

Effect of Maternal Separation and Transportation Stress on the Bovine Upper Respiratory Tract Microbiome and the Immune Response to Resident Opportunistic Pathogens

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Research Article

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Abstract

Background

The bovine upper respiratory tract (URT) microbiome includes opportunistic pathogens that cause respiratory disease and stress associated with maternal separation and transportation contributes to the severity of this respiratory disease. Stress is known to alter the gut microbiome but little is known regarding the effect of stress on the URT microbiota. This study used six-month old suckling beef calves to investigate whether maternal separation (weaned), by itself or combined with transportation (weaned + transport), altered the URT microbiome and host immune responses to resident opportunistic pathogens.

Results

Taxonomic and functional composition of the URT microbiome in suckling and weaned beef calves did not change significantly when serially sampled over a one-month period. Subtle temporal changes in the URT microbiome composition were observed in weaned + transport calves. Total bacterial density was lower ($p < 0.05$) on day 4 post-weaning in both the weaned and weaned + transport groups when compared to suckling calves. In addition, significant ($p < 0.05$) temporal changes in the density of the opportunistic pathogens, *M. haemolytica* and *P. multocida*, were observed independent of treatment but these changes did not correlate with significantly increased ($p < 0.05$) serum antibody responses to both of these bacteria in the weaned and weaned + transport groups. Weaning, by itself and in combination with transportation, had significant ($p < 0.05$) short- (2 to 8 days post-weaning) and long-term (28 days post-weaning) effects on the expression of adrenergic receptor genes in blood leukocytes when compared to age-matched suckling beef calves.

Conclusions

Maternal separation (weaning) and transportation has minor effects on the taxonomic and functional composition of the URT microbiome and temporal changes in the density of opportunistic pathogen residing in the URT did not correlate with the significant changes in immune responses to these bacteria. Significant changes in adrenergic receptor expression in blood leukocytes following weaning, with or without transportation, suggests altered neuroimmune regulation should be further investigated as a mechanism by which stress can alter host-microbiome interactions in the URT.

Background

In beef cattle the suckling period may last 5 to 7 months, leading to a strong dam-calf bond and separation of the calf from its dam, referred to as weaning, results in both physical and psychological stress [1]. Weaning transiently increases plasma cortisol [2] and both adrenaline and noradrenaline concentrations in beef calves [2, 3], confirming calves experience a stress response following maternal separation. Stress hormones can shape the gut microbial composition, while microbial metabolites alter host physiology through modulation of neurotransmitters [4], suggesting a bi-directional interaction

between host and its microbiota in the gut. It is not known, however, whether stress responses can shape or alter the composition of the upper respiratory tract (URT) microbial community. This question is of considerable importance since the URT microbial community includes many opportunistic pathogens that can colonize the lung and cause pneumonia during the post-weaning period.

Although there is a limited understanding of the effects of weaning stress, epidemiological studies have implicated stress as an important factor contributing to bovine respiratory disease (BRD). Respiratory disease is the most prevalent infectious disease in weaned beef cattle, accounting for the majority of morbidity and mortality in feedlots [5]. Stressors such as transportation and co-mingling of calves from different herds are associated with an increased risk of BRD in recently weaned beef calves [6, 7]. Use of next-generation sequencing has revealed that the URT microbial community changes significantly after calves enter feedlots [8, 9] and significant differences in the URT microbiota were also observed when comparing healthy dairy calves with age-matched cohorts diagnosed with respiratory infections [10, 11]. Studies with both beef and dairy cattle report changes in potentially pathogenic URT-resident bacterial groups (opportunistic pathogens) such as *Mannheimia* (*M.*), *Pasturella* (*P.*), *Moraxella* and *Mycoplasma* [8–11]. These studies suggested that observed changes in the URT microbiome may be a factor contributing to increased lung colonization by these opportunistic pathogens. There have been no studies, however, investigating whether the stress of maternal separation (weaning) and transportation significantly perturbs the composition of URT microbiome.

Studies of URT microbiome in calves following arrival in feedlots are complicated by animals experiencing multiple stressors, including weaning, transportation, co-mingling, dietary changes, a new environment, and respiratory virus infections [6]. The stress of weaning and transportation can double mortality in an experimental BRD model that combines a primary bovine herpesvirus-1 respiratory infection with a secondary *M. haemolytica* infection [12]. Weaning and transportation stress significantly altered host immune responses following viral and bacterial infection. Therefore, it is not known to what extent stress may contribute to differences reported for the URT microbiome when comparing sick calves (with clinical signs of BRD) versus healthy calves. Although a bi-directional interaction between gut microbiota and host during stress responses is well described [4, 13, 14], there is no information regarding a similar bi-direction interaction in the URT. Also, there is a lack of knowledge regarding the stability of the URT microbiome over time and the reliability of using single time point samplings to analyze URT microbial perturbations as weaned beef calves enter the feedlot.

Evidence is emerging that the mucosal immune system in the URT of healthy newborn calves responds to URT bacteria, including opportunistic pathogens such as *M. haemolytica* and *P. multocida* [15]. It is not known, however, whether alterations in the URT microbiome or stress can influence host responses to these opportunistic pathogens. Therefore, the current study was designed to address the question whether the stress of maternal separation (weaned), by itself or combined with transportation, significantly alters the URT microbiome or host responses to opportunistic pathogens within the microbiome. All calves in this study came from a single herd to eliminate comingling of animals from multiple sources as a potential factor contributing to microbiome changes and to prevent the introduction

of respiratory pathogens. The URT microbiome was serially sampled over a one month period to study microbiome stability in the URT of suckling beef calves and to determine if weaning and transportation were associated with either transient or sustained changes in the URT microbiome. Expression of adrenergic receptor (*ADR*) genes by blood leukocytes was also monitored to determine if weaning and transportation may have either short- or long-term effects on neuroimmune regulation of host responses by the stress hormones, epinephrine and norepinephrine. There are six known α -adrenergic receptors (*ADRA1A*, *ADRA1B*, *ADRA1D*, *ADRA2A*, *ADRA2B*, *ADRA2C*) and 3 known β -adrenergic receptors (*ADRB1*, *ADRB2*, *ADRB3*) but it is not known which ADRs regulate bovine immune function. Serum antibody responses to the URT commensal bacteria and opportunistic pathogens, *M. haemolytica* and *P. multocida*, were also monitored to determine if stress altered this microbial-host interaction.

Results

Clinical responses of calves following weaning

Rectal temperature was measured on experimental day 0 (D0) and every third day throughout the 28 day study period. Temperatures exceeding 40°C were considered a fever and a possible indication of a respiratory infection in the absence of other clinical signs of illness. All calves had temperatures below 40°C on D0. One calf in the Weaned + Transport group had a rectal temperature of 40.2°C on D3 post-weaning and two different calves in the Weaned + Transport group had rectal temperature of 40.2°C and 40.3°C on D12. All other calves in this group and the other two treatment groups had rectal temperatures below 40°C throughout the remainder of the study. Rectal temperatures provided evidence calves remained clinically normal throughout the study with only three calves in the Weaned + Transport group displaying a transient fever. No calves received antibiotic treatment throughout the study.

Weaning, with or without transportation, alters *ADR* gene expression in blood leukocytes

Analysis of *ADR* genes expressed in bovine blood leukocytes revealed detectable levels of transcript for all 9 genes. There were significant temporal changes in the expression of the three beta genes, *ADRB1*, *ADRB2*, and *ADRB3*, and one alpha gene, *ADRA2A*, when compared to time-matched samples from Suckling calves (Figure 1). Expression of the nine *ADR* genes did not change significantly when compared over time within the group of Suckling calves. However, expression of *ADRB1* was significantly ($p < 0.001$) elevated in Weaned + Transport calves when compared to Suckling calves on D28 post-weaning (Figure 1A). Expression of the *ADRB2* was significantly ($p < 0.05$) upregulated on D2 and D4 in Weaned calves and on D2 post-weaning in Weaned + Transport calves ($p < 0.001$) when compared to time-matched samples collected from the Suckling calves (Figure 1B). Expression of *ADRB3* tended to upregulate ($p = 0.06$) in Weaned + Transport calves when compared to the Suckling calves on D28 (Figure 1C). Finally, expression of *ADRA2A* was significantly upregulated in Weaned calves on D4 ($p = 0.03$) and D8 ($p = 0.05$) and Weaned + Transport calves on D8 ($p = 0.05$) when compared to time-matched samples from the group of Suckling calves (Figure 1D).

Serum antibody responses to *M. haemolytica* and *P. multocida*

All suckling calves had detectable but low serum antibody titers to both *M. haemolytica* leukotoxin (Figure 2A) and bacterial lysate proteins (Figure 2B) on experimental D0. These titers remained unchanged in the Suckling group throughout the 28-day observation period. In contrast, *M. haemolytica*-specific antibody titers were significantly ($p < 0.01$) increased on D28 (Figure 2A and B) within both the Weaned and Weaned + Transport groups when compared to the Suckling group. *M. haemolytica*-specific antibody titers on D28 in the Weaned + Transport group were significantly ($p = 0.03$) greater than the Weaned group.

A similar antibody response to *P. multocida* was observed when comparing the three treatment groups (Figure 2C). Suckling calves in all groups were seropositive for *P. multocida* on experimental D0 and antibody titers did not change significantly throughout the 28-day observation period within the Suckling group. There was, however, a significant ($p = 0.03$) increase in antibody titers specific for *P. multocida* on D28 in both the Weaned and Weaned + Transport groups when compared within each group to the D0 titers. Furthermore, the increase in antibody titers observed in these two groups on D28 were significantly ($p = 0.02$) greater when compared to the Suckling group but there was no difference when comparing Weaned and Weaned + Transport groups.

Colonization of the URT by opportunistic pathogens

The URT of beef calves was colonized primarily by bacteria (mean relative abundance: Suckling – 95.2-97.5%; Weaned – 92.4-97.5%; Weaned + Transport – 94.9-98.5%), followed by viruses (mean relative abundance: Suckling – 0.76-3.05%; Weaned – 0.64-6.73%; Weaned + Transport – 0.43-3.33%) and archaea (mean relative abundance: Suckling – 0.03-0.05%; Weaned – 0.02-0.05%; Weaned + Transport – 0.02-0.08%) at all time points sampled (Figure S1). When the microbial composition was compared at the domain level, significant temporal variations were identified from all samples regardless of the treatment group (Table 1). Comparison of the relative abundance of opportunistic pathogens at genus level revealed that the abundance of *Mannheimia*, *Pasteurella*, *Moraxella*, and *Histophilus* varied temporally regardless of treatment group (Table 1). There were no significant temporal changes in the abundance of opportunistic pathogens identified in the Suckling group. However, the median relative abundance of *Mannheimia* was highest on D4 after weaning in Weaned calves (Table 1). In Weaned + Transport calves, the median relative abundance of *Histophilus* was highest on D4, while that of Microvirus (*Enterobacteria* phage phiX174 sensu lato) was lowest on D2 (Table 1).

Time-dependent variation in the URT microbial community composition

PCA plot visualization of taxonomic profiles generated through metagenomics sequencing of samples from all calves at all time points revealed no effect of weaning, with or without transport, on the URT microbiome (Figure 3A, ANOSIM-R = 0.0091; $p = 0.18$). All microbial profiles clustered closely except a few individual animals were outliers from the population at individual time points. When the same analysis approach was used within treatment groups, taxonomic profiles clustered closely in both Suckling calves (Figure 3B, ANOSIM-R = 0.2662; $p < 0.01$) and Weaned calves (Figure 3C, ANOSIM-R = 0.1337; $p < 0.01$), regardless of sampling time point. In Weaned + Transport calves, however, the PCA plot

revealed the URT community no longer clustered closely together (Figure 3D, ANOSIM-R = 0.3418; $p < 0.01$) and sampling time had a significant effect on microbial community composition.

Comparison of the three treatment groups at individual sampling time points revealed no significant differences in the URT microbial taxonomic composition (Figure S2). In addition, when the same analysis approach was used to understand the effect of stressors and sampling time on microbial functions, all functional profiles were tightly clustered for all groups at all time points (Figure S3).

Colonization of the URT by opportunistic pathogens changes with time but not with stressors

Logistic regression analysis was used to understand the relationship between colonization of the URT by opportunistic pathogens and the possible impact of stressors relative to Suckling calves. This analysis revealed the abundance of most opportunistic pathogenic bacterial genera was not linked to weaning and associated stress, except for *Haemophilus* (Table 2). Colonization by *Haemophilus* was significantly decreased in Weaned calves compared to Suckling calves, reflecting a 70% decreased likelihood of high abundance (adjusted OR 0.30, 95%CI 0.11-0.81, $p = 0.02$; Table 2) when adjusted for sampling time. In addition, Microvirus (*Enterobacteria* phage phiX174 sensu lato) colonization tended to be increased in Weaned + Transport calves compared to suckling calves, reflecting more than a two-fold increased likelihood (adjusted OR 2.32, 95%CI 0.88-6.43, $p = 0.09$; Table 3) when adjusted for sampling time.

Colonization by opportunistic bacterial genera, *Mannheimia*, *Pasteurella*, *Histophilus*, *Haemophilus*, and *Moraxella* displayed significant temporal variations independent of stressors (Table 2). Colonization by *Mannheimia* increased significantly on days 2, 4, 8 and 14 when compared to D0, while colonization by *Pasteurella* increased significantly on days 2, 4, and 8 compared to D0 (Table 2). High abundances of *Histophilus* and *Haemophilus* were observed on D2 and D4 compared to D0 (Table 2). In addition, *Moraxella* colonization increased on D2 and tended to be increased on D4 when comparing to D0 (Table 2). The same analysis was performed using microbial colonization data after weaning (days 2, 4, 8, 14, and 28), which showed a reduction in the colonization of *Haemophilus* in Weaned calves compared to Suckling calves (adjusted OR 0.33, 95%CI 0.11-0.91, $p = 0.04$). A low abundance of *Haemophilus* was observed on D28 (adjusted OR 0.28, 95%CI 0.07-0.98, $p = 0.05$), whereas a lower abundance of *Histophilus* was observed on D8 and D14 than D2 (D8 – adjusted OR 0.21, 95%CI 0.50-0.76, $p = 0.02$, D14 – adjusted OR 0.18, 95%CI 0.05-0.66, $p = 0.04$).

When logistic regression analysis was performed within each treatment group to further understand the temporal variations in opportunistic pathogen colonization with stressors, use of neither whole microbial data (before and after treatment assignment) or after treatment assignment (post-weaning) data displayed temporal variation in Suckling calves. In contrast, Weaned calves had a higher abundance of *Moraxella* on D8 (adjusted OR 25, 95%CI 1.7-1058, $p = 0.04$) and D28 (adjusted OR 15, 95%CI 1.4-408, $p = 0.05$) as well as a higher abundance of *Histophilus* on D2 (adjusted OR 14, 95%CI 1.2-384, $p = 0.05$) compared to D0. In Weaned + Transport calves, *Pasteurella* and *Mannheimia* tended to be higher after weaning than D0 (*Pasteurella* D8 – adjusted OR 15, 95%CI 0.95-665, $p = 0.08$; *Mannheimia* D4 – adjusted OR 15, 95%CI 0.90-0.665, $p = 0.08$; *Mannheimia* D8 – adjusted OR 15, 95%CI 0.90-665, $p = 0.08$). Analysis

of only post-weaning data revealed a lower abundance of *Mannheimia* (adjusted OR 0.11, 95%CI 0.01-0.91, $p = 0.05$) and *Histophilus* (adjusted OR 0.02, 95%CI 0.005-0.26, $p = 0.01$) on D14 compared to D2 only in Weaned calves but there was no temporal variation in Weaned + Transport calves.

Microbial functions are related to weaning associated stressors

Use of logistic regression analysis to investigate the relationship between the presence/absence of a microbial function (KEGG Orthology at level 2) with weaning-associated stressors revealed that microbial functions related to “membrane transport”, “replication and repair”, and “metabolism of cofactors and vitamins” were linked to weaning (Table S1). Presence of microbial functions related to “membrane transport” (adjusted OR 0.38, 95%CI 0.14-1, $p = 0.05$) and “metabolism of cofactors and vitamins” (adjusted OR 0.23, 95%CI 0.08-0.63, $p = 0.01$) displayed a decreased likelihood in Weaned calves compared to Suckling calves. In contrast, the presence of microbial functions related to “replication and repair” (adjusted OR 3.7, 95%CI 1.3-10.8, $p = 0.01$) displayed an increased likelihood in Weaned calves compared to Suckling calves. Microbial functions related to “cell motility”, “transport and catabolism”, “signal transduction”, folding, sorting and degradation”, and “transcription” varied only with time when compared to D0 (before assigning treatments) (Table S1). A decreased likelihood of “membrane transport” function was evident post-weaning in both Weaned (adjusted OR 0.27, 95%CI 0.09-0.79, $p = 0.02$) and Weaned + Transport (adjusted OR 0.27, 95%CI 0.08-0.83, $p = 0.03$) calves compared to suckling calves. Microbial functions related to “replication and repair” were higher in Weaned calves (adjusted OR 3.4, 95%CI 1.1-10.6, $p = 0.03$) compared to suckling calves after weaning. “Cell communication”, “cell growth and death”, “cell mortality”, “carbohydrate metabolism”, and “xenobiotics biodegradation and metabolism” were linked only to sampling time points post-weaning (Table S1).

Weaning, with or without transportation, decreases total bacterial density

Estimation of bacterial densities collected via deep nasopharyngeal swabs revealed that calves assigned to the three treatments groups had similar bacterial densities (D0) prior to allocating treatments (Figure 4A). Bacterial densities were, however, significantly lower ($p < 0.01$) in weaned calves, with ($7.18 \pm 0.07 \log_{10}$ 16S rRNA gene copy/swab) or without ($7.10 \pm 0.07 \log_{10}$ 16S rRNA gene copy/swab) transportation, on day 4 after weaning when compared to Suckling ($7.76 \pm 0.07 \log_{10}$ 16S rRNA gene copy/swab) calves (Figure 4B). Bacterial densities were not statistically different on D8, D14, and D28 post-weaning when comparing among treatment groups (Figure 4B).

It is important to note that total bacterial density in URT samples also displayed temporal variation throughout the experimental period. In Suckling calves, bacterial density on D4 was higher than D2 and D28, whereas in Weaned and Weaned + Transport calves bacterial density on D8 was higher than D2, D4, and D28 (Figure 4B).

Density of opportunistic bacterial pathogens varies with time but not stressor

Estimation of the density of opportunistic pathogenic bacteria in the URT revealed *M. haemolytica* (MH) and *P. multocida* (PM) had colonized all suckling beef calves (Figure 4A). Densities of *M. haemolytica* and *P. multocida* were not statistically different among the three treatment groups either before (Figure 4A) or after allocation to treatments (Figure 4C and 4D). Similar to total bacterial density, the density of opportunistic bacterial pathogens also displayed temporal variations during the experimental period. The highest density of *M. haemolytica* in all treatment groups was observed on D7 and D14 (Figure 4C), while the highest density of *P. multocida* in all treatment groups was observed on D14 (Figure 4D).

Serum antibody responses to *M. haemolytica* and *P. multocida* are not related to bacterial abundance in the URT

A negative binomial (NB) regression analysis was used to analyze possible relationships between URT bacteria and serum antibody IgG responses to *M. haemolytica* and *P. multocida*. No significant association was identified between serum antibody responses and the relative abundance of *M. haemolytica* and *P. multocida* (Table S2). Consistent with previous analyses, the NB model also revealed an increase in antibody responses with time only in Weaned and Weaned + Transport calves but not in Suckling calves (Table S2).

Use of mediation analysis further suggested that the URT microbiota did not mediate the systemic immune responses to opportunistic pathogenic bacteria in any of the calf groups (Figure 5). Once again mediation analysis revealed that antibody responses varied in a time dependent manner in Weaned and Weaned + Transport calves but not in Suckling calves (Figure 5).

Discussion

This controlled study used age-matched Suckling, Weaned, and Weaned + Transport beef calves to serially sample the URT microbiome over a one-month period. Microbiome analysis revealed that the stressors of weaning and weaning + transportation had minimal effect on taxonomic and functional profiles of the URT microbiome. The microbiome of the Suckling and Weaned groups remained stable over the one-month period, while Weaned + Transport calves displayed subtle temporal changes post-weaning. These observations suggest that possibly other co-stressors, such as co-mingling, a new environment, or viral infections may be possible drivers of previously reported URT microbiome perturbations that occur following weaning of beef calves. Holman and colleagues [9] analyzed the URT microbiota using samples collected prior to and after arrival in the feedlot in the absence of co-mingling and reported significant changes in the URT microbiome 2 days after arrival at the feedlot. The present study also identified significant differences in total bacterial density and the abundance of *Mannheimia*, *Pasteurella*, *Histophilus*, *Haemophilus* and *Moraxella* when comparing consecutive samples on D0 (assignment to treatment group) and D2 after weaning. These significant differences were also observed, however, in the group of calves that continued to suckle their dams and the URT microbiome on subsequent days of sampling was not different in the three treatment groups when compared to D0. Bacterial density and microbial composition were also similar when comparing among the three groups

prior to allocation to treatment group. Thus, the observed microbiome variation observed on D2 versus D0 may indicate that the first sampling with DNS perturbed the URT mucosal surface and associated microbiome. Therefore, the reported striking changes in the URT microbiome of weaned beef calves immediately after arriving at the feedlot [9] may be an artifact of microbiome sampling and not a consequence of stressors associated with this transition. These observations reveal the importance of a control group to monitor possible effects of microbiome sampling and the current data support the conclusion that the stressors of weaning, with or without transportation, had a minimal effect on URT microbiome.

The stressors of weaning and transportation in calves have been shown to increase blood cortisol [2, 12] and noradrenaline concentrations [2, 3] in beef calves. Elevated cortisol levels have been linked to perturbations in both gut [16, 17] and oral microbiome [18]. Moreover, stress hormones such as epinephrine and norepinephrine, that mediate interactions between the neuroendocrine and immune system, have been reported to increase gut colonization by pathogens [19]. Collectively, these studies suggested stress hormones played a role in modulating host-associated microbiomes, especially the pathobiome (opportunistic pathogens). In the present study, stress hormones in the blood of calves were not measured but the expression of *ADR* genes was monitored in blood leukocytes to determine if stress may alter neuroimmune regulation of host responses. This study provides the first complete analysis of the expression of *ADR* genes in bovine blood leukocytes and significant ($p < 0.05$) stress-associated changes in the expression of *ADRB2* and *ADRA2A* were observed within the first week (day 2, 4, 8) and *ADRB1* and *ADRB3* on D28 post-weaning. These results suggest *ADRB2* and *ADRA2A* may modulate host immune responses during the first week post-weaning while *ADRB1* and *ADRB3* may modulate immune responses much later after weaning and transportation. An absence of *ADRB1* and *ADRB2* in mice promoted gut colonization by *Lactobacillaceae*, increased volatile fatty acid production, and reduced the frequency of IL17 producing T cells [20]. Microbial metabolites such as gamma-aminobutyric acid (GABA) has been shown to induce neurotransmitters [4]. Although gamma-aminobutyrate permease (a transporter of GABA) and glutamate decarboxylase (converts glutamate into GABA) were identified in the URT microbiome, their abundances were not differed among treatment groups or among sampling time points (data not shown). It may be informative to further investigate whether stress-induced changes in leukocyte *ADR* gene expression can alter host immune responses to opportunistic pathogens residing in the URT.

Opportunistic bacterial pathogens, such as *Mannheimia* and *Pasteurella* colonize the URT microbial community of neonatal calves shortly after birth [10] indicating they are autochthonous members of the URT microbiome. Amplicon sequencing based profiling of the URT microbial community revealed a high abundance of *Mannheimia* and *Pasteurella* in neonatal [10] and older [8] calves. Metagenomics sequencing in the present study also confirmed both *Mannheimia* and *Pasteurella* had colonized the URT of all suckling beef calves. However, similar to Gaeta et al. [10], who also used metagenomics sequencing, we observed the abundance of *Pseudomonas*, *Burkholderia*, *Mycoplasma*, and *Acinetobacter* in the URT microbiome exceeded that of the opportunistic pathogens. Colonization of the URT by these opportunistic pathogens has attracted much attention, as their colonization of the lungs can cause fatal

secondary infections during BRD. The present study revealed all suckling calves were seropositive for both *Mannheimia* and *Pasteurella* but in young calves, serum antibodies reacting with these opportunistic pathogens may reflect passive transfer of maternal antibody at the time of birth [15]. No significant change in the level of serum IgG reacting with *Mannheimia* and *Pasteurella* was observed in calves remaining with their dams throughout the study (Fig. 2).

Following weaning, there was a marked increase in *Mannheimia*- and *Pasteurella*-specific IgG antibody titres and weaning combined with transportation resulted in an even greater increase in IgG antibody reacting with *M. haemolytica* leukotoxin (Fig. 2A). Serum IgG antibody is generated primarily by the systemic immune system and the increase in serum antibody titres in weaned calves, with or without transportation, suggests increased systemic rather than mucosal exposure to *M. haemolytica* and *P. multocida*. These opportunistic pathogens can colonize the lungs of both healthy and diseased cattle [21], which is a possible site for systemic exposure and the induction of increased serum IgG antibody responses. The present study revealed no significant association between the density of *Mannheimia* and *Pasteurella* in the URT and serum antibody responses specific to these bacteria (Fig. 4). Therefore, the present data supports the conclusion that either increased exposure to *Mannheimia* and *Pasteurella* in the lower respiratory tract of weaned and transported calves or altered host immune responses to the bacteria contributed to increased antibody responses. It should be noted, however, that in the present study the altered antibody responses to *Mannheimia* and *Pasteurella* were not associated with fever, one of the cardinal clinical signs used to diagnose undifferentiated BRD. A previous study, using metagenomics sequencing, also revealed no significant difference in the abundance of opportunistic pathogens in the URT when comparing healthy dairy calves with calves diagnosed with BRD [11]. In contrast, following an analysis of the URT microbiome of weaned feedlot calves Timsit et al. [21] speculated that an increased abundance of opportunistic pathogens immediately after arrival at feedlots might result in increased susceptibility to BRD. Antibody responses to these bacterial pathogens were not monitored in this study [21]. Thus, it is not known if the apparent increase in bacterial abundance was also associated with increased host immune responses.

Conclusions

The present study revealed the URT microbiome is relatively stable over a one-month period in suckling beef calves and stressors, such as maternal separation (weaned) and transportation, resulted in minor microbial perturbations. When appropriate controls for microbiome sampling effects were included in the study then the stress associated with maternal separation and transportation did not significantly alter the URT microbiome. Maternal separation and transportation did, however, have a marked effect on serum antibody responses to the opportunistic pathogens, *M. haemolytica* and *P. multocida*, that reside in the URT. No significant association was apparent between the abundance of these opportunistic pathogens in the URT and host immune responses. However, altered expression of *ADR* genes in bovine leukocytes following weaning and transportation indicate neuroimmune regulation of host responses may be altered throughout the post-weaning period. Altered immune function should be considered as a possible mechanism mediating increased host responses to bacteria residing in either the upper or lower

respiratory tract. Further investigation is warranted to determine if stress hormones can enhance host defenses against opportunistic pathogens residing in the URT.

Materials And Methods

Animal experiment and sampling

Animal experiments were completed at the University of Saskatchewan following guidelines provided by the Canadian Council on Animal Care and approved by the University of Saskatchewan Animal Care Committee (Protocol #20170015). Calves recruited to the study were 5 to 6 month-old, suckling Hereford-cross females reared within the same herd (Goodale Farm, University of Saskatchewan, Saskatoon, SK Canada). Thirty (30) calves were randomly assigned to three treatment groups using Tufts Randomization plan. Experimental groups were: Suckling calves - calves remained with dams; Weaned - calves separated from dams on experimental day 0; Weaned + Transport – calves separated from dams on experimental day 0 (D0) and transported for 4.5 h before returning to research facility (Figure 6). Calves in the Weaned and Weaned + Transport group were co-mingled in a drylot with access to water and brome-alfalfa hay throughout the trial. Suckling calves remained with their dams in an adjacent paddock with access to water and brome-alfalfa hay throughout the trial. On D0, blood and deep nasal pharyngeal swabs (DNS) were collected from all calves at the time Weaned and Weaned + Transport calves were separated from their dams (Figure 6). Blood for serum samples and leukocyte isolation was collected from the jugular vein using 10 ml BD Vacutainer™ SST and EDTA blood collection tubes (Becton Dickenson, Franklin Lakes, NJ). DNS were collected using double guarded culture swabs (Jorgensen Laboratories Inc., Loveland CO). The guarded swab was inserted into the nasal cavity a distance approximately equal to the distance from the external nares to the medial canthus of the eye. The sterile swab was then extended beyond the sheath until it contacted an obstruction and the swab rotated three times against the mucosal surface. The swab was retracted into the protective sheath, removed from the nostril, placed in a sterile tube, transported on ice to the lab, and stored at -80°C until DNA was extracted. Blood and DNS were collected again from all calves on days 2, 4, 8, 14, and 28 following separation of the Weaned and Weaned + Transport calves from their dams (Figure 6). Duplicate one ml aliquots of serum and two snap-frozen pellets of 10 million blood leukocytes were stored at -20°C and -80 °C, respectively, until analyzed.

Isolation of Blood Leukocyte

Briefly, 12 mL of erythrocyte lysis buffer (0.17 M NH₄Cl, 10 mM KHCO₃, and 0.11 mM EDTA; pH 7.3) was added to 3 mL bovine blood. Cells were centrifuged at 325 g for 8 minutes and the supernatant discarded. Cell pellets were re-suspended in 1 mL Dulbecco's Modified Eagle Medium (Sigma Aldrich) containing 10% fetal bovine serum (FBS) and cells counted with a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Brea, CA). Aliquots of 10 million blood leukocytes were pelleted at 311 g for 8 minutes and cell pellets snap-frozen in liquid nitrogen and stored at -80°C.

RNA Isolation from Blood Leukocytes

RNA was extracted from blood leukocytes using a combined TRIzol/RNEasy Mini Kit extraction method. Frozen cell pellets were suspended in 1 mL TRIzol reagent (ThermoFisher Scientific, Waltham, MA) and 200 μ L chloroform (Sigma Aldrich, St. Louis, MO) was added to each sample. Samples were shaken for 15 seconds and incubated at room temperature for 2-3 minutes before centrifuging for 15 minutes at 13,282 g. Following centrifugation, the aqueous phase was removed, an equal amount of 70% ethanol added and samples were added to the silica columns provided in the RNEasy Mini Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany) and processed according to manufacturer's instructions.

cDNA Synthesis and Reverse Transcription PCR

Synthesis of complementary DNA (cDNA) from blood leukocyte RNA template was performed following manufacturer's instructions for the Quantitect Reverse Transcription Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany). A 30 minute cDNA synthesis incubation step (42°C) was used to remove excess RNA secondary structure. Briefly, 500 ng RNA was diluted in 6 μ L UltraPure DNase/RNase-Free Distilled Water (Invitrogen) and 1 μ L of 7x gDNA wipe-out buffer was added to remove genomic DNA. The GeneAmp 9700 PCR System (Applied Biosystems, California USA) was used to incubate this mixture for 2 min at 42°C. Following incubation, 3 μ L master mix was added to each reaction. The master mix for each reaction consisted of 2 μ L 5 \times Quantiscript RT buffer, 0.5 μ L of primer mix, and 0.5 μ L reverse transcriptase. Following addition of the master mix, each reaction was incubated for 30 minutes at 42°C, followed by 3 minutes at 95°C.

Reverse transcription PCR (RT-PCR) reactions were prepared with 25 ng of cDNA (5 μ L of 5ng/ μ L cDNA) and 10 μ L of master mix. The master mix consisted of 7.5 μ L 2 \times PERFECTA-IQ SYBR Green Supermix (QuantaBio), 2.2 μ L UltraPure DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA), and 0.3 μ L of 10 μ M forward and reverse primer (3 pmoles; Table S3). Reactions were run in Hard Shell Low-Profile 96-well semi-skirted, clear-shell, and clear-well PCR plates (BioRad, Hercules, CA). The CFX Connect Real Time System (BioRad, Hercules, CA) was used to run and quantify the real time PCR reactions. Reactions were first run at 95°C for 2 min to activate the hot-start Taq polymerase, then for 40 cycles at 95°C for 15 sec (denature), 60°C for 30 sec (anneal), and 72°C for 30 sec (extend). Following amplification, a melt curve was applied for detection of abnormal products. The melt curve started at 65°C, and the temperature held for 10 sec before increasing by 1°C. This pattern was repeated to a temperature of 95°C. Results were visualized using the CFX Manager/Maestro software and corrections for primer efficiency were included in Cq value calculations.

Serum Antibody Titres for *M. haemolytic* and *P. multocida*

Antibody capture enzyme-linked immunosorbent assays (ELISAs) were performed to quantify serum IgG antibody titers (Fig. 6) using the protocol described in Hill et al [22]. Briefly, the antibody capture antigens included recombinant *M. haemolytica* leukotoxin [23] and soluble bacterial lysates prepared from *M. haemolytica* and *P. multocida*. Serum titers are presented as the inverse of the final serum dilution

generating an OD reading exceeding the mean + 2 SD of the background OD value from triplicate wells containing negative serum samples.

Profiling of the URT Microbiome Using Metagenomics Sequencing

Total DNA was extracted from the DNS using PowerSoil DNA isolation kit (MO BIO Laboratory Inc., Carlsbad, CA). Briefly, the DNS was transferred into a PowerBeads tube containing the C1 solution and subjected to bead-beating using Mini-BeadBeater-16 (BioSpec Products, Bartlesville, OK) at 5000 rpm for 3 min. The supernatant was separated after centrifuging at 13000 rpm for 15 min and subsequently used to isolate DNA following the manufacturer's instructions. DNA quantity was measured using Qubit 3.0 fluorometer (Invitrogen, Carlsbad, CA) and Qubit dsDNA HS assay kit (ThermoFisher Scientific, Waltham, MA). Shotgun DNA libraries (Figure 6) were prepared using NEB Ultra II DNA Library Prep Kit for Illumina (New England Biolabs Inc., Massachusetts, USA) and sequenced using Illumina HiSeq4000 PE100 (Illumina, California, USA) at Genome Quebec (McGill, Quebec).

Analysis of Metagenomics Sequencing data

Demultiplexed raw data (229.8 Gb, Table S4) were first run through Trimmomatic version 0.39 [24] in paired-end mode to remove adapters, low quality sequences (Phred < 20) and short sequences (< 50 bp). Then, the host contaminations were removed using in Bowtie 2 [25], SAMtools [26] and BEDtools [27] by aligning to bovine genome (UMD 3.1). Host contamination removed unassembled sequences (53.3 Gb) were then uploaded into the MG-RAST metagenomic analysis server [28], version 4.0, and paired-ends were joined for each sample before submitting for processing. Artificial replicates, host (bovine) DNA and low-quality (Phred score < 25) sequences were removed from the raw data, and the remaining good-quality sequences were used to assign the microbial functions using the subsystems annotation source in the SEED hierarchy and KEGG Orthology and microbial taxa using RefSeq database. A maximum cut-off e-value of 1e-10, maximum identity of 70% and maximum alignment length of 80 was used as data selection criteria for the functions and taxa abundance analyses. In addition, MEGAHIT v1.1.1 [29] was used to assemble raw sequences with a minimum contig length of 200 bp and K-mer size 119. Assembled sequences were then used to assign taxonomic composition using Kraken2 database [30].

Estimation of total bacteria *M. haemolytica* and *P. multocida* densities

The density of total bacteria, *M. haemolytica* and *P. multocida* were estimated using quantitative real-time PCR (qPCR) and bacterial primers (Table S5). Total DNA extracted from DNS was diluted to 50 ng/μl and 1 μg of the template was used to perform qPCR with SYBR Green chemistry (Fast SYBR® Green Master Mix; Applied Biosystems, Foster City, CA) and StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA). The standard curve of total bacteria was constructed using purified PCR products amplified with 27F and 1492R primer pair, while standard curves for *M. haemolytica* and *P. multocida* were constructed using genomic DNA extracted from pure cultures of each bacterial species. Bacterial density (copy number of the 16S rRNA gene per DNS for total bacteria and *P. multocida* and copy number

of leukotoxin (lkt) gene per DNS for *M. haemolytica*) was calculated using the following equation: (quantity mean × DNA concentration × DNA elution volume)/DNA amount used in qPCR reaction.

Statistical analysis

The *ADR* gene expression data and ELISA data were analyzed using a two-way ANOVA with time and treatment as variables. Significant time and treatment effects were observed and a post-hoc test for multiple comparisons of factors was performed using Tukey's multiple comparison test to determine if there were significant treatment effects.

Taxonomic and functional compositions all metagenomes were first analyzed using principle component analysis (PCA) to understand the effect of stressors and sampling time point on URT microbiome. Analysis of similarities (ANOSIM) was used to test statistical significances of the PCA-based visualization. Then, the non-parametric Kruskal-Wallis test one way ANOVA by rank was performed to test the effect of stressors and sampling time point on the relative abundance of potentially pathogenic bacterial groups (*Mannheimia*, *Pasteurella*, *Haemophilus*, *Histophilus*, *Moraxella*, *Mycoplasma*) and bacteriophage (Microvirus, P2-like viruses). The same analysis was performed after stratifying data by stressor type to compare time points to test interaction effect between type of stressor and time point. A post-hoc test for multiple comparisons of factors was performed using pairwise Wilcoxon (Mann-Whitney U-tests) test and p values were adjusted using Benjamini and Hochberg method [31]. Data were presented as medians with 95% confidence intervals (CIs) and statistical differences were declared at p-value adjusted < 0.05.

A logistic regression analysis was performed to explore the relationship between the colonization of potential pathogens and type of stressor. High abundance of potential pathogens in the logistic regression model was defined as above (Yes) and below (NO) the median relative abundance and used to calculate the odds ratio (OR) of colonization (R package "questionr"). Associations between host immune responses to *M. haemolytica* and *P. multocida* and the abundance of genera *Mannheimia* and *Pasteurella* in the URT were explored using two different approaches. First, a negative binomial regression model was fitted for three groups together and within each stressor type. The colonization of *Mannheimia* and *Pasteurella* was defined as above (Yes) and below (NO) the median relative abundance. Then a mediation analysis (R package "mediation") was performed to test if changes in host immune responses were mediated by the respective bacterial group colonized in the URT. Bacterial densities estimated through qPCR were first normalized by log₁₀ transformation and then day 0 data were analyzed using a one-way ANOVA to test effect of calf group on initial bacterial densities. Post-weaning data (days 2, 4, 8, 14 and 28) were then analyzed using a repeated measure model with sampling point as the repeated measure and the generalized least square function using autoregressive of order 1 (AR1) covariance structure, which was selected as the best fit by the Bayesian information criterion. All data were analyzed using R package (version 4.0.0).

Abbreviations

ADR

Adrenergic receptor; ANOSIM-R:Analysis of similarities R value; BRD:Bovine respiratory disease; Cdna:complementary DNA; DNS:Deep nasopharyngeal Swabs; EDTA:Ethylenediaminetetraacetic acid; ELISA:Enzyme-linked immunosorbent assay; fbs:Fetal bovine serum; IgG:immunoglobulin G; M. haemolytica:Mannheimia haemolytica; NB:Negative binomial; OD:Optical density; OR; Odds ratio; PCA:Principle component analysis; P. multocida:Pasteurella multocida; qPCR:quantitative real-time polymerase chain reaction; RT-PCR:Reverse transcription polymerase chain reaction; rRNA Ribosomal RNA; URT:Upper respiratory tract.

Declarations

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Authors' contributions

NM contributed to study design, performed nucleic acid extraction, PCRs and qPCRs, analyzed and interpreted metagenomics data, visualized data, and wrote the manuscript. AH contributed to study design, designed ADR primers, performed nucleic acid extraction and RT-PCRs, and interpreted and visualized data. NA contributed to study design, archived samples, and performed data analysis and interpretation. PJG was involved in funding acquisition, study design and investigation, data analysis, interpretation and visualization, and manuscript editing.

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Availability of data and material

All microbial metagenome sequence data were deposited at NCBI Sequence Read Archive (SRA) (<https://www.ncbi.nlm.nih.gov/sra/PRJNA687519>) under accession number PRJNA687519.

Ethics approval and consent to participate

Animal experiments were completed at the University of Saskatchewan following guidelines provided by the Canadian Council on Animal Care and approved by the University of Saskatchewan Animal Care

Committee (Protocol #20170015).

Consent for publication

Not applicable

Competing interests

Authors declared no conflict of interest.

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Tables

Table 1

Differentially abundant microbial groups (metagenomics sequencing-based) in the URT of beef calves

Comparisons	Microbial group	Sampling time point (median, IQR %)						P value
		D0	D2	D4	D8	D14	D28	
All Samples	Bacteria (D)	97.1, 0.8 ^b	97.9, 1.1 ^b	97.5, 0.7 ^b	96.1, 4.4 ^a	97.1, 3.4 ^b	97.2, 1.4 ^b	0.01
	Viruses (D)	1.1, 0.9 ^b	0.6, 0.6 ^a	0.8, 0.6 ^a	2.1, 4.7 ^b	1.4, 3.2 ^b	1.7, 1.4 ^b	< 0.01
	Actinobacteria (P)	0.4, 0.1 ^a	0.4, 0.3 ^a	0.5, 0.2	0.5, 0.2	0.4, 0.2 ^a	0.6,0.3 ^b	< 0.01
	Bacteroidetes (P)	0.1, 0.2 ^a	0.1, 0.1 ^a	0.2, 0.4 ^a	0.2, 0.4 ^a	0.2, 0.3 ^a	0.7, 1.6 ^b	< 0.01
	Firmicutes (P)	0.2, 0.1 ^a	0.2, 0.1 ^a	0.2, 0.1 ^a	0.3, 0.2	0.2, 0.2 ^a	0.4, 0.4 ^b	< 0.01
	Tenericutes (P)	0.5, 0.8	1.0, 1.6	0.3, 1.2	1.4, 2.2 ^a	0.4, 0.7 ^b	0.1, 0.2 ^b	< 0.01
	<i>Mannheimia</i> (G)	0, 0.01 ^a	0.02, 0.1	0.2, 0.7 ^b	0.02, 0.04	0.01, 0.03 ^a	0, 0.02 ^a	< 0.01
	<i>Pasteurella</i> (G)	0, 0.01 ^a	0.02, 0.1 ^b	0.04, 0.07 ^b	0.01, 0.02 ^b	0, 0.02 ^a	0, 0.01 ^a	< 0.01
	<i>Moraxella</i> (G)	0.005, 0.02 ^a	0.1, 0.2 ^b	0.1, 0.3 ^b	0.03, 0.1 ^{ab}	0.01, 0.05	0.02, 0.08	0.02
	<i>Histophilus</i> (G)	0, 0.01 ^a	0.02, 0.04 ^b	0.06, 0.04 ^b	0, 0.02 ^b	0, 0.02 ^b	0.005, 0.01	< 0.01
Weaned only	<i>Mannheimia</i> (G)	0, 0 ^a	0.02, 0.04	0.3, 0.6 ^b	0.02, 0.08	0, 0.002 ^a	0.005, 0.01	< 0.01
Weaned + Transport only	<i>Histophilus</i> (G)	0.02, 0.02	0.03, 0.04	0.06, 0.02 ^a	0, 0 ^b	0, 0.03 ^b	0.005, 0.02	0.02
	Microvirus (G)	94.8, 9.2 ^a	86.7, 6.2 ^b	93.9, 6.0 ^a	98.5, 1.4 ^a	96.4, 2.0 ^a	96.9, 2.0 ^b	< 0.01
D – domain (median as a % of all assigned domains); P – Phylum (median as a % of all assigned phyla within bacteria), G – Genus (median as a % of all assigned genera within bacteria or viruses); IQR – inter quartile ration								
a,b median with different superscripts are different at $P < 0.05$ (Post-hoc test for multiple comparisons of sampling time point using pairwise Wilcox test)								

Table 2

Relationship between colonization by opportunistic pathogens and bacteriophage in the URT of beef calves with weaning and transportation stressors and sampling time points.

Microbial group	Factor	Comparison	Odds ratio (OR) ^c	95% CI		P - value
				25%	75%	
<i>Mannheimia</i>	Stressor ^a	W	0.70	0.27	1.80	0.46
		W + T	1.61	0.59	4.54	0.35
	Sampling time ^b	D2	15.04	2.91	120.85	< 0.01
		D4	34.85	5.10	409.30	< 0.01
		D8	9.79	1.96	75.76	0.01
		D14	9.16	1.92	68.71	0.01
		D28	4.99	1.03	37.16	0.07
<i>Pasteurella</i>	Stressor	W	0.06	0.36	2.47	0.90
		W + T	0.35	0.52	3.93	0.50
	Sampling time	D2	2.35	2.21	63.83	< 0.01
		D4	2.34	1.86	87.02	0.01
		D8	1.50	1.09	20.93	0.04
		D14	0.51	0.44	6.80	0.46
		D28	0.40	0.39	6.16	0.56
<i>Histophilus</i>	Stressor	W	1.31	0.52	3.39	0.57
		W + T	1.11	0.42	2.94	0.82
	Sampling time	D2	5.49	1.30	26.79	0.02
		D4	11.12	2.00	93.74	0.01
		D8	1.15	0.28	4.94	0.84
		D14	1.04	0.27	4.31	0.95

^a With reference to Suckling calves including all sampling time points (D0-D28)

^b With reference to D0 sampling including all 3 treatment groups

^c OR is calculated after adjusting logistic regression models for sampling time OR < 1 indicates a negative relationship (colonization of microbial group decreased); OR > 1 indicates a positive relationship (colonization of microbial group increased)

W – Weaned calves; W + T – Weaned + Transport calves

		D28	1.84	0.48	7.60	0.37
<i>Haemophilus</i>	Stressor	W	0.30	0.11	0.81	0.02
		W + T	0.67	0.25	1.80	0.43
	Sampling time	D2	9.26	1.97	56.00	<0.01
		D4	50.37	6.14	1161.44	<0.01
		D8	2.99	0.66	16.68	0.17
		D14	2.67	0.61	14.39	0.21
		D28	2.51	0.57	13.64	0.24
<i>Moraxella</i>	Stressor	W	1.16	0.47	2.88	0.75
		W + T	1.15	0.45	2.92	0.77
	Sampling time	D2	4.24	1.03	19.64	0.05
		D4	4.51	0.96	24.75	0.06
		D8	2.99	0.75	13.10	0.13
		D14	1.69	0.45	6.90	0.45
		D28	2.14	0.56	8.87	0.27
<i>Mycoplasma</i>	Stressor	W	1.91	0.79	4.76	0.15
		W + T	0.69	0.28	1.73	0.43
	Sampling time	D2	1.10	0.27	4.63	0.89
		D4	1.57	0.34	7.52	0.57
		D8	1.64	0.41	6.91	0.49
		D14	1.58	0.41	6.32	0.51
		D28	1.74	0.45	7.09	0.42
Microvirus	Stressor	W	1.57	0.62	4.10	0.35

^a With reference to Suckling calves including all sampling time points (D0-D28)

^b With reference to D0 sampling including all 3 treatment groups

^c OR is calculated after adjusting logistic regression models for sampling time OR < 1 indicates a negative relationship (colonization of microbial group decreased); OR > 1 indicates a positive relationship (colonization of microbial group increased)

W – Weaned calves; W + T – Weaned + Transport calves

		W + T	2.32	0.88	6.43	0.09
	Sampling time	D2	0.34	0.07	1.44	0.15
		D4	0.25	0.04	1.25	0.10
		D8	2.74	0.66	12.16	0.17
		D14	1.35	0.35	5.25	0.65
		D28	1.69	0.44	6.75	0.44
P2-like virus		Stressor	W	1.15	0.47	2.85
	W + T		0.73	0.28	1.84	0.50
	Sampling time	D2	1.85	0.46	7.78	0.39
		D4	2.69	0.57	14.16	0.22
		D8	0.51	0.12	2.04	0.34
		D14	0.94	0.25	3.54	0.92
		D28	0.73	0.19	2.80	0.65
		^a With reference to Suckling calves including all sampling time points (D0-D28)				
^b With reference to D0 sampling including all 3 treatment groups						
^c OR is calculated after adjusting logistic regression models for sampling time OR < 1 indicates a negative relationship (colonization of microbial group decreased); OR > 1 indicates a positive relationship (colonization of microbial group increased)						
W – Weaned calves; W + T – Weaned + Transport calves						

Figures

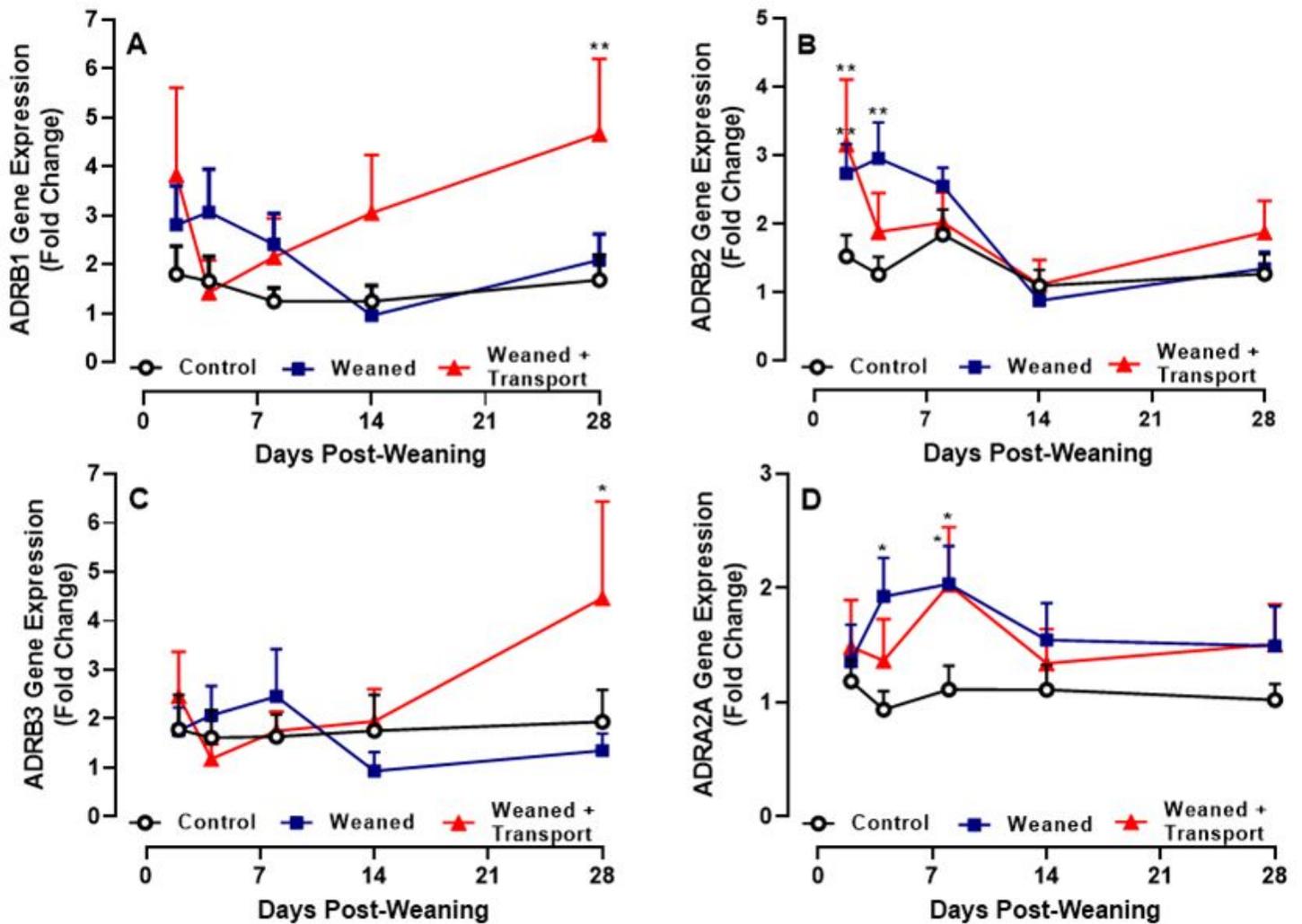


Figure 1

Analysis of adrenergic receptor gene expression in blood leukocytes of suckling calves (control), suckling calves separated from their dams (weaned), and weaned and transported (weaned+transport) calves. Gene expression was quantified with RT-PCR and data are expressed as fold change relative to Day 0 (prior to weaning). Gene expression was analyzed for 6 alpha- and 3 beta-adrenergic receptors (ADR) and data presented are for ADR gene where significant differences were observed when using a one-way ANOVA to compare weaned (n = 10) and weaned+transport groups (n = 10) to time-matched samples from the control group (n = 10). *p < 0.05; **p < 0.01

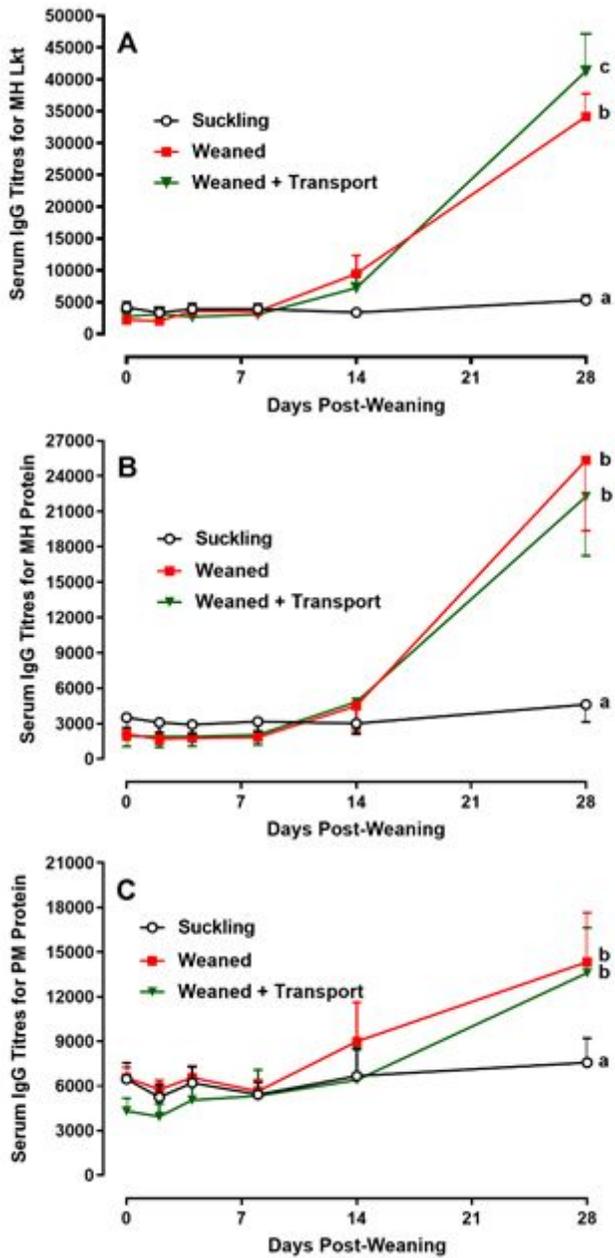


Figure 2

Serum antibody responses to *M. haemolytica* and *P. multocida* in suckling calves and in calves following weaning and transportation stress. Antibody titres to *M. haemolytica* were measured using both a recombinant leukotoxin (Lkt) protein (A) and soluble bacterial lysate (B). Antibody titres to *P. multocida* were measured using soluble bacterial lysate (C). Significant differences were observed in antibody titres on Day 28, when comparing among the three treatment groups (Suckling calves, Weaned calves, and Weaned+ Transport calves) and these differences are indicated by letters (a,b,c).

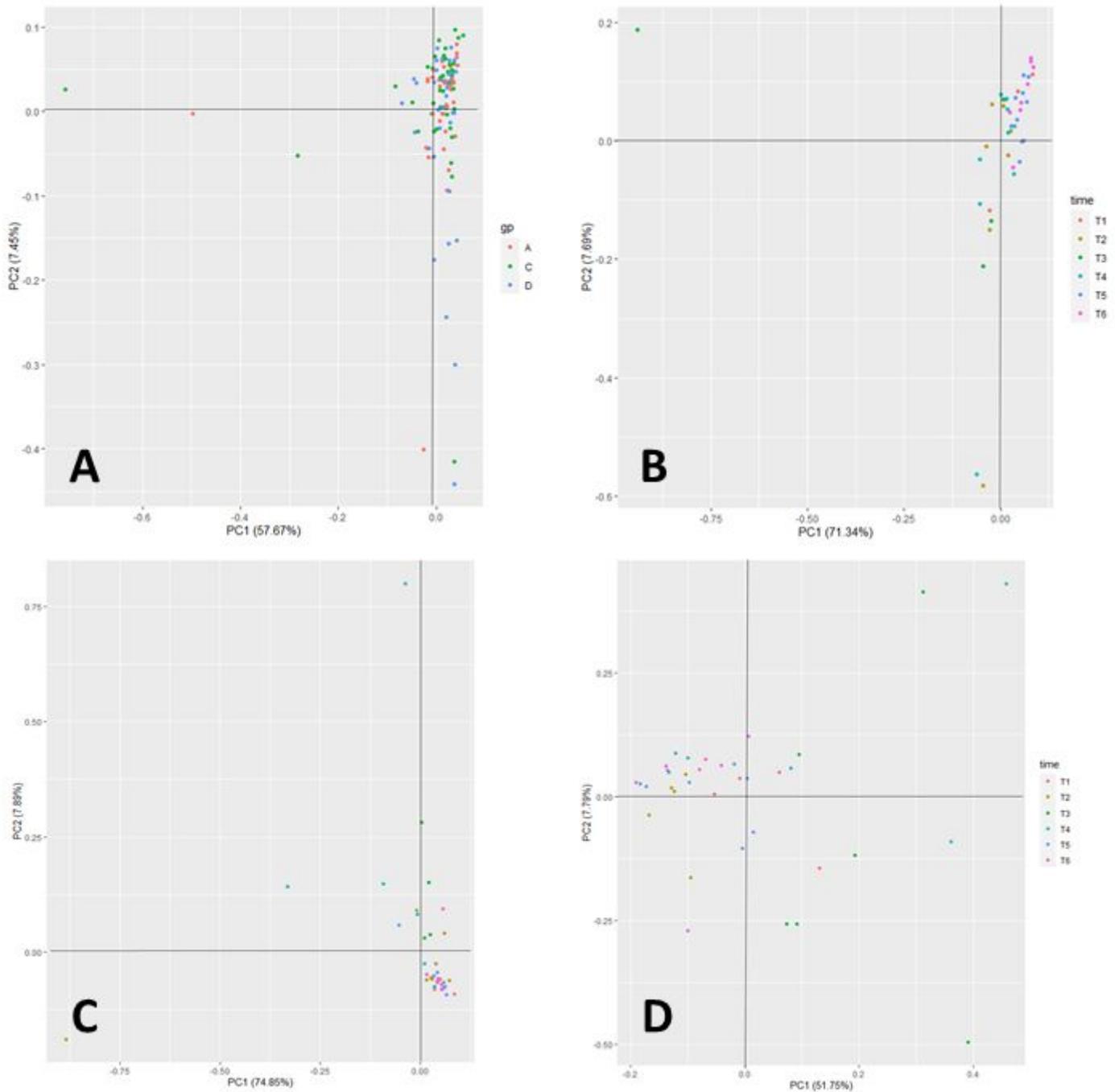


Figure 3

PCA-based visualization of microbial taxonomic profiles generated from the URT of beef calves. (A) All calves together comparing the effect of stressors independent of sampling point (ANOSIM $R = 0.009$; $p = 0.18$). (B) Suckling calves comparing sampling time (ANOSIM $R = 0.2662$; $p = 1e-04$). (C) Weaned calves comparing sampling time (ANOSIM $R = 0.1337$; $p = 0.006$). (D) Weaned + Transport calves comparing sampling time (ANOSIM $R = 0.3418$; $p = 1e-04$).

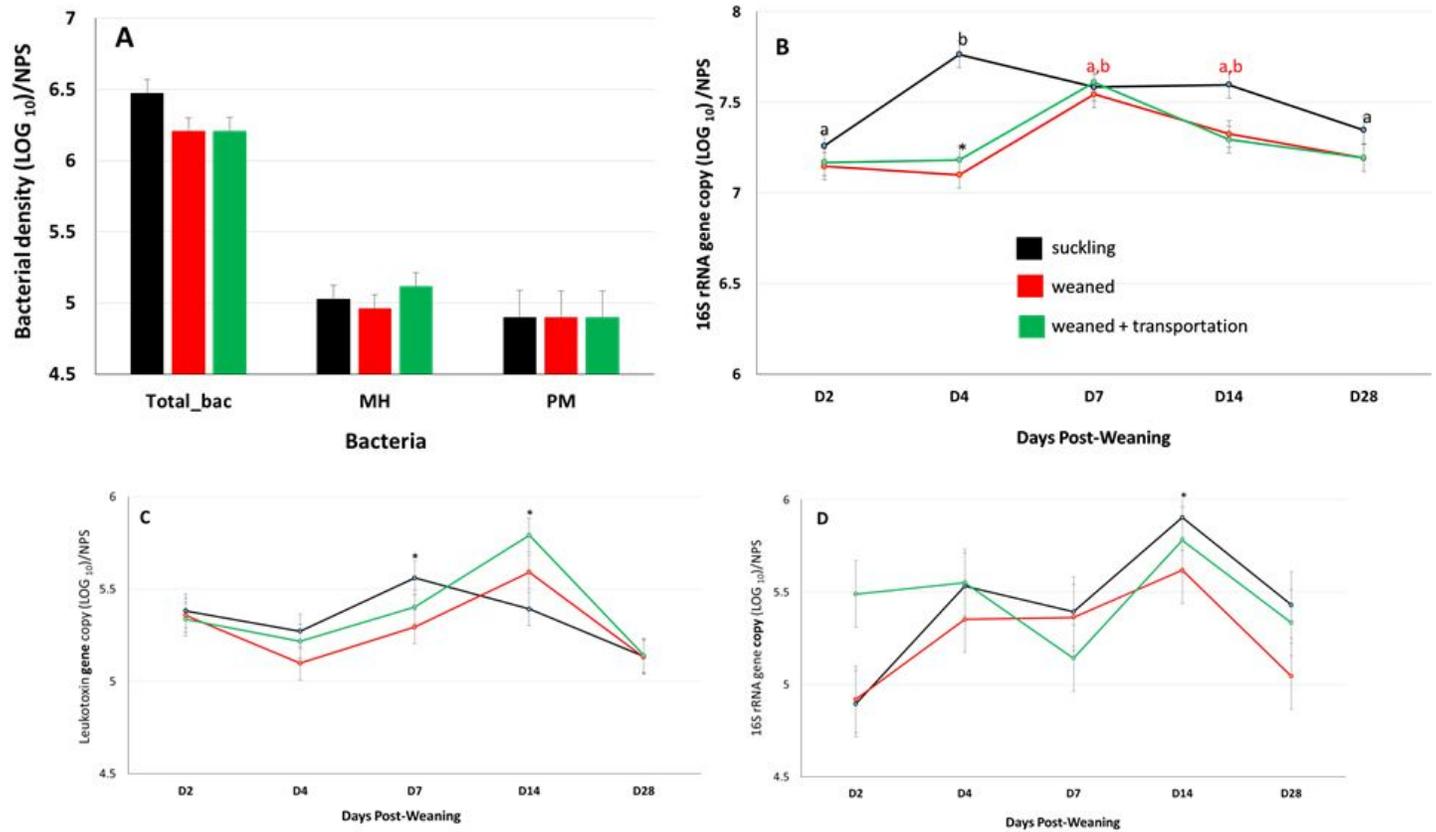


Figure 4

Estimated bacterial densities in the URT of beef calves. (A) Bacterial densities at the time suckling calves were assigned to treatment groups. Total bacterial density (Total_bac) and *P. multocida* (PM) density quantified as 16S rRNA gene copy/swab. *M. haemolytica* (MH) density quantified as leukotoxin (lkt) gene copy/swab. (B) Total bacterial density after allocation of calves to the respective treatment groups, ab – represents sampling points with significantly different bacterial densities ($P < 0.05$) within suckling calves, * - represents significant effect of weaning with or without transportation on bacterial density. (C) Density of *M. haemolytica* after allocation of calves to respective groups, * - represents sampling points with significantly higher density of *M. haemolytica*. (D) Density of *P. multocida* after allocation of calves to the respective stressors, * - represents sampling points with significantly higher density of *P. multocida*.

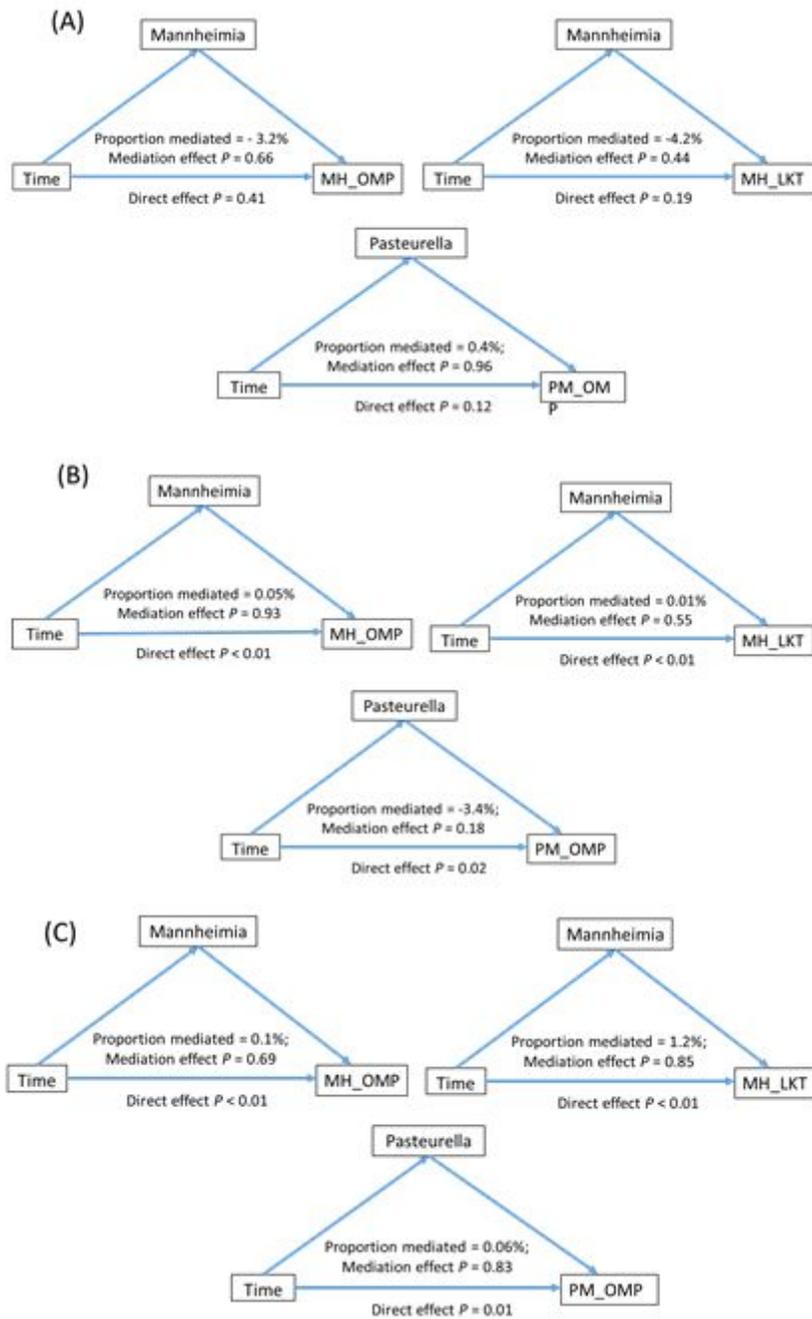
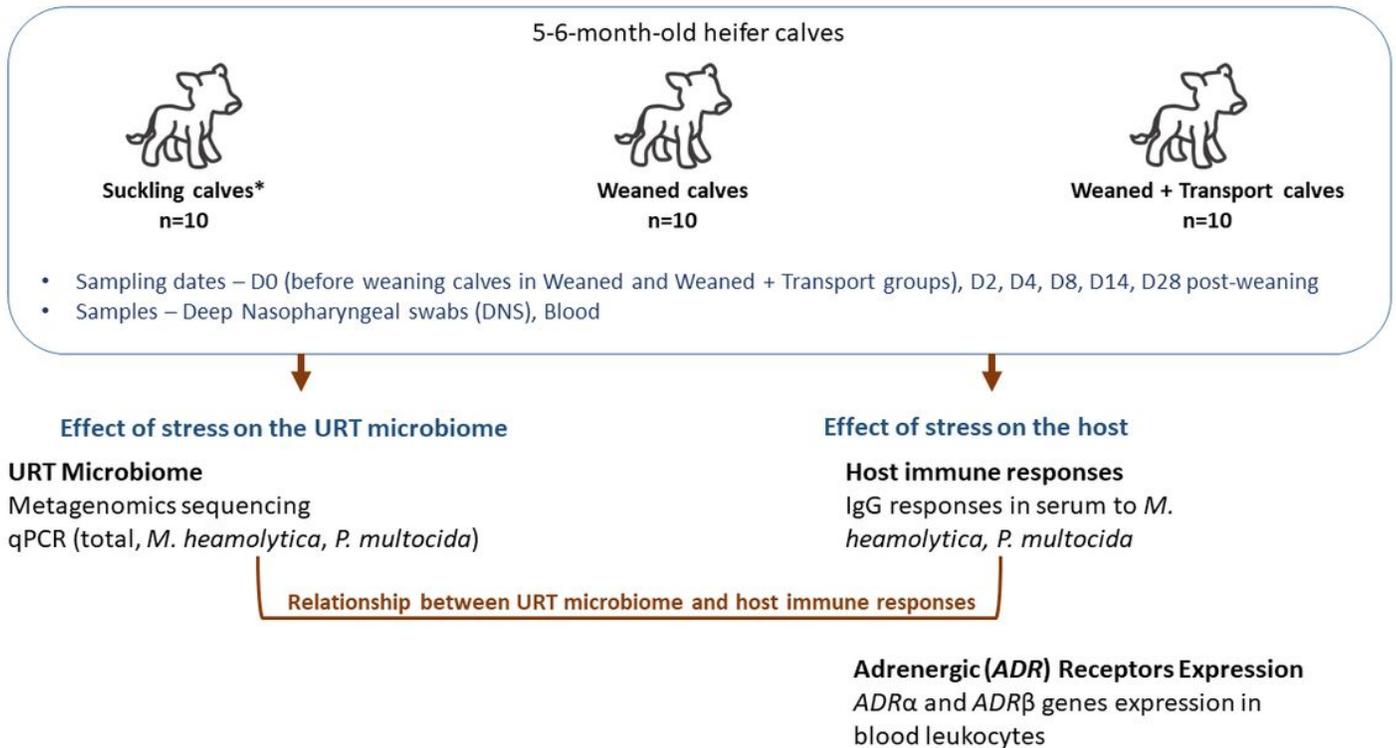


Figure 5

Mediation analysis to study the effect of the URT bacterial colonization on the development of antibody responses to *M. haemolytica* and *P. multocida*. (A) Mediation analysis within suckling calves (B) Mediation analysis within Weaned calves. (C) Mediation analysis within Weaned+Transportation calves. Proportion mediated represents the proportion of the effect of the independent variable (time) on the dependent variable (antibody response) that goes through the mediator (bacterial group). Mediation effect represents the p value of proportion mediated. Direct effect represents the P value of the effect of the independent variable (time) on the dependent variable (antibody response)



* Suckling calves- Control group stayed with dams throughout the experimental period

Figure 6

Flow chart depicting sampling process and approaches used to study the URT microbiome and host immune responses during weaning and transportation stress in beef calves.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Malmthugeetal.SupplementaryTable4.xlsx](#)
- [MalmthugeetalSupplementaryFiguresandTables.docx](#)