# Chapter 5

# Immunisation with either a live or a killed *S. bovis* vaccine against lactic acidosis in sheep

## 5.1 Introduction

One of the important considerations for developing an immunisation strategy against lactic acidosis was to select a suitable type of antigenic bacterial cells. Formalin killed bacterial cells have been widely used as antigens such as formalin killed streptococcal vaccine antigens described by Srivastava and Barnum (1981) and Holt *et al.* (1990). This suggests that formalin killed *S. bovis* cells may be suitable for the use as lactic acidosis vaccine antigens. Live microbial vaccines usually induce more durable and protective immune responses than do killed vaccines (Fuhrmann and Cebra, 1981; Holt *et al.*, 1990) but can be pathogenic. *S. bovis* is a normal inhabitant of the rumen, and does not cause lactic acidosis without the presence of high levels of soluble carbohydrates and suitable fermentation conditions (reviewed in Chapter 2). This suggests that using live *S. bovis* cells in an lactic acidosis vaccine may be possible.

It is clear that inducing a salivary antibody response is critical for any attempt to establish immunological control of rumen microbes, and immunisation primed intra-peritoneally (IP) has been successfully used to induce a salivary antibody response against rumen protozoan (Gnanasampanthan, 1993). However, at present IP is unlikely to be suitable for large scale commercial applications due to possible side effects such as adjuvant-induced mesenteric inflammatory lesions (Husband, 1985). Compared with IP, intramuscular injection is easier to apply and is considered to be commercially acceptable. Intramuscular immunisation is efficient

for inducing serum antibody responses in sheep (Watson, 1987) and the blood antibodies can be transported into saliva (see Chapter 2). Therefore, intramuscular immunisation was used in this experiment.

It is well-established that primary immunisation only induces a low level and short lasting antibody response, and that booster immunisation is necessary to stimulate a high-level and durable antibody response (Humphrey and White, 1970). However, an important consideration for developing an effective and practical immunisation strategy is to achieve the maximum antibody response with the minimal number of boosters. Clearly a single booster is ideal. Therefore, an important goal in developing a immunisation strategy is to induce the optimum immune response using a single booster following the primary immunisation.

As indicated in Chapter 1, the risk of lactic acidosis can be reduced through controlling the over production of lactic acid by antibiotics active against the rumen gram-positive bacteria such as *S. bovis*. It appears that *S. bovis* has a primary role in the onset of lactic acidosis (see Chapter 2). Accordingly, as an initial experiment to test the hypothesis described in Chapter 1, this study was based on the specific hypothesis that a salivary antibody response can be induced by immunisation with *S. bovis*, and that the over production of lactic acid in the rumen can be reduced by this antibody response. The experiment was also designed to determine whether live *S. bovis* cells induced a greater protective response than those killed by formalin treatment. Multiple boosters following the primary immunisation were to used in order to a) measure the maximum antibody response, and b) compare the levels of antibody response after the first booster with that after two or three boosters. Lactic acidosis was induced by suddenly introducing the animals to a pure wheat grain diet for three days. Protection against lactic acidosis was assessed by comparing the feed intake, rumen pH, rumen lactate concentration, and severity of diarrhoea in different treatment groups.

## 5.2 Materials and methods

### 5.2.1 Vaccine preparation

Killed-Sb — A killed-Sb bacterial suspension was obtained by formalin treatment based on the method described by Holt *et al.* (1990). The bacterial pellet described in Chapter 3 was resuspended in an equal volume of sterile PBS. Formaldehyde was added to the suspension to give a final concentration of 0.48%. The suspension of bacteria was held at 4°C for 16-20 hours and then checked for viability by culturing an aliquot of the suspension. The formalin treated cells were then washed twice in sterile PBS, and the number of bacterial cells were determined by direct microscopic count method (Gilstrap *et al.*, 1983) and adjusted to $1x10^{10}$ cells/ml in sterile PBS.

Live-Sb — The bacterial pellet described in Chapter 3 was washed twice in sterile PBS by suspension and centrifugation. The bacterial cell count was adjusted to $1x10^{10}$ cells/ml in sterile PBS.

Each vaccine batch was prepared using 7.0 ml of bacterial suspension containing $10^{10}$ cells/ml (Killed-Sb or Live-Sb) emulsified with 7.0 ml of Freund's complete adjuvant (Sigma Chemical Company), for the primary immunisation, and with 7.0 ml of Freund's incomplete adjuvant (Sigma Chemical Company) for the boosters. Each injection volume was 2 ml.

### 5.2.2 Experimental procedures

Fifteen 2-year old Merino wethers were selected from the UNE sheep flock at Kirby on the basis of health and appearance. Animals were run as a single flock, under paddock conditions, grazing improved phalaris/rye grass pastures before being brought into the animal house for grain feeding. The trial was conducted during the months of November 1993 to February 1994. There was regular rainfall during this

period and the pasture averaged approximately 2.5~4 t/ha green feed throughout the experiment.

The major experimental procedures are summarised in Table 5.1. The 15 sheep were randomly allocated to 3 treatment groups prior to immunisation: Killed-Sb group (n=5), immunised by formalin killed *S. bovis* vaccine; Live-Sb group (n=5), immunised by live *S. bovis* vaccine; and unvaccinated control group (n=5). Primary immunisation (on Day 0) was administered intramuscularly in the left medial thigh of sheep, while booster injections (on Days 28, 42, 56) were given intramuscularly in the right medial thigh (Watson, 1987). On day 62 (6 days after the third booster), the sheep were penned individually, and offered 600 g/day of chaffed lucerne hay with access to fresh water at all times. On the next day (Day 63), each sheep was fed 1,400 g wheat and on Days 64 and 65, they were offered 600 g wheat/sheep/day. The high dose (1,400 g/sheep/day) of wheat was used to ensure that acute lactic acidosis was induced. From Day 66 to 75, animals were offered 600 g/day of wheat and 600 g chaffed lucerne hay (/sheep/day) in two separate containers, while only chaffed lucerne hay (600g/sheep/day) was offered to animals eating below 100 g wheat/sheep/day (average intake of the group of animals) or if the average rumen pH dropped lower than 5.2. The diet was offered around 9.30 am daily after the uneaten feed was removed and weighted.

Samples of saliva from sheep were taken immediately prior to the primary immunisation (on Day 0). Further samples were taken on Days 42 and 56 (prior to each booster injection) and on Day 63 (prior to offering feed). The samples were used for the analysis of antibody concentration in saliva. Feed intake was measured daily after the animals were penned individually. Rumen fluid was collected via a stomach tube in the early morning prior to offering feed on Days 63, 64, and 65 and the pH of rumen fluid was measured. An aliquot of the rumen fluid was collected on Days 63 and 65 for measuring L-lactate concentration.

Table 5.1 Timetable of major events, sample collections, and measurements

| Day | Diet | Major events | Sample | Measurement |
|---|---|---|---|---|
| 0 | Pasture | Primary immunisation | Saliva | Antibody |
| 28 | Pasture | 1st booster | | |
| 42 | Pasture | 2nd booster | Saliva | Antibody |
| 56 | Pasture | 3rd booster | Saliva | Antibody |
| 62 | Chaffed lucerne hay | Sheep penned individually | | |
| 63 | Pure wheat | Grain challenge | Saliva, rumen fluid | Antibody, feed intake, rumen pH, L-lactate, diarrhoea score |
| 64 | Pure wheat | Grain challenge | Rumen fluid | Feed intake, rumen pH, diarrhoea score |
| 65 | Pure wheat | Grain challenge | Rumen fluid | Feed intake, rumen pH, rumen L-lactate, diarrhoea score |
| 66-75 | Chaffed lucerne hay or chaffed lucerne hay and wheat | Post challenge observation | | Feed intake, diarrhoea score |

## 5.3 Results

### 5.3.1 Antibody responses

Low levels of anti-*S. bovis* antibodies were detected in the saliva from all animals on Day 0 (prior to immunisation), and the control in the period Day 0-63 (Table 5.2).

Compared with the control group, higher levels ($P<0.01$) of salivary antibody concentrations were observed in the immunised animals from Day 42 (14 days after the first booster). The antibody concentration after the third booster in the sheep immunised with the live vaccine was significantly higher than that in the animals immunised with the killed vaccine ($P<0.05$).

Compared with the antibody level prior to immunisation, the salivary antibody concentrations in immunisation groups increased significantly after the first booster ($P<0.01$). There was a further increase in the antibody concentration after the second and the third booster ($P<0.05$), respectively. The salivary antibody concentrations in the control group remained unchanged ($P>0.05$) during the observation period.

Table 5.2 Mean salivary total antibody concentration (units/ml) of non-immunised sheep and sheep immunised with a vaccine containing either killed or live *S. bovis* cells. Primary immunisation was administered on Day 0, and boosters were given on Days 28, 42, and 56

| Day | Killed-Sb (n=5) | | Live-Sb (n=5) | | Control (n=5) | |
|---|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | Mean | SE |
| 0 | 2.6 $^{a}$ | 1.5 | 5.3 $^{a}$ | 1.0 | 5.7 $^{a}$ | 1.3 |
| 42 | 24 $^{b}$ | 3.4 | 23 $^{b}$ | 2.3 | 4.5 $^{a}$ | 1.3 |
| 56 | 53 $^{c}$ | 11 | 51 $^{c}$ | 13 | 5.0 $^{a}$ | 1.2 |
| 63 | 106 $^{d}$ | 11 | 197 $^{e}$ | 35 | 6.0 $^{a}$ | 1.1 |

Values within columns with different superscripts are significantly different ($P<0.05$).
Values between columns with different superscripts are significantly different ($P<0.05$).

### 5.3.2 Feed intake and mortality of animals

On Day 62 each of the animals ate 600 g chaffed lucerne hay. On Day 63 each of the animals in different treatment groups ate 1400 g of wheat. The average intake of wheat decreased significantly on the following 2 days ($P<0.01$) and no significant difference in the intake of wheat was found between treatment groups ($P>0.05$) (Table 5.3).

From Day 66 animals in the control and Killed-Sb groups were withdrawn from wheat and fed with chaffed lucerne hay only, because on Day 65 the average wheat intake in the groups dropped below 100 g/day and/or the average rumen pH dropped lower than 5.2 (see Section 5.2.2; Tables 5.3 and 5.5). The animals in the Live-Sb group were not completely withdrawn from wheat until Day 68 (see Section 5.2.2). One animal in the control group died on day 67, and an animal in the Killed-Sb group died on day 70 (Table 5.4). There was no significant difference between the average (over the period Day 66 to 75) feed intakes of the surviving animals in different treatment groups ($P>0.05$). However, significant treatment effects on feed intake were found on Days 66 and 67. On Day 66, the mean intake of Live-Sb group (188 g wheat + 257 g chaffed lucerne hay/sheep) was significantly higher than the control (43 g chaffed lucerne hay/sheep) ($P<0.05$), while the mean intake of Killed-Sb group (300 g chaffed lucerne hay/sheep) also tended to be higher than the control ($P=0.050$). On Day 67, the mean intake of the Live-Sb group (92 g wheat + 508 g chaffed lucerne hay/sheep) was significantly higher than the Killed-Sb (312 g chaffed lucerne hay) and the control (180 g chaffed lucerne hay/sheep) groups ($P<0.01$). From Day 68 to the end of the experiment (Day 75), all the animals were only fed with chaffed lucerne hay and there was no significant difference in the feed intake between treatment groups ($P>0.05$). The feed intakes (from Days 66 to 75) of the surviving animals are summarised in Figure 5.1.

Table 5.3 Mean feed intake (g/sheep) on Days 64 and 65 of non-immunised sheep and sheep immunised with a vaccine containing either killed or live *S. bovis* cells

| Day | Killed-Sb (n=5) | | Live-Sb (n=5 | | Control (n=5) | | P |
|---|---|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | Mean | SE | |
| 64 | 355 | 85 | 427 | 89 | 135 | 87 | >0.05 |
| 65 | 88 | 88 | 243 | 78 | 96 | 39 | >0.05 |

P>0.05, no significant difference between the three groups.

Table 5.4 Mortality after the grain feeding of animals in non-immunised sheep and sheep immunised with a vaccine containing either killed or live *S. bovis* cells

| | Killed-Sb (n=5) | Live-Sb (n=5) | Control (n=5) |
|---|---|---|---|
| Number of animals died | 1 | 0 | 1 |
| Mortality | 1/5 (20%) | | 1/5 (20%) |

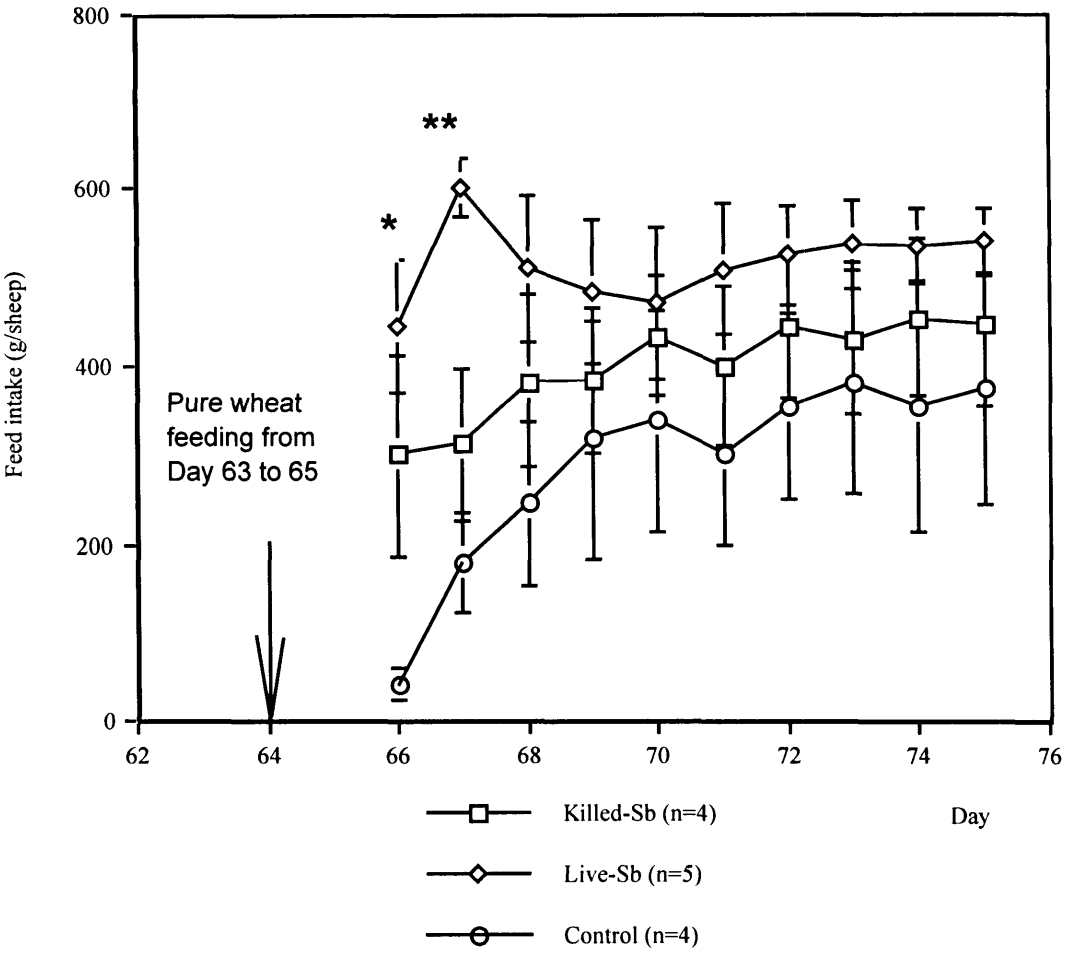



Figure 5.1 Mean daily feed intake (g/sheep) during the period following the grain challenge (Day 66 to 75) of non-immunised sheep and sheep immunised with a vaccine containing either killed or live *S. bovis* cells. Data are shown only for the surviving sheep in the immunised and control groups. Vertical error bars represent standard errors of the means

* On Day 66, the feed intake of Live-Sb group was significantly higher than that of the control ($P<0.05$); the intake of Killed-Sb group tended to be higher than the control ($P=0.050$). ** On Day 67, the feed intake of Live-Sb group was higher than those of the Killed-Sb and control groups ($P<0.01$)

### 5.3.4 Rumen pH and lactate concentration

The pH and L-lactate measurements of rumen fluid samples collected from immunised and control animals are summarised in Table 5.5. Compared with the pH at the beginning of grain feeding (Day 63), the mean rumen pH in all animals dropped significantly after the grain challenge ($P<0.01$). The mean L-lactate concentration in the animals increased significantly after the challenge ($P<0.01$). Significant differences between the rumen pH in the three treatment groups were observed on days 64 and 65. The rumen pH in animals immunised with Live-Sb vaccine was the highest, while that of the control group was the lowest. A lower ($P<0.05$) rumen L-lactate concentration was observed in the animals immunised with the Live-Sb vaccine, compared with that of the animals in the control group.

Table 5.5 Mean rumen pH and L-lactate concentration (mmol/L) during the grain challenge of non-immunised sheep and sheep immunised with a vaccine containing either killed or live *S. bovis* cells. Data are presented as Least Square Means with standard error

| | Killed-Sb (n=5) | Live-Sb (n=5) | Control (n=5) | SE |
|---|---|---|---|---|
| *pH* | | | | |
| Day 63 | 7.04 | 7.22 | 7.28 | 0.16 |
| Day 64 | 5.82 $^{a}$ | 6.32 $^{b}$ | 5.38 $^{c}$ | 0.13 |
| Day 65 | 5.38 $^{a}$ | 6.22 $^{b}$ | 5.02 $^{c}$ | 0.11 |
| *L-lactate* | | | | |
| Day 63 | NA | 0.98 | 1.04 | 0.64 |
| Day 65 | NA | 2.89 $^{b}$ | 15.2 $^{c}$ | 2.79 |

NA, data not available. Values within rows with different superscripts are significantly different ($P<0.05$).

### 5.3.5 Diarrhoea score

During the period of grain challenge, the diarrhoea score of the animals immunised with the live-Sb vaccine was significantly lower ($P<0.01$) than the animals immunised with the killed-Sb vaccine or the control animals on Days 63 and 64 (Table 5.6). Following the grain challenge, lower ($P<0.05$) diarrhoea scores in the immunised groups were observed on Day 67, compared with the control (Figure 5.2). The diarrhoea score (Table 5.6 and Figure 5.2) changed significantly over time (from Days 62 to 75, $P<0.01$).

Table 5.6 Mean diarrhoea scores during the grain challenge period of non-immunised sheep and sheep immunised with a vaccine containing either killed or live *S. bovis* cells

| Day | Killed-Sb (n=5) | | Live-Sb (n=5 | | Control (n=5) | |
|---|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | Mean | SE |
| 62 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| 63 | 2.0 [a] | 0.4 | 0.4 [b] | 0.2 | 2.4 [a] | 0.2 |
| 64 | 2.6 [a] | 0.5 | 0.4 [b] | 0.2 | 2.8 [a] | 0.5 |
| 65 | 1.0 | 0.5 | 0.6 | 0.2 | 1.6 | 0.6 |

Values within rows with different superscripts are significantly different ($P<0.01$).

## 5.4 Discussion

One of the main aims in this study was to examine whether immunisation using a vaccine containing rumen *S. bovis* cells can elicit a salivary antibody response. It is known that *S. bovis* is a normal inhabitant at the rumen and intestine (Latham *et al.*, 1979; Stewart and Bryant, 1988). Although Gnanasampanthan (1993) demonstrated that a specific saliva antibody response to rumen protozoa can

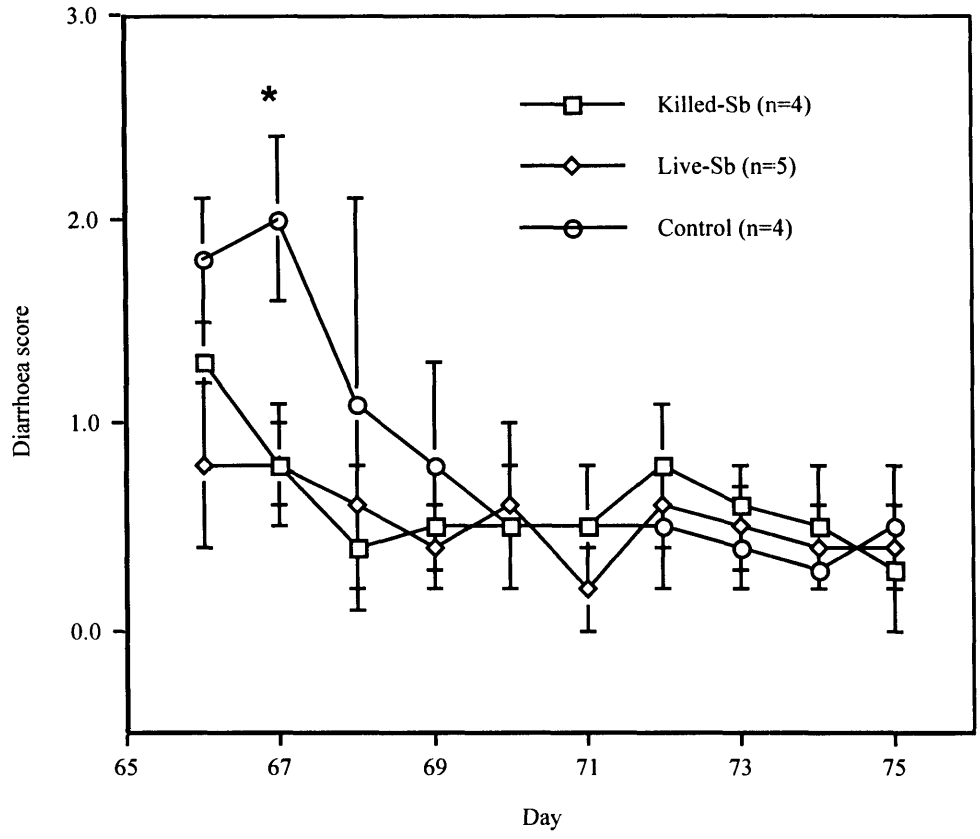



Figure 5.2 Mean daily diarrhoea score during the period following grain challenge (Day 66 to 75) of non-immunised sheep and sheep immunised with a vaccine containing either killed or live *S. bovis* cells. Data are shown only for the surviving sheep in the immunised and control groups. Vertical error bars represent standard errors of the means

* The diarrhoea scores of animals in the Live-Sb and Killed-Sb groups were significantly lower than the control on Day 67 ($P<0.05$)

be induced by immunisation, it was not clear at the commencement of this work whether early contact with *S. bovis* and presentation of the antigens of these organisms in the gut would lead to immune tolerance or unresponsiveness (Humphrey and White, 1970; Burnet, 1976). It was therefore very interesting that there was a significant rise in the concentration of salivary anti-*S. bovis* antibody in sheep immunised with either formalin killed or live *S. bovis* vaccines. This answered one of the key questions for developing an immunisation strategy against lactic acidosis, which was, that salivary anti-*S. bovis* antibody response could be induced by immunisation using a vaccine containing whole cells of *S. bovis*. Low levels of anti-*S. bovis* antibodies were detected in the saliva samples collected from all animals prior to immunisation. It was not clear, from the current experimental results, whether these low levels of antibodies reflected (1) the presence of natural non-specific antibodies or other immunoglobulin (Humphrey and White, 1970) which cross-reacted with *S. bovis*, or (2) the presence of specific antibodies which were induced by *S. bovis* during the normal contact with *S. bovis*.

As discussed at the beginning of this chapter, at least one booster immunisation is necessary to obtain a high level and lasting antibody response. An important goal in developing an immunisation strategy is to achieve the maximum immune response from a single booster following the primary immunisation. The significant increase in antibody concentration after each booster immunisation indicates that under these experimental conditions a single booster following the primary immunisation did not induce the maximum antibody response. This finding raises the question whether the 2nd and 3rd boosters are necessary to provide adequate protection against lactic acidosis. This issue is further explored in experiments reported later in this thesis.

Another component of the hypothesis is that the over production of lactic acid in the rumen can be reduced by elevated salivary anti-*S. bovis* antibodies. The over production and accumulation of lactic acid in the rumen causes the pH to drop to a low level, which adversely affects rumen function and can lead to lactic acidosis (Ahrens, 1967; Braun *et al.*, 1992). Compared with the control, the immunised animals had a significantly higher rumen pH, and lower L-lactate concentration during the grain challenge period. This suggests that less lactic acid was produced

in the rumen of immunised animals and the results directly support the hypothesis. The lower rumen L-lactate concentration of the immunised animals may be due to a reduced number and/or reduced activity of *S. bovis*. The possible mechanism may be similar to the immunisation against rumen protozoa (Gnanasampanthan, 1993), which is, that the antibodies specifically bind to these organisms and actively reduce their growth and/or interfere with other biological functions leading to a decrease in lactic acid production (Horacek *et al.*, 1977). The rapid increase in number of *S. bovis* in the rumen is one of the major factors contributing to the over production and uncontrolled build up of lactic acid (see Chapter 2). The effect of immunisation on the number of rumen *S. bovis* was not measured in this initial experiment but is reported in the later experiments described in Chapters 8 and 9.

Two of the typical clinical signs of lactic acidosis is decreased feed intake and the severe diarrhoea (Dawson and Allison, 1988; Feedlot Advisory Unit, 1990; Braun *et al.*, 1992). Therefore, a further positive effect of immunisation against lactic acidosis was also indicated by the significantly higher feed intake and less severity of diarrhoea particularly in those sheep immunised with the live vaccine. In the present experiment severe diarrhoea was observed within 24 hours, which is similar to the observation of Allison *et al.* (1975) when sheep were over-fed with grain. The results (Allison *et al.*, 1975; Shu *et al.*, unpublished data) also indicated that the appearance of severe diarrhoea was accompanied by low faecal pH, a high level of lactic acid, and the rapid build up of *S. bovis* in the hindgut. Accordingly, the lower severity of diarrhoea observed in the immunised sheep may have been partially due to antibodies from the blood circulation leaking into the hindgut and/or a local immune response in the gut system, which reduced the production of lactic acid by controlling the local *S. bovis* population in the hindgut. Unfortunately, this thesis was not able to explore the immunity in the gut system but concentrated on the results relating to the salivary antibody response.

A greater antibody response, higher rumen pH, higher feed intake, and lower diarrhoea score was observed in the group immunised with the live vaccine, compared with the group immunised with the killed vaccine. This is consistent with previous results showing that vaccines containing live antigen induce greater immunity and protective immune responses than vaccines containing killed antigen (Fuhrmann and Cebra, 1981; Holt *et al.*, 1990). One of the possible reasons for a

lower protective response induced by the killed vaccine could be that the formalin treatment caused some changes in the antigens of the vaccine *S. bovis* strain which may result in lower antigenicity, as it has been suggested by Holt *et al.* (1990) that an *in vitro* treatment for killing *S. suis* may destroyed the surface antigen. Multiplication of live *S. bovis* cells at the injection site was also a possibility but not being investigated in this thesis. No difference in the diarrhoea score between the control and the animals immunised with the formalin killed-Sb vaccine was found. This observation is not consistent with the higher antibody concentration and rumen pH in the immunised animals compared with the control. The reasons for this are not fully understood. One of the possibilities may be that the formalin killed vaccine stimulated little protective immune response in the hindgut.

All the animals in the group immunised with the live vaccine survived after the grain feeding, while one animal died due to lactic acidosis in both the Killed-Sb (8 days after introduction of grain) and control (5 days after introduction of grain) groups. There were too few animals in the experiment to determine if the pattern of deaths in the three treatment groups were statistically significant. This experiment was also not set out to determine, in detail, the possible adverse effect on the animal that could result from immunisation with live *S. bovis*. However, no animal health issues emerged during the period of immunisation other than those linked to grain poisoning, implying that it may be possible to develop a live vaccine without the risk of adverse effects on the animal.

In conclusion, this experiment demonstrated that a specific salivary antibody response can be induced by immunisation with *S. bovis* and that the over production of lactic acid in rumen can be reduced by the salivary antibody response to *S. bovis*. The results support the hypothesis that the risk of lactic acidosis in sheep can be reduced by immunisation against *S. bovis*, and also suggests that a vaccine containing live *S. bovis* cells may give better protection against lactic acidosis than a killed vaccine.

# Chapter 6

# Immunisation with a *S. bovis* vaccine primed either intramuscularly or intraperitoneally against lactic acidosis in sheep

## 6.1 Introduction

In Chapter 5 it was demonstrated that immunisation with live *S. bovis* induced a significant salivary antibody response and protected sheep against the development of lactic acidosis. In these studies, an intramuscular injection was used for the primary and booster immunisation. However, salivary glands belong to the mucosal immune system and most of the effective immunisation protocols for inducing mucosal immune responses (see Chapter 2) have used the intraperitoneal route for primary immunisation and oral and/or other mucosal immunisation for the booster(s), with inactive non-replicating antigen/vaccines (Pierce and Gowans, 1975; Husband, 1978; 1985; Husband *et al.*, 1979). The intraperitoneal immunisation has also been used successfully to induce a significant salivary antibody response against rumen protozoan (Gnanasampanthan, 1993). Therefore, in order to evaluate the efficacy of intramuscular immunisation, it was necessary to compare the efficacy of immunisation primed intramuscularly and intraperitoneally.

In this Chapter, an immunisation regime primed intraperitoneally (IP) was compared with one primed intramuscularly (IM). The boosters were based on those described in Chapter 5, and supplemented with oral immunisation when giving the 2nd and 3rd intramuscular injections. The comparison was based on the salivary antibody response and associated effects on feed intake, rumen pH, diarrhoea, and blood packed cell volume (PCV). The possible side effects of immunisation with

live *S. bovis* on animals were also examined by comparing the liveweight gain of the immunised and control sheep. Another objective of this experiment was to verify that the risk of lactic acidosis was reduced by immunisation against *S. bovis*. This study used more animals, a longer period of grain feeding, and measured a greater number of response variables than did the study reported in Chapter 4. To monitor response to grain feeding over a longer period, however, the pure wheat challenge described in Chapter 5 was considered unsuitable. In order to maximise the number of animals surviving through the experiment, two diets were used to induce lactic acidosis as follows. Firstly, the animals were suddenly introduced to a diet containing 75% wheat which was fed for 6 days. The diet was then switched to a ration containing 90% wheat for 4 days. After the challenge, the ration was again changed to the 75% wheat diet for another 21 days.

## 6.2 Materials and methods

### 6.2.1 Vaccine preparation

Each vaccine batch was prepared by emulsifying 40.0 ml of Sb-5 suspension (see Chapter 3) with 40.0 ml of Freund's complete adjuvant for the primary immunisation, and 40.0 ml of Freund's incomplete adjuvant for boosting. The suspension of formalin killed Sb-5 cells for oral administration was prepared using the method described in Chapter 5.

### 6.2.2 Experimental procedures

Forty-five approximately 6 month old Merino wethers were selected from the UNE sheep flock at Kirby on the basis of liveweight. Animals were run as a single flock, under paddock conditions, grazing improved phalaris/rye grass pastures prior to grain challenge. The trial was conducted during the months of May 1994 to August 1994. There was little rainfall during this period and only standing dry feed was available in the paddock.

The experimental procedures are summarised in Table 6.1. Before immunisation, the 45 sheep were stratified for liveweight, and randomly allocated to 3 treatment groups: IM (n=15), immunisation primed intramuscularly (Watson, 1987); IP (n=15), immunisation primed intraperitoneally (Gnanasampanthan, 1993); and control (n=15), no vaccination. For groups IM and IP, the booster injections were given as described in Chapter 5 except that in the second and third boosters, 50% of the Sb-5 cells were killed by formalin and administered orally without any adjuvant, while the other 50% live Sb-5 cells with Freund's incomplete adjuvant were injected intramuscularly. The dose volume of the vaccine for the intraperitoneal immunisation is also 2 ml.

On day 71, all the animals were transferred into an animal house, penned individually, and offered 600 g chaffed lucerne hay/day/sheep with access to fresh water at all times (Table 6.1). From day 74, sheep were offered 800g/d of a wheat diet (75% wheat, 25% chaffed lucerne hay). On Day 80 the ration was changed to 600 g/d/sheep of 90% wheat diet (90% wheat, 10% chaffed lucerne hay) for the following 4 days (Day 80 to 83). After the 90% wheat feeding, all the animals were again fed with the 75% wheat diet at 800 g/day/sheep for Days 84-104. The grain diet was offered around 9.30 am daily after the previous day's uneaten feed was removed and weighted.

Samples of saliva were taken from all the sheep immediately prior to the primary immunisation (Day 0) (see Table 6.1). Further saliva samples were taken on Days 28, 46, 67, 73, 75, 77, 87 and 105 for measuring antibody concentrations. Feed intake was measured daily after the introduction of grain diet. Rumen fluid was collected via a stomach tube before feeding on Days 73, 75, 77, 80, 83, 87, 95, and 105 for measuring pH. Diarrhoea score was recorded before feeding from Day 73 to 105. Blood (around 5 ml) was collected before feeding by jugular venipuncture into a plasma vacutainer (Becton Dickinson Vacutainer Systems, Rutherford, New Jersey) for measuring PCV (Jain, 1986) on days 73, 75, 77, 80, 87, 95, and 105. All the animals were weighed on Days 0, 28, 67, 75, 81, 89, 97, and 105.

During statistically testing the differences in feed intake, PCV, and liveweight between treatment groups (see Chapter 3), a covariate was used: (1) the intake on Day 74 as a covariate for analysing the feed intake, (2) the PCV on Day 73 for

analysing the PCV, and (3) the liveweight on Day 0 for analysing the liveweight change from Day 0 to 75, while the liveweight on Day 75 for the liveweight change from Day 75 to 105).

Table 6.1 Timetable of major events, sample collections, and measurements

| Day | Diet | Major events | Sample | Measurement |
|---|---|---|---|---|
| 0 | Pasture | Primary immunisation | Saliva | Ab, LW |
| 28 | Pasture | 1st booster | Saliva | Ab, LW |
| 46 | Pasture | 2nd booster | Saliva | Ab |
| 67 | Pasture | 3rd booster | Saliva | Ab, LW |
| 71 | LH | Sheep penned individually | | |
| 73 | LH | | Saliva, rumen fluid, blood | Ab, pH, DS, PCV |
| 75 | 75% wheat 25% LH | | Saliva, rumen fluid, blood | Ab, Intake, pH, DS, PCV, LW |
| 77 | 75% wheat 25% LH | | Saliva, rumen fluid, blood | Ab, Intake, pH, DS, PCV |
| 80 | 90% wheat 10% LH | | Rumen fluid, blood | Intake, pH, DS, PCV |
| 81 | 90% wheat 10% LH | | | LW |
| 83 | 90% wheat 10% LH | | Rumen fluid | Intake, pH, DS |
| 87 | 75% wheat 25% LH | | Saliva, rumen fluid, blood | Ab, Intake, pH, DS, PCV |
| 89 | 75% wheat 25% LH | | | LW |
| 95 | 75% wheat 25% LH | | Rumen fluid, blood | Intake, pH, DS, PCV |
| 97 | 75% wheat 25% LH | | | LW |
| 105 | End of experiment | | Saliva, rumen fluid, blood | Ab, Intake, pH, DS, PCV, LW |

Ab, concentration of total immunoglobulins;
Intake, daily feed intake on grain based diet was measured;
DS, daily diarrhoea score (DS) on grain based diet was measured; pH, rumen pH;
PCV, packed cell volume (PCV) of plasma; LW, liveweight; LH, chaffed lucerne hay.

## 6.3 Results

Two sheep in the IP group disappeared from the paddock and were not found until the end of the experiment. Therefore, the results reported here are based on the data collected from the other 43 animals.

### 6.3.1 Antibody response

Low levels of anti-*S. bovis* antibodies were detected in the saliva samples taken from all animals on Day 0 (prior to immunisation), and the controls on Days 0 to 105 (Table 6.2).

Compared with the control group, higher levels ($P<0.01$) of salivary antibody concentrations were observed in the immunised animals from Day 46 (18 days after the first booster) to the end of the experiment. IM animals had significantly higher salivary antibody concentrations than the IP group on day 75 and 77 ($P<0.05$). No significant differences in salivary antibody levels between the IM and IP groups were observed on other days. The average (over the period of experiment after the first booster) salivary antibody concentration in the IM group was significantly higher ($P<0.05$) than that of the IP group.

Compared with the antibody level prior to immunisation, the salivary antibody concentrations in both IM and IP groups increased significantly after the first booster (Day 46) ($P<0.01$). In the IM group, no significant difference was observed between the antibody levels on Day 46 (after the first booster) and Day 67 (after the 2nd booster) or Day 73 (after the 3rd booster) ($P>0.05$). However, significantly higher antibody levels were observed on Days 75, 77, and 87 (after the animals were challenged with grain) ($P<0.05$). In the IP group, no difference ($P>0.05$) was observed between the antibody levels on Day 46 (after the 1st booster) and Day 67 (after the 2nd booster), while the antibody concentrations after the 3rd booster were higher ($P<0.05$) than that on Day 46 (after the 1st booster). The antibody concentration in the control animals also increased ($P<0.01$) after grain feeding.

Table 6.2 Mean saliva antibody concentration (units/ml) of non-immunised sheep and sheep primed either intramuscularly or intraperitoneally. Primary immunisation was administered on Day 0, and the boosters were given on Days 28, 46, and 67

| Day | IM (n=15) | | IP (n=13) | | Control (n=15) | | P1 |
|---|---|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | Mean | SE | |
| 0 | 5.6 | 1.5 | 6.8 | 1.9 | 6.6 | 1.9 | >0.05 |
| 28 | 9.2 | 3.0 | 11 | 3.2 | 5.8 | 1.5 | >0.05 |
| 46 | 96 | 26 | 34 | 5.5 | 9.8 | 1.9 | <0.01 |
| 67 | 99 | 30 | 68 | 33 | 4.0 | 0.9 | <0.01 |
| 73 | 160 | 44 | 76 $_a$ | 15 | 9.6 | 3.0 | <0.01 |
| 75 | 224 $_a$ $^b$ | 34 | 103 $_a$ $^b$ | 26 | 18 | 4.6 | <0.01 |
| 77 | 238 $_a$ $^b$ | 73 | 124 $_a$ $^b$ | 60 | 18 | 4.2 | <0.01 |
| 87 | 307 $_a$ | 67 | 233 $_a$ | 51 | 16 | 3.4 | <0.01 |
| 105 | 116 | 30 | 90 $_a$ | 19 | 18 | 3.4 | <0.01 |
| P2 | <0.01 | | <0.01 | | <0.01 | | |

P1, significance of the differences between columns. All values in IM and IP groups (since Day 46) are significantly higher than those in the control; within the IM and IP groups, the values between the columns with a superscript "$^b$" are significantly different ($P<0.05$).

P2, significance of the differences within columns. The differences between values within columns are significant ($P<0.01$). Further compared with the antibody levels induced after the 1st booster (on Day 46), values (antibody concentrations after the 2nd and 3rd boosters) within columns with subscript "$_a$" are significantly higher ($P<0.05$).

### 6.3.2 Feed intake

The mean daily feed intake of sheep during the period of grain feeding is summarised in Figure 6.1. Significant treatment effect on feed intake was only found on Days 77 and 78: the mean feed intake of IM group was significantly higher than the intake of IP and control animals on Days 77 ($P<0.01$) and 78 ($P<0.05$). Average (over the whole period of experiment) intake between the three groups was not significantly different ($P>0.05$). A significant decrease in feed intake ($P<0.01$) in all animals was found after the first 6 days of grain feeding (75% wheat diet).

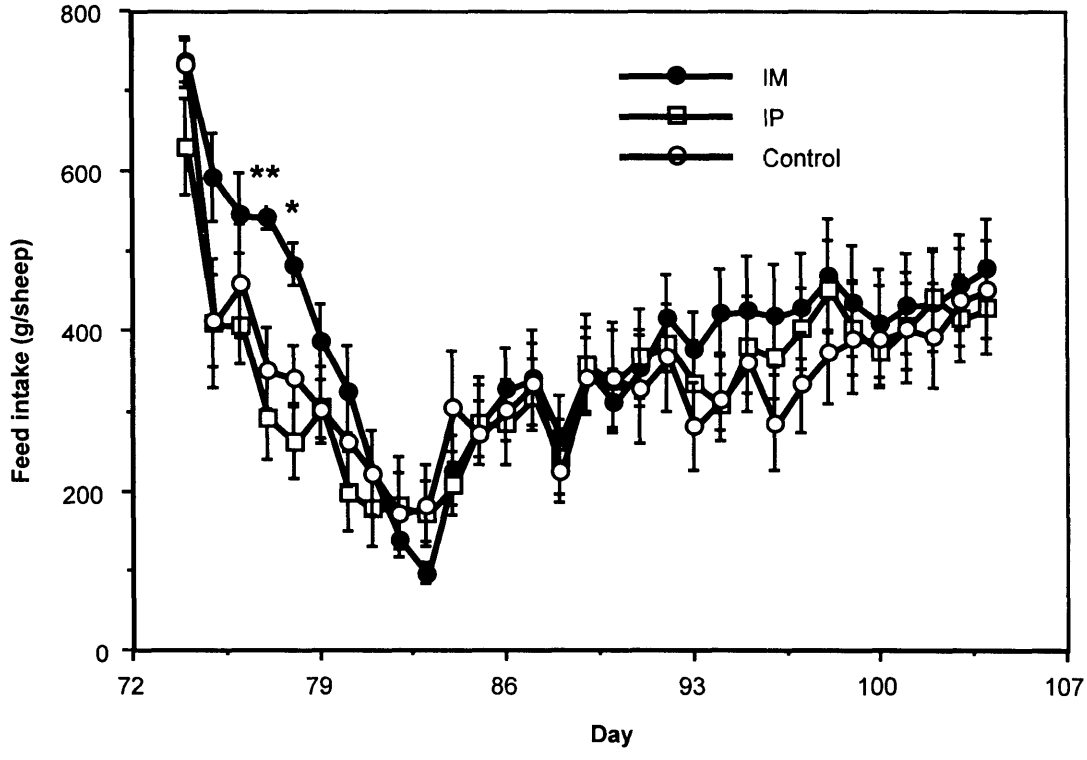



Figure 6.1 Mean daily feed intake (g/sheep) of non-immunised sheep and sheep primed either intramuscularly or intraperitoneally. Vertical error bars represent standard errors of the means. Data were from 15 animals in each of the IM and control groups, and from 13 IP animals
** The feed intake of IM group was significantly higher than those of IP and control animals on Day 77 ($P<0.01$). * The feed intake of IM group was significantly higher than those of IP and control animals on Day 78 ($P<0.05$)

### 6.3.3 Rumen pH, diarrhoea score, and blood PCV during grain feeding

The rumen pH in all of the three groups significantly decreased over the first 6 days of grain feeding ($P<0.01$) (Figure 6.2). No significant differences were found in rumen pH between treatment groups ($P>0.05$).

Figure 6.3 summarises the result of the diarrhoea scoring. A increase ($P<0.01$) in diarrhoea score occurred in all animals after grain feeding. Average (over the whole period of experiment) diarrhoea scores of both IM and IP groups were significantly lower than that of the control ($P<0.01$), and there were no differences between IM and IP groups ($P>0.05$). The highest diarrhoea scores were found during the first 6 days of wheat feeding ($P<0.01$), while the lowest diarrhoea scores were found at the end of experiment ($P<0.01$).

The PCV in the control group increased 0.99% after grain feeding, while those in the IM and IP groups decreased 2.56 and 2.07%, respectively. Significant effects of immunisation on the changes of PCV are summarised in Table 6.3.

Table 6.3 Changes of blood PCV (%) during the period of grain feeding of non-immunised sheep and sheep primed either intramuscularly or intraperitoneally. Data are shown as Least Square Means (SE)

| IM | IP | Control | Significance | | |
|---|---|---|---|---|---|
| | | | --- | --- | --- |
| (n=15) | (n=13) | (n=15) | IM vs. Control | IP vs. Control | IM vs. IP |
| -2.56 | -2.07 | 0.99 | * | P=0.062 | ns |
| (1.04) | (1.14) | (1.06) | | | |

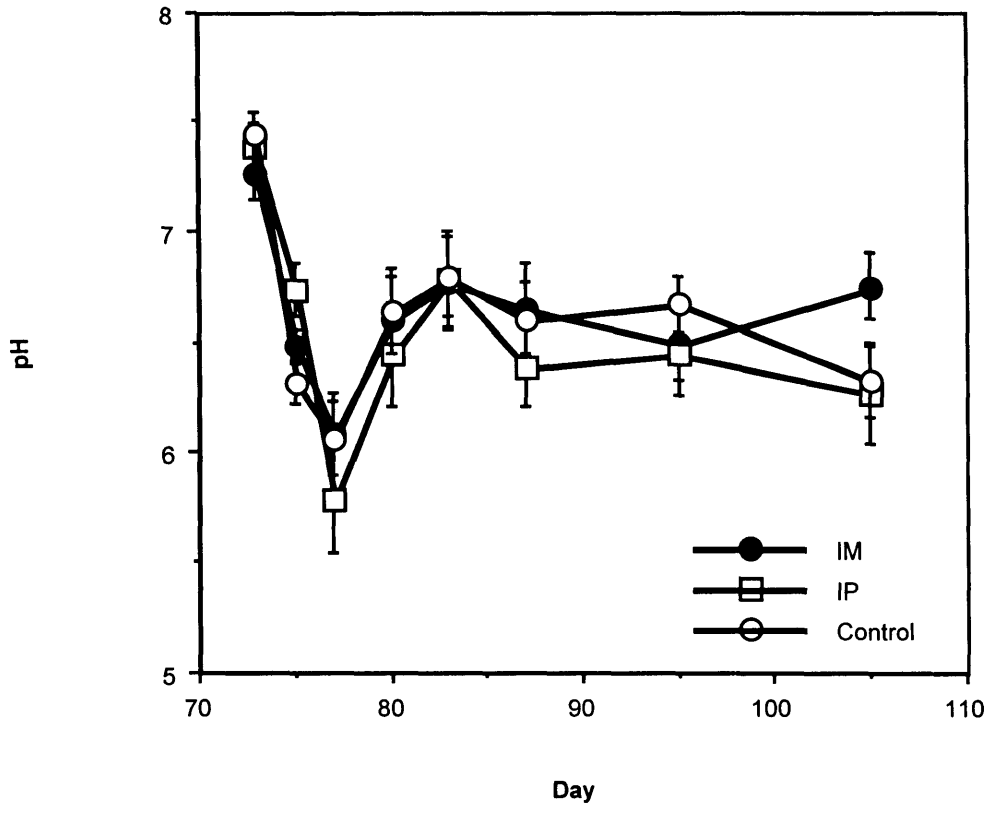



Figure 6.2 Mean rumen pH during the grain feeding period of non-immunised sheep and sheep primed either intramuscularly or intraperitoneally. Vertical error bars represent standard errors of the means. Data were from 15 animals in each of the IM and control groups, and from 13 animals in the IP group

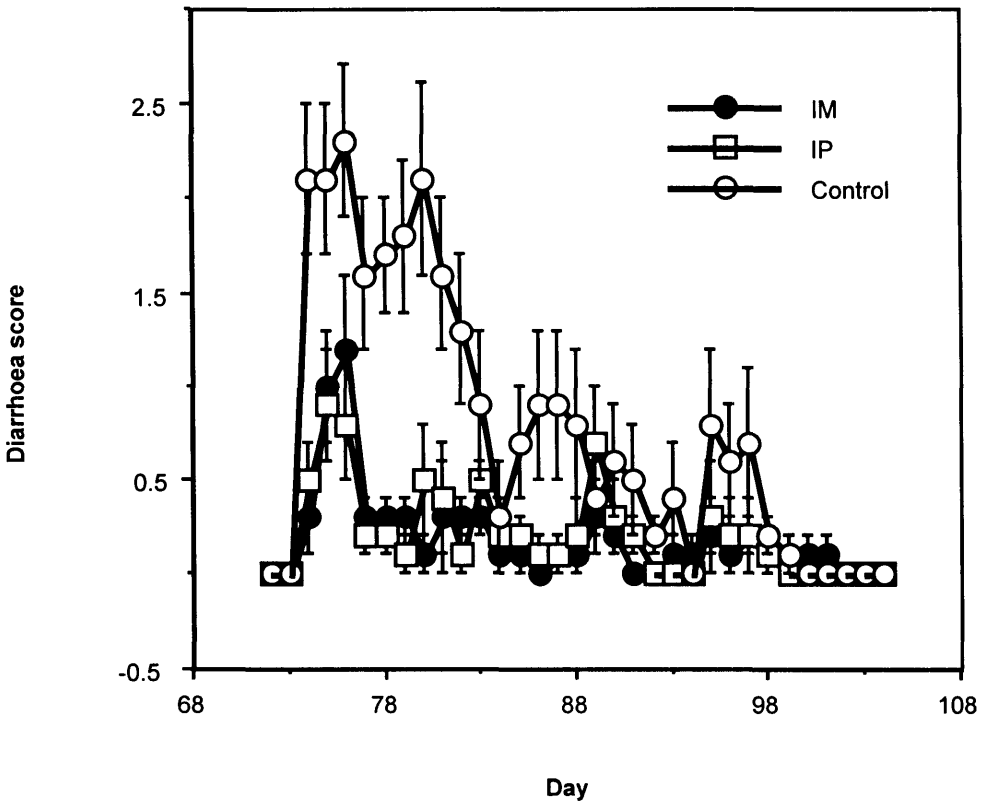



Figure 6.3 Mean diarrhoea score during the grain-feeding period of non-immunised sheep and sheep primed either intramuscularly or intraperitoneally. Vertical error bars represent standard errors of the means. Data were from 15 animals in each of the IM and control groups, and from 13 animals in the IP group

Diarrhoea scores in both IM and IP groups were significantly lower than in the control group ($P<0.01$), and there were no differences between IM and IP groups ($P>0.05$)

### 6.3.4 Liveweight gain

During the period Day 0-75 the average liveweight loss of IP group was significantly higher than those of the IM and the control groups ($P<0.05$) (Table 6.4). No significant treatment effect on liveweight gain from Day 75 to 105 was found ($P>0.05$).

Table 6.4 Liveweight change (kg/sheep) during the period Day 0-75 and Day 75-105 of non-immunised sheep and sheep primed either intramuscularly or intraperitoneally. Data are presented as Least Square Means (SE)

| Day | IM | IP | Control | Significance | | |
|---|---|---|---|---|---|---|
| | (n=15) | (n=13) | (n=15) | IM vs. Control | IP vs. Control | IM vs. IP |
| 0-75 | -1.73 | -3.76 | -1.22 | ns | ** | * |
| | (0.58) | (0.63) | (0.58) | | | |
| 75-105 | 0.18 | 0.14 | -0.61 | ns | ns | ns |
| | (0.74) | (0.84) | (0.76) | | | |

### 6.3.5 Correlations between the measurements during grain feeding

A negative linear correlation (r=-0.479, P<0.01) between the salivary antibody concentration and the diarrhoea score was found (Table 6.5 and Figure 6.4). There was a positive correlation (r=0.538, P<0.01) between the diarrhoea score and the change of PCV. Table 6.5 summarises the correlations between all the measurements made in this experiment.

Table 6.5 Correlations and the significance of the linear relationships between the measurements[a] of antibody, feed intake, rumen pH, diarrhoea score, PCV, and liveweight change during the period of grain feeding in non-immunised sheep and sheep primed either intramuscularly or intraperitoneally.

| | Antibody | Intake | Rumen pH | Diarrhoea | PCV | LWG |
|---|---|---|---|---|---|---|
| Antibody | 1.000 | ns | ns | ** | P=0.056 | ns |
| Intake | -0.021 | 1.000 | ns | ns | ns | ** |
| Rumen pH | 0.115 | -0.272 | 1.000 | ns | ns | * |
| Diarrhoea | -0.479 | -0.111 | -0.039 | 1.000 | ** | ** |
| PCV | -0.293 | -0.103 | -0.199 | 0.538 | 1.000 | ** |
| LWG | -0.035 | 0.619 | -0.381 | -0.469 | -0.486 | 1.000 |

a Antibody, average saliva antibody concentration over the period of grain feeding;
Intake, total feed intake over the period of grain feeding;
Rumen pH, average rumen pH of over the period of grain feeding;
Diarrhoea, total diarrhoea score over the period of grain feeding;
PCV, change of blood PCV over the period of grain feeding;
LWG, liveweight gain over the period of grain feeding.

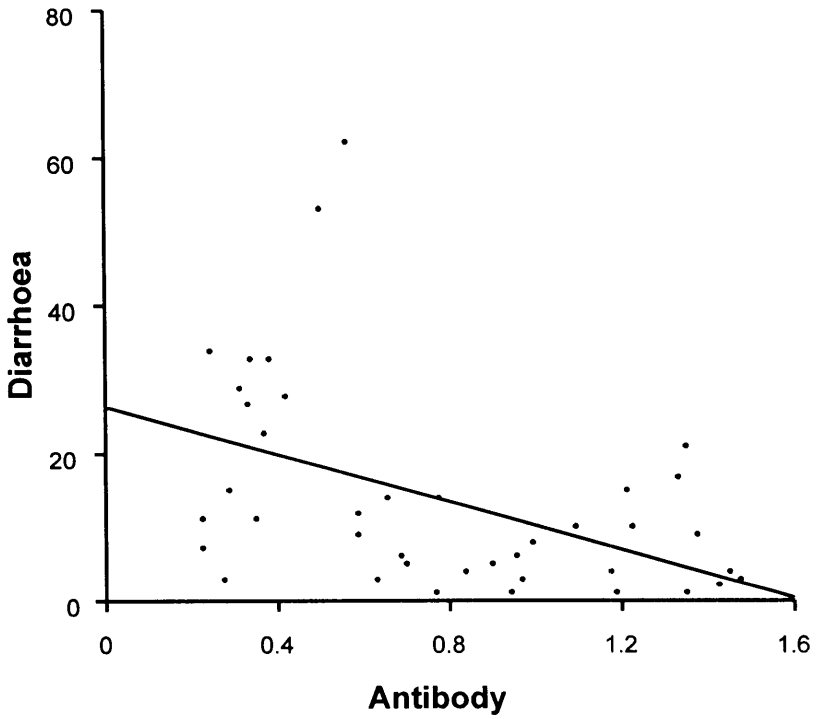



Figure 6.4 The overall relationship between the salivary antibody concentration and diarrhoea score in non-immunised sheep and sheep primed either intramuscularly or intraperitoneally. Antibody is the average saliva antibody concentration over the period of grain feeding, and diarrhoea is the total diarrhoea score over the period of grain feeding. The antibody data in the figure was transformed by log (1+x)

## 6.4 Discussion

This experiment shows that either the IM or the IP immunisation induced a significant salivary antibody response against *S. bovis*. Although a treatment effect on rumen pH was not observed in this experiment, the positive effect of immunisation against lactic acidosis was demonstrated by the higher feed intake and the lower diarrhoea score of immunised animals, which were consistent with the results in Chapter 5. It is clear that haemoconcentration, resulting from the loss of intravascular fluid and from osmotic diarrhoea, is another characteristic affect of lactic acidosis (reviewed in Chapter 2). The lower increase of PCV (blood packed cell volume percentage) reflected less loss of the intravascular fluid (Jain, 1986). Therefore, the lower increase of PCV of the immunised animals are consistent with reduced diarrhoea which is a positive effect of immunisation against lactic acidosis. These results further support the hypothesis that a salivary antibody response can be stimulated in sheep by immunisation against *S. bovis*, and immunisation against *S. bovis* is able to reduce the risk of lactic acidosis (see Chapter 5).

A significant difference in rumen pH between treatment groups was not demonstrated in this experiment, which is a direct measurement to confirm the hypothesis that the over-production of lactic acid in rumen can be reduced by the salivary antibody response. These results could be due to (1) the grain challenge was not serious enough to induce an excessively high level of lactic acid accumulation in the rumen, as shown in Figure 6.2 that the lowest average rumen pH was higher than 5.7; (2) the lowest rumen pH in the animals, with the highest level of lactic acid concentration in the rumen, was not detected because of the limitation of sampling frequency; (3) the difference in the responses between the two experiments might have also been due to difference in the nutritional status of animal used. In the trial reported in Chapter 5, the sheep had been grazing on good quality pasture whereas in the present trial sheep had been grazing a low quality pasture.

There was a positive correlation between diarrhoea score and change of PCV, and this is consistent with previous findings that diarrhoea is one of the significant factors causing haemoconcentration (reviewed in Chapter 2). A negative linear correlation between the salivary antibody concentration and the diarrhoea score

further indicated that the higher salivary antibody response led to less severity of diarrhoea and lower increase in blood packed cell volume. These results suggest that the level of protection against lactic acidosis is positively associated with the level of salivary antibody response against *S. bovis*. However, direct evidence of immunisation against lactic acid-producing bacteria in intestine had not obtained in this experiment.

The IM group had a significantly higher antibody concentration and feed intake than that of the IP group. However, no difference in diarrhoea score between IM and IP groups was found. The lack of difference in diarrhoea score between these two groups might be due to the IP immunisation inducing higher intestinal immune response (Husband, 1985 and 1987) against the local *S. bovis*, which was not examined in this experiment and may be important against diarrhoea (see Chapter 5). Previous results (Husband, 1978; 1980) have shown that immunisation primed intraperitoneally was effective in inducing intestinal immune responses against local *S. typhimurium*. A similar level ($P>0.05$) of blood PCV change occurred in the IM and IP groups, which was consistent with their severity of diarrhoea.

The liveweight change was not significantly different between groups during the period of grain feeding. However, the liveweight loss in the IP group was significantly greater than that of either the IM or the control group, while there was no significant difference between the IM and control groups. These results suggest that the intramuscular immunisation may be safe for the animals. On the other hand, the significantly greater liveweight loss in the IP group may be an indication that immunisation primed intraperitoneally with a vaccine containing live *S. bovis* can adversely affect animals' health. This is consistent with previous results as reviewed by Gnanasampanthan (1993) that few safe live vaccines primed intraperitoneally have been developed. The higher liveweight loss could be due to (1) infection of live *S. bovis* in the gut system, in particularly the mesenterium; and/or (2) adjuvant (FCA)-induced mesenteric inflammatory lesions (Husband, 1985). The liveweight loss in all three groups of sheep from Day 0 to 75 (see Table 6.4) may be due to the quality of feed getting lower and the weather becoming colder during the period May 1994 to August 1994, and the shift of animals from paddock to the animal house.

Although the antibody levels after the 3rd booster immunisation were significantly higher than that induced by the 1st booster in IP group, there was no significant difference between the antibody concentrations after the first, second and third boosters in IM group. This is different from the pattern of the antibody response found in the previous experiment reported in Chapter 5, in which a significant increase in antibody concentration was observed after each booster. The discrepancy could be due to the difference in experimental conditions, for example, in the previous experiment all the Sb-5 cells were administered by intramuscular injections, while in the present experiment 50% of the bacterial suspension used for the second and third boosters were killed Sb-5 and administered orally without any adjuvant. The difference in animal ages (2-year old sheep were used in the previous experiment, while 6-month old sheep were used in this experiment) may also affect the antibody response (Colditz *et al.*, 1996; Watson *et al.*, 1994a).

The highest salivary antibody concentration in immunised animals was observed after grain feeding (on Day 87), which was significantly higher than the antibody concentration after the first booster. However, we could not conclude that the significant increase in the antibody level resulted from the subsequent boosters, as the introduction of the grain-based diets resulted in a significant increase in antibody concentration in the control group. On the other hand, these results suggest that the second and third booster immunisation for the IM group may not significantly enhance the salivary antibody response under the experimental conditions, and the antibody response after the first booster may be able to achieve the same protection as that achieved by three boosters. The effect of multiple booster immunisation will be further explored in Chapter 8.

Compared with the intraperitoneal immunisation, higher salivary antibody response, higher feed intake, and smaller liveweight loss were observed in the animals immunised intramuscularly. In addition, it is suggested that a single booster following the primary immunisation may be as effective as multiple boosters.

# Chapter 7

# Comparison of adjuvants in sheep grazing pasture

## 7.1 Introduction

In Chapters 5 and 6 it was shown that immunisation using live *S. bovis* resulted in a significant increase in salivary antibody concentration, and a reduced risk of lactic acidosis. The studies described in Chapters 5 and 6 were based on the use of multiple boosters with Freund's complete/incomplete adjuvant. The results reported in Chapter 6 also suggested that one booster following the primary immunisation was likely to be successful against lactic acidosis. However, Freund's complete adjuvant is not acceptable for veterinary use because of side effects (Freund and McDermott, 1942; McCarthy *et al.*, 1977; Edelman, 1980), and the multiple booster immunisation regime is time-consuming and expensive. In order to develop an effective and practical immunisation strategy against lactic acidosis, it was necessary to test the efficacy of commercially acceptable adjuvants using just one booster following a primary immunisation.

The procedure for sampling and processing saliva for analysis of antibody response is difficult and time consuming. If serum antibody concentration could be used as an indicator of the efficacy of immunisation, then future analysis would be simpler and cheaper. Information reviewed in Chapter 2 showed that salivary antibodies originated from both blood and local production. Gnanasampanthan (1993) demonstrated that the pattern of salivary antibody response to rumen protozoan paralleled with the serum antibody response. Therefore, it may be possible

to monitor the salivary antibody response by analysing the serum antibody concentration.

The following experiment tested five commercially acceptable adjuvants (Freund's incomplete adjuvant, QuilA, Dextran, Imject Alum, and Gerbu) (reviewed in Chapter 2) for use in a vaccine against *S. bovis*. Freund's complete/incomplete adjuvant was included to act as positive control. One booster following a primary immunisation was employed. The efficacy of the commercial adjuvants was examined by comparing their antibody responses (antibody levels and duration of response) with those of the negative control (not immunised) and the positive control. These comparisons were based on weekly measurements, over a period of 77 days, of salivary antibody concentrations of sheep grazing pasture. The antibody concentrations in serum were also measured to explore the correlation between the salivary and serum antibodies. Liveweight gains of the sheep in different treatment groups were compared to evaluate the effects of the vaccines containing different adjuvants on the wellbeing of sheep.

## 7.2 Materials and methods

### 7.2.1 Vaccine preparation

Each vaccine batch was prepared by mixing 7.0 ml of the Sb-5 suspension (BS) (see Chapter 3) with a different adjuvant as follows:

FCA The 7.0 ml BS was emulsified with an equal volume of Freund's complete adjuvant for the primary immunisation and with an equal volume of Freund's incomplete adjuvant for the booster.

FIA The 7.0 ml BS was emulsified with an equal volume of Freund's incomplete adjuvant, and used for both primary and booster immunisation.

QuilA 7.0 mg QuilA (Superfos Biosector a/s) was dissolved in 7.0 ml sterile PBS, and then the 7.0 ml BS was added in drops over a period of 5 minutes with vigorous mixing. The mixing was continued for another 20 minutes.

Dex Dextran sulphate (Pharmacia) (43.8 mg) was mixed with 7.0 ml of sterile PBS, and then the 7.0 ml BS was added in drops over a period of 5 minutes with vigorous mixing. The mixing was continued for another 20 minutes.

Alum 3.5 ml of Imject Alum adjuvant (Pierce) was mixed with 3.5 ml sterile PBS, and then the 7.0 ml BS was added in drops over a period of 5 minutes with vigorous mixing. The mixing was continued for another 30 minutes.

Gerbu 300 μg of Gerbu Adjuvant (ScimR) was mixed with 7.0 ml sterile PBS, and then the 7.0 ml BS was added in drops over a period of 5 minutes with vigorous mixing. The mixing was continued for another 20 minutes.

### 7.2.2 Experimental procedures

Thirty five 1-year old Merino wethers were selected from the UNE sheep flock at Kirby on the basis of liveweight. Animals were run as a single flock, under paddock conditions, grazing improved phalaris/rye grass pastures. The trial was conducted during the months of November 1995 to February 1996. There was regular rainfall during this period and approximately 2.5~4 t/ha green feed was available throughout the experiment.

The experimental procedures are summarised in Table 7.1. Before immunisation, the 35 sheep were stratified for liveweight and randomly allocated to 7 treatment groups (5 animals/group). The primary immunisation was administered on Day 0, and one booster was given on Day 28. Both primary and booster injections were administered intramuscularly (Watson, 1987; see also Chapter 5). The adjuvant used in each treatment group was described as follows:

Control No immunisation;
FCA Freund's complete adjuvant for the primary immunisation and Freund's incomplete adjuvant for the booster ;
FIA Freund's incomplete adjuvant for both primary and secondary injections;
QuilA QuilA adjuvant for both primary and secondary injections;

| | |
|---|---|
| Dex | Dextran sulphate for both primary and secondary injections; |
| Alum | Imject Alum for both primary and secondary injections; |
| Gerbu | Gerbu adjuvant for both primary and secondary injections. |

Samples of saliva and blood were taken from the sheep in the early morning, at weekly intervals, from Day -7 to 70. Animals were also weighed on Days -7, 0, 14, 28, 42, 63 and 70. The liveweight on Day -7 was used as a covariate for statistically analyzing the difference in liveweight gains between treatment groups (see Chapter 3).

Table 7.1 Timetable of major events, sample collections, and measurements

| Day | Major events | Sample | Measurement |
|---|---|---|---|
| -7 | | Blood, saliva | Antibody, liveweight |
| 0 | Primary immunisation | Blood, saliva | Antibody, liveweight |
| 7 | | Blood, saliva | Antibody |
| 14 | | Blood, saliva | Antibody, liveweight |
| 21 | | Blood, saliva | Antibody |
| 28 | Booster immunisation | Blood, saliva | Antibody, liveweight |
| 35 | | Blood, saliva | Antibody |
| 42 | | Blood, saliva | Antibody, liveweight |
| 49 | | Blood, saliva | Antibody |
| 56 | | Blood, saliva | Antibody |
| 63 | | Blood, saliva | Antibody, liveweight |
| 70 | | Blood, saliva | Antibody, liveweight |

## 7.3 Results

### 7.3.1 Antibody responses

Low levels of anti-*S. bovis* antibodies were detected in the saliva samples taken from all animals on Days -7 and 0 (prior to immunisation), and the controls on Days -7 to 70 (over the whole period of experiment).

Compared with the control group, higher ($P<0.01$) salivary antibody concentrations in immunised animals were found from Day 14 (14 days following the primary immunisation) (Table 7.2). After the booster immunisation, higher antibody concentrations ($P<0.01$) were observed in all the immunised groups compared with the control, except that no difference ($P>0.05$) was found between the Gerbu and control groups on Days 35, 56, 63, and 70, and between the Dex and control groups on Days 42 and 56.

The statistical significance of the difference in antibody concentration between the FCA and other immunisation groups is also summarised in Table 7.2. The antibody levels induced by FCA were significantly higher than the other groups with the following exceptions: (1) there were no significant differences ($P>0.05$) in the antibody concentrations between the FIA and FCA groups on Days 21, 28, 35, 63, and 70; (2) the levels of antibody responses induced by the vaccine using QuilA adjuvant were also not significantly ($P>0.05$) different from those using FCA on Days 14, 21, 28, 35, and 42.

The average (over the period of experiment after the booster immunisation) salivary antibody concentration in the FCA group was the highest, and that of the Gerbu group was the lowest in the immunisation groups. There was no significant difference between the QuilA and FIA groups, which were second to the FCA group. These groups were followed by the Alum, and then the Dex groups (Figure 7.1).

From Day 35 (one week after booster immunisation) to Day 70, no significant change in antibody concentrations of the treatment groups ($P>0.05$) was found with the following exception: a significant decrease in antibody concentration was observed in the Gerbu group ($P<0.01$).

Table 7.2 Mean saliva antibody concentration (units/ml) of non-immunised sheep and sheep immunised with *S. bovis* vaccines containing FCA, FIA, QuilA, Dex, Alum, and Gerbu adjuvants. Data are presented as Least Square Means. Primary immunisation was administered on Day 0, and the single booster was given on Day 28

| Day | Control (n=5) | FCA (n=5) | FIA (n=5) | QuilA (n=5) | Dex (n=5) | Alum (n=5) | Gerbu (n=5) | SE | P |
|---|---|---|---|---|---|---|---|---|---|
| -7 | 8.0 | 5.8 | 5.6 | 5.6 | 3.4 | 6.2 | 3.4 | 2.1 | >0.05 |
| 0 | 6.2 | 7.2 | 6.2 | 6.4 | 4.2 | 16 | 3.8 | 3.7 | >0.05 |
| 7 | 13 | 11 | 14 | 9.2 | 7.4 | 17 | 4.2 | 3.9 | >0.05 |
| 14 | 4.8 | 23 | 11 | $34^{ns}$ | $13^{ns}$ | $23^{ns}$ | 7.2 | 7.3 | <0.01 |
| 21 | 12 | 42 | $35^{ns}$ | $45^{ns}$ | 16 | $29^{ns}$ | 8.0 | 7.6 | <0.01 |
| 28 | 41 | 136 | $79^{ns}$ | $46^{ns}$ | 25 | 24 | 8.6 | 27 | <0.01 |
| 35 | $30_a$ | 773 | $476^{ns}$ | $871^{ns}$ | 134 | 172 | $67_a$ | 254 | <0.01 |
| 42 | $24_a$ | 834 | 164 | $592^{ns}$ | $63_a$ | 198 | 118 | 144 | <0.01 |
| 49 | 12 | 1048 | 183 | 244 | 102 | 132 | 47 | 116 | <0.01 |
| 56 | $27_a$ | 984 | 292 | 253 | $56_a$ | 192 | $35_a$ | 141 | <0.01 |
| 63 | $25_a$ | 806 | $428^{ns}$ | 295 | 149 | 365 | $65_a$ | 121 | <0.01 |
| 70 | $29_a$ | 1547 | $528^{ns}$ | 272 | 73 | 169 | $36_a$ | 198 | <0.01 |

Compared with the antibody concentration (after booster) in the control, all the values (in FCA, FIA, QuilA, Dex, Alum, and Gerbu groups) within rows are significantly higher ($P<0.05$), except those values with subscript $_a$ which are not significantly different ($P>0.05$).

Compared with the antibody concentration (since Day 14) in FCA group, all the values (in FIA, QuilA, Dex, Alum, and Gerbu groups) within rows are significantly lower ($P<0.05$), except those values with superscript $^{ns}$ which are not significantly different ($P>0.05$).

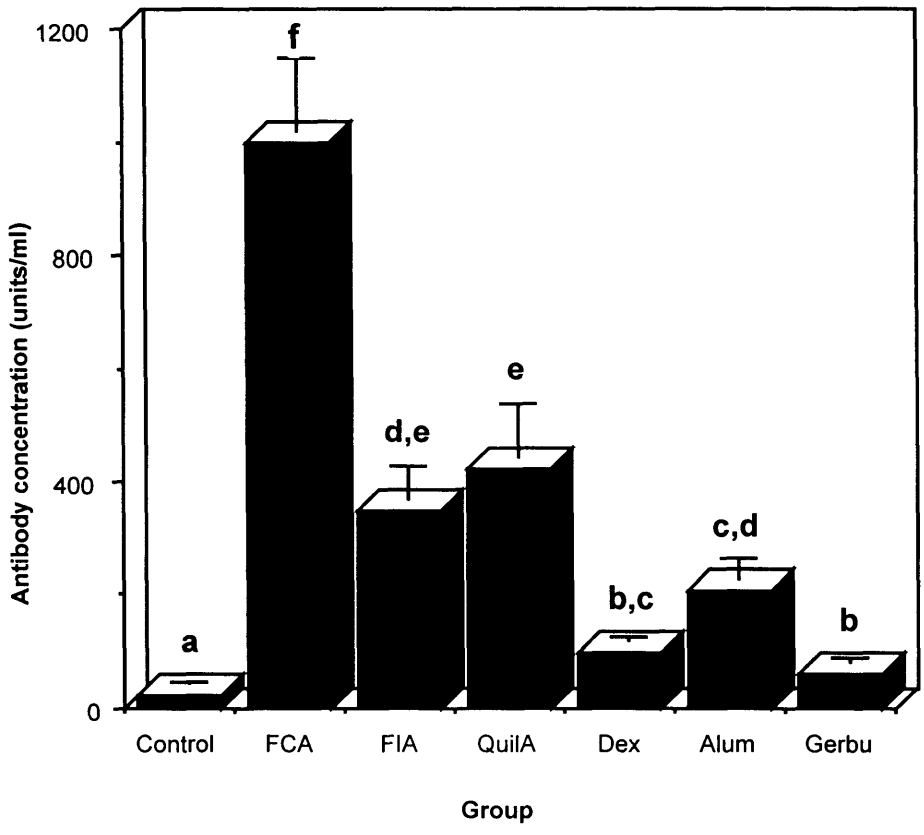


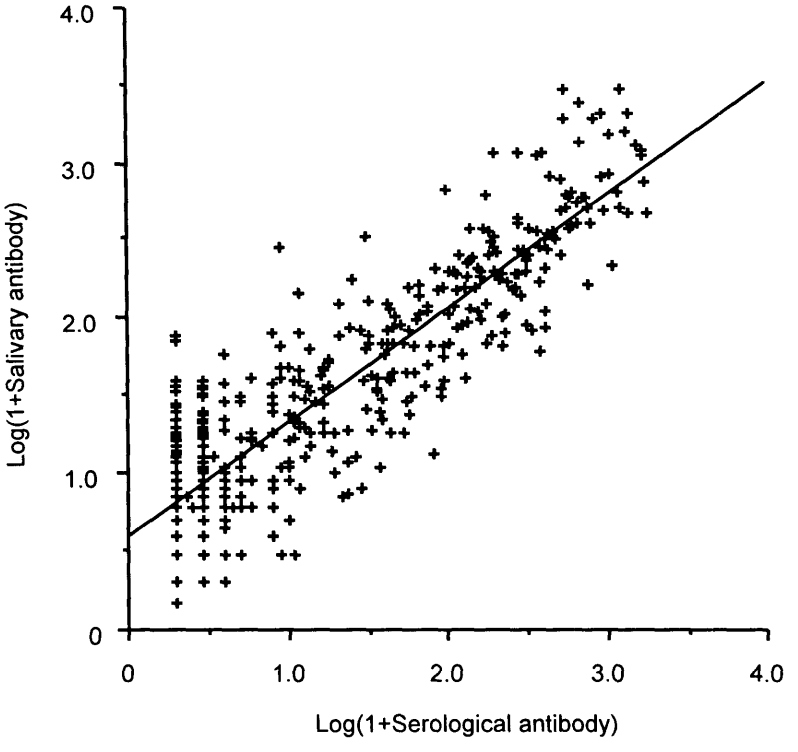

Figure 7.1 Average salivary antibody concentrations (units/ml) after booster immunisation (Day 35 to Day 70) of non-immunised sheep and sheep immunised with *S. bovis* vaccines containing FCA, FIA, QuilA, Dex, Alum, and Gerbu adjuvants. Vertical error bars represent standard errors of the means (each group has 5 sheep). Values with different letters (a, b, c, d, e, or f) are significantly different ($P<0.05$)

### 7.3.2 Correlation between salivary and serum antibodies

There was a poor correlation between the salivary antibody concentration and the serum antibody concentration in the control group ($R^2$=0.023) (Table 7.3). Strong positive linear correlations were found between the salivary and serum antibody concentrations in all the immunised groups with $R^2$ values ranging from 0.651 to 0.894. The overall linear relationship ($R^2$=0.770), between the salivary and serum antibodies in all the animals, is presented in Figure 7.2.

Table 7.3 The correlations between the salivary and serum antibody concentrations of non-immunised control and sheep immunised with *S. bovis* vaccines containing FCA, FIA, QuilA, Dex, Alum, and Gerbu adjuvants

| Group | Slope | Intercept | $R^2$ |
|---|---|---|---|
| Control | 0.321 | 1.006 | 0.023 |
| FCA | 0.853 | 0.317 | 0.894 |
| FIA | 0.727 | 0.514 | 0.823 |
| QuilA | 0.865 | 0.452 | 0.821 |
| Dex | 0.690 | 0.587 | 0.651 |
| Alum | 0.701 | 0.573 | 0.676 |
| Gerbu | 1.000 | 0.355 | 0.717 |




Log(1+Salivary antibody) = 0.604 + 0.728 Log(1+Serological antibody)

R^2 = 0.770

Figure 7.2 The overall relationship between serum (serological) antibody and salivary antibody concentrations (units/ml) of non-immunised sheep and sheep immunised with *S. bovis* vaccines containing FCA, FIA, QuilA, Dex, Alum, and Gerbu adjuvants. The line in the figure represents the regression line of the antibody concentration in serum and saliva. The equation is the regression equation of the antibody concentration in serum and saliva

### 7.3.3 Liveweight gain

No significant difference in liveweight gain between treatment groups was found (P>0.05) (Table 7.4).

Table 7.4 Mean liveweight gains (kg/sheep) over time (Day -7 to Day 70) of non-immunised sheep and sheep immunised with *S. bovis* vaccines containing FCA, FIA, QuilA, Dex, Alum, and Gerbu adjuvants. Data are presented as Least Square Means (SE)

| Control (n=5) | FCA (n=5) | FIA (n=5) | QuilA (n=5) | Dex (n=5) | Alum (n=5) | Gerbu (n=5) | P |
|---|---|---|---|---|---|---|---|
| 0.92 (0.57) | 1.08 (0.57) | 0.85 (0.57) | 0.67 (0.57) | 1.04 (0.57) | 1.32 (0.57) | 1.78 (0.57) | >0.05 |

## 7.4 Discussion

Results show that immunisation using a number of commercially acceptable adjuvants is effective in inducing a significant salivary antibody response to *S. bovis* in sheep, although none of the adjuvants induced the same high level and lasting antibody response as Freund's complete adjuvant. This result is similar to results reported in literature which indicate that FCA is the most potent available experimental adjuvant in terms of its ability to stimulate high level and lasting immunity (Edelman, 1980; Freund and McDermott, 1942; McCarthy *et al.*, 1977). QuilA and FIA induced similar levels of antibody responses, in which antibody concentrations were similar to FCA on several of the test days but the average was lower. In order of effectiveness (as measured by antibody levels) of the commercial

adjuvants QuilA and FIA were greatest, followed by Alum, Dextran, and Gerbu adjuvants. Except for the Gerbu, there were no significant decreases in the salivary antibody responses (after booster immunisation) observed in all the other groups, indicating that the duration of salivary antibody responses induced by using one of these adjuvants is greater than 42 days. The relatively low level and short duration of the antibody response in Gerbu group is similar to previous evidence provided by Thompson (personal communication) that multiple booster immunisation with vaccine using Gerbu adjuvant is necessary to achieve the antibody levels as FCA did by one booster following a primary immunisation. The pattern of effectiveness of the other adjuvants such as FIA>Dex is similar to that found by Watson (1987).

In the present experiment the antibody response to FCA was higher than that (2 weeks after the 1st booster) found in experiments reported in Chapters 5 and 6. This may be due to the different age of animals (Colditz *et al.*, 1996; Watson *et al.*, 1994a). The levels of antibody responses induced by vaccines containing various commercial adjuvants using only one booster were comparable to the levels induced by 3 boosters in the previous experiments. This suggests that the antibody responses achieved in this experiment could give comparable levels of protection against lactic acidosis, if the animals were challenged with grain as described in Chapters 5 and 6. It provided further evidence that one booster following a primary immunisation is likely to be successful against clinical lactic acidosis.

The large positive correlation between the salivary and serum antibody concentration is consistent with the observation of Gnanasampanthan (1993) that the pattern of salivary antibody response paralleled the serum antibody response to rumen protozoan. The practical implication of this result is that the serum antibody response to the rumen bacteria, *S. bovis,* could be used as an indicator of efficacy of immunisation. The salivary antibody response in saliva could be due to local production of antibodies and also due to transport from the blood circulation (Curtain *et al.*, 1971; Mach and Pahud, 1971; Watson and Lascelles, 1973a; 1973b). There was a poor correlation between the salivary and serum antibody concentration in the control group ($R^2$=0.023). This was probably due to the very low level of natural anti-*S. bovis* antibodies in these animals, and lack of a linear relationship between the salivary and serum anti-*S. bovis* antibodies prior to a specific immunisation.

Animals in all treatment groups showed a similar liveweight gain over the 77-day period of experiment, suggesting that immunisation with a live vaccine containing living *S. bovis* cells and one of the commercially acceptable adjuvants had no adverse effect on the wellbeing of animals.

In conclusion, the results suggest that immunisation (a single booster following the primary injection) with a live vaccine containing one of the commercially acceptable adjuvants (including FIA, QuilA, Alum, and Dex) can induce a high level and lasting antibody response, and is safe and likely to be successful against clinical lactic acidosis. Results from this study also indicate that the serum antibody response is a good indicator of efficacy of immunisation.

# Chapter 8

# Immunisation with a combination of *S. bovis* and *Lactobacillus* vaccine against lactic acidosis in cattle

## 8.1 Introduction

The previous experiments (Chapters 5, 6, and 7) have shown that immunisation with *S. bovis* resulted in a significant salivary antibody response and protection against lactic acidosis. In these studies only *S. bovis* was used as the vaccine antigen(s). However, *Lactobacillus* may also contribute to the development of lactic acidosis although its role has not been clearly identified (reviewed in Chapter 2). Accordingly, it was necessary to test the protective effect of immunisation with a combination of *S. bovis* and *Lactobacillus* in order to develop a suitable immunisation strategy against lactic acidosis.

An emerging tenet of vaccine formulation is that the antibody elicited in response to vaccination must be of a particular immunoglobulin isotype in order to contribute maximally to host defence against the pathogen (Kerlin and Watson, 1988; Watson, 1987). Although salivary antibodies contain several classes of immunoglobulin, a feature of the mucosal immune system in ruminants is the prominence of IgG (reviewed in Chapter 2). Gnanasampanthan (1993) demonstrated that immunisation stimulated a significant salivary IgG response against rumen protozoa and that there was no degradation of IgG molecule in rumen fluid for at least 4 hours. It is also well known that an intramuscular immunisation is particularly effective in inducing a high level and lasting blood IgG response (Watson, 1987), which is an important source of the salivary IgG (reviewed in Chapter 2).

Accordingly, investigation of the salivary IgG response to a lactic acidosis vaccine immunised intramuscularly is particularly important.

In order to test whether the risk of lactic acidosis can be reduced by immunisation against *S. bovis* and *Lactobacillus* and to verify our earlier observations in cattle, the study described below was based on the specific hypothesis that specific salivary antibody responses can be induced by immunisation with a combination of *S. bovis* and *Lactobacillus*, and that the over production of lactic acid in rumen can be reduced by immunisation against both *S. bovis* and *Lactobacillus*. The salivary antibody isotype IgG (anti-*S. bovis* IgG and anti-*Lactobacillus* IgG) responses were investigated using a multiple booster immunisation regime. Serum antibody IgG responses were measured to further explore the correlation between the salivary and serum antibody IgG response in cattle. Lactic acidosis was induced by suddenly introducing the animals to a diet containing 90% rolled wheat for 7 days. Effectiveness of protection against lactic acidosis was assessed by measuring feed intake, rumen pH, and lactate concentration in rumen fluid after grain challenge. It is clear that the over production of lactic acid in the rumen may be caused by the rapid build up of *S. bovis* and *Lactobacillus* in the presence of high levels of starch or soluble carbohydrates (Allison *et al.*, 1975; Shu and Liu, 1995a). Therefore, the numbers of *S. bovis* and *Lactobacillus* in the rumen fluid in immunised and control animals were also examined in this experiment.

It is worth noting that IgA, although a minor immunoglobulin in saliva in ruminants, is the major immunoglobulin in intestinal secretions and is designed to persist in functional form in external secretions (Cripps *et al.*, 1974). Unfortunately, it is not able to demonstrate the IgA response in this thesis mainly due to the lack of resources.

## 8.2 Materials and methods

### 8.2.1 Vaccine preparation

*S. bovis* strain Sb-5 and *Lactobacillus* isolate LB-27 suspensions were prepared as described in Chapter 3. The final bacterial suspension (BS) contained 3 x $10^{10}$ cells of Sb-5 and 1 x $10^{10}$ cells of LB-27 per 2.5 ml. Each vaccine batch was

emulsified 17.5 ml of the BS with an equal volume of FCA for the primary immunisation, and with an equal volume of FIA for the boosters. Each injection volume was 5 ml.

### 8.2.2 Experimental procedures

Ten 1-year old Hereford steers with cannula (from NSW Agriculture Grafton Research Station) grazing a kikuyu pasture were used in this experiment. The trial was conducted during the period from November 1995 to March 1995 with good regular rainfall during this period and approximately 3~5 t/ha green feed in the paddock was available throughout the experiment. The animals were randomly allocated to two treatment groups prior to the primary immunisation. One group (immunisation, n=5) was immunised and the other (control, n=5) group received no immunisation. The timetable of major events is summarised in Table 8.1. Following the primary immunisation (Day 0), booster injections were given on Days 30, 44, 59, and 73. Immunisation was administered intramuscularly into the medial thigh and neck of cattle. On Day 70 all the animals were transferred into an animal house and penned individually with access to fresh water at all times, and fed *ad libitum* with rye grass pasture hay (prior to the grain challenge (from Day 70 to 90). The animals had an average daily intake of 4.2±1 kg/head of dry matter of pasture hay in the week (Day 83 to 90) before grain challenge.

On Day 91, 6 kg/d/steer of 90% wheat diet (90% rolled wheat, 5% roughage, 1% urea, 1% limestone, 2.5% cottonseed, and 0.5% salt), with a dry matter content of 90%, was offered. After 8 hours of feeding, uneaten feed was artificially administered into the rumen of each animal through the rumen fistula in order to standardise intake. On the following 6 days (Day 92 to 97), 6 kg of the 90% wheat diet was offered daily to each animal and uneaten feed was removed and weighted. The feed was offered at around 9.30 am daily.

It was predetermined that animals would be withdrawn from the wheat diet if their rumen pH dropped below 5.2 during the challenge period. These animals would be fed with rye grass pasture hay. After the 7 days grain challenge all animals were

transferred to the paddock and supplemented with 2,300 g/head of wheat until the end of the trial.

Samples of saliva and blood were taken on Days 0, 28, 44, 59, 73, 90, 91, 92, 93, and 121 for the measurement of IgG. Rumen fluid was collected through cannula for the measurement of pH and lactate concentration (the sum of L-lactate and D-lactate concentrations) on Days 90, 91 (8 and 16 hours after grain feeding, respectively), 92, 93, 94, and 97. The samples were taken at around 9.00 am except those on Day 91. The numbers of *S. bovis* and *Lactobacillus* in the rumen fluid collected on Days 90 (animals were fed pasture hay) and 92 (animals were fed grain) were determined. The feed (dry matter) intake was measured every day during the period of grain challenge. Dry matter of the feed was determined by drying feed material to constant weight at 105±10°C. The average daily feed intake in the week before grain challenge was used as a covariate for statistically analyzing the difference in feed intake between treatment groups (see Chapter 3).

Table 8.1 Timetable of major events, sample collections, and measurements

| Day | Diet | Major event | Sample | Measurement |
|---|---|---|---|---|
| 0 | Pasture | Primary immunisation | Blood, saliva | Antibody |
| 30 | Pasture | 1st booster | Blood, saliva | Antibody |
| 44 | Pasture | 2nd booster | Blood, saliva, | Antibody |
| 59 | Pasture | 3rd booster | Blood, saliva | Antibody |
| 70 | Pasture | Cattle penned individually | | |
| 73 | Pasture | 4th booster | Blood, saliva | Antibody |
| 90 | Pasture | | Blood, saliva, rumen fluid | Antibody, pH, Lactate, rumen *S. bovis* and *Lactobacillus* |
| 91 | 90% Wheat | | Blood, saliva, rumen fluid | Antibody, pH, Lactate |
| 92 | 90% Wheat | | Blood, saliva, rumen fluid | Antibody, Intake, pH, Lactate, rumen *S. bovis* and *Lactobacillus* |
| 93 | 90% Wheat | | Blood, saliva, rumen fluid | Antibody, Intake, pH, Lactate |
| 94 | 90% Wheat | | Rumen fluid | Intake, pH, Lactate |
| 97 | 90% Wheat | | Rumen fluid | Intake, pH, Lactate |
| 98 | Pasture+wheat | Animals transferred to paddock | | |
| 121 | Pasture+wheat | | Blood, saliva | Antibody |

Antibody, salivary and serum anti-*S. bovis* and anti-*Lactobacillus* IgG concentrations; Intake, dry matter intake; pH, rumen fluid pH; Lactate, the sum of rumen L- and D-lactate concentration.

## 8.3 Results

### 8.3.1 Antibody responses

Low levels of anti-*S. bovis* and anti-*Lactobacillus* IgG were detected in saliva of all animals on Day 0 (prior to immunisation), and the control animals on Days 0 to 121 (Table 8.2).

Anti-*Lactobacillus* IgG concentrations of immunisation group were significantly higher than those of the control from Day 44 (2 weeks after the first booster immunisation) to Day 121 (the end of this experiment) with the following exceptions: (1) on Day 90, the anti-*Lactobacillus* IgG was not significant different; and (2) on Day 91, the anti-*S. bovis* IgG was not significantly different.

Compared with the IgG levels prior to immunisation, both anti-*S. bovis* IgG and anti-*Lactobacillus* IgG concentrations in the immunised animals on Day 44 were significantly higher ($P<0.01$). Further increases ($P<0.05$) in these values were observed on Day 59 and remained high on Day 73. On day 90, the IgG values decreased significantly ($P<0.05$) to the levels which were similar to those on Day 44. Then the values remained at a similar high level ($P>0.05$) from Day 90 to the end of the experiment.

Table 8.2 Mean salivary antibody concentrations (units/ml) of non-immunised cattle and cattle immunised with a vaccine containing *S. bovis* and *Lactobacillus*. Primary immunisation was administered on Day 0, and boosters were given on Days 30, 44, 59, and 73

| Day | Anti-*S. bovis* IgG | | | | | Anti-*Lactobacillus* IgG | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Immunisation (n=5) | | Control (n=5) | | P1 | Immunisation (n=5) | | Control (n=5) | | P2 |
| | Mean | SE | Mean | SE | | Mean | SE | Mean | SE | |
| 0 | 4.7 | 0.9 | 8.0 | 3.6 | >0.05 | 30 | 11 | 38 | 8.8 | >0.05 |
| 30 | 3.7 | 0.5 | 9.4 | 4.5 | >0.05 | 25 | 9.2 | 41 | 11 | >0.05 |
| 44 | 29 | 9.0 | 4.7 | 1.8 | <0.01 | 68 | 8.9 | 33 | 6.8 | <0.05 |
| 59 | $88_a$ | 26 | 5.5 | 1.2 | <0.01 | $207_a$ | 49 | 53 | 17 | <0.01 |
| 73 | $126_a$ | 26 | $21_a$ | 1.9 | <0.01 | $183_a$ | 31 | 57 | 12 | <0.01 |
| 90 | 45 | 8.9 | $13_a$ | 0.9 | <0.01 | 61 | 22 | 31 | 10 | >0.05 |
| 91 | 49 | 13 | $27_a$ | 7.9 | 0.09 | 136 | 36 | 62 | 12 | <0.05 |
| 92 | $122_a$ | 68 | $16_a$ | 5.0 | <0.05 | 150 | 40 | 55 | 14 | <0.05 |
| 93 | 61 | 9.5 | 12 | 3.5 | <0.01 | 84 | 13 | 45 | 10 | <0.05 |
| 121 | $107_a$ | 59 | $14_a$ | 4.1 | <0.05 | 85 | 17.0 | 59 | 11 | >0.05 |
| P3 | <0.01 | | <0.01 | | | <0.01 | | >0.05 | | |

P1, significance of the differences in anti-*S. bovis* IgG concentrations between columns.

P2, significance of the differences in anti-*Lactobacillus* IgG concentrations between columns.

P3, significance of the differences within columns. Further comparing with the antibody levels after the 1st booster (on Day 44), values (antibody concentrations after the 2nd, 3rd, and 4th boosters) within columns with subscript "$_a$" are significantly different (P<0.05).

### 8.3.2 Correlation of salivary and serum antibodies

In the immunised cattle, a positive linear correlation ($P<0.01$) was found between all of the antibodies with r values ranging from 0.445 to 0.790 (Table 8.3). There was a poor correlation between the salivary and serum antibodies in the control animals with r values ranging from -0.005 to 0.442.

Table 8.3 Correlations and the significance of the linear relationships between the salivary and serum antibodies in non-immunised cattle and cattle immunised with a vaccine containing *S. bovis* and *Lactobacillus*

| | Immunisation | | | | Control | | | |
|---|---|---|---|---|---|---|---|---|
| | Se-Sb | Sa-Sb | Se-LB | Sa-LB | Se-Sb | Sa-Sb | Se-LB | Sa-LB |
| Se-Sb | 1.000 | ** | ** | ** | 1.000 | * | ns | ns |
| Sa-Sb | 0.790 | 1.000 | ** | ** | 0.347 | 1.000 | ns | ** |
| Se-LB | 0.737 | 0.687 | 1.000 | ** | 0.222 | 0.095 | 1.000 | ns |
| Sa-LB | 0.459 | 0.680 | 0.445 | 1.000 | -0.005 | 0.442 | 0.128 | 1.000 |

Se-Sb, serum anti-*S. bovis* IgG;
Sa-Sb, salivary anti-*S. bovis* IgG;
Se-LB, serum anti-*Lactobacillus* IgG;
Sa-LB, salivary anti-*Lactobacillus* IgG.

### 8.3.3 Withdrawn animals

Three animals in the control group and 1 in the immunised group were withdrawn from the 90% grain diet on Day 93 due to their rumen pH being below 5.2 (Tables 8.4 and 8.5).

Table 8.4 Withdrawn animals from non-immunised cattle and cattle immunised with a vaccine containing *S. bovis* and *Lactobacillus*

| | Immunisation | Control |
|---|---|---|
| Number of animals | 5 | 5 |
| Number of animals withdrawn | 1 (20%) | 3 (60%) |

### 8.3.4 Feed intake

Eight hours (Day 91) after introduction to 90% wheat diet, all the animals had eaten the 6,000 g (5,416 g dry matter) of diet with the following exception: two animals in control group had left 58 and 529 g of dry matter, respectively. On the second day (Day 92) of the grain challenge, feed intake of the immunised group was higher ($P<0.05$) than that of the control. No significant difference in feed intake between the remaining animals in the immunised group (n=4) and the control (n=2) was observed on the other days ($P>0.05$) (Figure 8.1).

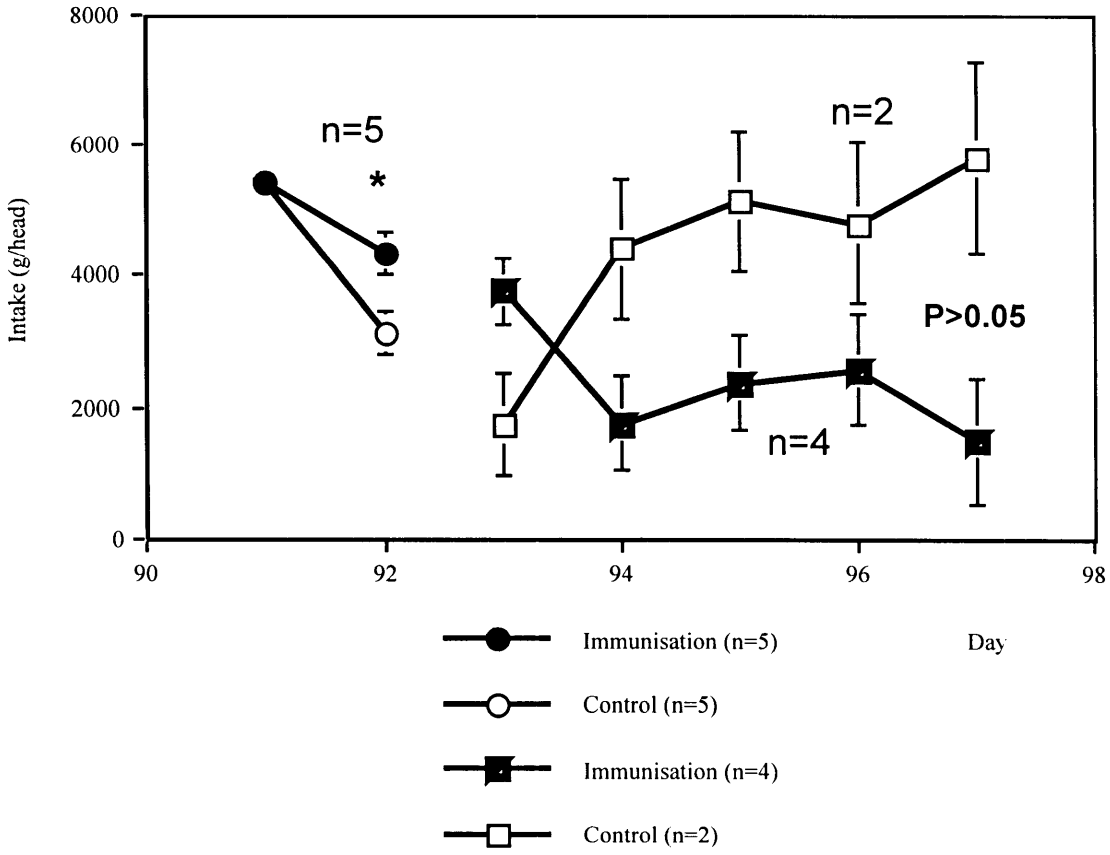



Figure 8.1 Mean daily dry matter intake (g/head) during the period of grain challenge of non-immunised cattle and cattle immunised with a vaccine containing *S. bovis* and *Lactobacillus*. Vertical error bars represent standard errors of the least square means

Three cattle from the control and 1 from the immunised group were withdrawn from the grain feeding on Day 93. *The intake of the immunised animals on Day 92 was significantly higher than that of the control ($P<0.05$). There was no significant difference ($P>0.05$) in feed intake between immunised (n=4) and control (n=2) groups from Day 93 to 97

### 8.3.5 Rumen pH and lactate concentration

Mean rumen pH decreased and lactate concentration increased as a result of grain challenge ($P<0.01$; Table 8.5; Figures 8.2 and 8.3). The lowest mean pH was observed on Day 93. A significant difference in rumen pH between groups (before and after the animals withdrawn) was not observed ($P>0.05$) but on Day 92 the immunised animals had a lower rumen lactate concentration ($P<0.05$). The change in the pH of the immunisation group was mainly due to a marked change in the pH of one animal that had a low pH of 4.02.

Table 8.5 Rumen pH on Day 93 in non-immunised cattle and cattle immunised with a vaccine containing *S. bovis* and *Lactobacillus*

| Cattle tag number | Treatment | pH | Fate |
|---|---|---|---|
| 1 | Immunisation | 5.29 | Remained on 90% wheat diet |
| 2 | Immunisation | 5.77 | Remained on 90% wheat diet |
| 4 | Immunisation | 5.95 | Remained on 90% wheat diet |
| 5 | Immunisation | 4.02 | **Withdrawn** |
| 19 | Immunisation | 5.37 | Remained on 90% wheat diet |
| | | 5.28 (0.34)[a] | |
| 10 | Control | 4.68 | **Withdrawn** |
| 11 | Control | 6.47 | Remained on 90% wheat diet |
| 12 | Control | 4.63 | **withdrawn** |
| 16 | Control | 5.58 | Remained on 90% wheat diet |
| 18 | Control | 4.86 | **Withdrawn** |
| | | 5.24 (0.35)[a] | |

Values with superscript "a" are the mean (SE) of the rumen pH of the immunised and control animals and not significant different ($P>0.05$).

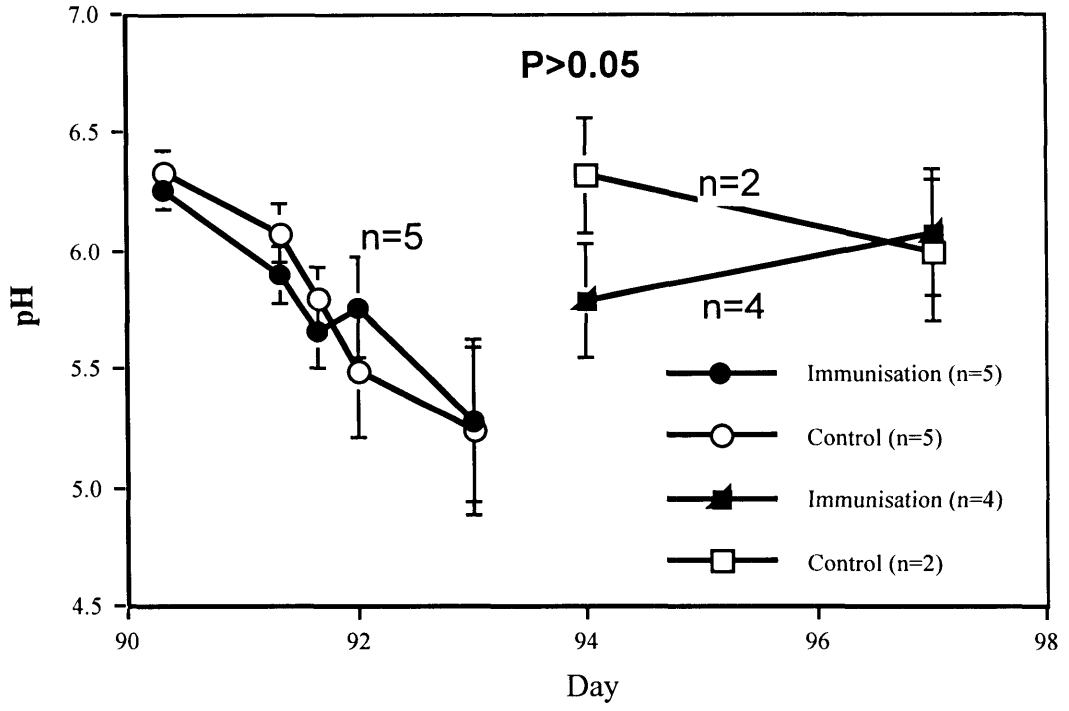



Figure 8.2 Mean rumen pH during the period of grain challenge of non-immunised cattle and cattle immunised with a vaccine containing *S. bovis* and *Lactobacillus*. Vertical error bars represent standard errors of the means

Three cattle from the control and 1 from the immunised group were withdrawn from the grain feeding on Day 93. The difference in rumen pH between the immunisation and control groups was not significant (P>0.05)

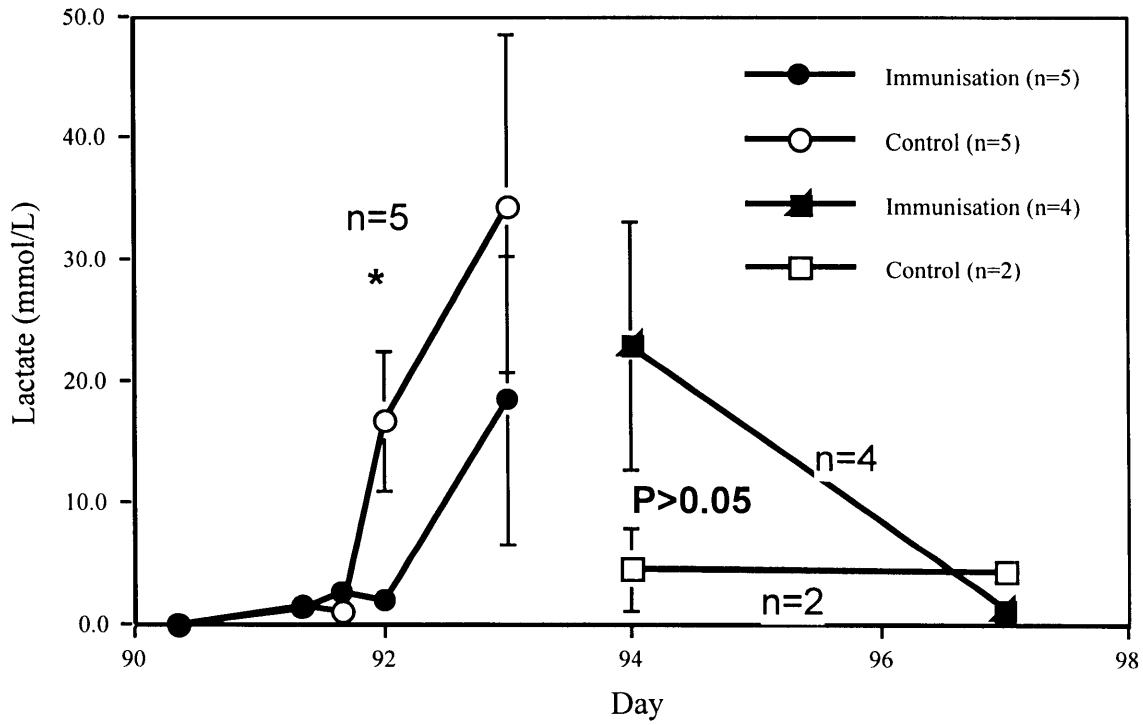



Figure 8.3 Mean rumen lactate concentration (mmol/L) during the period of grain challenge of non-immunised cattle and cattle immunised with a vaccine containing *S. bovis* and *Lactobacillus*. Vertical error bars represent standard errors of the means

Three cattle from the control and 1 from the immunised group were withdrawn from the grain feeding on Day 93. *The lactate concentration in immunised animals on Day 92 was significantly lower than in the control ($P<0.05$). The difference in lactate concentration between the immunisation (n=4) and control (n=2) groups was not significant ($P>0.05$)

### 8.3.6 Numbers of rumen *S. bovis* and *Lactobacillus*

The numbers of *S. bovis* and *Lactobacillus* in rumen fluid taken on Day 90 (16 hours before introduction of 90% grain diet) and Day 92 (24 hours after the introduction of grain diet) are summarised in Table 8.4. The number of *S. bovis* in the immunisation group was significantly lower than in the control group on Day 90 ($P<0.01$). The significant difference ($P<0.05$) in the number of *Lactobacillus* on Day 92 was also found between the two groups.

Table 8.6 Log numbers (CFU/ml) of *S. bovis* and *Lactobacillus* in the rumen fluid on Days 90 and 92 of non-immunised cattle and cattle immunised with a vaccine containing *S. bovis* and *Lactobacillus*. Data were converted using log10 transformation and presented as the Least Square Means. On Day 90 animals were fed with pasture hay and on Day 92 animals were fed with 90% grain

| Day | *S. bovis* | | | | *Lactobacillus* | | | |
|---|---|---|---|---|---|---|---|---|
| | Immunisation | Control | SE | P | Immunisation | Control | SE | P |
| 90 | 3.69 | 5.68 | 0.36 | ** | 3.07 | 3.70 | 0.54 | ns |
| 92 | 7.78 | 9.11 | 0.55 | ns | 6.33 | 8.23 | 0.40 | * |

## 8.4 Discussion

The cattle immunised with Sb-5 and LB-27 showed significant rises in salivary IgG concentrations after the 1st booster, and the IgG remained high until the end of the experiment. The important implication of these results is that high levels and

durable antibody responses can be induced by immunisation using a combination of *S. bovis* and *Lactobacillus* in cattle.

In Chapters 6 and 7 it was suggested that one booster following the primary immunisation was likely to be successful in inducing a protective response against clinical lactic acidosis. In this study, the anti-*S. bovis* and *Lactobacillus* antibody IgG concentration in saliva increased to a significantly high levels after the first booster. The second booster further increased the antibody concentration ($P<0.05$). No statistically significant difference was found between the IgG concentrations after the second and third boosters. A significant decrease in the antibody response was found after the fourth booster. These results suggest that the maximum salivary antibody response may have been reached after the 2nd or 3rd boosters. Although the reasons for this response pattern were not clearly identified, it may be due to: (1) short interval between booster immunisation, (2) antigen(s) from the booster immunisation being combined with the existing antibody and making it unavailable for the immune system. The salivary antibody levels after the first and fourth booster immunisations were not significantly different, this result further suggests that the protection conferred by the antibody responses induced by the 4 boosters may be equally achieved by only one booster immunisation. This implication is consistent with the observations by Srivastava and Barnum (1981) and Holt *et al.* (1990) that one booster following the primary immunisation was effective in protecting the animals from the streptococcal infection although multiple boosters following a primary immunisation induced higher levels of immune responses.

The significant positive correlation between the saliva and serum antibodies in the immunised animals confirmed the results in Chapter 7 and further suggests that the serum antibodies can be used as in indicator of efficacy of immunisation on the salivary IgG response. Furthermore, a significant positive correlation between the anti-*S. bovis* and anti-*Lactobacillus* IgG also suggests that measuring the serum anti-*S. bovis* IgG may be able to indicate the efficacy of the anti-*Lactobacillus* IgG response.

Another key aim of this experiment was to test whether the over production of lactic acid in the rumen can be reduced by the salivary antibody response to the *S. bovis* and *Lactobacillus*. Compared with the controls, Figure 8.3 indicates that the

immunised animals had a significantly lower rumen lactate concentration, which suggest that lactic acid production was lower in these animals. These results further support the hypothesis that the over production of lactic acid in rumen can be reduced by immunisation.

As discussed in Chapter 5, the possible mechanism of immunisation against lactic acidosis involves the binding of the antibodies to the organisms (Mathison *et al.*, 1984; Gnanasampanthan, 1993) and actively reduce their growth and/or interfere with other biological functions leading to a decrease in lactic acid production. The rapid increase in the number of *S. bovis* and *Lactobacillus* in the rumen are the major factors contributing to the over production and uncontrolled build up of lactic acid (Allison *et al.*, 1975; Dawson and Allison, 1988; Shu and Liu, 1995a). Accordingly, inhibition of the growth of the lactic acid producing bacteria should reduce lactic acid production thereby reducing the risk of lactic acidosis. The lower numbers of rumen *S. bovis* and *Lactobacillus* in the immunised animals suggest that the antibody response induced by immunisation can specifically reduce the growth of rumen *S. bovis* and *Lactobacillus*, reflecting a lower rumen lactic acid-producing capacity in the immunised animals compared with the control animals. Although the whole mechanism and the action model(s) of immunisation against *S. bovis* and *Lactobacillus* were not investigated in this program, the present results provide evidence indicating that direct reduction of the growth of the rumen lactic acid-producing bacteria is one of the consequences of immunisation. These results indicate that the immunisation did not completely remove either *S. bovis* or *Lactobacillus* in the rumen. The numbers of the *S. bovis* and *Lactobacillus* in the immunised animals were approximately $10^3$ to $10^4$ CFU/ml before grain feeding, which are comparable with the normal population range of these rumen bacteria in animals fed on forage-based diets (Allison *et al.*, 1975; Shu and Liu, 1995a; Shu et al unpublished data). When the population *S. bovis* and *Lactobacillus* in the control group increased to approximately $10^8$~$10^9$ CFU/ml in response to the grain challenge, the numbers of the bacteria in the immunised group also increased to around $10^6$ to $10^7$ CFU/ml, which are comparable with the normal population range of these rumen bacteria in animals fed on grain-based diets after gradual adaptation (Mackie et al, 1978; Shu et al, unpublished data). This characteristic may be important in terms of the normal rumen fermentation of grain fed animals, in which

*S. bovis* and *Lactobacillus* are important starch degraders (Cotta, 1988; McAllister et al, 1990). At this stage, it is not known if the biological functions of *S. bovis* and *Lactobacillus* in the immunised animals have changed in terms of the starch fermentation and lactic acid production, which may also contribute to the reduction of lactic acid in the rumen.

One of the clinical signs of lactic acidosis is the reduction in feed intake (reviewed in Chapter 2). Therefore, the positive effect of immunisation against lactic acidosis was further indicated by the significantly higher feed intake of the immunised cattle. Although there were too few animals (only five animals in each group) in the present experiment to determine if the different pattern of withdrawn animals in the immunised and control groups was statistically significant, in three of the five control animals (60%) rumen pH dropped to a range of 4.63-4.86 within 48 hours of grain challenge. This pH value indicated that severe acute lactic acidosis had occurred and the animals may die if not withdrawn from the 90% grain diet (Ahrens, 1967; Dawson and Allison, 1988; Newbold and Wallace, 1988). In contrast, only one of the five (20%) immunised animals had to be withdrawn. The animals remaining on the 90% diet had reasonably high rumen pH. The significant difference between rumen pH of animals after withdrawal was not found ($P>0.05$). This may be due to the remaining animals not being significantly affected by lactic acidosis. In this experiment it was not able to be determined whether immunisation with only *S. bovis* could achieve the same level of protection as a combination of *S. bovis* and *Lactobacillus*, which is one of the issues which need to be addressed in the future for further developing the lactic acidosis vaccine.

This experiment demonstrated that immunisation induced high levels and lasting salivary antibody responses against *S. bovis* and *Lactobacillus*, and suggests that the risk of lactic acidosis in cattle can be reduced by immunisation with a vaccine containing *S. bovis* and *Lactobacillus*. These results further showed that there was a positive correlation between salivary and serum antibodies, and that one booster following the primary immunisation is likely to be successful against clinical lactic acidosis.