Chapter 1

General introduction

Sheep and cattle are a major source of meat, milk and clothing for mankind. They are widely distributed throughout the world and can use a range of feeds, including temperate and tropical vegetation. Although most of the production systems are based on pasture and forage, grain-based diets are used to feed sheep and cattle and cereal grains are an important source of feed for sheep and cattle in Australia (Rowe and Pethick, 1994). Historically, the main use of cereal grain has been as a feed supplement during periods of drought or seasonal pasture shortage. This pattern has been changing with the rapid expansion of the beef cattle feedlot industry over the past decade (Feedlot Advisory Unit, 1990). Currently, some 7.4 million beef cattle are slaughtered annually in Australia, of these approximately 12.5 per cent are grain fed. This equates to approximately 922,000 head of feedlot beef cattle slaughtered annually (Meppem *et al.*, unpublished data).

In many parts of Australia cereal grains are a cheaper source of digestible energy than hay or silage, and are easier to store and handle. Cereal grains also offer the potential for greater intake of energy and higher levels of production than is achievable on forage-based diets (Rowe and Pethick, 1994). However, the usefulness of grains for feeding ruminants is restricted by the major problem associated with the rapid fermentation of grain starch, which may lead to the accumulation of lactic acid in the gut and reduce rumen pH to a point where health and productivity are affected (Braun *et al.*, 1992). This is known as lactic acidosis. Lactic acidosis can adversely affect animal production by reducing feed intake and growth rate as well as

increasing mortality and morbidity (Newbold and Wallace, 1988; Feedlot Advisory Unit, 1990; Braun *et al.*, 1992). For example, it has been estimated that the annual cost of lactic acidosis to the Australian Feedlot Industry is currently more than $9,441,280 (Meppem *et al.*, unpublished data). Details relating to the onset and clinical signs of lactic acidosis and the practical limitations of techniques for controlling the problem will be reviewed in Chapter 2.

Lactic acidosis is due to the over (or excessive) production of lactic acid, which is caused by lactic acid-producing bacteria *S. bovis* or a combination of *S. bovis* and *Lactobacillus* (reviewed in Chapter 2) when large quantities of soluble carbohydrates are present in the rumen (Ahrens, 1967; Braun *et al.*, 1992). Lactic acidosis is a condition in which excessive production is coupled with decreasesd rate of fermentation. Current practices to reduce the risk of lactic acidosis in livestock centre around management techniques based on introducing grain gradually to animals (Schwartz and Gilchrist, 1974; Mackie et al, 1978; Feedlot Advisory Unit, 1990). However, the procedure is time-consuming and expensive because it is involves numerous changes to the diet, frequent feeding and close monitoring of daily feed intake. Even with these methods the rations must be carefully formulated and there are still risks of lactic acidosis and poor animal performance. Feed additives and several other methods have been studied and used in conjunction with management practices to control lactic acidosis (reviewed in Chapter 2). At present, unfortunately, none of these feed additives and methods are considered satisfactory for application in livestock industries. For example, a couple of antibiotics are effective in preventing lactic acidosis by reducing the population of *S. bovis* and *Lactobacillus* thereby controlling the over production of lactic acid (Dennis *et al.*, 1981; Nagaraja *et al.*, 1981 and 1987; Tung and Kung, 1993; Thorniley *et al.*, 1996), but the use of antibiotics may be limited in practical situations due to the potential for emergence of drug-resistant microbial strains and the risk of antibiotic residues in animal products.

Immunisation enhances a specific immune response against the target micro-organisms (Humphrey and White, 1970), and is a sustainable method for preventing bacterial infections in livestock industries. Therefore, a practical alternative to the current methods may be immunisation against the lactic acid-producing bacteria, *S. bovis* or a combination of *S. bovis* and *Lactobacillus*, which are primarily

responsible for lactic acidosis. Gnanasampanthan (1993) has successfully demonstrated the feasibility of controlling rumen protozoa by an immunisation strategy, and has suggested that inducing a salivary antibody response is critical for any attempt to establish immunological control of rumen micro-organisms. Thus, the potential exists for developing a vaccine to reduce the risk of lactic acidosis by inducing salivary antibody responses against the *S. bovis* or *S. bovis* and *Lactobacillus*.

In summary: (1) lactic acidosis is due to the over production of lactic acid, (2) the over production of lactic acid can be reduced by antibiotics active against *S. bovis* or *S. bovis* and *Lactobacillus*, (3) it is possible to develop an immunisation strategy against *S. bovis* or *S. bovis* and *Lactobacillus*. Accordingly, the hypothesis of immunisation against lactic acidosis examined in this thesis is formulated as follows. Immunisation may be able to induce the salivary antibody responses against *S. bovis* or *S. bovis* and *Lactobacillus*, and thus reduce the over production of lactic acid in the rumen, when sheep and cattle are fed grain containing high levels of starch.

The literature relating to lactic acidosis and antibody responses is reviewed in Chapter 2. On the basis of the review of the literature, a series of experiments were designed to test the hypothesis and investigate several important factors for developing an immunisation strategy against lactic acidosis in sheep and cattle.

Chapter 2

Review of the literature

This chapter deals with the background knowledge of ruminant digestion, lactic acidosis, antibody-mediated immunity, and some important factors influencing specific immune responses.

2.1 Ruminant digestion

2.1.1 Digestive tract

Ruminants are a most developed and specialised group of herbivores able to use fibre and other carbohydrates unavailable to non-ruminant digestion (Van Soest, 1994). The ruminant digestive tract has evolved to handle large amounts of roughage through thousands of years. The ability to digest vegetation is conferred by the unique digestive tract, in particular, the rumen (Sutton, 1980).

The digestive tract of ruminants is mainly composed of: (1) the rumen, and (2) the other part of the gastrointestinal system, which includes the small intestine, caecum, colon and rectum. The rumen distinguishes the ruminants from other non ruminant herbivores. The reticulorumen volume is 9-13% of the whole body in both sheep and cattle, while the volume of any other part of the gastrointestinal system is not more than 2.3% (Table 2.1) (Parra, 1978). The volume of the colon and rectum of horse represents 8.8% of the whole body. Figure 2.1 illustrates the digestive tracts of sheep (ruminant) and horse (non ruminant) (Stevens, 1988). The rumen is the

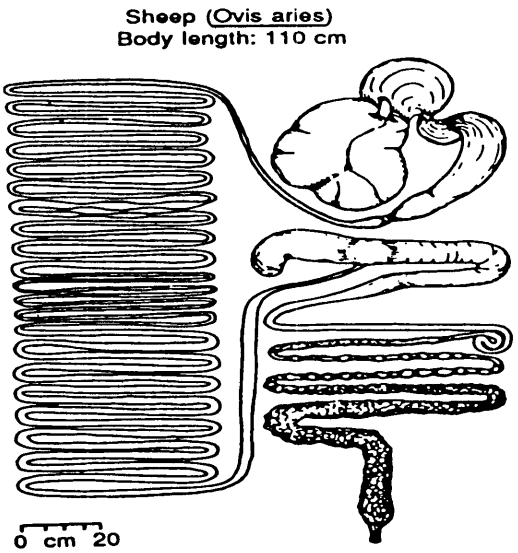
biggest part of the digestive tract of sheep, while the hind gut of the horse is the major part of the digestive tract. These large compartments support a dense and diverse population of microbes which ferment the feed, and the large size also allows for retention of material to facilitate the break down of fibres through fermentation (Parra, 1978; Stevens, 1988).

Table 2.1 Volume (weight) of various parts of the gastrointestinal tract of sheep, cattle (ruminant), and horse (non ruminant) expressed as a percentage of total body weight (from Parra, 1978)

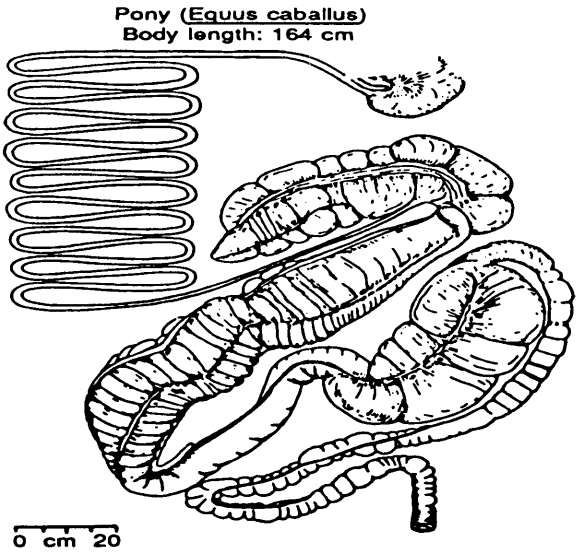
Species	Total contents (%)	Reticulorumen (%)	Omasum (%)	Abomasum (%)	Small intestine (%)	Caecum (%)	Colon and rectum (%)
Sheep	12-19	9-13	0.1-0.3	0.7-1.6	1.0-1.6	0.9-1.6	0.5-0.7
Cattle	13-18	9-13	1.1-2.8	0.5	0.9-2.3	0.8	0.8-1.5
Horse	16.4	-	-	1.3	2.6	2.4	8.8

2.1.2 Digestion and rumen microbes

Fibrous carbohydrates must be digested by gut micro-organisms in all higher animals that have not evolved cellulases, hemicellulases, or pectinases (Prins and Clarke, 1980; Sutton, 1980). The ruminant differs from other mammals in that its food is subjected to microbial fermentation in the rumen before it passes on to the true stomach and intestinal tract where normal mammalian digestion occurs (Figures 2.1 and 2.2) (Van Soest, 1994). For example, it is different to the horse which allows hind gut fermentation. The major benefit of ruminant digestion is that microbial protein synthesised in the rumen can be digested and absorbed from the small intestine, but the microbial protein produced in the hind gut is excreted in the faeces (Prins and Clarke, 1980; Sutton, 1980; Van Soest, 1994).



A) Digestive tract of sheep



B) Digestive tract of horse

Figure 2.1 The digestive tract of sheep (ruminant) and horse (non-ruminant) illustrating the large fermentation compartments of the rumen in the sheep and the hind gut of the horse (from Stevens, 1988)

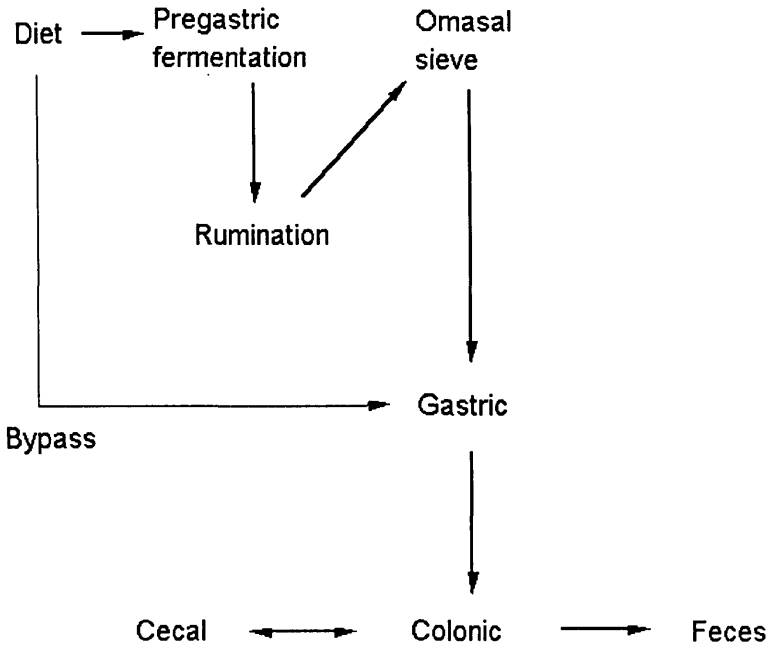


Figure 2.2 Sequence of digestion in ruminants (from Van Soest, 1994)

The microbes that inhabit the gastrointestinal tract of herbivorous vertebrates are the main agents for the digestion of complex carbohydrates in ingested plant material, and the microbial fermentation is critical to the digestion (Prins and Clarke, 1980; Sutton, 1980). In ruminants, most (around 70%) of the fermentation is performed in the rumen, which is structurally the largest part of the digestive tract, and around 10% occurs in the hind gut (Parra, 1978; see also Table 2.1 and Figure 2.1). This compares with around 70% of the total fermentative digestion which occurs in the hind gut of the horse (Parra, 1978).

The rumen microbes mainly include bacteria, protozoa, and anaerobic fungi. The relative numbers of rumen microbes vary depending on the diets. The very large

numbers of bacteria in the rumen (up to 10^{11} viable cells/ml) have been described by Hungate (1966). Although many species of bacteria are present in the rumen, "authentic" ruminal bacteria were found to consist of only about thirty species (Ogimoto and Imai, 1981). The criteria for "authentic" ruminal bacteria are that the bacteria can be shown to grow in the rumen and that they have a metabolism compatible with the reactions occurring and the environment present in the rumen. In addition to the bacteria in the rumen there are many larger (5-250 μm long) organisms, protozoa (Williams and Coleman, 1988). The rumen protozoa are divided into flagellates and ciliates, but almost all of them are ciliate protozoa (Ogimoto and Imai, 1981; Williams, 1986). Generally, these ciliates are non-pathogenic and anaerobic. The number of the ciliates range from 10^4 to 10^6 per millilitre of rumen contents in healthy animals (Ogimoto and Imai, 1981; Shu and Liu, 1995a). Yeasts and aerobic fungi have long been known to be normal inhabitants of the rumen but most species isolated are considered to be transient and non-functional under normal conditions, entering the rumen with the feed (Orpin and Joblin, 1988). However, some aerobic fungi are capable of growth under anaerobic conditions, and two other groups of fungi are now known to occur, one group parasitic upon ciliate protozoa, the other saprophytic on plant tissues (Ogimoto and Imai, 1981; Williams and Coleman, 1988).

The microbial population in the rumen is maintained in balance by the frequent introduction of nutrients and by the physiological regulation provided by the animal (nutrient absorption, flow of saliva and flow out of the rumen) (Dawson and Allison, 1988; Wolin and Miller, 1988). However, sudden changes in diet, or excessive intake of certain types of nutrients, can disrupt the normal microbial balance in the rumen and be detrimental to normal rumen function and to the health of the host animal (Dawson and Allison, 1988). During normal grazing conditions, the environment of the rumen is favourable for the maintenance of a large and diverse microbial population and its normal function to satisfy the nutrition requirements (Wolin and Miller, 1988). There is a relatively constant supply of feed (or substrate) and water. The temperature is held relatively constant at about 39°C. The pH of the rumen contents, usually slightly acidic (6.5 to 7.0) for pasture-fed cattle, is held relatively constant by the intake of food, water, heavily buffered saliva, and the absorption of volatile fatty acids (VFA) and the apparent tendency toward an

equilibrium between the rumen ingesta and the blood stream with regard to H ions (Dawson and Allison, 1988; Wolin and Miller, 1988; Van Nevel and Demeyer, 1988; Wolin and Miller, 1988). There is also constant removal of the other products of microbial reaction and growth via secondary fermentations, absorption through the rumen wall into the blood stream, and passage to the lower digestive tract (Prins and Clarke, 1980; Sutton, 1980; Van Nevel and Demeyer, 1988).

2.2 Fermentative lactic acidosis and its causative bacteria

2.2.1 Lactic acidosis and *S. bovis* and *Lactobacillus*

Lactic acidosis is the term commonly used to describe one of the major digestive disorders in ruminants, and the risk due to lactic acidosis to livestock industries is widely recognised. Lactic acidosis is mainly associated with high energy diets usually involving grain feeding systems. The acute condition is also known as "grain engorgement" or "grain overload" (Dawson and Allison, 1988). Lactic acidosis occurs when ruminants are overfed, or abruptly changed to, diets that contain large amounts of starch or other rapidly fermented carbohydrates.

The introduction of a large amount of easily fermentable carbohydrates into the rumen results in an increase in acid production in the rumen (Slyter, 1976). During the first few hours, the availability of excess fermentable carbohydrates causes a general increase in the growth rate of all rumen bacteria, and the production of VFA increases and causes a fall in rumen pH (Newbold and Wallace, 1988). At the same time, more lactic acid is produced which can be related to the increase in growth rate of bacteria. For example, at a low growth rate acetate and ethanol are the main products of *S. bovis* but at a high growth rate it produces lactic acid (Russell and Hino, 1985). In addition to regulation due to changes in growth rate, the lower pH in rumen environment also serves to shift the metabolism of *S. bovis* toward lactate by altering cellular regulation of lactate dehydrogenase (Dawson and Allison, 1988). In microbial cells, lactate production is advantageous when excess substrate is available, because in this situation oxidation of reduced nicotinamide adenine dinucleotide (NAD) and protection from the accumulation of excess acid (H^+ ions) inside the cell are particularly important (Dawson and Allison, 1988). The increase

in lactic acid production further decreases the rumen pH, which allows the acid tolerant *S. bovis* to proliferate which results in further lactic acid production.

Lactic acidosis occurs if the animal cannot remove the acid from the rumen and cope with the over (or excessive) production of lactic acid. Lactic acid is a stronger acid (pKa 3.1) than the volatile fatty acids (VFA) produced normally (average pKa 4.8) and is readily accumulated to reduce the rumen pH below 5.5 (Dawson and Allison, 1988; Newbold and Wallace, 1988). A pH of about 5.5 seems to represent the tolerance limit for many of the rumen microbial species, but the acid-tolerant species (*S. bovis* and *Lactobacillus*) will continue to grow in more acidic environments (Dawson and Allison, 1988). *S. bovis* is capable of very rapid growth, with population doubling times ranging from 21 minutes (Stewart, 1975) to between 24 and 27 minutes (Russell and Robinson, 1984) being recorded. Therefore, the decreased rumen pH enables *S. bovis* to outgrow other rumen bacteria on the excess substrate that is present, and its tolerance of low pH allows this organism to proliferate which leads to a further increase in lactic acid production. The increased lactate concentration progressively depresses rumen pH and inhibits the growth of other rumen bacteria. Thus *S. bovis* establishes a self-perpetuating cycle of lactic acid production and rapidly builds up as a major component of the rumen flora (Dawson and Allison, 1988). For example, Allison *et al.* (1975) and Shu and Liu (1995a) demonstrated that the number of rumen *S. bovis* increased around 1,000 fold after animals were fed with a large amount of wheat grain within 48 hours (Figure 2.3).

The rapid build up of *S. bovis* and the increase in the production of lactic acid is also accompanied by an increase in the number of *Lactobacillus* (Allison *et al.*, 1975; Shu and Liu, 1995a). The diverse species of bacteria normally found in the rumen are replaced by *S. bovis* and *Lactobacillus* (Bryant *et al.*, 1958; Newbold and Wallace, 1988). As *S. bovis* has an optimum pH>5.0 (Sims, 1964), a further drop of pH (such as pH<5.0) can inhibit the growth of *S. bovis* and the very acid tolerant lactate-producing *Lactobacillus* predominates (Mann, 1970; Jayne-Williams, 1979; Newbold and Wallace, 1988). At extremely low pH conditions a population that is almost a monoculture of *Lactobacillus* can develop (Dawson and Allison, 1988). However, the information from the current literature does not clearly identify the role of *Lactobacillus* at the initial stage of the development of lactic acidosis. Could

lactic acidosis occur if there were no *S. bovis* in the rumen? This question remains to be answered by future work.

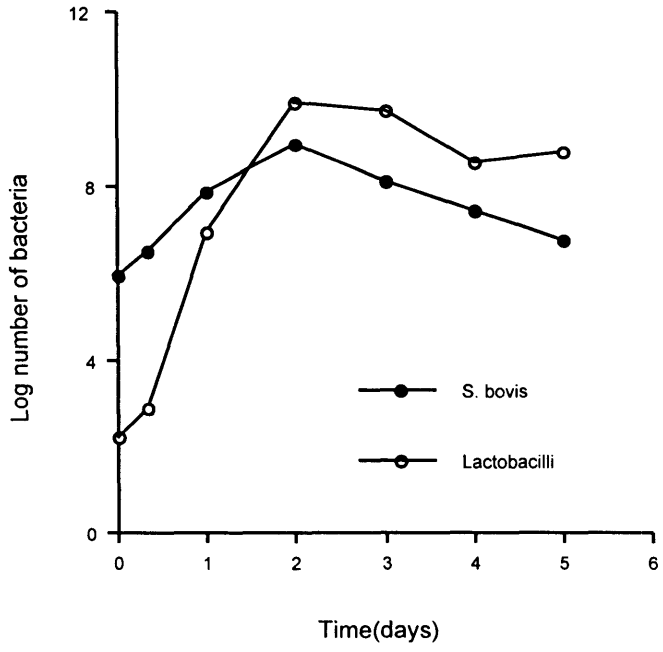


Figure 2.3 Changes in numbers of rumen *S. bovis* and *Lactobacillus* (lactobacilli) (CFU/ml) in sheep after overfeeding with wheat. Grain overfeeding was started on Day 0 (from Shu and Liu, 1995a).

Both D(-) and L(-) isomers of lactic acid are produced by the micro-organisms in the rumen (Ogimoto and Imai, 1981). The over production of lactic acid and the resultant low pH destroys the rumen function (Ahrens, 1967). As lactic acid accumulates the physical and chemical composition of the rumen contents changes and the production of volatile fatty acids is greatly reduced; the large quantities of lactic acid increase the osmolality of the rumen contents and intravascular and interstitial fluid is drawn into the rumen; the rumen contents also became watery, milky-grey in colour, and have a nauseous, potent, sour odour (Ahrens, 1967; Braun *et al.*, 1992). Enzymes for oxidation of L-lactate (L-lactate dehydrogenase) are widely distributed in the cytosol of all host-animal tissues (Dawson and Allison, 1988). The D-lactate must pass the mitochondrial membrane before it can be

oxidized by the D-2-hydroxy-acid dehydrogenase and it is more slowly degraded in the body (Giesecke and Stangassinger, 1980). Absorption of D- and L-lactic acids into the bloodstream leads to metabolic lactic acidosis (Braun *et al.*, 1992). Haemoconcentration, resulting from the loss of intravascular fluid into the rumen and from osmotic diarrhoea, is a characteristic finding in animals suffering from ruminal lactic acidosis. Haemoconcentration results in decreased renal blood flow, and pre-renal uraemia characterised by increased blood urea nitrogen and phosphorus concentrations develops. Blood glucose and pyruvate concentrations also increase because the absorbed lactic acid is used for gluconeogenesis (Giesecke and Stangassinger, 1980).

In addition to lactic acid, toxic substances such as histamine, tyramine and tryptamine may be absorbed from the rumen and contribute to the clinical signs and the release of endotoxins from normal rumen bacteria when they are exposed to the low pH has also been implicated in this condition (Dawson and Allison, 1988). However, it seems clear that the marked change in rumen pH and lactic acid concentration are of major importance (Braun *et al.*, 1992).

The clinical signs of lactic acidosis vary depending on the severity of the disease (Braun *et al.*, 1992). The condition of lactic acidosis can be acute, posing a life-threatening situation, or chronic (sub-acute), resulting in reduced feed consumption and weight gain. Generally gross symptoms of lactic acidosis include poor feed intake, diarrhoea, a listless or depressed or distressed appearance, founder or sore feet and death. Other symptoms that can be measured or observed after the onset of lactic acidosis include high incidence of abscessed livers and/or rumenitis at slaughter, altered blood metabolic profile and incidence of polioencephalomalacia (Kanoe *et al.*, 1976; Dawson and Allison, 1988).

In conclusion, although the current literature does not clearly identify the role played by the *Lactobacillus* at the initial stage of the development of lactic acidosis, it is clear that lactic acidosis is due to excessive production of lactic acid in the rumen, and that the over production of lactic acid can be attributed to *S. bovis* or *S. bovis* and *Lactobacillus* which proliferate under the acidic conditions associated with grain feeding (or high level of soluble carbohydrates).

2.2.2 Diversity of *S. bovis* and *Lactobacillus*

2.2.2.1 *S. bovis*

S. bovis is sometimes considered to be a facultative anaerobic streptococcus, although both strictly anaerobic and aerotolerant strains are found in the alimentary tract of ruminants (Latham *et al.*, 1979; Stewart and Bryant, 1988). *S. bovis* cells are gram-positive, non-motile, ovoid to coccal (0.8-1.5 μm) in shape, and occur in pairs, chains or in long chains when grown in broth (Ogimoto and Imai, 1981). Large capsules surround the cells of *S. bovis* in the rumen of animals. Although *S. bovis* has a high capacity for fermenting starch, it is also able to grow on water-soluble cellodextrins derived from crystalline cellulose (Russell, 1985; Shu and Liu, 1995a). Thus *S. bovis* is able to survive in the rumen of animals fed on forage (Hungate, 1966) since it can ferment a variety of substrates with the major product being lactate (L- and D-) and minor products being formate, acetate, ethanol, and CO_2 (Stewart and Bryant, 1988). Fermentation characteristics of *S. bovis* are summarised in Table 2.2. The fermentation of starch, which leads to the excessive production of lactic acid, is one of the main characteristics of *S. bovis* of interest in terms of the lactic acidosis. The utilization of starch is mainly dependent on the activity of amylase in *S. bovis* (Paje *et al.*, 1986; Cotta, 1988; McAllister *et al.*, 1990). The amylase activities of four *S. bovis* strains were studied by Paje et al (1986) and differences in the amylase activities between strains were found (Table 2.3).

Table 2.2 Fermentation characteristics of substrates of rumen *S. bovis* strains (from Stewart and Bryant, 1988)

Substrate	Fermentation of all the test strains	Fermentation varies between the test strains
Starch	+	
Cellulose	-	
Pectin		d
Maltose	+	
Cellobiose	+	
Sucrose	+	
D-xylose		d
L-arabinose		d
Glucose	+	
Fructose	+	
Galactose	+	
Mannose	+	
Lactose	+	
Mannitol		d
Glycerol		d
Lactate	-	
Aesculin hydrolysis		d

Abbreviations: +, positive reaction; -, negative reaction; d, positive reaction for some strains and negative reaction for some other strains.

Table 2.3 The amylase activities of four *S. bovis* strains (from Paje *et al.*, 1986)

Strain	Source	Amylase activity (EU/ml)
17	Rumen	6.30
20	Rumen	4.60
25	Rumen	4.63
TUA 148	NA	4.60

NA, data not available

Antigenic properties of *S. bovis*

Streptococci primarily includes antigenic groups A, B, C, D, and G, in which *S. bovis* was classified to belong to Lancefield's antigenic group D (Curtis and Krause, 1964a and 1964b; Elliott *et al.*, 1971; Hardie, 1986). Elliott (1960) reported the isolation of type-specific carbohydrates consisting of glucosamine, rhamnose and glucose from four serum types of group D streptococci. These antigenic carbohydrates were shown to be components of the cell wall and were considered to be the structural counterpart of the group-specific carbohydrates of group A, B, C, and G hemolytic streptococci. The cell-wall carbohydrate, which is a structural analogue of the group-specific antigens of most of the haemolytic streptococci, was also successfully isolated from *S. bovis* strains (Bleiweis and Krause, 1965; Kane and Karakawa, 1969a; 1969b; 1971). An immunogenic glycan isolated from clinical strains, and presumably a feature of rumen isolates also, has been shown to consist of a tetraheteroglycan consisting of 6-deoxy L-talose, D-galactose, L-rhamnose and D-glucuronic acid (Karakawa and Krause, 1966; Pazur and Forsberg, 1978). These findings are consistent with the observation of the capsular polysaccharides of rumen amylolytic streptococci (Hobson and Macpherson, 1954).

Medrek and Barnes (1962) have demonstrated that *S. bovis* can be separated into at least 12 serum types on the basis of precipitin reactions. The type-specificity of 75 strains of *S. bovis*, in most instances, was attributed to a capsular-like material. However, in view of the fact that the immunochemical basis for the antigenic specificity of these type-specific substances was not elucidated in their work, it is possible that the observed type-specific reactions may also be dependent upon components of the cell wall other than the capsular material. Further studies demonstrated that type-specific antigens could be found in both capsular material and cell-wall carbohydrate in *S. bovis* strains (Kane and Karakawa, 1969a; 1969b; 1971). The antigen variation between strains of *S. bovis* isolated from cattle and sheep was also shown by previous work (Shu and Liu, 1995b).

S. bovis is commonly present in ruminants, but strains having *S. bovis* phenotypic traits have also been isolated from other sources such as pigeons and humans (Ruoff *et al.*, 1984; De Herdt *et al.*, 1992). De Herdt *et al.* (1992 and 1993) have reported the divergences of antigenic and biochemical properties of *S. bovis* isolates from pigeons. The prevalence of the *S. bovis* biotypes and serotypes isolated from the intestinal tract of 37 healthy pigeons is summarised in Table 2.4. A total of 60 *S. bovis* isolates have been divided into 5 serotypes by cross agglutination tests. The cross-reactions between the different serotypes were observed in about 15% of the strains. These strains were biochemically classified into 5 biotypes and two sub-biotypes mainly on the basis of the different fermentation patterns of five carbohydrates, which were used in defining the biochemical properties of *S. bovis* in humans, cattle, and sheep (Medrek and Barnes, 1962; Ruoff *et al.*, 1984). The results in Table 2.4 demonstrate that the strains of *S. bovis* in different biotypes can be in the same serotype. On the other hand, the strains in the same biotype can be classified into different serotypes. The results also suggest that a variety of *S. bovis* strains having different antigenic properties are present in the intestinal tract of pigeons, with some serotypes predominating (Table 2.4). All the *S. bovis* strains from the pigeons were positive for raffinose fermentation. This is unlike human isolates, which were variable in their ability to ferment raffinose (Knight and Schlaes, 1985). The strains of *S. bovis* in humans have been classified into two biotypes (Ruoff *et al.*, 1984). Biotype 1 strains ferment mannitol and inulin, produce polysaccharide from saccharose, and hydrolyze starch. Biotype 2 strains do not

possess these properties. Human biotype 2 is divided into 2 sub-biotypes, based mainly on the fermentation of trehalose (Coykendall and Gustafson, 1985). De Herdt *et al.* (1992) further confirmed that *S. bovis* strains isolated from pigeons were biochemically different from the *S. bovis* strains which were isolated from humans. Medrek and Barnes (1962) found that *S. bovis* strains isolated from sheep and cattle all produced polysaccharide on saccharose-containing media and fermented lactose and inulin but did not produce acid from sorbitol. On the basis of hemolytic properties and the fermentation pattern of mannitol, arabinose, and raffinose, four biotypes (called S, RS, ARS, and MRS) could be distinguished. The ovine and bovine RS strains showed similarities to the pigeon biotype 5 strain.

Table 2.4 Prevalence of the *S. bovis* biotypes and serotypes isolated from the intestinal tract of healthy pigeons (from De Herdt *et al.*, 1992)

Serotype	Biotype	No of strains
1	1	8
2	1	16
2	4	1
3	2a	2
3	2b	6
4	3	0
4	5	0
5	1	4

Genetic homology of *S. bovis*

The strains of *S. bovis* , which were isolated from ruminants, differ genetically from many human strains (Klijn *et al.*, 1991). Some rumen *S. bovis* strains examined have been found to form a common DNA homology group (Farrow *et al.*, 1984) and to display a fairly common pattern of phenotypic traits (including minimal nutritional requirements, rapid growth rates, and fermentation of starch) (Medrek and Barnes, 1962; Russell and Robinson, 1984). In contrast, the human *S. bovis* strains display much more variation (Farrow *et al.*, 1984; Knight and Shales, 1985). Further studies were undertaken by Nelms *et al.* (1995) to differentiate the rumen and human *S. bovis* strains by DNA homology and 16s rRNA probes. The rumen strains were found to yield genomic DNA restriction endonuclease digest patterns different from the human strains when either the 16s rRNA gene amplified from the rumen *S. bovis* strain or a conserved universal 23s rRNA fragment was used as probes. A DNA probe based in the V1 region of the 16s rRNA of *S. bovis* JB1 was found to hybridize to DNAs of other rumen *S. bovis* strains. No hybridization occurred with the different strains representing the major human biotype or homology groups. The rumen *S. bovis* strains had a guanosine plus cytosine DNA content of 37.4 to 38.8 mol%. They also had less than 38% DNA homology to the human strains, indicating that rumen strains are clearly distinct from the human strains. High DNA homology of the rumen strains of *S. bovis* isolated from sheep and cattle in Australia have also been suggested by the results of DNA fingerprinting analysis (Klieve *et al.*, unpublished data).

Conclusion

So far, the current knowledge suggests that rumen *S. bovis* is a well established and unique microbe compared with the *S. bovis* from other sources such as humans and pigeons. A number of strains of rumen *S. bovis* have been found, which may be divided into several biotypes and serotypes. However, there is a high degree of genetic homology between the rumen *S. bovis* strains studied. It is therefore possible that the rumen strains of *S. bovis* have a reasonably high degree of overall antigenic homology, although they can be divided into a number of serotypes.

2.2.2.2 *Lactobacillus*

Lactobacillus includes several species of gram-positive rods (*Lactobacillus spp.*) and often proliferates in company with *S. bovis* in ruminants fed on rations containing large amounts of readily fermentable carbohydrates (Allison *et al.*, 1975; Stewart and Bryant, 1988; Shu and Liu, 1995a). *Lactobacillus ruminis* and *Lactobacillus vitulinus* have been described following their isolation from the rumen, and shown to require anaerobic conditions for growth, and contain meson-DAPA in the peptidoglycan (Sharpe *et al.*, 1973). Hungate *et al.* (1952) observed that organisms similar to *Lactobacillus brevis* were quite numerous (1.5 $x10^8$ per ml) in the rumen after a high level of glucose was given to sheep on a ration of alfalfa hay. Mann and Oxford (1954) identified *Lactobacillus brevis,* which was found in 1:3,000,000 and 1:10,000 dilutions of rumen contents from calves 54 and 32 days of age. Several other species of *Lactobacillus* isolated from the rumen have been recorded by Hobson and Mann (1957) and Hungate (1966). These include: *Lactobacillus lactis*, *Lactobacillus bifidus*, *Lactobacillus buchneri*, *Lactobacillus cellobiosus*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Lactobacillus acidophilus*.

Some similarities and differences between different species of *Lactobacillus* are easily found in a number of biological characteristics such as substrate fermentation and lactate production (Stewart and Bryant, 1988). These similarities and differences between *Lactobacillus ruminis* and *Lactobacillus vitulinus* are summarised in Table 2.5. Like *S. bovis*, *Lactobacillus spp.* also produces lactic acid as a major fermentation product. However, different species of *Lactobacillus* mainly produce different isomers of lactic acid after fermentation. It can be seen from Table 2.5 that the major product of *Lactobacillus ruminis* is L-lactic acid, while *Lactobacillus vitulinus* mainly produces D-lactic acid. Some of the other species such as *Lactobacillus acidophilus* produce both L-lactic acid and D-lactic acid (Ogimoto and Imai, 1981).

The amylase activity of the microflora in the rumen of calves was relatively high even in the first weeks after birth, and the main producers of this activity were

the amylolytic streptococci and *Lactobacillus* (Kmet *et al.*, 1986 and 1989). A high activity amylase was isolated and purified from *Lactobacillus plantarum*, which was found to have a pH optimum of 5.5 and Km value of 2.38 g/L with soluble starch substrate (Giraud *et al.*, 1993). This attribute suggests the importance of *Lactobacillus* in the course of lactic acidosis development in the rumen of animals on concentrate rations, because a pH of about 5.5 seems to represent the tolerance limit for many other rumen microbial species (Dawson and Allison, 1988).

In conclusion, *Lactobacillus* are comprised of several species, which may include many strains that can occur in the rumen. No studies of the antigenicity of these rumen organisms were found in the literature. A low degree of genetic homology has been found between some *Lactobacillus* species. It is therefore unlikely that a high degree of antigenic homology between a large number of different strains of *Lactobacillus spp.* isolated from the rumen will be found.

Table 2.5 Some characteristics of *Lactobacillus ruminis* and *Lactobacillus vitulinus* (from Ogimoto and Imai, 1981; Stewart and Bryant, 1988)

Characteristics	*Lactobacillus ruminis*	*Lactobacillus vitulins*
Microscopic observation	0.5-0.7 by 1.0-2.0 μm, occurring singly, with frequent tendency toward filamentation; Motile by peritrichous flagella.	0.5-0.6 by 0.8-3.0 μm, occurring singly or in pairs; Non-motile.
Mol% G+C in DNA	44-47	34-37
MIC of monensin (μg/ml)	1.5-3.0	0.38-1.5
MIC of lasalocid (μg/ml)	1.5	0.38-1.5
Acid from fermentation of each of the following substrates:		
Starch, maltose, cellobiose, sucrose, glucose, fructose, galactose, mannose	+	+
Lactose	d	d
Mannitol, glycerol, D-xylose, L-arabinose	-	-
Major product of fermentation	L-lactic acid	D-lactic acid

Abbreviations: +, positive reaction; -, negative reaction; d, positive reaction for some strains and negative reaction for some other strains.

MIC, Minimum inhibitory concentration.

2.2.3 Selective media for isolation of *S. bovis* and *Lactobacillus*

The choice of highly selective media is a key step for the isolation and enumeration of the rumen *S. bovis* and *Lactobacillus* which are important for lactic acidosis studies. A number of isolation procedures have been used to isolate *S. bovis* (Allison *et al.*, 1975; Ogimoto and Imai, 1981). However, confirmatory tests have to be performed to identify the isolates. This limits the application of the techniques to a small number of samples. Oragui and Mara (1981, 1984) developed a highly selective medium (mBA) for the isolation of *S. bovis*. The development of the mBA medium means that there is no need to perform the routine confirmatory tests for general enumeration of *S. bovis*. This medium was designed on the basis of the ability of *S. bovis* to utilize ammonium sulphate as its sole source of nitrogen. Other bacteria, in particular streptococci such as *S. faecalise, S. faecium, S. equinus, S. salivarius,* and *S. mitis*, which are closely related to *S. bovis*, are inhibited in mBA medium. Oragui and Mara (1981) reported that over 97% of the total 541 colonies grown on mBA were *S. bovis*. For the selective isolation and enumeration of *Lactobacillus*, the MRS medium (De Man *et al.*, 1960) has been the most widely used (Rose, 1985; Bridson, 1990). MRS supports good growth of *Lactobacillus* generally, and is a particularly good medium for a number of fastidious strains which grow only poorly in other general media (De Man *et al.*, 1960; Bridson, 1990). This medium has satisfied the general purpose for isolating and enumerating of *Lactobacillus* (Rose, 1985). However, complete selectivity for *Lactobacillus* was not achieved, and growth of some lactic acid bacteria (leuconostocs and pediococci) and some yeast occurred in this medium (Rose, 1985; Bridson, 1990). But the selectivity of MRS can be further enhanced by pH adjustment (for example, adjusting the medium pH to 5.5 which represents the tolerance limit of many rumen microbes), because *Lactobacillus* will tolerate lower pH levels than other bacteria including pediococci and leuconostocs (Dawson and Allison, 1988; Bridson, 1990).

2.3 Limitations of current techniques and prospects of immunology for controlling lactic acidosis

Lactic acidosis is due to the excessive production of lactic acid in the rumen, which is caused by lactic acid-producing bacteria *S. bovis* or a combination of *S. bovis* and *Lactobacillus* (see Section 2.2) when animals were overfed grain (Ahrens, 1967; Braun *et al.*, 1992). To overcome this problem, a number of approaches (Table 2.6) have been studied which mainly act on: (1) avoiding overfeeding grain, (2) removing the acid, (3) reducing the lactic acid production.

Table 2.6 Major methods for preventing lactic acidosis in sheep and cattle

Method	Action	Reference
Feed management	Avoiding overfeeding based on gradual introduction of grain	Mackie et al, 1978; Mackie and Gilchrist, 1979; Feedlot Advisory Unit, 1990
Inoculation of rumen fluid from grain-fed animals	Removing the lactic acid by providing a balanced rumen microbial population including lactic acid-utilizing bacteria	Cook *et al.*, 1977
Inoculation of lactic acid-utilizing bacteria	Removing the lactic acid by enhancing the lactic acid-utilizing bacterial levels in the rumen	Cook *et al.*, 1977; Wiryawan and Brooker, 1995
Buffers	Maintaining rumen pH within physiological limits by neutralising the acid	Huntington *et al.*, 1977; Stroud *et al.*, 1985; Kovacik *et al.*, 1986
Antibiotics	Reducing the lactic acid production by selectively controlling gram-positive bacteria including *S. bovis* and *Lactobacillus*	Dennis *et al.*, 1981; Wallace *et al.*, 1981; Nagaraja *et al.*, 1981 and 1987; Tung and Kung, 1993; Thorniley *et al.*, 1996

Current practices to reduce the risk of lactic acidosis in livestock centre around management techniques based on introducing grain gradually to animals (Feedlot Advisory Unit, 1990). This allows for adaptation of the rumen microbes to the new

diet (Schwartz and Gilchrist, 1974; Mackie et al, 1978). However, the procedure involves numerous changes to the diet, frequent feeding and close monitoring of daily feed intake. Even with these methods the rations must be carefully formulated and there are still risks of lactic acidosis and poor animal performance. The inoculation of rumen fluid from grain-fed animals is not practically acceptable because of the limitation of resources (Cook *et al.*, 1977). The inoculation of the rumen with lactic acid-utilizing bacteria has been studied for more than 20 years since the work reported by Cook *et al.* (1977). The method has been demonstrated to be able to provide excellent protection against lactic acidosis in the experimental animals (Cook *et al.*, 1977; Wiryawan and Brooker, 1995). Several patents have also been granted to protect the techniques relating to the use of the lactic acid-utilizing bacteria against lactic acidosis (e.g. US patent 5380525; US patent 4138498). However, at present no commercial products relating to the bacteria for controlling lactic acidosis are available in Australia. Feed additives have been used in conjunction with management practices to control lactic acidosis (Feedlot Advisory Unit, 1990; Tung and Kung, 1993; Thorniley *et al.*, 1996). These include: (1) nutrients to facilitate balanced and efficient fermentation; (2) dietary buffers; and (3) antibiotic feed additives (Rowe and Pethick, 1994). Unfortunately, none of the feed additives are considered satisfactory for application in livestock industries. For example, Rowe and Pethick (1994) indicated that buffers such as bicarbonate offer little protection against lactic acidosis when feeding rapidly fermentable carbohydrates to ruminants. The use of antibiotics may be limited in the practical situations because of the potential for emergence of drug-resistant microbial strains and the risk of antibiotic residues in animal products.

Immunisation enhances a specific immune response against the specific target micro-organisms (Humphrey and White, 1970), and is a sustainable approach. This approach is widely used in livestock industries for controlling pathogenic bacteria. Therefore, a practical alternative to the use of feed additives and lactic acid-utilizing bacteria may be immunisation against the lactic acid-producing bacteria, *S. bovis* or a combination of *S. bovis* and *Lactobacillus*, which are primarily responsible for lactic acidosis. It was demonstrated that substantial amount of antibodies to rumen enterococci were produced in a steer after immunisation (Hartman and Jacobson,

1971). The use of vaccination against *S. bovis* has also been suggested by Horacek et al. (1977) to control grain bloat in feedlot cattle.

It has been reported by Dobson *et al.* (1956) that the rumen wall constitutes a poor site for immune responses because the epithelium is non-glandular and highly keratinized. There is also little evidence for the presence of antibodies in the rumen wall (Mach and Pahud, 1971; Sharp *et al.*, 1975). However, saliva could provide a major source of antibodies in the rumen, due to the rumen having a high water content with more than 70% of the water derived from saliva (Church, 1988). The characteristics of rumination, secretion of saliva, and the survivability of antibodies in the rumen have been well reviewed by Gnanasampanthan (1993). His work further indicated that inducing a high level of salivary antibody response is the key point for the establishment of an immunological method to control specific rumen micro-organisms. He also demonstrated a salivary antibody response against rumen protozoa, showing that after immunisation anti-protozoa IgG steadily increased to a significantly high level. However, no significant increase in anti-protozoa IgA was observed after boosting. The increase in the salivary IgG followed a similar pattern to that of the increase in serum IgG. No rise in IgA was detected in the sera. The ability of the antibody response to interfere with the biological functions of rumen protozoa, such as immobilising effect on the organisms, had also been demonstrated by the experiments conducted by Gnanasampanthan (1993). Accordingly, the potential exists for developing a vaccine to reduce the risk of lactic acidosis by inducing salivary antibody responses against the *S. bovis* or a combination of *S. bovis* and *Lactobacillus*.

2.4 Antibody-mediated immunity in ruminants

The immune response can be classified into two broad categories (Humphrey and White, 1970): (1) antibody-mediated immunity; and (2) cell-mediated immunity. The cell-mediated immunity includes direct attack on foreign cells by lymphocytes and natural killer cells as well as phagocytosis and intracellular killing of foreign or infected cells by macrophages. Antibody-mediated immunity, otherwise known as humoral immunity, depends upon the capacity of immunoglobulins to bind

specifically to antigens, with or without participation of the complement system. Humoral immunity refers to the state of immune defence prevailing in extracelluar fluids like plasma, lymph, saliva, milk, bronchial fluid, tears, nasal, and uro-genital secretions (Humphrey and White, 1970; Watson *et al.*, 1994). It is mediated through the production of antigen-specific antibodies (immunoglobulins), which are effector molecules produced by B lymphocytes in response to an antigen (Humphrey and White, 1970; Lydyard and Grossi, 1989). Immunoglobulins belong to the class of proteins called globulins. The classes of immunoglobulin in ruminants include IgG, IgM, IgA and IgE. The IgG class is further subdivided into IgG1 and IgG2. A third subclass IgG3 and allotypic variants of IgG2 have also been reported (Babel and Lang, 1976; Heyermann *et al.*, 1992).

Although Schultze (1980) has argued persuasively that it must be recognised that protective immunity is mediated by both cellular and humoral immunity, Gnanasampanthan (1993) has suggested that salivary antibody-mediated immunity is critical for any attempt to establish immunological control of specific rumen micro-organisms. Therefore, the background knowledge of the common mucosal immune system (including salivary immunity) and some biological characteristics of antibodies in ruminants must be understood for developing an immunisation strategy against lactic acidosis. For this reason the bulk of this section will be devoted to the common mucosal immune system and transport of antibodies as well as their functional features. A suitable method for measuring an antibody response will be also indicated.

2.4.1 The common mucosal immune system and transport of antibodies

The mucosal immune system is associated with the mucosal surfaces of the gut, respiratory tract, urogenital tracts, pharynx, tonsils, the ducts of the salivary glands (Husband, 1985 and 1987; Gnanasampanthan, 1993). The mucosal surfaces represent the largest organ system in ruminants and exposure to exogenous agents including micro-organisms. These mucosal tissues are defended by a local immune system with properties and functions that in many respects are separate from the systemic immune system. The gut is the largest of the mucosal organs and the best

endowed with organized lymphoid structures capable of inductive events in immune responses. In addition there are large numbers of effector cells disseminated throughout the sub-epithelial tissues at the gut (Husband, 1985 and 1987). It has been reported that after antigen-induced proliferation and partial differentiation, both B and T cells enter the regional mesenteric lymph nodes, and after further differentiation they are transported through the thoracic duct into the circulation (Gowans and Knight, 1964; Craig and Cebra, 1971; Sheldrake *et al.*, 1985a and 1985b; Butcher, 1986; Sheldrake and Husband, 1988). It has also been demonstrated that these cells have surface determinants, called adressins, on endothelial cells in mucosal and glandular tissues (Streeter *et al.*, 1988). They appear to be involved in the recognition of mucosal tissues by immunoblasts originating in the gut-associated lymphoid tissue (GALT). While most of the activated B and T cells migrate from the circulation back to the lamina propria, a proportion (around 10-20%) end up in mucosal tissues outside the intestine (Rudzic *et al.*, 1975; Roux *et al.*, 1977; McDermott and Bienenstock, 1979; Weisz-Carrington *et al.*, 1979; Bennel and Husband, 1981a).

The concept of the common mucosal immune system refers to the inter-connected network of migration of activated lymphocytes between the distant mucosal sites (Bienenstock and Befus, 1980). The mucosae of different sites are provided with a common pool of recirculating lymphocytes and precursors of plasma cells from one tissue can seed the mucosa at other sites, resulting in the production of secretory antibodies in the alimentary, respiratory, genito-urinary and naso-lacrimal ducts, salivary glands, and mammary tissues (Rudzic *et al.*, 1975; Roux *et al.*, 1977; Husband 1978 and 1980; Husband and Seaman, 1979; Bienenstock and Befus, 1980; Husband, 1987). This concept has raised enormous interest in exploring possible ways of immunizing animals against infectious diseases related to mucosal organs. The mucosal immune interactions (in intestine, respiratory tract, and mammary gland) has been extensively reviewed by Husband (1985) who has suggested that problems of immunisation, particularly of mucosal organs lacking organized lymphoid tissue, may be overcome by concomitant immunization of the intestine.

It is known that the secretory IgA (SIgA) provides specific immune protection through inhibiting the adherence of pathogens to the mucosal epithelium (Husband,

1987). The IgA is secreted locally by plasma cells, for instance in the intestinal lamina propria (Vaerman *et al.*, 1973; Beh *et al.*, 1974; Cripps *et al.*, 1974; Quin *et al.*, 1975; Cripps and Lascelles, 1976; Pierce and Cray, 1982), and SIgA may also originate from serum (Sheldrake *et al.*, 1984). The distribution of various classes of immunoglobulins in cattle and sheep have been reviewed extensively (Butler 1969, 1980, and 1983; Lascelles *et al.*, 1986; Gnanasampanthan, 1993; Watson *et al.*, 1994b). Table 2.7 shows the concentrations of immunoglobulins in blood serum, colostral whey, milk whey, and intestinal fluid. IgG predominantly occurs in these body fluids.

Table 2.7 Concentrations of immunoglobulins (mg/ml) in blood serum and various secretions of cattle and sheep (from Gnanasampanthan, 1993)

Source	Species	IgG1	IgG2	IgM	IgA
Serum	Cattle	11.2	9.2	3.05	0.37
Serum	Sheep	17.9	6.3	2.40	0.16
Colostral whey	Cattle	48.2	3.98	7.10	4.70
Milk whey	Cattle	0.40	0.06	0.15	0.11
Parotid saliva	Sheep	0.0017	0.0003	0.0001	0.0012
Submaxillary saliva	Sheep	0.0572	0.0100	0.0049	0.5050
Thirty Vella loop fluid	Adult sheep	2.97	1.48	0.30	4.87
Normal intestinal fluid	Cattle	0.25	0.06	trace	0.24
Bile	Cattle	0.10	0.09	0.05	0.08
Tears	Cattle	0.32	0.01	0.18	2.72
Nasal secretion	Cattle	1.56	-	0.40	2.81

To clarify the possibility that colostral and milk IgG1 originates from production within the glandular tissue as well as from the serum, Newby and Bourne (1976) used radio-labelled IgG1 given intravenously to calculate changes in specific activity and established that nearly 100% of the colostral IgG1 was of serum origin in bovine milk. Similar transfer of IgG1 from blood into colostrum and milk occurs in ovine species (Brandon *et al.*, 1971; Sasaki *et al.*, 1976). However, local synthesis of IgG1 has been also demonstrated in organ cultures (Butler *et al.*, 1972).

In sheep and cattle, the higher ratio of IgG1/IgG2 in saliva when compared to serum indicated a combination of selective IgG1 transport and local synthesis (Curtain *et al.*, 1971; Mach and Pahud, 1971; Watson and Lascelles, 1973a). In the intestinal secretion of bovines, Newby and Bourne (1976) reported that IgG1 was selectively transported from the plasma. It has been reported that blood IgG2 is not selectively transported into ruminant intestinal secretions, bile or saliva (Watson and Lascelles, 1973a; Sheldrake *et al.*, 1984; Scicchitano *et al.*, 1986). However, in some studies, it has been observed that inflammation of the mammary gland resulted in serum transudation and elevation of albumin and IgG2 levels in milk (Mackenzie and Lascelles, 1968; Butler *et al.*, 1972; Guidry *et al.*, 1980). In the normal gland, levels of IgG2 are generally low, but local synthesis has been reported (Butler *et al.*, 1972).

It has been reported that the secretory component (SC)-dependent transport mechanism does exist for both dimeric IgA and for pentameric IgM in ruminants (Butler, 1983). In cattle, Newby and Bourne (1976) observed that more than 50% of the IgA in milk was serum origin. Butler (1980) indicated that injection of anti-SC into cattle resulted in a selective appearance of this antibody in both bile and saliva, suggesting that an SC-dependent pathway for IgA transport from serum is present in cattle. Levels of IgM in colostrum are often higher than levels of IgA (see Table 2.7). However, IgM is present in low concentration in most mucosal secretions (Butler, 1974; Lascelles and McDowell, 1974; Saif and Bohl, 1979). Newby and Bourne (1977) reported that less than half of the IgM in cows' milk was of serum origin. It is more than likely that IgM may be transported selectively into milk, saliva and other secretions by the SC mechanism (Scicchitano *et al.*, 1986). Polymeric IgM has been shown to bind successfully to SC with an affinity constant similar to that of polymeric IgA (Weicker and Underdown, 1975; Socken and Underdown, 1978).

2.4.3 Antibody functional features

Many important biological properties and the effector activities of antibodies have been well documented (Cohen and Porter, 1964; Humphrey and White, 1970; Watson, 1975b; Watson *et al.*, 1994) such as neutralization of toxins, immobilization of micro-organisms, interference with attachment of micro-organisms to cell receptors, neutralization of viral activity, antibody dependent cellular cytotoxicity (ADCC), activation of serum complements to facilitate the lysis of micro-organisms and opsonization to promote phagocytosis and destruction of micro-organisms by phagocytic cells.

IgG occurs predominantly as monomeric molecules in blood and all the other body fluids in ruminants in which they have been studied (Mach and Pahud, 1971; Duncan *et al.*, 1972; Tewari and Mukkur, 1975). Higher polymers of IgG are observed in both serum and secretions in cattle (Sullivan *et al.*, 1969; Hammer *et al.*, 1971). Its relatively long half-life compared to IgA and IgM, makes it an important molecule in the immune defence mechanisms. When bivalent IgG antibodies combine with micro-organisms, cross-linked complexes are formed by virtue of the multi-determinant nature of the antigens leading to the phenomenon of agglutination (clumping together). The clumped antigen-antibody complexes are phagocytized and destroyed by phagocytic cells. IgG plays an essential role in the colostrum of ruminants (Watson, 1980), where it provides antibody-mediated immunity to the neonate. It has been demonstrated that maternally derived IgG1 in the colostrum acts locally in the lumen of the gut of animals, when they were challenged with rotaviruses (Snodgrass and Wells, 1978; Snodgrass *et al.*, 1980; Fahey *et al.*, 1981). It has also been reported that IgG anti K99 pilus antibody in colostrum is more efficient on a molar basis than IgM derived from the same source (Altman and Mukkur, 1983). IgG1 is the major immunoglobulin in colostrum and milk, while IgG2 is of crucial importance in resistance to microbial infections in which phagocytosis is an important effector mechanism such as vaccination against staphylococcal mastitis (Watson, 1976). In the intestinal secretions, it has been speculated that in view of its recognized resistance to proteolysis by chymotrypsin (Brock *et al.*, 1977a and 1977b) IgG may play a role in prevention of bacterial

colonization or in toxin neutralization. The roles of IgG and SIgA have received considerable attention in caries research in laboratory animals. The oral cavities of these animals are continually exposed to various microbial organisms, and both saliva and the gingival fluid were shown to have immune functions mediated by these antibodies (Cole *et al.*, 1977; Michalek *et al.*, 1976; Kilian, 1981).

IgG1 and IgG2 have distinguishing biological functions, as well as having some in common. Both IgG1 and IgG2 have excellent immunoprecipitating properties (Butler, 1980), a feature which is explored effectively in many of the vaccines used in ruminant species (East *et al.*, 1992). Bovine macrophages have membrane Fc receptors for both IgG1 and IgG2 (Rossi and Kiesel, 1977), whereas the macrophages of sheep have receptors only for IgG1 (Yasmeen, 1981). In sheep, goats and cattle the neutrophil Fc receptor is specific for IgG2 (Watson, 1975a; Micusan and Borduas 1977; McGuire *et al.*, 1979). This provides a functional basis for the observation by Nansen (1972) that Danish Red cattle which were genetically deficient in IgG2, had greatly increased susceptibility to pyogenic infections. In contrast to the IgG subclasses, neither IgM nor IgA attach cytophilically to phagocytes, suggesting that IgG plays the major role as an opsonin in ruminants.

IgG1, IgG2, and IgM each fix homologous complement (Feinstein and Hobart, 1969; Hobart, 1976). Complement fixation mediates protection from pathogenic micro-organisms in two ways: (1) the complement cascade results in production of lytic enzymes which may kill the microbe; and (2) complement fixation results in enhanced phagocytosis (and intracellular killing) by both neutrophils and macrophages, via C_{3b} opsonisation. Therefore, IgM can promote phagocytosis of pathogens indirectly through its capacity to fix complement, although it does not opsonise via Fc acceptor-receptor affinity with phagocytes (Colditz *et al.*, 1988).

Although the functional significance of IgA antibody has been an enigma, its importance in common mucosal immunity has been documented (Vaerman *et al.*, 1973; Beh *et al.*, 1974; Cripps *et al.*, 1974; Quin *et al.*, 1975; Cripps and Lascelles, 1976; Pierce and Cray, 1982; Husband, 1985 and 1987). In the intestinal secretions the role of secretory IgA as an efficient agglutinating antibody has been reported in a number of studies (Walker *et al.*, 1972; Andre *et al.*, 1974; Cripps *et al.*, 1974; Stokes *et al.*, 1975). Ruminant IgA does not fix complement, nor is there any

compelling evidence that it can opsonise or contribute to ADCC (Watson *et al.*, 1994). However, IgA was a potent mediator of ADCC in a murine system in which *Shigella* organisms were the targets and lymphocytes from gut-associated lymphoid tissue were the effector cells (Tagliabue *et al.*, 1983). Evidence suggests that the major, if not exclusive, role of IgA antibody is to bind to adherence determinants of pathogens in order to prevent their entry to the body via specific attachment to epithelial cells (Husband 1987; Saif, 1987). This protective function occurs entirely in the secretions covering the epithelial membranes which line the respiratory, gastrointestinal and urinogenital tracts.

IgE normally is present in blood in extremely low concentrations in sheep and cattle (Thatcher and Gershwin, 1988; Engwerda *et al.*, 1992), and its biological significance has not been well explored. Recently, it has been shown that ovine IgE was associated with globule leucocytes and mast cells in intestinal sub-mucosa of sheep infected with gastrointestinal parasites (Gill *et al.*, unpublished data). It seems likely that ruminant IgE, like the IgE of other species (Urban, 1984), has a role in immunity to internal and external parasites, probably as reagin associated with basophils, mast cells, globule leucocytes and, perhaps, eosinophils and platelets.

In conclusion, four classes of immunoglobulins (IgG, IgM, IgA, and IgE) are present in a number of the body fluids such as blood, milk, intestinal fluid, and saliva in ruminants. These antibodies represent both local synthesis and blood origination (transport into saliva from blood circulation), and have different functional features.

2.4.4 Measuring an antibody response

The choice of a suitable technique for measuring antibody concentration in saliva samples is critical for the commencement of this project. A number of methods for measuring the specific antibody concentration in a variety of biological samples have been documented in details by Robin (1986), in which enzyme immunoassays based on enzyme-labelled immunoreactants are playing an increasingly important role in the diagnostic laboratory (Voller and Bidwell, 1986). Although radio-immunoassay has been much longer established, it is now largely confined to the endocrinological laboratory. The short shelf life of the isotopes used

for labelling (usually I^{125}) and the administrative inconveniences based on legislation (due to possible health risks) have restricted the further expansion of radio-immunoassays (Larsen and Odell, 1986). In microbiology, immunofluorescence is still widely used for the measurement of antibodies and increasingly, with monoclonal antibodies, for the specific detection of organisms in clinical samples. However, the necessity for well-trained staff and the subjective nature of immuno-fluorescence limit its use (Nakamura and Robbins, 1986). In contrast, the enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlmann, 1972) has stable reagents, is free from limiting legislation, and is adaptable to simple tests and to sophisticated automation. In addition, its variety of detection systems, which range from visual to photometric with coloured, fluorescent, or luminescent substrates, have all contributed to the dramatic expansion of ELISA procedures and applications over the decades (Voller and Bidwell, 1986).

The ELISA is based on the premise that one immunoreagent can be immobilized on the carrier surface while retaining its activity and the reciprocal immunoreagent can be linked to an enzyme in such a manner that both the enzymic reactivity and the immunoreactivity of this conjugate are retained (Engvall and Perlmann, 1972; Voller and Bidwell, 1986). The choice and preparation of the immunoreagent-sensitized solid phase and the enzyme conjugates deserve detailed consideration. The ELISA method has been used for measuring specific antibody concentrations in a number of samples such as blood and saliva taken from animals (Gill, 1991; De Herdt *et al.*, 1993; Gnanasampanthan, 1993). These include measuring specific antibody responses to *S. bovis* or other rumen bacteria as well as protozoa.

2.5 Some important factors influencing immune responses

There are some external as well as internal factors which determine the level of immune response that is manifested by an animal (Humphrey and White, 1970). Although the internal factors are an inherent property of an animal such as species and genotype as well as age (Glass *et al.*, 1991a and 1991b; Watson *et al.*, 1994; Colditz *et al.*, 1996), the immune response is very much dependent on a number of

external factors, for example, the types of antigens, booster regime, immunisation routes, choice of adjuvants, dose rate, and animal infection status (Humphrey and White, 1970; Bell and Torrigiani, 1984). The importance of numerous factors makes it complex and time consuming to optimise the immune response for any specific objective (Pierce, 1984). Ideally, a vaccine should induce high-level and lasting immunity following only a few doses of vaccine. In order to efficiently develop an immunisation strategy against lactic acidosis, it is a great advantage to review the background information related to the type of antigen, booster regime, immunisation route, and choice of adjuvant.

2.5.1 Antigen types and the requirement for booster immunisation

Most vaccines consist either of attenuated live organisms that proliferate in the recipient or of killed whole organisms that present a complex of different substances in a relatively large and complicated form (Bell and Torrigiani, 1984). Recently, subunit vaccines consisting of single antigens isolated from whole organisms have come into use. They have reduced the risks of untoward adverse effects as in the instance of toxic bacterial products, especially endotoxin, immunogens causing hypersensitivity or viruses with oncogenic potential. Now, polypeptide antigens are being produced by genetic recombination in cloned prokaryotic and eukaryotic cell cultures and very small hapten-like amino acid sequences that carry single epitope specificity have been produced synthetically. These new processes for production may yield products of low immunogenicity, and synthetic peptides in particular may require a larger-sized molecule as a carrier (Bell and Torrigiani, 1984; Rabinovich *et al.*, 1994). Although there are a number of forms of antigens, the simple and effective way to commence an immunisation study against bacteria is to use the whole organisms as antigens. This allows one to establish the principles and feasibility of the approach before refining the antigens. Therefore, whole bacterial cells were determined to be used in this study.

One of the important considerations for developing a vaccine against lactic acidosis is to select a suitable type of antigenic bacterial cells, either live or killed bacterial cells. It is well known that live microbial vaccines usually induce more

durable and protective immune responses than killed vaccines (Fuhrmann and Cebra, 1981; Holt *et al.*, 1990). However, the use of live bacteria has been limited, because some bacteria can be toxic or cause disease when animals are immunised with the live organisms as antigens. In these cases, the bacterial cells normally have to be killed or attenuated for the preparation of vaccines (Watson, 1984 and 1987; Holt *et al.*, 1990).

Formalin treatment has been commonly employed to prepare killed bacterial vaccines such as vaccines against *S. suis* and *S. equi* (Srivastava and Barnum, 1981; Holt *et al.*, 1990). Heat treatment was also used and compared with the use of formalin in some of the studies. Table 2.8 summarises the types of antigens and methods for killing vaccine cells in the studies of immunisation against streptococci that have close structural relationships to *S. bovis*. As shown by Holt *et al.* (1990) and Srivastava and Barnum (1981) both *S. suis* and *S. equi* vaccines killed by formalin treatment were effective in inducing immune responses in experimental animals. The vaccine treated by formalin also provided protection against pathogenic *S. equi* strain challenge infection in foals (Srivastava and Barnum, 1981). Immunisation using a formalin-killed *S. bovis* strain isolated from pigeons has also stimulated a significantly high level of antibody response in rabbits (De Herdt *et al.*, 1992). However, the heat-killed *S. suis* vaccine failed to stimulate a protective response in experimental animals (Holt *et al.*, 1990). Failure to enhance the protective response was attributed to some surface antigens of the bacterial cells being destroyed by heat.

Another well-established principle in immunology is that primary immunisation only induces a weak antibody response with short duration, and booster immunisation is necessary to stimulate a strong and lasting immune response (Humphrey and White, 1970). To induce a high level of antibody response, multiple boosters following a primary immunisation have often been used in experimental studies, in particular, in streptococcal vaccine studies (Table 2.9). For example, in order to induce a strong protective response in the streptococcal vaccine studies, Holt *et al.* (1990) have used repeated inoculation (7 inoculations) of pigs with a live *S. suis* vaccine. A similar protective response was obtained by a series of 10 inoculations of a formalin-killed culture of *S. suis*. Table 2.9 summarises the dose and immunisation regime of the streptococcal vaccines.

However, another important consideration for developing an practical vaccine is the ability to induce a high level and durable immune response using only a low number of immunisations, ideally, one booster following a primary vaccination. This objective can be achieved optimally by the manipulation of any of the many levels at which immune responsiveness is expressed. For example, in addition to the use of suitable type of antigen and booster regime, the choice of immunisation route and adjuvant is critical for facilitating antibody response and maintaining it. The background information related to immunisation route and adjuvant is given in the following sections.

Table 2.8 Types of antigen bacterial cells used in the studies of immunisation against streptococci

Types of antigen bacteria cells	For the preparation of	Animal	Reference
Live	*S. suis* vaccine	Pig	Holt *et al.*, 1990
Formalin-killed	*S. bovis* vaccine	Rabbit	De Herdt *et al.*, 1992;
	S. suis vaccine	Pig	Holt *et al.*, 1990;
	S. equi vaccine	foal	Srivastava and Barnum, 1981
Heat-killed	*S. suis* vaccine	Pig	Holt *et al.*, 1990;
	S. equi vaccine	foal	Srivastava and Barnum, 1981

Table 2.9 Numbers of immunisation and dose rate used in the studies of streptococcal vaccines

Vaccine	Number of immunisation	Dose rate of bacterial cells (cells/ml)	Volume of each immunisation (ml)	Reference
S. bovis vaccine for rabbits	21 days after primary injection, 4 boosters were performed at 3-day intervals	10^9-10^{10}	3.0 ml (including 1.5 ml adjuvant) for primary injection; 0.3-0.9 ml for booster injections	1
S. suis vaccine for pigs	10 inoculations were given at 2- or 3-day intervals	10^{10}	1 ml	2
S. equi vaccine for foals	4 inoculations were given at 10-day intervals	2.5×10^9	4 ml (including 1 ml adjuvant)	3

1, De Herdt *et al.*, 1992; 2, Holt *et al.*, 1990; 3, Srivastava and Barnum, 1981.

2.5.2 Immunisation routes

A number of immunisation routes, such as intramuscular and subcutaneous, are used to administer commercial or experimental vaccines for various objectives. However, there have been few studies on methods for inducing an immune response in saliva of sheep and cattle, although there have been a number of reports (in particular, those on streptococcal vaccines) related to the induction of salivary immune responses in non-ruminants by different immunisation routes. In order to determine the immunisation route to be used in this study, the present section is concentrated on the immunisation routes used in the studies related to enhance a salivary immune response.

These immunisation routes, which have been used for stimulating salivary immunity in non-ruminants, mainly include local injection and oral administration (Cohen *et al.*, 1979; Challocombe and Lehner, 1980). Local injections into the oral mucosa or feeding of specific antigens in both rats (Taubman and Smith, 1974 and 1977; Smith *et al.*, 1977) and monkeys (Bahn *et al.*, 1977) have resulted in the elevation of both IgG and IgA antibodies in the saliva. Other methods have included subcutaneous injections in the vicinity of salivary glands in either monkeys (Emmings *et al.*, 1975; Evans *et al.*, 1975) or rats (Taubman and Smith, 1974; McGhee *et al.*, 1974). Oral sub-mucosal immunisation with either live or killed *S. mutans* failed to elicit a significant SIgA antibody response in monkeys (Walker, 1981). In contrast, oral immunisation with killed *S. mutans* in rats was found to stimulate a specific SIgA antibody response in saliva. The ingestion by human volunteers of capsules containing killed *S. mutans* have been shown to enhance the antibody response in saliva. However, the salivary antibody titres fell rapidly (Mestechy *et al.*, 1978).

Attempts to enhance secretory antibody response have been made by altering immunisation protocols (Pierce, 1978; Beh *et al.*, 1979; Bennel and Husband, 1981b; Scicchitano *et al.*, 1984; Keren *et al.*, 1988). Pierce and Gowans (1975) demonstrated that intra-peritoneal administration of antigen in Freund's adjuvant followed by oral boosting with soluble antigen was more effective than oral or systemic immunisations alone. This immunisation protocol probably works by the

erosion of the serosal surface of Peyer's patches (PP) by adjuvant-induced mesenteric inflammatory lesions allowing direct and sustained access of antigen into PP from the oil depots (Husband, 1985). These investigators used combinations of parenteral injections of antigen and per enteric routes (either intra-peritoneal-oral or intramuscular-oral or intra-peritoneal-intra-duodental), and these have stimulated SIgA responses to levels previously attainable only after multiple oral immunisation. Although these immunisation routes were mainly used to stimulate gut-immune responses, direct application of them for inducing salivary antibody response in sheep has been made by Gnanasampanthan (1993). The immunisation routes primed intra-peritoneally have been demonstrated to be able to stimulate significant levels of salivary antibodies (in particular IgG) against rumen protozoa.

2.5.3 Adjuvants

The term adjuvant was first defined by Ramon (1925) as a substance which, when used in combination with antigen, enhances levels of immunity beyond those developed with the antigen alone. Since then many substances have been found to have immune stimulating properties (Freund and McDermott, 1942; Edelman, 1980; Vanselow, 1987; Gupta and Siber, 1995). Although the mechanisms by which adjuvants promote the immune response are poorly understood, there appear to be at least four general ways in which adjuvants promote the immune response:

(1) maintaining a depot of antigen at the injection site (Schroder and Stahl, 1984; Beh and Lascelles, 1985; Lascelles et at., 1989);
(2) promoting accumulation of immune-reactive cells at the site of injection and in the draining lymph nodes (Dresser *et al.*, 1970; Taub *et al.*, 1970; Bomford, 1982);
(3) modifying the activities of cells that are concerned with generating, promoting, and maintaining the immune response (Allison and Byars, 1986),
(4) improving the presentation of antigen to the immune system (Morein *et al.*, 1987; Shahum and Therien, 1988).

Freund's complete adjuvant (FCA)

FCA is composed of mineral oil containing tubercle bacilli (Freund and McDermott, 1942), and is extremely potent in terms of its ability to stimulate both humoral and cellular immunity (McCarthy *et al.*, 1977; Edelman, 1980). For example, after a single intramuscular injection, antibody formation was detected as early as 4 or 5 days later and continued for 8 or 9 months (McCarthy *et al.*, 1977; Edelman, 1980). When specific antigens are incorporated into FCA, the water-in-oil emulsion induces a granulomatous reaction in the tissues and the antigens within the granuloma slowly reach into the body and so provide prolonged antigenic stimulus (Davis *et al.*, 1970; Edelman, 1980). The FCA not only forms a depot, but the tubercle bacilli contains a compound called muramyl dipeptide which can stimulate the activity of immune cells. The antigens and adjuvant spread together to the draining lymph node, optimising the immune response. However, droplets of emulsion metastasise widely from the site of injection and intense, chronic, inflammatory responses develop around the inoculum and its metastases (Davis *et al.*, 1970). This intense inflammatory response and the induction of tuberculin sensitivity preclude the use of FCA in human and veterinary medicine. Its use is confined to experimental animals.

Oil emulsion

The most common way to achieve an antigen depot to help promote the immune response is to suspend the antigen in an oil-in-water emulsion. The best evidence of this effect is the correlation between persistence of antigen at the site of injection and maintenance of serum antibody levels (Humphrey, 1982; Beh and Lascelles, 1985). For example, the efficacy of FIA (Freund's incomplete adjuvant) was attributed to the depot formation at the site of injection and slow release of the antigen with stimulation of the antibody-producing cells. Without adding the tubercle bacilli as FCA (Freund and McDermott, 1942), FIA mainly contains a mineral oil. It acts as a repository adjuvant, delaying absorption of antigen and stimulating mononuclear cells to produce antibody at local and distant sites (Edelman 1980). The antigen must be trapped within the water droplet phase of the

emulsion for enhancement of the immune response (Herbert, 1968). FIA stimulates humoral immunity and weak cellular immunity. FIA has been used successfully in a number of veterinary vaccines (eg. hog cholera, foot-and-mouth disease, and Newcastle disease), although it has some side-effects such as local reactions: granuloma and sterile abscess formation (Ott *et al.*, 1962; Mckercher and Graves, 1977; Gupta *et al.*, 1993).

Saponins

Saponins are glycosides widely distributed in plant families. The crude saponins used in commercial FMD vaccines are derived from different sources and the side effects can be variable (Vanselow, 1987). Saponins may enhance the presentation of antigen to immunocompetent cells. Being detergents they may act in the same way as the addition of hydrophobic moieties to proteins enhances uptake by lymph node sinus macrophages and movement into thymus dependent areas (Waksman, 1979). The stimulation of cell-mediated immunity by saponins was also demonstrated by Bomford (1980b). In addition, saponins are very effective in promoting humoral responses (Bomford 1980a). Although a main drawback of the saponins is the formation of intense local reactions at the injection site, saponin has been included in a number of foot-and-mouth disease (FMD) vaccines, and a single well-characterised saponin adjuvant active substance (QuilA) has been used in some veterinary vaccines (Vanselow, 1987). The purification of QuilA has markedly reduced the intensity of the local reaction, although it is too irritant for use in horses (Vanselow, 1987).

Dextran

Dextran (including dextran sulphate and DEAE-dextran) has adjuvant activity. For example, dextran sulphate is strong adjuvant which appears to act directly on macrophages, enhancing antibody responses as well as delayed-type hypersensitivity responses (Vanselow, 1987). Dextran sulphate activates macrophages to be cytotoxic and cytostatic. It also induces an increase in cyclic GMP in conjunction

with B cell activation. It has been used in a number of veterinary vaccines such as the *Staphylococcus aureus* vaccine against mastitis (Watson, 1987, 1992a, 1992b).

Aluminium salts

The aluminium salts (including aluminium hydroxide, aluminium phosphate and calcium phosphate) are the most commonly used in human and veterinary vaccines. They are safe adjuvants, however they do occasionally produce sterile abscesses and persistent nodules, particularly if they are injected subcutaneously rather than intramuscularly (Edelman, 1980). The adjuvant action of aluminium salts is attributed to their 'depot' effect. In addition they produce local granulomas that contain antibody-producing plasma cells (Edelman, 1980). They have been shown to activate the alternative complement pathway which provokes chronic inflammation at the site of inoculation (Ramanthan *et al.*, 1979).

Choice of adjuvants

The selection of adjuvants for veterinary use is constrained by ethical considerations based on the side-effects. The side-effects of adjuvants are highly variable. The balance between toxicity and adjuvanticity is always an important consideration for developing an effective immunisation strategy. Some of the side effects can be ascribed to an unintentional stimulation of different mechanisms of the immune system whereas others may reflect general adverse pharmacological reactions. At present the choice of adjuvants reflects a compromise between a requirement for adjuvanticity and an acceptable low level of side effects.

Although there are a wide range of adjuvants under investigation including monophosphoryl lipid A, ISCOMs with Quil-A and Syntex adjuvant formulation, only a limited number of adjuvants such as aluminium salts are commonly accepted for human use at present (Gupta *et al.*, 1993). However, a wider range of adjuvants have been accepted by the veterinary medicine compared to the human vaccines. There are a number of extensive reviews that deal with adjuvanticity, the mode of

action, and side-effects of a wide range of adjuvants (Edelman, 1980; Vanselow, 1987; Gupta and Siber, 1995). So far, there has been little in the way of formal prohibition placed on the uses of a number of adjuvants in veterinary vaccines. Some of the commercially acceptable adjuvants are summarised in Table 2.10.

Table 2.10 Some of the commercially acceptable adjuvants for veterinary vaccines

Specific adjuvant	Example
Oil emulsions	Freund's incomplete adjuvant, mineral oil
Saponins	QuilA
Dextran	Dextran sulphate
Aluminium Compounds	Imject Alum
N-acetylglucosaminyl-N-actetylmuramyl-L-alanyl-D-isoglutamine	Gerbu adjuvant ('ScimaR', Life Sciences Specialities, Victoria, Australia)
Dextran plus mineral oil *	DEAE-Dextran combined with mineral oil

* Watson, 1992a.

2.6 Scope of the experimentation

The following experiments were conducted to test the hypothesis that the risk of lactic acidosis may be reduced by immunisation against *S. bovis* or *S. bovis* and *Lactobacillus*, and to investigate some key factors (as described above) for developing an immunisation strategy against lactic acidosis. These included:

(1) Isolates of *S. bovis* and *Lactobacillus* were obtained and selected for the use of vaccine antigens prior to the immunisation studies.

(2) Three sheep experiments were conducted to test the effect of immunisation against *S. bovis* on lactic acidosis. During these experiments we also compared: a) killed and live vaccines; b) immunisation routes; and c) various adjuvants.

(3) To further test the hypothesis, and verify the results obtained from sheep, two cattle experiments were performed to examine if the risk of lactic acidosis can be reduced by immunisation against *S. bovis* and *Lactobacillus*. During these experiments we also measured the correlation between serum and salivary IgG; and the effectiveness of various adjuvants.

(4) The last experiment in this thesis was designed to test the immunological cross-reactivity between the vaccine and other isolates of *S. bovis* and *Lactobacillus*. This provided a measure of the efficacy of the vaccine developed in this project against a number of isolates of *S. bovis* and *Lactobacillus* from both sheep and cattle.

The details and outcomes of these experiments are reported in the following chapters.

Chapter 3

General materials and methods

Several general materials and methods used in the following experiments are described in this chapter. Some specific materials and methods which apply to individual experiments are described in the respective chapters. All the experiments were done following approval of the Animal Ethics Committee of the University of New England.

3.1 Solutions and media for bacterial culture

For diluting samples, an anaerobic dilution solution (Ogimoto and Imai, 1981) was used. A selective medium called mBA (Orgagui and Mara, 1984) (pH 6.7±0.3) was used for isolating and enumerating *S. bovis*, while the MRS medium (De Man *et al.*, 1960) (Oxoid, England) with pH 5.6±0.1 was used for selective growth of *Lactobacillus*. The following solutions and media were also used throughout the experimental work:

Salt solution A: 0.3% KH_2PO_4, 0.6% NaCl, 0.3% $(NH_4)_2SO_4$, 0.03% $CaCl_2$, 0.03% $MgSO_4$ in distilled water.

Salt solution B: 0.3% K_2HPO_4 in distilled water.

VFA solution: 17 ml acetic acid, 6 ml propionic acid, 4 ml butyric acid, 1 ml isobutyric acid, 1 ml n-valeric acid, 1 ml isovaleric acid, and 1 ml D-L-a methyl butyric acid were mixed well and volumed to 100 ml with distilled water after pH adjusted to 7.5 with NaOH.

Mineral solution I: 0.6% K_2HPO_4 in distilled water.

Mineral solution II: 0.6% KH_2PO_4, 1.2% ammonium sulphate, 1.2% NaCl, 0.14% magnesium sulphate, anhydrous, 0.12% calcium chloride, 0.0027% cobalt chloride, 0.03% manganese chloride·$4H_2O$ in distilled water.

S. bovis growth medium A (SbM-A): 0.5% soluble starch, 0.2% yeast extract, 0.5% $NaHCO_3$, 16.5 ml salt solution A, 16.5 ml salt solution B, 33.0 ml cell-free rumen fluid, 34.0 ml distilled water. It was sterilised by autoclaving at 105°C for 45 minutes. pH 6.8±0.2.

S. bovis growth medium B (SbM-B): 1.0% soluble starch, 0.5% yeast extract, 0.5% $NaHCO_3$, 16.5 ml salt solution A, 16.5 ml salt solution B, 33.0 ml cell-free rumen fluid, 34.0 ml distilled water. It was sterilised by autoclaving at 105°C for 45 minutes. pH 6.8±0.2.

Lactobacillus growth medium (LBM): 0.9% soluble starch, 0.1% glucose, 0.5% yeast extract, 0.05% cellobiose, 7.5 ml mineral solution I, 7.5 ml mineral solution II, 5.0 ml of 8% Na_2CO_3 solution, 40.0 ml cell-free rumen fluid, 0.1 ml of 0.1% resazurin solution, 1.0 ml VFA solution, 0.05% cysteine-HCl, and 45 ml distilled water. It was sterilised by autoclaving at 105°C for 45 minutes. pH 6.8±0.2.

Medium for testing lactic acid-producing capacity (mRF-2): 2.0% trypticase peptone, 2.0% soluble starch, 0.5% glucose, 0.2% bacteriological peptone, 0.2% Yeast extract, 7.0 ml of 9.1% $NaHCO_3$ solution, 7.5 ml mineral solution I, 7.5 ml mineral solution II, 1.0 ml of 0.05% indigo carmine solution, 1.0 ml of 0.05% hemin solution, 40.0 ml cell-free rumen fluid, 0.02% cysteine-HCl, 1.0 ml VFA solution, and 40.0 ml distilled water. It was sterilised by autoclaving at 105°C for 45 minutes. pH 6.9±0.3.

Preservation medium (RF^+): 0.1% yeast extract, 0.1% bacteriological peptone, 0.2% soluble starch, 0.2% glucose, 0.2% cellobiose, 0.5% $NaHCO_3$, 16.5 ml salt solution A, 16.5 ml salt solution B, 33.0 ml cell-free rumen fluid, 0.1 ml of 0.1% resazurin solution, 1.0 ml VFA solution, 0.05% cysteine-HCl, 34.0 ml distilled water. It was sterilised by autoclaving at 105°C for 45 minutes. pH 6.9±0.3.

Broth media were prepared in roll-tubes (Hungate, 1969), or for larger volumes, in serum-bottles (10, 50, 100, or 200 ml) and Wheaton-bottles (500 or 1000 ml) fitted with butyl-rubber stoppers. Solid media were prepared by the addition of 1.5-2% (wt/vol) agar (Difco Laboratories) to liquid broth. Agar tubes were prepared by dispensing 5 ml of media per roll-tube. Anaerobic manipulations during the media preparation were based on those described by Hungate (1969).

3.2 Antigenic bacteria cells for vaccine preparation

3.2.1 *S. bovis* Sb-5

An aliquot of the *S. bovis* strain Sb-5 (see Chapter 4) culture was taken from the freezer, thawed in a cold water bath (20±5°C), and then cultured in RF^+ broth for around 24 hours at 38.5±0.5°C. The culture was further grown in SbM-A medium for around 6 hours. After incubation, the bacterial cells were harvested by centrifuging at 3,000 g (4°C) for 25 minutes, and at 10,000 g for 15 mins. (The 2nd centrifugation was carried out because the centrifugation at 3,000 g failed to yield the cell pellet). The supernatant was discarded. This preparation was used in the experiment reported in Chapter 5. In later experiments the growth medium was changed to SbM-B, which contained a higher level of starch than SbM-A and more closely represented that found in the rumen of animals fed high levels of starch, and the method of preparation of Sb-5 changed slightly: after culturing in RF^+ broth as described above, the Sb-5 was grown in SbM-B medium for 6 to 10 hours, and then the bacterial cells were harvested by centrifugation at 10,000 g for 25 minutes and washed 3 times with sterile phosphate-buffered saline pH 7.2-7.4 (PBS, Sigma Diagnostics). The number of bacterial cells were determined by the direct microscopic count using the method described by Gilstrap *et al.* (1983), and adjusted to $1x10^{10}$ cells/ml in sterile PBS. The suspension was stored at -80°C until use. Immediately prior to vaccine preparation, aliquots were thawed in a cold water bath (20-25°C) and mixed thoroughly for 10 mins using a vortex mixer. The bacterial cells in the mixed suspension were checked for viability by culturing an aliquot of the suspension. However, the % viability of the thawed *S. bovis* cells after storage at -80°C was not measured. Therefore, it is difficult to say what proportion of cells

were damaged during freezing, and if the antigen release had any effect on the immunogenicity of the vaccine. Anaerobic manipulation of *S. bovis* Sb-5 was based on the method described by Hungate (1969).

3.2.2 *Lactobacillus* LB-27

An aliquot of the LB-27 (see Chapter 4) culture was taken from the freezer, thawed in a cold water bath (20±5°C), and then cultured in RF^+ broth for 20 to 24 hours at 38.5±0.5°C. The culture was further grown in LBM medium for 18 to 24 hours. After incubation, the bacterial cells were harvested by centrifugation at 4,700 g for 25 minutes (at 4°C) and washed 3 times with sterile PBS. The number of bacterial cells were determined by the direct microscopic count method and adjusted to $2x10^{10}$ cells/ml in sterile PBS. The suspension was stored at -80°C until use. Immediately prior to vaccine preparation, aliquots were thawed in a cold water bath (20-25°C) and mixed thoroughly for 10 mins using a vortex mixer. The bacterial cells in the mixed suspension were checked for viability by culturing an aliquot of the suspension. Anaerobic manipulation of *Lactobacillus* LB-27 was based on the method described by Hungate (1969).

3.3 Antibody measurement

3.3.1 Samples for measuring antibody concentration

Saliva samples (approximately 0.5 g) were collected from sheep or cattle using a cotton swab, weighed and diluted to 1:80 in sterile PBS. Pilocarpine stimulation before sampling saliva (Gnanasampanthan, 1993) was not used in the present work. After centrifugation at 4°C (10,000 g for 15 mins), the supernatant was collected and stored at -20°C until needed.

Blood samples (approximately 10 ml) from sheep or cattle were collected by jugular venipuncture into a vacutainer (*SST* Gel and Clot Activator, New Jersey) and mixed well by gentle reversing 5 times. The serum was separated following

centrifugation (4°C and 2,000 g) for 15 minutes, and stored at -20°C until further use.

An aliquot of each of the samples was thawed in a cold room (4°C) overnight and then mixed thoroughly prior to analysis of antibody concentration.

3.3.2 Method for measuring antibody concentration

The determination of antibody concentration in saliva and serum samples was based on the Enzyme-Linked Immunosorbent Assays (ELISA) described by Gill (1991), Gnanasampanthan (1993), and De Herdt et al (1993) as summarised below.

Flat bottomed (96-well), microtiter plates (Dynatech Laboratories) were coated with 100 µl/well of Sb-5 ($5x10^7$ cells/ml) in 0.05 M carbonate buffer pH 9.6, by incubating overnight (16~20 hours) at 4°C.

The plates were washed 4 times in PBS containing 0.05% Tween 20 (PBST), and blocked with 150 µl/well of 2% albumin (Bovine, Sigma) in PBST for 1 hour at 37°C.

The plates were washed 3 times in PBST, and 100 µl/well of test saliva at a dilution of 1:80 (test serum at a dilution of 1:16,000) or serial dilutions of standard serum in PBST were added to wells. All the samples were run in duplicates and blanks were included with only PBST added to the wells.

After incubation for 1.5 hours at 37°C and washing 4 times in PBST, 100 µl of peroxidase-conjugated donkey anti-sheep immunoglobulin (Silenus Laboratories) diluted 1:3,000 in PBST was added to each well. The plates were incubated for 1 hour at 37°C and washed 4 times in PBST. Then 100 µl/well of freshly prepared substrate [0.06% o-phenylenediamine (Sigma Laboratories) containing 0.21% hydrogen peroxide in a citrate phosphate buffer pH5.0] was added.

The reaction was allowed to develop for 10 mins in dark at room temperature and then stopped by the addition of 50 µl/well of 2 N sulphuric acid.

The absorbance was measured with a Titertek Multiskan reader (Flow Laboratories) at a wavelength of 492 nm. The antibody concentration (unit/ml) was automatically calculated by the computer connected with the reader. The calculation was based on the absorbance at 492 nm which was negatively correlated with the standard serum dilutions. In each plate serial dilutions of standard serum were used, and the dilution (1:4,000) of the standard serum was assigned an arbitrary unit (3,200 units/ml). A standard curve from one of the ELISA plates is shown in Figure 3.1.

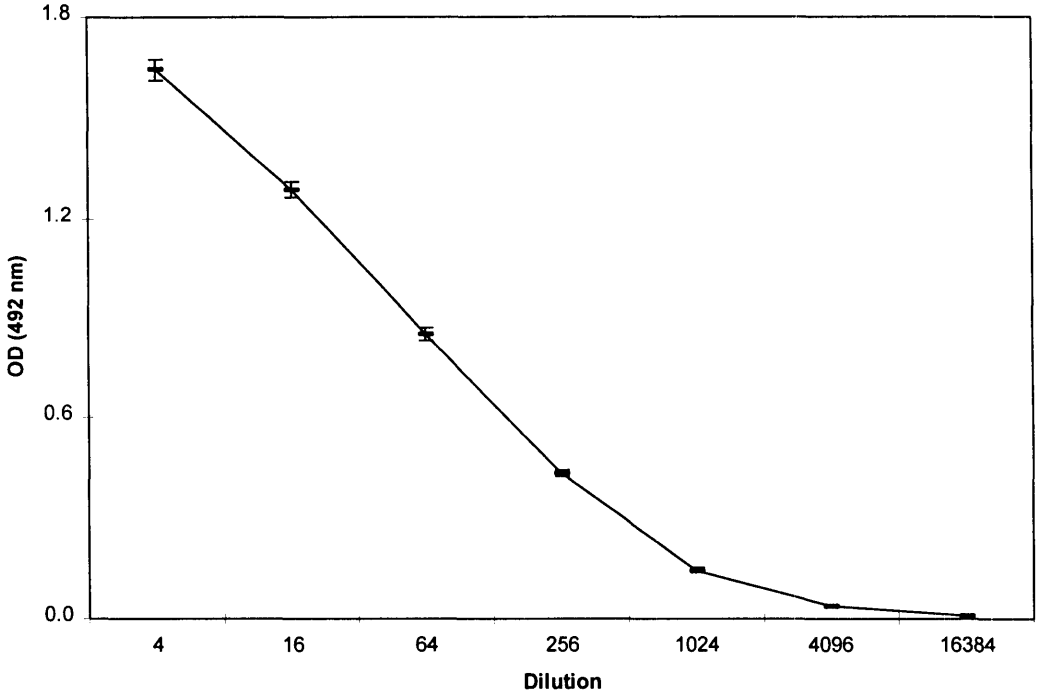


Figure 3.1 Standard curve of the relationship of different dilutions (x 1,000) of the standard serum and the absorbance values (OD).

The isotype anti-*S. bovis* IgG concentration of cattle was analysed using the same ELISA as described above except that Peroxidase-labelled Affinity Purified Antibody to Bovine IgG (Chemicon, Temecula) was used instead of the Peroxidase-

conjugated Donkey Anti-sheep Immunoglobulin. The isotype anti-*Lactobacillus* IgG concentration was analysed using the same method for anti-*S. bovis* IgG, except that LB-27 ($5x10^7$ cells/ml) suspension was used instead of the Sb-5 suspension for coating the plates.

3.4 Rumen pH, lactate concentration, and numbers of *S. bovis* and *Lactobacillus*

Rumen fluid samples from sheep or cattle were taken either via stomach tube or through rumen cannula as indicated in individual experiments described in the respective chapters.

3.4.1 Rumen pH

After sampling, an aliquot of rumen fluid (20-100 ml) was used for measuring rumen pH. The pH was measured immediately after sampling using a portable pH meter, which was calibrated with standard pH buffers (pH 4.0 and 7.0).

3.4.2 Rumen lactate concentration

After sampling, an aliquot of rumen fluid (around 10 ml) was immediately acidified using concentrated H_2SO_4 (3~5 drops/10 ml rumen fluid) and stored at -20°C for the measurement of lactate (L-lactate and/or D-lactate) concentration. Before measuring lactate concentration, rumen fluid samples were thawed at 4°C, mixed thoroughly, and centrifuged at 10,000 g for 15 mins. The concentrations of D- and L- lactate in the supernatant were analysed using a Cobas Mira Auto Analyser and the enzyme kits (Behring Diagnostics Inc.) (Thorniley *et al.*, 1996).

3.4.3 Numbers of rumen *S. bovis* and *Lactobacillus*

The procedure used to estimate the numbers of *S. bovis* and *Lactobacillus* in rumen fluid was as follows. One ml of rumen fluid was taken immediately after sampling using a sterile syringe (with 18Gx1 1/2 needle), and injected into a tube containing 9 ml of the dilution solution (see Section 3.1) and mixed vigorously, then kept at 37-38°C in a water bath. After sampling, all the samples were transported to the laboratory within two hours for further processing. Serial dilution was used to prepare 5 dilutions, which contained 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} times the original concentrations of rumen fluid. The numbers of *S. bovis* and *Lactobacillus* (CFU/ml) were determined using the method described by Ogimoto and Imai (1981). The selective media used were mBA and MRS agar (see Section 3.1). The media roll tubes were inoculated in triplicate for each dilution and incubated at 38.5°C for 2 (*Lactobacillus*) or 3 days (*S. bovis*). Anaerobic manipulations were based on those described by Hungate (1969).

3.5 Diarrhoea score

A scoring system of 0 to 4 based on faecal consistency was used to describe the severity of diarrhoea in animals: 0, formed pellets; 1, non-formed pellets, very soft; 2, faeces containing small amount of water-like faeces; 3, faeces containing more than half water-like faeces; 4, running diarrhoea.

3.6 Statistical methods

All the antibody data were transformed by log(1+x) prior to statistical analysis. The data of numbers of rumen *S. bovis* and *Lactobacillus* were converted by log10 transformation prior to statistical analysis.

Statistical significance of the lactic acid producing capacity (Chapter 4) and the cross-reactivity index (Chapter 10) were analysed by using ANOVA (StatView SE+Graphics, Abacus Concepts, Inc., Berkeley, CA, 1987).

Statistical significance of the antibody response, feed intake, rumen pH and lactate concentration, faecal pH and dry matter content, diarrhoea score, liveweight

gain, and numbers of rumen *S. bovis* and *Lactobacillus* were tested by analysis of variance using SuperANOVA (Abacus Concepts, Inc., Berkeley, CA, 1989).

Statistical significance of the effect of multiple boosters on the levels and duration of antibody responses were analysed by Single Factor Factorial - One Repeated Measure ANOVA (see StateView SE+Graphics, Abacus Concepts, Inc., Berkeley, CA, 1987).

Correlation between the salivary antibody response, feed intake, rumen pH, faecal diarrhoea score, blood PCV, and the liveweight gain (used in Chapter 5) was analysed by using the Correlation Matrix; and the significance of the linear relationship was tested by using the Fisher's r to z test (StateView, Abacus Concepts, Inc., Berkeley, CA, 1994). Correlation and significance of the linear relationship of anti-*S. bovis* and anti-*Lactobacillus* IgG in saliva and serum (used in Chapter 7) were also analysed by the same methods as above.

Correlation between antibodies in saliva and serum (used in Chapter 6), and the correlation between the anti-*S. bovis* and anti-*Lactobacillus* IgG (used in Chapter 8) were analysed by using Bivariate Plots (StateView, Abacus Concepts, Inc., Berkeley, CA, 1994).

The following descriptors are used in the text, tables, and figures to indicate statistical significance as follows (unless there is a specific explanation):

ns	not significant $P > 0.05$
*	$0.01 < P < 0.05$
**	$P<0.01$

Chapter 4

Isolation and selection of *S. bovis* and *Lactobacillus* for vaccine preparation

4.1 Introduction

As reviewed in Chapter 2, a number of strains of *S. bovis* and several species of *Lactobacillus* (*Lactobacillus* spp.) have been found in the rumen of sheep and cattle. Each species of *Lactobacillus* may contain many strains. The antigenic variation between *S. bovis* strains isolated from ruminants has been demonstrated by Medrek and Barnes (1962), and Shu and Liu (1995b). Therefore, in order to develop a vaccine which will be effective against a number of strains which are present in the rumen and able to cause lactic acidosis, it is necessary to select suitable strain(s) or species for the vaccine preparation. However, there are limited data on the immunological cross-reactivity (Watson and Franklin, 1988) between various strains of the lactic acid-producing bacteria. Thus, it was not possible to select the optimum strain(s) of *S. bovis* for preparation of the vaccine on the basis of the degree of immunological cross-reactivity from available information and resources. The selection of the optimum strain(s) for preparing a vaccine which would be effective against all *Lactobacillus* spp. found in the rumen was likely to be more difficult because the optimum vaccine may need to contain a combination of multiple strains which may be from several species of *Lactobacillus*. As an initial study in the development of an immunisation strategy against lactic acidosis, it was considered inappropriate to include either multiple strains of *S. bovis* or one strain from each of the species of *Lactobacillus* in a vaccine. This approach would involve enormous expenses and might be unnecessary.

The review (Chapter 2) on the diversities of *S. bovis* and *Lactobacillus* spp. indicated a high degree of genetic homology between rumen *S. bovis* strains, and a much lesser genetic homology between the species of *Lactobacillus*. It is possible that the rumen strains with a high degree of genetic homology may have a high degree of antigenic homology. Therefore, it was decided to use a single pure strain of *S. bovis*, and a isolate of *Lactobacillus* (a mixture of strains of *Lactobacillus spp.* isolated from a rumen content sample collected from a cattle fed a grain-based ration) as vaccine antigens for further studies. It is also clear that lactic acidosis is due to the over production of lactic acid in rumen (Ahrens, 1967; Braun *et al.*, 1992). Therefore, the selection of the single strain of *S. bovis* and the isolate of *Lactobacillus* was made on the basis of lactic acid-producing capacity when cultured in a rumen fluid-based medium. Growth rate of the bacteria may be one of the important factors for their lactic acid-producing capacities. However, the bacteria in this study were selected by comparing the lactic acid concentration in the different cultures on the basis of its ease and convenience. This chapter reports on the isolation and selection of the bacteria for the use of vaccine preparation in the following experiments.

4.2 Materials and methods

4.2.1 Isolation procedures

Samples (around 250 ml each) of rumen content were taken though rumen cannula from 3 cattle fed a grain-based diet, and rumen fluid via a stomach tube from 2 sheep grazing native grass-based pasture. They were transported to the laboratory immediately, and diluted by the procedures described by Ogimoto and Imai (1981). A single strain of *S. bovis* was isolated from each sample and purified using the roll tube method described by Hungate (1969). The selective mBA medium (see Chapter 3) was used for the roll tube agar, while RF^+ broth was used to grow the single clone picked from the mBA agar. Incubation was performed at 38.5±0.5°C.

The isolate of *Lactobacillus* was obtained by the following procedures. An aliquot of 0.10 ml of the diluted rumen content (1: 1,000 in the dilution solution)

was inoculated into 5 ml MRS broth (see Chapter 3), then incubated at 38.5±0.5°C for 36 hours. After incubation, a 0.01 ml aliquot of the culture was transferred into 5 ml MRS broth and incubated for 36 hours. Then six more transfers and incubations in MRS and RF^+ broths were performed, respectively. Anaerobic manipulations of bacteria were based on those described by Hungate (1969).

4.2.2 Selection procedures

The lactic acid producing capacities of the strains or isolates were measured *in vitro* using the following procedures. Each of the strains of *S. bovis* or isolates of *Lactobacillus* were cultured in RF^+ broth at 38.5°C for 20~24 hours. The number of bacterial cells in the culture was determined by direct microscopic count method (see Chapter 3) and adjusted to $1x10^{10}$ cells/ml in RF^+ broth. A 0.10 ml aliquot of the culture was transferred into 5 ml of mRF-2 broth and incubated for 24 hours; and a sterilized aliquot of 0.10 ml of the culture was used as a control. After incubation, the broth was centrifuged at 10,000 g for 15 mins, and the supernatant was collected. The concentration of D- or L- lactate in the supernatant was analysed as described in Chapter 3. Three replications were carried out.

The strain of *S. bovis* and isolate of *Lactobacillus* with the greater lactic acid producing capacities were selected and stored at -20°C in RF^+ broth with addition of 20% (wt/vol) glycerol (Teather, 1982).

4.3 Results

Five single strains of *S. bovis* and 5 isolates of *Lactobacillus* were obtained from the rumen contents of sheep and cattle (Table 4.1). There were very low levels of lactate in the controls (the broth incubated with sterilised bacterial culture): 0.32±0.03 (Mean±SE) mmol/L of L-lactate and 0.49±0.04 (Mean±SE) mmol/L of D-lactate, respectively. Table 4.2 summarises the results of lactate concentration in the mRF-2 broth after 24 hour's incubation with the bacterial cultures. The concentration of L-lactate in the broth incubated with *S. bovis* cultures ranged from

12.12 to 15.58 mmol/L, while the D-lactate ranged from 0.24 to 0.42 mmol/L. The Sb-5 culture had the highest ($P<0.01$) concentration of L- and D- lactate among the 5 strains of *S. bovis* tested. The isolates of *Lactobacillus* had L-lactate concentration ranging from 9.36 to 12.66 mmol/L, while the D-lactate ranged from 15.64 to 41.40 mmol/L. The broth incubated with LB-27 isolate had more L- and D-lactate than the other 4 isolates of *Lactobacillus* ($P<0.01$).

Table 4.1 The single strains of *S. bovis* and the isolates of *Lactobacillus* obtained from different animals

Strain of *S. bovis*	Isolate of *Lactobacillus*	Source	Diet
SB-I	LB-25	Sheep	Native pasture
SB-II	LB-26	Sheep	Native pasture
SB-III	LB-27	Cattle	Grain-based diet
Sb-5	LB-28	Cattle	Grain-based diet
SB-V	LB-29	Cattle	Grain-based diet

Table 4.2 Lactate concentration (mmol/L) in mRF-2 broth after incubation (at 38.5°C for 20~24 hours) with either a strain of *S. bovis* or a isolate of *Lactobacillus*

	L-lactate (n=3)		D-lactate (n=3)	
	Mean	SE	Mean	SE
S. bovis				
SB-I	12.12	0.35	0.24	0.01
SB-II	12.89	0.06	0.27	0.01
SB-III	12.49	0.22	0.33	0.01
Sb-5	15.58[a]	0.17	0.42[a]	0.02
SB-V	13.43	0.33	0.30	0.02
Lactobacillus				
LB-25	10.50	0.32	15.64	0.23
LB-26	9.36	0.21	20.55	0.62
LB-27	12.66[b]	0.06	41.40[b]	0.16
LB-28	12.31	0.08	35.11	0.20
LB-29	10.44	0.23	31.31	0.88

Compared with the lactate levels in the broth incubated with the other *S. bovis* strains, the values within columns with superscript [a] are significantly higher ($P<0.05$).

Compared with the lactate levels in the broth incubated with the other *Lactobacillus* isolates, the values within columns with superscript [b] are significantly higher ($P<0.05$).

4.4 Discussion

Five single strains of *S. bovis* and 5 isolates of *Lactobacillus* were successfully obtained from the rumen contents collected from sheep and cattle. The results suggest that Sb-5 had the highest lactate producing capacity among the 5 single strains of *S. bovis* examined, while LB-27 had the highest lactate producing capacity among the 5 isolates of *Lactobacillus*. Therefore, the *S. bovis* strain Sb-5 and *Lactobacillus* isolate LB-27 were selected. The Sb-5 was used as the *S. bovis* vaccine antigenic strain in all the following experiments. In addition to Sb-5, LB-27 was used as the *Lactobacillus* antigens in the two cattle experiments.

It was essential to obtain a pure bacterial strain or isolate, to serve as the antigen(s) for inducing the specific immune responses for the development of a vaccine. The purity of the single strain of *S. bovis* Sb-5 was guaranteed by the foregoing isolation technique. The selective medium for *S. bovis*, mBA, was designed on the basis of the ability of *S. bovis* to utilize ammonium sulphate as its sole source of nitrogen. The other streptococci such as *S. faecalise, S. faecium, S. equinus, S. salivarius,* and *S. mitis*, which are closely related to *S. bovis*, are inhibited in mBA medium (Oragui and Mara, 1981). It was reported that over 97% of the total 541 colonies grown on mBA were *S. bovis*, and that routine confirmatory tests on colonies growing on mBA were likely to be unnecessary (Oragui and Mara, 1981). However, to further confirm the identity and purity of Sb-5, a number of routine identification tests were carried out, and the results demonstrated that Sb-5 had typical biological characteristics and substrate fermentation pattern of *S. bovis* (Ogimoto and Imai, 1981). Also, it was further confirmed by the results of DNA fingerprinting analysis of the PCR amplified 16s rRNA genes of the *S. bovis* strain Sb-5 (Klieve, personal communication).

To obtain a isolate of *Lactobacillus*, the MRS media was used for the isolation in this experiment. It is known that MRS medium supports good growth of *Lactobacillus* spp. in general, even those strains which show poor growth in other *Lactobacillus* isolation media (De Man *et al.*, 1960; Bridson, 1990). Therefore, the isolate probably contained a mixture of strains of different species of *Lactobacillus*, which were present in the rumen samples. MRS is widely used as selective growth media for the enumeration of *Lactobacillus*, however, complete selectivity for

Lactobacillus is not attained. Growth of some other lactic acid-producing bacteria (leuconostocs and pediococci) in MRS is known to occur (Bridson, 1990). To overcome this problem, a repeated selection based on low pH (<4.3) (Bridson, 1990) was used in the isolation procedure of this experiment. A pH of about 5.5 seems to represent the tolerance limit of many rumen microbial species, and a dramatic reduction in rumen pH can result in a rumen microbial population that is almost a monoculture of *Lactobacillus* within 24 hours (Dawson and Allison, 1988). Accordingly, a pH below 4.3 is likely to select specifically for *Lactobacillus*. During the course of the isolation of *Lactobacillus*, the MRS medium started with a pH 5.6 ± 0.1, and dropped below 4.3 after 16 hours incubation at 38.5°C. The culture was then maintained at 38.5°C for another 20 hours (total of 36 hours). Therefore, the purity of the isolate of *Lactobacillus* was further enhanced by repeated cultures in the MRS broth.