Final report

Effect of curfew on the microbiology of sheep

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Abstract

The effect of 12 or 24h transport and food and water deprivation (FWD) for 12 and 24h prior to transport on the levels of *E. coli* in the faeces of sheep was investigated. The presence and numbers of *E. coli* O157 and *Salmonella* was also determined. Faecal samples were collected from each of 10 sheep before any treatment, after 12 or 24h FWD, after 12 or 24h transport and then after 24, 48 and 72h recovery after transport. A total of 680 faecal samples were collected and tested for the numbers of *E. coli* present and the presence and numbers of *E. coli* O157 and *Salmonella*. There appears to be no significant effect of 12 or 24h FWD or transport for 12 or 24h on the levels of *E. coli* found in the faeces of sheep. This suggests that applying these FWD and transport times will have little impact on the presence of *E. coli* and *Salmonella* in sheep faeces, and subsequently the risk of contamination of carcases.

Executive summary

Background

In order to protect public health and maintain consumer confidence and export markets for Australian meat, it is important to control pathogens through the food chain. This includes understanding the factors influencing the shedding of foodborne pathogens such as *Salmonella* and pathogenic strains of *Escherichia coli* by animals. There has been limited work investigating the impact of food and water deprivation (FWD) pre-slaughter on the shedding of pathogens by animals.

Objectives

The effect of 12 or 24h transport and food and water deprivation (FWD) for 12 and 24h prior to transport on the levels of *E. coli* in the faeces of sheep was investigated.

Methodology

The presence and numbers of *E. coli* O157 and *Salmonella* was also determined. Faecal samples were collected from each of 10 sheep before any treatment, after 12 or 24h FWD, after 12 or 24h transport and then after 24, 48 and 72h recovery after transport. A total of 680 faecal samples were collected and tested for the numbers of *E. coli* present and the presence and numbers of *E. coli* O157 and *Salmonella*.

Results/key findings

E. coli was enumerated in 677 samples as the number of *E. coli* could not be determined for 3 samples (either because of overgrowth by coliforms or because the count was below the level of detection which was <10 cfu/g). The mean $\log_{10}$ count of *E. coli* in the faecal samples was 6.05 cfu/g with counts ranging between $\log_{10}$ 1.7 to 8.61 cfu/g. Mean $\log_{10}$ counts of *E. coli* in the faeces of groups of sheep varied between 4.47 and 7.6 cfu/g throughout the experiment. The mean $\log_{10}$ count of *E. coli* in faecal samples of sheep before treatment was 5.9 cfu/g, after 12h FWD was 6.05 cfu/g, after 24h FWD was 6.15 cfu/g, after 12h transport was 5.76 cfu/g, after 24h transport was 6.33 cfu/g, then after 24, 48 and 72h recovery was 6.35, 6.03 and 5.87 cfu/g respectively. There appeared to be no consistent trend observed for mean counts of *E. coli* in the faeces of sheep at different stages of the experiment. There were no significant interactions between the mean $\log_{10}$ counts of *E. coli* in the faeces of sheep regardless of FWD or transport times. It appears the counts of *E. coli* sometimes fluctuate after FWD or transport but not in a consistent way.

E. coli O157 and *Salmonella* were not detected in this study and no conclusions can be offered about the effect of FWD and transport on these pathogens. This does suggest if there were very low numbers present they were not amplified by these potentially stressful activities. Testing for Shiga toxin genes (*stx*) in the faeces of sheep was performed to ascertain if there was any effect of FWD and transport times on the presence of these genes. Shiga toxins are predominantly harboured by *E. coli* and even though the total numbers of *E. coli* may not have varied significantly between treatments, the type of genes the *E. coli* possess may have been affected. The faecal samples collected from sheep were tested for both types of *stx*, stx_1 and stx_2. There were no significant differences observed in the prevalence of these genes between different FWD and transport treatments. In general, there was a decline in the prevalence of *stx* in the faeces of sheep after FWD

and transport, but this difference was not significant. No interactions between FWD or transport treatments were observed.

Benefits to industry

There appears to be no significant effect of 12 or 24h FWD or transport for 12 or 24h on the levels of *E. coli* found in the faeces of sheep. This suggests that applying these FWD and transport times will have little impact on the presence of *E. coli* and *Salmonella* in sheep faeces, and subsequently the risk of contamination of carcases

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1. Background

In order to protect public health and maintain consumer confidence and export markets for Australian meat, it is important to control pathogens through the food chain. This includes understanding the factors influencing the shedding of foodborne pathogens such as *Salmonella* and pathogenic strains of *Escherichia coli* by animals. There has been limited work investigating the impact of food and water deprivation (FWD) pre-slaughter on the shedding of pathogens by animals (Grau et al. 1969; Kudva et al. 1995). Sheep may become stressed by activities such as a change in diet (Kudva et al. 1995), food deprivation (Grau et al. 1969) and transport (Knowles 1998) which may lead to increased faecal pathogen shedding. Animals that shed higher numbers of pathogens at slaughter are more likely to be a source of cross contamination of other animals, the transport and lairage environments and the meat that is subsequently produced. It is therefore important for the industry to have information that can be used to assess the risks that transport and feed and water withdrawal times contribute to the microbiological quality of meat.

Project B.AHW.055 was set up to investigate the effect of pre-transport FWD on the behavioural and physiological responses resulting from transporting young sheep. This provided an opportunity to gather samples from sheep to test for effects of FWD and transport duration on the shedding of food borne pathogens. The numbers of *E. coli* and the presence of *Salmonella* on sheep carcases form part of the national *E. coli* and *Salmonella* monitoring program (ESAM). *E. coli* O157 is also an important pathogen with respect to the export beef industry. The aim of this study was to test faecal samples collected from the sheep enrolled in project AHW.055 for the numbers of *E. coli* and the levels and prevalence of *E. coli* O157 and *Salmonella* in their faeces. This study will determine if particular FWD and transport times lead to an increase or decrease in these microorganisms. FWD and transport times which lead to high counts of *E. coli* and pathogens may compromise the safety of meat produced from such animals and should be avoided. This study will provide information that can be used to guide the industry in providing curfew guidelines that will meet food safety requirements and animal health when combined with B.AWH.0055.

2. Objectives

The effect of 12 or 24h transport and food and water deprivation (FWD) for 12 and 24h prior to transport on the levels of *E. coli* in the faeces of sheep was investigated.

3. Methodology

3.1 Samples

Samples were collected as part of project AHW.055 which investigated the effect of transport duration on indicators of sheep welfare. The animal experiment conducted in AHW.055 was approved by the CSIRO Livestock Industries FD McMaster Laboratories Animal Ethics Committee (Approval No. 06/43). A factorial design was applied to the collection of samples from sheep, which comprised 2 replicates of 3 pre-transport FWD treatments (0, 12 and 24 h of feed and water deprivation) and 2 transport duration treatments (12 and 24 h). A total of 12 groups of sheep were

sampled with 10 sheep from each group sampled after each treatment (**Table 1**). Rectal faecal samples were collected by project staff in AHW.055 at the designated time points corresponding to other data collection (eg. blood samples etc). A fresh glove was used to collect faeces from each animal, which were placed into a specimen jar and stored chilled until processed. When faeces were not present in the rectum of an animal, a cotton tip swab was used to collect material from the rectal area. Swabs were placed in sterile 10ml containers and stored with other faecal samples. Samples were transported under chilled conditions via overnight courier to the Food Science Australia, Cannon Hill laboratories. The maximum time samples were stored in the chiller before processing was 5 days. Storage for this length of time has been shown to have no effect on the numbers of *E. coli* present in sheep faeces (data not shown).

Table 1. Treatment of different groups of sheep and points of faecal collection

	Animal group number											
Treatment	1	7	4	10	2	8	5	11	3	9	6	12
Before treatment	+[a]	+	+	+	+	+	+	+	+	+	+	+
12h FWD					+	+	+	+				
24h FWD									+	+	+	+
12h Transport	+	+			+	+			+	+		
24h Transport			+	+			+	+			+	+
24h Recovery	+	+	+	+	+	+	+	+	+	+	+	+
48h Recovery	+	+	+	+	+	+	+	+	+	+	+	+
72h Recovery	+	+	+	+	+	+	+	+	+	+	+	+

[a] + indicates where faecal samples were collected

3.2 Microbiological testing of samples

Only 10g of faeces was analysed when more than 10g of faeces was collected, when less than 10g was present, all of the faecal material were processed as follows. If less than 0.5g of faeces was present, the sample was treated as a swab. Faeces were diluted 1/10 with buffered peptone water (BPW), mixed using a bag mixer and 3ml was removed and stored for enumeration of pathogens. Swabs were processed by adding 10ml of BPW to the container and vortexed for 1 min to mix. For the purpose of enumeration, swabs were considered to contain 0.1g of faeces.

3.2.1 Enumeration of *E. coli*

Faecal and swab samples were serially diluted in BPW and plated onto Petrifilm™ *E. coli*/Coliform Count Plate (3M). Plates were incubated at 37°C for 24h and enumerated following the manufacturer's instructions.

3.2.2 Enumeration and presence of pathogens

Presence or absence of *E. coli* O157 and *Salmonella* was determined by enriching for 6 h at 42°C followed by testing using Dynabeads anti-*E. coli* O157 and Dynabeads anti-*Salmonella* (Dynal, Oslo, Norway) with Automated Immunomagnetic Separation (AIMS) following previously described protocols (Fegan et al. 2004b; Fegan et al. 2005). Enumeration of *E. coli* O157 and *Salmonella* in samples which tested positive was performed using a Most Probable Number and AIMS protocol (Fegan et al. 2004a; Fegan et al. 2004b).

3.2.3 Prevalence of Shiga toxin genes

DNA templates were prepared from each faecal sample by preparing a boiled cell lysate of the enrichment used for detection of *E. coli* O157 and *Salmonella*. Briefly, 1ml of enriched faeces was centrifuged at 17,000g for 3 min. The supernatant was removed and the pellet was resuspended in 1ml of sterile distilled water (SDW). The samples were centrifuged again (3 min at 17,000g) and the supernatant was removed. The pellet was resuspended in a final volume of 500µl of SDW and held in a heating block at 98°C for 10 min. The sample was mixed gently and centrifuged for 3 min at 17,000g. The supernatant was transferred to a fresh 1.5ml tube and stored at -20°C and used as DNA template in polymerase chain reactions (PCR). The presence of Shiga toxin genes (*stx*) was determined using the PCR protocol of Paton and Paton (Paton and Paton 1998) where only the primers targeting stx_1 or stx_2 were used. Separate PCR reactions were used for detecting each type of *stx*.

3.3 Statistical analysis

All counts were converted to $\log_{10}$ for statistical analysis. Results were analysed using the statistical computer package Minitab (Mintab Inc, PA). Swab samples were treated as if they contained 0.1g of faeces. When *E. coli* could not be enumerated, the number was considered to be one half the limit that could be determined. A one-way ANOVA was used to determine if there were differences in *E. coli* counts after different transport, curfew and recovery times. A two-way ANOVA was used to determine if there was any interaction between the different curfew and transport times on counts of *E. coli*. The Chi-square test was used to determine if the prevalence of Shiga toxin genes in the faeces of sheep differed between treatments. Results were considered significantly different if $p<0.05$.

4. Results

A total of 680 faecal samples were analysed during April and May 2007. *E. coli* was enumerated in all but 3 faecal samples. In two of these faecal samples, *E. coli* were overgrown by coliforms (coliform counts of $\log_{10}$ 5.11 and 7.46 cfu/g). At the lower dilution the plates were too overgrown to provide any counts of either *E. coli* or coliforms. For one sample there were no coliforms or *E. coli* detected on the Petrifilm plates. Of the 680 samples collected from the sheep, faeces were obtained for 619

samples and 61 swabs were taken. There were 34 outliers in relation to counts of *E. coli*, 3 of which were from swabs, therefore the swab samples were included in the statistical analysis.

4.1 Enumeration of *E. coli*

The counts of *E. coli* in the faeces of sheep ranged from $\log_{10}$ 1.7 to 8.61 cfu/g with an overall mean of $\log_{10}$ 6.05 cfu/g (SD 1.07cfu/g). The mean $\log_{10}$ counts of *E. coli* in the faeces of different groups of sheep after different treatments are shown in Figure 1. The mean $\log_{10}$ count of *E. coli* in the faeces of sheep from different groups prior to treatment ranged from 4.93 to 6.86 cfu/g, while after 72h recovery the counts ranged from 5.11 to 6.61 cfu/g. This variability continued throughout the treatments without any clear trends or significant relationships being observed for the effect of FWD or transport times on mean *E. coli* counts. The highest mean $\log_{10}$ count of *E. coli* was found in group 6 sheep after 24h transport ($\log_{10}$ 7.6 cfu/g) and the lowest was from group 12 after 48h recovery ($\log_{10}$ 4.47cfu/g). Both of these groups of sheep were subjected to 24h FWD and 24h transport, indicating that these FWD and transport treatments may cause fluctuations in the mean $\log_{10}$ counts of *E. coli*, but not in a consistent pattern associated with FWD or transport. The two-way ANOVA analysis on 48h recovery versus FWD and transport times indicated a small effect of transport on *E. coli* counts after 48h (p<0.1) and some interaction between the mean $\log_{10}$ counts of *E. coli* after 48h recovery and the period of FWD (p=0.01).

The changes in mean $\log_{10}$ counts of *E. coli* in the faeces of sheep from different treatments are shown in **Figure 2**. The variation in counts was less than a 1 log change either above or below the level prior to any treatment. It appears that in some cases the mean $\log_{10}$ *E. coli* count would increase after a treatment (e.g. groups 6/12 after 24h transport), while the same treatment for a different group would result in a decrease (e.g. groups4/10 after 24h transport). This was the case for mean $\log_{10}$ *E. coli* counts after 48h and 72h recovery and is probably why there was a small interaction observed between 48h recovery and FWD. Mean $\log_{10}$ *E. coli* counts after 24h recovery appeared in most cases to be higher or equal to those before any treatment, however this was not significant.

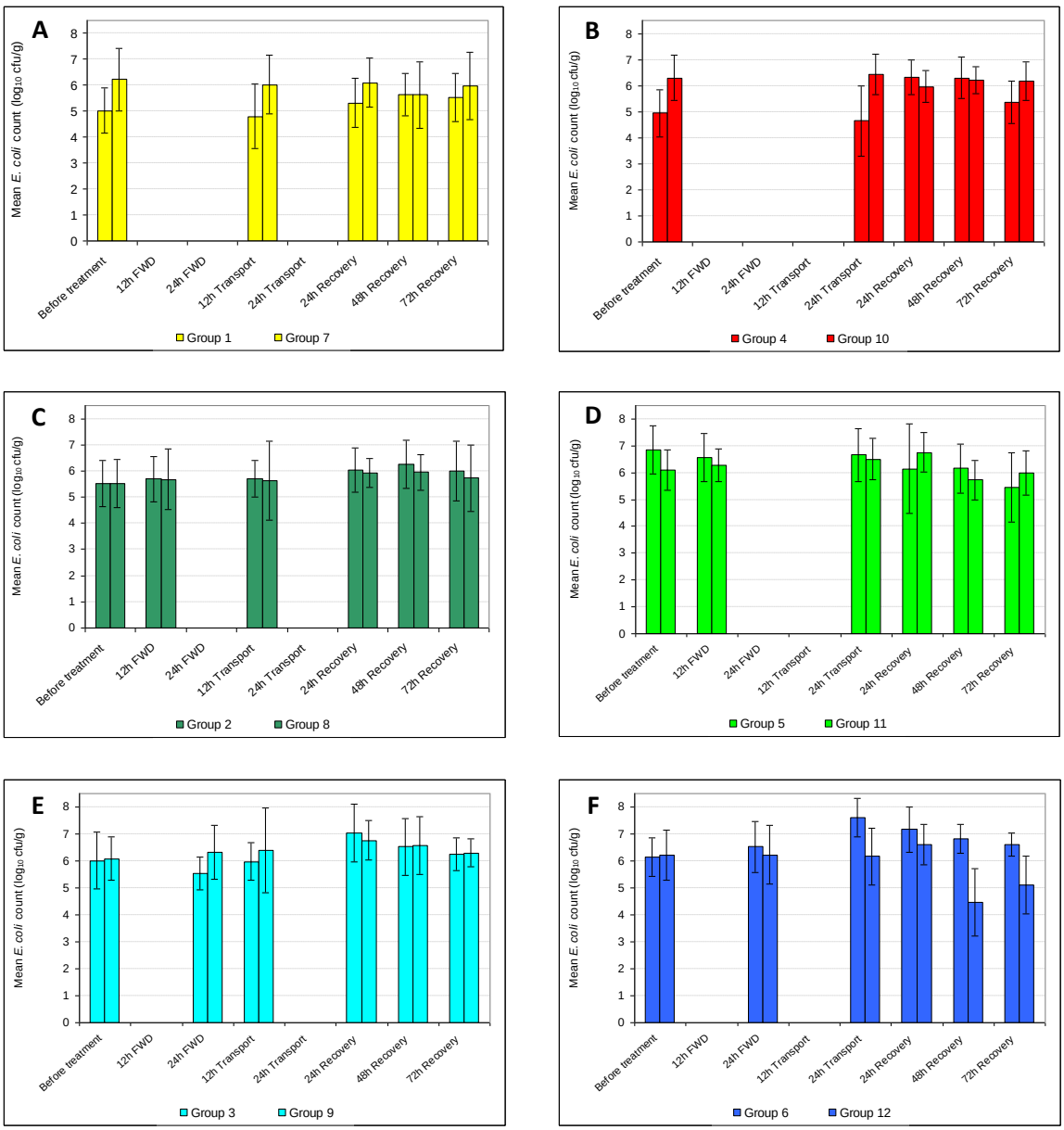

Figure 1. Mean log_{10} *E. coli* counts in the faeces of sheep in different groups after FWD and transport treatments (treatments were performed in duplicate). Mean log_{10} *E. coli* counts in the faeces of sheep which were not treated with FWD but were transported for 12h (A) and 24h (B) , 12h FWD with 12h (C) and 24h (D) transport and 24h FWD with 12h (E) and 24h (F) transport.

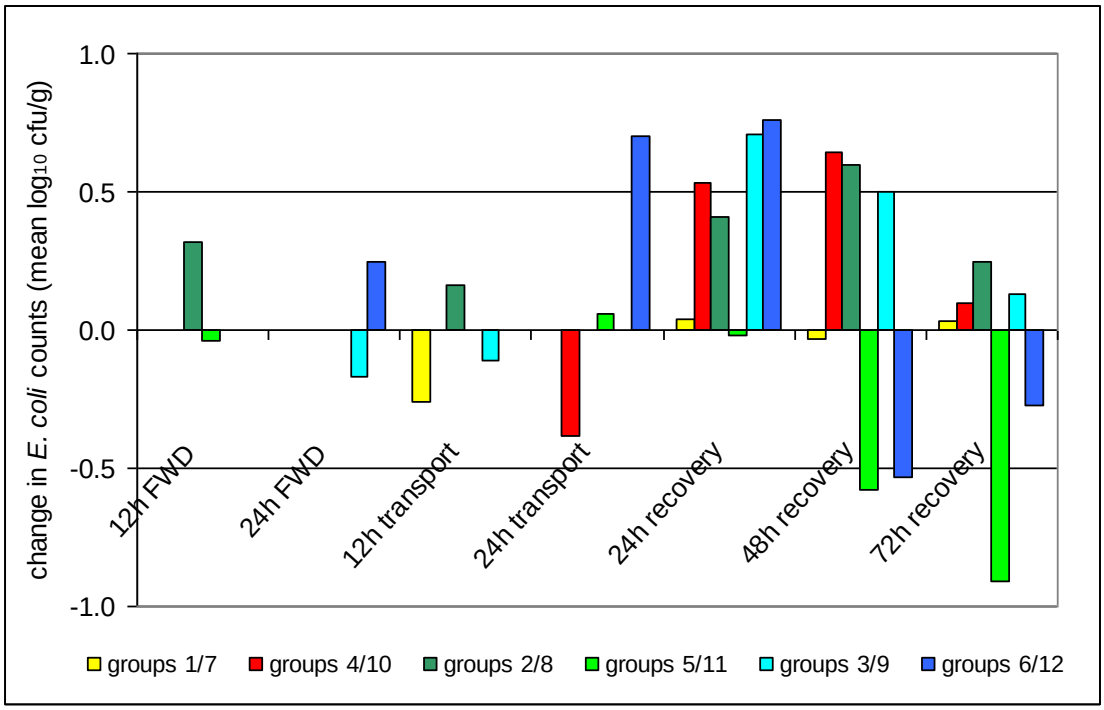

Figure 2. Change in mean log_{10} counts of *E. coli* in the faeces of sheep after different treatments.

Statistical analysis of the data revealed no other significant differences or interactions of FWD or transport times on the numbers of *E. coli* detected in the faeces of sheep. This is clearly demonstrated when all the data from different treatments are combined (**Figure 3**). The mean *E. coli* counts in the faeces of sheep varied from log_{10} 5.76 cfu/g (after 12h transport) to 6.35 cfu/g (after 24h recovery). This was a range of log_{10} 0.59 cfu/g across all treatments from samples collected prior to treatment, after different FWD and transport treatments and after recovery.

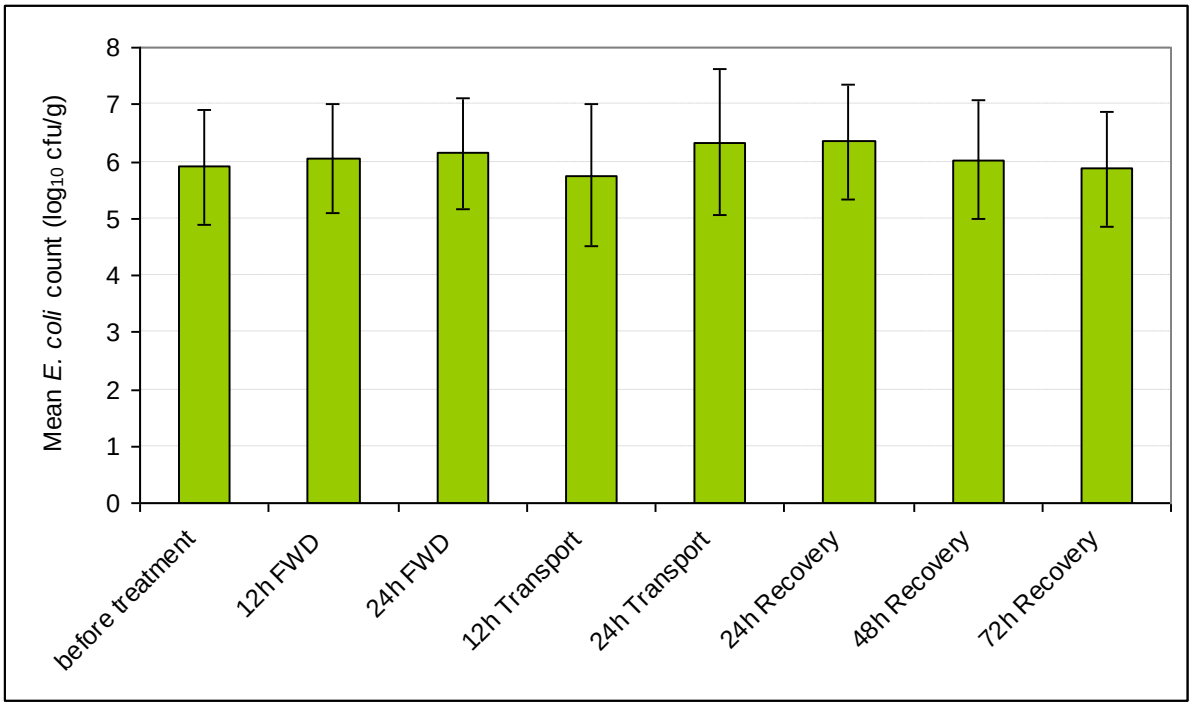

Figure 3. Mean log_{10} *E. coli* counts in the faeces of sheep when combined for each treatment.

4.2 *E. coli* O157 and *Salmonella*

E. coli O157 and *Salmonella* were not detected in any faecal samples with the methods used in this study.

4.3 Prevalence of Shiga toxin genes

Shiga toxin genes were detected in 561 (83%) of 679 sheep faeces tested (one sample was lost and not tested). Overall stx_1 was found in 534 (79%) of samples and stx_2 in 341 (50%). Both *stx* genes were detected in 322 (47%) faecal samples, stx_1 only in 216 (32%) and stx_2 only in 23 (3%). The changes in prevalence of stx_1 and stx_2 in the faeces of sheep after different treatments are shown in **Figure 4** and **Figure 5** respectively. There was only one significant difference found which was for animals treated with 24h FWD and 12h transport, (groups 3 and 9) which had a significantly lower prevalence of stx_1 after 12h transport than at any other time (p<0.05). No other interactions or correlations were found which may indicate this result was an anomaly. The variability of replicates was often high with prevalence ranges of 30% to 90% occurring with a replicate.

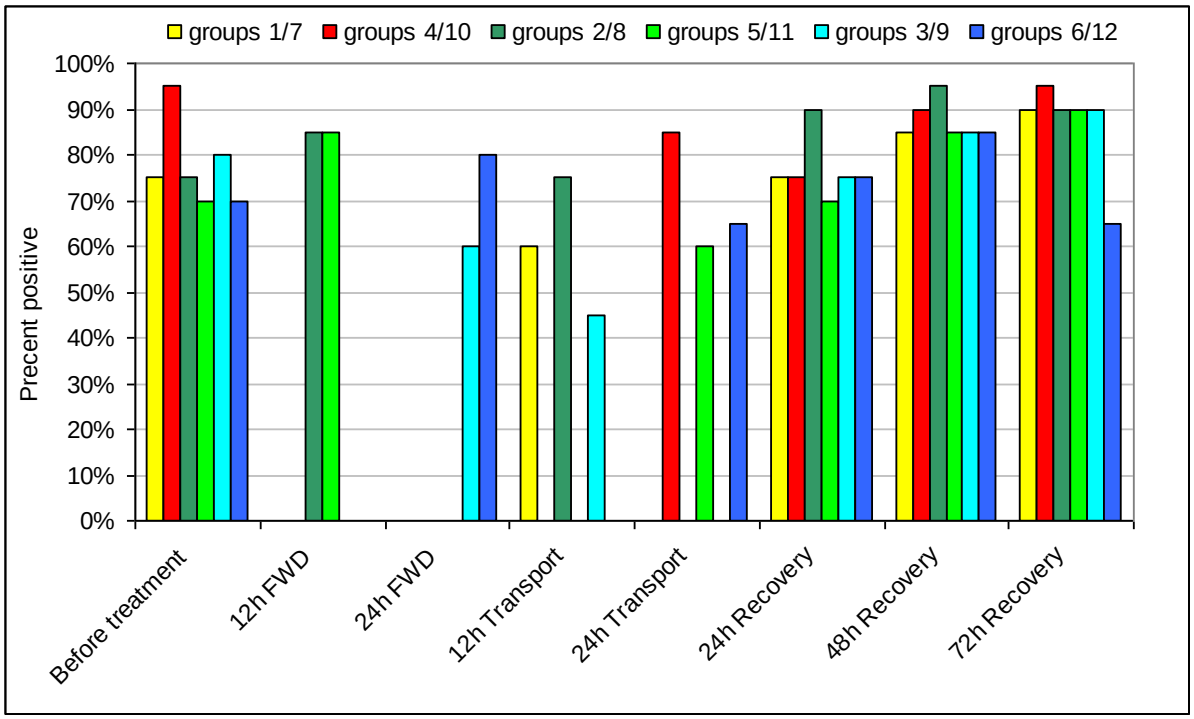

Figure 4. Prevalence of *stx*$_1$ in the faeces of sheep after different treatments. Coloured boxes represent different treatments and the numbers indicate the sheep group.

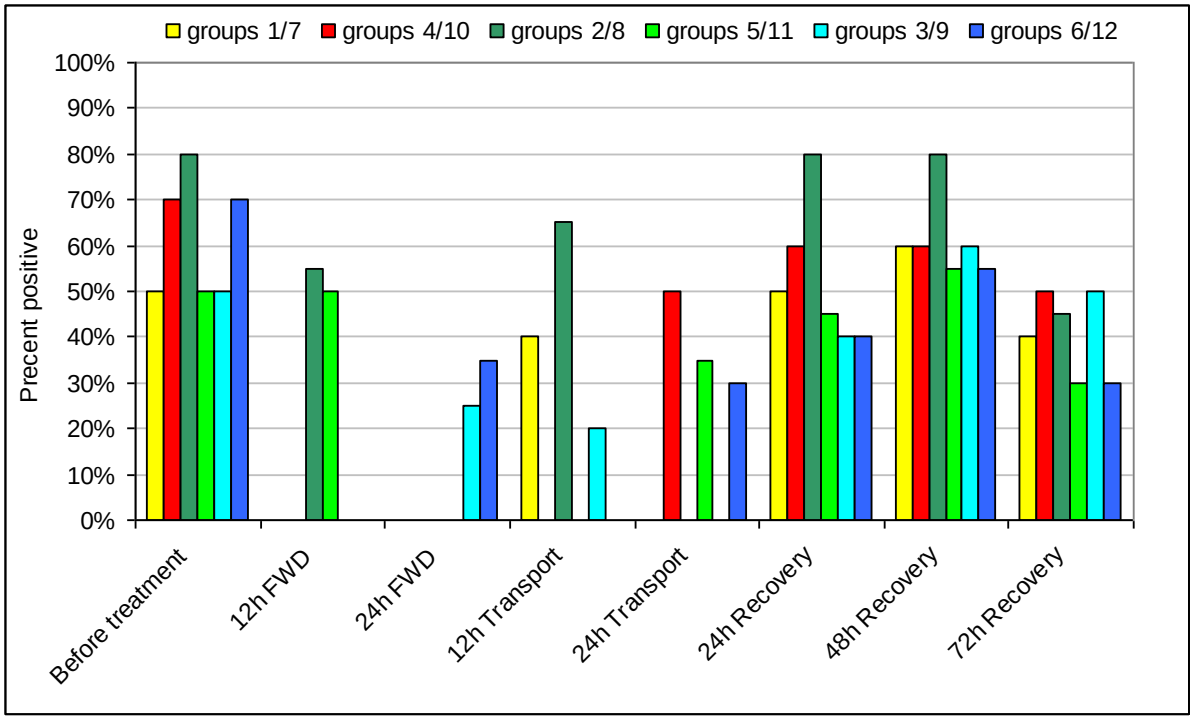

Figure 5. Prevalence of *stx*$_2$ in the faeces of sheep after different treatments. Coloured boxes represent different treatments and the numbers indicate the sheep group.

In general there was a trend for stx_1 prevalence to decrease after 12 and 24h transport and increase after 48 and 72h recovery (**Figure 6**), but these differences were not significant. The prevalence of stx_2 also decreased during FWD and transport treatments to increase again after 24 and 48h curfew (**Figure 6**), but these differences were not significant.

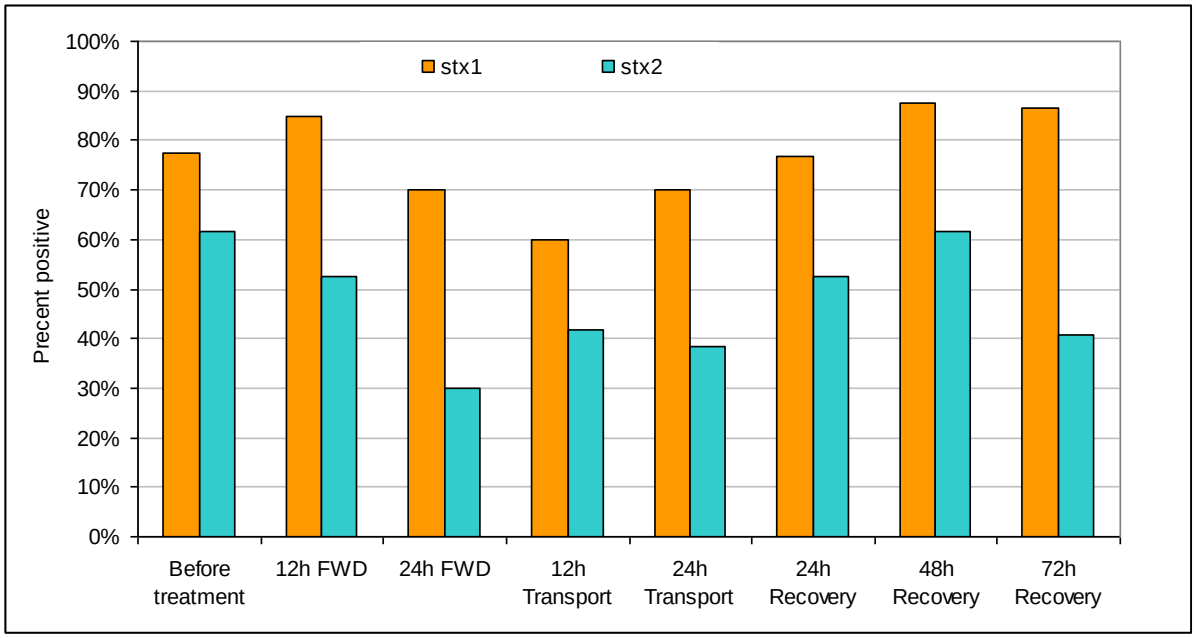

Figure 6. The prevalence of stx_1 or stx_2 or both genes in the faeces of sheep after different treatments

5. Discussion

E. coli counts were determined in 677 of the 680 faecal samples collected. The mean count of *E. coli* in faecal samples was $\log_{10}$ 6.05 cfu/g, with a minimum of 1.7 cfu/g and a maximum of 8.61 cfu/g. These counts are similar to those reported in a recent study where faecal *E. coli* numbers were determined for sheep at slaughter, with a mean count of $\log_{10}$ 6.23 cfu/g, minimum of 3 cfu/g and maximum of 8.81 cfu/g (A.MFS.0060). Mean $\log_{10}$ *E. coli* counts in sheep faeces fluctuated throughout the trial (range 4.47 to 7.6 cfu/g) regardless of treatment as there appeared to be no consistent effect of FWD, transport times or recovery times. Statistical analysis revealed only one significant interaction which was between counts after 48h recovery and FWD. However, this result was not considered to be relevant as it may have been skewed by the results of sheep groups 6 and 12 (e.g. highest mean count of *E. coli* in any group of sheep which occurred after 24h transport and lowest mean count which occurred after 48h recovery).

There were no pathogens detected in the faeces of animals enrolled in this study. The pathogens may have been present in levels below those that could be detected using the methods applied in this study. The prevalence of *E. coli* O157 and *Salmonella* in the faeces of different groups of sheep can be quite variable. *E. coli* O157 tends to be isolated less frequently from sheep than cattle, with sheep isolation rates generally below 10% (Chapman et al. 1997; Chapman et al. 2001; Keen et al. 2006; Zweifel et al. 2006). The prevalence of *Salmonella* also tends to be variable with between 0.1

and 42% of sheep reported positive for this organism (Samuel et al. 1981; Davies et al. 2004; Zweifel et al. 2004; Branham et al. 2005). *E. coli* O157 and *Salmonella* were isolated from the faeces of animals in 2 of 5 groups of sheep tested in a recent study from Australia (A.FMS.0060). The numbers of *E. coli* and *Salmonella* in the faeces of sheep starved for 3 days were found to increase during starvation (feed deprivation), then decrease again on return to normal feeding (Grau et al. 1969). The current study only used 12 and 24h of feed deprivation, along with deprivation and water, and it is possible that deprivation for longer periods may have resulted in significant increases in the *E. coli* populations and possibly the detection of *Salmonella*. However, such long periods of FWD are unlikely to occur in current industry practice.

There was only one significant effect of FWD and transport treatments on the prevalence of *stx* in the faeces of sheep, which was a significantly lower prevalence of stx_1 in the faeces of sheep after 12h transport following a 24h FWD period. The variability between different groups of sheep may indicate this result is an anomaly. In general, the prevalence of both Shiga toxin genes tended to decrease after FWD and transport, but the results were not significant. The majority of faeces contained both stx_1 and stx_2, which is consistent with the populations of STEC obtained from sheep in Australia which tend to carry both stx_1 and stx_2 genes (Fegan and Desmarchelier 1999; Djordjevic et al. 2001).

6. Conclusion

There appears to be little effect of FWD or transport treatments for the times studied in these experiments on the mean $\log_{10}$ counts of *E. coli* in the faeces of sheep indicating that these practices will have little impact on the shedding of *E. coli* in the faeces of sheep. The prevalence of *stx* in the faeces of sheep was not significantly affected by the FWD or transport treatments. The impact of FWD and transport up to 24h is minimal on the numbers of *E. coli* shed and the prevalence of *stx* in the faeces of sheep

6.1 Key findings

- There appears to be little effect of FWD or transport treatments for the times studied in these experiments on the mean $\log_{10}$ counts of *E. coli* in the faeces of sheep
- The prevalence of *stx* in the faeces of sheep was not significantly affected by the FWD or transport treatments

6.2 Benefits to industry

There appears to be no significant effect of 12 or 24h FWD or transport for 12 or 24h on the levels of *E. coli* found in the faeces of sheep. This suggests that applying these FWD and transport times will have little impact on the presence of *E. coli* and *Salmonella* in sheep faeces, and subsequently the risk of contamination of carcases

7. Future research and recommendations

Feed withdrawl for up to 24 hours and transport for up to 24 hours has little impact on the risk of contamination from sheep faeces.

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9. Appendix

The assistance of staff at CSIRO Livestock Industries, Armidale was vital for providing the experimental sampling plan, organising the FWD and transport treatments, ensuring collection of samples from animals for this study.