



Effect of an injectable trace mineral supplement on the immune response of dairy calves

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ARTICLE INFO

Keywords:

Trace mineral supplement
Immunity
Phagocytosis
Calf

ABSTRACT

On a spring calving, pastoral dairy farm, the first 40 heifer calves born after calving mid-point (50% of the herd calved) were blood sampled within 24 h. Thirty were selected, using stratified randomisation to form two equal groups (treatment and control) with the same distribution of serum total protein, copper, selenium, zinc, and manganese concentrations, age and breed. From the remaining 10 calves, five were randomly selected into a sentinel group to assess field exposure to *Salmonella* spp.

All calves received two injections of a killed vaccine containing *Salmonella* spp. antigens at 2 and 6 weeks of age. Concurrently, the treatment group were injected with 1 mL/50 kg trace mineral supplement (TMS) containing 40 mg zinc, 10 mg manganese, 5 mg selenium, 15 mg copper per mL. Sentinel animals received no injections. All animals were bled from 2 to 9 weeks for assay of immune function. At three and four weeks, white blood cells from TMS calves had an increased percentage of cells phagocytosing (effect size = 9.36 and 4.35) and increased number of bacteria ingested per cell (effect size = 0.93 and 1.52). No differences were detected in gamma interferon response (effect size < 0.15) or *Salmonella* sp. antibody titres (effect size < 0.20).

1. Introduction

Internationally, perinatal mortality (within 48 h of birth) incidence ranges from 3 to 9% of full-term births, with mortality after the perinatal period and up to weaning ranging from 5 to 11% (Compton et al., 2017). Within New Zealand, Cuttance et al. (2017a, 2017b, 2017c) reported similar levels of perinatal (5.7%; 95%CI: 5.4–6.1%) and postnatal mortality (4.1%; 95%CI: 3.6–4.6%) on pastoral dairy farms. Failure of passive transfer of immunoglobulin is a key driver of postnatal mortality (Cuttance et al., 2017b) as the neonatal calf has a naïve immune system requiring time to develop specific, adaptive antibody responses (Gelsinger and Heinrichs, 2017).

However, from birth, the calf does have a functioning innate cellular immune system in the form of peripheral leucocytes (Kampen et al., 2006). The functionality of the neonatal immune system can be heavily influenced by the availability of essential trace minerals that are important for multiple biochemical processes, including immune

response, cell replication, and skeletal development (Teixeira et al., 2014; Carroll and Forsberg, 2007). Understanding the internal and external forces that contribute to the immunological responses of calves to stressors has been identified as a key driver for reducing calf morbidity and mortality (Hulbert and Moisés, 2016).

Marginal concentrations of zinc, copper and selenium at the herd level were associated with an odds ratio for calf morbidity of 1.76–5.42 (Enjalbert et al., 2006) in a survey of 2080 commercial herds in France and Belgium. Teixeira et al. (2014) found that early postnatal injection of a trace mineral supplement (TMS) containing zinc, manganese, copper, and selenium at 3 and 30 days after birth reduced the incidence of calf diarrhoea (41.7% in TMS vs 49.7% in control) and calf pneumonia and or otitis (41.7% TMS, 49.1% controls) in the first 50 days of life. Under New Zealand pastoral conditions, supplementation of calves with an injectable TMS containing 40 mg zinc, 10 mg manganese, 5 mg selenium, 15 mg copper and 5 mg chromium per mL (Multimin, Virbac New Zealand Ltd) at 1 mL/50 kg body weight reduced the morbidity

Abbreviations: TMS, Trace mineral supplement; TP, Total protein; HEP, Sodium heparin; EDTA, Ethylene diamine triacetic acid; IFN- γ , Gamma interferon; PBS, Phosphate buffered saline; SEB, Staphylococcal enterotoxin B; OD, Optical density; PI, Predictive interval; ROPE, Region of probable equivalence

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<https://doi.org/10.1016/j.rvsc.2020.02.007>

Received 12 June 2019; Received in revised form 18 December 2019; Accepted 12 February 2020

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and mortality over the first 140 days of life (Bates et al., 2019).

These beneficial effects of TMS may be mediated by an improvement in immune function. As well as a reduction in early morbidity, Teixeira et al. (2014) reported an increased in neutrophil function and glutathione peroxidase activity at 14 days of age in TMS supplemented calves. Bittar et al. (2018) and Palomares et al. (2016) showed that injection of calves 3.5 months of age with a TMS containing zinc, copper, selenium and manganese increased the humoral and cellular immune response to an attenuated-live bacterin vaccine and a modified-live viral vaccine. Arthington and Havenga (2012) showed an increase in antibody production as a response to vaccination in yearling calves that had concurrently been given an identical TMS supplement containing zinc, copper, selenium and manganese.

However, most of these studies have looked at the effect of TMS on the immune response in older animals. The reduction in morbidity and mortality that we detected in our earlier study on the effects of TMS (Bates et al., 2019) occurred in calves 3–35 days of age. This will have been within the period where maternal immunity would potentially interact with vaccinal response and where the endogenous humoral response is still developing (Chase et al., 2008).

Thus, specific data on the effect of TMS supplementation on young calves' immune response is needed to identify the role that the immune response plays in reducing morbidity and mortality in young dairy calves.

The objective of the present study was therefore to measure the effect of an injectable TMS containing zinc, manganese, copper and selenium on the cellular and humoral immune response of dairy calves to an inactivated vaccine during the neonatal period of immune naivety (approximately the first 30 days after birth (Barrington and Parish, 2001)). Our null hypothesis was that there was no difference in the immune response to vaccination with *Salmonella* spp. antigens between calves receiving TMS and calves not receiving TMS.

2. Materials and methods

2.1. Animal use

The trial was conducted between July 2018 and December 2018 on a single convenience sampled, commercial, spring calving (July to October) pastoral dairy farm serviced by Vetlife Ashburton Ltd., New Zealand (NZ).

2.2. Ethics approval

All procedures were approved by Massey University Animal Ethics Committee, Palmerston North, New Zealand. Sample size was calculated for the dependent variables using GLIMMSE - <http://glimmse.samplesizeshop.org/#/> - (Kreidler et al., 2013) allowing for repeat measurements within calf for a 95% confidence interval and 80% power.

2.3. Housing and management

All calves were born at pasture, brought into the rearing facility within 24 h of birth and navels sprayed with iodine tincture spray containing 10% iodine (Ethical Agents, Manukau, NZ). Calves were housed in covered, open fronted bay-sheds with solid walls up to 1.5 m in groups of 10–12 and 1.5m² allocated space per calf. All calves were bedded on wood chips which were topped up weekly. Female, Jersey-Friesian cross-bred calves were used and all calves remained on the farm of origin for the period of study.

2.4. Nutrition

Calves were picked up twice a day at approximately 8:00 am and 3:00 pm. Calves picked up in the morning were fed 4 L/calf pooled

colostrum from cows that had calved overnight. Calves picked up in the afternoon were fed 3 L/calf pooled colostrum from cows that calved that day. This meant that all calves were fed colostrum from the first milking of cows calving within 7–15 h. Thereafter, until calves were 4 days old, calves were fed 2 L of pooled colostrum every 12 h from cows between 1 and 4 days calved. From 5 days to weaning, calves were fed pooled colostrum and saleable milk harvested from milking cows in the herd and had access to hay, water and calf meal (Enerpro20%, Enerpro Feeds, Merivale, Christchurch, NZ), containing contain 20% crude protein, 13 MJ metabolizable energy per kg dry matter. In addition, from 14 to 21 days of age calves had access to perennial ryegrass (*Lolium perenne*). Calves were fed two equal feeds (7:00 am and 3:00 pm) increasing from 2 to 3 L per feed for the first 14–21 days before switching to 6–8 L once a day for the remainder of the study.

2.5. Enrolment

The study sample population consisted of the first 40 heifer calves that were brought into the rearing facility from the expected mid-point of calving (13th August 2018, when 50% of the herd had calved). Within 24 h of birth, a 7 mL jugular blood sample was collected from all 40 calves into vacuum tubes without anticoagulant (Vacutainer, BD Diagnostics, Auckland, NZ). Samples were couriered to Gribbles Veterinary Pathology, Christchurch, NZ within 24 h and refrigerated overnight at 4 to 8 °C if necessary and assayed for serum selenium, zinc, copper, manganese and total protein (TP).

2.6. Treatment groups

The sample study population ($n = 40$) was ranked on serum TP, selenium, copper, zinc, manganese, and age. From the sample study population, a study population of 30 calves was randomly selected into either a control or treatment group using the random number generator function in Excel. From the remaining sample study population ($n = 10$), five calves were randomly selected to form a sentinel group to verify that no field exposure to *Salmonella* spp. occurred during the trial period.

At two weeks of age, 4 × 7 mL jugular blood samples were collected from the 30 study calves. One, into vacuum tubes with the sodium heparin (HEP), one with EDTA and two samples without anticoagulant (plain). A single jugular 7 mL blood sample was also collected from the 5 sentinel calves into a plain vacuum tube. The HEP sample from each study calf ($n = 30$) was couriered within 4 h to Microbiology department, University of Otago, Dunedin, NZ for assessment of neutrophil and monocyte function. The EDTA samples were couriered within 24 h to AgResearch, Hopkirk Research Institute, Palmerston North, NZ for in vitro assay of antigen induced gamma interferon (γ IFN) release by peripheral blood monocytes. One plain sample from each study calf ($n = 30$) and from each sentinel calf ($n = 5$) was also sent to AgResearch, Hopkirk Research Institute for assay of antibody levels to strains of *Salmonella* Typhimurium, *S. Bovis-Morbificans*, *S. Hindmarsh* and *S. Brandenburg*. The remaining plain samples ($n = 30$) were couriered within 24 h to Gribbles Veterinary, Christchurch, NZ for assay of serum selenium, copper, zinc concentrations.

After blood sampling, the control and treatment calves were injected with 2 mL of Salvexin B (MSD Animal Health, 33, Whakatiki Street, Wellington, NZ) s/c containing inactivated strains of *Salmonella* Typhimurium, *S. Bovis-morbificans*, *S. Hindmarsh* and *S. Brandenburg* into the anterior half of the neck. Treatment calves also received 0.75 mL of a subcutaneous injection containing 40 mg zinc (as disodium zinc EDTA), 10 mg manganese, (as disodium manganese EDTA), 5 mg selenium (as sodium selenite), 15 mg copper per mL (as disodium copper EDTA) - Multimin + Copper, Virbac NZ, Pukete, Hamilton, NZ). In each pen of 10–12 calves, there were treatment, control and sentinel calves and all farm and veterinary staff except for the lead investigator (AB) were blinded as to the treatment status of the calves. All calves

were otherwise treated identically for the duration of the study.

At 6 weeks of age, all calves received a second injection of 2 mL of Salvexin B and calves in the treatment group a second injection of TMS (1 mL/calf). Farm and veterinary staff remained blinded as to treatment status.

At 3, 4, 5, 6, 7, 8 and 9 weeks of age jugular blood samples were collected from the 30 study calves and processed as before for assessment of neutrophil and monocyte function, in vitro assay of antigen induced IFN- γ release by peripheral blood monocytes and antibody levels to combined strains of *S. Typhimurium*, *S. Bovis-morbificans*, *S. Hindmarsh* and *S. Brandenburg*. At the same time, a single jugular blood sample was collected from the 5 sentinel calves and assayed for antibody levels to combined strains of *S. Typhimurium*, *S. Bovis-morbificans*, *S. Hindmarsh* and *S. Brandenburg*.

At 4, 6 and 8 weeks of age one plain blood sample was also sent to Gribbles Veterinary, Christchurch, NZ for assay of serum selenium, copper and zinc concentrations.

2.7. Laboratory procedures

2.7.1. Assessment of total protein

Concentrations of total protein (TP) in serum have been validated as a measure of passive transfer of immunoglobulin in NZ dairy calves (Cuttance et al., 2017a) and were determined using the Biuret method (Busher, 1990). The colour intensity, which is directly proportional to the total protein concentration was determined photometrically using a Roche/Hitachi Modular analyser (Roche Diagnostics, Auckland, NZ) (Bakker and Mücke, 2007). The limits of the detection of the assay are 2.0–150 g/L.

2.7.2. Assessment of neutrophil and monocyte function

Neutrophil and monocyte function were assessed by measuring their phagocytic activity using the PHAOTEST kit (Orpegen Pharma GmbH, Heidelberg, Germany) containing fluorescein-labelled opsonised *Escherichia coli* (*E.coli* FITC). Cells were analysed using a FACSCalibur flow cytometer (Becton, Dickinson and Co) using a 488 nm argon-ion laser. Ten thousand events were collected for each cell population (neutrophils and monocytes) and the results reported as percentage of total number of cells in the granulocyte gate performing phagocytosis and as the mean increase of the green fluorescence of the gated cells corresponding to the number of ingested bacteria per cell (Teixeira et al., 2014).

2.7.3. Assessment of gamma interferon release

Whole blood was stimulated in vitro with *Salmonella* sp. antigens and the production of IFN- γ then quantified. For this stimulation, each whole blood sample was aliquoted at 1 mL/well into 4 wells of a 24-well plate tissue culture plates (Nunc, Roskilde, Denmark). One well per blood sample was left as unstimulated control and received 10 μ L of phosphate buffered saline (PBS). Cells in the remaining three wells were stimulated with (1) 10 μ L/well Salvexin B (MSD Animal Health, Wellington, New Zealand) 1:1, (2) 10 μ L/well Salvexin B 1:100, or (3) 10 μ L/well of Staphylococcal enterotoxin B (SEB) (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 200 ng/mL as positive control.

Plates were incubated for 20 \pm 0.5 h at 37 °C in 5% CO₂. Plates were then centrifuged at 2100 \times g for 15 min at room temperature before \geq 400 μ L of supernatant were transferred to microtitre tubes and stored at -20 °C until further analysis (\leq 8 weeks). Bovine IFN- γ was measured in supernatants using ELISA (Bovine IFN- γ ELISA development kit (HRP); Mabtech, Nacka Strand, Sweden) according to manufacturer's recommendations.

Results were examined following subtraction of the negative control (PBS) value for each animal from the corresponding antigen or mitogen values.

2.7.4. Assessment of antibody titres

Serum IgG antibodies binding to *S. Typhimurium*, *S. Bovis-morbificans*, *S. Hindmarsh* and *S. Brandenburg*. antigens were determined via an in-house ELISA at AgResearch, Hopkirk Research Institute, Massey University, Palmerston North, NZ. Commercial veterinary vaccine Salvexin+B (MSD, Wellington, New Zealand) was used as antigens. Vaccines were diluted 1:10 in carbonate buffer, pH 9.6, 50 μ L loaded into 96 well plates (Nunc MaxiSorp; Nunc, Roskilde, Denmark) and incubated overnight. The plates were then washed 3 \times with PBS and blocked with PBS Tween 20 (PBST), containing casein (1% w/v) and merthiolate (1:10,000 dilution; Sigma-Aldrich) for 30 min at room temperature. 50 μ L/well of serum samples (diluted 1:10 in PBST, containing casein and merthiolate) were added to the plates, and incubated for 2 h at room temperature. The plates were washed 3 \times with PBS, conjugate (1:6000 sheep anti-bovine IgG HRP, Biorad) was added (100 μ L/well), the plates incubated for 1 h at room temperature, and washed five times in PBST. TMB substrate (3,3',5,5'-Tetramethylbenzidine) was added and plates incubated until colour had developed (\leq 30 min at room temperature). Reactions were stopped by adding 50 μ L of 0.2 M sulfuric acid and plates read at 450 nm using an ELISA plate reader (VERSAmax microplate reader; Molecular Devices Corporation). Antibody levels were expressed as optical density (OD) readings at a serum dilution of 1:10.

2.7.5. Assessment of serum selenium, copper and zinc levels

On receipt, blood samples were centrifuged at 3000 rpm for three minutes and the serum stored at 2-8 °C pending analysis. Determination of serum copper, selenium and zinc levels was via inductively coupled plasma mass spectrometry (ICP-MS), using a Perkin-Elmer NexION 2000 P ICP Mass Spectrometer according to criteria laid out by the Association of Official Analytical Chemists (<https://www.aoac.org/official-methods-of-analysis-21st-edition-2019/>). Manganese levels were determined using graphite furnace atomic absorption spectrometry (Paschal and Bailey, 1988; Neve and Leclercq, 1991; FernándezLara et al., 2019).

Quality control measures indicated that the coefficient of variation between sample runs was 5.8% for zinc (range within runs 3.1%–6.3%), 8.3% for copper (range within runs 4.6%–9.2%), and for selenium, 5.2% (range within runs 2.8%–5.1%). Manganese was determined on a single occasion and the coefficient of variation was 14.3%.

2.8. Statistical analysis

2.8.1. Independent variables

Treatment (categorical) and week (categorical) were the main independent variables of interest, measured every week. Serum TP, copper, selenium, zinc concentrations and age measured every 14 days were considered as potential confounders and covariates.

2.8.2. Dependent variables

As dependent variables, serum copper, selenium, zinc status and white blood cell fluorescence were continuous and normally distributed. IFN- γ production was continuous but right skewed, antibody production was continuous but left skewed and white blood cell phagocytosis percentage was binomial.

2.9. Power analysis

Simulation was used to estimate the relationship between sample and effect size for the dependent variables of interest using methodology outlined by (Kurz, 2019). For the continuous and normally distributed variables, with a sample size of 15 animals per group, the minimum effect size we could detect with a probability in excess of 80% was 1.0. Thus, for each dependent variable we had an 80% probability of detecting a difference between the TMS and control calves so long as this difference was \geq one standard deviation for that variable. For the

Table 1

Priors used in a multivariable Bayesian analysis looking at the effect of TMS supplementation on the immune response of dairy calves allowing for repeated measurements from 2 to 9 weeks of age.

Intercept	Coefficients	Sigma ^a	Covariance
~ Student's t (3,0,2.5)	~ Student's t (3,0,2.5)	~ Exponential (0.32)	~ Decovariance (1,1,1,1)

All distributions are reported after the variables have been centred. To conform to normality, IFN-γ was log transformed and antibody optical density was cubed. No other variables were transformed.

^a Sigma: for linear models, sigma represents the variability with which the outcomes deviate from the predictions of the model.

right skewed variable, IFN-γ, the minimum effect size was 2.5 and 6.0 for the left skewed antibody production. For phagocytosis percentage, we were able to detect a difference of 20% between treatment and control groups.

2.10. Statistical method

Initial graphical analysis indicated a non-linear response of treatment with time. The effect of treatment over the entire period of follow up was investigated using Bayesian multivariable models allowing for repeat measures on calves with a variable intercept for calf and week and a varying slope for treatment. The priors used for these models are detailed in Table 1.

For multivariable models, predictive variables were added in a forward and backward step-wise manner and retained if their 95% posterior predictive interval (PI) excluded zero. Values inside the 95% PI are “the 95% most credible values of the parameter” (Kruschke, 2018). Treatment was forced into all models.

To investigate the effect of treatment at each week of study a univariable Bayesian comparison of TMS and control calves was made at each week. For the effect of TMS on serum copper, selenium, zinc concentrations and white blood cell fluorescence, the Bayes equivalent of a robust t-test was used for each week of data using models based on those described in Kruschke (2013). For the comparison of the effect of TMS on phagocytosis, an hierarchical binomial model was applied to each week's data using a model based on that described by Kruschke (2015). For the comparison of IFN-γ production, a robust log normal model was used based on Kruschke (2016). All these models were written in JAGS (Depaoli et al., 2016) and implemented in R (Core Team, 2013). For the univariable comparison of antibody levels at each week, a skew-normal model written in STAN (Carpenter et al., 2017) and based on the model described by Martin and Williams (2017) was used. The priors used for these models are detailed in Table 2.

To model antibody optical density, optical density was regressed on

Table 2

Priors used in a univariable Bayesian analysis looking at the effect of TMS supplementation on the immune response of dairy calves for each week of follow up from 2 to 9 weeks of age.

Variable y	Distribution of y	Mu (μ)	Sigma (σ)	Nu (ν)
Serum copper Serum selenium Serum zinc White blood cell fluorescence	$Y_{s c} \sim \text{Student's } t(\mu_c, 1/\sigma_c^2, \nu)$	$\mu \sim \text{norm}(\text{meanY}, 1/(100 \times \text{sdY})^2)$	$\sigma \sim \text{uniform}(\text{sdY}/1000, \text{sdY} \times 1000)$	$\nu \sim \text{exp.}(1/30)$
Variable z Phagocytosis %	Distribution of $Z_{i s,c}$ $Z_{i s,c} \sim \text{binomial}(\theta_{s c}, N_{s c})$	Theta (θ) $\theta_{s c} \sim \text{beta}(\hat{\omega}_c \times (\kappa_c - 2) + 1, (1 - \hat{\omega}_c) \times (\kappa_c - 2) + 1)$	Omega (ω) $\hat{\omega}_c \sim \text{beta}(\Omega_0 \times (K_0 - 2) + 1, (1 - \Omega_0) \times (K_0 - 2) + 1)$	Kappa (κ) $\kappa_c = (\kappa_c - 2) + 2$ $\kappa_c - 2 \sim \text{gamma}(0.01, 0.01)$
Variable log(y)	Distribution of log(y)	Mu of log(y) (μlogy)	Sigma of log(y) (σlogy)	
γIFN	$Y_{s c} \sim \text{lnorm}(\mu \log y_c, 1/\sigma \log y_c^2)$	$\mu \log y \sim \text{norm}(\text{mean of log(Y)}, 1/\text{sd of log(Y)})$	$\sigma \log y \sim \text{norm}(\text{mean of sd of log(Y)}, 1000)$	

Subscripts s|c refer to the sth subject (calf) in the jth treatment group.
Subscripts i|s|c refer to the ith observation on the sth subject in the jth treatment group.

treatment for each week using a skew-normal distribution, with the following parameters:

Zeta ~ norm (0,8) to describe the skew.

Gamma ~ Cauchy (0,10) to describe the residual standard deviation.

Intercept ~ norm (1.33, 2.66) set to the mean of the data and given a wide standard deviation.

Slope ~ norm (0,1.33) set to the centre and given a wide distribution.

To determine the size of any observed effects of TMS, we calculated the effect size (Cohen, 1988) using the formula suggested by Kruschke (2018). This relates the magnitude of the difference in means to the standard deviation of the population. An effect size of 0.2 was considered small and 0.8 was considered large.

To estimate the probability that the effects we observed were real, we calculated the 95% PI for each parameter value estimated by the models. To confirm a difference between treatment groups we looked at the predicted difference in outcome between the treatment groups, calculated in terms of the effect size relative to the standard deviation of the population. We set our null value for this difference at zero but with a range of values either side corresponding to the region of practical equivalence (ROPE, Kruschke, 2018). To compare the concentration of serum minerals and total protein between treatment groups, we used an effect size defined as +/- 1 standard deviation of the population. By deliberately using a wide ROPE, this gave us an indication of whether the values were broadly similar between treatment groups. In comparing the effect of treatment on immunity, we used a narrower ROPE. For effect size, we defined the ROPE at half of Cohen's conventional definition of a small effect size (δ = ± 0.1). For the difference in means (μ) and standard deviation (σ), we used the same value of effect size (0.1) translated onto the scale of measurement of the parameter. (ROPE for μ₁-μ₂ and ROPE for σ₁ - σ₂ = 0.1 x σ_{population} (Kruschke, 2018)).

For all models, an effective sample size of 10,000 was used and convergence of the MCMC chains, autocorrelation and comparison of the observed data with a random sample of replicates was assessed visually. Values of the Gelman and Rubin potential scale statistic (maximum tolerated value 1.1) and the standard error of the mean of the posterior draws (maximum tolerated value 10% of the posterior standard deviation) were assessed using ShinyStan (Stan Development Team, 2018).

3. Results

There were no losses to follow up during the study. Tissue reaction following injection with TMS or Salvexin B was not observed, and no clinical signs of disease were observed in any of the calves during the study.

Table 3

Descriptive statistics for total protein and serum minerals in calves ≤24 h enrolled in a study looking at the effect of trace mineral supplementation on immunity.

Descriptive statistics at birth				
Statistic	N	Median	25th percentile	75th percentile
Total protein ^a (g/L)	30	59	45	65.2
Serum manganese ^b (nmol/L)	30	40	29.2	54.5
Serum copper ^c (μmol/L)	30	5.5	5	6.8
Serum selenium ^d (nmol/L)	30	425	400	480
Serum zinc ^e (μmol/L)	30	13.5	9.5	17.8

^a Adequate range ≥ 52 g/L for calf serum and derived from Cuttance et al., (2017a).

^b Adequate range = 42-51 nmol/L for calf whole blood and derived from Suttle, (2010) and 18 nmol/L to 73 nmol/L for neonatal serum derived from Herdt and Hoff, (2011).

^c Adequate range = 8-26 μmol/L for adult cattle serum and derived from Black, (1982) and quoted in Laven et al., (2007) and 5–16 μmol/L for neonatal serum derived from Herdt and Hoff, (2011).

^d Adequate range = 200–240 nmol/L for calf serum and derived from Suttle and Underwood, (2010) and 250-900 nmol/L for neonatal serum derived from Herdt and Hoff, (2011).

^e Adequate range = 9.2-27 μmol/L neonatal serum derived from Herdt and Hoff, (2011).

3.1. Mineral and total protein levels

Summary statistics for the serum mineral and total protein concentrations within 24 h of birth are given in Table 3.

Changes in serum mineral concentrations over the period of follow up by treatment group are shown in Figs. 1-3 and the mean difference with 95%PI between treatment and control groups is given in Table 4.

All values were above the reference ranges except for serum copper in week 0. There were small differences between TMS and control calves in serum concentration of copper, selenium, zinc, manganese and total protein at birth, but compared to the spread of the data the effect size was < 0.2 in all cases. However, the 95% PI for the differences was wide so that a relatively wide ROPE (+/- σ_{population}) was required to contain the 95% PI.

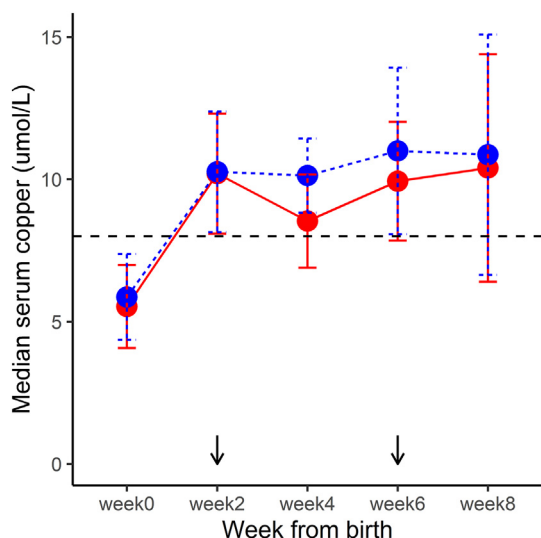


Fig. 1. Recorded serum concentrations of copper and their interquartile intervals by treatment group over the course of the study. Dotted line indicates trace mineral supplement^a (TMS) treated calves and solid line the control group. The minimum recommended normal limit for serum copper is indicated by the horizontal dashed line. Arrows indicate times for injection of TMS supplement in the TMS group.

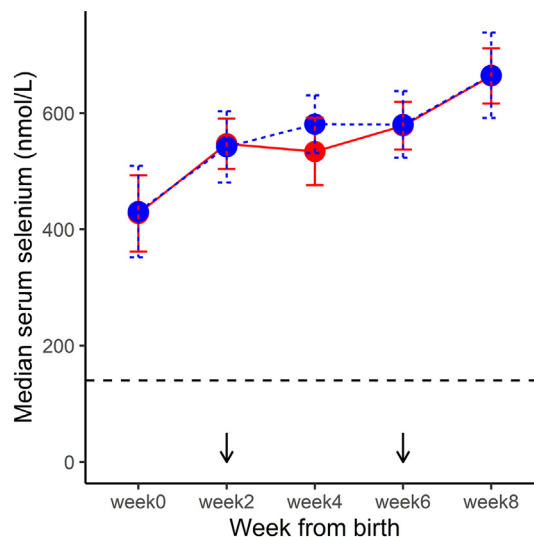


Fig. 2. Recorded serum concentrations of selenium and their interquartile intervals by treatment group over the course of the study. Dotted line indicates trace mineral supplement^a (TMS) treated calves and solid line the control group. The minimum recommended normal limit for serum selenium is indicated by the horizontal dashed line. Arrows indicate times for injection of TMS supplement in the TMS group.

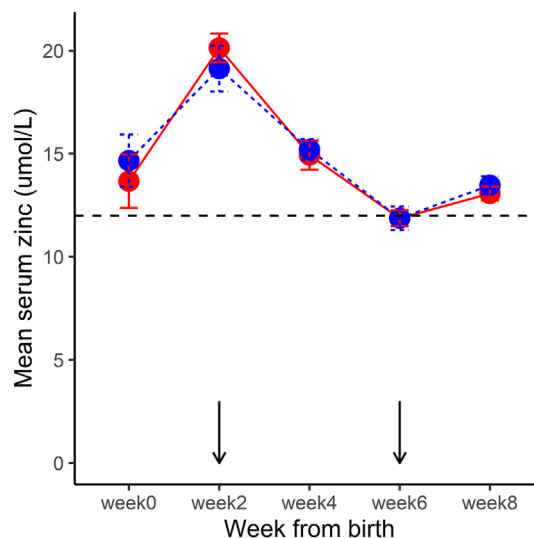


Fig. 3. Recorded serum concentrations of zinc and their interquartile intervals by treatment group over the course of the study. Dotted line indicates trace mineral supplement^a (TMS) treated calves and solid line the control group. The minimum recommended normal limit for serum zinc is indicated by the horizontal dashed line. Arrows indicate times for injection of TMS supplement in the TMS group.

Over the entire period of follow up, the multivariable models failed to confirm an effect of treatment on the serum concentration of copper, selenium or zinc or an interaction between treatment and week as the 95%PI for the coefficients included 0 in all cases.

From week 2 to week 8, serum copper and selenium concentrations were higher than in week 0, while serum zinc initially increased and then declined to similar levels at week 8 to week 0. Serum mineral concentrations over the follow up period are presented as supplementary material. For all mineral and week combinations, except for copper and selenium in week 4, the 95%PI for the difference in copper, selenium and zinc concentration between TMS and control calves was completely contained within the ROPE. The predicted percentage of occasions where the difference in serum copper, selenium and zinc

Table 4

Difference (95%PI) between mean values of total protein and serum copper, selenium, zinc and manganese for calves treated with a trace mineral supplement^a and untreated control calves.

	Week 0	Week 2	Week 4	Week 6	Week 8
Total protein (g/L)	1.2 (-10.86–13.33)	Not tested			
Serum copper (µmol/L)	0.3 (-0.95–1.47)	0.01 (-1.70–1.76)	1.54 (0.34–2.74)	0.89 (-1.16–2.88)	0.49 (-2.90–3.87)
Serum selenium (nmol/L)	3.7 (-55.21–61.79)	-11.2 (-52.31–31.79)	47.7 (3.35–91.92)	1.8 (-38.99–42.46)	-2.25 (-52.23–49.41)
Serum zinc (µmol/L)	1.1 (-3.02–5.10)	-1.40 (-4.05–1.42)	-0.8 (-1.90–0.74)	0.0 (-1.18–1.32)	0.4 (-0.87–1.55)
Serum manganese (nmol/L)	8.0 (-9.77–25.53)	Not tested			

^a Treated calves received a subcutaneous injection at 2 and 6 weeks of age of 1 mL/50 kg body weight of a trace mineral supplement. Each mL contained 40 mg zinc (as disodium zinc EDTA), 10 mg manganese, (as disodium manganese EDTA), 5 mg selenium (as sodium selenite), 15 mg copper per mL (as disodium copper EDTA) per mL (Multimin + Copper, Virbac NZ, Pukete, Hamilton, NZ).

Table 5

Predicted percentage of occasions where the difference in serum copper, selenium and zinc concentration between calves given a trace mineral supplement^a and untreated control calves was greater than zero.

	Week 2	Week 4	Week 6	Week 8
Serum copper (µmol/L)	50.5	99.3	80.8	61.4
Serum selenium (nmol/L)	29	98	53	46
Serum zinc (µmol/L)	16	16	55	71

^a See Table 1.

concentration between TMS and control calves was greater than zero is given in Table 5.

In week 4, re-setting the ROPE to $\delta = 0.1$, 99% and 97% of the most likely values of serum copper and selenium were greater than the upper limit of the ROPE ($\pm 0.17 \mu\text{mol/L}$ for copper and $\pm 5.8 \text{ nmol/L}$ for selenium). The effect size at week 4 for serum copper was 1.02 (95% PI = 0.21–1.87) and for serum selenium, 0.8 (95%PI = 0.07–1.67).

4. Measures of immunity

For all measures of immunity, when mineral concentrations were entered in the multivariable model, the 95%PI for their effect always included zero and the 95%PI for the other variables changed by < 10%. Consequently, mineral concentrations as potential confounders or covariates were excluded from analysis.

4.1. Fluorescence and phagocytosis by white blood cells

At week two prior to injection of TMS the difference in the fluorescence or phagocytosis of white blood cells between the two groups did not exceed one standard deviation (Table 6) and the 95% PI for the effect of TMS included zero.

4.1.1. White blood cell fluorescence

Changes in fluorescence intensity over the period of follow up by

Table 6

Summary statistics by treatment group for white blood cell fluorescence in calves measured prior to treatment allocation at two weeks^a.

Treatment group	N	Median	25th percentile	75th percentile
Control* - % phagocytosis	15	88	84.2	88.7
TMS* - % phagocytosis	15	89	85.7	90.6
Control* - fluorescence intensity	15	3306	3079	3851
TMS* - fluorescence intensity	15	3143	2902	3378

^a See Table 1.

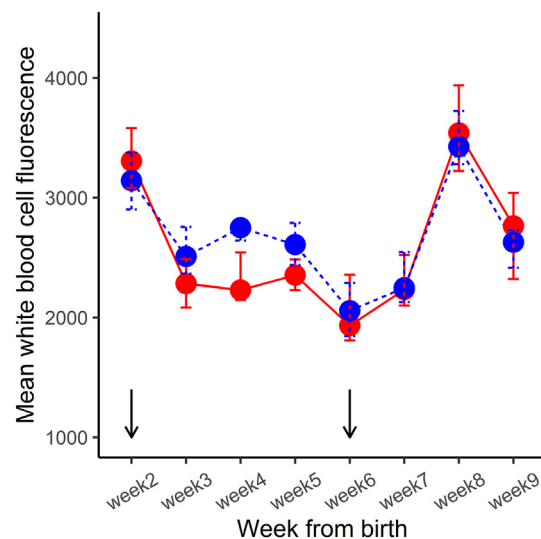


Fig. 4. Median white blood cell fluorescence and the inter quartile intervals by treatment group over the course of the study. Dotted line indicates TMS treated calves and solid line the control group^a. Arrows indicate times for injection of TMS supplement in the TMS group.

treatment group are shown in Fig. 4. The multivariable model suggested that there was an overall small decrease in fluorescence over the 8 weeks of follow up but that TMS increased fluorescence at weeks 3, 4 and 5 compared to control calves. The mean difference with 95%PI between treatment and control groups is given in Table 7 together with the predicted percentage of occasions when the difference exceeded zero and the ROPE.

The ROPE for which a difference was demonstrated with this data was $\pm 0.1 \cdot \sigma_{\text{pop}}$; the effect size at weeks 3,4 and 5 was 0.93 (95% PI = 0.13–1.72), 1.52 (95%PI = 0.52–2.58) and 0.78 (95%PI = -0.02–1.55), respectively. Together, this suggests a high probability that at week 3, 4 and 5 the increase in fluorescence between TMS and control calves was large (≥ 0.8) relative to the spread of the data.

4.1.2. White blood cell phagocytosis

The percentage of white blood cells phagocytosing and the inter quartile intervals by treatment group are given in Fig. 5.

The multivariable model suggested that overall there was an increase in the percentage of cells phagocytosing over the period of follow up but that TMS increased phagocytosis at weeks 3, 4 and 5 compared to control calves. The mean difference with 95%PI between treatment and control groups is given in Table 8 together with the predicted percentage of occasions when the difference exceeded zero and the

Table 7

Difference (95%PI) between mean values of fluorescence intensity for calves treated with a trace mineral supplement^a and untreated control calves, together with the predicted percentage of occasions when the difference exceeded zero in a study looking at the effect of trace mineral supplementation on immunity.

	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9
Difference in fluorescence intensity	-133 (-524-261)	281 (49-515)	455 (210-699)	248 (-2-487)	6 (-300-310)	9 (-220-230)	-94 (-493-305)	-86 (-407-238)
% of estimates > 0	24	99	100	98	52	53	31	29
% of estimates > ROPE (0.18)	17	98	99	96	42	43	23	21

^a See Table 1.

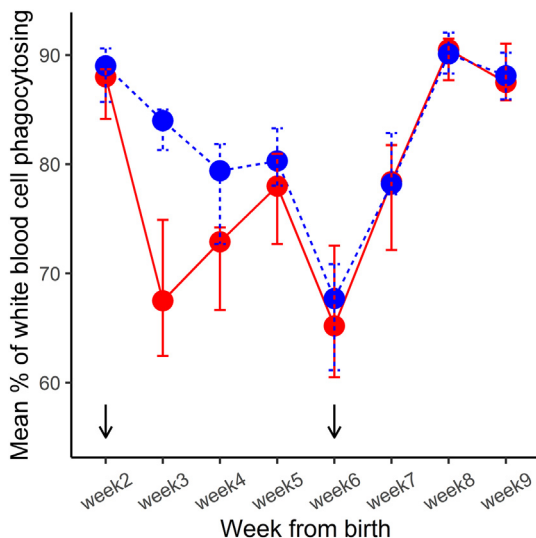


Fig. 5. Percentage of white blood cell phagocytosis and the inter quartile intervals by treatment group over the course of the study. Dotted line indicates TMS treated calves and solid line the control group^a. Arrows indicate times for injection of TMS supplement in the TMS group.

ROPE.

The effect size at weeks 3 and 4 was large and excluded zero (9.36 (95%PI = 6.12–12.48) and 4.35 (95%PI = 1.22–8.00) respectively. Thus, the data suggested a high probability that there was a large difference in phagocytosis relative to the spread of the data at weeks 3 and 4.

4.2. IFN-γ response

In the analysis of IFN-γ response the SEB positive control failed to elicit a consistent response. Consequently, we estimated the “antigen-specific response - unstimulated response” for each measurement. The summary statistics for the IFN-γ response by treatment group measured at two weeks and prior to TMS supplementation are given in Table 8. The difference between the two groups in the IFN-γ response prior to treatment did not exceed one standard deviation and the 95% PI for the effect of TMS included zero (Table 9).

Over the entire follow up period, there was no evidence that white blood cell IFN-γ production was different between TMS and control calves with an overall difference for the log transformed effect of

Table 8

Difference and 95%PI between percentage of phagocytosis by white blood cells for calves treated with a trace mineral supplement^a and untreated control calves, together with the predicted percentage of occasions when the difference exceeded zero in a study looking at the effect of trace mineral supplementation on immunity.

	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9
Difference in % phagocytosis	1.5 (-3.2-6.4)	15.3 (10.0-20.4)	7.5 (2.1-13.7)	5.2 (2.8-9.0)	0.3 (-1.5-8.0)	3.3 (-1.5-8.0)	0.6 (-2.8-3.8)	-0.1 (-3.7-3.6)
% of estimates > 0	73	100	99	86	54	91	64	48
% of estimates > ROPE (0.18)	69	100	99	83	49	90	60	44

^a See Table 1.

Table 9

Summary statistics by treatment group^a for white blood IFN-γ response in calves measured prior to treatment allocation at two weeks in a study looking at the effect of trace mineral supplementation on immunity.

Summary statistics by treatment				
Treatment group	N	Median	25th percentile	75th percentile
Control: IFN-γ	15	43	10.9	132.8
TMS: IFN-γ	15	83	28.1	227.3

^a See Table 1.

treatment of 0.70 (95%PI = -0.50-1.80). The 95% PI for the difference was neither entirely within or outside the ROPE (± 0.18) indicating that no differences were identified in this data set (Table 10). For all weeks of follow up, the calculated effect size was no greater than 0.15, with 95%PI that included zero suggesting that TMS was having little effect on IFN-γ production.

White blood cell IFN-γ production and the inter quartile intervals by treatment group are given in Fig. 6.

4.3. Salmonella antibody production

There was no evidence of field exposure to *Salmonella* antigen during the study. Optical density readings from sentinel calves showed a numerical decline in antibody titres from week 2 (median OD = 1.37, 95%PI = 1.36-1.48) to week 9 (median OD = 1.26, 95%PI = 1.17-1.42) with a difference in titres over this time of 0.95 (95%PI = -0.34-0.16).

The change in the OD over time for control and TMS calves is shown in Fig. 7.

The multivariable model suggested that the increase in antibody level from week 5 to week 9 was significant (mean increase = 0.15, 95%PI = 0.12-0.18). However, over the entire follow up period, there was no evidence of a difference between TMS and control calves in *Salmonella* antibody production with an overall difference in optical density of 0.00 (95%PI = -0.2-0.30) for the cube of the optical density.

The mean difference with 95%PI between treatment and control groups is given in Table 11 together with the predicted percentage of occasions when the difference exceeded zero and the ROPE. For all weeks of follow up, the calculated effect size was no greater than 0.2, with 95%PI that included zero suggesting that TMS was having little effect on *Salmonella* antibody production.

Table 10

Difference and 95%PI between mean values of IFN- γ production for calves treated with a trace mineral supplement^a and untreated control calves, together with the predicted percentage of occasions when the difference exceeded zero in a study looking at the effect of trace mineral supplementation on immunity.

	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9
Difference in IFN- γ production	232 (-267-770)	204 (-205-656)	78 (-193-372)	196 (-204-647)	138 (-1096-1484)	474 (-200-1332)	42 (-313-416)	93 (-230-425)
% of estimates > 0	84	85	72	84	59	93	59	73
% of estimates > ROPE (0.18)	79	80	63	80	55	90	53	63

^a See Table 1.

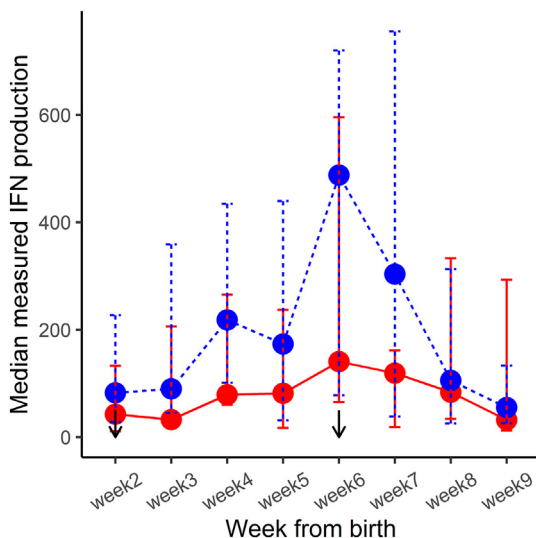


Fig. 6. White blood cell IFN- γ production and the inter quartile intervals by treatment group over the course of the study. Dotted line indicates TMS treated calves and solid line the control group^a. Arrows indicate times for injection of TMS supplement in the TMS group.

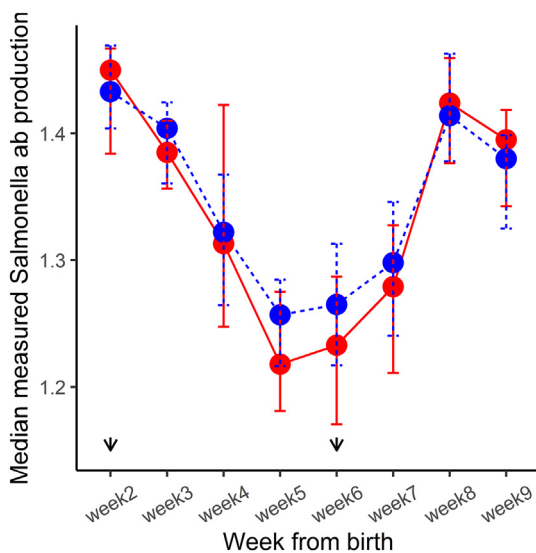


Fig. 7. Optical density readings with inter-quartile range for Salmonella antibodies by treatment group over the course of the study. Dotted line indicates TMS treated calves and solid line the control group^a. Arrows indicate times for injection of TMS supplement in the TMS group.

5. Discussion

This small-scale study has successfully demonstrated that differences in white blood cell immune function can be measured in response to TMS supplementation in new born dairy calves under NZ pastoral

farming conditions. The probability of a meaningful increase (effect size ≥ 0.8) in the number of bacteria ingested (white blood cell fluorescence) and the percentage of white blood cells phagocytosing was greater than 95% at weeks 3–5 of the study.

In this study, we did not have enough precision in our estimates of serum selenium and copper concentrations to confirm that TMS calves had anything more than a very small increase in serum copper and selenium concentrations at week 4. For all mineral and week combinations, except for copper and selenium in week 4, the 95%PI for the difference in copper, selenium and zinc concentration between TMS and control calves was completely contained within the ROPE. For serum copper and selenium at week 4, although the ROPE for which a high probability of a difference can be demonstrated is narrow, the mean effect size at week 4 for serum copper and selenium is enough to suggest that these increases in serum copper and selenium concentration between TMS and control calves may potentially be large relative to the spread of the data. However, with this small data set, we lack precision to confirm an effect of this size.

This contrasts with the findings from Palomares et al., (2016) where TMS supplementation increased liver copper and selenium concentrations. However, in our study, serum copper and selenium concentrations increased in both groups after birth compared to a net decrease in that study. In the current study, calves were younger and were colostrum and milk fed. Low plasma copper at birth was reported by McMurray et al. (1978) and appears to be a common finding in neonatal calves (Herdt and Hoff, 2011). Milk and colostrum are both rich sources of copper and selenium for the neonate (Suttle and Underwood, 2010) and the changes in concentration of serum copper and zinc that we observed are very similar to those reported by McMurray et al. (1978) in single suckled beef calves. Thus, any differences in serum concentrations of these minerals due to treatment with the TMS may have been confounded by extraneous sources of minerals or by utilization of these minerals in response to the challenge of vaccination (Arthington and Havenga, 2012; Palomares et al., 2016).

An increase in phagocytosis (% of cells phagocytosing and number of bacteria ingested per cell) was also demonstrated by Teixeira et al. (2014). Using neonatal calves, those workers demonstrated an increase in phagocytosis in white blood cells 14 days after supplementation at 3 days of age with an injectable TMS containing 60 mg of zinc, 10 mg of manganese, 5 mg of selenium, and 15 mg of copper. For that study, using 790 calves, TMS reduced the relative risk of diarrhoea in the first 50 days by 0.84 (95%CI = 0.76–0.92) and pneumonia or otitis by 0.85 (95%CI = 0.77–0.93) compared to negative controls. With a much smaller sample size, we did not expect to observe a differences in morbidity or mortality in our present study between treatment groups but have previously reported a reduction in morbidity and mortality for NZ dairy calves in the first 35 days when supplemented with TMS at birth (Bates et al., 2019).

The present study was undertaken to identify elements of the immune system responsive to TMS supplementation and that are potentially partly responsible for the differences in early morbidity and mortality observed in our earlier study. This early reduction in disease is not consistent with an antibody production response as there is insufficient time between birth and day 35 for TMS to effect antibody production and thereby morbidity and mortality. The lack of a

Table 11

Difference and 95%PI between mean values of Salmonella antibody production for calves treated with a trace mineral supplement^a, together with the predicted percentage of occasions when the difference exceeded zero in a study looking at the effect of trace mineral supplementation on immunity.

	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9
Difference in antibody production	0.012 (−0.037–0.062)	0.015 (−0.034–0.065)	−0.006 (−0.090–0.081)	0.016 (−0.041–0.088)	0.026 (−0.054–0.100)	0.024 −0.041- 0.088)	0.013 (−0.048–0.073)	−0.014 (−0.062–0.034)
% of estimates > 0	83	88	40	86	91	93	80	13
% of estimates > ROPE (0.18)	48	57	10	54	63	68	44	2

^a See Table 1.

demonstrable difference between TMS and control calves in vaccine antibody response in the present study is not inconsistent with this but contrasts with the findings of Teixeira et al., (2014 and Palomares et al., (2016) that antibody production in response to live viral vaccine challenge was increased in 3–4 month old calves supplemented with TMS. However, it is also possible that our antibody detection ELISA underestimated the immune response. Prior to isotype switching, the neonatal immune response is almost exclusively in the form of IgM (Gelsing and Heinrichs, 2017). As our antibody detection ELISA used an anti-bovine Ig G molecule as the secondary enzyme linked antibody, the optical density would have been reduced (because of IgM blocking the binding sites for the enzyme linked IgG).

Unlike Palomares et al. (2016), we did not see an increase in IFN- γ production for TMS treated calves that were vaccinated. This may reflect differences in the age of calves studied and the use of live viral versus dead bacterial vaccines.

In our statistical analysis we adopted a simple approach to the analysis and a conservative approach to the choice of prior, selecting weakly informative priors based on the data. Although similar studies have been performed by Teixeira et al. (2014) and Palomares et al. (2016) we felt that the use of older calves and live vaccine in these studies rendered them unsuitable for deriving prior information. Further, we were conscious of our small sample size and the risk that using too strongly an informative prior would mask information from the data. By identifying the magnitude and certainty associated with the differences in our data, we hope the reader can find the information useful and transparent.

In conclusion, injection of TMS to neonatal calves increased the phagocytic capability of their white blood cells with increases in the percentage of cells phagocytosing and the number of bacteria ingested per cell. This work adds to the evidence that injectable TMS supplementation at birth can improve immune function and reduce disease.

Acknowledgments

The authors would like to acknowledge the support of the farmer and staff in hosting this trial, and of Vetlife Ltd., NZ for continued interest and facilitation of research in clinical veterinary practice. Commercial sponsorship was provided by Virbac, NZ Ltd. We gratefully acknowledge the skill and help the project received in laboratory procedures from Disease Research Ltd., Otago NZ, the Microbiology Department at the University of Otago, Dunedin, NZ and AgResearch, Palmerston North, NZ and Gribbles Veterinary Pathology, NZ. The technical input of Dr.J Moffat, Merck, NZ and Aaron Yang, School of Veterinary Sciences, Massey University, NZ are also acknowledged.

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