METHYLOME PROFILING IN RESPONSE TO STRESS: MYCOTOXIN (SPORIDESMIN) EXPOSURE IN SHEEP

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SUMMARY

Epigenetic modifications, including DNA methylation, alter gene expression without changing the DNA sequence, allowing for immediate and reversible modulation of physiological responses to abiotic/biotic stress. Facial eczema (FE) is a metabolic disease, which causes liver damage in affected animals. It occurs as a result of ingestion of the mycotoxin sporidesmin, which is found in the spores of the pasture-dwelling fungus *Pseudopithomyces chartarum*. This pilot study investigated DNA methylation changes that occurred as a result of sporidesmin exposure and identified a number of differentially methylation genomic regions in animals with liver stress. Of note, the *HBA* gene showed differential methylation in the promoter region; the *HBA* co-subunit of haemoglobin *HBB* has previously been identified as a QTL for the disease in sheep. There may be potential for DNA methylation markers to be used as a diagnosis proxy for FE or as a selection marker for resilient animals in the future.

INTRODUCTION

Facial eczema (FE) is a metabolic disease responsible for major economic losses and animal welfare concerns in New Zealand. The disease is caused by ingestion of the mycotoxin sporidesmin, causing liver damage and leading to decreased productivity and reproduction in clinically and sub-clinically affected animals. Current strategies to reduce the severity of FE outbreaks include dosing animals with zinc, spraying pastures with fungicides, managing pastures, alternative feeds, and breeding for animals with increased tolerance to the disease.

Currently, ram breeders in NZ use an ethical dosing strategy using sporidesmin from laboratory-cultured *Pse. chartarum* to predict FE tolerance (RamGuard^{TM}; Aymes & Hawkes 2014). The physiological effects of the disease are assessed by measuring serum gamma-glutamyltransferase (GGT) at 21 days post-challenge (GGT21), which is recorded in the national genetic evaluation (h^2 = 0.44 ± 0.03) (McRae *et al*. 2021). While the underpinning genomics continues to be assessed, we have extended our investigation to include epigenetics profiles. Advances in "omics" technologies have fuelled investigation into the epigenome as a tool to enhance livestock selection and breeding practices. DNA methylation is an important epigenetic mark that is essential for genomic stability and maintenance throughout development and serves as a biomarker of chronological age and a biological fingerprint of a stress response (Clarke *et al*. 2021). A pilot study was conducted investigating changes in the methylome in response to a sporidesmin challenge to assess the potential application of methylation profiling for livestock breeding.

MATERIALS AND METHODS

Animals were managed following the provisions of the New Zealand Animal Welfare Act 1999, and the New Zealand Codes of Welfare developed under sections 68-79 of the Act. All work was undertaken with the approval of the AgResearch Ruakura Animal Ethics Committee (Approval number: 15059). Reduced-representation bisulphite sequencing (RRBS; Smith *et al*. 2009) was used to profile a cohort of sheep exposed to a controlled FE disease challenge with the identified stress-imposed changes to DNA methylation across two timepoints, day 0 (pre-challenge), day 21 (post-

challenge) presented. The animals used in this study consisted of 70 nine-month-old ram lambs from two breed groups. A total of 50 ram lambs (5 progeny per sire, 5 sires per breed) were challenged through the Ramguard™ (Amyes & Hawkes 2014) program. The remaining 20 rams (10 per breed) were selected from remaining unchallenged animals and were from a mixture of sires (1-2 progeny per sire). The Ramguard™ programme uses *Pse. chartarum* that is cultured in a laboratory to produce the toxic forms of mycotoxin sporidesmin, specifically sporidesmin A, B and E, with a >90% predominance of sporidesmin A. Animals were dosed with precise amounts of sporidesmin by intra-ruminal intubation at a volume that is dependent on the animal's live weight (mg per kg live weight). Blood samples were taken for GGT testing before dosing (for a base activity; d0) and at 21 days after dosing (d21) and processed for serum GGT activity (IU/L) through a commercial laboratory (IDEXX, Hamilton, New Zealand) (Johnson & Amyes 2021). Ear tissue punches (Allflex Tissue Sampling Unit; TSU samples) were also collected at d0 and d21 and genomic DNA was extracted from tissue samples using a high-salt method (Clarke *et al*. 2014).

The Zymo-seq RRBS Library Kit (Zymo Research, Irvine, CA, United States) was used for bisulphite conversion and library construction as per the manufacturer's instructions, using 500 ng of input DNA, and sequenced at AgResearch on a NovaSeq 6000 (Illumina Inc, San Diego, CA, USA.), yielding 101 bp single-end reads (minimum 5 x coverage). Data were processed and analysed as follows. Briefly, TrimGalore v.0.5.010 was used to trim raw reads to remove adapter oligos and poor-quality bases (Phred score < 20) with the flags: --non_directional --rrbs -q 20 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Trimmed reads were aligned to the reference sheep genome ARS-UI_Ramb_v2.0 (Davenport *et al*. 2022) using the BSSeeker23 script bs_seeker2-align.py and Bowtie2, with RRBS settings and allowing four mismatches (-m 4) (Guo *et al*. 2013). Methylation levels were called using the "bam2cgmap" function within CGmaptools with default options (Guo *et al* 2018).

After sequencing 7 samples were excluded due to low sequencing depth leaving 63 animals in the analysis. The challenged rams were categorized into high and low susceptibility groups based on their d21 GGT, which indicates liver/bile duct damage, with GGT<=300 IU/L classified as having a low GGT score (n=29) and GGT >=301 IU/L classified as having a high GGT score (n=14) (Johnson & Aymes 2021). Differentially methylated regions (DMR) were identified between groups and time points with MethylKit v1.12.0261 in R, which applies a sliding-window approach with a window of 1,000 bp and a step size of 500 bp (Akalin *et al*. 2012). The data were filtered for potential PCR duplicate reads by excluding bases with more than the 99.9th percentile of coverage in each sample (hi.perc=99.9). Read coverage distributions between samples were normalised using a scaling factor derived from differences between the median of the coverage distributions to avoid oversampling of reads from more highly sequenced individuals in downstream statistical analyses.

Methylated sites common between samples were identified and combined into a single R object for further analysis. To calculate differential methylation, groups were compared via a logistic regression model. P-values were adjusted to q-values using the Sliding Linear Model (SLIM) method (Wang *et al*. 2011). DMR were defined as regions with at least a 15% difference between the group being tested and the remaining samples and a Q-value ≤ 0.05, controlling for false discovery rate based on the SLIM method. DMRs were overlapped with annotated genes and gene promoters, defined as 1kb upstream flanks of genes in the ARS-UI_Ramb_v2.0 genome.

RESULTS AND DISCUSSION

Day 21 serum GGT activity ranged from 50 UI/L to 1056 UI/L (Figure 1). A total of 6 rams showed clinical signs of FE and had to be euthanised due to the severity of their symptoms, TSUs were collected from these animals on the day of euthanasia and used in this study. A total of 14 rams had GGT activity >=301 UI/L and were ranked as high GGT animals including the 6 euthanised animals. A series of group comparisons were performed to identify DMRs between time points and

GGT score groupings. These are summarised in Figure 2A. The number of DMRs between groups differed greatly. There was one DMR identified between the challenged and control groups at d0 which was also seen at d21, this DMR was located in the *FBXL17*gene which is involved in the mitotic cell cycle. There were a large number of DMRs (1330) identified in the comparison between d0 and d21 of the High GGT group.

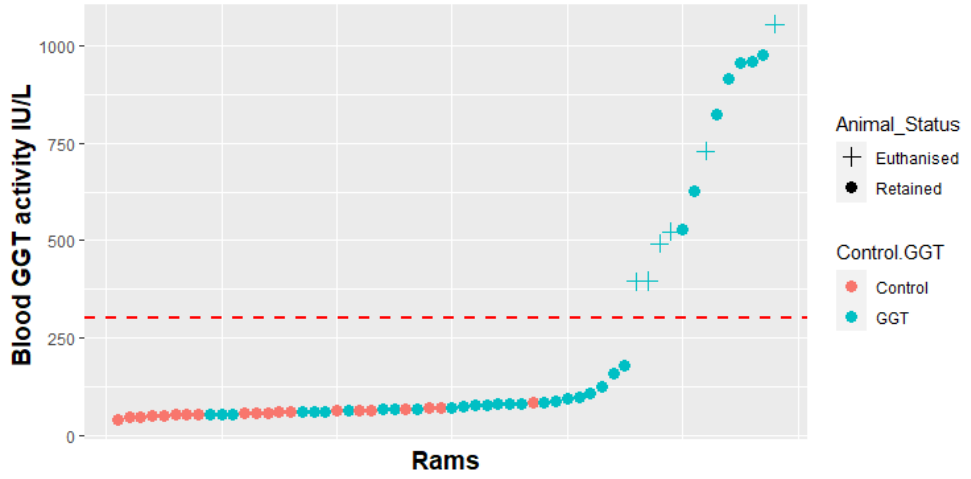

Figure 1. Blood GGT activity d21 post- Ramguard™ challenge, the red dashed line indicates the threshold for high GGT rank >=301 UI/L

A

Group 1	Group 2	# DMRs	# Genes
Control d0	Case d0	1	1
Control d0	Control d21	3	0
Case d0	Case d21	1	1
Control d21	Case d21	4	2
Low GGT d0	Low GGT d21	0	0
Low GGT d21	High GGT d21	66	45
High GGT d0	High GGT d21	1330	811

B

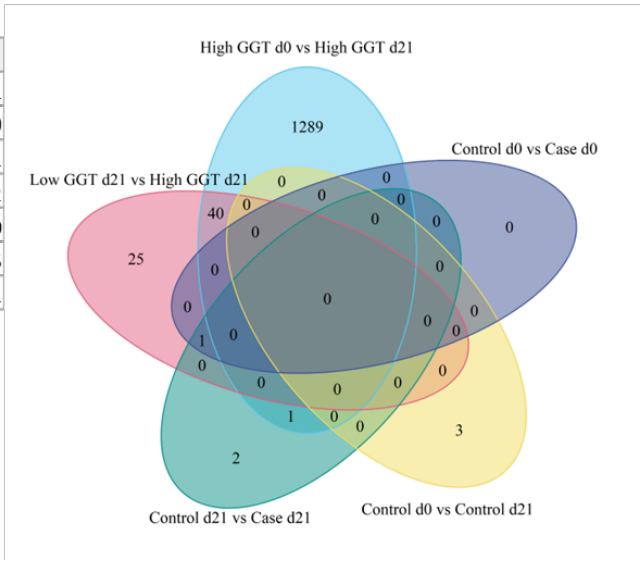

Figure 2. DMR identified between groupings; (A) number of DMRs identified between group 1 and group 2 in each comparison and number of gene regions (gene + 1KB upstream) identified in the DMRs. (B) common DMRs identified in each comparison

Interestingly there was only one DMR identified between d0 and d21 in the challenged group, however, when subsetted into low and high GGT groups a large number of DMRs were identified

between d0 and d21 in the high GGT group, suggesting that the low GGT responders have a different response mechanism compared to the high GGT responders. Two genes of note were identified as having DMRs in the promoter regions when comparing the high GGT d0 and d21 groups, *HBA* and *CARD11*. The β-globin gene, *HBB* has previously been identified as a QTL for FE susceptibility in a previous GWAS, explaining 5% of the phenotypic variance of resistance to FE (McRae *et al*., 2022). The *HBA* locus encodes an α-globin subunit that forms a haemoglobin complex with the β-globin subunit. The discovery of a DMR across the regulatory element of the *HBA* gene supports the notion that the haem complex is linked to FE susceptibility. Further investigations including proteomic mass spectrometry from blood samples of animals through the Ramguard™ program is currently underway to fortify this hypothesis. Another gene of note is *CARD11,* which has previously been associated with severe atopic dermatitis in humans (Ma *et al*. 2017). Clinical manifestation of FE in animals include flaking of exposed areas of the skin.

CONCLUSIONS

This pilot study indicates there are significant methylation changes that occur in animals with a poor response to mycotoxin challenge. The DMRs associated with a high GGT response have biological relevance and warrant further investigation. Methylation markers hold potential to be used as a diagnosis proxy for FE or as a selection marker for resilient animals.

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