Final report

Development of a novel oral vaccine for Bovine Respiratory Disease

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1 Abstract

This ambitious proof-of-principle project achieved its goal of providing evidence that an oral yeast-based vaccine is able to induce immune responses in cattle. The project also nominates candidate antigens for vaccines against BRD pathogens, focusing on Bovine Viral Diarrhoea Virus (BVDV). Bovine respiratory disease (BRD) is the major cause of production losses in feedlots globally and BVDV is a major contributor. We show that bovine macrophages can phagocytose a recombinant yeast vector with an ovalbumin (Ova) reporter molecule (OvaYeast). This leads to activation of the macrophages with accompanying expression of iNOS and TNFα genes and secretion of nitric oxide; effects not observed with non-transformed yeast. The prototype vaccine trial provides evidence that oral administration of yeast in cattle induces both humoral (antibodies) and cell-mediated immune responses (lymphocyte proliferation, IFNγ secretion) within the mucosal (IgA) and systemic (serum IgG, peripheral blood lymphocyte proliferation) immune pathways, indicating that this has potential as a feasible vaccine delivery vector for ruminants. Furthermore, OvaYeast itself appears to induce both cell-mediated (lymphocyte proliferation) and humoral (antibody) immune responses, the latter lasting at least 5 months post-vaccination. Oral vaccination also appears to induce immune reactions of cells from mucosal associated lymphoid tissue draining the respiratory tract (lymphocyte proliferation and IFNγ gene expression). The vaccine did not cause any adverse effects upon dosing or during the trial and it did not affect body weight gain. BVDV candidate antigens suitable for incorporation in vaccines were identified by bioinformatic screening the BVDV genome. Nine synthetic BVDV peptides based on the antigenic epitopes identified were screened, using sera from cattle that were known to be BVDV antibody ELISA positive or negative and the same peptides plus two additional BVDV peptides were screened for cell-mediated responses. When tested individually, antibodies to three peptides appeared to be higher in BVDV positive cattle; however, the combined response to all nine candidate antigens provides better differentiation of exposure to BVDV. There is provisional evidence of immunogenicity (cell-mediated immune response) for one of these candidate antigens with additional work required for confirmation. Yeast were transformed to express proteins containing single or multiple antigens for BVDV. Confirmation of successful transformation at both the DNA and protein level remains elusive, although putative positives have been found.

2 Executive summary

Background

Animal morbidity and mortality costs the Australian feedlot industry more than $50 million each year, with the greatest disease impact coming from respiratory disease (P.PSH.0547, 2013). Given the industry's commitment to antimicrobial stewardship, strategies to enhance immunity and reduce establishment of infection as well as new vaccines to prevent disease development are required. The major cause of production losses in feedlots globally is bovine respiratory disease (BRD) (Edwards 2010). Meat and Livestock Australia (MLA) have previously funded several projects to gain an understanding of the situation in Australian feedlots including the National Bovine Respiratory Disease Initiative. Around 18% of cattle develop BRD (Barnes et al. 2015) and it is responsible for 50% of deaths and 70% of clinical disease (Sackett et al. 2006). Respiratory disease also causes more than half (56%) of deaths in short-fed (up to 85 days) animals (Perkins 2013). The net loss to BRD mortality in Australia is estimated to be $1,647 per animal (Blakebrough-Hall et al. 2020). Based on earlier estimates, vaccination provides some relief with the cost of the disease in unvaccinated animals around $20 per head compared to $12 per head for vaccinated animals (Sackett, Holmes et al. 2006).

BRD is a multifactorial disease, where stressors and viral infections, e.g. due to bovine viral diarrhoea virus (BVDV), conspire to cause immunosuppression, which allows commensal bacteria in the respiratory tract to become opportunistic pathogens ultimately causing bronchopneumonia. Stressors include the exposure of young cattle to weaning, transportation, co-mingling and high stocking density, which contribute to suppression of the immune system (Caswell 2014). Beef cattle are most likely to be affected during the first 50 days after entry into a feedlot (Babcock et al. 2010). Pathogen profiles associated with BRD are similar in feedlots around the world despite differences in factors such as breed, age at feedlot entry and housing conditions: they are bovine viral diarrhoea virus (BVDV), bovine herpes virus (BoHV), bovine respiratory syncytial virus (BRSV), parainfluenza 3 (PI3), *Mannheimia haemolytica*, *Mycoplasma bovis*, *Pastuerella multocida* and *Histophilus somni* with a possible contribution of *Moraxella* in some parts of the world, whereby the relative contribution of the different microbial species may differ between individuals and farms (McMullen et al. 2020). Given its prevalence and impact on BRD within feedlots in Australia (Durham et al. 1991; Dunn et al. 1995; Hay et al. 2016; Hay et al. 2016) BVDV was selected as the pathogen of interest for this project as it had the most genomic information publicly accessible for Australian isolates and was utilised for the proof of concept of this approach.

There are 5 vaccines registered in Australia to protect against BRD, however, only one targets BVDV. Globally, BVDV vaccines have been available for more than 50 years (Griebel 2015) and have varying efficacy. In Australia, Pestigard®, was developed nearly 20 years ago using local BVDV isolates. An assessment in a previous MLA project final report (B.FLT.0225) suggests that vaccination with a primary and booster dose of this vaccine only offers a 20% reduction in the risk of developing BRD (Barnes, Hay et al. 2015; Hay et al. 2016). This may reflect the multifactorial nature of BRD and may be one of the reasons why uptake of BVDV vaccination is low (Lanyon et al. 2015). Vaccination is more commonly implemented to improve reproductive performance in a breeding herd than to prevent BRD at the fattening stage. Pestigard® vaccination of heifers does protect their calves against persistent infection (Morton, Phillips et al. 2013). In addition to the cost of the vaccine itself,

the requirement for a booster dose and additional animal handling to administer vaccine doses by injection could be an impediment to vaccine uptake.

Pestigard® vaccination prior to mating reduces the number of persistently infected calves; the odds of vaccinated heifers having BVDV ELISA positive progeny were 0.16 fold that of placebo-treated heifers (95% CI 0.04–0.59; P = 0.006) (Morton, Phillips et al. 2013). However, foetal or calf losses were similar in Pestigard® and placebo-treated groups (odds ratio 0.86; 95% CI 0.51–1.45; P = 0.568). Therein lies the dilemma of controlling a disease which can run relatively 'silently'. Persistently infected calves are unthrifty and, if not identified, will spread infection to other animals compounding economic costs over time (Taylor et al. 2001; Hessman et al. 2009).

Within this context, a more effective vaccine that is easier to administer is needed.

Protection against diseases such as BRD, where the pathogen route of entry is via a mucosal surface, requires activation of immune responses at a mucosal surface. Such vaccines are not well-developed for cattle. This project studied the potential for stimulating immune responses using an oral route of vaccination with a yeast-based formulation. Yeast have not been used as a vaccine delivery platform in livestock. In addition, BVDV antigens that are suitable for incorporation into candidate vaccines were evaluated. In assessing the potential of a yeast-based vaccine as a novel next generation immunostimulant for use in feedlot cattle, this project aligned with the Australian livestock industry's commitment to antimicrobial stewardship with a focus on BRD vaccines.

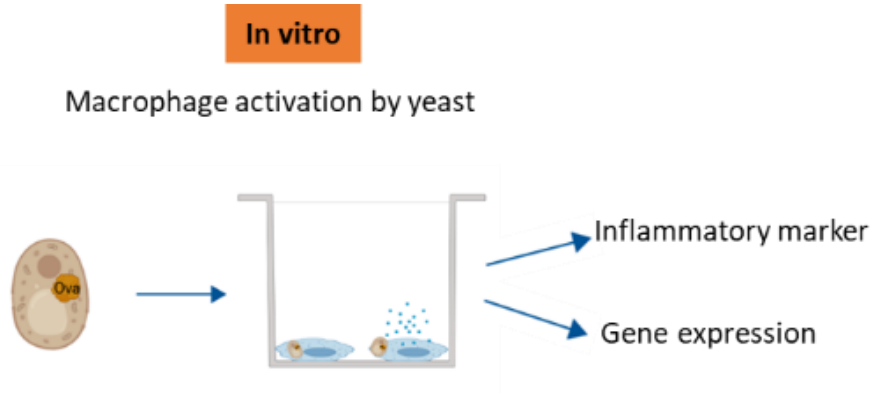
Outcomes

- The ability of a yeast-based vaccine system to activate cattle immune cells was successfully evaluated.
- The ability of a prototype yeast vaccine, given orally, to induce immune responses in cattle was successfully evaluated.
- Suitable candidate antigens for a BVDV vaccine are nominated.
- The ability of the candidate antigens (synthetic peptides) to be recognised by antibodies and immune cells from BVDV antibody positive cattle was successfully evaluated.
- Yeast-based prototype vaccines that contain the candidate BVDV antigens have been constructed; however, confirmation of successful transformation at both the DNA and protein level remains elusive.

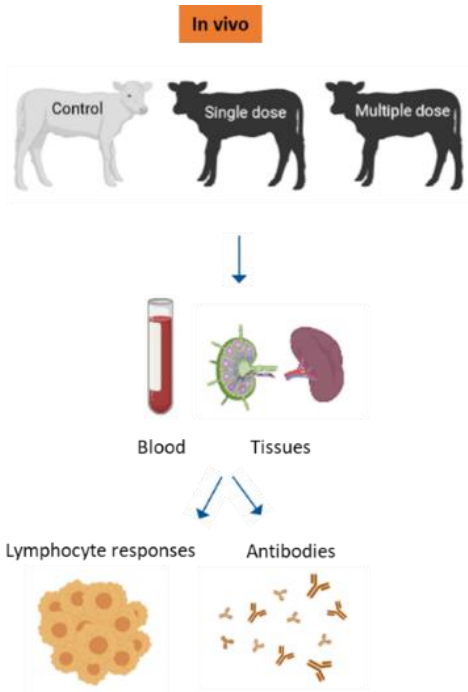
Methodology

Suitability and safety of yeast-based vaccines in cattle

- Yeast was modified to express a 'reporter' antigen (OvaYeast)
- The ability for OvaYeast to activate innate immune cells was assessed

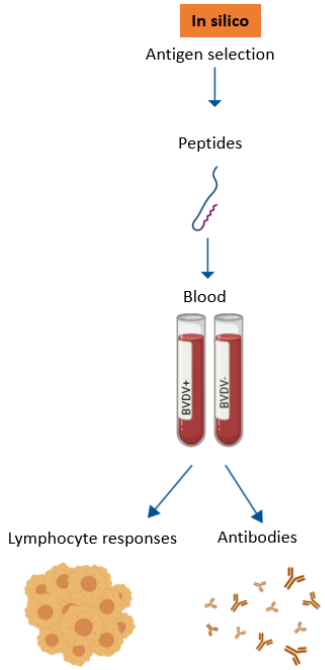


Ability of oral vaccination with OvaYeast to activate adaptive immune responses in cattle (3 groups of n=10)



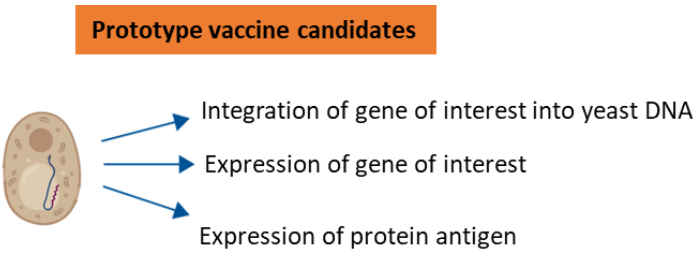
Suitability of candidate vaccine antigens

- Published literature and genomes of BRD pathogens including BVDV were evaluated to select candidate antigens
- The ability of candidate antigens (synthetic peptides) to induce lymphocyte responses and the presence of antibodies to the candidate antigens in BVDV seropositive and seronegative cattle was evaluated



Preparation of vaccine candidates

- Yeast was modified to carry genetic sequences of peptide epitopes
- Integration of DNA and expression of genes was assessed by PCR and qPCR
- Expression of antigens was assessed by Western blot and protein purification



Results/key findings

- The prototype vaccine (OvaYeast) activated innate immune cells
- Yeast is a suitable medium for oral vaccine delivery to cattle as it induced immune responses
- The prototype vaccine (OvaYeast) induced both cellular and antibody responses in cattle
- Antibody responses can last for at least 5 months after vaccination
- There is provisional evidence that 3 candidate BVDV antigens may be antigenic and 1 has the potential to be immunogenic, however no stably transformed clones of *K.lactis* yeast expressing these antigens were able to be obtained.

Benefits to industry

Yeast-based vaccines have the potential to be developed for BRD as well as other diseases that require mucosal immunity for protection. Production of yeast-based vaccines can be scaled up easily

and administration to cattle can be incorporated into normal husbandry procedures at backgrounding or at feedlot induction. Yeast-based vaccines can be given as a feed additive or in the style of an oral drench improving the ease of vaccination.

There is sufficient practical benefit and proof-of-concept to explore oral vaccination with yeast further but not quite enough evidence for large scale investment in cattle at the moment. Rather, refinement of the concept is recommended as the next step, to enable expression of the candidate antigens in a yeast or other vaccine delivery system.

Future research and recommendations

- Further improvements of yeast transformation to optimise use of this *K. lactis* expression system for production of recombinant antigen vaccine candidates or evaluation of alternate species of yeast.
- Expression of candidate antigens in another expression system (e.g. E. coli) to enable evaluation using other vaccine delivery systems e.g. nanoparticles.
- Further evaluation of yeast vaccine dose and route(s) of administration.
- Evaluation of combining multiple transformed yeasts containing multiple antigens from the major BRD pathogens in a single vaccine, to address the multifactorial nature of this disease.

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1 Background

1.1 Introduction

Bovine respiratory disease (BRD) is an important cause of production losses, poor welfare, and antimicrobial use in the beef industry, particularly in feedlots. To reduce the impact of BRD and reliance on antimicrobial use, prevention of disease through vaccination is desirable. This is particularly challenging due to the fact that BRD is a disease complex involving multiple viral and bacterial pathogens, and because of the production conditions in the feedlot industry. To be economically viable and practicable on-farm, vaccines must provide protection against multiple organisms, and the need to be administered in a simple, cost-effective way that minimises animal handling and the risk of injection site lesions that might affect beef quality. Oral administration of vaccine, as used in humans for e.g. polio vaccination would be desirable but this approach is in its infancy in ruminants. There is, however, evidence that compounds can be administered orally with beneficial impact on health and productivity in ruminants, e.g. oral selenium-yeast administration. Building on existing need and knowledge, the aims of this project were two-fold: to establish whether an orally administered yeast-based vaccine delivery system could induce specific immunity in cattle and to identify candidate antigens with potential to protect against causative agents of Bovine Respiratory Disease (BRD). Yeast has not previously been investigated as a vaccine platform in livestock. In assessing the potential of a yeast-based vaccine as a novel next generation immunostimulant for use in feedlot cattle, this project aligned with the Australian livestock industry's commitment to antimicrobial stewardship and to minimise the risk and impact of disease on animal welfare and productivity (LiveCorp 2020; MISP 2020; MLA 2020).

1.1.1 Bovine respiratory disease in the feedlot industry

The major cause of production losses, morbidity and mortality in feedlots globally is BRD (Edwards 2010). Meat and Livestock Australia (MLA) have funded several projects to gain an understanding of the situation in Australian feedlots: around 18% of cattle develop BRD (Barnes, Hay et al. 2015) and it is responsible for 50% of deaths and 70% of clinical disease (Sackett, Holmes et al. 2006). Respiratory disease also causes more than half (56%) of deaths in short-fed (up to 85 days) animals (Perkins 2013). The net loss to BRD mortality in Australia is estimated to be $1647 per animal (Blakebrough-Hall, McMeniman et al. 2020). Based on earlier estimates, vaccination provides some relief with the cost of the disease in unvaccinated animals around $20 per head compared to $12 per head for vaccinated animals (Sackett, Holmes et al. 2006).

Colonisation of the respiratory tract by microbes occurs soon after birth. The degree of colonization and the communities involved are influenced by a variety of factors including the surrounding environment, diet, transportation, and antimicrobial use (Zeineldin et al. 2017) . The upper respiratory tract (oral and nasal cavities and throat) is colonized by numerous microbiota while the lower respiratory tract (trachea and lungs) in healthy cattle have very low bacterial or viral loads. Respiratory microbiota composition is influenced by environment, age, diet, parenteral antimicrobials, and stressful events and changes from weaning to transport and then again in the first 10 weeks at feedlot (Timsit et al. 2016; Timsit et al. 2020).

BRD is a multifactorial disease, where stressors and viral infections, e.g. due to bovine viral diarrhoea virus (BVDV), conspire to cause immunosuppression, which allows commensal bacteria in the respiratory tract to become opportunistic pathogens ultimately causing bronchopneumonia. Stressors include the exposure of young cattle to weaning, transportation, co-mingling and high stocking density, which contribute to suppression of the immune system (Caswell 2014). Beef cattle are most likely to be affected during the first 50 days after entry into a feedlot (Babcock, Renter et al. 2010). Pathogen profiles associated with BRD are similar in feedlots around the world despite differences in factors such as breed, age at feedlot entry and housing conditions: they are bovine viral diarrhoea virus (BVDV), bovine herpes virus (BoHV), bovine respiratory syncytial virus (BRSV), parainfluenza 3 virus (PI3), *Mannheimia haemolytica*, *Mycoplasma bovis*, *Pastuerella multocida* and *Histophilus somni* with a possible contribution of *Moraxella* in some parts of the world, whereby the relative contribution of the different microbial species may differ between individuals and farms (McMullen, Alexander et al. 2020).

Prior exposure to BRD pathogens in Australian feedlot cattle disease is demonstrated by seropositivity to BVDV, BoHV-1, PI3 and BRSV at feedlot entry. The majority (>60%) of cattle were seropositive for BVDV (Dunn, Godwin et al. 1995; Hay, Barnes et al. 2016; Cusack et al. 2021). At feedlot entry seroprevalence to other pathogens ranged from 57-91% for BPI3 (Dunn, Godwin et al. 1995; Hay, Barnes et al. 2016) and 13-24% for BoHV (Dunn, Godwin et al. 1995; Hay, Barnes et al. 2016; Cusack, Bergman et al. 2021) and 27-89% for BRSV (Dunn, Godwin et al. 1995; Hay, Barnes et al. 2016).

In the study by Dunn et al (1995), disease was diagnosed in 6.8% of animals, with fever at the time of entry to the feedlot and BRD accounted for 66% of disease observed. Viral infections were identified in 72% of sick animals with BVDV and BoHV-1 significantly associated with cases of BRD. Bacteria were cultured from 19% of sick animals with the majority (7%) attributed to *M. haemolytica* or various *Salmonella* species (6.6%). In deceased animals, 41% of BRD cases were confirmed to have viral infections, with significant associations between both BVDV and BoHV-1 and respiratory disease. The recent study by Cusack et al (2021) found that seroprevalence to BVDV increased by 33.9% and to BoHV by 30.3% during backgrounding in cattle that were seronegative at entry to backgrounding.

1.1.2 Bovine viral diarrhoeal virus (BVDV)

BVDV is one of the top five endemic diseases that cause production losses for the red meat industries in Australia (Lane et al. 2015). Given its prevalence and impact on BRD within feedlots in Australia (Durham, Hassard et al. 1991; Dunn, Godwin et al. 1995; Hay, Ambrose et al. 2016; Hay, Barnes et al. 2016), in consultation with the MLA program manager, it was selected as the primary pathogen of interest for this project.

BVDV is an enveloped RNA virus with three distinct genotypes: BVDV1 or Pestivirus A, BVDV2 or Pestivirus B and BVDV3 or HoBi-like virus (Yesilbag et al. 2017; Evans et al. 2019; de Oliveira et al. 2021) with antigenic and geographic differences; BVDV subtypes 1a and 1b are more commonly detected in feedlots in north America and Europe, while the BVDV1c subtypes the most common in Australia (Ambrose et al. 2018). BVDV2 is not found in Australia or New Zealand.

Naïve cattle exposed to BVDV develop an acute transient infection originating in the nasal mucosa within 6 to 12 days following exposure, (Evans, Pinior et al. 2019). Transiently infected animals can shed low levels of the virus in bodily secretions and excretions for up to 3 weeks (Thurmond 2008). Once infection is cleared, shedding ceases and the animals develop antibody and T cell responses that are protective for life (Evermann et al. 2008; Brodersen 2014; Lanyon et al. 2014). *In utero* exposure is common and the timing of exposure is critical to disease outcome. Foetal exposure after day 30 and during the first trimester leads to persistently infected (PI) calves (Lanyon, Hill et al. 2014). The immune naivety of the foetus leads to immune tolerance to BVDV, resulting in an animal that has a lifelong viral infection and is capable of transmitting BVDV (Brownlie et al. 1998). From the pathogen's perspective this creates an ideal scenario where apparently healthy, persistently infected calves can surreptitiously spread infection to other cattle in the feedlot through the oronasal route (Goyal et al. 2008). Persistent infections are the major driver of BVDV epidemiology, whereas transient infections are the major driver of the economic impact of BVDV.

Cattle in feedlots where BVDV is present, either transiently or persistently, have a moderately higher risk of developing BRD than those in cohorts without BVDV (OR 1.7; 95% credible interval 1.1–2.5) (Hay et al. 2014; Barnes, Hay et al. 2015). Furthermore, the animals that have an increase in BVDV antibody titre following entry to the feedlot are at a higher risk of developing BRD compared with animals that have high titres at entry (Durham, Hassard et al. 1991). Therefore, exposure within the feedlot to BVDV either by transiently or persistently infected animals can increase the incidence of BRD, which could be prevented or reduced through boosting immunity to BVDV via feedlot entry vaccination.

1.1.3 BVDV vaccines

There are 5 vaccines registered in Australia to protect against BRD, however, only one targets BVDV. Globally, BVDV vaccines have been available for more than 50 years (Griebel 2015) and have varying efficacy. In Australia, Pestigard®, was developed nearly 20 years ago using local BVDV isolates. An assessment in a previous MLA project final report (B.FLT.0225) suggests that vaccination with a primary and booster dose of this vaccine only had a moderate effect on the incidence of BRD. This may reflect the multifactorial nature of BRD and may be one of the reasons why uptake of BVDV vaccination is low (Lanyon et al. 2015). Cost could be an issue for some producers. Pestigard® costs around $6 per dose; other vaccines with similar recommended dosage (two doses plus an annual booster) such as Ultravac® 5in1 (Zoetis) and Vibrovax® (Zoetis) cost around $0.60 and $10 respectively (www.fmb.com.au).

Pestigard® vaccination does moderately (20%) reduce the risk of BRD (odds ratio 0.8, 95% CI 0.5-1.1) (Barnes, Hay et al. 2015; Hay, Morton et al. 2016). Vaccination is more commonly implemented to improve reproductive performance in a female herd than to prevent BRD at the fattening stage. Pestigard® vaccination prior to mating reduces the number of persistently infected calves; the odds of vaccinated heifers having BVDV ELISA positive progeny were 0.16 fold that of placebo-treated heifers (95% CI 0.04–0.59; P = 0.006) (Morton, Phillips et al. 2013). However, foetal or calf losses were similar in Pestigard® and placebo-treated groups (odds ratio 0.86; 95% CI 0.51–1.45; P = 0.568). Therein lies the dilemma of controlling a disease which can run relatively 'silently'. Persistently

infected calves are unthrifty and, if not identified, will spread infection to other animals compounding economic costs over time (Taylor and Rodwell 2001; Hessman, Fulton et al. 2009).

Within this context, a more effective vaccine that is easier to administer is needed.

1.1.4 Candidate antigens for a BVDV vaccine

The evolution of vaccine design in recent years has led to the use of epitopes in vaccine formulations. Epitopes are short amino acid sequences of an immunogenic protein (e.g. from a pathogen) and are capable of inducing a direct and relevant immune response in the host organism (Kao et al. 2009). Epitope design for vaccine inclusion is increasingly reliant upon bioinformatic or *in silico* analysis (Fleri et al. 2017; Fleri et al. 2017) however a vital pre-requisite for this method of vaccine design is an accurate knowledge of disease pathogenesis i.e. it is vital to have access to genomic (DNA and protein sequences) information of the causative pathogen as well as have insight of the host response mechanisms that counter the pathogen.

1.1.5 A yeast-based vaccine for livestock

The mucosa that lines the respiratory and intestinal tracts is a physical barrier between the host and its environment. It comprises of epithelial cells, and innate and adaptive immune cells (Perez-Lopez et al. 2016). The largest reservoir of immune cells in the body is found within the mucosal immune system. These immune cells play a pivotal role in mucosal immunity, both by sampling the environment for pathogens and by being ready to respond to pathogen challenge. Manipulating these immune cells to 'remember' a pathogen through vaccination is a prudent strategy for ensuring better protection against disease. Currently most vaccines are administered by injection either subcutaneously or intramuscularly and this induces systemic immune responses. Effector T cells and immunoglobulins (antibodies) produced during mucosal immune stimulation differ from those generated from systemic immune stimulation; therefore, effective mucosal vaccination requires targeted delivery of the antigen to the mucosa (de Silva 2021). IgG antibodies are the main type found in the blood and are produced in response to systemic exposure to antigen via subcutaneous or intramuscular vaccination (Todd 1975). IgA antibodies are mainly found in mucosal secretions such as those in the respiratory and gastrointestinal tract. Production of IgA antibodies requires activation of mucosal immune cells; therefore, from a vaccine point of view, specifically requires mucosal vaccination (Kumar et al. 2014). For example, mucosal delivery of BoHV-1 vaccine by intranasal administration stimulates an IgA response and provides protection against secondary bacterial pneumonia (Hill et al. 2019).

There is a need for additional mucosal vaccines to prevent BRD, ideally with a simpler route of administration than intranasal vaccination. While mucosal immune cells prefer to home to the site of activation, they can migrate between different mucosal sites (for example between the respiratory and gut-associated mucosal tissues) as part of a common mucosal immune system (Holmgren et al. 2005). For a livestock vaccine to be readily adopted by producers, the method of vaccine administration is an important consideration. Thus, in this study, oral delivery was chosen as the preferred option. For ruminants, this can be challenging since antigen modification may occur in the rumen prior to encountering intestinal immune cells but the benefits of oral administration would be such that exploration of oral vaccination can be considered a "high risk, high reward" strategy. The potential for an oral vaccine to induce pulmonary immunity has been previously reported using

alginate microspheres as the delivery vehicle, indicating this may be achievable (Bowersock et al 1998).

Kluyveromyces lactis, a yeast found in bovine milk, has been successfully utilised for a variety of applications within the food and pharmaceutical industries (Spohner et al 2016). This yeast species is 'generally regarded as safe' (GRAS) for use in humans and animals. Yeast cell wall components (e.g. β-glucans) have adjuvant properties and can activate immune responses (Herre et al 2004); therefore, an additional adjuvant to stimulate immunity is unnecessary, making yeasts well-suited as vaccine vectors or 'carriers'. Whole yeast vaccines are considered to have potential (Roohvand et al 2017). *K. lactis* technology has been successfully utilised in livestock vaccine development, such as Infectious Bursal Disease (IBD) virus (Arnold et al, 2012) in poultry and porcine reproductive and respiratory syndrome virus (PRRSV) (Zhao et al, 2014) where it has been shown to elicit a favourable immune response. We therefore propose that this novel vaccine technology is amenable for formulation in vaccines against ruminant livestock diseases. In addition to the economic advantages, these yeast-vaccines are stable, alleviating cold-chain requirements, and are simple and safe to administer orally or nasally. To our knowledge, this is the first time this novel vaccine formulation has been proposed for assessment in cattle. In the pilot trial reported here, we provide evidence that the yeast-vaccine is able to stimulate both humoral (antibody) and cell-mediated immune responses. While aspects such as dose and longevity of responses still remain to be evaluated, this technology has huge potential to transform the production, administration and efficacy of ruminant livestock vaccines.

To provide better vaccines to support the Australian livestock industry, we propose a formulation that is cheaper and easier to administer; yeast is not only the delivery vehicle, but it is also the adjuvant. This offers a distinct advantage over traditional vaccines in that it lacks the toxicity of conventional adjuvants. Further, the vaccine modifications identified in this proposal will not only improve local immune protection, but also avoid safety issues of injection (needlestick injuries) as it can be administered orally, in feed or potentially as a nasal spray.

2 Objectives

The overall project objectives are to:

- Evaluate the ability of a yeast-based vaccine that contains a 'reporter' to activate cattle immune cells in a laboratory culture system
- Assess the ability of the reporter vaccine to induce an immune response in cattle
- Deliver a literature review identifying candidate antigens to BRD vaccines and selection for 10-15 'best-bet' antigens
- Evaluate the ability of the candidate antigens to be recognised by immune cells sourced from BRD infected feedlot cattle
- Produce and deliver an oral yeast-based prototype vaccine that contains the candidate BRD antigen/s

3 Methodology

3.1 Deliver a literature review identifying candidate antigens to BRD vaccines and selection for 10-15 'best-bet' antigens

3.1.1 BRD immunology and vaccines literature review

PubMed and Web of Science databases were searched for peer-reviewed literature on BRD and related pathogenesis, immune activation and vaccination. Data was accessed in 2020.

3.1.2 Literature review for candidate BRD antigens

PubMed and Web of Science databases were searched for peer-reviewed literature on BRD related candidate antigens. Additionally the search function within IEDB (http://www.iedb.org/home_v3.php) was utilised to identify previously published BVDV epitopes applicable to MHC I restriction, any host, and any epitope type or sequence. The immunogenicity of the selected protein sequences was determined through use of the Class I Immunogenicity tool (Calis et al. 2013) (http://tools.iedb.org/immunogenicity). Data was accessed in 2020. Preliminary analysis of IEDB to identify previously published (Rotzschke et al. 1993; Deregt et al. 1998; Collen et al. 2002) BVDV epitopes with MHC binding potential resulted in 13 epitopes (Table 4.1.2.1).

3.1.3 Selection of candidate antigens by reverse vaccinology

3.1.3.1 Selection of BVDV protein sequences

A keyword search was performed on the NCBI Taxonomy Browser (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi) to identify BVDV strain protein sequences. The resulting 1043 sequences were further sorted to include only those with specificity to BVDV sourced in Australia ("Bovine viral diarrhea virus 1"[Organism] OR ("Bovine viral diarrhea virus 1"[Organism] OR Bovine viral diarrhea virus [All Fields])) AND strain [All Fields] AND Australia [All Fields]. The resulting sequences were collated to a single table (Table 4.1.2.2).

3.1.3.2 TepiTool prediction of peptides binding to MHC class I molecules

Peptide sequences identified in 3.1.3.1 were uploaded to TepiTools (Paul et al. 2016) and individually matched to each of the 13 possible Bovine MHC class I alleles. The IEDB Consensus method was utilised as the binding prediction method. This method is based upon the availability of predictors and previously observed predictive performance. The Consensus method incorporates the algorithms ANN (Artificial neural network, also called as NetMHC, version 3.4), SMM (Stabilized matrix method), SMMPMBEC (SMM with a Peptide:MHC Binding Energy Covariance matrix) and Comblib_Sidney2008 (Scoring Matrices derived from Combinatorial Peptide Libraries) if any corresponding predictor is available for a molecule otherwise NetMHCpan (version 2.8) is used. These algorithms are utilised in decreasing order Consensus > ANN > SMM > NetMHCpan > CombLib. The resulting epitope sequences are arranged by predicted percentile rank and a lower value indicates better predicted binding affinity. All generated epitopes with the best predicted binding affinity with general consistency across multiple MHC Class I alleles were selected for immunogenicity analysis (data not shown as over 1000 epitopes were returned).

3.1.3.3 Analysis of MHC Class I binding epitope immunogenicity

The IEDB Class I Immunogenicity tool (http://tools.iedb.org/immunogenicity) was utilised to predict immunogenicity of a peptide (epitope) MHC complex. Calis *et al* have developed a prediction tool via a process of machine learning incorporating knowledge of mechanisms of action of amino acid sequences and T cell receptor affinity (Calis, Maybeno et al. 2013). Selected epitope sequences were uploaded and submitted for analysis. The resulting table categorises the prediction results by descending score values. Scores greater than 0 are indicative of a sequence likely to be located in a position relevant for T cell receptor ligand binding and are predicted to be capable of eliciting an immune response. The final list of proposed BVDV epitopes for further analysis is generated from this table (Table 4.1.2.3) based upon common incidence across multiple MHC alleles and high immunogenicity scores. Using this method, we have similarly generated epitope sequences for Bovine herpesvirus (type 1.2), Bovine Parainfluenza-3 virus, Bovine respiratory syncytial virus, *Mannheimia haemolytica, Mycoplasma bovis, Pasteurella multocida* and *Histophilus somni* (Table 4.1.2.4).

3.2 Evaluate the ability of a yeast-based vaccine that contains a 'reporter' to activate cattle immune cells in a laboratory culture system

In vitro assays using isolated primary bovine leukocytes from 3 healthy cattle were used to assess the ability of the recombinant yeast reporter-vaccine (OvaYeast) (Appendix 5) to activate an immune response. The dose and incubation time required for activation of cells was examined. The effects of heat inactivation and pH on innate immune cell activation were also assessed.

Detailed descriptions can be found in Appendix 2.

3.2.1 Bovine macrophage isolation and culture

Peripheral blood mononuclear cells (PBMC) were isolated from bovine peripheral blood collected into lithium heparin by density gradient centrifugation over Ficoll. Adherent monocytes were cultured in Dulbecco's Modified Eagle's Medium with supplementation with Macrophage Colony Stimulating Factor, bovine serum and antibiotics, at 37°C in 5% CO_2. Differentiation into macrophages during culture (7-10 days) was monitored microscopically.

3.2.2 Exposure of macrophages to yeast in vitro

3.2.2.1 Optimising dose and incubation time for in vitro experiments

Macrophage cultures were prepared from three Angus steers and incubated with the following stimulants:

1. Culture medium (macrophages alone, negative control)
2. Unmodified yeast (*K. lactis*) at multiplicity of infection (MOI) of 1:1
3. Unmodified yeast (*K. lactis*) at (MOI) of 5:1
4. OVA-yeast at MOI of 1:1

5. OVA-yeast at MOI of 5:1
6. Lipopolysaccharide (LPS, positive control)

Each of the above treatments had two replicates for each animal. Culture supernatants and macrophages were collected at 24, 48 and 72 hrs after stimulation.

Nitric oxide secreted by macrophages was detected by the Griess assay and expression of iNOS and TNFα genes was detected by qPCR. Assessment of these activation parameters for each macrophage culture sample was carried out in duplicate.

3.2.3 Effect of heat inactivation and pH on yeast responses

These data are from two experiments using cells from 3 Angus steers and includes four replicates per treatment per experiment. Lipopolysaccharide was included as a positive control in all experiments to ensure that the cultured macrophages had capacity to stimulate innate immune responses. Culture medium alone was included as a negative control.

Macrophages were cultured with yeast which had been subjected to a variety of pre-treatments:

1. Yeast inactivated at 100^{o}C for 10 minutes.
2. To mimic conditions of steam-flaked grains, inactivated yeast was incubated at 50^{o}C for 12 and 24 hours.
3. To mimic rumen conditions, yeast incubated at pH 4, 6 and 8 for 24 and 48 hrs.

Effects of several multiplicities of infection (MOI) (ratio of yeast to cells) were also assessed. Viability of treated yeast was assessed by culture. A positive stimulation control (lipopolysaccharide, LPS) was included in all experiments.

Each experiment was carried out with cells from three animals. Culture supernatant and lysed monocytes were collected to measure nitric oxide secretion and expression of the gene for inducible nitric oxide synthase (iNOS) respectively. The production of nitric oxide via activation of the iNOS gene is an indicator of macrophage activation towards a pro-inflammatory phenotype, capable of stimulating adaptive immune responses that are required for vaccine memory (Wang et al. 2019).

3.2.4 Detection of nitric oxide

Nitric oxide production in macrophage cultures was measured in culture supernatants by the Griess assay (Sigma-Aldrich), according to the manufacturer's instructions.

3.2.5 Detection of gene expression

Macrophage cultures were lysed with RNAzol followed by RNA extraction and cDNA synthesis as described previously (Plain et al. 2010). Specific quantitative PCR assays for bovine iNOS, tumour necrosis factor-alpha (TNF-α), interleukin-10 (IL-10) and multiple reference genes (GAPDH, β-actin, PPIA and H3F3a) were applied to detect the expression of these genes.

3.2.6 Statistical analysis

For each treatment type data were subjected to a two-factor ANOVA with replication to determine effects of period of incubation and treatments on nitric oxide secretion by macrophages. Gene expression analysis was conducted using the common base method (Ganger et al. 2017).

3.3 Assess the ability of the reporter vaccine to induce an immune response in cattle

3.3.1 Animals and general husbandry

Thirty Angus steers (25 Angus and 5 Angus-Hereford) from the Southern Tablelands, NSW region aged 8-9 months were transported to the University of Sydney farms at Camden, NSW and allowed to acclimatise for more than 7 days. Weight range of animals at purchase was 295-362 kg. Cattle had received the 5in1 booster vaccination prior to purchase.
Animal husbandry practices for all trial animals held at University of Sydney farms cover a wide range of activities which prioritise animal welfare, health and hygiene and were also adhered to for this study. Cattle were co-mingled in one paddock for the 'on-feed' portion of the trial where they had access to water, pasture and a feed pad. After completion of the feeding phase of the trial, cattle were rotationally grazed across several different paddocks to meet pasture demand and had access to water. Co-mingling of the cattle in all groups was to be representative of a single cohort inducted into the feedlot and to ensure that variables were kept constant between treatments. The feed pad had a large feed trough allowing for 30 cm of head room per animal following guidelines for required bunk headroom for feedlot cattle in Australia.

During the 21-day grain feeding period, cattle were fed once daily in the morning at approximately 8:30-9:30 am. Following feeding, residual feed was removed and discarded. To ensure cleanliness, the bunk was swept out and/or hosed as needed. The use of a feed pad also ensured that animals could be excluded from the area during the day to prevent soiling prior to feeding and ensure optimal bunk hygiene. Water troughs were checked daily for function and cleanliness and were cleaned at least once per day.

Cattle scales were calibrated prior to each use by using 100 kg calibrated weights and pre-weighed grain containers up to a total of 360 kg. Animals were weighed prior to sample collection and then allowed to access the feeding area. Steers were weighed weekly during the feeding period (day 0, 6 and 13) and then each time the animals were brought through the yards for sampling (at 1, 2 and 5 months).

3.3.2 Selecting vaccine dose

To estimate an appropriate vaccine dose, a literature search was carried out using Scopus and PubMed (NLM) using the key words 'yeast' AND 'cattle' AND 'immune' with the year of publication unrestricted. This retrieved a total of 132 publications from 1964 to 2019. None of these studies had used *Kluyveromyces lactis*, the yeast used in this project; the majority had used *Saccharomyces cerevisiae.* In addition, there were no publications on the use of yeast as a vaccine. For the majority of publications, the immune parameter being evaluated was acute phase proteins. In total there

were 15 publications that had included adult cattle (heifers or steers), yeast or a yeast fermentation product and measures of immune performance.

Further details of the published studies which were used as a general guide to select 10^{10} CFU of OvaYeast per dose for the *in vivo* trial are described in Appendix 3.

3.3.3 Vaccine safety

Vaccine safety was assessed initially in two animals. The steers were given one dose of the vaccine (10^{10} CFU of OvaYeast) orally and monitored for two weeks for any adverse effects. These animals were the source of blood cells for *in vitro* experiments (reported in Appendix 2). The two cattle were not included in the *in vivo* trial described in Section 3.3.4.

3.3.4 Study design

The protocol was approved by the University of Sydney Animal Ethics Committee (Protocol No. 2019/1582) and the *in vivo* use of biological materials by the Department of Agriculture, Water and the Environment (Approval No. 2020/042).

The animals were allocated to the three groups (Fig. 3.1) based on weight at the start of the trial in order of ascending body weight. These groups will be identified as 'treatment groups' to avoid confusion with 'stimulants' for in vitro experiments described later.

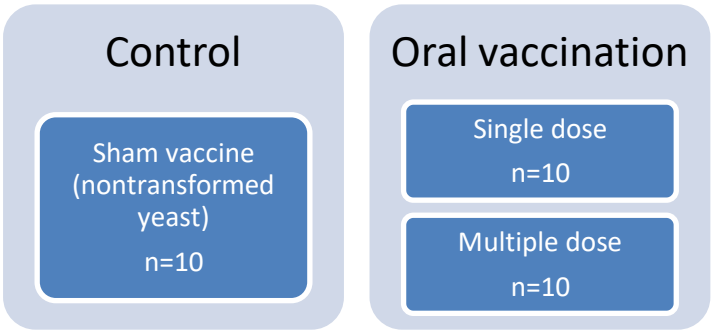


Figure 3.1 Experimental groups for establishing proof of principle in the *in vivo* phase of the project

Each treatment group was given either one (Single) or three (Multiple) doses of OvaYeast or one dose of nontransformed yeast (Control), orally. Each dose contained 10^{10} organisms.

Either heat-killed yeast or heat-killed OvaYeast was given orally, with delivery directly into the oesophagus of the animal using plastic tubing and a syringe to ensure the entire dosage was swallowed and not aspirated (n=10 per group). Each dose contained 10^{10} Yeast (Control group) or OvaYeast (Single and Multiple dose groups) in 10 mL of PBS on day 0. The Multiple dose group received two more doses on days 6 and 13, amounting to a total of $3x10^{10}$ OvaYeast. Multiple doses were to administer two different total doses of the vaccine, rather than to deliver a booster vaccination.

Internal secretion of ovalbumin (ova) protein in the vaccine doses was confirmed by Western blot and mass spectrometric analysis (Appendix 5).

For the first 3 weeks cattle from the time of the primary dose, cattle were fed a backgrounding diet (Beef Grower Pellets, MSM Stockfeeds) (see Appendix 3 Table 3.2). Residual feed was cleaned out daily and feed quantity was not increased until the feed had been consumed completely. The cattle were held on pasture and had *ad libitum* access to pasture both during and following the completion of the Grower pellet feeding period.

All 3 groups were monitored for 5 months post primary dose.

3.3.1 Sampling of animals

Blood samples were collected at 1, 2 and 5 months after the first dose. Blood was collected into tubes without anticoagulant and allowed to clot in order to obtain serum samples. The serum was aliquoted and stored at -20^{o}C until required. For cellular immune assays (lymphocyte proliferation and whole blood stimulation), blood was collected into lithium heparin containing blood collection vacutainers and processed immediately upon arrival at the laboratory (1-3 hours).

At the termination of the trial at 5 months post primary dose, the animals were subjected to a targeted necropsy to collect the desired tissues. On the day of the necropsy, blood and faecal samples were collected and blood was processed as above. Faeces was kept at 4^{o}C and stored at -80^{o}C immediately upon arrival at the laboratory. Tissue samples were collected at necropsy from multiple locations: lymph nodes (LN) from the ileal and jejunal regions of the intestine, the retropharyngeal LN, a section of the spleen and a section of the ileum that was tied off to retain the contents. The lateral retropharyngeal LN (Fig. 3.2) was selected as this drains the oral and nasal mucosal surfaces and forms part of the respiratory mucosal immune system.

For RNA extraction, a section of each LN was cut and immediately placed in DNA/RNA shield (Zymo) to preserve the RNA, such that the tissue:liquid volume was at minimum 1:9.

For intestinal mucus samples, the ileal section was opened and the contents were washed from the section using sterile phosphate buffered saline (PBS). A sterile paddle pop stick was used to gently scrape the mucus lining the ileal section and this was transferred to a sterile tube containing PBS and stored at -20^{o}C.

3.3.2 Cell isolation

3.3.2.1 Lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation, as previously described (de Silva et al. 2010). Briefly, blood collected from the jugular vein into lithium heparin was centrifuged to harvest buffy coats. These were diluted in PBS and layered over Ficoll and centrifuged for separation of mononuclear cells. The harvested white blood cells were resuspended in RPMI 1640/10% foetal bovine serum (FBS) (culture medium).

3.3.2.2 Lymph node and splenocytes

Sections (1–2 cm^2) of retropharyngeal (RLN), ileal (ILN), posterior jejunal (JLN) and spleen were collected into culture medium and processed immediately. The tissue was diced using a scalpel blade to release lymph node cells or splenocytes into the medium and the suspension strained through a 70 μm filter.

3.3.2.3 Lymphocyte proliferation

3.3.2.3.1 Total cell proliferation

PBMC or lymph node cells were labelled with the fluorescent tracking dye CFSE (5 mM), as previously described (de Silva, Begg et al. 2010). Triplicate aliquots of cells were then cultured in medium alone, or various stimulants (ovalbumin, yeast, OvaYeast) for 5 days at 37°C in 5% CO_2. Pokeweed mitogen (PWM) was included to evaluate general proliferative capacity of the cells.

At the end of the culture period, samples were fixed in 1% paraformaldehyde and stored at 4°C until data were acquired on a high throughput flow cytometer (Guava EasyCyte) and analysed using guavaSoft software (Luminex).

3.3.2.3.2 Subset proliferation

After 5 days of culture, replicate wells used for the total proliferation assay were labelled with cell surface markers to determine the phenotype of proliferating lymphocyte subsets. Culture medium was removed and these cells were incubated with antibodies to CD3 (a T cell marker), CD4 (T helper), CD8 (cytotoxic T), CD21 (a B cell marker), 86D (a γδ T cell marker) or CD45RO (a memory T cell marker) and then with a detection antibody conjugated to allophycocyanin (anti-IgG APC). Flow cytometric data were acquired on a high throughput system on a Guava EasyCyte and analysed using guavaSoft software (Luminex).

3.3.2.4 Flow cytometric data analysis

The lymphocyte population was selected based on cell size and granularity in a dot plot (Fig. 3.2 A) and subsequent analysis was on this gated population. For total lymphocyte proliferation, CFSE labelling was evaluated on data from the green fluorescence channel; nonproliferating cells do not lose CFSE retaining high fluorescence intensity (Fig. 3.2 B) while proliferating cells are dimmer (Fig. 3.2 C). For lymphocyte subset proliferation, the subset was identified on the second red fluorescence channel (Fig. 3.2 D). Proliferating and nonproliferating subset-specific cells were identified by CFSE intensity in the upper half of the dot plot.

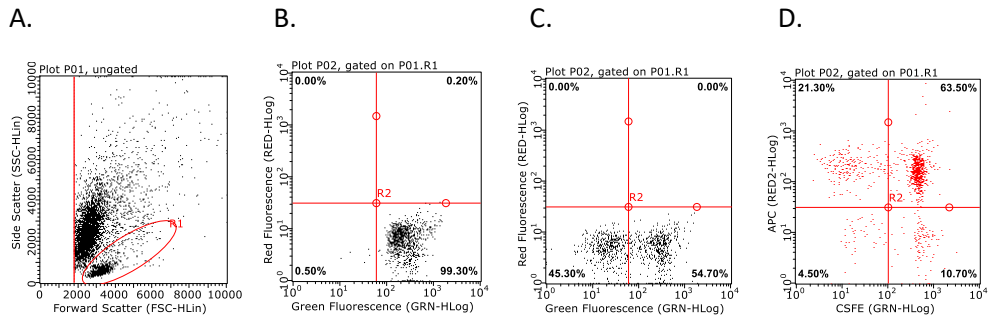


Figure 3.2 Flow cytometric data analysis A. The lymphocyte population was selected based on cell size and granularity (forward and side scatter) for downstream analysis. Lymphocytes labelled with

CFSE and are not proliferating retain high intensity green fluorescence (B and C, lower right quadrant) while intensity decreases in proliferating cells (C, lower left quadrant). Lymphocyte subsets labelled with allophycocyanin (APC) conjugated subset-specific markers are identified as high intensity red fluorescence (D, upper quadrants). These can be categorised as proliferating (D, upper left quadrant) and nonproliferating (D, upper right quadrant) lymphocytes for a specific subset.

The mean of replicate samples was used for further analysis. Data is presented as the percentage of the total lymphocyte or total subset-specific population.

3.3.3 Anti-ova antibodies

The coating antigens were ovalbumin, yeast lysate and OvaYeast lysate at 10 μg/mL. Serum samples were diluted 1 in 5 in PBS with 0.05% Tween20 while intestinal mucus samples were used without dilution. Detection was with anti-bovine IgG HRP and TMB substrate (3,3',5,5'-tetramethylbenzidine). Each sample was assessed in duplicate. The same positive and negative controls were included in all ELISA plates to normalize OD values across plates. Sample SP% was calculated as 100(Mean Sample OD – Mean plate negative OD)/(Mean plate positive OD – Mean plate negative OD).

3.3.4 Cytokine (interferon gamma) response

Equal volumes of blood and culture medium without or with various stimulants (ovalbumin, yeast, OvaYeast) were cultured for 48 hrs at 37^{o}C in an atmosphere of 5% CO_2. Pokeweed mitogen (PWM) was included to evaluate general responsiveness of whole blood cells. Culture supernatants were harvested and stored at -20^{o}C until used for detecting interferon gamma (IFNγ) by ELISA.

The bovine IFNγ ELISA was performed as previously described (Begg et al. 2018) using anti-bovine IFNγ (clone 6.19, a gift from Dr. Gregers Jungersen), anti-bovine IFNγ-biotin (clone CC302, Biorad), streptavidin HRP and TMB substrate. Each sample was assessed in duplicate. The same positive and negative controls were included in all ELISA plates to normalize OD values across plates. Sample SP% was calculated as 100(Mean Sample OD – Mean plate negative OD)/(Mean plate positive OD – Mean plate negative OD).

3.3.5 Gene expression in tissue samples

The tissue samples were transferred to frozen mortar and pestles that were kept on dry ice and pulverised into a powder, which was transferred to 1 ml RNAzol RT (Sigma) prior to thawing. Total RNA was extracted according to the manufacturer's recommendations. The quantity and integrity of the isolated RNA was verified on a NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific).

Complementary (c)DNA synthesis, or reverse transcription (RT), was performed on 1 μg RNA from each sample using the SensiFAST cDNA synthesis (Bioline) according to the manufacturer's recommendations, with the addition of 1 μl RNasin® RNase inhibitor (Promega) per reaction to

protect the RNA from degradation and both oligo dT and random priming. The resulting cDNA was diluted tenfold in nuclease-free water prior to quantitative (q)PCR to detect gene expression.

The qPCR was performed using an Mx3000P Real-time PCR system (Stratagene, Agilent) using the QuantiTect SYBR Green PCR kit (Qiagen). Assays were prepared in 96 well plates and included duplicate reaction of each sample. Reaction volumes of 20µl (including 4 µl of target cDNA at a 1/10 dilution) were prepared, including 300 nM forward and reverse primers. The bovine primers used for RT-qPCR are shown in Appendix 3 Table 1. Three non-changing genes were selected as reference genes; this follows MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Vandesompele et al. 2002). Amplification was performed under the following conditions: 95^{o}C for 15 min, then 40 cycles of 95^{o}C for 20 s, 56-58^{o}C for 30 s and 72^{o}C for 30 s, with fluorescence acquisition at the end of each annealing step. The specificity of the reaction was confirmed using melting curve analysis and standard curves were performed for each primer set to determine reaction efficiency.

3.3.6 Intestinal parasites

Faecal samples collected at necropsy 5 months post primary vaccination were analysed for parasite burden (faecal worm and fluke egg counts and larval differentiation) by a commercial veterinary parasitology laboratory (Elizabeth Macarthur Agricultural Institute).

3.3.7 Statistical analysis

3.3.7.1 Body weight

Since body weight was not able to be collected for some animals at some collection points, data were analysed by fitting a mixed model with Geisser-Greenhouse correction for sphericity and alpha at 0.1, rather than by repeated measures ANOVA (which does not account for missing values). Tukey's multiple comparisons test was used to compare the effect of treatment group at each time point.

3.3.7.2 Lymphocyte proliferation, cytokine and anti-Ova antibody data

Statistical analysis was by Repeated Measures two-way ANOVA with Geisser-Greenhouse correction for sphericity and alpha at 0.1. Tukey's multiple comparisons test was then used to compare the effect of each stimulant between treatment groups.

Where values for some stimulants were missing or excluded due to not meeting assay criteria, data were analysed by fitting a mixed model with Geisser-Greenhouse correction for sphericity and alpha at 0.1, rather than by repeated measures ANOVA (which does not account for missing values). Dunnett's multiple comparisons test was used to compare the effect of each stimulant between treatment groups.

3.3.7.3 Gene expression data

Data collected from qRT-PCR experiments were analysed utilising a modified comparative Ct (ΔΔCt) method termed the common base method (Ganger et al. 2020).

3.4 Evaluate the ability of the candidate antigens to be recognised by immune cells sourced from BRD infected feedlot cattle

3.4.1 Blood samples

3.4.1.1 For peptide ELISA

Cattle blood samples collected into serum tubes were obtained from commercial properties in NSW (Table 4.1). Breeds included Angus, Angus-cross, Simmental-cross, South Devon, South Devon-cross and English White. Serum was stored at -20^{o}C until required. Samples were categorised based on testing for the presence of anti-BVDV antibodies by ELISA (SVANOVIR®BVDV-AB) (data reported in Milestone 5 Report, Appendix 8.1). A total of 67 negative and 71 positive samples were subsequently screened by in-house peptide ELISA to detect the presence of serum antibodies to 9 predicted antigenic regions from BVDV. The current vaccine used in Australia contains killed virus from two BVDV strains, both of which were represented in the sequence dataset used to design the peptides (Appendix 1.3). Since the vaccine is not a marker vaccine, no distinction could be made between antibodies due to infection and those resulting from vaccination.

Table 4.1 Characteristics of blood samples from farms in NSW used for peptide screening

Source	Pestigard vaccination*	BVDV ELISA positive (n)	BVDV ELISA negative (n)	Antibody ELISA (antigenicity)	Cytokine ELISA (immunogenicity)
Quirindi (at induction)	No	11	28	Yes	No
Quirindi (at exit)	No	17	0	Yes	No
Bega (BF2)	No	4	8	Yes	No
Berry (BF3)	Unknown	6	6	Yes	No
Monaro (BF5)	No	12	0	Yes	No
Bega (BF8)	No	12	0	Yes	No
Kangaroo Valley (BF9)	No	0	12	Yes	Yes
Shoalhaven (BF10)	No	0	12	Yes	No
Shoalhaven (BF6)	No	4	3	No	Yes
Shoalhaven (BF11)	Yes	6	0	No	Yes
Shoalhaven (BF12)	No	0	1	No	Yes

*Information was provided by the sample collector

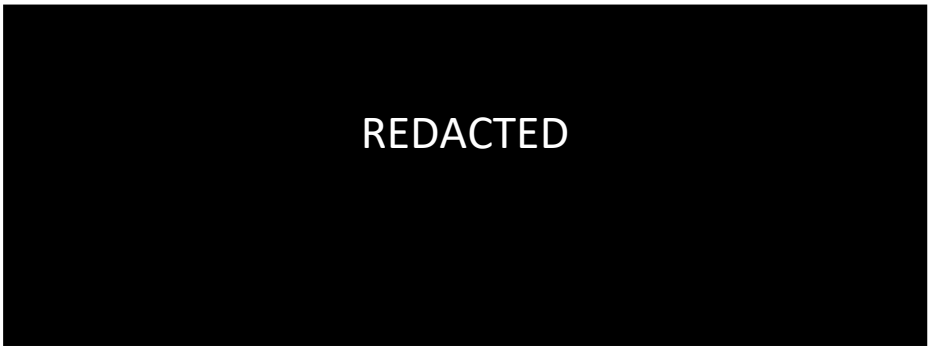
3.4.1.2 For cytokine ELISA

Blood samples were collected from cattle from four farms in NSW (Table 4.1, BF6, 9, 11 and 12) into lithium heparin and transported to the lab overnight. Whole blood stimulation assays were set up immediately. A total of 17 negative and 10 positive samples were screened by ELISA to detect the presence of serum antibodies to 11 peptides containing antigenic regions from BVDV. Because of the different sample collection and handling protocols for peptide vs cytokine ELISA, the two assays were conducted on different sample sets.

3.4.2 Peptides

Peptides were designed as described in the Milestone 3 report, using multiple BVDV genome sequences, including those of the BVDV strains used in PestiGard, the pestivirus vaccine available in Australia. Selected peptides (Table 4.2) synthesized by Mimotopes, VIC were used for screening serum samples. For the peptide ELISA, peptides were conjugated to biotin to facilitate binding to the ELISA plate, while nonbiotinylated peptides were used for whole blood stimulation.

Table 4.2 Peptides selected for screening for antigenicity and immunogenicity



3.4.3 Antibody ELISA (Humoral response)

Nunc Maxisorp plates were coated with streptavidin, biotinylated peptides (Table 4.2) added and subsequently blocked with wash buffer (PBS with 0.05% Tween20) with 1% bovine serum albumin (BSA) in wash buffer. Serum was at a dilution of 1 in 500 and detection was with anti-bovine IgG-horse radish peroxidase and TMB (3',3',5',5'-tetra-methyl-benzidine) substrate. Colour development was stopped with 2 M sulfuric acid and the optical density (OD) read at 450 nm.

The background level of serum binding in the absence of peptide was assessed for each sample. All samples were tested in duplicate and the mean value was used for subsequent analysis. The same BVDV ELISA positive sample (Positive) was included in all plates to normalise OD values across plates. SP was calculated as: Sample OD/ Positive OD. For each sample SP the relevant background SP (i.e. relevant sample without peptide) was subtracted.

3.4.4 Cytokine ELISA (Cell-mediated response)

3.4.4.1 Whole blood stimulation

Equal volumes of blood and culture medium without or with one of eleven peptides or pokeweed mitogen (PWM) were incubated for 48 hrs at 37^{o}C in an atmosphere of 5% CO_2. Culture supernatants were harvested and stored at -20^{o}C until used for detecting interferon gamma (IFNγ) by ELISA.

3.4.4.2 Interferon gamma assay

ELISA reagents were anti-bovine IFNγ (clone 6.19, a gift from Dr. Gregers Jungersen), anti-bovine IFNγ-biotin (clone CC302, Biorad), streptavidin HRP and TMB substrate (3,3',5,5'-tetramethylbenzidine). Each sample was assessed in duplicate. The same positive and negative controls were included in all ELISA plates to normalize OD values across plates. Sample SP% was calculated as 100(Mean Sample OD – Mean plate negative OD)/(Mean plate positive OD – Mean plate negative OD).

3.4.5 Statistical analysis

For each peptide, data for the BVDV positive and negative groups were compared by unpaired t-test (Graphpad Prism 8.4.2) for significant differences ($p<0.05$).

A two-way ANOVA with multiple comparisons (Graphpad Prism 8.4.2) was used to determine significant differences ($p<0.05$). To account for farm of origin data was analysed using a linear mixed model with farm and BVDV ELISA result as fixed and animal as random variables (Genstat 18th Edition).

3.5 Produce and deliver an oral yeast-based prototype vaccine that contains the candidate BVDV antigen/s

3.5.1 Antigen design

Described in detail in Appendix 1.2.

3.5.2 OvaYeast preparation and characterisation

Described in detail in Appendix 5

Yeast (*K. lactis*) was transformed to express the reporter antigen ovalbumin (Ova) following manufacturer's instructions for using the *K. lactis* protein expression kit (New England Biolabs). Briefly, a restriction digest was performed on the Ova-containing plasmid (InvivoGen) and the entire gene was ligated into PKLAC2 plasmid such that it was expressed internally. Insertion of the gene fragment in the plasmid was confirmed by PCR, prior to restriction digest and transformation of the competent *K.lactis* yeast.

3.5.2.1 Confirmation of successful transformation of recombinant OvaYeast

To confirm successful transformation of *K. lactis* with the Ova gene fragment, colonies grown on the selective media (3% 1 M sodium phosphate buffer, pH 7.5, 1.17% YCB medium supplied with the *K. lactis* Protein Expression kit [New England BioLabs], 2% agar and 0.005 M acetamide) were harvested and screened for presence of the Ova gene by qPCR. Prior to performing the qPCR, genomic DNA was extracted from the yeast clones to be screened. The DNA extraction method involved a cell wall digest in 300 µl of Cell Suspension Solution and 1.5 µl Lytic enzyme solutions from the GentraPuregene Yeast/Bact. kit (Qiagen) at 37oC for 1 hr, followed by DNA isolation of the cell pellet using the DNeasy kit (Qiagen). For the qPCR, primers were designed to target the Ova gene fragment; for 5'- Successfully transformed *K. lactis* isolates (OvaYeast) were aliquoted and stored at -80°C in 20% glycerol.

3.5.2.2 Confirmation of Ovalbumin expression by recombinant yeast

Automated Western Blotting of the Ova peptide sequence was performed using the WES Protein Simple system. Briefly, yeast cell suspensions confirmed for successful transformation by qPCR, was lysed in CelLytic Y cell reagent (Sigma). The protein concentration of the resulting lysates was quantified by micro-BCA assay (ThermoScientific) and diluted to the relevant concentration in 0.1x Sample Buffer (Protein Simple). The diluted samples were mixed to a 4:1 ratio with 5x Fluorescent Master Mix comprising of 1,4-dithiothreitol, sodium dodecyl sulphate and a fluorescence-labelled marker and incubated at 95°C for 5 minutes. The samples, biotin-labelled protein ladder, antibody diluent (blocking reagent), diluted primary antibody (A6075 Monoclonal Anti-Chicken Egg Albumin (Ovalbumin) antibody produced in mouse (1:100 dilution, Sigma), the horseradish peroxidase (HRP)-conjugated secondary antibody, chemiluminescent substrate (luminol -peroxidase), and stacking matrices were loaded into individual wells of the sample plate. The plate was centrifuged for 5 minutes at 2500 rpm at room temperature prior to loading into the WES equipment along with the relevant capillary cartridge. Separation electrophoresis and immunodetection steps taking place within the capillary system were fully automated within the WES system. The digital images obtained were analysed with Compass software (Protein Simple).

3.5.2.3 Preparation of OvaYeast Vaccine stocks

Stored OvaYeast aliquots were streaked onto selective agar and allowed to grow for 3 days. Colonies were selected and inoculated in 1 mL of 1% yeast, 2% soy-peptone and 4% galactose (YPGal) media overnight at 30°C with shaking (250 rpm). Overnight cultures were then added to 1 L of YPGal media and cultured overnight at 30°C with shaking (170 rpm) for batch growth of vaccine isolates. Aliquots of the bulk cultures were taken for cell counts and for qPCR to confirm retention of the Ova gene fragment following storage and re-culture. Remaining cultures were adjusted to the desired vaccine dose in phosphate buffered saline (PBS) and heat-killed at 100°C for 10 minutes. Inactivation of yeast was confirmed by streaking on selective agar and observing no growth after 7 days of incubation at 30°C. The control vaccine, wild-type *K. lactis* was prepared in the same way, with aliquots being streaked on non-selective agar (1% yeast extract, 2% soy-peptone, 2% glucose and 2% agar) prior to growth in YPGal. Vaccine dose aliquots (OvaYeast- Y222, Y243 and wild-type yeast Y238) were stored at -80°C till used.

Confirmation of expression of the Ovalbumin-specific protein sequence in the yeast vaccine batches used for the animal trial was performed using one-dimensional liquid chromatography mass spectrometry (1D LCMS) followed by data searches using PD 2.5 and Mascot 2.7 (performed by the Sydney Mass Spectrometry Core Facility, University of Sydney).

3.5.3 Antigen-yeast preparation and characterisation

Described in detail in Appendix 5.

3.5.3.1 Selection of regions for cloning

The process for candidate antigen selection and details of suggested BVDV epitopes are also shown in Appendix 1.2. Of the 15 suggested epitopes, 9 could be synthesised for antigenicity screening (Appendix 4) (Fig 5.2.2) and as such were used to select the regions for *K. lactis* cloning.

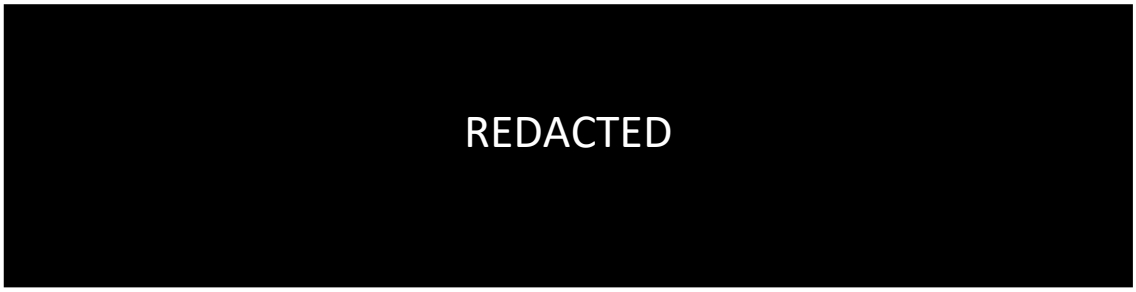


Figure 5.2.2 Schematic representation of the BVDV polyprotein and location of the peptide epitopes.

Four regions were selected for cloning based on the location of the epitopes identified (Fig. 5.2.2; Table 5.1). Region 1 is located within the E^{rns} protein and is 67 amino acids in length. The epitope associated with peptide 2 sits within this cloned region. Region 2a and 2b are two sequence variants located within the E2 protein that are 413 and 343 amino acids in length, respectively. Region 2a contains epitopes for peptides 3, 5 and 7 and Region 2b contains the epitopes for peptides 1, 4, and 8. Region 3, located in the HrpA section at the end of the NS3 protein, is 300 amino acids in length and contains the epitope associated with peptide 9. Region 4, located in NS2, is 345 amino acids long and contains the epitope associated with peptide 6.

Table 5.1 Peptides covered by regions selected for cloning into *K. lactis*

3.5.3.2 Cloning strategy to produce Recombinant antigen-yeast

'gBlock™' gene fragments were used to construct the plasmid vector for transformation of *K. lactis* to create the recombinant clones. The gBlocks™ are double-stranded DNA fragments corresponding to the regions of interest shown in Table 5.1.

The gBlock™ for each region underwent PCR modification to create two new gene fragments that when ligated into PKLAC2 and used to transform *K. lactis*, would each have the potential to produce recombinant yeast able to express BVDV proteins either intracellularly or extracellularly (Fig. 5.2.3).

In addition, a human influenza haemagglutinin (HA) molecule (corresponding to amino acids 98-106) was added to the modified gBlocks™. Recombinant proteins will express the HA tag allowing for identification using anti-HA antibodies and protein purification for antigenicity studies. The addition of the HA tag does not interfere with the bioavailability or biodistribution of the recombinant protein produced.

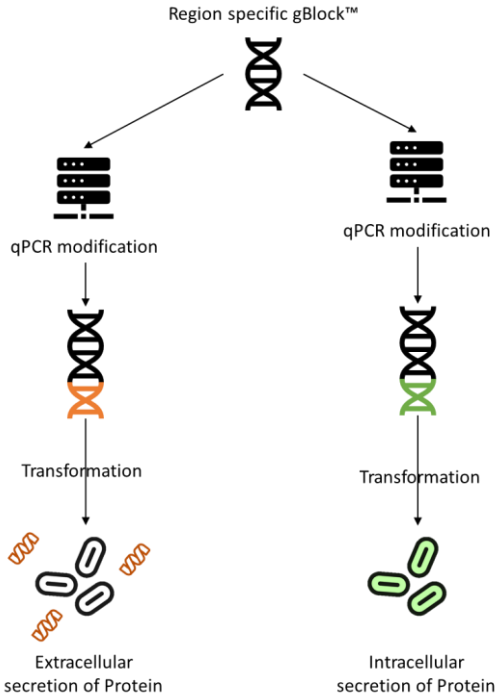


Figure 5.2.3 Schematic of the modification of gBlock™ regions to produce *K. lactis* clones capable of intracellular or extracellular protein secretion.

Modified gBlocks™ for each of the regions selected for cloning into *K. lactis* were ligated into the PKLAC2 plasmid vector for transformation of *K. lactis*, to generate a total of 10 plasmid vectors (four regions of interest with 2 sequence variants of Region 2(a/b) (n=5), cloned for intracellular and extracellular expression). Ligation was confirmed with PCR and gel electrophoresis, as shown in Fig 5.2.4.

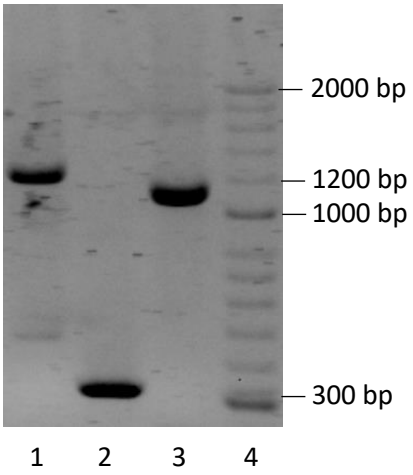


Figure 5.2.4. Representative gel electrophoresis image confirming plasmid insertion. A. Lane 1: Region 3 extracellular 1267 bp, Lane 2: Region 1 intracellular 293 bp, Lane 3: Region 2b intracellular 1112 bp. Lane 4 MW marker.

PKLAC2-antigen plasmid vectors for each region for both confirmations (intracellular and extracellular expression) were used to transform *K. lactis*. Following transformation, *K. lactis* was inoculated onto selective agar with growth of multiple pinpoint colonies forming a lawn after 3 days, with several larger colonies per plate. Large colonies were subcultured onto selective agar for 2 days to obtain a pure culture prior to inoculation into YPGal medium for culture overnight at 30°C with shaking (170 rpm). Hundreds of clones were screened by conventional and qPCR to identify successfully transformed isolates for each region.

3.5.3.3 Confirmation of successful transformation of recombinant antigen-yeast

Several methods were employed in parallel to screen for successful transformation of *K. lactis* recombinant antigen clones, including confirmation of the integrated DNA fragment, expression of RNA encoding the antigenic protein and identification of the antigen protein (in the *K. lactis* cell lysate).

Initially, antigen-yeast colonies for each region that had grown on selective agar were harvested and used to screen for successful transformation by conventional and qPCR amplification of the region of interest. Multiple sets of primers were designed for each region of interest in both confirmations, to identify recombinant antigen-yeast clones (Table 5.2). In qPCR reactions, a *K. lactis* reference genes (K.lactis Actin gene, forward 5'-TGGAAGCTGCTGGTATCGAC-3', reverse 5'-GAAGGAGCCAAGGCGGTAAT-3') was included to confirm quality of recombinant yeast DNA extracts (Fig. 5.2.5). In addition, standards constructed from the original gBlock™ gene fragments for each region were used as a test positive based on the fragment melt temperature (DNA size) and to determine DNA quantity in successfully transformed clones (Appendix 5 Figure 5.2.5).

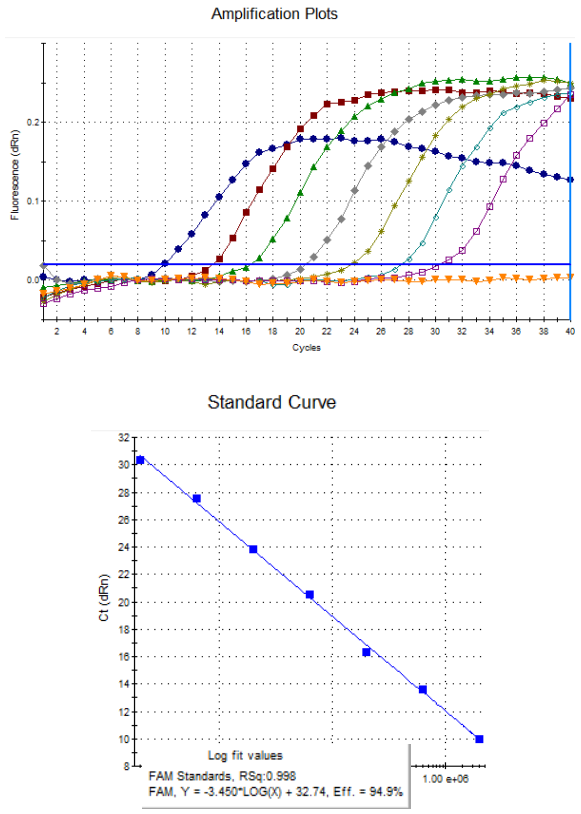


Fig. 5.2.5. Example standard curve using the Region 1 gBlock and qPCR primers used for screening Region 1 yeast clones. Standard 1 (blue) was at a very high concentration and hence there was an unusual amplification curve consequently, however the remaining standards all showed excellent amplification with a standard curve R^2=0.998 and reaction efficiency of 94.9%.

3.5.3.4 Gene Expression to confirm expression in recombinant K. lactis

A subset of the recombinant antigen yeast clones were screened for successful transformation via RNA extraction and gene expression. RNA was extracted using RNAzol RT (Sigma), according to the manufacturer's instructions. The RNA samples were subjected to an ethanol clean up to remove contaminants and the DNAse treated (RQ1 RNase-free DNase, Promega) to remove genomic DNA. These were converted to complementary (c)DNA using the SensiFast Reverse transcriptase kit (Bioline) and the cDNA was diluted 1 in 2 prior to qPCR. The same *K. lactis* reference gene described in section 5.2.2.3 was utilised for gene expression analysis and confirmation of RNA extract quality. The gene expression analysis was also used as an additional tool to examine isolates that had evidence of intracellular protein excretion by WES analysis but showed no DNA integration by qPCR.

3.5.3.5 Protein secretion in recombinant K. lactis lysates

As detailed in section 5.2.1.2, automated Western blotting of the HA-tagged antigen protein in recombinant *K. lactis* lysates was performed using the WES Protein Simple system. To identify the recombinant proteins of interest, the 51010 HA Tag Monoclonal Antibody (1:500 dilution, Invitrogen) was used.

In parallel, confirmation of expression of the antigen-specific protein sequences by the putative yeast clones was performed using 1D LCMS followed by data searches using PD 2.5 and Mascot 2.7 (performed by the Sydney Mass Spectrometry Core Facility, University of Sydney).

3.5.3.6 Purification of HA-tagged proteins from BVDV antigen-K. lactis lysates

BVDV protein-yeast clones where protein of appropriate size was detected by WES were selected for protein purification.

Yeast clones were pooled, lysed with CelLytic reagent (Sigma) and the lysate collected after centrifugation. Anti-HA magnetic beads (Thermo Scientific) were washed (0.05% Tween20 in TRIS-buffered saline) and incubated with yeast lysate for 30 min. The protein bound HA beads were collected magnetically and washed several times. The beads were removed with 50 mM NaOH and then neutralised in TRIS-Buffer. Protein in the eluate was quantified by micro-BCA assay.

3.5.3.7 Confirmation of BVDV antigen expression in recombinant K. lactis lysates by one-dimensional liquid chromatography mass spectrometry (1D LCMS)

1D LCMS was performed by the Sydney Mass Spectrometry Core Facility, University of Sydney. Concentrated supernatant samples were precipitated by the addition of 800 µL ice cold acetone to 200 µL of each sample and incubated at -30^{o}C freezer overnight. Acetone was removed and samples air dried, then 200 µL of lysis buffer was added to each sample. The protein concentration was quantified (QUBIT) and ~16 µg of each sample was taken out for reduction, alkylation and tryptic digestion (1:50). All samples were incubated at 37 ^{o}C overnight. The samples were then acidified with 10% TFA, ~1 µg was taken out for 'Zip Tipping'. Samples were eluted in 10 µL 50% ACN, 0.1% TFA and r/c in 8 µL loading buffer. Each sample was then subjected to 1D LCMS followed by data searches using PD 2.5 and Mascot 2.7.

4 Results

4.1 Deliver a literature review identifying candidate antigens to BRD vaccines and selection for 10-15 'best-bet' antigens

4.1.1 BRD immunology and vaccines literature review

4.1.1.1 Introduction

The major cause of production losses, morbidity and mortality in feedlots globally is bovine respiratory disease (BRD) (Edwards 2010). Meat and Livestock Australia (MLA) have funded several projects to gain an understanding of the situation in Australian feedlots; around 18% of cattle develop BRD (Barnes, Hay et al. 2015) and it is responsible for 50% of deaths and 70% of clinical disease (Sackett, Holmes et al. 2006). Respiratory disease also causes more than half (56%) of deaths in short-fed (up to 85 days) animals (Perkins 2013). A recent study concluded that the net loss to BRD mortality is estimated to be $1647 per animal and accounted for 73% of all deaths in an Australian feedlot (Blakebrough-Hall, McMeniman et al. 2020). Based on earlier estimates, vaccination provides some relief with the cost of the disease in unvaccinated animals around $20 per head compared to $12 per head for vaccinated animals (Sackett, Holmes et al. 2006).

BRD is primarily a disease of viral immunosuppression, which allows commensal bacteria in the respiratory tract to become opportunistic pathogens ultimately causing bronchopneumonia. Unavoidable exposure of young cattle to various stressors such as weaning, transportation, co-mingling and a high stocking density can also contribute to suppression of the immune system (Caswell 2014). Beef cattle are most likely to be affected during the first 50 days after entry into a

feedlot (Babcock, Renter et al. 2010). Pathogen profiles associated with BRD are similar in feedlots around the world despite differences in factors such as breed, age at feedlot entry and housing conditions: they are bovine viral diarrhoea virus (BVDV), bovine herpes virus (BoHV), bovine respiratory syncytial virus (BRSV), parainfluenza 3 (PI3), *Mannheimia haemolytica, Mycoplasma bovis, Pastuerella multocida* and *Histophilus somni*.

This review focuses on pathogens associated with BRD and host immunological responses with a view to formulating new ways of improving the health of feedlot cattle.

4.1.1.2 BRD epidemiology and prevalence in Australia

Prior exposure to BRD pathogens in Australian feedlot cattle disease is demonstrated by seropositivity to BVDV, BoHV-1, BPI3 and BRSV at feedlot entry. The majority (>60%) of cattle were seropositive for BVDV (Dunn, Godwin et al. 1995; Hay, Barnes et al. 2016; Cusack, Bergman et al. 2021). At feedlot entry seroprevalence to other pathogens ranged from 57-91% for BPI3 (Dunn, Godwin et al. 1995; Hay, Barnes et al. 2016) and 13-24% for BoHV (Dunn, Godwin et al. 1995; Hay, Barnes et al. 2016; Cusack, Bergman et al. 2021) and 27-89% for BRSV (Dunn, Godwin et al. 1995; Hay, Barnes et al. 2016).

In the study by Dunn et al (1995), disease was diagnosed in 6.8% of animals, with fever at the time of entry to the feedlot and BRD accounted for 66% of disease observed. Viral infections were identified in 72% of sick animals with BVDV and BoHV-1 significantly associated with cases of BRD. Bacteria were cultured from 19% of sick animals with the majority (7%) attributed to *M. haemolytica* or various *Salmonella* species (6.6%). In deceased animals, 41% of BRD cases were confirmed to have viral infections, with significant associations between both BVDV and BoHV-1 and respiratory disease. The recent study by Cusack et al (2021) found that seroprevalence to BVDV increased by 33.9% and to BoHV by 30.3% during backgrounding in cattle that were seronegative at entry to backgrounding.

The cause of cattle death was also examined by Dunn et al (1995) in the 0.9% of animals dying during the research period. BRD accounted for 53% of these deaths. In the deceased animals, 41% of BRD cases were confirmed to have viral infections, with significant associations between both BVDV and BoHV-1 and respiratory disease. Interestingly, there were no significant associations between viral infection and cause of death. In autopsy material from deceased animals 11.8% were positive for *P. multocida*, 7.2% positive for *M. haemolytica*; of these positive results 17.7% of cultured isolates came from the respiratory tract.

The seroprevalence of *M. bovis* at feedlot entry and later has been reported to range between 3-13.1% and 25.3-73.5% respectively (Wawegama et al. 2016; Schibrowski et al. 2018); this wide variation is likely to be due to differences in the diagnostic testing kits used. Regardless, it is clear that cattle are exposed to this pathogen during their time in the feedlot. The nasal prevalence of *H. somni, M. bovis, M. haemolytica*, and *P. multocida* in a sample of Australian live export cattle has been estimated at 42%, 4.8%, 13.4%, and 26%, respectively (Moore et al. 2015).

4.1.1.3 Pulmonary immune responses

The pulmonary epithelium provides an impermeable structural barrier against inhaled particles entering the host and interacts with airway macrophages as the first line of defence against respiratory pathogens and toxins (Llyod 2017; Iwasaki 2017). The epithelium comprises a variety of cell types with various functions. Ciliated cells make up the main proportion of the pulmonary epithelium. Goblet cells, which secrete mucous, decrease in number in the lower respiratory tract.

Club cells are functionally and structurally diverse and facilitate clearance functions in the lower respiratory tract. Basal cells can differentiate into the other cell types and replace cells in the epithelium. In healthy animals, mucous production is low but increases under severe inflammatory conditions. The mucins, defensins and other antimicrobial peptides in secretions from the respiratory epithelium form a protective layer. Inhaled toxins, pathogens and other particles are trapped by this mucous layer and are removed via the motion of cillia by a mechanism known as mucocilliary clearance. In deeper lung tissue, surfactants produced by pneumocytes prevent alveolar collapse and allow efficient gas exchange.

Airway macrophages are long-lived innate immune cells, which patrol the lumen of the respiratory tract. In the event the epithelial-macrophage response is unable to contain infection, chemokine and cytokine signalling from these cells can trigger activation of macrophages residing in interstitial tissues of the lungs. Additional innate immune cells such as neutrophils and natural killer cells as well as specialized lymphocytes, which circulate via lung lymphatics, are also recruited. Often, the balance between inflammatory responses and tissue healing responses can determine the extent of lung pathology. The induction of immunological memory leads to the generation of resident memory T cells (Trm). These Trm are an important component of adaptive immune responses as they reside in the tissue underlying the respiratory epithelium and can provide a rapid response when re-encountering the pathogen (Lloyd et al. 2017). $\gamma\delta$ T cells are a major component of mucosal epithelia, especially in young ruminants. These cells respond rapidly to signals from their microenvironment and co-ordinate adaptive immune responses. An understanding of these cellular responses to respiratory infections will support development of better vaccines.

4.1.1.4 Respiratory microbiome

Colonisation of the respiratory tract by microbes occurs soon after birth. The degree of colonization and the communities involved are influenced by a variety of factors including the surrounding environment, diet, transportation and antimicrobial use (Zeineldin 2019). The upper respiratory tract (oral and nasal cavities and throat) is colonized by numerous microbiota, while the lower respiratory tract (trachea and lung) is relatively sterile. There is a change in the respiratory microbiome from weaning to prior to cattle arriving at the feedlot and then again in the first 10 weeks at feedlot (Timsit 2016 Vet Micro 187).

4.1.1.5 Viral pathogens

The four most common viral pathogens associated with BRD are genetically distinct (Table 4.1.1) despite initiating similar respiratory symptoms in cattle.

Table 4.1.1. Viral pathogens

Virus	Family	Genus	Structure
BVDV	Flaviviridae	pestivirus	Single-stranded positive-sense RNA
BoHV	Herpesviridae	variellovirus	Double-stranded DNA
BRSV	Paramyxoviridae	pneumovirus	Single-stranded negative-sense RNA
PI3	Paramyxoviridae	respirovirus	Single-stranded negative-sense RNA

4.1.1.5.1 Bovine viral diarrhoea virus (BVDV)

BVDV has three distinct genotypes, BVDV1, BVDV2 and BVDV3 or HoBi-like virus (Yesilbag, Alpay et al. 2017; Evans, Pinior et al. 2019) with antigenic and geographic differences; BVDV1a and 1b are

more commonly detected in feedlots in north America and Europe, while the BVDV1c is the most common in Australia (Ambrose, Gravel et al. 2018) and BVDV2 is not found in Australia or New Zealand. In addition, there are two distinct biotypes within these two genotypes which are based on the effect of the virus on *in vitro* cultured cells (Bolin 2002). The cytopathic type, as the name suggests, induces cell death. Non-cytopathic BVDV is predominant in the field, however, both biotypes have a spectrum of virulence and both are capable of causing acute infection and respiratory disease *in vivo* (Booker et al. 2008). BVDV infects and affects the function of innate immune cells including phagocytic, microbicidal, and chemotactic functions; thus, hampering first line defence mechanisms (Goyal and Ridpath 2008). Lymphocytopenia, with a reduction in both T- and B-cells, and it's resulting immunosuppression is also typical in acute BVDV infection. The non-cytopathic type tends to induce an antibody-mediated response and traffics to lymphatic tissues associated with mucosal immunity while the cytopathic type drives a cell-mediated response and does not persist at immunological sites for long (Goyal and Ridpath 2008; Chase 2013).

Overall, BVDV is one of the top five endemic diseases that cause production losses for the red meat industries in Australia (Lane, Jubb et al. 2015). Naïve cattle exposed to BVDV develop an acute transient infection originating in the nasal mucosa within 6 to 12 days following exposure, (Evans, Pinior et al. 2019). Transiently infected animals can shed low levels of the virus in bodily secretions and excretions for up to 3 weeks (Thurmond 2008). Once infection is cleared, shedding ceases and the animals develop an antibody and T cell response that is protective for life (Evermann and Barrington 2008; Brodersen 2014; Lanyon, Hill et al. 2014). Low virulence strains are rapidly cleared unlike highly virulent ones which continue spreading into almost all organs and tissues (Goyal and Ridpath 2008). *In utero* exposure is common and the timing of exposure is critical to disease outcome. Fetal exposure after day 30 and during the first trimester leads to persistently infected (PI) calves (Lanyon, Hill et al. 2014). The immune naivety of the fetus leads to immune tolerance to BVDV, resulting in an animal that has a lifelong viral infection and is capable of transmitting BVDV (Brownlie, Hooper et al. 1998). From the pathogen's perspective this creates an ideal scenario where asymptomatically infected PI calves can surreptitiously spread infection to other cattle in the feedlot through the oronasal route (Goyal and Ridpath 2008).

Seropositivity to BVDV upon entry to feedlots is a risk factor associated with the development of BRD. In Australia seroprevalence of BVDV is widespread (Durham, Hassard et al. 1991; Dunn, Godwin et al. 1995; Hay, Ambrose et al. 2016; Hay, Barnes et al. 2016). Cattle in feedlots where BVDV is present, either transiently or persistently, have a higher risk of developing BRD than those in cohorts without BVDV (Barnes, Hay et al. 2015). Furthermore, the animals that have an increase in BVDV antibody titre following entry to the feedlot are at a higher risk of developing BRD compared with animals that have high titres at entry (Durham, Hassard et al. 1991). Therefore, exposure within the feedlot to BVDV either by transiently or persistently infected animals can increase the incidence of BRD, which could be prevented or reduced through boosting immunity to BVDV via feedlot entry vaccination.

A positive serological response indicates the presence of antibodies that can bind to the antigen(s) in the test kit. These antibodies may or may not be protective and are part of the humoral arm of the immune response. It is an indication that the animals have been exposed to the pathogen – not that they are protected if re-exposed. There can be many different antibodies to the pathogen (binding to different parts of the pathogen). Some of these will last longer than others. Similar to a booster vaccination, re-vaccination of an animal that is seropositive can make the immune response stronger. Therefore, the immune response of a seropositive animal can still be boosted.

Further to this, for some pathogens the cell-mediated arm of the immune response, including cytotoxic T cells that are able to lyse viral infected cells, is more efficacious at protecting from disease. The reverse vaccinology approach is targeting MHC I epitopes to facilitate CD8+T cell mediated immune responses. These epitopes will be present on the target antigens used to generate the yeast vaccine. Studies have shown that yeast presentation of vaccine antigens to dendritic cells in the gut-associated mucosal tissues (oral delivery) can lead to the development of a mucosal antibody response as well as an effective cell-mediated immune response, meaning that it may lead to dual protection by both arms of the immune response (Shin et al 2005; Kim et al 2014, Zhao et al 2014). We plan to measure these responses in the *in vivo* phase of the project for our reporter antigen as a proof-of-principle for the yeast vaccine delivery system in ruminants. However, efficacy studies are required for the antigens identified by the reverse vaccinology approach to determine if they will initiate protective immune responses to the specific target(s).

4.1.1.5.2 Bovine herpesvirus type 1

BoHV-1 has been noted to cause a wide range of clinical disease in Australia, including genital infection, conjunctivitis, encephalitis, gastrointestinal and respiratory disease (Rogers et al. 1978; Rogers et al. 1980; Babiuk et al. 1996). Unofficially BoHV-1 is grouped into 5 sub-groups based on restriction endonuclease profiles and broad clinical disease outcome (Smith et al. 1995). Subgroup 1.1 and 1.2b are associated with respiratory infection. Subgroup 1.2b causes milder disease than subgroup 1.1 and is the only type found in Australia (OGTR 2005). Although BoHV-1 has a predilection for mucosal tissue there are no differences in true tissue tropism between the different subgroups (Steukers et al. 2011). Similarly to other herpes viruses, pathogenesis of BoHV-1 follows the clinical steps of acute disease, latency and recurrence, with transmission possible during the acute and recurrence phases through aerosol, close contact of animals or contamination of feed stuffs and water (Turin et al. 1999). Although the acute clinical phase of BoHV-1 infection is commonly self-limiting, local lesions produced during this phase and immunosuppression facilitates secondary bacterial infections, commonly seen as part of the pathogenesis of BRD (Yates 1982; Turin, Russo et al. 1999; Leite et al. 2004).

A strong association exists in Australian feedlots between the occurrence of respiratory disease and infection with BoHV-1 (Dunn, Godwin et al. 1995). Infection of herds with BoHV-1 is common in the Australian cattle industry, however serological surveys have shown varying levels of past exposure, between 30 to 90% of herds across Australia being exposed (St George et al. 1967). On average 30% of cattle were seropositive to BoHV-1 on feedlot entry, with 76% seropositive by slaughter (Dunn, Godwin et al. 1995). Only animals seropositive for BoHV-1 in the first six weeks following entry into feedlots had a significant association with the development of BRD, with infection later in the feedlotting period not related to BRD occurrence (Dunn, Godwin et al. 1995). Furthermore, the proportion of cattle entering feedlots that were susceptible to BoHV-1 was significantly different depending on the feedlot tested (Dunn, Godwin et al. 1995). Therefore, it is likely that these differences suggest an influence of the geographical location from where cattle were sourced on potential exposure to BoHV-1 in Australia. The common practice of mixing cattle from multiple locations at feedlots would increase the chances of contact between naïve and infected animals and elevate the risk of susceptible animals developing BoHV-1 associated BRD.

4.1.1.5.3 Bovine parainfluenza-3 virus

Bovine PI3 is endemic in cattle populations worldwide; while it can infect ruminants and humans it is not pathogenic in humans. Its genomic material and capsid are surrounded by a lipid envelope derived from the host cell, aiding immune evasion (2017). Infection is restricted to the respiratory tract with the virus targeting epithelial cells of the nasal tract, pnemocytes and alveolar

macrophages. Clinical disease in cattle is of short duration (3-5 days) and generally resolves easily. Under conditions of stress, it can lead to severe bacterial bronchopneumonia, generally in conjunction with *M. haemolytica* or *Mycoplasma*. Animals that recover from infection have a strong humoral response with antibodies mainly to the haemagglutinin-neuraminidase viral protein. Infection of host cells require binding of this protein to sialic acid in mucous which it turn enables attachment of PI3 into cells in the host respiratory tract (Ellis 2010). BPI3 is considered to be only a minor contributor to BRD.

4.1.1.5.4 Bovine respiratory syncytial virus

BRSV is closely related to the human respiratory syncytial virus which is the major cause of lower respiratory tract illness in infants and older adults (CDC 2020). The presence of two non-structural proteins makes BRSV distinct from other viruses in the Paramyxoviridae family such as PI3 (Valarcher et al. 2007). These proteins play a role in regulating interferon alpha and beta (IFNα, IFNβ) by inhibiting transcription factors that activate these pathways. Similar to PI3, the BRSV genome is surrounded by a lipid envelope derived from the host. The glycoprotein (G protein) on the surface of the virus is immunodominant and is required for attachment to host cells. In its secreted form, the G protein neutralises antibodies.

Several antigenic and genetic BRSV subtypes have been identified (Valarcher and Taylor 2007). This includes four subgroups based on the G protein: A, B, AB, and untyped and six genetic subgroups based on G and five subgroups based on F (fusion) or N (nucleoprotein). The evolution of BRSV into subtypes may have been driven, in part, by selection pressure applied by vaccination. Assessing European BRSV isolates from a period of 32 years, Valarcher et al (Valarcher et al. 2000) found that there was a continuous evolution of the sequences of the N, G, and F proteins of BRSV with a strong positive selective pressure on the G protein and in specific sites of the N and F proteins in the countries where BRSV vaccines were widely used. The ability to vary the structure of the G protein allows the virus to successfully evade immune responses previously established by vaccination.

While particularly virulent strains exist, BRSV generally causes a mild disease. The main pathology is the destruction of the ciliated epithelium in the respiratory tract, which compromises mucociliary clearance and facilitates opportunistic bacterial infections from other BRD pathogens like *M. haemolytica.* It is likely to be the host response to the viral infection and subsequent severe inflammation that causes the destruction of lung tissue.

4.1.1.6 Bacterial pathogens

The composition of bacterial communities in the bovine respiratory tract changes over the course of their time spent in the feedlot (Holman et al. 2015). In addition, the presence of specific bacterial communities in the nasopharynx can prevent respiratory pathogens from establishing an infection (Holman, McAllister et al. 2015; Zeineldin, Lowe et al. 2017). In cattle that remain healthy, these communities are more diverse at feedlot entry than in those that develop BRD (Holman, McAllister et al. 2015) and these communities are distinct between the upper and lower respiratory tracts (Timsit et al. 2018). The nasopharynx and trachea in cattle with bronchopneumonia is enriched with *M. bovis, M. haemolytica* and *P. multocida*. The caveat is that some commensal bacteria can become opportunistic pathogens under the right conditions and the use of treatments (e.g. antibiotics) may drive some of the differences observed between health and disease.

4.1.1.6.1 Mannheimia haemolytica

In healthy cattle, *M. haemolytica* is naturally found in the upper respiratory tract (Confer et al. 2018). It is of the Pasteurellaceae family and is a Gram-negative, facultative anaerobic, coccobacillus.

Serotype 1 is most commonly associated with BRD and serotype 6 to a lesser extent (Klima et al. 2014). The immunogenic capacity of *Mannheimia* has been widely studied in relation to capsular polysaccharides, lipopolysaccharide, adhesins, outer membrane proteins, and leukotoxin (Confer and Ayalew 2018). Several antigens for incorporating into vaccines have been identified based on their immunoreactivity (Confer and Ayalew 2018; Klima et al. 2018) and include serine protease Ssa-1 (AC570_10970), a filamentous hemagglutinin (AC570_01600), a porin protein (AC569_05045), an outer membrane assembly protein YeaT (AC570_03060) and leukotoxin.

4.1.1.6.2 Mycoplasma bovis

Mycoplasma bovis, found as a commensal on mucosal membranes, can cause a variety of diseases in cattle including BRD, arthritis and mastitis and is one of the more pathogenic species of the genus Mycoplasma and Family *Mycoplasmataceae.* Mycoplasma have the smallest genome of any living cell and rely on the host for several metabolic requirements. They are anaerobic cells, which lack a cell wall making Mycoplasma resistant to beta-lactam antibiotics such as penicillin. *Mycoplasma bovis* strains contain variable membrane surface lipoprotein antigens (Vsps), which enable adherence to epithelial cells in the respiratory tract. (Calcutt et al. 2018) and are major immunogenic antigens. Vsps have a high rate of phase (on/off) and antigenic variation underpinned by DNA rearrangements at high frequency. Despite variation of these genes between different strains, a specific virulence profile for *M. bovis* has not been identified. To account for the variability of virulence, any vaccine may need to include antigens from several strains. *M. bovis* is an extracellular pathogen but it does have the capacity to infect cells and survive intracellularly as well. From a vaccine point of view this would require induction of both cellular and humoral immunity to overcome infection (Perez-Casal et al. 2017).

4.1.1.6.3 Pastuerella multocida

Pasteurella multocida is a zoonotic member of the family Pasteurellaceae. It is a Gram-negative coccobacillus that is part of the normal bacterial communities in the nasopharynx of many species including cattle. *P. multocida* is classified into five groups based on the structure of its capsule (A-F) and 16 serotypes (Dabo et al. 2007). Serogroup A is the most common cause of BRD worldwide with serotype A:3 most commonly isolated from lung tissue is animals with respiratory disease followed by D:3 (Confer 2009). *P. multocida* is often also detected in clinically normal cattle with a prevalence of 20-60% (Griffin et al. 2010); however, it is the most common bacterial pathogen isolated from lung lesions of animals with clinical BRD. It has a prevalence of 26% in nasal swabs of Australian live export cattle.

Virulence factors include adhesins (which enable adherence to cell surfaces), a thick polysaccharide capsule (prevents phagocytosis) and lipopolysaccharide (LPS, stimulates cytokine secretion and pulmonary inflammation) (Confer 2009).

4.1.1.6.4 Histophilus somni

Histophilus somni is another Gram-negative coccobacillus of the Pasteurellaceae family which is an opportunistic pathogen causing BRD in cattle. The main virulence factors are lipooligosaccharide and outer membrane proteins (Confer 2009). The lipooligosaccharide components trigger programmed cell death of endothelial cells and phase variations of these antigens facilitate immune evasion. Immunoglobulin binding proteins in the outer membrane of *H. somni* counteract complement-mediated host defence mechanisms. While the nasal prevalence of *H. somni,* in a sample of Australian live export cattle was relatively high at 42% (Moore, O'Dea et al. 2015) this bacterium was not associated with mortality due to BRD (Moore et al. 2014).

4.1.1.6.5 Trueperella pyogenes

Trueperella pyogenes is a facultative anaerobic Gram-positive bacillus of the family Actinomycetaceae, and was previously known as *Arcanobacterium pyogenes*. It is a ubiquitous commensal of the skin and mucosal surfaces and has the ability to cause opportunistic infections including pneumonia, mastitis and liver abscesses. Its virulence factors include a pyolysin which can lyse immune cells including macrophages and the ability to invade and survive intracellularly (Jost et al. 2005). *T. pyogenes* is one of the rare Gram-positive bacteria with fimbriae; the upregulation of one type of fimbriae, FimA, is thought to contribute to its pathogenicity. An understanding of the factors required for transformation of the commensal organism to a pathogen is still lacking (Rzewuska et al. 2019).

4.1.1.7 BRD control strategies

The pathogens causing BRD have been well-documented but there is scope to develop better treatments for this disease. In addition to management strategies, antibiotics and vaccines are often used as treatments to control or prevent BRD. There are several commercially available vaccines offering protection against BRD. In Australia, these include vaccines that offer protection against *M. haemolytica,* BVDV and BoHV (APVMA 2020). There are vaccines against PI3 and BRSV but are not licensed for use in Australia. A comprehensive review of published data from the USA on efficacy of commercially available vaccines for the four viral pathogens of BRD found that despite a low number of studies under natural conditions, there was a benefit to the use of multivalent vaccines in commercial feedlots (Theurer et al. 2014). However, the vaccine components that were responsible for better outcomes were not identified. A similar review on the bacterial pathogens *M. haemolytica, P. multocida* and *H. somni* found no benefit to vaccinate against the latter (Larson et al. 2012).

There is on-going concerted global effort to identify other candidate vaccine antigens for developing better vaccines. Combined with an understanding from the pathogen perspective of factors that are important for causing disease, it is essential that there is a better understanding of the host response, particularly the protective immunological response, as well as the effect of commensal microorganisms. The microbiota of the host affects the replication and transmission of a diverse array of viral pathogens. Therefore, it is tempting to speculate that the composition of an individual's microbiota can influence disease outcome during viral infection, making it a potential target for therapeutic intervention. Host microbiota can protect against or enhance susceptibility to viral infections (Wilks et al. 2013). This would mean that different treatment strategies would be needed depending on the viral pathogen's interaction with commensal organisms in the host. *Haemophilus influenzae,* like *M. haemolytica* and *P. multocida,* belongs to the Pasteurellaceae family. A recent study has demonstrated that a single dose of a commensal closely related to *H. influenzae* can prevent disease caused by this pathogen (Granland et al. 2020). It would be valuable to develop an understanding of the commensal respiratory microbiota within the context of Australian feedlots.

4.1.1.8 References

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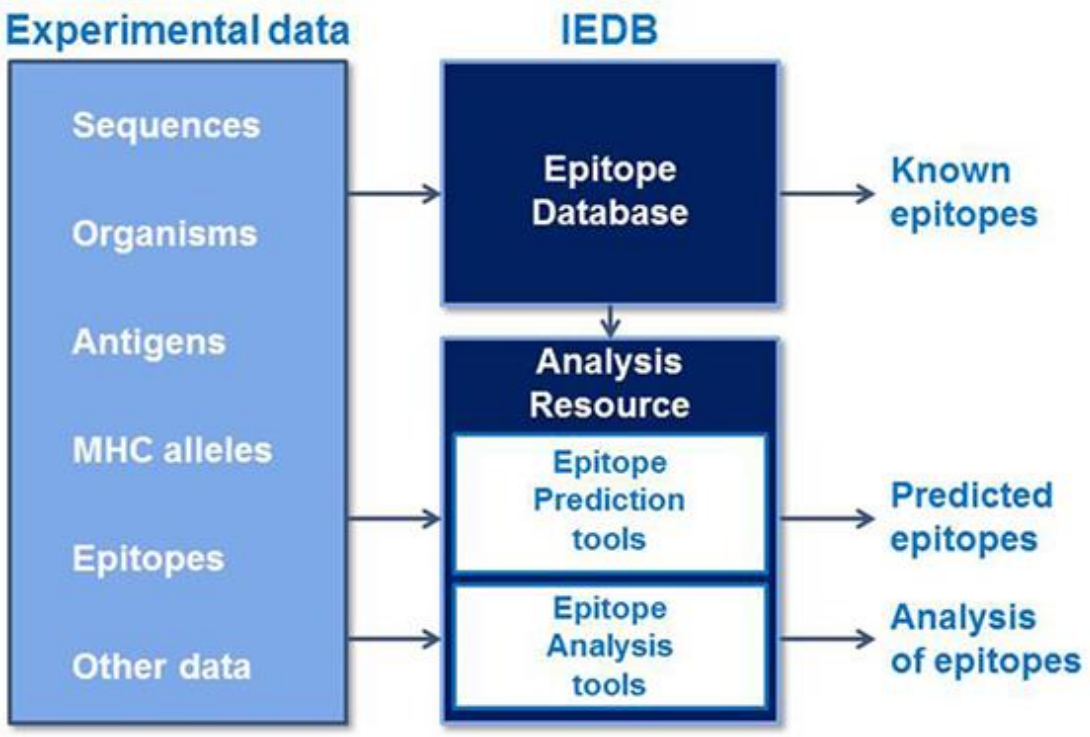
4.1.2 Literature review for candidate BRD antigens

Successful vaccines against parasite, bacterial or viral infections require insight of the cellular immune responses and to facilitate this in terms of T cell based epitope prediction; the Reverse Vaccinology approach (Burton 2017; Jensen et al. 2018) utilises the codon sequence data obtained from pathogenic DNA and translates it to a protein of interest via complementary cDNA. Experimentally (*in vivo*), this approach introduces putative antigenic proteins and evaluates the transition to antigen presenting cells (APC) and the subsequent processing to eventual presentation of antigens on Major Histocompatibility Complex (MHC) molecules to interact with T cell receptors. Smaller and potentially more immunogenic epitopes may be isolated by cloning the antigenic peptide sequences and utilising immune methodologies to identify the most relevant epitopes to take forward to vaccine formulations. Many of these *in vivo* steps may be eliminated by screening the whole protein sequence through use of *in silico* prediction programs that identify potential antigenic sequences and enable visualisation of those epitopes with highest predicted affinity, specificity and stability. Currently the *in silico* T cell epitope prediction tools are most successful in identification of MHC class I binding epitopes largely due to the structural variations inherent between MHC class I and class II. An epitope-based T cell inducing vaccine approach to viral diseases is increasingly favoured. This approach encompasses vaccines designed to induce CD4 and/or CD8 T cells that directly contribute to pathogen clearance via cell mediated effector mechanisms, rather than limiting the vaccine to target CD4 T cell mediated signals for B cell involvement leading to protective antibody responses. Several clinical trials have demonstrated the efficacy of T cell inducing vaccines (Rosa et al. 2006; Gilbert 2012). The selection and design of epitopes for vaccine use should take into account the desired immune response; for example a cytotoxic T cell response is prompted by a pathway that includes intracellular antigen processing with linear epitopes (Sun et al. 1991).

Viral exposure results in the virus and other components being taken into the host cells where the virus integrates within the host genome, replicates, and proliferates throughout the host organism. The host responds through the process of antigen presentation whereby secreted viral peptides are taken up by antigen presenting cells triggering a complex cascade of intracellular processes including proteasome processing, generation of antigenic peptides, transport to the endoplasmic reticulum, binding to MHC molecules and presentation through MHC molecules ultimately triggering recognition by CD8 T cells that are able to kill the cell, proliferate, produce cytokines and form memory T cell populations. Each step along the pathway has a corresponding efficacy and ultimately only 2.5% of the peptides that are generated will bind to an MHC molecule (Assarsson et al. 2007). Thus, there is a great deal of obsolescence in terms of immunogenicity potential for epitopes. The prediction algorithms developed by the IEDB Analysis Research team (Calis, Maybeno et al. 2013; Paul, Sidney et al. 2016) and others have been put to test against a range of pathogens including *Pseudomonas aeruginosa* (Kao and Hodges 2009) and *Leishmania donovani* (Alves-Silva et al. 2017; Barbosa Santos et al. 2017) with encouraging results showing that vaccines composed of epitopes specifically formulated to target immunogenic domains appear to optimise and potentially surpass the protective potential induced by whole protein vaccines. The success of these undertakings encourages use of a combination of immuno-informatics underpinned by experimental validation to identify epitopes for the formulation of novel T cell vaccine.

In silico B cell epitope prediction has to account for the fact that the majority of B cell epitopes are comprised from amino acid residues that are located on separate regions of the antigenic protein sequence and these are only joined together following the folding of the protein chain (Van Regenmortel 2016). This adds a level of complexity to the selection of putative epitopes and historically, unless there is *in vivo* or *in vitro* derived experimental data to support selection of antigenic sequences, *in silico* selection of B cell vaccine epitopes has not been very successful when translated to clinical trials. Since there is a paucity of accessible data available regarding verified antigen sequences relevant to bovine respiratory disease (BRD) in the Australian setting, the decision was made to concentrate on MHC class I T cell targeting epitopes however, we will continue to seek verified protein sequences to facilitate design of B cell specific epitopes. Previous B cell epitope design utilising an *in silico* approach has proven more sucessful when translated to clinical trials when utilising shorter regions of protein sequences from *in vitro* immunogenicity verified sources (Gurung et al. 2012).

The collection of bioinformatics tools that may be utilised in T cell and B cell epitope prediction and analysis are available at the Immune Epitope Database Analysis Resource (IEDB) (http://tools.iedb.org/main/). The process required for an *in silico* investigation utilising these tools is illustrated in the following diagram (Fleri, Paul et al. 2017).



Use of *in silico* methods does not negate the requirement for experimental analysis but it does provide a rapid method for identification of targeted viable epitope candidates and the vast number of bioinformatics tools currently available makes it possible to eliminate many unsuitable candidates prior to time consuming and expensive experimental research. This method has successfully been utilised within our research team to identify B cell epitopes. Previously we identified stress-regulated antigenic proteins associated with *Mycobacterium avium* subspecies *paratuberculosis* and then utilised these protein sequences for B cell epitope prediction. These *in silico*-identified epitopes were then produced as recombinant proteins and evaluated for immunogenicity via immune methodologies such as ELISA (Gurung et al. 2012; Gurung, Purdie et al. 2012).

In terms of antigen selection for a BRD vaccine, this multifactorial disease presents a challenge since BRD has multiple aetiologies. We sought to identify the primary pathogenic candidates relevant to the Australian feedlot setting while considering previously published research and these findings are presented as a review article (Appendix 1.2). Previous research shows that in the Australian setting, upon entry to the feedlot, antibodies of primary viral pathogens dominate with 68% positive for BVDV, 13% positive for BoHV-1, 57% positive for BPI3 and 27% positive for BRSV. The viral pathogens BVDV and BoHV-1 were significantly associated in those animals that succumbed to BRD during the finishing process and in 41% of deceased BRD cattle cases. The associations between BRD mortality and bacterial pathogens do not present a strong case; rather the data suggests that bacterial infections are an opportunistic infection. Further there is a lack of clarity in terms of bacterial serotype specificity within the Australian feedlots that would complicate the accurate selection of candidate for vaccine formulation and lack of access to respiratory disease associated Australian protein sequences for all relevant organisms apart from BVDV, *Pasteurella multicida* and Bovine herpesvirus type 1.2 strain B589. Thus, the epitope selection is targeted to identification of candidates for a vaccine against the viral pathogen BVDV1 since in Australia this pathogen is implicated as one of the primary risk factors associated with the development of BRD (Hay, Barnes et al. 2014).

The mechanisms of pathogenesis of bovine viral diarrhoea virus, a prototypic member of the genus Pestivirus, (family Flaviviridae) are reviewed in Appendix 1.1 and it must be acknowledged that there are currently available BVDV vaccines including conventional inactivated vaccines with adjuvants, modified live virus vaccines with reduced virulence or vaccines comprising genetically modified virus whereby virulence genes have been inactivated (Platt et al. 2017) however, the ongoing incidence of BVDV in cattle transiting the Australian feedlot system suggests that new vaccine approaches may be warranted.

Preliminary analysis of IEDB to identify previously published (Rotzschke, Falk et al. 1993; Deregt, Bolin et al. 1998; Collen, Carr et al. 2002) BVDV epitopes with MHC binding potential resulted in 12 epitopes (Table 4.1.2.1) sorted by descending score value. A higher score indicates a greater probability of eliciting an immune response (Calis, Maybeno et al. 2013). There was no overlap with the novel epitopes that we suggest taking forward.

Table 4.1.2.1 Previously published BVDV epitopes

Epitope sequence	Epitope Length	Immunogenicity Score[a]
KLLEIFHTI	9	0.38693
NDRIGPLGAEG	11	0.28554
NMLVFVPTRNM	11	0.25778
EQLAIIGKIHR	11	0.25619
YIFLDEYHCAT	11	0.15015
VTDTYENYS	9	0.12929
SSVDHVTAGKDLL	13	0.02556
QVIALDTKL	9	-0.00059
PAFFDLKNL	9	-0.01882
DTVVQVIALDTKLGPMP	17	-0.10222
VGRVKPGRY	9	-0.10326
IVRGKYNTTLLNG	13	-0.11698

a: an immunogenicity score greater than 0 is indicative of an epitope with greater capacity to elicit and immune response. The range for a successful epitope is generally between 0.2 and 0.6.

4.1.3 Selection of candidate antigens by reverse vaccinology

NCBI analysis identified 1048 protein sequences isolated from samples collected globally. Of these 22 were obtained from samples sourced in Australia (Table 4.1.2.2). There was a degree of overlap present in some of the sequences however this was addressed during the MHC class I processing epitope selection. Three sequences (9, 10 and 11) had to be excluded due to incompatibility with the IEDB analysis algorithms.

4.1.3.1 Prediction of BRD relevant MHC Class I epitopes and immunogenicity analysis

During this study, TepiTool was used to predict T cell epitopes for MHC Class I binding. This *in silico* tool allows the user to input separate sequences and these are then aligned to user selected MHC Class I alleles. In the case of the cattle MHC Class I alleles available on the database, the following choices were available for selection: BoLA-JSP.1, BoLA-AW10, BoLA-HD6, BoLA-N:00101, BoLA-N:00102, BoLA-N:00103, BoLA-N:00201, BoLA-N:00301, BoLA-N:00401, BoLA-N:00402, BoLA-N:00501, BoLA-N:00601, BoLA-N:00602. The collated protein sequences (Table 4.1.2.2) were submitted to TepiTool and individually aligned to each of the MHC Class I alleles. The predicted epitopes clustered to show multiple common epitopes between the MHC Class I alleles BoLA-JSP.1, BoLA-AW10, BoLA-HD6 (Group 1, Table 4.1.2.3) and separately to the MHC Class I alleles BoLA-N:00101, BoLA-N:00102, BoLA-N:00103, BoLA-N:00201, BoLA-N:00301, BoLA-N:00401, BoLA-N:00402, BoLA-N:00501, BoLA-N:00601, BoLA-N:00602 (Group 2, Table 4.1.2.3) suggesting that it would be advisable to determine incidence of allele variability of the MHC Class I locus in Australian feedlot cattle prior to finalising the choice for future vaccine development.

Over 1000 potential epitopes were generated against the viral pathogen BVDV1, selected following consultation with MLA as the most relevant pathogen candidate since in Australia this pathogen is implicated as one of the primary risk factors associated with the development of BRD (Hay, Barnes et al. 2014) and there is relevant Australian sourced proteomic sequence data available for *in silico* analysis ; in-depth examination of these epitope sequences to establish probability of immunogenicity enabled selection of 15 putative candidates for future analysis (Table 4.1.2.3).

Although not the focus of our analysis, we have additionally generated immunogenic epitopes for the BRD pathogens Bovine herpesvirus (type 1.2), Bovine Parainfluenza-3 virus, Bovine respiratory syncytial virus, *Mannheimia haemolytica, Mycoplasma bovis, Pasteurella multocida* and *Histophilus somni,* however we feel that additional primary input using verified Australian protein sequences would improve this selection (Table 4.1.2.4).

Table 4.1.2.2 BVDV protein sequences isolated from Australian sourced samples.

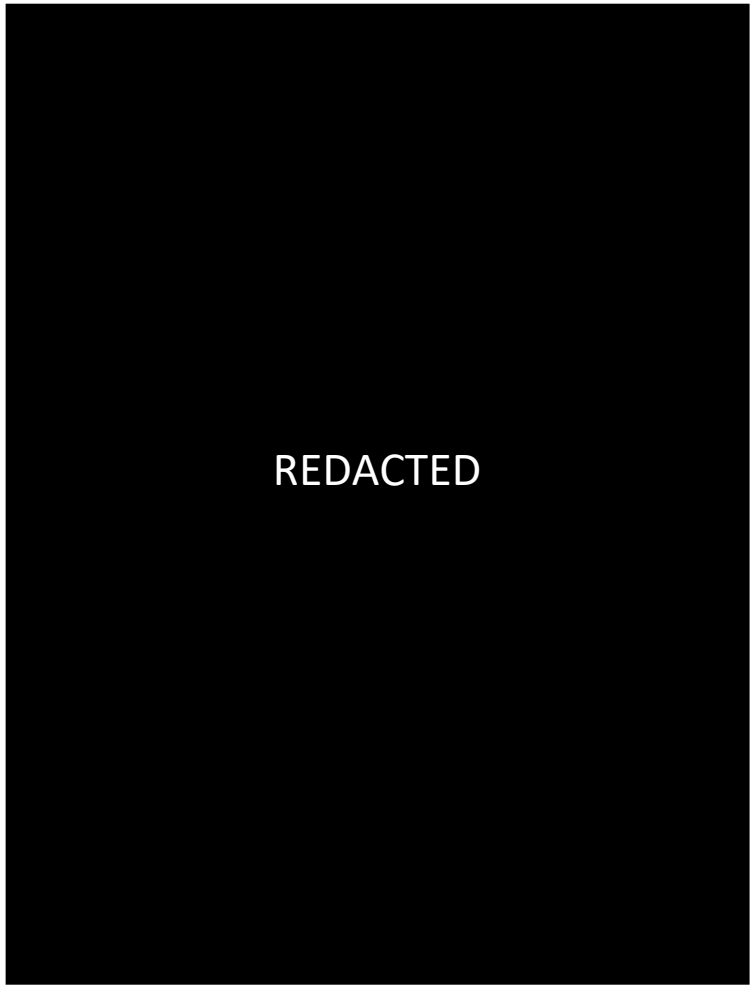


Table 4.1.2.3 Suggested epitopes for further analysis as potential BVDV vaccine candidates

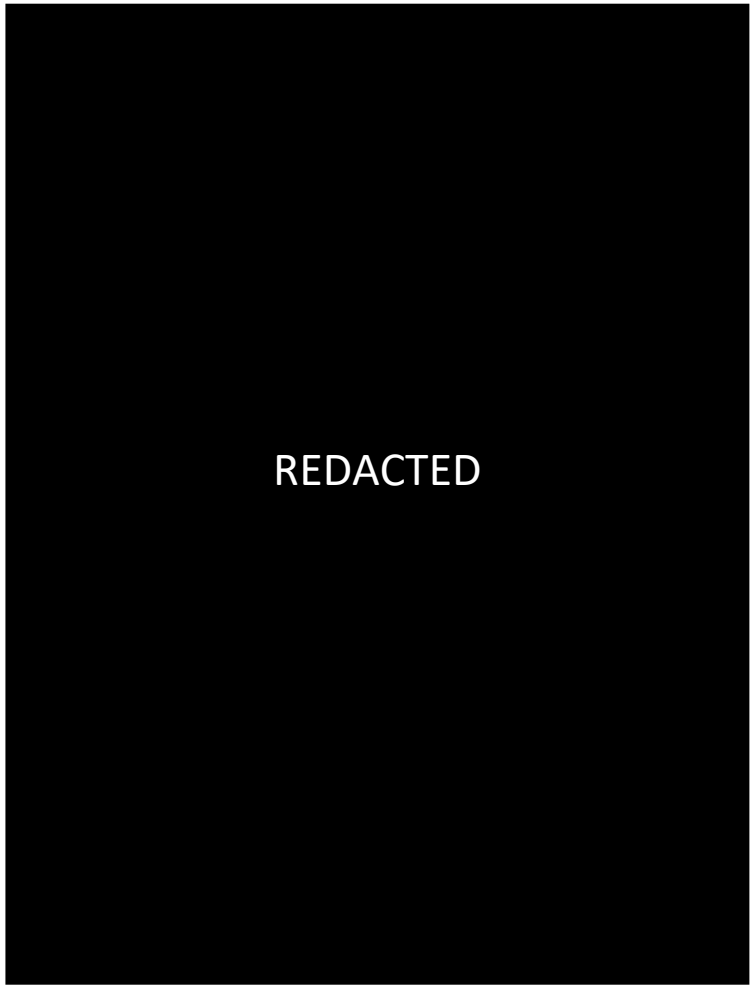
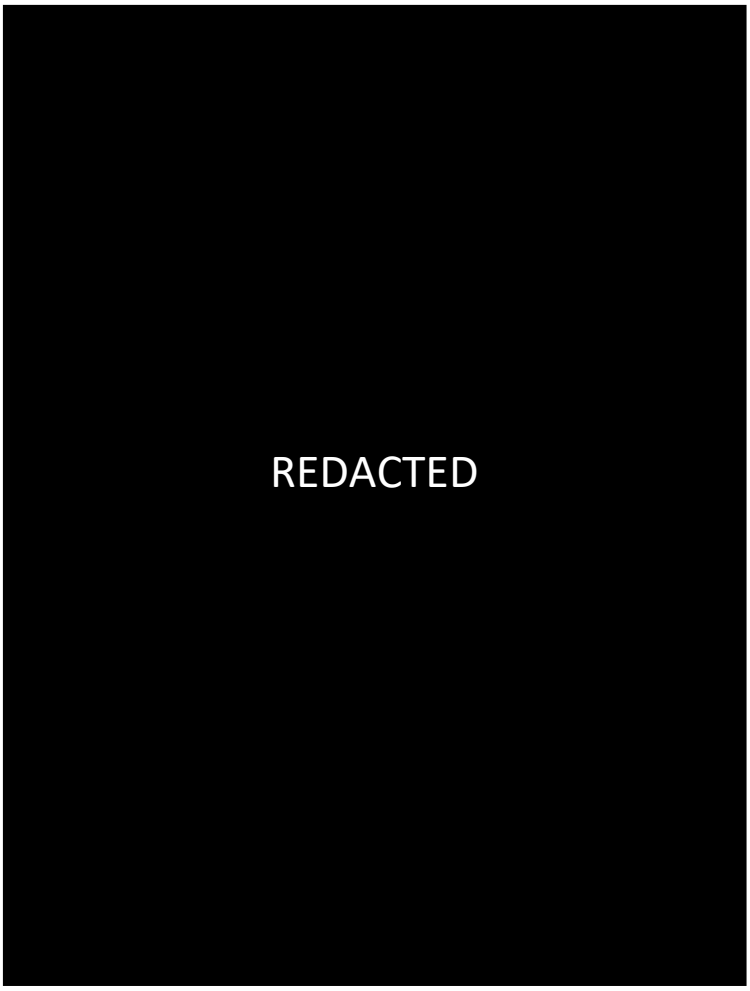


Table 4.1.2.4. Suggested epitopes for further analysis as potential BRD vaccine candidates



4.2 Evaluate the ability of a yeast-based vaccine that contains a 'reporter' to activate cattle immune cells in a laboratory culture system

Detailed descriptions can be found in Appendix 2.

The first phase of the project assessed the ability of OvaYeast to activate cattle macrophages.

Macrophages are innate immune cells responsible for detection, phagocytosis and destruction of pathogens. Phagocytosis activates microbicidal mechanisms such as induction of the respiratory burst. Nitric oxide is a product of this pathway and results from increased activity of the enzyme inducible nitric oxide synthase (iNOS) (McNeill et al. 2015). Macrophages also present antigens to lymphocytes (adaptive immune cells) and secrete cytokines such as interferon gamma (IFNγ) and TNF alpha (TNFα) to initiate inflammatory responses (Zelová et al. 2013).

Macrophages derived from peripheral blood of cattle (n=3) were cultured with untransformed yeast or OvaYeast and subsequently macrophage activation was detected as nitric oxide secretion as well as expression of genes for iNOS and TNFα. OvaYeast was pre-treated to simulate 'flaking' processes as well as the pH of rumen to determine if these conditions would alter the capacity of OvaYeast to activate macrophages.

4.2.1 Macrophage activation by yeast

While LPS-induced nitric oxide responses were detected as early as 24 hrs after in vitro incubation, yeast and OvaYeast responses required longer incubation periods (Fig. 2.3). The effect of stimulant, time and their interaction were significant ($p \leq 0.0001$). At the 72hr time point, LPS and OvaYeast at 5:1 induced significantly higher ($p<0.05$) nitric oxide compared to cells alone. In addition, at this time point, OvaYeast at 5:1 induced significantly higher ($p<0.05$) nitric oxide compared to unmodified yeast at 1:1 or 5:1 and OvaYeast at 1:1 (Fig. 2.3).

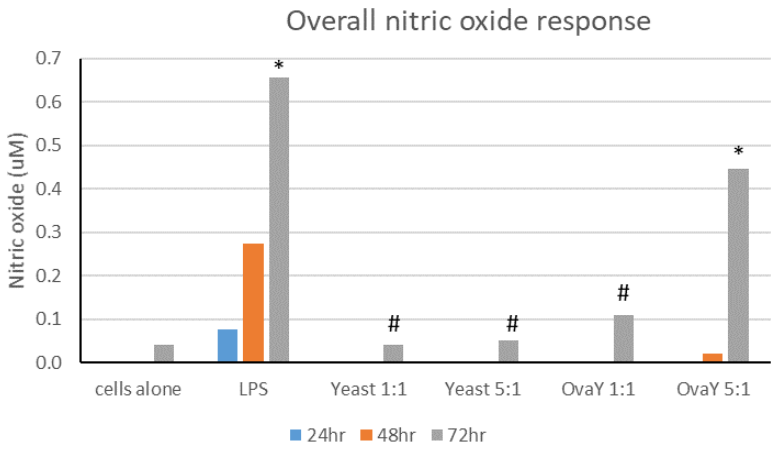


Figure 2.3 Overall nitric oxide response to yeast and OvaYeast
Bovine macrophages (n=3) were cultured with no stimulant (cells alone) or with lipopolysaccharide (LPS) as a positive control or with nontransformed yeast or OvaYeast. Nitric oxide in cell culture supernatants is shown *$p<0.05$ compared to cells alone at the same time point; #$p<0.05$ compared to OvaYeast at MOI 5:1 at the same time point

A similar pattern of responses was observed with iNOS and TNFa gene expression (Fig. 2.6); however, significantly higher responses compared to cells alone ($p<0.05$) were detected at the 24hr time point as well as with the lower OvaYeast MOI of 1:1.

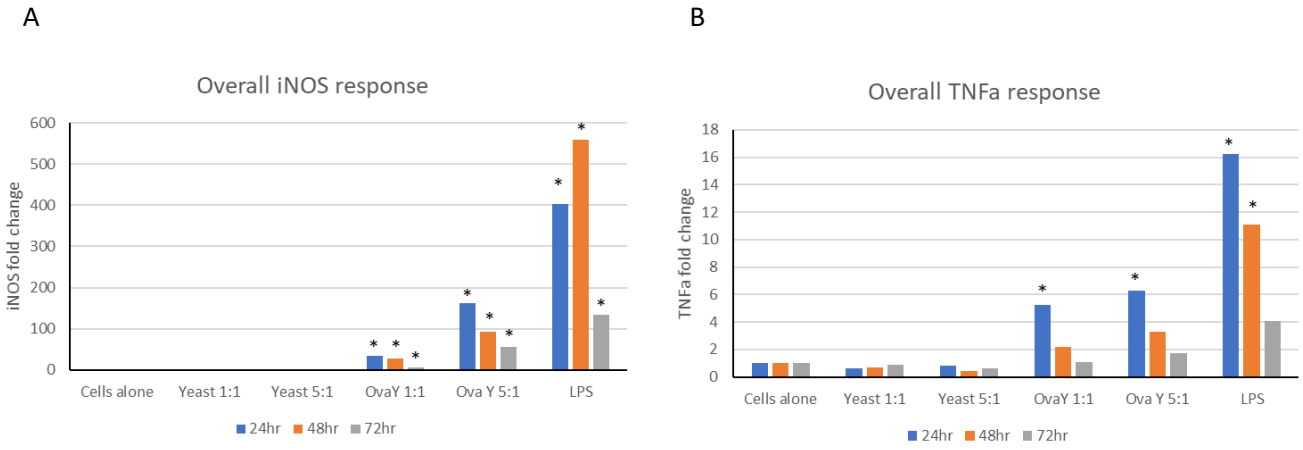


Figure 2.6 Overall iNOS (A) and TNFα (B) responses to yeast and OvaYeast
Bovine macrophages (n=3) were cultured with no stimulant (cells alone) or with lipopolysaccharide (LPS) as a positive control or with nontransformed yeast or OvaYeast. *Significant differences ($p<0.05$) between treatment and cells alone of the respective animal and timepoint are shown.

OvaYeast increases secretion of nitric oxide (Fig. 2.3) and upregulates the expression of TNFα and iNOS genes (Fig. 2.6) by macrophages, whilst there is no increase in secretion of nitric oxide or upregulation of gene expression in the presence of yeast. This demonstrates unequivocally that OvaYeast is immunostimulatory, and that this effect is specific to the Ova-modification.

The observation from these experiments was that shorter periods of incubation did not elicit a strong nitric oxide response. Therefore, the incubation time with OvaYeast was increased to enable detecting nitric oxide from cultured macrophages.

4.2.2 Comparison of live and heat-killed yeast on macrophage activation

The ability of live and inactivated OvaYeast to activate macrophages was assessed at two MOI.

Fig. 2.9 shows that exposure of macrophages to yeast, either live or heat-killed, induced secretion of nitric oxide compared to unstimulated macrophages. There were no significant differences between live and heat-killed OvaYeast at any of the MOI or time points assessed ($p>0.05$).

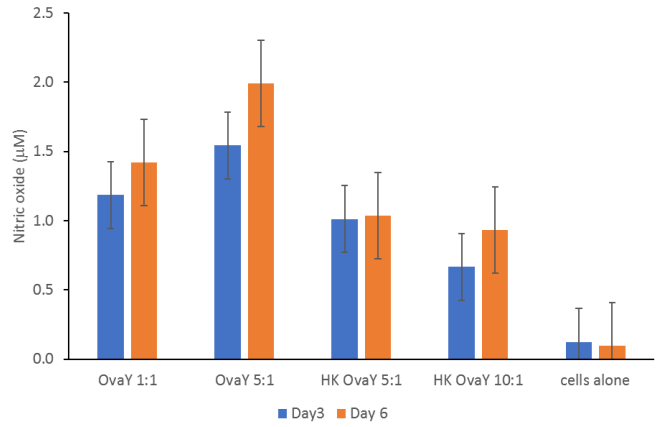


Figure 2.9. Activation of bovine macrophages by live or heat-killed yeast
Bovine macrophages (n=3) were cultured with no stimulant (cells alone) or with live OvaYeast (OvaY) or heat-killed OvaYeast (HK OvaY). Nitric oxide secretion is shown here as a measure of macrophage activation.

4.2.3 Effect of pH and heat treatments on OvaYeast activation of macrophages

OvaYeast was successfully heat-inactivated by incubating at 100°C as no viable organisms were detected by culture. Similarly, steam-flaking conditions at 50°C were also sufficient to inactivate the yeast. When OvaYeast were pre-incubated at pH 4, 6 and 8 the organisms remained viable. The OvaYeast at pH 4 and 8 were viable with no change in growth characteristics.

Nitric oxide secretion by macrophages exposed to 1:1 live 'flaked' OvaYeast was significantly lower than live OvaYeast (Fig. 2.10) ($p<0.05$). At the higher MOI, nitric oxide secretion was not significantly different between flaked and unflaked OvaYeast (Fig. 2.10). With heat-killed 'flaked' and unflaked OvaYeast, there were no significant differences between any of the treatment groups (Fig. 2.11).

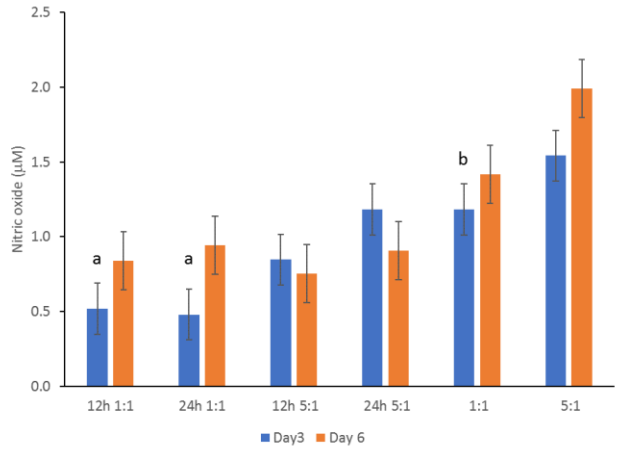


Figure 2.10 Effect of live 'flaked' OvaYeast on macrophage activation
Live OvaYeast were heat-treated at 50°C for either 12 or 24 hrs to simulating steam-flaking conditions prior to culturing with macrophages. Different letters above bars indicate a significant

difference ($p<0.05$). Data from live OvaYeast not subjected to 'flaking' simulations are shown in the two groups (1:1 and 5:1) on the right.

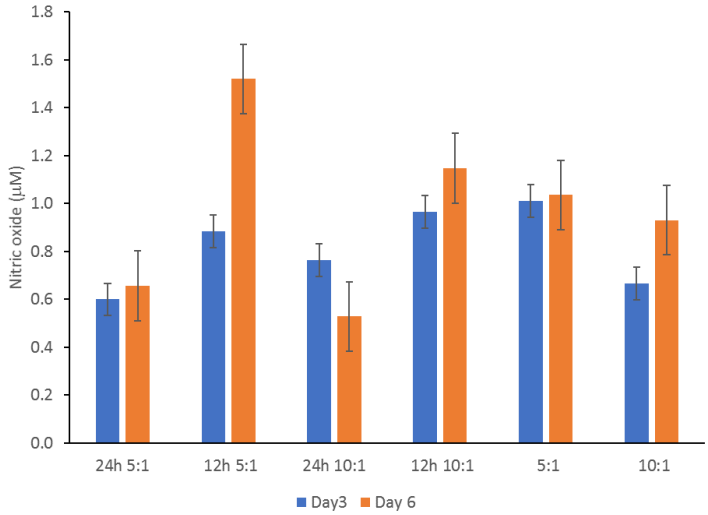


Figure 2.11 Effect of heat-killed 'flaked' yeast on macrophage activation
Heat-killed OvaYeast were heat-treated at 50°C for either 12 or 24 hrs to simulate steam-flaking conditions prior to culturing with macrophages. Data from heat-killed OvaYeast not subjected to 'flaking' simulations are shown in the two groups on the right.

At an MOI of 1:1, nitric oxide secretion was significantly lower ($p<0.05$) with live OvaYeast at both pH 4 and 8 compared to OvaYeast at pH 7.4 after 3 days of incubation (Fig. 2.12). This effect was not observed at the 6-day timepoint nor at an MOI of 5:1. For heat-killed OvaYeast, subjecting to pH variation did not induce any significant differences in nitric oxide production from bovine macrophages (Fig. 2.13). In addition, activation of macrophages by heat-killed or live OvaYeast subjected to pH variation was not significantly different.

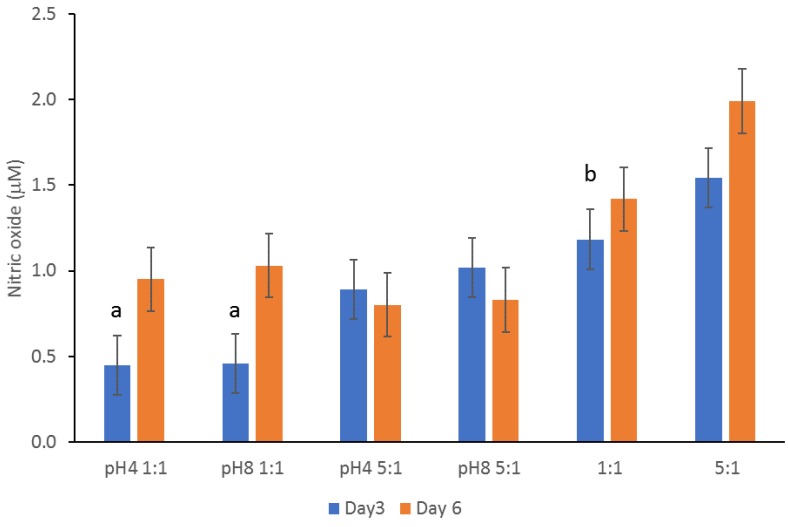


Figure 2.12 Effect of live OvaYeast subjected to pH variation on macrophage activation

Live OvaYeast were incubated at pH 4 or 8 prior to culturing with macrophages. Different letters above bars indicate a significant difference ($p<0.05$). Data from live OvaYeast not subjected to pH treatment are shown in the two groups on the right (1:1 and 5:1).

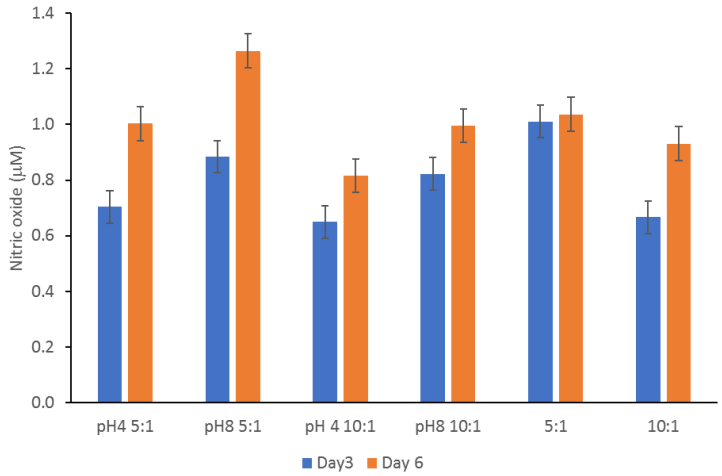


Figure 2.13 Effect of heat-killed OvaYeast subjected to pH variation on macrophage activation
Heat-killed OvaYeast were incubated at pH 4 or 8 prior to culturing with macrophages. Data from heat-killed OvaYeast not subjected to pH treatment are shown in the two groups on the right.

Macrophage activation was also assessed by the induction of genes in innate immune response pathways, specifically inducible nitric oxide synthase (iNOS) and tumour necrosis factor alpha (TNFα) (Fig. 6)

Results from one of two representative experiments are shown in Fig. 2.14.

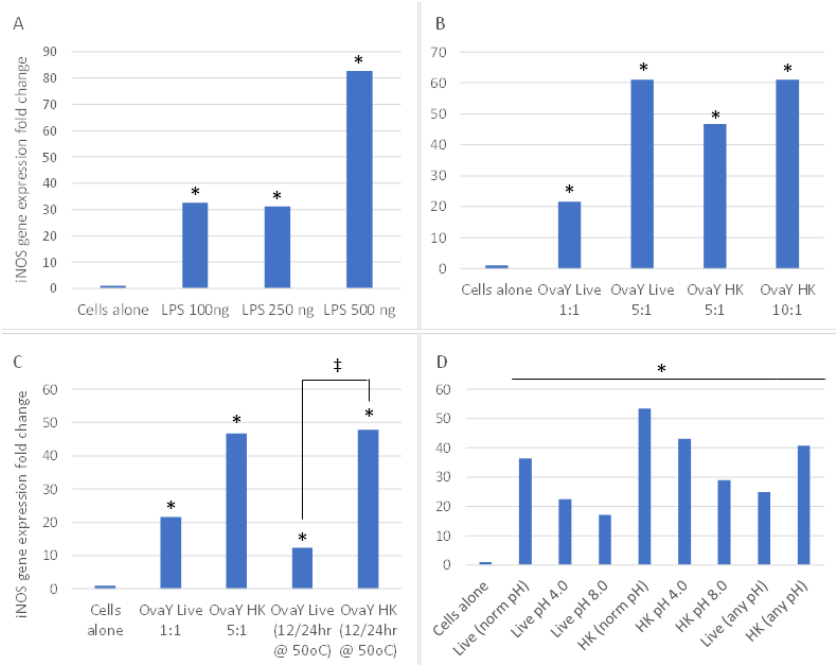


Figure 2.14 Expression of inducible nitric oxide synthase (iNOS) in bovine macrophages. Bovine macrophages were cultured with OvaYeast (OvaY) were subjected to a variety of treatments for 3-6 days prior to extracting cellular RNA. Varying LPS concentrations were included as the positive control (A), and cells alone were the negative control. Gene expression induced by live and heat-killed (HK) OvaY (B), 'flaking' pre-treatment (C) and pH pre-treatment (D) are shown. *indicates a significant difference compared to cells alone ($p<0.05$). ‡ indicates a significant difference between the two bracketed groups ($p<0.05$).

iNOS gene expression was significantly lower in macrophage cells alone (Fig. 2.14) compared to all other treatments ($p<0.05$). Both live and heat-killed OvaYeast, independent of the pH or 'flaking' pre-treatment, induced iNOS gene expression in bovine macrophages (between 10 and 50-fold increased expression). Increasing LPS concentrations led to higher expression levels of iNOS, as expected. There was no difference between live and heat-killed OvaYeast in the induction of iNOS for any condition, with the exception of live (12 or 24 hrs at 50°C) compared to heat-killed OvaYeast (12 or 24 hrs at 50°C); the heat-killed OvaYeast induced higher levels of iNOS expression.

TNFα is another cytokine expressed by activated macrophages. Gene expression changes induced by OvaYeast and its various pre-treatments are shown in Fig 2.15. As for iNOS gene expression levels, increased LPS concentrations led to a trend towards increased TNFα gene expression, though this did not reach statistical significance ($p>0.05$). Heat killed OvaY tended to induce higher expression levels of TNFα than live, though no specific trends in relation to pre-incubation at 50°C or at different pHs were seen.

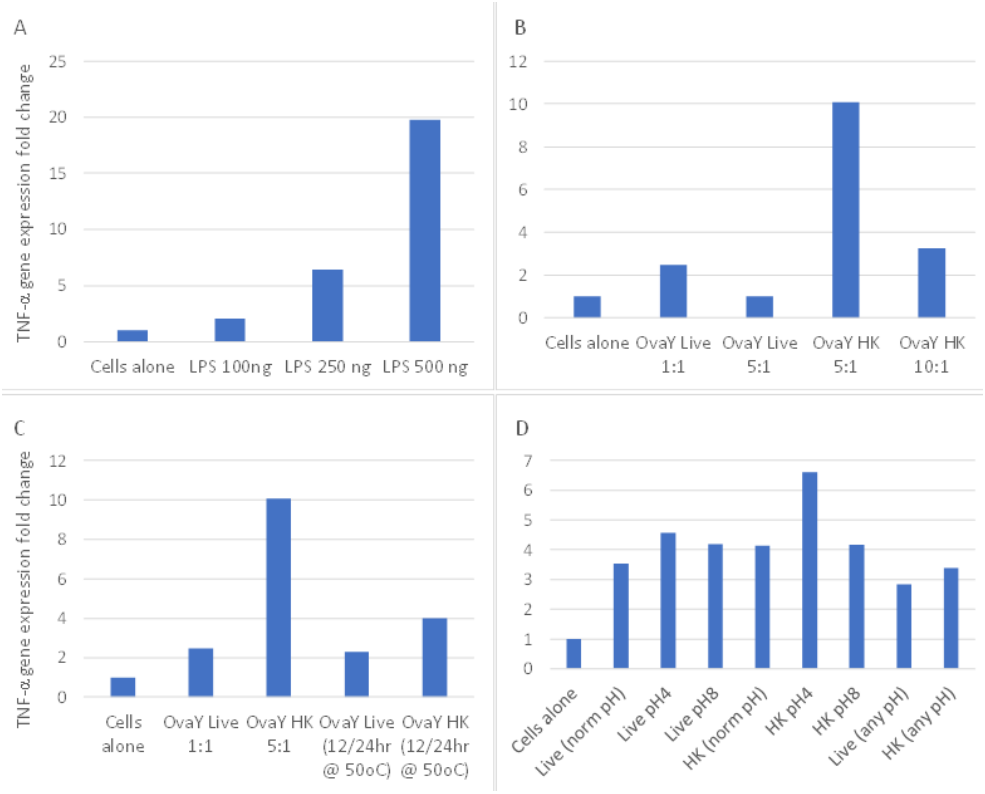


Figure 2.15 Expression of tumor necrosis factor alpha (TNFα) in bovine macrophages
Bovine macrophages were cultured with OvaYeast (OvaY) were subjected to a variety of treatments for 3-6 days prior to extracting cellular RNA. Varying LPS concentrations were included as the positive control (A), and cells alone were the negative control. Gene expression induced by live and heat-killed (HK) OvaY (B), 'flaking' pre-treatment (C) and pH pre-treatment (D) are shown.

These initial *in vitro* studies confirmed, at the cellular and molecular level, that bovine macrophages have the capacity to be activated by OvaYeast. Initial studies showed that responses were not detected or were low at shorter incubation periods (24 and 48 hrs). Culturing macrophages for 3-6 days was required for induction of activation by OvaYeast. Overall, OvaYeast increases secretion of nitric oxide and upregulates the expression of TNFα and iNOS by macrophages, whilst there is no increase in secretion of nitric oxide or upregulation of gene expression in the presence of unmodified yeast. This demonstrates unequivocally that OvaYeast is immunostimulatory, and that this effect is specific to the Ova-modification.

Importantly, there were no significant differences in this capacity between live and heat-inactivated OvaYeast. Extensive heat treatments or pH variation did not reduce the effect of heat-inactivated OvaYeast on bovine macrophages. These results indicate that heat-inactivated yeast has the potential to activate immune response pathways.

4.3 Assess the ability of the reporter vaccine to induce an immune response in cattle

Detailed descriptions can be found in Appendix 3.

This pilot vaccine trial used a 'reporter' molecule to assess immunological parameters and timing of a specific immune response, rather than targeting a particular pathogen.

In this proof-of-concept study, a recombinant yeast vaccine construct containing a 'reporter' molecule, ovalbumin (Ova), was evaluated as a mucosal vaccine delivery platform. Ovalbumin is the main protein found in egg white. It is a foreign protein for cattle and would be able to stimulate a specific immune response that can subsequently be measured in the laboratory using antibody-based or cellular assays. The study design incorporated groups of cattle given a non-transformed yeast (Control group), a single dose of the OvaYeast recombinant yeast vaccine (Singe dose group), or three doses (Multiple dose group) of the OvaYeast vaccine. The first dose was given to all animals on day 0 and the Multiple dose group received additional doses on days 6 and 13. The sampling time points refer to day 0 as the starting point and is defined as time 'post primary vaccination'. E.g. at one month post primary vaccination, the multiple dose group would have received its full complement of three vaccine doses 2 weeks prior.

The induction of cell-mediated memory responses by the OvaYeast vaccine was assessed by comparing the proliferation of circulating or lymph node lymphocytes in response to the reporter antigen (Ova), yeast or OvaYeast. The induction of a humoral (antibody based) immune responses was measured by enzyme linked immunosorbent assays (ELISA).

Vaccines protect against infection or disease by activating adaptive immune pathways (humoral and/or cell-mediated) and inducing memory of the pathogen. Upon vaccination, immune cells (lymphocytes) become activated and undergo proliferation resulting in clonal expansion of cells that are able to recognise the pathogen, or a component of the pathogen called an antigen. Some of these pathogen- or antigen-specific lymphocytes become long-lived memory cells. It is the presence of these cells that enables rapid reactivation of immune cells during subsequent pathogen exposure. If vaccine-mediated generation of immune memory has been successful, these cells can be reactivated under laboratory conditions (*in vitro*). When cells are cultured in the presence of vaccine antigens, vaccine-induced memory cells will become activated in a similar manner to when the animal is exposed to the pathogen and these parameters can be measured.

In this study, we used lymphocyte proliferation as a measure of cell-mediated memory responses. Lymphocytes consist of a diverse group of cell subsets originating from a common precursor; however, each subset can be identified by specific phenotypic and functional characteristics (Murphy et al. 2016). T lymphocytes express the CD3 molecule on the cell surface; these cells can be categorised into several subsets including cytokine-secreting T helper cells (CD4+) and cytotoxic T cells (CD8+). B cells mediate secretion of specific antibodies and can be characterised by cell surface markers such as CD21. $\gamma\delta$ T cells are a unique T cell subset which are rapid responders to infection. The molecule CD45RA is expressed on naïve T cells while antigen-activated memory T cells express the CD45RO isoform. We assessed proliferative responses of total lymphocytes as well as the lymphocyte subsets described here.

To detect lymphocyte proliferation, isolated cells were labelled with a tracking dye. This dye is readily able to enter cells. Viable cells process the dye into a form that is fluorescent and is unable to exit the cell. Therefore, viable cells can be detected as cells with high intensity fluorescence. When cells proliferate, the fluorescent intensity is halved in each subsequent generation and proliferation is associated with dimmer fluorescence. To identify proliferation in specific lymphocyte subsets, cell surface markers were identified using a separate fluorescent tag.

Induction of humoral adaptive immune responses by the OvaYeast vaccine was evaluated by detecting the presence of specific antibodies in serum as well as in intestinal mucus.

The immune changes associated with cellular activation that occur due to vaccination are also measurable at the level of gene expression. Essentially, gene expression is a measure of functional aspects of the genetic code written on our DNA. Gene expression studies enable us to determine when and where genes are turned on and off, enabling an understanding of an organism's response to changing conditions. Interferon-gamma (IFN-γ) is a key cytokine in the cell-mediated immune response. It is important for adaptive immunity, as triggered by a vaccine. The downstream actions of IFN-γ include increased antigen processing and presentation, upregulation of pathogen recognition, activation of microbicidal effector functions and antiviral responses, and leukocyte trafficking (Schroder et al. 2004). The chemokine CXCL10 is involved in T cell helper 1 mediated adaptive immune responses (Metzemaekers et al. 2018). Due to its functional properties, it was previously known as IFNγ-inducible protein 10 (IP-10). Tumour necrosis factor alpha (TNFα) and interleukin (IL)-10 and are two cytokines produced by macrophages, natural killer cells and dendritic cells (DC) that are associated with innate immune responses and antigen presentation to lymphocytes. TNF-α is associated with pro-inflammatory macrophage responses, whereas IL-10 is associated with anti-inflammatory responses (Benoit et al. 2008). Upon activation, antigen presenting cells such as macrophages, DCs and also B cells, upregulate the expression of the co-stimulatory molecules CD80 and CD86, which bind to CD28 on T cells (Sharpe et al. 2002). CD80-mediated co-stimulation preferentially favours cell-mediated immunity and T helper 1 cell differentiation. CD69 is a marker of lymphocyte activation and its expression on the cell surface is increased via transcriptional activation (Vazquez et al. 2009). CCR7, a lymphoid homing receptor present on the cell surface of lymphocytes, has been utilised in cattle as a marker of T central memory cell subsets (Maggioli et al. 2015); this cell type is induced in response to successful vaccination, reside in the lymphoid tissues and are rapidly activated in response to target antigens re-exposure.

4.3.1 Vaccine quality control and safety

Ovalbumin protein was confirmed to be expressed intracellularly in the yeast vaccine, using both Western blot analysis and 1D LCMS. The mass spectrometry sequence reads confirmed that the cloned ovalbumin protein sequence was in the top 10 'hits' from the analysis with the highest number of peptide spectral matches (PSMs), which is roughly proportional to the protein abundance in the sample.

There were no adverse signs observed following the oral dose of the vaccine in the two animals evaluated for vaccine safety.

4.3.2 Body weight

At the start of the trial the average body weight was 333.9 kg for the Control group, 326.5 kg for the Single dose group and 323.4 kg for the Multiple dose group. While time was a significant factor

(p<0.0001), there were no significant differences in body weight between treatment groups at any time during the trial (Fig. 3.3 and Appendix 3 Table 3.2.1).

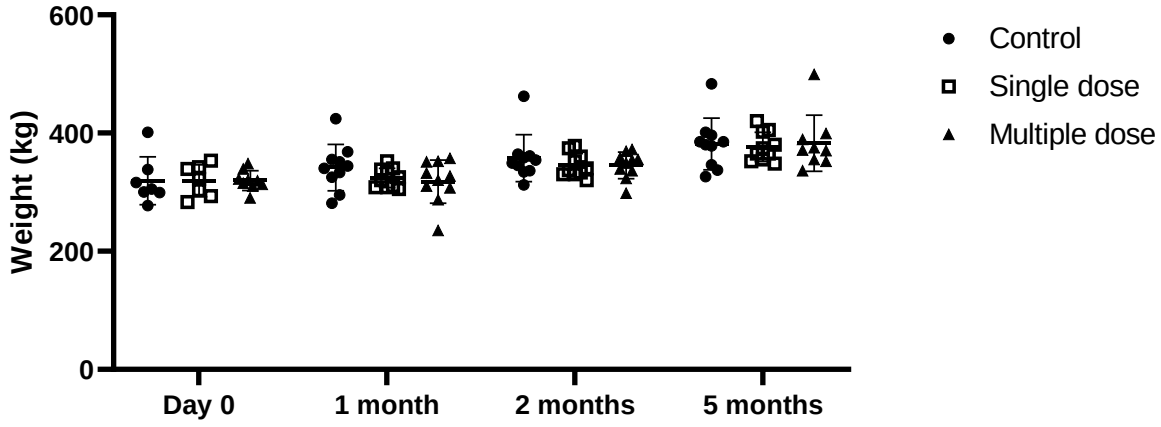


Figure 3.3 Body weights of animals during the trial
Animals in the treatment groups were weighed during the 4-month period of the trial. Control (one dose of nontransformed yeast), Single dose (one dose of OvaYeast vaccine) and Multiple dose (three doses of OvaYeast vaccine). n=10 per group

4.3.3 Preliminary investigations of lymphocyte proliferation

4.3.3.1 Comparison of lymphocyte phenotypes

The distribution of lymphocyte subsets in peripheral blood one month after the primary vaccine or control dose was given is shown in Fig. 3.4. As expected, the majority were CD3-expressing T cells. There was no difference in the proportion of lymphocyte subsets between control and vaccinated groups.

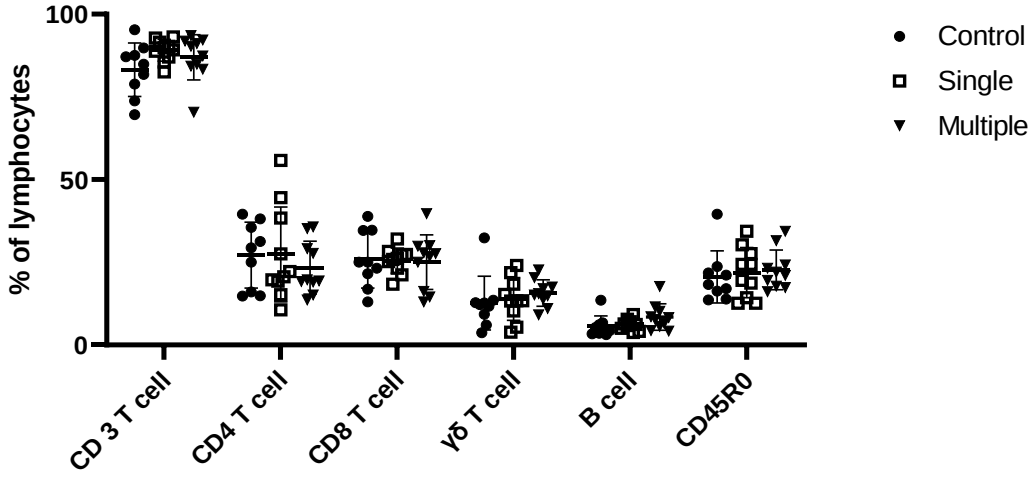


Figure 3.4 Distribution of lymphocyte subsets one month post primary vaccination

The proportion of lymphocyte subsets was determined by flow cytometry: CD3 (T cells), CD4 (T helper cells), CD8 (cytotoxic T cells), γδ T cells, B cells and CD45RO (memory cells). The treatment groups were: Control (one dose of nontransformed yeast), Single dose (one dose of OvaYeast vaccine) and Multiple dose (three doses of OvaYeast vaccine). n=10 per group

4.3.4 Lymphocyte proliferation responses

Proliferation assays assessed total lymphocytes as well as lymphocyte subsets and data were analysed for comparison of multiple stimulant groups within each treatment group. Several concentrations of each stimulant were included in the proliferation assay. The results did not always follow the same pattern for each treatment e.g. when there was significant proliferation to OvaYeast at an MOI of 1:1 proliferation in response to OvaYeast at 5:1 may have been similar to medium alone. All results are presented but interpretation of results is based on the response to a stimulant as a whole rather than to each concentration assessed.

ANOVA analysis was carried out using two models: 1) comparison of stimulant within each treatment group and 2) comparison of treatment groups for each stimulant.

4.3.4.1 Lymphocyte proliferation in peripheral blood at one month post primary vaccination

In all three treatment groups, the PWM response was significantly elevated ($p<0.01$) compared to the matched nontreated cells in Medium alone and there was no significant difference between treatment groups. This indicates that the assay was working, and that blood cells were viable and had the capacity to proliferate regardless of vaccination regime.

Overall, proliferative responses to all stimulants as well as to no treatment (medium alone) tended to be higher in the Control treatment group than in the two OvaYeast groups (Fig. 3.7). In the Control group, the response to yeast as well as to OvaYeast, but not to ovalbumin ($p>0.05$), was significantly ($p<0.05$) elevated (Fig. 3.7). The Control cattle were orally vaccinated with nontransformed yeast (i.e. without the reporter antigen Ova). In this group of cattle, there was no response to the reporter antigen ovalbumin which was not present in the 'sham' vaccine. It is likely that the lymphocyte proliferative response to yeast and OvaYeast stimulants reflects induction of immune memory to the yeast vector, whereby yeast is known to be immunostimulatory (hence it is of value as an adjuvant for oral vaccines).

In the Single dose treatment group, the response to the yeast (but not to ovalbumin or OvaYeast) stimulant was significantly higher ($p<0.05$) than to medium alone (Fig. 3.7), again indicating a response to yeast as immunostimulant. In the Multiple dose treatment group, the response to ovalbumin, yeast and OvaYeast all tended to be elevated ($p<0.1$) (Fig. 3.7). Overall, these results show that a repeated administration of OvaYeast (an overall greater quantity of OvaYeast) in the Multiple dose group has induced immune memory of exposure to yeast as well to the reporter antigen Ova.

The proliferative response to the stimulant OvaYeast tended to be higher ($p<0.1$) in the Control group compared to the Multiple dose group (Fig. 3.7). The reason for this effect is unclear.

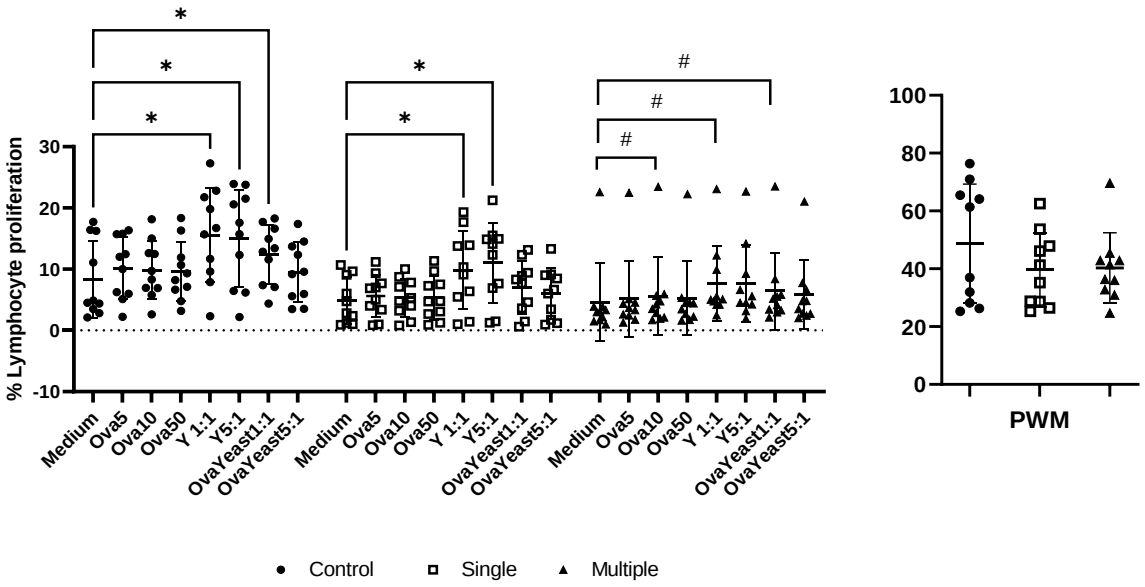


Figure 3.7 Total lymphocyte proliferation at one month post primary vaccination
Peripheral blood mononuclear cells were labelled with a fluorescent dye to track proliferation and cultured with a variety of stimulants: Ovalbumin (Ova) at 5-50 μg/mL, Yeast (Y) and OvaYeast at two MOI (1:1 and 5:1) or pokeweed mitogen (PWM; positive control for stimulation assays). Proliferation was assessed after 5 days of culture. The treatment groups were: Control animals (one dose of nontransformed yeast), Single dose (one dose of OvaYeast) and Multiple dose (three doses of OvaYeast) with n=10 per group. *p≤0.05 #p≤0.1

At this time point, i.e at one month post primary vaccination, for the CD3 T lymphocyte subset, data is available only for OvaYeast stimulant. OvaYeast induced significantly higher (p< 0.1) proliferation in the Single and Multiple dose treatment groups but not in the Control group (Fig. 3.8).

For the CD4 T cell subset, ovalbumin, yeast and OvaYeast stimulants all induced lymphocyte proliferation significantly (p<0.1) compared to Medium alone and the Single dose treatment group (Fig. 3.8). There were no significant differences in proliferation compared to Medium alone for any of the stimulants in the Control or Multiple dose groups for CD4 T cells. It is not clear why a single dose would induce a stronger response than multiple doses. To avoid loss of power or the risk of type II errors, we report results at p<0.10, so this result could potentially be a false positive (type I error).

CD8 T cell proliferation was significantly higher (p<0.1) in response to yeast compared to Medium alone (Fig. 3.8) in the Control treatment group. In the Single dose group, proliferation of this subset was significantly higher (p<0.1) in response to yeast and OvaYeast stimulants compared to Medium alone, whereas no significant responses were observed in the Multidose group. Results for the Control and Single dose groups suggest a response to yeast rather than to ova, but it is unclear why this result would not be replicated in the Multidose group.

B cells in animals from the Multidose group responded to the yeast stimulant but not to OvaYeast with a significantly (p<0.05) higher proliferative response than in the presence of medium alone (Fig. 3.85). The γδ T cell response to yeast stimulant was significantly higher in the Control (p<0.1), Single

($p<0.05$) and Multiple ($p<0.05$) dose treatment groups (Fig. 3.8). In the Single dose group this response was also significantly higher ($p<0.05$) in response to OvaYeast stimulant and in the Multiple dose group significantly higher ($p<0.1$) in response to OvaYeast as well as ovalbumin stimulants.

Proliferation of CD45RO lymphocytes was significantly ($p<0.1$) higher in response to ovalbumin, yeast and OvaYeast stimulants compared to medium alone for all three treatment groups (Fig. 3.8). The response to ovalbumin in the Control group is unexpected because control cattle were expected to be naïve to ovalbumin – a protein that is unlikely that pasture-raised cattle would have been exposed to. However, in hindsight, it would have been valuable to collect blood samples prior to vaccination.

A summary of significant differences in lymphocyte proliferation in response to stimulants compared to proliferation in medium alone for each treatment group is shown in Table 3.4. At one month after the primary dose was given, immune cell activation and memory cell generation to yeast and OvaYeast has occurred. CD4 and CD8 T cell activation is seen with the Single dose of the vaccine but not in the Multiple dose group. Induction of B cell responses is only seen in the Multiple dose group while induction of $\gamma\delta$ T and CD45RO lymphocyte responses are seen in both Single and Multiple dose groups. In the Control group, yeast stimulated $\gamma\delta$ T cell proliferation and ova stimulated CD45RO lymphocyte responses.

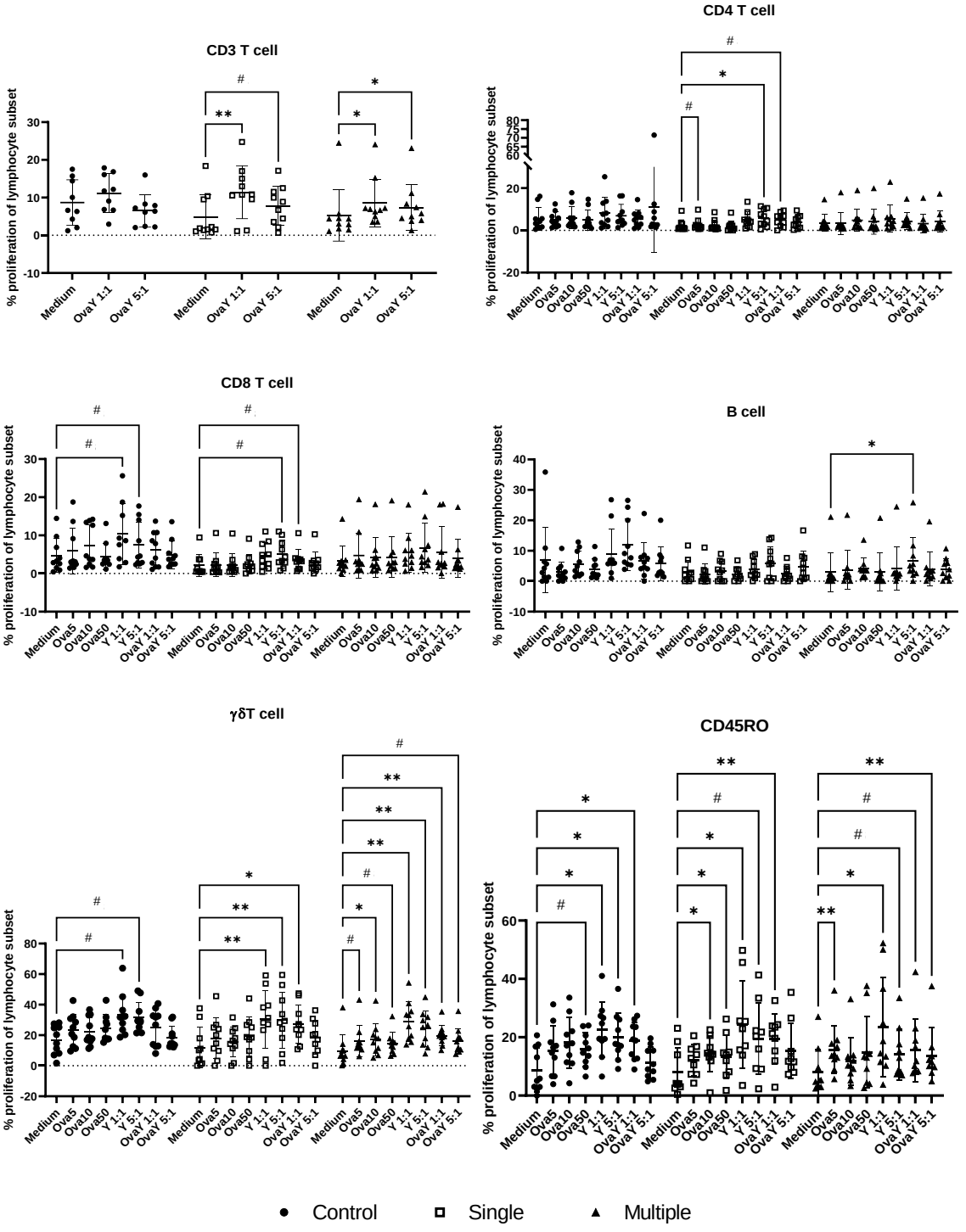


Figure 3.8 Lymphocyte subset proliferation at one month post primary inoculation with oral yeast vaccine

Peripheral blood mononuclear cells were labelled with a fluorescent dye to track proliferation and cultured with a variety of stimulants: Ovalbumin (Ova) at 5-50 μg/mL; Yeast (yeast) and OvaYeast at two multiplicities of infection (1:1 and 5:1). After 5 days of culture, cells were labelled to identify lymphocyte subsets prior to assessing proliferation. The treatment groups were: Control animals (one dose of nontransformed yeast), Single dose (one dose of OvaYeast) and Multiple dose (three doses of OvaYeast) with n=10 per group. **p≤0.01; *p≤0.05; #p≤0.1

Table 3.4 Summary of lymphocyte proliferation to stimulants within each treatment group at one month post primary vaccination

p values for statistically significant (p<0.1) responses which were higher compared to no stimulant (medium alone) for each treatment group are shown

Treatment group	Stimulant	Control	Single dose	Multiple dose
Total lymphocytes	Ova			0.073
	Yeast	0.011; 0.019	0.0401; 0.011	0.074
	OvaYeast	0.039		0.052
CD3 T cell	OvaYeast		0.004; 0.093	0.014; 0.024
CD4 T cell	Ova		0.0758	
	Yeast		0.0268	
	OvaYeast		0.0937	
CD8 T cell	Ova			
	Yeast	0.074; 0.132	0.0598	
	OvaYeast		0.0608	
B cell	Ova			
	Yeast			0.038
	OvaYeast			
γδT cell	Ova			0.074; 0.027
	Yeast	0.094; 0.085	0.003; 0.006	0.004; 0.0023
	OvaYeast		0.0108	0.008; 0.096
CD45RO	Ova	0.0971	0.026; 0.032	0.001
	Yeast		0.027; 0.070	0.024; 0.052
	OvaYeast		0.0036	0.053; 0.001

4.3.4.2 Lymphocyte proliferation in peripheral blood at two months post primary inoculation

Lymphocyte responses at two months after the first dose was administered to the treatment groups are shown in Fig. 3.9 and 3.10. The response to PWM in all treatment groups was significantly higher (p<0.1) than to medium alone and there were no significant differences between treatment groups. This indicates that blood cells were viable and had the capacity to proliferate regardless of vaccination regime.

In both OvaYeast treatment groups, total lymphocyte proliferation was significantly higher (p<0.1) in response to in vitro OvaYeast treatment (Fig. 3.9). The total lymphocyte response to ovalbumin stimulant in the Control treatment group was significantly lower (p≤0.1) than to medium alone.

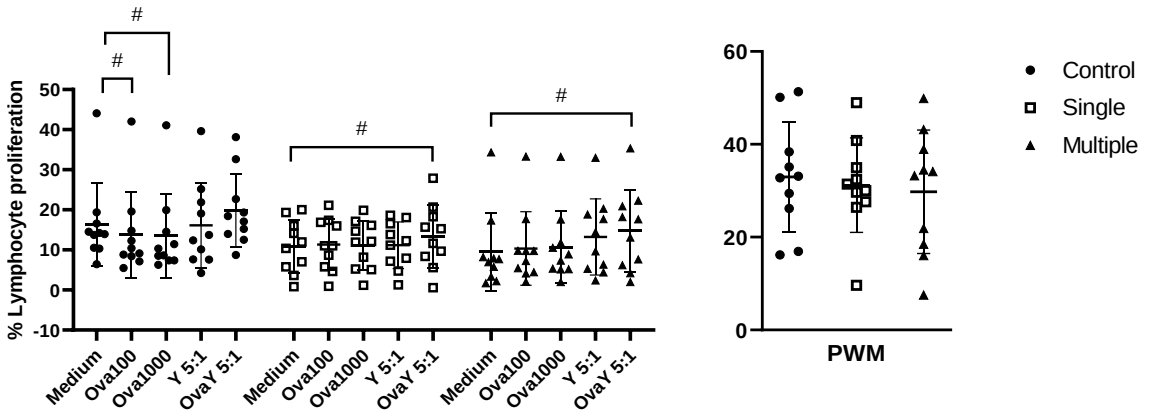


Figure 3.9 Lymphocyte proliferation at two months post primary vaccination
Peripheral blood mononuclear cells were labelled with a fluorescent dye to track proliferation and cultured with a variety of stimulants: Ovalbumin (Ova) at 100 and 1000 μg/mL; Yeast (Y) and OvaYeast (OvaY) at a multiplicity of infection of 5:1; pokeweed mitogen (PWM). Proliferation was assessed after 5 days of culture. The treatment groups were: Control animals (one dose of nontransformed yeast), Single dose (one dose of ova-yeast vaccine) and Multiple dose (three doses of ova-yeast vaccine) with n=10 per group. #p≤0.1

For any of the treatment groups, the CD3 T cell response to the stimulants yeast or OvaYeast was not significantly different ($p>0.1$) to medium alone (Fig. 3.10). The CD4 T cell response to both yeast and OvaYeast were significantly higher ($p<0.1$) compared to medium alone in the Multiple dose treatment group. Results were similar for the CD8 T cell response but only reached statistical significance ($p<0.1$) for the stimulant OvaYeast in the Multiple dose group.

The B cell response to the yeast stimulant was significantly higher ($p\leq0.1$) than medium alone in both the Control and Multiple dose treatment groups while response to OvaYeast was significantly higher ($p<0.05$) in the Single dose group compared to medium alone. The γδ T cell response to the OvaYeast stimulant was significantly higher ($p<0.1$) than medium alone in the Single dose treatment group. CD45RO proliferation was significantly higher in the OvaYeast treatment groups for Single ($p<0.01$) and Multiple dose treatment groups ($p<0.1$).

These results are summarised in Table 3.5.

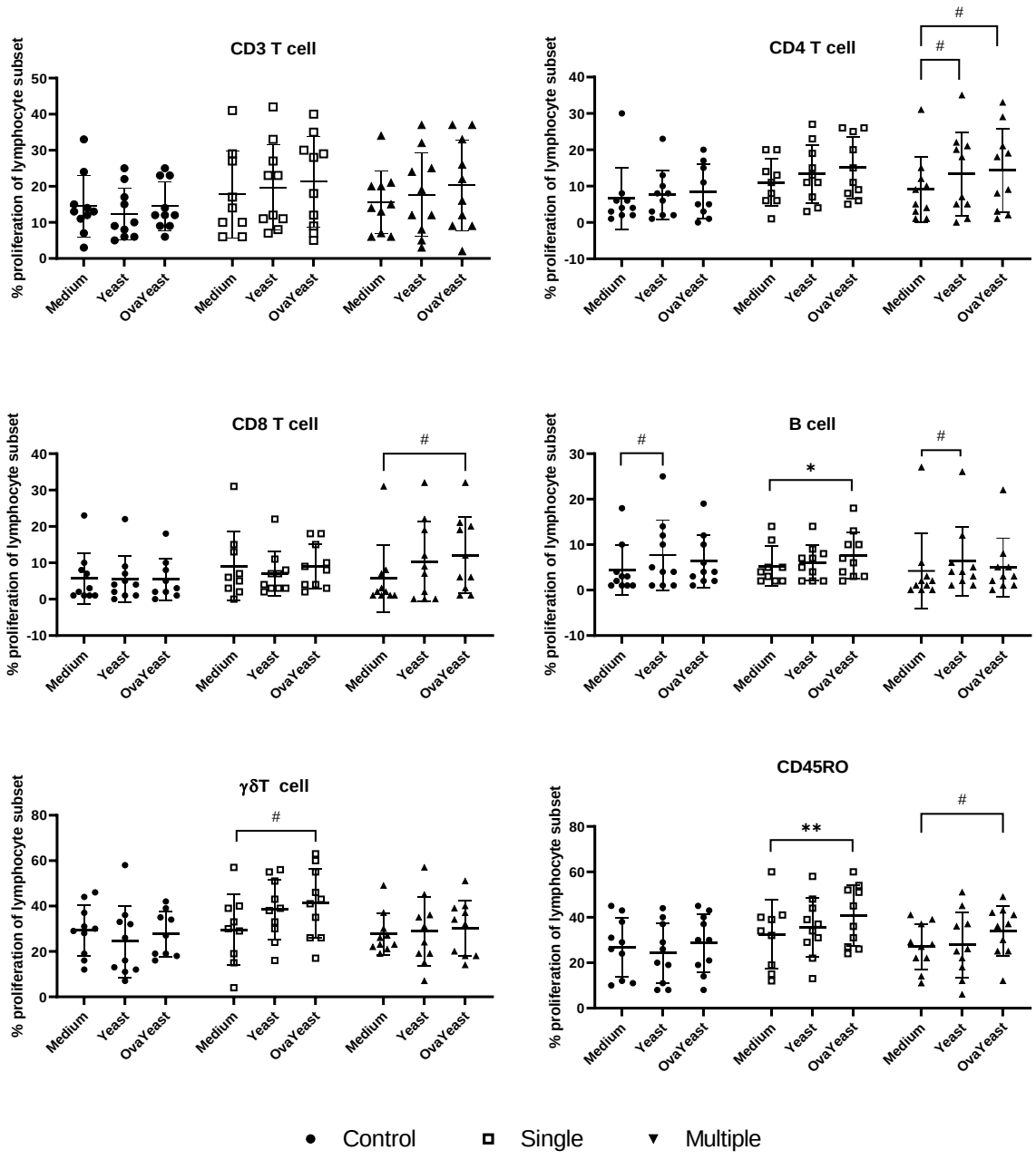


Figure 3.10 Lymphocyte subset proliferation at two months post primary vaccination
Peripheral blood mononuclear cells were labelled with a fluorescent dye to track proliferation and cultured a variety of stimulants: yeast (Yeast) and OvaYeast at a multiplicity of infection of 5:1. After 5 days of culture, cells were labelled to identify lymphocyte subsets prior to assessing proliferation. The treatment groups were: Control animals (one dose of nontransformed yeast), Single dose (one dose of ova-yeast vaccine) and Multiple dose (three doses of ova-yeast vaccine) with n=10 per group. **p≤0.05; *p≤0.05; #p≤0.1

Table 3.5 Summary of lymphocyte proliferation to stimulants within each treatment group at two months post primary vaccination

p values for statistically significant (p<0.1) responses compared to no stimulant (medium alone) for each treatment group are shown

Treatment group		Control	Single dose	Multiple dose
	Stimulant			
Total lymphocytes	Ova	0.0932*		
	Yeast			
	OvaYeast		0.053	0.088
CD3 T cell	Yeast			
	OvaYeast			
CD4 T cell	Yeast			0.069
	OvaYeast			0.088
CD8 T cell	Yeast			
	OvaYeast			0.069
B cell	Yeast	0.052		0.103
	OvaYeast		0.037	
γδT cell	Yeast			
	OvaYeast		0.079	
CD45RO	Yeast			
	OvaYeast		0.009	0.054

* significantly lower than medium alone

The overall pattern of peripheral blood lymphocyte proliferation responses seen at one-month post vaccination is reflected in the responses at two-months post vaccination. The ability of OvaYeast to activate lymphocyte proliferation in both Single and Multiple dose treatment groups suggests that the OvaYeast vaccine given to these animals have activated lymphocytes to retain memory of the vaccine. A two-months post vaccination, the memory responses are largely to OvaYeast rather than to yeast.

4.3.4.3 Lymphocyte proliferation in lymph nodes at five months post primary vaccination

To determine immunity at mucosal sites, tissue sections were taken at necropsy 5 months after the primary vaccination. Samples were taken from four tissue locations (retropharyngeal, ileal and jejunal lymph nodes (RLN, ILN, and JLN, respectively) and spleen. There was no difference in PWM-induced proliferation between treatment groups for the lymph nodes (Fig. 3.11) indicating that lymphocytes harvested from the tissues were viable and had the capacity to proliferation regardless of vaccination regime. Splenocyte proliferation was lower but this was due to lower proliferation capacity rather than a reduction in cell viability. Fig. 3.12 is a representative example of CFSE labelling in splenocytes which demonstrates that these cells are viable; cells have retained the dye CFSE (high CFSE) but there are few proliferating cells (dim CFSE).

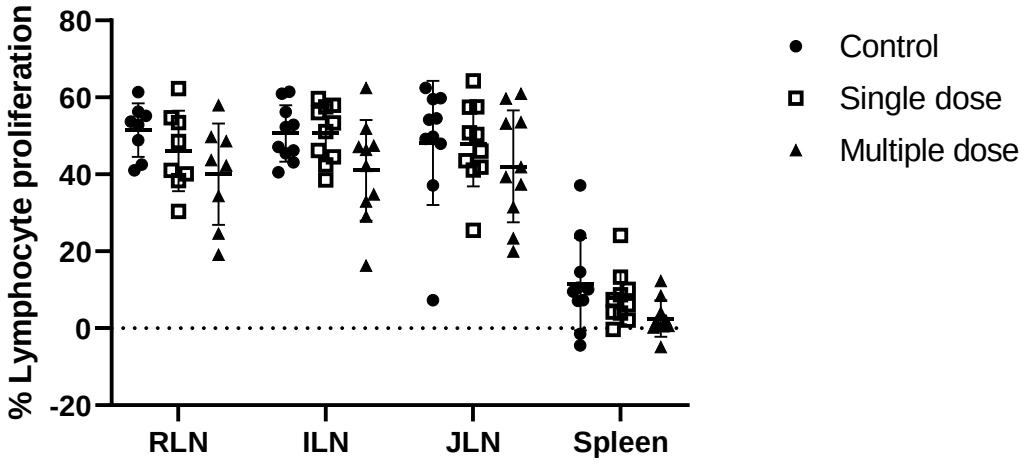


Figure 3.11 Proliferation of tissue lymphocytes in response to pokeweed mitogen (PWM).
Cells isolated from retropharyngeal lymph node (RLN), ileal lymph node (ILN), jejunal lymph node (ILN) and spleen were labelled with a fluorescent dye to track proliferation and cultured for 5 days with or without PWM. The treatment groups were: Control (one dose of nontransformed yeast), Single dose (one dose of OvaYeast vaccine) and Multiple dose (three doses of OvaYeast vaccine) with n=10 per group. Specific proliferation (medium alone subtracted) is shown.

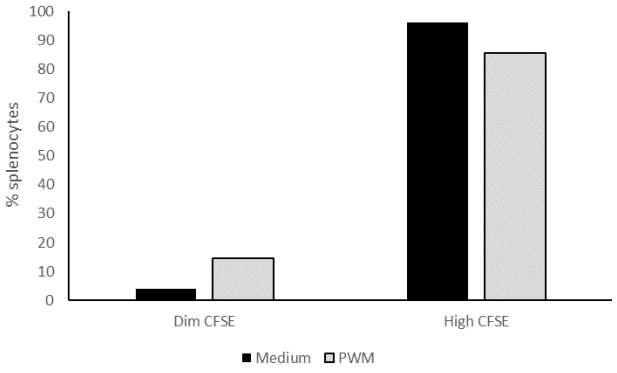


Figure 3.12 Example of splenocyte viability
Splenocytes were labelled with a fluorescent dye (CFSE) to track proliferation and cultured with pokeweed mitogen (PWM) or in medium alone and proliferation was assessed after 5 days of culture. CFSE fluorescence in splenocytes from one animal. Viable cells retain high CFSE fluorescence while proliferating cells lose CFSE intensity (dim CFSE) with each generation.

Proliferation of lymphocytes from tissues taken at necropsy in response to the stimulants yeast or OvaYeast is shown in Fig. 3.13. In the Control treatment group, the RLN proliferative response to stimulants yeast and OvaYeast were both significantly lower ($p<0.05$) compared to medium alone. The response in the Single dose treatment group was also significantly lower ($p<0.1$) to the OvaYeast stimulant. The response in the Control treatment group to yeast was significantly higher ($p<0.1$) compared to medium for splenocytes.

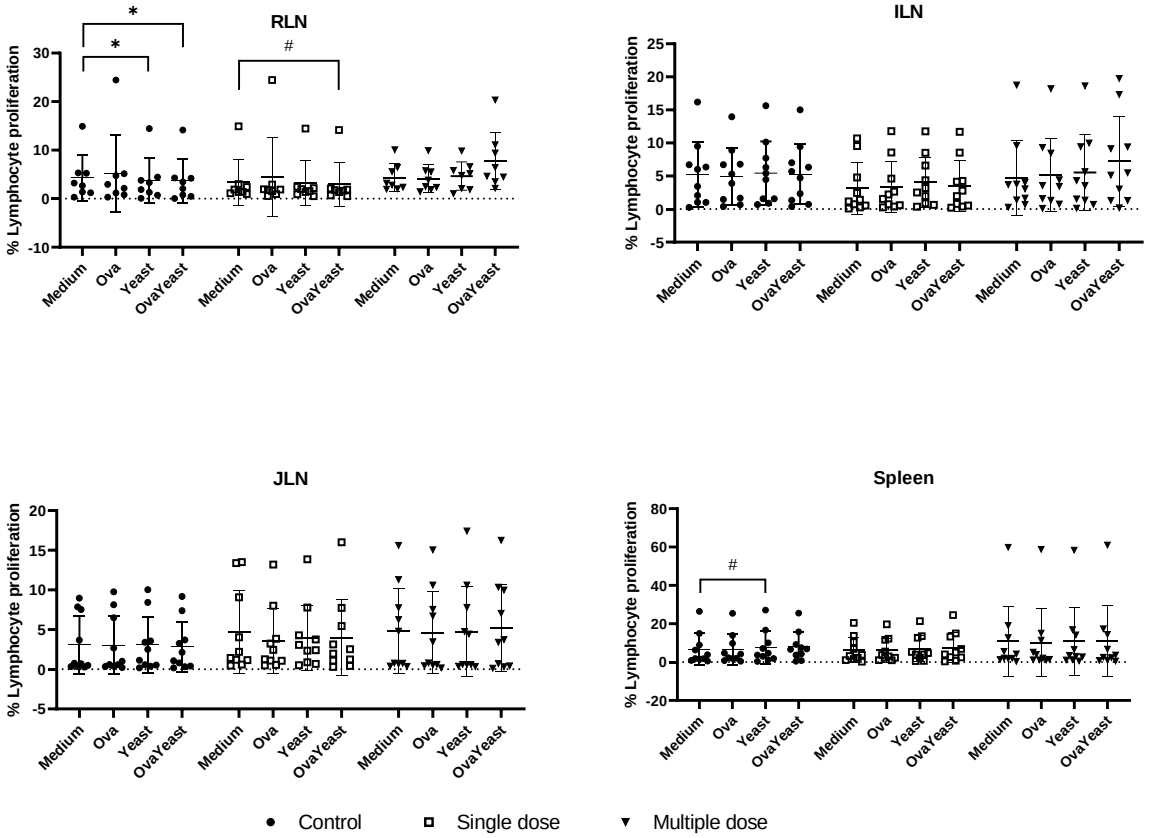


Figure 3.13 Proliferation of tissue lymphocytes in cattle given the yeast vaccine
Lymphocyte isolated from retropharyngeal lymph node (RLN), ileal lymph node (ILN), jejunal lymph node (ILN) and spleen were labelled with a fluorescent dye to track proliferation and cultured for 5 days with or without a variety of stimuli: ovalbumin (Ova), yeast, OvaYeast. The treatment groups were: Control (one dose of nontransformed yeast), Single dose (one dose of OvaYeast vaccine) and Multiple dose (three doses of OvaYeast vaccine) with n=10 per group

There were no significant differences ($p>0.1$) between yeast or OvaYeast stimulants for CD3 T cells, B cells or CD45RO cells compared to medium alone for RLN, ILN, JLN or splenocytes for any of the treatment groups (Fig. 3.14 and 3.15).

With the increase in the number of tissue samples selected for assessment at necropsy, the number of lymphocyte subsets analysed was reduced to enable sample processing and data acquisition without compromising sample quality. The CD3 T cell subsets CD4 and CD8 were not included and the immune memory cell marker CD45RO was included in preference to $\gamma\delta$ T cells.

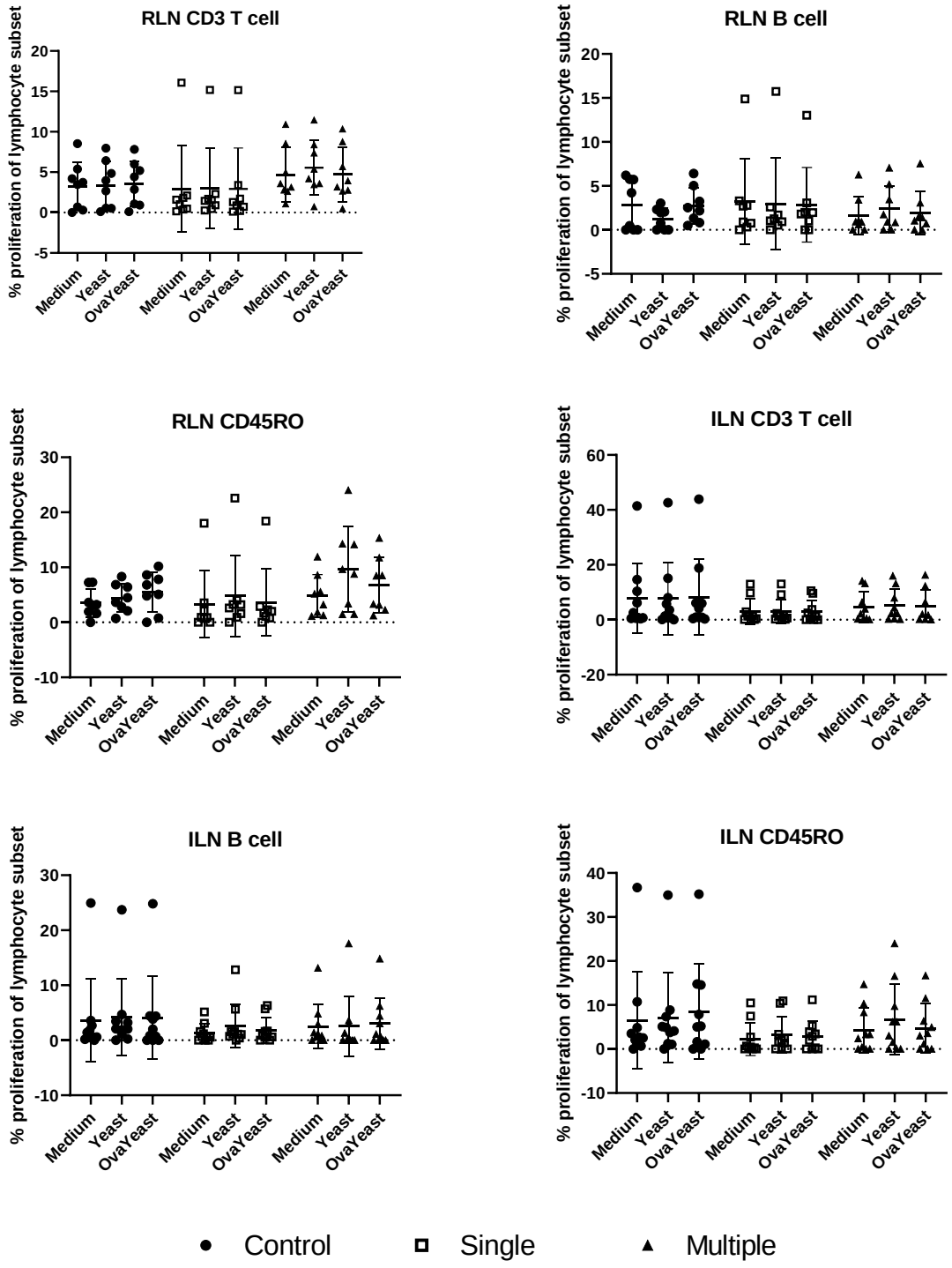


Figure 3.13 Proliferation of tissue lymphocyte subsets in cattle given the yeast vaccine
Lymphocytes isolated from retropharyngeal lymph node (RLN) and ileal lymph node (ILN) were labelled with a fluorescent dye to track proliferation and cultured for 5 days with or without yeast OvaYeast (stimulants) and labelled to identify T cells, B cells and CD45RO lymphocytes. The treatment groups were: Control (one dose of yeast), Single (one dose of OvaYeast) and Multiple dose (three doses of OvaYeast) with n=10 per group

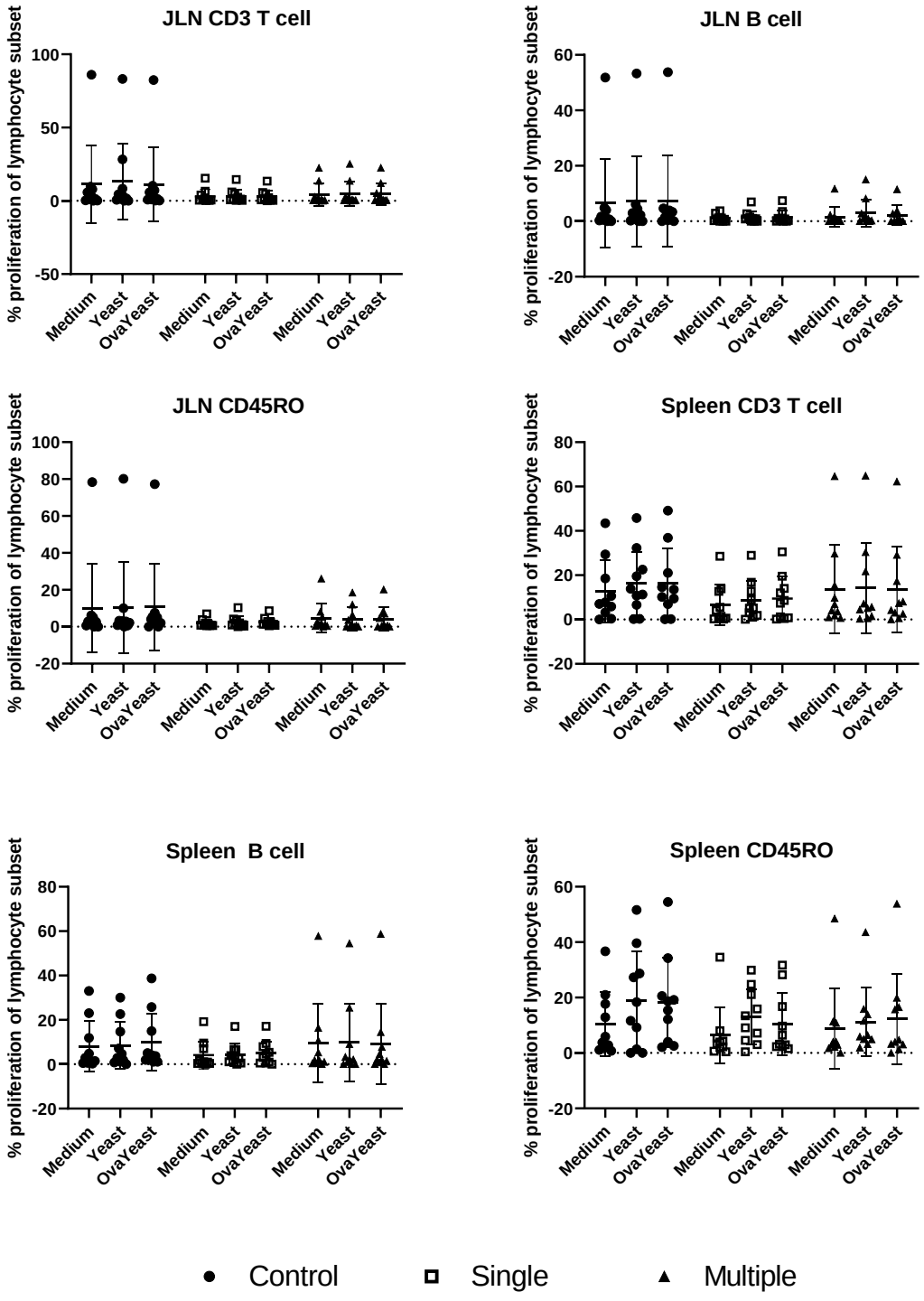


Figure 3.14 Proliferation of tissue lymphocyte subsets in cattle given the yeast vaccine
Lymphocytes isolated from jejunal lymph node (JLN) and spleen were labelled with a fluorescent dye to track proliferation, cultured for 5 days with or without yeast or OvaYeast (stimulants) and labelled to identify T cells, B cells and CD45RO lymphocytes. The treatment groups were: Control (one dose of yeast), Single (one dose of OvaYeast) and Multiple dose (three doses of OvaYeast) with n=10 per group.

Overall, we did not detect memory to yeast or OvaYeast in cells from the RLN, ILN, JLN or spleen despite detecting immune memory in circulating blood cells. The cattle intestinal system is lengthy, and we may not have sampled the specific sites of immune activation. At this time point, we did detect antibody responses in serum as well as in the mucus from the intestine (see Section 4.3.6).

4.3.5 Cytokine (interferon gamma) response

Induction of cell-mediated immune responses was also assessed by cytokine secretion, in this case, the cytokine interferon gamma. Cytokine responses in whole blood from the three treatment groups at one and two-months post primary vaccination is shown in Fig. 3.15. At both time points, interferon gamma responses were induced to yeast and OvaYeast stimulants in all three treatment groups. Similar to the lymphocyte proliferation responses it appears that immune memory to yeast has been induced supporting its potential value as an adjuvant for oral vaccines.

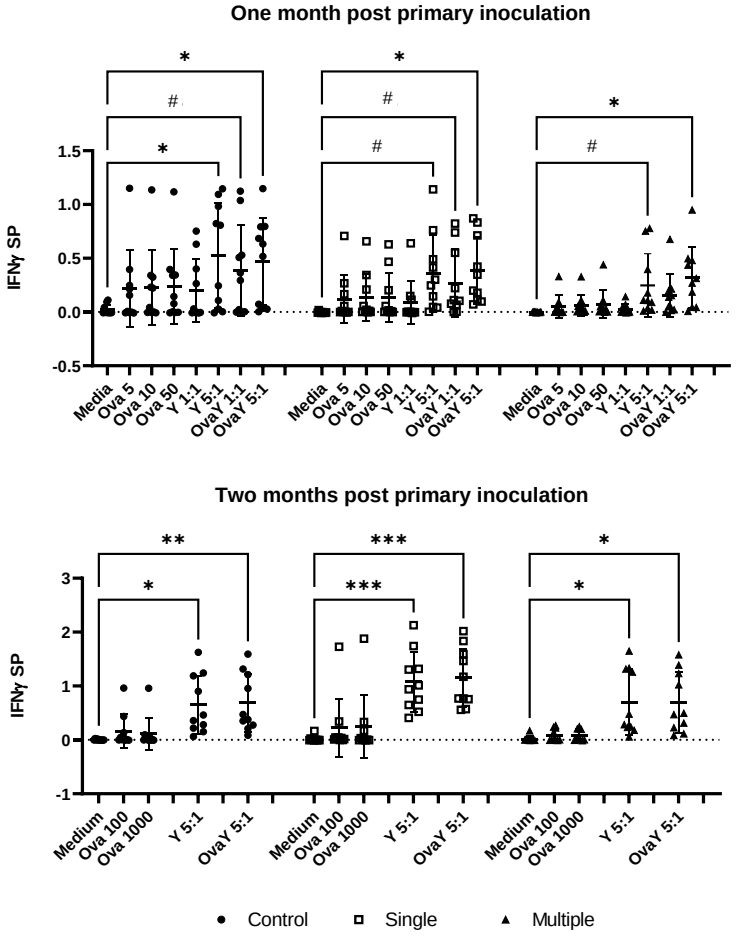


Figure 3.15 Interferon gamma secretion in whole blood
Whole blood taken at one- and two-month post primary vaccination was cultured with various stimuli (ovalbumin, Ova at 5-1000 μg/mL; lysed and intact yeast, Y; lysed and intact OvaYeast, OvaY; pokeweed mitogen) for 48 hrs. IFNγ in culture supernatants is shown. The treatment groups were: Control (one dose of nontransformed yeast), Single (one dose of OvaYeast) and Multiple dose (three doses of OvaYeast) with n=10 per group ***p<0.001; **p<0.01; *p<0.05; #p<0.1

4.3.6 Antibody responses

Presence of serum antibodies to ovalbumin and yeast and OvaYeast lysates was detected by ELISA, as a measure of activation of humoral immune pathways. Serum antibody levels at one, two and five months post primary vaccination are shown in Fig. 3.16. Similar protein concentrations were used for the coating the ova and yeast lysate antigens. While ovalbumin was a purified protein the two yeast lysates are a complex mix of yeast components thus the proportion of ova in the lysate protein and therefore coated on the plate is likely to be less than from the purified ovalbumin. This could be reflected in the overall higher ELISA SP for ovalbumin.

At one month post primary vaccination, the Multiple dose group had significantly higher ($p<0.05$) levels of antiOva antibodies compared to the Control group (Fig. 3.16). At five months post primary vaccination, antibodies to Ova and OvaYeast were higher ($p=0.082$ and $p=0.07$ respectively) in the Multiple dose group compared to the Control group.

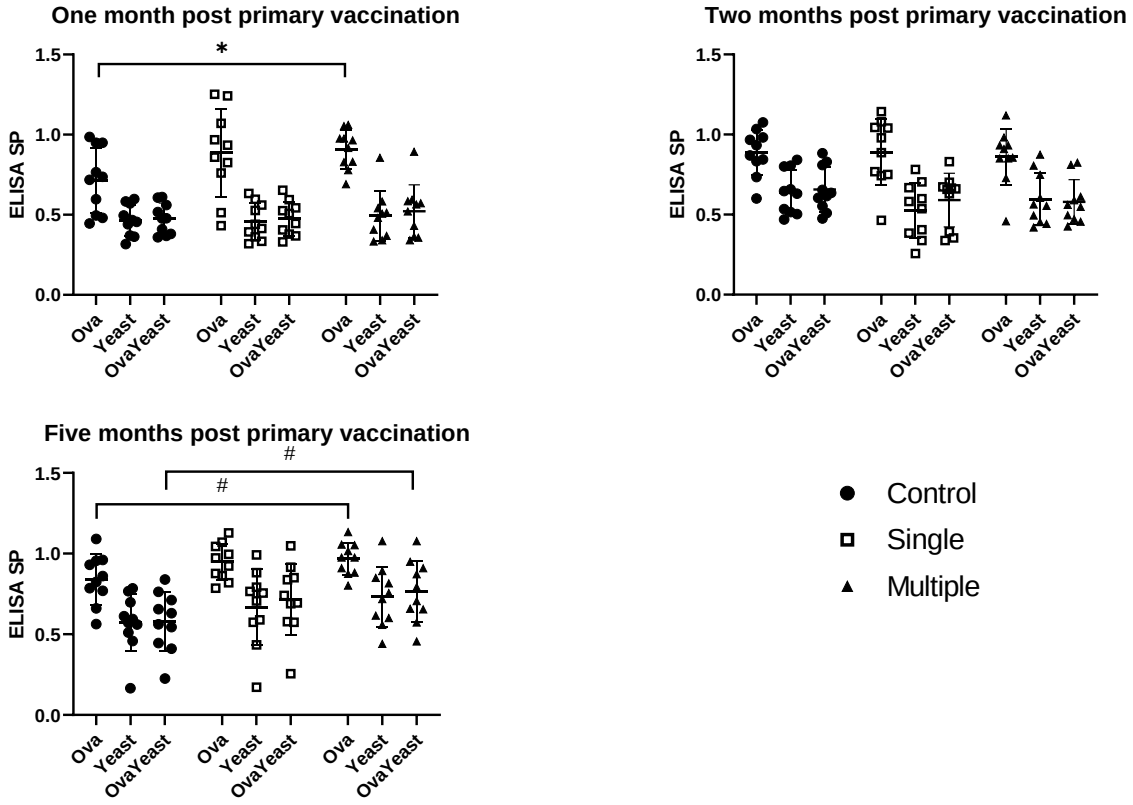


Figure 3.16 Serum IgG antibodies at three time points post primary vaccination
Serum samples were tested for antibodies to ovalbumin (Ova), lysed yeast and lysed OvaYeast. The treatment groups were: Control (one dose of nontransformed yeast), Single (one dose of OvaYeast) and Multiple dose (three doses of OvaYeast) with n=10 per group *$p<0.05$; #$p<0.1$

IgA antibodies to yeast and OvaYeast antigens in intestinal mucus samples taken at necropsy from the Multiple dose group was significantly higher compared to the Controls (Fig 3.17).

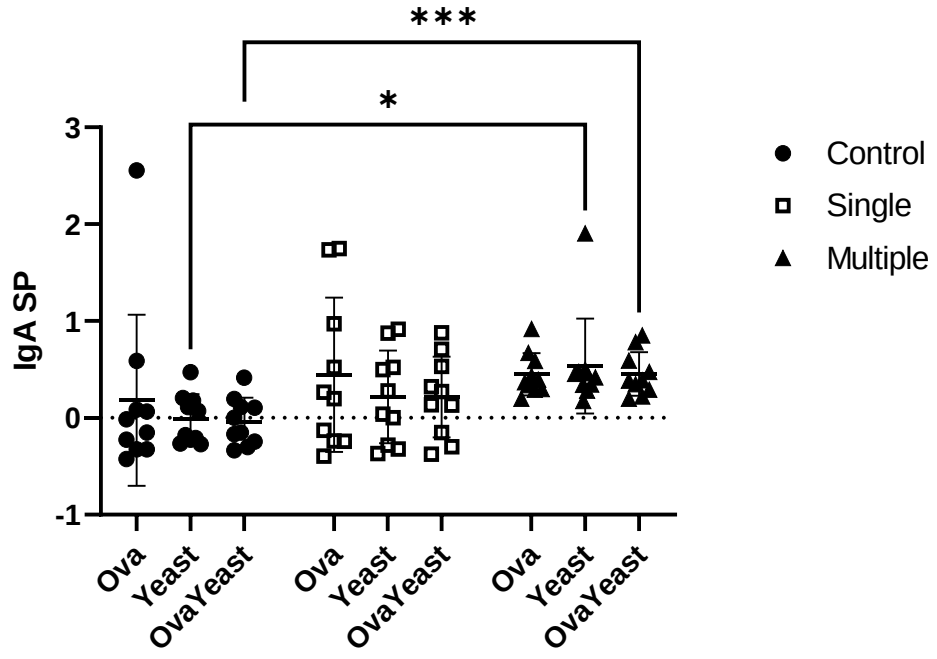


Figure 3.17 IgA antibodies in intestinal mucus 5 months post primary vaccination
Intestinal mucus samples were tested for IgA antibodies to ovalbumin (Ova), lysed yeast and lysed OvaYeast. The treatment groups were: Control (one dose of nontransformed yeast), Single (one dose of OvaYeast) and Multiple dose (three doses of OvaYeast) with n=10 per group ***p<0.001; *p<0.05

Significant IgG and IgA antibody results are tabulated in Appendix 3 Table 3.7.1A and B.

The overall cell-mediated and humoral immune responses in peripheral blood are summarised in Table 3.6. At one-month post vaccination it is clear that a humoral response to the reporter antigen in the vaccine (ovalbumin) has been induced in the Multiple dose group. The cell-mediated (proliferation and IFNγ) responses at this stage are more likely to be to the yeast component of the vaccine since both yeast and OvaYeast stimulants (but not ovalbumin) have generated recall responses in the Control as well as the Single and Multiple dose vaccine groups. This in itself is an important observation as it demonstrates that yeast, given orally, is an appropriate vehicle for delivering antigens that can stimulate immune pathways in cattle.

The lymphocyte proliferation response at the two-month time point provides evidence that a cell-mediated immune response has been induced to ovalbumin in both the Single and Multiple dose groups (Table 3.6). At this time point, recall of prior immune activation by the OvaYeast vaccine is evident in CD4+ helper T cells, CD8+ cytotoxic T cells in the Multidose group and in $\gamma\delta$ T cells in the Single dose group (Table 3.5). The proliferative capacity of CD45RO expressing cells to OvaYeast stimulant in both the Single and Multiple dose groups also demonstrates that the vaccine has been effective in inducing immune memory.

There was evidence of the initiation of an adaptive immune response, as evidenced by increased IFNγ gene expression within the LN of the animals in the Multiple dose group, though the expression

was only significantly different in the retropharyngeal LN. No other gene expression changes in the tissues at necropsy (5 months post vaccination) were significant.

The humoral response (serum IgG) to the reporter antigen lasts for at least five months post vaccination. At mucosal surfaces, IgA plays an important role in inhibiting adhesion of invading pathogens to epithelial cells. We show strong evidence of a mucosal IgA response triggered by yeast at the hypothesised site of vaccination induction (intestinal lining). It appears that while the lower dose of the vaccine given to the Single dose group is sufficient to induce cell-mediated immune responses, a higher dose was required for humoral responses.

The yeast vaccine does not alter weight gain in cattle; neither dose of the OvaYeast vaccine affected weight gain compared to animals given yeast alone. In summary, there is sufficient evidence to show that yeast-based vaccines given orally are capable of inducing both humoral and cell-mediated immune responses both systemic (in peripheral blood) and at mucosal sites (intestinal mucosal, retropharyngeal lymph node).

Table 3.6 Summary of significant immune responses to stimulants (rows) in the treatment groups (columns)

Time post primary vaccination	Immune response	Control	Single dose	Multiple dose	Interpretation
One month	Total lymphocyte proliferation	Medium <Yeast Medium < **OvaY**	Medium <Yeast	Medium<Yeast Medium<**OvaY**	Response to yeast?
	IFNγ	Medium <Yeast Medium < **OvaY**	Medium <Yeast Medium < **OvaY**	Medium<Yeast Medium<**OvaY**	Response to yeast?
	Antibodies			Anti-Ova antibodies greater than in Control group	Induction of ovalbumin specific humoral response
Two months	Total lymphocyte proliferation		Medium < **OvaY**	Medium<**OvaY**	Induction of ovalbumin specific cell-mediated response
	IFNγ	Medium <Yeast Medium < **OvaY**	Medium <Yeast Medium < **OvaY**	Medium<Yeast Medium<**OvaY**	Response to yeast?
	Antibodies			Medium<Yeast	
Five months	Total lymphocyte proliferation	Not done	Not done	Not done	NA
	IFNγ	Not done	Not done	Not done	NA
	Serum IgG antibodies			Anti-Ova and anti-OvaYeast antibodies greater than in Control group	Ovalbumin specific cell-mediated response present for 5 months

4.3.7 Gene expression

The ileal, jejunal and retropharyngeal LN from five animals were selected from the Control and the Multiple dose treatment groups as these potentially represented the groups with the most difference between treatments.

The expression of IFNγ was significantly elevated in the retropharyngeal LN of animals in the Multiple dose group (p=0.05), with a trend towards increased expression of IFNγ in the lymph nodes of animals in the multi dose group across all sites examined (Fig. 3.17). The other cytokines and chemokines analysed were not significantly different between the control and the multi dose group (Fig. 3.17).

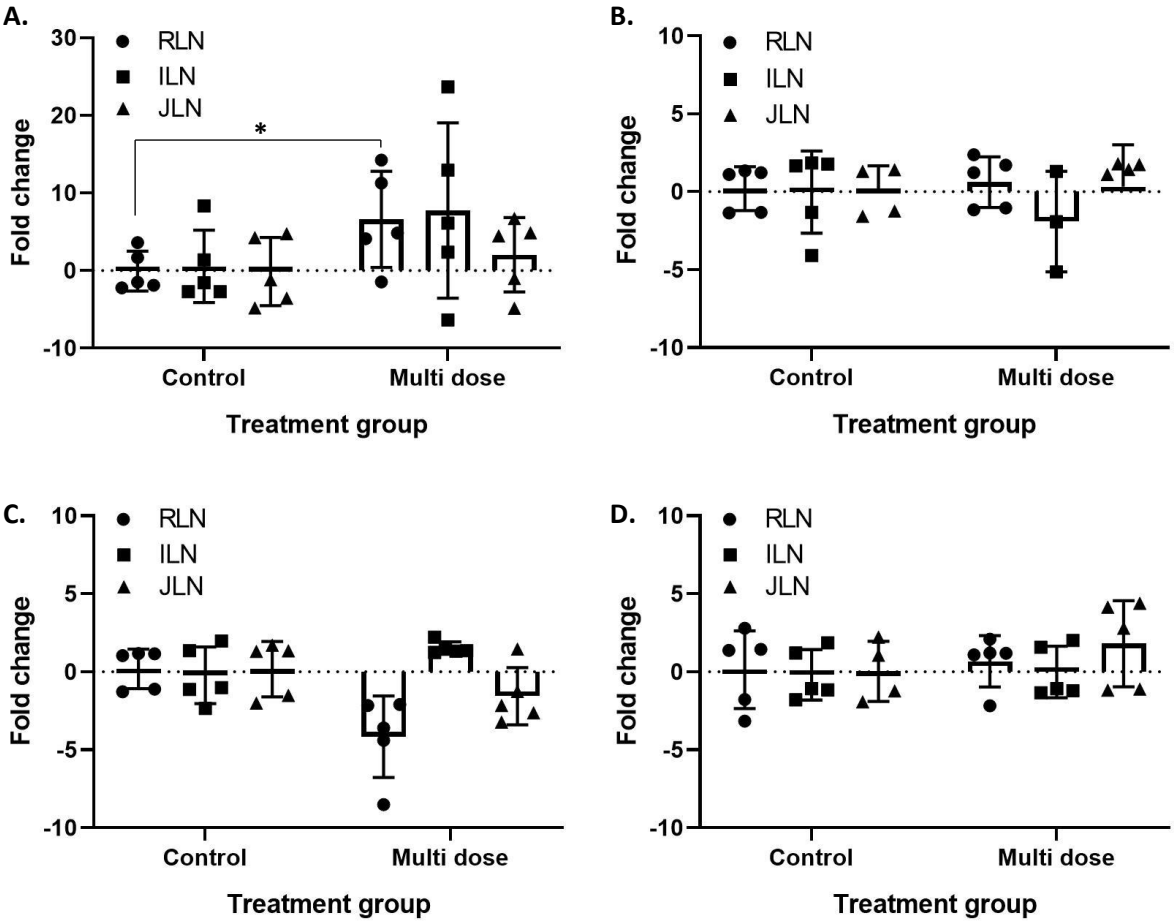


Figure 3.17: Individual animal fold change results for A. IFNγ, B. CXCL10, C. TNF-α and D. IL-10 gene expression in the retropharyngeal lymph node (RLN), ileal lymph node (ILN) and jejunal lymph node (JLN) (n=5/group), showing significantly (p=0.05) increased IFNγ in the RLN of the Multiple dose group compared to the Control group (n=5 per group).

4.4 Evaluate the ability of the candidate antigens to be recognised by immune cells sourced from BRD infected feedlot cattle

Detailed descriptions can be found in Appendix 4.

The suitability of peptide sequences from the cattle pathogen BVDV for incorporation into future vaccines was determined. Peptides are short protein segments and can act as antigens, i.e. induce an immune response. To assess antigenicity, the ability of antibodies in cattle sera to recognise the selected BVDV peptides was determined by ELISA (antibody ELISA). To assess immunogenicity, the ability of these peptides to induce secretion of the cytokine IFNγ by peripheral blood cells (cell-mediated immunity) was assessed.

4.4.1 Blood samples

Blood samples feedlot cattle (n=70) were provided by Dr Tony Batterham and from NSW commercial beef properties by Dr Karren Plain (Table 4.1).

All cattle were categorised based on a positive or negative result for BVDV using a commercially available ELISA. A positive result indicates the presence of antibodies to BVDV but is not definitive for disease. There is no data on health history for animals that were sampled with the exception of the 10 animals from the Quirindi case-control study. BVDV ELISA results for animals are shown in Table 4.1. Positive and negative cut-offs were as recommended by the BVDV ELISA manufacturer.

4.4.2 Antibody ELISA

Antibody ELISA results are shown in Fig. 4.2. The outlying data points were mainly due to the same animals across all peptides. There was one farm where the cohort of 12 animals tested were all BVDV ELISA positive and two farms where the 12 animals all tested negative. Therefore, it was unsurprising that farm of origin was a significant factor for all peptides when subjected to Linear Mixed Model analysis where farm was included as a fixed effect ($p<0.05$).

Without accounting for farm, i.e. based on the t-test, there were no statistically significant differences between animals that were BVDV ELISA positive or negative for any of the peptides (Fig. 4.2). When farm was included as a factor in the Linear Mixed Model, there were significant differences between commercial BVDV ELISA positive and negative groups for peptide 6 and 7 ($p<0.05$) and differences approached significance for peptide 8 ($p=0.06$), with higher values in BVDV-positive samples, indicating that these 3 peptides were antigenic.

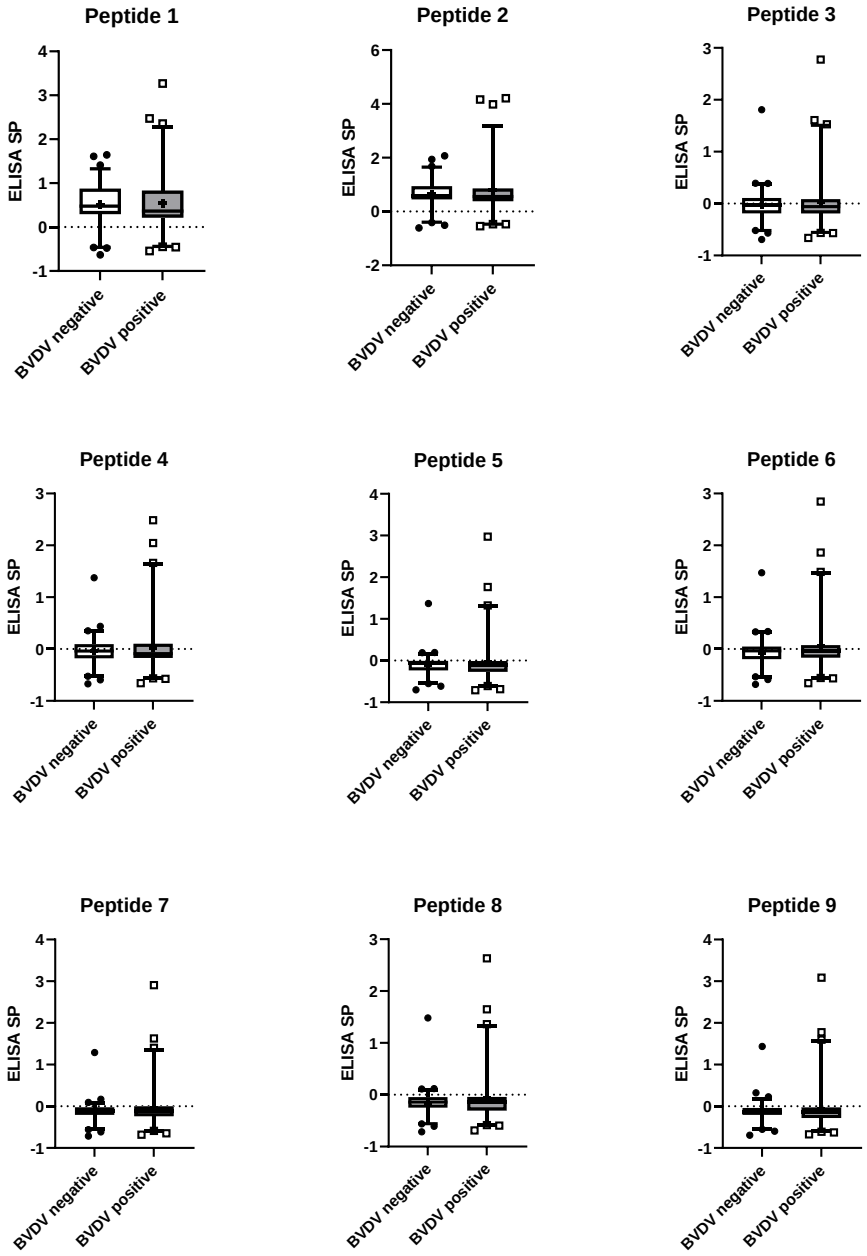


Figure 4.2 Presence of peptide-specific antibodies in BVDV ELISA positive and negative cattle
Values in the 25th to 75th percentile are shown in the box with the median marked by a line. The mean is marked as + within the box. The range ("whiskers") indicates values within the 5-95th percentile.

When the antibody ELISA results for all 9 peptides were combined, the BVDV ELISA positive group had significantly higher antibody levels compared to the BVDV ELISA negative group ($p<0.01$) (Fig. 4.3), albeit with a bimodal distribution in both groups.

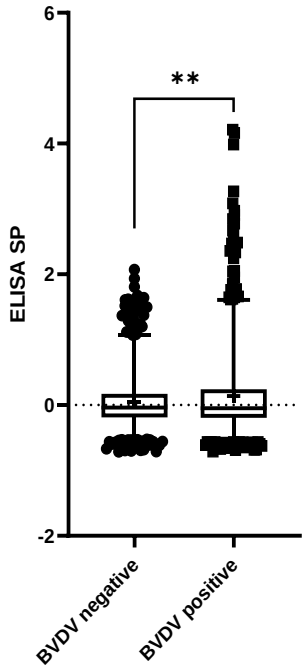


Figure 4.3 Collation of antibody ELISA results for all peptides
Values in the 25th to 75th percentile are shown in the box with the median marked by a line. The mean is marked as + within the box. The range indicates values within the 5-95th percentile **p<0.01

4.4.3 Cytokine ELISA

Induction of the inflammatory cytokine IFNγ by blood cells in response to BVDV peptides was assessed in cattle that were either BVDV ELISA positive or negative (Table 4.1). Tests for cell-mediated immune responses require viable cells. This requires rapid transport of blood tubes from the site of collection to the lab. This meant that a smaller sample set was tested for cell-mediated immune responses.

The blood samples for cell-mediated immune response studies were in good condition as shown by the elevated responses to the nonspecific mitogen PWM in Fig. 4.4.

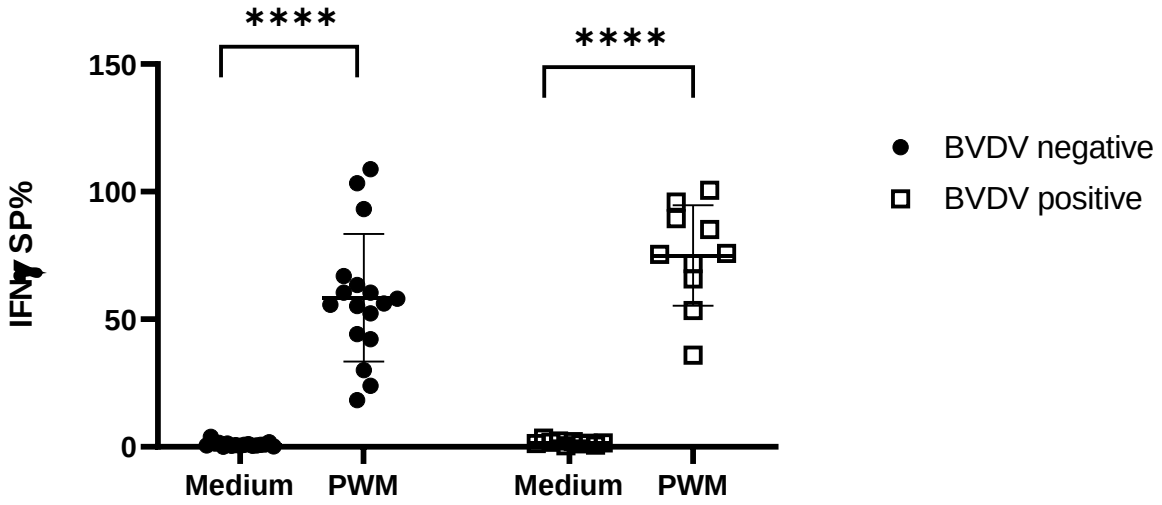


Figure 4.4 Blood cells from cattle were responsive to a mitogen
Whole blood was incubated with no stimulus (Medium) or with PWM. IFNγ in culture supernatants was measured by ELISA ****p<0.0001

The IFNγ response to stimulation by BVDV peptides was not significantly different between the BVDV ELISA positive and negative groups (Fig. 4.5). Outliers with high SP% are consistently associated with the same animals.

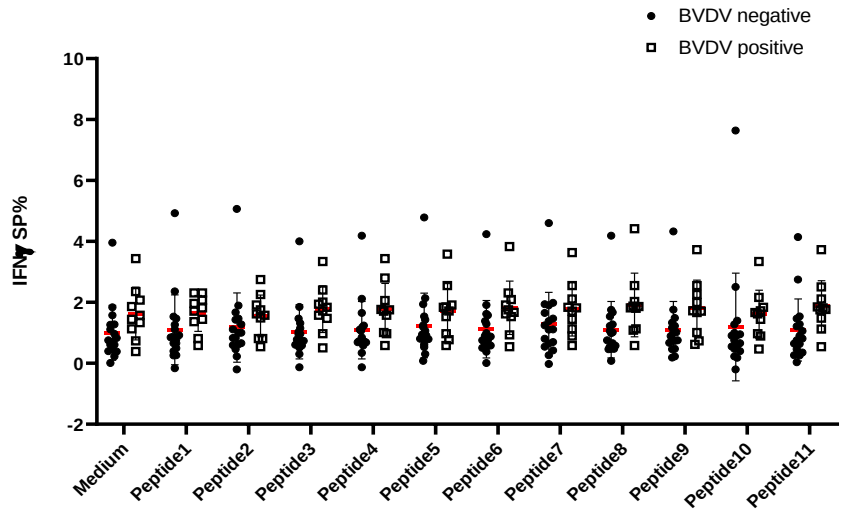


Figure 4.5 Cell mediated immune response to BVDV peptides
Whole blood from cattle was incubated with no stimulus (Medium) or with one of eleven BVDV peptides. IFNγ in culture supernatants was measured by ELISA. The red line indicates the mean.

When the results for all peptides was combined there was no significant difference between the two BVDV ELISA groups (p>0.05).

4.5 Produce and deliver an oral yeast-based prototype vaccine that contains the candidate BRD antigen/s

Detailed descriptions can be found in Appendix 5.

4.5.1 Recombinant OvaYeast

4.5.1.1 Confirmation of successful transformation

Fig. 5.3.1 shows growth of *K. lactis* on selective media; suggesting successful transformation.

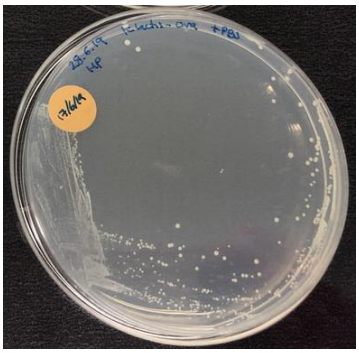


Figure 5.3.1. Growth of successfully transformed OVA-yeast colonies on selective media

Confirmation of successful transformation of *K. lactis* yeast with the *Ova* gene is shown in Fig. 5.3.2. The quantitative PCR amplification plot (Fig. 5.3.2A) shows the amplification of DNA extracted from a successfully transformed *K. lactis* colony, two positive controls (*Ova* gene in E. coli plasmid; *Ova* gene in *K. lactis* plasmid) and a negative control. The (cycle threshold) CT value in the amplification plot directly relates to the quantity of target DNA in the sample. The two positive control samples had a higher level of initial target DNA input, as it was directly extracted from the plasmid, compared to the transformed *K. lactis* colony, explaining the shift in CT value in the yeast isolate.

The dissociation curve (Fig. 5.3.2B) further confirms successful integration of the *Ova* gene into the yeast genomic DNA. DNA amplified from the transformed yeast colony has the same melt temperature (directly related to amplification fragment length) as the two positive controls. Non-transformed *K. lactis* yeast does not contain the ova gene or any sequences with similarity to the primer binding sites and shows no amplification in this qPCR (data not shown).

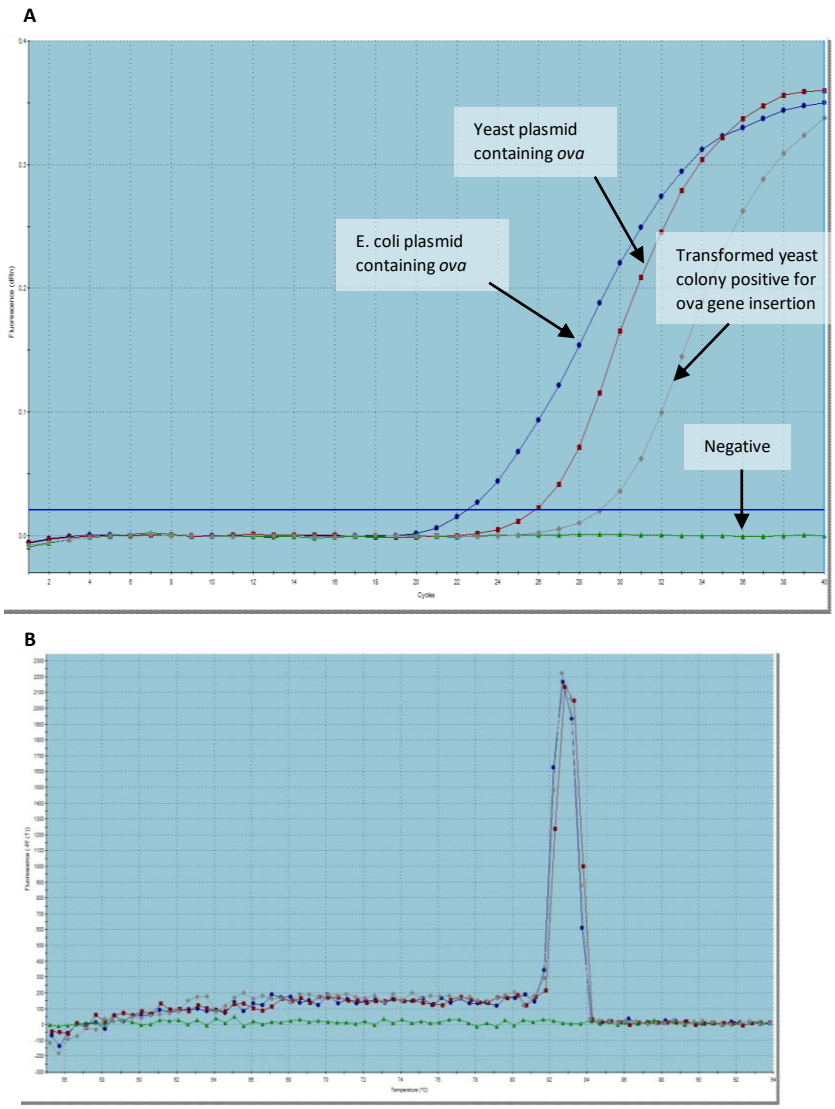


Figure 5.3.2 qPCR amplification plot and dissociation curve showing successful transformation of *K. lactis* yeast with an ova gene.
DNA was extracted from transformed *K. lactis* colonies following growth on selective media and analysed with qPCR to confirm integration of the ova gene. (A) Amplification of two positive controls for the ova gene, sample from a transformed *K. lactis* colony and a negative control containing no template DNA. (B) Integration of the correct fragment is confirmed in the melt analysis. Similar colour scheme to (A) is used for samples.

4.5.2 Confirmation of ovalbumin expression by recombinant OvaYeast

Evidence of integration and expression of the ovalbumin protein within the OvaYeast vaccine was obtained by Western Blot analysis. Fig. 5.3.3 shows appropriate sized bands (indicated by blue arrows) in one representative sample of protein expression from lysates prepared from a positive ovalbumin control (A5503 Albumin from chicken egg white, Sigma), a yeast control (nontransformed yeast Y238) and the OvaYeast vaccine (Y234).

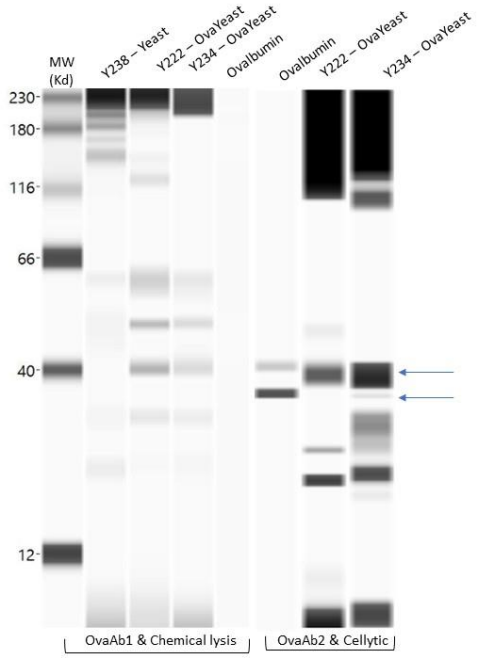


Figure 5.3.3 Evidence of protein expression was assessed by Western Blot.

Presence of ovalbumin in unmodified yeast and OvaYeast vaccine lysates were assessed by Western blot. Samples were subjected to either chemical (Fig. 5.3.3, left panel) or enzymatic (Fig. 5.3.3., right panel) lysis. The digital image (Figure 5.3.3) illustrates the presence of doublet bands (indicated by blue arrows) within the OvaYeast vaccine lysates that align to the doublet band produced by the ovalbumin positive control. This band is not replicated in the unmodified yeast lysate (Y238) however there is evidence of a band of a different (larger) size with unknown attribution within the unmodified yeast. Doublet ovalbumin bands may be due to the presence of ovalbumin splice variants and isoforms within the protein preparation and is commonly reported for this protein.

Mass spectrometry methods can be applied in metaproteomic studies of microbes (bacteria and yeast) to identify the proteins present in a sample (Hinzke et al. 2019). The workflow involves generation of a protein mixture from the sample that is digested into peptides, which are analysed in a mass spectrometer and then screened against a database using a dedicated search engine (e.g. MASCOT).

The results of the top 10 protein hits (excluding keratin) are shown. Ovalbumin was confirmed to be present in the yeast vaccine sample tested and was the most common protein hit as shown the table below.

Top ten protein hits for the OvaYeast vaccine (Y222, CelLytic lysis)

Accession	Description	Sum PEP Score	Coverage [%]	# Peptides	MW [kDa]	Score Mascot	Gene Symbol
OVA	Ovalbumin sequence	472.717	87	32	42.9	8349	OVA
Q70CP7	Kluyveromyces lactis Enolase	105.132	73	24	46.5	1210	ENO
Q6CQJ3	Kluyveromyces lactis Peptidyl-prolyl cis-trans isomerase	70.409	71	10	17.3	932	

P14828	Kluyveromyces lactis Phosphoglycerate kinase	45.325	42	10	44.5	297	PGK
Q6CNK3	Kluyveromyces lactis Phosphoglycerate mutase	48.09	51	12	27.5	487	
P20369	Kluyveromyces lactis Alcohol dehydrogenase 1	38.416	30	11	37.2	302	ADH1
P17819	Kluyveromyces lactis Glyceraldehyde-3-phosphate dehydrogenase 1	47.895	44	14	35.3	421	GAP1
P49383	Kluyveromyces lactis Alcohol dehydrogenase 2	20.386	19	6	37.1	200	ADH2
Q6CR18	Kluyveromyces lactis 60S ribosomal protein L36	23.202	49	6	11.1	268	
O13350	Kluyveromyces lactis ATP synthase subunit d, mitochondrial	37.995	49	8	19.7	462	ATP7

4.5.3 Recombinant antigen (BVDV) yeast

4.5.3.1 Screening of putative recombinant antigen-yeast

Recombinant antigen yeast clones were screened by qPCR (Table 5.3).

Table 5.3 Recombinant antigen yeast clones screened by qPCR, gene expression and/ or protein secretion

Region	No. screened with qPCR for DNA integration	No. confirmed for DNA fragment	No. screened for expression of antigen gene	No. positive for gene expression	No. screened by WES for secreted antigen	Secreted antigen present by western blot
1 extracellular expression	121	0	0	ND	0	ND
1 intracellular expression	141	0	4	0	4	3
2a extracellular expression	96	5*	0	ND	0	ND
2a intracellular expression	96	0	0	ND	0	ND
2b extracellular expression	65	0	0	ND	0	ND
2b intracellular expression	55	0	4	0	4	2
3 extracellular expression	96	4†	0	ND	0	ND
3	96	0	0	ND	0	ND

intracellular expression						
4 extracellular expression	96	2**	0	ND	0	ND
4 intracellular expression	96	0	0	ND	0	ND

* Three of these were pooled samples and 2 were individual clones. † Two of these are pooled samples and 2 were individual clones. **Both of these were pooled samples. ND=Not done.

There were a number of clones that showed evidence at the DNA level of the presence of the cloned gene in the qPCR screening (Figures 5.3.1 to 5.3.3). An example of qPCR results on positive pools (Figure 5.3.1, 5.3.2) and one individual clone (clone 595) from a positive pool transformed for extracellular secretion of antigen Region 2a (Figure 5.3.2) are shown. The Ct values were >30; a late Ct value often results from an artifact; however, the correct melt curve temperature was confirmed for each assay, suggesting this is consistent with the target region but a low number of copies. The copy number was lower than anticipated for clonal isolates, as a single copy *K.lactis* gene gave Ct values of 24 ± 1.6 (Mean ± SD) across all clones tested.

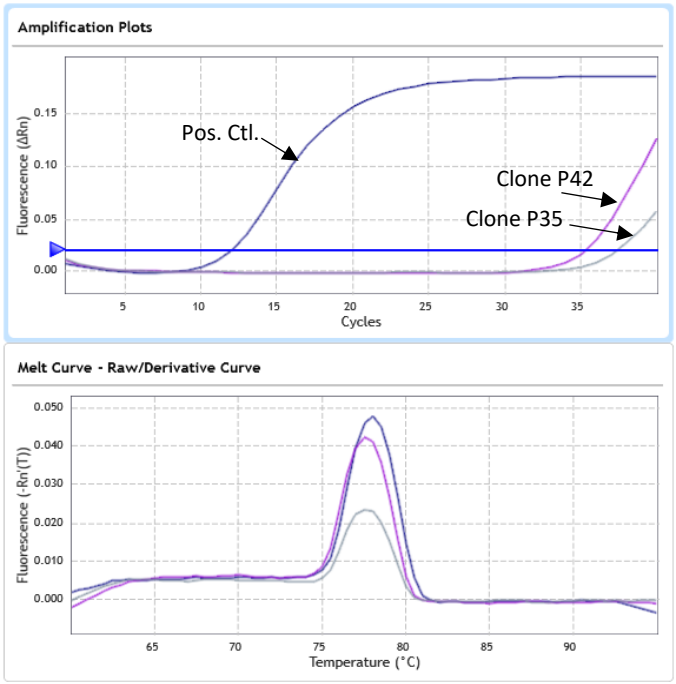


Fig. 5.3.1. Positive cloned yeast (pooled) by qPCR for Region 2a. Upper panel shows the amplification curves and the lower panel shows that the amplified product melt curves are consistent with the positive control (Pos. Ctl.).

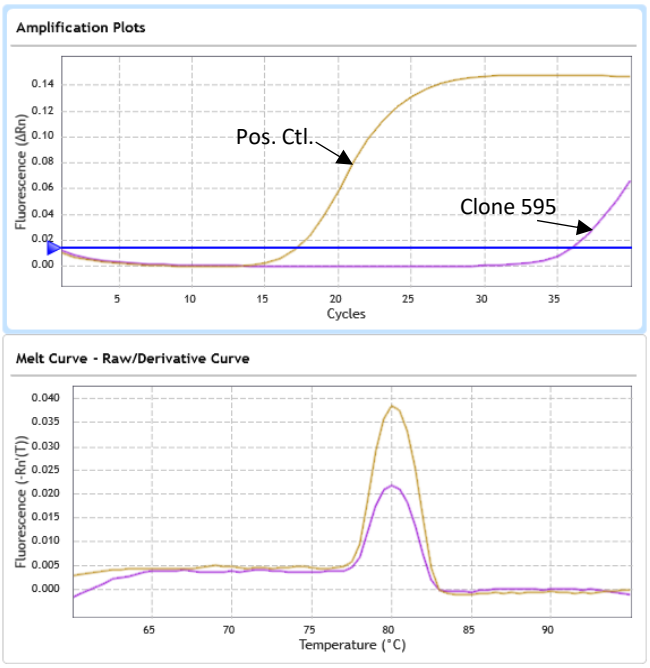


Fig. 5.3.2. Positive cloned yeast (individual) by qPCR for Region 2a. Upper panel shows the amplification curves and the lower panel shows that the amplified product melt curve is consistent with the positive control (Pos. Ctl.).

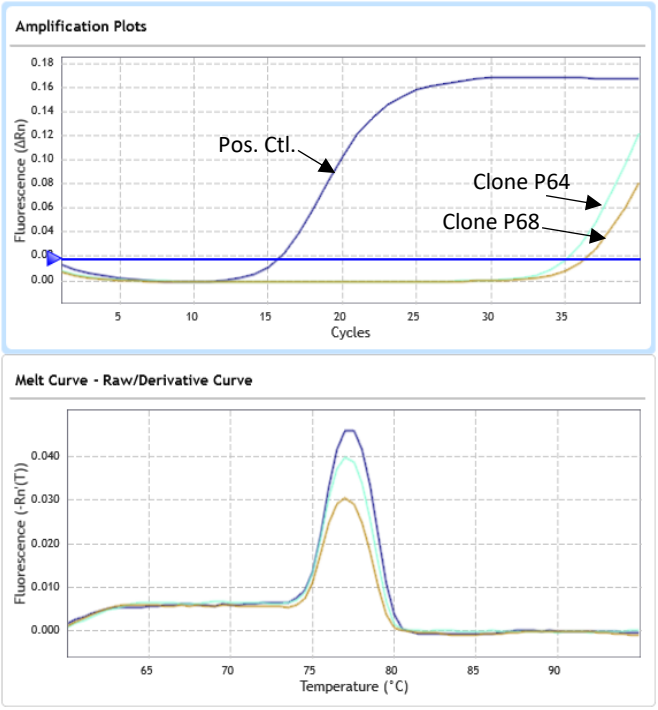


Fig. 5.3.3. Positive cloned yeast by qPCR for Region 3. Upper panel shows the amplification curves and the lower panel shows that the amplified product melt curves are consistent with the positive control (Pos. Ctl.).

4.5.3.2 Gene Expression analysis on clones with evidence of protein expression

RNA was extracted from several clones that were negative by qPCR on the extracted DNA but showed presumptive evidence of protein expression (see Section 5.3.3.3 below). The gene expression analysis showed strong amplification of the selected *K. lactis* reference gene by qPCR. However, there was no evidence of expression related to any of the antigenic regions in any of the clones selected for screening by gene expression (Fig. 5.3.4).

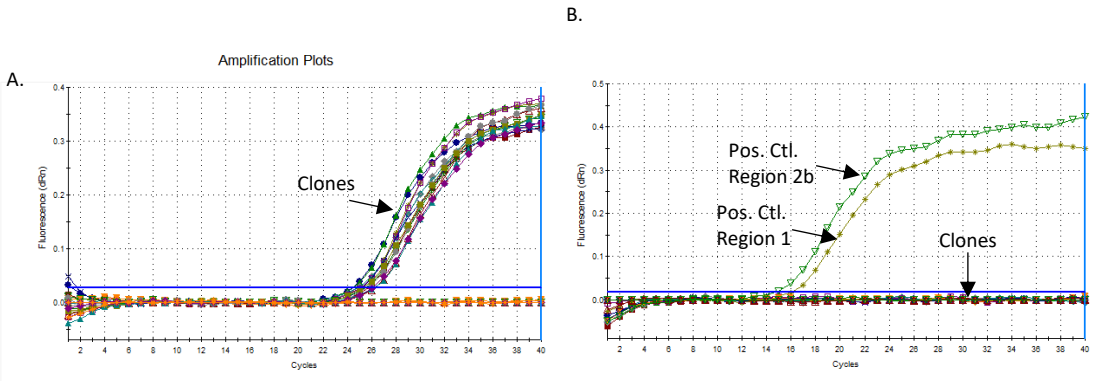


Fig 5.3.4. Gene expression screening of putative recombinant clones. Panel A shows the expression of the reference *K.lactis* gene, indicating successful isolation of the RNA from the yeast and reverse transcription into cDNA. Panel B shows the amplification plots using the gene expression primers for Region 1 and 2b. Only the positive control showed amplification, with no specific gene expression for these regions detected in the clones screened.

4.5.3.3 Antigen expression in recombinant antigen-yeast

Evidence of potential expression of the antigen proteins from yeast was obtained by Western Blot analysis. Fig. 5.3.5 illustrates appropriately sized bands (indicated by blue arrows) in one representative sample of antigen protein expression from lysates prepared from recombinant yeast transformed with gene fragments for Region 1 (expected size 7.3 kDa) and Region 2b (expected size 38.1 kDa).

Specific protein expression was unable to be confirmed by 1D-LCMS in the supernatant for any of the clones with evidence of integration at the DNA level. It is not clear if this related to lack of expression or other variables such as supernatant volume, incubation time or conditions, protein concentration method and/or protein precipitation method.

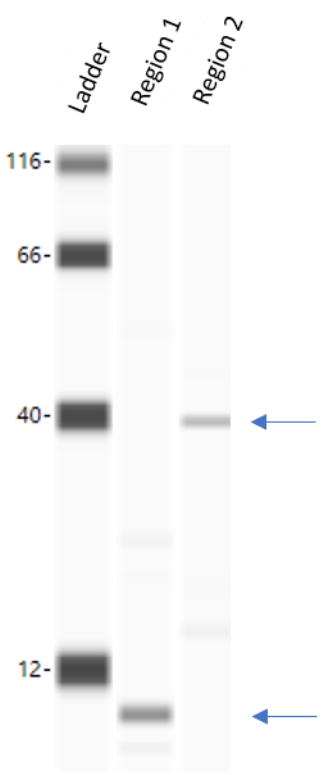


Figure 5.3.5 Evidence of potential antigen protein expression assessed by Western Blot. One sample of each antigen protein for region 1 and 2b is depicted. The blue arrows indicate bands at the approximate size expected for the HA tagged protein antigens.

4.5.3.4 Protein purification from recombinant antigen-yeast

Aliquots (40 µL) of recombinant antigen-yeast pellets (Fig. 5.3.5) showing putative evidence of the correct protein expression both yielded equivalent quantities (12-14 µg/mL) of eluted protein following HA-bead isolation. This was utilised for antigenic screening (see Appendix 4).

***Protein expression from recombinant antigen-yeast detected by** 1D LCMS*

Sample details for concentrated supernatant (sample numbers 3 to 11) assessed are shown below. Region 1 samples were not included as we were unable to detect integration of the gene at the DNA level.

Sample ID	Sample Name	Expected cloned protein*
3	Clone 463.6	Region 3
4	Clone 836.1	Region 4
5	Clone 463.4	Region 3
6	Clone 433.2	Region 3
7	Clone 771.1	Region 4
8	Clone 433.3	Region 3

9	Clone 595.1.2	Region 2a
10	Clone 770.2	Region 4
11	Clone 774.2	Region 4

* Protein sequences provided are described below.

For samples 3, 7 and 11, there were no protein hits after the post-Mascot filtering. This is likely due to the relatively low number of proteins identified. The pre-filtering (and still statistically significant) hits are presented. Results for Sample 3 and 4 are shown and were representative of the results for the supernatant samples (Samples 3 to 11).

None of the proteins for the specific cloned regions (Region 1, 2a, 2b, 3 or 4) was identified in any of the samples tested

Sample 3 (Clone 463.6, Region 3) Pre-filtered, top ten hits.

Accession	Description	Num. of sequences	MW [kDa]	Score Mascot	Gene Symbol
P17819	Kluyveromyces lactis Glyceraldehyde-3-phosphate dehydrogenase 1	10	35.302	2462	GAP1
Q6CX23	Kluyveromyces lactis Glyceraldehyde-3-phosphate dehydrogenase 2	5	35.524	1541	GAP2
P14828	Kluyveromyces lactis Phosphoglycerate kinase	5	44.487	859	PGK
P00761	Trypsin - Sus scrofa (Pig)*	4	24.394	782	
P60712	Actin, cytoplasmic 1 - Bos taurus (Cattle)*	11	41.71	464	ACTB
Q6CVQ6	Kluyveromyces lactis KLLA0B10197p	1	69.903	457	
P49383	Kluyveromyces lactis Alcohol dehydrogenase 2	7	37.075	451	ADH2
P20369	Kluyveromyces lactis Alcohol dehydrogenase 1	6	37.237	188	ADH1
P49385	Kluyveromyces lactis Alcohol dehydrogenase 4, mitochondrial	3	40.138	167	
Q6CTB1	Kluyveromyces lactis KLLA0C14047p	6	39.252	405	

*This is likely to be a constituent of the media.

Sample 4 (Clone 836.1, Region 4) Top ten protein hits.

Accession	Description	Num. of sequences	MW [kDa]	Score Mascot	Gene Symbol
P17819	Kluyveromyces lactis Glyceraldehyde-3-phosphate dehydrogenase 1	6	35.3	2166	GAP1

P14828	Kluyveromyces lactis Phosphoglycerate kinase	3	44.5	822	PGK
P00761	Trypsin - Sus scrofa (Pig)*	4	24.4	684	
Q6CTB1	Kluyveromyces lactis KLLA0C14047p	5	39.3	499	
P60712	Actin, cytoplasmic 1 - Bos taurus (Cattle)*	10	41.7	472	ACTB
Q6CLA9	Kluyveromyces lactis KLLA0F04433p	5	43	436	
Q6CW31	Kluyveromyces lactis KLLA0B07392p	5	36.8	392	
Q6CN67	Kluyveromyces lactis KLLA0E14939p	5	41.2	354	
Q6CYD7	Kluyveromyces lactis Adenosylhomocysteinase	3	49.2	298	
P27069	Kluyveromyces lactis 40S ribosomal protein S14	2	14.5	277	RPS14
P49383	Kluyveromyces lactis Alcohol dehydrogenase 2	5	37.1	269	ADH2

Expression of the ovalbumin protein was confirmed in the OvaYeast vaccine by Western and 1D LCMS analysis.

Confirmation of protein expression in the candidate vaccines, where BVDV epitope-carrying regions were incorporated into *K. lactis* was not successful. Of the two strategies used, gene integration was detected by PCR for multiple secretory BVDV antigens but none of the intracellular cloned antigens were detected. Specific protein expression was assessed in recombinant yeast designed to secrete BVDV antigens. We were unable to confirm secreted specific proteins by 1D-LCMS for any of the clones with evidence of integration at the DNA level. It is not clear what the reason for this may be. There are a number of potential reasons, including lack of expression of these proteins by the putative clones. Another possible reason relates to the method of sample preparation for this analysis, which is important in order to obtain full representation of the proteins in the sample. Two different methods were attempted, however neither was successful for the sample supernatants (Samples 3-11). Other potential methodological variables may include the supernatant volume used, culture incubation time or conditions, protein concentration method and/or protein precipitation method.

5 Conclusion

This was a bold, high risk project funded by MLA to ascertain proof-of-principle as to whether an oral vaccine could be suitable as a vaccine platform for cattle. Likelihood of producer uptake of new livestock vaccines relies heavily on the mode and ease of administration. Although oral vaccines provide a safe and easy route of administration, they encounter challenges when used in ruminant livestock. Vaccine delivered by the oral route most commonly engage with the host's immune system via intestinal immune cells; while antigen modifications in the rumen may impact immune engagement, we have shown that oral vaccination can induce IgA responses in the intestinal mucosa. This provides strong evidence to support further development of oral vaccines for ruminants, specifically cattle. The high occurrence of BRD in Australian feedlots, and its impact on animal health and welfare, coupled with the need for practical vaccine delivery has prompted investigation of this route of vaccine delivery for ruminants. Furthermore, there is a concerted effort by industry partners to reduce the use of antibiotics while addressing this issue, and vaccines can be a used as strategic tool.

Initial *in vitro* studies confirmed at the cellular and molecular level that bovine macrophages have the capacity to be activated by the prototype vaccine OvaYeast. These results supported the assessment of yeast as a method of vaccine antigen delivery, acting as a self-adjuvanting vaccine that would activate immune cells.

This study was unique in that, to the best of our knowledge, this is the first report of an oral yeast vaccine inducing antigen specific immune responses in cattle. Results from the pilot vaccine trial provides evidence of activation of both systemic and mucosal immune responses following oral vaccination with yeast. Immune cell activation and memory cell generation to yeast and OvaYeast occurred one month after the primary dose was given and antibodies against ovalbumin in the serum and mucus were detected at 5 months after the first dose of the vaccine was given. The yeast vaccine does not cause adverse effects or alter weight gain in cattle. There was evidence to suggest immune activation within lymph node cells of the respiratory tract (retropharyngeal lymph nodes) but we cannot verify whether this was related direct stimulation of lymphocytes in the oropharyngeal region or if lymphocytes from mucosa-associated lymphoid tissue from the intestinal

regions have migrated here. What it does show is that yeast vaccines have the potential to activate mucosal immune responses and other methods of delivery such as aerosolization and nasal administration should also be investigated the future.

Reverse vaccinology techniques were used to identify suitable antigens from BRD pathogens, BVDV in particular. There is evidence to suggest that three of the peptide antigens are potentially antigenic given that the specific antibody levels tended to be higher in the serum of BVDV ELISA-positive cattle, compared to BVDV ELISA-negative animals. The antibody response to a combination of antigens is likely to be of greater value than single antigens. There is also provisional evidence for immunogenicity, and we hypothesise that some peptides could be exploited to differentiate between different disease states; at this stage this is merely speculative and will require further detailed investigation to compare BVDV ELISA status with infection/disease status and longitudinal analysis.

The *K. lactis* expression system provides a useful platform for expression of recombinant antigens, with the option of capturing the protein intracellularly, giving the ability of using the whole heat-killed yeast cells as a self-adjuvating vaccine. The limitations of this system, including the low transformation efficiency, led to an inability to conclusively complete the cloning phase and positively identify successfully transformed clones at both the DNA (qPCR) and protein (Western blot) levels for recombinant antigen yeast for any single region of interest. Although some clones showed potential evidence of protein expression, there was no confirmation of DNA integration into the *K. lactis* genome or RNA expression. There were a number of putative transformed clones, as evidenced by the presence of the DNA for the particular gene region detected by qPCR. The *K. lactis* or other yeast-based expression system should be developed further. If not, the antigens identified here could be utilised with other vaccine adjuvants or delivery systems. This would however require further fundamental studies into the induction of immune responses similar to that reported here.

5.1 Key findings

- The prototype vaccine (OvaYeast) activated innate immune cells
- Yeast is a suitable medium for oral vaccine delivery to cattle as it induced immune responses
- The prototype vaccine (OvaYeast) induced both cellular and antibody responses in cattle
- Antibody responses can last for at least 5 months after vaccination
- There is provisional evidence that 3 candidate BVDV antigens may be antigenic and 1 has the potential to be immunogenic

5.2 Benefits to industry

Yeast-based vaccines have the potential to be developed for BRD as well as other diseases that require mucosal immunity for protection. Production of yeast-based vaccines can be scaled up easily and administration to cattle can be incorporated into normal husbandry procedures at backgrounding or at feedlot induction. Yeast-based vaccines can be given as a feed additive or in the style of an oral drench improving ease of administration.

There is sufficient practical benefit and proof-of-concept to explore oral vaccination with yeast further but not quite enough evidence for large scale investment in cattle at the moment. Rather, refinement of the concept is recommended as the next step to increase the number of parameters that can be investigated (e.g. dose, frequency, antigens) and group sizes at lower cost.

6 Future research and recommendations

- Further improvements of yeast transformation to optimise use of this *K. lactis* expression system for production of recombinant antigen vaccine candidates or evaluation of alternate species of yeast.
- Expression of candidate antigens in another expression system (e.g. E. coli) to enable evaluation using other vaccine delivery systems e.g. nanoparticles.
- Further evaluation of yeast vaccine dose and other route(s) of administration.
- Evaluation of combining multiple transformed yeasts containing multiple antigens from the major BRD pathogens in a single vaccine to address the multifactorial nature of this disease.

7 References

References are found following each section and in the Appendices.

8 Appendix

8.1.1 BRD pathogens and pathogenesis

8.1.2 Reverse vaccinology approaches and selection of candidate antigens from BRD pathogens

8.2 In vitro studies of cell activation by OvaYeast

8.3 Pilot study to assess the potential of an oral yeast vaccine to induce immune responses *in vivo* in beef cattle Preparation of recombinant yeast

8.4 Antigenicity of candidate antigens

8.5 Preparation of recombinant yeast

Appendix 1.1: BRD pathogens and pathogenesis

Introduction

The major cause of production losses, morbidity and mortality in feedlots globally is bovine respiratory disease (BRD) (Edwards 2010). Meat and Livestock Australia (MLA) have funded several projects to gain an understanding of the situation in Australian feedlots; around 18% of cattle develop BRD (Barnes et al. 2015) and it is responsible for 50% of deaths and 70% of clinical disease (Sackett et al. 2006). Respiratory disease also causes more than half (56%) of deaths in short-fed (up to 85 days) animals (Perkins 2013). A recent study concluded that the net loss to BRD mortality is estimated to be $1,647 per animal and accounted for 73% of all deaths in an Australian feedlot (Blakebrough-Hall et al. 2020). Based on earlier estimates, vaccination provides some relief with the cost of the disease in unvaccinated animals around $20 per head compared to $12 per head for vaccinated animals (Sackett, Holmes et al. 2006).

BRD is primarily a disease of viral immunosuppression, which allows commensal bacteria in the respiratory tract to become opportunistic pathogens ultimately causing bronchopneumonia. Unavoidable exposure of young cattle to various stressors such as weaning, transportation, co-mingling and a high stocking density can also contribute to suppression of the immune system (Caswell 2014). Beef cattle are most likely to be affected during the first 50 days after entry into a feedlot (Babcock et al. 2010). Pathogen profiles associated with BRD are similar in feedlots around the world despite differences in factors such as breed, age at feedlot entry and housing conditions: they are bovine viral diarrhoea virus (BVDV), bovine herpes virus (BoHV), bovine respiratory syncytial virus (BRSV), parainfluenza 3 (PI3), *Mannheimia haemolytica, Mycoplasma bovis, Pastuerella multocida* and *Histophilus somni*.

This review focuses on pathogens associated with BRD and host immunological responses with a view to formulating new ways of improving the health of feedlot cattle.

1.1 BRD epidemiology and prevalence in Australia

Prior exposure to BRD pathogens in Australian feedlot cattle disease is demonstrated by seropositivity to BVDV, BoHV-1, PI3 and BRSV at feedlot entry. The majority (>60%) of cattle were seropositive for BVDV (Dunn et al. 1995; Hay et al. 2016; Cusack et al. 2021). At feedlot entry seroprevalence to other pathogens ranged from 57-91% for BPI3 (Dunn, Godwin et al. 1995; Hay, Barnes et al. 2016) and 13-24% for BoHV (Dunn, Godwin et al. 1995; Hay, Barnes et al. 2016; Cusack, Bergman et al. 2021) and 27-89% for BRSV (Dunn, Godwin et al. 1995; Hay, Barnes et al. 2016).

In the study by Dunn et al (1995), disease was diagnosed in 6.8% of animals, with fever at the time of entry to the feedlot and BRD accounted for 66% of disease observed. Viral infections were identified in 72% of sick animals with BVDV and BoHV-1 significantly associated with cases of BRD. Bacteria were cultured from 19% of sick animals with the majority (7%) attributed to *M. haemolytica* or various *Salmonella* species (6.6%). In deceased animals, 41% of BRD cases were confirmed to have viral infections, with significant associations between both BVDV and BoHV-1 and respiratory disease. The recent study by Cusack et al (2021) found that seroprevalence to BVDV increased by 33.9% and to BoHV by 30.3% during backgrounding in cattle that were seronegative at entry to backgrounding.

The cause of cattle death was also examined in the 0.9% of animals dying during the research period. BRD accounted for 53% of these deaths. In the deceased animals, 41% of BRD cases were confirmed to have viral infections, with significant associations between both BVDV and BoHV-1 and respiratory disease. Interestingly, there were no significant associations between viral infection and cause of death (Dunn, Godwin et al. 1995). In autopsy material from deceased animals 11.8% were positive for *P. multocida*, 7.2% positive for *M. haemolytica*; of these positive results 17.7% of cultured isolates came from the respiratory tract.

The seroprevalence of *M. bovis* at feedlot entry and later has been reported to range between 3-13.1% and 25.3-73.5% respectively (Wawegama et al. 2016; Schibrowski et al. 2018); this wide variation is likely to be due to differences in the diagnostic testing kits used. Regardless, it is clear that cattle are exposed to this pathogen during their time in the feedlot. The nasal prevalence of *H. somni, M. bovis, M. haemolytica*, and *P. multocida* in a sample of Australian live export cattle has been estimated at 42%, 4.8%, 13.4%, and 26%, respectively (Moore et al. 2015).

1.2 Pulmonary immune responses

The pulmonary epithelium provides an impermeable structural barrier against inhaled particles entering the host and interacts with airway macrophages as the first line of defence against respiratory pathogens and toxins (Iwasaki et al. 2017; Lloyd et al. 2017). The epithelium comprises a variety of cell types with various functions. Ciliated cells make up the main proportion of the pulmonary epithelium. Goblet cells, which secrete mucous, decrease in number in the lower respiratory tract. Club cells are functionally and structurally diverse and facilitate clearance functions in the lower respiratory tract. Basal cells can differentiate into the other cell types and replace cells in the epithelium. In healthy animals, mucous production is low but increases under severe inflammatory conditions. The mucins, defensins and other antimicrobial peptides in secretions from the respiratory epithelium form a protective layer. Inhaled toxins, pathogens and other particles are trapped by this mucous layer and are removed via the motion of cilia by a mechanism known as mucocilliary clearance. In deeper lung tissue, surfactants produced by pneumocytes prevent alveolar collapse and allow efficient gas exchange.

Airway macrophages are long-lived innate immune cells, which patrol the lumen of the respiratory tract. In the event the epithelial-macrophage response is unable to contain infection, chemokine and cytokine signalling from these cells can trigger activation of macrophages residing in interstitial tissues of the lungs. Additional innate immune cells such as neutrophils and natural killer cells as well as specialized lymphocytes, which circulate via lung lymphatics, are also recruited. Often, the balance between inflammatory responses and tissue healing responses can determine the extent of lung pathology. The induction of immunological memory leads to the generation of resident memory T cells (Trm). These Trm are an important component of adaptive immune responses as they reside in the tissue underlying the respiratory epithelium and can provide a rapid response when re-encountering the pathogen (Lloyd and Marsland 2017). $\gamma\delta$ T cells are a major component of mucosal epithelia, especially in young ruminants. These cells respond rapidly to signals from their microenvironment and co-ordinate adaptive immune responses. An understanding of these cellular responses to respiratory infections will support development of better vaccines.

1.3 Respiratory microbiome

Colonisation of the respiratory tract by microbes occurs soon after birth. The degree of colonization and the communities involved are influenced by a variety of factors including the surrounding environment, diet, transportation and antimicrobial use (Zeineldin et al. 2017). The upper respiratory tract (oral and nasal cavities and throat) is colonized by numerous microbiota, while the lower respiratory tract (trachea and lung) is relatively sterile. There is as change in the respiratory microbiome from weaning to prior to cattle arriving at the feedlot and then again in the first 10 weeks at feedlot (Timsit et al. 2016).

1.4 Viral pathogens

The four most common viral pathogens associated with BRD are genetically distinct (Table 1) despite initiating similar respiratory symptoms in cattle.

Table 1. Viral pathogens

Virus	Family	Genus	Structure
BVDV	Flaviviridae	pestivirus	Single-stranded positive-sense RNA
BoHV	Herpesviridae	variellovirus	Double-stranded DNA
BRSV	Paramyxoviridae	pneumovirus	Single-stranded negative-sense RNA
PI3	Paramyxoviridae	respirovirus	Single-stranded negative-sense RNA

1.4.1 Bovine viral diarrhoea virus (BVDV)

BVDV has three distinct genotypes, BVDV1, BVDV2 and BVDV3 or HoBi-like virus (Yesilbag et al. 2017; Evans et al. 2019) with antigenic and geographic differences; BVDV1a and 1b are more commonly detected in feedlots in north America and Europe, while BVDV1c is the most common in Australia (Ambrose et al. 2018) and BVDV2 is not found in Australia or New Zealand. In addition, there are two distinct biotypes within these two genotypes which are based on the effect of the virus on *in vitro* cultured cells (Bolin 2002). The cytopathic type, as the name suggests, induces cell death. Non-cytopathic BVDV is predominant in the field, however, both biotypes have a spectrum of virulence and both are capable of causing acute infection and respiratory disease *in vivo* (Booker et al. 2008). BVDV infects and affects the function of innate immune cells including phagocytic, microbicidal, and chemotactic functions; thus, hampering first line defence mechanisms (Goyal et al. 2008). Lymphocytopenia, with a reduction in both T- and B-cells, and it's resulting immunosuppression is also typical in acute BVDV infection. The non-cytopathic type tends to induce an antibody-mediated response and traffics to lymphatic tissues associated with mucosal immunity while the cytopathic type drives a cell-mediated response and does not persist at immunological sites for long (Goyal and Ridpath 2008; Chase 2013).

Overall, BVDV is one of the top five endemic diseases that cause production losses for the red meat industries in Australia (Lane et al. 2015). Naïve cattle exposed to BVDV develop an acute transient infection originating in the nasal mucosa within 6 to 12 days following exposure, (Evans, Pinior et al. 2019). Transiently infected animals can shed low levels of the virus in bodily secretions and excretions for up to 3 weeks (Thurmond 2008). Once infection is cleared, shedding ceases and the animals develop an antibody and T cell response that is protective for life (Evermann et al. 2008; Brodersen 2014; Lanyon et al. 2014). Low virulence strains are rapidly cleared unlike highly virulent ones which continue spreading into almost all organs and tissues (Goyal and Ridpath 2008). *In utero* exposure is

common and the timing of exposure is critical to disease outcome. Fetal exposure after day 30 and during the first trimester leads to persistently infected (PI) calves (Lanyon, Hill et al. 2014). The immune naivety of the fetus leads to immune tolerance to BVDV, resulting in an animal that has a lifelong viral infection and is capable of transmitting BVDV (Brownlie et al. 1998). From the pathogen's perspective this creates an ideal scenario where asymptomatically infected PI calves can surreptitiously spread infection to other cattle in the feedlot through the oronasal route (Goyal and Ridpath 2008).

Seropositivity to BVDV upon entry to feedlots is a risk factor associated with the development of BRD. In Australia seroprevalence of BVDV is widespread (Durham et al. 1991; Dunn, Godwin et al. 1995; Hay et al. 2016; Hay, Barnes et al. 2016). Cattle in feedlots where BVDV is present, either transiently or persistently, have a higher risk of developing BRD than those in cohorts without BVDV (Barnes, Hay et al. 2015). Furthermore, the animals that have an increase in BVDV antibody titre following entry to the feedlot are at a higher risk of developing BRD compared with animals that have high titres at entry (Durham, Hassard et al. 1991). Therefore, exposure within the feedlot to BVDV either by transiently or persistently infected animals can increase the incidence of BRD, which could be prevented or reduced through boosting immunity to BVDV via feedlot entry vaccination.

1.4.2 Bovine herpesvirus type 1

BoHV-1 has been noted to cause a wide range of clinical disease in Australia, including genital infection, conjunctivitis, encephalitis, gastrointestinal and respiratory disease (Rogers et al. 1978; Rogers et al. 1980; Babiuk et al. 1996). Unofficially BoHV-1 is grouped into 5 sub-groups based on restriction endonuclease profiles and broad clinical disease outcome (Smith et al. 1995). Subgroup 1.1 and 1.2b are associated with respiratory infection. Subgroup 1.2b causes milder disease than subgroup 1.1 and is the only type found in Australia (OGTR 2005). Although BoHV-1 has a predilection for mucosal tissue there are no differences in true tissue tropism between the different subgroups (Steukers et al. 2011). Similarly to other herpes viruses, pathogenesis of BoHV-1 follows the clinical steps of acute disease, latency and recurrence, with transmission possible during the acute and recurrence phases through aerosol, close contact of animals or contamination of feed stuffs and water (Turin et al. 1999). Although the acute clinical phase of BoHV-1 infection is commonly self-limiting, local lesions produced during this phase and immunosuppression facilitates secondary bacterial infections, commonly seen as part of the pathogenesis of BRD (Yates 1982; Turin, Russo et al. 1999; Leite et al. 2004).

A strong association exists in Australian feedlots between the occurrence of respiratory disease and infection with BoHV-1 (Dunn, Godwin et al. 1995). Infection of herds with BoHV-1 is common in the Australian cattle industry, however serological surveys have shown varying levels of past exposure, between 30 to 90% of herds across Australia being exposed (St George et al. 1967). On average 30% of cattle were seropositive to BoHV-1 on feedlot entry, with 76% seropositive by slaughter (Dunn, Godwin et al. 1995). Only animals seropositive for BoHV-1 in the first six weeks following entry into feedlots had a significant association with the development of BRD, with infection later in the feedlotting period not related to BRD occurrence (Dunn, Godwin et al. 1995). Furthermore, the proportion of cattle entering feedlots that were susceptible to BoHV-1 was significantly different depending on the feedlot tested (Dunn, Godwin et al. 1995). Therefore, it is likely that these differences suggest an influence of the geographical location from where cattle were sourced on potential exposure to BoHV-1 in Australia. The common practice of mixing cattle from multiple locations at feedlots would increase the chances of contact between naïve and infected animals and elevate the risk of susceptible animals developing BoHV-1 associated BRD.

1.4.3 Bovine parainfluenza-3 virus

Bovine PI3 is endemic in cattle populations worldwide; while it can infect ruminants and humans it is not pathogenic in humans. Its genomic material and capsid are surrounded by a lipid envelope derived from the host cell, aiding immune evasion (2017). Infection is restricted to the respiratory tract with the virus targeting epithelial cells of the nasal tract, pnemocytes and alveolar macrophages. Clinical disease in cattle is of short duration (3-5 days) and generally resolves easily. Under conditions of stress, it can lead to severe bacterial bronchopneumonia, generally in conjunction with *M. haemolytica* or *Mycoplasma*. Animals that recover from infection have a strong humoral response with antibodies mainly to the haemagglutinin-neuraminidase viral protein. Infection of host cells require binding of this protein to sialic acid in mucous which it turn enables attachment of PI3 into cells in the host respiratory tract (Ellis 2010). BPI3 is considered to be only a minor contributor to BRD.

1.4.4 Bovine respiratory syncytial virus

BRSV is closely related to the human respiratory syncytial virus which is the major cause of lower respiratory tract illness in infants and older adults (CDC 2020). The presence of two non-structural proteins makes BRSV distinct from other viruses in the Paramyxoviridae family such as PI3 (Valarcher et al. 2007). These proteins play a role in regulating interferon alpha and beta (IFNα, IFNβ) by inhibiting transcription factors that activate these pathways. Similar to PI3, the BRSV genome is surrounded by a lipid envelope derived from the host. The glycoprotein (G protein) on the surface of the virus is immunodominant and is required for attachment to host cells. In its secreted form, the G protein neutralises antibodies.

Several antigenic and genetic BRSV subtypes have been identified (Valarcher and Taylor 2007). This includes four subgroups based on the G protein: A, B, AB, and untyped and six genetic subgroups based on G and five subgroups based on F (fusion) or N (nucleoprotein). The evolution of BRSV into subtypes may have been driven, in part, by selection pressure applied by vaccination. Assessing European BRSV isolates from a period of 32 years, Valarcher et al (Valarcher et al. 2000) found that there was a continuous evolution of the sequences of the N, G, and F proteins of BRSV with a strong positive selective pressure on the G protein and in specific sites of the N and F proteins in the countries where BRSV vaccines were widely used. The ability to vary the structure of the G protein allows the virus to successfully evade immune responses previously established by vaccination.

While particularly virulent strains exist, BRSV generally causes a mild disease. The main pathology is the destruction of the ciliated epithelium in the respiratory tract, which compromises mucociliary clearance and facilitates opportunistic bacterial infections from other BRD pathogens like *M. haemolytica.* It is likely to be the host response to the viral infection and subsequent severe inflammation that causes the destruction of lung tissue.

1.5 Bacterial pathogens

The composition of bacterial communities in the bovine respiratory tract changes over the course of their time spent in the feedlot (Holman et al. 2015). In addition, the presence of specific bacterial communities in the nasopharynx can prevent respiratory pathogens from establishing an infection (Holman, McAllister et al. 2015; Zeineldin, Lowe et al. 2017). In cattle that remain healthy, these communities are more diverse at feedlot entry than in those that develop BRD (Holman, McAllister et al. 2015) and these communities are distinct between the upper and lower respiratory tracts (Timsit et al. 2018). The nasopharynx and trachea in cattle with bronchopneumonia is enriched with *M. bovis, M. haemolytica* and *P. multocida*. The caveat is that some commensal bacteria can become opportunistic pathogens under the right conditions and the use of treatments (e.g. antibiotics) may drive some of the differences observed between health and disease.

1.5.1 Mannheimia haemolytica

In healthy cattle, *M. haemolytica* is naturally found in the upper respiratory tract (Confer et al. 2018). It is of the Pasteurellaceae family and is a Gram-negative, facultative anaerobic, coccobacillus. Serotype 1 is most commonly associated with BRD and serotype 6 to a lesser extent (Klima et al. 2014). The immunogenic capacity of *Mannheimia* has been widely studied in relation to capsular polysaccharides, lipopolysaccharide, adhesins, outer membrane proteins, and leukotoxin (Confer and Ayalew 2018). Several antigens for incorporating into vaccines have been identified based on their immunoreactivity (Confer and Ayalew 2018; Klima et al. 2018) and include serine protease Ssa-1 (AC570_10970), a filamentous hemagglutinin (AC570_01600), a porin protein (AC569_05045), an outer membrane assembly protein YeaT (AC570_03060) and leukotoxin.

1.5.2 Mycoplasma bovis

Mycoplasma bovis, found as a commensal on mucosal membranes, can cause a variety of diseases in cattle including BRD, arthritis and mastitis and is one of the more pathogenic species of the genus Mycoplasma and Family *Mycoplasmataceae.* Mycoplasma have the smallest genome of any living cell and rely on the host for several metabolic requirements. They are anaerobic cells, which lack a cell wall making Mycoplasma resistant to beta-lactam antibiotics such as penicillin. *Mycoplasma bovis* strains contain variable membrane surface lipoprotein antigens (Vsps), which enable adherence to epithelial cells in the respiratory tract. (Calcutt et al. 2018) and are major immunogenic antigens. Vsps have a high rate of phase (on/off) and antigenic variation underpinned by DNA rearrangements at high frequency. Despite variation of these genes between different strains, a specific virulence profile for *M. bovis* has not been identified. To account for the variability of virulence, any vaccine may need to include antigens from several strains. *M. bovis* is an extracellular pathogen but it does have the capacity to infect cells and survive intracellularly as well. From a vaccine point of view this would require induction of both cellular and humoral immunity to overcome infection (Perez-Casal et al. 2017).

1.5.3 Pastuerella multocida

Pasteurella multocida is a zoonotic member of the family Pasteurellaceae. It is a Gram-negative coccobacillus that is part of the normal bacterial communities in the nasopharynx of many species including cattle. *P. multocida* is classified into five groups based on the structure of its capsule (A-F) and 16 serotypes (Dabo et al. 2007). Serogroup A is the most common cause of BRD worldwide with serotype A:3 most commonly isolated from lung tissue is animals with respiratory disease followed by D:3 (Confer 2009). *P. multocida* is often also detected in clinically normal cattle with a prevalence of 20-60% (Griffin et al. 2010); however, it is the most common bacterial pathogen isolated from lung lesions of animals with clinical BRD. It has a prevalence of 26% in nasal swabs of Australian live export cattle.

Virulence factors include adhesins (which enable adherence to cell surfaces), a thick polysaccharide capsule (prevents phagocytosis) and lipopolysaccharide (LPS, stimulates cytokine secretion and pulmonary inflammation) (Confer 2009).

1.5.4 Histophilus somni

Histophilus somni is another Gram-negative coccobacillus of the Pasteurellaceae family which is an opportunistic pathogen causing BRD in cattle. The main virulence factors are lipooligosaccharide and outer membrane proteins (Confer 2009). The lipooligosaccharide components trigger programmed cell death of endothelial cells and phase variations of these antigens facilitate immune evasion. Immunoglobulin binding proteins in the outer membrane of *H. somni* counteract complement-mediated host defence mechanisms. While the nasal prevalence of *H. somni,* in a sample of Australian

live export cattle was relatively high at 42% (Moore, O'Dea et al. 2015) this bacterium was not associated with mortality due to BRD (Moore et al. 2014).

1.5.5 *Trueperella pyogenes*

Trueperella pyogenes is a facultative anaerobic Gram-positive bacillus of the family Actinomycetaceae, previously known as *Arcanobacterium pyogenes*. It is a ubiquitous commensal of the skin and mucosal surfaces and has the ability to cause opportunistic infections including pneumonia, mastitis and liver abscesses. Its virulence factors include a pyolysin which can lyse immune cells including macrophages and the ability to invade and survive intracellularly (Jost et al. 2005). *T. pyogoenes* is one of the rare Gram-positive bacteria with fimbriae; the upregulation of one type of fimbriae, FimA, is thought to contribute to its pathogenicity. An understanding of the factors required for transformation of the commensal organism to a pathogen is still lacking (Rzewuska et al. 2019).

1.6 BRD control strategies

The pathogens causing BRD have been well-documented but there is scope to develop better treatments for this disease. In addition to management strategies, antibiotics and vaccines are often used as treatments to control or prevent BRD. There are several commercially available vaccines offering protection against BRD. In Australia, these include vaccines that offer protection against *M. haemolytica,* BVDV and BoHV (APVMA 2020). There are vaccines against PI3 and BRSV but are not licensed for use in Australia. A comprehensive review of published data from the USA on efficacy of commercially available vaccines for the four viral pathogens of BRD found that despite a low number of studies under natural conditions, there was a benefit to the use of multivalent vaccines in commercial feedlots (Theurer et al. 2014). However, the vaccine components that were responsible for better outcomes were not identified. A similar review on the bacterial pathogens *M. haemolytica, P. multocida* and *H. somni* found no benefit to vaccinate against the latter (Larson et al. 2012).

There is on-going concerted global effort to identify other candidate vaccine antigens for developing better vaccines. Combined with an understanding from the pathogen perspective of factors that are important for causing disease, it is essential that there is a better understanding of the host response, particularly the protective immunological response, as well as the effect of commensal microorganisms. The microbiota of the host affects the replication and transmission of a diverse array of viral pathogens. Therefore, it is tempting to speculate that the composition of an individual's microbiota can influence disease outcome during viral infection, making it a potential target for therapeutic intervention. Host microbiota can protect against or enhance susceptibility to viral infections (Wilks et al. 2013). This would mean that different treatment strategies would be needed depending on the viral pathogen's interaction with commensal organisms in the host. *Haemophilus influenzae,* like *M. haemolytica* and *P. multocida,* belongs to the Pasteurellaceae family. A recent study has demonstrated that a single dose of a commensal closely related to *H. influenzae* can prevent disease caused by this pathogen (Granland et al. 2020). It would be valuable to develop an understanding of the commensal respiratory microbiota within the context of Australian feedlots.

A positive serological response indicates the presence of antibodies that can bind to specific antigen(s). These antibodies may or may not be protective and are part of the humoral arm of the immune response. It is an indication that the animals have been exposed to the pathogen – not that they are protected if re-exposed. There can be many different antibodies to the pathogen (binding to different parts of the pathogen). Some of these will last longer than others. Similar to a booster vaccination, re-vaccination of an animal that is seropositive can make the immune response stronger. Therefore, the immune response of a seropositive animal can still be boosted.

Further to this, for some pathogens the cell-mediated arm of the immune response, including cytotoxic T cells that are able to lyse viral infected cells, is more efficacious at protecting from disease. The reverse vaccinology approach proposed targets MHC I epitopes to facilitate CD8+T cell mediated immune responses. These epitopes will be present on the target antigens used to generate the yeast vaccine. Studies have shown that yeast presentation of vaccine antigens to dendritic cells in the gut-associated mucosal tissues (oral delivery) can lead to the development of a mucosal antibody response as well as an effective cell-mediated immune response, meaning that it may lead to dual protection by both arms of the immune response (Shin et al. 2005; Kim et al. 2014; Zhao et al. 2014). We plan to measure these responses in the *in vivo* phase of the project for our reporter antigen as a proof-of-principle for the yeast vaccine delivery system in ruminants. However, efficacy studies are required for the antigens identified by the reverse vaccinology approach to determine if they will initiate protective immune responses to the specific target(s).

Another strategy to improve immune-mediated protection against developing BRD during feedlotting is to vaccinate during backgrounding rather than at induction when animals are being subjected to multiple stressors. This early intervention has been trialled in Australia with some success (Condon 2017; Condon 2017). Oral vaccines are easy to administer and can be incorporated into normal husbandry practises are more likely to be adopted by producers.

1.7 References

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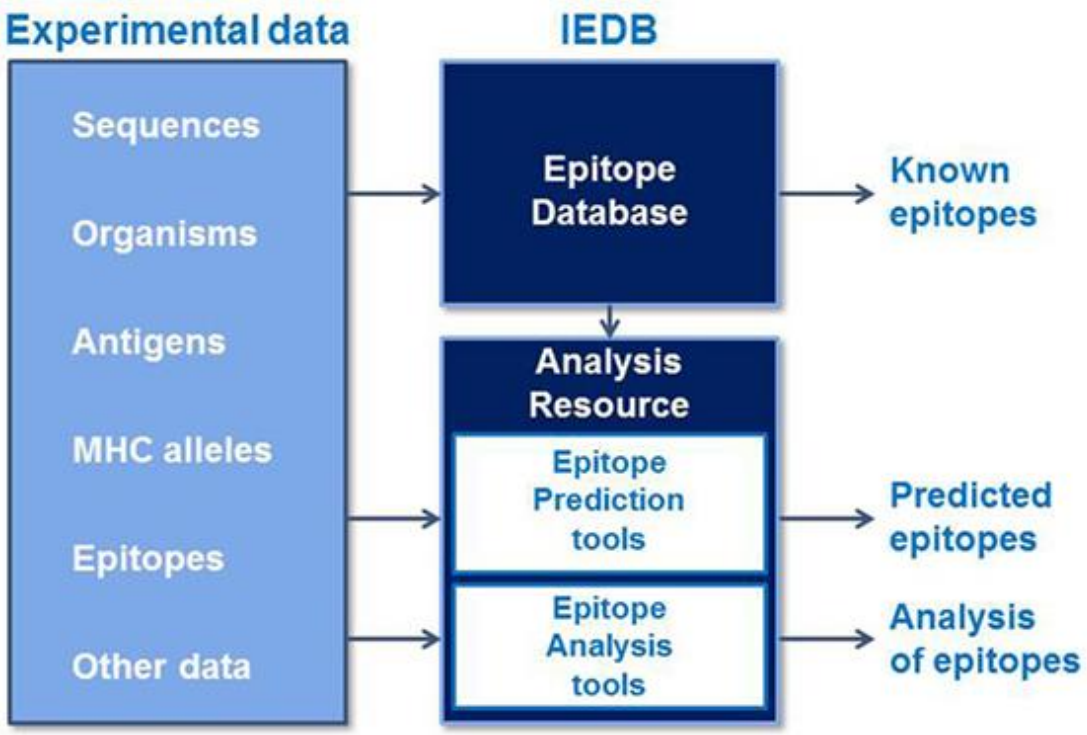
Appendix 1.2: Reverse vaccinology approaches and selection of candidate antigens from BRD pathogens

Introduction

The evolution of vaccine design in recent years has led to the use of epitopes in vaccine formulations. Epitopes are short amino acid sequences of an immunogenic protein (e.g. from a pathogen) and are capable of inducing a direct and relevant immune response in the host organism (Kao et al. 2009). Epitope design for vaccine inclusion is increasingly reliant upon bioinformatic or *in silico* analysis (Fleri et al. 2017; Fleri et al. 2017) however a vital pre-requisite for this method of vaccine design is an accurate knowledge of disease pathogenesis i.e. it is vital to have access to genomic (DNA and protein sequences) information of the causative pathogen as well as have insight of the host response mechanisms that counter the pathogen.

Successful vaccines against parasite, bacterial or viral infections require insight of the cellular immune responses and to facilitate this in terms of T cell based epitope prediction; the Reverse Vaccinology approach (Burton 2017; Jensen et al. 2018) utilises the codon sequence data obtained from pathogenic DNA and translates it to a protein of interest via complementary cDNA. Experimentally (*in vivo*), this approach introduces putative antigenic proteins and evaluates the transition to antigen presenting cells (APC) and the subsequent processing to eventual presentation of antigens on Major Histocompatibility Complex (MHC) molecules to interact with T cell receptors. Smaller and potentially more immunogenic epitopes may be isolated by cloning the antigenic peptide sequences and utilising immune methodologies to identify the most relevant epitopes to take forward to vaccine formulations. Many of these *in vivo* steps may be eliminated by screening the whole protein sequence through use of *in silico* prediction programs that identify potential antigenic sequences and enable visualisation of those epitopes with highest predicted affinity, specificity and stability. Currently the *in silico* T cell epitope prediction tools are most successful in identification of MHC class I binding epitopes largely due to the structural variations inherent between MHC class I and class II. An epitope-based T cell inducing vaccine approach to viral diseases is increasingly favoured. This approach encompasses vaccines designed to induce CD4 and/or CD8 T cells that directly contribute to pathogen clearance via cell mediated effector mechanisms, rather than limiting the vaccine to target CD4 T cell mediated signals for B cell involvement leading to protective antibody responses. Several clinical trials have demonstrated the efficacy of T cell inducing vaccines (Rosa et al. 2006; Gilbert 2012). The selection and design of epitopes for vaccine use should take into account the desired immune response; for example a cytotoxic T cell response is prompted by a pathway that includes intracellular antigen processing with linear epitopes (Sun et al. 1991). *In silico* B cell epitope prediction has to account for the fact that the majority of B cell epitopes are comprised from amino acid residues that are located on separate regions of the antigenic protein sequence and these are only joined together following the folding of the protein chain (Van Regenmortel 2016). This adds a level of complexity to the selection of putative epitopes and historically, unless there is *in vivo* or *in vitro* derived experimental data to support selection of antigenic sequences, *in silico* selection of B cell vaccine epitopes has not been very successful when translated to clinical trials. Since there is a paucity of accessible data available regarding verified antigen sequences relevant to bovine respiratory disease (BRD) in the Australian setting, the decision was made to concentrate on MHC class I T cell targeting epitopes however, we will continue to seek verified protein sequences to facilitate design of B cell specific epitopes. Previous B cell epitope design utilising an *in silico* approach has proven more sucessful when translated to clinical trials when utilising shorter regions of protein sequences from *in vitro* immunogenicity verified sources (Gurung et al. 2012).

The collection of bioinformatics tools that may be utilised in T cell and B cell epitope prediction and analysis are available at the Immune Epitope Database Analysis Resource (IEDB) (http://tools.iedb.org/main/). The process required for an *in silico* investigation utilising these tools is illustrated in the following diagram (Fleri, Paul et al. 2017).



Use of *in silico* methods does not negate the requirement for experimental analysis but it does provide a rapid method for identification of targeted viable epitope candidates and the vast number of bioinformatics tools currently available makes it possible to eliminate many unsuitable candidates prior to time consuming and expensive experimental research. This method has successfully been utilised within our research team to identify B cell epitopes. Previously we identified stress-regulated antigenic proteins associated with *Mycobacterium avium* subspecies *paratuberculosis* and then utilised these protein sequences for B cell epitope prediction. These *in silico*-identified epitopes were then produced as recombinant proteins and evaluated for immunogenicity via immune methodologies such as ELISA (Gurung et al. 2012; Gurung, Purdie et al. 2012).

In terms of antigen selection for a BRD vaccine, this multifactorial disease presents a challenge since BRD has multiple aetiologies. We sought to identify the primary pathogenic candidates relevant to the Australian feedlot setting while considering previously published research and these findings are presented as a review article (Appendix 1.1). Previous research shows that in the Australian setting, upon entry to the feedlot, antibodies of primary viral pathogens dominate with 68% positive for BVDV, 13% positive for BoHV-1, 57% positive for BPI3 and 27% positive for BRSV. The viral pathogens BVDV and BoHV-1 were significantly associated in those animals that succumbed to BRD during the finishing process and in 41% of deceased BRD cattle cases. The associations between BRD mortality and bacterial pathogens do not present a strong case; rather the data suggests that bacterial infections are an opportunistic infection. Further there is a lack of clarity in terms of bacterial serotype specificity within the Australian feedlots that would complicate the accurate selection of candidate for vaccine

formulation and lack of access to respiratory disease associated Australian protein sequences for all relevant organisms apart from BVDV, *Pasteurella multicida* and Bovine herpesvirus type 1.2 strain B589. Thus, in this report the epitope selection is targeted to identification of candidates for a vaccine against the viral pathogen BVDV1 since in Australia this pathogen is implicated as one of the primary risk factors associated with the development of BRD (Hay et al. 2014). We have however generated a list of suggested epitopes against each of the following BRD pathogens utilising protein sequences sourced from the NCBI (Bovine herpesvirus (type 1.2), Bovine Parainfluenza-3 virus, Bovine respiratory syncytial virus, *Mannheimia haemolytica, Mycoplasma bovis, Pasteurella multocida* and *Histophilus somni).*

The mechanisms of pathogenesis of bovine viral diarrhoea virus, a prototypic member of the genus Pestivirus, (family Flaviviridae) are reviewed in Appendix 1.1, and it must be acknowledged that there are currently available BVDV vaccines including conventional inactivated vaccines with adjuvants, modified live virus vaccines with reduced virulence or vaccines comprising genetically modified virus whereby virulence genes have been inactivated (Platt et al. 2017) however, the ongoing incidence of BVDV in cattle transiting the Australian feedlot system suggests that new vaccine approaches may be warranted.

1.1 Methods

1.1.1 Analysis of the IEDB to identify previously published epitopes

Utilised search function within IEDB (http://www.iedb.org/home_v3.php) to identify previously published BVDV epitopes applicable to MHC I restriction, any host, and any epitope type or sequence (Table.1.2.1) Determined immunogenicity of the selected protein sequences through use of the Class I Immunogenicity tool (Calis et al. 2013) (http://tools.iedb.org/immunogenicity).

1.1.2 Selection of BVDV protein sequences

A keyword search was performed on the NCBI Taxonomy Browser (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi) to identify BVDV strain protein sequences. The resulting 1043 sequences were further sorted to include only those with specificity to BVDV sourced in Australia ("Bovine viral diarrhea virus 1"[Organism] OR ("Bovine viral diarrhea virus 1"[Organism] OR Bovine viral diarrhea virus [All Fields])) AND strain [All Fields] AND Australia [All Fields]. The resulting sequences were collated to a single table (Table 1.2.2).

1.1.3 TepiTool prediction of peptides binding to MHC class I molecules

Peptide sequences identified in 1.2.2 were uploaded to TepiTools (Paul et al. 2016) and individually matched to each of the 13 possible Bovine MHC class I alleles. The IEDB Consensus method was utilised as the binding prediction method. This method is based upon the availability of predictors and previously observed predictive performance. The Consensus method incorporates the algorithms ANN (Artificial neural network, also called as NetMHC, version 3.4), SMM (Stabilized matrix method), SMMPMBEC (SMM with a Peptide:MHC Binding Energy Covariance matrix) and Comblib_Sidney2008 (Scoring Matrices derived from Combinatorial Peptide Libraries) if any corresponding predictor is available for a molecule otherwise NetMHCpan (version 2.8) is used. These algorithms are utilised in decreasing order Consensus > ANN > SMM > NetMHCpan > CombLib. The resulting epitope sequences are arranged by predicted percentile rank and a lower value indicates better predicted binding affinity. All generated epitopes with the best predicted binding affinity with general consistency across

multiple MHC Class I alleles were selected for immunogenicity analysis (data not shown as over 1000 epitopes were returned).

1.1.4 Analysis of MHC Class I binding epitope immunogenicity

The IEDB Class I Immunogenicity tool (http://tools.iedb.org/immunogenicity) was utilised to predict immunogenicity of a peptide (epitope) MHC complex. Calis *et al* have developed a prediction tool via a process of machine learning incorporating knowledge of mechanisms of action of amino acid sequences and T cell receptor affinity(Calis, Maybeno et al. 2013). Selected epitope sequences were uploaded and submitted for analysis. The resulting table categorises the prediction results by descending score values. Scores greater than 0 are indicative of a sequence likely to be located in a position relevant for T cell receptor ligand binding and are predicted to be capable of eliciting an immune response. The final list of proposed BVDV epitopes for further analysis is generated from this table (Table 1.2.3) based upon common incidence across multiple MHC alleles and high immunogenicity scores. Using this method, we have similarly generated epitope sequences for Bovine herpesvirus (type 1.2), Bovine Parainfluenza-3 virus, Bovine respiratory syncytial virus, *Mannheimia haemolytica, Mycoplasma bovis, Pasteurella multocida* and *Histophilus somni* (Table 1.2.4).

1.2 Results and Discussion

1.2.1 Previously published epitope sequences

Preliminary analysis of IEDB to identify previously published (Rotzschke et al. 1993; Deregt et al. 1998; Collen et al. 2002) BVDV epitopes with MHC binding potential resulted in 12 epitopes (Table 1.2.1) sorted by descending score value. A higher score indicates a greater probability of eliciting an immune response (Calis, Maybeno et al. 2013). There was no overlap with the novel epitopes that we suggest taking forward.

Table 1.2.1 Previously published epitopes

Epitope sequence	Epitope Length	Immunogenicity Score[a]
KLLEIFHTI	9	0.38693
NDRIGPLGAEG	11	0.28554
NMLVFVPTRNM	11	0.25778
EQLAIIGKIHR	11	0.25619
YIFLDEYHCAT	11	0.15015
VTDTYENYS	9	0.12929
SSVDHVTAGKDLL	13	0.02556
QVIALDTKL	9	-0.00059
PAFFDLKNL	9	-0.01882
DTVVQVIALDTKLGPMP	17	-0.10222
VGRVKPGRY	9	-0.10326
IVRGKYNTTLLNG	13	-0.11698

a: an immunogenicity score greater than 0 is indicative of an epitope with greater capacity to elicit and immune response. The range for a successful epitope is generally between 0.2 and 0.6.

Selection of BVDV protein sequences

NCBI analysis identified 1048 protein sequences isolated from samples collected globally. Of these 22 were obtained from samples sourced in Australia (Table B2). There was a degree of overlap present in some of the sequences however this was addressed during the MHC class I processing epitope selection. Three sequences (9, 10 and 11) had to be excluded due to incompatibility with the IEDB analysis algorithms.

1.2.2 Prediction of BRD relevant MHC Class I epitopes and immunogenicity analysis

Viral exposure results in the virus and other components being taken into the host cells where the virus integrates within the host genome, replicates, and proliferates throughout the host organism. The host responds through the process of antigen presentation whereby secreted viral peptides are taken up by antigen presenting cells triggering a complex cascade of intracellular processes including proteasome processing, generation of antigenic peptides, transport to the endoplasmic reticulum, binding to MHC molecules and presentation through MHC molecules ultimately triggering recognition by CD8 T cells that are able to kill the cell, proliferate, produce cytokines and form memory T cell populations. Each step along the pathway has a corresponding efficacy and ultimately only 2.5% of the peptides that are generated will bind to an MHC molecule (Assarsson et al. 2007). Thus, there is a great deal of obsolescence in terms of immunogenicity potential for epitopes. The prediction algorithms developed by the IEDB Analysis Research team (Calis, Maybeno et al. 2013; Paul, Sidney et al. 2016) and others have been put to test against a range of pathogens including *Pseudomonas aeruginosa* (Kao and Hodges 2009) and *Leishmania donovani* (Alves-Silva et al. 2017; Barbosa Santos et al. 2017) with encouraging results showing that vaccines composed of epitopes specifically formulated to target immunogenic domains appear to optimise and potentially surpass the protective potential induced by whole protein vaccines. The success of these undertakings encourages use of a combination of immuno-informatics underpinned by experimental validation to identify epitopes for the formulation of novel T cell vaccine.

During this study, TepiTool was used to predict T cell epitopes for MHC Class I binding. This *in silico* tool allows the user to input separate sequences and these are then aligned to user selected MHC Class I alleles. In the case of the cattle MHC Class I alleles available on the database, the following choices were available for selection: BoLA-JSP.1, BoLA-AW10, BoLA-HD6, BoLA-N:00101, BoLA-N:00102, BoLA-N:00103, BoLA-N:00201, BoLA-N:00301, BoLA-N:00401, BoLA-N:00402, BoLA-N:00501, BoLA-N:00601, BoLA-N:00602. The collated protein sequences (Table 1.2.2) were submitted to TepiTool and individually aligned to each of the MHC Class I alleles. The predicted epitopes clustered to show multiple common epitopes between the MHC Class I alleles BoLA-JSP.1, BoLA-AW10, BoLA-HD6 (Group 1, Table 3) and separately to the MHC Class I alleles BoLA-N:00101, BoLA-N:00102, BoLA-N:00103, BoLA-N:00201, BoLA-N:00301, BoLA-N:00401, BoLA-N:00402, BoLA-N:00501, BoLA-N:00601, BoLA-N:00602 (Group 2, Table 1.2.3) suggesting that it would be advisable to determine incidence of allele variability of the MHC Class I locus in Australian feedlot cattle prior to finalising the choice for future vaccine development.

Over 1000 potential epitopes were generated against the viral pathogen BVDV1, selected following consultation with the MLA as the most relevant pathogen candidate since in Australia this pathogen is implicated as one of the primary risk factors associated with the development of BRD (Hay, Barnes et al. 2014) and there is relevant Australian sourced proteomic sequence data available for *in silico* analysis ; in-depth examination of these epitope sequences to establish probability of immunogenicity enabled selection of 15 putative candidates for future analysis (Table 1.2.3).

Although not the focus of our analysis, we have additionally generated immunogenic epitopes for the BRD pathogens Bovine herpesvirus (type 1.2), Bovine Parainfluenza-3 virus, Bovine respiratory syncytial virus, *Mannheimia haemolytica, Mycoplasma bovis, Pasteurella multocida* and *Histophilus somni,* however we feel that additional primary input using verified Australian protein sequences would improve this selection (Table 1.2.4).

Table 1.2.2 BVDV protein sequences isolated from Australian sourced samples.

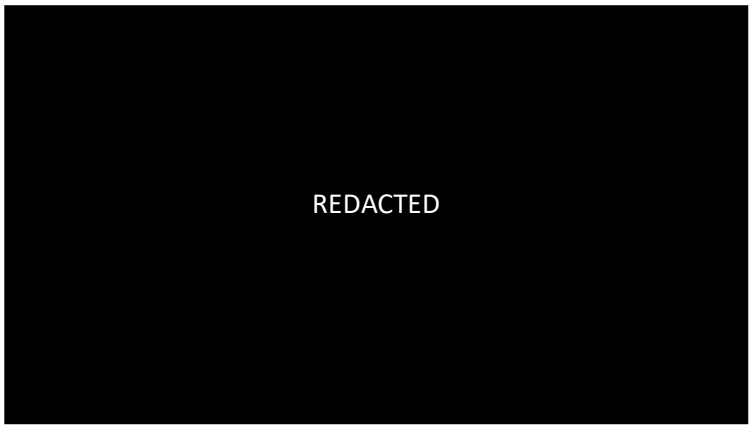


Table 1.2.3 Suggested epitopes for further analysis as potential BVDV vaccine candidates

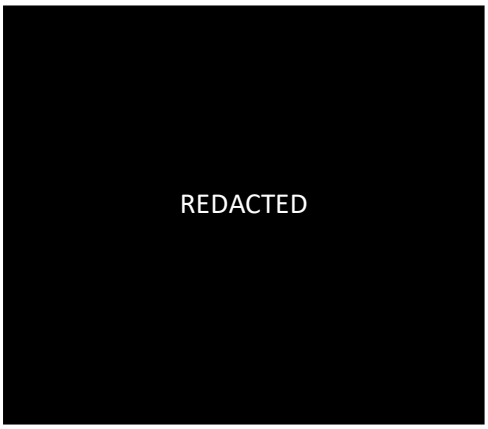
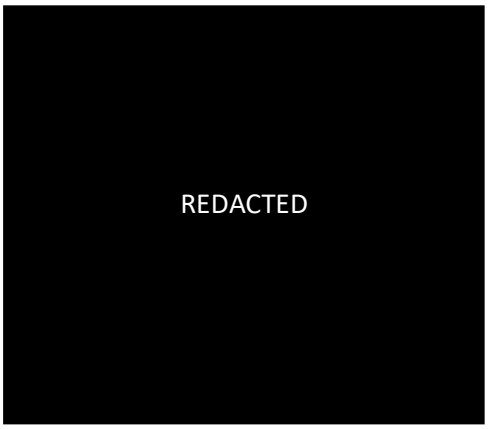


Table 1.2.4 Suggested epitopes for further analysis as potential BRD vaccine candidates



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Appendix 2: *In vitro* studies of cell activation by OvaYeast

1.1 Introduction

Initial studies were carried out to assess whether yeast and yeast expressing the reporter antigen (ovalbumin, ova) (OvaYeast) were able to interact with and activate cattle immune cells. Innate immune cells such as macrophages are able to capture and present antigens in a form that can activate adaptive immune pathways via lymphocytes. With a view to the yeast vaccine being administered via cattle feed, consideration was given to effects of heat during the flaking process, and to pH equivalent to rumen conditions on the yeast. Comparison of live and heat-inactivated yeast on macrophage activation was also evaluated.

1.2 Methods

In vitro assays using isolated primary bovine leukocytes from 3 healthy cattle were used to assess the ability of the recombinant yeast reporter-vaccine (OvaYeast) to activate an immune response. The dose and incubation time required for activation of cells was examined. The effects of heat inactivation and pH on innate immune cell activation were also assessed.

1.2.1 Bovine macrophage isolation and culture

Peripheral blood mononuclear cells (PBMC) were isolated from bovine peripheral blood collected into lithium heparin by density gradient centrifugation over Ficoll. Adherent monocytes were cultured in Dulbecco's Modified Eagle's Medium with supplementation with Macrophage Colony Stimulating Factor, bovine serum and antibiotics at 37°C in 5% CO_2. Differentiation into macrophages during culture (7-10 days) was monitored microscopically.

1.2.2 Exposure of macrophages to yeast *in vitro*

Optimising dose and incubation time for in vitro experiments

Monocytes were cultured under the following conditions:

Treatment	**Multiplicity of infection**	**Time of incubation**
No treatment	N/A	24 hrs, 48 hrs and 72 hrs
Live yeast	1:1 and 5:1	24 hrs, 48 hrs and 72 hrs
Live OvaYeast	1:1 and 5:1	24 hrs, 48 hrs and 72 hrs

Macrophage cultures were prepared from three Angus steers and incubated with the following treatments:

1. Culture medium (macrophages alone, negative control)
2. Unmodified yeast (*K. lactis*) at multiplicity of infection (MOI) of 1:1
3. Unmodified yeast (*K. lactis*) at (MOI) of 5:1
4. OvaYeast at MOI of 1:1
5. OvaYeast at MOI of 5:1
6. Lipopolysaccharide (LPS, positive control)

Each of the above treatments had two replicates for each animal. Culture supernatants and macrophages were collected at 24, 48 and 72 hrs after stimulation.

Nitric oxide secreted by macrophages was detected by the Griess assay and expression of iNOS and TNFα genes was detected by qPCR. Assessment of these activation parameters for each macrophage culture sample was carried out in duplicate.

NOTE: When used at 10:1 MOI, live OvaYeast multiplied rapidly in the macrophage cultures; therefore, the MOI for live OvaYeast was reduced to 1:1 and 5:1.

1.2.3 Effect of heat inactivation and pH on yeast responses

This data is from two experiments using cells from 3 Angus steers and includes four replicates per treatment per experiment. Lipopolysaccharide was included as a positive control in all experiments to ensure that the cultured macrophages had capacity to stimulate innate immune responses. Culture medium alone was included as a negative control.

Macrophages were plated into 96-well plates at 10^4-10^5/well and cultured with yeast which had been subjected to a variety of treatments:

1. Yeast was inactivated at 100°C for 10 minutes.
2. To mimic conditions of steam-flaked grains, inactivated yeast was incubated at 50°C for 12 and 24 hours.
3. Yeast was incubated at pH from 4, 6 and 8 for 24 and 48 hrs.

Effects of several multiplicities of infection (MOI) were also assessed. Viability of treated yeast was assessed by culture. A positive stimulation control (lipopolysaccharide, LPS) was included in all experiments.

Each experiment was carried out with cells from three animals. Culture supernatant and lysed monocytes were collected to measure nitric oxide secretion and expression of the gene for inducible nitric oxide synthase (iNOS) respectively. The production of nitric oxide via activation of the iNOS gene is an indicator of macrophage activation towards a pro-inflammatory phenotype, capable of stimulating adaptive immune responses that are required for vaccine memory (Wang et al. 2019).

1.2.4 Detection of nitric oxide

Nitric oxide production in macrophage cultures was measured in culture supernatants by the Griess assay (Sigma-Aldrich), according to the manufacturer's instructions.

1.2.5 Detection of gene expression

Macrophage cultures were lysed with RNAzol followed by RNA extraction and cDNA synthesis as described previously (Plain et al. 2010). Specific quantitative PCR assays for bovine iNOS, tumour necrosis factor-alpha (TNF-α), interleukin-10 (IL-10) and multiple reference genes (GAPDH, β-actin, PPIA and H3F3a) were applied to detect the expression of these genes.

1.2.6 Statistical analysis

For each treatment type data were subjected to a two-factor ANOVA with replication to determine effects of period of incubation (3 and 6 days) and treatments on nitric oxide secretion by

macrophages. Gene expression analysis was conducted using the common base method (Ganger et al. 2017).

1.3 Results

1.3.1 Effect of OvaYeast on macrophage activation

Phagocytosis

Bovine macrophages cultured in the presence OvaYeast phagocytose the yeast. As shown in Fig. 2.1B, these cells are larger compared to macrophages cultured in the absence of yeast (Fig 2.1A) and the circular yeast are seen intracellularly.

A. **B.**

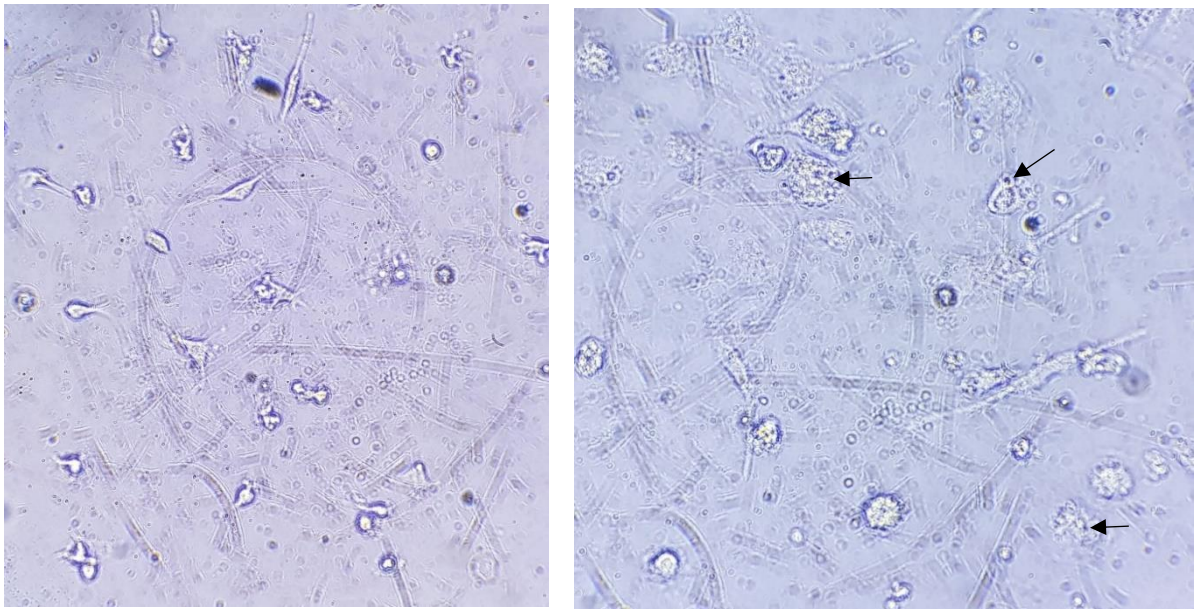

Figure 2.1 Bovine macrophages phagocytose OvaYeast
Bovine macrophages cultured in the absence (A) and presence (B) of OvaYeast. Some macrophages which have phagocytosed yeast are indicated by arrows.

1.3.2 Nitric oxide secretion

The nitric oxide response over time in individual animals is shown in Fig 2.2 and the combined response in Fig. 2.3. While LPS-induced nitric oxide responses were detected as early as 24 hrs after in vitro incubation, yeast and OvaYeast responses required longer incubation periods (Fig. 2.3). The effect of stimulant, time and their interaction were significant ($p \leq 0.0001$). At the 72hr time point, LPS and OvaYeast at 5:1 induced significantly higher ($p<0.05$) nitric oxide compared to cells alone. In addition, at this time point, OvaYeast at 5:1 induced significantly higher ($p<0.05$) nitric oxide compared to unmodified yeast at 1:1 or 5:1 and OvaYeast at 1:1 (Fig. 2.3).

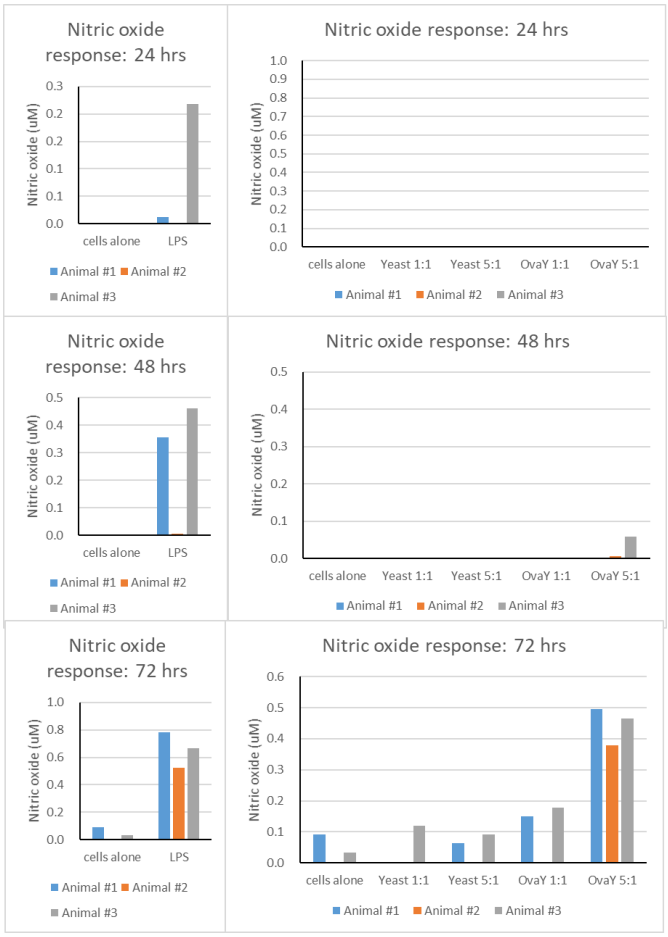

Figure 2.2 Nitric oxide response to yeast and OvaYeast for individual animals and timepoints. Bovine macrophages were cultured with no stimulant (cells alone) or with lipopolysaccharide (LPS) as a positive control or with nontransformed yeast or OvaYeast. Nitric oxide in cell culture supernatants is shown *p<0.05 compared to cells alone at the same time point; #p<0.05 compared to OvaYeast at MOI 5:1 at the same time point

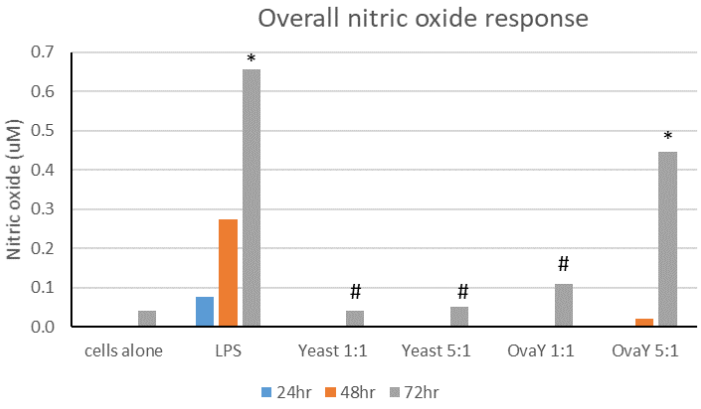

Figure 2.3 Overall nitric oxide response to yeast and OvaYeast
Bovine macrophages (n=3) were cultured with no stimulant (cells alone) or with lipopolysaccharide (LPS) as a positive control or with nontransformed yeast or OvaYeast. Nitric oxide in cell culture supernatants is shown *p<0.05 compared to cells alone at the same time point; #p<0.05 compared to OvaYeast at MOI 5:1 at the same time point

1.3.3 iNOS and TNFα gene expression

The macrophages gene expression responses to yeast control and OvaYeast are shown over time in individual animals is shown in Fig. 2.4 and 2.5, with overall responses shown in Fig. 2.6.

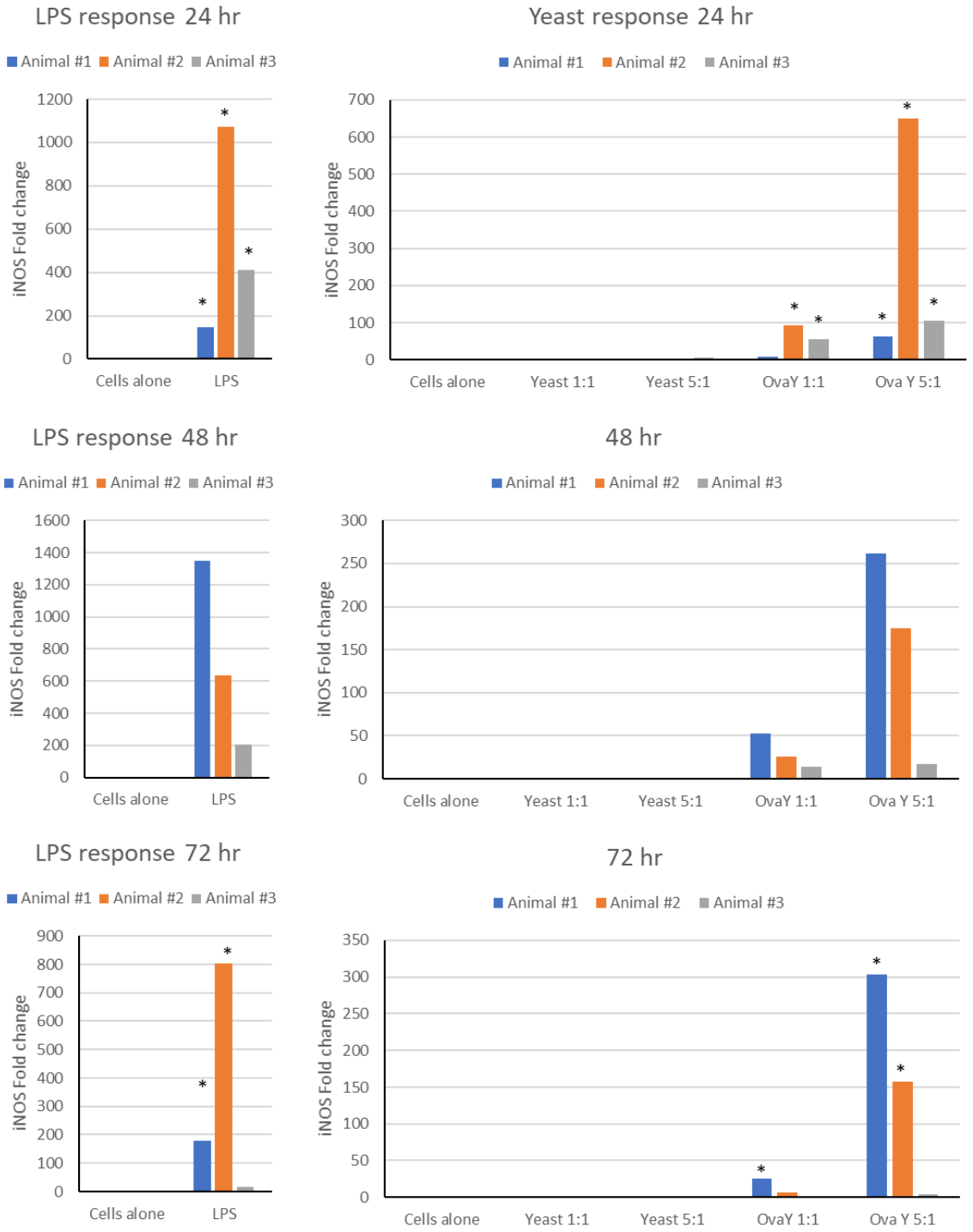

Figure 2.4 iNOS responses to yeast and OvaYeast for individual animals and timepoints. Bovine macrophages (n=3) were cultured with no stimulant (cells alone) or with lipopolysaccharide (LPS) as a positive control or with nontransformed yeast or OvaYeast. iNOS gene expression in

cultured macrophages is shown. *Significant differences (p<0.05) between treatment and cells alone of the respective animal and timepoint

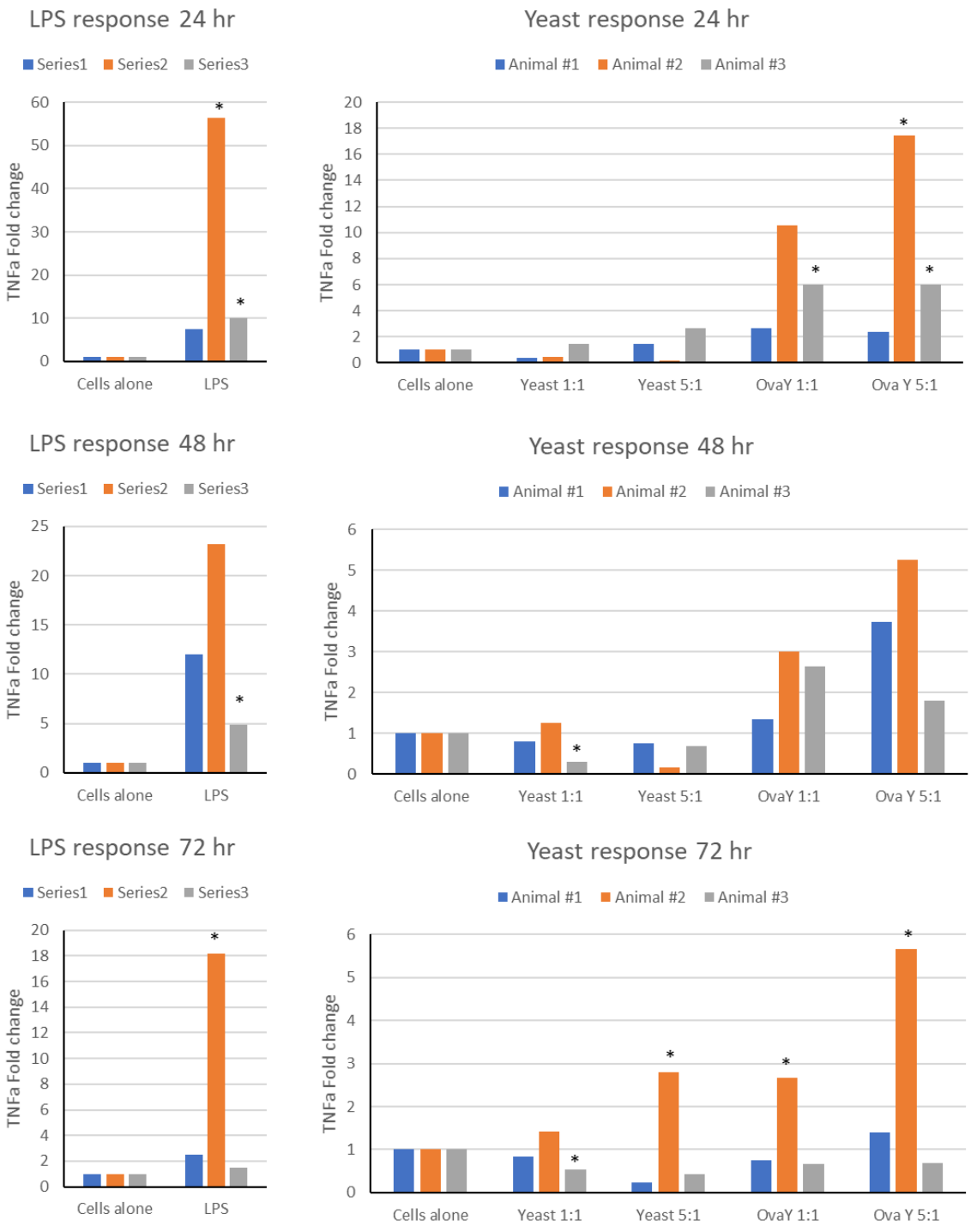

Figure 2.5 Individual TNFα responses to yeast and OvaYeast in individual animals
Bovine macrophages (n=3) were cultured with no *in vitro* treatment (cells alone) or with lipopolysaccharide (LPS) as a positive control or with nontransformed yeast or OvaYeast. TNFα gene expression in cultured macrophages is shown. *Significant differences (p<0.05) between treatment and cells alone of the respective animal and timepoint are shown.

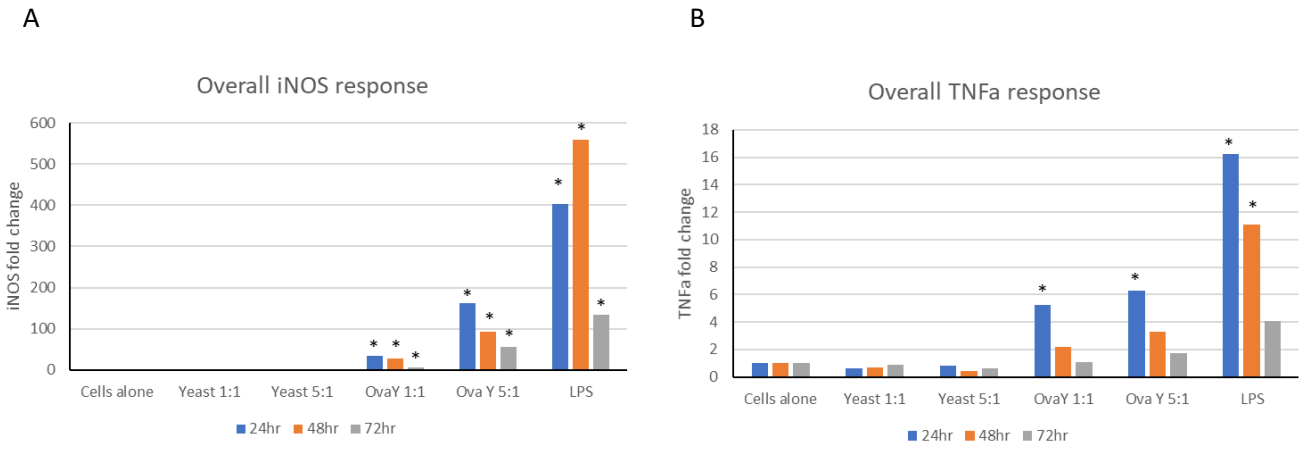

Figure 2.6 Overall iNOS (A) and TNFα (B) responses to yeast and OvaYeast
Bovine macrophages (n=3) were cultured with no stimulant (cells alone) or with lipopolysaccharide (LPS) as a positive control or with nontransformed yeast or OvaYeast. *Significant differences ($p<0.05$) between treatment and cells alone of the respective animal and timepoint are shown.

Overall, OvaYeast is increasing secretion of nitric oxide (Fig. 2.3) and upregulating the expression of TNFα and iNOS (Fig. 2.6) by macrophages, whilst there is no increase in secretion of nitric oxide or upregulation of gene expression in the presence of yeast. This demonstrates unequivocally that OvaYeast is immunostimulatory, and that this effect is specific to the OVA-modification.

The observation from initial experiments was that shorter periods of incubation did not elicit a strong nitric oxide response with LPS and the response to heat-inactivated OvaYeast was not measurable (Figs 2.3 and 2.7). Therefore, the incubation time with OvaYeast was increased to enable detecting nitric oxide from cultured macrophages.

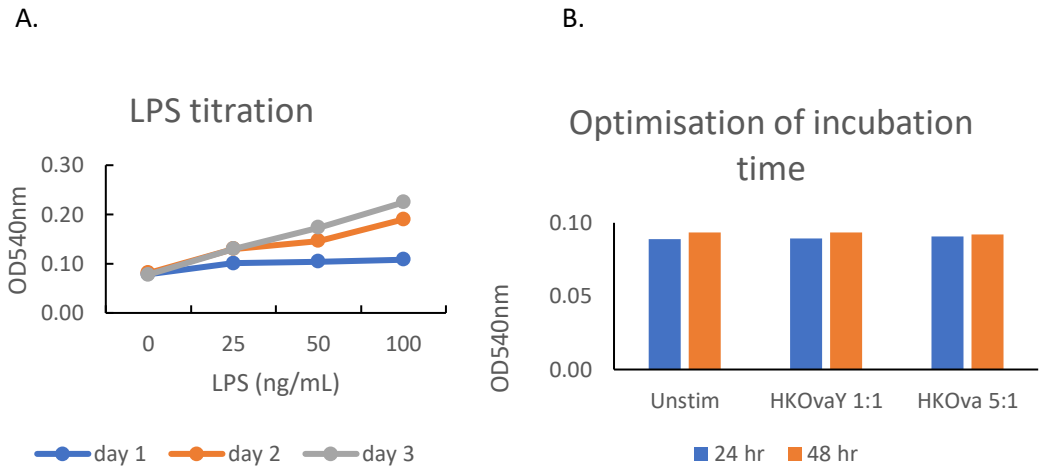

Figure 2.7 Culture of macrophages for less than 72 hrs of culture does not induce a strong nitric oxide response with either LPS (A) or heat-killed OvaYeast (B).

While there was no detectable increase in the nitric oxide response earlier than 72 hrs after exposure, this does not mean that there was no biological response prior to this time point. Microscopically we are able to visually see that the yeast is taken up by macrophages within 24 hrs suggesting that cellular interactions and activation has been initiated. Fig 2.6 shows that gene expression changes as a result of macrophage activation by live OvaYeast can be detected at earlier time points.

LPS treatment induced a significant nitric oxide response (Fig. 2.8) compared to unstimulated cells ($p<0.05$); however, neither time nor its interaction with treatment were significant. This indicates that under our *in vitro* conditions, the cultured bovine macrophages were able to become activated.

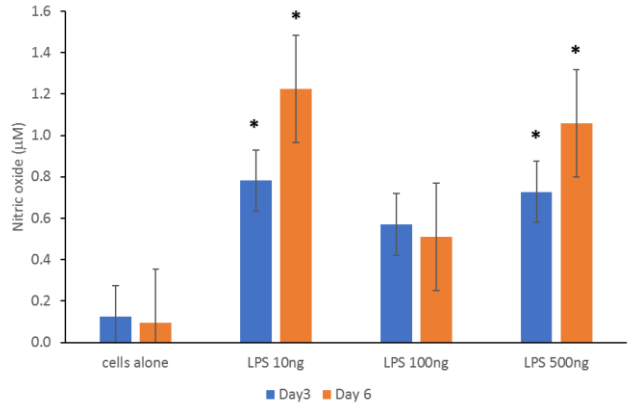

Figure 2.8 Activation of bovine macrophages in response to lipopolysaccharide (LPS)
Bovine macrophages (n=3) were cultured with no stimulant (cells alone) or with lipopolysaccharide (LPS) as a positive control at 10-500 ng/mL. *Significant differences ($p<0.05$) compared to the sample with cells alone at the relevant incubation time are shown.

The ability of live and inactivated OvaYeast to activate macrophages was assessed at two MOI. Initial experiments demonstrated that an MOI of 10:1 of live OvaYeast the macrophages were unable to phagocytose all OvaYeast in the culture medium (Milestone 3 Report Appendix D Figure D1). Therefore, live OvaYeast was used at a lower MOI than inactivated organisms. Heat-killed OvaYeast at an MOI of 1:1 did not induce elevated levels of nitric oxide. Fig. 2.9 shows that exposure of macrophages to yeast, either live or heat-killed, induced secretion of nitric oxide compared to unstimulated macrophages. There were no significant differences between live and heat-killed OvaYeast at any of the MOI assessed ($p>0.05$).

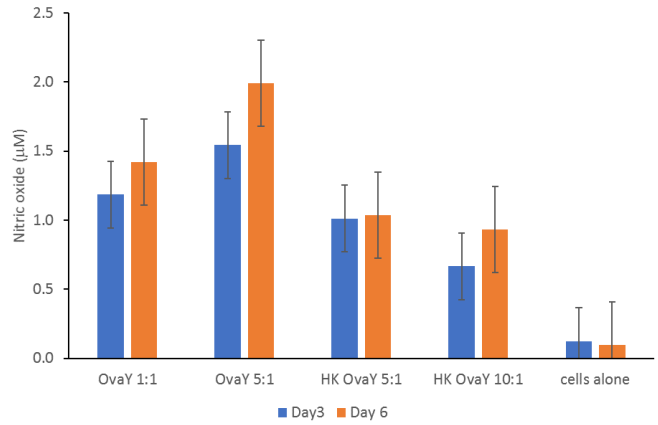

Figure 2.9. Activation of bovine macrophages by live or heat-killed yeast
Bovine macrophages (n=3) were cultured with no *in vitro* treatment (cells alone) or with lipopolysaccharide (LPS) as a positive control or with live OvaYeast (OvaY) or heat-killed OvaYeast (HK OvaY). Nitric oxide secretion is shown as a measure of macrophage activation.

1.3.4 Effect of pH and heat treatments on OvaYeast viability

OvaYeast was successfully heat-inactivated by incubating at 100°C as no viable organisms were detected by culture for up to 7 days. Similarly, steam-flaking conditions at 50°C were also sufficient to inactivate the yeast. When OvaYeast were pre-incubated at pH 4, 6 and 8 the organisms remained viable. The OvaYeast at pH 4 and 8 were viable with no change in growth characteristics.

Nitric oxide secretion by macrophages exposed to 1:1 live 'flaked' OvaYeast was significantly lower than live OvaYeast (Fig. 2.10) ($p<0.05$). At the higher MOI, nitric oxide secretion was not significantly different between flaked and unflaked OvaYeast (Fig. 2.10). With heat-killed 'flaked' and unflaked OvaYeast, there were no significant differences between any of the treatment groups (Fig. 2.11).

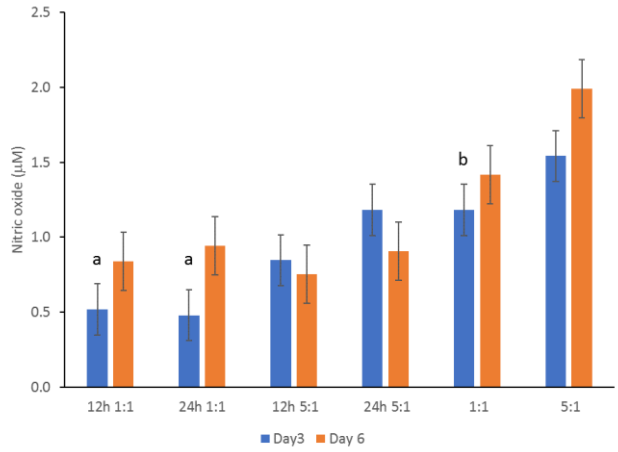

Figure 2.10 Effect of live 'flaked' OvaYeast on macrophage activation
Live OvaYeast were heat-treated at 50°C for either 12 or 24 hrs to simulating steam-flaking conditions prior to culturing with macrophages. Different letters above bars indicate a significant

difference ($p<0.05$). Data from live OvaYeast not subjected to 'flaking' simulations are shown in the two groups on the right (1:1 and 5:1).

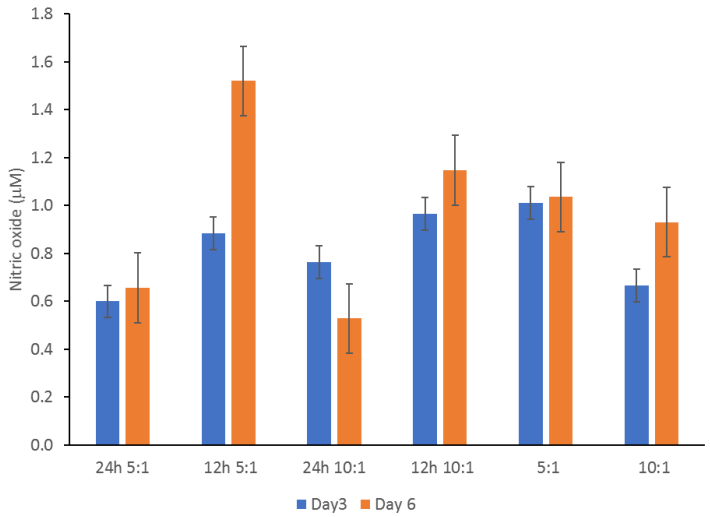

Figure 2.11 Effect of heat-killed 'flaked' yeast on macrophage activation
Heat-killed OvaYeast were heat-treated at 50°C for either 12 or 24 hrs to simulate steam-flaking conditions prior to culturing with macrophages. Data from heat-killed OvaYeast not subjected to 'flaking' simulations are shown in the two groups (5:1 and 10:1) on the right.

At an MOI of 1:1, nitric oxide secretion was significantly lower ($p<0.05$) with live OvaYeast at both pH 4 and 8 compared to OvaYeast at pH 7.4 after 3 days of incubation (Fig. 2.12). This effect was not observed at the 6-day timepoint nor at an MOI of 5:1. For heat-killed OvaYeast, subjecting to pH variation did not induce any significant differences in nitric oxide production from bovine macrophages (Fig. 2.13). In addition, activation of macrophages by heat-killed or live OvaYeast subjected to pH variation was not significantly different.

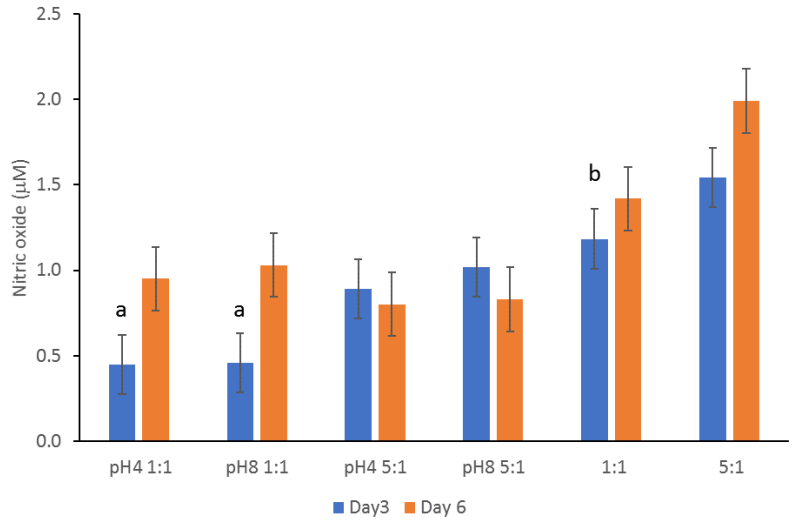

Figure 2.12 Effect of live OvaYeast subjected to pH variation on macrophage activation

Live OvaYeast were incubated at pH 4 or 8 prior to culturing with macrophages. Different letters above bars indicate a significant difference ($p<0.05$). Data from live OvaYeast not subjected to pH treatment are shown in the two groups on the right.

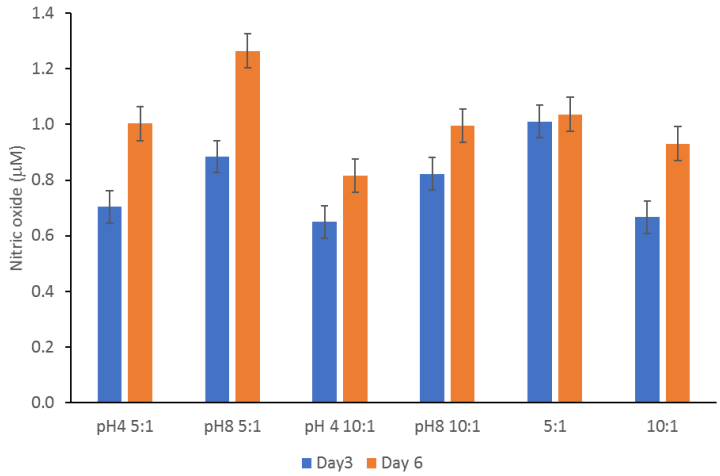

Figure 2.13 Effect of heat-killed OvaYeast subjected to pH variation on macrophage activation
Heat-killed OvaYeast were incubated at pH 4 or 8 prior to culturing with macrophages. Data from heat-killed OvaYeast not subjected to pH treatment are shown in the two groups on the right.

1.3.5 iNOS and TNFα gene expression

Macrophage activation was also assessed by the induction of genes in innate immune response pathways, specifically inducible nitric oxide synthase (iNOS) and tumour necrosis factor alpha (TNFα). Results from one of two representative experiments are shown in Fig. 2.14.

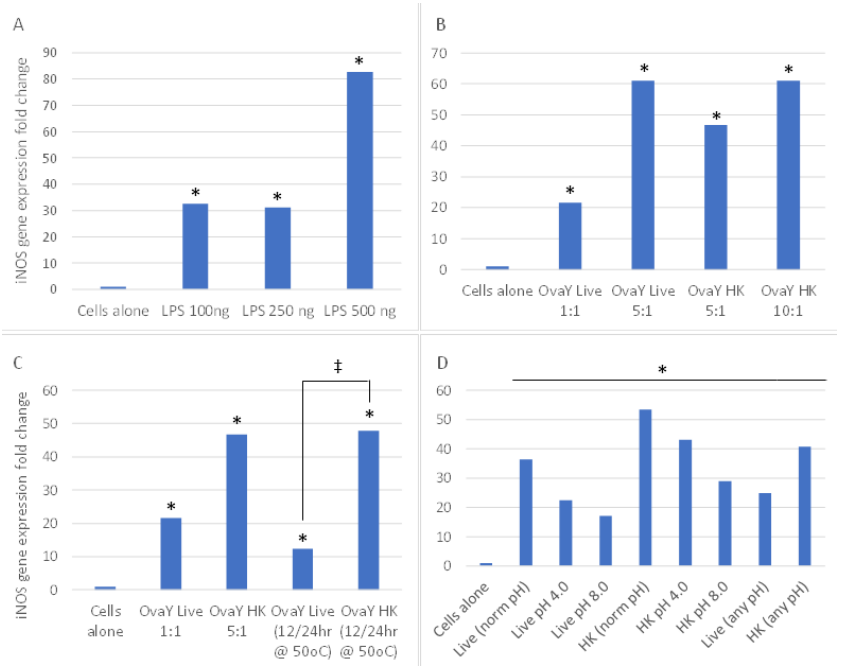

Figure 2.14 Expression of inducible nitric oxide synthase (iNOS) in bovine macrophages.
Bovine macrophages were cultured with OvaYeast (OvaY) were subjected to a variety of treatments for 3-6 days prior to extracting cellular RNA. Varying LPS concentrations were included as the positive control (A), and cells alone were the negative control. Gene expression induced by live and heat-killed (HK) OvaY (B), 'flaking' pre-treatment (C) and pH pre-treatment (D) are shown. *indicates a significant difference compared to cells alone ($p<0.05$). ‡ indicates a significant difference between the two bracketed groups ($p<0.05$).

iNOS gene expression was significantly lower in macrophage cells alone (Fig. 2.14) compared to all other treatments ($p<0.05$). Both live and heat-killed OvaYeast, independent of the pH or 'flaking' pre-treatment, induced iNOS gene expression in bovine macrophages (between 10 and 50-fold increased expression). Increasing LPS concentrations led to higher expression levels of iNOS, as expected. There was no difference between live and heat-killed OvaYeast in the induction of iNOS for any condition, with the exception of live (12 or 24 hrs at 50°C) compared to heat-killed OvaYeast (12 or 24 hrs at 50°C); the heat-killed OvaYeast induced higher levels of iNOS expression.

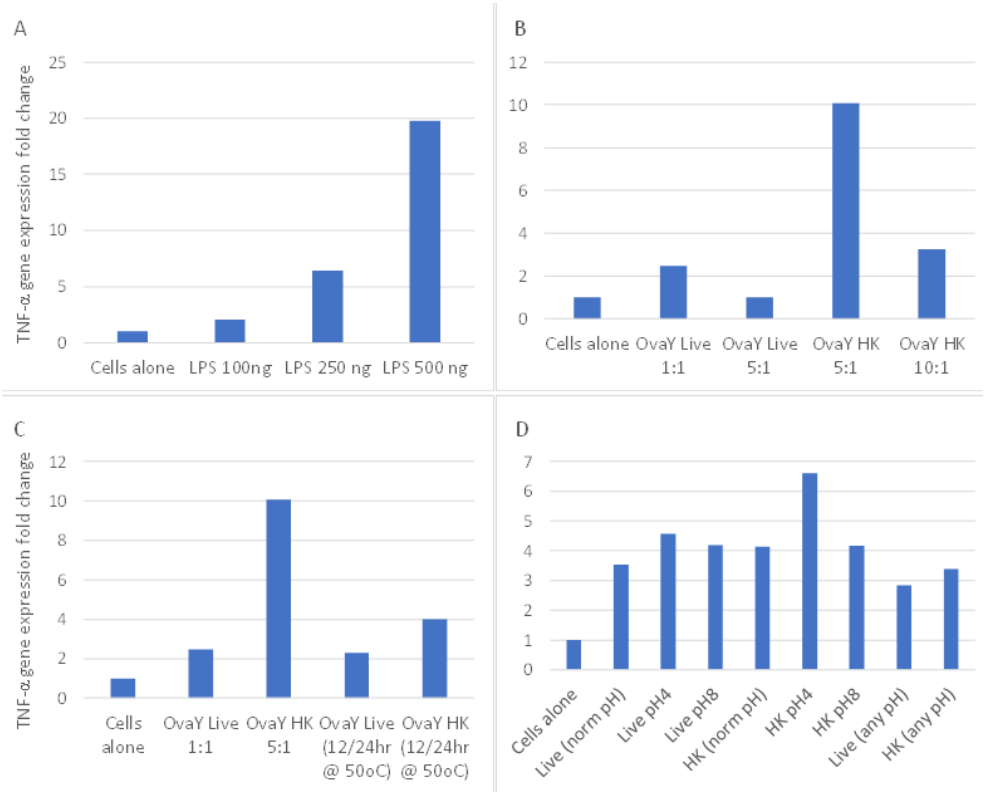

Figure 2.15 Expression of tumor necrosis factor alpha (TNFα) in bovine macrophages
Bovine macrophages were cultured with OvaYeast (OvaY) were subjected to a variety of treatments for 3-6 days prior to extracting cellular RNA. Varying LPS concentrations were included as the positive control (A), and cells alone were the negative control. Gene expression induced by live and heat-killed (HK) OvaY (B), 'flaking' pre-treatment (C) and pH pre-treatment (D) are shown.

TNFα is another cytokine expressed by activated macrophages. Gene expression changes induced by OvaYeast and its various pre-treatments are shown in Fig 2.15. As for iNOS gene expression levels, increased LPS concentrations led to a trend towards increased TNF-a gene expression, though this did not reach statistical significance ($p>0.05$). Heat-killed OvaYeast tended to induce higher expression levels of TNF-a than live, though no specific trends in relation to pre-incubation at 50°C or at different pHs were seen.

1.4 Discussion

These initial *in vitro* studies have confirmed at the cellular and molecular level that bovine macrophages have the capacity to be activated by OvaYeast. Overall, OvaYeast increases secretion of nitric oxide and upregulates the expression of TNFα and iNOS by macrophages, whilst there is no increase in secretion of nitric oxide or upregulation of gene expression in the presence of unmodified yeast. This demonstrates unequivocally that OvaYeast is immunostimulatory, and that this effect is specific to the OVA-modification.

Importantly, there were no significant differences in this capacity between live and heat-inactivated OvaYeast. Extensive heat treatments or pH variation did not reduce the effect of heat-inactivated OvaYeast on bovine macrophages. These results indicate that heat-inactivated yeast has the potential to activate immune response pathways.

1.5 References

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Appendix 3: Pilot study to assess the potential of an oral yeast vaccine to induce immune responses *in vivo* in beef cattle

1.1 Introduction

The mucosa which lines the respiratory and intestinal tracts is the physical barrier between the between the host and its environment. It comprises of epithelial cells, and innate and adaptive immune cells (Perez-Lopez et al. 2016). The largest reservoir of immune cells is found within the mucosal immune system. These immune cells are play a pivotal role in mucosal immunity, sampling the environment for pathogens and are ready to respond to pathogen challenge. Manipulating these immune cells to 'remember' a pathogen through vaccination is a prudent strategy for ensuring better protection against disease. Currently most vaccines are administered either subcutaneously or intramuscularly and this induces systemic immune responses. Effector T cells and immunoglobulins produced during mucosal immune stimulation differ from those generated from systemic immune stimulation; therefore, effective mucosal vaccination requires targeted delivery of the antigen to the mucosa (de Silva 2021). There is a need for developing mucosal vaccines.

While mucosal immune cells prefer to home to the site of activation, they can migrate between different mucosal sites (for example between the respiratory and gut-associated mucosal tissues) as part of a common mucosal immune system (Holmgren et al. 2005). For a livestock vaccine to be readily adopted by producers, the method of vaccine administration is an important consideration. Thus, in this study, oral delivery was chosen as the preferred option. For ruminants, this can be challenging since antigen modification may occur in the rumen prior to encountering intestinal immune cells.

Vaccines protect against infection or disease by activating adaptive immune pathways (humoral and/or cell-mediated) and inducing memory of the pathogen. Upon vaccination, immune cells (lymphocytes) become activated and undergo proliferation resulting in clonal expansion of cells that are able to recognise the pathogen. Some of these pathogen-specific lymphocytes become long-lived memory cells. It is the presence of these cells that enables rapid reactivation of immune cells during subsequent pathogen exposure. If vaccine-mediated generation of immune memory has been successful, these cells can be reactivated under laboratory conditions (in vitro). When cells are cultured in the presence of vaccine antigens, vaccine-induced memory cells will become activated in a similar manner to when the animal is exposed to the pathogen and these parameters can be measured.

Lymphocyte proliferation can be used as a measure of cell-mediated memory responses (de Silva et al. 2010). Lymphocytes consist of a diverse group of cell subsets originating from a common precursor; however, each subset can be identified by specific phenotypic and functional characteristics. T lymphocytes express the CD3 molecule on the cell surface; these cells can be categorised into several subsets including cytokine-secreting T helper cells (CD4+) and cytotoxic T cells (CD8+). B cells mediate secretion of specific antibodies and can be characterised by cell surface markers such as CD21. $\gamma\delta$ T cells are a unique T cell subset which are rapid responders to infection. The molecule CD45RA is expressed on naïve T cells while antigen-activated memory T cells express the CD45RO isoform. To detect lymphocyte proliferation, isolated cells can be labelled with a tracking dye (carboxy fluorescein diacetate succinimidyl ester; CFSE) that is readily able to enter cells. Viable cells process the dye into a form that is fluorescent and is unable to exit the cell.

Therefore, viable cells can be detected as cells with high intensity fluorescence. When the labelled cells proliferate, the fluorescent intensity is halved in each subsequent generation and hence proliferation is associated with dimmer fluorescence. To identify proliferation in specific lymphocyte subsets, cell surface markers can be used in conjunction with the intracellular dye identified using a separate fluorescent tag.

The immune changes associated with cellular activation that occur due to vaccination are also measurable at the level of gene expression. Essentially, gene expression is a measure of functional aspects of the genetic code written on our DNA. Gene expression studies enable us to determine when and where genes are turned on and off, enabling an understanding of an organism's response to changing conditions. Interferon-gamma (IFN-γ) is a key cytokine in the cell-mediated immune response. It is important for adaptive immunity, as triggered by a vaccine. The downstream actions of IFN-γ include increased antigen processing and presentation, upregulation of pathogen recognition, activation of microbicidal effector functions and antiviral responses, and leukocyte trafficking (Schroder et al. 2004). The chemokine CXCL10 is involved in T cell helper 1 mediated adaptive immune responses (Metzemaekers et al. 2018). Due to its functional properties, it was previously known as IFN-γ-inducible protein 10 (IP-10). Tumour necrosis factor alpha (TNF-α) and interleukin (IL)-10 and two cytokines produced by macrophages, natural killer cells and dendritic cells (DC) that are associated with innate immune responses and antigen presentation to lymphocytes. TNF-α is associated with pro-inflammatory macrophage responses, whereas IL-10 is associated with anti-inflammatory responses (Benoit et al. 2008). Upon activation, antigen presenting cells such as macrophages, DCs and also B cells, upregulate the expression of the co-stimulatory molecules CD80 and CD86, which bind to CD28 on T cells (Sharpe et al. 2002). CD80-mediated co-stimulation preferentially favours cell-mediated immunity and T helper 1 cell differentiation. CD69 is a marker of lymphocyte activation and its expression on the cell surface is increased via transcriptional activation (Vazquez et al. 2009). CCR7, a lymphoid homing receptor present on the cell surface of lymphocytes, has been utilised in cattle as a marker of T central memory cell subsets (Maggioli et al. 2015); this cell type is induced in response to successful vaccination, reside in the lymphoid tissues and are rapidly activated in response to target antigens re-exposure.

In this study, a recombinant yeast vaccine construct containing a 'reporter' molecule, ovalbumin (Ova), was evaluated in this proof-of-concept study as a vaccine delivery platform that would be able to stimulate mucosal immune responses in cattle. The study design incorporated groups of cattle given either the OvaYeast recombinant yeast vaccine, or nontransformed yeast which did not contain the reporter molecule. In addition, two doses of the OvaYeast were assessed. The induction of cell-mediated memory responses by the OvaYeast vaccine was assessed by comparing the proliferation of circulating and lymph node resident lymphocytes against the reporter antigen (Ova). We assessed proliferative responses of total lymphocytes as well as the lymphocyte subsets described above. The induction of humoral adaptive immune responses by the OvaYeast vaccine was evaluated by detecting the presence of Ova-specific antibodies. Gene expression changes (IFN-γ, CXCL10, TNF-α, IL-10) were also assessed in tissues of the mucosal immune system.

1.2 Methods

1.2.1 Animals and general husbandry

Thirty Angus steers (25 Angus and 5 Angus-Hereford) from the Southern Tablelands, NSW region aged 8-9 months were transported to the University of Sydney farms at Camden, NSW and allowed to acclimatise for more than 7 days. Weight range of animals at purchase was 295-362 kg. Animal husbandry practices for all trial animals held at University of Sydney farms cover a wide range of activities which prioritise animal welfare, health and hygiene and were also adhered to for this study. Cattle were co-mingled in one paddock for the 'on-feed' portion of the trial where they had access to water, pasture and a feed pad. After completion of the feeding phase of the trial, cattle were rotationally grazed across several different paddocks to meet pasture demand and had access to water. Co-mingling of the cattle in all groups was to be representative of a single cohort inducted into the feedlot and to ensure that variables were kept constant between treatments. The feed pad had a large feed trough allowing for 30 cm of head room per animal following guidelines for required bunk headroom for feedlot cattle in Australia.

During the grain feeding period, cattle were fed once daily in the morning at about the same time. Following feeding, residual feed was removed and discarded. To ensure cleanliness, the bunk was swept out and/or hosed as needed. The use of a feed pad also ensured that animals could be excluded from the area during the day to prevent soiling prior to feeding and ensure optimal bunk hygiene. Water troughs were checked daily for function and cleanliness and were cleaned as required.

Steers were weighed weekly during the feeding period and then each time the animals were brought through the yards for sampling. Cattle scales were calibrated prior to each use by using calibrated weights.

1.2.2 Selecting vaccine dose

To estimate an appropriate vaccine dose, a literature search was carried out using Scopus and PubMed (NLM) using the key words 'yeast' AND 'cattle' AND 'immune' with the year of publication unrestricted. This retrieved a total of 132 publications from 1964 to 2019. None of these studies had used *K. lactis*, the yeast used in this project; the majority had used *Saccharomyces cerevisiae.* In addition, there were no publications on the use of yeast as a vaccine. For the majority of publications, the immune parameter being evaluated was acute phase proteins. In total there were 15 publications that had included adult cattle (heifers or steers), yeast or a yeast fermentation product and measures of immune performance. Publication dates for these studies ranged from 2007-2019.

Five of these publications were excluded due to using yeast fermentation products rather than live or inactivated whole yeast. Five papers had information on colony forming units (CFU) as the dose (Guedes et al. 2008; Chaucheyras-Durand et al. 2016; Adeyemi et al. 2019; Crossland et al. 2019; Williams et al. 2019). Several studies had examined the effects of yeast supplementation on dry matter intake (DMI), feed conversion ratio (FCR) and average daily gain (ADG) in both beef and dairy cattle (Table 3.1). In contrast to our study design, these studies utilised dried yeast either as part of a commercial supplement or by itself, which was top-dressed onto feed daily for up to 8 weeks. Dosages had been calculated based on CFU of yeast present per gram of feed, with one study feeding as much as 6.2×10^{11} cfu/g per day. Effects on DMI, FCR and ADG have been noted in

animals that were supplemented with yeast in the diet, however only one study assessed the effects on basic immunological parameters (Adeyemi et al. 2019). In this study, a commercial yeast supplement (Select-TC, Alltech) was utilised and specific yeast quantities were not disclosed; usage was report only grams of supplement fed.

In the relevant studies, the highest daily doses were $1x10^{10}$, $1x10^{10}$, $6.2x10^{11}$, $6x10^{10}$, $3x10^{10}$ per animal per day. The average CFU dosage was $1.46x10^{11}$/h/d and the median CFU $3x10^{10}$ per head per day.

In a meta-analysis which examined 12 studies between 1994 and 2014, the average dose was $1.27x10^{9}$ CFU/h/d and the median $5.5x10^{8}$/h/d or on average 11.7 g/h/d with a median of 10 g/h/d (Sartori et al. 2017).

Based on the literature review, only one (Adeyemi, Harmon et al. 2019) study assessed the impact of yeast on immune factors and utilised a dosage in CFU, all other studies assessed effect on factors such as on dry matter intake, feed conversion ratios or other production traits. Therefore, even though there have been many previous studies published on feeding yeast and yeast derivatives to feedlot cattle, the differences in the yeast administration and the parameters assessed mean that dosages are only guidelines and cannot accurately inform dosage selection for the current vaccine study. However, the ability of the dosages stated in the studies to affect production parameters were considered in our decision. With estimated starting weights of 300 kg for the cattle in our study, the dose of recombinant yeast should be at least $3.3x10^{7}$ CFU/kg of body weight per day for the single dose group.

There were four studies that used live or inactivated whole yeast where the dose was in g/h/d with no information of CFU (Keyser et al. 2007; Van Bibber-Krueger et al. 2016; Kayser et al. 2019; Kayser et al. 2019). The highest daily doses were 0.5 g, 3.3 g, 25 g, 62.5g/h/d. The average was 22.8 g/h/d and the median 14.2 g/h/d.

Table 3.1 Published studies on the effect and dosage of yeast supplementation on cattle performance

Publication	Cattle type	No. animals	Dosage(s)
(Jiang et al. 2017)	Dairy cows (lactating)	4 groups of 4	Low dose live yeast (5.7×10^{7} CFU/cow per day; LLY), a high dose of live or killed yeast (6.0×10^{8} CFU/cow per day; HLY)
(Chaucheyras-Durand et al. 2016)	Dairy cows (non-lactating)	6	4-week acclimatisation then yeast daily for 4 weeks at $1x10^{10}$ CFU per day
(Guedes et al. 2008)	Dairy cows (non-lactating)	3	3 doses (0, 0.3 or 1 g) of $1x10^{10}$ CFU/g fed daily for 30 days

(Adeyemi, Harmon et al. 2019)	Newly weaned beef (Angus cross) steers	40	Basal diet or basal diet top dressed with 19 g commence feed additive (6.2×10^{11} CFU/g of S. cerevisiae) daily for 42 days
(Pukrop et al. 2018)	Mixed breed steers	80	Control or hydrolysed mannan and glucan rich yeast fraction (13g/steer daily) daily for 56 days

Kluyveromyces lactis, has been used as an oral vaccine in mice (Arnold et al 2012) at rate of 5% (w/w) of the feed. Animals were given the vaccine orally once per day for two days and repeated again 2 days later.

Using all of the above information as a general guide, we estimated 10^{10} CFU of OvaYeast per dose for the *in vivo* trial. OVA-yeast quantification by CFU provides an accurate measure of the dose and allows for easy delivery of an exact dose.

1.2.3 Vaccine safety

Vaccine safety was assessed initially in two animals. The steers were given one dose of the vaccine (10^{10} CFU of OvaYeast) orally and monitored for two weeks for any adverse effects. These animals were the source of blood cells for *in vitro* experiments (reported in Appendix 2). The two cattle were not included in the *in vivo* trial described in Section 3.2.4.

1.2.4 Study design

The protocol was approved by the University of Sydney Animal Ethics Committee (Protocol No. 2019/1582) and the *in vivo* use of biological materials by the Department of Agriculture, Water and the Environment (Approval No. 2020/042).

The animals were allocated to the three groups (Fig. 3.1) based on weight at the start of the trial. These groups will be identified as 'treatment groups' to avoid confusion with 'stimulants' for in vitro experiments described later.

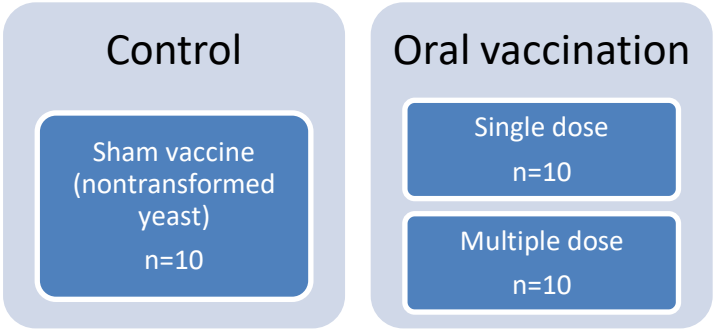

Figure 3.1 Experimental groups for establishing proof of principle in the *in vivo* phase of the project

Each treatment group was given either one (Single) or three (Multiple) doses of OvaYeast or one dose of nontransformed yeast (Control), orally. Each dose contained 10^{10} organisms.

Either yeast or OvaYeast was given orally, with delivery directly into the oesophagus of the animal using plastic tubing and a syringe to ensure the entire dosage was swallowed and not aspirated (n=10 per group). Each dose contained 10^{10} Yeast (Control group) or OvaYeast (Single and Multiple dose groups) in 10 mL of PBS. The first dose was given to all animals on day 0 and the Multiple dose group received additional doses on days 6 and 13 amounting to a total of $3x10^{10}$ OvaYeast for that group. Multiple doses were to administer two different total doses of the vaccine, rather than to deliver a booster vaccination. Internal secretion of ovalbumin (ova) protein in the vaccine doses was confirmed by Western blot analysis (Appendix 5).

For the first 3 weeks from the time of the primary dose, cattle were fed a backgrounding diet (Beef Grower Pellets, MSM Stockfeeds) which was approved, and a step-up feed regimen provided (Table 3.2), by MLA (Joe McMeniman). Residual feed was cleaned out daily and feed quantity was not increased until the feed had been consumed completely. The cattle were held on pasture and had *ad libitum* access to pasture both during and following the completion of the Grower pellet feeding period.

All three groups were monitored for 5 months post primary dose of vaccine.

Table 3.2 MSM Grower pellet starter chart

DM (Dry matter)	Nem (Net Energy of maintenance	MMEF (Multiple of Maintenance Energy Factor)	Day of Feed	Feed per animal based on starting weight of 360 kg
87.2	2.0015	1	0	3.6
87.2	2.0015	1.1	1	4
87.2	2.0015	1.2	2	4.4
87.2	2.0015	1.3	3	4.8
87.2	2.0015	1.4	4	5.2

87.2	2.0015	1.5	5	5.5
87.2	2.0015	1.6	6	5.9
87.2	2.0015	1.7	7	6.3
87.2	2.0015	1.8	8	6.7
87.2	2.0015	1.9	9	7.1
87.2	2.0015	2	10	7.5
87.2	2.0015	2	11	7.5
87.2	2.0015	2.1	12	7.9
87.2	2.0015	2.1	13	7.9
87.2	2.0015	2.2	14	8.3
87.2	2.0015	2.2	15	8.4
87.2	2.0015	2.3	16	8.8
87.2	2.0015	2.3	17	8.8
87.2	2.0015	2.4	18	9.2
87.2	2.0015	2.4	19	9.2
87.2	2.0015	2.5	20	9.7
87.2	2.0015	2.5	21	9.7

1.2.5 Sampling of animals

Blood samples were collected and individual weights recorded at 1 and 2 months after the first dose. Blood was collected into tubes without anticoagulant and allowed to clot in order to obtain serum samples. The serum was aliquoted and stored at -20^{o}C until required. For cellular immune assays (lymphocyte proliferation and whole blood stimulation), blood was collected into lithium heparin containing blood collection vacutainers and processed immediately upon arrival at the laboratory (1-3 hours).

At the termination of the trial at 5 months post primary dose, the animals were subjected to a full necropsy. On the day of the necropsy, blood and faecal samples were collected and blood was processed as above. Faeces was kept at 4^{o}C and stored at -80^{o}C immediately upon arrival at the laboratory. Tissue samples were collected at necropsy from multiple locations: lymph nodes (LN) from the ileal and jejunal regions of the intestine, the retropharyngeal LN, a section of the spleen and a section of the ileum that was tied off to retain the contents.. The lateral retropharyngeal LN (Fig. 3.2) was selected as this drains the oral and nasal mucosal surfaces and forms part of the respiratory mucosal immune system.

For RNA extraction, a section of each LN was cut and immediately placed in DNA/RNA shield (Zymo) to preserve the RNA, such that the tissue:liquid volume was at minimum 1:9.

For intestinal mucus samples, the ileal section was opened and the contents were washed from the section using sterile phosphate buffered saline (PBS). A sterile paddle pop stick was used to gently scrape the mucus lining the ileal section and this was transferred to a sterile tube containing PBS and stored at -20^{o}C.

1.2.6 Cell isolation

Lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation, as previously described (de Silva, Begg et al. 2010). Briefly, blood collected from the jugular vein into

lithium heparin was centrifuged to harvest buffy coats. These were diluted in PBS and layered over Ficoll and centrifuged for separation of mononuclear cells. The harvested white blood cells were resuspended in RPMI 1640/10% foetal bovine serum (FBS) (culture medium).

Lymph node and splenocytes

Sections (1–2 cm^2) of retropharyngeal (RLN), ileal (ILN), posterior jejunal (JLN) and spleen were collected into culture medium and processed immediately. The tissue was diced using a scalpel blade to release lymph node cells or splenocytes into the medium and the suspension strained through a 70 μm filter.

1.2.7 Lymphocyte proliferation

Total cell proliferation

PBMC or lymph node cells were labelled with the fluorescent tracking dye CFSE (5 mM), as previously described (de Silva, Begg et al. 2010). Triplicate aliquots of cells were then cultured in medium alone, or various stimulants (ovalbumin, yeast, OvaYeast) for 5 days at 37°C in 5% CO_2. Pokeweed mitogen (PWM) was included to evaluate general proliferative capacity of the cells.

At the end of the culture period, samples were fixed in 1% paraformaldehyde and stored at 4°C until data were acquired on a high throughput flow cytometer (Guava EasyCyte) and analysed using guavaSoft software (Luminex).

Subset proliferation

After 5 days of culture, replicate wells used for the total proliferation assay were labelled with cell surface markers to determine the phenotype of proliferating lymphocyte subsets. Culture medium was removed and these cells were incubated with antibodies to CD3 (a T cell marker), CD4 (T helper), CD8 (cytotoxic T), CD21 (a B cell marker), 86D (a γδ T cell marker) or CD45RO (a memory T cell marker) and then with a detection antibody conjugated to allophycocyanin (anti-IgG APC). Flow cytometric data were acquired on a high throughput system on a Guava EasyCyte and analysed using guavaSoft software (Luminex).

Flow cytometric data analysis

The lymphocyte population was selected based on cell size and granularity in a dot plot (Fig. 3.2 A) and subsequent analysis was on this gated population. For total lymphocyte proliferation, CFSE labelling was evaluated on data from the green fluorescence channel; nonproliferating cells do not lose CFSE retaining high fluorescence intensity (Fig. 3.2 B) while proliferating cells are dimmer (Fig. 3.2 C). For lymphocyte subset proliferation, the subset was identified on the second red fluorescence channel (Fig. 3.2 D). Proliferating and nonproliferating subset-specific cells were identified by CFSE intensity in the upper half of the dot plot.

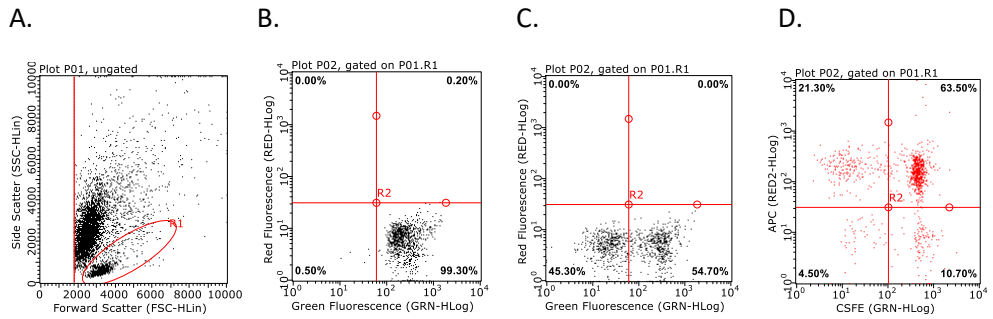

Figure 3.2 Flow cytometric data analysis

A. The lymphocyte population was selected based on cell size and granularity (forward and side scatter) for downstream analysis. Lymphocytes labelled with CFSE and are not proliferating retain high intensity green fluorescence (B and C, lower right quadrant) while intensity decreases in proliferating cells (C, lower left quadrant). Lymphocyte subsets labelled with allophycocyanin (APC) conjugated subset-specific markers are identified as high intensity red fluorescence (D, upper quadrants). These can be categorised as proliferating (D, upper left quadrant) and nonproliferating (D, upper right quadrant) lymphocytes for a specific subset.

The mean of replicate samples was used for further analysis. Data is presented as the percentage of the total lymphocyte or total subset-specific population.

1.2.8 Anti-ova antibodies

The coating antigens were ovalbumin, yeast lysate and OvaYeast lysate at 10 μg/mL. Serum samples were diluted 1 in 5 in PBS with 0.05% Tween20 while intestinal mucus samples were used without dilution. Detection was with anti-bovine IgG HRP and TMB substrate (3,3',5,5'-tetramethylbenzidine). Each sample was assessed in duplicate. The same positive and negative controls were included in all ELISA plates to normalize OD values across plates. Sample SP% was calculated as 100(Mean Sample OD – Mean plate negative OD)/(Mean plate positive OD – Mean plate negative OD).

1.2.9 Cytokine (interferon gamma) response

Equal volumes of blood and culture medium without or with various stimulants (ovalbumin, yeast, OvaYeast) were cultured for 48 hrs at 37°C in an atmosphere of 5% CO_2. Pokeweed mitogen (PWM) was included to evaluate general responsiveness of whole blood cells. Culture supernatants were harvested and stored at -20°C until used for detecting interferon gamma (IFNγ) by ELISA.

The bovine IFNγ ELISA was performed as previously described (Begg et al. 2018) using anti-bovine IFNγ (clone 6.19, a gift from Dr. Gregers Jungersen), anti-bovine IFNγ-biotin (clone CC302, Biorad), streptavidin HRP and TMB substrate. Each sample was assessed in duplicate. The same positive and negative controls were included in all ELISA plates to normalize OD values across plates. Sample SP%

was calculated as 100(Mean Sample OD – Mean plate negative OD)/(Mean plate positive OD – Mean plate negative OD).

1.2.10 Gene expression in tissue samples

The tissue samples were transferred to frozen mortar and pestles that were kept on dry ice and pulverised into a powder, which was transferred to 1 ml RNAzol RT (Sigma) prior to thawing. Total RNA was extracted according to the manufacturer's recommendations. The quantity and integrity of the isolated RNA was verified on a NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific).

Complementary (c)DNA synthesis, or reverse transcription (RT), was performed on 1 µg RNA from each sample using the SensiFAST cDNA synthesis (Bioline) according to the manufacturer's recommendations, with the addition of 1 µl RNasin® RNase inhibitor (Promega) per reaction to protect the RNA from degradation and both oligo dT and random priming. The resulting cDNA was diluted tenfold in nuclease-free water prior to quantitative (q)PCR to detect gene expression.

The qPCR was performed using an Mx3000P Real-time PCR system (Stratagene, Agilent) using the QuantiTect SYBR Green PCR kit (Qiagen). Assays were prepared in 96 well plates and included duplicate reaction of each sample. Reaction volumes of 20µl (including 4 µl of target cDNA at a 1/10 dilution) were prepared, including 300 nM forward and reverse primers. The bovine primers used for RT-qPCR are shown in Table 1. Three non-changing genes were selected as reference genes; this follows MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Vandesompele et al. 2002). Amplification was performed under the following conditions: 95°C for 15 min, then 40 cycles of 95°C for 20 s, 56-58°C for 30 s and 72°C for 30 s, with fluorescence acquisition at the end of each annealing step. The specificity of the reaction was confirmed using melting curve analysis and standard curves were performed for each primer set to determine reaction efficiency.

Table 1: Bovine primer sets used in the study.

Gene ID	Gene name	Accession code		Primer sequence (5' to 3')	Product length
ACTB*	*Bos taurus* actin beta	NM_173979.3	For	TGGGCATGGAATCCTG	196bp

			Rev	GGCGCGATGATCTTGAT	
H3F3A*	*Bos taurus* H3 histone, family 3A	NM_001014389.2	For	GAGGTCTCTATACCATGGCTC	151bp
			Rev	GTACCAGGCCTGTAACGATG	
PPIA*	Bos taurus peptidylprolyl isomerase A	NM_178320.2	For	TGACTTCACACGCCATAAT	181bp
			Rev	CTTGCCATCCAACCACTC	
TNF-α*	*Bos taurus* tumor necrosis factor	NM_173966.3	For	CCAGAGGGAAGAGCAGTCC	112bp
			Rev	GGCTACAACGTGGGCTACC	
IFN-γ*	*Bos taurus* interferon gamma	NM_174086.1	For	CAGAGCCAAATTGTCTCCTTC	168bp
			Rev	ATCCACCGGAATTTGAATCAG	
IL-10*	*Bos taurus* interleukin 10	NM_174088.1	For	CTTTAAGGGTTACCTGGGTTGC	262bp
			Rev	CTCACTCATGGCTTTGTAGACAC	
CXCL10†	*Ovis aries* C-X-C motif chemokine ligand 10	NM_001009191.1	For	CCCACGTGTCGAGATTATTGCC	283bp
			Rev	AGCTGATTTGGTGACTGGCTT	
CD80	*Bos taurus* CD80 molecule	NM_001206439.1	For	GGTCACACAATGAAGTGGGGA	112bp
			Rev	TCTTTGGGGTGATGCCTGAAC	
CD69	*Bos taurus* CD69 molecule	NM_174014.2	For	AGAGCTGAACACTGGATCGG	97bp
			Rev	ACCCCGTAAGGTTGAACCAG	
CCR7	*Bos taurus* C-C motif chemokine receptor 7	NM_001024930.3	For	GAGAGTCATGGACCTGGGGAAG	200bp
			Rev	ACATGATCGGGAGGAACCAG	

* Puech et al. (2015) BMC Veterinary Research 11:65. † Primer set is designed for ovine, but cross-reacts with bovine CXCL10.

1.2.11 Intestinal parasites

Faecal samples collected at necropsy 5 months post primary vaccination were analysed for parasite burden (faecal worm and fluke egg counts and larval differentiation) by a commercial veterinary parasitology laboratory (Elizabeth Macarthur Agricultural Institute).

1.2.12 Statistical analysis

Lymphocyte proliferation, cytokine and anti-Ova antibody data

Statistical analysis was by Repeated Measures two-way ANOVA with Geisser-Greenhouse correction for sphericity and alpha at 0.1. Tukey's multiple comparisons test was then used to compare the effect of each stimulant between treatment groups.

Where values for some stimulants were missing or excluded due to not meeting assay criteria, data were analysed by fitting a mixed model with Geisser-Greenhouse correction for sphericity and alpha at 0.1, rather than by repeated measures ANOVA (which does not account for missing values). Dunnett's multiple comparisons test was used to compare the effect of each stimulant between treatment groups.

Gene expression data

Data collected from qRT-PCR experiments were analysed utilising a modified comparative Ct (ΔΔCt) method termed the common base method (Ganger et al. 2020).

1.3 Results

1.3.1 Vaccine safety

There were no adverse signs observed following the oral dose of the vaccine in the two animals evaluated for vaccine safety.

1.3.2 Body weight

At the start of the trial the average body weight was 333.9 kg for the Control group, 326.5 kg for the Single dose group and 323.4 kg for the Multiple dose group. While time was a significant factor ($p<0.0001$), there were no significant differences in body weight between treatment groups at any time during the trial (Fig. 3.3).

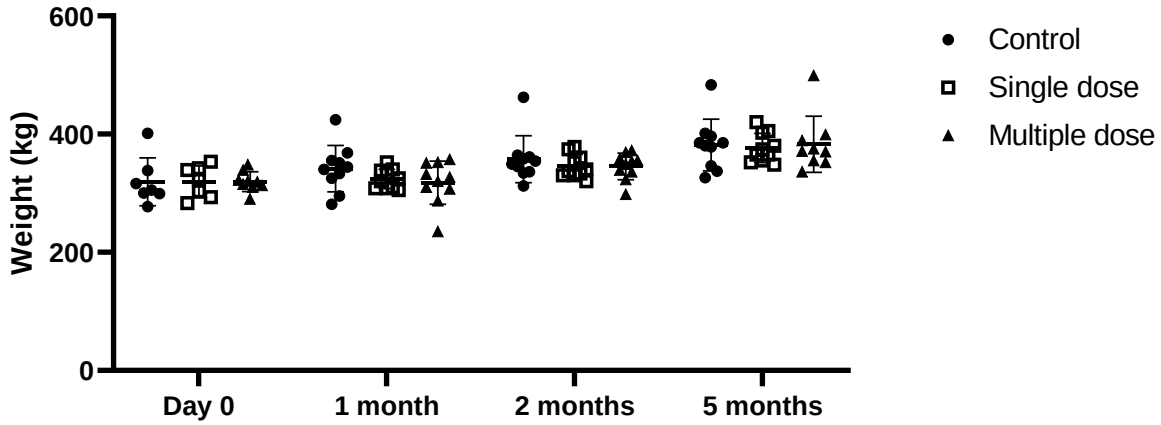

Figure 3.3 Weights of animals during the trial

Animals in the treatment groups were weighed during the 4-month period of the trial. Control (one dose of nontransformed yeast), Single dose (one dose of OvaYeast vaccine) and Multiple dose (three doses of OvaYeast vaccine). n=10 per group

Table 3.2.1 Body weight (kg) of individual animals

In some instances body weight was not recorded due to uncooperative behaviour by some cattle

	Control	Single dose	Multiple dose
Day 0	401	353	348
		342	339
	338	339	322
			320
	305	322	315
	316		319
	277		313
	300	303	310
	299	293	290
		283	
Day 6	419	362	360
	348	351	350
	357	331	336

	332	325	333
	332	321	321
	331	309	332
	314	302	320
	308	311	315
	316	316	315
	300	297	260
Day 13	407	360	355
	350	336	335
	350	328	332
		323	317
	334	320	330
	333	318	328
	321	301	316
	307	311	315
	327	310	300
	298	300	288
Day 21	443	375	
	357	348	359
		349	338
		329	333
		338	329
	340	330	345
	339	315	327
	318	318	
	335	328	314
	306	318	296
Day 28 (1 month)	424	340	357
	355	352	352
	333	305	235
	344	325	332
	340	338	351
	368	308	327
	325	310	310
	281	320	320
	351	334	307
	295	308	287
Day 60 (2 months)	462	378	358
	364	374	369
	349	360	355
	336	333	349
	361	352	354
	358	330	339
	354	320	372
	334	330	336

	345	337	323
	312	340	298
Day 151-155 (5 months)	483	420	371
	385	402	399
	401	405	375
	346	352	389
	380	365	370
	396	365	
	385	348	499
	337	355	355
	378	374	352
	326	380	336

1.3.3 Intestinal parasites

Cattle included in the trial were screened for parasites, including gastrointestinal nematodes, cestodes, protozoa (coccidia) and trematodes, both to check their health status and because high parasite burdens can cause immunosuppression (Howell et al., 2019; Snider et al., 1986). Screening was conducted individually for nematodes, cestodes, and coccidia, and in pools of 5 for liver – and rumen fluke (also known as stomach fluke). Larval cultures were conducted on pools of 10 faecal samples, with each pool representing one of the experimental groups (Table 3.3).

Table 3.3 Results of diagnostic testing for nematode, cestode, protozoan and trematode parasites in cattle cohort used for vaccine studies

Parasite	Unit	Animals per sample	Control group	Single dose group	Multidose group	Total cohort
Stongyles	epg	1	60 (0,260)	50 (0, 340)	45 (0,320)	40 (0, 340)
Nematodirus	epg	1	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)
Tapeworm	neg/pos	1	1 pos	All neg	1 pos	2 pos
Coccidia	neg/+/++	1	1 ++, rest +	All +	1 -, rest +	3 neg
Liver fluke	epg	5	11.5	3	9.5	7 (2, 14)
Rumen fluke	epg	5	0.5	7	0	0 (0, 14)
Larval culture	%	10				n/a
Cooperia			43	86	52	
Ostertagia			23	9	9	
Trichostrongylus			15	4	36	
Haemonchus			10	0	2	
Oesophagostomum			10	1	1	

Parasite burdens were low and similar across groups, especially for nematodes, cestodes and coccidia. Liver fluke epg was higher than expected based on clinical and post-mortem observations, and slightly above the NSW DPI reference level of 5 epg (NSW DPI, 2007). Shedding of liver fluke eggs can be variable due to potential storage and periodic release of liver fluke eggs in or from the gall bladder. Rumen fluke epg was higher in the single dose group than in the other groups, but rumen fluke is not known to have an immunosuppressive or immunomodulatory effect so this would not affect the outcome of the study. *Cooperia* (small intestinal worm) was the dominant strongyle species in each pool, as expected (NSW DPI, 2007), followed by *Ostertagia* (small brown stomach worm) and *Trichostrongylus* (black scour worm), with low levels of *Haemonchus* (Barber's pole worm) and *Oesophagostomum* (nodule worm). Levels of the latter species in the control group may appear comparatively high at 10%, but the number of larvae available for speciation from this sample was low, making the results subject to stochastic variability rather than indicative of true differences between groups. No clinical signs of parasitism were observed in any animals during the study, and no anthelminthic treatments were administered.

1.3.4 Preliminary investigations of lymphocyte proliferation

Comparison of lymphocyte phenotypes

The distribution of lymphocyte subsets in peripheral blood one month after the primary vaccine or control dose was given is shown in Fig. 3.4. As expected, the majority were CD3-expressing T cells. There was no difference in the proportion of lymphocyte subsets between control and vaccinated groups.

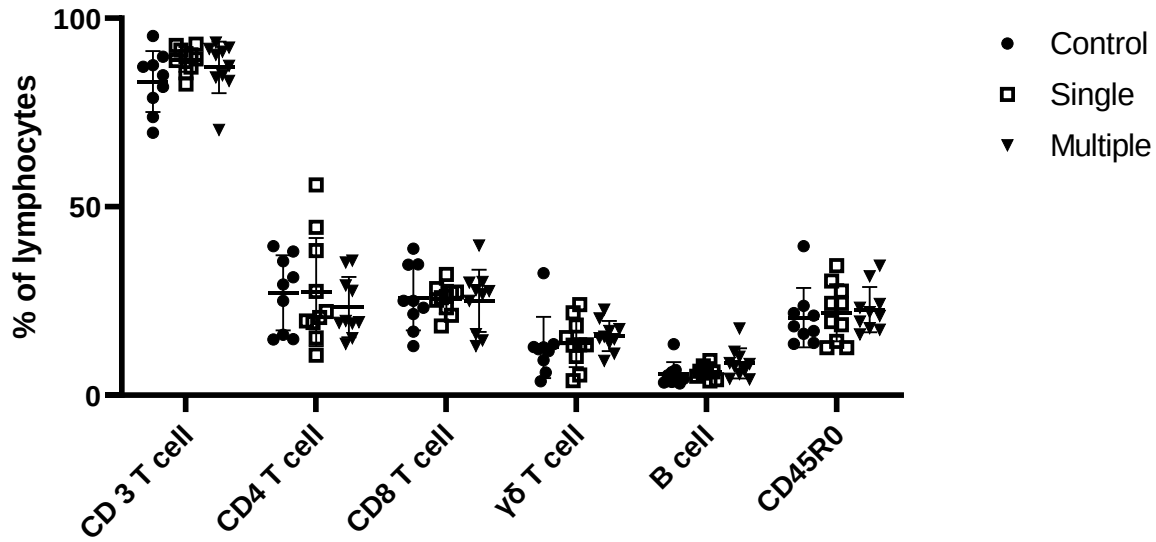

Figure 3.4 Distribution of lymphocyte subsets one month post primary vaccination
The proportion of lymphocyte subsets was determined by flow cytometry: CD3 (T cells), CD4 (T helper cells), CD8 (cytotoxic T cells) gd T cells, B cells and CD45RO (memory cells). The treatment groups were: Control (one dose of nontransformed yeast), Single dose (one dose of OvaYeast vaccine) and Multiple dose (three doses of OvaYeast vaccine). n=10 per group

Evaluation of in vitro stimulation protocol

The optimal protocol to monitor vaccine-induced immune responses in blood cells was evaluated. Isolated peripheral blood mononuclear cells (PBMC) were cultured in medium alone (no treatment) or with ovalbumin, (nontransformed) yeast or OvaYeast with and without 'priming' of monocytes. Monocyte presentation of antigens to lymphocytes enables immune cell activation. The 'priming' step was to allow time for monocytes to process and present antigens.

For 'priming', monocytes were first isolated from PBMC by adherence to the culture vessel overnight. Nonadherent lymphocytes were then removed after gentle mixing of the culture medium and the adherent cells 'primed' by incubation with an in vitro stimulant (ovalbumin, yeast or OvaYeast) for 2 hrs. The nonadherent cells were maintained in culture medium during this time. Autologous lymphocytes and the same stimulant were then added to the 'primed' monocytes and cultured for 5 days similar to the usual protocol for the proliferation assay. There was no difference in the proliferative response in primed and non-primed monocyte cultures (Fig. 3.5). Therefore, all lymphocyte cultures for the proliferation assay followed the non-primed protocol.

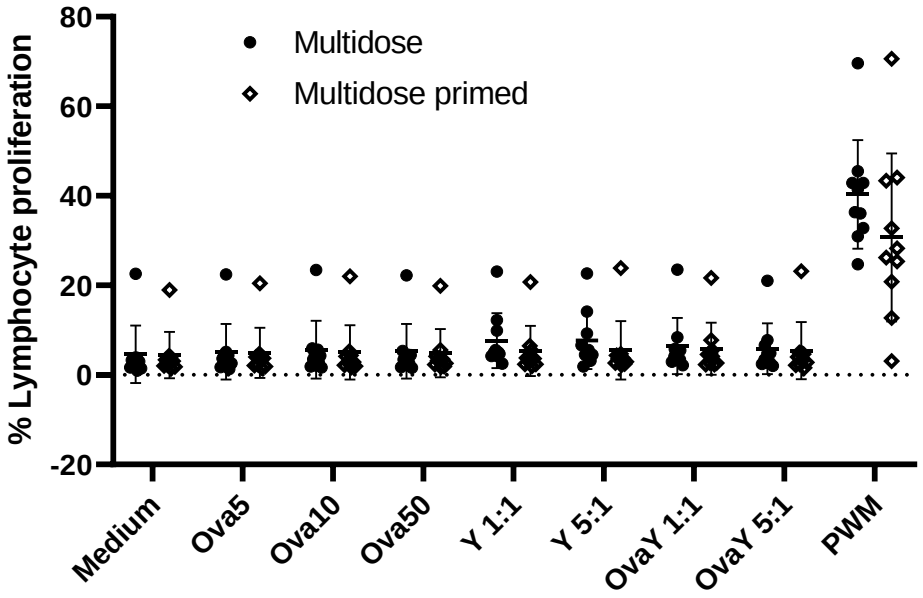

Figure 3.5 Effect of priming monocytes on lymphocyte proliferation

The requirement for priming monocytes for the proliferation assay was assessed using PBMC from the Multidose treatment group. Priming involved exposure of isolated monocytes to the stimulant (Ova, ovalbumin; Y, yeast; OvaY, OvaYeast, PWM, pokeweed mitogen) prior to starting the lymphocyte proliferation assay. Ova was assessed at 5, 10 an 50 μg/mL and Y and OvaY at MOI of 1:1 and 5:1.

Evaluation of lysed and intact yeast as an in vitro stimulant

Intact and lysed yeast and OvaYeast were assessed as stimulants (Fig. 3.6). There were no differences in treatment-specific lymphocyte proliferation (proliferation in medium alone subtracted) in response to intact or lysed yeast. Lysed yeast and lysed OvaYeast were omitted from subsequent analyses.

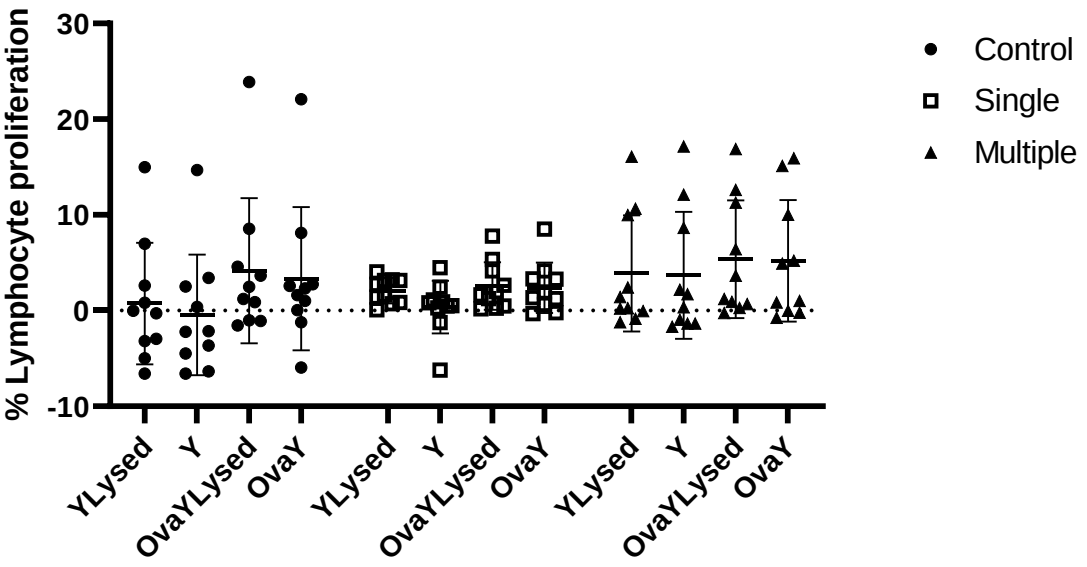

Figure 3.6 Effect of using lysed ovaYeast on the lymphocyte proliferative response
PBMC were labelled with a fluorescent dye to track proliferation and cultured with a variety of stimulants: Intact or lysed yeast (Y) and OvaYeast (OvaY) at a multiplicity of infection (MOI) of 5:1. Proliferation was assessed after 5 days of culture. The treatment groups were: Control (one dose of nontransformed yeast), Single dose (one dose of OvaYeast vaccine) and Multiple dose (three doses of OvaYeast vaccine). n=10 per group

1.3.5 Lymphocyte proliferation responses

Proliferation assays assessed total lymphocytes as well as lymphocyte subsets and data were analysed for comparison of multiple stimulant groups within each treatment group. Several concentrations of each stimulant were included in the proliferation assay. The results did not always follow the same pattern for each treatment e.g. when there was significant proliferation to OvaYeast at an MOI of 1:1 proliferation in response to OvaYeast at 5:1 may have been similar to medium alone. All results are presented but interpretation of results is based on the response to a stimulant as a whole rather than to each concentration assessed.

ANOVA analysis was carried out using two models: comparison of stimulant within each treatment group and comparison of treatment groups for each stimulant.

Lymphocyte proliferation in peripheral blood at one month post primary vaccination

In all three treatment groups, the PWM response was significantly elevated ($p<0.01$) compared to the matched nontreated cells in Medium alone and there was no significant difference between treatment groups. This indicates that blood cells were viable and had the capacity to proliferate regardless of vaccination regime.

Overall, proliferative responses to all stimulants as well as to no treatment (medium alone) tended to be higher in the Control treatment group than in the two OvaYeast groups (Fig. 3.7). In the Control group, the response to yeast as well as to OvaYeast, but not to ovalbumin ($p>0.05$), was

significantly (p<0.05) elevated (Fig. 3.7). The Control cattle were orally vaccinated with nontransformed yeast (i.e. without the reporter antigen Ova). In this group of cattle, there was no response to the reporter antigen ovalbumin which was not present in the 'sham' vaccine. It is likely that the lymphocyte proliferative response to yeast and OvaYeast stimulants reflects induction of immune memory to yeast and OvaYeast stimulants reflects induction of immune memory to the yeast vector, whereby yeast is known to be immunostimulatory (hence it is of value as an adjuvant for oral vaccines).

In the Single dose treatment group, the response to the yeast (but not to ovalbumin or OvaYeast) stimulant was significantly higher (p<0.05) than to medium alone (Fig. 3.7). In the Multiple dose treatment group, the response to ovalbumin, yeast and OvaYeast were all tended to be elevated (p<0.1) (Fig. 3.7). Overall, these results show that a higher oral dose of OvaYeast in the Multiple dose group has induced immune memory of exposure to yeast as well to the reporter antigen Ova.

It is unclear why the proliferative response induced by oral exposure to yeast (either nontransformed or OvaYeast) appear to be elevated in the Control group compared to the OvaYeast treatment groups.

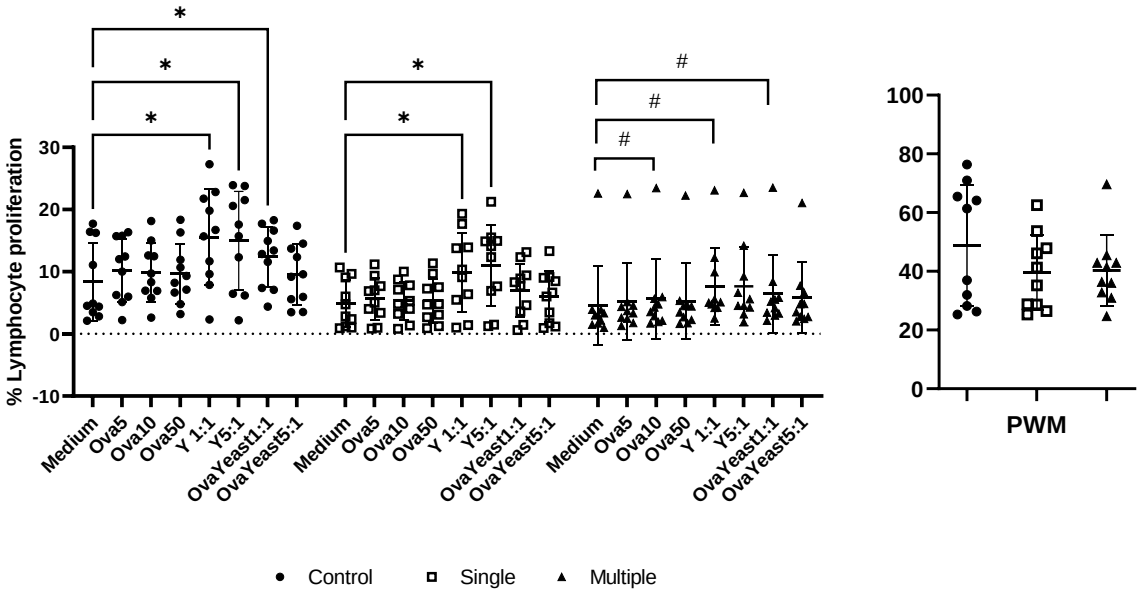

Figure 3.7 Total lymphocyte proliferation at one month post primary vaccination
Peripheral blood mononuclear cells were labelled with a fluorescent dye to track proliferation and cultured with a variety of stimulants: Ovalbumin (Ova) at 5-50 μg/mL, Yeast (Y) and OvaYeast at two MOI (1:1 and 5:1) or pokeweed mitogen (PWM; positive control for stimulation assays). Proliferation was assessed after 5 days of culture. The treatment groups were: Control animals (one dose of nontransformed yeast), Single dose (one dose of OvaYeast) and Multiple dose (three doses of OvaYeast) with n=10 per group. *p≤0.05 #p≤0.1

At this time point, for the CD3 T lymphocyte subset, data is available only for OvaYeast stimulant. OvaYeast induced significantly higher (p< 0.1) proliferation in the Single and Multiple dose treatment groups but not in the Control group (Fig. 3.8).

For the CD4 T cell subset, ovalbumin, yeast and OvaYeast stimulants all induced lymphocyte proliferation significantly ($p<0.1$) compared to Medium alone in the Single dose treatment group (Fig. 3.8). There were no significant differences in proliferation compared to Medium alone for any of the stimulants in the Control or Multiple dose groups for CD4 T cells. It is not clear why a single dose would induce a stronger response than multiple doses. To avoid loss of power or the risk of type II errors, we report results at $p<0.10$, so this result could potentially be a false positive (type I error).

CD8 T cell proliferation was significantly higher ($p<0.1$) in response to yeast stimulant compared to Medium alone (Fig. 3.8) in the Control treatment group. In the Single dose group, proliferation of this subset was significantly higher ($p<0.1$) in response to yeast and OvaYeast stimulants compared to Medium alone, whereas no significant responses were observed in the Multidose group. Results for the Control and Single dose groups suggest a response to yeast rather than to ova, but it is unclear why this result would not be replicated in the Multidose group.

In the Multiple dose group, B cells responded to yeast stimulant with a significantly ($p<0.05$) higher proliferative response than in the presence of medium alone (Fig. 3.85). The $\gamma\delta$ T cell response to stimulant with yeast was significantly higher in the Control ($p<0.1$), Single ($p<0.05$) and Multiple ($p<0.05$) dose treatment groups (Fig. 3.8). In the Single dose group this response was also significantly higher ($p<0.05$) in response to OvaYeast stimulant and in the Multiple dose group significantly higher ($p<0.1$) in response to OvaYeast as well as ovalbumin stimulants.

Proliferation of CD45RO lymphocytes was significantly ($p<0.1$) higher in response to ovalbumin, yeast and OvaYeast stimulants compared to medium alone for all three treatment groups (Fig. 3.8). The response to ovalbumin in the Control group is unexpected because control cattle were expected to be naïve to ovalbumin – a protein that is unlikely that pasture-raised cattle would have been exposed to. However, in hindsight, it would have been valuable to collect blood samples prior to vaccination.

A summary of significant differences in lymphocyte proliferation in response to stimulants compared to proliferation in medium alone for each treatment group is shown in Table 3.4. At one month after the primary dose was given, immune cell activation and memory cell generation to yeast and OvaYeast has occurred. CD4 and CD8 T cell activation is seen with the Single dose of the vaccine but not in the Multiple dose group. Induction of B cell responses is only seen in the Multiple dose group while induction of $\gamma\delta$ T and CD45RO lymphocyte responses are seen in both Single and Multiple dose groups. In the Control group, yeast stimulated $\gamma\delta$ T cell proliferation and ova stimulated CD45RO lymphocyte responses.

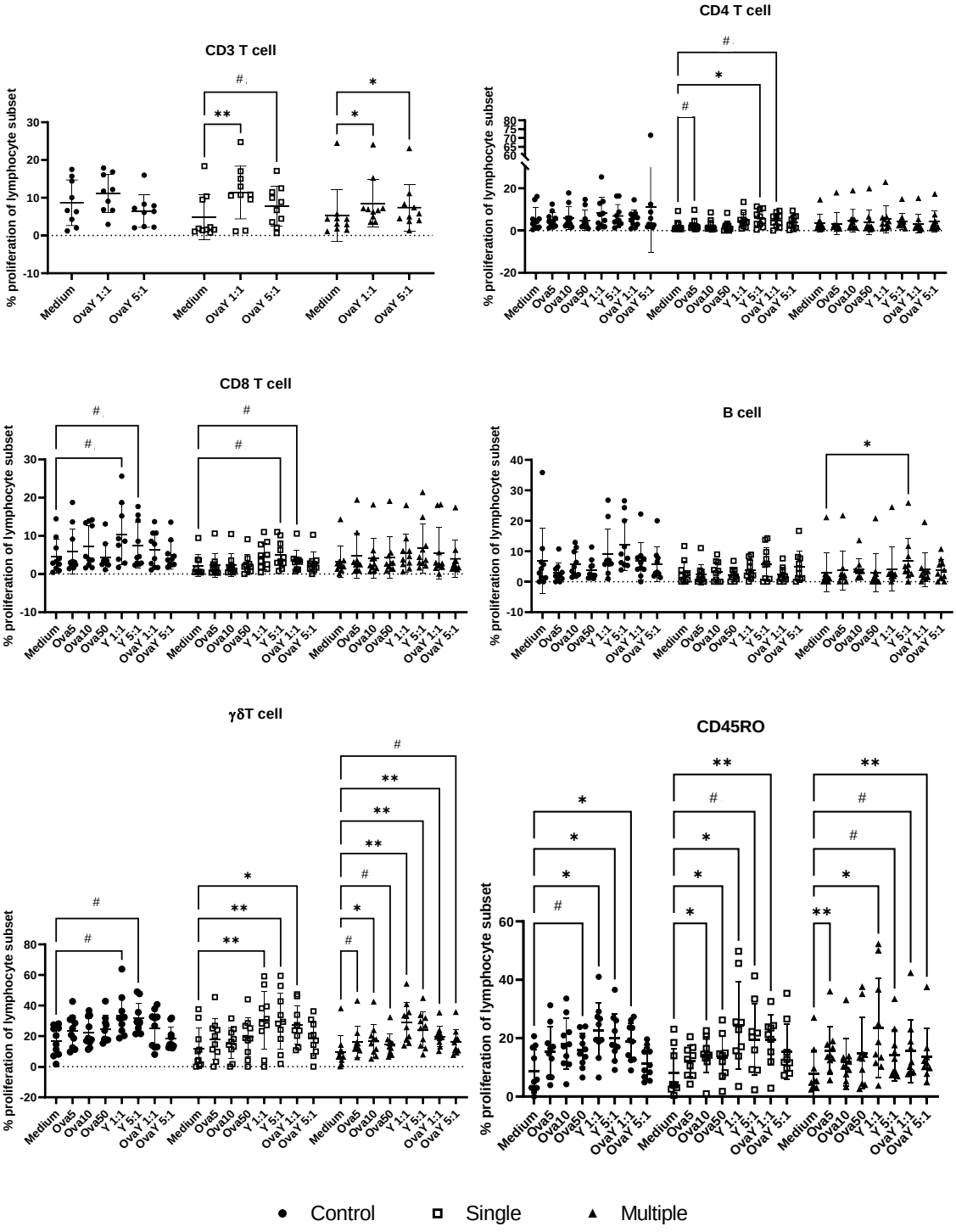

Figure 3.8 Lymphocyte subset proliferation at one month post primary inoculation with oral yeast vaccine

Peripheral blood mononuclear cells were labelled with a fluorescent dye to track proliferation and cultured with a variety of stimulants: Ovalbumin (Ova) at 5-50 μg/mL; Yeast (yeast) and OvaYeast at two multiplicities of infection (1:1 and 5:1). After 5 days of culture, cells were labelled to identify lymphocyte subsets prior to assessing proliferation. The treatment groups were: Control animals (one dose of nontransformed yeast), Single dose (one dose of OvaYeast) and Multiple dose (three doses of OvaYeast) with n=10 per group. **p≤0.01; *p≤0.05; #p≤0.1

Table 3.4 Summary of lymphocyte proliferation to stimulants within each treatment group at one month post primary vaccination

p values for statistically significant (p<0.1) responses which were higher compared to no stimulant (medium alone) for each treatment group are shown

Treatment group	Stimulant	Control	Single dose	Multiple dose
Total lymphocytes	Ova			0.073
	Yeast	0.011; 0.019	0.0401; 0.011	0.074
	OvaYeast	0.039		0.052
CD3 T cell	OvaYeast		0.004; 0.093	0.014; 0.024
CD4 T cell	Ova		0.0758	
	Yeast		0.0268	
	OvaYeast		0.0937	
CD8 T cell	Ova			
	Yeast	0.074; 0.132	0.0598	
	OvaYeast		0.0608	
B cell	Ova			
	Yeast			0.038
	OvaYeast			
γδT cell	Ova			0.074; 0.027
	Yeast	0.094; 0.085	0.003; 0.006	0.004; 0.0023
	OvaYeast		0.0108	0.008; 0.096
CD45RO	Ova	0.0971	0.026; 0.032	0.001
	Yeast		0.027; 0.070	0.024; 0.052
	OvaYeast		0.0036	0.053; 0.001

Lymphocyte proliferation in peripheral blood at two months post primary inoculation

Lymphocyte responses at two months after the first dose was administered to the treatment groups are shown in Fig. 3.9 and 3.10. The response to PWM in all treatment groups was significantly higher (p<0.1) than to medium alone and there were no significant differences between treatment groups. This indicates that blood cells were viable and had the capacity to proliferate regardless of vaccination regime.

In both OvaYeast treatment groups, total lymphocyte proliferation was significantly higher (p<0.1) in response to in vitro OvaYeast treatment (Fig. 3.9). The total lymphocyte response to ovalbumin stimulant in the Control treatment group was significantly lower (p≤0.1) than to medium alone.

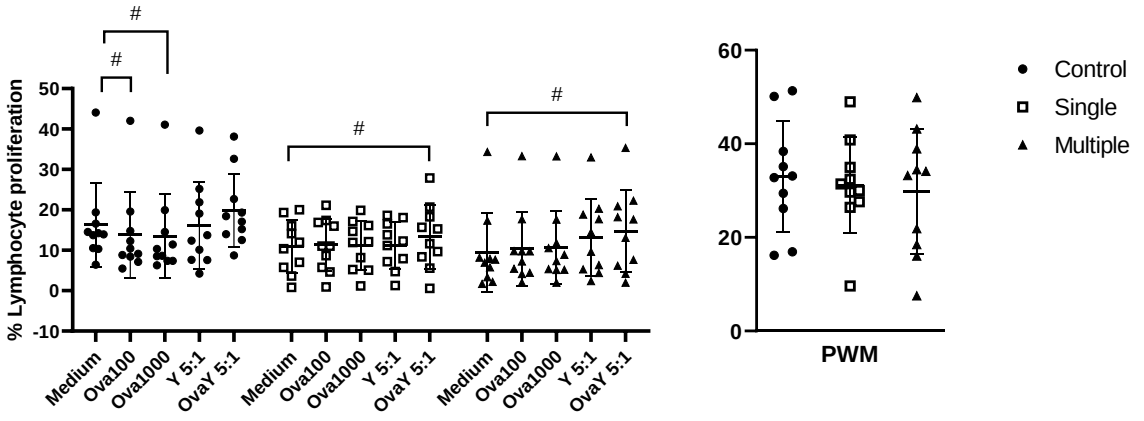

Figure 3.9 Lymphocyte proliferation at two months post primary vaccination
Peripheral blood mononuclear cells were labelled with a fluorescent dye to track proliferation and cultured with a variety of stimulants: Ovalbumin (Ova) at 100 and 1000 μg/mL; Yeast (Y) and OvaYeast (OvaY) at a multiplicity of infection of 5:1; pokeweed mitogen (PWM). Proliferation was assessed after 5 days of culture. The treatment groups were: Control animals (one dose of nontransformed yeast), Single dose (one dose of ova-yeast vaccine) and Multiple dose (three doses of ova-yeast vaccine) with n=10 per group. #p≤0.1

For any of the treatment groups, the CD3 T cell response to stimulation with either yeast or OvaYeast was not significantly different ($p>0.1$) to medium alone (Fig. 3.10). The CD4 T cell response to both yeast and OvaYeast stimulants were significantly higher ($p<0.1$) compared to medium alone in the Multiple dose treatment group. Results were similar for the CD8 T cell response but only reached statistical significance ($p<0.1$) for OvaYeast stimulant in the Multiple dose group.

The B cell response to yeast stimulation was significantly higher ($p\leq0.1$) than medium alone in both the Control and Multiple dose treatment groups while response to OvaYeast was significantly higher ($p<0.05$) in the Single dose group compared to medium alone. The γδ T cell response to OvaYeast stimulation was significantly higher ($p<0.1$) than medium alone in the Single dose treatment group. CD45RO proliferation was significantly higher in the OvaYeast treatment groups for Single ($p<0.01$) and Multiple dose treatment groups ($p<0.1$).

These results are summarised in Table 3.5.

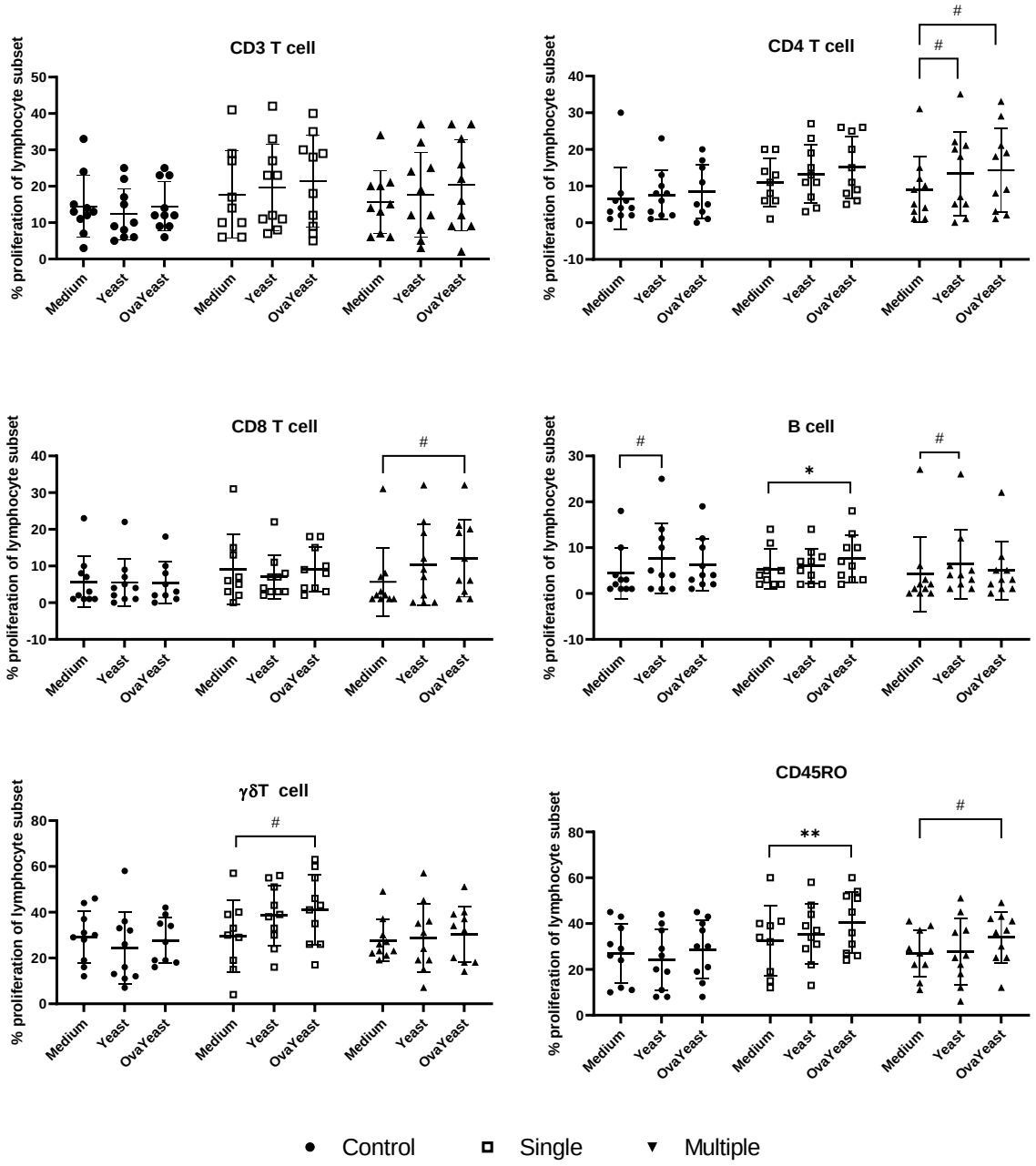

Figure 3.10 Lymphocyte subset proliferation at two months post primary vaccination
Peripheral blood mononuclear cells were labelled with a fluorescent dye to track proliferation and cultured a variety of stimulants: yeast (Yeast) and OvaYeast at a multiplicity of infection of 5:1. After 5 days of culture, cells were labelled to identify lymphocyte subsets prior to assessing proliferation. The treatment groups were: Control animals (one dose of nontransformed yeast), Single dose (one dose of ova-yeast vaccine) and Multiple dose (three doses of ova-yeast vaccine) with n=10 per group. **p≤0.05; *p≤0.05; #p≤0.1

Table 3.5 Summary of lymphocyte proliferation to stimulants within each treatment group at two months post primary vaccination
p values for statistically significant (p<0.1) responses compared to stimulant (medium alone) for each treatment group are shown

Treatment group	Stimulant	Control	Single dose	Multiple dose
Total lymphocytes	Ova	0.0932*		
	Yeast			
	OvaYeast		0.053	0.088
CD3 T cell	Yeast			
	OvaYeast			
CD4 T cell	Yeast			0.069
	OvaYeast			0.088
CD8 T cell	Yeast			
	OvaYeast			0.069
B cell	Yeast	0.052		0.103
	OvaYeast		0.037	
γδT cell	Yeast			
	OvaYeast		0.079	
CD45RO	Yeast			
	OvaYeast		0.009	0.054

* significantly lower than medium alone

The overall pattern of peripheral blood lymphocyte proliferation responses seen at one-month post vaccination is reflected in the responses at two-months post vaccination. The ability of OvaYeast to activate lymphocyte proliferation in both Single and Multiple dose treatment groups suggests that the OvaYeast vaccine given to these animals have activated lymphocytes to retain memory of the vaccine. At two-months post vaccination, the memory responses are largely to OvaYeast rather than to yeast.

Lymphocyte proliferation in lymph nodes at five months post primary vaccination

To determine immunity at mucosal sites, tissue sections were taken at necropsy 5 months after the primary vaccination. Samples were taken from four tissue locations (retropharyngeal, ileal and jejunal lymph nodes (RLN, ILN, and JLN, respectively) and spleen. There was no difference in PWM-induced proliferation between treatment groups for the lymph nodes (Fig. 3.11) indicating that these cells were viable and had the capacity to proliferation regardless of vaccination regime. Splenocyte proliferation was lower but this was due to lower proliferation capacity in these cells rather than a reduction in cell viability. Fig. 3.12 is a representative example of CFSE labelling in splenocytes which demonstrates that these cells are viable; cells have retained the dye CFSE (high CFSE) but there are few proliferating cells (dim CFSE).

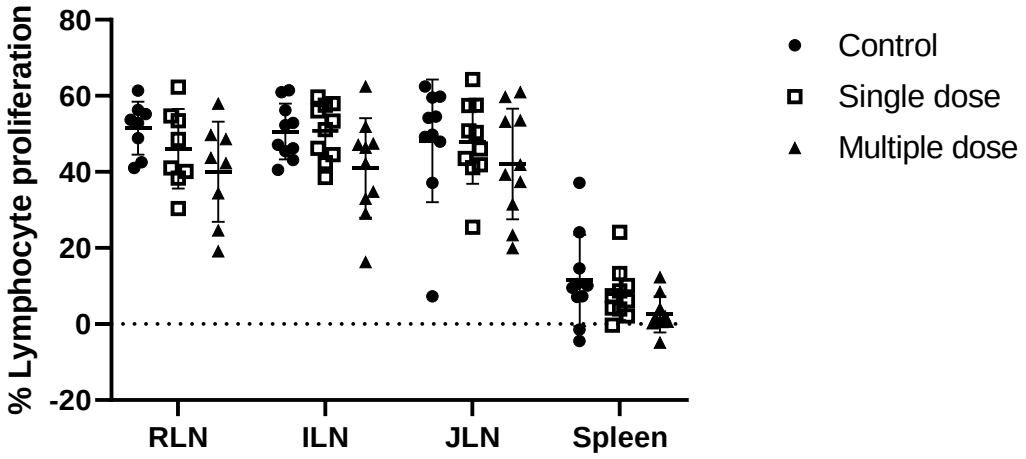

Figure 3.11 Proliferation of tissue lymphocytes in response to pokeweed mitogen (PWM).
Cells isolated from retropharyngeal lymph node (RLN), ileal lymph node (ILN), jejunal lymph node (ILN) and spleen were labelled with a fluorescent dye to track proliferation and cultured for 5 days with or without PWM. The treatment groups were: Control (one dose of nontransformed yeast), Single dose (one dose of OvaYeast vaccine) and Multiple dose (three doses of OvaYeast vaccine) with n=10 per group. Specific proliferation (medium alone subtracted) is shown.

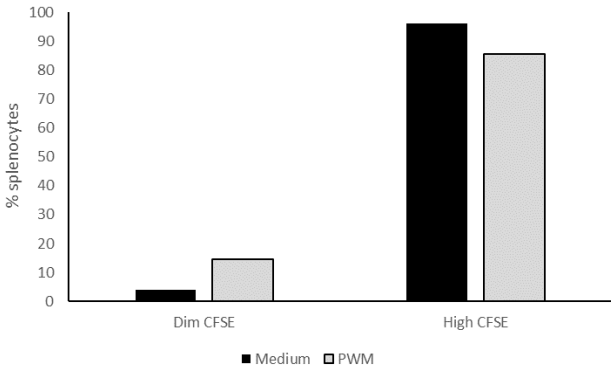

Figure 3.12 Example of splenocyte viability
Splenocytes were labelled with a fluorescent dye (CFSE) to track proliferation and cultured with pokeweed mitogen (PWM) or in medium alone and proliferation was assessed after 5 days of culture. CFSE fluorescence in splenocytes from one animal. Viable cells retain high CFSE fluorescence while proliferating cells lose CFSE intensity (dim CFSE) with each generation.

Proliferation of lymphocytes from tissues taken at necropsy in response to stimulation with yeast or OvaYeast is shown in Fig. 3.13. In the Control treatment group, the RLN proliferative response to stimulants yeast and OvaYeast were both significantly lower ($p<0.05$) compared to medium alone. The response in the Single dose treatment group was also significantly lower ($p<0.1$) in the OvaYeast stimulant. The response in the Control treatment group to the stimulant yeast was significantly higher ($p<0.1$) compared to medium for splenocytes.

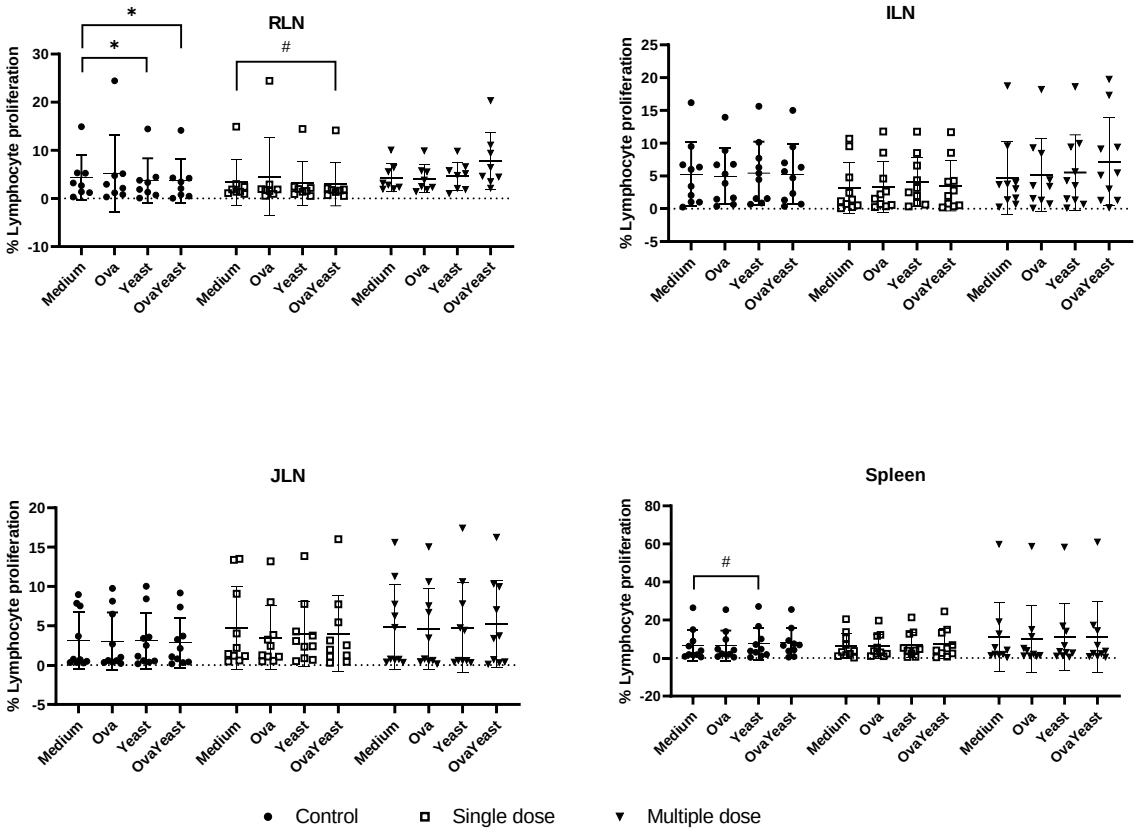

Figure 3.13 Proliferation of tissue lymphocytes in cattle given the yeast vaccine
Lymphocyte isolated from retropharyngeal lymph node (RLN), ileal lymph node (ILN), jejunal lymph node (ILN) and spleen were labelled with a fluorescent dye to track proliferation and cultured for 5 days with or without a variety of stimuli: ovalbumin (Ova), yeast, OvaYeast. The treatment groups were: Control (one dose of nontransformed yeast), Single dose (one dose of OvaYeast vaccine) and Multiple dose (three doses of OvaYeast vaccine) with n=10 per group

There were no significant differences ($p>0.1$) between yeast or OvaYeast stimulants for CD3 T cells, B cells or CD45RO cells compared to medium alone for RLN, ILN, JLN or splenocytes for any of the treatment groups (Fig. 3.14 and 3.15).

With the increase in the number of tissue samples selected for assessment at necropsy, the number of lymphocyte subsets analysed was reduced to enable sample processing and data acquisition without compromising sample quality. The CD3 T cell subsets CD4 and CD8 were not included and the immune memory cell marker CD45RO was included in preference to $\gamma\delta$ T cells.

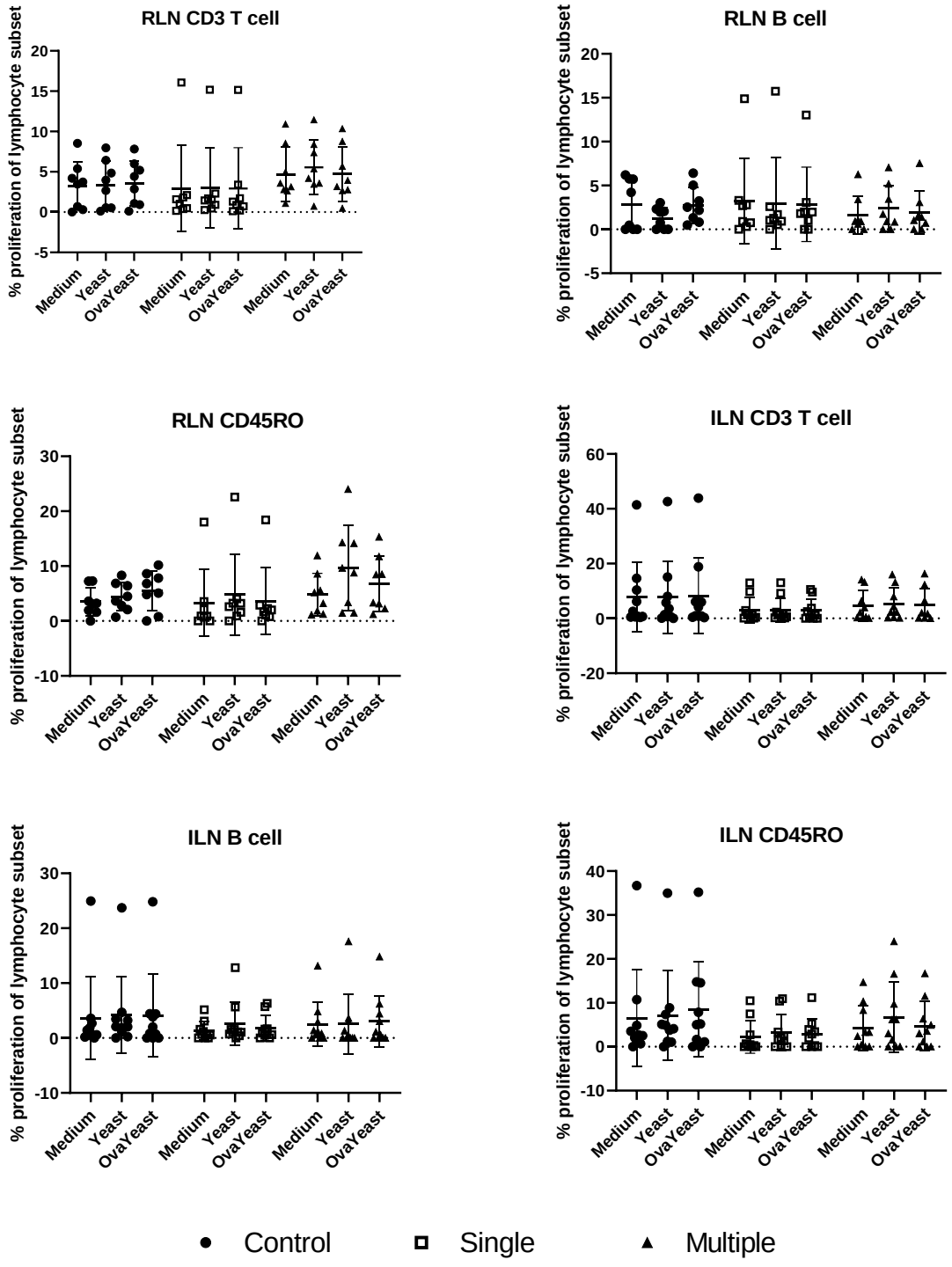

Figure 3.13 Proliferation of tissue lymphocyte subsets in cattle given the yeast vaccine
Lymphocytes isolated from retropharyngeal lymph node (RLN) and ileal lymph node (ILN) were labelled with a fluorescent dye to track proliferation and cultured for 5 days with or without yeast OvaYeast (stimulants) and labelled to identify T cells, B cells and CD45RO lymphocytes. The treatment groups were: Control (one dose of yeast), Single (one dose of OvaYeast) and Multiple dose (three doses of OvaYeast) with n=10 per group

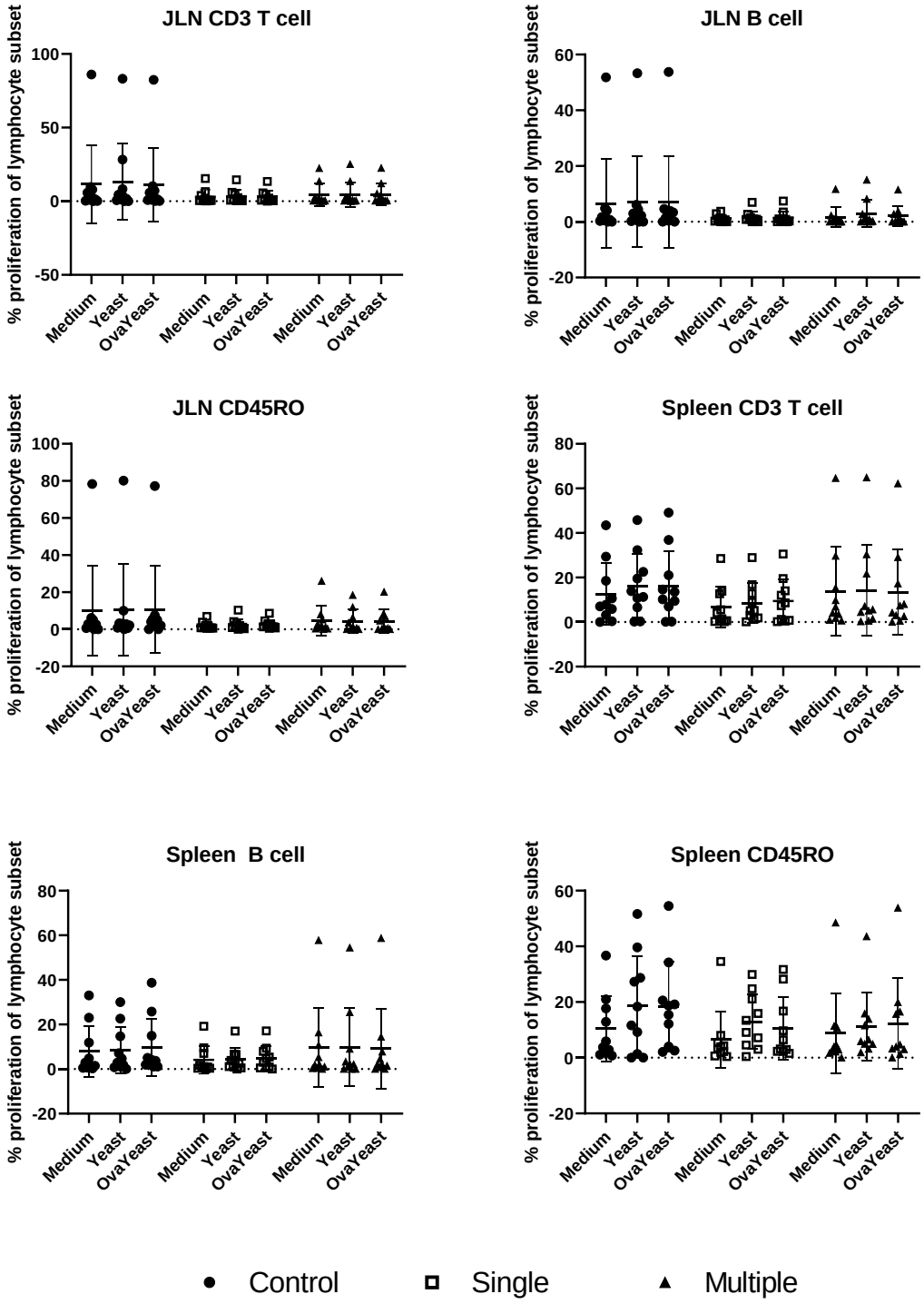

Figure 3.14 Proliferation of tissue lymphocyte subsets in cattle given the yeast vaccine
Lymphocytes isolated from jejunal lymph node (JLN) and spleen were labelled with a fluorescent dye to track proliferation, cultured for 5 days with or without yeast or OvaYeast (stimulants) and labelled to identify T cells, B cells and CD45RO lymphocytes. The treatment groups were: Control (one dose of yeast), Single (one dose of OvaYeast) and Multiple dose (three doses of OvaYeast) with n=10 per group.

Overall, we did not detect memory to yeast or OvaYeast in cells from the RLN, ILN, JLN or spleen despite detecting immune memory in circulating blood cells. The cattle intestinal system is lengthy, and we may not have sampled the specific sites of immune activation. At this time point, we did detect antibody responses in serum as well as in the mucus from the intestine (see Appendix 4).

1.3.6 Cytokine (interferon gamma) response

Induction of cell-mediated immune responses were also assessed by cytokine secretion, in this case, the cytokine interferon gamma. Cytokine responses in whole blood from the three treatment groups at one and two-months post primary vaccination is shown in Fig. 3.15. At both time points, interferon gamma responses were induced to yeast and OvaYeast stimulants in all three treatment groups. Similar to the lymphocyte proliferation responses it appears that immune memory to yeast has been induced supporting its potential value as an adjuvant for oral vaccines.

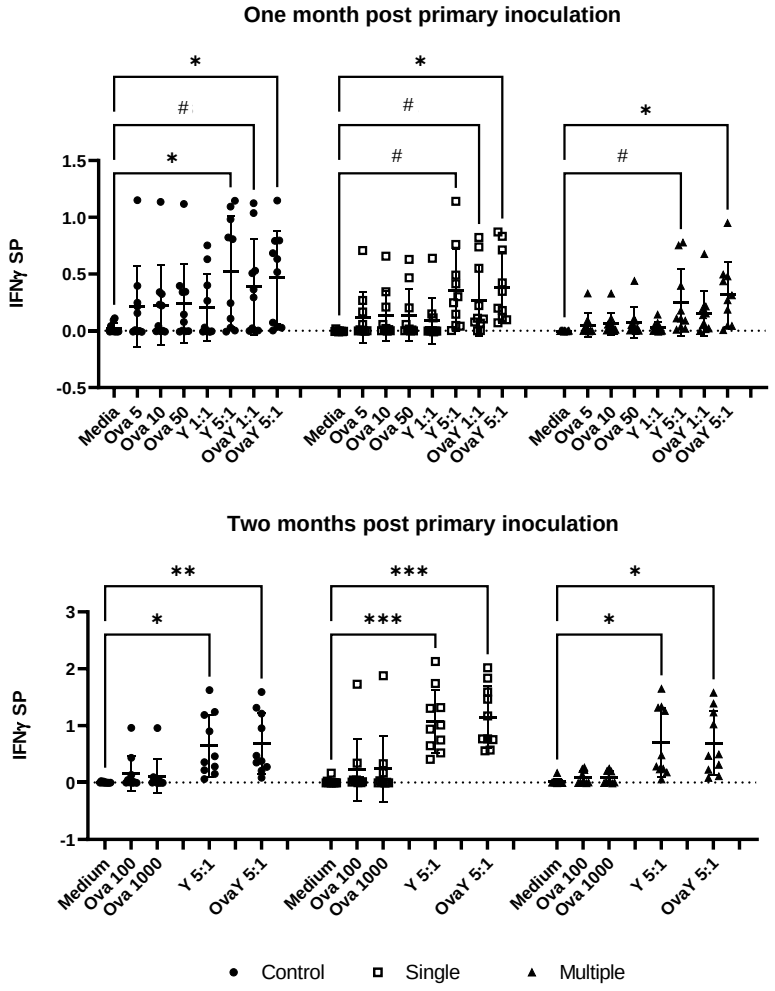

Figure 3.15 Interferon gamma secretion in whole blood

Whole blood taken at one- and two-month post primary vaccination was cultured with various stimuli (ovalbumin, Ova at 5-1000 μg/mL; lysed and intact yeast, Y; lysed and intact OvaYeast, OvaY; pokeweed mitogen) for 48 hrs. IFNγ in culture supernatants is shown. The treatment groups were: Control (one dose of nontransformed yeast), Single (one dose of OvaYeast) and Multiple dose (three doses of OvaYeast) with n=10 per group ***p<0.001; **p<0.01; *p<0.05; #p<0.1

1.3.7 Antibody responses

Presence of serum antibodies to ovalbumin and yeast and OvaYeast lysates was detected by ELISA, as a measure of activation of humoral immune pathways. Serum antibody levels at one, two and five months post primary vaccination are shown in Fig. 3.16. Similar protein concentrations were used for the coating antigens. While ovalbumin was a purified protein the two yeast lysates are a complex mix of yeast components thus the proportion of ova in the lysate protein and therefore coated on the plate is likely to be less than from the purified ovalbumin. This could be reflected in the overall higher ELISA SP for ovalbumin.

At one month post primary vaccination, the Multiple dose group had significantly higher ($p<0.05$) levels of antiOva antibodies compared to the Control group (Fig. 3.16). At five months post primary vaccination, antibodies to Ova and OvaYeast were higher ($p=0.082$ and $p=0.07$ respectively) in the Multiple dose group compared to the Control group.

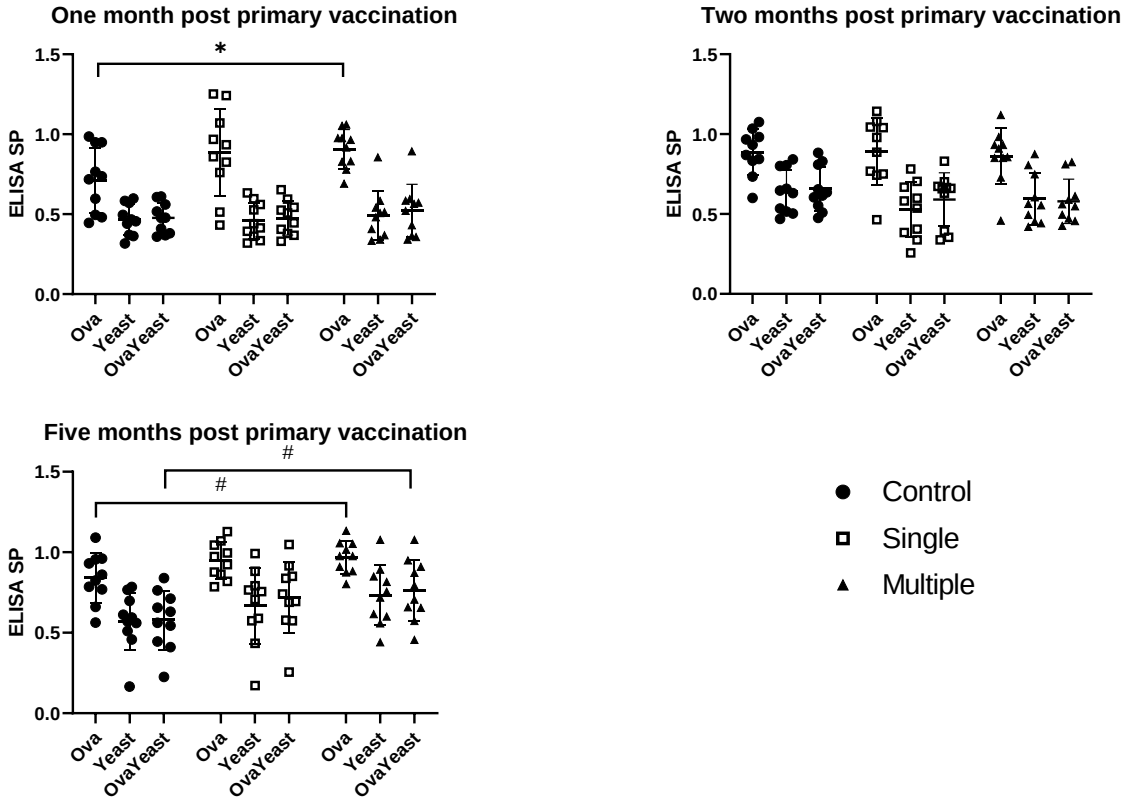

Figure 3.16 Serum IgG antibodies at three time points post primary vaccination
Serum samples were tested for antibodies to ovalbumin (Ova), lysed yeast and lysed OvaYeast. The treatment groups were: Control (one dose of nontransformed yeast), Single (one dose of OvaYeast) and Multiple dose (three doses of OvaYeast) with n=10 per group *$p<0.05$; #$p<0.1$

IgA antibodies to yeast and OvaYeast antigens in intestinal mucus samples taken at necropsy from the Multiple dose group was significantly higher compared to the Controls (Fig 3.17).

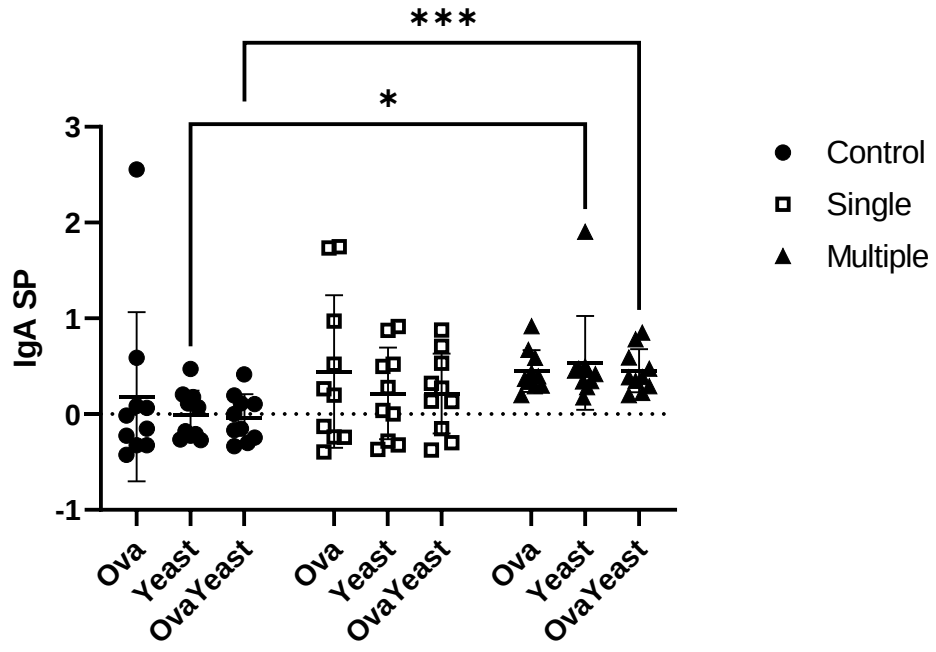

Figure 3.17 IgA antibodies in intestinal mucus 5 months post primary vaccination
Intestinal mucus samples were tested for IgA antibodies to ovalbumin (Ova), lysed yeast and lysed OvaYeast. The treatment groups were: Control (one dose of nontransformed yeast), Single (one dose of OvaYeast) and Multiple dose (three doses of OvaYeast) with n=10 per group ***p<0.001; *p<0.05

Significant serum IgG and mucosal IgA results are shown in Table 3.7.1A and B.

Table 3.7.1A Two-way Repeated Measures ANOVA results for serum IgG ELISA and mucosal IgA ELISA to specific antigens (ovalbumin, yeast or OvaYeast) for each vaccination group (Control, Single dose and Multiple dose)

Sample and time post primary vaccination	Factors	P value
1 month serum IgG	Antigen x Vaccination	0.041
	Antigen	<0.0001
	Vaccination	0.320
	Subject	<0.0001
2 month serum IgG	Antigen x Vaccination	0.477
	Antigen	<0.0001
	Vaccination	0.569
	Subject	<0.0001
5 month serum IgG	Antigen x Vaccination	0.932
	Antigen	<0.0001
	Vaccination	0.063

	Subject	<0.0001
5 month mucosal IgA	Antigen x Vaccination	0.5969
	Antigen	0.2264
	Vaccination	0.0849
	Subject	<0.0001

Table 3.7.1B Dunnett's multiple comparisons test results for antibody specificity to ovalbumin, yeast or OvaYeast where mean ELISA result was significant between vaccination groups

Sample and time post primary vaccination	Comparison group and antigen	P value	Control group mean	Multiple group mean
1 month serum IgG	Control vs. Multiple Ova	0.037	0.712	0.907
5 month serum IgG	Control vs. Multiple Ova	0.082	0.840	0.967
	Control vs. Multiple OvaYeast	0.070	0.579	0.765
5 month mucosal IgA	Control vs. Multiple Yeast	0.0199	-0.011	0.535
	Control vs. Multiple OvaYeast	0.0005	-0.037	0.454

1.3.8 Gene expression

The ileal, jejunal and retropharyngeal LN from five animals were selected from the Control and the Multiple dose treatment groups as these potentially represented the groups with the most difference between treatments.

The expression of IFNγ was significantly elevated in the retropharyngeal LN of animals in the Multiple dose group (p=0.05), with a trend towards increased expression of IFNγ in the lymph nodes of animals in the multi dose group across all sites examined (Fig. 3.17). The other cytokines and chemokines analysed were not significantly different between the control and the multi dose group (Fig. 3.17).

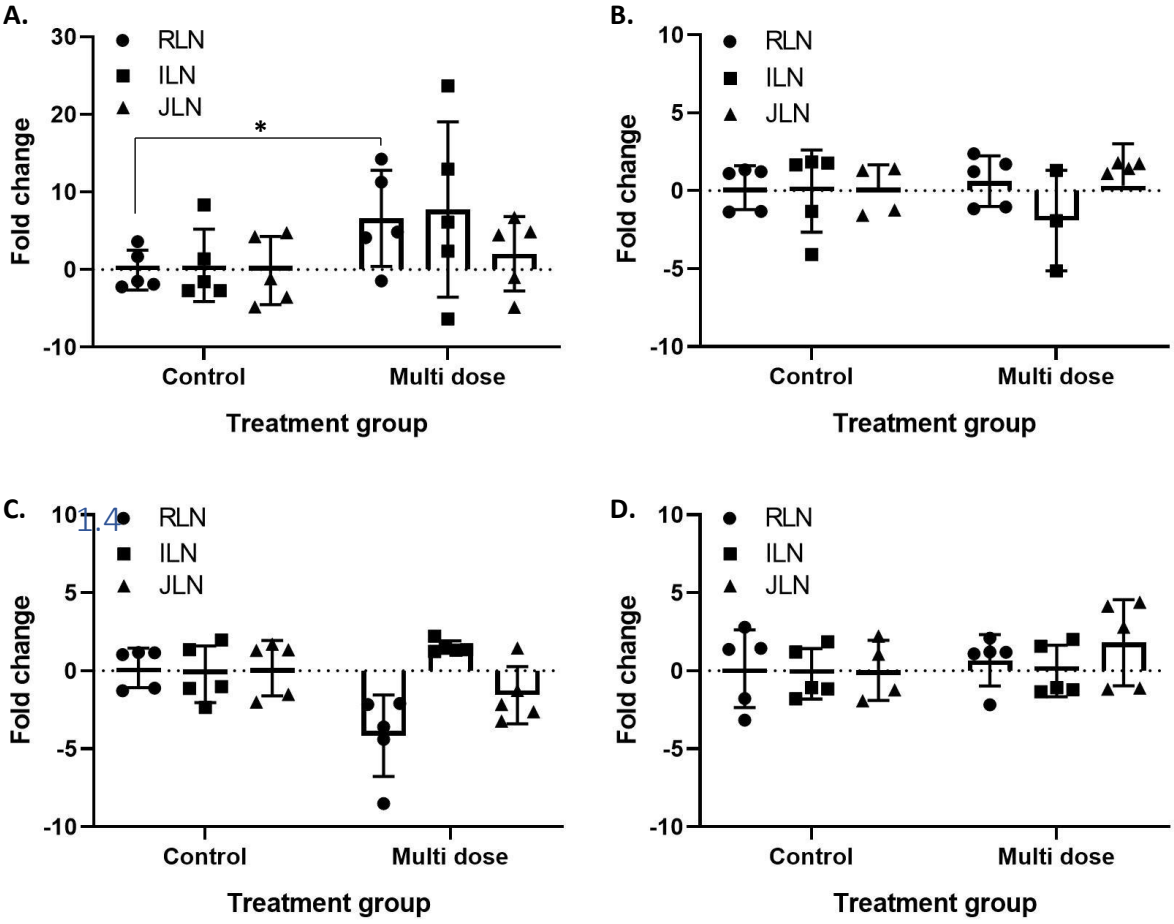

Figure 3.17: Individual animal fold change results for A. IFNγ, B. CXCL10, C. TNF-α and D. IL-10 gene expression in the retropharyngeal lymph node (RLN), ileal lymph node (ILN) and jejunal lymph node (JLN) (n=5/group), showing significantly (p=0.05) increased IFNγ in the RLN of the Multiple dose group compared to the Control group (n=5 per group).

1.5 Discussion

The overall cell-mediated and humoral immune responses in peripheral blood are summarised in Table 3.6. At one-month post vaccination it is clear that a humoral response to the reporter antigen in the vaccine (ovalbumin) has been induced in the Multiple dose group. The cell-mediated (proliferation and IFNγ) responses at this stage are more likely to be to the yeast component of the vaccine since both yeast and OvaYeast stimulants (but not ovalbumin) have generated recall responses in the Control as well as the Single and Multiple dose vaccine groups. This in itself is an important observation as it demonstrates that yeast, given orally, is an appropriate vehicle for delivering antigens that can stimulate immune pathways in cattle.

The lymphocyte proliferation response at the two-month time point provides evidence that a cell-mediated immune response has been induced to ovalbumin in both the Single and Multiple dose groups (Table 3.6). At this time point, recall of prior immune activation by the OvaYeast vaccine is evident in CD4+ helper T cells, CD8+ cytotoxic T cells in the Multidose group and in γδ T cells in the Single dose group (Table 3.5). The proliferative capacity of CD45RO expressing cells to OvaYeast

stimulant in both the Single and Multiple dose groups also demonstrates that the vaccine has been effective in inducing immune memory.

There was evidence of the initiation of an adaptive immune response, as evidenced by increased IFNγ gene expression within the LN of the animals in the Multiple dose group, though the expression was only significantly different in the retropharyngeal LN. No other gene expression changes in the tissues at necropsy (5 months post vaccination) were significant.

The humoral response (serum IgG) to the reporter antigen lasts for at least five months post vaccination. At mucosal surfaces, IgA plays an important role in inhibiting adhesion of invading pathogens to epithelial cells. We show strong evidence of a mucosal IgA response triggered by yeast at the hypothesised site of vaccination induction (intestinal lining). It appears that while the lower dose of the vaccine given to the Single dose group is sufficient to induce cell-mediated immune responses, a higher dose was required for humoral responses.

The yeast vaccine does not alter weight gain in cattle; neither dose of the OvaYeast vaccine affected weight gain compared to animals given yeast alone. In summary, there is sufficient evidence to show that yeast-based vaccines given orally are capable of inducing both humoral and cell-mediated immune responses both systemic (in peripheral blood) and at mucosal sites (intestinal mucosal, retropharyngeal lymph node).

Table 3.6 Summary of significant immune responses to stimulants (rows) in the treatment groups (columns)

Time post primary vaccination	Immune response	Control	Single dose	Multiple dose	Interpretation
One month	Total lymphocyte proliferation	Medium <Yeast Medium < **OvaY**	Medium <Yeast	Medium<Yeast Medium<**OvaY**	Response to yeast?
	IFNγ	Medium <Yeast Medium < **OvaY**	Medium <Yeast Medium < **OvaY**	Medium<Yeast Medium<**OvaY**	Response to yeast?
	Antibodies			Anti-Ova antibodies greater than in Control group	Induction of ovalbumin specific humoral response
Two months	Total lymphocyte proliferation		Medium < **OvaY**	Medium<**OvaY**	Induction of ovalbumin specific cell-mediated response
	IFNγ	Medium <Yeast Medium < **OvaY**	Medium <Yeast Medium < **OvaY**	Medium<Yeast Medium<**OvaY**	Response to yeast?
	Antibodies			Medium<Yeast	

Five months	Total lymphocyte proliferation	Not done	Not done	Not done	NA
	IFNγ	Not done	Not done	Not done	NA
	Serum IgG antibodies			Anti-Ova and anti-OvaYeast antibodies greater than in Control group	Ovalbumin specific cell-mediated response present for 5 months

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Appendix 4: Antigenicity of BVDV antigens in cattle with antibodies to BVDV

1.1 Introduction

In silico screening of the BVDV genome to select antigens considered suitable for generating immune responses is described in Appendix 1.2. Subunit vaccines, like the proposed antigen-yeast vaccine, include a fragment of the pathogen rather than incorporating the whole microorganism. Therefore, it is important to determine their ability to be recognised by the immune system prior to embarking on evaluating vaccine trials.

To minimise cost and accelerate the process of vaccine development, in this study, we determined if the candidate antigens have potential to generate relevant immune responses. Antigenicity was assessed using blood samples from cattle that had antibodies to BVDV and those that didn't. While the presence of BVDV antibodies does not indicate the presence of disease, it was used as a proxy for exposure to the virus.

1.2 Methods

1.2.1 Blood samples

For peptide and protein serum antibody ELISA

Cattle blood samples collected into serum tubes were obtained from commercial properties in NSW (Table 4.1). Breeds included Angus, Angus-cross, Simmental-cross, South Devon, South Devon-cross and English White. Serum was stored at -20^{o}C until required. Samples were categorised based on testing for the presence of anti-BVDV antibodies by ELISA (SVANOVIR®BVDV-AB). The current vaccine used in Australia contains killed virus from two BVDV strains, both of which were represented in the sequence dataset used to design the peptides Because the vaccine is not a marker vaccine, no distinction could be made between antibodies due to infection and those resulting from vaccination.

A total of 67 negative and 71 positive serum samples were subsequently screened by an in-house peptide ELISA to detect the presence of serum antibodies to 9 candidate antigenic regions from BVDV (see Section 4.2.3).

A total of 12 negative and 11 positive samples (BF5 and BF9 in Table 4.1) were screened by an in-house protein ELISA to detect serum reactivity to 2 recombinant proteins containing 4 of the candidate antigens from BVDV.

Table 4.1 Characteristics of blood samples from farms in NSW used for peptide screening

Source	Pestigard vaccination	BVDV ELISA positive (n)	BVDV ELISA negative (n)	Antibody ELISA (antigenicity)	Cytokine ELISA (immunogenicity)
Quirindi (at induction)	No	11	28	Yes	No
Quirindi (at exit)	No	17	0	Yes	No
Bega (BF2)	No	4	8	Yes	No
Berry (BF3)	Unknown	6	6	Yes	No
Monaro (BF5)	No	12	0	Yes	No
Bega (BF8)	No	12	0	Yes	No
Kangaroo Valley (BF9)	No	0	12	Yes	Yes
Shoalhaven (BF10)	No	0	12	Yes	No
Shoalhaven (BF6)	No	4	3	No	Yes
Shoalhaven (BF11)	Yes	6	0	No	Yes
Shoalhaven (BF12)	No	0	1	No	Yes

For cytokine ELISA

Blood samples were collected from cattle from four farms in NSW (Table 4.1, BF6, 9, 11 and 12) into lithium heparin and transported to the laboratory overnight. Whole blood stimulation assays were set up immediately. A total of 17 negative and 10 positive samples were screened using a peptide-stimulated whole blood stimulation followed by IFNγ ELISA to detect the presence of cell-mediated immune memory to 11 peptides containing antigenic regions from BVDV (See section 4.2.4).

Because of the different sample collection and handling protocols for the peptide serum ELISA versus the cytokine ELISA, the two assays were conducted on different sample sets.

From BRD case-control study (Quirindi pen trial)

Serum samples collected from a case-control study for BRD were generously provided by Tony Batterham, together with animal health and lung pathology (n=10). Cases and controls were held in the same environment.

1.2.2 Peptides and proteins

Peptides were designed as described in the Appendix 1.2, using multiple BVDV genome sequences, including those of the BVDV strains used in PestiGard® (Zoetis), the pestivirus vaccine available in Australia. Selected peptides (Table 4.2) synthesized by Mimotopes, VIC were used for screening serum samples. For the peptide serum antibody ELISA, peptides were conjugated to biotin to facilitate binding to the ELISA plate, while nonbiotinylated peptides were used in the whole blood stimulation for the cytokine ELISA.

Protein antigens were from Region 1 (peptide 2) and Region 2b (peptides 1, 4 and 8) as described in Appendix 5.

Table 4.2 Peptides selected for screening for antigenicity and immunogenicity

REDACTED

1.2.3 Antibody ELISA

Peptides

A sandwich ELISA method was followed for detecting antibodies to peptides from BVDV. As shown in Fig. 4.1, a streptavidin coated surface is used to attach biotinylated peptides to wells of an ELISA plate. Antibodies in cattle serum samples that recognise the BVDV-specific peptide will be bound, whereas other antibodies can be subsequently washed away. This binding is detected using a reagent (conjugated to peroxidase) that binds only to anti-bovine antibodies. A compound that is a substrate for the peroxidase enzyme is added to visualise the peptide-antibody binding which can be detected as a change in colour in the reaction mixture.

Optimisation of this ELISA is described in Milestone 5 Report Appendix 8.2.

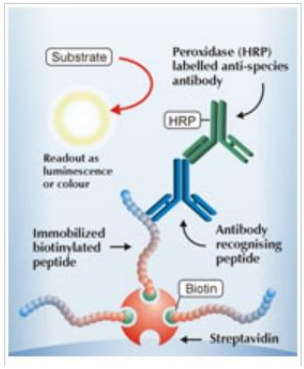

Figure 4.1 Schematic of sandwich ELISA method used for peptide screening

Nunc Maxisorp plates were coated with streptavidin by evaporating a 50 μL aliquot of 5 μg/mL at 37°C overnight. Biotinylated peptides (Table 4.2) were added at 5 μg/mL and incubated for 30 min.

Protein purified from antigen-yeast lysate

Purification of HA-tagged protein antigens is described in Appendix 5.

Nunc Maxisorp plates were coated with purified proteins at 2 μg/mL and incubated overnight at 4°C.

ELISA

Both biotinylated peptides and protein antigens were processed in a similar manner.

Wells were washed four times with wash buffer (PBS with 0.05% Tween20), blocked for 30 min with 1% bovine serum albumin (BSA) in wash buffer and washed four times prior to incubating with serum diluted 1 in 500 for 1 hr. Wells were then washed four times, incubated with anti-bovine IgG-horse radish peroxidase for 20 min and washed four times prior to incubation with TMB (3',3',5',5'-tetra-methyl-benzidine) substrate. Colour development was stopped with 2 M sulfuric acid and the optical density (OD) read at 450 nm.

The background level of serum binding in the absence of peptide was assessed for each sample. All samples were tested in duplicate and the mean value was used for subsequent analysis. The same BVDV ELISA positive sample (Positive) was included in all plates to normalise OD values across plates. SP was calculated as: Sample OD/ Positive OD. For each sample SP the relevant background SP (i.e. relevant sample without peptide) was subtracted.

1.2.4 Cytokine ELISA

1.2.5 Whole blood stimulation

Equal volumes of blood and culture medium without or with one of eleven peptides (10 μg/mL) or pokeweed mitogen (PWM) were incubated for 48 hrs at 37°C in an atmosphere of 5% CO_2. Culture supernatants were harvested and stored at -20°C until used for detecting interferon gamma (IFNγ) by ELISA.

1.2.6 Interferon gamma assay

A bovine IFNγ ELISA was performed on the stored plasma, as previously described (Begg et al. 2019). ELISA reagents were anti-bovine IFNγ (clone 6.19, a gift from Dr. Gregers Jungersen), anti-bovine IFNγ-biotin (clone CC302, Biorad), streptavidin HRP and TMB substrate (3,3',5,5'-tetramethylbenzidine). Each sample was assessed in duplicate. The same positive and negative controls were included in all ELISA plates to normalize OD values across plates. Sample SP% was calculated as 100(Mean Sample OD – Mean plate negative OD)/(Mean plate positive OD – Mean plate negative OD).

1.2.7 Statistical analysis

For each peptide or protein, data for the BVDV positive and negative groups were compared by unpaired t-test (Graphpad Prism 8.4.2) for significant differences ($p<0.05$).

A two-way ANOVA with multiple comparisons (Graphpad Prism 8.4.2) was used to determine significant differences ($p<0.05$). To account for farm of origin, data was analysed using a linear mixed model with farm and BVDV ELISA result as fixed and animal as random variables (Genstat 18th Edition).

1.3 Results

The suitability of peptide sequences from the cattle pathogen BVDV for incorporation into future vaccines was determined. Peptides are short protein segments; the peptides selected were based on

immune epitopes that were bioinformatically determined as regions from BVDV that could induce an immune response. Larger protein antigens incorporating these peptides that were cloned and produced in yeast were also assessed. One of these cloned antigens contained a single peptide within the sequence and the other contained 3 peptides that were in close proximity within the genome. To assess antigenicity, the ability of antibodies in cattle sera to recognise the selected BVDV peptides was determined by ELISA (antibody ELISA). To assess immunogenicity, the ability of these peptides to induce secretion of the cytokine IFNγ by peripheral blood cells was assessed.

1.3.1 Antibody ELISA

Antibody ELISA results are shown in Fig. 4.2. The outlying data points were mainly attributed to the same animals across all peptides. There was one farm where the entire cohort of 12 animals tested BVDV ELISA positive and two farms where all 12 animals tested negative. Therefore, it was unsurprising that farm of origin was a significant factor for all peptides when subjected to Linear Mixed Model analysis where farm was included as a fixed effect ($p<0.05$).

Without accounting for farm of origin, i.e. based on the t-test, there were no statistically significant differences between animals that were BVDV ELISA positive or negative for any of the individual peptides (Fig. 4.2). When farm was included as a factor in the Linear Mixed Model, there were significant differences between commercial BVDV ELISA positive and negative groups for peptide 6 and 7 ($p<0.05$) and peptide 8 ($p=0.06$), with higher values in BVDV-positive samples.

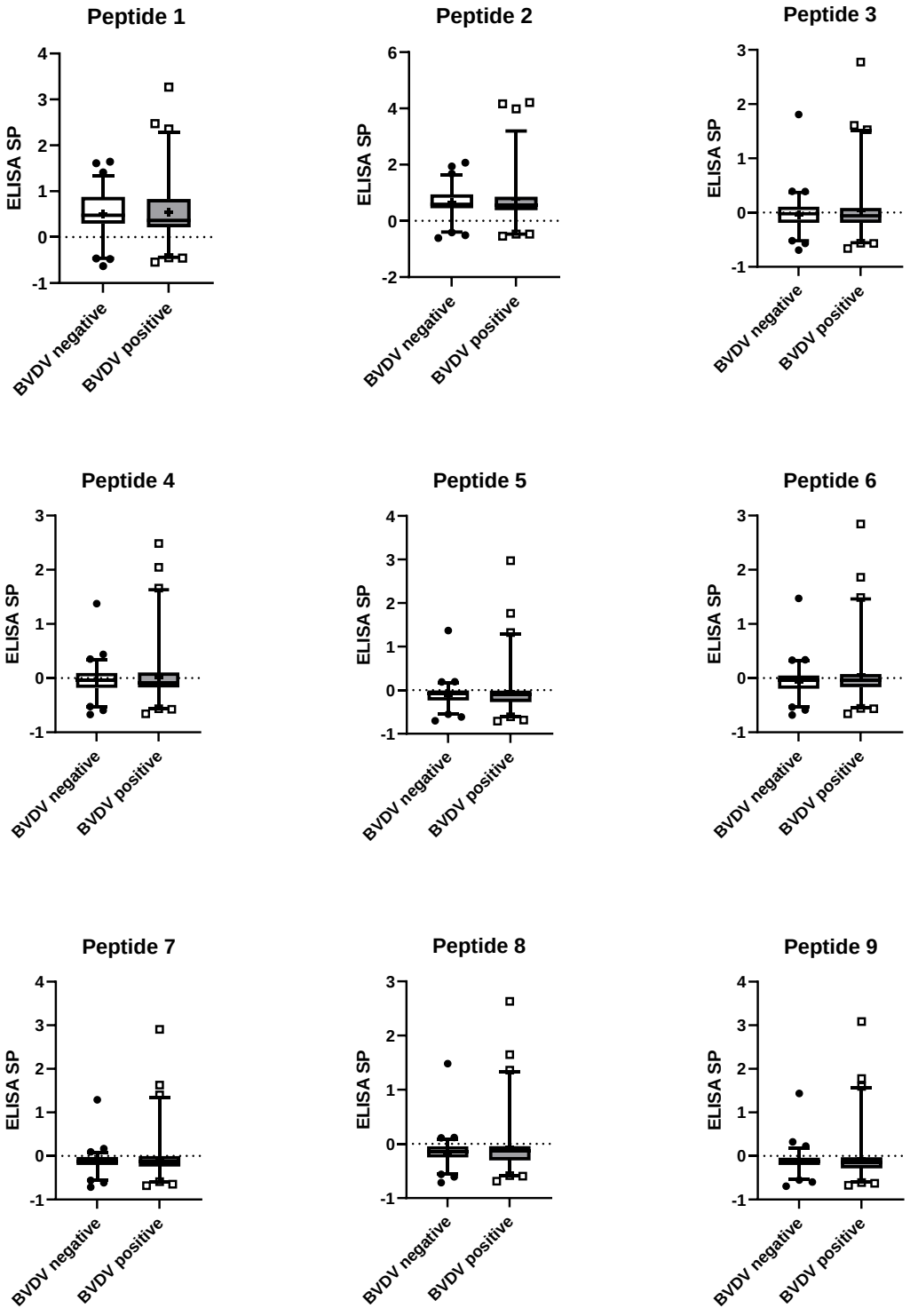

Figure 4.2 Presence of peptide-specific antibodies in BVDV ELISA positive and negative cattle
Antibodies to candidate antigens are shown as ELISA SP for BVDV antibody negative (n=67) and positive (n=71) cattle. Values in the 25th to 75th percentile are shown in the box with the median marked by a line. The mean is marked as + within the box. The range ("whiskers") indicates values within the 5-95th percentile.

When the antibody ELISA results for all 9 peptides were combined, the BVDV ELISA positive group had significantly higher antibody levels compared to the BVDV ELISA negative group ($p<0.01$) (Fig. 4.3), albeit with a bimodal distribution in both groups.

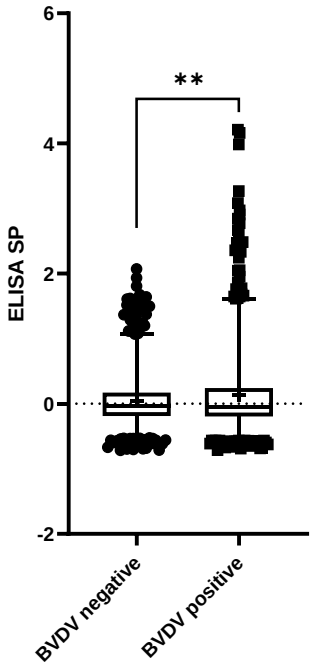

Figure 4.3 Collation of antibody ELISA results for all peptides
Antibodies to candidate antigens are shown as ELISA SP for BVDV antibody negative (n=67) and positive (n=71) cattle. Values in the 25th to 75th percentile are shown in the box with the median marked by a line. The mean is marked as + within the box. The range indicates values within the 5-95th percentile **p<0.01

There were no significant differences to the two candidate antigen-proteins produced in yeast (Fig. 4.4). The characterisation of protein expression in these yeast clones yielded puzzling results: while protein expression was detected by Western blot and purified based on HA-tag expression, integration of the gene into yeast and gene expression was not detected (Appendix 5). Therefore, the absence of a difference in antigenicity of these peptide-proteins in BVDV antibody positive and negative animals is inconclusive.

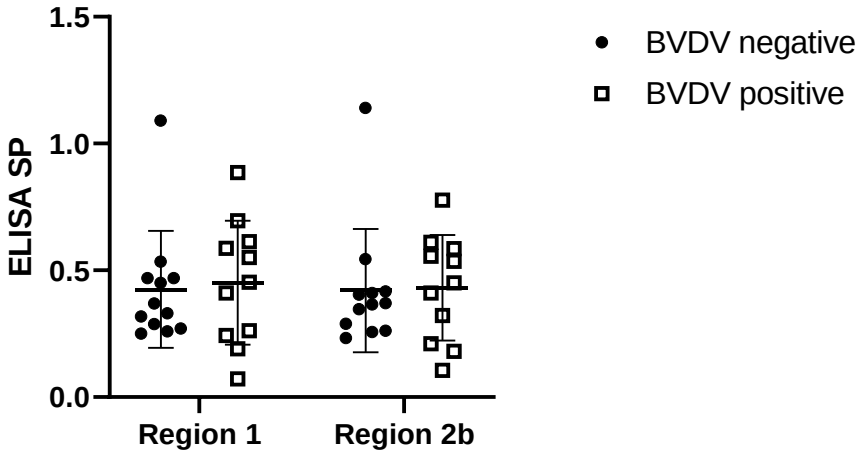

Figure 4.4 Presence of BVDV protein-specific antibodies in BVDV ELISA positive and negative cattle
Proteins from Region 1 and Region 2 of the BVDV genome contain peptide 2, and 1, 4 and 8 respectively. Antibodies to these proteins are shown as ELISA SP for BVDV antibody negative (n=12) and positive (n=11) cattle.

1.3.2 Cytokine ELISA

Induction of the inflammatory cytokine IFNγ by blood cells in response to BVDV peptides was assessed in cattle that were either BVDV ELISA positive or negative (Table 4.1). Tests for cell-mediated immune responses require viable cells. This requires rapid transport of blood tubes from the site of collection to the lab. The blood samples for cell-mediated immune response studies were in good condition as shown by the elevated responses to the nonspecific mitogen PWM in Fig. 4.5.

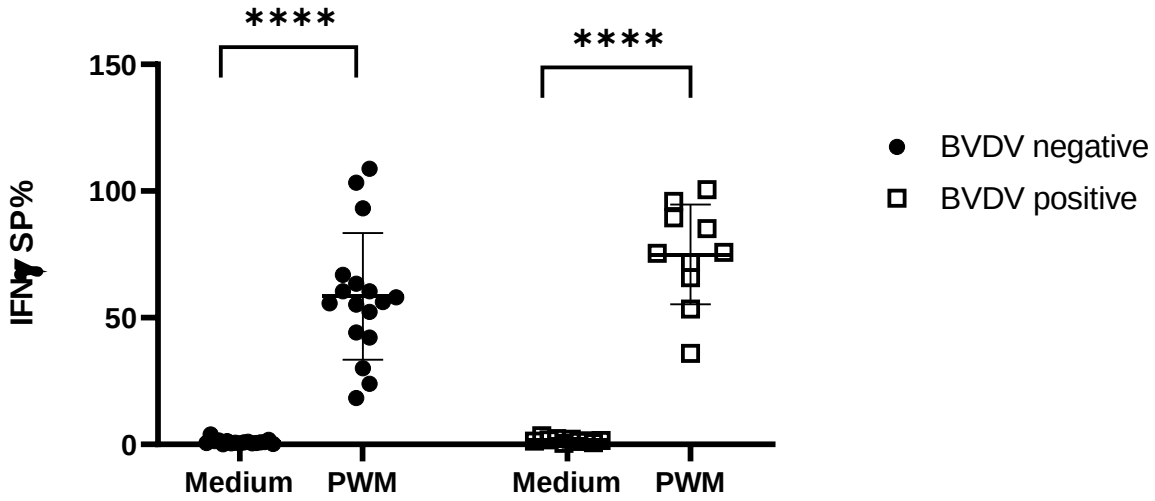

Figure 4.5 Blood cells from cattle were responsive to a mitogen
Whole blood was incubated with no stimulus (Medium) or with PWM. IFNγ in culture supernatants was measured by ELISA ****p<0.0001

The IFNγ response to stimulation by BVDV peptides was not significantly different between the BVDV ELISA positive and negative groups (Fig. 4.5). Outliers with a high IFNγ SP% were seen in both BVDV ELISA positive and negative groups across the different peptides and were consistently associated with the same animals from these respective groups. However, the response to peptide 11 in the BVDV positive group was significantly higher than to the matched Medium alone response ($p<0.05$). There was also a significant difference ($p<0.05$) between the responses to peptide 7 in the BVDV negative group, although the biological significance of this is unclear.

When the results for all peptides (with the medium alone value subtracted) was combined there was no significant difference between the two BVDV ELISA groups ($p>0.05$).

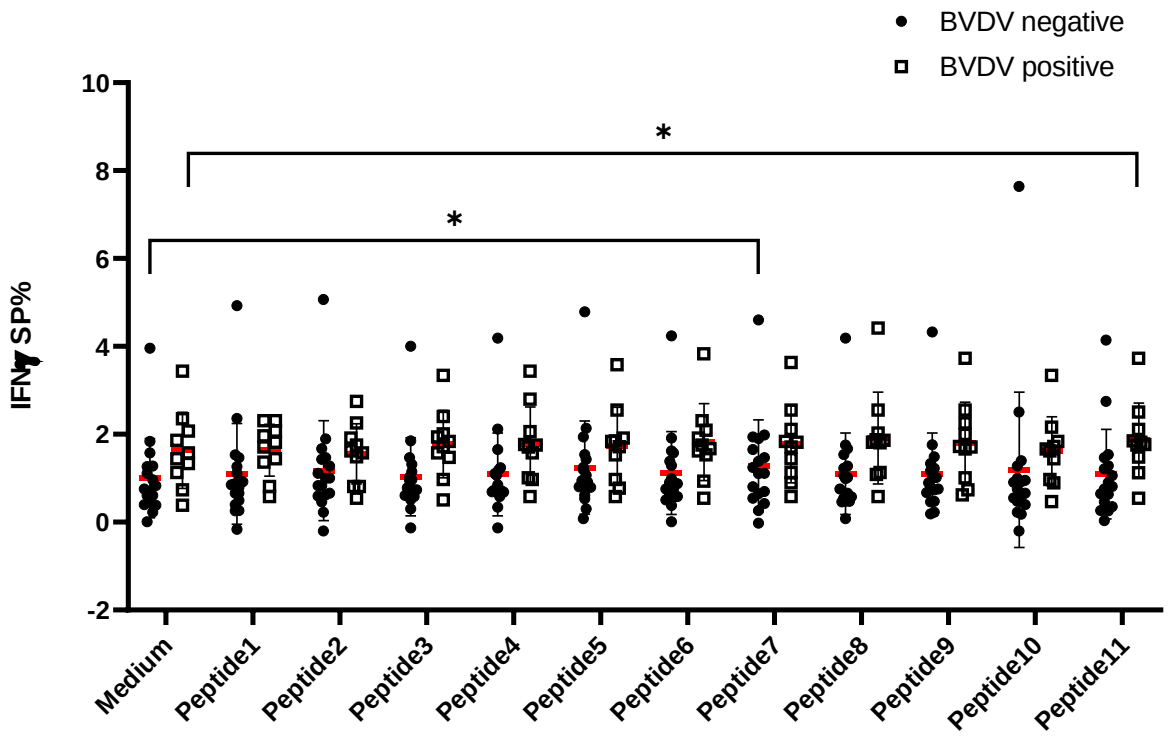

Figure 4.5 Cell mediated immune response to BVDV peptides
Whole blood from cattle was incubated with no stimulus (Medium) or with one of eleven BVDV peptides. IFNγ in culture supernatants was measured by ELISA. The red line indicates the mean. Animals were categorised based on BVDV ELISA result (BVDV negative n=17; BVDV ELISA positive n=10). *p<0.05)

1.3.3 BRD case-control (Quirindi pen trial) results

Serum samples collected from a case-control study for BRD were provided by Tony Batterham, together with health and lung pathology data (Table 4.3).

All cattle were categorised based on a positive or negative result for BVDV using a commercially available ELISA. A positive result indicates the presence of antibodies to BVDV but is not definitive for disease. All but one of the ten animals were BVDV ELISA positive. As shown in Table 4.3, animals with or without lung lesions at necropsy had a positive BVDV ELISA result. Lung pathology was detected in the one animal that was negative for BVDV antibodies in this cohort. This likely reflects

the multifactorial nature of BRD. For 3 of 10 animals, there was disagreement on disease status based on clinical signs versus necropsy results.

Table 4.3 Characteristics of blood samples from the Quirindi feedlot pen trial

BVDV ELISA result	Clinical signs	BRD lung lesions at necropsy
Positive	None	None
Positive	Present	Present
Negative	Present	Present
Positive	None	None
Positive	Present	None
Positive	Present	None
Positive	Present	Present
Positive	None	Present
Positive	None	None
Positive	Present	Present

Peptide ELISA results were analysed based on commercial BVDV ELISA result (Fig. 4.6). Meaningful interpretation cannot be made since there was only one animal that was BVDV ELISA negative.

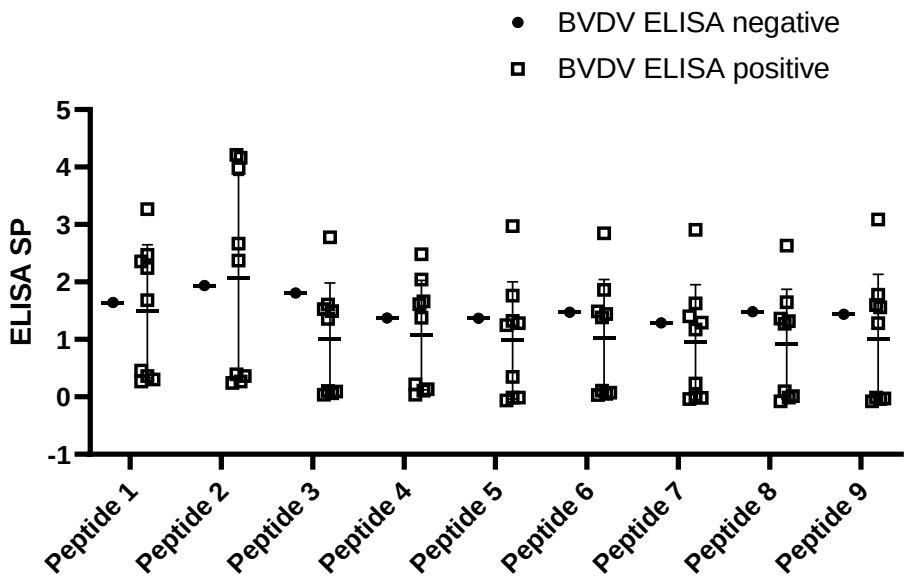

Figure 4.6 Antibodies to BVDV peptides in cattle from a case-control study grouped based on commercial BVDV ELISA result
BVDV negative (n=1) and BVDV positive (n=9)

Data was also analysed based on observation of clinical signs (illness or fever) during the trial (Fig. 4.7). Peptide ELISA data for cattle with clinical signs clustered into two groups while there was no such pattern for the animals without clinical signs of BRD. There were no statistically significant differences between groups.

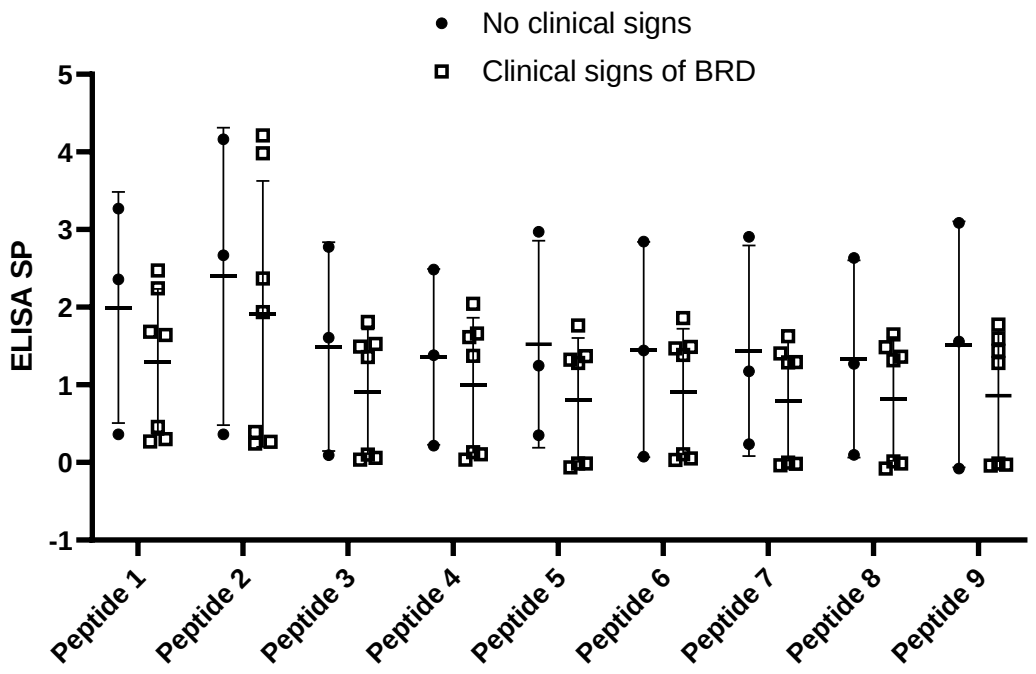

Figure 4.7 Antibodies to BVDV peptides in cattle grouped based on observation of clinical signs of BRD

No clinical signs (n=3) and Clinical signs present (n=7).

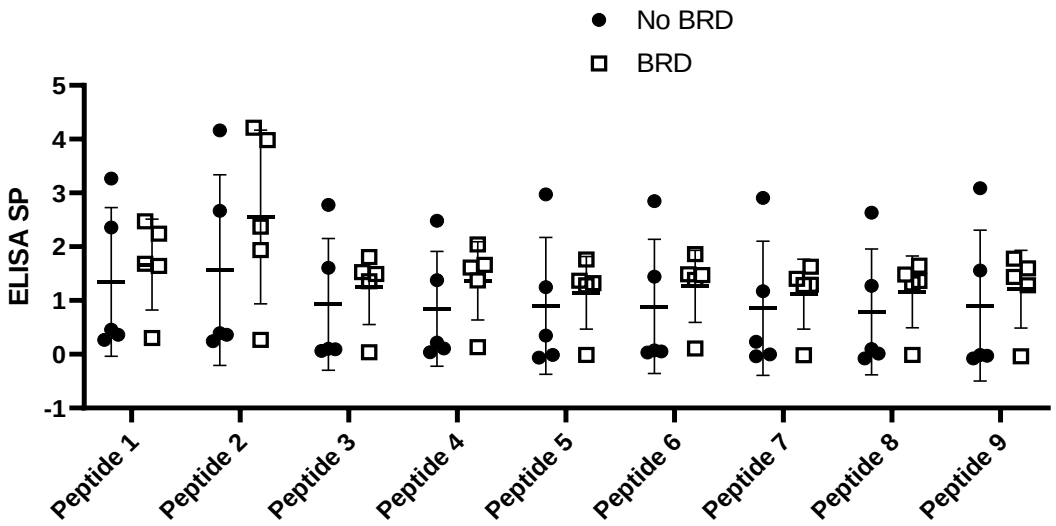

Figure 4.8 Antibodies to BVDV peptides in cattle grouped based on presence of lung lesions

No BRD (n=5) and Clinical signs present (n=5)

There was an overall trend for a higher level of peptide-specific antibodies in cattle with BRD compared to those without BRD-associated lung lesions (Fig. 4.8). In this small cohort of animals

there were no significant differences between the two disease outcomes for any of the peptides (p=0.0838), even without correction for clustering within farm.

1.4 Conclusions

In summary, there is evidence to suggest that peptides 6, 7 and 8 are potentially antigenic given that the specific antibody levels tended to be higher against these peptides in the serum of BVDV ELISA-positive cattle, compared to BVDV ELISA-negative animals. The antibody response to a combination of antigens is likely to be of greater value than single antigens. There is also provisional evidence for immunogenicity, given the stimulation of an IFNγ response to peptide 11 in the BVDV ELISA positive group.

It is conceivable that the response to some peptides could be exploited to differentiate between different disease states; at this stage this is merely speculative and will require further detailed investigation to compare BVDV ELISA status with infection/disease status and longitudinal analysis.

1.5 References

Begg, D. J., O. Dhungyel, A. Naddi, N. K. Dhand, K. M. Plain, K. de Silva, A. C. Purdie and R. J. Whittington (2019). "The immunogenicity and tissue reactivity of Mycobacterium avium subsp paratuberculosis inactivated whole cell vaccine is dependent on the adjuvant used." Heliyon **5**(6): e01911.

Appendix 5: Preparation of recombinant yeast

1.1 Introduction

Kluyveromyces lactis, a yeast found in bovine milk, has been successfully utilised for a variety of applications within the food and pharmaceutical industries (Spohner et al 2016). This yeast species is 'generally regarded as safe' (GRAS) for use in humans and animals. Yeast cell wall components (e.g. β-glucans) have adjuvant properties and can activate immune responses (Herre et al 2004); therefore, an additional adjuvant to stimulate immunity is unnecessary, making yeasts well-suited as vaccine vectors or 'carriers'. *K. lactis* technology has been successfully utilised in livestock vaccine development, such as Infectious Bursal Disease (IBD) virus (Arnold et al, 2012) in poultry and porcine reproductive and respiratory syndrome virus (PRRSV) (Zhao et al, 2014), where it has been shown to elicit a favourable immune response. We therefore proposed that this novel vaccine technology was amenable for formulation in subunit vaccines against ruminant livestock diseases. In addition to the economic advantages, these yeast-vaccines are stable, alleviating cold-chain requirements, and are simple and safe to administer orally or nasally.

The *K. lactis* expression system offers several advantages over other yeast and bacterial protein expression systems. First, K. *lactis* has been used to produce proteins at industrial scale in the food industry for over a decade due to its ability to rapidly achieve high culture densities and abundantly produce recombinant proteins (Spohner et al. 2016). Second, yeast expression is driven by a variant of the strong *LAC4* promoter that has been modified to lack background expression in *E. coli* (Colussi et al. 2005). Therefore, genes toxic to *E. coli* can be cloned into pKLAC2 in bacteria prior to their expression in yeast. Finally, proteins expressed in K. *lactis* have access to eukaryotic protein folding and glycosylation machinery that *E. coli* cells do not possess, making it an important alternative to bacterial expression systems. Folding and glycosylation are important factors in the immune response to foreign antigens.

The aim of this component of the project was to initially construct a yeast-based vaccine containing a 'reporter' that is suitable for use as an oral vaccine and then to construct yeast-based vaccines containing BVDV antigens (protein fragments). Recombinant *K. lactis* containing an ovalbumin (Ova) gene fragment (reporter gene) were produced following optimization of cloning strategies. The protocols for creation of OvaYeast and identifying successfully transformed clones were then optimized and adopted for creation of recombinant yeast that could produce BVDV antigens. Several strategies for verification of transformation and expression of the reporter gene/BVDV protein antigens were employed including Western Blot, magnetic bead-based purification and quantitative PCR. Screening of hundreds of recombinant antigen yeast clones was undertaken to identify putative transformed clones with evidence of gene integration or protein secretion.

1.2 Methods

1.2.1 Cloning strategy for preparation of yeast vaccine stocks expressing the ovalbumin antigen

Yeast (*K. lactis*) was transformed to express the reporter antigen ovalbumin (Ova) following manufacturer's instructions for using the *K. lactis* protein expression kit (New England Biolabs). Briefly, a restriction digest was performed on the Ova-containing plasmid (InvivoGen) and the entire gene was ligated into PKLAC2 plasmid such that it was expressed internally. Insertion of the gene fragment in the plasmid was confirmed by PCR, prior to restriction digest and transformation of the competent *K.lactis* yeast.

Confirmation of successful transformation of recombinant OvaYeast

To confirm successful transformation of *K. lactis* with the Ova gene fragment, colonies grown on the selective media (3% 1 M sodium phosphate buffer, pH 7.5, 1.17% YCB medium supplied with the *K. lactis* Protein Expression kit [New England BioLabs], 2% agar and 0.005 M acetamide) were harvested and screened for presence of the Ova gene by qPCR. Prior to performing the qPCR, genomic DNA was extracted from the yeast clones to be screened. The DNA extraction method involved a cell wall digest in 300 µl of Cell Suspension Solution and 1.5 µl Lytic enzyme solutions from the GentraPuregene Yeast/Bact. kit (Qiagen) at 37oC for 1 hr, followed by DNA isolation of the cell pellet using the DNeasy kit (Qiagen). For the qPCR, primers were designed to target the Ova gene fragment; for 5'- Successfully transformed K. lactis isolates (OvaYeast) were aliquoted and stored at -80°C in 20% glycerol.

Confirmation of ovalbumin expression by recombinant yeast by Western Blot analysis

Automated Western Blotting of the Ova peptide sequence was performed using the WES Protein Simple system. Briefly, yeast cell suspensions confirmed for successful transformation by qPCR, was lysed in CelLytic Y cell reagent (Sigma). The protein concentration of the resulting lysates was quantified by micro-BCA assay (ThermoScientific) and diluted to the relevant concentration in 0.1x Sample Buffer (Protein Simple). The diluted samples were mixed to a 4:1 ratio with 5x Fluorescent Master Mix comprising of 1,4-dithiothreitol, sodium dodecyl sulphate and a fluorescence-labelled marker and incubated at 95°C for 5 minutes. The samples, biotin-labelled protein ladder, antibody diluent (blocking reagent), diluted primary antibody (A6075 Monoclonal Anti-Chicken Egg Albumin (Ovalbumin) antibody produced in mouse (1:100 dilution, Sigma), the horseradish peroxidase (HRP)-conjugated secondary antibody, chemiluminescent substrate (luminol -peroxidase), and stacking matrices were loaded into individual wells of the sample plate. The plate was centrifuged for 5 minutes at 2500 rpm at room temperature prior to loading into the WES equipment along with the relevant capillary cartridge. Separation electrophoresis and immunodetection steps taking place within the capillary system were fully automated within the WES system. The digital images obtained were analysed with Compass software (Protein Simple).

Confirmation of ovalbumin expression by recombinant yeast by one-dimensional liquid chromatography mass spectrometry (1D LCMS)

1D LCMS was performed by the Sydney Mass Spectrometry Core Facility, University of Sydney. Yeast cell lysis was carried out using Cellytic reagent (Sigma) or TCA (trichloro acetic acid). Cell lysate samples were precipitated by the addition of 800 µL ice cold acetone to 200 µL of each sample and incubated at -30°C freezer overnight. Acetone was removed and samples air dried, then 200 µL of lysis buffer was added to each sample. The protein concentration was quantified (QUBIT) the entire sample was reduced alkylated, digested and incubated at 37 °C overnight. The samples were then acidified with 10% TFA. Samples were dried down to ~100 µL prior to 'Zip Tipping' and eluted in 10 µL 50%ACN, 0.1%TFA and r/c in 8 µL loading buffer. Each sample was then subjected to 1D LCMS followed by data searches using PD 2.5 and Mascot 2.7.

Preparation of OvaYeast vaccine stocks

Stored OvaYeast aliquots were streaked onto selective agar and allowed to grow for 3 days. Colonies were selected and inoculated in 1 mL of 1% yeast, 2% soy-peptone and 4% galactose (YPGal) media overnight at 30°C with shaking (250 rpm). Overnight cultures were then added to 1 L of YPGal media and cultured overnight at 30°C with shaking (170 rpm) for batch growth of vaccine isolates. Aliquots

of the bulk cultures were taken for cell counts and for qPCR to confirm retention of the Ova gene fragment following storage and re-culture. Remaining cultures were adjusted to the desired vaccine dose in phosphate buffered saline (PBS) and heat-killed at 100°C for 10 minutes. Inactivation of yeast was confirmed by streaking on selective agar and observing no growth after 7 days of incubation at 30°C. The control vaccine, wild-type *K. lactis* was prepared in the same way, with aliquots being streaked on non-selective agar (1% yeast extract, 2% soy-peptone, 2% glucose and 2% agar) prior to growth in YPGal. Vaccine dose aliquots (OvaYeast- Y222, Y243 and wild-type yeast Y238) were stored at -80°C till used.

1.2.2 Cloning strategy for preparation of recombinant yeast expressing BVDV antigens

The original cloning strategy of first cloning into *E.coli* and then into yeast was modified to clone directly into the yeast vector, as show in Fig. 5.2.1.

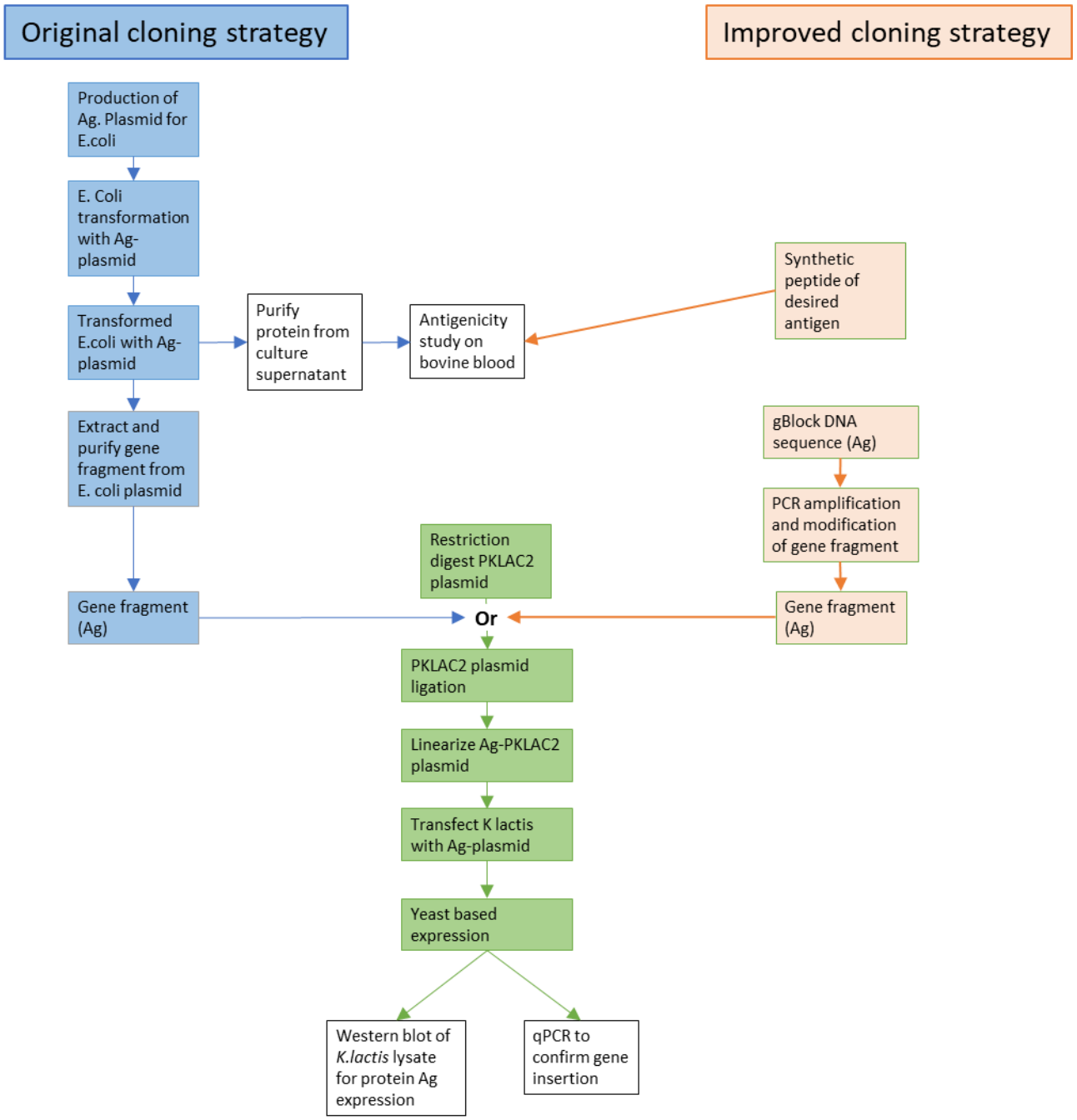

Fig. 5.2.1 Flow chart of the cloning strategy adopted. The improved cloning strategy removed the amplification of the gene sequence and expression in *E.coli* to screen antigenicity and this was replaced with the use of synthetic peptides to screen for antigenicity, thereby streamlining creation of the recombinant yeast clones.

Selection of regions for cloning

The process for candidate antigen selection and details of suggested BVDV epitopes are also shown in Appendix 1.2. Of the 15 suggested epitopes, 9 could be synthesised for antigenicity screening (Appendix 4) (Fig 5.2.2) and as such were used to select the regions for *K. lactis* cloning.

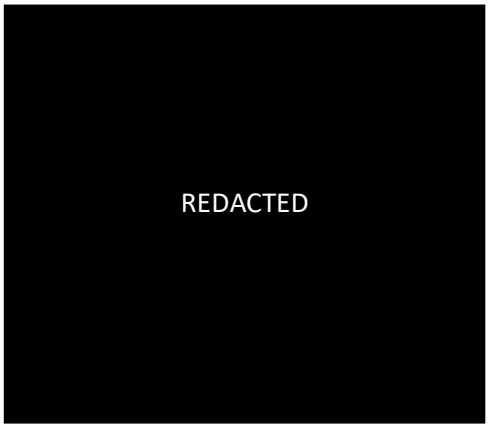

Figure 5.2.2 Schematic representation of the BVDV polyprotein and location of the peptide epitopes.

Four regions were selected for cloning based on the location of the epitopes identified (Fig. 5.2.2; Table 5.1). Region 1 is located within the E^{rns} protein and is 67 amino acids in length. The epitope associated with peptide 2 sits within this cloned region. Region 2a and 2b are two sequence variants located within the E2 protein that are 413 and 343 amino acids in length, respectively. Region 2a contains epitopes for peptides 3, 5 and 7 and Region 2b contains the epitopes for peptides 1, 4, and 8. Region 3, located in the HrpA section at the end of the NS3 protein, is 300 amino acids in length and contains the epitope associated with peptide 9. Region 4, located in NS2, is 345 amino acids long and contains the epitope associated with peptide 6.

Table 5.1 Peptides covered by regions selected for cloning into *K. lactis*

	Region 1	Region 2a	Region 2b	Region 3	Region 4
Number of peptides incorporated	2	7, 5, 3, 8	1, 4, 8	6	9
Size (bp)	198	1239	1029	900	1035
Protein product size (kDa)	7.33	45.84	38.07	33.3	38.3

Cloning strategy to produce recombinant BVDV antigen-yeast

'gBlock™' gene fragments were used to construct the plasmid vector for transformation of *K. lactis* to create the recombinant clones. The gBlocks™ are double-stranded DNA fragments corresponding to the regions of interest shown in Table 5.1.

The gBlock™ for each region underwent PCR modification to create two new gene fragments that when ligated into PKLAC2 and used to transform *K. lactis*, would each have the potential to produce recombinant yeast able to express BVDV proteins either intracellularly or extracellularly (Fig. 5.2.3). In addition, a human influenza haemagglutinin (HA) molecule (corresponding to amino acids 98-106) was added to the modified gBlocks™. Recombinant proteins will express the HA tag allowing for identification using anti-HA antibodies and protein purification for antigenicity studies. The addition of the HA tag does not interfere with the bioavailability or biodistribution of the recombinant protein produced.

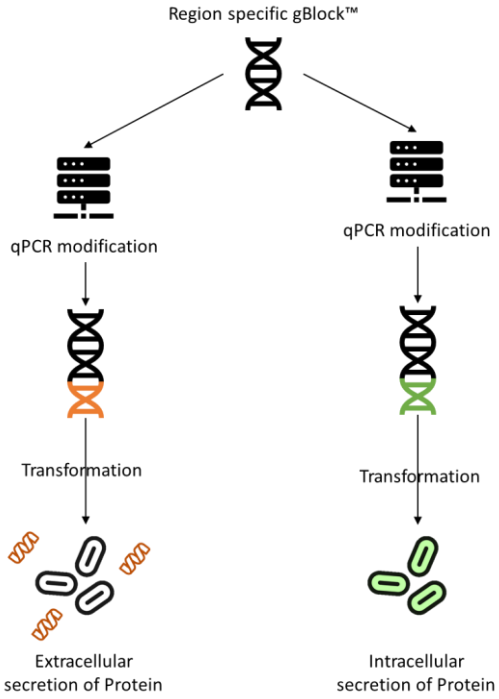

Figure 5.2.3 Schematic of the modification of gBlock™ regions to produce *K. lactis* clones capable of intracellular or extracellular protein secretion.

Modified gBlocks™ for each of the regions selected for cloning into K. lactis were ligated into the PKLAC2 plasmid vector for transformation of *K. lactis*, to generate a total of 10 plasmid vectors (four regions of interest with 2 sequence variants of Region 2(a/b) (n=5), cloned for intracellular and extracellular expression). Ligation was confirmed with PCR and gel electrophoresis, as shown in Fig 5.2.4.

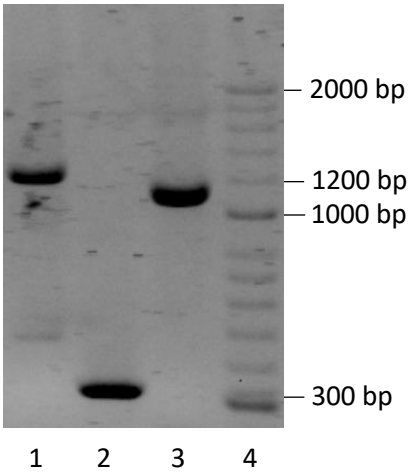

Figure 5.2.4. Representative gel electrophoresis image confirming plasmid insertion. A. Lane 1: Region 3 extracellular 1267 bp, Lane 2: Region 1 intracellular 293 bp, Lane 3: Region 2b intracellular 1112 bp. Lane 4 MW marker.

PKLAC2-antigen plasmid vectors for each region for both confirmations (intracellular and extracellular expression) were used to transform *K. lactis*. Following transformation, *K. lactis* was inoculated onto selective agar with growth of multiple pinpoint colonies forming a lawn after 3 days, with several larger colonies per plate. Large colonies were subcultured onto selective agar for 2 days to obtain a pure culture prior to inoculation into YPGal medium for culture overnight at 30°C with shaking (170 rpm). Hundreds of clones were screened by conventional and qPCR to identify successfully transformed isolates for each region.

Confirmation of successful transformation of recombinant antigen-yeast

Several methods were employed in parallel to screen for successful transformation of *K. lactis* recombinant antigen clones, including confirmation of the integrated DNA fragment, expression of RNA encoding the antigenic protein and identification of the antigen protein (in the *K. lactis* cell lysate).

Initially, antigen-yeast colonies for each region that had grown on selective agar were harvested and used to screen for successful transformation by conventional and qPCR amplification of the region of interest. Multiple sets of primers were designed for each region of interest in both confirmations, to identify recombinant antigen-yeast clones (Table 5.2). In qPCR reactions, a *K. lactis* reference genes (K.lactis Actin gene, forward 5'-TGGAAGCTGCTGGTATCGAC-3', reverse 5'-GAAGGAGCCAAGGCGGTAAT-3') was included to confirm quality of recombinant yeast DNA extracts (Fig. 5.2.5). In addition, standards constructed from the original gBlock™ gene fragments for each region were used as a test positive based on the fragment melt temperature (DNA size) and to determine DNA quantity in successfully transformed clones (Figure 5.2.5).

Table 5.2 Primers designed for optimisation of qPCR screening of recombinant antigen yeast

REDACTED

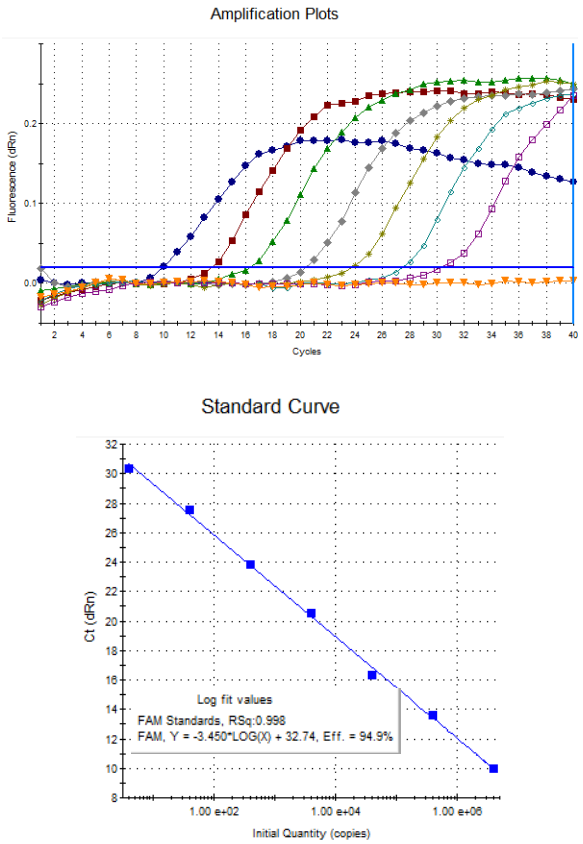

Fig. 5.2.5. Example standard curve using the Region 1 gBlock and qPCR primers used for screening Region 1 yeast clones. Standard 1 (blue) was at a very high concentration and hence there was an unusual amplification curve consequently, however the remaining standards all showed excellent amplification with a standard curve R^2=0.998 and reaction efficiency of 94.9%.

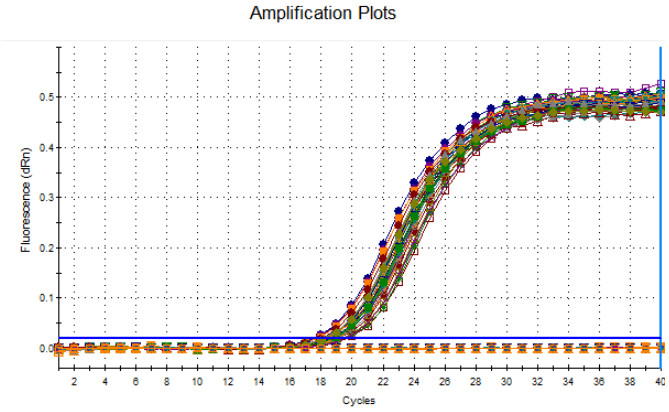

Fig. 5.2.6. Amplification of the *K.lactis* reference gene in DNA samples from yeast clones.

A pooled screening approach was adopted where a minimum of 55 and up to 141 yeast colonies were selected from the original selective agar culture for each region and type (intracellular/extracellular) and pooled (8 colonies per pool) prior to qPCR. Clone pools that were identified as positive then had individual colonies re-screened to identify isolates with successful transformation.

Gene Expression to confirm expression in recombinant K. lactis

A subset of the recombinant antigen yeast clones was screened for successful transformation via RNA extraction and gene expression. RNA was extracted using RNAzol RT (Sigma), according to the manufacturer's instructions. The RNA samples were subjected to an ethanol clean up to remove contaminants and the DNAse treated (RQ1 RNase-free DNase, Promega) to remove genomic DNA. These were converted to complementary (c)DNA using the SensiFast Reverse transcriptase kit (Bioline) and the cDNA was diluted 1 in 2 prior to qPCR. The same *K. lactis* reference gene described in section 5.2.2.3 was utilised for gene expression analysis and confirmation of RNA extract quality. The gene expression analysis was also used as an additional tool to examine isolates that had evidence of intracellular protein excretion by WES analysis but showed no DNA integration by qPCR.

Protein secretion in recombinant K. lactis lysates

As detailed in section 5.2.1.2, automated Western blotting of the HA-tagged antigen protein in recombinant *K. lactis* lysates was performed using the WES Protein Simple system. To identify the recombinant proteins of interest, the 51010 HA Tag Monoclonal Antibody (1:500 dilution, Invitrogen) was used.

Purification of HA-tagged proteins from BVDV antigen-K. lactis lysates

BVDV antigen-yeast clones with detection of antigen protein by WES were selected for protein purification.

Yeast clones were pooled, lysed with CelLytic reagent (Sigma) and the lysate collected after centrifugation. Anti-HA magnetic beads (Thermo Scientific) were washed (0.05% Tween20 in TRIS-buffered saline) and incubated with yeast lysate for 30 min. The protein bound HA beads were collected magnetically and washed several times. The beads were removed with 50 mM NaOH and then neutralised in TRIS-Buffer. Protein in the eluate was quantified by micro-BCA assay.

Confirmation of BVDV antigen expression in recombinant K. lactis lysates by one-dimensional liquid chromatography mass spectrometry (1D LCMS)

1D LCMS was performed by the Sydney Mass Spectrometry Core Facility, University of Sydney. Concentrated supernatant samples were precipitated by the addition of 800 µL ice cold acetone to 200 µL of each sample and incubated at -30^{o}C freezer overnight. Acetone was removed and samples air dried, then 200 µL of lysis buffer was added to each sample. The protein concentration was quantified (QUBIT) and ~16 µg of each sample was taken out for reduction, alkylation and tryptic digestion (1:50). All samples were incubated at 37 ^{o}C overnight. The samples were then acidified with 10% TFA, ~1 µg was taken out for 'Zip Tipping'. Samples were eluted in 10 µL 50% ACN, 0.1% TFA and r/c in 8 µL loading buffer. Each sample was then subjected to 1D LCMS followed by data searches using PD 2.5 and Mascot 2.7.

1.3 Results

1.3.1 Recombinant OvaYeast

Confirmation of successful transformation

Fig. 5.3.1 shows growth of *K. lactis* on selective media suggesting successful transformation.

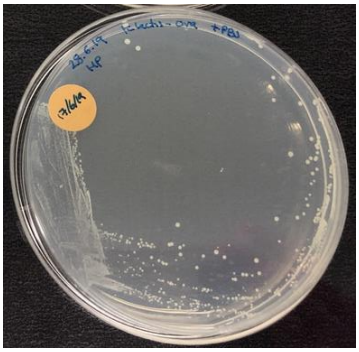

Figure 5.3.1. Growth of successfully transformed OVA-yeast colonies on selective media

Confirmation of successful transformation of *K. lactis* yeast with the *Ova* gene is shown in Fig. 5.3.2. The quantitative PCR amplification plot (Fig. 5.3.2A) shows the amplification of DNA extracted from a successfully transformed *K. lactis* colony, two positive controls (*Ova* gene in E. coli plasmid; *Ova* gene in *K. lactis* plasmid) and a negative control. The (cycle threshold) CT value in the amplification plot directly relates to the quantity of target DNA in the sample. The two positive control samples had a higher level of initial target DNA input, as it was directly extracted from the plasmid, compared to the transformed *K. lactis* colony, explaining the shift in CT value in the yeast isolate.

The dissociation curve (Fig. 5.3.2B) further confirms successful integration of the *Ova* gene into the yeast genomic DNA. DNA amplified from the transformed yeast colony has the same melt temperature (directly related to amplification fragment length) as the two positive controls. Non-transformed K. lactis yeast does not contain the ova gene or any sequences with similarity to the primer binding sites and shows no amplification in this qPCR (data not shown).

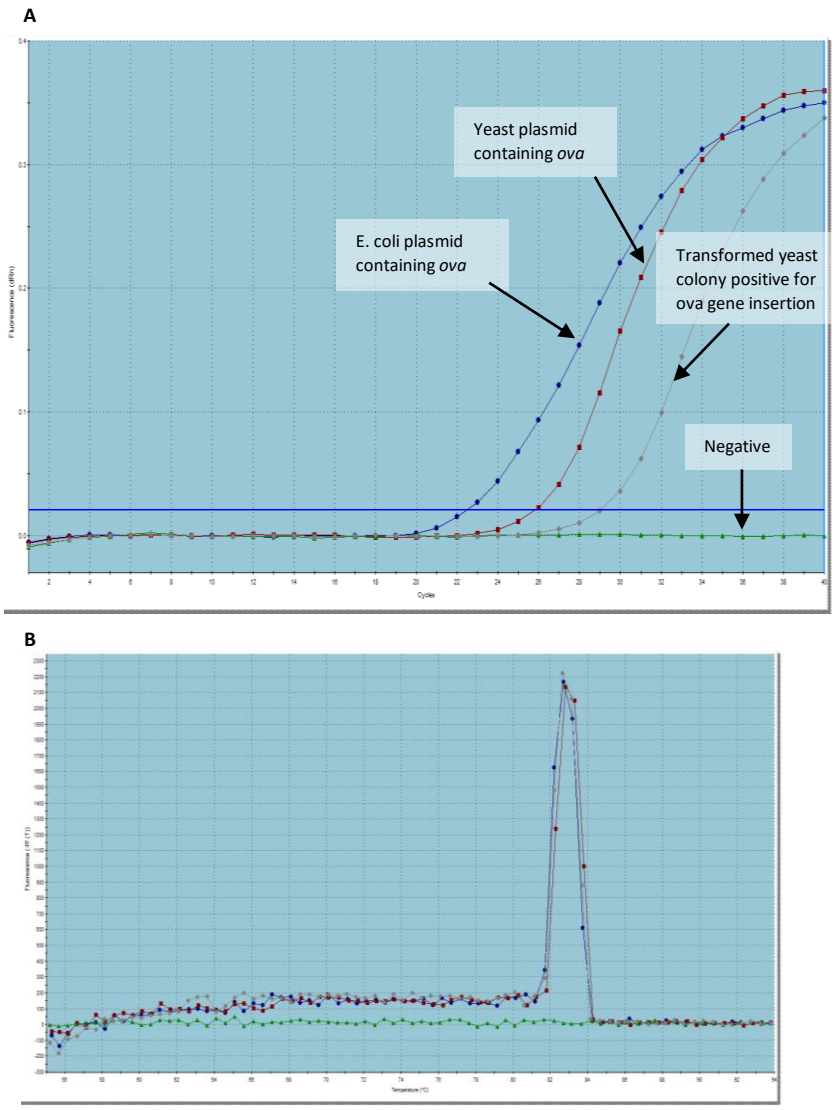

Figure 5.3.2 qPCR amplification plot and dissociation curve showing successful transformation of *K. lactis* yeast with an ova gene.

DNA was extracted from transformed *K. lactis* colonies following growth on selective media and analysed with qPCR to confirm integration of the ova gene. (A) Amplification of two positive controls for the ova gene, sample from a transformed *K. lactis* colony and a negative control containing no template DNA. (B) Integration of the correct fragment is confirmed in the melt analysis. Similar colour scheme to (A) is used for samples.

1.3.2 Confirmation of ovalbumin expression by recombinant OvaYeast

Evidence of integration and expression of the ovalbumin protein within the OvaYeast vaccine was obtained by Western Blot analysis. Fig. 5.3.3 shows appropriate sized bands (indicated by blue arrows) in one representative sample of protein expression from lysates prepared from a positive ovalbumin control (A5503 Albumin from chicken egg white, Sigma), a yeast control (nontransformed yeast Y238) and the OvaYeast vaccine (Y234).

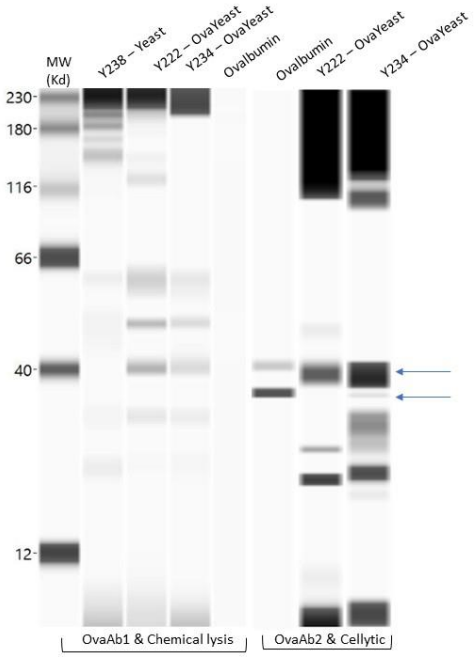

Figure 5.3.3 Evidence of protein expression was assessed by Western Blot.

Presence of ovalbumin in unmodified yeast and OvaYeast vaccine lysates were assessed by Western blot. Samples were subjected to either chemical (Fig. 5.3.3, left panel) or enzymatic (Fig. 5.3.3., right panel) lysis. The digital image (Figure 5.3.3) illustrates the presence of doublet bands (indicated by the blue arrows) within the OvaYeast vaccine lysates that align to the doublet band produced by the ovalbumin positive control. This band is not replicated in the unmodified yeast lysate (Y238, Fig. 5.3.3) however there is evidence of a band of a different (larger) size with unknown attribution within the unmodified yeast. Doublet OVA bands may be due to the presence of ovalbumin splice variants and isoforms within the protein preparation and is commonly reported for this protein.

Mass spectrometry methods can be applied in metaproteomic studies of microbes (bacteria and yeast) to identify the proteins present in a sample (Hinzke et al. 2019). The workflow involves generation of a protein mixture from the sample that is digested into peptides, which are analysed in a mass spectrometer and then screened against a database using a dedicated search engine (e.g. MASCOT).

Since the effect of the yeast cell lysis reagents on detection of proteins by mass spectrometry was unknown, the yeast vaccine (Y222) was subjected to two different lytic methods. The expected protein sequence for ovalbumin (pCpGfree OVA, InvivoGen) is:

MGSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAMVYLGAKDSTRTQINKVVRFDKLPGFGDSIEAQCGTSVNVHSSL
RDILNQITKPNDVYSFSLASRLYAEERYPILPEYLQCVKELYRGGLEPINFQTAADQARELINSWVESQTNGIIRNVLQPSSVDSQT
AMVLVNAIVFKGLWEKTFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTMSMLVLLPDEVSG
LEQLESIINFEKLTEWTSSNVMEERKIKVYLPRMKMEEKYNLTSVLMAMGITDVFSSSANLSGISSAESLKISQAVHAAHAEINEA
GREVVGSAEAGVDAASVSEEFRADHPFLFCIKHIATNAVLFFGRCVSP

Expected Molecular weight = 42.92 kDa

The results of the top 10 protein hits (excluding keratin) are shown. Ovalbumin was confirmed to be present in the two yeast vaccine samples tested and was the most common protein hit in each of these.

Sample 1 (Y222 Yeast vaccine, CelLytic) top ten protein hits

Accession	Description	Sum PEP Score	Coverage [%]	# Pepti
OVA	Ovalbumin sequence	472.717	87	
Q70CP7	Kluyveromyces lactis Enolase	105.132	73	
Q6CQJ3	Kluyveromyces lactis Peptidyl-prolyl cis-trans isomerase	70.409	71	
P14828	Kluyveromyces lactis Phosphoglycerate kinase	45.325	42	
Q6CNK3	Kluyveromyces lactis Phosphoglycerate mutase	48.09	51	
P20369	Kluyveromyces lactis Alcohol dehydrogenase 1	38.416	30	
P17819	Kluyveromyces lactis Glyceraldehyde-3-phosphate dehydrogenase 1	47.895	44	
P49383	Kluyveromyces lactis Alcohol dehydrogenase 2	20.386	19	
Q6CR18	Kluyveromyces lactis 60S ribosomal protein L36	23.202	49	
O13350	Kluyveromyces lactis ATP synthase subunit d, mitochondrial	37.995	49	

Sample 2 (Y222 Yeast vaccine, WES) top ten protein hits

Accession	Description	Sum PEP Score	Coverage [%]	# Pept
OVA	Ovalbumin sequence	298.454	79	
Q70CP7	Kluyveromyces lactis Enolase	106.485	60	
Q6CQJ3	Kluyveromyces lactis Peptidyl-prolyl cis-trans isomerase	76.607	71	
Q6CNK3	Kluyveromyces lactis Phosphoglycerate mutase	60.262	55	
Q6CRN6	Kluyveromyces lactis KLLA0D07634p	54.314	80	
P78700	Kluyveromyces lactis ATP synthase subunit delta, mitochondrial	52.775	43	
P00761	Trypsin - Sus scrofa (Pig)*	52.496	36	
Q6CKV3	Kluyveromyces lactis KLLA0F07865p	40.961	57	
P14828	Kluyveromyces lactis Phosphoglycerate kinase	38.406	37	
Q6CLU4	Kluyveromyces lactis 40S ribosomal protein S12	35.149	68	

*This is likely to be a constituent of the media.

1.3.3 Recombinant antigen (BVDV) yeast

Screening of putative recombinant antigen-yeast

Recombinant antigen yeast clones were screened by qPCR (Table 5.3).

Table 5.3 Recombinant antigen yeast clones screened by qPCR, gene expression and/ or protein secretion

Region	No. screened with qPCR for DNA integration	No. confirmed for DNA fragment	No. screened for expression of antigen gene	No. positive for gene expression	No. screened by WES for secreted antigen	Secreted antigen present by western blot

1 extracellular expression	121	0	0	ND	0	ND
1 intracellular expression	141	0	4	0	4	3
2a extracellular expression	96	5*	0	ND	0	ND
2a intracellular expression	96	0	0	ND	0	ND
2b extracellular expression	65	0	0	ND	0	ND
2b intracellular expression	55	0	4	0	4	2
3 extracellular expression	96	4†	0	ND	0	ND
3 intracellular expression	96	0	0	ND	0	ND
4 intracellular expression	96	0	0	ND	0	ND
4 extracellular expression	96	2**	0	ND	0	ND

* Three of these were pooled samples and 2 were individual clones. † Two of these were pooled samples and 2 were individual clones, ** Both of these were pooled samples. ND=not done

There were a number of clones that showed evidence at the DNA level of the presence of the cloned gene in the qPCR screening (Figures 5.3.1 to 5.3.3). One individual clone from a positive pool transformed for extracellular secretion of antigen Region 2a had positive qPCR cycle amplification results (Figure 5.4.2).

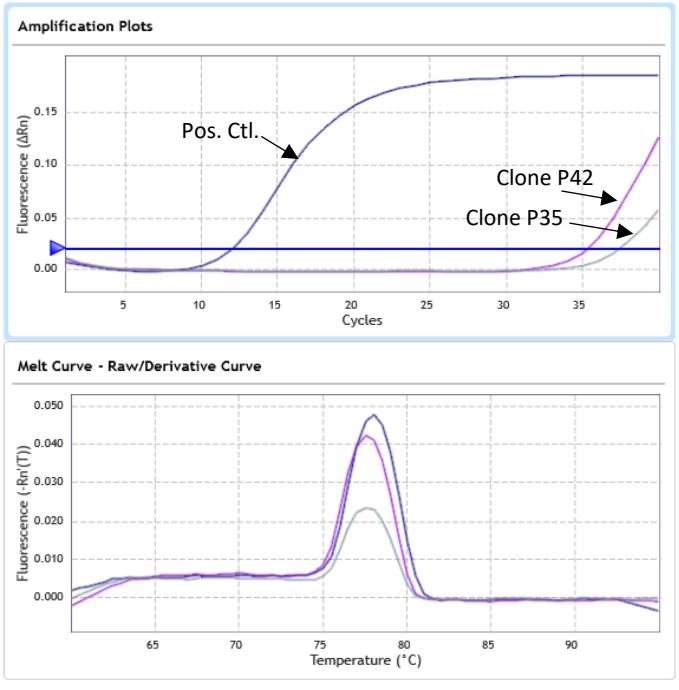

Fig. 5.3.1. Positive cloned yeast (pooled) by qPCR for Region 2a. Upper panel shows the amplification curves and the lower panel shows that the amplified product melt curves are consistent with the positive control (Pos. Ctl.).

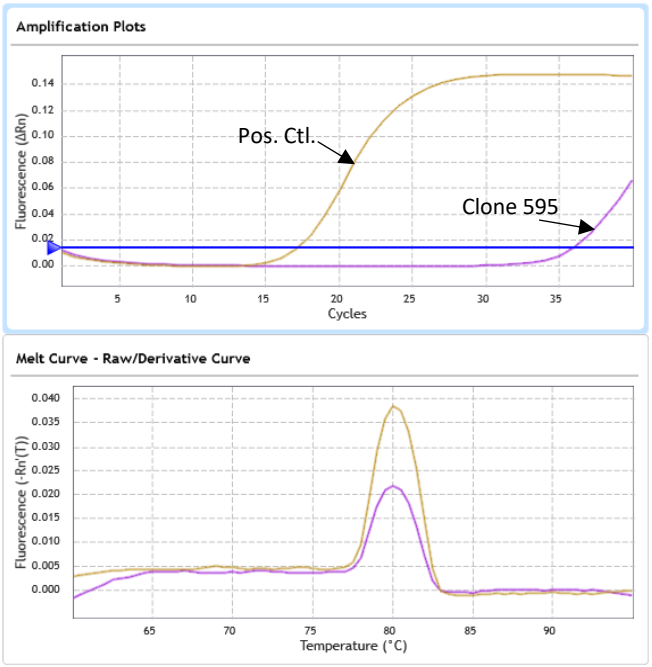

Fig. 5.3.2. Positive cloned yeast (individual) by qPCR for Region 2a. Upper panel shows the amplification curves and the lower panel shows that the amplified product melt curve is consistent with the positive control (Pos. Ctl.).

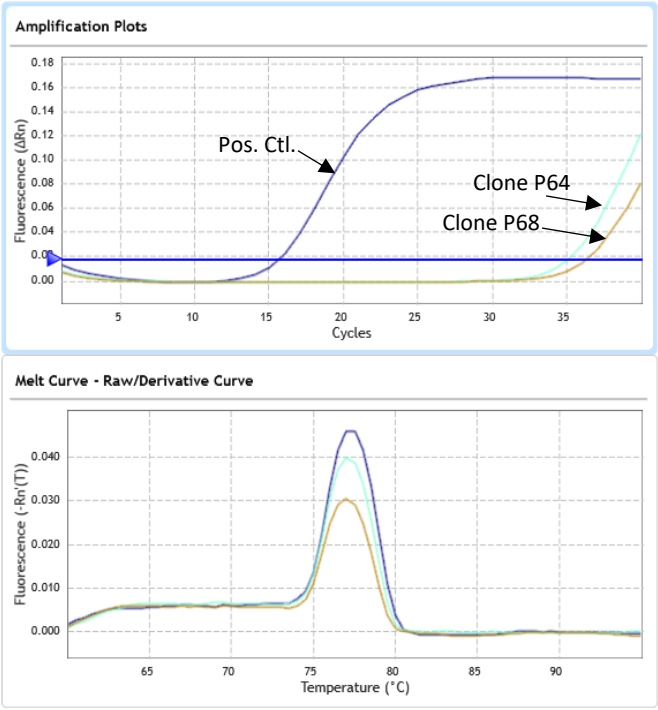

Fig. 5.3.3. Positive cloned yeast by qPCR for Region 3. Upper panel shows the amplification curves and the lower panel shows that the amplified product melt curves are consistent with the positive control (Pos. Ctl.).

Gene Expression analysis on clones with evidence of protein expression

RNA was extracted from several clones that were negative by qPCR on the extracted DNA but showed presumptive evidence of protein expression (see Section 5.3.3.3 below). The gene expression analysis showed strong amplification of the selected *K. lactis* reference gene by qPCR. However, there was no evidence of expression related to any of the antigenic regions in any of the clones selected for screening by gene expression (Fig. 5.3.4).

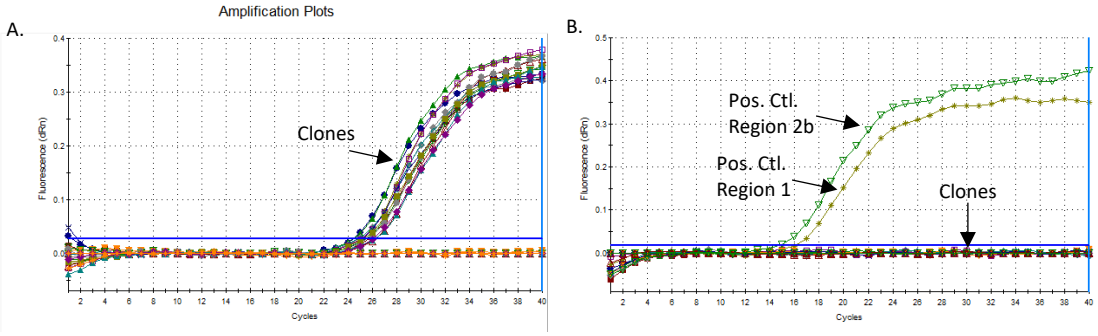

Fig 5.3.4. Gene expression screening of putative recombinant clones. Panel A shows the expression of the reference *K.lactis* gene, indicating successful isolation of the RNA from the yeast and reverse transcription into cDNA. Panel B shows the amplification plots using the gene expression primers for Region 1 and 2b. Only the positive control showed amplification, with no specific gene expression for these regions detected in the clones screened.

Antigen expression in recombinant antigen-yeast

Evidence of potential expression of the antigen proteins from yeast was obtained by Western Blot analysis. Fig. 5.3.5 illustrates appropriately sized bands (indicated by blue arrows) in one representative sample of antigen protein expression from lysates prepared from recombinant yeast transformed with gene fragments for Region 1 (expected size 7.3 kDa) and Region 2b (expected size 38.1 kDa).

Figure 5.3.5 Evidence of potential antigen protein expression assessed by Western Blot. One sample of each antigen protein for region 1 and 2b is depicted. The blue arrows indicate bands at the approximate size expected for the HA tagged protein antigens.

Protein purification from recombinant antigen-yeast

Aliquots (40 µL) of recombinant antigen-yeast pellets (Fig. 5.3.5) showing putative evidence of the correct protein expression both yielded equivalent quantities (12-14 µg/mL) of eluted protein following HA-bead isolation. This was utilised for antigenic screening (see Appendix 4).

Protein expression from recombinant antigen-yeast detected by 1D LCMS

Sample details for concentrated supernatant (sample numbers 3 to 11) assessed are shown below. Region 1 samples were not included as we were unable to detect integration of the gene at the DNA level.

Sample ID	Sample Name	Expected cloned protein*
3	Clone 463.6	Region 3
4	Clone 836.1	Region 4
5	Clone 463.4	Region 3
6	Clone 433.2	Region 3
7	Clone 771.1	Region 4

8	Clone 433.3	Region 3
9	Clone 595.1.2	Region 2a
10	Clone 770.2	Region 4
11	Clone 774.2	Region 4

* Protein sequences provided are described below.

Region 2a

Protein sequence:

REDACTED

Region 2b

Protein sequence:

REDACTED

Region 3

Protein sequence:

REDACTED

Region 4

Protein sequence:

REDACTED

For samples 3, 7 and 11, there were no protein hits after the post-Mascot filtering. This is likely due to the relatively low number of proteins identified. The pre-filtering (and still statistically significant) hits are presented. Results for Sample 3 and 4 are shown and were representative of the results for the supernatant samples (Samples 3 to 11).

None of the proteins for the specific cloned regions (Region 1, 2a, 2b, 3 or 4) was identified in any of the samples tested

Sample 3 (Clone 463.6, Region 3) Pre-filtered, top ten hits.

Accession	Description	Num. of sequences	MW [kDa]	Score Mascot	Gene Symbol
P17819	Kluyveromyces lactis Glyceraldehyde-3-phosphate dehydrogenase 1	10	35.302	2462	GAP1
Q6CX23	Kluyveromyces lactis Glyceraldehyde-3-phosphate dehydrogenase 2	5	35.524	1541	GAP2
P14828	Kluyveromyces lactis Phosphoglycerate kinase	5	44.487	859	PGK
P00761	Trypsin - Sus scrofa (Pig)*	4	24.394	782	
P60712	Actin, cytoplasmic 1 - Bos taurus (Cattle)*	11	41.71	464	ACTB
Q6CVQ6	Kluyveromyces lactis KLLA0B10197p	1	69.903	457	
P49383	Kluyveromyces lactis Alcohol dehydrogenase 2	7	37.075	451	ADH2
P20369	Kluyveromyces lactis Alcohol dehydrogenase 1	6	37.237	188	ADH1
P49385	Kluyveromyces lactis Alcohol dehydrogenase 4, mitochondrial	3	40.138	167	
Q6CTB1	Kluyveromyces lactis KLLA0C14047p	6	39.252	405	

*This is likely to be a constituent of the media.

Sample 4 (Clone 836.1, Region 4) Top ten protein hits.

Accession	Description	Num. of sequences	MW [kDa]	Score Mascot	Gene Symbol
P17819	Kluyveromyces lactis Glyceraldehyde-3-phosphate dehydrogenase 1	6	35.3	2166	GAP1
P14828	Kluyveromyces lactis Phosphoglycerate kinase	3	44.5	822	PGK
P00761	Trypsin - Sus scrofa (Pig)*	4	24.4	684	
Q6CTB1	Kluyveromyces lactis KLLA0C14047p	5	39.3	499	
P60712	Actin, cytoplasmic 1 - Bos taurus (Cattle)*	10	41.7	472	ACTB
Q6CLA9	Kluyveromyces lactis KLLA0F04433p	5	43	436	
Q6CW31	Kluyveromyces lactis KLLA0B07392p	5	36.8	392	
Q6CN67	Kluyveromyces lactis KLLA0E14939p	5	41.2	354	
Q6CYD7	Kluyveromyces lactis Adenosylhomocysteinase	3	49.2	298	

P27069	Kluyveromyces lactis 40S ribosomal protein S14	2	14.5	277	RPS14
P49383	Kluyveromyces lactis Alcohol dehydrogenase 2	5	37.1	269	ADH2

1.4 Discussion

The *K. lactis* expression system provides a useful platform for expression of recombinant antigens, with the option of capturing the protein intracellularly, giving the ability of using the whole heat-killed yeast cells as a self-adjuvating vaccine. The successful cloning of the *Ova* gene fragment into the *K. lactis* genome and evidence of protein secretion in whole cell lysates allowed assessment of this expression system for recombinant vaccine production. Successfully transformed *K. lactis* should contain the gene of interest, for example Ova, integrated into the genome. The plasmid used for incorporating the reporter gene contains a fungal gene, which can metabolise acetamide to ammonia for use as a nitrogen source. This allows initial selection of successfully transformed yeast on media where acetamide is the only source of nitrogen. Expression of the ovalbumin protein was confirmed in the OvaYeast vaccine by Western and 1D LCMS analysis.

Confirmation of protein expression in the candidate vaccines, where BVDV epitope-carrying regions were incorporated into *K. lactis,* was not successful. Of the two strategies used, gene integration was detected by PCR for multiple secretory BVDV antigens but none of the intracellular cloned antigens were detected. Specific protein expression was assessed in recombinant yeast designed to secrete BVDV antigens. We were unable to confirm secreted specific proteins by 1D-LCMS for any of the clones with evidence of integration at the DNA level. It is not clear what the reason for this may be. There are a number of potential reasons, including lack of expression of these proteins by the putative clones. Another possible reason relates to the method of sample preparation for this analysis, which is important in order to obtain full representation of the proteins in the sample. Two different methods were attempted, however neither was successful for the sample supernatants (Samples 3-11). Other potential methodological variables may include the supernatant volume used, culture incubation time or conditions, protein concentration method and/or protein precipitation method.

Although several other studies have used the same system for generation of clones excreting recombinant proteins, in our hands the transformation efficiency of *K. lactis* was very low (1.5%). Transformation efficacy can be impacted by the growth phase of the yeast, the yeast strain or the methodology (Yu et al. 2016). Chemical based methods of transformation are optimal for incorporation of DNA into cells in log phase growth (Tripp et al. 2013). Cells in the stationary growth phase, as was likely with the *K. lactis* used for transformation in this work, will have reduced transformation efficiency. Improvements to transformation efficiency can be achieved through the use of electroporation instead of chemical transformation methods, by the inclusion of nutrients and amino acids in the transformation media, or through changes to the duration of the heat shock step (Bolen et al. 1992; Russell et al. 1993; Sanchez et al. 1993; Tripp, Lilley et al. 2013). The low transformation efficiency noted in the OvaYeast clones is likely to be at a similar level for the recombinant antigen yeast, resulting in requirement to screen large numbers of clones to identify successful transformants. Therefore, further improvements of the transformation reaction are required to optimise the use of this *K. lactis* expression system for production of recombinant antigen vaccine candidates.

K. lactis, as with other non-conventional yeast, has some genetic intractability when compared to conventional yeast such as *Saccharomyces cerevisiae* (Wagner et al. 2016; Patra et al. 2021). In particular, *K. lactis* has a preference towards non-homologous end-joining (NHEJ) repair mechanisms when exogenous DNA is introduced (Wagner and Alper 2016). NHEJ results in random, spontaneous integrations of all or part of a cassette or vector instead of the precise integration that is possible with highly active homologous recombination in conventional yeast species (Rodicio et al. 2013; Spohner, Schaum et al. 2016). This preference can result in integration of part of the *PKLAC2* plasmid vector, conferring the ability of mutants to grown on selective media without insertion of the gene fragment

of interest (Iiizumi et al. 2008). This phenomenon would not only reduce the transformation rate, but also hamper the effectiveness of identification of successfully transformed clones based on growth on selective media. The possibility of integration of plasmid without the gene of interest or segments of the plasmid containing only the fungal gene necessary for growth on selective media necessitates additional screening by alternate methods for any clones that have grown. To ameliorate this issue, modification of the host strain to minimize NHEJ and flanking the integration cassette with longer homology sequences can be employed. However, both of these options result in alterations to growth rate, transformation rate, phenotypic stability and sensitivity to ultraviolet light (Kooistra et al. 2004; Wésolowski-Louvel 2011).

The ability to perform post-transcriptional modifications draws a distinction between eukaryotic and prokaryotic protein expression systems. Post-translational modifications involve processes that alter the protein composition such as addition of carbohydrates in glycosylation. In some applications, the ability of yeasts to express proteins with mammalian-like post translational modifications is beneficial (Vieira Gomes et al. 2018). However, glycosylation of proteins can impact immunogenicity, and hamper identification methods such as Western blot through changes to molecular weight and protein charge (Wang et al. 2017; Vieira Gomes, Souza Carmo et al. 2018). *K. lactis* has limited studies on post-translational glycosylation pattern but has been noted to add at least 30 mannose residues (Uccelletti et al. 2006; Liu et al. 2009; Zanni et al. 2009). This degree of glycosylation could impact protein migration during examination by WES, resulting in the appearance of incorrect band size or different band sizes amongst different clones. Therefore, assessment of the degree of glycosylation and potential removal of glycans prior to Western blot assessment would be advised.

In conclusion, generation of a recombinant vaccine with the *Ova* gene fragment with some evidence of protein expression has been successfully achieved with the *K. lactis* system. The limitations of this system, including the low transformation efficiency, preference for NHEJ and impacts of post-transcriptional modifications, has led to an inability to complete the cloning phase and positively identify successfully transformed clones at both the DNA (qPCR) and protein (Western blot) levels for recombinant antigen yeast for any single region of interest. Although some clones showed potential evidence of protein expression, there was no confirmation of DNA integration into the *K. lactis* genome or RNA expression. There are a number of putative transformed clones, as evidenced by the presence of the DNA for the particular gene region detected by qPCR, that have yet to be analysed by Western blot or other protein expression studies.

1.5 References

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