# ANTI-PARASITIC ACTIVITY OF BOVINE MILK

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fulfilment of the requirements
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# ERRATA

| Page | Paragraph | Line | Amendment |
|---|---|---|---|
| 14 | 4 | 4 | Should read "sheep and cows than in lambs and calves" |
| 17 | 3 | 10 | Should read " of infection with *O. ostertagi* in cattle (Fox *et al.*, 1988)". |
| 18 | 2 | 3 | Typographical error: "inflammation" |
| 23 | 2 | 4 | Lambs were between 21 and 25 days-of-age when first infected. |
| 28 | 1 | 2 | Second antibody was raised at Massey University and used undiluted |
| 29 | 3 | 2 | Should read " by a modification of the micro-method of Dorny and Vercruysse (1998)" |
| 30 | 4 | 11 | Centrifugation was at 10,000 *g* |
| 33 | 2 | 6 | Delete "of" |
| Facing 37 | Table 2.3 | - | Omasum/body weight for Solid-ex should read 2.8 ± 0.4 |
| Facing 37 | Table 2.3 | - | Body weight for Solid-ex and Solid-en should read 17.5 ± 1.7 and 15.3 ± 1.3 respectively |
| 39 | 2 | 2-3 | The height of the papillae (mean for each lamb) were in the range..... |
| 44 | 2 | 7 | Delete "not" |
| 61 | 2 | 2 | Replace "development" with "establishment" |

# ABSTRACT

Previous studies have shown that milk-fed ruminants were less susceptible to gastro-intestinal parasites compared with young animals that were given solid feed in the diet. The present studies were carried out to gain a better understanding of how milk feeding reduces worm establishment in new-born lambs. Two approaches have been taken: an in vivo study in which lambs were infected from three weeks of age with third-stage larval *Ostertagia circumcincta* and an in vitro examination of direct effects of bovine milk and some of its crude fractions on the motility of *O. circumcincta* larvae.

The in vivo experiment was designed to compare parasite establishment in lambs either fed entirely on milk from birth, weaned on to solid feed by three weeks of age, or provided with solid feed from two weeks of age and given a milk feed once a day. To examine whether lack of rumen development was a crucial factor, each diet group consisted of lambs given normal ensheathed third-stage larvae and an equal number of lambs given exsheathed larvae. A total of 24 lambs were included in the study, in six groups each of four lambs. All lambs were infected by tube (into the oesophagus) twice a week with either 1000 exsheathed or ensheathed third-stage *O. circumcincta* larvae. Infection began after the week taken to establish the lambs on their new diet, so that starting on week four of life, the lambs were trickle infected for six weeks.

There were highly significantly lower worm burdens at necropsy in the two milk-fed groups of lambs than in both the other groups, but no difference between the burdens in those completely fed solid food and in lambs receiving a 600 ml milk feed once a day along with solids. Irrespective of the diet, female worms made up half the total number of worms in each lamb, with males and immature stages equally making up the other half. Faecal egg counts in the Milk groups were also very low, three of the eight lambs never providing a faecal sample in which eggs were found. Also consistent with the lower worm burdens were the thinner abomasal mucosa and lower abomasal pH,

although these may also have been affected by the diet. Nodules were visible in the abomasa of all lambs in all groups.

All groups had increased serum gastrin and pepsinogen levels, with considerable variation between animals within all groups.

An important observation was that there was no significant difference between lambs receiving exsheathed or ensheathed larvae for any parameter measured. The immaturity of the reticulo-rumen and omasum does not appear to prevent ensheathed larvae from exsheathing and establishing in the abomasum of lambs with an underdeveloped rumen. The similar worm burdens in the milk-fed lambs given ensheathed and exsheathed larvae therefore does not support the conclusions from an earlier study in calves that lack of rumen function was the reason for lower worm burdens in non-ruminating calves. Instead, it would appear that the milk itself is reducing parasite establishment.

In vitro exposure to fresh bovine milk, commercial bovine milk with either 3.3% or 0.2% fat, the milk powder fed to the lambs, whey protein, casein or ultra low heat skim milk powder all reduced the motility of exsheathed third-stage *O. circumcincta* larvae. The effect on motility was concentration and time dependent for all milks. The active component appears to be associated with proteins and not with the lipid fraction and may be non-specific, as both whey and casein were effective. Different components may be responsible for inhibition of larvae by whey and casein proteins. The activity of whey protein increased as the pH increased; the whey was most active at pH 4.5 and above, when it would be in the anion form. In contrast, there was no difference in activity at pH 5.5 and 6.5 for casein. The effect of time of incubation also differed for whey and casein.

A possible explanation for the in vitro and in vivo effects of milk are the attachment of the proteins to the larvae. The lack of effect when milk and solid feed are ingested together may result from the protein attaching to the food particles in preference to the larvae or, alternatively, the milk may have left the abomasum before the larvae were administered. This suggests that practical

applications for milk proteins as anti-parasite agents may be limited in ruminants consuming solid feed.

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# INTRODUCTION

The neonatal mammal is dependent on milk for the wide variety of nutrients, particularly proteins, fats, lactose, minerals and vitamins, which are required for growth and development. Hormones and trophic agents in the milk also contribute to the growth and maturation of the gastro-intestinal tract. Specific antibodies, which are present in especially high concentrations in colostrum, allow the neonate to have some immediate protection from infectious diseases. Immunoglobulins can be absorbed directly from the intestine only for a few hours after birth, after which "closure" prevents most macromolecules from entering the circulation. A continuing intake of antibody in the milk is likely to be beneficial in combating pathogens in the gut lumen. In addition, there are a number of bactericidal compounds in milk, such as lactoferrin and lactoperoxidase, and others which have an anti-parasitic action.

Gastro-intestinal parasites are an important cause of disease in children in many parts of the world and a well-known cause of loss of production in farmed animals. In general, young animals are particularly at risk compared with adult animals that have developed natural immunity from prolonged exposure to the parasites. An exception is the reported reduced susceptibility of the unweaned animal to a range of parasites, both nematode and protozoal. Farmers do not routinely drench young stock until weaning and a small number of experiments on milk-fed animals support the relative resistance to parasitism of these animals. The exact mechanism appears to vary considerably between host-parasite systems, for example, the malarial parasite has an insufficient supply of an essential nutrient and *Giardia* is killed by fatty acids.

Abomasal nematodes are economically important parasites in New Zealand which are currently largely controlled by supplement chemical drenches. Any additional strategies for parasite control are therefore of considerable practical interest to livestock farmers. Reports that milk-fed calves have lower worm burdens (Porter, 1941; Rohrbacher et al., 1958; Satrija et al., 1991) and that feeding milk has an anthelmintic action (Leese, 1943; Spindler et al., 1944; Spindler & Zimmerman, 1944; Shorb & Spindler 1947)

suggest that there may be specific chemicals worthy of study. On the other hand, it may be that the stage of development of the gut is the critical factor and nematode establishment is reduced when rumination is absent (Satrija et al., 1991).

The experiments reported here are an attempt to understand how milk feeding reduces worm establishment in new-born ruminants. Two approaches have been taken: an in vivo study in which lambs were infected from three weeks of age *with Ostertagia circumcincta* (Chapter 2); and an examination of direct effects of bovine milk and some of its crude fractions on *O. circumcincta* larvae in vitro (Chapter 3).

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**Table 1.1** Compartments and their components in mature bovine milk (based on Jensen et al. (1990)).

| Compartment description | Major components |
|---|---|
| Aqueous phase | 1. Compounds of Ca, Mg, $PO_4$, Na, K, Cl, $CO_2$, citrate, casein<br>2. Whey proteins: a-lactalbumin, lactoferrin, IgA, lysozyme, and serum albumin B<br>3. Lactose and oligosaccharides<br>4. Nonprotein nitrogen compounds; glycocyamine, urea, amino acids<br>5. Miscellaneous: B vitamins, ascorbic acid |
| Colloidal dispersion | 6. Caseins: $\alpha$-, $\beta$-, $\kappa$-, Ca, $PO_4$ |
| Emulsion fat globules | 7. Fat globules: triacylglycerols, vitamins. |
| Fat globule membrane Absorbed layer | 8. Milk fat globule membrane: proteins, phospholipids, cholesterol, enzymes, trace minerals. |
| Cells and fragments | 9. Macrophages, neutrophils, lymphocytes, epithelial cells, leukocytes, cytoplasmic fragments |

# CHAPTER 1

# LITERATURE REVIEW

In this chapter, the principal topics reviewed are the composition of bovine milk, with particular emphasis on those components with anti-bacterial or anti-parasitic activity, and the effects of abomasal parasites on gastric function.

## 1.1 BOVINE MILK

### 1.1.1 COMPOSITION OF BOVINE MILK

All mammalian milks consist largely of water, in which all other components are dissolved or suspended. A small percentage of the water in milk is hydrated to lactose and salts, or is bound in the proteins (Jenness, 1988). Bovine milk contains about 87% water and 13% milk solids which are made up of proteins, carbohydrates, lipids, minerals, vitamins and non-protein nitrogen-containing compounds (NPN). The largest particles in milk are the cells, 98% of which are white blood cells and 2% are epithelial cells. Bovine milk contains about $10^4$ to $10^7$ cells/ml (Lipkin et al., 1993).

During milk synthesis and secretion, the components in milk become located in several compartments (Table 1.1), which can be separated by centrifugation of fresh milk (Neville, 1995). These compartments are: the aqueous phase, which contains dissolved proteins, carbohydrates, minerals and NPN (Johnson, 1974); a dispersed colloid phase largely made up of the caseins; the cells; and the fat globules which also have adsorbed substances on their membranes (Jensen et al., 1990). The water and lipid solubility of the many constituents of milk determine whether they will be in the aqueous phase, in fat globules or in a colloidal suspension.

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**Table 1.2** Lipid class composition of mature bovine milk during lactation (based on Bitman and Wood (1990)).

| Lipid Class | % of Total Lipid (g/100 g milk) |
|---|---|
| Phospholipid | 1.11 |
| Cholesterol | 0.46 |
| Triacylglycerol | 95.80 |
| 1,2-diacylglycerol | 2.25 |
| Free fatty acids | 0.28 |
| Monoacylglycerol | 0.08 |
| Cholesteryl ester | 0.02 |

Milk constituents may have a biological activity distinct from their importance as a nutrient. Included in the category of biologically active components are growth factors and hormones as well as the immunoglobulins, enzymes, proteins and lipid with anti-microbial or anti-parasitic actions.

#### 1.1.1.1 Carbohydrate

The principal carbohydrate in milk is lactose, a disaccharide which is found almost exclusively in milk. The concentration in bovine milk is 4.6%, whereas in human milk it is higher at 7.0%. There is a strong inverse correlation between the concentrations of protein and lactose in the milk of most species (Newburg & Neubauer, 1995). Glucose and galactose are monosaccharide precursors of lactose and are present in milk in concentrations of 13.8 mg/100 ml and 11.7 mg/100 ml respectively (Reineccius et al., 1970). The glucose concentration in milk from terrestrial mammals ranges from 0.1 to 0.8 mM/L (Faulkner et al., 1981; Marschke & Kitchen, 1984). Oligosaccharides are other carbohydrates which are present in milk and colostrum in widely varying concentrations in different species (Newburg & Neubauer, 1995).

#### 1.1.1.2 Lipids

The lipids of bovine milk, often simply called "fat", are almost all found in dispersed globules (Christie 1983; Jensen & Clark, 1988; Jensen et al., 1991). Average values for bovine milk lipids are given in Table 1.2. There are over 400 different fatty acids, which make up thousands of triacylglycerols (TG) and the many other lipids which are present in small quantities in milk.

There are only small amounts of free fatty acids in milk. Fatty acids vary in chain length, branching and degree of saturation. Saturated fatty acids contain no double bonds, monounsaturated fatty acids contain one double bond and polyunsaturated fatty acids contain two or more double bonds. Monounsaturated fatty acid is largely accounted for by oleic acid. Milk contains small quantities of saturated and branched-chain fatty acids and polyunsaturated fatty acids (Jensen &

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**Table 1.3** Amino acid composition of bovine milk (adapted from McBean & Speckmann, 1988).

| Amino acids | mg/100 g milk |
|---|---|
| Essential | |
| Histidine | 89.39 |
| Isoleucine | 199.24 |
| Leucine | 322.18 |
| Lysine | 261.14 |
| Methionine | 82.36 |
| Phenylalanine | 159.01 |
| Threonine | 148.43 |
| Tryptophan | 46.30 |
| Valine | 220.13 |
| Nonessential | |
| Alanine | 113.58 |
| Arginine | 119.30 |
| Aspartic acid | 249.61 |
| Cystine | 30.34 |
| Glutamic acid | 689.09 |
| Glycine | 69.71 |
| Proline | 318.88 |
| Serine | 179.12 |
| Tyrosine | 159.01 |

Clark, 1988; Ha et al., 1989). Although dairy cattle consume relatively large amounts of polyunsaturated fatty acids, the amounts in milk are low as a result of biohydrogenation in the rumen (Ha et al., 1989).

Triacylglycerols (TGs) make up 95% or more of the lipid in milk (Table 1.2). They are complex molecules formed by combining a glycerol with three fatty acids. Because of the large number of possible combinations of fatty acids, bovine milk contains thousands of TGs. Phospholipids differ from TGs in that one of the fatty acids attached to the glycerol is replaced by a phosphate-nitrogen grouping. They are found mainly in the membrane surrounding the milk fat globule or bound to caseins (Jensen & Clark, 1988). Milk also contains sterols, principally cholesterol, which is located mostly in the milk lipid globule membrane, and Vitamin D (Jensen & Clark, 1988; Bitman & Wood, 1990; Jensen et al., 1991). Other lipids which are present in small quantities in milk include glycosphingolipids, gangliosides, glycolipids and cerebrosides (Renner et al., 1989).

#### 1.1.1.3 Nitrogenous compounds

The nitrogenous components of bovine milk are largely the proteins in casein and whey. About 5% is non-protein nitrogen (NPN): amino acids, urea, creatine, nucleotides, nucleosides, acid-soluble nucleotides and nucleic acids.

There are a large number of both essential and non-essential amino acids in bovine milk (Table 1.3). Milk contains substantial quantities of derivatives of somatic cell DNA, reported to range from 5-20 mg/L The DNA content of the milk is correlated with the number of cells present (Bremel et al., 1977; Hutjens et al., 1979).

Milk contains a number of enzymes which are present either as unassociated forms in the aqueous phase, associated with the fat globule membrane or skim milk membrane vesicles, bound to casein micelles or as part of the microsomal particles (Shahani et al., 1973; Kitchen, 1985; Farkye, 1992). Lactoperoxidase is

**Table 1.4.** Protein composition of mature bovine herd milk (adapted from Swaisgood, (1995)).

| Protein | g/liter milk |
|---|---|
| Total protein | 36 |
| Casein protein | 29.5 |
| $\alpha_{s1}$-Casein | 11.9 |
| $\alpha s_2$-Casein | 3.1 |
| β-Casein | 9.8 |
| k-Casein | 3.5 |
| γ-Casein | 1.2 |
| Whey protein | 6.3 |
| α-Lactoglobulin | 1.2 |
| β-Lactoglobulin | 3.2 |
| Serum albumin | 0.4 |
| Immunoglobulin | 0.8 |
| Proteose- peptones | 1.0 |

the enzyme present in the highest concentration (3 mg/100 ml) (Jenness, 1982) while lysozyme has a concentration of 10-35 μg/100 ml (Shahani et al., 1973; Renner et al., 1989). There is also a relatively high ribonuclease content (1-2 mg/100 ml) (Chandan et al., 1968; Meyer et al., 1987), the activity of which is not reduced by commercial pasteurization (Meyer et al., 1987). Other enzymes in milk include amylase, xanthine oxidase and amine oxidase (Meyer et al., 1987). Some enzymes possess biological activities which will be discussed in Section 1.1.3.

The total protein concentration of bovine milk is 30-35 g/kg (Swaisgood, 1995). It is higher in winter than in summer, greatest in the first lactation and is not necessarily dependent on the dietary protein consumed (Bruhn & Franke, 1977). The two major milk protein groups, whey and caseins, can be identified by their behaviour in acids. Acid conditions cause caseins to coagulate whereas whey proteins remain in solution (Jenness, 1988). The protein composition of mature herd milk is listed in Table 1.4 (Swaisgood, 1995).

#### 1.1.1.3.1 Whey protein

Whey proteins make up about 16-18% of total bovine milk protein (Cerbulis & Faerrell, 1975; Swaisgood, 1995). They are a diverse group of proteins including β-lactoglobulin, α-lactalbumin, blood serum albumin, immunoglobulins and proteoses and peptones (Table 1.4). Lactoperoxidase has a concentration of 3 mg/100 ml (Jenness, 1982) and makes up about 0.5% of the total whey proteins. The time and temperature during pasteurization determine its loss during processing (Scott, 1989).

Immunoglobulins (Igs) belong to the smaller fractions of milk protein in mature milk. They account for about 2% of total milk protein and 12% (8-19%) of whey protein (Cerbulis & Farrell, 1975; Ng-Kwai-Hang & Kroeker, 1984). Their concentration is very high in colostrum (50-150 mg/ml), but it is reduced to less than 1 mg/ml in mature milk, although it increased slightly during the last months of the lactation period. IgG predominates in bovine colostrum, accounting for 80-

90% of the total immunoglobulins; the proportions of IgM and IgA are 7% and 5%, respectively. $IgG_1$ and $IgG_2$ account for 80-90% and 10-20% of IgG, respectively. The concentrations of all isotypes decrease rapidly postpartum, however, IgG predominates also in mature milk (Guidry & Miller, 1986).

#### 1.1.1.3.2 Casein Micelles

The characteristic opaque appearance of milk is due to the clumping of caseins to form larger particles (micelles) within which calcium and phosphorus are trapped (Jenness, 1988; Swaisgood, 1995). These particles make up 78- 82% of the protein in bovine milk (Cerbulis & Farrell,1975; Swaisgood, 1995). Caseins are precipitated from bovine milk by acidification to pH 4.6 at 20°C (Eigel et al., 1984). The casein micellar systems in milks differ considerably. There are four major types of caseins: $\alpha_{s1}$-, $\alpha_{s2}$-, $\beta$, and, $\kappa$-caseins in ratios of about 4:1:4:1 (Table 1.4) (Davies & Law, 1980; Farrell et al., 1990). Total casein N rapidly decreases after calving with the lowest concentration at 5-10 weeks, followed by a gradual increase to the end of lactation (Bruhn & Franke, 1977).

### 1.1.1.4 Vitamins, minerals and trace elements

The major ionic constituents of bovine milk are sodium, potassium, magnesium, calcium, chloride and phosphorus and the trace elements iron, copper and zinc. The sum of the monovalent ion (sodium, potassium and chloride) concentrations is more or less inversely proportional to the lactose concentration. The concentrations of ions in bovine milk are: sodium 30-70 mg/100 ml; potassium 100-200 mg/100 ml; chloride 80-140 mg/100 ml; calcium 90-140 mg/100 ml; magnesium 5-24 mg/100 ml and phosphorus 70-120 mg/100 ml (Renner, 1983).

Trace elements in mature milk are iron (0.2-0.6 µg/ml), zinc (4 µg/ml), copper (0.05-0.2 µg/ml) and manganese (21 ng/ml) (Anderson, 1992). The concentrations of selenium in tissues and body fluids are affected by the amount in

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**Table 1.6** Hormones and growth factors in bovine milk (Monk et al., 1975; Erb et al., 1977; Pope & Swinburne, 1980; Meisterling & Dailey, 1987; Campana & Baumrucker, 1995).

| | Hormones | Concentration |
|---|---|---|
| Steroids | 5-α Androstane-3,17-dione | 0.0-7.2 ng/ml |
| | Corticosteroids | 8-18 ng/ml |
| | Estrogen | 6-20 pg/ml |
| | estradiol-7β | 4-5 pg/ml |
| | Progesterone | 13 ng/ml |
| | Vitamin D | 45-105 IU/liter |
| Thyroid and parathyroid hormones | Parathyroid hormone-related peptide | 56 ± 12 ng/ml |
| | Thyroxin ($T_3$ and $T_4$) | trace |
| Hypothalamic hormones | Lutenizing hormone-releasing hormone (LHRH) | 3.9-11.8 ng/ml |
| | Gonadotropin hormone-releasing hormone (GnRH) | 0.1-3 ng/ml |
| | Thyrotropin-releasing hormones (TRH) | 16-34 ng/ml |
| Pituitary hormones | Growth hormone | < 1ng/ml |
| | Prolactin | 500-800 ng/ml |
| Others | Insulin-like growth factor-I | 5 ± 2 ng/ml |
| | Insulin-like growth factor-II | 1 ± 0.1 ng/ml |

**Table 1.5** The concentrations of vitamins in bovine milk (Scott et al., 1984; Woolard & Indyk 1989; Renner et al., 1989; Hidiroglou 1989; Fournier, et al., 1987).

| Vitamin | Content (μg/liter milk) |
|---|---|
| Water soluble vitamins | |
| Thiamine | 460 |
| Riboflavin | 1,780 |
| Pyridoxine | 610 |
| Cobalamin | 4 |
| Niacin | 710 |
| Folic acid | 60 |
| Pantothenic acid | 3,600 |
| Biotin | 20 |
| Ascorbic acid | 12,500 |
| Fat soluble vitamins | |
| Vitamin A (Retinyl ester) | 267 |
| Vitamin D | 0.2-0.8 |
| Vitamin E | 280 |
| Vitamin K | 19.7 |

food and therefore in the environment e.g. the concentration of selenium in milk is 5-7 ng/ml in New Zealand, whereas in South Dakota it is 160-1270 ng/ml (Dolamore et al., 1992; Casey et al., 1995). A large proportion of copper, zinc and manganese are bound to casein, and iron and manganese are partly bound to lactoferrin (Renner et al., 1989). Other trace elements include iodine, fluorine and cobalt.

Milk contains both water-soluble and fat-soluble vitamins (Table 1.5). Most of the thiamine (Vitamin $B_1$) in bovine milk is produced by micro-organisms in the rumen (Crenin & Power, 1982; Renner et al., 1989). As the concentration of vitamin D in milk largely depends on exposure of the animal to sunlight, it reaches a maximum during the summer months (National Research Council, 1989).

### 1.1.2 HORMONES AND GROWTH FACTORS IN BOVINE MILK

Bovine milk contains a variety of hormones and trophic agents which are capable of influencing the growth and maturation of the neonate (Table 1.6). Some of these agents have been shown to assist in the development of the gastrointestinal tract of the new-born animal. Lactoferrin and epidermal growth factor both promote cell growth (Berseth et al., 1983). Bioactive substances in milk are also capable of regulating mammary cell proliferation (Sporn & Roberts, 1985; Baumrucker & Blum, 1993).

### 1.1.3 DEFENCE AGENTS IN MILK

A substantial number of studies have demonstrated that milk has other biological activities as well as providing the nutritional requirements of the young. Milk contains immunoglobulins, a variety of different anti-microbial substances and even anti-parasitic agents. These help the young resist disease agents and some also protect the mammary gland from infection.

### 1.1.3.1 Immunological agents

There are several ways in which milk has been suggested to improve the immunity of the young. These include both the direct provision of Igs and the contribution of a number of milk components to stimulation of immune function. Average concentrations of the Igs in bovine milk are: IgG1, 59 mg/100 ml; IgG2, 20 mg/100 ml; IgA, 10 mg/100 ml; IgM, 5 mg/100 ml (International Dairy Federation, 1991). Leucocytes in milk may also play an active role against gastro-intestinal organisms. Fragments from $\alpha_{s1}$- and β-casein subunits have been identified as the immuno-stimulating peptide fractions of casein. It has been suggested that these immunopeptides stimulate the phagocytic activity of macrophages in vivo as well as the proliferation and maturation of T cells (Migliore-Samour & Jolles, 1988). Nucleotides in milk are also believed to increase the numbers of helper/inducer T lymphocytes (Van Buren et al., 1985).

### 1.1.3.2 Anti-tumour activity

Whey proteins have been shown to have beneficial effects as anti-tumour agents, both in vivo and in vitro. Addition of total whey protein to the diet is associated with the retardation of chemically induced cancers in several animal models, increasing survival times and stimulating the immune system. These effects may be mediated by the high content in low temperature-processed whey protein of glutamylcysteine, a dipeptide which can promote the synthesis of glutathione, an important antioxidant involved in cellular protection and repair (Bounous et al., 1988, 1989, 1991; Bounous & Gold, 1991; McIntosh et al., 1995). In another study, an α-lactalbumin was shown to be a calcium-modulating protein and thus may function as a metal carrier (Kronman et al., 1981; Lonnerdal & Glazier, 1985; Berliner et al., 1987). It was associated with apoptosis of transformed cancer cell lines in vitro, and therefore is considered a possible anti-tumour agent (Hakansson et al., 1995).

### 1.1.3.3 Antibacterial agents

Antibacterial agents in bovine milk include both specific antibodies and non-specific factors such as lysozyme, lactoferrin, lactoperoxidase, vitamin-binding proteins and lipids. These may act alone or have synergistic effects e.g. the activity of lactoferrin is enhanced by specific antibody (International Dairy Federation, 1991). Milk proteins which bind specifically vitamin $B_{12}$, folate or riboflavin (International Dairy Federation, 1991) inhibit the growth of micro-organisms with specific requirements for these vitamins. Antibodies act in different ways: some prevent adherence of the pathogen to the gut wall, whereas others neutralise bacterial toxins.

#### 1.1.3.3.1 Lysozyme

Lysozyme is reported to act both by modulating the activity of leucocytes (Reiter, 1985) and by directly acting on bacteria. It breaks up the cell walls of Gram-positive organisms by adhering to the basic bacterial surface in the presence of electrolytes and then hydrolysing glycosidic bonds (Reiter, 1985; International Dairy Federation, 1991)

#### 1.1.3.3.2 Lactoferrin

Milk contains 2-20 mg/100 ml of lactoferrin, an iron binding protein which limits the growth of micro-organisms with a specific requirement for iron (Rainard, 1986; Wakabayashi et al., 1992; Teraguchi et al., 1993). Significant amounts of lactoferrin are present in colostrums: >1 mg/ml on the first day postpartum, after which it declines over the next six months (Jenness, 1982). There appears to be an antimicrobial action in addition to deprivation of iron to the bacteria, as lactoferrin has a bactericidal domain (Saito et al., 1991) which is distinct from the iron-binding region (Bellamy et al., 1992).

Lactoferrin has several beneficial effects in vivo, including antibacterial activity (Dionysius et al., 1993), iron regulation (Quarterman, 1982; Davidson &

Lonnerdal, 1989) and immunomodulation (Kawakami et al., 1987; Hutchens et al., 1994). Rat milk stimulates proliferation of the small intestine, an effect ascribed to the trophic action of lactoferrin (Berseth et al., 1983). Human lactoferrin is also an antioxidant (Cohen et al., 1992) and has been associated with inhibition of metastases in mice when administered systemically (Bezault et al., 1994). Recently, lactoferricin, a derivative of lactoferrin, has been shown to have greater bactericidal activity than the native molecule (Tomita et al., 1991).

#### 1.1.3.3.3 Lactoperoxidase

Lactoperoxidase catalyses the conversion of hydrogen peroxide to water. The bactericidal effects of lactoperoxidase have been demonstrated by the addition of hydrogen peroxide and thiocyanate to raw milk: the thiocyanate is oxidised by the enzyme-peroxide complex to generate bactericidal compounds which destroy Gram-negative bacteria (Schmekel & Harnulv, 1986; Renner et al., 1989).

#### 1.1.3.3.4 Lipids

Gastric lipase releases microbicidal free fatty acids and monoacylglycerols from milk TGs (Jensen, 1995). The gangliosides in both human and bovine milk have been shown to inhibit cholera toxin: less than 1 ml of human milk inhibited 0.1 μg of cholera toxin in vitro and in vivo, whereas a 5- to 10-fold greater volume of bovine milk was needed to achieve comparable results (cited by Jensen & Newburg, 1995).

## 1.1.4 ANTI-PARASITIC ACTIVITY OF MILK

Over the last sixty years, a number of studies involving several different mammalian host-parasite systems have demonstrated that a milk diet can affect the course of a parasitism. There have also been a few in vitro experiments in which parasites have been exposed directly to milk or its components. Most studies have compared the levels of infection of milk-fed and weaned animals and therefore were not necessarily examining the effect of milk per se on the parasite. Instead,

lower infection rates may have resulted from an unfavourable environment in the stomach due to the physical immaturity of the tissues or a change in the physiological functioning of the stomach in the presence of milk.

Only experiments in which there is direct exposure of parasites to milk outside the gastro-intestinal tract will be discussed here. These studies appear to be confined to protozoan parasites. Parasitism in the milk-fed animal will be considered in Section 1.2.3. Human milk, but not bovine or goat milk, had in vitro anti-parasitic properties against *Giardia lamblia* trophozoites. There appeared to be two active fractions in non-immune human milk; the more active component was heat labile and the other was heat stable. Bile-stimulated lipase was proposed as the active agent in the labile fraction for two reasons: lipase had similar effects on *Giardia* to the milk fraction and lipase is present in human milk but absent from the milk of the other two species (Gillin et al., 1983). Further, human milk killed the protozoan parasite by generation of unsaturated free fatty acids from triglycerides when exposed to bile (Reiner et al., 1986), or by exposure to long-chain unsaturated fatty acids in the lipid fraction of milk (Hernell et al., 1986; Rohrer et al., 1986).

## 1.2 ABOMASAL NEMATODE PARASITES

Nematode parasites of the ruminant stomach inhabit the acid-producing fourth stomach, the abomasum, which is the equivalent structure to the monogastric stomach (Church 1969; Church & Pond, 1974; Dyce et al., 1987). The principal nematode species which parasitise the sheep stomach are *Ostertagia circumcincta* and *Haemonchus contortus*, a blood-sucking worm. Both belong to the Trichostrongyle family.

Abomasal parasites are an important cause of reduced productivity, as they impair the digestive function of the stomach, cause a negative nitrogen balance, reduce appetite and divert energy into mounting an immune response (reviewed by Coop & Kyriazakis, 1999). Blood sucking species cause anaemia in addition to the

loss of protein across the mucosa, increased mucus secretion and increased cell shedding which all abomasal worms cause in the affected stomach.

In individual animals, the level of larval intake, parasite pathogenicity and host genetics and immune status all play a part in determining the size of the worm burden (Michel et al., 1979). A group of animals in which parasitism has been studied relatively infrequently are unweaned ruminants, which have been reported to have lower levels of infection than weaned animals (Porter, 1941; Rohrbacher et al., 1958; Satrija et al., 1991). The study in calves by Satrija et al. (1991) suggested that the lack of development of the rumen was a critical factor in the smaller worm burdens in milk-fed animals. The role of the milk itself does not appear to have been examined.

## 1.2.1 LIFE CYCLE OF ABOMASAL PARASITES

The life cycle of the most common abomasal nematodes of sheep (*H. contortus* and *O. circumcincta*) and cattle (*Ostertagia ostertagi*) follow the same pattern, the differences being mainly in the duration of the different developmental stages. Eggs pass from the host to the ground with the faeces. There they hatch as first-stage larvae, and, under optimum conditions, *O. circumcincta* and *H. contortus* reach a sheathed infective stage (the third-stage) in 6-7 days and 4 days respectively (Sommerville, 1953). A new host acquires the parasite by eating the third-stage larvae with grass or other food.

The conditions provided by the microbial fermentation in the reticulo-rumen are favourable for the larvae to exsheath (Rogers & Sommerville, 1968). The carbon dioxide content is high and the pH is in excess of 5. The pH in the rumen of calves fed milk, concentrate and hay (pH 5.7-6.6 ) appears to be only slightly higher than in those fed milk alone (between 5.15 and 5.75) (Godfrey, 1961). In ruminants, there is a nearly continuous passage of digesta from the reticulum into the omasum and abomasum (Phillipson & Ash, 1965; Church 1969), carrying the

exsheathed larvae with it down to the abomasum where the parasites develop into adults.

In the abomasum, the larvae enter the gastric glands and undergo a second ecdysis and develop to fourth-stage larvae (Sommerville, 1953). The different species vary in the length of time spent within the gastric glands. Development of individual worms may proceed in one of three ways: most *O. circumcincta* grow within the mucosa, undergo a further moult and emerge as young adults into the abomasal lumen by about Day 8; some larvae may exit from the mucosa after the third ecdysis and live on the surface; others may not develop, but remain in the gastric pits and glands in an inhibited state for up to 2-3 months before developing into adult parasites (Sommerville, 1954; Armour et al., 1966). In contrast, *H. contortus* emerge after about 2-4 days (Stoll, 1943; Christie et al., 1967). The average time required for third-stage *O. circumcincta* larvae to develop into adults is about 16-28 days (Armour et al., 1966), therefore the whole life cycle of the parasite from infection to adult worms is from 22-34 days.

## 1.2.2 PATHOPHYSIOLOGY

Sheep infected with abomasal parasites have a decreased weight gain, disturbed protein metabolism, reduced digestibility, loss of appetite and, in some cases, diarrhoea (Sykes & Coop, 1977; Coop et al., 1977; Parkins et al., 1989; Coop & Kyriazakis, 1999). In addition, *H. contortus* causes anaemia (Hunter & Mackenzie, 1982; Rowe et al., 1988). Fat deposition may be markedly reduced and mineral deposition in the skeleton of the sheep decreased (Sykes & Coop 1977). Infected sheep may have hypoalbuminaemia and decreased gamma globulin and total circulating plasma protein concentrations (Coop et al., 1977). Serum pepsinogen and gastrin and abomasal pH levels are elevated in sheep which have been infected with larval *O. circumcincta* (Anderson et al., 1976, 1985, 1988; Coop et al., 1977; Lawton et al., 1996; Simcock, et al., 1999) or *H. contortus* (Nicholls et al., 1988; Rahman & Collins 1991; Simpson et al., 1997).

Histologically, there is mucous cell hyperplasia, loss of parietal cells and inflammation of the abomasal mucosa (Armour et al., 1966; Scott et al., 1998).

### 1.2.2.1 Hyperpepsinogenaemia

Pepsinogen is the biologically inactive proenzyme of pepsin. It is synthesised by the endoplasmic reticulum of the chief cell, stored in secretory granules and released through the apical surface by exocytosis on stimulation of the chief cell (Hirschowitz, 1991). Pepsinogen is converted to pepsin on contact with the acid of the gastric juice (Ross et al., 1995).

Increased serum pepsinogen concentration is a characteristic feature of abomasal parasitism and may reach ten times the pre-infection level (McLeay et al., 1973; Anderson et al., 1976; Michel et al., 1978; Shoo & Wiseman, 1986; Fox et al., 1988). Hyperpepsinogenaemia was reported to be an earlier indication of developing worm burdens than faecal egg counts in ruminants infected with *Ostertagia* spp. (Thomas & Waller, 1975). In general, there is a correlation between serum pepsinogen levels and total worm burdens in field infections with *O. ostertagi* in cattle and between the overall seasonal serum pepsinogen pattern and the mean worm count in tracer lambs (Brunsdon, 1971; Anderson, 1972; Xiao et al., 1991). In individual experimental animals, the increase in serum pepsinogen does not correlate well with the worm burden, particularly when there are sheep which have very low serum pepsinogen concentrations both before and after infection (Coop et al. 1977; Lawton et al., 1996; Simpson et al., 1997).

After a single infection with third-stage larval *O. circumcincta* in sheep, the circulating pepsinogen levels rose after 3-5 days, reached a maximum 16 days after infection and remained moderately elevated throughout the whole period of infection (Lawton et al., 1996). Even more rapid increases in circulating pepsinogen occur after transplantation of adult worms (Anderson et al., 1985; McKellar et al., 1986, 1987; Lawton et al., 1996). The transfer of adult *O. ostertagi* into calves caused serum pepsinogen to increase within 24 hours and remain

elevated until slaughter 4 to 21 days later (McKellar et al., 1986, 1987). Lawton et al. (1996) saw increased serum pepsinogen levels 8 hours after infection with adult *O. circumcincta* compared with 5-6 days after larval infection. Because circulating pepsinogen increased without the introduction of larvae, extensive invasion of the mucosa by parasites is not essential.

The exact cause of the increase in circulating pepsinogen levels in infected animals has not been established, although several different mechanisms have been proposed. These include increased permeability to macromolecules, direct stimulation of pepsinogen secretion by parasite chemicals and increased pepsinogen release due to the hypergastrinaemia usually seen in abomasal parasitism.

The increase in plasma pepsinogen has generally been ascribed to a "leak lesion", i.e. pepsinogen leaking back from the gland lumen through a mucosa damaged by the parasites. After infection with larvae, this damage would occur during development within the gastric glands and emergence of the later stages of the parasite. It was suggested, therefore, that the serum pepsinogen level indicated the degree of mucosal damage (Murray et al., 1970; Thomas & Waller, 1975; Shoo & Wiseman, 1986; Wiggin & Gibbs, 1987; Hilderson et al., 1991). A further contributing factor to increased back diffusion of pepsinogen may be the increased luminal pepsinogen concentration resulting from the failure of conversion of pepsinogen to pepsin when the abomasal pH was elevated. This was supported by the correlation between abomasal pH and plasma pepsinogen levels during ostertagiosis (Stringfellow & Madden, 1979).

Hypersensitivity reactions may contribute to the increased mucosal permeability and elevated serum pepsinogen level during challenge infections of immune sheep and cattle. The consistently higher serum pepsinogen levels in sheep than in lambs suggested a greater mucosal damage in older animals due to a hypersensitivity reaction (Reid & Armour, 1975; Armour et al., 1979; Pitt et al., 1988).

Parasite chemicals have been implicated in causing hyperpepsinogenaemia because of the rapidity of the increase and the absence of direct tissue damage after adult worm transfer. This does not rule out either chemical damage by the adult worms or disruption of the protective mucus layer adjacent to the mucosa. In addition, an excretory or secretory product of the parasites may directly stimulate the production and secretion of pepsinogen into the gland lumen or blood (McKellar et al., 1986, 1987). Because they failed to demonstrate increased diffusion from blood to abomasal lumen of systemically administered horse radish peroxidase in calves infected with *O. ostertagi*, Stringfellow & Madden (1979) suggested that mucosal permeability was not increased and that pepsinogen may be secreted directly into the blood.

Increased serum gastrin levels may contribute to the increased serum pepsinogen in infected animals, as gastrin is a natural stimulant of pepsinogen secretion. A role for gastrin was suggested by the increase in both serum pepsinogen and gastrin in omeprazole-treated calves (Fox et al., 1989). While hypergastrinaemia may be a contributing factor in some animals, serum pepsinogen can also increase without an increase in serum gastrin (McKellar et al., 1987; Pitt et al., 1988; Hilderson et al., 1992; Simcock et al., 1999) and serum pepsinogen often increases before either serum gastrin or abomasal pH (Holmes & MacLean 1971; McLeay et al., 1973; Lawton et al., 1996).

### 1.2.2.2 Reduced acid secretion

Acid secretion by the parietal cells is normally continuous in grazing ruminants as the primary stimulus appears to be the presence of digesta in the abomasum (Hill, 1960; Kay, 1983). There are fluctuations in the volume and acidity of gastric secretion under different feeding conditions: both acidity and volume increased after feeding and decreased following fasting and abomasal secretion was responsive both to neural stimuli and circulating hormones, particularly gastrin (McLeay & Titchen, 1975; 1977a,b; Reynolds et al., 1980).

Hypochlorhydria occurs after experimental infection of sheep with *O. circumcincta* or *H. contortus* (Anderson et al., 1976, 1981, 1985; Sykes & Coop, 1977; Nicholls et al., 1987, 1988; Lawton et al., 1996). Primary infection with *O. circumcincta* is accompanied by a marked rise in abomasal pH after 5-7 days. (Titchen & Anderson, 1977; Anderson et al., 1981; Lawton et al., 1996). After infection with *H. contortus* in sheep, abomasal pH rose within 2-4 days, reached a maximum between 4 and 10 days after infection, then gradually decreased (Christie et al., 1967; Simpson et al., 1997). Abomasal pH increased rapidly after the transfer of adult worms (Anderson et al., 1985; McKellar et al., 1986; Lawton et al., 1996; Simpson et al., 1997) and therefore does not depend on physical damage to the mucosa during larval development. Depending on the number of worms transferred, the pH may be high by the second day (Lawton et al., 1996; Simpson et al., 1997).

Parietal cell loss appears to contribute to the reduced acid secretion. During a normal infection with larvae, the glands containing the larvae lose their functional secreting cells and become lined with immature undifferentiated cells (Armour et al., 1966; Ritchie et al., 1966; Hunter & Mackenzie, 1982; Scott et al., 1998). These glands would be expected to secrete less HCl. After adult transplant, there is a generalised reduction in parietal cells: 8 days after *O. circumcincta* worms were transferred into recipient sheep, the number of parietal cells had been halved (Scott et al., 1998). Parietal cell loss is therefore likely to increase the effect of acid inhibitors on secretion by the parasitised abomasum.

The rapid rise in pH after adult worm transfer and the rapid restoration of acid secretion after drenching (Simpson et al., 1997) support earlier suggestions of a chemical mediation of the hypochlorhydria (McLeay et al., 1973; Titchen & Anderson, 1977). The presence of the parasite is essential for the maintenance of hypochlorhydria, as the abomasal pH began to fall soon after the worms were removed by anthelmintic treatment (Anderson et al., 1981; Simpson et al., 1997). Hertzberg et al. (1999) have recently reported that excretory/secretory (ES)

products *of O. circumcincta* inhibit enterochromaffin-like (ECL) cell function and therefore may indirectly inhibit the parietal cell. *H. contortus* ES products have also been shown to inhibit acid secretion by rabbit gastric glands in vitro, possibly through their ammonia content (Scott and Merkelbach, unpublished data). A further way that the parasites could inhibit or kill the parietal cells is through stimulating an acute inflammatory reaction. The rapid influx of neutrophils and eosinophils into parasitised tissues (Armour et al., 1966; Scott et al., 2000) could be a source of cytotoxic chemicals which damage parietal cells.

### 1.2.2.3 Hypergastrinaemia

Gastrin is mainly synthesised by the G-cells of the pyloric antrum and is released from granules by exocytosis at the base of the cell. Gastrin is the main stimulator of parietal cells to secrete acid during a meal (Blair et al., 1987; Kovacs et al., 1989) and is also a trophic agent for the fundic mucosa (Johnson et al., 1969), particularly the ECL and parietal cells (Crean et al., 1969; Axelson et al., 1988). The release of gastrin is regulated by the interaction of blood-born mediators, neural and paracrine agents and the nutrients from the gastric lumen (Schubert, 1996, 1997).

Hypergastrinaemia occurs in sheep experimentally infected with *O. circumcincta* (Anderson et al., 1976, 1985; Lawton et al., 1996) or with *H. contortus* (Nicholls et al., 1988; Simpson et al., 1997) and in cattle infected with *O. ostertagi* (Fox et al., 1988, 1993; Fox, 1993). The rise in serum gastrin after larval infection is seen around the time the parasites leave the gastric glands (Lawton et al., 1996; Simpson et al., 1997). Serum gastrin was elevated 2-4 days after an experimental infection with large doses of *H. contortus* larvae in sheep (Nicholls et al., 1988) and 1 week after a trickle infection with low doses of *H. contortus* larvae (Fox et al., 1988). Plasma gastrin may increase up to 10-fold within the third to fifth week of infection (Fox et al., 1988; Nicholls et al., 1988). After infection with O. *circumcincta* in sheep, the gastrin level increased in the second week after initial infection, reached maximum levels between 11 and 20

days and stayed elevated throughout the infection (Anderson et al., 1975, 1981). Since adult worm transfer rapidly caused an increase in serum gastrin concentration (Anderson et al., 1985; McKellar et al., 1987; Lawton et al., 1996; Simpson et al., 1997), larval development is not necessary for hypergastrinaemia to occur.

The aetiology of the hypergastrinaemia is not completely understood, but is likely to involve a loss of acid-feedback when the abomasal pH rises and possibly other contributing factors such as inflammtion. Because the early increases in serum gastrin concentration usually parallel the rise in abomasal pH, it was proposed that the initial hypergastrinaemia was secondary to the rise in gastric pH (Fox et al., 1988; Nicholls et al., 1988; Lawton et al., 1996). There is elevated serum gastrin before a rise in abomasal pH in some animals, and often serum gastrin remains high when abomasal pH returns to near normal around patency (Lawton et al., 1996), suggesting there may be other stimuli for the G-cells (Anderson et al., 1981, 1985; Hilderson et al., 1991; Lawton et al., 1996; Simcock et al., 1999). Inflammatory mediators are possible candidates, as gastrin secretion can be stimulated by histamine (Bado et al., 1994), tumour necrosis factor - α (TNF-α) (Lehmann et al., 1996; Weigert et al., 1996) and Interleukin (IL)-1 (Weigert et al., 1996). Parasite ES products have been suggested to cause increases in serum gastrin before abomasal pH (McLeay et al., 1973; McKellar et al., 1987), however, in vitro studies with ES products from *H. contortus* (Haag, 1995) or *O. circumcincta* (Lawton, 1995) have failed to find evidence of a direct parasite stimulant of gastrin secretion.

Antral tissue gastrin becomes depleted in parasitised animals (Fox et al., 1993; Purewal et al., 1997; Scott et al., 1998), even though there is increased gastrin synthesis (Purewal et al., 1997). As well as the depletion due to hypersecretion, in some cases there may be physical damage to the G-cells. Cell damage may explain the failure to see an increase in circulating gastrin in spite of an increase in abomasal pH in sheep infected with a strain of *O. circumcincta*

which forms nodules mainly in the pylorus and causes massive antral thickening and inflammation (Simcock et al., 1999).

Hypergastrinaemia is likely to explain the increased thickness of the abomasal mucosa in sheep infected with *H. contortus* (Hunter & MacKenzie 1982) or *O.circumcincta* (Coop et al., 1977; Anderson et al., 1988; Scott et al., 1998). It is also probably involved in the generation of new parietal cells seen in recovering abomasal tissue (Scott et al., 1998).

## 1.2.3 ABOMASAL PARASITISM IN MILK-FED RUMINANTS

There are several reports over the last 60 years that milk feeding may affect the level of parasitism in ruminants and several other mammalian species. Some were clinical reports only, such as that of Porter (1941), who found that the number and size of *H.contortus* worms at necropsy in calves fed only skim milk were less than in control calves fed "a normal diet". An anti-parasitic effect for bovine milk was suggested by the report that administration of bovine milk to colts infected with strongyles was highly beneficial even in the case of animals that "were almost dying before treatment was applied" (Leese, 1943). Parasitized pigs maintained on skim milk for three to five days expelled most of their nematode parasites and made better weight gains, attributed by the authors to freedom from parasitism (Spindler et al., 1944; Spindler & Zimmerman, 1944; Shorb & Spindler 1947). These observations in monogastric animals suggested that the milk itself, or the environment it created in the stomach, was detrimental to parasite survival.

A later study of expermental and natural infections of calves and rabbits with gastric and intestinal nematodes (Rohrbacher et al., 1958) lent further support to the anti-parasitic effects of milk. Calves fed milk alone were less susceptible to experimental infections of *Haemonchus placei* than were control calves fed grain and hay in addition to milk. Furthermore, fewer and smaller *H. placei* were found in unweaned calves than from weaned calves, regardless of whether they were experimentally or naturally infected. in addition, unweaned calves had fewer

*Cooperia spp.* and *Oesophagostomum radiatum* in natural infections. Also in this study, significantly larger numbers of *Trichostrongylus axei* and *Trichostrongylus colubriformis* were recovered from weaned rabbits than from unweaned rabbits 18 days after experimental infection.

In a more recent experiment, milk-fed calves and calves fed milk, hay and concentrate from birth were studied for 28 days after a single infection of 25,000 *O. ostertagi* at 7 weeks of age. (Satrija et al., 1991). The latter group were ruminating, whereas the milk-fed animals had negligible rumen function. Faecal egg counts and post-mortem worm counts were reported, but no biochemical data for the animals. All calves had abomasal lesions. A third group of milk-fed calves given 25,000 exsheathed larvae had a lower worm burden than those given ensheathed larvae. Even the milk-fed calves had significant worm burdens (3519 ± 1010 compared with 4846 ± 89 for the solid fed group). The authors concluded that the degree of ruminal development was the important factor in reducing the worm burden.

Experiments involving with protozoal infections in milk fed animals may not be directly relevant to nematode parasitism. In vitro studies (Section 1.1.4) have shown that the unsaturated fatty acids in human milk kills *G. lambis* (Reiner et al., 1986; Hernell et al., 1986; Rohrer et al., 1986). Malarial parasites are also affected by a milk diet, in this case possibly by a deficient supply of an essential nutrient. Rats maintained on milk diets (bovine milk, reconstituted dried milks or human milk) either failed to become parasitized or developed relatively light parasitaemia when infected with *Plasmodium. berghei* (Maegraith et al.,1952). When some animals in which malaria appeared to be suppressed were restored to the normal laboratory diet, parasites rapidly reappeared in the blood after the change of diet, but the resulting parasitaemia was seldom severe or sustained. A milk diet also suppressed the organism in experimental monkeys (Bray & Garnham, 1953). The first suggestion was that milk contains an inhibitor of the development of the asexual phase of *P. berghei* (Maegraith et al., 1952) but it was later proposed that

a milk diet causes a para-amino benzoic acid (PAB) deficiency in *P.berghei* (Hawking, 1953). Other clinical observations confirm that milk feeding protects against malaria (Murray et al., 1978; Hindsbo, 1996).

## 1.3 CONCLUSIONS

In addition to its nutrients, milk contains compounds with a vast array of biological activities which contribute to protection of the neonate against disease. Of particular interest in connection with nematode parasite control are the reports of anti-parasitic activity in human and bovine milk and the reduced worm burdens seen in milk-fed calves and rabbits. The small number of studies in ruminants have not clearly identified why the unweaned animal is less susceptible to nematode parasitism. In particular, they have not distinguished between a direct effect of the milk, as suggested by the early studies in pigs and horses, and failure of the parasites to establish because of immaturity of the gut, as suggested by the study in calves. Because of the possible practical applications for a milk component which could either to prevent parasite establishment or remove existing worm burdens, in vivo and in vitro experiments were designed to obtain further information on the anti-parasitic activity of bovine milk.

# Chapter 2

# EFFECT OF A MILK DIET ON *OSTERTAGIA CIRCUMCINCTA* INFECTION IN LAMBS

## 2.1 INTRODUCTION

Bovine milk has been reported to have an anthelmintic effect on existing strongyle infections in colts (Leese, 1943) and on nematode infections in pigs (Spindler et al., 1944; Spindler & Zimmerman, 1944; Shorb & Spindler 1947). Lesser worm burdens have also been reported in entirely milk-fed calves than in calves receiving solids in the diet, but there appear to be no comparable studies in sheep. There were fewer, smaller *H.contortus* worms in calves fed only skim milk than in control calves fed "a normal diet" (Porter, 1941) and fewer *H. placei*, *Cooperia spp.* and *O. radiatum* in unweaned calves than in weaned calves (Rohrbacher et al., 1958). Faecal egg counts and post-mortem worm counts were compared 28 days after a single infection of 25,000 *O. ostertagi* at 7 weeks of age in milk-fed calves and calves fed milk, hay and concentrate from birth (Satrija et al., 1991). A third group of milk-fed calves given 25,000 exsheathed larvae had a lower worm burden than those given ensheathed larvae. The authors concluded that the degree of ruminal development was the important factor in reducing the worm burden.

In New Zealand, as in other parts of the world, gastro-intestinal nematodes reduce productivity and cause economic losses to the pastoral industry. The protection that milk-feeding appears to afford the unweaned animal raises the possibility of isolating milk components with useful anthelmintic properties.

**Table 2.1** Experimental groups. Sex and body weight of lambs when assigned to groups (at 14 -18 days of age). (F: female, M: male)

| Group | n | Diet | Infected larvae | Body weight (kg) | | Sex | |
|---|---|---|---|---|---|---|---|
| | | | | Mean | Range | F | M |
| Milk-ex | 4 | Milk | Exsheathed | 5.3 | 4-6 | 2 | 2 |
| Milk-en | 4 | Milk | Ensheathed | 5.3 | 3.5-6.5 | 2 | 2 |
| Mixed-ex | 4 | Milk + solid | Exsheathed | 5.6 | 5-6.5 | 1 | 3 |
| Mixed-en | 4 | Milk + solid | Ensheathed | 5.4 | 4.5-6 | 2 | 2 |
| Solid-ex | 4 | Solid | Exsheathed | 5.9 | 4.5-7.5 | 2 | 2 |
| Solid-en | 4 | Solid | Ensheathed | 5.3 | 4-6 | 2 | 2 |

Alternatively, the reduced worm burdens may reflect a different environment in the underdeveloped stomach. The present experiments were conducted to investigate the susceptibility to parasitism of very young lambs given a bovine milk replacer during a trickle infection with *O. circumcincta*.

# 2.2 METHODS

## 2.2.1 EXPERIMENTAL DESIGN

At approximately 2 weeks of age. 24 lambs were weighed and 4 assigned to each of 6 experimental groups (Table 2.1) defined by the diet (Milk, Milk plus Solids or Solids only) and whether they were trickle infected with 1000 exsheathed or ensheathed third-*stage O. circumcincta* larvae twice a week from weeks 4 to 9:

Group 1 (Milk ex): fed milk only, infected with exsheathed larvae

Group 2 (Milk en): fed milk only, infected with ensheathed larvae

Group 3 (Mixed ex): fed milk once a day plus solid feed, infected with exsheathed larvae

Group 4 (Mixed en): fed milk once a day plus solid feed, infected with ensheathed larvae

Group 5 (Solid ex): fed solid feed only, infected with exsheathed larvae

Group 6 (Solid en): fed solid feed only, infected with ensheathed larvae.

Faecal egg counts and serum gastrin and pepsinogen concentrations were monitored during the infection, and abomasal pH, worm counts and weights and measurements of the stomachs were obtained after necropsy.

## 2.2.2 ANIMALS

Twenty four Finn-Dorset cross lambs, born at Flock House Agricultural Centre (AgResearch Grasslands, Bulls), were raised to be free of helminth

parasites. They were removed from their mothers at birth and housed indoors on grating at the Animal Physiology Unit, Massey University. They were weighed twice: at 14-18 days of age, when they were assigned to groups, and before necropsy after a further 7 weeks.

### 2.2.3 FEEDING

For approximately the first 2 weeks of life (14-18 days of life), all 24 lambs were bottle fed three times a day with 200-300 ml whole milk replacer ("Anlamb", New Zealand Dairy Co, Te Puke, New Zealand). The milk powder was made up in water in a concentration of 200g/L water. During the first experimental week (Week 3 of life), the lambs in the Solid groups were weaned by decreasing the milk offered each feed and providing them with high protein pellets and lucerne chaff. The milk intake was gradually increased for the Milk-fed groups and the number of feeds reduced from 3 to 2 per day. From Week 4 of life, the amounts and type of feed offered were as below:

Groups 1 and 2: (Milk ex and Milk en) were fed 600 ml milk replacer twice a day (at 8 am and 6 pm)

Groups 3 and 4 (Mixed ex and Mixed en) were fed 600 ml milk replacer (at 6 pm) and 300 g lucerne chaff and 300 g high protein cereal based sheep pellets (Pasture Plus, Harvey Farms, Wanganui, New Zealand) (at 8am)

Groups 5 and 6 (Solid ex and Solid en) were fed 500 g lucerne chaff and 700 g nuts (at 8 am daily).

Fresh water was available ad libitum to all lambs at all times.

### 2.2.4 INFECTION WITH *O. CIRCUMCINCTA* LARVAE

Infection began on Week 2, about 3 weeks of age, 7 days after the experimental diets were instituted. All lambs were infected with 1000 third-stage larvae, twice weekly for 6 weeks (Days 0, 3, 7, 10, 14, 17, 21, 24, 28 31, 35 and 38

after initial infection). Lambs in the Milk-ex, Mixed-ex and Solid-ex groups were infected with exsheathed larvae, and the other groups were infected with ensheathed larvae. A tube was inserted down the oesophagus and larvae injected through the tube with a syringe, followed by 10 ml of water.

## 2.2.5 COLLECTION OF BLOOD SAMPLES

Blood samples (2 ml) were collected by jugular venipuncture on Days 17, 24, 31, 38 and 41 after initial infection. Blood samples obtained from another 8 parasite-naive lambs (not included in experimental animals) were used as reference values for parasite-naive lambs. The blood samples were allowed to clot at room temperature and centrifuged at 3500 rpm for 20 minutes at 4°C (Beckman GPR refrigerated centrifuge). Serum was separated and stored at -20°C for subsequent determination of serum gastrin and pepsinogen concentrations.

## 2.2.6 COLLECTION OF FAECAL SAMPLES

Faeces were collected per rectum for faecal floats and faecal egg counts (FEC) before the first infection and twice weekly from Day 14 after initial infection. FEC were carried out when there was a positive faecal float.

## 2.2.7 NECROPSY

On Day 43 after the initial infection (Week 7 of the experiment), lambs were weighed and euthanased by captive bolt and exsanguination. The duodenal bulb and abomasum (at the omasal junction) were ligated. The oesophagus and duodenum were cut and the reticulo-rumen, omasum and abomasum were removed intact and placed on a tray. The abomasum with the contents intact were removed from the rest of the stomachs and placed in a plastic bag.

The omasum was cut from the reticulo-rumen and weighed on a Mettler AT200 balance (Watson Victor N.Z. Ltd). The reticulo-rumen was opened, its contents removed by washing with tap water, dried with paper towels and weighed.

Five pieces of the wall of the rumen from the area with long papillae were collected for mearsurement of the height of the rumen papillae. Five pieces of the ventral part of the reticulum were collected for measurement of the reticular cells.

The abomasum was dissected within the plastic bag to avoid loss of worms. An incision was made in the wall along the greater curvature and a small piece of antral mucosa and a section of a fundic fold were removed for histology and tissue pepsinogen estimation. A small volume of the contents was collected and placed on ice until the pH was measured. The abomasum and its contents were then frozen and stored at -20°C for worm counts.

The piece of antral mucosa and small pieces of the fundic fold were placed in Bouins's fixative. A piece of fundic fold was removed with a cylindrical punch and kept on ice until homogenised (Section 2.2.8) and the homogenate stored at -20°C for tissue pepsinogen determination.

## 2.2.8 TISSUE FOR PEPSINOGEN ESTiMATION

The fundic tissue piece was gently rinsed with saline, blotted dry and the wet weight obtained.The tissue was then homogenised on ice (Sorvall Omni-mixer,.Ivan Sorvall Inc., Newtown, U.S.A.) in 8 ml of a solution containing 0.1% Triton X-100 (BDH Chemicals, Poole, England) in 0.01 M HCl, pH 2.0. After centrifugation at 1500 r.p.m. for 30 minutes at 4°C , the supernatant was aspirated and stored at -20 °C for later pepsinogen determination (Section 2.2.12.2).

## 2.2.9 ABOMASAL pH

The pH of the abomasal contents was measured with a glass electrode and standard pH meter (PHM82 Standard pH Meter) (Radiometer, Copenhagen).

## 2.2.10 GROSS MEASUREMENTS OF THE RETICULO-RUMEN

For each lamb, the heights of 5 papillae were measured from each of the 5 tissue pieces, using a ruler and a low power microscope. A mean value was obtained from the 25 values.

Five reticular "cells" were measured with a ruler for each of the 5 tissue pieces. Three measurements were made across each width of the hexagonal shaped cells. The 75 measurements were used to calculate a mean value.

## 2.2.11 HISTOLOGY AND MORPHOMETRY

Abomasal tissue was removed from the Bouin's fluid after 6-8 hours fixation and placed in 70% alcohol. Samples were routinely dehydrated and embedded in paraffin using an automatic tissue processor (Leica, Jung TP 1050, Germany). Sections 6-μm thick were cut and stained with Haematoxylin and Eosin (H & E).

Mucosal thicknesses were measured with a digitising tablet (Sketchmaster, GTCO Corporation) and an associated computer program (SigmaScan: Jandel Scientific). Ten measurements were made to obtain the average mucosal thickness for each sample.

## 2.2.12 ASSAYS

### 2.2.12.1 Gastrin radioimmunoassay

Serum gastrin was estimated by the method of Hansky & Cain (1969) with the modifications described by Simpson et al. (1993). The primary antibody was Hansky's Ab 74 which recognises all forms of sheep gastrin equipotently, used in a final dilution of 1:100,000. Standards were made from synthetic human nsG17 (Peninsular Laboratories, California U.S.A.), which was also used to prepare the

$^{125}I$ labelled G17 tracer. Bound and free fractions were separated with an anti-rabbit second antibody raised in sheep. The detailed procedure is as follows:

Assay buffer (0.02M Veronal Buffer, pH 8.6):

8.24 g sodium barbiturate

1.488 g barbitone

10 g bovine serum albumin (BSA)

400 mg thiomerosal

40 mg neomycin sulphate

Made up to 2 litres with distilled water and adjusted to pH 8.6.

Antiserum:

Ab 74 was made up at 1:40,000 dilution in assay buffer containing Normal Rabbit Serum (NRS) at 1:100 dilution.

Tracer:

$^{125}I$ G17 tracer was diluted with assay buffer to contain 2000 cpm. per 0.5 ml.

Assay procedure:

Samples, internal standard serum samples and gastrin standards were assayed in triplicate. Standards contained 2, 5, 10, 20, 50, 100, 200, and 300 pM gastrin.

Assay tubes contained:

Total: 500 µl tracer (total counts per tube).

Blank: 500 µl buffer plus NRS, 500 µl tracer (non-specific binding)

Standards: 100 µl standard solution, 400 µl antiserum, 500 µl tracer.

Samples: 100 µl sample, 400 µl antiserum, 500 µl tracer

Assay tubes were incubated for 2 days at 4 °C. Free and bound fractions were separated by the addition to each tube (except totals) of 200 µl of second antibody and incubation for a further 3 days at 4 °C. All tubes, except the totals, were centrifuged at 2000 g for 30 minutes and the supernatant aspirated. The pellet was counted for 5 minutes in a Wallac gamma counter. The attached computer generated a standard curve and calculated the gastrin concentrations in each sample (expressed as pM).

**2.2.12.2 Pepsinogen assay**

The pepsinogen concentrations of serum and tissue homogenates were determined by the micro-method of Dorny & Vercruysse (1998). Tyrosine is liberated from the BSA substrate by the pepsin formed by acid-activation of the pepsinogen. Tissue homogenates were diluted 1:1000 before assay.

BSA buffer (glycine-NaCl-HCl-buffer):

(1) Stock solution A (0.1 M glycine-0.1M NaCl): 5.844 g NaCl and 7.507 g of glycine were each dissolved separately in 500 ml distilled water and the two solutions mixed to make 1000 ml.

(2) Stock solution B (0.27 N HCl): 270 ml of 1 N HCl were added to 730 ml distilled water.

(3) Glycine-NaCl-HCl: 2 volumes of stock solution A were mixed with 1 volume of stock solution B.

(4) BSA-buffer: 2 g BSA was added to 100 ml glycine-NaCl-HCl and stirred at room temperature until dissolved. The solution was then filtered through glass wool.

Assay procedure:

(1) Incubated samples:100 µl of sample was thoroughly mixed with 500 µl of BSA-buffer. 300 µl of this mixture were placed into an Eppendorf tube and incubated in a water bath at 37 °C for 24 hours.

(2) Non-incubated blanks: the remaining 300 µl was mixed with 500 µl of trichloroacetic acid (4 g/100 ml) in an Eppendorf tube and centrifuged at 10,000 rpm for 5 minutes. 200 µl of supernatant was placed in a tube and kept at 4 °C.

(3) Assay blanks for O.D. readings: several tubes were set up with 300 µl of distilled water instead of sample and treated as in (1).

(4) After 24 hours, the reaction in the incubated tubes was terminated by adding 500 µl of trichloroacetic acid (4 g/100 ml) and centrifuged at 10,000 rpm for 5 minutes. 200 µl of supernatant was placed in a tube.

(5) To each 200 µl of supernatant was added 2 ml of 0.25 N NaOH followed by 300 µl of diluted Folin and Ciocalteu's reagent (1:3, v/v) and mixed.

(6) Tyrosine standards: Tyrosine solutions of 0.1 µmol/ml, 0.2 µmol/ml, 0.3 µmol/ml were prepared from a sterile stock solution. The standards were treated as for the supernatants in (5).

(7) The optical densities (O.D.) of samples and standards were measured at 680 nm wavelength within 25 minutes of the colour developing. The assay blank (3) was used as the reference to zero the spectrophotometer.

(8) Pepsinogen concentrations (expressed in mU tyr/L (i.e. µmole tyrosine released per litre per minute × 1000) were calculated from the formula:

$$mU\ tyr/L = (OD_{sample} - OD_{blank}) \times F \times 11.11 \times 1000$$

where:

F is the calculation factor derived from the tyrosine standards:

$F = (0.1/OD_{tyr\ 0.1\ \mu mol/ml} + 0.2/OD_{tyr\ 0.2\ \mu mol/ml} + 0.3/OD_{tyr\ 0.3\ \mu mol/ml}) /3$

11.11 is the conversion factor for the sample dilution and the incubation time.

## 2.2.13 PARASITOLOGY

### 2.2.13.1 Larval culture

A ram lamb, obtained off pasture, was treated orally with 0.4 mg/kg ivermectin (Ivomec; Merck and Co. Inc., Rahway, NJ., U.S.A.) and 8 mg/kg ricobendazole and levamisole HCl (Leviben; Young's Animal Health Ltd, N.Z.). Faecal floats performed 1 and 2 weeks after drenching showed no parasite eggs in the faeces. The sheep was then infected by ruminal intubation with 50,000 larvae of a pure strain of *O. circumcincta*. Faeces were collected daily in a faecal bag when the egg count exceeded 200 eggs per gram (e.p.g.).

Faeces were macerated, mixed with approximately half their volume of vermiculite, and moistened with water. The mixture was incubated at 27°C in a plastic tray covered with a piece of glass. The moisture level of the mixture was checked occasionally to ensure it did not dehydrate.

After 10 days, larvae were recovered using a modified Baermann technique (Ministry of Agriculture, Fisheries and Food, 1986). The apparatus consisted of a large funnel with a short length of rubber tubing attached to its spout. The tubing was clamped to retain water in the funnel. The faecal mixture was placed on a single layer of tissue paper (Snowtex, Caxton Products Pty, Ltd., Australia) in a large sieve. The sieve was placed on top of the funnel so that the mixture was immersed in water at room temperature. The larvae moved through the tissue paper and sank to the bottom of the funnel. After 24 hours, the clamp at the bottom of the funnel was opened, and about 50 ml of fluid containing most of the larvae were collected and stored at 4°C.

### 2.2.13.2 Counting larvae and assessing viability

A 1:10 dilution of the larval suspension was made with water and 10 subsamples each of 1 ml was placed in a well on a glass slide. A few drops of iodine were added to kill and stain the larvae. A coverslip was placed over the meniscus of the fluid in the well. Two minutes later, larvae were counted under a microscope and the 10 counts averaged to obtain the number of larvae per ml stock suspension.

The viability was assessed from the percentage of motile larvae in a drop of larval suspension placed on a slide and covered with a glass coverslip. A total of 100 larvae were observed.

### 2.2.13.3 Larval exsheathment

Equal volume of larval stock suspension and 0.4% sodium hypochlorite were mixed and placed in a water bath at 37°C for 15 minutes. Exsheathment was confirmed by microscopic examination. The mixture was filtered using a 45 µm millipore filter paper (Micro Filtration Systems, U.S.A.). The larvae retained on the filter were rinsed several times with distilled water, then washed off the filter paper with a small volume of distilled water and counted.

### 2.2.13.4 Faecal floats

Two grams of faeces were macerated, mixed with approximately 30 mls saturated NaCl solution and the mixture poured into a vial. A coverslip was placed on the meniscus of the liquid surface. After 15 minutes, the coverslip was carefully uplifted and placed on a microscope slide and examined at low magnification.

### 2.2.13.5 Faecal egg counts

Two grams of faeces were macerated and mixed with approximately 30 mls of saturated NaCl solution. The mixture was used to fill the two chambers of a

counting slide. After 2 minutes, the eggs within the grids of both chambers were counted under a microscope. Each egg counted represented 50 e.p.g.

#### 2.2.13.6 Postmortem worm counts

The method of the Ministry of Agriculture, Fisheries and Food (1986) was used. The frozen abomasum was thawed at room temperature, opened and the contents collected. The abomasum was washed thoroughly under a stream of water while rubbing the mucosa to remove any adherent worms. The plastic bag was also washed. The abomasal contents and all washings were poured through a sieve with a of 38 μm mesh and the retained contents and worms washed until all small particles had passed through the sieve. The material on the screen was then washed off thoroughly into a container and the volume made up to 6000 ml with water. Two 150 ml samples were taken while agitating vigorously. Each 150 ml sample was examined separately, diluted with water if necessary, and the total number of worms counted under a dissecting microscope. Numbers of male, female and immature worms were recorded separately.

The washed abomasum was dried, weighed and the immature worms recovered by peptic digestion. The abomasum was incubated at 37 °C for 2 hours in a digestion fluid consisting of 4 g pepsin and 4 ml concentrated HCl in 600 ml water.

The digested abomasal tissue was then poured through two wire-mesh screens, the upper having an aperture of 75 μm and the lower an aperture of 38 μm, and washed with a jet of water. Immature worms were then resuspended in 300 ml water and one 15 ml sample was removed and the immature worms counted.

### 2.2.14 STATISTICS

Normal values for serum gastrin and pepsinogen of parasite-naive lambs were derived from serum samples from 8 lambs aged about 2 months. The means

+ 2 standard deviations were considered the upper limit of the normal ranges of these parameters. Any value above the defined upper limit was considered to be raised.

Faecal egg counts and serum gastrin and pepsinogen were compared using the General Linear Model (GLM) Repeated Measures programme; worm counts, abomasal weight, omasal weight, reticulo-rumen weight, abomasal pH and mucosal thickness of the abomasum were compared by the GLM Univariate programme of SPSS (Statisitc Program for Social Science) version 9.0 (SPSS Inc., Chicago, U.S.A.) using Fisher's least significant difference (LSD). P values less than 0.05 were considered to be significant.

# 2.3 RESULTS

## 2.3.1 PARASITOLOGY

### 2.3.1.1 Faecal Egg Counts

Group mean ± s.e.m. faecal egg counts (FEC) are shown in Fig 2.1 and FEC for individual lambs in each group are shown in Fig 2.2. The FEC of the Milk groups were significantly lower than in the Mixed groups ($P < 0.05$) and Solid groups ($P < 0.001$). Very few eggs were seen in the faeces of the Milk lambs (Fig 2.2). There was a significant effect of time after initial infection on FEC ($P < 0.001$). There was no significant difference between the Solid and Mixed groups ($P = 0.2$) nor between exsheathed and ensheathed larvae ($P = 0.97$).

FEC from individual lambs within groups varied considerably. Eggs were first recovered on Day 34 after initial infection in both Milk groups, compared with Days 17 and 20 for Mixed-ex and Mixed-en respectively and Day 24 for both Solid-ex and Solid-en groups. No eggs were seen in the faeces in 3 of the 8 milk-

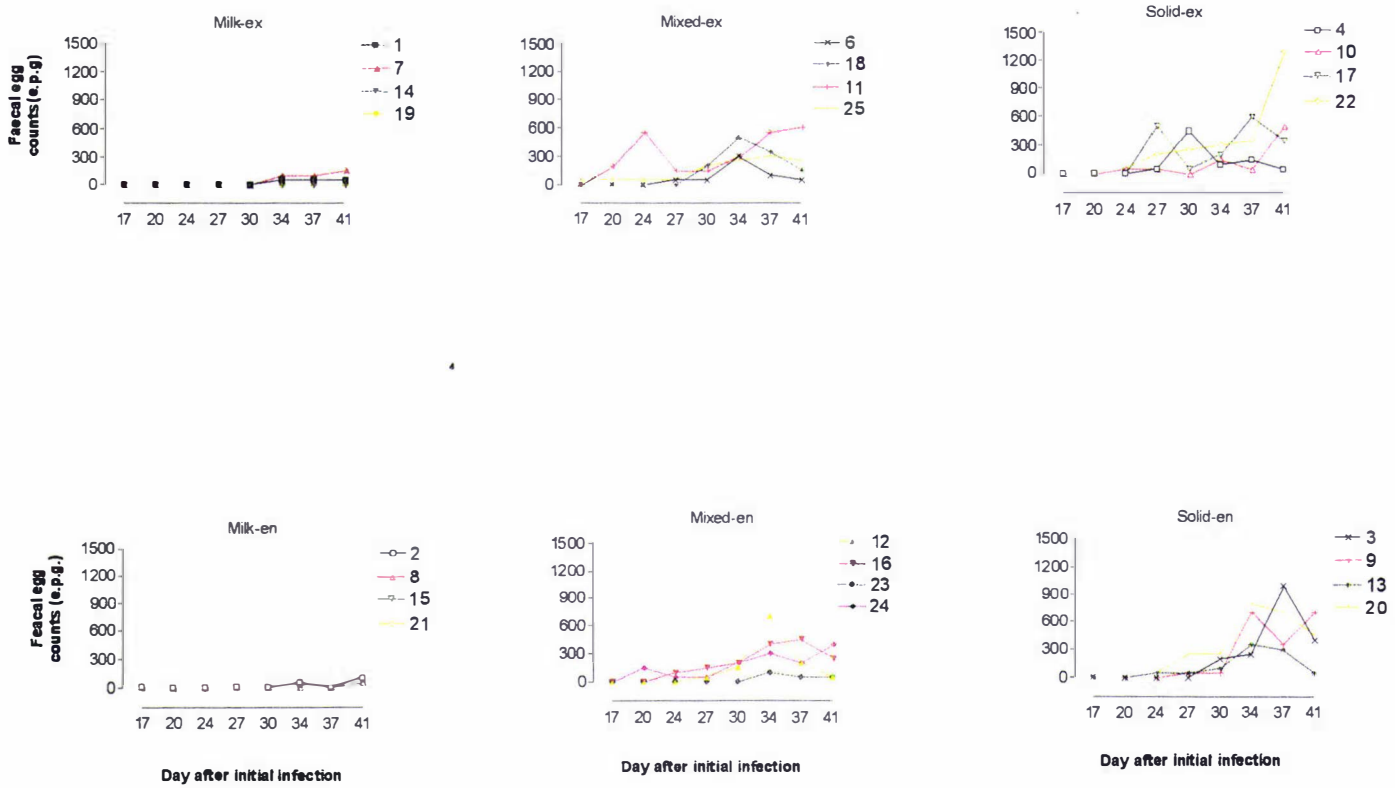


**Figure 2.2** Faecal egg counts in individual lambs following infection with *O. circumcincta* larvae.

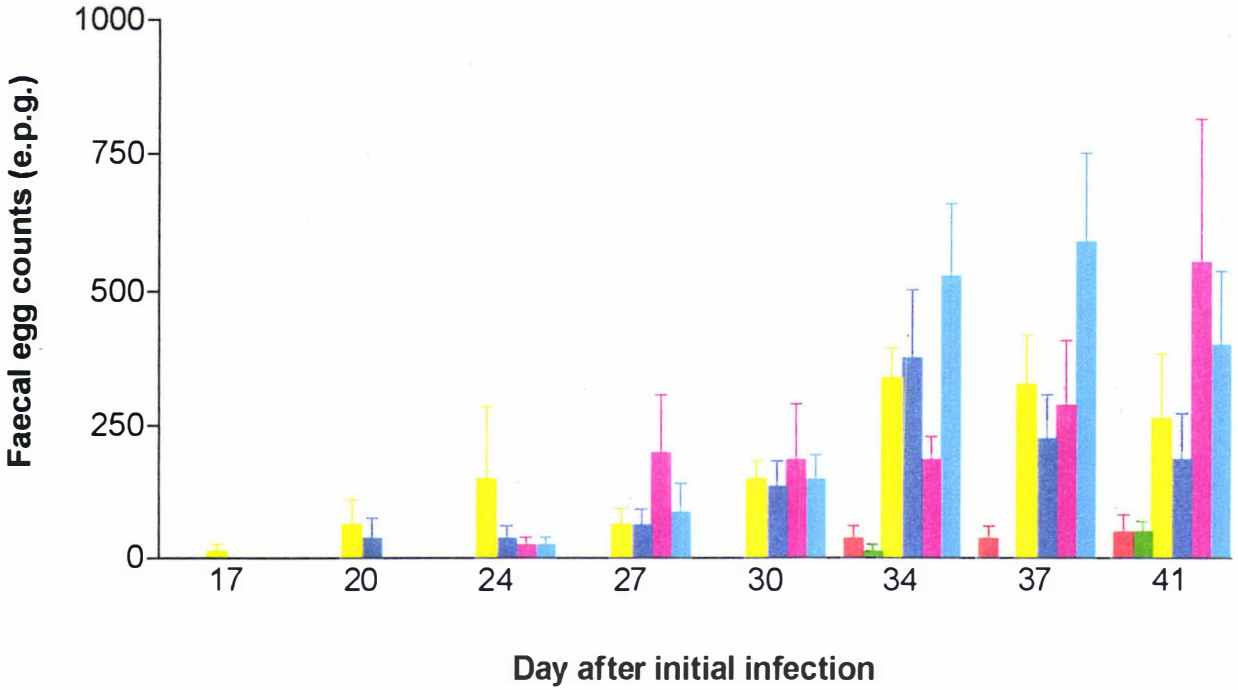


**Figure 2.1** Faecal egg counts (mean ± s.e.m.) in 6 groups of lambs following infection with *O. circumcincta* larvae.

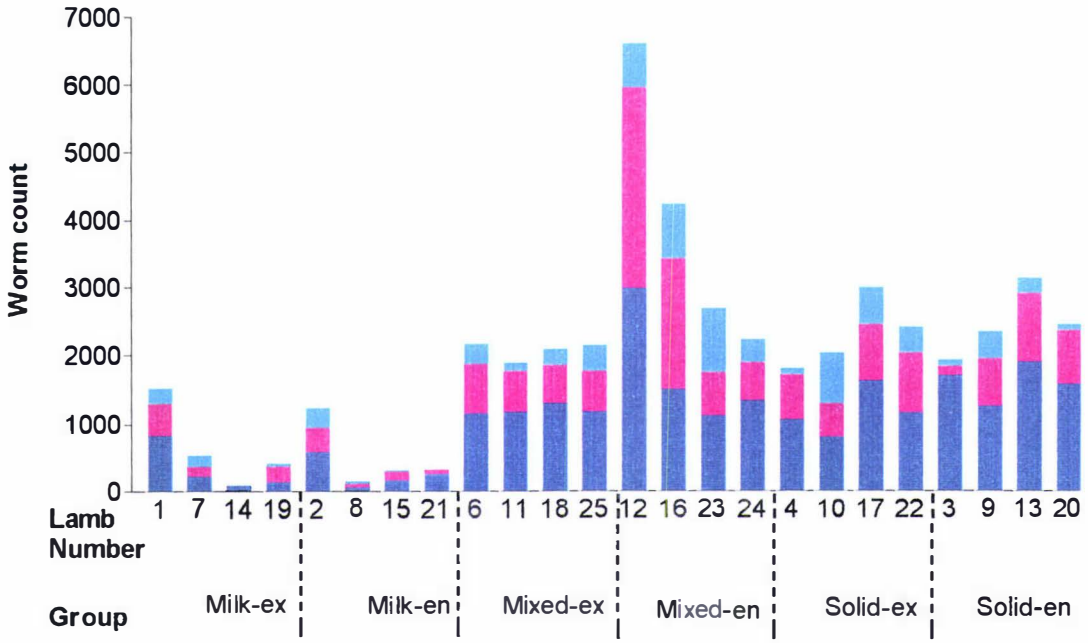


**Figure 2.3** Worm counts at necropsy in individual lambs following infection with *O.circumcincta* larvae.

**Table 2.2** Abomasal worm counts (mean ± s.e.m.) at necropsy in 6 groups of lambs following infection with *O. circumcincta* larvae.

| | Group | | | | | |
|---|---|---|---|---|---|---|
| | Milk-ex | Milk-en | Mixed-ex | Mixed-en | Solid-ex | Solid-en |
| Female | 318 ± 173 | 260 ± 114 | 1185 ± 32 | 1715 ± 422 | 1145 ± 168 | 1590 ± 134 |
| Male | 218 ± 97 | 158 ± 73 | 620 ±35 | 1530 ± 578 | 715 ± 92 | 665 ± 187 |
| Immature | 110 ± 51 | 88 ± 68 | 260 ± 55 | 685 ± 128 | 440 ± 135 | 200 ± 73 |
| Total | 645 ± 306 | 505 ± 248 | 2065 ± 64 | 3930 ± 987 | 2300 ± 258 | 2455 ± 249 |

fed lambs (#14 and #19 in Milk-ex and #21 of Milk-en group). The greatest group mean FEC were seen in the Solid groups (Figure 2.1).

### 2.3.1.2 Postmortem worm counts

Group mean ± s.e.m. worm counts are shown in Table 2.2 and for individual lambs in each group in Fig 2.3. In all except 2 lambs, there were more female than male worms. All lambs, including the three lambs in which no eggs were detected in the faeces, had worms in the abomasum at necropsy. The worms found in animals fed on milk only generally appeared to be slightly shorter in length and smaller in diameter than worms from lambs in the other groups.

The total worm counts for the Milk groups were significantly lower than in the Mixed groups ($P < 0.001$) and Solid groups ($P < 0.001$). There was no significant difference between the Solid and Mixed groups ($P = 0.19$), nor between exsheathed and ensheathed larvae ($P = 0.11$).

The female worm counts for the Milk groups were significantly lower than in the Mixed groups ($P < 0.001$) and Solid groups ($P < 0.001$). There was no significant difference between the Solid and Mixed groups ($P = 0.70$), nor between exsheathed and ensheathed larvae ($P = 0.09$).

The male worm counts for the Milk groups were significantly lower than in the Mixed groups ($P < 0.01$) and approaching significance for Solid groups ($P = 0.065$). There was no significant difference between the Solid and Mixed groups ($P = 0.15$) nor between exsheathed and ensheathed larvae ($P = 0.22$).

The immature worm counts for the Milk groups were significantly lower than in the Mixed groups ($P < 0.001$) and Solid groups ($P < 0.05$). There was no significant difference between the Solid and Mixed groups ($P = 0.11$), nor between exsheathed and ensheathed larvae ($P = 0.48$).

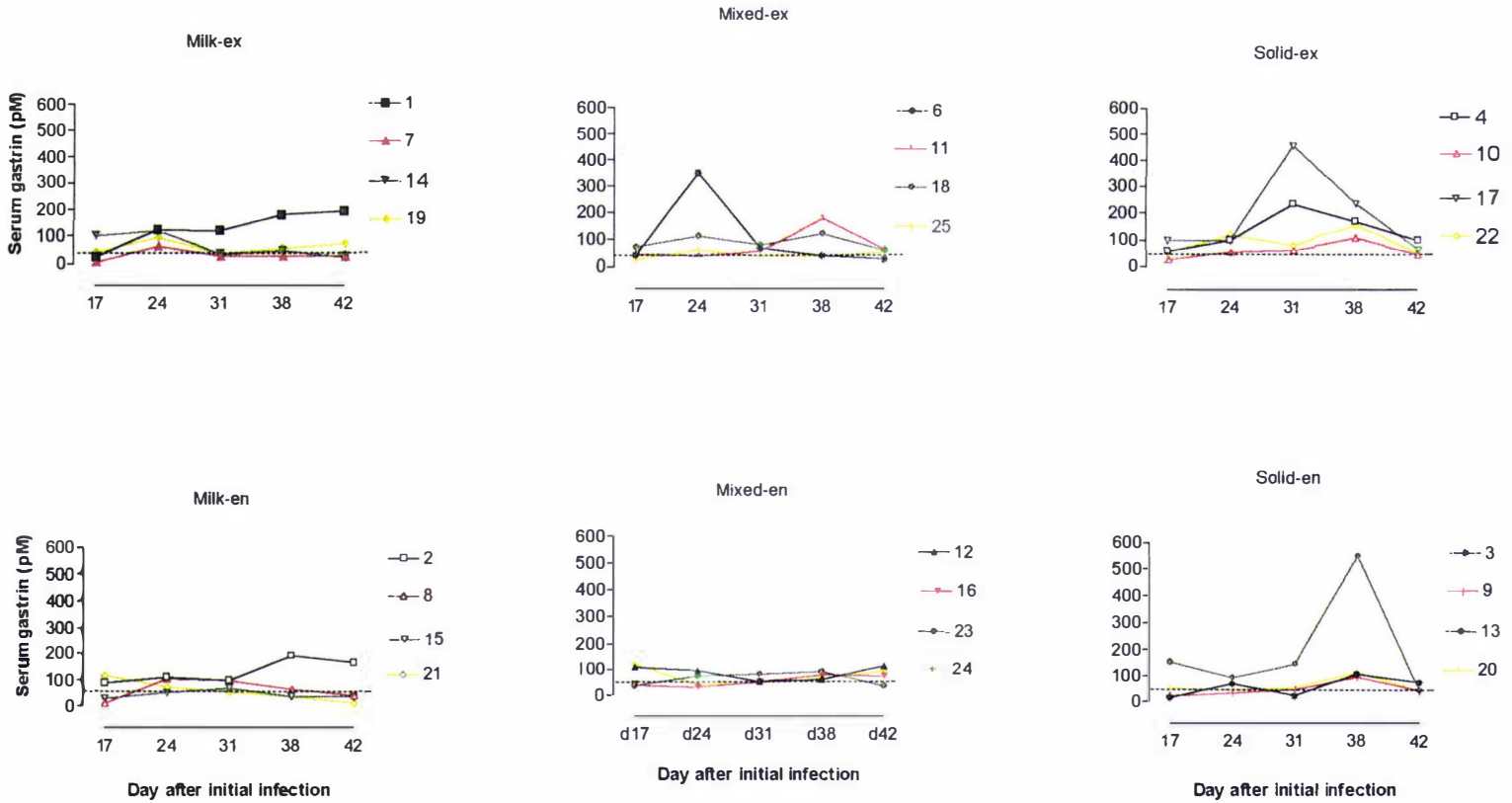


**Figure 2.11** Serum gastrin concentration in individual lambs following infection with *O. circumcincta* larvae. The horizontal dotted line denotes the upper limit of the normal range, defined as the mean plus two standard deviations of values determined in unifected lamb.

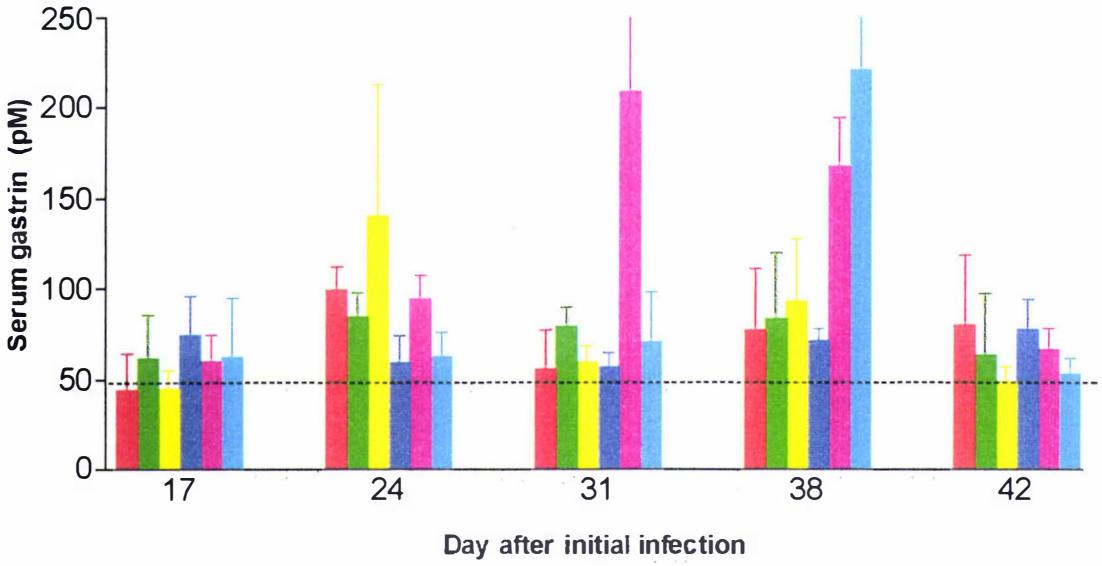


**Figure 2.10** Serum gastrin concentration (mean ± s.e.m.) in 6 groups of lambs following infection with O. circumcincta larvae. The horizontal dotted line denotes the upper limit of the normal range, defined as the mean plus two standard deviations of values determined in unifected lambs.

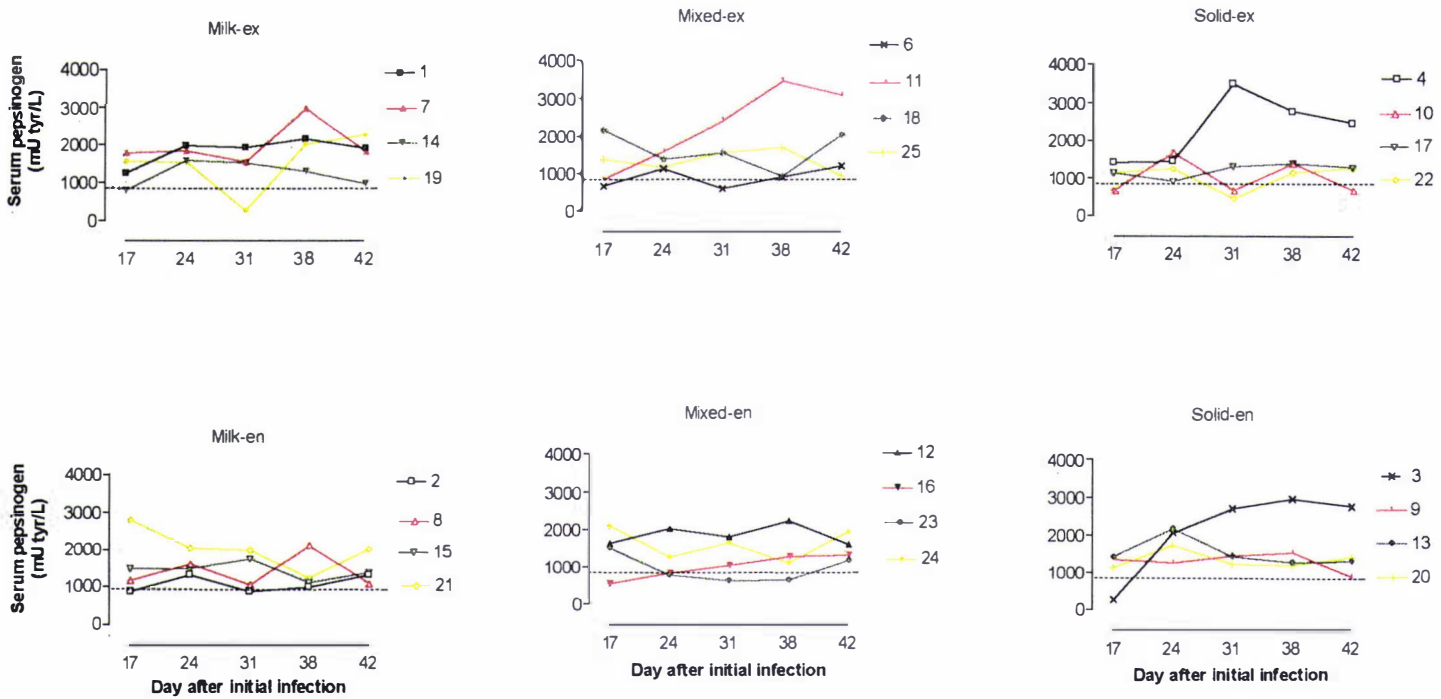


**Figure 2.9** Serum pepsinogen concentration in individual lambs following infection with *O. circumcincta* larvae. The horizontal dotted line denotes the upper limit of the normal range, defined as the mean plus two standard deviations of values determined in uninfected lambs.

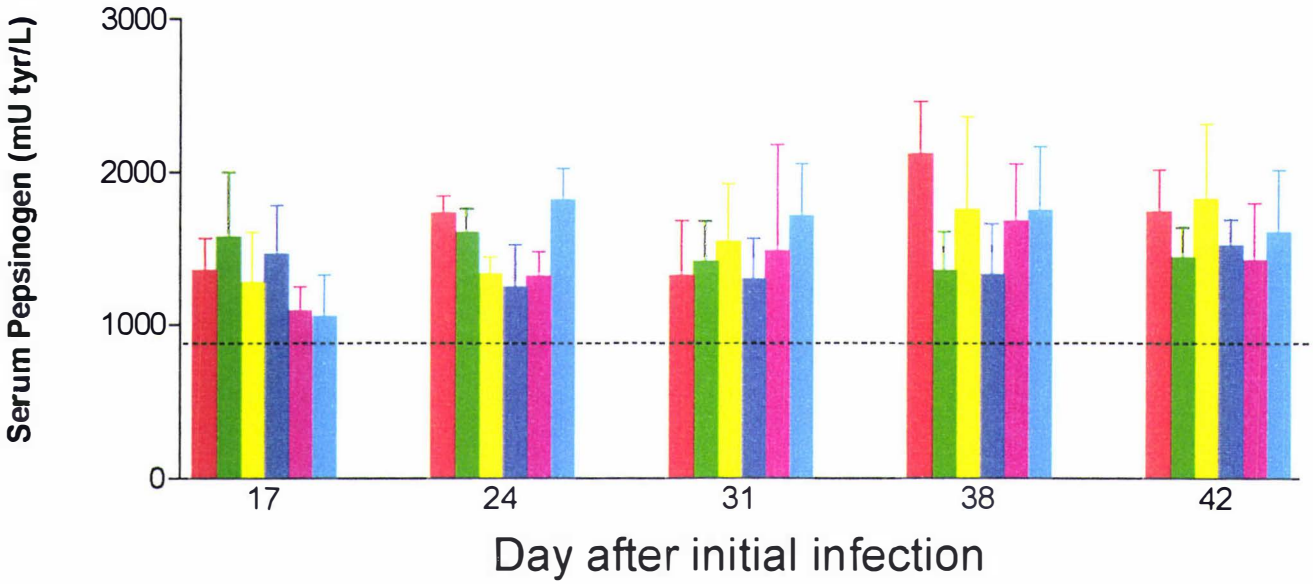


**Figure 2.8** Serum pepsinogen concentration (mean ± s.e.m.) in 6 groups of lambs following infection with *O. circumcincta* larvae. The horizontal dotted line denotes the upper limit of the normal range, defined as the mean plus two standard deviations of values determined in uninfected lambs.

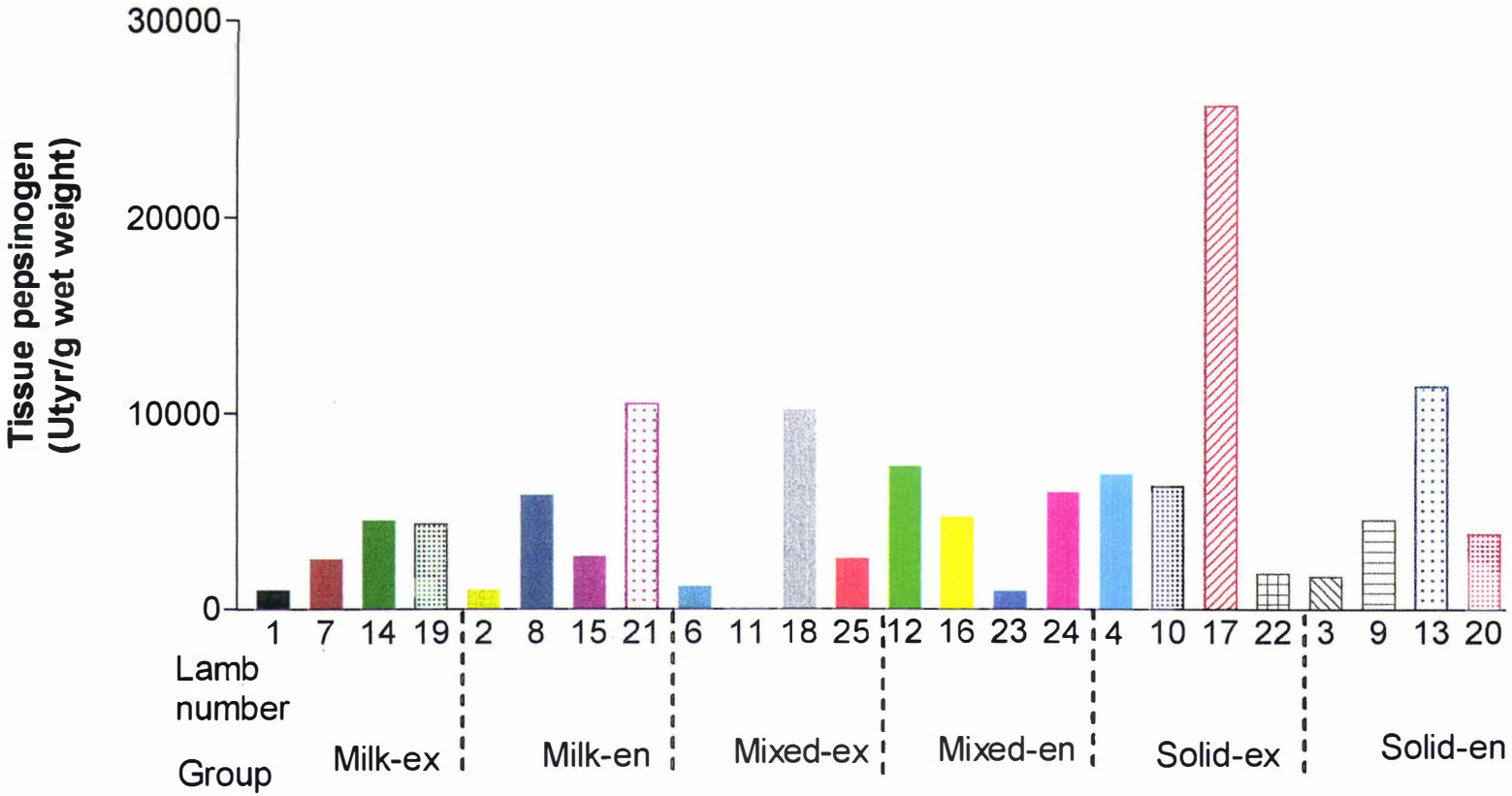


**Figure 2.7** Tissue pepsinogen content at necropsy in individual lambs following infection with *O. circumcincta* larvae.

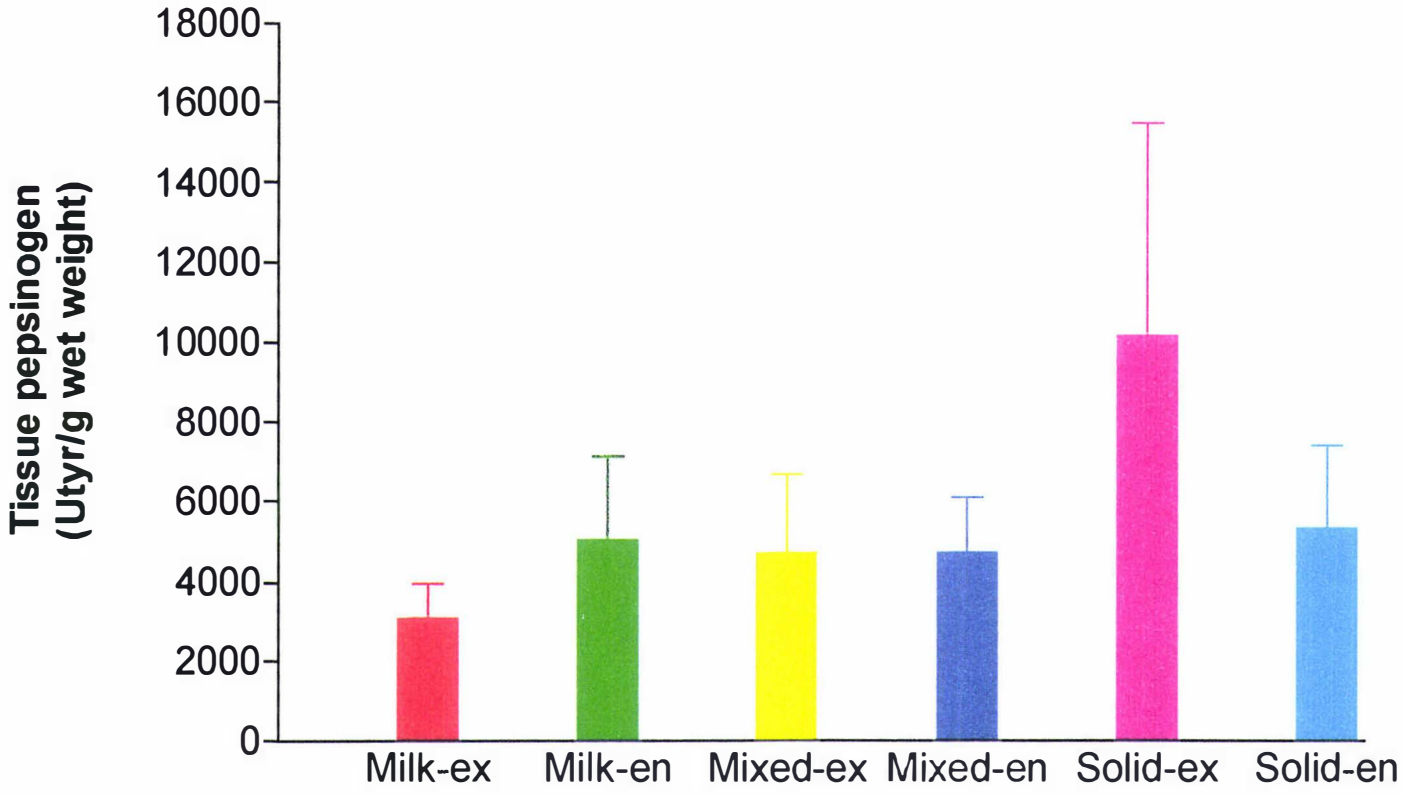


**Figure 2.6** Tissue pepsinogen content (mean ± s.e.m.) at necropsy in 6 groups of lambs following infection with *O. circumcincta* larvae.

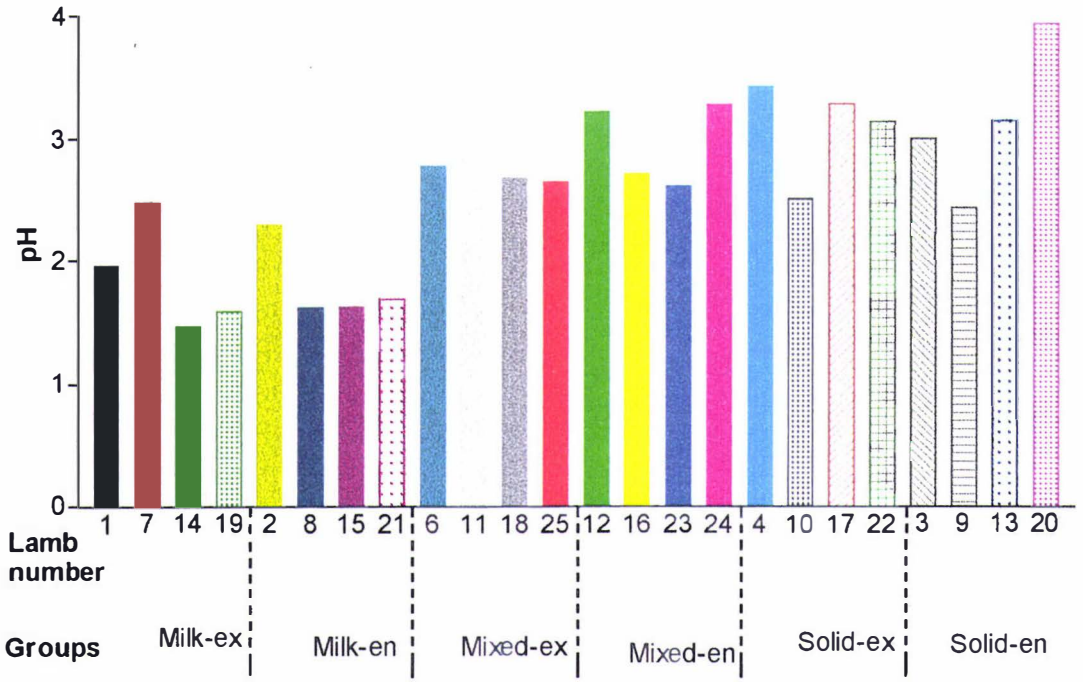


**Figure 2.5** Abomasal pH at necropsy in individual lambs following infection with *O. circumcincta* larvae.

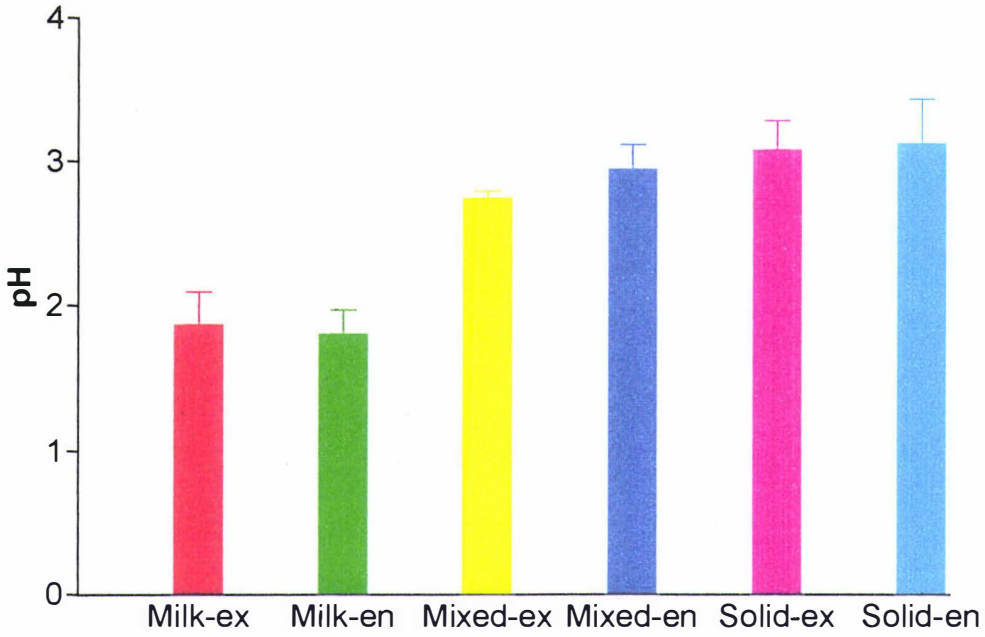


**Figure 2.4** Abomasal pH (mean ± s.e.m.)at necropsy in 6 groups of lambs following infection with *O. circumcincta* larvae.

## 2.3.2 ABOMASAL pH

Group mean ± s.e.m. abomasal pH are shown in Fig. 2.4 and for individual lambs in each group in Fig 2.5. The mean pH for the Milk-ex and Milk-en were 1.87 and 1.81, compared with 2.75 and 2.95 for the corresponding Mixed groups and 3.08 and 3.13 for the Solid groups. The abomasal pH for the Milk groups were significantly lower than in the Mixed groups ($P < 0.001$) and Solid groups ($P < 0.001$). There was no significant difference between the solid and mixed diets ($P = 0.22$), nor between exsheathed and ensheathed larvae ($P = 0.72$).

## 2.3.3 TISSUE PEPSINOGEN

Group mean ± s.e.m. tissue pepsinogen contents are shown in Fig. 2.6 and for individual lambs in each group in Fig 2.7. There were no effects of either diet ($P = 0.36$) or type of larva used for infection ($P = 0.66$).

## 2.3.4 SERUM PEPSINOGEN

Group mean ± s.e.m. serum pepsinogen concentrations are shown in Fig. 2.8 and for individual lambs in each group in Fig 2.9. The mean concentrations in all groups were above the upper limit of the normal range from Day 17 after initial infection to the end of the experiment. There was considerable variation within and between groups, however, serum pepsinogen was elevated in all lambs at one time point, at least, after infection. There were no effects of either diet ($P = 0.90$), time after infection within subjects ($P = 0.11$) or type of larva used for infection ($P = 0.78$).

## 2.3.5 SERUM GASTRIN CONCENTRATION

Group mean ± s.e.m. serum gastrin concentrations are shown in Fig. 2.10 and for individual lambs in each group in Fig 2.11. The mean concentrations in all groups were above the upper limit of the normal range from Day 17 after initial

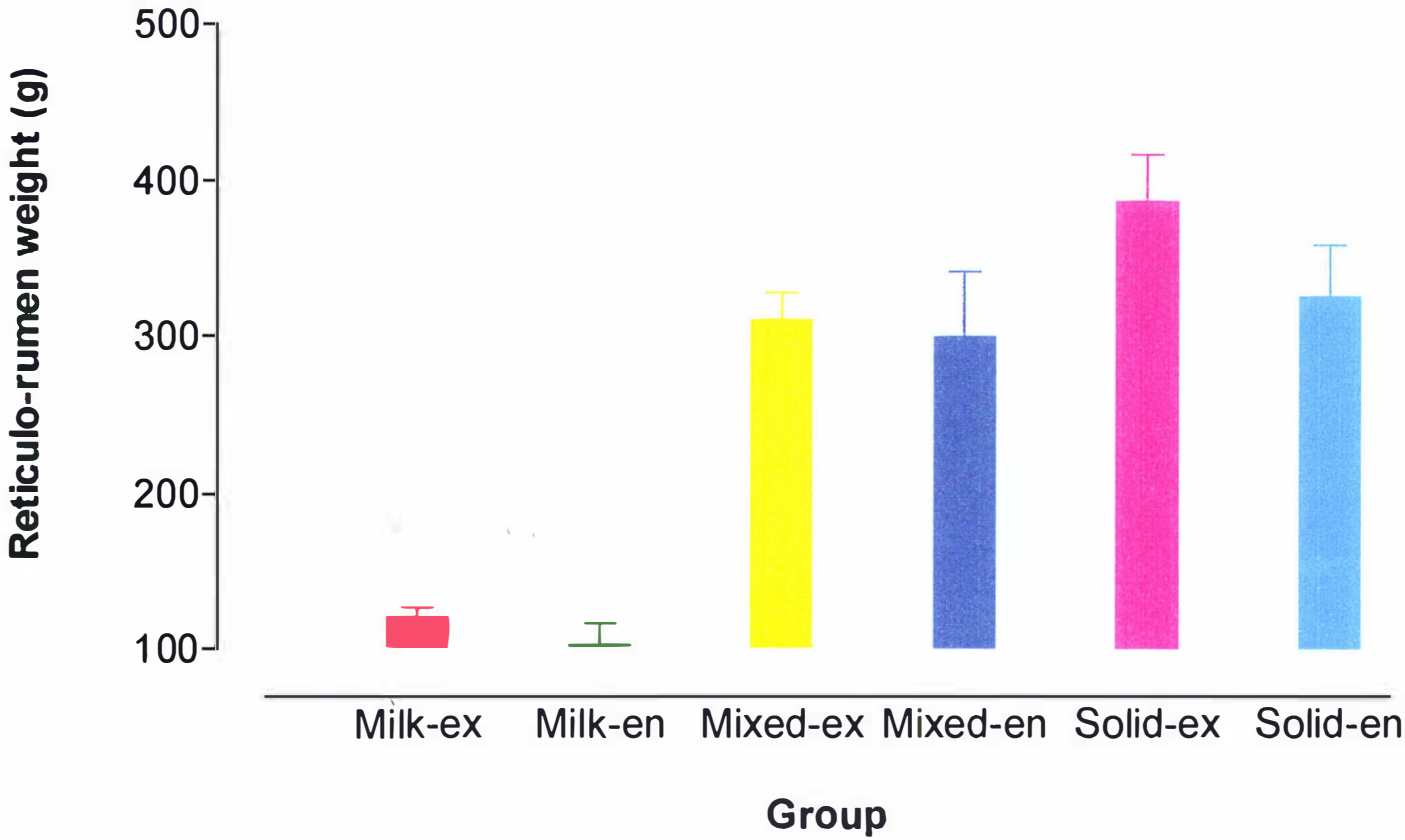


**Figure 2.12** reticulo-rumen weight (mean ± s.e.m.) at necropsy in 6 groups of lambs following infection with *O. circumcincta* larvae.

**Table 2.3** Body, reticulo-rumen, omasum and abomasum weight and organ/body weight (mean ± s.e.m.) at necropsy in 6 groups of lambs following infection with *O. circumcincta* larvae.

| | Group | | | | | |
|---|---|---|---|---|---|---|
| | Milk-ex | Milk-en | Mixed-ex | Mixed-en | Solid-ex | Solid-en |
| Reticulo-rumen (g) | 121 ± 6 | 102 ± 14 | 309 ± 17 | 300 ± 41 | 387 ± 29 | 325 ± 33 |
| Reticulo-rumen/body weight (g/kg) | 9 ± 1 | 7 ± 1 | 17 ± 1 | 17 ± 2 | 22 ± 1 | 21 ± 1 |
| Omasum (g) | 20 ± 2 | 16 ±3 | 33 ± 4 | 36 ± 6 | 49 ± 10 | 37 ± 4 |
| Omasum/body weight (g/kg) | 1± 0.1 | 1.1 ± 0.1 | 1.8 ± 0.2 | 2.0 ± 0.3 | 2.5 ±0.4 | 2.38 ± 0.11 |
| Abomasum (g) | 127 ± 19 | 117 ± 4 | 152 ± 15 | 135 ± 11 | 143 ± 12 | 131 ± 9 |
| Abomasum/body weight (g/kg) | 10 ± 1 | 8 ± 1 | 9 ± 1 | 8 ± 0 | 9 ± 2 | 9 ± 0 |
| Body weight (kg) | 13.5 ± 1.7 | 14.5 ± 1.3 | 18.0 ± 1.5 | 17.6 ± 0.9 | 16.4 ± 1.7 | 16.3 ± 0.9 |

infection to the end of the experiment except groups Milk-ex and Mixed-ex in which the group means were just below the limit on day 17. There was considerable variation within and between groups, however, serum gastrin was elevated in all lambs at one time point, at least, after infection. There were no effects of either diet ($P = 0.25$) or type of larva used for infection ($P = 0.57$). Within subjects, there was a significant effect of time after infection, the relationship being quadratic ($P < 0.01$) and the mean highest on Day 38.

## 2.3.5 BODY WEIGHT

Group mean ± s.e.m. body weights at necropsy are shown in Table 2.3. The body weights of the Milk groups were significantly lower than in the Mixed groups ($P < 0.05$) but not the Solid groups ($P = 0.11$). There was no significant difference between the Solid and Mixed groups ($P = 0.32$), nor between exsheathed and ensheathed larvae ($P = 0.64$).

## 2.3.6 ORGAN WEIGHTS

### 2.3.6.1 Reticulo-rumen

Group mean ± s.e.m. reticulo-rumen weights are shown in Table 2.3 and Fig. 2.12 and weights per kg body weight in Table 2.3.

The absolute weights for the Milk groups were significantly lower than in the Mixed groups ($P < 0.001$) and the Solid groups ($P < 0.001$). There was no significant difference between the Solid and Mixed groups ($P = 0.068$), nor between exsheathed and ensheathed larvae ($P = 0.18$).

The weights per kg body weight for the Milk groups were significantly lower than in the Mixed groups ($P < 0.001$) and the Solid groups ($P < 0.001$) and the Mixed groups were significantly lower than the Solid groups ($P < 0.001$). There

was no significant difference between exsheathed and ensheathed larvae (P = 0.12).

### 2.3.6.2 Omasum

Group mean ± s.e.m. omasal weights and weights per kg body weight are shown in Table 2.3.

The absolute weights of the omasum were significantly lower in the Milk groups than in the Mixed groups ($P < 0.05$) and the Solid groups ($P < 0.001$). There was no significant difference between the Mixed and Solid groups (P = 0.16), nor between exsheathed and ensheathed larvae (P = 0.34).

The weights per kg body weight were significantly lower in the Milk groups than in the Mixed groups ($P < 0.05$) and the Solid groups ($P < 0.001$) and the Mixed groups were significantly lower than in the Solid groups ($P < 0.05$). There was no significant difference between exsheathed and ensheathed larvae (P = 0.35).

### 2.3.6.3 Abomasum

Group mean ± s.e.m. abomasal weights and weights per kg body weight in Table 2.3. For the absolute weights of the abomasum, there were no effects of either diet (P = 0.24) or type of larva used for infection (P = 0.22). For the weights of the abomasum per kg body weight, there were no effects of either diet (P = 0.68) or type of larva used for infection (P = 0.36).

**Table 2.4** Thickness of abomasal mucosa (mean ± s.e.m.) at necropsy in 6 groups of lambs following infection with *O. circumcincta* larvae.

| Group | Mucosal thickness ( μm) |
|---|---|
| Milk-ex | 369 ± 31 |
| Milk-en | 359 ± 33 |
| Mixed-ex | 506 ± 43 |
| Mixed-en | 488 ± 23 |
| Solid-ex | 458 ± 64 |
| Solid-en | 536 ± 28 |

## 2.3.7 ORGAN MORPHOLOGY

### 2.3.7.1 Reticulo-rumen

The reticulo-rumen of the Mixed and Solid groups were considerably larger, darker in colour and the walls were noticeably thicker than in the Milk groups. The reticulo-rumen had a whitish appearance in the lambs fed only milk.

The mean heights of the rumen papillae in individual lambs in the different groups were: 0.9-1 mm (Milk-ex); 1-1.5 mm (Milk-en); 2-4 mm (Mixed-ex); 3-4 mm (Mixed-en); 3-5 mm (Solid-ex); 3-5 mm (Solid-en).

The mean diameters of the reticular cells in individual lambs in the different groups were: 8.3 mm (Milk-ex); 8.4 mm (Milk-en); 16.3 mm (Mixed-ex); 15.9 mm (Mixed-en); 15.6 mm (Solid-ex); 14.3 mm (Solid-en).

### 2.3.7.2 Abomasum

The walls of the abomasa of the milk-fed lambs appeared thinner allowing the contents within to be seen in the intact organ. The mucosal folds also appeared to be shorter and thinner than in both groups of lambs eating solid feed.

There were many small (2 ~ 3 mm in diameter) nodules on the mucosal surface in all lambs.

### 2.3.7 Abomasal histology

Typical nodules were seen in H.& E. stained sections of the fundic mucosa in all lambs irrespective of group.

Group mean ± s.e.m. abomasal fundic mucosal thicknesses are shown in Table 2.4. The mucosal thicknesses for the Milk groups were significantly lower

than in the Mixed and Solid groups ($P < 0.01$) but there was no significant difference between the Mixed and Solid groups ($P = 0.995$). There was no significant difference between exsheathed and ensheathed larvae ($P = 0.61$).

## 2.4 DISCUSSION

This experiment clearly demonstrated that milk-fed lambs were less susceptible to parasitism by abomasal nematodes than fully weaned lambs or lambs fed a mixed milk and solid diet. This was reflected in the worm count at necropsy and by the low number of eggs seen in the faeces. In spite of the lack of development of the reticulo-rumen, ensheathed larvae were able to exsheath in milk-fed lambs, and there were no greater worm burden when the larvae were exsheathed before administration. Although there were considerably fewer worms in milk-fed lambs, there appeared to be no benefit from a single daily feed of milk for lambs fed hay and high protein pellets.

### 2.4.1 PARASITE ESTABLISHMENT

The reduced susceptibility of the milk-fed lambs was very clear-cut (Table 2.2). At necropsy, only two of the eight milk fed lambs had total worm burdens over 1000, and four lambs had very small numbers of worms. No eggs were detected in the faeces of the milk-fed lambs until Day 34, and none was ever found in 3 of the lambs. This is not surprising, given the small number of female worms present in the Milk groups and the small faecal samples taken to estimate FEC. Over all groups, the FEC did not reflect the worm burden at necropsy in individual animals. In contrast to the Milk groups, in the other groups, the first eggs were seen on Day 17 and, by Day 24, some lambs in all 4 other groups were excreting eggs (Fig. 2.2). This is consistent with immediate establishment of larvae from the first infections and a prepatent period for *O. circumcincta* of 16-21 days (Armour et al., 1966). It is difficult to assess from the FEC whether the larvae were slower to establish in milk-fed lambs or just in smaller numbers.

Female worms recovered from the milk-fed lambs were visibly smaller, although they were not measured because of the small number in the aliquots. There also were few eggs per worm. Reduced worm length was also seen in *H. placei* recovered from milk-fed calves (Rohrbacher et al., 1958). In all lamb groups, the number of female worms exceeded the number of male worms and usually the male and immature worms together about equalled the number of mature females. This imbalance of the sexes suggests a less than optimal environment for the parasites in these very young lambs.

## 2.4.2 ABOMASAL FUNCTION

On the fundic folds, there were small numbers of nodules where larvae were developing or in which they had previously developed. Visible nodules are typical of abomasal parasitism (Armour et al., 1966; Rogers & Sommerville, 1968; Scott et al., 1998). At necropsy, mean abomasal pH values for the Mixed and Solid groups exceeded pH 3, suggesting a mild inhibition of acid secretion. Abomsal pH rises in the abomasum of older parasitised animals (Coop et al., 1977; Anderson et al., 1975, 1985, 1988; Lawton et al., 1996; Simcock, et al., 1999). The abomasal pH at necropsy may not reflect the changes in abomasal pH during the preceding weeks of infection, and it also may not represent the pH of the contents for much of the day in milk-fed animals as the abomasal pH was measured 14 hours after the last milk feed.

Serum gastrin also increased in all lambs, although only for a few days in some animals, and in most not to the very high levels seen in older animals (Lawton et al., 1996). Within the Milk groups, lambs #1 and #2 were the two with worm burdens over 1000 at necropsy and these lambs also had serum gastrin concentrations over 200 pM in the last two weeks of the infection. At necropsy, these two lambs had abomasal pH values higher than average for their groups. The abomasal pH in the milk-fed lambs was well below those usually seen in parasite-naïve older ruminants (Lawton et al., 1996).

The infection of lambs with larval *O. circumcincta* resulted in increased serum pepsinogen concentration at some time point in all animals, regardless of the worm burden. There was no difference in serum pepsinogen between the Milk groups and the other lambs. It would appear that the increased appearance of pepsinogen in the circulation is related more to the presence of larvae in the abomasum than to the numbers which develop into adult worms.

## 2.4.3 DEVELOPMENT OF THE STOMACH

The reticulo-rumen and omasum were much less developed in the milk-fed lambs than in those fed solid food or a mixture of milk and solids. The wall of the reticulo-rumen was visibly thinner, the rumen papillae were shorter, the reticular cells were smaller and the wet weights of the organs were significantly lower. This was expected, as development of the reticulum, rumen and omasum depends on the consumption of solid food in young ruminants (Church & Pond, 1974).

In the present study, there was no evidence of impaired exsheathing in the milk-fed lambs in spite of the reticulo-rumen being small and probably non-functional. There is also the possibility of altered rumen function in the Mixed groups, since milk can cause defaunation of the rumen (Kreuzer & Kirchgessner, 1987). These authors reported defaunation in lambs and heifers fed with pelleted concentrates and milk, which was caused by the milk fat. The conclusion from a previous study on milk-fed calves was that the degree of development of ruminal function influenced the establishment of gastrointestinal nematodes (Satrija et al., 1991) although their data showed a greater establishment of ensheathed than exsheathed larvae. Abomasal nematodes normally exsheath in the rumen where the carbon dioxide tension is considered to be a major factor in initiating exsheathment of third stage larvae by stimulating the secretion of exsheathing enzymes (Rogers & Sommerville, 1968). The present study, and that of Satrija et al. (1991), both suggest that larvae are able to exsheath in the absence of a developed functional rumen. Therefore, the low establishment of *O. circumcincta*

in milk-fed ruminants cannot be attributed to the concurrent lack of rumen development.

## 2.4.4 EFFECT OF MILK IN THE ABOMASUM

Since conditions in the rumen did not appear to be crucial for worm establishment, the effects of the milk in the abomasum must have presented an unfavourable environment for the development of *O. circumcincta.* This may be due to a direct adverse effect on the larvae of the milk or by creating adverse conditions which do not favour development of the larvae to adult worms. Alternatively, the immaturity of the gastro-intestinal tract may present tissues to the incoming parasites which do not induce entry into the gastric glands.

A variety of explanations have been offered for the resistance to parasites of milk-fed animals: high abomasal pH was suggested to protect against nematodes in milk-fed calves (Rohrbacher et al., 1958); nutrient (PAB) deprivation to explain the reduced infection with malarial parasites in humans Jacobs (1964); or increased gut motility to explain expulsion of nematodes from skim milk-fed pigs (Spindler et al., 1944). In the milk-fed lambs, there was no evidence of scouring as suggested by the study in pigs. The effect of the high pH of milk is a possible contributing factor.

At necropsy, the abomasal pH was lower in the Milk groups than in the Mixed and Solid groups and also below that normally seen in parasite-naïve ruminating lambs (Lawton et al., 1996). These pH values contrast with the high pH recorded from milk-fed calves (Rohrbacher et al., 1958), but were measured over 14 hours after the last milk feed. In the calf study, milk in the diet tended to neutralize the acidity of the abomasa: whereas the pH of the abomasal contents of calves fed solid feed was between 2.5 and 2.9, for animals fed solid food in addition to milk, the pH was 6.3 30 minutes after the milk intake and was between 2.5 to 2.9 within 6 to 18 hours after milk feeding. The abomasal pH of calves fed

on milk alone was 5.75 30 minutes after feeding and decreased to 3.1 18 hours after milk was fed. When the lambs in present study were killed 14 hours after milk feeding, the abomasal pH of the Milk group lambs was between 1.47 and 2.48, the pH was 2.61 to 3.61 in the lambs fed on mixed diet, and in the Solid groups, the pH was 2.43 to 3.94.

Reduced parasite survival because of the high pH of milk is not consistent with in vitro survival of larvae incubated in HBSS in the experiments reported in Chapter 3. It is also not supported by survival studies with fourth-stage larvae and adult *H. contortus* (Haag, 1995), in which survival was best at higher pH and poor at low pH. In addition, abomasal parasites rapidly inhibit acid secretion (Christie et al., 1967; Titchen & Anderson, 1977; Anderson et al., 1981, 1985; Lawton et al., 1996; Simpson et al., 1997), presumably because it is not detrimental to their survival. There may however, be an optimal pH which is lower than that of milk, as maximum egg laying by *H. contortus* was observed at pH 4-4.5 (Honde & Bueno, 1982).

Anti-parasitic chemicals may be present in the milk itself. While there is no evidence either for or against involvement of a number of substances with known biological activity, they cannot be excluded at this stage. Casomorphins, derived from β-casein in milk, have been found to have opioid activity (Regester et al., 1997), which could slow the motility of the nematodes. Milk contains vitamin-binding proteins which bind vitamin $B_{12}$, folate, and riboflavin (International Dairy Federation, 1991) and inhibit the growth of micro-organisms that require these vitamins for growth. The addition of phosphate to the basal salt ration of sheep experimentally-infected with *H. contortus*, was reported to reduce FEC, mortality and loss of haemoglobin to the same extent as the addition of phenothiazine to the salt (Emerick et al., 1956).

The Igs in milk are worthy of consideration as the protective agent in milk. Suckled young can be protected against helminth parasites by specific Igs in the

milk of mothers with gastro-intestinal parasites (Musoke et al., 1975) through passive transmission of specific IgG in the milk (Smith & Herbert 1976) or antigen-specific T cells (Kumar et al., 1989). Specific secretory IgA in milk from mice infected with *G. lamblia* induced a specific parasiticidal effect on trophozoites in their sucking offspring (Stager et al., 1998). Since antibodies to *O. ostertagi* are likely to be present in bovine milk, the increased resistance to *O. circumcincta* infection in the lambs could be the result of cross-reactivity between the two *Ostertagia* species.

A further possible explanation for poorer worm establishment in the milk-fed lamb is the non-specific attachment of proteins to the larvae, rather than the action of specific milk components. If this were the case, the worm burdens would be expected to be lower in the lambs fed a Mixed diet compared with the Solid groups. The reason this did not occur may be the timing of the infection and the milk feed. Larvae were given in the morning, whereas the milk was fed in the evening and there was continuous access to solid food. When the larvae were administered, very little milk may have remained in the abomasum or milk proteins may have already attached to the solid food. Another possibility is that the milk may adhere to the large number of micro-organisms in the rumen of the lambs ingesting solid food (Ensiminger et al., 1990) when they pass down to the abomasum.

In summary, lambs fed entirely with milk were less susceptible to *O.circumcincta* infection, although there was no benefit from feeding a milk plus solid diet over a solid diet. The cause of the lower worm establishment was not the failure to exsheath in the immature gastro-intestinal tract, as there were similar worm burdens in lambs in all groups when ensheathed and exsheathed larvae were administered. This suggests that the milk itself is responsible for the adverse effects on the parasites. This question is addressed in the experiments described in Chapter 3, in which larvae were exposed in vitro.

# Chapter 3

# THE EFFECT OF MILK AND MILK COMPONENTS ON THE MOTILITY OF *OSTERTAGIA CIRCUMCINCTA* IN VITRO

## 3.1 INTRODUCTION

Milk feeding has been shown to be associated with reduced nematode burdens in calves (Porter, 1941; Rohrbacher et al., 1958; Satrija et al., 1991), rabbits (Rohrbacher et al., 1958), horses (Leese, 1943), pigs (Spindler et al., 1944; Spindler & Zimmerman, 1944; Shorb & Spindler, 1947) and now also in lambs. In these in vivo experiments, it has been difficult to determine whether or not it is the milk itself which adversely affects the parasites, although, in most of the experiments, the presence of a parasiticidal component in milk cannot be excluded.

There have been relatively few *in vitro* experiments in which parasites have been exposed directly to milk or its components, and apparently none involving nematodes. *Giardia* trophozoites are killed by long chain unsaturated fatty acids in milk (Gillin et al., 1983; Rohrer et al., 1986), and lactoferrin increases the uptake and killing of *Trypanosoma cruzi* by mouse peritoneal macrophages and human blood monocytes (Lima & Kierszenbaum, 1985). Milk is known to contain other biologically active agents including immunoglobulins (IgG and IgA), anti-tumour activity (whey and calcium) and antibacterial agents (lysozyme, lactoferrin, lactoperoxidase, vitamin-binding proteins and lipids), which could potentially damage parasites.

The experiment reported in the previous Chapter has demonstrated that a milk diet can influence the worm burden in lambs trickle-infected with larval *O.*

*circumcincta*, but it is not clear whether the anti-parasitic activity of milk is dependent upon the change of environment brought by the milk or by the milk itself. Therefore, exsheathed *O. circumcincta* larvae have been exposed to different milks in vitro to ascertain if bovine milk contains specific components which can inhibit the activity of the parasites.

## 3.2 MATERIALS AND METHODS

### 3.2.1 LARVAL MOTILITY ASSAY

The motility of exsheathed third-stage larvae was assessed after incubation in the test solution at 37°C for periods of 1 hour, 2 hours, 4 hours, 24 hours or 48 hours. From each incubation tubes, 50 larvae were examined for visible motility in a McMaster slide. Systematic viewing of the chambers aided assessing larvae once only.

Before each assay, the viability of the larvae was assessed (Section 2.2.13.2) to ensure it was greater than 90%. The larvae were exsheathed as described previously (Section 2.2.13.3), washed and resuspended in Hank's Balanced Salt Solution (HBSS) at about 3000 larvae/ml. The larval suspension was mixed well before 25 µl (containing at least 50 larvae, usually about 75) was added to each of the tubes containing 1 ml of the test solutions. The tubes were covered with plastic film (Parafilm, American National Can, USA) and placed in a 37 °C incubator (Clayson, New Zealand). A number of tubes were made up for each solution, and after examination of the larvae, the incubation solution and larvae were discarded.

At the end of the incubation period, the mixture in the tubes was mixed well and used to fill the two chambers of a counting slide. The motility of 50 larvae was assessed under a microscope (Olympus, CH-2. Japan) and recorded as percentage immotile.

## 3.2.2. TEST SOLUTIONS

### 3.2.2.1. Hanks Balanced Salt Solution (HBSS)

The control medium was HBSS made with powdered HBSS without $NaHCO_3$ (9.8 g/litre, GIBCO BRL, USA), to which was added 0.35 g of $NaHCO_3$, made to 1 litre with distilled water and adjusted to pH 7.4.

### 3.2.2.1. Fresh bovine milk

Fresh bovine milk was obtained on the morning of the experiment from cows milked at the No 3. Dairy at Massey University. Serial dilutions were made with HBSS. The concentrations tested were: 100%, 50%, 25%, 12.5%, 6.25%, 3.12% and 1.56%. The incubation periods were 1 hour, 24 hours and 48 hours. The experiment was carried out three times.

### 3.2.2.2. Commercial homogenised bovine milk (3.3% fat)

Commercial milk ("Tararua" family fresh homogenised milk), containing 3.3% fat was purchased from a supermarket and diluted with HBSS. The concentrations tested were: 100%, 50%, 25%, 12.5%, 6.25%, 3.12% and 1.56%. The incubation periods were 1 hour, 24 hours and 48 hours. The experiment was carried out three times.

### 3.2.2.3. Commercial low fat bovine milk (0.2% fat)

Commercial milk ("Tararua" trim milk, containing 0.2% fat) was purchased from a supermarket and diluted with HBSS. The concentrations tested were: 100%, 50%, 25%, 12.5%, 6.25%, 3.12% and 1.56%. The incubation periods were 1 hour, 24 hours and 48 hours. The experiment was carried out three times.

### 3.2.2.4. Bovine milk replacer for lambs

Lamb milk replacer ("Anlamb", New Zealand Dairy Co, Te Puke, New Zealand) was made by adding 50 g of powder to 500 ml of distilled water and gently stirring until dissolved. Further dilutions were made with HBSS. The concentrations tested were: 100%, 50%, 25%, 12.5%, 6.25%, 3.12% and 1.56%. The incubation periods were 1 hour, 24 hours and 48 hours. The experiment was carried out three times.

### 3.2.2.5. Whey protein

Dried whey protein was kindly supplied by Prof. H. Singh. The powder was dissolved in water with gentle stirring to make a series of concentrations from 10% (w/v) (4 g/40ml) to 30% (12 g/40 ml), A series of HBSS solutions were adjusted to pH 2.5, 3.5, 4.5, 5.5 and 6.5 with 1M HCl. Each whey solution was mixed with an equal volume of HBSS to give final whey solutions of 5%, 7.5%, 10%, 12.5% and 15%, each at pH 2.5, 3.5, 4.5, 5.5 and 6.5. The series of HBSS at each pH was also tested as control solutions. The incubation periods were 2 hours, 4 hours and 24 hours. The experiment was carried out three times.

### 3.2.2.6. Casein

Dried casein was kindly supplied by Prof. H. Singh. The powder was dissolved in water with gentle stirring to make concentrations of 10% (w/v) (4 g/40ml) and 15% (6 g/40 ml). HBSS solutions were adjusted to pH 5.5 and 6.5 with HCl. Each whey solution was mixed with an equal volume of HBSS to give final casein solutions of 5% and 7.5%, each at pH 5.5 and 6.5. HBSS at pH 5.5 and 6.5 were also tested as control solutions. The incubation periods were 2 hours, 4 hours and 24 hours. The experiment was carried out three times.

### 3.2.2.7. Ultra low heat skim milk powder (ULHSMP)

Dried ULHSMP was kindly supplied by Prof. H. Singh. The powder was dissolved in water with gentle stirring to make a series of concentrations from 10% (w/v) (4 g/40ml) to 30% (12 g/40 ml). HBSS solutions were adjusted to pH 5.5 and 6.5 with 1M HCl. Each ULHSMP solution was mixed with an equal volume of HBSS to give final ULHSMP solutions of 5%, 7.5%, 10%, 12.5% and 15%, each at pH 5.5 and 6.5. HBSS at pH 5.5 and 6.5 were also tested as control solutions. The incubation periods were 2 hours, 4 hours and 24 hours. The experiment was carried out three times.

### 3.2.1.4.4 Soybean trypsin inhibitor

Trypsin inhibitor (Type I-S: From soybean) was purchased from the Sigma Chemical Co., USA. Final concentrations of 500 μg/ml, 100 μg/ml, 20 μg/ml and 4 μg/ml were each made up in HBSS adjusted to pH 5.5 and 6.5. HBSS at pH 5.5 and 6.5 were also tested as control solutions. The incubation periods were 2 hours, 4 hours and 24 hours. The experiment was carried out once.

In another experiment, the trypsin inhibitor was made in a concentration of 7.8 mg/ml in HBSS adjusted to pH 5.5 and 6.5. HBSS at pH 5.5 and 6.5 were also tested as control solutions. The incubation period was 24 hours. The experiment was carried out once.

## 3.2.3 STATISTICS

The percentages of immotile larvae in the different solutions were compared using the General Linear Model (GLM) Univariate (Two way ANOVA) and Repeated Measures programme of SPSS (Statistics Program for Social Science) version 9.0 (SPSS Inc., Chicago, U.S.A.) using Fisher's least significant difference (LSD). P values less than 0.05 were considered to be significant.

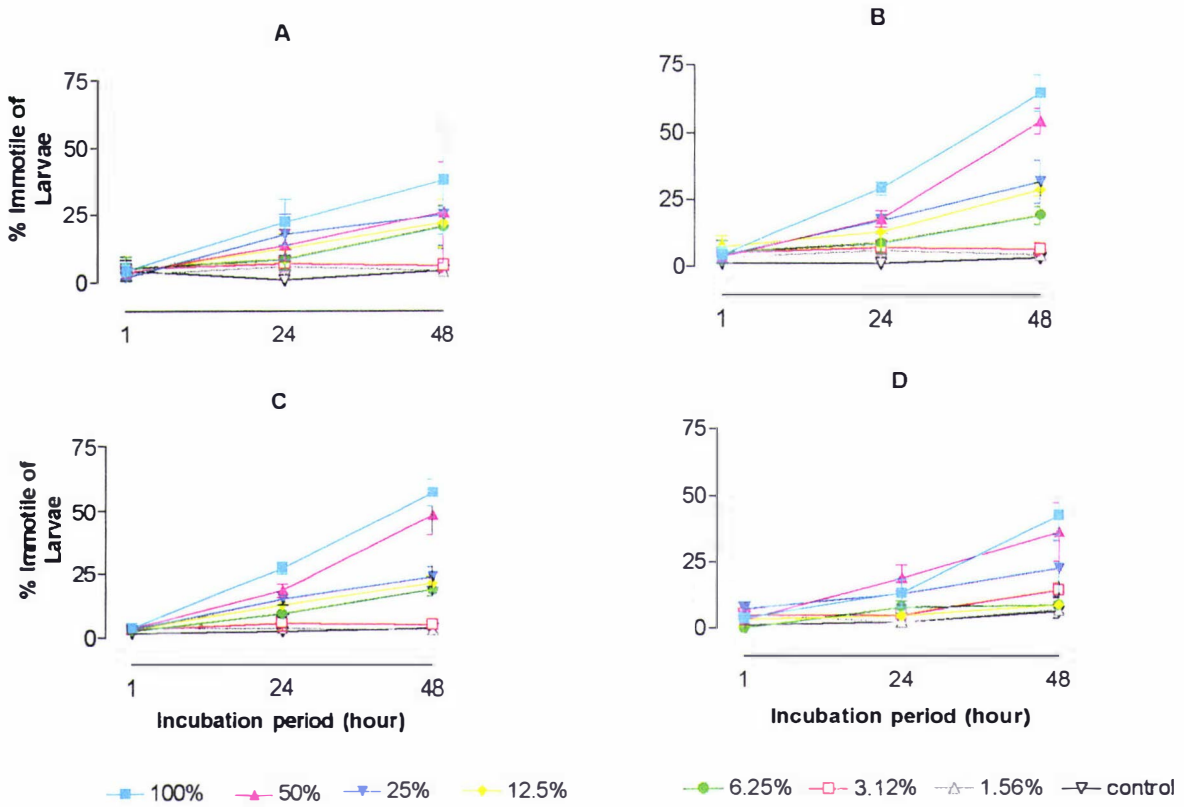


**Figure 3.1** Percentage of immotile *O. circumcincta* larvae (mean ± s.e.m.) differnet concentrations of milk for incubation periods of 1, 24 and 48 hours.
A: Fresh bovine milk.
B: Commercial homogenised bovine milk (3.3% fat).
C: Commercial low fat bovine milk (0.2% fat).
D: Bovine milk replacer for lambs.

**Table 3.2** Percentage of immotile larval *O. circumcincta* (mean ± s.e.m) in different concentrations of commercial homogenised bovine milk (3.3% fat) for incubation periods of 1, 24 and 48 hours.

| Milk concentration (%) | Incubation time | | |
|---|---|---|---|
| | 1 hour | 24 hours | 48 hours |
| 100 | 5 ± 3 | 30 ± 2 | 65 ± 7 |
| 50 | 4 ± 2 | 18 ± 3 | 54 ± 5 |
| 25 | 4 ± 2 | 17 ± 2 | 32 ± 8 |
| 12.5 | 7 ± 4 | 12 ± 5 | 28 ± 2 |
| 6.25 | 5 ± 4 | 9 ± 4 | 19 ± 3 |
| 3.12 | 5 ± 4 | 7 ± 3 | 7 ± 2 |
| 1.56 | 3 ± 2 | 6 ± 3 | 5 ± 2 |
| Control | 1 ± 1 | 1 ± 1 | 3 ± 1 |

**Table 3.1** Percentage of immotile larval *O. circumcincta* (mean ± s.e.m) in different concentrations of fresh bovine milk for incubation periods of 1, 24 and 48 hours.

| Milk concentration (%) | Incubation time | | |
|---|---|---|---|
| | 1 hour | 24 hours | 48 hours |
| 100 | 5 ± 3 | 23 ± 9 | 38 ± 20 |
| 50 | 4 ± 2 | 14 ± 6 | 26 ± 19 |
| 25 | 2 ± 2 | 18 ± 8 | 25 ± 11 |
| 12.5 | 4 ± 2 | 13 ± 5 | 22 ± 9 |
| 6.25 | 5 ± 4 | 9 ± 4 | 21 ± 8 |
| 3.12 | 5 ± 4 | 7 ± 3 | 7 ± 2 |
| 1.56 | 3 ± 2 | 6 ± 3 | 5 ± 2 |
| Control | 5 ± 4 | 1 ± 1 | 5 ± 1 |

# 3.3 RESULTS

## 3.3.1 LARVAL *O. CIRCUMCINCTA* MOTILITY IN FRESH BOVINE MILK

The group mean ± s.e.m percentages of immotile larvae in different concentrations of fresh bovine milk and for different incubation periods (n = 3) are shown in Table 3.1 and Fig 3.1A. There was a significant increase with time: compared with the percentage immotile at 1 hour, the percentage at 24 hours was approaching significance ($P = 0.065$) and was significantly increased at 48 hours ($P < 0.001$). The difference in percentages at 24 and 48 hours was also approaching significance ($P = 0.06$) Compared with the control HBSS, the percentage immotile was increased in 100% milk ($P < 0.01$) and approaching significance in concentrations of 50% ($P = 0.074$) and 25% ($P = 0.064$).

## 3.3.2 LARVAL *O. CIRCUMCINCTA* MOTILITY IN COMMERCIAL HOMOGENISED BOVINE MILK (3.3% FAT)

The group mean ± s.e.m percentages of immotile larvae in different concentrations of homogenised milk and for different incubation periods (n = 3) are shown in Table 3.2 and Fig. 3.1B. There was a significant increase with time: compared with the percentage immotile at 1 hour, the percentage was significantly increased at 24 hours ($P < 0.001$) and at 48 hours ($P < 0.001$). ) and between 4 and 24 hours ($P < 0.001$). Compared with the control HBSS, the percentage immotile was increased in concentrations of milk of 100% ($P < 0.001$), 50% ($P < 0.001$), 25% ($P < 0.001$), 12.5% ($P < 0.001$) and 6.25% ($P < 0.01$).

**Table 3.3** Percentage of immotile larval *O. circumcincta* (mean ± s.e.m) in different concentrations of commercial low fat bovine milk (0.2% fat) for incubation periods of 1, 24 and 48 hours.

| Milk concentration (%) | Incubation time | | |
|---|---|---|---|
| | 1 hour | 24 hours | 48 hours |
| 100 | 4 ± 2 | 27 ± 2 | 57 ± 5 |
| 50 | 4 ± 2 | 19 ± 3 | 49 ± 8 |
| 25 | 3 ± 1 | 15 ± 2 | 24 ± 4 |
| 12.5 | 3 ± 1 | 13 ± 2 | 21 ± 3 |
| 6.25 | 3 ± 1 | 9 ± 2 | 19 ± 2 |
| 3.12 | 3 ± 1 | 6 ± 3 | 5 ± 2 |
| 1.56 | 4 ± 1 | 4 ± 1 | 3 ± 1 |
| Control | 2 ± 0 | 3 ± 1 | 4 ± 1 |

### 3.3.3 LARVAL *O. CIRCUMCINCTA* MOTILITY IN COMMERCIAL LOW FAT BOVINE MILK (0.2% FAT)

The group mean ± s.e.m percentages of immotile larvae in different concentrations of low fat milk and for different incubation periods (n = 3) are shown in Table 3.3 and Fig. 3.1C. There was a significant increase with time: compared with the percentage immotile at 1 hour, the percentage was significantly increased at 24 hours ($P < 0.001$) and at 48 hours ($P < 0.001$) and between 24 and 48 hours ($P < 0.001$). Compared with the control HBSS, the percentage immotile was increased in concentrations of milk of 100% ($P < 0.001$), 50% ($P < 0.001$), 25% ($P < 0.001$), 12.5% ($P < 0.001$) and 6.25% ($P < 0.01$).

### 3.3.4 LARVAL *O. CIRCUMCINCTA* MOTILITY IN BOVINE MILK REPLACER FOR LAMBS

The group mean ± s.e.m percentages of immotile larvae in different concentrations of milk replacer and for different incubation periods (n = 3) are shown in Table 3.4 and Fig. 3.1D. There was a significant increase with time: compared with the percentage immotile at 1 hour, the percentage was significantly increased at 24 hours ($P < 0.05$) and at 48 hours ($P < 0.001$) and between 24 and 48 hours ($P < 0.05$). Compared with the control HBSS, the percentage immotile was increased in concentrations of milk of 100% ($P < 0.001$), 50% ($P < 0.001$) and 25% ($P < 0.01$).

### 3.3.5 COMPARISON OF EFFECT OF THE BOVINE MILKS ON LARVAL *O. CIRCUMCINCTA* MOTILITY

The data for the 4 different bovine milks (group mean ± s.e.m) are compared in Fig 3.1. The maximum % of immotile larvae for all was seen in 100% milk after 48 hours incubation; these percentages were respectively, fresh milk (38 ± 20),

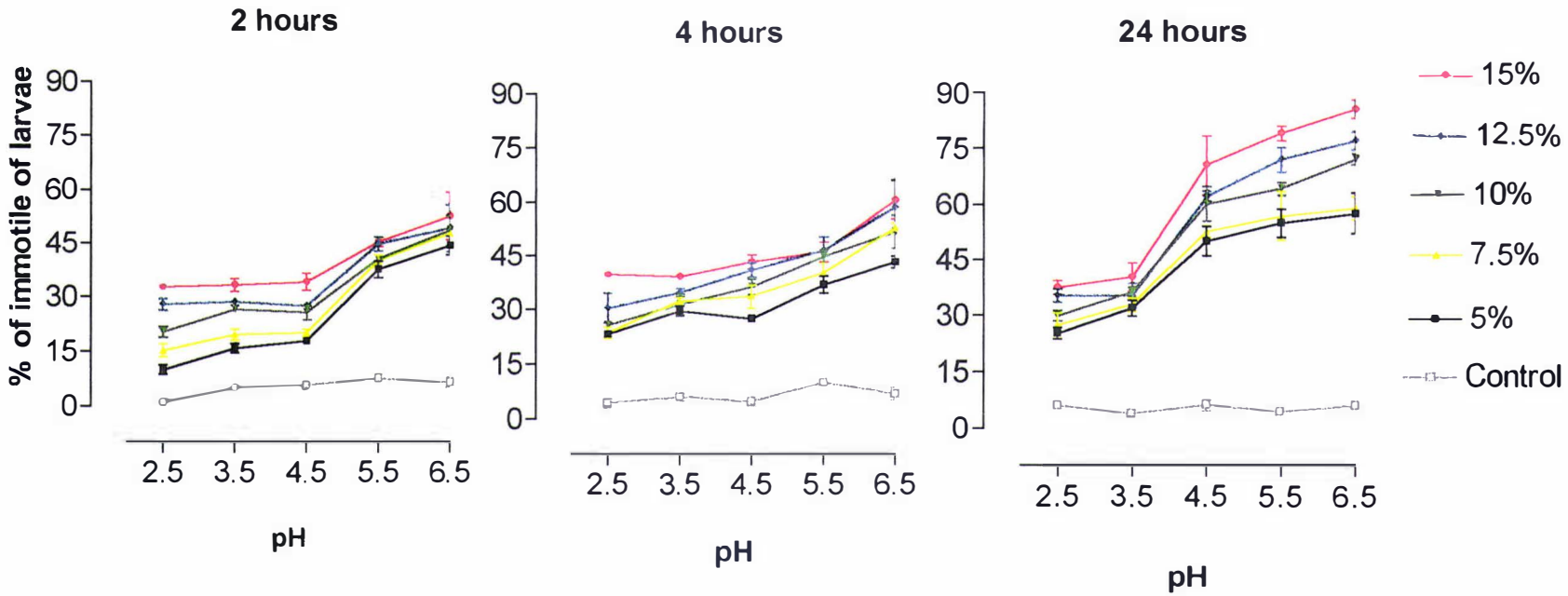


**Figure 3.2** The Percentage of immotile *O. circumcincta* larvae (mean ± s.e.m.) in different concentrations of whey proteins and pH from 2.5 to 6.5 for incubation periods of 2, 4 and 24 hours.

**Table 3.5** Percentage of immotile larval *O. circumcincta* (mean ± s.e.m) in different concentrations of whey protein and pH from 5.5 to 6.5 for incubation periods of 2, 4 and 24 hours.

| Incubation period | pH | Control | Concentration (%) | | | | |
|---|---|---|---|---|---|---|---|
| | | | 5 | 7.5 | 10 | 12.5 | 15 |
| 2 hours | 6.5 | 1 ± 1 | 3 ± 1 | 13 ± 1 | 16 ± 0 | 15 ± 1 | 17 ± 1 |
| | 5.5 | 2 ± 1 | 4 ± 1 | 5 ± 1 | 5 ± 1 | 4 ± 2 | 7 ± 1 |
| 4 hours | 6.5 | 4 ± 0 | 14 ± 1 | 17 ± 1 | 17 ± 2 | 23 ± 2 | 25 ± 2 |
| | 5.5 | 2 ± 2 | 10 ± 2 | 14 ± 2 | 13 ± 1 | 26 ± 7 | 22 ± 2 |
| 24 hours | 6.5 | 1 ± 1 | 33 ± 1 | 46 ± 2 | 50 ± 4 | 61 ± 2 | 66 ± 3 |
| | 5.5 | 3 ± 1 | 42 ± 2 | 41 ± 4 | 43 ± 5 | 46 ± 3 | 60 ± 1 |

homogenised milk (3.3% fat) (65 ± 7), low fat milk (0.2% fat) (57 ± 5) and lamb milk replacer (43 ± 7).

All 4 sets of data were analysed together. The percentage of immotile larvae in homogenised milk (3.3.% fat) and low fat milk (0.2% fat) were not significantly different ($P = 0.19$). The percentages immotile in fresh milk ($P<0.05$) and lamb milk powder ($P < 0.01$) were both lower than in commercial homogenised milk (3.3% fat) but not in the low fat milk (0.2% fat).

### 3.3.6 LARVAL *O. CIRCUMCINCTA* MOTILITY IN WHEY PROTEIN SOLUTIONS

The group mean ± s.e.m percentages of immotile larvae in different concentrations of whey protein and pH and for different incubation periods ($n = 3$) are shown in Table 3.5 and Fig.3.2. There were significant effects of time of incubation, pH of the solution and whey concentration. There was a significant increase with time: compared with the percentage immotile at 2 hours, the percentage was significantly increased at 4 hours ($P < 0.001$) and at 24 hours ($P < 0.001$) and between 4 and 24 hours ($P < 0.001$). Compared with the control HBSS, the percentage immotile was increased in all concentrations of whey: 15% ($P < 0.001$), 12.5% ($P < 0.001$), 10% ($P < 0.001$), 7.5% ($P < 0.001$) and 5% ($P <0.001$). The percentage of immotile larvae increased as the pH increased and there was a significant difference ($P < 0.001$) between all pairs of solutions of different pH.

### 3.3.7 LARVAL *O. CIRCUMCINCTA* MOTILITY IN CASEIN SOLUTIONS

Casein was soluble in HBSS only at pH 5.5 and 6.5 but not at pH 4.5 and below. The highest concentration in which the casein dissolved was 15%, giving a final concentration of 7.5% after mixing with HBSS.

**Table 3.7** Percentage of immotile larval *O. circumcincta* (mean ± s.e.m) in different concentrations of ULHSMP and pH from 5.5 to 6.5 for incubation periods of 2, 4 and 24 hours.

| Incubation period | pH | Control | Concentration (%) | | | | |
|---|---|---|---|---|---|---|---|
| | | | 5 | 7.5 | 10 | 12.5 | 15 |
| 2 hours | 6.5 | 1 ± 1 | 3 ± 2 | 13 ± 1 | 16 ± 0 | 15 ± 1 | 17 ± 1 |
| | 5.5 | 2 ± 1 | 4 ± 1 | 5 ± 1 | 5 ± 1 | 4 ± 2 | 7 ± 1 |
| 4 hours | 6.5 | 4 ± 0 | 14 ± 1 | 17 ± 1 | 17 ±2 | 23 ± 2 | 25 ± 2 |
| | 5.5 | 3 ± 2 | 10 ± 2 | 14 ± 2 | 13 ± 1 | 16 ± 7 | 23 ± 2 |
| 24 hours | 6.5 | 1 ± 1 | 33 ± 1 | 46 ± 2 | 50 ± 4 | 61 ± 2 | 66 ± 3 |
| | 5.5 | 3 ± 1 | 42 ± 2 | 42 ± 4 | 42 ± 5 | 46 ± 3 | 60 ± 1 |

**Table 3.6** Percentage of immotile larval *O. circumcincta* (mean ± s.e.m) in different concentrations of casein protein and pH from 5.5 to 6.5 for incubation periods of 2, 4 and 24 hours.

| Incubation period | pH | Control | Concentration (%) | |
|---|---|---|---|---|
| | | | 5 | 7.5 |
| 2 hours | 6.5 | 2 ± 1 | 3 ± 2 | 3 ± 1 |
| | 5.5 | 1 ± 1 | 3 ± 1 | 3 ± 1 |
| 4 hours | 6.5 | 2 ± 1 | 46 ± 1 | 57 ± 4 |
| | 5.5 | 2 ± 1 | 48 ± 3 | 57 ± 4 |
| 24 hours | 6.5 | 3 ± 2 | 48 ± 1 | 56 ± 2 |
| | 5.5 | 3 ± 2 | 50 ± 2 | 68 ± 2 |

The group mean ± s.e.m percentages of immotile larvae in different concentrations of casein and pH and for different incubation periods (n = 3) are shown in Table 3.6. There were significant effects of time of incubation and casein concentration, but not pH of the solution. There was a significant increase with time: compared with the percentage immotile at 2 hours, the percentage was significantly increased at 4 hours ($P < 0.001$) and at 24 hours ($P < 0.001$), but was no different at 4 and 24 hours ($P = 0.097$). Compared with the control HBSS, the percentage immotile was increased in both concentrations of casein: 5% ($P < 0.001$) and 7.5% ($P < 0.001$) and 7.5% was significantly greater than 5% ($P < 0.001$).

### 3.3.8 LARVAL *O. CIRCUMCINCTA* MOTILITY IN ULTRA LOW HEAT SKIM MILK POWDER (ULHSMP)

The group mean ± s.e.m percentages of immotile larvae in different concentrations of ULHSMP and pH and for different incubation periods (n = 3) are shown in Table 3.7. There were significant effects of time of incubation, pH of the solution and ULHSMP concentration. There was a significant increase with time: compared with the percentage immotile at 2 hours, the percentage was significantly increased at 4 hours ($P < 0.001$) and at 24 hours ($P < 0.001$) and between 4 and 24 hours ($P < 0.001$). Compared with the control HBSS, the percentage immotile was increased in all concentrations of ULHSMP: 15% ($P < 0.001$), 12.5% ($P < 0.001$), 10% ($P < 0.001$), 7.5% ($P < 0.001$) and 5% ($P <0.001$). The percentage of immotile larvae was greater at pH 6.5 than at pH 5.5 ($P < 0.001$).

### 3.3.9 LARVAL *O. CIRCUMCINCTA* MOTILITY IN HBSS CONTAINING TRYPSIN INHIBITOR

The viability of the larvae remained over 90% in all concentrations of trypsin inhibitor at pH 5.5 and 6.5 and after incubation for 24 hours.

## 3.4 DISCUSSION

The motility of exsheathed third-stage *O. circumcincta* larvae was reduced by exposure to different bovine milk products and to whey, casein and skim milk proteins. In all cases with the exception of casein, the effect on motility was concentration and time dependent. The pH of the solution also influenced the activity of the component protein fractions, presumably through the effect on the electrostatic charge of proteins and perhaps also the degree of polymerization. The lowest concentration of whey tested was similar to the whey concentration in whole milk, whereas the casein concentrations (5 and 7.5 g/100 ml) were only about 20% of the concentration in milk.

Fresh bovine milk and three processed milks had similar effects on larval motility (Fig 3.1), indicating that the activity survives the pasteurization and drying processes and the removal of most of the fat. Indeed, the fresh milk had a slightly lower activity than the processed forms. In addition, the activity remained after processing of milk to whey and casein fractions and production of skim milk powder. Of relevance to the in vivo experiment reported in Chapter 2, is the reduction in larval motility by the milk replacer which was fed to the lambs.

The active component in the milk appears to be associated with proteins and not with the lipid fraction. Involvement of milk lipids appears unlikely for several reasons. First, the similar levels of activity in 3.3% fat milk and 0.2% fat milk and its presence in skim milk powder and casein, neither of which contains significant amounts of lipid, all point to the activity not being associated with milk lipid. Pasteurization of milk can reduce the free fatty acid content in milk (Renner, 1983), yet the pasteurized commercial milks were just as effective as the farm milk (Table 3.1). The immobilisation of nematode larvae therefore appears to be unrelated to the adverse effects of milk on *Giardia* which has been attributed to the free fatty acids (Gillin et al., 1983; Rohrer et al., 1986; Hernell et al., 1986).

The milk proteins contain several compounds with different types of biological activity, some of which could possibly contribute to the loss of larval motility in these experiments. Lactoferrin in human milk increased the uptake and killing of *T. cruzi* by mouse macrophages and human monocytes in vitro (Lima & Kierszenbaum, 1985) and lactoferrin also limited bacterial growth by binding iron (Renner et al., 1989; Jensen, 1995). These mechanisms are probably unrelated to the in vitro effects of milk on nematodes. Both lactoferrin and lactoperoxidase are present in the whey proteins, but not the casein fraction, in which they would only be minor contaminants. Since both protein fractions had similar effects on larval motility, this would probably eliminate them as the active substances. Calcium is trapped in the proteins, particularly in the casein fraction and is a further specific component of milk worthy of consideration as the inhibitor as it has been suggested to be responsible for the inhibition of parasites *in vivo* by a milk diet (Emerick et al., 1956).

A trypsin inhibitor from soybean was examined for inhibitory activity, as milk contains trypsin inhibitors in the whey fraction (Christensen & Sottrup-Jensen, 1994; Precetti et al., 1997). Although the milk and soybean trypsin inhibitors are different enzymes, it was thought that the similarity was sufficient to be indicative of proteinase inhibition. There was no evidence of any inhibition of larval motility.

None of these specific components appeared to be a strong candidate as the milk inhibitor, therefore, a more general physico-chemical property of the milk proteins should be considered. The pH of the solution did not affect the activity of the casein, but had a marked on the activity of the whey proteins. Casein is completely insoluble in the acid form but is completely soluble above pH 5.5, whereas whey proteins are positively charged below pH 4.6 and negatively charged above this isoelectric point (Morr, 1982; Mulvihill, 1992). This may explain the effect of pH on the anti-parasitic activity. A possible mode of action is adhesion of the protein to the larvae, either to the cuticle or to the gut of the parasite.

The mode of action can only be the subject of speculation as this was not investigated. Not all larvae were affected, the maximum percentage being 86% in 15 g/100 ml whey protein at pH 6.5. In general, more larvae were affected with increasing time of exposure. There were differences in the time course of loss of motility with the different milk products. All four milks were without effect within the first hour, but had caused 10-30% of larvae to be immotile by 24 hours. Whey had significant effects by 2 hours, when the first observations were made, but effects with casein began only after 4 hours, when the full effect was apparent. These differences may reflect the properties of the different proteins and the extent to which they adhere to surfaces.

In summary, these in vitro experiments have demonstrated a direct detrimental effect of milk on the motility of exsheathed third-stage *O. circumcincta* larvae. The number of larvae affected increased as the milk concentration and time of exposure increased. The active fraction appeared to be protein not lipid. Whey and casein proteins also had inhibitory activity, although they did not share the same characteristics with respect to pH and exposure time. It is suggested that the reduction of larval motility may be a non-specific effect of milk proteins, possibly through adhesion to the larvae.

# Chapter 4

# GENERAL DISCUSSION

Earlier studies have shown that milk has anti-parasitic activity in vitro and that milk feeding reduces susceptibility to protozoan and nematode parasites in young animals. The active agents have not been identified. Milk contains Igs and, in addition, there are defence agents which protect against bacterial diseases (discussed in Chapter 1), some of which may prove in the future to be active also against parasites. Milk has been reported to reduce nematode burdens in foals (Leese, 1943), pigs (Spindler et al., 1944; Spindler & Zimmerman, 1944; Shorb & Spindler 1947) and calves (Porter, 1941; Rohrbacher et al., 1958; Satrija et al., 1991), but these studies have not established the mechanism involved. Satrija et al. (1991) concluded that the absence of rumination was the important factor in milk-fed calves, in spite of obtaining a higher worm burden when the calves were given ensheathed than exsheathed larvae. Their exsheathed larvae were effective in an older animal, so were not damaged by the chemical exsheathing.

The present studies were carried out to gain a better understanding of how milk feeding reduces worm establishment in newborn ruminants. There appear to be no previous observations of the effects of milk feeding to sheep. Two approaches have been taken: an in vivo study in which lambs were infected from three weeks of age with third-stage larval *O. circumcincta* and an in vitro examination of direct effects of bovine milk and some of its crude fractions on the motility of *O. circumcincta* larvae. Both experiments confirmed the anti-parasitic effects of milk.

There were highly significantly lower worm burdens at necropsy in the two milk-fed groups of lambs than in both the other groups, but no difference between the burdens in those completely fed solid food and in lambs receiving a milk feed

once a day along with solids. Irrespective of the diet of the lamb, female worms made up about half the total worm population in most lambs, with males and immature stages equally making up the other half. Faecal egg counts in the Milk groups were also very low, three of the eight lambs never providing a faecal sample in which eggs were found. Also consistent with the lower worm burdens were the thinner abomasal mucosa and lower abomasal pH, although these may also have been affected by the diet. Nodules were visible in the abomasa of all lambs in all groups. All groups had increased serum gastrin and pepsinogen levels, with considerable variation between animals within all groups.

An important observation was that there was no significant difference between lambs receiving exsheathed or ensheathed larvae in any parameter measured, even in the lambs in the two Milk groups. In these lambs, the reticulo-rumen and omasum were smaller and more immature than in those lambs receiving solid food. The similar worm burdens in the Milk lambs given ensheathed and exsheathed larvae does not support the conclusions from an earlier study in calves that lack of rumen function was the reason for lower worm burdens in non-ruminating calves (Satrija et al., 1991). This suggests that it is the milk which interferes with parasite establishment in the abomasum. This could be through creating an unfavourable environment for the parasites or by directly damaging the parasites.

It has been suggested that the high pH in the abomasum protects against nematodes in milk-fed calves (Rohrbacher et al., 1958). The pH of bovine milk is usually between pH 6.5 and 6.7 (Jenness et al., 1974), which would raise the abomasal pH immediately after milk ingestion and for some hours afterwards (Rohrbacher et al., 1958). Reduced parasite survival because of the high pH of milk is not consistent with in vitro survival of larvae in HBSS in the present experiments, nor with survival studies with fourth-stage larvae and adult *H. contortus* (Haag, 1995), in which survival was best at higher pH and poor at low pH. In addition, abomasal parasites rapidly inhibit acid secretion (Christie et al.,

1967; Titchen & Anderson, 1977; Anderson et al., 1981, 1985; Lawton et al., 1996; Simpson et al., 1997), presumably because it is not detrimental to their survival.

The direct in vitro effects of milk on larval motility would rather support a direct effect of milk on the parasites. These experiments further indicated that the active component is associated with proteins and not with the lipid fraction. All milk products and crude fractions, including both whey and casein proteins, were inhibitors of the motility of exsheathed third-stage *O. circumcincta* larvae. Igs are part of the whey fraction and could account for at least some of the anti-parasitic activity of the milk fraction, but would only be expected in the casein fraction in contaminating amounts. As the casein was active at about 20% the concentration in whole milk, and the Ig content of mature milk is relatively low, it is unlikely that Ig explains the in vitro effects of milk on larval motility.

The reason that there is anti-parasitic activity in both whey and casein may be because it is a non-specific effect of proteins in general. Alternatively, there may be sufficient in the casein fraction because of the way the proteins are processed, which results in some whey contaminating the casein. The maximum precipitation of bovine casein occurs at pH 4.6, although this method also induces some co-precipitation of whey proteins or non-casein proteins within the caseins (Hambraeus 1982). There were, however, some differences in the experiments with whey and casein proteins. The effect on motility was concentration and time dependent for both proteins, but the effect of pH differed. The activity of whey protein increased as the pH increased; the whey was most active at pH 4.5 and above, when it would be in the anion form (Morr, 1982; Mulvihill, 1992). The effect on larval motility of time of incubation differed for whey and casein and there was no difference at pH 5.5 and 6.5 for casein, whereas there was for whey. Therefore, different components may be responsible for inhibition of larvae by whey and casein proteins.

A possible explanation for the in vitro and in vivo effects of milk is the non-specific attachment of proteins to the larvae rather than the action of specific milk components. If this were the case, the worm burdens would be expected to be lower in the lambs fed a mixed diet compared with the Solid groups. The reason this did not occur may be the timing of the infection and the milk feed. Larvae were given in the morning, whereas the milk was fed in the evening and there was continuous access to solid food. When the larvae were administered, very little milk may have remained in the abomasum or milk proteins may have already attached to the solid food. Another possibility is that the milk may adhere to the large number of micro-organisms in the rumen of the lambs ingesting solid food (Ensiminger et al., 1990) when they pass down to the abomasum.

In conclusion, the experiments conducted in this study have shown that bovine milk suppresses the development of *O. circumcincta* in lambs and also inhibits the motility of *O. circumcincta* larvae in vitro. Determining why the larvae become immotile and identifying the active agents by further purification are both important further experiments to be carried out before the value of milk as an anti-parasitic agent can be assessed. An impediment may prove to be loss of activity in the presence of digesta other than milk, which would limit its value in ruminants consuming solid feed.

# REFERENCES

Anderson, N. (1972). Trichostrongylid infections of sheep in a winter rainfall region I. Epizootiological studies in the western district of Victoria, 1966-1967. Australian Journal of Agricultural Research. 23, 1113-1129.

Anderson, N., Hansky, J.& Titchen, D.A. (1975). Hypergastrinaemia during a parasitic gastritis in sheep. Journal of Physiology. 256, 51–52 P.

Anderson, N., Hansky, J.& Titchen, D.A. (1985). Effects on plasma pepsinogen, gastrin and pancreatic polypeptide of *Ostertagia* spp. transferred directly into the abomasum of sheep. International Journal for Parasitology.15, 159-165.

Anderson, N., Blake, R. & Titchen, D.A. (1976). Effects of a series of infections of *Ostertagia circumcincta* on gastric secretion of sheep. Parasitology. 72, 1-12.

Anderson, N., Hansky, J. & Titchen, D.A. (1981). Effects of *Ostertagia circumcincta* infections on plasma gastrin in sheep. Parasitology. 82, 401-410.

Anderson, N., Reynolds, G.W. & Titchen, D.A. (1988). Changes in gastrointestinal mucosal mass and mucosal and serum gastrin in sheep experimentally infected with *Ostertagia circumcincta.* International Journal for Parasitology. 18, 325-331.

Anderson, R. R. (1992). Comparison of trace elements in milk of four species. Journal of Dairy Science. 75, 3050-3055.

Armour, J., Bairden, K., Duncan, J.L., Jennings, F.W. & Parkings, J.J. (1979). Observations on ostertagiasis in young cattle over two grazing seasons with special reference to plasma pepsinogen levels. The Veterinary Record. 105, 500-503.

Armour, J., Jarrett, W.F.H. & Jennings, F.W. (1966). Experimental *Ostertagia circumcincta* infections in sheep: Development and pathogenesis of a single infection. American Journal of Veterinary Research. 27, 1267-1278.

Axelson, J., Hakanson, R., Rosengren, E. & Sundler, F. (1988). Hypergastrinaemia induced by acid blockade evokes enterochromaffin-like (ECL) cell hyperplasia. Cell Tissue Research. 254, 511-516.

Bado, A., Moizo, L., Laigneau, J-P., Delwaide, J. & Lewin, M.J.M. (1994). $H_3$-receptor regulation of vascular gastrin and somatostatin releases by the isolated rat stomach. Yale Journal of Biology and Medicine. 67, 113-21.

Baumrucker, C. R. & Blum, J. W. (1993). Effects of dietary recombinant human insulin-like growth factor I on concentrations of hormones and growth factors in the blood of newborn calves. The Journal of Endocrinology. 140, 15-21.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K. & Tomita, M. (1992). Identification of the bactericidal domain of lactoferrin. Biochimica et Biophysica Acta. 1121, 130-136.

Berliner, L.J., Koga, K., Nishikawa, H. & Scheffler, J.E. (1987). High resolution proton and laser photochemically induced dynamic nuclear polarization NMR studies of cation binding to bovine α-lactalbumin. Biochemistry. 26, 5769-5775.

Berseth, C.L., Lichtenberger, L.M. & Morriss, F.H. (1983). Comparison of the gastroinestinal growth-promoting effects of rat colostrum and mature milk in newborn rats *in vivo*. American Journal of Clinical Nutrition. 37, 52-60.

Bezault, J., Bhimani, R. & Wiprovnick, J., et al. (1994). Human lactoferrin inhibits growth of solid tumors and development of expeimental metastases in mice. Cancer Research. 54, 2310-2312.

Bitman, J. & Wood, D.L. (1990). Changes in milk phospholipids during lactation. Journal of Dairy Science. 73, 1208-1216.

Blair, A.J., Richardson, C.T., Walsh, J.H. & Feldman, M.(1987). Variable contribution of gastrin to gastric acid secretion after a meal in humans. Gastroenterology. 92, 944-949.

Bounous, G. & Gold, P. (1991). The biological activity of undenatured dietary whey proteins: role of glutathione. Clinical and Investigative Medicine. 14, 296-309.

Bounous, G. Batist, G. & Gold, P. (1991). Whey proteins in cancer prevention. Cancer Letters 57, 91-94.

Bounous, G., Gervais, F., Amer, V., Batist, G. & Gold, P. (1989). The influence of dietary protein on tissue glutathione and the diseases of aging. Clinical and Investigative Medicine. 12, 343-349.

Bounous, G., Kongshavn, P.A.L. & Gold, P. (1988). The immunoenhancing property of dietary whey protein concentrate. Clinical and Investigative Medicine. 11, 271-278.

Bray, R.s. & Garnham, P.C.C. (1953). Effect of milk diet on *P. cynomolgi* infections in monkeys. British Medical Journal. 1, 1200- 1201.

Bremel, R.D., Schultz, L. H. & Gablet, R. & Peters, J. (1977). Estimating somatic cells in milk samples by the membrane-filter-NA procedure. Journal of Food Protection. 40, 32-38.

Bruhn, J.C. & Franke, A.A. (1977). Monthly variations in gross composition of California herd milks. Journal of Dairy Science. 60, 696-700.

Brunsdon, R.V. (1971). Trichostrongyle worm infection in cattle: further studies on problems of diagnosis and on seasonal patterns of occurrence. New Zealand Veterinary Journal. 19, 203-212.

Campana, W.M. & Baumrucker, C.R. (1995). Nitrogenous components of milk. I. Hormones and growth factors in bovine milk. In "Handbook of Milk Composition". Edited by Jensen, R.G. Academic Press. San Diego. pp 476-489.

Casey, C. E., Smith, A. & Zhang, P. (1995). Minerals, ions, and trace elements in milk. C. Microminerals in human and animal milks. In "Handbook of Milk Composition". Edited by Jensen, R.G. Academic Press. San Diego. pp 622-674.

Cerbulis, J. & Farrell, H.M. (1975). Composition of milks of dairy cattle. I. Protein, lactose, and fat contents and distribution of protein fraction. Journal of Dairy Science. 58, 817-827.

Chandan, R.C., Parry, R.M., Shahani, K.M. (1968). Lysozyme, lipase and ribonuclease in milk and various species. Journal of Dairy Science. 51, 606-610.

Christensen, S. & Sottrup-Jensen, L. (1994). Characterization of two serpins from bovine plasma and milk. Biochemistry Journal. 303, 383-390.

Christie, M.G., Brambell, M.R. & Mapes, C.J. (1967). Effects of young *Haemonchus contortus* on abomasal pH in sheep. Veterinary Research. 80, 207-208.

Christie, W.C. (1983). The composition and structure of milk lipids. In "Developments in Dairy Chemistry-2. Lipids". Edited by Fox, P.F. Applied Sciences. New York. pp 1-36.

Church, D.C. & Pond, W.G. (1974). The gastro-intestinal tract and nutrition. In "Basic Animal Nutrition and Feeding". O & B Books. U.S.A. pp 14-26.

Church, D.C. (1969). Anatomy of the ruminant stomach. In "Digestive Physiology and Nutrition of Ruminants. Volume 1 – Digestive Physiology". Church, D.C., U.S.A. pp 5-26.

Cohen, M. Jhinge, M. & Rasmussen, G.T., Serody, J.S. & Britigan, B.E. (1992). Interaction of lactoferrin and lipopolysaccharide (LPS): effect on the antioxidant

property of lactoferrin and the ability of LPS to prime human neutrophils for enhanced superoxide formation. Journal of Infectious Diseases. 166, 1375-1378.

Coop R.L., Sykes A.R.& Angus K.W. (1977). The effect of a daily intake of *Ostertagia circumcincta* larvae on body weight, food intake and concentration of serum constituents in sheep. Research in Veterinary Science. 23, 76-83.

Coop, R.L. & Kyriazakis, I. (1999). Nutrition-parasite interaction. Veterinary Parasitology. 84, 187-204.

Crean, G.P., Marshall, M.W. & Rumsey, R.D.E. (1969). Parietal cell hyperplasia induced by the administration of pentagastrin (ICI 50,123) to rats. Gastroenterology. 57, 147-155.

Crenin, F.M. & Power, P. (1982). Vitamins in bovine and human milks. In "Developments in Dairy Chemistry-3". Edited by Fox, P. F. Elsevier. New York. pp 337-398.

Davidson. L.A. & Lonnerdal, B. (1989). Specificity of the intestinal lactoferrin receptor. In "Milk proteins: nutritional, clinical, functional and technological aspects. Edited by Barth, C.A. & Schlimme, E. Springer-Verlag. New York. pp76-82.

Davies, D.T. & Law, A.J.R. (1980). The content and composition of protein in creamery milks in south west Scotland. Journal of Dairy Research. 47, 83-90.

Dionysius, D.A., Grieve, P.A. & Milne, J.M. (1993). Forms of lactoferrin; their antibacterial effect on enterotoxigenic *Escherichia coli*. Journal of Dairy Science. 76, 2597-2606.

Dolamore, B.A., Brown, J. & George, P.M. (1992). Selenium status of Christchurch infants and the effect of diet. New Zealand Medical Journal. 105, 139-142.

Dorny, P. & Vercruysse, J. (1998). Evaluation of a micro method for the routine determination of serum pepsinogen in cattle. Research in Veterinary Science. 65, 259 - 262.

Dyce, K.M., Sack, W.O. & Wensing, C.J.G. (1987). The abdomen of the ruminants. In "Textbook of Veterinary Anatomy". 1st edition. W.B.Saunders Company. Philadelphia. pp 671-694.

Eigel, W.N., Butler, J.E., Ernsrom, C.A., Farrell, H.M., McL Whitney, R, Harwalkar, V.R. & Jenness, R. (1984). Nomenclature of proteins of cow's milk. Journal of Dairy Science. 67, 1599-1631.

Emerick, R.J., Bemrick, W.J., Pope, A.L., Herrick, C.A. & Phillips, P.H. (1956). Mineral vs. phenothiazine supplementation in the control of stomach worms in grazing sheep. Journal of Animal Parasitology. 15, 1266. (Abstract).

Ensiminger, M.E., Oldfield, J.E. & Heinemann, W.W. (1990). Digestion/absorption. In "Feeds & Nutrition". 2nd edition. The Ensiminger Publishing Company. California. pp 47-65.

Erb, R.E., Chew, B. P. & Keller, H.F. (1977). Relative concentrations of extrogen and progesterone in milk and blood, and excretion of estrogen in urine. Journal of Animal Science.45, 617-626.

Farkye, N.Y. (1992). Other enzymes. In "Advanced Dairy Chemistry. 1. Proteins". Edited by Fox, P.F. Elsevier. London. pp 339-367.

Farrell, H.M., Pessen, H., Brown, E.M. & Kumosinski, J.F. (1990). Structural insights into the bovine casein micelle: Small angle X-ray scattering studies and correlations with spectroscopy. Journal of Dairy Science. 73, 3592-3601.

Faulkner, A., Chaiyabutr, N, & Peaker, M. (1981). Metabolic significance of milk glucose. Journal of Dairy Research. 48, 51-56.

Fournier, B., Sann, L., Guillaumont, M. & Leclercq, M. (1987). Variations of phylloquinone concentration in human milk at various stages of lactation and in cow's milk at various seasons. American Journal of Clinical Nutrition. 45, 551-558.

Fox, M.T. (1993). Pathophysiology of infection with *Ostertagia ostertagi* in cattle. Veterinary Parasitology. 46, 143-58.

Fox, M.T., Carroll, A. P., Hughes, S.A., Uche, U.E., Jacobs, D.E. & Vaillant, C. (1993). Gastrin and gastrin-related responses to infection with Ostertagia ostertagi in the calf. Research in Veterinary Science. 54, 384-391.

Fox, M.T., Gerrelli, D., Shivalkar, P. &, Jacobs, D.E. (1989). Effect of omeprazole treatment on feed intake and blood gastrin and pepsinogen levels in the calf. Research in Veterinary Science. 46, 280-282.

Fox, M.T., Pitt, S.R., Gerrelli, D., Jacobs, D.E., Adhikari, D.R. & Coddard, P.J. (1988). Use of blood gastrin assay in the diagnosis of ovine haemonchosis. Veterinary Research. 122, 136-137.

Gillin, F.D., Reiner, D.S. & Wang, C.S. (1983). Killing of Giardia lamblia Trophzoites by Normal human milk. Journal of Cellular Biochemistry. 23, 47-56.

Godfrey, N.W. (1961). The functional development of the calf II. Development of rumen function in the calf. Journal of Agricultural Science. 57, 177-183.

Guidry, A. J. & Miller, R. H. (1986). Immunoglobulin isotype concentrations in milk as affected by stage of lactation and parity. Journal of Dairy Science. 69, 1799-1805.

Ha, Y.L., Grimm, N. K. & Pariza, M.W. (1989). Newly recognized anticarcinogenic fatty acids: identification and quantification in natural and processed cheese. Journal of Agricultural and Food Chemistry. 37, 75-81.

Haag, E. (1995). The effect of *Haemonchus contortus* excretory/secretory products on abomasal secretion. Dr med vet Thesis. Hannover.

Hakansson, A., Zhivotovsky, B., Orrenius, S., Sabharwal, H. & Svanborg, C. (1995). Apoptosis induced by a human milk protein. Proceedings of the National Academy of Sciences of the USA. 92, 8064-8068.

Hambraeus, L. (1982). Nutritional aspects of milk proteins. In "Developments in Dairy Chemistry. 1. Proteins". Edited by Fox, P. F. Applied Sciences. New York. pp 289-313.

Hansky, J. & Cain, M.D. (1969). Radioimmunoassay of gastrin in human serum. The Lancet. 27, 1388 - 1390.

Hawking, F. (1953). Milk diet, p-aminobenzoic acid, and malaria (*P. berghei*). British Medical Journal. May, 1201.

Hernell, O., Ward, H., Blackberg, L. & Pereira, M. E.A. (1986). Killing of *Giardia lamblia* by human milk lipases: an effect mediated by lipolysis of milk lipids. Journal of Infectious Diseases. 153, 715 - 720.

Hertzberg, H., Lindström, E., Chen, D. & Håkanson, R. (1999). Excretory/secretory products of *Haemonchus contortus* suppress stimulation of parietal cells by inhibiting secretory activity of enterochromaffine-like (ECL) cells. Proceedings 17th International Conference of the WAAVP. Copenhagen. Abstract a.202.

Hidiroglou, M. (1989). Mammary transfer of vitamin E in dairy cows. Journal of Dairy Science. 72, 1067-1071.

Hilderson, H., Dorny, P., Berghen, P., Vercruysse, J., Fransen, J. & Braem, L. (1991). Gastrin and pepsinogen changes during an *Ostertagia ostertagi* challenge infection in calves. Journal of Veterinary Medicine. Series B. 38, 25-32.

Hilderson, H., Vercruysse, J., Berghen, P., Dorny, P. & McKellar, Q.A. (1992). Diagnostic value of gastrin for clinical bovine ostertagiosis. Journal of Veterinary Medicine. Series B. 39, 187-192.

Hill, K.J. (1960). Abomasal secretion in the sheep. Journal of Physiology. 154, 115 – 132.

Hindsbo, O. (1996). Milk controls malaria. Naturens Verden. 5, 194- 199.

Hirschowitz, B.I. (1991). Control of pepsinogen secretion. Current Opinion in Gastroenterology. 7, 863-869.

Holmes P.H. & Maclean, J.M. (1971). The pathophysiology of ovine ostertagiasis: a study of the changes in plasma protein metabolism following single infections. Research in Veterinary Research. 12, 265-271.

Hunter, A.R. & MacKenzie, G. (1982). The pathogenesis of a single challenge dose of *Haemonchus contortus* in lambs under six months of age. Journal of Helminthology. 56, 135-144.

Hutchens, T.W., Rumball, S.V. & Lonnerdal, B. (1994). Lactoferrin: structure and function. Advances in Experimental Medicine and Biology. 357, 1-298.

Hutjens, M. F., Schultz, L. H. & Ward, G. E. (1979). Estimation of somatic cells in milk using membrane filter separation and DNA determination with diphenylamine. Journal of Milk and Food Technology. 33, 227-231.

International Dairy Federation (1991). Significance of the indigenous antimicrobial agents of milk to the dairy industry. Bulletin of the International Dairy Federation. 264, 2-19.

Jacobs, R.L. (1964). Role of p-aminobenzoic acid in *Plasmodium berghei* infection in the mouse. Experimental Parasitology. 15, 213-225.

Jenness, R. (1982). Inter-species comparison of milk proteins. In "Developments in Dairy Chemistry-1. Proteins". Edited by Fox, P.F. Applied Science Publishers. London. pp 87-114.

Jenness, R. (1988). Composition of milk. In "Fundamentals of Dairy Chemistry". Edited by Wong, N. P. 3rd edition. Van Nostrand-Reinhold Company. New York. pp1-38.

Jensen R.G. & Clark, R.M. (1988). Lipid composition and properties. In "Fundamentals of Dairy Chemistry". Edited by Wong, N. P. 3rd edition. Van Nostrand-Reinhold. New York. pp 171-214.

Jensen, R. G. & Newburg, D. S. (1995). Milk lipids. B. Bovine milk lipids. In "Handbook of Milk Composition". Edited by Jensen, R.G. Academic Press. San Diego. 543-573.

Jensen, R. G. (1995). Defense agents in milk. B. Defense agents in bovine milk. In "Handbook of Milk Composition". Edited by Jensen, R.G. Academic Press. San Diego. pp746-748.

Jensen, R. G., Ferris, A. M. & Lammi-Keefe, C. J. (1991). The composition of milk fat. Journal of Dairy Science. 74, 3228-3243.

Jensen, R.G., Ferris, A.M., Lammi-Keefe, G.J., Henderson, R.A. (1990). Lipids of bovine and human milks: A comparison. Journal of Dairy Science. 73, 223-240.

Johnson, A. H. (1974). The compopsition of milk. In "Fundamentals of Dairy Chemistry". Edited by Webb, B. H., Johnson, A. H. & Alford, J. A. 2nd edition. The AVI Publishing Company, Inc. Westport, Connecticut. pp1-57.

Johnson, R., Aures, D. & Yuen, L. (1969). Pentagastrin-induced stimulation of protein synthesis in the gastrointestinal tract. American Journal of Physiology. 217, 251-254.

Kawakami, H., Shinmoto, H., Dosako, S. & Sogo, Y. (1987) One-step isolation of lactoferrin using immobilized monoclonal antibodies. Journal of Dairy Science. 70, 752.

Kay, R.N.B. (1983).Rumen function and physiology. The Veterinary Record. 113, 6-9.

Kitchen, B.J. (1985). Indigenous milk enzumes. In "Developments in dairy chemistry 3. Lactose and minor constituents". Edited by Fox, P.F. Elsevier. London. pp 239-279.

Kovacs, T.O.G., Walsh, J.H., Maxwell, V., Wong, H.C., Azuma, T. & Katt, E. (1989). Gastrin is a major mediator of the gastric phase of acid secretion in dogs: Proof by monoclonal antibody neutralization. Gastroenterology. 97, 1406-1413.

Kreuzer, M. & Kirchgessner, M. (1987). The nutritive defaunation of the rumen in ruminants. Archiv fur Tierernahrung. 37 (6), 489-503.

Kronman, M.J., Sinha, S.K. & Brew, K. (1981). Characteristics of the binding of $Ca^{2+}$ and other divalent metal ions to bovine α-lactalbumin. Journal of Biological Chemistry. 256, 8582-8587.

Kumar, S.N., Stewart, G.L., Steven, W.M. & Seelig, L.L. (1989). Maternal to neonatal transmission of T-cell mediated immunity to Trichinella spiralis during lactation. Immunology. 68, 87-92.

Lawton, D.E.B. (1995). Abomasal secretion in parasitised sheep. PhD Thesis. Massey University.

Lawton, D.E.B., Reynolds, G.W., Hodgkinson, S.M., Pomroy, W.E. & Simpson, H.V. (1996). Infection of sheep with adult and larval *ostertagia circumcincta*: effects on abomasal pH and serum gastrin and pepsinogen. International Journal for Parasitology. 26 (10), 1063 – 1074.

Leese, A.S. (1943). Parasitic aneurism in foal (letter to editor dated sept.26). Veterinary Journal. 99, 276.

Lehmann, F.S., Golodner, E.H., Wang, J., Chen, M.C., Avedian, D., Calam, J., Walsh, J.H., Dubinett, S. & Soll, A.H. (1996). Mononuclear cells and cytokines stimulate gastrin release from canine antral cells in primary culture. American Journal of Physiology. 270, G783-788.

Lima, M.F. & Kierszenbaum, F. (1985). Lactoferrin effects on phagocytic cell function. I. Increased uptake and killing of an intracellular parasite by murine macrophages and human monocytes. Journal of Immunology. 134, 4176-4183.

Lipkin, E., Shalom, M., Khabib, H., Soller, M. & Friedmann, A. (1993). Milk as a source of deoxyribonucleic acid and as a substrate for the polymerase chain reaction. Journal of Dairy Science. 76, 2025-2032.

Lonnerdal, B. & Glazier, C. (1985). Calcium binding by α-lactalbumin in human milk and bovine milk. Journal of Nutrition. 115, 1209-1216.

Maegraith, B.G., Deegan, T., & Jones, E.S. (1952). Suppression of malaria (*P. berghei*) by milk. British Medical Journal. 2, 1382-1384.

Marschke, R. J. & Kitchen, B. J. (1984). Glucose levels in normal and mastitic milk. Journal of Dairy Research. 51, 233-237.

McBean, L.D. & Speckmann, E.W. (1988). Nutritive value of dairy foods. In "Fundamentals of Dairy Chemistry". Edited by Wong, N. P. 3rd edition. Van Nostrand-Reinhold Company. New York. pp 343-407.

McIntosh, G.H., Regester, G.O., Le Leu, R.K., Royle, P.J., Smithers, G.W. (1995). Dairy proteins protect against dimethylhydrazine-induced intestinal cancers in rats. Journal of Nutrition. 125, 809-816.

McKellar, Q., Duncan, J.L., Armour, J. & McWilliam, P. (1986). Response to transplanted adult *Ostertagia ostertagi* in calves. Research in Veterinary Science. 40, 367-371.

McKellar, Q., Duncan, J.L., Armour, J., Lindsay, F.E.F. & McWilliam, P. (1987). Further studies on the response to transplanted adult *Ostertagia ostertagi* in calves. Research in Veterinary Science. 42, 29-34.

McLeay, L.M. & Titchen, D.A. (1975). Gastric, antral and fundic pouch secretion in sheep. Journal of Physiology. 248, 595– 612.

McLeay, L.M. & Titchen, D.A. (1977a). Inhibition of hydrochloric acid and pepsin secretion from gastric pouches by antral pouch acidification in sheep. Journal of Physiology. 273, 707-716.

McLeay, L.M. & Titchen, D.A. (1977b). Acid and pepsin secretion of separated gastric pouches during perfusion of antral pouches with cholinergic stimulating and blocking agents and lignocaine. Journal of Physiology. 264, 215-227.

McLeay, L.M., Anderson, N., Bingley, J.B. & Titchen, D.A. (1973). Effects on abomasal function of *ostertagia circumcincta* infections in sheep. Parasitology. 66, 241 – 257.

Meisterling, E.M. & Dailey, R.A. (1987). Use of concentrations of progesterone and estrodiol-17 β in milk in monitoring postpartum ovarian function in dairy cows. Journal of Dairy Research. 70, 2154-2161.

Meyer, D.H., Kunin, A.S., Maddalena, J. & Meyer, W.L. (1987). Ribonuclease activity and isoenzymes in Raw and Processed cows' milk and infant formulas. Journal of Dairy Science. 70, 1797.

Michel, J. F., Lancaster, M. B. & Hong, C. (1979). The effect of age, acquired resistance, pregnancy and lactation on some reactions of cattle to infection with *Ostertagia ostertagi*. Parasitology. 79, 157-168.

Michel, J.F., Lancaster, M.B., Hong, C. & Berrett, S. (1978). Plasma pepsinogen levels in some experimental infections of *Ostertagia Ostertagi* in cattle. Veterinary Research. 103, 370-373.

Migliore-Samour, D. & Jolles, P. (1988). Casein, A prohormone with an immunomodulating role for the newborn? Experientia. 44, 188-192.

Ministry of Agriculture, Fisheries and Food. (1986). Manual of Veterinary Parasitological Laboratory Techniques. Reference Book 418. London.

Monk, E.L., Erb, R.E. & Mollet, T.A. (1975). Relationships between immunoreactive estrone and estradiol in milk, blood, and urine of dairy cows. Journal of Dairy Science. 58, 34-40.

Morr, C.V. (1982). Functional properties of milk proteins and their use as food ingredients. In "Developments in Dairy Chemistry – 1. Proteins". Edited by Fox, P.F. Applied Science Publishers. London. pp 375-400.

Mulvihill, D.M. (1992). Production, functional properties and utilization of milk protein products. In " Advanced Dairy Chemistry. 1. Proteins". Edited by Fox, P.F. Elsevier Applied Science. London. pp 369-404.

Murray, M., Jennings, F.W. & Armour, J. (1970). Bovine ostertagiasis: structure, function and mode of differentiation of the bovine gastric mucosa and kinetics of the worm loss. Research in Veterinary Science. 11, 417-427.

Murray, M.J., Murray, A.B., Murray, N.J. & Murray, M.B. (1978). Diet and cerebral malaria: the effect of famine and refeeding. American Journal of Clinical Nutrition. 31(1), 57-61.

Musoke, A.J., Williams, J.F., Leid, R.W. & Williams, G.S.F. (1975). The immunological response of the rat to infection with *Taenia Taeniaeformis*. IV. Immunoglobulins involved in passive transfer of resistance from mother to offspring. Immunology. 29, 845.

National Research Council, U. S. A. (1989). Committee on dietary allowances. Fat-soluble vitamins. In "Recommended Dietary Allowances (RDA)". 10th edition. National Academy Press. Washington DC. pp 78-104.

Neville, M.C. (1995). The structure of milk: implications for sampling and storage. C. Sampling and storage of human milk. In "Handbook of Milk Composition". Edited by Jensen, R.G. Academic Press. San Diego. pp64-77.

Newburg, D. S. & Neubauer, S. H. (1995). Carbohydrates in milks: analysis, quantities, and significance. In "Handbook of Milk Composition". Edited by Jensen, R.G. Academic Press. San Diego. pp273-350.

Ng-Kwai-Hang, K. F. & Kroeker, E. M. (1984). Rapid seperation and quantification of major caseins and whey proteins of bovine milk by polyacrylamide gel electrophoresis. Journal of Dairy Science. 67, 3052-3056.

Nicholls, C.D., Hayes, P.R. & Lee, D.L. (1987). Physiological and microbiological changes in the abomasum of sheep infected with large doses of *Haemonchus Contortus*. Journal of Comparative Pathology. 97, 299-308.

Nicholls, C.D., Lee, D.L., Adrian, T.E., Bloom, S.R. & Care, A.D. (1988). Hypergastrinaemia of sheep infected with *Haemonchus contortus*. Research in Veterinary Science. 45, 124-126.

Parkins, J.J. & Holmes, P.H. (1989). Effects of gastrointestinal helminth parasites on ruminant nutrition. Nutrition Research Reviews. 2, 227-46.

Phillipson, A.T. & Ash, R.W. (1965). Physiological mechanisms affecting the flow of digesta in ruminants. In " Physiology of Digestion in the Ruminant". Edited by

Dougherty, R.W., Allen, R.S., Burroughs, W., Jacobson, N.L. & McGilliard, A.D.. Butterworths Inc. Washington. pp 97–107.

Pitt, S.R., Fox, M.T., Gerrelli, D. & Jacobs, D.E. (1988). Blood gastrin and pepsinogen responses to subclinical infection with *Ostertagia ostertagi* in adult dairy cattle. Research in Veterinary Science. 45, 130-131.

Pope, G.S. & Swinburne, J.K. (1980). Reviews of the progress of dairy science: Hormones in milk: their physiological significance and value as diagnostic aids. Journal of Dairy Research. 47, 427-449.

Porter, D.A. (1941). The effect of milk diet on the development of Haemonchus Contortus in calves. Journal of Parasitology. 27(supplement), 18-19. (Abstract).

Precetti, A.S., Oria, M.P. & Nielsen, S.S. (1997). Presence in bovine milk of two protease inhibitors of the plasmin system. Journal of Dairy science. 80, 1490-1496.

Purewal, A., Fox, M.T., Shivalkar, P., Carroll, A.P., Uche, U.E., Vaillant, C. & Watkinson, A. (1997). Effects of *Ostertagia ostertagi* on gastrin gene expression and gastrin-related responses in the calf. Journal of Physiology. 498, 809-816.

Quarterman, J. (1982). Milk, lactoferrin and heavy-metal absorption. Proceedings of the Nutrition Society. 40, 45A.

Rahman, W.A. & Collins, G.H. (1991). Infection of goats with *Haemonchus contortus* and *Trichostrongylus colubriformis*: histopathology and pH changes. British Veterinary Journal. 147, 569-574.

Rainard, P. (1986). Bacteriostatic activity of bovine milk lactoferrin against mastitic bacteria. Veterinary Microbiology. 11, 387-392.

Reid, J.F.S. & Armour, J. (1975). Seasonal variations in the gastro-intestinal nematode populations of Scottish hill sheep. Research in Veterinary Science. 18, 307-313.

Reineccius, G. A., Kavanagh, T. E. & Keeney, P. G. (1970). Identification and quantitation of free neutral carbohydrates in milk products by gas-liquid chromatography and mass spectrometry. Journal of Dairy Science. 53, 1018-1022.

Reiner, D.S., wang, C.S. & Gillin, F.D. (1986). Human milk kills *Giardia lamblia* by generating toxic lipolytic products. The Journal of Infectious Diseases. 154, 825-833.

Reiter, B. (1985). The biological significance and exploitation of the non-immunoglobulin protective proteins in milk: lysozyme, lactoferrin, lactoperoxidase, xanthineoxidase. Bulletin International Dairy Federation. 191, 3-35.

Renner, E. (1983). Milk and Dairy Products in Human Nutrition. W-GmbH, Volkswirtschaftlicher Verlag. Munchen. pp 190-256.

Renner, E., Schaafsma, G. & Scott, K.J. (1989). Micronutrients in milk. In "Micronutrients in Milk and Milk-Based Products". Edited by Renner, E. Elsevier. New York. pp1-70.

Reynolds, G.W., Hansky, J. & Titchen, D.A. (1980). Effects of vagotomy on serum gastrin levels and gastric secretion during insulin hypoglycaemia in the sheep. New Zealand Medical Journal. 10, 444.

Ritchie, J.D.S., Anderson, N., Armour, J., Jarrett, W.F.H., Jennings, F.W. & Urquhart, G.M. (1966). Experimental Ostertagia ostertagi infections in calves; parasitology and pathogenesis of a single infection. American Journal of Veterinary Research. 27 (118), 659-667.

Rogers, W.P. & Sommerville, R.I. (1968). The infectious process and its relationship to development of early parasitic stages of nematodes. Advances in Parasitology. 6, 327-348.

Rohrbacher, G.H., Porter, D.A. & Herlich, H. (1958). The effect of milk in the diet of calves and rabbits upon the development of Trichostronglylid nematods. American Journal of Veterinary Research. July, 625-631.

Rohrer, L., Winterhalter, K.H., Eckert, J & Kohler, P. (1986). Killing of Giardia lamblia by human milk is mediated by unsaturated fatty acids. Antimicrobia Agents and Chemotherapy. Aug. 254-257.

Ross, M.J., Romrell, L.J. & Kaye, G.I. (1995). Digestive system II: esophagus and gastrointestinal tract. In "Histology, A Text and Atlas". 3rd edition. Williams & Wilkins. Baltimore. pp 440-495.

Rowe, J.B., Nolan, J.V., Chaneet, G.D.E. & Teleni, E. (1988). The effect of haemonchosis and blood loss into the abomasum on digestion in sheep. British Journal of Nutrition. 59, 125-139.

Saito, H., Miyakawa, H., Tamura, Y., Shimamura, S. & Tomita, M. (1991). Potent bactericidal activity of bovine lactoferrin hydrolysate produced by heat treatment at acidic pH. Journal of Dairy Science. 74, 3724-3730.

Satrija, F., Nansen, P. & Christensen, C.M. (1991). Ostertagia ostertagi in neonatal calves: establishment of infection in ruminating and non-ruminating calves. Research in Veterinary Science. 51, 344-346.

Schmekel, J. & Harnulv, G. (1986). Activation of the Lactoperoxidase as a means of saving milk in tropical countries for early spoilage. In "Antimicrobial Systems in Milk". International Dairy Federation. pp75-79.

Schubert, M.L. (1996). Gastric exocrine and endocrine secretion. Current Opinion in Gastroenterology. 12, 493-502.

Schubert, M.L. (1997). Regulation of gastric secretion. Current Opinion in Gastroenterology. 13, 441-450.

Scott, I., Hodgkinson, S.M., Khalaf, S., Lawton, D.E.B., Collett, M.G., Reynolds, G.W., Pomroy, W.E. & Simpson, H.V. (1998). Infection of sheep with adult and larval *ostertagia circumcincta*: abomasal morphology. International Journal for Parasitology. 28, 1383– 1392.

Scott, I., Khalaf, S., Simcock, D.C., Knight, C.G, Reynolds, G.W., Pomroy, W.E. & Simpson, H.V. (2000). A sequential study of the pathology associated with the infection of sheep with adult and larval *Ostertagia circumcincta*. Veterinary Parasitology. (in press)

Scott, K.J. (1989). Micronutrients in milk products. In "Micronutrients in Milk and Milk-Based Products". Edited by Renner, E. Elsevier. New York. pp71-124.

Scott, K.J., Bishop, D.R. & Zechalko, A.. (1984). Nutrient content of liquid milk I. Vitamins A, D-3, C and of the B complex in pasteurized bulk liquid milk. Journal of Dairy Research. 51, 37-50.

Shahani, K., Harper, W., Jensen, R., Perry, R. & Zittle, C. (1973). Enzymes in bovine milk: A review. Journal of Dairy Science. 56, 531-543.

Shoo, M.K. & Wiseman, A. (1986). Changes in serum pepsinogen and haemoglobin concentrations in calves infected with *Haemonchus contortus*. Research Veterinary Science. 41, 124-125.

Shorb, D.A. & Spindler, L.A. (1947). Growth rate of pigs fed skim milk to control intestinal parasites. Proceedings of the Helminthological Society of Washington. 14 (1), 30-34.

Simcock, D.C., Joblin, K.N., Scott, I., Burgess, D.M., Rogers, C.W., Pomroy, W.E. & Simpson, H.V. (1999). Hypergastrinaemia, abomasal bacterial population densities and pH in sheep infected with *Ostertagia circumcincta*. International Journal for Parasitology. 29, 1053–1063.

Simpson, H.V., Lawton, D.E.B., Simcock, D.C., Reynolds, G.W. & Pomroy, W.E. (1997). Effects of adult and larval *Haemonchus contortus* on abomasal secretion. International Journal for Parasitology. 27 (7), 825-831.

Simpson. H.V., Reynolds, G.W. & Carr, D.H. (1993). Low tissue gastrin content in the ovine distal duodenum is associated with increased percentage of G34. Comparative Biochemistry and Physiology. 104A, 461-468.

Smith, H.V. & Herbert, I.V. (1976). The passive transfer of humoral immunity from sows infected with Hyostrongylus rubidus (Hassal and Stiles, 1892), the red stomach worm, to their offspring and its significance in the conferring of protective immunity. Immunity. 31, 213-219.

Sommerville R.I. (1953). Development of Ostertagia circumcincta in the abomasal mucosa of the sheep. Nature. 171, 482-483.

Sommerville R.I. (1954). The histrotrophic phase of the nematode parasite *Ostertagia circumcincta.* Australian Journal of Agricultural Research. 5, 130-144.

Spindler, L.A. & Zimmerman, H.E. (1944). Effect of skim milk on the growth and acquisition of parasites by pigs under conditions of constant exposure to infection. Proceedings of the Helminthological Society of Washington. 11 (2), 49-54.

Spindler, L.A., Zimmerman, H.E. & Hill, C.H. (1944). Preliminary observations of the control of worm parasites in swine by the use of skim milk. Proceedings of the Helminthological Society of Washington. 11 (1), 9-12.

Sporn, M. B. & Roberts, A. B. (1985). Introduction: Autocrine, paracrine and endocrine mechanisms of mechanisms of growth control. Cancer Surveys. 4, 627-632.

Stager, S., Gottstein, B., Sager, H., Jungi, T.W. & Muller, N. (1998). Influence of antibodies in mother's milk on antigenic variation of *Giardia lamblia* in the murine mother's offspring model of infection. Infection and Immunity. 66 (4), 1287-1292.

Stoll, N.R. (1943). The wandering of *Haemonchus contortus* in the sheep. Journal of Parasitology. 29, 407-416.

Stringfellow, F. & Madden, P.A. (1979). Effects of Ostertagia ostertagi on pepsinogen granules of chief cells from calf abomasa correlated with selected plasma and abomasal proteins. Proceedings of the Helminthological Society of Washington. 46, 233-239.

Swaisgood, H.E. (1995). Nitrogenous components of milk. F. Protein and amino acid composition of bovine milk. In "Handbook of Milk Composition". Edited by Jensen, R.G. Academic Press. San Diego. 464-468.

Sykes, A.R. & Coop, R.L. (1977). Intake and utilization of food by growing sheep with abomasal damage caused by daily dosing with *ostertagia circumcincta* larvae. Journal of Agricultural Science. 88, 671–677.

Teraguchi, S., Ozawa, K., Yasuda, S., Fukuwatari, Y. & Shimamura, S. (1993). Effects of orally administered bovine lactoferrin on the faecal Enterobacteriaceae of SPF mice fed milk. Bioscience, Biotechnology, And Biochemistry. 57, 360-361.

Thomas, R.J. & Waller, P.J. (1975). Significance of serum pepsinogen and abomasal pH levels in a field infection of *Ostertagia circumcincta* in lambs. Veterinary Research. 97, 468-471.

Titchen, D.A. & Anderson, N. (1977). Aspects of the physio-pathology of parasitic gastritis in the sheep. Australian Veterinary Journal. 53, 369-373.

Tomita, M., Bellamy, W. & Takase, M., et al. (1991). Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. Journal of Dairy Science. 74, 4137-4142.

Van Buren, C.T., Kulkarni, A.D., Fanslow, W.C. & Rudolph, F.B. (1985). Dietary nucleotides: A requirement for helper/inducer T lymphocytes. Transplantation. 40, 694-697.

Wakabayashi, H., Bellamy, W., Takase, M. & Tomita, M. (1992). Inhibition and inactivation of Listeria monocytogenes by lactoferricin, a potent antimicrobial peptide derived from cow's milk. Journal of Food Protection. 55, 238-240.

Weigert, N., Schaffer, K., Schusdziarra, V., Classen, M. & Schepp, W. (1996). Gastrin secretion from primary cultures of rabbit antral G cells: stimulation by inflammatory cytokines. Gastroenterology. 110, 147-54.

Wiggin, C.J. & Gibbs, H.C. (1987). Pathogenesis of simulated natural infections with *Ostertagia ostertagi* in calves. American Journal of Veterinary Research. 48, 274-280.

Woolard, D. C. & Indyk, H. (1989). The distribution of retinyl esters in milks and milk products. Journal of Micronutrient Analysis. 5, 35-52.

Xiao, L., Gibbs, H.C. & Yang, C. (1991). Pathophysiologic effects of *Ostertagia ostertagi* in calves and their prevention by strategic anthelmintic treatments. American Journal of Veterinary Research. 52, 1706-1711.