Final report

Evaluation of Mycobacterium Cell Wall Fraction to reduce Bovine Respiratory Disease.

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Abstract

The aim of this project was to evaluate the potential for an innate immune stimulant, 'Amplimune' that is comprised of a Mycobacterial cell wall fraction, to provide protection for feedlot cattle against BRD during the induction period. We conducted a pilot trial to determine safe and effective doses of Amplimune in weaner cattle. Results suggested that Amplimune, administered either subcutaneously or intramuscularly, at a dose rate of 2mL or 5mL is 'safe' to use in weaner cattle of approximately 220kg liveweight. Furthermore, administration of Amplimune did not result in a) inappetence (assessed indirectly by measuring liveweight change over the trial period), b) changes in hydration levels (assessed indirectly by measuring blood haematocrit), c) severe fever (assessed by measuring core body temperature) or excessive inflammation (assessed by measuring cytokine production). A trial was also conducted to investigate the mechanism by which Amplimune stimulated immune responses in cattle. Results from the mechanistic trial suggested that Amplimune has anti-inflammatory properties and created an environment conducive to stimulation of both innate and adaptive type responses, but no evidence of a strong pro-inflammatory mediator driven innate response was observed. The ability of Amplimune to protect feedlot cattle against BRD either directly, by providing short term protection against the disease during the induction period, or indirectly, by improving the efficacy of BRD vaccination at the feedlot when co-administered at induction was not assessed here as the project was terminated by MLA.

Executive summary

Background

Feedlot cattle are at a heightened risk of contracting diseases, such as BRD, during the induction period due to stress-induced suppression of immune system function which occurs at this time. This is especially the case for cattle not vaccinated against disease prior to feedlot entry. Despite financial incentives for producers to pre-vaccinate cattle against BRD on-farm, it remains that large numbers of cattle are not vaccinated prior to feedlot entry. Protective responses to vaccination take time to develop meaning these animals are not protected against BRD during the high-risk period immediately following induction. Therefore we proposed to investigate the potential of an innate immune stimulating compound, 'Amplimune', when administered at feedlot induction, to provide short term, broad-based disease protection for feedlot cattle until such time as protective responses from vaccination develop and thereby reducing reliance on antibiotics to prevent/treat disease in Australian feedlots. The Amplimune product was expected to induce 'trained immunity' in treated animals, stimulating the animal's immune system to eliminate pathogens. We also proposed to investigate the potential for 'Amplimune', when co-administered with BRD vaccines, to enhance responses to vaccination. Amplimune is currently registered for use in beef cattle in Canada and the US where it has also been certified for use in organic herds. Registration of 'Amplimune' with the APVMA in Australia, for treatment of scours in calves, is pending and is expected to fast-track future registration of the product for the applications in feedlot cattle.

Aims/objectives

The overall aims of the current project were to assess the potential for the immunostimulant, Amplimune to:

1) Provide short term protection for unvaccinated feeder cattle against disease (including BRD) during the high-risk induction period.
2) Improve the efficacy of vaccination (including against BRD) when administered at induction to improve long term protection against disease.

Methodology

- Negotiate a royalty stream agreement between MLA, NovaVive and CSIRO. NOTE: Discussions were had between the various parties and the notion of a royalty stream agreement was agreed to in principle. Following these discussions and it was decided that a royalty stream agreement should be finalised later.
- Phase 1 – Part 1: Evaluate safe dosage and the preferred route of administration of the Amplimune product. NOTE: Completed.
- Phase 1 – Part 2: To investigate the mechanism by which Amplimune stimulates the immune system of beef cattle by assessing a variety of innate immune responses induced by administration of Amplimune using both in-vivo and ex-vivo immunological assays. Note: Largely completed. Such activities could not be finished due to Covid-19 restrictions on staff movements between CSIRO sites.
- Phase 2 – Part 1: Assess the ability of Amplimune to reduce incidence and/or severity of BRD infections using an artificial infection model. NOTE: Not commenced as project terminated by MLA.
- Phase 2 – Part 2: Investigate the impacts of Amplimune treatment on feed/water intake during the induction period. NOTE: Not commenced as project terminated by MLA.
- Phase 3: Assess the ability of Amplimune to enhance responses to BRD vaccines when co-administered at feedlot induction. NOTE: Not commenced as project terminated by MLA.

Results/key findings

Phase 1 – Part 1: Evaluate safe dosage and the preferred route of administration of the Amplimune product.

The first phase in the process of evaluating the Amplimune product was to identify safe and effective doses of the product. In this study, different doses of Amplimune (2mL vs 5mL) were administered via different routes (subcutaneous vs intramuscular) and physiological responses induced by the treatment compared to those induced by saline (control). For Amplimune to be deemed 'safe' it is important that no adverse reactions to administration of the product are observed. For Amplimune to be deemed 'effective' the product should stimulate the immune system to a level which increases the ability of the system to fight off disease challenges while 'not' overstimulating the immune system to a level that induces severe fever or excessive inflammatory responses, both of which would be detrimental to the health, welfare and performance of the animal. Results from the study showed that Amplimune, administered either subcutaneously or intramuscularly at a dose rate of 2mL or 5mL, is 'safe' to use in calves of approximately 220kg liveweight. Furthermore, administration of Amplimune did not result in inappetence (assessed indirectly by measuring liveweight change over the trial period) or changes in hydration levels (assessed indirectly by measuring HCT). Results demonstrated that when Amplimune was administered subcutaneously at a dose of 2mL the product induced physiological responses indicative of innate immune system activation without causing severe fever or excessive inflammation.

Phase 1 – Part 2: To investigate the mechanism by which Amplimune stimulates the immune system of beef cattle

The next phase in the process of evaluating the Amplimune product was to attempt to identify the mechanism by which the product stimulates immune cells in cattle. In this study, Amplimune was administered (1.5mL/100 kg LWT) subcutaneously and physiological responses induced by the treatment compared to those induced by saline (control). Key observations from the study were that a) serum TNF-α concentrations were lower in steers following administration of Amplimune and b) numbers of neutrophils present at injection sites were lower in steers injected with Amplimune than in steers injected with saline. Taken together these results suggested that Amplimune may have an anti-inflammatory effect, down regulating production of pro-inflammatory cytokines by immune cells. Based on these findings we hypothesised that neutrophils which encountered mycobacterial antigens, from the Amplimune formulation at the injection site, may have migrated from the site of injection, possibly via afferent lymphatic vessels, to draining lymph nodes and sites of immune cell activation, where they play a role in antigen presentation to T cells and other immune cells triggering 'memory' responses in innate immune system cells (known as trained immunity) and also in adaptive immune system cells (traditional memory response). Findings from the study suggested that Amplimune has anti-inflammatory properties and created an environment conducive to stimulation of both innate and adaptive type responses, but no evidence of a strong pro-inflammatory mediator driven innate response was observed.

Phase 2 – Part 1: Assess the ability of Amplimune to reduce incidence and/or severity of BRD infections using an artificial infection model.

This phase of the project did not commence as the project was terminated by MLA

Phase 2 – Part 2: Investigate the impacts of Amplimune treatment on feed/water intake during the induction period.

This phase of the project did not commence as the project was terminated by MLA

Phase 3: Assess the ability of Amplimune to enhance responses to BRD vaccines when co-administered at feedlot induction

This phase of the project did not commence as the project was terminated by MLA

Recommendations

The ability of Amplimune to protect feedlot cattle against BRD either directly, by providing short term protection against the disease during the induction period, or indirectly, by improving the efficacy of BRD vaccination at the feedlot when co-administered at induction was not assessed here as the project was terminated by MLA. A pilot safety trial was completed which established safe dose rates for Amplimune in weaner cattle. At the request of MLA, a further study, aimed at determining the mechanism by which Amplimune stimulates immune cells in cattle was also undertaken. Due to the complexity of the immune system and the short time frame provided to complete the study, a clearly defined mechanism of action could not be determined. As the potential of Amplimune to reduce the incidence of BRD in Australian feedlot cattle, and consequently, reduce reliance on antibiotics and improve animal health & welfare, was not assessed in the current study we are unable to make any recommendation about the suitability of the Amplimune product for this application. We recommend that future studies be conducted to assess the ability of products like Amplimune to reduce the incidence of BRD in commercial feedlot settings which involve treatment of animals, not previously vaccinated against BRD, at feedlot induction and close monitoring of disease incidence during feedlot finishing. The Amplimune product has been shown to significantly improve responses to vaccination when co-administered with various vaccines. Therefore, we further recommend that future studies be conducted to assess the ability of Amplimune to enhance the efficacy of BRD vaccinations. when co-administered with the vaccine at induction, in commercial feedlot settings.

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1. Background

Infectious disease in food producing animals is currently prevented/controlled through the use of antibiotics and vaccines. Antibiotics are used therapeutically, to eliminate the target pathogen, or prophylactically, to prevent infection occurring in the first place, while vaccination induces protective immune responses in the host preventing subsequent infections. However, several converging issues have arisen that limit both the success of these disease control strategies and also our ability to implement such strategies.

- Vaccines take time to generate protective responses and are disease specific, meaning that many different vaccines are required to protect any individual against the plethora of potential pathogens they are exposed to in their production environment. Vaccination can be expensive and requires time to develop protective responses. Some of the most economically important diseases (such as bovine respiratory disease, BRD) arise through opportunistic infection and as such can potentially involve many different pathogenic species making it difficult to vaccinate against all causative agents. Immediate protection cannot be achieved, and this can be problematic when animals are first vaccinated when moved into new environments where new disease threats are faced.

- The efficacy of antibiotic treatments has reduced due to the rise of antibiotic resistance in target bacterial species, and as a consequence, infectious diseases that were once dismissed to the history books have returned with a vengeance as antibiotic control strategies fail. In response to this threat, there has been pressure to significantly reduce the use of antibiotics in animal production systems, especially as blanket preventative treatments, as this is considered a major contributor to the emergence of antibiotic resistance. This response is appropriate, however, infectious diseases will continue to have negative impacts on the productivity and welfare of food-producing animals, highlighting a critical need for reliable non-antibiotic alternatives to prevent and treatment of disease.

A previously under-utilised arm of the natural immune system, the innate immune system, has the ability to protect animals against disease and may prove to be the alternative disease control option that animal production systems are seeking. In the classical view of immunity, mammals generate a two-step immune response that is initiated with an “innate” response before culminating with the “acquired” response. Vaccination targets the acquired immune response to generates a highly antigen specific antibody-based response that protects the host from subsequent infection with the same pathogen. Vaccination is credited with reducing morbidity and mortality in billions of people and animals alike {Greenwood, 2014 #1437}. Not surprisingly, vaccination and the accompanying acquired immunity that drives the protective response, have been the focus of intensive investigation (A search of PubMed for ‘vaccine’ returns over 1.2 million hits). However, the overwhelming success of vaccination has reduced the imperative to consider the innate immune response as a potential tool to protect and treat disease. In addition, there has also been a misconception that innate responses are nonspecific and uncontrollable and therefore are not good targets for disease control strategies. However, there is increasing evidence to suggest that the innate immune response is not as random as once thought and is, in fact, highly regulated being induced by molecular patterns that allow the body to discriminate foreign invaders from self {Werling, 2003 #179}. Further, the nonspecific nature

of the effector response, involving antimicrobial peptides, reactive oxygen and nitrogen species and complement may be advantageous in providing broad based disease resistance against a range of pathogens for a specific period of time {Finlay, 2004 #1438}.

Evidence of a new immune activation pathway termed 'trained immunity', that is a variant of the innate immune response but with a memory component, has emerged in recent years. The discovery of this pathway was based on a collection of observations that certain infections or immune stimulant compounds were able to induce reprogramming of innate immune cells such as macrophages, dendritic cells and natural killer cells in a manner that heightens subsequent immune responses {Sanchez-Ramon, 2018 #1434}{Netea, 2016 #1435}{Uthayakumar, 2018 #1436}. Unlike acquired immunity the subsequent infection does not need to be with the same pathogen, and the response is not characterised by production of antigen- specific antibody. The speed of initiation of the trained immune response is rapid with preliminary evidence from our lab showing that compounds which stimulate trained immunity induction can protect mice from a lethal bacterial (Listeria monocytogenes) challenge 24 hrs after treatment. While the response duration is unclear at this stage, and may vary depending on the agent used for stimulation, it but may last for as long as three months as has been demonstrated in calves given the BCG vaccine {Guerra-Maupome, 2019 #1439}. Therefore, compounds which induce 'trained immunity' and stimulate the animal's own immune system to eliminate pathogens have the potential to be used as alternatives to antibiotics to prevent and treat disease.

Feedlot cattle are at a heightened risk of contracting diseases, such as BRD, during the induction period due to stress-induced suppression of immune system function which occurs at this time. This is especially the case for cattle not vaccinated against disease prior to feedlot entry. Despite financial incentives for producers to pre-vaccinate cattle against BRD on-farm, it remains that large numbers of cattle are not vaccinated prior to feedlot entry. Protective responses to vaccination take time to develop meaning these animals are not protected against BRD during the high-risk period immediately following induction. Therefore we proposed to investigate the potential of an innate immune stimulating compound, 'Amplimune', when administered at feedlot induction, to provide short term, broad-based disease protection for feedlot cattle until such time as protective responses from vaccination develop and thereby reducing reliance on antibiotics to prevent/treat disease in Australian feedlots. The Amplimune product is expected to induce 'trained immunity' in treated animals, stimulating the animal's immune system to eliminate pathogens. We also proposed to investigate the potential for 'Amplimune', when co-administered with BRD vaccines, to enhance responses to vaccination. Amplimune is currently registered for use in beef cattle in Canada and the US where it has also been certified for use in organic herds. Registration of 'Amplimune' with the APVMA in Australia, for treatment of scours in calves, is pending and is expected to fast-track future registration of the product for the applications in feedlot cattle.

2. Objectives

The overall aim of this work is to deliver to industry a registered non-antibiotic based product for use in Australian feedlots which will reduce the incidence of disease, and in particular BRD, during the high risk period immediately following feedlot induction. By reducing the incidence of disease, such a product is expected to reduce reliance on the use of antibiotics to treat disease (in alignment with the National Strategy for Antimicrobial Resistance, 2015). It is also well recognised that changing consumer confidence can have a significant effect on the profitability of livestock industries. Consumers are increasingly conscious of the health and welfare of the animals producing their food and are demanding the highest possible standards of animal welfare through purchasing choices. Consumers are also increasingly concerned with the use of antibiotics in food-producing animals. Therefore, products capable of reducing the incidence of BRD in Australian feedlots, and as a consequence, improving the health and welfare of feedlot cattle and reducing reliance on antibiotics to treat disease are expected to improve consumer confidence in Australian grain fed beef products. Reducing the incidence of disease in feedlot cattle is also expected to provide significant economic benefits for feedlot operators. It has been estimated that BRD alone costs the Australian feedlot sector in excess of $40 million annually, with losses estimated to be in excess of $20 per head for unvaccinated cattle (MLA Project AHW.087) suggesting even a small reduction in the incidence of BRD would be expected to result in significant economic benefits for the feedlot industry.

Amplimune is an innate stimulating compound which is expected to induce trained immunity in treated animals to stimulate the animal's immune system to eliminate pathogens. Amplimune has a proven track record in the effective treatment of several diseases including scours in calves and respiratory disease in horses. Therefore, the overall aims of the current project were to assess the potential for the immunostimulant, Amplimune to:

3) Provide short term protection for unvaccinated feeder cattle against disease (including BRD) during the high-risk induction period.
4) Improve the efficacy of vaccination (including against BRD) when administered at induction to improve long term protection against disease.

The specific aims of the project were to:

1) Negotiate a royalty stream agreement between MLA, NovaVive and CSIRO. NOTE: Discussions were had between the various parties and the notion of a royalty stream agreement was agreed to in principle. Following these discussions and it was decided that a royalty stream agreement should be finalised later.
2) Evaluate safe dosage and the preferred route of administration of the Amplimune product (Phase 1 – Part 1). NOTE: Completed.
3) To investigate the mechanism by which Amplimune stimulates the immune system of beef cattle by assessing a variety of innate immune responses induced by administration of Amplimune using both in-vivo and ex-vivo immunological assays (Phase 1 – Part 2). Note: Largely completed. Such activities could not be finished due to Covid restrictions on staff movements between CSIRO sites.
4) Assess the ability of Amplimune to reduce incidence and/or severity of BRD infections using an artificial infection model (Phase 2 – Part 1). NOTE: Not commenced as project terminated by MLA.
5) Investigate the impacts of Amplimune treatment on feed/water intake during the induction period (Phase 2 – Part 2). NOTE: Not commenced as project terminated by MLA.
6) Assess the ability of Amplimune to enhance responses to BRD vaccines when co-administered at feedlot induction. NOTE: Not commenced as project terminated by MLA.

3. Methodology

3.1 Phase 1 – Part 1- Evaluating safe dose rates and the preferred route of administration of the Amplimune product

3.1.1 Trial Details

All experimental procedures conducted as part of this trial were pre-approved by the CSIRO, Chiswick Animal Ethics Committee (Animal Research Authority 18/26). The trial was conducted at the CSIRO, F.D McMaster field station located near Armidale in Northern NSW. The trial commenced on the 22nd March 2019 and concluded on the 30th of March 2019. As the Amplimune product is not currently registered with the APVMA for use in Australian cattle, the trial was conducted under the CSIRO, F.D McMaster field station APVMA 7250 permit. Under this permit, a mandatory 12-month WHP is applied to all animals treated with a non-registered drug.

3.1.2 Animal Details

A total of 60 mixed sex Angus weaners (36 heifers & 24 steers), which were progeny of the CSIRO Chiswick Angus Performance Register (APR) herd, were enrolled in the safety trial. Weaners had an average liveweight of 216.5kg (range 183-272kg). Due to the drier than average seasonal conditions, calves were lighter than the initial target feeder cattle weight. Prior to enrolment in the study, all calves were yard-weaned (as per standard industry practice) for a period of seven days (12th -19th March 2019). Following weaning calves were briefly returned to the paddock prior to the trial commencing on the 22nd March 2019.

3.1.3 Treatment Group Details

Calves were assigned to one of six treatment groups (n=10 per grp) as outlined below. Treatment groups were balanced for the liveweight, sex and sire of calves.

- Grp 1 – 2mL dose administered intramuscularly (2mLIM)
- Grp 2 – 2mL dose administered subcutaneously (2mLSC)
- Grp 3 – 5mL dose administered intramuscularly (5mLIM)
- Grp 4 – 5mL dose administered subcutaneously (5mLSC)
- Grp 5 - Placebo (saline 5mL) administered intramuscularly (Sal5mLIM)
- Grp 6 - Placebo (saline 5mL) administered subcutaneously (Sal5mLSC)

To ensure consistent timing of sample collection, and to minimise the impact of ambient temperature changes on core body temperature measures, calves were assigned a number (1-60) which was painted on the calf for easy identification (Figure 1), allowing calves to be processed in a specific order. Two calves from each of the six treatment groups were randomly assigned numbers 1-12, then a further 2 calves from each treatment group were randomly assigned numbers 13-24, 25-36, 37-48 or 49-60. At each sampling time, animals 1-12 were processed first, followed in order by animals 13-24, 25-36, 37-48, 49-60.

Figure 1. Calves on feed in the holding yard

3.1.4 Experimental Procedures

For a detailed timetable of experimental procedures see Table 1.

Briefly, four days prior to Amplimune administration (day -4) all calves were weighed, fitted with i-button temperature loggers and a blood sample collected from all animals for haematology and cytokine analysis to provide baseline parameters. Over the following two days prior to Amplimune administration (days -3 and -2) calves were allowed to graze in a small paddock undisturbed to allow baseline diurnal temperature profiles to be recorded on individual animals. On the day prior to Amplimune administration (day -1), all cattle were transported by road (6hrs, from 12pm to 6pm) and then yarded overnight with access to lucerne hay and water (Figure 2). On the day of Amplimune treatment (day 0) all animals were again transported by road (6hrs, from 6am to 12pm). Following transportation, all calves were assessed by a veterinarian to ensure they were in good health. Clinical measures assessed included body condition, respiration rate, rectal temperature and heart rate (Figure 3). Following clinical assessment, calves were put through an induction protocol designed to mimic induction protocols commonly used at the feedlot (weighing, and standard health treatment administration, including vaccination against clostridial diseases and anthelmintic application for control of internal parasites). Transportation and induction procedures were undertaken to mimic stressors likely encountered by animals entering a commercial feedlot environment. Administration of Amplimune or placebo (as detailed above) coincided with other induction procedures.

Figure 2. Calves during road transportation

All calves were closely monitored by a veterinarian following Amplimune treatment in case of any adverse reactions to the product being observed. Blood samples were collected at various time points including just prior to treatment (0hrs) and post-treatment (+6, +12, +24, +48, +72 & +96hrs relative to treatment) for haematology and cytokine concentration analysis. All calves were also weighed at various time points including just prior to treatment (0hrs) and post-treatment (+48, & +96hrs relative to treatment). The i-button temperature loggers were removed 72hrs post-treatment and logged data downloaded. At 72hrs post-treatment (day 3) all calves were again assessed by a veterinarian to ensure they were in good health. Clinical measures assessed included body condition, respiration rate, rectal temperature and heart rate.

All calves were confined to a large single pen following treatment with Amplimune (day 0) until the end of the trial period (day 4). During this time calves always had access to clean drinking water and were fed a feedlot starter ration (sourced from Tullimba feedlot) once daily starting at a rate of 3kg/hd (day 0) and increasing incrementally to 6kg/hd (day 4).

Figure 3. Assessment of clinical scores prior to Amplimune administration.

Table 1: Timetable of Experimental Activities

Day relative to Amplimune treatment	Time	Experimental Activity
-4	12pm	Liveweight (D-4)
		Blood sample (D-4)
		Calves identified with Paint
		i-buttons inserted
-3		Nil
-2		Nil
-1	12pm	Transported for 6 hours
0	6am	Transported for 6 hours
	12pm	Vet health check – clinical scores
		Liveweight (D0)
		Induction protocol (drench/vaccinate)
		Blood sample (T0)
		Administer treatment (Amplimune or saline)
	6pm	Blood sample (T6)
	12am	Blood sample (T12)
1	12pm	Blood sample (T24)
2	12pm	Liveweight (D2)
		Blood sample (T48)
3	12pm	Blood sample (T72)
		i-buttons removed
		Vet health check – clinical scores
4	12pm	Liveweight (D4)
		Blood sample (T96)

Liveweight Recording – Liveweights were recorded in two separate locations during the trial. At the start (D-4) and conclusion (D4) of the trial period, liveweights were recorded at the highway (Hway) yards using scales which were calibration certified. As these Hway yards did not contain sufficient yard space to accommodate all calves in a single pen during the trial period, all experimental work was conducted at an alternate set of yards (K3 yards) with calves walked between yards at the start and conclusion of the trial period. At both locations liveweights were recorded using a TruTest weighing system with the weigh platform built into the crush (Hway yards) or into a separate weigh box (K3 yards). Calibration weights were used to monitor the performance of scales in the Hway yards prior to, during and at the conclusion of each weighing event.

Blood Sample Collection and Processing – Blood samples were collected at each time point using jugular venepuncture. A total of 2*10mL serum tubes (vacutainers) and 1*6mL tube containing EDTA anticoagulant were collected at each time point. Serum was collected from coagulated blood by centrifugation (700 x *g*, 20 min, RT) and stored in multiple aliquots at -80°C for subsequent cytokine assays. Blood collected in EDTA tubes was mixed upon returning to the laboratory and immediately analysed on an automated haematology analyser (Cell-Dyn 3500R, Abbott Diagnostics, North Ryde, NSW, Australia) with a specialized veterinary package installed. Parameters measured included total white blood cell count (WBC), neutrophil count (NEU), lymphocyte count (LYM) and haematocrit (HCT; = (RBC × MCV)/10 as %).

i-Button Temperature Loggers - Probes were prepared to house i-Button temperature loggers and these probes inserted into the rectum of calves to record core body temperature as outlined in

the standard operating procedure (SOP) attached (see Appendix A). Loggers recorded core body temperature every 5 minutes from the time they were inserted (D-4) until they were removed (D3).

Cytokine Assays – ELISA assays were conducted to determine changes in the concentration of various pro-inflammatory cytokines in serum samples collected at various time points post-treatment using either bovine specific ELISA kits or in-house developed assays. Cytokines analysed included Tumour Necrosis Factor-α (TNF-α), Interleukin 1-β (IL-1β), Interleukin 12 (IL-12) and Interleukin 6 (IL-6).

3.1.5 Statistical Analysis

Statistical analysis was conducted using Rx64 3.5.0 (R Core Team, 2018). For parameters measured over time, repeated measures linear mixed models, fitting animal as a random effect, were used to estimate variance components using the REML method. For parameters measured only once, simple linear models were used to estimate variance components using the REML method. Residuals generated from models were tested for normality by assessing skewness and kurtosis and data transformed where required to improve normality. Fixed effects assessed in all models included treatment, sex, treatment*sex and liveweight (at commencement of the trial, D-4). For repeated measures time and time*treatment were also fitted as fixed effects. Where appropriate, baseline parameter values were fitted as covariates in statistical models. For details of fixed effects fitted to statistical models when analysing parameters see Table 3. Where fixed effects were clearly not significant (P>0.1 for single factors or P>0.2 for interactions) they were removed from the final statistical model. Where significant treatment effects were observed, specific linear contrasts were undertaken to compare Amplimune treated versus saline treated (control group) animals when delivered via the same route of administration (ie. subcutaneously or intramuscularly).

For graphical representation of results, least squares means (LSMs) were generated from the models for effects and interactions using raw untransformed data. However, the significance of fixed effects were determined using transformed data, where transformation was required to improve normality.

3.2 Phase 1 – Part 2- Investigating the mechanism by which Amplimune stimulates the immune system of beef cattle

3.2.1 Trial Details

All experimental procedures conducted as part of this trial were pre-approved by the CSIRO, Chiswick Animal Ethics Committee (Animal Research Authority 19/20). The trial was conducted at the CSIRO, F.D McMaster field station located near Armidale in Northern NSW. As the Amplimune product is not currently registered with the APVMA for use in Australian cattle, the trial was conducted under the CSIRO, F.D McMaster field station APVMA 7250 permit. Under this permit, a mandatory 12-month withhold period (WHP) is applied to all animals treated with a non-registered drug. In accordance with this CSIRO will continue to hold and care for the treated cattle until the end of the mandatory WHP at which time they can be sold.

3.2.2 Animal Details

A total of 18 Angus yearling steers, which were progeny of the CSIRO Chiswick Angus Performance Register (APR) herd, were enrolled in the mechanistic trial. At the commencement of the trial the steers had an average liveweight of 262kg (range 236-305kg).

3.2.3 Treatment Group Details

Steers were randomly assigned (within weight categories) to one of two treatment groups (n=9 per grp) as outlined below. The treatment groups were balanced for the liveweight of steers to the extent possible. Steers within each treatment group were then randomly assigned to one of three cohorts for the duration of testing (n=6 per cohort including 3 steers from each treatment). Steers were split into these cohorts for logistical reasons, specifically to ensure that samples collected during testing were able to be promptly stored or processed where immediate laboratory analysis was required. Each cohort was tested over consecutive weeks.

- Grp 1 – Amplimune administered subcutaneously high on the neck (AMP, 1.5mL/100kg liveweight)
- Grp 2 - Saline administered subcutaneously high on the neck (SAL, 1.5mL/100kg liveweight)

To ensure consistent timing of sample collection, steers within each cohort were assigned a number (1-6) which was painted on the steer for easy identification, allowing steers to be processed in a specific and repeated order. Within each cohort, a steer from each treatment was randomly assigned number 1 or 2, a further steer from each treatment number 3 or 4 and a further steer from each treatment number 5 or 6 to minimise any bias that the timing of sample collection may have on treatment effects.

3.2.4 Experimental Procedures

For a detailed timetable of experimental procedures see Table 2.

Briefly, three days prior to Amplimune administration (day -3) all steers within a given cohort were weighed and fitted with i-button temperature loggers to provide baseline temperature measures. Over the following two days (days -2 and -1), prior to Amplimune administration steers were allowed to graze in a small paddock undisturbed to allow baseline diurnal temperature profiles to be recorded on individual animals. On the day of Amplimune treatment (day 0) all steers were assessed by experienced personnel to ensure they were in good health. Clinical measures assessed included body condition, respiration rate and rectal temperature. Following clinical assessment, Amplimune or placebo (saline) was administered as detailed above.

All steers were closely monitored by experienced personnel for the initiation of any adverse reactions following Amplimune treatment. Blood samples were collected at various time points, including just prior to treatment (0hrs) and post-treatment (+3, +6, +12, +24, +48 & +96hrs relative to treatment) for haematology (all time points), serum cytokine concentration analysis (all time points), gene expression in circulating white blood cells (all time points) and for *in-vitro* cell culturing procedures (0, 24 & 96hrs post-treatment). Skin biopsies were collected at the site of injection and a matched control site on the opposite side of the neck at 24hrs post-treatment. The i-button temperature loggers were removed 96hrs post-treatment and logged data was downloaded. At 96hrs post-treatment (day 4) all steers were again assessed by experienced personnel to ensure they were

in good health. Clinical measures assessed included body condition, respiration rate and rectal temperature.

All steers were allowed to graze in a small paddock adjacent to the yards following treatment with Amplimune (day 0) until the end of the trial period (day 4). During this time steers always had access to clean drinking water and were supplemented with good quality hay.

Table 2: Timetable of Experimental Activities

Day relative to Amplimune treatment	Time	Experimental Activity
-3	12pm	Liveweight (D-3)
		Calves identified with Paint
		i-buttons inserted
-2		Nil
-1		Nil
0	7am	Health check – clinical scores
		Blood sample (T0)
		Administer treatment (Amplimune or saline)
	10am	Blood sample (T3)
	1pm	Blood sample (T6)
	7pm	Blood sample (T12)
1	7am	Collect skin biopsies (T24)
		Blood sample (T24)
2	7am	Blood sample (T48)
4	7am	i-buttons removed
		Blood sample (T96)

Liveweight Recording

Liveweights were recorded at the start of testing of each cohort (D-3) using calibration certified scales (TruTest weighing system with the weigh platform built into a weigh box) to allow accurate dose rates to be determined. Calibration weights were used to monitor the performance of scales prior to, during and at the conclusion of weighing.

Blood Sample Collection and Processing

Blood samples were collected at each time point using jugular venepuncture. A total of 2*10mL serum tubes (vacutainers) were collected at all time points to prepare serum for cytokine analyses and allowed to clot at RT for 1hr. A further 2*10mL tubes containing EDTA anticoagulant were collected at each time point for haematology (1 tube) and preparation of buffy coat samples (1 tube) for gene expression studies. At time points T0, T24 & T96 a further 3*10mL tubes containing EDTA anticoagulant were collected for *in vitro* cell culturing procedures. Tubes containing EDTA were immediately chilled on ice following collection.

Serum was prepared from coagulated blood by centrifugation (700 x *g*, 20 min, RT) and stored in multiple aliquots at -80°C for subsequent cytokine assays. Buffy coats (layered white blood cells) were prepared from blood by centrifugation (700 x *g*, 20 min, RT), immediately snap frozen in liquid nitrogen and stored in multiple aliquots at -80°C for subsequent gene expression studies. Blood collected in EDTA tubes for haematology was mixed upon returning to the laboratory and immediately

analysed on an automated haematology analyser (Cell-Dyn 3500R, Abbott Diagnostics, North Ryde, NSW, Australia) with a specialized veterinary package installed. Parameters measured included total white blood cell count (WBC), neutrophil count (NEU), lymphocyte count (LYM) and haematocrit (HCT; = (RBC × MCV)/10 as %).

Skin Biopsy Collection

Skin biopsies were collected at the site of Amplimune/saline injection high on the neck (test site) and from an equivalent site on the opposite side of the neck (control site) at T24. Prior to injection of Amplimune or saline at T0, an injection site (test site) was identified by shaving a small ($4cm^2$) square on the side of the neck using animal clippers and injections administered in the centre of the shaved square. An area was shaven at an equivalent site on the alternate side of the neck (control site) but no injection administered. Test and control sites were swabbed with antiseptic spray and 1.0mL of local anaesthetic (2% lignocaine) injected subcutaneously at the sites to be biopsied. Approximately 5mins later, the skin biopsy was collected with a 1cm trephine (biopsy punch) at test and control sites. Subcutaneous tissue beneath the disc of skin cut by the biopsy punch was severed with sharp scissors to release the biopsy. Biopsies were cut in half with a sharp scalpel. Half the biopsy was then immediately snap frozen in liquid nitrogen for subsequent gene expression analysis and the remaining half fixed in 10% buffered formalin for histological examination.

Histological Examination

Skin sections, preserved in 10% buffered saline, were embedded in paraffin blocks and sections (5µm) mounted on slides. Slides were then stained with H&E to identify individual cell types present and numbers of mononuclear (lymphocytes/monocytes), eosinophils and neutrophils in 10 contiguous high-powered fields (40x) enumerated per section. Cells were counted in the superficial and deep dermis. Cells in blood vessels were excluded from counts.

i-Button Temperature Loggers

Probes were prepared to house i-Button temperature loggers and these probes inserted into the rectum of steers to record core body temperature as outlined in the standard operating procedure (SOP) attached (see Appendix A). Loggers recorded core body temperature every 10 minutes from the time they were inserted (D-3) until they were removed (D4).

In vitro Cell Culturing

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood drawn 0, 24 and 96hrs post-vaccination using ficoll paque (GE Health, USA) density centrifugation according to the manufacturer's instructions. The isolated PBMC were counted using a haematological Cell-Dyn autoanalyser (Abbott, USA). A total of 1×10^5 cells per well were cultured in 24 well plates (Sarstedt, Germany) in complete culture media (High-glucose DMEM, 10% FCS, 1× P/S, 1× glutamax; ThermoFisher Scientific, USA) and incubated for 24hrs at 37˚C in a humidified 5% CO2:95% air atmosphere. The cultures were then stimulated with:-

- CTRL - media only (unstimulated control)
- BHV-1 - inactivated bovine herpesvirus-1 virus (MOI = 0.01)
- AMP - Amplimune (4µL/mL, NovaVive)
- AMP+BHV-1 - Amplimune + inactivated BHV-1 virus (MOI = 0.01) or
- ConA - Concanavilin A (5µg/mL, Sigma, USA) (positive control).

Following treatment cells were incubated for a further five days at 37˚C in a humidified 5% CO2:95% air atmosphere. During the last 18 hours of incubation, Brefeldin A (3µg/ml; Sigma USA) and Monensin (5µg/mL; Sigma USA) were added and the cultures and returned to the incubator. At the conclusion of the incubation period culture supernatants were collected and stored in multiple aliquots at -80°C for subsequent cytokine assays and cells were harvested for flow cytometric analysis.

Flow Cytometry

All staining procedures were performed in 1.5 mL microfuge tubes. Adherent and suspended cells were harvested from culture wells at the end of the incubation period by scraping with a cell scraper (BD, USA). The cells were centrifuged (350 x *g*, 5 min, 4°C), supernatant discarded and the cell pellet resuspended in 100µL of ice-cold FACs buffer (PBS (pH 7.4), 0.1% BSA). The cells were again centrifuged (350 x *g*, 5 min, 4°C), supernatant discarded and the cells pellet resuspended in 100µL of ice-cold FACs staining buffer containing a CD-staining antibody cocktail (mouse anti-bovine CD4-Alexa Fluor 647 (BioRad, USA), mouse anti-bovine CD8-RPE (BioRad, USA) or fluorescent isotype control (BioRad, USA) antibody cocktail and incubated for 30 minutes on ice in the dark. The cells were washed by adding 1mL of ice-cold FACs staining buffer, centrifuged (350 x *g*, 5 min, 4°C) and the supernatant discarded. The cells were resuspended in 100µL of FACs buffer and 150µL of Cyto-Fast Perm Wash solution (Biolgend, USA) and fixed and permeabilised according to the manufacturer's instructions. Fixed and permeabilised cells, suspended in 100µL of Cyto-Perm wash buffer, were stained with 1µg of mouse anti-bovine IFNg-FITC antibody (IFN9, CSIRO, Australia) for 30 minutes at RT in the dark. Cells were washed by adding 1mL of Cyto-Perm wash buffer and centrifuged (350 x *g*, 5 min, 4°C). The supernatant was discarded and the cells resuspended in 100 µl of FACs buffer and then stored on ice in the dark until flow cytometric acquisition on a FlowSight imaging flow cytometer (Merck, USA).

Serum and Culture Media Cytokine ELISA Assays

ELISA assays were conducted to determine the concentrations of pro-inflammatory cytokines in serum samples collected at various time points post-treatment and in cell culture supernatants following the stimulation of cells. ELISA assays were conducted using either bovine specific ELISA kits or in-house developed assays. Cytokines to be investigated were Tumour Necrosis Factor-α (TNF-α), Interleukin 1-β (IL-1β), Interleukin 12 (IL-12), Interleukin 6 (IL-6) and Interferon gamma (IFN-γ).

Gene Expression Analysis

-RNA Extraction & Purification

For each tissue sample, 50 mg of frozen tissue was wrapped in thick aluminium foil and immediately transferred to liquid nitrogen. The tissue was then manually pulverised with a hammer on a cold block, flash chilled again in liquid nitrogen and the tissue powder scraped into RLY-2ME Lysis buffer (BioLine, Meridian LifeScience Inc., Memphis, Tennessee) and immediately vortexed twice for 60s.

Total RNA was isolated from the homogenized tissues and from buffy coat samples using an Isolate II RNA Mini Kit (BioLine, Meridian LifeScience Inc., Memphis, Tennessee) as per manufacturer's instructions. In contrast, total RNA was isolated from cells stimulated in cell culture using an Isolate II RNA Micro Kit (BioLine, Meridian LifeScience Inc., Memphis, Tennessee) as per manufacturer's instructions. Both kits incorporate the use of genomic DNA eliminator spin columns. The quantity of total RNA isolated was determined using a Nanodrop 8000 Spectrophotometer (v2.3.2, Thermo Fisher Scientific™ Inc.). The 260:280nm absorbance ratios for prepared samples were between 1.7 and 2.6

and the 260:230nm ratios were between 2.0 and 2.2. For qualitative and quantitative RT-PCRs, cDNA was generated from 1000ng of total RNA isolated from cells or 150ng of total RNA isolated from skin biopsies by reverse transcription. To prepare cDNA, total RNA was added to 5x TransAmp buffer containing anchored oligo(dT), random hexamer primers and Reverse Transcriptase (BioLine, Meridian LifeScience Inc., Memphis, Tennessee) in a final volume of 20 µL, and samples cycled through an RT protocol using a Veriti 96-well Thermal Cycler (Applied Biosystems), as per manufacturer's instructions. The resulting single-stranded cDNA was then diluted 10-fold with RNAse free H20 (BioLine, Meridian LifeScience Inc., Memphis, Tennessee) for use in RT-PCR.

-Real-Time PCR

Real time PCR (RT-PCR) assays were used to quantify the expression levels of four pro-inflammatory cytokine genes, TNF-α, IL6, INF-γ and IL-1β relative to two housekeeping genes, ribosomal protein large subunit P0 (RpLP0) and 18S ribosomal RNA (18S) as previously described (Strandberg et al., 2005; Vuocolo, Cockett & Tellam, 2005).

Briefly, RT-PCR assays were performed using the ABI qPCR thermocycler ViiA7 (Applied Biosystems). Each reaction contained 2 × SensiFAST SYBR® Lo-ROX Master Mix (BioLine Meridian), 400nM each primer and a constant amount of cDNA (corresponding to 1000 ng of reverse transcribed RNA from cells or 150ng of reverse transcribed RNA from skin biopsies). Four technical replicates were evaluated for each candidate gene. For each of the biological samples, gene expression was quantified by normalizing the expression of each target gene to the expression of the reference genes, RpLP0 and 18S, using the Q-GENE statistical analysis package (Simon, 2003). The mean of technical replicates was determined (minimum of triplicate values) and the result expressed as Mean Normalised Expression (MNE) ± Standard Error (SE).

3.2.5 Statistical Analysis

Statistical analysis of haematology, cytokine and body temperature data were performed using Rx64 3.5.0 (R Core Team, 2018). For parameters measured over time, repeated measures linear mixed models, fitting animal as a random effect, were used to estimate variance components using the REML method. For parameters measured only once, simple linear models were used to estimate variance components using the REML method. Residuals generated from models were tested for normality by assessing skewness and kurtosis and data transformed where required to improve normality. Fixed effects assessed in all models included treatment and cohort along with liveweight as a covariate. For repeated measures analysis time and time*treatment were also fitted as fixed effects. Where appropriate, baseline parameter values were fitted as covariates in statistical models. For details of fixed effects fitted to statistical models when analysing parameters see Table 11. Where fixed effects were not significant (P>0.05 for single factors or P>0.1 for interactions) they were removed from the final statistical model. For analysis of data from in vitro cell culture assays, where multiple treatments were used to stimulate cytokine production, specific linear contrasts were undertaken to compare relevant treatments where a significant treatment effect was observed. An alpha value of 0.05 was considered significant.

Statistical analyses of flow cytometric data were performed using the SPSS statistical analysis package, version 25 (IBM, USA). Flow data, CD4+ interferon bright and CD8+ interferon bright gated cells, were considered normally distributed with a skew value of -0.016 and -0.383 respectively. Data describing numbers of CD4+, CD8+, interferon bright, cells were analysed using a MANOVA followed

by univariate linear regression ANOVA analyses with Tukey's post hoc tests. An alpha value of 0.05 was considered significant. Box's Test of Equality of Covariance Matrices was not significant ($p > 0.330$) and demonstrated that the estimated CD4+ and CD8+ variances in the MANOVA were equal.

For graphical representation of results, least squares means (LSMs) were generated from the models for effects and interactions using raw untransformed data. However, the significance of fixed effects were determined using transformed data, where transformation was required to improve normality.

3.3 Phase 2 -Part 1&2 - Assess the ability of Amplimune to reduce incidence and/or severity of BRD infections using an artificial infection model & investigate the impacts of Amplimune treatment on feed/water intake during the induction period

NOTE: This phase of the project did not commence as MLA terminated the project. Therefore, methodology outlined here was proposed methodology only.

The challenge trial was designed to investigate:

1) The ability of Amplimune to induce responses in the host which enhance their ability to resist a controlled BRD infection as measured by body temperature, clinical score and pathology at slaughter.
2) The ability of Amplimune to induce responses at different stages of the BRD infection model.

A total of 30 animals (including 6 spares), confirmed free of BRD, will be enrolled in the study. Cattle will be approx. 240kgs liveweight, of Hereford or Angus breed and will be sourced from the New England region to increase likelihood of BRD free status. Cattle will be purchased and transported to the QASP facility at Gatton in two separate lots (15 calves per lot) to arrive at the facility approximately 4-6 weeks apart. Calves will be allocated to each lot based on weight, with the heaviest 15 animals assigned to lot 1 to be delivered first. On arrival at QASP the animals (in each lot) will be rested for 7 days. During this period they will be inspected daily for general health and well-being and fed a starter feedlot ration (to be designed by Joe McMeniman). After this rest period, 12 cattle (from each lot of 15) will be chosen for entry to PC2 trial based on behavioural compatibility (day -9). Animals will be weighed and placed into 4 weight categories and then randomly allocated within weight category to 3 treatment groups (n=4 per treatment group) as outlined below. These cattle will then be acclimatised to the PC2 facility for 7 days prior to challenge during which time individual feed intake and clinical scores (as outlined below) will be recorded daily (to provide a baseline for post-challenge period). At the end of the acclimation period in the PC2 facility (day -2), calves will be transported by road (6hrs) and then yarded overnight with access to water. The following day (day -1) calves will again be transported by road (6hrs) back to the QASP facility and will return immediately to the PC2 facility. Please note transportation of calves prior to challenge will only be possible if approval from the relevant animal ethics committee can be obtained.

The challenge trial will then commence the day after calves have been transported back to the PC2 facility (day 0). The challenge trial will be replicated twice with 4 animals from each of treatment groups 1, 2 & 3 below challenged in each trial. Equal numbers of animals from each treatment (n=2) will be assigned to each room (n=6 per room) to minimise room effects (two rooms at QASP will be used during each individual challenge trial giving a total of 12 animals per trial). Trial will be replicated, with trials staggered by approximately 4-6 weeks with a total of 24 animals to be tested across both trials (n=8 per treatment group). Please note that implementation of the proposed challenge study design will be reliant on obtaining approval from the relevant animal ethics committee.

- Grp 1 - Amplimune treatment (dose and route of administration to be finalised following safety trial) at the time of viral challenge and saline treatment at time of bacterial challenge.
- Grp 2 - Amplimune treatment (dose and route of administration to be finalised following safety trial) at the time of bacterial challenge and saline treatment at time of viral challenge
- Grp 3 - Placebo treatment (saline, same volume and route of administration) at the time of viral challenge and bacterial challenge.
-

Animals will be fed a starter feedlot ration (to be designed by Joe McMeniman) for the duration of the challenge trial. Animals will be housed in individual pens inside the PC2 building and individual feed intake will be recorded daily on all individual animals throughout the challenge trial period (day 1 to day 23).

On day 1 the following data/samples will also be collected from all animals:

1. Record rectal temperature, respiration rate
2. Collect blood sample for cell isolation
3. Collect blood sample for serology
4. Collect nasal swabs from each nostril

Following sample collection, animals will be treated with Amplimune (Trt Grp 1) or saline (Trt Grps 2 & 3) and all 12 cattle (Grps 1, 2 & 3) will receive a BRD challenge consisting of (BHV-1: Challenge with $10^{\hat{}7}$ TCID50 1 to 2 ml per nostril). Immediately following challenge animals will be monitored hourly for 4 hrs. From this time all animals will also be observed daily (or more frequently if required).

On days 2-4, nasal swabs and blood samples will be collected and temperature & respiration rate data recorded on all animals. Once a day all animals will also be clinically assessed according to the following criteria - clinical score in brackets:

- Feed residue – Nil (0), 0–25% ration (1), 25–50% ration (2), 50-75% ration (3), 75-100% (4)
- Temperature (C) - Normal 37.8 to 39.4 (0), 39.5 to 40.1 (1); 40.2 to 40.7 (2); 40.8 to 41.3 (3); >41.4 (4)
- Coughing – no (0), cough due to exercise (1), coughing in pen (2)
- Nasal discharge – nil (0), mild (1), medium (2), heavy (3)
- Respiration rate – determined by number of breaths per 15 sec x 4
- Demeanour – – normal (0), dull/quieter than normal (1), lethargic requiring encouragement to leave pen (2), recumbent (3)

On day 5 the following data/samples will also be collected from all animals:

1. Record rectal temperature, respiration rate
2. Collect blood sample for cell isolation
3. Collect nasal swabs from each nostril

Following sample collection, animals will be treated with Amplimune (Grp 2) or saline (Grps 1 & 3) and all 12 cattle (Grps 1, 2 & 3) will then receive BRD Bacterial Challenge (5 x $10^{\wedge 9}$ cfu of *M. haemolytica* 1 to 2 ml per nostril). Blood samples will be collected at 6 and 12 hours post-bacterial challenge.

On days 6-7: Collect nasal swabs and inspect for nasal lesions, blood samples (24, 36, 48 & 60 hours post-bacterial challenge) and complete full clinical assessment (as described above)

On days 8-20: Collect nasal swabs and inspect for nasal lesions, blood samples and complete full clinical assessment (as described above).

On day 21 the following data/samples will also be collected from all animals:

1. Complete Clinical assessment
2. Collect blood sample for cell isolation
3. Collect blood sample for serology
4. Nasal swabs (separate swab for each nostril)
5. Inspect for nasal lesions

Following sample collection on day 21 all 12 cattle will be humanely euthanased and their lungs and injection site lesions inspected and scored. An expert will be contracted to conduct blinded & objective scoring of lung damage and histopathological assessment. They will have extensive experience in assessment of lung damage and will have experience with the use of an appropriate scoring system (details to be supplied). A sample of lung tissue will collected from a standardised site from all animals for histopathology assessment to accompany lung tissue damage scores.

Haematology parameters (white cell differential counts) and pro-inflammatory cytokine (IL-1 & TNFα) production will be assessed in blood samples collected at 0, 6, 12, 24, 36, 48, 60 & 72 hours post bacterial challenge. Please note collection of these serial blood samples will only be possible if approval from the relevant animal ethics committee can be obtained to collect such samples. Serology, to assess response to BHV1 and confirm infection will be conducted on blood samples collected pre-viral challenge (day 1, baseline) and post-viral challenge (days 7, 9, 11, 13, 15, 17, 19 & 21).

The entire challenge protocol (described above) will then be repeated on second lot of calves (n=12). Please note that following challenge death as an end point is not acceptable for animal ethics reasons. Therefore, should clinical scores suggest that an animal will die without intervention as a result of the challenge, the animal will be humanely euthanased at that time and the standard post-euthanisation protocols described above followed. Scales used to measure liveweight will be calibrated by BesTech Pty Ltd prior to use in the trial (periods between calibrations not to exceed 6 months).

3.4 Phase 3 - Assess the ability of Amplimune to enhance responses to BRD vaccines when co-administered at feedlot induction

NOTE: This phase of the project did not commence as MLA terminated the project. Therefore, methodology outlined here was proposed methodology only.

The vaccine efficacy trial was designed to investigate:

1) the ability of Amplimune to enhance responses to both modified live and inactivated BRD vaccines when co-administered at feedlot induction
2) the potential for Amplimune administration to result in injection site lesions

Ruth Davis (RedCap Solutions) will be consulted on final trial design to ensure trial fulfils requirements for APVMA registration.

A total of 60 cattle will be enrolled in the vaccine efficacy trial (these will not be same cattle as used in the safety trial). The day prior to vaccine/amplimune administration, all cattle will be transported by road (6hrs) and then yarded overnight with access to water. On the day of vaccine/amplimune treatment all animals will be again transported by road (6hrs) and put through a standard feedlot induction protocol (weighing, tagging and administration of standard health treatments including vaccination against clostridial diseases (Ultravac 7in1, Zoetis) and BRD (Rhinogard modified live vaccine, Zoetis OR Bovilis MH+IBR inactivated vaccine, Coopers) and anthelmintic application for control of internal parasites) to mimic stressors encountered at feedlot induction. The exact induction protocol, including specific health treatment products to be administered, will be finalised in consultation with MLA.

Animals will be weighed 72hrs pre-treatment with Amplimune. Animals will be placed into 5 weight categories and then randomly allocated within weight category to 4 treatment groups (n=15 per treatment group) for the trial as outlined below:

- Grp 1 – Rhinogard + Amplimune treatment (dose and route of administration to be finalised following safety trial)
- Grp 2 – Rhinogard + Placebo treatment (saline, same dose and route of administration)
- Grp 3 – Bovilis (MH+IBR) + Amplimune treatment (dose and route of administration to be finalised following safety trial)
- Grp 4 – Bovilis (MH+IBR) + Placebo treatment (saline, same dose and route of administration)

Post-treatment, animals from all treatment groups will be run together. Blood samples will be collected at various time points pre- and post-treatment (day 0, 14, 21 & 28) to assess responses to BRD & clostridial vaccination by measuring levels of antigen-specific serum antibodies in individual animals. Injection sites will be assessed in Amplimune treated animals at day 14, 21, 28, 56, 84 & 112 post-treatment for signs of injection site lesions. Injection sites will be palpated and any lesions detected will be measured, photographed and visually appraised using a standardised scoring system.

4. Project outcomes

4.1 Phase 1 – Part 1- Evaluating safe dose rates and the preferred route of administration of the Amplimune product

4.1.1 General Observations

All calves enrolled in the study were inspected by a veterinarian following transportation and prior to Amplimune administration (Day 0) and again three days post-treatment (Day 3). All calves were deemed to be 'healthy' and in 'good' condition on both Day 0 and again on Day 3 with all clinical measures assessed (included body condition, respiration rate, rectal temperature and heart rate) within normal range for calves of their age. No adverse reactions to Amplimune administration were observed in any calves. Following treatment, no signs of inappetence were observed in any calves during the duration of the trial period.

4.1.2 Haematology Parameters

Haematology parameters were assessed in blood samples collected at the start of the trial (D-4), at the time of treatment with Amplimune or saline (T0) and again at 6, 12, 24, 48, 72 and 96 hours post-treatment. Although numerous haematology parameters were assessed, specific parameters selected for analysis are listed below:-

- Total White Blood Cell count (WBC)
- Neutrophil count (NEU)
- Lymphocyte count (LYM)
- Neutrophil:Lymphocyte ratio (NEU:LYM)
- Haematocrit (HCT)

For analysis of haematology parameters, baseline values for each parameter observed either at the start of the trial (D-4) or at the time of treatment with Amplimune or saline (T0, post transportation) were fitted as covariates in statistical models. The significance of fixed effects when baseline values observed at D-4 were fitted as covariates to models are presented in Table 3 and treatment group LSMs (over time) for WBC, NEU, LYM, NEU:LYM and HCT are presented in Figures 4, 5, 6, 7 and 8, respectively. The significance of fixed effects when baseline values observed at T0 were fitted to models are presented in Table 4 and treatment group LSMs (over time) for WBC, NEU, LYM, NEU:LYM and HCT are presented in Figures 9, 10, 11, 12 and 13, respectively.

Parameter	Transformation	Weight (D-4)	Sex	Time	Treatment	Baseline Covariate (D-4)	Treatment *Time	Treatment *Sex
WBC	Log	NS	NS	***	NS	***	NS	*
NEU	Log	NS	NS	***	NS	NS	NS	**
LYM	Log	NS	NS	***	NS	***	NS	**
NEU:LYM	Nil	NS	NS	***	NS	***	NS	*
HCT	Nil	NS	NS	***	NS	***	NS	NS

Table 3: Significance of fixed effects (including baseline covariate recorded at D-4) when analysing haematology parameters (NS = non-significant P>0.05, * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

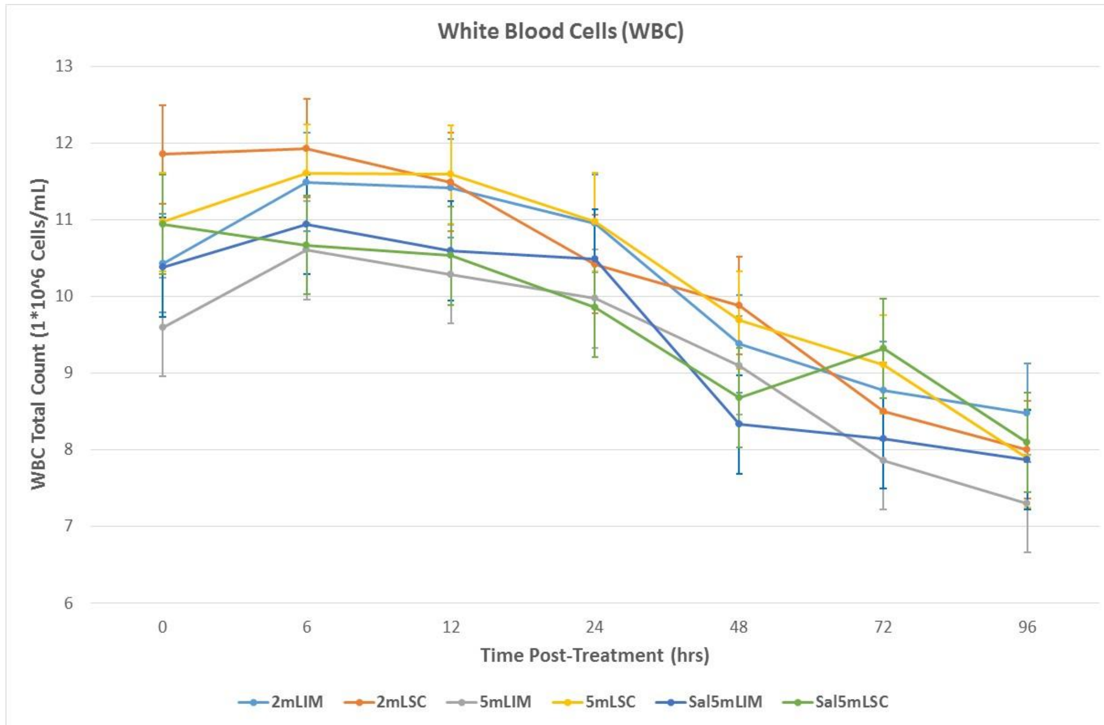

Figure 4. Treatment group LSMs (±SEM) for total White Blood Cell counts (WBC). When calculating LSMs, baseline values observed at D-4 were fitted as a covariate in statistical models.

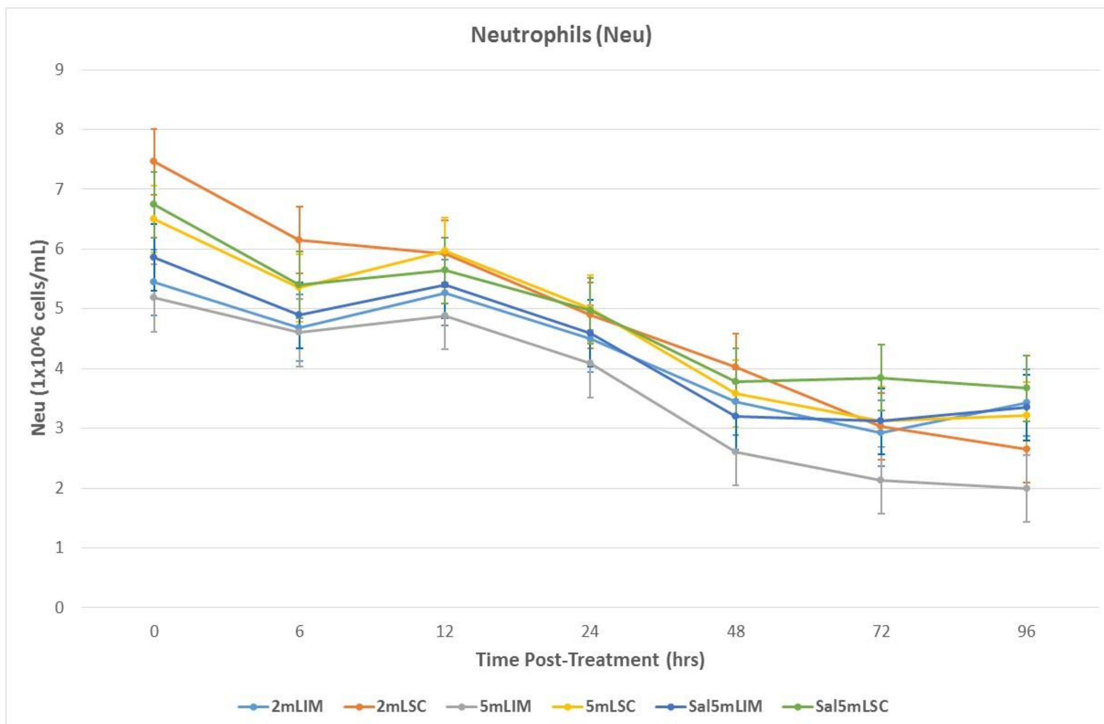

Figure 5. Treatment group LSMs (±SEM) for Neutrophil counts (NEU). When calculating LSMs, baseline values observed at D-4 were fitted as a covariate in statistical models.

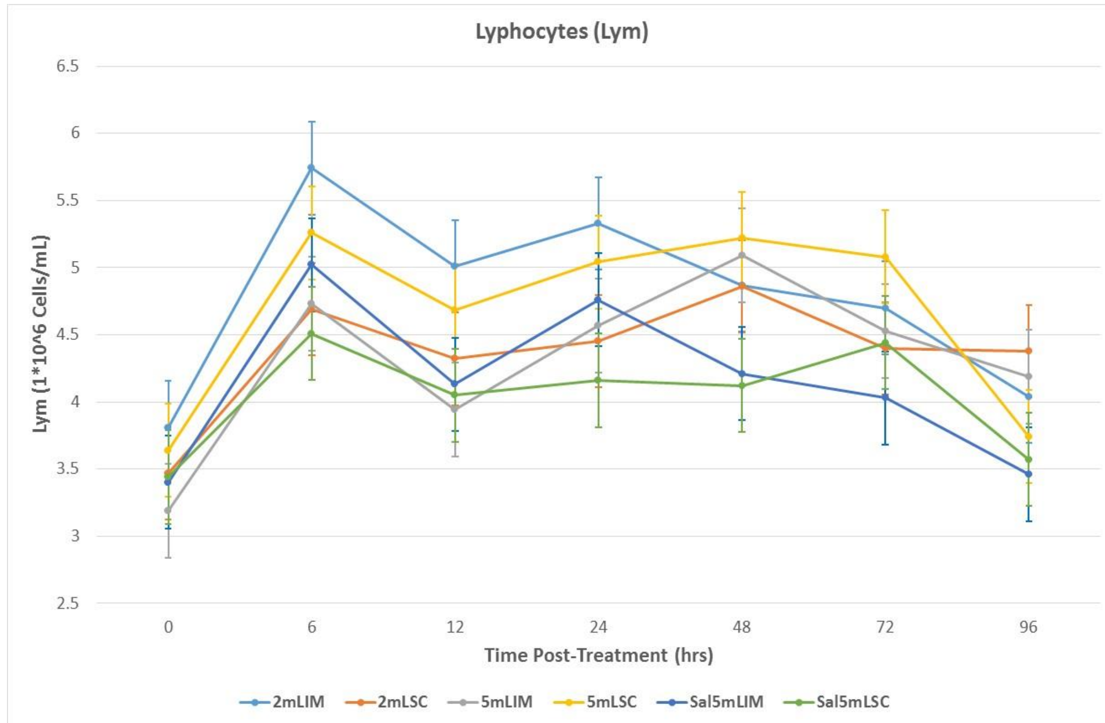

Figure 6. Treatment group LSMs (±SEM) for Lymphocyte counts (LYM). When calculating LSMs, baseline values observed at D-4 were fitted as a covariate in statistical models.

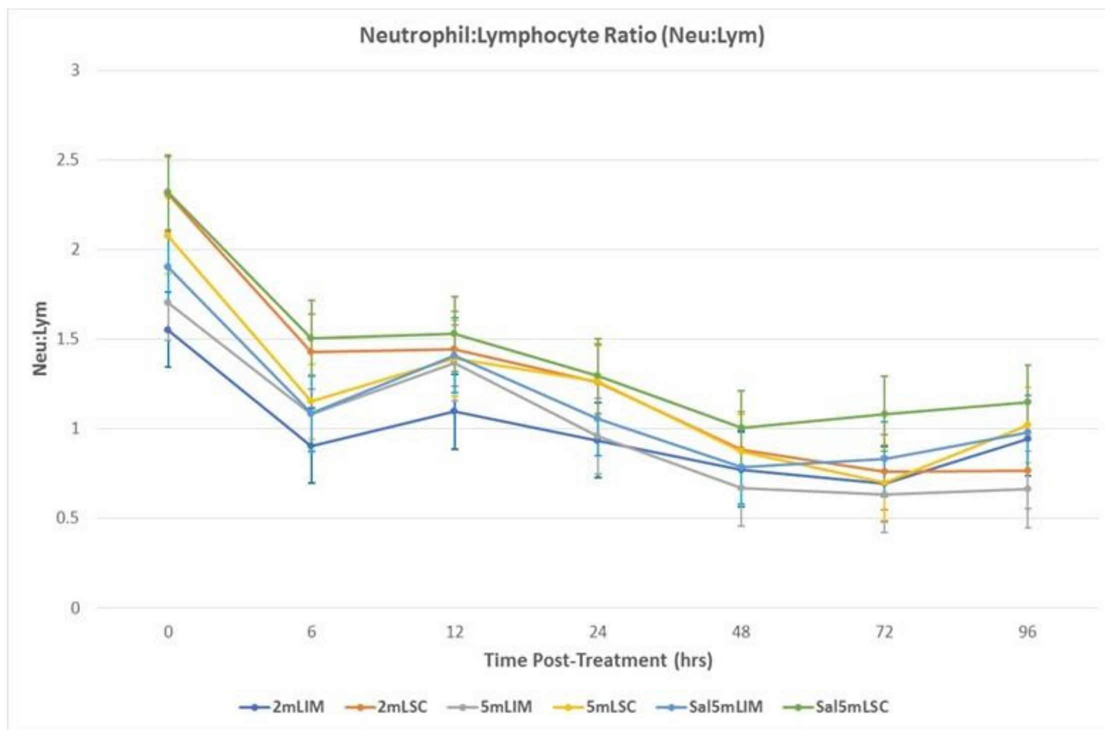

Figure 7. Treatment group LSMs (±SEM) for Neutrophil:Lymphocyte ratio (NEU:LYM). When calculating LSMs, baseline values observed at D-4 were fitted as a covariate in statistical models.

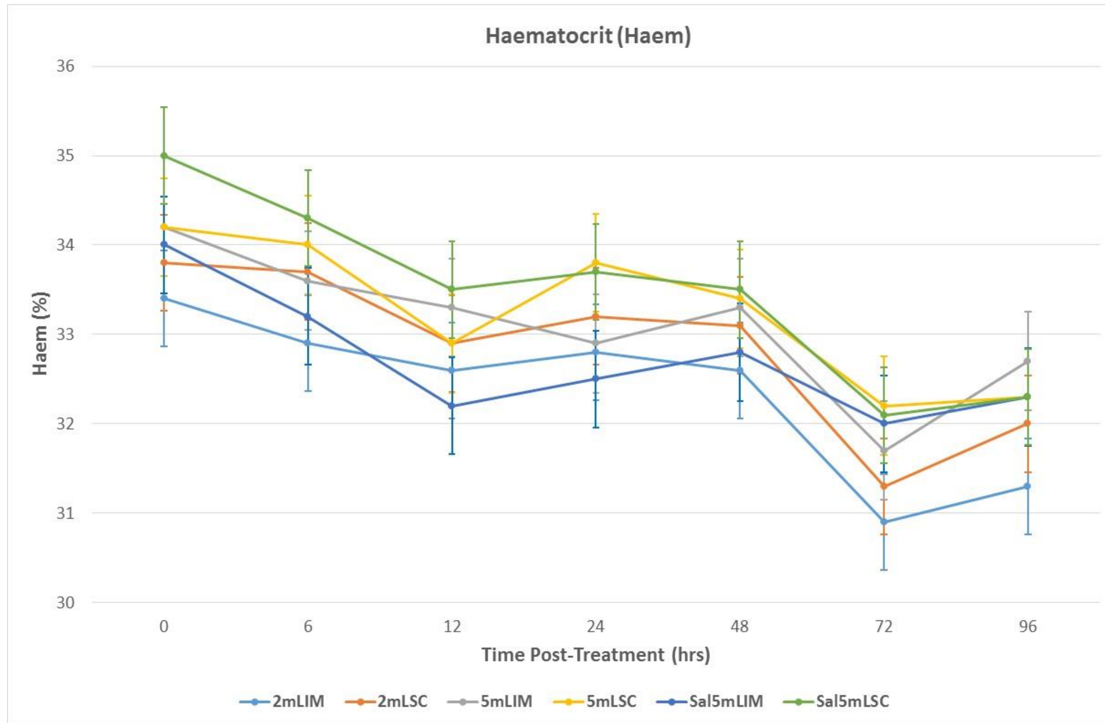

Figure 8. Treatment group LSMs (±SEM) for Haematocrit (HCT). When calculating LSMs, baseline values observed at D-4 were fitted as a covariate in statistical models.

Parameter	Transformation	Weight (D-4)	Sex	Time	Treatment	Baseline Covariate (T0)	Treatment *Time	Treatment *Sex
WBC	Log10	NS	NS	***	NS	***	NS	NS
NEU	Log10	NS	NS	***	NS	***	NS	NS
LYM	Log10	NS	NS	***	NS	***	NS	NS
NEU:LYM Ratio	Nil	NS	NS	***	NS	***	NS	NS
HCT	Nil	NS	NS	***	NS	***	NS	NS

Table 4: Significance of fixed effects (including baseline covariate recorded at T0) when analysing haematology parameters (NS = non-significant P>0.05, * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

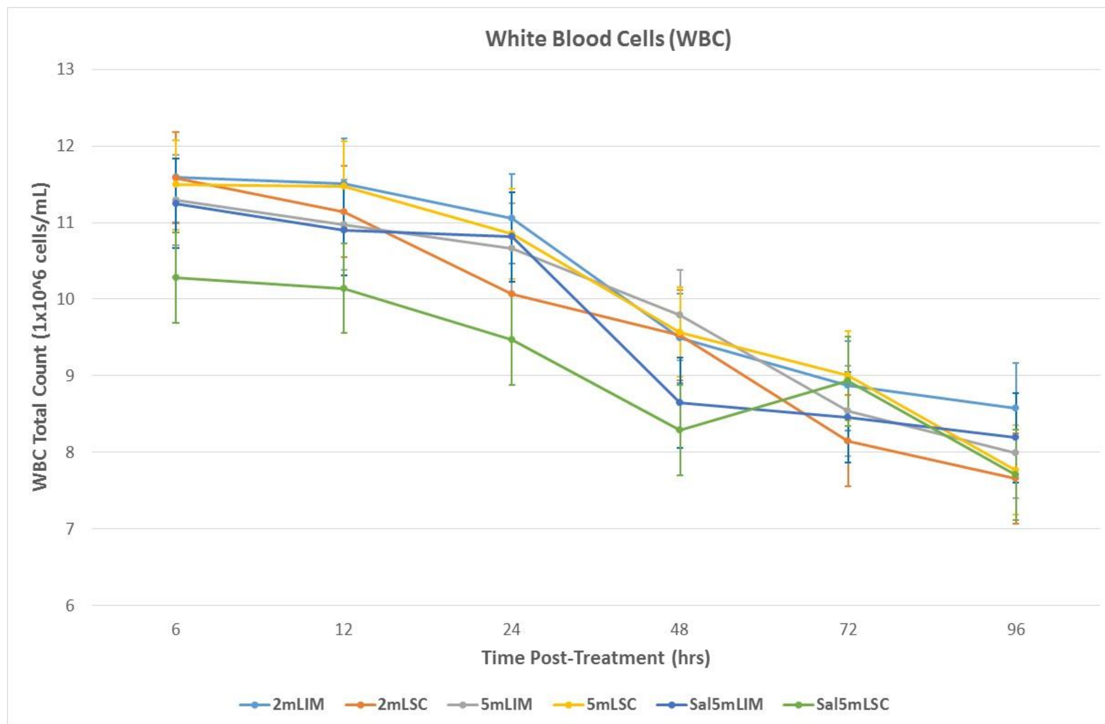

Figure 9. Treatment group LSMs (±SEM) for total White Blood Cell counts (WBC). When calculating LSMs, baseline values observed at T0 were fitted as a covariate in statistical models.

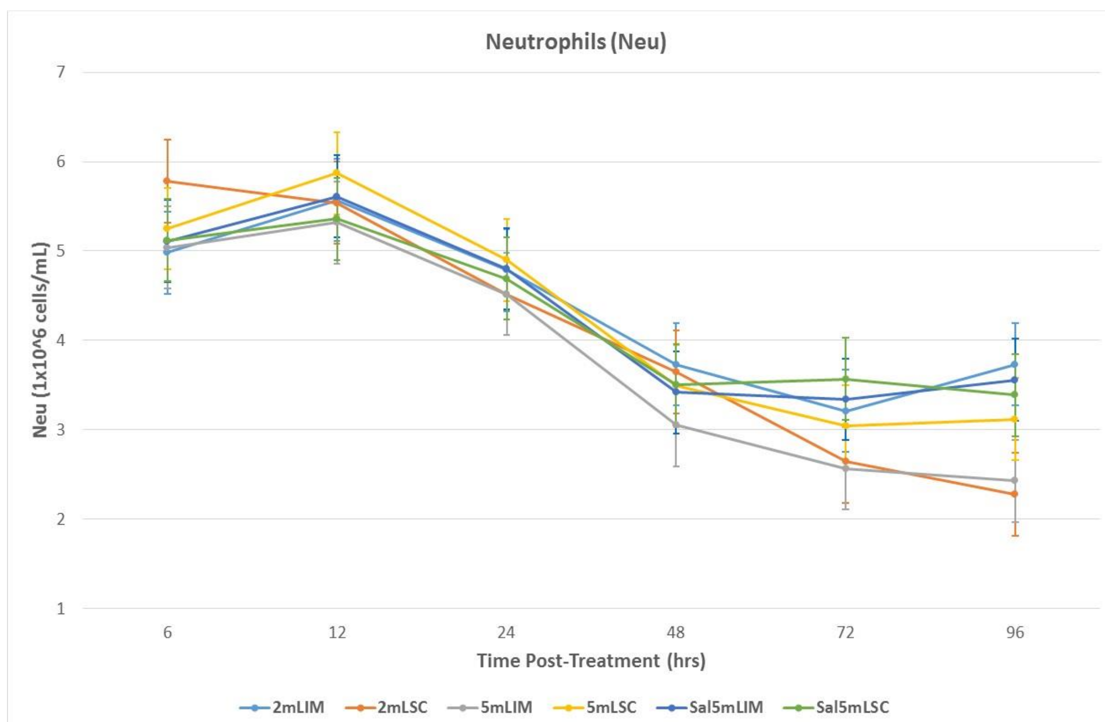

Figure 10. Treatment group LSMs (±SEM) for Neutrophil counts (NEU). When calculating LSMs, baseline values observed at T0 were fitted as a covariate in statistical models.

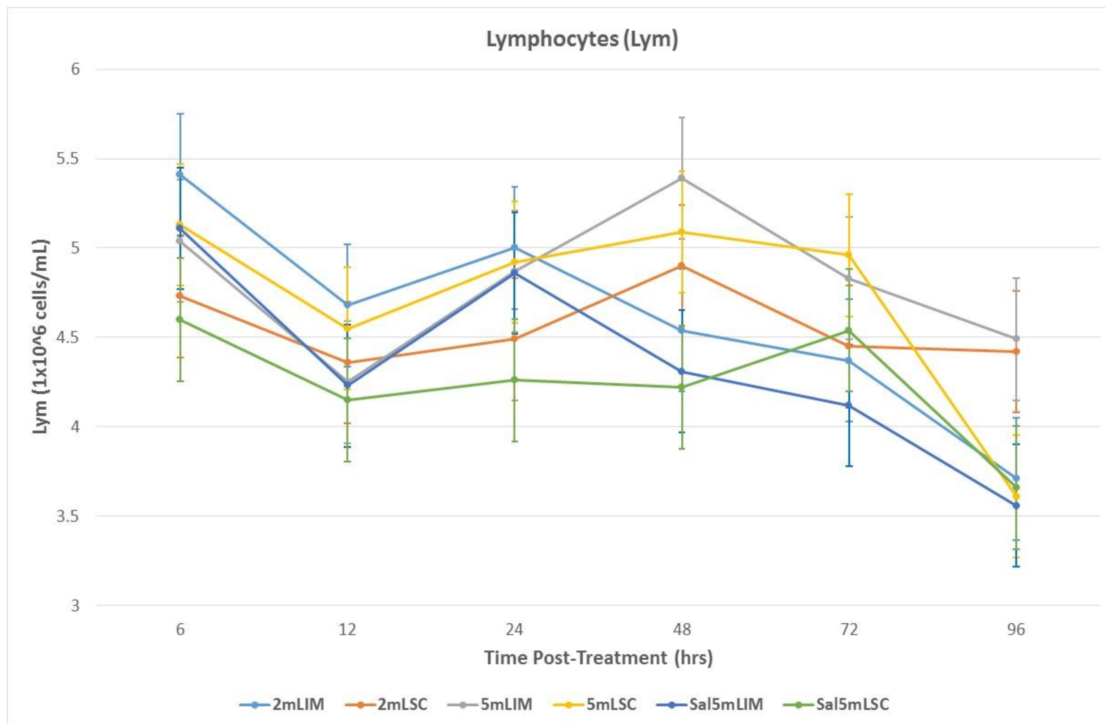

Figure 11. Treatment group LSMs (±SEM) for Lymphocyte counts (LYM). When calculating LSMs, baseline values observed at T0 were fitted as a covariate in statistical models.

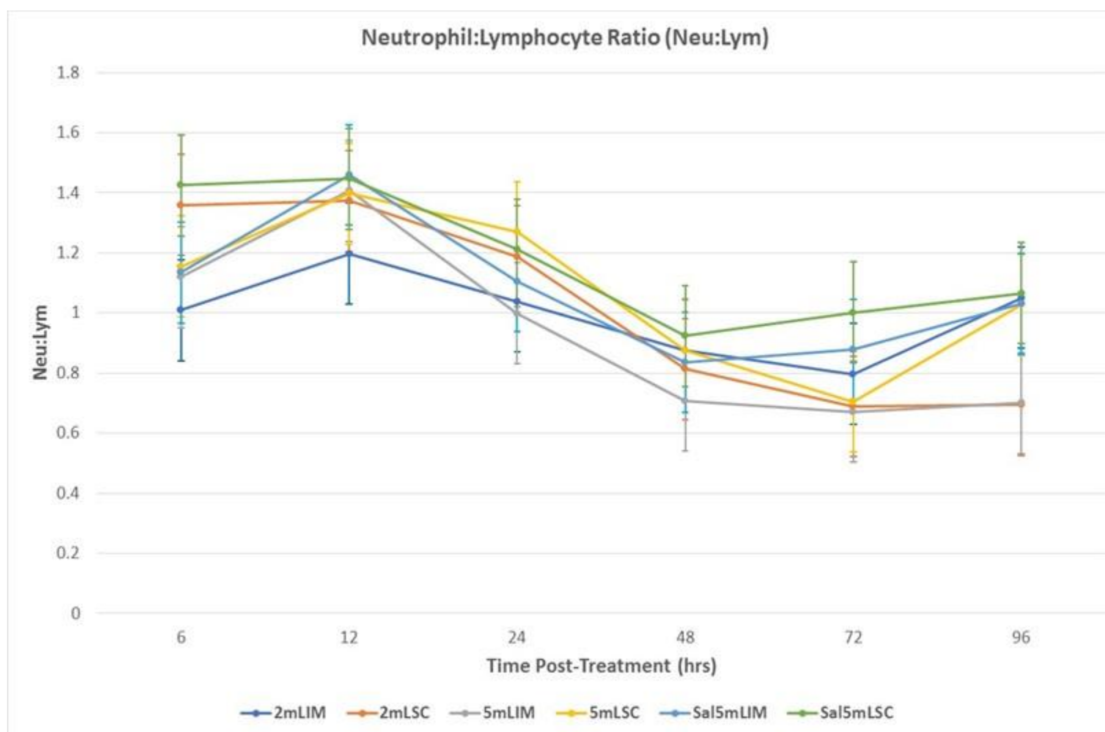

Figure 12. Treatment group LSMs (±SEM) for Neutrophil:Lymphocyte ratio (NEU:LYM). When calculating LSMs, baseline values observed at T0 were fitted as a covariate in statistical models.

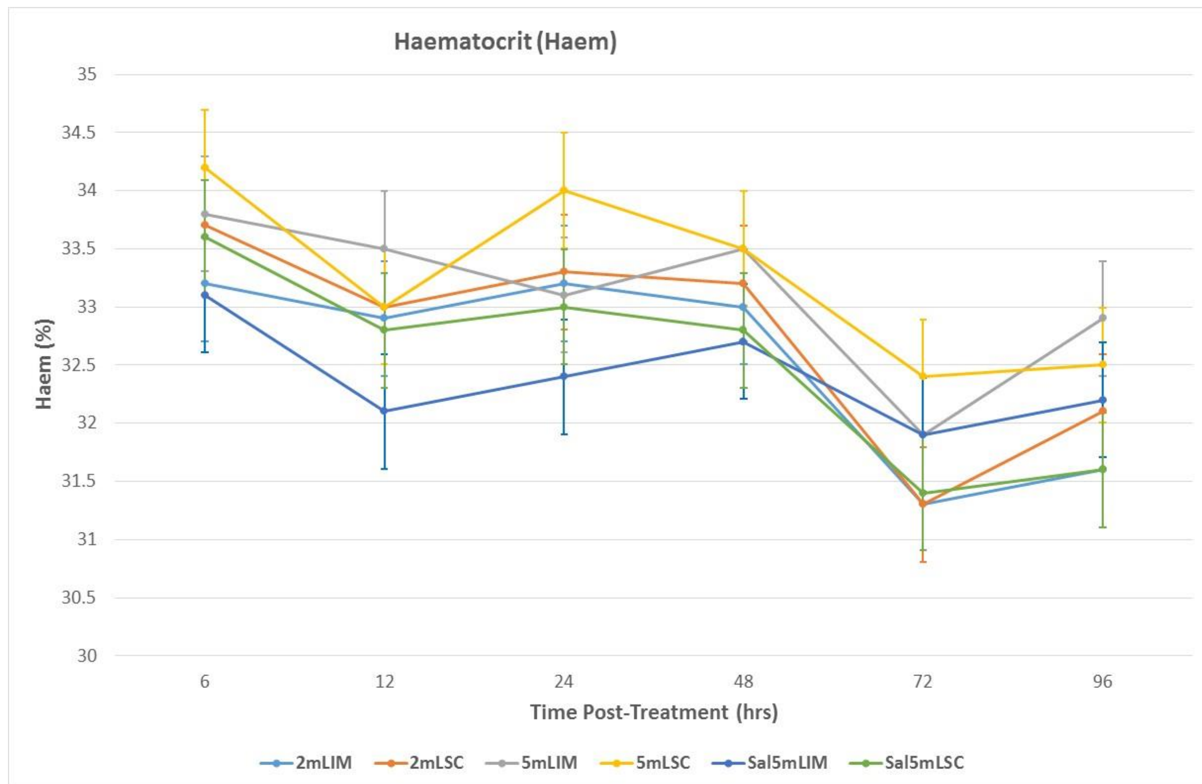

Figure 13. Treatment group LSMs (±SEM) for Haematocrit (HCT). When calculating LSMs, baseline values observed at T0 were fitted as a covariate in statistical models.

4.1.3 Changes in Core Body Temperature

Core body temperature was continually assessed (in 5 minute increments) from the start of the trial (D-4) until 3 days post treatment with Amplimune or saline (Day 3) using i-button temperature loggers inserted into the rectum of calves. When analysing body temperature data it is important to consider both the variation which exists in the normal diurnal temperature profiles of individual animals (under non-experimental conditions) and the effects of ambient temperature. To identify the normal diurnal temperature profiles of individual calves, temperature measures logged on days when calves were grazing in a small paddock undisturbed (days -3 & -2) were assessed to generate baseline body temperature parameters which could be fitted to statistical models. Adjustments for ambient temperature on core body temperature measures were not made in the current study; however, calves were assigned a number and processed in a specific order in an attempt to minimise any differential effects of ambient temperature on calves in any given treatment group.

To analyse changes in core body temperature, logged temperature readings were grouped into blocks of data which represented a 6hr, 12hr or 24hr time frame commencing at a time equivalent to when Amplimune or saline was administered. For example, if a calve received Amplimune at 1.40pm on day 0 and we were investigating a 6hr time frame, temperatures logged from 1.40pm to 7.40pm on each consecutive day that temperature loggers were fitted (starting at D-3 and finishing at D2 relative to treatment) were grouped into a block of data. Similarly, when investigating a 12hr time frame, temperatures logged from 1.40pm to 1.40am on each consecutive day were analysed and so on. For each block of time (representing a 6hr, 12 hr or 24hr time window) the mean and maximum temperature observed for each calf was calculated and used for analysis. Baseline temperature

measures (assessed across days -3 & -2) were fitted as covariates in statistical models. Therefore body temperature parameters analysed were as follows:-

- Mean body temperature - 6hr window (Mean 6hr)
- Max body temperature - 6hr window (Max 6hr)
- Mean body temperature - 12hr window (Mean 12hr)
- Max body temperature - 12hr window (Max 12hr)
- Mean body temperature - 24hr window (Mean 24hr)
- Max body temperature - 24hr window (Max 24hr)

The significance of fixed effects when baseline temperature measures (assessed across days -3 & -2) were fitted as a covariate to models are presented in Table 5 and treatment group LSMs (over time) for Mean 6hr, Max 6hr, Mean 12hr, Max 12hr, Mean 24hr and Max 24hr are presented in Figures 14, 16, 18, 20, 22 and 24, respectively. A significant overall treatment effect was observed for all body temperature parameters (with the exception of Mean 12hr); however, this effect varied depending on the sex of calves (as evidenced by the highly significant treatment*sex interaction observed when analysing each parameter) (Table 5). The significant treatment group and treatment*sex interaction effects reported in Table 5 were confirmed to be a result of changes in body temperature induced by treatment with Amplimune (rather than as a result of differential body temperature changes between treatment groups induced by transport), by repeating analyses excluding body temperature data collected during the transportation period. As significant treatment group effects were observed, specific linear contrasts were undertaken to compare LSMs for Amplimune treated versus saline (control) treated steers and heifers (independently) as detailed below at each individual timepoint (D0, D1 & D2):-

- 2mLSC versus Sal5mLSC
- 2mLIM versus Sal5mLIM
- 5mLSC versus Sal5mLSC
- 5mLIM versus Sal5mLIM
-

Treatment group LSMs (over time) for Mean 6hr, Max 6hr, Mean 12hr, Max 12hr, Mean 24hr and Max 24hr parameters showing the significance of specific linear contrasts are presented in Figures 15, 17, 19, 21, 23 and 25, respectively.

Parameter	Transformation	Weight (D-4)	Sex	Time	Treatment	Baseline Covariate	Treatment *Time	Treatment *Sex
Mean 6hr	Nil	*	NS	***	*	***	NS	***
Max 6hr	Nil	*	NS	***	*	***	NS	***
Mean 12hr	Nil	NS	NS	***	NS	***	NS	***
Max 12hr	Nil	*	NS	**	*	***	NS	***
Mean 24hr	Nil	*	*	***	*	***	NS	**
Max 24hr	Nil	**	NS	***	*	***	NS	***

Table 5: Significance of fixed effects (including baseline covariate recorded across D-3 and D-2) when analysing core body temperature parameters (NS = non-significant P>0.05, * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

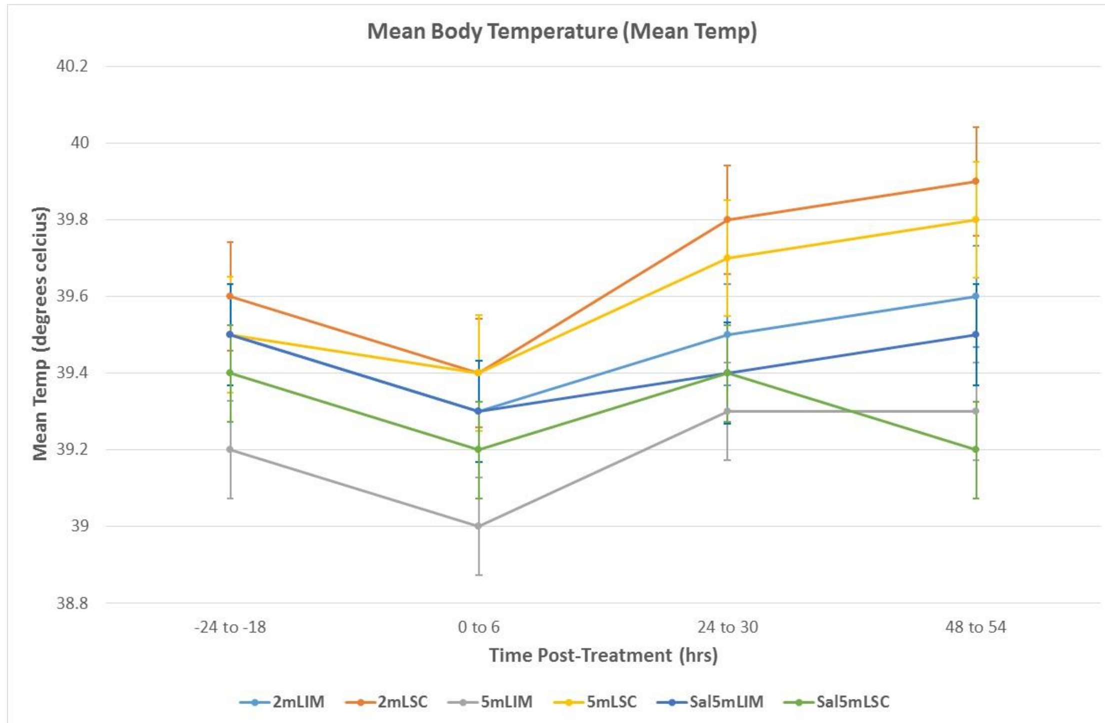

Figure 14. Treatment group LSMs (±SEM) for Mean Body Temperature -6hr. When calculating LSMs, baseline values observed across D-3 and D-2 were fitted as a covariate in statistical models.

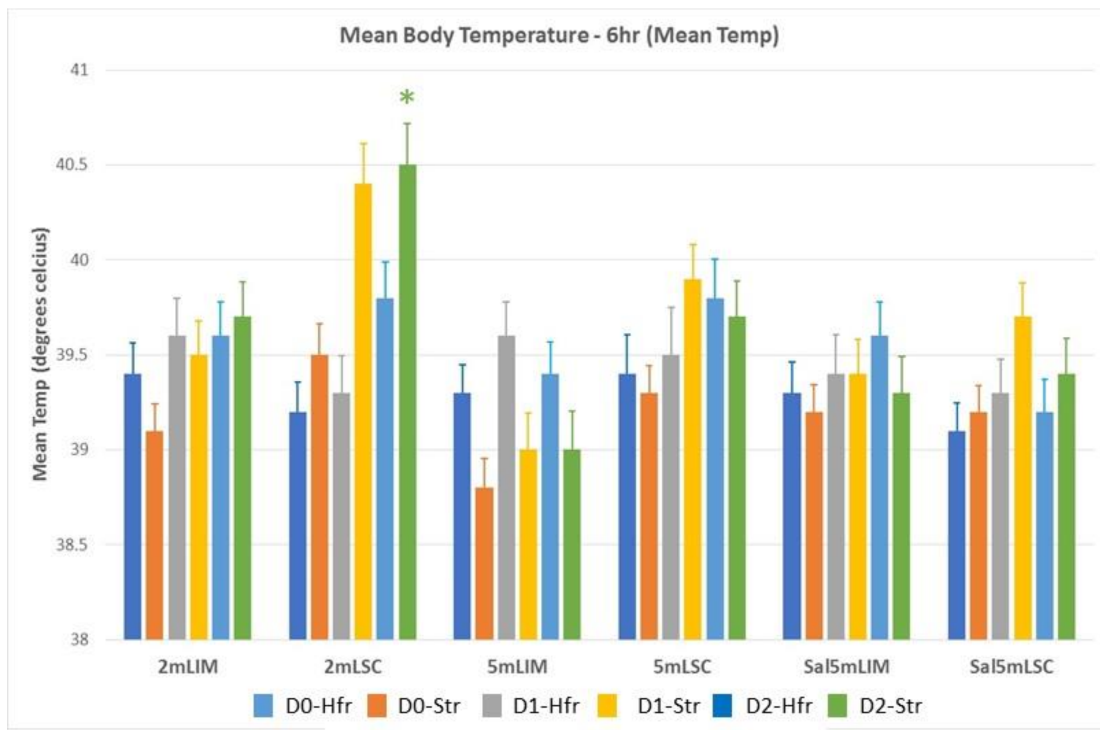

Figure 15. Treatment group LSMs (±SEM) for Mean Body Temperature -6hr. Significance of specific linear contrasts within sex and within day (as described above) * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

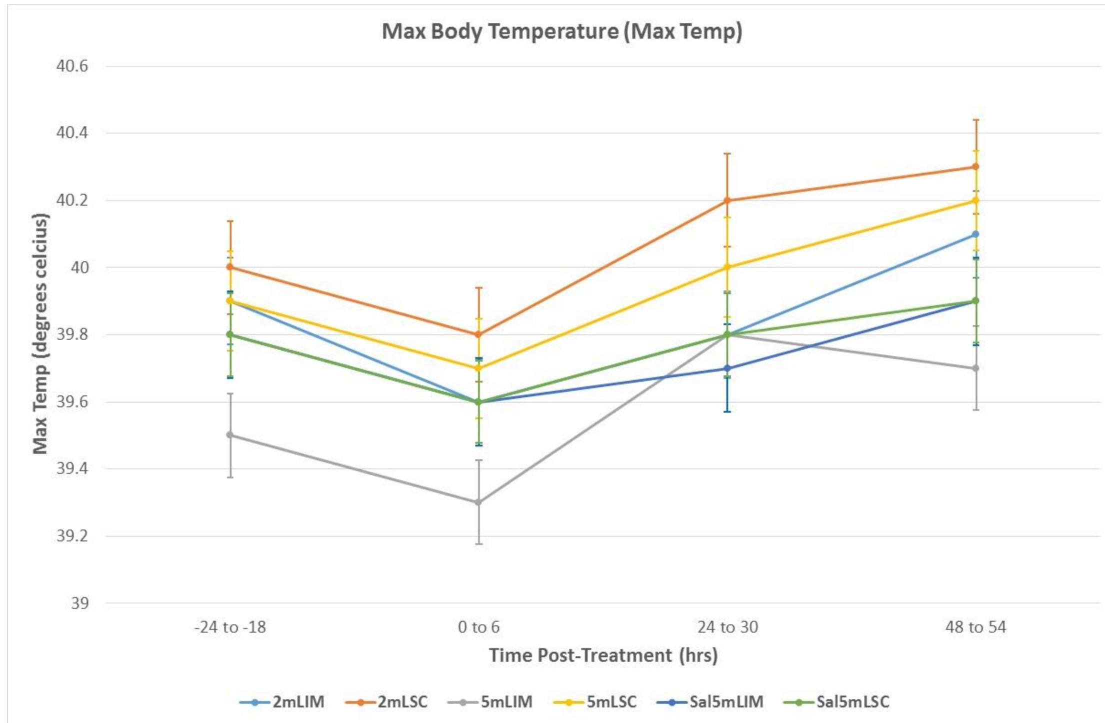

Figure 16. Treatment group LSMs (±SEM) for Max Body Temperature -6hr. When calculating LSMs, baseline values observed across D-3 and D-2 were fitted as a covariate in statistical models.

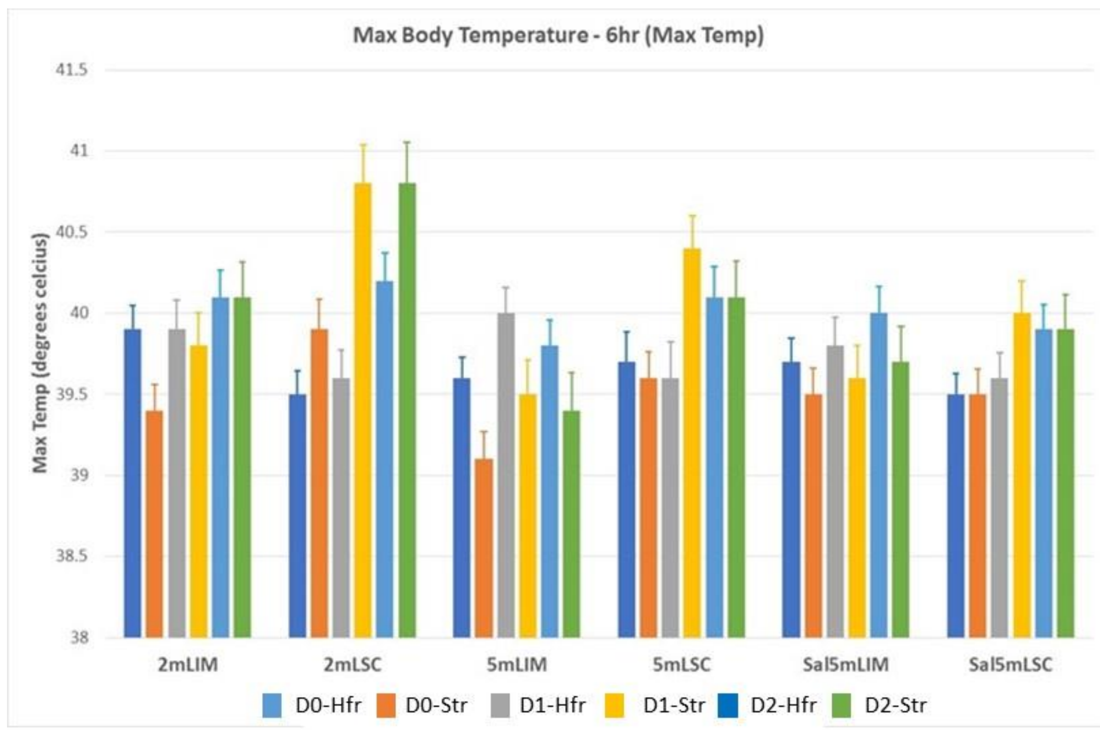

Figure 17. Treatment group LSMs (±SEM) for Max Body Temperature -6hr. Significance of specific linear contrasts within sex and within day (as described above) * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

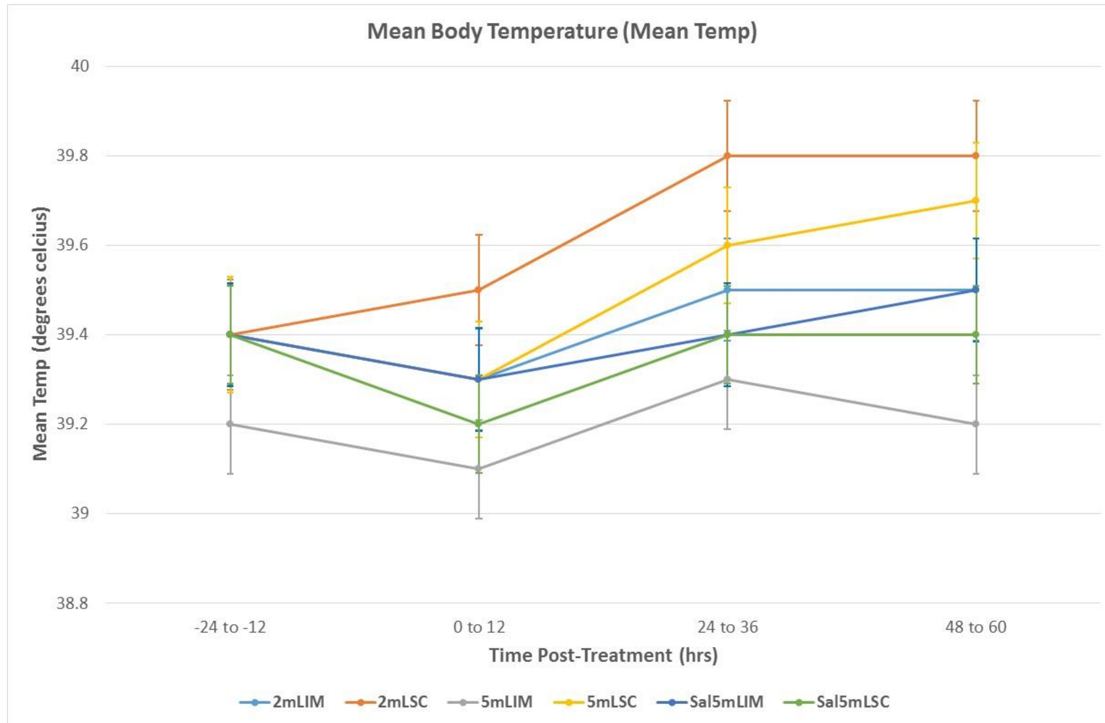

Figure 18. Treatment group LSMs (±SEM) for Mean Body Temperature -12hr. When calculating LSMs, baseline values observed across D-3 and D-2 were fitted as a covariate in statistical models.

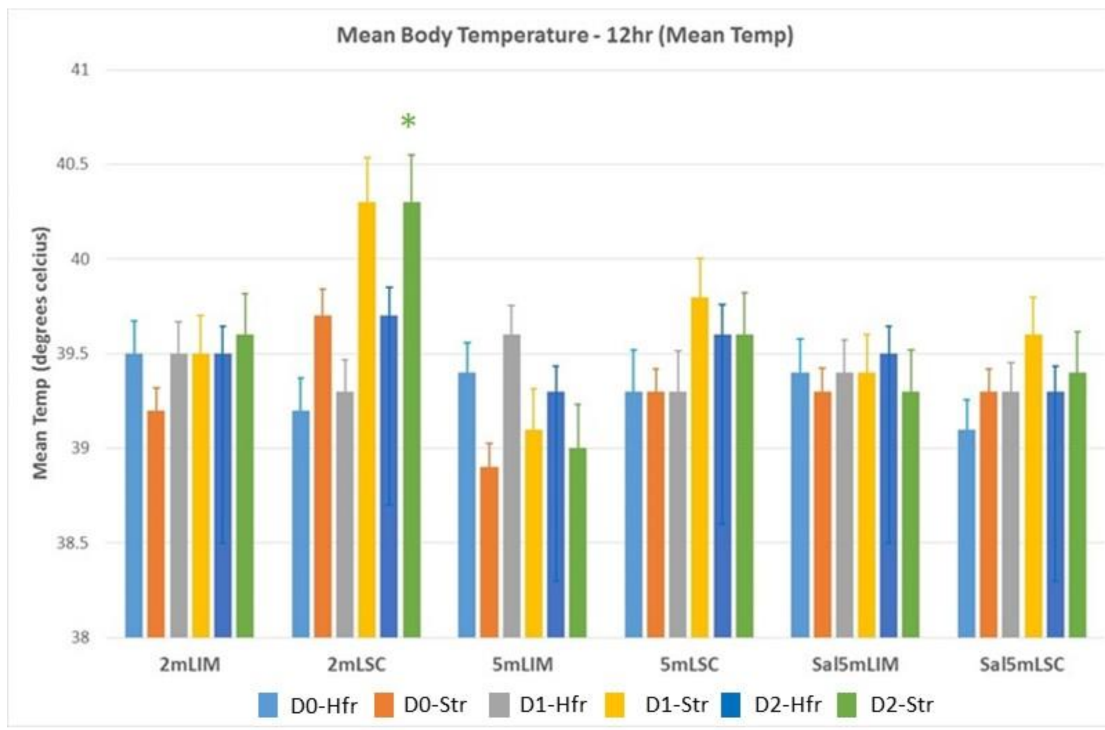

Figure 19. Treatment group LSMs (±SEM) for Mean Body Temperature -12hr. Significance of specific linear contrasts within sex and within day (as described above) * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

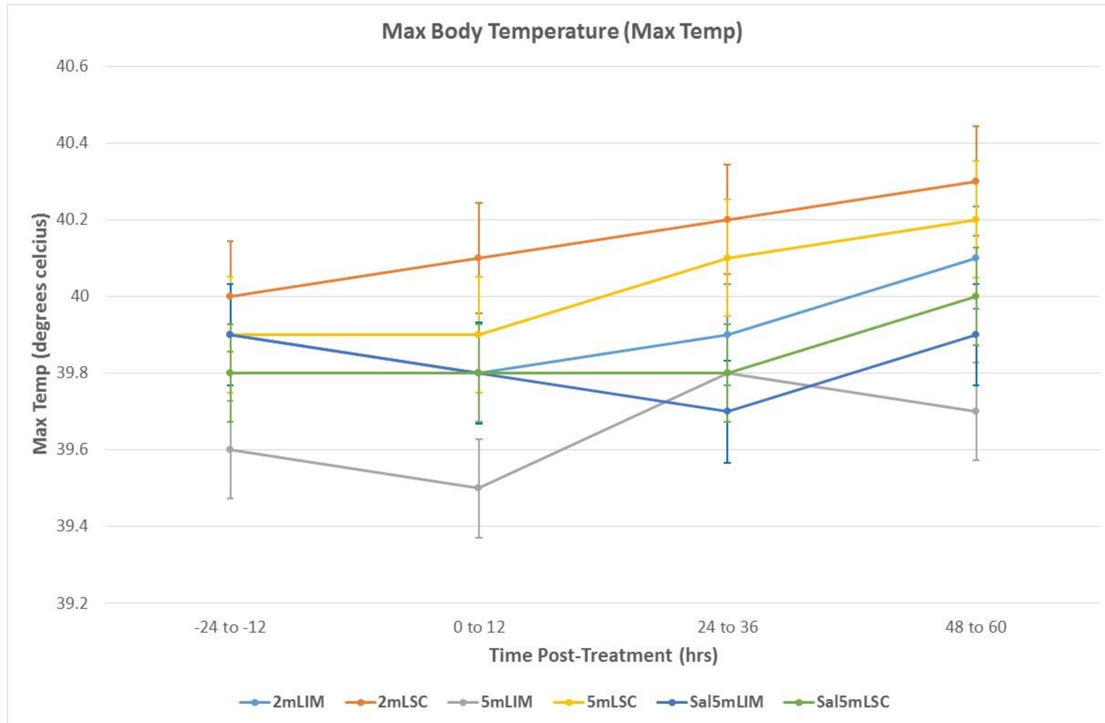

Figure 20. Treatment group LSMs (±SEM) for Max Body Temperature -12hr. When calculating LSMs, baseline values observed across D-3 and D-2 were fitted as a covariate in statistical models.

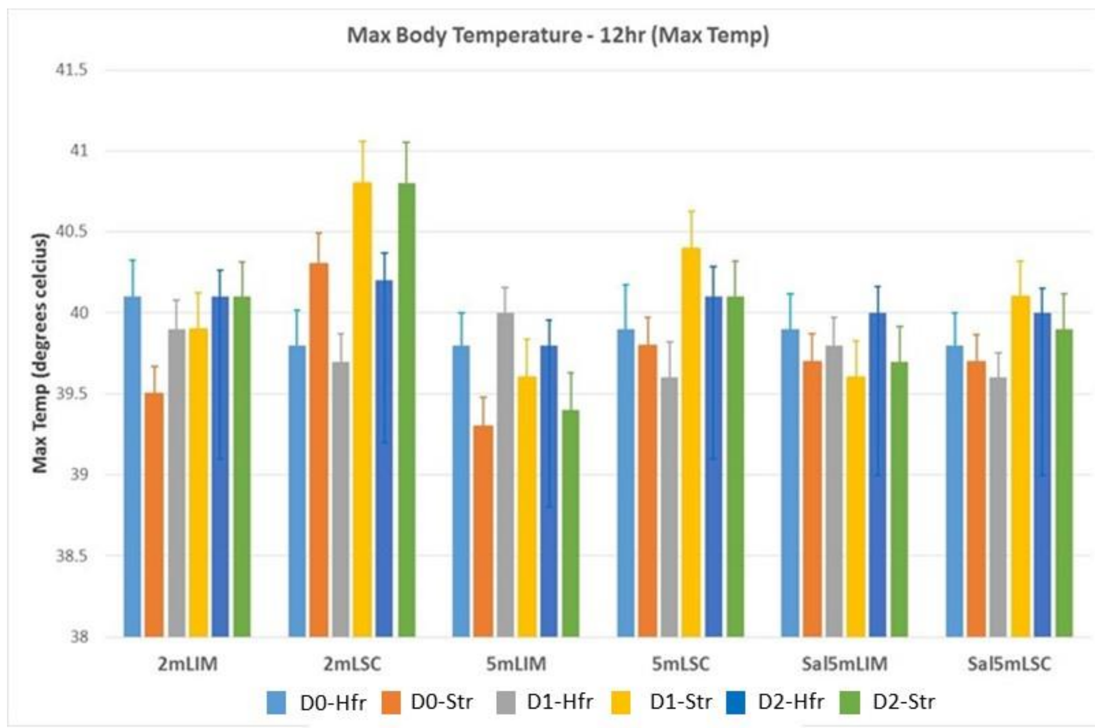

Figure 21. Treatment group LSMs (±SEM) for Max Body Temperature -12hr. Significance of specific linear contrasts within sex and within day (as described above) * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

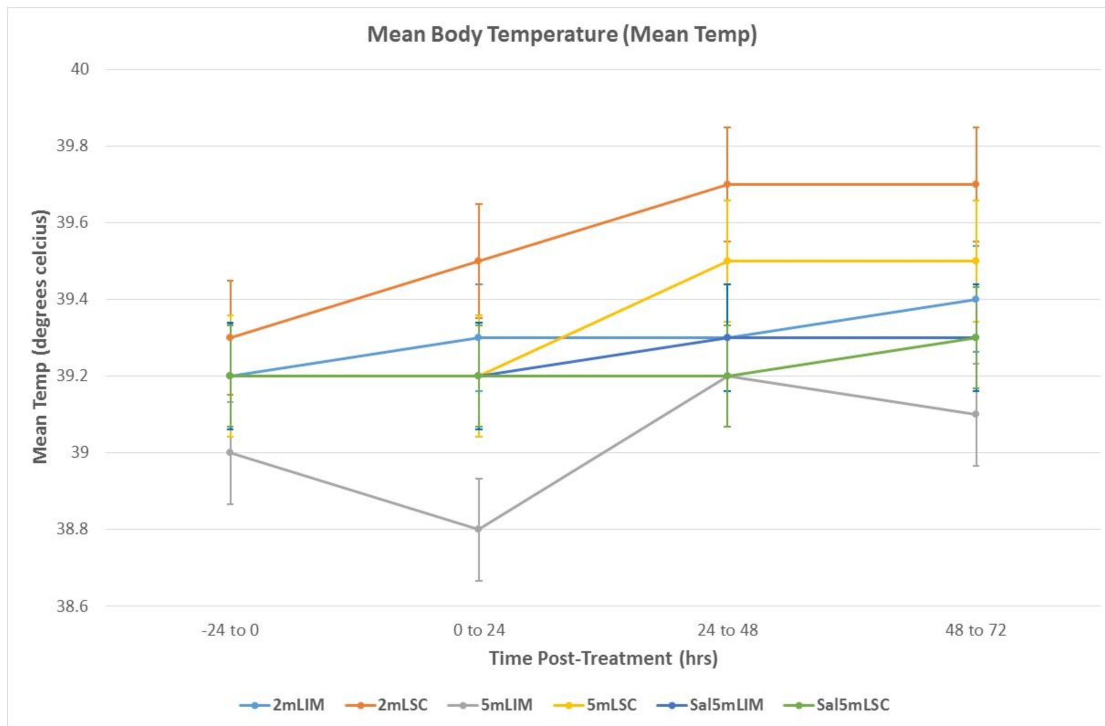

Figure 22. Treatment group LSMs (±SEM) for Mean Body Temperature -24hr. When calculating LSMs, baseline values observed across D-3 and D-2 were fitted as a covariate in statistical models.

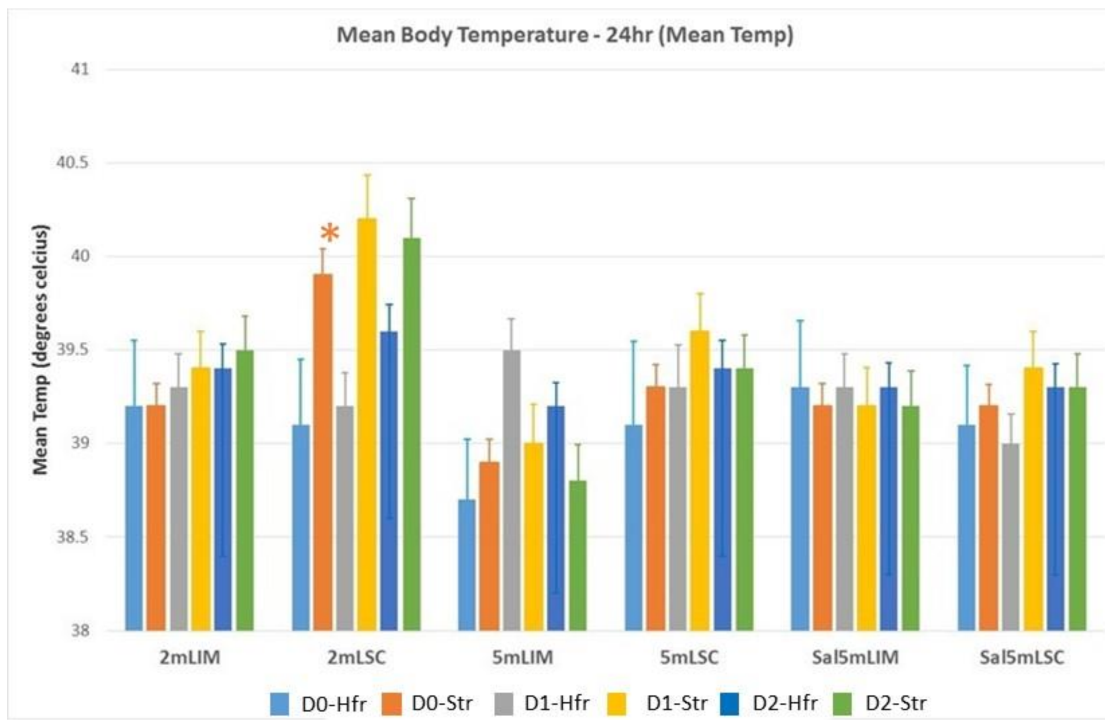

Figure 23. Treatment group LSMs (±SEM) for Mean Body Temperature -24hr. Significance of specific linear contrasts within sex and within day (as described above) * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

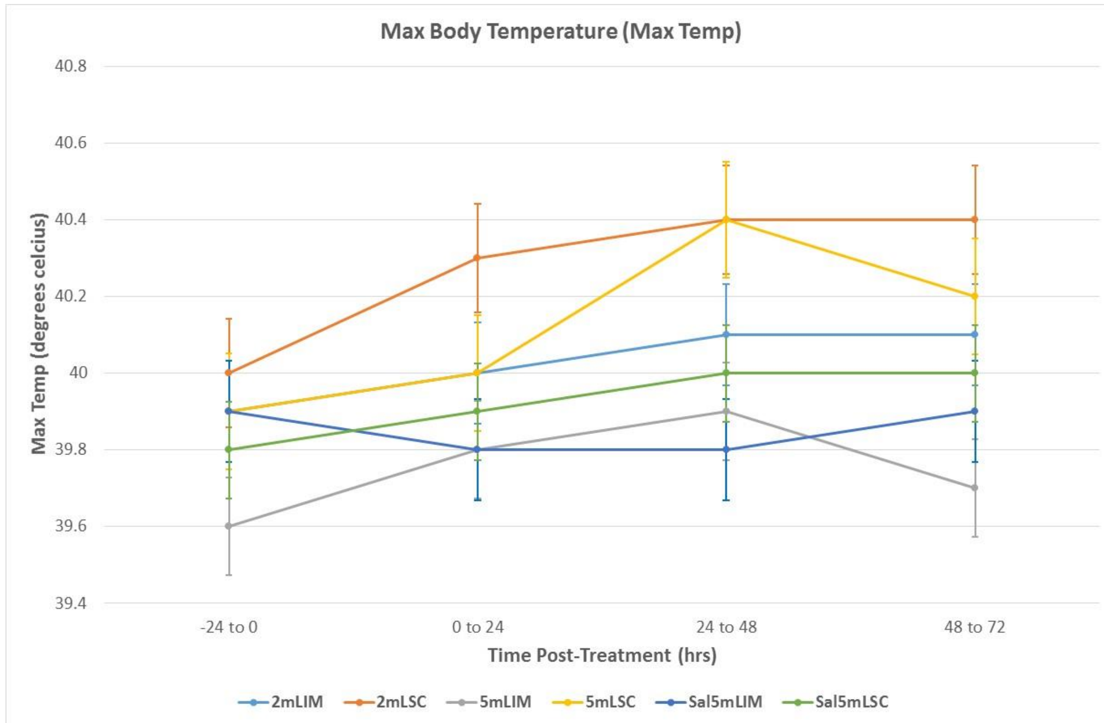

Figure 24. Treatment group LSMs (±SEM) for Max Body Temperature -24hr. When calculating LSMs, baseline values observed across D-3 and D-2 were fitted as a covariate in statistical models.

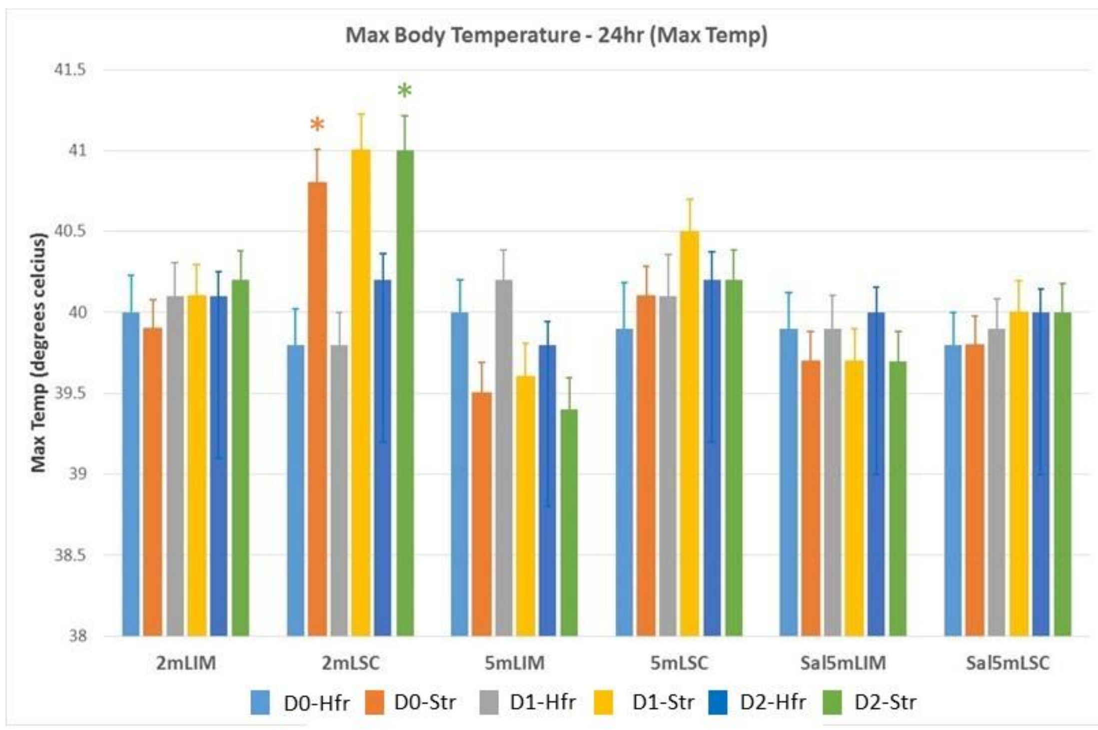

Figure 25. Treatment group LSMs (±SEM) for Max Body Temperature -24hr. Significance of specific linear contrasts within sex and within day (as described above) * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

4.1.4 Changes in Liveweight

Liveweight was assessed at the start of the trial (D-4) and end of the trial (D4) in the Hway yards which contained calibration certified scales. During the trial period, liveweights were also assessed on D0, D2 and D4 in the K3 yards where calves were housed during the trial period. Therefore specific liveweight parameters analysed were:-

- Liveweight change Hway yards (calibrated scales)
- Liveweight change K3 yards

For analysis of liveweight parameters, baseline liveweight either at the start of the trial (D-4) or at the time of treatment with Amplimune or saline (D0, post transportation) were fitted as covariates in statistical models. The significance of fixed effects when baseline values observed at D-4 were fitted as covariates to models and liveweight was measured at Hway yards is presented in Table 6 and treatment group LSMs are presented in Figure 26. The significance of fixed effects when baseline values observed at D-4 or T0 were fitted to models and liveweight was measured at K3 yards are presented in Tables 7 and 8, respectively, and treatment group LSMs (over time) are presented in Figures 27 and 28, respectively.

A significant overall treatment effect was observed when liveweights were recorded at Hway yards (Table 6). Therefore specific linear contrasts were undertaken to compare LSMs for Amplimune treated versus saline (control) treated calves as detailed below:-

- 2mLSC versus Sal5mLSC
- 2mLIM versus Sal5mLIM
- 5mLSC versus Sal5mLSC
- 5mLIM versus Sal5mLIM

No significant differences were observed when specific group contrasts were undertaken (Figure 26).

Parameter	Transformation	Sex	Time	Treatment	Baseline Covariate (D-4)	Treatment* Time	Treatment* Sex
Liveweight (Hway yards)	Nil	NS	NA	**	***	NA	NA

Table 6: Significance of fixed effects (including baseline covariate recorded D-4) when analysing liveweights measured in Hway yards (NS = non-significant P>0.05, * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

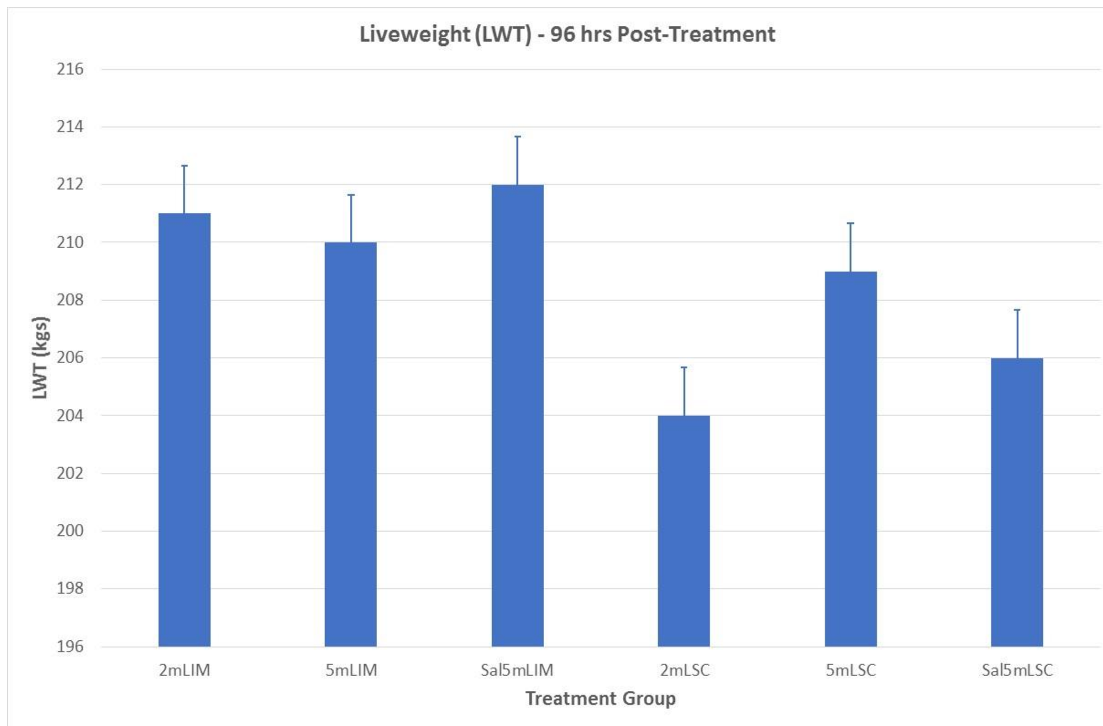

Figure 26. Treatment group LSMs (±SEM) for liveweight measured at the Hway yards. When calculating LSMs, baseline liveweight recorded at D-4 was fitted as a covariate in statistical models.

Parameter	Transformation	Sex	Time	Treatment	Baseline Covariate (D-4)	Treatment* Time	Treatment* Sex
Liveweight (K3 yards)	Nil	NS	***	NS	***	NS	NS

Table 7: Significance of fixed effects (including baseline covariate recorded D-4) when analysing liveweights measured in K3 yards (NS = non-significant P>0.05, * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

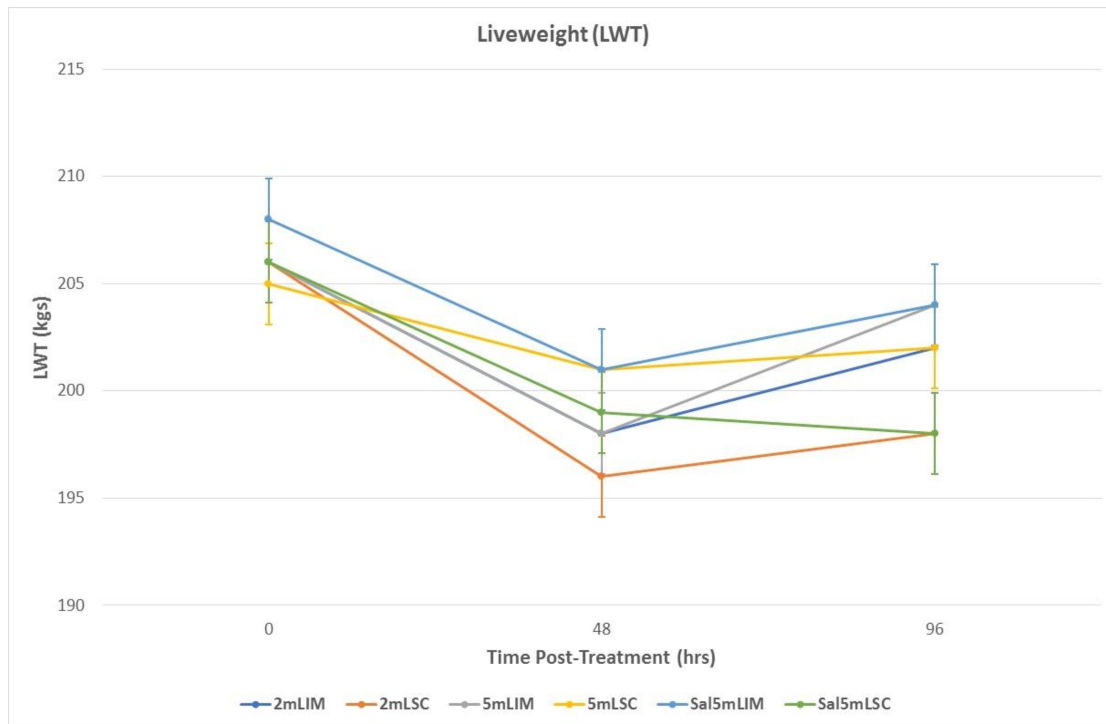

Figure 27. Treatment group LSMs (±SEM) for liveweight measured at the K3 yards. When calculating LSMs, baseline liveweight recorded at D-4 was fitted as a covariate in statistical models.

Parameter	Transformation	Sex	Time	Treatment	Baseline Covariate (D0)	Treatment* Time	Treatment* Sex
Liveweight (K3 yards)	Nil	NS	**	NS	***	NS	NS

Table 8: Significance of fixed effects (including baseline covariate recorded D0) when analysing liveweights measured in K3 yards (NS = non-significant P>0.05, * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

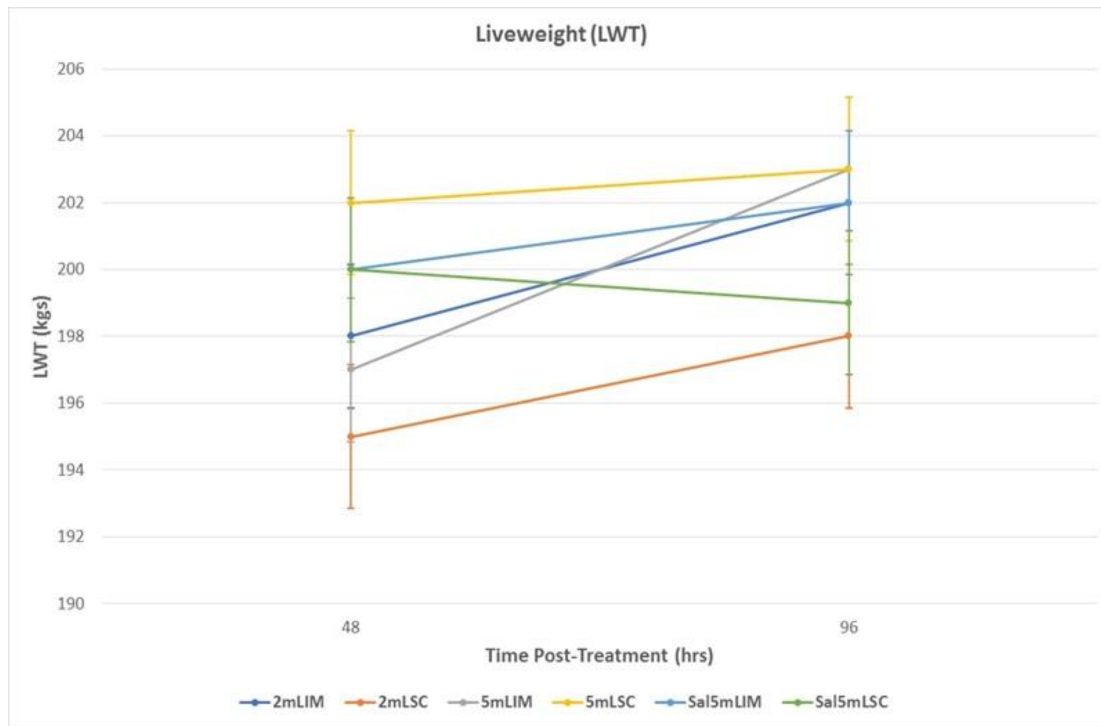

Figure 28. Treatment group LSMs (±SEM) for liveweight measured at the K3 yards. When calculating LSMs, baseline liveweight recorded at D0 was fitted as a covariate in statistical models.

4.1.5 Changes in Circulating Cytokine Concentrations

Circulating TNF-α, IL-1β and IL-6 cytokine concentrations were assessed in blood samples collected at the start of the trial (D-4), at the time of treatment with Amplimune or saline (T0) and at 6, 12, 24, 48, 72 and 96 hours post-treatment from all calves. Due to a lack in availability of required reagents to conduct ELISA assays, circulating IL-12 cytokine concentrations were assessed in a subset of blood samples (collected at D-4, T0 and 6, 12, 24 and 48 hours post-treatment in calves from the 2mLSC, 5mLSC and Sal5mLSC treatment groups). Therefore cytokine parameters analysed were:-

- Tumour Necrosis Factor-α (TNF-α)
- Interleukin 1-β (IL-1β)
- Interleukin 6 (IL-6)
- Interleukin 12 (IL-12) (subset of samples)

(Please note: Initially we proposed to assess changes in TNF-α & IL-1β cytokine concentrations in samples collected during the current trial; however, based on advice from NovaVive it was decided to also evaluate changes in IL-6 and IL-12 cytokine concentrations).

For analysis of cytokine concentrations, baseline concentrations for each cytokine observed either at the start of the trial (D-4) or at the time of treatment with Amplimune or saline (T0, post transportation) were fitted as covariates in statistical models. The significance of fixed effects when baseline cytokine concentrations observed at D-4 were fitted as covariates to models are presented

in Table 9 and treatment group LSMs (over time) for TNF-α (0-96hrs), TNF-α (48-96hrs), IL-1β, IL-6 and IL-12 are presented in Figures 29, 30, 31 and 32, respectively. The significance of fixed effects when baseline values observed at T0 were fitted to models are presented in Table 10 and treatment group LSMs (over time) for TNF-α (6-96hrs), TNF-α (48-96hrs), IL-1 β, IL-6 and IL-12 are presented in Figures 33, 34, 35, 36 and 37, respectively. As a significant treatment*time interaction was observed when analysing TNF-α concentration data (fitting baseline cytokine concentrations observed at either D-4 or T0 as covariates to models) and differences between groups were increasingly evident from 48-96hrs post treatment, repeated measures of TNF-α concentration at time points from T48 to T96 were analysed separately to measures of TNF-α concentration observed at all time points.

A significant overall treatment effect was observed when analysing TNF-α concentration at time points T48 to T96 (Table 9). Therefore specific linear contrasts were undertaken to compare LSMs for Amplimune treated versus saline (control) treated calves as detailed below:-

- 2mLSC versus Sal5mLSC
- 2mLIM versus Sal5mLIM
- 5mLSC versus Sal5mLSC
- 5mLIM versus Sal5mLIM

No significant differences were observed when specific group contrasts were undertaken (Figure 36).

Parameter	Transformation	Weight (D-4)	Sex	Time	Treatment	Baseline Covariate (D-4)	Treatment *Time	Treatment *Sex
TNF-α (0-96hrs)	Sqrt	NS	NS	NS	NS	***	**	NS
TNF-α (48-96hrs)	Sqrt	NS	NS	NS	*	***	NS	NS
IL-1β	Log	NS	NS	***	NS	***	NS	NS
IL-6	Log	NS	NS	NS	NS	***	NS	NS
IL-12	Log	NS	NS	NS	NS	***	NS	NS

Table 9: Significance of fixed effects (including baseline covariate recorded at D-4) when analysing cytokine concentrations (NS = non-significant P>0.05, * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

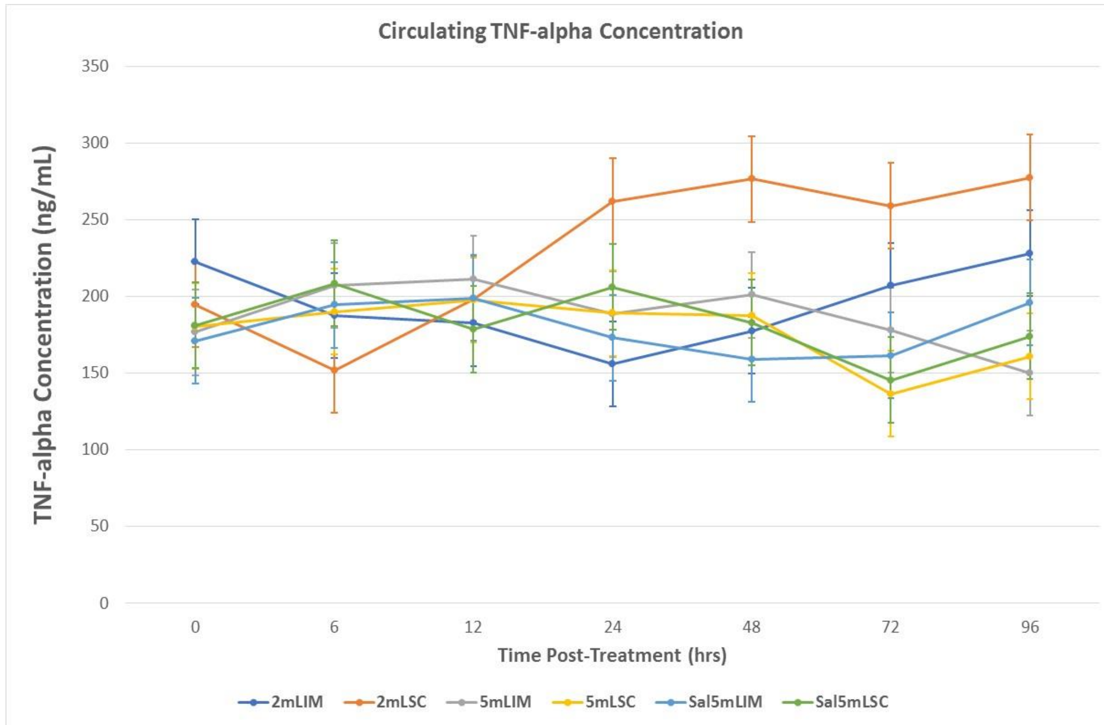

Figure 29. Treatment group LSMs (±SEM) for circulating TNF-α concentrations. When calculating LSMs, baseline liveweight recorded at D-4 was fitted as a covariate in statistical models.

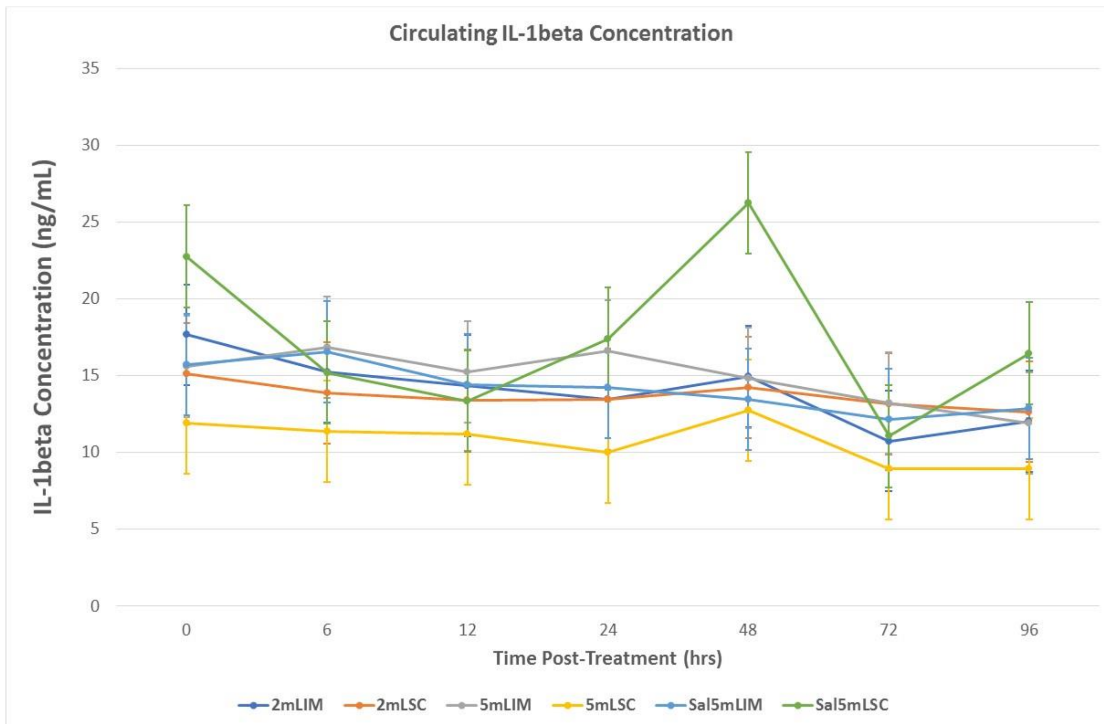

Figure 30. Treatment group LSMs (±SEM) for circulating IL-1β concentrations. When calculating LSMs, baseline liveweight recorded at D-4 was fitted as a covariate in statistical models.

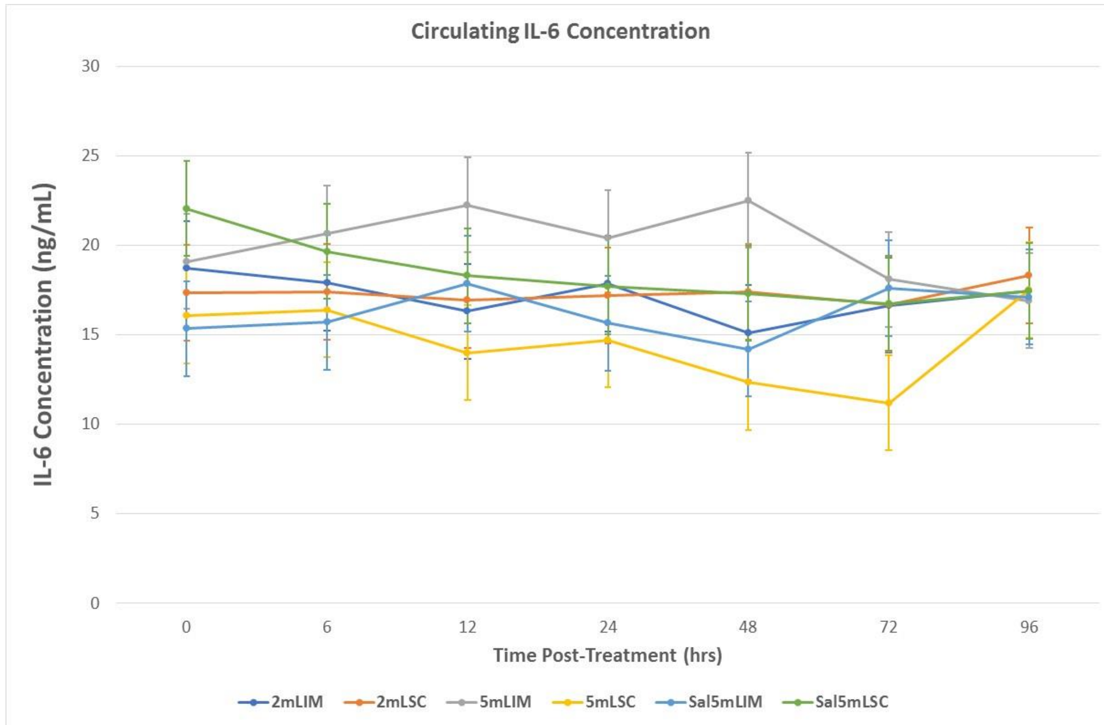

Figure 31. Treatment group LSMs (±SEM) for circulating IL-6 concentrations. When calculating LSMs, baseline liveweight recorded at D-4 was fitted as a covariate in statistical models.

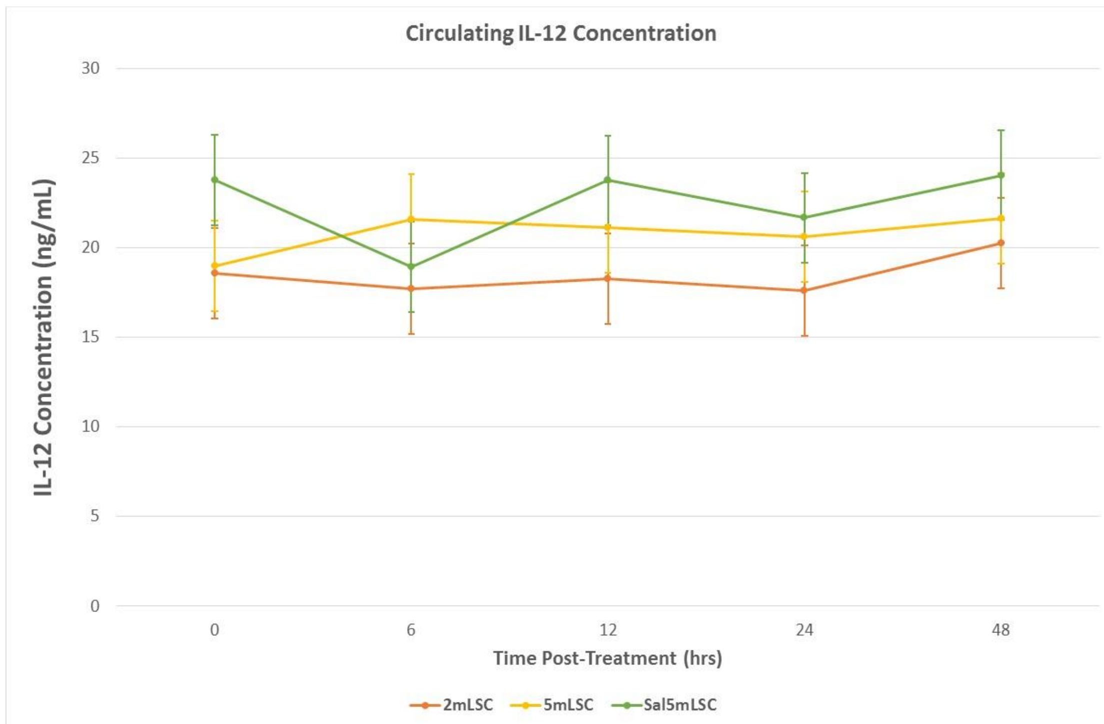

Figure 32. Treatment group LSMs (±SEM) for circulating IL-12 concentrations. When calculating LSMs, baseline liveweight recorded at D-4 was fitted as a covariate in statistical models.

Parameter	Transformation	Weight (D-4)	Sex	Time	Treatment	Baseline Covariate (D-4)	Treatment *Time	Treatment *Sex
TNF-α (6-96hrs)	Sqrt	NS	NS	NS	NS	***	**	NS
TNF-α (48-96hrs)	Sqrt	NS	NS	NS	NS	***	NS	NS
IL-1β	Log	NS	NS	***	NS	***	NS	NS
IL-6	Log	NS	NS	NS	NS	***	NS	NS
IL-12	Log	NS	NS	NS	NS	***	NS	NS

Table 10: Significance of fixed effects (including baseline covariate recorded at T0) when analysing cytokine concentrations (NS = non-significant P>0.05, * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

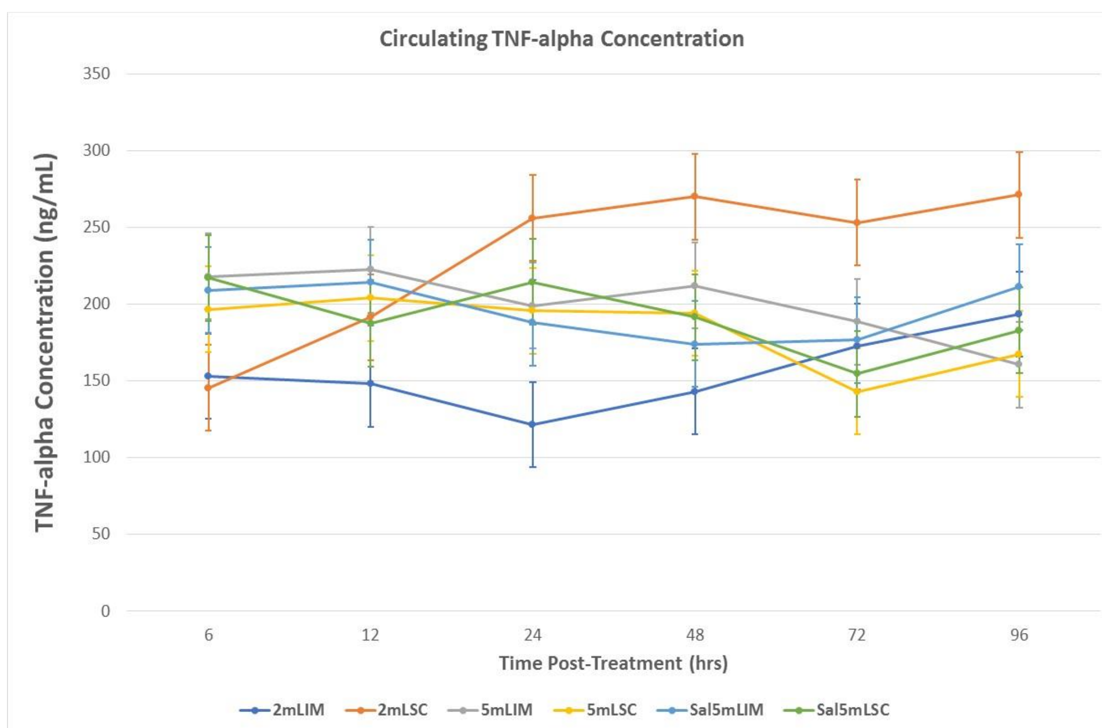

Figure 33. Treatment group LSMs (±SEM) for circulating TNF-α concentrations. When calculating LSMs, baseline liveweight recorded at T0 was fitted as a covariate in statistical models.

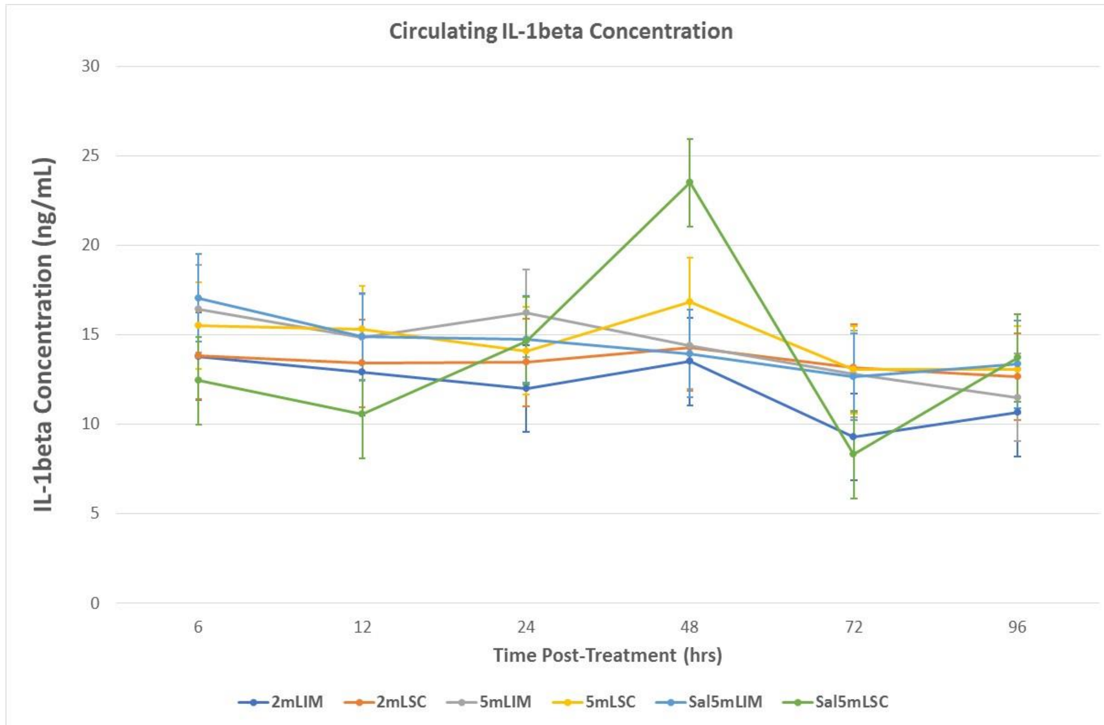

Figure 34. Treatment group LSMs (±SEM) for circulating IL-1β concentrations. When calculating LSMs, baseline liveweight recorded at T0 was fitted as a covariate in statistical models.

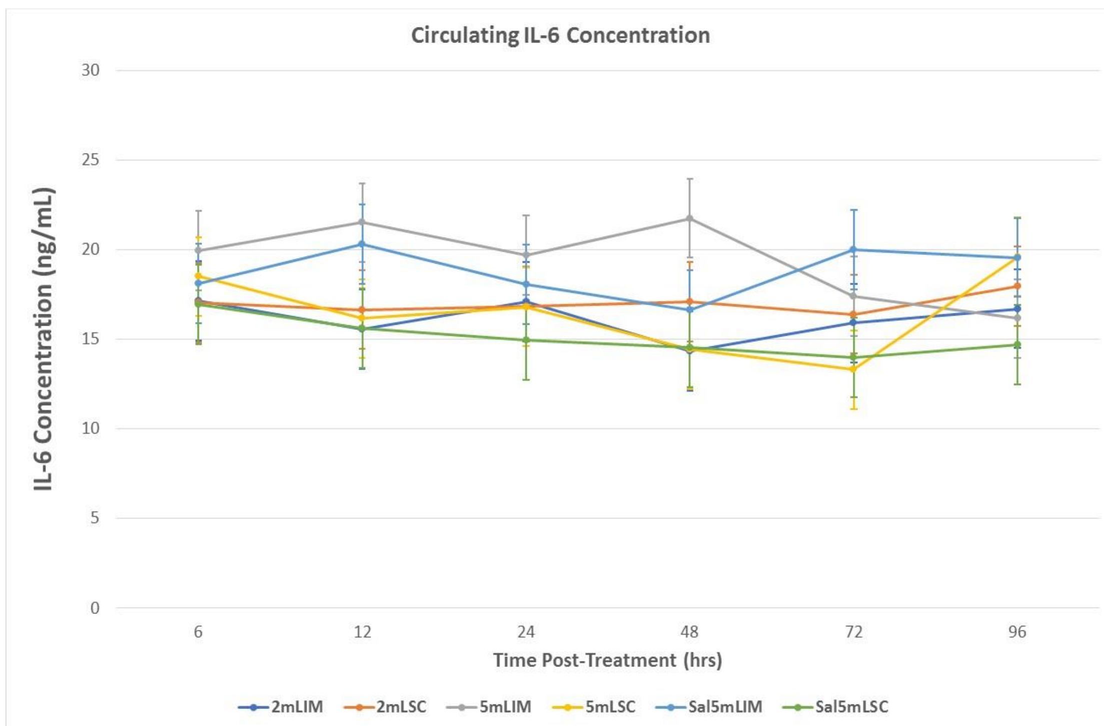

Figure 35. Treatment group LSMs (±SEM) for circulating IL-6 concentrations. When calculating LSMs, baseline liveweight recorded at T0 was fitted as a covariate in statistical models.

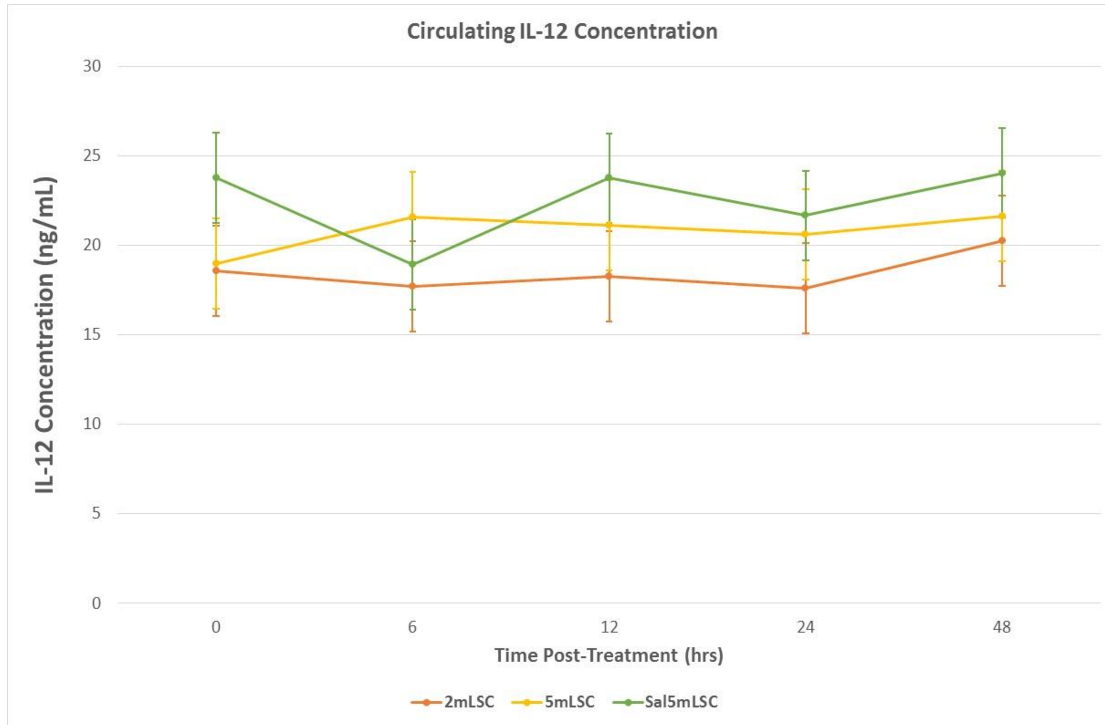

Figure 35. Treatment group LSMs (±SEM) for circulating IL-12 concentrations. When calculating LSMs, baseline liveweight recorded at T0 was fitted as a covariate in statistical models.

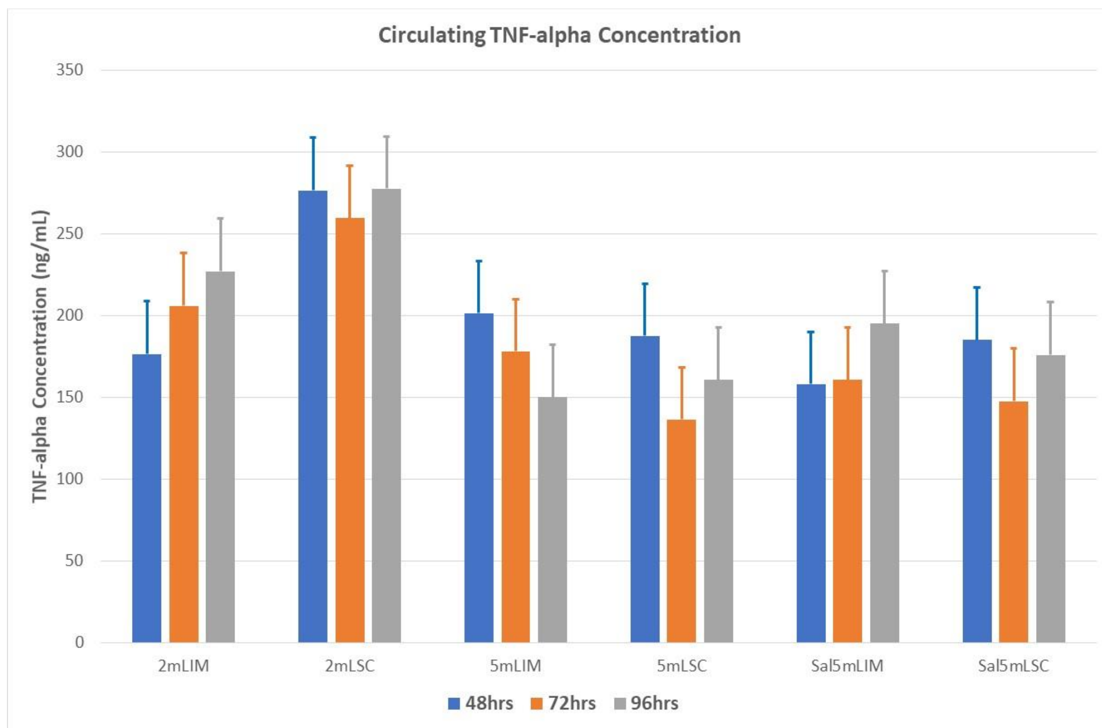

Figure 36. Treatment group LSMs (±SEM) for circulating TNF-α concentrations. Significance of specific linear contrasts within day (as described above).

4.2 Phase 1 – Part 2- Investigating the mechanism by which Amplimune stimulates the immune system of beef cattle

4.2.1 General Observations

All steers enrolled in the study were inspected by experienced personnel prior to Amplimune administration (Day 0) and again at the end of the trial period (Day 4). All steers were deemed to be ‘healthy’ and in ‘good’ condition on both Day 0 and again on Day 4 with all clinical measures assessed (included body condition, respiration rate and rectal temperature) within normal range for steers of their age. No adverse reactions to Amplimune administration were observed in any steers.

4.2.2 Haematology Parameters

Haematology parameters were assessed in blood samples collected at the time of treatment with Amplimune or saline (T0) and again at 3 (T3), 6 (T6), 12 (T12), 24 (T24), 48 (T48) and 96 (T96) hours post-treatment. Although numerous haematology parameters were assessed, specific parameters selected for analysis are listed below:-

- Total White Blood Cell count (WBC)
- Neutrophil count (NEUabs) and neutrophil % (NEU%)
- Lymphocyte count (LYMabs) and lymphocyte % (LYM%)
- Monocyte count (MONabs) and Monocyte % (MON%)
- Neutrophil:Lymphocyte ratio (NEU:LYM)

For analysis of haematology parameters, baseline values for each parameter observed either at the time of treatment with Amplimune or saline (T0) were fitted as covariates in statistical models. The significance of fixed effects are presented in Table 11. No significant effect of treatment was observed for any of the haematology parameters assessed. Treatment group LSMs (over time) for WBC/NEU:LYM, NEUabs/NEU%, LYMabs/LYM% and MONabs/MON% are presented in Figures 37, 38, 39 and 40, respectively.

Parameter	Transformation	Weight	Cohort	Time	Baseline Covariate (T0)	Treatment	Treatment *Time	Treatment *Cohort
WBC	Log	NS	NS	***	***	NS	NS	NS
NEUabs	Log	NS	**	***	***	NS	NS	NS
LYMabs	Log	NS	NS	***	***	NS	NS	NS
MONabs	Sqrt	NS	*	***	***	NS	NS	NS
NEU:LYM	Nil	NS	NS	*	***	NS	NS	NS
NEU%	Nil	NS	NS	***	**	NS	NS	NS
LYM%	Nil	NS	NS	***	***	NS	NS	NS
MON%	Nil	NS	NS	***	***	NS	NS	NS

Table 11: Significance of fixed effects (including baseline covariate recorded at D-4) when analysing haematology parameters (NS = non-significant P>0.05, * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

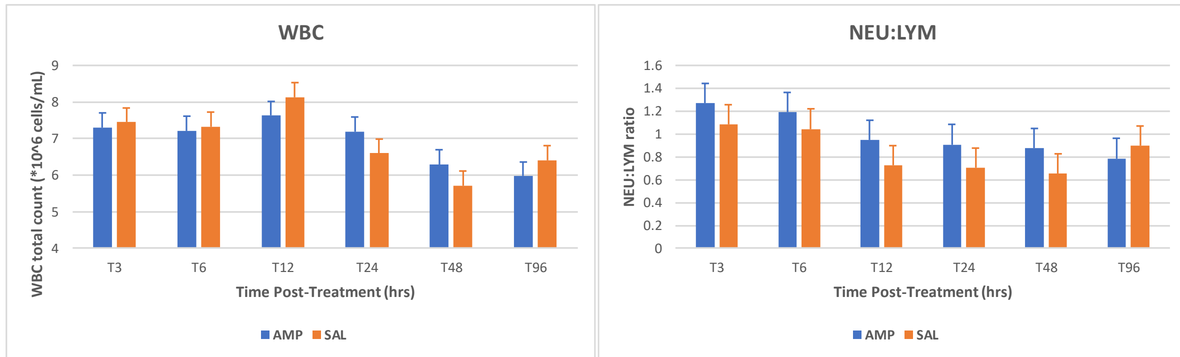

Figure 37. Treatment group LSMs (±SEM) for total White Blood Cell count (WBC) and Neutrophil:Lymphocyte ratio (NEU:LYM). When calculating LSMs, baseline values observed at T0 were fitted as a covariate in statistical models. No significant effect of treatment or treatment*time on WBC or NEU:LYM were observed.

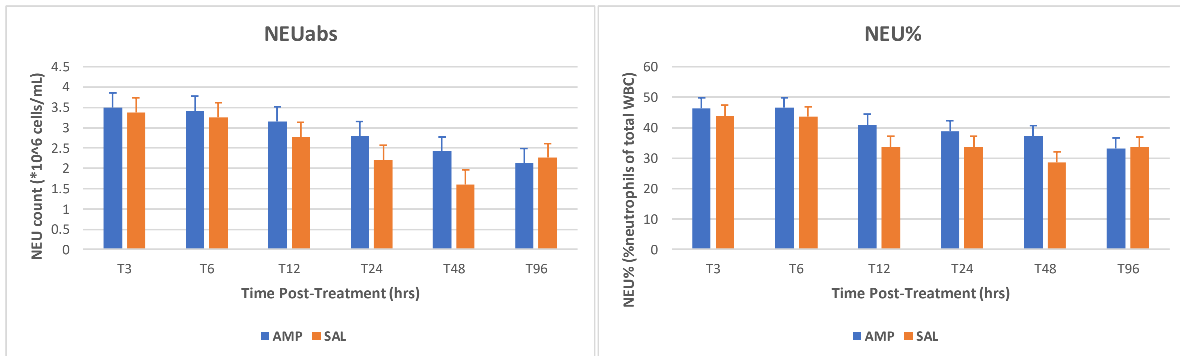

Figure 38. Treatment group LSMs (±SEM) for Neutrophil count (NEUabs) and for Neutrophil % (NEU%). When calculating LSMs, baseline values observed at T0 were fitted as a covariate in statistical models. No significant effect of treatment or treatment*time on NEUabs or NEU% were observed.

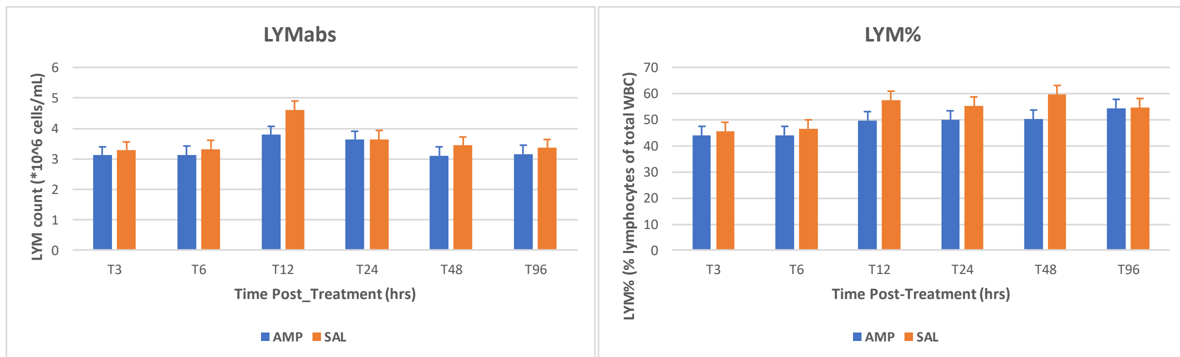

Figure 39. Treatment group LSMs (±SEM) for Lymphocyte count (LYMabs) and for Lymphocyte % (LYM%). When calculating LSMs, baseline values observed at D-4 were fitted as a covariate in statistical models. No significant effect of treatment or treatment*time on LYMabs or LYM% were observed.

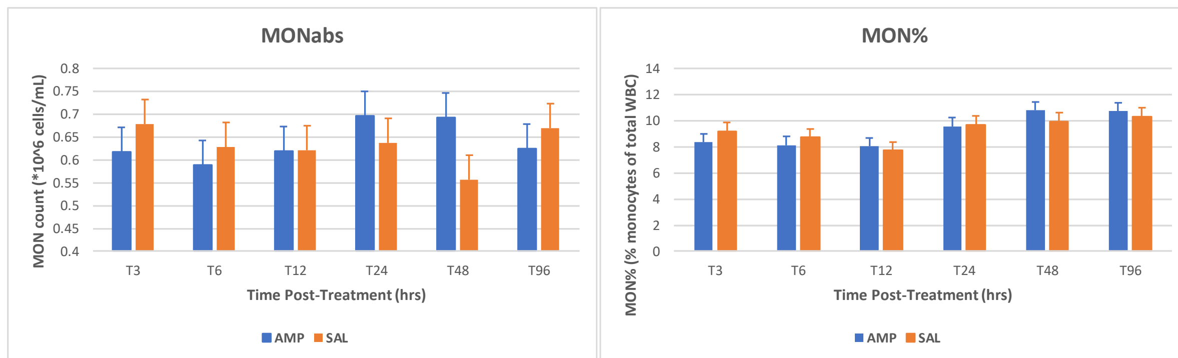

Figure 40. Treatment group LSMs (±SEM) for Monocyte count (MONabs) and for Monocyte % (MON%). When calculating LSMs, baseline values observed at D-4 were fitted as a covariate in statistical models. No significant effect of treatment or treatment*time on MONabs or MON% were observed.

4.2.3 Changes in Core Body Temperature

Core body temperature was continually assessed (in 10 minute increments) from the start of the trial (D-3) until 4 days post treatment with Amplimune or saline (Day 4) using i-button temperature loggers inserted into the rectum of steers. When analysing body temperature data it is important to consider both the variation which exists in the normal diurnal temperature profiles of individual animals (under non-experimental conditions) and the effects of ambient temperature. To identify the normal diurnal temperature profiles of individual steers, temperature measures logged on days when steers were grazing in a small paddock undisturbed (days -2 & -1) were assessed to generate baseline body temperature parameters which could be fitted to statistical models. Adjustments for ambient temperature on core body temperature measures were not made in the current study; however, steers were assigned a number and processed in a specific order in an attempt to minimise any differential effects of ambient temperature on steers in any given treatment group.

To analyse changes in core body temperature, logged temperature readings were grouped into blocks of data which represented 0-3hr, 0-6hr, 0-12hr, 0-24hr, 24-48hr, 48-72hr and 72-96hr time frames commencing at a time equivalent to when Amplimune or saline was administered. For example, if a steer received Amplimune at 7.40am on day 0 and we were investigating the 0-6hr time frame, temperatures logged from 7.40am on day 0 (0hrs post-treatment) to 1.40pm on day 0 (6hrs post-treatment) were grouped into a block. Similarly, when investigating the 48-72hr time frame, temperatures logged from 7.40am on day 2 (48hrs post-treatment) to 7.20am day 3 (72hrs post-treatment). For each block of time (representing a 3hr, 6hr, 12 hr or 24hr time window) the mean and maximum temperature observed for each steer was calculated and used in analysis. Baseline temperature measures (from an equivalent start and end time period of 3, 6, 12 or 24hrs), averaged across days -2 & -1, were fitted as covariates in statistical models in an attempt to adjust for diurnal effects on body temperature parameters. Accordingly the body temperature parameters analysed as repeated measures over time were as follows:-

Mean body temperature

- 0-3hr window (Mean 3hr)
- 3-6hr window (Mean 6hr)
- 6-12hr window (Mean 12hr)
- 12-24hr window (Mean 24hr)
- 24-48hr window (Mean 48hr)
- 48-72hr window (Mean 72hr)
- 72-96hr window (Mean 96hr)

Max body temperature

- 0-3hr window (Max 3hr)
- 3-6hr window (Max 6hr)
- 6-12hr window (Max 12hr)
- 12-24hr window (Max 24hr)
- 24-48hr window (Max 48hr)
- 48-72hr window (Max 72hr)
- 72-96hr window (Max 96hr)

The significance of fixed effects when baseline temperature measures (assessed across days -2 & -1) were fitted as a covariate to models are presented in Table 12. No significant treatment effects on Mean or Max body temperature were observed. Treatment group LSMs for Mean and Maximum body temperature over time are presented in Figures 41 and 42, respectively.

Parameter	Transformation	Weight	Cohort	Time	Baseline Covariate (T0)	Treatment	Treatment *Time	Treatment *Cohort
Mean Body Temperature	Log	NS	NS	NS	***	NS	NS	NS
Maximum Body Temperature	Nil	NS	NS	***	***	NS	NS	NS

Table 12: Significance of fixed effects (including baseline covariate recorded across D-2 and D-1) when analysing core body temperature parameters (NS = non-significant P>0.05, * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

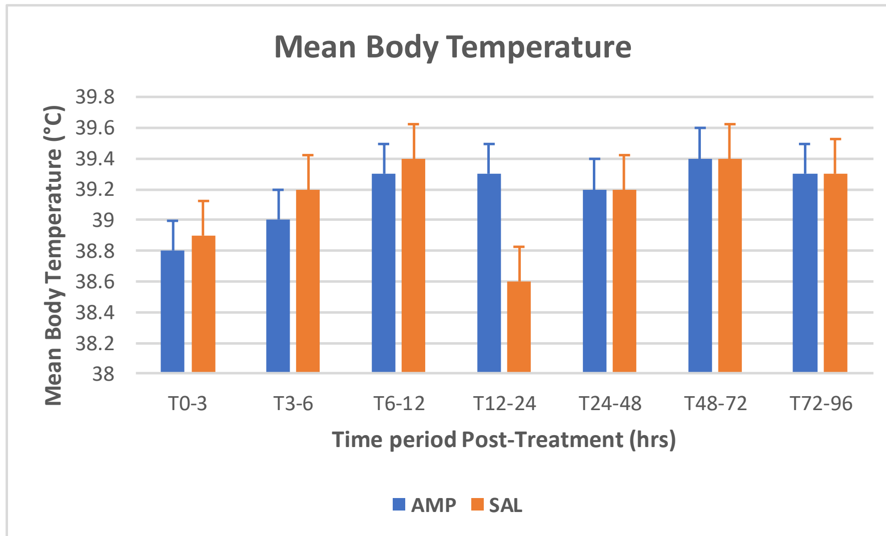

Figure 41. Treatment group LSMs (±SEM) for Mean Body Temperature. When calculating LSMs, baseline values observed across D-2 and D-1 were fitted as a covariate in statistical models. No significant effect of treatment or treatment*time on Mean Body Temperature were observed.

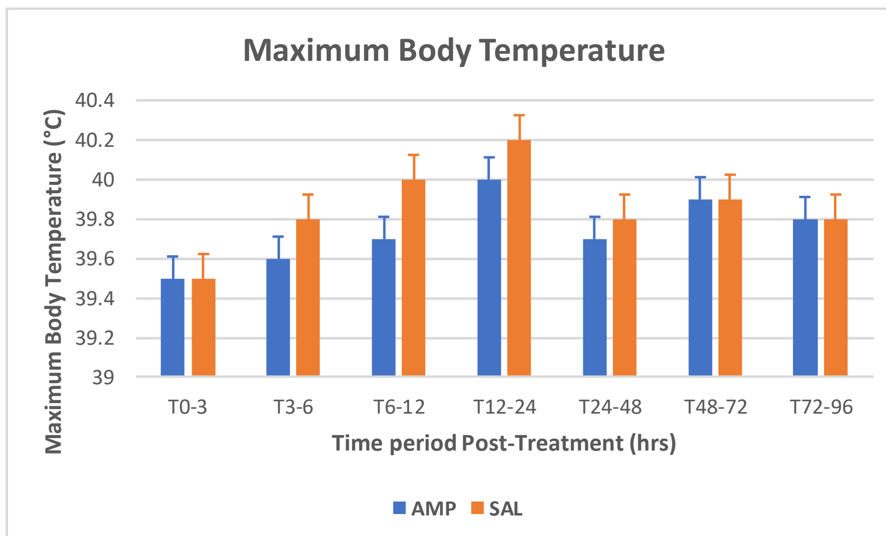

Figure 42. Treatment group LSMs (±SEM) for Maximum Body Temperature. When calculating LSMs, baseline values observed across D-2 and D-1 were fitted as a covariate in statistical models. No significant effect of treatment or treatment*time on Maximum Body Temperature were observed.

4.2.4 Circulating Cytokine Concentrations

Circulating TNF-α and IL-1β cytokine concentrations were assessed in serum from blood samples collected at the time of treatment with Amplimune or saline (T0) and at 3, 6, 12, 24, 48, 72 and 96hrs post-treatment from all steers. We had planned to assess concentrations of IL-6, IL-12 and IFN-γ in serum also; however, restrictions on staff movements as a result of the current COVID-19 situation have prevented us from completing these assays. Once restrictions on staff movements have been lifted, we will complete these assays as soon as practicable. Therefore cytokine parameters analysed to date and reported here only include:-

- Tumour Necrosis Factor-α (TNF-α)
- Interleukin 1-β (IL-1β)

For analysis of cytokine concentrations, baseline concentrations for each cytokine observed at the time of treatment with Amplimune or saline (T0) were fitted as covariates in statistical models. The significance of fixed effects when baseline cytokine concentrations observed at T0 were fitted as covariates to models are presented in Table 13. A significant overall treatment group effect on serum TNF-α concentrations was observed. Treatment group LSMs (overall) and treatment group LSMs (over time) for TNF-α are presented in Figures 43 and 44, respectively. A significant overall treatment group effect on serum IL-1β concentrations was also observed; however, this effect varied with cohort (as evidenced by the significant treatment*cohort interaction). Treatment group LSMs (across cohorts) and treatment group LSMs (over time) for IL-1β are presented in Figures 45 and 46, respectively

Parameter	Transformation	Weight	Cohort	Time	Baseline Covariate (T0)	Treatment	Treatment *Time	Treatment *Cohort
TNF-α	Cube root	NS	*	NS	***	*	NS	NS
IL-1β	Square root	*	*	NS	***	*	NS	**

Table 13: Significance of fixed effects (including baseline covariate recorded at T0) when analysing serum cytokine concentrations (NS = non-significant P>0.05, * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

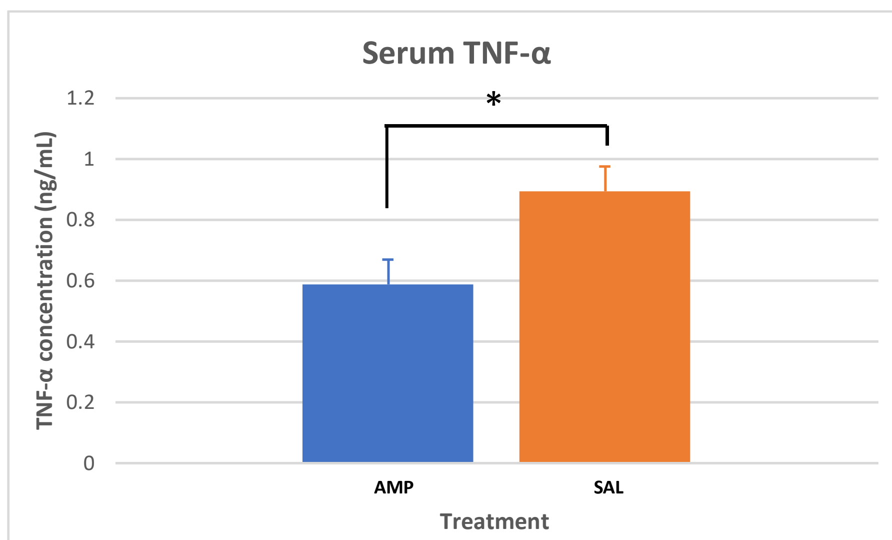

Figure 43. Treatment group LSMs (±SEM) for serum TNF-α concentrations. When calculating LSMs, baseline serum cytokine concentrations recorded at T0 were fitted as a covariate in statistical models. A significant overall treatment effect on serum TNF-α concentrations was observed (* = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

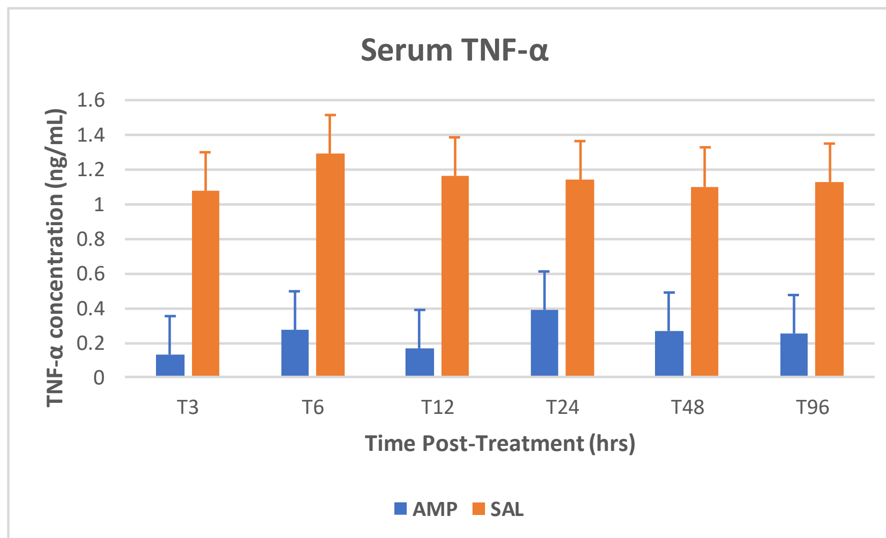

Figure 44. Treatment group LSMs (±SEM) for serum TNF-α concentrations. When calculating LSMs, baseline serum cytokine concentrations recorded at T0 were fitted as a covariate in statistical models. A significant effect of treatment on serum TNF-α concentration was observed which did not vary over time.

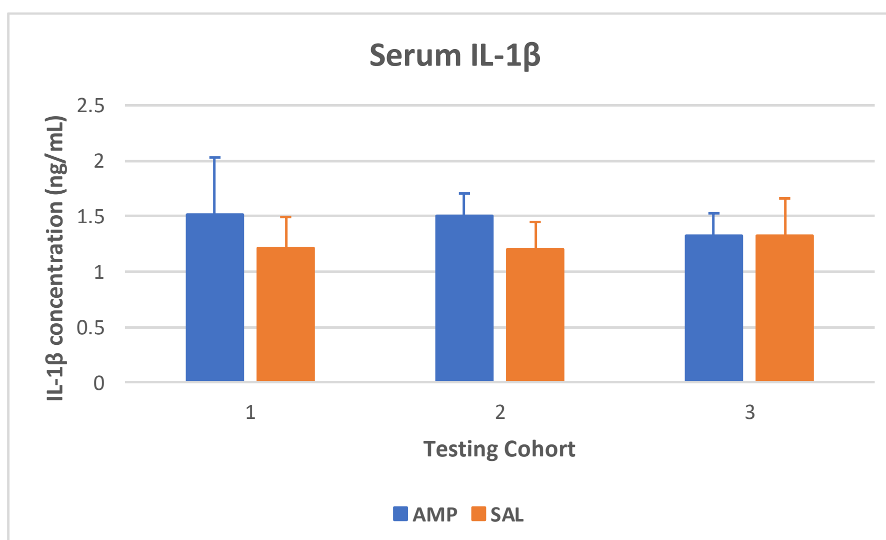

Figure 45. Treatment group LSMs (±SEM) for serum IL-1β concentrations. When calculating LSMs, baseline serum cytokine concentrations recorded at T0 were fitted as a covariate in statistical models.

A significant effect of treatment on serum IL-1β concentration was observed, however, this effect varied across cohorts. Specific contrasts of treatment (AMP vs SAL) within each cohort showed no significant effect of treatment on serum IL-1β concentration within cohort.

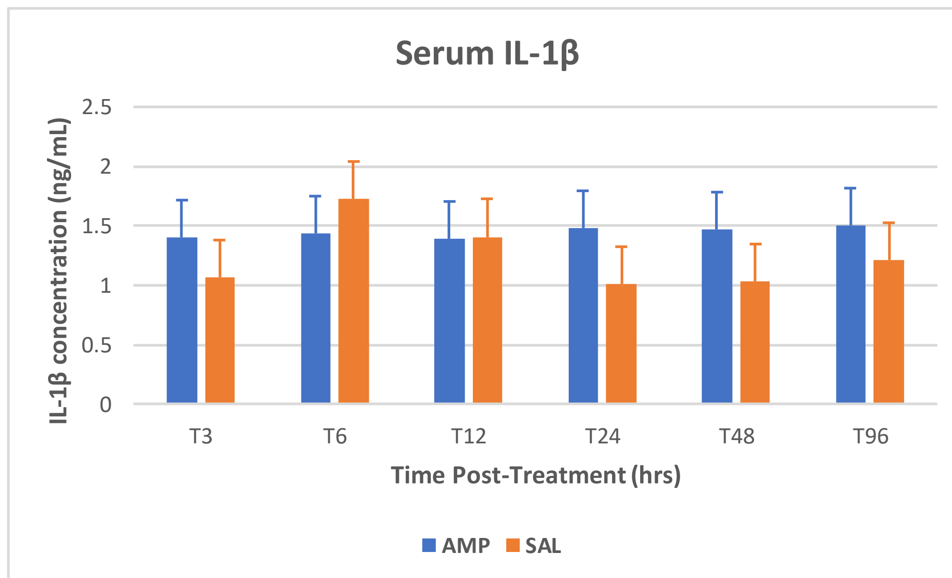

Figure 46. Treatment group LSMs (±SEM) for serum IL-1β concentrations. When calculating LSMs, baseline serum cytokine concentrations recorded at T0 were fitted as a covariate in statistical models. A significant effect of treatment on serum IL-1β concentration was observed which did not vary over time.

4.2.5 Cell Culture Supernatant Cytokine Concentrations

Concentrations of TNF-α and IL-1β were assessed in cell culture supernatants following stimulation of cells collected at the time of treatment with Amplimune or saline (T0) and at 24 and 96hrs post-treatment. Cells were stimulated in culture with:-

- CTRL - media only (unstimulated control)
- BHV-1 - inactivated bovine herpesvirus-1 virus (MOI = 0.01)
- AMP - Amplimune (4µL/mL, NovaVive)
- AMP+BHV-1 - Amplimune + inactivated BHV-1 virus (MOI = 0.01) or
- ConA - Concanavilin A (5µg/mL, Sigma, USA) (positive control).

However, restrictions on staff movements as a result of the current COVID-19 situation have prevented us from completing assays to assess the concentrations of TNF-α and IL-1β in culture supernatants stimulated with AMP and AMP+BHV-1. We had planned to assess concentrations of IL-6, IL-12 and IFN-γ in culture supernatants also; however, restrictions on staff movements as a result of the current COVID-19 situation have prevented us from completing these assays as well. Once restrictions on staff movements have been lifted, we will complete these assays as soon as practicable. Therefore cytokine parameters analysed to date and reported here only include:-

- Tumour Necrosis Factor-α (TNF-α) (CTRL, BHV-1 & ConA only)
- Interleukin 1-β (IL-1β) (CTRL, BHV-1 & ConA only)

The significance of fixed effects when fitted to models used to analyse supernatant cytokine concentration data are presented in Table 14. No significant overall treatment group effect on serum

TNF-α or IL-1β concentrations in culture supernatants following stimulation of cells were observed, however the effect of treatment did vary across cohorts for TNF-α. A significant effect of culture treatment (CTRL, BHV-1, ConA) on supernatant TNF-α concentration (but not IL-1β concentrations) was observed, however the effect varied across cohorts and with time. Treatment group LSMs (across cohorts) and treatment group LSMs (over time) for TNF-α are presented in Figures 47 and 48, respectively. Culture treatment group LSMs (over time) for TNF-α are presented in Figure 49. Treatment group LSMs (over time) for IL-1β are presented in Figures 50. Culture treatment group LSMs (over time) for IL-1β are presented in Figure 51.

Parameter	Transformation	Cohort	Time	Treatment	Culture Treatment	Treatment *Time	Treatment *Cohort	Culture Treatment *Time	Culture Treatment *Cohort
TNF-α	Nil	*	***	NS	***	NS	***	**	*
IL-1β	Square root	*	NS	NS	NS	NS	NS	NS	*

Table 14: Significance of fixed effects when analysing cytokine concentrations in cell culture supernatants (NS = non-significant P>0.05, * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

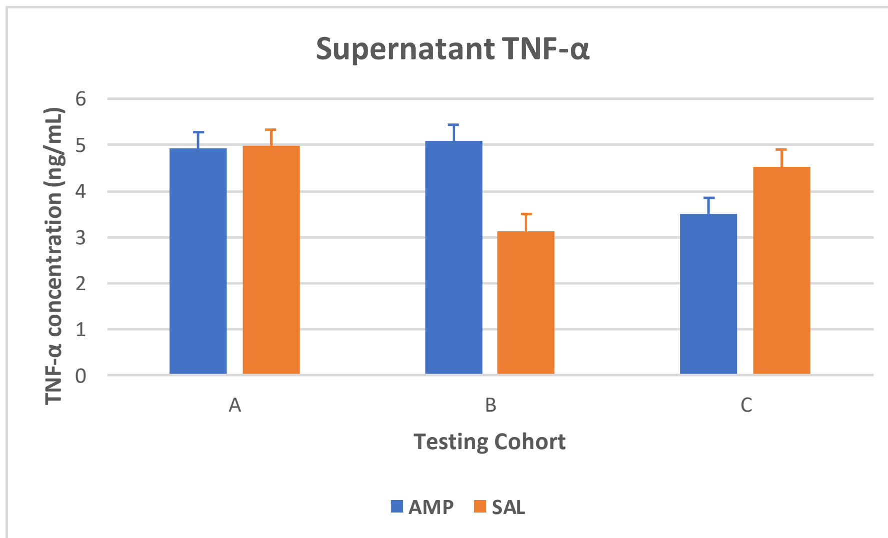

Figure 47. Treatment group LSMs (±SEM) for supernatant TNF-α concentrations. The effect of treatment on serum IL-1β concentration varied across cohorts. Specific contrasts of treatment (AMP vs SAL) within each cohort showed no significant effect of treatment on cytokine TNF-α concentration within cohort.

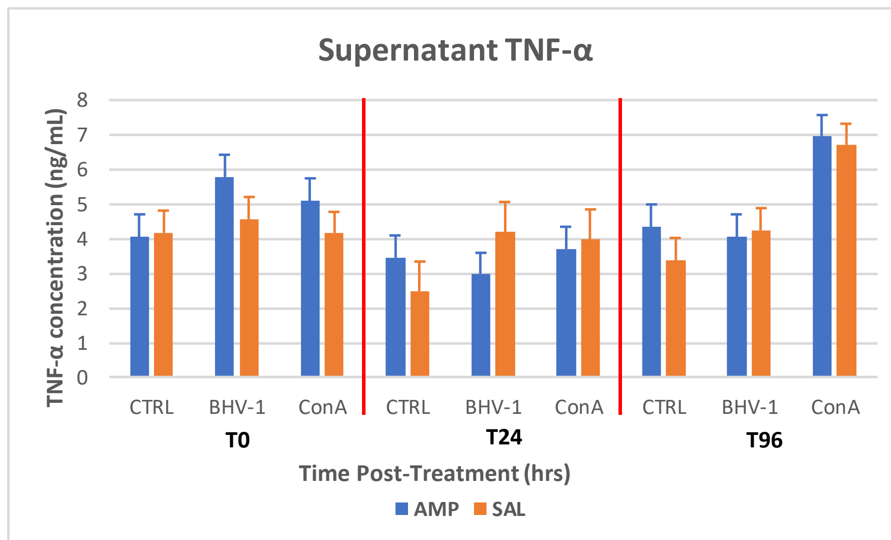

Figure 48. Treatment group LSMs (±SEM) for supernatant TNF-α concentrations. No significant effect of treatment or treatment*time on supernatant TNF-α concentrations were observed.

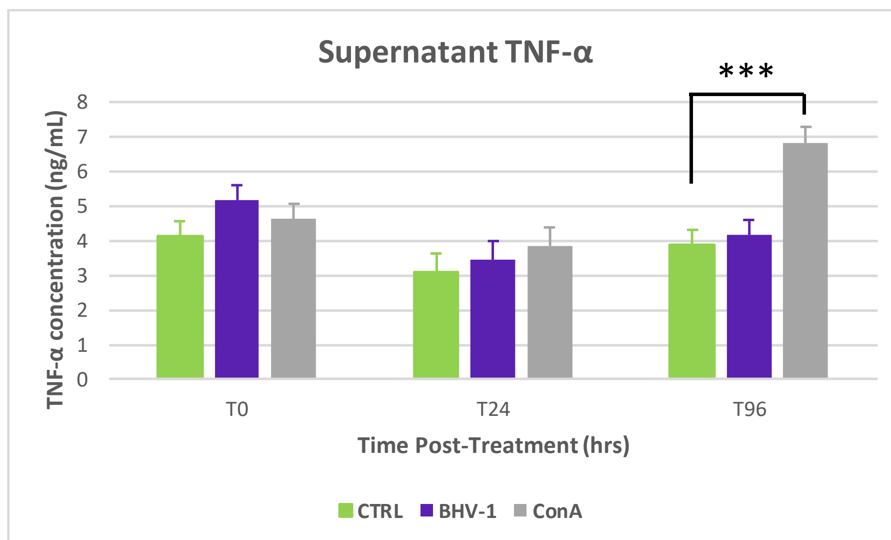

Figure 49. Culture treatment LSMs (±SEM) for supernatant TNF-α concentrations. A significant effect of culture treatment on supernatant TNF-α concentrations was observed, however, this effect varied across cohorts and time.

(* = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001)

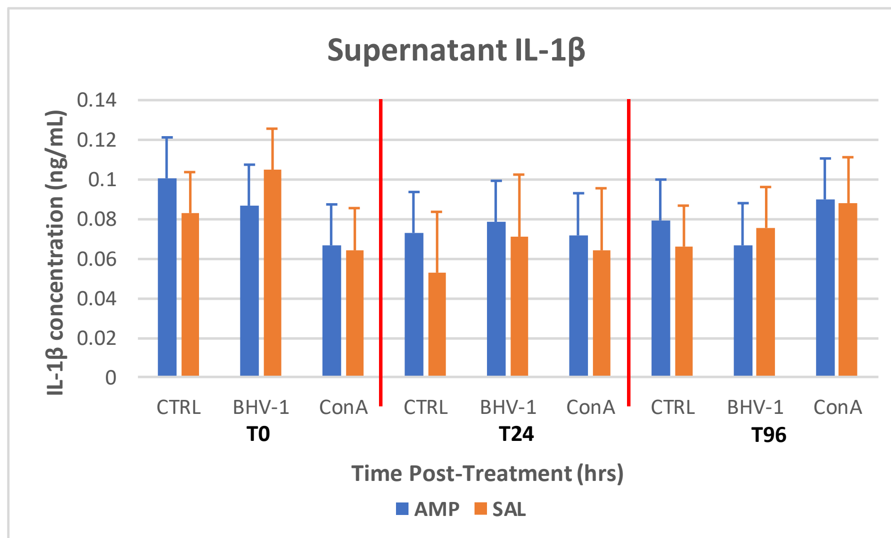

Figure 50. Treatment group LSMs (±SEM) for supernatant IL-1β concentrations. No significant effect of treatment or treatment*time on supernatant TNF-α concentrations were observed.

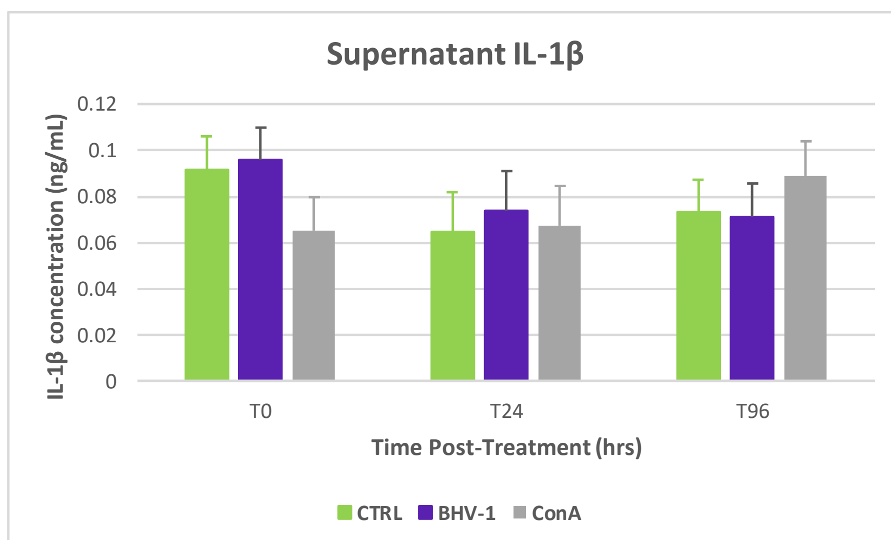

Figure 51. Culture treatment LSMs (±SEM) for supernatant IL-1β concentrations. No significant effect of culture treatment on supernatant IL-1β concentrations was observed.

4.2.6 Cell Population Analysis using Flow Cytometry

The ability of treatments (culture treatments) to stimulate intracellular expression of IFN-γ in CD4 and CD8 T-lymphocyte cell populations in PBMCs collected and cultured invitro at the time of treatment with Amplimune or saline (T0) and at 24 and 96hrs post-treatment. Cells were stimulated in culture with:-

- CTRL - media only (unstimulated control)
- BHV-1 - inactivated bovine herpesvirus-1 virus (MOI = 0.01)
- AMP - Amplimune (4µL/mL, NovaVive)
- AMP+BHV-1 - Amplimune + inactivated BHV-1 virus (MOI = 0.01) or

- ConA - Concanavilin A (5µg/mL, Sigma, USA) (positive control).

Due to technical issues with the flow cytometer requiring service agent, analysis of cell populations from only a single cohort of animals (cohort C) could be assessed. Therefore cell population parameters reported here include:-

- % CD4+/IFN-γ+ cells (cohort C)
- % CD8+/IFN-γ+ cells (cohort C)

The significance of fixed effects when fitted to models used to analyse cell population data are presented in Table 15. No significant overall treatment group effect on the proportion of CD4+/IFN-γ+ and CD8+/ IFN-γ+ cells in culture following stimulation were observed. A significant effect of culture treatment (CTRL, BHV-1, AMP, AMP+BHV-1, ConA) on proportion of both CD4+/IFN-γ+ and CD8+/IFN-γ+ cells was observed, however the effect varied with time. Treatment group LSMs (across culture treatments) and treatment group LSMs (over time) for CD4 cells are presented in Figures 52 and 53, respectively. Culture treatment group LSMs (over time) for CD4 cells are presented in Figure 54. Treatment group LSMs (across culture treatments) and treatment group LSMs (over time) for CD8 cells are presented in Figures 55 and 56, respectively. Culture treatment group LSMs (over time) for CD8 cells are presented in Figure 21.

Parameter	Transformation	Time	Treatment	Culture Treatment	Treatment* Time	Culture Treatment *Time
CD4+/IFN-γ+	Nil	***	NS	***	NS	***
CD8+/IFN-γ+	Nil	***	NS	***	NS	**

Table 15: Significance of fixed effects when analysing cultured cell populations (NS = non-significant P>0.05, * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

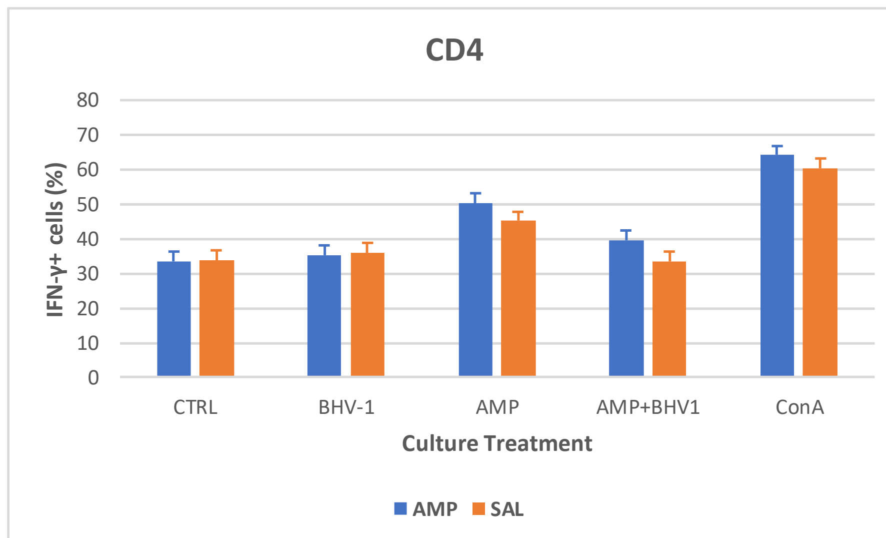

Figure 52. Treatment group LSMs (±SEM) for proportion of CD4+/IFN-γ+ cells in culture following stimulation. No significant effects of treatment on proportion of CD4+/IFN-γ+ cells were observed

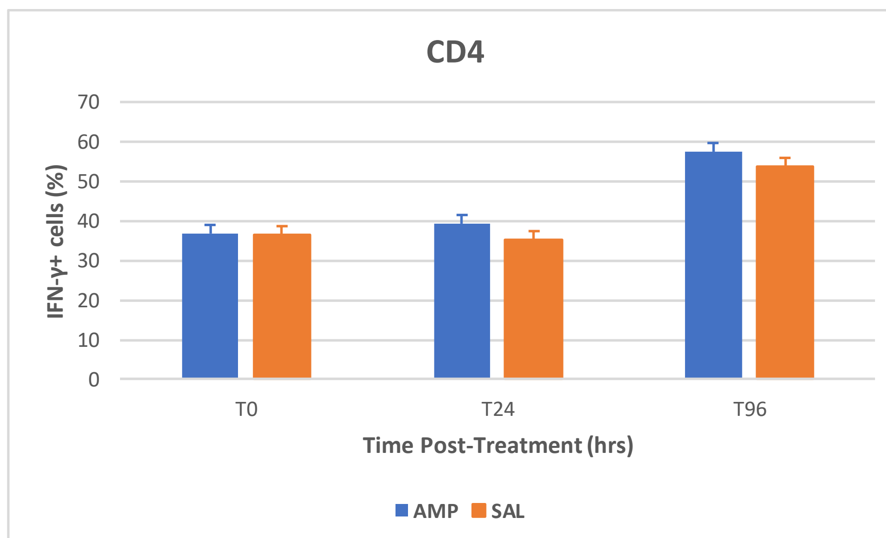

Figure 53. Treatment group LSMs (±SEM) for proportion of CD4+/IFN-γ+ cells in culture following stimulation. No significant effect of treatment or treatment*time on proportion of CD4+/IFN-γ+ cells were observed.

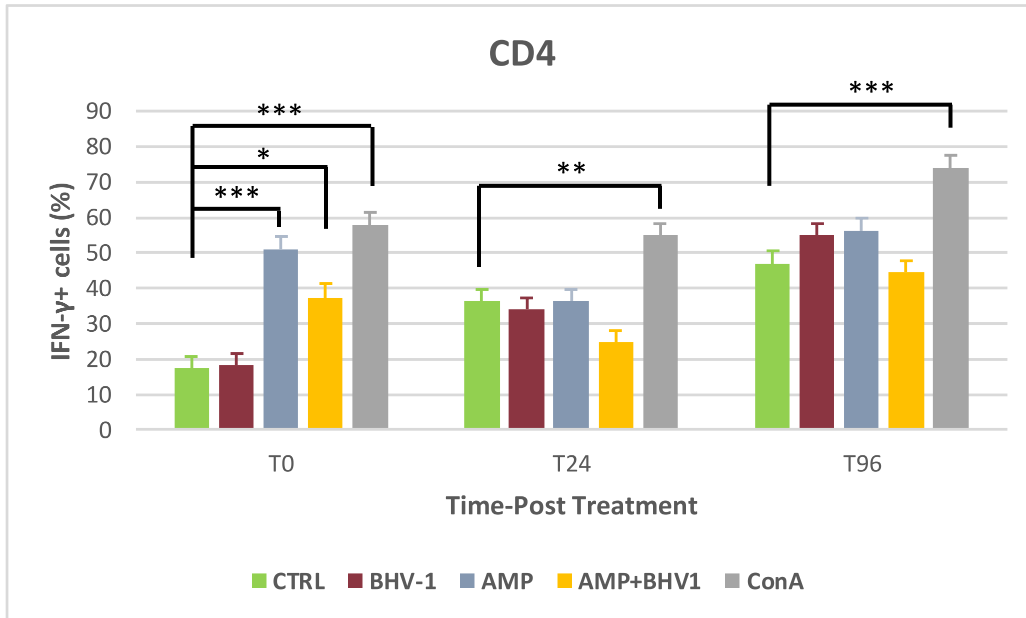

Figure 54. Culture treatment LSMs (±SEM) for proportion of CD4+/IFN-γ+ cells in culture following stimulation. A significant effect of culture treatment on the proportion of CD4+/IFN-γ+ cells was observed, however, this effect varied across time.

(* = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001)

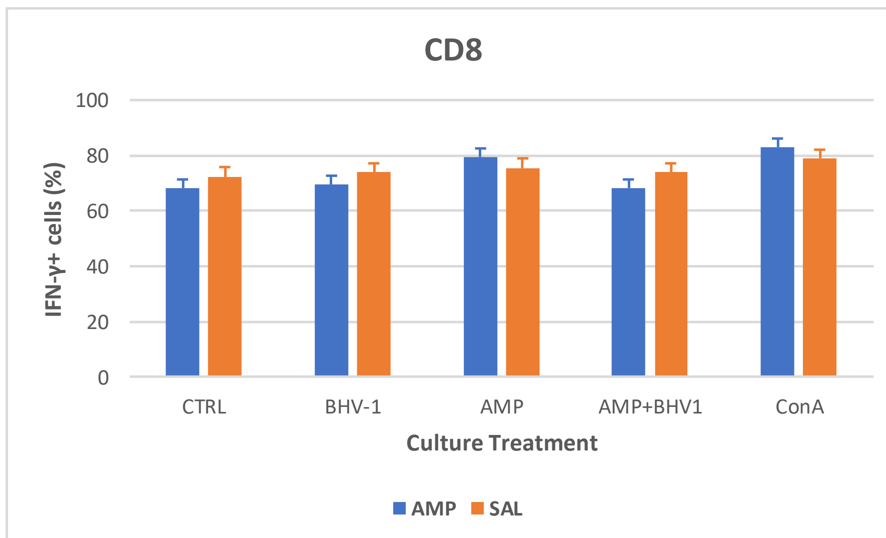

Figure 55. Treatment group LSMs (±SEM) for proportion of CD8+/IFN-γ+ cells in culture following stimulation. No significant effects of treatment on proportion of CD8+/IFN-γ+ cells were observed

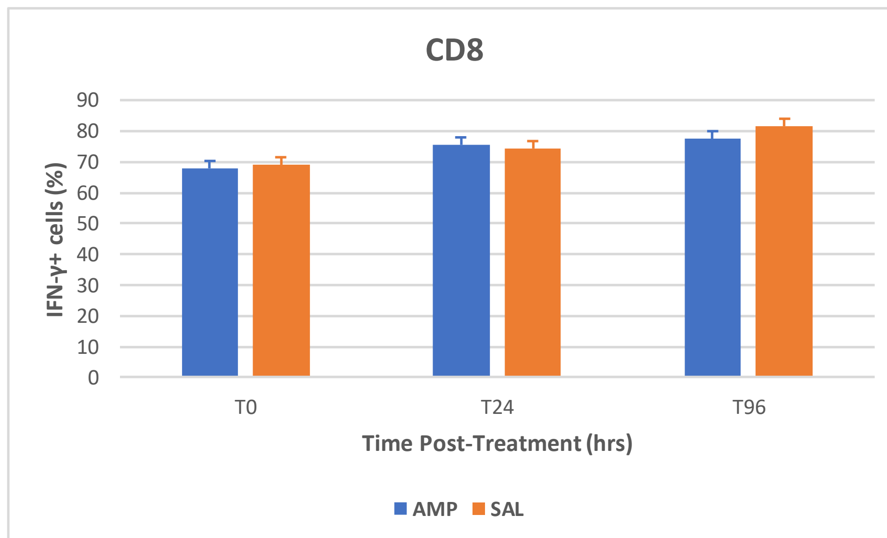

Figure 56. Treatment group LSMs (±SEM) for proportion of CD8+/IFN-γ+ cells in culture following stimulation. No significant effect of treatment or treatment*time on proportion of CD8+/IFN-γ+ cells were observed.

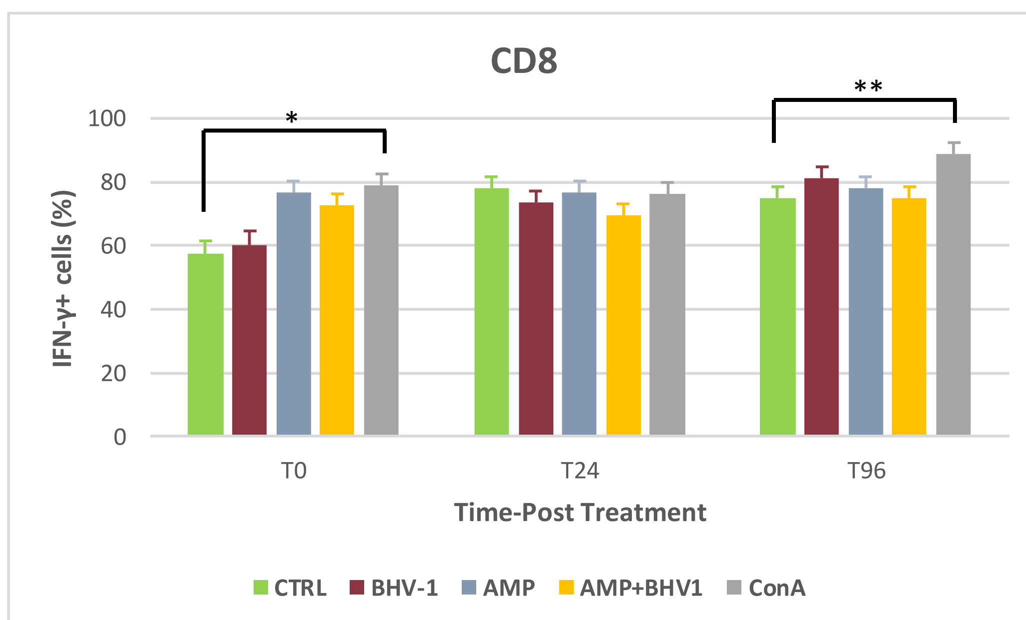

Figure 57. Culture treatment LSMs (±SEM) for proportion of CD8+/IFN-γ+ cells in culture following stimulation. A significant effect of culture treatment on the proportion of CD8+/IFN-γ+ cells was observed, however, this effect varied across time.

(* = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001)

4.2.7 Histology

Numbers of mononuclear (lymphocytes/monocytes), eosinophils and neutrophils cells in skin biopsies taken at the site of injection with Amplimune or Saline (or equivalent control site on opposite side of the neck) at 24hrs post-treatment were enumerated. Therefore histological parameters analysed and reported here include:-

- Mononuclear cell numbers at injection (and control) sites at 24hrs post-treatment
- Eosinophil cell numbers at injection (and control) sites at 24hrs post-treatment
- Neutrophil cell numbers at injection (and control) sites at 24hrs post-treatment

For analysis of cell populations in skin biopsy samples taken at injection sites 24hrs post-treatment, numbers of cells at control sites (equivalent site on opposite side of neck which was not injected) at 24hrs post-treatment baseline concentrations for each cytokine observed at the time of treatment with Amplimune or saline (T0) were fitted as covariates in statistical models. The significance of fixed effects when cell numbers at control sites were fitted as covariates to models are presented in Table 16. A significant treatment group effect on numbers of neutrophils at injection sites 24hrs post-treatment was observed. No significant effects of treatment were observed for mononuclear and eosinophil cell numbers at injection sites 24hrs post-treatment. Treatment group LSMs for mononuclear, eosinophil and neutrophil cell numbers at injection sites are presented in Figure 58.

Parameter	Transformation	Control Site Cell Numbers	Treatment
Mononuclear Cells	Nil	***	NS
Eosinophils	Nil	***	NS
Neutrophils	Nil	***	*

Table 16: Significance of fixed effects (including control site cell numbers as a covariate) when analysing cell numbers in skin biopsies taken at injection sites at 24hrs post-treatment. (NS = non-significant P>0.05, * = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

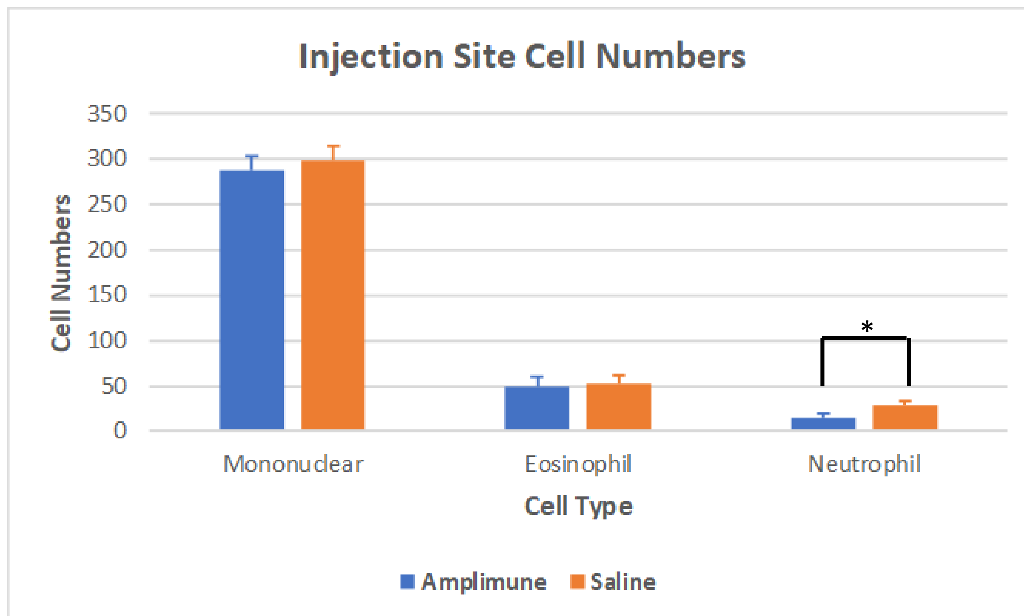

Figure 58. Treatment group LSMs (±SEM) for cell numbers in skin biopsies taken at injection sites at 24hrs post-treatment. A significant effect of treatment on numbers of neutrophil cells was observed, however, no significant effects of treatment on numbers of mononuclear or eosinophil cells were observed (* = P≤0.05>0.01, ** = P≤0.01>0.001, *** = P≤0.001).

4.2.8 Cytokine Gene Expression

Real time PCR (RT-PCR) assays were planned to quantify the expression levels of four pro-inflammatory cytokine genes, TNF-α, IL6, INF-γ and IL-1β in a) circulating WBCs at various times post-treatment (0, 3, 6, 12, 24, 48 & 96hrs), b) in skin biopsy samples taken from control and test sites at 24hrs post-treatment and c) cultured PBMCs stimulated with various culture treatments (CTRL, BHV-1, AMP, AMP+BHV-1 and ConA). However, restrictions on staff movements as a result of the current COVID-19 situation have prevented us from completing these assays. To date cDNA has been prepared from all samples but RT-PCR assays are yet to be run. Once restrictions on staff movements have been lifted, we will complete these assays as soon as practicable.

5. Conclusion and recommendations

5.1 Phase 1 – Part 1- Evaluating safe dose rates and the preferred route of administration of the Amplimune product

5.1.1 Summary of Key Findings

The overall aim of this work is to assess the potential for the immunostimulant, Amplimune to a) provide short term protection for unvaccinated feeder cattle against disease (including BRD) during the high risk induction period and b) enhance responses to vaccination (including against BRD) when administered at induction to improve long term protection against disease. The first phase in the process of evaluating Amplimune for the above applications is to identify safe and effective doses of the product. In the current study, different doses of Amplimune (2mL vs 5mL) were administered via different routes (subcutaneous vs intramuscular) and physiological responses induced by the treatment compared to those induced by saline (control). For Amplimune to be deemed 'safe' it is important that no adverse reactions to administration of the product are observed. For Amplimune to be deemed 'effective' the product should stimulate the immune system to a level which increases the ability of the system to fight off disease challenges while 'not' overstimulating the immune system to a level that induces severe fever or excessive inflammatory responses, both of which would be detrimental to the health, welfare and performance of the animal. Results from the current study suggest that Amplimune administered either subcutaneously or intramuscularly at a dose rate of 2mL or 5mL is 'safe' to use in calves of approximately 220kg liveweight. Furthermore, administration of Amplimune did not result in inappetence (assessed indirectly by measuring liveweight change over the trial period) or changes in hydration levels (assessed indirectly by measuring HCT). Results demonstrated that when Amplimune was administered subcutaneously at a dose of 2mL the product induced physiological responses indicative of innate immune system activation without causing severe fever or excessive inflammation. In summary:-

- Haematology Parameters - No significant differences in WBC, NEU, LYM and NEU:LYM were observed between treatment groups suggesting that administration of Amplimune did not result in systemic changes in white blood cell parameters. It is expected that administration of Amplimune results in significant changes in immune cell populations locally at the site of injection but localised changes in cell populations were not assessed in the current study. Hydration levels have a major impact on HCT parameter values. No significant differences in HCT were observed between treatment groups suggesting that administration of Amplimune did not impact on water intake of calves.

- Core Body Temperature – Significant differences in Mean6hr, Max6hr, Max12hr, Mean24hr and Max24hr were observed between treatment groups. However, treatment group effects were influenced by the sex of the animal. Specific linear contrasts were undertaken to compare LSMs for Amplimune treated versus relevant saline (control) treated steers and heifers (independently) at each individual timepoints (Day 0, Day 1 & Day 2). Contrasts demonstrated that Mean24hr and Max24hr parameters were higher in 2mLSC treatment group steers as compared to Saline5mLSC (control) treatment group calves following administration of treatments (Day 0). Mean6hr, Mean12hr and Max24hr parameters were also higher in 2mLSC treatment group steers as compared to Saline5mLSC (control) treatment group calves 2 days post-treatment (Day 2).

- Liveweight - Significant differences in liveweight change between treatment groups were observed when assessed at the end of the trial period (Day 4) at the Hway yards; however, no significant differences in liveweight change between treatment groups were observed when assessed during the trial (Day 0, Day 2 & Day 4) at the K3 yards. Specific linear contrasts were undertaken to compare liveweight LSMs for Amplimune treated versus relevant saline (control) treated calves at Day 4 (assessed at Hway yards). No significant differences were observed when specific group contrasts were undertaken, suggesting that administration of Amplimune did not adversely impact on weight gain during the trial period and did not cause inappetence.

- Cytokine concentrations - No significant differences in the concentration of the cytokines IL-1β, IL-6 and IL-12 were observed between treatment groups suggesting that administration of Amplimune did not result in systemic changes in the concentration of these pro-inflammatory cytokines. It is noteworthy that IL-12 concentrations were only compared in a subset of treatment groups (2mLSC, 5mlSC and Sal5mLSC). In contrast, a significant treatment*time interaction was observed when analysing TNF-α concentration changes across the entire trial period and a significant treatment effect observed when TNF-α concentrations at time points from 48hrs to 96hrs were analysed. Although no significant differences were observed when specific linear contrasts were undertaken to compare TNF-α concentration LSMs for Amplimune treated versus relevant saline (control) treated calves at 48hrs, 72hrs & 96hrs post-treatment, a trend was evident in the data suggesting that the concentration of TNF-α was higher in calves in the 2mLSC group as compared to the Sal5mLSC control group with p-values for comparisons within day approaching significance. While increases in the pro-inflammatory cytokine TNF-α observed in response to administration of Amplimune in the current study are indicative of innate immune system activation, such increases were not expected to be of sufficient magnitude to have caused 'excessive' inflammation which would be detrimental to the animal.

5.2 Phase 1 – Part 2- Investigating the mechanism by which Amplimune stimulates the immune system of beef cattle

5.2.1 Summary of Key Findings

The overall aim of this work is to assess the potential for the immunostimulant, Amplimune to a) provide short term protection for unvaccinated feeder cattle against disease (including BRD) during the high risk induction period and b) enhance responses to vaccination (including against BRD) when administered at induction to improve long term protection against disease. The first phase in the process of evaluating Amplimune for the above applications was to identify safe and effective doses of the product. Trial work conducted during this initial phase demonstrated that Amplimune can be safely administered to young cattle either subcutaneously or intramuscularly at a dose rate ranging from 1mL to 2.5mL/100 kg liveweight. Further, treatment with Amplimune did not result in appetence or dehydration in treated calves. As part of that trial work an initial investigation was undertaken to determine the mechanisms by which Amplimune induces immune responses in treated animals. Results suggested that administration of Amplimune does not induce a strong pro-inflammatory response, as evidenced by only minor changes in the serum concentrations of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-12 following treatment, or excessive fever, as evidenced by only minor changes in mean and maximum core body temperature following treatment. This was an important finding as for Amplimune to be deemed 'effective' the product should stimulate the immune system to a level which affords protection against disease challenges while 'not'

overstimulating the immune system to a level that induces severe fever or excessive inflammatory responses, both of which would be detrimental to the health, welfare and performance of the animal.

Results from the initial study failed to identify a clear mechanism by which Amplimune stimulates immune responses in treated animals. Therefore, a more detailed mechanistic study was undertaken to further elucidate such mechanisms, the results of which are reported here. Key findings from the current study are summarised below:-

• Haematology Parameters - No significant differences in the absolute numbers of white blood cells, the absolute numbers or proportions of neutrophils, lymphocytes or monocytes or the ratio of neutrophils to lymphocytes were observed between treatment groups following administration of Amplimune or saline (control) in the current study suggesting that administration of Amplimune does not result in systemic changes in white blood cell parameters. These results are consistent with those observed in the initial safety trial.

• Core Body Temperature – No significant differences in mean or maximum body temperature were observed between treatment groups following administration of Amplimune or saline (control) suggesting that administration of Amplimune does not result in changes in core body temperature parameters in the current study.

In the initial safety trial, significant differences in Mean6hr, Max6hr, Max12hr, Mean24hr and Max24hr were observed between treatment groups, however, treatment group effects were influenced by the sex of the animal, with significant differences observed in steers but not in heifers. Specific linear contrasts within sexes of calves demonstrated that mean and max body temperatures were higher in steers treated with 2mL of Amplimune subcutaneously, as compared to steers treated with 5mL Amplimune subcutaneously or control group steers receiving saline, during periods up to 48 hours post-treatment. Although significant, differences observed would be considered small in a biological context, are were not expected to cause significant fever or deleterious effects on the health of the animals.

• Serum Cytokine Concentrations – A significant overall treatment group effect on serum TNF-α concentrations post-treatment were observed in the current study, with Amplimune treated steers having consistently lower serum TNF-α concentrations than their counterparts treated with saline (control). A significant overall treatment group effect on serum IL-1β concentrations was also observed in the current study; however, this effect varied with cohort with specific linear contrasts conducted within cohort, demonstrating that treatment had no significant effect on serum IL-1β concentrations within any given cohort.

In the initial safety trial, no significant overall treatment effect on serum concentrations of TNF-α or IL-1β were observed, however, a significant treatment*time interaction was observed when analysing TNF-α concentration changes, suggesting that the effect of treatment on serum TNF-α concentrations changed over time. Specific linear contrasts were undertaken to compare TNF-α concentration LSMs in Amplimune versus saline (control) treated calves at 48hrs, 72hrs & 96hrs post-treatment. No significant differences between Amplimune and saline treated animals were observed with time points.

- Supernatant Cytokine Concentrations - No significant overall treatment (*in vivo* treatment with Amplimune or saline (ctrl)) group effect on serum TNF-α or IL-1β concentrations in culture supernatants following stimulation of cells were observed, however the effect of treatment did vary across cohorts for TNF-α. Specific linear contrasts conducted within cohort, demonstrated that treatment (Amplimune versus saline) had no significant effect on supernatant TNF-α concentrations within any given cohort.

A significant effect of culture treatment (*in vitro* treatment with media only (unstimulated, CTRL), inactivated virus (BHV-1) or Concanavilin-A (positive control, ConA)) on supernatant TNF-α concentrations was observed, however the effect varied across cohorts and with time. Specific linear contrasts conducted within time and cohort, demonstrated that supernatant TNF-α concentrations were significantly higher in ConA treated versus unstimulated cells when cultured at 96hrs post-treatment. No other significant effects of culture treatment on supernatant TNF-α concentrations within cohort or time were observed. No significant effect of culture treatment on supernatant IL-1β concentrations was observed.

- Intracellular Expression of Interferon-gamma (IFN-γ) by T-cell Populations – No significant overall treatment (*in vivo* treatment with Amplimune or saline (ctrl)) group effect on the proportion of CD4+/IFN-γ+ and CD8+/ IFN-γ+ in PBMCs cells stimulated in culture were observed.

A significant effect of culture treatment (*in vitro* treatment with CTRL, BHV-1, AMP, AMP+BHV-1, ConA) on proportion of both CD4+/IFN-γ+ and CD8+/IFN-γ+ cells was observed, however the effect varied with time. Specific linear contrasts conducted within time, demonstrated that there was a significantly higher proportion of CD4+/IFN-γ+ cells in PBMCs stimulated with Con A versus CTRL when cultured at 0, 24 and 96hrs post-treatment. A significantly higher proportion of CD4+/IFN-γ+ cells in PBMCs stimulated with AMP and AMP+BHV-1 versus CTRL when cultured at 0hrs post-treatment. A significantly higher proportion of CD8+/IFN-γ+ cells in PBMCs stimulated with ConA versus CTRL when cultured at 0 and 96hrs post-treatment was also observed.

- Cell Populations at Injection Sites - A significant treatment group effect on numbers of neutrophils at injection sites 24hrs post-treatment was observed. No significant effects of treatment were observed for mononuclear and eosinophil cell numbers at injection sites 24hrs post-treatment.

- Expression of Genes Encoding Pro-Inflammatory Cytokines – Due to restrictions on staff movements as a result of the current COVID-19 situation gene expression assays could not be completed.

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