ABSTRACT

Transportation significantly impacts the health and welfare of surplus dairy calves largely due to the various stressors and pathogen exposures encountered during the process. Concurrently, the animal’s microbiome is known to correlate with its health status, with stress-induced alterations in the microbiota potentially precipitating various diseases. This study aimed to compare the impacts of transportation durations of 6, 12, or 16 h on the fecal microbiota in young surplus dairy calves. We used a randomized controlled design, where surplus dairy calves aged 1–19 d old from 5 commercial dairy farms in Ontario were allocated into one of 3 (6, 12, and 16 h of continuous transportation) transportation groups. Health assessments were conducted before, immediately after, and for 2 weeks following transportation. Fecal samples were collected before, immediately after, and at 24- and 72 h post-transportation and subjected to 16S rRNA sequencing. Alpha diversity metrics showed no significant differences between the 3 transportation groups at any of the sampling time points. Although β diversity metrics revealed no clustering by transportation groups, they indicated significant differences across sampling time points within each group. The overall analysis revealed a total of 22 phyla and 353 genera, with Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria being the most abundant phyla. Bacteroides, Escherichia/Shigella, Lactobacillus, Collinsella, and Bifidobacterium were the most abundant genera. The reduction in Fusobacteria abundance before and after transport was significantly larger in the 16-h transportation group when compared with the 6-h transportation group. We also identified several genus-level and ASV-level taxa that displayed significant differences in their abundances across various transportation groups, observed at all sampling time points investigated. In conclusion, this research identifies microbiota changes due to varying transportation durations in surplus dairy calves providing a broad understanding of the microbial shifts in surplus dairy calves’ post-transportation across varying durations. While these variations may not directly correlate with overall calf health or indicate dysbiosis, these results emphasize the importance of further investigating transportation practices to enhance calf health and well-being. Further studies are warranted to elucidate the relationship between microbiota and calf health.

Keywords: Surplus calves, microbiota, transportation

INTRODUCTION

Transportation of young dairy calves from the dairy farm of origin to the calf-raising facility is a common practice in surplus calf production systems. This process exposes calves to multiple stressors such as temperature fluctuations, vehicle movement, commingling, and deprivation of feed and water. These factors can contribute to weakened immune systems and increased susceptibility to disease (reviewed by Creutzinger et al., 2021). Calves often arrive at calf raisers with health issues such as dehydration, low body weight, and navel inflammation (Pempek et al., 2017; Renaud et al., 2018b, 2018a; Wilson et al., 2000), which likely result from long periods without access to milk or water during transportation and at auction facilities, and from suboptimal care on the dairy farm of origin (reviewed by Creutzinger et al., 2021).

The gut microbiota serves as a sentinel of health, directly interacting with the host’s immune system and metabolism. Stressors, such as those encountered during transportation, can induce marked shifts in the microbiota composition, impacting the balance and...
functionality of this microbial community (Freestone and Lyte, 2010). Imbalances in the microbial community are associated with a wide variety of diseases in young and adult animals (Neuman et al., 2018). Stress hormones such as catecholamines, and cortisol, chiefly produced via the sympathetic adrenal medullary and hypothalamus-pituitary-adrenal axis, have profound impacts on the gut microbiota of livestock, altering microbial composition and functions. For instance, in dairy calves, stress-related to dehorning and castration is known to cause significant shifts in the fecal microbiota, particularly impacting lighter-weight individuals (Mir et al., 2019). Similarly, the correlation between cortisol and the gut microbiota has been illustrated in studies involving pigs, where the presence of *Ruminococcus* in the gut was found to have a negative correlation with serum cortisol concentrations (Mudd et al., 2017). However, less is known about the impact of transportation on the fecal microbiome. A study in beef cattle showed that changes in the external environment during transportation caused significant changes in fecal microbial richness and phylogenetic diversity following feedlot placement (Maslen et al., 2022). This occurs mainly because transportation exposes animals to multiple stressors such as alterations in diet and water intake and exposure to potential pathogens, each contributing to significant alterations in their microbiota. These changes can, in turn, impact their health and growth (Malmuthuge et al., 2021). Furthermore, stress during transportation is associated with increased shedding of *Pasteurella haemolytica* and altered microbial structure in the bovine respiratory ecosystems of recently weaned beef calves (Frank and Smith, 1983; Taylor et al., 2010). The duration of transportation also plays a significant role in altering the microbiome. Long-distance transportation (3-d transportation) had greater impacts on the nasopharyngeal microbiota of beef calves and increased the risk of bovine respiratory disease pathogens than those transported for shorter distances (4.5 h) (Chai et al., 2022). The gut and respiratory tracts are intricately linked via the mucosa-associated lymphoid tissue (MALT) and the gut-lung axis (GLA). Changes in the gastrointestinal tract (GIT) microbiota, as observed post-transportation, may thus have repercussions on respiratory health and vice versa. The microbiota in the GIT significantly influences the immune system and by extension, the respiratory tract (Enaud et al., 2020). In surplus calves, little is known about how transportation impacts the microbiota despite the high level of stressors and feed and water deprivation that occurs before arriving atveal and dairy-beef facilities. It is likely that these factors can impact the establishment of region- and site-specific microbial communities of the GIT in neonatal calves. Microbial colonization follows a sequential pattern that is influenced by various factors, including host physiological state, age, diet, and environment (Gomez et al., 2019). Multiple studies have shown that development of healthy gastrointestinal microbiota in calves can benefit their overall health and prevent disease (Adak and Khan, 2019; Dill-McFarland et al., 2019; Oikonomou et al., 2013). Research on calf diarrhea has revealed that a greater diversity of microbial communities is associated with better health (Ma et al., 2020; Oikonomou et al., 2013), and certain taxa have been identified as markers for the disease (Hennessy et al., 2021; Ma et al., 2020; Oikonomou et al., 2013). Given the intertwined relationship between stress, the gut microbiome, and health, identifying the components of a healthy microbiota and the perturbations that occur during transportation is especially important to promote calf health and prevent the onset of disease, like calf diarrhea, in later life. However, the microbiota in young surplus dairy calves following transportation has yet to be explored. The primary objective of this study was to investigate the effects of transportation on the fecal microbiota of surplus calves after 6, 12, or 16 h of continuous road transportation using 16S rRNA sequencing. We hypothesized that calves transported for 16h would exhibit more pronounced alterations in their fecal microbiota when compared with calves transported for 6h, potentially indicating a higher likelihood of microbiota disturbances with longer transportation durations.

**MATERIALS AND METHODS**

**Experimental design and selection of animals**

The study was conducted between October 2020 and June 2021 as a component of a randomized controlled trial to determine the effect of transport duration on neonatal calf health (Goetz et al., 2023). Briefly, surplus dairy calves were sourced from 5 commercial dairy farms located in southern Ontario, Canada. The source dairy farms were visited 14 d before the day of the transportation, and surplus dairy calves born during the 14-d period were selected for the study. Therefore, the calf age ranged from 1 to 19 d at the start of transportation. Immediately before transportation, the calves were randomly allocated, while balancing by age (using a treatment allocation sequence generated in Microsoft Excel version 16.54) to 3 groups with different durations of continuous transportation: 6h transportation group (T1), 12h transportation group (T2) and 16h transportation group (T3). Surplus calves were blocked by farm and assigned to this sequence from youngest to oldest. Unloading times were standard-
ized to ensure that calves assigned to each group were transported for the full duration of their transport. All 177 calves enrolled in the study were transported to a single grain-fed veal producer. Among those calves, 52% were transported during spring, 24% during fall, 14% in the summer, and 10% during winter. Most the calves were male (85%) and were either Holstein (69%) or crossbred Holstein-beef breed.

Health assessment of calves

From the day of birth through 14 d after transportation, health checks were conducted on all the sampling time points in calves. Health assessment of the calves consisted of examining fecal consistency and scoring for signs of respiratory disease, dehydration, and umbilical inflammation. A 0 to 3-point scale was used for measuring fecal consistency based on visual observations: 0 = normal (firm but not hard), 1 = soft (does not hold form, piles but spreads slightly), 2 = runny (spreads readily), and 3 = watery (liquid consistency, splatters) (Renaud et al., 2020). Calves with a fecal score of 2 or 3 on 1 or more days were classified as having diarrhea (Renaud et al., 2020). Respiratory illness was scored based on the scoring system developed by Love et al., 2014b which identified the following predictors and assigned scores respectively: coughing (spontaneous or induced, 2 points), nasal discharge (any, 4 points), eye discharge (any, 2 points), ear droop or head tilt (any, 5 points), fever (>39.2°C, 2 points), and respiratory rate (abnormal respiration, 2 points). Calves were categorized as having respiratory illness if their total score on a single day was ≥5. Dehydration scoring included assessing the presence of a prolonged skin tent (>2 s) or eye recession (recession >2 mm). Navels were scored as normal (<1.3 cm in diameter at the umbilical stalk) or abnormal (enlarged, painful or >1.3 cm) (adapted from Fecteau et al., 1997).

Transportation of calves

Before transportation, calves were individually housed within the designated calf barns and cared for according to producer protocols. For transportation, a single 20.9 m² (9.1 m long x 2.3 m wide) trailer was used, resulting in 0.54–1.23 m² space allowance per calf. On each day of transport, this trailer visited all the enrolled commercial farms to pick up the surplus calves. All the calves were then loaded from each farm at one time but were dropped off at the calf-rearing facility at different times depending on the duration of transport assigned to them. There were a total of 7 cohorts of calves consisting of 17–39 calves per cohort. The sex distribution of calves is given in Table 1. The mean age before transportation was 10 d in all the 3 groups. Across all 7 cohorts of calves, 35%, 33%, and 32% belonged to T1, T2, and T3, respectively. Before each transport, the trailer was disinfected and deeply bedded with clean chopped straw. Space allowance was maintained throughout the study by introducing a sliding wall after each drop-off to approximately consistent space allowance. Calves were fed within 1 h of loading and unloading but were not provided with feed or water during the entire duration of transportation. During transportation, the temperature inside the trailer ranged from –13 to 31°C (median 4.6°C).

Fecal sample collection

Fecal samples were collected directly from all the calves by rectal stimulation into 15 mL polystyrene conical centrifuge tubes (Corning, Falcon) at 4 time points: immediately before transportation (0), immediately after transportation (AT), and at 24 h, and 72 h post transportation. They were immediately placed in a cooler and kept on ice until transported to a freezer where they were stored at −20°C until 16S rRNA analysis.

DNA extraction

The bacterial DNA from 200 μg of wet-weighted fecal samples was extracted using the E.Z.N.A Stool DNA kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer’s instructions. DNA concentrations were adjusted to 5 ng/μL before library preparation either by dilution or by concentrating using a SpeedVac (Thermo Fisher Scientific, Waltham, MA, USA). The V4 hypervariable region of the 16S rRNA gene was amplified using the primer set 515F/806R, 5′-GAGT-Vinayamohan et al.: Exploring the effects…

Table 1. Sex distribution of calves across different sample time points and at different transportation groups. T1, 6h transportation group; T2, 12h transportation group; T3, 16h transportation group; AT, immediately after travel

<table>
<thead>
<tr>
<th>Time point</th>
<th>Transportation group</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>T1</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>22</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>AT</td>
<td>T1</td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>23</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td>24</td>
<td>T1</td>
<td>26</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>72</td>
<td>T1</td>
<td>27</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>18</td>
<td>5</td>
</tr>
</tbody>
</table>
Bioinformatics and statistical analysis

To maintain the accuracy and reliability of our microbial profiling, we incorporated both negative and positive controls in our methodology. Negative controls, consisting of DNA extracted from reagents, were used to detect and monitor potential contamination during the sequencing process, and a well-characterized mock community was included as a positive control to validate the precision and reliability of our sequencing data. The R/Bioconductor package DADA2 (version 1.16, (Callahan et al., 2016)) was used to generate a count table with counts of each inferred Amplicon Sequence Variation (ASV) for each sample. Taxonomic assignment was performed using DADA2 (assignTaxonomy and addSpecies functions) using the Silva (Quast et al., 2013) database (version 138.1, available at (Martin, 2011)). Next, ASV counts for technical replicates were summed, and several filtering steps were performed: ASVs were filtered by length (retaining only ASVs with lengths between 250 and 255 bp), contaminants were inferred and removed with the R/Bioconductor package decontam (version 1.14.0, (Davis et al., 2018)) using a negative control and DNA concentration measurements, ASVs that had been assigned to the order Chloroplast, the family mitochondria, or the domain Eukaryota were removed, and samples with a total ASV count below a 1,000 were removed.

A phylogenetic tree for all ASVs was inferred using the R package phangorn (version 2.8.1, (Schliep, 2011). The R/Bioconductor package phyloseq (version 1.38.0, (McMurdie and Holmes, 2013) was used to store the resulting count table, taxonomy table, phylogenetic tree, and a metadata table as a single R object and to perform several downstream analyses. In our study, we presented the data with and without rarefaction. Rarefied data can reduce the biases introduced by variable sequencing depth across samples, enabling a more balanced comparison of microbial communities. On the other hand, unrarefied data preserve the original richness and depth of the sequencing data, potentially capturing the natural variability and complexity of microbial ecosystems more accurately. For the rarefied data set, we standardized the sampling effort across all samples by rarefying the data set to the smallest library size, identified using phyloseq. The α diversity of the 3 transportation groups were estimated using the Chao1 index, which measures richness, and the Shannon and Simpson’s indices for measuring both richness and evenness. The α diversity boxplots reflected the minimum, maximum, degree of dispersion, and outliers of microbial diversity within each transportation group. The effect of transportation time on the Shannon diversity index was investigated using linear mixed-effect regression models. Potential confounders were identified using a directed acyclic graph (DAG, Figure 1, https://www.dagitty.net/dags.html#). Note that only calf characteristics and health status before the transportation (i.e., measured on farm) were considered potential confounders and included in the model analysis. Identified confounders were screened by constructing univariable models with the calf identification as the random effect. For all the analyses, variables with $P < 0.2$ were included as independent variables in the multivariable model. The Shannon diversity index was included as the outcome, and the transportation time, sampling time, and their interaction term were independent variables of interest. Interaction terms of the transportation time and selected variables were included to explore interaction effects. If included, the calf health status and characteristics would be modeled as categorical variables except the age and body temperature. Therefore, backward Akaike Information Criterion (AIC) selection was performed to screen variables based on model fit. Variables with the variance inflation factor (VIF) larger than 10 were removed from the model to control multicollinearity, and the normality of model residuals was assessed by distribution histograms. Results were reported as the increment/decrement of the Shannon diversity index by 1 unit increase in continuous variables (e.g., transportation time, age, body temperature, serum total protein (STP)) or compared with the references for categorical variables (e.g., fecal score, respiratory score, dehydration). The reference categories were set at score $= 0$ for health assessment (e.g., fecal consistency, respiratory illness), absence for clinical signs (e.g., weak, dehydration), male for sex, and beef for breed (beef versus Holstein). Additionally, the relationship between the model-predicted diversity and sampling time by the transportation groups was plotted conditioned on the random effect (calf identification).
Likewise, potential confounders of the effect of transportation time on the change of bacterial abundances were identified and screened using linear regression models. The changes in bacterial abundances after transportation were taken as a relative measure by subtracting abundances before Transportation from the after-transportation sampling time points. Calf identification was not included as a random effect because bacterial abundances at different sampling points were fitted into separate models. Similar to the Shannon diversity model, potential confounders were identified using the DAG (Figure 2), modeled as continuous and categorical independent variables, and screened by univariable models and backward AIC selection. The final model included the bacterial abundances as the outcome, the transportation time as the variable of interest, and the selected confounders. Multicollinearity and residual normality were assessed following the Shannon diversity model. The results were reported as the increment of abundance change by one unit increase in transportation time.

Additionally, the Kruskal-Wallis test was used to evaluate pairwise differences in α diversity across transportation groups. The fecal scores were found to be different among the transportation groups even before the start of Transportation. Therefore, to account for changes that occurred before Transportation due to the abnormal fecal score, the changes in diversity and abundances at later sampling time points were taken as a relative measure by subtracting diversity and abundances before Transportation from the after-Transportation sampling time points. Pair-wise, Kruskal-Wallis comparisons were performed on the adjusted data as previously stated.

Bray-Curtis dissimilarity was utilized to analyze β-diversity among microbial communities, assessing variations in microbial composition between samples. This distance matrix was derived employing the phyloseq package in R, providing a quantitative measure of community compositional dissimilarity between different microbial ecosystems. Subsequently, analysis of similarities (ANOSIM) and permutational multivariate ANOVA (PERMANOVA) were performed using the vegan package in R to statistically validate observed dissimilarities between groups. Principal coordinates analysis (PCoA) was conducted using the ordinate function from the phyloseq package to visualize and spatially represent microbial community structures across samples. For our analysis, a prevalence threshold of 10% was considered appropriate, as per the method established by Nearing et al., 2022 allowing for a balanced inclusion of taxa. This threshold means that the taxa or features were included in the analysis if they were present in at least 10% of the samples, aiming to incorporate significant taxa that are representative of the microbial diversity and interactions in the studied communities. This was done using the R/Bioconductor package DESeq2 version 1.30.1 where the Wald test was used to identify statistically significant differences (M. I. Love et al., 2014a). Reported p-values were adjusted using the Benjamini-Hochberg multiple-testing correction method, and we used a significance threshold of 0.05.

Figure 1. Directed acyclic graph to identify potential confounders for the effect the transportation time on shannon diversity index.
RESULTS

Health assessment of calves before transportation

Comprehensive results describing the impact of transportation on the health of calves have been previously published (Goetz et al., 2023). Health data, specifically from calves with microbiota measurements, is shown in Table 2. Among the calves enrolled, the results demonstrated that some calves suffered from respiratory illness (33.9% (60/177), respiratory score ≥5) or had an abnormal navel (35.6% (63/177), enlarged, painful, or ≥1.3cm at the umbilical stalk) for at least one day before transport. In addition, most the calves (59.3%, (105/177)) had diarrhea with a fecal score of ≥2 for at least one day before transportation (Figure 3). The mean body weight before the time of transport was 51 kg, and the mean arrival weight was 47 kg (Rot et al., 2022).

Table 2. Health assessment of calves at different sampling time points. The table shows the percentage of calves with abnormal respiratory, navel, and fecal scores. The number within the parenthesis indicates the number of calves with abnormal scores out of the total calves in that transportation group. T1, 6h transportation group; T2, 12h transportation group; T3, 16h transportation group; AT, immediately after travel.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Transportation Group</th>
<th>Abnormal Respiratory score</th>
<th>Abnormal Navel score</th>
<th>Abnormal Fecal score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>T1</td>
<td>8% (2/26)</td>
<td>31% (8/26)</td>
<td>19% (5/27)</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>0% (0/23)</td>
<td>43% (10/23)</td>
<td>20% (5/25)</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>7% (2/27)</td>
<td>19% (5/27)</td>
<td>30% (8/27)</td>
</tr>
<tr>
<td>AT</td>
<td>T1</td>
<td>13% (3/23)</td>
<td>13% (3/23)</td>
<td>29% (7/24)</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>10% (2/20)</td>
<td>25% (5/20)</td>
<td>20% (4/20)</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>10% (2/20)</td>
<td>20% (4/20)</td>
<td>35% (7/20)</td>
</tr>
<tr>
<td>24</td>
<td>T1</td>
<td>9% (2/23)</td>
<td>21% (5/23)</td>
<td>8% (2/24)</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>20% (4/20)</td>
<td>30% (6/20)</td>
<td>30% (6/20)</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>0% (0/20)</td>
<td>25% (5/20)</td>
<td>50% (10/20)</td>
</tr>
<tr>
<td>72</td>
<td>T1</td>
<td>5% (1/22)</td>
<td>23% (5/22)</td>
<td>35% (8/23)</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>5% (1/20)</td>
<td>15% (3/20)</td>
<td>25% (5/20)</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>6% (1/16)</td>
<td>13% (2/16)</td>
<td>33% (6/18)</td>
</tr>
</tbody>
</table>

Sequencing analysis

A total of 268 fecal samples were collected from 177 animals from 4 different sampling time points (0, AT, 24h, and 72h). Several samples were excluded due to mislabeling, which resulted in 79 samples before transportation, 64 samples immediately after transportation, 64 samples 24h after transportation, and 61 samples 72h after transportation, respectively (Table 3).

From the V3-V4 hyper-variable region of the DNA samples, non-chimeric reads were obtained with a per-sample minimum of 71,263, a median of 134,222, and a maximum of 212,444. All the samples were then rarefied to a minimum read of 71263. Furthermore, rarefaction curves were generated and assessed to ensure that the chosen depth was reflective of true microbial diversity, and a stable plateau was reached at this depth, ensuring that the majority of the species diversity was captured (Supplementary file - Figure S1). The assembly process resulted in 3012 unique ASVs belonging to the bacteria domain. Of these, 96.65% could be success-
fully classified at the phylum level. The ability to assign classifications decreased at lower taxonomic resolutions (Supplementary file - Figure S2). Overall, 67.64% of the ASVs were identifiable to the genus level, and only a relatively small fraction, 7.83%, could be resolved to the species level.

**Microbial diversity across different transportation groups**

Our study investigated microbial diversity across different transportation groups and across different sampling time points for each travel group. We utilized
α diversity measurements with and without rarefaction and found no statistically significant differences between the 3 transportation groups at any of the sampling time points, as demonstrated by the Shannon index (linear mixed effect model; Supplementary file - Table S1 and S2; Figures S3A -S3B). Non-significant effects of calf diarrheic and healthy statuses were reported on the Shannon index (Table S2 and S3, Figures 4, S4). However, the Shannon index was found to increase by 0.07 for one day increase in calf age at transportation \( (P = 0.004, \text{Table S2}) \). Likewise, there were no significant differences observed in α diversity across the various sampling time points within each travel group and between diarrheic and healthy calves (Figure 5, S5, Table S2 -S4).

We assessed the baseline microbial diversity before transportation, thus accounting for any changes that might have occurred due to variations in fecal scores. No significant differences were found in the changes in α diversity measures at any of the later sampling time points (Supplementary file - Table S5), although a degree of dispersion was observed at 24 and 72h post-transportation across all transportation groups (Figure 6, S6).

PCoA plots are shown for each individual sampling time point (Figures 7, S7, Table S6). Separate plots for healthy and diarrheic calves at each time point are also shown in Figures 8, Table S7. Across all sampling time points, we observed no significant clustering of microbial communities between the transportation groups (Figures 7–8, Table S5-S6) by ANOSIM and PERMANOVA. However, an analysis of dissimilarities across the 4 sampling time points within each travel group revealed significant differences \( (P < 0.001) \) in all 3 transportation groups (Figure 9, Table S8). These measurements provided insights into community composition differences between samples based on ASV counts, irrespective of taxonomic assignments.

### Table 3. The total number of fecal samples collected from each transportation group during each time point. T1, 6h transportation group; T2, 12h transportation group; T3, 16h transportation group; AT, immediately after travel

<table>
<thead>
<tr>
<th>Time point</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>27</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>AT</td>
<td>24</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>24</td>
<td>24</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>72</td>
<td>23</td>
<td>20</td>
<td>18</td>
</tr>
</tbody>
</table>

### Table 4. The number of differentially abundant ASVs (\( \text{Padj} \leq 0.05 \)) between transportation groups at four different sampling time points. T1, 6h transportation group; T2, 12h transportation group; T3, 16h transportation group; AT, immediately after travel

<table>
<thead>
<tr>
<th>Sampling time points</th>
<th>T1 vs T2</th>
<th>T1 vs T3</th>
<th>T2 vs T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>AT</td>
<td>2</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>72</td>
<td>20</td>
<td>13</td>
<td>16</td>
</tr>
</tbody>
</table>

**Differential abundance across time and between travel groups**

Overall, a total of 22 different bacterial phyla were identified in the fecal samples. The 5 most abundant phyla detected across the transportation groups were Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria in varying group abundances (Figures 10, S8). An analysis was conducted to explore the relationship between health scores before transportation and phyla abundance after transportation. Overall, calves with mild eye recession (score = 1) were estimated to have lower Firmicutes abundance change (relative to which measured before transportation) at the 24th hour after transportation compared with those without eye recession (score = 0) \( (\beta = -0.33175, P = 0.02, \text{Table S10}) \). Likewise, diarrheic calves showed lower Actinobacteria abundance change at the 72nd hour after transportation compared with the healthy calves \( (\beta = -0.13043, P = 0.04, \text{Table S10}) \). Calves with certain respiratory (2 and 8) and skin tent (2) scores showed mild increase in the Chlamydia abundance change compared with those with respiratory and skin tent scores equal to zero, and for each unit increase in the transportation period (in hours), the abundance change increases by 0.00056 \( (\beta = 0.00056, 0.01749, \text{and } 0.00069, P = 0.03, < 0.001, \text{and } 0.02, \text{Table S10}) \). Finally, lower Fusobacteria abundance change was estimated for calves with longer transportation period \( (\beta = -0.00231, P = 0.04) \), respiratory score equal to 4 \( (\beta = -0.05160, P = 0.004) \), equal to 6 \( (\beta = -0.28323, P < 0.001) \), equal to 9 \( (\beta = 0.09999, P = 0.02) \), and weak appearance \( (\beta = -0.11419, P < 0.001) \) (Table S10).

There was a decrease in the abundance of Firmicutes over the 72-h sampling time points in all the transportation groups (Figures 10B, S8B). However, Kruskal-Wallis pair-wise comparisons showed no significant differential abundance between the 3 transportation groups at any of the sampling time points. Taxonomic assignments at the genus level revealed the presence of 353 distinct genera. The distribution of the top 15
most abundant genera across all the samples is shown in Figures 11, S9, and these included *Bacteroides*, *Escherichia/Shigella*, *Lactobacillus*, *Collinsella*, and *Bifidobacterium* (Figures 11A-S9B). When investigating the distribution patterns of microbial genera across the different sampling time points, a gradual decrease in *Lactobacillus* and an increase in *Bacteroides* and *Butyrivibrio* over time was observed (Figures 11B-S9B).

Further, to account for abundance differences before the start of transportation, microbial abundances at the start of transportation were taken as the baseline, and relative abundances were calculated (Figures...)
12–13, S10-S11). The results demonstrated that the reduction in Fusobacteria abundances before and immediately after transport were significantly larger in the T3 transportation group compared with the T1 transportation group ($P = 0.028$, Supplementary file - Table S9), and their abundances remained lower in 24 and 72h sampling time points when compared with T1 and T2 transportation groups (Figure 12).

In our analysis at the ASV level using DESeq2, differential abundance analysis across transportation groups revealed distinct variations in microbial compositions at each sampling time point. Specifically, genera *Bacteroides*, and *Lactobacillus* showed consistent variations across comparisons. Each time point presented a unique set of differentially abundant genera, reflecting the adaptability of microbial communities in response to travel conditions and durations (detailed in Supplementary file Tables S11 -S21). The 72-h mark showed a significant shift with 20 differentially abundant ASVs in the T1 vs T2 comparison, demonstrating the microbial community’s adaptability over extended travel. These findings suggest that specific genera respond in a time-dependent manner to travel-related stress.

**DISCUSSION**

Previous work within the surplus calf production system showed that transportation could induce physiological, immunological, and behavioral changes in young calves (Masmeijer et al., 2019; Van de Water et al., 2003; Wilson et al., 2020). Nevertheless, the influence of transportation duration on the gut microbiota of surplus dairy calves remains unexplored, despite the crucial role of gut microbiota in maintaining host health and well-being (Jandhyala et al., 2015). In this study, we followed surplus calves from multiple source dairy farms to a commercial veal facility to evaluate the effect of transportation on the microbiota and to determine whether the duration of transportation (i.e., 6, 12, 16 h) influenced the structure and composition of the surplus calf microbial communities.

We chose the immediately after transportation (AT), 24 h and 72 h post-transportation sampling points based on evidence suggesting that microbiomes quickly adjust to stress and then stabilize, as highlighted by Moya and Ferrer, 2016. Capturing the immediate, transient microbiome changes post-stress is vital since these shifts, though possibly short-lived, can impact host health, metabolism, and immune response. While the microbiome may reach a new stability post-stress, this state could differ from the original, affecting its functionality. Such periods can provide insights for strategies to counter stress effects. Moreover, while no direct studies address early life transportation stress on

*Figure 5. Shannon index (after rarefaction) of calves shown across sampling time points for A) T1 (6hour transportation group), B) T2 (12hour transportation group), C) T3 (16hour transportation group), respectively. $P > 0.05$.*
calf health, research indicates that early life interventions can long-term affect gut health in animals (Ma et al., 2020). Our findings revealed that α diversity indices did not differ among any of the transportation groups throughout the study period. We included both healthy and diarrheic calves in our study with an objective to match the natural heterogeneity in health status typically present within herds. In real-world scenarios, herds are usually composed of individual animals with interconnected microbiomes, exhibiting varying degrees of health status. By representing this diversity within our study, we aimed to render our findings more broadly applicable and reflective of actual herd conditions (Lozupone et al., 2012). Calf health before transportation appears to play an essential role in influencing their microbiota composition post-transportation. Specific pre-existing conditions, such as eye recession or the presence of diarrhea, can correlate with apparent shifts in certain microbial phyla as shown in our analysis, suggesting that initial health status can modulate the microbiome’s response to transportation stress. While previous research, including studies by (Gomez et al.,

**Figure 6.** Alpha diversity (after rarefaction) when calculated as a relative measure. Here abundances at later sampling time points were subtracted from 0h sampling time points, and α diversity was measured using A) Shannon index, B) Chao1 Index, C) Simpson index. T1, 6h transportation group; T2, 12h transportation group; T3, 16h transportation group; AT, immediately after travel.
2022, 2017; Schoster et al., 2017), has highlighted the influence of health status, especially diarrhea, on microbial α diversity, our study uniquely establishes a pre-transport microbial diversity baseline to account for these potential variances. This ensured that observed differences could be primarily attributed to the effects of travel stressors rather than pre-existing health conditions. Previous studies conducted on donkeys (Jiang et al., 2021) reported a significant reduction in α diversity after long-distance transportation (~21 h). Maintaining a diverse microbiota is crucial for promoting host immune defenses and metabolism, as a decrease in species richness has been linked to various gastrointestinal diseases such as diarrhea, acute enteric infections, and colitis in adult horses (Jiang et al., 2021). As outlined in the companion paper (Goetz et al., 2023), nearly all calves experienced diarrhea within 14 d after transport. Although our study did not find significant changes in the gut microbiota, some alterations could be observed at later time points. Moreover, the incidence of abnormal fecal scores was higher in calves transported for 16 h compared with those transported for 6 h (Goetz et al.: Exploring the effects…)

Figure 7. PCoA plots (after rarefaction) comparing β diversity across various sampling time points, assessed using ANOSIM and PERMANOVA. Points colored by transportation times, showing no significant clustering or diversity. A) 0 sampling time point; B) at immediately after travel (AT) sampling time point; C) at 24h sampling time point; D) at 72h sampling time point. T1, 6h transportation group; T2, 12h transportation group; T3, 16h transportation group; AT, immediately after travel.
et al., 2023). These findings suggest that transportation duration may have a modulating effect on the gut microbiota of surplus calves. Yet, it is essential to note that diet and new environments can also play a role in microbiota changes, acting as potential confounding factors. These calves were introduced to a new milk type and environment, both of which would have impacted their gut microbiota and explained some of the changes after arrival at the grower facility. However, it remains unknown whether these observed initial alterations are directly associated with the development of diarrhea. At the same time, the α diversity across the different sampling time points for each transportation group remained consistent, suggesting that the overall richness and evenness of microbial species within each sample did not show significant variation over time. This constancy in α diversity indicates that the total number and distribution of species remained relatively stable throughout the different phases of transportation. Further investigation is warranted to explore this potential link and better understand the relationship between transportation, gut microbiota, and calf health.

The PCoA analysis did not detect any clustering of microbial communities between the 3 transportation groups at any of the sampling time points. In a study conducted by Klein-Jöbstl et al. (2014), a high variance in microbial communities, as shown by a principal coordinate analysis was observed in calves until 40 d of birth. This suggests that individual differences in microbiota development may have contributed to a

![Figure 8](image_url)

*Figure 8.* PCoA plots (after rarefaction) of healthy (a) and diarrheic calves (b) before the start of transportation at A) 0h (immediately before travel), B) AT (immediately after travel), C) 24h and D) 72h after travel respectively. T1, 6h transportation group; T2, 12h transportation group; T3, 16h transportation group; AT, immediately after travel.
high variance and masked changes in β diversity due to transport duration. While our analyses indicate a substantial constancy in the β diversity between travel groups across the varying sampling time points, a clear distinction was observed in the β diversity across the sampling time points for each transportation group. Beta diversity measures the variation in species composition between different samples, and a change in this metric can suggest alterations in the types of species present, potentially due to environmental or external factors. The observed differences in β diversity across our transportation time points hint at shifts in the microbial community structures. While the overall number and distribution of species (α diversity) remained consistent, the specific species present and their relative abundances did change. These findings points to the complex interactions of microbial communities under transportation stress. While the overall microbial richness and evenness might remain stable, the specific microbial interactions, their competitive nature, or external influences during transportation might result in changes in community composition. Such alterations in microbial communities could have implications on host health, metabolism, and overall well-being, emphasizing the importance of understanding these interactions during transportation. Furthermore, the lack of clustering between transportation groups likely indicates that transportation alone may not have a significant impact on the gut microbiota of the calves. It is possible that longer sampling periods may be needed to

**Figure 8 (Continued).** PcoA plots (after rarefaction) of healthy (a) and diarrheic calves (b) before the start of transportation at A) 0h (immediately before travel), B) AT (immediately after travel), C) 24h and D) 72h after travel respectively. T1, 6h transportation group; T2, 12h transportation group; T3, 16h transportation group; AT, immediately after travel.
accurately determine when the microbiota changes and when diarrhea occurs, as well as to better understand the relationship between transportation duration and gut microbiota alterations in surplus calves.

In our study, the calf fecal microbiota was clearly dominated by Firmicutes, followed by Bacteroidetes and Proteobacteria. These findings are comparable to studies in calves and adult cattle (Fan et al., 2021; Zhang et al., 2022). In all transportation groups, there was a decrease in Firmicutes and an increase in Bacteroidetes over time. The Firmicutes phylum has been shown to play a crucial role in regulating host immunity by promoting the production of IL-10, which is essential for the host defense system. Furthermore, this phylum has been associated with the breakdown of fiber and resistant starch in the gut and its abundance has been linked to improved host health (Cheng et al., 2016; Kosiewicz et al., 2011; Ze et al., 2012).

At the genus level, we observed a decrease in Lactobacillus with a simultaneous increase in Bacteroides

![Figure 9](image-url)
and *Butyricicoccus* over time. *Lactobacillus* is a well-known probiotic genus that plays a crucial role in maintaining gut health by producing lactic acid and other antimicrobial compounds (Gaggìa et al., 2010). Therefore, a decrease in *Lactobacillus* abundance might suggest a potential detrimental effect of transportation stress on gut health. *Bacteroides*, one of the most abundant genera in the gut, has a symbiotic relationship with its host, providing essential benefits such as the digestion of complex polysaccharides, enhancement of the immune system, and competition against potential pathogens (Wexler, 2007). However, alterations in its relative abundance can disrupt this delicate balance, potentially leading to dysbiosis and associated health implications. *Bacteroides* are obligate anaerobes and their surge in abundance with extended transportation time may imply an increasingly anaerobic gut environment, which can occur under conditions of reduced gut

---

**Figure 10.** Abundance of the 6 most abundant phyla (after rarefaction) A) abundances for the 3 transportation groups graphed along the y-axis and transportation group along the x-axis; B) Distribution of the phyla at different sampling time points for each transportation group graphed along the y-axis and transportation groups faceted by sampling time points along the x-axis. T1, 6h transportation group; T2, 12h transportation group; T3, 16h transportation group; AT, immediately after travel.
motility – a phenomenon often associated with long-duration travel (Wexler, 2007). Conversely, an increase in *Veillonella* in the T2 and T3 groups compared with the T1 group suggests a possible suppression or out-competition of these taxa with extended travel duration. As some species within these genera are associated with the production of beneficial short-chain fatty acids, this reduction could potentially have implications for host energy metabolism and gut health (Ríos-Covián et al., 2016).

Of particular interest, the abundance of *Fusobacteria* was significantly reduced in the T3 transportation group immediately after transport compared with the T1 transportation group. Recent studies have suggested that *Fusobacteria* may play a role in exacerbating *Cryptosporidium*-induced diarrhea in calves (Ichikawa-Seki et al., 2019). The observed reduction in *Fusobacteria* abundance in the T3 transportation group indicates that prolonged transportation can impact the gut microbiota composition of pre-weaned calves. Further research is required to understand the consequences of these changes on the health and welfare of transported calves.

Several calves developed diarrhea despite the absence of significant changes in microbiota, prompting further investigation into the potential connection between

---

**Figure 11.** The abundance of the 15 most abundant genera (after rarefaction): A) genus abundances for the 3 transportation groups graphed along the y-axis and transportation group along the x-axis; B) Distribution of the genus at different sampling time points for each transportation group graphed along the y-axis and transportation groups faceted by sampling time points in the x-axis. T1, 6h transportation group; T2, 12h transportation group; T3, 16h transportation group; AT, immediately after travel.
transportation, diarrhea, and microbiota. A study conducted by Antonopoulos et al. (2009) suggested that the gut microbial community is relatively resistant to perturbations by various ecological stressors. Moreover, it is possible that the gut microbiota community exhibited resilience, where the community structure shifted back toward the baseline state following the transportation stressor. This resiliency could explain why we did not observe significant changes in the microbiota, even though many calves developed diarrhea. Alternatively, subtle alterations in the relative abundance of certain taxa, as observed in this study, could have contributed to diarrhea development, even without significant changes in α and β diversity (Rigsbee et al., 2012). To better understand this relationship, future studies could be designed to capture the possible link between transportation, diarrhea, overall calf health and microbiota. This might involve more frequent sampling of the calves’ gut microbiota before, during, and after transportation and correlating these data with the incidence of diarrhea and calf health parameters. In our analyses, the inclusion or exclusion of rarefaction did not result in significant differences, indicating the strength of our findings irrespective of the rarefaction process. Exploring the functional capabilities of the microbial communities, such as through metagenomic analysis, could provide a deeper understanding of how transportation-induced stress might influence gut microbiota function and its potential role in the development of diarrhea. Nonetheless, the study provides important insights into the health status and microbial diversity of calves before and after transportation, which could inform the development of strategies to minimize the negative impacts of transportation on calf health and welfare. Interestingly, the study did not find significant differences in α and β diversity, but it did reveal minor changes in the relative abundance of certain taxa. This indicates that although the overall microbiota remained

![Figure 12](attachment:fig12.png)

**Figure 12.** The overall difference in the proportional abundance of bacteria across phyla relative to the sampling time 0 (after rarefaction), immediately after travel (AT), at 24h after travel, and 72h after travel. Here the sum of bacterial abundances of all calves at each of the later sampling time points were subtracted from the bacterial abundances of all calves at 0h sampling time point. T1, 6h transportation group; T2, 12h transportation group; T3, 16h transportation group; AT, immediately after travel.
**Figure 13.** The mean difference in proportional abundance of bacteria across phyla relative to the sampling time 0 (after rarefaction). (A) immediately after travel (AT), (B) at 24h after travel, (C) and 72h after travel. Each bar represents the mean difference in proportional abundance. Error bars represent the standard error of the mean. Here the bacterial abundances of individual calves at each of the sampling time points were subtracted from the bacterial abundances of the same calf at 0h sampling time point. * indicate $P < 0.05$. T1, 6h transportation group; T2, 12h transportation group; T3, 16h transportation group; AT, immediately after travel.
relatively stable, subtle alterations in specific microbial populations might have occurred.

An important limitation to note is that the microbiome analysis in this study had a relatively small sample size, as it was determined based on hematological parameters as the primary objective and more studies with larger sample sizes may be necessary to further investigate the impact of transportation on the microbial diversity of calves.

CONCLUSION

In conclusion, our study revealed no significant differences in the α and β diversity measures between the 3 transportation groups, but we observed subtle changes in microbial abundances and community structure. While these alterations might not have a direct association with the overall health of the calf or signify dysbiosis, it is important to conduct more detailed studies to clarify the relationship between microbiota alterations and the calf health. Further studies are also warranted to elucidate the functional consequences of these changes and to develop strategies to mitigate the negative effects of transportation on calf health and well-being.

ACKNOWLEDGMENTS

This work was supported by funding from the Dairy Farmers of Canada, Veal Farmers of Ontario, and the Ontario Ministry of Food, Agriculture and Rural Affairs. The authors have not stated any conflicts of interest.

Supplementary Materials: https://data.mendeley.com/drafts/rkrz57dmd2

REFERENCES


Ichikawa, S., M. Motosoka, A. Kimami, F. Murakoshi, Y. Takashiki, J. Aita, K. Hayashi, A. Tashibi, S. Nakamura, T. Iida, T. Horii, and Y. Nishikawa. 2019. Specific increase of Fusobacterium in the faecal microbiota of neonatal calves infected with Crypt-