



# final report

Project code: W.LIV.0163  
Prepared by: F R Murdoch and M Laurence  
Murdoch University  
Date published: September 2014  
ISBN: 9781740362733

PUBLISHED BY  
Meat & Livestock Australia Limited  
Locked Bag 991  
NORTH SYDNEY NSW 2059

## **Antibiotic medication for the treatment of Infectious Ovine Keratoconjunctivitis (IOK) in pre-export feedlots.**

## **The pharmacology and clinical efficacy of in-water and in-feed oxytetracycline.**

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

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## Abstract

Infectious ovine keratoconjunctivitis (IOK) is an ongoing challenge within the live export industry. A number of experiments were conducted to determine the most effective treatment for sheep with differing grades of IOK infection. Treatments that were tested included Oxytetracycline (OTC) delivered in-water, in-feed or by intramuscular injection.

Both in-water and in-feed OTC are absorbed into the bloodstream at detectable levels. In-water OTC at a dose of 22mg/kg was found to cause a significant reduction in both feed and water intake. The reduced water intake resulted in poor intake of the drug and therefore a poor clinical response. In-feed OTC at a dose of 20 mg/kg was an effective treatment for sheep with IOK up to and including grade 3. Feed intakes were reduced, but not to a level below maintenance. Injectable OTC was the most effective treatment when sheep are given 2 injections, 4 days apart at a dose of 20mg/kg. Injectable OTC is effective for all grades of IOK, up to and including 5.

The cheapest and easiest way of treating of sheep with IOK (Grade 3 and below) in pre-embarkation feedlots is with OTC medicated pellets, for at least 5 days.

## Executive summary

Within the live export industry infectious ovine keratoconjunctivitis (IOK) remains an ongoing challenge. With increasing scrutiny on the health and welfare of animals within the industry it is important to address persistent issues. With an estimated 0.5% of sheep rejected from the export trade annually and additional costs related to holding sheep back from shipments for treatment, this work was undertaken to determine a treatment that was effective and easily administered to the numbers of sheep involved. As IOK is a progressive disease it is important to establish the benefits of treating cases at different stages of the disease to ensure the best outcome for the sheep. IOK is a multifactorial disease, in addition to the bacterial agents involved a number of environmental factors play a role in the development of disease. Attempts were made to identify some of these factors with the hope of finding ways to reduce them.

Experimental work focused on treating sheep with oxytetracycline (OTC), a broad spectrum antibiotic which is widely used within the animal health industry. Previous work by Chapman *et al* (2010) highlighted the efficacy of injectable OTC, however given the numbers of sheep involved, the researchers and the industry were keen to find a solution that would be easier to administer on a larger scale. Initial work looked at the efficacy of in-water OTC as this is a relatively easy method of medicating large numbers of sheep; following on from this in-feed OTC was investigated.

In-water OTC was found to cause a significant decrease in both feed and water intake during treatment; this effect did not appear to be dose dependant. Although a drop in feed intake can be acceptable with some treatments, the decreases seen with this treatment were such that sheep would lose weight. The decreased water intake resulted in low intakes of OTC and therefore sheep were receiving a sub-therapeutic dose that was considered unlikely to treat the disease and could potentially lead to the development of antibiotic resistance. Initially it was postulated that this decrease in water intake was due to an unpleasant taste, however work looking at mitigating the taste using a dextrose additive showed no improvement. Studies on the effect of in-water OTC on the rumen microflora indicated a significant change in the bacterial, archael and fungal populations. This, coupled with the decrease in feed intake and the poor water intake, rendered this treatment an unsuitable option for the treatment of IOK in the pre-export feedlot.

A small pilot experiment was carried out to establish the clinical effectiveness of OTC medicated feed and also to determine whether any negative effects on feed and water intake were observed as with in-water OTC. In-feed OTC at a dose of 20 mg/kg daily for 5 days resulted in a significant improvement of clinical IOK. Although feed intakes were reduced during the treatment phase, these reductions were not to the degree seen with in-water OTC and quantities consumed still remained above that required for maintenance.

Following on from the success of the pilot experiments, a large scale feedlot experiment was carried out to compare the two treatments known to be effective: in-feed OTC and two injections of OTC (20mg/kg) 4 days apart. These were given intramuscular into the neck. This large scale experiment included animals with varying grades of IOK to establish cut-offs for when to treat and what treatment to use. Both the injectable OTC and in-feed OTC proved to be effective treatments for sheep with IOK up to and including grade 3 and injectable OTC also resulted in good clinical improvement in sheep with IOK of grade 4 and 5.

Attempts to identify risk factors and to quantify numbers of cases arriving and leaving the feedlot were not successful. Historical data was obtained from one exporter that highlighted a seasonal pattern; cases increase over summer. Reports in the literature would support the increase in cases during the summer months due to the hot, dry, dusty conditions experienced during a Western Australian summer.

W.LIV.0163 - Antibiotic medication for the treatment of Infectious Ovine keratoconjunctivitis (IOK)  
in pre-export feedlots

From this work it can be concluded that IOK is a treatable disease. Sheep in pre-export feedlots with IOK up to and including grade 3 should be treated with in-feed OTC (20mg/kg) for a minimum of 5 days; those with IOK grade 4 or 5 should be treated with 2 injections of OTC (20mg/kg) 4 days apart, the interval between injections may vary with proprietary product used. Given the increase in cases seen during the summer months, inspectors should pay particular attention to eye inspections during these months in order to identify cases early allowing for prompt treatment. As with many diseases, early treatment is beneficial in ensuring a successful outcome and minimising the impact of the disease on animal welfare.

Although this work was focused on sheep within a pre-export feedlot, the researchers believe that these treatment protocols could be used across the industry: commercial lamb feedlots; commercial flocks; potentially onboard livestock vessels. Given the limited access for administering injections to sheep on board vessels, medicated feed would be a suitable option. The limitations of medicating feed onboard will be isolating individual feeders and the ability of the vessel to carry medicated feed separately to non-medicated feed.

At present, the in-feed product used is registered for use in sheep however there is no label claim for the treatment of IOK. Additionally, to the researchers' knowledge there is no export slaughter interval (ESI) established in sheep for this medication. An ESI of 90 days has been established for this product in cattle. Since January 2012, Russia has imposed a 90 day ESI on all OTC products therefore use of this product within 90 days of export to Russia would exclude animals from the Russian market. This, and any concerns regarding withdrawal periods would need to be addressed before blanket use of the in-feed treatment could be recommended.

## Contents

### Table of Contents

## Contents

<b>Executive summary</b> .....	<b>3</b>
<b>1 Background – Section</b> .....	<b>7</b>
<b>2 Project objectives</b> .....	<b>7</b>
<b>3 Determining the efficacy of in-water medication</b> .....	<b>7</b>
<b>3.1 Clinical efficacy of in-water OTC</b> .....	<b>7</b>
3.1.1 Methods	7
3.1.2 Results	10
3.1.3 Discussion	16
<b>3.2 Optimising the in water dose and mitigation of palatability issues</b> <b>18</b>	
3.2.1 Methods	18
3.2.2 Results	18
3.2.3 Discussion	21
<b>3.3 Determining the effect of in-water OTC on rumen health</b> .....	<b>22</b>
3.3.1 Methods	22
3.3.2 Results	24
Bodyweight	26
Feed Intake	26
Water Intake	28
Plasma Beta-hydroxybutyrate (BHB) concentrations	29
Faecal Score	30
Rumen pH	31
Plasma OTC concentrations	32
Rumen fluid parameters	32
3.3.3 Discussion	34
<b>4 Bioavailability of OTC when given in-water or in-feed</b> .....	<b>35</b>

<b>4.1</b>	<b>Methods</b> .....	<b>35</b>
<b>4.2</b>	<b>Results</b> .....	<b>36</b>
<b>4.3</b>	<b>Discussion</b> .....	<b>36</b>
<b>5</b>	<b>Determination of clinical efficacy of in-feed medication ..</b>	<b>37</b>
<b>5.1</b>	<b>Impact of In-Feed medication on feed and water intake.....</b>	<b>37</b>
5.1.1	Methods	37
5.1.2	Results	38
5.1.3	Discussion	39
<b>5.2</b>	<b>Clinical efficacy of in-feed OTC .....</b>	<b>39</b>
5.2.1	Methods	39
5.2.2	Results	41
5.2.3	Discussion	43
<b>6</b>	<b>Determining the clinical effect of in-feed and intra- muscular OTC in a feedlot experiment .....</b>	<b>44</b>
<b>6.1</b>	<b>Methods</b> .....	<b>44</b>
<b>6.2</b>	<b>Results</b> .....	<b>44</b>
<b>6.3</b>	<b>Discussion</b> .....	<b>48</b>
<b>7</b>	<b>Determination of ocular fluid levels of OTC.....</b>	<b>48</b>
<b>7.1</b>	<b>Methods</b> .....	<b>48</b>
<b>7.2</b>	<b>Results</b> .....	<b>48</b>
<b>7.3</b>	<b>Discussion</b> .....	<b>48</b>
<b>8</b>	<b>Determining prevalence and risk factors associated with outbreaks.....</b>	<b>49</b>
<b>8.1</b>	<b>Methods</b> .....	<b>49</b>
<b>8.2</b>	<b>Results</b> .....	<b>49</b>
<b>8.3</b>	<b>Discussion</b> .....	<b>50</b>
<b>9</b>	<b>General Discussion and Conclusion .....</b>	<b>50</b>
<b>10</b>	<b>Conclusions .....</b>	<b>51</b>

## 1 Background – Section

Infectious ovine keratoconjunctivitis (IOK) causes conjunctivitis, ocular discharge and, in severe cases, corneal ulceration in sheep. In cases where corneal oedema and/or ulceration occur, animals are likely to be in pain and have reduced vision, thus compromising welfare.

Currently it is estimated that 0.5% of sheep are rejected from the live export trade due to IOK (G. Robinson, pers. comm.). The disease is infectious and the Australian Standards for the Export of Livestock states that affected animals must be rejected for export until the condition is treated and resolved. This has significant financial implications for the export industry. By identifying an effective and easily administered treatment it is probable that this figure will be reduced. Furthermore, instigating treatment in the early stages of disease will improve recovery rates. Early treatment will limit the number of animals that progress to the more severe stages at which point animal welfare becomes compromised and success of treatment reduced.

Chapman *et al.* (2010) (Project W.LIV.0361) looked at a number of different treatment options in small groups of sheep. This work indicated that two intramuscular injections of oxytetracycline (20mg/kg) (OTC) given four days apart was the most effective treatment but the timing of the treatment may vary depending on the proprietary brand used. Results also indicated that treating IOK with in-water OTC at a dose of 11 mg/kg resulted in clinical improvement of the disease. A key recommendation from this project was to investigate the efficacy of in-water OTC treatments at dose rates (22mg/kg liveweight) suggested by (Davidson, 2009)

Although it is known from previous studies that intra-muscular injection is a very effective treatment, the costs associated with this treatment both in costs of drug, labour and time to administer may limit its use in the feedlots. In-water and in-feed treatments could be a cost-effective way of treating IOK, owing to the reduced cost of the drug, the reduced labour input and the reduced animal handling associated with these routes of administration.

## 2 Project objectives

Project Objectives

1. Determine the efficacy of in-water OTC (22 mg/kg) for the treatment of IOK in sheep.
2. Assess the impact of in-water OTC on rumen health, function and animal welfare.
3. Determine an optimal therapeutic treatment regime for particular grades of IOK severity in a commercial feedlot environment
4. Identify control points that lead to increased incidence of IOK in pre embarkation feedlots.

## 3 Determining the efficacy of in-water medication.

### 3.1 Clinical efficacy of in-water OTC

#### 3.1.1 Methods

Animals

Animal Ethics Committee approval was obtained for this project prior to commencement (R2394/11)

27 merino and merino cross, mixed age and mixed sex sheep were selected from Emmanuel's Peel pre-embarkation feedlot in Mundijong, SW Western Australia. Sheep selected had clinical disease graded 2 (conjunctivitis, epiphora) or 3 (corneal oedema, conjunctivitis, epiphora) on the scale developed by Chapman *et al* 2010.

## W.LIV.0163 - Antibiotic medication for the treatment of Infectious Ovine keratoconjunctivitis (IOK) in pre-export feedlots

Sheep were housed in the Animal House at Murdoch University during the experiment to enable close monitoring and measurement of individual feed and water intake. Sheep were housed in individual raised pens with visual contact on three sides. There was a two-day acclimatisation period prior to the experiment starting. Liveweights were recorded on day 0 and again on day 10.

Sheep were fed a pellet ration during the experiment. These pellets were sourced from pre-embarkation feedlots. Feed and water intake was measured for all three treatments daily. Faecal scores were also recorded. Faecal scores were measured as a visual assessment of faecal consistency using a 1-5 scale, with 1 being firm pellets and 5 being diarrhoea.

### Treatments

Sheep were randomly assigned to one of three treatments (n=9 per treatment):

- Treatment 1 (Control) consisted of the control animals and received no treatment.
- Treatment 2 (IM) was treated with OTC (Alamycin LA 300, Norbrook Laboratories Australia) by intramuscular injection at a dose of 20 mg/kg on day 0 and 4.
- Treatment 3 (Oral) was treated with water soluble OTC (CCD OTC, CCD Animal Health Australia) in the drinking water. Water concentration was calculated to provide a dose of 22 mg/kg OTC for an animal that drank 10% of live-weight per day.

Liveweights of the Oral treatment of sheep were averaged for this calculation.

### Determining Ocular Flora

Ocular swabs were taken from all sheep on day 0, 5 and 10 for bacteriology. A sterile, cotton tipped wood shafted swab was placed into the medial canthus and held between the third eyelid and conjunctiva. Swabs were directly plated onto blood agar plates in the manner for achieving a single culture and the swab tip was then placed in Mycoplasma broth. Samples were submitted to the Animal Health Laboratory at the Department of Agriculture and Food WA in South Perth.



### Clinical Eye Grading

All sheep were closely examined every second day during the experiment to assess the clinical grade of their ocular disease. Grading was done using the eye grading scale developed by Chapman *et al* (2010).



Grade – 0 : Normal eye



Grade 1 : epiphora (weeping eye)



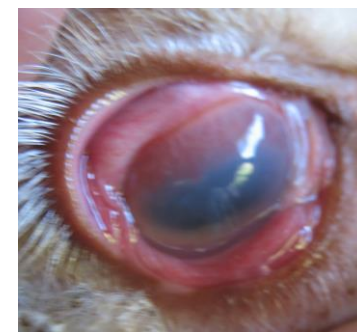
Grade 2 : Conjunctivitis eye)



Grade 3 : Corneal oedema (clouding of the



Grade 4 : corneal ulceration



Grade 5 : neo-vascularisation



Grade 6 : Chronic eye damage

## OTC Pharmacology

Lacrimal fluid samples were collected from sheep in treatments 2 and 3 for analysis of OTC concentrations. A Schirmer tear test strip (Haag-Streit UK) (STT) was placed in the fornix of the eye and held in place for 15 seconds. Strips were then placed in a plain tube and frozen prior to analysis. Samples were taken on days 0, 1, 3, 5, 7 and 10.

Blood samples were taken from sheep in treatments 2 and 3 for analysis of OTC concentrations. A 3 mL sample was taken from the jugular vein using a 20-gauge, 1 inch needle collecting into an EDTA vacutainer tube. Samples were centrifuged for 3 minutes at 4000 rpm and the plasma was transferred into a cryovial tube and stored at -80°C. Samples were taken on days 0, 1, 3, 5, 7 and 10.

Samples were submitted to the Separation Science Laboratory at Murdoch University for analysis. Analysis was done to determine the concentrations of OTC in the plasma samples and the lacrimal fluid.

Tear strips were extracted in methanol, while plasma samples were cleaned up using solid phase extraction (SPE) prior to analysis. Samples were analysed using a specially developed liquid chromatography-tandem mass spectrometry method (LC-MS/MS). The method was developed using OTC solution to determine the appropriate LC conditions and MS/MS transitions. In total, five transitions were monitored to reduce the chance of matrix interference.

## Statistical Analysis

Linear mixed models (LMM) that included treatment effects, covariates and appropriate random effects were used to analyse the data. Covariance structures were defined for random terms as required and simplified where likelihood ratio tests indicated that this was possible. Hierarchical tests (Type I sums of squares) and a 5% level of significance were used to assess whether treatment and covariate effects were significant. When covariates were fitted after treatment effects they explained within-treatment variance; when they were fitted before treatment effects, treatment effects were adjusted for covariance.

### 3.1.2 Results

Predicted treatment means and standard errors (SE) were corrected to mean and covariate values where appropriate.

Animals in the IM treatment received a dose of 20 mg/kg of OTC on days 0 and 4. Those in the Oral treatment received a variable dose over time as a result of variable water intake as shown in Table 1.

	Day 1	Day 2	Day 3	Day 4
Mean mg OTC consumed	662.4	372.0	353.9	254.1
Average dose mg/kg	14.7	8.2	7.8	5.6

Table 1.OTC dose received by the Oral treatment.

Table 2 shows the structure of the models used for the analysis of water and feed intake, and clinical eye score.

<b>Linear models and P-values: Experiment 1</b>				
	<b>Water Intake</b>	<b>Feed Intake</b>	<b>Pink eye score</b>	<b>Plasma OTC</b>
<b>Fixed term</b>	<b>P-Value</b>	<b>P-Value</b>	<b>P-Value</b>	<b>P-Value</b>
Liveweight	<b>&lt;0.001</b>	<b>&lt;0.001</b>	0.095	<b>&lt;0.001</b>
Treatment	<b>0.008</b>	0.162	0.136	<b>&lt;0.001</b>
OTC Dose		<b>0.001</b>	<b>&lt;0.001</b>	0.062
Day	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>0.006</b>	0.077
Day.Treatment	<b>0.002</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	0.275

Table 2: Linear models and P-values for the analysis of water intake, feed intake, clinical eye score and plasma OTC concentration after treatments.

The live-weight range was 30.5 kg to 57.5 kg (mean 44.15 kg) at the beginning of the experiment and 33.5 kg to 67.5 kg (mean 46.25 kg) at the end of the experiment.

Feed intake was significantly affected by liveweight (p value <0.001), data was corrected for liveweight and a significant difference in feed intake was seen between the treatments on different days (see Figure 1).

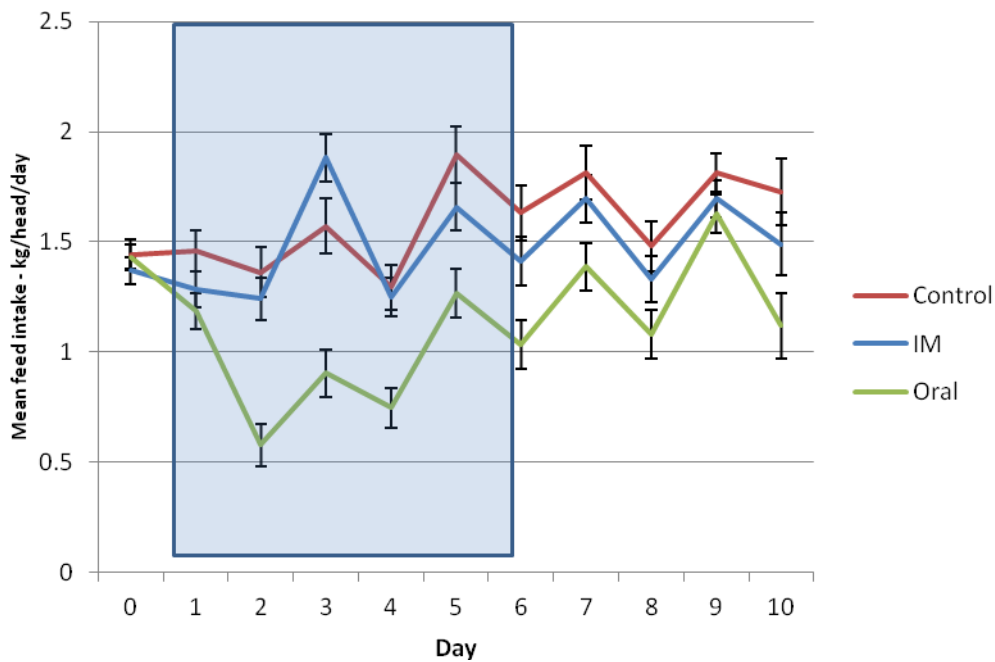


Figure 1. Mean feed intake (kg/head/day) over time. Shaded area represents the duration of Oral treatment. Error bars represent standard errors.

W.LIV.0163 - Antibiotic medication for the treatment of Infectious Ovine keratoconjunctivitis (IOK)  
in pre-export feedlots

<b>Feed intake – kg/head/day</b>			
<b>Day</b>	<b>Control</b>	<b>IM</b>	<b>Oral</b>
0	1.442	1.37	1.429
1	1.458a	1.285	1.185b
2	1.361a	1.241a	0.579b
3	1.572a	1.882a	0.903b
4	1.295a	1.25a	0.746b
5	1.896a	1.659a	1.268b
6	1.632a	1.414	1.033b
7	1.812a	1.697	1.387b
8	1.48	1.33	1.081
9	1.815	1.695	1.627
10	1.728a	1.49	1.12b

Table 3. Mean feed intake (kg/head/day) for treatments on different days. Within Day, means with different letters differ significantly ( $P < 0.05$ ).

Feed intake was reduced in the Oral treatment during the treatment period but returned to levels on a par with the other treatments following cessation of treatment. but returned to maintenance levels following cessation of treatment.

Feed intake was significantly lower in the Oral treatment compared to the Control treatment on days 1, 2, 3, 4, 5, 6, 7 and 10 (Table 4). Feed intake was significantly lower in the Oral treatment compared to the IM treatment on day 2, 3, 4 and 5 (Table 3). There was no significant difference in feed intakes between the Control and the IM treatments on any of the days (Table 3).

Water intake was significantly affected by live-weight ( $P < 0.001$ ). There was a significant difference in water intake between the treatments and on different days ( $p = 0.002$ ), Figure 2.

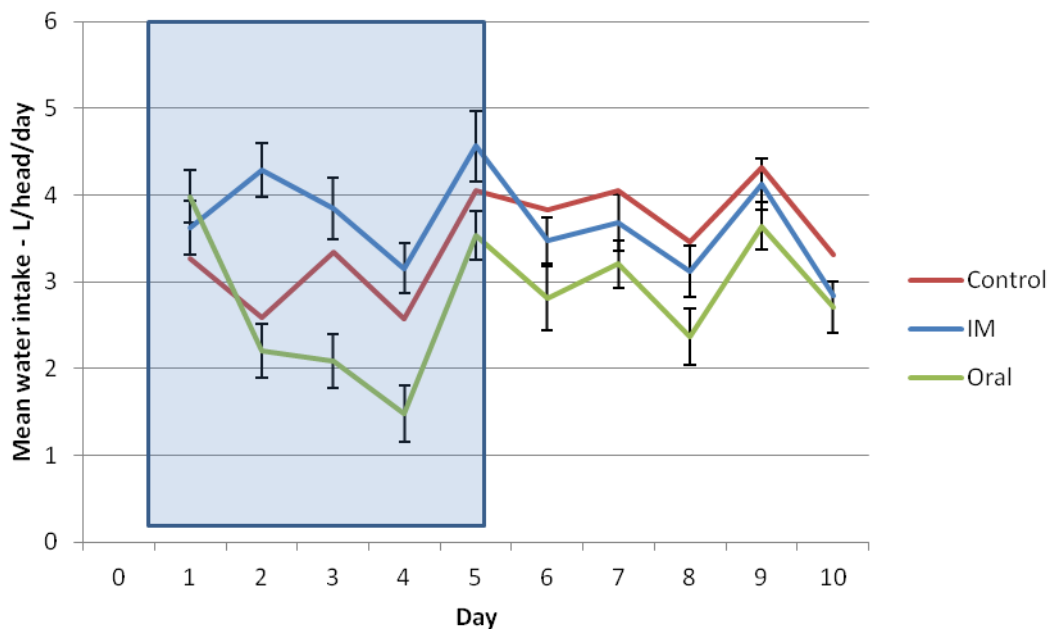


Figure 2. Mean water intake (L/head/day) over time. Shaded area represents the duration of oral treatment. Error bars represent standard errors.

<b>Water intake – L/head/day</b>			
<b>Day</b>	<b>Control</b>	<b>IM</b>	<b>Oral</b>
0			
1	3.273	3.623	3.981
2	2.586a	4.289b	2.203a
3	3.336a	3.845a	2.092b
4	2.577	3.158a	1.481b
5	4.053a	4.567a	3.537b
6	3.836	3.475	2.814
7	4.046	3.678	3.204
8	3.461a	3.123	2.37b
9	4.324	4.126	3.645
10	3.308	2.845	2.703

Table 4. Mean water intake (L/head/day) for treatments on different days. Within Day, means with different letters differ significantly ( $P < 0.05$ ).

Water intake in the Oral treatment was significantly lower than the Control or IM treatment during the in-water treatment period. Following cessation of in-water treatment, water intake was on the whole no different to the other treatments (day 8 being the exception). Water intake in the Oral treatment returned to pre-treatment levels following cessation of treatment. Day 8 is the exception where intake of the Oral treatment reduced for one day before returning to similar levels to the other groups for the remainder of the monitoring period.

The oral treatment drank significantly less than the IM treatment on days 2, 3, 4 and 5 and drank significantly more than the Control treatment on day 2 (Table 4). The Oral treatment drank significantly less water than the Control treatment on day 3, 5 and 8 (Table 4).

Clinical eye grades were seen to change over the course of the experiment, Figure 3. A significant difference was seen between the different treatments on different days throughout the experiment ( $p < 0.001$ ).

W.LIV.0163 - Antibiotic medication for the treatment of Infectious Ovine keratoconjunctivitis (IOK) in pre-export feedlots

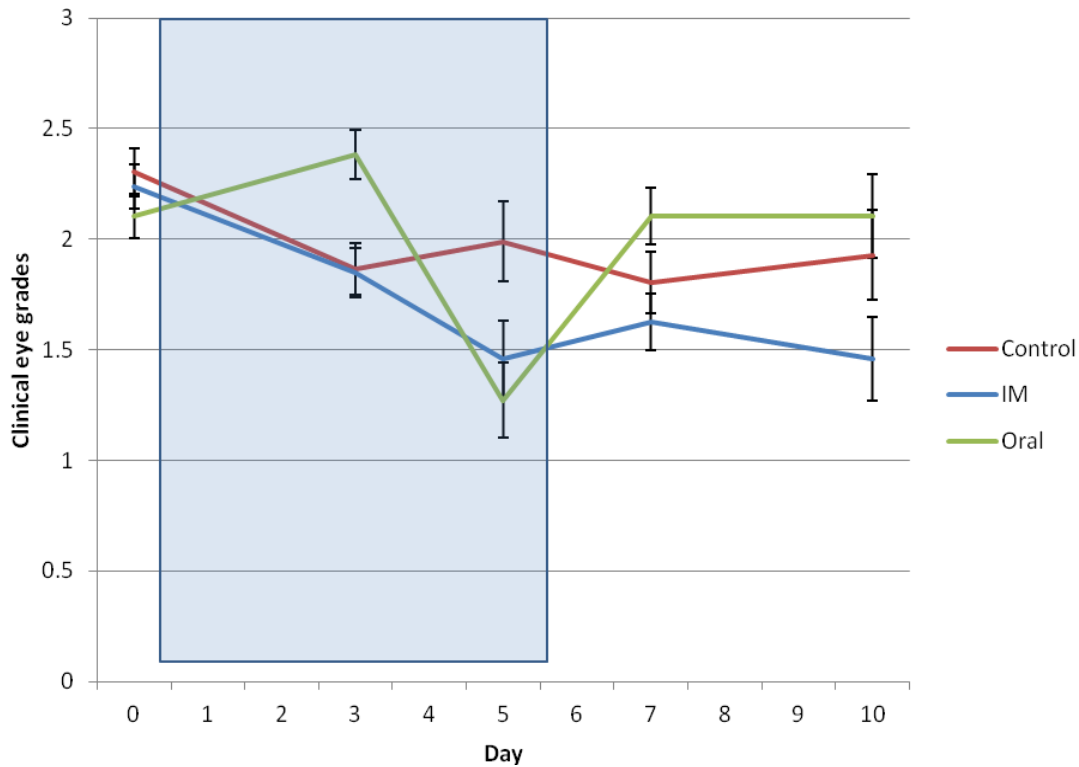


Figure 3: Mean clinical eye grade (average both eyes) over time. Shaded area represents the duration of Oral treatment. Error bars represent standard errors.

Clinical eye grades did not differ at the end compared to the start for both the Control and the Oral treatments. There was a significant improvement at the end of the experiment in clinical eye grade in the IM treatment (Table 5). After initially worsening, eye grades of the Oral treatment group made a marked improvement on day 5, this improvement however was not sustained and clinical eye grades in this group returned to levels similar to those at the start of treatment.

<b>Clinical Eye Grades</b>			
<b>Day</b>	<b>Control</b>	<b>IM</b>	<b>Oral</b>
0	2.303	2.238	2.104
1			
2			
3	1.865a	1.849a	2.382b
4			
5	1.99a	1.46	1.271b
6			
7	1.803	1.626a	2.106b
8			
9			
10	1.928	1.46a	2.104b

Table 5 .Mean clinical eye grade (average of both eyes) for treatments on different days. Within Day, means with different letters differ significantly (P<0.05).

Plasma OTC concentrations (measured on day 1 and then day 5 of the treatment) differed between Oral and IM treatments (P <0.001) but were not significantly different over the course of the treatment period. The IM treatment resulted in a significantly higher mean plasma OTC concentration than the Oral treatment (see Figure 4). Plasma OTC concentrations measured on day 1 and day 5 of treatment differed between the Oral and IM treatments. Serial measurements taken between these two points over the treatment period were not significantly different between the groups. Looking at mean plasma OTC concentrations over the treatment period, IM treatment was significantly higher than Oral treatment (Figure 4).

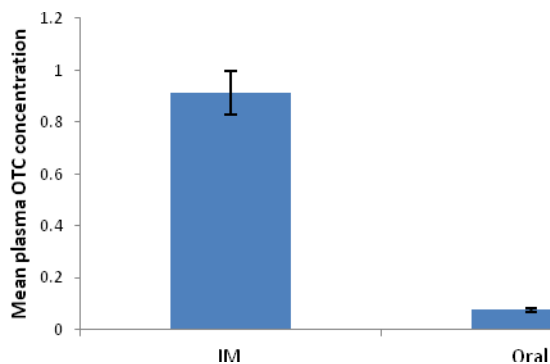


Figure 4: Mean plasma OTC concentration during treatment period for the Oral and the IM treatment groups

### 3.1.3 Discussion

As expected, liveweight was a significant covariate in the analysis of feed and water intake, whereby heavier animals ate and drank more. As such all analyses were corrected for liveweight.

Feed intake decreased during the in-water treatment period in the Oral treatment. This seemed to coincide with a decrease in water intake in the same animals during this time. It is hypothesised



W.LIV.0163 - Antibiotic medication for the treatment of Infectious Ovine keratoconjunctivitis (IOK) in pre-export feedlots

that the decrease in feed intake is a consequence of the reduced water intake. All animals were fed a pellet based ration which was dry. As the water intake decreased in these animals the hydration state would also decrease resulting in a decreased palatability of a dry feed and a decreased appetite.

It is also hypothesised that the decrease in water intake during the in-water treatment period in this treatment was an effect of the palatability of the in-water OTC. It is suggested that the concentration of soluble OTC was high enough to cause the taste to limit the water intake. Supporting this is the fact that after the in-water treatment period ended, both feed and water intake increased to be no different from the IM or control treatments. The return of water intake to levels equivalent to those in the other treatments once in-water treatment finished implies that medicated water was relatively unpalatable. Both feed and water intakes were seen to reduce following the commencement of treatment in the Oral treatment, it is thought this is related to an unpleasant taste inferred on the water by the OTC powder. Following cessation of treatment, feed and water intakes did increase. Although these new levels fluctuated over the monitoring period, the trend was towards levels similar to those prior to treatment.

An improvement in the clinical eye score was only seen in the IM treatment. In contrast to Chapman *et al.* (2010) there was no improvement in the Oral treatment. no overall improvement despite some initial improvement. It is suggested that the unpalatable taste of the OTC dissolved in water at a concentration that would deliver a dose of 22mg/kg if an animal drank 10% of its liveweight, caused the animals to drink too little water to deliver an effective dose of OTC. The benefits of giving IM medication over medication in the water or feed is that a known concentration is given, whereas in-water or in-feed doses are very variable depending on intake. However, optimising the in-water concentration such that animals received an effective dose would mean that in a feedlot, in-water treatment would be far less labour intensive than giving injections.

Changes in clinical appearance can be attributed to the difference in OTC dose. In the Oral treatment the highest average dose is 14 mg/kg on a single day. It is difficult to compare this directly with the intramuscular dose of 20 mg/kg which is a long acting preparation. Shorter acting preparations, those designed to last 24 hours, are given daily at a dose of 8 mg/kg (Engemycin Intervet/Schering-Plough Animal Health Australia). Sheep in the Oral treatment received an effective dose orally only on days 1 and 2 therefore the remainder of the course was at sub-therapeutic levels.

The oral bio-availability of OTC in sheep has not been reported in the literature. Castro *et al* studied the oral bioavailability of doxycycline in sheep (Castro *et al.*, 2009). Doxycycline is a modification of the OTC drug molecule with a high lipophilicity and therefore an increased distribution and tissue penetration. The oral bioavailability of doxycycline was found to be around 35% which is relatively low. The low bioavailability was attributed to the loss of drug in the forestomachs of the ruminant. It is interesting to note that this study found plasma concentrations of drug to be similar to those seen in pre-ruminants and monogastrics, chickens and pigs. This concurs with the results of this experiment which show that OTC is absorbed into the bloodstream following both IM and oral administration but plasma concentrations are higher after the IM route of administration. This is not an effect of dose because *OTC dose* was not a significant covariate in the model ( $P = 0.062$ , see Table 3), hence the hypothesis that the difference is due to the different bioavailability of the drug from the different routes of administration.

Unfortunately, analysis of the lacrimal fluid has failed to isolate OTC to date. No analysis of the ocular flora has been done at this stage but the results of this analysis are not relevant in the absence of any difference in clinical response to the different treatments.

### 3.2 Optimising the in water dose and mitigation of palatability issues

#### 3.2.1 Methods

Thirty, adult merino cross ewes were randomly selected for this experiment from the Murdoch University teaching flock. Animals were housed in a purpose built animal house in individual pens with sheep contact on at least one side.

The sheep were randomly assigned to 5 treatments (n=6, see Table 6). All sheep were weighed on entry to the animal house and again on leaving the animal house. A two-day acclimatisation period was allowed prior to commencing measurements to allow the sheep to adapt to the pellet ration and the individual pens. All sheep were fed a maintenance pellet ration during this period.

The concentrations of the water were calculated such that a 50kg sheep drinking 4 litres per day would consume the dose stated. Treatments started after 2 days of baseline measurements and lasted for 5 days. Following this there were a further 5 days of monitoring. Measurements of feed and water intake were taken daily.

Treatment	Dose rate and water additive
1	2% dextrose
2	2% dextrose and 11 mg/kg OTC
3	2% dextrose and 16.5 mg/kg OTC
4	11mg/kg OTC
5	16.5 mg/kg OTC

Table 6. Treatments in experiment to optimise dose and palatability

#### Statistical analysis

For each animal all pre-treatment feed and water intakes were averaged over the acclimation period and change from the pre-treatment average was calculated for each post-treatment date. A linear mixed model was fitted to the calculated changes on all post-treatment dates. The model included fixed effects for pre-treatment liveweight, treatment, post-treatment date and treatment by date interaction; and random effects for animal and the animal by date interaction. An autoregressive model allowed for correlations between measurements made on the same animal on different dates and different residual variance on each date. 5% LSD's were calculated to compare treatment means to zero, i.e. whether treatment caused a change from the pre-treatment mean, and to compare treatments.

#### 3.2.2 Results

The addition of dextrose made no difference to feed or water intake, with or without the addition of OTC (feed intake  $P = 0.099$ , water intake  $P = 0.154$ , see Table 7).

Adding OTC to water at a dose rate of 16 mg/kg and 11mg/kg caused both feed and water intake to fall significantly after treatment began (feed intake  $P = 0.003$ , water intake  $P = 0.007$ , see Table 7 and Figure 5). Water intake fell more when at the 6 mg/kg dose rate compared to the 11mg/kg dose rate ( $P = 0.051$ , see Table 7 and Figure 6).

Over time, particularly after OTC treatment ended, feed and water intake recovered to near the level of the no OTC treatment.

Total plasma OTC concentrations per day were no different for the 11mg/kg treatment compared to the 16 mg/kg dose rates (see Figure 7).

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in pre-export feedlots

<b>Term</b>	<b>Change in feed intake</b>	<b>Change in water intake</b>
weight	0.846	0.323
OTC (with or without OTC)	<b>0.003</b>	<b>0.007</b>
OTC.dextrose (OTC with or without dextrose)	0.099	0.154
OTC dose (11 or 16mg/kg)	0.951	<b>0.051</b>
OTC.dextrose.dose (11 or 16mg/kg ± dextrose)	0.626	0.437
Time	<b>&lt;0.001</b>	<b>&lt;0.001</b>
time.OTC	<b>0.004</b>	<b>0.005</b>

Table 7: Significance levels (P-Values) for terms in model with respect to change in feed or change in water intake.

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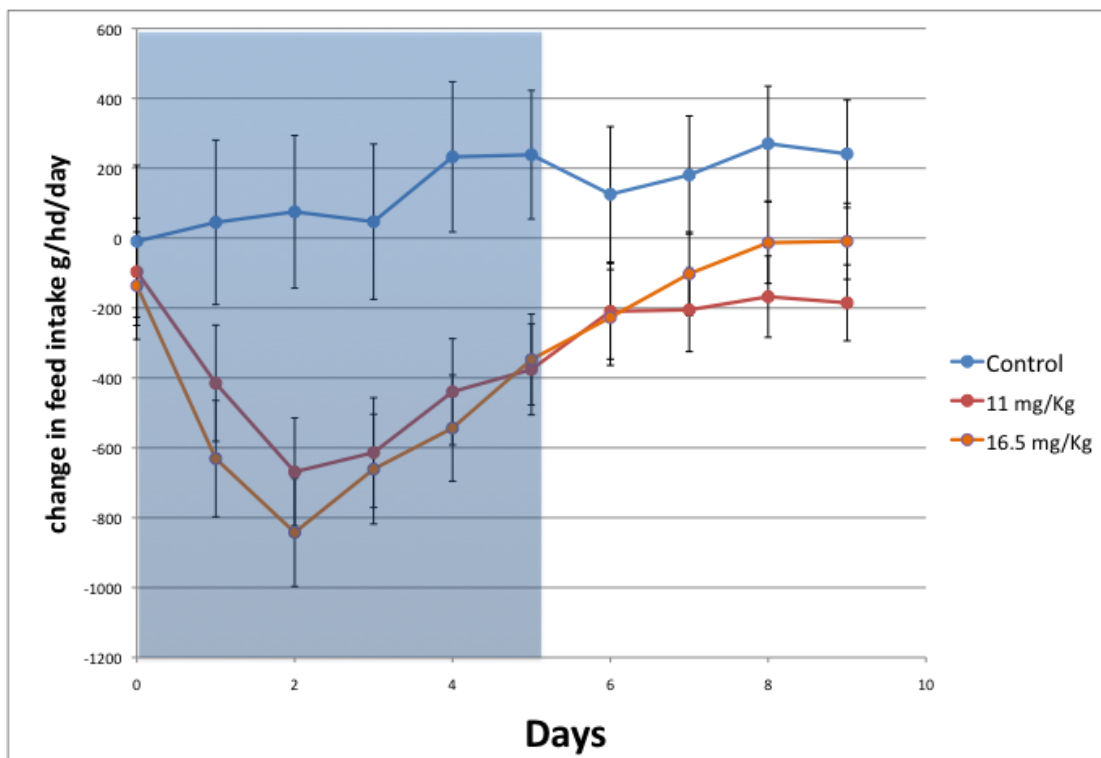


Figure 5: change in feed intake (g/head/day) over time. Shaded box represents treatment period Error bars represent standard errors.

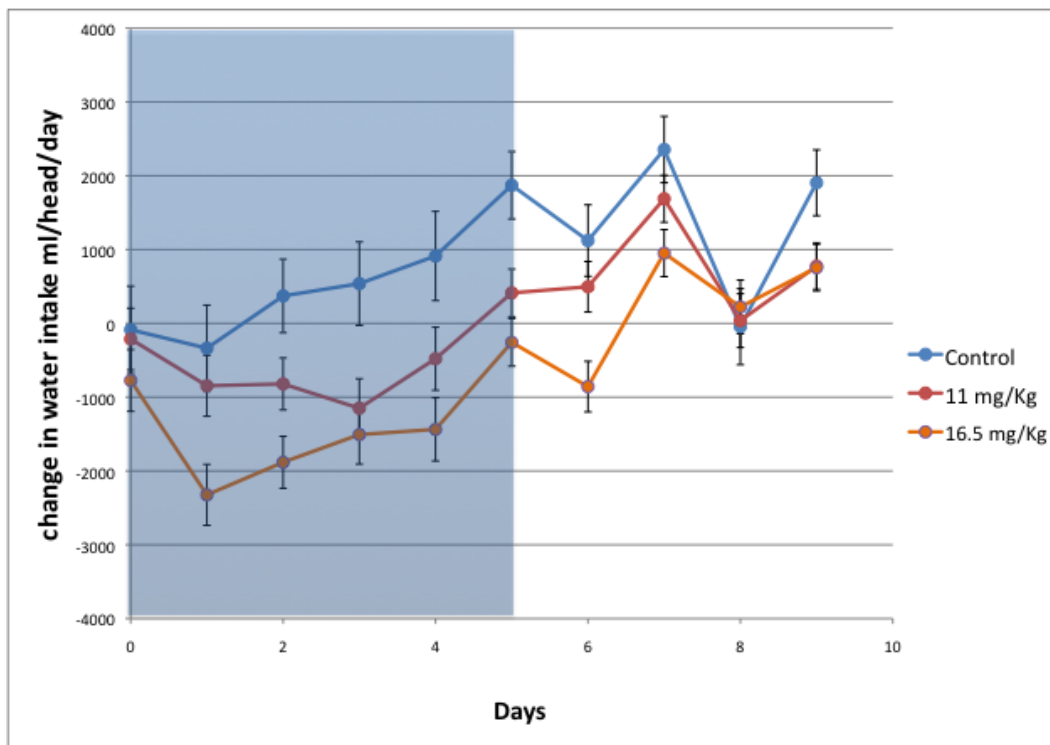


Figure 6: Change in water intake (ml/head/day) over time. Shaded box represents treatment period. Error bars represent standard errors.

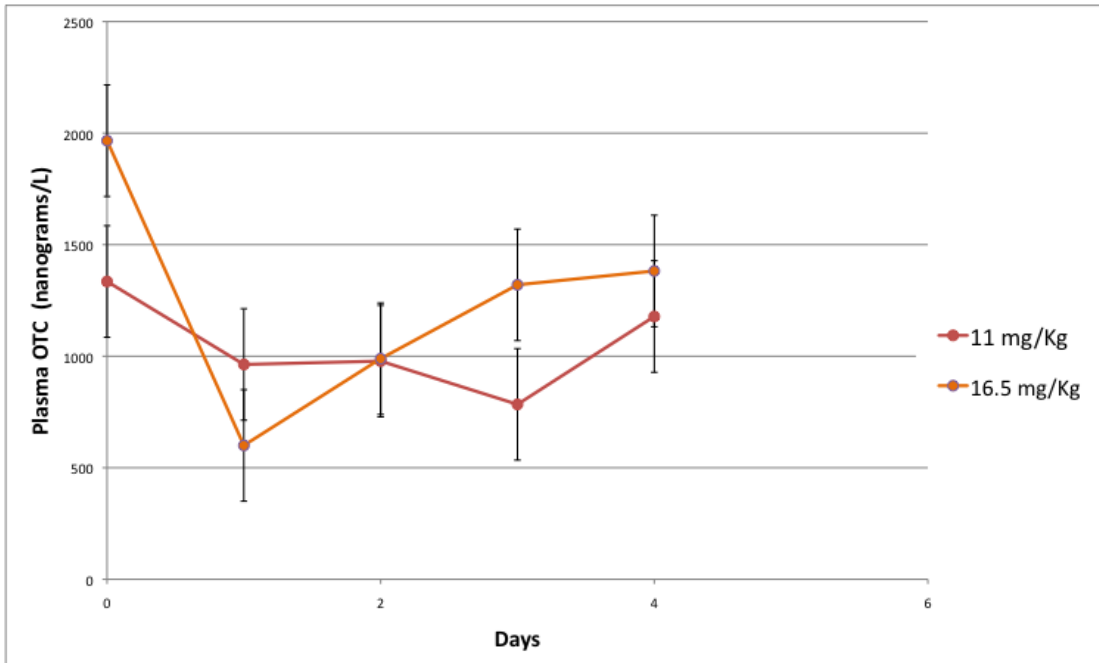


Figure 7: Plasma OTC concentration (based on Experiment 1) for different doses of OTC during treatment period.

### 3.2.3 Discussion

Following results from the previous experiment, it was decided to try and mitigate the presumed negative palatability effects of including OTC IW. Dextrose was chosen as an additive as anecdotal evidence exists of its use in the poultry industry as a supplement in medicated water to improve water intake. Dextrose is readily soluble IW and has a sweet taste. It is not fully understood if this is a taste that would be attractive to sheep. Lower doses were selected to include in the trial with the hypothesis that if sheep liked the taste they would consume more water with the potential of ingesting a therapeutic dose.

The results showed that at two differing dose rates of IW OTC, dextrose had no effect on feed or water intake (which both fell during the 5 day treatment period as expected and consistent with previous experimental results).

The addition of IW OTC reduced feed and water intake to different levels. To determine the impact on plasma OTC concentrations the results for Experiment one were used to produce the graph in Figure 5. This showed that there was no difference in plasma OTC concentrations at differing dose rates of OTC suggesting there is no optimal dose to add to water. The higher the dose, the less the sheep drink and the lower the effective plasma concentration.

### **3.3 Determining the effect of in-water OTC on rumen health.**

#### **3.3.1 Methods**

Fourteen, mixed age wethers with permanent rumen fistulas in place were used in this experiment. The sheep had previously been fistulated using a standard two-stage process. The fistula was located in the left paralumbar fossa and allowed direct access to the rumen. All sheep were housed in a purpose-built animal house at CSIRO Floreat, WA. Sheep were housed in individual pens and contact between sheep through pens was allowed. Sheep were housed one week prior to commencement of the experimental period for adaptation to pelleted feed. Sheep were weighed and body conditions were measured by the same operator on entry to the animal house. This was also done once during the experiment and again at the end of the experiment. The sheep were split into two treatments and to balance the treatments each group consisted of three older and three younger wethers. Individual feed and water intake and faecal scores were measured on a daily basis throughout the experiment. Faecal scores were measured as a visual assessment of faecal consistency using a 1-5 scale, with 1 being firm pellets and 5 being diarrhoea.

Alternative feedstuffs (chaff) were offered to sheep to mitigate the decrease in feed intake when it occurred during the experiment.

#### **Treatments**

Both Treatments were treated with water soluble OTC for 5 days. Treatment 1 was given 22mg/kg liveweight of OTC and Treatment 2 were given 11mg/kg liveweight OTC. OTC powder (CCD OTC, CCD Animal Health, Geelong VIC, Australia) was diluted in water and administered via the rumen fistula each day for 5 days. 22mg/kg oral OTC has been suggested as the effective concentration for the treatment of IOK in sheep (Davidson 2009). 11mg/kg was also chosen such that, were there to be a severe impact on rumen health at the 22mg/kg dose, it would be possible to assess the health impact of a half-dose and possibly develop recommendations based on these results

#### **Rumen fluid samples**

Rumen fluid samples were taken on days -5, -3, -1, 0, 1, 2, 3, 4, 5, 6, 7, 9, 11, 14, 16 and 18. On day 0 a rumen fluid sample was taken immediately prior to administration of medication and a follow up sample was taken 1 hour after medication. Rumen fluid samples were taken by inserting a rigid plastic tube through the fistula and removing fluid by capillary action. Samples were placed on ice after collection. Samples taken prior to commencement of treatment were used as controls.

Rumen pH was measured immediately using an electronic pH meter which was calibrated daily. Rumen fluid samples were frozen and analysed later for Volatile Fatty Acid (VFA) and Ammonia concentrations. Separate samples were taken immediately prior to drug administration on day 0 and on day 5. These were frozen at -80°C and sent for microbial profiling.

#### **Rumen Microbial profiling**

The microbial profiling methodology used to investigate rumen bacterial, archaeal, fungal and protozoan communities was based on the terminal restriction fragment length polymorphism (T-RFLP) technique. T-RFLP is a culture independent technique for profiling microbial communities based on differences at the nucleic acid or genome level. T-RFLP has been widely used to investigate gut bacterial communities within poultry. This tool has being used to investigate changes in gut bacterial communities associated with dietary modification, such as addition of feed enzymes, prebiotics, organic acids and antimicrobials, as a means of developing alternatives to in-

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feed antibiotics for the poultry industry. (Geier et al., 2010; Geier et al., 2009; Torok et al., 2011; Torok et al., 2008a) The advantage of the technique is that it is high-throughput, high resolution and capable of providing a “snap shot” of the entire microbial community at any particular time. Hence, it is an ideal initial screening tool that requires no prior knowledge of the actual microorganism present within the community. Where significant treatment differences are detected then other molecular techniques can be used to identify organisms involved. This method was deemed appropriate for the purposes of this experiment, namely to assess the impact of oral OTC on rumen microflora.

## DNA Analysis

Total nucleic acid, including that of the representative microbial population, was extracted from 24 freeze-dried rumen samples by a modified South Australian Research & Development Institute (SARDI) proprietary method (Torok et al., 2008b). 12 samples were from sheep prior to oxytetracycline treatment and acted as controls. The bacterial ribosomal DNA from the extracted material was amplified with universal 16S bacterial primers, one of which was labelled with the fluorescent dye (Torok et al., 2008b). Archaeal, fungal and protozoan communities were also amplified using universal group specific primers (Torok et al., manuscript in preparation). The resulting amplicons were restricted with a specific recognition sequence restriction enzymes and electrophoretically separated on a capillary DNA sequencer (ABI 3730, Applied Biosystems). Data were analysed using GeneMapper (Applied Biosystems) to determine positions of terminal restriction fragments (TRF).

## Limitations of the rumen profiling technique

T-RFLP analysis alone does not assign a microbial classification to operational taxonomic units (OTU). However, other techniques can be employed to isolate and sequence OTU of interest or to pyrotag sequence the entire microbial community from the same nucleic acid template as used for T-RFLP analysis.

## Blood samples

Blood samples were collected from the jugular vein using a 20 gauge, 1” needle and a vacutainer tube, plain tube for OTC concentrations and lithium heparin for BHB analysis. Blood samples were centrifuged at 2000 rpm for 15 minutes and the plasma titrated off. Plasma was frozen and analysed at a later date. Blood samples collected on days -3, 0, 1, 2, 3, 4, 5, 7, 9, 11, 14, 16 and 18 were analysed for BHB concentrations. Blood samples were also collected on days 0, 1, 2, 3, 4, 5, 7, 9 and 11 for measurement of plasma OTC concentrations.

## Statistical Analysis

For all data except those associated with rumen microbe profiling the following analysis was carried out. For each animal all pre-treatment values were averaged and change from the pre-treatment average was calculated for each post-treatment date. A linear mixed model was fitted to the calculated changes on all post-treatment dates. The model included fixed effects for Treatment (11 vs 22), post-treatment date and their interaction; and random effects for animal and the animal by date interaction. An autoregressive model allowed for correlations between measurements made on the same animal on different dates. 5% LSD's were calculated to compare Treatment means to zero, i.e. whether treatment caused a change from the pre-treatment mean, and to compare the two Treatments. For pellet and total ME intake the analysis has also been performed with water intake included in the fixed model, i.e. as a covariate.

For the rumen profiling analysis, data points from GeneMapper analysis were validated and outputs generated for statistical analysis using queries within a custom built database (Torok et al., 2008b). Queries in the database were used to compare duplicate T-RFLP profiles and identify synonymous fragment sizes ( $\pm 2$  bp). The resulting fragments were treated as OTU, representing particular microbial species or taxonomically related groups. OTU obtained from the rumen of the 24 rumen samples were analysed using multivariate statistical techniques (PRIMER 6, PRIMER-E Ltd., Plymouth, UK). These analyses were used to examine similarities in sheep rumen microbial communities and to identify OTU accounting for the differences observed in microbial communities (Torok et al., 2008b). Bray-Curtis measures of similarity (Bray and Curtis, 1957a) were calculated to examine similarities between rumen microbial communities from the T-RFLP generated (OTU) data matrices, following standardization and fourth root transformation. The Bray-Curtis similarity co-efficient (Bray and Curtis, 1957b) is a reliable measure for biological data on community structure and is not affected by joint absences that are commonly found in microbial data (Clarke, 1993). Analysis of similarity (ANOSIM) (Clarke, 1993) was used to test if rumen microbial communities were significantly different between treatments. The R-statistic value describes the extent of similarity between each pair in the ANOSIM analysis, with values close to unity indicating that the two groups are entirely separate and a zero value indicating that there is no difference between the groups.

Similarity percentages (SIMPER) (Clarke, 1993) analyses were done to determine which OTU contributed most to the dissimilarity between treatments. SIMPER identifies individual species (OTU) contributing to the overall dissimilarity between treatments. The overall average dissimilarity ( $\delta_{\square}$ ) between microbial communities of ruminants on two treatments shown to significantly differ were calculated and the average contribution of the  $i$ th OTU ( $\delta_i$ ) to the overall dissimilarity determined. Average abundance ( $\bar{y}$ ) of important OTU in each of the groups were determined. OTU contributing significantly to the dissimilarity between treatments were calculated ( $\delta_i/SD(\delta_i) > 1$ ). Percent contribution of individual OTU ( $\delta_i\%$ ) and cumulative percent contribution ( $\sum \delta_i\%$ ) to the top 65% of average dissimilarities were also calculated.

Unconstrained ordinations were done to graphically illustrate relationships between treatments using non-metric multidimensional scaling (nMDS) (Kruskal, 1964; Shepard, 1962). nMDS ordinations attempt to place all samples in an arbitrary two-dimensional space such that their relative distances apart match the corresponding pair-wise similarities. Hence, the closer two samples are in the ordination the more similar are their overall microbial communities. "Stress" values (Kruskal's formula 1) reflect difficulty involved in compressing the sample relationship into the 2-D ordination.

### 3.3.2 Results

In all cases for each animal all pre-treatment values were averaged and change from the pre-treatment average was calculated for each post-treatment date. Table 8 shows the level of significance for all the measurements taken.

	<b>Intake - kilo's of feed</b>	<b>Intake - MJ ME</b>	<b>Water intake</b>	<b>Faecal score</b>	<b>rumen pH</b>	<b>Plasma BHB</b>	<b>Plasma OTC</b>
<b>Term</b>	<b>P-value</b>	<b>P-value</b>	<b>P-value</b>	<b>P-value</b>	<b>P-value</b>	<b>P-value</b>	<b>P-value</b>
Time	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Water	<0.001	<0.001					
Treatment	0.646	0.745	0.670	0.948	0.184	0.265	0.154



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in pre-export feedlots

Time.Treatment	0.455	0.164	0.019	0.858	0.140	0.122	0.258
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Table 8: Significance table for measurements in determining effect of OTC on rumen health experiment.

### Bodyweight

The mean bodyweight of Treatment 1 (11mg/kg OTC) was 72.7 kg prior to commencement of treatment compared to 65.3kg for Treatment 2 (22 mg/kg OTC). The wethers were of varying ages and sizes, attempts were made to put equal numbers of old and young animals in each group to minimise overall differences in weight. In both groups, bodyweight decreased up to day 3 before increasing again. The mean weight loss in both Treatments was 2.8 kg. There was no significant difference between the Treatments.

### Feed Intake

Feed intake reduced over time following commencement of treatment. It returned to pre-treatment levels following cessation of treatment (Figure 8). There was no significant difference between the two Treatment groups. When water was fitted as a co-variant it was highly significant ( $p < 0.001$ ), suggesting a significant association between feed and water intake.

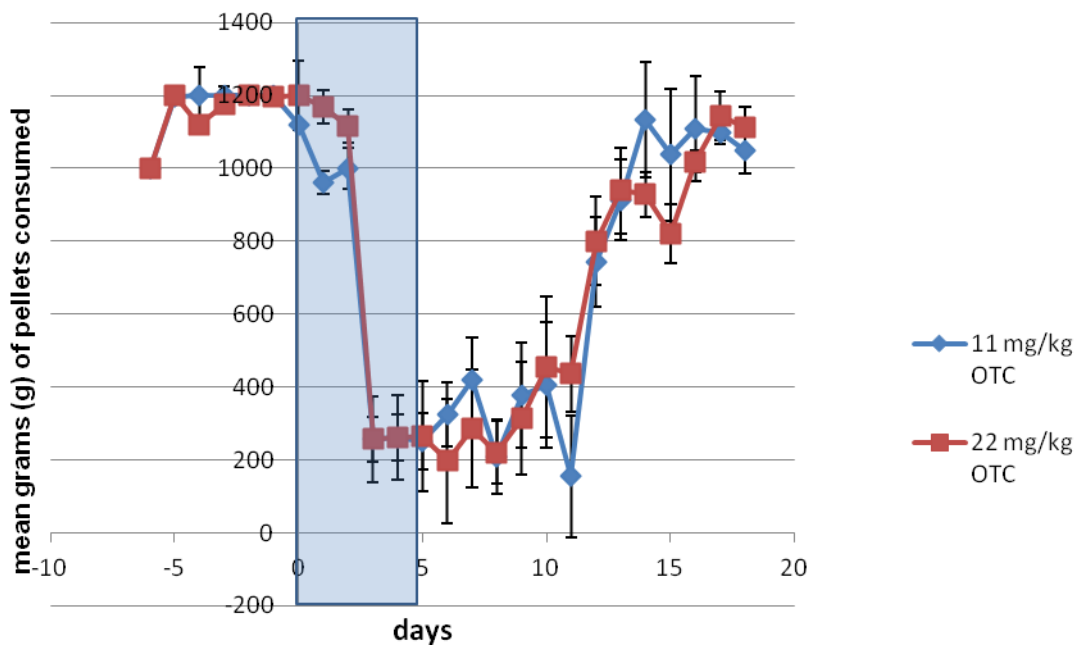


Figure 8: Graph of mean feed intake over time for Treatments (11mg/kg and 22mg/kg oral OTC). Shaded box represents OTC treatment period. Error bars represent standard errors.

### Metabolisable energy

The total mega-joules (MJ) of metabolisable energy (ME) were calculated (Figure 9) to account for the addition of chaff when intake dropped significantly. As with feed intake, energy intake decreased following start of treatment and recovered after treatment finished. It appeared that sheep preferentially ate chaff over pellets during the treatment period. As with pellet intake, there was no significant difference between the Treatments over time.

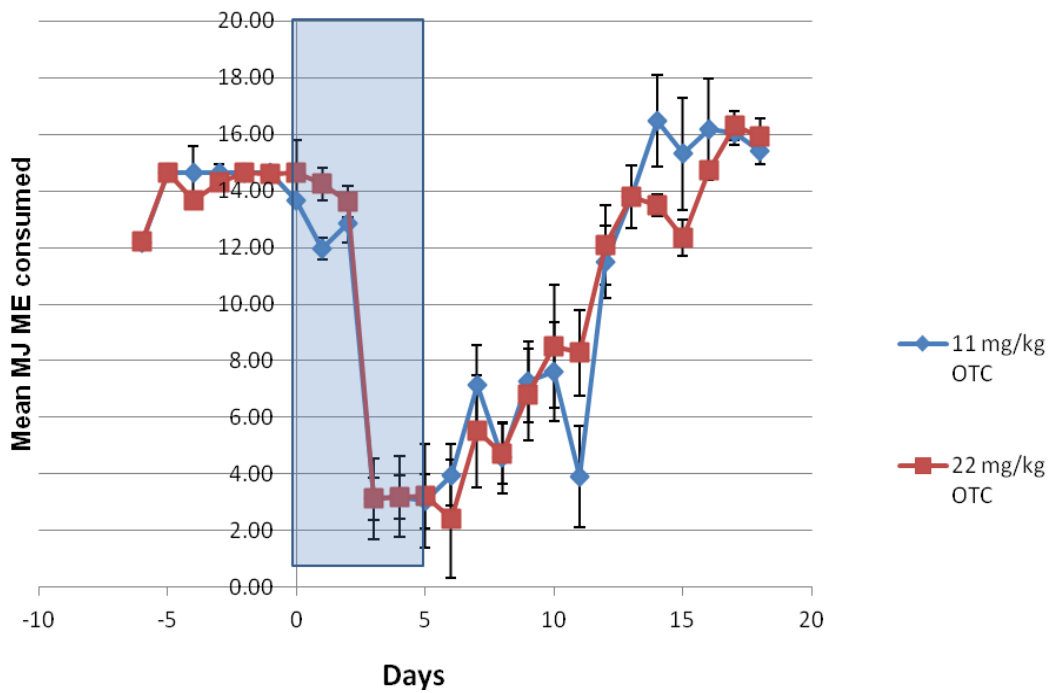


Figure 9: Graph of mean ME intake over time for Treatments (11mg/kg and 22mg/kg oral OTC). Shaded box represents OTC treatment period. Error bars represent standard errors.

### Water Intake

Mean water intake fell significantly following the start of treatment (Figure 10). There was a significant difference in mean water intake over time and also a significant difference between the Treatments on days 4, 10, 14 and 15.

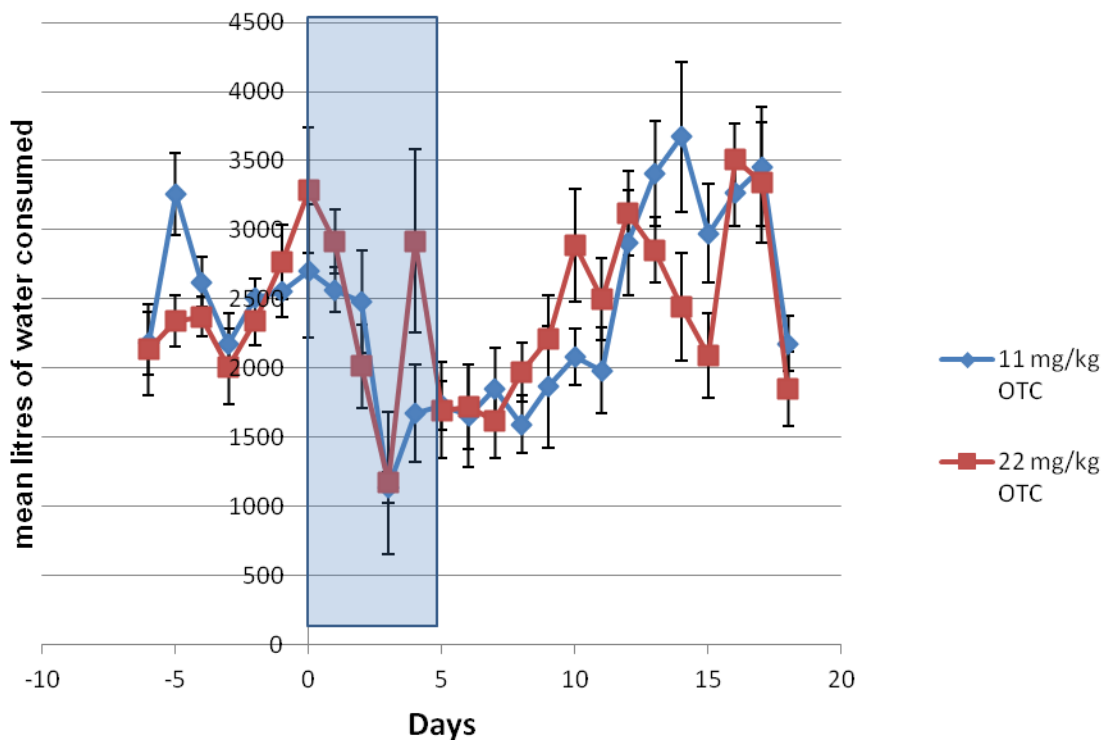


Figure 10: Graph of mean water intake over time for Treatments (11mg/kg and 22mg/kg oral OTC). Shaded box represents OTC treatment period. Error bars represent standard errors.

### Plasma Beta-hydroxybutyrate (BHB) concentrations

Mean BHB concentrations rose significantly following commencement of treatment. Concentrations peaked at day 8 and returned to pre-treatment on approximately day 13 (Figure 11). There was no difference between Treatments over time.

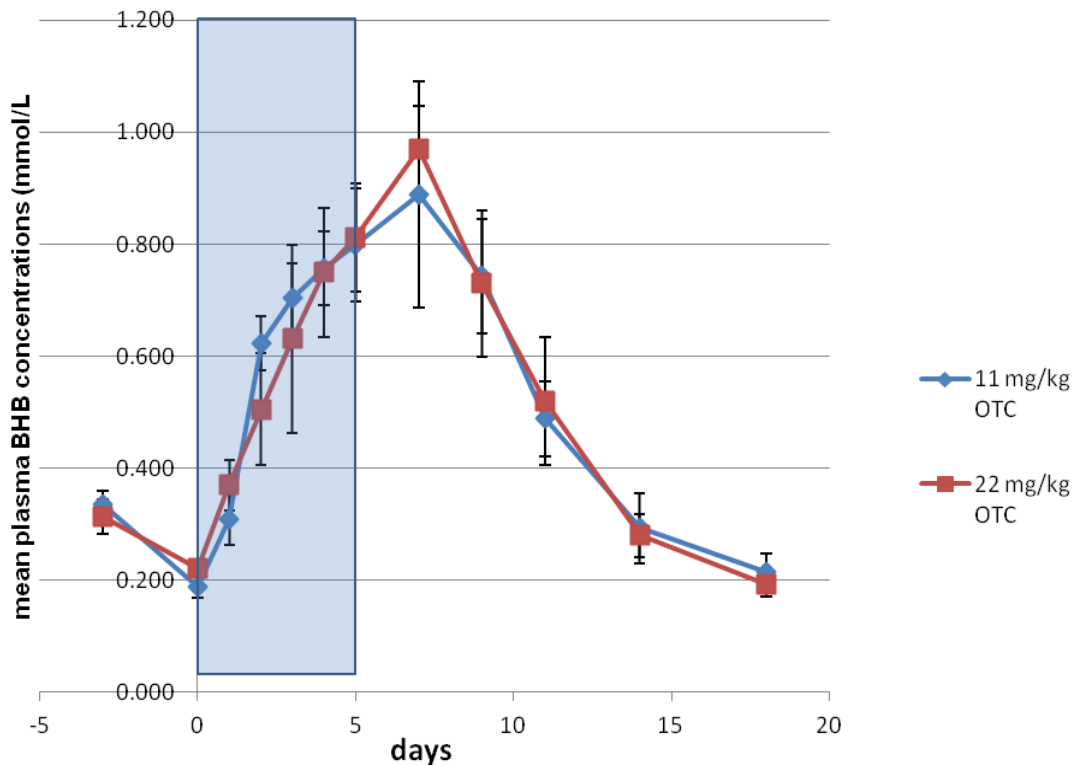


Figure 11: Graph of mean plasma BHB concentrations over time for Treatments (11mg/kg and 22mg/kg oral OTC). Shaded box represents OTC treatment period. Error bars represent standard errors.

### Faecal Score

Faecal score changed significantly over time and increased significantly after the start of OTC treatment (Figure 12). There was no significant difference between the Treatments over time.

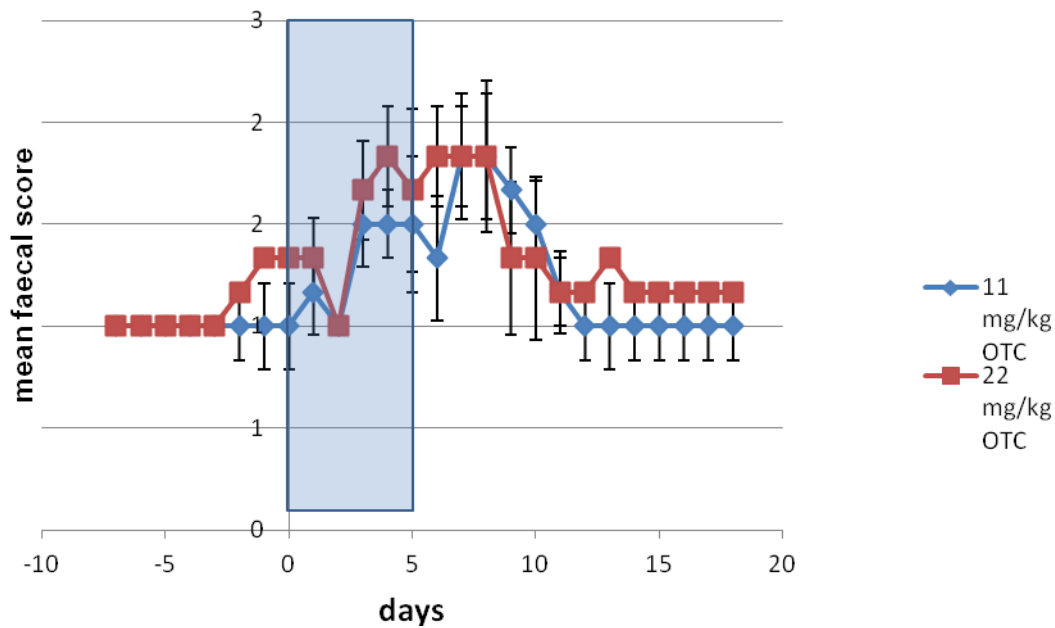


Figure 12: Graph of mean faecal score over time for Treatments (11mg/kg and 22mg/kg oral OTC). Shaded box represents OTC treatment period. Error bars represent standard errors.

### Rumen pH

Rumen pH changed significantly over time and increased significantly after the start of OTC treatment (Figure 13). There was no difference between the Treatments over time.

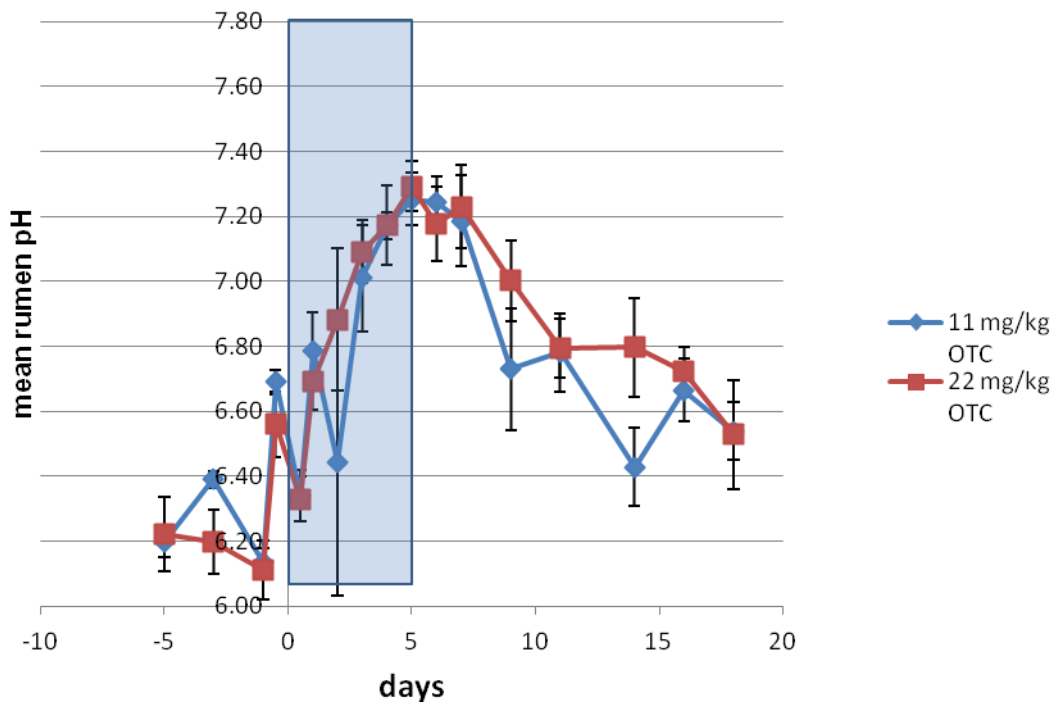


Figure 13: Graph of mean rumen pH over time for Treatments (11mg/kg and 22mg/kg oral OTC). Shaded box represents OTC treatment period. Error bars represent standard errors.

### Plasma OTC concentrations

OTC was detected in the blood of all animals during OTC treatment period. Concentrations peaked at day 5 and low levels were still detected on day 9, 4 days after treatment ceased (Figure 14). There was no difference between Treatments over time.

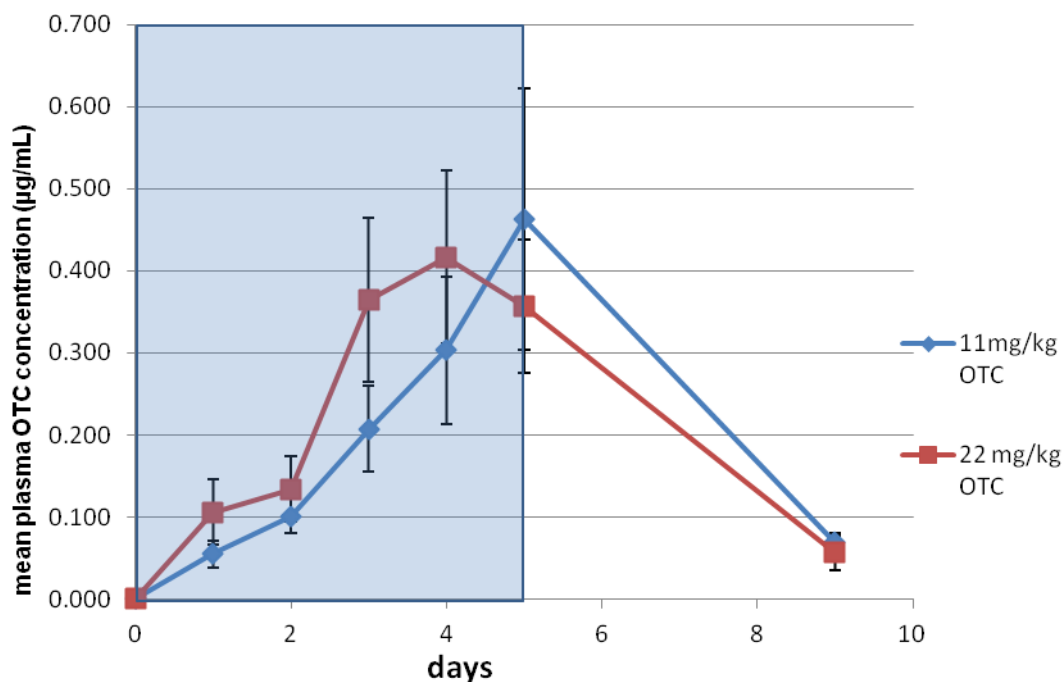


Figure 14: Graph of mean plasma OTC over time for Treatments (11mg/kg and 22mg/kg oral OTC). Shaded box represents OTC treatment period. Error bars represent standard errors.

### Rumen fluid parameters

There was a significant effect of OTC treatment on rumen bacterial, archaeal and fungal populations, however no effect was seen on protozoal populations. What is evident from these depictions is that the rumen microbiota are variable among animals on the same treatment and that there is a need for robust statistical analysis when investigating treatment differences. Despite this inter-animal variability, a subjective judgement can be made for the bacterial, archaeal and fungal communities in that the rumen communities of animals pre-treatment compared to after OTC treatment starts. This difference relates to both presence/absence of unique OTU, as well as, shifts in abundance of common OTU. The number of OTU shared among the animals post-treatment is reduced compared with the number of OTU observed pre-treatment, indicating a reduced biodiversity in micro-organisms within the rumen after OTC treatment starts.



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Multivariate statistical analysis was used to investigate differences in rumen bacterial, archaeal, fungal and protozoan communities associated with OTC treatment. Significant differences ( $P=0.001$ ) associated with OTC treatment were detected in the rumen bacterial, archaeal and fungal communities (Table 9). OTC treatment did not influence the rumen protozoan communities (Table 9). Both Treatments altered the rumen bacterial, archaeal and fungal communities when compared with pre-treatment populations. However, it was only within the bacterial communities that there was also a significant difference associated with OTC dose (Table 9). The influence of OTC dose on rumen bacterial communities is shown in Figure 15.

<b>Bacteria (R=0.703, P=0.001)</b>			
	<b>pre-treatment</b>	<b>11 OTC<sup>a</sup></b>	<b>22 OTC<sup>b</sup></b>
<b>pre-treatment</b>		<b>0.774</b>	<b>0.816</b>
<b>11 OTC</b>	<i>0.001</i>		<b>0.141</b>
<b>22 OTC</b>	<i>0.001</i>	<i>0.050</i>	
<b>Archaea (R=0.448, P=0.001)</b>			
	<b>pre-treatment</b>	<b>11 OTC</b>	<b>22 OTC</b>
<b>pre-treatment</b>		<b>0.618</b>	<b>0.616</b>
<b>11 OTC</b>	<i>0.001</i>		<b>0.009</b>
<b>22 OTC</b>	<i>0.001</i>	<i>0.366</i>	
<b>Fungi (R=0.489, P=0.001)</b>			
	<b>pre-treatment</b>	<b>11 OTC</b>	<b>22 OTC</b>
<b>pre-treatment</b>		<b>0.434</b>	<b>0.689</b>
<b>11 OTC</b>	<i>0.004</i>		<b>0.083</b>
<b>22 OTC</b>	<i>0.002</i>	<i>0.177</i>	
<b>Protozoa (R=0.063, P=0.203)</b>			

\* For each pairwise comparison the R value (bold) and P value (italics) are indicated.  $P < 0.05$  is significant.

<sup>a</sup> 11 OTC = Daily oral dose of 11 mg OTC per kg live weight

<sup>b</sup> 22 OTC = Daily oral dose of 22 mg OTC per kg live weight

TABLE 9: One-way ANOSIM of rumen microbial communities associated with OTC treatment. For each microbial group the influence oral OTC treatment was investigated. Where significant differences in rumen microbiota were detected, the pairwise\* differences between treatments were investigated further.

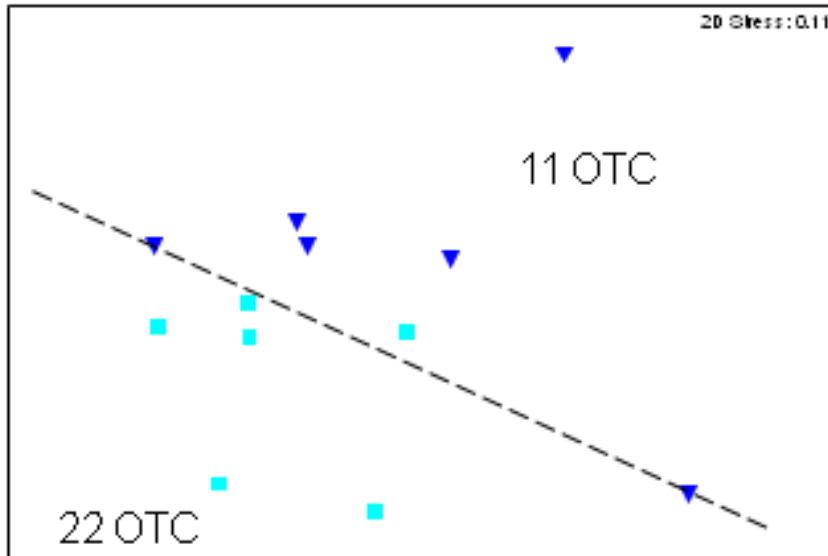


Figure 15: non-metric multidimensional scaling nMDS of rumen bacterial communities associated with oral dose of OTC. Treatments are: ▼= 11mg OTC /kg LW/day; and ■=22mg OTC /kg LW/day. Each point in the ordination shows the overall microbial profile of an individual animal. The closer two points are in the ordination the more similar are their profiles.

### 3.3.3 Discussion

Feed intake decreased with the introduction of oral OTC treatment. In the previous work it was hypothesised that feed intake reduced in response to a decreased water intake as a result of palatability issues resulting from the addition of medication to the water. In this trial sheep were offered un-medicated water and the OTC given to each Treatment was administered directly into the rumen via the fistula. This was done to ensure each sheep received the correct dose to allow a true assessment of the effect of the two doses on intake and rumen health.

It is postulated that the decrease in feed intake is partially related to the effects of the OTC on the rumen microflora. Analysis of the data from the rumen fluid showed that the Treatments significantly changed the populations of rumen microbes. The two Treatments caused the populations to change in different ways such that the rumen microbe profile at the end of the experiment differed between Treatments. This, however, did not lead to a difference between Treatments in any of the other variables, namely intake, energy intake, faecal score, BHB concentrations or rumen pH. Pyrosequencing has been done to speciate microflora populations that have been affected and the report is included as Appendix 3.

Both Treatments lost weight following the introduction of OTC. This weight loss is probably attributable to loss of rumen mass as opposed to loss of muscle mass. This can be confirmed by only a mild increase in BHB concentrations that indicates a relatively low level of fat catabolism. None of the animals entered a state of negative energy balance which is evident when the BHB level exceeds 1.5 mmol/L.

The decrease in feed intake appeared to be offset to a degree by the introduction of chaff. Chaff was mixed through the pellet ration and sheep appeared to preferentially eat the chaff during the treatment period and for a number of days following treatment. Further work would be required to definitively say that feeding chaff during the treatment period would maintain feed and energy

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Faecal score increased over time. Faecal score can be used as a crude assessment of rumen health, increasing faecal score indicates a softening of faecal pellets. Increased faecal score can be seen with a change in diet which can alter the rumen microbial dynamics. In this case it is postulated that the change was related to the alteration of rumen microflora as a result of the treatments administered.

Rumen pH increased over time but the mean pH did not exceed the pH of a healthy sheep which should be in the range of 6.5-7.5. Prior to introduction of OTC the pH was lower than 6.5. This is to be expected in sheep on a diet consisting of only pelleted feed.

It is encouraging that OTC was absorbed following administration directly into the rumen. Plasma concentrations of OTC were comparable to those in the previous experiment even though the OTC dose was administered directly into the rumen as opposed to relying on water intake.

There were no significant differences between the two different doses of OTC given in this experiment and it appears that the responses to soluble OTC were not dose dependant. There was a cumulative dose effect whereby peak plasma OTC concentrations were achieved after 4 days treatment in the 22 mg/kg Treatment and 5 days in the 11 mg/kg Treatment. Plasma concentrations were lower than those found following intra-muscular injections of OTC, namely 0.94 µg/mL.

## 4 Bioavailability of OTC when given in-water or in-feed.

### 4.1 Methods

Six adult merino cross ewes were selected at random for the experiment. A three-way cross over study design was used with a 10 day rest period between study periods allowed. The sheep were randomly assigned into 3 groups of 2 animals per group. They remained in that assigned group of 2 for all 3 study periods.

The three treatments were:

**IV.** 8mg/kg OTC (Engemycin, MSD Animal Health, Bendigo, VIC, Australia) given by **intravenous** injection

**IW.** 22mg/kg OTC (CCD OTC, CCD Animal Health, Geelong VIC, Australia) given orally **in-water**

**IF.** 20 mg/kg OTC (Terramycin 200, Phibro Animal Health, Girraween, NSW, Australia) given orally **in-feed**.

The two sheep in each group were rotated for each experiment period meaning that all sheep were used in each treatment group once. All animals were weighed and housed in individual, raised pens with sheep contact on at least one side. Indwelling catheters were placed in the jugular vein of each sheep and an extension set was attached to facilitate easier sampling. Catheters were sutured in place using Nylon sutures and a net bandage was used to provide additional protection over the neck area.

Sheep were housed for 36 hours prior to the start of the experiment to allow them to become accustomed to the housing and feed. All animals were offered a diet of oaten chaff and pellets. The 2 sheep in the IF treatment had feed withheld for 12 hours prior to commencement of the

W.LIV.0163 - Antibiotic medication for the treatment of Infectious Ovine keratoconjunctivitis (IOK) in pre-export feedlots experiment to improve feed intake over a short period of time. Animals IW treatment were given their dose by a drench using a McGrath feeder tube to ensure accurate dosing.

Blood samples were taken via the catheter. On each occasion the first 2 mL of blood was discarded as this removed for any blood or flush fluid that remained in the extension set. A blood sample was taken from all the sheep 5 minutes prior to any treatment being given as a base-line value. Serial blood samples were then taken at 1,2,3,5,10,15,20,30,40,60 minutes and 2,4,6,8,12 and 24 hours following treatment.

The catheter and extension set were flushed using heparinased saline following each sample. Blood was transferred to a plain vacutainer and then centrifuged at 2000rpm for 15 minutes. Serum was siphoned off and stored in cryovials at -80°C.

### Statistical analysis

Plasma OTC values over a 24 hour period for animals in each treatment were examined graphically. Average OTC values for each treatment at each time of measurement were estimated using split plot analysis of variance and these values were used to calculate the total plasma OTC over a 24 hour period (the area under the concentration vs. time curve). OTC bioavailability for IF and IW treatments was calculated by comparing their total plasma OTC over a 24 hour period to the same value for the IV treatment:

$$\text{Bioavailability}_{in\text{-}feed} = 100 * \frac{\text{TotalPlasmaOTC}_{in\text{-}feed}}{\text{TotalPlasmaOTC}_{IV}}$$

### 4.2 Results

OTC was detected in the plasma of sheep in the IV IF and IW treatments. Serum concentrations peaked within minutes following IV injection and steadily declined over the monitoring period. In both the IF and IW treatments, serum concentrations were not detected within the first 30 minutes of monitoring and peak concentrations were seen around 6 hours after administration.

The oral bioavailability of OTC in the IF treatment was 18% of the IV treatment within a 24 hour time period.

The bioavailability of OTC in the IW treatment was 27% of the IV treatment within a 24 hour time period.

### 4.3 Discussion

Oral bioavailability is defined as the fraction of the drug that reaches the systemic system unchanged following oral administration. This measurement is considered to be of greater clinical importance than the rate of drug absorption. From the data in Experiment 1, OTC was shown to be absorbed and detectable in plasma following administration both IF and IW. IW medication was bioavailable than IF (27% vs 18%). This may be explained by the increased time taken to release the drug from the pelleted feed particles through the process of digestion. In addition to this delay, increased time within the rumen may cause increased denaturing of the drug through the action of the rumen pH.

OTC is absorbed through the glandular stomach, the abomasum, therefore transit time through the forestomachs would have a strong influence on both the rate of absorption and the quantity of drug available for absorption. It is expected that water would have a faster transit time than feed which may in part explain the difference in oral bioavailability. OTC is found in the plasma following oral (IW & IF administration but concentrations are lower than those following IV injection. But, OTC is detectable in plasma for longer periods following IF or IW administration.

By knowing that only a fraction of drug given orally is absorbed it may in theory be possible to increase the dose so that enough is absorbed to reach clinically significant concentrations in the blood. There is a paucity of data relating to the concentrations required in blood to have a clinical effect against the *Moraxella* species and *Mycoplasma* species associated with clinical IOK infection. However, an impression can be formed from examining clinical results. The limiting factor of increasing dose appears to be the effect on palatability of both medicated pellets and water.

## 5 Determination of clinical efficacy of in-feed medication

### 5.1 Impact of In-Feed medication on feed and water intake

#### 5.1.1 Methods

Thirty, adult merino cross ewes were sourced from the Murdoch University teaching flock. Sheep were housed in individual pens in a purpose built animal house with sheep contact on at least one side. All sheep were weighed on entry to the animal house and a 2 days acclimatisation period was observed prior to commencement of the experiment.

The sheep were randomly assigned to 2 treatments.

**IF.** This treatment received non-medicated pellets for the first 2 days followed by 5 days of medicated pellets and then 5 days of non-medicated pellets.

**Control.** This treatment received only non-medicated pellets throughout.

Feed and water measurements were recorded daily at the same time each day for all animals. All sheep were weighed at the end of the experiment.

Preparation of medicated pellets (in-feed medication).

Medicated pellets were specially manufactured so that a 50 kg sheep eating 2% of its bodyweight in pellets per day would consume a dose of 20mg/kg OTC. Pellets were manufactured by Wellard Rural Exports at their feedmill. The pellets consisted of a mixture of lupins, grain (Triticale) and chopped straw. The OTC powder (Terramycin, Phibro Animal Health Pty Ltd, Girraween, Australia) was mixed in with the cereal and 15 minutes was allowed in the mixer to ensure adequate distribution of the powder throughout the mixture. Following this, chopped straw was added and a further 10 minutes of mixing occurred. The mixture is then heated to approximately 70°C for 2 minutes. The heat is generated by application of steam which moistens the mixture to enable better compaction into the pellet shape.

#### Statistical analysis

For each animal all pre-treatment feed and water intakes were averaged. A linear mixed model was fitted to the feed and water intakes on all post-treatment dates. The model included fixed effects for pre-treatment liveweight and intakes, treatment, post-treatment date and treatment by

date interaction; and random effects for animal and the animal by date interaction. An autoregressive model allowed for correlations between measurements made on the same animal on different dates and different residual variance on each date. 5% LSD's were calculated to compare the two treatments.

### 5.1.2 Results

Both feed and water intakes were significantly impacted by the introduction of medicated feed to the sheep in the IF treatment when compared to those in the control group ( $P < 0.001$ , see Figure 16 and 17).

Feed intake fell after the introduction of medicated feed and recovered to near the levels of the control animals by the end of the experiment. Similarly, water intake fell after the introduction of pellets, recovered and then fell slightly again before the end of the experiment.

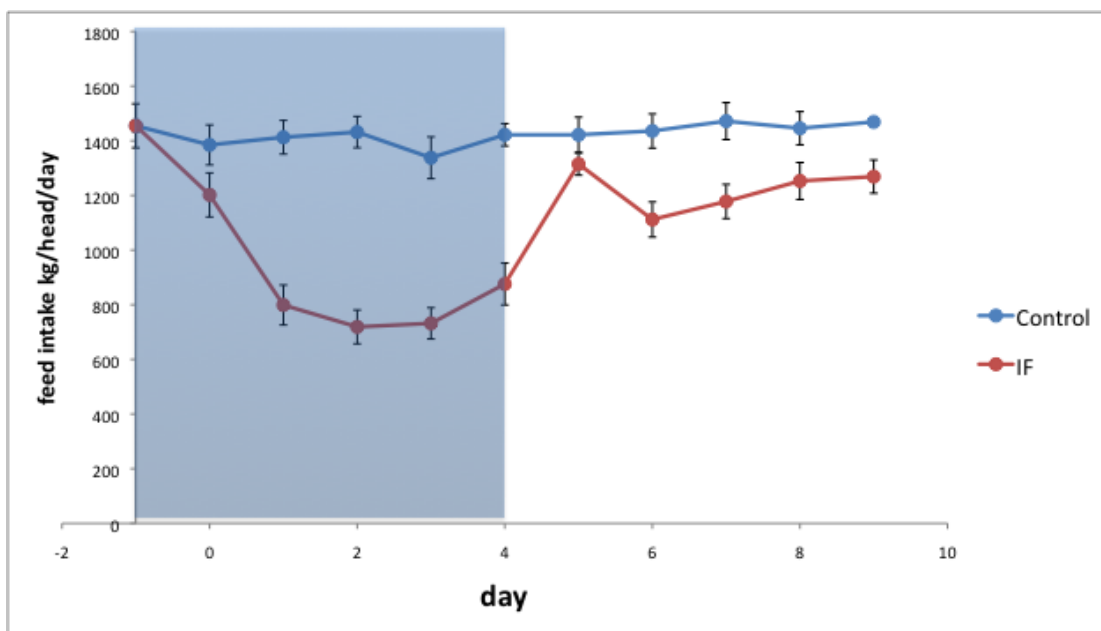


Figure 16: Feed intake (g/head/day) over time. Shaded box represents treatment period Error bars represent standard errors.

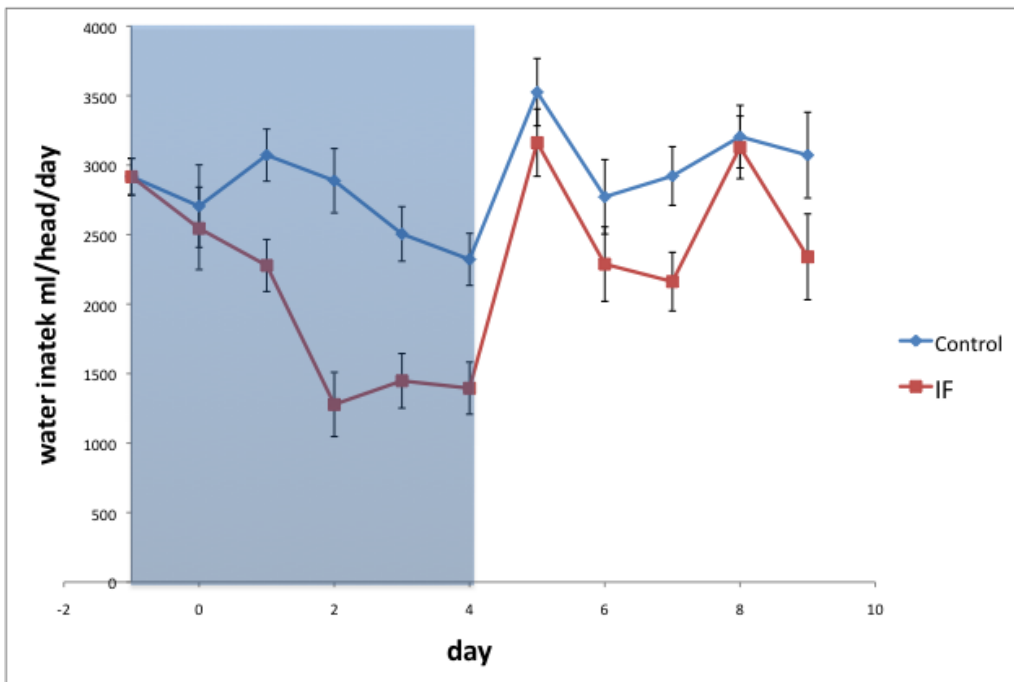


Figure 17: Water intake (kg/head/day) over time. Shaded box represents treatment period. Error bars represent standard errors

### 5.1.3 Discussion

This experiment has shown that feed and water intake fall following IF administration of OTC but recovers after treatments end.

All sheep were offered a pelleted ration at a standard weight of 1.5 kg. This represented approximately 4% of bodyweight that is above maintenance. Although feed intake fell after the addition of OTC to feed, it is important to note that intake on average only fell to the level of maintenance. Given that maintenance and not growth is the aim for sheep during pre-embarkation, and given the recovery of intake to previous levels following cessation of treatment, this reduction in intake may be an acceptable consequence of treatment.

As with feed intake, water intake was seen to drop but still remained within acceptable concentrations for maintenance.

## 5.2 Clinical efficacy of in-feed OTC

### 5.2.1 Methods

Thirty, mixed age and mixed breed sheep with clinical IOK were selected from a pre-export feedlot. Sheep were selected with either grade 2 (conjunctivitis) or 3 (conjunctivitis and corneal oedema)

W.LIV.0163 - Antibiotic medication for the treatment of Infectious Ovine keratoconjunctivitis (IOK) in pre-export feedlots infection in both eyes. No treatment had been given to the sheep prior to selection for this experiment. The sheep were transported to a purpose built animal house facility at Murdoch University where they were housed in individual pens with sheep contact on at least one side.

All sheep were weighed on arrival at the animal house and on leaving. Sheep were accustomed to the pelleted ration prior to selection therefore a 24 hour acclimatisation period was allowed for the change in housing.

The sheep were randomly assigned into 2 treatments:

**Control.** These sheep received non-medicated pellets throughout the duration.

**IF.** These sheep received medicated pellets for 5 days followed by 5 days of un-medicated pellets.

Medicated pellets were as described in Section 3.3.1.

Swabs for bacterial culture were taken from all sheep on arrival at the animal house and again on leaving. Swabs were taken by placing a sterile cotton tipped wooden shafted swab in the fornix of the eye between the third eyelid and the conjunctiva, these swabs were then plated on sheep blood agar in the standard manner to obtain a single culture before the tip was broken off into a *Mycoplasma* broth. Plates and *Mycoplasma* broths were submitted to the Department of Agriculture microbiology laboratory in South Perth for culture growth and analysis.

Feed and water intake were recorded for individual sheep daily. Eye grades were assessed and recorded in all sheep on day 0, 1, 3, 5, 7 and 10.

### Statistical analysis

For each animal all pre-treatment feed and water intakes were averaged. A linear mixed model was fitted to the feed and water intakes on all post-treatment dates. The model included fixed effects for pre-treatment liveweight and intakes, treatment, post-treatment date and treatment by date interaction; and random effects for animal and the animal by date interaction. An autoregressive model allowed for correlations between measurements made on the same animal on different dates and different residual variance on each date.

For each eye on each animal all pre-treatment eye grades were averaged. A linear mixed model was fitted to the eye grade data on all post-treatment dates ( days 0,2,4 and 7). The model included fixed effects for pre-treatment eye grade, treatment, post-treatment date and treatment by date interaction; and random effects for animal, the animal by date interaction and eyes within dates and animals. An autoregressive model allowed for correlations between measurements made on the same animal and eyes on different dates and different residual variance on each date.

A split plot analysis of covariance was used to analyse bacterial scores from each eye of each animal made at the end of the experiment. Pre-treatment score for the appropriate bacteria was used as a covariate measurement and eye within animal was used as the split factor.

5% LSD's were calculated to compare the two treatments.

### Serum sample analysis

Serum samples were analysed for concentrations of OTC. The method used for sample analysis is as described in Section 3.1.1.



## 5.2.2 Results

Feed intakes reduced significantly during the treatment period in the IF treatment but recovered after treatment ended (P=0.049, see Table 10 and Figure 18)

Clinically, sheep in the IF treatment showed a significant improvement in eye grade during treatment compared to those in the control group (P=0.007, see Table 10 and Figure 20). Following cessation of treatment there was a degree of worsening of eye grades in the treatment group but they still remained significantly lower than those in the control group.

Sheep in the treatment group showed an improvement in bacterial load for *Moraxella ovis* only following IF treatment compared to the control group (P=0.004, see table 10). There was no difference seen between the treatments in *Mycoplasma conjunctivae* load.

<b>Term</b>	<b>Feed intake</b>	<b>Water intake</b>	<b><i>M.ovis</i></b>	<b><i>Mycoplasma</i></b>	<b>Eye grade</b>
eye grade (cov)					<b>0.035</b>
feed intake (cov)	0.056	0.130			
water intake (cov)	0.361	<b>0.034</b>			
weight	0.826	0.914			
day (time)	<b>&lt;0.001</b>	<b>&lt;0.001</b>			<b>&lt;0.001</b>
treatment (cont v IF)	<b>0.049</b>	0.285	<b>0.004</b>	0.231	<b>0.007</b>
day.treatment	<b>&lt;0.001</b>	<b>0.054</b>			<b>0.008</b>

Table 10: Significance levels (P-values) for terms used in models to analyse data in Experiment 4.

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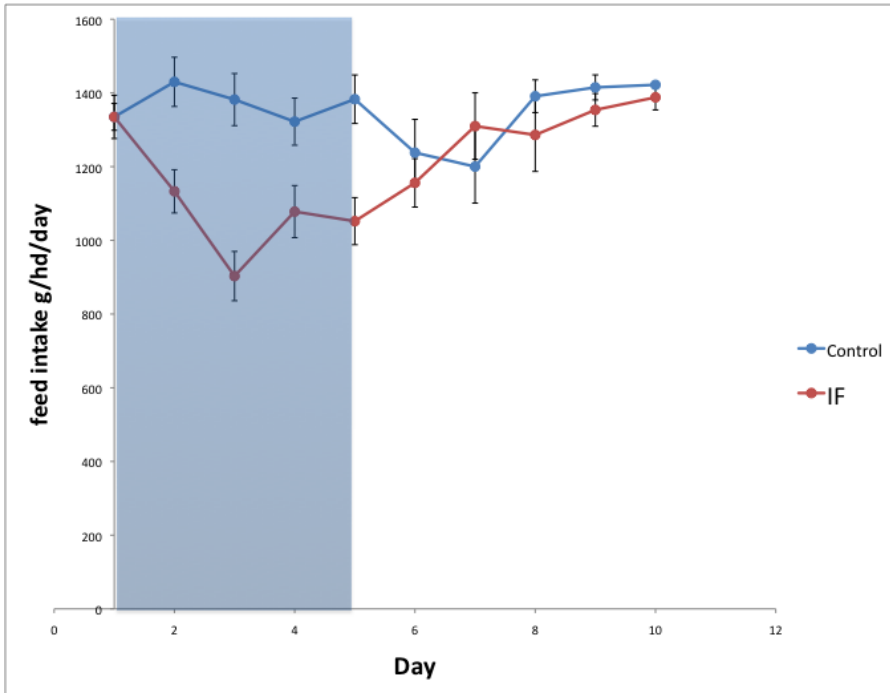


Figure 18: Feed intake(g/hd/day) of Control vs. IF treatments over time. Shaded box represents treatment period. Error bars represent standard errors.

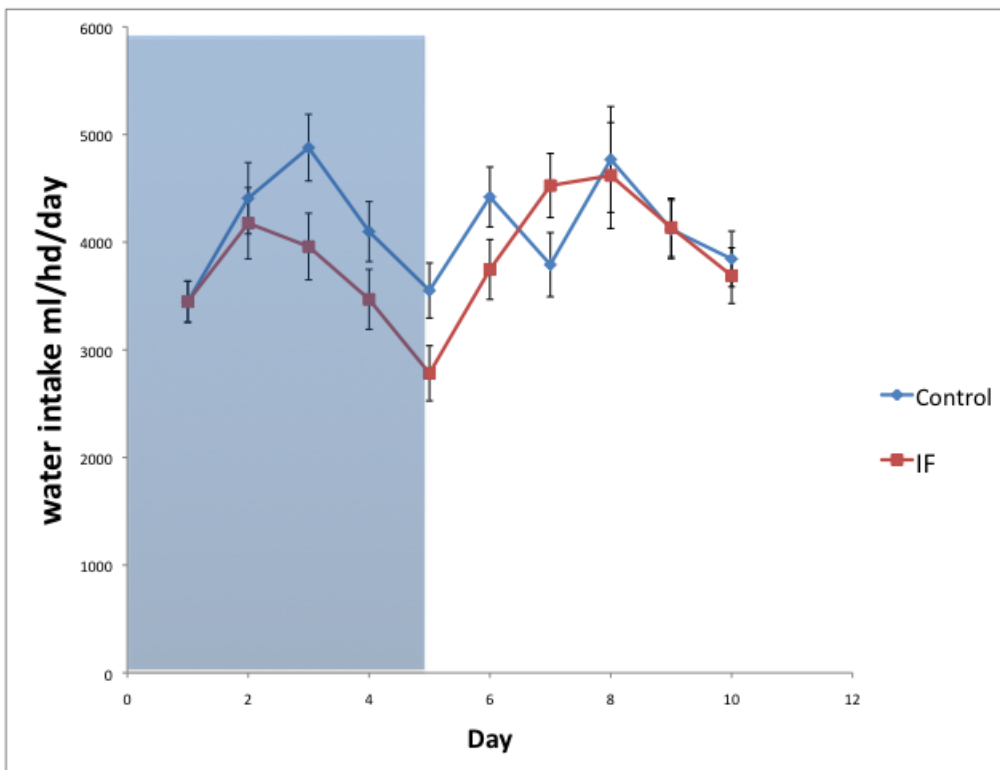


Figure 19: Water intake(ml/hd/day) of Control vs. IF treatments over time. Shaded box represents treatment period. Error bars represent standard errors

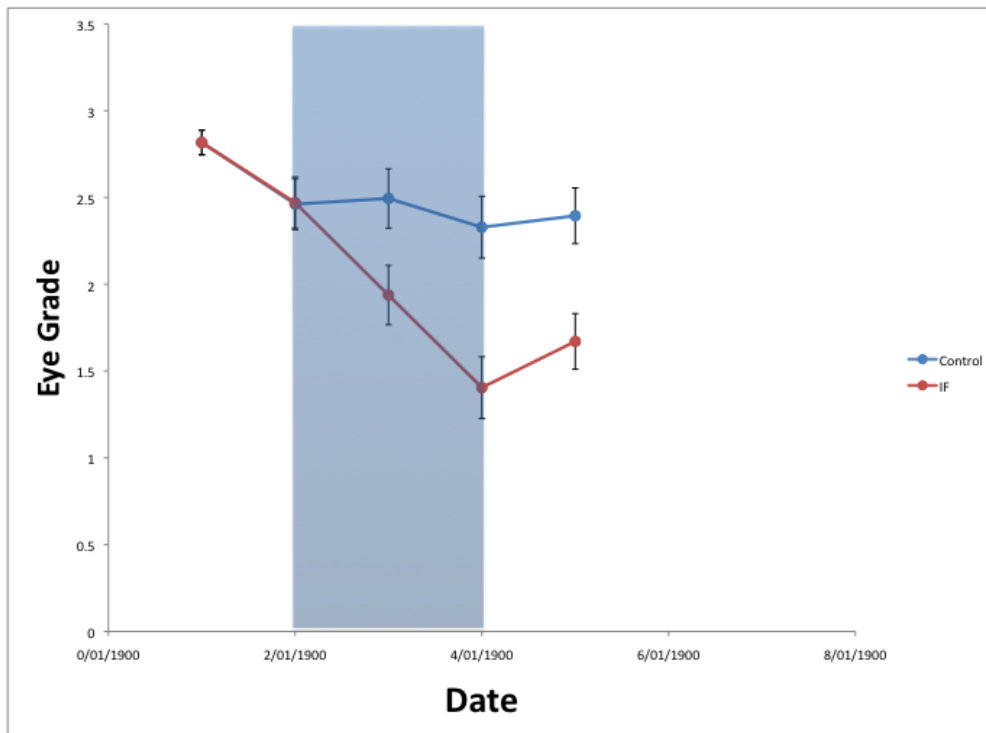


Figure 20: Change in eye grade over time - Control vs. IF treatments. Shaded box represents treatment period. Error bars represent standard errors

### 5.2.3 Discussion

This experiment was designed to show if in-feed (IF) medication had any effect on clinical IOK. Despite the reduction in intake of medicated pellets there was a significant improvement in clinical eye grade within the IF treatment group (see Figure 20).

Bacteriology measured before and after treatment was also different indicating that effective concentrations of OTC were reaching the ocular tissue. Notably, although there was a decrease in *Moraxella ovis* colonies following IF treatment, there appeared to be very few other organisms grown from the final sample and in some cases a pure culture of *M. ovis* were present following IF treatment. This may, in part, explain the slight deterioration in clinical eye grades following cessation of treatment.

OTC is a bacteriostatic antibiotic, whereby it doesn't kill bacteria outright, rather it halts their growth allowing the body's own immune system to kill off the bacteria. We suggest that it may be beneficial to give a longer course of treatment which may enable the immune system to more effectively target the remaining *M. ovis* populations. Given that sheep are generally in the feedlot for around 7 days this may be a feasible option. Further studies would need to be done to assess the effect of this.

## 6 Determining the clinical effect of in-feed and intra-muscular OTC in a feedlot experiment

### 6.1 Methods

207 merino cross, mixed age sheep with clinical pink eye were selected from those rejected at a pre-export feedlot. No sheep had received treatment prior to selection.

Sheep eyes were graded using the grading system developed in the work of Chapman *et al.* (2010), Appendix 1. Following grading sheep were drafted into two groups, one group with clinical eye grades 2-4 and the other with clinical eye grades 5-6. From these groups, sheep were randomly drafted into 3 treatment groups:

- Group 1 - control group receiving no treatment (n=69 with 40 grade 2-4 and 29 grade 4-5)
- Group 2 intra-muscular injection OTC (Alamycin 300 LA, Nobrook Laboratories Australia PTY Ltd, Tullamarine, VIC, Australia) (2 doses of 20mg/kg 2 days apart) (n=70 with 45 grade 2-4 and 25 grade 4-5)
- Group 3 in feed OTC (Terramycin 200, Phibro Animal Health, Girraween, NSW, Australia) (n= 68 with 36 grade 2-4 and 32 grade 4-5).

Sheep were housed in a standard feedlot raised pen in three separate pens. Free access to water was given to all and those in groups 1 and 2 had ad lib access to pelleted feed as would be typical in a feedlot. Sheep in group 3 received medicated feed, as described in Section for 5 days. Accurate measurements of feed intake were not taken, however subjective assessment of residuals were made on a daily basis. Accurate measurement of residuals removed on a daily basis was not possible due to contamination of feed and crumbling of pellet diet. *Ad lib* access to non-medicated pellets was given following cessation of medicated pellets.

Eye grades were recorded every second day following baseline grades on day 0 up to day 10. For a treatment to be effective, eye grades should reduce over time and return towards grade 0.

### 6.2 Results

Eye score on Day 0 significantly affected pinkeye scores post treatment ( $P < 0.001$ ). When treatment means are corrected for Score on Day 0 there is still a significant effect of Treatment ( $P < 0.001$ ) on daily pink eye score but this effect interacts with day post treatment such that the effect of treatment on pink eye score is different on different days post treatment ( $P < 0.001$ ). Daily treatment eye score means (corrected for Score on Day 0) are shown in Figure 21

At the end of the experiment animals treated with injections had lower eye scores than those treated with in-feed medication. Both treatments had lower mean eye scores than the control animals.

Fixed Term	P Value
Day	<0.001
Score on Day 0	<0.001
Treatment	<0.001
Day.Treatment	<0.001
Score on Day 0.Treatment	<0.001

Table 11. Significance levels (P Values) for analysis of pink eye scores on each day post-treatment (control vs in-feed vs injection)

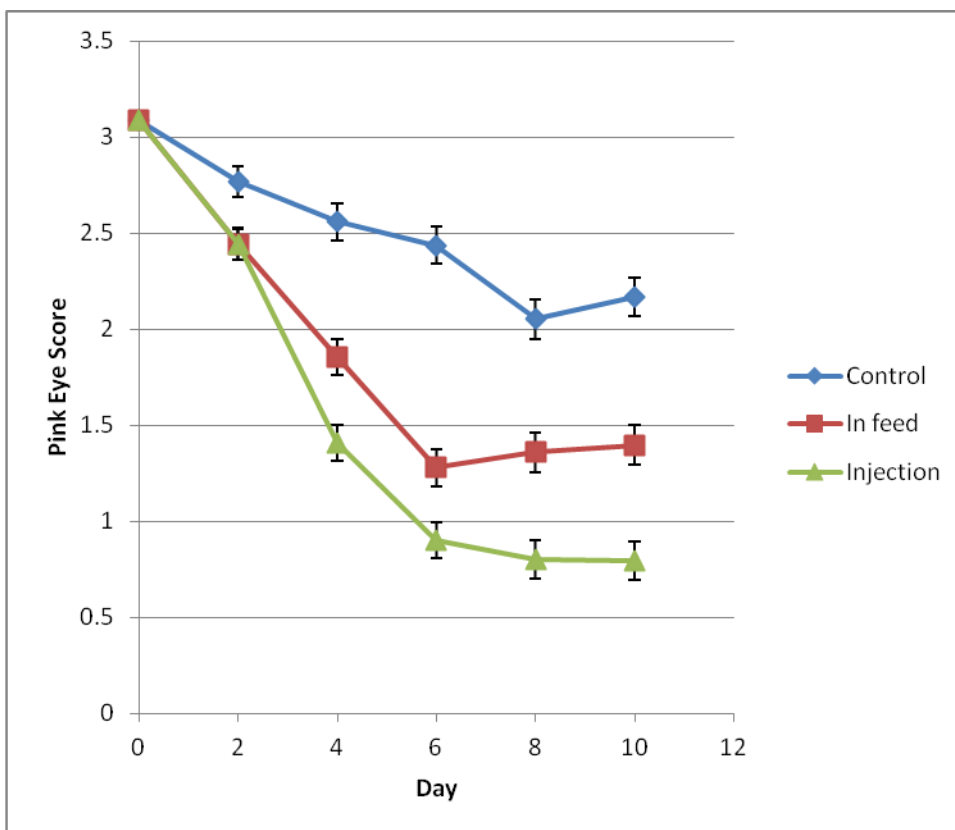


Figure 21 . Adjusted pink eye mean scores for each treatment and day. In feed treated daily from day 0-5 and Injection treated on day 0 and 4.

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in pre-export feedlots

Fixed Term	P value
Score on day 0	<0.001
Treatment	<0.001
Score_0.Treatment	0.048

Table 12 Significance levels (P values) for analysis of change in pink eye score over time

The pink eye score on Day 0 significantly affected the degree of change of eye score over time (P<0.001). The effect is shown in below.

Treatment	Control	In feed	Injection
	-0.898	-1.691	-2.318
<i>Standard errors</i>	0.1316	0.1322	0.1304

At an average Score\_0 value of 3.1, the decrease in Pink Eye score from Day 0 to Day 10 for the In-feed group is 0.793 ( $\pm 0.186$ ) more than for the Control group (-0.898 vs -1.691). The decrease in Pink Eye score from Day 0 to Day 10 for the Injection group is 1.4206 ( $\pm 0.186$ ) more than for the Control group ((-0.898 vs -2.318). The Injection group has a significantly higher decrease in Pink Eye score than the In-Feed group.

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The eye score means according to the starting eye score are depicted in Figure 22

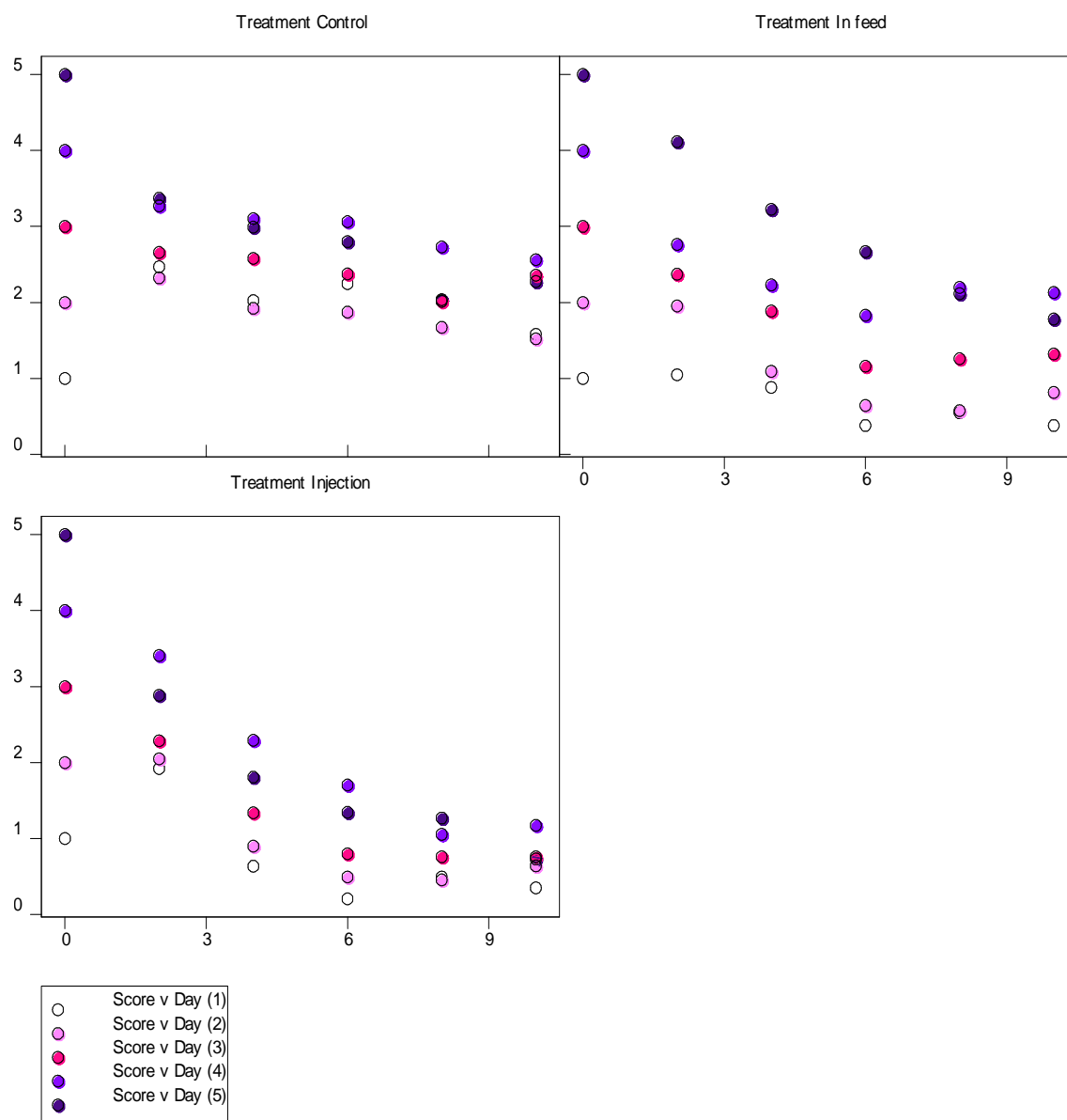


Figure 22. Average Pink eye scores on each day, grouped according to Score on Day 0.

In-feed OTC for 5 days is an effective treatment for sheep with IOK up to and including grade 3, whereas injectable OTC is effective in treating IOK up to and including grade 5, Table 13.

Score	In-feed	Injection
1	Treat	Treat
2	Treat	Treat
3	Treat	Treat
4	Not effective	Treat
5	Not effective	Treat

Table 13: Treatment recommendations (Treat vs Not effective) of different routes of administration (In-feed vs Injection) at different degrees of severity of disease (Score) on day 8 of experiment.

### 6.3 Discussion

The results of the feedlot experiment follow results seen in smaller preliminary experiments. Although feed intake in the medicated feed group did decrease, the residuals left were small. In-feed OTC is considered effective in treating sheep with eye grades up to and including 3, however those with grades 4 and 5 need to be treated with intra-muscular OTC to have the best chance of recovery to the point where they could be shipped.

## 7 Determination of ocular fluid levels of OTC

### 7.1 Methods

Fifteen, clinically healthy sheep which had had no recent exposure to OTC were selected from the Murdoch University teaching flock. Sheep were housed in either individual raised pens or group pens depending on which treatment group they fell into. Sheep were randomly assigned to 3 treatment groups: group 1 was a control and received no treatment; group 2 received one intra-muscular injection of OTC ((Alamycin 300 LA, Nobrook Laboratories Australia PTY Ltd, Tullamarine, VIC, Australia) (20mg/kg) and group 3 received OTC (Terramycin 200, Phibro Animal Health, Girraween, NSW, Australia) medicated pellets (effective dose 20mg/kg). Only animals in group three were housed in individual pens to ensure consistent intake of medicated pellets. Lacrimal fluid samples were taken by inserting a sterile disc (Susceptibility Discs, Oxoid Ltd, Basingstoke, England) in between the conjunctiva and the eyeball and leaving in place for 15 seconds to ensure saturation. Discs used were blank discs that are used in bacteriology sensitivity testing. Ophthalmic forceps were used to aid placement and removal of the disc. Discs were frozen immediately prior to analysis.

Blood agar plates were lawn inoculated with a pure culture of *Moraxella ovis*, the discs were placed on the plate and then incubated at 37°C overnight. Zones of growth inhibition were read following 24 hours incubation.

### 7.2 Results

A pure culture of *Moraxella ovis* grew successfully on the blood agar plates. No zones of inhibition were seen around any of the discs following 24 hours of incubation.

### 7.3 Discussion

The experiment to assess the ocular concentrations of OTC was designed to follow on to the work done previously using liquid chromatography-tandem mass spectrometry as described in Section 3.1. The experiment design used methods similar to Konig's experiment (Konig, 1983). The Minimum Inhibitory Concentration (MIC) of *Moraxella ovis* is reported to be 1 µg/mL of OTC (N.



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Buller DAFWA Pers. Comm.). Given no zones of inhibition were seen it can be assumed that this concentration of OTC was not present in the samples taken. Sampling times were based on peak plasma concentration times as seen in the experimental data from the bioavailability experiment. It could be possible that ocular levels do not correlate with plasma concentrations, additionally sheep used in this experiment had no clinical signs of IOK and therefore no inflammation of the conjunctiva. Blood flow increases to an inflamed area therefore higher concentrations of OTC may be found in the lacrimal fluid of sheep with clinical signs of IOK. Treating sheep clinically affected with IOK with both intra-muscular injection and in-feed OTC appear to clinically improve therefore sufficient OTC must reach the ocular tissues and fluid to have an effect on the bacterial populations.

Further experimental work may be warranted to determine ocular concentrations. The next approach would involve catheterizing the lacrimal ducts of sheep and harvesting lacrimal fluid over a longer period of time and more frequently. The researchers feel that this would be unnecessary work given the clinical response seen.

## 8 Determining prevalence and risk factors associated with outbreaks

### 8.1 Methods

Historical data was collected from managers at a pre-export feedlot. This data reflected records kept by inspectors of arrivals at the feedlot. Data received comes from 9 shipments.

All sheep are inspected on arrival at the feedlot to identify animals that are deemed to be “of no commercial value” to the exporter, sheep with advanced IOK are included in this group.

### 8.2 Results

Table 14 shows the historical data received from the feedlot in relation to IOK cases seen on arrivals.

Date	Number in shipment	Number cases IOK	Percentage of shipment (%)
August 2012	74268	7	0.01
August 2012	19421	5	0.03
October 2012	73541	13	0.02
November 2012	69641	101	0.15
December 2012	67303	62	0.09
January 2013	73456	47	0.06
February 2013	29450	40	0.12
February 2013	28224	25	0.09
March 2013	75170	60	0.08

Table 14. Historical data showing numbers of IOK cases rejected on arrival at feedlot

### 8.3 Discussion

Data received from the exporter highlights a seasonal pattern in the occurrence of IOK cases, with more cases seen in the warmer and dryer summer months. This could be attributed to the higher likelihood of sheep being exposed to sunlight. This has been reported to be a risk factor in establishing disease (Hosie, 1988). Disease can be spread between sheep by a number of factors: flies (Beveridge, 1942); physical contact (Hosie, 1988) and nasal discharge (Beveridge, 1942). The effect of a dusty environment has been considered as a risk factor by feedlot staff, however this is not supported in the literature (Hosie, 1988). It is worth noting that most studies into the epidemiology of IOK have been done in Northern hemisphere flocks where both environmental and husbandry conditions are different. Although total numbers of cases seen in each shipment are relatively low, it is thought that these numbers will be lower than the true numbers of cases present. Inspectors record and mark animals which are deemed to have “no commercial value” and as such payment is not made to the producer for these animals. Only animals with more advanced IOK would be included in these figures, these sheep would typically have: an obvious ulcer on one or both eyes; severe corneal oedema resulting in a cloudy eye or evidence of visual deficits. Many other sheep with milder cases will arrive in the feedlot, these sheep will be treated and in most cases clinical signs will resolve within the quarantine period.

It was hoped that these figures could be verified by researchers inspecting a whole shipment on arrival and again on load out to determine the numbers of sheep with any form of IOK arriving at the feedlot. The staff numbers involved to be able to accurately do this was prohibitive and there was concern from the management that the presence of too many personnel around the loading ramps and receival area would hinder their work. Inspections would have been carried out by a researcher on either side of the loading ramp allowing inspection of both eyes. Depending on the number of lines of sheep on a truck, the rate of sheep passing ranged between 2 per second up to 6 per second which would make accurate inspection of the eye near impossible.

Feedlot management have further data that breaks down the shipments into sheep lines including ages, breed and source. It is hoped that this data will be made available to researchers in the future. It is a time consuming process to extract this data and at this stage it has not been made available to us. Additionally the exporters have concerns releasing detailed data due to the sensitive nature of such data.

## 9 General Discussion and Conclusion

IOK continues to affect the Live Export industry, both in pre-export feedlots and onboard voyages. With ever increasing scrutiny on the industry it is important to address anything that may compromise animal welfare and give the public reason for concern. IOK is a multifactorial disease with several aetiological agents and is associated with numerous risk factors. These along with the potential for the development of a carrier state make treating and remaining disease free within the export chain a significant challenge.

The work thus far had highlighted that injectable OTC at a dose of 20mg/kg on at least one occasion, but preferably two, is the most effective treatment available to exporters. The logistics of injecting the number of sheep involved in outbreaks proves challenging.

In-water medication initially showed promise in the pilot work done by Chapman *et al* (2010), however further experiments with this treatment have highlighted problems. The addition of medication appears to have a deleterious effect on the palatability of water resulting in intakes of negligible amounts during the treatment period. This leads to very low concentrations of OTC being consumed resulting in poor clinical response. Additionally changes are observed in the rumen

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microflora, the significance of these have not been fully elucidated as the poor clinical response and adverse effect on feed and water intakes is sufficient to discount this as a suitable treatment.

In-feed medication has consistently shown to have a good clinical effect. Feed intake is reduced, but not to a level that would result in significant weight loss. Maintenance intakes are still maintained. Feed intake consistently returns to levels seen pre-treatment following the cessation of treatment. In-feed medication is not superior to injectable medication in clinical effect, however it is cheaper and easier to administer to large groups. To date experimental work has focused on a 5-day treatment period and it has been noted that following initial improvement in clinical signs, clinical signs can begin to worsen in the 5-day period following treatment. The researchers would recommend testing a longer period of offering medicated feed – possibly seven days. Sheep are typically in the pre-export feedlot for around 7 days and it may be possible to extend the treatment to this time. The stability of in-feed OTC following incorporation in pellets is unknown. All feed used in these experiments was made within 1 week of being fed to sheep. Further work may be required to determine how long medicated feed can be stored. It is known that injectable OTC reacts with sunlight so medicated pellets may also be affected and should be kept out of direct sunlight. Although the in-feed medication used in these experiments is registered for use in sheep in Australia, it does not have a label claim for the treatment of IOK. Additionally no export slaughter interval has been established to the researchers' knowledge. These issues would need to be considered prior to widespread use of this treatment strategy.

This work has shown that treatment can be effective in sheep with pink eye grades of up to 5. This data should be of use to feedlotters and it is hoped that instigating the most effective treatment at the earliest possible opportunity will result in higher recovery rates and fewer culls owing to infection with pink eye.

## 10 Conclusions

Early identification and treatment of IOK cases is key to success in resolving infection.

OTC is detectable in plasma following in-water and in-feed administration.

In-water medication results in a reduction in feed and water intake and has a marked impact on rumen microflora.

Taste mitigation using dextrose in conjunction with in-water OTC is not effective in increasing the intake of feed and water.

In-feed OTC is effective in the treatment of IOK. Animals with eye scores of up to and including grade 3 will improve if given for 5 days or longer.

Injectable OTC (2 injections of 20mg/kg given 4 days apart) is usually effective at treating all grades of IOK up to and including grade 5.

Detectable levels of OTC were not found in lacrimal fluid by either bioassay or liquid chromatography-tandem mass spectrometry but the clinical response to OTC treatment suggests that OTC is present in the eye in concentrations sufficient to treat IOK.

Accurate measurement of the prevalence of IOK cases is not possible on entry or exit from a pre-embarkation feedlot.

More cases of IOK are seen on arrival at the feedlot in summer than in winter.