



**The use of bioactive forages towards organic/sustainable
control of gastrointestinal parasites in sheep**

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Declaration

I hereby declare that the work presented in this thesis is the product of my own efforts, and has not been submitted in any previous application for a degree. The work on which it is based is my own except where stated in the text and in acknowledgement section.

Ouranios Tzamaloukas

*To my parents, Othona and Alexandra,
and my brothers, Kosta and Foti*

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Abstract

The aim of this thesis was to investigate the potential use of bioactive plants as alternative grazing forages to control gastrointestinal parasites of growing lambs. For this purpose four different grazing experiments were conducted using the following bioactive forages: *Lotus pedunculatus* (lotus), *Onobrychis viciifolia* (sainfoin), *Hedysarum coronarium* (sulla), and *Cichorium intybus* (chicory); the forages were tested against two parasitic ovine nematode species, *Teladorsagia circumcincta* and *Trichostrongylus colubriformis*. The first two experiments tested the effects of short-term grazing on bioactive forages against the viability and fecundity of adult, and the establishment and development of larval nematodes of *T. colubriformis* (Chapter Two) and *T. circumcincta* (Chapter Three) species. In the first experiment, grazing on bioactive forages showed no evidence of forage effects upon incoming *T. colubriformis* larvae or against adult *T. colubriformis* worms during a two-week period. In contrast, the results from the second experiment showed significant reductions in adult *T. circumcincta* worm burdens of lambs grazing chicory for a two-week period compared to lambs grazing grass/clover (*Lolium perenne*/*Trifolium repens*). Immature worm burdens were affected by physiologically and/or immunologically mediated mechanisms, which reduced larval establishment in all treatments. The following experiment (Chapter Four) aimed to investigate the effects of sequential grazing on bioactive forages, firstly on chicory followed by sulla, towards an established adult *T. circumcincta* worm population of growing lambs. The results showed that grazing on chicory reduced the faecal egg counts (FEC) of infected lambs, whilst the following grazing on sulla provided little evidence of additional benefits. During this grazing period, evidence of emergence of immunity, declining FEC and low worm establishment rate, were observed across all treatments. The last experiment (Chapter Five) aimed to separate the direct (anthelmintic) and indirect (immunological/physiological) mediated effects of bioactive forages and investigate their effects on acquired immunity against the abomasal nematode *T. circumcincta*. The results showed elevated immune responses, in terms of nematode larval development and mucosal cell counts, of growing lambs grazing on either sulla or chicory compared to those grazing on grass/clover. It is therefore suggested that the effects of these two bioactive forages are due to a combination of their direct and indirect properties. The potential application of bioactive forages as an alternative to control gastrointestinal parasitic infections in organic/sustainable sheep production systems is considered in the General Discussion (Chapter Six).

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List of abbreviations

Condensed tannins	<i>CT</i>
Crude protein	<i>CP</i>
Dry matter	<i>DM</i>
Eosinophils	<i>EOS</i>
Faecal output	<i>FO</i>
Fecal egg counts	<i>FEC</i>
Globule leukocytes	<i>GL</i>
Herbage intake	<i>HI</i>
In vitro dry matter digestibility	<i>IVDMD</i>
Metabolisable energy	<i>ME</i>
Metabolisable protein	<i>MP</i>
Mucosal mast cells	<i>MMC</i>
Neutral cellulase and gammanase digestibility	<i>NCGD</i>
Neutral detergent fibre	<i>NDF</i>
Periparturient relaxation of immunity	<i>PPRI</i>
Plant secondary metabolites	<i>PSM</i>
Sheep mast cell proteases	<i>SMCP</i>
Standard error of the mean	<i>SEM</i>
Total phenolics	<i>TP</i>
Water soluble carbohydrate	<i>WSC</i>

Chapter One

General Introduction

1.1 Introduction

Parasitic gastroenteritis, induced by nematodes, is one of the main sources of economic loss in small ruminant production systems in both temperate and tropical regions (Waller, 1997; Coop and Jackson, 2000). The control of this disease has been successful over the past 30 years or so, using regularly, highly effective doses of anthelmintic drugs (Radostits, et al., 2000). However, the emerging, widespread development of anthelmintic resistance, i.e. the heritable ability of the parasite to tolerate a normally effective dose of the anthelmintic, renders such control method unsustainable and threatens the economic viability of small ruminant production worldwide (Jackson and Coop, 2000; Sangster and Dobson, 2002; Abbott et al. 2004). There is, therefore, the need to develop alternative non-chemical control measurements, which are also imposed by the regulated requirements of special low-input farming systems, such as the organic production, which restricts the use of prophylactic chemotherapy in livestock farming practices (UKROFS, 2001). Promising alternative control measures that are currently being investigated, include vaccine development (Smith, 1999; Knox, 2000), selective breeding (Stear et al., 1997, 2001; Bishop et al., 2004), biological control using nematophagous fungi (Githigia et al., 1997; Larsen, 1999; Chandrawathani et al., 2004), and the use of nutrition, either through supplementation strategies (Coop and Kyriazakis, 1999, 2001) or using special forages that may have biological activity against nematodes (bioactive forages). The latter alternative control method is the focus of the present thesis.

1.2 Gastrointestinal nematodes

This section will focus on the sheep gastrointestinal nematode species *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (trichostrongylidae family), which are the main species responsible for parasitic gastro-enteritis in temperate climates (Coop and Jackson, 2000; Abbott et al., 2004), and have been used throughout the experiments of the present thesis.

1.2.1 Life cycle

Nematode species *T. circumcincta* and *T. colubriformis* have simple direct life cycles (i.e. there is no intermediate host). Adult female worms in the gastrointestinal tract of the sheep lay eggs that pass out of the host with the faeces. Eggs, under suitable environmental conditions, hatch within the faecal pat to produce the free-living first larval stage (L1). The L1 develop and undergo a moult to the second stage larvae L2. The L1 and L2 are active and feed on bacteria in the faeces. L2 moult to the infective third larval stage (L3). The time taken to hatch and develop to infective larvae may be as early as 14 days and is related to the microclimatological conditions. L3 retain the shed cuticle from the previous moult (L2), which remains as a sheath protecting but also preventing them from feeding (Urquhart et al. 1996). Ensheathed L3 migrate on the herbage, where they can be ingested by sheep. Within minutes or few hours after ingestion most of the ensheathed L3 larvae lose their sheath (exsheathment), a fact that marks the transition from the free-living to parasitic phase, where the larvae start to feed from the host (Sommerville et al., 1953, 1954a,b; Hertzberg et al., 2002). Exsheathment takes place in the gastrointestinal part immediately anterior to the habitat of the adult parasite (Sommerville et al., 1953; Rogers and Sommerville 1957; Hertzberg et al., 2002). Thus, exsheathment of *T. circumcincta* L3 larvae occurs quickly, after the larvae reach rumen fluid, while most of the larval *T. colubriformis* L3 stages exsheath in the abomasum (Sommerville, 1954b; Hertzberg et al., 2002).

After exsheathment, *T. circumcincta* L3 larvae move to the abomasum and invade the abomasal gastric glands, where they develop and moult two more times before emerging from the gland as adult (L5) parasite, which may be as early as 8 days post infection (Armour et al., 1966, Denham, 1969). The adult worms develop further and reside in the lumen and close to the abomasal mucosal surface (Armour et al., 1966). Similarly, *T. colubriformis* exsheathed L3 penetrate intestinal walls and undergo a third moult to L4. Developing larvae coil in the gastric

pit, which distends due to larval growth. Adult *T. colubriformis* emerge into the lumen and remain in close association with the intestinal mucosa (Soulsby, 1982; Coop and Jackson, 2000). Sexually mature adult worms may then copulate and female worms lay eggs, which are deposited on the pastures. The time from ingestion of larvae to appearance of egg laying females (prepatent period) is normally about 16 to 21 days for both species (Radostitis, et al., 2000; Abbott et al., 2004). This period may become extended, due to an adaptation mechanism that nematodes have developed to survive adverse pasture conditions, delaying development and emergence from the mucosa (hypobiosis) for weeks or months (Soulsby, 1982; Urquhart et al. 1996; Eysker, 1997).

1.2.2 Epidemiology

The epidemiology of the infection and the importance of nematode species vary with locality and season (Armour, 1980). In the area where the experiments of the present thesis took place (Southeast Scotland), *Teladorsagia* and *Trichostrongylus spp* infestation follows the typical pattern of nematode infection in cool temperate regions. This includes a rise in pasture larval availability in early summer, a peak in pasture contamination by larvae in mid-summer or early autumn and a progressive reduction in larval numbers in pastures during winter (Armour, 1980; Abbott et al., 2004). Rainfall and temperature influence the infectivity of pastures. Egg hatching and rate of larval development to the infective stage (L3) are dependent on ambient temperature, being optimal between 18 to 26°C, while rain assists the movement of L3 out of the faecal pats and migration onto the herbage (Urquhart et al., 1996; Stromberg, 1997).

Possible sources of infection by parasitic L3 larvae in sheep are: (i) the over-wintering larvae on pastures, (ii) the hypobiotic larvae that resume their development within the host, and (iii) the larvae deriving from eggs shed by ewes during the periparturient relaxation of immunity (PPRI) (Michel, 1969, 1974; Eysker, 1978; Armour, 1986). In late winter or early spring, over-

wintering larvae could be a major source of pasture contamination, which, however, do not survive long on pastures after the rise of environmental temperatures in late spring or early summer (Michel, 1969; Stromberg, 1997). At the start of grazing season, the major source of pasture contamination is the eggs shed by the hypobiotic larvae that have resumed their development (Urquhart et al., 1996; Coop and Jackson, 2000; Abbott et al., 2004).

The cessation of nematode growth in the early parasitic development, L4 stages for *Teladorsagia* and L3 for *Trichostrongylus* spp (Eysker, 1978), is a well-known adaptation phenomenon for ovine parasitic nematodes, called hypobiosis, and occurs predominately during the unfavourable environmental conditions for the survival of the free-living stages (Michel, 1974; Eysker, 1978; Armour, 1980, 1986). Another possible cause of hypobiosis, which may affect epidemiology to a greater extent than the environmental stimuli, is the development of immunity (Eysker 1997). The arrested development in immature worm stages is associated with the protection of larvae from hostile, immunologically affected, gastrointestinal tract. Thus, the arrested larvae provide a reservoir for the replacement of ageing or expelled adults, resuming their development and maintaining an adult worm population in the host (Michel, 1974; Balic et al., 2000). Although highly immune animals can overcome this barrier and eliminate the total worm population (Eysker, 1997), in natural infections by gastrointestinal nematodes, where there are different degrees of immunity within groups, hypobiosis is a successful survival strategy.

The final source of pasture contamination, and possibly the most important, is the eggs shed early in spring by ewes during periparturient relaxation of immunity (PPRI) (Michel 1969; Gibbs and Barger 1986; Gibbs, 1986). The PPRI in ewes, i.e. the loss of previously acquired immunity against gastrointestinal nematodes, occurs before or at the time of parturition and reaches a peak six to eight weeks after lambing. This phenomenon contributes greatly to

nematode survival/epidemiology, and among the various hypotheses that have been suggested the most likely is that PPRI has a nutritional background (Coop and Kyriazakis, 2001, Houdijk et al., 2003). A large body of evidence supports the view that the relaxation of ewe immunity may result from prioritised scarce nutrients, in particular metabolisable protein (MP), towards reproductive functions rather than immune functions (Houdijk et al., 2000, 2001a,b,c, 2005, 2006).

In conclusion, nematode epidemiology is a complex phenomenon with specific characteristics that have to be taken into account when designing alternative control management measures. Thus, the seasonal rise in pasture infectivity suggests that future control methods should focus on specific grazing periods, the significance of hypobiotic larvae highlights the need for effective control measures against both adult and immature parasitic stages, whilst the possible nutritional basis of the PPRI points towards alternative methods that may well improve the nutritional status of the host. The latter will further be developed in the following section where evidence suggests that nematode pathogenicity and impaired animal productivity are both connected with alterations in the nutritional status of the host.

1.2.3 Pathogenicity - productivity

Abomasal *T. circumcincta* infections cause extensive pathological and biochemical changes, which result in impaired nutrient metabolism and manifest when adult worms start to emerge from the distended gastric glands as early as 8 days post infection (Armour et al., 1966, Denham, 1969). This emergence triggers the hyperplastic reaction in neighbouring glands and surrounding cells, which lose their differentiation and functionality. The reduced functionality of parietal cells leads to reduced gastric acid secretion and increased gastric pH, which may rise to 6-7 (Lawton et al., 1996; Fox, 1997). The elevated gastric pH and the hyperplastic mucosal reaction induces other pathophysiological changes, which include increased circulating

pepsinogen and gastrin levels, reduced conversion of pepsinogen to pepsin, reduction in abomasal protein digestion, permeability of the abomasal epithelial sheet and protein loss in the gastric lumen (Holmes, 1985; Gruner et al., 1994; Fox, 1997; Scott et al., 1998; Sykes and Greer, 2003).

Intestinal *T. colubriformis* infections are associated with alterations of the mucosa such as inflammatory reactions, stunting or flattening of the intestinal villi and reduced epithelial enzyme activity. Lesions are confined in the anterior small intestine, while larval *T. colubriformis* localized intraepithelially and adult are found loosely associated with the surface mucus causing damage and sloughing of the epithelial cell layers, leakage of plasma and extracellular fluids and increasing mucus production (Holmes and Coop, 1994; Fox, 1997). Disrupted nutrient absorption and utilization, in particular of protein, is also observed during this parasitic infection (Poppi et al., 1981, 1985, 1986; Bown et al., 1989, 1991).

Although each trichostrongylid species differs in the habitat that it occupies and consequently in the damage and the disease that it causes, infections are commonly characterised by similar clinical signs, such as anorexia, diarrhoea and production deficits (MacRae, 1993; Sykes, 1994; Coop and Holmes, 1996; Fox, 1997). Anorexia, i.e. the depression of voluntary food intake, is a constant feature of both intestinal and abomasal parasitic infection and may account for over 60% of the differences in weight gain between infected and worm-free sheep (Sykes and Coop 1977; Coop and Kyriazakis, 1999). Significantly depressed appetite has been observed even in parasitised animals showing no clinical signs (Kimambo et al., 1988; Holmes and Coop, 1994) suggesting that anorexia is an important contributor to the reduced performance in both clinical and subclinical infections. Pair-feeding studies have established that anorexia is not the only cause of production loss (Sykes and Coop, 1976) and may be accompanied by changes in animal metabolism, which can

also account for impaired productivity (Fox, 1997, Sykes and Greer, 2003). The studies of Yu et al. (2000) have shown that, in sheep infected with *T. colubriformis*, the utilisation of the absorbed amino acids in the gastrointestinal tissue increases by as much as 40% and thus made them unavailable for peripheral body tissues. It is, therefore, suggested that the detrimental consequences of parasitism on animal productivity are mainly due to both anorexia and increased nutrient demand of gastrointestinal tissue (Coop and Kyriazakis, 2001; Sykes and Coop, 2001; Sykes and Greer, 2003). These nutrient demands of gastrointestinal tissue are due to the suggested pathophysiological alterations, as well as due to the needs to mount an immune response towards parasites. The severity of productivity loss is dependent on the level of infection, the age of the animals as well as on their nutritional and immunological status (Fox, 1997; Sykes and Coop, 2001).

1.2.4 Development of immunity to gastrointestinal parasites

Following the first contact with gastrointestinal worms, hosts become sensitised by recognising the parasite antigens (macromolecules such as proteins and polysaccharides) as non-self and gradually develop immunity against them (Balic et al., 2000; McClure et al., 2000). This phase of acquisition of immunity to gastrointestinal parasites is relatively long compared, for example, to bacterial or blood-born protozoan infections (Abbas et al., 2000; Janeway et al., 2000). Development of lamb immunity against *T. circumcincta* or *T. colubriformis* parasites may take between 4 to 8 weeks of continuous and repeated challenge (Seaton et al., 1989; Dobson et al., 1990, 1992; Barnes and Dobson, 1993; Coop et al., 1995; Stear et al., 2000). This delay in immune expression may occur due to the stage-specific nature of resistance and the changing of antigenic profile of developing/moulting infective larvae (Keith et al., 1990; Knox, 2000; Harisson et al., 2003), or may reflect the loose degree of contact between parasites and the host tissue (Wakelin, 1996).

The acquisition of immunity to gastrointestinal nematodes is also affected by the level of infection and the age of the host, with growing lambs having slower rate of acquired immunity than the older sheep (Smith et al., 1985; Dobson et al., 1990, 1992). Lambs start to demonstrate immunity against parasite from 4 to 5 months old, while immunity increases in strength with the continued exposure to parasites. Nutrition appears to have no effect on the rate of acquisition of immunity, while increased protein intake has been demonstrated to significantly improve the strength or expression of acquired immunity (Coop et al. 1995; van Houtert et al, 1995; van Houtert and Sykes, 1996; see next section).

Generally, in parasite-naïve hosts, as the protection develops, animals first acquire the ability to inhibit the development of infective larvae, then become able to inhibit the establishment of incoming larvae, to reduce the fecundity of adult female worms and finally to expel adult worms (Seaton et al. 1989 for *T. circumcincta*, and Dobson et al., 1990, 1992 for *T. colubriformis*). The host achieves these effects on the parasitic population using variable magnitudes of the same or combination of immune mechanisms. Such mechanisms, cellular or humoral, responsible for the resistance against parasitic infections are: (i) the mucosal mast cell (MMC) and globule leukocyte (GL) hyperplasia, (ii) the proliferation of eosinophils in both blood and local mucosal sites, (iii) the increase of mucus production and appearance in mucus substances inhibitory to parasites and (iv) the production of nematode specific IgG1, IgG2, IgE and IgA antibodies (Huntley et al., 1992, 1998; Harrison et al., 1999, 2003; Balic et al., 2003).

The immunisation process and acquisition of resistance is not yet fully understood. However, recent reviews on gastrointestinal nematode immunobiology (Balic et al., 2000; McClure et al., 2000) have suggested a model of immune response reconciling most of the present knowledge. According to this model, immunisation under a repeated infection regime with trichostrongylid L3 results in an increase of MMC and GL numbers in the gut mucosal

tissue over the first 5 to 6 weeks after the start of challenge infection (Pfeffer et al., 1996; Seaton et al., 1989; Huntley, et al., 1992, 1995, 1998; Coop et al., 1995). These elevated MMC and GL numbers reach a plateau at a level significantly higher than the uninfected animals and remain elevated on the presence of parasitic challenge. These particular cells have been negatively correlated with the worm burden in a number of studies (Smith et al., 1983; Coop et al., 1995; Stear et al., 1999) and their effect was also associated with their released cell mediators (such as sheep mast cell proteases, SMCP), which derive from their activation/degranulation (Huntley et al., 1992). Eosinophil cells increase rapidly following the repeated challenge but they will drop abruptly from both tissue and blood, when larvae become rejected from the tissue (Buddle et al., 1992; Pfeffer et al., 1996; Stear et al., 2002). Although the role of eosinophils in immunoregulation is still debatable (Rothwell 1989; Huntley et al., 1995), a number of studies conducted in sheep have shown a correlation between eosinophilia and reduced gastrointestinal worms (Dawkins et al., 1989; Buddle et al., 1992; Stear et al., 2002). Other anti-parasitic effector mechanisms such as larval inhibitory substances in the mucus, antibodies, lymphocytes and lymph node activation will also develop gradually during the continuing larval challenge affecting and enhancing the immune development. However, in the absence of any further parasite challenge, MMC and GL numbers are the main effector mechanism remaining for considerable time after the cessation of the infection and gradually declining in a variable period of time to pre-infected levels. In contrast all other effector mechanisms will drop abruptly in the face of absence of stimuli (Sutherland et al., 1999; Balic et al., 2000). The decline in host immune mechanisms with time, in worm-free environments, has practical significance in parasite elimination strategies since it suggests that small parasite presence on pastures are important in continuing to stimulate and maintain an effective immunity (Abbott et al., 2004).

1.3 Control of gastrointestinal parasites

The objective of any parasite control programme is to limit the parasite populations or, at least, the contact between the infective stage of the parasite and the host. At present, there are two main strategies to achieve this, namely chemotherapy and grazing management, which are commonly used in combination. The use of broad or narrow spectrum anthelmintic drugs aims to prevent or limit the pasture contamination, while grazing management aims to reduce the rate of host infection from pastures by means of several practices. Such practices may include changes in stocking rates, timing of parturition/weaning, rotational grazing, alternate grazing either with different host species (mixed cow/sheep grazing) or hosts from the same species with different susceptibility (older animals with acquired immunity) and the use of aftermaths or new sown paddocks (Barger, 1997, 1999; Abbott et al., 2004). Unfortunately, the widespread development of anthelmintic resistance (Jackson et al., 1992; Sangster, 1999; Jackson and Coop, 2000) and the shortage of grazing land per animal render these measures ineffective. Among the suggested alternative control methods, the use of nutrition, either through supplementation strategies or using specific bioactive forages, has attracted major research effort, and will be discussed in detail in the following paragraphs, since it is relevant to the experimental chapters of this thesis.

1.3.1 Effects of host nutrition on immunity

It has long been established that malnutrition of the host increases susceptibility to parasitism (Gibson, 1963). Due to protaineous nature of the humoral and cellular immune response, coupled with the anorexia and general pathological nutrient metabolism that accompanies gastrointestinal infections, parasites elevate the requirements for nutrients and in particular metabolisable protein (MP) of their hosts (Coop and Holmes, 1996; Coop and Kyriazakis 1999; Walkden-Brown and Khan, 2002). Thus, there is a large body of evidence to support the view that protein supplementation of sheep enhances immune response against

gastrointestinal nematodes. The focus of the current section will be on lambs infected with *T. colubriformis* and *T. circumcincta* although similar observations have been reported for older sheep (ewe supplementation studies; see review of Houdjik and Athanasiadou, 2003) and other nematode species such as *Haemonchus contortus*, *Nematodirus spp.*, (see review of Steel et al., 2003).

The initial work of Bown et al., (1991) demonstrated that supplementation with MP reduced the worm egg output by 36% and worm burdens by 55% of growing *T. colubriformis* infected lambs, while no effect was detected when energy intake was increased. Such observations were confirmed by Kambara et al., (1993), who used trickle infected lambs with *T. colubriformis* and two levels of dietary protein with similar levels of metabolisable energy. The results showed that lambs fed previously the high protein diet had higher levels of resistance in terms of faecal egg counts, worm burdens and mesenteric lymphocyte numbers. Similarly, van Houtert et al. (1995) showed that MP supplementation of lambs increased immune resistance against trickle *T. colubriformis* infection, as indicated by the significantly reduced worm egg excretion and total worm burdens, and further supported by the enhanced lymphocyte stimulation, eosinophilia and higher concentrations of SMCP. More recently, Kahn et al. (2000) tested the effect of protein and energy supply and especially the role of increased volatile fatty acids, in the development and the expression of resistance. In contrast to previous studies, they reported that digestible energy supply lowered the *T. colubriformis* burdens, while the MP supply was ineffective. The authors concluded that for better gastrointestinal control the nutrition of young growing sheep must be improved in general, aiming to provide sufficient amount of protein and energy as well, rather than increase the supply of a particular component.

Enhanced immune expression in response to protein supplementation has also been reported in studies with *T. circumcincta* (Coop et al., 1995) and mixed *T. circumcincta* and *T.*

colubriformis infections, (Smith et al., 1996). In the first study, (Coop et al., 1995), the development of immunity was assessed by following trickle-infected lambs, supplemented with bypass protein for 8 weeks. Animals were then treated with anthelmintic and given a single challenge dose of *T. circumcincta* larvae. Supplemented animals had lower worm burdens, increased GL/MMC numbers, higher concentration of SMCP and a significantly higher proportion of inhibited larval development. Smith et al. (1996) tested the effects of MP supplementation in lambs with concurrent infections of intestinal (*T. colubriformis*) and abomasal (*T. circumcincta*) infections. The results showed a more rapid decline of faecal egg counts in the supplemented lambs compared to non-supplemented control lambs. These observations provide further support for the association between better nutritional status and a more effective immune response against nematode infection in young lambs.

1.3.2 Forages with anti-parasitic properties

Plant species with anti-parasitic properties have been used for centuries, and in some parts of the world, are still used to treat parasitic infections of humans or animals (Guarrera, 1999; Waller and Thamsborg, 2004). Endless lists of plant species or plant compounds claiming biological activity (bioactive plants) against internal or external parasites have been reported in literature (Houdijk and Athanasiadou, 2003; Athanasiadou and Kyriazakis, 2004). In most cases the bioactivity of these plants has been attributed to organic compounds, which appear to have no direct function in growth, reproduction or development of plants, (i.e. primary metabolism) and are known as plant secondary metabolites (PSM). In general, secondary metabolites differ from primary metabolites in terms of chemical diversity and distribution in the plant kingdom. More than 80,000 different PSM compounds have been described, while more than 100,000 compounds are estimated to exist in plants, giving a large diversity of these compounds, which are found only in a small proportion of plant species (Taiz and Zeiger 2002; Acamovic et al., 2005). The present section will discuss some aspects of the PSM-containing species, such as

chicory or the rich in condensed tannins (CT) legumes, sulla, sainfoin and lotus, that have been recently purported to exert some bioactivity against gastrointestinal parasites.

Evidence supporting the antiparasitic activity of the PSM-rich plant chicory (*Cichorium intybus*) was first reported in grazing experiments in New Zealand. They showed that chicory, as grazing forage, could affect trichostrongylid infections in ruminants in terms of worm egg excretion, worm burdens or improved animal productivity. The grazing studies of Scales et al. (1994) showed that naturally infected lambs grazing chicory had fewer worm *T.circumcincta* burdens and better growth rates compared to lambs grazing grass-based pastures. Also in the same work, it was reported that fewer infective larvae per kg DM were recovered from chicory forage than from grasses. Similar results on pasture infectivity were previously reported by Moss and Vlassoff (1993), where nematode infective larvae recovered, in similarly contaminated pastures, were lower in chicory than those recovered from grass or other herbage species. Later work by Hoskin et al., (1999) with grazing deer reported that the withdrawal of regular anthelmintic treatment dramatically reduced growth rates of deer grazing perennial grasses, but not in deer grazing chicory, which sustained high performance and low FEC. More recently, Marley et al., (2003b) investigated the effects on naturally infected lambs grazing chicory as opposed to ryegrass/white clover pastures. The results showed that sheep grazing on chicory for five weeks had fewer abomasal, but not intestinal nematode worm burdens, while the liveweight gain was significantly higher for chicory-fed lambs than those grazing ryegrass/white clover.

Evidences supporting the antiparasitic activity of CT-containing legumes such as lotus (*Lotus pedunculatus*) or sulla (*Hedysarum coronarium*) were initially reported by Niezen et al., (1994; 1995 and 1998b). In the first study (Niezen et al., 1994) parasite-naïve lambs were either infected naturally (undosed) or experimentally (dosed with 9,000 *T. colubriformis* and 9,000 *T.*

circumcineta larvae per week), for a six-week period and slaughtered 9 weeks post infection. The lambs grazing sulla had significantly reduced *T. circumcineta* worm numbers and significantly reduced *T. colubriformis* worm burdens in naturally infected animals compared to animals grazing grass/clover. The same study also reported significant lower FEC and higher live-weight gains for the lambs grazing on lotus (*Lotus pedunculatus*) or chicory relative to those grazing grass/clover forage; however, worm burdens were not recovered from these treatments. These promising results were further investigated in the following work of Niezen et al., (1995), where lambs, single dosed with 20,000 *T. colubriformis* infective larvae, grazed either sulla or lucerne for six weeks and were slaughtered thereafter for worm recoveries. The results showed that lambs grazing sulla had significantly higher live-weight-gains, lower FEC and lower worm burdens at slaughter, whilst it was claimed by the authors that parasite induced anorexia was evident only in lambs grazing lucerne (*Medicago sativa*). Later work by the same group (Niezen et al., 1998a) reported that parasite-naïve lambs, infected at the start of the experiment with both 10,000 *T. circumcineta* and 10,000 *T. colubriformis* infective larvae, and fed lotus for five-week period, had lower FEC and lower *T. circumcineta*, while *T. colubriformis* worm burdens were unaffected, when compared to lambs fed ryegrass. The absence of any effect against the intestinal *T. colubriformis* parasite contrasted to previous results obtained with sulla forage, suggesting that different CT-containing legumes might act differently against parasite species (species specificity).

However, the beneficial effects of CT-containing forages against gastrointestinal parasitism were not confirmed by subsequent studies of the same group (Niezen 1998b, Niezen 2002a). In the study of Niezen et al., (1998b), naturally infected animals, carrying substantial numbers of abomasal and intestinal worm burdens, were allocated for a six-week period onto monospecific forages; birdsfoot trefoil (*L. corniculatus*), lotus (*L. pedunculatus*), sulla, plantaine (*Plantago lanceolata*) and the control grass/clover to evaluate the effects of forage type on

worm burdens and liveweight gains. The results showed that none of the tested forages reduced the parasitic species burden, either intestinal or abomasal, whilst the lambs grazing on lotus or sulla had better weight gains relative to grass/clover, despite the similar FEC and high worm burdens recorded. Similar inconsistencies related to purported sulla anti-parasitic effects were also reported in the later study of Niezen et al., (2002a). The two experiments of this study reported contradictory results regarding the effects of sulla forage against gastrointestinal parasitism, while, according to the authors, the study was inconclusive in relation to the mode of action of this bioactive forage.

Nevertheless, these studies stimulated further investigation regarding the CT-action against gastrointestinal nematodes in sheep (Butter et al., 2000, Athanasiadou et al., 2000a,b, 2001a,b; Waghorn and Molan, 2001; Marley et al., 2003a,b; Hounzangbe-Adote et al., 2005; Max et al., 2005b), goat (Paolini et al., 2003a,b,c, 2005; Min et al., 2004, 2005; Max et al., 2005a; Pomroy and Adlington, 2006), rat (Butter et al., 2001), or deer hosts (Max et al., 2005a). The above investigations showed that the CT effects varied in terms of (i) their efficacy, (ii) the affected parasite species and (iii) the affected developmental parasitic stage. For example, the study of Marley et al., (2003b) reported that lambs fed the CT-rich forage, birdfoot trefoil, for five weeks had reduced the numbers of both intestinal and abomasal adult worms compared to grass/clover fed lambs. In contrast, the immature populations of the same species remained unaffected. This suggests that the bioactive forages may exert their anti-parasitic properties in a stage-specific manner (Paolini et al., 2003a,b,c), and specifically designed *in vivo* experiments are needed to test such hypothesis in sheep. Furthermore, other studies have suggested that the anti-parasitic effects of CT forages may be exerted against female worm fecundity rather than total worm population. For example, recently published studies of Paolini et al. (2003b, 2005) reported reductions in FEC of dairy goats fed sainfoin (*Onobrychis viciifolia*) hay in *T. colubriformis* infected animals, without concurrent reduction in female worm burdens

suggesting a bioactive forage effect on worm fecundity.

The conflicting evidence of forage CT action against parasites was also reported by *in vivo* studies using CT-substances extracted from CT-rich plants (CT-supplementation studies). This is another experimental way to test the possible use of bioactive forages, where the CT-substances are extracted from CT-rich plants and either given to the animals as a gavage or added to their feed. Thus, the study of Athanasiadou et al., (2001a) investigated the CT-parasite interaction *in vivo*, using quebracho (a CT-extract from *Schinopsis spp*) as a supplement against abomasal and intestinal infection of sheep. Their results showed that tannin-rich foods lowered the intestinal *T.colubriformis* parasite worm burdens by 50% compared to unsupplemented animals but had no effect on abomasal species. In contrast, the *in vivo* study of Max et al. (2005b), which also tested the effects of quebracho-extract on parasitised lambs, showed significant reductions only in abomasal but not on intestinal *T. colubriformis* worm numbers, while, in addition, the effects were greater on adult rather than on immature worm stages. These studies support the need for more investigations into the CT-action of forages against parasites.

Further investigation is also required into the action of the previously mentioned bioactive forage, chicory, since similar species-specific action against some parasite species has also been reported. For example, the grazing studies of Scales et al. (1994) and Marley et al. (2003) have both reported chicory effects only against abomasal worm burdens, but not against the intestinal parasite population residing in the same naturally infected sheep. In conclusion, the previously reported studies have provided useful information regarding the bioactive forages and their extracts against parasites or parasite-induced detrimental effects on the host. It is, however, evident that more research is needed related to the bioactive forage effects against specific parasite species or developmental stages as well as regarding the hypothesised mode of bioactive action.

1.3.3 Hypothesised mode of action of the bioactive plants

It has been suggested that the bioactive plants owe their anti-parasitic activity to their secondary metabolites, such as the CT from the tanniferous legumes or the sesquiterpene lactones from chicory forage (Molan et al., 2000a,b,c, 2002, 2003a,b; Paolini et al., 2004). *In vitro* studies using PSM-compounds extracted from lotus, sulla, sainfoin, chicory, *L. cuneata*, *Scinopsis spp* or woody plants (Molan et al., 2000a; Molan et al., 2000b; Molan et al., 2002, Athanasiadou et al., 2001a, Paolini et al., 2004), have demonstrated an anthelmintic effect against immature stages of several nematode species (Cervine species: *Dictyocaulus* and *Ostertagia*; Ovine species: *H. contortus*, *T. colubriformis* and *T. circumcincta*). In these studies the anthelmintic efficacy of the PSM-extracts was judged by the inhibitory effects of different PSM concentrations on the ability of the infective stage of the parasite to move, feed, develop or survive. However, the bioactive forage action under grazing conditions seems to be more complicated and several mechanisms have been put forward to account for the phenomenon. The suggested mechanisms fall into those affecting: (i) the free-living parasitic stages within the grazing pastures, (ii) the nematodes within the host through an anthelmintic-like effect, and (iii) the nematodes through an immunologically/physiologically mediated effect on the parasitised host.

i) Effects on free-living parasitic stages

The suggested effects on free-living larvae within pastures are attributed to either the PSM content or the sward structure of bioactive plants. PSM compounds are largely indigestible in the gastrointestinal tract of the sheep (Makkar et al., 2003; Acamovic and Brooker, 2005) and therefore are present in large amounts within the faecal pat. It is, thus, suggested that these compounds affect the nematode egg hatching, larval development and survival within the faeces, due to the PSM anthelmintic properties, which consequently reduce the numbers of

infective parasitic larvae consumed by the animals (Marley et al., 2003b). In addition, it is suggested that the same result may occur due to bioactive forage structure and sward canopy. It has been hypothesised that the specific bioactive sward characteristics may affect (i) the decomposition of the sheep faeces in different vegetation types, (ii) the larval migration in the stratum that is consumed by grazing animals and (iii) the larval survival within the pastures (Moss and Vlassoff, 1993; Barry 1998; Hoskin et al. 1999; Marley et al., 2003a). Nevertheless, the investigation of such mechanisms was beyond the scope of the present thesis, which focused on the bioactive forage effects against gastrointestinal nematodes within the parasitised host through a direct, anthelmintic-like, effects of PSM compounds or the indirect effect related to physiological/immunological effects on the parasitised host.

ii) Direct effects of bioactive plants within the animal host

This hypothesis on bioactive forage action has been supported by *in vitro* experimentation, where purified extracts from bioactive plants exerted anthelmintic activity against larval stages of gastrointestinal parasites. For example Molan et al. (2000a,b), showed that CT extracts from *L. pedunculatus*, *L. corniculatus*, *Hedysarum coronarium*, and *Onobrychis viciifolia* forages reduced *in vitro* (i) the rate of larval development (eggs to L3 larvae), (ii) the number of eggs hatching and (iii) the motility of L3 larvae. Such studies have suggested that the anti-parasitic effects of the PSM-rich forage consumption are due to *in vivo* anthelmintic activity. However, *in vitro* results are not always confirmed in *in vivo* studies, when the same forages or extracted PSM-substances were tested (Athnasiadou et al., 2001b; Athnasiadou and Kyriazakis, 2004; Waghorn et al., 2006). This may be due to different PSM concentrations, PSM bioavailability or different mechanism of PSM action involved in animal experimentation studies.

Regarding the PSM anti-parasitic action, there is a large body of evidence suggesting

the dependence of PSM efficacy to PSM concentration/bioavailability. Invariably, in almost all the previously referred *in vitro* studies the PSM anthelmintic effects were exerted in a dose-related manner. For example, the CT extracted from lotus (*L. pedunculatus*) inhibited the mobility of *T. colubriformis* L3 larvae by 20% and 57% at 100µg/ml and 1000µg/ml CT-concentrations, respectively. Similarly, sulla CT-extracts inhibited the mobility of the *T. colubriformis* larvae by 10% and 43% in corresponding CT-concentrations (Molan et al., 2000b). It is therefore appreciated that the conflicting evidence between *in vitro* and *in vivo* tests of the same PSM substance may occur due to different PSM concentrations *in vivo*, affecting their anthelmintic efficacy. One example of possible misinterpretation of the *in vitro* PSM-concentrations and extrapolation to *in vivo* conditions can be seen in the study of Molan et al. (2003b) regarding the chicory PSM compounds. These authors showed that rumen fluid containing 400 or 1000 µg chicory-CT/ml inhibited the mobility of deer gastrointestinal larvae by 28 and 41%, respectively. However, the CT content of chicory is very low, approximately 0.5 g/kg DM (Barry, 1998), and it therefore seems unlikely that CT-chicory-concentration *in vivo* could ever reach the concentrations tested *in vitro* (Barry et al., 2002; Athanasiadou and Kyriazakis, 2004). Furthermore, even if the CT-concentration is present within the ruminal fluid in high amounts, such those observed in sheep fed CT-rich legumes *Lotus spp* or sulla (between 5,000 to 1,000 µg CT/ml; Terrill et al., 1994), their true bioavailability is unknown. It has been demonstrated that the majority of CT *in vivo* are found in complexes with several molecule types, such as protein, carbohydrates, enzymes etc. (Muller-Harvey, 1999), and therefore, may be unavailable for action against parasite in gastrointestinal tract.

In addition, differences between *in vitro* and *in vivo* results may occur due to differences in parasitic-stage susceptibility to PSM compounds. The parasitic stage tested, in most *in vitro* studies, is the free-living larval stage (Molan et al., 2000a; Athanasiadou et al., 2000; 2001b). During the parasitic phase, nematodes exsheath, moult, change antigenic profile

and occupy different habitat niche. It is therefore possible that *in vitro* anthelmintic effect cannot be exerted *in vivo* due to a special niche in which the larval stages dwell and consequently the absence of contact between larvae and PSM compound. Such a mechanism has been suggested by Butter et al. (2001) in rats to account for the differences in species susceptibility to CT-action. In conclusion, since the exact mode of PSM action or the PSM concentration *in vivo* is generally unknown, it is recommended that the extrapolation of *in vitro* properties to *in vivo* conditions should be made with caution taking always into account other likely modes of action such as through nutritional, physiological or immunological effects that the bioactive forage may have.

iii) Indirect effects of bioactive plants

This hypothesis of bioactive action has been supported by the *in vivo* nutritional studies, which suggested specific bioactive forages, such as the CT-rich legume sulla and the herbaceous forage chicory, as nutritionally superior forages (Min et al., 2003; Li and Kemp 2005) compared to grasses or grass-based pastures. Therefore, they have raised the possibility that their anti-parasitic effects are mediated through a nutritional effect on sheep immunity.

Nutritional studies have suggested that legumes rich in CT have higher nutritional value than other non-CT legumes, grasses or grass/legume mixtures. This was attributed to their CT, which can bind dietary proteins, reduce their solubility and degradation by the rumen micro-organisms and consequently optimise the rumen function (Min et al, 2003). When sheep fed high quality, nitrogen (N)-rich fresh forages, degradation of the forage N is excessive, resulting in surplus levels of ammonia, which is ultimately absorbed by the rumen and excreted as urea in the urine (Van Soest, 1994). In contrast, when sheep fed forages containing moderate CT levels (20 to 40 g of CT/kg DM), such as sulla or *L. corniculatus*, the rates of both N solubilisation and N degradation by the rumen bacteria slows down, with concurrent increases

in amino acid concentration and non-ammonia-N outflow from the rumen (Min et al., 2003). The formation of CT-protein complex is influenced by pH in a manner that CT can bind to protein at near neutral pH (pH between 3.5 to 7.5), while disassociate and release protein at pH less than 3.5 (Asquith and Butler, 1986; Mangan, 1988). It was, therefore, suggested that in rumen natural pH (pH between 6.0 to 7.0), CT from tanniferous plants bind to proteins, protecting them from rumen degradation. During the subsequent move of rumen digesta into abomasum, where the pH is less than 3.5, the CT-protein complex disassociates, resulting in an increase of protein supply to the abomasum and small intestine (McNabb et al., 1996; Min et al., 2000; Barry et al., 1999; Min et al., 2003). It has also been shown that free CT in the rumen could also bind to other sources of protein such as rumen bacteria or bacteria secreted enzymes, changing the rumen microflora environment (Jones et al., 1994; Molan et al., 2001; McSweeney et al., 2001) and consequently inhibit further the protein degradation in the rumen (Min et al., 2003). Studies with sheep fed certain CT-forages, such as birdfoot trefoil and sulla, have suggested an overall increase of protein supply to the animal without reducing the amount of microbial protein synthesized (Aerts et al., 1999). It is, therefore, hypothesised that such improvement in host nutrition, and particularly in protein, may enhance immune response against gastrointestinal parasites (Niezen et al., 1995; 1998a; 2002a), as has been shown previously in several protein supplementation studies referred (Bown et al., 1991; van Hourtert et al, 1995; Coop et al., 1995).

As far as chicory is concerned, there is a large body of evidence suggesting that chicory forage has high nutritional and feeding value for ruminants resulting in high voluntary feed intake and animal productivity. The superior nutritional value of the chicory forage has been supported by several studies testing the effects on sheep, cattle and deer productivity and reported greater production in terms of live-weight gains (Fraser et al., 1988; Hoskin et al., 1995; Scales et al., 1994), carcass weight (Min et al., 1997), milk production (Jung et al., 1996)

and wool production (Fraser and Rowarth, 1996) relative to grass-based pastures. Studies of Crush and Evans, (1990); Scales et al., (1994); Hoskin et al., (1995); Barry (1998); Jung et al., (1996); Belesky et al., (2001) Rumball et al., (2003); reported that chicory led to (i) high voluntary feed intake, (ii) high concentration in most macro and micro-minerals, similar to or higher than those found in grasses and legumes, (iii) higher metabolisable energy (ME) content relative to perennial ryegrass or grass/legume mixtures and (iv) variable concentrations in crude protein levels, between 134 to 244 g/kg DM. Studies on its feeding value reported that chicory digestibility was higher than the grass-based pastures due to higher concentrations of readily fermentable-soluble carbohydrates and lower concentrations of structural carbohydrates than the perennial ryegrass (Hoskin et al., 1995; Kusmartono et al., 1996a; 1997). The higher carbohydrate solubility along with the lower levels of chicory NDF has been suggested by Barry (1998) as the reasons of higher voluntary feed intake associated with animals grazing chicory. Kusmartono et al., (1996a,b, 1997) demonstrated that chicory degradation and disappearance from the rumen were approximately twice as fast compared to perennial ryegrass in deer, supporting the view that chicory high feeding value is also related to its high digestibility and high voluntary feed intake. It is therefore hypothesised that the chicory-based pasture, due to its nutritional and feeding properties, may supply the grazing animals with extra nutrients, resulting in an enhancement of the immune response against gastrointestinal parasites similar to those observed in supplemented animals (Bown et al., 1991; Coop et al., 1995; Datta et al., 1998, 1999; Kahn et al., 2000, 2003a,b).

In conclusion, evidence from the previously referred research on bioactive forages supports the view that the potential anti-parasitic use of these plants merits more investigation. Further studies are needed to identify the bioactive efficacy against specific parasite species and/or developmental stages, as well as to elucidate more fully the underlying mechanisms, through which they may be beneficial to sheep health and/or productivity.

1.4 Aims of the present study

The present thesis used *in vivo* experimentation to test for the potential use of bioactive plants as grazing forages to control gastrointestinal parasites of growing lambs. As these alternative grazing forages are not commonly used in the mainstream agricultural practices, it has been suggested that their incorporation into existing grazing regimes will probably be confined to short-term grazing, as de-worming paddocks (Coop and Kyriazakis, 2001). For this reason, the effectiveness of short-term grazing on bioactive forages, such as lotus, sulla, sainfoin and chicory, was primarily tested. The hypothesis that different parasitic species show different degrees of susceptibility to bioactive plants, as suggested previously from grazing trials (Niezen et al., 1998a) or *in vivo* PSM-supplementation trials (Athanasidou et al., 2001a,b), was investigated on two different parasitic species, *T. colubriformis* (Chapter Two) and *T. circumcincta* (Chapter Three). In order to address the hypothesis of parasitic stage specificity, the experiments used a novel design to investigate the effect of the bioactive plants against both established adult and incoming larval populations. The findings from these experiments formed the basis of a more detailed investigation that aimed to determine the possible combination of bioactive forages as a short-term grazing regime to optimize the anti-parasitic effects of these plants (Chapter Four). Finally, the last experiment (Chapter Five) aimed to investigate whether bioactive forages have any effects on the acquired immunity against gastrointestinal parasites of lambs.

The aims of the present study were:

1. To investigate the potential effects of short-term grazing on bioactive forages towards (i) the worm number and fecundity of established intestinal *T. colubriformis* adult parasites and (ii) the establishment and development of incoming *T. colubriformis* larval population (Chapter Two)
2. To investigate the potential effects of short-term grazing on bioactive forages towards

- (i) the worm number and fecundity of established abomasal *T. circumcincta* adult parasites and (ii) the establishment and development of incoming *T. circumcincta* larval population (Chapter Three)
3. To test the effects of sequential, short-term grazing on the bioactive forages chicory (*Cichorium intybus*) and sulla (*Hedysarum conarium*), towards an established adult *T. circumcincta* worm population of growing lambs (Chapter Four).
 4. To investigate the effects of grazing different bioactive forages on the acquired immunity against *T. circumcincta* infection of growing lambs (Chapter Five).

Chapter Two

**The effects of short-term grazing of bioactive forages on
established adult and incoming larvae populations of**

Trichostrongylus colubriformis in lambs

Abstract

The aim of the present study was to investigate the potential effects of short-term grazing on bioactive forages towards (i) worm numbers and fecundity of established intestinal adult *Trichostrongylus colubriformis* population and (ii) the establishment and development of incoming *T. colubriformis* larvae. Forty-eight parasite-naïve lambs were infected with 8,000 *T. colubriformis* L3 on day 1 of the experiment. On day 28, lambs entered one of the experimental plots, which had been sown either with *Lotus pedunculatus* (lotus), or *Hedysarum coronarium* (sulla), or *Cichorium intybus* (chicory), or the control forage *Lolium perenne*/*Trifolium repens* (grass/clover). On day 35 of the experiment, all sheep were re-infected with 8,000 *T. colubriformis* L3 and were killed on day 42. Sheep grazing on lotus tended to have a lower FEC compared to sheep grazing on grass/clover ($P = 0.06$), however they also produced more faeces and thus daily faecal output was higher in these sheep grazing lotus compared to those grazing on the other forages ($P < 0.05$). Consequently, daily egg output was similar in all sheep suggesting that the reduction in faecal egg count was due to dilution effect of the faecal output. By day 42, all parasites of the primary infection would have been recovered as adults, whereas those of the secondary challenge would have only developed to the fourth stage larvae within a week (i.e. days 35-42). Grazing on the bioactive forages showed no evidence of forage effect upon incoming *T. colubriformis* larvae within a week or against adult *T. colubriformis* worms during a two-week period. Comparisons between adult (primary) and immature (secondary challenge infection) worm burdens showed no reduction in parasite establishment of the second challenge dose suggesting that no effective immunity against *T. colubriformis* had been acquired in sheep.

Keywords: *Cichorium intybus*; *Lotus pedunculatus*; *Nematoda*; *Plant secondary metabolites*; *Sheep*; *Hedysarum coronarium*

2.1 Introduction

Widespread anthelmintic resistance in parasite populations (Sangster and Dobson 2002) has increased the need for alternatives to chemoprophylaxis to control gastrointestinal parasitic infections in sheep. This need has also been urged by regulations in organic farming, which prohibit the prophylactic use of anthelmintic drugs in sheep production (UKROFS, 2000). Amongst the proposed alternatives is the use of bioactive forages, such as *Cichorium intybus* or *Hedysarum coronarium*, which have reputedly shown beneficial effects on parasitised ruminants in terms of reduced worm burdens or improved performance (Niezen et al. 1995; Scales et al., 1994; Hoskin et al., 1999; Min and Hart, 2003; Marley et al., 2003b). These effects have been attributed to the plant secondary metabolites (PSM) of these species suggesting a direct anthelmintic-like PSM effect or an immune related effect. The last hypothesis has been suggested due to the condensed tannin (CT) characteristics of some of the PSM-rich forages, such as *Hedysarum coronarium* or *Lotus corniculatus*. CT from these forages have shown to offer nutritional benefits to the ruminants as they can improve the protein availability to the host (Min et al., 2003). In turn protein supplementation has been shown to enhance immune response against gastrointestinal parasites (Bown et al., 1991; van Houtert 1995; Datta et al., 1998, 1999; Houdijk et al., 2003a,b,c). Evidence for the potential direct anthelmintic effects of PSM comes mainly from *in vitro* studies. It has been shown that PSM-compounds extracted from *Cichorium intybus* (Molan et al., 2003), *Lotus pedunculatus*, *Hedysarum coronarium*, and *Onobrychis viciifolia* (Molan et al. 2000a,b,c, 2002; Schreurs et al. 2004) can reduce egg hatching and larval migration, development and viability of several nematode species (Cervine: *Dictyocaulus sp.* and *Ostertagia sp.*; and Ovine: *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Teladorsagia circumcincta*). Despite the evidence available from *in vitro* studies, there is no information on the potential bioactive forage effect on different developmental stages of *T. colubriformis in vivo*, in sheep. Such study will confirm the effects against larval stages

observed *in vitro* and elucidate more fully the underlying mechanisms of bioactive forage action.

The objective of the present study was to investigate whether the short-term grazing on selected bioactive forages can affect (i) worm numbers and fecundity of adult *T. colubriformis* already established in the gastrointestinal tract of parasitised lamb and (ii) the ability of incoming L3 *T. colubriformis* larvae to establish and develop within the host. This study aimed to enhance understanding of the mechanism involved and the worm stages affected, as well as to suggest candidate species in pasture management practices to control *T. colubriformis* infections. The bioactive forages used, were *C. intybus* (chicory), *L. pedunculatus* (lotus; greater lotus), *O. viciifolia* (sainfoin) and *H. coronarium* (sulla).

2.2 Materials and methods

2.2.1 Experimental plots

Ten experimental plots of 0.1 ha each were established in spring 2001 on fields that had always been used for arable production and thus were considered parasite-free. The plots were sown as monocultures using the following sowing rates: *L. pedunculatus* (greater lotus; lotus) 12,5 kg/ha, *H. coronarium* (sulla) 15 kg/ha, *O. viciifolia* (sainfoin) 50 kg/ha, *C. intybus* (chicory) 7 kg/ha and a mixture of *Lolium perenne*/*Trifolium repens* (grass/clover) 37 kg/ha (Frame et al., 1998). The grass/clover swards provided the control treatment. The 10 experimental plots contained two replicates from each forage species and their position was completely randomised. Fencing divided the plots and each plot contained an automated water trough.

During the 2001 grazing season, the experimental pastures were not grazed, but were twice mechanically topped to remove reproductive stem material in order to maintain the vegetative state of the plants. Nitrogen fertiliser (Yara UK Ltd; $N < 50$ kg/ha) was applied to all experimental plots in summer 2001, to enhance growth. In spring 2002, the vegetation growth in all experimental swards was considered satisfactory with the exception of the sulla swards, which were re-sown. The rest of the plots, which had successfully established in the previous year, were mechanically topped in June 2002 to maintain the vegetative state of the plants. In July 2002, nitrogen fertiliser and a phosphorus-potassium mix (Yara UK Ltd; 100 kg/ha) were applied, following soil analysis, to enhance plant growth. No insecticides or herbicides were used throughout the pre- and experimental period.

2.2.2 Animals

Sixty Texel x Scottish Greyface sheep were used for this experiment (30 wethers and 30 ewe lambs). Until weaning (at 10 weeks of age), sheep were reared outdoors under conditions

that reduced nematode exposure (parasite-free grass/clover pastures). Prior to weaning, sheep were prophylactically drenched with levamisole hydrochloride (Levacide 3% drench; Norbrook Laboratories Ltd) at 7.5 mg/kg bodyweight. Following weaning sheep were drenched with ivermectin (Oramec, Merial, Animal Health Ltd; 0.2 mg/kg liveweight) and were grazed for two weeks on the same grass/clover pasture until the start of the experiment.

2.2.3 Parasites

Infective larvae from a Moredun ovine anthelmintic susceptible isolate of the intestinal nematode *Trichostrongylus colubriformis* were cultured from monospecifically infected donor sheep according to standard procedures (Christie and Jackson, 1982). The infective larvae were stored at 4°C and used within 4 weeks of being harvested.

2.2.4 Experimental design

One day prior to the start of the experiment, sheep were moved onto a newly seeded grass pasture, which had been sown on land previously used for arable production, and thus was considered worm-free. On the first day of the experiment (day 1: mean liveweight \pm standard error of the mean (S.E.M.): 33.9 ± 0.54 kg), lambs were dosed with 8,000 L3 of *T. colubriformis* and were maintained on the parasite-free grass pasture until day 28 of the experiment. The dose of 8,000 infective larvae was used to comply with the size of infection suggested by the World Association for the Advancement of Veterinary Parasitology for evaluation of the efficacy of anthelmintics (Wood et al., 1995). On day 28 of the experiment, when their worm populations would have consisted largely of adult *T. colubriformis*, lambs were allocated into one of the five treatment groups (12 animals per forage) balanced for sex, liveweight and faecal egg counts (FEC) on day 21 of the experiment (mean (\pm SEM): $33.8 (\pm 0.53)$ kg and $444 (\pm 7.3)$ eggs/g faeces, respectively). All lambs were maintained on these plots until the end of the experiment (day 42). This design enabled us to test for a potential anti-

parasitic effect of the bioactive forages on the adult established population, as well as on the fecundity of female parasites.

On day 35 of the experiment, sheep were infected with a second single dose of 8,000 *T.colubriformis* L3 and slaughtered one week later (day 42 of the experiment). The second challenge of larvae was administered to the animals to investigate whether grazing on the bioactive forages could affect the establishment of the incoming infective larvae. By day 42, surviving worms from the primary infection (8,000 *T.colubriformis* L3 administered on day 1) would have been recovered as adults, whereas the secondary challenge would have only developed to the fourth stage larvae over a week (i.e. days 35-42).

2.2.5 Sward measurements

Twenty randomised sward samples were obtained from each experimental plot on day 28 of the experiment, the day sheep entered the plots. These samples were pooled and analysed in duplicates for determination of dry matter (DM), crude protein (CP; Kjeldahl method), total phenolics (TP; Price and Butler, 1977), extractable condensed tannin content (CT; Porter et al., 1985), and *in vitro* dry matter digestibility (IVDMD; Tilley and Terry, 1963). Pre- and post grazing sward heights of the plots were measured on days 27 and 42, respectively using a sward stick (Barthram, 1985). Botanical composition of the experimental swards was determined using 20-forage gripper harvests (Barthram et al., 2000) taken to ground level (sampling area 9x2 cm) from each plot, on days 27 and 42 of the experiment. The samples were stored at 4°C, and subsequently were sorted into three fractions: the forage species that was sown on each plot, dead plant material and other plant species present in the sward. Dry matter of each fraction was determined following drying the samples at 60°C for 18 h.

2.2.6 Animal measurements

Sheep were weighed weekly early in the morning. Faecal samples were collected directly from the rectum, weekly until day 28 and twice a week thereafter until the end of the experiment. Numbers of nematode eggs per gram fresh faeces (faecal egg count, FEC) were determined by a modified floatation technique (Christie and Jackson, 1982). Total faecal output was determined by using C-36 alkane as an indigestible marker, as described by Mayes et al., 1986. In brief, each sheep was administered daily a paper pellet that contained a known amount of C-36, between days 28 and 42 of the experiment. During the last five days of the alkane administration (days 37-42) faecal samples were collected directly from the rectum of each sheep. Samples were pooled within individuals across days and the pooled samples were analysed for C-36 determination in duplicate (Mayes et al., 1986; Dove and Mayes, 1991). Herbage samples were also analysed to ensure that C-36 was not present in the herbage. Faecal output (FO) was then calculated based on the following formula:

$$\text{FO (kg DM per day)} = (\text{D36} \times 0.95) / \text{F36}$$

Where D36 and F36 are the dosed amount (mg/day) and faecal concentrations (mg/kg DM) of alkane C-36, respectively and 0.95 is the correction factor for incomplete faecal recovery of C-36 (Mayes et al., 1986).

Herbage intake was calculated taking into account the estimates of *in vitro* dry matter digestibility (IVDMD) and faecal output (FO) estimates based on the following formula:

$$\text{Herbage Intake (HI, kg DM per day)} = \text{FO} / (1 - \text{IVDMD})$$

Following the estimates of total faecal output, total egg output was determined by multiplying the total faecal output by the FEC for each sheep. Total egg output represented the total amount of *T. colubriformis* eggs excreted daily (eggs per day). At slaughter the small

intestines of the sheep were removed and nematode parasites were recovered from digesta and intestinal mucosa after a 4 h incubation in saline at 37°C. Total immature and adult worm burdens were estimated from 2% aliquots of digesta and mucosa digest for each sheep. *Per capita* fecundity was calculated by dividing the FEC recorded at the last day of the experiment by the total number of female worms recovered.

2.2.7 Statistical analysis

All experimental data collected were analysed by Minitab, version 14 (Minitab Inc. 2000. MINITAB Statistical Software, Release 14 for Windows, State College, Pennsylvania). Liveweight gain (g/day) for each animal was estimated by linear regression of the weights on days 28, 35, 42 (least square method) and are reported as arithmetic means (\pm S.E.M.). Worm numbers, FEC, total worm egg output and per capita fecundity were log-transformed ($\log_{10}(x+1)$) before the statistical analysis to remove positive skewness and are reported as back-transformed means with 95% confidence intervals (lower and upper limit). The effects of the feeding treatments on liveweight gain, faecal output, FEC, total worm egg output, worm numbers and per capita fecundity of the female worms were investigated using one-way analysis of variance. Any apparent treatment effects were further investigated using Dunnett's test (Zar, 1984; Matthews et al., 1990; Wilson, 1997) with grass/clover treatment as the control. Comparisons between adult and immature worm burdens were performed using paired t-tests. No statistical analysis was performed on the sward measurements, as they only aimed to describe the experimental plots.

2.3 Results

2.3.1 Sward measurements

On the day the sheep entered the experimental plots, lotus, sulla, chicory and grass/clover represented 61, 69, 79, and 83% of the sward dry matter, whereas sainfoin was poorly represented in the sainfoin plots (22% of the swards; Figure 2.1). As a consequence the animal measurements taken on this treatment are not considered here. At the end of the experiment (day 42), the representation of the species sown in each of the experimental swards was reduced in all treatments. On day 28 of the experiment mean (\pm standard deviation) sward heights were 21 (\pm 7.1), 23 (\pm 8.6), 21 (\pm 7.8), and 21 (\pm 4.0) cm for lotus, sulla, chicory and grass/clover swards respectively. On the last day of the experiment (day 42), mean sward heights were 6 (\pm 4.5), 6 (\pm 6.6), 3 (\pm 2.5) and 8 (\pm 3.9) cm respectively.

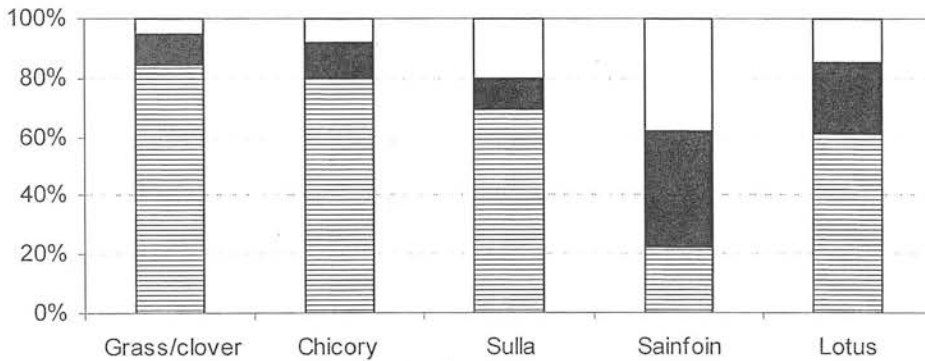





Figure 2.1 Pre-grazing proportion (% of the dry matter) of the sown species () *Lolium perenne/Trifolium repens*, *Cichorium intybus*, *Hedysarum coronarium*, *Onobrychis viciifolia* and *Lotus pedunculatus* in grass/clover, chicory, sulla, sainfoin and lotus experimental plots, respectively. Percentages of the dead () or other forage species () in the experimental plots are also shown.

The chemical composition of the experimental swards is shown in Table 2.1. Grass/clover swards had the highest and chicory swards the lowest dry matter (DM) content. Lotus swards had the highest whereas sulla swards had the lowest crude protein contents (CP). Lotus and sulla swards contained the highest concentration of condensed tannins (CT), while chicory and grass/clover contained only traces of CT amounts. Chicory swards had the highest content of total phenolics (TP), almost two-fold higher than those observed in the other swards.

Table 2.1 Composition of the experimental swards measured on the day 28 of the experiment. Each sample was analysed in duplicate and the numbers in the table represent the mean value of the two replicates for each forage species. For details on the experimental design, see text.

Experimental swards	DM ^a (g/kg FM)	CP ^b (g/kg DM)	CT ^c (g/kg DM)	TP ^d (g/kg DM)	IVDMD ^e (g/kg DM)
Grass/clover	272	97	0.3	129	689
Chicory	131	95	0.5	262	764
Sulla	164	73	15.8	158	682
Lotus	193	195	15.9	166	571

^a Dry Matter; ^b Crude Protein; ^c Extractable Condensed Tannins; ^d Total Phenolics; ^e In Vitro Dry Matter Digestibility

2.3.2 Animal measurements

On the day lambs were turned out onto the experimental plots (day 28) they had a mean (\pm SEM) weight of 34.1 (\pm 0.52) kg. Following two weeks of grazing on the experimental plots lambs reached a mean (\pm S.E.M.) final weight of 37.7 (\pm 0.57) kg with no significant differences observed in the liveweight gains between forage treatments (overall mean (\pm SEM) daily weight

gains: 279 (\pm 26.9) g). Herbage intake tended to be higher in sheep grazing chicory (1.49) and sulla (1.61) than in those grazing on grass/clover (1.13) or lotus (1.21) kg DM per day, although the means did not differ significantly.

During days 35-42 of the experiment, sheep grazing on lotus and sulla had significantly higher ($P < 0.05$) calculated faecal outputs than sheep grazing on chicory and grass/clover. Respective mean values (with 95% C.I.) for lotus, sulla, chicory and grass/clover were 558 (519-599), 567 (528-609), 364 (339-391) and 364 (339-392) g dry faeces per day.

Backtransformed FECs of sheep grazing on the forages are shown in Figure 2.2. The infection became patent by day 21 in all groups and FECs remained high until the end of the experiment. Sheep grazing on lotus tended to have lower FEC compared to sheep grazing on the control grass/clover or the chicory swards after day 31 of the experiment: day 42 FEC were 439 (399-483), 522 (475-575), 738 (671-812), 689 (625-757) for sheep grazing on lotus, sulla, chicory and grass/clover respectively, ($P = 0.06$). Sheep grazing on sulla and chicory had similar FEC to those of sheep grazing on the grass/clover swards.

The total egg output of sheep parasitised with *T.colubriformis* was similar on days 35 and 38 across the grazing forages (Figure 2.3). Although sheep grazing on lotus had lower total egg output compared to sheep grazing on any other forages, the observed difference was not statistically significant (day 38: 804 (694-931), 1,122 (969-1,299), 909 (785-1,053), and 990 (854-1,146) $\times 10^3$ eggs per day in sheep grazing lotus, sulla, chicory and grass/clover respectively).

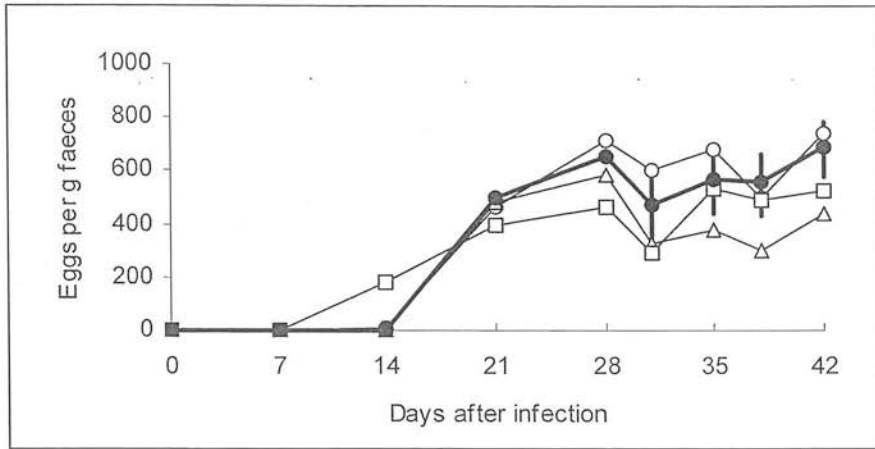


Figure 2.2. Backtransformed means of faecal egg counts (eggs per g faeces) of sheep infected with two single doses of 8,000 L3 of *T.colubriformis* on days 1 and 35 of the experiment and grazing one of the grass/clover (●), chicory (○), lotus (Δ) and sulla (□) swards for a period of two weeks. The vertical bars indicate the confidence intervals (95%) of the control group.

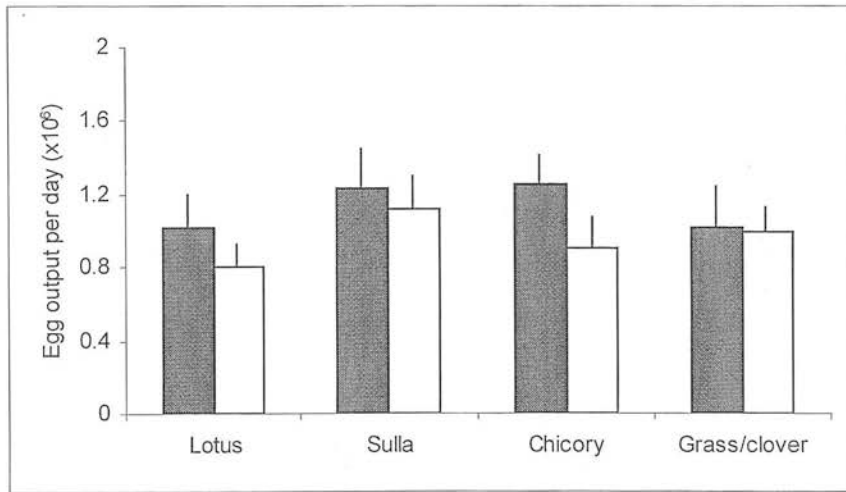


Figure 2.3 Backtransformed total egg output on days 35 (■) and 38 (□) of the experiment of sheep infected with two single doses of 8,000 L3 of *T.colubriformis* on days 1 and 35 of the experiment and grazing one of chicory, sulla, lotus, and grass/clover sward for a period of two weeks. The vertical bars indicate the confidence intervals (95%).

The worm counts of sheep grazing on the experimental plots are shown on Figure 2.4. Although the number of adult worms found in sheep on the chicory swards was lower than in sheep grazing on the other forages, the differences were not significant. Similarly, sheep grazing on chicory swards had significantly lower numbers of immature worms compared to sheep grazing on any other swards. No differences were observed in male or female parasite population in sheep grazing the different experimental swards. No differences were found between adult worm numbers derived from the challenge dose on day 1 and the immature numbers originating from the second challenge infection dosed on day 35, in all groups (Figure 2.4).

Per capita fecundity of female worms in sheep grazing chicory was similar to that of sheep grazing on other experimental swards. Eggs per female worm were 0.14 (0.046-0.233); 0.175 (0.081-0.268); 0.297 (0.204-0.390) and 0.186 (0.092-0.279) for lotus, sulla, chicory and grass/clover, respectively.

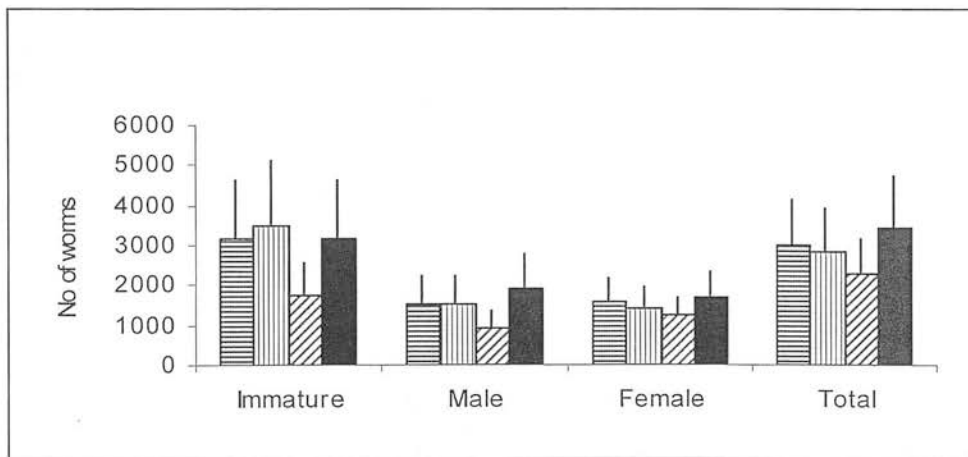


Figure 2.4 Backtransformed immature and adult (male, female and total adult) worm burdens recovered from the gastrointestinal tract of sheep infected with two single doses of 8,000 L3 of *T.colubriformis* on days 1 and 35 of the experiment and grazing one of the lotus (▨),sulla (▧),chicory (▩) and grass/clover (■) swards. The vertical bars indicate the confidence intervals (95%).

2.4 Discussion

The aim of the present study was to examine the effects of a short-term grazing of bioactive forages (chicory, sulla or lotus) on *T. colubriformis* infections in growing lambs. The results showed no evidence of forage effect exerted towards the incoming *T. colubriformis* larvae within a week or against the established adult *T. colubriformis* worms following a two-week grazing period. Although eggs per gram faeces (FEC) of the lotus group tended to be lower ($P < 0.06$) than those of grass/clover group, the daily egg outputs produced over the same period were similar between the groups. Therefore, the reduced FEC observed on this group was attributed to dilution effect of the faecal output rather than to a forage effect on either female worm numbers or female fecundity.

The results of the tannin-rich forages, in the present experiment, contradicts previous studies suggesting that grazing on tannin-rich forages, such as sulla, can result in a reduction of the level of parasitism in grazing sheep (Niezen et al., 1994, 1995). However, the observed reductions on these studies may be attributed to an enhanced immune response of the animals grazing the bioactive forages, whereas such effector mechanism has been minimised by the design of the current experiment. In the study presented here, parasite-naive lambs were infected with two single doses of 8,000 L3 *T. colubriformis*, administered on day 1 and day 35 of the experiment respectively. On day 42 (slaughter day) the worm establishment of both the primary and secondary challenge infections were around 35% of the worms administered. As there was no reduction in parasite establishment of the second challenge dose in animals grazing grass/clover, it is reasonable to suggest that no effective immunity against *T. colubriformis* had been acquired at that point in time by those animals. On the contrary, in the studies of Niezen et al., (1994, 1995), it is possible that the reduction on the level of parasitism observed was the result of the sheep acquired immunity. In the first study (Niezen et al., 1994) parasite-naive lambs were continuously infected, naturally or experimentally (9,000 L3 *T.colubriformis* per

week), for a six-week period and slaughtered 9 weeks post infection. Given that the immunity against *T. colubriformis* becomes apparent after 8 weeks of comparable continuous infection (7,000 L3 *T.colubriformis* per week; Dobson et al., 1990, 1992), it is possible that immune related effects were present at the end point of that trial. In the second study (Niezen et al., 1995), five- or six-month-old sheep had been grazed post-weaning on grass/clover and had acquired a natural worm burden prior to the commencement of the trial. These animals were challenged with a single dose of 20,000 L3 *T. colubriformis* on day 1 of the experiment and were grazed either sulla or lucerne for six weeks. They were killed 42 days later for worm recoveries. It is therefore possible that the reduced worm numbers in the sulla group, compared to the lucerne one, was due, either wholly or partly, to immunologically mediated responses. The observed effect, as suggested by the authors, was attributed to sulla CT. Tannins from sulla may decrease protein degradation in the rumen, increase protein availability to the host and consequently reduce worm burdens, since protein supplementation is associated with improved immune responses against gastrointestinal parasites (Bown et al., 1991; van Houtert et al., 1995; Houdijk et al., 2001). The present experimental design minimised such nutritional effects on immunity and the absence of any effect on worm burdens was more likely due to the lack of a direct anthelmintic effect of tannin-rich forages sulla and lotus.

However, the lack of evidence on a direct anthelmintic effect of tannin-rich forages, in the present study, can be attributed to low levels of CT concentration in sheep digesta. Based on herbage intake and the CT content of the experimental swards, sheep grazing on lotus and sulla ingested around 21 and 25 g CT per day, respectively. This relatively low CT content (Table 2.1) was partly the outcome of weed infestation in the swards, which resulted in dilution of the tannin-rich forages, although other factors, such as the geographical area or seasonal effects upon plant PSMs, may have contributed to low CT levels (Mueller-Harvey, 1999). Previous evidence from studies using housed artificially infected sheep has shown that the

minimum concentration of CT that was tested and found active against *T. colubriformis* intestinal nematodes was 35 g per day (Athanasiadou et al., 2000a,b, 2001a). Although the CT used in the studies of Athanasiadou et al. was from a different source (quebracho tannins from the tropical tree *Schinopsis*), the findings from that study support the view that the amount of CT ingested in the present study was insufficient to initiate large scale direct anthelmintic effects against *T. colubriformis*.

The present study also tested the effects of short term grazing on chicory, which is a PSM-rich forage as indicated by the higher phenolic content (Table 2.1) and suggested by previous studies (Rees and Harborne, 1985). Although this forage had a high representation in the experimental swards (70% of the sward DM) and resulted in high sheep feed intake (1.49 kg DM per day), no direct anthelmintic effect was exerted against incoming or established *T. colubriformis* populations. Similar results have been reported previously (Niezen et al., 1994; Scales et al., 1994; Marley et al., 2003b), where chicory forage was used for longer time periods. In those studies, sheep, grazing chicory swards and infected with a mixed population of abomasal and intestinal nematodes, showed reduced abomasal worm burdens but no reductions in the intestinal *T. colubriformis* worm burdens. From the evidence available to date from the *in vivo* studies, chicory has shown specificity towards gastric parasites affecting the abomasal worm species, through direct-anthelmintic and/or indirect immunologically mediated mechanisms, but not the intestinal nematodes.

Currently, potential direct anthelmintic effects of bioactive forages has been mainly obtained from *in vitro* studies, where PSM compounds extracted from chicory (Molan et al., 2003) lotus and sulla (Molan et al. 2000, 2000b, 2002; Schreurs et al. 2004) reduced egg hatching and larvae migration, development and viability of several nematode species (Cervine: *Dictyocaulus* and *Ostertagia*; and Ovine: *Haemonchus contortus*, *Trichostrongylus*

colubriformis and *Teladorsagia circumcincta*). However, despite the evidence deriving from *in vitro* studies, no direct anthelmintic effect was obtained in the present study with sheep grazing the bioactive forages. This could be due to variable concentrations of the anthelmintic forage compounds within grazing seasons or due to the different conditions that apply between *in vitro* and *in vivo* studies which may affect the bioavailability of the active compounds (Terril et al., 1994). Such discrepancies between *in vivo* and *in vitro* studies have been reported previously (Athanasiadou et al., 2001), and it seems likely that under certain circumstances parasitised animals might not be able to experience the beneficial effects of anthelmintic compounds observed *in vitro*.

In conclusion, the present study investigated the potential effects of a two-week grazing period on either chicory or on the tannin rich forages sulla and lotus towards an intestinal nematode. There was no evidence for any effect of the forages tested towards an establishing or an established *T. colubriformis* population. The lack of evidence of bioactive effect in this short-term study was more likely due to an absence of a direct anthelmintic effect of PSM of these forages. However, this outcome can not be generalised, as the PSM content of these forages, and likely substances with anthelmintic properties, can be variable within and across grazing seasons (Muller-Harvey, 1999). Further long-term studies examining the effects of the bioactive forages upon immunity against different gastrointestinal nematode species are required to determine their true potential for incorporation in parasite control strategies.

Chapter Three

**The effects of short-term grazing of bioactive forages on
established adult and incoming larvae populations of
Teladorsagia circumcincta in lambs.**

Abstract

The objective of this study was to investigate the consequences of short-term grazing on bioactive forages on a) the worm numbers and fecundity of established adult *Teladorsagia circumcincta* population and b) the establishment and development of incoming *T. circumcincta* infective larvae. Forty-eight, parasite naive, three-month old, grazing lambs were artificially infected with 8,000 infective larvae of *T. circumcincta* on day 1 of the experiment. On day 21, post infection, lambs were allocated to one of three bioactive forage grazing treatments; chicory (*Cichorium intybus*), sulla (*Hedysarum coronarium*), lotus (*Lotus pedunculatus*), and the control grass/clover (*Lolium perenne/Trifolium repens*) forage (twelve animals per group). On day 28 of the experiment a second dose of 8,000 *T. circumcincta* infective larvae was administered to the lambs to investigate the effects of forages on the ability of infective larvae to establish within the host. All animals were slaughtered for worm recovery on day 35, while liveweight gain, faecal egg counts (FEC) and total worm egg output were monitored regularly throughout the experiment. Although FEC or total egg output were similar among the groups, adult worm burdens at slaughter were significantly affected ($P < 0.05$) by forage treatment during the two week grazing period. Lambs grazing chicory had the lowest adult worm burdens and significantly lower numbers of male worms compared to those grazing on grass/clover ($P < 0.01$), while the lambs grazing on sulla or lotus had similar adult populations to grass/clover fed animals. The results from the worm recoveries of the second dose (immature worm burdens) were affected by the emergence of acquired immunity, which reduced larval establishment in all treatments. Nevertheless, immature worm burdens at slaughter were similar between chicory, sulla and grass/clover group, while the immature worm recoveries from the lotus group were significantly higher ($P < 0.05$) compared to those from lambs grazing grass/clover. Overall, the results of the present study support the view that chicory can be a promising candidate species in pasture management practices to control *T. circumcincta* burdens.

Keywords: *Teladorsagia circumcincta*; Sheep; Nematoda; *Cichorium intybus* ; *Hedysarum coronarium*; *Lotus pedunculatus*

3.1 Introduction

The need for alternative, non chemical, control strategies in sheep production systems has increased in the last decade due to development of anthelmintic resistant strains of parasitic nematodes (Jackson and Coop, 2000). Proposed alternative approaches against gastrointestinal nematode infections include vaccination (Meeusen and Piedrafita, 2003), nematophagous fungi (Larsen, 1999), exploitation of genetic resistance of sheep (Bishop et al., 2004), dietary supplementation of growing lambs (Steel, 2003) and the use of bioactive forages (Waller and Thamsborg, 2004; Nguyen et al., 2005).

For *Teladorsagia circumcincta* infections, field studies have suggested that grazing sheep on certain bioactive forages can reduce their worm burdens compared to sheep grazing on conventional forages. Sheep grazing on chicory (*Cichorium intybus*) have reduced *T. circumcincta* burdens in mixed parasite infections (Scales et al., 1994; Marley et al., 2003b), while similar reductions have been observed in sheep given access to tanniferous legumes like sulla (*Hedysarum coronarium*) (Niezen et al., 1994; Niezen et al., 2002a), lotus (*Lotus pedunculatus*) (Niezen et al., 1998a) and birdsfoot trefoil (*Lotus corniculatus*) (Marley et al., 2003b). The mode of action of these forages is not known, but it is suggested to be either a direct anthelmintic-like effect or an indirect effect as a consequence of nutritional effects upon immunity and/or resilience. *In vitro* studies using compounds extracted from chicory (Molan et al., 2003), lotus, sulla and sainfoin (*Onobrychis viciifolia*) (Molan et al., 2000a; Molan et al., 2000b; Molan et al., 2002) have demonstrated an anthelmintic like effect against immature stages of several nematode species (Cervine *Dictyocaulus* and *Ostertagia*; and Ovine *Haemonchus contortus*, *Trichostrongylus colubriformis* and *T. circumcincta*). To date, there is no report studying *in vivo* the bioactive forage effects on specific developmental stages of *T. circumcincta*. Such study could enhance knowledge of the mechanism of bioactive-forage action as well as suggest opportunities for optimum pasture management practices.

This study used a short-term grazing trial and artificially infected growing lambs to examine the effects of selected bioactive forages on i) the worm numbers and fecundity of adult *T. circumcincta* already established in the lamb abomasum and ii) the ability of incoming L3 *T. circumcincta* larvae to establish and develop within the host. The bioactive forages used, were *C. intybus* (chicory), *L. pedunculatus* (lotus; greater lotus), *O. viciifolia* (sainfoin) and *H. coronarium* (sulla).

3.2 Materials and Methods

3.2.1 Experimental plots

In spring 2001, ten experimental plots of 0.1 ha each were established on fields that had never previously been used for grazing livestock (parasite free pastures). Replicate plots of 0.1 ha were sown as monocultures using the following rates: *C. intybus* (chicory) 7 kg/ha, *H. coronarium* (sulla) 15 kg/ha, *O. viciifolia* (sainfoin) 50 kg/ha, *L. pedunculatus* (lotus) 12.5 kg/ha and *Lolium perenne*/*Trifolium repens* (grass/clover) 37 kg/ha. The grass/clover plots were the control treatment against which all other plots were compared. Nitrogen fertiliser (Yara UK Ltd.; N < 50 kg/ha) was applied to all experimental plots in late August 2001, while in October 2001 all forages were topped to overwinter. In April 2002, all species started to re-grow, with the exception of sulla, which had to be re-sown. In July 2002, nitrogen fertiliser and a phosphorus-potassium mix (Yara UK Ltd.; 100 kg/ha) were applied, following soil analysis. All swards were in the vegetative stage of growth throughout the experiment. No herbicides were used throughout the pre- and experimental period.

3.2.2 Animals

Thirty-one female and twenty-nine castrated male Texel x Scottish Greyface lambs were used. They were reared outdoors until 3 months of age (from April to July 2002), on grass pastures that had been grazed by cattle for more than a year. Prior to weaning, lambs were treated orally with levamisole (Levacide 3% drench; Norbrook Laboratories Ltd., UK; 7.5 mgs/kg liveweight). All lambs were also treated with ivermectin (Oramec drench, Merial Animal Health Ltd.; 0.2 mg/kg liveweight), following weaning, at ten weeks of age (10th of July) and moved to clean grass/clover pasture until the start of the experiment (23rd of July).

3.2.3 Parasites

The infective larvae used were of a strain of *T. circumcincta*, which has been

maintained at the Moredun Research Institute for 5 years. Larvae were harvested from faeces of a monospecifically infected donor sheep using a standard Baerman procedure. Following harvesting, larvae (L3) were stored at 4°C in tap water (1,000 L3 / ml) and used within three weeks of collection.

3.2.4 Experimental design

On day 1 of the experiment, all lambs were infected with a single dose of 8,000 L3 *T. circumcincta*, weighed and faecal sampled. The larval dose (8,000 L3) was based on the recommendations of the World Association of the Advancement of Veterinary Parasitology for the size of infection to evaluate the efficacy of anthelmintics (Wood et al., 1995). From days 1 to 21, lambs remained on the parasite-free grass/clover pastures. On day 21, they were allocated to one of the five treatment groups (twelve animals per forage) based on both their liveweight and faecal egg counts on day 14 (mean \pm S.E.M.: 33.5 \pm 0.66 kg and 16 \pm 7 eggs/g, respectively). On day 28, a second single dose of 8,000 L3 *T. circumcincta* was administered to the animals to investigate the effects of the bioactive forages on the establishment of infective L3. The animals remained on the experimental plots until day 35, when they were slaughtered for worm recovery (26 August 2002).

3.2.5 Sward measurements

Pre-grazing sward composition of the experimental plots was estimated by taking 20 randomised sward cuts from each plot (0.1 ha) on day 21 of the trial, using a herbage-gripping device (Barthram et al., 2000). The cut material from each plot was placed in nylon bags in the field and was later separated manually to the following fractions: the sown species of the plot, dead material and “other” species. Each component was separately weighed, oven-dried (60 °C for 18 h) and re-weighed. Sown species representation in the plot was calculated as the dry matter (DM) percentage of the sown forage in the total sward DM of the plot. Sward heights

were taken one day before lambs entered the experimental plots (day 21) and on the last day of the experiment (day 35) by taking 20 measurements from each plot using a sward stick (Barthram, 1985).

The samples from the experimental plots were also used for the chemical analysis of the swards. The cut samples from each plot were pooled and analysed in duplicates for determination of crude protein (Kjeldahl method), total phenolics (Price and Butler, 1977), extractable condensed tannin content (Porter et al., 1985) and in vitro dry matter digestibility (IVDMD) (Tilley and Terry, 1963).

3.2.6 Animal measurements

The weights of the animals were taken at the start of the experiment (day 1) and at weekly intervals thereafter. In order to evaluate the effects of different forage species on lamb performance, the liveweight gain (g/day) was estimated for each animal during the period from day 21 to day 35. Faecal output was determined for each animal using n-alkanes (C-36) as indigestible markers according to the technique described by Mayes et al. (1986). One alkane pellet (C-36) was administered to each lamb every day for two consecutive weeks (days 21-32). Rectal faecal samples (10-15 g of weight) were collected during the second week (days 28-32), pooled per animal, oven dried for 24 hours (100 °C), and ground. Duplicate sub-samples (0.5g each) of the dried faeces were analysed for alkane determination in faeces (F36). Six alkane pellets were subjected to the same analytical procedures (Mayes et al., 1986) to determine the amounts of dosed alkane per pellet (D36) and faecal output was then calculated using the following equations:

$$\text{Faecal output (kg DM / day)} = (\text{D36} * 0.95) / \text{F36}$$

where D36 and F36 dosed amount (mg/day) and faecal concentration (mg/kg DM) of alkane C-36, respectively, 0.95 is the correction factor for incomplete faecal recovery of C-36.

For the faecal worm egg count (FEC) determination, faecal samples were taken from the rectum of all the lambs at weekly intervals between days 1 to 21 and twice a week thereafter (days 21 to 35). The samples were processed either immediately or stored at 4 °C and then processed within 2 days. The number of eggs per gram of fresh faeces were determined using a modified flotation technique (Christie and Jackson, 1982) in which polyallomer centrifuge tubes (Beckman) were used to separate the egg bearing layer after flotation in saturated sodium chloride solution. In order to estimate total worm egg output, FEC's were multiplied by the faecal output.

The lambs were slaughtered on day 35 of the experiment and immediately after their abomasa were ligated and removed. Adult and immature stages of *T. circumcincta* were recovered from digesta and abomasal mucosa following a 4-hour incubation in saline at 37 °C (Jackson et al., 1984). Adult and immature total worm burdens were estimated from 2% (v/v) aliquots of digesta and mucosal digest for each lamb. Per capita fecundity of female worms was calculated by dividing the FEC recorded at the last day of the experiment by the total female worm numbers recovered.

3.2.7 Statistical analysis

Liveweight gain (g/day) for each animal was estimated by linear regression of the weights on days 21, 28, 35 (least square method) and are reported as arithmetic means (\pm S.E.M.). Worm numbers, FEC, total worm egg output and per capita fecundity were log-transformed ($\log_{10}(x+1)$) before the statistical analysis to remove positive skewness and are reported as back-transformed means with 95% confidence intervals (lower and upper limit). The effects of the feeding treatments on liveweight gain, faecal output, FEC, total worm egg output, worm numbers and per capita fecundity of the female worms were investigated using one-way

analysis of variance (Zar, 1984). Any apparent treatment effects were further investigated using Dunnett's test (Minitab Inc., 2000 MINITAB Statistical Software, Release 14 for Windows, State College, Pennsylvania) with grass/clover treatment as the control. No statistical analysis was performed on the sward data since these measurements aimed only to describe the plots.

3.3 Results

3.3.1 Sward measurements

Sward chemical analysis and sward composition of the experimental plots are shown in Table 3.1 and Figure 3.1, respectively. Legume forages had higher extractable CT content (15.8 and 16 g/kg DM for sulla and lotus, respectively) than the grass/clover or chicory (CT extractable values less than 0.5 g/kg DM), while chicory swards had the highest concentration of total phenolics (262 g/kg DM) compared to other swards. Sward composition results revealed differences in viability of the sown forages. Chicory, sulla, lotus and grass/clover represented 87, 73, 60 and 80% of the sward DM, respectively, while sainfoin forage was only the 3% of the collected sample and therefore the sward chemical analysis and the animal measurements for this treatment are excluded. Sward heights were similar among the different plots and were reduced after the two-week grazing period across all plots. Overall mean (\pm S.E.M.) on day 21 was 16.0 (\pm 4.77), which was reduced to 4.8 (\pm 2.02) by the end of the experiment (day 35).

Table 3.1. Mean values of two determinations of dry matter (DM), crude protein (CP), condensed tannin (CT) and total phenolic (TP) content as well as in vitro dry matter digestibility (IVDMD) of the samples collected from grass/clover, chicory, sulla, and lotus experimental plots.

	Grass/clover	Chicory	Sulla	Lotus
DM (g/kg fresh matter)	235	137	171	166
CP (g/kg DM)	97	96	73	195
CT (g/kg DM)	0.3	0.5	15.8	16.0
TP (g/kg DM)	130	262	159	167
IVDMD (g/kg DM)	689	765	682	571

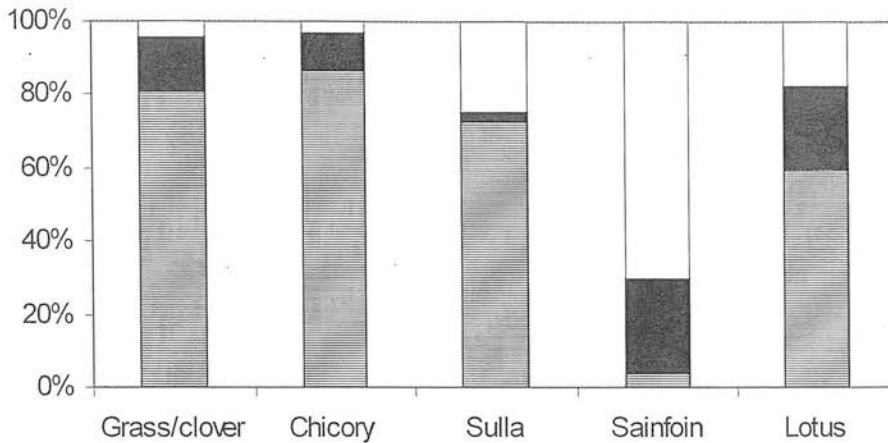


Figure 3.1. Pre-grazing proportion (% of the dry matter) of the sown species (▨), *Lolium perenne*/*Trifolium repens*, *Cichorium intybus*, *Hedysarum coronarium*, *Onobrychis viciifolia* and *Lotus pedunculatus* in grass/clover, chicory, sulla, sainfoin and lotus experimental plots, respectively. Percentages of the dead (■) or other forage species (□) in the experimental plots are also shown.

3.3.2 Animal measurements

The mean liveweight of the lambs on the day 21 of the experiment was 33.7 ± 0.59 kg (overall mean \pm S.E.M.) and their growth rate remained similar among the different grazing groups during the following two-week period (overall mean \pm S.E.M.: 231 ± 26.2 g/day). Faecal output was significantly ($P < 0.001$) affected by the forage treatment with the lambs grazing on grass/clover having lower faecal output (292 g DM/day) compared to lambs grazing on chicory, sulla and lotus (mean values of 447, 439 and 528 g DM per day, respectively).

FEC's during the course of the experiment are shown in Figure 3.2. Worm eggs were first observed in the faeces of parasitised lambs on day 14, rising and reaching 105 eggs/gram (overall backtransformed mean) at day 21, when animals moved into experimental plots. The FEC in all groups had large variation within groups and there were no significant differences between the group means at any time point (days 24, 28, 31, and 35).

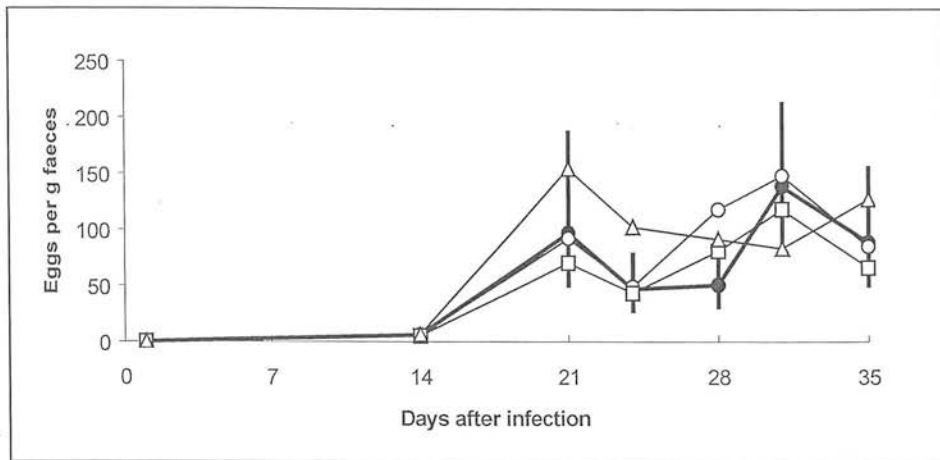


Figure 3.2. Backtransformed means of faecal egg counts (eggs per g faeces) of lambs infected with two single doses of 8,000 L3 of *Teladorsagia circumcincta* on days 1 and 28 of the experiment and grazing one of the grass/clover (●), chicory (○), lotus (Δ) and sulla (□) sward for a period of two weeks. The vertical bars indicate the confidence intervals (95%).

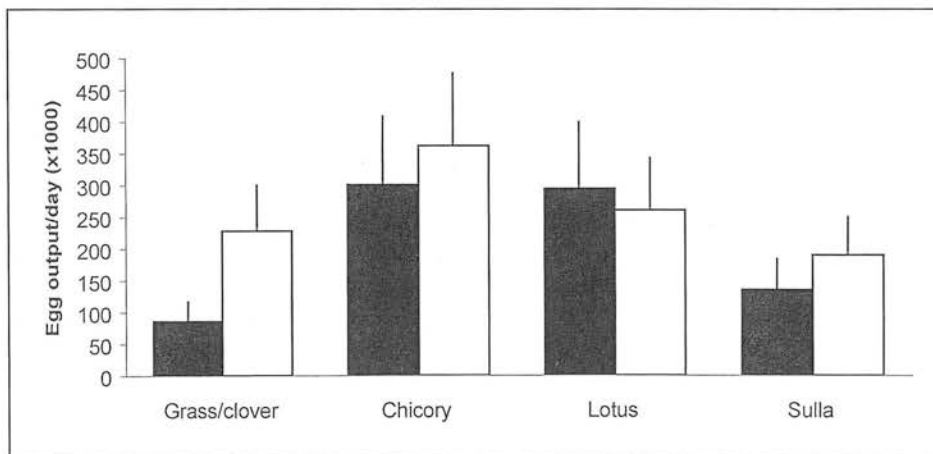


Figure 3.3. Backtransformed means of the total worm egg output on days 28 (■) and 31 (□) of the experiment. Lambs were infected with two single doses of 8,000 L3 of *Teladorsagia circumcincta* on days 1 and 28 of the experiment and grazing one of grass/clover, chicory, lotus and sulla sward for a period of two weeks. The vertical bars indicate the confidence intervals (95%).

Since the faecal output varied significantly among the lambs grazing on different forages, ranging from 292 g DM/day in grass/clover treatment to 528 g DM/day in lotus, the total worm egg output on days 28 and 31 of the experiment was estimated (Figure 3.3). Statistical analysis showed no significant differences in the total worm egg output between the treatments.

The immature worm burdens recovered from the lambs at the end of the experiment (day 35) were affected by forage treatment offered ($P < 0.01$) (Figure 3.4). The lowest immature worm numbers were recorded in lambs on sulla, whereas the highest were found in lambs grazing lotus. Comparisons with the control, grass/clover group, revealed no significant differences between sulla, chicory and grass/clover immature worm burdens. However, immature worm recoveries from lambs grazed on lotus were significantly higher ($P < 0.05$) than grass/clover lambs.

The bioactive forages significantly ($P < 0.05$) affected the number of adult worms recovered. Comparisons with the control showed that total adult worm burdens of lambs grazing chicory tended to be lower from those recovered from lambs grazing grass/clover (2,543 (2,033-2,582) vs. 4,426 (3,511-5,580) respectively; $P = 0.14$), and this effect was more profound in the number of adult male worms recovered ($P < 0.01$). By way of contrast, the adult worm burdens recovered from lambs grazing on sulla and lotus were not different to those in animals grazing on grass/clover (Figure 3.4).

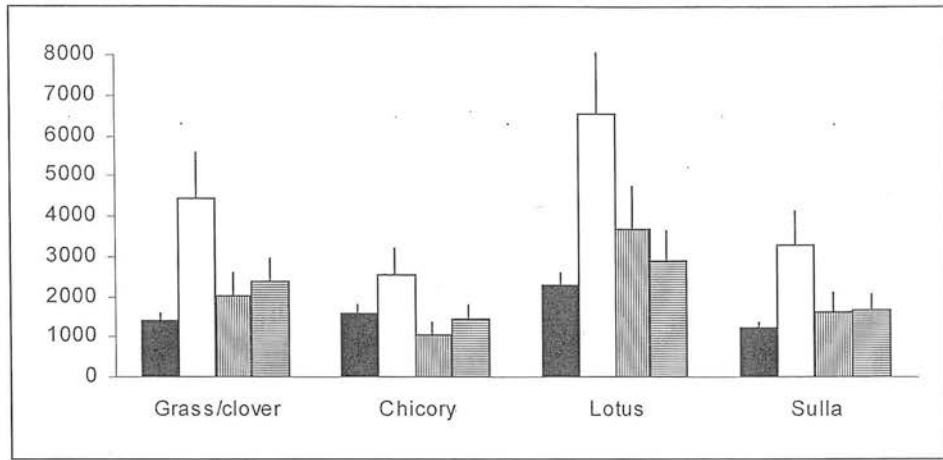


Figure 3.4. Backtransformed means of immature (■), total adult (□), male adult (▨) and female adult (▩) worm burdens recovered from the gastrointestinal tract of lambs infected with two single doses of 8,000 L3 of *Teladorsagia circumcincta* on days 1 and 28 of the experiment and grazing one of the grass/clover, chicory, lotus and sulla sward. The vertical bars indicate the confidence intervals (95%).

Comparing the adult worm numbers derived from the single dose of 8,000 L3 *T. circumcincta* at the start of the experiment (day 1) and surviving after introduction to the forages with the immature population originating from the second single dose of 8,000 L3 *T. circumcincta* (day 28), reduction in worm establishment was found in all groups. Lotus, sulla and grass/clover, had significant ($P < 0.05$) reductions of around 65%, 63% and 68%, respectively, while the chicory-fed animals had a reduction of 37% (non significant).

Per capita fecundity of female worms in lambs offered the bioactive forages was not significantly different from that of the controls. Eggs per female worm were 0.062 (0.051-0.073); 0.076 (0.065-0.087); 0.052 (0.041-0.063) and 0.054 (0.043-0.065) for sulla, chicory, lotus and grass/clover, respectively.

3.4 Discussion

The results from the present study examining the effects of different forages on adult and immature *T. circumcincta*, have shown differences between the effects of the different forage crops. Adult worm recoveries at slaughter indicated that in a two week grazing period, chicory fed lambs tended to have lower adult burdens and significantly reduced numbers of male adult worms compared to lambs grazing on grass/clover pastures. Previous grazing studies (Scales et al., 1994; Marley et al., 2003b) using chicory and mixed natural infections have also shown reduced *T. circumcincta* burdens in growing lambs compared to use of conventional forages, whilst there was no reduction in the intestinal worm *Trichostrongylus* spp. burdens of the same animals. These results suggest differences in species susceptibility to the effects of chicory and are further supported by the absence of any effect of chicory on *Trichostrongylus colubriformis* in mono-specifically infected lambs (Chapter Two).

The mechanism(s) responsible for these reduced *T. circumcincta* worm burdens in chicory fed lambs are not clear. Possible explanations for the observed effects would include (i) direct, anthelmintic-like effects of chicory compounds and/or (ii) possible indirect effects through enhanced immune responses. In a previous grazing trial, where animals were exposed to infection from pasture (Scales et al., 1994), a third mechanism may have been implicated; the effects of chicory on larval development, migration and survival on the pasture (Scales et al., 1994; Marley et al., 2003b). Since the present study used artificially infected lambs grazing clean pastures, this third mechanism cannot apply. However, the present study could not determine whether the observed effects on adult worm burdens were direct anthelmintic effects or indirect through an enhanced immunologically mediated response that may have become established in the lambs within the 5 week period of the trial. A previous study using continuously infected lambs challenged with *T. circumcincta* (Seaton et al., 1989) showed maximum worm recruitment in animals killed after 6 weeks of exposure to infection when

almost 40% of the challenge dose was recovered as established worms. In the current study average total adult worms present after exposure to bioactive forages ranged from 32% (Chicory) to over 81% for lotus compared to 55% of the challenge dose in the grass/clover control animals. This suggests that there were no immune mediated effects against the adult worms in the controls and the lotus fed animals, while the reduced male worm burdens in the chicory group may have been attributable to either a direct anthelmintic or an indirect nutritional mediated immunoregulatory or immunomodulatory effect. Chicory is a plant rich in secondary metabolites as indicated by the higher concentration of total phenolics (Table 3.1) and suggested by previous studies (Rees and Harborne, 1985). The role of these metabolites in parasite-nutrition interaction is still not known and further research is needed to identify their *in vivo* properties. Nevertheless, irrespective of the mechanism, the lower adult burdens and significantly reduced male worm numbers reported here suggest that chicory may offer a possible future opportunity for manipulating *T. circumcincta* infections in sheep.

In contrast to chicory, the results from two-weeks grazing of lotus or sulla did not affect the established adult worm populations compared to those grazing on grass/clover. These results contradict previous *in vivo* studies, which showed “anti-parasitic” effects of tannin-containing legumes on *T. circumcincta* populations. Lambs grazing lotus had reduced *T. circumcincta* burdens compared to the lambs grazing grass (*Lolium perenne*), in a 5-week experiment (Niezen et al., 1998a), while other, 6-week long trials, suggested that growing lambs had reduced *T. circumcincta* worms when grazed on sulla compared to grass/clover (*L. perenne/T. repens*) (Niezen et al., 1994) or lucerne (*Medicago sativa*) (Niezen et al., 2002a). The lack of any effect on established adult *T. circumcincta* populations in the present study might be attributed to the shorter length of exposure (only 2 weeks) or to low levels of CT in the experimental swards. As shown in Table 3.1, the extractable CT content of the samples collected from lotus and sulla plots were 16 and 15.8 g/kg DM, respectively. The chemical analysis of the swards in the

previously cited studies showed much higher extractable CT values than those observed in the present study. Reduced *T. circumcincta* burdens were observed in lambs grazing lotus (56 g/kg DM extractable CT content, Niezen et al. 1998a) or sulla (31 g/kg DM total CT content, Niezen et al. 2002a) in studies where animals grazed 5 and 6 weeks, respectively. Chemical analyses of the same species of plants in other studies have also demonstrated around twice the CT content observed in the present study. Lotus CT extractable content was 59 ± 0.4 g/kg DM (mean of six determination \pm S.E.M.; Terrill et al. 1992b), while sulla CT extractable fraction was 23 ± 1.1 , 29 ± 1.5 and 33.4 ± 0.6 g/kg DM (mean of six determination \pm S.E.M.) in three different experiments, respectively (Terrill et al. 1992a,b). Difference in CT levels of the sward DM could be attributed to i) seasonal changes, ii) geographical area of the trial, iii) changes during leaf development (Mueller-Harvey, 1999) or iv) due to weed infestation and poor agronomic viability of these Mediterranean legumes in the present experimental plots, prohibiting any definitive conclusions to be drawn.

The present study also tested the effects of bioactive forages, lotus, sulla or chicory, on incoming infective *T. circumcincta* larvae. As shown in Figure 3.4, the establishment of the second infection of 8,000 L3 (immature worms) measured on one week post challenge was reduced in comparison to the establishment figures of the first challenge infection (adult worms). These reductions could be due to (i) regulation of establishment of incoming larvae by the established adults, (ii) immunologically mediated mechanisms acting against third and fourth larval stages or (iii) due to differences between larvae batch infectivity, which has not been tested using infectivity control lambs in the present experiment. Nevertheless, the results from the immature worm recoveries at slaughter showed that short-term grazing on either chicory or tanniferous legumes (lotus and sulla) did not reduce the establishment of incoming *T. circumcincta* larvae compared with grass/clover treatment. In contrast, immature worm burdens recovered from animals grazed on lotus were significantly higher ($P < 0.05$) compared

to the animals grazed on grass/clover. However, the design of the current study makes it impossible to determine whether the differences between the groups are attributable to different establishment rates or rates of loss of established worms and whether those differences are mediated directly or are the result of an indirectly mediated mechanism. Further research examining population changes over time would be required in order to determine if these effects have an anthelmintic or nutritional/immunological aetiology.

Other results from the present study such as liveweight, FEC, total worm egg output and per capita worm fecundity of the parasitised lambs did not show any differences between the treatments over the two-week grazing period. Liveweight gain was similar in all treatments, a result that is not surprising given the short-term grazing on different feeding treatments. The FEC measurements did not show any tendency of reduced worm eggs in the chicory group compared to the grass/clover group. This was probably due to significantly reduced male and not female worms (Figure 3.4). In addition, FEC are usually considered to give poor correlation with levels of infections for the less fecund abomasal parasites such as *T. circumcincta* (Jackson and Christie, 1979; Stear et al., 1996; Eysker and Ploeger, 2000).

The present study investigated the effects of short-term grazing of selected bioactive forages on different developmental stages of *T. circumcincta*. The results suggest that within a two-week grazing period, chicory reduced the established adult worm population but there was little evidence for similar effects on incoming larvae. Although, the effects of the bioactive forages on sheep gastric parasites were relatively small in the short term of this trial, the possibility that there may also be long term benefits in terms of reduced parasitism and increased productivity supports the need for further research in this area.

Chapter Four

**The effects of sequential short-term grazing of different
bioactive forages on established adult population of
Teladorsagia circumcincta in lambs**

Abstract

The aim of the experiment was to test the effects of sequential short-term grazing on the bioactive forages, chicory (*Cichorium intybus*) and sulla (*Hedysarum coronarium*), towards an established adult *Teladorsagia circumcincta* worm population of growing lambs. Forty-eight, parasite naive, three-month-old, Texel-cross lambs were each dosed with 30,000 infective larvae of *T. circumcincta* at the start of the experiment and given access to worm-free grass/clover pastures for a two-week period. On day 14, the animals were randomly allocated to one of four groups balanced for sex and liveweight and moved into experimental grazing plots for the following four weeks. During the first two-weeks (period I: days 14-28) lambs grazed on either chicory (C) or grass/clover (GC) plots (24 animals per forage group). For the following two-weeks (period II: days 29-42), lambs were moved into either sulla (S) or grass/clover (GC) experimental plots according to the design (period I-II): C-S, C-GC, GC-S, GC-GC. All animals were slaughtered on day 42 for worm recovery, whilst measurements of live-weights and faecal egg counts (FEC) were monitored regularly. During period I, lambs grazing on chicory had reduced FEC compared to lambs grazing grass/clover. No additional benefits in terms of FEC were observed when sulla was grazed for the following two-weeks (period II). On the last day of the experiment (day 42), no effect of grazing treatment was detected on either worm numbers or worm per capita fecundity. A decline in FEC during the period II and a low worm establishment rate (overall mean establishment of 13%) were observed across all groups. Lamb growth rates during the four weeks grazing (period I and II: days 14-42), was not affected by the grazing treatments and the overall liveweight gains averaged 220 g/day. The results suggest that grazing on chicory may reduce FEC of *T. circumcincta* infected lambs, while provide little evidence of additional benefits of short-term grazing on sulla towards established adult worm populations.

Keywords: bioactive forages, *Cichorium intybus*, *Hedysarum coronarium*, single infection, *Teladorsagia circumcincta*

4.1 Introduction

The development of anthelmintic resistant strains amongst the major nematode parasite species of small ruminants threatens the sustainability of sheep production systems (Waller 1997, 2003, Sangster 1999). Alternative, non-chemotherapeutic strategies, such as grazing management practices or the use of bioactive forages have been proposed to compliment anthelmintics for nematode control (Waller and Thamsborg 2004). Management grazing strategies, such as clean or mixed grazing improve the efficiency of parasite control in livestock (Barger, 1997), whilst the use of bioactive forages, such as taniferous legumes or chicory (*Cichorium intybus*), has been reported to lower the parasite worm numbers or worm egg excretion (Niezen et al., 1994, 1995, 1998a,b, 2002; Scales et al., 1994; Marley et al., 2003b; Chapter Three).

Since bioactive forages are not yet used in conventional grazing systems, their establishment requires extra cost and special knowledge associated with their agronomic management. It is therefore considered that their incorporation into existing grazing regimes is likely to be confined to short-term grazing as de-worming paddocks (Coop and Kyriazakis, 2001). In a previous experiment (Chapter Three), two-weeks grazing on chicory showed promising results in reducing *Teladorsagia circumcincta* worm burdens in growing lambs. The objective of the present experiment was to test whether animals carrying an established adult *T. circumcincta* worm population and previously grazing on chicory would further benefit from a consecutive two-week grazing period on sulla (*Hedysarum coronarium*), a forage considered bioactive due to its high condensed tannin (CT) content. *In vitro* studies with CT extracts from sulla, have shown anthelmintic properties against gastrointestinal larvae (Molan et al., 2000a, 2000b, 2002), while *in vivo* trials have supported a nutritional/immunological mediated effect of this forage due to its CT-rich content (Niezen et al., 1995, 1998a,b). CT from sulla have been reported to bind with plant proteins, reduce their degradation in the rumen, and overall increase

the protein supply to the sheep (Min et al., 2003). This increased protein supply may improve the ability of the animals grazing on sulla to cope with parasitism, and also trigger enhanced immune responses against gastrointestinal parasites (Brown et al., 1991; Van Hourtert et al., 1995; Houdijk et al., 2001b).

4.2 Materials and Methods

4.2.1 Experimental plots

The experimental plots (0.1 ha each) were established at the Scottish Agricultural College's Thomson fields (Midlothian, Southeast of Scotland), where a 2.4 ha site was allocated to the experiment. The area, which had been grazed by animals in the previous year was re-ploughed (winter 2003) and cultivated to prepare a firm seedbed (spring 2004). The experimental forages were sown (late spring 2004) using the rates of 7 kg/ha for *C. intybus* (chicory), 15 kg/ha for *H. coronarium* (sulla) and 37 kg/ha for *Lolium perenne*/*Trifolium repens* (grass/clover). Fertiliser (50 kg/ha of P & K; Yara UK Ltd.) was applied to all experimental area, prior to the sowing, while no insecticides or herbicides were used once plots were sown. Livestock fencing divided the experimental plots, all of which had automated water troughs. All swards established well and were in the vegetative stage of growth when the experiment started (24 July 2003).

4.2.2 Experimental design

Twenty-four female and twenty-four castrated male, three-an-a-half-month-old, Texel cross lambs (mean liveweight \pm standard error of the mean (S.E.M): 29.8 ± 0.49 kg) were reared indoors and used in the experiment. All animals were dosed with 15,000 infective larvae of *T. circumcincta* on days 0 and 1 of the experiment (30,000 L3 per animal, in total) and given access to parasite-free grass/clover pastures for a two-week period. On day 14, the animals were randomly allocated to one of four groups (12 animals per group), balanced for sex and liveweight, and moved into the experimental plots for the following four weeks. During the first two-weeks (period I: days 14-28) lambs grazed on either chicory (C) or grass/clover (GC) plots (24 animals per forage group). The following two-weeks (period II: days 29-42), lambs were moved into either sulla (S) or grass/clover (GC) experimental plots according to the design (period I- period II): C-S, C-GC, GC-S and GC-GC, which resulted in four grazing treatments.

All animals were slaughtered on day 42 for worm recovery, while measurements of live-weights and faecal egg counts (FEC) were monitored regularly throughout the experiment.

4.2.3 Parasites

The infective larvae used were of an ovine susceptible strain of *T. circumcincta*, which has been maintained at the Moredun Research Institute for 6 years. Larvae were harvested from faeces of a monospecifically infected donor sheep using a standard Baerman procedure. Following harvesting, L3 larvae were stored at 4°C in tap water (1,000 L3 / ml) and used within three weeks of collection.

4.2.4 Sward measurements

Pre-grazing herbage mass availability and botanical composition of the experimental swards were determined by cutting 2 random quadrats (0.25 m²) per plot to ground level one day before the animals entered the plots. The cut material was separated manually to either the sown species of the sward (forage treatment) or “other material”, such as dead material or other species, present in the plots. The fractions were then weighed, oven-dried (60 °C for 18 h) and re-weighed to determine sown forage representation in the experimental plots. Twenty measurements of pre- and post-grazing sward heights were taken from each plot, one day before and one day after the two-week grazing period, respectively, by using a sward stick device (Bathram, 1985).

The samples taken from the pre-grazed experimental plots were pooled and analysed in duplicates for determination of dry matter (DM), crude protein (CP; Kjeldahl method), extractable condensed tannin (CT; Porter et al. 1985) and neutral cellulase and gamma-nase digestibility (NCGD; MAFF, 1986) value. Estimates of the metabolisable energy (ME) value of all forage species were calculated from the NCGD values, using the equation for the prediction

of ME of grasses (Agricultural and Food Research Council, 1993), since there are no equations available for the bioactive forages tested in the present experiment.

4.2.5 *Animal measurements*

The lambs were weighed weekly throughout the experiment and the daily liveweight gain of each animal over the four week experimental period (days 14-42) was used to estimate the effect of feeding treatment on lamb performance. Faecal samples were taken weekly from the rectum of each lamb for faecal egg counts (FEC, eggs per g faeces) determination using a modified flotation technique described by Christie and Jackson (1982). Faeces were scored for consistency on a scale of 1 to 5, where the score of 1 was given to loose, diarrhoeic faeces. Watery samples, consisting largely of faecal debris were given a score of 2. Soft unformed faeces were given the score of 3 and faeces forming moist or hard dry pellets were given the scores of 4 and 5, respectively. Total daily faecal output was determined for each animal using N-alkane (C-36) as indigestible marker (Mayes et al., 1986; Dove and Mayes, 1991). Total worm egg output on day 42 was calculated by multiplying the daily faecal output with the FEC recorded on that day. Immediately after slaughter and post-mortem, the abomasum and its contents were subjected to a saline digest to harvest *T. circumcincta* worms. Worm numbers were determined from 2% (v/v) aliquots of the digest (Jackson et al., 1984). Female worm per capita fecundity was determined by dividing the total worm egg output estimated at slaughter (day 42) with the total female worms recovered.

4.2.6 *Statistical analysis*

Liveweight gain (g/day) for each animal was estimated by linear regression (least square method) of the cumulative liveweights measured weekly and are reported as arithmetic means (\pm SEM). FEC, total egg output, worm numbers, worm per capita fecundity and daily faecal output values were log-transformed ($\log_{10}(x+1)$) prior to statistical analysis to remove

positive skewness and are reported as backtransformed means with 95% confidence intervals (lower and upper limit). The effects of the feeding treatments on parasitological and production measurements were analysed using one-way analysis of variance (Minitab Inc. 2000. MINITAB Statistical Software, Release 14 for Windows, State College, Pennsylvania). No statistical analysis was performed on the agronomic or chemical composition data since these measurements aimed only to describe the plots and the forages on offer.

4.3 Results

4.3.1 Sward measurements

Sward chemical analysis and botanical composition of the experimental plots are shown in Table 4.1 and Figure 4.1, respectively. Samples collected from chicory plots had higher crude protein and lower dry matter content compared to those of grass/clover or sulla samples. Measurements on samples collected from sulla plots showed higher extractable CT content and lower digestibility and metabolisable energy values compared to the samples collected from the chicory and grass/clover plots (Table 4.1).

Experimental plots contained 1,136-1,540 kg DM per hectare of total sward biomass and the sown species, grass/clover, chicory, and sulla, represented the 89, 81 and 67% of the total sward dry matter, respectively (Figure 4.1). Clover constituted the 7% of the total dry matter in the grass/clover plots. Pre-grazing sward heights averaged (\pm SEM) 13 cm (\pm 0.5), 24 cm (\pm 0.6) and 15 cm (\pm 0.8) for grass/clover, chicory and sulla plots, respectively, and were reduced to 6 cm (\pm 0.2), 9 cm (\pm 0.6) and 4 cm (\pm 0.2), respectively, after the two week grazing period.

Table 4.1 Mean values of dry matter (DM), crude protein (CP), extractable condensed tannin (CT), neutral cellulase and gammanase digestibility (NCGD) and calculated metabolisable energy (ME) of the samples collected from grass/clover, chicory and sulla swards. Each sample was analysed in duplicate and the numbers in the table represent the mean value (\pm standard error of the mean) for 4 samples per forage.

	Grass/clover (n ^a = 8)	Chicory (n ^a = 4)	Sulla (n ^a = 4)
DM (g/kg fresh matter)	299 (\pm 15.8)	131 (\pm 6.3)	232 (\pm 29.3)
CP (g/kg DM)	110 (\pm 11.8)	154 (\pm 19.5)	120 (\pm 6.1)
CT (g/kg DM)	3.9 (\pm 0.98)	1.7 (\pm 0.31)	38.4 (\pm 9.11)
NCGD (g/kg DM)	672 (\pm 25.2)	630 (\pm 19.5)	575 (\pm 14.8)
ME ^b (MJ/kg DM)	10.7 (\pm 0.28)	10.2 (\pm 0.22)	9.6 (\pm 0.17)

^an: number of samples collected

^bME = 3.24 + 0.0111 NCGD (AFRC, 1993)

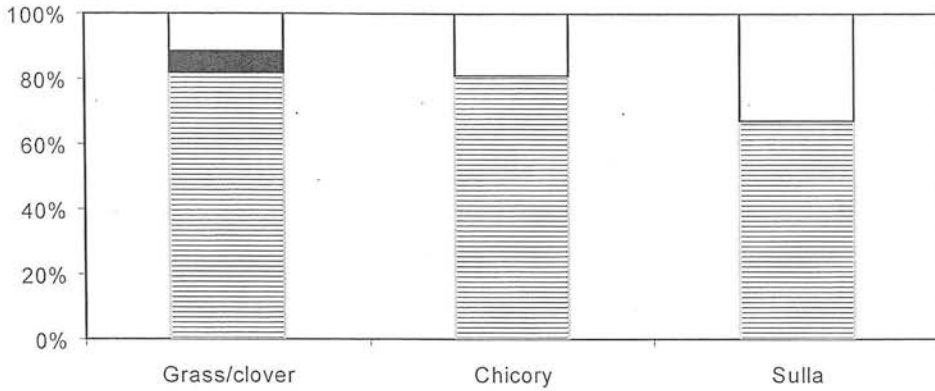


Figure 4.1 Pre-grazing proportion (% of the dry matter) of the main species (horizontal lines), *Lolium perenne*, *Cichorium intybus* and *Hedysarum coronarium* in grass/clover, chicory and sulla experimental plots, respectively. Percentage of the *Trifolium repens* (black) in the grass/clover plots and other material (white), such as dead matter or other species, found in the experimental plots are also shown.

4.3.2 Animal measurements

The mean daily liveweight gain of the different lamb groups during the four-week period (period I and II; days 14-42) is shown in Figure 4.2. No significant differences were observed between treatments, while all lambs gained weight during the experiment (average value \pm SEM: 5.3 ± 0.42 kg). No overt clinical symptoms of parasitic gastro-enteritis were observed on any animal throughout the experiment.

Mean faecal egg counts (FEC) during period I (days 14-28) and period II (days 29-42) of the experiment are shown in Figure 4.3. During period I, FEC of the animals grazing on chicory were lower ($P < 0.05$) compared to those fed on grass/clover (GC). Following the change in pastures, during period II, FEC declined in all treatments with no significant differences between the groups at any time point.

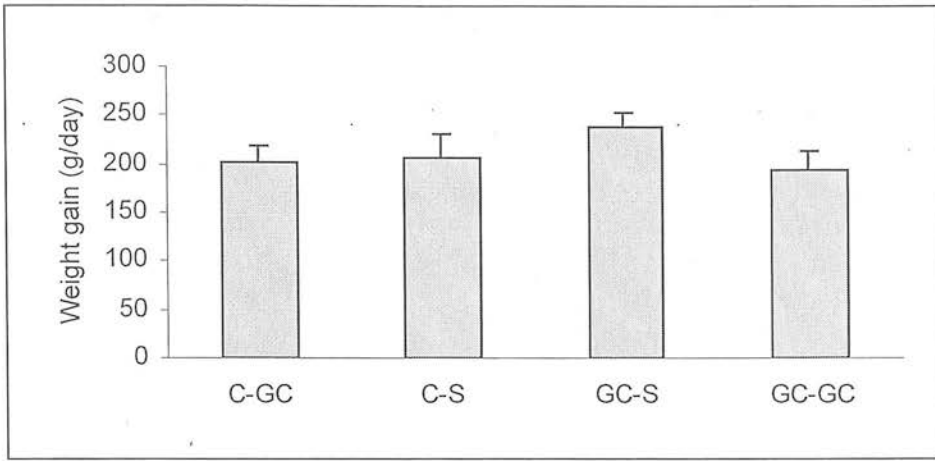


Figure 4.2 Mean daily liveweight gains of lambs infected with 30,000 L3 of *Teladorsagia circumcincta* at the start of the experiment and grazed chicory (C) or grass/clover (GC) for a two-week period (days 14-28, post-infection) and sulla (S) or grass/clover for the following two weeks (days 29-42, post-infection). The vertical bars indicate the standard error of the mean.

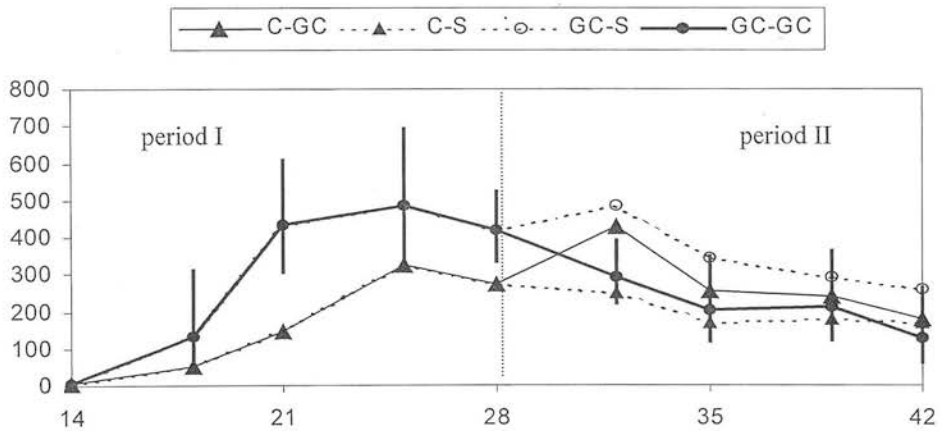


Figure 4.3 Backtransformed means of faecal egg counts (eggs per g faeces) of lambs infected with 30,000 L3 of *Teladorsagia circumcincta* at the start of the experiment and grazed on chicory (C) or grass/clover (GC) for two weeks (period I: days 14-28, post-infection) and sulla (S) or grass/clover (GC) for the following two weeks (period II: days 29-42, post-infection). The vertical bars in the control group (GC-GC) indicate the confidence intervals (95%).

No differences were observed between treatments in faecal consistency scores throughout the experiment, with all faecal samples being between soft unformed or formed moist pellets (scores of 3 and 4, respectively). Daily faecal output was similar between the different groups. Respective values (95% confidence intervals), on dry matter basis, were 324 g (260-390) for C-GC, 376 g (324-430) for C-S, 379 g (293-470) for GC-S and 305 g (276-335) for GC-GC group. Similarly, total worm egg output on day 42 was not affected by the grazing treatment. Corresponding values (95% confidence intervals) were 56,652 eggs (25,636-125,190) for C-GC, 62,580 eggs (32,717-112,806) for C-S, 92,076 eggs (56,515-150,012) for GC-S and 48,408 eggs (28,238-80,879) per day for GC-GC.

Male, female and total adult worm numbers at the end of the experiment are shown in Figure 4.4. No immature worms were recovered. The recoveries of adult worms showed large variation within the groups with no significant difference between treatments in male, female or total worm burdens. Per capita fecundity values of the female worms recovered at the end of the experiment (day 42) are shown in Figure 4.5. There were no significant differences between treatments in worm fecundity values.

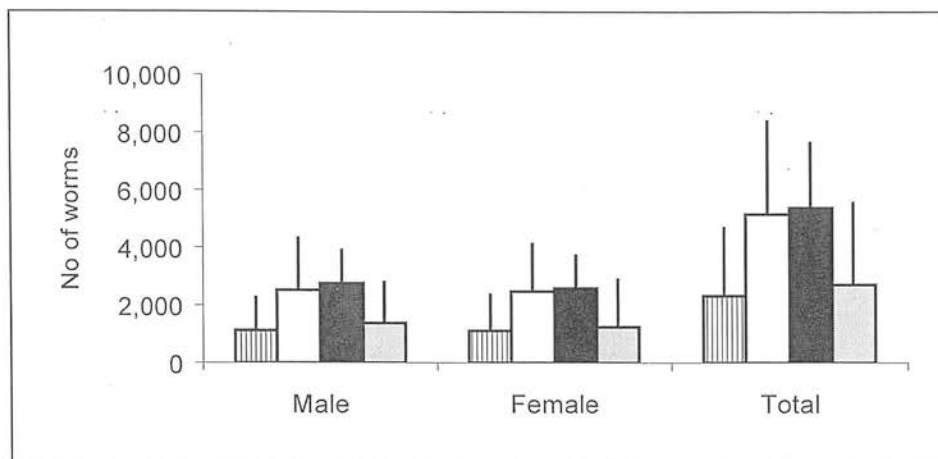


Figure 4.4 Backtransformed means of male, female and total adult worm burdens recovered from the gastrointestinal tract of lambs infected with 30,000 L3 of *Teladorsagia circumcincta* at the start of the experiment and grazed chicory (C) or grass/clover (GC) for two weeks (days 14-28, post-infection) and sulla (S) or grass/clover (GC) for the following two weeks (days 29-42, post-infection). The animal groups were C-GC (vertical lines), C-S (white), GC-S (black) and GC-GC (grey), while the vertical bars indicate the confidence intervals (95%).

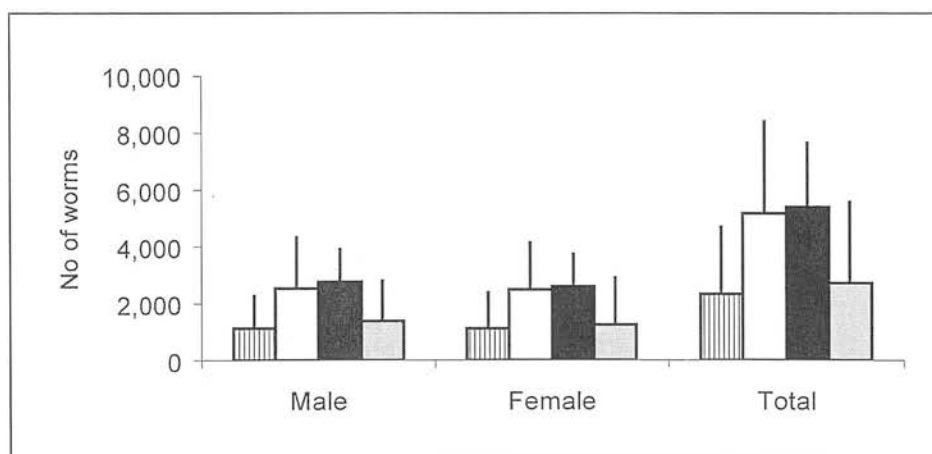


Figure 4.5 Backtransformed means of per capita fecundity (egg output/worm) of the female worms recovered from lambs at slaughter (day 42). The lambs were single infected with 30,000 *Teladorsagia circumcincta* larvae and grazing chicory (C) or grass/clover (GC) for a two-week period (days 14-28 post-infection) followed by an other two-week grazing period on sulla (S) or grass/clover (days 29-42, post-infection).

4.4 Discussion

The aim of the present study was to investigate the potential effects of a short-term sequential grazing, first on chicory and then on sulla, towards established *T. circumcincta* infection in lambs. Previous *in vivo* studies have suggested that ruminants fed forages rich in plant secondary metabolites (such as condensed tannins) or dosed with compounds extracted from such plants, could affect either worm egg excretion or worm population numbers (Niezen et al., 1994, 1995, 2002a; Athanasiadou et al., 2001a,b; Marley et al., 2003b). The study was expected to provide guidelines for the optimum grazing management practices using these plant species as short-term alternative grazing forages affecting the parasite epidemiology and/or survival (Coop and Kyriazakis, 2001). Thus, the first two weeks grazing on chicory (period I: days 14-28, post infection) was used to investigate for any possible anti-parasitic effects against established adult worms, such those observed in previous experiment (Chapter Three), whilst the second two-week grazing on sulla (period II: days 28-42, post infection) was used to test for additional benefits in terms of reduced worm populations or worm fecundity. The results showed that the FEC of lambs grazing chicory for the first two-week period were lower than the FEC of animals grazing in the control grass/clover forage, while during the following two weeks (period II) FEC was reduced across all groups with no significant effect of grazing treatment. Worm recoveries at the end of the experiment (day 42) were very low in all treatments (overall mean establishment of 13%) with no significant difference between groups.

The reduction of FEC in lambs grazing chicory forage during period I compared to those grazing grass/clover suggests an effect on worm fecundity and/or survival of adult female worms. Previous grazing studies (Scales et al. 1994; Marley et al. 2003b; Athanasiadou et al., 2004a, 2005a; Chapter Three) have shown similar effects of chicory forage against *T. circumcincta* infections supporting the present results. However, in the present study, the differences on FEC observed over the first two weeks (period I: days 14-28 post infection) did

not persist after the change over in the grazing treatment (period II: days 29-42), where animals grazed either sulla or grass/clover. No differences were found in worm numbers or fecundity of female worms recovered from the groups that had experienced chicory (C-GC or C-S) at the end of the trial (day 42; Figure 4.4 and 4.5). This suggests either that the effects were reversible or that other mechanisms were affecting worm populations and/or fecundity during the second period (days 28-42), which masked the previously shown differences in FEC. Such a mechanism could be the emergence of immunity against *T. circumcineta* worms during period II, affecting the FEC and worm numbers throughout the treatments. Evidence of the emergence of immunity during the period II could be seen, firstly, in the declining FEC (around five-fold reduction across all the treatments, Figure 4.3), and secondly, in the worm establishment, which averaged at 9% of the dosed infection in the control group (GC-GC) at the end of the experiment. In a previous study of Hong et al. (1986) on development of immunity of the three-and-a-half-month-old parasite-naive lambs towards a, similar to the present, mono-specific single-dose infection (33,000 L3 *T. circumcineta*) showed that the adult worm population started to decline after 27 days of infection, with increasing proportion of flapless female worm recoveries. Therefore, the presence of such an effective mechanism, such as an immune response, over the course of the present study would have masked any possible effects that took place at an earlier stage of the infection (period I), prohibiting any significant effect to be demonstrated on worm egg excretion or worm recoveries at the end of the trial.

The results obtained during period II (days 28-42, post infection) suggest that the two-week allocation on sulla treatment did not further benefit the parasitised lambs in terms of FEC or worm numbers of adult *T. circumcineta* population. This result contradicts previous observations on the effects of feeding sulla on worm populations of growing lambs (Niezen et al., 1994, 1998a, 2002a), whilst it agrees with the observations reported in Chapter Three. In the last study, two weeks grazing on sulla had no effects against the adult *T. circumcineta* worm

populations and this was partly attributed to the low CT concentrations of the forage samples collected from the sulla plots (16 g/kg DM). This cannot apply to the present experiment, since the extractable CT of sulla samples, measured by the same methodology (Porter et al. 1985), were relatively high at 38 g/kg DM (Table 4.1). The lack of any effect of sulla forage in the present study, as opposed to the studies of Niezen et al. (1994, 1998a, 2002a), was likely due to the differences in the experimental design. Firstly, the animals, in Niezen's studies, were allocated to the sulla grazing treatment for five or six-weeks, a much longer period than the two-week allocation time used in the present study. Thus, the length of parasite exposure to a harsh gastrointestinal environment could have accounted for the differences in survival rates of parasites, as has been shown in a number of *in vitro* studies (Molan et al. 2000a,b,c, 2002, 2004, Shreurs et al., 2002). Secondly, the observed antiparasitic effects in these previous studies were not stage specific, and could have been exerted against any developmental stage of *T. circumcincta* worms. The animals were single (Niezen et al., 1998a, 2002a) or trickle infected (Niezen et al., 1994) with *T. circumcincta* infective larvae, after they had already been allocated to the sulla grazing treatment. Therefore, the observed effects of the sulla forage may have occurred against larval establishment and larval viability, as well as towards adult worms. In contrast, the present study was designed to test the potential effects against adult stages only.

The mode of action of bioactive plants against the gastrointestinal parasite is not well established. *In vitro* studies have shown a direct anthelmintic-like effect against larval parasitic stages (Molan et al, 2000a,b,c, 2002, 2003; Athanasiadou et al., 2001a; Paolini et al., 2004), while *in vivo* studies have supported a nutritionally/immunologically mediated effect of the CT-rich forages (Niezen et al., 1995). The present study tested the bioactive forage effects towards an established adult *T. circumcincta* population, since this knowledge could help to use these forages in common practice, for example as short term de-worming paddocks for heavily parasitised animals. The results suggested that the immunity interferes and such an effector

mechanism has to be considered when testing bioactive plants as alternative grazing forages. Experiments' testing for the effect of bioactive forages through an immunological mediated mechanism seems to be missing from the literature and in order to use these forages in grazing management practices, further *in vivo* studies are needed to address such a possible mode of action.

With regards to lamb performance, the present short-term experiment showed similar growth rates between the parasitised animals grazing different forages with no significant differences in liveweight gains at any time point (Figure 4.2). Previous studies testing the performance of parasitised lambs (Scales et al, 1994, Niezen et al., 1998a) suggested that lambs grazing chicory or tannin-rich forages, such as lotus (*Lotus pedunculatus*) or sulla, sustained better growth rates compared to other conventional forages, such as reygrass or reygrass/clover. Nevertheless, grazing on bioactive forages in the previous studies was for longer periods (six to fourteen weeks) than the two (C-GC and GC-S) or four week (C-S treatment) grazing periods used in the present study, which due to its short duration focused on parasitological rather than on performance data. Further longer-term studies are needed to test these forages for the production benefits of parasitised lambs taking always into account the plant grazing tolerance and extra costs related with the bioactive forage management.

The present study confirmed previous observations on the reductions of FEC in parasitised lambs grazing chicory (Scales et al., 1994; Marley et al., 2003b; Athanasiadou et al., 2004a, 2005a). However, it provides little evidence of additional benefits of short-term grazing on sulla against an established adult *T. circumcineta* population. Further *in vivo* studies using bioactive forages are needed to investigate the effects on other *T. circumcineta* developmental stages, testing for the possible underlying mechanisms.

Chapter Five

The effect of chicory (*Cichorium intybus*) and sulla
(*Hedysarum coronarium*) on larval development and mucosal
cell responses of growing lambs challenged with
Teladorsagia circumcincta

Abstract

The aim of the present study was to investigate the effects of grazing different bioactive forages on acquired immunity against *Teladorsagia circumcincta* infection. The development of immunity was assessed by following the response of trickle-infected lambs grazing chicory (*Cichorium intybus*; IC), sulla (*Hedysarum coronarium*; IS) or grass/clover (*Lolium perenne*/*Trifolium repens*; IGC), to a single challenge infection. Parasite-naive lambs, grazing grass/clover, were also challenged with the single infection dose providing the uninfected control (UGC) group. Trickle infection significantly reduced worm establishment, inhibited larval development and increased mucosal mast cell (MMC) and globule leucocyte (GL) cells. Grazing treatment (chicory, sulla or grass/clover) significantly affected adult worm ($P < 0.05$), late-L4 ($P < 0.01$) and mid-L4 ($P < 0.01$) larval stage recoveries of the trickle infected lambs, with IGC group always carrying higher worm burdens than either IC or IS lambs. MMC and GL cells of trickle-infected lambs were positively correlated with the proportion of early-L4 worms recovered and negatively correlated with both the proportion of adult worms recovered and the total worm establishment, suggesting that the observed effects were due to an enhanced immune response. The results suggest elevated immune responses against *T. circumcincta* infections in growing lambs grazing on either sulla or chicory compared to those grazing on grass/clover.

Keywords: *Teladorsagia*; Sheep; Immunity; *Cichorium intybus*; *Hedysarum coronarium*; gastrointestinal nematode

5.1 Introduction

The widespread development of anthelmintic resistance in sheep (Jackson and Coop, 2000) and the demand for non-chemical (organic, ecological, low-input) farming of livestock have increased the need for alternative ways to control gastrointestinal parasites. One of the proposed alternatives is the use of bioactive forages. Previous studies using natural or experimental challenges with *Teladorsagia circumcincta* have suggested that lambs grazing on bioactive forages, such as chicory (*Cichorium intybus*) or sulla (*Hedysarum coronarium*), have reduced parasite burdens, compared to lambs grazing on conventional forages (Scales et al., 1994; Niezen et al. 2002a; Marley et al. 2003b; Chapter Three). However, the mode of action of these forages remains unknown and possible explanations of the observed reductions may include: (i) direct, anthelmintic-like effect of plant compounds, (ii) indirect, immune related effects of the forage, and/or (iii) effects on free-living larval development, migration and survival on the pasture. In a previous experiment the use of worm-naïve animals and controlled experimental infections (Chapter Three) excluded the effects on free-living stages. However, the experimental design was unable to determine whether the observed effects of grazing on bioactive forages were direct anthelmintic effects, or indirect immunologically or physiologically mediated responses.

The objective of the present study was to investigate the effects of grazing bioactive forages, chicory or sulla, on the rate of development of immunity in growing lambs infected with *T. circumcincta*. The immune response was assessed by following the response of trickle-infected lambs to a single challenge infection, using an established experimental model (Smith et al. 1983, 1985; Barrett et al., 1998; Macalodowie et al. 2003).

5.2 Materials and Methods

5.2.1 Experimental swards

The experiment was carried out at Scottish Agricultural College's Thomson fields (Midlothian, Southeast Scotland) where a 2.4 ha site was allocated for the experimental plots. The area was prepared by spraying existing vegetation (mixed grasses, white clover and herbs) with glyphosate (N-(phosphono-methyl) glycine) at a 1.2 kg/ha (autumn 2003), ploughed (winter 2003) and cultivated to prepare a firm seedbed (spring 2004). The experimental plots were sown (late spring 2004) using the rates of 7 kg/ha for *C. intybus* (chicory), 15 kg/ha for *H. coronarium* (sulla) and 37 kg/ha for *Lolium perenne*/*Trifolium repens* (grass/clover). Fertiliser (50 kg/ha of P & K; Yara UK Ltd.) was applied to all experimental area, while no insecticides or herbicides were used once plots were sown. Fencing divided the experimental area into 24 plots of 0.1 ha each, all of which had automated water troughs. All swards grew well and were in the vegetative stage of growth when the experiment started (August 2004).

5.2.2 Experimental design

Twenty female and twenty castrated male crossbred lambs, three-and-a-half months old (mean liveweight \pm standard error of the mean (S.E.M.): 32 ± 0.5 kg) were reared worm-free from birth and used in the present experiment. The animals were allocated into four groups of ten animals each balanced in terms of liveweight and sex. Three groups were randomly assigned to one of the three different grazing treatments: chicory (*C. intybus*; IC), sulla (*H. coronarium*; IS) and grass/clover (*L. perenne*/*T. repens*; IGC), and trickle infected with *T. circumcincta* larvae, three times per week (Monday-Wednesday-Friday), for six weeks with a total of 10,000 L3 per week. The fourth group was kept uninfected and given access to grass/clover pastures (UGC) for the same period. All pastures used were parasite-free prior to the trial and animals were moved every two weeks to a new plot to prevent autoinfection. On day 40 post-infection, lambs were moved indoors and fed hay ad libitum for the rest of the trial. On day 42, all lambs,

including the uninfected controls (UGC), were treated with levamisole (Levacide 3% drench; Norbrook Laboratories Ltd., UK; 7.5 mgs/kg liveweight) and ivermectin (Oramec drench, Merial Animal Health Ltd.; 0.2 mg/kg liveweight) to remove all gastrointestinal nematodes. One week later (day 50), the lambs were challenged with 50,000 L3 *T. circumcincta* and slaughtered 10 days later (day 60).

5.2.3 Sward measurements

Pre-grazing herbage mass availability and botanical composition of the experimental swards were determined by cutting 2 random quadrats (0.25 m²) per plot to ground level. The cut material was separated manually to the following fractions: sown species of the sward (forage treatment), dead material and “other” species, and each fraction was then weighed, oven-dried (60 °C for 18 h) and re-weighed. Twenty measurements of pre- and post-grazing sward heights were taken from each plot one day before and one day after grazing, respectively, by using a sward stick device.

The samples cut from the pre-grazed experimental plots were pooled and analysed in duplicates for determination of dry matter (DM), ash, crude protein (CP), neutral detergent fibre (NDF), water soluble carbohydrate (WSC), neutral cellulase and gammanase digestibility (NCGD), potassium (K), calcium (Ca), phosphorus (P), sodium (Na), magnesium (Mg) and sulphur (S) (MAFF, 1986). The extractable condensed tannin content (CT) of the samples was measured according to Porter et al. (1985). The chemical composition of the hay offered to the animals indoors (days 40 to 60) was also analysed for DM, ash, CP, NDF, WSC content and NCGD value. Metabolisable energy (ME) values of the fresh forage samples were calculated using the equation for the prediction of ME of grasses (Agricultural and Food Research Council, 1993), since there are no equations available for the bioactive forages tested in the present experiment. ME value of the hay was also calculated according to AFRC (1993).

5.2.4 Animal measurements

The lambs were weighed weekly throughout the experiment and the daily liveweight gain of each animal over the 60-day experimental period was used to estimate the effect of feeding treatment on lamb performance. Faecal samples were taken weekly from the rectum of each lamb for faecal egg counts (FEC) determination using a modified flotation technique described by Christie and Jackson (1982). Faeces taken were scored for consistency on a scale of 1 to 5. The scores of 1 and 5 were given to loose diarrhoeic faeces and dry hard pellets, respectively, while the other scores were given to intermediate states of consistency. Immediately after post-mortem, the abomasum was removed from each lamb and small sections of abomasal folds were taken for histological enumeration of mucosal mast cells (MMC), globule leucocytes (GL) and eosinophils (EOS) (expressed per 0.1mm^2), as described previously (Huntley et al. 1992). The abomasum and its content were subjected to a saline digest to harvest all developmental stages (adult and larval stages) of *T. circumcincta* (Jackson et al., 1984). The worms recovered from 1% (v/v) aliquots of the digest were counted and classified as early-L4 (EL4), middle-L4 (ML4), late-L4 (LL4) and adult-L5 stages using the morphological criteria defined by Denham (1969).

5.2.5 Statistical analysis

Liveweight gain (g/day) for each animal was estimated by linear regression of the weekly liveweights against time (least squares method) and analysed using one-way analysis of variance. FEC, worm recoveries (EL4, ML4, LL4, and adults) and total worm establishment (combined L4 and adult worms) were log-transformed ($\log_{10}(x+1)$) prior to statistical analysis to remove positive skewness and are reported as backtransformed means with 95% confidence intervals (lower and upper limit). Cell counts (MMC, GL, EOS) were normally distributed and are reported as means (\pm S.E.M.). The effects of the feeding treatments on FEC, worm numbers,

worm establishment, and cell counts were compared using one-way analysis of variance, while the effect of trickle infection (trickle infected animals vs. uninfected controls) was investigated using t-test. Correlations between log-transformed cell counts and log-transformed worm numbers were estimated (Minitab Inc. 2000. MINITAB Statistical Software, Release 14 for Windows, State College, Pennsylvania). No statistical analysis was performed on the agronomic or chemical composition data since these measurements aimed only to describe the plots and the forages on offer.

5.3 Results

5.3.1 Sward measurements

Pre-grazing herbage mass availability and botanical composition of the experimental plots are presented in Figure 5.1. Pastures contained 2130-2537 kg DM/ha of total herbage biomass and the sown species (forage treatment) represented between 76-84% of the total dry matter (DM) available in all plots. Other species found in the experimental pastures (other grasses and weeds) contributed less than 9% in chicory and grass/clover plots, while comprised the 19 % of the DM in sulla plots. Clover existed in small proportion in the grass/clover mixtures representing 9 and 5% of the DM in grass/clover plots grazed by UGC and IGC animals, respectively (Figure 5.1). Pre-grazing sward heights were similar among the different treatments, overall mean (\pm standard deviation) of 21 cm (± 5.9), 19 cm (± 6.7), 17 cm (± 5.3) and 15 cm (± 4.5) for chicory, sulla, grass/clover (UGC) and grass/clover (IGC) plots, respectively. Sward heights were reduced by the end of the grazing period to 8 cm (± 3.4), 6cm (± 2.3), 10 cm (± 4.3) and 9 cm (± 3.6), respectively.

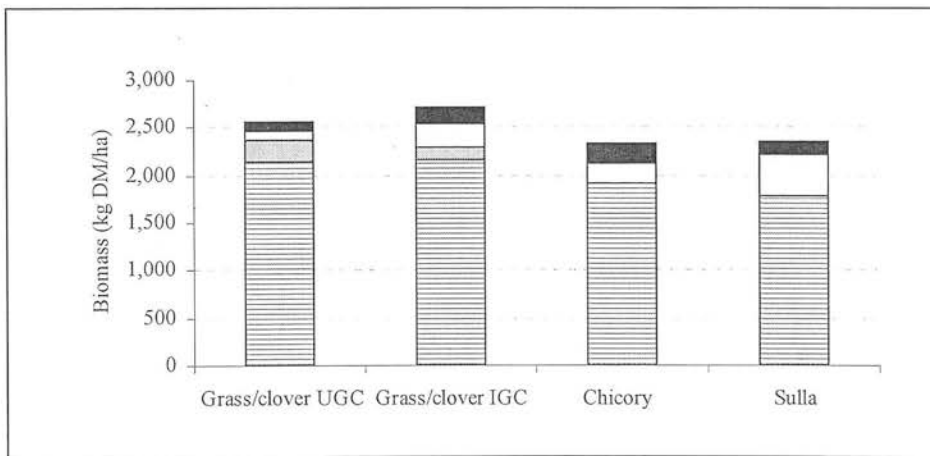


Figure 5.1 Pre-grazing herbage mass availability and botanical composition of the grazing swards. Main forages (horizontal lines) were *Lolium perenne*, *Cichorium intybus* and *Hedysarum coronarium* for grass/clover, chicory and sulla swards, respectively. Dry matter (DM) availability of *Trifolium repens* (grey) in the grass/clover plots as well as other species (white) and dead matter (black) in all experimental swards are also shown.

The chemical analysis of the experimental swards grazed by the animals for the first 6 weeks of the experiment is shown in Table 5.1. Chicory swards had the lowest and grass/clover swards the highest dry matter content. Samples collected from chicory and sulla plots had higher crude protein (CP) content than those collected from grass/clover plots. The energy supply to the animals, as estimated by the metabolisable energy (ME) of the collected samples, was similar between the different treatments ranging from 11.2 to 12.1 (MJ/kg DM). Grass/clover had higher values of neutral detergent fibre (NDF) and water soluble carbohydrate (WSC) content than the other forages. Chicory and grass/clover swards contained traces of condensed tannins (CT), whereas sulla had the highest amount of extractable CT. Grass/clover samples tended to be higher in most minerals measured (except sodium), than those of chicory and sulla samples. The DM content of the hay was 847 (g/kg fresh) while its chemical composition was 80, 44, 659, 110, 458 and 8.16 for Ash, CP, NDF, WSC, NCGD (g/kg DM) and ME (MJ/kg DM), respectively. Analysis of the hay samples suggested that the hay on offer was of lower nutritional value, in terms of CP, NDF and ME content, compared to fresh forages.

5.3.2 *Animal measurements*

Mean daily liveweight gains of the different animal groups are shown in Figure 5.2. The weight gains of the chicory group (IC) were significant higher ($P < 0.05$) than any other group (trickle infected, IGC and IS, or uninfected control UGC). During the course of the experiment all animals gained weight (average value \pm SEM: 6 ± 0.5 kg) except animals No. 852 (from IS group) and No. 860 (IGC group), which did not gain any weight, and animal 854 (IS group) which gained less than a kilogram. One lamb from the IGC group developed severe diarrhea and lost weight during the first 3 weeks of the trickle infection and was removed from the experiment for humane reasons. All data from this animal have been excluded from the current and subsequent analysis.

Table 5.1 Chemical composition of the grazing swards

	Grass/clover UGC swards ^a	Grass/clover IGC swards ^b	Chicory IC swards ^c	Sulla IS swards ^d
Dry matter (DM) (g/kg fresh)	166	161	68	103
<i>Analysis (g/kg DM) and calculated metabolisable energy(MJ/kg DM)</i>				
Ash	84	90	151	110
Crude protein	111	113	183	185
Neutral detergent fibre	490	469	332	388
Water soluble carbohydrate	90	102	55	69
Condensed tannins	7.9	6.8	8.3	36.9
Potassium	49.9	44.8	40.2	17.8
Calcium	18.1	12.8	10.1	12.7
Phosphorus	5.1	4.7	2.8	2.1
Sodium	1.2	0.9	5.0	5.3
Magnesium	3.9	3.1	2.1	2.6
Sulphur	3.3	3.2	4.3	5.2
Neutral cellulase and gammanase digestibility	713	743	797	743
Metabolisable Energy	11.2	11.5	12.1	11.5

Each sample was analysed in duplicate and the numbers in the table represent the mean value. For details on the chemical analysis and the prediction of metabolisable energy see text.

^{a, b} samples collected from grass/clover swards grazed by the uninfected (UGC) and trickle-infected (IGC) animal groups respectively.

^{c, d} samples collected from chicory and sulla swards respectively.

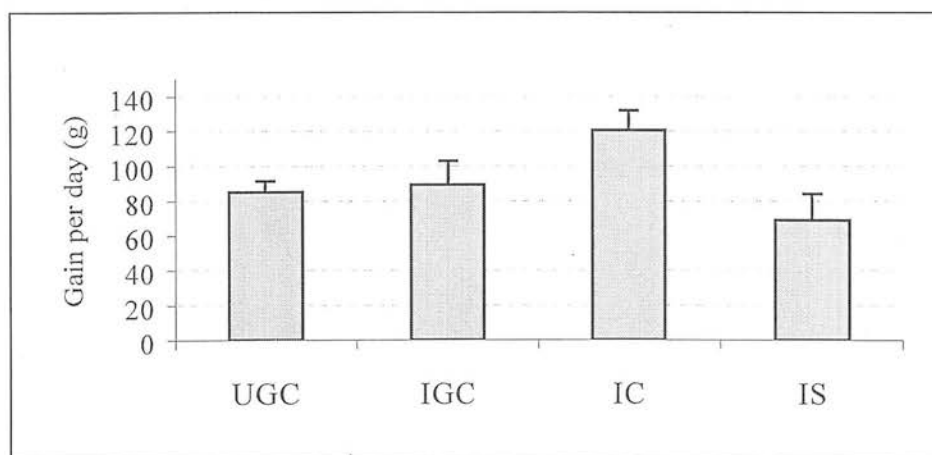


Figure 5.2 Mean (S.E.M.) daily liveweight gains of trickle infected lambs grazing different forages for a six-week period (grass/clover, chicory and sulla for IGC, IC and IS group, respectively) and uninfected control lambs grazing grass/clover (UGC group).

The average FEC of the different groups are shown in Figure 5.3. The FEC of the trickle infected animals were apparent after the second week of infection, varied markedly within the groups and followed similar pattern with no significant difference between groups at any time point. FEC from the previously uninfected group (UGC) were zero throughout the experiment. No differences were observed between treatments in faecal consistency scores, with all faeces falling into soft unformed or formed moist pellet categories (scores of 3 and 4, respectively).

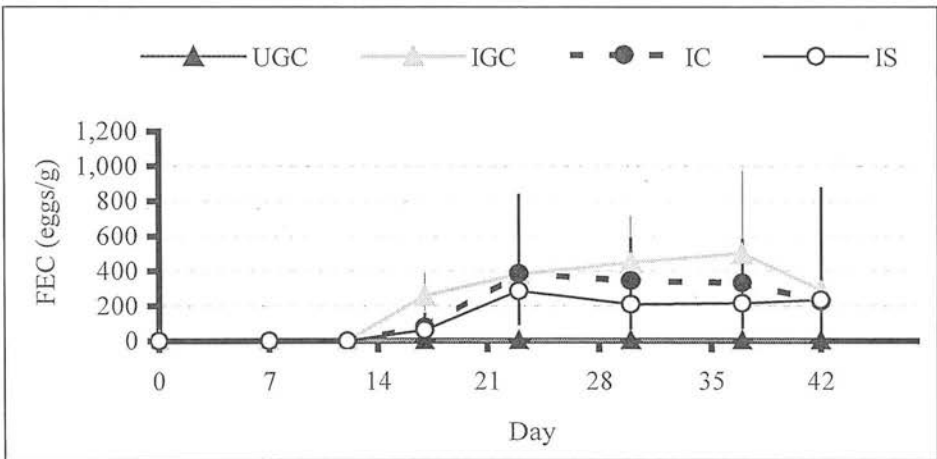


Figure 5.3 Backtransformed group means (95% confidence intervals) of the eggs per gram faeces (eggs/g) recovered from lambs trickle infected with 10,000 L3 *Teladorsagia circumcincta* per week (IGC, IC and IS group) or kept uninfected (UGC group). IGC and UGC animals were grazing on grass/clover while IC and IS were grazing on chicory and sulla swards, respectively.

Table 5.2 shows the mean *T. circumcincta* worm numbers recovered from lambs and the mean cell (MMC, GL and EOS) numbers counted in abomasal fold sections. Comparing the previously uninfected controls (UGC) with the trickle-infected animals (IS, IC, and IGC), UGC animals had higher ($P < 0.01$) total worm establishment (combined EL4, LL4, ML4 and adult numbers) and lower ($P < 0.001$) MMC and GL cell counts. Significant differences were also

observed in the attained stage of development of the recovered worms. UGC animals had higher adult ($P < 0.001$), higher LL4 ($P < 0.01$) and lower EL4 ($P < 0.001$) worm populations compared to trickle infected groups, most of whose worms were in early- or mid-L4 stage of development. Comparisons between trickle infected groups (IS, IC, and IGC) showed that the grazing forage treatment significantly affected the adult worm ($P < 0.05$), late-L4 ($P < 0.01$) and the mid-L4 ($P < 0.01$) larval stage recoveries of the trickle infected lambs, with the IGC group always carrying higher worm burdens than either IC or IS lambs. The total worm establishment tended to be higher and the GL cell counts tended to be lower for IGC animals compared to IS and IC lambs (Table 5.2), although these differences were not statistically significant. Correlations of the cell counts and the worm establishment, using trickle-infected animals only, investigated the relationships between cells recruited on the abomasal tissue and worm recoveries. Thus, higher GL and MMC counts were negatively correlated with the total worm numbers (correlation coefficient (r) values of -0.63 and -0.51, respectively; $P < 0.01$). Similar analysis on the relationship between GL and MMC counts with worm development demonstrated positive relationships with the worms being in early-L4 stage (r values of 0.68 and 0.73 for GL and MMC, respectively; $P < 0.001$) and negative relationships with the worms in the adult stage (r values of -0.74 and -0.71 for GL and MMC, respectively; $P < 0.001$). No relationship was found between worm establishment or stage of development with the mucosal EOS counts.

Four trickle infected animals, three from IS group (No. 842, 852 and 854) and one from IGC group (No. 860), had 10-fold higher numbers of adult worm establishment (recoveries of 31,300, 28,600, 31,200 and 33,100, respectively) compared to other trickle infected animals. These animals also had zero early-L4 worm numbers and zero GL cell counts, values very similar to those observed in non-trickle-infected animals (UGC). With the omission of these four animals from the analysis, the variation within the groups was reduced and the forage effect

became significant ($P < 0.05$) for both the total worm establishment (values of 33%, 20% and 9% for IGC, IC and IS group, respectively), and GL counts (mean \pm S.E.M. cells per 0.1mm²: 4 \pm 1.1, 7 \pm 1.5 and 15 \pm 3.7 for IGC, IC and IS group, respectively). Individual worm recoveries and cell counts of the trickle infected lambs grazing sulla (IS group) are shown in Table 5.3.

Table 5.2 Backtransformed mean worm burdens (95% confidence intervals), backtransformed percentage of total worm established (combined early-L4, mid-L4, late-L4, and adult numbers) and mean (\pm S.E.M.) cell numbers (mucosal mast cells (MMC), globule leucocytes (GL) and eosinophils (EOS) per 0.1 mm² tissue) counted in lambs challenged with

50,000 L3 *Teladorsagia circumcincta*

Group (n)	Early-L4	Mid-L4	Late-L4	Adults-L5	Total worms	Established	MMC	GL	EOS
UGC (10)	8 (0-65)	974 (534-1,775)	3,276 (1,919-5,591)	21,234 (15,932-28,300)	27,751 (24,029-32,049)	56%	4.5	0.0	4.0
IGC (9)	1,547 (135-17,581)	3,049 (1,432-6,489)	1,644 (518-5,219)	1,007 (220-4,610)	17,927 (13,506-23,794)	36%	13.3	1.4	4.1
IC (10)	6,691 (3,957-11,313)	1,081 (164-7,099)	53 (3-719)	15 (1-146)	9,790 (5,226-18,339)	20%	18.7	2.1	6.8
IS (10)	266 (14-4,901)	63 (6-577)	18 (1-188)	34 (0-1,149)	6,982 (2,306-21,131)	16%	13.5	2.3	10.7
Infection effect	P < 0.001	NS	P < 0.01	P < 0.001	P < 0.01	P < 0.01	P < 0.001	P < 0.001	NS
Forage effect	NS	P < 0.01	P < 0.01	P < 0.05	NS	NS	NS	NS	NS

n: number of animals per group, NS: non-significant differences

Table 5.3 Worms recovered from 1% (v/v) aliquots of the abomasal digest and mucosal mast cell (MMC), globule leucocyte (GL) and eosinophil (EOS) cell numbers counted per 0.1 mm² abomasal tissue of the trickle infected lambs grazing sulla (*Hedysarum coronarium*, IS group) and challenged with 50,000 L3 *Teladorsagia circumcincta*

Animal No	Early-L4	Mid-L4	Late-L4	Adults-L5	Total worms	MMC	GL	EOS
197	32	0	0	0	32	91	38	20
478	101	2	0	1	104	66	26	6
600	88	37	19	0	144	53	10	37
614	27	4	0	0	31	101	101	13
838	1	1	0	0	2	82	74	22
842	0	0	3	313	316	14	0	10
852	0	0	4	286	290	22	0	17
854	0	1	3	312	316	16	0	21
858	73	13	0	0	86	85	9	10
861	33	3	1	0	37	95	55	32

5.4 Discussion

There was evidence in the present study that grazing on sulla or chicory encouraged the development of immunity against *T. circumcincta*. The trickle infected lambs grazing on sulla and chicory had reduced establishment of the challenge worms compared to those grazing on grass/clover pastures, while the worms recovered from lambs grazing these bioactive forages had also inhibited development. The finding that the reduced worm establishment and inhibited larval development were correlated with higher numbers of GL and MMC in abomasal tissues suggests that the observed effects were due to an enhanced immune responses, since these cells are associated with immune responses against gastrointestinal parasites (Balic et al., 2000). The chemical composition of the grazing forages (Table 5.1) showed that bioactive forages had higher nutritional value than grass/clover in terms of their crude protein content (184, 185 and 113 g/kg DM for chicory, sulla and grass/clover (IGC) samples, respectively). Higher dietary protein content and metabolisable protein supply of growing lambs have enhanced immune responses against gastrointestinal parasites in several past studies (Bown et al., 1991; van Hourtert et al. 1995), and the present findings are consistent with the enhanced immune response to *T. circumcincta* infection observed in lambs supplemented with protein (Coop et al., 1995).

Previous studies using either *T. circumcincta* or *Trichostrongylus colubriformis* have reported reduced worm establishment in lambs grazing sulla or chicory (Scales et al. 1994; Niezen et al. 1995, 2002a; Marley et al. 2003b; Chapter Three). These effects on parasite burdens have been attributed to the plant secondary metabolite (PSM) content of these forages. Suggested potential ways of PSM action include an indirect immune-related effect and/or a direct anthelmintic effect. Further studies have investigated the possible PSM anthelmintic attributes of these forages either *in vitro* or *in vivo*. *In vitro* studies (Molan et al., 2000a, 2003a), using compounds extracted from chicory and sulla, have suggested an anthelmintic-like effect

against immature stages of gastrointestinal parasites, although the study in Chapter Two did not demonstrate similar effects on *T. colubriformis* infections of sheep *in vivo*. The present study aimed to investigate the immunological effects of sulla and chicory, and in order to avoid the direct contact of the potentially PSM rich digesta with the challenge infection, the animals were removed from the bioactive forages and were offered basal hay, 10 days before the administration of the challenge dose. Given the relatively short retention times of sheep ingesta (less than 3 days for this type of forage; Van Soest, 1994), the time elapsed between the consumption of the bioactive forage and the administration of the challenge larvae should have minimised the possible PSM anthelmintic effects exerted through direct contact with the infection larvae, although this does not rule out the longer-term effects of any likely persistent active compound. However, given that in this study worm burdens correlated with the numbers of mucosal cells (MMC and GL) and the fact that the observed effects were more pronounced on larval development rather than on worm establishment, the findings support the view that the results seen were the outcome of enhanced immune responses. This also agrees with previous studies with *T. circumcincta* infections (Smith et al. 1985; Coop et al. 1995; Stear et al. 1999), where the stage of development or worm length of the recovered worms was a more consistent indicator of development of immunity than the total worm establishment.

The effects of the bioactive forages on *T. circumcincta* populations in the present study may be due to the forage nutritional value and the possible effect on lamb immune response. Chicory and sulla had higher crude protein content compared to the control, grass/clover, forage (Table 5.1). Although this was not observed in previous studies with chicory and sulla (Scales et al. 1994, Chapter Three) both are considered forages with high nutritional value. Chicory forage has been associated with higher voluntary feed intake and higher apparent digestibility compared to grasses (Barry, 1998), whilst sulla is a tannin-rich forage with CT characteristics that can improve the protein availability to the host (Min et al. 2003). CT from sulla can bind

dietary proteins and reduce their degradation in the rumen, without reducing the microbial protein produced, resulting in an overall increase of protein supply in the animal (Min et al. 2003). Sulla samples in the present study had higher extractable CT concentration than grass/clover (37 and 7 g/kg DM, respectively) and consequently the lambs of this group may also have benefited from a higher metabolisable protein supply, which has been shown to enhance immune response against gastrointestinal parasites (Coop et al. 1995; Van Houtert et al. 1995; Houdijk et al. 2001a,b,c).

A further finding of the present study was the significantly higher liveweight gain of the trickle-infected lambs grazing chicory compared to both the other trickle-infected groups (IS and IGC) and the previously uninfected lambs grazing grass/clover. However, the finding that the weight gains of trickle-infected (IGC) lambs were similar to their previously uninfected (UGC) counterparts grazing the same forage, suggests that the six-week infection regime of the present experiment did not penalise performance profoundly. This has also been observed in previous studies, where comparable infection regimes (infections of 10000 L3 *T. circumcincta* per week), resulted in similar growth rates between trickle infected and uninfected control, three-and-a-half-month-old, lambs over an eight-week infection period (Coop et al., 1982; Richardson PhD thesis, 2000). Nevertheless, in previous studies, lambs grazing chicory have shown superior growth rates compared to those grazing grasses under parasite challenge or not (Fraser et al. 1988; Komolong et al. 1992; Niezen et al. 1994; Scales et al. 1994; Marley et al. 2003b). Barry (1998) suggested that these differences could be explained by the higher voluntary feed intake and the digestibility characteristics of this forage. This may also apply to the present experiment and could explain the differences in weight gains or immune responses since grasses constituted more than the 90% of the control forages. However, food intake measurements or digestibility data were not taken in the current study and further long-term feeding trials with chicory are required in order to confirm these findings.

In conclusion, the design of the present study has enabled the separation of direct (anthelmintic) and indirect (immunological/physiological) mediated effects on host responsiveness against the gastric nematode *Teladorsagia*. The findings support the view that some of the benefits from grazing bioactive forages derive from their effects on the development and expression of immunity. Further studies are needed both to confirm these effects, elucidate more fully the underlying mechanism and also to develop ways of incorporating these forages within grazing management systems, so as to reduce our reliance upon chemoprophylaxis.

Chapter Six

General Discussion

6.1 Introduction

Widespread helminth resistance to drugs threatens the sustainability of the sheep industries in both the developed and developing countries (Waller, 1997; Sangster and Dobson, 2002). This, together with the advent of organic systems of production, raises the requirement for alternatives to chemoprophylaxis to control parasitism (Cabaret et al., 2002a,b). The present work has provided novel information on one of the proposed alternative means to control gastrointestinal parasites in sheep: the use of bioactive forages.

To investigate the use of bioactive forages grazing experiments, as opposed to indoor feeding trials, were used, while growing lambs were the parasitised hosts due to their higher susceptibility to nematode infections (Smith et al., 1985; Dobson et al., 1990). All experiments used short-term grazing periods on bioactive forages; two weeks allocation on bioactive forages in the first two experiments (Chapter Two and Three); four weeks grazing period in the third experiment (Chapter Four); and six week allocation on bioactive plants in the final study (Chapter Five). The short duration was chosen since most of the bioactive forages are not commonly used in mainstream agricultural practice in the UK and questions were raised not only about their agronomic viability, grazing persistence and their adoption in the long term by the farmers, but also about the extra costs related to their management. It was therefore suggested that their use would more likely be confined to short-term periods, when they can be used as de-worming paddocks (Coop and Kyriazakis, 2001).

To test the hypothesis of different nematode species susceptibility to different bioactive plant species specific studies and experimental designs were developed. The first two experiments, Chapters Two and Three, tested the possible bioactive effect of four different forage species against the intestinal *T. colubriformis* and abomasal *T. circumcincta* worm populations, respectively. Based on the findings of these studies, two subsequent experiments,

Chapters Four and Five, focused on the use of selected forage species, namely sulla and chicory forages, against abomasal *T. circumcincta* infections, testing for possible mechanisms of bioactive forage action.

To investigate the affected developmental stage (immature larvae or established adults), the experiments in Chapters Two and Three tested different bioactive forage species against both immature and adult worm populations. The subsequent experiments focused on the effects of selected forage species against either adult (Chapter Four) or immature (Chapter Five) populations only. Chapter Four tested the possible combination of bioactive forages in a sequential short-term grazing regime, investigating the additive beneficial effect that these forages may have on lambs carrying a substantial adult worm population. Chapter Five tested the effects of bioactive forages on lamb immunity against incoming infective larvae giving insights on the immune related bioactive action.

The present Chapter will discuss the principal issues raised by the experimental chapters, which are the use of bioactive forages as alternative parasitic control method and the possible future application of these forages in sheep production systems.

6.2 *The mode of action of bioactive forages against parasites*

It has long been hypothesised that bioactive forage activity against gastrointestinal parasites is likely to be attributed to their PSM-rich content (Niezen et al., 1994, 1995). Possible active secondary metabolites have been suggested, such as the condensed tannins in the tanniferous legumes and the sesquiterpene lactones in chicory forage, mainly due to their anthelmintic properties demonstrated by several *in vitro* studies (Molan et al., 2000a,b,c, 2002, 2003a,b; Paolini et al., 2004). More recently other PSM compounds in sainfoin, such as flavonol glycosides, have also shown anthelmintic properties *in vitro*, suggesting that other non-CT compounds may also be involved in the anti-parasitic activity of such legumes (Barrau et al., 2005).

Chicory forage is a plant rich in secondary metabolites, particularly phenolics and terpenoids, which have demonstrated a feeding deterrent role against insects (Reese and Harbone, 1985; Pickman, 1986). Among the various chemical entities, the major secondary constituents of chicory, in terms of their presence in chicory leaves throughout the seasons, are the sesquiterpene lactones, lactucin, lactupicrin and 8-Deoxylactucin, and the phenolic compounds, cichoriin and chicoric acid (Rees and Harbone, 1985). To date, only crude sesquiterpene lactone extracts from chicory have been tested against parasitic stages *in vitro* (Molan et al., 2003a), and the results demonstrated a significant antiparasitic effect. It is therefore suggested that more research is needed to isolate specific secondary constituents from PSM-rich plants and test their properties against different developmental parasitic stages *in vitro*. However, the bioactive mode of action cannot be identified only from *in vitro* studies since other factors may be implicated in grazing conditions and several hypotheses have been suggested to account for the phenomenon. These include: (i) the effects on free-living nematode stages, (ii) the effects on parasitic nematodes through direct, anthelmintic-like, effects of PSM compounds, and (iii) the effects on parasitic nematode population through physiological/immunological effects in the parasitised host.

The investigation of the first hypothesis was beyond the scope of the present thesis, which focused on the bioactive forage effects within the parasitised host. To achieve this, specific measures and experimental practices were applied throughout the present study. First of all, experimental pastures were considered parasite free at the start of each study. We ensured this by ploughing and reseeded the pastures every spring. Second, animals that were used in all experiments were previously parasite naïve. Third, to ensure clean grazing throughout the experimental period, sheep were moved into clean pastures every two weeks. These practices resulted in controlled infections, which excluded any possible effects of pasture contamination (free-living nematode populations) on the present data. This can be seen throughout the present thesis in the FEC and worm burdens recovered from the control animals, which largely corresponded to the experimental infection regime. For example, during the longer grazing period used in the present thesis (six-week grazing on experimental plots; Chapter Five), none of the non-infected control animals (UGC group) showed any signs of parasitic infection, monitored by FEC during the six weeks grazing (Figure 5.3) or by worm burdens and histological examination of the tissue at the end of the experiment (Table 5.1). It is therefore suggested that the present thesis investigated only the possible bioactive forage effect, due to their consumption by the animals, while any parasite contamination of the experimental plots or the parasite-plant interactions within the pasture, and outside of the host, had no effect on the present results.

As mentioned above, the consumption of bioactive forages may affect the parasitic stages of helminths mainly through direct, anthelmintic-like, effects of PSM plant compounds and/or indirect effect through nutritional/immunological mechanisms. Exploring further the latter hypothesis, the last experiment of the present thesis (Chapter Five) enabled the separation of direct and indirect mediated effects of bioactive forages providing novel data on forage effect

on lamb immunity against *T. circumcincta* infection. The findings support the view that the consumption of bioactive forages, at least the tanniferous legume sulla and the herb chicory, enhance the development and expression of immunity against the gastric nematode *Teladorsagia*. The ways through which these bioactive forages can enhance the immune response of sheep against nematode infection have not been identified. Suggested modes of action relate to higher nutritional supplementation of the host due to specific CT characteristics of the sulla legume, or the nutritional and feeding properties of chicory forage. Evidence of the effects of CT-rich plant species and chicory forage on animal nutrition/metabolism exist in literature and the nutritional attributes of these forages will be developed in the following sections.

6.3 Effects of CT-rich forages on sheep nutrition

It has been shown by Bermingham et al., (2001) that sulla increases the overall protein supply to the animals due to its specific CT characteristics. Sheep fed sulla had increased both the amino acid flux from their abomasums and the amino acid absorption in the small intestine compared to sheep fed sulla but infused intraruminally with polyethylene glycol (PEG), which inactivated the CT (Bermingham et al., 2001). Nutritional supplementation, and particularly with protein, has long been proven to enhance sheep immune response against *Trichostrongyloidea* parasites (Bown et al., 1991; Coop et al., 1995). It is therefore suggested that a similar mode of action may account for the observed enhancement of immune response in sheep fed sulla, which, in addition, had a higher protein content relative to the grass/clover forage in the present study of Chapter Five (CP content of 185 vs 112 g/kg DM for sulla and grass/clover swards, respectively). This mode of action of the CT forages appears to be very promising for the control of parasites or the animal productivity of parasitised hosts. However, the effects on animal digestion/performance are not the same for all CT, and therefore generalisation of their mode of action must be made with caution, as it might not apply to all CT-containing plant species, but depend upon the concentration and structure of the CT.

It was suggested by nutritional studies that CT from temperate species could bind to dietary proteins, reduce their solubilization and degradation by the rumen micro-organism and consequently optimise the rumen function (Min et al, 2003). In the subsequent move of rumen digesta into abomasum, where the pH is less than 3.5 and favours the disassociation of CT-protein complex (Martin et al., 1985; Mangan, 1988), a net increase of protein supply to the abomasum and small intestine occurs (MacNabb et al., 1996; Min et al., 2000). However, tropical forages, with high CT concentrations, have anti-nutritional effects, such as reduced proportion of the diet being available for utilisation, reduced rate of digestion, depressed feed intake, with an overall decrease to the nutrient supply to the animals (McSweeney et al., 2001;

Silanikove et al., 2001; Waghorn and McNabb, 2003). In contrast to what is observed with temperate forages, feeding tropical forages can result even in protein (N) deficiency, especially when fed with poor-quality grasses (Waghorn and McNabb, 2003). This has been attributed to their high CT concentration, which binds strongly with protein reducing their availability for both the ruminant microflora and the host ruminant throughout the gastrointestinal tract (Min et al., 2003; Waghorn and McNabb, 2003).

Min et al., (2003) concluded that high forage CT concentration (>55g CT/kg DM) generally reduces nutrient digestibility and feed intake, whilst low concentrations of CT (20-45g CT/kg DM) bind protein in reversible manner, protects protein from degradation and ultimately increases net protein absorption and animal productivity. However, little evidence exists that tropical forages with low levels of CT have beneficial effects on sheep digestion or production (Makkar, 2003; Silanikove et al., 2001), while even the use of some temperate forages with moderate CT levels, such as sainfoin or *L. pedunculatus*, resulted in no net increase of protein supply. The last was demonstrated by Waghorn et al., (1987, 1994) and Bermingham et al. (2001) when tested the absorption of essential amino acids from the small intestine of sheep fed on fresh *L. corniculatus*, *L. pedunculatus*, sulla and sainfoin with or without the intraruminal infusion of PEG. The studies of Waghorn et al. (1987, 1994) showed that *L. corniculatus* increased the absorption of amino acids from the small intestine, relative to PEG supplemented sheep, whilst *L. pedunculatus* resulted in no net increase. Similarly, Bermingham et al., (2001) demonstrated no net increase of amino acid absorption from the small intestine of sheep fed sainfoin (CT-concentration of 38 g/kg DM), whilst feeding sulla increased amino acid supply, relative to PEG supplemented sheep, under the same experimental procedures.

These differences on nutritional supply and/or animal metabolism of the CT-rich legumes species have been attributed to the forage CT structure rather than the CT concentration

(Min et al., 2003). Structurally, CT consist of complexes of oligomers and polymers of flavonol units that are linked by carbon-carbon bonds forming high molecular weight molecules (Asquith and Butler, 1986; Hagerman et al., 1992; Mueller-Harvey, 1999). Due to several chemical parameters such as the total number of flavonol units (i.e. molecular weight), position and type of linkage between the flavonol units, position of OH groups etc., it is appreciated that an infinite variety of chemical structures can be produced (Mueller-Harvey, 1999). As a result a vast array of different CT molecules is synthesised by plants of the same family or even of the same genera. For example, differences in CT of *L. pedunculatus* and *L. corniculatus* (*Lotus spp.*) has been found in their molecular weights, 2,200 vs. 1,900 respectively, as well as in their chemical structure. The CT from *L. pedunculatus* contains predominantly prodelphinin-type subunits, whereas the CT from *L. conriculatus* consists predominantly of procyanidin-type subunits (Foo et al., 1996; 1997). It is therefore concluded that, due to specific CT characteristic, forages with similar CT-concentration present different chemical and biological properties and therefore more analytical information regarding the levels and structure of CT must be provided when CT-forages are tested on animal health or productivity (Makkar, 2003; Min et al., 2003; Min and Hart, 2003; Waghorn and McNabb, 2003).

6.4 Effects of chicory forage on sheep nutrition

There is a large body of evidence suggesting that chicory is a forage with high nutritional and feeding value. Studies by Hoskin et al., (1995); Barry (1998); Scales et al., (1994); Jung et al., (1996); Belesky et al., (2001) have reported that most of the macro-minerals in chicory were similar to or exceeded those found in grasses and legumes. A higher micro-mineral content (high concentration of the elements S, B, Mn and Zn; Li and Kemp, 2005) has also been reported. Chicory has variable crude protein levels, between 134 to 244 g/kg DM (Crush and Evans, 1990), while its metabolisable energy (ME) concentration is higher than that observed in perennial ryegrass (Barry, 1998). Similar observations were reported in the present thesis, where chicory had similar mineral content, higher CP and higher ME concentration relative to grass/clover pastures (i.e. Table 5.1, Chapter Five). Previous studies on feeding value reported that chicory digestibility was also higher than grass-based pasture (Holden et al., 2000; Kusmartono et al., 1997) due to higher concentrations of readily fermentable-soluble carbohydrates and lower concentrations of structural carbohydrates than perennial ryegrass (Hoskin et al., 1995; Kusmartono et al., 1996a). The higher carbohydrate solubility along with the lower levels of chicory NDF have been suggested by Barry (1998) as the reasons for higher voluntary feed intake associated with animals grazing chicory. Kusmartono et al., (1996a,b) demonstrated that chicory degradation and disappearance from the rumen were approximately twice as fast compared to perennial ryegrass, supporting the view that high feeding value of chicory is also related to its high digestibility and high voluntary feed intake. In the present study measurements on chicory digestibility (IVDMD or NCGD in Chapters Two, Three, Four or Five, respectively) and feed intake (N-alkane methods in Chapters Two and Three) tended to agree with these observations. The superior nutritional value of the chicory forage has also been supported by several studies testing for its beneficial effects on sheep, cattle and deer productivity and reported greater production in terms of live-weight gains (Fraser et al., 1988; Hoskin et al., 1995; Scales et al., 1994), carcass weight (Min et al., 1997), milk production

(Jung et al., 1996) and wool production (Fraser and Rowarth, 1996) relative to grass-based pastures. In the present study similar observations reported in the Chapter Five where *T. circumcinta* trickle-infected lambs grazing on chicory for a six-week period had higher liveweight gains compared both to the trickle infected and previously uninfected lambs grazing on grass/clover pastures. From the present and previous data, it is concluded that chicory is forage with high nutritional value that may offer an alternative in grazing management practice.

6.5 Problems associated with the use of bioactive grazing forages against nematode infections

The use of bioactive forages as a control measure against gastrointestinal parasitism has the advantage of providing a readily applicable method to practitioners, as opposed to other long-term alternative control methods, such as the breeding for resistance. It is, however, evident from the present thesis and previous studies that such a control method is liable to two major disadvantages, namely the moderate efficacy and the inconsistent effectiveness against parasitism. The bioactive efficacy against parasitic population of lambs in the present thesis, although significant, did not exceed the 60% reduction in absolute worm egg counts or in worm numbers (Figures 3.4, 4.3 and Table 5.2), with similar efficacies being observed in previous studies with bioactive forages (Niezen et al., 1995, 1998a; Marley et al., 2003b). Furthermore, previous experimentation with bioactive forages demonstrated inconsistent results regarding their anti-parasitic effects. For example, the CT-rich forages birdsfoot trefoil (*Lotus corniculatus*) or sulla reduced the level of parasitism of grazing lambs in some studies (Niezen et al., 1995; Marley et al., 2003b), but had no effects on parasitised sheep in other grazing trials (Niezen et al., 1998a,b, 2002a; Bernes et al., 2000). Similarly, the chicory forage has been reported to exert anti-parasitic properties in some grazing studies (Scales et al., 1994; Hoskin et al., 1999; Marley et al., 2003b), whilst had no effect on subsequent grazing experimentation (Thamsborg, 2003).

One possible reason for such discrepancies may be the varying bioactive plant representation within the experimental swards in different grazing trials. This could be caused by variation in forage establishment, re-growth or weed infestation and hence variation in its representation in the diet. Such was the case in the present thesis, where the establishment of the bioactive forages differ markedly between bioactive species. For example in Chapter Two, the bioactive forage representation in the plots was 61, 69, 79 and 22 % DM for the lotus, sulla, chicory and sainfoin forages, while the establishment of the same bioactive species also varied

slightly between experimental years (sulla forage represented the 65 and 76 % of the total sward DM in experimental plots of Chapter Two and Five, respectively). As a consequence such variation may result in different PSM concentration in the diet offered, across the years. For example, CT content of the samples collected from sulla plots was 15.8 and 36.9 g/kg DM in the experimental swards of Chapter Two and Chapter Five, respectively.

Apart from the bioactive forage representation within the plots, the different PSM concentration within the same plant species may also be attributed to variation of specific secondary metabolite across the different experimental years. It has been shown, for example, that CT formation within the same plant species can be affected by factors such as the weather conditions, the extent of herbivory, pathogen infestation or even the date of sampling, which consequently can result in different CT concentration and/or CT structure (Mueller-Harvey, 1999). The study of Haring et al. (2005) reported two-fold differences in CT concentration of sainfoin and birdsfoot lotus between the samples taken on 5th and the 20th week after sowing. Similarly, Koupai-Abyzani et al. (1993) demonstrated a five-fold difference in CT concentration during sainfoin leaf development, while also substantial was the alteration in the chemical composition of CT isolated at different developmental stages. Similar variation in PSM content has also been reported for chicory forage. Rees and Harbone (1985) demonstrated the seasonal variation of chicory PSM and particularly for sesquiterpene lactones, the secondary metabolites showing bioactivity against insects (Rees and Harbone, 1985) and parasites (Molan et al., 2003a) *in vitro*. In the study of Rees and Harbone, (1985), samples of chicory leaves collected on different months had four-fold difference in the total sesquiterpene lactone content (concentration of 0.1 and 0.4 % DM on April and June, respectively). It is, therefore, appreciated that such magnitude of PSM concentration difference in the consumed forages may result in different magnitude of biological activity or even contradicting evidences of their action, which consequently may restrict the application of such alternative forages in

mainstream agricultural practice.

6.6 The potential use of bioactive forages in sustainable/organic production systems

The lack of repeatable anti-parasitic effects in the studies referred to above, along with the moderate efficacy of the bioactive forages renders such a control measure unable to dispense the need for the timely intervention of effective anthelmintic treatment. Similar deficiencies have also been reported with all other alternative methods currently being investigated, such as nematophagous fungi (Chandrawathani et al., 2004), grazing management (Barger, 1997, 2006) or breeding resistant sheep (Gruner et al., 2002). It is therefore suggested that the issue of sustainable helminth control rests with the preservation of anthelmintic effectiveness, through the implementation of these alternative methods, aiming to reduce heavy selection for anthelmintic resistance (Miller et al., 2004). Within this framework, bioactive forages could be used in sustainable production systems as a means to reduce the number of anthelmintic applications and the preservation of susceptible worms on the farm.

The beneficial use of bioactive forages could be achieved through the incorporation of these forages in grazing management practices of specific seasons, aiming to reduce the accumulation of parasitic burdens that can lead to loss of production. Pasture contamination with infective larvae is governed by the climatic conditions. Thus, in cool temperate climate conditions, such as those prevailing in the area of the present experimentation, a succession of individual parasitic species with *Teladorsagia* and *Trichostrongylus* spp is observed, which contributes increasingly in pasture infectivity in late summer and autumn months. It is therefore suggested that bioactive forage use should be applied in early summer, where the prevailing species is the gastric *Teladorsagia* nematode, affecting in some degree the load of pasture contamination. Furthermore, in a number of studies, it has been demonstrated that the effect of parasitism had a threshold level of infection, below which there is no obvious effect on productivity loss. Coop et al. (1977, 1981, 1982 and 1985) using four-month-old lambs and different levels of infection concluded that this threshold level for *T. circumcincta* infection was

a trickle larval dose between 1,000 to 1,500 L3 per day. The existence of such threshold level, below which there is no obvious performance penalty, has a significant meaning from a practical point of view, since bioactive forages could be used complementarily to other methods, such as selective drenching with anthelmintics and close flock monitoring tools (i.e. estimation of sheep FEC).

The use of bioactive forages, an alternative control method with moderate efficacy against parasites, will result in the presence of some parasites on the pastures. However, this control method allows the lambs to build up immunity, which, as suggested by the present results (Chapter Five), is further enhanced by the use of these special forage species. The maintenance of effective immunity in animals and consequently the reduction on anthelmintic dependence has been highlighted as a significant factor to sustainable worm control strategies by recent guidelines (Abbott et al., 2004). Furthermore, the moderate efficacy of bioactive forages has the advantage of low selective pressure on the parasite population, a fact that marks the sustainability of the bioactive forage use, as a control method, particularly in organic production systems that require the use of grazing pastures (Cabaret et al., 2002a,b).

Other benefits of the use of bioactive forages, such as the CT legumes, particularly in low-input organic systems, are the increase of N utilisation efficiency and reductions of CH₄ and NH₃ produced. (Rochon et al., 2004; Ramirez-Restrepo and Barry, 2005). Similarly, chicory forage, being a deep-rooted perennial, can be used in warm seasons producing a large quantity of high quality feed, while at the same time can reduce nitrate leaching, deep drainage and therefore the rate of soil acidification and occurrence of dryland salinity (Li and Kemp, 2005).

6.7 Future directions

The present study provided new evidence of the anti-parasitic activity of bioactive grazing forages, such as sulla and chicory, while demonstrating the first, *in vivo*, evidence of immune related effects from these bioactive forages. However, the exact mode of the enhancement of immune response has not been fully identified. The existing literature to date has focused on the effects of bioactive forage on the immune response through a nutritional mediated mechanism resulting from certain PSM, such as the CT, or other plant properties such as the forage feeding value. There is no research, however, on forage PSM substances with immunostimulatory properties *per se* towards the animal local or systemic, humoral or cellular immune responses. Evidence exists in the human research literature, where immunostimulatory agents were extracted from plants and tested on human lymphocytes *in vitro*. Amirghorfran et al., (2000) evaluated the immunomodulatory effects of five herbal plants, and reported that extracts from chicory enhanced the proliferation of human lymphocytes *in vitro*. Furthermore, sesquiterpene lactones appear to have such immunomodulatory activity stimulating the mast cell degranulation (Picman, 1986), while other compounds, such as low molecular weight phenolics, extracted from several plants also show similar effects (Wagner, 1999; Singh et al., 2003). Regarding the present study, it is appreciated that such mechanisms cannot be excluded since the observed bioactive effects, such as the reduced larvae establishment/development (Chapter Five), affected FEC (Chapter Four), or reduced worm numbers (Chapter Three), could all be related to enhanced host immune response by immunomodulatory effects of PSM compounds. Nevertheless, such mechanisms of the PSM compounds should be evaluated by future studies. A summary of the up to date suggested ways of action of bioactive plants against gastrointestinal parasites is given in Figure 6.1.

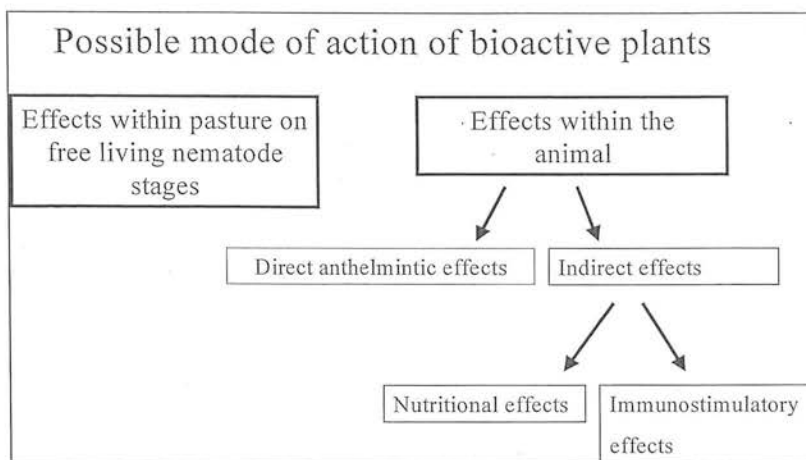


Figure 6.1 The proposed mode of action of bioactive forages against the gastrointestinal parasites of small ruminants

The present thesis focused on the investigation of the effects of bioactive forages on parasitised growing lambs, since they are more susceptible to nematode infections due to their slow development of immunity. It will, however, be very relevant to investigate their bioactive effect on adult sheep, and particularly ewes around parturition, a period that has a significant role in parasite epidemiology and the initiation of the infection of growing lambs (Michel, 1969; Abbott et al., 2004). Recent evidence suggests that the periparturient relaxation of ewe immunity (PPRI) has a nutritional basis (Houdijk et al., 2000, 2001a,b,c, 2005, 2006) and therefore any likely nutritional and/or immunological effect of bioactive forages, such those suggested by the present study, may well be proven beneficial for ewes around parturition.

In addition an interesting future research topic, with practical significance, would be to investigate the possible use of bioactive forages in conserved form (hay or silage). This would have practical benefits in regions without access to all year round grazing pasture such as those that exist in subtropical or Mediterranean areas. Recent studies testing sainfoin hay have shown promising results, with observed reductions on worm egg output (Paolini et al. 2003b,c, Athanasiadou et al., 2005b). There is however the need for further research in this area, testing

several bioactive species as possible sources of hay, while investigating the changes of PSM content and structure during conservation stages.

Furthermore, as has been mentioned earlier in this thesis, the bioactive forages are alternative plants with limited application in mainstream agricultural practices. It is, therefore, suggested that further research related to bioactive forage use is required, especially in order to enhance our knowledge of soil-plant-animal interaction for a wide range of bioactive species under different soil types and climatic conditions. Research is needed on the incorporation of these special species as a component of annual and perennial pastures. New cultivars with specific attributes, such as higher production in low input farming conditions, higher feed quality or even enhanced concentration in specific secondary metabolites, may well contribute towards sustainable animal production.

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