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Production responses of reproducing ewes to a by-product-based diet inoculated with the probiotic *Bacillus amyloliquefaciens* strain H57

Oanh T. Le[A], *Benjamin Schofield*[B], *Peter J. Dart*[B], *Matthew J. Callaghan*[C], *Allan T. Lisle*[B], *Diane Ouwerkerk*[D], *Athol V. Klieve*[B] *and David M. McNeill*[A,E]

[A]School of Veterinary Science, The University of Queensland, Gatton, Qld 4343, Australia.
[B]School of Agriculture and Food Sciences, The University of Queensland, Gatton, Qld 4343, Australia.
[C]Ridley AgriProducts Pty Ltd, Toowong, Qld 4066, Australia.
[D]Department of Agriculture, Fisheries and Forestry, Dutton Park, Qld 4102, Australia.
[E]Corresponding author. Email: d.mcneill@uq.edu.au

Abstract. The potential application of the spore-forming probiotic *Bacillus amyloliquefaciens* strain H57 (H57) as a novel probiotic for ruminants was evaluated in reproducing ewes. Performance responses were determined by delivering H57 in a pelleted diet based mainly on palm kernel meal (PKM) and sorghum grain. PKM is an agro-industrial by-product with a reputation for poor palatability and the availability of the starch in sorghum grain can be limited in ruminants. The hypothesis was that H57 improves the feeding value of a relatively low quality concentrate diet. Twenty-four first-parity white Dorper ewes were fed PKM-based pellets manufactured with or without H57 (10^9 cfu/kg pellet) in late pregnancy. During this phase of late pregnancy, the H57 ewes ate 17% more dry matter (1019 vs 874 g/day, $P = 0.03$), gained more weight (194 vs 30 g/day, $P = 0.008$) and retained more nitrogen (6.13 vs 3.34 g/day, $P = 0.01$), but produced lambs with a similar birthweight (4.1 vs 4.2 kg, $P = 0.73$). Rumen fluid collected from H57 ewes in late pregnancy had higher pH (7.1 vs 6.8, $P = 0.07$), acetate : propionate ratio (3.4 vs 2.7, $P = 0.04$), lower ammonia (69 vs 147 mmol/L, $P = 0.001$) and total volatile fatty acid concentrations (40 vs 61 mg/L, $P = 0.02$). The digestibility of dry matter, organic matter and fibre were similar between the two groups. The lambs of the H57 ewes grew faster than those of the Control ewes for the first 21 days of lactation (349 vs 272 g/day, $P = 0.03$), but not thereafter. H57 can improve feed intake and maternal liveweight gain in late pregnancy of first-parity ewes fed a diet based on PKM.

Additional keywords: feed intake, liveweight, palm kernel meal, pregnant ewes.

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Introduction

Probiotic supplements are single or mixed strain cultures of live microorganisms that benefit the host by improving the ecological balance of the indigenous microflora (Newbold 1996). Probiotics have been widely used in non-ruminants to reduce the risk of diarrhoea and the stress of weaning and to improve both feed efficiency and growth performance (Mohan *et al*. 1996; Alexopoulos *et al*. 2001; Hong *et al*. 2005; Chaucheyras-Durand and Durand 2010). Studies into the potential for probiotics in ruminant diets are increasing but focus mainly on pre-weaned animals. In immature ruminants, probiotics can advance rumen development by increasing the length and density of rumen papillae (Sun *et al*. 2011), reduce the frequency of diarrhoea (Timmerman *et al*. 2005; Jatkauskas and Vrotniakiene 2010; Signorini *et al*. 2012), increase dry matter intake (DMI) (Kowalski *et al*. 2009; Frizzo *et al*. 2010), daily weight gain (DWG) (Galina *et al*. 2009; Bayatkouhsar *et al*. 2013) and feed efficiency (Sun *et al*. 2010).

Compared with pre-weaned ruminants, the effects of probiotics on more mature ruminants are inconsistent. Supplementation with probiotics can increase milk yield and milk protein in lactating cows (Qiao *et al*. 2010; Peng *et al*. 2012) and lactating ewes (Kritas *et al*. 2006). However, most studies have found no effects of probiotics on growth performance (Garza-Cázares *et al*. 2001; Antunovi *et al*. 2006; Raeth-Knight *et al*. 2007; Galina *et al*. 2009; Khalid *et al*. 2011). Inconsistent responses of mature ruminants to probiotics could be due to the variety of different types of probiotics used (Malik and Bandla 2010) or nature of the background diet (Frizzo *et al*. 2011). Moreover, studies have generally evaluated probiotics in ruminants fed conventional diets rather than diets based on low quality by-products. As worldwide demand for the incorporation of by-products into ruminant diets is expected to increase, strategies to improve the nutritional value of these alternative feedstuffs are required (McNeill 2013). The *Bacillus amyloliquefaciens* strain H57

(H57) could provide such a strategy, as by-products are commonly at risk of spoilage and H57 has been shown to ameliorate this risk in other roughages such as hay. In research at the University of Queensland, H57 spores isolated from fresh lucerne leaves were shown to reduce the risk of hay spoilage (Brown and Dart 2005). In a subsequent pilot study, H57 also improved feed intake and the efficiency of nitrogen utilisation in pregnant ewes fed the treated hay, for a short period (Norton *et al*. 2008). The following study evaluated the impact of the probiotic H57 on pregnant ewes fed a diet comprised of a high concentration of palm kernel meal (PKM), an agro-industrial by-product with a reputation for poor palatability. The hypothesis tested was that H57 improves the feeding value of a pelleted diet based on PKM through improved feed intake in pregnant and lactating ewes.

Materials and methods

Animals and management

The use of the animals and the experimental procedure were approved by the Animal Ethics Committee of the University of Queensland.

Thirty-two first-parity white Dorper ewes (liveweight: 47.3 ± 6.9 kg and age: 15 ± 4.6 months) were allocated to a completely randomised experiment with two treatments (with or without H57). Ewes were selected from a stud flock following a synchronised breeding program in which all ewes were impregnated by artificial insemination on the same day. From Day –120 (120 days before parturition), in addition to grazing, the ewes were offered a PKM and sorghum-based pelleted diet (Table 1) at 200 g/day.ewe in self-feeders as a group. At Day –100, the ewes were relocated into individual pens (1.5 m width × 2.1 m length) with metal mesh floor in an animal house at the Queensland Animal Science Precinct, The University of Queensland (Gatton, Qld, Australia), and so from this point onwards were individually fed, all on the PKM-based Control diet. On entry to the pens, the ewes were treated for gastrointestinal parasites with Cydectin long-acting injection for sheep (Virbac, Milperra, NSW, Australia) and vaccinated against clostridial diseases with Glanvac 6 (Zoetis, Sydney, NSW, Australia).

In the first 2 weeks of the adjustment period, in the pens, eight ewes were removed from the study due to particularly poor voluntary feed intake, leaving 24 ewes. The intake problem was thought to be due to the stress of the new environment, the reputed poor palatability of PKM, and a possible metabolic acidosis. To optimise the appetite of the ewes, oaten chaff was placed over the top of pellets (100 g chaff/ewe.day), the adjustment period was extended from 2 weeks to 7 weeks (Day –105 to –58) and NH_4Cl removed from the pellets. The NH_4Cl was initially added to reduce the risk of urinary calculi development but can also impede appetite and reduce metabolic pH. Urine pH can indicate the level of decline and when tested on Day –84, urine pH was found to be more acidic than expected (mean of all ewes was pH = 5.9). Consequently the diet was reformulated by replacing the NH_4Cl with an iso-nitrogenous amount of urea, which elevated urine pH to a normal level (pH = 7.9) within 3 days and the ewes were given a few more weeks to adjust before treatment were imposed. On Day –58, the 24 ewes were allocated to one of two treatment groups according to liveweight. The treatment groups were 'H57' (n = 12, fed pellets containing 2.85×10^9 cfu of H57 spores/kg pellets, as fed) and 'Control' (n = 12, fed pellets without added H57). The H57 spores were incorporated into the pellets during the steam pelleting process at the Ridley Agriproducts Pty Ltd production facility (Toowoomba, Qld, Australia).

Table 1. The ingredients and chemical composition of the experimental diet

DM, dry matter; CP, crude protein; OM, organic matter; NDF, neutral detergent fibre; ADF, acid detergent fibre

	Pregnancy diet	Lactation diet
	Ingredients (% *DM*)	
Palm kernel meal	37.5	–
Sorghum grain ground	39.6	4.0
Chickpea hull	9.5	–
Urea	0.3	–
Oaten chaff	8.0	48.0
Lucerne chaff	–	48.0
Molasses	2.5	–
Limestone	1.5	–
Salt	0.5	–
Ammonium sulfate	0.5	–
Mineral/Vitamin premix[A]	0.2	–
H57 spores (cfu/kg DM)	$+/-2.85 \times 10^9$	$+/-4.3 \times 10^{10}$
	Composition (% *DM*)	
DM (%)	91.1	83.1
Crude protein	12.7	13.7
Organic matter	93.5	88.6
Neutral detergent fibre	36.8	45.3
Acid detergent fibre	24.7	28.5
Lignin	7.15	5.62
Calcium	10.2	9.90
Phosphorus	3.31	3.61

[A]Mineral/Vitamin premix (mg/kg, unless stated): Vitamin A, 3000 IU/g; Vitamin D_3, 250 IU/g; Vitamin E, 2500; iron, 7500; zinc, 25000; manganese, 1000; selenium, 50; molybdenum, 500; cobalt, 500; iodine. 500.

During pregnancy, ewes were individually offered an amount of feed calculated to meet their energy and protein requirements for the developing conceptus plus 70 g/day of maternal tissue weight gain, according to their liveweight and stage of pregnancy (Freer 2007). The amount of feed offered was reset weekly. The amount for the coming week was pre-weighed into a separate storage bin for each ewe, from which fresh pellets were transferred twice daily, in two approximately equal amounts that represented 1/14th of the week's feed using pre-calibrated feed scoops, at 7 a.m. and 4 p.m., to the ewe's feed trough, so that by the end of the week the fresh pellets bin was emptied. Refusals were removed once daily from the ewe's feed trough, before the morning feeding, and bulked in a separate bin for each ewe across the week, and weighed weekly. Samples of pellets were collected weekly and stored at −20°C. At the end of the trial, weekly samples were combined and two subsamples from each treatment group were taken for analysis of the compositions (Table 1). The weekly samples of feed offered and refused were analysed for DM to determine DMI. Feed intake was measured weekly (except during two digestibility periods

when intake was measured daily) and divided by 7 to express it on a daily basis.

During lactation, from Days 7 to 14 the ewes were changed to a pellet diet reformulated to account for lactation requirements and with less PKM. Within this week, the diets were also accidentally swapped between treatment groups such that the Control ewes received the H57 treatment and vice versa. Within the same week, a ewe showed signs of acute copper toxicity and a generalised copper toxicity was quickly confirmed that was suspected to be due to the PKM in the pellets. Hence, from Day 14 to Day 63, the diet was replaced by a 50 : 50 mix of lucerne : oaten chaff, fed *ad libitum*, plus 100 g/ewe.day of ground sorghum grain with or without H57 (4.3×10^9 cfu spores/100 g DM). The grain was fed in a separate feeder to the chaff to ensure complete intake of the grain.

The ingredients and chemical composition of experimental diets are presented in Table 1.

Measurements

Liveweight of ewes and lambs was measured weekly before feeding in the morning. Lamb birthweight was measured as soon as the dam completed grooming their lambs (within 1 h after birth). The daily weight gain of each sheep was calculated by regressing each set of weights against their age in days across three periods (adjustment, pregnancy, lactation), so that the slope of the line indicated liveweight gain in grams per day. Body condition score (BCS) was measured in the adjustment period (Day 51), pregnancy (Day 7) and lactation (Day 52) on a scale of 1 (emaciated) to 5 (obese).

Nitrogen balance and digestibility were measured twice during pregnancy, first in the adjustment period (from Day –70 to –60) and second, 3 weeks after transition onto the treatment diets (from Day –36 to –26). Ewes were moved into metabolism crates for 10 days, the first 3 days for acclimation and the following 7 days for total collection. About 80 mL of 5% sulfuric acid was added to urine collection buckets to keep pH below 3.5 in order to stabilise urinal nitrogen. Faeces and urine for each ewe were collected and weighed daily, at 6 a.m. Faeces were separated from urine by attaching a device underneath the metabolism crate for the duration of the collection period. The separation device comprised a funnel directing all faeces and urine onto a wire sieve set at an angle sufficient to allow the faeces to roll off in one direction, to a plastic collection bag, whereas the urine flowed vertically into a bucket. About 10% subsamples of the daily feed offered, refusal, faeces and urine were collected and stored at – 20°C. At the end of the collection period, daily samples were bulked and mixed thoroughly, then 10% subsamples were collected and oven-dried at 60°C for 48 h and ground through a 1-mm screen (Retsch ZM 200; Haan, Germany) for subsequent chemical analysis.

Rumen fluid was collected by a stomach tube before the morning feeding during pregnancy (Day –58 and –21) and lactation (Day 52). The pH of rumen fluid was measured using a portable pH meter (Elmetron IP67, Wincentego Witosa 10, Zabrze, Poland) immediately after collection and then two subsamples (4 mL/sample) were taken: one for the analysis of volatile fatty acids (VFA) concentration (4 mL rumen fluid + 1 mL of 20% metaphosphoric acid), and one for ammonia analysis (4 mL rumen fluid + 2 mL of 20% sulfuric acid). The concentration of ruminal VFA was determined by gas liquid chromatography (GC17, Shimadzu Kyoto, Honshu, Japan) fitted with a polar capillary column (ZB-FFAP, Phenomenex Lane Cove, NSW, Australia) using the method described by Cottyn and Boucque (1968) and Playne (1985). The ruminal ammonia concentration was determined by distillation using a Buchi 321 distillation unit (Flawill, St Gallen, Switzerland) using the manufacturer's guidelines.

Chemical analyses

Dry matter and organic matter were measured using the AOAC (1990) methods. Nitrogen content of feed offered, feed residue, faecal and urine samples were measured using the AOAC (1990) method adapted for an automatic distillation of the Kjeldahl digestion product (Kjeltec, 8400 FOSS Hillerod, North Zealand, Denmark). Neutral detergent fibre and acid detergent fibre were determined using an Ankom fibre digestion unit using guidelines described by the manufacturer (Ankom Technology, Macedon, NY, USA).

Statistical analyses

From the 12 ewes per treatment group, one H57 ewe was removed from the analysis as it was the only ewe to produce twin lambs, all others produced single lambs. From parturition onwards, several more ewes were removed from the study: two Control ewes due to the loss of their lambs through dystocia, one H57 ewe due to poor brain development in its lamb, one H57 ewe due to its refusal to suckle its lamb for several days after birth and one H57 ewe died due to copper toxicity in the second week after parturition. Consequently, the final statistical analysis was completed on 23 single-bearing ewes for the pregnancy period (H57, $n = 11$; Control, $n = 12$), and 18 ewes, each with a single lamb, for the lactation period (H57, $n = 8$; Control, $n = 10$).

Analyses of DMI of the ewes and liveweight change of both ewes and lambs were conducted using a general linear model analysis in STATISTICA 8.0 (Weiß 2007). Sum of squares were partitioned into effects for treatment and time along with possible interactions. The initial liveweight of ewes was used as a covariate for the analysis of ewe liveweight change and DMI, ewes within treatment were included as a random effect and time was considered as a repeated factor within each ewe. Differences between treatment means were tested for significance at the level of $P < 0.05$ using Tukey HSD. All means were expressed as least square mean and standard errors (s.e.). Data of rumen characteristics, digestibility, nitrogen utilisation and BCS were analysed using a mixed model ANOVA in STATISTICA 8.0 (2008), Statsoft. The model included treatment as a fixed effect, ewes within treatment and residual errors as random effects.

Results

Dry matter intake

During the adjustment period (Day –105 to –58) DMI was similar between the treatments. Differences in DMI between two groups became significant ~5 weeks after supplementation with H57 began (Fig. 1). For the entire supplementation period

in pregnancy (Day –57 to parturition) the DMI of the H57 ewes was 14.6% higher than the Control ewes (P = 0.03, Fig. 1, Table 2).

One week post-parturition (from Day 7 to 14), the dietary treatments were accidentally reversed for a week. That problem was rectified but at the same time a copper toxicity became evident in both groups and so within the space of the following week all ewes were progressively adjusted onto the predominately chaff diet with or without H57 added in ground sorghum grain fed in a separate feeder. No difference in DMI was recorded due to treatment throughout lactation (P = 0.66, Table 2). Post-parturition, DMI in both groups increased rapidly, by 2.5 times for the H57 ewes and 3.2 times for the Control ewes compared with that in late pregnancy, over the first 4 weeks (Fig. 1).

Ewe and lamb liveweight change

Over the adjustment period, the liveweight gain of ~5 kg was similar between both groups such that each started the late pregnancy treatment period at similar liveweight (P = 0.89 Table 2, Fig. 2). By parturition, after 8 weeks of H57 supplementation, the H57 ewes were 17% heavier than the Control ewes (P = 0.001). Over late pregnancy, the H57 ewes gained 11.3 kg compared with 2.1 kg in the Control ewes. The liveweight advantage in the H57 ewes persisted for ~4 weeks post-lambing and tended to be higher (4.6 kg, P = 0.08) at the end of the lactation. Despite this, for the entire lactation period, the DWG of the Control ewes tended to be 53.5 g/day higher than the H57 ewes (P = 0.07). Consistent with the liveweight response, BCS increased during gestation in H57 ewes and this difference remained through lactation. By the end of lactation the H57 fed ewes had a 14.6% higher BCS than the Control ewes (P = 0.02, Table 2).

Despite the higher liveweight gain of the H57 ewes during late pregnancy, there was no effect of supplementation on lamb birthweight (Table 3). However, from birth to Day 21, lambs produced by the H57 ewes grew 25% faster than those of the Control ewes (P = 0.03). Thereafter, from Day 21 to Day 63, lamb growth rate was similar between the two treatment groups so that by Day 63, a treatment effect on lamb liveweight was not detectable.

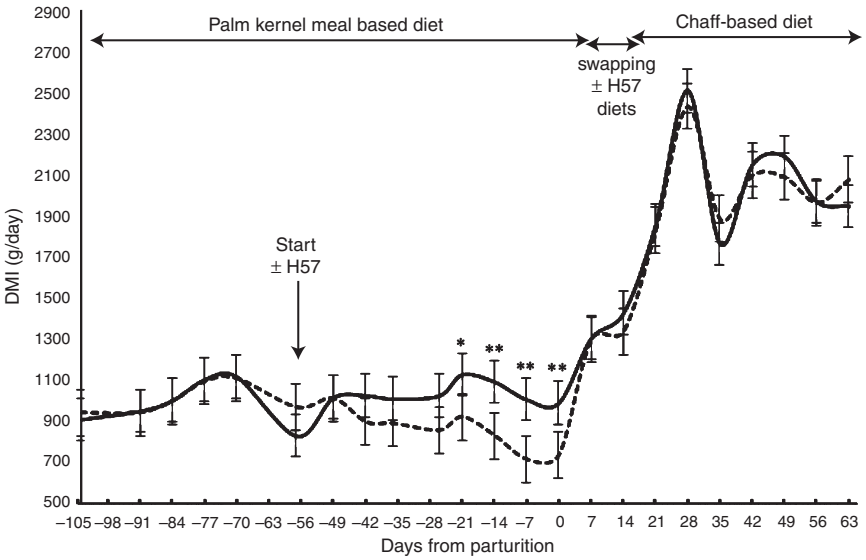

Fig. 1. Effect of supplementation with *Bacillus amyloliquefaciens* strain H57 on the dry matter intake of pregnant and lactating ewes. Solid line: H57 group; dashed line: Control group. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (between treatments within weeks).

Table 2. Ewe growth parameters due to supplementation with *Bacillus amyloliquefaciens* strain H57
DMI, dry matter intake, DWG, daily weight gain, BCS, body condition score

	Adjustment				Late pregnancy				Lactation			
	Control	H57	s.e.m.	P	Control	H57	s.e.m.	P	Control	H57	s.e.m.	P
n	12	11			12	11			10	8		
Initial weight (kg)	47.4	47.2	1.99	0.96	52.2	51.8	1.82	0.89	45.2	53.9	1.20	0.001
Final weight (kg)	49.6	50.3	1.81	0.79	54.3	63.6	1.60	0.001	50.2	54.8	1.40	0.08
DMI (g/day)	1033	1002	28.3	0.45	873	1018	44.2	0.03	1865	1850	21.4	0.66
DWG (g/day)	95.2	108.0	13.6	0.52	29.8	194	24.9	0.001	68.2	14.7	19.3	0.07
BCS	3.19	3.15	0.97	0.78	3.20	3.59	0.12	0.04	2.40	2.75	0.09	0.02

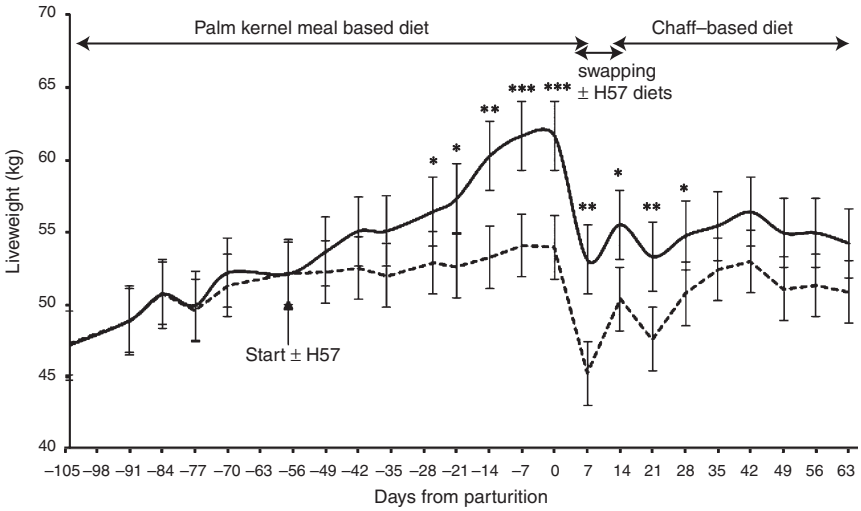

Fig. 2. Effect of supplementation with *Bacillus amyloliquefaciens* strain H57 on the liveweight trajectory of pregnant and lactating ewes. Solid line: H57 group; dashed line: Control group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (between treatments within weeks).

Table 3. Effect of *Bacillus amyloliquefaciens* strain H57 on lamb growth rate
DWG, daily weight gain

	Control	H57	s.e.m.	*P*
n	10	8		
Birthweight (kg)	4.07	4.20	0.19	0.73
Final liveweight (kg)	23.3	24.5	0.73	0.40
DWG (0–21 days old) (g/day)	272	349	21.0	0.03
DWG (22–63 days old) (g/day)	320	310	12.7	0.57
DWG (0–63 days old) (g/day)	309	318	11.5	0.64

Digestibility and nitrogen retention in pregnancy

Nitrogen retention was similar across the planned treatment groups in the adjustment period. After 21 days of treatment, nitrogen retention in the H57 group increased to double that of the Control group ($P = 0.01$), despite nitrogen intake and nitrogen digestibility being similar. In that period, urinary nitrogen, expressed as percentage of nitrogen intake, was 31.6% lower in H57 ewes than in the Control ewes ($P = 0.02$) but digestibility of DM, organic matter, and neutral detergent fibre remained unaffected by treatment (Table 4).

Table 4. Effect of *Bacillus amyloliquefaciens* strain H57 on digestibility and nitrogen utilisation of pregnant ewes
DMD, dry matter digestibility; OMD, organic matter digestibility; NDFD, neutral detergent digestibility; CPD, crude protein digestibility

	Adjustment				Pregnancy			
	Control	H57	s.e.m.	*P*	Control	H57	s.e.m.	*P*
				Nitrogen intake				
g/day	18.8	18.1	0.72	0.51	17.5	20.4	1.20	0.11
				Urinary nitrogen				
g/day	9.99	8.97	0.56	0.23	9.27	8.23	0.68	0.15
% intake	53.7	53.3	2.37	0.90	53.7	40.8	3.52	0.02
				Faecal nitrogen				
g/day	6.41	5.22	0.46	0.67	4.91	6.03	0.54	0.17
% intake	32.8	31.0	1.41	0.37	27.1	28.9	1.67	0.47
				Nitrogen retention				
g/day	2.59	2.53	0.43	0.92	3.34	6.13	0.49	0.01
% intake	13.5	15.6	2.51	0.53	19.2	30.3	2.94	0.02
				Digestibility (%)				
DMD	63.2	65.0	1.32	0.33	66.5	68.3	1.79	0.48
OMD	67.6	69.3	1.23	0.32	69.6	70.8	1.66	0.62
NDFD	44.7	45.8	2.59	0.61	45.7	47.0	3.22	0.78
CPD	67.2	68.8	1.41	0.42	72.7	71.1	1.63	0.48

Rumen fermentation

During the adjustment period in pregnancy, on Day –58, rumen fermentation parameters were similar across the planned treatment groups. But when tested on Day –21, 36 days after treatment began, the rumen pH in the H57 ewes tended to be higher than the Control ewes (0.33 pH units, $P = 0.07$, Table 5), the concentration of ammonia in the rumen was approximately half that of Control ewes ($P = 0.008$), and total VFA concentration was 34% lower than in the Control ewes ($P = 0.02$). The molar percentages of acetate were similar in both treatment groups, but the H57 ewes had 30% less propionate ($P = 0.02$) and 90% more valerate ($P = 0.01$), and a tendency for 21% more butyrate ($P = 0.09$) in their rumen fluid. The acetate : propionate ration was 21% higher in the H57 ewes compared with the Control ewes ($P = 0.04$). During lactation, rumen fermentation products and rumen pH were unaffected by H57 treatment.

Discussion

H57-inoculated feed pellets improved the feeding value of a PKM-based diet. The labour-saving consequence of probiotic

Table 5. Effect of supplementation with *Bacillus amyloliquefaciens* strain H57 on rumen fermentation in pregnant and lactating ewes (±s.e.m.)
VFA, volatile fatty acids; A : P ratio, acetate : propionate ratio

	Adjustment				Pregnancy				Lactation			
	Control	H57	s.e.m.	*P*	Control	H57	s.e.m.	*P*	Control	H57	s.e.m.	*P*
Rumen pH	6.98	6.84	0.10	0.38	6.79	7.11	0.11	0.07	7.08	7.07	0.06	0.95
Ammonia (mg/L)	120	101	14.6	0.35	147	69	19.5	0.01	125	113	11.8	0.71
Total VFA (mmol/L)	59.8	71.4	7.66	0.28	61.4	40.5	5.9	0.02	68.9	77.6	6.13	0.12
					Molar VFA (% total)							
Acetate	32.3	38.93	3.86	0.23	59.2	60.9	1.85	0.46	79.4	79.8	0.59	0.90
Propionate	18.6	21.51	2.86	0.48	23.8	18.2	1.58	0.02	11.3	10.7	0.63	0.77
n-Butyrate	6.91	9.17	1.32	0.23	14.2	17.2	1.27	0.09	5.56	5.85	0.28	0.37
Iso-Butyrate	0.66	0.61	0.05	0.50	0.98	0.87	0.17	0.75	1.5	1.43	0.09	0.58
Iso-Valerate	0.67	0.57	0.10	0.45	1.06	1.12	0.09	0.87	1.75	1.68	0.14	0.69
n-Valerate	0.62	0.66	0.16	0.85	0.81	1.54	0.15	0.01	0.56	0.5	0.07	0.43
A : P ratio	1.87	1.93	0.15	0.77	2.7	3.4	0.14	0.04	7.41	7.58	6.23	0.82

addition in the pellet during the manufacturing stage, afforded by the spore forming capacity of H57, also represents a valuable advance on current practice whereby probiotics are commonly added daily. This study is one of few that have tested and shown a benefit of probiotic supplementation in older rather than pre-weaned ruminants. During late pregnancy, the H57 ewes ate 16.6% more pellets, retained 45% more nitrogen when estimated between Day –36 and Day –26, and were 17% heavier by parturition. The present study not only corroborated the potential for an improvement in nitrogen balance, as found by Norton *et al*. (2008), but highlighted a greater efficiency in the use of nitrogen by reducing the rate of excretion of nitrogen in the urine. Moreover, H57 can also improve completely different types of diets. In the present study, H57 improved the feeding value of a concentrate pellet based on PKM and sorghum grain. By contrast, Norton *et al*. (2008) fed only hay diet, and only over a few weeks. The present study also shows that the benefit of H57 can persist over a relatively long period, at least over the last third of pregnancy and possibly into early lactation. These are the first reports of a benefit in ruminants from the probiotic H57.

Unfortunately, the testing of the H57 during lactation was compromised due to the development of an unexpected copper toxicity in both treatment groups plus an accidental feeding of the Control ewes with the H57 supplement in early lactation. In retrospect, focusing now on the background diet rather than the H57 outcomes, the experiment was over-ambitious in that the H57 was tested with a relatively novel background diet. It would have been easier to have used a standard commercial pellet but in using the PKM-rich pellet valuable background data was gained for future studies on how to incorporate PKM into ruminant diets. A salient lesson for example was not to use PKM in sheep diets. However, it still holds great potential for use in cattle diets. One ewe died in the week after parturition and subsequent blood and liver tissue biopsy confirmed a copper toxicity. The plasma copper from all ewes was in upper marginal band (20–25 mmol/L) indicating of chronic copper toxicity (Underwood and Suttle 1999), the details of which will be reported in a subsequent paper. The risk of copper toxicity in sheep fed PKM has been documented (Alimon *et al*. 2011). However, we had expected that the level of inclusion of PKM in the diet would have mitigated the risk, given that the dietary copper level in our pellets was estimated at 12.5 mg/kg DM, and the range within which chronic copper toxicity is likely to occur has been defined as 12–36 mg/kg DM (Underwood and Suttle 1999). Unfortunately, the exceptional sensitivity of sheep prevailed, compared with more tolerant ruminants such as cattle (Suttle 2010). In addition, the diets were accidentally swapped for a week, 7 days after parturition. The swap plus chronic copper toxicity likely negated any advantages the probiotic H57 could have conferred in lactation. However, the lamb growth response in those first 3 weeks may be due to an immediate effect of the probiotic in the lactating ewes, or more likely a carry-over effect consequential to the extra maternal weight gain in the H57 ewes advantaging milk production (not measured) in the ewes. Such a possibility should be investigated in future studies. Because of the accidental swap, we focus the discussion and conclusions of the benefits of H57 on the late pregnancy period.

We found DMI responses to the H57 in older pregnant ewes, whereas published literature indicates DMI responses to probiotics are more likely in pre-weaned calves. Kowalski *et al*. (2009) reported a 13% increase in starter diet intake in pre-weaned calves that had spores of *B. licheniformis* and *B. subtilis* added to their feed daily. Similarly, pre-weaned calves supplemented with *Lactobacillus* strains increased DMI by 29% resulting in increased liveweight gain (Frizzo *et al*. 2010). Other studies on pre-weaned calves showed much smaller or no increase in DMI and liveweight gains with *Lactobacillus*-based probiotics e.g. Timmerman *et al*. (2005) or a *Bacillus*-based probiotic (Riddell *et al*. 2010) but all previous studies that we are aware of in weaned older cattle found no effect on DMI (Garza-Cázares *et al*. 2001; Kumagai *et al*. 2004; Raeth-Knight *et al*. 2007; Malik and Bandla 2010; Qiao *et al*. 2010; Peng *et al*. 2012). If H57 can show benefits in reproducing ewes, further evaluation in weaned and more mature ruminants is warranted.

Consistent with the improvement in DMI in pregnancy, H57 stimulated a greater liveweight gain. By removing weight gain due to the gravid uterus (~11 kg for singleton-bearing ewes (Freer 2007)), the H57 ewes were estimated to deposit ~5 kg of maternal tissue whereas the Control ewes lost in the order of 4.3 kg, before parturition. Gravid uterus weight is likely to be similar between treatment groups as treatment had no effect on

lamb birthweight. Others have also shown improvements in liveweight gain, although not necessarily related to DMI responses, indicative of improved feed conversion efficiency, but the improvements were mainly found in pre-weaned cattle (Timmerman *et al*. 2005; Adams *et al*. 2008; Sun *et al*. 2010).

The ewes in the present study were relatively immature having been mated when they were between 10 and 18 months of age. Hence all ewes in the present study had the demand for nutrient for growth of their own maternal tissue in addition to the demand of the gravid uterus. Therefore, during early lactation the H57 ewes had more maternal reserves to mobilise to better overcome the expected, delayed improvement of DMI in the transition period of the first 3 weeks of lactation, and thereby to improve the potential for milk production. The potential for more milk is consistent with the higher growth rate observed in the H57 lambs during the first 3 weeks of life. Others have also noted probiotics improved milk yield and quality, in lactating ewes (Kritas *et al*. 2006) and cows (Qiao *et al*. 2010) due to probiotics, without a concomitant improvement in DMI. The higher growth rate of H57 lambs in our study was similar to the results of Thompson *et al*. (2011) who found that the growth rate of pre-weaned lambs was affected by liveweight gain during late pregnancy of ewes. Although the better growth of the H57 lambs in the first 3 weeks of life did not translate to heavier lambs at weaning, the result could mean more lambs per ewe at weaning in a commercial flock. A lamb that is well fed in the first few weeks of life will have a greater chance of survival, the first weeks of life being the time where lamb mortality is greatest (Hinch and Brien 2014).

Consistent with the argument that maternal tissue growth was enhanced by H57 fed in late pregnancy, the BCS of the H57 ewes was 0.39 units better than the Control ewes at parturition. The BCS is positively related to both fat and muscle reserves in a ewe. We propose that this difference in BCS is mainly due to a difference in muscle, as supported by the nitrogen balance data.

The H57 ewes retained more nitrogen during pregnancy. Given DMI was enhanced throughout late pregnancy, it is reasonable to expect that an important component of this improvement was a greater intake of nitrogen. During the nitrogen balance period, a DMI response was not detected, perhaps not surprising given that intake is commonly reduced in stressful situations and the move from the pens to metabolism crates likely stressed the ewes (Van Soet 1989). Despite that, an improvement in the efficiency of nitrogen utilisation was still evident. The H57 ewes retained more nitrogen per unit of nitrogen digested as also found by Norton *et al*. (2008) and Khalid *et al*. (2011). The improved efficiency of use may be due to a greater flow of microbial and/or rumen escape protein resulting in an improved quality of amino acid absorbed or it may be that a lesser proportion of absorbed or mobilised amino acids were degraded to produce energy or glucose.

The digestibility of nitrogen and other feed components including DM, organic matter and neutral detergent fibre in late pregnant ewes were similar between treatment groups. Kumagai *et al*. (2004) for ewes and Masucci *et al*. (2011) for buffalo calves also found no difference in digestibility due to the addition of probiotics. In contrast, Galina *et al*. (2009) for goats, Qiao *et al*. (2010) for Holstein cows and Khalid *et al*. (2011) for lambs, found that a supplement of probiotic increased fibre digestibility. The greater DMI of the H57 ewes may have masked any effect of H57 on digestibility as faster rate of passage of a given diet consequent on increased DMI is likely to limit opportunity for digestion, especially in the reticulo-rumen (Van Soet 1989).

Despite no effect on digestibility, the rumen environment was modified during late pregnancy, after 35 days of exposure to H57. Rumen pH tended to be higher in the H57 ewes ($P = 0.07$), and total VFA and ammonia concentrations were lower ($P = 0.02$). The improvement of rumen pH with high concentrate diets is often a targeted effect of probiotics (Chaucheyras-Durand *et al*. 2008, 2012). Stabilisation of the rumen pH is considered to be a primary mechanism by which probiotics can improve the balance of the rumen microbial community to improve nutrient output from the rumen to the ruminant host. Sun *et al*. (2010) found that the addition of *B. subtilis* to pre-weaned calves reduced VFA and ammonia concentrations and suggested this was a consequence of a greater absorptive capacity of the rumen as evidenced by a greater surface area of rumen papillae. Papillae surface area could not be measured in the present study but should be considered in future studies. Despite being lower, the concentration of ammonia in the H57 group remained in the optimal range of 60–80 mg/L for the activity of ruminal microbiota, although the total VFA concentration was lower than expected (40 compared with 61 mmol/L in the Control ewes). The high concentration of rumen ammonia in the Control group may have enhanced ammonia absorption from the rumen to the blood to account for the increased urinary nitrogen excretion observed. Despite the effect of H57 on ruminal fermentation, the low concentration of total VFA in both treatment groups (a normal range: 70–130 mmol/L (Dijkstra *et al*. 2005)) could be a consequence of the relatively low digestibility of organic matter in the PKM component of the diet (Chanjula *et al*. 2011) or a consequence of sampling time being too distant from feeding time when the production of VFA is lowest (Pitt and Pell 1997).

The molar percentages of individual VFA in pregnant ewes were also affected by H57. Lower propionate and consequently an increased ratio of A : P was recorded in the H57 group. By contrast, supplementation with similar probiotics such as *B. subtilis* decreased A : P ratio in lactating cows (Peng *et al*. 2012) and weaned calves (Sun *et al*. 2011). Valerate and butyrate are preferentially metabolised in the epithelium of the rumen, therefore higher concentration of these acids can stimulate the development of rumen papillae (Kristensen *et al*. 1998; Shen *et al*. 2005). In our study, the H57 ewes had 1.9 times more valerate in their rumen fluid ($P = 0.01$) and tended to have higher butyrate concentrations ($P = 0.09$). The higher concentration of these VFA could explain a greater ruminal absorptive capacity and thereby explains the lower total VFA level in the H57 ewes. The rumen fluid was also collected to evaluate changes in rumen ecology due to H57 supplement, and will be reported in a subsequent paper.

Conclusion

The probiotic *B. amyloliquefaciens*, strain H57 improved feed intake and maternal liveweight gain in late pregnancy of first-parity ewes fed a diet based on PKM. The potential for improved

subsequent lactation should be assessed through further experimentation.

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