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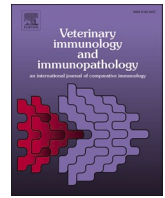
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# Long term dietary vitamin D<sub>3</sub> supplementation impacts both microbicidal and inflammatory responses to *ex-vivo* *Mycobacterium bovis* BCG challenge in dairy calves

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## ABSTRACT

Vitamin D deficiency (VDD) is associated with enhanced susceptibility to multiple respiratory diseases in humans, including tuberculosis. However, the consequences of VDD for disease susceptibility in calves are unknown. Previously we developed a model to drive divergent circulating 25OHD concentrations in cattle, where animals were supplemented with vitamin D<sub>3</sub> (vit D<sub>3</sub>) from birth to 7 months of age. Calves in the control group (Ctl) received a diet containing a standard vit D<sub>3</sub> concentration, whereas the vit D group (VitD) received a diet with the highest vit D<sub>3</sub> concentration allowed under EU guidelines. Here, we assessed the microbicidal activity and immunoregulatory effect of divergent 25OHD circulating levels to *Mycobacterium bovis* BCG challenge *ex-vivo*. Blood samples from Ctl and VitD calves were taken at 1-, 3- and 7-months of age. 25OHD concentrations were significantly different at 7 months (but not at 1 or 3 months) with animals from the VitD group having higher serum levels. Differences in microbicidal activity followed the same pattern, with no significant differences observed at 1 and 3 months, but at 7 months a significant increase in the percentage of bacteria killed was detected. Furthermore, analysis of the reactive oxygen species (ROS) and nitric oxide (NO) in serum showed a higher production of ROS and NO in VitD-supplemented calves. In contrast, serum concentrations of IL-1 $\beta$  and IL-8 were significantly lower. A similar anti-inflammatory profile was observed after gene expression analysis, with a significant downregulation of a cluster of genes including *IL1B*, *IL1R1*, *CXCL1*, *CXCL2*, *CXCL5*, *MMP9* and *COX2* and an upregulation of *CXCR1*, *CX3CR1* and *NCF1*, in VitD calves after BCG challenge relative to Ctl animals. Collectively, these results suggest that dietary vit D<sub>3</sub> boosts antimicrobial and innate immune responses and thereby could improve host anti-mycobacterial immunity.

## 1. Introduction

Bovine tuberculosis (BTB) is one of the most important bovine zoonoses globally, not only due to its threat to animal and public health but also for the economic losses to agriculture and costs of national control programmes (Zinsstag et al., 2006). BTB is caused mainly by the animal adapted mycobacteria *Mycobacterium bovis* (*M. bovis*) producing granulomatous lesions in the lungs, draining lymph nodes, and other organs

depending on the bacilli's point of entry (Cassidy, 2006). Cattle are considered the main host for *M. bovis*, although other livestock and wildlife species can be infected and act as reservoirs, representing an obstacle for control and eradication programmes (Skuce et al., 2012). Control programmes based on test and cull have decreased the BTB burden in many countries; however, BTB is still a significant problem in developing countries, but also in some federal states of the United States of America (USA), New Zealand, UK, and Ireland (Ramos et al., 2020).

**Abbreviations:** Vit D<sub>3</sub>, vitamin D<sub>3</sub> or cholecalciferol; VDD, vitamin D deficiency; Ctl, control group; VitD, vitamin D-supplemented group; ROS, reactive oxygen species; NO, nitric oxide; BTB, bovine tuberculosis.

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The presence of wildlife reservoirs as well as the high cost of the eradication programmes call for the implementation of additional measures to decrease the incidence of BTB and reduce cattle-to-cattle transmission.

Nutrition has a profound impact on animal health, and micronutrients (vitamins and minerals) play a determining role in regulating and shaping the immune response (Calder, 2013). Recently, vitamin D (vit D) has gained interest for its association with infectious and non-infectious diseases. Vit D is a secosteroid hormone synthesized in the skin after sunlight exposition, and it can also be obtained from the diet through natural foods and supplements (Bishop et al., 2020). The role of vit D in tuberculosis (TB) has been widely investigated with data showing vit D deficiency (VDD) as an independent risk factor for TB infection in humans (Huang et al., 2017). In cattle, low 25(OH)D serum concentrations are associated with BTB, but whether the reduction in circulating vit D levels are the cause or consequence of the disease remains unclear (López-Constantino et al., 2022). Vit D induces pro- and anti-inflammatory effects against mycobacteria that restrain bacillary growth and limit an excessive inflammatory response (Hewison, 2011). The pro-inflammatory activities are associated with the production of cathelicidin and  $\beta$ -defensins which can directly damage the bacterial wall (Drayton et al., 2021; Merriman et al., 2015). Another way in which vit D modulates the microbicidal activity of macrophages is by upregulation of the ROS and NO production, as well as by the induction of autophagy in infected cells (Fabri et al., 2011; Garcia-Barragan et al., 2018; Nelson et al., 2011; Nelson et al., 2010; Waters et al., 2001; Yang et al., 2009). On the other hand, the anti-inflammatory actions of vit D are linked with the reduction of pro-inflammatory cytokines like IFN- $\gamma$  and IL-1 $\beta$ , as well as with the decrease of chemokine production, and with expansion of T reg lymphocytes which in turn limit the activity of Th1 cells (Reeme and Robinson, 2016; Selvaraj et al., 2015; Selvaraj et al., 2012).

In cattle, the vit D pathway is activated in the same manner as in humans, and research suggests that after *M. bovis* exposure there is a rapid mobilization of the vit D metabolite 1,25(OH) $_2$ D $_3$  in blood, which might be necessary for granuloma formation (Nelson et al., 2012; Rhodes et al., 2003). Studies on bovine macrophages have suggested that 1,25(OH) $_2$ D $_3$  stimulates NO and  $\beta$ -defensin production which restrain *M. bovis* replication (Garcia-Barragan et al., 2018; Nelson et al., 2010). Similar studies on *Mycobacterium avium* ssp. *paratuberculosis* (MAP) infected cattle have shown the benefits of 1,25(OH) $_2$ D $_3$  on limiting MAP survival (Wherry et al., 2022). Therefore, these findings suggest that vit D has a high potential for increasing cattle's intrinsic resistance against mycobacteria. However, studies are lacking on investigating the effects of vit D supplementation as a prophylaxis in bovines.

Our research has shown a negative association between the seasonal changes in 25(OH)D serum levels and IL-8 production (O'Brien et al., 2021). Additionally, low 25(OH)D serum levels were associated with a perturbed circulating leukocyte profile in comparison to calves with sufficient 25(OH)D concentration (Flores-Villalva et al., 2021); however, the consequences for disease susceptibility have not been clearly defined. In this study, using our previous model to drive divergent vit D status (Flores-Villalva et al., 2021), we assessed the microbicidal activity and immunoregulatory effect of differing 25(OH)D circulating levels to an *ex-vivo* *M. bovis* BCG challenge. Our data shows that dietary vit D improves mycobacterial growth control by peripheral blood leukocytes and promotes a controlled inflammatory response which can be beneficial to prevent tissue damage.

## 2. Methods

### 2.1. Ethical statement

All experimental procedures were approved by the Teagasc Ethics Committee (TAEC237–2019) and were conducted under the experimental license (AE19132/P105) from the Health Products Regulatory

Authority in accordance with the cruelty to Animals Act (Ireland 1876) and the European Community Directive 2010/63/EU. Reporting in the manuscript follows the recommendations in the ARRIVE guidelines.

### 2.2. Experimental design and treatments

The study was conducted using samples from animals in the indoor control (Ctl) and vitamin D (VitD) groups from our previously described model (Flores-Villalva et al., 2021). Briefly, animals were supplemented in the milk replacer (MR) and ration with vit D $_3$  from birth to 7 months age and were kept indoors during the duration of the trial. Calves from Ctl group were fed with 6000 IU/Kg of vit D $_3$  in MR +2000 IU/Kg of vit D $_3$  in ration. Whereas VitD calves received 10,000 IU/Kg of vit D $_3$  in MR +4000 IU/Kg of vit D $_3$  in ration.

For this study blood samples were taken at three time points corresponding to the age of 1, 3 and 7 months of age.

### 2.3. *M. bovis* BCG whole blood challenge

The BCG whole blood infection method was adapted from Pepponi et al. (Pepponi et al., 2017). Briefly, 300  $\mu$ l of whole blood was placed in a 2 ml tube and 300  $\mu$ l of RPMI medium containing  $1 \times 10^6$  CFU of BCG Denmark added. The tubes were placed in a rotatory platform and incubated at 37°C for 24 h. The tubes were then centrifuged at 500 x g for 5 min, and the supernatant removed and stored at -20 °C. The cell pellets were lysed with 200  $\mu$ l of H $_2$ O with 0.05 % Tween 80 and used to determine the bacterial load by CFU counting. The cell lysate was diluted in ten-fold serial dilutions with 7H9 media. Then, 50  $\mu$ l of each serial dilution was plated on a 7H11 agar petri dish. Plates were put inside a plastic bag (to prevent them drying out) and stored at 37 °C for 2–3 weeks, until visible colonies were counted. All samples were run in duplicate. The CFU/ml was determined with the formula: CFU/ml = (number of colonies x dilution factor)/ volume plated. Percentage of bacterial killing was calculated by the ratio of bacteria used for challenge relative to those killed after 24 h.

A similar aliquot of whole blood was prepared for RNA extraction, but the cell pellet was lysed with 0.8 ml Trizol and stored at -80 °C until analysis. A non-infected (null) sample was prepared in the same way and used as negative control.

### 2.4. Bacterial killing in serum

Analysis of the bacterial killing in serum was done with two types of bacteria, *E. coli* and BCG. The assay was conducted on a 96 well plate in a total volume of 100  $\mu$ l,  $10^8$  CFU of bacteria was incubated with 90 % serum and incubated at 37 °C for 1 h. The number of viable bacteria was determined by serial dilution on agar plates. Enumeration of *E. coli* colonies was done after 24 h, whereas for BCG was carried after 2–3 weeks as previously stated. Analysis was done by duplicate using PBS as a control.

### 2.5. Determination of reactive oxygen metabolites and nitric oxide concentrations

The analysis of reactive oxygen species (ROS) production was carried out using the D-ROM assay (Diacron International, Grosseto, Italy). This assay measures the reactive oxidative metabolites in serum as a reference for ROS production and results are expressed as arbitrary 'Carratelli Units' (CarrU).

Concentration of nitric oxide (NO) was analysed using the Griess reagent system (Promega) following the manufacturer's recommendations. This system measures nitrite (NO $_2$ ) as an indicator of NO production. Absorbance at 550 nm was measured using microplate reader (Clariostar Plus, BMG Labtech). The micromolar concentration ( $\mu$ M) of NO $_2$  was calculated with a nitrites' standard curve using fresh RPMI medium enriched with 10 % FBS.

## 2.6. Haematology, serum 25-hydroxyvitamin D, IL-1 $\beta$ and IL-8 by ELISA

Haematology and determination of 25(OH)D serum concentrations was carried out as part of our previous study (Flores-Villalva et al., 2021). The bovine IL-8 ELISA used to measure IL-8 concentration was carried out as previously described by Cronin et al., (Cronin et al., 2015). The IL-1 beta bovine uncoated ELISA kit (ThermoFisher) was used as per manufacturer's instructions.

## 2.7. RNA extraction, cDNA synthesis and Fluidigm analysis

RNA was isolated from cells using a combination method of Trizol and the NucleoSpin® Mini kit (Macherey-Nagel). Chloroform was added to cells containing Trizol in a 2 ml microfuge and shaken vigorously. The solution was then centrifuged at 12,000 x g for 15 min at 4 °C. The aqueous layer containing RNA was transferred to a clean microfuge tube. An equal amount of 70 % ethanol was then added and mixed vigorously. This solution was then transferred to a kit column, and from this point the manufacturer's instructions were followed. RNA integrity was assessed using the RNA 6000 Nano Kit (Agilent Technologies) on the Bioanalyser according to manufacturer instructions. All samples had a RIN value  $\geq 7.5$ , cDNA was synthesised using the iScript™ cDNA synthesis kit (Bio-Rad) with 15 ng/ $\mu$ l of RNA. Then, gene expression was assessed with the Fluidigm Biomark HD system using a 96.96 chip format according to the manufacturer's instructions. Briefly, a pool of 96 primers was prepared by combining 1  $\mu$ l of each 100  $\mu$ M stock of forward and reverse primers, then a DNA suspension buffer (Fluidigm) is added to make the final volume of 200  $\mu$ l with a final concentration of 500 nM. Then, a pre-mix is prepared by adding 1  $\mu$ l of PreAmp Master Mix (Fluidigm), 0.5  $\mu$ l of the pool of primers and 2.25  $\mu$ l of water. Next, 1.25  $\mu$ l of the cDNA (at 1:5 dilution) for each sample was added to the pre-mix and a pre-amplification is performed in a thermal cycler. The cycling conditions for the pre-amplification consisted of 1 cycle at 95 °C for 2 min, followed by 14 cycles at 95 °C for 15 s, with an extension step at 60 °C for 4 min. The preamplification allows for multiplex amplification of the targets of interest and it is used to increase the number of copies to a detectable level. Then, an Exonuclease-1 (Fluidigm) treatment was performed to remove the unincorporated primers. Samples were diluted 5X in TE buffer and mixed with 2X SsoFast EvaGreen supermix and 20X DNA Binding Dye (Fluidigm) Next, they were loaded in the right-hand side of the 96.96 IFC plate, whereas, in the left-hand side one pair of primers mixed with 2X assay loading reagent (Fluidigm) per well (96 assays) was added. Then, the IFC run was carried out in the Biomark HD Fluidigm. Data were analysed with Fluidigm Real-Time PCR software to determine the cycle threshold (Ct) values. Gene expression was normalized to the mean expression of two reference genes (*PPIA* and *ACTB*) [expression of *GAPDH* was not detected, thus it was removed from the analysis] to obtain the  $\Delta$ Ct value. For each animal, values from BCG challenged samples were normalized to the uninfected-unstimulated sample to obtain the relative gene expression or fold change using the 2- $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001). Lowly expressed or non-detected genes were removed before statistical analysis; the remaining genes and their primers' sequences are shown in suppl. Table 1.

## 2.8. Statistical analysis

GraphPad Prism software version 8 was used for data presentation and statistical analysis, except for analysis of Fluidigm data. Data is presented as mean  $\pm$  SEM, unless otherwise stated. Statistical test and number of samples for each experiment is indicated in the figure legends. Evidence of statistical significance was considered at  $P \leq 0.05$ .

For Fluidigm data, multiple T tests and principal component analysis (PCA) were done in R studio (Version 4.0.3) with the packages rstatix, factoextra, and tidyverse. Standardized log<sub>2</sub> fold change was used for PCA, whereas the differences between groups were assessed on log<sub>2</sub> fold

change by unpaired T test using the Benjamini-Hochberg method with a false discovery rate (FDR) of 0.1.

## 3. Results

### 3.1. Long-term dietary vit D<sub>3</sub> supplementation improves mycobacterial growth control by circulating leukocytes

This study was carried out using samples from our previously published model (Flores-Villalva et al., 2021). Samples from Ctl and VitD groups that had been kept indoors during the trial were used.

No differences in circulating 25(OH)D levels were observed at 1 or 3 months. However, at 7 months animals from the VitD group had significantly higher 25(OH)D concentrations, with a mean of 46 ng/ml in comparison to 38 ng/ml in the Ctl group (Fig. 1 A). Differences in microbicidal activity followed the same pattern, with no significant differences in the number of viable bacteria and the proportion of bacterial killing during month 1 and 3. However, at 7 months a significant drop in the CFU counts and a corresponding rise in bacterial killing was detected in the VitD group (Fig. 1 B). The percentage of bacteria killed in the VitD calves was on average 48.0 % in contrast to 29.5 % in the Ctl group (Fig. 1C and Table 1). Therefore, subsequent analysis was done on samples from 7 months.

A bactericidal assay with serum from 7 months was carried out to assess the microbicidal activity by serum components. Results shown that there were no differences in bacterial growth between serum from Ctl and VitD calves (Suppl. Fig. 1). Therefore, this result suggests that the bactericidal activity against BCG was due to cellular components in the blood. Furthermore, no differences in the cellular profile were observed at this timepoint (Suppl. Fig. 2).

### 3.2. Production of ROS and NO is significantly increased by dietary vit D<sub>3</sub>

Results shown that both ROS and NO were significantly higher in null samples from VitD calves. Mean ROS production in null samples from VitD animals was 123.6 CARR units, in contrast to 54.2 CARR units in Ctl calves. Null NO levels were on average 1  $\mu$ M in Ctl and 5.6  $\mu$ M in VitD group.

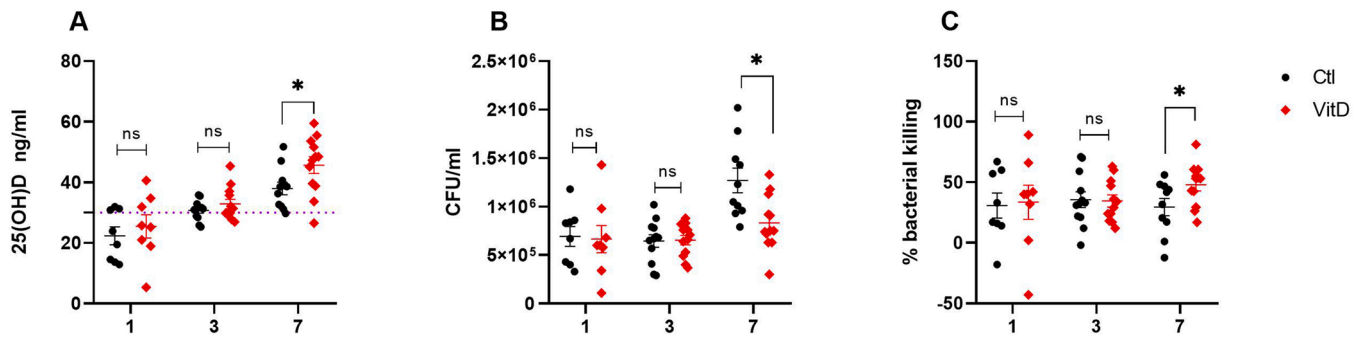
After BCG infection, differences between groups were only significant for ROS production, but not for NO. However, there was no effect of BCG challenge in ROS or NO production since there were no differences between the null and BCG treatment within any group (Fig. 2).

### 3.3. Lower production of IL-1 $\beta$ and IL-8 was observed in animal supplemented with vit D<sub>3</sub>

Levels of IL-1 $\beta$  and IL-8 in null samples were not significantly different between Ctl and VitD groups; however, after BCG challenge the concentration of both cytokines increased although at a significantly lower concentration in the VitD calves. After BCG challenge the mean concentration of IL-1 $\beta$  in Ctl was 2.0 ng/ml in comparison to 0.41 ng/ml in VitD group. For IL-8 a similar pattern was observed, with mean concentrations of 25.02 ng/ml and 14.85 ng/ml in Ctl and VitD calves, respectively (Fig. 3).

### 3.4. Dietary vit D<sub>3</sub> downregulates the inflammatory response

The expression of 96 genes was assessed across samples using the Fluidigm platform. After removal of lowly expressed or non-detected genes, a PCA analysis was performed to analyse the overall structure of the dataset. Fig. 4 shows the representation of four dimensions that explain 57 % of the variance. The results show an overlap in the response between animals from Ctl and VitD group along dimension 1. A group of animals from Ctl treatment separates alongside dimension 2 that explains 11 % of the variance. The separation along dimension 2 from Ctl group is explained by their differential response to *CXCL2*, *CXCL1*,



**Fig. 1.** Microbicidal activity of whole blood from Ctl and VitD calves at 1, 3 and 7 months. Blood from Ctl and VitD groups was infected with  $1 \times 10^6$  CFU/ml of BCG, after 24 h cells were washed and lysed to determine the number of intracellular bacteria. Analysis was done at 1-, 3- and 7-months age. (A) Serum 25(OH)D levels. (B) number of viable bacteria and (C) percentage of bacteria killing at 24 h post-infection. Data is shown as mean  $\pm$  SEM of n = 8–12. Mixed effect model with Bonferroni correction to assess differences between Ctl and VitD groups \*P < 0.05 was considered statistically significant.

**Table 1**  
Mean bactericidal killing in PBL from Ctl and VitD calves.

Time	Ctl			VitD			*P value
	n	Mean	SD	n	Mean	SD	
1 month	8	30.75	29.1	8	33.5	39.89	0.877
3 months	12	35.58	22.26	12	34.58	17.13	0.903
7 months	10	29.5	22.35	12	47.97	17.59	0.049

\* Comparison between groups at each time point with Bonferroni correction

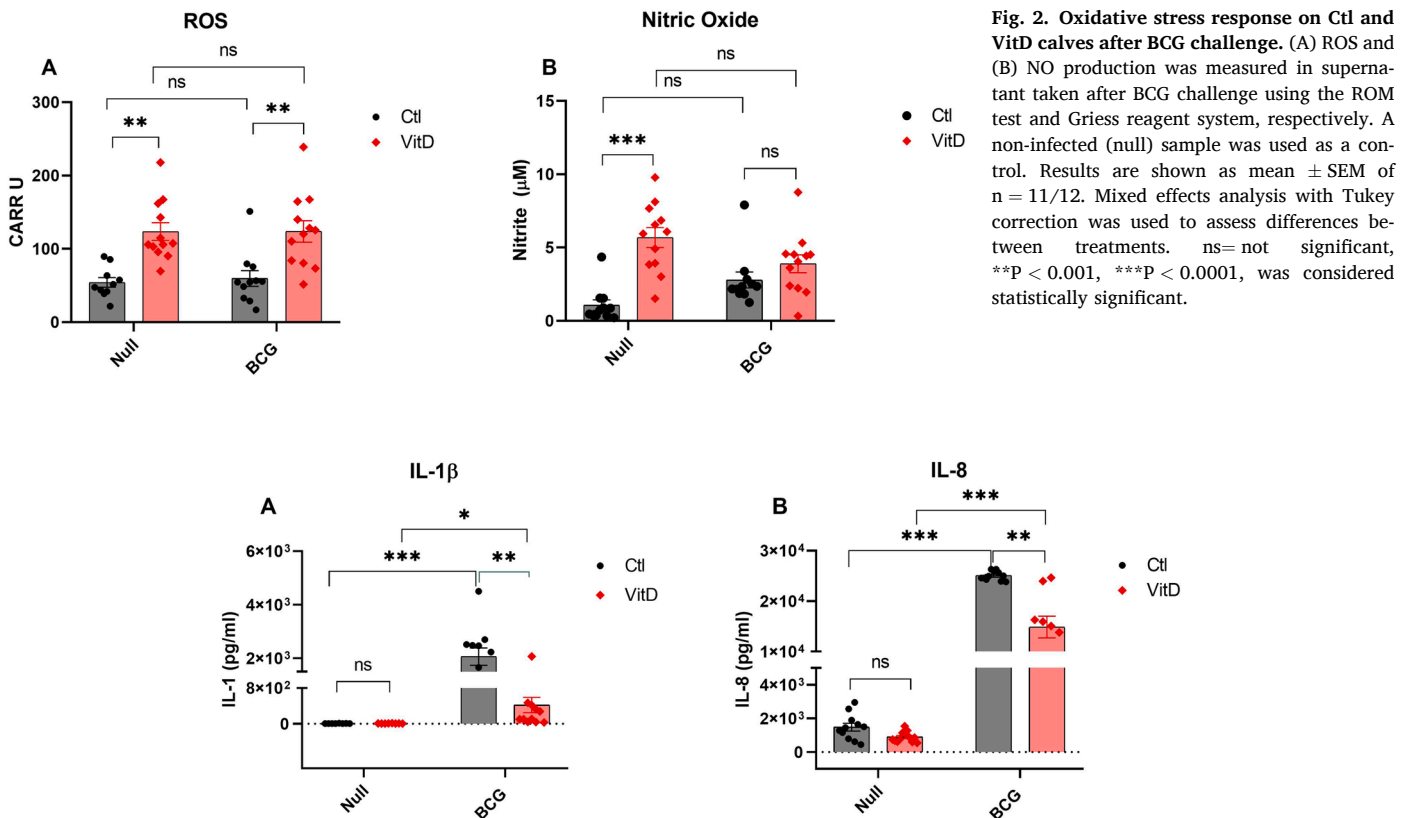
*CXCL8*, *IL1B*, *COX2* and *TNFA* (Fig. 4A). The response to genes such as *DEFB7*, *DEFB4*, *DEFB6* and *NCF1* explain the divergence between Ctl and VitD groups along dimension 3 (Fig. 4B).

Multiple genes were significantly differentially regulated between

Ctl and VitD groups, including *CXCL1*, *CXCL2*, *CXCL5*, *CXCR1*, *CX3CR1*, *NCF1*, *MMP9* and *COX2* (Fig. 5). Similarly, analysis of genes associated with the IL-1 pathway showed a significantly lower expression of *IL1B* and *IL1R1* in VitD calves in comparison to Ctl group (Suppl. Fig. 3). No differential response was observed for *IL1A* and *IL1RN*. Additionally, this data showed a biological trend for higher expression of *IFNB*, *IL10*, *NLRP3* and *CASP13* in VitD calves; however, animal-to-animal variability did not allow this difference to reach statistical significance after P value adjustment (Suppl. Fig. 3).

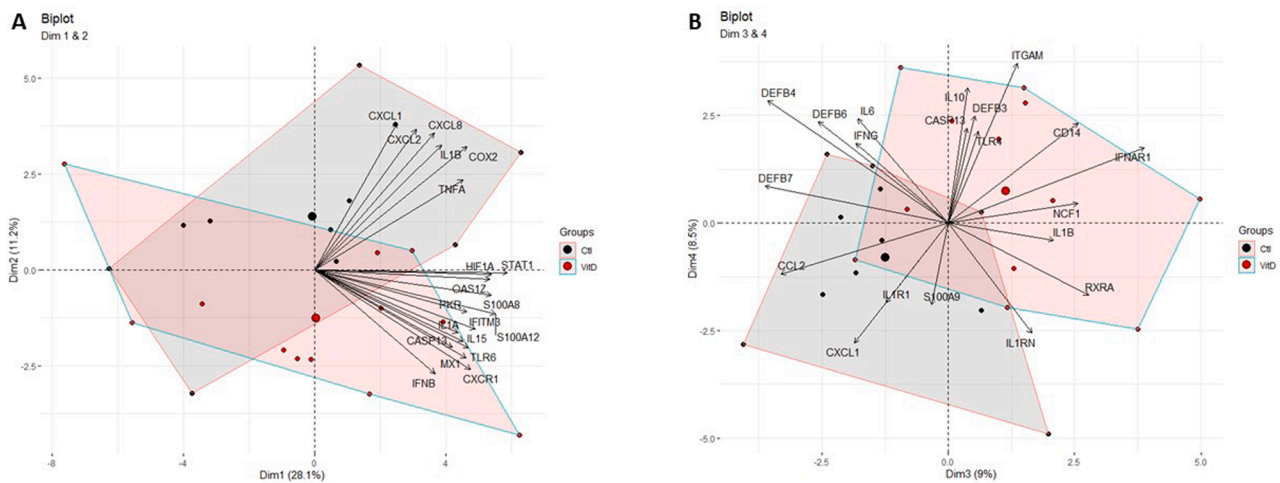
#### 4. Discussion

Despite the growing appreciation of the beneficial role of vit D for health and resistance against infectious diseases, research has



**Fig. 2.** Oxidative stress response on Ctl and VitD calves after BCG challenge. (A) ROS and (B) NO production was measured in supernatant taken after BCG challenge using the ROM test and Griess reagent system, respectively. A non-infected (null) sample was used as a control. Results are shown as mean  $\pm$  SEM of n = 11/12. Mixed effects analysis with Tukey correction was used to assess differences between treatments. ns= not significant, \*\*P < 0.001, \*\*\*P < 0.0001, was considered statistically significant.

**Fig. 3.** IL-1 and IL-8 production on Ctl and VitD calves after BCG challenge. (A) IL-1 and (B) IL-8 levels were measured in supernatants taken after BCG challenge by ELISA. A non-infected (null) sample was used as a control. Results are shown as mean  $\pm$  SEM of n = 11/12. Mixed effects analysis with Tukey correction was used to assess differences between treatments. ns= not significant, \*P < 0.05, \*\*P < 0.001, \*\*\*P < 0.0001 was considered statistically significant.



**Fig. 4.** Biplot of individuals from PCA of gene expression effects on Ctl and VitD groups after BCG challenge. (A) Biplots for dimension 1 and 2, and (B) dimension 3 and 4, showing the representation of individuals and variables within the principal component. Animals are grouped by a convex hull to show the clustering of the groups. Ctl (n = 11), VitD (n = 12).

predominantly focused on murine and human species. This represents a significant gap in the bovine immunology research, and particularly in relation to BTB. In this study we used an *ex-vivo* BCG challenge model to assess the antimycobacterial activity and immunoregulatory effects in calves-fed with standard or high vit D<sub>3</sub> diets for 7 months (Flores-Villaiva et al., 2021). Our results demonstrate that dietary vit D<sub>3</sub> modulates the immune response to BCG by boosting the microbicidal activity and controlling excessive inflammatory response.

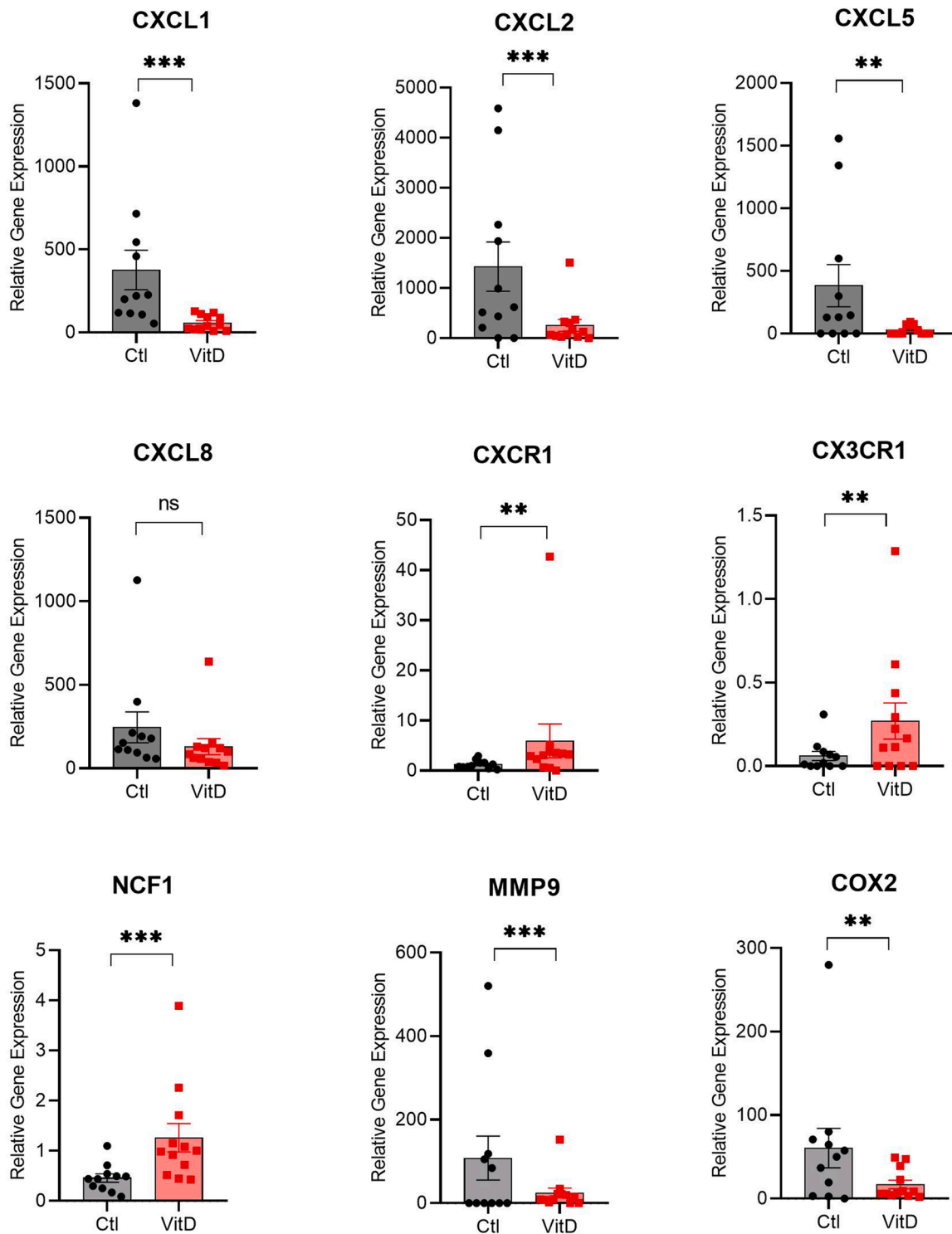
Previous studies have analysed the effects of vit D supplementation on disease susceptibility in cattle. Sacco et al. (Sacco et al., 2012) did not observe an effect on the pathogenesis of respiratory syncytial virus (RSV) infection in calves with differential 25(OH)D circulating levels. In this latter study, new-born calves were fed with a milk replacer containing low or high vit D<sub>3</sub> (1700 or 17,900 IU of vit D/Kg, respectively), and after 10-weeks calves were challenged with RSV. Animals in the high vit D group had on average 177.3 ng/ml of serum 25(OH)D, in contrast to 32.5 ng/ml 25(OH)D levels in the low vit D group. Despite the divergent vit D status, there were no differences in severity of lung lesions between groups. Nonetheless, the authors showed that expression of *IL8*, *IL12B* and *IFNG* were upregulated in the vit D group relative to the low vit D group. However, it remained unclear whether the increased production of pro-inflammatory cytokines would be beneficial in the long term (Sacco et al., 2012). Another study found no differences in the severity of MAP infection in calves fed with colostrum supplemented or not with vit D<sub>3</sub> (or vit ADE) for 14 days (Krueger et al., 2016). However, in this study calves were challenged with MAP after one day of birth, and differences in 25(OH)D circulating levels were observed after 7 days of birth. Thus, it is still uncertain if increased 25(OH)D circulating concentrations are protective against MAP infection (Krueger et al., 2016).

In comparison with our model, the above studies provided vit D<sub>3</sub> supplementation over short time periods, from days to a maximum of 10 weeks. However, data from clinical trials in humans have shown that standard dosage interventions for 6–12 months are more beneficial to prevent acute respiratory infections (Jolliffe et al., 2020; Martineau et al., 2017). Our vit D<sub>3</sub> supplementation study is in line with this evidence and shows that animals receiving a diet with high vit D<sub>3</sub> concentrations (VitD) for 7 months had better ability to restrict BCG than animals fed with low vit D<sub>3</sub> concentrations (Ctl). This was reflected in the fact that we did not observe differences in the BCG killing at earlier time points, but only after 7 months of vit D<sub>3</sub> supplementation. This opens the question of whether increasing the vit D status of calves at younger ages will improve their resilience to infections during the

pre-weaning period (Hulbert and Moisés, 2016). For instance, Martineau et al. (Martineau et al., 2007) showed that a single high dose (2.5 mg) of Vit D<sub>2</sub> enhanced the ability of PBL from TB contact patients to restrict BCG growth. This dose of supplementation corrected VDD in all individuals and reduced BCG-lux luminescence after 24 h of infection (Martineau et al., 2007). Thus, results from this study suggest that raising 25(OH)D serum levels is an effective intervention to enhance immunity to mycobacteria, an effect that might be extended against other bacteria.

Analysis of potential microbicidal mechanisms regulated by vit D included the assessment of NO and ROS production. Our results showed that calves in the VitD group had a higher oxidative stress response than Ctl calves. In addition, our results showed that *NCF1* (which encodes a component of the NADPH oxidase) was significantly upregulated in the VitD group. Thus, collectively these results suggest that after a stressful condition like hypoxia or bacterial challenge, animals with higher serum 25(OH)D concentrations had a differential regulation of the redox signalling pathway which could be advantageous for bacterial control (Weiss and Schaible, 2015). Besides their direct bactericidal killing effect on mycobacteria, ROS and RNS work as secondary messengers on diverse signalling pathways including NF- $\kappa$ B, AP-1, MAPK, PI3K, influencing the expression of genes involved in inflammation, cell proliferation, differentiation, and apoptosis (Gwinn and Vallyathan, 2006). Moreover, ROS plays an important role in regulating autophagy and cathelicidin production, thereby potentially influencing the vit D mediated antimicrobial effects (Yang et al., 2009). Although autophagy was not evaluated in this work, research has shown that this defence mechanism inhibits *M. bovis* survival in bovine neutrophils (Wang et al., 2013). Thus, modulation of ROS production seems to be an important avenue to understand the antimicrobial mechanism of vit D in cattle.

Our results also showed that dietary vit D<sub>3</sub> enhanced NO production in VitD calves in comparison to Ctl animals; however, in contrast to other studies, we did not observe expression of *NOS2* in our study. This might be explained by the different model of infection used, namely PBL instead of macrophages. Previous research has shown that vit D enhances *NOS2* expression in macrophages and monocytes (García-Barragan et al., 2018; Nelson et al., 2010), while gene expression data from PBMC showed no differences after 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment (Nelson et al., 2011). Therefore, it is possible that the use of PBL (or PBMC) allows for crosstalk between monocytes, neutrophils and T cells which influence the dynamics of *NOS2* gene expression. Nonetheless, our data supports the evidence of other research groups which suggests that vit D enhances *M. bovis* killing in bovine cells via a NO-dependent mechanism



**Fig. 5.** Gene expression of differentially expressed genes between Ctl and VitD groups after BCG challenge. Results are expressed as relative gene expression compared to a non-infected sample. Data is represented as mean  $\pm$  SEM of Ctl (n = 11), VitD (n = 12). Difference between groups were assessed by multiple paired T-test, significance is after p-adjustment by Benjamini-Hochberg method. ns = not significant, \*P < 0.1, \*\*P < 0.05. \*\*\*P < 0.01 was considered statistically significant.

(Garcia-Barragan et al., 2018; López-Constantino et al., 2022; Nelson et al., 2010; Waters et al., 2003; Waters et al., 2001).

The pattern of gene expression in PBL after BCG challenge showed a high inter-animal variability, with some animals showing a high response to vit D and others mid or null responses. Furthermore, the correlation analysis did not show a relationship with 25OHD serum concentrations and gene expression or mycobacterial killing. Oral vit D<sub>3</sub>

supplementations studies in human have shown a similar response (Carlberg and Haq, 2018; Seuter et al., 2017; Vukić et al., 2015). It has been shown that the characterization of the response to up to 36 parameters including changes in gene expression, chromatin accessibility and serum proteins and metabolites, allowed the segregation of individuals into high, mid, and low responders to vit D. However, this classification was based on the response inducible by vit D<sub>3</sub> and did not

reflect the 25OHD serum level. Thereby, an individual with high vit D index was a participant that showed a response to most of the parameters evaluated (Carlberg and Haq, 2018; Seuter et al., 2017; Vukić et al., 2015). Our results suggest that calves could be classified by a similar method, but the analysis of more parameters would be necessary to confirm our findings. If it were true, identification of animals with high vit D responsiveness would be of interest since they might be less affected by low 25OHD serum levels.

Analysis of gene expression showed an anti-inflammatory profile in VitD calves in comparison to Ctl animals after BCG infection. Remarkably, a significant lower production of IL-1 $\beta$  and IL-8 was observed at protein and mRNA level in the VitD group. IL-1 $\beta$  is recognized for its role in mediating control of *M. tuberculosis* infection, although an overactive response can contribute to pathology and disease severity (Liu et al., 2009; Verway et al., 2013). IL-1 $\beta$  production is regulated by multiple mechanisms including IFNs and the inflammasome NLRP3. Studies in vitro have shown that 1,25(OH) $_2$ D $_3$  and 25(OH)D boost IL-1 $\beta$  production in a bimodal fashion, by increasing *IL1B* transcription and by promoting the maturation of the protein through the NLRP3 inflammasome (Tulk et al., 2015; Verway et al., 2013). On the other hand, studies in mice showed that IFN- $\beta$  inhibits IL-1 $\beta$  production in macrophages and dendritic cells by an upregulation of the *IL1R* antagonist (IL-1Ra) and by promoting IL-10 expression (Mayer-Barber et al., 2011). Likewise, data from patients with multiple sclerosis has shown that vit D synergises with IFN- $\beta$  to reduce the inflammatory response by CD4 $^+$  T cells lymphocytes (Bianchi et al., 2020). Overall, these data suggest that there is a complex interplay between vit D, type I IFN signalling, inflammasome and cytokine expression that together promotes a controlled inflammatory immune response. Our results suggest a similar biological mechanism, where VitD calves had a reduction in IL-1 response but not a complete inhibition. IL-1 $\beta$  production was significantly upregulated after BCG challenge in both Ctl and VitD in comparison to non-infected treatments. However, dietary vit D promoted a controlled IL-1 $\beta$  response in VitD calves. NLRP3 was upregulated in VitD but not in Ctl which suggest that inflammasome activation induced an efficient IL-1 $\beta$  maturation adequate to control BCG growth. Furthermore, *IL1B* expression was regulated by *IFNB* and *IL10* in VitD calves, whereas an uncontrolled IL-1 $\beta$  response was observed in Ctl animals. However, analysis of IL-1 modulation by vit D for disease outcome and severity requires further investigation.

The anti-inflammatory profile observed in VitD calves was not only limited to the IL-1 pathway, but to a cluster of genes *CXCL1*, *CXCL2*, *CXCL5*, *COX2* and *MMP9*. *CXCL1*, *CXCL2* and *CXCL5* are involved in neutrophil recruitment, and *COX2* and *MMP9* are regulatory enzymes in pathways of inflammation, known to promote lung tissue damages. This transcriptomic signature suggests a control of neutrophil recruitment and inflammation by VitD. Suppression of CXC chemokines by 1,25(OH) $_2$ D $_3$  was reported on PBMCs from patients with active TB (Selvaraj et al., 2012). Our data shows that although IL-8 production was reduced in VitD calves, expression of the IL-8 receptor (*CXCR1*) was upregulated in this group of animals, suggesting that dietary vit D promotes a differential regulation of leukocyte recruitment upon infection to prevent an overzealous inflammatory immune response, which is also supported by the lower expression of *COX2* and *MMP9* in VitD calves.

Finally, given the low statistical significance of BCG killing between VitD and Ctl groups ( $P = 0.049$ ), another plausible explanation is that the result occurred by chance (type 1 error). However, based on data from other studies on bovine and human cells (Garcia-Barragan et al., 2018; Liu et al., 2006; Liu et al., 2007; López-Constantino et al., 2022; Verway et al., 2013; Wang et al., 2004; Yang et al., 2009), the results suggest that the increased BCG killing in VitD calves reflects a meaningful enhancement of global antimycobacterial host response. And even the results in bacterial killing were modest, the effects on the inflammatory immune response were substantially different between groups. These differences could shape the outcome to infection by dampening the potentially harmful inflammatory T cell responses.

Furthermore, vit D can modulate the function of other immune cells including neutrophils and thereby limit the inflammatory response and participate in the control of bacilli growth (Rhodes et al., 2003; Selvaraj et al., 2012).

## 5. Conclusion

Collectively our results have shown that vit D mediates protection against BCG by enhancing microbicidal activity of innate cells through a ROS/RNS mechanism. Likewise, dietary vit D exerts anti-inflammatory effects by inducing a controlled production of inflammatory agents including *IL1B*, *IL8*, and *CXC* chemokines. Because of the similarities between cattle and humans, the bovine model provides an opportunity to delve into the complexities of the vit D effects in response to mycobacterial infections, research that would have positive impacts for public health and the agriculture sector.

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## CRediT authorship contribution statement

Conceived and designed the study: SFV, KM, SG. Performed the experiments: SFV, AR, CR. Statistical analysis: SFV and CR. Wrote and edited the paper: SFV, and KM. Interpretation of results: all authors. All authors read and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetimm.2023.110575.

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