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# A Novel Direct-Fed Microbial for Beef Cattle Has a Supportive Effect against *Clostridium perfringens* In Vitro and In Vivo

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**Abstract:** Two experiments were conducted to evaluate the in vitro and in vivo effects of a novel direct-fed microbial (DFM) containing *Lactobacillus animalis* LA-51, *Propionibacterium freudenreichii* PF-24, *Bacillus licheniformis* CH-200, and *Bacillus subtilis* King (BOVAMINE DEFEND® Plus) against *Clostridium perfringens* pathogenic strains. In Experiment 1 (in vitro), an agar diffusion assay was performed to qualitatively evaluate the in vitro inhibitory effects of the DFM against *C. perfringens* types A and C. Including the DFM in the tested yielded inhibition zones with greater than three ring diameters in a 96-well plate. In Experiment 2 (in vivo), twenty 1-day-old beef calves were allocated to control (n = 10) or DFM (n = 10) for 21 days. All calves were orally challenged with $1.0 \times 10^8$ colony forming units of *C. perfringens* type A strain S-107 per head. The procedures such as general health scores, body weight, and fecal sample collections were performed following the *C. perfringens* challenge. Daily feeding of DFM significantly reduced the incidence of diarrhea while improving general impression and appearance scores of calves. Overall, these results highlight the ability of the DFM containing *L. animalis* LA-51, *P. freudenreichii* PF-24, *B. licheniformis* CH-200, and *B. subtilis* (BOVAMINE DEFEND® Plus) to inhibit *C. perfringens* types A and C under different experimental settings.

**Keywords:** *Bacillus* spp.; beef cattle; *Clostridium perfringens*; direct-fed microbial; *Lactobacillus animalis*; pathogen inhibition; *Propionibacterium freudenreichii*



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## 1. Introduction

Direct-fed microbials (DFM), or probiotics, have been gaining more attention from the scientific community and ruminant production segments, as these have been shown to support the health and performance of beef and dairy calves, as well as mature feedlot beef animals [1–5]. Regardless of the DFM strains being fed, support for gastrointestinal tract (GIT) health is one of the main targets of commercially available DFM products, as health benefits to the host are the core features of DFM, or probiotics [6,7].

In beef cattle, gastrointestinal tract (GIT) diseases represent up to 9.6% of all calf death losses, whereas these numbers also vary depending on the size of the beef operation (7.3 to 14.3%) [8]. *Clostridium perfringens* has been associated with GIT diseases and early postnatal mortality in calves, as well as contributing to GIT upsets that also lead to sudden death in feedlot cattle [9]. In brief, *Clostridium perfringens* is a rod-shaped, Gram-positive, spore-forming, anaerobic bacterium that produces toxins and exoenzymes, which, in turn, are responsible for the disease occurrences [10,11]. Moreover, *C. perfringens* has been shown to damage the integrity of intestinal cells under an in vitro assay [12]. Previous studies from our research group reported positive effects, though by different modes of action, of different DFM on the inhibition of *C. perfringens*. As an example, incubation of *Lactobacillus animalis* LA-51 counteracted the in vitro intestinal integrity damage of *C. perfringens* [12].

Moreover, Segura et al. [13] demonstrated that *Bacillus* spp. (*B. licheniformis* CH-200 and *B. subtilis* CH-201) can reduce in vitro gas production of *C. perfringens* type A and pathogenic counts, suggesting a direct inhibitory effect of these DFM trains against *C. perfringens*. Lastly, Cull et al. [4] recently demonstrated that dairy calves orally challenged with *C. perfringens* type A and fed a DFM mixture containing *L. animalis* LA-51, *Propionibacterium freudenreichii* PF-24, *B. licheniformis* CH-200, and *B. subtilis* CH-201 had a greater number of days with normal fecal score, as well as improved calf survival rates when compared with non-supplemented calves that were also challenged with the latter pathogen.

Based on this rationale, we hypothesized that *C. perfringens* types A and C would be inhibited, under in vitro and in vivo settings, by a novel DFM mixture containing *L. animalis* LA-51, *P. freudenreichii* PF-24, *B. licheniformis* CH-200, and *B. subtilis* King (BOVAMINE DEFEND® Plus). Therefore, our objective was to evaluate the effects of the aforementioned DFM mixture on in vitro inhibition of *C. perfringens* types A and C (Experiment 1), as well as health scores of newborn beef calves challenged with *C. perfringens* type A (Experiment 2).

## 2. Materials and Methods

### *2.1. Experiment 1—In Vitro*

The agar diffusion assay was performed by evaluating both *C. perfringens* types A (CHCC #14327) and C (CHCC #18121), following the methodology described by Santano et al. [14]. On d 1 of the assay, *C. perfringens* types A and C were inoculated on trypticase soy agar with sheep blood (**TSA-SB**) medium and incubated, separately, at 37 °C anaerobically for 18 h. On the same day, independent cultures in brain heart infusion (**BHI**) broth of direct-fed microbial (**DFM**) strains (*Lactobacillus animalis*, *Propionibacterium freudenreichii*, *Bacillus licheniformis*, and *Bacillus subtilis*; BOVAMINE DEFEND® Plus; Chr. Hansen A/S, Hørsholm, Denmark) were prepared and incubated overnight.

On d 2, Perfringens Agar Base (Oxoid) was melted and cooled to 50 °C, while the pathogenic cultures (types A and C) were suspended by using a cotton swap with a 1.3 MacFarland suspension in a maximum recovery diluent (**MRD**) medium. Following this step, a 35 mL melted agar and 10 µL of pathogen suspension were mixed in a 50 mL Falcon tube (Corning Life Sciences, Corning, NY, USA), and the mixture was cast in Omnitray plates with an immediate application of the Nunc™Immuno TSP (Thermo Fisher Scientific, Waltham, MA, USA). Then, the plates were left to solidify for 20 min before removing the Nunc™ Immuno TSP lid. Thereafter, plates were allowed to dry with normal lid on for additional 20 min. Lastly, 10 µL of the DFM strains overnight culture were mixed (final pH mix = 6.5) and applied to the selected wells in the agar.

Samples were analyzed in sextuplicates (n = 6). After anaerobic incubation at 42 °C for 24 h, the inhibition zone was measured via photoshop from full growth to full growth, with a lower limit of 3.5 mm (width of each well in the plate). The inhibition zone was measured at 5 and 24 h post-DFM strains addition on the agar plate and the zones were classified as follows: (0): clear agar in the bottom of the wells demonstrating no pathogen inhibition, (+): clear agar in slim line around the well, (++): clear agar bigger than (+) and up to about twice the size of the well, (+++): clear agar bigger than (++) and up to about three times the size of the well, and (++++): clear agar bigger than (+++).

### *2.2. Experiment 2—Challenge*

All activities related to this study were reviewed and approved by the Institutional Animal Care and Use Committee of Midwest Veterinary Services, Inc. prior to study initiation (IACUC number AC18046B)

#### 2.2.1. Animals and Study Design

Twenty (n = 20) healthy newborn male beef calves were initially selected for inclusion in the study (initial body weight = 41.4 kg). These calves were a day old, had been fed the colostrum at birth, and did not receive any vaccines or antibiotics, and all animals were born in a single day. Each calf passed an examination from a veterinarian, which

deemed them to be healthy for enrollment into the study. Calves were commercially sourced from Firth, NE. The study was conducted in a randomized design. Calves were individually housed indoors on concrete floors with no nose-to-nose contact. Housing conditions were per "Guide for the Care and Use of Agricultural Cattle in Research and Teaching by the Federation of Cattle Science Societies" [15]. The individual calf was considered the experimental unit. Study personnel involved in the collection, recording, or interpretation of any data were masked to the treatment assignment of cattle. The test material dispenser(s), test material administrator, and quality control personnel were unmasked to study treatments and were the only study personnel with access to the randomization and treatment assignments. Unmasked study personnel were not involved in clinical observations, including recording of those observations. Calves were in overall good health with no complicating diseases reported at the time of enrollment. All calves enrolled in the study had access to veterinary care as needed. All veterinary care was at the discretion of the site veterinarian or investigator in consultation with the study monitor when possible. The study also consisted of thorough euthanasia guidelines with humane endpoints as per the IACUC governing bodies, veterinarians, and trained personnel. When animals met the clinical criteria of moribund at any observation, the veterinarian would intervene, and those animals would be euthanized using an AVMA-approved method. Mortality within the paper would include both animals found dead, and/or euthanized; however, Clostridial injections can be challenging as the disease/death can progress quickly. Due to the possible disease progression, a veterinarian and/or trained staff observed the animals at minimum twice a day.

#### 2.2.2. Treatments

The study consisted of two groups of calves allocated randomly to two different treatments: (1) Control: no probiotic supplementation (CON = n = 10) and (2) DFM containing a mixture of *Lactobacillus animalis*, *Propionibacterium freudenreichii*, *Bacillus licheniformis*, and *Bacillus subtilis* at a rate of $6.0 \times 10^9$ colony forming units/head per day (50 mg/head per day; BOVAMINE DEFEND® Plus; Chr. Hansen Inc., Milwaukee, WI, USA; DFM; n = 10). Control calves did not receive any probiotic in the milk replacer, whereas DFM was added in the milk replacer at a rate of 50 mg/head per day.

The study lasted for 25 days with 4 days of acclimation (d-11 to d-7), 7 days of probiotic feeding (pre-challenge period; d-7 to d-0), oral *Clostridium perfringens* type A S-107 challenge (d-0), and 14 days of probiotic feeding (post-challenge; d-1 to d-14), following a methodology recently published [4]. A description of the experimental period is described in Figure 1. Calves were exposed to approximately 12 h of light per day. Calves were fed twice daily a commercially available, nonmedicated milk replacer (crude protein min. = 21%, crude fat min. = 20%, crude fiber max. = 0.15%, CalfCare, North Manchester, IN, USA) and received water ad libitum. Throughout the study, calves were observed twice per day and findings were recorded.

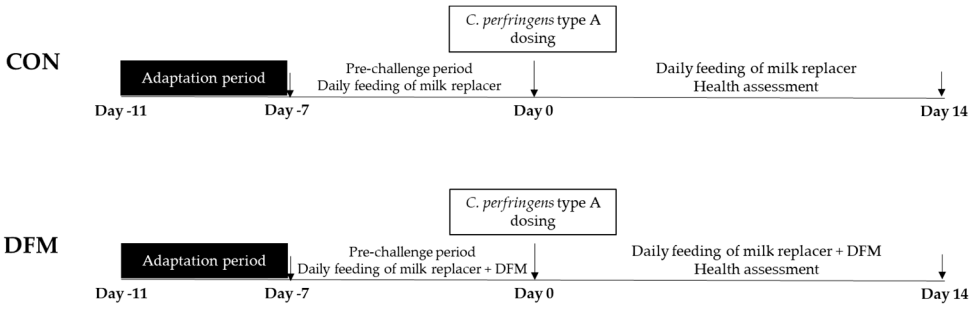



**Figure 1.** Timeline of the present experiment. CON = milk replacer without supplementation of a direct-fed microbial; DFM = mixture containing *Lactobacillus animalis* LA-51, *Propionibacterium freudenreichii* PF-24, *Bacillus licheniformis* CH-200, and *Bacillus subtilis* King (BOVAMINE DEFEND® Plus; Chr. Hansen A/S, Hørsholm, Denmark).

#### 2.2.3. Experimental Challenge

The *C. perfringens* type A S-107 (ATCC 13124 was available and based on preliminary challenge model development work; derived from bovine source) challenge was prepared at the CSRC, Veterinary Diagnostic Laboratory (Oakland, CA, USA). The challenge material was prepared in anaerobic BHI broth. The final concentration of the challenge material was adjusted with anaerobic BHI broth to obtain a target dose of $1 \times 10^8$ colony forming units (**CFU**) per mL. The concentration of *C. perfringens* in the challenge material was performed by serial dilution (i.e., $10^{-1}$ to $10^{-6}$) in 9 mL of sterile phosphate buffer saline (PBS). From each dilution, 0.1 mL was spread plated on duplicate Perfringens agar plates supplemented with Kanamycin and Polymyxin B. The plates were incubated at 37 °C for 48 h in an anaerobic chamber, with final counts being as follows: pre-challenge concentration = $1.16 \times 10^8$ CFU/mL and post-challenge concentration = $9.70 \times 10^7$ CFU/mL. All calves were challenged with 300 mL on day 0. This dosage was required to obtain clinical and reproducible outcome variables of interest.

#### 2.2.4. General Health Monitoring

Routine daily observations for the general health of the calves occurred during the study and were performed as recently reported and described by Cull et al. [4]. Observations for clinical signs of disease associated with Clostridial infection included, at a minimum, general health, hunger, skin tent, dehydration, calf appearance, and diarrhea based on fecal consistency. A description of the scoring system is shown below (Table 1), whereas fecal scoring and consistency was described as follows: 0 = normal feces with retained form; 1 = form is a puddle with sufficient water content to easily flow across or down a smooth surface, while leaving some adherent material; 2 = moderate, feces with sufficient water content to easily flow across or down a smooth surface, while leaving some adherent material; and 3 = severe, part or all of feces watery, draining away while leaving little or no residue on a smooth surface.

**Table 1.** Health score used during the present study.

| Score | General Health | Hunger | Skin Tent | Dehydration | Appearance |
|---|---|---|---|---|---|
| 0 | Good | Normal suckle, drinking all the MR [b] | 0–1 s | None | Clean backside, tail, and legs |
| 1 | Mildly depressed | Moderate suckle, but still drinking all the MR | 2–3 s | Mild: possible doubtful eyes, with skin and fur dull | Backside and tail slightly dirty with some sticky feces or dry fecal material |
| 2 | Moderately depressed | Weak suckle, requiring assistance to consume the MR | 3–4 s | Moderate: sunken eyes, reduced skin elasticity, with a dull fur | Backside and tail very dirty, not wet, dying |
| 3 | Severely depressed | Unwilling to suckle, must tube the animal to consume the MR | >4 s | Severe: eyes lie very deep, with a dull fur | Backside, tail, and legs dirty from watery diarrhea |
| 4 [a] | Moribund or dead | | | | |

[a] Calf unlikely to recover, requiring euthanasia. [b] MR = milk replacer.

#### 2.2.5. Body Weight

All calves were weighed at arrival and at the conclusion of the trial. A daily scale check was performed before weighing cattle by placing calibrated (within the past 12 months) check weights on the scale in the following increments: 0 pounds, 50 pounds, 100 pounds, 150 pounds, and 200 pounds (1 kg = 2.2 pounds), to determine a within ±5% error. The scale weigh checks were within a ±5% error of the actual weight.

2.2.6. Fecal Sample Collection

Fecal samples were collected directly from the rectum of each calf using a new glove. All samples were labeled with the calf identification, study number, and date of collection. Fecal samples were transferred to the laboratory at ambient temperature and all fecal samples were tested for *C. perfringens* using microbial plating methods. All fecal samples were stored at −70 °C or colder after the initial testing was performed.

2.2.7. Fecal Concentration of Clostridium Perfringens

Approximately 1 g of fecal sample from each animal was weighed and to it was added 9 mL of PBS. After vortexing for 30 s, a 10-fold serial dilution was performed in PBS starting from $10^{-1}$ to $10^{-6}$ by transferring 0.1 mL of the material from tube 1 to tube 2 containing 0.9 mL of PBS. This step was repeated until $10^{-6}$ dilution. One hundred microliters of each dilution were plated in duplicate onto Perfringens agar plates supplemented with Kanamycin and Polymyxin B. All plates were incubated at 37 °C for approximately 48 h in an anaerobic chamber. The plates were evaluated for viable counts and the results were noted on the data capture form. The CFU/gram counts were based on the following equation:

$$\text{CFU per gram} = \frac{(\text{weight of fecal sample} + \text{total volume of broth added})}{\text{Weight of fecal sample}} \times \text{no. of colonies} \times \text{dilution factor}$$

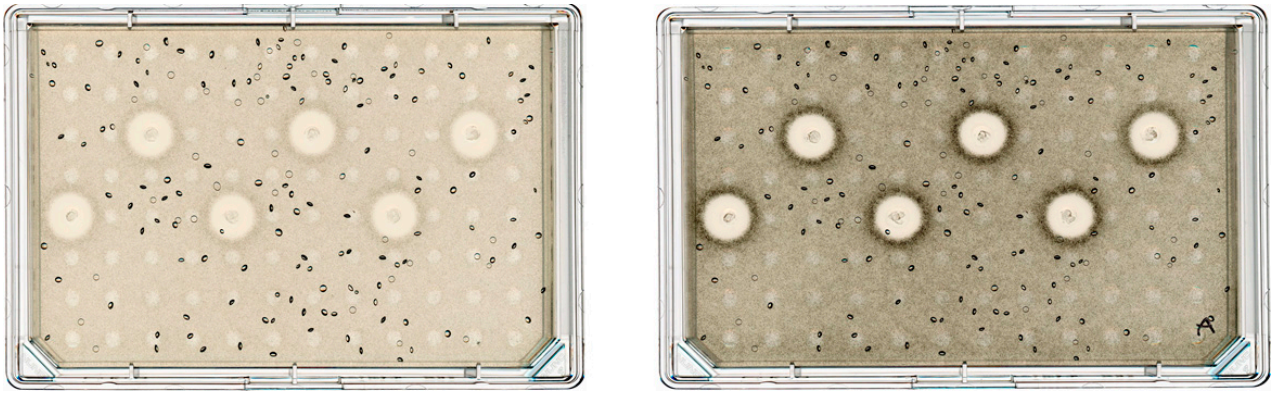
2.2.8. Statistical Analysis

Primary outcome variables associated with Clostridial infection included mortality, diarrhea, health scores, depression, dehydration, and Clostridial fecal concentration. Secondary outcome variables were body weight and body weight change. The mixed and generalized linear mixed models (MIXED and GLIMMIX) were used to estimate the effect of treatment over time on production, diagnostic, and clinical outcomes. Continuous outcomes such as concentration of bacteria in feces among enumerable samples (concentration in log10 CFU/g of bacteria in feces among enumerable samples (samples with at least one CFU/g) and body weight were modeled with a Gaussian distribution, identity link, and maximum likelihood estimation. Dichotomous outcomes (yes/no; 1/0) including the presence of at least one CFU of bacteria in feces and clinical scores (presence of abnormal diarrhea, hunger, general impression, skin tent, and appearance scores), were modeled with a binary distribution, logit link, restricted pseudo-likelihood estimation, and Kenward–Rogers degrees of freedom estimation, using the PROC GLIMMIX of SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). To estimate the effect of treatment over time on diagnostic and clinical outcomes, multivariable models including fixed effects for treatment, study day, and a two-way interaction term between treatment group and day were fitted. When the interaction term was not significantly associated with the outcome ($p > 0.05$), a model with main effects only (treatment group and study day) was fitted. Models included a first-order autoregressive or a heterogeneous first-order autoregressive covariance structure for animal ID to account for repeated measures at the animal level (for measures equally and unequally spaced over time, respectively). Significances were set at $p < 0.05$, whereas means and mean percentages, standard error of the means, 95% confidence intervals, and *p*-values were reported. For interpretation of interaction terms, analyses of simple effects were computed (slice and slice by options in LSMEANS statement, PROC GLIMMIX).

## 3. Results

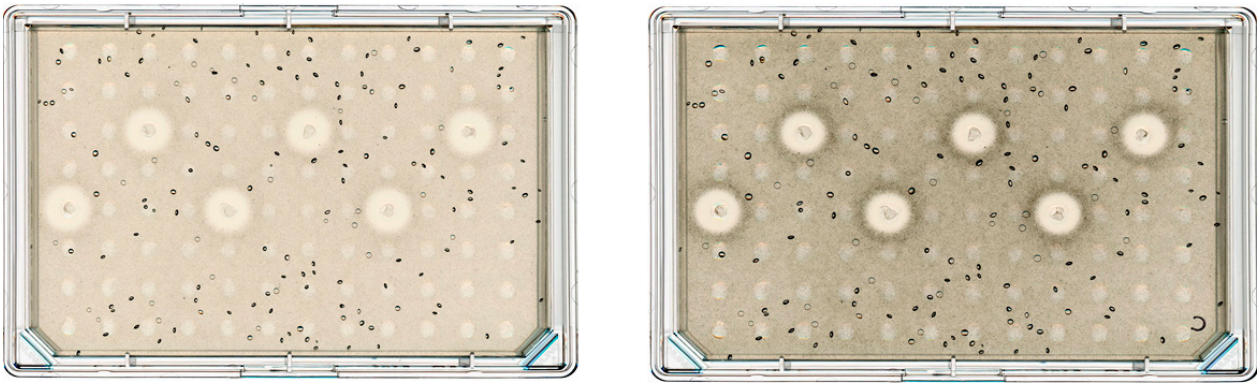
### *3.1. Experiment 1—In Vitro*

Figures 2 and 3 demonstrate the images of the plates that were read and evaluated in the computer and that measured the inhibition zone of the samples containing the pathogens (*C. perfringens* types A and C, respectively) with the DFM strains incubated for 5 and 24 h.



(**A**) (**B**)

**Figure 2.** Inhibition zones of the direct-fed microbial (DFM) mixture containing *Lactobacillus animalis* LA-51, *Propionibacterium freudenreichii* PF-24, *Bacillus licheniformis* CH-200, and *Bacillus subtilis* King (BOVAMINE DEFEND® Plus; Chr. Hansen A/S, Hørsholm, Denmark) against *Clostridium perfringens* type A at 5 (**A**) and 24 (**B**) h post-incubation.



(**A**) (**B**)

**Figure 3.** Inhibition zones of the direct-fed microbial (**DFM**) mixture containing *Lactobacillus animalis* LA-51, *Propionibacterium freudenreichii* PF-24, *Bacillus licheniformis* CH-200, and *Bacillus subtilis* King (BOVAMINE DEFEND® Plus; Chr. Hansen A/S, Hørsholm, Denmark) against *Clostridium perfringens* type C at 5 (**A**) and 24 (**B**) h post-incubation.

Overall, incubation of the DFM strains with the pathogens for 24 h resulted in greater and more visible inhibition zones than at 5 h, regardless of *C. perfringens* serotype, supporting the efficacy of such DFM mixture against these pathogenic bacteria. Following the classification score mentioned above, at 24 h the inhibition score against both *C. perfringens* types A and C was ++++.

### *3.2. Experiment 2—Challenge*

On day 9 post-challenge, two calves from the CON group died and were removed from the study. Therefore, some of the results reported below contain eight calves in the statistical analysis for the CON group, whereas no calves had died from the DFM treatment group.

Table 2 reports the final BW and BW change of calves receiving or not the DFM mixture during the 21-day experimental period. No treatment effects were observed on initial and final BW ($p \geq 0.10$).

**Table 2.** Final body weight (BW) of newborn beef calves challenged with *Clostridium perfringens* type A and receiving or not (CON; n = 10) a direct-fed microbial mixture (DFM; n = 10) for 21 days [1].

| Item | CON | DFM [2] | SEM | *p*= |
|---|---|---|---|---|
| Final BW, kg | 52.6 | 52.8 | 1.94 | 0.96 |
| BW change, kg | 10.0 | 12.7 | 1.93 | 0.35 |

[1] Calves were assigned to treatments on day 7 and orally challenged with *C. perfringens* type A on day 0 of the study. [2] DFM contained *Lactobacillus animalis* LA-51, *Propionibacterium freudenreichii* PF-24, *Bacillus licheniformis* CH-200, and *Bacillus subtilis* King (BOVAMINE DEFEND® Plus; Chr. Hansen Inc.).

For Clostridial shedding, no observations were detected at the beginning and immediately before the *C. perfringens* oral challenge. Moreover, no treatment × day or main treatment effects were observed on Clostridial shedding when analyzed as % of animals positive per day and/or for the CFU of Clostridial per day ($p \geq 0.20$; Tables 3 and 4). Nonetheless, as expected, day effects were observed for both analyses ($p \leq 0.01$).

**Table 3.** The proportion of Clostridial-positive newborn beef calves challenged with *Clostridium perfringens* type A and receiving or not (CON; n = 10) a direct-fed microbial mixture (DFM; n = 10) for 21 days [1].

| Item | Treatments | | SEM | *p*= [2] | | |
|---|---|---|---|---|---|---|
| | CON | DFM [3] | | T | D | T × D |
| Daily proportion of calves positive for Clostridial, % | | | | 0.21 | 0.01 | 0.40 |
| Day 1 | 80.0 | 70.0 | 14.91 | | | |
| Day 2 | 80.0 | 80.0 | 14.91 | | | |
| Day 3 | 80.0 | 60.0 | 14.91 | | | |
| Day 4 | 70.0 | 50.0 | 14.91 | | | |
| Day 7 | 70.0 | 20.0 | 14.91 | | | |
| Day 14 | 40.0 | 40.0 | 14.91 | | | |

[1] No observation on days -7 and 0 (relative to the *C. perfringens* challenge); [2] T = treatment effect; D = day effect; T × D = treatment × day interaction; [3] DFM contained *Lactobacillus animalis* LA-51, *Propionibacterium freudenreichii* PF-24, *Bacillus licheniformis* CH-200, and *Bacillus subtilis* King (BOVAMINE DEFEND® Plus; Chr. Hansen Inc.).

**Table 4.** Mean fecal colony forming units (CFU) counts of Clostridial in newborn beef calves challenged with *Clostridium perfringens* type A and receiving or not (CON; n = 10) a direct-fed microbial mixture (DFM; n = 10) for 21 days [1].

| Item | Treatments | | SEM | *p*= [2] | | |
|---|---|---|---|---|---|---|
| | CON | DFM [3] | | T | D | T × D |
| Mean fecal CFU of Clostridial, log CFU/gram of feces | | | | 0.20 | <0.0001 | 0.51 |
| Day 1 | 4.84 | 4.24 | 0.821 | | | |
| Day 2 | 4.78 | 4.47 | 0.821 | | | |
| Day 3 | 4.14 | 3.26 | 0.821 | | | |
| Day 4 | 3.36 | 2.31 | 0.821 | | | |
| Day 7 | 3.68 | 0.95 | 0.821 | | | |
| Day 14 | 1.92 | 1.23 | 0.821 | | | |
| Overall mean | 3.79 | 2.74 | 0.561 | | | |

[1] No observation on days -7 and 0 (relative to the *C. perfringens* challenge); [2] T = treatment effect; D = day effect; T × D = treatment × day interaction; [3] DFM contained *Lactobacillus animalis* LA-51, *Propionibacterium freudenreichii* PF-24, *Bacillus licheniformis* CH-200, and *Bacillus subtilis* King (BOVAMINE DEFEND® Plus; Chr. Hansen Inc.).

A treatment × day interaction was observed ($p < 0.0001$) on the occurrence of abnormal diarrhea scores in newborn beef calves following the *C. perfringens* type A challenge (Figure 4). From days 1 to 11, more CON calves had an abnormal diarrhea score when compared with DFM ($p < 0.01$), whereas no differences were observed on day 12 post-challenge

($p$ = 0.42; Figure 4). Moreover, a greater proportion of CON calves presented an abnormal diarrhea score over the 14 days post-challenge when compared with the DFM cohorts ($p < 0.0001$; 65.5 vs. 16.0% for CON and DFM, respectively; SEM = 5.80). Conversely, no cases were observed on days 0, 13, and 14 post-*C. perfringens* type A challenge.

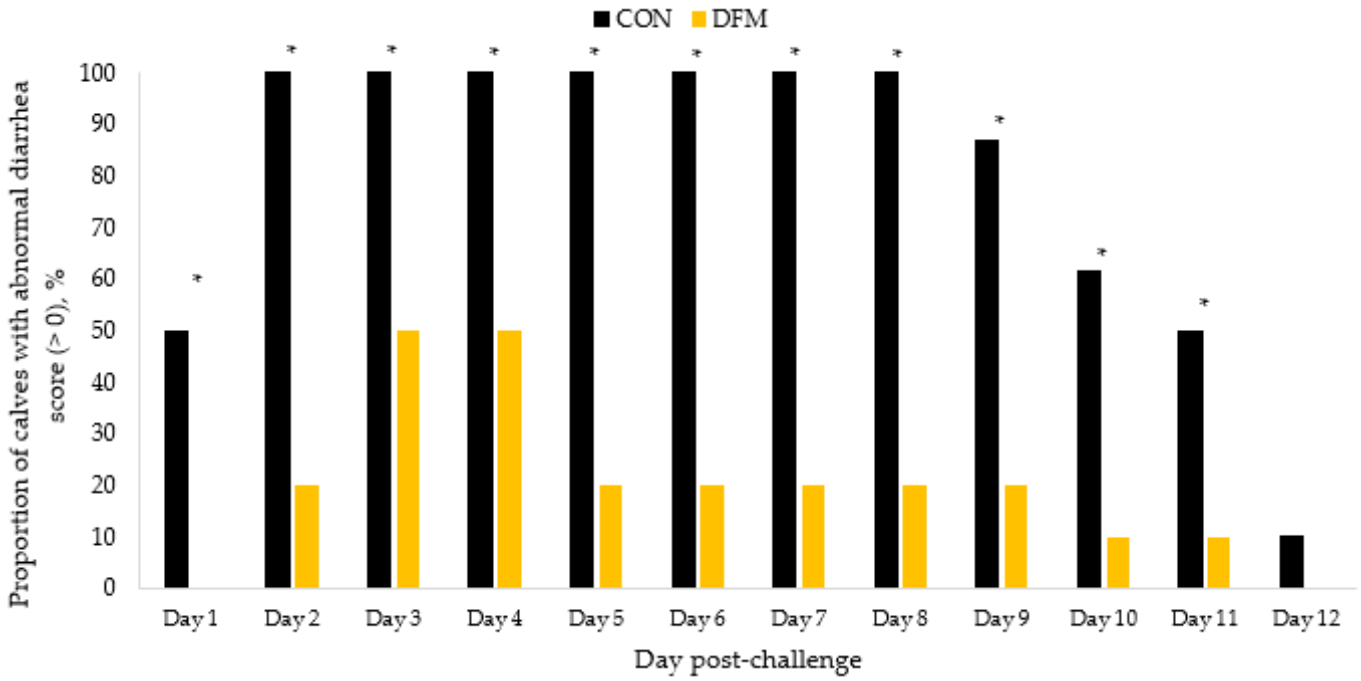



**Figure 4.** Proportion of abnormal diarrhea score of newborn beef calves supplemented or not (CON; n = 10) with a direct-fed microbial (DFM; n = 10) and orally challenged with $1.0 \times 10^8$ CFU of *Clostridium perfringens* type A on day 0. A treatment × day interaction was observed ($p < 0.0001$); SEM = 10.5; * denotes differences at $p < 0.01$ level.

Similarly, a treatment × day interaction was also observed ($p \leq 0.01$) for scores of general impression and appearance (Figures 5 and 6), as CON had a greater proportion of calves with abnormal scores from days 3 to 9 for general impressions ($p \leq 0.03$) and from days 3 to 10 for appearance ($p < 0.01$). For general impression, no abnormal cases were observed on days 0 and 1, whereas no cases were observed on days 0 and 14 for abnormal appearance. Lastly, no further treatment × day interactions or main treatment differences were observed for hunger, skin tent, and dehydration scores ($p \geq 0.17$).

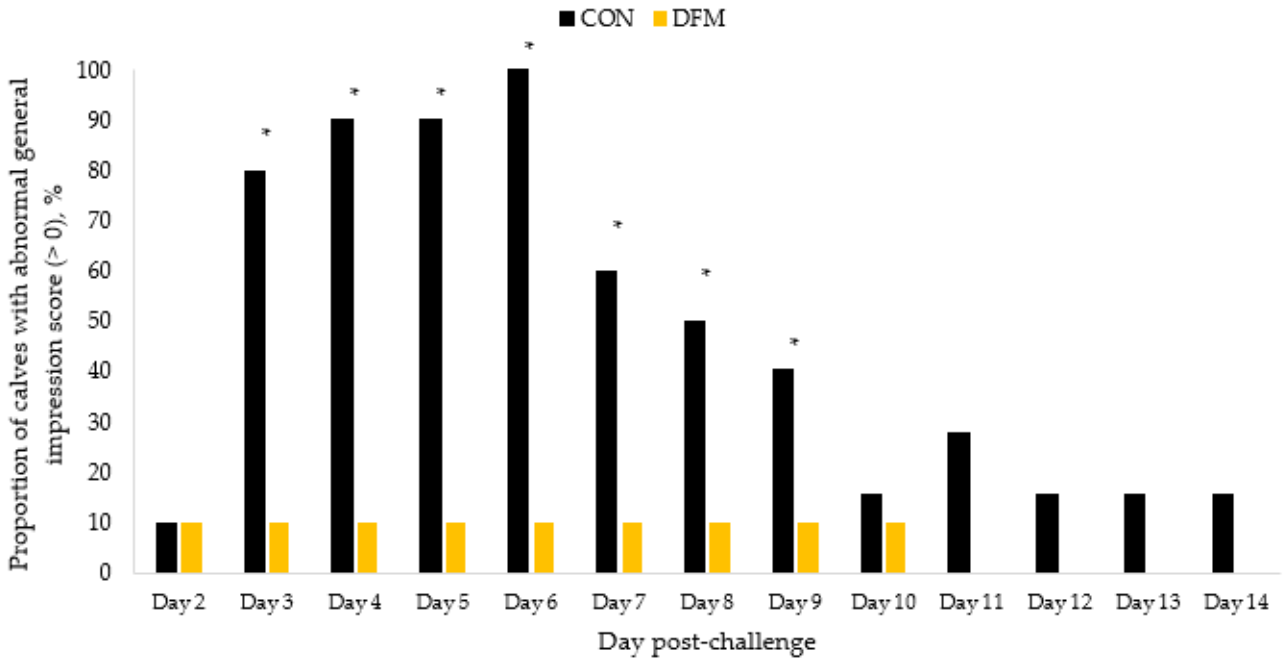



**Figure 5.** Proportion of abnormal general impression score of newborn beef calves supplemented or not (CON; n = 10) with a direct-fed microbial (DFM; n = 10) and orally challenged with $1.0 \times 10^8$ CFU of *Clostridium perfringens* type A on day 0. A treatment × day interaction was observed ($p < 0.001$); * denotes differences at $p < 0.01$ level.

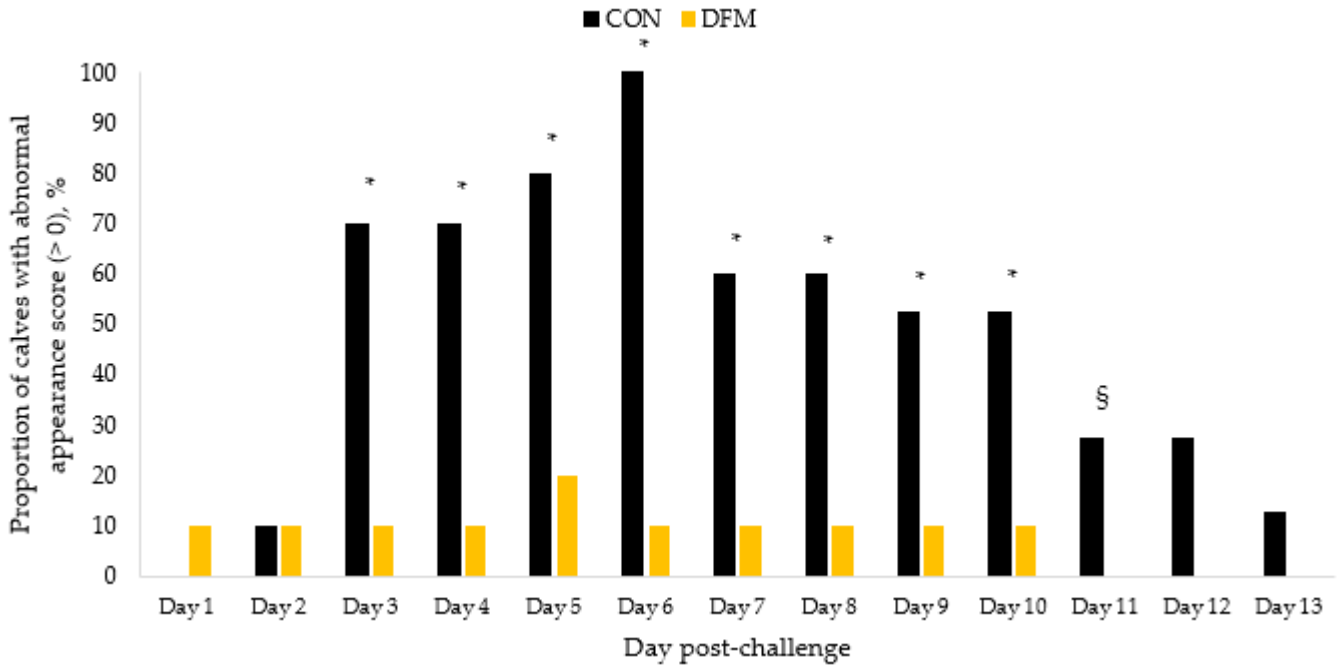



**Figure 6.** Proportion of abnormal appearance score of newborn beef calves supplemented or not (CON; n = 10) with a direct-fed microbial (DFM; n = 10) and orally challenged with $1.0 \times 10^8$ CFU of *Clostridium perfringens* type A on day 0. A treatment × day interaction was observed ($p < 0.01$); SEM = 10.1; * denotes differences at $p \leq 0.01$ level; § denotes differences at $0.05 < p \leq 0.10$ level.

## 4. Discussion

The main goal of the present article was to evaluate the effects of a novel direct-fed microbial (DFM) on the in vitro and in vivo inhibition of *Clostridium perfringens* types A (Exp. 1 and 2) and C (Exp. 1 only). To the best of our knowledge, this is the first scientific

report evaluating the DFM mixture containing *Lactobacillus animalis*, *Propionibacterium freudenreichii*, *Bacillus licheniformis*, and *B. subtilis* (BOVAMINE DEFEND® Plus) efficacy against gastrointestinal pathogens of interest for ruminants, such as *C. perfringens* types A and C.

Direct-fed microbials, or probiotics, must bring health benefits to the host, whereas healthier animals are more productive and profitable, regardless of being beef [16] or dairy [5] cattle. The combination of different bacterial strains in a DFM mixture might bring additional benefits to the host, as these may also present different modes of action to support the health of the herd. As an example, Cull et al. [17] demonstrated that the combination of *L. animalis* LA-51 and *P. freudenreichii* PF-24 reduced the adverse health effects in *Salmonella*-challenged beef calves. In a feedlot trial, the feeding of *L. animalis* LA-51 and *P. freudenreichii* PF-24 improved average daily gain and feed efficiency of commercial beef cattle [18].

In a similar approach and experimental design, Cull et al. [4] validated the *C. perfringens* type A oral challenge for dairy calves that was used herein. Moreover, different formulations of DFM were tested by the authors, and the feeding of the DFM mixture that contained the same strains, except for *B. subtilis*, as reported herein resulted in greater survival rates (100%) and reduced the number of calves with abnormal health scores when compared with a non-supplemented control group that was also challenged with *C. perfringens* type A, showing that a combination of lactic-acid-producing and utilizing bacteria with Bacilli might support the health of the animals.

At least five different serotypes of *C. perfringens* have been reported to produce toxins that cause enterotoxaemia in several animal species [19]. In previous studies, feces from neonatal calves were tested (n = 103) and *C. perfringens* was detected in 25.2% of the samples (26/103) [20]. The same authors also reported that *C. perfringens* type A was the most predominant serotype in the feces of the calves (92.3%), whereas others also demonstrated the high prevalence of this serotype [21]. *Clostridium perfringens* type A produces major toxin (CPA) [22,23], which is the most studied toxin, being an important antigen involved in the pathogenesis of enterotoxaemia as well as in the induction of necrotic lesions in the calf intestinal loop model [24]. In the end, the resulting damage to the intestinal wall and its components (i.e., tight junctions) will disrupt the normal paracellular permeability barrier of the intestinal epithelium, which may contribute to necrotic enteritis (or diarrhea) [25]. On the other hand, *C. perfringens* type C produces a toxin (CPB) that is responsible for the fatal hemorrhagic dysentery in humans and livestock species [26].

Our results demonstrated the ability of the novel DFM mixture in inhibiting *C. perfringens* type A under an in vitro and in vivo setting. In vitro, it can be speculated that the bacteria strains included in the DFM produce specific antimicrobial compounds that directly impact growth and survival of *C. perfringens* types A and C. Luise et al. [27] reported that *Bacillus* spp. can (1) reduce the pH of a medium by stimulating the production of short-chain fatty acids (SCFA), (2) can produce a wide range of bacteriocins (i.e., subtilin, bacteriocin-like substance, bacillocin, subtilosin) that have direct effects against Gram-positive bacteria, and (3) can produce enzymes, such as elastase and endopeptidases, that lyse the cell wall of potentially harmful bacteria [28,29]. As reported before, the pH of the final mixture was 6.5, which prevents us from concluding that the drop in pH was one of the modes of action involved in *C. perfringens* types A and C inhibition for Exp. 1. Moreover, lactic-acid-producing bacteria, such as the *L. animalis* fed herein, also are effective in reducing the pH of a medium [30,31]. Altogether, the in vitro results from Exp. 1 support the fact that the DFM mixture containing *L. animalis*, *P. freudenreichii*, *B. licheniformis*, and *B. subtilis* (BOVAMINE DEFEND® Plus) have direct mechanisms that could be involved in the inhibition of *C. perfringens* types A and C.

In Exp. 2 (in vivo challenge), we observed that no calves being fed DFM died during the 21-day study, whereas two CON calves died. Probiotics, or DFM, support gut health through different and, at some level, complementary synergistic mechanisms. For example, *L. animalis* has alleviated the negative effects of *C. perfringens* type A on intestinal integrity

by using a leaky gut assay (transepithelial electrical resistance assay) [12]. Moreover, different *Bacillus* spp. have differing abilities to stimulate the expression and, therefore, production of secreted and membrane-bound mucin [14], as well as stimulate the formation of biofilm in intestinal cells [13] that supports the competitive exclusion of pathogenic bacteria. Feeding a mixture of *L. animalis* LA-51 and *P. freudenreichii* PF-24 to Holstein calves supported the development of intestinal cells during the pre-weaning period [2]. Lastly, it is noteworthy to mention that the support of the health of the host against *C. perfringens* serotypes cannot solely rely on vaccination, as it has been demonstrated that only 40–50% of vaccinated lactating dairy cows develop an effective memory against this pathogen [32]. This can be even more critical if we consider that newborn calves do not have a developed immune system from a systemic or gut-located standpoint [33] and are not vaccinated early in life, meaning that they are at high risk if they encounter *C. perfringens*. Therefore, additional supportive tools must be fed to the animals in order to alleviate the occurrence and the severity of *C. perfringens*-related observations in animals from all categories. One of these tools, as reported in both experiments., could be the novel DFM mixture containing lactic-acid-producing and utilizing bacteria, and Bacilli.

## 5. Conclusions

The novel mixture of *Lactobacillus animalis* LA-51, *Propionibacterium freudenreichii* PF-24, *Bacillus licheniformis* CH-200, and *Bacillus subtilis* King (BOVAMINE DEFEND® Plus) effectively inhibited *C. perfringens* types A and C (in vitro) as well as *C. perfringens* type A when fed at 50 mg/head per day under an in vivo challenge setting. The in vivo inhibition of *C. perfringens* type A led to a greater proportion of healthier animals, reducing diarrhea and increasing general impression score. Additional research efforts are warranted to understand the long-term health and performance effects of the presented novel DFM mixture for beef animals.

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**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** Not applicable.

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