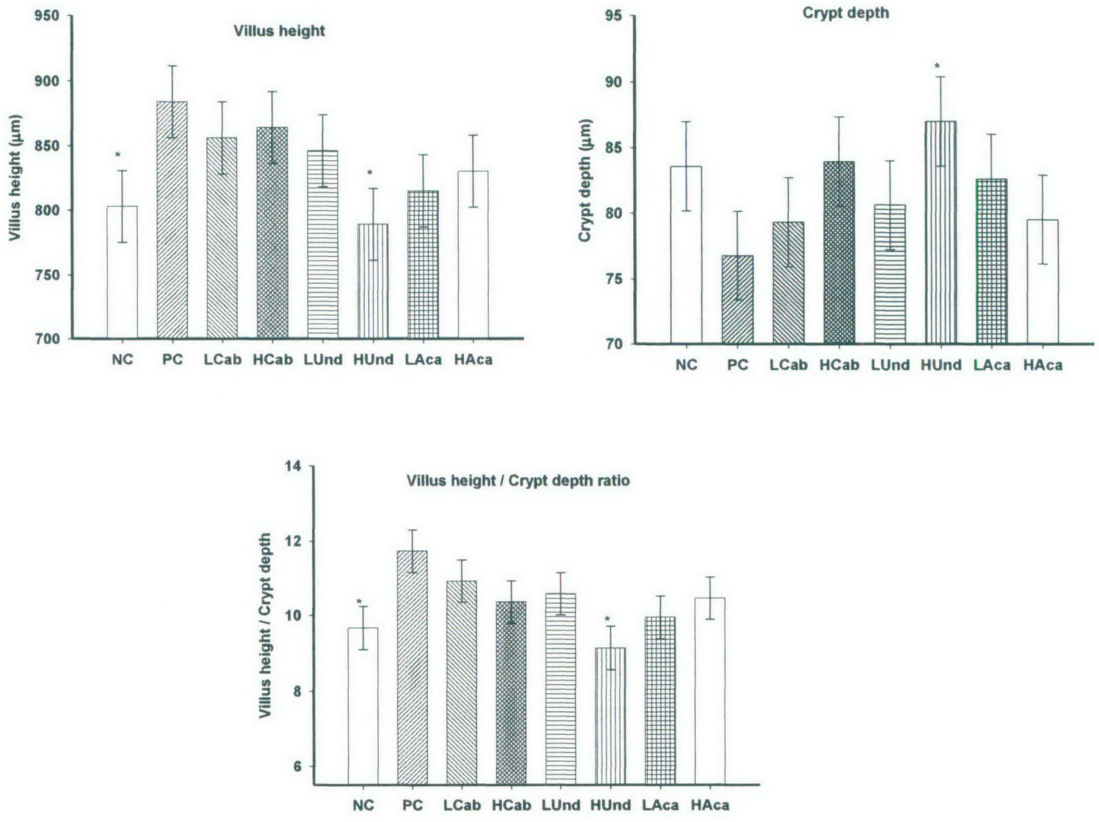


Figure 5.1 Effects of dietary plant extracts on the morphometric parameters of ileum at 35 days of age

Negative control (NC), positive control (PC), low level of Cabbage tree extract (LCab), high level of Cabbage tree extract (HCab), low level of *Undaria* extract (LUnd), High level of *Undaria* extract (HUnd), low level of *Acacia* extract (LAca) and high level of *Acacia* extract (HAca). (Results are given as least square means, n=6; error bars indicate pooled standard error of the least square means; bars with asterisks are significantly different from the positive control

5.4 DISCUSSION

5.4.1 Bird performance

In the present study, supplementing diets with any of the three types of prebiotic plant extracts did not improve the BWG or FI of broilers. This is consistent with published reports of Patterson *et al.* (1997) and Zdunczyk *et al.* (2005) who also found no beneficial effects on the performance of poultry due to diets supplemented with prebiotic and bioactive compounds. Other researchers have reported that supplementation with prebiotic compounds similar to those used in the current study, resulted in increased BWG and FCR in broilers (Ao & Choct, 2003; Kleessen *et al.*, 2003a). Kleessen *et al.* (2003a) reported that fructans extracted from Jerusalem artichoke (0.5%) and administered via drinking water resulted in increased BWG in broilers at d 35. Similarly, Ao and Choct (2003) reported that broilers given drinking-water supplemented with oligofructose (0.05%) were heavier and had more efficient FCR up to d 35 compared to the control birds. It is likely that these variations in the efficacy of prebiotics on broiler performance are due to differences in the content and degree of polymerisation of the active ingredients, and differences in management practices, such as the type of basal ingredients used in diets and the health status of birds.

The poor BWG of birds given the diet supplemented with *Undaria* extract at the high dose compared with the negative control group may be attributed to the high ileal digesta viscosity and low apparent ileal fat digestibility. This is in agreement with the findings of Maisonnier *et al.* (2001) who found that increased intestinal digesta viscosity causes lower apparent lipid digestibility in the intestine of broilers. Maisonnier *et al.* (2002) reported that absorption of lipid end-products such as free fatty acids is lower when the ileal viscosity is high in broiler chickens. It is generally considered that the detrimental effects of the soluble polysaccharides contained in plant materials are associated with the viscosity of these polysaccharides and their physiological and morphological effects on the digestive tract (Choct *et al.*, 1992). For instance, viscous polysaccharides can increase the residence time of digesta, which may result in increased microbial fermentation activities within the intestine (Choct *et al.*, 1996; Smits *et al.*, 1998). *Undaria* extract is composed predominantly of α-1,2 linked galactofucan-sulphate oligosaccharides with glucuronic acid and mannuronic acid as minor constituents. Therefore, relatively high levels of galactofucan-sulphate oligosaccharides, glucuronic acid and mannuronic acid would have increased ileal digesta

viscosity in broilers fed with the high dosage of *Undaria* extract. However, growth depression associated with high levels of dietary *Undaria* extract in birds may also be in part explained by reduced FI.

Although the population of lactobacilli was larger in the ileal and caecal contents of the treatment groups fed plant extracts, the current study did not demonstrate an improvement in growth of birds due to supplementation with plant extracts. Microorganisms present in the small intestine contribute significantly to small intestinal function, including digestion and absorption of nutrients, which are limiting factors to the growth rate of the host animal (Smits *et al.*, 1998; Maisonnier *et al.*, 2003). The impact of lactobacilli on animal health and performance is controversial. Many *Lactobacillus* species have been shown to act via a number of mechanisms, including CE to reduce the number of pathogenic bacteria in GIT, thereby improving the performance of broilers (Jin *et al.*, 1998a; Schneitz *et al.*, 1998). However, increased bacterial activity in the small intestine of chickens can cause deconjugation of bile salts, leading to poor lipid digestion (Smits *et al.*, 1998; Maisonnier *et al.*, 2003). Lactobacilli inhabiting the small intestine have been identified as largely responsible for the hydrolysis of bile salts (Feighner & Dashkevicz, 1987; Tannock *et al.*, 1989). In the current study, the *Undaria* extract (10 g/kg)-supplemented group had the highest number of lactobacilli in the ileum. It is possible that the increased number of lactobacilli has contributed to the lowered apparent ileal fat digestibility and poor performance observed in this study. Hence, the trade-off between benefits and costs of bacterial species such as lactobacilli on broiler performance need to be interpreted with caution.

Despite the fact that ileal viscosity and lactobacilli counts in birds on the high level of *Acacia* extract were higher than those observed with the negative control group, the FCR was improved with the same dietary treatment group up to d 35. Moreover, the apparent ileal fat digestibility of this group was not different from that of other treatment groups. This shows that factors such as high viscosity and large number of intestinal lactobacilli may not always contribute to impaired apparent ileal fat digestibility as observed. It can be speculated that a difference in species composition of the lactobacilli population between the *Undaria* extract and *Acacia* extract-fed group in the ileal and caecal digesta could be the reason for this. As reported in Section 6.3, the *Acacia* extract-fed group had a higher population of *L. johnsonii* in the ileal and caecal digesta, whereas the *Undaria* extract-fed group had predominantly *L. salivarius*. Bile salt hydrolase, the enzyme which is responsible for bile-

salt deconjugation, has been detected in *L. salivarius* (Gilliland & Speck, 1977; Knarreborg *et al.*, 2002a). Conversely, *L. johnsonii* is not known to have this enzyme. Therefore the large number of *L. johnsonii* in the ileum of high level of *Acacia* extract-supplemented birds would not reduce the apparent ileal digestibility of fat. In order to draw firm conclusions regarding the above hypothesis, further studies need to be carried out to test the deconjugation ability of bile salts by the *Lactobacillus* species isolated and identified in the current study. Another possible explanation for the improved FCR in birds fed *Acacia* extract may be an increased digestibility of nutrients other than fat, which was not analysed in the current study.

In the current study, birds receiving a diet supplemented with Zn-bacitracin had improved growth performance throughout the experimental period. The mode of action of Zn-bacitracin in improving the growth of chickens is well known. Zinc-bacitracin is active mainly against Gram-positive bacteria such as *Cp.* forming a complex with C_{55}-isoprenyl pyrophosphate, a carrier for the *N*-acetylmuramyl pentapeptides, which are intermediates in the synthesis of peptidoglycan in bacterial cell wall (Butaye *et al.*, 2003). A number of studies have reported enhancement of performance by dietary supplementation of Zn-bacitracin in broiler chickens (Elwinger *et al.*, 1998; Engberg *et al.*, 2000; Ravindran *et al.*, 2006).

5.4.2 Ileal and caecal microflora and microbial activity

Prebiotics are mainly known to favour the growth and activity of certain intestinal bacteria, such as bifidobacteria and lactobacilli, generally regarded as beneficial to the host. In the present study, dietary supplementation with plant extracts significantly increased the population of lactobacilli in the ileum and the caecal contents of birds fed plant extracts. Similar results have been shown in caecal contents of broiler chickens and faecal matter of humans fed diets supplemented with prebiotic compounds (fructans and aranbinogalactans) such as those present in Cabbage tree extract (fructans) and *Acacia* extract (arabinogalactans) in this experiment (Cherbut *et al.*, 2003; Yusrizal & Chen, 2003b). Patterson *et al.* (1997) reported that caecal *Lactobacillus* counts in ketose oligosaccharide-treated broilers were increased sevenfold compared to the control group.

Results from the current study demonstrated that bifidobacteria make up a minor proportion of the ileal and caecal microflora in broilers. Prebiotic plant extracts did not have any effect

on the bifidobacteria counts in the ileum and the caeca. In agreement with this, Zhu *et al.* (2002) also found that the bifidobacteria are relatively rare in caecal digesta (1.3 %) of broiler chickens as confirmed by 16S rRNA gene analysis. Another molecular study (16S rRNA gene sequencing) also revealed that the genus *Bifidobacterium* is substantially low in both ileum (0.19 %) and caeca (0 %) of broilers (Lu *et al.*, 2003). In contrast, Thitaram *et al.* (2005) observed *Bifidobacterium* populations as high as 9-10 $\log_{10}$ CFU/g caecal digesta in broilers fed a diet supplemented with isomaltooligosaccharide as a prebiotic compound. Although these authors mentioned that they have confirmed the identity of bifidobacteria using F6PPK activity, it is unclear how many isolates they have tested by the enzymatic assay. Furthermore, the medium (transoligosaccharide propionate agar supplemented with 1% v/v glacial acetic acid) used for enumeration of bifidobacteria by Thitaram *et al.* (2005) is different from that used in this study and it did not contain the antibiotic mupirocin. The selectivity of MTPY agar is due to the presence of mupirocin, an antibiotic to which bifidobacteria are resistant and to which many lactobacilli are susceptible (Rada *et al.*, 1999).

The most reliable non-molecular test for distinguishing bifidobacteria from the related genera is F6PPK activity. In the current study, isolation and identification of bifidobacteria by the F6PPK activity revealed a low recovery of bifidobacteria from the plates. It was found that microorganisms other than the bifidobacteria (gas producers) can also grow on the MTPY agar medium. These results are similar to those of Hartemink & Rombouts (1999) who found that non-bifidobacteria (clostridia, lactobacilli and different cocci) are capable of growing on three selective media [Beernes propionic acid medium (PROP), Raffinose-Bifidobacterium medium (RB), and Neomycin-Paromomycin-Nalidixic acid-Lithium chloride agar (NPNL)]. Furthermore, Mikkelsen *et al.* (2003) reported that characteristic bifid-shape *Actinomyces* spp. grow well on the modified MTPY medium and they further concluded that morphology is not sufficient for identifying bifidobacteria on selective agar media.

Many studies have indicated that dietary prebiotic and bioactive compounds could increase the ileal and caecal bifidobacteria numbers in broiler chickens (Orban *et al.*, 1997; Patterson *et al.*, 1997; Guo *et al.*, 2004b; Cao *et al.*, 2005). The selectivity of the agar media used in some other published studies was not verified or accounted for (Patterson *et al.*, 1997; Guo *et al.*, 2004b; Cao *et al.*, 2005) and as pointed out by the present results, bifidobacterial numbers in broiler chickens need to be interpreted with caution due to potential methodological biases.

Similar to findings in this study, Engberg *et al.* (2000) observed that population of *Cp* was significantly reduced in the ileum and caeca of broilers fed a diet supplemented with an even lower level of Zn-bacitracin than that used in this study (20 mg/kg *vs.* 45 mg/kg). The finding of the current study that the Zn-bacitracin-supplemented diet reduced the population of Gram-negative coliforms in the ileal content of broiler chickens agrees with the reports of Stanley *et al.* (2004) and Engberg *et al.* (2000). This observation is somewhat surprising because Zn-bacitracin is active mainly against Gram-positive bacteria (Engberg *et al.*, 2000; Butaye *et al.*, 2003).

In the current study, total organic acid concentrations and the molar proportion of caecal butyrate were generally higher in birds fed any of the three plant extracts compared to the negative control group. These results indicate that the plant extracts were fermented by the caecal microflora. Previous studies have shown that feeding prebiotic compounds such as fructooligosaccharides, oligofructose and plant extracts increased caecal butyrate concentration (Le Blay *et al.*, 1999; Cross *et al.*, 2005; Zdunczyk *et al.*, 2005). Therefore, the growth and activity of butyrate-producing bacteria in the caeca are likely promoted by the prebiotic plant extracts. *C. perfringens* is known to produce a large amount of butyrate from the fermentation of carbohydrates (Engberg *et al.*, 2002). However, *Cp* numbers were significantly lower in plant extract-supplemented groups, thus, other butyrogenic bacteria, which have not been enumerated in the current study, may be responsible for the observed butyrate production. A recent study found that the butyrate-producing bacteria in the ileum and the caeca of broilers were predominantly ruminococci, clostridia, *Enterococcus cecorum* and some other unidentified butyrate-producing bacteria (Gong *et al.*, 2002b).

Lactate was the most predominant organic acid in the ileal content while acetate was predominant in the caecal digesta. Lactate is an electron sink, further oxidized to other SCFAs such as acetate, propionate and butyrate in the caeca due to the longer retention time of the caeca digesta. As observed in the current study, a number of prebiotic oligosaccharides have been shown to decrease the caecal pH in poultry due to an increase in the concentration of organic acids such as acetate and butyrate (Terada *et al.*, 1994; Zdunczyk *et al.*, 2005). An increased SCFA production through bacterial fermentation of non-digestible carbohydrates such as oligosaccharides and the resulting decrease in intestinal pH, is an accepted mechanism for the inhibition of acidophobic bacteria such as

enterobacteria and *Cp* in broiler chickens (Terada *et al.*, 1994; Orban *et al.*, 1997). In the current study, the mean pH of the caecal contents decreased by 0.28 to 0.52 units in the groups consuming plant extracts in the diet. Therefore, such low pH values may have increased the undissociated form of organic acids in the caeca of birds fed plant extracts. This lower pH resulting from the fermentation of plant extracts may explain the lower numbers of *Cp* in the caecal contents of birds fed with plant extracts. Another possibility could be a CE effect resulting from the increased number of *Lactobacillus* species in plant extract-supplemented groups. A recent study indicated that acetate, isovalerate, and succinate decrease the sporulation of *Cp* while isobutyrate decreases the vegetative cell numbers as well as sporulation of this species (Wrigley, 2004).

An increased lactate production by the greater number of *Lactobacillus* in the ileum of *Undaria* extract and *Acacia* extract supplemented groups may be responsible for the reduced numbers of coliforms observed in the present study. Lactate is mainly produced by enterococci and lactobacilli, and increased lactate concentration has been found in intestinal and faecal contents of animals fed prebiotic oligosaccharides (Le Blay *et al.*, 1999). Lactate can inhibit the growth of many bacteria, including pathogenic Gram-negative organisms *in vitro* (Adams & Hall, 1988) and *in vivo* in broiler chickens (Byrd *et al.*, 2001). The early studies of Fuller (1977) demonstrated a relationship between increased number of lactobacilli and declining number of coliforms in the crop of conventional and gnotobiotic chicks. Apart from the above mentioned mechanisms, de Vaux *et al.* (2002) recently observed that *E. coli* growth could be inhibited completely by adding prebiotic carbon sources such as sorbitol, L-arabinose, rhamnose and trehalose into *in vitro* growth media.

5.4.3 Gut histomorphology

The villi and the crypts of Lieberkuhn play an important role in the digestion and absorption of nutrients in animals. The effects of prebiotics and bioactive compounds on epithelial morphology of broilers are variable. Some workers have observed that these additives may have a marked beneficial effect on the gut morphology (Iji *et al.*, 2001b; Xu *et al.*, 2003; Santos *et al.*, 2005), whereas others could not find any effect on the mucosal morphology (Owens *et al.*, 2003). These discrepancies in research findings may be attributed to the physico-chemical characteristics of different prebiotics, their level of incorporation in the diet, the duration of ingestion, the age of birds and site of activity (Montagne *et al.*, 2003). It has been hypothesised that the beneficial effects of prebiotic compounds on gut morphology

are due to indirect effects such as the production of SCFAs from the fermentation of prebiotics by beneficial gut microflora (Xu *et al.*, 2003; Santos *et al.*, 2005). However, in the current study, plant extracts did not have any positive effects on the ileal mucosal structure. In birds supplemented with high level of *Undaria* extract, the ileal villus height was significantly lower compared with the positive control group, and tended to be lower compared to the negative control. The same group also had deeper crypt depths compared to that of birds fed with diet containing Zn-bacitracin.

In the current study, dietary supplementation with Zn-bacitracin increased the villus height and villus height/crypt depth ratio in the ileum. Antibiotic-treated farm animals are known to have longer villi and villi/crypt depth ratios in the small intestine compared with their antibiotic-free counterparts (Nousiainen, 1991; Ao, 2004). Morphologically, long villi result in an increased surface area capable of greater absorption of nutrients. The presence of shorter and more tongue-shaped instead of finger-shaped villi may reduce the effective surface area for nutrient absorption, particularly at the villus tips (Jeurissen *et al.*, 2002). Therefore, the improvement in performance of the Zn-bacitracin-fed group in the present study could also be explained by the changes in the intestinal morphology.

5.5 CONCLUSIONS

In conclusion, plant extracts had no effect on growth performance of broilers during the 5 week experimental period. Growth suppression observed with high level of *Undaria* extract fed birds may be linked to an increased ileal digesta viscosity and a lower fat digestibility observed with this group. However, supplementation with *Acacia* extract (both levels) produced an improvement in FCR of broilers during the first three weeks, a feature that could be economically beneficial. Inclusion of plant extracts in broiler diets increased the populations of *Lactobacillus* and decreased the levels of *Cp* and coliforms which may have beneficial influence on birds' health. Dietary plant extracts did not affect the ileal and caecal populations of *Bifidobacterium* and counts were in general substantially low.

CHAPTER 6 MOLECULAR AND BIOCHEMICAL CHARACTERISATION OF LACTOBACILLI ISOLATED FROM ILEAL AND CAECAL DIGESTA OF BROILERS FED PREBIOTIC PLANT EXTRACTS

ABSTRACT

Molecular and biochemical characterisations were carried out for lactobacilli isolated from ileal and caecal digesta of broiler chickens fed with water-soluble prebiotic carbohydrate extracts (10 g/kg) obtained from Cabbage tree (Cordyline australis), seaweed, Undaria pinnatifida, and exudates from Acacia pycnantha. Genomic DNA was extracted from lactobacilli isolated from Rogosa agar; the 16-23S rDNA intergenic spacer regions were amplified and subjected to Amplified Ribosomal DNA Restriction Analysis (ARDRA) using HaeIII enzyme. Partial 16S rRNA gene sequences of major genotypic groups were determined and compared to sequences in the GeneBank using the Basic Local Alignment Search Tool (BLAST) algorithm. The ARDRA and partial 16S rRNA gene sequencing revealed four distinctive groups of lactobacilli: L. salivarius group (group I), L. crispatus group (group II), unidentified Lactobacillus species (group III), and L. johnsonii (group IV). These Lactobacillus species are dominant in the ileal and caecal digesta of broilers. The L. johnsonii group was mainly detected in the ileal and caecal digesta of the Acacia extract-supplemented group. The Ph-48 generalized PhenePlate system was used to test the fermentation characteristics of the four groups of lactobacilli. All four groups fermented the monosaccharide; galactose at varying levels and disaccharides; sucrose, trehalose, and palatinose. Sucrose was the substrate which was the most fermented by isolates from all four groups.

6.1 INTRODUCTION

The gut microflora of chickens is a complex ecosystem composed of a large variety of bacteria (Lan *et al.*, 2002; Zhu *et al.*, 2002; Lu *et al.*, 2003). It has been proposed that the gastrointestinal microflora of chickens comprises around 640 different species of bacteria, mostly unknown species which may play a role in the nutrition and health of the host

(Apajalahti *et al.*, 2004). The genus *Lactobacillus* is the predominant bacterial group in the ileum of chickens (Lu *et al.*, 2003), where they may be present at 10^8 to 10^9 CFU per gram of digesta (Engberg *et al.*, 2000; Knarreborg *et al.*, 2002b). The recent work of Guan *et al.* (2003) found that *L. gallinarum*, *L. crispatus*, *L. reuteri*, *L. salivarius*, and *L. johnsonii* are present in the crop throughout the life cycle of broilers raised under commercial farming conditions. *L. salivarius* is reported as the dominant *Lactobacillus* species in chicken intestinal samples (Engberg *et al.*, 2000).

The ability of selected gut microflora to use substrates not digested by the host may play an important role in their ability to successfully colonize the avian GIT. Furthermore, dietary composition directly influences the initial acquisition, developmental succession, and eventual stability of microflora in the alimentary tract of chickens (Mead, 1997; Rubio *et al.*, 1998; Apajalahti, 2005). Hence, there is considerable interest in understanding how the gut microflora can be modified, particularly through diet. Some dietary carbohydrates, that are selectively fermented by beneficial microorganisms in the lower part of the GIT, are generally known as prebiotics (Gibson & Roberfroid, 1995).

Prebiotics and bioactive compounds have been shown to beneficially modulate the composition of the gut microflora of chickens *in vivo* and specially to increase bifidobacteria and lactobacilli populations (Yusrizal & Chen, 2003b; Guo *et al.*, 2004b; Cao *et al.*, 2005). These beneficial bacteria are thought to create conditions unfavourable to the growth of pathogens, such as *C. perfringens* (La Ragione *et al.*, 2004), *E. coli* (Watkins *et al.*, 1982) and salmonella (Hinton *et al.*, 2002) in chicken GIT. As a result, the use of prebiotic compounds and of several *Lactobacillus* species have recently gained further interest in the poultry industry as an alternative to the use of in-feed antibiotic growth promoters (Jin *et al.*, 1998a; Dalloul *et al.*, 2003; Torres-Rodriguez *et al.*, 2005).

In the previous feeding experiment on caged broilers (Chapters 5) the inclusion of prebiotic plant extracts in the diets resulted in increased *Lactobacillus* counts in the ileal and caecal digesta (Vidanarachchi *et al.*, 2006). However, a substantially lower number of bifidobacteria was observed in the ileal and caecal contents of broilers in the same study (Chapter 5) than in other published data for broiler chickens (Vidanarachchi & Mikkelsen, 2006).

Culture-based techniques are used to isolate culturable bacteria from excreta or intestinal digesta. Upon isolation of bacterial colonies, it is important to identify the genus and further characterise species or strains (O'Sullivan, 2000). A number of DNA-based (genotypic) fingerprinting techniques have been developed for molecular characterization of gut microflora, and accurate identification of unknown isolates is now achieved by sequence analysis of 16S rRNA (Knarreborg *et al.*, 2002b; Zhu *et al.*, 2002; Guan *et al.*, 2003).

Measurement of specific metabolites produced by certain species of bacteria can indirectly give more information on the presence of specific microflora, or to be more precise, on the metabolic activities of specific groups of bacteria (O'Sullivan, 2000). The biochemical phenotyping of bacterial populations is a reliable tool that has been used to study the metabolic activities of bacteria by assessing their capacity *in vitro* to metabolize a number of substrates (Katouli *et al.*, 1997; Kuhn *et al.*, 2003). Many prebiotic compounds can be degraded by a variety of lactic acid bacteria (Kaplan & Hutkins, 2000; Kneifel *et al.*, 2000; Al-Tamimi *et al.*, 2006). Thus, correct identification and characterization of *Lactobacillus* species are of fundamental importance for screening prebiotic compounds. Furthermore, characterization of the metabolic potential of intestinal lactobacilli contributes to the understanding of important functional information on the fermentation characteristics of prebiotic compounds in intestinal habitats.

The aim of the present study was to perform a molecular characterization of lactobacilli isolated from ileal and caecal digesta of broiler chickens fed three different prebiotic plant extracts using Amplified Ribosomal DNA Restriction Analysis (ARDRA) and 16S rRNA gene sequence analysis and to evaluate the *in vitro* fermentative capacity of isolated lactobacilli using a generalized microplate (PhP-48) technique.

6.2 MATERIALS AND METHODS

6.2.1 Enumeration and isolation of lactobacilli

Enumeration of lactobacilli from ileal and cecal digesta samples was carried out as described in Section 5.2.6. Twenty randomly selected colonies from Rogosa agar from the highest dilution of samples from the treatments: negative control, and each of the water-soluble carbohydrate extracts (10 g/kg) supplemented with cabbage tree (*Cordyline australis*), a seaweed from *Undaria pinnatifida*, and exudates from *Acacia pycnantha* were carefully

isolated and sub-cultured in MRS broth (De Man, Rogosa, Sharpe; Oxoid, CM0359). Isolates were grown at 39°C in the anaerobic cabinet (Model SJ-3, Kaltec Pty. Ltd., Edwardstown, SA, Australia) and stored at -20°C in 30% (v/v) sterilized glycerol until further analysis. Five isolates from each replicate (30 isolates per treatment group) were randomly selected for molecular characterization of lactobacilli from the ileum (n = 120) and the caeca (n = 120).

6.2.2 Extraction of genomic DNA for PCR amplification

The isolates (240) were thawed, streaked on Rogosa agar (Oxoid, CM0627) and incubated at 39°C for 48 h in anaerobic jars (Oxoid Ltd, Hampshire, UK) in an anaerobic environment consisting of <1 % O_2 and 9-13 % CO_2, generated using anaerobic AnaeroGen™ sachets (AN0025A, Oxoid Ltd, Hampshire, UK). A single colony was then picked, inoculated to 10 mL MRS broth in a sterile screw cap tube. The cells were grown overnight at 39°C and 1.0 mL of bacterial suspension transferred into an Eppendorf tube and harvested by centrifugation (14,500 x *g*, 5 min) in an Eppendorf centrifuge (Eppendorf 5415 D, Eppendorf AG, Hamburg, Germany). The pellet was washed with 1.0 mL TES buffer (50 mM Tris-HCl, 50 mM NaCl, 5mM EDTA, pH 8.0) and resuspended in 0.5 mL TES buffer, with 5 µL of lysozyme (10 mg/mL) added and incubated at 37°C for 30 min. Subsequently, 5 µL each of proteinase K (10 mg/mL) and ribonuclease (10 mg/mL) were added and the mixture was incubated at 65°C for 60 min. Finally, lysis was achieved by addition of 50 µL 20 % (w/v) sodium dodecyl sulphate (SDS) and incubation at 65°C for 10 min. The cells were subjected to bead beating with ~ 0.5 g glass-beads cell disruption media (0.5-mm diameter) in a mini bead-beater (Disruptor Genie™, Scientific Industries Inc., New York, USA) at 5,000 x g for 5 min. After recovering the supernatants, precipitation and purification of DNA was carried out using the DNeasy® Tissue kit (QIAGEN Pty Ltd., Doncaster, VIC, Australia) following the manufacturer's instructions.

6.2.3 PCR amplification of 16-23S rDNA

The 16-23S rDNA (16S rRNA gene and the entire 16S-23S rRNA intergenic region) were amplified from DNA extracted from isolates by PCR using the primers Lb16a and 23-1B, (Table 6.1) reported by Guan *et al.* (2003). The reaction mixture (50 µL) contained a 2.5 mM deoxynucloside triphosphate (dNTP), 3.0 mM $MgCl_2$, 1.0 U/µL *Taq* (*Thermus aquaticus*) DNA polymerase supplied with the 10x PCR buffer (all from Fisher Biotec, West Perth, WA, Australia), 5 pmol/µL both forward and reverse primers (Proligo Australia Pty Ltd., Lismore,

NSW, Australia) (Table 6.1) and 2.0 µL purified template DNA sample. The reaction mixtures were amplified in an Eppendorf PCR Thermal Cycler (MasterCycler®, Eppendorf AG, Hamburg, Germany) under the following conditions: initial cycle of 1 min denaturation at 95°C, followed by 30 cycles of 30 sec denaturation at 95°C, 30 sec of annealing at 57°C and 45 sec elongation at 72°C, followed by a final extension of 10 min at 72°C. Amplified PCR products were electrophoresed on a 1 % agarose gel containing 5 µL of GelStar® nucleic acid gel stain (BioWhittaker Molecular Applications, Rockland, ME, USA), viewed by UV transillumination and digitised on an Infinity CN-3000 Gel Documentation System (Vilber Lourmat, Cedex, France).

Table 6.1 Primers used for amplification and sequencing of 16-23S rDNA

Primer[a]	Direction	Nucleotide sequence (5'→ 3')	Use
Lb16a	Forward	GTG CCT AAT ACA TGC AAG TCG	ARDRA
23-1B	Reverse	GGG TTC CCC CAT TCG GA	ARDRA
THOO8	Forward	AGR GTT YGA TTM TGG CTC AG	Sequencing
PH1522	Reverse	AAG GAG GTG ATC CAG CCG CA	Sequencing

[a]Guan *et al.* (2003) and Mikkelsen *et al.* (2003).

6.2.4 Amplified Ribosomal DNA Restriction Analysis (ARDRA) of 16-23S rDNA

The amplified 16-23S rDNA intergenic spacer regions of *Lactobacillus* isolates were digested with the restriction endonuclease *Hae*III enzyme (restriction enzyme isolated from *Haemophilus aegptius*) according to the manufacturer's instructions (New England BioLabs, Brisbane, QLD, Australia). *Hae*III restriction enzyme recognizes and cleaves directly the centre of the 5'....GG/CC....3', 3'....CC/GG....5' DNA sequence. Restriction digestion was carried out for 2 h at 37°C in 40 µL final volume containing 4 µL 10x buffer, 15 µL MilliQ water, 1 µL enzyme (10 U/µL) and 20 µL of amplified PCR product. Restriction digestion products were electrophoretically resolved in a 2 % agarose gel containing 5 µL of GelStar® nucleic acid gel stain (BioWhittaker Molecular Applications, Rockland, ME, USA) for 4 h at 90 V and band patterns were viewed by UV transillumination and digitised on an Infinity CN-3000 Gel Documentation System (Vilber Lourmat, Cedex, France). Infinity Capture version 12.6 for Windows software was used for image analysis.

6.2.5 Sequencing of 16S rRNA gene

Amplified PCR products generated with the primer pair TH008-PH1522 were purified and concentrated using the QIAquick® PCR purification kit (QIAGEN Pty Ltd., Doncaster, VIC, Australia) as described by the manufacturer. DNA concentrations of the purified products were determined with an ND-1000 NanoDrop Spectrophotometer (Biolab Ltd, Mulgrave, VIC, Australia) in order to adjust the DNA concentrations in sequencing mixtures. Partial sequences of 16S rRNA gene of lactobacilli isolates were determined with the primer TH008 (Table 6.1) and the sequencing reactions were performed with a GenomeLab™ Dye Terminator cycle sequencing quick start kit (Beckman Coulter, Inc., Fullerton, CA, USA) as described by the manufacturer. The reaction mixtures were amplified in an Eppendorf PCR Thermal Cycler (MasterCycler®, Eppendorf AG, Hamburg, Germany) under the following conditions: thirty (30) cycles of 20 sec denaturation at 96°C, 20 sec of annealing at 50°C and 4 min elongation at 60°C. Sequencing was carried out using the Beckman Coulter CEQ 8000 Genetic Analysis System and sequences were analysed using the CEQ 8000 software package (Beckman Coulter, Inc., Fullerton, CA, USA). Resulting 16S rRNA gene sequences were subjected to sequence comparisons in the GeneBank (National Center for Biotechnology Information, Bethesda, MD, USA) using the Basic Local Alignment Search Tool (BLAST) algorithm. The sequences of the 16S rRNA genes determined in this study were deposited with the GeneBank nucleotide database under the accession numbers DQ676989 for isolate group I, DQ676990 for isolate group II, DQ832760 for isolate group III, and DQ676991 for isolate group IV.

6.2.6 Fermentation characteristics of lactobacilli

Fermentation characteristics of each group of *Lactobacillus* species were performed with the Ph-48 generalized PhenePlate system (BactusAB, Huddinge, Sweden). This is based on interval measurements of colour changes, visualized by the pH indicator (bromothymol blue), resulting from bacterial metabolism of two sets of 46 freeze-dried substrates including low–molecular-weight carbohydrates (mono-, di-, and trisaccharides), carbohydrate derivatives (sugar alcohols, sugar acids, and glucosides), organic acids, urea, and ornithine (Table 6.2). Two control wells in each set contained only control buffers at pH 5.5 and pH 7.4 (Table 6.2). The PhP plates were filled with 150 µL suspending medium (BactusAB, Huddinge, Sweden) containing lactobacilli isolates. The plates were incubated anaerobically at 39°C and the intensity of each colour reaction was measured as absorbance at 620 nm (A_{620}) after 64 h with a microplate reader (Titertek Multiskan® Plus, Pathtech Digasnostic Pty Ltd., Balwyn, Victoria, Australia). The absorbance values were multiplied by 10, resulting in values ranging

from 0 to 30 for each reaction, where low values (0-12) indicate acidic reactions (green to yellow; pH < 6.6) and high values (12-30) indicate alkaline reactions (bluish green to deep blue; pH > 7.2). In the current study, four representative isolates from each lactobacilli group were analysed and each isolate was analysed in duplicate.

Table 6.2 Substrates/reagents used in the Ph-48 PhenePlate system for measuring the fermentation capacity of lactobacilli groups isolated from ileum and caeca of broiler chickens

1 Mannonic acid lacton	17 Adonitol	33 Arbutin
2 L-Arabinose	18 Inositol	34 β-Methyl-glucoside
3 D-Xylose	19 D-Arabitol	35 5-Ketogluconate
4 Galactose	20 Glycerol	36 Gluconate
5 Maltose	21 Maltitol	37 Melbionate
6 Cellobiose	22 Sorbitol	38 Galactouronic lacton
7 Trehalose	23 Dulcitol	39 Salicine
8 Palatinose	24 pH 7.4 Control	40 pH 5.5 Control
9 Sucrose	25 Sorbose	41 Citrate
10 Lactose	26 Deoxy-glucose	42 Fumarate
11 Melibiose	27 Deoxy-ribose	43 Malinate
12 Lactulose	28 Rhamnose	44 Malonate
13 Gentobiose	29 D-Fucose	45 Pyruvate
14 Melezitose	30 L-Fucose	46 L-Tartarate
15 Raffinose	31 Tagatose	47 Urea
16 Inosine	32 Amygdalin	48 Ornithine

6.3 RESULTS

Two hundred and forty *Lactobacillus* isolates from ileal and caecal digesta were identified using the *Hae*II-ARDRA method, and the results are summarised in Table 6.3. In the present study, distinct results of ARDRA analysis of the 16-23S rRNA genes divided the *Lactobacillus* isolates (240) into four genotypic groups (Plate 6.1): group I (161 isolates), group II (40 isolates), Group III (4 isolates), and group IV (33 isolates) (Table 6.3). The genotypic group I was the most abundant group of *Lactobacillus* species in both ileum and caeca. Genotypic group II was observed in ileal isolates of 4 (13%) from the negative control treatment, 11 (37%) from 10 g/kg cabbage tree extract-supplemented group, and 3 (10%) each from the 10 g/kg seaweed extract- and *Acacia* extract-supplemented groups. The genotypic groups III and IV were not detected in ileal and caecal digesta of birds fed with the negative control and 10 g/kg cabbage tree extract-supplemented diets. Of the 240 16-23S rDNA ARDRA profiles analysed, group IV was the most abundant in the 10 g/kg *Acacia* extract-supplemented group at 43% in the ileum and 63% in the caecum. The genotypic group III was found in one isolate in ileal and two isolates in caecal contents of 10 g/kg *Undaria* seaweed extract-supplemented group. From the caecal isolates (30) of 10 g/kg *Acacia* extract-fed group, only one isolate had ARDRA band patterns similar to genotypic group III. Two isolates from ileal content (Table 6.3) of 10 g/kg *Acacia* extract-fed group generated *Hae*III-ARDRA banding patterns that did not match any of the four genotypic groups reported in this study.

The partial sequence analysis of 16S rRNA gene in the four major types of ARDRA groups resulted in sequences with significant similarity (94 to 99%) to the 16S rDNA of known *Lactobacillus* species (Table 6.4). The group I isolates showed the closest relationship to *L. salivarius*, with high sequence similarities (>97%). The group II isolates had a high level of sequence similarities (>98%) to *L. crispatus*. Group III, which was observed only in four out of 240 isolates, showed a close relationship, with high levels of sequence similarities (98%) to an uncultured *Lactobacillus* species from chicken intestine (DQ057431) and comparatively low sequence similarities (94%) to a known species, *L. reuteri*. Additionally, the 16S rRNA gene sequences of this group are very similar to *L. vaginalis* (97%). The group IV isolates showed a close relationship, with high levels of sequence similarities, to *L. johnsonii* (99%).

Table 6.3 Distribution of major genotypic groups of lactobacilli isolated from ileum and caeca of broilers at d 35

Treatment	Groups in ileum				Groups in caeca			
	I	II	III	IV	I	II	III	IV
Negative control	26	4	0	0	23	7	0	0
10 g/kg Cabbage tree extract	19	11	0	0	24	6	0	0
10 g/kg Seaweed extract	25	3	1	1	27	1	2	0
10 g/kg Acacia extract	12	3	0	13	5	5	1	19

Table 6.4 BLAST search results for 16S rDNA sequences obtained for major genotypic groups of lactobacilli isolated from ileum and caeca of broiler chickens

Genotypic group	Closest relative in GeneBank (accession no.)	Percentage identity
Group I	*Lactobacillus salivarius* (DQ193532)	97%
Group II	*Lactobacillus crispatus* (AY335495)	98%
Group III	Uncultured bacterium from chicken intestine (DQ057431)	98%
Group IV	*Lactobacillus johnsonii* (AE017198)	99%

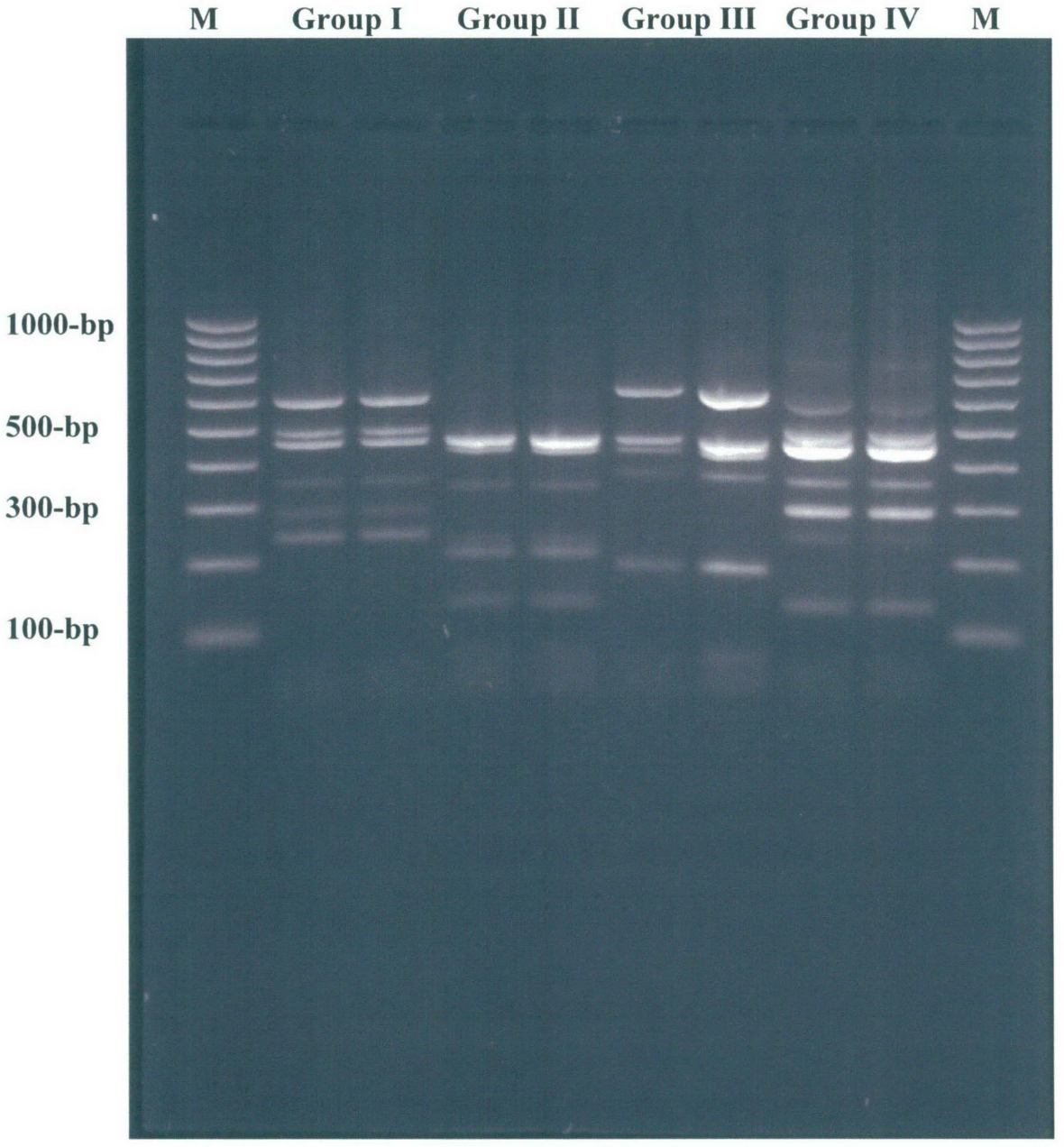


Plate 6.1 ARDRA patterns of 16-23S rRNA genes from lactobacilli isolated from ileum and caeca of broiler chickens

The amplified 16-23S rRNA gene fragments were digested with the restriction endonuclease *Hae*III enzyme and resolved by electrophoresis through a 2 % agarose gel. Lanes M, molecular weight marker (100-1000bp ladder).

The colony characteristics of four genotypic groups of lactobacilli isolated from the ileal and caecal digesta revealed different size, shape and appearance of surface colonies on MRS agar plates (Plate 6.2). Colonies of genotypic group I (*L. salivarius*) were large (4 to 6 mm in diameter), circular entire margins and glistening white with convex elevation. Surface colonies of group II appeared as circular, entire margins, smaller than group I (2 to 3 mm in diameter), occurred singly, glistening white, and showed convex elevation. Colonies of genotypic group III were usually small (1.5 to 2.0 mm in diameter), off-white, with entire margins. Genotypic group IV had slightly irregular margins and small off-white colonies (2 to 3 mm in diameter) with flat elevation. All of the isolates studied were Gram-positive and cells were short to long rods (0.5-1.0 μm in width and 2.0-8.0 μm in length) and occurred singly, in pairs, and some times in short chains.

The biochemical phenotyping results of the four groups of lactobacilli are shown in Table 6.5. All four groups fermented a monosaccharide, galactose and the disaccharides, sucrose, trehalose, and palatinose to varying degree. Sucrose was the substrate which was preferentially fermented by isolates from all four groups. Isolates from all groups, except those from group III, were able to ferment the disaccharides lactose and melibiose and the trisaccharide, raffinose. The group III isolates differed from other groups by their ability to ferment inosine and deoxy-ribose. Only group I and II isolates were able to ferment the disaccharide, maltose. The group IV isolates differed from other groups by their ability to ferment the disaccharide, cellobiose. The pentose sugar, arabinose was fermented at low levels by groups III and IV isolates.

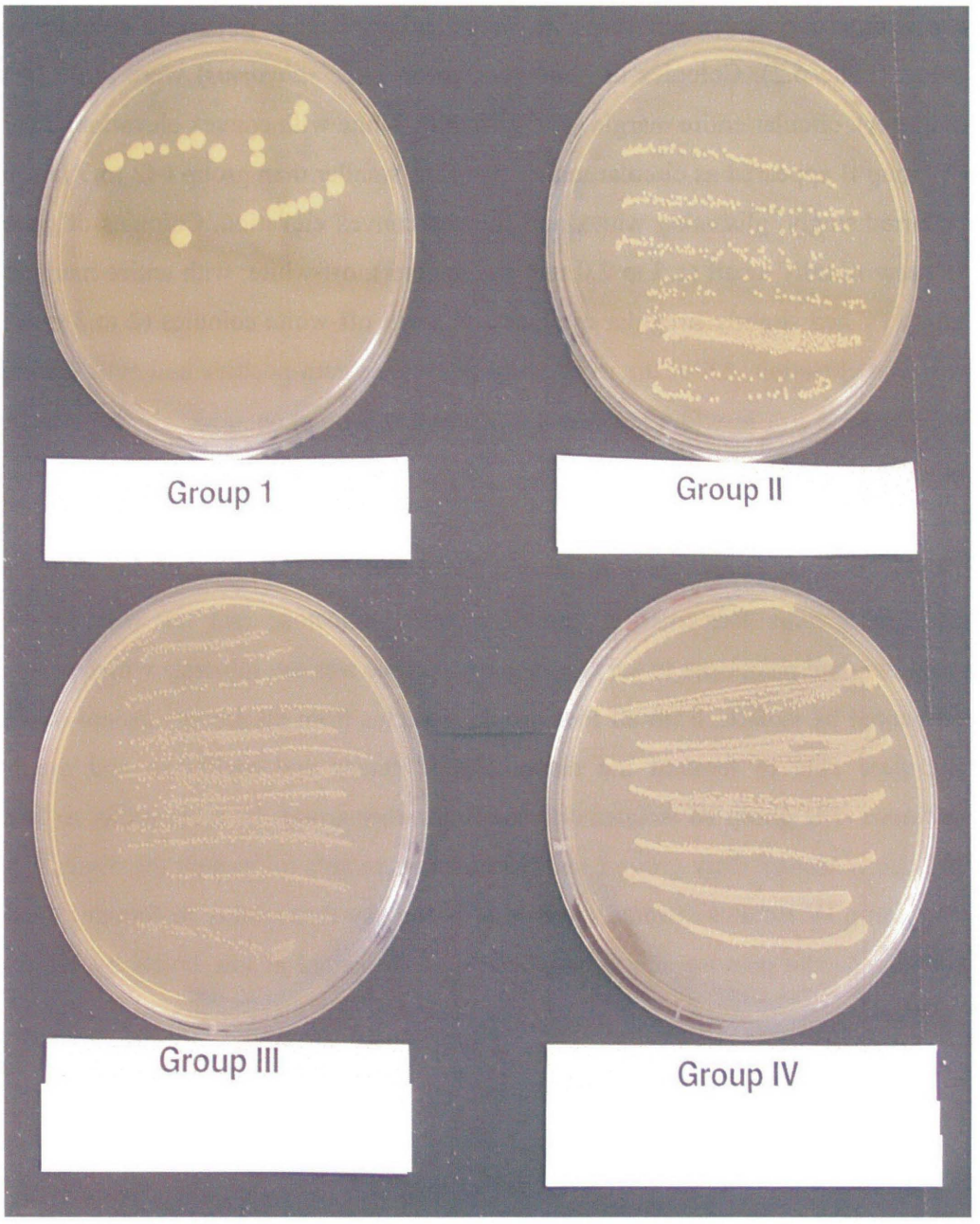


Plate 6.2 Colony characteristics of four genotypic groups of lactobacilli isolated from ileum and caecal digesta.

Table 6.5 Fermentation characteristics of four genotypic groups of lactobacilli isolated from broiler chickens[1]

Substrate	Lactobacilli isolates			
	Group I	Group II	Group III	Group IV
L-Arabinose	–	–	+/– (9.29)	+/– (9.98)
Galactose	+ (6.42)	+ (6.42)	+ (4.84)	+ (2.40)
Maltose	+ (3.15)	+ (3.30)	–	–
Cellobiose	–	–	–	+ (5.92)
Trehalose	+ (4.48)	+ (7.35)	+ (6.12)	+ (4.95)
Palatinose	+ (10.28)	+ (10.41)	+ (8.21)	+ (8.71)
Sucrose	+ (3.13)	+ (2.93)	+ (2.21)	+ (3.24)
Lactose	+ (8.10)	+ (7.35)	–	+ (6.07)
Melibiose	+ (8.66)	+ (7.68)	–	+ (7.40)
Raffinose	+ (6.03)	+ (6.97)	–	+ (9.04)
Inosine	–	–	+ (6.98)	–
Deoxy-ribose	–	–	+ (8.16)	–
D-fucose	–	+ (8.81)	–	+/– (10.19)
Amygdalin	–	+ (5.60)	–	–

[1]Four representative isolates from each group were analysed. +, ability to ferment indicated substrate; – inability to ferment indicated substrate; +/–, variable results within a group. Values in brackets are absorbance values 620 nm x 10; 25-30 = deep blue = pH >8.0; 18-25 = clear blue = pH 7.5; 12-18 = bluish green = pH 7.2; 7-12 = green = pH 6.6; 3-7 = yellowish green = pH 5.5; 0-3 = yellow = pH < 5.5.

None of the groups fermented mannonic acid lacton, D-xylose, lactulose, gentobiose, melezitose, adonitol, inositol, D-arabitol, glycerol, maltitol, sorbitol, dulcitol, sorbose, deoxy-glucose, rhamnnose, L-fucose, tagatose, arbutin, β-methyl-glucoside, 5-keto-gluconate, gluconate, melbionate, galactouronic lacton, salcine, citrate, fumarate, malinate, malonate, pyruvate, L-tatarate, urea and ornithine.

6.4 DISCUSSION

6.4.1 Molecular characterisation of lactobacilli

The *Hae*II restriction enzyme digestion of the amplified 16-23S rDNA intergenic region from the *Lactobacillus* isolates produced four different restriction patterns. Sequencing of representatives from these genotypic groups revealed that *L. salivarius*, *L. crispatus*, unidentified *Lactobacillus* species and *L. johnsonii* are dominant in the ileal and caecal digesta of broilers. Results from the present study are in agreement with the previous observations that *L. salivarius* is the predominant *Lactobacillus* spp. present in the intestinal tract of adult broilers (Engberg *et al.*, 2000). Similar to the findings in this experiment, Knarreborg *et al.* (2002b) detected four *Lactobacillus* species; *L. johnsonii*, *L. crispatus*, *L. salivarius* and *L. reuteri* in the ileum of broiler chickens. Knarreborg *et al.* (2002b) also reported that *L. johnsonii*, *L. reuteri* and *L. crispatus* were detected in all age groups, whereas *L. salivarius* was found only in 35-day-old broilers. Recent molecular characterisation has also revealed that the composition of lactobacilli in ileal digesta varies with the age of the broilers; predominantly *L. delbrueckii* at 3 days to *L. acidophilus* from d 7 to 21 and *L. crispatus* from d 28 to 49 (Lu *et al.*, 2003). These authors also observed that the predominant *Lactobacillus* species on d 49 is *L. salivarius* in ileal digesta. Results from the current study also revealed that *L. salivarius* was the predominant *Lactobacillus* species observed in ileal and caecal digesta of 35-day-old broilers.

L. salivarius is an obligatory homofermentative species which belongs to the *L. casei* group (Sarra *et al.*, 1985). In agreement with the findings of Sarra *et al.* (1985), the present study also showed that homofermentative lactobacilli are clearly predominant in the ileal and caecal digesta of broilers. Of the 240 isolates obtained from ileal and caecal digesta, 236 were homofermentative. The production of gas was observed only with the group III isolates during sub-culturing of bacteria and it is very likely attributed to the heterofermentation of carbohydrates which produce CO_2. Out of the four major genotypic groups observed in this study two groups (group II; *L. crispatus*, and group IV; *L. johnsonii*) belong to the *L. acidophilus* group (Klein *et al.*, 1998). According to the current reclassification of the *L. acidophilus* group, it can be divided into two DNA-homology groups (A and B) containing six related species; homology group A consist of *L. acidophilus* sensu stricto (A1 or Ia), *L. crispatus* (A2 or Ib), *L. amylovorus* (A3 or Ic), and *L. gallinarum* (A4 or Id); DNA homology

group B consists of *L. gasseri* (B1 or IIa) and *L. johnsonii* (B2 or IIb) (Fujisawa *et al.*, 1992; Klein *et al.*, 1998).

6.4.2 Fermentation characteristics of lactobacilli

The genus *Lactobacillus* contains non-spore-forming, Gram-positive and usually catalase-negative bacteria which are either facultative or obligate anaerobes (Kandler & Weiss, 1986; Klein *et al.*, 1998). Lactobacilli are extremely fastidious bacterial species which possess efficient carbohydrate fermentation pathways coupled to substrate-level phosphorylation and therefore can utilize various complex organic substrates (Kandler & Weiss, 1986; Axelsson, 1998). In general, the term homofermentative lactobacilli refers to those that use the glycolytic pathway (Embden-Meyerhof pathway) to generate lactate as the sole product of fermentation, whereas heterofermentative lactobacilli use the 6-phosphogluconate/phosphoketolase pathway (pentose phosphoketolase pathway) to produce a mixture of CO_2, ethanol, acetate and lactate (Kandler & Weiss, 1986; Axelsson, 1998). A variety of *Lactobacillus* species have been shown to utilize several prebiotic compounds such as arabinogalactans and fructooligosaccharides *in vitro* (Kaplan & Hutkins, 2000; van Laere *et al.*, 2000; Al-Tamimi *et al.*, 2006). Furthermore, recent *in vivo* studies have shown that some prebiotic and bioactive compounds may specifically stimulate intestinal lactobacilli (Konstantinov *et al.*, 2004; Humblot *et al.*, 2005).

The plant extracts which were used as dietary supplements in the current study mainly contain water-soluble carbohydrates with monosaccharides, disaccharides, oligosaccharides and polysaccharides (Section 3.3). Although it was not tested in this study, *Lactobacillus* spp. possess α- and β-glucosidase, α- and β-galactosidase and β-fructofuranosidase enzymes and thus are not only able to utilize simple sugars but also complex carbohydrates such as oligosaccharides and polysaccharides (Mital *et al.*, 1973; Li & Chan, 1983; Sasaki *et al.*, 1993; Otieno *et al.*, 2005). The majority of disaccharides and oligosaccharides are taken up by lactobacilli, with the help of specific permeases, and are split by the respective glycosidases prior to phosphorylation of the resulting monosaccharides (Kandler & Weiss, 1986; Sasaki *et al.*, 1993). Therefore, it could be suggested that the ability to hydrolyse various prebiotic carbohydrates in the plant extracts through the production of glycosidases by lactobacilli could lead to increase in the numbers of lactobacilli in ileal and caecal digesta.

Lactobacillus species belonging to four genotypic groups in the current study had some differences in fermenting various substrates in PhP plates. To my knowledge, this is the first study that used PhP plates for biochemical phenotypic characterization of lactobacilli isolated from broiler chickens. All four groups fermented galactose, a monosaccharide, and the disaccharides, sucrose, trehalose, and palatinose. Isolates from group I (*L. salivarius*) and II (*L. crispatus*) were able to ferment the disaccharide maltose. Li and Chan (1983) found intracellular α-glucosidase produced from *L. acidophilus* has a high specificity to hydrolyse maltose. Except for the genotypic group III (unidentified *Lactobacillus* species) all other groups were able to ferment the disaccharides, lactose and melibiose, and the trisaccharide, raffinose. The group I (*L. salivarius*) and II (*L. crispatus*) isolates differed from group III isolates (Unidentified *Lactobacillus* species) in their ability to ferment L-arabinose, inosine, and deoxy-ribose.

The major sugars present in the water-soluble carbohydrate extracts obtained from the cabbage tree shoots are fructose and glucose. Water-soluble carbohydrates from *Undaria* seaweed extract contain mainly fucose and galactose (Section 3.3). In the current study, all of the above-mentioned monosaccharides present in plant extracts, except fucose were fermented by isolates from all four groups of lactobacilli. *Lactobacillus* isolates which belong to group II (*L. crispatus*) and group IV (*L. johnsonii*) were able to ferment fucose. Hexoses other than glucose, such as galactose, mannose, and fructose, and disaccharides such as lactose, maltose, and sucrose are fermented by many lactic acid bacteria (Axelsson, 1998). Similar to findings in this experiment Klein *et al.* (1998) also found that disaccharides such as trehalose, melibiose and trisaccharides like raffinose are fermented by *L. acidophilus*-group species such as *L. crispatus* and *L. johnsonii*. The β-fructofuranosidase-positive *L. salivarius* was able to hydrolyse melibiose, raffinose, and stachyose (Mital *et al.*, 1973). Recently, Barrangou *et al.* (2003) confirmed that *L. acidophilus* has a gene locus which is important for regulation of complex carbohydrates such as fructooligosaccharide-degrading enzymes, β-fructosidase and sucrose phosphorylase. Complete genome sequencing of *L. acidophilus* revealed that it possesses a genome which encodes a large variety of genes related to carbohydrate utilization, including phosphoenolpyruvate sugar-transferase systems and five ABC families of transporters (Altermann *et al.*, 2005). Furthermore, these authors confirmed that *L. acidophilus* can utilize carbohydrates such as fructose, glucose, mannose, maltose trehalose, melibiose, cellobiose, raffinose, and fructooligosaccharides, because of the presence of the above-mentioned cellular transport systems.

According to the ARDRA patterns and gene sequence analysis results, the genotypic group IV (*L. johnsonii*) was predominantly detected in the ileal and the caecal digesta of *Acacia* extract-supplemented group. These results suggest that prebiotics such as arabinogalactans (*Acacia* extract) may have promoted the growth of *Lactobacillus* species *L. johnsonii*. It could be assumed that such specific groups would have fermented the water-soluble carbohydrates present in the *Acacia* extract. However, Pridmore *et al.* (2004) reported that *L. johnsonii* may not be able to depolymerize higher order complex polysaccharides due to lack of enzymes such as xylanases, amylases and arabinofuranosidases. Therefore, it is unclear how *L. johnsonii* numbers were increased in both ileal and caecal digesta of arabinogalactan-containing *Acacia* extract-supplemented groups. One possible explanation may be because of the presence of the heterofermentative bacterial group, *Bacteroides* or other *Lactobacillus* species, such as *L. reuteri* or *L. fermentum* in the GIT of broilers. These may initially hydrolyse the complex polysaccharides, arabinogalactans, into simple sugars such as mono- and di-saccharides. Such simple sugars which are mainly composed of arabinose and galactose may have been utilised by *L. johnsonii* as observed in this study. For example, Kneifel *et al.* (2002) observed excellent growth stimulation of *L. reuteri* when the growth medium contained galacto-oligosaccharides. *Acacia* extract is mainly composed of arabinogalactans, where linear chains of galactose monomer units are linked with β-(1→3)- and (1→6)- bonds. In a recent study, Al-Tamimi *et al.* (2006) found that arabino-oligosccahrides (arabinan) obtained from sugar-beet pulp can be degraded *in vitro* by lactobacilli. Van Laere *et al.* (2000) also found that arabinogalactan-enriched polysaccharides and arabinogalactooligosaccharides can be fermented by *L. acidophilus* and *L. fermentum* isolated from swine faeces. Pridmore *et al.* (2004) noted that *L. johnsonii* has the ability to ferment sugars such as ribose and possibly, arabinose and xylose due to the presence of gene loci that are important for the synthesis of aldolase epimerase and xylulose-5-phosphate phosphoketolase.

According to the previous classification of lactobacilli, based on sugar fermentation ability, *L. johnsonii* is categorised under the obligatory homofermentative group, meaning the hexose sugars can only be fermented through glycolysis and that pentoses cannot be utilised by this group (Kandler & Weiss, 1986). This classification is debatable because recent studies have shown that *Lactobacillus* species that are capable of fermenting hexoses are also able to utilize pentoses (Iyer *et al.*, 2000). Hence, it can also be argued that certain homofermentative *Lactobacillus* species are able to change from homolactic to heterolactic fermentation because of a shift in metabolic pathways in response to environmental conditions as suggested by Charteris *et al.* (1997).

6.5 CONCLUSIONS

In conclusion, lactobacilli isolated on Rogosa agar from the ileum and caeca of broilers fed prebiotic plant extracts could be identified using amplified ribosomal DNA restriction analysis (ARDRA) and partial sequencing of the 16S rRNA gene. The predominant *Lactobacillus* species in ileal and caecal digesta of broiler chickens fed prebiotic plants extracts were *L. salivarius*, *L. crispatus*, unidentified *Lactobacillus* species and *L. johnsonii*. The current study suggests that *L. salivarius* is the predominant *Lactobacillus* species in ileal and caecal digesta of 35-day-old broiler chickens. The results also indicated that supplementation with *Acacia* extract supports the growth *of L. johnsonii* in the ileum and caeca of broilers. The assimilation ability of carbon sources in the PhP plates were different among four genotypic groups of lactobacilli isolated from ileum and caeca of broilers.

CHAPTER 7 NATURAL PLANT EXTRACTS AND PREBIOTIC COMPOUNDS AS ALTERNATIVES TO ANTIBIOTICS IN BROILERS IN A NECROTIC ENTERITIS CHALLENGE MODEL

ABSTRACT

An experiment was conducted to determine the effects of two different water-soluble carbohydrate extracts (Rengarenga lily extract and Acacia extract), and two commercially available prebiotic compounds, Fibregum® and Raftifeed®-IPE, with similar chemical compositions to the plant extracts on performance, gut microflora composition, gut morphology and humoral immune responses of broiler chickens subjected to a necrotic enteritis (NE) challenge model involving oral inoculation with Clostridium perfringens (Cp). The plant extracts and prebiotic compounds were added (10 g/kg) to a wheat-based diet. There were three control treatment groups: basal diet without Cp challenge (unchallenged control), basal diet with Cp challenge (negative control) and basal diet supplemented with 45 ppm active ingredients of Zn-bacitracin and 100 ppm monensin (positive control). The diets were fed to six groups of 150 broilers (n = 6) for 5 weeks. All chicks, except the unchallenged control group were challenged on d 14, 15, and 16 with 3.5 x 10^8 CFU Cp by oral gavage. Addition of plant extracts or prebiotic compounds neither improved performance nor reduced NE lesion scores in disease-challenged groups. The birds on positive control diet showed better performance throughout the experimental period. An overall 8.8 % NE-related mortality was recorded, with mean jejunal and ileal lesion scores in dead birds ranging from 3.03 to 3.90 in all challenged groups except the positive control groups. Necrotic enteritis-specific deaths or clinical abnormalities were not observed with unchallenged control and positive control groups. Chi-square analysis revealed that the Fibregum-supplemented group had a lower (X^2 = 7.61, df = 1, P < 0.05) NE related mortality compared to the Acacia extract-supplemented group, but this mortality was not different (X^2 = 3.06, df = 1, P < 0.08) when compared to the negative control group. Fibregum decreased (P < 0.05) the Cp numbers in caecal contents before Cp challenge but this effect was not significant after Cp challenge, although the values tended to be lower than those of the negative control group. Birds fed the plant extracts and Fibregum had

higher ($P < 0.05$) Lactobacillus counts in the ileal digesta than those of the negative control group on d 21. At seven days post-challenge, the concentration of specific IgY antibodies against the α-toxin of Cp in the serum was lower ($P < 0.05$) in birds fed the positive control and Fibregum-supplemented diets in comparison with those from the negative control group. The total serum IgY response did not differ between treatment groups of chickens before challenge. However, birds fed Fibregum had increased ($P < 0.05$) IgM concentration compared to those fed with Acacia extract and lily extract. The Fibregum-fed group also had higher ($P < 0.05$) IgA levels in serum than the positive control and lily extract-supplemented groups at 14 days but this effect did not persist to 21 days. The results from this study demonstrated that supplementation with water-soluble carbohydrates from the two plant sources was not effective in controlling NE. However, the prebiotic compound Fibregum was found to be effective in reducing NE-associated mortality and can be considered a novel prebiotic with some immunomodulatory effects. Addition of Zn-bacitracin, and monensin, was highly effective in counteracting the negative effects of the disease challenge.

7.1 INTRODUCTION

Necrotic enteritis (NE), is an acute or chronic enterotoxaemia caused by *Cp* (van der Sluis, 2003; Williams, 2005). *C. perfringens* is a Gram-positive, obligate anaerobic, spore-forming bacterium readily found in soil, dust, used poultry litter and as a normal inhabitant of the gut microflora of healthy birds. Most often the only sign of an outbreak of clinical NE in broilers is a sudden increase in mortality which usually occurs from 2-5 weeks of age. Mortality rate within a flock is usually 2-10 % but can be as high as 40-50% (Hofacre, 2005). The proliferation of *Cp* in the intestine and increase in its toxin are considered the main cause of hemorrhagic necrosis of the intestinal mucosa. A subclinical form has been associated with increased FCR and retarded growth rate in birds (Kaldhusdal & Hofshagen, 1992). This disease has been reported in most areas of the world where broilers are produced under intensive management conditions, and it has significant economic impact on the poultry industry (van der Sluis, 2000b).

A variety of AGPs such as viginiamycin, bacitracin, penicillin and tylosin have been used in feed to effectively control and prevent NE (Watkins *et al.*, 1997; Collier *et al.*, 2003). However, many countries are moving towards a reduction in the use of AGPs in animal diets because large-scale use of antibiotics can cause resistant bacterial strains to develop (Barton,

1998). As a consequence, the use of most AGPs in poultry feed in Europe has been banned. However, this has resulted in problems such as increased mortality in poultry flocks, occurrence of ill-defined intestinal dysbacteriosis, a decline in bird welfare, an increase in the use of anticoccidial drugs and the incidence of food-borne human illness (Pattison, 2002; Casewell *et al.*, 2003). In the light of the situation where fewer, if any, antibiotics will be allowed in feeds in the future, it is important for the Australian poultry industry to find alternative ways to control NE.

Most strategies towards finding alternatives to AGPs aim at either modulating the natural bacterial population of the intestine in broilers through nutritional manipulation such as selection of specific feed ingredients or by using alternative feed supplements (Hofacre *et al.*, 2003; Mitsch *et al.*, 2004). Cross *et al.* (2004) found that caecal counts of *Cp* were reduced when broiler diets were supplemented with thyme and yarrow extracts. Recently, Mitsch *et al.* (2004) observed that supplementation with a specific blend of essential oil components can control *Cp* colonization and proliferation in the gut of broilers and therefore may be of help in preventing NE. Feeding oligosaccharides derived from the cell wall of the *Saccharomyces cerevisiae* (mannan-oligosaccharide), has also been reported to decrease mortality due to NE (Hofacre *et al.*, 2003).

It is believed that immune stimulation in meat-type chickens is important because these birds have lower antibody responses and non-specific proliferative responses compared to layer-type strains (Koenen *et al.*, 2004). Recently, McReynolds *et al.* (2004) reported that immunosuppression increases the severity of NE in broilers. Therefore, increasing the immunomodulating capacity of broilers to respond effectively to the diversity of antigens during early life is important. Guo *et al.* (2004a) observed that mushroom and herb extracts had significant impacts on the inductive immune responses against *E. tenella* infection in broilers, by enhancing both cellular and humoral immunity. Prebiotic compounds such as oligosaccharides may also act as immunomodulators at the intestinal level. Kudoh *et al.* (1999) reported that IgA secretion from caecal mucosa was promoted by orally administered highly fermentable, indigestible saccharides. Kleessen *et al.* (2003a) reported that fructans-rich Jerusalem artichoke syrup administered via drinking water resulted not only significant reduction in the numbers of *Cp* in caecal chyme, but also decreased the levels of microbial endotoxins in the blood of broilers. However, there is very little information available in relation to the influence of most of these prebiotic and bioactive substances on immune responses in chickens challenged with *Cp* or *Eimeria* spp. In view of the growing interest in

identifying potential alternatives for AGPs in poultry feed, testing novel forms of prebiotic and bioactive compounds is of utmost importance.

It was hypothesized in this study that dietary supplementation with *Acacia* extract, Rengarenga lily extract and commercially available prebiotic compounds with similar chemical composition to the plant extracts, Fibregum® and Raftifeed®-IPE, would exert prebiotic effects and thereby control NE in birds challenged with *Cp*. The objectives of the present study were to evaluate the effects of these prebiotic compounds on production performance, gut microflora composition, gut morphology and humoral immune responses of broiler chickens subjected to a NE disease challenge model involving oral inoculation with *Cp*.

7.2 MATERIALS AND METHODS

7.2.1 Bird husbandry

One thousand and fifty day-old male, commercial broilers (Cobb), vaccinated against Marek's disease, infectious bronchitis, and Newcastle disease were obtained from a local hatchery (Baiada hatchery, Kootingal, NSW, Australia). The research facility was thoroughly cleaned and disinfected prior to bird placement. Day-old chicks were placed in 42 floor pens in a semi-commercial broiler shed located at the University of New England (UNE) Kirby Research Station, Armidale, NSW, Australia. The pens were randomly assigned to seven treatments of six floor pens (replicates). Each pen (1.5 x 1.5 m) was stocked with 25 chicks. The pens were enclosed with metal plates at the sides. Between challenged and unchallenged pens, a space of at least two pens was kept empty to avoid cross-contamination. The shed temperature was set at 33-34°C during the first week and gradually decreased by 3°C per week until 24-25°C was reached by the third week. Relative humidity was between 65 and 70 %. Chicks were subjected to artificial fluorescent illumination of 23 h from 1 to 21 d of age, and 18 h from 22 to 35 d of age. Each pen was equipped with a separate feeding trough and water was supplied through nipple drinkers. Water and feed were provided *ad libitum*.

Weekly weight gain and FI per pen were measured and FCR, adjusted for mortality, calculated on a pen basis. Birds were observed twice daily for general health. All dead birds and culls (due to unhealthy condition), were weighed and necropsied.

7.2.2 Experimental diets

The composition of the basal diets is shown in Table 7.1. The seven treatment groups were: 1) an unchallenged group fed basal diet (UC; unchallenged control); 2) a challenged group fed basal diet (NC; negative control); 3) a challenged group fed basal diet supplemented with antibiotic (PC; positive control) (Zn-bacitracin, 45 mg/kg) and coccidiostat (monensin, 100 mg/kg); 4) a challenged group fed basal diet supplemented with 10 g/kg water-soluble carbohydrates from golden wattle exudate (*Acacia pycnantha*) (*Acacia* extract, 76 % (w/w) arabinogalactans); 5) a challenged group fed basal diet supplemented with 10 g/kg prebiotic arabinogalactan product Fibregum® (Fibregum, Colloïdes Naturels International, Rouen Cedex, France); 6) a challenged group fed basal diet supplemented with 10 g/kg water-soluble carbohydrates from Rengarenga lily (*Arthropodium cirratum*) rhizomes (Lily extract, 65 % (w/w) inulins), and 7) a challenged group fed basal diet supplemented with 10 g/kg prebiotic inulin compound, Raftifeed®-IPE (Raftifeed, Orafti active food ingredients, Tienen, Belgium). The extraction of water-soluble carbohydrates from the two plant sources has been described previously (Sections 3.2.1.2 and 3.2.1.3). Additions of plant extracts and prebiotic products were made in place of wheat, and all supplements were added to the respective diets for the entire experimental period at the inclusion rates indicated above. Raftifeed®-IPE contains mainly inulin (>700 g/kg fructo-oligosaccharides) extracted from chicory root and some glucose, fructose and sucrose (100 g/kg). The average chain length (degree of polymerisation) of the inulin in Raftifeed®-IPE is about 2-60. Fibregum® is a naturally-occurring arabinogalactan extracted from the exudate of *Acacia senegal*. The Fibregum® contained >900 g/kg arabinogalactans.

The basal diets were prepared by a commercial feed miller (Ridley Agriproducts Pty Ltd., Tamworth, NSW, Australia) with the same batch of ingredients and bulk-shipped to UNE. Upon arrival, the various supplements were properly mixed in according to the treatments, and then cold-pelleted (52-63°C).

Table 7.1 Composition (g/kg as received) of experimental diets

Ingredient (g/kg)	Starter (d1-7) and (d15-21)	Hi-Protein starter (d8-14)	Finisher (d22-d35)
Wheat	475	342	500
Oats	100	100	100
Wheat offal	--	40	--
Rice pollard	26	--	26
Tallow	35	--	62
Soybean meal (48% CP)	190	--	150
Meat-bone meal	80	--	75
Peas	75	--	72
Fish meal	--	500	--
Limestone (38 % Ca)	5.0	5.0	4.0
Sodium bicarbonate	3.4	3.4	2.0
Salt (NaCl)	1.0	1.0	1.5
Lysine-HCl	2.2	2.2	2.3
DL-methionine	3.3	3.3	2.5
L-threonine	1.0	1.0	0.3
Choline chloride	0.6	0.6	0.4
Premix[1]	2.0	2.0	2.0
Nutrient composition			
Metabolisable energy (MJ/kg)	12.8	12.3	13.4
Crude protein (g/kg)	230	448	210
Crude fat (g/kg)	69	72	94
Lysine (g/kg)	14	32	11
Methionine (g/kg)	6	13	5
Available phosphorous (g/kg)	4	17	4
Calcium (g/kg)	10	25	10

[1]Vitamin mineral premix (Ridley Agriproducts Pte Ltd., Tamworth, NSW) contained the following minerals in milligrams per kilogram of diet: Mn, 80; Zn, 60; Fe, 60; Cu, 8; I, 1.2; Co, 0.3; Se, 0.1; Mo, 1.0 and the following vitamins per kilogram of diet: Vitamin A, 12,000 IU from all *trans*-retinyl acetate; cholecalciferol D_3, 3.500 IU; vitamin E, 44.7 IU from DL-α-tocopherol; vitamin B_{12}, 12.75 μg; riboflavin, 6.0 mg; niacin, 50 mg; pantothenic acid, 12 mg; folic acid, 2 mg; biotin, 0.1 mg; thiamine, 2 mg; vitamin K, 2 mg and pyridoxine, 5 mg.

7.2.3 Necrotic enteritis challenge model

From one day after hatching until 7 d of age, the birds were fed with starter diets. From d 8 to d 14 prior to inoculation with *Cp*, the birds were fed a high-protein diet based on 50 % (w/w) fish meal (with the full dose of supplements). After d 14, the starter diets were returned until d 21. The starter feed was replaced by the finisher feed on d 22 and birds were kept until d 35. On d 9 all the birds, except those in the unchallenged controls, were given, *per os*, a suspension of 2,500 oocysts of *Eimeria acervulina*, *E. maxima* and *E. tenella* in 1 mL PBS. Unchallenged birds received sterile PBS. The *Eimeria* isolates originated from a commercial broiler farm and were obtained from Bioproperties Pty Ltd., Glenorie, NSW, Australia. The three species of *Eimeria* had been purified by serial passages through 3-week-old *Eimeria*-free chickens, and the sporulated oocysts were stored in 2 % (w/v) potassium dichromate at 10°C before inoculation. A primary poultry isolate of *Cp* type A was obtained from the CSIRO laboratory, Geelong, VIC, Australia, and maintained in thioglycollate broth (USP alternative, Oxoid, CM391) with 30 % (v/v) glycerol at -20°C. The challenge inocula were prepared fresh by growing the bacterium overnight at 39°C in 1000 mL of thioglycollate broth with added starch (10 g/L) and casitone (5 g/L). The stock culture of *Cp* had been previously subcultured in cooked meat media (Oxoid, CM81) and thioglycollate broth. On d 14, 15 and 16, birds in challenged groups were individually inoculated *per os* with 1 mL of *Cp* suspended in thioglycollate broth at a concentration of 3.5 x 10^8 CFU/mL, and birds in unchallenged cages were gavaged with 1 mL of sterile thioglycollate broth. Unchallenged birds were serviced first to lessen the likelihood of cross contamination and feed was withdrawn from all pens for 3 h on all days, prior to commencement of inoculation.

A gross pathologic diagnosis of NE in all dead birds and sampled birds was based on the presence of intestinal lesions typical of naturally occurring and experimentally induced NE, as described by Prescott *et al.* (1978) and Broussard *et al.* (1986): gas-filled small intestines, with confluent necrosis and sloughing of the mucosal surface of the intestinal tract which appeared as tan-orange pseudomembrane ("dirty turkish towel"-like appearance). The small intestine from each bird was incised longitudinally and examined for evidence of gross necrotic lesions. Small intestinal lesions were scored according to the criteria of Prescott *et al.* (1978) with slight modifications as illustrated in Plate 7.1. All birds were examined twice daily and all dead chickens were immediately collected for postmortem analysis.

7.2.4 Collection and processing of samples

On d 14 and 21, twelve chickens per treatment were randomly selected for necropsy. The intestines were removed aseptically. To synchronise the feeding pattern of the birds, light was switched off for 2 h, followed by at least 1 h of light before the chickens were sacrificed. Subsequently, the chest cavity and the abdomen were opened and the small intestine was ligated and removed from the bird. The contents of the ileum and caeca were collected by gently finger-stripping the respective intestinal segments into plastic containers. Around 0.2 g ileal and caecal contents were suspended in 0.8 mL of distilled water, and the pH was measured with a glass pH electrode (EcoScan 5/6 pH meter, Eutech Instruments Pte Ltd., Singapore) corrected for temperature. Remaining ileal and caecal digesta samples were frozen immediately after collection in order to measure organic acid contents (only on d 21). For histomorphological analysis, approximately 2.5 cm of the middle portion of the ileum was excised, flushed with PBS buffer (pH 7.6) and fixed in 10 % (v/v) neutral buffered formalin. The bursa of Fabricius and spleen were removed from 12 birds of each treatment at d 14 and 21 and weighed to the nearest gram. The data were expressed as a percentage of body weight.

7.2.5 Gut histomorphology

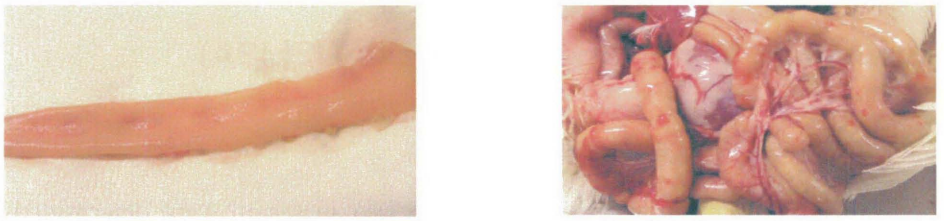
The morphology of the ileal tissues was determined as described in Section 4.2.12.

7.2.6 Enumeration of intestinal bacteria

Enumeration of lactobacilli, coliforms, lactose-negative enterobacteria and *C. perfringens* were carried out as described in Section 5.2.6. Total anaerobic bacteria were enumerated on Wilkins-Chalgren anaerobic agar (Oxoid, CM0619) after incubation at 39°C for 7 d using the Hungate roll-tube technique (Hungate, 1969). Bacterial numbers were expressed as $\log_{10}$ CFU/g digesta.

7.2.7 Measurement of organic acids

Short-chain fatty acids and organic acids in the ileal and caecal digesta were determined as described in Section 5.2.8.



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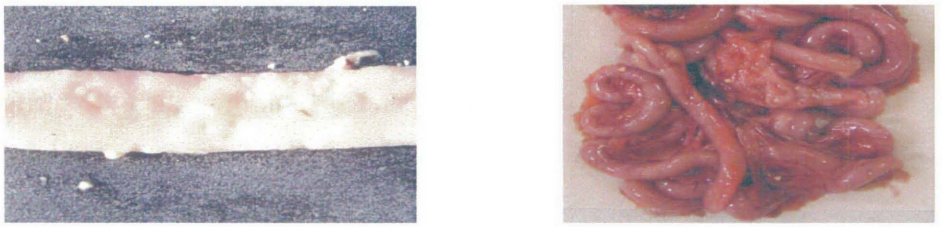

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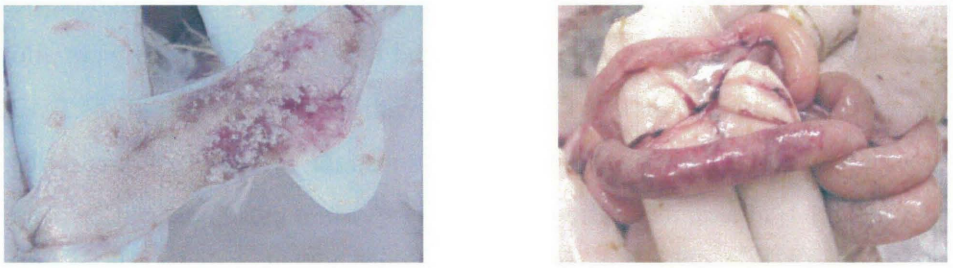

Score = 4

Plate 7.1 Gross appearance of the jejunal and ileum lesions, showing criteria for assigning necrotic enteritis scores

0 = no gross lesions, normal intestinal appearance; 1 = thin walled or friable and few whitish plaques in the serosal surface (mild); 2 = thin-walled, focal necrosis or ulceration, small amounts of gas production (moderate); 3 = thin-walled, large patches of necrosis, gas-filled intestine, small flecks of blood (marked/severe); 4 = severe extensive necrosis, marked hemorrhage, excessive amounts of gas in the intestine (very severe).

7.2.8 Enzyme-linked immunosorbent assay (ELISA)

Total serum antibody concentrations

Total antibody titre concentrations of IgY, IgM, and IgA in serum were determined before *Cp* challenge (14 d), and at 7 d after first challenge (21 d) using a sandwich ELISA. Blood samples were collected from the jugular vein into 7-mL serum tubes and clotted at room temperature (RT) (25°C) for 2 h, and serum was separated from the cells by centrifugation (Beckman Instruments Inc., Palo Alto, CA, USA) at 2,300 x *g* for 5 min and stored at -20°C.

Microtitre plates (96 wells, Nunc-Immuno Plate, Nunc A/S, Denmark) were coated with 100 µL per well of 0.05 M carbonate coating buffer (pH 9.6) containing 1 µL goat-anti-chicken coating antibody-Fc fragment (Bethyl Laboratories Inc., Montgomery, TX, USA), and incubated in the dark at room temperature for 1 h followed by three washes with washing buffer (50 mM Tris, 0.14 M NaCl, pH 8.0 containing 0.05 % (v/v) Tween 20). Blocking was performed with 200 µL of 1 % (w/v) bovine serum albumin (BSA, Sigma Chemicals, St. Louis, MO, USA) in Tris-NaCl buffer (pH 8.0) in each well, and the microtitre plates were left for 30 min in the dark at RT. The plates were washed 3 times with washing buffer, and the diluted sera (1:50,000, 1:10,000, and 1:2,000 for IgY, IgM, and IgA assays, respectively) or standard samples were applied to microtiter plates at 100 µL per well, incubated in the dark at RT for 1 h, and then washed five times with washing buffer. Thereafter, 100 µL of goat anti-chicken horseradish peroxidase-labelled conjugate (Bethyl Laboratories Inc., Montgomery, TX, USA) was added to each well, and the microtitre plates were allowed to incubate for a further 1 h in the dark. After washing with buffer (x5), 100 µL of TMB (*3, 3', 5, 5'*-tetramethylbenzidine) microwell peroxidase substrate system (0.4 g/L TMB in an organic base and H_2O_2 at a concentration of 0.02 % (v/v) in a citric acid buffer, Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA) was added to each well, and the plates were covered and allowed to incubate for 15 min at RT. The reaction was stopped by the addition of 100 µL of 2 M H_3PO_4 per well. The optical density (OD) was read at 450 nm with an ELISA Microplate Reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA). Antibody reagent dilutions and sample dilutions were carried out in 50 mM Tris, 0.14 M NaCl, pH 8.0 containing 1 % (w/v) BSA and 0.05 % (v/v) Tween 20. The standard chicken reference serum samples (Bethyl Laboratories Inc., Montgomery, TX, USA) were analysed in duplicate for each ELISA run. A standard curve expressing antibody level (ng) versus

absorbance was drawn, based on the overall run mean absorbance, from each dilution of the standard. Sample results were read only in the absorbance range where the standard curve was linear. Results were expressed as antibody titres in mg/mL of sera.

Specific IgY antibodies against C. perfringens α-toxin

The specific IgY antibodies against the α-toxin of *Cp* in blood serum were determined as described by Heier *et al.* (2001), with some modifications: briefly, the microtitre plates were coated with 1 μg of phospholipase C type I (7633; Sigma Chemicals) in 100 μL of carbonate buffer, pH 9.6 for 1 h at RT, washed three times with phosphate-buffered saline pH 7.4 with 0.05 % Tween 20 (PBST) and blocked with 200 μL of 1 % bovine serum albumin (BSA)/PBST for 1 h at RT. After washing as above, the plates were incubated with 100 μL of serum diluted 1:250 in PBST, washed and incubated with 100 μL horseradish peroxidase-labelled goat anti-chicken IgY (Bethyl Laboratories Inc., Montgomery, TX, USA) diluted 1:200 in 1 % BSA/PBST for 1 h at RT. After washing, the colour reaction was developed with 100 μL of *3, 3', 5, 5'*-tetramethylbenzidine. The reactions were stopped by adding 100 μL of 2M H_3PO_4 and the absorbance read at 450 nm using an ELISA microplate reader. At least two separate determinations were carried out for each serum sample, the samples being analysed in duplicate each time. Finally, results were expressed as average optical density values from two separate analyses.

7.2.9 Animal ethics

All experimental procedures were approved by the University of New England Animal Care and Ethics Committee (Approval No.: AEC05/109), and throughout the experiments, health and husbandry practices complied with the *Code of Practice for the Care and Use of Animals for Scientific Purposes* (National Health and Medical Research Council, 2004), for the Commonwealth of Australia and the *Australian Model Code of Practice for the Welfare of Animals: Domestic Poultry* (Primary Industries Standing Committee, 2002).

7.2.10 Statistical analysis

Bacterial counts were transformed to $\log_{10}$ values, and molar proportions of organic acids were subjected to arcsine transformation before statistical analysis; organic acids data are presented as natural numbers (Steel & Torrie, 1981). Each variable (except mortality data

and lesion scores) was analysed as a completely randomized design with a pen of broilers composing an experimental unit. The experiment consisted of 7 dietary treatments and data were analysed according to the following model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij};$$

where,

Y_{ij} = observed dependent variable;

μ = overall mean;

α_i = fixed effect of treatment, i = 1,2,3,4,5,6,7;

β_j = random effect of replicates, j = 1,2,3,4,5,6;

ε_{ij} = residual error for treatment 'i' of replicate 'j' ~ N (0, σ_ε^2).

All possible interactions were tested for significance ($P \leq 0.05$) and were eliminated from the models because they were not significant. The errors were assumed to be independently and normally distributed with a mean of zero and variance of σ_ε^2. All data were analysed by using ANOVA option of PROC MIXED procedure (SAS Institute Inc., 2000). Treatment least-squares means were compared using predetermined contrasts and considered significant at $P \leq 0.05$. Results are reported as least-squares means (n = 6) and pooled standard error (SE). The mortality data were compared using a chi-square test.

7.3 RESULTS

7.3.1 Bird performance

The mean BWG, FI, and FCR of chickens fed the experimental diets are shown in Table 7.2. There were no differences in growth performance between plant extracts- or prebiotics-supplemented groups challenged with *Cp* and the negative control group during the entire experimental period. The BWG of challenged chickens fed the control diet and plant extracts/prebiotic diets was markedly reduced compared to the unchallenged control group. This effect was most pronounced in the first three weeks. At the end of the five-week period, BWG was decreased by 14 % ($P < 0.0001$) in the negative control group compared with the unchallenged control group. Over the same period, FCR was impaired by 16 points due to *Cp* challenge in the negative control group. The FCR of the unchallenged control group was similar to that of broilers fed the positive control diet during the fourth week of experiment. Over the whole experimental period, there were no treatment effects on cumulative FI of birds.

Table 7.2 Mean body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) of broiler chickens fed diets supplemented with plant extracts and prebiotic compounds[2]

Treatment	Body weight gain (BWG; g/bird)			Feed Intake (FI; g/bird)			Feed conversion ratio (FCR)		
	d 1 to 21	d 21 to 35	d 1 to 35	d 1 to 21	d 21 to 35	d 1 to 35	d 1 to 21	d 21 to 35	d 1 to 35
Unchallenged-control (UC)	837	1068	1993	1177	2242	3551	1.41	2.10	1.78
Negative-control (NC)	690	902	1741	1055	2065	3366	1.53	2.32	1.94
Positive-control	805	1128	1998	1092	2113	3296	1.36	1.88	1.65
Acacia extract	725	969	1817	1099	2119	3611	1.52	2.20	1.99
Fibregum	713	941	1841	1069	2148	3534	1.50	2.29	1.92
Lily extract	692	882	1740	1087	2165	3533	1.57	2.48	2.03
Raftifeed	684	906	1712	1036	2066	3300	1.51	2.29	1.93
S.E.M.[1]	14.35	36.71	44.47	23.59	48.71	112.43	0.02	0.09	0.05
Orthogonal contrasts	Probability level of contrasts								
UC vs. NC	<0.0001	0.002	<0.0001	0.0003	0.01	NS	0.0002	NS	0.02
NC vs. PC	<0.0001	<0.0001	<0.0001	NS	NS	NS	<0.0001	0.0005	<0.0001
NC vs. *Acacia* extract	NS	NS	NS	NS	NS	NS	NS	NS	NS
NC vs. Fibregum	NS	NS	NS	NS	NS	NS	NS	NS	NS
NC vs. Lily extract	NS	NS	NS	NS	NS	NS	NS	NS	NS
NC vs. Raftifeed	NS	NS	NS	NS	NS	NS	NS	NS	NS

[1]S.E.M. = Pooled standard error of the least square means. [2]Results are given as least square means (n = 6).

However, FI of challenged birds during the first three weeks was reduced ($P < 0.0003$) compared to that of the unchallenged control group. The addition of Zn-bacitracin and monensin increased ($P < 0.05$) average BWG and improved FCR throughout the experimental period. At d 21 and d 35, broilers fed positive control diet had a 17 % and 15 % higher BWG, respectively, than chickens from the negative control group.

Mortality data and the incidence of jejunal and ileal lesion scores in broiler chickens which died due to NE are depicted in Table 7.3. Mortality resulting from NE started at 1 d after birds was first challenged with *Cp*. Total necrotic enteritis-associated (NEA) mortalities of 8.8 % were observed for the whole experimental period. Necrotic enteritis-associated mortalities occurred during the first four days and continued for a maximum of 7 days after the first challenge began (Figure 7.1). Necrotic enteritis-specific deaths or clinical abnormalities were not observed with unchallenged control and positive control groups. A chi-square analysis revealed that a highly significant difference ($X^2 = 51.68$, df = 6, $P < 0.0001$), existed in mortality among the seven treatment groups (Table 7.3). However, chi-square analysis excluding unchallenged control and positive control groups demonstrated no difference ($X^2 = 8.44$, df = 4, $P < 0.08$) in mortality among the other five treatment groups. The highest NEA mortality, 18.2 %, was observed in the *Acacia* extract-supplemented group. Similar NEA mortality percentages occurred in birds fed with lily extract (18/146), and those supplemented with Raftifeed (16/148). Among the treatment groups which had mortality due to NE, only the Fibregum-supplemented group had a lower ($X^2 = 7.61$, df = 1, $P < 0.006$) mortality rate than the *Acacia* extract-supplemented group. However, the mortality rate in Fibregum fed group was not different ($X^2 = 3.06$, df = 1, $P < 0.08$) from that of the negative control group. The non-NE-related mortalities for the entire experimental period were 6.2 %. The main causes of non-NEA mortalities in this experiment were due to acute death syndrome and ascites. Until seven days after first inoculation, most of the birds in all challenged groups showed depression, anorexia and huddled together. Birds that died due to severe NE (lesion score 4) had friable and distended intestines filled with gas and focal to massive necrosis and sloughing of the mucosal surface of the intestinal tract which appeared as tan-orange pseudomembrane (diphtheritic membrane), caused by the α- and β-toxins. Moreover, there was often a greenish tinge in the small intestine of birds that died from NE. There were no significant differences in jejunal and ileal scores in birds which died due to NE.

Table 7.3 Mortality percentages and jejunal and ileal lesion scores in broiler chickens which died due to necrotic enteritis

Treatment	No. of Birds[1]	NE Mortality % (dead/total)	Distribution of jejunal lesion scores in dead birds[2]					Distribution of ileal lesion scores in dead birds[2]					Mean jejunal lesion score in dead birds	Mean ileal lesion score in dead birds
			0-1.0	1.0-1.9	2.0-2.9	3.0-3.9	≥4	0-1.0	1.0-1.9	2.0-2.9	3.0-3.9	≥4		
Unchallenged-control (UC)	146	0 (0/146)	0	0	0	0	0	0	0	0	0	0	0.00	0.00
Negative-control (NC)	145	13.8 (20/145)	1	2	3	6	8	1	3	1	7	8	3.18	3.18
Positive-control	147	0 (0/147)	0	0	0	0	0	0	0	0	0	0	0.00	0.00
Acacia extract	148	18.2 (27/148)	1	1	4	16	5	2	3	3	15	4	3.30	3.90
Fibregum	147	7.5 (11/147)	0	0	2	7	2	0	0	1	7	3	3.34	3.36
Lily extract	146	12.3 (18/146)	1	2	4	9	2	1	5	2	8	2	3.03	3.24
Raftifeed	148	10.8 (16/148)	0	0	2	9	5	1	0	3	8	4	3.31	3.06

[1]Birds that died before *C. perfringens* challenge were subtracted from the total number of birds (150) in each treatment group.

[2]Jejunal and ileal lesion scores were scored on a 0-4 scale where: 0 = no gross lesions, normal intestinal appearance; 1 = thin walled or friable and few whitish plaques in the serosal surface (mild); 2 = thin-walled, focal necrosis or ulceration, small amounts of gas production (moderate); 3 = thin-walled, large patches of necrosis, gas-filled intestine, small flecks of blood (marked/severe); ≥4 = severe extensive necrosis, marked hemorrhage, excessive amounts of gas in the intestine (very severe).

The mean jejunal and ileal lesion scores ranged from 3.03 to 3.90 in all challenged groups except in positive control groups. Lesions were absent when the birds were sampled seven days post challenge.

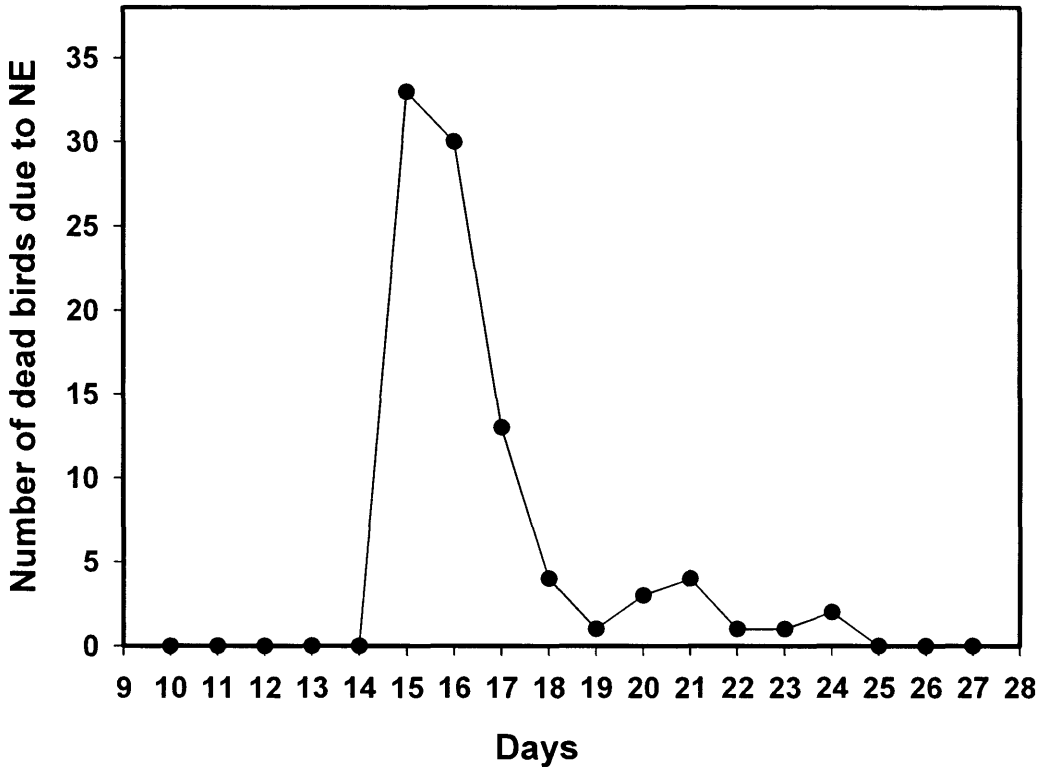

Figure 7.1 Mortality pattern of broiler chickens challenged with *C. perfringens*

7.3.2 Gut histomorphology

Results from the histomorphological analysis of ileal segments on d 14 and d 21 are presented in Table 7.4. Supplementation the diets with plant extracts or prebiotic compounds had no effect on villus height or crypt depth of the ileum compared to the negative control group on either sampling day. At d 14 and 21, the birds fed the positive control diet had longer villi, shallower crypts and greater villus/crypt ratio in the ileum compared to the negative control group. However, there was no difference ($P > 0.05$) in villus heights of all prebiotic and plant extracts supplemented groups compared to the positive control group before *Cp* challenge. In the current study, the villus height and crypt depth of unchallenged control birds and negative control birds were different ($P < 0.05$) on d 21 (after challenge).

Table 7.4 Ileal morphometric parameters of birds fed diets supplemented with plant extracts and prebiotic compounds at d 14 and d 21[2]

Treatment	Before challenge (d 14)			After challenge (d 21)		
	Villus height (μm)	Crypt depth (μm)	Villus/Crypt ratio	Villus height (μm)	Crypt depth (μm)	Villus/Crypt ratio
Unchallenged-control (UC)	611	82	7.54	705	75	9.36
Negative-control (NC)	605	81	7.68	624	85	7.39
Positive-control	702	62	11.54	720	73	9.92
Acacia extract	641	79	8.25	631	88	7.19
Fibregum	657	78	8.43	658	93	7.13
Lily extract	636	87	7.43	631	88	7.24
Raftifeed	664	75	8.93	663	91	7.34
S.E.M.[1]	22.50	4.26	0.56	25.14	2.97	0.42
Orthogonal contrasts	Probability level of contrasts					
UC vs. NC	NS	NS	NS	0.02	0.01	0.0001
NC vs. PC	0.005	0.004	<0.0001	0.01	0.006	0.0002
NC vs. *Acacia* extract	NS	NS	NS	NS	NS	NS
NC vs. Fibregum	NS	NS	NS	NS	NS	NS
NC vs. Lily extract	NS	NS	NS	NS	NS	NS
NC vs. Raftifeed	NS	NS	NS	NS	NS	NS

[1]S.E.M. = Pooled standard error of the least square means [2]Results are given as least square means (n = 6).

The histomorphological analysis of ileal tissues on 21 d also revealed that detachment and disruption of the normal apical microvilli of enterocytes, which is characteristic of NE, was observed in two sections out of the gut.

7.3.3 Ileal and caecal microflora

The results of the bacterial counts in ileal and caecal contents are shown in Tables 7.5, 7.6, 7.7, and 7.8. In general, coliforms, lactose-negative enterobacteria and *Cp* counts were higher in both ileal and caecal contents on d 14 (before challenge) than d 21. Total anaerobic counts in caecal digesta were decreased ($P < 0.05$) by ~0.5 log unit in birds fed positive control vs. negative control, on d 14. Compared with the negative control group, no difference ($P > 0.05$) was detected among various dietary treatment groups with respect to total anaerobic bacterial numbers in ileal and caecal contents on d 21 (Table 7.7 and Table 7.8).

The *Lactobacillus* counts in ileal and caecal digesta on d 14 tended to be higher in *Acacia* extract-, lily extract- and Raftifeed-supplemented treatment groups compared to the negative control group. Birds fed with the same supplements had higher ($P < 0.05$) *Lactobacillus* counts in ileal digesta than those of the negative control group on d 21. Furthermore, birds supplemented with *Acacia* extract had higher ($P < 0.05$) counts of lactobacilli in caecal digesta on d 21 compared to the negative control group. The counts of lactobacilli decreased by 9.5- and 8.3-fold in the ileal digesta of positive control vs. negative control groups on d 14 and 21, respectively.

Compared to the negative control group, ileal and caecal lactose-negative enterobacteria numbers were not different between dietary treatment groups. Both *Acacia*- and Fibregum-fed groups had lower ($P < 0.05$) coliform numbers in ileal and caecal digesta on d 14. Neither the ileal nor caecal coliform counts were affected by dietary supplementation of plant extracts or prebiotic compounds on d 21.

C. perfringens counts in ileal and caecal digesta in all treatment groups, except in the positive control group before *Cp*-challenge were higher than those after *Cp*-challenge.

Table 7.5 Bacterial counts in ileal digesta of birds fed diets supplemented with plant extracts and prebiotic compounds at d 14 (before challenge)[2]

Treatment	Total anaerobic bacteria	Lactobacilli	Lactose-negative enterobacteria	Coliform bacteria	*Clostridium perfringens*
	Log_{10} CFU/g digesta				
Unchallenged-control (UC)	9.76	7.19	7.55	8.76	9.00
Negative-control (NC)	9.72	7.64	7.38	8.72	9.03
Positive-control	9.31	6.66	6.95	8.75	3.53
Acacia extract	9.64	8.24	7.00	8.21	9.29
Fibregum	9.30	7.29	6.78	8.18	8.35
Lily extract	9.53	8.00	6.82	8.72	9.03
Raftifeed	9.86	8.08	7.18	8.90	9.04
S.E.M.[1]	0.16	0.24	0.28	0.17	0.26
Orthogonal contrasts	Probability level of contrasts				
UC vs. NC	NS	NS	NS	NS	NS
NC vs. PC	NS	0.006	NS	NS	<0.0001
NC vs. *Acacia* extract	NS	NS	NS	0.04	NS
NC vs. Fibregum	NS	NS	NS	0.02	NS
NC vs. Lily extract	NS	NS	NS	NS	NS
NC vs. Raftifeed	NS	NS	NS	NS	NS

[1]S.E.M. = Pooled standard error of the least square means. [2]Results are given as least square means (n = 6).

Table 7.6 Bacterial counts in caecal digesta of birds fed diets supplemented with plant extracts and prebiotic compounds at d 14 (before challenge)[2]

Treatment	Total anaerobic bacteria	Lactobacilli	Lactose-negative enterobacteria	Coliform bacteria	*Clostridium perfringens*
	Log_{10} CFU/g digesta				
Unchallenged-control (UC)	10.78	8.28	8.34	9.51	7.89
Negative-control (NC)	10.51	8.26	8.11	9.55	8.88
Positive-control	10.07	8.36	8.26	9.28	3.50
Acacia extract	10.29	8.98	7.70	8.90	8.13
Fibregum	10.27	8.25	7.73	8.85	6.97
Lily extract	10.51	8.65	8.06	9.15	8.09
Raftifeed	10.31	8.71	7.73	9.27	8.13
S.E.M.[1]	0.09	0.32	0.26	0.17	0.37
Orthogonal contrasts	Probability level of contrasts				
UC vs. NC	NS	NS	NS	NS	NS
NC vs. PC	0.002	NS	NS	NS	<0.0001
NC vs. *Acacia* extract	NS	NS	NS	0.005	NS
NC vs. Fibregum	NS	NS	NS	0.003	0.001
NC vs. Lily extract	NS	NS	NS	NS	NS
NC vs. Raftifeed	NS	NS	NS	NS	NS

[1]S.E.M. = Pooled standard error of the least square means. [2]Results are given as least square means (n = 6).

Table 7.7 Bacterial counts in ileal digesta of birds fed diets supplemented with plant extracts and prebiotic compounds at d 21 (after challenge)[2]

Treatment	Total anaerobic bacteria	Lactobacilli	Lactose-negative enterobacteria	Coliform bacteria	*Clostridium perfringens*
	Log_{10} CFU/g digesta				
Unchallenged-control (UC)	9.08	8.23	6.92	7.99	6.11
Negative-control (NC)	9.06	8.36	6.98	7.82	6.70
Positive-control	8.42	7.44	6.39	7.59	3.33
Acacia extract	8.98	8.83	7.00	7.74	6.44
Fibregum	9.02	8.34	6.99	7.13	6.58
Lily extract	8.92	8.94	7.04	7.50	6.80
Raftifeed	8.73	8.90	6.99	7.56	6.74
S.E.M.[1]	0.16	0.16	0.32	0.31	0.32
Orthogonal contrasts	Probability level of contrasts				
UC vs. NC	NS	NS	NS	NS	NS
NC vs. PC	NS	0.0002	NS	NS	<0.0001
NC vs. *Acacia* extract	NS	0.04	NS	NS	NS
NC vs. Fibregum	NS	NS	NS	NS	NS
NC vs. Lily extract	NS	0.01	NS	NS	NS
NC vs. Raftifeed	NS	0.02	NS	NS	NS

[1]S.E.M. = Pooled standard error of the least square means. [2]Results are given as least square means (n = 6).

Table 7.8 Bacterial counts in caecal digesta of birds fed diets supplemented with plant extracts and prebiotic compounds at d 21 (after challenge)[2]

Treatment	Total anaerobic bacteria	Lactobacilli	Lactose-negative enterobacteria	Coliform bacteria	*Clostridium perfringens*
	Log_{10} CFU/g digesta				
Unchallenged-control (UC)	10.25	9.29	7.17	9.02	6.44
Negative-control (NC)	10.12	9.23	7.22	9.03	6.50
Positive-control	9.99	8.67	6.98	8.57	3.87
Acacia extract	9.87	9.80	7.16	8.78	6.67
Fibregum	10.09	9.54	7.30	8.59	6.00
Lily extract	10.17	9.69	7.25	8.97	6.79
Raftifeed	9.96	9.42	7.43	8.95	6.36
S.E.M.[1]	0.11	0.19	0.23	0.19	0.40
Orthogonal contrasts	Probability level of contrasts				
UC vs. NC	NS	NS	NS	NS	NS
NC vs. PC	NS	0.04	NS	NS	<0.0001
NC vs. *Acacia* extract	NS	0.04	NS	NS	NS
NC vs. Fibregum	NS	NS	NS	NS	NS
NC vs. Lily extract	NS	NS	NS	NS	NS
NC vs. Raftifeed	NS	NS	NS	NS	NS

[1]S.E.M. = Pooled standard error of the least square means. [2]Results are given as least square means (n = 6).

The mean *Cp* count in the ileum decreased from 8.96 to 6.56 log10 CFU/g of digesta in all treatment groups 7 d after first *Cp*-challenge. Corresponding values for caecal contents decreased from 8.02 to 6.46 log10 CFU/g of digesta. Fibregum decreased ($P < 0.05$) the *Cp* numbers in caecal contents before *Cp*-challenge but this effect was not significant after *Cp*-challenge, although the values tended to be lower in that group as compared to the negative control group. The Raftifeed and two plant extracts did not affect ($P > 0.05$) *Cp* populations before and after *Cp*-challenge. The *Cp* counts in luminal contents from the ileum and caeca of birds fed the positive control diet was significantly lower than those from the negative control groups at both sampling days.

7.3.4 Organic acids and pH

The ileal pH and molar proportions of organic acids in 21-day-old broilers are shown in Table 7.9. The positive control group had higher ($P < 0.05$) ileal digesta pH compared to the negative control group. The same group also tended to have lower ileal total organic acids compared to plant extract-supplemented groups and a lower ($P < 0.05$) molar proportion of lactate compared to the negative control group. The concentration of total ileal organic acids in the lily extract-fed group was higher ($P < 0.05$) than that of the negative control group. Table 7.10 shows the caecal pH and organic acid concentrations at d 21. Dietary supplementation with *Acacia* extract and Raftifeed resulted in lower ($P < 0.05$) pH in the caecal digesta compared to the negative control group. The total organic acids and molar proportions of organic acids in the caecal digests were not affected ($P > 0.05$) by the experimental diets.

7.3.5 Lymphoid organ weights and humoral immune responses

The indices of two immune organs are shown in Figure 7.2. There were no differences in relative weights of spleen or bursa resulting from supplementation by either of the two plant extracts or of Raftifeed on either sampling day (before and after challenge). However, the relative weight of bursa of birds supplemented with Fibregum was higher ($P < 0.05$) than that of the negative control group on d 21.

Table 7.9 pH and molar proportions of various organic acids in ileal digesta of 21 d-old broiler chickens fed the experimental diets[2]

Treatment	pH	Total organic acids (μmol/g digesta)	Molar proportions of organic acids	
			Acetate	Lactate
			mole/100 mole	
Unchallenged-control (UC)	7.85	9.2	29.3	70.7
Negative-control (NC)	7.58	12.8	20.7	79.4
Positive-control	8.39	3.6	41.4	58.6
Acacia extract	7.26	34.8	12.3	83.8
Fibregum	7.59	32.4	12.0	88.0
Lily extract	7.47	41.6	11.4	80.8
Raftifeed	7.41	16.7	13.0	72.4
S.E.M.[1]	0.13	8.80	4.10	5.33
Orthogonal contrasts	Probability level of contrasts			
UC vs. NC	NS	NS	NS	NS
NC vs. PC	0.0002	NS	0.0008	0.01
NC vs. *Acacia* extract	NS	NS	NS	NS
NC vs. Fibregum	NS	NS	NS	NS
NC vs. Lily extract	NS	0.03	NS	NS
NC vs. Raftifeed	NS	NS	NS	NS

[1]S.E.M.= Pooled standard error of the least square means. [2]Results are given as least square means (n = 6).

Table 7.10 pH and molar proportions of various organic acids in caecal digesta of 21d-old broiler chickens fed the experimental diets[2]

Treatment	pH	Total organic acids (μmol/g digesta)	Molar proportions of organic acids					
			Acetate	Propionate	*n*-Butyrate	*n*-Valerate	*iso*-But+ *iso*-Vale	Succinate
			mole/100 mole					
Unchallenged-control (UC)	7.09	106.8	75.2	8.0	11.4	1.7	0.9	2.5
Negative-control (NC)	7.33	111.3	79.9	6.0	9.0	1.5	1.0	2.5
Positive-control	7.47	101.5	79.2	6.5	9.0	1.4	1.1	2.5
Acacia extract	6.25	115.6	78.1	7.7	9.3	1.2	1.0	2.5
Fibregum	6.98	108.1	77.0	7.9	10.1	1.7	0.9	2.0
Lily extract	7.00	110.0	76.5	6.9	12.3	1.3	1.0	1.7
Raftifeed	6.82	112.8	80.9	5.9	8.4	1.7	0.7	2.0
S.E.M.[2]	0.14	9.95	2.05	1.38	1.41	0.26	0.27	0.47
Orthogonal contrasts	Probability level of contrasts							
UC vs. NC	NS	NS	NS	NS	NS	NS	NS	NS
NC vs. PC	NS	NS	NS	NS	NS	NS	NS	NS
NC vs. *Acacia* extract	<0.0001	NS	NS	NS	NS	NS	NS	NS
NC vs. Fibregum	0.06	NS	NS	NS	NS	NS	NS	NS
NC vs. Lily extract	NS	NS	NS	NS	NS	NS	NS	NS
NC vs. Raftifeed	0.007	NS	NS	NS	NS	NS	NS	NS

[1]S.E.M. = Pooled standard error of the least square means [2]Results are given as least square means (n = 6).

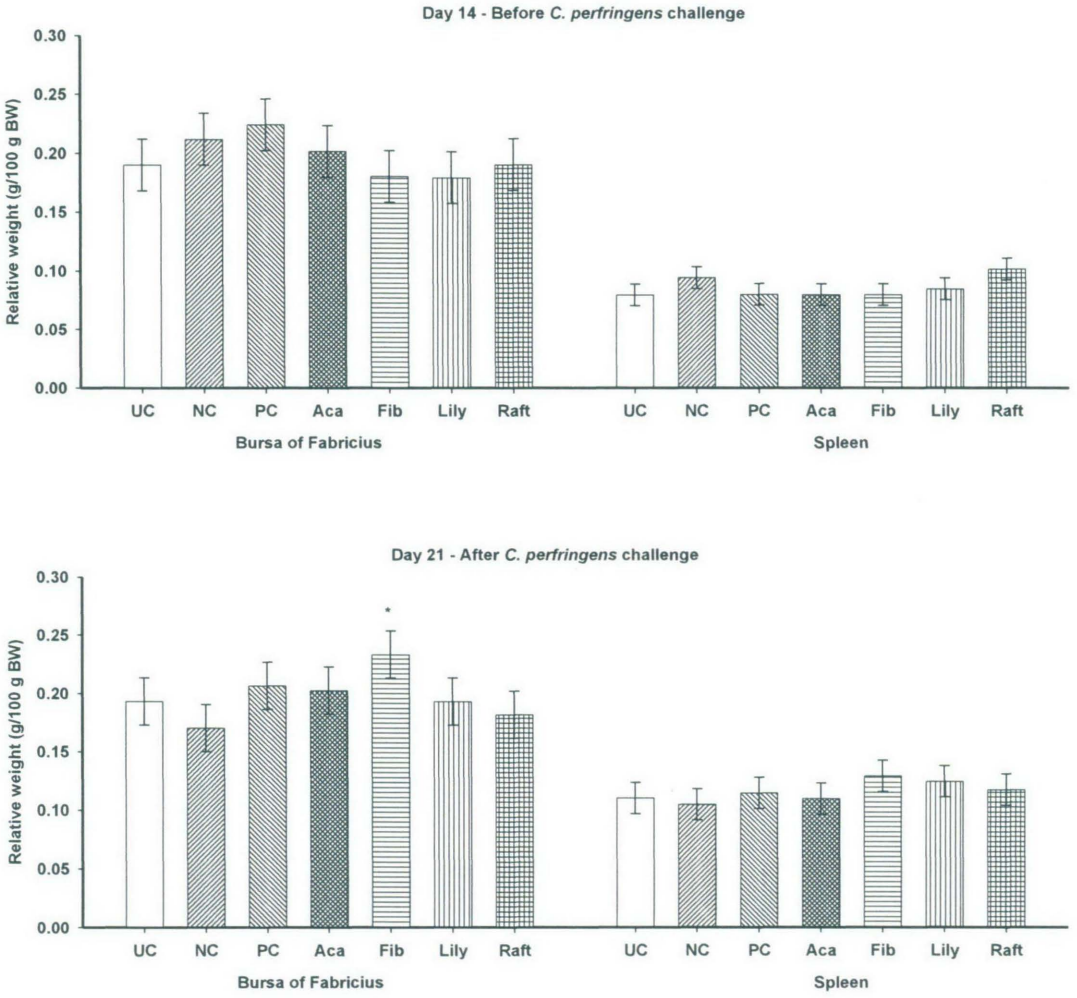

Figure 7.2 The effects of plant extracts and prebiotic compounds on relative immune organ weights in broiler chickens at d 14 (before *C. perfringens* challenge) and at d 21 (after *C. perfringens* challenge)

Unchallenged control (UC), Negative control (NC), Positive control (PC), Acacia extract (Aca), Fibregum (Fib), Lily extract (Lily) and Raftifeed (Raft). (Results are given as least square means, n = 6; error bars indicate pooled standard error of the least square means; bars with asterisks are significantly different from the negative control).

Figure 7.3 shows the serum optical density values of the specific serum IgY antibodies against α-toxin (phospholipase C) analysed by ELISA technique. At seven days post-challenge with *Cp*, the concentration of the specific IgY antibodies against the α-toxin of *Cp* in the serum, as represented in optical density values (Figure 7.3), was significantly lower ($P < 0.05$) in unchallenged birds and challenged birds fed the positive control and Fibregum diets in comparison with those from the challenged negative control group.

The total antibody responses to *Cp* before and after challenge are shown in Figure 7.4. The serum IgY response was not different between treatments before challenge. However, birds fed Fibregum had a higher IgM response compared to the *Acacia* extract- ($P < 0.05$) and lily extract- ($P < 0.05$) supplemented groups. The Fibregum-fed group also had higher ($P < 0.05$) amounts of IgA in serum than the positive control-and lily extract-supplemented groups at 14 d, but this effect did not persist at 21 d. The concentration of IgY antibody titres in sera from the positive control group was lower ($P < 0.05$) than in the negative control group on d 21.

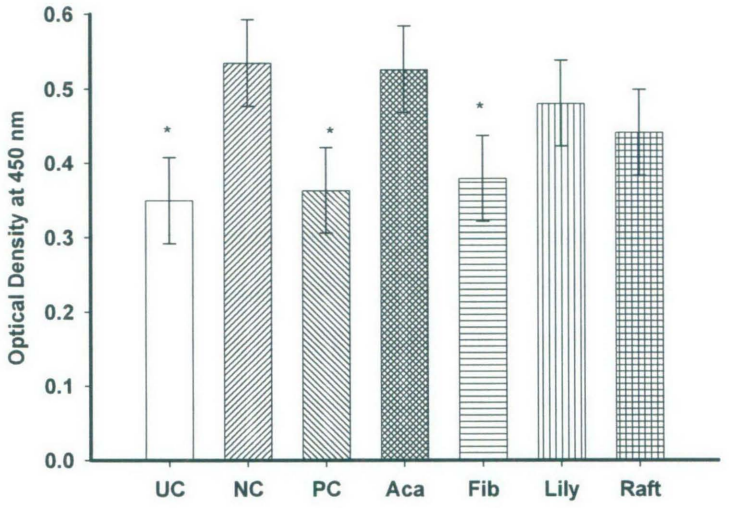

Figure 7.3 The effects of plant extracts and prebiotic compounds on specific IgY antibody titres against *C. perfringens* α-toxin at d 21 (after *C. perfringens* challenge)

Unchallenged control (UC), Negative control (NC), Positive control (PC), Acacia extract (Aca), Fibregum (Fib), Lily extract (Lily) and Raftifeed (Raft). (Results are given as least square means of optical density values, n = 6; error bars indicate pooled standard error of the least square means; bars with asterisks are significantly different from the negative control).

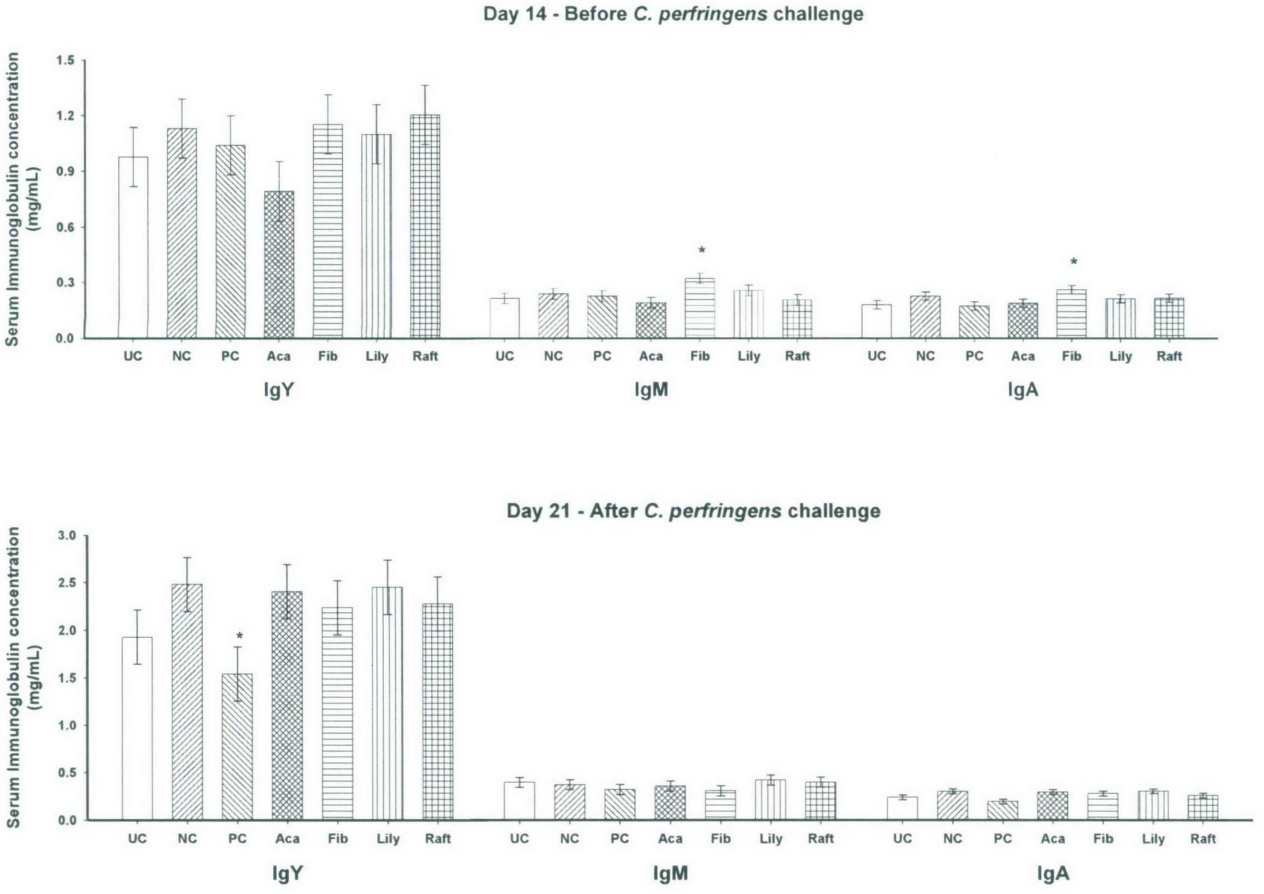

Figure 7.4 The effects of plant extracts and prebiotic compounds on total serum immunoglobulins (IgY, IgM and IgA) titres in broiler chickens at d 14 (before *C. perfringens* challenge) and at d 21 (after *C. perfringens* challenge)

Unchallenged control (UC), Negative control (NC), Positive control (PC), Acacia extract (Aca), Fibregum (Fib), Lily extract (Lily) and Raftifeed (Raft). (Results are given as least square means, n = 6; error bars indicate pooled standard error of the least square means; bars with asterisks are significantly different from the negative control).

7.4 DISCUSSION

7.4.1 Bird performance

Necrotic enteritis caused by *Cp* is considered a sporadic disease problem in the broiler industry. Feed additives such as organic acids, probiotics, prebiotics and plant extracts have all been reported to reduce the colonisation of the intestine by *Cp*, the causative organism for NE, and subsequently reduce the incidence of the disease, with varying success (Kaldhusdal *et al.*, 2001; Hofacre *et al.*, 2003; Mitsch *et al.*, 2004; Jamroz *et al.*, 2005). In the current study, except in the positive control group, there was a significant decline in the growth performance of birds challenged with *Cp* in all treatment groups compared to unchallenged birds. The impaired FCR observed with all *Cp*-challenged groups can be explained by decreased FI and BWG in the first three weeks of the period. Hofacre *et al.* (1998) and Dahiya *et al.* (2005) also reported decreased BWG and impaired FCR in broiler chickens challenged with *Cp*. The benefits to chick growth and FCR resulting from supplementation with Zn-bacitracin and monensin in the present study were similar to those reported by Wicker *et al.* (1977) and Elwinger *et al.* (1998). In the current study, any of the plant extracts or prebiotic products failed to prevent the outbreak of NE or to improve broiler growth in a necrotic enteritis challenge model. These results are in agreement with the findings of Hofacre *et al.* (2003) who found no effect of fructooligosaccharide on growth performance or reduction in lesion scores in *Cp*-challenged boilers. In contrast, Butel *et al.*(2001) reported that *Cp*-induced necrotizing enterocolitis in gnotobioic quails could be controlled by dietary supplementation with oligofructose. Mitsch *et al.* (2004) reported that a specific blend of essential oils (thymol, eugenol, curcumin, piperin, and carvacrol) could control the proliferation of *Cp* in the intestine of broilers and thereby help to control NE.

The Zn-bacitracin/monensin supplementation successfully prevented an outbreak of NE, while mortality due to NE occurred in spite of all the other supplements. Birds on Fibregum showed lower mortality from NE than those fed the *Acacia* extract, which had the highest mortality due to NE. High mortality of birds in 24 hours post-challenge suggested that the experimental disease had a rapid onset in challenged animals and continued for a maximum of 7 days after the first challenge began. The α-toxin which is produced by the *Cp* is a phospholipase-C spingomyelinase, which is believed to cause membrane disorganization and disintegration, thus activating various inflammatory mediators and finally causing blood

vessel contraction, platelet aggregation and myocardial dysfunction, leading to acute death (van Immerseel *et al.*, 2004). The lack of confluent necrosis and sloughing of the epithelial lining of the jejunum and ileum among the treatment groups was most likely influenced by the very low mortality among the challenged treatment groups seventh days post challenge. Al-Sheikhly and Truscott (1977) found that NE lesions developed at close time intervals during the first 24 h after infection and concluded that scores were highest 12 h post challenge. This agrees with the findings from this experiment where the highest mortality and maximal lesion scores were observed within first three days post challenge. In contrast, Williams *et al.* (2003) observed that maximal lesion scores appeared after 8 days of first inoculation. Variations could be due to differences in dosage rates of *Cp* given, strain differences, dietary composition and management practices.

7.4.2 Gut Histomorphology

Diseases such as NE and coccidiosis caused by pathogenic microorganisms can produce necrotic enterocytes on the top or side of an intestinal villus (Al-Sheikhly & Truscott, 1977). The premature death of an enterocyte requires replacement by another recently divided cell. However, the immature replacement cells lack the specific functions of the mature cells and thereby affect the integrity and functionality of enterocytes, causing poor digestion and absorption of nutrients (Hoerr, 1998; Jeurissen *et al.*, 2002). The contribution to digestion from the brush border enzymes is lost and the villi contract and shorten, yielding an overall reduction in absorptive surfaces. Such changes in morphology in the intestine due to *Cp*-associated NE are more pronounced because of damage caused to the mucosal layer of the intestinal tract (Kaldhusdal & Hofshagen, 1992).

For investigation of intestinal integrity, *in situ* measurement of villus height and crypt depth and their ratio has been used by many researchers. In the current study, histomorphological examination of ileal sections indicated that unchallenged birds had longer villi and shallower crypts than negative control birds. This effect was only observed after challenge. Similarly, Al-Sheikhly & Truscott (1977) noted that birds which recovered from NE regenerated the exposed surfaces of the gut epithelium by formation of short and blunt villi. The presence of shorter and more tongue-shaped, instead of finger-shaped, villi may reduce the effective surface area for nutrient absorption, particularly at the villus tips (Jeurissen *et al.*, 2002). Furthermore, changes in the gut morphometric parameters such as deeper crypts of Lieberkuhn and shorter villi have been associated with the presence of harmful or toxic

substances (Xu *et al.*, 2003). Miles *et al.* (2006), suggested that deeper crypts of Lieberkuhn are an indication of increased cell turnover to permit renewal of villi as needed in response to sloughing or inflammation from pathogens like *Cp* and their toxins. The energy conserved by the reduced turnover rate of the gut epithelial cells in unchallenged birds in the current study would have been utilised for production of lean tissue mass. This may partly explain the growth depression associated with *Cp*-challenged birds in this study compared to unchallenged-control birds even in the later stage of growth.

In the current study, it was hypothesised that plant extracts and prebiotic compounds in broiler diets challenged with *Cp* would preserve the villus structure and villus length. However, the results showed that villus length was not affected by the plant extracts or prebiotic compounds. Dietary supplements such as prebiotic and bioactive compounds have been shown to have a trophic effect on the gut morphology in chickens (Iji *et al.*, 2001b; Santin *et al.*, 2001; Xu *et al.*, 2003; Yamauchi *et al.*, 2006). It should be noted that these researchers assessed compounds under hygienic husbandry practices and not in a disease-challenged condition as in the case of the present study. However, fructooligosaccharides have been observed to alleviate salmonella-induced necrosis of the caecal mucosa and enhance the ileal micro-villus length of broilers (Choi *et al.*, 1994).

In the current study, birds fed with the positive control diet had a longer villus height and shallower crypt depth in the ileum, thus increasing the villus/crypt depth ratio, both before and after challenge. Supplementation of broiler diets with Zn-bacitracin and monensin effectively reduced the numbers of *Cp* in the ileal and caecal contents during the experimental period. Hence, the damage that would have been caused in the intestinal morphometry by NE may have been prevented. Such morphological changes in Zn-bacitracin- and monensin fed birds may also be responsible for the improved growth performance as seen in the current study in the disease-challenged groups. Antibiotic-treated farm animals are known to have longer villi and villus/crypt depth ratios in the small intestine compared with their antibiotic-free counterparts (Visek, 1978; Nousiainen, 1991; Ao, 2004).

7.4.3 Ileal and caecal microflora

The numbers of *Cp* in the healthy chicken digestive tract are in the range of 10^5-10^7 CFU/g intestinal contents (Craven, 2000; Engberg *et al.*, 2002). This is substantially lower than *Cp*

counts of 10^9 CFU/g observed before *Cp* challenge observed in the present study. However, in agreement with the present results Mitsch *et al.* (2004) reported jejunal *Cp* counts as high as 10^{10} CFU/g digesta in unchallenged broilers at 21 d.

Different factors may have contributed to an apparently higher *Cp* count in digesta of birds before challenge in this study. The most possible explanation may be the inclusion of 50% (w/w) fish meal in diets from d 8 until d 14. Large amounts of dietary protein from animal sources have been reported to exacerbate the proliferation of *Cp* and cause outbreaks of NE (Baba *et al.*, 1992; Drew *et al.*, 2004; Wilkie *et al.*, 2005). It can be argued that the high nutrient density, in particular high levels of protein, alters the microflora and creates favorable conditions in the intestine for the proliferation of *Cp*. Recent studies of Dahiya *et al.* (2005) and Wilkie *et al.* (2005) suggested that high levels of glycine in animal protein sources, such as fish meal, could be the factor that enhances the proliferation of *Cp* in the GIT of chickens. It has also been observed that fish-meal is an important source of *Cp* contamination in poultry diets (Wijewanta & Seneviratne, 1971). Therefore, feeding fish meal diets (45% crude protein) may have resulted in conditions favourable to the proliferation of *Cp* just before the challenge, leading to very high numbers of *Cp* in ileal and caecal digesta.

Although high *Cp* counts (6.5-7.0 $\log_{10}$ CFU/g) were observed in ileal and caecal digesta, focal necrotic lesions in the jejunum and ileum were not observed seven days post challenge. This demonstrates that high population of *Cp* may not always be associated with subclinical or clinical NE. However, many authors have indicated that intestinal *Cp* counts ranging from 10^7-10^9 CFU/g could cause a clinical form of NE (Craven, 2000; Drew *et al.*, 2004). Since NE is a complex, multifactorial disease with many unknown factors influencing its occurrence and the severity of outbreaks, the presence of high numbers of *Cp* may not necessarily lead to development of NE (Kaldhusdal *et al.*, 1999; van Immerseel *et al.*, 2004). Another explanation could be the TSC & SFP agar medium used in this study may also favour the growth of physiologically similar clostridia species resembling *Cp* as suggested by Adams and Mead (1980). *Clostridium* species such as *C. lituseburense* and *C. irregularis*, that have not been shown to be pathogenic to chickens can be present in large numbers in the ileal digesta of broilers (Lu *et al.*, 2006). In the present study, *Clostridium* strains grown in Perfringens agar base (TSC & SFP) were not further identified in order to confirm their specificity.

In the present study, dietary supplementation with *Acacia* extract and lily extract and the prebiotic product Raftifeed significantly increased or tended to increase the number of lactobacilli in the ileum at both sampling periods. These results are consistent with other work performed with plant extracts in Chapter 5. Several mechanisms may account for the enhanced growth of lactobacilli in response to dietary supplementation with plant extracts. Certain dietary carbohydrates such as fructooligosaccharides and arabinogalactans are not digested in the small intestine, since the chicken lacks the enzymes to hydrolyse them. Therefore, these carbohydrates are more likely degraded by the gastrointestinal microflora which they selectively stimulate. Lactobacilli have been reported to hydrolyse and to utilise the galactose- and fructose- containing prebiotic compounds such as arabinogalactans and fructooligosaccharides (Kneifel *et al.*, 2000; van Laere *et al.*, 2000). Recently, Al-Tamimi *et al.* (2006) found that fermentation of arabino-oligosaccharides in a batch culture fermentation system increases the lactobacilli and bifidobacteria counts while decreasing *Cp* numbers.

Results from the previous *in vivo* study (Chapter 5) showed that lactobacilli in the ileum and caeca were stimulated, while the growth of *Cp* was suppressed in the caeca by supplementing the broiler diets with *Acacia* extract (Vidanarachchi *et al.*, 2005; Vidanarachchi *et al.*, 2006). In the current study, although the *Lactobacillus* numbers were increased in caeca of birds fed with *Acacia* extract on d 21, there was no reduction in *Cp* counts in the same group. The exact reason for this dissimilarity is unclear. The starter diet given in the second week of this experiment (prior to *Cp* challenge) had 50 % w/w fish meal and the basal diet used in this study was composed mainly of wheat instead of sorghum which was used in previous experiments. Wheat contains considerable amounts of non-digestible oligosaccharides (Henry & Saini, 1989). Therefore, the use of basal ingredients with high levels of proteins and oligosaccharides in this experiment may have diluted or masked the effects of the water-soluble carbohydrate extracts or prebiotic compounds added. Furthermore, in order to have greater proliferation of beneficial bacteria in the gut environment, the requirements for their growth need to be fulfilled and the physiological conditions need to be optimum for their viability (Apajalahti, 2005). Such effects may also be altered due to high infection pressure in the current challenge study and may have failed to show beneficial effects on gut microflora composition as previously observed (Chapter 5), when birds were fed with plant extracts and reared under adequately hygienic conditions without NE challenge.

In the present study, feeding of *Acacia* extract or the prebiotic compound Raftifeed caused reduction in coliform counts in the ileal and caecal digesta on d 14, before *Cp* challenge. The *Lactobacillus* counts in the same groups were significantly higher or tended to be higher than negative control group. Many studies have demonstrated a relationship between an increase in numbers of lactobacilli and decline in numbers of coliforms in chickens (Fuller, 1977; Watkins *et al.*, 1982; Jamroz *et al.*, 2005).

Unlike *Acacia* extract, the Fibregum showed some beneficial effects in controlling NE in broiler chickens. This finding implies that although both of these products contain arabinogalactans, the magnitude of their response to the *Cp* challenge was different. The beneficial effects of Fibregum on NE associated mortalities could be due to some factors other than the prebiotic effect, such as the immune-enhancing properties described in the section below.

As expected, the present study demonstrates that supplementation of broiler diets with Zn-bacitracin and monensin significantly reduced the *Cp* counts in ileal and caecal digesta with consequent significant improvement in body performance and pathophysiological indices of NE. The counts of *Cp* in luminal contents from the ileum and caeca of birds fed the positive-control diet were as low as 3-4 $\log_{10}$ CFU/g digesta on both sampling days, indicating that the AGPs used in this study were highly effective in controlling *Cp* proliferation in the gut of broilers. These findings are consistent with previous studies which demonstrated that dietary supplementation with antibiotics such as Zn-bacitracin, bacitracin methylene disalicylate and tylosin, causes a significant reduction in lesion scores and mortality caused by NE (Prescott *et al.*, 1978; Brennan *et al.*, 2003; Collier *et al.*, 2003).

Many ionophore-type substances used as anticoccidials, including lasalocid, monensin, maduramycin, narasin and salinomycin, have been proven to have inhibitory effects on the growth of *Cp* (Elwinger *et al.*, 1998; Brennan *et al.*, 2001; Knarreborg *et al.*, 2002b; Martel *et al.*, 2004). The coccidiostat used in this study, monensin, is a monovalent carboxylic ionophorous polyether antibiotic that has been shown to be effective against *Cp* (Elwinger *et al.*, 1998; Martel *et al.*, 2004; Lu *et al.*, 2006).

7.4.4 Organic acids and pH

The supplementation of broiler diets with Zn-bacitracin and monensin in the present study significantly increased or tended to increase pH of ileal and caecal digesta. Correspondingly, the same treatment group had the lowest total organic acids and molar proportion of lactate in the ileal digesta. Since the positive control group had significantly lower *Lactobacillus* counts, compared to the challenged control group, it can be speculated that low microbial fermentation activities in the ileal digesta resulted in less volatile fatty acid production. Engberg *et al.* (2000) also observed higher pH and lower lactate concentrations in ileal digesta of broilers fed diets supplemented with salinomycin alone or salinomycin and Zn-bacitracin. The present results also confirmed those of Engberg *et al.* (2002) who found that lactate and acetate are the predominant organic acids produced due to bacterial fermentation in the ileum. The molar proportion of the acetate indicates that the positive control group had significantly higher values compared to the negative control group. However, this is due to lower total organic acid concentrations and not to the high acetate concentration in the ileum.

In spite of the fact that lower pH and higher *Lactobacillus* counts were observed in caecal digesta of the *Acacia* extract-fed group compared to the negative control group, the results could not be related to total organic acids or to any of the proportions of organic acids detected. Because of the high inter-replicate (cage) variability, statistically significant differences were not observed in many of the estimations made related to organic acid production in ileal and caecal digesta. Therefore, it is difficult to draw any firm conclusions based on the organic acid analysis in this study.

7.4.5 Lymphoid organ weights and humoral immune responses

The first week of life is a period of rapid seeding of lymphoid organs and expansion of the leukocyte population that will mediate immunity later in life. It has been reported that proper development of lymphoid organs such as the bursa can positively impact on the immune system (Klasing, 1998) and that an enlarged bursa may suggest an alteration in B-cell antibody production (Schat & Myers, 1991). In the current study, when birds were reared in a challenged environment, the bursa of the Fibregum-fed birds was heavier than that of the negative control group on d 21. Therefore, the increase in the relative weight of the bursa by Fibregum-treatment could be associated with an improvement in immune responses in the birds after *Cp* challenge. The results of the present study are in agreement with the findings of Kleessen *et al.* (2003a) who observed that increase in the relative weight of bursa of

broilers in response to the consumption of Jerusalem artichoke fructans syrup (0.5%) administered via drinking water. Chen *et al.* (2003) noted a significantly larger bursa and greater immunomodulatory effect in broilers fed diets supplemented with two Chinese herbal polysaccharides, astragalan and achyranthan.

Birds from the unchallenged control, positive control and Fibregum-supplemented treatment groups had lower serum levels of IgY antibody against *Cp* α-toxin and less mortality due to *Cp*-associated NE. This indicates that antigenic stimulation was lower in these three groups. In agreement with this, *Cp* counts in ileal and caecal digesta from the same three groups were either significantly lower or tended to be lower than those of the other *Cp*-challenged treatment groups. These immunological findings are in accordance with those of Løvland *et al.* (2003) who observed a higher level of IgY antibody against *Cp* α-toxin in the serum of broilers with a sub-clinical form of NE or *Cp*-associated hepatitis. Detection of IgY antibody against *Cp* α-toxin in birds in the unchallenged-control group may be due to a natural exposure of these birds to *Cp* from the pen environment. The same group also had *Cp* counts between 6.00-6.44 log CFU/g digesta in both ileal and caecal contents at seven days post-challenge. Løvland *et al.* (2003) also suggested that birds may become seropositive without exhibiting any *Cp*-associated enteric gut lesions. The minimum and maximum optical density ratios of the specific serum IgY antibodies detected against α-toxin in commercial flocks in Norway at slaughtering stage were 0.04 and 0.14 (OD values), respectively (Heier *et al.*, 2001). The higher OD values observed in this study are mainly due to the challenging of birds with *Cp*. Animal performance may be reduced as the immune system is stimulated due to infections or inflammations in the GIT (Thomke & Elwinger, 1998). As suggested by Thomke and Elwinger (1998), the current study has also proven that AGPs alleviate the changes in the humoral immune system through their gut microbial regulating effects.

Results from the current study showed that Fibregum elevates serum IgM and IgA levels and enhances systemic immune capacity in chickens. Perhaps these elevated levels of IgM and IgA may be due to the increased proportion of B lymphocytes in Firbregum-fed groups. These results indicate that further work on the determination of cell-mediated immune responses such as the proportions of different subpopulations of B and T lymphocytes is warranted. Elevated levels of serum IgA have been shown to correlate well with higher secretory IgA in the intestine (Brito *et al.*, 1993) and may explain the mechanism of *Cp* reduction from the intestinal lumen in the Fibregum-fed group. The same treatment group also had lower *Cp* counts in the caecal contents on d 14, and lower NE-associated

mortalities. In the current study, the possibility that Fibregum stimulated the immune system directly is feasible because the molecular weight of the arabinogalactan polysaccharide is 350, 000 Daltons. As it has a high molecular weight and a highly ramified structure, the water-soluble, anionic polysaccharide may act as an immune-stimulating substance. On the other hand the molecular weight of the *Acacia* extract is lower (30,000 Da; Section 3.3) and such a low molecular weight compound may not be able to act as an immuno-stimulant.

7.5 CONCLUSIONS

Neither the plant extracts nor the commercial prebiotic products were effective in controlling NE. The present study also showed no effect of dietary plant extracts on humoral immunomodulation in broiler chickens challenged with *Cp.* Although some beneficial effects were observed with Fibregum, the product did not improve performance parameters, as BWG and FCR were similar to those of the negative control. Under the conditions of the present study, Fibregum was effective in reducing NE-associated mortalities and in stimulating some immune response to the NE infection in broiler chickens. Dietary supplementation with Zn-bacitracin, and monensin was highly effective in counteracting the negative effects of the disease challenge. Finally, it can also be concluded that the NE model used in this study was effective in inducing clinical and sub-clinical NE in broiler chickens by inducing intestinal gross lesions, and poor performance and death.

CHAPTER 8 GENERAL DISCUSSION

8.1 INTRODUCTION

The findings of the current study add to the plethora of information available on alternatives to AGPs in animal feed. Typical of the nature of the research field, the plant extracts used in the current study yielded mixed results in terms of both bird performance and gut microflora. This highlights the challenges that researchers have to deal with when it comes to investigating multi-disciplinary areas of science, such as identification of alternatives to AGPs. Finding viable alternatives to AGPs will require a great knowledge and tools of nutrition, chemistry, immunology, microbiology, gut physiology, and disease. The current study certainly attempted to cover many of these disciplines and investigate their interactions. Most of the results obtained in the current study have been discussed under relevant chapters, in line with the factors involved. However, it will be worthwhile to look at the various areas of response, and synthesise the discussion further, towards the derivation of overall conclusions to the study.

8.2 CHEMICAL PROPERTIES OF EXTRACTS

Water-soluble carbohydrates from the three plant sources and the seaweed differ in their physicochemical properties such as carbohydrate content, sugar composition, molecular weight, distribution of molecules (polydispersity) and degree of polymerisation. These are properties that would determine the biological effect of the extracts. For example, the Rengarenga lily and Cabbage tree extracts both contain fructans as their main carbohydrate but their degrees of polymerisation are different; Cabbage tree extract contains short chain fructans (oligofructans), while Rengarenga lily extract has long chain fructans. *Acacia* extract contains the highest amount of water-soluble carbohydrates of all the extracts, of which the main components are arabinogalactans. The water-soluble carbohydrates extracted from *Undaria* seaweed are mainly galactose- and fucose- (galactofucans) containing carbohydrates. On the basis of current physicochemical analyses and literature findings (Chapter 2) the water-soluble carbohydrate compounds described above are prebiotic and bioactive in nature. Similar compounds have been shown to stimulate the growth of bifidobacteria and lactobacilli *in vitro* and *in vivo* in animals and humans (Kuda *et al.*, 1998; Cherbut *et al.*, 2003; Xu *et al.*, 2003; Al-Tamimi *et al.*, 2006). In the current study, the

impact of the extracts on gut microflora was mixed. This could be due to genuine differences in chemical properties or failure to identify optimal levels of inclusion.

8.3 BIOLOGICAL RESPONSE OF BIRDS TO SUPPLEMENTS

In the present experiments performed in cages none of the plant extracts or commercial prebiotic products at low dosage (5 g/kg) affected the FI of birds, whereas, at high dosage (10 g/kg), *Undaria* extract and Frutafit significantly reduced the FI of the birds compared with the negative control group.

In the current studies, plant extracts and prebiotic products had a marginally positive effect, no effect or a negative effect on bird performance, depending on the type of plant extract or prebiotic product and its inclusion rate in the diet. This occurred despite the fact that the dietary inclusion levels (5 g and 10 g/kg) of plant extracts and prebiotic compounds were similar to the amounts used by some other researchers (Yusrizal & Chen, 2003a; Guo *et al.*, 2004b). The BWG of birds given the diet supplemented with *Undaria* extract and Frutafit at the high dose was significantly lower compared to the negative control group, probably as a result of the effect of the former two supplements on FI. Although supplementation with *Acacia* extract (both low and high levels) produced an improvement in FCR of broilers reared in a clean environment (cages) during the first three weeks, the same extract failed to show any positive effect on performance or control of NE when the birds were challenged with *Cp*. The major hypothesis in many of the studies on similar products is their ability to improve health and growth of broiler chickens, and this has been realised in some of the studies (Kleessen *et al.*, 2003a; Guo *et al.*, 2004b; Cao *et al.*, 2005). However, many studies have also showed that these products have either no effect or a reduction in performance of broilers, depending on the type of product and its inclusion rate, as was the case in the current study (Patterson *et al.*, 1997; Wu *et al.*, 1999; Gajewska *et al.*, 2002).

When an antibiotic (Zn-bacitracin) was added to diets, the performance of birds was improved both under unchallenged conditions and challenged (with *Cp*) conditions. In the present study none of the supplements or the commercial products supported productivity or health to the same standard as the antibiotic supplement although the prebiotic product, Fibregum (arabinogalactans), tended to lower the levels of IgY antibody against *Cp* α–toxin, in agreement with lower *Cp* numbers and lower mortality. Experimental findings indicate

that arabinogalactans can act as immunomodulators in animals because of the highly branched β–galactan fraction (Taguchi *et al.*, 2004).

8.4 INTESTINAL DEVELOPMENT AND FUNCTION

In general, the plant extracts and commercial prebiotic products did not alter the ileal mucosal structure of birds reared under cage experiments or when subjected to *Cp* challenge. The high level of Frutafit (10 g/kg diet) impaired growth, reduced dietary AME and lowered the apparent ileal digestibility of fat and protein. Similar effects were observed with the higher level (10 g/kg) of *Undaria* seaweed extract used in the current thesis. These effects are consistent with the impaired biological responses observed in the same treatment groups. It appears that the microbial fermentation of large amounts of certain extracts or prebiotic products can impair nutrient utilisation in birds. This is further examined below.

8.5 MICROBIAL DYNAMICS AND FUNCTION

Although most of the exact mechanisms by which antibiotics improve poultry productivity are unknown, antibiotics generally tend to reduce the population of target microbes and may also alter the species composition of the gastrointestinal microflora (Visek, 1978; Thomke & Elwinger, 1998; Gaskins *et al.*, 2002). It is therefore logical to hypothesise that any bioactive compound that achieves the same effects may be able to improve productivity and health of birds when included in the diet. Results from the present study demonstrate that plant extracts and the commercial prebiotic products modulated the microflora in the ileum and caeca of broilers reared under clean environmental conditions as well as under disease-challenged conditions. In the cage experiment (Chapter 5), the inclusion of all three plant extracts in feed increased the lactobacilli population in the ileal and caecal digesta of the birds and also reduced the number of *Cp* and coliforms in the caeca. These findings are in agreement with other studies showing that prebiotic and bioactive compounds from plants, herbs and mushrooms are fermented by lactobacilli *in vivo* and *in vitro*, leading to significant shifts in the bacterial community in the GIT of broilers and in fermentation medium, respectively (van Laere *et al.*, 2000; Gajewska *et al.*, 2002; Al-Tamimi *et al.*, 2006). However, results from the NE challenge study in this thesis showed an increase in the lactobacilli numbers but no significant reduction in *Cp* numbers in the caeca when birds were fed with a diet supplemented with the *Acacia* extract. This shows that although the prebiotic plant extracts stimulated the *Lactobacillus* populations under both unchallenged

and challenge conditions, they were not effective in controlling *Cp* numbers in the face of *Cp*-associated NE challenge. The reason for these results is not clearly understood, and is an area that requires further research.

In the present study, one of the most interesting findings was that the inclusion of *Acacia* extract in the diets supported the growth *of L. johnsonii* in the ileum and caeca of the birds (Chapters 5 and 6). As previously mentioned, *Acacia* extract improved the FCR during the first three weeks and reduced the population of intestinal *Cp* in the same cage experiment. This may be of practical importance because it appears that *L. johnsonii* as such is an indicator organism for "good microflora". In support of this, an *in vivo* study by La Ragione *et al.* (2004) revealed that a single oral dose of *L. johnsonii* is sufficient to suppress all aspects of colonization and persistence of *Cp* in chickens. The increased population of lactobacilli in birds fed the other plant extracts (Cabbage tree extract and *Undaria* extract) was dominated specially by *L. salivarius* and also *L. crispatus* (Chapter 6). These birds had impaired growth performance and it was noted (Chapter 5) that when the birds were fed a diet supplemented with a high level (10 g/kg) of *Undaria* seaweed extract, beside reduced performance, the apparent ileal digestibility of fat was decreased. These findings suggest that an increase in lactobacilli numbers and microbial fermentation activities in the GIT of birds resulting from supplementation with high levels of certain plant extracts or prebiotic products may not necessarily result in positive effects, instead they can have a negative impact on nutrient utilisation and performance. This may be due to the fact that certain *Lactobacillus* spp., such as *L. salivarius* are able to deconjugate bile acids and thereby reduce the fat digestion in animals (Gilliland & Speck, 1977; Knarreborg *et al.*, 2002a). Results from Chapter 6 also demonstrate that out of the 240 *Lactobacillus* isolates tested, *L. salivarius* was the most abundant (67 %), followed by *L. crispatus* (17 %) and *L. johnsonii* (14 %).

It is claimed that prebiotic substances can stimulate the growth of bifidobacteria in the GIT of humans and animals and it is generally believed that they are helpful in maintaining a proper balance in gut microflora (Mitsuoka, 2002). Results reported in Chapter 5 indicate that bifidobacteria numbers in ileum and caeca of birds fed with plant extracts were very low and in most of the replicates values were below detection limit.

8.6 CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER STUDIES

The plant extracts and commercial prebiotic products tested in the current study do not appear to be suitable as alternatives to AGPs in broiler diets. Although the products modulated the gut microflora composition of birds, this did not lead to an improvement in bird performance. The major contribution of this study to on-going research in the area is its breadth, with the evaluation of a few plant extracts and commercial products. This has laid a foundation for further studies, in which focus should be directed at one product at a time. This will facilitate the evaluation of a range of levels of supplementation, and the possibility of identifying an optimal level for use in diets.

There is a also a need for future research to elucidate the interaction between compounds that are present in different sources, their interactions with the gut microflora, and their effect on macronutrient utilisation in broilers. For instance, by conducting further studies to test the deconjugation ability of bile salts by the *Lactobacillus* spp. isolated and identified in the current study, one could determine their role in fat digestibility of broilers. It would also be interesting to look at how different *Lactobacillus* species that were isolated in this study affect the performance of broilers *in vivo*. In the quest for alternatives to antibiotic growth promoters, it should be considered whether combinations of different prebiotics and bioactive compounds can elicit diverse beneficial effects, exert synergistic effects, or perhaps have negative effects. In order to screen and test the prebiotic and bioactive compounds under clean environmental conditions, an experimental model should be developed which takes into account animal factors, dietary factors and management factors so that reproducible results can be translated to practice.

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