Chapter 9

Further comparison of adjuvants in cattle under feedlot conditions

9.1 Introduction

The study reported in Chapter 8 demonstrated that salivary antibody IgG response and protection against lactic acidosis can be induced by immunisation with a vaccine containing a combination of *S. bovis* and *Lactobacillus* in cattle. The study was based on the use of animals immunised before being introduced to a grain-based diet, and the use of Freund's complete/incomplete adjuvant under a multiple booster regime. A number of acceptable adjuvants for veterinary use (reviewed in Chapter 2) were compared with Freund's complete/incomplete adjuvant in sheep grazing green pasture (see Chapter 7) for the use in lactic acidosis vaccines. Immunisation using these adjuvants induced high levels and durable salivary antibody responses against *S. bovis*. The adjuvants included QuilA, FIA, Alum, and Dex adjuvants. Watson (1992a) found that the Dextran adjuvant combined with mineral oil was the most promising adjuvant for a *S. aureus* vaccine. He used it to prevent mastitis in cattle grazing pasture and given a supplementary grain ration. The previous experiment showed that one booster following the primary immunisation is successful for inducing a significant antibody response, and that the serum antibody concentration can be used as an indicator of the salivary antibody response. Positive correlation between the anti-*S. bovis* and anti-*Lactobacillus* IgG was also observed.

Feedlot industry is a major area associated with lactic acidosis (Dawson and Allison, 1988; Feedlot Advisory Unit, 1990). In particular, for cattle adapted to a

finishing ration, subclinical lactic acidosis can be a main adverse factor for reducing the animal production (Feedlot advisory unit, 1990; Meppem *et al.*, unpublished data). In order to use the immunisation strategy against lactic acidosis in the feedlot industry, there was a practical need to further verify the results of adjuvant studies in feedlot cattle, and to select commercially acceptable adjuvants under feedlot conditions.

This study was designed to compare the efficacy of QuilA, Alum, Dextran combined with mineral oil (Dex), and Freund's complete/incomplete adjuvants under feedlot conditions. The regime of one booster following the primary immunisation was used. The comparison was based on the level and duration of serum IgG response, correlation between the anti-*S. bovis* and anti-*Lactobacillus* IgG, and the effects of immunisation on rumen pH, faecal pH and dry matter content, and liveweight gain. The numbers of rumen *S. bovis* and *Lactobacillus* were also determined in rumen fluid samples collected from the control and the animals immunised with vaccine containing either QuilA or Dex adjuvant, in order to further confirm the observation in Chapter 8 that the immunisation can inhibit the growth of rumen *S. bovis* and *Lactobacillus* in cattle.

9.2 Materials and methods

9.2.1 Vaccine preparation

The *S. bovis* (Sb-5) and *Lactobacillus* (LB-27) suspensions were prepared as described in Chapter 3. The final bacterial suspension (BS) contained 2 x 10^{10} cells of Sb-5 and 1 x 10^{10} cells of LB-27 per 2.5 ml. Each vaccine batch was prepared using 17.5 ml of the BS (a mixture with the ratio of 14.5 ml of the Sb-5 suspension and 3.50 ml of the LB suspension) and a different adjuvant:

FCA The 17.5 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.

QuilA 7.0 mg QuilA was dissolved in 17.5 ml sterile PBS, and 17.5 ml of the BS was added in drops over a period of 5 minutes while being vigorously mixed. The mixing was continued for another 20 minutes.

Dex 14.5 ml Sb-5 suspension was centrifuged at 4°C (10,000 g for 20 minutes), and then 12.25 ml of the supernatant was removed. The pellet was re-suspended by adding 3.50 ml of the LB suspension and mixed thoroughly. The concentrated suspension was mixed (vortex) with 5.25 ml of 20% DEAE-Dextran (Pharmacia Biotech) solution (pH 7.5). This mixture was then emulsified with 24.5 ml of the mineral oil (Whittrex-307).

Alum 8.75 ml of the Imject Alum adjuvant was mixed well with 8.75 ml sterile PBS, and 17.5 ml of the BS was added to it dropwise over a period of 5 minutes while being vigorously mixed. The mixing was continued for another 30 minutes.

9.2.2 Experimental procedures

Twenty four 1-year old Angus steers (in Tullimba Feedlot Facility of CRC for cattle and beef industry) fed a finishing ration (75% Barley, 10% Lucerne hay, 8% Molafos, 5% Protein meal, 1% Limestone, 0.5% Bicarbonate, and 0.5% Ammonium sulphate) were used in this experiment. They were penned together with access to water at all times under the feedlot conditions. The animals were fed in the morning (8:00 am) and in the afternoon (4:00 pm), and were maintained on the ration until the end of the experiment. The trial was conducted during the period from November 1995 to May 1996. The experimental procedures are summarised in Table 9.1.

Prior to immunisation the animals were randomised into 5 treatment groups, and a primary injection was administered on Day 0, while the booster injection was given on Day 26. Each injection was administered intramuscularly into the neck of cattle. The adjuvant used in each treatment group was described as follows:

Control (n=4) Control group receiving no immunisation;

FCA (n=5) Freund's complete adjuvant for the primary immunisation and Freund's incomplete adjuvant for the booster;

QuilA (n=5) QuilA for both primary and secondary injections;

Dex (n=5) DEAE-Dextran combined with mineral oil as the adjuvant for both primary an secondary injections; and

Alum (n=5) Imject Alum for both primary an secondary injections.

Sample collection and weighing of animals were performed in the morning on the days described follows. Samples of blood were collected on Days -9, 12, 26, 33, 47, 61, 75, 103, and 138 for measuring IgG antibody responses. Samples of rumen fluid were taken via a stomach tube on Days -9, 12, 26, 47, and 138 for the measurement of rumen pH. A rectal sample of faeces (1 g) was weighed immediately after collection on Days 12, 26, 33, 40, 47, 54, 61, 75, 89, 103, and 138 and mixed thoroughly with 8 ml of distilled water (pH 7.0) for measuring pH. The dry matter content of faeces collected on Days -2, 54, 61, 75, 89, 103, and 138 was determined by drying the faeces to a consistent weight at 70^{o}C. The animals were weighted on Days -2, 5, 12, 19, 26, 33, 40, 47, 54, 61, 75, 89, 103, and 138, and the liveweight on Day -2 was used as a covariate for statistically analyzing the difference in liveweight gains between treatment groups. The numbers of *S. bovis* and *Lactobacillus* in the rumen fluid collected on Days -9, 47, and 138 from the animals in the Control, QuilA, and Dex groups were determined. Due to the cost and time involved in these sampling and in the analysis, rumen samples were not collected from the FCA and Alum groups for the analysis of the rumen bacteria population.

Table 9.1 Timetable of major events, sample collections, and measurements

Day	Immunisation	Sample	Measurement
-9		Blood, rumen fluid	Antibody, RpH, numbers of rumen *S. bovis* and *Lactobacillus*
-2		Faeces	FDM, LW
0	Primary immunisation		
5			LW
12		Blood, rumen fluid, faeces	Antibody, RpH, FpH, LW
19			LW
26	Booster	Blood, rumen fluid, faeces	Antibody, RpH, FpH, LW
33		Blood, faeces	Antibody, FpH, LW
40		Faeces	FpH, LW
47		Blood, rumen fluid, faeces	Antibody, RpH, FpH, LW, numbers of rumen *S. bovis* and *Lactobacillus*
54		Faeces	FpH, FDM, LW
61		Blood, faeces	Antibody, FpH, FDM, LW
75		Blood, faeces	Antibody, FpH, FDM, LW
89		Faeces	FpH, FDM, LW
103		Blood, faeces	Antibody, FpH, FDM, LW
138		Blood, rumen fluid, faeces	Antibody, RpH, FpH, FDM, LW numbers of rumen *S. bovis* and *Lactobacillus*

Antibody, serum anti-*S. bovis* and anti-*Lactobacillus* IgG; RpH, rumen fluid pH; FpH, faecal pH; FDM, faecal dry matter content; LW, liveweight.

9.3 Results

9.3.1 Antibody responses

Low levels of anti-*S. bovis* and anti-*Lactobacillus* IgG were detected in the serum of all animals on Day -9 (prior to immunisation), and the control on Days -9 to 138 (over the whole period of experiment) (Tables 8.2 and 8.3).

Compared with the control group, significantly higher serum antibody (anti-*S. bovis* and anti-*Lactobacillus* IgG) concentrations were observed on Day 12 (12 days following the primary immunisation), in FCA, QuilA, and Dex groups ($P<0.05$). The level of the anti-*Lactobacillus* IgG in the Alum group was significantly higher than the control on Day 12. Significantly higher levels of the anti-*S. bovis* and anti-*Lactobacillus* IgG concentrations in serum were observed in all of the immunised groups from Day 26 to 75. However, no significant difference in the anti-*S. bovis* IgG concentration was found on Day 103 ($P>0.05$), while the anti-*Lactobacillus* IgG levels in all the immunised groups remained significantly higher than the control until the end of the experiment. On Day 138, the levels of anti-*S. bovis* IgG in FCA and Alum were not different from the control ($P>0.05$).

The significant difference in antibody concentration between the FCA and other immunisation groups is also summarised in Tables 8.2 and 8.3. The anti-*S. bovis* IgG and anti-*Lactobacillus* IgG concentrations in the QuilA group were not significant different from those in the FCA group ($P>0.05$). There were similar or higher levels ($P<0.05$) of anti-*S. bovis* and/or anti-*Lactobacillus* IgG concentrations in the Dex and Alum groups than the FCA group.

The average (over the period Day 33 to 138) antibody concentration in the Dex group was the highest ($P<0.05$) in the treatment groups in terms of both anti-*S. bovis* and anti-*Lactobacillus* IgG, and that there were no significant differences between the QuilA, Alum, and FCA groups.

The anti-*S. bovis* IgG and anti-*Lactobacillus* IgG levels remained constant ($P>0.05$) until Day 103 when significant decreases in the IgG concentrations occurred in all immunisation groups ($P<0.05$) with the following exceptions: (1) a

significant decrease in anti-*S. bovis* IgG concentration was observed on Day 61 in the QuilA and Dex groups (P<0.05), and (2) there was no a further decrease (P>0.05) in anti-*S. bovis* IgG level of the QuilA group after Day 61.

Table 9.2 Mean serum anti-*S. bovis* IgG concentration (units/ml) of non-immunised cattle and cattle immunised with vaccines using FCA, QuilA, Dex, and Alum adjuvants. Data are presented as Least Square Means. Primary immunisation was administered on Day 0, and the booster was given on Day 26

Day	Control (n=4)	FCA (n=5)	QuilA (n=5)	Dex (n=5)	Alum (n=5)	SED	P
-9	3.8	4.2	5.0	7.1	5.4	2.1	>0.05
12	4.8	14	20	19	11_{ns}	4.2	<0.01
26	3.1	10	14	17	8.4	2.4	<0.01
33	3.3	30	24	41	19	13	<0.01
47	3.3	31	19	45	23	16	<0.01
61	4.7	17	11	29*	9.7_{ns}	6.9	<0.01
75	2.0	28	12	31	15	12	<0.01
103	6.7	17_{ns}	11_{ns}	15_{ns}	9.9_{ns}	7.4	>0.05
138	4.7	8.4_{ns}	11	17	7.1_{ns}	4.1	<0.05

Compared with the antibody concentration (since Day 12) in the control, all the values (in FCA, QuilA, Dex, and Alum groups) within rows are significantly higher (P<0.05 or P<0.01), except those values with subscript $_{ns}$ are not significantly different (P>0.05).

Compared with the antibody concentration (since Day 12) in FCA group, all the values (in QuilA, Dex, and Alum groups) within rows are not significantly different (P>0.05), except the value with superscript * is significantly higher (P<0.05).

Table 9.3 Mean serum anti-*Lactobacillus* IgG concentration (units/ml) of the groups of non-immunised cattle and cattle immunised with vaccines using FCA, QuilA, Dex, and Alum adjuvants. Data are presented as Least Square Means. Primary immunisation was administered on Day 0 and the booster was given on Day 26

Day	Control (n=4)	FCA (n=5)	QuilA (n=5)	Dex (n=5)	Alum (n=5)	SED	P
-9	8.1	11	15	18	12	6.7	>0.05
12	9.3	17	18	23	20	4.7	<0.05
26	6.7	13	18	26*	19	4.7	<0.01
33	9.2	29	53	88*	49	21	<0.01
47	8.8	23	40	66*	64*	16	<0.01
61	6.8	23	34	65*	46*	12	<0.01
75	12	48	59	109*	70	17	<0.01
103	5.6	27	27	52	32	12	<0.01
138	6.2	22	26	44	25	7.2	<0.01

Compared with the antibody concentration (since Day 12) in the control, all the values (in FCA, QuilA, Dex, and Alum groups) within rows are significantly higher ($P<0.05$ or $P<0.01$).

Compared with the antibody concentration (since Day 12) in FCA group, all the values (in QuilA, Dex, and Alum groups) within rows are not significantly different ($P>0.05$), except those values with superscript * are significantly higher ($P<0.05$).

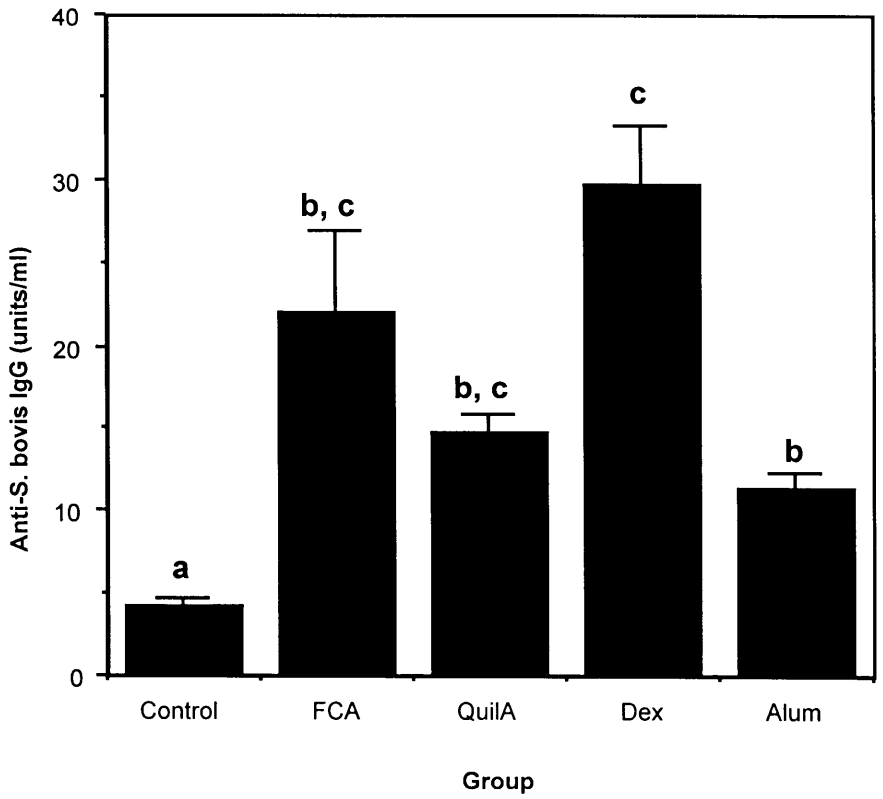


Figure 9.1 Average (after booster immunisation, from Day 33 to 138) serum anti-*S. bovis* antibody IgG concentrations (units/ml) of non-immunised cattle and cattle immunised with vaccines using FCA, QuilA, Dex, and Alum adjuvants. Vertical error bars represent standard errors of the means. Values with different letters (a, b, or c) are significantly different ($P<0.05$)

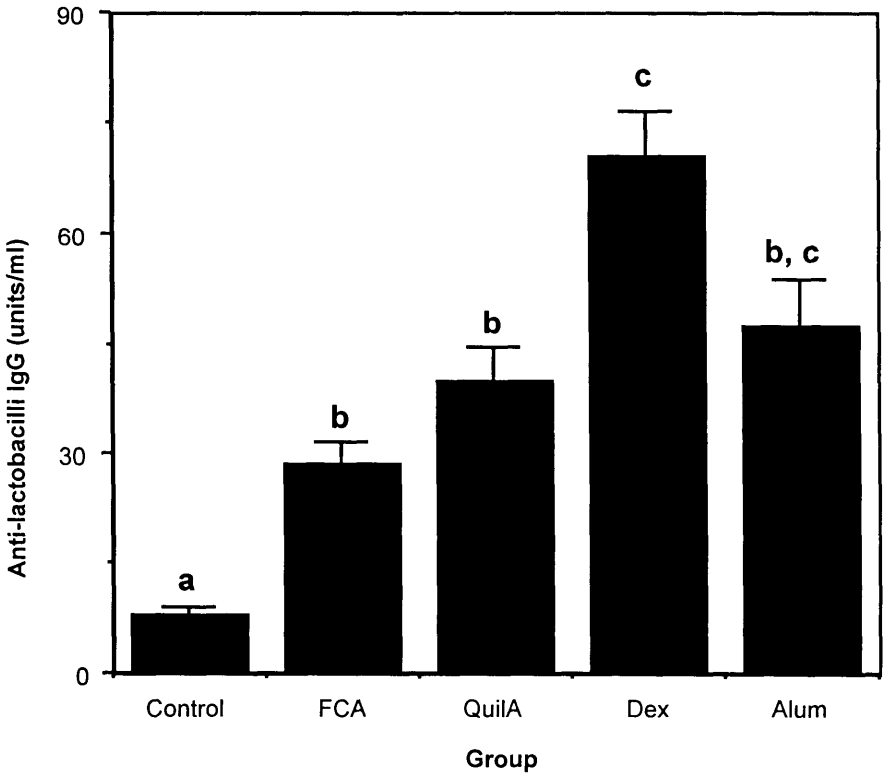


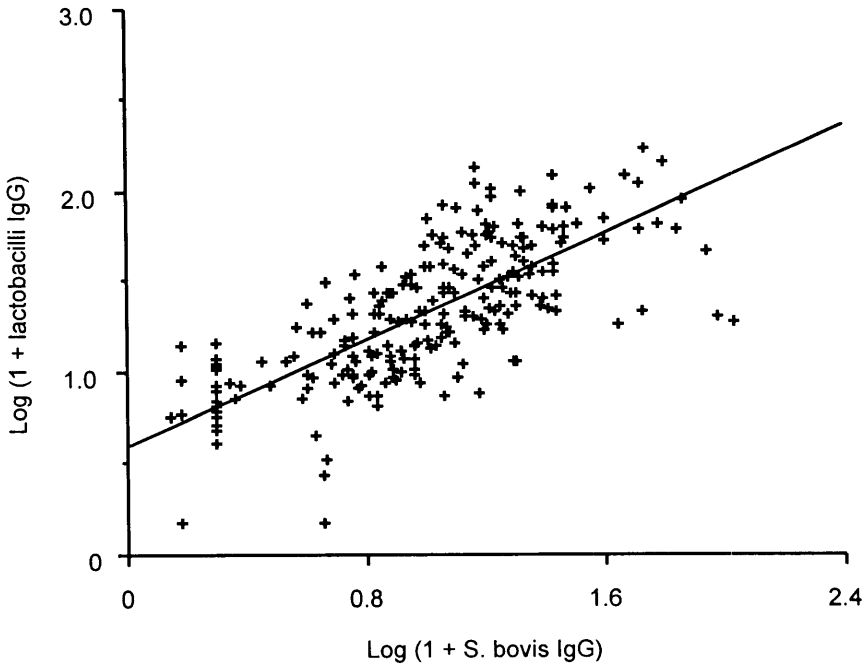
Figure 9.2 Average (after booster immunisation, from Day 33 to 138) serum anti-*Lactobacillus* (lactobacilli) antibody IgG concentrations (units/ml) of non-immunised cattle and cattle immunised with vaccines using FCA, QuilA, Dex, and Alum adjuvants. Vertical error bars represent standard errors of the means. Values with different letters (a, b, or c) are significantly different ($P<0.05$)

9.3.2 Correlation between anti-*S. bovis* and anti-*Lactobacillus* IgG

There was a poor correlation between the anti-*S. bovis* and anti-*Lactobacillus* IgG in the control group (R^2=0.176). Positive linear correlations were found between the anti-*S. bovis* and anti-*Lactobacillus* IgG responses in all the immunised animals, with R^2 values ranging from 0.341 to 0.726 (Table 9.4). The correlation between the antibodies in the animals immunised with the DEAE-Dextran combined with mineral oil adjuvant was the strongest (R^2=0.726). The overall linear relationship (R^2=0.563), between the anti-*S. bovis* and anti-*Lactobacillus* IgG in all the animals, is presented in Figure 9.3.

Table 9.4 The correlations between the anti-*S. bovis* and anti-*Lactobacillus* IgG concentrations of non-immunised cattle and cattle immunised with vaccines using FCA, QuilA, Dex, and Alum adjuvants

Group	Slope	Intercept	R^2
Control	0.392	0.636	0.176
FCA	0.402	0.857	0.341
QuilA	0.628	0.719	0.416
Dex	0.822	0.563	0.726
Alum	0.807	0.633	0.526



Log (1 + lactobacilli IgG) = 0.593 + 0.733 Log (1 + S. bovis IgG); R^2 = 0.563

Figure 9.3 The overall relationship between anti-*S. bovis* and anti-*Lactobacillus* (lactobacilli) IgG in serum taken from non-immunised cattle and cattle immunised with vaccines using FCA, QuilA, Dex, and Alum adjuvants. Line in the figure represents the regression line of the anti-*S. bovis* and anti-*Lactobacillus* antibody IgG. The equation in the figure is the regression equation of the anti-*S. bovis* and anti-*Lactobacillus* antibody IgG

9.3.3 Rumen pH, faecal pH and dry matter content, and liveweight gain

There were no treatment effects on rumen pH, faecal dry matter content, and liveweight gain ($P>0.05$). However, a significant treatment effect on faecal pH ($P<0.05$) was found: the average (over time) faecal pH in the QuilA and Dex groups

were significantly higher than the control. Table 9.5 summarises the average rumen pH, faecal pH, faecal dry matter content, and the liveweight gain of the cattle.

Table 9.5 Average (over time) rumen pH, faecal pH, and faecal dry matter content, and liveweight gain of non-immunised cattle and cattle immunised with vaccines using FCA, QuilA, Dex, and Alum adjuvants. Data were presented as Least Square Means (SE)

Measurement	Control	FCA	QuilA	Dex	Alum
Rumen pH	6.21	6.31	6.31	6.27	6.23
	(0.10)	(0.09)	(0.09)	(0.09)	(0.09)
Faecal pH	6.67	6.79	6.92 *	6.93 *	6.84
	(0.08)	(0.07)	(0.07)	(0.07)	(0.07)
Faecal dry matter content (%)	17.3	17.4	16.5	16.6	17.5
	(0.74)	(0.66)	(0.66)	(0.66)	(0.66)
Liveweight gain (Kg)	174	160	159	168	158
	(11.2)	(9.9)	(10.0)	(9.9)	(9.9)

Values with the superscript "*" were significantly higher than the value in the control group within the same row ($P<0.05$)

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

No significant difference in the number of either *S. bovis* or *Lactobacillus* between the immunised and control groups was observed prior to immunisation (on Day -9) ($P>0.05$). The number of rumen *S. bovis* in the immunised cattle was lower ($P<0.05$) than the control on Day 47 (21 days after the booster immunisation), and the number of rumen *Lactobacillus* in the immunised cattle tended to be lower

(P=0.050) than the control. On Day 103 (77 days after the booster immunisation) the difference in the number of *S. bovis* or *Lactobacillus* between the groups was not significantly different (P>0.05). The numbers of the rumen bacteria in the FCA and Alum groups were not investigated.

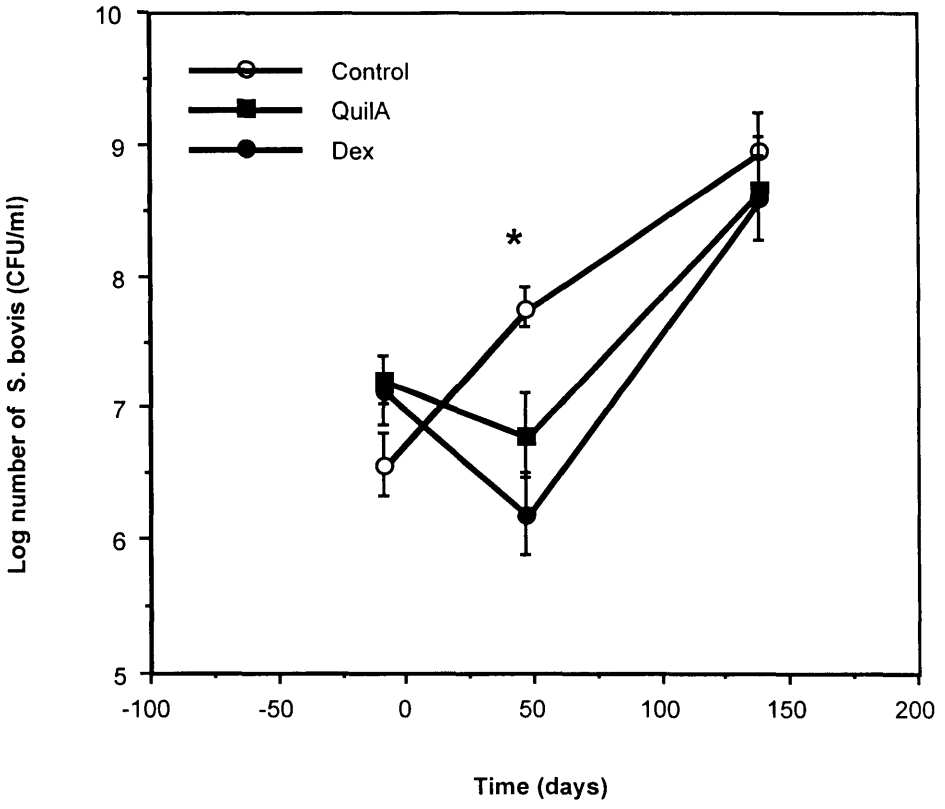


Figure 9.4 Mean log number of *S. bovis* in rumen fluid collected from the control animals and cattle immunised with vaccines using QuilA and Dex adjuvants. Vertical error bars represent standard errors of the means (n=5 for QuilA or Dex group, n=4 for the Control). * The number of rumen *S. bovis* in the immunised cattle was significantly lower than in the control (P<0.05) on Day 47

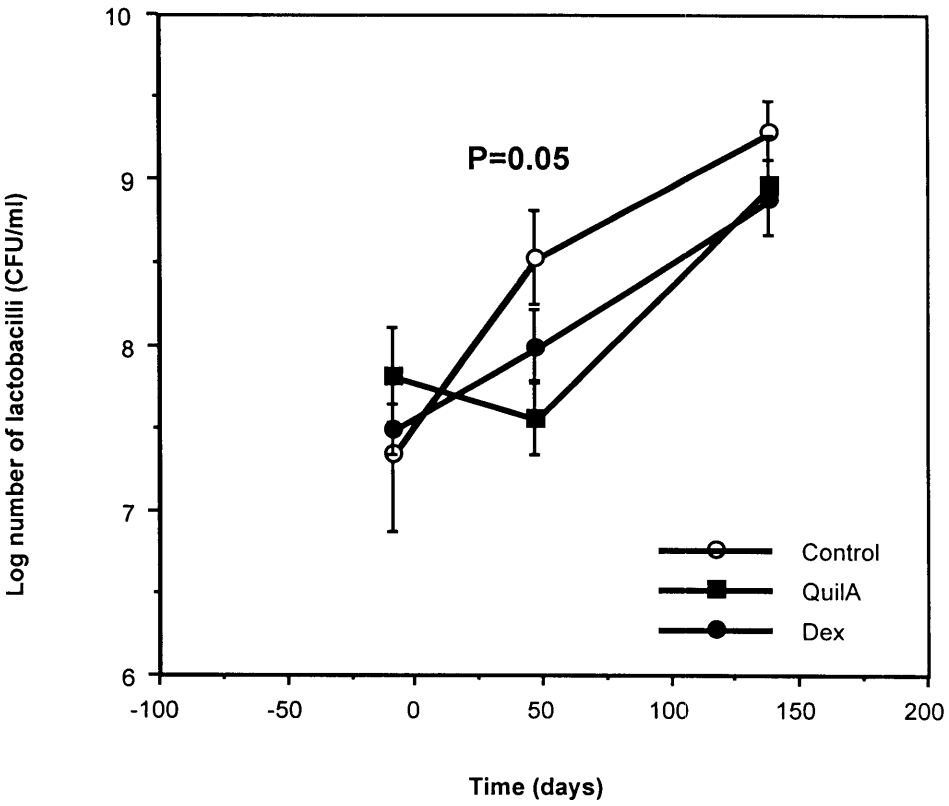


Figure 9.5 Mean log number of *Lactobacillus* (lactobacilli) in rumen fluid collected from non-immunised cattle and cattle immunised with vaccines using QuilA and Dex adjuvants. Vertical error bars represent standard errors of the means (n=5 for QuilA or Dex group, n=4 for the Control). The number of rumen *Lactobacillus* in the immunised cattle tended to be significantly lower than in the control on Day 47 (P=0.050)

9.4 Discussion

This experiment shows that the three commercially acceptable adjuvants are effective in inducing high levels and lasting serum IgG responses to *S. bovis* and *Lactobacillus* in cattle. The results also indicated that a vaccine using QuilA adjuvant induced a similar level of IgG antibody response as the vaccine using Freund's complete/incomplete adjuvant, and a higher IgG level in the Alum group was observed on several occasions compared with the FCA group. The vaccine using Dextran-DEAE combined with mineral oil adjuvant induced the highest antibody IgG responses, which is consistent with the findings of Watson (1992a) who reported that the most promising mastitis vaccine had dextran sulphate combined with mineral oil as the adjuvant. This is probably due to the combined effects of the mineral oil and the Dextran adjuvants, because both the mineral oil and the Dextran adjuvant can induce significant antibody responses on their own (see Chapter 7). Similar or higher levels of IgG responses were induced by either QuilA or Alum groups compared with the FCA. The reasons for these results are not fully understood because it is well known that FCA is the most potent experimental adjuvant in terms of its ability to stimulate high level and lasting immunity (Edelman, 1980; Freund and McDermott, 1942; McCarthy et al, 1977). This pattern of the efficacy of the adjuvants is apparently different from that described in Chapter 7, where the FCA induced the highest antibody response compared with the other adjuvants (including QuilA and Alum). The difference could be due to (1) the difference of responsiveness to the vaccine of animal species (sheep in Chapter 7, and cattle in this study), (2) the difference of antibody measurements (total antibody concentration was measured in Chapter 6, and IgG in the present study).

The durable antibody responses found in this experiment are in agreement with the antibody duration observed in the study described in Chapter 7. Even though there was a decrease in anti-*S. bovis* IgG concentrations in the QuilA and Dex groups on Day 61, the levels were still significantly higher ($P<0.01$) than in the control, and higher ($P<0.05$) than or equal to ($P>0.05$) the IgG level of the FCA group (see Tables 9.2 and 9.3). These results suggest that the lasting antibody response may be able to confer protection from lactic acidosis to feedlot cattle over a two-month period. The period of feedlot cattle on grain normally ranges from 80 to 150 days

(Feedlot Advisory Unit, 1990). Accordingly, the above results further suggest that a second booster may be administered following 2 months after the first booster, in order to maintain high antibody levels until the cattle are finished. However, a 2-month period of grain feeding may allow the animals to become well adapted to the concentrate diet and lactic acidosis would not be a problem in reducing production (Schwartz and Gilchrist, 1974; Mackie *et al.*, 1978; Mackie and Gilchrist, 1979; Shu *et al.*, 1996). On the basis of this consideration, one booster following a primary immunisation regime may provide adequate protection against lactic acidosis for the whole period of grain-feeding in a feedlot situation.

The positive linear correlation between the anti-*S. bovis* and anti-*Lactobacillus* IgG (see Table 9.4 and Figure 9.3), particularly in the Dex group (R^2=0.726), is in agreement with the observation made in Chapter 8 and suggests that the anti-*S. bovis* IgG response can be used as an indicator of the efficacy of anti-*Lactobacillus* IgG response. The antibody levels attained in cattle under feedlot conditions in this experiment are similar to those induced (after the 1st booster) in the cattle grazing pasture (see Chapter 8). The implication of these results is that in practical situations an lactic acidosis vaccine can be administered in animals fed either a forage-based or grain-based diet. It is worth noting that the risk of lactic acidosis is generally accompanied with the introduction of grain (Ahrens, 1967; Braun *et al.*, 1992), and whenever possible immunisation should be conducted prior to the introduction of grain in order to achieve the maximum benefits against lactic acidosis.

Compared with the FCA group, the similar or higher levels of IgG responses in QuilA, Alum, or Dex groups suggest that using these adjuvants should provide at least a similar level of protection against acute lactic acidosis in cattle as FCA. The direct biological benefits of the immune responses against *S. bovis* and *Lactobacillus* are seen in this experiment. In animals adapted to high grain rations, some grain can still pass undigested to the hind gut where fermentation can lead to some accumulation of lactic acid (Rowe and Pethick, 1994). The higher faecal pH of the cattle immunised using vaccines with the Dex and QuilA adjuvants against *S. bovis* and *Lactobacillus*, are therefore important as it suggests a subtle, yet significant effect on acid accumulation in the hindgut (Allison *et al.*, 1975; Shu *et al.*, unpublished data). Figures 9.4 and 9.5 show that there were lower numbers of rumen *S. bovis* and *Lactobacillus* in the immunised animals 3 weeks after the booster,

compared with the control. These results provide further evidence supporting the observation in Chapter 8 that the immunisation can directly reduce the rumen *S. bovis* and *Lactobacillus* populations thereby reducing the lactic acid-producing capacities.

The results also confirmed the previous observations reported in Chapter 8 that immunisation did not completely remove either *S. bovis* or *Lactobacillus* in the rumen. The reduced numbers of *S. bovis* and *Lactobacillus* in immunised animals still remained at levels comparable with those found in the normal animals adapted to high concentrate rations (Mackie *et al.*, 1978; Shu *et al.*, unpublished data). *S. bovis* and *Lactobacillus* actively degrade starch and are important in terms of normal rumen flora (Cotta, 1988; McAllister *et al.*, 1990). The importance of these bacteria is indicated by the high proportion (25-70%) of rumen *S. bovis* and *Lactobacillus* in the total amylolytic bacteria in animals which have been well adapted to grain-based diets (Mackie et al, 1978; Shu et al, unpublished data). Immunisation confers two apparent benefits: (1) the lower numbers of lactic acid-producing bacteria in the immunised animals (compared with the control) which may lead to lower lactic acid production thereby reducing the risk of lactic acidosis, and (2) the reasonably high levels of rumen *S. bovis* and *Lactobacillus* in the immunised animals being able to contribute to the normal starch fermentation when animals are fed grain-based diets. Again, it is worth mentioning that it is not known if the functions of *S. bovis* and *Lactobacillus* in the immunised animals have changed under the present experimental conditions, in terms of the fermentation of starch and the production of lactic acid. For example it has been shown that salivary anti-protozoa antibodies can immobilise protozoa in the rumen fluid and reduce the rate of predation by the ciliates on radio-labelled bacteria (Gnanasampanthan, 1993). Therefore, it is possible that the antibody responses to *S. bovis* and *Lactobacillus* may also affect other functions of the bacteria in the rumen rather than just inhibit the growth.

No significant difference in the numbers of the bacteria between the groups was found in the rumen fluid collected on Day 138 (112 days after booster immunisation). This result could be due to the significant decrease in the antibody concentration in the immunised animals. However, this may be of minor importance in terms of lactic acidosis, as by then the animals would be expected to be well adapted to the grain ration and will be killed soon for meat production (Schwartz and

Gilchrist, 1974; Mackie et al, 1978; Mackie and Gilchrist, 1979; Feedlot Advisory Unit, 1990; Shu et al, 1996). No significant treatment effects on rumen pH and faecal dry matter content were observed during the experimental period. These results are not surprising, as rumen pH over the experimental period did not fall below 6.0 in the control group. A rumen pH greater than 6.0 suggests that lactic acidosis is not a problem for these animals (Ahrens, 1967; Braun *et al.*, 1992). Another possible reason is that due to limitation of sample size and frequency, the differences in the rumen pH or faecal dry matter content between the groups were not statistically significant or not found.

Animals in all treatment groups showed a similar liveweight gain over the 5-month period of experiment. This observation is consistent with the results described in Chapters 6 and 7, which suggests that immunisation with a vaccine containing live *S. bovis* and *Lactobacillus* cells and one of the commercially acceptable adjuvants, has no adverse effect on the health of animals.

Although saliva samples were collected for measuring the antibody responses during the experiment. There could not be analyzed due to lack of resources. More useful information would be drawn from the saliva antibody measurements.

In conclusion, the results suggest that immunisation with vaccines using the commercially acceptable adjuvants may provide equal or superior protection against lactic acidosis when compared to Freund's complete/incomplete adjuvant. The group vaccinated with the DEAE-dextran combined with mineral oil adjuvant had the highest IgG response and the strongest correlation between the anti-*S. bovis* and anti-*Lactobacillus* IgG, and it might be the most promising adjuvant tested.

Chapter 10

Immunological cross-reactivity between the vaccine and other isolates of *S. bovis* and *Lactobacillus*

10.1 Introduction

Results from the experiments reported in Chapters 4, 5, 6, 7, and 8 have supported the hypothesis that the risk of lactic acidosis can be reduced by immunisation against *S. bovis* or *S. bovis* and *Lactobacillus*, and indicated that a number of practically acceptable adjuvants in the use of lactic acidosis vaccines have been successful in stimulating significant antibody responses in sheep and cattle. These studies have been based on the use of the single strain Sb-5 of *S. bovis* or a combination of the Sb-5 and isolate of *Lactobacillus* LB-27 (see Chapter 3). However, a large number of strains of *S. bovis* and *Lactobacillus spp.* have been found in the rumen, and antigenic variation between strains has been shown (reviewed in Chapter 2; Shu and Liu, 1995b). During the development of an immunisation strategy it is therefore necessary to consider the potential of the vaccine to protect sheep and cattle from a number of strains of *S. bovis* and *Lactobacillus spp.* which may cause lactic acidosis.

Watson and Franklin (1988) have successfully developed an *in vitro* method to assess the potential for the *S. aureus* vaccine to protect animals from numerous strains of *S. aureus* which may cause mastitis. Their method assesses the degree of immunological cross-reactivity between the vaccine strain of *S. aureus* and field isolates from cases of bovine mastitis. Accordingly, in order to determine the

efficacy of the vaccine developed in this project against a number of strains of *S. bovis* and *Lactobacillus spp.*, the following experiment was designed to investigate the degree of immunological cross-reactivity between the vaccine strain (Sb-5) and 8 other strains of *S. bovis*, or the vaccine isolate (LB-27) and 4 other isolates of *Lactobacillus*.

10.2 Materials and methods

10.2.1 Isolates of *S. bovis* and *Lactobacillus*

Nine strains of *S. bovis* (including Sb-5) and 5 isolates of *Lactobacillus* were used in this experiment. These strains or isolates were isolated previously from the rumen content collected from sheep and cattle fed with grain-based diets in this laboratory. The procedures were the same as described in Chapter 3. The DNA fingerprint data (Restriction Fragment Length Polymorphism data: CfoI, HaeIII gpl, Table 10.1) was kindly provided by Klieve (personal communication). The DNA analysis was based on PCR amplified 16S rRNA genes of the strains. The animal sources and some characteristics of the bacteria are also summarised in Table 10.1.

10.2.2 Experimental procedures

Suspensions of the above strains of *S. bovis* (1 x 10^{10} cells/ml) and the isolates of *Lactobacillus* (1 x 10^{10} cells/ml) were prepared as described in Chapter 3. Antiserum was collected from cattle immunised with a vaccine containing Sb-5 and LB-27 cells reported in Chapter 9, and the antiserum was a pooled serum collected from 5 cattle in the FCA group (2 weeks after the booster immunisation). The determination of immunological cross-reactivity was based on the method described by Watson and Franklin (1988) and is summarised below.

Antiserum was diluted to 1:8,000 in sterile PBS, and a 5 ml aliquot was transferred into a 10 ml sterile tube. A 25 µl aliquot of 1 x 10^{10} cells/ml bacterial suspension was then added to the tube, mixed thoroughly, and rotated for 1 hour at 37°C in a water bath. Following incubation the mixture was then centrifuged at 4°C for 30 minutes (11,000 g), and the supernatant was tested by ELISA for any residual

Table 10.1 Sources and some characteristics of strains of *S. bovis* and isolates of *Lactobacillus* obtained from sheep and cattle

Bacteria	Animal type	Gram reaction	Capsule	RFLP data
S. bovis				
SI	Sheep	+	-	CfoI, HaeIII gp1
SII	Sheep	+	-	CfoI, HaeIII gp1
SIII	Cattle	+	-	CfoI, HaeIII gp1
Sb-5	Cattle	+	+	CfoI, HaeIII gp1
SVIII	Cattle	+	+	CfoI, HaeIII gp1
SIX	Cattle	+	-	CfoI, HaeIII gp1
SX	Cattle	+	-	CfoI, HaeIII gp1
SXI	Cattle	+	-	CfoI, HaeIII gp1
SXII	Cattle	+	+	CfoI, HaeIII gp1
Lactobacillus				
LB-G1	Cattle	+	NA	NA
LB-G2	Cattle	+	NA	NA
LB-T1	Cattle	+	NA	NA
LB-T2	Cattle	+	NA	NA
LB-27	Cattle	+	NA	NA

RFLP, Restriction Fragment Length Polymorphism; NA, data not available.

antibody IgG activity against Sb-5 and LB-27. An unabsorbed antiserum (no added bacterial cells) was included as positive control. On each ELISA plate the unabsorbed antiserum (positive control) was assayed as well as the antiserum which had been absorbed with the Sb-5 or LB-27 (negative control) or the test strains. Each preparation was assayed in triplicate. A cross-reactivity index (CRI) was calculated for each strain of *S. bovis* or isolate of *Lactobacillus*:

$$\text{CRI (\%)} = \frac{\text{Antibody units (per ml) in antiserum after absorption}}{\text{Antibody units (per ml) in unabsorbed antiserum}} \text{ X } 100$$

Thus, the CRI would have a range of 0-100%. A low CRI indicates a high degree of immunological cross-reactivity between: the vaccine strain (Sb-5) and other strains of *S. bovis*, or the vaccine isolate (LB-27) and other isolates of *Lactobacillus*.

10.3 Results

10.3.1 Antigenic cross-reactivity between the vaccine strain (Sb-5) and test strains of *S. bovis*

When tested by ELISA for anti-*S. bovis* antibody IgG (induced by Sb-5), the unabsorbed antiserum (positive control) contained 98.9 (SE=1.3) antibody units/ml. Antiserum absorbed with the Sb-5 strain (negative control) had an antibody concentration of 9.3 (SE=0.6) units/ml, giving a CRI of 9.4% (Figure 10.1). The other 2 encapsulated strains of *S. bovis* (SVIII and SXII) had CRIs of 7.3% and 12.4%, respectively. The other 6 (non-encapsulated) strains of *S. bovis* had CRIs ranging from 28.9% to 56.1%. The CRI values of the encapsulated strains (included Sb-5) were significantly lower than the CRI values of the non-encapsulated strains of *S. bovis* ($P<0.01$).

10.3.2 Antigenic cross-reactivity between vaccine isolate (LB-27) and the test isolates of *Lactobacillus*

When tested by ELISA for the anti-*Lactobacillus* antibody IgG (induced by

LB-27), the unabsorbed antiserum (positive control) contained 102.4 (SE=2.0) antibody units/ml. Antiserum absorbed with the LB-27 isolate (negative control) had an antibody concentration of 11.8 (SE=0.6) units/ml, giving a CRI of 11.5%. The four test isolates of *Lactobacillus* had CRIs ranging from 13.1 to 72.2% (Figure 10.2).

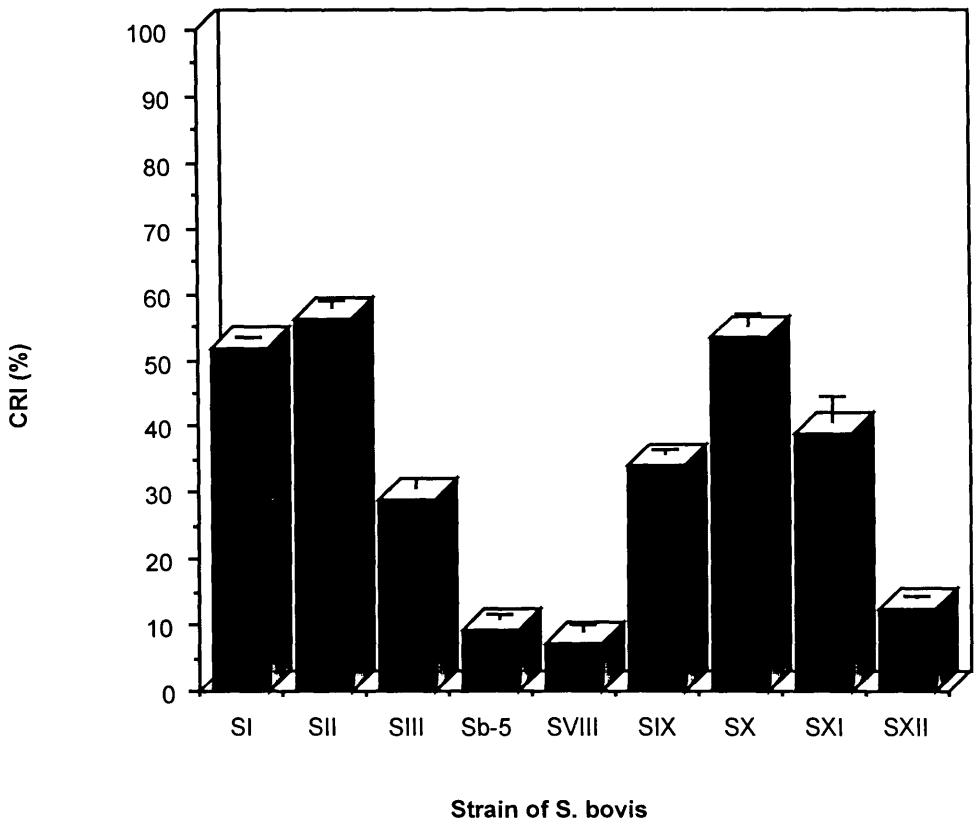


Figure 10.1 Mean cross-reactivity index of the vaccine strain Sb-5 and 8 other strains of *S. bovis* isolated from sheep and cattle. Vertical error bars represent standard errors of the means
9.4% is the cross-reactivity index of the antiserum absorbed by the vaccine strain Sb-5 itself. 100% is nil cross-reactivity

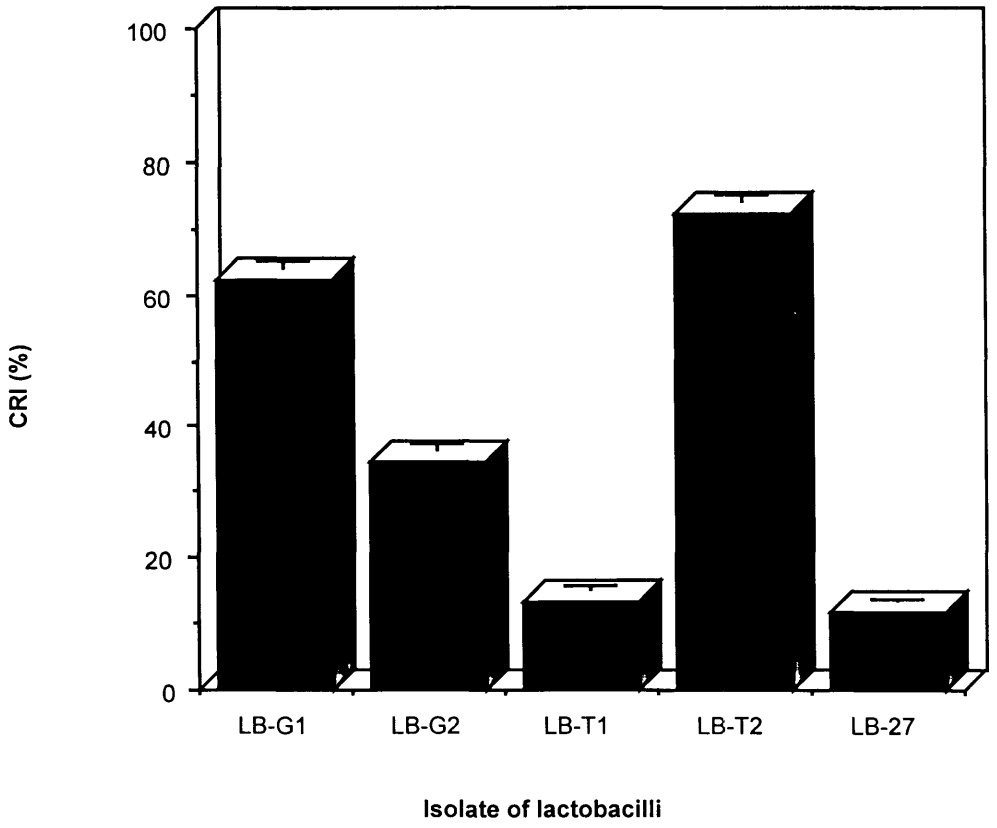


Figure 10.2 Mean cross-reactivity index of the vaccine isolate LB-27 and 4 other isolates of *Lactobacillus* (lactobacilli) obtained from sheep and cattle. Vertical error bars represent standard errors of the means
11.5% is the cross-reactivity index of the antiserum absorbed by the vaccine isolate LB-27 itself. 100% is nil cross-reactivity

10.4 Discussion

The results of DNA fingerprint analysis (Klieve, personal communication) suggest that there is a high degree of genetic homology between them. However, there is no experimental evidence that genetic homology correlates with a high

degree of immunological cross-reactivity, which is an indicator of the potential ability for a vaccine to be active against a wide range of pathogenic bacteria strains (Watson and Franklin, 1988). On the other hand, antigenic variation between *S. bovis* strains isolated from sheep and cattle has also been demonstrated (Medrek and Barnes, 1962; Shu and Liu, 1995b) although a high degree of genetic homology has been found between rumen *S. bovis* strains (Farrow *et al.*, 1984; Klijin *et al.*, 1991; Nelms *et al.*, 1995). Accordingly, in order to determine the efficacy of the vaccine developed in this project against several strains of *S. bovis* and isolates of *Lactobacillus* from both sheep and cattle, there was a need to test the degree of immunological cross-reactivity between the vaccine and other isolates of *S. bovis* and *Lactobacillus* which may cause lactic acidosis.

Evidence of considerable antigenic variation and immunological cross-reactivity have been demonstrated with *S. bovis* strains isolated from pigeons (De Herdt et al, 1992). These authors identified 5 serotypes, with immunological cross-reactivity between only 15% of the strains. Figure 9.1 shows that the CRIs ranged from 7.3 to 56.1%, which provides further evidence that strains of *S. bovis* from sheep and cattle in Australia have considerable antigenic variation. However, the results also indicate that although there were large differences between strains in the degree to which they cross-react with the vaccine reference strain, all the strains tested cross-reacted to some extent.

A feature of the results was that the 3 encapsulated strains of *S. bovis* (including Sb-5) had similar CRIs (range: 7.3 to 12.4%), which were significantly lower than the CRIs of the non-encapsulated strains (range: 28.9 to 56.1%). There is evidence indicating that both cell wall and capsule materials may contribute to the antigenicity of *S. bovis* (Kane and Karakawa, 1969a; 1969b; 1971). The higher CRIs of the non-capsulate strains may be due to the lack of capsular antigens of these strains, because the antiserum was prepared from the cattle immunised with a vaccine containing encapsulated *S. bovis* strain Sb-5 cells. The non-encapsulated strains might lose their capsules when cultured *in vitro* (Watson, 1982; Shu *et al.*, unpublished data). Under rumen conditions, however, it is believed that *S. bovis* cells are surrounded by a large capsule (Ogimoto and Imai, 1981). Therefore, *in vivo*, the degree to cross-reactivity between the Sb-5 and the 8 other strains of *S. bovis* may be even greater (with lower CRIs). These results suggest that the vaccine containing Sb-

5 cells may be effective against a wide range of strains of *S. bovis* in sheep and cattle. However, the current study only examined 9 strains of *S. bovis*. The degree of the cross-reactivity, between Sb-5 and a large number of strains of *S. bovis*, needs to be investigated to further confirm this suggestion.

Figure 9.2 shows that the CRIs ranged from 11.5 to 72.2%, which provide evidence that the isolates of *Lactobacillus* obtained from sheep and cattle have considerable antigenic variation. However, the results also indicate that although there are large differences between isolates in the degree to which they cross-react with the vaccine reference isolate, all the isolates tested cross-reacted to some extent. The degree of cross-reactivity, between the vaccine LB-27 and the test isolates of *Lactobacillus*, was more variable (CRIs from 11.5 to 72.2%) than the strains of *S. bovis* (CRIs from 7.3 to 56.1%). This is probably due to the diversity of several species of *Lactobacillus*, which may contain many strains (Chapters 2 and 3). The results suggest that the vaccine containing LB-27 may not adequately control all species (and strains) of *Lactobacillus* found in sheep and cattle. However, there is a reasonable chance of enhancing the degree of cross-reactivity by optimising the *Lactobacillus* vaccine strains such as using a combination of multiple strains from different *Lactobacillus spp.* because that would enable the vaccine to cover a greater range of strains in different species of *Lactobacillus* (reviewed in Chapter 2).

Finally, the assay used in this experiment for testing the degree of immunological reactivity will be able to serve as a useful resource for further optimising vaccine strains and assessment of future lactic acidosis vaccination programmes. Strain(s) having a high degree of immunological cross-reactivity with a reasonable large number of clinical isolates should be carefully selected to be used in preparing vaccines. In the practical situations, should cases of lactic acidosis appear in vaccinated animals, it will be a straightforward matter to test the isolates for the degree of cross-reactivity with the vaccine strain(s).

Chapter 11

General discussion

Specific aspects of the work reported in this thesis have been discussed in relevant chapters. This chapter collates the main findings and discusses the work in a broader context.

11.1 Overview of the immunisation studies

The results of the sheep experiments support the hypothesis that the risk of lactic acidosis can be reduced by immunisation against *S. bovis*, and the cattle experiment provides further evidence that the risk of lactic acidosis can be reduced by immunisation against *S. bovis* and *Lactobacillus*. Together, these results indicate a sound potential for the use of immunisation against *S. bovis* or a combination of *S. bovis* and *Lactobacillus* as a method for preventing lactic acidosis. The results also provide further evidence to support the suggestion made by Gnanasampanthan (1993), that control of rumen microbes by immunisation is possible. The mechanism suggested by Gnanasampanthan (1993) is that the antibodies specifically bind to the organisms and actively reduce their growth and other functions. Although the effects of immunisation on the other functions of *S. bovis* and *Lactobacillus* were not examined in the work reported in this thesis, direct reduction of the lactic acid-producing bacteria population was shown in the experiments reported in Chapters 7 and 8. The results have also demonstrated that the immunisation did not completely remove either *S. bovis* or *Lactobacillus* in the rumen, and the reduced numbers of *S. bovis* and *Lactobacillus* in immunised animals still remained at the levels

comparable with those in normal animals adapted to high concentration rations (Mackie *et al.*, 1978; Shu *et al.*, 1996). This feature has practical importance in terms of the normal rumen fermentation of grain fed animals, in which *S. bovis* and *Lactobacillus* are major starch degraders (Eadie and Mann, 1970; Hungate, 1975; Mackie *et al.*, 1978; Cotta, 1988; McAllister *et al.*, 1990).

One of the main objectives of this work was to examine several key parameters for developing an effective and practical immunisation strategy against lactic acidosis. The following points related to immunisation strategy can be summarised from the present studies. a) The live *S. bovis* vaccine was more effective than the killed *S. bovis* vaccine. b) The intramuscular immunisation route (using live vaccine bacteria cells) was safe and more effective than which the immunisation primed intraperitoneally. c) A number of commercially acceptable adjuvants were shown to be able to induce a high level and lasting antibody response. Dextran-DEAE combined with mineral oil adjuvant was found to be the most promising adjuvant in cattle. d) One booster following the primary immunisation regime was successful in inducing significant antibody responses and positive biological effects. Accordingly, an immunisation strategy for further work in development of a commercially acceptable vaccine against lactic acidosis is recommended as follows.

(1) Antigen type: Live bacteria cells.

(2) Immunisation route: Intramuscular injection into either medial thigh or/and neck. *Note:* Although intramuscular immunisation is often administered into the thigh of an animal (Watson, 1987 and 1992a and 1992b) and the effects of different injection sites were not compared in this thesis, injections into the thigh and/or neck muscles in animals have been used and induced significant immune responses (Chapter 5, 6, 7, 8, and 9). Therefore, the choice of an injection site depends on practical need. For example, the neck is considered to be preferable to the thigh as an injection site in beef cattle, because this can prevent the risk of any possible local reaction in the thigh and avoid the adverse effects on its meat quality which is important for beef production.

(3) Adjuvant: DEAE-dextran combined with mineral oil (QuilA or Alum adjuvant could also be used).

(4) Booster regime: One booster following the primary immunisation.

Some positive effects of immunisation in reducing the risk or severity of lactic acidosis has been observed in these experiments. These results suggest that the immunisation strategy may be possible to be used in practical situations, although further work need to be carried out to refine the technique. In particular, during drought conditions where grain is often the only food source available for livestock. To place animals on a pure grain diet under these harsh conditions can cause a high incidence of lactic acidosis often leading to deaths. There is a need to have an economical and efficient means of protecting animals from the incidence of lactic acidosis. Immunisation may provide significant protection against lactic acidosis in sheep and cattle in this situation.

It has been demonstrated that the age and genotype of animals influence the immune responsiveness (Glass *et al.*, 1991a and 1991b; Watson *et al.*, 1994a; Colditz *et al.*, 1996). The present immunisation studies were conducted in sheep and cattle of different ages (Chapters 5, 6, and 7) and breeds (Chapters 7 and 8), and reasonably high levels of protective responses were found in all of these animals. This suggests that immunisation may be applicable over a wide range of ages and breeds of sheep and cattle, and it may be possible to be extended to all ruminants and even other herbivorous animals (such as horses) that are fed grain.

11.2 Immunisation and grain-feeding management

The results from the current work have shown that the risk of lactic acidosis can be reduced by immunisation against *S. bovis* or *S. bovis* and *Lactobacillus*. However, complete protection against acute lactic acidosis was not achieved under extreme grain challenge conditions, in which the animals were suddenly introduced to high levels of grain diets, and the immunised animals still suffered from a degree of lactic acidosis (Chapters 4, 5, and 7). A significant indication of this was the decrease in feed intake of the animals (Chapters 4, 5, and 7), which is one of the

typical clinical signs of lactic acidosis (Dawson and Allison, 1988; Feedlot Advisory Unit, 1990; Braun *et al.*, 1992).

Feed intake is fundamental to nutrition and dependent on the digestibility and utilisation of nutrients (Nolan, *et al.*, 1975; Van Soest, 1994). As reviewed in Chapter 2, it is clear that microbial fermentation is an key component of the digestive processes in ruminants. The microbial population in the rumen is maintained in balance by frequent introduction of nutrients and by the physiological regulations provided by the animal (nutrient absorption, flow of saliva and flow out of the rumen). A sudden change in diet can disrupt the normal microbial balance in the rumen and be detrimental to normal rumen function and to health of the host animal (Dawson and Allison, 1988; Van Soest, 1994). One of the indications of these consequences is the decrease in feed intake. In order to prevent the adverse effects, gradual adaptation (introducing a new feed such as grain slowly to the diet of the animals) is normally needed for gradual build up of a new balanced microbial ecology for fermenting the changed diet efficiently (Mackie and Gilchrist, 1978).

As discussed in Chapter 1, in order to reduce the risk of lactic acidosis in livestock, current practices centre around grain-feeding management based on introducing grain slowly to the diet of the animals allowing for adaptation to ferment and digest the new diet. The established microbial population in the rumen of animals fed with concentrated rations is remarkably different from that of animals fed high-forage diets (Schwartz and Gilchrist, 1974; Mackie *et al.*, 1978; Shu *et al.*, 1996). When animals are gradually adapted from a high-forage to a high-concentrate ration, the key change in rumen microbial ecology is the build up of starch-utilising and lactate-utilising bacteria, which contribute to the fermentation of dietary starch and remove lactic acid produced as a result of the rapid fermentation of the starch (Mackie *et al.*, 1978; Mackie and Gilchrist, 1979; Shu *et al.*, 1996). The increase in starch- and lactate- utilising bacteria is critical for the adaptation of the animal from a forage-based diet to a diet containing high level of starch without the risk of lactic acidosis (Mackie and Gilchrist, 1979; Shu *et al.*, 1996). In addition to *S. bovis* and *Lactobacillus*, other starch-utilising bacteria such as *Bacteroides, Selenomonas, Butyrivibrio, and Eubacterium* also play important roles in the fermentation of starch (Mackie and Gilchrist, 1979) in grain fed animals.

As reviewed in Chapter 2, a sudden change from a roughage-based diet to a high concentrate ration can result in the uncontrolled build up of *S. bovis* and *Lactobacillus*, leading to a significant increase in lactic acid-producing capacity and the accumulation of lactic acid (Allison *et al.*, 1975; Dawson and Allison, 1988; Shu and Liu, 1995a). The accumulation of lactic acid in the rumen may drop the rumen pH to below 5.5 and inhibit the growth of other rumen bacteria (including the other types of normal starch utilising bacteria and the lactate-utilising bacteria). The dysfunction of the lactate-utilising bacteria results in an even lower capacity for removing the lactic acid from the rumen. This break down in the balance of the rumen microbial population may lead to lactic acidosis.

The results reported in Chapters 8 and 9 show that *S. bovis* and *Lactobacillus* were still present in the immunised animals, and could rapidly build up in large numbers after grain feeding. The grain challenge (sudden introduction of a diet containing 75-100% wheat as described in Chapters 5, 6, and 8) might disrupt the normal microbial balance in the rumen and be detrimental to normal rumen function of the immunised animal. The significant decrease in feed intake of both sheep and cattle observed as a result of the grain challenge is probably a direct consequence of rumen microbial population unbalance. Therefore, in addition to the immunisation strategy, a gradual adaptation procedure is needed to allow animals to develop a balanced microbial ecology. It appears that immunisation may not achieve the maximum production benefits if the animals are not gradually adapted from a high-forage diet to a high-concentrate ration. However, it may be possible to simplify the grain-feeding management procedures in conjunction with the immunisation strategy as these will be significantly less risk of lactic acidosis. For example, the coupling of the immunisation program with management could halve the adaptation period from 2~4 weeks (Feedlot Advisory Unit, 1990) to 1~2 weeks through improving the rumen conditions by immunisation against the lactic acid-producing bacteria. The reduced lactic acid accumulation may prevent the development of very low pH in the rumen and allow the build up of other starch degraders and lactic acid-utilising bacteria in the period of adaptation. The simplified grain-feeding management procedure would need less labour and reduce the other costs normally involved in the management techniques. Thus, in addition to the production benefits due to reduced risk of lactic acidosis, there may also be other benefits. The optimal

way to combine immunisation with feed management techniques remains to be investigated.

If animals in the feedlot industry are immunised with a lactic acidosis vaccine, under current grain-feeding management, production is expected to be enhanced by the reduced risk of lactic acidosis. Vaccination may also provide an alternative to feed additives such as dietary buffers and antibiotics and the use of lactic acid-utilizing bacteria (reviewed in Chapter 2) for reducing the risk of lactic acidosis in the feedlot industry. It is well known that vaccination is a sustainable and widely used strategy for preventing bacterial diseases in livestock industries. Therefore, the immunisation approach should be acceptable for practical use, in particular, for the prevention of lactic acidosis in feedlot industry which is the major area associated with lactic acidosis (Dawson and Allison, 1988; Feedlot Advisory Unit, 1990; Meppem *et al.,* unpublished data). However, in this work the advantage of immunisation, in terms of the effective protection against lactic acidosis, has not been compared with the other methods as reviewed in Chapter 2.

In conclusion, the forgoing discussion suggests that the immunisation strategy may be used in combination with grain-feeding management techniques in order to achieve maximum benefits in livestock production.

11.3 Immunisation and hindgut lactic acidosis

The study of lactic acidosis is made difficult by the variability between animals. In response to the same amount of carbohydrates, some animals will maintain a normal pattern of fermentation while others develop high levels of lactic acid in the rumen and/or severe diarrhoea (Allison *et al.,* 1975; Shu *et al.*, unpublished data). The exact factors contributing to this variation have never been studied or clearly identified (Rowe and Pethick, 1994). Most research on lactic acidosis in ruminants has concentrated on changes in the rumen with little reference to possible problems of lactic acidosis in the hindgut (Rowe and Pethick, 1994). This is probably due to the rumen being the major area of fermentation in ruminants (Parra, 1978). In a number of studies of animals fed high levels of starch, however, there have been numerous cases of normal rumen fermentation in animals with

severe scouring (Allison *et al.*, 1975; Shu *et al.*, unpublished data) indicating possible abnormalities in the hindgut. Rowe and Pethick (1994) have indicated that hindgut lactic acidosis can be more common and as harmful as the better known problems of lactic acidosis in the rumen. Accordingly, in order to assess the effectiveness of any strategy against lactic acidosis, hindgut lactic acidosis should be taken into account.

When sheep and cattle are over fed with grain, hindgut lactic acidosis may occur and is probably associated with a rapid build up of local *S. bovis* or *S. bovis* and *Lactobacillus* (Allison et al, 1975; Shu et al, unpublished data). As discussed in Chapter 5, immunisation against the lactic acid-producing bacteria in the hindgut may be mediated by antibodies in blood circulation leaking into the hindgut and/or by the local immune responses in the hindgut. High levels of antibody responses have been demonstrated in this thesis, and the antibodies can be transported to the intestine (reviewed in Chapter 2) to act against the bacteria. Thus, reducing the risk or severity of hindgut acidosis. The existence of a protective response in the hindgut against the lactic producing bacteria is also suggested by the indirect experimental evidence including less severe diarrhoea and higher faecal pH observed in the immunised animals (Chapters 4, 5, and 8).

11.4 Future research

11.4.1 Exploring the immunological mechanisms

As indicated earlier by Gnanasampanthan (1993) the induction of responses of salivary antibodies, which enter the rumen and are active against specific rumen micro-organisms, is critical for any attempt to establish immunological control of rumen microbes. This thesis provides evidence that immunisation can reduce the numbers of the rumen lactic acid-producing bacteria. However, there are a number of basic questions that remain to be answered in order to understand the immunological mechanisms by which the vaccine "works". For example: Is the antibody bacteriostatic or bactericidal? Is complement involved? Is there antibody-dependent bacterial killing/phagocytosis by leucocytes? Do the antibodies inhibit the other functions of the bacteria such as lactic acid-producing capacity? Because the

rumen is very complicated and there are few published data on "rumen immunology" related to rumen bacteria, the knowledge of the effector mechanisms may be a great advantage for the development of the immunisation strategy against lactic acidosis. Therefore, it is necessary to conduct further research for exploring the immunological mechanisms.

The literature review (Chapter 2) clearly indicates that saliva is a main source of antibodies in rumen and that IgG is the predominant antibody in saliva which may play an important role against lactic acidosis (see also Chapters 7 and 8). However, the functional features of IgG are different, to some extent, from the other classes of antibodies including IgM, IgA, and IgE (Watson, 1975a; Micusan and Borduas 1977; McGuire *et al.*, 1979; Husband 1987; Watson *et al.*, 1994b). The antibody responses reported in this study were based on the responses of total antibodies (Chapters 4, 5, and 6) and IgG (Chapters 7 and 8). The responses of IgM, IgA and IgE to the lactic acid-producing bacteria have not been explored, and these antibodies may also contribute to the effectiveness of immunisation against rumen microbes, particularly IgA response. The pattern and roles of these antibodies remain to be investigated in order to understand fully the mechanisms by which the immunisation may confer the protection against lactic acidosis. It is difficult to quantify the amount of saliva produced by each animal and if it had any influence on antibody concentration is not clear.

11.4.2 Improving media for producing antigen cells

The media used in this work for producing antigen cells of *S. bovis* and *Lactobacillus* are based on cell free rumen fluid (Chapters 3 and 4). It may not be possible to use rumen fluid for preparing commercial vaccines for the following reasons: (1) the components in rumen fluid are very complex and not easy to define; (2) the components of rumen fluid vary depending on individual animals and their diets; (3) composition of media have been shown to influence the nature of bacterial antigens (Watson, 1989a). Therefore, a suitable medium may need to be developed for preparing vaccine bacteria cells.

11.4.3 Optimising the vaccine bacteria strain(s) and dose rate

During the development of a vaccine, it is important to consider the potential for the vaccine to protect the animals against a wide range of the pathogenic strains of bacteria (Watson and Franklin, 1988). A number of strains of *S. bovis* and *Lactobacillus spp.* have been found in the rumen of sheep and cattle (Chapter 2). The antigenic variation between isolates of either *S. bovis* or *Lactobacillus* has also been demonstrated (Medrek and Barnes, 1962; Shu and Liu, 1995b; Chapter 10). Therefore, to develop a vaccine which will be effective against a large number of strains of *S. bovis* or *S. bovis* and *Lactobacillus*, it is important to select suitable antigen strain(s) on the basis of the degree of immunological cross-reactivity (see Chapter 10).

The isolates used for the vaccine preparation in this thesis were not selected on the basis of antigenic homology, and had considerable antigenic variation with the other lactic acid bacteria (see Chapters 4 and 10). Accordingly, this may have been one of the reasons that the immunised animals (Chapters 5, 6, and 8) suffered from a degree of lactic acidosis (as discussed in Section 11.2). It is likely that the vaccines used in these studies were not effective against all strains of *S. bovis* and *Lactobacillus* in terms of antigenic variation. The large numbers of the lactic acid-producing bacteria present in the rumen after immunisation and the same levels ($p>0.05$, compared with those in the control group) of *S. bovis* (following grain feeding) and *Lactobacillus* (prior to grain feeding) numbers (see Chapters 8 and 9; Hartman and Jacobson, 1971) may have been strains which had a low degree of immunological cross-reactivity with the vaccine antigen(s) and were not affected by the immune responses. These strains were still able to produce a high level of lactic acid in the rumen. It is interesting to ask if there would have been such large numbers of the lactic acid bacteria in the rumen if the vaccine antigen(s) had a greater degree of cross-reactivity with lactic acid-producing bacteria in the rumen?

Only a limited number of isolates were tested (see Chapter 10) and it is therefore essential to investigate the immunological cross-reactivity between the vaccine strain Sb-5 and a wider range of other strains of *S. bovis*, in order to assess if Sb-5 can be used as a vaccine strain against a great number of *S. bovis* which may potentially cause lactic acidosis. We also need to answer the question as to whether

Lactobacillus need to be included in an lactic acidosis vaccine and how important the immunisation against *Lactobacillus* is? As indicated in Chapter 2, the role of *Lactobacillus* in the development of lactic acidosis is not clear. The current study demonstrates that the risk of lactic acidosis can be reduced by immunisation against either *S. bovis* or both *S. bovis* and *Lactobacillus*. However, the current study provides no means of understanding the role of immunisation against *Lactobacillus* to prevent lactic acidosis. Therefore, further studies are needed to investigate the importance of immunisation against *Lactobacillus* in the prevention of lactic acidosis. If the vaccine containing only *S. bovis* offers a similar level of protection against lactic acidosis as the vaccine containing both *S. bovis* and *Lactobacillus*, the preparation of an lactic acidosis vaccine will be much simpler and cheaper. On the other hand, if *Lactobacillus* need to be included in the vaccine, reconstruction of the *Lactobacillus* candidates for a vaccine preparation will be needed as there is a large degree of antigenic variation between isolates (see Chapter 10). The vaccine antigen *Lactobacillus* LB-27 is a mixture of *Lactobacillus spp.* (see Chapter 3), and the composition and proportion of the possible strains (or species) involved in the LB-27 are not known. To have an effective commercial vaccine containing *Lactobacillus*, along with *S. bovis*, it is important to use identified strains (as vaccine antigens) having high degrees of immunological cross-reactivity with a great number of *Lactobacillus spp.*. Therefore, it is necessary to investigate the immunological cross-reactivity between single strains of different species of *Lactobacillus* by the same methodology as used in the study for *S. bovis*, in order to select the most suitable *Lactobacillus spp.* strains for the preparation of vaccine antigens (see Chapter 3).

The dose rate of antigenic bacteria cells for the vaccine is an important factor in the induction of the immune responses (see Chapter 2). The study reported here did not attempt to determine the optimum dose rate of vaccine bacteria cells. All the experiments in this work were based on a level of 10^{10} cells of organisms (Chapter 5, 5, 6, 7, and 8). Therefore, there is a need to compare the protective responses using different dose rates in order to obtain the optimum. Another question will rise if both *S. bovis* and *Lactobacillus* strains need to be included in the vaccine: what is the optimum ratio? Two ratios (Sb-5 : LB-27) have been used in this work: 3:1 (see Chapter 8) and 2:1 (see Chapter 9). The results suggest that the ratio (2:1) induced a

more balanced antibody responses to *S. bovis* and *Lactobacillus* in terms of the antibody levels enhanced (by comparing the highest IgG concentration after immunisation with the lowest IgG concentration prior to immunisation) (see Tables 7.3 and 8.3). However, it may not be the optimum ratio. Accordingly, in order to maximise the effectiveness of the vaccine and the possible commercial benefits, further work for optimising the vaccine dose rate is suggested before the commencement of any large scale field trials.

The present studies (Chapters 5, 6, 7, and 8) have shown that one booster following a primary immunisation was successful in inducing significant antibody responses and positive biological effects. However, the protection against acute lactic acidosis under this regime was not investigated following a single booster. Also the minimum level of antibody response, which is sufficient to give protection against lactic acidosis, was not determined. The results from this thesis provided no means to understand if the antibody levels induced in the animals were high enough to provide complete protection against lactic acidosis regardless of the other possible factors, because a complete protection against acute lactic acidosis was not observed in any of the grain challenge experiments (see Chapter 5, 5, and 6). Therefore, there is a need to test the effectiveness against acute lactic acidosis by using one booster following a primary immunisation, and compare a range of dose rates which are also important to optimise the immunisation strategy (see Chapter 2). As indicated in Chapter 2, an important consideration for developing a practical vaccine is the ability to induce a high level and durable immune response using only a low number of immunisations, ideally, one booster following a primary vaccination. Therefore, optimising the dose rate under one booster following a primary immunisation is highlighted. Even if the one booster regime will not induce the maximum antibody response, it may still be possible to be sufficient to reduce the risk of lactic acidosis if a suitable vaccine dose rate is used (see Chapters 2 and 7).

While many questions remain unanswered in the development of a commercial vaccine, the results show very encouraging evidence that vaccination is a potentially useful way to reduce the risk of gut lactic acidosis in sheep and cattle. As the problems associated with lactic acidosis become more widely understood, and as concerns rise in connection with the use of feed additives, the use of vaccines is sure to become increasingly attractive.

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