**General microbiota composition and impact of enteric challenge on diversity**

Sequencing of the 16S rRNA gene yielded an average of 57,437 reads per sample. After rarefaction to 24,791 reads to normalize sequencing depth, 65 of 70 samples (92.9%) were retained for diversity analysis, identifying 2,391 unique ASVs across all samples. Consistent with previous studies in broilers (Borda-Molina et al., 2018; Oakley et al., 2014; Pan & Yu, 2014), the jejunal microbiota was heavily dominated by Firmicutes (>93%), primarily the family Lactobacillaceae (~90%) and genus *Lactobacillus* (~90%) (Fig. 4, 5, 6). Proteobacteria (mainly *Escherichia-Shigella*) constituted the second most abundant phylum (~6%), while Bacteroidota and Actinobacteriota were minor members (<0.2%).

Alpha diversity analysis revealed significant differences among treatment groups in Observed ASV richness (Kruskal-Wallis, P = 0.001) and Fisher's alpha diversity (P = 0.002), while Shannon and Simpson diversity indices showed no significant differences (P > 0.10) (Fig. 7). Post-hoc Dunn's tests indicated that both LF and RC treatments had significantly lower Observed ASV richness compared to the unchallenged control (NC) group (Padj = 0.008 and Padj = 0.017, respectively) and the adequate calcium, fine particle challenged group (AF) (Padj = 0.008 and Padj = 0.012, respectively). Dietary calcium concentration also significantly influenced jejunal microbial richness (Observed ASVs, P = 0.040; Fisher's alpha, P = 0.037; Kruskal-Wallis main effect test excluding NC). Adequate calcium diets supported higher microbial richness compared to low calcium diets (Fig. 7A, 7D), with post-hoc Dunn's tests showing adequate calcium had significantly higher Fisher's alpha than low calcium (Padj = 0.043). Limestone particle size did not significantly influence alpha diversity measures (Kruskal-Wallis P > 0.31). These findings suggest that the enteric challenge reduced richness, particularly under specific low and reduced calcium conditions, while adequate dietary calcium helped maintain richness (Paiva et al., 2013).

The *Eimeria* spp. and *C. perfringens* co-infection significantly altered the overall jejunal microbial ecosystem structure. Beta diversity analysis revealed distinct clustering based on treatment groups across multiple distance metrics, including Bray-Curtis (PERMANOVA, P = 0.001), Jaccard (P = 0.001), and Unweighted UniFrac (P = 0.009), while Weighted UniFrac showed no significant difference (P = 0.335) (Fig. 8). Pairwise PERMANOVA indicated that the NC group differed significantly from challenged treatments, particularly LF (Bray-Curtis Padj = 0.001) and RC (Bray-Curtis Padj = 0.018), but also RF (Bray-Curtis Padj = 0.001), AC (Bray-Curtis Padj = 0.005), and LC (Bray-Curtis Padj = 0.005). Although calcium concentration and particle size did not show statistically significant main effects on overall community structure (PERMANOVA P > 0.10 and P > 0.27, respectively), specific pairwise comparisons between challenged groups revealed dietary influences, such as AF clustering separately from RF (Bray-Curtis Padj = 0.043) and LF (Bray-Curtis Padj = 0.039) (Fig. 8). This clear separation between NC and challenged groups signifies a substantial challenge-induced shift in microbial structure, a common feature of enteric diseases and dysbiosis (Antonissen et al., 2016; Ducatelle et al., 2018), aligning with the observed jejunal pathology (Fig. 2, 3).

**Dietary calcium and challenge status drive differential taxon abundance**

Differential abundance analysis using DESeq2 identified specific ASVs responsive to dietary treatments and challenge status (Fig. 9). Comparing NC to challenged groups revealed significant shifts. Notably, *Romboutsia* was highly abundant in NC but significantly depleted in AF (Padj < 0.001, log2FC = -44.74). Conversely, several *Lactobacillus* and *Bacillus* were significantly depleted in NC compared to specific challenged groups (e.g., NC vs AF: *Lactobacillus* Padj < 0.001, *Bacillus* Padj < 0.001 and Padj = 0.043; NC vs AC: *Lactobacillus* Padj < 0.001, *Staphylococcus* Padj = 0.019; NC vs LC: *Bacillus* Padj < 0.001). At the phylum level, Bacteroidota abundance was significantly higher in NC compared to challenged groups RF (Padj = 0.001) and RC (Padj = 0.014). The higher relative abundance of potentially beneficial butyrate-producing families Lachnospiraceae and Ruminococcaceae, as well as the immunomodulatory *Candidatus Arthromitus* (Bortoluzzi et al., 2018) in the NC group compared to most challenged groups (Fig. 5, 6), further underscores the challenge effect, although these differences were not significant at the family level in the DESeq2 analysis (Padj > 0.10).

Within challenged groups, dietary calcium levels drove significant changes. Several *Lactobacillus* were significantly enriched in adequate (AF) and reduced (RF) calcium groups compared to the low calcium group (LF) when using fine particles (Padj < 0.001), suggesting adequate calcium supports these dominant commensals (Pan & Yu, 2014). *Clostridium sensu stricto 1* showed a strong positive association with calcium, being significantly higher in AC compared to RC (Padj < 0.001, log2FC = 21.88) but depleted in RC compared to LC (Padj < 0.001, log2FC = -12.33). This genus includes important butyrate producers, aligning with predicted functional data (Bortoluzzi et al., 2018). Additionally, the *Romboutsia* mentioned above was higher in RC compared to RF (Padj < 0.001, log2FC = 40.97). Certain potentially pathogenic *Gallibacterium* were enriched in RF compared to LF (Padj = 0.001 and Padj = 0.002). These ASV level differences reflect highly specific microbial responses to dietary calcium levels and the enteric challenge.

**Predicted functional consequences of microbiota shifts**

PICRUSt2 analysis predicted functional differences across treatment groups based on 16S rRNA data (Fig. 11). Comparing the challenged AF group to the unchallenged NC group showed significant downregulation of predicted pathways related to bacterial secretion systems and cell cycle regulation (e.g., Cell cycle - Caulobacter, FoldEnrichment = 26.48, P < 0.001) in AF, suggesting altered bacterial activity post-infection. Adequate calcium treatments significantly upregulated predicted starch and sucrose metabolism compared to lower calcium diets (Padj < 0.01, Fold enrichment > 4.2). Crucially, predicted butanoate (butyrate) metabolism pathways were significantly enriched in higher calcium treatments (AF, AC, RC), particularly with coarse particles (e.g., AC vs RC, P < 0.001, Fold enrichment 7.0), consistent with the higher abundance of *Clostridium sensu stricto 1* ASVs. This suggests adequate calcium, especially from coarse limestone, promotes potential butyrate production, vital for gut health (Dittoe et al., 2018). Adequate calcium treatments also enriched predicted pathways for biofilm formation (e.g., AF vs RF, P < 0.001) and antimicrobial peptide resistance (e.g., AF vs RF, P < 0.001), potentially contributing to community resilience. Furthermore, the enrichment of secondary bile acid biosynthesis in adequate calcium groups (e.g., AF vs RF, Padj < 0.01) suggests an enhanced capacity to modulate host bile acids, potentially impacting pathogen resistance (Ducatelle et al., 2018).