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**Efficacy of DNA Adenine Methylase  
Salmonella Vaccines in Livestock**

**By**

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**A thesis submitted in fulfilment of  
the requirements for the degree of  
Doctor of Philosophy (Veterinary)**



**Livestock Teaching and Research Unit**

**Faculty of Veterinary Science**

**University of Sydney**

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## **AUTHOR'S DECLARATION**

This work represents the original work of the author apart from where acknowledgements of assistance and references are made in the text. The work presented herein has not been submitted for any other degree or diploma at this University or any other institution.

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Date:

## EXECUTIVE SUMMARY

Intensive livestock production and management systems are associated with increased faecal-oral pathogen transmission which can contribute to a high prevalence of multiple *Salmonella* serovars in large dairy farms and feedlots. Outbreaks of salmonellosis in livestock often reflect a series of events that compromise host immunity and increase pathogen exposure. High risk groups in cattle include neonates and post partum cows (Anderson, *et al.*, 2001; House, *et al.*, 2001a; Fossler, *et al.*, 2005a) and variation in susceptibility to salmonella infection has been observed in sheep entering feedlots according to property of origin, body condition, and time of year (Norris, *et al.*, 1989a; Norris, *et al.*, 1989b; Richards and Hyder, 1991; Kelly, 1995; Makin, 2011). The associated increase in the incidence of disease and contamination of livestock-derived food products imposes a significant risk to food safety via consumption of contaminated meat, milk, eggs and vegetables. The development and application of effective *Salmonella* vaccines offers a potential means of reducing industry associated losses and public health risks.

Effective *Salmonella* vaccination therefore requires induction of protection against several *Salmonella* serovars and stimulation of both innate and acquired immune mechanisms. Vaccine prophylaxis is normally achieved through vaccinating animals several weeks prior to virulent pathogen exposure. This is not possible in neonates where exposure occurs at birth and in feedlots where livestock are sourced from diverse locations and vendors. Additionally, direct physical handling of livestock to administer vaccines contributes to stress and may lead to carcass damage. Conducting stressful procedures at feedlot induction when there is concurrent exposure to a diversity of pathogens contributes to an increased risk of disease.

Traditional vaccination methods are labour intensive and associated with carcass damage and adverse reactions. Oral delivery of vaccines and medications via drinking water is a common practice in intensively managed poultry. Oral vaccine delivery via drinking water avoids the stress of additional handling and provides a means of rapidly vaccinating large numbers of animals.

The efficacy of *Salmonella* vaccination is largely influenced by the diversity of *Salmonella* serovars encountered and the interval between immunisation and pathogen exposure, which may be short in field settings, e.g., following birth, during transport and following introduction into feedlots. The timing of virulent pathogen exposure may also have an impact on the safety of a *Salmonella* vaccine. It is imperative to develop livestock vaccines that are capable of safely eliciting potent states of cross-protective immunity against a diversity of serovars.

This thesis examines the capacity of the *dam* *S. Typhimurium* vaccine (serogroup B) to elicit cross-protection against a virulent challenge in models of neonate and adult ruminant models of salmonellosis, as well as investigating in-water vaccine delivery. Cross-protective efficacy of the vaccine was evaluated against an emerging, clinically relevant, and multi-drug resistant strain of serovar Newport (serogroup C<sub>2</sub>-C<sub>3</sub>) that had been associated with clinical disease in calves and humans (CDC, 2002; Clark, 2004). Vaccinated calves challenged with *S. Newport* exhibited a significant attenuation of clinical disease and a concomitant reduction in *S. Newport* faecal shedding and colonisation of mesenteric lymph nodes and lungs compared to non-vaccinated control animals. The safety and efficacy of a *S. Typhimurium dam* vaccine in adult sheep was demonstrated via novel oral delivery in drinking water (*ad libitum*). The capacity of *S. Typhimurium dam* vaccine to be delivered in drinking water to protect livestock from virulent *Salmonella* challenge offers an effective, economical, stressor free *Salmonella* prophylaxis for intensive livestock production systems.

Oral delivery of live attenuated *Salmonella* vaccines minimises vaccine production costs, provides an opportunity for mass medication via drinking water, avoids carcass damage and reduces animal stress. The capacity to elicit cross-protective immunity in calves and sheep suggests that *dam* mutant vaccines have potential application toward the prevention and control of *Salmonella* infection in commercial livestock production systems wherein livestock are exposed to a diversity of *Salmonella* serovars

## **RESEARCH WORK AND/OR AUTHORSHIP**

This thesis includes two original papers published in a peer reviewed journal (Chapters 5 and 8) and one original paper that has been submitted to a peer reviewed journal for consideration (Chapter 7). The literature review of this thesis (Chapter 2) includes excerpts from an original book chapter and an original paper published in a non-peer reviewed journal.

The core themes of the thesis are the development of ruminant models of salmonellosis, the establishment of safety and cross protective efficacy of the modified live *dam* attenuated *Salmonella* Typhimurium UK-1 vaccine in ruminants, and the development of vaccine delivery systems for livestock. The ideas, development and writing of all the chapters and papers in this thesis were the principal responsibility of the candidate, working independently within the Livestock Veterinary Teaching and Research Unit, Faculty of Veterinary Science under the supervision of Associate Professor John. K. House (principal supervisor) and Dr Keith H. Walker (associate supervisor) from Meat and Livestock Australia. Additional supervision was provided by Professor Michael J. Mahan (associate supervisor) and Dr Douglas Heithoff (associate supervisor) from the Department of Molecular, Cellular and Developmental Biology, University of California Santa Barbara; Dr Michael A. Hornitzky and Dr Les Gabor at the Elizabeth Macarthur Agricultural Institute, NSW Department of Industry and Investment; and Associate Professor Peter C. Thomson from the Faculty of Veterinary Science provided assistance with ordinal data analysis and supervision of continuous data analysis.

The inclusion of co-authors in the authorship of Chapters 2, 5, 7 and 8 reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

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## PUBLICATIONS

### Refereed Publications

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Mohler, V.L., Heithoff, D.M., Mahan, M.J., Walker, K.H., Hornitzky, M.A., Shum, L.W.C., Makin, K.J., House, J.K. (2008). Cross-protective immunity conferred by a DNA adenine methylase deficient *Salmonella enterica* serovar Typhimurium vaccine in calves challenged with *Salmonella* serovar Newport. *Vaccine*, **26**(14): 1751-1758 (accepted January 2008).

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## ABBREVIATIONS

°C	Degrees Celsius
-	Minus or Negative
±	Plus/minus
+	Positive
< or ≤	Less than or less than or equal to
> or ≥	Greater than or greater than or equal to
ANOVA	Analysis of Variance
AOAC	Association of Official Agricultural Chemists Standards
AQIS	Australian Quarantine and Inspection Service
ASR	Antimicrobial susceptibility and resistance
AVMA	American Veterinary Medical Association
BWT	Body Weight
CDC	Centres for Disease Control and Prevention
CFU	Colony Forming Unit(s)
CI	Competitive Index
d	Day(s)
<i>dam</i>	DNA Adenine Methylase
<i>dam</i> <sup>-</sup>	<i>S. Typhimurium dam</i> mutant
<i>dam</i> <sup>+</sup>	<i>S. Typhimurium dam</i> overproducer
dL	Decilitre(s)
DTH	Delayed Type Hypersensitivity
EMAI	Elizabeth Macarthur Agricultural Institute
GALT	Gut Associated Lymphoid Tissue(s)
h	Hour(s)
H <sub>2</sub> S	Hydrogen Sulfide
IC	Intracardiac
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin 1
IL-12	Interleukin 12

IL-2	Interleukin 2
IM	Intramuscular
IMVS	Institute of Medical and Veterinary Science, <i>Salmonella</i> Reference Laboratory, South Australia,
INF- $\gamma$	Interferon type II - gamma
IV	Intravenous
IW	In-water
Kg	Kilogram(s)
L	Litre(s)
LA	Luria Agar
LB	Luria Broth
LD <sub>50</sub>	Lethal Dose <sub>50</sub>
LD <sub>90-100</sub>	Lethal Dose <sub>90-100</sub>
LFB	Lactose Fermenting Bacteria
LPS	Lipopolysaccharide
MLN	Mesenteric Lymph Node(s)
NATA	Australian National Association of Testing Authorities
NIH	National Institutes of Health
mg	Milligram(s)
MHC	Major Histocompatibility Complex
MIC	Minimal Inhibitory Concentration
min	Minute
mL	Millilitre(s)
mm	Millimetre(s)
MSB	Mannitol Selenite Broth
NLIS	National Livestock Identification Scheme
NSAID	Non-Steroidal Anti-Inflammatory Drug(s)
NSW	New South Wales
OB	Oral Bolus - Per os
OGTR	Office of the Gene Technology Regulator
ONPG	<i>o</i> -nitrophenyl- $\beta$ -D-galactopyranoside
PBS	Phosphate Buffered Saline
PC2	Physical Containment Level 2

PO	Per os
QC2	AQIS Quarantine Control Level 2
REML	Residual (or Restricted) Maximum Likelihood Analysis
rpm	Rotations per minute
RT	Rectal Temperature(s)
SBM 06-225	<i>Salmonella</i> Bovismorbificans 06-225 challenge
SN 03-721	<i>Salmonella</i> Newport 03-721 challenge
ST 06-131	<i>Salmonella</i> Typhimurium 06-131 challenge
SPI	<i>Salmonella</i> Pathogenicity Island(s)
<i>spv</i>	<i>Salmonella</i> Plasmid Virulence
SE	Standard Error(s)
T-cell	T lymphocytes
Th	T helper cells
Th1	T <sub>1</sub> helper cells
Th2	T <sub>2</sub> helper cells
TNF	Tumour Necrosis Factor
TRT	Tetrathionate Broth
TSI	Triple Sugar Iron Agar
USDA	United States Department of Agriculture
Vaccine	<i>Salmonella</i> Typhimurium UK-1 <i>dam</i> <sup>-</sup> MT2313
WHO	World Health Organization
XLD	Xylose lysine deoxycholate plating media
XLD-CHL	Xylose lysine deoxycholate with chloramphenicol
XLD-NEO	Xylose lysine deoxycholate with neomycin sulfate
XLD-XNL	Xylose lysine deoxycholate with ceftiofur sodium
$\alpha$	Alpha
$\beta$	Beta
$\gamma$	Gamma
$\Delta$	Delta
$\mu\text{g}$	Microgram(s)
$\mu\text{L}$	Microlitre(s)

### Terminology used in the thesis in reference to *Salmonella*

The nomenclature for *Salmonella* is still evolving and under debate within the scientific and medical community (Euzeby, 1999; Brenner, *et al.*, 2000). The nomenclature system based on the Kauffman-White scheme that involves allocation of *Salmonella* to serovars or serotypes based on the serological identification of O (somatic/LPS), H (flagellar), and K (capsular) antigens (Kauffman, 1973) were applied to this document in reference to *Salmonella enterica* subspecies *enterica* as outlined in Table 1. This system is generally accepted by the Centres for Disease Control and Prevention (CDC), the World Health Organization (WHO) Collaborating Centre and *Bergey's Manual of Systematic Bacteriology* (Garrity, *et al.*, 2004; Dougan, *et al.*, 2011). The common names for *Salmonella* serovars were applied to this document.

Table 0.1. Examples of *Salmonella* Nomenclature

Complete Name	Common Name	O-Antigen Serogroup/Serotype
<i>Salmonella enterica</i> subspecies <i>enterica</i> serovar Bovismorbificans	<i>Salmonella</i> Bovismorbificans <i>S. Bovismorbificans</i>	C <sub>2</sub> -C <sub>3</sub>
<i>Salmonella enterica</i> subspecies <i>enterica</i> serovar Dublin	<i>Salmonella</i> Dublin <i>S. Dublin</i>	D <sub>1</sub>
<i>Salmonella enterica</i> subspecies <i>enterica</i> serovar Newport	<i>Salmonella</i> Newport <i>S. Newport</i>	C <sub>2</sub> -C <sub>3</sub>
<i>Salmonella enterica</i> subspecies <i>enterica</i> serovar Typhimurium	<i>Salmonella</i> Typhimurium <i>S. Typhimurium</i>	B
<i>Salmonella enterica</i> subspecies <i>enterica</i> serovar Typhimurium UK-1 <i>dam</i> <sup>-</sup>	<i>Salmonella</i> Typhimurium <i>dam</i> <sup>-</sup> <i>S. Typhimurium dam</i> <sup>-</sup>	B

## CHAPTER 1. INTRODUCTION

This thesis describes the development of experimental salmonella challenge models in neonatal calves and adult sheep. These models were then utilised to evaluate the protective efficacy of a *S. Typhimurium dam* attenuated vaccine and the efficiency of in-water delivery systems in adult ruminants.

Chapter 2 of this thesis contains a review of the current literature relating to salmonellosis in ruminants including pathophysiology, risk factors, treatment, control, and salmonella vaccination. The review highlights the current status of *Salmonella* vaccines and approaches to assessing protective immunity.

In Chapter 3, materials and methods used to conduct these experiments are described. The vaccine evaluated in this research is an attenuated modified live *Salmonella* that was developed by our collaborators in California. The conduct of this work required AQIS, OGTR, and animal ethics approvals prior to initiation of research. Methods applied across multiple experiments are presented and are referred to in subsequent chapters of the thesis.

In Chapter 4, the development of a heterologous neonate model of salmonellosis and methodology for collection of observational and quantifiable data to be used in future vaccine trials is described. The objective was to evaluate the effect of a clinically relevant, multidrug resistant strain of *S. enterica* serovar Newport on neonatal calves and to determine the challenge dose that induces clinical disease with minimal mortalities in neonatal calves. The optimal dose of *S. enterica* serovar Newport in neonatal calves was determined to be  $10^{10}$  CFU which was subsequently used to evaluate the cross-protective efficacy of the *dam S. Typhimurium* vaccine (Chapter 5).

Chapter 5 presents the results of a vaccination-challenge trial where the cross-protective efficacy of the *S. Typhimurium dam* vaccine was demonstrated in neonatal calves using the heterologous neonatal model of salmonellosis described in Chapter

4. In this experiment, the capacity of the *S. Typhimurium dam* vaccine (serogroup B) to elicit cross-protection against a virulent, multi-drug resistant strain of *S. Newport* (serogroup C<sub>2</sub>-C<sub>3</sub>) that has been associated with clinical disease in recent salmonellosis outbreaks in cattle and calves was demonstrated. These results were published in the journal *Vaccine* in March 2008.

Chapter 6 describes the development of adult ruminant models of salmonellosis in sheep and the methodology for collection of observational and quantifiable data to be used in future vaccine trials is described. The objective was to determine a challenge dose of industry relevant strains of *S. enterica* serovar Typhimurium and *S. enterica* serovar Bovismorbificans that would induce clinical disease with minimal mortalities in adult Merino sheep. The optimal dose of *S. enterica* serovar Bovismorbificans heterologous challenge was determined to be 10<sup>9</sup> CFU in adult Merino sheep and was used to evaluate the cross-protective efficacy of a *S. Typhimurium dam* vaccine in future trials. The optimal dose of *S. enterica* serovar Typhimurium homologous challenge was determined to be 10<sup>9</sup> CFU in adult Merino sheep and used to evaluate the protective efficacy of the vaccine in Chapter 8.

Chapter 7 describes a series of experiments conducted to determine the viability and stability of the *S. Typhimurium dam* vaccine in water and development of an in-water vaccine delivery model. Oral delivery of vaccines via drinking water has the potential to rapidly immunise large numbers of animals with minimal stress, to avoid carcass damage due to injection site reactions and to reduce production costs. Hence, oral delivery via drinking water allows for cost effective salmonella prophylaxis in susceptible populations. The objective was to determine if the *S. Typhimurium dam* vaccine would be viable in drinking water and to determine if adult ruminants could be effectively vaccinated with *S. Typhimurium dam* vaccine via oral delivery in drinking water, providing a potential means to effectively vaccinate large numbers of livestock. These studies were submitted for publication in the journal *Vaccine* in July of 2011.

Chapter 8 presents the results of an in-water vaccination-challenge trial. Adult sheep administered *S. Typhimurium dam* vaccine in water were challenged with virulent *S. Typhimurium* utilising the homologous salmonellosis challenge model established in Chapter 6. This study included assessments of vaccine safety and efficacy. Adult merino sheep were vaccinated in drinking water -28 d, -7 d and 24 h pre and 24 h post-virulent *S. Typhimurium* challenge. Significant attenuation of clinical disease (temperature, appetite, and attitude) as well as reductions in mortality, virulent *S. Typhimurium* faecal shedding and tissue colonisation were observed in sheep vaccinated at 28 and 7 d pre-challenge. Furthermore, vaccination did not pose an increased risk of mortality in stock concurrently infected with virulent salmonellae. These results were published in the journal *Vaccine* in April 2011.

The thesis concludes with a general discussion, conclusions and further research goals in Chapter 9.



## CHAPTER 2. LITERATURE REVIEW

Sections of this literature review were in part published in two book chapters on the topic of salmonellosis in ruminants. These chapters were executed and reported by the candidate under the general supervision with the candidate's appointed supervisor, Associate Professor John K. House.

Mohler, V.L. and House, J.K. (2009), 'Salmonellosis in Ruminants', in D.E. Anderson and D.M. Rings (eds.), *Current Veterinary Therapy: Food Animal Practice* (5<sup>th</sup> edn.; St. Louis: Saunders), 106-111.

Mohler, V.L., Izzo, M.M., House, J.K. (2009). *Salmonella* in Calves. *Veterinary Clinics of North America-Food Animal Practice* **25**(1): 37-54

### 2.1 Introduction

There are over 2,500 different serotypes of *Salmonella*; all are potentially capable of causing disease in livestock and humans (Le Minor and Bockemuhl, 1989; Gelberg, 2001; Popoff, *et al.*, 2004; Popoff and Le Minor, 2005; WHO, 2005). The majority of *Salmonella enterica* subspecies *enterica* belong to one of ten distinct O-somatic antigen serogroups: A, B, C<sub>1</sub>, C<sub>2</sub>, D<sub>1</sub>, D<sub>2</sub>, E<sub>1</sub>, E<sub>2</sub>, E<sub>3</sub> and E<sub>4</sub> (Iwen, 1998; Old and Threlfall, 1998; Brenner, *et al.*, 2000; Wray and Davies, 2000). The most common serogroups isolated from cattle being B, C, D and E.

*Salmonella* are endemic on many large intensive dairy farms and feedlot production systems. Livestock manure management systems reduce, but do not eliminate, *Salmonella* which are subsequently recycled, promoting faecal-oral transmission. Most *Salmonella* infections in livestock are sub-clinical (Norris, *et al.*, 1989a; Higgs, *et al.*, 1993; Kelly, 1995; Anderson, *et al.*, 2001). Salmonellosis in livestock usually reflects a variety of management events and environmental stressors that contribute to compromised host immunity and increased pathogen exposure. While a diversity of *Salmonella* serovars may be endemic in a given production facility, the virulence of

these serovars is variable, reflected by a disparity among *Salmonella* isolates from surveillance and clinical submissions (Glynn, *et al.*, 1998; Anderson, *et al.*, 2001; Zhao, *et al.*, 2007). In the literature, *S. enterica* serovars Typhimurium and Dublin are most frequently associated with disease in cattle worldwide with the virulence of other serovars being more variable. Conversely, the most commonly isolated serovars from cattle in Australia are *S. enterica* serovars Bovismorbificans, Typhimurium and Anatum (IMVS, 2010). Typhimurium and Bovismorbificans are the serovars most frequently associated with disease in sheep (Kelly, 1995; Wray and Davies, 2000; Wray and Davies, 2004; IMVS, 2010).

The diversity of *Salmonella* serovars present on farms and the potential for different serovars to possess virulence attributes requires the implementation of broad prophylactic strategies that are efficacious for all salmonellae. At the farm level, producers may implement programs to promote host immunity through provision of good nutrition and a comfortable environment. When this is accomplished, livestock health and productivity is promoted. In practice, consistent day to day implementation of these apparently simple principles is paradoxically complex, with numerous variables such as changes in weather conditions, mechanical breakdowns, variable feedstuff availability and quality, and labour compliance issues all having the potential to adversely impact management programs and, subsequently, host immunity.

There has been significant progress toward developing an effective *Salmonella* vaccine using genetically modified *Salmonella* organisms, with the development of vaccines that provide good protection against homologous strains (Rankin and Taylor, 1967; Cameron and Fuls, 1976; Wray, *et al.*, 1977; Meyer, 1980; Jelinek, *et al.*, 1982a; Mukkur, *et al.*, 1987; Wray and McLaren, 1987; Mukkur, *et al.*, 1991a; Mukkur, *et al.*, 1991b; Smith, *et al.*, 1993; Hassan and Curtiss, 1994a; Segall, *et al.*, 1994; Hormaeche, *et al.*, 1996; Dueger, *et al.*, 2001, 2003a). A continued limitation has been the limited capacity to induce protection against challenge with unrelated or

heterologous strains (Rankin, *et al.*, 1966; Rankin, *et al.*, 1967; Mukkur, *et al.*, 1987; Mukkur, *et al.*, 1991b). The need to develop a *Salmonella* vaccine that protects against infection with the diverse array of *Salmonella* strains that challenge livestock continues.

## **2.2 *Salmonella enterica subspecies enterica (Salmonella)***

### ***2.2.1. Physical characteristics***

*Salmonella* are a member of the Enterobacteriaceae family. They are gram-negative, mobile, non-lactose fermenting, facultative anaerobic rods classified as a facultative intracellular parasite in humans and many animals (Old and Threlfall, 1998; Quinn, *et al.*, 1999). Salmonellae are capable of growth in minimal media at pH range from 4.5 to 9.5 (Chung and Goepfert, 1970). However, the optimal pH for the growth of *Salmonella* has been reported to range between 6.5 and 7.5 (Chung and Goepfert, 1970; Park, *et al.*, 2004). Interestingly, *S. Typhimurium* has developed mechanisms for surviving exposures to pH levels as low as 3.0 (Goepfert and Hicks, 1969; Chung and Goepfert, 1970; Huhtanen, 1975; Foster and Spector, 1995) which is of considerable concern to food safety.

### ***2.2.2. Distribution and ecological characteristics***

*Salmonella* are ubiquitous in the environment and can survive for extended periods of time, which contributes to the transmission and re-infection of animals. Additionally, salmonellae are considered parasites of the gastrointestinal tract of mammals, birds, reptiles, fish and insects (Venkateswaran, *et al.*, 1989; Levine and Levine, 1994; Alcamo, 2001; Beuchat, 2002; Coleman, *et al.*, 2003; Leriche, *et al.*, 2003; Miller, *et al.*, 2006; Heithoff, *et al.*, 2008b; Lynch, *et al.*, 2009). *Salmonella* typically infect the host via the oro-faecal route in the form of contaminated feedstuffs or polluted water (Levine and Levine, 1994; Smith, *et al.*, 1994; Mead, *et al.*, 2000; Alcamo, 2001; Davison, *et al.*, 2006; You, *et al.*, 2006). Several surveys of dairy farms in the US report *Salmonella* contamination of the environment and up to 75% prevalence in

animals in the absence of disease or current outbreak (Smith, *et al.*, 1994; Huston, *et al.*, 2002; Woodward, *et al.*, 2002). Laboratory experiments have demonstrated that *S. Dublin* survives in faeces for almost six years (Plym-Forsell and Ekesbo, 1996); while McLaren and Wary (McLaren and Wray, 1991) reported that *Salmonella* persisted in disinfected calf units for up to two years; and Kelly (1995) observed infection of previously naive sheep within 14 days (d) of exposure to salmonella contaminated sheep yards. Salmonellae are capable of proliferating in moist faecal material (Arrus, *et al.*, 2006; Holley, *et al.*, 2006; You, *et al.*, 2006). Soils and bedding with a mean dry matter content of 80% have been reported to support *Salmonella* growth for up to 25 weeks (Chandler and Craven, 1978, 1980; Arrus, *et al.*, 2006; You, *et al.*, 2006; Danyluk, *et al.*, 2008). In laboratory experiments, *Salmonella* were demonstrated to rapidly proliferate in sheep yard material at 20% moisture content (Chandler and Craven, 1980; Ingram, 2008). This demonstrates that a wetting event, e.g. rain, overflowing water trough, and urine can promote the growth of salmonellae in the environment.

The two *Salmonella* serovars most commonly reported to cause salmonellosis in cattle are *S. Dublin* and *S. Typhimurium* (Smith, *et al.*, 1994; Wray and Davies, 2000; Davis, *et al.*, 2007; Zhao, *et al.*, 2007; Adhikari, *et al.*, 2009). *Salmonella* serovars commonly isolated from sheep internationally include *S. Typhimurium*, *S. Arizonae*, *S. Derby*, and *S. Montevideo* (Wray and Davies, 2000). In Australia *S. Bovismorbificans* has been the most commonly reported cause of salmonellosis in both cattle and sheep for the last 10 years (IMVS, 2010). Serovars *S. Typhimurium*, *S. Bovismorbificans*, *S. Havana*, and *S. Anatum* have been the predominant serotypes associated with disease in Australian sheep feedlots for decades (Kelly, 1995).

### **2.2.3. Public health concerns**

The potential for *Salmonella* in livestock production to contaminate livestock-derived food products and other food products via effluent imposes a risk to food safety. The

CDC reported that an estimated 1.4 million human *Salmonella* infections occurred in the United States, with only 40,000 reported at a cost of 5 billion dollars (Mead, *et al.*, 1999; Mead, *et al.*, 2000; Thorns, 2000; Voetsch, *et al.*, 2004; CDC, 2005, 2006, 2010). In 2004 the WHO reported that over 3 million deaths were attributed to *Salmonella* infections worldwide (WHO, 2005). Most cases of human salmonellosis in developed countries are derived from contaminated foods such as eggs, milk, beef, and poultry or products that have been exposed to animal faeces, including fruits and vegetables (OzFoodNet, 2003; CDC, 2005; NIH, 2005; WHO, 2005). Recent statistics show that 40.3 cases of salmonellosis per 100,000 population were reported in Australia (OzFoodNet, 2003), while 13 per 100,000 population were reported in the United States between 2003 and 2004 (CDC, 2005). These statistics emphasise the growing need for food safety reform and implementation of *Salmonella* control measures in Australia.

During investigations of salmonellosis, it is prudent to inform animal handling personnel of the human health risks *Salmonella* pose. Multidrug resistant strains of *Salmonella* are frequently implicated in disease outbreaks in calves, sheep and occasionally people. These risks have been highlighted in reports of human salmonellosis caused by the multidrug resistant *S. Newport* (CDC, 2002; Devasia, *et al.*, 2005; Varma, *et al.*, 2006); *S. Typhimurium* DT104 (Glynn, *et al.*, 1998); and *S. Brandenburg* (Clark, *et al.*, 2003; Clark, *et al.*, 2004; Baker, *et al.*, 2007) derived from livestock. Additionally, surveys report that hospitalisation rates are higher in human outbreaks of salmonellosis associated with antimicrobial resistant *Salmonella* than those outbreaks associated with pansusceptible *Salmonella* strains (Varma, *et al.*, 2005).

Current research indicates that *Salmonella* isolates associated with human salmonellosis patients are distinct from those of animal origin (Heithoff, *et al.*, 2008b). This indicates that selective pressure within a host may give rise to bacteria

strain variants that show signs of enhanced fitness in the current host relative to the parental host from which the strain was derived or originated.

### **2.3 Salmonella in Cattle and Sheep**

Salmonellosis is a common disease in intensively managed domestic animals, with consequences ranging from subclinical carrier status to acute fatal septicaemia. Disease manifestations associated with salmonellosis include: pneumonia, septic arthritis, meningitis, gangrene, ill-thrift and sudden death (Jones, 1992; Higgs, *et al.*, 1993; Mohler and House, 2009; Mohler, *et al.*, 2009; Makin, 2011). Outbreaks of disease in calves typically occur during the first 4 weeks of life (Anderson, *et al.*, 2001). In sheep, salmonellosis is more common in intensive production systems, live export (Norris and Richards, 1989; Norris, *et al.*, 1989a; Kelly, 1995; Makin, 2011) and lamb fattening. Conversely, salmonellosis is relatively uncommon in pastoral and wool sheep operations.

#### ***2.3.1. Clinical presentation***

Severity and duration of clinical disease is related to virulence of the strain, challenge dose, age of animal, immune status, nutrition and management practices. Infections in calves are aggravated by poor husbandry, inadequate nutrition, immature immune response, inadequate passive transfer and undeveloped microflora in the gastrointestinal tract. Variables that impact the risk for salmonellosis in sheep include season, dietary change, prior exposure to *Salmonella*, stocking density, new introductions, transport and lambing.

Common findings in clinical cases include fever, depressed mentation, loss of appetite and scours containing blood and mucus (Deignan, *et al.*, 2000; Wray and Davies, 2000; Gelberg, 2001; Quinn, *et al.*, 2002; Smith, 2002; Mohler and House, 2009). *Salmonella* Dublin also has a propensity to cause respiratory disease in calves while *S. Typhimurium* is commonly associated with enteric forms of the disease in younger calves (Wray and McLaren, 1987; Mee, 1995; Tsolis, *et al.*, 1999; Wray and

Davies, 2000; Quinn, *et al.*, 2002; Smith, 2002; Mohler and House, 2009). The pathophysiology of diarrhoea produced by salmonellae infection involves local release of enterotoxins and endotoxin leading to inflammation, submucosal infiltration of neutrophils into the intestinal mucosa, necrosis of enterocytes and villous atrophy (Wray and Davies, 2000). Sloughing of the intestinal epithelial cells leads to acute haemorrhage, fibrin production, and increased fluid in the gastrointestinal tract due to poor absorption and hypersecretion (Wray and Davies, 2000; Hodgson, *et al.*, 2003). Mediators of inflammation characterised by Th1-like cytokines such as interleukins (IL-1, IL-6, IL-12 and IL-18), interferon type II gamma (INF- $\gamma$ ), and tumour necrosis factor alpha (TNF- $\alpha$ ) (Ramarathinam, *et al.*, 1991; Ramarathinam, *et al.*, 1993; Elhofy and Bost, 1999b; Elhofy, *et al.*, 2000a; Mastroeni, *et al.*, 2000; Tizard, 2000; Tam, *et al.*, 2008; Dougan, *et al.*, 2011); macrophage inhibitory factors and inducible nitric oxide synthases (iNOS) (Mastroeni, *et al.*, 1998; Schwacha, *et al.*, 1998; Mastroeni, *et al.*, 1999); and lactic acid can affect ion flux within the intestine creating a hyperosmotic state that draws fluid into the intestinal tract. This in turn leads to a net loss of water, sodium, potassium, and bicarbonate and contributes to the diarrhoea. Afflicted animals lose body condition, become weak, dehydrated and emaciated.

### ***2.3.2. Risk factors***

The main risk factors associated with *Salmonella* infections in calves include poor health at birth, poor colostrum immunity, receiving pooled colostrum, poor nutrition, presence of carrier or infected animals, level of environmental contamination, and inadequate hygiene. With adult ruminants the main risk factors include mixing of animals from multiple sources, stocking density, transport, diet, and environmental contamination.

#### ***i. Health at birth***

Dystocia and asphyxia during birth can compromise the normal physiological

transition phases that occur after birth and increase chances of mortality (Vaala and House, 2002a). Calves with low viability scores commonly suffer hypothermia, malnutrition and fail to consume adequate amounts of colostrum. Additionally, calves that are compromised at birth due to dystocia, premature birth and caesarean section, have lower uptakes of colostral immunoglobulins from the intestinal tract which can lead to failure of passive transfer (Sangild, 2003)

### *ii. Colostral immunity*

Disease resistance is greatly influenced by passive transfer of maternal antibodies and cells via colostrum (Sangild, 2003; Cortese, 2009). Calves are born agammaglobulinaemic and immunologically naïve as no intrauterine transfer of immunoglobulin occurs in ruminants (Vaala and House, 2002b). Colostrum provides a concentrated source of immunoglobulins that are optimally absorbed from the intestinal tract of a neonatal calf during the first 24 hours (h) of life. Failure of passive transfer of immunoglobulins is related to time of colostrum ingestion, amount of colostrum consumed, the immunoglobulin and microbial quality of the colostrum and health of the calf at birth (Stewart , *et al.*, 2005; Godden, *et al.*, 2006; Chigerwe, *et al.*, 2008; Chigerwe, *et al.*, 2009; Mohler, *et al.*, 2009). Calves devoid of passive immunoglobulins are highly susceptible to bacterial septicaemia (Barrington and Parish, 2002; Fecteau, *et al.*, 2009).

### *iii. Nutrition and feeding*

Adequate nutrition is critical for host immunity and calorie deprived animals are more likely to succumb to disease. Poor nutrition and hygiene have been identified as risk factors for salmonellosis in calves (de Jong and Ekdahl, 1965; Warnick, *et al.*, 2001; Smith, 2002; McGuirk and Peek, 2003; Mohler and House, 2009; Smith, 2009). Inadequate caloric intake can lead to increased cortisol release, poor immune function and increased susceptibility to disease (Pollock, *et al.*, 1993; Drackley, 2008; Nonnecke, *et al.*, 2009; Khan, *et al.*, 2011). When considering the energy and



nutritional needs of calves, it is important to factor in seasonal conditions and exposure to the elements (Tomkins and Jaster, 1991; Danyluk, *et al.*, 2008).

The microbial quality of milk fed to calves is also important. On farms where calves are fed milk collected from recently calved and sick or “hospital” cows, it is not uncommon to isolate *Salmonella* from over 25% of the samples (Mohler, *et al.*, 2009). *Salmonella* contamination and proliferation in calf milk can also be associated with poor cleaning and sanitation of milk feeding equipment and a lack of refrigeration, which carries significant risk when residual milk is held over for the next feeding. This is particularly pertinent during hot weather when proliferation of *Salmonella* is favoured. Pasteurisation is effective in eliminating *Salmonella* contamination and, when properly executed, provides a robust risk management strategy for controlling transmission via colostrum and milk (Stewart, *et al.*, 2005; Godden, *et al.*, 2006).

Feeding utensils, equipment and personnel often play a significant role in transmitting *Salmonella* between both calves and adult ruminants (Pacer, *et al.*, 1989; Hardman, *et al.*, 1991; Smith, *et al.*, 1994; Heinrichs and Radostits, 2001; Fossler, *et al.*, 2005a; Fossler, *et al.*, 2005b; Oliver, *et al.*, 2007). *Salmonella* infects the salivary glands and is shed in saliva and nasal secretions, thereby contaminating nipples, bottles, buckets, and oesophageal feeders (Richards and Fawcett, 1973; Nolan, *et al.*, 1995). Adequate cleaning and disinfection of feeding utensils is necessary to remove *Salmonella* contamination.

Poor rumen fill due to fasting or anorexia and rumen imbalance have been identified as risk factors for salmonellosis in adult ruminants (Norris, *et al.*, 1989a; Norris, *et al.*, 1989b; Richards, *et al.*, 1989; Richards and Hyder, 1991; Higgs, *et al.*, 1993; Kelly, 1995; Lenahan, *et al.*, 2010). It is common for sheep and cattle to be held off feed for prolonged periods of time during assembly and transport and then undergo a rapid change in diet - from one typically of pasture, to a grain-pellet ration following

entry to feedlots. *Salmonella* are rapidly cleared from the rumen of regularly fed ruminants, but maintain or increase their numbers when feed intake is decreased or interrupted for one or more days (Brownlie and Grau, 1967; Chambers and Lysons, 1979; Mattila, *et al.*, 1988; Lenahan, *et al.*, 2010). Feeding after a period of starvation is associated with proliferation of *Salmonella* in the rumen (Grau, *et al.*, 1968; Frost, *et al.*, 1988) which is likely to be associated with provision of substrates for growth. Growth of *Salmonella* in rumen fluid is influenced by the type of feed consumed (Brownlie and Grau, 1967; Grau, *et al.*, 1969; Lenahan, *et al.*, 2010). Anorexia in ruminants is associated with low concentrations of volatile fatty acids and a high rumen pH that favours the growth of salmonellae (Chung and Goepfert, 1970; Park, *et al.*, 2004). Upon entering a new environment, it is not uncommon for sheep to not consume the diet offered due to lack of prior exposure or experience with the feed source. Norris *et al.* (1990) reported 0.2% to 23% of sheep were non-feeders (classified as neophobic animals) following entry into feedlots.

#### ***iv. Environmental contamination***

With the global trend toward intensive management of livestock, contamination of the environment is difficult to avoid (Anderson, *et al.*, 2001; Huston, *et al.*, 2002). The resilience of *Salmonella* and its capacity to survive for extended periods of time in the environment further contribute to the difficulties associated with attempts to eliminate *Salmonella* from the environment (Robertsson, *et al.*, 1983; Wray, *et al.*, 1987). Management practices in the calving barns and calf rearing areas can directly affect the incidence of disease (Robertsson, *et al.*, 1983; Wray, *et al.*, 1987; Heinrichs and Radostits, 2001; McGuirk, 2008). One study identified employment of part-time staff and lack of biosecurity protocols on farm as risk factors for isolation of salmonella (Davison, *et al.*, 2006). Scrupulous attention to hygiene in the calving areas, reduction in faecal contamination and isolation of sick animals can reduce the environmental exposure of calves and assist in the control of *Salmonella* infection.

Facilities used to handle large numbers of livestock are prone to becoming contaminated with *Salmonella* and are subsequently a risk for pathogen transmission (Norris, *et al.*, 1989a; Higgs, *et al.*, 1993; Kelly, 1995; Davison, *et al.*, 2006; Vanselow, *et al.*, 2007a). Higgs *et al.* (1993), Kelly (1995), Makin (2011), and Vanselow *et al.*, (2007a) all reported a low prevalence of *Salmonella* faecal shedding by sheep and cattle entering abattoirs and feedlots. This low prevalence of salmonella in the general ruminant population in Australia is supported by a slaughterhouse study by Vanselow *et al.* (2007a). During the feedlot period, the prevalence of *Salmonella* excretion has been reported to rise from 0-2% to 26 - 97% within two weeks of entry (Higgs, *et al.*, 1993; Richards, *et al.*, 1993; Kelly, 1995; Makin, 2011). Faecal shedding contributes to environmental contamination, with infected animals shedding  $10^6$  to  $10^{13}$  salmonellae per day in faeces (de Jong and Ekdahl, 1965; Nazer and Osborne, 1977; Mukkur, *et al.*, 1987; Mukkur and Walker, 1992; Fecteau, *et al.*, 2003; Dueger, *et al.*, 2003b; Mohler, *et al.*, 2006; Mohler, *et al.*, 2008).

Environmental sampling conducted in sheep feedlots conducted by Makin *et al* (2011) demonstrated that loading and unloading yards and holding paddocks are frequently contaminated with salmonella. Infection of sheep with *S. Typhimurium*, *S. Bovismorbificans* and *S. Brandenburg* via holding in contaminated yards from 3 days to 6 weeks has been documented in New Zealand (Robinson, 1967; Clark, *et al.*, 2004; Li, *et al.*, 2005). Scenarios such as feeding hay on ground, mixing groups of sheep, weather changes, and overcrowding of pens can contribute to the contamination of yarding facilities with salmonellae and increase exposure to naive animals.

*Salmonella* persistence and multiplication in the environment may contribute to outbreaks of salmonellosis in livestock that cannot be attributed to a recent source of infection. *Salmonella* has been reported to survive in manure-amended soils and manure slurries from 14 days to 7 years (Smith, *et al.*, 1994; Baloda, *et al.*, 2001; Huston, *et al.*, 2002; Woodward, *et al.*, 2002). Holley *et al.* (2006) demonstrated that

an increasing inoculation dose of *Salmonella* increased environmental survival time in manure-amended soils. Laboratory experiments demonstrated that *S. Dublin* could survive in faeces for almost six years (Plym-Forshell and Ekesbo, 1996), while McLaren and Wray (1991) reported that *Salmonella* persisted in disinfected calf units for up to two years.

The survival of *Salmonella* in the environment is dependent upon environmental factors such as temperature and moisture. High soil moisture and low soil temperature increase *Salmonella* survival time, whilst freeze-thaw cycles significantly reduced *Salmonella* viability (Holley, *et al.*, 2006). You *et al.* (2006) demonstrated that *S. Newport* was capable of a 4-fold increase in numbers following inoculation into a manure substrate over a 3 day period. Under freezing conditions, *Salmonella* were shown to survive up to 7 years in ice cream (Wallace, 1938).

#### **v. *Stress factors***

Transportation, high ambient temperatures, time of year, water deprivation, overcrowding, parturition, concurrent infections and sudden dietary changes are stress factors that have been known to contribute to *Salmonella* infections in ruminants (Counter and Gibson, 1980; Wray and Roeder, 1987; Giles, *et al.*, 1989; Norris, *et al.*, 1989a; Richards, *et al.*, 1989; Richards and Hyder, 1991; Higgs, *et al.*, 1993; Kelly, 1995; Evans and Davies, 1996; Losinger, *et al.*, 1997; Higgs and Norris, 1999; Fossler, *et al.*, 2005a; Fossler, *et al.*, 2005b; Makin, 2011). When immunity is compromised by concurrent disease, physiological or dietary stress, the infectious dose of salmonellae can be reduced to as few as several hundred organisms (Grau, *et al.*, 1969).

#### **vi. *New introductions and mixing of animals***

Poor quarantine procedures can contribute to the transmission of salmonellae (House and Smith, 1998; Anderson, *et al.*, 2001; Warnick, *et al.*, 2001; Huston, *et al.*, 2002; Fossler, *et al.*, 2005a; Fossler, *et al.*, 2005b; Davison, *et al.*, 2006; Vanselow, *et al.*,

2007a). Most *Salmonella* infections in livestock are sub-clinical (Anderson, *et al.*, 2001). *Salmonella* shedding by adult cattle can provide a source of infection for calves (Smith, *et al.*, 1989; Nielsen, *et al.*, 2004a). A single infected asymptomatic shedder may shed one million *Salmonella* per gram of faeces which can then contaminate the environment and other animals (Smith, *et al.*, 1989; Huston, *et al.*, 2002). Carrier animals usually appear normal and will go on to shed *Salmonella* in faeces and milk, maintaining a source of infection in the herd (Smith, *et al.*, 1989; House, *et al.*, 1993).

Source of sheep has been identified as a risk factor for mortality associated with salmonellosis in the live sheep export industry (Norris and Richards, 1989; Norris, *et al.*, 1989b; Richards, *et al.*, 1989; Richards and Hyder, 1991; Higgs and Norris, 1999; Makin, 2011). The cause for this increased susceptibility for mortality has not been determined but is hypothesised to reflect differences in host immunity. Pathogen exposure is inherent when livestock from various properties are introduced into feedlots and live export assembly depots.

### **2.3.3. Pathogenesis of salmonellosis**

Faecal-oral transmission is the primary route of infection; other reported routes include the mucosa of the upper respiratory tract and conjunctiva. Following ingestion, *Salmonella* colonise the intestinal tract and invade the bowel through specialised cells in intestinal lymphoid tissue called M-cells, enterocytes (Holt, 2000; Wray and Davies, 2000; Hamada, *et al.*, 2002; Reis, *et al.*, 2003; Pabst, *et al.*, 2005) and tonsillar lymphoid tissue (de Jong and Ekdahl, 1965; Gelberg, 2001). In the lymphoid tissue, *Salmonella* gain entry into mononuclear phagocytes and are rapidly disseminated throughout the body (Vazquez-Torres, *et al.*, 1999; Worley, *et al.*, 2006). The capacity of *Salmonella* to infect calves via the tonsils was demonstrated by experimental challenge studies in oesophagectomised calves (de Jong and Ekdahl, 1965). In these calves *Salmonella* were isolated from tissues within 3 h of oral

challenge, indicating lymphoid involvement in systemic distribution. Other reported routes of infection include the mucosa of the upper respiratory tract through aerosol transmission and intranasal inoculation (Nazer and Osborne, 1977; Wathes, *et al.*, 1988; Hardman, *et al.*, 1991). The conjunctival route of infection has been utilised to experimentally infect sheep with *S. Brandenburg* (Li, *et al.*, 2005). This route is suspected to be an important mode of transmission relevant to sheep passing through contaminated yards.

The basic virulence mechanisms of *Salmonella* species include the ability to invade the intestinal mucosa, to multiply in the lymphoid tissues and to evade host defence systems leading to systemic disease. The diarrhoea associated with salmonellosis is largely believed to be mediated by the inflammatory response to infection. *Salmonella* Typhimurium requires a functional type III secretion system encoded by *Salmonella* pathogenicity islands 1 and 5 (SPI1 and SPI5) to cause diarrhoea (Wray and Roeder, 1987; Tsohis, *et al.*, 1999; Zhang, *et al.*, 2002; Zhang, *et al.*, 2003). The main function of the invasion-associated type III secretion system is to translocate effector proteins into the cytosol of a host cell. The resulting cell death is associated with a pro-inflammatory response and influx of neutrophils into the intestinal mucosa (Wray and Davies, 2000). A positive correlation is observed between the severity of histopathological lesions detected in the ileal mucosa and the level of fluid secretion (Zhang, *et al.*, 2002). Release of endotoxin, prostaglandins, and pro-inflammatory cytokines, e.g., IL-1 and TNF- $\alpha$  (Tizard, 2000) also promote vascular permeability and hypersecretion. Sloughing of the intestinal epithelial cells leads to acute haemorrhage, fibrin production, maldigestion and malabsorption (Wray and Davies, 2000). The resulting hyperosmotic state within the lumen of the intestine draws fluid into the intestinal tract, contributing to a net loss of water, sodium, potassium, and bicarbonate. Mucosal damage also contributes to protein loss and a resulting hypoproteinemia.

The bovine host adapted *S. Dublin* and some strains of *S. Typhimurium* carry a plasmid that contains the *Salmonella* plasmid virulence (*spv*) gene. The *spv* gene enhances virulence by promoting the intracellular survival of *Salmonella* in reticuloendothelial cells of liver and spleen, lymph nodes and macrophages (Nazer and Osborne, 1977; Counter and Gibson, 1980; Mee, 1995; Baumler, *et al.*, 1998; Mastroeni, *et al.*, 2000). Other non-adapted serovars may carry the virulence plasmid, however it is less common and the virulence attributes of these serovars are more variable.

#### **2.3.4. Pathology of salmonellosis**

Although pathologists associate salmonellosis with enteric lesions such as diphtheritic membranes, peracute infections often have few pathological findings (Tsolis, *et al.*, 1999; Wray and Davies, 2000; Gelberg, 2001). Lesions observed with peracute salmonellosis include pulmonary congestion and submucosal and subserosal petechial haemorrhages of multiple organs including the intestines and heart.

Acute salmonellosis is typically characterised by diffuse catarrhal haemorrhagic enteritis with diffuse fibrinonecrotic ileotyphlocolitis (Tsolis, *et al.*, 1999; McGavin and Carlton, 2001). The intestinal contents are watery, malodorous and may contain mucous or whole blood. Inflammation of the gall bladder is common and histopathological evidence of fibrinous cholecystitis is considered pathognomonic for acute enteric salmonellosis in calves. Enlargement, oedema and haemorrhage are commonly observed in the mesenteric lymph nodes (Tsolis, *et al.*, 1999; McGavin and Carlton, 2001). Abomasal mucosal erosions may be observed, particularly with *S. Dublin* infections. Microscopic thickening of the intestinal wall with macroscopic yellow-grey necrotic material overlying a red mucosal surface may be observed with chronic lesions (McGavin and Carlton, 2001). However, in the case of carrier animals, especially cattle infected with *S. Dublin*, gross pathologies are rare (Wray and Davies, 2000; Nielsen, *et al.*, 2004a).

### 2.3.5. *Development of a carrier state*

Animals chronically infected with salmonella are sometimes referred to as “Salmonella Carriers” (Nielsen, *et al.*, 2004a). In the literature, prevalence of carrier animals within herds ranged from two to eight percent (House, *et al.*, 1993; Losinger, *et al.*, 1997; Huston, *et al.*, 2002; Nielsen, *et al.*, 2004a; Nielsen, *et al.*, 2004b) with variations attributed to herd size, *Salmonella* serotype, population sampled and method of detection. *Salmonella* Dublin, which is host adapted to cattle, is more likely to produce a carrier state (Losinger, *et al.*, 1997; Wray and Davies, 2000; Nielsen, *et al.*, 2004a), however, multiple *Salmonella* serotypes have been reported to produce chronic infections in cattle (Losinger, *et al.*, 1997; Wray and Davies, 2000; Wray and Linklater, 2000; Smith, 2002; Nielsen, *et al.*, 2004a). Carrier animals are capable of shedding high numbers of *Salmonella* either continuously or intermittently into faeces (Smith, *et al.*, 1989; House, *et al.*, 1993; Wray and Davies, 2000; Nielsen, *et al.*, 2004b). It has been reported that stress may trigger the shedding of bacteria. Stressors include parturition and overcrowding (Counter and Gibson, 1980; Smith, *et al.*, 1989; House, *et al.*, 1993; Wray and Davies, 2000; Nielsen, *et al.*, 2004b; Wray and Davies, 2004), transportation (Wray and Davies, 2000), introduction of new animals (Losinger, *et al.*, 1997), and concurrent diseases such as bovine viral diarrhoea virus and parasitism (Wray and Roeder, 1987; Penny, *et al.*, 1996). Carrier animals provide a reservoir for continuous infection of the herd and perpetuation of the carrier state within the herd.

Chronic infections have experimentally been induced in lactating dairy cows through low dose intramammary inoculation of *S. Dublin* by Spier *et al* (1991). House *et al* (1993) observed chronic *S. Dublin* infections in a group of heifers that were infected as neonates during a disease outbreak on a farm. Chronic infections in the calves were associated with dissemination of *S. Dublin* to mammary glands, lymph nodes and other tissues. Several studies have demonstrated that acutely infected animals that survive the initial *Salmonella* challenge are better able to clear the infection



completely and are less likely to become carriers (Wray and Davies, 2000; Wells, *et al.*, 2001).

## **2.4 Therapy**

Dehydration, electrolyte imbalances, endotoxaemia and bacteraemia are common clinical features of *Salmonella* infections in ruminants. The treatment of salmonellosis in calves is directed at replacing fluid and electrolyte losses, limiting inflammatory cascades through use of non-steroidal anti-inflammatory drugs, and the judicious use of antimicrobials (Smith, 2002; Fecteau, *et al.*, 2003; Constable, 2004; McGuirk, 2008; Constable, 2009; Mohler and House, 2009). Bacteraemia is a common feature of salmonellosis in calves; prompt aggressive treatment with antimicrobials early in the infection is recommended (Wray and Davies, 2000; Constable, 2004; McGuirk, 2008; Constable, 2009; Fecteau, *et al.*, 2009). In sheep, the diversity of *Salmonella* serovars present during an outbreak and the potential for variable virulence between serovars requires the implementation of broad prophylactic strategies that are efficacious for all *Salmonellae*. There are no reports of controlled clinical trials evaluating treatment options for sheep.

There is some controversy surrounding the use of antimicrobials to treat salmonellosis in livestock. Concern relates to selection for antimicrobial resistance and questions regarding its necessity and efficacy have been derived from experience in human medicine where invasive *Salmonella* infections are uncommon and routine use of antimicrobial therapy is not recommended. While there are a wide range of antimicrobials that include a gram negative spectrum that may appear appropriate for treatment of salmonellosis, most are not labelled for use in ruminants or not registered at a dose rate that provides therapeutic drug concentrations against all *Salmonella* strains. In Australia, amoxicillin, amoxycyclavulonic acid, trimethoprim-sulphadoxine and neomycin sulphate are registered for use in the treatment of salmonellosis in ruminants (APVMA, 2010). Conversely in the US, there are no antimicrobials

specifically labelled for the treatment of salmonellosis in ruminants.

Antimicrobial resistance is common in virulent *Salmonella* serotypes (CDC, 1996, 2002; Hoelzer, *et al.*, 2010). Increased virulence has been observed in some antimicrobial resistant strains of *Salmonella* (Glynn, *et al.*, 1998; Davis, *et al.*, 2007; Zhao, *et al.*, 2007; Adhikari, *et al.*, 2009). This is reflected by the finding that calves infected by *S. Typhimurium* DT104 are 13 times more likely to die than calves infected with antibiotic sensitive strains of *S. Typhimurium* (Evans and Davies, 1996). The spread of resistance genes by transformation, transduction and conjugations is well documented in *Salmonella* species and the Enterobacteriaceae family (Cardenas and Clements, 1993; Wray and Davies, 2000; Bueno, *et al.*, 2009). Multi-drug resistant strains of *Salmonella* are frequently implicated in disease outbreaks in calves and occasionally people.

Antimicrobial selection ideally should be based on the results of susceptibility testing utilising a *Salmonella* isolate recovered from the tissues of infected animals. Broad spectrum antimicrobials are usually employed pending the availability of susceptibility test results (Fecteau, *et al.*, 2003; McGuirk and Peek, 2003; Bell, *et al.*, 2004; Constable, 2004; McGuirk, 2008; Constable, 2009). It has been reported in the literature that *Salmonella* show variable resistance to ampicillin, amoxicillin, amoxyclovulonic acid, ceftiofur, florphenicol, neomycin, sulphonamides, tetracycline, and trimethoprim-sulphonamides and resistance to penicillin, erythromycin, and tylosin. In a recent Australian study, no resistance to amikacin, amoxicillin-clavulanic acid and nalidixic acid was observed in *Salmonella* isolated from diarrhoeic calves and only 14% of isolates exhibited multiple resistance patterns (Izzo, *et al.*, 2011) when compared to 26% from a US study (Berge, *et al.*, 2006). Additionally, overseas studies have demonstrated that *Salmonella* isolates from healthy animals are susceptible to most antimicrobials whereas the isolates obtained from clinically affected animals have higher levels of antimicrobial resistance (Blau, *et al.*, 2005; Lundin, *et al.*, 2008).

Because *Salmonella* are facultative intracellular pathogens, selecting an antimicrobial with good tissue penetration and the ability to attain intracellular therapeutic drug concentrations within macrophages is desirable. A number of experimental studies have evaluated the efficacy of antimicrobial agents in the treatment of salmonellosis in cattle. In a comparative experimental trial, amoxicillin and trimethoprim sulphoxidine were shown to have equivalent efficacy in the treatment of calves with *Salmonella* infections via oral (PO), intravenous (IV) and intramuscular (IM) routes when administered at doses based on minimal inhibitory concentration (MIC) and peak blood levels (Groothuis and van Miert, 1987). Similarly in an experimental challenge study, extra-label use of ceftiofur at 5 mg/Kg IM was shown to attenuate the severity of clinical disease and reduce faecal shedding of *Salmonella* (Fecteau, *et al.*, 2003). It is important to note that beta lactams, ceftiofur and amoxicillin, have poor intracellular activity (Tulkens, 1991; Rang, *et al.*, 2001a). Prudent use of antimicrobial drugs is recommended with an emphasis on establishing a herd diagnosis, susceptibility testing of invasive *Salmonella* serotypes, and using the narrowest spectrum of antibiotic indicated by the susceptibility data (Helmuth, 2000; Constable, 2004, 2009).

Non-steroidal anti-inflammatory drugs (NSAIDs) have been utilised to attenuate endotoxin-induced inflammatory cascades. NSAIDs inhibit endotoxin-induced inflammation by blocking the arachidonate cyclo-oxygenase pathway reducing the formation of thromboxanes and prostaglandin (Rang, *et al.*, 2001b; MacKay, 2002; Constable, 2009). Analgesic and antipyretic effects may persist after serum concentrations have dropped below detection due to extensive tissue binding (George, 2003). The therapeutic efficacy of NSAIDs in the treatment of salmonellosis in calves has not been documented in the literature. Extralabel use of the NSAID flunixin meglumine at 2.2 mg/kg IV once a day to 0.25 -0.33 mg/Kg three times a day for up to 3 days or meloxicam at 0.5 mg/kg IV/SC for a maximum of three days, has been anecdotally reported in the literature to improve outcomes and reduce

morbidities in calves and cows with non-specific diarrhoea (Rantala, *et al.*, 2002; Barnett, *et al.*, 2003; Todd, *et al.*, 2007; Constable, 2009; Fecteau, *et al.*, 2009). Precautions should be taken when administering NSAIDs to dehydrated calves as hypotension and reduced renal perfusion increase the risk of toxicity (George, 2003). Therefore, implementation of appropriate fluid therapy may reduce adverse consequences often associated with the administration of NSAIDs to dehydrated patients. In Australia, meloxicam, flunixin meglumine, ketoprofen and tolfenamic acid have been approved for use in cattle with meat withholding periods of 8, 7, 4 and 7 days, respectively (MIMS, 2007; Smith, *et al.*, 2008).

## **2.5 Salmonella Vaccines and Vaccination**

Vaccination provides a relatively simple means of enhancing pathogen-specific immunity. In regard to salmonellae, historically the efficacy of vaccination has been limited. There are three general classes of *Salmonella* vaccines: killed whole cell (bacterin), bacterial fractions (subunit), and attenuated modified live (MLV).

While the use of *Salmonella* vaccines is common, most of the products on the market are bacterins of limited efficacy. The potential benefit of attenuated live *Salmonella* vaccines has been recognised for many years and a small number are available commercially (Curtiss, *et al.*, 1993).

### **2.5.1. Bacterin vaccines**

Bacterin *Salmonella* vaccines are the most widely used vaccines in cattle. Bacterins are killed *Salmonella* whole cells, components and by-products. This ensures that there is no reverting to virulence and no risk of environmental contamination (Griffen, *et al.*, 2002). Bacterins are mixed with adjuvants which enhance the immune response by increasing the stability of the antigens and targeting the antigen to antigen processing cells (Mastroeni, *et al.*, 2000; Griffen, *et al.*, 2002). There are conflicting reports regarding the efficacy of *Salmonella* bacterins. Experimental studies in cattle have produced equivocal results (Curtiss, *et al.*, 1993; House, *et al.*,

2001a; Anderson, 2005). The perceived limitations of killed *Salmonella* vaccines are the failure of these products to present antigens expressed *in vivo*, induce cellular immunity, and induce mucosal immunity (Robertsson, *et al.*, 1983; Curtiss, *et al.*, 1993; Mastroeni, *et al.*, 2000).

Under field conditions neonatal exposure often occurs during the first few days of life, limiting the opportunity to stimulate acquired immunity in calves (House, *et al.*, 2001a). In the US, it is reported that up to 75% of calves are exposed to pathogens within their first 48 h of life which is an insufficient amount of time to induce protective acquired immunity using bacterins (Griffen, *et al.*, 2002; Dueger, *et al.*, 2003b). Additionally, research has demonstrated that neonatal calves have a dramatic decrease in systemic immune responses following birth, due to suppression of lymphocyte proliferation and diminished cytokine production (IFN- $\gamma$ ) (Rajaraman, *et al.*, 1997; Cortese, 2009) which can further reduce response to vaccination as well as lead to increased susceptibility. Passive immunity acquired from maternal *Salmonella* bacterin vaccination and colostrum transfer is limited, however partial protection has been reported in some experimental challenge trials (Jones, *et al.*, 1988; Mortola, *et al.*, 1992; Cortese, 2009).

Adverse reactions in the form of anaphylactic reactions are occasionally reported in ruminants vaccinated with *Salmonella* bacterins (Mamak and Aytakin, 2009). The cause of these reactions is unknown but has been suggested to be associated with lipopolysaccharide (LPS) content of these products (Mastroeni, *et al.*, 1994; Mamak and Aytakin, 2009). While it has been demonstrated that transient tolerance to LPS can be induced with administration of sub-lethal doses LPS (Gupta and Reed, 1971; Fahmi and Chaby, 1993; Randow, *et al.*, 1995), hypersensitivity reactions to LPS are more common (Hormaeche, *et al.*, 1981; Mukkur, *et al.*, 1987; Matsuura and Galanos, 1990; Mukkur, *et al.*, 1991a; Mukkur, *et al.*, 1991b; Mukkur and Walker, 1992; Brennan, *et al.*, 1994; Mastroeni, *et al.*, 1994; Kweon, *et al.*, 1998). Generally, *Salmonella* vaccines are used in herds where it is likely that some animals may have

been recently infected with *Salmonella*. Following sub-lethal *Salmonella* infections, the sensitivity of animals to the lethal activity of LPS increases exponentially (Matsuura and Galanos, 1990). Other disadvantages of bacterin vaccines include injection site reactions and the requirement for multiple injections to induce adequate protection (Hjerpe, 1990; Griffen, *et al.*, 2002).

Despite their limitations, bacterins are still the most widely available and accepted vaccines. In Australia, a mixed bacterin vaccine, BOVILIS S Inactivated *Salmonella* Vaccine (Coopers Animal Health, AU) is registered for use in adult cattle (MIMS, 2007; APVMA, 2010). There are currently no registered *Salmonella* bacterin vaccines for use in calves or sheep.

### **2.5.2. Subunit vaccines**

Subunit vaccines are composed of bacterial fractions, surface antigens or flagella. These vaccines consist of a single protein that is purified using either conventional methods or recombinant DNA technology. These vaccines are designed to isolate *Salmonella* antigens that will elicit an immune response without inducing adverse reactions or immunosuppression (Griffen, *et al.*, 2002). Attempts to immunise mice, sheep and cattle with subunit vaccines have resulted in the induction of moderate immunity in innately resistant animals or against moderately virulent organisms (Mastroeni, *et al.*, 2000; Dougan, *et al.*, 2011).

*Salmonella* Newport Bacterial Extract vaccine (AgriLabs® Inc., USA), a relatively new subunit cattle vaccine composed of highly purified extracts of specialised bacterial proteins known as siderophore receptors and porins (SRP), is currently available in the US (Emery, *et al.*, 2002; Anderson, 2005). This vaccine works on the absolute requirement for iron in order for the bacteria to survive. Identification of iron capturing mechanisms (transferrin-binding proteins) allowed development of vaccine candidates (Babiuk, 2002). These proteins are common to all strains of *Salmonella* and offer the potential to induce immunity to homologous and heterologous

*Salmonella* serovars. Field trials with *Salmonella* Newport SRP have been conducted on healthy beef and dairy cattle. In beef cattle, SRP had no effect on faecal prevalence of *Salmonella* or health or performance (Dodd, *et al.*, 2011). However, dairy cattle administered *Salmonella* Newport SRP proteins prior to parturition demonstrated increased milk production, even in cattle without detectable shedding of *Salmonella* spp. or clinical signs of salmonellosis (Hermesch, *et al.*, 2008). No controlled clinical trials documenting the efficacy of this product at preventing salmonellosis in cattle have been reported in the literature.

### **2.5.3. Attenuated or modified live vaccines**

The observation that calves exposed to low doses of virulent *Salmonella* were protected against subsequent high dose virulent challenge suggested that prevention of salmonellosis via vaccination with live vaccines was possible (Curtiss, *et al.*, 1993; Barrow and Wallis, 2000; Mastroeni, *et al.*, 2000). Modified live vaccines induce a broad immune response via stimulation of cellular, humoral and mucosal immunity similar to natural infection (Mastroeni, *et al.*, 2000; Villarreal-Ramos, *et al.*, 2000a). There are a number of naturally occurring and genetically manipulated attenuated *Salmonella* strains that have been used to immunise cattle against salmonellosis. The naturally occurring rough mutants were the first modified live *Salmonella* vaccines to be used in cattle (Mastroeni, *et al.*, 2000). Although live rough mutants induce protective immunity, their stability is questionable and genetically altered attenuated strains have become more popular.

The most widely tested genetically altered *Salmonella* mutant vaccines in cattle are auxotrophic and contain mutations that interfere with regulating genes needed for virulence. Villarreal-Ramos *et al.* (1998) showed that SC administration of an avirulent *Salmonella* mutant induced protective immunity when compared to the more traditional oral route. It is generally accepted that the ability to deliver the

attenuated vaccine via the natural route of infection, orally in the case of *Salmonella*, and limited replication in the host, induces a longer duration of immunity.

Table 2.1. Genes Targeted in the Development of Attenuated *Salmonella* Vaccines

Genes Target For Mutation	Gene Function
<i>galE</i>	Conversion of UDP-galactose to UDP- Glucose
<i>aro</i>	Biosynthesis of PABA, DHB and aromatic amino acids
<i>pur</i>	Biosynthesis of adenine
<i>htrA</i>	Protection against heat stress and oxidative stress
<i>cya/crp</i>	Biosynthesis of cAMP and expression of AMP receptor
<i>PhoP/PhoQ</i>	Regulation of a number of virulence genes
<i>ompR/ompC/ompF</i>	Regulation and biosynthesis of outer membrane proteins
<i>dam</i>	Expression of DNA adenine methylase

A list of the genes that have been targeted in the construction of avirulent and/or immunogenic *Salmonella* vaccines is presented in **Error! Reference source not found.** (adapted from Curtiss *et al* (1993) and Mastroeni *et al* (2000)). Vaccine constructs have included aromatic amino acid dependent (*aro*) (Smith, *et al.*, 1984; Begg, *et al.*, 1990; Mukkur, *et al.*, 1991a; Mukkur and Walker, 1992; Smith, *et al.*, 1993; Mukkur, *et al.*, 1995; Mukkur, *et al.*, 1998; Villarreal-Ramos, *et al.*, 1998; George, 2003), *htrA* (Villarreal-Ramos, *et al.*, 2000a), *purE* (Villarreal-Ramos, *et al.*, 2000a) and *galE* mutants (Wray, *et al.*, 1977; Jelinek, *et al.*, 1982a; Nnalue and Stocker, 1986; Wray and McLaren, 1987). These vaccines are generally effective at inducing acquired protective immunity against homologous *Salmonella* challenge but have limited protective efficacy against heterologous *Salmonella* serogroups. Modified live *Salmonella* are capable of reducing faecal shedding of salmonellae and limiting the severity of disease in ruminants (Cameron and Fuls, 1976; Meyer, 1980; Robertsson, *et al.*, 1983; Wray and McLaren, 1987; Jones, *et al.*, 1991; Segall and Lindberg, 1993; Smith, *et al.*, 1993; Smith, *et al.*, 1994).



Attenuated *Salmonella* vaccines currently on the market provide homologous but not heterologous protection. An *aro* auxotroph *S. Dublin* vaccine (Entervene-D, Fort Dodge Animal Health, US) is available in the United States for the control of *S. Dublin* infections in calves and there is an *aroA Salmonella* Typhimurium vaccine (Vaxsafe® ST Vaccine, Bioproperties Pty Ltd, AU) available for use in poultry.

Experimental studies with *aro Salmonella* vaccines in calves have demonstrated protective immunity to homologous and heterologous *Salmonella* when calves were challenged within 3 weeks of vaccination (Rankin, *et al.*, 1966; Smith, *et al.*, 1984). The heterologous protection is afforded by transitory T-cell independent non-specific protection which disappears about 1 month after immunisation, following clearance of the organisms from the reticuloendothelial system. Thereafter, protection is limited to homologous challenge with recall of immunity presumably involving specific antigen recognition (Collins, 1974; Hormaeche, *et al.*, 1991).

It has been demonstrated that *S. Typhimurium* containing mutations in the *dam* gene which prevents DNA adenine methylase expression are avirulent, yet confer cross-protective immunity to multiple *Salmonella* strains when used as MLV in murine (Garcia-Del Portillo, *et al.*, 1999; Heithoff, *et al.*, 1999; Heithoff, *et al.*, 2001; Heithoff, *et al.*, 2008a) and avian models of typhoid fever (Dueger, *et al.*, 2001, 2003a). Additionally, *S. Typhimurium dam* vaccine has elicited both early (competitive exclusion) and late onset (adaptive immune) immunity in the protection of calves from virulent homologous *S. Typhimurium* challenge (Dueger, *et al.*, 2003b). The capacity of *S. Typhimurium dam* vaccine to confer cross-protective immunity in calves has been demonstrated using a virulent heterologous *S. Dublin* challenge (Mohler, *et al.*, 2006). These data are promising, as salmonellosis in livestock is associated with a diverse array of *Salmonella* serotypes (Kelly, 1995; Huston, *et al.*, 2002; Fossler, *et al.*, 2005b; Cummings, *et al.*, 2009; Cummings, *et al.*, 2010) which necessitates cross protective efficacy which is lacking in current vaccine candidates.

Another potential benefit of the *S. Typhimurium dam* vaccine is that, in contrast to *aroA Salmonella* vaccines, *dam* mutant vaccines do not induce a transient state of immunosuppression following antigen exposure (Heithoff, *et al.*, 2001). Vaccine-induced immunosuppression is particularly undesirable from the standpoint of livestock immunisation, as producers often administer multiple vaccines simultaneously. It is important that one vaccine does not limit the immune response to concurrently administered vaccines. Immunosuppression is also undesirable from the standpoint of increasing susceptibility to disease, which is a potential issue for animals vaccinated upon entry to a feedlot.

The stability of attenuations is of primary importance to avoid reversion to virulence and brings about issues of safety and efficacy (Nnalue and Stocker, 1986; Miller and Mekalanos, 1990; Curtiss, *et al.*, 1993; Mastroeni, *et al.*, 2000; Bueno, *et al.*, 2009). Additionally, some mutations demonstrated to be avirulent in one species may be virulent in another. For example, *htrA* and *purE* *S. Typhimurium* mutants, demonstrated to be avirulent in mice, were highly virulent in calves (Villarreal-Ramos, *et al.*, 2000a). Work to develop genotypically stable vaccines with two or three irreversible attenuating deletions to improve stability and increase immunogenicity is under development. However, some combinations could prove to be too effective at attenuating a vaccine and reduce the ability of the vaccine to persist in the tissues and to induce protective immunity (Wilson, 1967; Curtiss, *et al.*, 1993; Hone, *et al.*, 1999; Mastroeni, *et al.*, 2000; Babiuk, 2002; Anderson, 2005; Bueno, *et al.*, 2009).

#### **2.5.4. Vaccine immunology**

The field of immunology is one of active research in veterinary and human medicine. New and advanced methods for assessing immune function using cytokine expression, antibodies and lymphocytes are being developed. Much of the research on immune mechanisms of *Salmonella* infection has been conducted in the murine

typhoid model (Tam, *et al.*, 2008; Dougan, *et al.*, 2011). Murine models are very useful in the study of immune mechanisms; unfortunately, observations from these models have not always correlated with other species, including humans (Tam, *et al.*, 2008; Dougan, *et al.*, 2011).

Humoral and cellular responses of mice to infection with *Salmonella* have been characterised by the stage of infection. The initial stage occurs when *Salmonella* invade the host tissues and extracellular organisms are targeted by antibody and complement, while those *Salmonella* that move into the intracellular space are removed by phagocytic cells (VanCott, *et al.*, 1998; Mastroeni, *et al.*, 2000; Dougan, *et al.*, 2011). Route of infection, host species/strain and virulence of the infective strain all influence immune responses to a *Salmonella* challenge. The second phase of infection begins once the *Salmonella* have evaded the innate and humoral immune responses and become intracellular. Natural resistance-associated macrophage proteins (Nramp) and cytokines (IFN- $\gamma$ , TNF- $\alpha$ , IL-1, IL-6, IL-12 and IL-18) play a critical role in controlling infection and preventing proliferation. Failure of the host to attenuate proliferation is associated with death. In surviving animals the final stage of infection involves clearance of the infection from the tissues via CD4<sup>+</sup> T-cells, CD8<sup>+</sup> T-cells, cytokines, IFN- $\gamma$  and TNF- $\alpha$ , and to a lesser degree B cells and antibody production. Measurements of different components of the immune system to salmonella antigens have failed to predict the efficacy of *Salmonella* vaccines (Stabel, *et al.*, 1993; Kweon, *et al.*, 1998; Elhofy and Bost, 1999a; Sirard, *et al.*, 1999; Elhofy, *et al.*, 2000b; Holt, 2000; Mastroeni, *et al.*, 2000; Tam, *et al.*, 2008; Dougan, *et al.*, 2011).

In ruminant models of salmonellosis, antibody response has been used to measure the immune response. High levels of *Salmonella* specific-serum IgM, IgG and IgA as well as some intestinal-specific IgA antibodies are produced in response to vaccination or natural challenge with salmonellae (Wijburg, *et al.*, 2006). Variable humoral immune responses to LPS, LPS fragments, somatic O antigens (cell wall),

porins, surface antigens (Vi antigen) and flagellar H antigens have been observed in response to infections with different salmonella serovars (Mukkur, *et al.*, 1987; Smith, *et al.*, 1989; Begg, *et al.*, 1990; Mukkur and Walker, 1992; House, *et al.*, 1993; Berthon, *et al.*, 1994; Brennan, *et al.*, 1994; Mukkur, *et al.*, 1995). There are 67 known somatic O antigens. Each serogroup carries one specific O antigen and several non-specific O antigens. Salmonella serology in cattle has demonstrated that antibody response to LPS antigens are highly specific to somatic O antigens (Smith, *et al.*, 1989; House, *et al.*, 1993; Smith, *et al.*, 1995; House, *et al.*, 2001b; House, *et al.*, 2001c). However, antibody responses do not reliably predict protective immunity.

In all animal models, route of infection has an effect on the type of response elicited and intensity of the response. Oral administration of salmonellae vaccines are associated with lower serum and intestinal antibody responses when compared to IM and SC routes of vaccination (Lascelles, *et al.*, 1988; Begg, *et al.*, 1990; Mukkur, *et al.*, 1991a; Mukkur and Walker, 1992). In one study, oral administration of an *aroA* *Salmonella* mutant showed absence of vaccine specific immune response in sheep when compared to mice but ultimately provided significant protection from virulent challenge in both species (Brennan, *et al.*, 1994). Both Brennan *et al* (1994) and Lascelles *et al* (1988) observed that oral vaccination with an *aroA* mutant elicited protective immunity in sheep that was not attributable to local intestinal IgA production. Further research by Mukkur *et al* (1995) in sheep demonstrated only minimal contribution of both mucosal and systemic IgA antibodies following vaccination with an *aroA* mutant via IM or oral routes of administration. This finding indicates that secretory and circulatory IgA can contribute to the control of *Salmonella* infection but do not have major roles in providing protective immunity against salmonellae challenge (Wijburg, *et al.*, 2006; Dougan, *et al.*, 2011).

Delayed type hypersensitivity reactions (DTH) have been used to assess cellular immune responses to *Salmonella* vaccination. DTH is typically the expression of T-

cell mediated immunity (Mastroeni, *et al.*, 2000). DTH responses to proteins, porins, LPS and Vi surface antigens have been reported in mice (Hormaeche, *et al.*, 1981; Mukkur, *et al.*, 1987; Cao, *et al.*, 1992; Mukkur and Walker, 1992; Brennan, *et al.*, 1994), sheep (Mukkur, *et al.*, 1987; Begg, *et al.*, 1990; Mukkur and Walker, 1992; Brennan, *et al.*, 1994; Mukkur, *et al.*, 1995), calves (Robertsson, *et al.*, 1982; Segall and Lindberg, 1993) and pigs (Stabel, *et al.*, 1993). In one study, DHT persisted in sheep following IM vaccination with an *aroA* mutant for a period of 6 months, but offered limited protective immunity against virulent challenge at 6 and 12 m post vaccination (Mukkur and Walker, 1992). DTH responses have not been found to differentiate between immune and susceptible (unsensitised) individuals or predict vaccine efficacy.

Previous studies indicate there are numerous methods available to measure the immune response to *Salmonella* and numerous antigens that can be investigated. The results of these investigations have failed to identify the antigenic responses that equate with a protective immune response. As such, vaccination challenge experiments are required to assess vaccine efficacy. The body of work presented in this research was directed at developing an effective *Salmonella* vaccine for use in livestock and focused on studies that would provide a robust measure of efficacy of the *dam* *S.* Typhimurium vaccine in neonate and adult ruminant models of salmonellosis.

## **2.6 Study Objectives**

This thesis presents research that is an extension of a project to develop an effective salmonella vaccine to prevent disease in livestock and promote human food safety initiated by Associate Professor John K. House and Professor Michael J. Mahan in 2000. The research was funded by the United States Department of Agriculture (USDA). Previous work conducted by the research team has demonstrated that *S.*

Typhimurium *dam* vaccine protects mice, chickens and calves against heterologous and homologous salmonella challenges. The objectives of this research were:

- To develop models of neonatal salmonellosis using clinically relevant multidrug resistant *Salmonella* isolates that are heterologous to the *S. Typhimurium dam* vaccine.
- To further evaluate the cross protective efficacy of the *S. Typhimurium dam* vaccine in neonatal calves.
- To develop models of salmonellosis in adult sheep and to establish methods for evaluating clinical outcomes of ovine salmonellosis using clinically relevant *Salmonella* isolates.
- To investigate the feasibility of in-water vaccine delivery as an option for inexpensively vaccinating livestock populations.
- To evaluate the safety and efficacy of the *S. Typhimurium dam* vaccine when administered in drinking water to adult ruminants.

## CHAPTER 3. GENERAL METHODS

### 3.1 Introduction

This chapter describes the general methods that were employed during the conduct of the project. Microbiological techniques used in the culture, growth, identification and re-isolation of the vaccine and challenge bacteria were core to each experiment. Animal husbandry and monitoring were critical to the overall success of the project. The protocols for cleaning, feeding, monitoring, handling and housing of animals needed to meet the requirements of the *Animal Research Act 1985* (NHMRC, 2004) for the purpose of animal welfare and ethics; the Office of the Gene Technology Regulator (OGTR) to comply with the *Gene Technology Act 2000*, and section 46 A of the *Quarantine Act 1908*, AQIS Quarantined Approved Premises (QAP) (AQIS/DAFF, 2010) for the purpose of the importation and use of a quarantine approved biological. Protocols were established early in the project to facilitate training of support and animal care staff to ensure compliance with regulatory agencies and to ensure accurate data collection.

### 3.2 Bacterial strains

#### 3.2.1. Vaccine strain

Immunisations were carried out with *Salmonella* Typhimurium UK-1 *dam*- vaccine strain MT2313 (vaccine; *S. Typhimurium dam*) (Dueger, *et al.*, 2001; Heithoff, *et al.*, 2001; Dueger, *et al.*, 2003a, 2003b; Mohler, *et al.*, 2006) which contained an insertion element, *dam102::Mud-Cm*, encoding chloramphenicol [Cm<sup>r</sup>] resistance in strain UK-1 [obtained from Roy Curtiss III (Hassan and Curtiss, 1994a)]. The vaccine was imported into Australia following approval by AQIS (Import Permit no: 200203550) and maintained at EMAI under AQIS Quarantine.

#### 3.2.2. Challenge agents

***i. Neonatal model of heterologous salmonellosis***

The challenge agent selected for the neonate model of salmonellosis was a clinically relevant *Salmonella enterica* serovar Newport (*S.* Newport 03-721) a multi-drug antimicrobial resistant strain (ampicillin, ceftiofur, chloramphenicol, clindamycin, oxytetracycline, spectinomycin, sulphonamides, tiamulin, tilmicosin, trimethoprim/sulphamethoxazole, tylosin) isolated from a dairy cow with a history of chronic diarrhoea. The unique resistance to ceftiofur allowed this organism to be easily isolated and identified following administration to animals. The organism was obtained from Ms Jo Ann J. Yee from the Microbiology Laboratory at the Veterinary Medical Teaching Hospital, at University of California, Davis. The *S.* Newport 03-721 was imported into Australia following approval by AQIS (Import Permit: 200519069) and maintained at EMAI under AQIS Quarantine.

The *S.* Newport 03-721 was passaged through a calf via oral administration at  $1 \times 10^{11}$  CFU. The resulting challenge strain, *S.* Newport 03-721 -SP, was recovered from the spleen of the infected calf at 72 h post-ingestion. The isolate was identified by biochemical testing, antimicrobial susceptibility, and agglutination with serogroup C<sub>2</sub> specific antisera as described in sections 3.3.3 to 3.3.6

***ii. Ovine models of homologous salmonellosis***

The challenge agent selected for the ovine homologous model of salmonellosis was a *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium 06-131) multi-drug antimicrobial resistant strain (apramycin, erythromycin, neomycin, novobiocin, sulphonamides, streptomycin) isolated from the tissues of a dead sheep during a mortality investigation at a feedlot in Western Australian by Dr John K. House from the Livestock Teaching and Research Unit at the University of Sydney, Camden, NSW in 2006. The unique resistance to neomycin sulphate allowed this organism to be easily isolated and differentiated from the vaccine following administration to animals. The virulence of the isolate was determined by Professor Mike Mahan and



Dr Doug Heithoff at the University of California Santa Barbara using a competitive index assay as described in Heithoff *et al* (2008b). Competitive index (CI) scores are based on the results of oral and intraperitoneal lethal dose<sub>50</sub> (LD<sub>50</sub>) testing in mice (Conner, *et al.*, 1998) and identification of the *spv* genes (Jones, *et al.*, 1982; Gulig, *et al.*, 1992), as well as genes encoding the *spvB* actin cytotoxin, required for systemic survival (Gulig, 1990; Matsui, *et al.*, 2001), and the *pef* fimbriae implicated in adherence to the murine intestinal epithelium (Baumler, *et al.*, 1996). The CI score of an isolate is useful in predicting virulence of an isolate when compared to the index strain, *S. Typhimurium* UK-1 wild type. It was determined that this isolate carried the *spvB* and *pefA* virulence genes and that it was virulent in a mouse inoculation assay (Heithoff, *et al.*, 2008b) with a competitive index score of 0.545. The isolate was identified by biochemical testing, antimicrobial susceptibility, and agglutination with serogroup B specific antisera as described in *Sections* 3.3.3 to 3.3.6.

### *iii. Ovine models of heterologous salmonellosis*

The challenge agent selected for the ovine heterologous model of salmonellosis was a *Salmonella enterica* serovar Bovismorbificans (SMB 06-225) a multi-drug antimicrobial resistant strain (apramycin, erythromycin, neomycin, novobiocin, sulphonamides, streptomycin) isolated from the tissues of a dead sheep during a mortality investigation at a feedlot in Western Australian by Dr John K House from the Livestock Teaching and Research Unit at the University of Sydney, Camden, NSW in 2006. The unique resistance to neomycin sulphate allowed this organism to be easily isolated and differentiated from the vaccine following administration to animals. This isolate also carries the *spvB* and *pefA* virulence genes and was determined to be virulent in mouse inoculation studies (Heithoff, *et al.*, 2008b) with a competitive index score of 2.716. The isolate was identified by biochemical testing, antimicrobial susceptibility, and agglutination with serogroup C<sub>2</sub> specific antisera as described in *Sections* 3.3.3 to 3.3.6.

### **3.3 Microbiology**

#### ***3.3.1. Culture and growth of bacteria***

##### ***i. Storage of Salmonella isolates***

A frozen culture bank was established for the vaccine and each of the challenge strains in order to preserve isolates for use in future experimental procedures and to prevent genetic drift such as loss of virulence and resistance. Colour coded Protect™ Bacterial Preservers (Technical Service Consultants Ltd, Lancashire, UK) were employed to cryopreserve the isolates.

Several colonies from a culture plate containing the isolate were inoculated into sterile saline to form a thick suspension. One to two drops of the suspension were deposited aseptically into a Protect™ cryovial labelled with the isolate name, date prepared and contact details. Vials were closed and inverted several times to ensure uniform contact of the bacteria with the Protect™ cryopreservation beads and Protect™ cryoprotectant fluid. Prior to placement into a -70°C freezer or liquid nitrogen tank located in the Physical Containment Level 2 (PC2)/Quarantine Containment (QC2) Laboratories in Block 3 at EMAI, excess cryoprotectant solution was aseptically removed from each vial. Multiple vials were prepared for each isolate and stored in multiple locations to ensure culture bank security in the event of equipment malfunction or power failure. Quality control was performed on each cryopreserved isolate to confirm viability and purity of each isolate following procedures outlined in *Section 3.3*.

##### ***ii. Preparation of bacterial culture plates***

A cryopreservation bead coated with isolate was aseptically removed from the appropriately labelled cryopreservation vial and allowed to thaw on a Luria agar (LA) (OXOID Ltd, AU) plate containing a selective antibiotic appropriate for re-isolation of the strain. Care was taken to ensure that the beads remaining within the cryopreservation vial did not thaw and were returned to the freezer or liquid nitrogen

as soon as samples were collected. The LA plate was labelled with the name of the isolate, date revived and expiration date. Once thawed, the sample was streaked across the LA plate for single colonies and incubated aerobically at 37°C overnight. Following incubation, the bacterial culture plate was stored at 4-8°C for no more than 14 d. Quality control was performed on each revived isolate to confirm identity and purity. The procedures outlined in *Section 3.3* were performed on the revived isolates prior to initiation of experimental procedures to confirm identity.

### ***iii. Bacteria solution preparation***

Vaccine and challenge bacterial strains were grown following procedures outlined in Appendix I: Bacteria Growth and Dose Preparation Worksheet. Vaccine and challenge dose titres were confirmed via serial dilutions plated onto LA plates containing appropriate antibiotics; colony serotype was confirmed via antisera agglutination (described in *Section 3.3.4.*) and biochemical testing (described in *Section 3.3.5.*).

### ***3.3.2. Salmonella selective media***

*Salmonella* enrichment and selective media were used to culture, isolate and identify *Salmonella enterica* subspecies *enterica*. The media were applied according to the Australian National Association of Testing Authorities (NATA) and Association of Analytical Communities (AOAC) guidelines (MacFaddin, 2000; Waltman, 2000; AOAC, 2005)

#### ***i. Xylose lysine desoxycholate agar***

Xylose lysine desoxycholate agar (XLD) is a *Salmonella* selective and differential plating media. Sodium desoxycholate is the selective agent within the agar while the combination of sugars (xylose, lactose, and sucrose), the amino acid lysine and an H<sub>2</sub>S indicator system are used to identify *Salmonella* (Waltman, 2000). On XLD, *Salmonella* colonies are pink to red with black centres, which indicate H<sub>2</sub>S

production. A colour change in the plating media from orange-red to hot pink indicates fermentation of xylose, an alkaline reaction that is typical of *Salmonella*.

To enhance the selectivity of the plates further, antibiotics were added to the XLD plating media. Chloramphenicol, at 20 µg/mL, was added to the XLD media (XLD-CHL) to select for the vaccine strain. When selecting for the *S. Newport* 03-721 challenge agent for the calf trials, ceftiofur sodium was added to XLD media at 8 µg/mL (XLD-XNL). When selecting for the *S. Typhimurium* 06-131 and *S. Bovismorbificans* 06-225 challenge agents for the sheep trials, neomycin sulphate was added to XLD media at 10 µg/mL (XLD-NEO).

Samples suspected of containing *Salmonella* were inoculated onto appropriate XLD agar plates and incubated overnight at 37°C. After overnight incubation, plates were examined for the presence of ‘black’ colonies with ‘pink rings’. Further biochemical testing was conducted on all suspect colonies as false positive results could be elicited by *Proteus* spp. and *Citrobacter* spp. (Waltman, 2000) and occasionally *E. coli*.

#### **ii. Mannitol selenite enrichment media**

Mannitol selenite (MSB) is a selective enrichment broth that uses sodium biselenite and mannitol to promote *Salmonella* growth and inhibit other enteric pathogens like *E. coli*. MSB utilises the ability of bacteria to reduce selenite to create an alkaline environment and the ability of *Salmonella* to utilise mannitol as a carbohydrate source (Waltman, 2000).

Samples suspected of containing *Salmonella* were inoculated into vials containing MSB and incubated aerobically overnight at 37°C. After overnight incubation, samples were streaked onto XLD agar plates containing the appropriate selection antibiotic.

#### **iii. Tetrathionate enrichment media**

Tetrathionate (TRT) is a selective enrichment broth that uses tetrathionate, formed by

iodine and sodium thiosulphate, to produce an environment in which *Salmonella* can proliferate. The mode of action of TRT broth is poorly understood, but it is generally accepted that tetrathionate suppresses the growth of non-*Salmonella* through inactivation of the sulphydryl groups of enzymes (Waltman, 2000).

Samples suspected of containing *Salmonella* were inoculated into vials containing TRT broth at a ratio of 1:10 and incubated aerobically overnight at 37°C. After overnight incubation, samples were streaked on appropriate XLD agar plates. TRT was only used in the initial study phases of the project in conjunction with MSB as enrichment media. Previous research conducted with the vaccine strain had used TRT (Dueger, *et al.*, 2001, 2003a, 2003b). However, variable results were observed with TRT when compared to MSB selective enrichment, a finding consistent with reports in the literature (Waltman, 2000; Feldsine, *et al.*, 2002; AOAC, 2005). Therefore, the use of TRT was phased out of the project in preference to MSB; it gave more consistent and reliable results and is the generally accepted standard for enrichment of *Salmonella* spp. (AOAC, 2005).

### **3.3.3. Presumptive *Salmonella* identification and serotyping**

Prior to initiating presumptive *Salmonella* identification and serotyping, a single colony from a *Salmonella* suspicious isolate was sub-cultured onto a sheep blood agar plate (SBA) and incubated aerobically at 37°C overnight. Sub-culturing onto a non-selective growth media such as SBA, reduced the chance of cross-reactivity and inhibition of flagellar antigens from compounds in the selective media and ensured a pure culture from which multiple biochemical tests could be performed.

#### **i. Presumptive *Salmonella* identification**

Presumptive *Salmonella* identification was performed using the OXOID *Salmonella* Test Kit (DR1108; OXOID Ltd, UK). The OXOID *Salmonella* Test Kit detects the majority of common *Salmonella* spp. including *S. Typhimurium* and *S. Enteritidis*. In order to minimise cross reactions with other Enterobacteriaceae, antibodies to the

principal somatic antigens are removed during preparation of the antisera used in this assay. Consequently, the reactions are predominantly with flagellar antigens, however non-motile species *S. Pullorum* and *S. Gallinarum* will elicit positive reactions. Each kit contained a polyvalent antisera prepared against a wide range of *Salmonella* flagellar antigens which were used to coat latex particles. When mixed with a suspension of *Salmonella*, the latex particles coated with the antigen rapidly agglutinated to form visible blue clumps, indicating a positive reaction.

On a clean microscope slide, three or four large circles were drawn with a water insoluble marker. A 15 µL drop of sterile saline was placed within each circle. The isolate to be identified was selected from a colony from a pure culture that had been inoculated onto an SBA. The colony was emulsified into the drop of sterile saline using a sterilised loop. Then a 15 µL drop of the *Salmonella* Latex agglutinating sera (OXOID Ltd., UK) was pipetted into the suspension and mixed thoroughly. The slide was rocked after the addition of the latex agglutination sera and monitored for agglutination within the suspension for approximately two minutes. Agglutination of the suspension, appearing as blue clumps within a clear liquid, indicated that the isolate was positive for *Salmonella* flagellar antigens. Positive isolates were subjected to further serotyping and biochemical testing to confirm presumptive identification and to eliminate false-positives such as oxidase-positive bacteria species and *E. coli* (OXOID, 2004).

Quality assurance was performed each day using a positive and negative control to ensure integrity of the test kit and validate test results. The positive control was a *Salmonella* Typhimurium ATCC® 14028 and the negative control was *Escherichia coli* ATCC® 25922 as recommended by the manufacturer's guidelines (OXOID, 2004). Additionally, the vaccine and challenge strains were assessed alongside the positive and negative controls.

#### **3.3.4. *Salmonella* serogrouping**

On a clean microscope slide, large circles were drawn with a water insoluble marker to form wells. A 15 µL drop of sterile saline was pipetted into each well on the slide and the test culture was emulsified into each drop of saline with a sterilised loop. To the first well on the slide, 15µL of sterile saline was added to the emulsion and acted as a negative control. To the second well, a 15µL of somatic O antisera of the suspected serogroup of the *Salmonella* isolate was added to the emulsion. In the event two serogroups were suspected, e.g., a cross-protective vaccination-challenge trial, then a third well was tested with the other suspected serogroup. The *Salmonella* somatic O antisera were: group B, factors 1, 4, 12, and 27 (229731; BD Diagnostics, USA); group D, factors 1, 9, and 12 (229511; BD Diagnostics, USA); and group C<sub>2</sub>, factors 6 and 8 (229501 BD Diagnostics, USA).

The slide was then rocked to mix the emulsion after the addition of the antisera for one minute and observed for agglutination (white clumps). Isolate identification was verified following agglutination patterns shown in Table 3.1. Quality assurance was performed each day using a positive and negative control for each antisera used to ensure integrity of the substrate. Pure cultures of the vaccine and challenge strains were also assessed alongside the positive and negative controls.

Table 3.1. *Salmonella enterica* subspecies *enterica* Serotype Identification Scheme

Isolate Identification		Antisera			
Serogroup	Strain	Saline (Control)	B	C2	D
B	Typhimurium	NEG	POS	NEG	NEG
C <sub>2-3</sub>	Bovismorbificans	NEG	NEG	POS	NEG
C <sub>2-3</sub>	Newport	NEG	NEG	POS	NEG
D	Dublin	NEG	NEG	NEG	POS
Test Failure – Repeat		POS	NEG or POS	NEG or POS	NEG or POS
POS – Agglutination (clumps in the suspension) observed; NEG – No agglutination					

### 3.3.5. *Biochemical identification of salmonella*

Since no one biochemical test can conclusively identify salmonellae, a combination of biochemical tests was used to classify and differentiate members of the Enterobacteriaceae family. The tests commonly utilised in the identification of salmonellae included fermentation of glucose, mannitol and dulcitol; inability to ferment sucrose, salicin and lactose; inability to hydrolyse urea and form indole from tryptophan; o-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) negative and production of hydrogen sulphide ( $H_2S$ ) (Old and Threlfall, 1998; Quinn, *et al.*, 1999; Jones, *et al.*, 2000; Waltman, 2000; Quinn, *et al.*, 2002). The following biochemical tests were used to confirm that isolates were a *Salmonella enterica* subspecies *enterica*: urea, triple sugar iron agar, ONPG, and indole. *Salmonella* suspicious or presumptuous isolates were sub-cultured onto SBA and incubated aerobically at 37°C overnight to ensure a pure culture from which multiple biochemical tests could be performed.

#### *i. Urea*

This test is used to determine the ability of the isolate to hydrolyse urea and confirm the presence or absence of the urease enzyme (MacFaddin, 2000). A colony from the sub-cultured isolate to be identified was inoculated onto Christensen's urea agar slant with 2% urea and phenol red pH indicator (Difco, AU) and then incubated overnight at 37°C. A colour change in the media from buff-yellow to pink indicated a positive (alkaline) reaction and a urease positive isolate. *Salmonella* are unable to hydrolyse urea (Old and Threlfall, 1998; Jones, *et al.*, 2000) and therefore produce a negative reaction (i.e., no colour change). The urea test is useful to differentiate *Salmonella* spp. from *Proteus* spp. and *Citrobacter* spp.

#### *ii. Triple sugar iron agar (TSI)*

TSI is a composite medium for the differentiation of Enterobacteriaceae based on varying abilities to ferment sucrose, lactose and glucose and ability to produce hydrogen sulphide ( $H_2S$ ) from an inorganic sulphur source (Holmes, 1998; Jones, *et*



*al.*, 2000; MacFaddin, 2000). The medium was inoculated by stabbing the isolate into the base (butt) then streaking the isolate onto the slope. The TSI slants were incubated overnight at 37°C with loosened caps (aerobic). A yellow colour change in the media indicated an acid reaction due to the utilisation of glucose and production of lactic acid. A red-purple colour change in the media indicated an alkaline reaction caused by the breakdown of protein. The production of H<sub>2</sub>S was characterised by blackening of the TSI medium. Gas bubble formation in the butt of the media indicated anaerobic fermentation. The interpretation key used for TSI media reactions is depicted in Table 3.2 (adapted from Jones *et al.*(2000)).

Table 3.2. TSI Reaction Interpretation Key

TSI Reaction Regions		<i>Salmonella</i>	<i>E. coli</i>	<i>Proteus</i>
Butt	Anaerobic metabolism	AG	AG	AG
Slope	Aerobic metabolism	ALK or NC	A	A
H <sub>2</sub> S	Produce H <sub>2</sub> S from sodium thiosulphate	+	-	+

AG, acid (yellow) and gas formation; A, acid (yellow); NC, No change in media colour; ALK, alkaline reaction (red); +, H<sub>2</sub>S (black); -, no H<sub>2</sub>S (no blacking of media)

### iii. ONPG

This test is used to determine the presence or absence of the enzyme β-galactosidase in order to differentiate lactose delayed organisms from lactose negative organisms (MacFaddin, 2000). A colony from the sub-cultured isolate to be identified was inoculated into a vial containing 2.5 mL of 0.15% ONPG in 1% peptone water and incubated overnight at 37°C. A colour change in the media from clear to yellow would indicate the presence of β-galactosidase and confirm that the isolate was a lactose fermenter. *Salmonella enterica* subspecies *enterica* are generally non-lactose fermenters and ONPG negative (Holmes, 1998; Jones, *et al.*, 2000; MacFaddin, 2000). The ONPG test is useful for differentiating *Salmonella enterica* subspecies *enterica* from *Citrobacter* spp. and other *Salmonella* subspecies (Old and Threlfall, 1998; Jones, *et al.*, 2000; MacFaddin, 2000).

#### ***iv. Indole***

The indole test is used to determine whether an organism has the ability to split indole from the amino acid tryptophan. A single colony from the sub-cultured isolate was inoculated into a vial containing 10 mL of tryptone broth (Difco, USA) and incubated overnight at 37°C. After incubation, 1 mL of Kovac's Reagent (TM246; OXOID Ltd., AU) was added to the vial and observed for the appearance of a red ring on the surface of the medium. A red ring indicated a positive indole test result. *Salmonella* spp. are unable to form indole. The indole test is useful for differentiating *Salmonella* spp. from *Edwardsiella* spp. and *E. coli* (Jones, *et al.*, 2000; MacFaddin, 2000).

#### ***3.3.6. Susceptibility and resistance testing procedures***

Antimicrobial susceptibility and resistance (ASR) testing was performed following the CDS Test method (Bell, *et al.*, 2004; Bell, *et al.*, 2008). *Salmonella* isolates, confirmed by biochemical and antisera testing, were sub-cultured onto either LA or SBA plates and incubated aerobically at 37°C overnight prior to initiating ASR testing.

#### ***i. Preparation of samples***

A single colony forming unit (CFU) from a fresh culture plate was tapped with a sterile straight wire and inoculated into a tube containing 2.5 mL of sterile saline. The tube was capped and inverted several times to thoroughly mix the bacteria. The contents of the tube were poured onto a plain sensitivity agar (PSA) (Sensitest Agar CM0409; OXOID Ltd, AU) plate that was labelled with the isolate identification number, date and antibiotic test series. The plate was gently swirled to ensure the entire surface of the PSA plate was coated with bacterial solution. Excess bacterial solution was removed from the PSA plate using a sterile transfer pipette and discarded. The inoculated PSA plate was then placed face-up under a Bunsen burner to dry for at least 15 min but no more than 45 min.

Table 3.3. Susceptibility and Resistance Testing Antibiotics

<b>Antibiotic Tested</b>	<b>Disc Label</b>	<b>Concentration (<math>\mu\text{g}</math>)</b>
Amikacin	AK	30
Amoxicillin/Clavulanic Acid	AMC30	30
Ampicillin	AMP	20
Apramycin	APR	15
Ceftiofur	EFT	30
Cefuroxime	CXM	30
Chloramphenicol	C	10
Erythromycin	E	5
Kanamycin	K	50
Nalidixic Acid	NA	30
Neomycin	N	30
Novobiocin	NV	5
Penicillin	P	0.5 units
Streptomycin	S10	10
Sulphonamides Compound	S3	300
Sulphamethoxazole	SF	300
Sulphamethoxazole/Trimethoprim 19:1	SXT	25 (23.75/1.25)
Tetracycline	TE	30
Trimethoprim	W	5

*ii. Antibiotic challenge of isolates*

Following drying, antibiotic challenge discs (OXOID Ltd, AU)(Table 3.3) were applied to the inoculated PSA plates using an OXOID™ Disc Dispenser MKII (OXOID Ltd, AU). No more than eight antibiotic discs were applied to any one inoculated PSA plate. The PSA plates were then covered and placed right-side up in an incubator at 37°C for 16-18 h. At the end of the incubation period, the plates were removed from the incubator and the elapsed time was calculated to the nearest quarter of an hour.

### *iii. Antibiotic susceptibility and resistance*

The zone of inhibition, the area free from bacterial growth, was measured from the edge of the antibiotic disc to the edge of confluent bacterial growth (radii measurement) to the nearest 0.1 mm using a ruler or microcalipers following standardised techniques developed by Bell *et al.* (2004; 2008) and the standard protocol used by the Region Veterinary Laboratory at EMAI. Radii measurements < 6 mm indicated that an isolate was resistant to the antibiotic impregnating the disc. Radii measurements  $\geq$  6 mm indicated that the isolate was susceptible to the antibiotic impregnating the disc. *Escherichia coli* ATCC 25922 was used as a quality control to assess antibiotic discs.

## **3.4 Neonate Models of Ruminant Salmonellosis**

### *3.4.1. Animal ethics and biosecurity approvals*

In vivo usage of the vaccine and *S. Newport* challenge strains was approved by AQIS using *Approval for In Vivo Use (Direct or Indirect) of Restricted Imported Biological Material in Non-Laboratory Animals Permits (In vivo Approval Numbers: vaccine; 2003/077 and 2006/020 and challenge, 2006/006)* as required by AQIS conditions for the use of imported biological products in non-laboratory animals (AQIS/DPIE, 2010).

The use of calves in this research was approved by the Animal Ethics Committee (AEC) at EMAI (AEC Reference: M05/029).

### *3.4.2. Test system*

#### *i. Species/strain*

Holstein-Friesian bull calves (*Bos taurus*) were the test system used in the conduct of this project. Cattle were chosen for this study as they will be one of the target species for application of the vaccine and the efficacy of attenuated live *Salmonella* vaccines is host species dependent. Calves were selected as this is a target subpopulation and

logistically easier to handle than adults. The mechanism by which live vaccines confer immunity is unknown and the strain requirements for an effective live vaccine are unknown. Previous studies have demonstrated species specific variation in response to vaccination, e.g., avirulent in one but pathogenic in another.

*ii. Source*

The calves used in the conduct of this project were obtained from either Moxey Dairies, Pty. Ltd., Gooloogong, NSW, AU or the EMAI Dairy Number 9, EMAI, Department of Investment & Industry, Menangle, NSW, AU.

**3.4.3. Selection criteria**

The criteria used to select calves were passive transfer of colostral antibodies and an observation of no physical abnormalities or deformities during physical examination.

*i. Passive transfer of antibodies*

Passive transfer of antibodies of colostral antibodies was assessed via measurement of serum proteins using a TS Meter® handheld refractometer, (Catalogue #10400, American Optical Company, USA). Blood was collected from the jugular vein of each calf into a 10 mL red top serum separation vacutainer tube (BD Vacutainer® 36643; BD, USA). Approximately 2.5 mL was transferred into a polypropylene microcentrifuge tube and allowed to stand for 30 minutes (min). Tubes were centrifuged for 10 min in a Beckman™ Microcentrifuge. Sera were placed onto the sample window of the refractometer and a total solids reading was recorded on a calf collection record. Two readings were recorded for each calf and the average of these readings was used to determine serum protein level. Calves with a serum protein less than 5.0 g/dL at 48 h of age were excluded on the basis of failure of passive transfer (Heinrichs and Radostits, 2001; Vaala and House, 2002b).

## *ii. Health and vigour*

Each calf underwent a veterinary health check prior to selection. Any calf with conformational defects, congenital abnormalities or poor vigour was excluded from selection. If a calf became injured or ill after selection, it was removed from the study and humanely euthanised (as described in *Section 3.4.11*).

### *3.4.4. Animal identification*

Calves were individually identified with colour-coded ear tags. At birth, each calf received a National Livestock Identification System (NLIS) tag. The tag was affixed to the right ear of the calf as required by the National Livestock Identification System, Stock Disease Regulation 2004, Part 3 (Stock Diseases Act 2004) (NLIS, 2004). In addition to the NLIS tag, a white plastic ear tag with temporary study identification number was placed into the right ear following selection for inclusion in the trial. At study initiation, each calf received a colour-coded ear tag with a unique study identification number following randomisation that was placed in the left ear.

### *3.4.5. Management and housing of calves at source dairy*

Bull calves were collected from the maternity pen at the dairy and placed into a designated calf nursery area following birth. In the calf nursery area the calves were fed 2 L of pooled colostrum immediately after birth and then again at 12 h and 24 h. To reduce the chance of navel infection, the umbilical region of each calf was dipped in 7% iodine solution on arrival in the calf nursery and at 24 h of age. Following the third colostrum feeding, the calves were moved from the calf nursery to an isolation nursery to prevent contact with older animals and potential exposure to enteric pathogens.

In the isolation nursery, the calves were placed into small groups of approximately 3-4 calves of similar age. Each calf was trained to drink milk replacer from a plastic bucket. Calves were bucket fed Palastart Blue® (Probiotec Pty. Ltd., AU), a non-

medicated milk-replacer that was 20% fat and 24% protein. To reduce the chance of cross-contamination between calves of varying ages in the isolation facility, the youngest animals were fed first, gloves were changed between groups of calves, and any calves with loose stool or diarrhoea were fed last.

Any calf that did not meet selection criteria during the collection period was removed from the isolation nursery area and excluded from the trial. All excluded calves were returned to the on-farm bull calf rearing facility for further assessment and treatment if appropriate. Any calf that met euthanasia criteria as described in *Section 3.4.10* was humanely euthanised following on-farm protocols.

### ***3.4.6. Neonate animal management and husbandry***

#### ***i. Housing***

Calves were transported to EMAI Medium Security Animal Facility, Menangle, NSW and housed in PC2/QC2 Animal Rooms. Within the animal rooms, calves were individually housed in wire mesh pens on a raised mesh flooring. To ensure comfort and improve traction, a tubular matting material was used to line the back two-thirds of each pen. Each pen was equipped with a bucket holder and a plastic all-purpose bucket for administration of milk replacer and water. Colour-coded cage cards were affixed to each pen and included the following information: animal identification number, study identification, test articles/bacterial strains, group assignment, study initiation date, name of the study director and study monitor. Individual animal clinical assessments and observation records were maintained for each calf on the *Individual Daily Animal Observation Records* (see Appendix II: Individual Daily Animal Observation Record for Calves for sample form) during the conduct of each experiment.

#### ***ii. Nutrition***

Calves were bucket fed Palastart Blue® (Probiotec Pty. Ltd., AU), a non-medicated,

milk-replacer that was 20% fat and 24% protein. Milk replacer was prepared following manufacturer’s instructions and fed at approximately 12-15% of estimated body weight of the calves over two feedings using Table 3.4. as a guideline. Calves were provided access to tap water *ad libitum*. All milk replacer preparation equipment was dedicated to a single animal room and milk buckets were assigned to individual calves to prevent cross contamination.

Table 3.4. Milk Replacer Feeding Volume Schedule

Study Week	Age of Calves (Days)	Approximate Weight of Calves (Kg)	Total Daily Volume (L)
Acclimation	1-14	35 to 40	5.0
1	7-21	38 to 43	6.0
2	14-28	41 to 46	7.0
3	21-35	44 to 49	7.0
4	28-49	47 to 52	8.0
5	35-56	50 to 55	8.0
6	49-63	55 to 60	9.0
7	56-70	60 to 65	9.0
8	63-77	65 to 75	9.0

**iii. Husbandry and sanitation**

Prior to initiating each trial, the animal rooms used to house the calves and all equipment used in the care and maintenance of the calves were thoroughly cleaned and sanitised to ensure the environment was free from common scour pathogens and any other infectious agents that may have been harboured on equipment or within the room from previous experiments following procedures outlined in SOP 80.03.01, Schedule for Animal Room Housekeeping During the Conduct of *Salmonella* and *Salmonella* Vaccine Trials (see Appendix IV: Standard Operating Procedure Animal Room Husbandry). Animal room floors and individual pens were flushed with water daily and with quaternary disinfectant at least twice a week following procedures outlined in Appendix IV. A quaternary ammonium disinfectant was selected due to its



stability in the presence of organic matter, non-corrosive to metal, and high efficacy against bacteria, moulds and viruses, as well as reduced risk to staff and animals with daily use (Walker, 2003; Mueller-Doblies, *et al.*, 2010). Additionally, quaternary ammonium disinfectants have been shown to be useful in the disinfection of surfaces and equipment contaminated with salmonellae and coliforms (Payne, *et al.*, 2005; Lee, *et al.*, 2007; Mueller-Doblies, *et al.*, 2010). Faecal material within the pen that could not be removed with low pressure hosing was scrubbed with a brush. Care was taken to avoid wetting the calves during this process. Equipment used in the preparation of milk replacer was cleaned after each feeding. Milk buckets were rinsed after each feeding and cleaned with warm soapy water. Weekly, the feeding equipment was sanitised with a quaternary disinfectant following cleaning. All husbandry procedures were recorded on the *Daily Housekeeping Record* (see Appendix IV).

#### **3.4.7. Biosecurity procedures**

Biosafety protocols recommend by Office of the Gene Technology Regulator (OGTR), *Gene Technology Regulations Act 2001* (Government, 2010) for the handling of PC2 organisms as well as AQIS QAP regulations for handling a QC2 organism (AQIS/DAFF, 2010; AQIS/DPIE, 2010) were followed to prevent release of the vaccine into the environment, to prevent cross contamination between vaccinated and non-vaccinated groups, and to ensure safety of all personnel working on this project. In compliance with OGTR regulations and AQIS QAP, standard operating procedures used for entry and exit into the PC2/QC2 Animal Rooms at EMAI during the conduct of the studies were developed and outlined in Appendix V: Standard Operating Procedure for Exit and Entry into Animal Rooms. The Institutional Biosecurity Committee (IBC) at EMAI approved the biosecurity protocols, as well as the use of animals and micro-organisms in these research (IBC Reference No: M05/029). All staff and researchers received biosecurity training and hazard awareness training prior to entry into the animal house facility.

### 3.4.8. Clinical assessment of calves

#### i. Attitude/mentation

Attitude and mentation status of the calves was assessed daily at feeding time using the scoring system shown in Table 3.5 and recorded on the *Individual Daily Animal Observation Record* (Appendix II). An accumulative daily attitude score was developed to evaluate the mentation status of each calf. Daily accumulative attitude scores greater than 2 were considered abnormal.

Score	Attitude Observation
0	Standing; ready to be fed
1	Stands with stimulus
2	Stands with assistance
3	Unable to stand but maintains sternal recumbency
4	Lateral recumbency

#### ii. Appetite

The appetite of calves was monitored at feeding time. A schedule of the milk replacer feeding volumes offered to calves throughout the study is shown in Table 3.4. The volume of milk replacer consumed by each calf at each feeding was recorded to the nearest 0.1 L and recorded on the *Individual Daily Animal Observation Record* (Appendix II). Total daily intake was determined for each calf.

#### iii. Rectal temperature

The calves had rectal temperatures (RT) measured following morning feeding with a calibrated digital clinical thermometer (Livingstone Pty. Ltd., AU) and recorded to the nearest 0.1°C (range of 32.0 to 42.9°C) and recorded on the *Individual Daily Animal Observation Record* (Appendix II). During the acclimation and vaccination periods, RT was record weekly. The acclimation temperature readings were used to establish normal baseline temperature ranges for the calves. Vaccinated calves were

monitored for at least three days post vaccination to evaluate the effect of vaccination on RT (e.g., evidence of pyrexia). After virulent *Salmonella* challenge, RT were recorded daily during the post-challenge period.

**iv. Faecal composition score**

The faeces texture and composition of each calf was monitored twice daily. Faecal characteristics were evaluated using the scoring system shown in and recorded on the *Individual Daily Animal Observation Record* (Appendix II). A daily accumulative faecal score greater than 2 was considered abnormal. A dichotomous scale using 0 for normal (daily accumulative faecal score less than or equal to 2) and 1 (daily accumulative faecal score greater than 2) for abnormal was used to analyse faecal composition.

Table 3.6. Faecal Scoring System

Score	Faecal Characteristics
0	Normal colour and texture
1	Soft stool/runny
2	Blood or casts in stool
3	Diarrhoea (watery/increased frequency)

**3.4.9. Additional welfare observations**

Additional welfare observations were performed during the first 7 d post-challenge. Calves were observed between the morning and evening feedings and at midnight. During these observation periods it was considered normal for a calf to be standing or lying in sternal recumbency and abnormal to be in lateral recumbency. All observations were recorded on the *Individual Daily Animal Observation Record* (Appendix II).

#### ***3.4.10. Criteria for intervention and euthanasia of calves***

Criteria for intervention and euthanasia were established in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2004), NSW *Animal Research Act 1985* and approved by the EMAI AEC. Procedures for intervention and euthanasia criteria were as follows:

##### ***i. Attitude:***

Assistance was provided to calves found in lateral recumbency. Animals were placed into sternal recumbency and then re-assessed. Any calf that failed to maintain sternal recumbency received an attitude score of 4 and was humanely euthanised (as described in *Section 3.4.11*).

##### ***ii. Appetite:***

Calves that failed to consume milk replacer or were unable to reach a milk bucket were offered feeding assistance and encouraged to drink by study personnel. Any calf that failed to consume milk replacer, e.g., zero feed intake over two consecutive feedings, was humanely euthanised (as described in *Section 3.4.11*).

#### ***3.4.11. Euthanasia of calves***

Euthanasia was performed when an animal met the criteria for euthanasia (described in *Section 3.4.10*) or at the completion of study. Calves were euthanised via IV injection of 60-80 mg/kg of pentobarbitone sodium. Intracardiac (IC) administration of pentobarbitone sodium was employed in sedated and unconscious animals. Euthanasia methods were approved by the EMAI AEC and followed guidelines set forth in the American Veterinary Medical Association (AVMA) Panel on Euthanasia (AVMA, 2001).

### **3.5 Ovine Models of Ruminant Salmonellosis**

#### ***3.5.1. Animal Ethics and Biosecurity Approvals***

*In vivo* usage of the vaccine was approved by AQIS on Approval for *In Vivo* Use (Direct or Indirect) of Restricted Imported Biological Material in Non-Laboratory Animals Permits (In vivo Approval Number: 2008/067) as required by AQIS import conditions for the use of imported biological products in non-laboratory animals (AQIS/DPIE, 2010).

The use of animals in this research was approved by the AEC at EMAI (AEC Reference No: M09/013).

### **3.5.2. Test system**

#### ***i. Species/Strain***

Merino wethers (*Ovis aries*) were the test system used in the conduct of this project. Adult male castrated sheep - wethers - were chosen as sheep are one of the target species for application of the vaccine and the logistics of evaluating the vaccine in sheep is more practical than adult cattle.

The efficacy of attenuated live *Salmonella* vaccines is host species dependent. The mechanism by which live vaccines confer immunity is unknown and the strain requirements for a good live vaccine are unknown. Previous studies have demonstrated different responses in different species as well as age groups.

#### ***ii. Source***

All sheep used in the conduct of this project were obtained from J.M. Stephen from the property "Warrane" in Armidale, NSW, Australia.

### **3.5.3. Selection criteria**

The selection criteria for the sheep used in these research trials were: Australian Johne's Disease Market Assurance Program (MAP) status, health and sex.

*i. Australian Johne's disease MAP*

The sheep flock maintained at the Warrane property had a MAP flock status of MN3. This status was obtained in 2003 and identifies the property to be of low risk of Johne's infection based on annual screening of sheep held on the property. Selection of sheep from a MAP certified property ensured that the gastrointestinal tract and immune system of the sheep were not compromised by Ovine Johne's disease. Johne's disease is associated with the infection of sheep with *Mycobacterium avium* subspecies *paratuberculosis*. Symptoms of Johne's disease may not manifest for many months to years and are typically associated with chronic wasting, ill thrift and scouring.

*ii. Health and vigour*

Prior to shipment the sheep were: shorn; drenched with a broad spectrum anthelmintic, Q-drench® (Jurox Pty. Ltd., AU); treated for external parasites with Zapp pour-on lousicide (Bayer Australia Ltd., AU); and vaccinated with a commercial clostridial vaccine, e.g., 5-in-1. Each sheep underwent a veterinary health check on arrival. Any sheep with conformational defects, congenital abnormalities or poor vigour following shipment, were excluded from selection. If a sheep became injured or ill after selection, the animal was removed from the study and humanely euthanised (as described in *Section 3.5.10.*).

**3.5.4. Animal identification**

Sheep were individually identified with colour-coded ear tags. Each sheep had a NLIS tag affixed to the right ear which was labelled with a property identification code as required by the National Livestock Identification System, Stock Disease Regulation 2004, Part 3 (*Stock Diseases Act 2004*) (NLIS, 2004). In addition to the NLIS tag, a white plastic ear tag with a temporary study identification number was placed into the right ear following selection for inclusion in the trial. At study

initiation, each sheep received a colour-coded ear tag with a unique study identification number following randomisation.

### **3.5.5. Sheep management and husbandry**

#### ***i. Housing***

Sheep were transported to EMAI Medium Security Animal Facility, Menangle, NSW and housed PC2/QC2 Animal Rooms. Within the animal rooms sheep were individually housed in wire mesh pens on raised mesh flooring. To ensure comfort and improve traction, tubular matting material was used to line the back two-thirds of each pen. Each pen was equipped with three plastic all-purpose buckets for administration of water and feed. Colour-coded cage cards were affixed to each pen and included the following information: animal identification number, study identification, test articles/bacterial strains, group assignment, study initiation date, name of the study director and study monitor. Individual animal clinical assessments and observation records were maintained for each sheep on the *Individual Daily Animal Observation Record* (Appendix III) during the conduct of each experiment.

#### ***ii. Nutrition***

Sheep received a diet of oaten chaff (8 mega joules of metabolisable energy per kilogram of dry matter [MJ ME/kg DM]) and commercially prepared grain pellet (12 MJ ME/kg DM) during the acclimation, vaccination and challenge phases of the experiment. Sheep were gradually introduced onto the grain pellet during the acclimation and vaccination periods. The amount of pellets and chaff fed to the sheep was approximately 1-2% body weight and adjusted to ensure that each animal received roughly 10 MJ ME/d/50 kg of body weight (BWT). Pellets and chaff were offered in separate feeders to facilitate monitoring of the eating habits of sheep with and without salmonellosis, as well as to determine if there were any feeding preferences.

Any sheep that refused to consume chaff and pellets during the acclimation period was offered 100-200 g of fresh rye or kikuyu grass in addition to regular diet to facilitate transition onto the diet. During the challenge phase of the trial, any sheep that became inappetent or anorexic was offered 100-200 g of fresh grass to stimulate appetite, to provide a familiar feed stuff, and to prevent anorexia.

**iii. Water intake**

Sheep were provided 6 L of tap water at the morning feeding period and at afternoon observation period. The volume of water consumed after each offering was recorded daily to the nearest 0.5 L and total daily water consumption was determined for each sheep from these data.

**iv. Husbandry and sanitation**

Prior to initiating each trial, the animal rooms used to house the sheep and all equipment used in the care and maintenance of the sheep were thoroughly cleaned and sanitised to ensure the environment was free from common scour pathogens and any other infectious agents that may be harboured on equipment or the room from previous experiments, following procedures outline in SOP 80.03.01, *Schedule for Animal Room Housekeeping During the Conduct of Salmonella and Salmonella Vaccine Trials* (see Appendix IV). Animal room floors and individual pens were flushed with water daily and with quaternary disinfectant at least twice a week, following procedures outlined in Appendix IV. Faecal material within the pen that could not be removed with low pressure hosing was scrubbed with a brush. Care was taken to avoid wetting the sheep during this process. Weekly, the feeding equipment was cleaned with a quaternary disinfectant and mats were scrubbed. All husbandry procedures were recorded on the *Daily Housekeeping Record* (see Appendix IV).

**3.5.6. Biosecurity procedures**

Biosafety protocols recommend by OGTR, *Gene Technology Regulations Act 2001* (Government, 2010) for the handling of PC2 organisms as well as AQIS QAP



regulations for handling a QC2 organism (AQIS/DAFF, 2010; AQIS/DPIE, 2010) were followed to prevent release of the vaccine into the environment, to prevent cross contamination between vaccinated and non-vaccinated groups, and to ensure safety of all personnel working on this project. In compliance with OGTR regulations and AQIS QAP, standard operating procedures used for entry and exit into the PC2/QC2 Animal Rooms at EMAI during the conduct of the studies were developed and outlined in Appendix V. The IBC at EMAI approved the biosecurity protocols as well as the use of animals and micro-organisms in these research (IBC Reference No: M09/013). All staff and researchers received biosecurity training and hazard awareness training prior to entry into the animal house facility.

### ***3.5.7. Clinical assessment of sheep***

#### ***i. Attitude/Mentation***

Attitude and mentation status of the sheep was assessed daily at feeding time using the scoring system shown in Table 3.7. and recorded on the *Individual Daily Animal Observation Record* (Appendix III). An accumulative daily attitude score was used to evaluate the mentation status of each sheep.

Table 3.7: Attitude Scoring System for Sheep

<b>Score</b>	<b>Attitude Observation</b>
0	standing and ready to be fed (BAR)
1	standing and quiet and alert (QAR)
2	standing with head low
3	stands with stimulus or standing but leaning on pen
4	unable to stand or lateral recumbency

#### ***ii. Appetite***

Individual feed intake of the sheep was monitored daily. Fresh feed was provided to the sheep each morning following observations, utilising feeding guidelines outlined

in Table 3.8. Each morning any chaff or pellets remaining from the previous feeding were weighed using a digital scale and refusal of pellets and chaff was recorded to the nearest gram onto the *Individual Daily Animal Observation Record* (Appendix III).

Table 3.8: Sample Feeding Plan for sheep

<b>Stage of Study</b>	<b>Pellets per head/d</b>	<b>Chaff per head/d</b>
Arrival	0 grams	800 -1000 grams
Acclimation	100-300 grams	800-700 grams
Challenge	200 - 400 grams	600-700 grams

### *iii. Rectal temperature (RT)*

The sheep had RT measured prior to morning feeding with a calibrated digital clinical thermometer (Livingstone Pty Ltd, AU) and recorded to the nearest 0.1°C (range of 32.0 to 42.9°C) onto the *Individual Daily Animal Observation Record* (Appendix III). During the acclimation period, RT was recorded twice weekly. The acclimation RT readings were used to establish normal baseline ranges for the sheep housed in the research facility. Vaccinated sheep were monitored for at least 4 d post vaccination. After the challenge with virulent *Salmonella*, RT was recorded daily.

### *iv. Faecal composition score*

The faeces from each sheep were observed and scored twice daily. Faecal characteristics were evaluated using the scoring system shown in and recorded on the *Individual Daily Animal Observation Record* (Appendix III).

An accumulative daily faecal score was used to evaluate the faecal composition of the sheep following challenge with virulent *Salmonella*.

### **3.5.8. Additional welfare observations**

Additional welfare observations were performed during the first 7 d post-challenge. Sheep were observed midmorning following the morning feeding and at midnight. During these observation periods it was considered normal for a sheep to be standing

or lying in sternal recumbency and abnormal to be in lateral recumbency. Additional welfare observations were recorded on the *Individual Daily Animal Observation Record* (Appendix III).

### **3.5.9. Criteria for intervention and euthanasia of sheep**

Criteria for intervention and euthanasia were established in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004), NSW Animal Research Act 1985 and approved by the EMAI AEC. Procedures for intervention and euthanasia criteria for sheep used in these research trials were as follows:

#### ***i. Attitude***

Any sheep that was unable to stand or found in lateral recumbency at any time received an attitude score of 4 and was humanely euthanised (as described in *Section 3.5.10*). Any sheep that would not stand unless stimulated or leaned against pen gates/panels over a 48 h period, received an attitude score of 3 and was humanely euthanised (as described in *Section 3.5.10*).

#### ***ii. Appetite***

Sheep that refused to eat chaff or pellets during the challenge phase of the trial were encouraged to eat by offering fresh grass. Any sheep that had failed to consume chaff, pellets or grass over three consecutive days (100% refusal) was humanely euthanised (as described in *Section 3.5.10*).

### **3.5.10. Euthanasia of sheep**

Euthanasia was performed when an animal met the criteria for euthanasia (described in *Section 3.5.9*.) or at completion of the study. Sheep were euthanised via injection of 60-80 mg/kg of pentobarbitone sodium IV or IC in sedated or unconscious animals when IV injections were not possible. Euthanasia methods were approved by the

EMAI AEC and followed guidelines set forth in the AVMA Panel on Euthanasia (AVMA, 2001).

### **3.6 Sample collection and processing**

#### ***3.6.1. Qualitative Salmonella faecal cultures***

Faecal samples were collected from animals prior to enrolment and once a week for qualitative detection of *Salmonella*. Cotton tipped swabs were inserted into the rectum of each calf or several pellets were collected from the rectum of each sheep and subsequently placed into a 10 mL enrichment broth, e.g., MSB. The broth containing the rectal swabs/pellets was incubated aerobically for 24 h at 37°C, and then plated onto XLD media. Plates were incubated aerobically for 24 h at 37°C. Identification and serotyping of *Salmonella* suspicious organisms isolated from the XLD plates was initiated following procedures outlined in *Sections* 3.3.3 to 3.3.5. *Salmonella* isolates collected from calves and sheep during the acclimation phase were submitted to the Institute of Medical and Veterinary Science, *Salmonella* Reference Laboratory, South Australia, Australia (IMVS) for comprehensive serotyping and phage identification.

#### ***3.6.2. Quantitative Salmonella faecal cultures***

Quantitative faecal culturing was performed in order to quantify the number of *Salmonella* organisms shed into the faeces of an animal following vaccination and challenge. Prior to collection of faecal samples, sterile tubes were weighed and labelled. Faeces were collected per rectum from each animal using a clean disposable latex glove. Approximately 2 to 5 g of faeces were deposited into the appropriately labelled tube and placed on ice until processing.

##### ***i. Sample processing***

Each tube was re-weighed to determine actual weight of sample collected then diluted approximately 1:4 with sterile, chilled PBS. The weight of the faeces within the tube

and volume of PBS added to the tube were recorded onto a data collection sheet (Appendix VI: Quantitative/Qualitative Faecal Culture Worksheet). A dilution factor for each sample was calculated using these values and used to determine the CFU/g faeces. Samples were homogenised with an electronic homogeniser until a smooth emulsion was formed, and then placed on ice to await further processing.

Table 3.9: Dilution Scheme for Homogenised Faecal and Tissue Samples

Tube ID	Description	Dilution in Tube	Plating Factor
1	Homogenised Sample (#1)	TBD*	10 & 100*
2	1:10 of #1	10*	1 x 10 <sup>3</sup> *
3	1:10 of #2	100*	1 x 10 <sup>4</sup> *
4	1:10 of #3	1 x 10 <sup>3</sup> *	1 x 10 <sup>5</sup> *
5	1:10 of #4	1 x 10 <sup>4</sup> *	1 x 10 <sup>6</sup> *
6	1:10 of #5	1 x 10 <sup>5</sup> *	1 x 10 <sup>7</sup> *

\* Final dilution and plating factors were based on the initial dilution calculated for Homogenised sample (Tube #1) based on sample weight and volume of PBS added.

#### ii. *Direct quantification plating*

Following homogenisation, the faecal samples were serially diluted into PBS following procedures outlined in Table 3.9. One hundred µL of the homogenised sample was applied onto an XLD plate (spin plate) and 10 µL of the homogenised sample and each dilution of the homogenate were applied onto an appropriate section of XLD plate (dot plate). For the quantification of the vaccine, XLD-CHL plates were used to select for the vaccine strain. When quantification of the challenge agents was performed, XLD plates containing either ceftiofur sodium (XLD-XNL) or neomycin sulphate (XLD-NEO) were used.

Spin and dot plates were incubated aerobically at 37°C for 24-48 h and black, H<sub>2</sub>S producing, CFU were counted (see Figure 3.2). The number of *Salmonella* organisms present in the original faecal sample in CFU per gram (CFU/g) was calculated based

on the sample weight, dilution factor and number of CFU counted. The detection limit of this quantification method was approximately 40 CFU/g of faeces.

### ***iii. Selective enrichment quantification plating***

One millilitre of the homogenised faeces was inoculated into 9 mL of enrichment broth to give a 1:10 dilution. The inoculated broth was incubated aerobically for 24 h at 37°C, and then subcultured onto XLD with appropriate antibiotics. Plates were incubated aerobically for 24-48 h at 37°C. Presence of black, H<sub>2</sub>S producing, CFU indicated the presence of *Salmonella* and were recorded as a positive sample. When the concentration of *Salmonella* was below the detection limit of the direct quantification plating technique and positive on selective enrichment, samples were assigned a value of 10 CFU/g. Methods for selective enrichment were adapted from techniques reported by Dueger *et al.* (2003b) and Fecteau *et al.* (2003).

Samples that were culture negative on selective enrichment were assigned a value of 4 CFU/g which was determined to be the detection limit of selective enrichment method based on dilution factor.

### ***3.6.3. Quantification of Salmonella in tissues***

The quantification of *Salmonella* in tissues was performed at the end of the challenge phase at necropsy. Quantitative tissue culturing was performed in order to determine the number of *Salmonella* organisms that had colonised in the tissues of an animal following vaccination or challenge. Prior to collection of tissue samples, sterile tubes were weighed and labelled with the animal identification number and tissue type. Tissues were collected using aseptic technique during necropsy. Approximately 2 to 5 g of tissue were collected from each target organ and deposited into the appropriately labelled sterile tube and placed onto ice until processing. Each tube was re-weighed to determine actual weight of sample collected, and then diluted approximately 1:4 with sterile, chilled PBS. Bile samples were processed undiluted. The weight of the tissue within the tube and volume of PBS added to the tube were recorded onto a data

collection sheet (Appendix VII: Individual Animal Necropsy Quantitative Culture Worksheet). Tissue samples were processed following procedures outlined in *Section 3.6.2.*

#### ***3.6.4. Confirmation of isolate identity***

Identification and serotyping was initiated on at least two colonies from each *Salmonella* positive culture following procedures outlined in *Sections 3.3.3. to 3.3.5.* All results for isolate identification, serotyping and biochemical tests were recorded on the *Salmonella Identification Testing Results Worksheet* (Appendix VIII).

### **3.7 Statistical Programs**

Statistical software programs used to analyse the data collected during the conduct of this research were Genstat, 12<sup>th</sup> Edition, VSN International, UK; StatsDirect statistical software®, Version 2.4.1., StatsDirect Limited, England (Buchan, 2000); and SPSS for Windows (SPSS Inc., Chicago, Illinois)

Continuous data were analysed using residual (or restricted) maximum likelihood (REML) analysis (Genstat, 12<sup>th</sup> Edition, VSN International, UK). Following analysis, data were presented as predicted model based means. Predicted means were obtained from the fitted model rather than the raw sample means.

StatsDirect statistical software®, Version 2.4.1., StatsDirect Limited, England (Buchan, 2000) was used to analyse repeated measures of non-parametric clinical data using the Wei-Lachin test (Wei and Lachin, 1984; Davis, 1991), Mann Whitney U, Students T test, analysis of variance (ANOVA) and Chi squared methods of analysis.

For all statistical analyses, a significance level (*P*) of less than 0.05 was used to reject the null hypothesis. Data were presented in figures as group predicted means  $\pm$  the standard error of the mean (SE).

Figure 3-1. Faecal Scoring System for Sheep






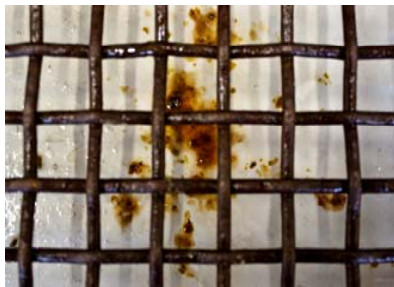
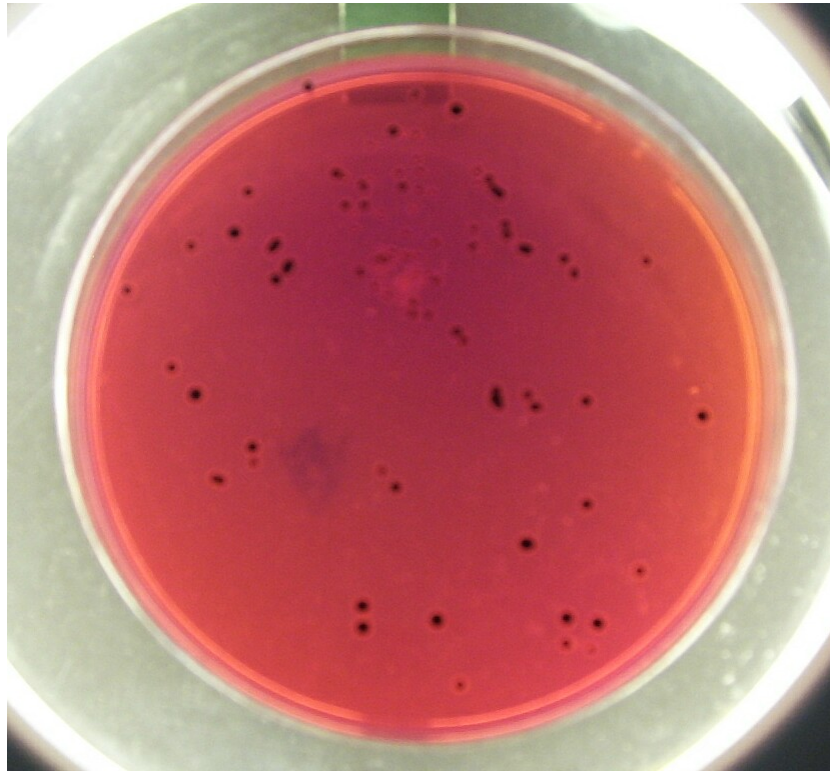
Score	Faecal Characteristics		Score	Faecal Characteristics	
0	Normal pellets		1	Soft (clumped pellets)	
2	Very Soft (dog poo)		3	Pile (cow pat)	
4	Diarrhoea (watery, casts)		4	Diarrhoea (watery, blood)	

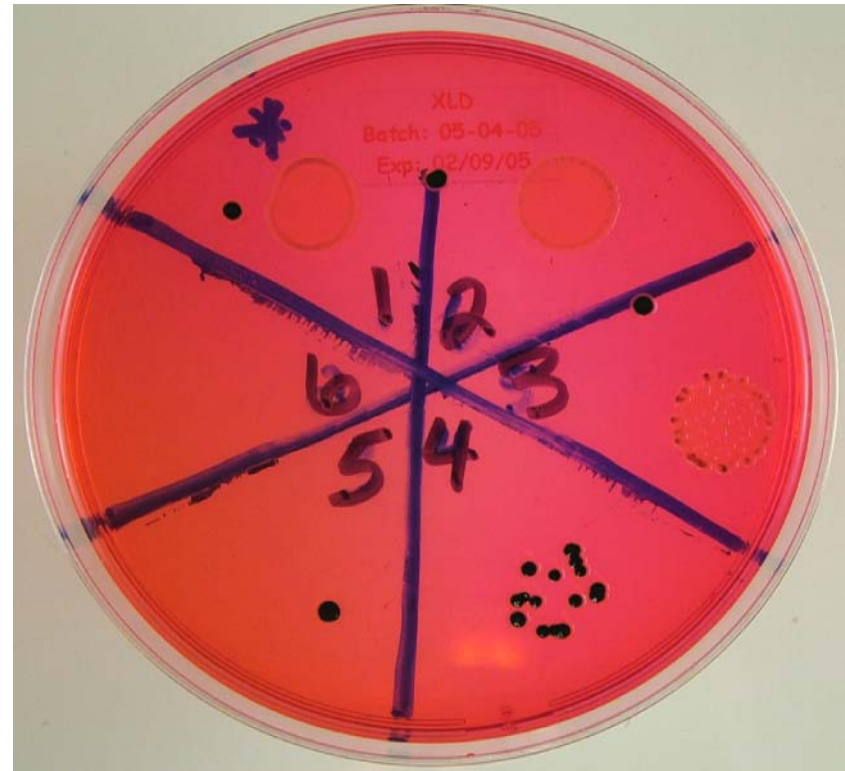


Figure 3-2. Direct Quantification Plating Technique for *Salmonella*

Spin Plates



Dot Plate



## **CHAPTER 4. Dose Range Finding Study of *Salmonella enterica* subspecies *enterica* serovar Newport in Six-Week-Old Calves**

### **4.1 Introduction**

According to published reports of *Salmonella* challenge studies in calves, between  $10^9$  and  $10^{10}$  organisms are required to induce salmonellosis in six-week-old calves (Wray and Sojka, 1978; Jones, *et al.*, 1991; Deignan, *et al.*, 2000; Fecteau, *et al.*, 2003; Dueger, *et al.*, 2003b; Mohler, *et al.*, 2006). However, the most common problems encountered when working with a new challenge organism is lack of virulence and variable virulence amongst strains. This challenge dose trial was conducted in order to characterise the response of six to seven week-old calves to challenge with a multi-drug resistant *Salmonella* Newport 03-721 (SN 03-721).

Outcomes commonly used to evaluate vaccine efficacy in models of salmonellosis include attenuation of clinical signs, reductions in *Salmonella* faecal shedding and mortality. To demonstrate a beneficial response to vaccination it is important that calves in the non-vaccinated group shed the challenge strain in faeces, display clinical signs commonly associated with neonatal salmonellosis, and for a proportion to succumb to infection. While all of these outcomes could be induced by selecting a lethal dose, an excessively high challenge dose is likely to mask the beneficial effects of the vaccination and rapidly reduce sample size and ability to measure outcomes. Another important consideration is the consideration of animal ethics committees, which do not look favourably on mortality models. Therefore, conducting challenge dose experiments to select the minimum dose required to achieve the desired model outcomes while minimising mortality and thus preserving statistical power, are critical to establishing vaccine efficacy.

### **4.2 Experimental Design**

#### ***4.2.1. In vivo passage of S. Newport 03-721 (SN 03-721)***

*In vivo* passage of *S. Newport* 03-721 (SN 03-721) through the target species was conducted on the isolate. Passage of the organism was considered important for induction of virulence factors that might not be expressed during extended storage and ensured that the selected organism was still capable of tissue invasion. Additionally, expression of virulence by the isolate and the ability of the isolate to establish infection in the target species could be verified prior to initiation of large scale dose range and vaccine efficacy evaluation trials.

The challenge SN 03-721, described in *Section 3.2.2.i*, was passaged through a four-day-old Holstein-Friesian bull calf. The calf received  $1.5 \times 10^{11}$  CFU of the challenge candidate prepared following procedures outlined in Appendix I, in 500 mL of LB administered PO on day 1 of the study period. After receiving the challenge, clinical assessments were performed on the calf every 6 h following procedures outlined in *Section 3.4.8*.

When the calf demonstrated signs of salmonellosis (lethargy, pyrexia, diarrhoea, depressed mentation, inappetance) it was humanely euthanised and necropsied. At necropsy, tissue from the liver, spleen and lung were harvested using aseptic technique for culture and re-isolation of SN 03-721 following procedures outlined in *Section 3.6* with media containing 8 µg/mL ceftiofur sodium. The tissue derived isolate was used as the challenge agent in the subsequent dose range and vaccine efficacy trials.

#### ***4.2.2. Dose range trial of SN 03-721-SP in calves***

##### ***i. Calf selection***

Twelve calves meeting the study selection criteria described in *Section 3.4.3* were transported to EMAI via commercial animal transport. On arrival, calves were placed into the PC2/QC2 Medium Security Animal House, Block 14.5, at EMAI and housed following procedures outlined in *Section 3.4.6*.

Calves were acclimatised for six weeks prior to initiation of the dose range study such that the calves would be of similar age and have experienced similar husbandry conditions as calves that were to be included in the subsequent vaccine efficacy challenge trials. Clinical assessment of the calves was performed twice a day during the acclimation period and rectal temperatures were recorded bi-weekly to ensure that calves were healthy following procedures outlined in *Section 3.4.8*. A normal range for RT was established for the calves, based on mean RT  $\pm$  two standard deviations as 37.9 – 39.4°C. Rectal temperature was recorded daily following virulent *Salmonella* challenge. Faecal swabs were collected for qualitative faecal cultures on all calves and then weekly through the acclimation period following procedures outlined in *Section 3.6.1*. *Salmonella* Dublin was isolated from the faeces of two calves on arrival to the isolation facility. These two calves were culture negative for the subsequent five qualitative cultures and no abnormalities or signs of clinical disease were detected during the acclimation period and enrolled in the study. Another calf was removed from study during the acclimation period after becoming entrapped in a pen gate and suffering a fractured limb.

At the end of the six week acclimation period (day 0), calves were randomly placed into two groups, low dose (n = 6) and high dose (n = 5). The average serum proteins [low dose 5.69 g/dL and high dose 5.81 g/dL ( $P = 0.09$ )], and age distribution [low dose 43.2 d and high dose 44.7 d ( $P = 0.12$ )], were similar between groups. The target dose level of the SN 03-721-SP challenge agent for the low and high dose groups was  $5 \times 10^9$  and  $5 \times 10^{10}$  CFU/calf respectively. The SN 03-721-SP challenge agent was administered PO in 20 mL of PBS. Following challenge, calves were clinically assessed following procedures outlined in *Section 3.4.8*. Qualitative and quantitative faecal culturing was conducted 3, 7, 10, and 15 d post-challenge, following procedures outlined in *Section 3.6.2*.

### *ii. Necropsy and tissue collection*

On day 15 post-challenge, surviving calves were humanly euthanised and necropsied. Approximately 2 to 5 g of tissue was obtained from the liver, lung, spleen, and mesenteric lymph node (MLN) of each calf to evaluate tissue colonisation of the challenge agent, as well as approximately 5 mL of bile from the gall bladder and contents from the lumen of the caecum and jejunum. Samples were processed following procedures outlined in *Section 3.6.3*. All gross pathological lesions were recorded on a necropsy record.

### *iii. Statistical analysis*

Continuous data were analysed using REML analysis (Genstat, 12<sup>th</sup> Edition, VSN International, UK). A single variate, repeated measures model was fitted for the factors of time and treatment for variables CFU, temperatures, chaff and pellet refusal, and water intake. Following analysis, data are presented as predicted model based means. Predicted means are those obtained from the fitted model rather than the raw sample means. This is an important distinction, as predicted means represent means adjusted to a common set of variables, thus allowing valid comparison between means. A *P* value less than 0.05 was considered to be statistically significant. Differences between the individual means were determined by calculating an approximate least significant difference (LSD). A difference of means that exceeded the calculated LSD was considered significant.

Repeated measures of non-parametric clinical data, faecal composition and mentation, were analysed using the Wei-Lachin test (Wei and Lachin, 1984; Davis, 1991) using StatsDirect statistical software, <http://www.statsdirect.com>, 2002, CamCode, England (Buchan, 2000). Wei Lachin analysis of the data performed univariate group comparisons for individual time points and a multivariate comparison over the period analysed. Data are presented as the percentage of calves that displayed an abnormal attitude (cumulative daily score >

3) or had an abnormal faecal score (cumulative daily score > 3) over the course of the challenge period. Mann-Whitney U test was used to analyse non parametric tissue colonisation data that failed to meet conditions of normality with the Kolmogorov-Smirnov test. For all statistical analyses, a significance level (*P*) of less than 0.05 was used to reject the null hypothesis. Data is presented in figures as the group mean  $\pm$  SE.

### **4.3 Results**

#### ***4.3.1. In vivo passage of SN 03-721***

Twenty-four hours following the administration of  $1.5 \times 10^{11}$  CFU of SN 03-721 PO, the calf began to scour. By 60 h post-challenge the calf was moribund, anorexic, unable to maintain sternal recumbency and had a watery diarrhoea containing blood. The calf was euthanised following procedures outlined in *Section 3.4.11*.

SN 03-721 was isolated from the liver, lung and spleen of the calf upon direct culture on to XLD-XNL. The isolates recovered from the liver, lung and spleen of the calf retained their antimicrobial susceptibility patterns. The isolate collected from the spleen was selected as the challenge strain for use in future trials and assigned the identifier of *S. Newport 03-721-SP* (SN 03-721-SP). This isolate was cryopreserved and maintained as a frozen culture bank following procedures outlined in *Section 3.3.1*.

#### ***4.3.2. Effect of SN 03-721-SP challenge on calves.***

The actual dose of the SN 03-721-SP orally administered to calves was  $3 \times 10^9$  and  $3 \times 10^{10}$  CFU/calf in the low and high dose groups, respectively. While these levels were 60 % of the target, the doses were sufficient to produced clinical disease in all calves and two mortalities were observed in the high dose group. One calf died on day 3 post-challenge and the other calf was euthanised after

meeting euthanasia criteria on day 10 post-challenge.

Significant differences in the mean RT of calves in the high and low dose groups were observed on days 1 through 3 and day 11 post-challenge ( $P < 0.05$ ) and over the 14 d observation period ( $P < 0.001$ ). The mean RT in both the low and high dose groups were above the normal range 48 h post-challenge (Figure 4-1). The mean RT of the low and high dose groups peaked on days 3 and 2 post-challenge respectively, and returned to the normal range by day 6 post-challenge in both groups.

Significant differences in the mentation scores were observed in the high and low dose groups from day 7 to 14 post-challenge ( $P < 0.05$ ) and over the 14 d observation period ( $P < 0.005$ ) (Figure 4-2). The incidence of abnormal mentation was significantly higher in the high dose group when compared to the low dose group and abnormal mentation was observed in the high dose group 24 h post-challenge and mentation scores remained elevated until the end of the challenge period.

Diarrhoea was observed in the calves 24 h post-challenge with peak incidence occurring on study day 4 and 2 in the low and high dose groups, respectively. There were significant differences in the incidence of diarrhoea in the low and high dose groups on days 1 and 2 post-challenge ( $P < 0.05$ ) (Figure 4-3). The incidence of diarrhoea was increased in the high dose group when compared to the low dose group over the 14 d observation period ( $P < 0.001$ ).

Faecal shedding of SN 03-721-SP was measured on days 3, 7, 10 and 15 post-challenge (Figure 4-4). The high dose group shed significantly higher numbers of the challenge strain when compared to the low dose group ( $P < 0.05$ ). On day 15 post-challenge, the challenge strain was recovered from 2 of 6 and 2 of 3 calves, from the low and high doses groups respectively.

Calves demonstrated reduced appetites following challenge with SN 03-721-SP (Figure 4-5). Onset of appetite reduction was 48 h post-challenge in both dose groups. The high dose group consumed significantly less milk replacer when compared to the low dose group on days 2, 6, 7, and 8 post-challenge ( $P < 0.05$ ) and over the 14 d observation period ( $P < 0.001$ ). The appetites of the calves in the low dose group returned to pre-challenge consumption levels by study day 6, while the appetites of the surviving high dose calves remained depressed until the end of the 14 d observation period.

#### ***4.3.3. SN 03-721-SP colonisation of tissue in calves***

Tissues were collected from six low dose calves and three surviving high dose calves. Gross pathological findings included enlarged mesenteric lymph nodes, ulcerated bile ducts, fibrin deposits in the small intestine, caecum and abomasum, ulcerations of the pylorus, mucosal erosions of the abomasum, small intestine and caecum, as well as serosal petechial haemorrhages (Figure 4.6). Additionally, trichobezoars, 5 to 12 cm in diameter, were observed in the abomasum and small intestines of the calves from both dose groups. The challenge organism was not isolated from the bile of calves in the low dose group using both direct and indirect selective enrichment culture techniques. There were no differences in tissue colonisation between the low and the high dose groups (Figure 4-7) and SN 03-721-SP was recovered from at least one tissue in all calves.

#### **4.4 Discussion**

The data from the dose range finding study of the passaged SN 03-721-SP demonstrated that the isolate was capable of inducing clinical disease in six week old Holstein-Friesian bull calves following oral challenge. All calves in the low and high dose challenge groups developed clinical signs of salmonellosis which included fever, diarrhoea, decreased appetite and depressed mentation during the 14 d post-challenge observation period. The clinical signs were biologically



significant in both groups with dose dependent changes in time to onset of and severity of changes in mentation, appetite, faecal composition, and rectal temperature.

The aims of this study were to confirm virulence of the SN 03-721-SP challenge agent and to determine the dose that would induce clinical disease with low mortality in six-week-old calves. Dose range finding studies often incorporate several doses of the challenge agent at half log intervals which allows for statistical estimation of the lethal dose (LD<sub>90-100</sub>). One of the difficulties of developing a good *Salmonella* challenge model for evaluation of vaccines or antimicrobial efficacy studies is the identification of a suitable challenge strain. Occasionally, potential challenge strains prove to be avirulent in the test subjects. This issue was discussed with the EMAI AEC and it was decided that two dose groups would be utilised using doses that would be expected to cause disease based on doses published in the literature (Rankin and Taylor, 1966; Nazer and Osborne, 1977; Jones, *et al.*, 1988; Mukkur, *et al.*, 1991a; House, *et al.*, 2001a; Fecteau, *et al.*, 2003; Dueger, *et al.*, 2003b; Mohler, *et al.*, 2006). The logic of this decision was to minimise the number of calves that were challenged and to avoid the scenario of including larger numbers of calves, only to find that the strain was avirulent. The low dose ( $3 \times 10^9$  CFU) of the challenge agent in this study produced disease in 100% of the calves with no mortality and a rapid recovery, while the high dose ( $3 \times 10^{10}$  CFU) produced disease in 100% of the calves with a 40% mortality rate. The two dose levels verified that the challenge agent was virulent in 6 week-old calves and that the optimal oral dose was greater than  $3 \times 10^9$  CFU but less than  $3 \times 10^{10}$  CFU per calf.

Necropsy findings demonstrated that gross pathology in the lymph nodes, gall bladder and intestines occurred after the administration of the challenge agent. However, the lungs, liver and spleen were grossly normal despite colonisation

with the challenge agent. *Salmonella* infections have been associated with colonisation of the lymph nodes, lung, liver, spleen, gall bladder and salivary glands in cattle (Wray and Davies, 2000; Fecteau, *et al.*, 2003; Dueger, *et al.*, 2003b). In previous research, reduced tissue colonisation in calves challenged with *S. Dublin* was shown to correlate with resolution of clinical signs (Mohler, *et al.*, 2006). Results of this trial demonstrate that the minimum oral challenge dose of SN 03-721-SP required to produced clinical disease and colonisation of tissues in calves with minimal mortalities is  $10^{10}$  CFU/calf over an observation period of 14 d. This dose was targeted in future *Salmonella* vaccine efficacy trials.

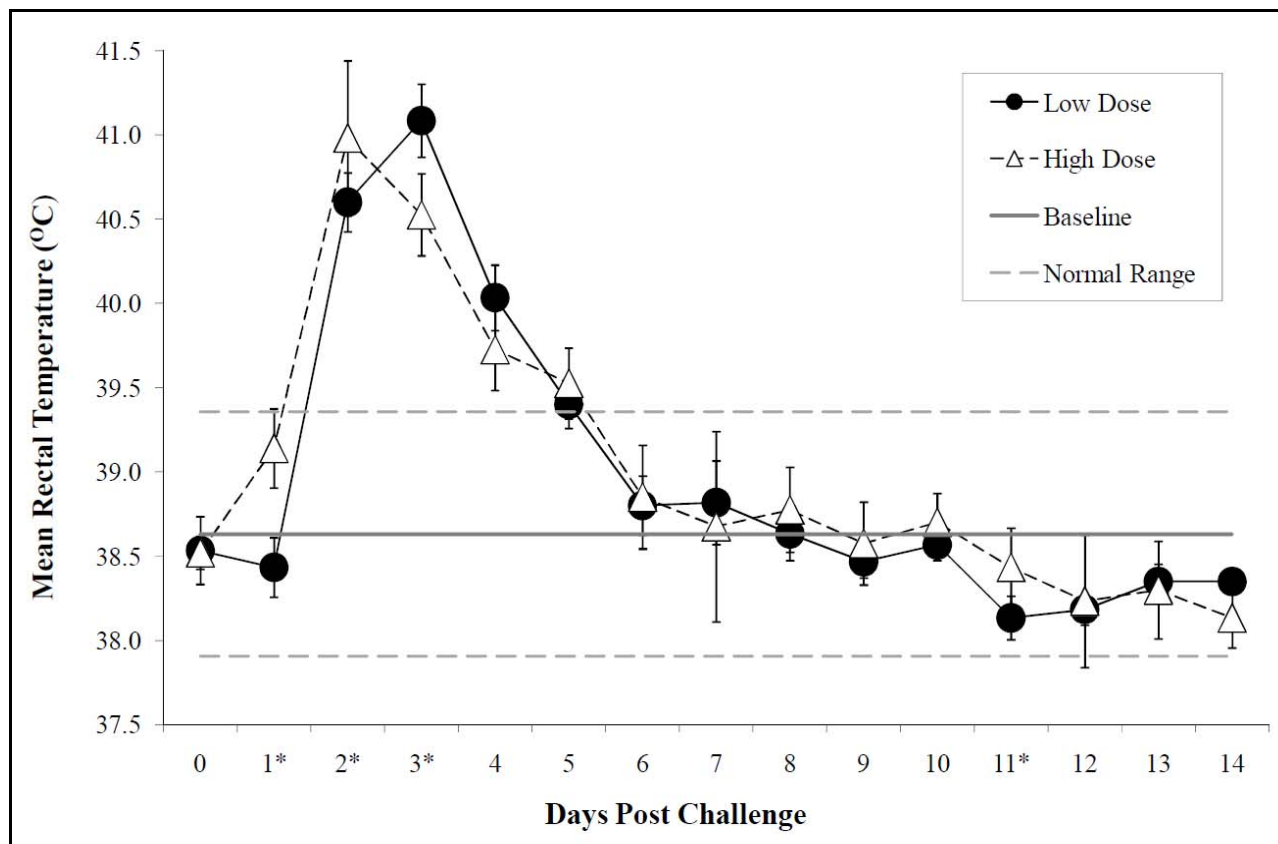


Figure 4-1. Mean rectal temperatures of calves challenged with virulent SN 03-0721-SP

RT were measured daily for 14 d following challenge. Data are depicted as mean RT  $\pm$  SE. The data are presented in the context of the normal range (dashed grey lines) and baseline (solid grey line) established pre-challenge. \*RT in the low dose group were significantly lower on days 1, 2 and 11 post-challenge and significantly higher on day 3 post-challenge when compared to the high dose group ( $P < 0.05$ ).

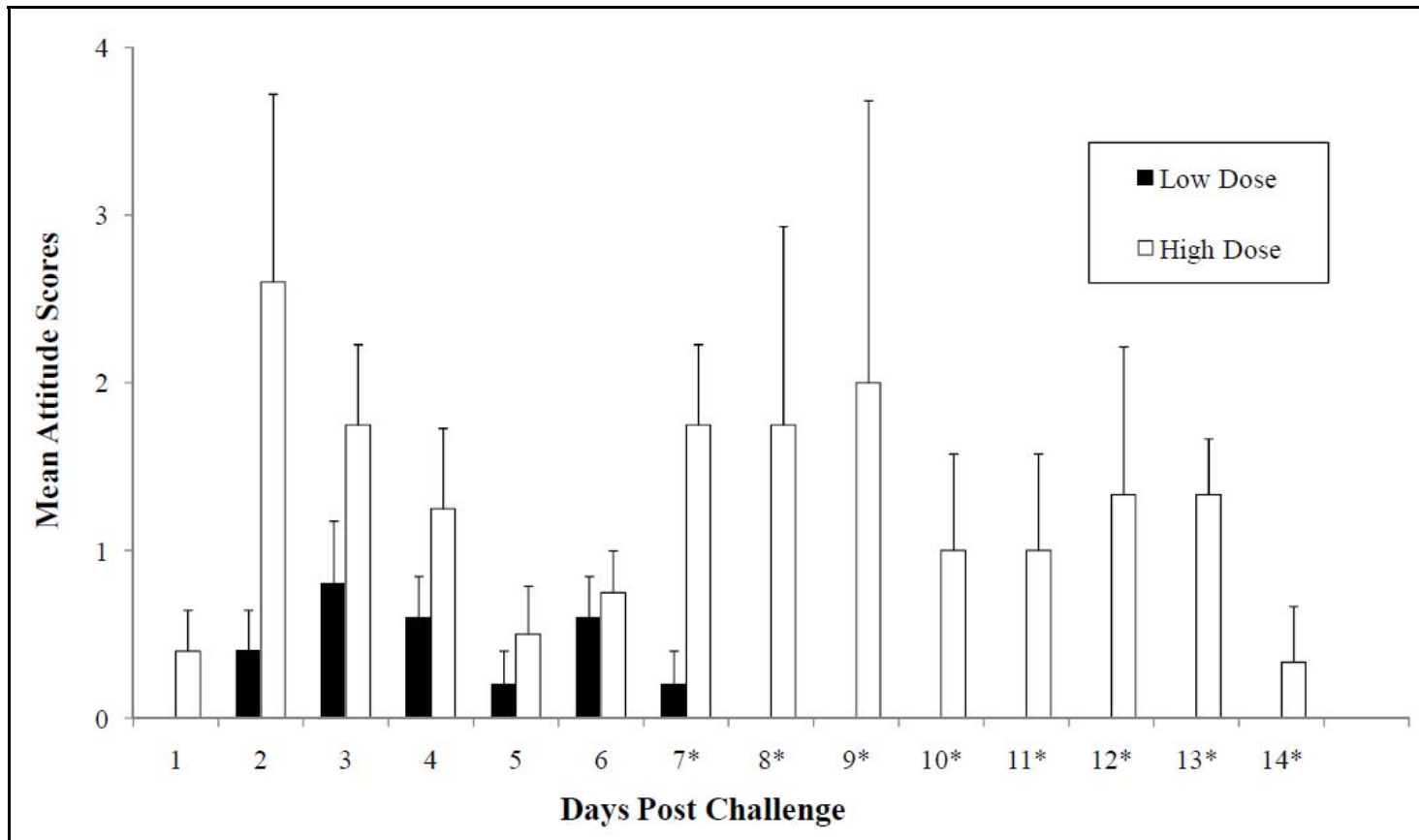


Figure 4-2. Mentation scores of calves challenged with virulent SN 03-0721-SP

Attitudes of the calves were scored twice a day. Data are presented as mean daily mentation score post virulent challenge  $\pm$  SE. \*The low dose group had significantly lower attitude scores when compared to the high dose group from day 7 to 14 post-virulent challenge ( $P < 0.05$ ).

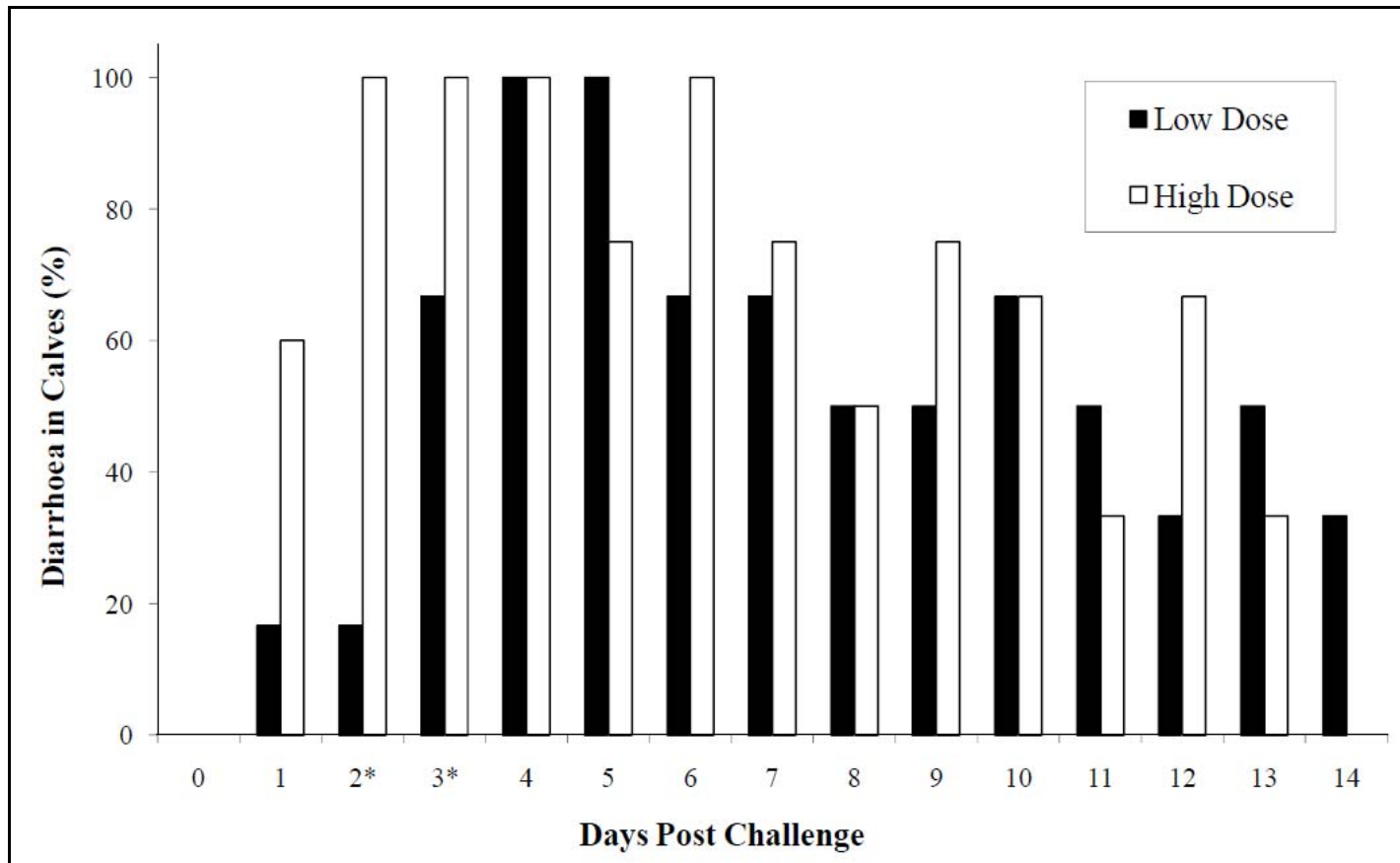


Figure 4-3. Incidence of diarrhoea in calves challenged with virulent SN 03-0721-SP

Faecal composition was assessed twice a day. Data are presented as the percentage of calves with diarrhoea following virulent challenge. \*Significantly lower incidence in diarrhoea was observed in the low dose group on days 2 and 3 post-challenge when compared to the high dose group ( $P < 0.05$ ).

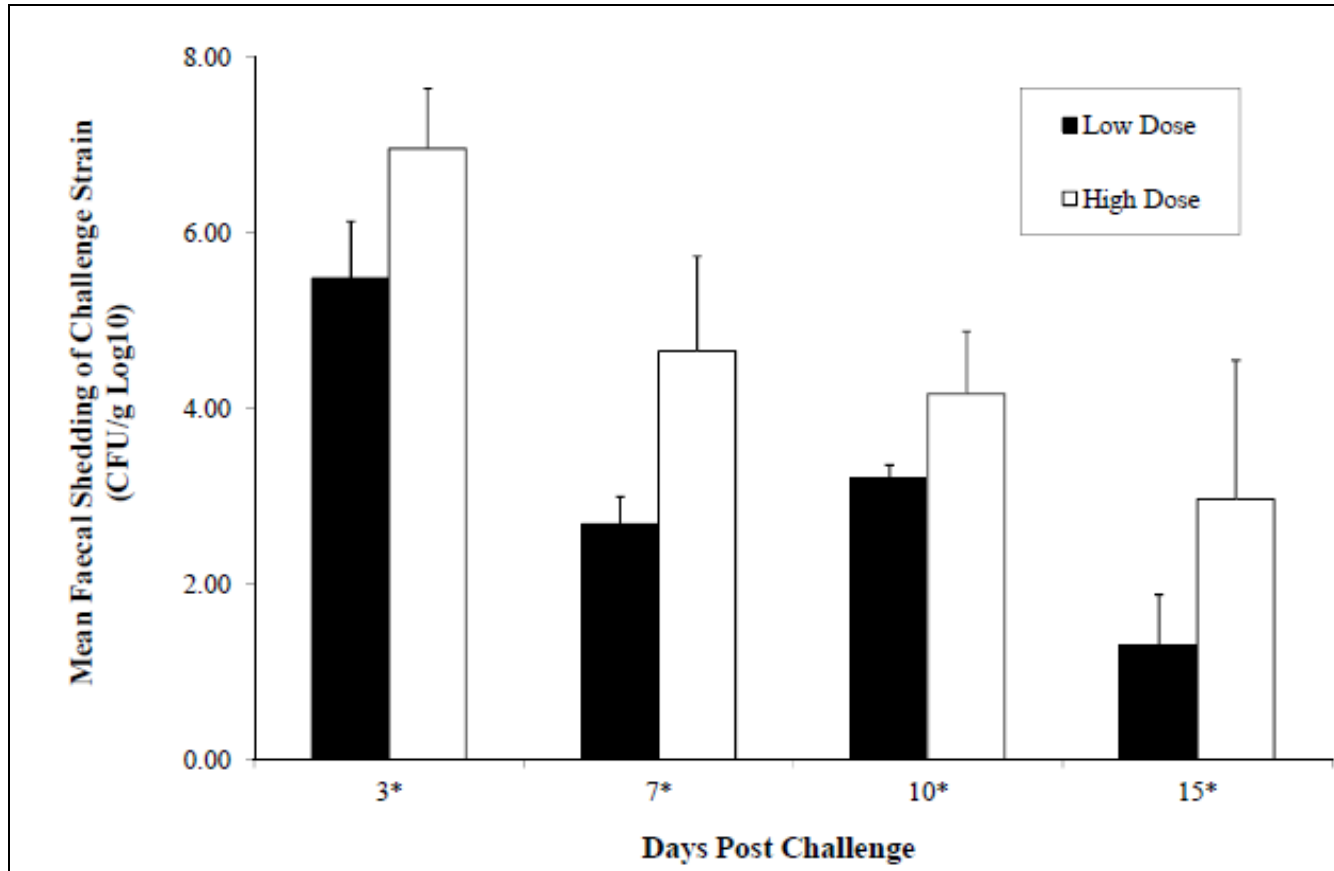


Figure 4-4. Mean faecal shedding of virulent SN 03-721-SP in calves following challenge.

Data are depicted as mean log<sub>10</sub> CFU of SN 03-721-SP per g of faeces post-challenge ± SE. \*Significant differences in faecal shedding were observed on days 3, 7, 10 and 14 post-challenge ( $P < 0.05$ ).

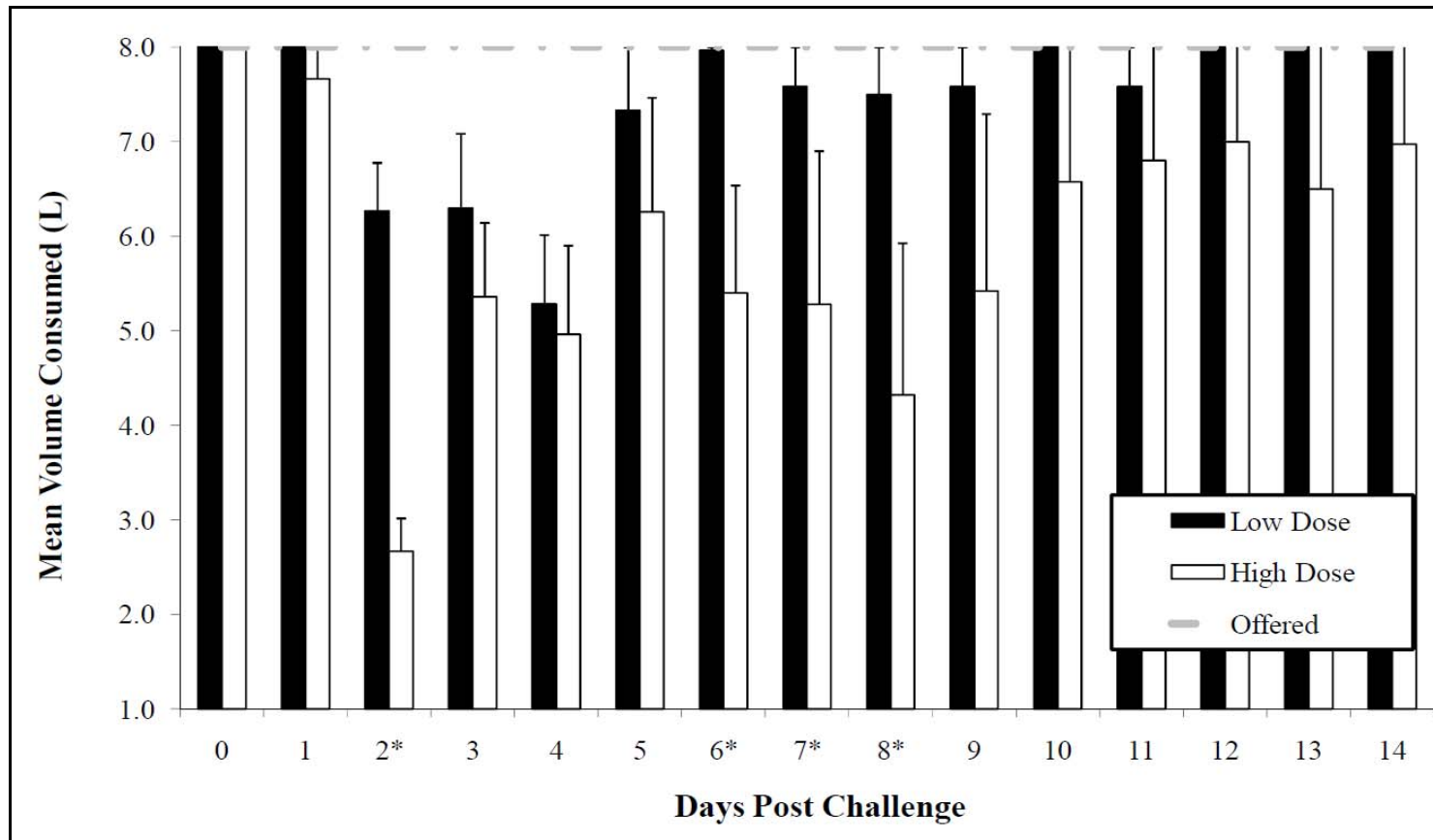


Figure 4-5. Mean appetite of calves following challenge with virulent SN 03-0721-SP

Mean milk replacer consumption in L is depicted  $\pm$  SE. Each calf was offered 8 L of milk replacer a day. \*Milk replacer consumption was significantly lower in the high dose group when compared to the low dose group on days 2, 6, 7, and 8 post-challenge ( $P < 0.05$ ).

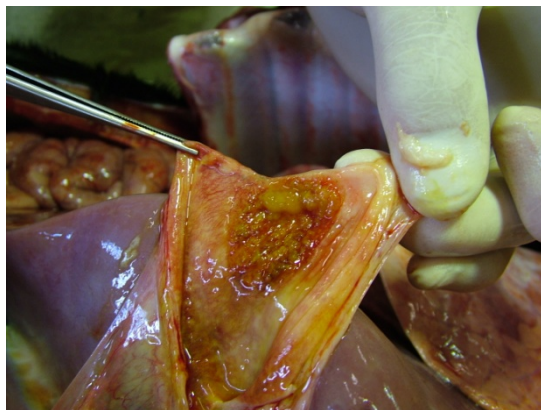
Figure 4-6. Necropsy findings of calves challenged with SN 03-721-SP



(A) Enlarged mesenteric lymph node with multiple irregular pink to red areas on the serosa suggesting serosal haemorrhage



(B) Fibrin deposits in the small intestine



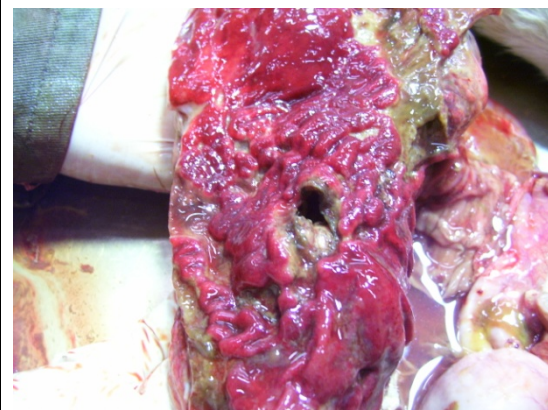
(C) Ulcerated gall bladder



(D) Ulceration of the pylorus



(E) Trichobezoars removed from the abomasum



(F) Abomasum with severe subacute active ulcerative abomasitis with perforation



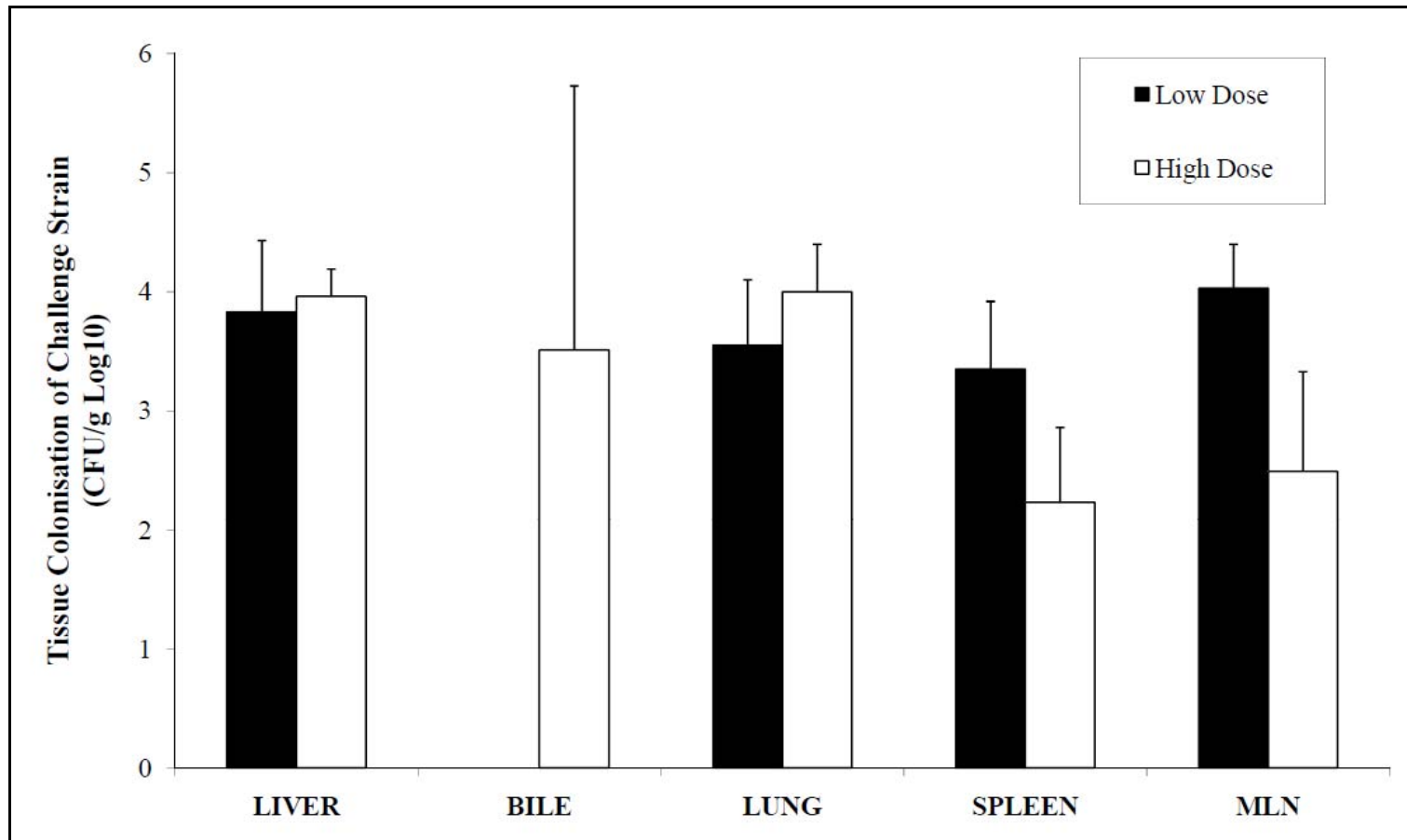


Figure 4-7. Colonisation of tissues in calves with SN 03-721-SP following challenge.

Tissue colonisation data are depicted as mean log<sub>10</sub> CFU ± SE of virulent SN 03-721-SP, recovered by organ.

## **CHAPTER 5. Cross-protective Immunity conferred by a DNA Adenine Methylase Deficient *Salmonella enterica* serovar Typhimurium Vaccine in Calves Challenged with *Salmonella* serovar Newport**

The following is a re-formatted published manuscript:

Mohler, V.L., Heithoff, D.M., Mahan, M.J., Walker, K.H., Hornitzky, M.A., Shum, L.W.C., Makin, K.J., House, J.K. (2008). Cross-protective Immunity conferred by a DNA adenine methylase deficient *Salmonella enterica* serovar Typhimurium vaccine in calves challenged with *Salmonella* serovar Newport. *Vaccine* 26: 1751-1758

The study was designed, executed and reported by the candidate under the general supervision of the remaining authors, who were the candidate's appointed supervisors (principal and associate) with the exception of Drs Kate J. Makin and Lucy W.C. Shum, who provided assistance with calf husbandry, necropsy procedures and laboratory assistance.

### **5.1 Introduction**

Salmonellosis in livestock usually reflects a variety of management and environmental events that contribute to compromised host immunity and increased pathogen exposure. Recent epidemiological studies of salmonellae on dairy farms have been able to identify risk factors that influence shedding of salmonellae. Those animals at highest risk include neonates and post-partum cows (Anderson, *et al.*, 2001; House, *et al.*, 2001a; Fossler, *et al.*, 2005a; Fossler, *et al.*, 2005b). Increased herd size is considered a principal risk factor influenced by housing type, rodent/pest control, ration storage facilities, isolation protocols for sick animals, maternity pen practices, and surface water management (Huston, *et al.*, 2002; Fossler, *et al.*, 2005a; Fossler, *et al.*, 2005b). Additionally, livestock manure management systems reduce, but do not eliminate, salmonellae, and the on-farm practice of recycling manure promotes faecal-oral pathogen transmission.

Although the implementation of sound nutritional and environmental programs that maintain and/ or promote broad-based host immunity contributes to livestock health and productivity, reliable implementation of these management programs is a formidable challenge, with numerous variables to consider such as weather, mechanical failures, variable feedstuff availability and quality, and labour compliance issues. Thus, although such epidemiological and management studies have enhanced our understanding of the risk factors resulting in salmonellae shedding, effective and reliable strategies for salmonellae control have not been established (Fossler, *et al.*, 2005b).

*Salmonellae* control strategies are further complicated by the fact that most infections of livestock are subclinical, as evidenced by the disparity among salmonellae isolates from surveillance and clinical submissions (Anderson, *et al.*, 2001). Additionally, some isolates are capable of asymptomatic colonisation and/ or persistence in a particular animal species while causing acute disease in another animal species (e.g., different types or classes of stock) (Counter and Gibson, 1980; Gray, *et al.*, 1995; Xercavins, *et al.*, 1997; Uzzau, *et al.*, 2000; Reen, *et al.*, 2005; Badie, *et al.*, 2007). Thus, the diversity of salmonellae present on farms and feedlots, and the potential for different serovars to possess an array of virulence attributes, necessitates the use of broad prophylactic strategies that are efficacious for many serovars simultaneously. An effective approach for a number of years has been the therapeutic and prophylactic administration of antibiotics to livestock, but this option has become limited due to the emergence of multi-drug resistant pathogenic strains that present a bona fide risk to human health (CDC, 2007).

Vaccination is one of the best forms of prophylaxis against the development of disease caused by infectious agents. Although vaccination is generally highly specific in the protection conferred in immunised hosts (protection is limited to a

specific strain or closely-related set of strains), recent advancements have resulted in the development of vaccines that elicit cross-protective immunity to multiple strains of the same species. Many of these vaccine candidates are *Salmonella* modified live vaccines that contain mutations in global regulatory networks (Hassan and Curtiss, 1994a; Heithoff, *et al.*, 2001; Nagy, *et al.*, 2004). The molecular basis of cross-protection for these regulatory-mutant based vaccines appears to depend on the over expression of a number of potential antigens that may be shared among heterologous serotypes; they are also sufficiently attenuated for their capacity to cause acute disease in immunised animals.

*Salmonella* Typhimurium containing a loss of function mutations in the gene encoding *dam* expression are avirulent, and confer significant protection to homologous and heterologous *Salmonella* challenge when used as modified live vaccines in murine (Garcia-Del Portillo, *et al.*, 1999; Heithoff, *et al.*, 1999; Heithoff, *et al.*, 2001) and avian models of typhoid fever (Dueger, *et al.*, 2001, 2003a), as well as in a bovine model of salmonellosis (Dueger, *et al.*, 2003b; Mohler, *et al.*, 2006). The objective of this study was to evaluate the cross-protective efficacy of a *S. Typhimurium dam* vaccine in calves challenged with a clinically relevant strain of *S. Newport*, which has recently emerged as a significant pathogen causing disease in cattle and humans (CDC, 2002; Clark, 2004; Devasia, *et al.*, 2005; Varma, *et al.*, 2006; You, *et al.*, 2006).

## **5.2 Materials and Methods**

### ***5.2.1. Bacterial strains and growth conditions***

Immunisations were carried out with *dam*-102::Mud-Cm Typhimurium UK-1 vaccine strain MT2313 (*S. Typhimurium dam*) as described in *Section 3.2.1*. Challenge studies were performed with a derivative of a serovar Newport strain, 03-721, a multi-drug antimicrobial resistant strain (SN 03-721-SP) as described in *Section 3.2.2.i*. Strains used in infection studies were grown overnight in LB at

37°C following procedures outline in Appendix I.

### **5.2.2. Calf selection and husbandry**

Holstein-Friesian bull calves used in the study originated from a single dairy in NSW, Australia. Calves were fed 2 L of colostrum from the dairy of origin at birth and again at 12 and 24 h of age. Passive transfer was assessed via measurement of serum protein using a handheld refractometer (American Optical Company, USA). Calves with a serum protein less than 5.0 g/dL at 48 h of age were excluded on the basis of failure of passive transfer. At 3 to 7 d of age, calves were transported to the Medium Security Animal Housing Facility at EMAI (Menangle, NSW, AU), randomly assigned to experimental groups and housed following procedures outlined in *Section 3.4.6*. Vaccinated and non-vaccinated calves were housed in separate isolation rooms. Biosafety protocols approved by OGTR for containment of PC2 level organisms were followed to prevent cross contamination between groups and prevent release of the vaccine into the environment.

Calves were bucket fed Palastart Blue® (Probiotec Pty. Ltd., AU), a 20% fat, 24% protein, non-medicated milk-replacer, twice daily at 15% of body weight as outlined in *Section 3.4.6* and given tap water ad libitum. All milk-replacer preparation equipment was dedicated to a single room and milk buckets to a single calf. Equipment was cleaned and disinfected after each feeding.

### **5.2.3. Clinical assessment**

Clinical parameters evaluated twice daily included attitude, appetite, and faecal characteristics following procedures outlined in *Section 3.4.8*. Rectal temperatures were recorded following the morning feeding for 3 d following each vaccination, once a week prior to and daily following virulent *Salmonella* challenge. Attitude and faecal composition were scored on an ordinal scale as

described in *Sections* 3.4.8.i and 3.4.8.iv respectively. Appetite was recorded to the nearest 0.1 L of milk-replacer consumed at each feeding. An attitude score of 4 at any time of the day, a failure to consume milk replacer, or an appetite score of 0 over two consecutive feedings, constituted grounds for euthanasia. Post-virulent challenge, calves showing signs of salmonellosis were evaluated every 6 h. Calves were weighed prior to vaccination, prior to virulent challenge, and day 15 post-challenge. Average daily weight gains were calculated for post-vaccination and post-challenge intervals. The study protocol was approved by the AEC at EMAI.

#### **5.2.4. Faecal sampling**

Sterile cotton-tipped applicators were used to collect faecal samples from all calves prior to enrolment and once a week for qualitative detection of *Salmonella*. Rectal swabs were placed into 10 mL of TRT and MSB broths, incubated for 24 h at 37°C and plated onto XLD and XLD plates containing chloramphenicol (20 µg/mL) (XLD-CHL), to select for the vaccine strain. Both plates were incubated for 24 h at 37°C. *Salmonella* suspect colonies were sub-cultured for pure growth following procedures outlined in *Sections* 3.3.3 to 3.3.5. *Salmonella* isolates were sent to the IMVS, *Salmonella* Reference Laboratory (South Australia, AU) for serotyping.

Faecal samples were assessed for shedding of the *S. Typhimurium dam* vaccine strain on day 2 following each vaccination and following procedures outlined in *Section* 3.6.2. The homogenised faecal material was plated onto XLD-CHL, which was selective for the vaccine strain. Following virulent challenge, faecal samples were assessed for shedding of the SN 03-721-SP on days 3, 7, 10, 13 and 15 post-challenge, using procedures outlined in *Section* 3.6.2. The homogenised faecal material was plated onto XLD plates containing ceftiofur sodium (8 µg/mL) (XLD-XNL) which was selective for the challenge strain.

The numbers of *Salmonella* organisms present in faecal samples were calculated based on the sample weight, dilution factor, and number of colonies counted. Two colonies from each *Salmonella* positive faecal culture were sub-cultured and tested with O-antigen specific antisera to verify isolate identification following procedures outlined in *Section 3.6.4*.

#### **5.2.5. *Salmonella* isolation from bovine tissues**

Calves were euthanised and immediately necropsied using standard techniques 15 d post virulent challenge. Approximately 1 to 2 g of tissue was obtained from the MLN, liver, lung and spleen, and 5 mL of bile was collected from the gall bladder of each calf. Samples were processed following procedures outlined in *Section 3.6.3*.

#### **5.2.6. *Statistical analysis***

Mean age, total plasma protein concentrations and body weight of vaccinated and non-vaccinated calves were compared using a student's T test. Clinical and quantitative *Salmonella* faecal culture data collected following SN 03-721-SP challenge reflected repeated measures of non-parametric data with uneven group sizes, and were analysed using the Wei-Lachin test (Wei and Lachin, 1984). Analysis of *Salmonella* faecal shedding was based on the results of quantitative *Salmonella* faecal cultures performed on study days 3, 7, 10, 13, and 15. Wei-Lachlin analysis of the clinical data performs univariate group comparisons for each time point (d post-challenge) and a multivariate comparison over the post-challenge period. The statistical program utilised to perform student's T test, Mann-Whitney U, and Wei Lachlin test was StatsDirect statistical software (Buchan, 2000)(<http://www.statsdirect.com>, 2002, CamCode, England).

### **5.3 Results**

#### **5.3.1. *Challenge dose trial***

A challenge dose trial was conducted in order to determine an optimum dose that

would induce disease while minimising mortality in order to maintain sample size and statistical power for measured outcomes. Similar to previous calf-challenge studies (Fecteau, *et al.*, 2003; Dueger, *et al.*, 2003b; Mohler, *et al.*, 2006), seven-week old calves were orally administered *S. Newport* challenge strain, 03-0721-SP, at  $3 \times 10^9$  (n = 6) and  $3 \times 10^{10}$  organisms (n = 5) for low and high dose, respectively. All of the calves in the low- and high- dose challenge groups developed clinical signs of salmonellosis (fever, diarrhoea, decreased appetite and depressed attitude) during the 14 d post-challenge observation period. The severity of these signs was greater in calves challenged at the high dose. There were significant differences in the severity of these outcomes ( $P < 0.05$ ). One of the five calves administered the high dose died on day 3, and another was euthanised on day 10 post-challenge after meeting euthanasia criteria. Therefore, to reduce mortality and maintain sample size, a challenge dose of  $1 \times 10^{10}$  was targeted for the vaccine efficacy trial.

### ***5.3.2. Cross-protective efficacy of *S. Typhimurium dam* vaccine strain against virulent Newport challenge in calves***

More than 2500 serovars of *S. enterica* have been identified and classified typically by serotyping, based on antigenic variation in the lipopolysaccharide (O-antigen) and phase 1 (H1) and phase 2 (H2) flagella on the surface of the organism (Ewing, 1986; Le Minor and Bockemuhl, 1989; Popoff, *et al.*, 2004). These O- and H- antigens not only allow the classification of *Salmonella* into distinct serogroups (B, C<sub>1</sub>, C<sub>2</sub>-C<sub>3</sub>, D<sub>1</sub>, etc), they also constitute immunodominant antigens that contribute to virulence and protective immunity in infected animals (reviewed in (Dorsey, *et al.*, 2005; Salazar-Gonzalez and McSorley, 2005)). Immunisation of calves with a *S. Typhimurium dam* vaccine strain (serogroup B) has been shown to elicit cross-protective immunity to serovar Dublin (serogroup D<sub>1</sub>) as evidenced by a significant reduction in clinical disease and reduced



colonisation of bovine tissues (Mohler, *et al.*, 2006). Here we questioned whether vaccination of calves with *S. Typhimurium dam* can confer cross-protection against an emerging pathogen of livestock, *S. Newport*, strain SN 03-0721-SP. This strain is classified in the C<sub>2</sub>-C<sub>3</sub> serogroup and has been associated with clinical disease in recent salmonellosis outbreaks in commercial dairy farms in California, USA, and with multi-drug resistance against a variety of clinically relevant antibiotics (ampicillin, ceftiofur, chloramphenicol, clindamycin, oxytetracycline, spectinomycin, sulphonamides, tiamulin, trimethoprim/sulphamethoxazole, tilmicosin, tylosin). Moreover, *S. Newport* expresses significantly different O- and H- antigens (O<sub>6,8,20</sub>: e,h; 1,2:[Z67]) from that expressed by the *S. Typhimurium dam* vaccine strain (O<sub>1,4,[7],12</sub>: i; 1,2) or that expressed by *S. Dublin* (O<sub>1,9,12</sub>[Vi]: g,p monophasic (Popoff and Le Minor, 2005).

Thirty calves were randomly assigned to two dose groups of 15 calves each. The average serum proteins (non-vaccinates 5.65 g/dL and vaccinates 5.62 g/dL [ $P = 0.861$ ]), age distribution (non-vaccinates 13.40 d and vaccinates 13.53 d [ $P = 0.936$ ]), and body weights (non-vaccinates 46.3 kg and vaccinates 44.8 kg [ $P = 0.348$ ]) were similar between groups. Calves in the vaccinate group were orally vaccinated twice with *S. Typhimurium dam* ( $5 \times 10^7$  CFU) - once at 7 to 21 d of age and again 7 d following the initial vaccination. All calves were orally challenged with  $2 \times 10^{10}$  CFU (actual dose) of virulent *S. Newport* strain SN 03-721-SP four weeks following the second vaccination.

Vaccinated calves exhibited significantly lower mean rectal temperatures compared to non-vaccinates on days 5 and 6 post-challenge ( $P < 0.05$ ) and over the 14 d study period ( $P < 0.0001$ ; Figure 5-1); temperatures returned to the normal baseline on day 5 and day 7 for vaccinates and non-vaccinates respectively. Vaccinated calves also exhibited significantly lower (improved)

attitude scores relative to non-vaccinates 24 h (day 1) post-challenge ( $P = 0.0473$ ; Figure 5-2), and over the 14 d post-challenge observation period ( $P = 0.0161$ ); a significantly lower incidence of diarrhoea in vaccinated versus non-vaccinated animals was also observed on day 6 post-challenge ( $P = 0.001$ ) and over the 14 d post-challenge observation period ( $P = 0.0012$ ; Figure 5-3). Additionally, vaccination resulted in a reduction of *S. Newport* faecal shedding compared to non-vaccinates on days 13 and 15 post-challenge ( $P = 0.0322$ ;  $P = 0.0019$ ), and over the 15 d faecal sample collection period ( $P = 0.004$ ) (Figure 5-4). Moreover, vaccination resulted in a higher average daily weight gain over the challenge period, day 3 to day 15 post-challenge ( $P = 0.0012$ ; Figure 5-5).

There were no statistically significant differences in the appetites of the vaccinated and non-vaccinated calves following virulent challenge. A reduction in appetite of the calves was observed starting from 48 h post-challenge and persisted until study day 11. There was no statistical difference in mortalities between non-vaccinates and vaccinates challenged with *S. Newport* 03-0721-SP ( $P = 0.6121$ ), although calf mortalities ( $n=1$  and  $n=2$ , respectively) were observed during the course of the trial.

### ***5.3.3. Immunisation with *S. Typhimurium dam* confers cross-protection against virulent *S. Newport* colonisation of mesenteric lymph nodes and lungs of calves***

The ability of *S. Typhimurium dam* to confer protection against colonisation of lymph nodes and visceral organs was evaluated in calves. Vaccine efficacy was determined by enumeration of *S. Newport* strain SN 03-721-SP recovered from MLN, lungs, spleen, liver, and bile 2 weeks post-challenge. Significantly lower numbers of *S. Newport* were recovered from MLN ( $P = 0.0232$ ) and lungs ( $P = 0.0496$ ) of vaccinates compared to non-vaccinates (Figure 5-6). Although *S. Newport* was isolated only from the spleen and liver of non-vaccinates ( $n = 1$  and

n = 2, respectively), there was no statistically significant difference between vaccinates and non-vaccinates for these organs ( $P = 0.9630$  and  $P = 0.5185$  respectively), or from bile contents derived from these two animal groups ( $P = 0.9422$ ). Taken together with the data in Section 5.3.2, these data suggest that immunisation with *S. Typhimurium dam* conferred significant cross-protection against virulent *S. Newport* challenge as evidenced by attenuation of clinical disease, reduced faecal shedding and reduced colonisation of the MLN and lung.

#### **5.4 Discussion**

The global trend towards intensive livestock production is associated with an increased incidence of disease and contamination of livestock-derived food products with a wide array of *Salmonella* serovars, which poses a significant increase in public health risk and industry-associated losses (CDC, 1996; Roels, *et al.*, 1997; CDC, 2007). Vaccination represents a sustainable approach to prevention and control of *Salmonella* infection in commercial livestock production systems. Although vaccination is generally highly specific in the protection afforded to immunised hosts (e.g., the influenza vaccine is a trivalent inactivated vaccine that confers protection against the same three viral serotypes comprised within the vaccine formulation), recent advancements have resulted in the development of vaccines that confer cross-protective immunity to multiple strains of the same species. Herein, we show that vaccination of calves with *dam* mutant Typhimurium confers cross-protective immunity against an emerging, clinically relevant and multi-drug resistant Newport challenge, as evidenced by a significant attenuation of clinical disease, faecal shedding, and tissue colonisation. The development and application of *Salmonella* vaccines offers a potential means of promoting the health and productivity of livestock by reducing infection and clinical disease, thereby reducing *Salmonella* contamination of livestock, livestock-derived food products, and enhancing food safety.

On-farm studies have demonstrated that livestock are exposed to a diversity of serovars and that calves may be infected with salmonellae within hours of birth (Anderson, *et al.*, 2001; House, *et al.*, 2001a; Fossler, *et al.*, 2005a; Fossler, *et al.*, 2005b). Effective *Salmonella* vaccination in livestock therefore requires induction of homologous and heterologous protection and the capability to stimulate both early- and late- onset innate and acquired immune mechanisms. Although the use of *Salmonella* vaccines is relatively common (Smith, *et al.*, 1994), the majority of the products on the market are bacterins (inactivated *S. enterica* organisms) of limited efficacy. The rationale is that, under field conditions, neonatal exposure often occurs during the first few hours after life (House, *et al.*, 2001a), minimising the opportunity of bacterins to confer protective immune mechanisms which may require stimulation by a stable source of diverse antigens for sufficient time to transition to the development of strong adaptive immune responses.

The potential benefit of modified live attenuated *Salmonella* vaccines as immune cells has been recognised for many years and a small number are available commercially (Curtiss, *et al.*, 1993). Vaccine constructs have included aromatic amino acid dependent (*aro*), streptomycin-dependent, and *galE* mutants (Habasha, *et al.*, 1985; Da Roden, *et al.*, 1992; Smith, *et al.*, 1993; Villarreal-Ramos, *et al.*, 1998). These vaccines are typically effective at inducing acquired protective immunity against homologous *Salmonella* challenge. However, such live vaccines (e.g., *aroA* Typhimurium) have a limited capacity to confer protective immunity against heterologous *Salmonella* serovars as most cross-protective responses reported are highly dependent on transient, non-specific immune responses attributed to the persistence of the vaccine strain within the immunised animal (reviewed in (Hormaeche, *et al.*, 1991; Hormaeche, *et al.*, 1996; Harrison, *et al.*, 1997)) and thus, most cross-protective responses are negligible soon after the vaccine strain is cleared from the animal.

An ideal livestock *Salmonella* vaccine that is safe, stimulates rapid and sustained immunity in all classes of stock, and provides protection against the diverse array of *Salmonella* serovars encountered in these production systems, has yet to be developed. Currently, the most promising *Salmonella* live vaccine candidates that confer potent cross-protective immune responses include strains harbouring mutations in regulatory genes that control the expression of a number of potential antigens, including regulatory-mutant based vaccines involved in catabolite repression (*cya crp*; (Hassan and Curtiss, 1994a; Hassan and Curtiss, 1994b)), DNA methylation (*dam*; (Heithoff, *et al.*, 2001)), and LPS core and O-antigen synthesis (*rfaH* (Nagy, *et al.*, 2004)). Thus, although different *Salmonella* serovars possess different antigen repertoires, some of the antigens ectopically expressed by these modified live vaccines may be shared among heterologous serotypes, and expression of these shared diverse antigens may lead to potent cross-protective responses. Consistent with this notion, *Salmonella dam* mutant vaccines ectopically express a number of potential antigens that are normally induced during infection (Garcia-Del Portillo, *et al.*, 1999; Heithoff, *et al.*, 1999; Heithoff, *et al.*, 2001), and have been shown to confer cross-protective immunity to multiple salmonellae in murine (Heithoff, *et al.*, 2001) and avian models of typhoid fever (Dueger, *et al.*, 2001, 2003a). Additionally, *dam* mutant vaccine technology has been applied to bovine salmonellosis, wherein *S. Typhimurium dam* vaccine conferred significant immunity to clinically relevant strains of Typhimurium (serogroup B) (Dueger, *et al.*, 2003b) and Dublin (serogroup D<sub>1</sub>) (Mohler, *et al.*, 2006), two serovars that are frequently associated with disease in cattle (Anderson, *et al.*, 2001).

Herein we evaluated whether vaccination of calves with *S. Typhimurium dam* mutant can confer cross-protection against an emerging pathogen of livestock, *S. Newport* (serogroup C<sub>2</sub>-C<sub>3</sub>), which expresses a significantly different O-antigen from that expressed by *S. Typhimurium* or *S. Dublin*, and has been associated

with multi-drug resistance and clinical disease in recent salmonellosis outbreaks in commercial dairy farms. Vaccination of calves with *S. Typhimurium dam* conferred cross-protective immunity against a Newport challenge, as evidenced by a significant attenuation of clinical disease (improved attitude scores, increased daily weight gains and reduced fever and diarrhoea) and a reduction in Newport faecal shedding and colonisation of mesenteric lymph nodes and lungs compared to non-vaccinated control animals. Such *Salmonella* heterologous cross-protection suggests the potential application of this vaccine toward the prevention and control of *Salmonella* infection in commercial livestock production systems.

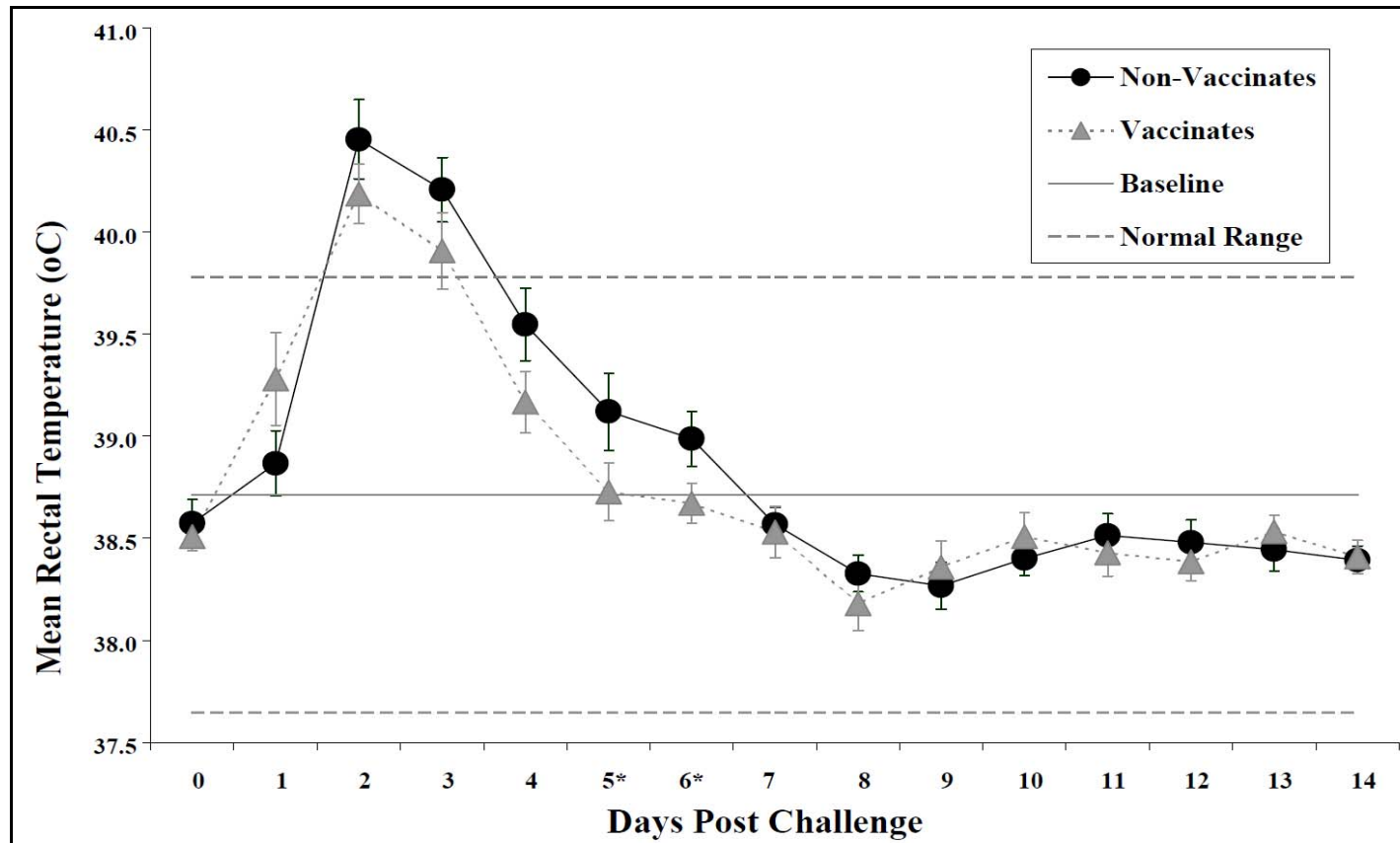


Figure 5-1. Immunisation with *S. Typhimurium dam* confers protection against pyrexia in calves following virulent SN 03-721-SP challenge.

Data are depicted as mean RT ± SE. The data are presented in the context of the normal range (dashed grey lines) and baseline (solid grey line) established pre-challenge. \*RT were significantly lower in vaccinated animals than in non-vaccinates on days 5 and 6 post-challenge (\* $P < 0.05$ ), and over the entire 14 day study period ( $P < 0.0001$ )

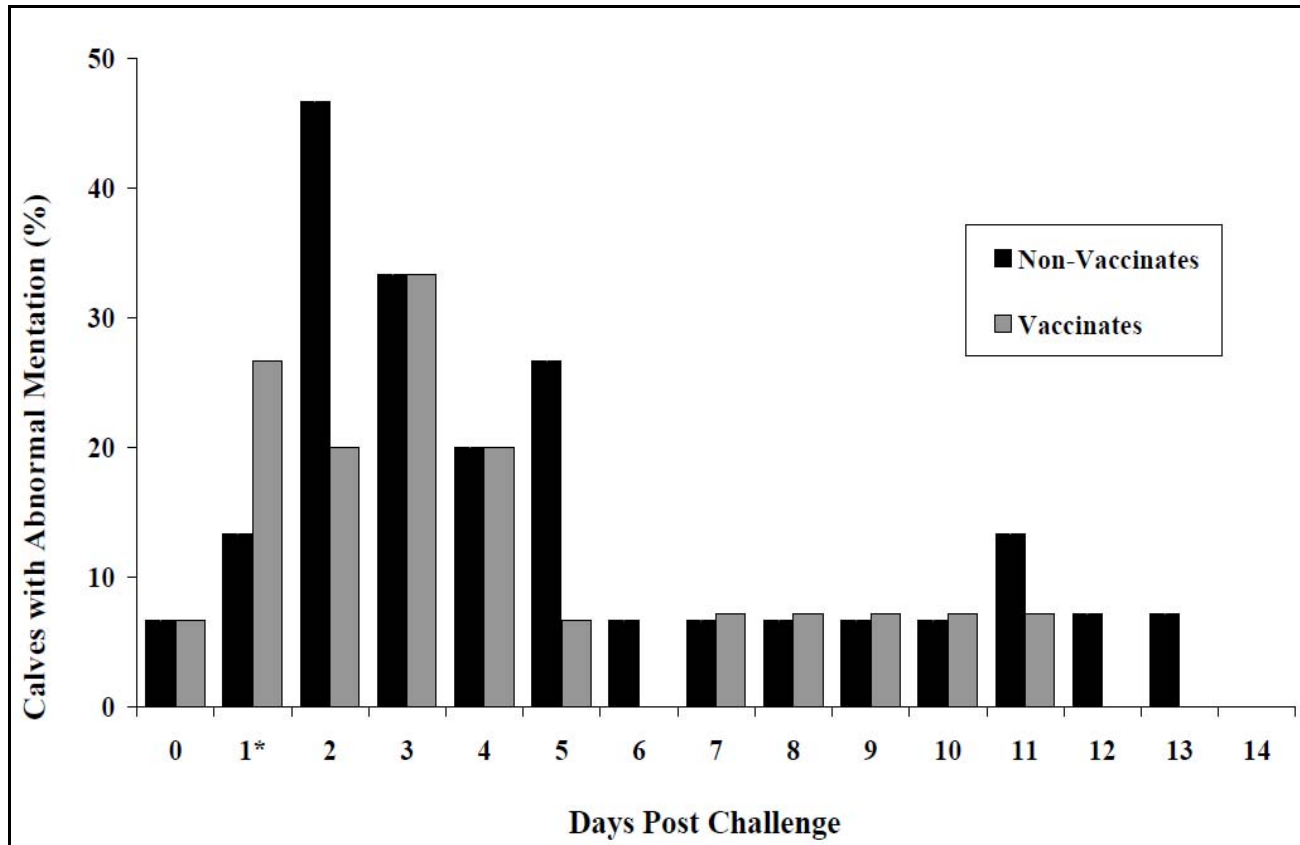


Figure 5-2. Immunisation with *S. Typhimurium dam* improves mentation in calves following virulent SN 03-721-SP challenge. Data are depicted in percentage of calves with abnormal mentation. \*Attitude scores were significantly lower in vaccinated animals than in non-vaccinated controls on day 1 post-challenge (\* $P = 0.0473$ ), and over the entire 14 day study period ( $P = 0.0161$ ).



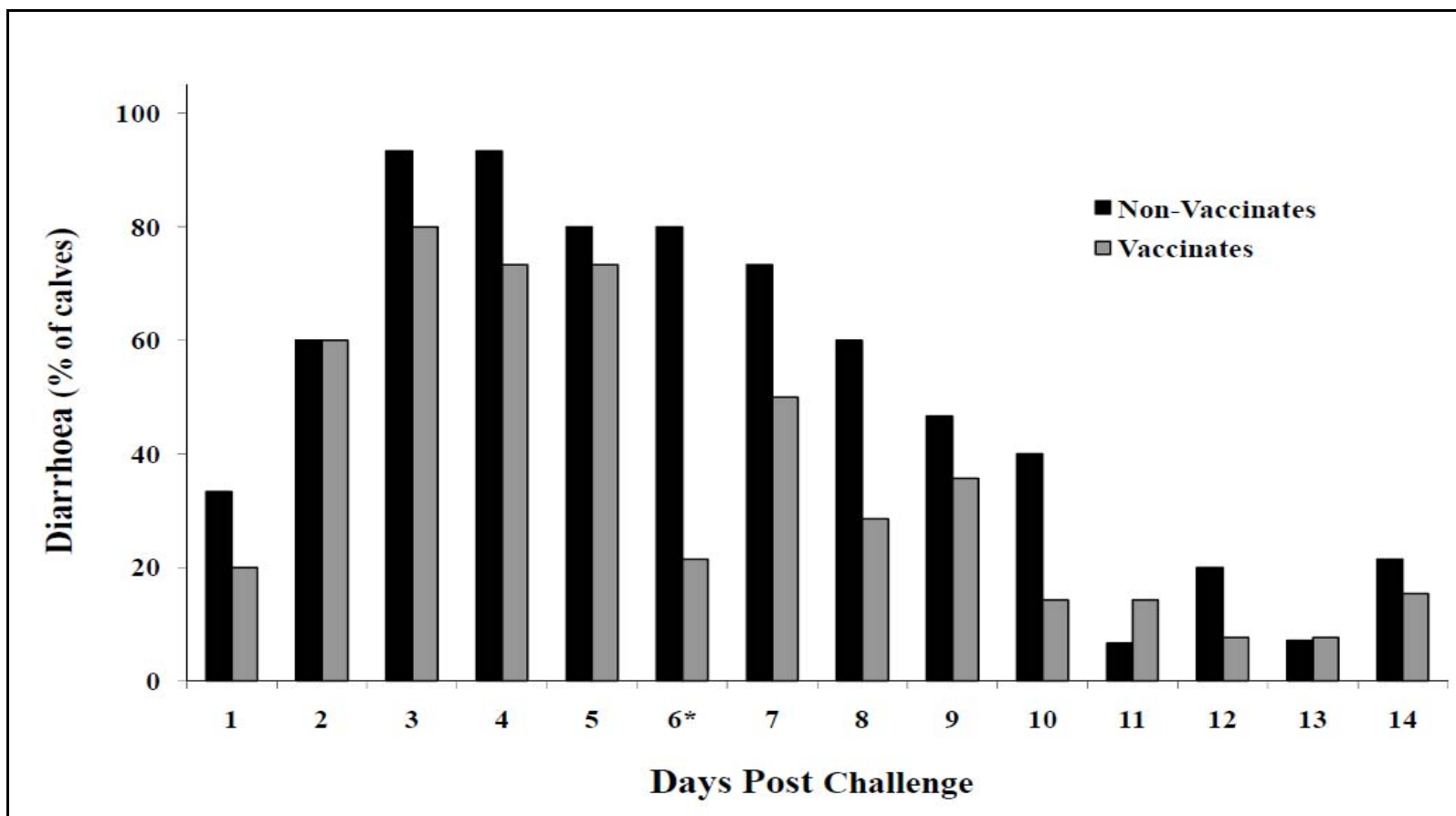


Figure 5-3. Immunisation with *S. Typhimurium dam* confers protection against diarrhoea in calves following virulent SN 03-721-SP challenge. Percent of calves with diarrhoea were scored on dichotomous scale of 0 or 1 for grossly normal stool or diarrhoea. \*Incidence of diarrhoea was significantly lower in vaccinated animals than in non-vaccinated controls on day 6 post-challenge (\* $P = 0.0001$ ), and over the entire 14 d study period ( $P = 0.0012$ ).

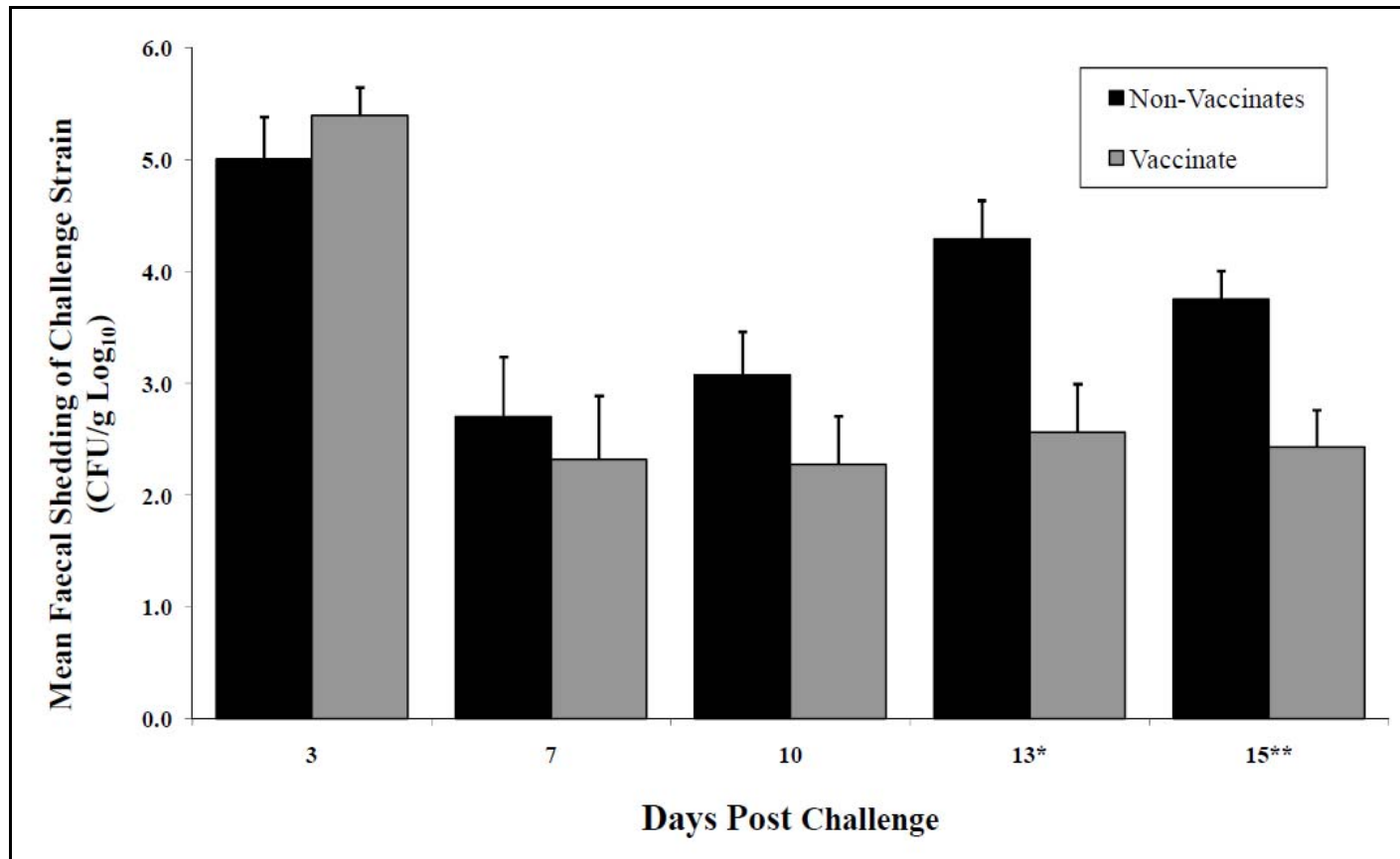


Figure 5-4. Immunisation with *S. Typhimurium dam* confers protection against shedding of virulent SN 03-721-SP in calves following challenge. Data are depicted as a mean log<sub>10</sub> CFU of SN 03-721-SP per g of faeces post-challenge ± SE. Vaccinated calves shed less SN 03-721-SP in the faeces than non-vaccinated controls. Significant differences in faecal shedding were observed on days 13 and 15 post-challenge (\**P* = 0.0322 and \*\**P* = 0.0019), and over the cumulative post-challenge period (*P* = 0.004).

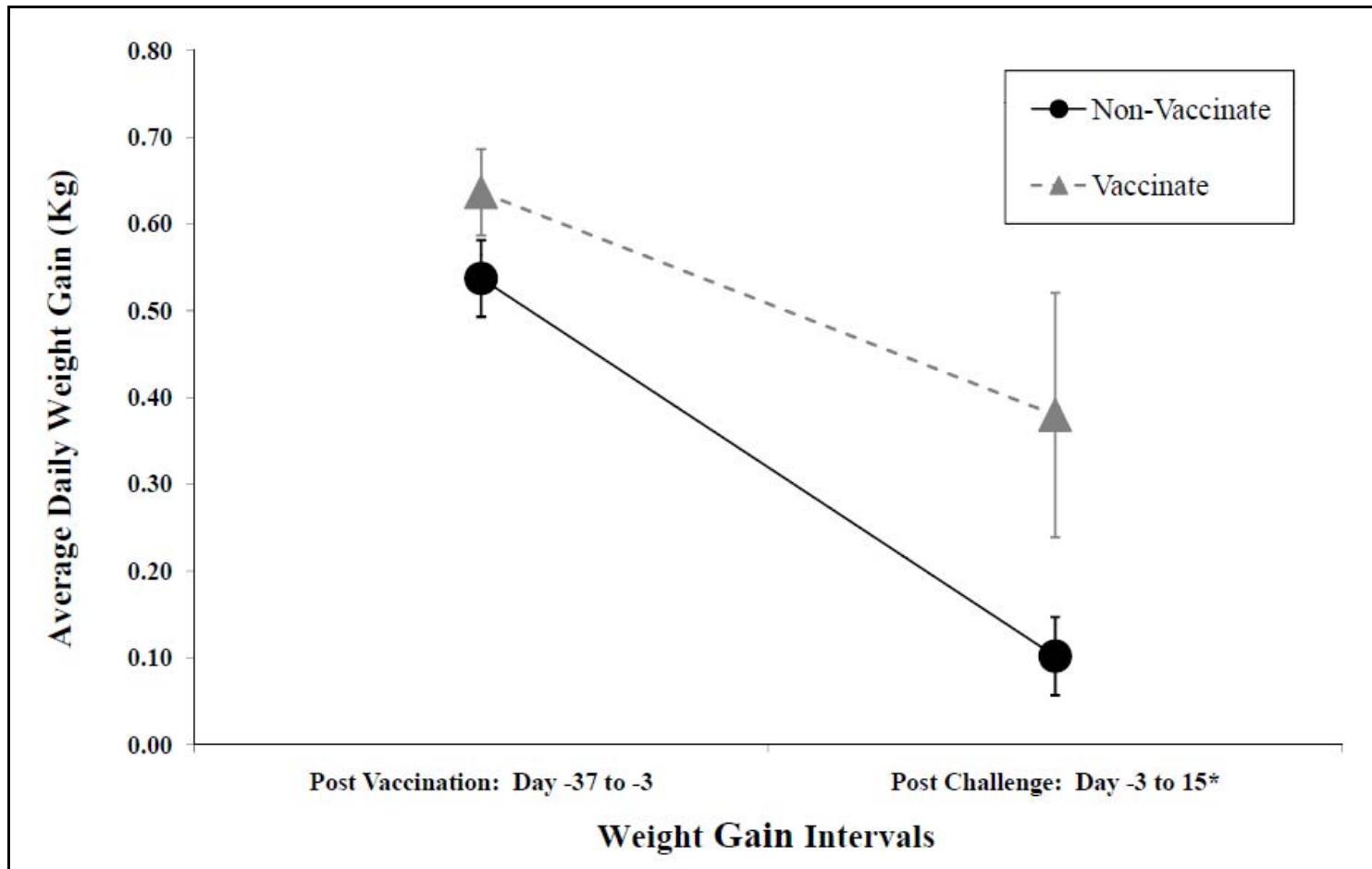


Figure 5-5. Immunisation with *S. Typhimurium dam* improves weight gain in calves following virulent SN 03-721-SP challenge.

Data are depicted in average daily weight gain in Kg per d  $\pm$  SE over the post vaccination and post-challenge intervals. The reduction in average daily weight gains following virulent challenge was significantly less in the vaccinated calves (\* $P = 0.0012$ ).

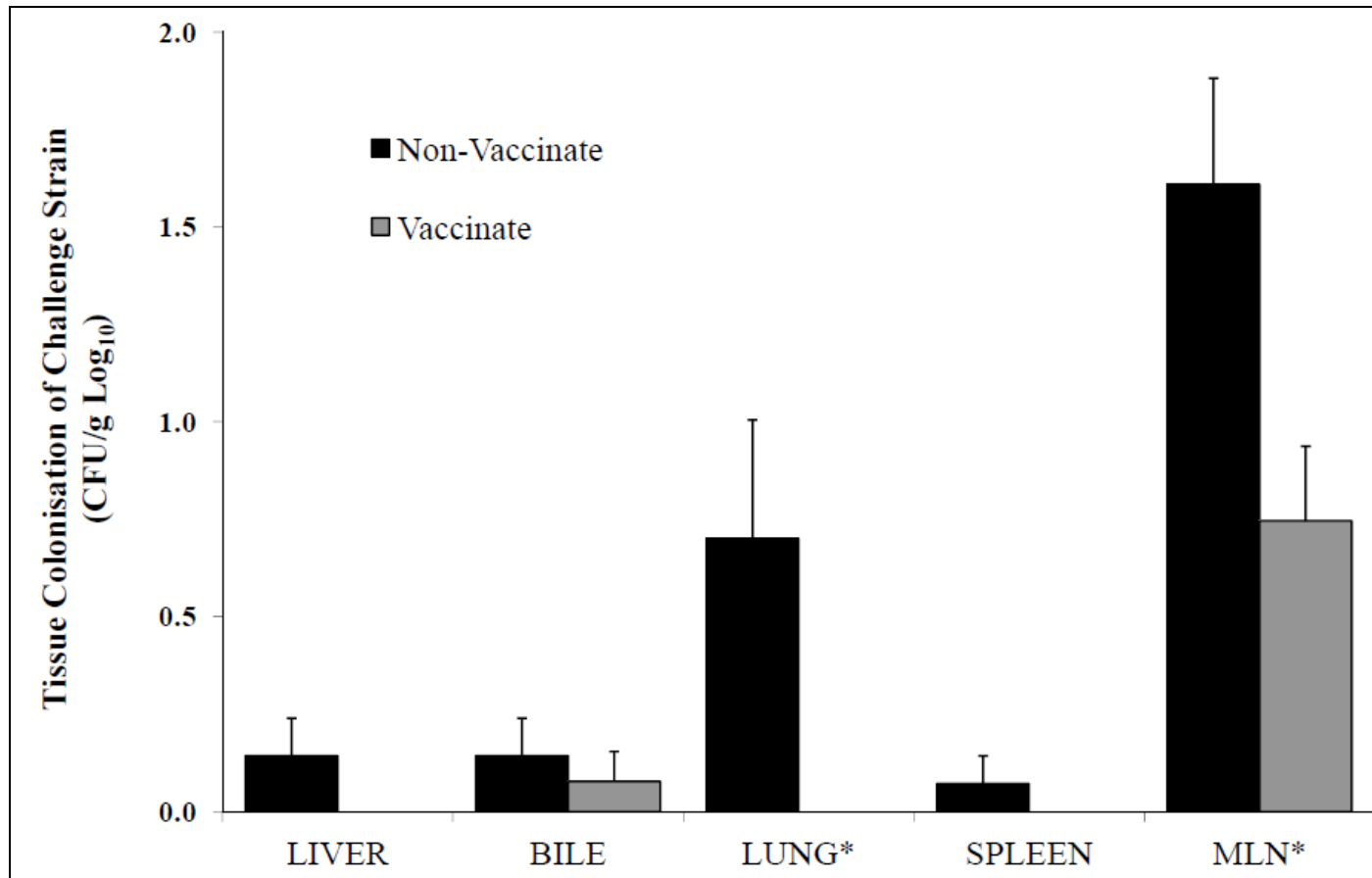


Figure 5-6. Immunisation with *S. Typhimurium dam* confers heterologous protection against SN 03-721-SP colonisation of mesenteric lymph nodes and lungs in calves.

Data are depicted as mean CFU log<sub>10</sub> by organ ± SE. \*Significantly lower CFU/g were recovered from MLN ( $P = 0.0232$ ) and lungs ( $P = 0.0496$ ) of vaccinated versus non-vaccinated calves.

## **CHAPTER 6. Adult Ruminant Models of Salmonellosis**

### **6.1 Introduction**

According to published reports of *Salmonella* challenge studies in sheep, between  $10^9$  and  $10^{12}$  organisms are required to induce salmonellosis in adult sheep (Jelinek, *et al.*, 1982a; Mukkur, *et al.*, 1987; Begg, *et al.*, 1990; Mukkur, *et al.*, 1991b; Mukkur and Walker, 1992; Mukkur, *et al.*, 1995; Wray and Linklater, 2000). Mortality, diarrhoea, and rectal temperature are commonly used to assess clinical outcomes of salmonellosis in group housed animals. Individual outcomes for appetite, water consumption, and behaviour have not been reported in sheep.

These challenge dose trials were conducted in order to ascertain the optimum dose of *Salmonella* that would induce clinical disease in adult sheep, while minimising mortality, to maintain sample size and statistical power for measured outcomes. Additionally, establishing protocols for monitoring salmonellosis in sheep allows for better understanding of the pathogenesis of salmonella infection which has not been reported in the literature and facilitates prediction of outcomes in clinical cases (i.e., mortality, morbidities) which could then be applied to salmonella outbreaks in feedlot situations.

### **6.2 Experimental Design**

#### ***6.2.1. Isolate selection***

Isolates were selected from an outbreak of salmonellosis at a feedlot in Western Australia and considered industry and clinically relevant. The isolates included serotypes *S. Bovismorbificans*, *S. Bredeney*, *S. Havana*, *S. Infantis*, *S. Kottbus*, *S. Singapore* and *S. Typhimurium*. Eleven isolates, three *S. Bovismorbificans* and eight *S. Typhimurium*, were selected as candidates for use in the challenge models based on virulence testing that was conducted by the Department of Molecular, Cellular and Developmental Biology at the University of California, Santa

Barbara using procedures outlined in Heithoff *et al.* (2008b). A competitive index (CI) score was assigned for each isolate based on results of oral and intraperitoneal LD<sub>50</sub> testing in mice (Conner, *et al.*, 1998) and identification of the *spv* genes (Jones, *et al.*, 1982; Gulig, *et al.*, 1992) as well as genes encoding the *spvB* actin cytotoxin, required for systemic survival (Gulig, 1990; Matsui, *et al.*, 2001), and the *pef* fimbriae, implicated in adherence to the murine intestinal epithelium (Baumler, *et al.*, 1996). Antimicrobial sensitivity and resistance (ASR) testing following procedures outlined in *Section 3.3.6* was performed on the selected isolates in order to establish resistance and susceptibility profiles for use in re-isolation protocols.

#### **6.2.2. Verification of isolate resistance**

Eight of the eleven virulent isolates were identified to have resistance to neomycin sulphate, allowing differentiation from the *S. Typhimurium dam* vaccine: five *S. Typhimurium* and three *S. Bovismorbificans*. These isolates were grown overnight on LA plates containing neomycin sulphate at 5, 10, 15, and 20 µg/mL to identify resistant clones and to determine the level of antibiotic required to ensure re-isolation of the virulent isolates from tissues, faeces and solutions containing the isolate, microbial flora and the vaccine strain. To ensure that isolates maintained resistance to antimicrobials, colonies were stored frozen and re-cultured following procedures outlined in *Section 3.3.1* by plating onto media containing neomycin, then serial diluted onto selective media at incremental concentrations of neomycin and chloramphenicol. Plates were incubated aerobically overnight at 37°C and colonies were enumerated to determine recovery rate.

#### **6.2.3. Challenge dose trials in Merino wethers**

Strains used in these challenge studies were grown overnight in LB at 37°C following procedures outlined in Appendix I, and delivered to the sheep in a 10 mL PO bolus resuspended in PBS.

***i. Homologous dose range finding study***

Homologous challenge studies were performed with a derivative of a *S. Typhimurium* strain, 06-131, an industry-relevant, multi-drug antimicrobial resistant strain (apramycin, erythromycin, neomycin, novobiocin, sulphonamides, streptomycin) that was isolated from an outbreak of salmonellosis in sheep on a commercial feedlot in Western Australia (ST 06-131) as described in *Section 3.2.2.ii*.

Fourteen sheep were randomly placed into two groups, low dose and high dose, of seven sheep each. Sheep were orally administered ST 06-131 challenge strain at  $5 \times 10^8$  and  $5 \times 10^9$  organisms for low and high dose, respectively.

***ii. Heterologous dose range finding study***

Heterologous challenge studies were performed with a derivative of a *S. Bovismorbificans* strain, 06-225, an industry-relevant, multi-drug antimicrobial resistant strain (apramycin, erythromycin, neomycin, novobiocin, sulphonamides, streptomycin) that was isolated from an outbreak of salmonellosis in sheep on a commercial feedlot in Western Australia (SBM 06-225) as described in *section 3.2.2.iii*.

Fifteen sheep were randomly placed into two groups, low dose and high dose, of eight and seven sheep respectively. Sheep were orally administered SBM 06-225 challenge strain at  $1.7 \times 10^8$  and  $1.7 \times 10^9$  organisms for low and high dose respectively.

***iii. Sheep selection and husbandry***

Merino wethers, approximately 2 to 3 years in age, were sourced from a single flock in New South Wales, Australia (see *Section 3.5.2*). Faecal samples were collected from all sheep prior to enrolment in the study and underwent qualitative detection of *Salmonella* following procedures outlined in *Section 3.6.1*. No

salmonellae were detected in the sheep during the acclimation period. Sheep were transported to the Medium Security Animal Housing Facility at EMAI (NSW, AU), and randomly assigned to experimental groups and housed following procedures outlined in *Section 3.5.5*. Sheep received a diet of oaten chaff (8 MJ ME/kg DM) and commercially prepared grain pellet (12 MJ ME/kg DM) during the acclimation and challenge phases of the experiment. Sheep were gradually introduced to the grain pellet during the acclimation period. Sheep were offered 600 g of oaten chaff and 300-400 g of grain pellets at the morning observation period during the challenge periods (roughly 9 MJ ME/d).

*iv. Clinical assessment of sheep*

Sheep were acclimated for approximately two weeks prior to initiation of the dose range study in order to establish normal ranges for behaviour, faecal scores and rectal temperatures, as well as to introduce the sheep to a diet of pellets and chaff. Clinical assessment of the sheep was performed twice a day during the acclimation period and RT was recorded bi-weekly to ensure that sheep were healthy, following procedures outlined in *Section 3.5.7*. The normal range of RT for the sheep used in these experiments based on the pre-challenge mean (baseline) RT  $\pm$  two standard deviations was 37.5 – 39.8°C. RT was recorded daily following virulent *Salmonella* challenge.

During the challenge phase, sheep were clinically assessed following procedures outlined in *Section 3.5.7*. Quantitative faecal culturing was conducted 3, 7, 10, and 12 d post-challenge following procedures outlined in *Section 3.6.2*. Appetite was recorded in g of chaff and pellets not consumed over each 24 h feeding period. Percent refusal of chaff and pellets was determined for each animal during the challenge period based on pre-challenge consumptions. An attitude score of 4 at any time of the day, or failure to consume chaff or pellets (100% refusal of feed) for three consecutive feedings, constituted grounds for euthanasia as



described in *Section 3.5.10*. Sheep were weighed during the acclimation period prior and 12 d post-challenge.

***v. Necropsy and tissue collection***

On day 12 post-challenge, surviving sheep in the homologous and heterologous challenge studies were humanely euthanised and necropsied. Approximately 2 to 5 g of tissue was obtained from the liver, ileum, spleen, and mesenteric lymph node of each sheep to evaluate tissue colonisation of the challenge agent.

***vi. Statistical analysis***

Continuous data were analysed using REML analysis (Genstat, 12<sup>th</sup> Edition, VSN International, UK). A single variate, repeated measures model was fitted for the factors of time and treatment for variables CFU, temperatures, chaff refusal, pellet refusal, and water intake. The Wald chi-square test was used to determine significant individual effects and/or significant interactions between factors. Any non-significant terms were dropped from the model and analysis repeated. The following analysis data are presented as predicted model based means. Predicted means are those obtained from the fitted model rather than the raw sample means. This is important as predicted means represent means adjusted to a common set of variables, thus allowing valid comparison between means. A *P* value less than 0.05 was considered to be statistically significant. Differences between the individual means were determined by calculating an approximate LSD. A difference of means that exceeded the calculated LSD was considered significant.

Repeated measures of non-parametric clinical data, faecal composition and mentation, were analysed using the Wei-Lachin test (Wei and Lachin, 1984; Davis, 1991) using StatsDirect statistical software, <http://www.statsdirect.com>, 2002, CamCode, England (Buchan, 2000). Wei Lachin analysis of the data performed univariate group comparisons for individual time points and a multivariate comparison over the study period analysed. Data are presented as the

percentage of sheep that displayed an abnormal attitude (cumulative daily score > 3) or have abnormal faecal score (cumulative daily score > 3) over the course of the challenge period. Tissue colonisation and were evaluated using ANOVA, the vaccine dose and body weight of sheep that did and did not develop pyrexia following vaccination was compared using unpaired t-test, and mortality results were compared using chi-square.

## **6.3 Results**

### ***6.3.1. Selection of isolates***

Virulence testing aided in the identification of virulent candidates for use in the challenge models. Three *S. Bovismorbificans* and five of the *S. Typhimurium* were selected as candidates for use in the development of ovine models of heterologous and homologous salmonellosis based on the results of the CI score and detection of virulence genes. Based on the results of the ASR testing, the eight selected isolates displayed neomycin sulphate resistance and chloramphenicol susceptibility (Table 6.1). This ASR profile was ideal, as it allow differentiation of the *S. Typhimurium dam* vaccine strain from these challenge isolates in a challenge -vaccination trial.

### ***6.3.1. Confirmation of isolate resistance***

When the isolates were plated for single colonies onto LA or XLD media containing neomycin sulphatesulfate at 30 µg/mL, no growth was observed. However, when the level of neomycin was reduced to 10 µg/mL, consistent growth from one of the *S. Typhimurium* isolates, *S. Typhimurium* 06-131 (ST 06-131), and two of the *S. Bovismorbificans* isolates, *S. Bovismorbificans* 06-174 and *S. Bovismorbificans* 06-225 (SMB 06-225) was observed. Serial dilution plating of the isolates onto selective media containing neomycin sulphatesulfate at 10 µg/mL was performed, and ST 06-131 and SBM 06-225 retained neomycin

Table 6.1: Results of antimicrobial susceptibility and resistance testing and virulence scores of salmonella challenge agent candidates

Antibiotic Disc Identification <sup>a</sup>																		
ISOLATE ID	Serotype	TE	AMP	AK	W	NA	SXT	S3	N	AMC	S10	APR	K	E	NV	C	EFT	Virulence
		30 µg	10 µg	30 µg	5 µg	30 µg	25µg	300 µg	30 µg	30 µg	10 µg	15 µg	50 µg	5 µg	5 µg	10 µg	30 µg	Competitive Index Score
<i>S. Typhimurium</i> <i>Vaccine</i> MT2313	B	S <sup>b</sup>	S	S	S	S	S	R	S	S	R	R	S	R	R	R	S	
<i>S. Bovismorbificans</i> 174	C <sub>2</sub> -C <sub>3</sub>	S	S	S	S	S	S	R	R	S	R	R	S	R	R	S	S	1.733
<i>S. Bovismorbificans</i> 220	C <sub>2</sub> -C <sub>3</sub>	S	S	S	S	S	S	R	R	S	R	R	S	R	R	S	S	0.29
<i>S. Bovismorbificans</i> 225	C <sub>2</sub> -C <sub>3</sub>	S	S	S	S	S	S	R	R	S	R	R	S	R	R	S	S	2.716
<i>S. Typhimurium</i> 131	B	S	S	S	S	S	S	R	R	S	R	R	S	R	R	S	S	0.545
<i>S. Typhimurium</i> 146	B	S	S	S	S	S	S	S	R	S	R	R	S	R	R	S	S	2.03
<i>S. Typhimurium</i> 150	B	S	S	S	S	S	S	R	R	S	R	R	S	R	R	S	S	0.951
<i>S. Typhimurium</i> 222	B	S	S	S	S	S	S	R	R	S	R	R	S	R	R	S	S	0.726
<i>S. Typhimurium</i> 230	B	S	S	S	S	S	S	R	R	S	R	R	S	R	R	S	S	2.32
<i>S. Typhimurium</i> UK-1 Wild type parent strain	B	N/A																2.37

<sup>a</sup> AMP - Ampicillin; S3 - Sulfonamides Compound; W - Trimethoprim; TE - Tetracycline; N - Neomycin; NA - Nalidixic Acid; AK - Amikacin; SXT - Sulphamethoxazole/trimethoprim 19:1; K - Kanamycin; E - Erythromycin; NV - Novobiocin; C - Chloramphenicol; S10 - Streptomycin; AMC -Amoxycillin/Clavulanic Acid; APR - Apramycin (APR) and EFT - Cefiofur

<sup>b</sup> Antimicrobial resistance result: S = Susceptible and R = Resistant (grey)

resistance and the antibiotic did not inhibit growth (Table 6.2). When the level of neomycin was increased to 15 or 20 µg/mL, partial to complete inhibition in growth was observed in all of the isolates. From these data, ST 06-131 was the isolate selected as the challenge strain for the homologous salmonellosis model and SBM 06-225 was the isolate selected as the challenge strain for the heterologous salmonellosis model. .

### ***6.3.1. Dose range of S. Typhimurium 06-131 in sheep***

A challenge dose trial was conducted in order to determine an optimum dose that would induce disease while minimising mortality, in order to maintain sample size and statistical power for measured outcomes. All of the sheep developed clinical signs of salmonellosis (fever, diarrhoea, decreased appetite and depressed attitude) during the 12 d post-challenge observation period. No mortalities were observed in the low dose group. There were four mortalities in the sheep administered the high dose of ST 06-131 on days 6, 7, 8 and 11 post challenge, respectively. As expected, the severity of clinical signs was greater in sheep challenged at the high dose of ST 06-131.

Sheep receiving the ST 06-131 challenge strain had significantly elevated predicted mean RT when compared to pre-challenge baseline RT from days 2 to 8 post-challenge in the low dose and days 2 to 10 in the high dose ( $P < 0.05$ ; Figure 6-1). However, by day 8 post-challenge, the RT of the challenged sheep had returned to the normal range. Dose level of the challenge strain did not have an effect on the predicted mean RT in the sheep over the 12 d observation period ( $P = 0.494$ )

The dose of ST 06-131 had a significant effect on the mentation of the sheep during the 12 d observation period ( $P < 0.005$ ). The incidence of abnormal mentation were significantly increased in the high dose group when compared to the low dose on days 7 to 10 post-challenge ( $P < 0.05$ ; Figure 6-2).

Table 6.2: Results of growth of isolates on selective media containing antibiotics.

Salmonella Isolate Identification																		
Plate Media	ST 06-131						SBM 06-174						SBM 06-225					
	1 <sup>a</sup>	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
Plain XLD	+	+	38	5	1	NG	+	+	74	5	1	NG	+	+	13	1	NG	NG
XLD+ NEO 10µg/mL	+	+	36	3	NG	NG	+	+	42	4	NG	NG	+	+	13	1	NG	NG
XLD+ NEO 15µg/mL	+	12	1	NG	NG	NG	NG	NG	NG	NG	NG	NG	+	89	7	NG	NG	NG
XLD+ NEO 20µg/mL	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
XLD+ NEO 30µg/mL	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
Luria + CHL 10µg/mL	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG

a: Series of six serial 1:10 dilution of stock solution (see *Section 3.6*)  
 +: Too many colonies to count; NG - No growth

There was a significant increase in the incidence of abnormal faeces in the high dose group when compared to the low dose on days 7 to 11 post-challenge ( $P < 0.05$ ; Figure 6-3). The shedding of ST 06-131 challenge strain in faeces of the sheep was measured on days 3, 7, 10 and 12 post challenge (Figure 6-4). The predicted mean CFU/g of ST 06-131 shedding was significantly higher in the faeces of sheep from high dose group on days 10 and 12 post challenge ( $P < 0.05$ ).

The dose level of ST 06-131 had a significant effect on the chaff appetite of the sheep over the course of the study ( $P < 0.001$ ). Chaff refusals in the low dose group were significantly lower when compared to the high dose group on days 6 to 11 post-challenge ( $P < 0.05$ ; Figure 6-5). Pellet refusals were significantly lower in the low dose group when compared to the high dose on days 8 and 11 post-challenge ( $P < 0.05$ ; Figure 6-6). Nearly 100% refusal of pellets was observed from day 3 post challenge to completion of the study in the high dose group.

Water consumptions were significantly reduced from days 2 to 11 post-challenge in the high dose group and days 2 to 9 post-challenge in the low dose when compared to pre-challenge water consumption levels ( $P < 0.05$ ; Figure 6-7). Water consumptions were significantly higher in the low dose group on days 10 and 11 when compared to the high dose group ( $P < 0.05$ ). Interestingly, following the challenge, water consumption was  $2.43 \pm 0.20$  L, and  $2.07 \pm 0.20$  L for the low and high dose groups, respectively, which was 144.2% and 115.5% of the mean daily water consumption during the five days preceding challenge.

There was no difference in the mean body weights of the low and high dose groups over the course of the study ( $P = 0.158$ ). Significant loss in predicted mean body weight was observed on day 12 post challenge at both dose levels when compared to acclimation and pre-challenge predicted mean body weights ( $P <$

0.05; Figure 6-8).

Colonisation of tissues was determined by enumeration of ST 06-131 recovered from MLN, spleen, liver, and ileum wall 12 d post-challenge. ST 06-131 was recovered from 7 of 7 and 3 of 3 sheep from the low and high dose groups, respectively. There was no difference between the mean CFU/g of ST 06-131 recovered from the tissues of sheep from the low and high dose groups (Figure 6-9).

These data suggest that the ST 06-131 isolate was capable of producing clinical salmonellosis in adult sheep when administered as an oral bolus as evidenced by pyrexia, altered mentation, diarrhoea, reduced appetites, weight loss, faecal shedding and colonisation in tissues. The isolate retained neomycin resistance following *in vivo* oral challenge in sheep and was successfully re-isolated from the faeces and tissues when placed onto XLD plates containing 10 µg/mL neomycin sulphate (XLD-NEO).

### **6.3.2. Dose range of *S. Bovismorbificans* 06-225 in sheep**

This challenge dose trial was conducted in order to determine an optimum dose that would induce disease while minimising mortality, in order to maintain sample size and statistical power for measured outcomes for use in a heterologous model of salmonellosis in sheep. All of the sheep developed clinical signs of salmonellosis (fever, diarrhoea, decreased appetite and depressed attitude) during the 12 d post-challenge observation period. There were three mortalities in the sheep administered the high dose of SBM 06-225 on days 6 (n=2) and 9 (n=1) post challenge, respectively. No mortalities were observed in the low dose group.

Sheep receiving the SBM 06-225 challenge strain had significantly elevated predicted mean RT when compared to pre-challenge baseline from days 2 to 10 post-challenge in the low dose group and in the high dose group from day 1 to 12

post challenge ( $P < 0.05$ ; Figure 6-10). While the dose of SBM 06-225 challenge did not have an effect on the predicted mean RT over the course of the 12 d observation period ( $P = 0.338$ ), mean RT of sheep in the low dose group was significantly lower than the high dose group on day 2 post challenge ( $P < 0.05$ ).

Administration of SBM 06-225 had a significant effect on the mentation of the sheep over the course of the study ( $P = 0.0001$ ). Abnormal mentation was first noted on day 2 post-challenge in both groups with peak incidence occurring on day 4 and day 7 in the low and high dose groups respectively (Figure 6-11).

There was a significant increase in the incidence of abnormal faeces in sheep receiving the SBM 06-225 challenge strain over the course of the study ( $P < 0.0001$ ). The incidence of abnormal faeces was significantly increased in the high dose group on days 3 and 4 post-challenge when compared to the low dose group ( $P < 0.005$ ; Figure 6-12). Shedding of SBM 06-225 challenge strain in faeces was evaluated on days 3, 7, 10 and 12 post challenge (Figure 6-13). The predicted mean shedding of the SBM 06-225 challenge strain was significantly higher in the high dose group on day 3 post challenge when compared to the low dose group ( $P < 0.05$ ). There was no difference in the shedding of SBM 06-225 in the low and high dose groups on days 7, 10, and 12 post challenge.

The SBM 06-225 challenge had a significant effect on appetite of the sheep over the course of the study ( $P < 0.012$ , chaff;  $P < 0.001$ , pellets). Chaff refusals in the low dose group were significantly reduced when compared to the high dose group day 2 post-challenge ( $P < 0.05$ ; Figure 6-14). Additionally, the chaff refusals were significantly increased when compared to pre-challenge refusals (day 0) from day 3 to 10 and day 2 to 11 post-challenge in the low and high dose groups respectively ( $P < 0.05$ ). Pellet refusals were significantly less in the low dose group when compared to the high dose group on day 2 post-challenge (Figure 6-15). And pellet refusals were significantly increased when compared to pre-



challenge refusals (day 0) from day 3 to 10 and day 1 to 8 post-challenge in the low and high dose groups respectively ( $P < 0.05$ ).

Dose level of the SBM 06-225 challenge did not have a significant effect on water consumption of the sheep over the course of this study ( $P = 0.055$ ), however, water consumption of the sheep was significantly reduced when compared to pre-challenge consumptions on days 3, 4, 10 and 11 post-challenge in the low dose sheep and day 3 post-challenge in the high dose sheep ( $P < 0.05$ ). Significantly improved water consumptions were observed in the low dose group on days 1 and 2 when compared to the high dose group ( $P < 0.05$ ; Figure 6-16). Following the administration of the SBM 06-225 challenge, mean water consumption of the sheep was  $4.56 \pm 0.94$  L, and  $2.94 \pm 0.44$  L in the low and high dose groups, respectively, which was 143.9% and 105.6 % of the mean water consumption during the five days preceding challenge for each group.

There was no difference in the mean body weights of the low and high dose groups following the challenge ( $P = 0.963$ ). Over the course of the study both groups showed a similar decrease in predicted mean body weight when compared to pre-challenge body weights ( $P < 0.05$ ; Figure 6-17).

Colonisation of tissues was determined by enumeration of SBM 06-225 recovered from MLN, spleen, liver, lung and ileum wall 12 d post challenge. SBM 06-225 was recovered from 7 of 7 and 4 of 4 sheep from the low and high dose groups respectively. There was no difference in the mean CFU/g of SBM 06-225 recovered from the tissues of sheep from the low and high dose groups (Figure 6-18).

### ***6.3.3. Ovine salmonellosis gross pathology findings***

Gross pathological findings included enlarged mesenteric lymph nodes, ulcerated bile ducts, fibrin deposits in the small intestine, caecum and abomasum, ulceration of the pylorus, mucosal erosions of the abomasum, small intestine and caecum as

well as ecchymotic haemorrhages on the serosal surface intestines and mesentery (Figure 6-19). These findings were commonly associated with animals that were found dead or euthanised. Interestingly, very few lesions were observed in the sheep that survived to the end of the study. Given that the sheep had shown clinical signs consistent with salmonellosis it suggests that the bowel is capable of healing quickly once the immune response controls the infection.

#### **6.4 Discussion**

The aims of this research were to identify virulent *Salmonella* candidates for use in the development of ovine models of homologous and heterologous salmonellosis. Dose range finding studies often incorporate several doses of a challenge agent at half log intervals to enable statistical estimation of the lethal dose (LD<sub>90-100</sub>). One of the difficulties of developing a good *Salmonella* challenge model for evaluation of vaccines or antimicrobial efficacy is the identification of a challenge strain. It has been well documented that prevalence of an isolate during an outbreak of salmonellosis does not always correlate with virulence (Rice, *et al.*, 1997; Tsolis, *et al.*, 1999; Mahan, *et al.*, 2000; Adaska, *et al.*, 2008). Every effort was made to ensure candidates used in the challenge models were virulent and industry relevant prior to *in vivo* usage. Unfortunately, potential challenge strains occasionally prove to be avirulent in the test subject due to variable expression of virulence within a particular host. Two dose levels, one log difference between dose levels for each of the challenge isolates, were selected based on published ruminant models of salmonellosis (Rankin and Taylor, 1966; Nazer and Osborne, 1977; Jones, *et al.*, 1988; Mukkur, *et al.*, 1991a; House, *et al.*, 2001a; Fecteau, *et al.*, 2003; Dueger, *et al.*, 2003b; Mohler, *et al.*, 2006). The logic of this decision was to avoid the scenario of including large numbers of sheep only to find that the strain was avirulent.

The data from the dose range finding studies demonstrated that ST 06-131 and

SBM 06-255 were capable of inducing clinical disease in Merino wethers. And both isolates retained neomycin resistance following *in vivo* oral challenge in sheep and was successfully re-isolated from the faeces and tissues. All sheep in the low and high dose challenge groups developed clinical signs of salmonellosis which included fever, diarrhoea, decreased appetite and depressed mentation during the 12 d post-challenge observation period. However, there are no reported ovine salmonella challenge trials where attitude, appetite, and water consumption have been monitored in individual sheep with which to compare these results. The mortalities in the sheep could be attributed to the development of septicæmia, acidosis (depressed mentation), diarrhoea, dehydration and anorexia. The hypothermia observed in these sheep prior to death was associated with release of endotoxin, acidosis, cardiovascular collapse and septic shock. From these results, a challenge dose of  $1 \times 10^9$  CFU of ST 06-131 was selected as a target for use in homologous vaccine efficacy trials and  $1 \times 10^9$  CFU of SBM 06-225 for use in future cross protective vaccine efficacy trials, as these doses are likely to provide predictable induction of clinical disease with minimal mortality. The dose levels required to induce mortality, pyrexia and diarrhoea in sheep were within the ranges reported in the literature (Mukkur, *et al.*, 1987; Mukkur, *et al.*, 1991b; Wray and Linklater, 2000).

One interesting outcome of the ASR testing was the relative lack of antimicrobial resistance in the isolates. Antimicrobial resistance is a common feature in salmonellosis outbreaks (CDC, 1996, 2002; OzFoodNet, 2003). The isolates from the feedlot outbreaks were sensitive to tetracycline, trimethoprim-sulphonamide, amoxycyclavulonic acid, ceftiofur and amikacin which are all approved for use in livestock (APVMA, 2010). None of the isolates tested were resistant to nalidixic acid which is not surprising as fluoroquinolones are not allowed to be used in livestock species in Australia (APVMA, 2010). It was interesting to note that all of the isolates from the outbreak were resistant to the antibiotic apramycin which

is commonly used in feedlots.

Inanition and salmonellosis are the most common causes of death in exported sheep and contributes to 75% of mortalities reported (Jelinek, *et al.*, 1982b; Norris and Richards, 1989; Norris, *et al.*, 1989a; Norris, *et al.*, 1989b; Richards, *et al.*, 1989; Higgs and Norris, 1991; Richards and Hyder, 1991; Higgs, *et al.*, 1993; Kelly, 1995; Makin, 2011). During the conduct of this research it was observed that post virulent *Salmonella* challenge, feed intakes of the sheep were significantly reduced within 48 h of challenge and were anorectic within 72 h post challenge. Another observation was that challenged sheep preferentially selected the chaff component of the diet over pellets. This phenomenon persisted following clearance of the organism and resolution of clinical signs in the challenged sheep. Interestingly, it is reported that inappetence and poor rumen fill in feedlot sheep often precedes infection with *Salmonella* (Richards, *et al.*, 1989; Richards and Hyder, 1991; Higgs, *et al.*, 1993; Kelly, 1995). In contrast, the sheep in these research trials displayed inappetence following the virulent *Salmonella* challenge, not preceding it. These studies demonstrate that inappetence and feed refusal, often called 'neophobia' in feedlot industry, may actually be attributed to salmonellosis.

The commonly reported observation that sheep in a new environment may not consume a novel diet due to lack of experience with the feed source (neophobia), is believed to be a contributing factor to the observation of inanition. The number of non-feeders has been reported to range between 0.2% to 50% in various groups of sheep under varying conditions (Norris and Richards, 1989; Norris, *et al.*, 1989a; Norris, *et al.*, 1989b; Norris, *et al.*, 1990; Higgs and Norris, 1991; Richards and Hyder, 1991; Bailey and Fortune, 1992; Kelly, 1995; Higgs and Norris, 1999). During the acclimation phase of these studies, one of the 29 sheep (3.4%) displayed neophobia to pellets and chaff while 5 of 29 (17.9%) refused to

consume pellets. With poor rumen fill, feed withholding, anorexia and rumen imbalance identified as risk factors for salmonellosis in feedlot sheep (Norris, *et al.*, 1989a; Norris, *et al.*, 1989b; Richards, *et al.*, 1989; Richards and Hyder, 1991; Higgs, *et al.*, 1993; Kelly, 1995; Lenahan, *et al.*, 2010), ensuring that sheep accept the diet offered is essential to maintaining the health of a flock. Finding ways to improve transition from pasture to pellet reduces the risk of neophobia and inanition. However, in this sample population, sheep demonstrated dietary preferences with avoidance of pellets, despite previous exposure and transition. Although a small sample size, these data suggest that animal preferences and salmonellosis may also be important factors in neophobia and inanition.

Necropsy findings demonstrated that gross pathology in the lymph nodes and intestines occurred following the administration of the challenge agent. However the lungs, liver and spleen were grossly normal, despite colonisation with the challenge agent. *Salmonella* infections have been associated with colonisation of the lymph nodes, lung, liver, spleen, gall bladder and salivary glands in ruminants (Jelinek, *et al.*, 1982a; Jelinek, *et al.*, 1982b; Mukkur, *et al.*, 1987; Norris, *et al.*, 1989a; Richards, *et al.*, 1989; Higgs, *et al.*, 1993; Mukkur, *et al.*, 1995; Wray and Linklater, 2000; Vanselow, *et al.*, 2007a). In previous dose range finding studies, low yield tissue colonisation of *S. Dublin* and *S. Newport* in calves were shown to be time dependent and correlated with resolution of clinical signs (Mohler, *et al.*, 2006; Mohler, *et al.*, 2008). The sheep that survived to the end of the observation period were noted to have few to no gross lesions. It has been reported that acutely infected animals shed fewer *Salmonella* for shorter periods of time and these animals are less likely to become carriers (Smith, *et al.*, 1979; Wray and Davies, 2000; Smith, 2002; Mohler, *et al.*, 2009).

This study outlines the development of homologous and heterologous models of salmonellosis for use in future challenge-vaccination efficacy trials with the

modified live *S. Typhimurium dam* attenuated vaccine in adult Merino wethers. Importantly, ST 06-131 and SBM 06-225 are both isolates that are relevant to the Australian sheep industry. Both isolates were capable of producing clinical disease in adult sheep which allowed for the development of criteria for assessing clinical outcomes (mentation, appetite, diarrhoea, pyrexia) and maintained antimicrobial resistance, which facilitated evaluation of faecal shedding and tissue colonisation.

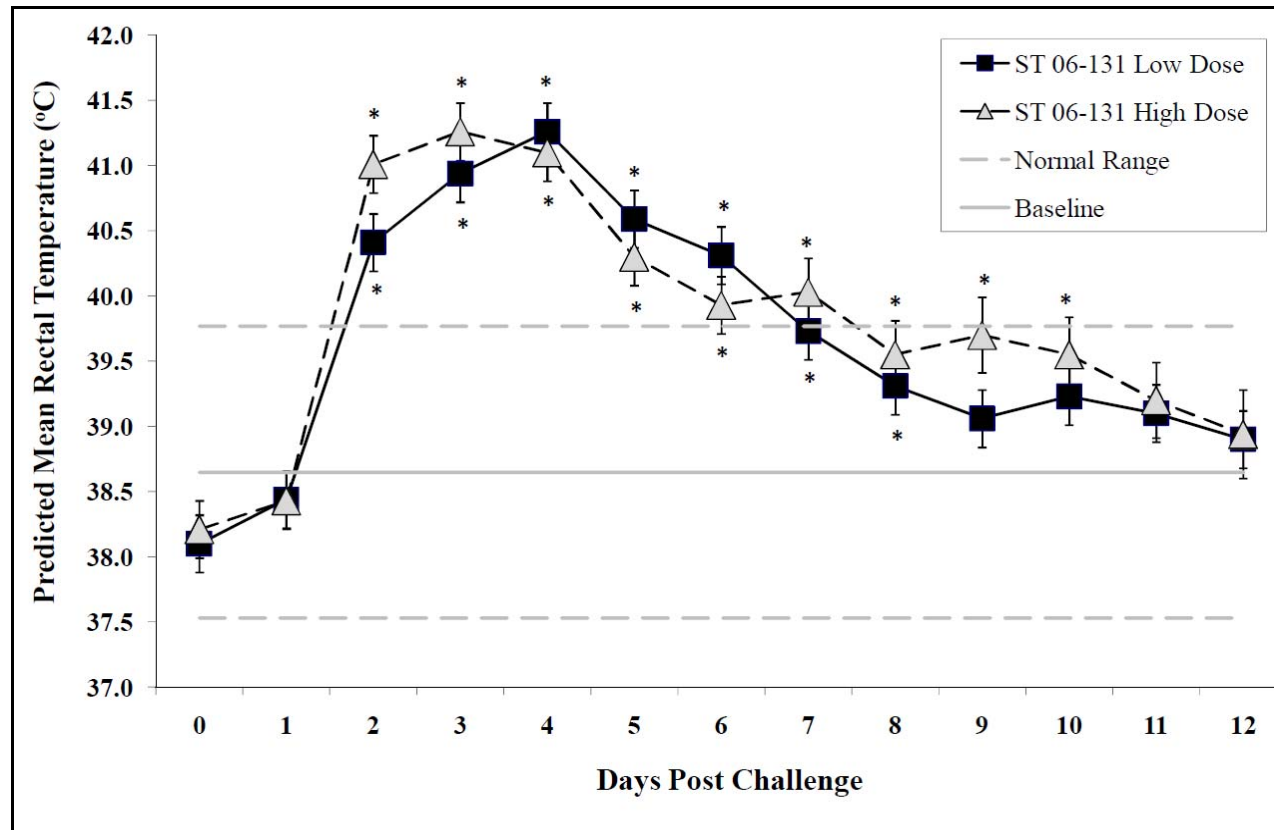


Figure 6-1. Predicted mean rectal temperatures of sheep following challenge with ST 06-131

RT were measured daily for 12 d following challenge. Data are depicted as a predicted mean RT  $\pm$  SE. The data are presented in the context of the normal range (dashed grey lines) and baseline (solid grey line) established pre-vaccination. \*RT were significantly increased when compared to baseline on days 2 to 10 and on days 2 to 8 post-virulent challenge in the high and low dose groups, respectively ( $P < 0.05$ ).

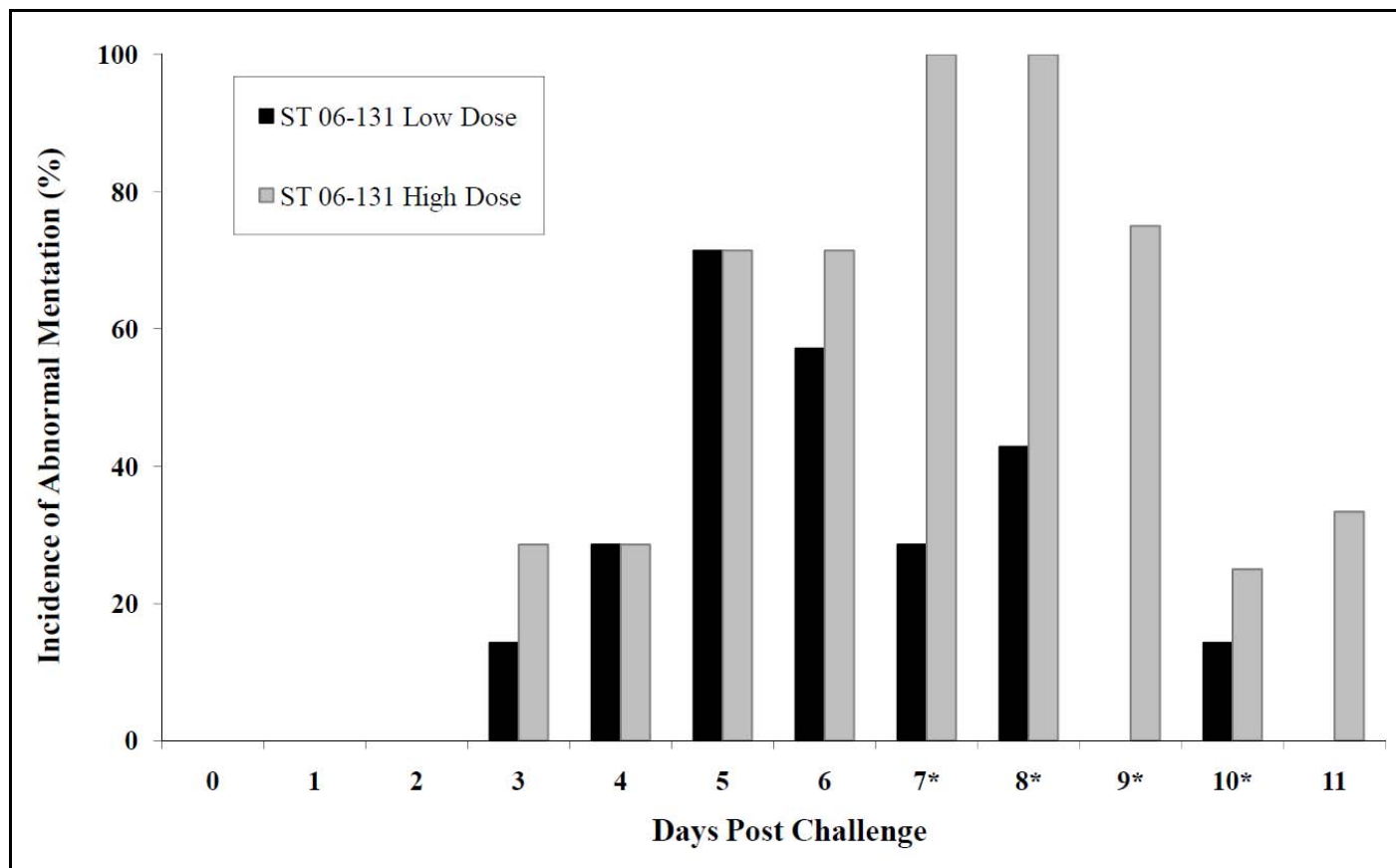


Figure 6-2. Incidence of abnormal mentation in sheep following challenge with ST 06-131

Data are depicted in percentage of sheep with abnormal mentation. \*The incidence of sheep with abnormal mentation following challenge was significantly increased in the high dose group on days 7 to 10 post-challenge (\* $P = 0.05$ ), and over the 12 d observation period ( $P < 0.005$ ).



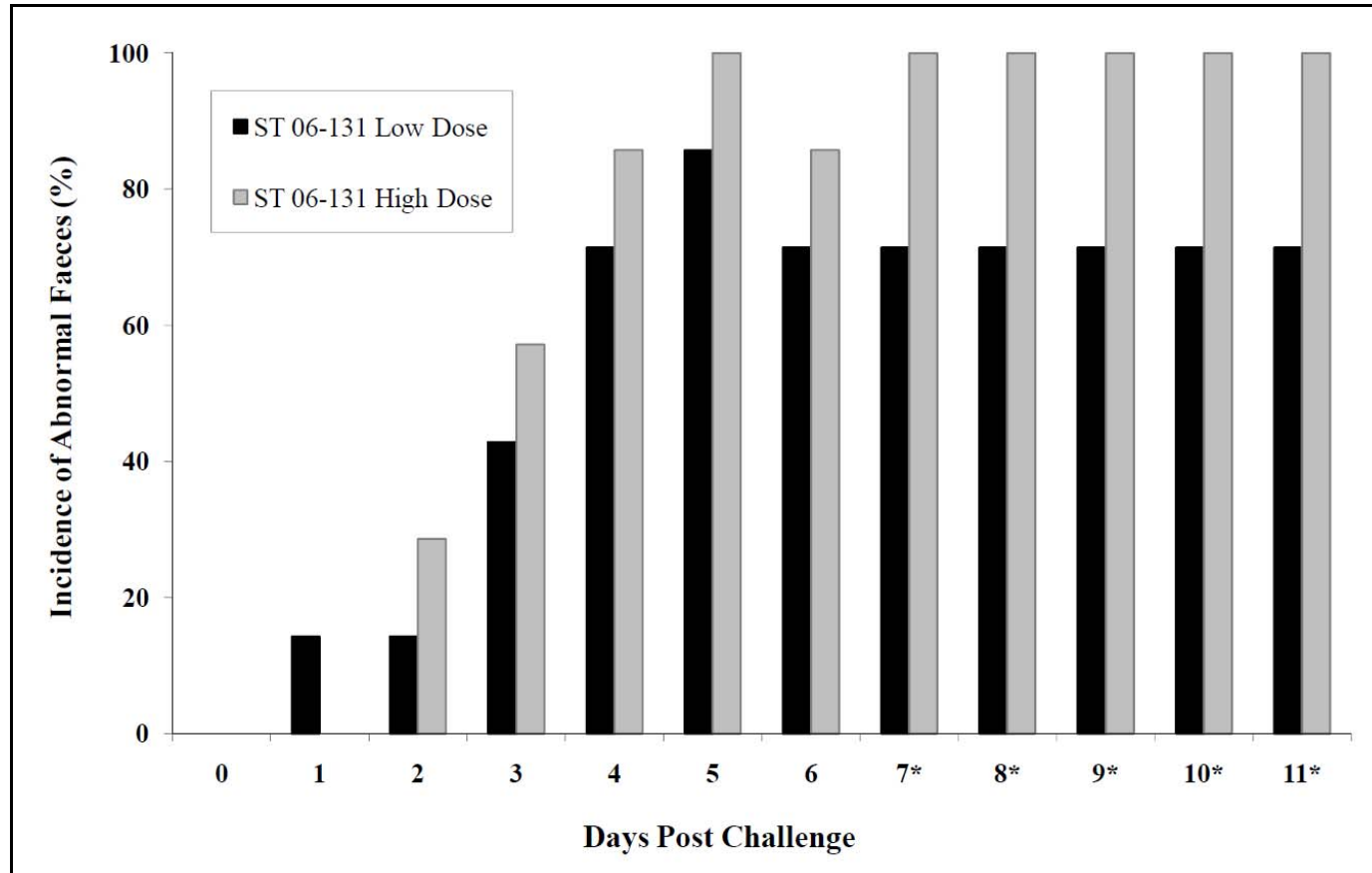


Figure 6-3. Incidence of abnormal faeces in sheep following challenge with ST 06-131

Faecal composition was assessed twice a day. Data are depicted as percentage of sheep with abnormal faeces following challenge. \*Significantly lower incidence of diarrhoea was observed in the low dose group when compared to the high dose group days 7 to 11 post challenge ( $P < 0.05$ ).

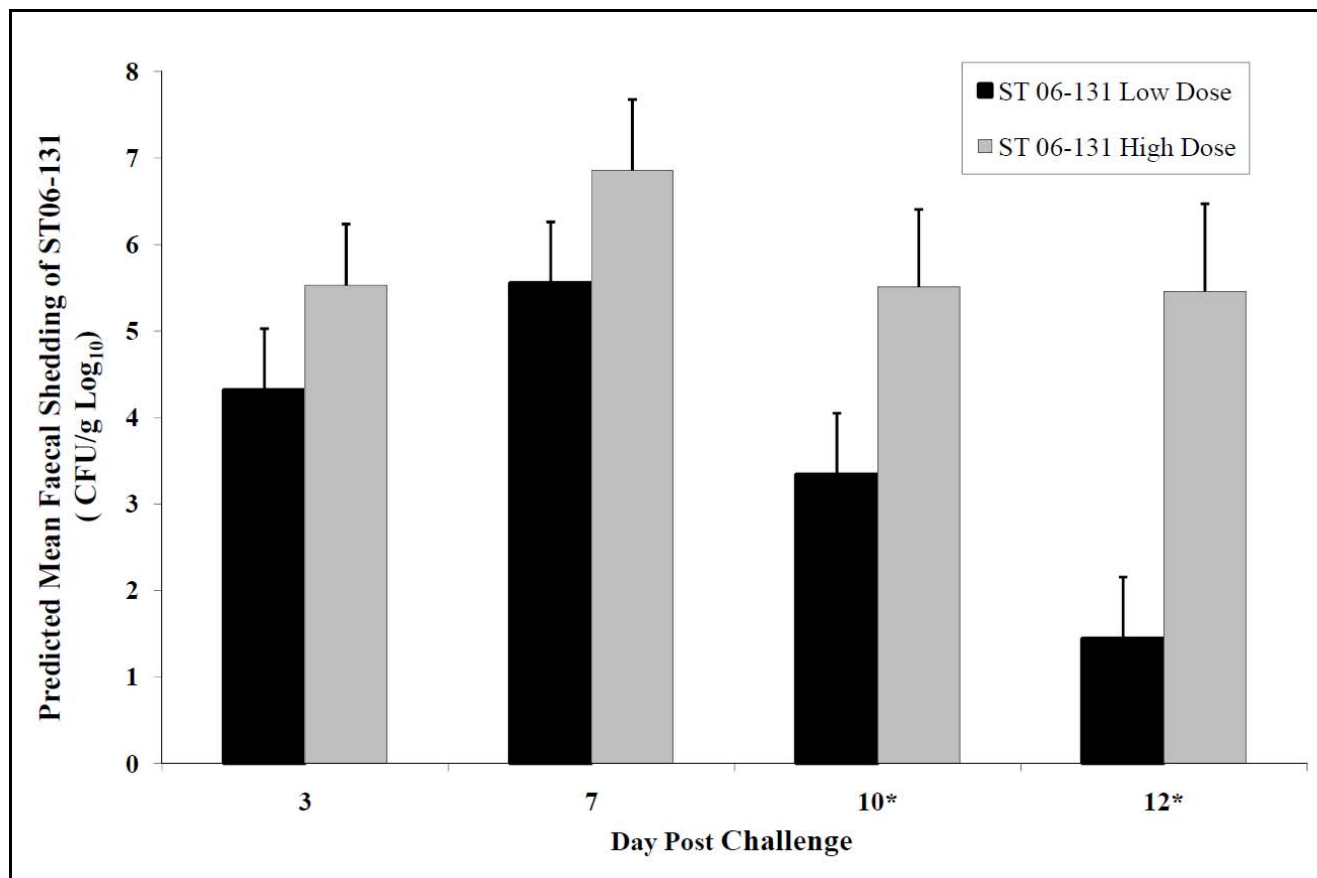


Figure 6-4. Predicted mean faecal shedding of following challenge with ST 06-131 in sheep.

Data are depicted as a predicted mean log<sub>10</sub> CFU of ST 06-131 per g of faeces post-challenge ± SE. \*Significant differences in faecal shedding were observed on days 10 and 12 post-challenge between the low and high dose group ( $P < 0.05$ ).

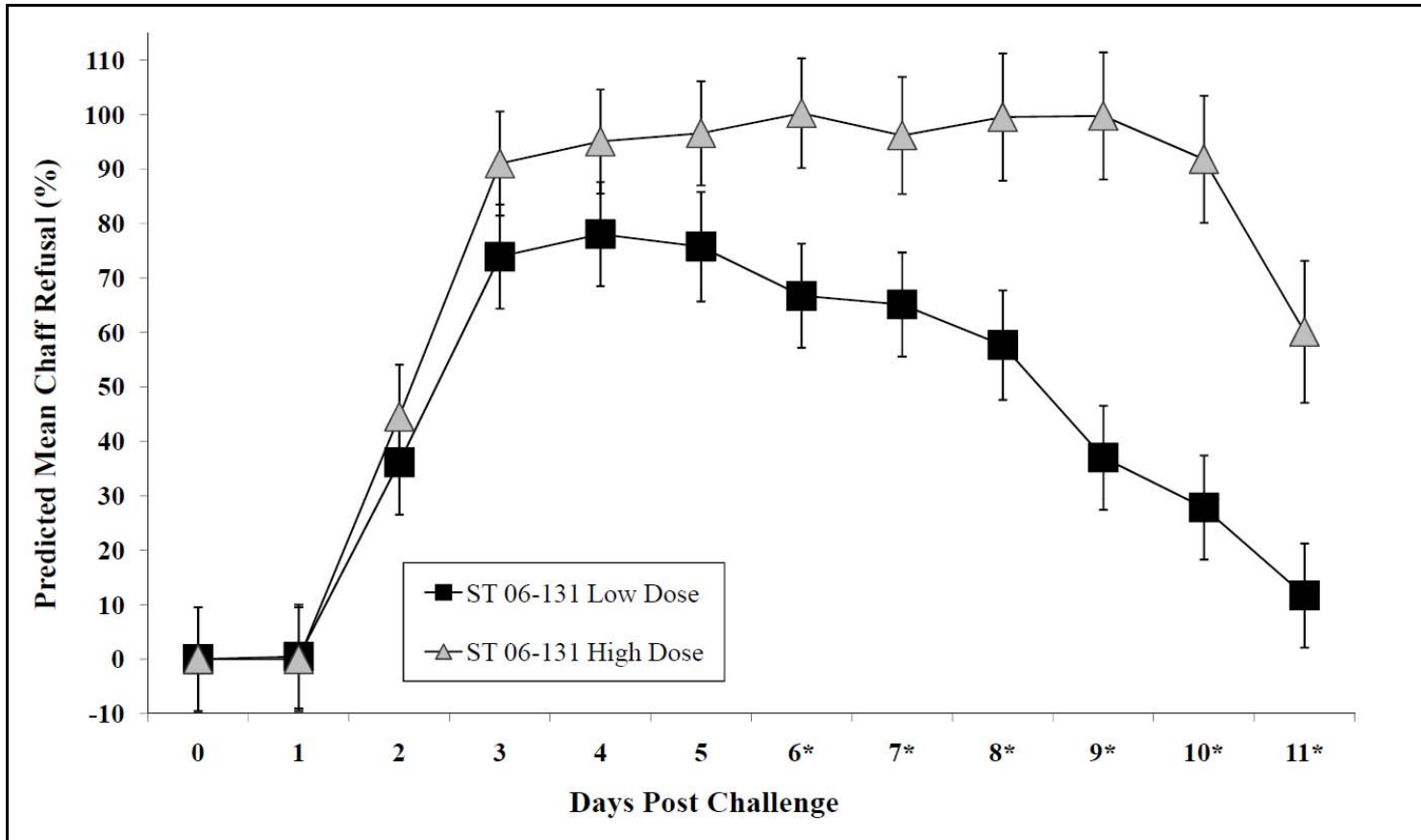


Figure 6-5. Predicted mean chaff refusal in sheep following ST 06-131 challenge.

Predicted mean chaff refusal is depicted  $\pm$  SE. \*Chaff refusal was significantly lower in the low dose group when compared to the high dose from days 6 to 11 post-challenge ( $P < 0.05$ ) and over the study period ( $P < 0.001$ ).

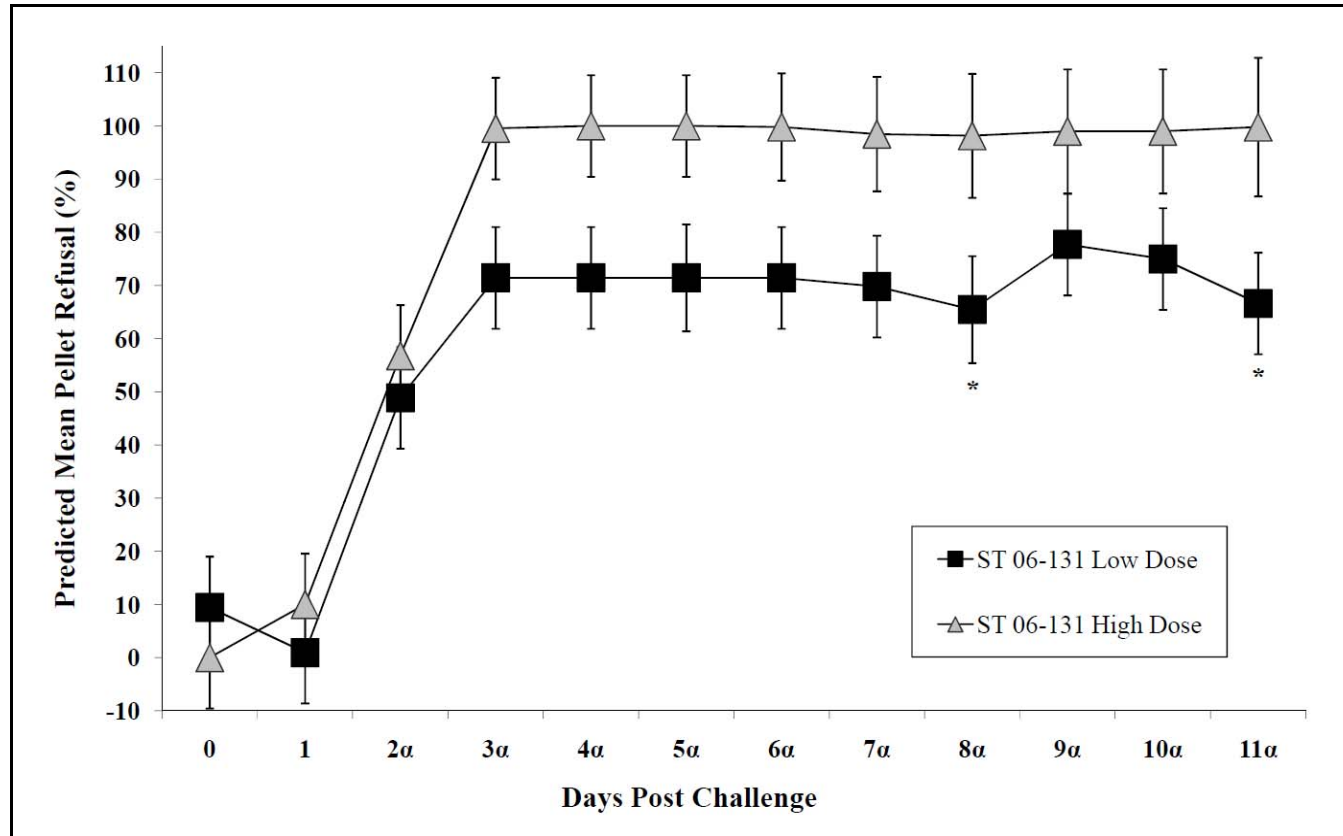


Figure 6-6. Predicted mean pellet refusal in sheep following ST 06-131 challenge

Predicted mean pellet refusal is depicted  $\pm$  SE. Pellet refusal was significantly reduced in the low dose group when compared to the high dose on days 8 and 11 post-challenge ( $\alpha P < 0.05$ ). Pellet refusals in both groups was significantly increased days 2 to 11 when compared to day 0 refusals ( $\alpha P < 0.05$ ).

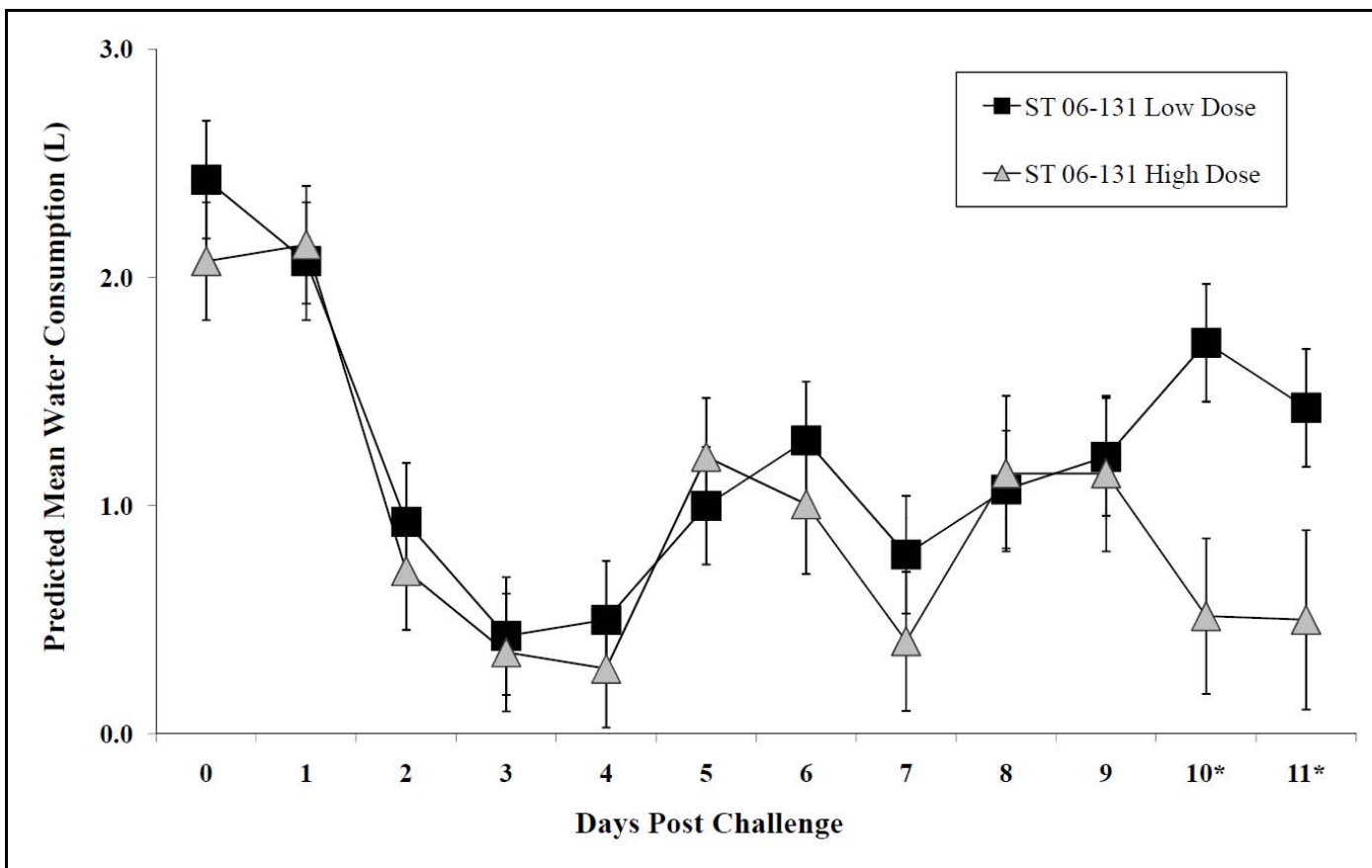


Figure 6-7. Predicted mean water consumption in sheep following ST 06-131 challenge

Predicted mean water consumption in L is depicted  $\pm$  SE. Water consumption was significantly improved in the low dose group when compared to the high dose on days 10 and 11 post-challenge ( $\alpha P < 0.05$ ).

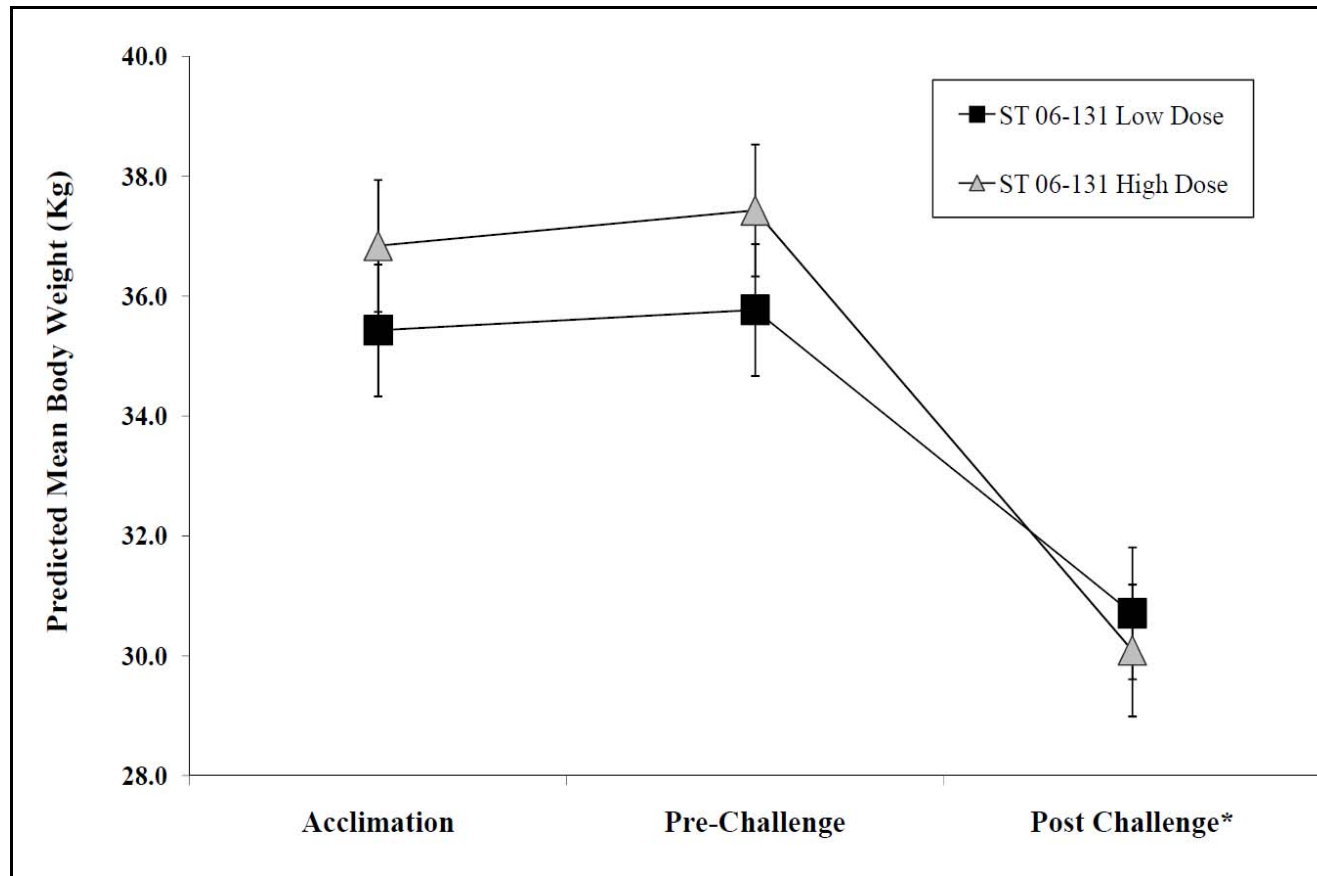


Figure 6-8. Predicted mean body weights of sheep following ST 06-131 challenge

Data are depicted in predicted mean BWT in Kg  $\pm$  SE at acclimation, pre-challenge and day 12 post-challenge. \*BWT of the low and high dose groups were significantly decreased on day 12 post challenge when compared to pre-challenge and acclimation weights ( $P < 0.05$ ).

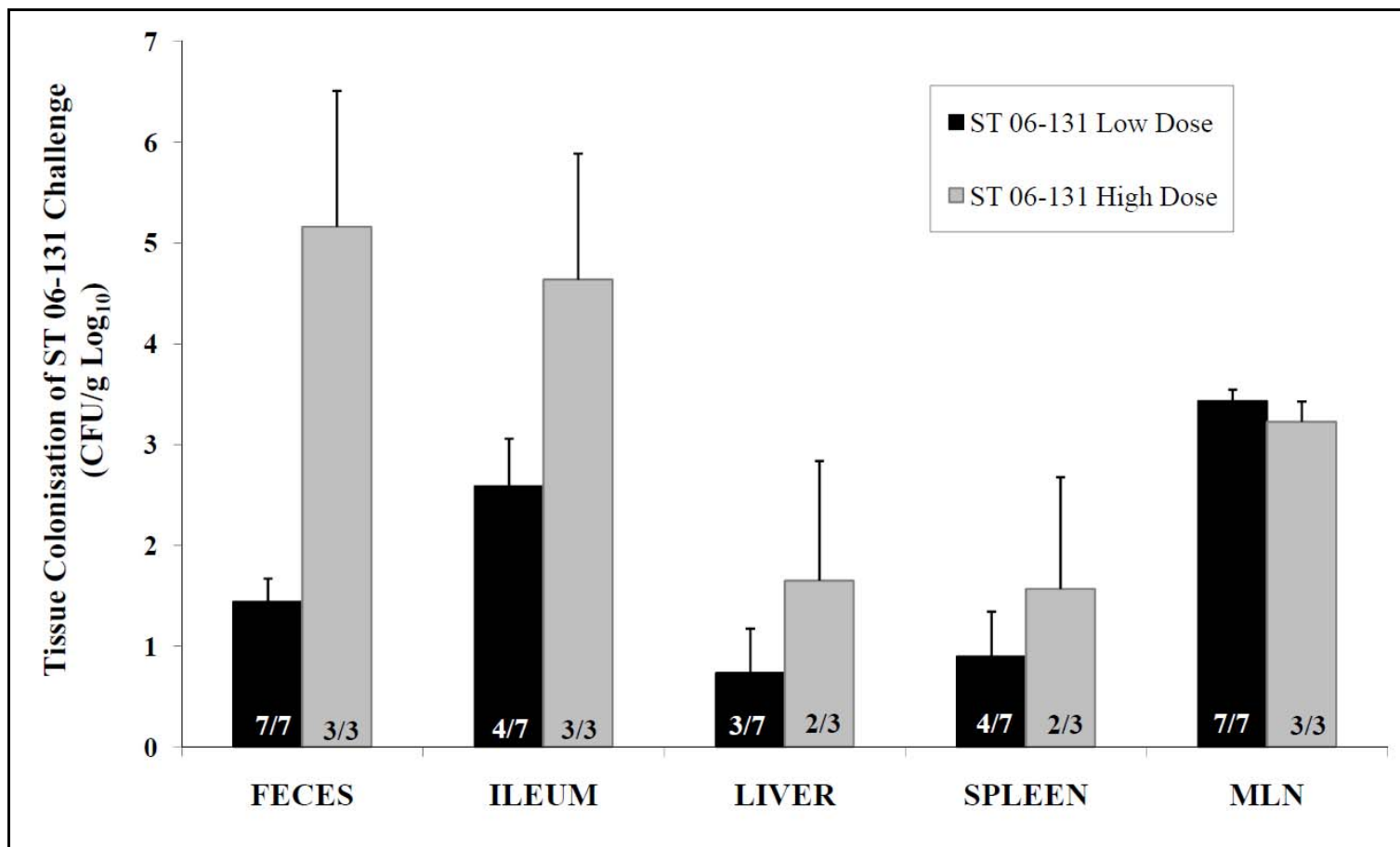


Figure 6-9. Colonisation of tissues with ST 06-131 in challenged sheep.

Tissue colonisation data are depicted as mean log<sub>10</sub> CFU ± SE of ST 06-131, recovered by organ.

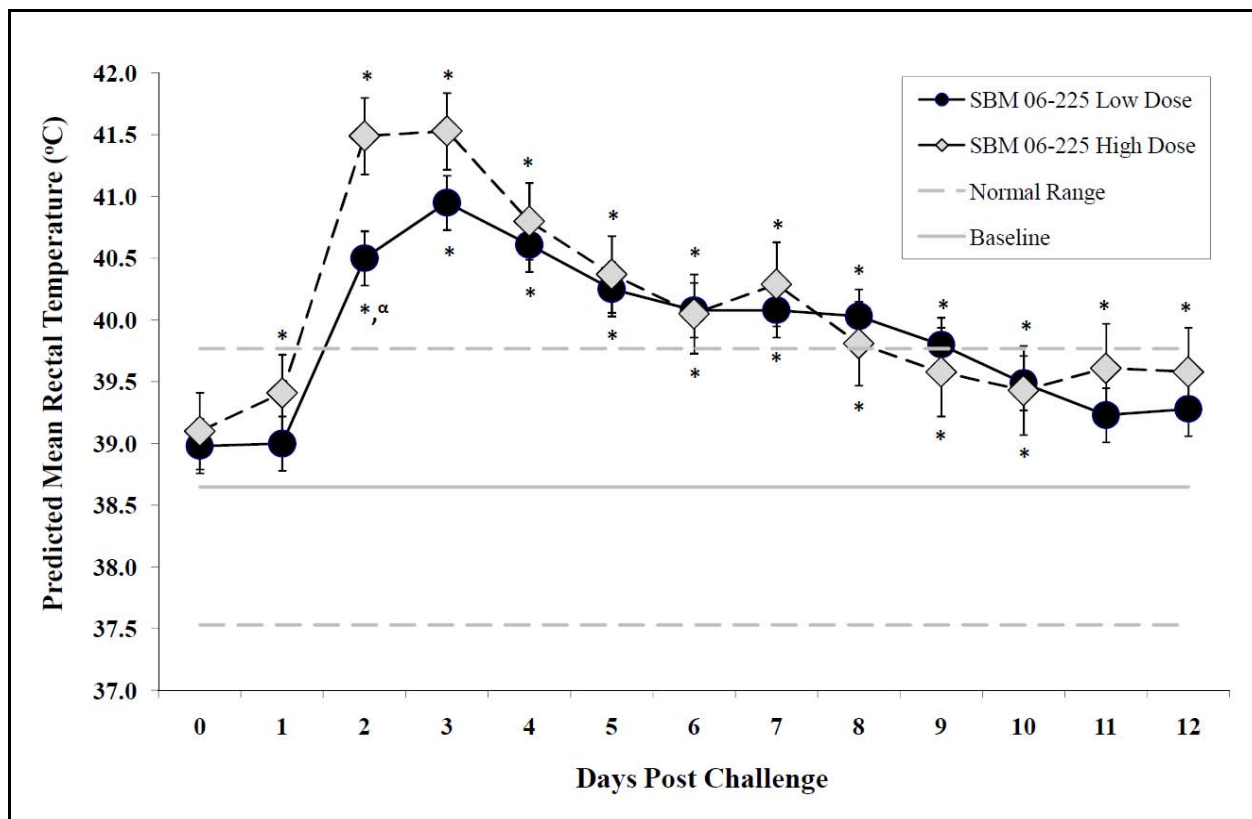


Figure 6-10. Predicted mean rectal temperatures of sheep following challenge with SBM 06-225

RT were measured daily for 12 d following challenge. Data are depicted as a predicted mean RT  $\pm$  SE. The data are presented in the context of the normal range (dashed grey lines) and baseline (solid grey line) established pre-challenge. \*RT were significantly increased when compared to baseline on days 1 to 12 and on days 2 to 10 post-virulent challenge in the high and low dose groups, respectively ( $P < 0.05$ ). And the low dose was significantly lower when compared to the high dose on day 2 post-challenge ( $\alpha P < 0.05$ ).



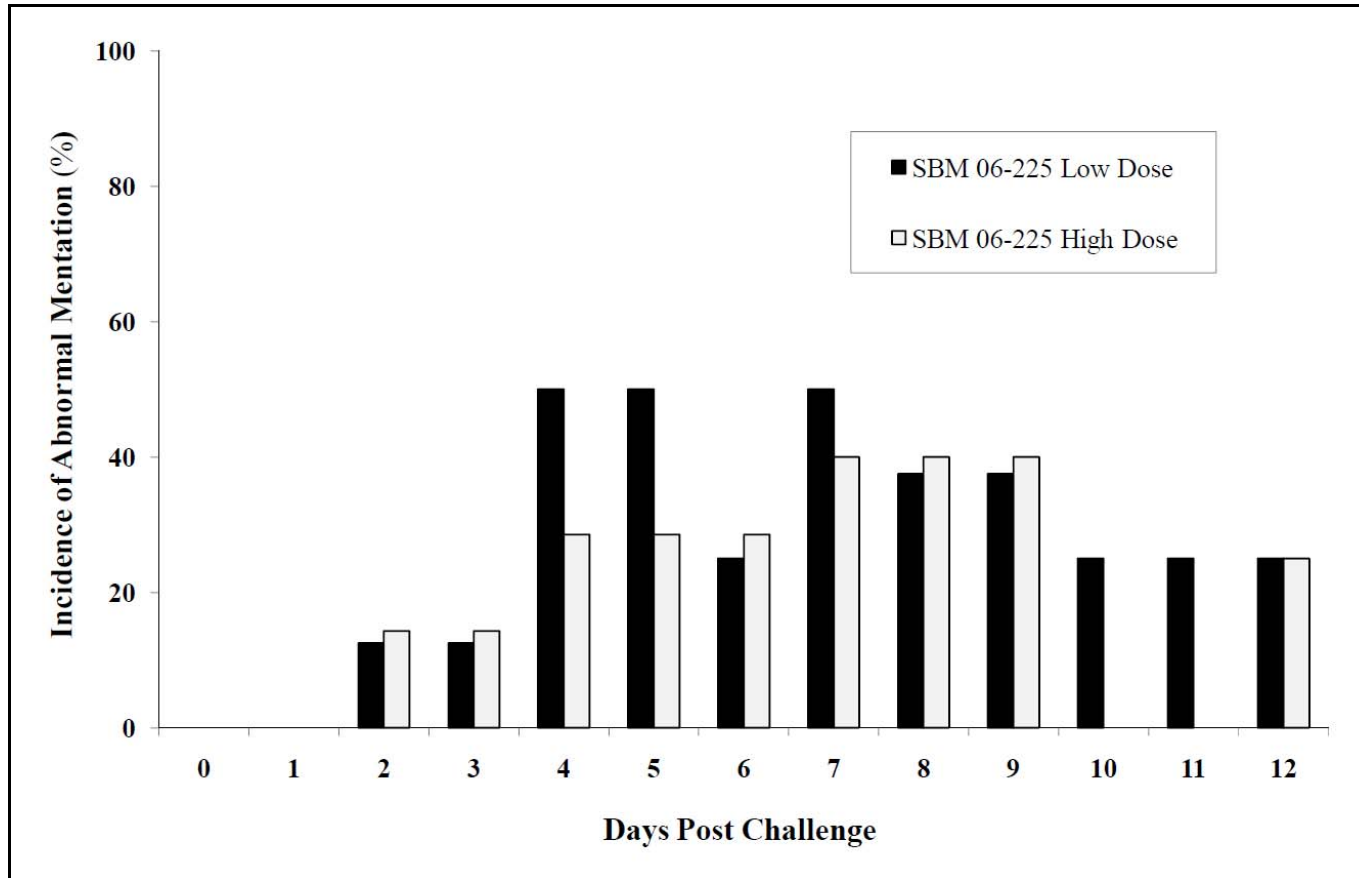


Figure 6-11. Incidence of abnormal mentation in sheep following challenge with SBM 06-225

Data are depicted in percentage of sheep with abnormal mentation. Administration of the SBM 06-225 had an effect on mentation over the 12 d observation period ( $P = 0.0001$ ).

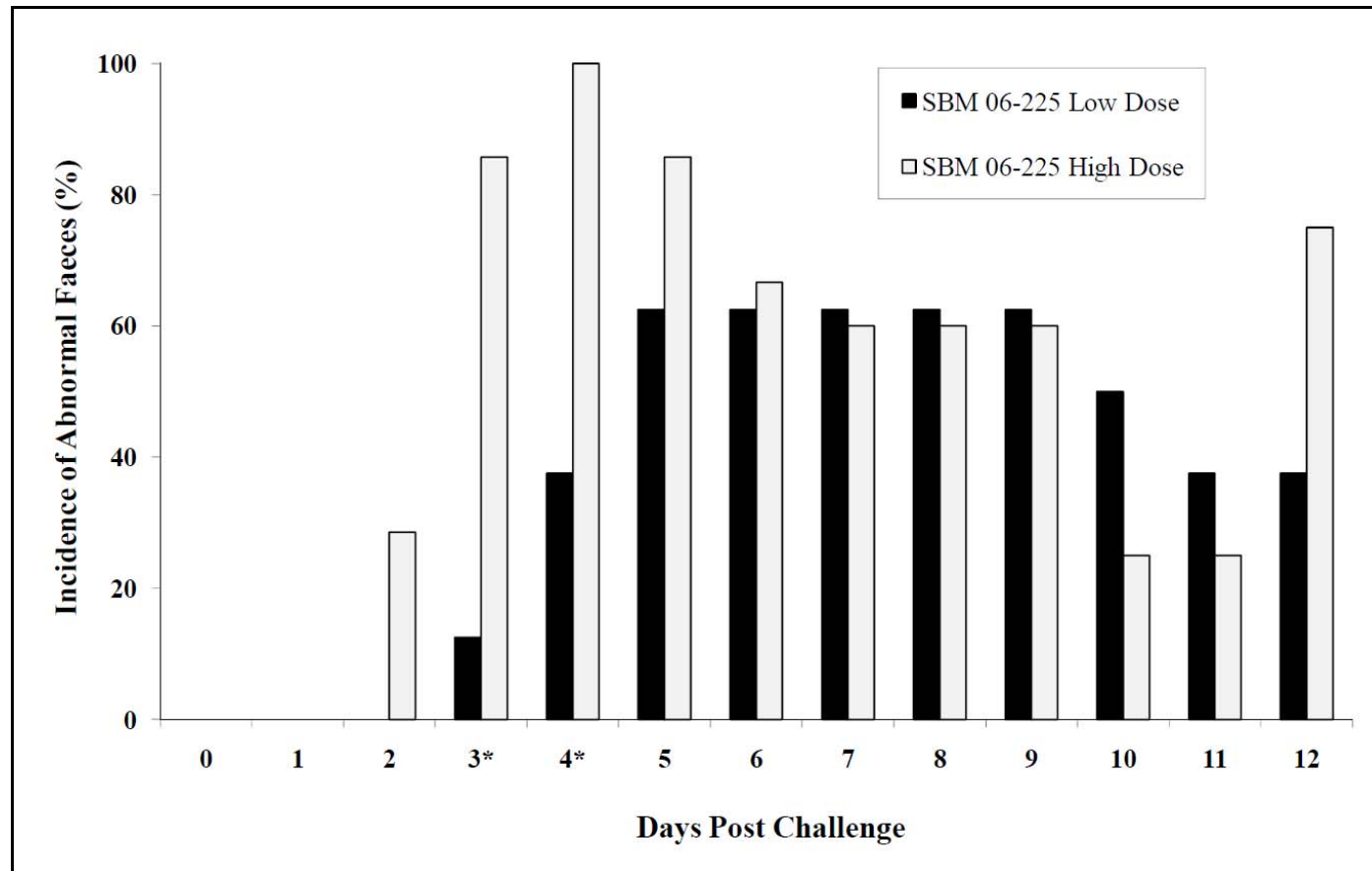


Figure 6-12. Incidence of abnormal faeces in sheep following challenge with SBM 06-225  
 Faecal composition was assessed twice a day. Data are depicted as percentage of sheep with abnormal faeces following challenge. \*Significantly lower incidence of diarrhoea was observed in the low dose group when compared to the high dose group days 3 and 4 post challenge ( $P < 0.05$ ).

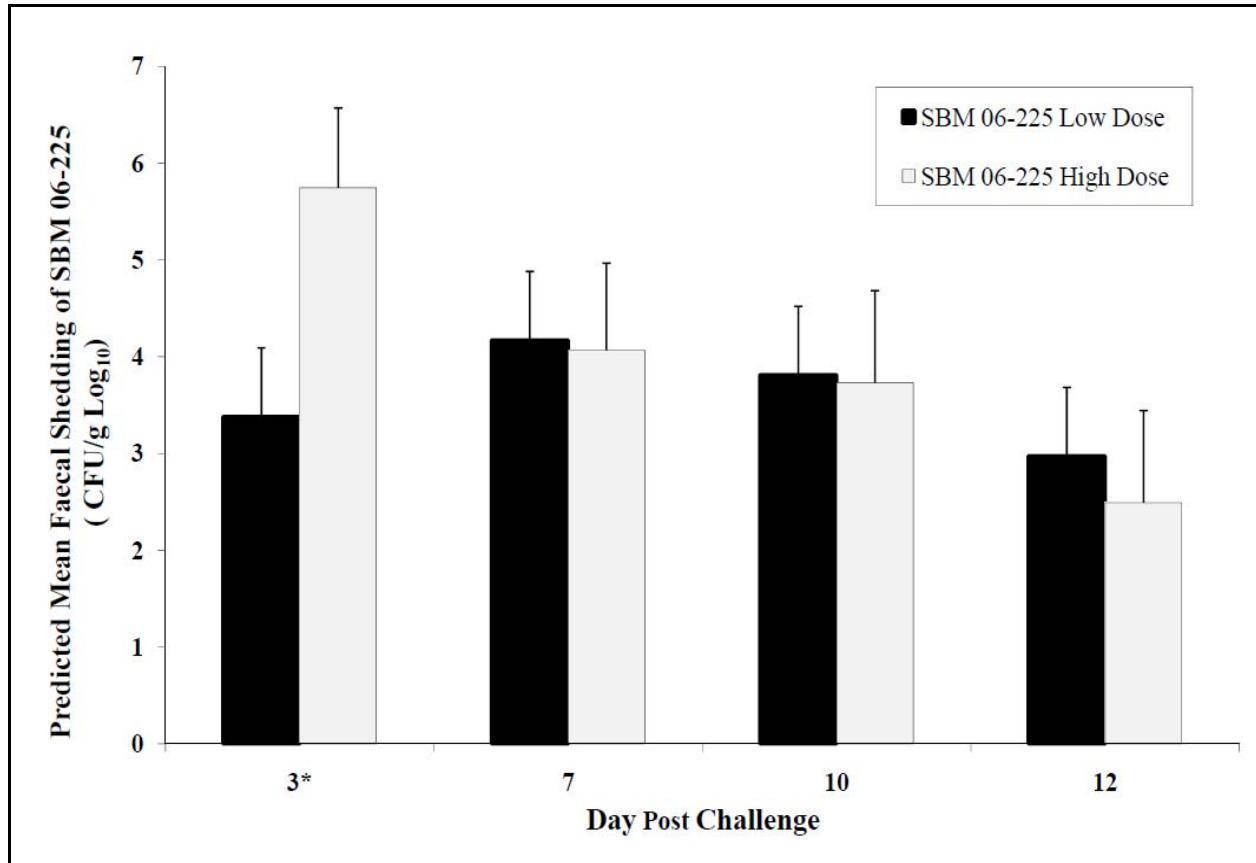


Figure 6-13. Predicted mean faecal shedding of following challenge with SBM 06-225 in sheep.

Data are depicted as a predicted mean log<sub>10</sub> CFU of SBM 06-225 per g of faeces post-challenge ± SE. \*Significant differences in faecal shedding were observed between the low and high dose group on day 3 post-challenge ( $P < 0.05$ ).

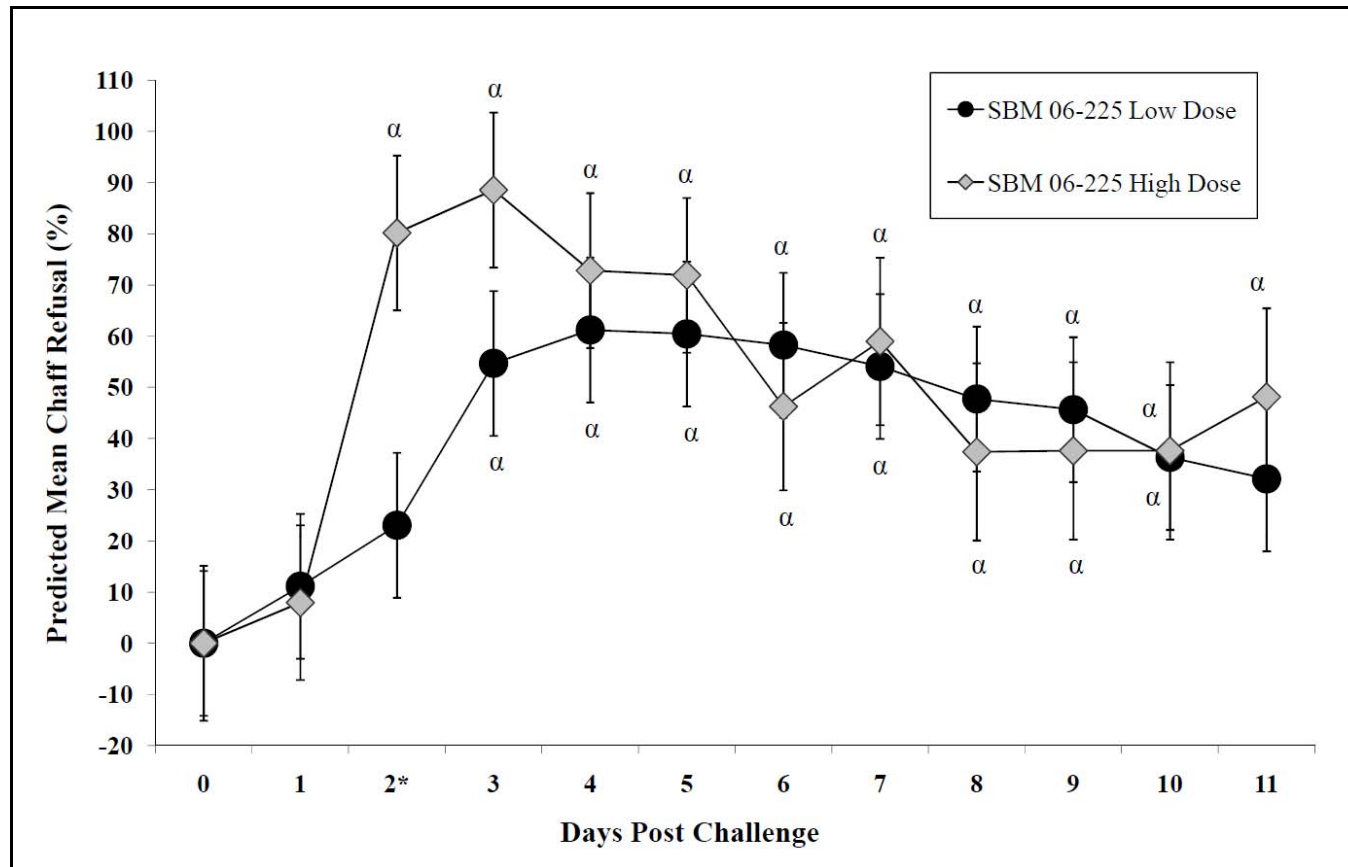


Figure 6-14. Predicted mean chaff refusal in sheep following SBM 06-225 challenge

Predicted mean chaff refusals are depicted  $\pm$  SE. Chaff refusal was significantly reduced in the low dose group when compared to the high dose on day 2 post-challenge ( $*P < 0.05$ ) and significantly increased compared to day 0 refusal ( $\alpha P < 0.05$ ).

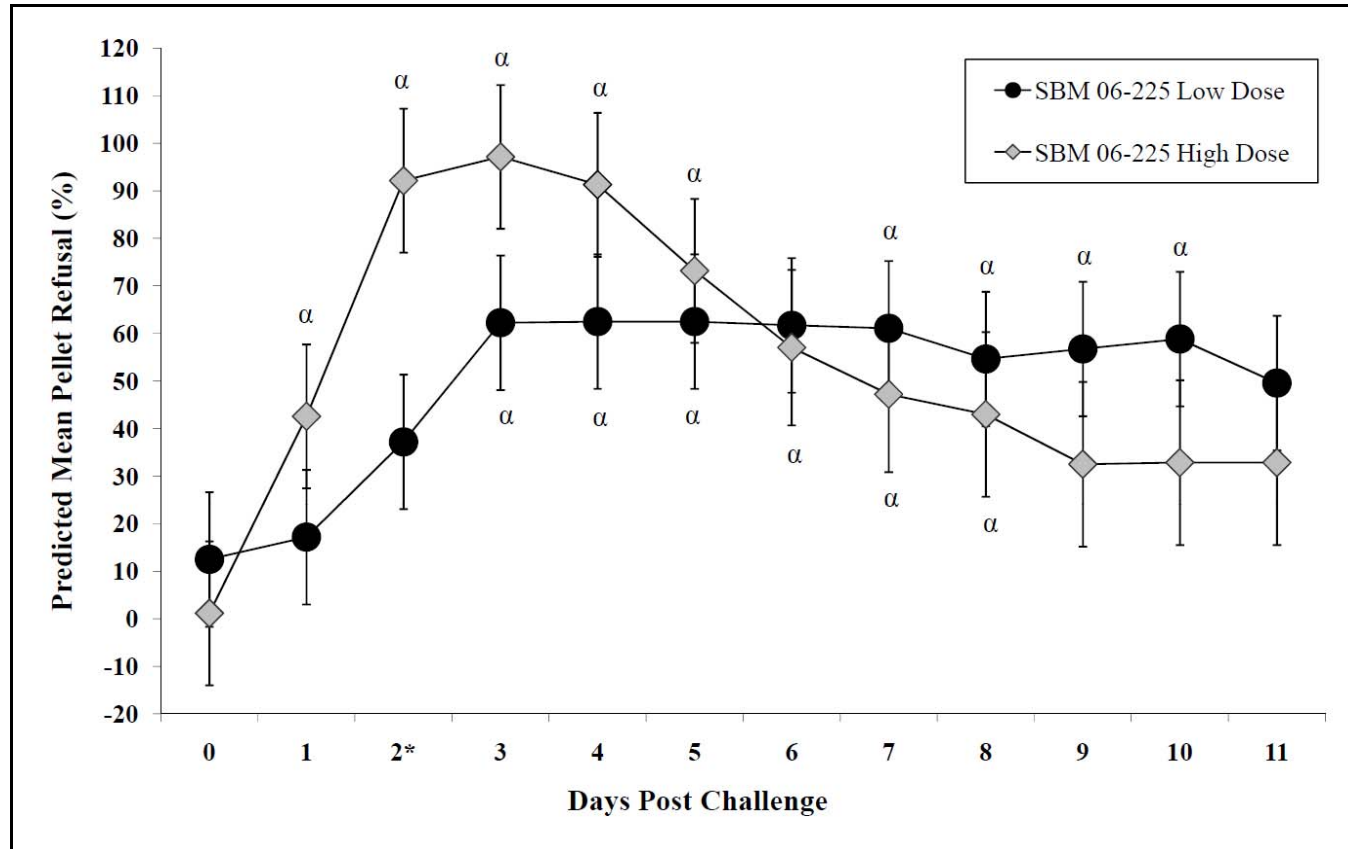


Figure 6-15. Predicted mean pellet refusal in sheep following SBM 06-225 challenge.

Predicted mean pellet refusals are depicted  $\pm$  SE. Pellet refusal was significantly reduced in the low dose group when compared to the high dose on day 2 post-challenge ( $*P < 0.05$ ) and significantly increased when compared to day 0 refusal ( $\alpha P < 0.05$ ).

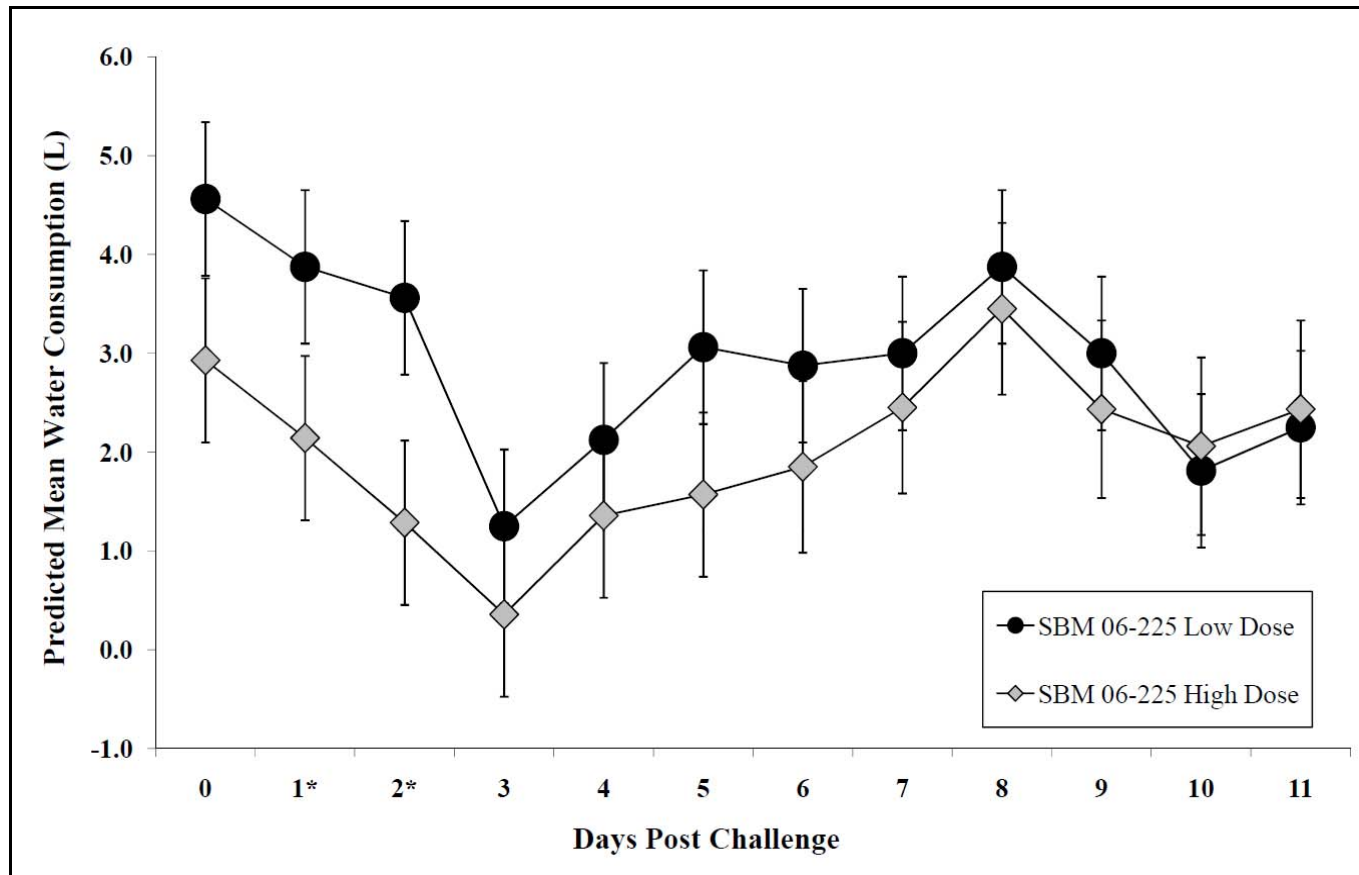


Figure 6-16. Predicted mean water consumption in sheep following SBM 06-225 challenge

Predicted mean water consumption in L is depicted  $\pm$  SE. \*Water consumption was significantly improved in the low dose group when compared to the high dose on days 1 and 2 post-challenge ( $P < 0.05$ )

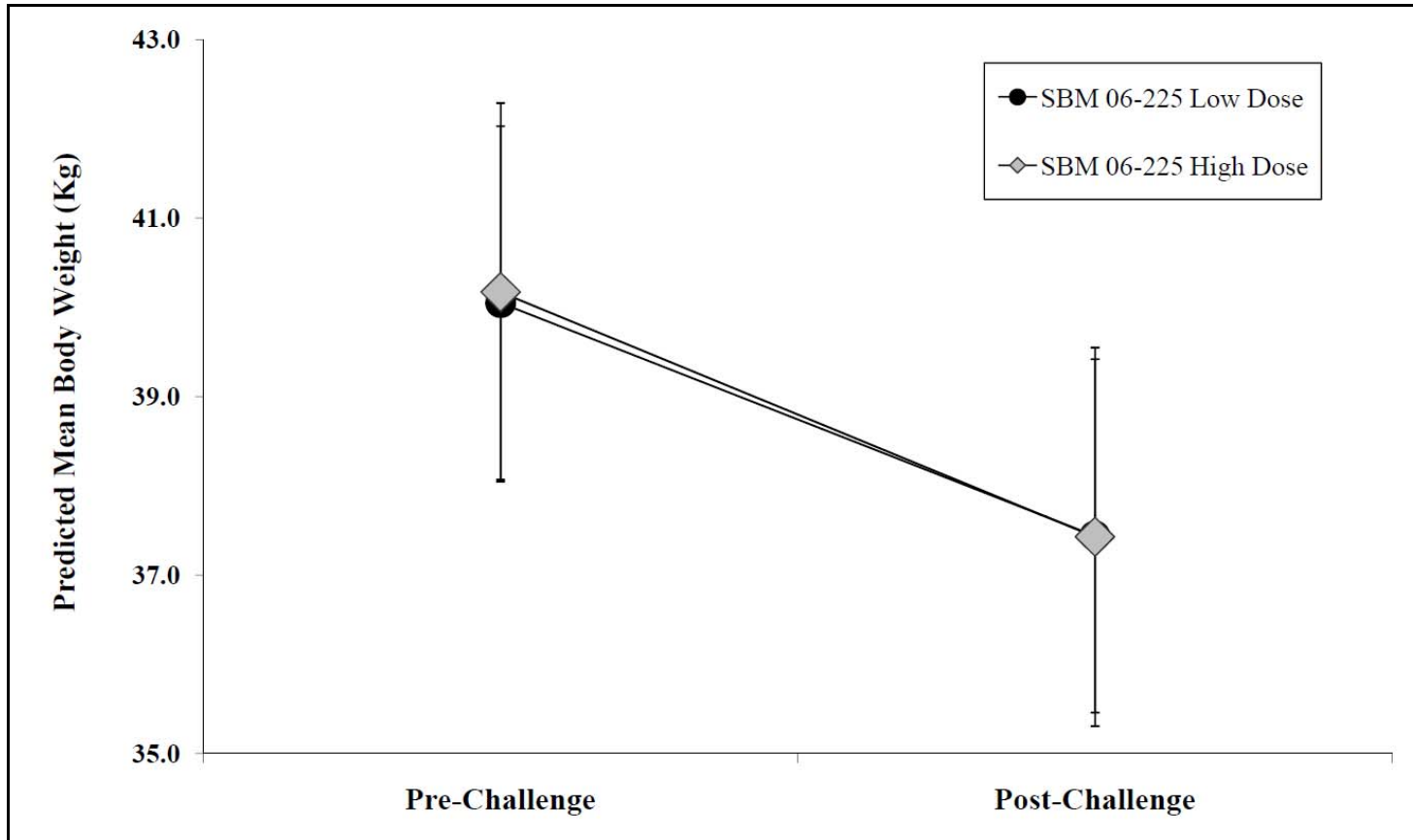


Figure 6-17. Predicted mean body weights of sheep following SBM 06-225 challenge

Data are depicted in predicted mean BWT in Kg  $\pm$  SE pre-challenge and on day 12 post-challenge. No differences were observed in the body weights of the sheep post-challenge ( $P = 0.963$ ).

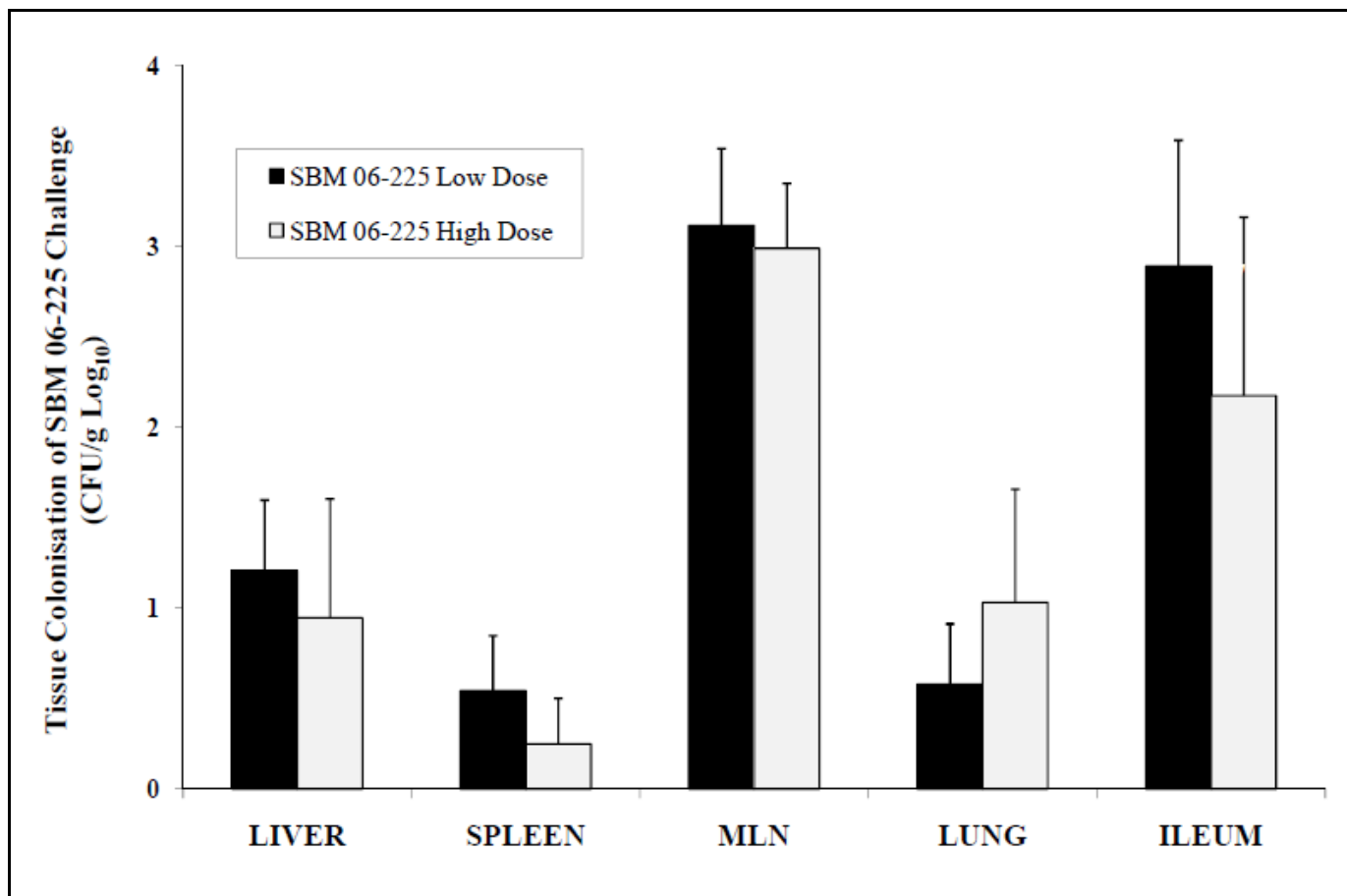


Figure 6-18. Colonisation of tissues with SBM 06-225 in challenged sheep.

Tissue colonisation data are depicted as mean log<sub>10</sub> CFU/g ± SE of SBM 06-225, recovered by organ.



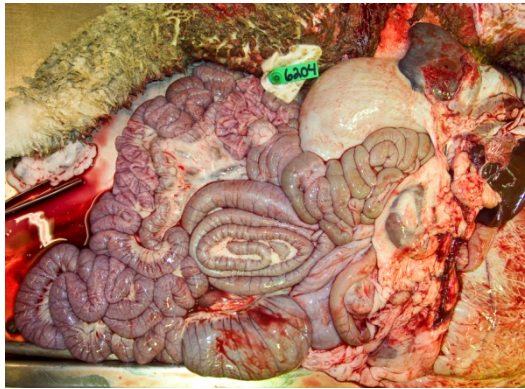
Figure 6-19. Necropsy findings of sheep challenged with *Salmonella*



(A) Enlarged mesenteric lymph node



(B) Haemal lymph nodes within the mesentery



(C) Enteritis in small and large intestines



(D) Serosal petechiation and reddening



(E) Caecum - mucosal congestion ("tiger striping")

## **CHAPTER 7. Development of a Novel In-Water Vaccination Protocol for DNA Adenine Methylase Deficient *Salmonella enterica* serovar Typhimurium Vaccine in Adult Sheep**

The following is a re-formatted manuscript, currently under review for publication:

Mohler, V.L., Heithoff, D.M., Mahan, M.J., Hornitzky, M.A., Thomson, P.C., House, J.K. (2011). Development of a Novel In-Water Vaccination Protocol for DNA Adenine Methylase Deficient *Salmonella enterica* serovar Typhimurium Vaccine in Adult Sheep. *Vaccine* (currently under review)

The study was designed, executed and reported by the candidate under the general supervision of the remaining authors, who were the candidate's appointed supervisors (principal and associate).

### **7.1 Introduction**

*Salmonella* continues to be one of the most common causes of gastroenteritis in the developed world. There are over 2,500 different serotypes of *Salmonella*; all are potentially capable of causing disease in livestock and humans (Garrity, *et al.*, 2004; Popoff, *et al.*, 2004; WHO, 2005). Human disease is commonly linked to the consumption of contaminated foodstuffs such as poultry, eggs, milk, meat and vegetables (Pacer, *et al.*, 1986; CDC, 1996; Glynn, *et al.*, 1998; Mead, *et al.*, 1999; Mead, *et al.*, 2000; CDC, 2002, 2005; WHO, 2005; CDC, 2006). Animal production management strategies to prevent disease have not been successful and antimicrobial use in salmonellae contaminated facilities is causing growing concern regarding emerging antimicrobial resistance (WHO, 1997; CDC, 2002; Mølbak, 2005; WHO, 2005; CDC, 2008).

Recent innovations have resulted in the development of modified live *Salmonella* vaccines that elicit cross-protective immunity to homologous and heterologous

salmonellae serovars (Jelinek, *et al.*, 1982a; Smith, *et al.*, 1984; Begg, *et al.*, 1990; Hassan and Curtiss, 1994a; Heithoff, *et al.*, 2001; Nagy, *et al.*, 2004; Heithoff, *et al.*, 2008a). These vaccine candidates are attenuated live *Salmonella* that contain mutations in global regulatory networks that are thought to increase antigen exposure and/or modulate the immune response to confer protection. While vaccination is generally a useful strategy to control infectious diseases, it has also been associated with increased production costs, carcass damage and adverse reactions (Bowersock and Martin, 1999; Giudice and Campbell, 2006; Murphy, *et al.*, 2008; Savini, *et al.*, 2008; Mamak and Aytakin, 2009).

To address these livestock-production challenges, the use of in-water medication and vaccination to control and prevent disease is a common practice in intensively managed poultry (Vermeulen, *et al.*, 2002) and swine (Kolb, 1996; Agerso, *et al.*, 1998; Walter, *et al.*, 2001). Additionally, use of impregnated baits and sachets have been employed to successfully vaccinate feral dogs and wildlife against rabies (Bergman, *et al.*, 2008; Cliquet, *et al.*, 2008; Niin, *et al.*, 2008) and wild deer populations against tuberculosis (Nol, *et al.*, 2008). Conversely, very little information has been reported on the use of in-water or oral vaccination in livestock species such as cattle, goats and sheep, wherein the use of such practices may improve animal health and well-being, as well as conferring significant reductions in pre- and post- harvest bacterial loads.

*Salmonella* Typhimurium containing loss of function mutations in the gene encoding the DNA adenine methylase (*dam*) are attenuated for virulence and confer significant protection to homologous and heterologous *Salmonella* challenge when applied as modified live vaccines in mice (Garcia-Del Portillo, *et al.*, 1999; Heithoff, *et al.*, 1999; Heithoff, *et al.*, 2001; Heithoff, *et al.*, 2008a), poultry (Dueger, *et al.*, 2001, 2003a), and calves (Dueger, *et al.*, 2003b; Mohler, *et al.*, 2006; Mohler, *et al.*, 2008). The objective of the current studies was to

evaluate the stability and viability of the *S. Typhimurium dam* vaccine over a 24 h period for use in a novel in-water vaccination delivery system for livestock and to demonstrate the efficacy of in-water delivery to adult sheep.

## **7.2 Materials and Methods**

### ***7.2.1. Bacterial strains and growth conditions***

Immunisations were carried out with *dam*-102::Mud-Cm Typhimurium UK-1 vaccine strain MT2313 (*S. Typhimurium dam*) as described in *Section 3.2.1*. The wild-type Typhimurium strain MT2315 is a virulent derivative of strain UK-1, containing a Lac<sup>+</sup> MudJ transcriptional fusion which is used to discern it from other *Salmonella* which are inherently Lac<sup>-</sup> (Conner, *et al.*, 1998; Dueger, *et al.*, 2001).

Strains used in these stability studies were grown overnight in Luria broth at 37°C. Cells were re-suspended in phosphate buffered saline (PBS), 137mM 8.0 g/L of NaCl, (8.45mM) 1.2g/L Na<sub>2</sub>HPO<sub>4</sub>, (1.5mM) 0.2 g/L KH<sub>2</sub>PO<sub>4</sub>, and (2.7mM) 0.2g/L of KCl pH 7.2-7.4 for these studies, unless otherwise described. Dose titres were confirmed via serial dilutions plated on Luria agar plates; colony serotype was confirmed via standard biochemical tests (urea, TSI, ONPG) and agglutination with serogroup B specific antisera [somatic O antisera group B, factors 1, 4, 5, 12, and 27 (BD Diagnostics, USA)].

### ***7.2.2. Salmonella isolation from test solutions***

Samples were serially diluted into PBS 1:10, plated onto XLD and XLD containing chloramphenicol at 20 µg/mL, to select for the *S. Typhimurium dam* vaccine (XLD-CHL) and incubated for at least 24 h at 37°C. Several colonies from each experiment were evaluated using standard biochemical tests (urea, TSI, ONPG) and agglutinated with serogroup B specific antisera [somatic O antisera group B, factors 1, 4, 5, 12, and 27 (BD Diagnostics, USA)].

### **7.2.3. Stability of *S. Typhimurium dam* vaccine strain in water**

Twenty 50 mL polypropylene tubes were filled with 9 mL of sterile Milli-Q® water (DIH<sub>2</sub>O). Three tubes each were inoculated with 1 mL of either *S. Typhimurium dam* vaccine (*dam*<sup>-</sup>) or *S. Typhimurium dam*<sup>+</sup> (*dam*<sup>+</sup>) strains prepared following methods in *Section 2.1* and reconstituted in PBS to dose concentrations of 10<sup>4</sup>, 10<sup>6</sup> and 10<sup>8</sup> CFU/mL. The remaining two tubes served as negative controls. Samples were incubated at room temperature with loose caps. Viability of the bacteria strains was assessed 24, 48, 72, and 120 h post inoculation. Fixed effects used to analyse viability and stability of the organisms were time, strain and inoculation dose.

### **7.2.4. Stability of the *S. Typhimurium dam* vaccine in trough water with and without faecal contamination**

Ten litres of trough water were collected from the Mayfarm Sheep Yards at the University of Sydney (Camden, NSW, Australia) for use in this experiment. Faeces used in this trial were obtained from sheep and determined to be *Salmonella* free via inoculation into enrichment cultures. At time zero ( $t = 0$ ), 3 mL of *S. Typhimurium dam* vaccine was prepared following methods in *Section 2.1* and reconstituted in PBS, then inoculated into 500 mL polypropylene jars containing solutions of either 297 mL of sterile DIH<sub>2</sub>O ( $n=6$ ), 297 mL trough water (TRW;  $n=6$ ) or 294 mL of trough water with 3 g sheep faeces (pellets) (TRW+F;  $n=6$ ). The final concentration of the *S. Typhimurium dam* vaccine in each jar was approximately 10<sup>4</sup> CFU/mL. Samples were incubated at room temperature with loose caps. To determine the homogeneity of the *S. Typhimurium dam* vaccine in drinking water, 3 jars from each group were vortexed prior to sampling (vortex) and the remaining 3 jars from each group were sampled from the upper layer of the sample (still) at each time point. Viability of the vaccine was assessed at 6, 24 and 48 h post inoculation. Fixed effects used to

analyse CFU/mL of the *S. Typhimurium dam* vaccine were time, solution and sampling method.

#### ***7.2.5. Stability and viability of the *S. Typhimurium dam* vaccine in buffered water***

In this experiment, the *S. Typhimurium dam* vaccine was prepared following methods in *Section 7.2.1* and reconstituted in sterile de-ionised water, then inoculated into 50 mL polypropylene tubes containing 19 mL of either DIH<sub>2</sub>O, PBS or PBS serial diluted 1:1 with DIH<sub>2</sub>O, to give solutions of 0.5X PBS, 0.25X PBS, 0.125X PBS, 0.062X PBS or 0.031X PBS. The final concentration of the *S. Typhimurium dam* vaccine in each tube was approximately 10<sup>5</sup> CFU/mL at  $t = 0$ . Viability of the *S. Typhimurium dam* vaccine was assessed at 24 and 48 h post inoculation. Fixed effects used to analyse CFU/mL of the *S. Typhimurium dam* vaccine were time and PBS concentration (solution).

#### ***7.2.6. Effect of temperature on the viability of the *S. Typhimurium dam* vaccine in buffered water***

The *S. Typhimurium dam* vaccine was prepared following methods in *Section 7.2.1* and reconstituted in PBS, then inoculated into 500 mL polypropylene tubes containing 199 mL of either PBS (n=6), 0.2X PBS [PBS dilute 1:5 in DIH<sub>2</sub>O] (n=6) or DIH<sub>2</sub>O (n=6) test solutions. The final concentration of the *S. Typhimurium dam* vaccine in each jar was approximately 10<sup>4</sup> CFU/mL. Three jars from each test solution were maintained at 37°C in an incubator and 3 jars were kept at room temperature (range: 22-26°C). Viability of the *S. Typhimurium dam* vaccine and the pH of each the test solutions (CyberScan pH 510, Eutech Instruments, AU) were assessed at 6, 12 and 24 h post inoculation. One 200 mL jar of each solution without vaccine served as the controls. The controls were maintained at room temperature throughout the experiment and pH measurements were performed in quadruplet and sterility was monitored at each time point.

Fixed effects used to analyse CFU/mL of the *S. Typhimurium dam* vaccine were time, test solution, temperature and pH.

**7.2.7. Delivery of *S. Typhimurium dam* vaccine to adult Merino wethers via drinking water**

**i. Sheep and husbandry**

Merino wethers, approximately 2 to 4 years in age, were sourced from a single flock in New South Wales, Australia. The flock was routinely monitored for, and determined to be free of, Johne's and virulent foot rot. Faecal samples were collected from all sheep prior to enrolment for qualitative detection of *Salmonella*. Approximately 1 g of faeces was placed into 10 mL mannitol selenite (MSB) broths, incubated for 24 h at 37°C, and then plated onto plain XLD. Suspect *Salmonella* colonies were sub-cultured for pure growth, tested for agglutination with *Salmonella* Test Kit [for detection of *Salmonella* Antigen (DR1108A) (Oxoid Ltd, UK)] and then assessed using standard biochemical tests (urea, TSI, ONPG). No *Salmonella* were detected in the sheep during the acclimation period. Sheep were transported to the Medium Security Animal Housing Facility at Elizabeth MacArthur Agricultural Institute (EMAI), Department of Industry & Investment (NSW, AU), and randomly assigned to experimental groups. Sheep were individually housed in wire mesh pens on raised mesh flooring. Each pen was equipped with three plastic all-purpose buckets for administration of water and feed. Biosafety protocols approved by the Australian Office of the Gene Technology Regulator (OGTR) for containment of PC2 level organisms were followed to prevent cross contamination between groups and prevent release of the vaccine into the environment. Sheep received a diet of oaten chaff (8 MJ ME/Kg DM) and commercially prepared grain pellet (12 MJ ME/Kg DM). Sheep were offered 600 g of oaten chaff and 300 g of grain pellets during morning, roughly 8-9 MJ ME/d.

## **ii. Clinical assessment**

Clinical parameters evaluated twice daily included attitude, water consumption, and faecal characteristics. Rectal temperatures were recorded in the mornings prior to feeding - twice a week during the week prior to vaccination, and daily following vaccination. The normal range of rectal temperatures for the sheep used in these experiments based on the pre-vaccination mean  $\pm$  two standard deviations was 37.6 – 39.5°C. Clinical pyrexia was defined as a sheep with a rectal temperature above 40°C (Radostits, *et al.*, 2007; Terra, 2009). Attitude and faecal composition were scored on an ordinal scale outlined in *Section 3.5.7*. Appetite was recorded in grams of chaff and pellets not consumed over each 24 h feeding period. Percent refusal of chaff and pellets was determined for each animal following vaccination. An attitude score of 4 at any time of the day, or failure to consume chaff or pellets (100% refusal of feed) for three consecutive feedings, constituted grounds for euthanasia. The study protocol was approved by the Animal Ethics Committee at EMAI.

## **iii. Experimental design**

A total of 22 sheep were used in the conduct of this study. Two vaccination trials were conducted using fifteen and seven sheep, respectively. In experiment A, fifteen sheep were randomly assigned to two dose groups on day -8:  $10^7$  CFU oral bolus vaccinates (n=7; [ $10^7$ OB]) and  $10^7$  CFU in-water vaccinates (n=8; [ $10^7$ IW]). In experiment B, seven sheep were assigned to a  $10^6$  CFU in-water vaccinate group (n=7; [ $10^6$ IW]). On day 6 during the acclimation period, all sheep received drinking water containing 20% phosphate buffered saline (0.2X PBS) to determine if the buffering agent would affect consumption prior to use during the vaccine trial.

Prior to vaccination, all sheep were food and water fasted overnight. The *S. Typhimurium dam* vaccine was delivered in the drinking water of the  $10^6$ IW and  $10^7$ IW vaccinates *ad libitum* over a 24 h period ( $t = -24$  h to 0 h) via plastic drinking buckets maintained within each pen. The vaccine was inoculated into



fresh water containing 20% PBS to a final volume of 6 L at the morning and evening observation periods. The target concentration of the vaccine was  $10^6$  and  $10^7$  CFU/L for the  $10^6$ IW and  $10^7$ IW vaccinate groups respectively. Consumption of water containing the *S. Typhimurium dam* vaccine was recorded to the nearest 0.1 L on vaccination day. The sheep in  $10^7$ OB vaccination group received the vaccine *per os*, reconstituted into 10 mL of PBS at  $t = 0$  h.

**iv. Faecal sampling**

Prior to initiating the study, faecal samples collected from the sheep were cultured for *Salmonella* as described in Section 3.6.1., *Salmonella* was not detected prior to vaccination of the sheep used in this trial. Faecal samples were assessed for shedding of the *dam* vaccine at 72 h post-vaccination. Between 2 and 5 g of faeces were collected per rectum of each sheep using individual disposable latex gloves. The faecal material was homogenised as a 1:4 dilution in sterile PBS, serially diluted, plated on XLD-CHL to select for the vaccine strain, and incubated for 24 h at 37°C. Additionally, 1 mL of the homogenised sample was placed into 9 mL of MSB, incubated for 24 h at 37°C, and streaked for single colonies onto XLD-CHL. The number of *Salmonella* organisms present in the faecal sample was calculated based on the sample weight, dilution factor, and number of colonies counted. Two colonies from each *Salmonella* positive faecal culture were sub-cultured and tested with O-antigen specific antisera to verify isolate identification.

**v. Isolation of *S. Typhimurium dam* vaccine from ovine tissues**

Sheep were euthanised and immediately necropsied using standard techniques 96 h following vaccination. Approximately 1 to 2 g of tissue was obtained from the mesenteric lymph node (MLN), liver, and spleen of each animal. Tissue sections were homogenised as a 1:4 dilution in sterile PBS, serially diluted, plated onto XLD-CHL, and incubated for 24 h at 37°C. Additionally, 1 mL of the

homogenised sample was placed into 9 mL of MSB enrichment media, and incubated for 24 h at 37°C. Colony counts were enumerated and MSB enrichments were streaked onto XLD-CHL. Samples that were positive by selective enrichment in MSB, were recorded as 10 CFU/g and negative samples were recorded as 4 CFU/g.

*vi. Statistical analysis*

The statistical program Genstat (12<sup>th</sup> Edition, VSN International, UK) was utilised to perform residual (or restricted) maximum likelihood (REML) analysis. Data from the stability and viability studies were analysed using a single variate repeated measures model where fixed effects of the model were time, treatment or temperature and their interactions, and the random effect was sample tube/jar, outcome variables included CFU/mL and pH. Results of the sheep vaccination trial were analysed using a single variate, repeated measures model where the fixed effects of the model were time, treatment and their interaction, and random effects were animal and experiment (A vs. B), outcome variables included faecal shedding (CFU/g), rectal temperature, chaff & pellet refusal, and water intake. Prior to analysis, CFU data was converted to log base 10. The Wald chi-square test was used to determine significant individual effects and or significant interactions between factors. Any non-significant terms were dropped from the model and analysis repeated. Following analysis, data are presented as predicted model based means. Predicted means are those obtained from the fitted model rather than the raw sample means. This is important, as predicted means represent means adjusted to a common set of variables, thus allowing valid comparison between means.

A *P* value less than 0.05 was considered to be statistically significant. Differences between the individual means were determined by calculating an approximate least significant difference (LSD). A difference of means that

exceeded the calculated LSD was considered significant.

The statistical program StatsDirect (version 2.7.8., <http://www.statsdirect.com>, England: StatsDirect Ltd 2008 (Buchan, 2000)) was utilised to perform analysis of variance and unpaired t-tests. Tissue colonisation was analysed using analysis of variance. An unpaired t-test was employed to analyse the following: the effect of vaccine dose on pyrexia in sheep following vaccination, the effect of 0.2X PBS on the water consumption of sheep, and the effect of in-water vaccination on the water consumption of sheep.

### 7.3 Results

#### 7.3.1. Viability and stability of *S. Typhimurium dam* vaccine strain in water

In-water delivery of a modified live vaccine requires vaccine viability and stability in drinking water for effective livestock immunisation. Here, vaccine viability and stability were initially examined in DIH<sub>2</sub>O to eliminate confounders such as pH, water hardness and bacterial contaminants. Note that, since the bacterial strains were reconstituted in 1X PBS, the final concentration was equivalent to 0.1X PBS in these experiments. Three salient observations were made (Figure 7-1): 1). The CFU/mL of both the *S. Typhimurium dam* vaccine and isogenic *dam*<sup>+</sup> strains was markedly stable at a 10<sup>8</sup> inoculation dose over the 120 h study period with 100.2% and 99.0% of the original inoculums remaining viable, respectively. 2). The *dam* vaccine and *dam*<sup>+</sup> strains exhibited relatively mild, but significant reductions in viability over time at lower inoculation doses over the 120 h study period, with 82.2% and 70.4% of the original inoculums remaining viable respectively ( $P < 0.05$ ). 3) Significant CFU/mL differences were observed between *dam*<sup>-</sup> and *dam*<sup>+</sup> strains at 24 h and from 24 to 72 h post inoculation at the 10<sup>6</sup> and 10<sup>8</sup> dose levels, respectively ( $P < 0.05$ ); however, no differences were observed in the predicted mean CFU/mL between the dose levels at 120 h post

inoculation, and strain alone did not have a significant effect on predicted mean CFU/mL ( $P = 0.728$ ). These data demonstrate that the *S. Typhimurium dam* vaccine has similar viability to the *dam*<sup>+</sup> strain when inoculated into DIH<sub>2</sub>O, and inoculation dose has a significant effect on stability over the 5 d study period.

### ***7.3.2. Viability of S. Typhimurium dam vaccine in trough water with and without faecal contamination***

*Salmonellae* are capable of proliferating in moist faecal material (Arrus, *et al.*, 2006; Holley, *et al.*, 2006) and, thus, faecal contamination may alter dose delivery for livestock immunisation. Here, we assessed *S. Typhimurium dam* vaccine CFU/mL in DIH<sub>2</sub>O; trough water (TRW); and TRW + faeces (TRW + F) as a function of time at a dose concentration of 10<sup>4</sup> CFU/mL. Note that the final concentration of PBS was equivalent to 0.01X PBS following inoculation with the *S. Typhimurium dam* vaccine reconstituted in PBS. The CFU/mL of the *S. Typhimurium dam* vaccine in solutions of TRW+F was significantly higher than that observed in TRW at 24 to 48 h post inoculation ( $P < 0.05$ ; Figure 7-2). The predicted mean CFU/mL of the *S. Typhimurium dam* vaccine in TRW declined over the course of the experiment with 98%, 72.3% and 53.1% of the original inoculum viable at 6, 24 and 48 h post inoculation, respectively; the predicted mean CFU/mL of the *S. Typhimurium dam* vaccine in TRW+F was relatively stable over the course of the 48 h experiment with 102.2%, 105.9% and 93.8% of the original inoculum viable at 6, 24 and 48 h respectively. Further, sampling of the *S. Typhimurium dam* vaccine from the surface of the trough versus vortexed samples did not have an effect on predicted mean CFU/mL over the course of the study ( $P = 0.94$ ; Figure 7-3). These data indicate that the vaccine was uniformly distributed within the trough water following inoculation and, thus, water consumption from the surface of the trough will not limit delivery of an effective vaccination dose. Taken together, these data establish the viability and stability of the *S. Typhimurium dam* vaccine in trough water with and without faecal

contamination, suggesting that such vaccine persistence should be sufficient for oral dosing of livestock. Moreover, the observed decline in vaccine viability in clean trough water over time provides an inherent mechanism for elimination of the vaccine following trough inoculation.

### ***7.3.3. Viability of the *S. Typhimurium dam* vaccine in buffered water***

Vaccine persistence may be prolonged by the addition of buffers to enhance vaccine stability (Kolb, 1996; Leigh, *et al.*, 2008). Thus, we examined the minimum concentration of PBS required for stability of the *S. Typhimurium dam* vaccine when inoculated into drinking water. The concentration of PBS had a significant effect on the viability of the vaccine over the 48 h study period ( $P = 0.005$ ; Figure 7-4). Solutions containing greater than or equal to 0.062X PBS had significantly increased predicted mean CFU/mL when compared to the DIH<sub>2</sub>O ( $P < 0.05$ ), with solutions of PBS, 0.5X PBS and 0.25X PBS containing 103.2%, 103.0 and 90.5% of the initially seeded  $10^5$  dose at 48 h post inoculation. The minimum concentration of PBS required for improved viability of the *S. Typhimurium dam* vaccine in drinking water over a 24 h period was greater than or equal to 0.125X PBS. Note that PBS concentrations of 0.2X PBS in combination with the *S. Typhimurium dam* vaccine does not compromise water intake in sheep (Mohler, *et al.*, 2011). These data suggest that inclusion of a buffering agent in vaccine formulation facilitates *S. Typhimurium dam* vaccine persistence, without compromising livestock water consumption.

### ***7.3.4. Effect of temperature and buffering agents on the viability of the *S. Typhimurium dam* vaccine***

Under field conditions, trough water temperatures are likely to vary and may affect viability or favour proliferation of the vaccine strain. Here, we evaluated the effect of water temperature on viability and/or proliferation of the *S. Typhimurium dam* vaccine in drinking water with and without buffering agents.

Although the viability of the *S. Typhimurium dam* vaccine declined post inoculation, no significant differences in the predicted mean number of CFU/mL were observed across all solutions incubated at either room temperature (20-25°C) or 37°C over the 24 h time course ( $P = 0.22$ ; Figure 7-5). The capacity for the addition of PBS to significantly improve *S. Typhimurium dam* vaccine viability over the 24 h time course when compared to DIH<sub>2</sub>O, was independent of incubation temperature with a greater predicted mean CFU/mL in PBS and 0.2X PBS solutions when compared to the DIH<sub>2</sub>O control from 6 to 24h post inoculation ( $P < 0.05$ ; Figure 7-6).

The improved *S. Typhimurium dam* vaccine viability conferred by PBS was correlated, in part, to stabilisation of pH, which was significantly lower in DIH<sub>2</sub>O relative to that observed in PBS and 0.2X PBS solutions from 6-24 h post inoculation ( $P < 0.05$ ; Figure 7-7). Control solutions of DIH<sub>2</sub>O and PBS without inoculation of the *S. Typhimurium dam* vaccine exhibited only a mild decrease in pH over the experimental time course (data not shown). Thus, the buffering capacity of PBS likely influences vaccine viability by preventing vaccine-mediated acidification of the solution over the 24 h study period. Note that the mild (half log) decrease in *S. Typhimurium dam* vaccine viability at the 6 h time point in buffered solutions (Figure 7-6) was not attributed to pH as no measurable change in pH was observed over the entire time 24 h time course. Taken together, these data demonstrate that the viability of *S. Typhimurium dam* vaccine is similar during the first 24 h in solutions maintained at room temperature or 37°C and that the vaccine does not proliferate when inoculated into warm water. Moreover, the buffering capacity of PBS in solution likely influences vaccine viability by preventing acidification and, thus, vaccine persistence can be controlled via inclusion/exclusion of buffering agents and stabilisers, providing an effective means for vaccine delivery in drinking water for *ad libitum* oral delivery under on-farm conditions.

### 7.3.5. *Delivery of S. Typhimurium dam vaccine to adult Merino wethers via drinking water*

Delivery of an effective *S. Typhimurium dam* vaccine in drinking water to adult sheep requires that water consumption is not compromised, no adverse clinical reactions are manifested, effective colonisation occurs in host lymphoid tissues, and the vaccine is subsequently cleared, with minimal vaccine shedding from the immunised animals. To address the potential issue of buffering agents on water consumption, sheep were offered drinking water containing 0.2X PBS during the acclimation period. Water consumptions were compared to the mean water consumptions from the previous 5 days. No differences in consumption of PBS treated water were observed when compared to mean untreated drinking water consumptions ( $P = 0.86$ ). These data establish that 0.2X PBS does not affect palatability or inhibit consumption of water by adult sheep. Further, there was no evidence of refusal of the *S. Typhimurium dam* vaccine when delivered in-water containing 0.2X PBS ( $P = 0.81$ ). On the day of vaccination, water consumption of the in-water vaccinates was  $1.9 \pm 0.24$  L and  $2.4 \pm 0.22$  L for the  $10^7$ IW and  $10^6$ IW groups respectively, which was 85.4 and 105.6 percent of the mean daily water consumption during the five days preceding vaccination for each group. Moreover, the mean in-water vaccination dose consumed was:  $1.1 \times 10^6$  CFU (range  $8.7 \times 10^5$  to  $1.5 \times 10^6$  CFU/sheep) in the  $10^6$ IW group and  $1.5 \times 10^7$  CFU (range  $1.2 \times 10^7$  to  $1.8 \times 10^8$  CFU/sheep) in the  $10^7$ IW group. The sheep in the OB $10^7$  group received  $9.4 \times 10^6$  CFU/sheep in a 10 mL oral bolus. These data indicate that the *S. Typhimurium dam* vaccine was effectively delivered in water to sheep under field conditions.

Next, we assessed clinical manifestations as a consequence of vaccination. Pyrexia (rectal temperature  $> 40.0^\circ\text{C}$ ) (Radostits, *et al.*, 2007; Terra, 2009) was not observed in sheep from the  $10^6$ IW vaccinate group (Figure 7-8). This group

remained within the normal temperature range throughout the study and had significantly lower predicted mean rectal temperatures relative to that exhibited by the  $10^7$ OB and  $10^7$ IW vaccinates from 24 to 72 h post vaccination ( $P < 0.05$ ). In contrast, pyrexia was observed in 4 of 7 sheep in the  $10^7$ OB group and 7 of 8 sheep in the  $10^7$ IW group at 48 and 72 h following vaccination ( $P < 0.001$ ). There was no difference in the predicted mean rectal temperatures of  $10^7$ OB and  $10^7$ IW vaccinates, even though the  $10^7$ IW vaccinates consumed over a log more vaccine when compared to the  $10^7$ OB vaccinates. Taken together, these data indicate that the  $10^6$ IW vaccinate group remained within the normal temperature range throughout the study period; moreover, no significant pyrexia differences were observed between the  $10^7$ IW and  $10^7$ OB vaccinate groups.

There was no significant effect of vaccination treatment on chaff appetite (Figure 7-9), pellet appetite (Figure 7-10), and water consumption (Figure 7-11) of the sheep over the course of the experiment. Moreover, the attitude and faecal scores of all sheep remained at baseline values over the course of the study period. However, chaff refusal of the  $10^7$ OB vaccinates was significantly increased when compared to the  $10^6$ IW vaccinates at 72 h post vaccination ( $P < 0.05$ ; Figure 7-9); and the water consumption of the sheep in the  $10^7$ OB vaccinates was significantly higher than the  $10^6$ IW and  $10^7$ IW vaccinates during vaccination period,  $t 0$  h ( $P < 0.05$ ; Figure 7-11). Taken together, these data indicate that vaccine treatment had minimal effects on chaff appetite, pellet appetite, and water consumption.

Colonisation of lymphoid tissue (e.g. intestine [Peyer's patches]; mesenteric lymph nodes [MLN]; spleen) and visceral organs (e.g., liver) leads to the initiation of protective innate and adaptive immune responses. Here we assessed the ability of the *S. Typhimurium dam* vaccine to colonise such host tissue sites via enumeration of CFU recovered from faeces, MLN, spleen, and liver at 96 h post-vaccination. Faecal shedding Significantly lower numbers of *dam* vaccine



shedding (2 logs less CFU/g faeces) were observed in the  $10^6$ IW vaccinates when compared to the  $10^7$ OB and  $10^7$ IW vaccinates ( $P < 0.01$ ; Figure 7-12); no differences were observed between  $10^7$ OB and  $10^7$ IW vaccinates ( $P = 0.9579$ ). Moreover, the *dam* vaccine was recovered from the faeces of 1 of 7 of the  $10^6$ IW vaccinates as compared to 8 of 8 and 7 of 7 sheep from the  $10^7$ IW and  $10^7$ OB vaccinates, respectively. MLN colonisation Significantly lower numbers of the *dam* vaccine were observed in the MLN of the  $10^6$ IW vaccinates (2 logs less CFU/g) when compared to the  $10^7$ IW vaccinates ( $P < 0.005$ ); no differences were observed between the  $10^7$ IW and  $10^7$ OB vaccinates ( $P = 0.0854$ ) and between the  $10^7$ OB and  $10^6$ IW vaccinates ( $P = 0.2439$ ). Moreover, the *S. Typhimurium dam* vaccine was recovered from the MLN of 2 of 7, 8 of 8 and 4 of 7 sheep from the  $10^6$ IW,  $10^7$ IW and  $10^7$ OB vaccination groups respectively. Spleen and liver colonisation The *S. Typhimurium dam* vaccine was not recovered from the spleen and liver of the  $10^6$ IW vaccinates, with only very low CFU recovered from one sheep from the  $10^7$ IW and  $10^7$ OB vaccinates groups. Collectively, these data indicate that in-water delivery of the *S. Typhimurium dam* vaccine to sheep over a 24 h period is a viable administration route in adult ruminants, with minimal colonisation of spleen and liver and low-level shedding in faeces.

#### **7.4 Discussion**

The global trend towards intensive livestock production is associated with an increased incidence of salmonellosis via faecal-oral transmission. Vaccination is a useful tool in the control of infectious diseases without the use of antimicrobials. Unfortunately, the most common methods of vaccination in livestock require animals to be handled by personnel that can further stress susceptible populations and cause carcass damage, adverse reactions, and resultant increased production costs (Bowersock and Martin, 1999; Giudice and Campbell, 2006; Murphy, *et al.*, 2008; Savini, *et al.*, 2008; Mamak and Aytakin, 2009).

Delivery of an effective attenuated live vaccine in drinking water for livestock production requires that the vaccine is stable in drinking water under field conditions; does not affect water consumption or alter vaccine dosing and does not proliferate in the environment. Here we show that the *S. Typhimurium dam* attenuated vaccine was stable in the drinking water of adult ruminants with or without buffering agents and remained viable under temperatures between 20°C and 37°C; does not compromise water consumption with the addition of buffering agents, and does not proliferate in drinking water contaminated with animal faeces.

From a farm-management perspective, many of these features would have general utility in intensive livestock production systems and could be modified accordingly depending on the specific management/environmental strategies implemented. For example, if the planned application called for a more prolonged vaccination period, the *S. Typhimurium dam* vaccine maintained highest viability in solutions containing at least 0.2X PBS. Indeed, PBS has been used to enhance survival of live *Mycoplasma gallisepticum* vaccine in aerosol delivery systems to chickens (Bowersock and Martin, 1999; Giudice and Campbell, 2006; Murphy, *et al.*, 2008; Savini, *et al.*, 2008; Mamak and Aytakin, 2009) and did not negatively impact water consumption by sheep in our study. The sheep consumed 4 to 8% of their body weight in water per day, which was within the reported normal daily range of 5 to 20% (Singh, *et al.*, 1976; Savage, *et al.*, 2008). If the planned application were to deliver the vaccine over a 24 h period, it would not be necessary to include a buffering agent; moreover, replenishing water troughs with un-buffered water would promote elimination of the vaccine following the delivery period.

Environmental parameters such as temperature and faecal contamination may adversely affect vaccine dosing of the animals. For example, although our data

show that the *S. Typhimurium dam* vaccine is stable under temperatures between 20°C and 37°C and does not adversely affect water consumption, ambient temperatures may need to be considered when delivering the *S. Typhimurium dam* vaccine to sheep, as higher environmental temperatures (40°C) encourage increased water consumption (Savage, *et al.*, 2008) and could affect vaccine viability/stability. Faecal contamination may affect dosing as it is not uncommon to find faeces or contaminated bedding/feed material in troughs used to supply animals with water, and *Salmonellae* are capable of proliferating in moist faecal material (Arrus, *et al.*, 2006; Holley, *et al.*, 2006). Our studies show that although exposure to faecal-contaminated trough water increased the stability of the *S. Typhimurium dam* vaccine, no increase in vaccine CFU was observed.

A potential limitation to oral vaccine delivery in ruminants is the capacity of volatile fatty acids produced in the rumen to attenuate the survival of *Salmonella* (Chambers and Lysons, 1979; Mattila, *et al.*, 1988). The establishment of the *S. Typhimurium dam* vaccine in the MLN of adult sheep demonstrated that the vaccine is capable of bypassing the rumen and surviving in the gastrointestinal tract of adult sheep, indicating the opportunity for successful in-water prophylaxis in adult ruminants.

The focus of these studies has been to evaluate the viability and vaccine dose stability of the *S. Typhimurium dam* attenuated vaccine in livestock drinking water over a 24 h delivery period. The results of these studies indicate that drinking water is a feasible vaccine delivery option for adult ruminants.

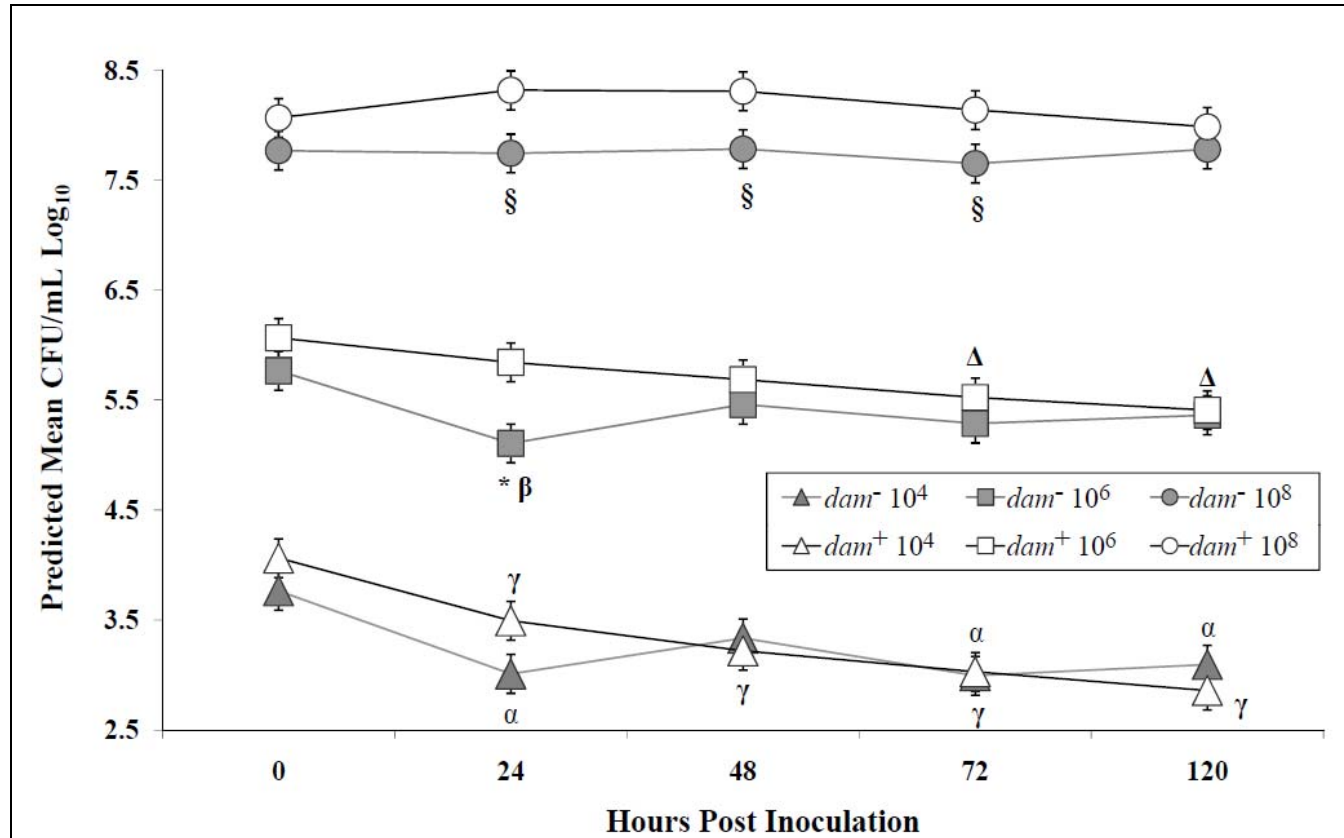


Figure 7-1. Viability of a *S. Typhimurium dam* vaccine following inoculation into de-ionised water at room temperature.

Data are depicted as a predicted mean log<sub>10</sub> CFU/mL ± SE. Significant difference between strains at same dose level are denoted by \* for 10<sup>6</sup> and § for 10<sup>8</sup> CFU/mL ( $P < 0.05$ ). A significant difference from  $t = 0$  inoculation dose level is denoted by <sup>α</sup> for *dam*<sup>-</sup> 10<sup>4</sup>, <sup>β</sup> for *dam*<sup>-</sup> 10<sup>6</sup>, <sup>γ</sup> for *dam*<sup>+</sup> 10<sup>4</sup> and <sup>Δ</sup> for *dam*<sup>+</sup> 10<sup>6</sup> CFU/mL

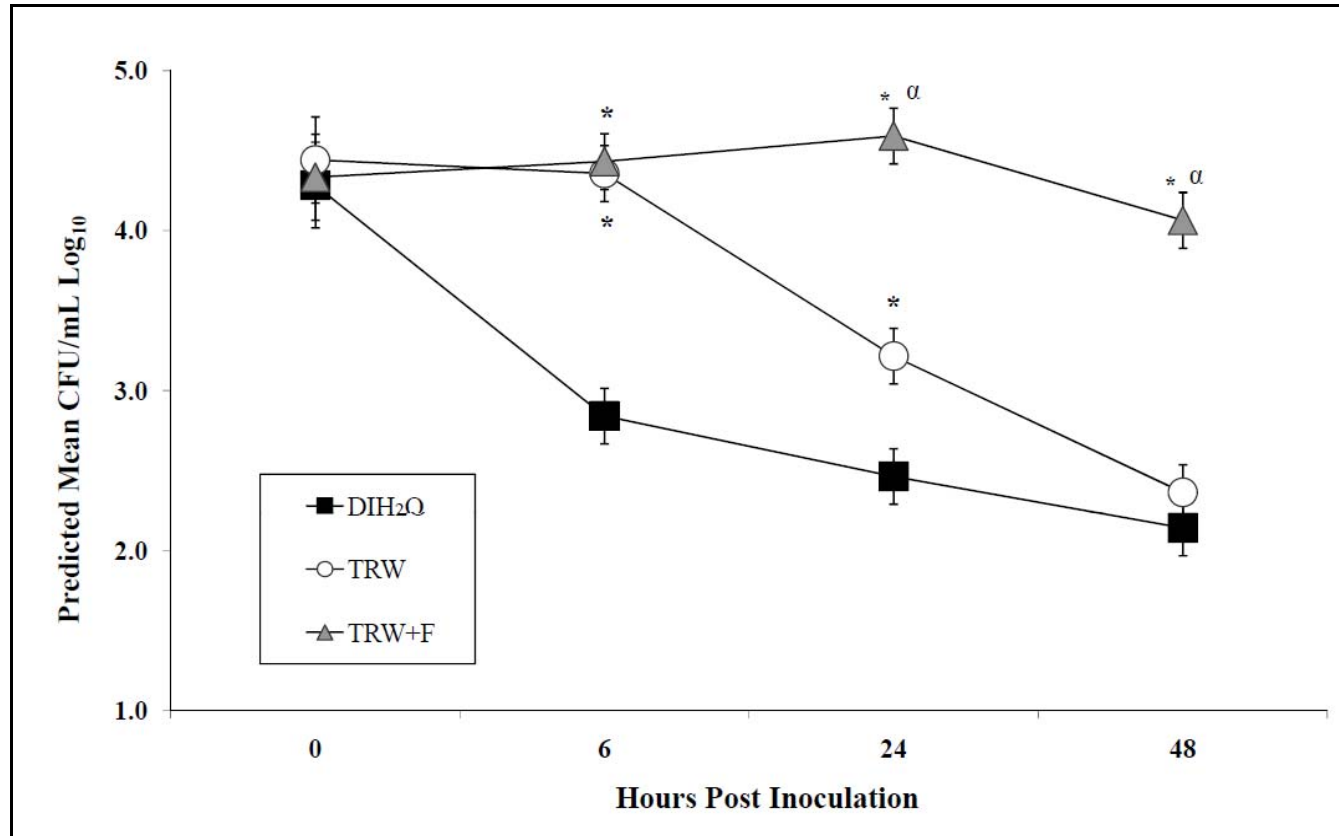


Figure 7-2. Viability of a *S. Typhimurium dam* vaccine when inoculated into trough water at room temperature.

Six jars, each containing DIH<sub>2</sub>O (black square), TRW (white circle) and TRW+F (grey triangle) were inoculated with *S. Typhimurium dam* vaccine at a dose of 10<sup>4</sup> CFU/mL at *t* = 0. Data are depicted as a predicted mean log<sub>10</sub> CFU/mL ± SE. \* Denotes a significant difference from DIH<sub>2</sub>O control (*P* < 0.05) and <sup>α</sup> denotes a significant difference between TRW and TRW+F (*P* < 0.05).

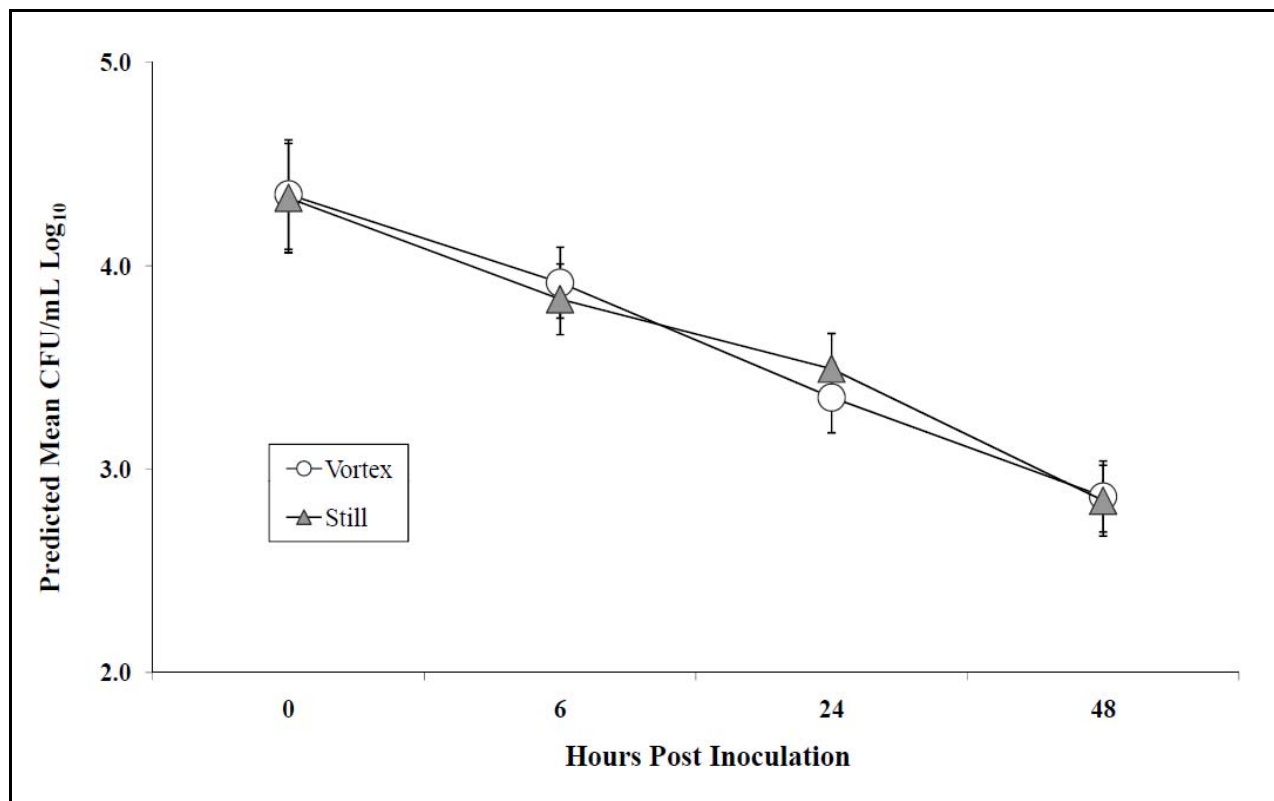


Figure 7-3. Effect of sampling technique on a *S. Typhimurium dam* vaccine following inoculation into trough water at room temperature.

Six jars, each containing either de-ionised water, trough water and trough water with faeces were inoculated with *S. Typhimurium dam* vaccine at a dose of  $10^4$  CFU/mL at  $t = 0$ . Three jars from each solution ( $n = 9$ ) were vortexed prior to sample collection (white circle) while samples were collected from the surface of the remaining three jars each ( $n = 9$ ) (grey triangle). Data are depicted as a predicted mean  $\log_{10}$  CFU/mL  $\pm$  SE.

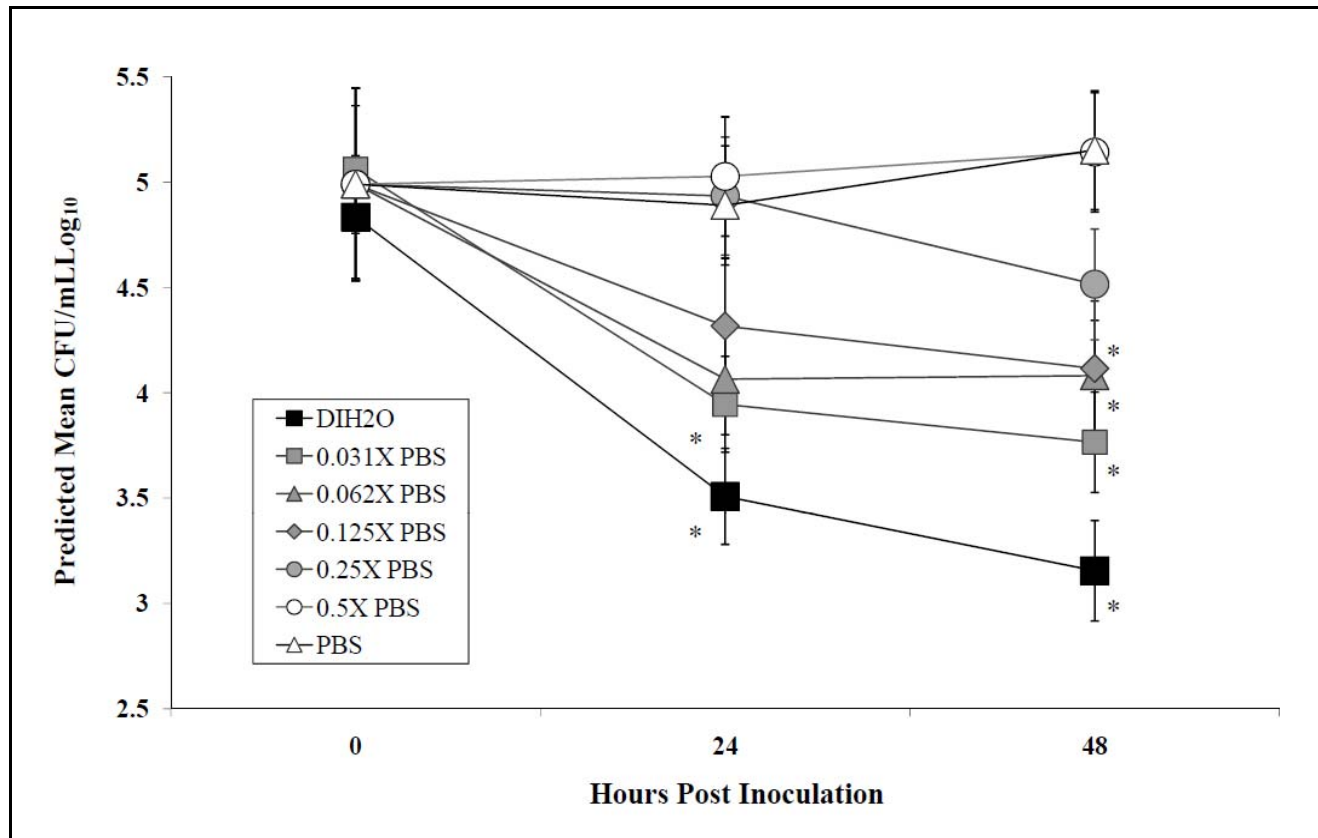


Figure 7-4 Effect of PBS concentration on the viability of a *S. Typhimurium dam* vaccine at room temperature.

*S. Typhimurium dam* vaccine was inoculated into tubes containing either DIH<sub>2</sub>O (black square), PBS (white triangle) and 0.031X PBS (grey square), 0.062X PBS (grey triangle), 0.125X PBS (grey diamond), 0.25X PBS (grey circle), 0.5X PBS (white circle) at a concentration of 10<sup>5</sup> CFU/mL at *t* = 0. Data are depicted as a predicted mean log<sub>10</sub> CFU/mL ± SE. \*Denotes significantly different from PBS control (*P* < 0.05).

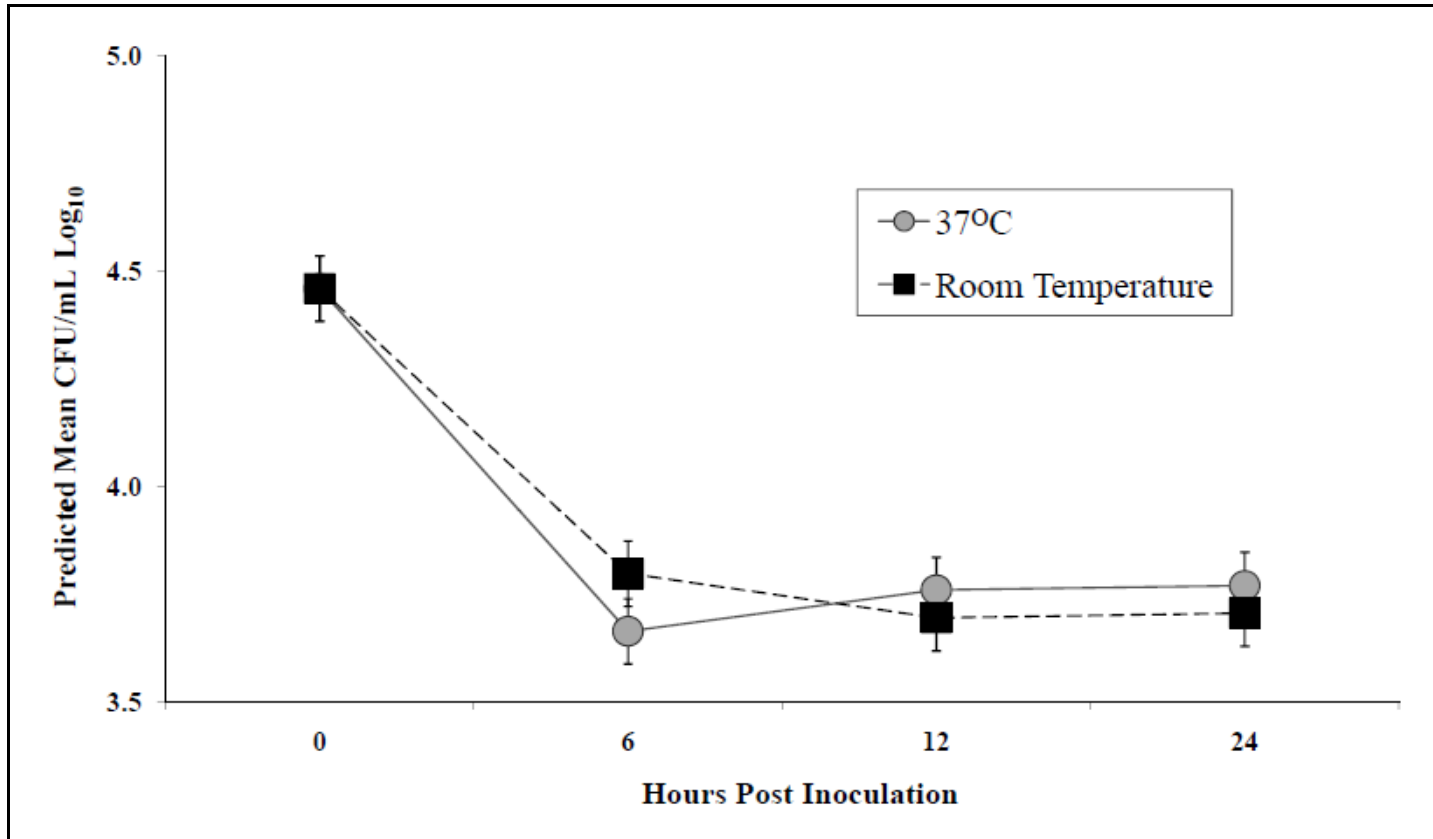


Figure 7-5. Effect of incubation temperature on viability of a *S. Typhimurium dam* vaccine.

*S. Typhimurium dam* vaccine was inoculated into six jars, each containing either PBS; 0.2X PBS or DIH<sub>2</sub>O to concentration of  $2.5 \times 10^4$  CFU/mL at  $t = 0$ . Three jars from each solution were incubated at room temperature (20-26°C) (black square, dashed lines) or 37°C (grey circle; solid lines). Data are depicted as a predicted mean log<sub>10</sub> CFU/mL  $\pm$  SE. No differences between incubation temperatures were observed ( $P = 0.22$ ).



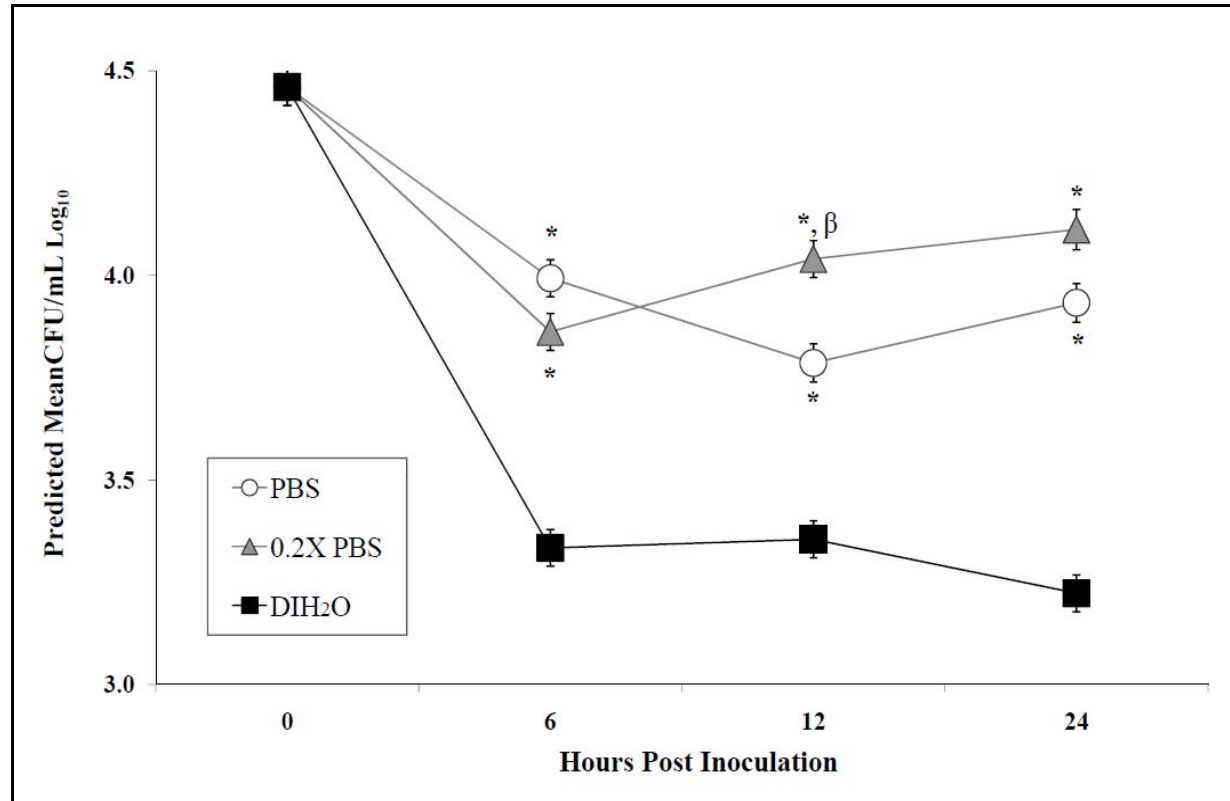


Figure 7-6. Effect of PBS on viability of a *S. Typhimurium dam* vaccine following incubation at room temperature and 37°C.

Six jars, each containing either PBS (white circle); 0.2X PBS (grey triangle) or DIH<sub>2</sub>O (black square) were inoculated with *S. Typhimurium dam* to concentration of  $2.5 \times 10^4$  CFU/mL at  $t = 0$ . Three jars from each solution ( $n = 9$ ) were incubated at RT or 37°C. Incubation temperature (RT vs. 37°C) did not have a significant effect on viability ( $P = 0.22$ ). Data are depicted as a predicted mean  $\log_{10}$  CFU/mL  $\pm$  SE. \*Denotes significant difference from DIH<sub>2</sub>O and <sup>β</sup> denotes significant difference between 0.2X PBS and PBS ( $P < 0.05$ ).

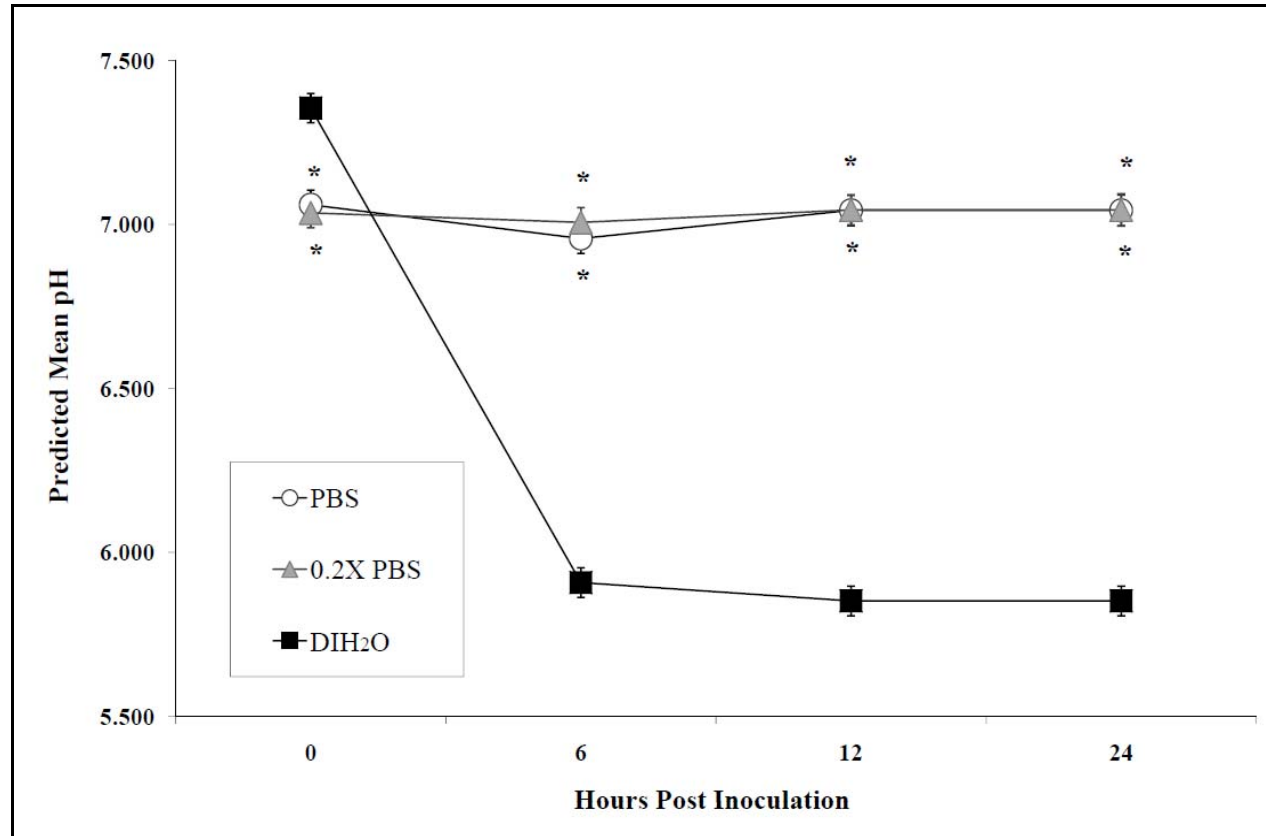


Figure 7-7. Effect of a *S. Typhimurium dam* vaccine on solution pH.

Six jars, each containing either PBS (white circle); 0.2X PBS (grey triangle) or DIH<sub>2</sub>O (black square) were inoculated with *S. Typhimurium dam* vaccine to a concentration of  $2.5 \times 10^4$  CFU/mL at  $t = 0$ . Three jars from each solution ( $n = 9$ ) were incubated at RT or 37°C. Data are depicted as a predicted mean pH  $\pm$  SE. \*Denotes significant difference from DIH<sub>2</sub>O ( $P < 0.05$ ).

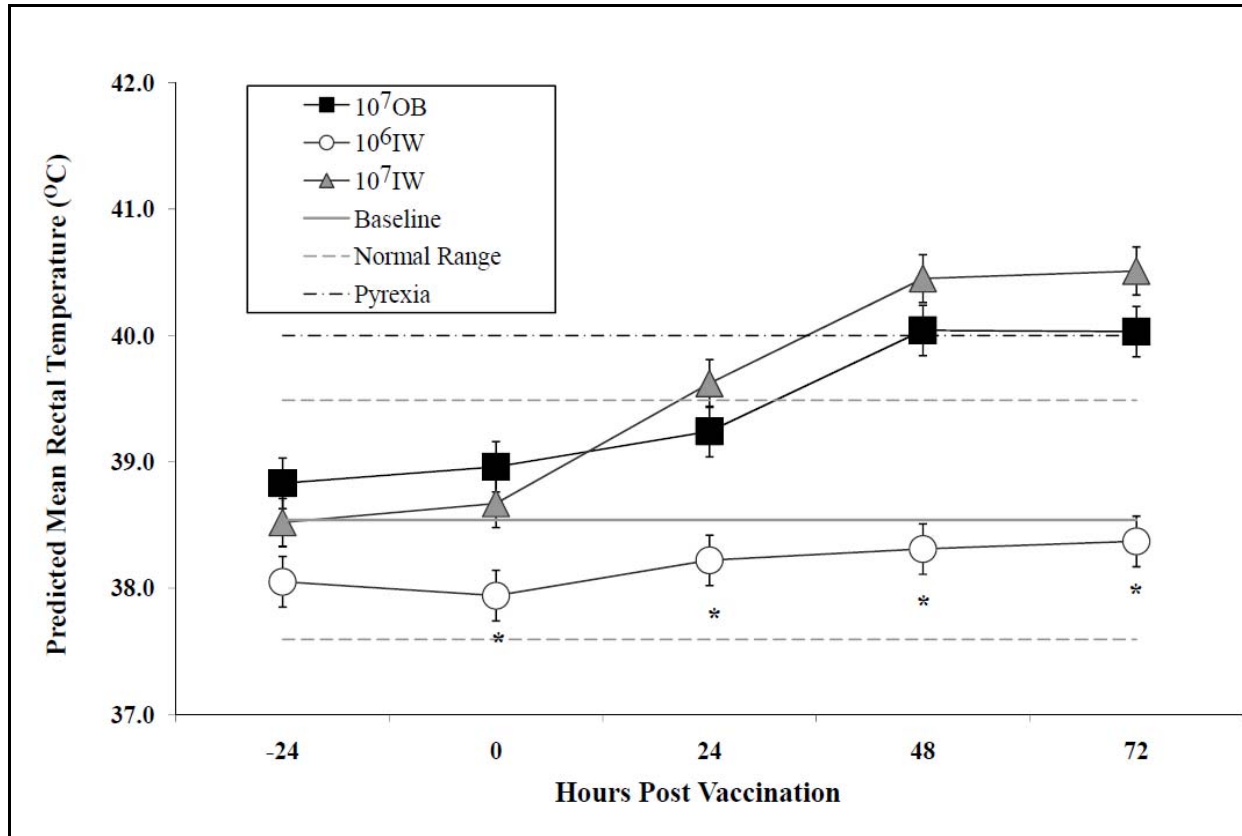


Figure 7-8. Predicted mean rectal temperatures following in-water immunisation of adult sheep with *S. Typhimurium dam*

Data are presented as predicted mean RT  $\pm$  SE; in the context of the normal range (dashed grey lines) and baseline (solid grey line) established pre-vaccination; and the threshold for pyrexia (dashed black line). \*RT of the 10<sup>6</sup>IW vaccinates were significantly lower than the 10<sup>7</sup>IW and 10<sup>7</sup>OB vaccinates from time 0 to 72 h post vaccination ( $P < 0.05$ ). There was no difference in RT of the 10<sup>7</sup>IW and 10<sup>7</sup>OB vaccinates.

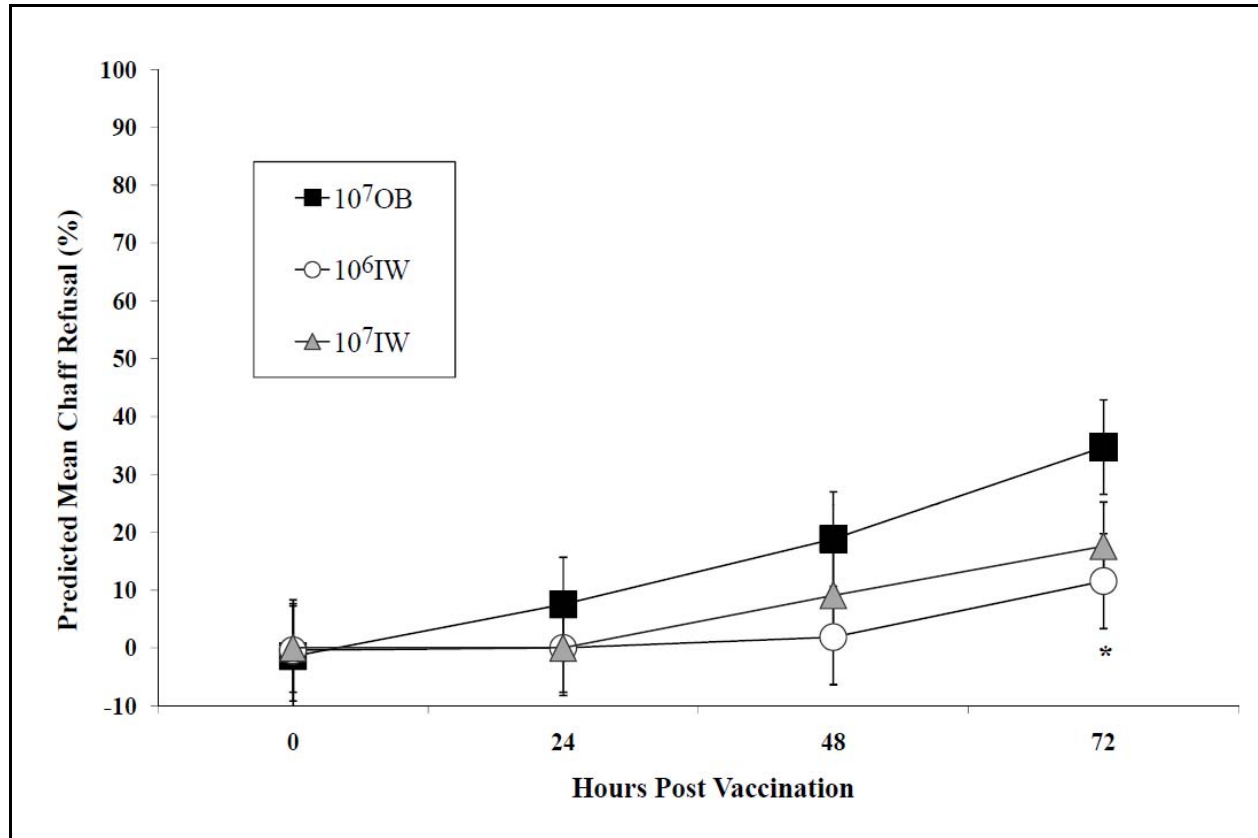


Figure 7-9. Predicted mean chaff refusals following in-water immunisation of adult sheep with *S. Typhimurium dam*.

Percent of chaff refused was determined for each animal during the challenge period based on amount of offered chaff refused. Data are depicted as predicted mean chaff refusal  $\pm$  SE. \*Chaff refusal was significantly lower in the 10<sup>6</sup>IW when compared to the 10<sup>7</sup>OB vaccinates at 72 h post vaccination ( $P < 0.05$ ). There was no difference in the chaff refusals of the 10<sup>6</sup>IW and 10<sup>7</sup>IW vaccinates.

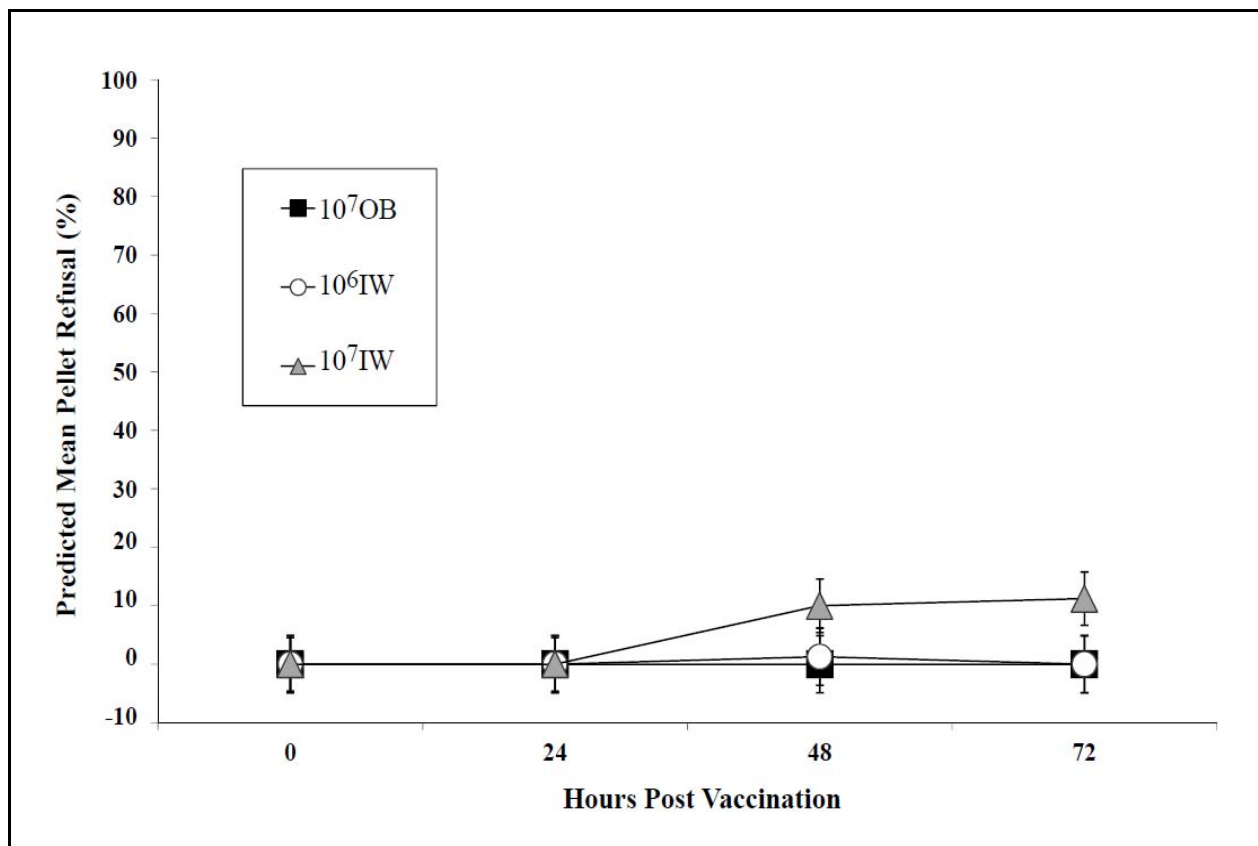


Figure 7-10. Predicted mean pellet refusals following in-water immunisation of adult sheep with *S. Typhimurium dam*.

Percent of pellet refused was determined for each animal during the challenge period based on amount of pellet refused over offered. Predicted mean pellet refusal  $\pm$  SE is depicted. There was no difference in the pellet refusals of the 10<sup>7</sup>OB, 10<sup>6</sup>IW and 10<sup>7</sup>IW vaccinates over the 72 h observation period.

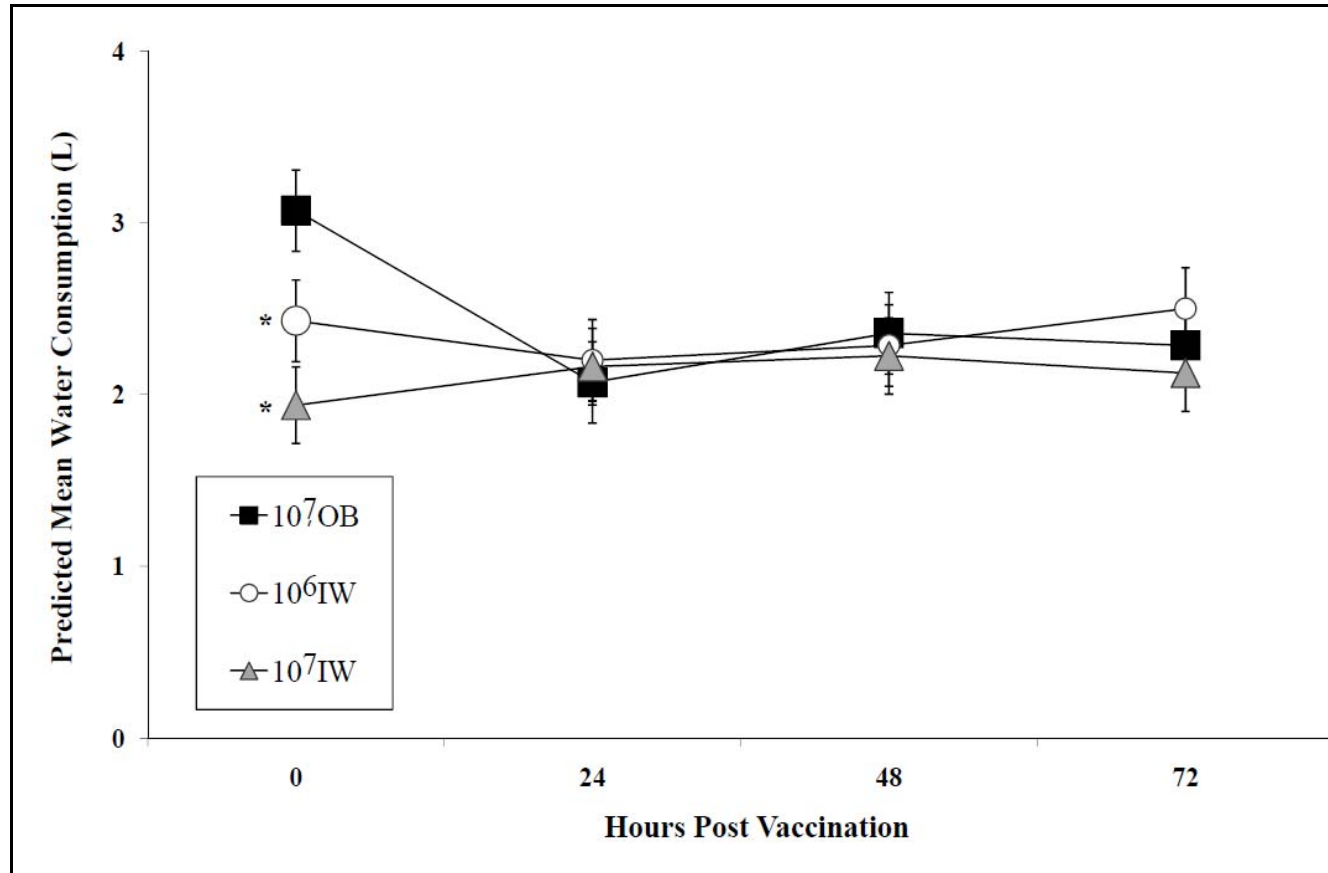


Figure 7-11. Predicted mean water consumption following in-water immunisation of adult sheep with *S. Typhimurium dam*.

Data are depicted as predicted mean water consumption in L  $\pm$  SE. \*The water consumption of the 10<sup>6</sup>IW and 10<sup>7</sup>IW vaccinates was significantly lower than the 10<sup>7</sup>OB vaccinates during 24 h post-vaccination period ( $P < 0.05$ )

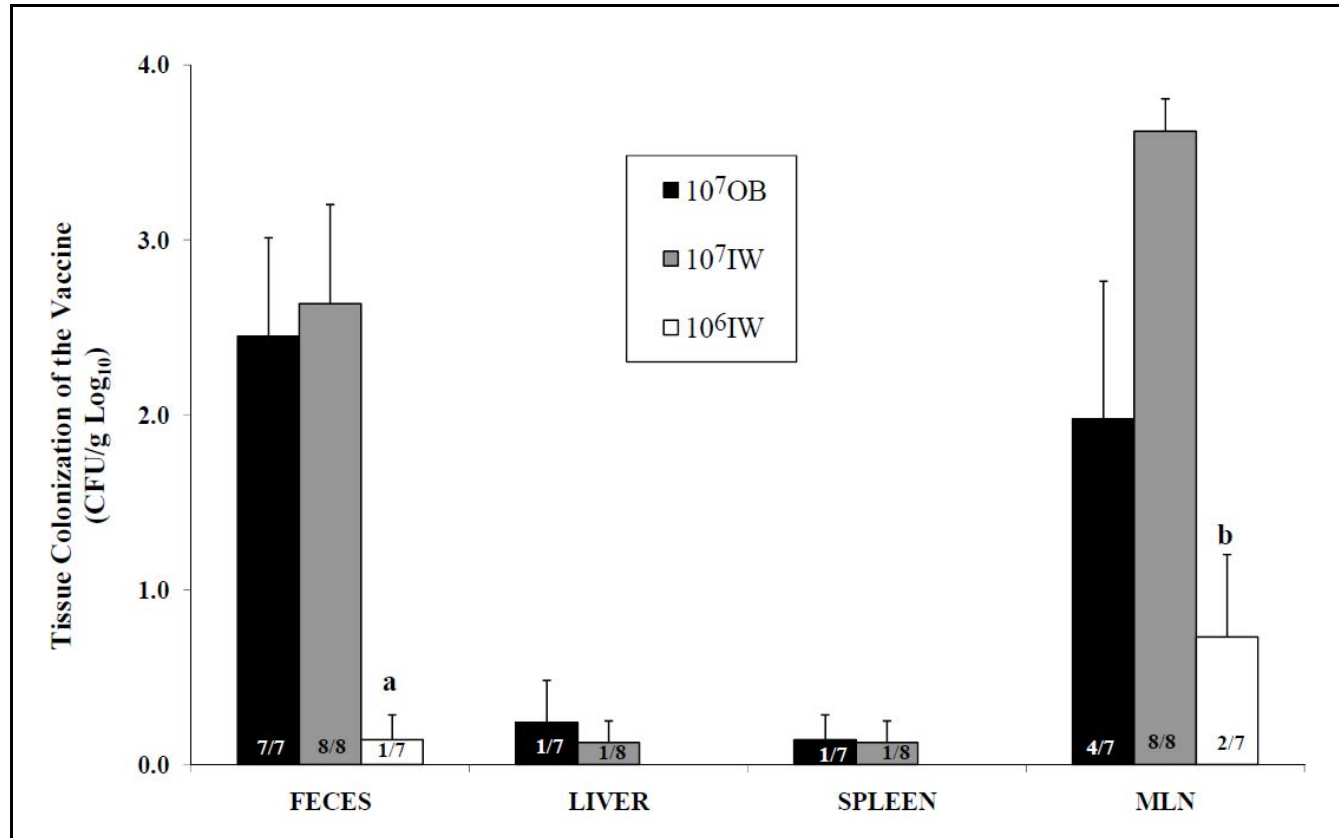


Figure 7-12. Colonisation of lymphoid tissue and visceral organs in adult sheep following in-water delivery of a *S. Typhimurium dam*.

Data are depicted as mean log<sub>10</sub> CFU/g by tissue ± SE. Significantly lower numbers of the *S. Typhimurium dam* were shed into the faeces of the 10<sup>6</sup>IW vaccinates when compared to the 10<sup>7</sup>OB and 10<sup>7</sup>IW vaccinates (<sup>a</sup>*P* < 0.01). Significantly higher numbers of the *S. Typhimurium dam* were isolated from the MLN of the 10<sup>7</sup>IW vaccinates when compared to the 10<sup>6</sup>IW vaccinates (<sup>b</sup>*P* < 0.005). Limit of detection is 4 CFU/g tissue.

**CHAPTER 8. Protective Immunity Conferred by a DNA Adenine Methylase Deficient *Salmonella enterica* serovar Typhimurium Vaccine When Delivered In-water to Sheep Challenged with *Salmonella enterica* serovar Typhimurium**

The following is a re-formatted manuscript, currently in press:

Mohler, V.L., Heithoff, D.M., Mahan, M.J., Walker, K.H., Hornitzky, M.A., Gabor, L., Thomson, P.C., Thompson, A., House, J.K. (2011). Protective Immunity Conferred by a DNA Adenine Methylase Deficient *Salmonella enterica* serovar Typhimurium Vaccine When Delivered In-water to Sheep Challenged with *Salmonella enterica* serovar Typhimurium. *Vaccine* (*in press*: accepted March 2011)

The study was designed, executed and reported by the candidate under the general supervision of the remaining authors, who were the candidate's appointed supervisors (principal and associate) with the exception of Drs Andrew Thomson and Les Gabor who provided assistance with post mortem procedures.

**8.1 Introduction**

*Salmonella* is the most commonly isolated infectious enteric bacterial pathogen of dairy cattle (1994; Vanselow, *et al.*, 2007b) and the most common zoonotic disease associated with human consumption of beef and dairy products (Donnelly, 1990; Troutt and Osburn, 1997). In recent years, there has been a rise in the incidence and severity of human cases of salmonellosis and emergence of multidrug resistant strains of *Salmonella* (CDC, 2002; Mølbak, 2005; CDC, 2008, 2009). Prevalence studies indicate 16 to 73% of U.S. dairy farms are infected with *Salmonella* (Pacer, *et al.*, 1986; Pacer, *et al.*, 1989; Besser, *et al.*, 1997; Dunne, *et al.*, 2000; Kiessling, *et al.*, 2002) and up to 50% of cull dairy cows are contaminated with *Salmonella* at slaughter (Troutt, *et al.*, 2001). Naïve animals introduced to intensive production systems by either birth or transport are often



exposed to virulent salmonellae shortly after arrival (Gay and Hunsaker, 1993; House, *et al.*, 2001a). One dairy field study demonstrated the potential magnitude of the exposure to salmonellae by demonstrating 67% of calves shedding salmonellae within 24 h of birth (House, *et al.*, 2001a). Similar findings have been reported in sheep feedlots, where the prevalence of salmonellae faecal shedding was observed to increase from 1-2 to 17-89% within 14 d of entry (Higgs, *et al.*, 1993; Kelly, 1995).

Vaccine prophylaxis is normally achieved through vaccinating animals several weeks prior to virulent pathogen exposure. This is not possible in neonates and often not practically possible in feedlots where livestock are sourced from diverse locations and vendors. Vaccination of animals on arrival to feedlot facilities is an alternative approach to reduce the incidence and severity of disease. However, implementing an effective *Salmonella* vaccination program upon arrival to feedlot facilities poses a number of technical and logistical challenges, as livestock have often undergone variable periods of feed deprivation and varying degrees of stress due to transport, altered environmental conditions, and changes in social grouping. These stresses and concurrent exposure to a diversity of pathogens contribute to the risk of disease. Direct physical handling of livestock to administer vaccines may contribute further stress and negatively impact stock health and welfare. In contrast, oral vaccine delivery in drinking water significantly reduces the stress of additional handling, and is a means of rapidly vaccinating large groups of animals. However, the efficacy of *Salmonella* vaccination in feedlots is largely influenced by the interval between immunisation and pathogen exposure, which may be short in field settings. Therefore it is desirable for the onset of immunity to be rapidly initiated post vaccination. Additionally, exposure of livestock to virulent *Salmonella* during transport may negatively impact vaccine efficacy when administered upon arrival at the feedlot. Finally, the timing of virulent pathogen exposure may also impact on the safety of *Salmonella* vaccines as administration

of aro attenuated *Salmonella* vaccines either 24 h prior or 48 h following virulent *Salmonella* exposure has been reported to exacerbate salmonellosis disease manifestations (Foster, *et al.*, 2008).

Modified live attenuated *Salmonella* vaccines deficient in the *dam* gene have been shown to confer cross-protective immunity to multiple salmonellae in murine (Heithoff, *et al.*, 2001; Heithoff, *et al.*, 2008a), avian (Dueger, *et al.*, 2001, 2003a) and bovine models of salmonellosis (Dueger, *et al.*, 2003b; Mohler, *et al.*, 2006; Mohler, *et al.*, 2008) and have been demonstrated as safe. Protective immunity in *S. Typhimurium dam* immunised mice directly correlated with increased levels of cross-reactive opsonising antibodies and memory T cells and a diminished expansion of myeloid-derived suppressor cells (MDSCs) that are responsible for immune suppression associated with several conditions of host stress including chronic microbial infections, trauma and cancer (Heithoff, *et al.*, 2008a). Additionally, infection of mice with *S. Typhimurium dam* showed reduced multi-tissue innate immune cytokine responses relative to wild type, which may contribute to the reduced disease manifestations and protective immunity observed in vaccinated animals (Shtrichman, *et al.*, 2002; Simon, *et al.*, 2007). Thus, *S. Typhimurium dam* vaccines have been demonstrated to be safe and efficacious in experimental models of salmonellosis and therefore are suitable candidates for application in livestock production systems. Delivery of vaccines in drinking water provides a potential means of effectively vaccinating large numbers of livestock in a feedlot setting. The objective of this study was to determine if adult ruminants could be effectively vaccinated and protected from virulent homologous challenge via oral delivery of *S. Typhimurium dam* vaccine in drinking water.

## **8.2 Materials and Methods**

### ***8.2.1. Bacterial strains and growth conditions***

Immunisations were carried out with *dam*-102::Mud-Cm Typhimurium UK-1 vaccine strain MT2313 (*S. Typhimurium dam*) as described in *Section 3.2.1*. Challenge studies were performed with a derivative of a *Salmonella* Typhimurium strain, 06-131 (ST 06-131, challenge) as described in *Section 3.2.2.ii*.

Strains used in infection studies were grown overnight in LB at 37°C, following procedures outline in Appendix I. Vaccine and challenge dose titres were confirmed via serial dilutions plated on Luria agar plates, colony serotype was confirmed via standard biochemical tests (urea, TSI, ONPG) and agglutination with serogroup B specific antisera [somatic O antisera group B, factors 1, 4, 5, 12, and 27 (BD Diagnostics, USA)].

The vaccine strain was delivered to vaccinated sheep via drinking water over 24 h. Each sheep had an individual bucket of drinking water. The vaccine strain was inoculated into fresh water containing 20% phosphate buffered saline (PBS) to a final volume of 5L and target concentration of  $1 \times 10^7$  CFU/L at the morning and evening feeding. Water consumption was recorded to the nearest 0.1 L for the 24 h vaccination periods.

### **8.2.2. Sheep selection and husbandry**

Merino wethers, approximately 2 to 4 years in age, were sourced from a single flock in New South Wales, Australia, using selection criteria outlined in *Section 3.5.3*. Faecal samples were collected from all sheep prior to enrolment in the study and underwent qualitative detection of *Salmonella* following procedures outlined in *Section 3.6.1*. No salmonellae were detected in the sheep during the acclimation period. Sheep were transported to the Medium Security Animal Housing Facility at EMAI, and randomly assigned to experimental groups and housed following procedures outlined in *Sections 3.5.5* and *3.5.6*. Sheep received a diet of oaten chaff (8 MJ ME/kg DM) and commercially prepared grain pellet (12 MJ ME/kg DM) during the acclimation, vaccination and challenge phases of

the experiment. Sheep were gradually introduced to the grain pellet during the acclimation and vaccination periods. Sheep were offered 700 g of oaten chaff and 250 g of grain pellets at the morning observation period during the challenge periods (roughly 9 MJ ME/d).

### **8.2.3. Clinical assessment**

Clinical parameters evaluated twice daily included attitude, water consumption, and faecal characteristics. Rectal temperatures (RT) were recorded in the mornings prior to feeding twice a week during the acclimation period, once daily for seven days following vaccination and then daily following virulent *Salmonella* challenge. The normal range of RT for the sheep used in these experiments based on the pre-vaccination mean  $\pm$  two standard deviations was 38.4 – 39.6°C. Clinical pyrexia was defined as a sheep with a RT above 40°C (Radostits, *et al.*, 2007; Terra, 2009). Attitude and faecal composition were scored on an ordinal scale twice a day using procedures outlined in *Sections* 3.5.7.i and 3.5.7.iv, respectively. Appetite was recorded in grams of chaff and pellets not consumed over each 24 h feeding period. Percent refusal of chaff and pellets was determined for each animal during the challenge period. An attitude score of 4 at any time of the day or failure to consume chaff or pellets (100% refusal of feed) for three consecutive feedings constituted grounds for euthanasia. Sheep were weighed during the acclimation period, prior to virulent challenge and 13 d post-challenge. Average daily weight gains were calculated for pre-challenge and post-challenge intervals. The study protocol was approved by the AEC at EMAI.

### **8.2.4. Experimental design**

A total of 100 sheep were used in the conduct of this study. An initial challenge dose trial was conducted in order to determine an optimum dose that would induce disease while minimising mortality, in order to maintain sample size and statistical power for measured outcomes. Sheep were orally administered ST 06-

131 challenge strain at  $5 \times 10^8$  (n = 7) and  $5 \times 10^9$  organisms (n = 7) for low and high dose, respectively. Following this, two vaccination-challenge trials (Experiments A and B) were conducted using 42 and 44 sheep respectively, although the results from these were analysed together. In Experiment A, 44 sheep were randomly assigned to three dose groups on day -28: non-vaccinates (n = 15), 7 d pre-challenge vaccinates (n = 14; challenged 7 d post-vaccination [7 d]) and 28 d pre-challenge vaccinates (n = 15; challenged 28 d post-vaccination [28 d]). In Experiment B, 42 sheep were randomly assigned to three dose groups on day -7: non-vaccinates (n = 14), 24 h pre-challenge vaccinates (n = 14, vaccinated 24 h prior to challenge [24 h pre-challenge]) and post-challenge vaccinates (n = 14; vaccinated 24 h post-challenge [24 h post-challenge]).

The vaccine strain was offered to the 28 d, 7 d and 24 h pre-challenge groups in drinking water for 24 h following overnight food and water fasting. The 12 h fasting prior to vaccination was intentional, in order to mimic the fasting livestock experience with mustering and road transport to feedlot facilities. The sheep in the 24 h post-challenge vaccination group received the vaccine as an oral bolus delivered via a syringe (on day 1) to ensure that the sheep consumed an adequate dose of the vaccine. This group was included to assess safety of the vaccine in livestock that are already infected with virulent salmonellae.

All sheep were orally challenged with  $1 \times 10^9$  CFU (actual dose) of ST 06-131 on day 0 which was 28 d, 7 d, and 24 h following vaccinations for the 28 d, 7 d, and 24 h pre-challenge vaccination groups, respectively, and 24 h preceding vaccination of the 24 h post-challenge vaccination group.

#### **8.2.5. Faecal sampling**

During the acclimation phase, faecal samples were collected from sheep and screened for *Salmonella* following procedures outlined in Section 3.6.1. *Salmonella* suspect colonies were sub-cultured for pure growth and identity

confirmed following procedures outlined in *Section 3.6.4*. *Salmonella* isolates were sent to the IMVS, *Salmonella* Reference Laboratory (SA, Australia) for serotyping.

Faecal samples were assessed for shedding of the *S. Typhimurium dam* vaccine strain 3 d post vaccination and days 3, 7, 10 and 13 post virulent challenge. Shedding of the challenge strain, ST 06-131, was assessed on days 3, 7, 10, and 13 post-challenge. Between 2 and 5 g of faeces were collected per rectum of each sheep. The faecal material was homogenised as a 1:5 dilution in PBS, serially diluted, plated on XLD plates containing chloramphenicol (20 µg/mL) (XLD-CHL) to select for the *S. Typhimurium dam* vaccine strain, or XLD plates containing neomycin sulphate (10 µg/mL) (XLD-NEO) to select for the challenge strain, and incubated for 24-48 h at 37°C. Additionally, 1 mL of the homogenised sample was placed into 9 mL of MSB enrichment media, incubated for 24h at 37°C, and streaked for single colonies on XLD-CHL and XLD-NEO. The estimated number of *Salmonella* organisms present in the original faecal sample was calculated based on the sample weight, dilution factor, and number of colonies counted. Two colonies from each *Salmonella* positive faecal culture were sub-cultured and tested with O-antigen specific antisera to verify isolate identification following procedures outlined in *Section 3.6.4*.

#### **8.2.6. *Salmonella* isolation from ovine tissues**

Sheep were euthanised and immediately necropsied using standard techniques 13 d post virulent challenge. Approximately 1 to 2 g of tissue were obtained from the ileum wall, MLN, liver, lung, and spleen for each animal. Tissue sections were homogenised as a 1:4 dilution in PBS, serially diluted, plated on XLD-NEO and XLD-CHL, and incubated for 48 h at 37°C. Additionally, 1 mL of the homogenised sample was placed into 9 mL of MSB enrichment media, and incubated for 24 h at 37°C. Colony counts were enumerated and MSB

enrichments were streaked onto XLD-NEO and XLD-CHL. Samples positive by selective enrichment in MSB were recorded as 40 CFU/g and negative samples were recorded as 1 CFU/g.

### **8.2.7. Statistical analysis**

Continuous data were analysed using REML analysis (Genstat, 12<sup>th</sup> Edition, VSN International, UK). A single variate, repeated measures model was fitted for the factors time and treatment for variables CFU, RT, chaff & pellet refusal, and water intake. The Wald chi-square test was used to determine significant individual effects and or significant interactions between factors. Any non-significant terms were dropped from the model and analysis repeated. Following analysis, data are presented as predicted model based means. Predicted means are those obtained from the fitted model rather than the raw sample means. This is an important distinction, as predicted means represent means adjusted to a common set of variables, thus allowing valid comparison between means.

A P value less than 0.05 was considered to be statistically significant. Differences between the individual means were determined by calculating an approximate LSD. A difference of means that exceeded the calculated LSD was considered significant.

Ordinal data (attitude and faecal scores) was analysed using a proportional odds ordinal logistic regression model with random effects (Agrestis, 2002) using ASRemL 3.0 statistical software (Gilmour, *et al.*, 2009). The fixed effects of the model were time, treatment and their interaction, and random effects were animal and experiment (A vs. B). Significance of fixed effects was assessed using Wald chi-square tests. Comparisons of specific treatments were conducted using Wald z tests. P values less than 0.05 were considered statistically significant. This analysis established the significance of any differences between the treatments and determined a probability of each response for each treatment (Dobson, 2002).

Data are presented as the probability as determined by the model that an animal would display an abnormal attitude (cumulative daily score > 5) or have diarrhoea (cumulative daily score > 5) over the course of the challenge period.

Tissue colonisation and pre-challenge clinical assessment of the 24 h pre-challenge vaccinates were evaluated using ANOVA; the vaccine dose and body weight of sheep that did and did not develop pyrexia following vaccination was compared using unpaired t-test; mortality results were compared using chi-square (Buchan I, StatsDirect statistical software <http://www.statsdirect.com>, 2002, CamCode, England).

## **8.3 Results**

### ***8.3.1. Challenge dose trial***

The virulent challenge strain, ST 06-131, is an industry relevant, multi-drug antimicrobial resistant strain that was isolated from an outbreak of salmonellosis in sheep at a commercial feedlot in Western Australia. A challenge dose trial was conducted in order to determine an optimum dose that would induce disease while minimising mortality, in order to maintain sample size and statistical power for measured outcomes. All of the sheep in the low ( $5 \times 10^8$  CFU) and high ( $5 \times 10^9$  CFU) dose challenge groups developed clinical signs of salmonellosis (fever, diarrhoea, decreased appetite and depressed attitude) during the 13 d post-challenge observation period. As expected, the severity of these signs was greater in sheep challenged at the high dose. There were significant differences in the severity of these outcomes ( $P < 0.001$ ) when comparing low to high dose groups. There were four mortalities in the sheep administered the high dose on days 6, 7, 8 and 11 post-challenge, respectively. No mortalities were observed in the low dose group. A challenge dose of  $1 \times 10^9$  CFU of ST 06-131 was targeted for the vaccine efficacy trials to provide for predictable induction of disease with minimal mortality.



### **8.3.2. Effect of a *S. Typhimurium* dam vaccine strain when delivered in drinking water to adult sheep**

There was no evidence of refusal of the vaccine when delivered in-water, as indicated by significantly increased water consumption ( $P < 0.05$ ). On vaccination day, water consumption was  $3.5 \pm 0.47$  L,  $3.7 \pm 1.05$  L and  $4.0 \pm 1.37$  L for the 28 d, 7 d, and 24 h pre-challenge vaccination groups respectively, which was 132.4, 140.3 and 151.5 percent of the mean daily water consumption during the 3 days preceding vaccination for each group. Sheep have been reported to consume between 5 and 20% of their body weight in water daily (Singh, *et al.*, 1976; Savage, *et al.*, 2008). In this experiment, sheep consumed 7-12% of their body weight per day, which was within the normal range. The mean in-water vaccination dose of the vaccine consumed by the sheep was:  $4.2 \times 10^7$  CFU (range 2.9 to  $5.8 \times 10^7$  CFU/sheep) for the 28 d vaccinates,  $6.7 \times 10^7$  CFU (range 3.1 to  $9.5 \times 10^7$  CFU/sheep) for the 7 d vaccinates and  $1.9 \times 10^7$  CFU (range  $9.7 \times 10^6$  to  $3.1 \times 10^7$  CFU/sheep) for the 24 h pre-challenge vaccinates. The sheep in the 24 h post-challenge vaccination group received  $1 \times 10^7$  CFU/sheep in a 10 mL oral bolus.

The vaccine strain was recovered 72 h post vaccination from the faeces of 11 of 15 and 10 of 14 sheep from the 28 d and 7 d vaccinates, respectively. During the challenge phase of the experiment, the faeces of the sheep were monitored for the shedding of the vaccine. Low numbers of the vaccine strain were isolated on enrichment culture. The vaccine strain was isolated from the faeces of 5 of 14, 6 of 13, and 2 of 12 of the 24 h pre-challenge vaccinates and 7 of 14, 4 of 12 and 0 of 11 sheep from the 24 h post-challenge vaccinates on days 3, 7 and 10 post-challenge, respectively. The vaccine strain was isolated from the faeces of 4 of 14 sheep from the 7 d vaccinates on day 3 post-challenge only. The vaccine strain was not isolated from the 28 d vaccinates during the 13 d challenge period. The

vaccine strain was not isolated from the faeces of the vaccinated sheep at necropsy (day 13), which was 42, 21, 15 and 12 d post vaccination in the 28 d, 7 d, 24 h pre-challenge and 24 h post-challenge vaccination groups, respectively.

There were no biologically significant changes in attitude or faecal scores following vaccination in the 28 d and 7 d vaccinated sheep. No mortalities were associated with administration of the vaccine. It was not possible to assess the clinical response to vaccination in the 24 h pre- and post-challenge groups due to the association with virulent challenge. However, the rectal temperatures ( $P = 0.7386$ ), chaff appetites ( $P = 0.0745$ ), pellet appetites ( $P = 0.7345$ ), water consumption ( $P = 0.0907$ ), attitude ( $P = 0.6501$ ) and faecal scores ( $P = 0.7252$ ) of the 24 h pre-challenge vaccinates were not significantly different from the controls during the 48 h period preceding virulent challenge.

Transient pyrexia ( $RT > 40.0^{\circ}\text{C}$  (Radostits, *et al.*, 2007; Terra, 2009)) was observed following vaccination with a significant interaction observed between time and vaccination treatment ( $P < 0.001$ ) (Figure 8-1). Pyrexia was observed in 5 of the 15 sheep in the 28 d vaccinates and 4 of 14 sheep in the 7 d vaccinates during the seven day period following vaccination for a period of 1 to 3 days. One sheep in the 7 d vaccinates maintained a fever for 5 days ( $40.4 - 41.2^{\circ}\text{C}$ ). There was no significant interaction between vaccine dose consumed and pyrexia ( $P = 0.97$ ), however there was a trend for pyrexia to be associated with lower body weight ( $P = 0.09$ ).

A significant interaction between time and vaccination treatment was observed over the whole 7 d post-vaccination periods for chaff ( $P < 0.001$ ) but not for pellets ( $P = 0.08$ ). The 7 d vaccinates consumed less chaff than non-vaccinated controls from 24 to 96 h post vaccination ( $P < 0.05$ ) (Figure 8-2) and fewer pellets at 48 h and 96 to 168 h post vaccination ( $P < 0.05$ ) (Figure 8-3). Although there was a reduction in pellet and chaff consumption following vaccination

relative to the non-vaccinated sheep, the reduction was not considered biologically significant as the pellet and chaff consumption of vaccinated sheep remained within the normal range established during the 7 d acclimation period preceding vaccination.

**8.3.3. Immunisation with *S. Typhimurium dam* confers improved clinical disease outcomes against virulent *S. Typhimurium* in adult sheep when delivered in drinking water**

Here we questioned whether in-water vaccination of sheep with a *S. Typhimurium dam* can confer homologous protection against a virulent *S. Typhimurium* strain 06-131. There was a significant interaction between time and vaccination treatment on RT during the 14 d study period ( $P < 0.001$ ). Sheep receiving the vaccine had significantly lower mean RT when compared to non-vaccinated controls from days 2 to 13 post-challenge for the 28 and 7 d vaccinates, days 7 to 10 post-challenge for the 24 h pre- challenge vaccinates and on day 7 for the 24 h post-challenge vaccinates ( $P < 0.05$ ) (Figure 8-4). The temperatures of the 28 d and 7 d vaccinated sheep remained within the normal range throughout the challenge period.

A significant time by vaccination treatment interaction on attitude scores was observed during the 14 d observation period ( $P < 0.001$ ). The probability that non-vaccinated controls, 24 h pre-challenge vaccinates, and 24 h post-challenge vaccinates had an abnormal attitude score (daily attitude score greater than 3) was increased when compared to the 28 d and 7 d vaccinates (Figure 8-5). The 28 and 7 d vaccinates had significantly lower attitude scores when compared to the non-vaccinated sheep from day 3 to 13 post-challenge ( $P < 0.05$ ). The attitude scores of the 24 h pre-challenge and 24 h post-challenge vaccinates were significantly lower than the non-vaccinated controls on day 8 post-challenge ( $P < 0.05$ ).

There was a significant time by vaccination treatment interaction on faecal score during the 14 d observation period ( $P < 0.001$ ). There was no significant difference between the non-vaccinated controls, 24 h pre-challenge and 24 h post-challenge vaccinates, which experienced an increase in faecal scores during the middle phase of the experiment and then a decline (Figure 8-6). However the faecal scores of the 24 h post-challenge vaccinates were significantly increased when compared to the controls on day 4 post-challenge, and significantly lower on day 10 ( $P < 0.05$ ). The 7 d vaccinates maintained significantly lower faecal scores than the non-vaccinated controls from days 5 to 12 post-challenge, while the 28 d vaccinates were significantly lower than the non-vaccinated controls from days 4 to 13 post-challenge ( $P < 0.05$ ). Additionally, the 28 d vaccinated group had significantly improved faecal scores when compared with the 7 d vaccinates from day 3 to 6 and day 11 to 13 post-challenge ( $P < 0.05$ ).

A significant interaction was observed between time and vaccination treatment on the faecal shedding of ST 06-131 ( $P < 0.001$ ). Sheep receiving the vaccine at 28 d and 7 d pre-challenge had significantly reduced faecal shedding of ST 06-131 when compared to non-vaccinated controls on days 3, 7, 10 and 13 post-challenge ( $P < 0.05$ ) (Figure 8-7). The 24 h post-challenge vaccinates had significantly reduced faecal shedding of ST 06-131 when compared to non-vaccinated controls on day 10 post-challenge ( $P < 0.05$ ).

There were significant interactions between time and vaccination treatment on chaff and pellet appetite and water consumption during the 13 d observation period ( $P < 0.001$ ). The 28 d and 7 d vaccinates had significantly lower chaff and pellet refusals when compared to the non-vaccinated controls from days 2 to 12 post-challenge ( $P < 0.05$ ) (Figure 8-8 and Figure 8-9). The 24 h pre-challenge vaccinates had significantly lower chaff refusal than controls on days 3, 4, and 6 to 8 d post-challenge, as well as significantly lower pellet refusals on days 8 to 10

( $P < 0.05$ ). The 24 h post-challenge vaccinates had significantly increased chaff refusal on day 2 post-challenge and significantly lower refusal on day 9 post-challenge ( $P < 0.05$ ) (Figure 8-8). Additionally, 24 h post-challenge vaccinates had significantly increased pellet refusals on days 2 and 3 post-challenge and significantly lower refusal on day 8 post-challenge ( $P < 0.05$ ) (Figure 8-9). Water consumption when compared to the non-vaccinated controls was significantly increased in the 28 d vaccinates from day 4 to 9 post-challenge ( $P < 0.05$ ) (Figure 8-10). The 7 d vaccinates had significantly increased water consumption from day 2 to 11 post-challenge ( $P < 0.05$ ). The 24 h pre-challenge vaccinates had significantly increased water consumption when compared to the non-vaccinated controls on days 3, 6 and 10 post-challenge, while the 24 h post-challenge vaccinates were increased on days 9 and 10 post-challenge ( $P < 0.05$ ).

There were significant interactions between time and vaccination treatment on body weight over the study period ( $P < 0.001$ ). Weights day 13 post-challenge in the 28 d and 7 d vaccinate groups were significantly higher than those of the non-vaccinated controls ( $P < 0.05$ ) (Figure 8-11). There was no difference between the weights of the 24 h pre-challenge vaccinates and the non-vaccinated controls on day 13 post-challenge, however, the 24 h post-challenge vaccinate body weights were significantly less than the non-vaccinated controls ( $P < 0.05$ ).

#### ***8.3.4. Immunisation with *S. Typhimurium dam* confers protection against virulent *S. Typhimurium* colonisation of tissue in adult sheep***

The ability of *S. Typhimurium dam* to confer protection against colonisation of lymph nodes and visceral organs with ST 06-131 was evaluated in sheep. Vaccine efficacy was determined by enumeration of ST 06-131 recovered from mesenteric lymph nodes, lungs, spleen, liver, and ileum wall 13 d post-challenge. Significantly lower numbers of ST 06-131 were recovered from mesenteric lymph

nodes and ileum of the 28 d and 7 d vaccinates when compared to the non-vaccinated controls (Figure 8-12;  $P < 0.0001$ ). Significantly lower numbers of ST 06-131 were recovered from the liver ( $P < 0.005$ ), spleen ( $P < 0.05$ ) and lungs ( $P < 0.05$ ) of the 28 d vaccinates and from the liver ( $P < 0.005$ ) and spleen ( $P < 0.01$ ) of the 7 d vaccinates when compared to non-vaccinates. There was no significant difference in tissue colonisation of ST 06-131 in the 24 h pre- and post-challenge vaccinates when compared to the controls ( $P > 0.5$ ). The vaccine strain was isolated from the MLN of 1 of 14 sheep from the 7 d vaccinates and 1 of 10 sheep from 24 h pre-challenge vaccinates following necropsy using enrichment culture.

These data suggest that immunisation with *S. Typhimurium dam* conferred significant protection against a virulent *S. Typhimurium* challenge as evidenced by attenuation of clinical disease, improved appetite, weight gains, reduced faecal shedding and reduced colonisation in tissues when administered 28 d and 7 d prior to virulent challenge.

#### ***8.3.5. Immunisation with S. Typhimurium dam confers protection against mortality in adult sheep challenged with virulent S. Typhimurium***

Sixteen sheep mortalities were observed during the conduct of these trials. No mortalities were seen in the 28 d and 7 d vaccination groups. Eight mortalities were observed in the non-vaccinated sheep on days 5 ( $n = 1$ ), 8 ( $n = 3$ ), 11 ( $n = 3$ ) and 12 ( $n = 1$ ) post-challenge. Four mortalities were observed in the 24 h pre-challenge vaccinates on days 6 ( $n = 1$ ), 7 ( $n = 1$ ), and 12 ( $n = 2$ ) post-challenge, and four mortalities were observed in the 24 h post-challenge vaccinates on days 6 ( $n = 1$ ), 7 ( $n = 2$ ), and 11 ( $n = 1$ ) post-challenge. When compared to the non-vaccinated controls, improved survival was observed in the 28 d vaccinates ( $P = 0.006$ ) and 7 d vaccinates ( $P = 0.007$ ). There was no statistical difference in the

mortality rates of the non-vaccinates when compared to the 24 h pre- and post-challenge vaccinates following challenge with virulent ST 06-131 ( $P = 0.95$ )

#### **8.4 Discussion**

The increase in the incidence of disease and contamination of livestock-derived food products imposes a significant risk to food safety via consumption of contaminated meat and milk products (CDC, 1996; Roels, *et al.*, 1997; CDC, 2002, 2006). *Salmonella* infections are estimated to be the cause of 1.4 to 1.6 million food borne illnesses in the U.S. annually (Mead, *et al.*, 1999; CDC, 2006, 2009, 2010; Scharff, 2010) at an estimated economic cost of between \$2.6 and \$14.6 billion (CDC, 2010; Scharff, 2010). Epidemiological studies of salmonellae on dairy farms and feedlots have failed to identify effective and reliable avenues for salmonellae control (Norris, *et al.*, 1989a; Higgs, *et al.*, 1993; Fossler, *et al.*, 2005a; Vanselow, *et al.*, 2007b). The development and application of an effective *Salmonella* vaccine offers a means of reducing industry *Salmonella*-associated losses and public health risks. The challenge is to develop an ideal livestock *Salmonella* vaccine that is safe, affordable, practical to administer, stimulates rapid and sustained immunity and provides protection against the diversity of salmonellae serovars that may be encountered.

It has previously been reported that *S. Typhimurium dam* vaccines provide homologous and heterologous protection against diverse *Salmonella* serovars when administered to mice, chickens, and calves (Norris, *et al.*, 1989a; Dueger, *et al.*, 2001; Heithoff, *et al.*, 2001; Dueger, *et al.*, 2003a, 2003b; Mohler, *et al.*, 2006; Mohler, *et al.*, 2008). Oral delivery of vaccines via drinking water to livestock has a number of potential advantages. Large numbers of animals can be immunised rapidly, no stressful handling is required for vaccination, no carcass damage is induced by injection site reactions and the cost of vaccination should be minimised due to the minimal packaging requirements. However, the volatile fatty

acids produced in the rumen have previously been demonstrated to attenuate the survival of *Salmonella* in vivo (Chambers and Lysons, 1979; Mattila, *et al.*, 1988) and bile salts have been shown to reduce the ability of *Salmonella* to invade epithelial cells (Heithoff, *et al.*, 2001; Begley, *et al.*, 2005). Thus, either environmental condition could potentially limit the efficacy of oral delivery of live attenuated *Salmonella* vaccines in adult ruminants and had to be investigated.

The *S. Typhimurium dam* vaccine was readily consumed by sheep when delivered in drinking water. Sheep consume between 5 and 20% of their body weight in water daily (Singh, *et al.*, 1976; Savage, *et al.*, 2008). In this experiment, the water consumption of sheep was 7-10% of their body weight per day during the three days prior to vaccination and 10-12% of their body weight during the 24 h vaccination period. The *S. Typhimurium dam* vaccine was recovered from the faeces of a high proportion of sheep 72 h following vaccination, indicating the organism was capable of bypassing the rumen and establishing a vaccination response. At post mortem, the vaccine was isolated at low concentrations using enrichment cultures from the lymph nodes of two sheep at 14 d and 21 d post vaccination. The *S. Typhimurium dam* vaccine was not isolated from the lung, liver, spleen, ileum or faeces of any sheep at necropsy. These low concentrations suggest minimal carcass contamination if appropriate withholding periods are applied to sheep destined for slaughter.

Pyrexia was observed in nine of the 29 vaccinated sheep following vaccination prior to virulent challenge. Mild pyrexia was observed over 1 to 3 days in 8 of 9 pyretic animals and was not associated with significant changes in attitude or appetite. In the current experiment there was no association between pyrexia and the vaccine dose consumed, however, there was a trend for pyrexia to be observed in the lighter weight sheep. In previous studies conducted in neonatal calves, no fevers were observed in calves receiving an oral vaccine bolus of  $10^7$  CFU



(Mohler, *et al.*, 2006; Mohler, *et al.*, 2008). The pyrexia observed was consistent with responses to commercially available vaccines (Allen, *et al.*, 1996; Villarreal-Ramos, *et al.*, 1998).

This experiment was designed to reflect realistic scenarios commonly encountered during feedlot entry. Feedlots typically acquire livestock from multiple sources, often from diverse geographical locations. Such diversity of sources is an important variable for prior pathogen exposure. The purpose of administering a vaccine to livestock on entry to a feedlot is to provide protection against an infectious agent that is likely to be encountered in the feedlot. It is, however, possible that some stock may be infected prior to arrival on the feedlot. In this experiment, the *S. Typhimurium dam* vaccine was administered to sheep pre- and post-virulent *Salmonella* challenge. Experiments conducted with aro attenuated *Salmonella* vaccines have suggested the potential for vaccination to exacerbate virulent disease (Foster, *et al.*, 2008). Although the sheep vaccinated with *S. Typhimurium dam* following virulent challenge had reduced appetites for 2 d following vaccination, the trend for the group across attitude, appetite and faecal score variables was for a more rapid return to clinical normality when compared to non-vaccinated controls. There was no evidence that administration of the *S. Typhimurium dam* vaccine as a prophylaxis or concurrent with infection exacerbated clinical disease or mortality in adult sheep. This may reflect the fact that *S. Typhimurium dam* infection is associated with reduced multi-tissue innate immune cytokine responses relative to wild type (Shtrichman, *et al.*, 2002; Simon, *et al.*, 2007), and does not induce the immune suppression associated with virulent challenge and aro attenuated *Salmonella* vaccines (Alramadi, *et al.*, 1991; Heithoff, *et al.*, 2008a).

The time taken for the development of protective immunity following vaccination of livestock entering feedlots is a significant issue. The interval from arrival to

pathogen exposure is likely to be short and highly variable. In this study, administration of the *S. Typhimurium dam* vaccine via drinking water prevented clinical disease associated with virulent *S. Typhimurium* infection within seven days of vaccination. *Aro* attenuated *Salmonella* vaccines have previously been demonstrated to elicit early onset of protection via intra-peritoneal vaccination of mice and intramuscular vaccination of sheep (Mukkur and Walker, 1992; Schafer and Eisenstein, 1992). However, onset of protective immunity has been shown to be delayed (21 d) following oral vaccination (Mukkur and Walker, 1992). The difference in the time to onset of protective immunity between *dam* and *aro* attenuated *Salmonella* vaccines may be related to the expansion of myeloid-derived suppressor cells and resultant immune suppression associated with *aro* *Salmonella* infection (Heithoff, *et al.*, 2001; Heithoff, *et al.*, 2008a). Vaccination with *S. Typhimurium dam* vaccine protected against mortality and permitted the sheep to maintain appetites and body weight in spite of virulent challenge. While there was some evidence of protection associated with vaccination 24 h prior to challenge, it was limited and insignificant compared to the robust protection observed within 7 d. Salmonellosis and inanition are common causes of sheep mortality in feedlots and during live export (Norris, *et al.*, 1989a; Richards, *et al.*, 1989; Higgs, *et al.*, 1993; Richards, *et al.*, 1993; Vanselow, *et al.*, 2007a). In these studies, administration of the *S. Typhimurium dam* vaccine protected sheep from inanition associated with virulent *Salmonella* challenge within seven days of administration. Causes of inanition in feedlot animals are multi-factorial, however, protection of susceptible populations of animals from salmonellosis could significantly reduce losses associated with inanition and feed refusal in addition to protecting against virulent salmonella carcass contamination.

The study demonstrates that vaccination of adult ruminants with *S. Typhimurium dam* confers protective immunity against a homologous *S. Typhimurium* virulent challenge assessed by attenuation of clinical disease, reduced faecal shedding,

reduced tissue colonisation, increased appetite and weight gain, and reduced mortality. Additionally, the above results indicate that vaccination via in-water inoculation of *S. Typhimurium dam* is a viable alternative to parenteral administration. Homologous *Salmonella* protection supports the potential validity of in-water vaccination for the prevention and control of *Salmonella* infections in commercial livestock production systems.

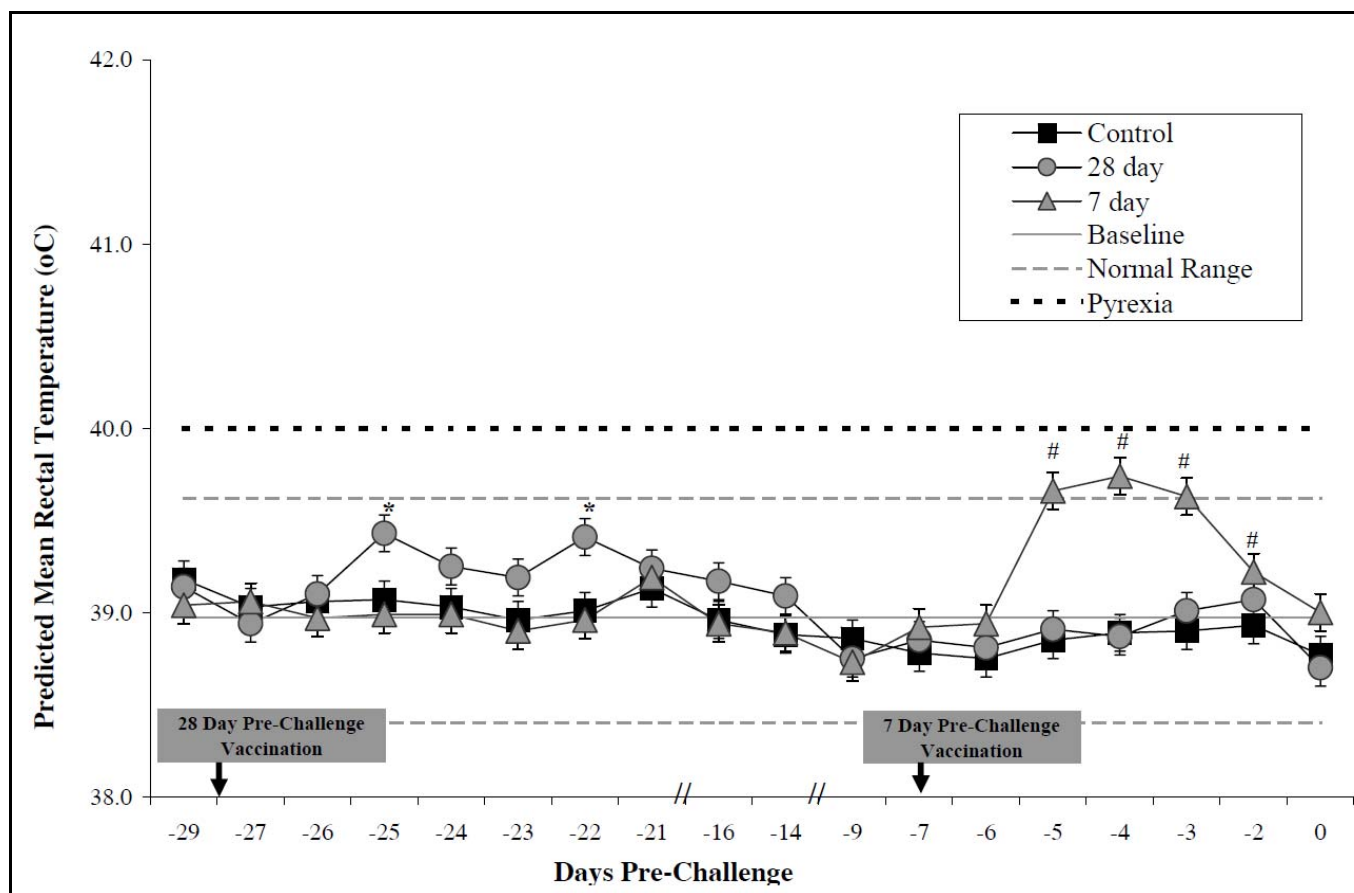


Figure 8-1. Predicted mean rectal temperatures following in-water immunisation of adult sheep with *S. Typhimurium dam*

Data are presented as predicted mean RT  $\pm$  SE; in the context of the normal range (dashed grey lines) and baseline (solid grey line) established pre-vaccination; and the threshold for pyrexia (dashed black line). \*RT of the 28 d vaccinates when compared to the unvaccinated sheep, 7 d and non-vaccinated controls, were significantly different at day -25, and day -22 pre-challenge ( $P < 0.05$ ). #RT of 7 d vaccination group exhibited significantly elevated RT when compared to the non-vaccinated controls days -5 to -2 pre-challenge ( $P < 0.05$ ).

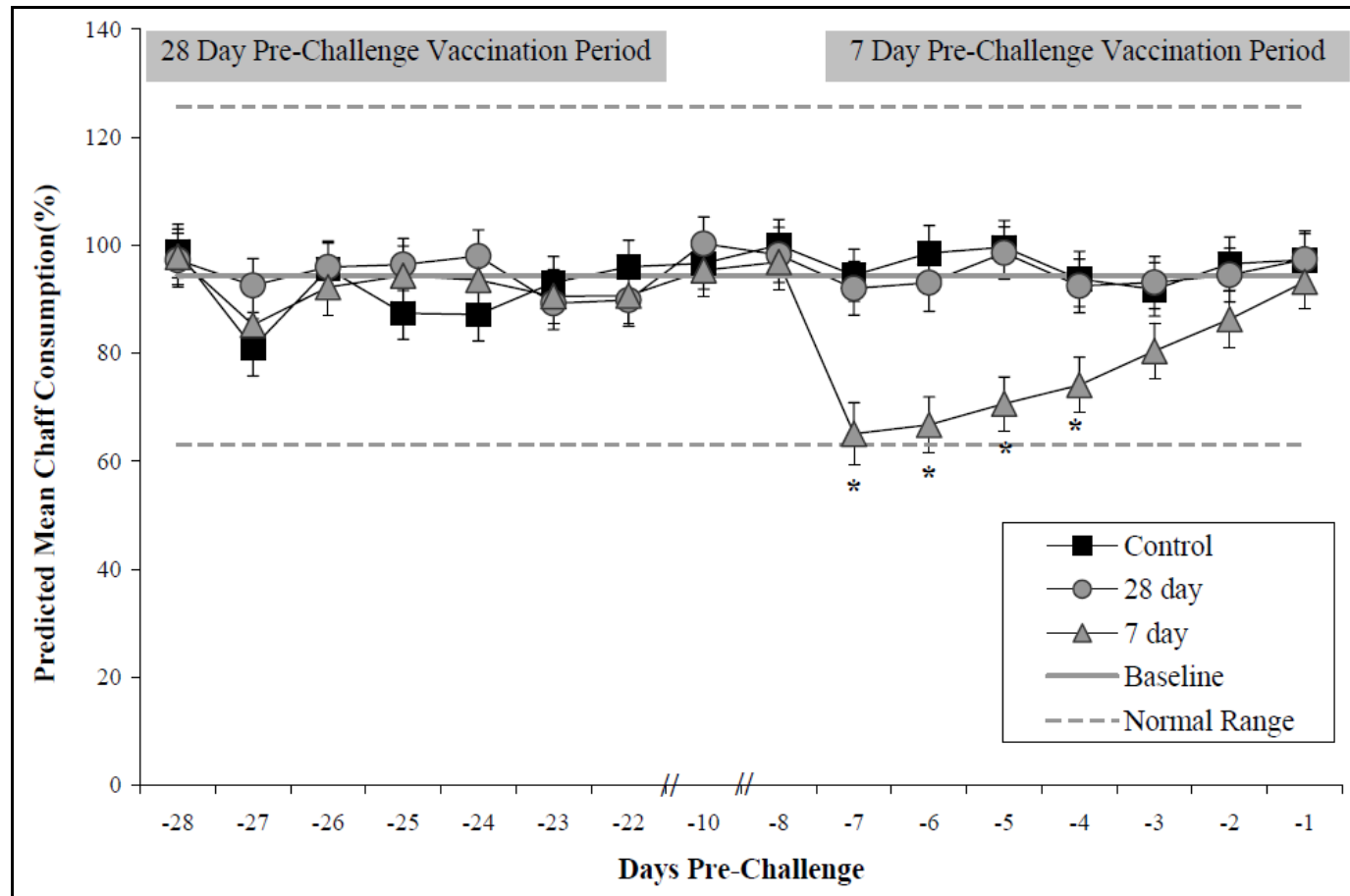


Figure 8-2. Predicted mean chaff consumption following in-water immunisation of adult sheep with *S. Typhimurium dam*

Chaff appetite is presented as the predicted mean chaff consumption  $\pm$  SE. The normal range for chaff consumption for sheep used in these experiments was based on the pre-vaccination mean  $\pm$  two standard deviations (dashed grey lines). \*Chaff consumption was significantly reduced in the 7 d vaccination group when compared to the non-vaccinated controls from days -7 to -4 pre-challenge ( $P < 0.05$ ).

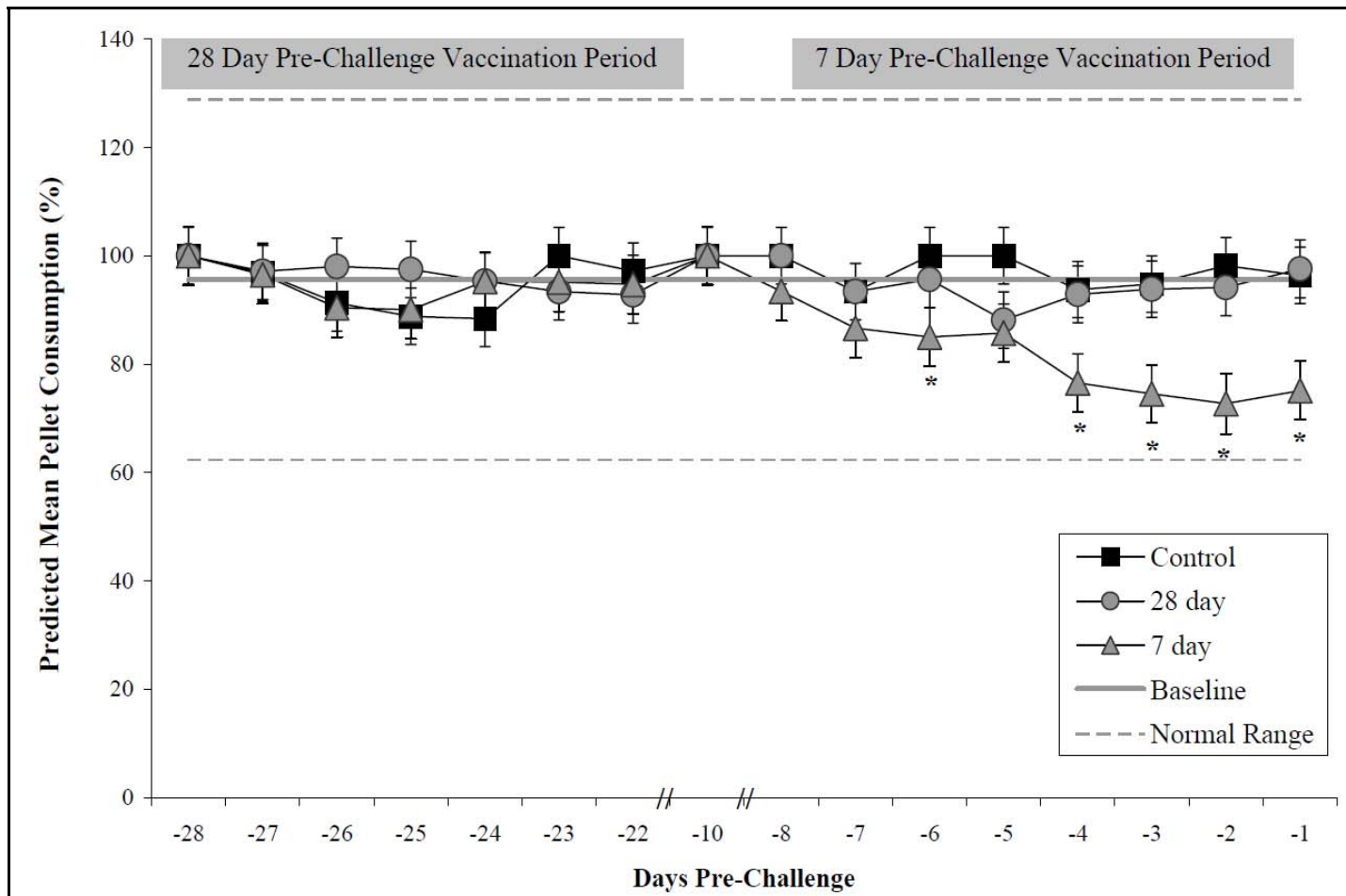


Figure 8-3. Predicted mean pellet consumption following in-water immunisation of adult sheep with *S. Typhimurium dam*.

Pellet appetite is presented as the predicted mean pellet consumption  $\pm$  SE. \*Pellet consumption was significantly reduced in the 7 d vaccination group when compared to the non-vaccinated controls at days -6 and -4 to -1 pre-challenge ( $P < 0.05$ ).

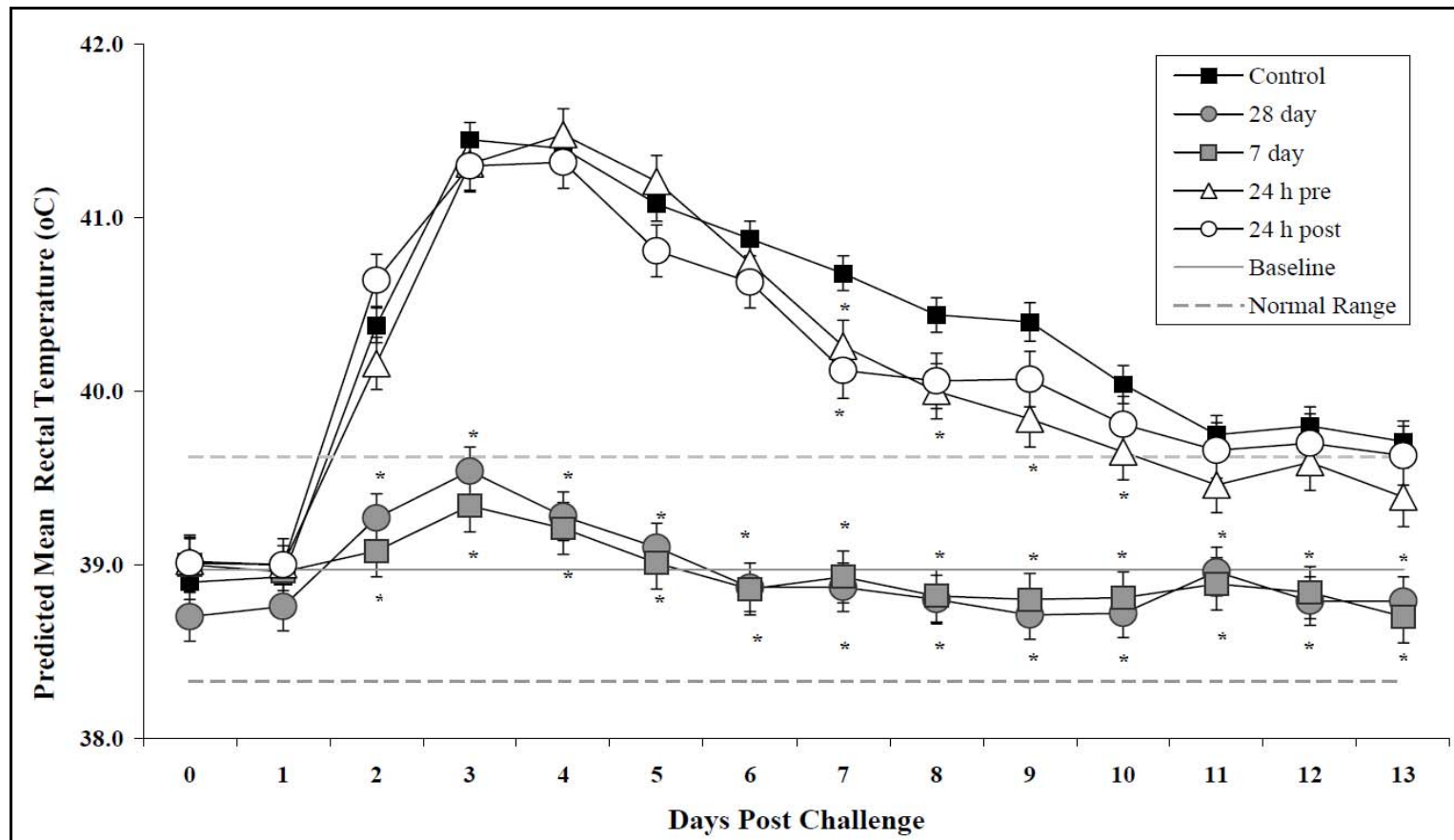


Figure 8-4. Predicted mean rectal temperatures of *S. Typhimurium dam* immunised sheep following virulent Typhimurium challenge.

RT were measured daily for 13 d following challenge. Data are depicted as a predicted mean RT  $\pm$  SE. The data are presented in the context of the normal range (dashed grey lines) and baseline (solid grey line) established pre-vaccination. \*RT were significantly lower in 28 d and 7 d vaccinates on days 2 to 13 post-virulent challenge, and the 24 h pre vaccinates on days 7 to 10 post-virulent challenge and on day 7 in the post-challenge vaccinates when compared to the non-vaccinated controls ( $P < 0.05$ ).

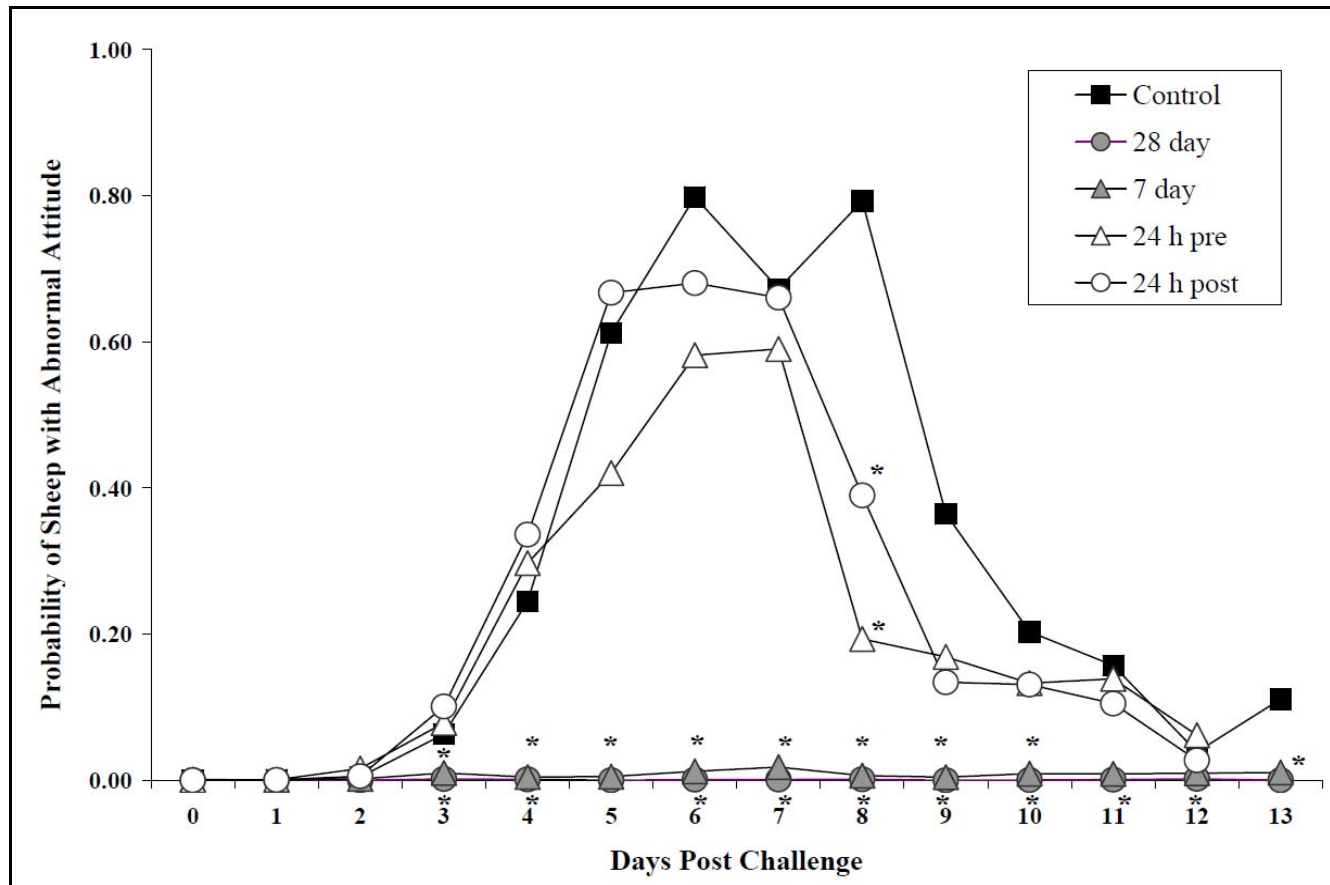


Figure 8-5. Probability of abnormal attitude score in *S. Typhimurium dam* immunised sheep following virulent Typhimurium challenge

Attitude was scored twice a day. Data are presented as the probability that at a sheep would display an abnormal attitude following virulent challenge.

\*The 28 d and 7 d vaccinates had significantly lower attitude scores when compared to the non-vaccinated sheep from day 3 to 13 post-virulent challenge ( $P < 0.05$ ). \*The attitude scores of the 24 h pre and 24 h post vaccinates were significantly lower than the non-vaccinated controls on day 8 post-virulent challenge ( $P < 0.05$ ).



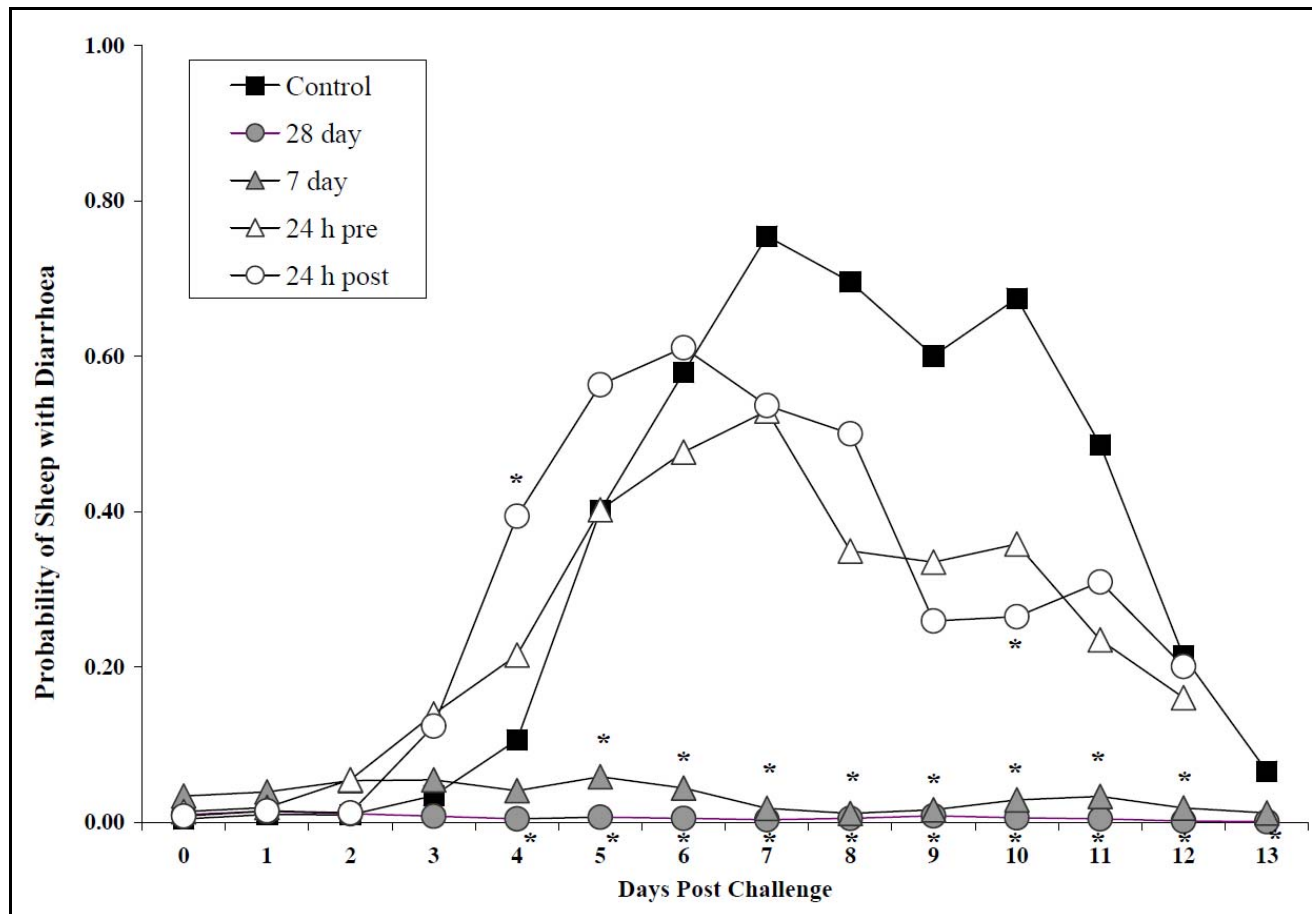


Figure 8-6. Probability of diarrhoea in *S. Typhimurium dam* immunised sheep following virulent Typhimurium challenge

Faecal scores were assessed twice a day. Data are presented as the probability that a sheep would have diarrhoea following virulent challenge. \*The 7 d vaccinates maintained significantly lower faecal scores than the non-vaccinated controls from days 5 to 12 post-virulent challenge while the 28 d vaccinates were significantly lower than the non-vaccinated controls from days 4 to 13 post-virulent challenge ( $P < 0.05$ ).

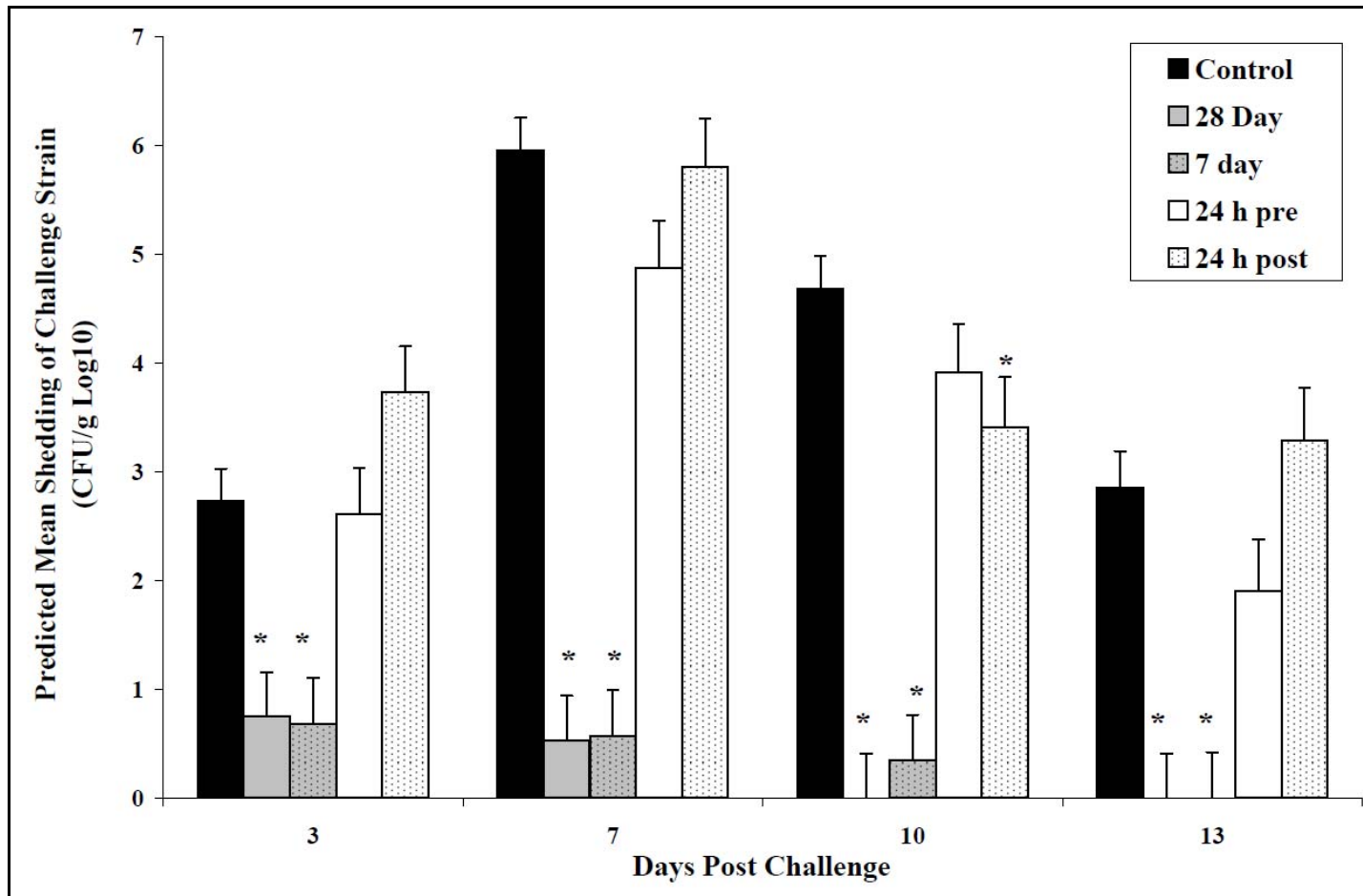


Figure 8-7. Predicted mean faecal shedding of virulent Typhimurium following challenge in *S. Typhimurium dam* immunised sheep

Data are depicted as a predicted mean  $\log_{10}$  CFU of ST 06-131 per g of faeces post-challenge  $\pm$  SE. \*Significant differences in faecal shedding were observed on days 3, 7, 10 and 13 post-challenge in the 28 d and 7 d vaccinates and on day 10 post-challenge in the 24 h post vaccinates ( $P < 0.05$ ).

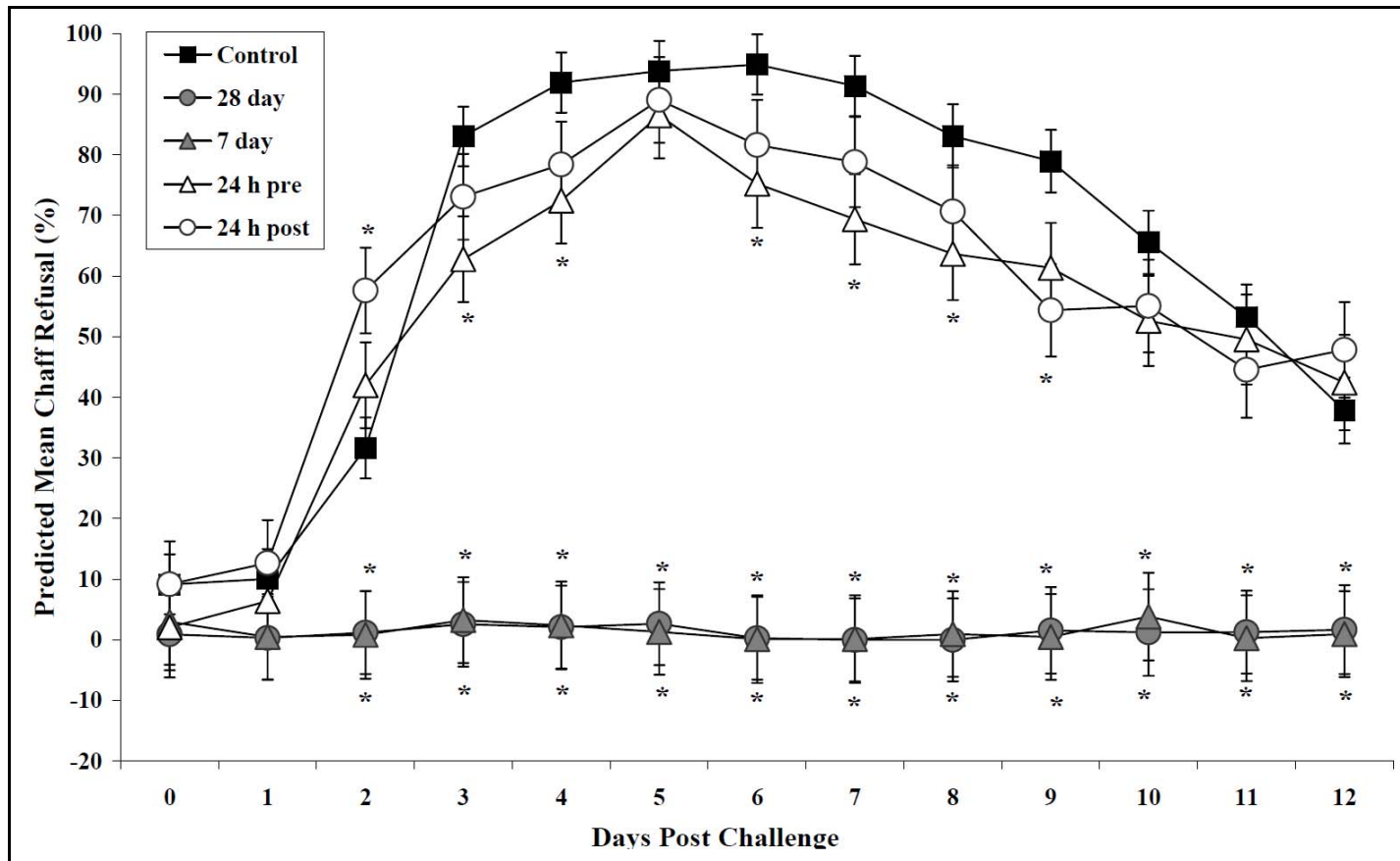


Figure 8-8. Predicted mean chaff refusal in *S. Typhimurium dam* immunised sheep following virulent Typhimurium challenge

Predicted mean chaff refusal is depicted  $\pm$  SE. \*Chaff refusal was significantly lower in the 28 d and 7 d vaccinates when compared to the non-vaccinated controls from days 2 to 12 post-virulent challenge ( $P < 0.05$ ). \*The 24 h pre-vaccinates had significantly lower chaff refusal than controls on days 3, 4, and 6 to 8 d post-virulent challenge, as well as significantly lower pellet refusals on days 8 to 10 ( $P < 0.05$ ). \*The 24 h post-vaccinates had significantly increased chaff refusal on day 2 post-virulent challenge and significantly lower refusal on day 9 post-virulent challenge ( $P < 0.05$ ).

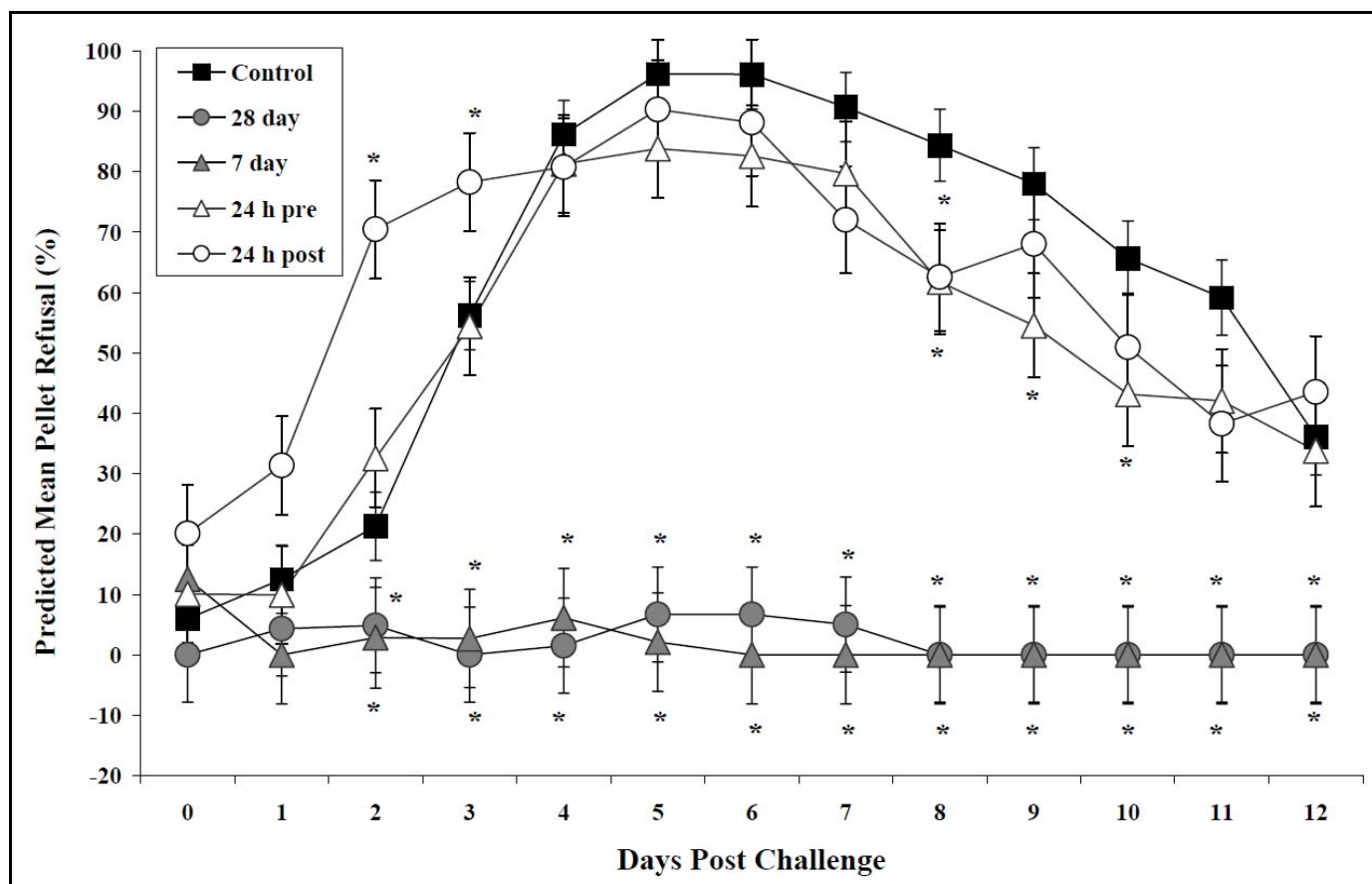


Figure 8-9. Predicted mean pellet refusal in *S. Typhimurium dam* immunised sheep following virulent Typhimurium challenge.

Predicted mean pellet refusal is depicted  $\pm$  SE. \*The pellet refusal of the 28 d and 7 d vaccinates was significantly lower when compared to the non-vaccinated controls from days 2 to 12 post-virulent challenge ( $P < 0.05$ ). \*The 24 h pre-vaccinates pellet refusals were significantly lower on days 8 to 10 ( $P < 0.05$ ). \*The 24 h post-vaccinate pellet refusals were significantly increased on days 2 and 3 post-challenge and significantly lower on day 8 post-virulent challenge ( $P < 0.05$ ).

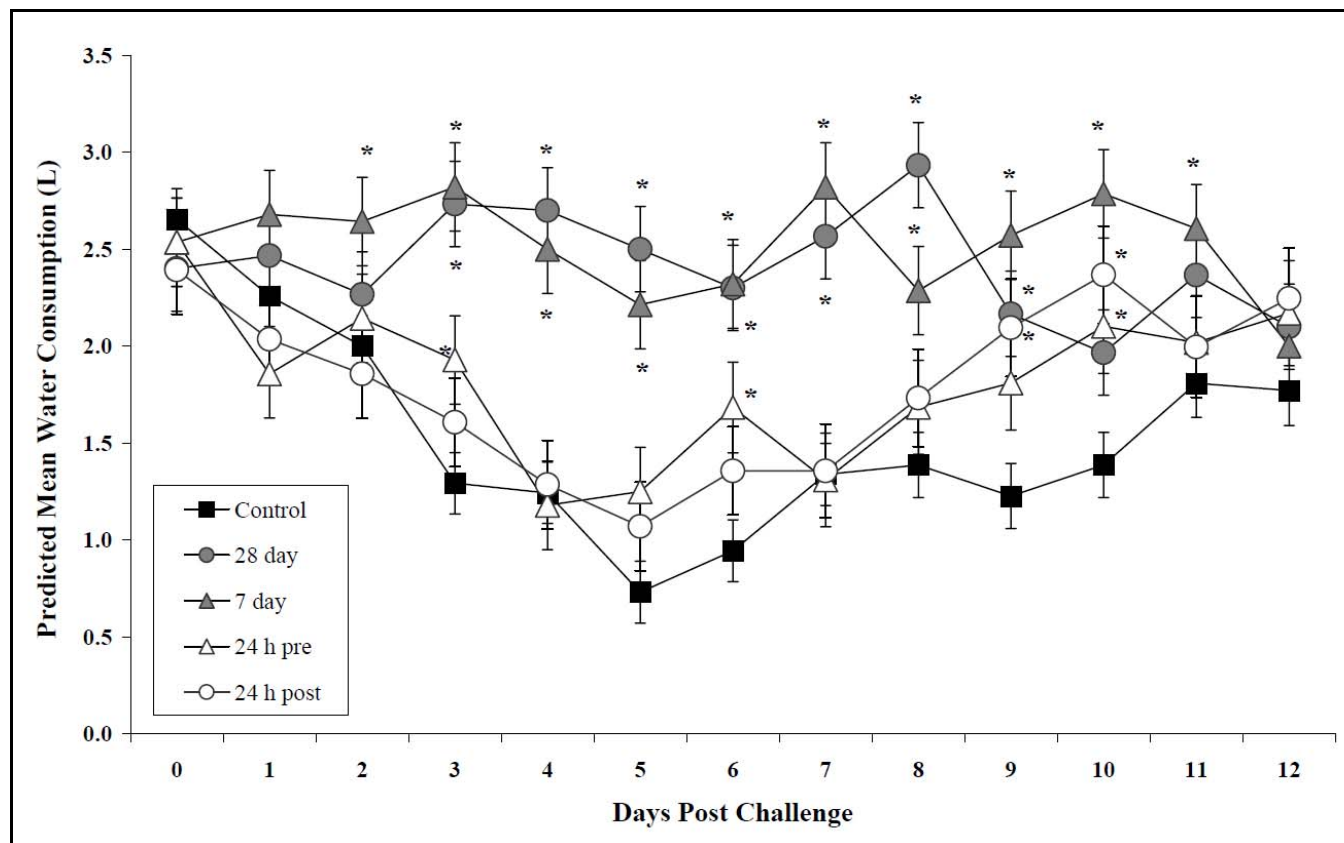


Figure 8-10. Predicted mean water consumption in *S. Typhimurium dam* immunised sheep following virulent Typhimurium challenge.

Predicted mean water consumption in litres is depicted  $\pm$  SE. \*The water consumption of the 28 d vaccinates when compared to the non-vaccinated controls was significantly higher days 3 to 9 post-virulent challenge ( $P < 0.05$ ). \*The water consumption of the 7 d vaccinates was significantly increased from day 2 to 11 post-virulent challenge ( $P < 0.05$ ) when compared to the control. \*The 24 h pre-vaccinates were significantly increased when compared to the non-vaccinated controls on days 3, 6 and 10 post-virulent challenge while the 24 h post vaccinates were increased on days 9 and 10 post-virulent challenge ( $P < 0.05$ ).

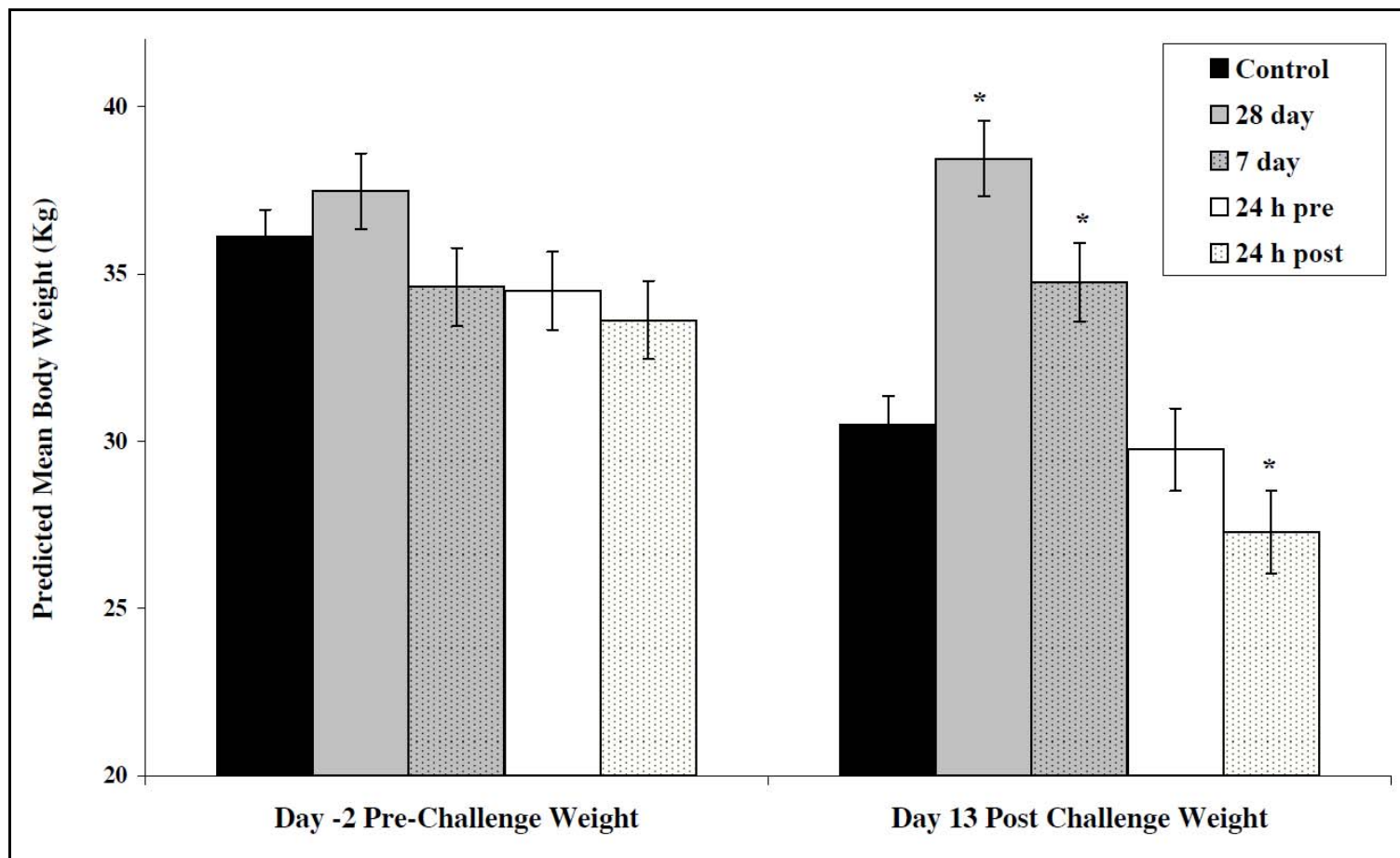


Figure 8-11. Predicted mean body weight of *S. Typhimurium dam* immunised sheep following virulent Typhimurium challenge.

Data are depicted in predicted mean BWT in Kg  $\pm$  SE at 2 days pre-challenge and day 13 post-challenge. \*BWT of the 28 d and 7 d vaccinates were significantly higher than the non-vaccinated controls 13 d following virulent challenge ( $P < 0.05$ ) and the BWT of the 24 h post-vaccinates were significantly less than that of the non-vaccinated sheep ( $P < 0.05$ ).

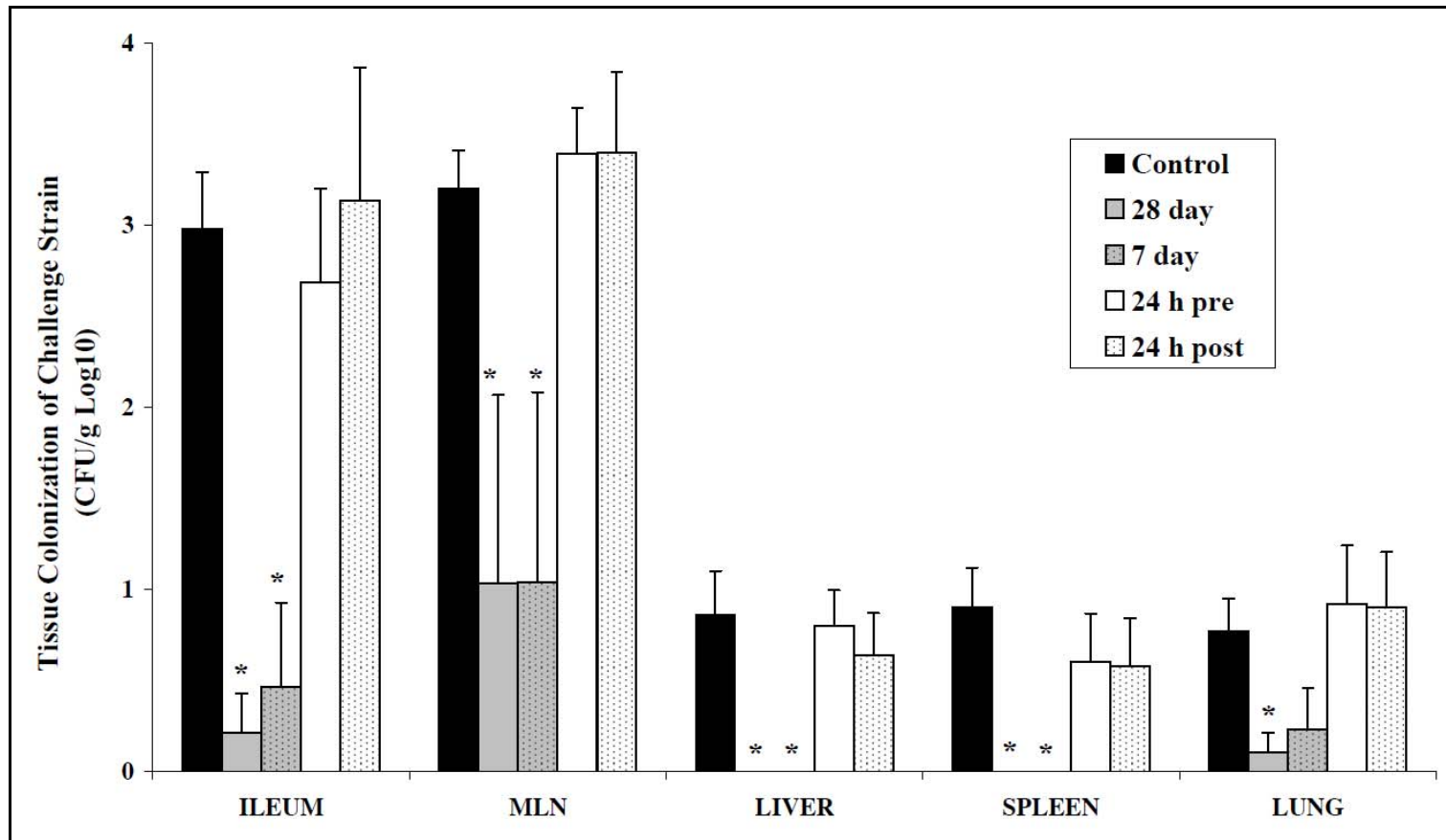


Figure 8-12. Immunisation with *S. Typhimurium dam* confers homologous protection against virulent Typhimurium, ST 06-131, colonisation of tissues in sheep

Tissue colonisation data are depicted as mean log<sub>10</sub> CFU ± SE of ST 06-131, recovered by organ. \*Significantly lower CFU were recovered from MLN ( $P < 0.0001$ ), liver ( $P < 0.005$ ), spleen ( $P < 0.05$ ) and ileum ( $P < 0.0001$ ) of the 28 d and 7 d vaccinates when compared to the non-vaccinated controls. \*Significantly lower CFU were also recovered from the lungs ( $P < 0.05$ ) of the 28 d vaccinates when compared to non-vaccinated controls.

## CHAPTER 9. DISCUSSION AND CONCLUSIONS

### 9.1 Discussion

#### *9.1.1. Cross protective efficacy of S. Typhimurium dam vaccine in neonate calves*

These studies were conducted as a continuation of research into the cross protective efficacy of the *S. Typhimurium dam* vaccine in neonate calves using a multi-drug antimicrobial resistant serovar of *S. Newport*. The vaccine had previously been shown to provide cross protective immunity to a host adapted *S. Dublin*, serotype D<sub>1</sub> (Mohler, *et al.*, 2006). Further assessment of the breadth of cross protective efficacy was required to determine if the vaccine would be likely to provide the broad protection required in livestock production systems where a diversity of *Salmonella* serovars are likely to be encountered.

In Chapter 5, the *S. Typhimurium dam* vaccine attenuated clinical disease, reduced faecal shedding and tissue colonisation in neonatal calves challenged with a virulent multiple antimicrobial resistant *S. Newport* strain. These results, along with those from Mohler *et al.* (2006) and Dueger *et al.* (2003b), provide convincing evidence that the *S. Typhimurium dam* vaccine provides robust protection against homologous and heterologous salmonella challenge in neonatal calves. This is encouraging as the most common isolates reported to infect cattle in Australia are *S. Typhimurium* (B), *S. Dublin* (D<sub>1</sub>), *S. Bovismorbificans* (C<sub>2</sub>-C<sub>3</sub>), and *S. Anatum* (E<sub>1</sub>) (IMVS, 2010). Future challenge efficacy trials in the neonatal salmonellosis model should include *S. Bovismorbificans* (C<sub>2</sub>-C<sub>3</sub>), and *S. Anatum* (E<sub>1</sub>) to further demonstrate the cross protective efficacy of the *S. Typhimurium dam* vaccine and its relevance in the Australian livestock industry.

#### *9.1.2. Salmonellosis in sheep*

The dose range finding studies described in Chapter 6 established comprehensive



models of salmonellosis in sheep. Previously published ruminant models of salmonellosis utilised mortality, rectal temperature and observation of diarrhoea as outcome assessments. Some studies also measured cytokine and antibody responses, however, these did not equate with protective immunity. Previous studies have not measured attitudes (mentation), appetite and water consumption, nor established normal baselines prior to initiation of challenge. These dose range finding studies in sheep established a comprehensive model for evaluating the effects of salmonellosis on adult ruminants that included behaviour, feeding preferences, water consumption and body weight change, in addition to body temperature and faecal composition. These models of ruminant salmonellosis will be useful in studying animal behaviour, treatment protocols and future vaccination trials.

Interesting findings in the development of the salmonellosis models were the observations of food preference in sheep during the acclimation period along with inappetence and anorexia following virulent salmonellae challenge. Prior to virulent challenge, several of the sheep demonstrated food preferences and avoidance of either pellets or chaff. This was expected as the animals were raised on pastures and most had never been exposed to pellets or eating from feeders for that matter. However, following entry into the housing facility, none of the sheep in these trials were observed with poor rumen fill. Most of the animals readily adapted to the diets offered, accepting both components, however, some did refuse parts of the diet.

Following virulent salmonellae challenge, inappetence and anorexia were observed in all sheep within 48 h of challenge. Taken together with the observation that the prevalence of *Salmonella* excretion in sheep during the feedlot period rises from 0-2% to 26 - 97% following entry (Higgs, *et al.*, 1993; Richards, *et al.*, 1993; Kelly, 1995; Makin, 2011), the interval between

*Salmonella* exposure and inappetence in sheep could explain the 'neophobia' observed in sheep entering feedlots. Additionally, the observation that sheep infected with *Salmonella* preferred chaff over a pelleted high energy diet during the post challenge period, even when animals were considered recovered, may also explain avoidance of pellets by feedlot sheep classified as 'neophobic'. The significance of this finding deserves further research, as it may be useful in explaining the observation of 'neophobia' in feedlot sheep. Additionally, the feeding preferences observed in these models of ovine salmonellosis may be useful in the management of convalescing sheep during, and following, a salmonellae outbreak.

### ***9.1.3. Development of novel in-water vaccination protocol for ruminants***

In Chapter 7 the feasibility of in-water vaccination with *S. Typhimurium dam* attenuated vaccine was investigated. The *S. Typhimurium dam* was stable in drinking water with or without buffering agents for up to 24 h, remained viable under temperatures between 20°C and 37°C and did not proliferate in drinking water contaminated with animal faeces. A potential limitation to oral vaccine delivery in ruminants is the capacity of volatile fatty acids produced in the rumen to attenuate the survival of bacteria, e.g., *Salmonella*. The *S. Typhimurium dam* successfully established in the MLN of adult sheep demonstrated that the vaccine was capable of bypassing the rumen and surviving in the gastrointestinal tract of adult sheep. These results indicated the in-water prophylaxis of *S. Typhimurium dam* in adult ruminants was feasible.

Application of an in-water vaccination protocol on-farm will provide additional challenges that go beyond the scope of this research. On-farm or in feedlot scenarios, water is supplied to animals via communal water troughs. This could introduce more variability in dose of vaccine consumed. Ensuring that all animals consume an effective dose of the vaccine from trough water may be a limiting

factor. Environmental factors such as ultraviolet radiation, ambient temperature and water quality may play important roles in delivering an effective, safe dose of the vaccine. Unfortunately, due to the current QC2 quarantine status of the *S. Typhimurium dam* vaccine, in-field trials could not be performed. Future research with the vaccine in-water protocols should include administration of the vaccine from trough water to group housed sheep; vaccine-challenge efficacy trials following in-trough vaccination; as well as determination of the minimal dose of vaccine required to induce cross-protective efficacy in sheep.

#### ***9.1.4. Homologous efficacy of the vaccine in sheep***

In Chapter 8 the safety and efficacy of a *S. Typhimurium dam* vaccine administered to sheep via oral delivery in drinking water (*ad libitum*) was assessed. The *S. Typhimurium dam* vaccine significantly attenuated clinical disease (temperature, appetite, and attitude), reduced mortality, faecal *Salmonella* shedding and tissue colonisation following virulent *S. Typhimurium* ST 06-131 challenge within 7 d of vaccination. Biologically, animals receiving the vaccine 28 d or 7 d prior to virulent challenge with *S. Typhimurium* ST 06-131 were clinically normal during the post-challenge period. In fact, these animals did not display any signs of clinical disease and, more importantly, gained or maintained body weight over the post challenge period. Further, vaccination with *S. Typhimurium dam* did not pose a risk to stock previously infected with virulent *Salmonella*, which makes this vaccine useful in outbreak scenarios where animals are already immunologically compromised. The capacity of *dam* attenuated *Salmonella* vaccines delivered in drinking water to protect livestock from virulent *Salmonella* challenge offers an effective, economical, stressor free *Salmonella* prophylaxis for intensive livestock production systems.

## **9.2 Future Research**

Controlled vaccine-challenge trials are critical in establishing efficacy and safety

of a vaccine candidate. However, field trials are essential in establishing efficacy of the vaccine candidate in production systems where salmonellae prevalence and exposure are unpredictable and host immunity may be variable. Under field conditions, the vaccine would be applied to a diversity of host genetics and the animals in the field are exposed to varying risk factors, e.g., season, social stress, parturition, transport, feed changes, and concurrent infections that can increase risk to salmonellae infections. The research with the attenuated *S. Typhimurium dam* vaccine has been conducted in a controlled laboratory setting to prevent introduction of such confounders. Future research of the vaccine should include continued safety and efficacy testing in livestock species in larger field trials. Application of the vaccine to production systems where salmonellosis is endemic is the next logical step.

#### ***9.2.1. Further efficacy testing of the vaccine***

Further efficacy testing of *S. Typhimurium dam* vaccine is warranted, however the current vaccine formulation is problematic for commercial application. In the conduct of this research, vaccinations were carried out with *S. Typhimurium dam* vaccine strain MT2313 that contained an insertion element that allowed the vaccine to express resistance to chloramphenicol (*Cm<sup>r</sup>*). This antibiotic resistance gene was inserted into the MT2313 vaccine strain in order to facilitate re-isolation, enumeration and identification. With growing concern regarding multi-drug resistant strains of salmonellae, regulatory agencies such as OGTR, AQIS and APVMA would not favour a vaccine candidate carrying antimicrobial resistance.

Therefore, commercial application of the vaccine will necessitate formulation of the *dam* attenuated *S. Typhimurium* vaccine without the *Cm<sup>r</sup>* gene. While removal of the *Cm<sup>r</sup>* gene from the *dam S. Typhimurium* vaccine is unlikely to have a significant effect on the efficacy, verification of the genetic construct and

additionally safety and efficacy trials that verify performance, as well as environmental stability and persistence, are going to be necessary prior to application in field trials.

Additionally, a second attenuating deletion should also be considered to improve safety of the vaccine, as well as to ensure that vaccine is unable to revert to the level of virulence observed in the parent strain. A second attenuating deletion is likely to have more of an effect on performance of the vaccine and would require multiple safety and efficacy trials in murine, avian, and bovine models of salmonellosis. While this may seem an unnecessary duplication from an animal welfare perspective, it is absolutely vital to demonstrate that the new formulation of the *dam* *S. Typhimurium* is safe in livestock and, even more importantly, efficacious prior to initiating field trials.

#### ***9.2.1. Efficacy of the S. Typhimurium dam vaccine in swine***

*Salmonella* is an important pathogen of pigs and is an important food safety consideration for pork producers. Between 5% and 30% of all cases of foodborne salmonellosis list pork as the source of infection (Berends, *et al.*, 1997). In one Australian study, *Salmonella* spp. were isolated from the caecal contents of more than 20% of slaughtered pigs (Jackowiak, *et al.*, 2006). There are no published efficacy trials using a *S. Typhimurium dam* mutant vaccine in pigs. The development of models of porcine salmonellosis similar to those outlined in Chapters 4 and 6 and the conduct of efficacy trials in pigs would provide wider application of the vaccine in promoting animal health and welfare and food safety.

#### ***9.2.2. Safety of the S. Typhimurium dam vaccine in the environment***

The *S. Typhimurium dam* vaccine has been safely administered to mice, chickens, calves and sheep and demonstrated to provide robust protection against virulent *Salmonella* challenge. Further studies are required to evaluate persistence of the *S. Typhimurium dam* vaccine in the environment. As with all modified live attenuated vaccines there is a potential for reversion to virulent form (Cardenas

and Clements, 1993; Mastroeni, *et al.*, 2000; Babiuk, 2002) by mechanisms of transformation, transduction and conjugations (Cardenas and Clements, 1993; Wray and Davies, 2000; Bueno, *et al.*, 2009). Environmental passage experiments are indicated in order to evaluate the stability of the *S. Typhimurium dam* when exposed to the environment.

### **9.2.3. Future application of the *S. Typhimurium dam***

Another potential application for the *S. Typhimurium dam* is the use of the bacteria as a novel antigen delivery system in the construction of multivalent vaccines. *Salmonella* can be used as a targeted antigen delivery system capable of presenting antigen by way of major histocompatibility complex (MHC) classes I and II. *Salmonella* can be used to directly transfer plasmids that encode foreign antigens to antigen presenting cells, e.g., macrophages, dendritic cells and B-cells (Paglia, *et al.*, 1998; Mastroeni, *et al.*, 2000; Tizard, 2000), thus allowing the antigen to be produced within the host cells. Some of the difficulties associated with the use of plasmids are instability of the plasmid, toxicity of the proteins expressed to the host bacteria, degradation within the host bacteria, and incorrect expression of the antigen (Mastroeni, *et al.*, 2000). Recombination of *Salmonella* through blending or insertion of foreign antigens into the genome has been used to overcome difficulties associated with the use of plasmids (Cardenas and Clements, 1993; Bueno, *et al.*, 2009). Fimbrial colonisation factors of enterotoxigenic *Escherichia coli* (Morona, *et al.*, 1994) and non-toxic fragment C of tetanus toxins (Villarreal-Ramos, *et al.*, 2000b) have been successfully incorporated into *Salmonella*, where they have been expressed and elicited protective immune responses. Future applications of the *dam* attenuated vaccine to human, poultry and livestock medicine are promising.

### **9.2.4. Development of PC2/QC2 laboratory and animal housing facilities at the University of Sydney**

Vaccination-efficacy trials are labour intensive and require facilities that are approved for the use of infectious pathogens and can handle large numbers of livestock. In Australia, PC2 and QC2 compatible animal house facilities are limited and demand for use is high. Unfortunately, at the University of Sydney, there are no such facilities available and researchers are required to seek space in facilities which can uphold these standards at considerable cost. As of 2011, in New South Wales there are no QC2 animal research facilities capable of handling livestock due to changes in AQIS animal facility accreditation requirements. The EMAI PC2/QC2 animal house no longer meets QC2 standards and EMAI has elected to downgrade the facility to PC2/QC1 status.

Emerging infectious diseases are becoming more relevant to Australian aquaculture, agriculture and livestock, requiring development and research into control strategies. The limited availability of animal research housing facilities capable of handling quarantine organisms and infectious agents seriously hampers the ability of researchers to investigate emerging disease pathogens. The candidate experienced an 18 month delay due to researchers at EMAI being given preferential access to the animal research facilities over outsiders. In a government sponsored facility such as EMAI, research into emerging diseases or emergency disease outbreaks will always take precedence over an outside researchers project. There are now no longer any facilities in the greater Sydney Area accredited to handle livestock infected with an AQIS QC2 biological agent. Therefore, it is strongly recommended that the University of Sydney establish livestock housing facilities suitable for the research of PC2 and QC2 class organisms.

### **9.3 Conclusions**

The global trend toward intensive livestock production is associated with increased oro-faecal pathogen transmission resulting in a high prevalence of

*Salmonella*. Outbreaks of salmonellosis in livestock often reflect a series of events that compromise host immunity and increase pathogen exposure. In many livestock industries, these events are inherent and difficult to overcome through husbandry and management. The associated increase in the incidence of disease and contamination of livestock-derived food products imposes a significant risk to food safety via consumption of contaminated meat and milk products (Roels, *et al.*, 1997).

The development and application of an effective *Salmonella* vaccine offers a potential means of reducing industry associated losses and public health risks. The principal challenge in meeting this goal is that multiple *Salmonella* serovars are endemic to many livestock production systems and high risk groups that include neonates, post partum cows, and inappetent and stressed sheep. Effective *Salmonella* vaccination therefore requires induction of early onset, adaptive, cross protective immune mechanisms in order to provide protection against several *Salmonella* serovars in these high risk groups as well as stimulation of both innate and acquired immune mechanisms.

This research demonstrates rapid induction of cross protective immunity in ruminants vaccinated with *dam* attenuated *S. Typhimurium* challenged with virulent homologous and heterologous *Salmonella* and establishes an affordable, stress-free vaccine delivery option with in-water vaccination of adult ruminants. These data, along with research conducted in murine and avian models, provide robust evidence that support application of the *dam* attenuated *S. Typhimurium* in the control and prevention of salmonellosis in livestock and food producing species.



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Appendix I: Bacteria Growth and Dose Preparation Worksheet.

**BACTERIA GROWTH AND DOSE PREPARATION WORKSHEET**

1. Study #:
2. Bacteria Culture Plate Information:
  - a. Strain:
  - b. Source:
  - c. Date Plated: \_\_\_\_\_ By: \_\_\_\_\_
3. Seed \_\_\_\_\_ (number seeded) \_\_\_\_\_ mL bottle(s) of Luria growth media with at least one colony from the culture plate and place in the incubator at 37°C overnight. Initial/Date: \_\_\_\_\_
4. Add an equal or slightly greater volume of pre-warmed Luria growth media and return to the incubator for 2 hours ± 15 minutes.
  - a. Time returned to incubator: \_\_\_\_\_
  - b. Time removed: \_\_\_\_\_
5. Centrifuge bacteria at 2000 rpm at 4°C for 20 minutes in order to form bacteria "pellets". Discard supernatant media taking care not to disturb the bacteria pellet.
6. Combine pellets and wash bacteria with PBS. Once bacteria has been resuspended repeat Step # 5.
7. Resuspend washed pellet in PBS (20-30 mLs) and then dilute 1:10 in PBS to create stock solution.
8. Compare the stock solution to McFarland Standards. Record McFarland standard that correlates to the stock. If stock is too concentrated, serial dilute using PBS. Record McFarland Standard Tube # and dilution factor on the work sheet.

McFarland I.D. \_\_\_\_\_ Dilution factor \_\_\_\_\_

McFarland Tube I.D.	Theoretical CFU/mL	McFarland Tube I.D.	Theoretical CFU/mL
0.5	$1.04 \times 10^8$	1	$1.2 \times 10^8$
2	$1.4 \times 10^8$	3	$1.6 \times 10^8$
4	$1.8 \times 10^8$	5	$1.0 \times 10^9$

9. Using the theoretical value for CFU/mL of the McFarland Standard and the dilution factor if applicable, calculate the concentration of the stock bacteria solution using the following equation:

Concentration of Stock Bacteria = \_\_\_\_\_ CFU/mL

10. Stock bacteria solution \_\_\_\_\_ mL \* \_\_\_\_\_ CFU/mL  
= \_\_\_\_\_ Total CFU

Revised 21-JAN-2010

**BACTERIA GROWTH AND DOSE PREPARATION WORKSHEET**

**11. Dose Preparation:**

<b>A</b>	Dose Solution Volume Needed		mLs
<b>B</b>	Dose Solution Concentration		CFU/mL
<b>C</b>	Amount of Bacteria Needed:		CFU
<b>D</b>	Concentration of Stock Bacteria (Step 9)		CFU/mL
<b>E</b>	Volume of Stock Bacteria Needed (C/ D)		mLs
<b>F</b>	Volume of PBS (diluent) Needed (A - E):		mLs
Calculations Performed By:			Date:
Calculations Verified By:			Date:

**12. Remove 1 mL of the final "Dose Solution" for plating.**

- a. Prepare two identical dilution sets (label "A" and "B") and perform serial 5-fold dilutions (200 µL into 800 µL) on the "Dose Solution" until no (theoretical) CFU exist in the last dilution.
- b. Plate 100 µL from each of the last 3 to 5 dilutions from each set.
- c. Incubate at 37°C overnight.

**13. Count the plates 24 hours after plating to verify CFU/mL of "Dose Solution":**

PLATE #	#CFU "A"	#CFU "B"	AVERAGE CFU	DILUTION FACTOR	CFU/mL
4				1250	
5				6250	
6				31250	
7				156250	
8				781250	

+ = Too many colonies to count on plate (merging colonies)  
 - = Too few colonies to count on plate (less than 3)

<b>Expected CFU/mL</b>		<b>Actual CFU/mL</b>	
<b>Percent Deviation (+/-)</b>			

**Study Coordinator:** \_\_\_\_\_ **Date:** \_\_\_\_\_

Revised 21-JAN-2010

Appendix II: Individual Daily Animal Observation Record for Calves

<b>Animal Number: 316</b>		<b>Study Number: USDA07-VAC-001</b>						<b>Test Article: <i>S. Typhimurium</i> UK-1 dam</b>						
<b>Age: 5-8 weeks</b>		<b>Dose Level: 0 CFU/Calf (Control)</b>						<b>Principal Investigator: John House/Keith Walker</b>						
<b>Species/Strain/Sex: Bovine/Holstein/Male</b>		<b>Group: #1 (Non-Vaccinate)(GREEN)</b>						<b>Study Coordinator: Jennie Mohler</b>						
<b>PROCEDURES/DATE</b>	4-Mar-07	5-Mar-07	6-Mar-07	7-Mar-07	8-Mar-07	9-Mar-07	10-Mar-07	11-Mar-07	12-Mar-07	13-Mar-07	14-Mar-07	15-Mar-07	16-Mar-07	17-Mar-07
<b>DAILY:</b>														
<b>Morning Health Check (~8AM)</b>														
Attitude (0-4)														
Appetite (L)														
Faecal Score (0-3)														
Rectal Temperature (°C)														
INITIALS														
<b>Afternoon Health Check (~12PM)</b>														
Attitude (S or L)														
INITIALS														
<b>Evening Health Check (~4PM)</b>														
Attitude (0-4)														
Appetite (L)														
Faecal Score (0-3)														
INITIALS														
<b>Night Health Check (~12AM)</b>														
Attitude (S or L)														
INITIALS														
<b>WEEKLY:</b>														
Faecal Sample Collection (3 g or swab)														
Blood Sample														
Body Weight (Kg)														
<b>COMMENTS:</b> NA = Not Applicable ND = No Data Available Faecal Score: 0 = Normal; 1 = Soft/Runny; 2 = Blood or Casts; 3 = Diarrhoea (Watery/increased frequency) Attitude Scores: 0 = standing to be fed; 1 = stands w/stimulus; 2 = stands w/assistance; 3 = unable to stand/maintains in sternal recumbancy; and 4 = lateral recumbancy 12 am/pm Attitude Scores: S = standing or sternal recumbancy and L = lateral recumbancy assistance required														

Appendix III: Individual Daily Animal Observation Record for Sheep

Animal Number:		Study Number: USDA10-VAC-002					Test Article: <i>S. Typhimurium</i> UK-1 dam-								
Species/Strain/Sex/Age: Ovine/Merino/Wether/~2 yrs		Dose Level: 0 CFU/Sheep Group: 1 (Blue)					Principal Investigator: John House/Jennie Mohler								
PROCEDURES/DATE	28-Feb-10	1-Mar-10	2-Mar-10	3-Mar-10	4-Mar-10	5-Mar-10	6-Mar-10	7-Mar-10	8-Mar-10	9-Mar-10	10-Mar-10	11-Mar-10	12-Mar-10	13-Mar-10	
Morning Health Check (~8AM)					Necropsy										
Attitude (0-4)															
Faecal Score (0-4)															
Rectal Temperature (°C)															
Pellets Not Eaten (g)															
Chaff Not Eaten (g)															
Water Consumed after 6 L Offer (L)															
Observed Eating/Ruminating (Y or N)															
Pellets Offered AM (g)	250	250	250	250	N/A										
Chaff Offered AM (g)	700	700	700	700	N/A										
INITIALS															
Afternoon Health Check (~12PM)															
Attitude (S or L)															
INITIALS															
Evening Health Check (~4PM)															
Attitude (0-4)															
Faecal Score (0-4)															
Water Consumed after 6 L Offer (L)															
Observed Eating/Ruminating (Y or N)															
INITIALS															
Night Health Check (~12AM)															
Attitude (S or L)															
INITIALS															
WEEKLY:															
Faecal Sample Collection (3 g or swab)															
Weigh															

COMMENTS: NA = Not Applicable ND = No Data Available Faecal Score: 0 = Normal (Pellets); 1= Soft; 2= Soft (dog poo); 3= pile (cow pat); or 4 = Diarrhoea (Watery/increased frequency) Attitude Scores: 0 = standing to be fed (BAR); 1 = stand quietly (QAR); 2 = stands with head low (QAR); 3= Stands with stimulus (3A) or leaning on pen gates (3B); and 4 = Unable to Stand

12 am/pm Attitude Scores: S = standing or sternal recumbancy and L = lateral recumbancy assistance required Σ: Sheep food and water fasted overnight prior to challenge

\* Food and water fasted overnight. Feed and water removed at 4PM check

## Appendix IV: Standard Operating Procedure Animal Room Husbandry

### STANDARD OPERATING PROCEDURE

TITLE: Schedule for Animal Room Housekeeping During the Conduct of Salmonella and Salmonella Vaccine Trials.		
SOP No.: 80.03.01	R00	Effective Date:
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#### 1. PURPOSE

This SOP outlines the schedule for performing housekeeping in the animal rooms.

#### 2. SCOPE

2.1. This SOP is to be followed by the Animal Care Staff and/or Study Technicians to maintain a clean environment in the occupied animal rooms.

#### 3. POLICY

- 3.1. It is the policy of EMAI and LVS that personnel follow established procedures.
- 3.2. It is the policy of the EMAI Animal Care and Ethics Committee (ACEC) that appropriate personnel be trained in animal care and use procedures.
- 3.3. It is a requirement of OGTR and the Gene Technology Regulations Act (2001) that procedures are established for PC2 research and that personnel received training in these procedures.
- 3.4. It is a requirement of United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA) that animal care and use procedures are established and followed at all times.

#### APPROVALS

AUTHOR:	NAME:	NAME:
SIGNATURE:	SIGNATURE:	SIGNATURE:
DEPARTMENT NAME:	DEPARTMENT NAME:	DEPARTMENT NAME:
DATE:	DATE:	DATE:
NAME:	NAME:	NAME:
SIGNATURE:	SIGNATURE:	SIGNATURE:
DEPARTMENT NAME:	DEPARTMENT NAME:	DEPARTMENT NAME:
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NAME:	NAME:	NAME:
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DEPARTMENT NAME:	DEPARTMENT NAME:	DEPARTMENT NAME:
DATE:	DATE:	DATE:

3/16/11



Appendix IV (continued)

STANDARD OPERATING PROCEDURE

TITLE: Schedule for Animal Room Housekeeping During the Conduct of Salmonella and Salmonella Vaccine Trials.

SOP No.: 80.03.01

R00

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**4. DEFINITIONS**

- 4.1. Animal Room – designation for room where animals are housed/maintained during the conduct of a PC2 study
- 4.2. Anteroom - the annex that connects the Animal Room to the breezeway that is used for entry and exit into the Animal Rooms and study preparation
- 4.3. Technician - individual performing animal housekeeping procedures
- 4.4. Authorized Personnel – individuals approved by the study director or principal investigator to enter PC2 study room
- 4.5. Breezeway – main corridor through Animal Block 14.5
- 4.6. “Clean” Gum boot – Gum boots that never enter into the animal rooms and used by technicians to move from breezeway to anteroom
- 4.7. EMAI - Elizabeth Macarthur Agricultural Institute
- 4.8. Inner Coverall – Coveralls donned in the Storage Room prior to entry into PC2 Anterooms.
- 4.9. Outer Coverall – Coveralls placed over inner coveralls prior to entry into a PC2 Animal Room
- 4.10. OGTR – Office of the Gene Technology Regulator
- 4.11. PC2 – Physical Containment Level 2
- 4.12. PPE – Personal Protective Equipment
- 4.13. Technician – authorized personnel
- 4.14. LVS – Livestock Veterinary Services, University of Sydney, Camden

**5. RESPONSIBILITIES**

- 5.1. All procedures performed should be recorded on the Animal Room Housekeeping Records (FD 80.03.01A-C) by the Technician performing the procedure.
  - 5.2. It is the responsibility of authorized study personnel to follow and understand cleaning and husbandry procedures for PC2 Animal Rooms as outlined in this SOP.
  - 5.3. It is the responsibility of the Study Director/Study Coordinator to ensure that all authorized personnel receive training in cleaning and husbandry procedures.
  - 5.4. It is the responsibility of the Department Manager/Supervisor to ensure that:
    - 5.4.1. Personnel receive training in procedures outlined in this SOP and training is documented.
    - 5.4.2. Ensure compliance with OGTR regulations and guidelines.
-

Appendix IV (continued)

STANDARD OPERATING PROCEDURE

TITLE: Schedule for Animal Room Housekeeping During the Conduct of Salmonella and Salmonella Vaccine Trials.

SOP No.: 80.03.01

R00

Effective Date:

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**6. FREQUENCY**

6.1. As required

**7. PRECAUTIONS**

7.1. Read all signs and door cards and understand all safety precautions prior to entering an animal room.

**8. MATERIALS/EQUIPMENT**

8.1. Animal Housekeeping Record

8.2. Bucket(s) various sizes

8.3. Ball-point Pen (Blue)

8.4. Cleaning Equipment in Each Animal Room: broom, dustpan, squeegee or equivalent

8.5. Disinfectant Solution: quaternary ammonia (diluted 1:20 in water), 4-5% sodium hypochlorite (bleach) or equivalent

8.6. Dish washing detergent or equivalent

8.7. Footbath (es) containing disinfectant solution

8.8. Clinical Waste Bags or equivalent

8.9. Hand Sanitizing Agent: Instant foam or surgical scrub antimicrobial agent

8.10. High Pressure Water Hose

8.11. Paper toweling

8.12. Personal Protective Equipment (PPE): Disposable Tyvek® Coverall, latex gloves, calf rubber gum boots, safety glasses (optional)

8.13. Measuring containers: 1-5 litre capacity

8.14. Mop Bucket or equivalent

8.15. Sponge

8.16. Spray Bottle

8.17. Trash Receptacle

8.18. Waste Bin(s)

8.19. Yellow Hazardous Waste Wheelie Bin(s)

## Appendix IV (continued)

### STANDARD OPERATING PROCEDURE

TITLE: Schedule for Animal Room Housekeeping During the Conduct of Salmonella and Salmonella Vaccine Trials.

SOP No.: 80.03.01

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#### 9. PROCEDURE

##### 9.1. Daily Animal Room Housekeeping Activities

- 9.1.1. Check the water supply to each animal or group of animals if automatically watered. Record initials in the "Check Water" box.
- If water supply is contaminated with faeces or debris, clean trough and replenish with fresh water. Record initials in the "Clean Water Troughs" box.
  - For calves, milk and water are supplied with the same bucket. Once calves have finished milk, ensure that the bucket is rinsed prior to filling with fresh water.
- 9.1.2. Rinse pen floors with water using a high pressure hose to deposit excreta and animal waste into floor drain. Record initials in the "Rinse Pen Floors" box.
- 9.1.3. Ensure that sink area in the ante-room is free from debris and cleaned as required. Record initials in the "Clean Sink Area" box.
- 9.1.4. When calves are present, clean and sanitize all milk replacer preparation equipment and buckets after each feeding.

##### 9.2. Weekly/Biweekly Animal Room Housekeeping Activities

- 9.2.1. When a weekly activity is performed, record initials in the appropriate activity box on the day performed.
- 9.2.2. Clean and disinfect water feed and watering troughs (or buckets) at least once per week.
- 9.2.3. Clean and disinfect sink area in the ante-room at least once per week.
- 9.2.4. Flush floor drains with 40-50 litres of disinfectant solution at least once per week.
- Dilute quaternary ammonia solution 1:20 in clean water (1L of disinfectant into 19L of water or;
  - Dilute sodium hypochlorite (bleach) 1:20 in clean water (1L of disinfectant into 19L of water)
- 9.2.5. Refresh foot bathes with fresh disinfectant solutions at least once per week
- Dilute quaternary ammonia solution 1:20 in clean water (1L of disinfectant into 19L of water)
-

Appendix IV (continued)

STANDARD OPERATING PROCEDURE

TITLE: Schedule for Animal Room Housekeeping During the Conduct of Salmonella and Salmonella Vaccine Trials.			
SOP No.: 80.03.01	R00	Effective Date:	Page 5 of 7

- 9.2.6. Refresh disinfectant spray bottles with fresh disinfectant solution weekly
  - Dilute quaternary ammonia solution 1:20 in clean water (1L of disinfectant into 19L of water)

9.3. Weekly/Biweekly Animal Room Housekeeping Activities

- 9.3.1. Clean and disinfect floors of the ante-room with disinfectant at least once per week.
- 9.3.2. Disinfect animal room floors biweekly.
  - Can be flooded with disinfectant solution when drains are flushed.
- 9.3.3. Clean and disinfect tubular matting, if present, biweekly.

9.4. Monthly Animal Room Housekeeping Activities

The following activities are performed once a month:

- 9.4.1. Clean and disinfect doors as well as handles and fixtures.
- 9.4.2. Disinfect broom(s), dustpan, and squeegee.

9.5. As Needed Animal Room Housekeeping Activities

- 9.5.1. Empty trash receptacles and discard water material into yellow Hazardous Waste Wheelie Bin(s). Ensure that trash receptacle has two (2) fresh clinical waste or biohazardous waste bin liner.
- 9.5.2. Replace sponge mop heads, broom heads, and dust pans.
- 9.5.3. Clean and polish stainless steel fixtures, e.g., sink, door kick plates.

9.6. Animal Room Housekeeping Activities Prior to Initiating and Upon Completion of an AQIS Quarantine or PC2 Study:

- 9.6.1. Empty trash receptacles and disinfect.
- 9.6.2. Remove paper towels/products from room and place into trash.
- 9.6.3. Ensure that all work station drawers are empty and that all electronic devices that might be damaged by moisture have been disinfected and protected from damage
- 9.6.4. Clean and disinfect walls and floors of animal room
- 9.6.5. Clean and disinfect walls and floors of ante room.
- 9.6.6. Clean and disinfect all animal equipment, mats, pens, gates, buckets, bucket holders, troughs, etc.

## Appendix IV (continued)

### STANDARD OPERATING PROCEDURE

TITLE: Schedule for Animal Room Housekeeping During the Conduct of Salmonella and Salmonella Vaccine Trials.

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- 9.6.7. Clean and disinfect all gum boots and footwear used inside animal room
- 9.6.8. Allow room to stand for 24 hours post final disinfection
- 9.6.9. Collect 5 to 10 environmental swabs from each room and ante-room and initiate *Salmonella* isolation cultures (See S.O.P. 30.51.20).
- 9.6.10. If *Salmonella* cultures are negative, room can be dismantled and cleared from quarantine or animals can safely enter the facility.
- 9.6.11. If *Salmonella* cultures are positive for any room, repeat steps 9.6.4. to 9.6.9 until cultures are negative.

#### 10. REFERENCES/RELATED DOCUMENTS

- 10.1. S.O.P. 30.51.20, "Isolation and Identification of *Salmonella sp.*"
- 10.2. OGTR Guidelines: [www.ogtr.gov.au](http://www.ogtr.gov.au)
  - 10.2.1. Gene Technology Act (2000): [www.ogtr.gov.au/pubform/legislation.htm](http://www.ogtr.gov.au/pubform/legislation.htm)
  - 10.2.2. Gene Technology Regulations Act (2001)
- 10.3. Code of Federal Regulations, Title 21, Part 58
- 10.4. *Guide for the Care and Use of Laboratory Animals*. Institute of Laboratory Animal Resources. 1996. National Academy Press. Washington, D.C.

#### 11. ATTACHMENTS

- 11.1. Form Example 1: "Daily Animal Room Housekeeping Record"
-

Appendix IV (continued)

**STANDARD OPERATING PROCEDURE**

TITLE: Schedule for Animal Room Housekeeping During the Conduct of Salmonella and Salmonella Vaccine Trials.			
SOP No.: 80.03.01	R00	Effective Date:	Page 7 of 7

**FORM EXAMPLE 1: Daily Animal Room Housekeeping Record**

**DAILY ANIMAL ROOM HOUSEKEEPING RECORD**

ROOM NO.	SUITE NO.	FREQ.														ID.
PROCEDURE		SUN	MON	TUE	WED	THU	FRI	SAT	SUN	MON	TUE	WED	THU	FRI	SAT	
<b>DAILY CLEANING</b>																
Wash Trays																
Clean Water Transfer Batches																
Clean Fan Filters																
Clean Sub Area																
Clean M.B. Rabbit Transport Equipment																
<b>WEEKLY/BIWEEKLY CLEANING</b>																
Clean Trough Feed Bins																
Disinfect Fish Area - Anteroom																
Disinfect Anteroom Floor																
Disinfect Floor																
Wash Trays with Disinfectant																
Empty Trays/Equipment																
Refill Food Bins																
Clean and Disinfect Fan/Misting Material																
<b>MONTHLY/QUARTERLY CLEANING</b>																
Clean/Disinfect Anteroom Floor & Walls																
Clean/Disinfect Animal Room Floor & Walls																
Clean/Disinfect Animal Room Equipment																
Credit Environment Monitoring																

COMMENTS: \* = Except on weekends and holidays    NA = Not Applicable    ND = Not Determinable    Δ = Insufficient    ⊙ = Not desired

## Appendix V: Standard Operating Procedure for Exit and Entry into Animal Rooms

STANDARD OPERATING PROCEDURE			
TITLE: Entry Into and Exit From PC2/QC2 Animal Rooms in Block 14.5 During the Conduct of Salmonella and Salmonella Vaccine Trials			
SOP No.: 80.55.01	R02	Effective Date: 11-JAN-2010	Page 1 of 8

- 1. PURPOSE**  
To describe the procedures for entry into Animal Block 14.5 and PC2/QC2 animal rooms used for the conduct of the Salmonella Vaccine Research.
- 2. SCOPE**
  - 2.1. This SOP applies to all studies conducted in PC2/QC2 designated animal rooms in Animal Block 14.5
- 3. POLICY**
  - 3.1. It is the policy of EMAI and LVS that personnel follow established procedures.
  - 3.2. It is the policy of the EMAI Animal Care and Ethics Committee (ACEC) that appropriate personnel be trained in animal care and use procedures.
  - 3.3. It is a requirement of OGTR and the Gene Technology Regulations Act (2001) that procedures are established for PC2 research and that personnel received training in these procedures.
  - 3.4. It is a requirement of United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA) that animal care and use procedures are established and followed at all times.
- 4. DEFINITIONS**
  - 4.1. Animal Room – designation for room where animals are housed/maintained during the conduct of a PC2/QC2 study
  - 4.2. “Animal Room” Gum Boots – gum boots designated for use in specified animal room that should not be used in the breezeway or shared between rooms.
  - 4.3. Anteroom - the annex that connects the Animal Room to the breezeway that is used for entry and exit into the Animal Rooms and study preparation

APPROVALS		
AUTHOR:	NAME:	NAME:
SIGNATURE:	SIGNATURE:	SIGNATURE:
DEPARTMENT NAME:	DEPARTMENT NAME:	DEPARTMENT NAME:
DATE:	DATE:	DATE:
NAME:	NAME:	NAME:
SIGNATURE:	SIGNATURE:	SIGNATURE:
DEPARTMENT NAME:	DEPARTMENT NAME:	DEPARTMENT NAME:
DATE:	DATE:	DATE:

## Appendix V (continued)

### STANDARD OPERATING PROCEDURE

TITLE: Entry Into and Exit From PC2/QC2 Animal Rooms in Block 14.5 During the Conduct of Salmonella and Salmonella Vaccine Trials

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- 4.4. Authorized Personnel – individuals approved by the study director or principal investigator to enter PC2/QC2 study room
- 4.5. Breezeway – main corridor through Animal Block 14.5
- 4.6. “Clean” Gum boot – Gum boots that never enter into the animal rooms and used by technicians to move from breezeway to anteroom
- 4.7. EMAI - Elizabeth Macarthur Agricultural Institute
- 4.8. Inner Coverall – Coveralls donned in the Storage Room prior to entry into PC2/QC2 Anterooms.
- 4.9. Outer Coverall – Coveralls placed over inner coveralls prior to entry into a PC2/QC2 Animal Room
- 4.10. OGTR – Office of the Gene Technology Regulator
- 4.11. PC2 – Physical Containment Level 2
- 4.12. PPE – Personal Protective Equipment
- 4.13. QC2 - AQIS Quarantine Containment Level 2
- 4.14. Technician – authorized personnel
- 4.15. LVS – Livestock Veterinary Services, University of Sydney, Camden
5. **RESPONSIBILITIES**
  - 5.1. It is the responsibility of authorized study personnel to follow and understand entry and exit procedures for PC2/QC2 Animal Rooms as outlined in this SOP.
  - 5.2. It is the responsibility of all non-authorized personnel to contact appropriate authorized study personnel if entry into PC2/QC2 Animal Rooms is required.
  - 5.3. It is the responsibility of the Study Director/Study Coordinator to ensure that all authorized personnel receive training in proper entry and exit procedures.
  - 5.4. It is the responsibility of the Department Manager/Supervisor to ensure that:
    - 5.4.1. Personnel receive training in procedures outlined in this SOP and training is documented.
    - 5.4.2. Ensure compliance with OGTR/AQIS regulations and guidelines.
6. **FREQUENCY**
  - 6.1. This SOP is to be followed each time personnel enter into or exit from a PC2/QC2 Anteroom/Animal Room in Animal Block 14.5
7. **PRECAUTIONS**
  - 7.1. *Salmonella* spp are potentially capable of causing disease in humans. Most cases of human salmonellosis are derived from contaminated food.
    - 7.1.1. No eating, drinking or storing food for human consumption is permitted in Animal Block 14.5.
    - 7.1.2. No smoking is permitted in Animal Block 14.5.



## Appendix V (continued)

### STANDARD OPERATING PROCEDURE

TITLE: Entry Into and Exit From PC2/QC2 Animal Rooms in Block 14.5 During the Conduct of Salmonella and Salmonella Vaccine Trials			
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- 7.2. Ensure that hands are washed and sanitized with an antimicrobial agent designed for cleaning hands upon exit from Animal Rooms.
    - 7.2.1. It is also suggested that hands be washed/sanitized upon exit from Animal Block 14.5.
  - 7.3. If immunocompromised, e.g., pregnant, undertaking antimicrobial or anticancer therapy or HIV positive, DO NOT ENTER Animal Block 14.5.
  - 7.4. Read all signs and door cards and understand all safety precautions prior to entering any animal room.
- 8. MATERIALS/EQUIPMENT**
- 8.1. Bio-hazardous/Clinical waste bin liners
  - 8.2. Disinfectant Solution: JAMAC Disinfectant (quaternary ammonia 1:20), 4% sodium hypochlorite (bleach) or equivalent
  - 8.3. Footbath (es) containing disinfectant solution
  - 8.4. Hand Sanitizing Agent: Instant foam or surgical scrub antimicrobial agent
  - 8.5. Paper toweling
  - 8.6. Personal Protective Equipment (PPE): Disposable Tyvek® Coverall, latex gloves, calf rubber gum boots, safety glasses (optional)
  - 8.7. Room Entry Signs
  - 8.8. Scrub Brush with long handle
  - 8.9. Spray Bottle
  - 8.10. Waste Bin(s)
  - 8.11. Yellow Hazardous Waste Wheelie Bin(s)
- 9. PROCEDURE**
- 9.1. Entering Animal Block 14.5 – The Breezeway and Store Room
    - 9.1.1. The breezeway is considered a clean corridor. No additional PPE or gowning requirements apply to personnel working in the breezeway.
    - 9.1.2. The Store Room is considered a clean area. No additional PPE or gowning requirements apply to personnel working in the storeroom.
      - The Store Room is used to maintain supplies for studies and as a changing area for authorized personnel.
  - 9.2. Animal Room Entry Order
    - 9.2.1. All personnel must follow Anterooms/Animal Room entry order in order to prevent cross contamination of rooms.
    - 9.2.2. Each Anteroom/Animal Room has been assigned an entry order using numbers and colour-codes.
-

## Appendix V (continued)

### STANDARD OPERATING PROCEDURE

TITLE: Entry Into and Exit From PC2/QC2 Animal Rooms in Block 14.5 During the Conduct of Salmonella and Salmonella Vaccine Trials

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- Numbers: 1 ⇒ 2 ⇒ 3 ⇒ 4 ⇒ 5 ⇒ etc.
  - Colour-codes: WHITE ⇒ BLUE ⇒ RED
- 9.2.3. Colour-coded signs are affixed to the Breezeway and Animal Room doors of each room.
- 9.2.4. Enter all WHITE colour-coded rooms first. After entering a WHITE room, you may enter another WHITE, BLUE or RED room without having to change inner coveralls (See 9.3).
- 9.2.5. If you enter a BLUE room, you may enter another BLUE or RED room without having to change into fresh pair of inner coveralls (see 9.3).
- You must change your inner coveralls prior to entering a WHITE room.
  - Showering prior to re-entry into a WHITE room is recommended.
- 9.2.6. If you enter a RED room, you may enter another, RED room without having to change into fresh pair of inner coveralls (see 9.3).
- You must change your inner coveralls prior to entering a WHITE or BLUE room.
  - Showering prior to re-entry into a WHITE room is recommended
- 9.3. Entering PC2/QC2 Anterooms in Animal Block 14.5
- 9.3.1. PC2/QC2 Animal Rooms are clearly marked with appropriate signage and doors are to remain locked when study personnel are not present.
- 9.3.2. Prior to entering the anterooms of a PC2/QC2 animal room, the technician must gown-up into a pair of clean disposable coveralls (inner coverall layer), latex gloves (or equivalent) and “clean” gum boots in the Store Room gowning area.
- 9.3.3. Prior to entering the anteroom, ensure that proper “Room Entry Order” is being followed (see 9.2)
- 9.3.4. Unlock the door of the PC2 animal room and step into footbath filled with appropriate disinfectant solution.
- Footbaths are changed when overly soiled or at a minimum of weekly.
- 9.3.5. Step out of footbath into the anteroom and close the door to the breezeway.
- Technician is now appropriately gowned for working in the anteroom of the PC2/QC2 Animal Room Complex.
  - If the technician intends to enter the Animal Room, proceed to step 9.3
- 9.4. Entering Into PC2/QC2 Animal Room in Animal Block 14.5
- 9.4.1. Follow procedures outlined in Step 9.3
- 9.4.2. Ensure that door between the Anteroom and the Breezeway is closed.
- 9.4.3. Remove “clean” gum boots and put on “animal room” gumboots located
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## Appendix V (continued)

### STANDARD OPERATING PROCEDURE

TITLE: Entry Into and Exit From PC2/QC2 Animal Rooms in Block 14.5 During the Conduct of Salmonella and Salmonella Vaccine Trials			
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inside the Anteroom.

9.4.4. Obtain a second pair of disposable white coveralls (outer coveralls) hanging from hooks on the wall in the Anteroom and put on over first pair and “animal room” gum boots.

- If outer coveralls are excessively soiled or damaged, obtain a fresh pair of outer coveralls.
- Discard soiled outer coveralls into a yellow hazardous waste wheelie bins or appropriate biohazard trash bins inside the Animal Room on entry.

9.4.5. Ensuring that door to Breezeway is closed, open door to the Animal Room and enter.

9.4.6. Close Animal Room door after last person has entered the room.

- Door into Animal Room may remain open if door to Breezeway is locked.

9.5. Exiting From PC2/QC2 Animal Rooms in Animal Block 14.5

9.5.1. Step into foot bath located within the Animal Room.

- The footbath should be located near the door but out of main foot path to avoid injuries due to tripping.

9.5.2. Using a scrub brush, remove all debris from gum boots. Pay special attention to the sides and the soles of the boots.

9.5.3. Open the door of the Animal Room, ensuring Breezeway door is closed, and step into the Anteroom.

- If outer coveralls are excessively soiled, remove while in the Animal Room and place into the yellow hazardous waste wheelie bin. As an added precaution, spray inner coveralls with disinfectant solution prior to entering the Breezeway.

9.5.4. If coveralls are relatively clean and free from faecal material, spray lightly with disinfectant solution.

- Spray the front of the outer coveralls with a disinfectant solution and remove.
- Then hang outer coverall on appropriate hooks in the Anteroom and spray back with disinfectant solution.

9.5.5. Remove “animal room” gum boots and put on “clean” gumboots.

9.5.6. Remove latex gloves and deposit into the waste bin lined with a hazardous waste bin liner in the Anteroom.

9.5.7. Thoroughly wash hands using a scrub solution, or equivalent, and dry with disposable paper toweling and apply waterless hand sanitizing agent.

- It is recommended that a clean pair of gloves be obtained if the technician must remain in the anteroom to perform procedures for any

## Appendix V (continued)

### STANDARD OPERATING PROCEDURE

TITLE: Entry Into and Exit From PC2/QC2 Animal Rooms in Block 14.5 During the Conduct of Salmonella and Salmonella Vaccine Trials

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period.

- 9.5.8. Prior to exiting Anteroom, ensure that work stations are clean and tidy following procedures outline in SOP 80.03.01 - "*Schedule for Animal Room Housekeeping During the Conduct of Salmonella and Salmonella Vaccine Trials*".
- 9.5.9. Ensuring that Animal Room door is closed, open the door to the Breezeway and step into the Anteroom footbath.
- 9.5.10. Step out of the Anteroom into the Breezeway
- 9.5.11. Close the door to the Anteroom.
  - If technician is the last person exiting, ensure that door is locked.
- 9.5.12. Return to the store room, remove inner coveralls, and place into "Coverall Autoclave Bin". Inner coveralls are recycled and autoclaved once a week.
  - If inner coveralls are damaged, soiled or damp, place into a garbage bin located in the Store Room.
- 9.5.13. Remove 'clean' gum boots and don street shoes.
- 9.5.14. If technician is the last person in the Store Room, ensure that door is closed and locked.
- 9.6. Exiting Animal Block 14.5
  - 9.6.1. When exiting Animal Block 14.5, ensure that the door is closed and secure.
  - 9.6.2. Do not exit Animal Block 14.5 wearing inner or outer coveralls.
  - 9.6.3. Do not exit Animal Block 14.5 wearing "clean" or "animal room" gum boots.
  - 9.6.4. If possible, wash hands after exit from the area.
- 9.7. Removal of Items from Animal Rooms, Ante Rooms and Animal Block
  - 9.7.1. Any items to leave Animal Block 14.5, e.g., yellow hazardous waste bins, trash, samples, equipment, must be thoroughly disinfected and wiped down prior to removal from Animal Room and Anteroom.
  - 9.7.2. All carcasses or animal parts must be contained in a leak proof, spill proof container for transport. Ensure than container is be thoroughly disinfected and wiped down prior to removal from Animal Room and Anteroom.

#### 10. REFERENCES/RELATED DOCUMENTS

- 10.1. S.O.P. 80.03.01, "*Schedule for Animal Room Housekeeping During the Conduct of Salmonella and Salmonella Vaccine Trials.*"
  - 10.2. Code of Federal Regulations, Title 21, Part 58
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Appendix V (continued)

STANDARD OPERATING PROCEDURE			
TITLE: Entry Into and Exit From PC2/QC2 Animal Rooms in Block 14.5 During the Conduct of Salmonella and Salmonella Vaccine Trials			
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<p>10.3. OGTR Guidelines: <a href="http://www.ogtr.gov.au">www.ogtr.gov.au</a></p> <p>10.3.1. Gene Technology Act (2000): <a href="http://www.ogtr.gov.au/pubform/legislation.htm">www.ogtr.gov.au/pubform/legislation.htm</a></p> <p>10.3.2. Gene Technology Regulations Act (2001)</p> <p>10.4. <i>Guide for the Care and Use of Laboratory Animals</i>. Institute of Laboratory Animal Resources. 1996. National Academy Press. Washington, D.C.</p> <p><b>11. ATTACHMENTS</b></p> <p>11.1. SOP Review and Training Sign Off Sheet</p>			

EXAMPLE SOP

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Appendix VI: Quantitative/Qualitative Faecal Culture Worksheet

QUANTITATIVE/QUALITATIVE FAECAL CULTURE WORKSHEET

Research Project #:	NLRD 1443-2004	Principal Investigator:	John K. House
Study Number:		Study Coordinator:	Jennie Mohler
Test Article:		Group #:	
Species/Strain/Sex		Collection Date:	
MSB Batch #:		XLD Plate Batch #:	

Animal ID#:	EMPTY TUBE WEIGHT (g)	TUBE + SAMPLE WEIGHT (g)	Volume PBS (mLs)
CFU PLATE #1 <sup>a</sup>		CFU PLATE #4	SPIKE PLATES <sup>b</sup>
CFU PLATE #2		CFU PLATE #5	MSB
CFU PLATE #3		CFU PLATE #6	
100µL CFU PLATE	PLATES READ BY (Initials & Date):		PLATES READ BY (Initials & Date):

Animal ID#:	EMPTY TUBE WEIGHT (g)	TUBE + SAMPLE WEIGHT (g)	Volume PBS (mLs)
CFU PLATE #1 <sup>a</sup>		CFU PLATE #4	SPIKE PLATES <sup>b</sup>
CFU PLATE #2		CFU PLATE #5	MSB
CFU PLATE #3		CFU PLATE #6	
100µL CFU PLATE	PLATES READ BY (Initials & Date):		PLATES READ BY (Initials & Date):

Animal ID#:	EMPTY TUBE WEIGHT (g)	TUBE + SAMPLE WEIGHT (g)	Volume PBS (mLs)
CFU PLATE #1 <sup>a</sup>		CFU PLATE #4	SPIKE PLATES <sup>b</sup>
CFU PLATE #2		CFU PLATE #5	MSB
CFU PLATE #3		CFU PLATE #6	
100µL CFU PLATE	PLATES READ BY (Initials & Date):		PLATES READ BY (Initials & Date):

Animal ID#:	EMPTY TUBE WEIGHT (g)	TUBE + SAMPLE WEIGHT (g)	Volume PBS (mLs)
CFU PLATE #1 <sup>a</sup>		CFU PLATE #4	SPIKE PLATES <sup>b</sup>
CFU PLATE #2		CFU PLATE #5	MSB
CFU PLATE #3		CFU PLATE #6	
100µL CFU PLATE	PLATES READ BY (Initials & Date):		PLATES READ BY (Initials & Date):

Animal ID#:	EMPTY TUBE WEIGHT (g)	TUBE + SAMPLE WEIGHT (g)	Volume PBS (mLs)
CFU PLATE #1 <sup>a</sup>		CFU PLATE #4	SPIKE PLATES <sup>b</sup>
CFU PLATE #2		CFU PLATE #5	MSB
CFU PLATE #3		CFU PLATE #6	
100µL CFU PLATE	PLATES READ BY (Initials & Date):		PLATES READ BY (Initials & Date):

a: ++ = Too many colonies to count (merging colonies); - = No *Salmonella* colonies to count; and NS = Non-*Salmonella* colonies  
 b: + = Positive for *Salmonella* Growth; Neg = Negative for *Salmonella* (other bacteria present); and NG = No Growth

Appendix VII: Individual Animal Necropsy Quantitative Culture Worksheet

INDIVIDUAL ANIMAL QUANTITATIVE CULTURE WORKSHEET						
Research Project #	NLRD 1443-2004		Principal Investigator:	John K. House		
Study Number:			Study Coordinator:	Jennie Mohler		
Test Article:			Group #			
ANIMAL ID#:			XLD Plate Batch #			
<b>LIVER</b>						
	EMPTY TUBE WEIGHT (g)		TUBE + SAMPLE WEIGHT (g)		Volume PBS (mLs)	
CFU PLATE #1 <sup>a</sup>		CFU PLATE #3		SPIKE PLATES <sup>b</sup>		
CFU PLATE #2		CFU PLATE #4		MSB		
100µL CFU PLATE						
<b>LUNG</b>						
	EMPTY TUBE WEIGHT (g)		TUBE + SAMPLE WEIGHT (g)		Volume PBS (mLs)	
CFU PLATE #1 <sup>a</sup>		CFU PLATE #3		SPIKE PLATES <sup>b</sup>		
CFU PLATE #2		CFU PLATE #4		MSB		
100µL CFU PLATE						
<b>SPLEEN</b>						
	EMPTY TUBE WEIGHT (g)		TUBE + SAMPLE WEIGHT (g)		Volume PBS (mLs)	
CFU PLATE #1 <sup>a</sup>		CFU PLATE #3		SPIKE PLATES <sup>b</sup>		
CFU PLATE #2		CFU PLATE #4		MSB		
100µL CFU PLATE						
<b>ILEUM WALL</b>						
	EMPTY TUBE WEIGHT (g)		TUBE + SAMPLE WEIGHT (g)		Volume PBS (mLs)	
CFU PLATE #1 <sup>a</sup>		CFU PLATE #4		SPIKE PLATES <sup>b</sup>		
CFU PLATE #2		CFU PLATE #5		MSB		
CFU PLATE #3		CFU PLATE #6				
100µL CFU PLATE						
<b>FECES</b>						
	EMPTY TUBE WEIGHT (g)		TUBE + SAMPLE WEIGHT (g)		Volume PBS (mLs)	
CFU PLATE #1 <sup>a</sup>		CFU PLATE #4		SPIKE PLATES <sup>b</sup>		
CFU PLATE #2		CFU PLATE #5		MSB		
CFU PLATE #3		CFU PLATE #6				
100µL CFU PLATE						
<b>LYMPH NODE</b>						
	EMPTY TUBE WEIGHT (g)		TUBE + SAMPLE WEIGHT (g)		Volume PBS (mLs)	
CFU PLATE #1 <sup>a</sup>		CFU PLATE #4		SPIKE PLATES <sup>b</sup>		
CFU PLATE #2		CFU PLATE #5		MSB		
CFU PLATE #3		CFU PLATE #6				
100µL CFU PLATE				PLATES READ BY (Initials & Date):		
PLATES READ BY (Initials & Date):						

a. ++ = Too many colonies to count (merging colonies); - = No *Salmonella* colonies to count; and NS = Not *Salmonella*  
 b. + = Positive for *Salmonella* Growth, Neg = Negative for *Salmonella* (other bacteria present), and NG = No Growth<sup>1</sup>. Non-*Salmonella* colonies



