

Project number: 6311
Funding source: EU PLF (311825)

Date: Dec 2016
Project dates: Nov 2012 to Dec 2016

Animal and farm-centric approach to precision livestock farming in Europe (EU PLF 311825).



Key external stakeholders:

Beef and Dairy farmers; Bord Bia; Department of Agriculture, Food and the Marine (DAFM).

Practical implications for stakeholders:

Gradual weaning of artificially reared dairy calves is welfare friendly and does not induce a stress response. Knowledge of breed-specific immune responses and superior bovine respiratory disease (BRD) diagnostics could enable improved health management practices better tailored towards specific disease sensitivities of Holstein-Friesian and Jersey calves. Novel bacterial species present in the lungs and lymph nodes of calves which died from bovine respiratory disease (BRD) were identified, and consequently, may be implicated in BRD development and progression.

Main results:

The overall objective of this project was to characterise health and immunity in two commonly used breeds of artificially reared dairy calves with a specific focus on responses to gradual weaning and develop a novel diagnostic method for the identification of bacteria associated with BRD. The results demonstrated unique haematological profiles and reduced transcription of immune response associated genes in Jersey compared with Holstein-Friesian calves during the peri-weaning period. The gradual weaning practiced was welfare-friendly and did not induce global differential gene expression in whole blood or evoke a physiological stress response in dairy calves. A novel, culture-free, bacterial 16S rRNA gene amplicon sequencing method was developed for BRD diagnostics.

Opportunity / Benefit:

Gradual weaning of artificially reared dairy calves was observed to be welfare friendly as it did not induce a stress response. Knowledge of breed-specific immune responses and superior BRD diagnostics could enable improved health management practices better tailored towards specific disease sensitivities of Holstein-Friesian and Jersey calves. Furthermore, the results of this research highlight that as of yet unknown bacterial species are present in the lungs and lymph nodes of calves which died from BRD, and consequently, may be implicated in BRD development and progression

Collaborating Institution: UCD

Teagasc project team: Dr. Bernadette Earley (PI), Dr. Dayle Johnston, Dr. David Kenny, Dr. Sinead Waters, Dr. Mark McGee, Dr. Matthew McCabe, Dr. Paul Cormican
External collaborators: Dr. Alan Kelly (UCD)

1. Project background:

High morbidity and mortality associated with bovine respiratory disease (BRD) in dairy calves results in significant economic loss for farmers in Ireland, and worldwide. Infectious causes of the BRD include viral and bacteriological agents with calves being more susceptible during stressful rearing periods such as weaning. The effect of weaning from milk can be examined using artificially reared dairy calves, as dairy calves are normally removed from their dams at birth and fed by artificial means thereafter. However, within dairy breeds, there is a paucity of published research on the immune response, particularly the molecular level response, to gradual weaning from a milk based diet. Therefore, the overall objective of this project was to characterise health and immunity in two commonly used breeds of artificially reared dairy calves with a specific focus on responses to gradual weaning. An additional objective was to develop a novel diagnostic method for the identification of BRDC causing bacteria. These objectives were achieved by conducting a series of studies using physiological, molecular and next generation sequencing approaches with artificially reared dairy calves. In study one, the overall health of Holstein-Friesian and Jersey calves, was characterised during the pre- and post-weaning periods. Additionally, calves' pre-weaning feeding behaviour recorded by electronic calf feeders was examined for alterations during incidents of BRD. This was done by performing clinical assessments and relating them to calf feeding behaviour pre, during, and post, the BRD event. Calves with the BRD were found to have, and had a tendency, for reduced unrewarded visits to the electronic milk feeder, pre and post the BRD event, respectively, compared with healthy calves. In the second study, haematological and molecular (gene expression) responses to gradual weaning were investigated in Holstein-Friesian and Jersey calves. RNA-seq technology was used in a third study to further examine global changes in the whole blood mRNA transcriptome, between Holstein-Friesian and Jersey calves, in response to gradual weaning.

2. Questions addressed by the project:

The objectives of this project were to:

1. Characterise the clinical health, and to determine if electronic feeding behavioural data has potential to act as indicators of the BRDC, in Irish dairy calves
2. Examine the effect of breed and plane of nutrition, on the haematological profiles of artificially reared dairy calves in response to gradual weaning, and the effect of breed and gradual weaning on expression of selected immunological genes in bovine whole blood using real-time quantitative PCR (qPCR)
3. Examine differences in global gene expression, using an RNA-seq approach in whole blood, between artificially reared H-F and J calves, in response to gradual weaning
4. Develop a single universal assay, based on high throughput phylogenetic (16S ribosomal RNA (rRNA) gene) PCR amplicon sequencing, and accurately identify and differentiate bacteria in post-mortem lung (cranial lobe) and mediastinal lymph node tissue samples, from beef and dairy calves with fatal BRDC, and from healthy dairy calves.

3. The experimental studies:

Detection of the BRDC by monitoring clinical health and feeding behaviour

The objectives of this study were (i) to characterise clinical health in dairy calves during the artificial calf rearing period on an Irish research farm and (ii) to determine if calves' pre-weaning intakes and feeding behaviour recorded by electronic calf feeders changes in response to incidents

of bovine respiratory disease (BRD). Holstein-Friesian (H-F) and Jersey (J) calves were group housed for a 56 day pre-weaning and a 28 day post-weaning period. They were fed by automatic milk replacer (MR) and concentrate feeders. Feeding behaviour, including MR consumption, drinking speed, number of rewarded visits to the feeder, number of unrewarded visits to the feeder and concentrate consumption, were recorded by the feeders. Clinical assessments were carried out on all calves twice weekly during the pre-weaning period and once weekly during the post-weaning period. A modified version of the Wisconsin health scoring criteria was used to score the calves clinical measurements and identify incidences of BRD (Table 1).

Calves were considered to have BRD when they presented simultaneously with a rectal temperature of ≥ 39.5 °C and a Wisconsin health score of 5 or greater. Mortality was low (2.4 %) but 40 % of calves had at least one incident of BRD. Feeding behaviour recorded by electronic feeders was altered during incidents of BRD. The number of unrewarded visits to the feeder was reduced, by approximately four visits, for calves with BRD during the three days prior to the identification of a BRD event ($P < 0.05$) and tended to be reduced during the seven days following the identification of a BRD event ($P = 0.05$), compared with healthy calves. Additionally, calves with BRD had a tendency for reduced net energy intake (approximately 8 %) during the three days prior to the identification of BRD event ($P=0.09$) and for reduced concentrate intake (approximately 18 %) on the day that BRD was identified ($P=0.09$), compared with healthy calves. Therefore, calf feeding behavioural data recorded by electronic feeders during the pre-weaning period, can indicate cases of BRD.

Table 1. The number of calves from each breed and plane of nutrition with a sickness event (BRD, enteritis, gastrointestinal disorder) during the pre-weaning sub periods, weaning and post-weaning periods.

| Breed | H-F | | | J | | |
|---|-------------|-------------|-------------|-------------|------------|---------|
| | H (n=11) | M (n=14) | L (n=14) | H (n=10) | M (n=9) | L (n=9) |
| <i>Pre-weaning period (start)</i> | | | | | | |
| Calves with a BRD event | 1 | 3 | 2 | 0 | 2 | 2 |
| Calves with an enteritis event | 1 | 1 | 0 | 1 | 0 | 0 |
| Calves with a gastrointestinal disorder event | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>Pre-weaning period (mid)</i> | | | | | | |
| Calves with a BRD event | 0 | 4 | 2 | 1 | 3 | 1 |
| Calves with an enteritis event | 0 | 1 | 0 | 0 | 1 | 0 |
| Calves with a gastrointestinal disorder event | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>Pre-weaning period (end)</i> | | | | | | |
| Calves with a BRD event | 3 | 2 | 0 | 2 | 1 | 1 |
| Calves with an enteritis event | 0 | 0 | 0 | 0 | 0 | 0 |
| Calves with a gastrointestinal disorder event | 0 | 1 | 0 | 0 | 0 | 0 |
| <i>Weaning period</i> | | | | | | |
| Calves with a BRD event | 2 | 1 | 0 | 2 | 1 | 2 |
| Calves with an enteritis event | 0 | 0 | 0 | 0 | 0 | 0 |
| Calves with a gastrointestinal disorder event | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>Post-weaning period</i> | | | | | | |

| | | | | | | |
|--|---|---|---|---|---|---|
| Calves with a BRD event | 0 | 1 | 2 | 1 | 0 | 0 |
| Calves with an enteritis event | 0 | 0 | 0 | 0 | 0 | 0 |
| Calves with a gastrointestinal disorder event | 0 | 0 | 0 | 0 | 0 | 0 |

H-F = Holstein-Friesian; J = Jersey.

H = high plane of nutrition; M = medium plane of nutrition; L = low plane of nutrition.

Pre-weaning period (start) = days -53 to -37, relative to weaning (day 0); pre-weaning period (mid) = days -36 to -35, relative to weaning (day 0); pre-weaning period (end) = days -24 to -14, relative to weaning (day 0); weaning period = days -14 to -1; post-weaning period = days 0 to 28.

The effect of breed and plane of nutrition, on the haematological profiles of artificially reared dairy calves in response to gradual weaning, and the effect of breed and gradual weaning on expression of selected immunological genes in bovine whole blood using real-time quantitative PCR (RT-qPCR)

Haematological profiles indicate the health status of an animal and can be used to identify sub-clinical stress responses. The objectives of the study were to examine (i) the effect of breed and plane of nutrition, on haematological profiles of artificially reared Holstein-Friesian and Jersey bull calves in response to gradual weaning, and (ii) the effect of breed on immune response genes in bovine whole blood using real-time quantitative PCR (qPCR). Holstein-Friesian and Jersey bull calves were group housed indoors and individually fed using an automatic feeder. They were allocated to a high, medium or low planes of nutrition, based on milk replacer (MR) and concentrate. The nutrition treatments were calculated using NRC guidelines in order to achieve a high, medium or low growth rate for each respective breed. During the weaning phase MR was gradually reduced over a 14 day (d) period (d -13 to d 0). Calves were blood sampled on d -14, -6, -3, 0, 1, 3, 8, and 14 relative to weaning (d 0) for subsequent haematological analysis. On d -14, 1 and 8, a subset of eight Holstein-Friesian calves randomly selected from the medium nutrition treatment and eight Jersey calves randomly selected from the high nutrition treatment, were blood sampled for gene expression profiling, targeting biomarkers of weaning stress. These two treatment groups were chosen to examine the effect of breed on expression of the genes of interest, as energy intake and animal performance was similar. There was no effect of breed × plane of nutrition interaction nor effect of plane of nutrition on any variable measured ($P > 0.05$). Gradual weaning produced differential biological responses in the two breeds evidenced by breed × time interactions for lymphocyte, monocyte, and red blood cell (RBC) number, plasma haemoglobin (HGB) and haptoglobin concentrations ($P < 0.05$). The typical stress response consisting of neutrophilia and lymphopaenia was not observed for any treatment. An immune response to gradual weaning was observed as the relative gene expression level of the pro-apoptotic gene, Fas, increased on d 1 relative to d -14 ($P < 0.05$). Relative gene expression levels were greater in Jersey calves compared with Holstein-Friesian for the pro-inflammatory cytokine CXCL8 ($P = 0.05$) and the glucocorticoid receptor, GRα ($P < 0.05$) (Figure 1). The increased levels of these transcripts suggest that Jersey calves may have a more sensitive immune system compared to Holstein-Friesian.

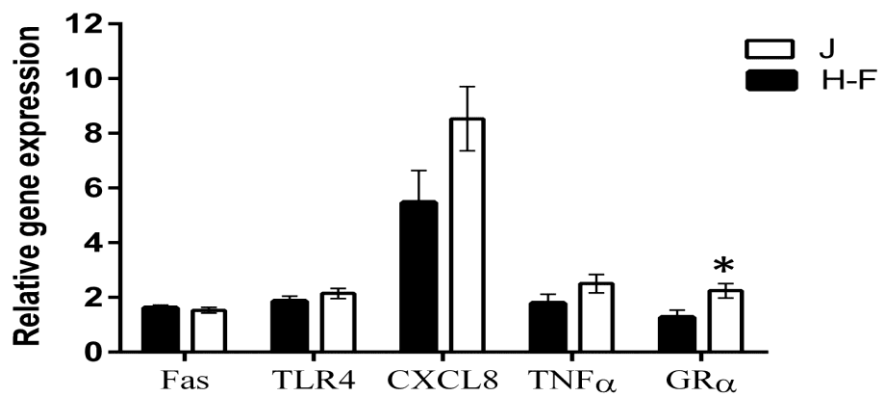


Figure 1. Effect of breed on immunological gene expression levels. Relative gene expression levels of both CXCL8 ($P = 0.05$) and GR α ($P < 0.05$) were greater in J calves. * $P < 0.05$.

Examination of the differences in global gene expression, using an RNA-seq approach in whole blood, between artificially reared H-F and J calves, in response to gradual weaning

The objectives of the study were to use RNA-seq technology to examine the effect of (i) breed and (ii) gradual weaning, on the whole blood mRNA transcriptome of artificially reared Holstein-Friesian and Jersey calves. The calves were gradually weaned over 14 days (day (d) -13 to d 0) and mRNA transcription was examined one day before gradual weaning was initiated (d -14), one day after weaning (d 1), and 8 days after weaning (d 8). On d -14, 550 genes were differentially expressed between Holstein-Friesian and Jersey calves, while there were 490 differentially expressed genes (DEG) identified on d 1, and 411 DEG detected eight days after weaning ($P < 0.05$; FDR < 0.1) (Figure 2). No genes were differentially expressed within breed, in response to gradual weaning ($P > 0.05$). The pathways, gene ontology terms, and biological functions consistently over-represented among the DEG between Holstein-Friesian and Jersey were associated with the immune response and immune cell signalling, specifically chemotaxis. Decreased transcription of several cytokines, chemokines, immunoglobulin-like genes, phagocytosis-promoting receptors and g-protein coupled receptors suggests decreased monocyte, natural killer cell, and T lymphocyte, chemotaxis and activation in Jersey compared to Holstein-Friesian calves. Knowledge of breed-specific immune responses could facilitate health management practices better tailored towards specific disease sensitivities of Holstein-Friesian and Jersey calves. Gradual weaning did not compromise the welfare of artificially-reared dairy calves, evidenced by the lack of alterations in the expression of any genes in response to gradual weaning.

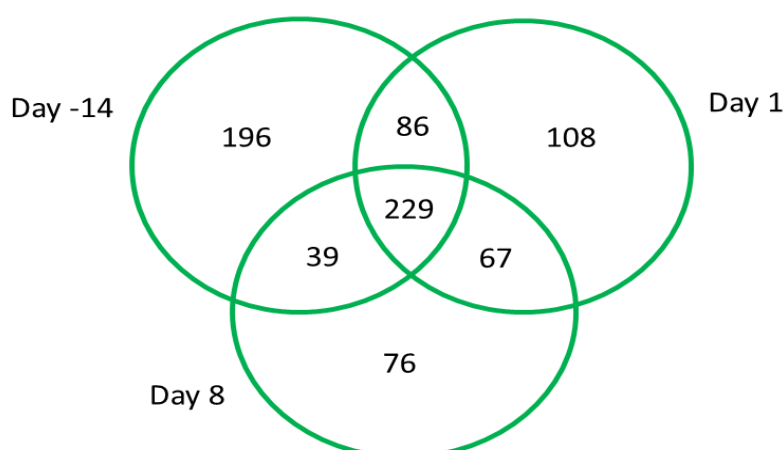


Figure 2. Venn diagram of the DEG between Holstein-Friesian and Jersey calves at each time-point. Each circle represents a time-point relative to weaning (i.e. day -14, day 1 and day 8).

A novel diagnostic tool for the bovine respiratory disease complex

A novel, convenient, single universal bacterial 16S rRNA gene amplicon sequencing assay which can detect the bacterial operational taxonomic units (OTU)s present in a tissue sample was developed. The microbiomes of the cranial lung lobe and mediastinal lymph node from calves which died from the BRDC and from healthy H-F calves were characterised using the 16S rRNA gene amplicon sequencing assay. Bacterial OTUs were demonstrated to be present within the lung and lymph node tissues of healthy calves as well as those calves which died from the BRDC. However, the bacteria associated with the BRDC (*Mycoplasma*, *Pasteurellaceae*) found within the lungs and lymph nodes of healthy calves were present less often, and had lower relative abundances compared with the bacteria associated with the BRDC found within the corresponding tissues from calves which died from the BRDC. A bacterial family taxonomic level OTU (*Leptotrichiaceae*) was identified to be abundant in the lungs and mediastinal lymph nodes from calves with the BRDC as a confirmed cause of death (Figure 2). Additionally, it was not present in any lung or mediastinal lymph node post-mortem tissue sample collected from healthy H-F calves without lung lesions observed at slaughter. This family taxonomic level OTU contains a novel species member possibly implicated in the BRDC as sequence contigs assembled from a post-mortem lung sample, belonging to this bacterial family, were not identical to any known bacterial species.

Additional to *Leptotrichiaceae*, many bacteria which are not currently screened for by PCR or cultured at veterinary laboratories, were found to be present within the cranial lung lobes (Figure 3) and mediastinal lymph nodes from calves which died from the BRDC, including *Fusobacterium*, *Helcococcus*, *Mycoplasma* (only *Mycoplasma bovis* is generally screened for), *Ureaplasma*, *Bacteroides* and *Streptococcus*. The 16S rRNA gene amplicon sequencing assay, has identified within the cranial lung lobe and mediastinal lymph node microbiomes in calves which died from the BRDC, unknown bacteria and bacteria which are not commonly screened in BRDC cases, which may be implicated in the onset and progression of the BRDC. Consequently, this 16S rRNA gene

amplicon sequencing assay has potential to expedite BRDC diagnosis and identify more, as of yet unknown, bacteria which may be key players in BRDC development and progression. Furthermore, results from the 16S rRNA gene amplicon sequencing assay, highlight that the high rates of mortality and morbidity due to the BRDC will continue if current diagnostics remain focused on bacterial agents that have been commonly associated with the BRDC and neglect both common and presently unknown commensals that may offer a pathogenic threat.

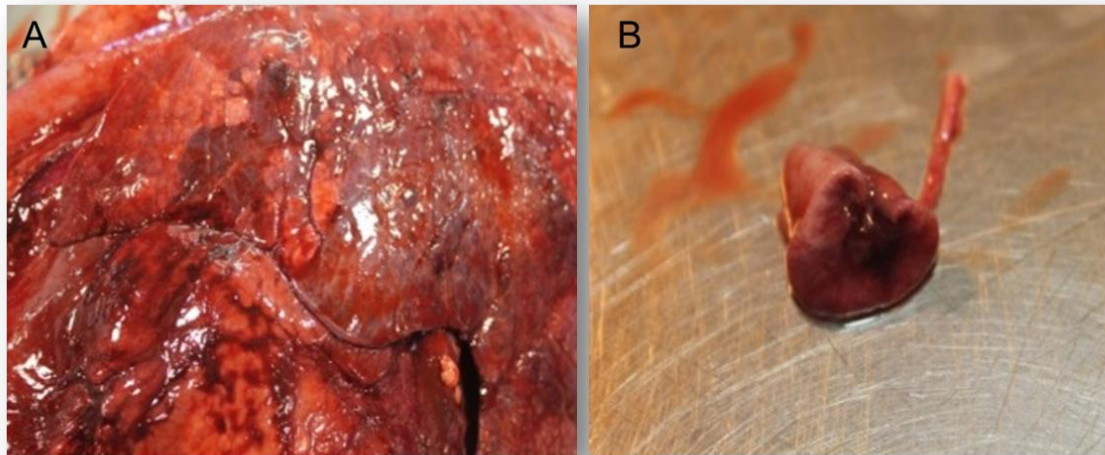


Figure 2. Post-mortem tissue types collected from calves. A = cranial lung lobe from a calf which died from bovine respiratory disease. B = mediastinal lymph node from a calf which died from bovine respiratory disease.

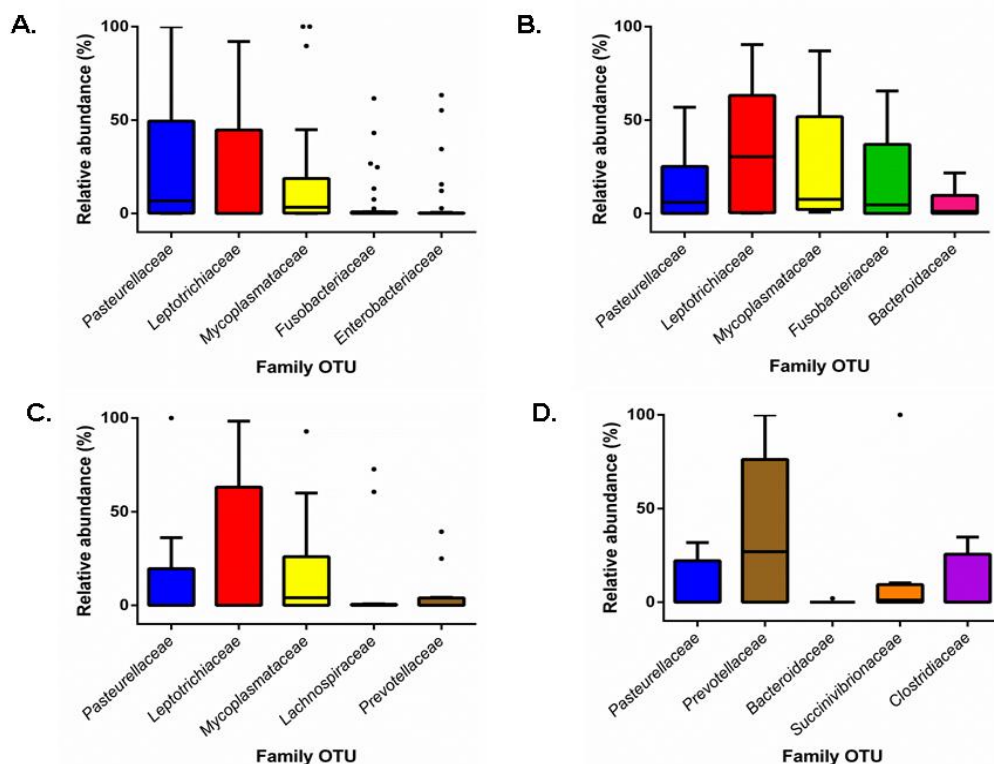


Figure 3. Percentage relative abundance of the top five most abundant bacterial family level OTUs in post-mortem lung tissues. A = Percentage relative abundance of bacterial family level OTUs in lungs of beef calves which died from bovine respiratory disease (n = 32). B = Percentage relative abundance of bacterial family level OTUs in lungs of beef calves which died from bovine respiratory disease (n = 32). C = Percentage relative abundance of bacterial family level OTUs in lungs of beef calves which died from bovine respiratory disease (n = 32). D = Percentage relative abundance of bacterial family level OTUs in lungs of beef calves which died from bovine respiratory disease (n = 32).

family level OTUs in lungs of dairy calves which died from bovine respiratory disease (n = 6). C = Percentage relative abundance of bacterial family level OTUs in lungs of healthy H-F calves with lung lesions (n = 12). D = Percentage relative abundance of bacterial family level OTUs in lungs of healthy H-F calves with no lung lesions (n = 8). Boxplot = Tukey boxplot (lowest datum (whisker) is within a 1.5 interquartile range of the lower quartile, highest datum (whisker) is within a 1.5 interquartile range of the upper quartile, outliers (individual points) fall above and below these whiskers).

4. Main results:

The main findings of this project are summarised as follows:

1. Calves with BRDC had altered feeding behaviour. The number of visits to the automatic feeder unrewarded with MR was reduced during the three days prior to the identification of the BRDC event and tended to be reduced during the seven days following the identification of the BRDC event. Net energy intake tended to be reduced during the three days prior to the identification of the BRDC event and concentrate intake tended to be reduced on the day that the BRDC was identified
2. The gradual weaning of artificially reared dairy calves from milk to a solid feed based diet, practiced in this study, is welfare friendly and does not induce a pro-inflammatory stress response. This is evidenced by the absence of neutrophilia and lymphopaenia, no changes in gene transcription of pro-inflammatory cytokines, in the whole blood mRNA transcriptome, or in the level of the APP haptoglobin, in artificially reared H-F and J calves, in response to gradual weaning
3. The H-F and J calves have breed specific haematological profiles and concentrations of the APP, haptoglobin
4. Holstein-Friesian and J calves have different levels of expression of several genes involved in immune responses and cell signalling activity. Transcriptional down-regulation of several cytokines, chemokines, immunoglobulin-like genes, phagocytosis-promoting receptors and g-protein coupled receptors suggests decreased monocyte, natural killer cell and T lymphocyte chemotaxis and activation in J compared to H-F calves
5. A convenient, single universal bacterial 16S rRNA gene amplicon sequencing assay was developed which can detect the OTUs present in a post-mortem tissue sample. This assay has potential to expedite BRDC diagnosis and identify, as of yet, unknown bacteria, which may be key players in BRDC development and progression
6. The cranial lung lobe and the mediastinal lymph node tissue microbiomes have been characterised for calves with a confirmed cause of death from the BRDC and for healthy H-F calves
7. The OTUs representing bacteria currently associated with the BRDC are present more often and in greater relative abundances within the lungs and mediastinal lymph nodes of calves which died from the BRDC compared to within the lungs and mediastinal lymph nodes from the healthy H-F calves
8. A family of bacteria (Leptotrichiaceae) has been identified to be the most abundant OTU within the post-mortem cranial lobe lung and mediastinal lymph node tissue samples obtained from calves which died from the BRDC. Leptotrichiaceae was not present in samples from healthy calves. The Leptotrichiaceae family contains a novel species member which is suggested to be implicated in the BRDC.

Opportunity/Benefit:

Gradual weaning of artificially reared dairy calves was observed to be welfare friendly as it did not induce a stress response. Knowledge of breed-specific immune responses and superior BRD

diagnostics could enable improved health management practices better tailored towards specific disease sensitivities of Holstein-Friesian and Jersey calves. Furthermore, the results of this research highlight that as of yet unknown bacterial species are present in the lungs and lymph nodes of calves which died from BRD, and consequently, may be implicated in BRD development and progression.

5. Dissemination:

1. Johnston, D., Kenny, D. A., Kelly, A. K., McCabe, M. S., McGee, M., Waters, S. M. and Earley, B. (2016) 'Characterisation of haematological profiles and whole blood relative gene expression levels in Holstein-Friesian and Jersey bull calves undergoing gradual weaning', *Animal*, 10(9); 1547-1556.
2. Johnston, D., Earley, B., Cormican, P., Kenny, D. A., McCabe, M. S., Kelly, A. K., McGee, M. and Waters, S. M. (2016) 'Characterisation of the whole blood mRNA transcriptome in Holstein-Friesian and Jersey calves in response to gradual weaning', *PLoS One*, 11(8), e0159707.
3. Johnston, D., Kenny, D.A., McGee, M., Waters, S.M., Kelly, A.K. and B. Earley. (2016) 'Electronic feeding behavioural data as indicators of health status in dairy calves', *Irish Journal of Agriculture and Food Science* (In press October 2016).
4. Vandermeulen, J., Bahr, C., Johnston, D., Earley, B., Tullo, E., Fontana, E., Guarino, M., Exadaktylos, V., Berckmans, D. 2016. Early recognition of bovine respiratory disease in calves using automated continuous monitoring of cough sounds. *Computers and Electronics in Agriculture*, 129; 15-26.

6. Compiled by: Dr Bernadette Earley
