dairy

MDPI

Article

Non-Specific Cross Protection of BCG Vaccination in Dairy Calves

Renata López [1], Valentina Villarroel [2], Víctor Neira [2], Carolina Aguayo [3], Karina Saadi [3], Katherinne Orozco [2], Pedro Abalos [2] and Patricio Retamal [2,*]

[1] Programa de Magister en Ciencias Animales y Veterinarias, Universidad de Chile, Santiago 8820808, Chile; renalo93@gmail.com
[2] Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Santiago 8820808, Chile; v.villarroel.1@ug.uchile.cl (V.V.); victorneira@u.uchile.cl (V.N.); korozcob@gmail.com (K.O.); pabalos@uchile.cl (P.A.)
[3] Servicio Agrícola y Ganadero de Chile, Santiago 9020000, Chile; carolina.aguayo@sag.gob.cl (C.A.); karina.saadi@sag.gob.cl (K.S.)
* Correspondence: pretamal@uchile.cl; Tel.: +56-229785690

Abstract

Bovine tuberculosis (bTB) is mainly caused by *Mycobacterium bovis*, which affects cattle, leading to significant economic losses. In Chile, the vaccination with the *M. bovis* Bacillus Calmette-Guérin (BCG) strain has been implemented in dairy herds with high prevalence of bTB. This study evaluated non-specific protection associated with BCG on the detection of pathogen-associated genes (*nsp5*, *stx1*, *stx2*, *invA*, IS1081) and mortality related to diarrhea and pneumonia in calves. A total of 186 calves from a commercial dairy farm were enrolled and grouped as vaccinated (n = 96) and non-vaccinated (n = 90). The BCG Russia strain (2–5 $\times$ 10^5 UFC) was inoculated subcutaneously within the first 30 days after birth. Animals were monitored through fecal sampling at 3 and 6 months of age for molecular detection of gene sequences. A logistic regression analysis showed differences in detection rates of the *stx1* sequence at 3 months, with a higher risk for the non-vaccinated individuals (OR 2.91, CI 1.42–5.94, $p = 0.03$) and for those born in the cold season (OR 9.55, CI 2.02–45.11, $p = 0.004$). A Kaplan–Meier survival analysis showed a significant difference in deaths in vaccinated calves compared with non-vaccinated animals ($p = 0.018$), suggesting that BCG confers non-specific protection during the first 3 months after birth, in field conditions.

Keywords: BCG; vaccination; calves; non-specific; protection

check for updates

Academic Editor: Erminio Trevisi

Received: 25 July 2025
Revised: 18 September 2025
Accepted: 14 October 2025
Published: 16 October 2025

Citation: López, R.; Villarroel, V.; Neira, V.; Aguayo, C.; Saadi, K.; Orozco, K.; Abalos, P.; Retamal, P. Non-Specific Cross Protection of BCG Vaccination in Dairy Calves. *Dairy* **2025**, *6*, 60. https://doi.org/10.3390/dairy6050060

Copyright: © 2025 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Bovine tuberculosis is a chronic, zoonotic bacterial disease mostly caused by *Mycobacterium bovis*. With a high host range, among domestic reservoirs it primarily affects cattle, leading to significant economic losses in the livestock industry due to decreased productivity in beef and dairy herds, as well as export restrictions, mandatory culling of animals, condemnations, and price penalties faced by producers [1]. This disease has a worldwide distribution and is of great importance in veterinary medicine and public health, especially as an occupational hazard. Various countries have implemented eradication programs that have successfully controlled tuberculosis in cattle [2]. However, this disease remains prevalent in developing countries or in regions with persistent infection hotspots in wildlife [2,3].

Historically, in human populations, the *M. bovis* Bacillus Calmette-Guérin (BCG) vaccine has been included in the immunization programs of various countries to confer protection against pediatric tuberculosis, being currently the only available vaccine against *M. tuberculosis* [4]. Furthermore, multiple studies have documented a reduction in infant mortality attributable to the heterologous or non-specific effects of the vaccine, conferring cross protection against tumoral cells and unrelated pathogens [5–7]. Additional evidence about its adjuvant properties shows enhancement of immune responses to influenza A (H1N1) and hepatitis B vaccines, as well as reductions in viral replication and viral load in experimental models using the yellow fever vaccine virus [6]. During the SARS-CoV-2 pandemic, retrospective analyses indicated that individuals vaccinated within the preceding five years exhibited lower infection rates, reduced disease duration, and milder clinical manifestations [8], although these findings remain subject to ongoing debate due to conflicting reports [9].

Recently, the vaccination of cattle with BCG has been recommended for areas with a high prevalence of bovine tuberculosis [10], a measure that may reduce the incidence in animals and the occupational risk for farmers. In cattle, multiple experimental and field studies have been conducted to determine the efficacy of BCG vaccination, inducing significant protection against *M. bovis* infection and disease [11]. Furthermore, non-specific effects have also been documented, stimulating the cellular immune response against antigens and infections associated with other pathogens under experimental settings [12,13]. In studies performed under field conditions, the BCG vaccination improves health indicators during the lactation period, reducing the incidence of post-partum diseases and clinical mastitis, as well as allowing higher milk yields when compared with non-vaccinated animals [14,15]. These non-specific effects have been linked to myeloid cells of the innate immune system through epigenetic mechanisms, including DNA methylation, histone modifications, and non-coding RNA expression [16]. This phenomenon, which is known as trained immunity, is mediated by monocytes, macrophages, and natural killer cells [16,17], resulting in an increased response against secondary pathogens, greater production of inflammatory mediators, and an enhanced capacity to eliminate infections [16].

In Chile, bTB is an endemic disease with a variable geographical distribution. To address this, the official plan for its control and eradication, launched in 2011 [18], works under the eradication goal in the northern and southern areas, in which both animal- and herd-level prevalence are low. Conversely, central Chile has the highest national prevalence, where the efforts are focused in disease control [19]. This area is characterized by dairy herds with intensive production systems, whose owners are unwilling to cull reactor animals due to lack of compensation and, consequently, the plan faces difficulties for cleaning up infected farms, ultimately hindering the eradication of the disease [19]. This epidemiological context prompted the implementation of BCG vaccination in 2016, specifically for dairy herds with high bTB prevalence within the Metropolitan Region, as a complementary tool for controlling the disease. Consequently, neonatal calves and 12-month-old heifers from several farms have been vaccinated, experiences that have allowed the description of specific and non-specific effects associated with the BCG vaccine [15,20]. Because the immune response interferes with traditional diagnosis, vaccinated animals have been tested using the interferon gamma release assay with DIVA (Differentiating Infected from Vaccinated Animals) antigens, such as ESAT-6, CFP-10, and Rv3615 [20].

On the other hand, the most common infectious disorders affecting unweaned calves are diarrhea and respiratory disease, which lead to economic losses for farmers due to deaths or growth delays and medical treatment costs [21]. Diarrhea has a complex and multifactorial etiology and is the main cause of calf mortality in dairy farms [22]. Among the most identified infectious agents are *Escherichia coli* pathotypes, *Salmonella enterica*,

and Rotavirus, although mixed infections involving multiple pathogens are frequently observed [23]. The prevalence and incidence of each pathogen vary depending on geographic location, management practices, and herd size [23]. The aim of this study was to evaluate the non-specific cross protection of BCG vaccination in calves from a commercial dairy farm in central Chile, through comparing the occurrence of mortality and the presence of pathogen-associated genes between vaccinated and non-vaccinated animals.

2. Materials and Methods

The herd

The dairy herd, located in the Metropolitan Region, was operated as an intensive production system with permanent confinement and was classified as a tuberculosis-infected farm. It had 320 Holstein Friesian (HF) and hybrid HF-Swedish Red (HFSR) breeding cows in milk production. Males were bred between one and two years until they were sold to the slaughterhouse. The historical prevalence of bTB or the presence of other infectious diseases was unknown in males, because these animals were not routinely checked.

Animals and BCG vaccination schedule

Between January 2022 and May 2023, monthly visits were conducted at the farm, enrolling male calves up to 30 days old (0 to 4 weeks) in a double-blinded study. A vaccinated group (n = 96) and a control group (n = 90) were selected with the aim of identifying differences in the presence of virulence-associated genes and death rates, using a confidence level of 0.95, p1 = 0.15, p2 = 0.05, and a power of 0.8. After birth, calves were immediately separated from their mothers and were bred in individual cages until 4 months old, when they were moved to collective pens.

Animals were randomly assigned to each group. Those vaccinated received 2–8 $\times$ 10^5 colony-forming units (CFU) (0.1 mL) of the BCG Russia strain (Serum Institute, Pune, India) by the subcutaneous route. Calves in the control group received 0.1 mL of NaCl 0.9% (vaccine diluent).

Molecular diagnosis

Fecal samples of all animals were collected individually in sterile tube (10–20 g), directly from the rectum using disposable gloves, at 3 and 6 months of age. For viral detection, a fraction of the fecal sample was placed in a tube with 2 mL of viral transport medium (Minimal Essential Medium, TPCK-treated trypsin 1×, 0.3% bovine serum albumin, and 1% antibiotic-antimycotic solution containing penicillin, streptomycin, and amphotericin B) to maintain viral integrity. Each sample was properly labeled, transported, and refrigerated at 4 °C. Once at the laboratory, samples were processed within a maximum of 24 h. The DNA extraction was performed using a commercial column-based kit (Stool DNA Isolation Kit®, Norgen Biotek, Thorold, ON, Canada), following the manufacturers' instructions, and stored at −20 °C until use. The RNA extraction was performed using a protocol described previously [24]. Briefly, samples were vortexed to dissolve any remaining material in the swab, followed by centrifugation to separate and remove larger particles. The supernatant was collected and aliquoted into 1.5 mL tubes. Then, 1 mL of Chomczynski's solution with phenol was added and cells were lysed by repeated pipetting. After an incubation for 5 min at room temperature (RT), 0.2 mL of chloroform were added and samples were shaken vigorously for 15 sec and then incubated for 3 min at RT. A centrifugation at 12,000× *g* for 15 min at 4 °C was performed, and the upper phase was collected into a new 1.5 mL tube. The RNA was precipitated by adding 0.5 mL of isopropyl alcohol, followed by an incubation for 10 min at RT. Samples were centrifuged at 12,000× *g* for 15 min at 4 °C, the supernatant was removed, and the precipitated RNA was washed with 1 mL 75% ethyl alcohol. Then, after a new centrifugation at 7500× *g* for 5 min at 4 °C, the supernatant was

removed, the precipitate was air dried for 10 min, the RNA was dissolved in 100 mL of nuclease-free water by repeated pipetting and stored at –80 °C until use.

The extracted genetic material was subjected to reverse transcription real-time PCR (rt-RTPCR) and real-time PCR (RTPCR) to detect virulence-associated genes from pathogens causing disease and mortality in calves, following protocols described previously (Table 1). The targets included the *S. enterica invA* invasion gene, the *E. coli* Shiga toxins *stx1* and *stx2* genes, the bovine rotavirus *nsp5* gene, and the insertion sequence IS1081, associated with the *Mycobacterium tuberculosis* complex (MTC). The *stx1*, *stx2*, and *invA* PCR reactions were performed in a 30 µL volume using 15 µL of TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA), 1.5 µL primer-probe mix, 10.5 µL milli-Q pure water, and 3 µL of template DNA. The *nsp5* rt-RTPCR and RTPCR reactions were performed in a 20 µL volume using 11 µL of AzuraQuant® Probe 1-Step qPCR Mix NoRox (Azura Genomics, Raynham, MA, USA), 2.5 µL primer-probe mix, 1.5 µL milli-Q pure water, and 5 µL of template DNA. The IS1081 RTPCR assay was performed in a final volume of 20 µL using KAPA Fast 2× Master Mix (Roche Holding AG, Basel, Switzerland), and 5 µL of template DNA. All reactions contained 0.5 µM of each primer and 0.25 µM of probe. Thermal cycling conditions are described in the Supplementary Table S1. The assay detection limit was Ct 36. The amplification was performed using the respective protocols in a Real-Time PCR Instrument (QuantStudio™3, Applied Biosystems, Marsiling, Singapore). Positive controls for RTPCR were *E. coli* reference strain 933 J (*stx1*, *stx2*), *Salmonella* Enteritidis SARB18 (*invA*), a previously confirmed bovine rotavirus positive sample (*nsp5*), and the *M. bovis* BCG strain (IS1081).

Table 1. Pathogen-associated sequences detected by real-time PCR.

Target	Oligo Sequences (5′ to 3′)	Amplicon Size (bp)	Reference
invA	F′-CGTGTTTCCGTGCGTAATA R′-GCCATTGGCGAATTTATG Probe-FAM/ATTATGGAAGCGCTCGCATT/BHQ1	138	[25]
stx1	F-ATGTCAGAGGGATAGATCCA R-TATAGCTACTGTCACCAGACAAT Probe-FAM/CGCTTTGCTGATTTTTCACATGTTACC/BHQ1	185	[26]
stx2	F-AGTTCTGCGTTTTGTCACTGTC R-CGGAAGCACATTGCTGATT Probe-TET/CACTGTCTGAAACTGCTCCTGT/BHQ1	160	[26]
nsp5	F-AACGATCCACTCACCAGCTTT R-ATTGCTTGATGGTCGTGATTG Probe-FAM/TGAATCCATAGACACGCCAGC/BHQ1	105	[27]
IS1081	F-GAGGGCTACCGAGAGATCCT R-GACCAGGTCGCGGAAGAA Probe-FAM/TCCAGGTCACCTCCGCCGAG/BHQ1	84	[28]

Statistical analysis

Generalized linear models with a logistic regression approach were fitted to evaluate the relationship between predictor variables and the binary response variable (absence or presence of gene sequences) [29]. The predictor categorical variables included the vaccination status (BCG or control), the season of birth (warm months from October to March, or cold months from April to September), and the breed (HF or HFSR). Statistical significance was established at $p < 0.05$. Model selection was based on Akaike's Information Criterion, utilizing the glmulti algorithm. Additional chi-square tests were conducted to further explore significant relationships in the model.

The farm personnel recorded animal deaths and their causes. When they observed symptoms like respiratory distress, increased respiratory rate, reduced feeding, nasal discharge, coughing, and fever, the attributed cause was pneumonia. If symptoms were loose or watery stools, weakness and dehydration, the recorded cause was diarrhea. Diseased animals received broad-spectrum antibiotics (oxytetracycline and amoxicillin) and parenteral fluids for hydration in cases of diarrhea. Due to economic issues of the farm, the etiological diagnosis was not performed. With death records, a Kaplan–Meier survival curve was generated and compared using the Log-Rank test to determine statistically significant differences in survival rates between BCG and control groups. Finally, Welch's t-test was used to compare the mean Ct values of detected genes between vaccinated and control groups. All analyses were performed using RStudio 4.3.3 and Infostat 2020v software®.

3. Results

A total of 186 male calves were enrolled and then analyzed for detection of target genes in their feces. However, samplings at 3 and 6 months recovered a lower number of individuals due to mortality (Table 2 and Supplementary Tables S2 and S3).

Table 2. Frequency of pathogen-associated genes and diarrhea- and pneumonia-related deaths according to animal age.

		3 Months N (%)	**6 Months N (%)**
Target sequences			
	invA	1/149 (0.7)	0/122 (0)
	stx1	57/149 (36.3)	22/122 (18)
	stx2	113/149 (69.1)	34/122 (27.9)
	stx1/stx2	43/149 (28.9)	17/122 (13.9)
	nsp5	25/149 (16.8)	ND
	IS1081	0/149 (0)	0/122 (0)
Deaths			
	Diarrhea	5/163 (3.1)	2/148 (1.4)
	Pneumonia	10/163 (6.1)	0/148 (0)
	Total	15/163 (9.2)	2/148 (1.4)

The PCR results showed a higher detection rate of target genes at 3 months than at 6 months, with Shiga toxin-coding genes being the most prevalent sequences in this study (Table 2). Significant odds ratio was found for the *stx1* gene detection in the control non-vaccinated group at 3 months (OR 2.91, CI 1.42–5.94) (Table 3, Supplementary Table S4). Being born during the colder season was another predictor variable associated with higher detection of *stx1* (OR 8.52, CI 1.84–39.42), as well as the *stx1*/*stx2* combination (OR 4.12, CI 1.50–11.26) (Table 3, Supplementary Table S5). No interaction between vaccination status and season was detected ($p > 0.05$). Breed was not associated with the detection frequency of any gene (Table 3, Supplementary Table S6). Neither the presence of other pathogens' sequences (*invA*, *nsp5*, IS1081) nor the mean Ct values of the real-time PCR assays differed by vaccination status, season, or breed ($p > 0.05$). It was not possible to process samples at 6 months for *nsp5* detection as the RNA extracts were unavailable due to storage-related issues.

The analysis of at least one gene sequence detection (*nsp5*, *stx1*, *stx2*, *invA*) at 3 months showed that calves born during the cold season had a higher risk of carrying any of these virulence genes (OR = 2.49, 95%CI 1.06–5.81, $p = 0.035$).

In samples collected at 6 months, only *stx1* and *stx2* sequences were detected (Table 2), without any significant OR associated with the explanatory variables ($p > 0.05$).

Table 3. Odds ratios (OR) for molecular detection of Shiga toxin-coding genes at 3 months according to vaccination group, season of birth, and breed of animals.

Sequence	Explanatory Variable	OR	95% CI	*p*-Value
stx 1	Group: Control	2.91	1.42–5.94	0.003
	Season: Cold	8.52	1.84–39.42	0.006
	Breed: HFSR	0.64	0.31–1.30	0.214
stx2	Group: Control	1.19	0.59–2.42	0.629
	Season: Cold	2.13	0.81–5.61	0.126
	Breed: HFSR	1.10	0.54–2.23	0.796
stx1/stx2	Group: Control	1.41	0.63–3.28	0.407
	Season: Cold	4.12	1.50–11.26	0.006
	Breed: HFSR	1.05	0.46–2.38	0.906

OR, odds ratio; CI, confidence interval; HFSR, Holstein Friesian × Swedish Red crossbred.

To evaluate the potential non-specific cross protection of BCG, deaths associated with diarrhea or pneumonia were compared between vaccination groups. Other non-infectious causes of death, such as traumatic injuries, food-associated disorders (ruminal bloat, acidosis) or unknown causes were not included in the analysis (Supplementary Table S3). Thus, 15/163 (9.2%) and 2/148 (1.4%) of calves died by diarrhea or pneumonia at 3 and 6 months, respectively (Table 2), showing a significant difference in survival rates in the follow up at 3 ($p = 0.018$) but not at 6 ($p = 0.129$) months between the vaccinated and the control groups (Figure 1).

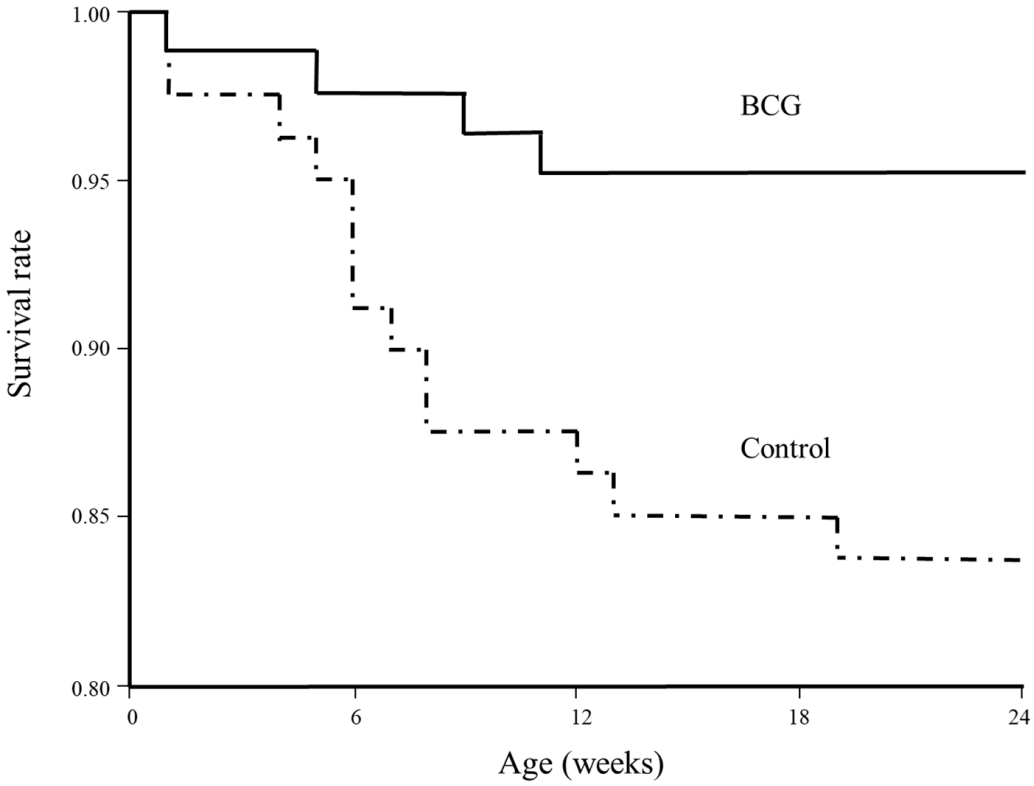

Figure 1. Kaplan–Meier analysis comparing survival rates between BCG vaccinated (solid line) and non-vaccinated control group (segmented line), including deaths associated with diarrhea or pneumonia in calves ($p = 0.018$).

4. Discussion

The BCG vaccine has been associated with several non-specific effects in humans and animals, encouraging the research on immunological responses and protection after its inoculation [30]. In cattle, the BCG vaccination has also exhibited epigenetic and functional changes in immune system cells [12,31], prompting us to analyze BCG cross protection in calves, whose survival rates became a critical indicator of health and welfare in dairy farms [32]. Although several factors are involved in calf health, infectious diseases represent a great proportion of morbidity and mortality, especially those caused by diarrhea and pneumonia [33]. In Chile, there is a lack of reliable data about the incidence of mortality in dairy calves attributed to these diseases' symptoms. In this work, diarrhea and pneumonia records corresponded to 17.2% and 34.5% of total deaths during the first 3 months, respectively, which are within the range observed in other studies [34–36]. In the first few weeks of life, the immune system of calves is not entirely functional and relies on passive immunity provided by colostrum, but by 4–6 months, it progressively matures and responds more efficiently [37]. Despite this, in this work, the BCG vaccination showed an early protective effect against infections, decreasing mortality associated with pneumonia and diarrhea during the first 3 months ($p < 0.05$) (Figure 1).

Except for IS1081, the targeted sequences of this study were related to diarrheagenic pathogens in calves. Diarrhea is a common gastrointestinal disorder in unweaned calves, representing economic losses for farmers due to high mortality rates, growth delays, and medical treatment costs [21]. Among causal agents, pathogenic *Escherichia coli*, *Salmonella enterica*, and Rotavirus are frequently observed, both in single and mixed infections. The prevalence and incidence of each pathogen vary depending on geographic location, management practices, and herd size [38]. In this work, the most frequent gene sequences detected were *stx1* and *stx2*, which have been identified in several bacterial species [39], although in cattle they are mostly associated with the Shiga toxin-producing *E. coli* (STEC) pathotype. At 3 months, 79% of samples had any or both sequences, in agreement with other South American studies that show a high bacterial prevalence [40,41]. At 6 months, the detection rate decreased to 32%, consistent with previous reports indicating a lower prevalence when microbiota stabilizes and the immune system matures [42]. Both Stx1 and Stx2 are virulence factors whose presence in *E. coli* strains is relevant to public health, due to the clinical consequences of STEC in humans, inducing life-threatening hemorrhagic colitis and hemolytic uremic syndrome (HUS) conditions [43]. In the epidemiology of this bacterium, cattle are considered the main animal reservoir and the infection source for humans, usually as asymptomatic carriers of this pathogen [39]. In this work, only the frequency of the *stx1* gene was variable between groups at the age of 3 months, suggesting that the BCG vaccination modifies the host–pathogen interaction with bacteria expressing this particular toxin type. Such BCG effect may be related with the induction of a trained immunity phenotype, and by changes in the gut microbiota observed after vaccination [44], although this study did not give clues in this issue and no reports of these changes exist in vaccinated cattle. Shiga toxins act as immunosuppressive factors, targeting cells of the innate and adaptive immune response [45]. Moreover, when STEC has been associated with calf diarrhea and mortality, the *stx1* gene seems to be more incidental than *stx2* [46,47]. It can therefore be speculated that the non-specific protective mechanisms stimulated by BCG primarily targeted Stx1, decreasing its frequency in the vaccinated group (Table 2). How these mechanisms cause this effect is a question that remains to be elucidated.

Detection rate of *nsp5*, the Rotavirus-associated sequence, was lower at 3 months than Shiga toxin genes, and did not show significant differences between vaccinated (15.4%) and control animals (18.3%) (Table 2). Reported prevalence ranges from 10% to 63% in diarrheic calf samples [48–50], aligning with the detection rate of tested animals in this

work. However, scarce information about bovine rotaviruses at a national level precludes any comparison with other local data. The only sample with the *Salmonella invA* gene detection suggests a low incidence of this pathogen, in agreement with another study from southern Chile [51].

The MTC was analyzed by RTPCR using the IS1081 target sequence (Table 1), because the dairy farm was categorized as positive by the official surveillance system. Calves were immediately separated from their mothers after birth and were raised in individual cages during the first 4 months, and their infection status at the beginning of the study was unknown. The RTPCR results suggested that these animals were probably not infected or had a very low incidence of MTC bacteria during the period of this study.

The colder season (autumn–winter) was another factor associated with the detection of *stx1* and the *stx1*/*stx2* pair (Table 3). In this context, seasonality has not demonstrated a consistent pattern in other studies, with variations in the months when Shiga toxin genes are most frequently detected, even among farms sampled during comparable time periods [52,53]. Since neonatal calves do not adequately regulate their body temperature in extreme environmental temperatures, they are susceptible to hypothermia or hyperthermia, particularly when pen conditions are inadequate, negatively affecting their immune status [38,54,55]. Thus, the interplay between management practices and environmental temperatures may be critical for the incidence of pathogens expressing Shiga toxins.

The third independent variable analyzed was the breed of the animals, because the host's genetics has shown relevance in the BCG response [56]. In addition, a previous study in cows with the same breed categories as this work showed a differential non-specific effect after BCG vaccination [15]. However, in this study, the breed was not a significant factor (Table 3), suggesting that the relationship between the genetic background and the BCG response is diverse and requires further analyses along the productive cycle of dairy cattle.

5. Conclusions

In conclusion, these results demonstrated a significant influence of BCG vaccination in calves for inducing cross protection against mortality associated with pneumonia and diarrhea in the first three months. The differential detection of the *stx1* gene between vaccinated and control groups suggests that BCG may influence host–pathogen interactions, through yet unknown non-specific mechanisms, probably linked to trained immunity or microbiota modulation. The high detection rates of Shiga toxin genes highlight their epidemiological and public health importance, reinforcing the role of cattle as reservoirs for bacteria carrying these sequences. Seasonal conditions also appeared to influence pathogen detection, underlining the interplay between environmental factors and calf immunity. These findings collectively support the hypothesis that BCG vaccination can confer early, non-specific protection against infectious diseases in calves, reducing the incidence of MTC and other zoonotic agents, and consequently improving the health status in the animal–human interphase.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/dairy6050060/s1.

Author Contributions: Conceptualization, R.L. and P.R.; methodology, R.L., V.V., C.A., K.O. and P.A.; formal analysis, P.R., R.L. and V.V.; investigation, K.S. and V.N.; resources, K.S., V.N. and K.O.; data curation, R.L., V.V. and P.R.; writing—original draft preparation, P.R.; writing—review and editing, R.L., V.V., V.N., K.S., C.A., K.O. and P.A.; project administration, P.R.; funding acquisition, P.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Fondecyt N° 1221818 project.

Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of University of Chile (protocol code N° 22555-VET-UCH, 28 April 2022).

Informed Consent Statement: The study was performed after an informed consent was signed by the farm owner.

Data Availability Statement: The original contributions presented in this study are included in the article and the Supplementary table. Further inquiries can be directed to the corresponding author.

Acknowledgments: We thank Oscar Crespo, Natalie Hultazo, Angela Ortíz, María Isabel Stevenson, for their support in laboratory and fieldwork procedures. We also thank the personnel of Animal Virology Lab at FAVET, and the Biotechnology Lab at SAG.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

BCG	Bacillus Calmette-Guérin
bTB	Bovine tuberculosis
HF	Holstein Friesian
HFSR	Hybrid Holstein Friesian—Swedish Red
HUS	Hemolytic uremic syndrome
MTC	*Mycobacterium tuberculosis* complex
RTPCR	Real-Time PCR
STEC	Shiga toxin-producing *Escherichia coli*

References

1. Pérez-Morote, R.; Pontones-Rosa, C.; Gortázar-Schmidt, C.; Muñoz-Cardona, Á. Quantifying the Economic Impact of Bovine Tuberculosis on Livestock Farms in South-Western Spain. *Animals* **2020**, *10*, 2433. [CrossRef] [PubMed]
2. Glanville, R. Australia's colourful path to tuberculosis freedom. *Ir. Vet. J.* **2023**, *76* (Suppl. 1), 15. [CrossRef]
3. Singh, B.; Dhand, N.; Cadmus, S.; Dean, A.; Merle, C. Systematic review of bovine and zoonotic tuberculosis in the Western Pacific and the Southeast Asia regions of the World Health Organization. *Front. Public Health* **2024**, *12*, 1345328. [CrossRef] [PubMed]
4. Lange, C.; Aaby, P.; Behr, M.; Donald, P.; Kaufmann, S.; Netea, M.; Mandalakas, A. 100 years of *Mycobacterium* bovis bacille Calmette-Guérin. *Lancet Infect. Dis.* **2022**, *22*, E2–E12. [CrossRef]
5. Covián, C.; Fernández-Fierro, A.; Retamal-Díaz, A.; Díaz, F.; Vasquez, A.; Lay, M.; Riedel, C.; González, P.; Bueno, S.; Kalergis, A. BCG-Induced Cross-Protection and Development of Trained Immunity: Implication for Vaccine Design. *Front. Immunol.* **2019**, *10*, 2806. [CrossRef]
6. Moorlag, S.; Arts, R.; van Crevel, R.; Netea, M. Non-specific effects of BCG vaccine on viral infections. *Clin. Microbiol. Infect.* **2019**, *25*, 1473–1478. [CrossRef]
7. Schaltz-Buchholzer, F.; Berendsen, M.; Roth, A.; Jensen, K.; Bjerregaard-Andersen, M.; Kjær Sørensen, M.; Monteiro, I.; Aaby, P.; Stabell Benn, C. BCG skin reactions by 2 months of age are associated with better survival in infancy: A prospective observational study from Guinea-Bissau. *BMJ Glob. Health* **2020**, *5*, e002993. [CrossRef]
8. Moorlag, S.; van Deuren, R.; van Werkhoven, C.; Jaeger, M.; Debisarun, P.; Taks, E.; Mourits, V.; Koeken, V.; de Bree, L.; Ten Doesschate, T.; et al. Safety and COVID-19 Symptoms in Individuals Recently Vaccinated with BCG: A Retrospective Cohort Study. *Cell Rep. Med.* **2020**, *1*, 100073. [CrossRef]
9. Upton, C.; van Wijk, R.; Mockeliunas, L.; Simonsson, U.; McHarry, K.; van den Hoogen, G.; Muller, C.; von Delft, A.; van der Westhuizen, H.; van Crevel, R.; et al. Safety and efficacy of BCG re-vaccination in relation to COVID-19 morbidity in healthcare workers: A double-blind, randomised, controlled, phase 3 trial. *EClinicalMedicine* **2022**, *48*, 101414. [CrossRef] [PubMed]
10. OMSA. Guidelines for the Control of Mycobacterium Tuberculosis Complex in Livestock. In *Beyond the Test and Slaughter*; World Organisation for Animal Health: Paris, France, 2024. [CrossRef]
11. Srinivasan, S.; Conlan, A.J.K.; Easterling, L.A.; Herrera, C.; Dandapat, P.; Veerasami, M.; Ameni, G.; Jindal, N.; Raj, G.D.; Wood, J.; et al. A Meta-Analysis of the Effect of Bacillus Calmette-Guérin Vaccination Against Bovine Tuberculosis: Is Perfect the Enemy of Good? *Front. Veter. Sci.* **2021**, *8*, 637580. [CrossRef]

12. Guerra-Maupome, M.; Vang, D.X.; McGill, J.L. Aerosol vaccination with Bacille Calmette-Guerin induces a trained innate immune phenotype in calves. *PLoS ONE* **2019**, *14*, e0212751. [CrossRef]
13. Díaz, F.; Guerra-Maupome, M.; McDonald, P.; Rivera-Pérez, D.; Kalergis, A.; McGill, J. A Recombinant BCG Vaccine Is Safe and Immunogenic in Neonatal Calves and Reduces the Clinical Disease Caused by the Respiratory Syncytial Virus. *Front. Immunol.* **2021**, *12*, 664212. [CrossRef] [PubMed]
14. Retamal, P.; Ábalos, P.; Alegría-Morán, R.; Valdivieso, N.; Vordermeier, M.; Jones, G.; Saadi, K.; Perez Watt, C.; Salinas, C.; Ávila, C. Vaccination of Holstein heifers with *Mycobacterium* bovis BCG strain induces protection against bovine tuberculosis and higher milk production yields in a natural transmission setting. *Transbound. Emerg. Dis.* **2022**, *69*, 1419–1425. [CrossRef]
15. Contreras, C.; Alegría-Moran, R.; Duchens, M.; Ábalos, P.; López, R.; Retamal, P. Specific and non-specific effects of *Mycobacterium* bovis BCG vaccination in dairy calves. *Front. Veter. Sci.* **2023**, *10*, 1278329. [CrossRef]
16. Sun, S.; Aguirre-Gamboa, R.; de Bree, L.; Sanz, J.; Dumaine, A.; van der Velden, W.; Joosten, L.; Khader, S. BCG vaccination alters the epigenetic landscape of progenitor cells in human bone marrow to influence innate immune responses. *Immunity* **2024**, *57*, 2095–2107. [CrossRef]
17. Chen, J.; Gao, L.; Wu, X.; Fan, Y.; Liu, M.; Peng, L.; Song, J.; Li, B.; Liu, A.; Bao, F. BCG-induced trained immunity: History, mechanisms and potential applications. *J. Transl. Med.* **2023**, *21*, 106. [CrossRef] [PubMed]
18. Max, V.; Paredes, L.; Rivera, A.; Ternicier, C. National control and eradication program of bovine tuberculosis in Chile. *Veter. Microbiol.* **2011**, *151*, 188–191. [CrossRef]
19. Valdivieso, N.; Retamal, P. Is it possible to control bovine tuberculosis without compensation? Reviewing ten years of the Chilean program and its progress. *Ir. Veter. J.* **2023**, *76*, 20. [CrossRef] [PubMed]
20. Abalos, P.; Valdivieso, N.; Pérez de Val, B.; Vordermeier, M.; Benavides, M.; Alegría-Morán, R.; Saadi, K.; Wistuba, M.; Ortega, C.; Sánchez, N.; et al. Vaccination of Calves with the Mycobacterium bovis BCG Strain Induces Protection against Bovine Tuberculosis in Dairy Herds under a Natural Transmission Setting. *Animals* **2022**, *12*, 1083. [CrossRef]
21. Maier, G.; Breitenbuecher, J.; Gomez, J.; Samah, F.; Fausak, E.; Van Noord, M. Vaccination for the Prevention of Neonatal Calf Diarrhea in Cow-Calf Operations: A Scoping Review. *Veter. Anim. Sci.* **2022**, *15*, 100238. [CrossRef]
22. Probo, M.; Veronesi, M. Clinical Scoring Systems in the Newborn Calf: An Overview. *Animals* **2022**, *12*, 3013. [CrossRef]
23. Jessop, E.; Li, L.; Renaud, D.; Verbrugghe, A.; Macnicol, J.; Gamsjäger, L.; Gomez, D. Neonatal Calf Diarrhea and Gastrointestinal Microbiota: Etiologic Agents and Microbiota Manipulation for Treatment and Prevention of Diarrhea. *Veter. Sci.* **2024**, *11*, 108. [CrossRef] [PubMed]
24. Chomczynski, P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *BioTechniques* **1993**, *15*, 532–534. [PubMed]
25. Bai, J.; Trinetta, V.; Shi, X.; Noll, L.; Magossi, G.; Zheng, W.; Porter, E.; Cernicchiaro, N.; Renter, D.; Nagaraja, T. A multiplex real-time PCR assay, based on *invA* and pagC genes, for the detection and quantification of Salmonella enterica from cattle lymph nodes. *J. Microbiol. Methods* **2018**, *148*, 110–116. [CrossRef]
26. Guy, R.; Tremblay, D.; Beausoleil, L.; Harel, J.; Champagne, M. Quantification of *E. coli* O157 and STEC in feces of farm animals using direct multiplex real time PCR (RTPCR) and a modified most probable number assay comprised of immunomagnetic bead separation and RTPCR detection. *J. Microbiol. Methods* **2014**, *99*, 44–53. [CrossRef]
27. Meng, W.; Chen, Z.; Jiang, Q.; Chen, J.; Guo, X.; Ma, Z.; Jia, K.; Li, S. A multiplex real-time fluorescence-based quantitative PCR assay for calf diarrhea viruses. *Front. Microbiol.* **2024**, *14*, 1327291. [CrossRef] [PubMed]
28. Dykema, P.; Stokes, K.; Beckwith, N.; Mungin, J.; Xu, L.; Vickers, D.; Reising, M.; Bravo, D.; Thomsen, B.; Robbe-Austerman, S. Development and validation of a direct real-time PCR assay for *Mycobacterium bovis* and implementation into the United States national surveillance program. *PeerJ* **2016**, *4*, e1703v1. [CrossRef]
29. Dohoo, R.; Martin, W.; Stryhn, H. *Veterinary Epidemiologic Research*, 2nd ed.; Wiley: Hoboken, NJ, USA, 2009.
30. Jurczak, M.; Druszczynska, M. Beyond Tuberculosis: The Surprising Immunological Benefits of the Bacillus Calmette-Guérin (BCG) Vaccine in Infectious, Auto-Immune, and Inflammatory Diseases. *Pathogens* **2025**, *14*, 196. [CrossRef]
31. Samuel, B.; Diaz, F.; Maina, T.; Corbett, R.; Tuggle, C.; McGill, J. Evidence of innate training in bovine γδ T cells following subcutaneous BCG administration. *Front. Immunol.* **2024**, *15*, 1423843. [CrossRef]
32. Roche, S.; Genore, R.; Renaud, D.; Shock, D.; Bauman, C.; Croyle, S.; Barkema, H.; Dubuc, J.; Keefe, G.; Kelton, D. Describing mortality and euthanasia practices on Canadian dairy farms. *J. Dairy Sci.* **2020**, *103*, 3599–3605. [CrossRef]
33. Calderón-Amor, J.; Gallo, C. Dairy Calf Welfare and Factors Associated with Diarrhea and Respiratory Disease Among Chilean Dairy Farms. *Animals* **2020**, *10*, 1115. [CrossRef]
34. Ahmedin, U.; Assen, A. Calf morbidity, mortality, and management practices in dairy farms in Jimma City, Southwestern Ethiopia. *BMC Veter. Res.* **2023**, *19*, 249. [CrossRef]
35. Compton, C.; Heuer, C.; Thomsen, P.; Carpenter, T.; Phyn, C.; McDougall, S. A systematic literature review and meta-analysis of mortality and culling in dairy cattle. *J. Dairy Sci.* **2017**, *100*, 1–16. [CrossRef]
36. Cuttance, E.; Laven, R. Perinatal mortality risk factors in dairy calves. *Veter. J.* **2019**, *253*, 105394. [CrossRef]

37. Chase, C.; Hurley, D.; Reber, A. Neonatal immune development in the calf and its impact on vaccine response. *Veter Clin. N. Am. Food Anim. Pract.* **2008**, *24*, 87–104. [CrossRef]
38. Cho, Y.; Yoon, K.J. An overview of calf diarrhea–infectious etiology, diagnosis, and intervention. *J. Veter. Sci.* **2014**, *15*, 1–17. [CrossRef] [PubMed]
39. Wang, X.; Yu, D.; Chui, L.; Zhou, T.; Feng, Y.; Cao, Y.; Zhi, S. A Comprehensive Review on Shiga Toxin Subtypes and Their Niche-Related Distribution Characteristics in Shiga-Toxin-Producing E. coli and Other Bacterial Hosts. *Microorganisms* **2024**, *12*, 687. [CrossRef] [PubMed]
40. Fernández, D.; Sanz, M.; Parma, A.; Padola, N. Short communication: Characterization of Shiga toxin-producing Escherichia coli isolated from newborn, milk-fed, and growing calves in Argentina. *J. Dairy Sci.* **2012**, *95*, 5340–5343. [CrossRef]
41. Ferreira, M.; Freitas, F.; Pinto, J.; Dias, M.; Moreira, C. Isolation, prevalence, and risk factors for infection by shiga toxin-producing Escherichia coli (STEC) in dairy cattle. *Trop. Anim. Health Prod.* **2014**, *46*, 635–639. [CrossRef] [PubMed]
42. Auvray, F.; Bièche-Terrier, C.; Um, M.; Dupouy, V.; Nzuzi, N.; David, L.; Allais, L.; Drouet, M.; Oswald, E.; Bibbal, D.; et al. Prevalence and characterization of the seven major serotypes of Shiga toxin-producing Escherichia coli (STEC) in veal calves slaughtered in France. *Veter. Microbiol.* **2023**, *282*, 109754. [CrossRef]
43. Mainil, J.; Nakamura, K.; Ikeda, R.; Crombé, F.; Diderich, J.; Saulmont, M.; Piérard, D.; Thiry, D.; Hayashi, T. Emerging hybrid shigatoxigenic and enteropathogenic Escherichia coli serotype O80:H2 in humans and calves. *Clin. Microbiol. Rev.* **2025**, *38*, e0001125. [CrossRef] [PubMed]
44. Jeyanathan, M.; Vaseghi-Shanjani, M.; Afkhami, S.; Grondin, J.; Kang, A.; D'Agostino, M.; Yao, Y.; Jain, S.; Zganiacz, A.; Kroezen, Z.; et al. Parenteral BCG vaccine induces lung-resident memory macrophages and trained immunity via the gut-lung axis. *Nat. Immunol.* **2022**, *23*, 1687–1702. [CrossRef]
45. Menge, C. The Role of Escherichia coli Shiga Toxins in STEC Colonization of Cattle. *Toxins* **2020**, *12*, 607. [CrossRef] [PubMed]
46. Fakih, I.; Thiry, D.; Duprez, J.; Saulmont, M.; Iguchi, A.; Piérard, D.; Jouant, L.; Daube, G.; Ogura, Y.; Hayashi, T. Identification of Shiga toxin-producing (STEC) and enteropathogenic (EPEC) Escherichia coli in diarrhoeic calves and comparative genomics of O5 bovine and human STEC. *Veter. Microbiol.* **2017**, *202*, 16–22. [CrossRef] [PubMed]
47. Fernández, M.; Casaux, M.; Fraga, M.; Vignoli, R.; Bado, I.; Zunino, P.; Umpiérrez, A. Shiga Toxin-Producing Escherichia coli (STEC) Associated with Calf Mortality in Uruguay. *Microorganisms* **2023**, *11*, 1704. [CrossRef]
48. Geletu, U.; Usmael, M.; Bari, F. Rotavirus in Calves and Its Zoonotic Importance. *Veter. Med. Int.* **2021**, *2021*, 6639701. [CrossRef]
49. Bertoni, E.; Aduriz, M.; Bok, M.; Vega, C.; Saif, L.; Aguirre, D.; Cimino, R.; Miño, S.; Parreño, V. First report of group A rotavirus and bovine coronavirus associated with neonatal calf diarrhea in the northwest of Argentina. *Trop. Anim. Health Prod.* **2020**, *52*, 2761–2768. [CrossRef]
50. Louge Uriarte, E.; Badaracco, A.; Spetter, M.; Miño, S.; Armendano, J.; Zeller, M.; Heylen, E.; Späth, E.; Leunda, M.; Moreira, A.; et al. Molecular Epidemiology of Rotavirus A in Calves: Evolutionary Analysis of a Bovine G8P[11] Strain and Spatio-Temporal Dynamics of G6 Lineages in the Americas. *Viruses* **2023**, *15*, 2115. [CrossRef]
51. Dueñas, F.; Rivera, D.; Toledo, V.; Tardone, R.; Hervé-Claude, L.; Hamilton-West, C.; Switt, A. Short communication: Characterization of Salmonella phages from dairy calves on farms with history of diarrhea. *J. Dairy Sci.* **2017**, *100*, 2196–2200. [CrossRef]
52. Stanford, K.; Johnson, R.; Alexander, T.; McAllister, T.; Reuter, T. Influence of Season and Feedlot Location on Prevalence and Virulence Factors of Seven Serogroups of Escherichia coli in Feces of Western-Canadian Slaughter Cattle. *PLoS ONE* **2016**, *11*, e0159866. [CrossRef]
53. Lambertini, E.; Karns, J.; van Kessel, J.; Cao, H.; Schukken, Y.; Wolfgang, D.; Smith, J. Dynamics of Escherichia coli Virulence Factors in Dairy Herds and Farm Environments in a Longitudinal Study in the United States. *Appl. Environ. Microbiol.* **2015**, *81*, 4477–4488. [CrossRef] [PubMed]
54. Neves, L.; Gomes, M.; Campolina, J.; Campos, M.; Souza, E.; Diavão, J.; Silva, A.; Tomich, T.; Carvalho, W.; Lage, H.; et al. Impact of Heat Stress on Intake, Performance, Digestibility, and Health of Neonatal Dairy Calves. *Animals* **2025**, *15*, 1876. [CrossRef] [PubMed]
55. Roland, L.; Drillich, M.; Klein-Jöbstl, D. Invited review: Influence of climatic conditions on the development, performance, and health of calves. *J. Dairy Sci.* **2016**, *99*, 2438–2452. [CrossRef] [PubMed]
56. Lai, R.; Gong, D.; Williams, T.; Ogunsola, A.; Cavallo, K.; Arlehamn, C.S.L.; Acolatse, S.; Beamer, G.; Ferris, M.; Sassetti, C.; et al. Host genetic background is a barrier to broadly effective vaccine-mediated protection against tuberculosis. *J. Clin. Investig.* **2023**, *133*, e167762. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.