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THE EFFECTS OF HOPS (*Humulus lupulus* L.) AND SILYMARIN ON PERFORMANCE AND HEALTH OF NEWLY WEANED PIGS

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A thesis submitted in fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy

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ABSTRACT

The ban of antimicrobial growth promoters in pig production has resulted in an increased interest in investigating the effects of different alternatives, which can reduce the problems post weaning and improve the performance in piglets. Initially two botanical extracts with different properties were investigated: hops, which have antibacterial properties and silymarin, which is used for liver disorders.

The first experiment showed that hops and silymarin had beneficial effects on FCR in piglets (P = 0.014) by 9.6% and 15.8%, respectively. Furthermore, hops significantly improved some of the measured liver enzymes, so it was decided to focus on hops only.

Two further experiments were carried out examining the effects of different concentrations of hops and isolated hop compounds (iso-α acids and β-acids), and the combination with organic acids in weaner pigs. A higher inclusion level of hops was associated with a better FCR, and the isolated hop compounds also resulted in an improved FCR (P = 0.027) by 9.2%. The addition of an organic acid mixture did not affect the performance.

An *in vitro* experiment confirmed that hops and isolated hop compounds had antibacterial properties. To try and elucidate the mechanism by which the hops and the isolated hop compounds improved the FCR, the effects on the gut flora were studied in the piglets. Both the hops and the iso-α acids and β-acids reduced the level of lactic acid bacteria and the level of *Bacteriodes* in one of the two experiments, but no effects were seen on the other bacteria. This lead to investigate the effects of hops/isolated hop compounds on the level of volatile fatty acids, digestibility and level of digestive enzymes and level of liver enzymes. However, none of these parameters gave conclusive results about the mode of action of hops/isolated hop compounds in the piglet.

DECLARATION

This thesis was composed by the author and is a record of work carried out by her in an original line of research. All sources of information are shown in the text and listed in the references; all help given by others is indicated in the acknowledgements.

None of this work has been presented in any previous application for a degree.

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LIST OF ABBREVIATIONS

AGP	Antimicrobial growth promoter
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
CFU	Colony forming units
DLWG	Daily live weight gain
ETEC	Enteropathogenic *E. coli*
FCR	Feed conversion ratio
GGT	Gamma-glutamyl transferase
LT	Heat labile
MRS	De Mann, Rogosa, Sharpe
NDF	Neutral detergent fibre
ST	Heat stable
VFA	Volatile fatty acids

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INTRODUCTION

Weaning is generally considered as a stressful period for the piglet, often resulting in problems such as diarrhoea and reduced growth rates (Bruininx *et al.*, 2001; Pierce *et al.*, 2005a). Antimicrobial growth promoters (AGP) have been used to improve feed intake, maximise daily live weight gain and reduce the level of diarrhoea and other post weaning diseases of pigs since the 1950s (Done, 2001). However, public concern with regards to the long term use of AGP has resulted in a ban in the EU from 1st January 2006 of all AGP used in animal feed.

The concerns arising from prolonged use of AGP are the applied genetic selection pressure on the normal population in the gut flora, which in the long term may lead to the development of extremely virulent pathogenic bacterial strains (Baynes and Varley, 2001), and the potential of development of resistance to the AGP (Walton, 1996; Aarestrup, 1999). This would result in a health risk, not only to the pigs but to humans as well.

In Sweden, AGP have been banned as growth promoters since 1986. As a result of the ban, they experienced increased levels of diarrhoea, higher mortality rates and lower daily liveweight in piglets (Göransson, 2001; Wierup, 2001b), ultimately affecting the profitability. The same could happen in the UK and the rest of Europe with the ban if no action is taken.

This has led to a search for potential alternatives which meet the standards for quality, safety and efficacy. The main interest in alternatives has been organic acids, probiotics, prebiotics, enzymes, plant extracts and essential oils. The problems so far have been that the results obtained with the alternatives are inconsistent.

Botanical extracts have been used in human medicine for many years for the prevention and cure of various diseases and disorders. The main problems associated with these are the lack of consistency of the products, a shortage of knowledge about all the different plants, their components and active ingredients. A large range of mixtures including herbs are indeed available, but with very little or no scientific evidence of modes of action and efficacy. Many of the products have been tested *in vitro,* where they have shown antimicrobial/antioxidant properties (Kohlert *et al.*, 2000; Losa, 2000), but the effect in animals has not been determined.

The objectives of this study were to review the literature and identify the problems associated with piglets during the early post weaning period which may arise from the ban of AGP. Select a few plant extracts, as potential alternatives to AGP, based on their potential mechanisms and study the responses in piglets, to see if they can help in overcoming the problems seen at weaning and improve the performance of the piglets and finally elucidate their potential mode of action. This thesis examines such effects and the the results and their potential influence on the piglets have been described.

1. LITERATURE REVIEW

1.1 Problems associated with weaning and early pig production

Weaning is naturally a gradual process, but in modern pig production it is abrupt, occurring at a time when the digestive and immune system of the piglet is relatively immature (Li *et al.*, 2003; Pierce *et al.*, 2005a). The piglets experience a range of changes at weaning including removal from the sow, moving to a new environment, change in diet and often mixing with other piglets (Jonsson and Conway, 1992). This often results in low feed intake, poor performance and it makes the piglets more susceptible to diseases and diarrhoea (Baynes and Varley, 2001; Broom *et al.*, 2003; Pickard and Wiseman, 2003). This is a major economical problem for the pig industry, since between 20 and 50% of newly weaned pigs in a herd may be affected with diarrhoea (King, 2003). Up until January 2006 AGP were routinely incorporated in pig feed to minimise the effects of the growth check seen at weaning, and improvements in DLWG has been reported to range between 5 and 15% for piglet and 5 to 8% for piglets (Done, 2001). With the ban different strategies and alternatives are studied.

1.2 Effects of management

1.2.1 Management at weaning

Good management practices and health are some of the most important factors for optimised animal performance (Wenk, 2000; Wierup, 2000; Baynes and Varley, 2001). In several studies, Callesen (2002a; 2002c; 2002b) compared the addition of 40 mg/kg Avilamycin with optimised management, such as reduced stocking density, improved ventilation, better feeding practices, sufficient water supply, good cleaning and disinfection practices and prevention of draught from 3 to 7 weeks on weaner piglets. The optimised management resulted in lower incidence of diarrhoea, lower mortality (1.6% *vs.* 3.7%) and

better productivity (DLWG of 352 g/day from 3 to 10 weeks *vs.* 310 g/day for pigs fed Avilamycin (40 mg/kg)).

Weaning piglets at a too early age and too low weight also influences the level of postweaning diarrhoea. Svensmark *et al.* (1989) found that piglets weaned at less than 3 kg had a much higher risk of developing diarrhoea than piglets weaned at a higher weight, see figure 1.1. However, in 2004 in the UK piglets weighed an average of 7.8 kg at weaning and were weaned at an average of 30 days of age (BPEX, 2005), which compared to figure 1.1 is at a weight where the incidence of diarrhoes is not that high.

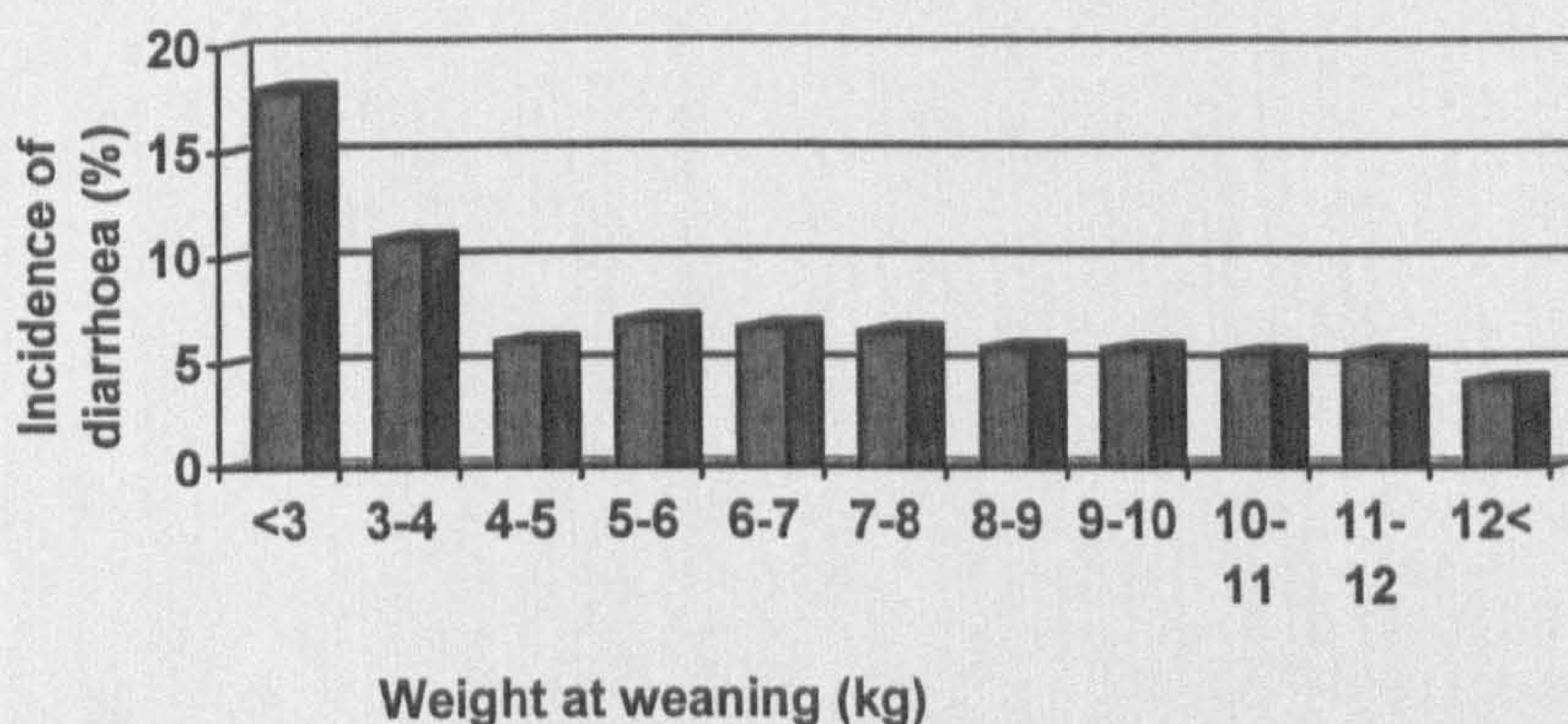


Figure 1.1: The incidence of post-weaning diarrhoea in relation to weaning weight in weaner pigs.

Source: Svensmark *et al.* (1989).

1.2.2 Environmental variables

The environment the piglets are weaned into influences their health and performance. Temperature and ventilation are important factors, as cold or fluctuating temperatures have also shown to predispose postweaning diarrhoea (Hampson, 1994; Wierup, 2000). For example, Jones *et al.* (2001) found that different stressors (removal from the sow, a cold stressor i.e. 12°C for 48 hours and mixing with non-litter mates) significantly increased the

faecal shedding of enterotoxigenic *E. coli* (ETEC) and reduced the weight gain in stressed piglets.

Furthermore, temperature may affect feed intake (Le Dividich and Sève, 2001). Too high temperature i.e. around 30-32°C has resulted in lower feed intake and too low temperature, i.e. around ++++18-20°C has resulted in higher feed intake (Ferguson, 2001). Other environmental variables affecting the feed intake and therefore the performance include hygiene, group size and space allowance (Le Dividich and Sève, 2001).

1.3 Pig health

Many predisposing factors happen prior to weaning. The most common neonatal diseases and disorders occurring before weaning are listed in table 1.1.

Table 1.1: Neonatal disorders and diseases that occur before weaning and subsequently affect the weaner pig.

Disorders	Disease
Starvation	Rotavirus
Poor body weight	Transmissible gastroenteritis
Low daily gain	Coccidia
Poor maternal antibody provision and absorption	*Clostridium perfringens* type A
	Escherichia coli

Source: adapted from Done (2001).

1.3.1 Gut health

Gut health is defined as the ability of the immune system to discriminate between harmful and harmless antigens and accurately express appropriately responses to them (Bailey *et al.*, 2001). It represents a difficult balancing act between microorganisms acting as invading opportunists and the piglets own immune system defending the integrity of the gut (Baynes and Varley, 2001). Many different criteria have been used as a measure for gut health, for example the different digestive enzymes, gut morphology, pH, microbial

activity and bile salts (van der Klis and Jansman, 2002). These will be covered in sections 1.3. and 1.4.

1.3.1.1 Changes in gut microflora after weaning

The stress associated with weaning prolongs gut transit time and lets the undigested food particles enter the lumen of the small intestine and supply substrate for bacterial growth and multiplication (Pluske *et al.*, 2002; Hopwood and Hampson, 2003; Snoeck *et al.*, 2004). The colonic flora of humans and other mammals is a highly complex ecosystem comprising of more than 400 different bacterial species (Rowland, 1992). In the small intestine and stomach the transit of fluid is rapid and does only allow little or no accumulation of food (Bach Knudsen *et al.*, 1991), but there is a constant population of *Lactobacilli* and *Streptococci.* In the large intestine the retention time is much longer (20 to 38 hours), which allows the bacteria to proliferate (Bach Knudsen *et al.*, 1991; Borg Jensen, 2001). An example of the distribution of bacteria in the gut of pigs is shown in table 1.2.

Table 1.2: The distribution of different bacteria in the digestive tract of pigs.

Organ	Bacteria	Population level (CFU/g content)
Stomach	*Lactobacilli*	10^9
	Streptococci	10^6
	E. coli	10^2
	Yeasts	10^4
Small bowel	*Lactobacilli*	10^7
	Streptococci	10^4
	E. coli	10^4
	Yeasts	10^4
Large bowel	*Lactobacilli*	10^9
	Streptococci	10^7
	E. coli	10^7
	Yeasts	10^4
	Obligate anaerobes	10^{10}

Source: adapted from Tannock (1992).

The number of *E. coli* in healthy pigs increases significantly from the anterior portion of the small intestine to the posterior portion, where the haemolytic (which often are the pathogenic) coliforms are present (Robinson *et al.*, 1981; Hampson *et al.*, 1985; Zoric *et al.*, 2001).

1.3.1.2 Post weaning diarrhoea

Post weaning diarrhoea is caused by a change in the faecal flora from one of mainly non-pathogenic bacteria such as *Lactobacillus* to one of pathogenic bacteria such as *E. coli* (Lecce *et al.*, 1983; Pluske *et al.*, 2002; Hopwood and Hampson, 2003). The aetiology of post-weaning diarrhoea is multi-factorial, complex and poorly understood (Miller *et al.*, 1984b; Hampson *et al.*, 2001; Melin *et al.*, 2004). It involves an interaction between different stressors associated with weaning, exposure to bacteria and viruses, changes in the gut and genetics of the pigs (Göransson *et al.*, 1995; Jones *et al.*, 2001; King, 2003).

Piglets most commonly develop diarrhoea 3 to 10 days after weaning, which is most often caused by *Escherichia coli* (*E. coli*) and rotavirus (Pluske *et al.*, 1997). Relatively few pigs die from diarrhoea (Svendsen, 1974; van Beers-Schreurs *et al.*, 1992), but it does affect the gut morphology and immunity, and therefore also the performance of the pigs as described in sections 1.3.2 and 1.3.3.

1.3.1.3 Rotavirus

Rotavirus is a common cause of diarrhoea in animals and humans (Hess and Bachmann, 1981; Gomez, 1997; Yuan and Saif, 2002). Young piglets are particularly susceptible to rotavirus and symptoms normally arise in 1 to 8 week old pigs (Hess and Bachmann, 1981; King, 2003). The mortality rate is less than 15% and the morbidity between 10% and 20% (King, 2003).

Rotavirus belongs to the family *Reoviridae* and the genus *Rotavirus* (King, 2003). There are seven serogroups of rotavirus (A to G) and the groups A to E have been diagnosed in pigs (King, 2003). Type A is the predominant type in nursing pigs (Done, 2001). Immunity to one type of rotavirus does not prevent infection from other types (King, 2003).

Diarrhoea normally starts two to three days after introduction with rotavirus (Gomez, 1997). It is normally transferred via faecal-oral route (King, 2003). Rotavirus infects epithelial cells in the upper part of villi of the small intestine and causes villous atrophy as well as inferring with the normal clearing mechanisms of the gut (Hess and Bachmann, 1981; Gomez, 1997). The symptoms normally last two to three days if the disease is not complicated by other enteric pathogens (King, 2003). Rotavirus often occurs at the same time as an *E. coli* infection. For example, Wilson and Francis (1986) found that 63% of mixed infections involved both rotavirus and *E. coli.* After infection the piglets have diarrhoea, become anorexic, loose weight and loose body condition, they suffer from dehydration and they may vomit (Gomez, 1997; Yuan and Saif, 2002; King, 2003).

1.3.1.4 Escherichia coli

E. coli is the most important enteric pathogen of weaned pigs (Jonsson and Conway, 1992; Frydendahl, 2002). Colonisation with Enteropathogenic *E. coli* (ETEC) primarily spreads via the faecal-oral route but also by aerosols.

The pathogenic strains of ETEC colonize mainly the anterior small intestine by means of fimbrias (Garabal *et al.*, 1997; Frydendahl, 2002; Hopwood and Hampson, 2003). After colonisation, specific enterotoxins produced by the ETEC are released. The fimbrial structures on the surface of ETEC strains contain colonization antigens (K88, K99, F41 and 987P) which enable bacteria to colonise the epithelial surface by combining with highly specific receptors on the intestinal mucusosa (Blanco *et al.*, 1997; Fang *et al.*, 2000;

King, 2003). The number of receptors increases as the piglet gets older (from 0 to 35 days old pigs) (Conway *et al.*, 1990). Different strains of ETEC have different fimbrial adhesions. The ones causing diarrhoea in the piglet are K88, K99, 987P and F41, also known as F4, F5, F6 and F41, respectively (Wilson and Francis, 1986; van Beers-Schreurs *et al.*, 1992; Jin *et al.*, 2000). They all cause diarrhoea in the neonatal pig, and K88 is the main one involved in diarrhoea after weaning (Osek, 2003).

There are two types of enterotoxins produced, the heat-labile (LT), which has a prolonged onset and irreversibly bound to mucosal cells, and heat-stable (ST), which has a rapid onset of action and it is reversibly bound to intestinal mucosal receptors (Argenzio, 1992). The two subtypes of ST are STa and STb, which appear to be unrelated. The differences are that STa only induces intestinal secretion in the newborn pig, whereas the STb induces secretion in both the newborn pig and older pigs (eight-week old pig) (van Beers-Schreurs *et al.*, 1992). LT is consistently produced only by K88-positive ETEC (van Beers-Schreurs *et al.*, 1992). K88 fimbrial antigens are classified into three sets designated K88ab, K88 ac and K88ad (Smyth *et al.*, 1994; Jeyasing *et al.*, 1999; Fang *et al.*, 2000).

The most common strain of *E. coli* to cause diarrhoea in piglets is the K88. However, some piglets do not possess the receptors for this *E. coli* and some only have weak receptors (Gibbons *et al.*, 1977; Chandler and Mynott, 1998; Pluske *et al.*, 2002). The presence of these receptors have also been found to be age dependent, the newborn piglet seems more susceptible to K88 fimbriae than older pigs (Conway *et al.*, 1990; Fang *et al.*, 2000).

Five different phenotypes of pigs exist, distinguished by the K88 adhesin variants that bind to their intestinal brush border membranes *in vivo* and *in vitro* (Conway *et al.*, 1990; Billey *et al.*, 1998). These phenotypes and the K88 adhesins that bind them are:

1. K88ab and K88ac

2. K88ab and K88ad

3. K88ad

4. All three variants

5. None of the variants (Morris and Sojka, 1985; Smyth *et al.*, 1994; Billey *et al.*, 1998).

1.3.1.5 Lactobacillus

The pig harbour large populations of *lactobacilli* in the proximal regions of their digestive tract (Tannock, 1992). These are thought to enhance growth and health of animals and maintain normal intestinal microflora by suppressing the *E. coli* (Ahn *et al.*, 2002; Snel *et al.*, 2002; Manzanilla *et al.*, 2004). Their inhibitory activity against intestinal pathogens is mainly due to their production of organic acid, hydrogen peroxide, carbon dioxide, bacteriocins and other metabolites (Tannock, 1990; Blomberg *et al.*, 1993; Ahn *et al.*, 2002).

Lactic acid bacteria are also often used as probiotics. Several strains of lactobacilli of porcine origin produce components that reduce or interfere with the adherence of K88-bearing cells to porcine ileal mucus (Blomberg *et al.*, 1993; Jin *et al.*, 2000). Jin *et al.* (2000) studied the antagonistic action of intestinal *Lactobacillus spp.* against several ETEC *in vitro* with an agar spot test by spotting *Lactobacillus* onto the surface of agar and the following day *E. coli* was poured onto the plate and the radius of the inhibition was determined after incubation. The radii of inhibition ranged from 3.8 to 11.4 mm (see table 1.3).

Table 1.3: Antagonistic action of several intestinal *Lactobacillus* spp. against a range of ETEC isolated from piglets.

Lactobacillus isolate	Inhibition zone radius (mm)							
	K88ab	K88ac	K88-nh	K88	K88+MB	K99-F	K99-K12	987P
L. brevis	7.1	6.4	3.8	8.8	8.0	7.5	7.3	9.0
L. paracasei	8.8	9.6	6.9	11.4	10.4	9.3	11.1	10.8
L. acidophilus	7.9	10.0	4.9	7.8	8.5	8.1	8.4	8.0
L delbrueckii	10.0	7.6	6.5	8.6	6.8	8.5	9.6	7.6

Source: adapted from Jin *et al.* (2000).

1.3.1.6 Salmonella

Salmonella spp., particularly *enterica* and *typhimurium* are often associated with diarrhoea after weaning (Hopwood and Hampson, 2003). The pigs usually become infected with *Salmonella* after consumption of contaminated protein sources or through exposure to infected faeces from rodents or wild birds (Hopwood and Hampson, 2003). Diarrhoea caused by *Salmonella* usually occurs in 6 to12 weeks old pigs and produces yellow diarrhoea but the mortality rate is generally low (Done, 2001).

1.3.1.7 Clostridium

Clostridium bacteria can also cause diarrhoea in piglets. *Clostridium perfringes* type C is a widespread problem in neonatal pigs and type A has been linked with enteric disease in suckling and feeding pigs with mild necrotic enterocolitis and villous atrophy (Klaasen *et al.*, 1999; King, 2003). The bacteria cause disease within 12 hours (King, 2003). *Clostridium difficile* spores germinate in the large intestine and where they multiply and produce toxins (Nagy and Bilkei, 2003).

Infection with *Clostridium* often reflects lack of hygiene, stress and dietary changes (Nagy and Bilkei, 2003). The clinical signs include diarrhoea and respiratory diseases (Nagy and Bilkei, 2003).

1.3.2 Changes in immunity after weaning

Weaning at 2 to 4 weeks of age means removal of the passive immunity provided by the sow's milk against pathogens such as *E. coli*, before the immune system of the piglets is fully developed (normally at 5 to 8 weeks of age) (Svensmark *et al.*, 1989; Pluske *et al.*, 1997; Partridge and Gill, 2001). It disturbs the crucial balance between the regulatory effect or function and ability of the mucosal immune system to discriminate harmless and harmful antigens (Bailey *et al.*, 2001).

A well developed immune system with optimal responsiveness is important for the overall productivity and welfare of the animal. To achieve high performance, the gut function, digestion and intestinal barrier should be optimised by minimum use of nutrition for immune and inflammatory responses, for example to food antigens (Bailey *et al.*, 2001; van der Klis and Jansman, 2002). Inflammation consists of a complex cascade of non specific events known as acute-phase response. The systemic part of the acute-phase response is induced by pro-inflammatory cytokines IL-1, IL-6 and TNF-α, which have locally been released from the activated macrophages (Goddeeris *et al.*, 2002).

Acute phase proteins are used as potential indicators of the health status. The major ones are C-reactive protein, haptoglobin and serum amyloid A (Heegaard *et al.*, 1998; Chen *et al.*, 2003; Hulten *et al.*, 2003). These are liver-derived plasma proteins, which are a part of the early defence mechanisms involving systemic and metabolic changes. They increase rapidly in response to abnormal events that disturb the physiologic homeostasis, including tissue injury and infection (Petersen *et al.*, 2002; Chen *et al.*, 2003). An increase in haptoglobin has been observed after weaning (Petersen *et al.*, 2002).

Chen *et al.* (2003) and Petersen *et al.* (2002) found that pigs with lameness, tail biting and diarrhoea had highly elevated haptoglobin concentrations and pigs with fever and inflammatory reaction had elevated C-reactive protein.

1.3.3 Changes in gut morphology

The gut morphology and function changes drastically after weaning, this includes villous atrophy and crypt hyperplasia, which are directly related to the absorptive capacity of the mucous membranes (Hampson, 1986b; Kelly, 1990; Pluske *et al.*, 1997). This is associated with reductions in brush-border enzyme activities, and also a potential proliferation of ETEC (Miller *et al.*, 1984a; Hampson *et al.*, 1988). These changes are claimed to play a major role in the post weaning growth check and level of clinical diarrhoea (Baynes and Varley, 2001).

Villous height is reduced around 3 to 7 days after weaning, and increases again at 5 to 12 days after weaning (Dunsford *et al.*, 1989; Pluske *et al.*, 1997; Pluske, 2001). Table 1.4 illustrates the villous height at different ages before and after weaning. It illustrates how the villous height was reduced after weaning and that it was almost 21 days before it was back to the original height it was at weaning.

Table 1.4: The effect of age and weaning on small intestinal villous height of pigs.

Age of pigs (days)	**Suckling**	**21 days weaning**
	Villous height (µm)	
2	718 ± 95	
10	703 ± 32	
21	527 ± 35	
24		183 ±17
28	416 ± 41	216 ± 17
35	410 ± 31	313 ± 14
42		429 ± 38
49		437 ± 16

Source: adapted from Cera *et al.*(1988).

The villous height and crypt depth can be affected by different factors. After weaning villous shortening happens due to the low (or no) feed intake (Makkink *et al.*, 1994b; Zijlstra *et al.*, 1996; Vente-Spreeuwenberg *et al.*, 2004). For example, the supply of creep feed has been found to result in longer villi post weaning (Makinde *et al.*, 1997). A continuous supply of nutrition to the gut after weaning is considered to prevent detrimental changes to gut structure and function after weaning (Kelly *et al.*, 1991; Bruininx *et al.*, 2004). Furthermore, piglets from specific pathogen free herds had significantly greater villous height than piglets from herds with diarrhoea (Nabuurs *et al.*, 1993; van der Klis and Jansman, 2002), possibly due to to the the inflammation caused by bacteria and/or rotavirus in pigs with diarrhoea (Kelly, 1990). It has also been suggested that the morphological changes are a product of a transient hypersensitivity to antigenic compounds in the diet, i.e. compounds that induce an antibody response in the animal, especially soyabean is a problem (Kelly, 1990; Zijlstra *et al.*, 1996; Partridge and Gill, 2001). Soyabean meal has shown to result in malformed villi (Dunsford *et al.*, 1989).

1.3.3.1 Cell changes

In the pig, there is a separation of T cells in the villi and plasma cells around the crypts within the villi, the cytotoxic T cells (CD8+) are in and immediately underlying the epithelium, and the deeper in the lamina properia lie T helper cells (CD4+) (Bailey *et al.*, 2001). From birth to 14 days of age, the intestine is readily colonised with lymphocytes, but not CD4+ and CD8+ lymphocytes (Varley and Miller, 2002). The intestine normally becomes colonised with CD4+ cells between day 14 and 28 (Goddeeris *et al.*, 2002; Varley and Miller, 2002). Normally, the cell population in the small intestine is replaced every 1 to 3 days (Chandler and Mynott, 1998). The cells originating in the villous crypts migrate and differentiate up the length of the villous to replace epithelial cells lining at tips (Chandler and Mynott, 1998).

Sarmiento *et al.* (1988) found that tissues obtained from pigs given a suspension of ETEC (containing 10^{10} CFU/ml) to induce acute phase of diarrhoea had significantly lower number of eosinophils and a higher number of neutrophils than control pigs not induced with the ETEC. ETEC cause disease without major structural changes in the mucosa of the small intestine, but with changes such as partial villous atrophy and neutrophil infiltration (see table 1.5).

Table 1.5: The histological features of intestinal mucosa from piglets during the acute phase of postweaning diarrhoea after inoculation with ETEC.

Segment of intestine	Group	Eosinophils §	Neutrophils §
Proximal portion of jejunum	*Acute diarrhoea*	7 ± 2*	2.5 ± 1.0*
	Control	10 ± 1	1.5 ± 0.5
Distal portion of jejunum	*Acute diarrhoea*	9 ± 2*	3.3 ± 1.3*
	Control	16 ± 1	0.9 ± 0.3
Ileum	*Acute diarrhoea*	12 ± 1*	3.0 ± 1.5*
	Control	17 ± 1	0.2 ± 0.1

§ Mean number of cells/microscopic field (100x magnification).
* means are significantly ($P < 0.05$) different from the respective means in the control group.

Source: adapted from Sarmiento *et al.* (1988).

1.4 Effects of diet in the newly weaned pig

1.4.1 Changes in digestive processes after weaning

At weaning the digestive and immune systems of piglets are immature and ill-prepared to handle the changes from milk based diet to a plant diet. The digestive tract is not fully developed until around 5 weeks of age (Bolduan *et al.*, 1988a; Risley *et al.*, 1991; Ravindran and Kornegay, 1993). The pigs experience poor digestibility as a result of inadequate pancreatic enzymes in the gastric stomach to digest plant protein after weaning, as a result of an increased pH due to insuffient HCl secretory capacity (Ravindran and Kornegay, 1993; Makkink *et al.*, 1994b; Li *et al.*, 1999). The pancreatic enzymes decline

after weaning and the development of them may depend on the protein source and feed intake (Makkink *et al.*, 1994a).

Digestion of feed involves a series of processes along the gastrointestinal tract by which the dietary components are broken down into smaller particles and with a combination of mechanical and enzymatic processes along the tract (Thomke and Elwinger, 1998c). Nutrients are first absorbed through the action of specific hydrolytic enzymes before being absorbed (table 1.6).

Table 1.6: Major digestive enzymes in the gastrointestinal tract of pigs.

Mouth	Stomach	Small intestine	Pancreas
Amylase	Chymosin	Enterokinase	Amylase
Lingual	Gastricsin	Glucoamylase	Trypsin
Lipase	Pepsin	Disaccharides (e.g. Isomaltase,	Chymotrypsin
	Gastric	Sucrase, Lactase)	Carboxypeptidase
	Lipase	Peptidases (e.g. Dipeptidase	Elastase
		Aminooligopeptidase)	Lipase-colipase
			Phospholipase A2
			Cholesterol esterase

Source: adapted from Shen and Liechty (2003).

The process of digesting proteins is limited in the young pig (Easter and Kim, 2000). Proteins are generally hydrolysed to free amino acid or short peptide. The absorption starts in the gastric lumen through the actions of hydrochloric acid and gastric proteases, continues in the small intestinal lumen and is completed at the brush border membrane of the enterocytes (Shen and Liechty, 2003). The source of protein in the diet affect the development of the digestive capacity of the piglet (Aumaitre *et al.*, 1995). Fish meal, fish protein concentrate and high level of soyabean concentrate reduce activity of trypsin and chymotrypsin (Aumaitre *et al.*, 1995). The trypsin and chymotrypsin activity in both pancreatic tissue and stomach contents increases as the pigs matures (Easter and Kim, 2000).

The enzyme responsible for the digestion of starch is amylase, which is present in both saliva and the pancreas (Shen and Liechty, 2003). Shields *et al.* (1980) found that the total amylase activity was higher in pigs weaned at 2 weeks of age than in pigs weaned at 4 weeks of age. The combined amylase activity increased most from 6 to 10 weeks of age, when the feed intake increased. However, the total enzyme activity is not affected by weaning age. See table 1.7.

Table 1.7: Effect of age and enzyme site on amylase activity in piglets (gram starch hydrolysed/minute ± SE).

	Age in weeks					
Amylase activity	**0**	**2**	**4**	**6**	**8**	**10**
Total	15 ± 4	52 ± 8	98 ± 14	208 ± 16	540 ± 82	1222 ± 174
Pancreas	5 ± 2	10 ± 3	48 ± 16	164 ± 14	317 ± 48	939 ± 171
Intestinal content	1 ± 0	20 ± 3	21 ± 6	15 ± 3	158 ± 42	207 ± 34
Intestinal mucosa	9 ± 3	22 ± 3	29 ± 4	29 ± 2	65 ± 8	76 ± 4

Source: adapted from Shields *et al.* (1980).

Lipid digestion starts in the gastric lumen by the action of gastric contractions to form an emulsion, it then continues in the small intestine through the action of bile acids and various pancreatic enzymes. The lipids are generally absorbed in the jejunal region in the small intestine (Xu and Cranwell, 1991; Platel and Srinivasan, 2000b; Shen and Liechty, 2003). Young animals with limited bile acid synthesis may have lower fat digestibility (van der Klis and Jansman, 2002). Secreted bile acids are readily absorbed from the small intestine and recirculated (van der Klis and Jansman, 2002). In the distal parts of the intestine the enzyme concentrations and activities are reduced and bile acids are deconjugated or reabsorbed (van der Klis and Jansman, 2002).

Fibre is thought to contribute to increased feed intake, as the animal is trying to maintain sufficient energy intake. This may explain why an increase in fibre, non starch polysaccharides, stimulates the VFA production, decrease the rate of digesta in the upper

part of the digestive tract, lowers pH and also reduce the number of *E. coli* (Dierick *et al.*, 1989; Le Dividich and Sève, 2001; O'Connell *et al.*, 2005b).

1.4.1.1 VFA

Dietary fibre is the main substrate for microbial fermentation (Montagne *et al.*, 2003). The main product from microbial fermentation is short chain fatty acids (acetic, propionic and butyric acids) and various gases (hydrogen, carbon dioxide, methane) (Bach Knudsen *et al.*, 1991; Mathew *et al.*, 1996; Borg Jensen, 2001).

The VFA produced are rapidly absorbed from the gut lumen and contribute to energy supply of the animal (Bach Knudsen *et al.*, 1991; Freire *et al.*, 2000; Borg Jensen, 2001). They may also inhibit the growth of pathogens as well as improve epithelial cell proliferation and stimulate the immune response (van der Klis and Jansman, 2002). Control of the bacterial activity in the small intestine is important to avoid undesired competition for nutrients between the bacteria and the animal (van der Klis and Jansman, 2002). Franklin *et al.* (2002) found that the VFA concentration declines after weaning. This may have significant effects on the energy available to the intestinal mass, increase the pH of the colonic lumen and allow the pathogenic bacteria to proliferate more easily (Mathew *et al.*, 1996; Topping *et al.*, 1997; Franklin *et al.*, 2002).

Freire *et al.* (2000) studied the effects of feeding different fibre sources: wheat bran, beet pulp, soyabean hulls and alfala meal to weaner pigs 58 days after weaning on the VFA. They found that acetic, buteric and total VFA concentration was significantly affected by fibre source (see table 1.8). They were higher with the soya bean hulls. The pH of the caecal content was also affected, it was higher for alfalfa meal and lowest for wheat bran and soyabean meal.

Table 1.8: The effect of dietary fibre source on the concentration of VFAs and pH in caecal content of weaner pigs.

	Diets			
	Wheat bran	**Sugar beet pulp**	**Soya bean meal**	**Alfalfa meal**
pH	6.17	6.55	6.28	6.74
VFA (mg/g of caecal content)				
Acetic	2.36	2.11	3.03	2.19
Propionic	1.35	1.09	1.38	1.28
Butyric	0.74	0.60	0.56	0.43
Total	4.46	3.80	4.96	3.90

Source: adapted from Freire *et al.* (2000).

1.4.2 Nutrient requirements

Diets for weaner pigs are normally specialised with high content of milk products and processed raw materials making them expensive (Partridge and Gill, 2001). Cooking the wheat and maize has showed to improve the availability of the starch fraction and make it more susceptible to enzymatic digestion in the gut, which is important in the newly weaned pig (Lawlor *et al.*, 2003; Medel *et al.*, 2004).

1.4.2.1 Protein

Dairy products have consistently shown to improve the performance of weaner pigs as they are a highly digestible protein source for pigs (Le Dividich and Sève, 2001; Pierce *et al.*, 2004). However, due to the high costs involved with using them other protein sources are also used in weaner diets. For example, soyabean meal is a cheap source of protein, but a high level of inclusion of soyabean meal in weaner diets results in poor growth performance and feed conversion ratio. This has been attributed to its poor digestibility and the hypersensitivity of the intestinal mucosa in weanling pigs, as described in section 1.3.3 (Li *et al.*, 2003; Pierce *et al.*, 2004).

Salgado *et al.* (2002) looked at feeding different legume protein sources; control (C), soyabean meal (SBM), pea (P), faba bean (FB) and blue lupin seeds (L) to weaned pigs. They found that the digestibility values for total tract apparent digestibility for dry matter (DM), crude protein (CP), organic matter (OM) and energy were significantly lower with legume-containing diets than the control. This is may be due to their high level of indigestible neutral detergent fibre (NDF), apart from the faba bean where there is no difference (see table 1.9).

Table 1.9: Total tract digestibility coefficients of different legume protein sources in pigs aged between 32 and 39 days.

	Control	Soyabean meal	Pea	Faba bean	Blue lupin seeds
Dry matter	0.887^{a}	0.841^{b}	0.839^{b}	0.812^{bc}	0.801^{c}
Organic matter	0.917^{a}	0.864^{b}	0.863^{b}	0.835^{bc}	0.822^{c}
Crude protein	0.932^{a}	0.857^{bc}	0.866^{b}	0.841^{bc}	0.833^{c}
Crude fat	0.913	0.892	0.907	0.905	0.900
Minerals	0.457	0.538	0.519	0.492	0.506
Fat	0.903^{a}	0.844^{bc}	0.853^{b}	0.825^{bc}	0.816^{c}
NDF	0.207^{c}	0.371^{ab}	0.452^{a}	0.219^{c}	0.310^{bc}
ADF	0.180	0.190	0.266	0.056	0.241

$^{a-c}$ values with different superscript letters in the same row differ significantly at $P < 0.05$.

Source: adapted from Salgado *et al.* (2002).

1.4.2.2 Fats and oils

Fats are often utilised in weaner diets to enhance the energy intake after weaning, as the piglets on an average only eat 200 g/day in the first week post weaning (Partridge and Gill, 2001). However, just after weaning the utilisation of fat may be reduced by the lower level of lipase activity (Partridge and Gill, 2001). The digestibility of fats which contain high levels of short- and medium-chain unsaturated fatty acids is greater than that of fats which are rich in long chain saturated fatty acids (Li *et al.*, 2003). Also fat digestibility decreased with decreasing free fatty acid content (Powles *et al.*, 1994). Fat is known to decrease the

rate of passage of the digesta in the upper part of the digestive tract (Le Dividich and Sève, 2001).

1.4.2.3 Fibre

Fibre comprises a variety of different compounds including cellulose and hemicellulose in the cell walls and other complex polymers. There is some controversy regarding the use of fibre in pig diets. Dietary fibre stimulates the secretion of saliva, gastric juice, bile and pancreatic juice, which in combination with the effect of reducing nutrient density may help to maintain a satisfactory balance between feed intake and digestive capacity of the young pig (Sissons, 1989). It possibly affects the yield of VFA from fermentation of fibre in the colon alter the absorption of water, and cause firmer faeces (Sissons, 1989).

Addition of insoluble fibre has been reported to reduce the level of post weaning diarrhoea and excretion of haemolytic *E. coli* (Sissons, 1989; Hampson *et al.*, 2001; Hopwood and Hampson, 2003). Fibre can have adverse effects on digestibility. For example, Jørgensen and Jensen (1994) studied the effects of dietary fibre in pigs from 45 to 105 kg on ileal and faecal digestibility. They included high fibre diet 260 g/kg dietary fibre (based on barley supplemented with pea fibre and pectin) and low fibre content 60 g/kg dietary fibre (based on barley and wheat starch). Table 1.10 shows how the higher fibre content lowered the digestibility of protein and energy. However, this might depend on the type of fibre used, for example barley is often preferred to maize or wheat as it has shown to result in lower levels of diarrhoea, although the reason for this is not well understood (Le Dividich and Sève, 2001). There is some controversy regarding the use of fibre in pig diets. Some fibres sources are thought to adversely affect the nutrient uptake (Montagne *et al.*, 2003). For example, oat hulls have at 150 g/kg, 300 or 400 g/kg shown to reduce feed intake in broilers (Nielsen *et al.*, 2003; Sandilands *et al.*, 2005; Sandilands *et al.*, 2006).

Table 1.10: The influence of high (260 g/kg) and low (60 g/kg) fibre content on apparent ileal and faecal digestibility in finisher pigs (%).

Fibre level	Digestibility (%)					
	Low		High		Significance	
	Ileal	Faecal	Ileal	Faecal	Ileal	Faecal
Protein	76	91	64	74	***	***
Starch	100	100	95	100	***	-
Non-starch polysaccharides	7	59	2	76	NS	***
Energy	87	94	58	83	***	***

Source: adapted from Jørgensen and Jensen (1994).

Durmic *et al.* (1998) found that pigs fed different fibre in diets had different bacteria in the colonic wall. For example, in a diet containing guar gum no *Clostridium* were found, but they were found in the control diet, and the diets containing oaten chaff and novolose. In fact the only bacteria they all had in common were *E. coli* and *Lactobacillus.*

1.5 Feed additives

1.5.1 Antibiotic growth promoters

AGP are used to improve performance of piglets after weaning and thereby reduce the production costs (Thomke and Elwinger, 1998a). These have been included in animal feed since the late 1940s (Walton, 2001). Most antibiotics are isolated from naturally growing fungi and bacteria, although some are synthetically produced antimicrobials (Walton, 2001). They can inhibit the growth of microorganisms either by interfering with their growth, metabolism or actually killing them (Walton, 1996; Anadón and Martínez-Larrañaga, 1998; Li *et al.*, 2003).

1.5.1.1 Effectiveness of antimicrobial growth promoters

The responses of AGP are variable. It depends on type of antibiotic agent, the nature of the diet (the greatest benefits are seen when cereals and plant proteins are used in the diets) and the health status of the animal (germ-free animals showed no response to antimicrobial growth promoters) (Thomke and Elwinger, 1998b; Gaskins *et al.*, 2002; Pierce *et al.*, 2005a). They are included at rates of 50 to 100 ppm, which is much less than used for treating diseases. The typical responses in weaner pigs are around 5 to 8% better FCR and between 5 and 15% better growth rate (Baynes and Varley, 2001), see table 1.11.

Table 1.11: Typical effects of antibiotic growth promoters on daily liveweight gain and FCR in different classes of pigs.

Type of pigs	**Improvement (%)**	
	Average DLWG	**FCR**
Piglets	5-15	5-8
Grower pigs	3-8	2-4
Finisher pigs	0-3	0-2

Source: adapted from Baynes and Varley (2001).

Broom *et al.* (2003) found that when 40 mg/kg Avilamycin and 3100 mg/kg zinc oxide was included in weaner pig diets, there was a significant better weight gain and feed intake in pigs receiving antibiotics and zinc oxide compared to the control after seven days. The FCR was significantly better overall for the whole period for the treated group (see table 1.12). After seven days there was a significant difference in weight gain and feed intake with the group receiving antibiotics and zinc oxide performing better. The FCR was significantly better for the treated group for the whole period (0-20 days). However, the FCR seems to be unbelievably good (between 0.83 as the best to 1.35) and the authors do not mention this in the article, this raises questions to their calculation/measurement of FCR.

Table 1.12: The effects of feeding zinc oxide and avilamycin to weaned piglets on average feed intake, daily liveweight gain and FCR.

	Treatment		
Days after weaning	**ZnO + avilamycin**	**Control**	**s.e.**
Feed intake (g/pig/day)			
1-7 days	116.4	109.0	7.9
8-14 days	280.2	212.8*	13.6
15-20 days	534.1	443.1***	5.1
all (1-20)	299.0	245.5***	3.7
Daily live weight gain (g/pig/day)			
1-7 days	101.0	79.8	11.2
8-14 days	338.8	247.8**	14.8
15-20 days	510.2	413.8***	11.1
all (1-20)	306.6	236.3***	2.1
FCR			
1-7 days	1.20	1.35	0.09
8-14 days	0.83	0.86	0.02
15-20 days	1.05	1.08	0.03
all (1-20)	0.98	1.04**	0.01

Source: adapted from Broom *et al.* (2003).

No significant difference was found in faecal bacterial counts of aerobes, *E. coli* and Lactobacilli between the two groups (see table 1.13). Overall the experiment showed that removing antibiotics and zinc oxide from weaner diets did not affect the bacterial count, but did have detrimental effects on the performance of pigs.

Table 1.13: Faecal bacterial counts (log $_{10}$ cfu) of aerobes, *E. coli* and Lactobacilli in piglets fed a control diet or a diet containing zinc oxide and Avilamycin on different days post weaning.

		Treatment	
	Day	ZnO + Avilamycin	Control
Aerobes	0	7.51	7.39
	5	7.40	7.06
	19	5.62	5.84
	25	6.90	6.61
	60	6.78	6.70
	118	6.88	6.88
E. coli	0	5.28	5.45
	5	5.93	5.82
	19	4.86	4.90
	25	4.85	4.47
	60	5.62	5.28
	118	4.57	4.46
Lactobacilli	0	7.70	7.24
	5	7.32	7.12
	19	7.36	7.75
	25	7.63	7.63
	60	7.33	7.25
	118	7.08	7.03

Source: adapted from Broom *et al.* (2003).

1.5.1.2 Mode of action for antibiotic growth promoters

The exact mode of action of antibiotic growth promoters has not been determined (Jamroz *et al.*, 2003; Li *et al.*, 2003). Different types of AGP have different modes of action. The proposed mechanisms for antibiotic growth promoters include:

1) Reduce potentially harmful bacteria and maintain a balance between the different intestinal bacteria

2) Reduce microbial use of nutrients

3) Inhibit the sub-clinical infections

4) Reduce growth-depressing microbial metabolites or toxins

5) Enhance uptake and use of nutrients though the thinner intestinal wall

6) Reduce microbial deconjugation of bile acids

7) Reduce the metabolic faecal energy and nitrogen losses

(Thomke and Elwinger, 1998b; Bach Knudsen, 2001a; Gaskins *et al.*, 2002). Other proposed mechanisms include decreased food transit time, better fatty acid, glucose, calcium and trace element absorption (Gaskins *et al.*, 2002).

1.5.1.3 The problems associated with using AGP

One of the major concerns, regarding usage of antibiotics in animal feed, is the potential bacterial resistance that can develop and transfer to man and the possible antibiotic residues in the meat (Göransson, 1997; Wierup, 2000; Bach Knudsen, 2001a). It was first discovered in the 1960s, and recommendations were made in the "Swann Report" in 1969 that antibiotics used in human medicine should not be used as feed additives in animal husbandry (Greko, 2001; Witte, 2001; Salisbury *et al.*, 2002).

The AGP are also thought to cause antibiotic resistance amongst the non-pathogenic gut bacteria normally resident in the gut lumen (Baynes and Varley, 2001). Tetracycline resistance is frequently found in zoonotic pathogenic intestinal bacteria such as *E. coli, Enterococcus faecum* and *faecalis.* This is likely to be a result of the extensive use of tetracyclines in animal production (Sengeløv *et al.*, 2003).

Several studies report about bacterial resistance to antibiotics. Up to 86% of the *E. coli* isolated from pigs in the UK have proven resistant to tetracycline, 53% to sulphamethoxazole/trimethoprim and 42% to ampicilin (Blake *et al.*, 2003). Furthermore, the prevalence of resistance in faecal *Escherichia coli* from pigs to Olaquindox increased from 0.004% to 6% in three years (van den Bogaard and Stobberingh, 2000). Table 1.14 shows the prevalence of vancomycin and erythromycin resistant enterococci in the faecal flora of healthy humans and animals in the Netherlands.

Table 1.14: The prevalence (%) of vancomycin and erythromycin resistant enterococci in the faecal flora of healthy humans and animals in the Netherlands.

Population	Number of animals	Prevalence of resistance *Vancomycin Van A*	*Erythromycin*
Pigs	282	84	75
Veal calves	539	-	-
Broilers	51	94	98
Turkeys	47	-	-
Dogs and cats	23	-	75
Hospital patients	3	-	-
Urban residents	117	50	30

Source: adapted from van den Bogaard and Stobberingh (2000).

Tylosin was the most commonly used antibiotic growth promoter in pigs and in 1996 around 90% of all *Entercoccus faecium* were resistant (Aarestrup, 1999). Between 2 and 5% of European people have been reported to carry vancomycin resistant enterococci, and the major source of resistance is suggested to be from food animals to man, as the enterococci from man and animal species contain the same genetic coding for resistance (Greko, 2001).

1.5.1.4 Implication of antibiotic growth promoter ban

Many growth promoting antibiotics have already been excluded from the pig diets (Pluske *et al.*, 2002). The last AGP to be banned in January 2006 were Avilamycin, Flavophospholipol, Monensin and Salinomycin (Manzanilla *et al.*, 2004; Straub *et al.*, 2005).

Sweden was the first country to ban the usage of antimicrobial growth promoters in 1986. As a result, they experienced a higher mortality rate, higher level of diarrhoea and a lower daily live weight gain in weaner pigs and higher mortality rates (an increase around 1.5%) (Göransson, 1997; Bywater, 1998; Wierup, 2001b). No obvious clinical problems were seen in finisher pigs, but the time to reach 25 kg took 5 to 6 days more (Wierup, 2001b).

Laine *et al.* (2004) found no major problems caused by withdrawing antibiotic growth promoters from weaner pigs fed in Finland neither on the incidence of diarrhoea nor on the age at weaning.

A voluntary ban of antibiotic growth promoters was implemented in 2000 in Denmark, they reported poorer performance and increased levels of diarrhoea during the first week after weaning (Wierup, 2001a; Højberg *et al.*, 2005). However, the prevalence of antibiotic resistance was reduced once the antibiotic growth promoters were no longer in use (Wierup, 2001b). It would therefore be expected that the ban of all antimicrobial growth promoters would result in reduced performance in the pig and poultry industry.

1.5.1.5 Alternatives to AGP

Many different alternatives to antimicrobial growth promoters are being investigated in animal production, with variable results. These include nutritional supplementation, such as organic acids, enzymes and probiotics, prebiotics, zinc oxide and botanical extracts. Each of these is discussed in the following sections.

1.5.2 Organic acids

Organic acids are widely distributed in nature in plants and animal tissue (Partanen and Mroz, 1999). Some organic organic acids have shown to improve animal performance after weaning (Gabert and Sauer, 1994; Partanen and Mroz, 1999; Dibner and Buttin, 2002). However, the results from different experiments are variable, which may be due to differences in type and dose of acid used, composition of basal diet and the buffering capacity of the diet, age and level of performance of animals (Gabert and Sauer, 1994; Partanen and Mroz, 1999; Partridge and Gill, 2001). The buffering capacity of the diets influence the response to organic acids, as it compensates for the reduction in gastric pH (Blank *et al.*, 1999; Partanen and Mroz, 1999). Acids with higher pK_a values have

improved antimicrobial efficacy, mainly due to their increased chain length and degree of unsaturation (see table 1.15) (Partanen and Mroz, 1999).

Table 1.15: Formulas, physical and chemical characteristics of organic acids used in pig diets.

Acid	Formula	MM (g/mol)	Density (g/ml)	pK_a
Formic	$HCOOH$	46.03	1.220	3.75
Acetic	CH_3COOH	60.05	1.049	4.76
Propionic	CH_3CH_2COOH	74.08	0.993	4.88
Butyric	$CH_3CH_2CH_2COOH$	88.12	0.958	4.82
Lactic	$CH_3CH(OH)COOH$	90.08	1.206	3.83
Sorbic	$CH_3CH:CHCH:CHCOOH$	112.14	1.04	4.76
Fumaric	$COOHCH:CHCOOH$	116.07	1.635	3.02
Malic	$COOHCH_2CH(OH)COOH$	134.09		3.40
Tartaric	$COOHCH(OH)CH(OH)COOH$	150.09	1.760	2.93
Citric	$COOHCH_2C(OH)(COOH)CH_2COOH$	192.14	1.665	3.13

Source: adapted from Partanen and Mroz (1999) and Dibner and Buttin (2002).

1.5.2.1 Effects of organic acids on animal physiology

The exact mode of action of organic acids is unclear (Partanen and Mroz, 1999; Li *et al.*, 2003). It is thought that the organic acids lower the gastric pH. This results in activity of proteolytic enzymes and gastric retention time, and act on pathogenic bacteria and potentially modifies the gastrointestinal microflora. This can result in a reduction in microbial fermentation. However, some acids e.g. lactic acid may stimulate microbial fermentation (Partanen and Mroz, 1999; Partridge and Gill, 2001; Li *et al.*, 2003). Weaker organic acids, i.e. citric acid and fumaric acid, may not modify the acidity of the gastrointestinal tract, even if a lower pH of the feed is seen (Aumaitre *et al.*, 1995; Pierce *et al.*, 2005b) (see also section 1.5.2.3). They are also thought to enhance nutrient digestibility (Ravindran and Kornegay, 1993).

Organic acid can improve absorption of minerals, particularly Ca, Mg, zinc and P and retention of calcium and phosphorus (Partanen and Mroz, 1999; Partanen, 2001). Dietary

minerals act as a buffer in the stomach and can make more favourable conditions for the proliferation of *E. coli* (Partanen, 2001).

1.5.2.2 Citric and fumaric acid

Citric and fumaric acid have shown to improve growth rates in weaner pigs (Giesting and Easter, 1981; Falkowski and Aherne, 1984; Partanen, 2001). Although, the results obtained with feeding citric acid to piglets are highly variable (Ravindran and Kornegay, 1993). Table 1.16 and 1.17 summarises some of the experiments carried out with citric acid and fumaric acid, respectively. Henry *et al.* (1985) found that feeding citric (30 g/kg) in the feed increased the feed intake and growth rate. Maribo and Sparre Ibsen (2003) found that citric acid added at 45 g/kg at 4 to7 week and 10 g/kg at 7 to 12 weeks had no effect on the daily liveweight gain, but there was a slight improvement in FCR in 4 to7 weeks old pigs. Broz and Schulze (1987) showed that citric acid included at levels of 5, 10 and 20 g/kg in the diet all improved the feed conversion ratio, probably by an improvement in digestion of energy and organic matter. There was no improvement in the digestibility of crude protein.

Citric acid has generally little or no effect against microbial growth, partly because many microorganisms can metabolise it and also because of its low pK_a (Gabert and Sauer, 1994; Partanen and Mroz, 1999; Boling *et al.*, 2000). Risley *et al.* (1992) found that citric acid 15 g/kg and fumaric acid at 15 g/kg in diets were unable to reduce the digesta pH and it failed to reduce the *E. coli* or *Lactobacilli* population of the gut and consequently did not prevent diarrhoea in weaner pigs (Risley *et al.*, 1992) (see table 1.16 and 1.17). This may partly be explained by the little effect citric acid has on the diet pH. At 20 g/kg inclusion rate citric acid resulted in a decrease in pH of the diet by 1.38 units and 0.26 unit decrease in the digesta after slaughter (Radcliffe *et al.*, 1998). The exact mode of action of citric acid is unknown, it is thought to improve pepsin digestion and protein digestion (Falkowski and Aherne, 1984; Broz and Schulze, 1987).

Table 1.16: The effect of different concentrations of citric acid in piglet feed on performance (DLWG and FCR), pH in the stomach and level of *E. coli* and *Lactobacilli* in the stomach.

g/kg diet	Age (weeks)	DLWG (g/day)	FCR	pH	*E. coli* cfu/g	*Lactobacilli* cfu/g	Reference
0	3-6			4.07	5.4	7.5	Risley *et al.* (1992)
15				3.82	6.0	8.0	
0	4-9	359	1.78	4.73			Risley *et al.* (1991)
15		372	1.67	4.83			
0	4-8	313	2.21	3.81			Radcliffe *et al.* (1998)
15		336	2.09	3.48			
30		340	2.00	3.31			
0	2-5	189	1.04				Henry *et al.* (1985)
30		216	1.03				
0	4-8	797	2.14	3.60			Radcliffe *et al.* (1998)
20		786	2.17	3.33			
0	4-8	371	1.49				Broz and Schulze (1987)
5		355	1.42				
10		362	1.42				
20		365	1.42				
0	4-8	407	1.61				Falkowski and Aherne (1984)
10		423	1.53				
20		437	1.45				
0	4-7	163	1.81				Maribo and Sparre Ibsen (2003)
10	4-7	165	1.70				
0	7-12	613	1.90				
45	7-12	596	1.88				
0	4-8	307	1.97				Radecki *et al.* (1988)
15		282	2.03				
30		304	1.87				

Fumaric acid has been shown to stimulate feed intake (by 5.4 %) and improve weight gains (by 9.7%) and feed efficiency (by 4.4 %) (Kirchgessner and Roth, 1982). Giesting and Easter (1981) found that inclusion of 30 or 40 g/kg of fumaric acid tended to improve the daily gain and FCR in the 4 weeks after weaning, as shownin table 1.17. Contrary, Henry *et al.* (1985) found that the inclusion of fumaric acid (15 g/kg) in piglet feed tended to decrease the growth rate.

Table 1.17: The effect of different concentrations of fumaric acid in piglet feed on performance (DLWG and FCR), pH in the stomach and level of *E. coli* and *Lactobacilli* in the stomach.

(g/kg) diet	Age (weeks)	DLWG (g/day)	FCR	pH	*E. coli* cfu/g	*Lactobacilli* cfu/g	Reference
0	3-6			4.07	5.4	7.5	Risley *et al.* (1992)
15				3.87	6.0	7.7	
0	4-9	359	1.78	4.73			Risley *et al.* (1991)
15		367	1.69	4.30			
0	2-5	189	1.04				Henry *et al.* (1985)
15		170	1.16				
0	2-6	221	1.30				Bosi *et al.* (1999)
5		220	1.32				
0	4-8	407	1.61				Falkowski and Aherne, (1984)
10		431	1.52				
20		426	1.49				
0	4-8	245	1.83				Radecki *et al.* (1988)
15		244	1.83				
30		216	1.94				
0	4-8	261	1.92				Giesting and Easter, (1981)
10		261	1.85				
20		257	1.75				
30		296	1.67				
40		297	1.67				

Improvements in nutrient, energy, amino acid, protein and dry matter digestibility and utilisation have been reported when diets are supplemented with fumaric acid (Ravindran and Kornegay, 1993; Blank *et al.*, 1999; Partanen, 2001). Blank *et al.* (1999) found that inclusion of 10, 20 or 30 g/kg of fumaric acid had a positive effect on the ileal digestibilities of crude protein, gross energy and amino acids in early weaned pigs. Better results were seen in weaner pigs than in grower pigs, which may be due to the immaturity of the digestive tract in weaner pigs. In contrast, Giesting and Easter (1991) found that addition of 20 g/kg fumaric acid did not significantly improve the ileal digestibily and performance of weaner pigs. Gabert and Sauer (1995) found that 30 g/kg fumaric acid decreased ($P < 0.05$) the ileal digestibility coefficients of energy, crude protein and the amino acids arginine, glycine and tyrosine.

1.5.2.3 Effectiveness of other organic acids

The results of experiments with propionic and formic acids are variable (Ravindran and Kornegay, 1993; Gabert and Sauer, 1994). Bolduan *et al.* (1988b) studied the effects of a propionic acid at 3 g/kg and 10 g/kg and formic acid at 3 g/kg and 10 g/kg inclusion level in diets for weaned pigs. They did not find significantly improved growth rates from the propionic acid, although 3 g/kg formic acid did increase the growth rate. Table 1.18 summarises some other experiments carried out with formic, acetic, propionic, lactic and sorbic acid.

Table 1.18: Experiments with formic, acetic, propionic, lactic and sorbic acid included in diets fed to weaner pigs.

Acid	g/kg inclusion	Initial body weight (kg)	DLWG (g/day)	% change*	FCR	% change
Formic	6	6.1	463	+21.8	1.47	-5.6
	12	6.1	468	+22.1	1.43	-7.5
	18	6.1	401	+4.6	1.53	-1.0
	24	6.1	325	-15.1	1.60	+3.9
Acetic	9	5.6	415	-2.1	1.77	+1.1
	18	5.6	429	+1.2	1.72	-1.7
	27	5.6	441	+4.0	1.70	-2.9
Propionic	10	5.6	388	-3.2	1.78	+1.2
	20	5.6	385	-4.0	1.80	+2.2
	30	5.6	395	-1.5	1.74	-1.1
Lactic	8	6.8	489	+4.7	1.65	+1.1
	16	6.8	505	+8.7	1.60	+2.2
	24	6.8	501	+7.3	1.60	-1.1
Sorbic	12	7.2	490	+13.7	1.63	-4.1
	18	7.2	523	+21.3	1.60	-5.9
	24	7.2	546	+26.7	1.59	-6.5

* % increase/decrease in relation to control.

Source: adapted from Roth and Kirchgessner (1998).

Addition of formic acid to piglet diets has resulted in improved daily liveweight gain either due to improvement in digestibilities of nutrients and energy or due to suppression of growth of pathogenic bacteria (Bolduan *et al.*, 1988c; Gabert and Sauer, 1994; Gabert *et al.*, 1995). Bolduan *et al.* (1988b) found that inclusion level of 3.5 g/kg and 12 g/kg formic

acid inhibited the growth of *E. coli* and the 12 g/kg significantly improved the growth rate and digestibility in newly weaned pigs.

Thacker and Bowland (1980) found that propionic acid decreased the feed intake and also the daily liveweight gain in growing pigs. Lactic acid has been reported to increase the feed intake in piglets from weaning to between 51 and 58 days of age, but it did not seem to improve the performance (Partanen *et al.*, 2002b). Inclusion levels of 8 g/kg lactic acid in drinking water has been shown to improve growth and feed efficiency of weaner pigs and result in a reduction of haemolytic *E. coli* in the duodenum and jejunum (Ravindran and Kornegay, 1993; Partanen and Mroz, 1999).

Malic acid is naturally found in apples and many other fruits and is active against bacteria and yeasts (Partanen and Mroz, 1999). Malic acid has in some experiments resulted in depression in performance and feed intake in weaner pigs (Ravindran and Kornegay, 1993; Krause *et al.*, 1994). Sorbic acid at 0.8 to 2.4 g/kg feed has resulted in 14 to 27% higher growth rates than has non-acidified diets, but the price of sorbic acid is too high to justify including it in pig diets (Partanen *et al.*, 2002a).

1.5.2.4 Effectiveness of inorganic acids

Attempts to acidify diets with inorganic acids have given disappointing results (Ravindran and Kornegay, 1993). HCl resulted in severe depressions in intake and growth, sulphuric acid depression feed efficiency and phosphoric acid improved growth and feed efficiency in one trial but showed no effect in later trials (Ravindran and Kornegay, 1993).

1.5.3 Enzymes

Enzymes (such as carbohydrases, proteases, lipases and phytases) are added to try and overcome the potential shortfalls of exogenous hydrolytic enzymes at weaning. Enzymes

have been shown to increase the availability of nutrients in the animal. They have resulted in improved feed utilisation, improved nutrient availability, reduced level of diarrhoea, lowered excretion of nutrients and a limited nutrient discharge in the environment (Thomke and Elwinger, 1998c; Wenk, 2000; Li *et al.*, 2003). Enzymes may enhance the feed digestibility by breaking down antinutritional factors, such as non-starch polysaccharides and protease inhibitors (Li *et al.*, 2003). Some other desirable effects of the addition of enzymes are the increased production of VFA and reduced manure and nitrogen excretion (O'Connell *et al.*, 2005b). The exact mode of action has not been identified (Thomke and Elwinger, 1998c). The results of carbohydrase addition are inconsistent, see table 1.19.

Table 1.19: The effects of supplementation with carbohydrases on the nutrient digestibility and growth of weaner pigs.

Basal diet	Enzymes	Responses	Reference
Barley and soya-bean meal based diet	*β-glucanase, xylanase and amylase 1 g/kg*	No significant effects on growth performance and protein digestibility	Gill *et al.* (2000)
Barley and soyabean meal based diet	*Xylanase, amylase and pectinase 1 g/kg*	No significant effects on growth performance and protein and energy digestibility	Gill *et al.* (2000)
Dehulled barley and soyabean meal based diet	*β-glucanase 2 g/kg*	Significant improvement in feacal digestibility of protein and energy	Li *et a.l* (1996)
Wheat and soya-bean meal based diet	*β-glucanase 2 g/kg*	No significant improvement in faecal digestibility or energy and protein	Li *et al.* (1996)
Corn and soya-bean meal based diet	*β-glucanase, pectina-se, amylase, protease and cellulose 0.7 g/kg*	Tendency for improved performance and increased levels of trypsin, chymotrypsin and amylase	Li *et al.* (1999)

1.5.4 Probiotics

Probiotics are defined as "live-microorganisms", which optimise the colonisation and composition of gut microflora in both animals and humans and stimulate the digestive processes and the immunity and maintain normal intestinal microflora by suppressing the harmful bacteria (Havenaar *et al.*, 1992; Hillman and McFarland, 1999; Snel *et al.*, 2002).

Probiotics are thought to have growth promoting effects, improve FCR, have antimutagenic and anticarcinogenic activity and to stimulate the immune response (Havenaar *et al.*, 1992; Thomke and Elwinger, 1998c; Annuk *et al.*, 2003).

Bacterial species used as probiotics include lactobacilli, bifidobacteria and enterococci, although bacteria such as *E. Coli* and certain yeasts have been used for their probiotic effects (Famularo *et al.*, 1999; Shanahan, 2003; Broom *et al.*, 2006). Probiotics are used for prevention and therapy of some diseases such as diarrhoea (Hillman, 2001; Bogovic Matijasic *et al.*, 2004). The results with probiotics are variable (Sissons, 1989; Fuller, 1992). For example, Broom *et al.* (2006) found that the addition of *Enterococcus faecium* SF68 did not improve the performance of weaner pigs. Some species of lactobacilli have greater capacity than others to inhibit the establishment of pathogens by the production of antagonistic substances (Jonsson and Conway, 1992). The organisms selected as probiotics should ideally be able to inhibit pathogenic bacteria, they must be able to survive in the intestinal tract, and have bile salt resistance and acid resistance (Hillman, 2001; Broom *et al.*, 2006). Table 1.20 compares some of the bacterial probiotics for pigs with different ages with antibiotic feed additives.

Table 1.20: The positive (+) or negative (-) effects of bacterial probiotics for pigs with antibiotic feed additives.

Additive	Effect on growth	Effect on FCE
Virginiamycin	+	-
Freeze-dried *Lactobacillus acidophilus*	-	-
Furazolidone	+	+
Bacillus cereus	+	+
Zn-Bacitracin	+	++
B. coagulans	++	+
Carbadox	+	
***B. licheniformis* + *B. subtilis* (BioPlus 2B)**	+	
Caradox + BioPlus 2B	++	

Source : adapted from Hillman (2001).

Ahn *et al.* (2002) investigated *Lactobacillus acidophilus in vitro* and *E. coli* K88 and K99 strains *in vitro*. They found that *L. acidophilus* retarded the growth of K88 and K99 at pH 4.45 and 4.46. They concluded that *L. acidophilus* grew well in the gastrointestinal tract of piglets and chickens as it has acid and bile tolerance and specific adhesion to host cells.

Another study with gnotobiotic mice showed that lactobacilli drastically reduced the number of *E. coli* in the stomach, but the lactobacilli had relatively little effect on the number of *E. coli* in the large intestine (Freter, 1992).

1.5.4.1 Modes of action of probiotics

Probiotics have been suggested to have the following properties and functions:

- adhere to the host epithelial tissue
- acid resistance
- bile tolerance
- elimination of pathogens (reduction in pathogenic adherence and neutralisation of toxins such as the toxin release from *E. coli*)
- production of acids, hydrogen peroxide and bacteriocins antagonistic to pathogen growth

- safe to use (non-pathogenic and non-carcinogenic)
- production of nutrients such as short chain fatty acids
- stimulation of immunity

(Thomke and Elwinger, 1998c; Bomba *et al.*, 2002; Kaur *et al.*, 2002).

1.5.5 Prebiotics

Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by stimulating the growth activity of bacteria or affect the bacteria already adapted to the gut environment (Snel *et al.*, 2002; Li *et al.*, 2003). Some oligosaccharides are regarded as soluble fibre which can resist attack by digestive enzymes (Monsan and Paul, 1995; Li *et al.*, 2003). A list of some oligosaccharodes are shown in table 1.21. The fermentation of β-glucan in the large intestine and the fermentation of lactose to lactic acid has increased the production of lactic acid and level of VFA and lowered the pH, which is associated with a decrease in pathogenic bacteria in the gut (O'Connell *et al.*, 2005a).

Table 1.21: Some commercially available oligosaccharides and their mode of production.

Product	Method of production
β-fructo-oligosaccharides	a) Transfructosyltation b) Hydrolysis of inulin
α-galacto-oligosaccharides	Isolation from soyabean whey
β-galacto-oligosaccharides	Transgalactosyltation
Maltotetraose	Enzymatic hydrolysis of starch

Source: adapted from Perry (1995).

Fructo-oligosaccharides (FOS) belong to the carbohydrates which are not enzymatically digested in the gut, but are fermented by the microflora (Pierce *et al.*, 2005a). The results with oligosaccharides are variable, but they have proven to improve weight gain, improve appetite and reduce the level of diarrhoea in some experiments when included in the diet at amounts less than 10 g/kg (Monsan and Paul, 1995; Li *et al.*, 2003). Table 1.22 shows

results from an experiment using fructo-oligosaccharides (FOS) at 1.65 g/kg of feed compared with a control diet containing 50 ppm Olaquindox. There was no significant difference between the two treatments, indicating the FOS gives similar performance to the Olaquindox. Both β-glucan and lactose have shown prebiotic effects on lactic acid bacteria in the gut (O'Connell *et al.*, 2005a). Similarly, fructo-oligosaccharides and sugarbeet pulp either fed separately or combined have shown to help maintain a healthy gastrointestinal tract environment through greater colonization of bifidobacteria and a reduction in the number of *E. coli* (Klein Gebbink *et al.*, 2001).

Table 1.22: The effect of FOS and Olaquindox in feed for pigs day 0 to 28 after weaning on performance.

	Treatment		
Parameter	**FOS**	**Olaquindox**	***P*-value**
DLWG (g/day)	389 ± 61	369 ± 35	0.130
Feed intake (g/day)	583 ± 79	563 ± 67	0.172
Feed efficiency	0.667 ± 0.035	0.659 ± 0.042	0.424

Source: adapted from Mul and Perry (2001).

1.5.6 Zinc oxide

Zinc oxide has proven to work as growth promoter at high concentrations. At inclusion rates of 2500 ppm ZnO is comparable with Olaquindox (Jensen-Waern *et al.*, 1998; Hill *et al.*, 2001b). It has also been shown to improve performance and help in preventing diarrhoea without associated toxic symptoms in the animals (Hill *et al.*, 2001a; Wierup, 2001b; Broom *et al.*, 2006).

The growth promoting effects of ZnO are thought to be mainly due to its ability to control enteric diseases (Pluske *et al.*, 2002; Højberg *et al.*, 2005). However, concerns over excessive excretion and accumulation of zinc in the environment may affect future use of zinc oxide (Broom *et al.*, 2006).

1.5.7 Botanical extracts

Many plants have been used in human medicine, for foods and perfumes for many years (Srinivasan, 2005; Tepe *et al.*, 2005a). Plant extracts are important for the preservation of food and control of animal and plant diseases of microbial origin (Baratta *et al.*, 1998; Tepe *et al.*, 2005a). The ban of AGP in animal production has led to a higher interest in plant secondary metabolites in animal diets. Plant extracts have demonstrated antimicrobial activities *in vitro*, but their influence on growth performance on farm animals is not well documented and the results are contradictory or inconsistent (Hernandez *et al.*, 2004).

Beneficial effects from including herbs and botanicals in diets for animals include:

- activation of feed intake and secretion of digestive secretions
- immune stimulation
- antimutagenic
- anti-microbial and anti-viral
- antihelmintic and coccidiostatic
- improved protein digestion
- hypolipidemic/hypocholesterlemic
- anti-inflammatory effects
- antioxidant activity

(Singh *et al.*, 2003; Wenk, 2003; Srinivasan, 2005).

Some spices have digestive stimulating activities, for example garlic is thought to be a gastric stimulant (Platel and Srinivasan, 2000b).

The secondary plant metabolites are often only produced by specific plants or groups of plants and there can be great variability in quantity and quality of them (Greathead, 2003; Wenk, 2003). The difference in composition will affect the properties of the plants and

spices. The biological activity of the plant preparations depends on the following factors: species of plant, phytogenic substances, their stability and the level of purity (Grant, 1999; Bast *et al.*, 2002; Jamroz *et al.*, 2003). The secondary plant metabolites serve, in many cases as a plant defence mechanism against microorganisms, insects, herbivores, inter-plant competition and abiotic stress (Trevisan *et al.*, 1997; Cowan, 1999; Greathead, 2003). Manzanilla *et al.* (2004) studied the effects of a diet containing herbal mixture (XT), consisting of 50 g/kg carvacol (*Oreganum spp.*), 30 g/kg cinnamaldehyde (*Cinnamonum spp.*) and 20 g/kg capsicum oleoresin (*Capsicum annum*) in combination with two levels of formic acid (0 and 5 g/kg) in weaner pigs. No effects was seen on the performance of the piglets, except the formic acid diets improved the FCE ($P = 0.040$). The pH of the stomach was significantly lower for the formic acid treatment, and the ratio of lactobacilli: *E. coli* was significantly higher with the XT treatment (see table 1.23).

Table 1.23: The effects of adding a herbal mixture XT at 0, 150 and 300 mg/kg and formic acid (FA) at two levels (0 and 5 g/kg) on pH in stomach, performance and microbial counts (log 10 cfu/g) of newly weaned pigs.

FA (g/kg)		0			5			*P*-value	
XT (mg/kg)	0	150	300	0	150	300	SEM	XT	FA
Performance									
DLWG (g/day)	452	403	423	417	411	447	11.3	0.139	0.967
FCE	0.65	0.63	0.66	0.66	0.67	0.68	0.010	0.285	0.040
pH									
Stomach	2.4	3.4	3.6	3.2	3.0	3.4	0.27	0.051	0.900
Caecum	5.5	5.5	5.5	5.6	5.6	5.6	0.09	0.963	0.264
Colon	6.0	5.9	5.6	5.6	5.6	6.0	0.09	0.653	0.153
Bacteria									
Lactobacilli	7.3	7.9	8.7	7.9	7.6	7.9	0.30	0.090	0.615
E. coli	5.9	6.2	5.6	5.8	5.9	5.3	0.24	0.088	0.233
Lacto:E. coli	0.93	1.61	3.44	2.02	1.83	2.72	0.34	0.002	0.563

Source: adapted from Manzanilla *et al.* (2004).

Corrigan *et al.* (2001) compared three growth promoting agents in newly weaned pigs: zinc oxide (to provide 3000 ppm added zinc), antibiotic CSP 250 (at 2.5 g/kg of the diet to

provide 110 mg of chlortetracycline, 110 mg of sulfamethazine and 55 mg penicillin per kg) and the plant extract mixture containing mixed herbs, essential oils and a natural form of allicin from garlic APEX^{TM} 3050 (at 1.0 g/kg of the diet). They found that the pigs fed the zinc oxide performed the best followed by the antibiotic and APEX fed pigs.

Results from other experiments looking at the effects of spices and herbs on digestive enzymes are variable. For example, Platel *et al.* (2002) studied the effect of three different spices mixtures included in diets for rats (containing Mixture I: 400 g/kg coriander, 200 g/kg red pepper, 50 g/kg black pepper, 200 g/kg turmeric, 50 g/kg cumin, 10 g/kg ginger, 50 g/kg mustard, 20 g/kg fenugreek, 10 g/kg cinnamon and 10 g/kg clove; Mixture II: 50 g/kg coriander, 200 g/kg red pepper, 200 g/kg black pepper, 150 g/kg turmeric, 50 g/kg cumin, 200 g/kg ginger, 50 g/kg cinnamon, 50 g/kg clove and 50 g/kg bay leaves; mixture III: 390 g/kg coriander, 200 g/kg red pepper, 50 g/kg black pepper, 100 g/kg turmeric, 50 g/kg cumin, 10 g/kg ginger and 200 g/kg onion). Mixture I and II resulted in significantly higher levels of amylase in the small intestine and Mixture III in significantly lower amylase values. Mixture I resulted in significantly lower lipase in the small intestine. Furthermore, the trypsin was significantly lower for all three mixtures in the pancreas and the chrymosin was higher for Mixture I and III.

Ramakrishna Rao *et al.* (2003) studied the effects of different spices on the *in vitro* activity of different enzymes isolated from pancreas and small intestine isolated from rats. They found that the lipase, chymotrypsin and amylase activity increased with different spices such as garlic, ginger, funigrek and mint. Jang *et al.* (2004) studied the effects of commercial blends of essential oils in broiler chickens. The activity of trypsin and amylase in the pancreas was higher in chickens fed a combination of different concentrations (25 ppm and 50 ppm) of essential oils and 1 g/kg lactic acid indicating a synergistic effect compared to only lactic acid, only essential oils and to only antibiotics.

1.5.7.1 Active ingredients in botanical extracts

1.5.7.1.1 Essential oils

Essential oils are complex mixtures of lipophilic, liquid and volatile and often terpenoid compounds present in higher plants (Kohlert *et al.*, 2000; Losa, 2000; Wills *et al.*, 2000). Many essential oils have shown antimicrobial and antimyotic properties in *in vitro* experiments, mainly due to their monoterpene content (Cox *et al.*, 2000; Kohlert *et al.*, 2000; Losa, 2000).

1.5.7.1.2 Saponins

Saponins are found in many plants (Cheeke, 2001). Saponins are glucosidic compounds of steroid and triterpenoid (Johnson *et al.*, 1986; Singh *et al.*, 2003). Haemolytic saponins are highly toxic when given intravenously and some are known to cause growth failure in poultry and monogastric animals (Johnson *et al.*, 1986). They have beneficial as well as non-beneficial activities, including:

- antioxidant properties
- depression of tumour cell proliferation and cytotoxocity
- stimulate immunoglobulin production
- cardioprotective effects
- antidiabetic affects and anti-inflammatory effects

(Ilsley *et al.*, 2003; Singh *et al.*, 2003; Ilsley and Miller, 2005).

Saponins have shown to suppress ruminal protozoa and have antibacterial activity, particularly against gram positive bacteria (Cheeke, 1999; Cheeke, 2001). Saponins found in *Quillaja* have shown to reduce the number of stillborn piglets when 2.5 g of *Quillaja saponaria* was added from day 72 to day 93 of gestation (see table 1.24) (Ilsley and Miller, 2005).

Table 1.24: Effect of supplementation of sow diet with 2.5 g/day *Quillaja saponaria* on mean sow litter weight at birth and weaning, percentage of stillbirth and mortality.

	Control	*Quillaja saponaria*	*s.e.*
Litter size (birth)	11.88	10.81	0.64
Stillborn (%)	13.25	7.67*	1.93
Number died	1.12.	0.64	0.30
Number weaned	9.18	9.36	0.33

* $P < 0.001$.

Source: adapted from Ilsley and Miller (2005).

1.5.7.1.3 Terpenes

The number of characterised terpenoids approaches 20,000 and exceeds that of any other group of plant product (Harborne, 1999). They are widely distributed amongst flowering plants (Harborne, 1999). Terpenes primary function in plants is to protect the plants from herbivores (Harborne, 1999). They have antimicrobial activity and may result in antinutritional effects in ruminants (Harborne, 1999). Terpenoids are the primary components of essential oils and provide the flavour in the herbs (Cross *et al.*, 2003).

1.5.7.1.4 Phenolics and polyphenolics

Phenols are aromatic structures bearing one or more hydroxyl group (Harborne, 1999). Most phenolics are broadly toxic or have other harmful effects (Harborne, 1999). Phenolic compounds can react as antioxidants in three ways: they can trap free radicals, they can inhibit lipogenases and they can react in their capacity as chelating agents (Mikyška *et al.*, 2002). The mechanism thought to be responsible for the phenolic toxicity to microorganisms may be enzyme inhibition by oxidized compounds (Cowan, 1999).

1.5.7.1.5 Flavonoids

Flavonoids are polyphenolic compounds that occur in foods of plant origin (Rodriguez *et al.*, 2001). There are more than 500 known flavonoids in the plant and animal kingdom.

They are the best known group of polyphenols (Mira *et al.*, 1994; Valenzuela and Garrido, 1994; Stevens *et al.*, 1998). Flavonoids are a group of phenolic compounds which naturally occur in fruits, vegetables, nuts, seeds and flowers (Yilmazer *et al.*, 2001a). They exhibit a wide range of biological activities in mammals such as antioxidative, anti-inflammatory, antiviral, anti-proliferative, antiallergenic, antifungal, antibacterial and anti-carcinogenic (Stevens *et al.*, 1998; Yilmazer *et al.*, 2001a; Yilmazer *et al.*, 2001b).

Flavonoids have antioxidant properties, and some have been widely used in humans for providing protection against toxic agents and for reducing capillary fragility and permeability problems (Mira *et al.*, 1994; Valenzuela and Garrido, 1994; Jenkins and Atwal, 1995). Furthermore, flavonoids are known to affect the activity of enzymes that are involved in the metabolism of xenobiotic compounds like drugs and carcinogens (Stevens *et al.*, 1998; Yilmazer *et al.*, 2001b).

1.5.7.1.6 Tannins

Plants contain tannins, which may at high levels exert antinutritional effects in animals, such as lower the feed intake and result in impaired growth and metabolism and health of animals (Grant, 1999; Jamroz *et al.*, 2003; Mariscal-Landin *et al.*, 2004). They may adversely affect the microflora (Singh *et al.*, 2003) and reduce digestibility of protein in ruminants and reduce the metabolisable energy level in broilers (Vernon, 1999; Singh *et al.*, 2003; Mariscal-Landin *et al.*, 2004). However, they may have some positive effects such as reduce gastrointestinal parasites and they contain a compound that is well known to possess inhibitory activity against viruses, bacteria and fungi, i.e. *Bacteriodes fragilis, Clostridium perfringens, E. coli* and *Salmonella typhymirium* (Singh *et al.*, 2003; Acamovic and Brooker, 2005).

Tannins are natural constituents of many foods and drinks, such as grapes in wine, the apples in cider and the hops in beers (Mueller-Harvey, 1999). There is a large variation in the amounts and chemical composition of tannins between plant species (Mueller-Harvey, 1999). There are two types of tannins, hydrolysed and condensed (also known as proanthocyanidins), which can have antinutritional and toxic properties when consumed by animals (Cowan, 1999; Mueller-Harvey, 1999; Singh *et al.*, 2003). Longstaff and McNab (1991) studied the effects of different inclusion rates of tannin-rich bean hulls on the activity of digestive enzymes and lipid and starch digestibility in broiler chickens. They found that at high concentrations the lipase and α-amylase and the digestibility of energy and starch was reduced with higher concentrations of the tannins, see table 1.25.

Table 1.25: The influence of tannin-rich hulls on metabolizable energy (ME) of diets and on lipid and starch digestibilities (coefficient) in 2-3 week-old male broiler chicks.

Hulls (g/kg)	0	20	50	100	300	s.e.d.	*P*-value
ME (MJ/kg)	13.68	13.81	13.15	12.64	10.31	0.415	<0.001
Lipid digestibility	0.876	0.892	0.887	0.899	0.877	0.125	<0.05
Starch digestibility	0.993	0.990	0.991	0.985	0.956	0.005	<0.001

Source: adapted from Longstaff and McNab (1991).

Different varieties of sorghum (containing different levels of tannins) have also shown to have a negative effect on the digestibility of nitrogen and energy in weaner pigs and decreased trypsin activity (Lizardo *et al.*, 1995). It has been suggested that a variety of tannin-containing beverages such as green tea and red wine can prevent different illnesses, and they stimulate phagocytic cells, host mediated tumour activity, antimicrobial and a wide range of anti-infective actions (Cowan, 1999).

1.6 Specific selected botanical extracts

Two herbs with different modes of action and different composition were selected for investigation in this thesis. Hops, which are used for its antibacterial properties in the

brewing industry and silymarin, which is used for its antioxidant properties in human medicine, these are described in sections 1.6.1 and 1.6.2.

1.6.1 Silymarin

1.6.1.1 Use of silymarin

Silymarin is isolated from milk thistle (*Silybum marianum* (*L.) Gaertner*), which is a member of the Aster family (Asteraceae or Compositae). It is commonly found on uncultivated ground and waste places throughout much of Europe (Foster, 1991; Flora *et al.*, 1998; Pepping, 1999). It has been cultivated in European gardens as a vegetable, where the young leaves were used in spring salads and as a spinach substitute (Foster, 1991).

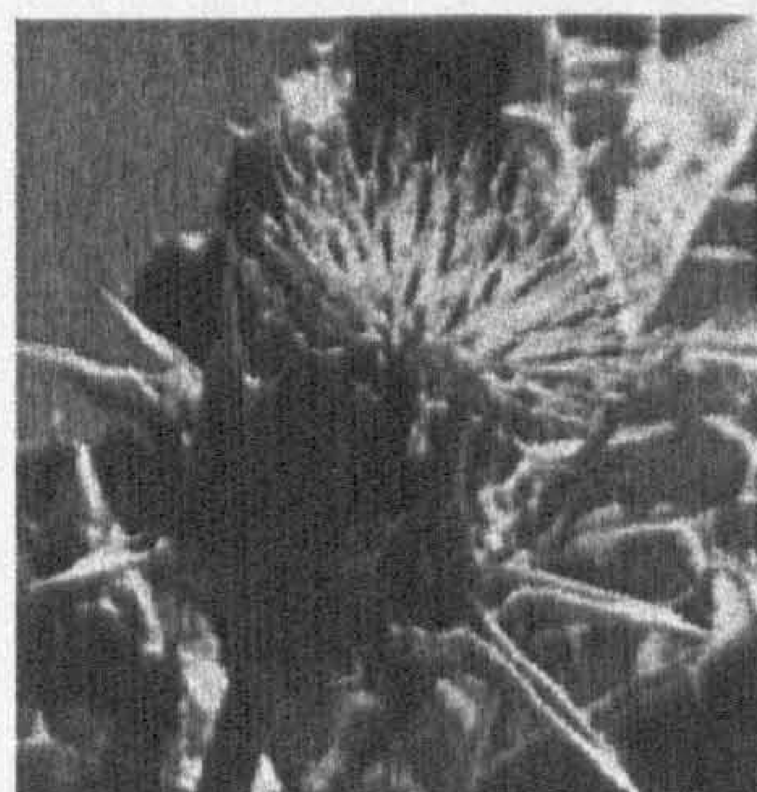


Figure 1.2: Milk thistle flower.

The annual or biannual milk thistle is a 1 to 2 m high plant. The mature plant has large purple flowers and the head can have a diameter up to 6 cm, these have black and shiny seeds (see figure 1.2) (Leung and Foster, 1996; Flora *et al.*, 1998; Pepping, 1999). Milk thistle has been used medically for more than 2000 years, and it is still used today (Foster, 1991). In human medicine, the isolated compounds, silymarin, has been used as supportive treatment of chronic inflammatory liver disorders such as cirrhosis, hepatitis and fatty infiltration due to alcohol and toxic chemicals (Fintelmann, 1991; Flora *et al.*, 1998; Kvasnicka *et al.*, 2003). Silymarin has proven to be 100% effective in humans exposed to liver damage by the poisonous mushrooms *(Amanita phalloides*), but only when administered prior to the doses of poisonous mushrooms (Valenzuela and Garrido, 1994;

Pepping, 1999; Kvasnicka *et al.*, 2003). This is due to its protective effects on the liver, when exposed to various toxic compounds, such as intoxication with the phalloidin, galactosamine, thioacetamide, thioacetamide, halothane and carbon tetrachloride, but it has also shown beneficial effects for upper gastrointestinal disturbances, menstrual disorders and it has shown positive effects on the immune system (Valenzuela and Garrido, 1994; Saller *et al.*, 2001; Johnson *et al.*, 2003). Fraschini *et al.* (2002) showed to improve liver function tests and liver morphology in patients with increased serum activities of AST, ALT and GGT in patients intoxicated with paracetamol. In contrast, He *et al.* (2002) did not find any significant effect of silymarin on liver enzymes, they found a dose-dependent, but not significant decrease in AST and ALT was seen. Salmi and Sarna (1982) found the mean percentage decrease of AST and ALT was significantly lower for silymarin treated patients than controls.

Generally silymarin is considered as non toxic to humans and only few adverse effects have been recorded. These include a mild laxative effect at high dosages (more than 1500 mg/day) in a few cases, because of the increased bile flow and secretion (Pepping, 1999; Wellington and Jarvis, 2001; Singh and Agarwal, 2002). Other studies have found reaction to milk thistle extracts including intermittent episodes of severe sweating, abdominal cramping, nausea, vomiting, diarrhoea and weakness in humans (Pepping, 1999; Wellington and Jarvis, 2001).

1.6.1.2 Components of milk thistle and their properties

Milk thistle is a mixture of many flavonolignans including: silymarin which is a mixture of three compounds silychristine, silyadianine and silybin. Other flavnolignans are found in the seeds such as silydianin, silybin and silychristin, dehydrosilybin, desoxysilychristin, desoxysilydianin, silandrin, silybinome, silyhermin and neosilyhermin. The chemical structure of silybin, one of the major constituents of silymarin, is shown in figure 1.3.

Other constituents such as taxofoline, apigenin, silybonol, consist largely of linoleic and oleic acids plus myristic, palmitic and stearic acids, as well as betaine hydrochloride, triamine and histamine (Singh and Agarwal, 2002; Kvasnicka *et al.*, 2003; Skottova *et al.*, 2003). However, silybin appears to have the most biological activity (Valenzuela and Garrido, 1994; Pepping, 1999).

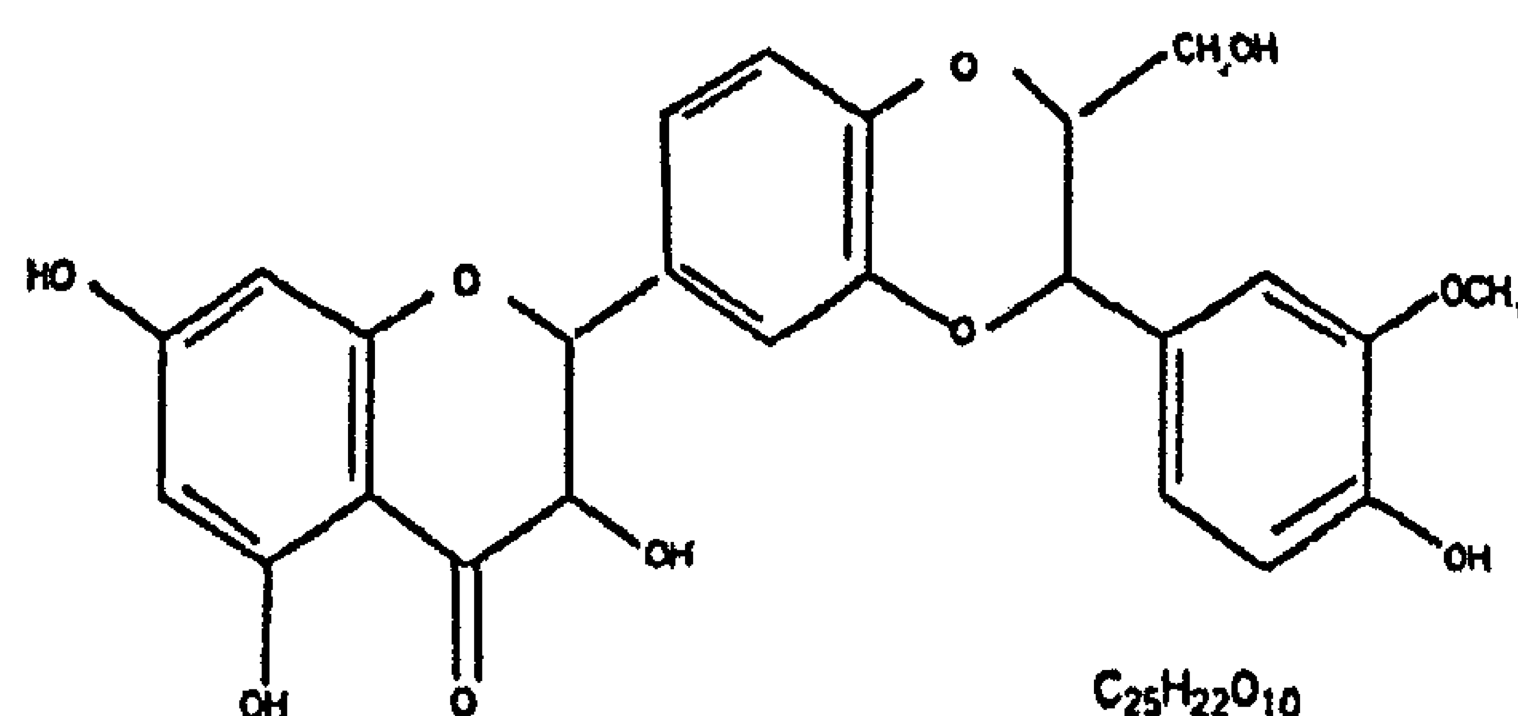


Figure 1.3: The chemical structure of silibin

Several experiments have shown that silymarin is a strong antioxidant that in short-term (hours) *in vitro* experiments will scavenge the reactive oxygen species produced by inflammation. This could mean that higher doses of silymarin treatment may increase the ability of the immune system to fight bacterial infections. The ability of silymarin to protect against oxidative stress induced hepatocellular damage is mainly associated with its free radical scavenging properties and its ability to enhance endogenous antioxidant defencences (Bhatia *et al.*, 1999; Lahiri-Chatterjee *et al.*, 1999; Fraschini *et al.*, 2002).

The different compounds of silymarin are often quantified by high performance liquid chromatograph (HPLC), and the amounts of the various compounds vary between different samples (Quaglia *et al.*, 1999; Kvasnicka *et al.*, 2003).

1.6.1.3 Uses in animal production, human medicine and in vitro

Feeding oil from *S. marianum* seeds to mice has shown to increase their weight gains, which may be due to the extra supply of energy (Khan *et al.*, 1986). Increasing level of *S. marianum* resulted in increasing levels of weight gain, up to 100 g/kg was included in the diets, see table 1.26. This level showed no adverse effect on degenerative effects of the organs (Khan *et al.*, 1986).

Table 1.26: The effect of different inclusion levels of seed oil from silymarin on groups with five mice in each on weight gain and feed intake.

Inclusion	Feed intake (g)			Body weight (g)		
(g/kg)	0	50	100	0	50	100
Week						
0	-	-	-	144	158	142
1	120	160	168	149	169	155
2	136	192	196	160	176	166
3	144	201	239	162	181	176
4	108	153	201	156	188	183
5	90	130	108	157	202	182
6	98	120	128	153	198	186
% increase				6	25	31

Source: adapted from Khan *et al.* (1986).

Johnson *et al.* (2002) found that treating mice with silymarin by injections for five consecutive days (up to 250 mg/kg) did not cause any signs of toxicity, behavioural changes, weight gain and relative thymus weight. However, their food consumption was reduced.

Both silymarin and silibinin alone have protective properties for hepatocytes both in animal studies and *in vitro* studies (Beckmann-Knopp *et al.*, 2000). In animal studies, silymarin protected animals against various hepatotoxins. Experiments have shown that treatment of rats with either silymarin or silibinin alone protects the animals against the hepatic oxidative stress and acute intoxication with ethanol or acetaminophen (Valenzuela

and Garrido, 1994; Fraschini *et al.*, 2002; Kittur *et al.*, 2002). Crocenzi *et al.* (2000) found that silymarin had little or no impact on liver integrity in rats, which was suggested by the lack of effect on the AST, ALT and alkaline phosphatase. However, a dose-dependent decrease in total body weight was seen, but not in liver weight (see table 1.27).

Table 1.27: The effect of different concentrations of silymarin on the live weight, liver weight of rats for 5 days.

Silymarin (mg/kg body weight)	Initial body weight (g)	Final body weight (g)	Liver weight (g)	Final liver/body weight ration (%)
0	330 ± 6	332 ± 6	11.0 ± 1.1	3.3 ± 0.2
25	326 ± 7	320 ±7	10.9 ± 1.5	3.4 ± 0.2
50	332 ± 9	320 ± 8*	11.1 ± 0.8	3.5 ± 0.3
100	329 ± 8	303 ± 7*†	11.0 ± 1.0	3.7 ± 0.2†
150	325 ± 8	294 ± 8*†	10.9 ±1.2	3.7 ± 0.2†

* Significantly different from initial values ($P < 0.05$).
†Significantly different from control group ($P < 0.05$)
Source: adapted from Crocenzi *et al.* (2000)

Silymarin has shown to posses significant chemopreventive and anti-cancer activity, and several animal and *in vitro* experiments have shown that silymarin may have applications in the prevention and treatment of certain cancers, including breast, prostate, skin tumours and protection against UVB radiation (Zhao and Agarwal, 1999; Fraschini *et al.*, 2002; Singh and Agarwal, 2002). Bhatia *et al.* (1999) found that silymarin and silibin showed a significant growth inhibition of prostate carcinoma, breast carcinoma and cervical carcinoma cells, with silymarin having marginally inhibitory effect after five days of treatment (significantly different from silibinin). They concluded that silymarin exhibits comparable inhibition of DNA synthesis in human prostate, breast and cervical carcinoma cells. Furthermore, it has been shown that silymarin has chemo preventive efficacy in urinary bladder carcinogenesis in mice, and that silibinin and silymarin is a potential preventative and therapeutic agent against prostate cancer in rats (Tyagi *et al.*, 2002; Vinh *et al.*, 2002).

Studies indicate that silibin may have a therapeutic potential for non-insulin-dependant diabetes mellitus (Schönfeld *et al.*, 1997). Velussi *et al.* (1997) found that when insulin-treated patients were given silymarin, the mean daily insulin requirement decreased significantly and constantly from 55 ± 5 IU/day at the beginning of experiment to 42 ± 2 IU/day after 12 months.

Skottova *et al.* (2003) studied the effects of silymarin on the liver, the plasma lipid content (cholesterol and triacylglycerols), the plasma lipoprotein profile and blood and liver glutathione in rats fed a high cholesterol diet with either lard or currant oil. They found that both fat types caused the same accumulation of cholesterol in the liver, and silymarin actually decreased the cholesterol content in both groups. The suggested mode of action for silymarin and its polyphenolic fraction on cholesterol metabolism was thought to be due to inhibition of cholesterol absorption from the intestine.

Potkanski *et al.* (2001) looked at the effect of feeding the endosperm from milk thistle to dairy cows. They found that substituting 200 g/kg of the concentrate with the milk thistle endosperm resulted in a significantly higher fat percent in the milk than for the control group, although milk yield and protein content was not significantly affected.

Breschi *et al.* (2002) found that silymarin showed a moderately protective effect against the bronchospasm induced by aerosol antigen challenge in sensitised guinea pigs. This could indicate that silymarin could potentially be used as a protective agent in the management of asthmatic disorders.

Silymarin has been found to be immunostimulatory and dose-dependent, a higher dose results in higher lymphocyte proliferation (Wilasrusmee *et al.*, 2002). Johnson *et al.* (2003) studied the effects of silymarin on the immune system. Mice were treated with different

doses of silymarin: control group, 10 mg/kg, 50 mg/kg and 250 mg/kg. The low dose resulted in a decrease in the T-lymphocyte population in the spleen, whereas high doses promoted inflammatory response in mice.

Anti-inflammatory effects of silymarin have been found in the liver tissue, as it exerts a number of effects including inhibition of Kupffer cells, marked inhibition of leukotriene synthesis, inhibitory effect of inducible nitric oxide synthase (iNOS) gene expression and formation of prostaglandins (Fraschini *et al.*, 2002; Kang *et al.*, 2002).

1.6.2 Hops

1.6.2.1 Composition of hops and general use

Hops (*Humulus lupulus* L.) belong to the family Cannabinaceae. They have for many years been used for they bitter flavour, enhancing beer foam and preservation of alcoholic beverages, such as beer, through bacteriostatic properties (Fukao *et al.*, 2000; Moir, 2000; Suzuki *et al.*, 2002). They are cultivated for other purposes as well such as medicinals, pharmaceuticals, bread making, salad greens, ornament, pillow stuffing, textile fibres and fodder (Hampton *et al.*, 2001). Hops are known to cause allergic contact dermatitis, due to the myrcene in fresh hop oil (Estrada *et al.*, 2002).

Until recently, only the hop cones were used in beer production. In the 1960s the hop cones were extracted with organic solvent and the extracts obtained were used instead. Later hop pellets and their liquid or supercritical carbon dioxide extracts became popular (Lu-Ping Ting and Goldstein, 1996). There are many different species of hops with different properties. Particular varieties are grown in different parts of the world, for example *H. lupulus* var. *cordifolius* in Japan, *H. lupulus* var. *lupuloides* in central and eastern North America and *H. lupulus* var. *lupulus* from Europe and North America (Hampton *et al.*, 2001). The European variety *H. lupulus* var. *lupulus* has been the main genetic resource for

modern hop cultivars (Hampton *et al.*, 2001). *Humulus lupulus* is a perennial vine, which is widely found in Europe including England, it can climb on trees and shrubs to a length of six metres or more (Omar, 1992; Hampton *et al.*, 2001; Estrada *et al.*, 2002). Figure 1.4 shows a hop plant.

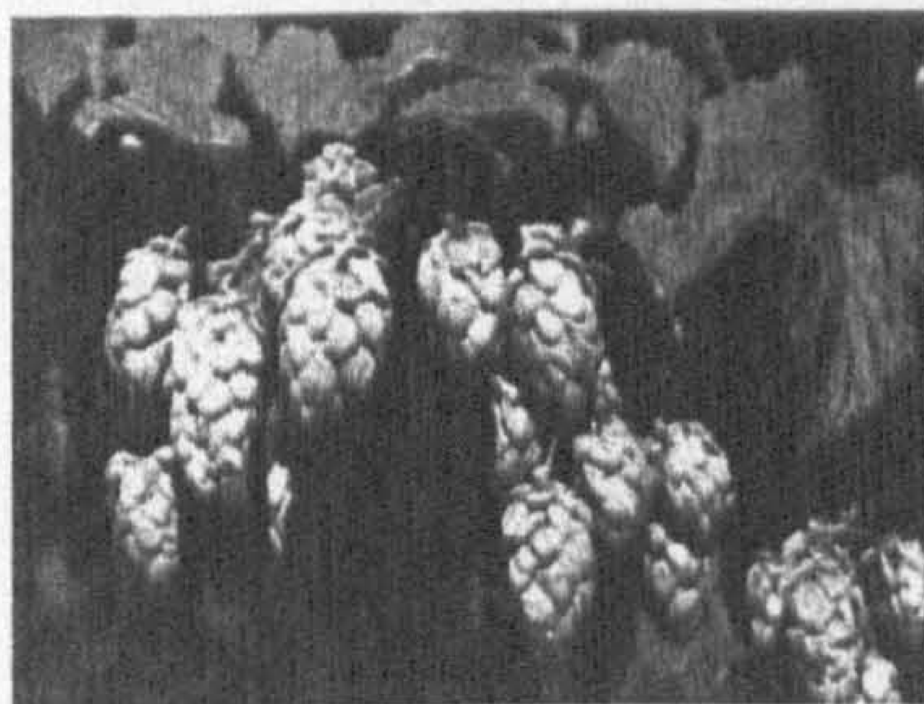


Figure 1.4: Hop plant.

The cones, the greenish female flowers are used in breweries for their bittering flavouring and aroma-enhancing powers (Sabo *et al.*, 2001; Estrada *et al.*, 2002). A cone consists of a stem, spindle and two types of paery scales – bracts and bracteoles. The scales are covered with lupulin glands, whose secreta contain resinous matter and essential oils (Likens *et al.*, 1978; Sabo *et al.*, 2001). The secreta of lupulin glands consists of soft resins, hard resins and essential oils (Sabo *et al.*, 2001).

1.6.2.2 Components of hops and their properties

Hops contain volatile oil, tannins, sugars, fatty acids and resin, but the quality of hops is mainly indicated by the bitter resin and essential oils (Kralj *et al.*, 1991; Omar, 1992). Resins and essential oils are found in the cones of female hop plants (Simpson and Smith, 1992; Larson *et al.*, 1996; Moir, 2000).

1.6.2.2.1 Resins

The resins are found in the lupulin glands and transferred to beer by adding whole hop cones, hop pellets or hop extract (Roberts and Lewis, 2002). Resins from the cones are primarily used as a preservative and as a flavouring agent in beer, due to humulone and

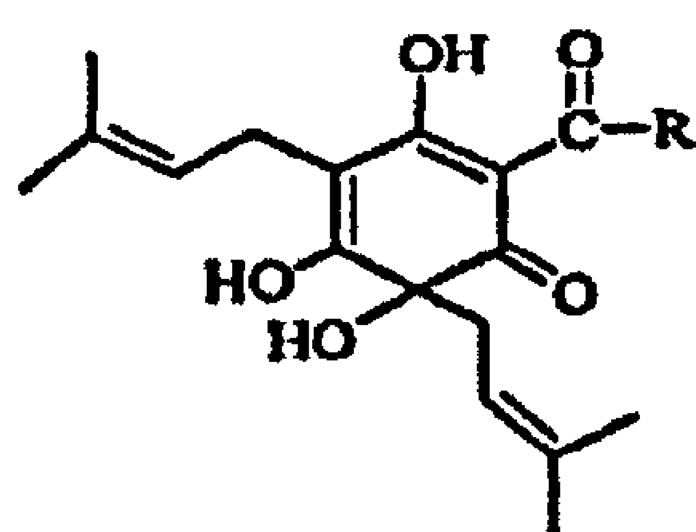
lupulone and their derivatives (Haas and Barsoumian, 1994; Milligan *et al.*, 2000; Hampton *et al.*, 2001). There are hard resins and soft resins. The soft resins are the most important as they consist of α-acids and β-acids, which have various potent biological activities (Sabo *et al.*, 2001; Chen and Lin, 2004), see sections 1.6.2.2.2 and 1.6.2.2.3.

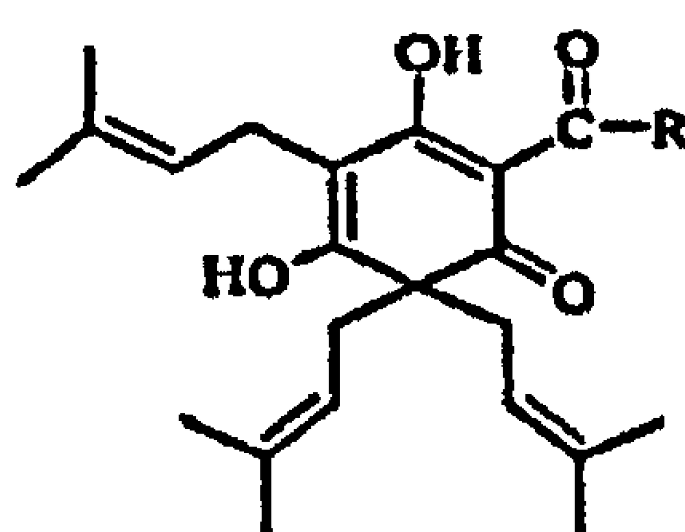
Sabo *et al.* (2001) determined that the content of total resin α-acids in hop cones depended on the number of lupulin glands in the cones of particular genotypes. They found variation in different varieties from 92.3 g/kg to 237.2 g/kg in total resins.

1.6.2.2.2 α-acids

The α-acids, up to 70 to 140 g/kg hops in bitter genotypes, are represented by humulone and its congeners (cohumulone, adhumulone, prehumulone, posthumulone) see figure 1.6 for chemical structure, and their isomerization products (isohumulones), which are the most important component of hops for the brewing industry (Simpson and Smith, 1992; Lu-Ping Ting and Goldstein, 1996).

In the brewing process the α-acids in hops are converted by a isomerization process during the wort boiling stage to the previously mentioned iso-α-acids, which occur both in *cis-* and *trans-* isomers and are more soluble and bitter (Simpson and Smith, 1992; Haas and Barsoumian, 1994; Moir).





α-acids (humulones)   **β-acids (lupulones)**

Figure 1.5: The chemical structure of alpha and beta acids.

1.6.2.2.3 β-acids

The β-acids (42 to 55 g/kg hops for Phoenix variety) (Sabo *et al.*, 2001; Chen and Lin, 2004), also known as lupulones, are represented by lupulone and its congeners (colupulone, adlupulone, prepulone, postlupulone) (see figure 1.5 for chemical structure) (Simpson and Smith, 1992; Lu-Ping Ting and Goldstein, 1996; Moir, 2000). The β-acids are poorly soluble in wort and beer and they cannot undergo the same isomerization reaction as the α-acids (Moir, 2000). Therefore, the β-acids have no direct brewing value. These are said to have higher antimicrobial activity than the iso-alpha resins (Haas and Barsoumian, 1994; Shipp *et al.*, 1994).

1.6.2.2.4 Essential oils

Each hop variety has a typical essential oil pattern, the essential oils are found in the lupulin glands (Kovacevic and Kac, 2002; Roberts and Lewis, 2002). Gas chromatography has aided in identifying many of the components of hops. Hydrocarbons usually constitute between 40 and 80% of the oil, and apart from a few simple alkanes, are terpenoid in origin (Moir, 2000). A complex group of at least 40 acyclic, monocyclic, bicyclic, and tricyclic sesquiterpenes are usually dominanted by α-humulene and β-caryophyllene (Moir, 2000).

Dry hops contain between 0.5 and 2% of essential oil, which is mainly terpene hydrocarbons and their oxidation products (Kovacevic and Kac, 2002). The volatile oils, normally between 0.3 and 1.0%, consists mainly of the terpene humulene (-caryophyllene), with –caryophyllene, myrecene, farnesene, 2-methylbut-3-ene-2-ol, 3-methyl-but-2-ene-1-al, 2,3,5-trihiahexane with traces of acids such as 2-methyl-propanoic and 3-methylbutanoic which increases in concentration in stored extracts (Omar, 1992). Kralj *et al.* (1991) studied the essential oils in 95 different genotypes of hops. They found that the total percentage of essential oil varied from year to year. They also found that not all

component of essential oil is present in every type of hop, but myrcene, linalool, α-humulene, β-caryophyllene, undecanone-2 and α-selinene were found in all samples.

1.6.2.2.5 Tannins

Hops are rich in condensed tannins (Stevens and Page, 2004). The proanthocyanidins (condensed tannins), defined as oligomers and polymers, are one of two major categories of vegetable tannins widely distributed in the plant kingdom, and these occur in the hop cones (Stevens *et al.*, 2002).

The total amount of proanthocyanidins is estimated to be in the range 5 and 50 g/kg on a dry weight basis, and human intake of the component is mainly through the intake of beer (Stevens *et al.*, 2002). Proanthocyanidins from various sources exhibit a wide range of biological activities: they are effective antioxidants and offer protection against cardiovascular disease, immune disorders, neurodegenerative diseases and they have strong free radical scavenging activities (Stevens *et al.*, 2002).

1.6.2.2.6 Polyphenols

Polyphenolic compounds comprise of 30 to 60 g/kg of the dry weight of hop cones. They are a large group of secondary plant metabolites (Papagiannopoulos and Mellenthin, 2002). Their antioxidant properties may prevent staling and account partly for the aroma stability of beer. Due to their antioxidative capacity they have a health potential, but little is known about their individual compounds and their potential effects (Papagiannopoulos and Mellenthin, 2002).

1.6.2.2.7 Flavonoids

Some flavonoids have been isolated from hops, i.e. chalcones and flavanones. Rodriguez *et al.* (2001) investigated their potential antioxidant propeties. They found that some of the

components in hops have antioxidant activity. The main flavonoid in hops is xanthahumol (Yilmazer *et al.*, 2001a). There is great interest in xanthohumol and related prenylflavonoids found in hops. These may have:

- anticarcinogenic effects in humans
- antioxidant properties
- antiviral properties against bovine viral diarrhoea
- potential inhibitors of herpes virus HSV-1 and HSV-2 and potentially inhibitor of HIV-1 (Stevens *et al.*, 1998; Wang *et al.*, 2004).

Experiments have shown that some of the flavonoids in hops such as xanthohumol, isoxanthohumol and dehydrocycloxanthohumol, can partially inhibit growth proliferation of human breast cancer cells, colon cancer cells, and ovarian cancer cells (Stevens *et al.*, 1998; Yilmazer *et al.*, 2001b).

1.6.2.3 Uses of hops in animal production and in vitro

Hops have been claimed to have anticarcinogenic effects against leukaemia, ovarian, colon and tumours in the colon of rats, effects on lipid metabolism and estrogenic as well as antimicrobial effects (Stevens *et al.*, 1998; Herath *et al.*, 2003; Chen and Lin, 2004).

Cornelison *et al.* (2006) studied the effects of feeding hops at inclusion levels 227, 454, 680 and 907 g/t in broiler chickens *versus* a negative control and a postive control containing antibiotics. They found that the hops improved the performance, starting at inclusion level 227 g/t compared with the negative control, but was not significantly different to the antibiotic treatment, see table 1.28.

Table 1.28: The effects of hops at 227 g/t, 453 g/t, 680 g/t and 907 g/t included in broiler diets on performance.

	Penicillin (g/t)		Hops g/t				
	0	50	227	453	680	907	*P*-value
Body wt (kg)							
14 days	0.404[c]	0.438[a]	0.427[ab]	0.422[b]	0.414[bc]	0.416[bc]	0.0002
35 days	2.162[b]	2.268[a]	2.168[b]	2.172[b]	2.184[b]	2.176[b]	0.0101
42 days	2.778[b]	2.892[a]	2.837[ab]	2.813[b]	2.809[b]	2.817[b]	0.0377
FCR							
14 days	1.266[a]	1.210[b]	1.229[ab]	1.254[a]	1.257[a]	1.258[a]	0.0121
35 days	1.575[ab]	1.486[c]	1.551[b]	1.581[a]	1.555[b]	1.577[ab]	<0.0001
42 days	1.716[a]	1.614[d]	1.669[c]	1.706[ab]	1.698[b]	1.707[ab]	<0.0001

Source: adapted from Cornelison *et al.* (2006).

1.6.2.3.1 Estrogenic activity of hops

A range of phytoestrogens have been identified in hops including 8-prenylnaringenin, 6-prenylnaringen, xanthohumol and isoxanthohumol (Coldham and Sauer, 2001). The lupulin glands of the hop flower contain 8-prenylnaringenin, which is a potential phytoestrogen in hops, along with other prenylflavonoids and the hop acids essential in brewing (Milligan *et al.*, 2000; Promberger *et al.*, 2001).

It has been suggested that hops have strong estrogenic activity, they have been suggested to cause menstrual disturbances and they have been used for treatment of gynaecological disorders (Milligan *et al.*, 2000; Promberger *et al.*, 2001). However, there are many controversies regarding the potential estrogenic activities of hops (Stevens *et al.*, 1998). Milligan *et al.* (2000) studied the estrogenic activity of hop extracts. They found that the endocrine activity of hops and hop products is mainly due to the high estrogenic activity of 8-prenylnaringenin.

1.6.2.3.2 Antimicrobial effects of hops

Hops have also shown to have antimicrobial activity. The α- and β-acids in the hops are antimicrobial (Omar, 1992). Colupulone is an antibiotic and antifungal agent (Mannering

et al., 2002). Shipp *et al.* (1994) investigated whether cupulone could induce cytochrome production in rats. No significant differences were found in hepatic activity compared to the control group.

In 1937, Shimwell (1937a) showed that hops had antibacterial properties against some bacteria, but not all. He assumed that some of the resistance from the bacteria occurred due to long contact with hop antiseptic under brewing conditions. He concluded that gram negative bacteria continued to grow as rapidly in hopped wort as in unhopped wort, whereas gram positive bacteria were either totally or partially inhibited in the presence of the hop extracts. Similarly, Teuber (1970) found that lupulone and humulone have antimicrobial activities against gram positive, but not gram negative bacteria. They also compared the antibiotic properties of isohumulone with humulone and lupulone by measuring the inhibitory concentration. Table 1.29 shows the concentrations of isohumulone necessary to produce a 50% reduction of gram positive bacteria. These were 15 to 30 times higher than those of humulone. The gram negative bacteria were not markedly inhibited.

Table 1.29: Inhibitory concentrations of lupulone, humulone and isohumulone which produce a 50% reduction in bacterial growth.

	Resin, inhibition concentration(μg/ml)		
Organism	**Lupulone**	**Humulone**	**Isohumulone**
Bacillus subtilis	20	16	300
Staphylococcus aureus	32	16	500
Micrococcus lysodeikticus	62	32	450
Escherichia coli	>500	>500	>500
Proteus mirabilis	>500	>500	>500

Source: adapted from Teuber (1970).

However, a potential problem of using hops as an antibacterial is the potential resistance of microorganisms to hop extracts (Larson *et al.*, 1996). Fernandez and Simpson (1993)

investigated the resistance of beer spoiling organisms to hops by measuring the minimum inhibitory concentrations for *trans*-isohumulone, humulone, colupulone and other antibacterial agents. They found that *trans*-humulone –sensitive organisms were also sensitive to humulone and colupulone and those resistant to *trans*-humulone were also resistant to humulone and colupulone.

Larson *et al.* (1996) studied the effects of hop resins on inhibition of *Listeria monocytogenes* in foods. They studied the effects of four different hop acid extracts (see table 1.29). They found that HE I only moderately affected *L. monocytogenes* in trypticase soy broth (TSB), whereas HE II showed strong inhibition of *L. monocytgens* at concentrations 10µg/ml (see table 1.309). HE III also inhibited *L. monocytogens* in TBS and brain heart infusion broth. However, HE IV was a poor inhibitor of *L. monocytogens* and only slightly affected growth at concentrations 50-500 µg/ml. The experiment showed that hop extracts were more effective in acidic foods than in foods with higher pH.

Table 1.30: Inhibition of *L. monocytogenes* Scott A by hop extracts in trypticase soy broth (TSB) or brain heart infusion broth (BHI).

Medium	Hop extract	Carrier	Concentration (µg/ml)	% inhibition (24h)
TSB	**HE I:** *iso-acids 30% w/v in aqueous suspension as potassium salts*	Tween 20	10	15
			30	11
			100	61
			300	99
TSB	**HE I:** *iso-acids 30% w/v in aqueous suspension as potassium salts*	Ethanol	10	19
			30	25
			100	64
			300	66
TSB	**HE II:** *41% β-acids, 12% α-acids, and desoxy-α-acids, hop oils and hop waxes*	Ethanol	0.3	12
			1	56
			3	62
			10	100
			30	100
			100	100
BHI	**HE III:** *29.7% colupulone, 65% β-acids, 8% desoxy-α-acids, 7% H_2O, and 0.6% iso-α-acids*	Ethanol	0.1	0
			0.3	31
			1	96
			3	98
			10	100
			30	100
TSB	**HE IV:** *post-β-acids (a serie of peaks that eluded after the β-acid (6% w/v)*	Ethanol	5	1
			10	4
			50	36
			100	47
			500	54
			1000	55

Source: adapted fom Larson *et al.* (1996).

Fukao *et al.* (2000) studied the antimicrobial effects of hop resins that are free of α-acids in combination with antibacterial agents, such as glycerol monocarpate, lysozyme, and sodium hexametaphosphate against *E. coli.* These agents have strong antimicrobial effects against gram-postitive bacteria, but very little is known about their antimicrobial effect against gram negative bacteria (Fukao *et al.*, 2000). They measured the antimicrobial activity by monitoring the turbidity of liquid cultures. They found that hop resins inhibited the growth of the six gram-positive bacteria shown in table 1.31 at concentrations <50µg/ml, whereas the gram negative bacteria were not inhibited at >10,000µg/ml.

Table 1.31: Antimicrobial activity of hop resins against different bacterial strains.

Species	Minimum inhibitory concentration (μg/ml)
Gram positive	
Bacillus cereus	<10
B. subtilis	40
Staphylococcus aureus	20
Micrococcus flavus	50
Lactobacillus brevis	20
Leuconostoc mesenteroides	20
Gram negative	
Escherichia coli	>10,000
Pseudomonas fluorescens	>10,000
Klebsiella pneumoniae	>10,000

Source: adapted fom Fukao *et al.* (2000).

Xanthohumol and prenylnaringenins have shown active against the fungi *Trichophyton mentagrophytes* and *T. rubrum.* The minimum inhibitory concentrations were in the range 3.13 to 12.5 μg/ml (Mizobuchi and Sata, 1984; Stevens *et al.*, 1998).

Mizobuchi and Sata (1984) studied the possible antifungal activities of different components from hard resins in hops; 6-Isopentenylnaringnin, xanthohumol, isoxanthohumol and sophoraflavanone. 6-Isopentenylnaringenin and xanthohumal had antifungal activities against the *Trichophyton* spp. None of the components were active against *E. coli, Candida albicans* and *Fusarium oxysporum.* In agreement with Stevens *et al.* (1998) none of the flavonoids showed activity against *Candida albicans, Fusarium oxysporum* or *E. coli.*

1.6.2.3.3 Other uses of hops

The hop products are used as sedative, tranquilizers, hypnotics, tonics, diuretics, anodyne and aromatic bitters (Wohlfart *et al.*, 1983; Omar, 1992; Estrada *et al.*, 2002). The sedative and tranquillizing activity has been well established from different tests with animals. This is partly due to the 2-methylbut-3-ene-2-ol contents of hops (Omar, 1992). Furthermore,

hops are often used for sleeplessness in the form of hop pillows (Omar, 1992). Hops are used in commercial products such as blood pressure tablets and Nervine Medicinal tea bags (Omar, 1992).

Chappel *et al.* (1998) conducted a 90 day oral toxicity study in dogs to provide safety information of tetrahydroisohumulone and hexahydroisohumulone isolated from hops. They concluded that both compounds were well tolerated by the dogs (Chappel *et al.*, 1998).

1.7 Conclusions

The ban of AGP may result in lower performance and higher level of diarrhoea after weaning in piglets. After evaluating different alternatives to AGP it was decided to select two herbal additives, which have not previously been used in piglets, and investigate their effects in piglets after weaning. Based on the evidence from human medicine it was decided to test the silymarin compound in pigs to see if it with its antioxidant effects could stimulate the immunity and thereby improve the performance in piglets just after weaning. Similarly, it was decided to test the effects of hops in piglets to see if they could improve the performance and test their potential antimicrobial effects.

2. GENERAL MATERIALS AND METHODS

2.1 Animal husbandry

All the experiments were carried out at Harper Adams University College's pig unit. The piglets used were PIC Camborough 15 (50% Large White, 25% Duroc and 25% Landrace). The selection of piglets for each experiment was balanced for weight, sex and dam.

The piglets were weaned at approximately 28 days of age. Standard commercial procedures of teeth clipping, tail docking and ear tagging were carried out approximately 24 hours after birth and an iron injection was given at three days of age. No creep feed was given to the piglets during the lactation period.

2.2 Feed

The same basal diets were used in all of the experiments. They were provided by Ian Hollows (Whitchurch, UK). There were two diets. A first stage diet fed from weaning to day 11 or 12 (day 12 for experiment 2), followed by a second stage diet fed from day 12 or 13 to day 26 or 28 (28 days were only in experiment 1). In experiment 1 the diets were mixed and pelleted (3 mm) by Ian Hollows (Whitchurch, UK). For the other experiments, the diets were provided by Ian Hollows but the additives mixed in and the diets were pelleted (3 mm) at Harper Adams University College, see experiments 2 and 3 for further details. No additional antimicrobials, enzymes, acids or herbs were included in the basal diets. The composition of ingredients and the estimated nutrient composition are shown in table 2.1.

Table 2.1: Composition and formulation of first and second stage basal diet used in the experiments (g/kg).

Ingredients	Stage 1	Stage 2
Cooked maize	200	100
Maize		100
Cooked wheat	164	140
Wheat		99
Porridge oats	100	100
Skimmed milk	100	50
Whey Powder	100	75
Fish meal	85	50
Full fat soya	160	145
Soya bean meal	20	100
Soya oil	30	15
Lysine HCl	2.5	1.5
Methionine	1	0.5
Threonine	2	1.5
Tryptophan	0.5	0
DiCal Phosphate	5	8
Vit/mineral premix*	15	15
Glucose	15	
Braes optisweet 2097	0.3	0.3
Vitamin E	0.2	
Nutrients		
Protein (g/kg)	226.1	219.4
Oil (g/kg)	84.6	65.7
GE (MJ/kg)	16.9	16.0
Total lysine (g/kg)	16.7	14.8
T. lysine/DE ratio	0.10	0.09

* Compostion of vit/mineral premix: 10 g/kg LysHCl, 42 g/kg Limestone, 44 g/kg MCP granules, 19 g/kg salt, 0.3 g/kg Vit A 500TFL, 0.04 g/kg Vit D, 3 g/kg Vit E 50%, 0.06 g/kg Vit B1, 0.2 g/kg Vit B12, 0.8 g/kg Copper sulphate 25%, 4 g/kg Choline chloride 50%, 5 g/kg zinc bioplex, 0.05 g/kg biotin 2, 0.3 g/kg nicotinic acid, 0.04 g/kg Vit B2 80%, 0.04 g/kg Vit B6, 0.05 g/kg Vit K 44%, 0.04 g/kg folic acid, 15 g/kg iron sulphate 20%TFL, 0.8 g/kg manganese oxide 62%, 2.7 g/kg zinc oxide 74%, 0.03 g/kg calcium iodate 62%, 0.25 g/kg selenium premix 1% TFL, 0.4 g/kg cobalt BMP 5%, 0.45 g/kg Cal pan 45%, 1 g/kg copper bioplex, 1 g/kg selplex 0.1%.

2.3 Feed analysis

Feed samples were collected at the beginning and the end of each experiment and pooled prior to analysis. The samples were analysed in duplicates for dry matter, ash, fat, protein and neutral detergent fibre content.

2.3.1 Dry matter

Dry matter content was analysed according to Watson (1994) by weighing approximately 100 g of the sample into a foil tray and drying for 24 hours at 100°C in an oven (Phillips

Harris Ltd). The sample was then weighed again and this was continued until a constant weight was reached. The samples were cooled in a dessicator and the dry matter calculated according to the following equation:

DM (g/kg) = (Dry sample weight (g)/Fresh sample weight (g)) x 1000

2.3.2 Ash

Ash was determined according to Watson (1994) by weighing approximately 1 g of dried sample into a porcelain crucible and ashed at 500°C in a furnace (Muffle Furnace size 3, Gallenkamp) for 16 hours. After cooling down in a dessicator the samples were reweighed and the ash content calculated according to the equation below:

Ash (g/kg DM) = (Ashed sample weight (g)/Original dry sample weight (g)) x 1000

2.3.3 Ether extract

Crude fat content of experimental diets and faeces were measured according to the AOAC (2000) method (no 920.39). Fat was determined by weighing 1 g of milled sample into extraction thimbles and boiling in petroleum ether for 60 minutes using an extraction unit (Soxtec, Foss). After 60 minutes of rinsing, the condensed petroleum ether was collected in the extraction cups and allowed to evaporate. The cups were reweighed for calculation of fat content according to the equation:

Ether extract (g/kg DM) =
(Weight of extracted fat (g)/Weight of original sample (g)) x 1000

For experiment 3 the feed and faecal samples were analysed with acid hydrolysis. The samples were boiled in hydrochloric acid, filtered and dried before ether extraction with

petroleum ether as described by Salgado *et al.* (2002). This was carried out by Central Labs (Banbury, UK).

2.3.4 Neutral detergent fibre (NDF)

The neutral detergent fibre content of the feed samples was determined by the Van Soest (1991) method. 0.5 g of dried milled sample was weighed and placed in a crucible and digested within a fibretec apparatus (Tecator 1020, Foss UK Ltd). The crucible was placed in the fibretec apparatus with 25 ml of cold neutral detergent fibre solution (93 g disodium ethylene diamine tetra-acetate dehydrate (EDTA), 34 g sodium borate, 150 g sodium lauryl sulphate, 50 ml 2-ethoxy ethanol and 22.8 g anhydrous disodium phosphate made up to 5 litres with distilled water) plus 0.5 ml octanol (antifoaming agent) and boiled for 30 minutes. Cold neutral detergent fibre solution and 2 ml of alpha amylase solution (2.0 g α-amylase (BHD) from *Bacillus subtilis* dissolved with 10 ml 2-ethoxy ethanol and 90 ml water) were added, and the mixture was boiled for a further 30 minutes. The sample was filtered and washed with hot water. Another 25 ml of hot water and 2 ml of alpha amylase solution was added and allowed to stand for 15 minutes. The samples were washed three times with deionised water, once with 20 ml acetone and dried at 100°C overnight, then cooled in a dissicator and reweighed. The samples were heated to 550°C in a furnace (Muffle Furnace size 3, Gallenkamp) for four hours, cooled in a dessicator and then reweighed. The neutral fibre content was calculated as:

NDF (g/kg DM) =
(Residual weight (g) – Ash content (g)/ Original sample weight (g)) x 1000

2.3.5 Protein

The total nitrogen content was measured on a LECO (FD-528) analyser and the results were multiplied by 6.25 to calculate the protein content of the sample. According to Dumas

(AOAC method 990.03) as described by Simonne *et al.* (1994) and Matejovic (1995) a 150 mg sample was weighed into a capsule and dropped into a 850°C furnace purged with O_2 gas. The combustion products of CO_2, H_2O and NO_x were filtered. The O_2 was removed and the NO_x gases converted to N_2, which was then measured by a thermal conductivity cell against a He background and the result displayed as weight percentage of nitrogen.

2.4 Liver enzymes

An intracardial blood serum sample was taken after slaughter (intravenous in experiment 1) (the blood sampling procedure is detailed in each experimental chapter: section 3.3.5 for experiment 1, section 5.3.6 for experiment 2 and section 6.3.4 for experiment 3) in 7 ml vacutainers containing no additives (BD Vacutainer, Plymouth) for analysis of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), total and direct bilirubin. The samples were centrifuged at 3100 rpm (rotations per minute) (Sanyo Centaur 2) for 15 minutes and the serum was removed and stored in aliquots at - 20°C until subsequent analysis. All the samples were analysed in duplicate.

2.4.1 Alanine aminotransferase (ALT)

The serum samples were analysed for ALT (EC 2.6.1.2 UV method) on a Cobas Mira Plus blood analyser (ABX Diagnostics, Montpellier, France) with a Randox test kit (Crumlin, UK). The principle of the method is that ALT catalyses the reversible transamination of α-oxoglytarate and L-alanine to pyruvate and α-glutamate. The pyruvate is then reduced to lactate in presence of lactate dehydrogenase (LDH) with concurrent oxidation of NADH to NAD. The rate of change in absorbance at 340 nm is monitored over a fixed time, and this is directly related to the ALT activity. For daily quality control Randox Assayed multisera level 2 and 3 were used.

2.4.2 Gamma-glutamyl transferase (GGT)

The GGT in the serum samples was analysed by a Colorimetric method according to the European Committee for Clinical Laboratory Standards (method EC 2.3.2.2) with a Randox test kit (Crumlin, UK) on a Cobas Mira Plus blood analyser (ABX Diagnostics, Montpellier, France). The method used is based on the reaction of the substrate L-γ-glutamyl-3-carboxy-4-nitroanilide in presence of glycylglycine is converted by γ-GT to 5-amino-2-nitrobenzoate, which can be measured at 405 nm. For daily quality control Randox Assayed multisera level 2 and 3 were used.

2.4.3 Aspartate aminotransferase (AST)

The level of AST in the serum was analysed with the International Federation of Clinical Chemistry (Method EC 2.6.1.1) with a Randox test kit (Crumlin, UK) on a Cobas Mira Plus blood analyser (ABX Diagnostics, Montpellier, France). The principle of the test is that in the reaction the AST catalyses the reversible transamination of α-oxoglutarate and L-aspartate to oxaloacetate and L-glutamate. The oxaloacetate is then reduced to malate in the presence of maltate dehydrogenase with the concurrent oxidation of NADH to NAD. The rate of change in absorbance is directly proportional to the AST activity and can be read at 340 nm. For daily quality control Randox Assayed Multisera level 2 and 3 (Crumlin, UK) were used.

2.4.4 Total and direct bilirubin

The serum sample was analysed for direct bilirubin (Colorimetric method) on a Cobas Mira Plus blood analyser (ABX Diagnostics, Montpellier, France) with a Randox test kit (Crumlin, UK). Direct bilirubin is determined in the absence of an accelerator by the reaction with diasotised sulphanilic acid and read at 546 nm as described by Walters and Gerarde (1970). Total bilirubin in the serum (Colorimetric method) was measured on a Cobas Mira Plus blood analyser (ABX Diagnostics, Montpellier, France) with a Randox

test kit (Crumlin, UK) as described by Winsten and Cehelyk (1969) and Walters and Gerarde (1970). The method used was based on the principle that total bilirubin is determined in the presence of dimethylsulphoxide by the reaction with diasotised sulphanilic acid and read at 546 nm. For daily quality control Randox Assayed multisera level 2 and 3 and elevated bilirubin (Crumlin, UK) were used.

2.5 Volatile fatty acids (VFA)

Volatile fatty acids (VFA) were quantified by the method described by Otto *et al.* (2003), Franklin *et al.* (2002) and van Laar *et al.* (2000). Digesta samples were collected from the caecum and the colon after slaughter (the slaughtering procedure is detailed in each experimental chapter section 5.3.6 for experiment 2 and section 6.3.4 for experiment 3) and diluted with 1.0 M HCl to lower the pH to below 2. These samples were frozen at -20°C until VFA analysis was performed. The acidified digesta samples were mixed thoroughly with a vortex mixer. Approximately 10 ml was centrifuged at 16,500 rpm (Beckham Avanti 30 centrifuge) for 22 minutes at 4°C, the supernatant taken off and then centrifuged again at 16,500 rpm (Beckham Avanti 30 centrifuge) for 22 minutes at 4°C.

An internal standard of 25 mmol phenol was mixed with the supernatant at the ratio 1 in 10 and then filtered through a Whatman nitrocellular membrane filter, 25 mm diameter, 0.2 µm pore size (Merck). The samples were analysed by gas chromatography (Perker-Elmer 8500) with a DB-FFAP capillary column.

An external standard was used consisting of 2.35 g phenol in 10 ml acetic acid, 8 ml propionic acid, 5 ml butyric acid, 1 ml iso-butyric acid, 1 ml iso-valeric acid, 1 ml valeric acid and 1 ml caproic acid.

2.6 Gut histology

After slaughter (the slaughtering procedure is detailed in each experimental chapter section 5.3.6 for experiment 2 and section 6.3.4 for experiment 3), two 1 cm sections of the small intestine were removed at approximately 1 m from either end. These were placed in 10% neutral buffered formalin. After fixation the samples were sent to Precision Histology International (Norfolk, UK) for hematoxylin and eosin (H & E) staining of tissue. They were then embedded in paraffin wax, sectioned, dehydrated and placed on a slide. They were stained with haematoxylin and eosin as described by Pluske *et al.* (1996a).

The slides were examined using an Olympus CH20 microscope and the measurements were made with an eyepiece graticule with at a total magnification x10. The villous height, crypt depth and villous height plus crypt depth was measured on 10 intact villi. The villous height was measured from the tip to the crypt-villous junction, and the crypt depth was measured from the crypt-villous junction to the crypt base as described by Pluske *et al.* (1996a).

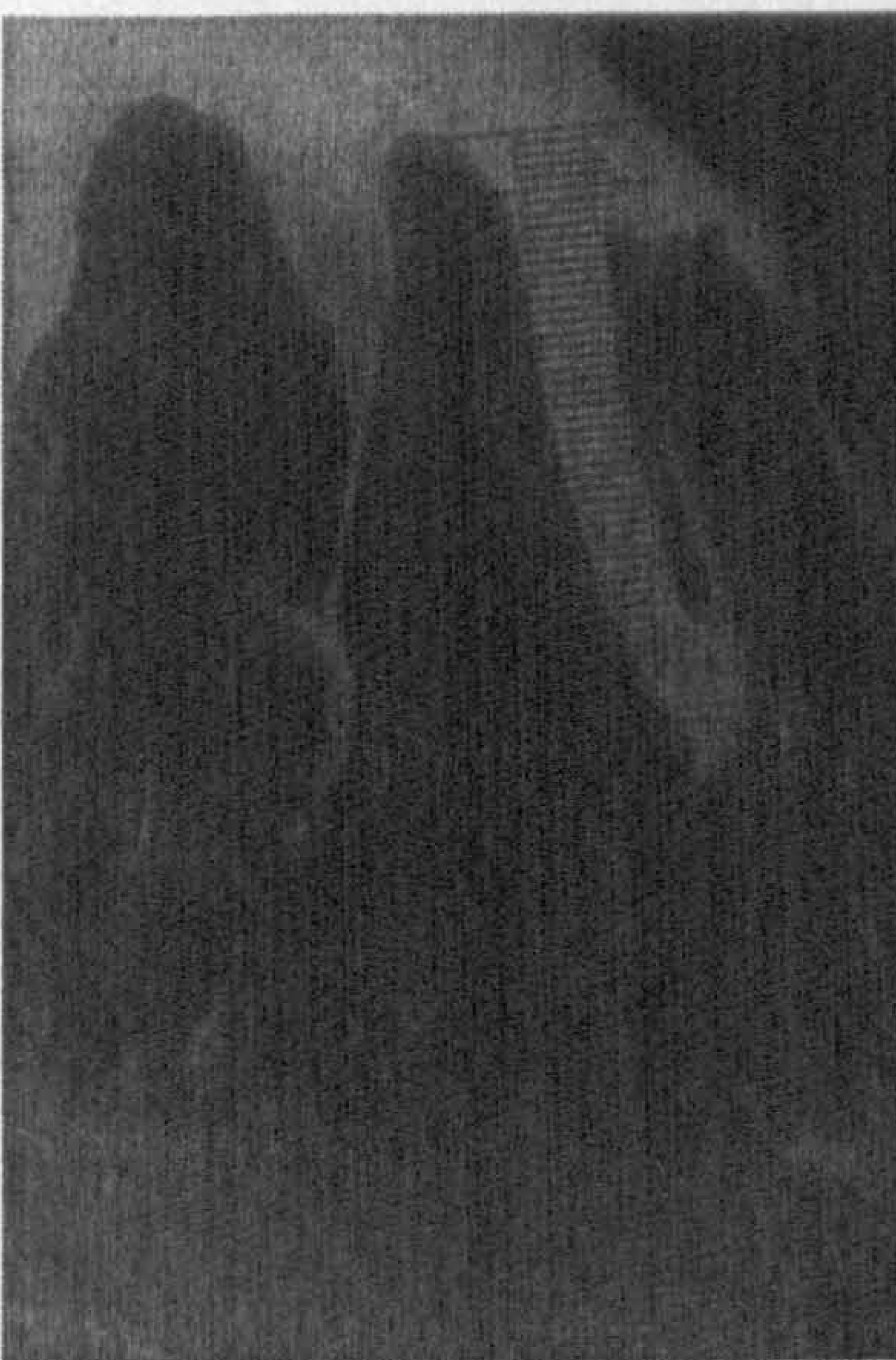


Figure 2.1: The measurement of the villous height and crypt depth of a four week old weaner pig in the microscope.

The number of eosinophils and the lymphocytes were counted in each of the measured villous and crypts under at a total magnification of x40.

2.7 Microbiology

Quantitative counts of *E. coli,* Lactic acid bacteria, *Bacteriodes, Clostridia* and *Streptococci* and presence of *Salmonella* and *Campylobacter* in pig faeces, the small intestine and colon were carried out by Devan Labs (Telford, UK). After slaughter (the slaughtering procedure is detailed in each experimental chapter section 5.3.6 for experiment 2 and section 6.3.4 for experiment 3) the pigs were dissected and a section of 5 to 10 cm was sealed of with small cable ties at approximately the middle of the small intestine and the middle of the large intestine. The sections were cut off and stored anaerobically (BBL GasPak, Becron Pickerson Systems, Sparks, USA). Faecal samples were collected in sterile pots by grab sampling. The samples were transported directly to Devan labs (Telford, UK) for bacterial analysis on the same day.

The intestinal content was extracted using cotton wool through an incision made into the tied section of the segment. The samples (faecal as well as intestinal) were diluted 1/10 in Maximum recovery diluent (MRD) (Oxoid) and vortexed to achieve an even suspension. Subsequent 1/10 serial dilutions were made by pipetting 0.5 ml of suspension of the dilutions into 4.5 ml of MRD and vortexing. This procedure was repeated to achieve a series from 10^{-2} to 10^{-11}. The diluted samples were inoculated onto the different culture media depending on the bacteria they were analysed for, see below.

2.7.1 Salmonella species

The presence/absence of *Salmonella* was found by mixing approximately 1 g of material into 10 ml selenite cysteine broth (Merck) and incubated at 37°C for 24 hours. The mixture was plated onto Brilliant Green agar and Xylose Lysine Decarboxylase agar (Oxoid). The

plates were then incubated at 37°C for a further 24 hours. *Salmonella* colonies were confirmed with oxidase test (positive results indicated presence of *Salmonella*) followed by plating on urea agar (negative result indicated *Salmonella*) and API20E Biomerieux followed by polyvalent and monovalent serotyping.

2.7.2 E. coli

E. coli were quantified by inoculating the samples onto petrifilm (3M) and incubating them at 37°C for 24 hours. The colonies were identified as typically blue coloured colonies with associated gas bubbles on the petrifilm.

2.7.3 Lactic acid bacteria

Samples were incubated on De Mann, Rogosa, Sharpe (MRS) agar (Oxoid) at 37°C for 72 hours. White colonies were checked by microscopy to confirm lactic acid bacteria.

2.7.4 Gram negative facultative anaerobes (Bacteriodes)

Samples were incubated on Wilkins Chalgren agar (with GN supplement and 5% horse blood) GN (Oxoid) in anaerobic gas jars with anaerobic Gaspaks (Oxoid) at 37°C for 48 hours. The total number of colonies was counted.

2.7.5 Sulphide reducing Clostridia

Samples were incubated on Tryptose-sulfite-cycloserine (TSC) agar (Merck) in anaerobic gas jars with anaerobic Gaspaks (Oxoid) at 37°C for 24 hours. The colonies of *Clostridia* were identified as large black colonies.

2.7.6 Streptococci

Samples were incubated on Kanamycin aesculin azide agar (Merck) at 37°C for 48 hours. *Streptococci* were identified as small colonies with black halos which usually pit the agar

and were catalase negative. Further examination with microscope typically showed as oval chain formation.

2.7.7 Campylobacter

For the presence/absence of *Campylobacter*, approximately 1 g of faecal material was placed into 10 ml Prestons Campylobacter broth +7% lysed horse blood (Oxoid) and incubated for 48 hours at 42°C in a micro-aerobic atmosphere (7 to 10% oxygen) in Campylobacter gaspaks (Oxoid). Conformation of typical colonies was made by microscopial examination (very small and highly motile organisms) and an oxidase test (Positive).

2.8 Faecal score

In the experiments the level of diarrhoea was measured using a subjective score from 1 to 5 for each individual pig. The scale was adapted from Gill *et al.* (2000), as shown in table 2.2.

Table 2.2: Description of faecal score on a scale from 1 to 5 for weaner pigs.

Score	Description
1	Watery/runny
2	Wet/loose
3	Moist/firm
4	Dry
5	Dry/hard

3. EXPERIMENT 1: THE EFFECTS OF HOPS AND SILYMARIN IN WEANER PIG DIETS OF DIFFERENT ENERGY LEVELS

3.1 Introduction

Experiments with herbal additives have shown that some herbs and spices can improve the performance of animals by various mechanisms. For example, yarrow has shown to have a positive effect on the performance of broiler chickens by improving the fat digestion (Lewis, 2005), and other experiments have as described in the literature review (section 1.5.7) shown that herbs/herbal mixtures resulted in better performance, similar to that achieved with AGP.

After weaning piglets experience a period of low feed intake and poor performance. It makes the piglets more susceptible to diseases, especially diarrhoea (Baynes and Varley, 2001; Broom *et al.*, 2003; Pickard and Wiseman, 2003), which is most often caused by *E. coli* and rotavirus (Pluske *et al.*, 1997), as described in the literature review section 1.3.1.2 The AGP, which were previously used to minimise the problems are now banned, and the industry is looking for alternatives.

Silymarin and hops were identified in the literature review as having the potential to improve performance, and were chosen for investigation in weaner pigs. They are thought to have different mechanisms and modes of action. Hops are mainly used for their antimicrobial activities, but they also have anticarcinogenic effects against leukaemia, ovarian, colon and breast cancer cells, as well as lipid metabolic and estrogenic effects, see also section 1.6.2 (Stevens *et al.*, 1998; Herath *et al.*, 2003; Chen and Lin, 2004), and have proven to improve performance in broilers (Cornelison *et al.*, 2006). Silymarin has antioxidant and protective effects on the liver, when exposed to various toxic compounds, such phalloidin, galactosamine, thioacetamide, thioacetamide, halothane and carbon

tetrachloride. It has also been shown to have beneficial effects on upper gastrointestinal disturbances, menstrual disorders, the immune system and it has been shown to improve the weight gain of mice, see also section 1.6.1 (Khan *et al.*, 1986; Valenzuela and Garrido, 1994; Johnson *et al.*, 2003).

Several authors have discussed that there is a possibility that different feed additives fail to exhibit growth promotion in feeding experiments, when the animals are fed highly digestible diets and already exhibit high performance (Botsoglou *et al.*, 2002; Lee *et al.*, 2003; Jang *et al.*, 2004). It was therefore decided to try and compromise the condition by "diluting" the diets. Addition of dietary fibre is generally considered to have "anti-nutrious" properties (Montagne *et al.*, 2003). High fibre intake has shown to increase intestinal transit time, delay gastric emptying, delay glucose absorption, increase pancreatic secretion, assist faecal bulking and supply fermentative substrates to the large intestine (Le Dividich and Sève, 2001; Pluske *et al.*, 2001; Montagne *et al.*, 2003). There is some controversy regarding the use of fibre in pig diets (see also section 1.4.2.3). Some researchers suggest that increased dietary fibre is associated with higher incidence of diarrhoea (McDonald *et al.*, 1999), and in growing pigs "fibrous" compounds have been associated with negative influence on energy and protein digestibility (Pluske *et al.*, 2001).

In the present experiment the effects of diluting the diets with oat hulls was tested. Oat hulls were chosen, as they are insoluble lignified fibres, which are usually to a high degree resistant to degradation in the large intestine, whereas soluble fibres are usually well degraded (for example sugar beet fibre) (Bach Knudsen, 2001a). Oat hull meal has a high lignin content (148 g/kg dry matter compared to sugar beet pulp 35 g/kg dry matter and a high concentration of non-cellulosic polysaccharides (Bach Knudsen, 2001b). They have been used to "dilute" diets in broilers at 150 g/kg (Nielsen *et al.*, 2003) and 300 or 400 g/kg, which showed to reduce the feed intake (Sandilands *et al.*, 2005; Sandilands *et al.*,

2006). The dilution in the current experiment was made to investigate if the hops and silymarin had more pronounced effects on weaner pigs if the diets were less digestible.

3.2 Aims and objectives

The hypothesis to be tested in the current experiment is that hops and silymarin can reduce the negative effects seen after weaning in piglets (see section 1.1), and that the effects of hops or silymarin are more pronounced with a lower nutrient density diet from 0 to 28 days. To evaluate the potential different mechanisms of hops and silymarin (see section 1.6) by which this could happen, the following parameters were measured:

1) growth performance
2) liver enzymes and blood metabolites
3) immune status (inflammation and infection)
4) gut health

3.3 Materials and methods

The experiment was a 3 x 2 factorial design, with three different dietary regimes: control (with no additives), hops at 1 kg/t and silymarin (BFI, Chester) at 0.30 kg/t. The silymarin was in a mixture containing 300 g/kg of 80 % silymarin, 300 g/kg olive oil, 250 g/kg corn cob and 150 g/kg silica, which gives an inclusion level of 72 g silymarin/t feed. The inclusion level of silymarin compound was selected assuming an intake between 5 mg/kg bodyweight and 20 mg/kg bodyweight, as in humans mushroom poisoning with the *Amanita phalloides* is treated with between 5 mg/kg/day and 20 mg/kg/day (Wellington and Jarvis, 2001). For the hops (variety Phoenix) (BFI Chester) the levels were chosen at which they exhibit antimicrobial effects. *In vitro* studies were carried out with hops to test their antibacterial properties at different concentration (see chapter 4, where the *in vitro* experiments are described), they showed that at 1 g/kg they had slightly inhibitory effects against *Staphylococcus, Streptococcus* and certain strains of *E. coli*. It has to be considered

that silymarin is an isolated compound, whereas the hops are the whole hops grinded. Each of the treatments was "diluted" with 100 g/kg oat hulls. The composition of the diets is shown in section 2.2 (table 2.1) and table 3.1 and the proximate analysis in table 3.2.

Table 3.1: The composition of the diluted diets.

	Stage 1 Diluted	Stage 2 Diluted
Cooked maize	182	91
Maize		91
Cooked wheat	149	127
Wheat		90
Porridge oats	91	91
Skimmed milk	91	45
Whey Powder	91	68
Fish meal	77	45
Full fat soya	145	132
Soya bean meal	18	91
Soya oil	27	14
Lysine HCl	2.3	1.4
Methionine	0.91	0.45
Threonine	1.82	1.36
Tryptophan	0.45	0.45
DiCal Phosphate	4.5	7.27
Vit/mineral premix	14	14
Glucose	14	
Braes optisweet 2097	0.27	0.27
Vitamin E	0.18	
Oat hulls	91	91
Total	**1000**	**1000**

Table 3.2: Nutritional values of the diets.

	Dry matter (g/kg)	Ash (g/kg)	Protein (g/kg)	Oil (g/kg)	NDF (g/kg)	Calculated DE (MJ/kg dry matter)
Stage 1						
St control	877.8	53.1	228.9	87.2	120.6	17.21
St hops	879.6	61.8	243.7	86.3	130.4	16.89
St silymarin	883.6	59.0	247.5	85.9	145.7	16.79
D control	880.0	60.0	234.8	78.8	161.0	16.33
D hops	883.7	58.1	231.8	79.5	163.5	16.35
D silymarin	884.9	55.1	231.4	87.3	156.7	16.66
Stage 2						
St control	874.3	57.0	248.0	60.7	125.7	16.74
St hops	876.4	59.9	240.8	58.6	124.2	16.58
St silymarin	871.0	58.8	243.2	62.6	129.2	16.62
D control	880.2	57.0	235.4	55.3	182.7	15.76
D hops	880.6	56.0	234.0	54.5	175.4	15.87
D silymarin	877.1	55.4	226.6	59.5	177.1	15.89

St= standard diet, D= diluted with 10% oathulls

192 PIC piglets were weaned at approximately 28 days of age in three batches. There were 96 piglets in the first batch and 48 piglets in second and the third batch. The piglets were allocated as described in section 2.1. At weaning the piglets were randomly allocated into one of the six treatments with four pigs per pen. The diets were fed *ad libitum* for 28 days. There were two diets; a first stage diet was fed from weaning to day 11 and a second stage diet from day 11 to day 28, as described in section 2.2. Water was available *ad libitum.*

3.3.1 Recordings

The live weight of the pigs was measured at weaning, day 4, 11, 14, 21 and 28 on a on a balance ranging from 0 to 30 kg with a crate for the piglets (Pharmweigh, Bury, St Edmunds). The feed intake was measured on a pen basis at the same time as the piglets were weighed.

3.3.2 Faecal sampling and faecal score

Faecal scores were determined at weaning, day 11 and day 28 using the method described in section 2.8. Faecal samples were collected by grab sampling on days 11 and 28 from each pig. The samples were pooled from all the pigs in a pen and instantly frozen with liquid nitrogen. They were stored at -80°C until further analysis.

3.3.3 E. coli analysis

Only the control and hops treatments were analysed for total viable count of *E. coli,* as the silymarin has not been reported to affect bacterial growth. 0.5 g of the faecal samples were thawed and diluted to from 10^5 to 10^7 in sterile ringer solution and inoculated by overlay method onto Harlequin *E. coli/Coliform* (HAL 008) media (Lab M, Bury). The samples were incubated for 24 hours at 37° C. The colonies could then be identified as blue/purple colonies and were counted.

3.3.4 Rotavirus analysis

The faecal samples from day 11 were also screened for rotavirus to see if rotavirus was present in the piglets. This was performed with an indirect ELISA Rotavirus kit (Biovet, Surrey). The samples were diluted 1/50 in deionised warm water, homogenised and further diluted with a buffer. The samples were added to the rotavirus antibody coated wells and incubated for 30 minutes. The wells were washed out and a conjugate was added and incubated for a further 30 minutes. They were washed again and two substrates were added to allow a colour change to show where the virus was present. The samples were visually assessed for presence of rotavirus.

3.3.5 Blood sampling and analysis

Blood samples were taken by venipuncture in the supine position from two pigs per pen on days 7, 18 and 27 under the home office license "Factors affecting pigs health and welfare" PPL 40/2109. The serum was obtained as desribed in section 2.4. The samples were analysed for liver enzymes; ALT, GGT and AST, and direct and total bilirubin, as described in section 2.4. All of the serum samples were also analysed for urea nitrogen on a Technicon RA-1000 spectrometer (product no. T01-1821-56). The reaction is initiated by the addition of the serum. The amount of urea in the sample is directly proportional to the decrease in absorbance at 340 nm due o the formation of NAD from NADH.

The level of glucose in all serum samples were measured on a Technicon RA-1000 spectrometer (product no. T01-1833-56). The test involves the phosphorylation of glucose by hexokinase and the action of glucose-6-phosphate dehydrogenase on the product.

The haptoglobin level of the serum (Tridelta Ltd, Ireland) was measured on day 27 on a Cobas Mira Plus analyser. The principle of the test is based on the fact that the peroxidase activity of free haemoglobin is preserved at a low pH level. The haemoglobin peroxidase is directly proportional to the haptoglobin present in the serum and the absorbance is measured at 630 nm.

3.3.6 Statistical analysis

The data was analysed with Genstat 5th edition as a 2 x 3 factorial design. The performance, feed intake, blood analysis and faecal score of the piglets were analysed using ANOVA (analysis of variance). The rotavirus results with Chi-square test. Significant differences are based on probability of less than 0.05.

3.4 Results

3.4.1 Performance

Two individual pigs did not grow very well, it was assumed to be due to illness, and their data was taken out of the analysis and adjusted for in the pen data.

No statistically significant difference was seen in the feed intake between the different treatments at any time during the experiment. The average feed intake for all treatments is shown in table 3.3.

Table 3.3: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on voluntary feed intake (g/pig/day) in newly weaned pigs.

	No added fibre			Added fibre			*Treatment x fibre*	
Day	**C**	**H**	**S**	**C**	**H**	**S**	**s.e.d.**	***P***
0-4	227	202	208	226	206	209	38.5	0.996
4-11	323	294	305	320	287	294	30.1	0.984
11-14	502	467	461	498	479	477	52.5	0.956
14-21	693	743	650	719	739	679	81.7	0.949
21-28	924	920	913	994	931	902	83.4	0.780

The daily live weight gain was not affected by treatments (see table 3.4). The dilution of the diets did not result in a significant difference at any time period during the experiment. Similarly, the live weight at the different days post weaning was not affected by treatment (see table 3.5). The FCR was unaffected by dietary treatment in the period 4 to 11 days post weaning. Overall, the silymarin and the hops treatments had better FCR than the control in the periods between day 11 and 28 ($P = 0.056$) and day 21 and 28 after weaning ($P = 0.014$), see table 3.6 and 3.7.

Table 3.4: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on daily live weight gain (g/day) in newly weaned pigs.

	No added fibre			Added fibre			*Treatment x fibre*	
Day	**C**	**H**	**S**	**C**	**H**	**S**	**s.e.d.**	***P***
4-11	232	184	201	215	216	196	30.2	0.489
11-28	499	502	478	475	531	490	45.8	0.709
14-21	481	468	421	457	523	433	54.7	0.601
21-28	562	580	578	530	593	587	50.4	0.790
0-21	326	301	291	314	337	293	39.9	0.686
4-28	421	409	397	399	439	404	38.4	0.635

Table 3.5: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on liveweight (kg) in newly weaned pigs.

	No added fibre			Added fibre			*Treatment x fibre*	
Day	**C**	**H**	**S**	**C**	**H**	**S**	**s.e.d.**	***P***
0	9.67	9.71	9.63	9.70	9.65	9.63	0.772	0.997
4	10.35	10.29	10.26	10.43	10.34	10.19	0.888	0.992
11	11.98	11.57	11.67	11.93	11.85	11.57	1.015	0.959
14	13.16	12.76	12.81	13.10	13.07	12.75	1.181	0.969
21	16.52	16.04	15.75	16.30	16.73	15.79	1.504	0.906
28	20.45	20.10	19.79	20.01	20.88	19.89	1.669	0.874

Table 3.6: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on feed conversion ratio (FCR) in newly weaned pigs.

	No added fibre			Added fibre			*Treatment x fibre*	
Day	**C**	**H**	**S**	**C**	**H**	**S**	**s.e.d.**	***P***
4-11	1.40	1.72	1.53	1.42	1.42	1.71	0.169	0.136
11-28	1.52	1.53	1.44	1.67	1.46	1.51	0.075	0.127
14-21	1.46	1.58	1.58	1.56	1.41	1.59	0.096	0.148
21-28	1.65	1.59	1.45	1.88	1.60	1.53	0.128	0.453
4-28	1.50	1.53	1.44	1.64	1.44	1.51	0.075	0.092
0-21	1.40	1.50	1.49	1.48	1.34	1.52	0.079	0.096

Table 3.7: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on feed conversion ratio in newly weaned pigs.

	Treatment		*Added fibre*	
Day	**s.e.d.**	***P***	**s.e.d.**	***P***
4-11	0.120	0.186	0.098	0.719
11-28	0.053	0.056	0.043	0.284
14-21	0.068	0.351	0.055	0.788
21-28	0.091	**0.014**	0.074	0.157
4-28	0.053	0.184	0.044	0.373
0-21	0.057	0.317	0.046	0.708

Table 3.8 shows the average intake of the herbal additives per kg bodyweight of pig at the different time periods. The calculations are based on the average pig weight in each period and the average feed intake, knowing there is 0.30 kg/t silymarin in the diet and 1 kg/t of hops.

Table 3.8: The average intake of hops and silymarin at different time periods in weaner pigs (mg/kg bodyweight).

Day	**Standard hops**	**Fibre hops**	**Standard silymarin**	**Fibre silymarin**
0-4	19.53	19.92	6.08	6.15
4-11	25.45	24.39	7.84	7.60
11-14	36.60	36.57	10.77	11.22
14-21	46.38	44.17	12.38	12.90
21-28	45.77	44.54	13.84	13.59

3.4.2 Faecal score and gut health

The faecal scores were not statistically significantly different between the herbal additives at any time (tables 3.9 and 3.10). However, for the diets with added fibre, the score was significantly higher ($P = 0.005$) indicating firmer faeces on the last day of measuring.

Rotavirus was analysed with a chi-square analysis. No significant difference was seen between the different treatments (table 3.11). However, a trend was seen for lower level of

rotavirus in the diluted diet (P = 0.083) (table 3.12). The number of *E. coli* was not affected by dietary treatment at any of the days it was measured (table 3.13).

Table 3.9: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on faecal score in newly weaned pigs.

	No added fibre			Added fibre			*Treatment x fibre*	
Day	**C**	**H**	**S**	**C**	**H**	**S**	**s.e.d.**	***P***
0	4.28	3.97	4.06	4.16	3.97	4.03	0.203	0.902
11	3.06	2.78	2.95	3.19	2.76	2.78	0239	0.683
28	2.94	2.84	2.83	3.47	3.25	3.13	0.241	0.782

Table 3.10: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on faecal score in newly weaned pigs.

	Treatment		*Added fibre*	
Day	**s.e.d.**	***P***	**s.e.d.**	***P***
0	0.142	0.215	0.117	0.658
11	0.169	0.109	0.098	0.871
28	0.170	0.411	0.139	0.005

Table 3.11: Effect of control, hops and silymarin in diets on the number of pens where rotavirus samples were positive or negative on day 11 in newly weaned pigs.

	Control	Hops	Silymarin	X^2 value	*P*-value
Positive	8	7	9	0.50	0.779
Negative	8	9	7		

Table 3.12: Effect of adding fibre in the diets on the number of pens where rotavirus samples were positive or negative on day 11 in newly weaned pigs.

	Standard	Fibre	X^2 value	*P*-value
Positive	15	9	3.00	0.083
Negative	6	15		

Table 3.13: Effect of control (C) and hops (H) with or without added fibre in the diets on the number of *E. coli* in faeces on day 11 and day 28 in newly weaned pigs (log 10 cfu per gram faeces/ digesta).

	No Fibre		Added fibre		*Treatment x fibre*	
Day	**C**	**H**	**C**	**H**	**s.e.d.**	***P***
11	5.93	5.76	5.73	5.73	0.445	0.759
28	5.19	4.76	4.89	4.68	0.510	0.749

3.4.3 Blood metabolites

The analysis of the blood glucose level is shown in tables 3.14 and 3.15. A significant difference was seen on day 7 ($P = 0.005$), where hops diets were lower than the other treatments. No other significant differences were seen. The levels of blood glucose were within the reported normal ranges for pigs (Rushton, 1981; Kaneko, 1997; He *et al.*, 2001).

Blood urea levels were not significantly different between the treatments at any time period (table 3.16). All the treatments were within the normal range for weaner pigs (3.57 to 10.7 mmol/l) (Rushton, 1981; Finco, 1997).

Table 3.14: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in diets on level of blood glucose in newly weaned pigs (umol/l).

	No added fibre			Added fibre			*Treatment x fibre*	
Day	**C**	**H**	**S**	**C**	**H**	**S**	**s.e.d.**	***P***
7	6.04	5.51	5.98	5.88	5.51	5.70	0.198	0.601
18	5.88	5.66	5.74	5.95	5.89	5.64	0.201	0.499
27	6.17	5.90	6.06	6.07	5.91	5.99	0.241	0.950

Table 3.15: Main effect of control (C), hops (H) and silymarin (S) with or without added fibre in diets on level of blood glucose in newly weaned pigs.

	Treatment		*Added fibre*	
Day	**s.e.d.**	***P***	**s.e.d.**	***P***
7	0.140	0.005	0.115	0.197
18	0.142	0.287	0.116	0.571
27	0.170	0.455	0.139	0.684

Table 3.16: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on level of blood urea in newly weaned pigs (umol/l).

	No added fibre			Added fibre			*Treatment x fibre*	
Day	**C**	**H**	**S**	**C**	**H**	**S**	**s.e.d.**	***P***
7	4.20	3.42	3.92	3.25	3.60	3.79	0.356	0.073
18	4.37	4.08	4.38	4.09	3.68	4.23	0.461	0.928
27	4.17	3.61	3.89	3.53	3.43	3.87	0.384	0.492

3.4.4 Liver enzymes

The level of ALT was not affected by treatment on day 7 and 18, but on day 27 post weaning there was a trend for the hop treatments to be lower than the control and the silymarin (P = 0.068) (see table 3.17 and 3.18). The average values of ALT found were higher than the reported normal values by Rushton (1981) for pigs between 10 and 18 U/l. However, they were within the range reported by Tennant (1997) of 32 to 84 U/l .

No significant difference was seen between the herbal additives and the control on day 7 and 18 post weaning in AST levels (table 3.19 and 3.20). However, on day 27 the hop treatments had significantly lower AST levels (P = 0.011) than the control and the silymarin. The level of AST found was higher than the normal ranges between 10 and 22 IU/l reported by Rushton (1981), but within the ranges between 18 to 65 U/l for pigs found in other experiments (Tennant, 1997; He *et al.*, 2001).

The GGT serum level was not significantly affected by the herbal additives on any day after weaning (table 3.21). The silymarin treatments were the highest on day 27, thus it was lower than on day 7 and 18. The diluted treatments showed significantly lower GGT levels on day 7 post weaning (P = 0.048) but they were not significantly lower later on in the trial. The level of GGT was within the levels reported by Rico *et al.* (1977) of 36 ± 14 U/l and the level of between 10 and 60 U/l (Tennant, 1997).

Table 3.17: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on level of ALT in newly weaned pigs (U/l).

	No added fibre			Added fibre			*Treatment x fibre*	
Day	**C**	**H**	**S**	**C**	**H**	**S**	**s.e.d.**	***P***
7	33.50	32.13	32.00	32.00	32.94	30.62	2.380	0.743
18	40.25	38.56	42.56	41.13	43.75	40.50	2.964	0.222
27	45.31	40.81	48.13	46.63	43.38	46.50	3.256	0.648

Table 3.18: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on level of ALT in newly weaned pigs.

	Treatment		*Added fibre*	
Day	**s.e.d.**	***P***	**s.e.d.**	***P***
7	1.683	0.656	1.374	0.618
18	2.083	0.921	1.701	0.435
27	2.302	0.068	1.880	0.691

Table 3.19: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on level of AST in newly weaned pigs (U/l).

	No added fibre			Added fibre			*Treatment x fibre*	
Day	**C**	**H**	**S**	**C**	**H**	**S**	**s.e.d.**	***P***
7	39.7	38.7	35.8	36.9	38.1	37.5	5.53	0.844
18	42.8	49.4	52.6	41.4	49.4	45.5	6.15	0.768
27	50.3	40.2	59.2	52.3	40.1	53.4	7.57	0.756

Table 3.20: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on level of AST in newly weaned pigs.

	Treatment		*Added fibre*	
Day	**s.e.d.**	***P***	**s.e.d.**	***P***
7	3.91	0.879	3.19	0.866
18	4.35	0.212	3.55	0.326
27	5.35	**0.011**	4.37	0.769

Table 3.21: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on level of GGT in newly weaned pigs (U/l).

	No added fibre			Added fibre			*Treatment x fibre*	
Day	C	H	S	C	H	S	**s.e.d.**	***P***
7	25.06	28.50	26.69	24.31	23.69	22.75	2.731	0.544
18	23.44	27.12	26.37	25.31	23.62	23.69	2.625	0.301
27	25.19	24.56	25.19	23.50	21.44	21.94	2.373	0.326

The direct bilirubin was at no time influenced by the herbal additives or the addition of fibre (table 3.22). The values were within the ranges reported by Rushton (1981). Similarly, the total bilirubin levels were not affected by the addition of herbal additives at any time (table 3.23), but on day 17, there was a trend ($P = 0.050$) towards the pigs fed the added fibre having lower values than the control ones. All the ranges were within reported values (Rushton, 1981).

Table 3.22: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on level of direct bilirubin in newly weaned pigs (mmol/l).

	No added fibre			Added fibre			*Treatment x fibre*	
Day	C	H	S	C	H	S	**s.e.d.**	***P***
7	2.28	2.27	2.33	2.08	2.07	1.98	0.336	0.936
18	2.19	2.26	2.56	1.97	2.33	2.08	0.309	0.466
27	2.21	2.38	2.73	2.37	2.29	2.36	0.290	0.443

Table 3.23: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on level of total bilirubin in newly weaned pigs (mmol/l).

	No added fibre			Added fibre			*Treatment x fibre*	
Day	C	H	S	C	H	S	**s.e.d.**	***P***
7	3.28	3.50	3.44	3.34	3.36	2.97	0.413	0.646
18	3.03	3.53	3.38	2.65	2.85	2.87	0.458	0.898
27	4.49	4.55	4.80	4.12	4.13	4.79	0.486	0.810

3.4.5 Haptoglobin

The level of haptoglobin was not affected by the addition of herbal additives, but the addition of fibre significantly reduced the level, particularly in the control and hops fed pigs, but not in the silymarin treatments (table 3.24 and 3.25). The values found were similar to those reported by Amory *et al.* (2001) for slaughter pigs, and only slightly higher than the ranges reported prior to challenge (between 0.19 and 0.52 mg/ml) by Heegaard *et al.* (1998).

Table 3.24: Effect of control (C), hops (H) and silymarin (S) with or without added fibre in the diets on level haptoglobin on day 27 in newly weaned pigs (mg/ml).

	No added fibre			Added fibre			*Treatment x fibre*	
Day	**C**	**H**	**S**	**C**	**H**	**S**	**s.e.d.**	***P***
27	1.43	1.43	1.15	0.69	0.65	1.13	0.260	0.075

Table 3.25: Effect of control (C), hops (H) and Silymarin (S) with or without added fibre in the diets on level haptoglobin on day 27 in newly weaned pigs.

	Treatment		*Added fibre*	
Day	**s.e.d.**	***P***	**s.e.d.**	***P***
27	0.184	0.835	0.150	<.001

3.5 Discussion

Fibrous components of feedstuffs may have negative effects on energy and protein digestibility, and sometimes growth and feed efficiency (Pluske *et al.*, 2001) (see also the literature review section 1.4.2.3). However, in this experiment the addition of fibre, resulted in an interaction between the hops and the fibre on FCR, where the combination of hops and fibre resulted in better feed utilisation. The fibre also significantly reduced the level of haptoglobin in the pigs, indicating a lower immune response and possible less infection and inflammation (Heegaard *et al.*, 2000). It is possible that the lower level of rotavirus can have contributed to this, and also the significantly higher faecal score was seen on day 28, indicating firmer faeces. A potential explanation might be that the rate of

digestion is slower when fibre is fed and it reduces the incidence of diarrhoea (Le Dividich and Sève, 2001).

Hops and silymarin did not have any significant effect on the growth rate during the first 11 days post weaning. Some experiments have shown similar effects with antibiotics i.e. Broom *et al.* (2003) found that including Avilamycin at 40 mg/kg feed and zinc oxide at 3100 mg/kg feed did not result in significantly better growth rates at day 1 to 7 after weaning. Broom *et al.* (2003) reported DLWG ranging from 236 to 307 g/day from day 0 to 20 was for pigs fed control diet and Avilamycin and zinc oxide, respectively. This is slightly lower than the DLWG in the same period for the current experiment (ranging between 291 to 337 g/day) and slightly lower than Gill *et al.* (2000) which were ranging between 329 and 363 g/day in the 4 weeks after weaning. Overall the pigs had high growth rates and good FCR, indicating they were in good health, which may explain the little response to the hops and silymarin, as little or no response is often seen under these circumstances (Pierce *et al.*, 2005a).

The FCR for the hops treatments was better than the control on the second stage diet. This is in agreement with Cornelison *et al.* (2006) and may be due to the potential antibacterial effect of hops. Post weaning diarrhoea is mainly caused by gram negative bacteria such as *E. coli* (Done, 2001). Several *in vitro* experiments, for example both Shimwell (1937a) and Langezaal *et al.* (1992) have shown that hops can inhibit some bacteria, particularly gram positive bacteria and gram negative at a pH lower than 5.18. Other studies have shown hops do not inhibit gram negative bacteria, i.e. Fukao *et al.* (2000) showed that *E. coli, Pseudomonas fluorescens and Klebsiella pneumonia* were not inhibited.

The faecal score was not affected by silymarin or hops, and no effects were seen on the level of rotavirus and the faecal number of *E. coli*. The number of *E. coli* in the faeces,

ranging between 4.68 and 5.93 log 10 cfu/g, were within ranges reported for weaner pigs 5 to 25 days post weaning fed either control diet or AGP and ZnO (Broom *et al.*, 2003). Broom *et al.* (2003) also reported the number of faecal *E. coli* to range between 4.47 and 5.93 log 10 cfu/g. However, the samples had been frozen in the current experiment due to practical reasons. The freezing had previously been carried out at Harper Adams University College by Lewis (2005) and it was initially assumed not to influence the analysis, as all the samples across the treatments had been treated the same. However, other researchers have reported that it could be expected that the freezing and thawing of samples may affect the microbial populations to some extent when compared to fresh samples (Sims *et al.*, 2004). Furthermore, freezing of ruminal fluid has been shown to result in relatively more destruction of gram negative than gram positive bacteria (Hsu and Fahey, 1990). Therefore, it was decided to avoid freezing any samples for microbiological analysis in subsequent experiments.

Cornelison *et al.* (2006) studied the effects of feeding 227 g/t, 453 g/t, 680 g/t and 907 g/t hops to broilers, they found that the inclusion of 227 g/t resulted in improved FCE, improved body weight at day 14 and 42 compared to a control diet containing no additives. Higher levels of hops resulted in some improvements as compared to the negative control (see also section 1.6.2.3). In this experiment 1 g/kg hop was used indicating a positive response in piglets, but higher amounts may result in more conclusive effects. *In vitro* studies indicate that a higher concentration of hops increases their ability to inhibit bacteria (Haas and Barsoumian, 1994; Fukao *et al.*, 2000).

A few studies have evaluated the potential effect of hops on liver function. They have shown that hops had no effect on liver, on cytochrome P-450 enzyme or on rat hepatocytes (Shipp *et al.*, 1994; Rodriguez *et al.*, 2001). However, this experiment showed hops might

influence the liver enzymes AST and ALT after three to four weeks. It would be interesting to see if the improvement in liver enzymes had contributed to the better FCR.
Silymarin did not affect the daily live weight gain at any time, but resulted in a better FCR than the control in the second half of the experiment. The maximum pigs ate was 13.84 mg/kg body weight silymarin (standard diet day 21 to 28 post weaning), and at the beginning 6.08 mg/kg. In humans mushroom poisoning with *Amanita phalloides* is treated with 5 mg/kg/day to 20 mg/kg/day (Wellington and Jarvis, 2001). The ranges used in this study are within these limits. Feeding silymarin to mice has shown to increase their weight gains when fed 50 g/kg and 100 g/kg of silymarin oil (Khan *et al.*, 1986) (see also the literature review section 1.6.1.3). Increasing levels of *Silymarin marianum* resulted in increasing levels of weight gain. This level showed no adverse effect on degenerative effects of the organs (Khan *et al.*, 1986). In contrast, Crocenzi *et al.* (2000) found in a dose dependent study that the body weight of rats decreased with increasing levels of silymarin. However, their inclusion rates were higher, as they fed 25, 50, 100 or 150 mg/kg/day, which also is higher than in the present study. Furthermore, Crocenzi *et al.* (2000) found that silymarin had an additive effect on the bile salt output and the bile flow. More than 1500 mg/day in humans has shown to produce loose stools due to the increased bile flow (Pepping, 1999). Lewis (2005) studied the inclusion rates of 9 mg/kg feed silymarin and 18 mg/kg feed silymarin in broiler chickens. The higher inclusion rate of the silymarin resulted in a higher daily live weights.

Some experiments showed that silymarin could improve liver function tests in patients with increased serum activities of AST, ALT and GGT (Salmi and Sarna, 1982; Fraschini *et al.*, 2002; Ramadan *et al.*, 2002) or prevent an increase in AST and ALT (Oliveira *et al.*, 2001). Whereas other did not find any significant evidence of decreased liver enzyme levels with silymarin (Pares *et al.*, 1998; Crocenzi *et al.*, 2000; He *et al.*, 2002). The latter is in agreement with this experiment, as no reduction in the level of liver enzymes was

seen at any time due to the inclusion of silymarin. The level of liver enzymes in the experiment did not reflect hepatotoxicity.
Soto *et al.* (1998) looked at silymarin as a potential medicine for diabetes. However, they found that on its own silymarin did not change the plasma glucose levels. This agrees with the blood glucose level for silymarin treatment not being significantly different from control treatment at any time during the experiment. On day 7 the blood glucose was significantly lower for the hops diets than the control diets, but it was not significantly lower at any other time, so it was not considered as a constant effect. Although, the blood urea was not affected by treatment, it was interesting to see how the blood urea first increased at day 18 in all treatments and then decreased at day 27. Valencia and Chavez (1997) found that the blood urea levels were lower four weeks after weaning compared to earlier.

The experiment indicated a potential for both hops and silymarin to improve FCR in the later stage of the experiment. The combination of hops and fibre seemed to result in better FCR than hops on their own. The hops also showed to improve the AST and ALT levels in the pigs, which was surprising as hops are known for their antimicrobial activity (see section 1.6.2.3.2) rather than improving liver function. In contrast, silymarin, which is a well-known medication in humans with liver problems (see section 1.6.1.3), did not show any effect on the liver function. Addition of fibre to the diets did not affect the performance, but it resulted in a better faecal score after 28 days and lower immune response. The addition of fibre also tended to lower the blood urea seven days after weaning, but not later on. Overall no significant interaction was seen between the herbal additives and the addition of fibre.

Both hops and silymarin resulted in an improved FCR in the seond part of the experiment, for the hops treatment an improvement in the level of AST and ALT may partly explain

this, but no effects on the liver or other measurements were seen for the silymarin. Finally, the addition of fibre to the diet resulted in firmer faeces at 28 days, which was accompagnied by a reduction in the level of haptoglobin in the blood at the same age.

4. *IN VITRO* EXPERIMENT: EFFECT OF HOPS AND HOP COMPOUNDS AT TWO PH LEVELS ON THE INHIBITION OF REPRESENTATIVE GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

4.1 Introduction

Results from the first experiment (chapter 3) showed that hops improved the feed conversion ratio of weaned pigs. It was hypothesised that the primary reason for this was due to the potential antimicrobial activities of some of the compounds in hops. Thus in the present experiments the antibacterial effects of hops and some of their isolated compounds were tested *in vitro* against a range of bacteria present in the pig gut. Schmalreck *et al.* (1975) studied the chemical structure of hops and isolated compounds that had potential antimicrobial properties. The compounds were investigated in an agar diffusion test against a set of representative Gram-negative and Gram-positive bacteria. They found that when compared to a group of several chemically unrelated compounds, such as carboxylic polyethers, polyene-, polypeptide-, and marcolide-antibiotics, the hop resins showed similar action against gram positive bacteria.

Many herbs and spices contain antibacterial compounds (see section 1.5.7), which may be important for the preservation of food and for the control of human, animal and plant diseases of microbial origin, as they can inhibit bacterial growth (Baratta *et al.*, 1998). For example, tea tree oil has shown to have inhibitory effects against *E. coli* and *Staphylococcus aureus* due to its ability to disrupt the permeability of microbial membrane structures and it also inhibits the respiration of the bacteria (Cox *et al.*, 2000). These compounds could play a significant clinical role in inhibiting antibiotic resistant microbes, such as *E. coli, Salmonella typhi, Staphylococcus aureus* and *Streptococcus faecilis* (Barbour *et al.*, 2004).

The α-acids and β-acids have demonstrated antimicrobial activities, see section 1.6.2.2 in the literature review, and they are available as isolated compounds. Separation and purification of α-acids and β-acids is performed with CO_2 extraction (Lu-Ping Ting and Goldstein, 1996). The α-acids can be selectively extracted into an aqueous solution, leaving behind the insoluble material (Lu-Ping Ting and Goldstein, 1996). A subsequent caustic extraction of the remaining material gives a pure β-acid aqueous solution (Lu-Ping Ting and Goldstein, 1996).

Haas and Barsoumian (1994) studied the antimicrobial effects of iso-α-acid and β-acid from hops by a turbidity method against *Staphylococcus aureus, Streptococcus salivarius, Bacillus megaterium, E. coli* and *Bacillus subtilis.* They found that *E. coli* and *Bacillus subtilis* were resistant to iso-α-acid and β-acid, but the growth of the other tested bacteria was inhibited after 24 hours. Similarly, Fukao *et al.* (2000) found that hop resins inhibited the growth of *Bacillus cereus, B. subtilis, Staphylococcus aureus, Lactobacillus brevis* and *Leuconostoc mesenteroides* but not the tested gram negative bacteria (five different *E. coli* strains, *Pseudomonads fluorescens* and *Klebsiella pneumoniae*) (see literature review section 1.6.2.3.2 for more details).

Shimwell (1937b) showed that the pH of hops or hop compounds is an important factor in the inhibition of gram negative bacteria such as *E. coli*, which showed to be unaffected at pH 5.18, but was inhibited at pH 4.40. Similarly, Simpson and Smith (1992) found that the antibacterial activity of the hop compounds humulone, colupulone, *trans*-isohumulone and *trans*-humulinic acid towards *Lactobacillus brevis* was increased at a lower pH, when tested at a pH range of from 4 to 7. In contrast, Fernandez and Simpson (1993) found no difference in their pH range for growth of hop-resistant bacteria and hop-sensitive bacteria with different *Lactobacillus* and *Pedicoccus* strains, most strains grew well at pH 4-7. In both the latter experiments they used the minimum inhibitory concentration method. In

which the test organism in broths containing a range of hop/hop compounds are inoculated, and then the growth of the organism determined spectophotometrically after incubation.

4.2 Objectives

Section 4.1 suggests that hops have potential inhibitory effects against, particularly, gram positive bacteria, but also gram negative when the pH is lowered. Since the results are variable and dependent on different factors (e.g. hop sompounds, pH, media), it was decided to test the antimicrobial activity with two different methods (sensitivity method and total variable count method). The objectives of these experiments were to:

1) investigate the potential antibacterial effects of hops and the isolated hop compounds (iso-α-acid extract and β-acid) against a range of gram positive and gram negative bacteria some which can be found in the microflora of the pig
2) test the effect of different concentrations of the hops compounds on the growth of selected bacteria
3) study the effect of adding different types of acid and the effect of pH on the growth of selected bacteria

4.3 General material and methods for sensitivity tests and the different concentration experiments

The preparation of the cultures was the same for both the *in vitro* experiments and is described in this section. Not all the bacteria were tested in each experiment, but the materials and methods for each experiment describe the organisms tested.

4.3.1 Selected bacteria for the tests

Bacteria selected for the tests were:

Gram negative:

- *Escherichia coli* K88 (strain 2496)
- *Escherichia coli* K88 (strain 2551)
- *Salmonella typhimurium*

Gram positive:

- *Streptococcus agalas*
- *Streptococcus faecilis*
- *Staphylococcus albus*

The bacteria were stock cultures from the laboratory at Harper Adams University College, except the *E. coli* K88 strains, which were provided by Don Whitley Scientific (Shipley, West Yorkshire).

4.3.2 Preparation of media

Sheep blood agar (CM0854, Oxoid, Hampshire) was mixed with deionised water and sterilised in the autoclave for 15 minutes at 120°C. After cooling down to approximately 40°C, 5% sterile sheep blood (SR00511, Oxoid, Hampshire) was aseptically added, and the plates were poured aseptically.

Antibiotic sensitive agar plates (LAB 12, LAB M, Lancashire) was mixed with deionised water and sterilised in the autoclave for 15 minutes at 120°C. After cooling to around 50°C, the plates were poured aseptically.

Nutrient broth (No 2, Oxoid, Hampshire) was mixed with deionised water and sterilised in tubes (either with 2, 4 or 10 ml in each) for 15 minutes at 120°C.

The pH of the broths was adjusted to 5 and 7 in some of the tests prior to sterilisation. In the initial sensitivity test buffer tablets (pH 4 phtalate and pH 7 phosphate) (Fisons, Loughborough) were used for this, and in the sensitivity test (*in vitro* experiment 1) an organic acid mixture Ultracid ® (INVE, Belgium) was used, the composition is shown in table 4.1. The Ultracid mixture was chosen to lower the pH, as it is an organic acid mixture which is used in animal production, and the aim was to test it *in vitro* first with the hops/hop compounds and if an effects was found test it *in vivo* later. In the initial test as well as the different concentration experiment (*in vitro* experiment 2) hydrochloric acid (HCl) or an organic acid mixture Ultracid ® (INVE, Belgium) were used. The pH was measured with a pH meter (Jenway 3510).

Table 4.1: The composition of Ultracid ®.

Ingredients	Inclusion (g/kg)
Phosphoric acid (85%)	625.0
Citric acid	20.0
Fumaric acid	2.5
Malic acid	2.5
Carrier	350.0

4.3.3 Inoculation of purity plates

One colony of each bacterial strain was inoculated onto the blood agar plates (CM0854, Oxoid, Hampshire). These were incubated overnight at 37°C. The following day the plates were visually assessed for contamination and only pure plates were used for further analysis.

4.3.4 Preparation of bacteria

The *Salmonella typhimurium, Staphylococcus Sp., Escherichia coli Sp.* and *Streptococcus Sp.* were prepared by picking one colony off the purity plate and inoculating it in sterile nutrient broth (No 2, Oxoid, Hampshire) overnight at 37°C.

4.3.5 Analysis of hops for quantification of α-acid and β-acids

To determine the quantity of α-acid and β-acids in the hops variety Phoenix (Hopsteiner, Epping, England) used in the experiments, the hops were analysed by high performance liquid chromatography (HPLC) by Hopsteiner (Epping, England). The procedure was the European Brewing Convention (EBC) method 7.7 using International Calibration extract.

The hops contained 105.3 g/kg α-acid and 43.9 g/kg β-acid, which was within the expected ranges between 85 and 120 g/kg w/w for α-acids and between 42 and 55 g/kg w/w for β-acids ((Roberts, 2004) Steiner Hops Ltd, Epping, Essex, personal communication).

4.3.6 The composition of the iso-α-acid and β-acid solutions

The isolated hop compounds, iso-α-acid (Hopsteiner, Epping) contained 30% iso-α-acid in an aqueous solution. The β-acid solution (Hopsteiner, Epping) contained 20% β-acid in propylene glycol.

4.4 Experiment 1: Sensitivity test with hops, iso-α-acid and β-acid extracts at pH 5 and pH 7

The sensitivity of pure hops and the hops compounds iso-α-acid extract and β-acid extract were tested for their antimicrobial properties at pH 7 and pH 5 (lowered Ultracid ®) against a range of bacteria.

4.4.1 Material and methods

4.4.1.1 The selected bacteria

The bacteria tested were *E. coli* K88 (strains 2496 and 2551), *Salmonella typhimurium*, *Streptococcus agalas* (pH 7), *Streptococcus faecalis* (pH 5) as the *Streptococcus agalactiae* did not grow well at pH 5 and *Staphylococcus albus* at pH 7 and pH 5.

4.4.1.2 Inoculation of bacteria

The sensitivity plates were prepared by pipetting 0.1 ml of the prepared bacteria in the nutrient broth (No 2, Oxoid, Hampshire) onto sterile antibiotic sensitive agar plates (LAB 12, LAB M, Lancashire) and spread with a sterile swab.

4.4.1.3 Total bacterial count of initial bacteria

The number of bacteria in the nutrient broth (No 2, Oxoid, Hampshire) was prior to inoculation estimated by total bacteria counts. 1 ml of the broth with the bacteria was serial diluted in ringer solution 10^{-1} to 10^{-9}. The solutions were plated out on nutrient agar containing 0.01g 2,3,5-triphenyltetrazolium chloride/100ml for identification of colonies and incubated for 24 hours before counting. The results are shown in table 4.2.

Table 4.2: The mean number of bacteria grown in the nutrient broth.

	pH 5	pH 7
Salmonella typhimurium	5.05E+08	1.13E+09
Escherichia coli **K88 (2551)**	2.50E+08	3.00E+08
Escherichia coli **K88 (2496)**	4.35E+08	4.35E+08
Staphylococcus albus	6.00E+07	1.05E+07
Streptococcus agalactiae	-	2.30E+08
Streptococcus faecilis	2.15E+08	-

4.4.1.4 Preparation of hops solution

Grinded hops variety Phoenix (Hopsteiner, Epping, England) were dissolved at concentrations of 1 mg/ml and 10 mg/ml into a sterile water with the pH adjusted to pH 5 or pH 7 with buffer tablets (Fisons, Loughborough). This was done by mixing the hops in and leave to dissolve for 30 to 50 minutes in a waterbath at 55°C.

These concentrations were chosen because 1 mg/ml was equal to the amount used in the pig feed in experiment 1 and Haas and Barsoumian (1994) showed that hops did not dissolve at concentrations higher than 10 mg/ml in aqueous solutions.

3.5 mg/ml of iso-α-acid extract was included and mixed with 2.2 mg/ml β-acid extract in sterile water. These concentrations were used because that was the equivalent to the amounts in the 10 mg/ml hops treatment (see section 4.3.6 for the analysis of hops). Furthermore, 10 mg/ml of iso-α-acid extract in aqueous solution and 10 mg/ml of β-acid in aqueous solution were tested for antimicrobial activity.

4.4.1.5 Preparation of discs

The sensitivity of the isolated bacteria was tested by adding 20 µl of the hops/hop compounds (pH 7) onto 6 mm paper discs (Whatman) and let them dry for 30 to 60 minutes at ambient temperature. Although hops contain antioxidant compounds, which potentially could be affected by the drying in air, antibacterial effects were seen, indicating leaving the hops/hop products drying did not destry this effect. These were placed onto the sensitivity plates with the tested bacteria spread onto it using aseptic methods. The plates were incubated for 24 hours. The method was adapted from Tepe *et al.* (2005b). Antibiotic discs were used as control. Different antibiotic discs were used for different bacteria, as some bacteria are resistant to certain antibiotics. 5 µg vancomycin (Oxoid, Basingstoke) for the *Streptococci* and *Staphylococcus* and 10 µg colistin (Oxoid, Basingstoke) for *E. coli* and *Salmonella.*

4.4.1.6 Preparation of sensitivity test pH 5

The procedure was repeated at pH 5 with the pH of all the media i.e. the nutrient broth, sensitivity agar and the diluted hop products lowered with the acid mixture Ultracid ® (see table 4.1 for composition). The inhibitory zones were measured accurately with a ruler. Each treatment was repeated ten times.

4.4.2 Results

4.4.2.1 Effects of hops and hop compounds

The gram negative bacteria *Salmonella typhimurium* and *Escherichia coli* K88 were not inhibited by the hops or the hop compounds, but the gram positive were, particularly *Staphylococcus albus* (table 4.3 and see also figure 4.1). The iso-α-acid extract and the hops at 10 mg/ml had antimicrobial activities at pH 5. An inclusion level of 1 mg/ml of hops did not seem to inhibit any of the tested organisms.

Table 4.3: Diameter of inhibition for hops and hop compounds iso-α-acid extract (α) and β-acid (β) at pH 7 and pH 5 on different bacteria (mm including 6mm disc).

Concentration	10 mg/ml hops	1 mg/ml hops	Mix α & β	10 µl/ml α	10 µl/ml β	AB
pH 5						
Salmonella typhimurium	-	-	-	-	-	16
Escherichia coli K88 (2551)	-	-	-	-	-	16
Escherichia coli K88 (2496)	-	-	-	-	-	16
Staphylococcus albus	10	-	20	25	15	15
Streptococcus faecalis	14	-	19	24	13	17
pH 7						
Salmonella typhimurium	-	-	-	-	-	14
Escherichia coli K88 (2551)	-	-	-	-	-	14
Escherichia coli K88 (2496)	-	-	-	-	-	14
Staphylococcus albus	-	-	12	-	17	15
Streptococcus agalactiae	-	-	-	-	-	-

AB = antibiotic control

4.4.2.2 The effects of pH

The mean inhibition zones are shown in table 4.3. The hops and hop compounds had greater inhibitory effects at pH 5 than at pH 7. The strongest activity was exhibited by the iso-α-acid extract at pH 5 and the combination of iso-α-acid extract and β-acid extract at pH 5.

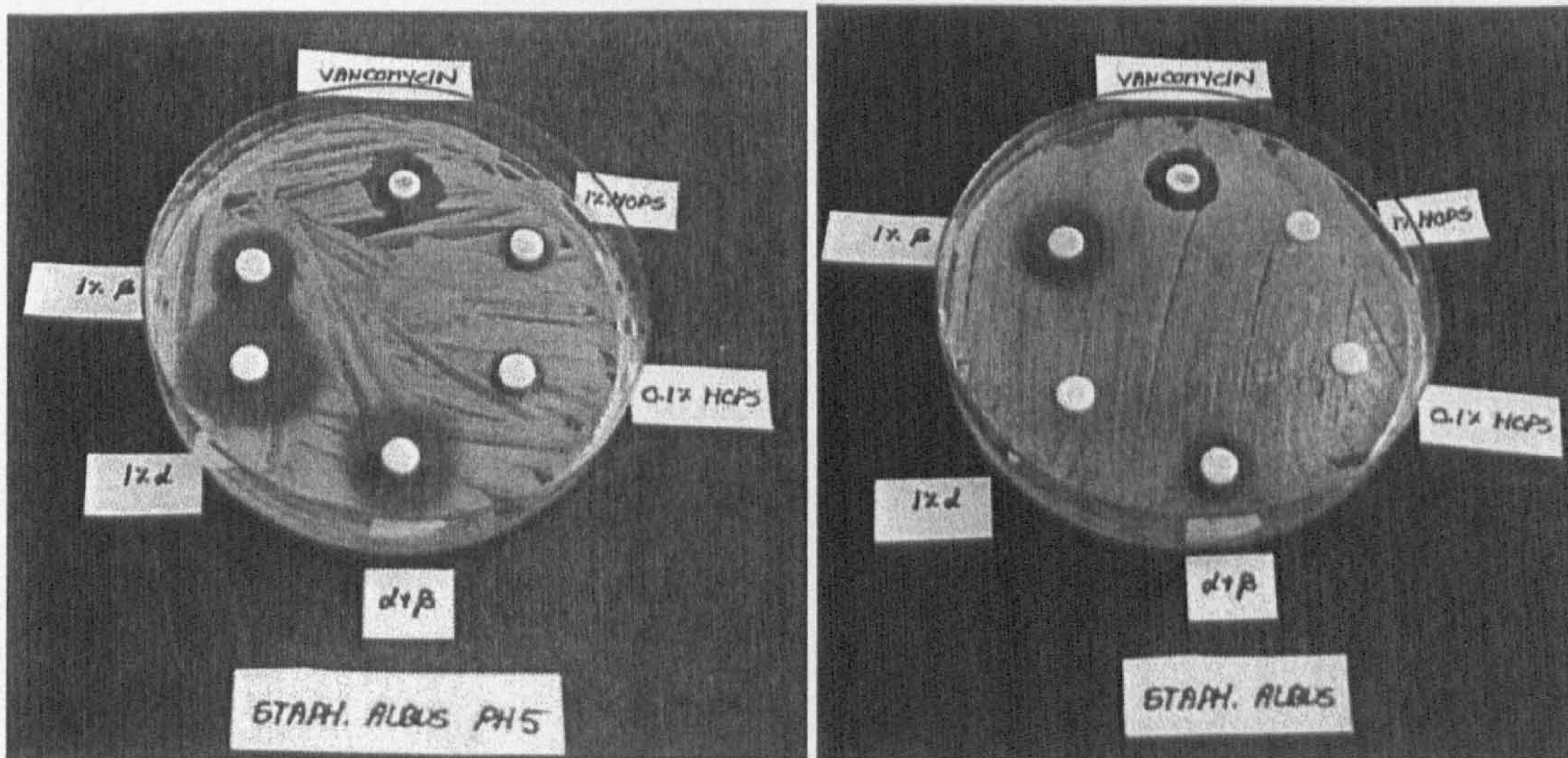


Figure 4.1: The inhibition of *Staphylococcus albus* at pH 5 (on the left) and pH 7 (on the right) by 10 mg/ml iso-α-acid, 10 mg/ml β-acid, 1 and 10 mg/ml hops, a combination of 3.5 mg/ml iso-α-acid and 2.2 mg/ml β-acid and vancomycin.

4.5 Experiment 2: The effect of different concentrations of hops and hop compounds at pH 5 and pH 7 on various bacteria

It was decided to test K88, the 2496, which is one of the main bacteria causing diarrhoea in piglets and showed a small response to the hop compounds in the sensitivity test, in sufficient numbers to carry out statistical analysis.

4.5.1 Materials and methods

4.5.1.1 Selection of bacteria

The inhibitory concentration of pure hops (Hopsteiner, Epping) and the hops compounds iso-α-acid extract (Hopsteiner, Epping) and β-acid (Hopsteiner, Epping) were tested against *E. coli* K88 (2496).

4.5.1.2 Concentrations of hops and hop compounds tested

The range of different concentrations of hops tested at pH 5 and pH 7 (using either Ultracid ® or HCl, as desribed in section 4.3.2) were 10 mg/ml, which was ten times what was used

in experiment 1 in the piglets, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.6 mg/ml, 0.3 mg/ml and 0 (control). The different concentrations of iso-α-acid extract and β-acid tested at pH 7 and pH 5 (using either Ultracid ® or HCl) were 10 μg/ml, 5 μg/ml, 2.5 μg/ml, 1.25 μg/ml, 0.6 μg/ml, 0.3 μg/ml and 0 (control). These were made by transferring 40 μl of the iso-α-acid extract or β-acid (or 40 mg hops) (see also section 1.6.2.2 for description of the hop components) into 4 ml nutrient broth (No 2, Oxoid, Hampshire). These were serial diluted until a concentration of 0.3 was reached. This gave tubes containing 2 ml of nutrient broth (No 2, Oxoid, Hampshire) with the tested hop product.

4.5.1.3 Inoculation of bacteria and bacterial counts

Each of the hops/hop compounds dilutions in the test tubes were inoculated with 40 μl of the broth containing the *E. coli* K88, which was incubated for 24 hours at 37°C. Each of the test tubes was inoculated with 40 μl of the prepared broths containing the bacteria. The pH was adjusted to 5 with either Ultracid ® or HCl and measured (as described in section 4.3.2). The tubes containing the bacteria were incubated for 24 hours at 37°C.

Afterwards, the mixture containing the bacteria was serial diluted in ringer solution from 10^{-1} to 10^{-7} dilutions (Oxoid BR0052G, Oxoid, Hampshire). The dilutions were plated onto nutrient agar (LAB 8, LAB M, Bury, England) containing 0.01g 2,3,5-triphenyl-tetrazolium chloride (BHD, Poole)/100ml agar for identification of colonies. These were plated out in duplicate. The plates containing test bacteria between 20 and 250 colonies were counted after 24 hours in the incubator at 37°C.

All the different concentrations of each hop compound and acid were repeated 8 to 10 times for statistical analysis.

4.5.1.4 Statistical analysis

The results were analysed with analysis of variance (ANOVA) in Genstat 5th edition as factorial designs. Firstly, to test if there was a significant difference between the treatments and the different acid types (not taking the concentration into account). Secondly, to test the difference of the compounds with the concentration of hops or hop compounds as a contrast. Significant differences are based on probability of less than 0.05.

4.5.2 Results

4.5.2.1 The effects of hops and hop compounds and pH on the inhibition of E. coli *K88*

The log (10) cfu of K88 (2496) was analysed first for the four different treatments (control, hops, iso-α-acid extract and β-acid extract) and the effects of the different pH and acids used (pH 7, pH 5 with either HCl or the Ultracid ® mixture). The results showed that the treatments with hops or hop compounds significantly affected the number of *E. coli* K88 (see table 4.4 and 4.5). The control had statistically significantly higher number of *E. coli* K88 bacteria than the other treatments, and the β-acid extract resulted in a lower number of bacteria followed by the iso-α-acid extract. Neither the HCl nor the Ultracid ® affected the number of *E. coli* K88. For hops and the β-acid extract the lowest bacterial count was found at pH 7, whereas a statistical interaction effect was seen between the iso-α-acid extract and the organic acid mixture, reducing the log number of *E. coli* by 0.1 log number.

Table 4.4: The effects of hops, iso-α-acid extract and β-acid extract and acids on the log (10) cfu of *E. coli* K88 (2496) bacteria.

Treatment	Control	Hops	Iso-α-acid	β-acid
Acid				
pH 7	8.95	8.76	8.43	7.70
Ultracid pH 5	8.97	8.82	8.34	8.26
HCl pH 5	8.97	8.87	8.46	8.46
Mean	8.97	8.81	8.41	8.12

Table 4.5: The statistical analysis for the effects of different hops products and acids on the log (10) number of *E. coli* K88 (2496) bacteria.

Treatment s.e.d.	*Treatment P-value*	*Acid s.e.d.*	*Acid P-value*	*Treat x acid s.e.d.*	*Treat x acid interaction*
0.086	<.001	0.057	<.001	0.154	<.001

4.5.2.2 The effects of concentration of hops and hop compounds on the inhibition of E. coli K88

The log (10) number of K88 (2496) was analysed for the effect of the different treatments (hops, iso-α-acid and β-acid) the control was excluded from this analysis and the effect of concentration of the products (0.3 mg/ml, 0.6 mg/ml, 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml) was analysed. The data was analysed with concentration as polynomial contrast (see table 4.6 and 4.7).

Table 4.6: The effects of different concentrations of hops (mg/ml), iso-α-acid extract (μl/ml) and β-acid extract (μl/ml) and their concentrations on the log (10) no of K88 (2496).

Concentration	Hops	Iso-α-acid	β-acid
0.3	9.05	8.65	8.49
0.6	8.90	8.54	8.47
1.25	8.90	8.59	8.20
2.5	8.68	8.57	8.20
5	8.68	8.48	7.89
10	8.66	7.54	7.60
Means	8.81	8.40	8.14

Table 4.7: Statistical analysis of the effects of hops, iso-α-acid extract and β-acid extract and their concentrations on the log (10) cfu of K88 (2496).

Treatment s.e.d.	*Treatment P-value*	*Conc. s.e.d.*	*Conc. P-value*	*Treat x conc. s.e.d.*	*Treat x conc interaction*
0.058	<.001	0.082	<.001	0.140	<.001

The increasing concentration of hops and the hop compounds reduced the number of *E. coli* K88 for the β-acid extract and iso-α-acid extract. For hops a reduction from log number 9.05 *E. coli* K88 to 8.66 was seen and stronger inhibition with higher concentration was seen with the iso-α-acid extract (from log number 8.65 to 7.54) and the β-acid extract (from log number 8.49 at 0.3 mg/ml to 7.60 at 10 mg/ml).

4.6 Discussion

Both the sensitivity and the different concentrations experiment showed that hops and their isolated compounds have inhibitory effects against some bacteria, in agreement with Haas and Barsoumian (1994) who found isolated hop compounds inhibited the growth of *Streptococcus* and *Staphylococcus*, but that *E. coli* and *Bacillus subtilis* were resistant. In agreement with the results from the effect of different concentration experiments 4.5), they found that β-resins exerted greater inhibition of bacteria than iso-α-extract.

In contrast, the sensitivity tests (experiment 4.4) showed that iso-α-acid produced greater inhibitory zones than the other compounds. They also showed a greater inhibition of gram positive (*Staphylococcus* and *Streptococcus*) bacteria than gram negative. No inhibition zones were seen for the *E. coli* and *Salmonella.* Furthermore, the pH influenced the degree of inhibition. Higher inhibition zones were found at lower pH as in agreement with Shimwell (1937b) and Simpson and Smith (1992). The different concentrations experiment (section 4.5) showed a reduction in the number of *E. coli* K88 by the hops and the hop compounds although they did not completely inhibit the growth of *E. coli* K88.

A reduction in the number of *E. coli* K88 was found by the β-acids in the different concentration experiment (section 4.5), whereas in the sensitivity experiment (section 4.4), the iso-α-acid extract showed greater inhibition. However, this was towards gram positive

bacteria. The fact that hops/ hop compounds have greater effects towards gram positive bacteria might be explained by:

1) the mechanisms of the antibacterial compounds in the hops
2) the difference in the cell structure of gram positive and gram negative bacteria

Some antibiotic growth promoters only work against gram positive or gram negative bacteria and some work against both (Gaskins *et al.*, 2002). The mechanism of action of antibiotics varies, for example the oligosaccharide such as avilamycin and macrolide such as tylosin inhibit protein synthesis of gram positive bacteria. The polyether such as salinomycin alters the membrane of gram positive bacteria. Penicillin inhibits cell wall synthesis of gram positive bacteria, whereas the broad spectrum products i.e. quinoxalines such as carbadox inhibit DNA synthesis. Sulfonamides such as sulfamethazine inhibit metabolism and tetracylines such as terramycin inhibit protein metabolism (Gaskins *et al.*, 2002). Hops contain a group of several unrelated antimicrobial compounds, similar to the ones found in the antibiotics, such as carboxylic polyethers, polyene-, polypeptide- and macrolide-antibiotics (Schmalreck *et al.*, 1975). These are mainly active against gram positive bacteria.

Gram positive bacteria have a relatively simple cell envelope consisting of the cytoplasmic membrane, the peptidoglycan or murein layer and various other structures may be present around the cell such as protein capsules (Walton, 1985). Gram negative bacteria cell have a very complex structure consisting of the innermost layer, the cytoplasmic membrane, which is under a layer of peptidoglycan with a combination of peptidoglycan with lipoprotein molecules and the outer membrane which provides a permeability barrier limiting the outward and inward movement of various molecules, particularly the penetration of various antibiotics (Walton, 1985). The hop resins cause leakage of the cytoplasmic membrane of the susceptible bacteria resulting in inhibition of active transport

of sugar and amino acids leading to inhibition of respiration and synthesis of protein, RNA and DNA which is mainly due to a hydrophobic part (Schmalreck *et al.*, 1975; Langezaal *et al.*, 1992; Sakamoto and Konings, 2003).

These experiments showed that hops contain some antimicrobial compounds which can potentially inhibit or reduce the number of certain potential pathogenic as well as non-pathogenic bacteria isolated from pigs, either on their own or in combination with acids. Like the AGP, the antimicrobial compounds of hops work mainly against gram positive bacteria. It was therefore hypothesised that similar effects to the AGP could be expected with the hops fed to weaner pigs, and that further research was required to determine the optimal concentration and the active compounds in the hops. The first test, the sensitivity test showed that the number of gram positive bacteria was reduced by the hops/hop compounds, and that lowering the pH enhanced this antibacterial effect. However, no effect was seen on gram negative bacteria with this test. The second test, the inhibitory concentration did show that the number of *E. coli* K88 were reduced by the addition of hops and different hop compounds, it also identified a stronger effect at higher concentrations.

5. EXPERIMENT 2: THE EFFECTS OF DIFFERENT CONCENTRATIONS OF HOPS ON THE PERFORMANCE, GUT MORPHOLOGY, MICROFLORA AND LIVER ENZYME ACTIVITY OF NEWLY WEANED PIGLETS

5.1 Introduction

Experiment 1 (chapter 3) showed that hops included in the diet at 1 kg/t improved the FCR of newly weaned pigs two weeks post weaning. This was thought to be due to the antimicrobial activity of hops, which has been demonstrated in several *in vitro* studies. For example, the *in vitro* experiments (see chapter 4) indicated that higher inclusion levels of hops (or isolated hop compounds) resulted in a reduction or complete inhibition of several bacteria, in agreement with other experiments, such as Haas and Barsoumian (1994) and Teuber (1970). It was therefore hypothesised that hops may act on a range of bacteria within the intestine of the pig. This may include gram negative bacteria such as pathogenic strains of *E. coli,* which are often associated with diarrhoea after weaning, and thereby improve the performance and overall health status (Bywater, 1998; Hopwood and Hampson, 2003).

Hops have a bitter taste, and pigs have more than 20,000 taste buds, and high inclusion levels of hops could result in a lower feed intake (del Castillo *et al.*, 2002). Prior to this experiment, an investigation (see appendix I) was carried out to see if concentration of hops caused palatability problems in piglets. An inclusion level of 10 kg/t of hops were found not to affect the intake, so it was decided to investigate the effects of an inclusion rate of 1 kg/t and 10 kg/t of hops and restrict feed the pigs to ensure even intakes.

After weaning changes in gut morphology and function are seen as villous atrophy and crypt hyperplasia resulting in poorer absorption and growth (Hampson, 1986b; Pluske, 2001; Vente-Spreeuwenberg *et al.*, 2004). It has been suggested that villous shortening

happens due to the low (or no) feed intake just after weaning and/or due to disease/diarrhoea (Makkink *et al.*, 1994b; Zijlstra *et al.*, 1996; Vente-Spreeuwenberg *et al.*, 2004). The shortening and fusion of the villi results in loss of surface area for digestion and absorption of food and causes undigested nutrients to enter the hind gut, where they act as substrates for microbial fermentation (Van Dijk *et al.*, 2002; Bruininx *et al.*, 2004).

VFA are the major anionic products of bacterial intermediary metabolites and are largely the end products of carbohydrate fermentation (Rowland, 1992; Conway, 1994). They contribute to the maintenance energy of the pig and the VFA concentration is an important factor determining the pH of the colonic lumen and reducing opportunistic bacteria, such as *E. coli* and thereby preventing diarrhoea (Bach Knudsen *et al.*, 1991; Mathew *et al.*, 1996; Franklin *et al.*, 2002). It was hypothesised that hops may affect the digestion and absorption of nutrients in piglets, so it was decided to test the level of VFA in the ceacum and colon, as well as the gut morphology.

Experiment 1 (chapter 3) also showed that hops had a positive influence on some of the liver enzymes (AST and ALT). It is possible that this may have contributed to the improved FCR. Therefore, it was decided to measure the liver enzymes in this experiment, to see if a higher concentration of hops had an effect.

5.2 Objectives

The objectives were to identify the mechanisms by which hops may have resulted in the improved FCR experienced in chapter 3 and to test if a higher concentration of hops influences:

1) growth performance
2) gut health

3) hindgut fermentation

4) liver function

5.3 Material and methods

5.3.1 Animals

The experiment was carried out at Harper Adams University College's pig unit. A total of 30 piglets were used, 15 males and 15 females, see section 2.1 for breed and husbandry.

5.3.2 Housing and feeding

The experiment was carried out in four identical weaner rooms with eight pens in each. At weaning the piglets were randomly allocated to one of three treatments in individual pens. This gave a replication of ten pigs per treatment. The selection was balanced for weight, sex and dam.

The pigs were restrict fed with pellets for the whole experiment. The diets were the standard diets described in section 2.2 and the treatments shown in section 5.3.3. They were fed twice a day (morning and afternoon) according to the scale shown in table 5.1. The feed scale was based on the recommendation for guidelines in Nutrients Requirements (Whittemore *et al.*, 2003) standards for pigs. Any feed left was weighed back and the feed intake was calculated on a daily basis. Water was available *ad libitum*.

Table 5.1: Feed scale for the pigs during the trial day 0 to 28 post weaning.

Days after weaning	Morning (g)	Afternoon (g)
0	Weaning	100
1-2	100	100
3-5	150	100
6-7	150	150
8	200	150
9-14	200	200
15	200	250
16-17	250	250
18	300	300
19	350	300
20	350	350
21	350	400
22-28	400	400

5.3.3 Treatments

There were three treatments in the trial:

1) Control: basal diet with no additives
2) 1 kg/t: basal diet containing 1 kg/t of hops
3) 10 kg/t: basal diet containing 10 kg/t of hops

There were two diets. A first stage diet, which was fed from weaning to day 12 after weaning, and a second stage diet, which was fed from day 12 to day 28. The hops were the same variety (Phoenix) as used in the first experiment (chapter 3).

5.3.4 Sampling

The liveweight of the pigs was measured at weaning, five days after weaning, day 12, 15, 22 and 26 post weaning on a balance ranging from 0 to 30 kg (Pharweight, Bury, St Edmunds). Mortality and morbidity were recorded. Faecal scores were measured at weaning, day 11 and day 25 according to the scale in section 2.8.

5.3.5 Blood samples

An intracardial blood sample was taken from the animal immediately after euthanization at day 26 to 28 after weaning. The samples were analysed for ALT, AST, GGT and total and direct bilirubin as described in section 2.4.

5.3.6 Slaughtering

Pigs were slaughtered over a period of three days on day 26 to 28 of the experiment. The pigs were injected with an intracardial injection of 0.5 ml Pentobarbatione sodium (Dodge Animal Health, Southampton) for dissection and sampling of gut and liver samples. No home office licence was required for this procedure. After killing the whole intestine was removed from the animal and the different samples different samples could be taken for microbacterial analysis (see section 2.7), VFA analysis (see section 2.5) and gut histology (see section 2.6).

5.3.7 Gut morphology and histology

The villous height and crypt depth were measured as described in section 2.6. The number of eosinophils and lymphocytes were counted on each measured villous and associated crypt, as described in section 2.6.

5.3.8 Microbiology

5.3.8.1 Bacterial counts

Faecal samples were collected on days 11 and 26 and samples from the contents of the small intestine and the colon were taken at slaughter. The sample analysis consisted of: *E. coli,* lactic acid bacteria, *Campylobacter spp., Streptococcus spp., Clostridium spp., Salmonella spp.* and *Bacteriodes.* The sampling and bacterial analysis is described in section 2.7.

5.3.9 Volatile fatty acids

Digesta samples were collected from the caecum and the colon. The pH was lowered for VFA analysis and the samples were stored at -20°C, as described in section 2.5. The samples were analysed as described in section 2.5.

5.3.10 Statistical analysis

The results were analysed with one-way ANOVA (Genstat, 5th edition) using the hop concentration as polynomial contrast for the performance, bacteria and VFA data. The presence/ absence of *Salmonella* and *Campylobacter* were analysed using chi-square test. Significant differences are based on probability of less than 0.05.

5.4 Results

5.4.1 Performance

The feed intake was not significantly different between the treatments at any time (see table 5.2). There was no significant difference between treatments in daily live weight gain, liveweight and FCR for any time period throughout the experiment (table 5.2). However, in the last period (from 22 to 26 days) there was a trend for the FCR to improve ($P = 0.094$) with increasing levels of hops (1.17 for inclusion level of 10 kg/t *vs.* 1.41 for control group).

Table 5.2: Effect of different concentrations of hops in the diets on feed intake, DLWG, liveweight and FCR in newly weaned pigs.

Level of hops (kg/t)	0	1	10	s.e.d	*P*-value
Feed intake (g/pig/day)					
Day 0-5	190	169	176	21.7	0.777
Day 5-12	355	354	355	3.1	0.812
Day 12-15	397	393	400	6.1	0.379
Day 15-22	564	564	564	0.0	1.000
Day 22-26	800	780	800	10.9	0.382
FCR					
Day 5-12	1.40	1.33	1.33	0.140	0.601
Day 12-26	1.16	1.15	1.12	0.055	0.670
Day 0-22	1.16	1.17	1.19	0.053	0.523
Day 22-26	1.41	1.32	1.17	0.139	0.094
Day 5-26	1.19	1.18	1.15	0.049	0.381
DLWG (g/day)					
Day 5-12	269	274	283	27.4	0.621
Day 12-26	521	519	535	24.9	0.586
Day 5-26	437	437	451	18.3	0.393
Day 0-22	340	332	329	16.8	0.542
Day 5-26	437	437	451	18.3	0.393
Live weight (kg)					
Day 0 (weaning)	8.78	8.67	8.77	0.272	0.903
Day 5	9.48	9.30	9.34	0.286	0.797
Day 12	11.36	11.21	11.32	0.388	0.960
Day 15	12.35	12.17	12.17	0.407	0.765
Day 22	16.25	15.97	16.01	0.437	0.743
Day 26	18.66	18.48	18.81	0.568	0.652

5.4.2 Liver function tests

The liver enzymes and bilirubin results for day 26 to 28 were not significantly affected by the different concentrations of hops (table 5.3). However, there was a trend for the liver weight to be higher for the 10 kg/t hops treatment and for the total bilirubin to be lower for the 10 kg/t hops treatment.

The level of AST was higher than the normal ranges of 10 to 22 U/l reported by Rushton (1981), and only the control treatment was slightly above the average of 18 to 65 U/l found in pigs in other experiments (Tennant, 1997; He *et al.*, 2001). The level of ALT was slightly higher than the normal values by Rushton (1981) of ranging between 10 and 18 U/l. However, they were within the range reported by Tennant (1997) of 32 to 84 U/l. The

level of GGT was within the normal levels for pigs reported by Rico *et al.* (1977) of 36 ± 14 U/l and the level of 10 to 60 U/l (Tennant, 1997). The total and the direct bilirubin levels found were within the reported normal ranges of 0 to 8.55μmol/l for pigs (Rushton, 1981).

Table 5.3: Effect of different concentration of hops in the diets on liver weight and liver enzymes in newly weaned pigs.

Level of hops (kg/t)	0	1	10	s.e.d	*P*-value
Liver weight (g)*	589	599	650	33.2	0.061
AST (u/l)	69.2	45.9	58.1	21.19	0.945
ALT (u/l)	46.5	41.6	44.3	5.80	0.983
GGT (u/l)	30.8	32.7	34.0	6.76	0.683
T. Bilirubin (mmol/l)	7.87	7.40	6.00	1.105	0.093
D. bilirubin (mmol/l)	2.79	2.64	2.68	0.375	0.883

* analysed with body weight as covariate

5.4.3 Faecal score

The faecal score was not affected by the level of hops included in the diet at day 12 or day 26, see table 5.4.

Table 5.4: Effect of different concentration of hops in the diet on faecal score in newly weaned pigs.

Level of hops (kg/t)	0	1	10	s.e.d	*P*-value
Day 12	2.80	2.90	2.70	0.444	0.716
Day 26	2.90	2.90	3.10	0.141	0.116

5.4.4 Microbiology

The number of *E. coli* was not affected by the concentration of hops at any day or position in the gut (see table 5.5).

Table 5.5: Effect of different concentration of hops in the diets on *E. coli* counts (log 10 cfu per gram faeces/ digesta in faeces (day 11 and 26), small intestine and colon in newly weaned pigs.

Level of hops (kg/t)	0	1	10	s.e.d	*P*-value
Faeces day 11	7.90	8.16	8.38	0.386	0.277
Faeces day 26	8.13	7.89	8.49	0.386	0.189
Small intestine	3.69	4.27	3.72	0.495	0.622
Colon	9.06	8.70	9.31	0.795	0.569

On day 11 ($P = 0.006$) and day 26 ($P = 0.021$) the number of faecal lactic acid bacteria were significantly reduced by the hops treatments (see table 5.6), but they were not significantly affected in the small intestine and the colon at slaughter. This resulted in a significantly lower ratio of lactic acid bacteria:*E. coli* in the faeces for day 11 ($P < 0.001$) and day 26 ($P = 0.04$) and in the colon ($P = 0.05$) for the pigs fed 10 kg/t of hops (table 5.7).

Table 5.6: Effect of different concentration of hops in the diets on lactic acid bacteria counts (log 10 cfu per gram faeces/ digesta in faeces (day 11 and 26), small intestine and colon in newly weaned pigs.

Level of hops (kg/t)	0	1	10	s.e.d	*P*-value
Faeces day 11	9.43	9.02	8.27	0.380	**0.006**
Faeces day 26	8.47	8.42	7.41	0.488	**0.021**
Small intestine	4.59	5.41	4.35	0.485	0.176
Colon	8.98	8.80	8.00	0.736	0.167

Table 5.7: Effect of different concentration of hops in the diets on lactic acid bacteria:*E. coli* (log 10 cfu per gram faeces/ digesta) in faeces (day 11 and 26), small intestine and colon in newly weaned pigs.

Level of hops (kg/t)	0	1	10	s.e.d	*P*-value
Faeces day 11	1.21	1.11	0.99	0.050	**<.001**
Faeces day 26	1.05	1.08	0.87	0.068	**0.04**
Small intestine	1.30	1.38	1.22	0.186	0.489
Colon	1.02	1.03	0.87	0.086	**0.05**

As shown in table 5.8, the concentration of hops did not affect the number of *Clostridium* at any time. Similarly, no effects were seen on the number of *Bacteriodes* at any time during the experiment (table 5.9) or the number of *Streptococci* (table 5.10).

Table 5.8: Effect of different concentration of hops in the diets on *Clostridium* (log 10 cfu per gram faeces/ digesta in faeces (day 11 and 26), small intestine and colon in newly weaned pigs.

Level of hops (kg/t)	0	1	10	s.e.d	*P*-value
Faeces day 11	4.02	3.66	4.22	0.491	0.422
Faeces day 26	3.03	3.25	3.51	0.273	0.113
Small intestine	2.03	2.00	2.03	0.035	0.678
Colon	2.13	2.25	2.43	0.187	0.143

Table 5.9: Effect of different concentration of hops in the diets on *Bacteriodes* (log 10 cfu per gram faeces/ digesta) in faeces (day 11 and 26), small intestine and colon in newly weaned pigs.

Level of hops (kg/t)	0	1	10	s.e.d	*P*-value
Faeces day 11	7.04	7.37	6.94	0.577	0.629
Faeces day 26	8.11	8.42	7.01	0.839	0.104
Small intestine	5.67	5.69	5.28	0.982	0.641
Colon	7.66	6.68	6.77	0.662	0.408

Table 5.10: Effect of different concentration of hops in the diets on *Streptococci* (log 10 cfu per gram faeces/ digesta) in faeces (day 11 and 26), small intestine and colon in newly weaned pigs.

Level of hops (kg/t)	0	1	10	s.e.d	*P*-value
Faeces day 11	7.09	7.11	6.92	0.446	0.641
Faeces day 26	6.82	6.93	6.66	0.340	0.481
Small intestine	3.40	2.73	2.63	0.446	0.216
Colon	5.06	5.16	4.81	0.458	0.474

Salmonella was not present in any of the analysed samples at any point in time. No *Campylobacter* were found in any of the samples from the small intestine. *Campylobacter* were present in all faecal samples on day 26. The results for the faecal samples day 11 and

the samples from the colon are shown in table 5.11. A chi-square test showed no significant difference between treatments in the incidence of *Campylobacter* in the faeces and colon.

Table 5.11: Effect of different concentration of hops in the diets on the frequency of *Campylobacter* in faces (day 11) and the colon.

Level of hops	0 kg/t		1 kg/t		10 kg/t			
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	χ^2 value	*P*-value
Faeces day 11	9	1	10	0	7	3	4.04	0.133
Colon	7	3	10	0	9	1	4.04	0.133

5.4.5 Gut morphology

The data for villous, crypt and gut cells was analysed using the pig as a block. Gut architecture (villous height and crypt depth) was not significantly affected by the concentration of hops (table 5.12).

Table 5.12: Effect of different concentration of hops in the diets on villous height and crypt depth at 1 m from either end of small intestine of piglets 28 days after weaning (μm).

Inclusion level of hops	Control	1 kg/t	10 kg/t	s.e.d.	*P*-value
Crypt + villous height 1	593.8	600.8	613.7	36.2	0.595
Crypt + villous height 2	692.2	712.8	708.1	33.52	0.806
Crypt depth 1	152.5	173.9	157.5	23.07	0.844
Crypt depth 2	215.1	236.3	213.1	26.22	0.635
Villous height 1	455.1	424.0	451.6	29.85	0.716
Villous height 2	483.2	482.1	504.5	29.55	0.405

1=proximal small intestine, 2=distal small intestine

5.4.5.1 Lymphocytes and eosinophils in villi and crypts

The counts of lymphocytes were not significantly different at any time, but the eosinophils tended to increase with higher concentrations of hops in the distal part of the small intestine (see table 5.13).

Table 5.13: Effect of hops at different concentration of the diets on number of eosinophils and lymphocytes per villous in distal and proximal end the small intestine of piglets 28 days after weaning.

Inclusion level of hops	Control	1 kg/t	10 kg/t	s.e.d.	*P*-value
Eosinophils 1/villi	23.81	27.90	30.83	4.062	0.145
Eosinophils 2/villi	13.74	16.05	18.97	2.736	0.085
Lymphocytes 1/villi	84.4	86.2	88.4	6.25	0.559
Lymphocytes 2/villi	102.9	104.4	110.4	7.78	0.319

1=proximal small intestine, 2=distal small intestine

A regression analysis has shown a significant positive relationship between the number of lymphocytes in the distal part of the small intestine and the villous height ($R^2 = 0.2812$, $P = 0.003$) (figure 5.1).

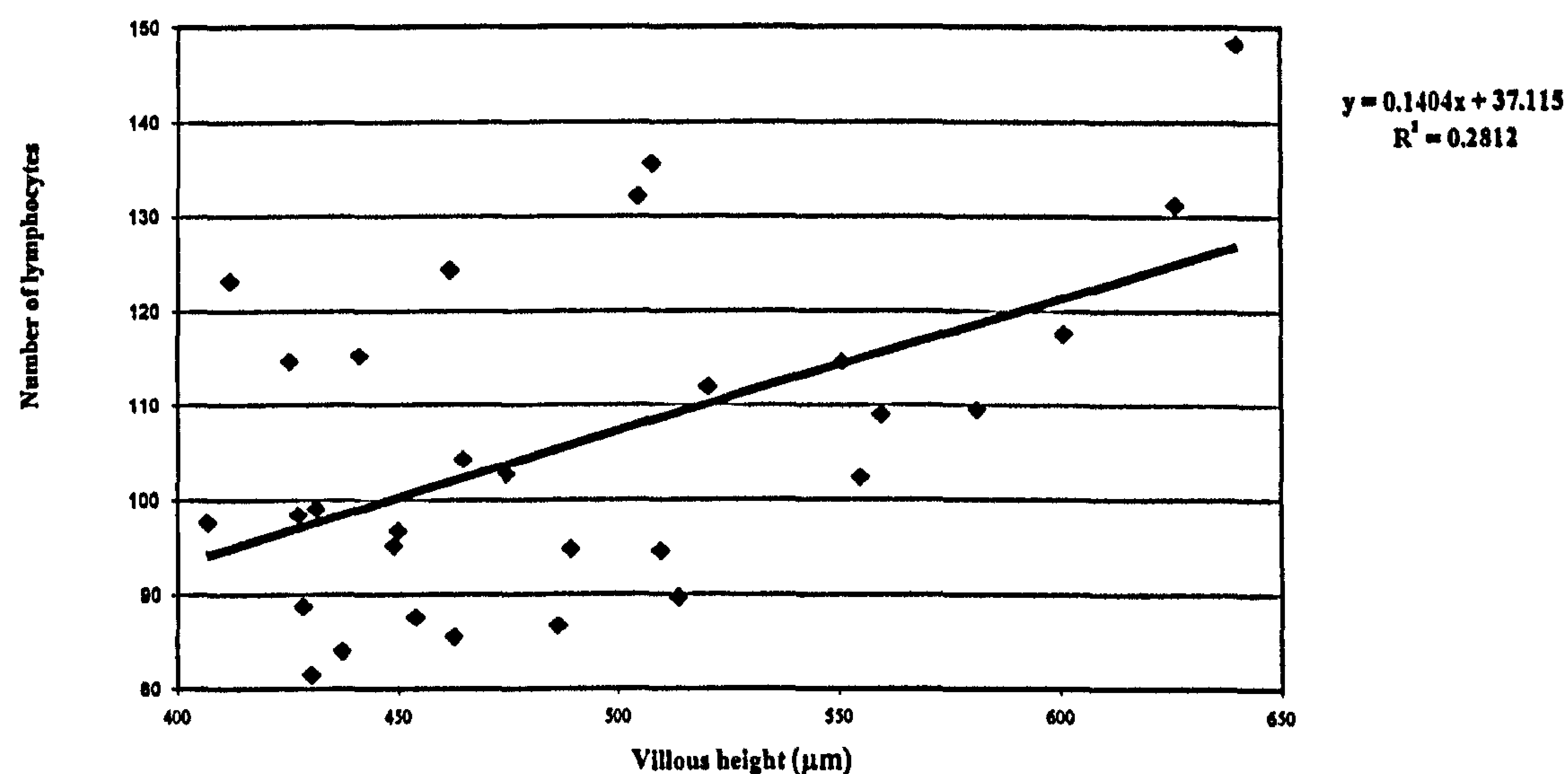


Figure 5.1: The relationship between the number of lymphocytes and villous height in the distal part of the small intestine

5.4.6 Volatile fatty acids

The total amount of VFA in the colon was significantly higher for the pigs fed 10 kg/t hops than the other treatments (see table 5.14), which may partly be explained by the significant higher level of acetic acid in the colon for the pigs receiving 10 kg/t of hops ($P = 0.011$). There was a tendency for butyric to be higher in the colon in the pigs fed the 10 kg/t hops

diet ($P = 0.091$) than the other treatments. There was no significant difference in any of the analysed VFA in the caecum (see table 5.15).

Table 5.14: Effect of hops at different concentrations in the diets on the VFA (mmol/l of supernantant from colonic content analysed) in the colon in weaner pigs 28 days after weaning.

Inclusion level of hops	Control	1 kg/t	10 kg/t	s.e.d.	*P*-value
Acetic	26.3	29.4	37.7	4.22	0.011
Propionic	12.21	13.86	14.99	1.676	0.164
Isobutyric	0.77	0.90	0.72	0.140	0.382
Butyric	7.33	7.02	8.99	1.181	0.091
Isovaleric	1.06	1.20	0.92	0.204	0.272
Valeric	2.57	1.66	1.80	0.566	0.431
Total	50.5	54.1	65.2	6.82	0.034

Table 5.15: Effect of hops at different concentrations in the diets on the VFA (mmol/l of supernantant from caecal content analysed) in the caecum in weaner pigs 28 days after weaning.

Inclusion level of hops	Control	1 kg/t	10 kg/t	s.e.d.	*P*-value
Acetic	30.5	36.9	33.8	4.88	0.887
Propionic	13.09	16.88	16.28	2.358	0.444
Isobutyric	0.433	0.475	0.457	0.095	0.936
Butyric	6.65	8.14	9.01	1.618	0.228
Isovaleric	0.502	0.513	0.507	0.114	0.999
Valeric	1.077	1.242	1.224	0.231	0.702
Total	52.6	64.2	61.6	8.55	0.586

5.5 Discussion

No significant difference was seen in daily live weight gain at any time throughout the experiment. In the experiment the DLWG ranged from 329 to 340 g/day (from day 0 to 22) which is similar to rates reported by Gill *et al.* (2000) which ranged between 329 and 363 g/day in the four weeks after weaning for piglets fed wheat, barley or sugar beet pulp based diets, and within those reported by Pierce *et al.* (2005a) of between 280 and 367 g/day day 0 to 21 for pigs fed different concentrations of avilamycin or inulin with different concentrations of lactose. The overall good growth rates might be explained by the overall

good health of the pigs or the control of feed intake for all the pigs. High health status and good performance often results in a lack in response to growth promoters (Pierce *et al.*, 2005a).

No effect of the hop concentration on feed intake was seen during the first stage of the experiment. Low feed intake or no feed intake is normally seen during the first few days after weaning (Pluske, 2001), and low intake was also noted in this experiment during the first 5 days (178 g/pig/day), as illustrated in figure 5.1. This means the intake of hops and other compounds will be low in this period, and it would take longer for the compound to have an effect. For example, Broom *et al.* (2003) found that antibiotic growth promoters did not show improved growth rates until between 8 and 14 days post weaning, and the FCR was improved after a period of 20 days, but not immediately after weaning. Later in the present experiment (day 22 to 26) the FCR tended ($P = 0.094$) to be better for the pigs fed 10 kg/t of hops. A similar trend was seen in the first experiment (chapter 3), where the FCR was improved with 1 kg/t hops in the period 12 to 28 days after weaning.

A major problem post weaning is diarrhoea caused by haemolytic *E. coli* (Nabuurs *et al.*, 1993; Pluske *et al.*, 2001). The number of *E. coli,* as well as faecal score, were considered to be important indicators of gut health. However, these were not significantly affected by treatment on day 11 or day 26 in the faeces in the current experiment. The number of *E. coli* were within the range reported by others for weaner pigs at this age (Kenworthy and Crabb, 1963; Mathew *et al.*, 1997; Durmic *et al.*, 1998). This is in agreement with experiment 1 (chapter 3) where hops had no effect on the level of *E. coli* and faecal score. *In vitro* experiments (chapter 4) have shown that hops can have weak antibacterial effects on *E. coli* and other gram negative bacteria (Langezaal *et al.*, 1992; Haas and Barsoumian, 1994; Fukao *et al.*, 2000). However, an antibacterial effect was seen towards the beneficial lactic acid bacteria. The hops significantly reduced the number of lactic acid bacteria in the

faeces on day 11 (P=0.006) and day 26 (P=0.021), which is similar to the results from the *in vitro* experiment looking at the effect of different concentrations of hops (section 4.5). The number of lactic acid bacteria was similar to those reported by Durmic *et al.* (1998) for healthy pigs (between 9.06 and 9.36 log10 cfu/g) in the colon, and slightly lower in faeces than results reported by White *et al.* (2002) (between 8.86 and 9.72 log 10 cfu) and slightly lower than the number of ileal *Lactobaillus* (from 6.51 to 8.93 log 10 cfu) reported by Mathew *et al.* (1997). Several *in vitro* experiments, including the one carried out prior to this experiment (chapter 4) have shown that hops have antibacterial effects against a variety of gram positive bacteria. Fernandez and Simpson (1993) and Fukao *et al.* (2000) showed that hop resins had antimicrobial activity against *Lactobacillus brevis.* The reduction in the number of lactic acid bacteria could have resulted in a higher flow of readily fermentable substances to the caecum and proximal part of the colon and thereby increased the level of VFA (Højberg *et al.*, 2005).

The other bacteria analysed were not significantly affected by the hops. The number of *Clostridium* was not affected by dietary treatment. The number was slightly higher than those reported by White *et al.* (2002) in faeces of pigs fed yeast, acids and carbadox (ranging from 2.62 to 3.73 log10 cfu/g), but within the ranges reported by Conway (1994) in one week old pigs in the jejunum between 2.7 and 6.6 log10 cfu/g, 2.0 and 9.5 log10 cfu/g in the faeces and in the colon 0 and 9.2 log10 cfu/g of one to eight weeks old pigs. Similarly, the number of *Bacteriodes* was not affected by the concentration of hops at any time. They were within the ranges reported by Conway (1994) (between 0 and 8.6 log10 cfu/g in the colon and between 0 and 10.5 log10 cfu/g in faeces of piglets aged one to eight weeks, and in the duodenum 4.2 to 5.2 log10 cfu/g of one week old piglets) and slightly lower than found by Mul and Perry (2001) (ranging from around 6 and 7 log10 cfu/g in the small intestine to around 9 log10 cfu/g in the colon). The number of *Streptococci* was slightly lower than ranges reported by Mathew *et al.* (1997), who found 8.19 log10 cfu/g

for the control and 9.17 log10 cfu/g for pigs fed 5 g/kg galactosyl lactose in the ileum of pigs of the same age, but within the ranges reported by Conway (1994) (5.1 cfu/g in the duodenum and 7.1 cfu/g in the colon).

Mathew *et al.* (1991) reported VFA caecal values much higher than in the present experiment. The values in this experiment are lower than those reported by Borg Jensen (2001) who tested different *in vitro* and *in vivo* methods. Reductions in the level of VFA has been reported to coincide with an increased number of *E. coli* and fewer total lactic acid bacteria (Mathew *et al.*, 1996). In this experiment there was no reduction in the number of *E. coli* with the increasing amount of VFA at 10 kg/t hops in the colon, but there was a lower number of lactic acid bacteria.

Higher levels of butyric and acetic acid were found in the colon. Butyric acid is thought to have important implications for the metabolism, structure and function of the epithelial cells lining the large intestine and propionic acid might modify hepatic metabolism (Bach Knudsen *et al.*, 1991; Borg Jensen, 2001; Montagne *et al.*, 2003). Acetic acid acts as an energy substrate for muscle tissue (Borg Jensen, 2001; Montagne *et al.*, 2003). The total amount of VFA was higher in the colon for the pigs fed 10 kg/t hops, which might indicate that the higher level of hops has potential beneficial effects on the fermentation in the colon. The VFA produced are rapidly absorbed from the gut lumen and contribute to the energy supply of the animal, between 5 to 28% of the total maintenance requirement of the animal is provided by VFA fermentation (Bach Knudsen *et al.*, 1991; Borg Jensen, 2001; Franklin *et al.*, 2002). It could be speculated that the higher level of VFA in the colon has contributed to the energy supply to the pig and thereby the tendency for improved FCR.

No changes in villous height and crypt depth were seen 26 days post weaning. The results for the villous height was within averages of 400 and 550 μm reported by van der Klis and

Jansman (2002). They were, however, lower than those reported by Dunsford *et al.* (1989) of 500 and 640 µm in the upper part of the small intestine and within those in the lower part of the intestine of 430 and 540 µm. Dunsford *et al.* (1989) reported crypt depths between 360 and 410µm in the upper part of the small intestine and 260 and 330 µm in the lower. Both of which are higher than observed in the current experiment.

No other experiment was found which examined the effects of hops on the same type of cells in the villi as the current experiment. Other experiments have counted the number of epithelial cells on the column of the villous and crypt in weaned pigs between 22 and 26 days old. These reports range between 162 and 123 for the villous and the crypt (Hampson, 1986a), which is slightly higher than the numbers found in this experiment.

A positive relationship was found between the number of lymphocytes and the villous height ($r = 0.2812$, $P=0.003$). This contradicts results from Ganessunker *et al.* (1999) who found that $CD4^+$and $CD8^+$ T-lymphocytes tended to be negatively correlated with villous height. However, they were looking for specific types of lymphocytes, the intraepithelial T cells ($CD4^+$ and $CD8^+$). These represent around 50% of all intestinal lymphocytes in the mature pig (King *et al.*, 2003). In the current experiment different types were not identified.

Supplementing weaner pig diets with 1 kg/t or 10 kg/t hops for 26 days post weaning had a small effect on the performance in this period, which was seen as an improved FCR in the last week of the experiment. This was in agreement with the first experiment and the general high health status of the animals may mean they were already performing to their highest potential. The high daily live weight gains would support this hypothesis. Inclusion of hops did result in higher levels of acetic acid and total VFA in the colon. However, this did not reduce the number of *E. coli,* as it would have been expected, but it may have

contributed to the energy supply to the pig. The hops resulted in an undesirable reduction in the number of *Lactobacilli* in the faeces and the colon, which was not associated with a higher level of diarrhoea at any time nor did it seem to affect the performance. Overall the physiology and function of the gut was not markedly affected by inclusion of hops.

A tendency for improved FCR was found with higher inclusion levels of hops towards the end of the experiment, but no other performance characteristics were affected. Furthermore, a reduction in the number of lactic acid bacteria and the ratio between lactic acid bacteria and *E. coli* was seen. Finally the level of VFA in the colon was increased by the higher inclusion level of hops.

6. EXPERIMENT 3 – THE EFFECTS ON WEANER PIGS OF HOPS AND HOP EXTRACTS SUPPLEMETATION EITHER WITH OR WITHOUT THE ADDITION OF ORGANIC ACIDS

6.1 Introduction

Results from the previous experiments (chapter 3 and 5) indicate that hops improve feed utilisation in piglets a few weeks after weaning. The actual mechanism by which this happened was not identified. The *in vitro* experiments (chapter 4) showed that hops or isolated hop compounds had antimicrobial effects against some bacteria, particularly gram positive.

The antimicrobial properties of hops have been reported to be found in the β-resins and iso-α resins (see also section 1.6.2.3.2) (Haas and Barsoumian, 1994; Larson *et al.*, 1996; Fukao *et al.*, 2000) and these have proven to exhibit improved antimicrobial effects at lower pH (Simpson and Smith, 1992; Larson *et al.*, 1996). This was confirmed by the *in vitro* experiment (chapter 4) where the β-acid and iso-α –acid extract resulted in a reduced number of *E. coli* K88 and complete inhibition of some gram positive bacteria (i.e. *Streptococcus*), particularly at a lower pH.

After weaning the pigs experience poor digestibility as a result of insufficient HCl secretory capacity and inadequate pancreatic enzymes, such as amylase, trypsin and chymotrypsin (Bolduan *et al.*, 1988a; Burnell *et al.*, 1988; Li *et al.*, 1999). Organic acids are often added as an alternative to or in combination with AGP in piglets diets, as acidification of the diet of the weaned pig has been reported to reduce the gastric pH, affect the digestibility of energy and protein and stimulate the activity pancreatic enzymes (Eidelsburger, 1998; Valencia and Chavez, 2002), see also section 1.5.2. Furthermore, some other herbal additives have already been shown to affect the activity of pancreatic

enzymes and bile acid secretion in the rat (Platel and Srinivasan, 2000a; Platel and Srinivasan, 2000b) and digestive enzyme activity in broiler chickens (Lee *et al.*, 2003). For example, yarrow has been shown to increase the level of lipase in broiler chickens (Lewis, 2005).

Lactic acid bacteria were significantly reduced in the faeces with increasing levels of hops in the previous experiment (chapter 5), although this did not seem to be linked to any of the performance parameters. The addition of an organic acid and potentially lowering of gastric pH may provide a better environment for the lactobacilli to survive in the gastrointestinal tract, reduce the number of pathogenic bacteria (Pierce *et al.*, 2005b), and thereby potentially prevent the reduction in the number of lactic acid bacteria when hops/hop compounds were added. Lactic acid bacteria have high acid tolerance, which permits their successful inhabitation of the pig stomach, their growth is only ceasing when pH is reduced below 4 (Tannock, 1990). The *in vitro* experiment (chapter 4) showed that some of the tested *Lactobacilli* grew well when the Ultracid ® was added compared to HCl. In the previous experiments (chapter 3 and 5) hops have shown variable results on the liver enzymes. It was decided to investigate if the isolated hop compounds or the combination with organic acids and the hops/hop compounds would affect the level of liver enzymes.

6.2 Objectives

The objectives of this study were to identify the compounds and mechanisms responsible for the improved piglet performance in the previous trials, and to investigate the efficacy of hops and isolated hop compounds with or without an organic acid on:

1) growth performance
2) hindgut fermentation
3) digestibility and digestive enzymes

4) liver function

5) gut health

6.3 Materials and methods

6.3.1 Animal husbandry

The experiment was carried out at Harper Adams University College's pig unit. A total of 62 PIC piglets were used in two replicates. The piglets were weaned at approximately 28 days of age (see section 2.1 for breed and husbandry).

6.3.2 Housing and feeding

The pigs were housed in four identical weaner rooms with eight pens in each. At weaning the piglets were randomly allocated into one of the six treatments placed in individual pens. This gave a replication of ten pigs per treatment. The pigs were restrict fed with pellets for the whole period twice daily (morning and afternoon) according to the scale shown in table 5.1. The feed scale was based on the recommendation for guidelines in Nutrient Requirement Standards for Pigs (Whittemore *et al.*, 2003) and was the same as for experiment 2 (see chapter 5, section 5.3.2).

The hops, iso-α extract and β-extract were provided by Steiner Hops Ltd (Epping). The Ultracid 45 ® acid mixture was provided by INVE (Kasterlee, Belgium). The composition of the acid mixture is the same as used in the *in vitro* experiment (see table 4.1, section 4.3.3). The Ultracid 45 ® mixture was included at 3 kg/t according to manufacturers recommendations for use in piglets.

The experiment was a 3 x 2 factorial design with the three treatments being: hops, iso-α and β-mixture and a control diet, either with or without the Ultracid ®. The hops (variety Phonix) were analysed for α-acid and β-acid content by Steiner Hops Ltd, these were 105.3

g/kg 43.9 g/kg, respectively, see section 4.3.5. The iso-α extract and β-acid extract were included at the same levels as provided by the hops. The treatments are given below:

1) control
2) 10 kg/t hops
3) 3.51kg/t iso-α extract and 2.20 kg/t β-acid extract
4) control with 3 kg/t Ultracid ®
5) 10 kg/t hops with 3 kg/t Ultracid ®
6) 3.51kg/t iso-α extract and 2.20 kg/t β-acid extract with 3 kg/t Ultracid ®

There were two diets, stage 1 and 2 basal diets. These were supplied in meal form by Ian Hollows (Whitchurch) they were the basal diets used in experiment 1 and 2, see section 2.2 for the composition. The first stage diet fed from day 0 to 11 after weaning, and the second stage diet fed from day 12 to day 25. The second stage diet contained an indigestible marker, titanium dioxide (BHD, Poole, UK), added at 1 g/kg for digestibility analysis. The hops and iso-α and β-mixture were added at Harper Adams University College and mixed in a horizontal mixer in 25 kg batches. The diets were "cold" pelleted (3 mm) at maximum 40°C.

6.3.3 Analysis of diets

Proximal analysis of the diets was carried out (see section 2.3). The results are shown in table 6.1.

Table 6.1: The proximate analysis of the fresh diets (corrected for dry matter).

	DM (g/kg)	Ash (g/kg)	Protein (g/kg)	Oil (g/kg)	NDF (g/kg)	GE (MJ/kg)	pH
Stage 1							
Control	913.1	64.2	244.9	106	65	19.67	6.10
Control + acid	918.9	68.0	251.3	108	68	19.62	5.92
10 kg hops/t	917.3	65.0	237.2	111	65	19.59	6.00
10 kg hops/t + acid	916.4	64.2	238.7	108	65	19.75	5.83
α- and β	914.2	65.1	251.2	105	60	19.56	6.13
α- and β + acid	915.6	62.9	239.6	114	64	19.78	5.89
Stage 2							
Control	906.4	61.6	243.9	82	81	19.29	6.04
Control + acid	902.5	58.1	233.9	90	76	19.41	5.80
10 kg hops/t	905.0	60.4	237.8	88	77	19.35	5.98
10 kg hops/t + acid	907.6	61.5	242.1	86	67	19.26	5.81
α- and β	902.5	62.1	245.8	85	73	19.39	6.03
α- and β + acid	903.9	61.8	240.9	84	79	19.40	5.85

6.3.3.1 pH of diets

The pH of the feed was measured by mixing 20 g of the fresh feed into 100 ml distilled water. This was shaken for one hour and then the pH was measured (Jenway 3510) as described by Risley *et al.* (1992).

6.3.4 Sampling

The liveweight of the pigs was measured at weaning, 4, 11, 14, 21 and 25 days after weaning on a balance ranging from 0 to 30 kg (Pharmweigh, Bury St Edmunds). This was done at the same time of the day each time. The feed intake was measured daily.

On day 26 to 28 after weaning the pigs were slaughtered with an intracardial injection of 0.5 ml Pentobarbatione sodium (Dodge Animal Health, Southampton). The whole of the intestine and the liver were taken out for dissection and sampling of gut and liver samples. No home office licence was required for this procedure.

Faecal scores were measured at weaning, days 11 and day 25 according to the scale used in previous experiments (see section 2.8).

6.3.5 Bacterial counts

Faecal samples were collected on days 11 and 26 after weaning for bacterial counts. Samples were taken at slaughter from the small intestine and colon, as described in section 2.7, they were analysed for: *E. coli,* lactic acid bacteria, *Clostridium* spp., *Salmonella* spp. and *Bacteriodes*

6.3.6 Blood samples

An intracardial blood sample was taken from the animal after euthanization with Pentobarbatione sodium. The samples were analysed for AST, ALT, GGT, total and direct bilirubin as described in section 2.4.

6.3.7 Volatile fatty acids

Digesta samples were collected from the caecum and the colon for VFA analysis and diluted with HCl, stored and later analysed for VFA as described in section 2.5.

6.3.8 pH of digesta

The pH of the digesta was measured in the small intestine, the colon and the caecum directly with a pH meter Jenway 3510.

6.3.9 Digestive enzymes and bile acids

Digesta samples were taken from the small intestine and stored at -20°C until analysis. Before analysis these were thawed at 4°C and then one gram of digesta sample was mixed in 9 ml ice-cold water, these were further diluted and in some cases to 1:100 for the analysis. These were homogenised and centrifuged at 6500 RPM for 15 minutes at 4°C

(Lee *et al.*, 2003). The supernatant samples were analysed for trypsin (EC 3.4.21.4) and chymotryopsin (EC 3.4.21.1), amylase (EC 3.2.1.1), lipase (EC3.1.1.3) and bile acids.

6.3.9.1 Amylase

Amylase (EC 3.2.1.1) activity was measured using a Synermed amylase test kit VI400 (Synermed Europe Ltd, West Sussex) on a Cobas Mira blood analyser (ABX Diagnostics, Montpellier, France). This method is with 2-chloro-*p*-nitrophenyl-α-D-maltoside (CNPG3), which reacts direct with α-amylase. CNPG3 is hydrolysed by α-amylase to release 2-chloro-*p*-nitrophenol, which is measured spectrophotometrically at 405 nm.

6.3.9.2 Lipase

Lipase activity (EC 3.1.1.3) was measured using a Randox lipase kit (Crumlin, UK) on a Cobas Mira blood analyser (ABX Diagnostics, Montpellier, France) using a turbidimetric method. The principle of the test is that triolein and water form monoglyceride and oleic acid when lipase is present. The decrease in turdidity is measured at 340 nm.

6.3.9.3 Trypsin

Trypsin activity (EC 3.4.21.4) was measured using *p*-toluenesulphonyl-L-arginine methyl ester (TAME, Sigma Chemical Company) as a substrate as described by Hummel (1959). The rate of hydrolysis of TAME was measured by the increase in absorbance at 247 nm (Beckman DU640). One unit of trypsin activity was defined as 1 μmole of TAME hydrolysed per minute at 37°C and pH 8.1 in presence of 10 minutes $CaCl_2$/l.

6.3.9.4 Chymotrypsin

Chymotrypsin activity (EC 3.4.21.1) was measured by the method of Hummel (1959) using benzoyl-L-tyrosine ethyl ester (BTEE, Sigma Chemical Company) as a substrate.

The rate of BTEE was measured by the change in absorbance at 256 nm. One unit of chymotrypsin is defined as 1 μmole BTEE hydrolysed per minute at 37°C at pH 7.8.

6.3.9.5 Bile acids

Bile acid concentrations were measured using a Randox test (Crumlin, UK) on a Cobas Mira blood analyser (Montpellier, France). The principle of the test is that 3α-hydroxy bile acids are converted to corresponding 3-keto bile acids in presence of NAD. The NADH formed reacted with nitrotetrazolium blue and formed a blue dye which was measured at 540 nm.

6.3.10 Liver

The liver was removed after slaughter and weighed.

6.3.11 Gut morphology and histology

Samples were taken from the gut and the villous height and crypt depth were measured as described in section 2.6. The number of eosinophils and lymphocytes were counted on each measured villous and associated crypt, as described in section 2.6.

6.3.12 Apparent digestibility

For the digestibility study the pigs had a five day adaptation period to the diets containing titanium and were fed at 7.30 and 19.30 (day 13 to day 17). From day 18 to 22 faecal samples were collected by grab sampling from each pig four times daily (10.30, 13.30, 16.30 and 19.30) as described by Powles *et al.* (1994) into sterile pots and frozen immediately. The faecal output was bulked for each pig for the whole period and stored at -20°C.

At the end of the sampling period, the faecal samples were freeze dried for 7 days and finely ground.

6.3.12.1 Analysis of titanium dioxide

The diets and faeces were analysed for amount of titanium dioxide. The method used for measuring titanium dioxide was based on the method by Short *et al.* (1996). One gram of dried faeces and diets was weighed out and ashed overnight at 580°C. The sample was boiled gently for 30 minutes with 1.5 g anhydrous sodium sulphate and 10 ml concentrated sulphuric acid. The content was diluted with pure water to 100 ml and 5 ml of the contents was mixed with 0.2 ml of hydrogen peroxide (30% w/v). The colour change was measured on a spectrophotometer (Jenway 6305 UV/Vis spectrophotometer) at 408 nm. A titanium standard (99 µg/ml in H_2O) (Aldrich Chemical Company, USA) was used as control.

6.3.12.2 Gross energy

The gross energy of the dried faeces and feed was analysed with an Isoperibol calorimeter (Parr Instruments 1261).

6.3.12.3 Fat extraction, protein, nitrogen content

Fat was measured in diets and faeces using the acid hydrolysis method, as described in section 2.3.3. This was carried out by Central Labs (Banbury, UK). The protein and nitrogen content was analysed using a LECO analyser (FP 528) as described for the feed analysis in section 2.3.5.

6.3.12.4 Calculation of digestibility

The apparent dry matter digestibility of the diets was calculated using equation 1. A correction factor of 1.06 was used to allow for the recovery of the titanium marker

(titanium dioxide), and was calculated from the standard samples. The apparent digestibility for protein, fat, energy and ash is shown in equation 2 to 5, respectively.

Equation 1: dry matter digestibility:

$$\textit{Digestibility coefficient} = 1\text{-}(Tif/(Tifa/1.06)$$

Equation 2: Apparent protein digestibility (g/kg dry matter):

$$\textit{Apparent digestible protein} = 1\text{-}((1\text{-}X) \times Pfa)/Pf) \times Pf$$

Equation 3: Apparent fat digestibility (g/kg dry matter):

$$\textit{Apparent digestible fat} = 1\text{-}((1\text{-}X) \times Ffa)/Ff) \times Ff$$

Equation 4: Apparent ash digestibility (g/kg dry matter):

$$\textit{Apparent digestible ash} = 1\text{-}((1\text{-}X) \times Ashfa)/Ashf) \times Ashf$$

Ashfa = ash of faeces

Ashf = ash of feed

GEfa = gross energy faeces

GEf = gross energy feed

Tif = titanium feed

Tifa = titanium faeces

Pfa = protein faeces

Pf = protein feed

X = coefficient of digestibility

6.3.13 Statistical analysis

All the data was analysed with ANOVA (Genstat 5th edition) as a 3 x 2 factorial design for unbalanced treatments with the replication as a block. Chi-square analysis was used for faecal scores. Orthogonal contrasts were used to determine differences between the control and hops plus iso-α and β-mixture and between the iso-α and β-mixture and the hops. Significant differences are based on probability of less than 0.05.

6.4 Results

6.4.1 Performance

The feed intake was not significantly affected by treatment at any time during the experiment. An interaction between acid and treatment was seen in th period d 11-14 (P = 0.032), but not at any other time.The results are shown in table 6.2.

Table 6.2: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on feed intake of newly weaned pigs (g/pig/day).

	No acid			Acid			*Treatment x acid*	
Day	**C**	**H**	**A & β**	**C**	**H**	**α & β**	**s.e.d.**	***P***
0-4	132	115	129	131	131	89	23.3	0.224
4-11	269	237	256	260	259	241	25.9	0.565
11-14	389	362	361	352	390	387	19.6	0.032
14-21	534	536	525	501	539	542	19.9	0.178
21-25	777	756	770	744	780	781	20.4	0.124

The liveweight of the piglets was not significantly affected by treatment at any time (table 6.3) However, the daily live weight gain was affected. In agreement with previous experiments (chapter 3 and 5), hops affected the performance of piglets two to three weeks post weaning. There was a trend for higher DLWG for the pigs fed the hops and iso-α and β-mixture in the period day 14 to 21 (P = 0.055). This resulted in a significant effect on the FCR (P = 0.027) in the same period. The orthogonal contrast analysis identified the hops

and iso-α and β-mixture as a cause of the improved FCR. There was a significant acid x hops/ iso-α and β-mixture interaction on DLWG and FCR day 14 to 21 (P = 0.041) (see table 6.4, 6.5, 6.6 and 6.7).

Table 6.3: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on liveweight of newly weaned pigs (kg).

	No acid			Acid			*Treatment x acid*	
Day	**C**	**H**	**A & β**	**C**	**H**	**α & β**	**s.e.d.**	***P***
0	8.77	8.76	8.81	8.79	8.73	8.61	0.541	0.958
4	9.02	8.92	9.03	9.10	9.07	8.60	0.551	0.721
11	10.49	10.03	10.31	10.44	10.11	10.13	0.559	0.948
14	11.47	11.01	11.33	11.36	11.33	11.20	0.562	0.814
21	14.90	14.47	14.68	14.22	14.78	14.75	0.605	0.488
25	17.68	17.10	17.47	17.31	17.56	17.61	0.657	0.834

Table 6.4: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on DLWG in newly weaned pigs (g/day).

	No acid			Acid			*Treatment x acid*	
Day	**C**	**H**	**α & β**	**C**	**H**	**α & β**	**s.e.d.**	***P***
4-11	197	159	183	191	148	201	42.1	0.868
11-25	479	471	477	465	497	489	20.5	0.370
14-21	490	494	479	409	492	508	31.0	0.041
4-25	405	389	402	401	404	418	19.9	0.730
0-21	289	272	280	258	288	288	22.5	0.297

Table 6.5: Main effect and analysis of contrast on the effects of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on DLWG.

	Treatment		*Acid*		**Probability of contrasts**	
Day	**s.e.d.**	***P***	**s.e.d.**	***P***	**C *vs.* H + α & β**	**H *vs.* α & β**
4-11	29.7	0.312	24.3	0.992	0.403	0.177
11-25	14.5	0.662	11.8	0.513	0.354	0.981
14-21	21.9	0.055	17.9	0.320	0.021	0.957
4-25	14.0	0.655	11.5	0.443	0.999	0.340
0-21	15.9	0.750	13.0	0.883	0.462	0.808

Table 6.6: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on FCR in newly weaned pigs.

	No acid			Acid			*Treatment x acid*	
Day	C	H	α & β	C	H	α & β	s.e.d.	*P*
4-11	1.51	1.42	1.38	1.31	1.69	1.26	0.225	0.316
11-25	1.12	1.12	1.10	1.11	1.09	1.11	0.036	0.744
14-21	1.11	1.10	1.10	1.28	1.10	1.07	0.063	0.054
4-25	1.17	1.17	1.14	1.15	1.17	1.11	0.032	0.747
0-21	1.24	1.23	1.24	1.27	1.21	1.17	0.063	0.564

Table 6.7: Main effect and analysis of contrast on the effects of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on FCR.

	Treatment		*Acid*		Probability of contrasts	
Day	s.e.d.	*P*	s.e.d.	*P*	C *vs.* H + α & β	H *vs.* α & β
4-11	0.159	0.317	0.129	0.805	0.260	0.875
11-25	0.026	0.848	0.020	0.648	0.568	0.866
14-21	0.044	**0.027**	0.036	0.208	**0.011**	0.737
4-25	0.023	0.192	0.018	0.373	0.706	0.064
0-21	0.044	0.514	0.036	0.514	0.260	0.662

6.4.2 Liver weight and function

There was no significant effect of hops or iso-α and β-mixture on liver weight (see table 6.8). Analysis of the levels of bilirubin showed that both direct and total bilirubin levels were unaffected by dietary treatment. The total and the direct bilirubin levels found were within the reported normal level of 0 to 8.55 μmol/l for pigs (Rushton, 1981).

Table 6.8: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on total and direct bilirubin (mmol/l) and liver weight (g) in newly weaned pigs.

	No acid			Acid			*Treatment x acid*	
	C	H	α & β	C	H	α & β	s.e.d.	*P*
Direct bilirubin	2.92	2.45	2.88	3.02	2.57	2.69	0.428	0.849
Total bilirubin	4.21	3.50	4.10	4.31	3.67	3.95	0.487	0.885
Liver weight	552	555	552	545	555	553	25.6	0.973

The analysis of liver enzymes in the serum showed that the level of AST was not affected by addition of hops or the hops compounds, but a trend was found (P = 0.095) for increased level of AST with the addition of acid to the diets See table 6.9). The level of AST found was slightly higher than the normal ranges between 10 and 22 U/l reported by Rushton (1981). The level of ALT was not affected by dietary treatment, but the average values found were higher than the reported normal values by Rushton (1981) of 10 and 18 U/l. There was a trend for the level of GGT to be higher in the hops treatment (P = 0.061). Orthogonal contrast analysis showed that the hops treatment was higher than the iso-α and β-mixture. The level of GGT was within the levels reported by Rico *et al.* (1977) of 36 ± 14 U/l.

Table 6.9: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on liver enzymes AST, ALT and GGT (U/l) in newly weaned pigs.

	No acid			Acid			*Treatment x acid*	
	C	H	α & β	C	H	α & β	s.e.d.	*P*
AST	38.2	38.4	32.7	43.3	42.0	48.4	8.74	0.550
ALT	41.7	40.7	39.5	42.4	46.5	43.8	5.01	0.764
GGT	25.2	25.7	26.3	24.4	33.6	21.3	3.69	0.052

6.4.3 Faecal score

The faecal score was analysed with a chi-square analysis. The score was not affected at weaning and day 11, but on day 25 there was a tendency (P = 0.089) for the pigs receiving the hops treatments to have a lower score indicating looser faeces, see table 6.10.

Table 6.10: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diet on faecal score at weaning, day 11 and day 25 in newly weaned pigs.

		Faecal score		
Acid	**Treatment**	**Day 0**	**Day 11**	**Day 25**
-	**Control**	3.7	2.7	3.5
-	**Hops**	4.4	2.6	2.7
-	**α & β**	4.4	3.0	3.5
+	**Control**	4.0	3.3	3.6
+	**Hops**	3.6	2.3	3.0
+	**α & β**	4.0	3.5	3.9
	DF	20	20	20
	χ^2	11.94	17.53	28.92
	P	0.918	0.618	0.089

6.4.4 Analysis of bacteria

All the samples were negative for *Salmonella* spp. Dietary treatments did not significantly affect the number of *E. coli* in the faeces or digesta at any time point (table 6.11). An interaction between the treatment and the acid was seen in the faeces on day 11 ($P = 0.043$). Orthogonal contrast identified a significantly higher number of *E. coli* ($P = 0.036$) in iso-α and β-mixture with the acid than the iso-α and β-mixture without the acid in the faecal samples on day 11. The iso-α and β-mixture without the acid was significantly ($P = 0.023$) lower than the hop treatment without the acids in the faeces on day 11.

Table 6.11: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diet on number of *E. coli* in faeces, small intestine (S.I.) and colon in newly weaned pigs (log 10 cfu per gram faeces/ digesta).

	No acid			Acid			*Treatment x acid*	
	C	H	α & β	C	H	α & β	**s.e.d.**	***P***
Faeces d11	7.38	7.78	6.72	7.14	7.18	7.68	0.445	0.043
Faeces d25	7.03	7.70	7.12	7.14	6.95	7.72	0.575	0.267
S.I.	5.43	5.03	5.09	5.05	4.95	4.99	0.583	0.923
Colon	7.16	7.30	7.47	7.17	7.19	7.16	0.494	0.898

The number of lactic acid bacteria was affected by the addition of hops and hop compounds (see table 6.12 and 6.13). No interactive effects were seen between any of the

treatments. Orthogonal contrast identified the hops and iso-α and β-mixture as being responsible for the reduction in lactic acid bacteria in the faeces on day 11 ($P = 0.058$) and in the colon ($P = 0.064$). On day 25 the hops treatment resulted in a significantly ($P = 0.001$) lower number of lactic acid bacteria. In contrast, in the small intestine the hop treatment resulted in significantly higher ($P = 0.013$) levels of lactic acid bacteria compared to the control and iso-α and β-mixture.

As a result of the affected number of lactic acid bacteria, the ratio between the *E. coli*:lactic acid bacteria was significantly affected on day 25 ($P = 0.002$) in the faeces. An orthogonal contrast analysis identified that the hops and iso-α and β-mixture had the lowest ratio significantly different from the control and a trend for the iso-α and β-mixture to be higher than the hops (table 6.14 and 6.15).

Table 6.12: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diet on number of *lactic acid bacteria* in faeces, small intestine (S.I.) and colon in newly weaned pigs (log 10 cfu per gram faeces/ digesta).

	No acid			Acid			*Treatment x acid*	
	C	H	α & β	C	H	α & β	s.e.d.	*P*
Faeces d11	8.02	7.43	7.61	8.32	7.65	7.81	0.380	0.982
Faeces d25	8.68	7.34	8.20	8.48	7.29	8.14	0.466	0.968
S.I.	6.68	7.04	6.49	6.46	7.20	6.24	0.358	0.659
Colon	8.88	8.32	8.47	8.74	7.97	8.56	0.390	0.731

Table 6.13: Main effect and analysis of contrast on the effects of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on number of *lactic acid bacteria* in faeces, small intestine (S.I.) and colon in newly weaned pigs.

	Treatment		*Acid*		Probability of contrasts	
	s.e.d.	*P*	s.e.d.	*P*	C *vs.* H + α & β	H *vs.* α & β
Faeces d11	0.269	0.058	0.219	0.280	0.019	0.507
Faeces d25	0.329	0.001	0.269	0.701	0.003	0.009
S.I.	0.253	0.013	0.206	0.606	0.413	0.004
Colon	0.275	0.064	0.224	0.565	0.046	0.171

Table 6.14: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diet on the ratio of lactic acid bacteria:*E. coli* in faeces, small intestine (S.I.) and colon in newly weaned pigs.

	No acid			Acid			*Treatment x acid*	
	C	H	α & β	C	H	α & β	s.e.d.	*P*
Faeces d11	1.12	0.96	1.15	1.18	1.09	1.03	0.082	0.081
Faeces d25	1.25	0.97	1.17	1.22	1.06	1.08	0.082	0.310
S.I.	1.45	1.63	1.58	1.56	1.96	1.50	0.324	0.684
Colon	1.27	1.17	1.16	1.25	1.13	1.22	0.106	0.766

Table 6.15: Main effect and analysis of contrast on the effects of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on number of lactic acid bacteria:*E. coli* in faeces, small intestine (S.I.) and colon in newly weaned pigs.

	Treatment		*Acid*		Probability of contrasts	
	s.e.d.	*P*	s.e.d.	*P*	C *vs.* H + α & β	H *vs.* α & β
Faeces d11	0.058	0.112	0.047	0.657	0.068	0.335
Faeces d25	0.058	**0.002**	0.047	0.779	**0.001**	0.057
S.I.	0.229	0.396	0.187	0.545	0.420	0.256
Colon	0.074	0.383	0.061	0.982	0.189	0.593

The number of *Streptococci* was unaffected by dietary treatment (table 6.16) and found to be within the reported ranges for one to eight weeks old pigs (Conway, 1994; Mul and Perry, 2001). The number of *Bacteriodes* was significantly reduced by the iso-α and β-mixture on day 11 ($P < 0.001$) and day 25 ($P = 0.003$) in the faeces and in the colon ($P < 0.001$). In the small intestine the *Bacteriodes* were significantly reduced by the iso-α and β-mixture ($P = 0.011$) and increased by the hops (table 6.17 and 6.18).

Table 6.16: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diet on number of *Streptococcus* in faeces, small intestine (S.I.) and colon in newly weaned pigs (log 10 cfu per gram faeces/ digesta).

	No acid			Acid			*Treatment x acid*	
	C	H	α & β	C	H	α & β	s.e.d.	*P*
Faeces d11	5.44	5.05	5.56	5.09	5.08	5.70	0.487	0.756
Faeces d25	4.42	4.60	3.89	4.39	4.09	4.49	0.402	0.158
S.I.	4.47	5.03	4.62	4.72	4.48	4.56	0.580	0.625
Colon	5.24	5.34	5.35	5.75	5.73	5.74	0.579	0.985

Table 6.17: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diet on number of *Bacteriodes* in faeces, small intestine (S.I.) and colon in newly weaned pigs (log 10 cfu per gram faeces/ digesta).

	No acid			Acid			*Treatment x acid*	
	C	H	A & β	C	H	α & β	s.e.d.	*P*
Faeces d11	8.01	7.37	6.91	8.42	7.49	7.10	0.344	0.822
Faeces d25	7.52	7.04	6.80	7.44	6.98	6.91	0.255	0.836
S.I.	6.18	6.29	5.60	5.96	6.24	5.42	0.356	0.942
Colon	8.81	7.85	7.22	8.84	7.74	7.77	0.477	0.596

Table 6.18: Main effect and analysis of contrast on the effects of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on number of *Bacteriodes* in faeces, small intestine (S.I.) and colon in newly weaned pigs.

	Treatment		*Acid*		Probability of contrasts	
	s.e.d.	*P*	s.e.d.	*P*	C *vs.* H + α & β	H *vs.* α & β
Faeces d11	0.243	<.001	0.198	0.237	<.001	0.087
Faeces d25	0.180	0.003	0.147	0.949	<.001	0.380
S.I.	0.252	0.011	0.205	0.942	0.402	0.003
Colon	0.337	<.001	0.275	0.559	<.001	0.392

There was a tendency for the number of *Clostridium* to be lower in the hops and iso-α and β-mixture in the faeces on day 11 ($P = 0.086$), but not in any other of the samples, table 6.19. Orthogonal analysis showed that the control treatments had significantly higher number of *Closridium* than the hops and iso-α and β-mixture ($P = 0.036$). The interaction

was due to the number of *Clostridium* increasing with iso-α and β-mixture when acid was added, but the number was decreasing with hops and control when acid was added.

Table 6.19: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diet on number of *Clostridium* in faeces, small intestine (S.I.) and colon in newly weaned pigs (log 10 cfu per gram faeces/ digesta).

	No acid			Acid			*Treatment x acid*	
	C	H	A & β	C	H	α & β	s.e.d.	*P*
Faeces d11	4.12	3.31	2.54	3.51	2.52	3.69	0.577	0.038
Faeces d25	2.24	2.53	2.42	2.55	2.93	2.42	0.606	0.889
S.I.	2.15	2.39	2.59	2.40	2.01	2.18	0.474	0.549
Colon	3.86	3.85	3.91	3.88	4.23	4.39	0.463	0.763

6.4.5 Volatile fatty acids

The level of VFA in the caecum was not affected by treatment apart from propionic acid which was significantly higher in the iso-α and β-mixture ($P = 0.020$). The inclusion of acid tended to increase the propionic, acetic, isobutyric, butyric and valeric acid (table 6.20 and 6.21).

No effects of the addition of organic acids were seen on the VFA in the colon. Isobutyric and isovaleric were higher for the control treatments than the other treatments (table 6.22 and 6.23).

Table 6.20: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on the VFA in the caecum in weaner pigs 28 days after weaning (mmol/l of supernantant in caecal content).

	No acid			Acid			*Treatment x acid*	
	C	H	α & β	C	H	α & β	s.e.d.	*P*
Acetic	46.32	46.71	50.15	55.20	48.06	56.82	5.448	0.610
Propionic	19.56	21.42	22.64	25.62	24.61	33.08	3.004	0.234
Isobutyric	0.45	0.46	0.49	0.66	0.50	0.56	0.089	0.352
Butyric	9.25	11.47	10.25	12.64	12.17	11.44	1.723	0.510
Isovaleric	0.50	0.56	0.49	0.75	0.51	0.53	0.109	0.158
Valeric	1.61	2.38	1.59	2.49	2.48	2.54	0.387	0.250
Total	77.74	83.20	85.70	97.48	88.26	105.10	8.974	0.426

Table 6.21: Main effect and analysis of contrast on the effects of control, hops and iso-α and β-mixture with or without acid in diets on the VFA in the caecum in weaner pigs 28 days after weaning.

	Treatment		*Acid*	
	s.e.d.	*P*	s.e.d.	*P*
Acetic	3.851	0.273	3.143	0.078
Propionic	2.124	**0.020**	1.733	**<.001**
Isobutyric	0.063	0.505	0.051	**0.043**
Butyric	1.218	0.691	0.994	0.085
Isovaleric	0.077	0.288	0.063	0.200
Valeric	0.274	0.345	0.223	**0.004**
Total	6.344	0.243	5.178	**0.006**

Table 6.22: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on the VFA in the colon weaner pigs 28 days after weaning (mmol/l of supernantant in colonic content).

	No acid			Acid			*Treatment x acid*	
	C	H	α & β	C	H	α & β	s.e.d.	*P*
Acetic	35.44	41.24	43.70	36.69	42.03	36.20	6.655	0.578
Propionic	15.07	16.48	17.21	16.95	18.38	16.73	2.956	0.809
Isobutyric	1.04	1.11	0.79	1.19	0.93	0.94	0.142	0.183
Butyric	7.97	10.03	8.73	8.92	11.25	8.22	1.633	0.723
Isovaleric	1.45	1.47	1.00	1.74	1.22	1.20	0.229	0.234
Valeric	1.87	2.59	2.00	2.31	2.28	2.35	0.290	0.382
Total	63.15	73.09	73.64	67.88	73.95	68.49	11.40	0.821

Table 6.23: Main effect and analysis of contrast on the effects of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on the VFA in the colon in weaner pigs 28 days after weaning.

	Treatment		*Acid*		Probability of contrasts	
	s.e.d.	*P*	s.e.d.	*P*	C *vs.* H + α & β	H *vs.* α & β
Acetic	4.702	0.492	3.839	0.630	0.253	0.690
Propionic	2.089	0.797	1.705	0.519	0.516	0.822
Isobutyric	0.101	**0.043**	0.082	0.636	**0.045**	0.139
Butyric	1.153	0.106	0.942	0.568	0.272	0.060
Isovaleric	0.162	**0.012**	0.132	0.530	**0.009**	0.154
Valeric	0.290	0.498	0.237	0.488	0.398	0.399
Total	8.056	0.613	6.570	0.987	0.331	0.745

6.4.6 pH of the intestinal contents

The pH in the small intestine, colon and caecum was unaffected by the addition of hops or iso-α and β-mixture and also the addition of acid (table 6.24).

Table 6.24: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on pH in the small intestine (SI), colon and caecum on weaner pigs 26 days post weaning.

	No acid			Acid			*Treatment x acid*	
	C	H	α & β	C	H	α & β	s.e.d.	*P*
SI	5.64	5.70	5.74	5.72	5.55	5.68	0.165	0.635
Colon	6.14	6.00	6.14	5.99	6.10	5.95	0.201	0.564
Caecum	5.73	5.68	5.63	5.63	5.69	5.58	0.125	0.862

6.4.7 Digestibility studies

The results from the digestibility study are shown in tables 6.25 and 6.26. The digestibility of dry matter was significantly higher in the control treatment than for the hops and iso-α and β-mixture, and it was also significantly higher for the iso-α and β-mixture treatments than in the hops treatment. The digestibility coefficient for energy was significantly higher for the control and the iso-α and β-mixture than for the hops ($P = 0.002$) with and without an acid (table 6.26). There was a trend for the fat digestibility coefficients to be affected by dietary treatment ($P = 0.058$) and by acid treatment ($P = 0.028$). With an average of 0.914

the acid treatments were significantly higher than the coefficient of 0.903 (P = 0.028) for the non-acid treatments. The protein digestibility was significantly higher in the control and iso-α and β-mixture (P = 0.009) than the control. There was a trend for the coefficient for ash digestibility to be higher in the iso-α and β-mixture (P = 0.083) compared to the hops. No significant difference was seen in the ratio between the digestibility coefficient for energy and protein.

Table 6.25: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets coefficient of digestibility of energy, protein, fat and ash of weaner pigs day 18 to 22 after weaning.

	No acid			Acid			*Treatment x acid*	
	C	H	α & β	C	H	α & β	s.e.d.	*P*
DM	0.919	0.907	0.909	0.919	0.903	0.918	0.0050	0.200
Energy	0.918	0.912	0.915	0.924	0.907	0.923	0.0046	0.110
Fat	0.898	0.901	0.909	0.912	0.904	0.918	0.0070	0.558
Protein	0.894	0.884	0.890	0.900	0.877	0.897	0.0077	0.357
Ash	0.711	0.702	0.709	0.712	0.700	0.738	0.0147	0.263
Energy: protein	1.027	1.032	1.028	1.027	1.035	1.029	0.0046	0.871

Table 6.26: Main effect and analysis of contrast on the effects of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets coefficient of digestibility of energy, protein, fat and ash of weaner pigs day 18 to 22 after weaning.

	Treatment		*Acid*		Probability of contrasts	
	s.e.d.	*P*	s.e.d.	*P*	C *vs.* H + α & β	H *vs.* α & β
DM	0.0036	**0.007**	0.0030	0.226	**0.037**	**0.029**
Energy	0.0032	**0.002**	0.0027	0.192	**0.022**	**0.005**
Fat	0.0049	0.058	0.0040	**0.028**	0.493	**0.026**
Protein	0.0054	**0.009**	0.0044	0.670	**0.035**	**0.018**
Ash	0.0104	0.083	0.0085	0.270	0.895	**0.028**
Energy: protein	0.0032	0.150	0.0026	0.545	0.137	0.174

6.4.8 Digestive enzymes

There was no statistically significant difference between treatments for the amount of lipase and amylase in the intestinal content of the pigs. A trend (P = 0.077) was found for

the trypsin to be higher in the control treatments compared with the other treatments. Orthogonal analysis showed a trend (P = 0.077) for the control to have higher trypsin values than the hops and iso-α and β-mixture. The chymotrypsin was not influenced by dietary treatment (table 6.27 and 6.28).

Table 6.27: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on level of lipase, amylase, trypsin and chymotrypsin in the small intestine of weaner pigs day 26 after weaning (U/g wet digesta).

	No acid			Acid			*Treatment x acid*	
	C	H	α & β	C	H	α & β	s.e.d.	*P*
Lipase	128.5	108.8	88.6	138.4	88.0	102.3	31.65	0.705
Amylase	221.7	187.2	138.6	199.9	150.7	158.9	59.38	0.782
Trypsin	37.29	35.15	23.72	34.29	28.99	23.50	7.591	0.860
Chymo.	3.37	2.48	2.52	3.19	2.49	2.33	0.967	0.987

Table 6.28: Main effect and analysis of contrast on the effects of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on level of lipase, amylase, trypsin and chymotrypsin in the small intestine of weaner pigs day 26 after weaning (U/g wet digesta).

	Treatment		*Acid*		Probability of contrasts	
	s.e.d.	*P*	s.e.d.	*P*	C *vs.* H + α & β	H *vs.* α & β
Lipase	22.36	0.172	18.26	0.946	0.058	0.905
Amylase	41.97	0.330	34.23	0.729	0.154	0.614
Trypsin	5.365	0.077	4.380	0.479	0.086	0.156
Chymo.	0.684	0.383	0.558	0.828	0.112	0.924

6.4.9 Bile acids

The bile acid values in this experiment were not affected by dietary treatment, as shown in table 6.29.

Table 6.29: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on level of bile acids in the small intestine of weaner pigs day 18 to 26 after weaning (U/g wet digesta).

	No acid			Acid			*Treatment x acid*	
	C	H	α & β	C	H	α & β	s.e.d.	*P*
Bile acids	11.44	13.31	11.75	12.22	11.48	11.75	2.826	0.794

6.4.10 Gut morphology

The villous height and crypt depth were unaffected by dietary treatment at any point in the small intestine (table 6.30). Similarly, the counts of lymphocytes and eosinophils on the villous and crypt were not significant different for any treatment (table 6.31).

Table 6.30: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on villous height (μm) and crypt depth (μm) 1m from upper (1) and lower (2) end of small intestine of piglets 26 days after weaning.

	No acid			Acid			*Treatment x acid*	
	C	H	α & β	C	H	α & β	s.e.d.	*P*
Villous 1	627.5	590.4	558.1	576.6	612.7	553.2	41.58	0.460
Villous 2	410.0	446.2	425.3	412.6	419.6	419.5	32.96	0.816
Crypt 1	189.2	195.9	208.4	208.2	206.6	207.7	17.31	0.720
Crypt 2	153.8	159.7	158.3	157.3	166.6	153.7	15.53	0.867
Crypt + Vil 1	815.5	786.5	766.3	784.9	819.7	752.9	38.62	0.491
Crypt + Vil 2	563.6	607.2	583.6	569.4	586.2	574.1	32.31	0.843

Table 6.31: Effect of control (C), hops (H) and iso-α and β-mixture (α & β) with or without acid in diets on number of eosinophils and lymphocytes per villous in upper (1) and lower (2) part of the small intestine of piglets 26 days after weaning.

	No acid			Acid			*Treatment x acid*	
	C	H	α & β	C	H	α & β	s.e.d.	*P*
Lymphocytes 1	74.3	68.7	74.4	66.9	76.0	72.9	5.06	0.127
Lymphocytes 2	47.2	46.8	49.3	47.2	46.8	50.7	3.94	0.962
Eosinophils 1	15.3	13.1	12.6	12.3	12.1	12.4	2.45	0.700
Eosinophils 2	25.2	22.3	25.2	22.8	24.5	24.5	2.56	0.446

6.5 Discussion

This experiment clearly demonstrated that hops and the hops compounds improved the performance in newly weaned pigs two to three weeks after weaning. This is in agreement with previous experiments (see chapters 3 and 5) and Cornelison *et al.* (2006), who found that hops improved the performance in broilers. The DLWG is within the ranges reported by Broom *et al.* (2003) who found an average of 236 and 307 g/day in the period 0 to 20 days for pigs fed a control diet and Avilamycin and zinc oxide, respectively. The isolated iso-α and β-mixture resulted in similar performance of the pigs as hops, indicating they could be the components in hops responsible for the improved feed utilisation ($P = 0.027$) day 14 to 21.

The addition of an acid mixture lowered the pH of the feed by between 0.1 and 0.24 units. This had no significant effect on pH of the digesta. In fact, Manzanilla *et al.* (2004) found that inclusion of 5 g/kg of formic acid increased the pH in the stomach, although organic acids are generally believed to lower the gastric pH (Partanen and Mroz, 1999). This might be due to differences in type and dose of acid used, buffering capacity and composition of basal diet, age of animals and existing levels of performance (Partanen and Mroz, 1999; Manzanilla *et al.*, 2004; Pierce *et al.*, 2005b). The acid mixture used in this experiment, included at 3 kg/t feed, consisted mainly of citric acid, included at 20 g/kg, fumaric acid at 2.5 g/kg and malic acid at 2.5 g/kg. Fumaric acid has been shown to improve the gain and efficiency of feed utilisation in the first few weeks after weaning, whereas citric acid did not have any effect (Radecki *et al.*, 1988). In contrast, Broz and Schulze (1987) showed that citric acid included at levels of 5, 10 and 20 g/kg all improved the feed conversion ratio in weaner pigs. The pH values found in the caecum and the colon were similar to the values reported by Montagne *et al.* (2004) of 5.6 to 6.5. They were also similar to values in the colon, the caecum and for the small intestine reported by Li *et al.* (1999), Houdijk *et al.* (2002) and Pluske *et al.* (2003). Malic acid is thought to have antibacterial activity, but has

in some experiments resulted in depression in performance and feed intake in weaner pigs (Ravindran and Kornegay, 1993; Krause *et al.*, 1994) (see also the literature review section 1.5.2).

The exact mode of action of organic acids is unclear (Partanen and Mroz, 1999; Li *et al.*, 2003), see also section 1.5.2.1 for their potential mode of action. It is thought that the organic acids stimulate the activity of pepsin and pancreatic enzymes, reduce the number of pathogenic bacteria and improve the digestibility (Partanen and Mroz, 1999; Valencia and Chavez, 2002; Li *et al.*, 2003). These effects were not observed in this experiment. In agreement with this experiment, Risley *et al.* (1992) reported that citric and fumaric acid at 1.5% (wt/wt) in diets did not reduce the digesta pH and had no effect on the *E. coli* population of the gut. In the current experiment the acid mixture had a positive effect on fat utilisation (P = 0.028), and increased fat digestibility from an average of 0.903 to 0.911, but digestibility measures of the other parameters were not affected. The results observed by other workers investigating acids are variable. Some researchers found that fumaric acid and propionic acid did not influence the digestibility of dry matter, crude protein and energy (Thacker *et al.*, 1992). Giesting and Easter (1991) found that addition of 19.9 g/kg fumaric acid did not significantly improve the ileal digestibility and performance of weaner pigs. In contrast, Blank *et al.* (1999) tested 0, 10, 20 and 30 g/kg inclusion of fumaric acid. They found that 20 g/kg inclusion level had a positive effect on the digestibility of crude protein (0.736 for control *vs.* 0.809 for inclusion of 20 g/kg fumaric acid), gross energy (0.718 for control *vs.* 0.745 for inclusion of 20 g/kg fumaric acid) and various indispensable and dispensable amino acids in early weaned pigs.

Overall the digestibility values were high in comparison to other published values. However, they were high for all the treatments and could therefore be compared against each other without problems. The digestibility study showed that neither the hops nor the

iso-α and β-mixture improved the feed utilisation by increasing the digestibility of protein or energy, as the piglets on these treatments had lower digestibility coefficients than the control. The digestibility of energy ranged between 0.907 to 0.924, which is within the ranges reported in other studies for pigs (Pettersson and Lindberg, 1997; Le Bellego and Noblet, 2002; Wang *et al.*, 2002), but this is higher than reported for weaner pigs (0.848 to 0.870) by Pierce *et al.* (2005a). The results were higher than other reported for ether extract and apparent digestibility in piglets (between 0.852 and 0.865) by Jansman *et al.* (1993). The digestibility coefficients ranged between 0.880 and 0.900 for protein. The values for ash digestibility ranged from 0.700 to 0.738, which was higher than the 0.570 to 0.590 reported by Jansman *et al.* (1993) in piglets. The dry matter digestibility ranged between 0.903 to 0.919, which is higher than the 0.850 to 0.866 reported by Jansman *et al.* (1993). The improved digestibility for fat and ash in pigs supplemented with iso-α and β-mixture could potentially explain the better performance for the pigs on that treatment on day 14 and 21. Fat utilisation is normally linked with energy utilisation and it could be expected that a higher fat utilisation would be linked with a better energy utilisation (Le Dividich and Sève, 2001). However, the energy utilisation was better for the control as well as the iso-α and β-mixture, so it was not consistent for this experiment.

The better performance for hops and iso-α and β-mixture and the better fat digestibility by the iso-α and β-mixture could not be explained by a higher level of pancreatic enzymes, as they were not affected by dietary treatment. Only trypsin was significantly lower in the iso-α and β-mixture fed pigs and hops fed pigs. Van Baak *et al.* (1991) reported values of 12 U/g for trypsin in pigs, which is lower than in this experiment, and 3 U/g for chymotrypsin in pigs, which is higher, in fresh ileal chyme samples. The variability between the results for the digestive enzymes in this experiment may be due to method of analysis.

Hops contain an average of 74 g/kg of tannins (Canbas *et al.*, 2001) and at the inclusion level used in this study of 10 g/kg this would result in an increase of 0.74 g/kg tannins in the hop treatments, this was not thought to influence the performance, although some have reported no significant effects and others negative effects (Longstaff and McNab, 1991). However, feeding tannins to newly weaned piglets who's digestive tract is not fully developed could result in impaired digestion (Lizardo *et al.*, 1995), see also section 1.5.7.1.6. For example, Jansman *et al.* (1993) fed different varieties of field beans with different tannin content (ranging from 0.57 g/kg to 1.57 g/kg). They found the apparent ileal and faecal digestibility of protein was better for the control diet than the field beans containing a high (1.52 to 1.55 g/kg) or medium (1.19 g/kg) level of tannins.

In this experiment, there was a tendency for the pigs fed the hop diets to have looser faeces on day 25 ($P = 0.089$). This could be due to the significant lower number of lactic acid bacteria in the faeces for the hops treatment ($P = 0.001$). Similar results were found in experiment 2 (see chapter 5). Lactic acid bacteria normally enhance growth and health of animals and maintain normal intestinal microflora by suppressing the *E. coli* (Ahn *et al.*, 2002; Snel *et al.*, 2002) (see also section 1.3.1.5). The number of lactic acid bacteria was slightly lower than those reported by Durmic *et al.* (1998) for healthy pigs (9.06 to 9.36 log10 cfu/g) in the colon and slightly lower in faeces than results reported by White *et al.* (2002) (8.86 to 9.72 log 10 cfu) and within the number of ileal *Lactobacillus* (6.51 to 8.93 log 10 c.f.u.) reported by Mathew *et al.* (1997). It does not seem to be due to *E. coli* induced diarrhoea, as the number of *E. coli* was unaffected. The numbers of *E. coli* were similar to those found in experiment 2. This contradicts the *in vitro* experiment, where the number of K88 was reduced by hops and hop compounds.

The impact of the lower number of *Bacteriodes* in the treatments containing the hops or the iso-α and β-mixture is not known. *Bacteriodes* spp. are part of the anaerobic gut flora in

the healthy pig (Thomke and Elwinger, 1998c) and certain strains are listed as safe for incorporation in animal feeds (Li *et al.*, 2003). The numbers of *Bacteriodes* were similar to the ones found in the previous experiment (chapter 5). However, in experiment 2 the hops did not reduce the number of *Bacteriodes.* The number of *Bacxteriodes* were within the ranges reported by Conway (1994) (between 0 and 8.6 log10 cfu/g in colon and 0 and 10.5 log10 cfu/g in faeces of piglets aged one to eight weeks).

The number of *Clostridium* were similar to those reported in the second experiment chapter 5) and the numbers are also within the ranges reported by Conway (1994) and White *et al.* (2002). The trend for a lower number of *Clostridium* in the faeces on day 11 in the hops and hop extract treatments did not continue beyond that day and was therefore not thought to influence the performance or other parameters.

A high concentration of VFA may reduce the pH of the colonic lumen and reduce opportunistic bacteria, thereby preventing diarrhoea (see also literature review, section 1.4.1.1) (Rowland, 1992; Mathew *et al.*, 1996; Topping *et al.*, 1997). The total amount of VFA in the caecum was higher than what was found in the second experiment (chapter 5). In fact, the main ones (acetic, propionic and butyric) were between 3 to 17 mmol/l higher. Borg Jensen (2001) evaluated different experiments using different methods i.e. the *in vivo – in vitro* and absorption in the portal vein. Their reported values for acetic, propionic, butyric and valeric acid were close to the ones found in this experiment. The pH in the caecum and colon decreases as a result of the fermentation process (Bach Knudsen *et al.*, 1991). The higher VFA values in the caecum due to the organic acid treatment did not seem to influence the level of potential pathogenic bacteria or lower the pH in the caecum.

The villous height and the crypt depth were not influenced by hops and hops compounds in this experiment indicating that the dietary treatments did not influence the enzyme activity

and the absorptive capacity of mucuous membranes (see section 1.3.3). The results for the villous height was slightly higher than the averages of 400 and 550 μm reported by van der Klis and Jansman (2002). This indicates a healthy gut and that the pigs eat, as a positive relationship between the feed intake and villous height has been found (Pluske *et al.*, 1996b). No other experiment was found where they looked at the same type of cells. However, Hampson (1986a) looked at epithelial cells on the column of the villous and crypt in weaned pigs between 22 and 26 days old. Their numbers ranged between 162 and 123 for the villous and the crypt, which is slightly higher than the numbers found in this experiment.

This experiment showed that the hop products iso-α and β-mixture could be the compounds in hops responsible for the improved performance of piglets 14 to 21 days post weaning, as the pigs on those two treatments had better performance than the control group. The physiology behind this could not be explained purely by improved digestibility of nutrients, as only iso-α and β-mixture improved the digestibility of fat and ash, but the hops did not. The digestive enzymes were lower in the hops and isolated hop compounds treatments and it could be speculated if a complex is formed between the tannins in the hops and the trypsin and lipase making the protein in the feed less available to the animals. The antimicrobial effects of hops did not seem to influence the gut microflora in the pigs. Hence the improved feed utilisation may not be due to a reduction in pathogenic bacteria. The addition of acids to the diets did not improve performance nor did it prevent the reduction in lactic acid bacteria. The only positive response of the acids was the higher level of VFA in the caecum.

The DLWG and the FCR was improved by the hops and the hop products in the period 14 to 21 days. The number of lactic acid bateria were significantly reduced by the hops and to a lesser extend by the hop products, similarly the number of *Bacteriodes* were reduced by

the hops and the hop products. The digestibility of DM, energy and protein were reduced for the hops and hop products, only a tendendcy for improved fat digestibility was seen. The addition of an organic acid mixture did only significantly affect the level of VFA in the caecum.

7. GENERAL DISCUSSION

7.1 The ban of AGP

The ban of AGP in animal feed in the EU has resulted in an increased interest in other alternatives which can minimise the detrimental effects on animal performance and potential economical losses. Herbal additives and spices have received much interest as they are "natural" products, some of which have been exploited in humans and animals for centuries, even millennia. Herbs and botanicals may improve feed intake and digestive secretions, immune stimulation, antimutagenic, anticarcinogenic, anti-microbial, antihelmintic, antiviral, protein digestion, coccidiostatic, anti-inflammatory effects and antioxidant activity (see also literature review, section 1.5.7).

The literature review highlighted the problems associated with the weaning of pigs and the physiology behind it, with links to the potential effects of different growth promoters. It also highlighted a great variability in the responses, the use of and results obtained with herbal additives, mainly due to variation in composition of the plants and their different components and the lack of knowledge regarding active components within the plants. This is also seen with other growth promoting agents and can be influenced by high health status, good performance, nature of diet and the environment (Pierce *et al.*, 2005a). The performance, general health and the digestibility of the piglets was considered good in all the experiments undertaken, and this may explain the little response to the hops, the isolated hop compounds, silymarin and the acids. Little or no response has been observed by other workers under these circumstances, for example Pierce *et al.* (2005a). In the first experiment it was hypothesised that lowering the density of the diets would improve the action of the herbs. However, no differences were seen from "diluting" the diets with 100 g/kg oathulls.

The first experiment tested two different herbal additives, hops and silymarin, with different modes of action and demonstrated that they both resulted in better feed utilisation than the control diet from day 11 to 28, in particular the last week of the experiment. It was postulated that the beneficial effects of the hops treatments were due to their antimicrobial properties and the silymarin due to its antioxidant properties.

7.2 Effects of silymarin and hops on liver function

Silymarin is normally used to treat liver disorders in humans, but it failed to improve the level of liver enzymes in experiment 1, although there were no liver problems to improve (section 3.4.4). The results from human medicine with silymarin are also variable (see literature review section 1.6.1).

Interestingly, the hops lowered AST and ALT in the first experiment. The effect of hops on the liver seems to be an area which had not received much attention, the few studies found, looking at the effects of hops on the liver function, showed that hops had no effect on the liver, on cytochrome P-450 enzyme and on rat hepatocytes (Shipp *et al.*, 1994; Rodriguez *et al.*, 2001). Comparison of experiment 1, 2 and 3 showed that the results with the liver enzymes were inconsistent. In the first experiment the inclusion of 1 kg/t of hops reduced the level of liver enzymes, but in the following experiments with higher inclusion levels of hops, no effects on the liver enzymes were seen. It appears that the improved performance of hops/hop compounds is not related to the improved liver function. Table 7.1 summarises the effects of hops, silymarin and isolated hop compounds on performance, liver enzymes, bacteria in the faeces, colon and small intestine, VFA and digestibility in pigs in experiment 1, 2 and 3.

Table 7.1: A summary of the effects of hops (1 kg/t and 10 kg/t), isolated hop compounds (α & β) and silymarin on performance, liver enzymes, bacteria, VFA and digestion in weaner pigs compared to the control diets in the different experiments (exp) (% change compared to control).

Measure (day)	Exp.	Treatment				Reference	*P-value*
		1 kg/t	10 kg/t	α & β	Sily-marin		
Performance							
FCR (21-28)	1	↓ 9.6			↓ 15.8	Table 3.4	0.014
FCR (11-28)	1	↓ 6.3			↓ 7.5	Table 3.4	0.056
FCR (22-26)	2	↓ 6.4	↓ 17.0			Table 5.2	0.094
FCR (14-21)	3		↓ 8.3	↓ 9.2		Table 6.5	0.027
DLWG (14-21)	3		↑ 9.6	↑ 9.8		Table 6.3	0.055
Liver							
ALT (28)	1	↓ 8.5			↑ 2.8	Table 3.15	0.068
AST (28)	1	↓ 21.6			↑ 9.7	Table 3.17	0.011
T. bilirubin (26)	2	↓ 6.0	↓ 23.8			Table 5.3	0.093
Liver weight (26)	2	↑ 1.7	↑ 10.4			Table 5.3	0.061
Bacteria in the							
LAB faeces (11)	2	↓ 4.3	↓ 12.3			Table 5.6	0.006
LAB faeces (26)	2	↓ 0.5	↓ 12.5			Table 5.6	0.021
LAB:*E. coli* faeces (11)	2	↓ 8.3	↓ 18.3			Table 5.7	<.001
LAB:*E. coli* faeces (26)	2	↑ 2.9	↓ 17.1			Table 5.7	0.04
LAB:*E. coli* colon	2	↑ 0.98	↓ 15.5			Table 5.7	0.05
LAB faeces(11)	3		↓ 7.7	↓ 5.6		Table 6.11	0.058
LAB faeces (25)	3		↓ 14.3	↓ 4.8		Table 6.11	0.001
LAB SI	3		↓ 8.2	↓ 3.0		Table 6.11	0.013
LAB colon	3		↓ 7.5	↓ 3.3		Table 6.11	0.064
LAB:*E. coli* faeces (25)	3		↓ 17.7	↓ 8.9		Table 6.13	0.002
Bacteriodes faeces (11)	3		↓ 9.6	↓ 14.8		Table 6.16	<.001
Bacteriodes faeces (25)	3		↓ 6.3	↓ 8.3		Table 6.16	0.003
Bacteriodes SI	3		↑ 3.3	↓ 9.2		Table 6.16	0.011
Bacteriodes colon	3		↓11.8	↓ 15.1		Table 6.16	<.001
VFA							
Total colon	2	↑ 7.1	↑ 29.1			Table 5.14	0.034
Propionic caecum	3		↑ 1.9	↑23.3		Table 6.19	0.020
Digestion							
Trypsin (26)	3		↓ 10.4	↓ 34.0		Table 6.26	0.077
Energy digestibility	3		↓ 1.2	↓ 0.2		Table 6.24	0.002
Fat digestibility	3		↓ 0.3	↑ 0.9		Table 6.24	0.058
Protein digestibility	3		↓ 1.8	↓ 0.3		Table 6.24	0.009

Although, the results were variable in experiment 1, 2 and 3, all the experiments indicated that hops have the ability to improve the feed utilisation (see also table 7.1). Cornelison *et al.* (2006) similarly found that hops improved the performance of broilers at all tested

inclusion rates (0.5, 1.0, 1.5 and 2.0 lb/t) compared to a negative control. However, they found that with penicillin at 50 g/t the broilers performed better than with the hops, indicating that although hops improve performance, they are not quite as efficient as penicillin. At an inclusion rate of 1 kg/t the hops resulted in an improved FCR by 9.6% (experiment 1, section 3.4.1). In the second experiment day 22 to 26 the 10 kg/t of hops resulted in a 17% improved FCR (P = 0.094) compared to control, this was seen in the last experiment as well, where the same amount of hops also resulted in improved FCR by an average of 8.3% for the hops day 14 to 21 (P = 0.027). It has to be taken into consideration that in the first experiment the conditions were different than in the other two. The pigs were group housed in pens of four with *ad lib* feed intake, whereas in the second and third experiment the pigs were individually housed with restricted feed intake. A difference in feed intake has been observed in group housed and individually housed pigs, mainly due to social interaction in the group housed pigs (Bruininx *et al.*, 2001). A higher intake would be expected in *ad libitum* fed piglets, and therefore also a potential higher FCR. The difference in conditions did not seem to greatly affect the results.

7.3 Antibacterial effects of hops *in vitro*

The *in vitro* experiments were designed to test the hypothesis that the improved FCR seen in the first experiment was due to the antibacterial properties of hops and some of their isolated compounds, as there are many controversies regarding the antimicrobial activity of hops. The hops reduced the number of some of the bacteria tested, the results were variable. However, other workers have shown some bacteria to have resistance to some of the compounds in hops. Fernandez and Simpson (1993) and Simpson and Fernandez, (1994) investigated the resistance of beer spoiling organisms to hops by measuring the minimum inhibitory concentrations for *trans*-isohumulone, humulone, colupulone and other antibacterial agents. They found that *trans*-humulone –sensitive organisms were also

sensitive to humulone and colupulone and those resistant to *trans*-humulone were also resistant to humulone and colupulone.

7.4 Antibacterial effects of hops *in vivo*

The results found *in vitro* are often different in animals as many other factors influence the activity of the herbs. Within the animal there is more competition with the other major nutrients in the gastrointestinal tract whereas *in vitro* there is less competition (Wenk, 2003; Hernandez *et al.*, 2004). There are several possible explanations for the resistant organisms. Firstly, cells may prevent the antibacterial agent from reaching the target site. Secondly, the cells might inactivate the antibacterial agent by enzymatic modification. Thirdly, the cells may grow in the presence of the antibacterial agent by responding in a way that overcomes its effects. Fourthly, the cells might dispense with the function that is affected by antibacterial agent (Simpson and Fernandez, 1994).

The effect of hops and isolated hop compounds were tested *in vivo*, to see if the bacteria generally living in the gut, such as *E.coli, Lactobacilli* and *Streptococci*, were also affected in the small intestine, colon and faeces of the piglets. The treatment with the iso α-acids and β-acids resulted in a better feed utilisation than the control, by 9.2% as well as daily weight gain, which could mean that these compounds are the ones responsible for the improved FCR in all the experiments. The consistent results from the analysis of the bacteria in the faeces and the gastrointestinal tract indicate that it is not a reduction in potential pathogenic bacteria which is responsible for the improved performance. A higher concentration of hops resulted in a consistent lower level of lactic acid bacteria, the other bacteria were unaffected. Similarly, in experiment 3, where isolated hop compounds were tested in combination with an organic acid mixture, to try and provide a more suitable environment for the lactic acid bacteria to survive, the number of lactic acid bacteria was reduced both by the isolated compounds and the hops, and no difference was seen from the

addition of organic acids. *In vitro* experiment have shown that the growth of lactic acid bacteria is only inhibited when the pH is reduced below 4 (Tannock, 1990). *Lactobacilli* are important to maintain a good intestinal health and balance, as they are able to control potential pathogenic bacteria such as pathogenic *E. coli* (Manzanilla *et al.*, 2004) by producing organic acid, hydrogen peroxide, carbon dioxide, bacteriocins and other metabolites (Tannock, 1990; Ahn *et al.*, 2002). However, the reduction in lactic acid bacteria did not seem to have an adverse effect on the pigs in any way, which may be due to the good health status of the pigs, as they still had improved FCR. The decrease in the number of lactic acid bacteria did not result in an increased in the number of *E. coli*, and the hops or hop compounds did not succeed to reduce the *E. coli*.

7.5 Effects of organic acids

In contradiction to the hypothesis the addition of acids did not further improve the performance of the piglets (see section 6.4). The acids had no effects on most of the parameters measured, apart from an improved fat digestion and 18% higher level of VFA in the caecum. It was the control and the isolated hop compounds that exhibited the increase in the level of total VFA in the caecum. Manzanilla et al. (2004) also found that the addition of formic acid to the herbal mixture XT (see literature review section 1.5.7) did not reduce the number of enterococcobacteria further and neither did it increase the number of lactobacilli or change the ratio between the two of them.

7.6 The effects of hops on the hindgut fermentation

The level of VFA in the colon did increase linearly with the amount of hops in the second experiment, however this was not seen in the third experiment. It could be speculated that the higher level of VFA contributed to the energy supply to the pig and thereby contributed to the improvement in performance, as identified in the review (section 1.4.1.1) that VFA supply energy to the pigs. This would allow them to utilise otherwise undigested

components of the feed. However, in the last experiment the level of VFA in the colon did not significantly differ between the treatments.

In the last experiment the isolated hop compounds resulted in a lower number of *Bacteriodes* with the hops and iso-α and β-mixture, but the effects within the piglets of this was not known. *Bacteriodes* spp. are part of the anaerobic gut flora in the healthy pig (Thomke and Elwinger, 1998c). They are therefore regarded as harmless bacteria, they may however influence the hindgut fermentation.

7.7 Effects of hops on digestion and digestive enzymes

The elimination of an antibacterial effect and improved liver function lead to the hypothesis that hops/isolated hop compounds may affect the digestion of the piglets. The digestive enzymes and bile acids were not significantly affected by the hops/isolated hop compounds, but they had great variability, which makes statistical differences difficult to achieve. However, the digestibility of the nutrients was affected. The fat digestibility was improved for the isolated hop compounds, but not for the hops, which suggests that the mechanism by which hops improved the performance is different from the isolated hop compounds. The fat digestion can also be influenced by the amount of bile secreted. Many spices have shown to enhance the bile secretion and thereby improve the fat digestion (Srinivasan, 2005). However, the hops or hop compounds showed no effect on the level of bile acids in the intestinal digesta. Platel *et al.* (2002) studied the effects of feeding different spices mixtures (including coriander, red and black pepper, tumeric, cumin, ginger, onion, mustard, fenugreek, cinnamon, clove and bay leaves) to rats and their effects on bile acid concentration in the bile. They showed that the bile flow rate was increased by the spice mixtures and the bile secretion as well.

7.8 Tannins in hops

The energy digestibility was better for the isolated hop compounds and the control, and again lower for the hops. Similarly the protein digestibility was lowest for the hops, therefore it appears that hops did not improve digestibility. This could be due to the amount of tannins in hops, which in the isolated hop compounds are assumed not to be present. Tannins and proteins interact through hydrogen and hydrophobic bonds (Mueller-Harvey, 1999). Tannins form complexes with proteins, either by binding to the digestive enzymes (e.g. trypsin) or a substrate such as dietary protein and rendering them unavailable to the host. This has been reported in rabbits, pigs and broilers (Cowan, 1999; Singh *et al.*, 2003; Mariscal-Landin *et al.*, 2004). Inclusion levels of 0.6 g/kg, tannins have been reported to form tannin-protein soluble and insoluble complexes in the grain or digestive tract, which may result in a lower digestibility of protein, energy and starch in various species, such as weaner pigs, chickens and rabbits. They may also reduce the level of trypsin and α-amylase (Longstaff and McNab, 1991; Jansman *et al.*, 1993; Al-Mamary *et al.*, 2001).

However, if tannins present in the hops adversely affected the digestibility, it would have been expected that the performance also was adversely affected, which was not the case any of the experiments. Several experiments, such as Longstaff and McNab (1991) (table 1.25, section 1.5.7.1.6) have shown that a certain level of tannins can be tolerated before a negative effect is observed (Vernon, 1999).

7.9 Future research

Other components in hops such as flavonoids are effective antioxidants, and they have strong free radical scavenging activities (Rodriguez *et al.*, 2001; Stevens *et al.*, 2002). This may affect the performance of pigs fed hops and would mean further research in other areas such as antioxidant status in the piglets.

This study comprised of identifying herbs with potential beneficial effects, testing them in piglets and trying to investigate their potential mode of action. The results with silymarin from the first experiment (chapter 3) showed that silymarin improved the FCR, but no other beneficial effects were seen in the piglets, and it was decided to discontinue studying the effects of silymarin. Furthermore, as described in the literature review (section 1.6.1.3) the results with feeding silymarin to different animal species has given variable results, such as a lower feed intake in mice.

The hops results in an improvement in the FCR in all three experiments.The next stage would be to test the hops/isolated compounds in commercial conditions to see if the performance is improved in these kind of conditions. It would be interesting to know if the improvement in performance continues after the 28 days and whether the hops/hop compounds have beneficial effects in grower/finisher pigs as well. Furthermore, it would be interesting to see what effects the hops would have on pigs with poorer health status.

Assuming an average improvement in FCR with the hops/hop compounds of 0.15 units up to 35 kg (the rearing period) could be achieved, taking the average BPEX figures for compound fed pigs of 36.51 p/kg produced (BPEX, 2005), with the improvement of 0.15 the cost would be 33.46 p/kg, saving 3 p/kg. Further studies that give greater understanding of the mechanisms by which hops and their isolated compounds act within the piglets, and their interactions with diet composition are required. Finally, it would be interesting to test the hops/isolated hop compounds in combination with other alternatives, particularly probiotics would be interesting to see if the lactic acid bacteria could be increased and further improvements would be seen.

CONCLUSIONS

Two different herbal additives, silymarin and hops, were tested in weaned piglets. In one experiment, silymarin resulted in improved feed utilisation, but no other benefits were seen, and it was decided to discontinue with silymarin.

It was instead decided to focus on hops. The inclusion of hops in piglet diets at different levels consistently improved the FCR in three out of three experiments after two weeks of feeding and there was a tendency for improved DLWG in one experiment. At a higher inclusion rate of hops, a better performance was found. This could in commercial conditions result in economical benefits.

In order to identify the active ingredients in hops, the isolated compounds iso-α- acid and β-acid from hops were tested *in vitro* and in piglets, they also improved the FCR and the DLWG.

To elucidate the potential mechanism of hops and the iso-α extract and β-extract both *in vitro* and *in vivo* experiments were carried out. *In vitro* both the hops and the iso-α extract and β-extract showed antibacterial effects against a range of both gram positive and gram negative bacteria, whereas *in vivo* a consistent reduction of lactic acid bacteria was seen, and in one out of two experiment a lower level of *Bacteriodes* was seen. This lead to the conclusion that it was not due to their antimicrobial activity that hops and iso-α extract and β-extract improved the performance of piglets.

Hops increased the level of VFA in the colon in one experiment, but not in the other, and the iso-α extract and β-extract had no effects on the level of VFA in the colon and caecum, so this was not the reason for the improved performance.

No beneficial effects were seen on liver enzymes, gut morphology, digestibility and digestive enzymes, only a negative effect on energy digestibility and protein digestibility with the hops and -α extract and β-extract.

The interaction with other compounds was also tested. "Dilution" of diets with oat hulls did not result in more significant improvements of neither the silymarin nor the hops. No additional improvement in performance was seen when an organic acid mixture was added in combination with hops or iso-α extract and β-extract.

Therefore, further investigations are required to determine the mechanism by which the performance is improved by the hops and the iso-α extract and β-extract and also to identify the optimal inclusion rate of the iso-α extract and β-extract and the hops for pigs and their potential synergistic effects with other additives.

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APPENDIX I

The effect of different inclusion levels of hops on the palatability of the diet

Prior to experiment 2 a small test was carried out to see at which concentration the hops caused palatability problems in piglets.

Objectives

The objectives of this study were to investigate the palatability of hops at three different inclusion levels in diets for weaner pigs.

Treatments

Standard weaner pig diets in meal form. Hops at three different inclusion levels 1 kg/t, 5 kg/t and 10 kg/t were mixed a commercial pig diet.

Trial procedures

At weaning the 24 piglets were randomly allocated into six pens (4 pigs/pen) with four pigs in each, giving two pens of each treatment. The pigs were fed a commercial diet for five days (or 7 for group 2), and then the diets were changed to the hop diets.

There were two groups of pigs with three pens in each. In the first one the intake of hops was measured from day 6-8 and for the second one (three pens) the intake was measured from day 8 to 11.

Recordings

The liveweight of the pigs was measured at weaning, and on day six and eight for group 1 and on day eight and eleven for group 2.

The feed intake was measured at the same time as the weighing of the pigs.

Results

There was not sufficient number of replicates for statistical analysis, as it was only a test. The results are shown in table I.

Table I: The effects of different concentrations of hops on the palatability of weaner pigs

	Group 1			Group 2		
	1 kg/t	**5 kg/t**	**10 kg/t**	**1 kg/t**	**5 kg/t**	**10 kg/t**
DLWG (g/day)						
Day 6-8	350	388	195			
Day 8-11				242	232	232
Feed intake (kg/pen/day)						
Day 6-7	1.06	1.66	0.90			
Day 7-8	1.60	1.44	1.02			
Day 8-9				1.34	1.38	1.22
Day 9-11				1.90	1.76	1.98
FCR						
Day 6-8	0.95	1.00	1.23			
Day 8-11				1.34	1.35	1.09

An inclusion level of 10 kg/t was found not to affect the intake, so it was decided to include that amount of hops as in the second pig experiment (chapter 5).