Effects of waste milk on growth performance, immunity, and gut health of dairy calves

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Research

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Abstract

Background

Liquid feed are the major nutrient source that can have a significant impact on the growth and development of immune system of calves before weaning. Waste milk containing antibiotic residue has been produced because of the continuous expansion of dairy farms. In order to reduce economic loss and prevent environmental pollution, most farms seem waste milk as one of the calves' liquid feeds. However, there is limited information to report the effects of waste milk on growth performance, especially immunity function of calves. Thus, the objective of this study was to investigate the effects of waste milk on growth, immunity and gut health of dairy calves.

Results

Feeding WM improved hip width, hip height, heart girth, final body weight, and feed efficiency of dairy calves compared to MR. Plasma concentrations of IgA, IgM, IgG and IL-10 were higher and TNF-α was lower in WM group. In addition, treatment and time interactively affected plasma concentrations of IgG and IL-2, which increased and decreased in WM group but decreased and increased in MR group, respectively, from 49 to 70 d of age. There was no difference in diarrhea case and average days of diarrhea among treatments. Difference in fecal microbiota was observed between MR and WM groups only at 49 d of age. Analysis of differential abundance showed that the increase in the relative abundance of *Prevotellaceae* NK3B31 group and the decrease in that of *Bacteroides* was higher in WM than MM group from 49 to 70 d of age.

Conclusions

WM had beneficial effects on growth performance and did not affect health statue, which might be explained by enhanced immune function modulated by fecal microbiota.

Study Design

This trial was performed according to randomized complete block design and calves were assigned to 4 blocks based on arrival date. Within each block, calves were randomly allocated to three dietary treatments including 100% milk replacer (MR, Land O' Lakes, Arden Hills, MN), 50% pasteurized waste milk mixed with 50% milk replacer (MM), as well 100% pasteurized waste milk (WM, including milk with antibiotic and transition milk of 2~3 d of postpartum cows at Nestle Dairy Farm Institute).

Background

Calves are born with an immature immune system (1) and thus rely on colostrum to acquire passive immunity during the first days of life (2–3). After successful transfer of passive immunity, liquid feed is the main source of nutrition that plays an essential role in the development of immune functions of calves (3–4). Whole milk, milk replacer, and waste milk are the most common liquid feeds for calves on dairy farms (5). Although whole milk is generally considered as the best liquid feed for calves, it is mainly produced for human consumption (6). On the other hand, milk replacer is made up from high quality materials that are easy to digest and can meet the nutritional requirements of calves (7). In addition, due to the continuous expansion of dairy farms, a large amount of waste milk including transitional milk, abnormal milk and milk with antibiotic residue has been produced. In the United States, calves in 87.2% of dairy farms are fed waste milk (8) and in England and Wales, this proportion is 83% (9). In China, although it is estimated that the annual production of waste milk accounts for 2 ~ 4% of the total milk production, that is, 0.8 ~ 1.6 million tonnes (10), the application of waste milk is limited.

Immunoglobulins (Ig), including IgG, IgA and IgM, can bind antigen and activate complement (11), and protect calves from pathogens. A study showed that serum concentrations of IgG and IgA of pre-weaned calves fed with waste milk was significantly higher than those fed with raw milk (12). On the contrary, another study showed that no difference in serum concentration of IgM between waste milk and raw milk was observed, although IgG concentration was lower in calves fed with waste milk (13). Additionally, tumor necrosis factor alpha (TNF-α) participates in cell-mediated immune responses and defenses against intracellular viruses and mycoplasma (14–15). Interleukins (IL) are a class of immune factors that play an important role in regulating inflammation and immune responses induced by infection and injury (15). Tarradas reported that serum concentrations of TNF-α and IL-1β of calves fed waste milk were significantly higher than those fed whole milk (16). However, above studies did not explain the reason of difference in immune parameters caused by different liquid feeds, which we speculate that might be related to the change of gut microbiota.

Most liquid feeds flow directly into the abomasum, and thus the intestine is the major digestion site for pre-weaned calves (5). The intestinal tract microbiota is considered to play an important role in establishing immune system and protecting the host against pathogens (17). For example, *Clostridium* could enhance the anti-inflammatory effect in acquired immunity and contribute to the expansion of Foxp3 + Treg cells in the peripheral circulation (18). Treg cells can express IL-10, IL-2, TGF-β and other interleukins, and induce immune tolerance and immunosuppression (19). Effect of waste milk on intestinal microbiota are inconsistent among studies. For example, some studies showed that the amount of antibiotic-resistant *Escherichia coli* increased in the feces of calves fed waste milk compared with raw milk and milk replacer (20–21), while there was no difference in the percentage of antibiotic-resistant *E. coli* in feces between calves fed waste milk and milk replacer (22). Compared to raw milk, waste milk tended to increase the abundance of cecal mucosa-associated bacteria (10). On the contrary, another study showed that feeding waste milk did not change the relative abundance of fecal bacterial composition compared to raw milk (23). However, there is no study investigating if and how waste milk modulate growth and health by regulating intestinal microbiota in dairy calves. The objective of this study was therefore to evaluate the effects of waste milk on growth, health, plasma immune parameters,

and fecal microbial composition of dairy calves. We hypothesized that feeding waste milk could improve the growth performance and immune functions via regulating the gut microbiota of dairy calves.

Materials And Methods

This experiment was carried out from November 7, 2020 to February 2, 2021 at Nestle Dairy Farm Institute (DFI, Harbin, Heilongjiang, China, E 125°41', N 45°08'). The average temperature and humidity were −12.7°C and 69.7%, respectively. Treatment of disease followed the standard operating procedures at DFI and sick calves were treated by a veterinarian accordingly.

Calves, Housing, and Diets

A total of twenty-four Holstein male calves were separated from their dams after birth and placed in individual pens (1.8 m × 0.8 m) on straw beddings. Calves were fed 4 L colostrum within 3 h after birth and another 4 L colostrum within 12 h to ensure the successful passive transfer of immunity. Then 4 L of pasteurized whole milk were provided twice daily (1100 h and 1900 h) until 7 d of age. After that, calves were bottled-fed three times a day at 0730 h, 1430 h and 2200 h the whole study with an adjusted step-up/step-down milk feeding protocol as 5, 6, 7, 6, 5, 4 and 3 L at wk 1, 2, 3, 4, 5, 6, 7 and 8, respectively. All calves were weaned at 56 d of age and the experiment was terminated at 70 d of age.

This trial was performed according to randomized complete block design and calves were assigned to 4 blocks based on arrival date. Within each block, calves were randomly allocated to three dietary treatments including 100% milk replacer (MR, Land O' Lakes, Arden Hills, MN), 50% pasteurized waste milk mixed with 50% milk replacer (MM), as well 100% pasteurized waste milk (WM, including milk with antibiotic and transition milk of 2~3 d of postpartum cows at DFI). Milk replacer was mixed with warm water (46 ℃) at a ratio of 1:7 and waste milk was pasteurized by heating up to 72 ℃ for 15 s and cooled down to 38~40 ℃ for bottle feeding. During the experiment, starter and fresh water were fed *ad libitum* from 8 d of age. Intake of starter was recorded daily, and samples were taken every two weeks, which were kept frozen for subsequent analyses.

Measurements, Sampling, and Analyses

Body weight (BW), height, length, hip width, hip height and heart girth were measured as described by Mirzaei (24) before morning feeding at 8 d, 56 d and 70 d of age. Starter intake were analyzed every two weeks, and average daily gain (ADG), dry matter intake (DMI) and feed efficiency (FE) were calculated from 8 to 56 d (pre-weaned period), 57 to 70 d (post-weaned period) and 8 to 70 d (whole experiment period) of age. Fecal consistency was scored daily before morning feeding according to the guideline by Larson (25): 1 = firm, well-formed (not hard); 2 = soft, pudding like; 3 = runny, pancake batter; 4 = liquid, splatters, pulpy orange juice. Feces were estimated abnormal when the score ≥ 3, and a diarrhea case was defined when fecal score was ≥ 3 for at least 2 days.

Blood samples were obtained from the jugular vein into heparin sodium tubes before morning feeding at 8, 49, and 70 d of age. Following collection, blood samples were separated by centrifuging at 3500 × g for 10 min to obtain plasma, which was divided into several aliquots and stored at −20°C for subsequent analyses. The concentration of IgG, IgM, IgA, IL-2, IL-6, IL-10 and TNF-α were measured using ELISA assays (Enzyme-linked Biotechnology Co., Ltd, Shanghai, China).

Fresh feces were obtained by-hand from the rectum of calves using clean gloves at 49 d and 70 d of age. After collection, part of samples was directly stored at −80 °C for analysis of 16S rRNA gene copies of *E. coli* and *Lactobacillus* species. Pour plate method was used for counting bacteria (26) using Maconkey Agar Medium (Aoboxing, Beijing, China) for *E. coli* and MRS Agar Medium (Aoboxing, Beijing, China) for *Lactobacillus* species (21, 27). The remaining fecal samples were diluted with water (1:4) for measuring pH using a glass electrode (Sartorius, Göttingen, Germany).

The bacterial community was profiled by sequencing V3–V4 hypervariable region of 16S rRNA genes using 314F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATATAAT-3') primers (28). The amplicons were sequenced (2 × 250 bp) using the nova PE250 in Novaseq 6000 platform (Novogene, Tianjin, China). Analysis of the sequence data were performed using Quantitative Insights Into Microbial Ecology 2 (29) (QIIME2, version 2021.04). Amplicon sequence variants were generated using DADA2 (30) workflow to remove the barcodes, primers and low-quality sequences. The taxonomic classification was performed using the SILVA database (SILVA Release 138) based on 99% sequence similarity. All sequences were deposited in the NCBI Sequence Read Archive under the project number PRJNA752817.

Statistical Analysis

Growth performance, plasma immune parameters, fecal scores, health-related indices, and copy numbers of *E. coli* and *Lactobacillus*) analyses were conducted using PROC MIXED procedure of SAS (version 9.4; SAS Institude Inc., Cary, NC), with treatment and sampling time as fixed effect, and block as random effect. Initial value of body weight, structural measures and blood immune indices were considered as covariates. Treatment, age, and their interactions were included in the model as fixed effects.

Fecal bacterial alpha diversity and relative abundance of bacterial taxa were analyzed using Kruskal-Wallis test in R (version 4.0.2; R Foundation for Statistical Computing, Vienna, Austria), and the *P*-value was adjusted based on false discovery rate (FDR) using the Benjamini-Hochberg algorithm. Principal coordinate analysis (PCoA) of the fecal microbial profiles was conducted using Bray-Curtis distance and permutational analysis of variance (PERMANOVA) in QIIME2. Statistical significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. One calf became ill and dead at 14 d of age and thus the data for this calf was excluded from the dataset before analysis.

Results

Growth Performance

No interactive effect of treatment and age was observed on starter intake ($P = 0.87$; Figure 1). Starter intake increased significantly with age ($P < 0.001$), however, which was not different among treatments ($P = 0.27$). Both treatment ($P = 0.01$) and age ($P < 0.001$) significantly impacted BW of calves, which was not affected by their interactions ($P = 0.92$) (Figure 2). BW of calves in WM group was highest at 70 d of age. No interactive effect of treatment and age was found on body measurements, which increased over age ($P < 0.001$) (Table 2). Hip width ($P = 0.007$), hip height ($P = 0.03$) and heart girth ($P = 0.008$) were higher in WM group than other two groups. Compared with MM, calves fed WM had greater body length ($P = 0.04$). No difference was observed in body height ($P = 0.13$) among treatment.

No interactive effect of treatment and age was observed on ADG ($P = 0.89$; Table 3), DMI ($P = 0.69$), and FE ($P = 0.92$). No difference was showed in ADG and DMI among treatments ($P > 0.05$). FE was significant higher in WM group during the whole period ($P = 0.04$). All these measurements increased significantly over age ($P \leq 0.05$).

Plasma immune parameters

Interactive effect of treatment and age was found on plasma concentration of IgG ($P = 0.05$) and IL-2 ($P < 0.001$; Table 4), but not observed on IgA, IgM, IL-6, IL-10 and TNF-α. Specifically, plasma concentrations of IgG and IL-2 increased and decreased in WM group, but decreased and increased in MR group, respectively, from 49 to 70 d of age. Concentrations of IgA ($P < 0.001$) and IgG ($P < 0.001$) of calves fed WM were significantly higher than those of calves fed MM and MR, and was also higher in MM than MR group ($P < 0.001$) regardless of age. Plasma concentration of IgM ($P < 0.001$) and IL-10 ($P < 0.001$) were lower in MR group at 49 and 70 d of age. Concentration of IL-2 was lower ($P = 0.006$) and higher ($P = 0.006$) at 49 and 70 d of age, respectively, in MR than MM and WM groups. At 70 d of age, concentration of IL-2 was higher in MR group ($P < 0.001$) but not different ($P = 0.11$) in WM group compared with 49 d of age. Plasma concentration of TNF-α was highest in MR treatment and lowest in WM treatment at 49 and 70 d of age ($P < 0.001$). Concentration of IL-6 tended to decrease in calves fed with WM ($P = 0.06$).

Fecal score and health status

There was no interactive effect of treatment and age on fecal score and health-related indicators except for oral electrolyte treatment times (Table 5), which was higher in MM group during pre-weaned period ($P = 0.001$). Fecal score ($P = 0.01$) and abnormal fecal days ($P = 0.03$) were significantly higher in WM group during pre-weaned and post-weaned period. The average days ($P = 0.08$) and longest diarrhea days ($P = 0.07$) tended to be higher in WM group. We observed no difference in diarrhea case ($P = 0.13$), average days to recover from diarrhea ($P = 0.22$), treated with antibiotic times ($P = 0.26$) and fecal pH ($P = 0.32$) among treatments throughout the experimental period.

Fecal bacterial diversity and taxonomic compositions

Copy numbers of *E. coli* and *Lactobacillus* were not different among treatments at 49 or 70 d of age (Figure 3). Shannon ($P = 0.01$) and Simpson ($P = 0.002$) indices were significantly higher in MR group at

49 d of age, but no difference was found among treatments at 70 d of age ($P > 0.05$; Table 6). Similarly, no difference was observed in Chao 1 index at 49 or 70 d of age ($P > 0.05$). Beta diversity based on Bray-Curtis distance showed clear separation of samples among treatments at 49 ($P = 0.002$) but not 70 d of age ($P = 0.80$, PERMANOVA; Figure 4). Bacterial taxa with a relative abundance > 0.1 % in at least 4 calves per group was considered as identified in this study. Bacteroidota and Firmicutes were predominant phylum at 49 and 70 d of age in all treatments and Actinobacteriota did not exist in MR group at 49 d (Figure 5). At genus level, the relative abundances of *Prevotellaceae* NK3B31 group ($P = 0.01$) and *Eubacterium coprostanoligenes* group ($P = 0.03$) were highest in MR group, meanwhile, the relative abundances of *UCG-005* ($P = 0.01$), *Christensenellaceae* R-7 group ($P = 0.03$), and *Bacteroides* ($P = 0.02$) were highest in WM group at 49 d of age. Meanwhile, above differences did not discover at 70 d.

Differential abundance was analyzed to compare the temporal changes in bacterial diversity and composition among treatments. We found that the difference in the relative abundance of only two genera (*Prevotellaceae* NK3B31 group and *Bacteroides*) between 49 and 70 d of age were different among treatments. Specifically, the increase in the relative abundance of *Prevotellaceae* NK3B31 group ($P = 0.03$) and the decrease in that of *Bacteroides* ($P = 0.04$) was higher in WM than MM from 49 to 70 d of age (Figure 6).

Discussion

Li (31) reported that adding antibiotics to MR did not affect starter intake of Holstein × Angus crossbred calves, which is similar to our findings that antibiotic in WM group has no difference in comparison with MR group. However, BW of calves in WM group was higher than MR group in the present study, which is in accordance with the findings in calves from 4 to 13 week of age reported by Brunton (22). The result might due to that MR contains lower energy compared with WM, and the calves fed MR may only have maintenance energy to cope with the cold condition. Conversely, WM had more energy to support the growth of calves (32). Body skeletal growth is an important indicator to reflect overall development of calves, as well as an intuitive reflection of body growth and feeding level. Our study showed that calves fed WM had greater skeletal growth and development, which may be associated with the change of BW, as Heinrichs reported that there was a significant correlation between BW and heart girth (33).

Han (12) reported that the serum concentrations of IgG and IgA of calves fed with WM at 60 d of experiment were significantly higher than those fed with whole milk, which is similar to our result. This may be due to the existence of antibiotic residue in WM that can improve the phagocytic activity of granulocytes in blood (34). On the other hand, inclusion of transition milk can lead to a high amount of blood immunoglobulin, which may be associated with high BW of calves (35). In addition to immunoglobulins, cytokines are essential to the immune function of neonatal calves (36). As a proinflammatory factor, higher plasma concentration of IL-2 in MM and WM at 49 d but not 70 d of age might indicate that calves fed WM were more resistant to inflammation as they grew. IL-6 is associated with the growth and differentiation of lymphocytes and B cells and TNF-α often induces partial inflammatory changes and mediate systemic acute-phase responses to tissue injury and microbial

invasion (37). Lower plasma concentration of IL-6 and TNF-α in calves of WM group showed that WM could improve the level of immune parameters before or after weaning, and the increasing immunity might be related to high milk protein content. Han (12) suggested that high protein level in diet could enhance the anti-infection ability by affecting the T cell immune response. Indeed, higher concentration of IL-10 in WM group was found in our experiment. IL-10 is an anti-inflammatory cytokine that plays a pivotal role in the function of regulatory T cells that control inflammatory responses in the intestine (38). Furthermore, Gifford (39) claimed that acute phase inflammatory reactions have been linked with lower BW gain in calves, which might be the reason why calves in WM group had higher body weight. In addition, the change of IgG and IL-2 between 49 and 70 d of age potentially illustrate that liquid feed may have a long-term influence on growth performance of calves, which warrants further investigation.

The goal of a successful calf-rearing program is to minimize morbidity and mortality (40). In the current study, fecal scores were monitored daily, which has been frequently used to evaluate calves' health status (41–42). Consistent with previous studies, calves had high incidence of diarrhea during first weeks of life (43–44). Godden (32) reported that WM containing antibiotic residue could help calves to overcome the challenging period of early life and decrease the incidence of diarrhea and mortality in comparison to MR. However, no difference was found in the incidence of diarrhea case in our study, which might be related to the concentration of antibiotic residue in WM (45).

Gut microbiota during early life plays a vital role in modulating host intestinal barrier function, immune system development, metabolism, and health (46). Richness describes the number of unique taxa present in a sample, whereas evenness expresses the distribution of the taxa present in a sample (47). In this present study, the result of Simpson index demonstrates that MM and WM groups are associated with reduced microbial evenness at 49 d of age compared with MR group. A potential explanation for the decreased bacterial diversity observed in MM and WM groups may be related to antimicrobial effects of antibiotics as shown by Deng (10). In addition, results based on PCoA suggest that different feeding strategies was associated with dissimilarities in microbial composition and community structure after considering presence, abundance, and phylogenetic relationships between taxa, but no difference was observed until 70 d of age. Moreover, failure to observe the change of differential abundance in Shannon index and Bray-curtis distance of fecal bacteria among treatments suggest that no effect of liquid feed source on the change of fecal bacterial diversity between pre-weaned and post-weaned.

Firmicutes is regarded as the most predominant phylum during the first 49 d of life in fecal samples of dairy calves (48–49), while others reported that *Bacteroidetes* is the most predominant phylum in feces of pre-weaned calves (50–51). Similar to later, *Bacteroidetes* is also detected as the most abundant phylum in the present study. These inconsistent results may be due to different calf feeding strategies, and breed or sampling methods (36). Few studies analyzed microbial profile using local intestinal tissue and content samples (52–54), because fecal samples are noninvasive and easy to collect repeatedly (55), and it has been recently investigated that fecal microbial composition could be a good indicator of the gastrointestinal microbiome (56). Therefore, selecting the representative intestinal samples to explore the microbial profile is encouraged in the future.

Prevotellaceae NK3B31 group can promote anti-inflammatory responses (57). The differential abundance of this genus may explain the reason of the change in plasma concentrations of IgG and IL-2 between pre-weaned and post-weaned. Similarly, *Bacteroides* is considered as biomarker in inflammatory bowel diseases, which can impair intestinal barrier and subsequently invade body as well as induce endotoxemia (58–59). After weaning, the decrease in the relative abundance of *Bacteroides* also suggested that calves fed WM may establish more resistant to pathogens. Furthermore, the genus shifting from *Bacteroides* to *Prevotellaceae* NK3B31 group may indicate a metabolism change, including amino acid, and carbohydrate and lipid, and nucleotide (60). In addition, previous study has reported that increased relative abundance of *Proteobacteria* has been correlated with antibiotic use (61). Unfortunately, the antibiotic resistance genes (ARG) were not measured in this trial. It is therefore essential to investigate profiles of ARG in gut microbiome and observe differential abundance between supplying WM with antibiotic residue and whole milk.

Conclusions

In current study, we demonstrated that WM had beneficial effects on growth performance and a limited influence on health statue in comparison of MR, possibly by improving plasma immune parameters regulated by gut microbiota. Further studies should be performed to investigate the long-term effect of use of WM on growth and development of immune functions of calves.

Abbreviations

Ig: Immunoglobulins

TNF-α: Tumor necrosis factor alpha

IL: Interleukins

DFI: Nestle Dairy Farm Institute

MR: 100% milk replacer

MM: 50% pasteurized waste milk mixed with 50% milk replacer

WM: 100% pasteurized waste milk

BW: Body weight

Average daily gain: ADG

Dry matter intake: DMI

FE: Feed efficiency

False discovery rate: FDR

Principal coordinate analysis: PCoA

Declarations

Availability of data and materials

The data sets from the current study are available from the corresponding author on reasonable request.

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Author Contributions

XH, ZY and JX contributed to conception and design of the study. XH and MT organized the database. XH, MT and ZX performed the statistical analysis. ZX, CC, LJ, and BH raised calves during the whole experiment. ZX wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Ethics approval and consent to participate

The animal study was reviewed and approved by the Ethical Committee of the College of Animal Science and Technology, Northeast Agriculture University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Tables

Table 1 Nutrient levels of different liquid feeds

Items	Diets	
	MR	WM
Protein (%)	3.13 ± 0.01	3.33 ± 0.01
Fat (%)	2.59 ± 0.006	4.41 ± 0.08
Total solid (%)	13.12 ± 0.02	12.76 ± 0.02
Lactose (%)	6.67 ± 0.01	4.48 ± 0.01
Energy (MJ/kg)	19.43 ± 0.13	22.37 ± 0.09

MR, milk replacer; WM, waste milk.

Table 2 Effects of waste milk on body measurements of dairy calves

Items		Diets [1]			SEM	*P*-value		
		MR	MM	WM		Treatment	Age	Treatment × Age
Body height (cm)	8 d	82.3	80.4	81.9				
	56 d	90.8	91.1	91.5	0.72	0.13	<0.001	0.54
	70 d	92.7	94.0	94.8				
Body length (cm)	8 d	68.0	66.0	68.4				
	56 d	79.2^{ab}	77.8^{b}	80.5^{a}	1.03	0.04	<0.001	0.78
	70 d	84.7^{ab}	82.5^{b}	87.0^{a}				
Hip width (cm)	8 d	25.7	25.5	24.7				
	56 d	30.9^{b}	30.9^{b}	32.5^{a}	0.52	0.007	<0.001	0.70
	70 d	33.8^{b}	33.0^{b}	35.6^{a}				
Hip height (cm)	8 d	76.8	75.0	77.0				
	56 d	83.8^{b}	84.2^{b}	86.0^{a}	0.66	0.03	0.0025	0.96
	70 d	85.9^{b}	86.4^{b}	88.4^{a}				
Heart girth (cm)	8 d	87.4	86.9	87.8				
	56 d	100.8^{b}	100.3^{b}	103.4^{a}	1.18	0.008	<0.001	0.62
	70 d	106.8^{b}	106.4^{b}	111.7^{a}				

MR, 100% milk replacer; MM, 50% milk replacer mixed with 50% waste milk; WM, 100% waste milk.

Table 3 Effects of waste milk on growth performance of dairy calves

Items		Diet			SEM	*P*-value		
		MR	MM	WM		Treatment	Age	Treatment × Age
ADG (kg/d)	8-56 d	0.57	0.57	0.63	0.09	0.54	<0.001	0.89
	57-70 d	1.08	1.00	1.15				
	8-70 d	0.68	0.66	0.77	0.07	0.33		
DMI (kg/d)	8-56 d	1.02	0.95	1.01	0.17	0.45	<0.001	0.69
	57-70 d	2.08	1.74	2.03				
	8-70 d	1.26	1.13	1.25	0.11	0.50		
FE	8-56 d	0.54	0.60	0.66	0.03	0.06	0.05	0.92
	57-70 d	0.49	0.51	0.58				
	8-70 d	0.5^{b}	0.59^{ab}	0.63^{a}	0.03	0.04		

MR, 100% milk replacer; MM, 50% milk replacer mixed with 50% waste milk; WM, 100% waste milk.

[1]FE, feed efficiency; FE = kilogram of BW gain per kilogram of total DMI (DMI: milk solid and starter).

Table 4 Effects of waste milk on plasma immune indices of dairy calves

Items		Diets [1]			SEM	*P*-value		
		MR	MM	WM		Treatment	Age	Treatment × Age
IgA (μg/ml)	8 d	265.3	285.6	313.0				
	49 d	240.1^{c}	301.1^{b}	384.9^{a}	8.30	<0.001	0.44	0.41
	70 d	205.6^{c}	313.5^{b}	372.7^{a}				
IgM (μg/ml)	8 d	203.8	247.7	277.4				
	49 d	222.6^{b}	279.2^{a}	287.8^{a}	5.65	<0.001	0.15	0.54
	70 d	222.0^{b}	297.0^{a}	312.5^{a}				
IgG (μg/ml)	8 d	806.7	980.3	995.0				
	49 d	872.4^{c}	1064.7^{b}	1195.5^{a}	22.96	<0.001	0.37	0.05
	70 d	772.0^{c}	1141.1^{b}	1314.6^{a}				
IL-2 (pg/ml)	8 d	153.1	151.6	130.5				
	49 d	147.3^{b}	161.3^{a}	150.8^{a}	3.11	0.006	0.78	<0.001
	70 d	190.7^{a}	141.6^{b}	132.3^{b}				
IL-6 (pg/ml)	8 d	23.1	18.8	15.7				
	49 d	18.8	18.8	15.8	0.45	0.06	0.33	0.64
	70 d	20.4	18.6	16.7				
IL-10 (pg/ml)	8 d	8.3	10.6	11.6				
	49 d	8.5^{b}	11.4^{a}	11.8^{a}	0.24	<0.001	0.10	0.75
	70 d	8.8^{b}	12.1^{a}	12.9^{a}				
TNF-α (pg/ml)	8 d	29.5	25.8	22.0				
	49 d	28.6^{a}	24.2^{b}	19.8^{c}	0.49	<0.001	0.72	0.77

	70 d	28.3[a]	24.3[b]	20.9[c]

MR, 100% milk replacer; MM, 50% milk replacer mixed with 50% waste milk; WM, 100% waste milk.

Table 5 Effects of waste milk on fecal score and health-related indices of dairy calves

Items		Diets [1]			SEM	*P*-value		
		MR	MM	WM		Treatment	Age	Treatment × Age
Fecal score	8-56 d	1.6[b]	1.7[a]	1.9[a]	0.11	0.01	0.07	0.17
	57-70 d	1.2[b]	1.8[a]	1.7[a]				
Abnormal fecal days (d)	8-56 d	8.0[b]	10.6[a]	13.0[a]	1.04	0.03	<0.001	0.64
	57-70 d	0.1[b]	2.6[a]	2.7[a]				
Diarrhea case (times)	8-56 d	1.5	2.0	2.3	0.32	0.13	<0.001	0.90
	57-70 d	0	0.6	0.6				
Average days of diarrhea (d)	8-56 d	1.9	2.8	3.4	0.45	0.08	<0.001	0.61
	57-70 d	0	1.6	0.9				
Longest days of diarrhea (d)	8-56 d	2.1	4.0	4.9	0.66	0.07	<0.001	0.59
	57-70 d	0	1.6	1.0				
Average days to recover from diarrhea (d)	8-56 d	0.7	1.1	1.7	0.32	0.22	0.01	0.33
	57-70 d	0	0.8	0.2				
Treated with antibiotic times (times)	8-56 d	1.5	2.4	2.5	0.31	0.26	<0.001	0.45
	57-	0	0.3	0				

	70 d							
Treated with oral electrolyte times (times)	8-56 d	0[b]	0.9[a]	0[b]	0.09	0.001	0.008	0.001
	57-70 d	0	0	0				
Fecal pH	8-56 d	6.8	6.8	6.9	0.23	0.32	<0.001	0.08
	57-70 d	7.5	7.1	7.2				

MR, 100% milk replacer; MM, 50% milk replacer mixed with 50% waste milk; WM, 100% waste milk.

[1] Abnormal fecal days means fecal score ≧ 3.

[2] Diarrhea means abnormal fecal days of calves last at least two days.

Table 6 Effects of waste milk on fecal microbial alpha diversity indices for the bacterial communities

Items		Diets [1]			SEM	*P*-value
		MR	MM	WM		
Chao1	49 d	561.0	516.0	517.0	9.84	0.15
	70 d	482.0	480.0	477.0	12.32	0.94
Shannon	49 d	7.41[a]	6.76[b]	6.96[b]	0.09	0.01
	70 d	6.56	6.36	6.63	0.14	0.58
Simpson	49 d	0.99[a]	0.96[b]	0.98[b]	0.01	0.002
	70 d	0.96	0.95	0.96	0.01	0.54

MR, 100% milk replacer; MM, 50% milk replacer mixed with 50% waste milk; WM, 100% waste milk.

Figures

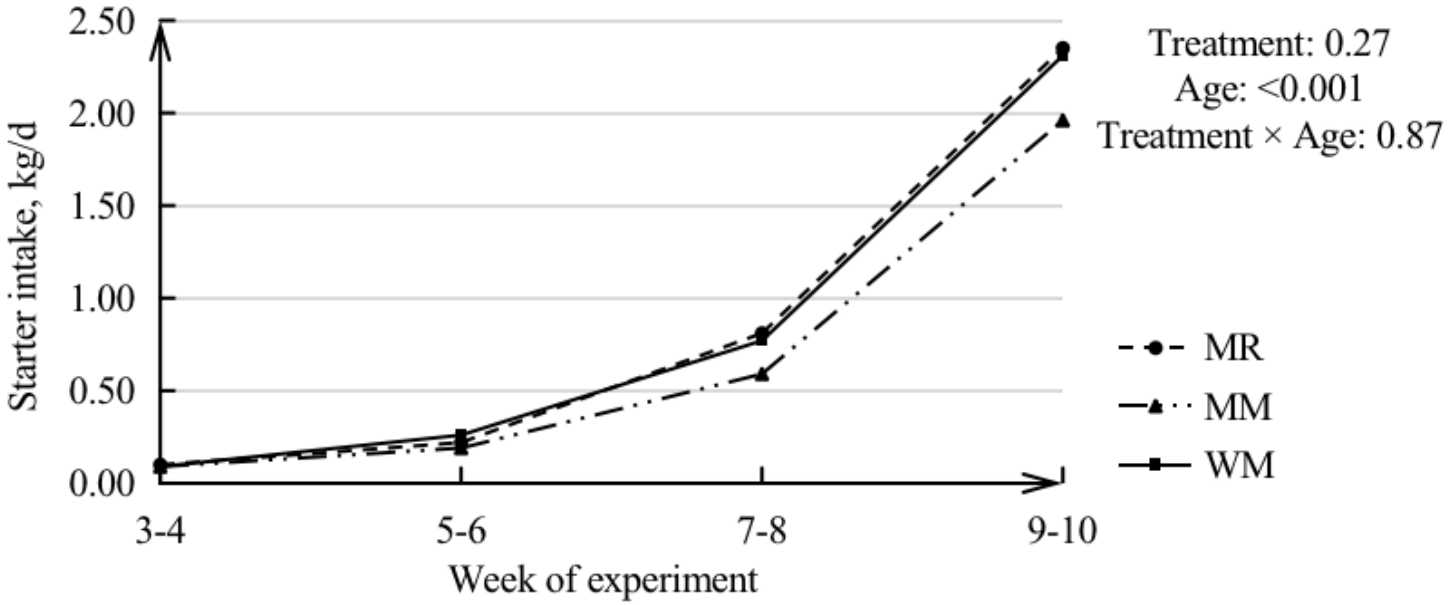

Figure 1

Effect of waste milk on starter intake of dairy calves

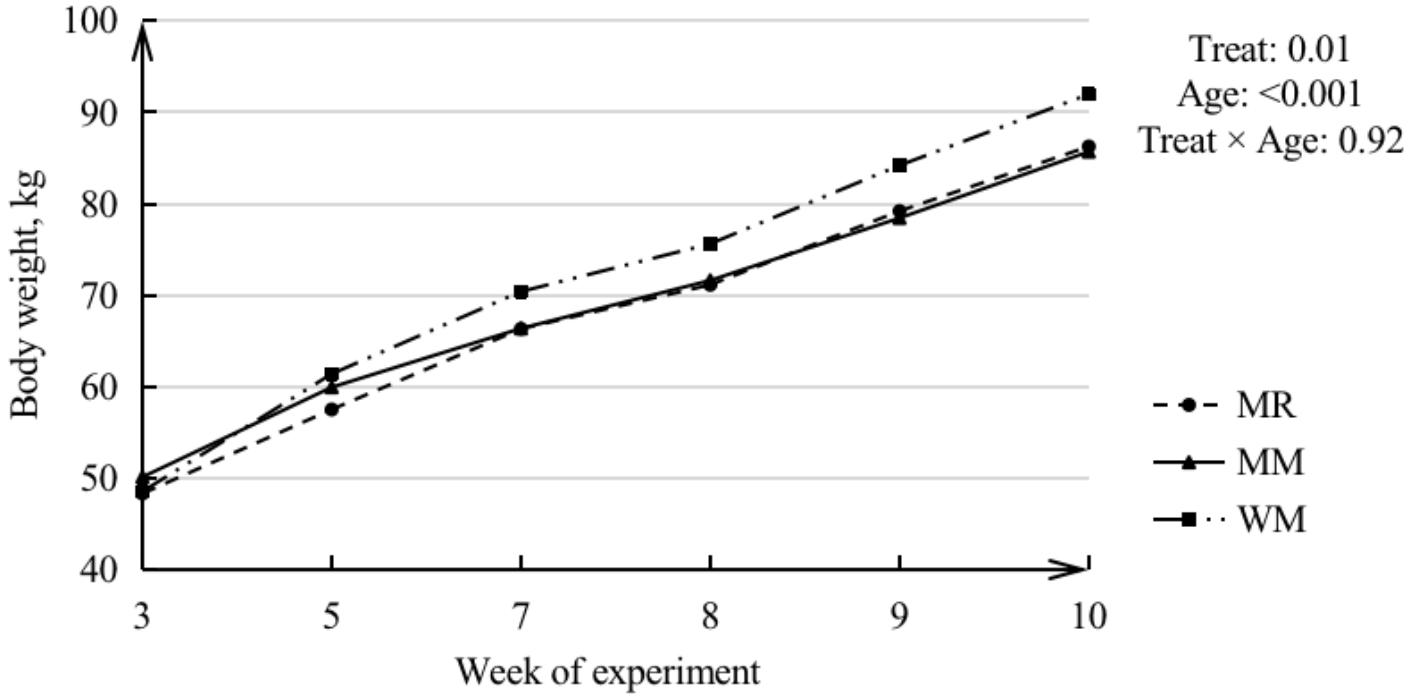

Figure 2

Effects of waste milk on body weight of dairy calves

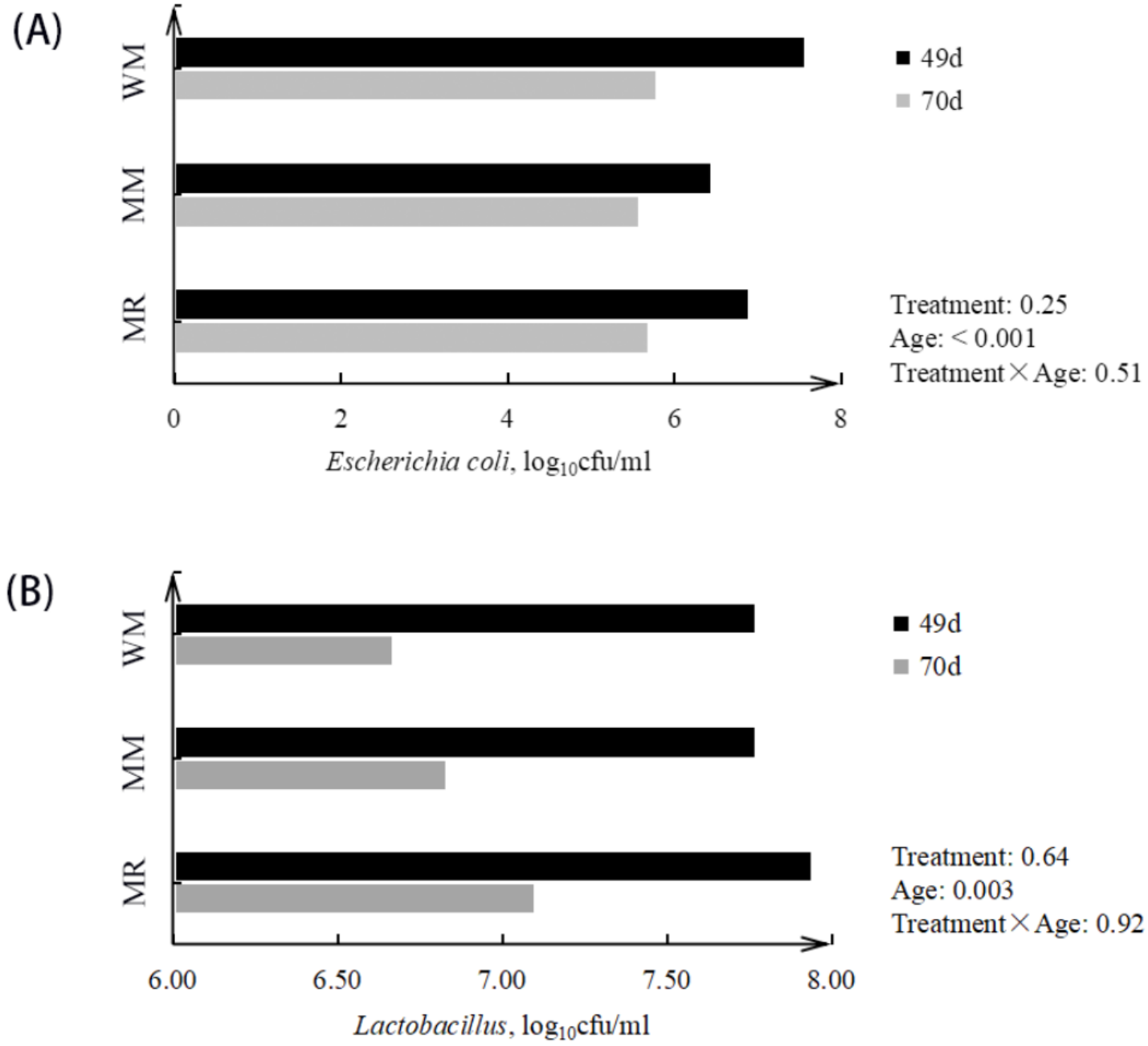

Figure 3

Effects of waste milk on fecal population of E. coli and Lactobacillus of dairy calves at 49 d and 70 d of age

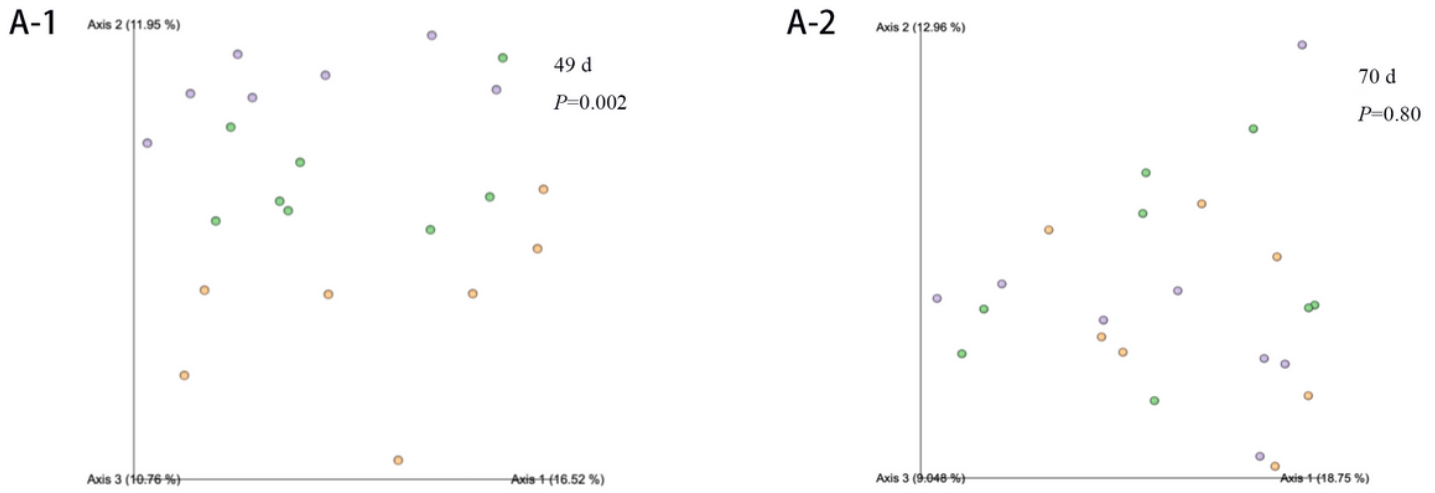

Figure 4

Principal Co-ordinates Analysis of beta-diversity of the fecal bacterial community of calves 1 (A-1): Bray-Curtis distance matrix values at 49 d of age; (A-2): Bray-Curtis distance matrix values at 70 d of age of calves 2 Treatment groups are as follows: MR, purple circles; MM, green circles; WM, orange circles.

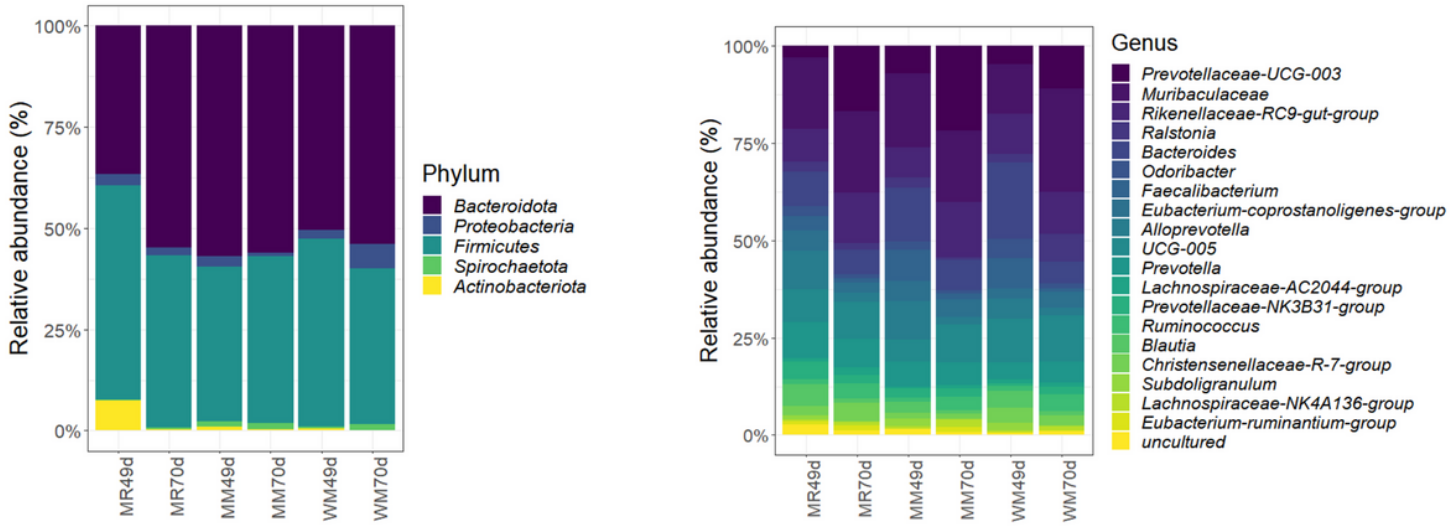

Figure 5

Relative abundance of fecal bacteria composition at 49 d and 70 d of age 1 Only bacterial phylum of top 5, family of top 10, genus of top 20 are included.

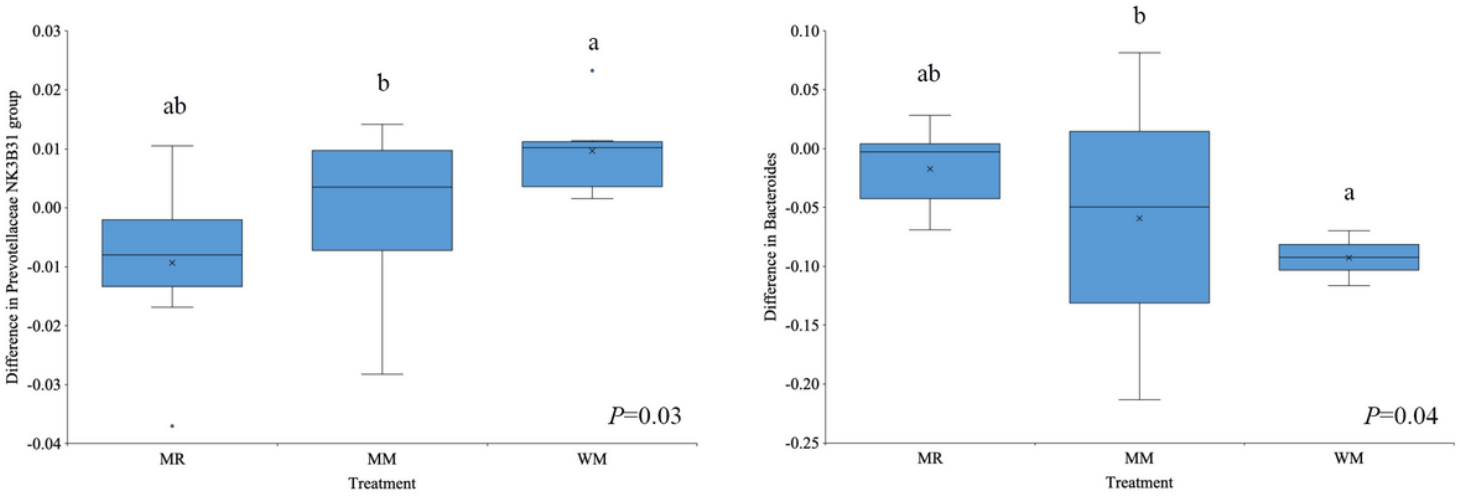

Figure 6

Differential abundance of Prevotellaceae NK3B31 group and Bacteroides between 49 d and 70 d of age.