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# The Effect of Postbiotic Supplementation on Body Weight Changes, Blood Metabolic Profiles, Rumen Microbiome Changes, and Immune Responses in Dairy Calves

**Byeng Ryel Min[1*], Mariline Hilaire[1], Honghe Wang[1], Anthony Kumi[1], Santoshes Chaudhary[1], Vivian Kanyi[1], Isaac Min[2], EunYoo Kim[2], Isaac Ha[2], Heba Abdo[1], Hosaam Ismael[1], Sean Yang[3], Richard Puchala[4,5]**

[1]Department of Agricultural and Environmental Sciences, Tuskegee University, Tuskegee, AL, USA
[2]Loveless Academic Magnet School, Montgomery, AL, USA
[3]Verdesian Life Sciences LLC., South Salt Lake City, UT, USA
[4]Military Institute of Hygiene and Epidemiology, Kozielska, Poland
[5]America Institute for Goat Research, Langston University, Langston, OK, USA
Email: *bmin1@tuskegee.edu

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## Abstract

Limited information exists about the effects of postbiotic supplementation (dried *Lactobacillus* Fermentation Product) on animal performance, blood metabolites, immune responses, and changes in the rumen microbiome in pre- and post-weaning dairy calves. This study aims to clarify how postbiotic supplementation (in non-viable cells) affects growth performance, blood metabolites, rumen microbiome dynamics, immune response, and immune-related gene expression in pre-weaned dairy calves over a 95-day period. The experiment involved 30 male healthy pre-weaned Holstein (*Bos taurus*) calves (5 days old, weighing 40 - 50 kg), randomly divided into three groups. The control group received no postbiotics (preferred as a control [0.0 mg]), while the other groups received 1.75 g/d and 3.5 g/d postbiotic supplements in their diet for 50 days (period I). During the post-weaning period, calves in the control group received increasing postbiotic doses (0.0%, 3.5 g/d, and 7.0 g/d per calf) for 45 days, for a total of 95 days of the study (period II). Calves were housed individually in pens with free access to water. Data were analyzed using the Mixed procedure of SAS (version 9.4). Results showed that, with postbiotic supplementation, animal body weight and cell-mediated immune response

were significantly higher in the higher-dose group than in the control group ($P < 0.01$). Dry matter intake (DMI) did not differ among treatments. An increase ($P < 0.05$) was observed in Ca, blood urea (mg/dL), glucose, and lymphocyte (%) with higher postbiotic doses. Among bacterial phyla, Bacteroidetes decreased ($P < 0.05$) in the higher-dose group compared with the control group, whereas Pseudomonadota increased ($P < 0.05$). Most bacterial and methanogen species remained unchanged across treatments. Immune cells from the blood of supplemented calves showed increased expression of TNF-*a*, IL-6, and IFN genes. In conclusion, postbiotic feeding improved animal health, strengthened immune responses, caused minor changes in rumen microbiome diversity, and affected blood chemistry in dairy calves compared to controls.

## Keywords

Postbiotic, Animal Performance, Animal Health, Immune Response

## 1. Introduction

The International Scientific Association of Probiotics and Prebiotics (ISAPP) has reported that probiotics are, by definition, alive and must contain an effective amount of viable bacteria at the time of administration to the host, while effective postbiotics must include inactivated microbial cells or cell components, with or without metabolites, that contribute to observed health benefits [1]. Postbiotic products derived from the fermentation of *Saccharomyces cerevisiae* and *S. acidophilus* are commonly used as feed additives for animals. They contain compounds such as oligosaccharides, organic acids, amino acids, and peptides, which can positively influence various rumen microorganisms, including bacteria [2], protozoa [3], and fungi [4], leading to improvements in milk production [5], rumen fermentation parameters (e.g., volatile fatty acids; VFA; [6]), ruminal pH [7], and dry matter intake (DMI; [8]). In young calves, supplementing with postbiotics has also been shown to improve survival rates, boost animal performance (e.g., average daily gain [ADG]; [9] [10], and promote the development of ruminal morphology [11]. However, the effects of postbiotic supplementation (e.g., dried *Lactobacillus* fermentation product) in calf diets on animal performance, immune-related gene expression, and microbial community changes remain poorly studied. Additionally, discrepancies between the FAO/WHO Expert Consultation Report and the FAO/WHO Guidelines have been clarified to incorporate scientific advances and new applications.

Recent evidence suggests that microbial viability is not necessary to achieve such effects [12] [13]. It has been proposed that inactivated or non-viable probiotic cells, along with their metabolic byproducts (referred to here as "postbiotics" and "para-probiotics"), can also benefit the host's health [13]. Specifically, they may demonstrate anti-inflammatory, immunomodulatory, anti-proliferative, and

antioxidant activities, positively influencing various host metabolic and signaling pathways [13] [14]. Based on this premise, postbiotics and paraprobiotics have potential applications across several fields, primarily in food and nutrition (e.g., dietary supplements and feed additives) [15]. Regarding the gastrointestinal tract, prior studies have shown that piglets [16] 2009) and broilers [17] fed postbiotics exhibited greater improvements in morphology; however, similar research is lacking in calves. Therefore, we hypothesize that including postbiotics in both milk (during the pre-weaning phase) and starter (during the post-weaning phase) fed to growing calves could accelerate animal performance, enhance immune development, and alter the rumen microbiome. The goal of this project is to examine the effects of postbiotic supplementation (dried *Lactobacillus* fermentation product) on growth performance, blood metabolites, rumen microbiome dynamics, immune response, immune-related gene expression, and overall health in dairy calves over 95 days.

## 2. Materials and Methods

### 2.1. Animals and Management

The study was carried out at the Caprine Research and Education Center within the Department of Animal Science at Tuskegee University in Tuskegee, AL. The Tuskegee University Animal Care and Use Committee approved the procedures for animal care, handling, and sampling for this experiment (# RD 03-2023-4). The experimental design and time schedule are shown in **Figure 1**. Thirty healthy, gonadally intact Holstein male calves, 5 days old, with an average body weight of 45.0 kg ± 0.23 kg, were randomly assigned to three treatment groups (n = 10). All the calves received 1.5 liters of high-quality colostrum obtained from the mother cow twice a day (8:00 and 16:00), for a total of 3 liters per calf per day for 4 days, using a clean bottle with a nipple. This amount provides sufficient immunoglobulin (IgG) levels to support the calf's immune system. Quality of colostrum (e.g., IgG) was not measured. The control group received no postbiotics (preferred as a control [0.0 g]). In contrast, the other groups received 1.75 g/d and 3.5 g/d of a postbiotic (Lumensa LFM: dried *Lactobacillus* fermentation product, Verdesian Life Sciences LLC., 2700 S 600 W, South Salt Lake, UT 84115, USA) added to their diet for 50 days during the pre-weaning (collection period I) period (see **Figure 2**). Calves received concentrates at two weeks to 1 kg/day, followed by a gradual weaning period. This helps in preparing the calves for the transition to free-range feeding. During the post-weaning period (collection period II), calves in the control group received no postbiotics (0.0%), whereas the other groups received escalating doses of 3.5 g/d and 7.0 g/d per calf for 45 days, for a total of 95 days due to increased daily dry matter intake and higher body weight. Each treatment group included calves that remained in the same group across both phases, as a single study with two phases. It is essential to provide a balanced diet that meets the calves' energy and protein requirements, and to monitor calves by a veterinarian familiar with the herd's needs, especially during

the transition period.

The calves were housed individually in slatted-floor pens (1.5 m × 1.5 m) and fed three different diets across two growth stages. During the collection period (days 0 - 50), all calves were fed milk replacer twice daily (8:00 a.m. and 4:00 p.m.) until weaning. The replacer was formulated with 22% protein, 20% fat, calcium (1.25%), phosphorus (0.7%), sodium (0.5%), and vitamins A, D, and E to serve as a complete and balanced substitute for fresh milk (Suckle Pro Milk Replacer, 1118 Pony Express Hwy, Marysville, Kansas). Ingredients included dried whey, dried whey product, dried whey protein concentrate, soy protein concentrate, animal and vegetable fats (preserved with BHA and BHT), dried skimmed milk, lecithin, hydrolyzed yeast, brewer's dried yeast, calcium carbonate, dicalcium phosphate, dl-methionine, l-lysine monohydrochloride, vitamin A supplement, vitamin D3 supplement, vitamin E supplement, ascorbic acid, dried Bacillus subtilis fermentation product, dried Enterococcus faecium fermentation product, vitamin B12 supplement, folic acid, choline chloride, riboflavin supplement, niacin supplement, calcium pantothenate, thiamine mononitrate, pyridoxine hydrochloride, biotin, ferrous sulfate, copper sulfate, cobalt sulfate, zinc sulfate, manganese sulfate, magnesium oxide, ethylenediamine dihydriodide, dextrose, mono- and diglycerides of edible fats or oils, sodium silico aluminate, selenium yeast, and artificial flavor. The amount of milk replacer fed was 1.5% of each calf's body weight (BW). The dairy cattle NRC [18] provides formulas to estimate daily energy and protein needs for calves. Replace these with calf-appropriate standards and ensure the stated study duration and measurement windows are consistent across sample types (e.g., the timing of sample collection). To prepare the milk, dissolve the milk replacer powder in warm water (about 1300 g of powder per 20 L of water at 45˚C - 50˚C). After dissolving, allow it to cool to room temperature until it reaches an optimal drinking temperature of 39˚C, then feed the calves.

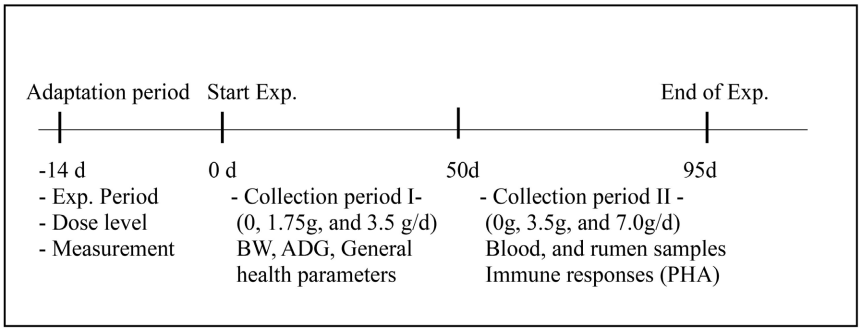


**Figure 1.** Experimental (Exp.) design. Time schedule of experiments, including general health parameters (fecal consistency and nasal discharge score; twice weekly), body weight (BW), and average daily gain (ADG) measurement every 14 days during the collection period I. During collection period II, a cell-mediated immune response was assessed using phytohemagglutinin A (PHA) on days 83 to 85, and blood and rumen samples were collected on day 90. Composite diet samples were collected weekly. Post-biotic supplementation was conducted between collection period I (control [0 g/d], 1.75 g, and 3.5 g/d/hd) and collection period II (control [0 g], 3.5 g, and 7.0 g/d/hd).

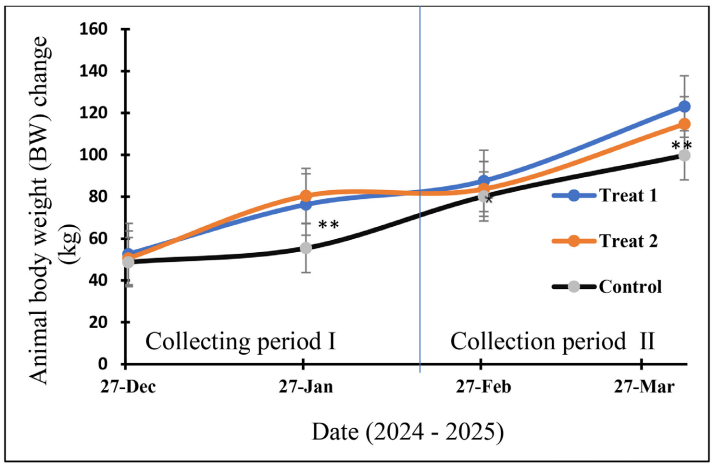


**Figure 2.** Monthly animal body weight (BW; kg) changes in different supplementation treatments (Treat.) of postbiotics supplementation in dairy calves. Sampling and supplementation were conducted in collection period I (control [0 g/d], treat 1 [1.75 g/d], and treat 2 [3.5 g/d]) and collection period II (control [0 g/d], treat 1 [3.5 g/d], and treat 2 [7.0 g/d]), respectively. Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$; values without asterisks are not significant ($P > 0.05$).

## 2.2. Measurement

The pens had a raised mesh floor design that allowed daily flushing of feces and urine into a lagoon system. Daily feed intake and refusals were monitored and recorded, with intake adjusted weekly over a 90-day period. Body weights were measured after a 4-hour withdrawal from feed and water, and this process was repeated every 14 days. General health parameters, including fecal consistency and nasal discharge score, were assessed twice weekly. The average daily gain (ADG) for each calf was calculated as the difference between the initial and final body weights (BW) over the performance interval. Composite diet samples were collected weekly, labeled by month, placed in secure zip-lock bags, and stored in a dry, room-temperature area until further chemical and mineral analysis.

The collection period II (51 - 95 days) was fed commercially available diets (T320CR calf starter, Kalmbach Feeds, 7148 State Hwy 199, Upper Sandusky, OH). Starter concentrates/rations were introduced from day 51 onward and gradually increased during 5 days. Blood chemical parameters, rumen samples, and cell-mediated immune responses were measured on day 90 during the second period. Blood samples were collected from each animal at the end of the study to determine trace mineral levels and other blood metabolites. Two mL EDTA vacutainer blood collection tubes were used for a complete blood count. Ten mL non-EDTA Vacutainer blood collection tubes were used for serum blood chemistry, and serum lipid-proof, acid-washed, non-EDTA Vacutainer tubes were used for serum analysis. Blood samples were kept on ice immediately after collection and transported to the Tuskegee University Clinical Diagnostics Laboratory for further processing.

The amount of grass and concentrate provided was adjusted weekly based on 1.5% of that week's body weight. Bermuda grass hay and post-weaning diets were supplied at 8:00 a.m. and 4:00 p.m. each day. Fresh drinking water and a mineral

block were continuously available in each pen. The animals' body weight was recorded before morning feeding at the start of the trial and weekly thereafter. Rumen fluid was collected (about 10 mL per calf) from each calf 2 hours after feeding [19]-[21]. The ruminal contents were filtered through four layers of cheesecloth. The resulting sample was used for VFA, pH, and microbiome analyses (Min *et al.*, 2019a, b, c). A 6 mL rumen fluid sample was collected using a stomach tube for the analysis of pH, VFA, and microbial populations. Ruminal pH was measured shortly after collection with a pH meter (Mettler-Toledo Ltd., England, UK).

### 2.3. Cell-Mediated Immunity

On day 83, the cell-mediated immune response in goats was evaluated using an intradermal skin test, and skinfold thickness at the injection site was measured, following the protocol of Agazzi *et al.* [22]. Animals in each treatment group received intradermal injections of 200 µl of phytohemagglutinin A (PHA). A 0.025 g sample of PHA (Sigma Chemical Co., St. Louis, Missouri) was mixed with 5 mL of phosphate-buffered saline (PBS, pH 7.4) to create a 250 mg/100mL solution. The injection site was shaved, and skinfold thickness measurements were recorded before injection (Day 83) and at 24 hours (Day 84) and 48 hours (Day 85) after injection using scientific micrometric calipers.

### 2.4. Laboratory Analysis

Dairy One Forage Testing Laboratory (Ithaca, NY) analyzed the chemical compositions and mineral content. The dry matter (DM) concentrations of the experimental diet samples were determined by oven-drying the samples at 105˚C for 24 hours [23]. Minerals, starch, and ether extract (crude fat) were measured using procedures outlined by AOAC [23]. Nitrogen concentrations were assessed with an organic elemental analyzer (Flash 2000; CE Elantech Inc., Lakewood, NJ, USA; [23]). The pH of the fluid was measured using a Laqua F-73G (Horiba Scientific; Kyoto, Japan). The manufacturer's method, as described by Van Soest *et al.* [24], was used to determine the concentrations of acid detergent fiber (ADF) and neutral detergent fiber (NDF) using an ANKOM200/220 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA). Heat-stable amylase (Type XI-A from Bacillus subtilis; Sigma-Aldrich Corporation, St. Louis, MO, USA) was used to pretreat the sodium sulfite before adding it to the mixture for NDF analysis [25].

### 2.5. Rumen Fermentation

For VFA analysis, rumen fluids were acidified with 25% metaphosphoric acid (w/v) at a 4:1 (v/v) ratio and then centrifuged at 3000×g for 10 minutes. The supernatant was collected, filtered, and used to determine VFA. 4-methyl-n-valeric acid (Sigma, St. Louis, MO) served as the internal standard. Next, 0.5 mL of clear supernatant was mixed with an equal volume of 4-methylvaleric acid (Sigma-Aldrich, St. Louis, MO) as the internal standard. The VFA profile of the rumen fluid was analyzed using a 6890 N Network GC System (Agilent Technologies) according to the method of Filípek and Dvořák [26]. The separation of the VFA profile

was achieved using a Quadrex 007 Series (Quadrex Corp., New Haven, CT 06525, USA) bonded phase fused silica capillary column (15 m, 0.250 mm ID, 0.25 μm film thickness), coupled with the 6890 N Network GC System gas chromatograph equipped with a flame ionization detector.

### 2.6. Rumen Microbial Population

Following the manufacturer's protocol, microbial DNA was extracted from rumen fluid using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). The DNA concentration was measured with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Only samples with DNA concentrations above 30 ng/μL and high purity were selected for further analysis. Changes in the microbiome community were assessed [19].

### 2.7. RNA Extraction and RT-PCR of Immune-Related Genes

Total RNA was extracted from blood samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. RNA concentration and purity (measured by the 260/280 nm absorbance ratio) were assessed with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Approximately 100 ng/μL of purified RNA was reverse transcribed into complementary DNA (cDNA) using the Quantitect reverse transcription kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Genomic DNA was removed before reverse transcription of RNA into cDNA. Real-time qPCR was performed using the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, CA, USA). **Table 1** details the targeted genes, primer sequences, and product sizes. Quantitative real-time PCR for immune-related gene expression (**Table 1**) was performed as described by Puech *et al.* [27] and Min *et al.* [20].

**Table 1.** Cytokine relative expression in bovine; Primer information on target and reference genes. Primer pairs and optimized conditions used for determining bovine gene expression by quantitative real-time PCR.

| Target gene | Primer sequence | primer concentration (nM) | Amplicon size (bP) | Tm (˚C) |
|---|---|---|---|---|
| INF-$\gamma$ | F-CAGAGCCAAATTGTCTCCTTC | 300 | 167 | 80.2 |
| | R-ATCCACCGGAATTTGAATCAG | 300 | | |
| TNF-$\alpha$ | F-CCA GAG GGA AGA GCA GTC C | 300 | 111 | 84.5 |
| | R-GGC TAC AAC GTG GGC TAC C | 300 | | |
| IL-10 | F-CTT TAA GGG TTA CCT GGG TTG C | 300 | 239 | 86.6 |
| | R-CTC ACT CAT GGC TTT GTA GAC AC | 300 | | |

References: [24]-[27]. Tm (˚C) = melting temperature; Interferon-gamma (IFN-$\gamma$); tumor necrosis factor-alpha (TNF-$\alpha$); interleukin (IL)-10.

### 2.8. Statistical Analysis

The entire statistical analysis was performed using the mixed model in SAS (SAS

Institute Inc., Cary, NC) for longitudinal outcomes (e.g., body weight, intake, and health scores) with calf as a random effect and time and treatment-by-time as fixed effects. The linear dose level of the dietary treatment was identified as the cause of the observed effects. SAS PROC GLM was used to estimate coefficients for orthogonal contrasts. The data were presented as least-squares means with standard errors of the mean (SEM).

## 3. Results and Discussion

In calves, the first few months of life are critical for future productivity. Nutritional management plays a key role in minimizing the impact of early growth stages on the digestive system and supporting optimal animal performance. In pre-ruminants, focus should be on developing the digestive system and its microbiota. This study hypothesized that postbiotics modify the gut microbiota and improve calf growth, immune function, and microbiome diversity in newborn calves. Although the highest dose level (±7.0 g/d/calf) resulted in greater BW gain, positive effects on the immune system—such as immune-related gene expression—and on overall health (e.g., fecal score and respiratory disease) may still be observed. Therefore, using postbiotics may benefit dairy calves.

### 3.1. Nutritional Composition of the Diet

**Table 2.** Chemical composition (% DM) of experimental diets fed to post-weaned dairy calves.

| Ingredient | Diets, % DM[1] | | | |
|---|---|---|---|---|
| | Control group | Treat. 1 | Treat. 2 | SEM |
| Nutrient composition, % | | | | |
| Dry matter (DM) | 92.2 | 90.4 | 90.4 | 0.43 |
| Crude protein (CP) | 20.6 | 19.4 | 19.4 | 0.01 |
| Acid detergent fiber (ADF) | 26.0 | 27.1 | 27.1 | 0.45 |
| Neutral detergent fiber (NDF) | 35.4 | 36.0 | 36.0 | 0.74 |
| Total digestible nutrient (TDN) | 71.3 | 72.0 | 72.0 | 0.98 |
| Minerals | | | | |
| Ca | 1.0 | 0.9 | 0.9 | 0.06 |
| P | 0.5 | 0.4 | 0.4 | 0.005 |
| Mg | 0.4 | 0.3 | 0.3 | 0.01 |
| K | 1.2 | 1.3 | 1.3 | 0.02 |
| Zn | 76.1 | 77.1 | 77.1 | 2.69 |

[1]Diets in the control group did not contain postbiotics, and the treatment group was the same as the control group, but contained postbiotics (dried *Lactobacillus* fermentation product). The basal diet in both treatment (Treat.) groups was the same diet with different levels of postbiotics.

The ingredients and chemical composition of the experimental diets are summarized in Table 2. The diets were formulated to meet the dietary requirements of

multiparous dairy goats (NRC, 2007; Table 1). Dietary treatments had similar nutrient contents, including dry matter (DM), crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), and mineral levels. There was no significant difference in chemical composition among treatments.

### 3.2. Animal Performance

Thirty healthy Holstein male calves were housed individually in metabolism cages for approximately 95 days during both pre- and post-weaning periods. Changes in animal body weight (BW) resulting from various post-biotic supplements during December, January, February, and March are shown in Figure 2. Supplementation with a postbiotic (Diamond V XP Yeast Culture; Diamond V Mills, Inc., Cedar Rapids, IA) has been shown to improve body weight (BW) and average daily gain (ADG) in broilers [17] and pre-weaned dairy calves [9]. Lesmeister *et al.* [9] reported that ADG increased by 15.6% with the 2% yeast treatment in dairy calves. It is consistent with previous results, and current data show an increased BW gain from 27% to 31% in the postbiotic-supplemented groups compared to the control group over the experimental period (Figure 2).

### 3.3. Cell-Mediated Immune Response

Bovine respiratory disease causes illness and death in cattle of all ages. Supplementing with postbiotic products derived from dried Lactobacillus fermentation has been shown to boost growth and provide metabolic support essential for immune activation in calves. During early life, as immunity develops, calves are exposed to multiple external stressors, including transportation, the transition from liquid to solid feed, and dehorning. These stressors increase the risk of illness and death by causing greater variability in the immune system, leading to digestive and respiratory diseases, as well as immune-related problems [28]. The findings of this study align with those of a previous study [28]. The cell-mediated immune response (Figure 3) to PHA injection was significantly improved ($P < 0.05$) by supplementation with 3.5 and 7.0 g/day of postbiotic, compared with a basal diet without postbiotics, showing that postbiotic supplementation notably enhanced cell-mediated immune responses in young calves during the post-weaning period (Table 3).

Table 3. The effect of postbiotic supplementation on immune-related gene expression in immune cells from the peripheral blood of dairy calves was determined by RT-PCR in post-weaning dairy calves.

| Item | Treatment (g post-biotic/d) | | | | $P$ – value |
|---|---|---|---|---|---|
| | 0 | 3.5 | 7.0 | SEM | |
| INF-$\gamma$ | 22.85$^{b}$ | 17.45$^{c}$ | 31.92$^{a}$ | 2.191 | 0.05 |
| IL-10 | 17.45$^{b}$ | 28.34$^{a}$ | 27.49$^{a}$ | 2.224 | 0.01 |
| TNF-$\alpha$ | 20.82$^{b}$ | 32.96$^{a}$ | 32.56$^{a}$ | 2.311 | 0.01 |

$^{a, b, c}$Means that the different superscripts within the diet column represent different values. SEM = Standard error of the mean. Interferon-gamma (IFN-$\gamma$); tumor necrosis factor-alpha (TNF-$\alpha$); interleukin (IL)-10.

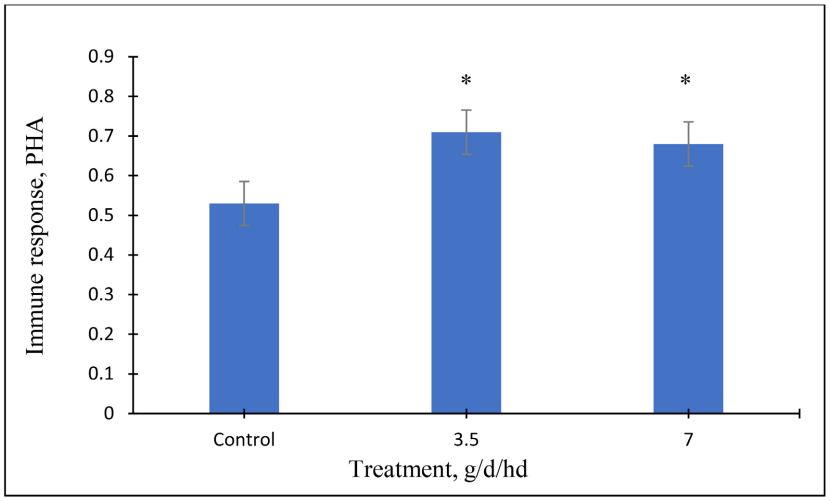


**Figure 3.** The effect of different levels of post-biotic supplementation on immune response by intradermal phytohemagglutinin (PHA, cell-mediated) in post-weaning dairy calves. Statistical significance: * $P < 0.05$.

### 3.4. Immune-Related Gene Expression

Postbiotics primarily play immunomodulatory roles, activating both innate and adaptive immune systems, protecting the intestinal mucosal barrier, and combating pathogens via antimicrobial compounds similar to those in probiotics [29]. Increasing postbiotics, especially dried Lactobacillus fermentation products in this study, significantly raised ($P < 0.05$ - 0.01) the expression of immune-related genes (INF-$\gamma$, IL-10, and TNF-$\alpha$) in the peripheral blood of post-weaning dairy calves (**Table 4**). Izuddin *et al.* [30] reported that post-weaning lambs fed postbiotics showed a fivefold increase in hepatic IGF-1 mRNA compared with controls. Immune cells from the peripheral blood of calves given postbiotics produced more IL-6 when stimulated with toll-like receptors. Conversely, cells from the bronchoalveolar lavage (BAL) of postbiotic-treated calves secreted fewer proinflammatory cytokines, including lower levels of TNF-$\alpha$ and IL-6, when similarly stimulated. Compared to probiotics, postbiotic Lactobacillus gasseri TMC 0356 caused a greater increase in IL-12 production in macrophages, indicating that postbiotic supplementation boosts IL-12 production, and that the postbiotic form has a stronger immunomodulatory effect than the probiotic [31]. Non-living microorganisms such as Lactobacillus acidophilus A 2, *Lactobacillus gasseri* A 5, and *Lactobacillus salivarius* A 6 (heat-inactivated and suspended at $10^6$ cells/mL in PBS) can induce changes in Th 1-mediated immune responses by stimulating IL-10 and IL-12 p 70 proliferation, IFN-$\gamma$ production in splenocytes, and IL-12 p 70 secretion in dendritic cells, respectively [31]. Additionally, Del-Immune V® (Pure Research Products LLC, Boulder, CO, USA) is a commercial postbiotic that contains cell lysate (muramyl peptides) and DNA fragments from the probiotic strain *Lactobacillus rhamnosus* V (DV; [32]). Clinical studies with inbred laboratory mice supplemented with *Lactobacillus rhamnosus* V postbiotic showed it effectively stimulates the innate immune system, mainly by promoting cytokine produc-

tion such as IFN-*γ*, TNF-*α*, IL-1, IL-2, IL-6, IL-8, and IL-12 [32]. In this study (Table 4), doses of 3.5 mg and 7.0 mg per day per calf of the postbiotic actively induced IFN-*γ*, IL-10, and TNF-*α*, similar to previous studies [31] [32], highlighting its significant potential as an immunomodulating agent.

**Table 4.** Effects of high levels of post-biotic supplementation on blood serum metabolites and plasma minerals in post-weaning dairy calves.

| Item | Treatment (g post-biotic/d) | | | | *P*-value |
|---|---|---|---|---|---|
| | 0 | 3.5 | 7.0 | SEM | |
| Blood serum minerals (mg/dL) | | | | | |
| Ca | 5.8[b] | 9.9[a] | 9.1[a] | 0.12 | 0.05 |
| Phosphorus | 5.8 | 6.6 | 6.8 | 1.28 | 0.61 |
| Blood serum electrolytes (mM/L) | | | | | |
| Na | 141 | 142 | 143 | 0.55 | 0.51 |
| K | 4.53 | 4.73 | 4.70 | 0.18 | 0.21 |
| Cl | 96.6 | 98.0 | 97.9 | 0.54 | 0.21 |
| Blood serum proteins (g/dL) | | | | | |
| Total protein | 5.8 | 6.4 | 6.6 | 0.14 | 0.48 |
| Albumin | 2.5 | 2.5 | 2.6 | 0.11 | 0.91 |
| Blood serum metabolites (mg/dL) | | | | | |
| Total bilirubin | 0.2 | 0.2 | 0.2 | 0.03 | 0.89 |
| Creatine | 0.9 | 0.9 | 0.9 | 0.09 | 0.43 |
| Blood urea nitrogen | 4.8[b] | 7.4[ab] | 9.8[a] | 0.72 | 0.05 |
| Glucose | 84.9[b] | 80.6[b] | 93.7[a] | 1.93 | 0.05 |
| Cholesterol | 95.5 | 96.3 | 99.2 | 5.11 | 0.78 |
| Blood serum enzymes (IU/L) | | | | | |
| Amylase | 37.1 | 26.3 | 29.3 | 5.61 | 0.73 |
| Alanine Aminotransferase | 23.9 | 24.9 | 24.5 | 0.19 | 0.12 |
| Alkaline Phosphatase | 125.1 | 131.8 | 174.0 | 0.91 | 0.18 |
| Gamma Glutamyl Transferase | 24.1 | 25.7 | 50.8 | 1.98 | 0.45 |
| Blood serum (μg/mL) | | | | | |
| White blood cell | 9.4 | 8.5 | 9.4 | 0.85 | 0.44 |
| Red Blood Cell | 9.7 | 9.0 | 10.4 | 0.65 | 0.87 |
| Lymphocyte (%) | 40.9[b] | 45.1[a] | 43.3[ab] | 0.92 | 0.05 |
| Monocyte (%) | 11.9 | 12.6 | 17.8 | 0.72 | 0.07 |
| Eosinophil (%) | 0.9 | 0.5 | 2.9 | 0.55 | 0.12 |
| Basophil (%) | 0.2 | 0.2 | 0.2 | 0.93 | 0.85 |
| Hemoglobin (Hgb) | 11.6 | 11.0 | 12.6 | 0.47 | 0.49 |
| Hematocrit | 34.1 | 31.1 | 36.3 | 0.89 | 0.83 |
| Mean Corpuscular Vol | 34.9 | 34.7 | 34.9 | 0.53 | 0.86 |
| Red Cell Distribution width | 40.7 | 40.7 | 42.3 | 0.89 | 0.09 |

[a, b]Means with different superscripts within the diet column are different. SEM = Standard error of the mean.

### 3.5. Blood Serum Metabolites and Plasma Minerals

Blood serum metabolites and plasma minerals are listed in Table 5. In this study, the hemogram and serum chemistry showed no significant differences between the experimental groups, except for increases in serum calcium (linear; $P < 0.05$), blood urea nitrogen ($P < 0.05$), glucose ($P < 0.05$), and lymphocytes ($P < 0.05$). Similarly, lambs receiving postbiotic supplementation in their diet had higher blood total protein, urea nitrogen, and glucose concentrations; however, no significant differences were observed in blood triglyceride and cholesterol levels between the groups [30].

**Table 5.** The predominant bacterial phylum (cell numbers) observed in the rumen samples of dairy calves fed post-biotic supplementation was determined by pyrosequencing of the 16S rDNA1 during the post-weaning period.

| Item[1] | Treatment (g post-biotic/d) | | | | |
|---|---|---|---|---|---|
| | Control (0 g/d) | 3.5 (g/d) | 7.0 (g/d) | SEM | *P*-value |
| Bacteroidota | 2182.0 | 1942.3 | 1503.0 | 271.99 | 0.12 |
| Actinomycetota | 837.4 | 1793.2 | 690.0 | 509.9 | 0.19 |
| Proteobacteria | 277.1 | 417.7 | 406.0 | 151.5 | 0.49 |
| Firmicutes | 3354.4 | 2875.0 | 2859.0 | 526.91 | 0.54 |
| Bacteroidetes | 9002.1[a] | 5269.5[b] | 7472.1[ab] | 1213.7 | 0.05 |
| Bacillota | 9812.7 | 11920.8 | 14272.0 | 1704.1 | 0.11 |
| Candidatus Melainabacteria | 1412.0 | 1107.7 | 736.0 | 422.2 | 0.31 |
| Pseudomonadota | 1022.0[b] | 2771.8[a] | 1173.8[ab] | 587.4 | 0.05 |

[1]This table presents the relative abundances of the seven most abundant bacterial phyla (> 1.0%). [a-b]Means within a row with different superscripts differ ($P < 0.05$). SEM = Standard error of the mean.

It seems that when a basal diet provides an adequate amount of postbiotics (3.5-7.0 g/day), plasma does not show a significant response to additional postbiotics. However, earlier research has shown that feeding postbiotics reduces serum haptoglobin levels during the periparturient period, thereby limiting excessive immune activation after calving [2] [12]. Cows that received a postbiotic challenge had reduced levels of serum acute-phase proteins and less ruminal lipopolysaccharide compared to the control group, indicating that the postbiotic supplement helped stabilize the rumen microbiota and decrease inflammation [33] [34].

### 3.6. Rumen Microbiome Diversity

Improving the microbiome is a key strategy for controlling ruminal fermentation and boosting production efficiency in ruminants. The use of probiotics, prebiotics, synbiotics, and postbiotics (biotics) in animal feeds has become a common approach to achieve these objectives. Unlike monogastric animals, the ruminant gastrointestinal system is designed to ferment dietary nutrients in the rumen, gen-

erating energy for growth and production. Promoting microbiome development is a key strategy for influencing ruminal fermentation and enhancing ruminant efficiency [35]. Additionally, a calf's early life is a critical period because its digestive and immune systems are still developing. Colonization of the gastrointestinal microbiota is a complex process, and establishing a stable population of beneficial microbes is essential for high-producing dairy cows, especially for immune system development [26]. Incorporating probiotics, prebiotics, and postbiotics into animal feed additives is a common approach to achieving these goals [35]-[37]. Our study revealed notable diversity in ruminal bacterial phyla at multiple taxonomic levels in dairy calves fed diets supplemented with postbiotics (**Table 5**; **Figure 4** and **Figure 5**). The dominant microbiota phyla across treatments in the rumen of post-weaning dairy calves included Bacteroidota, Actinomycetota, Proteobacteria, Firmicutes, Bacteroidetes, Bacillota, Candidatus Melainabacteria, and Pseudomonadota. Among these, Bacteroidetes populations decreased ($P < 0.05$), while Pseudomonadota populations increased ($P < 0.05$) when calves received postbiotic supplementation, especially at dosages of 1.7, 3.5, and 7.0 g daily. Across the dietary treatments, the most predominant phyla after normalizing the data were Firmicutes, Bacteroidetes, Euryarchaeota, Proteobacteria, Spirochaetes, Actinobacteria, and Chloroflexi in the rumen of dairy calves (**Figure 4**). This was similar to the results reported by other researchers [38] [39], in which dairy cows consuming postbiotics had higher concentrations of total anaerobic and cellulolytic bacteria in the rumen. In addition, **Figure 4** compares rumen bacterial phyla from 16S pyrosequencing of dairy calves fed different doses of postbiotic supplements and analyzes the data using principal component analysis (PCA). Principal component analysis accounted for 75.6% and 15.1% of the overall variation in postbiotic supplementation (**Figure 5**). A greater distance between points indicated a greater difference in the bacterial community principal components (PC1 and PC2), which explained 75.6% and 15.1% of the variation, respectively. In this study, bacterial diversity and composition of the rumen microbiota differed across postbiotic dose levels when the same basal diets were fed. Therefore, the microbiome composition was altered, and a distinct clustering pattern was observed in dairy calves following postbiotic supplementation. These technologies provide valuable insights into how dietary postbiotics affect the gastrointestinal microbiota and their impact on ruminant performance and health.

Most bacterial species showed no significant differences among treatments at the species level; however, Prevotella ruminicola (**Table 6**) exhibited a quadratic response to postbiotic inclusion ($P < 0.05$). These results suggest that daily feeding of postbiotic-rich diets helps maintain a relatively stable microbial community in growing calves compared with those fed non-postbiotic diets. Nonetheless, Xiao *et al.* [39] reported that postbiotic supplementation improved gastrointestinal morphology, likely by altering bacterial diversity, reducing the prevalence of the Prevotella genus, and increasing the abundance of Butyrivibrio spp. The enhancement of cellulolytic bacteria, such as Fibrobacter succinogenes and Ruminococcus

flavefaciens, through postbiotic supplementation has been previously documented [4] [38]. Conversely, research on the effects of postbiotics on cellulolytic species like Ruminococcus albus and Butyrivibrio spp. remains limited (**Table 6**), similar to findings by Izuddin *et al.* [30]. The primary role of Ruminococcus albus and Butyrivibrio spp. in the gut is to ferment complex polysaccharides into volatile fatty acids (VFAs), including butyrate, propionate, and acetate [40].

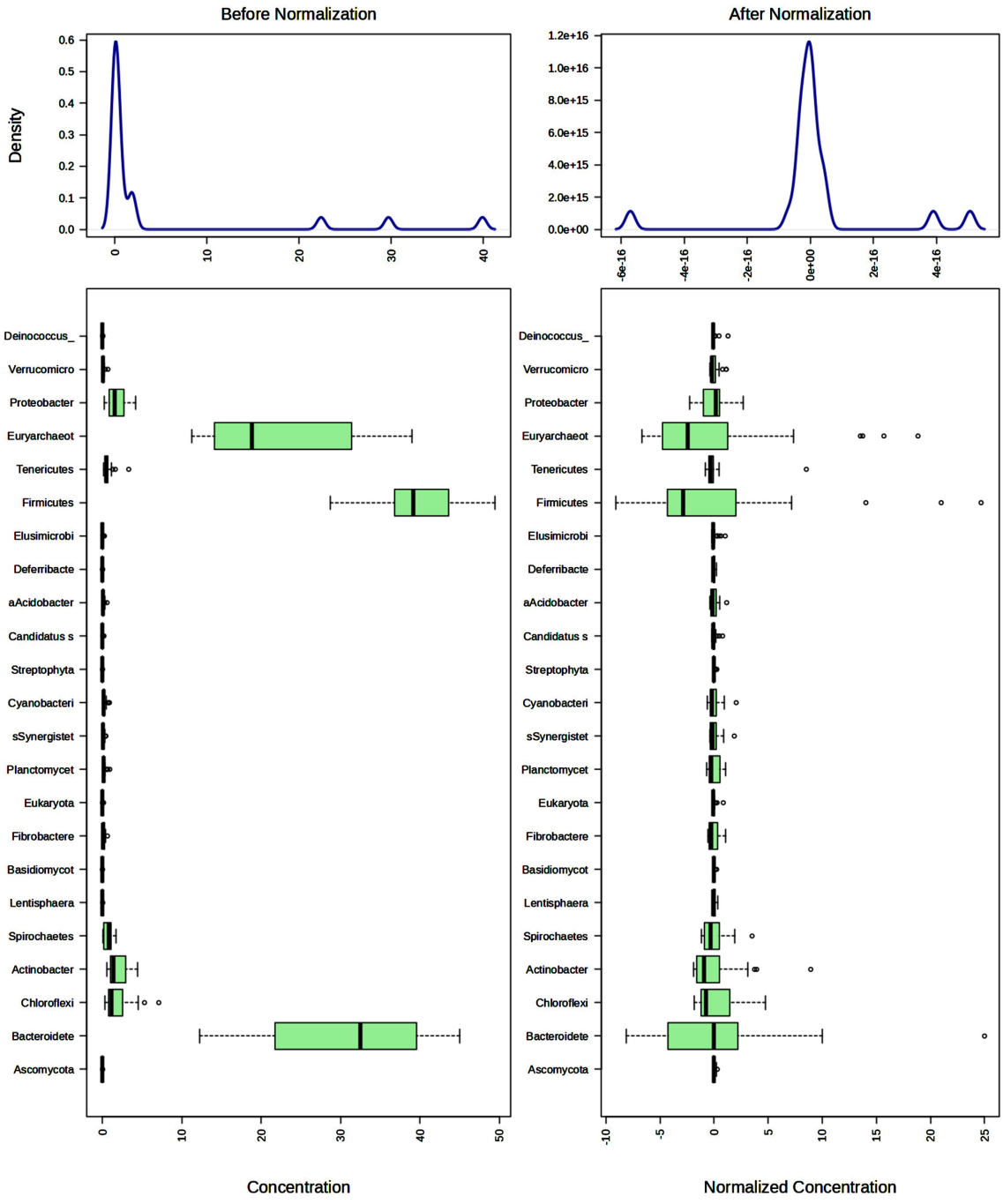


**Figure 4.** Diversities across the treatment groups in bacterial community compositions in dairy calves fed. Richness and diversity were measured by operational taxonomic units (OUTs).

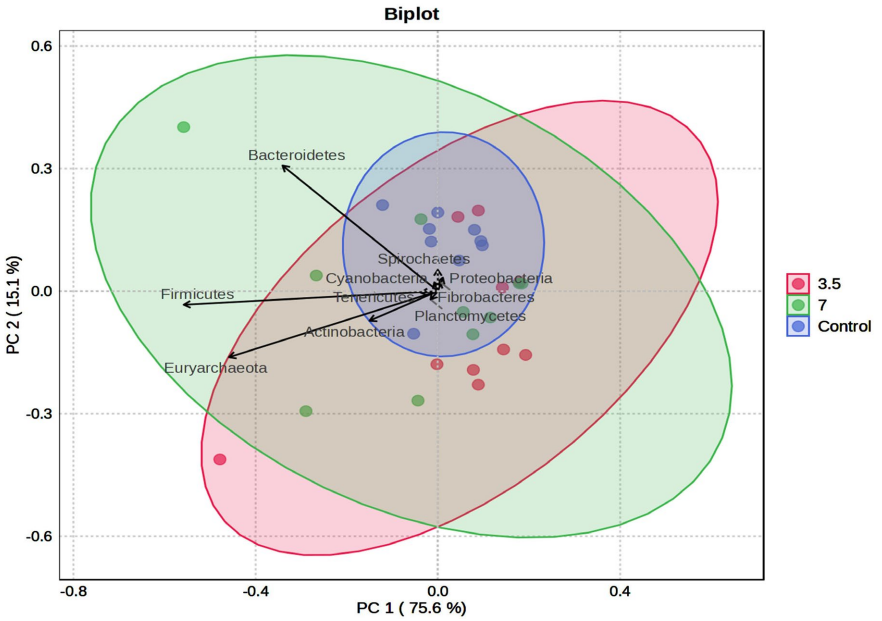


**Figure 5.** Principal component analysis of 16S bacterial profiles from rumen contents collected from dairy calves fed postbiotic with control, 0.0 g/hd/d(O), 3.5 g/hd/d (O), and 7.0 g/hd/d (O). Richness and diversity were measured by operational taxonomic units (OUTs). There is a distinct clustering pattern for dairy calves by diets. Results indicated that dairy calf samples showed a high degree of variability in community composition. The percentage of variation in the data explained by the analysis is shown in *brackets* (PC1 vs. PC2).

**Table 6.** The most abundant bacterial species diversity (cell number) in the rumen samples of dairy calves fed postbiotic supplementation based on pyrosequencing of the 16S rDNA during the post-weaning period.

| Item[1] | Treatment (g post-biotic/d) | | | | |
|---|---|---|---|---|---|
| | Control (0 g/d) | 3.5 (g/d) | 7.0 (g/d) | SEM | *P*-value |
| *Aristaeella hokkaidonesis* | 1684.3 | 2546.3 | 1684.4 | 783.01 | 0.46 |
| *Vampirovibrio chlorellavorus* | 1412.0 | 1107.7 | 736.4 | 422.2 | 0.31 |
| *Ruminococcus albus* | 106.1 | 114.2 | 85.8 | 27.9 | 0.50 |
| *Provotella ruminicola* | 8758.4[a] | 5029.0[b] | 7266.0[ab] | 11923.9 | 0.05 |
| *Pseudobutyrivibrio ruminis* | 1072.0 | 1397.5 | 2251.8 | 704.90 | 0.46 |
| *Butyrivibrio clostridium proteoclasticum* | 929.7 | 621.7 | 1539.2 | 501.99 | 0.27 |
| *Xylanibacter ruminicola* | 317.0 | 267.2 | 183.6 | 119.6 | 0.66 |

[1]This table presents the relative abundances of the seven most abundant bacterial species (> 1.0%). [a-b]Means within a row with different superscripts differ ($P < 0.05$). SEM = Standard error of the mean.

A lower population of methanogens in *Methanosphaera stadmanae* was observed in the rumen ($P = 0.07$; Table 7) following supplementation with postbiotics in this study and in post-weaning Dorper lambs [30]. Izuddin *et al.* [30] and

Jeyanathan *et al.* [41] reported that increased ruminal propionate production in the postbiotic group was associated with reduced methane formation and greater energy retention from the diet. Some studies using Saccharomyces cerevisiae have demonstrated a methane-lowering effect; however, to date, no systematic investigation of direct-fed microbials as modulators of rumen methanogenesis has been conducted [41]. Pathways that redirect $H_2$ away from methanogenesis and those that produce less H2 during feed fermentation are preferred options [41] [42]. This may explain the reduction in the ruminal methanogen population in the postbiotic group, as $H_2$ depletion, a methane source for methanogens, reduces methane production. Our results suggest that postbiotic supplementation could be a promising alternative for reducing rumen methanogenesis, and further research on its practical application is warranted.

**Table 7.** Diversity of rumen methanogen populations (cell numbers) in the rumen samples of dairy calves fed postbiotic supplementation, based on pyrosequencing of the 16S rDNA during the post-weaning period.

| Item[1] | Control (0 g/d) | 3.5 (g/d) | 7.0 (g/d) | SEM | *P*-value |
|---|---|---|---|---|---|
| *Methanobrevibacter millerae* | 584.4 | 127.0 | 296.6 | 119.06 | 0.17 |
| *Methanosphaera stadtmanae* | 79.7 | 24.3 | 48.2 | 19.38 | 0.07 |

[1]This table presents the relative abundances of the seven most abundant bacterial species (>1.0%). SEM = Standard error of the mean.

## 3.7. Postbiotic and Calf Health

Fecal consistency and nasal discharge scores, which are linked to bovine respiratory disease, serve as valuable tools for diagnosing, monitoring, and treating sick calves, as shown in **Figure 5** and **Figure 6**. In this study, postbiotic supplementation significantly reduced ($P < 0.05$ - 0.01) both the fecal consistency score (**Figure 6**) and the nasal discharge score (**Figure 7**) in pre- and post-weaning dairy calves. Management practices targeting these scores consistently yield positive outcomes when combined with postbiotic supplementation. Bovine respiratory disease remains a major challenge to animal welfare, causing significant economic losses for producers due to morbidity, mortality, and decreased productivity in adult dairy cows [43] [44]. It is now the second leading cause of death in pre-weaning heifers and the top cause of mortality in weaned heifers [43]. These losses include treatment costs, higher mortality rates, early culling, slower growth, impaired fertility, and reduced milk production [45]. Prior research [46] indicates that heifers that experienced diarrhea as calves had lower average daily gains at weaning and lower milk production during their first lactation. Supplementing with postbiotics has been shown in this and earlier studies to improve animal health during pre- and post-weaning periods, potentially by enhancing immune function during these critical times [11] [33] [47] [48]. These management practices can help develop protective strategies for Holstein dairies and other dairy calf systems.

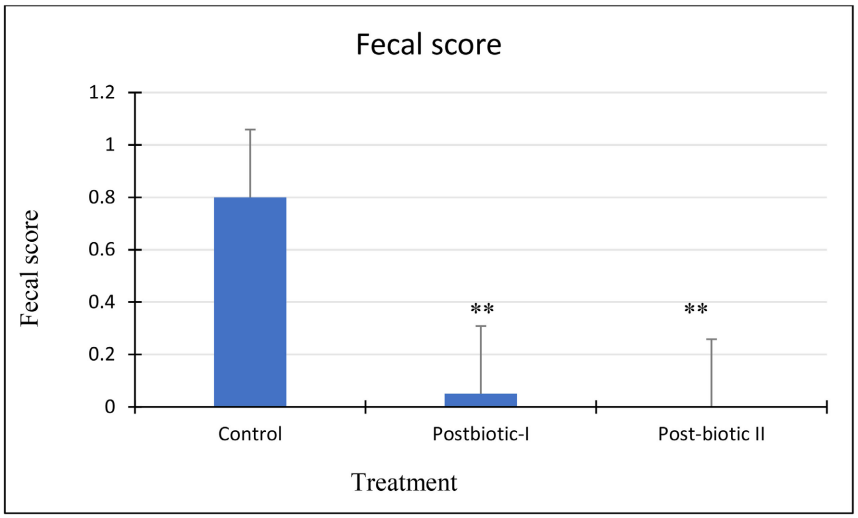


**Figure 6.** Effects of increasing levels of postbiotic on the fecal consistency score of dairy male calves during the 30 days following calving (n = 10). Fecal consistency score: 0: Normal consistency, firm, but not hard; Fecal score 1: semi-formed, soft, or pasty stool; fecal score 2: Loose or runny stool that spreads quickly; fecal score 3: watery feces that splatter. Postbiotic I = pre-weaning period/collection period I. Postbiotic II refers to the post-weaning (collection period II). Statistical significance: ** $P < 0.01$.

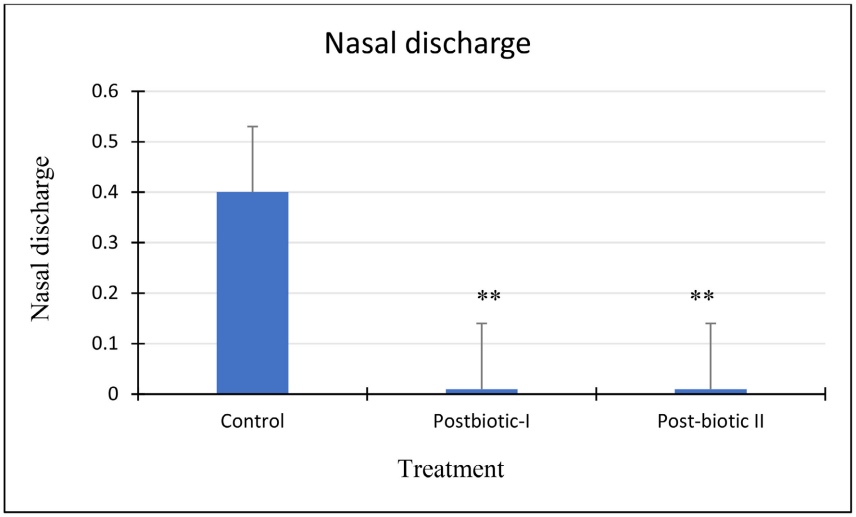


**Figure 7.** Effects of increasing levels of postbiotic on the nasal discharge score of dairy male calves during the 30 days following calving (n = 10). Nasal discharge score: 0 = normal serous discharge (none), 1 = coughs upon movement (slightly mucous discharge), 2 = excessive nasal discharge, and 3= copious bilateral mucopurulent discharge. Postbiotic I = pre-weaning period (collection period I). Postbiotic II refers to the post-weaning period (collection period II). Statistical significance: ** $P < 0.01$.

## 4. Conclusion

In calves, the first months of life are crucial for future production. Although no significant effects on dairy calves' performance, such as body weight changes, were observed, potential positive effects on animal health, including enhanced immune function and immune-related gene expression, may occur without negative effects

on rumen microbiome community changes or blood chemical parameters. However, these effects warrant further investigation in future studies. Therefore, research is needed to determine the optimal inclusion rate of Lactobacillus culture-based postbiotics in dairy calf diets.

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## Conflicts of Interest

There are no competing interests in this study.

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