Beneficial changes in rumen bacterial community profile in sheep and dairy calves as a result of feeding the probiotic *Bacillus amyloliquefaciens* H57

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Abstract

Aims: The probiotic *Bacillus amyloliquefaciens* H57 increased weight gain, increased nitrogen retention and increased feed intake in ruminants when administered to the diet. This study aims to develop a better understanding of this probiotic effect by analysing changes in the rumen prokaryotic community.

Methods and Results: Sequencing the 16S rRNA gene PCR amplicons of the rumen microbiome, revealed that ewes fed H57 had a significantly different rumen microbial community structure to Control sheep. By contrast, dairy calves showed no significant differences in rumen community structure between treatment groups. In both instances, H57 was below detection in the rumen community profile and only present at low relative abundance as determined by qPCR.

Conclusions: The altered rumen microbial community in sheep likely contributes to increased weight gain through more efficient digestion of plant material. As no change occurred in the rumen community of dairy calves it is suggested that increased weight gain may be due to changes in community function rather than structure. The low relative abundance of H57 as determined by qPCR, suggests that weight gain was not directly mediated by the probiotic, but rather by influencing animal behaviour (feed consumption) and/or altering the native rumen community structure or function.

Significance and Impact of Study: This study provides a novel look at the rumen prokaryotic community in both sheep and dairy calves when fed H57. These findings improve our understanding for the potential rumen community involvement in H57-enabled weight gain. The study reveals that the probiotic *B. amyloliquefaciens* H57 is capable of benefiting ruminants without colonising the rumen, suggesting an indirect mechanism of action.

Keywords

Rumen; *Bacillus amyloliquefaciens* H57; probiotic; community profile; sheep; dairy calves.

Introduction

In the 1950's the use of antibiotics in agriculture increased, as farmers discovered that antibiotics such as avoparcin, streptomycin, penicillin and tetracycline, could promote growth when administered with feed in sub therapeutic quantities (Marshall and Levy, 2011). However, the increased use of avoparcin as a feed additive resulted in the emergence of avoparcin and vancomycin resistant enterococci (Aarestrup, 1995), leading to a ban on the use of avoparcin in Denmark in 1995. This was followed by a European-wide ban on use of medically-important antibiotics as feed supplements (Wielinga *et al.*, 2014), resulting in increased interest in probiotics as alternative growth promotants in animal agriculture.

In ruminants, probiotics have increased milk yields and meat production. Milk yield was increased by 7.6% in dairy cows fed a combination of *Lactobacillus acidophilus* NP51 and *Propionibacterium freudenreichii* NP24 (Boyd *et al.*, 2011). A combination of *Lactobacillus casei* Zhang and *Lactobacillus plantarum* P-8 also significantly increased milk production in dairy cows (Xu *et al.*, 2017). Xu *et al.* (2017) observed that after 30 days, the difference in the amount of milk produced between the control and treated cows averaged at 9.08 kg. In Holstein bull calves fed *Propionibacterium jensenii* 702, a 25% increase in weight gain was reported, and was hypothesised to be a result of increased ruminal propionate production (Adams *et al.*, 2008). The increase in daily weight gain of Holstein bull calves was also found when fed a supplement of *Bacillus subtilis* natto (Sun *et al.*, 2010). In each instance, it was not clear whether the probiotic colonised the gastrointestinal tract, directly influencing the host nutrition, or whether the probiotics acted indirectly on either the feed or rumen microbiome.

Bacillus species are popular as probiotics because they produce antimicrobial compounds and form spores. Spores prolong shelf life, provide protection from heat, UV radiation, desiccation and low pH, such as in the gastric stomach (Riesenman and Nicholson, 2000). Antimicrobial compounds such as surfactin, fengycin and bacillomycin D, have strong anti-fungal, -bacterial and -viral properties (Koumoutsi *et al.*, 2004; Huang *et al.*, 2011; Liu *et al.*, 2011). The probiotic *B. amyloliquefaciens* strain H57 was selected as an inoculant to prevent mould development on hay (Brown and Dart, 2005). The freeze-dried powdered product contains a large number of spores and has a long shelf life at room temperature. Ewes fed grass-clover hay inoculated with H57 ate more hay, had greater weight gain and retained more nitrogen, than ewes fed uninoculated hay (Brown and Dart, 2005).

Le *et al.* (Le *et al.*, 2016b), showed that pregnant Dorper ewes fed a poor quality concentrate diet supplemented with H57 at 10^9 cfu kg^{-1} pellets, had significantly increased weight gain (194 vs 30 g day^{-1}) coupled with an increased uptake of dry matter (1,019 vs 874 g day^{-1}) and increased nitrogen retention (6.13 vs 3.34 g day^{-1}). The beneficial effect of H57 was also demonstrated in dairy calves (Le *et al.*, 2016a) fed sorghum based concentrate pellets, with and without H57 as they weaned. Not only was there a 39% increase in live weight gain, a 16.2% increase in feed conversion efficiency and a reduction in weaning age by nine days, but the H57 fed calves also had a reduction in the occurrence and duration of diarrhoea. The probiotic H57 not only increased meat production but also improved animal health.

The current study investigates the impact of H57 on the rumen microbiome in these latter studies, to evaluate possible means by which H57 modifies rumen metabolism and promotes more efficient feed utilisation.

Materials and methods

Feeding trials

Samples were collected from the ewes and dairy calves involved in the H57 feeding trials reported by Le *et al.* (2016b) and Le *et al.* (2016a). The Animal Ethics Committee of the University of Queensland approved the use of these animals and experimental procedures under the approval numbers of SVS/022/13/ARC and SVS/280/14/ARC for the sheep and calves respectively.

In the sheep trial (Le *et al.*, 2016b), 24 first parity white Dorper ewes (liveweight: 47.3 ± 6.9 kg and age: 15 ± 4.6 months) were randomly divided into two treatment groups (Control and H57). All ewes were fed a palm kernel meal and sorghum based pellet diet. From week eight of the trial, pellets fed to the H57 treated group contained 2.85 x 10^9 cfu kg^{-1} of H57 spores. Rumen fluid samples were collected at week eight (day -58 before parturition), immediately before the H57 treatment group were started on H57 inoculated pellets, and at week 13 (day -21 before parturition) five weeks later.

The calf trial (Le *et al.*, 2016a) involved 24 Holstein-Friesian calves (12 heifers and 12 bulls: liveweight, 51.4 ± 5.7 kg; age, 28 ± 3 days). The calves were randomly divided into two treatment groups (Control and H57) with an equal number of bulls and heifers in each group and balanced starting weight. From 24 h after birth until four weeks of age the calves were separated from their dams and fed whole milk by a robotic milk feeder (maximum five feeding times day^{-1}, maximum 2 L each feed) and *ad libitum* sorghum-based, starter concentrate pellets that were antibiotic free. From 4 to 12 weeks of age the calves were fed 6 L of whole milk per day and *ad libitum* starter pellets. The H57 group were fed pellets of the same ingredient composition as starter pellets but containing H57 spores at 3.2 x 10^8 cfu kg^{-1}. When the calves reached a liveweight of 70 kg and a pellet intake of 700 g day^{-1} for three consecutive days, they were individually weaned onto a 100% pellet diet. Rumen fluid was collected at week four,

prior to feeding H57-starter pellets and week 12, after eight weeks of feeding H57-starter pellets and after the majority of the calves were weaned.

Sample collection and DNA extraction

Rumen fluid samples from both sheep and dairy calves were collected using a custom made stomach tube. The stomach tube consisted of a 1.5 m Powaflex TPR hose with an internal diameter of 8 mm (Advanced Industrial Products, Darra, QLD, Australia) and custom-made brass filter with 1 mm pore diameter holes. Rumen fluid contents were withdrawn into a 60 mL catheter tip syringe (Terumo, Somerset, NJ, USA) attached to the stomach fluid collection tube and placed into sterile 250 mL containers (Thermo Fisher Scientific, Waltham, MA, USA). After collection the rumen fluid was divided into 1 mL aliquots in sterile 1.5 mL microcentrifuge tubes (Eppendorf, Hamburg, Germany). The 1 mL aliquots were then immediately flash frozen in liquid nitrogen and stored on dry ice for transportation back to the laboratory. Final storage of rumen fluid samples was at -80°C. Genomic DNA was extracted from both sheep and calf rumen fluid samples via the RBB + C method of Yu and Forster (2005). The concentration and purity of DNA samples were then determined using the Qubit® dsDNA BR Assay Kit with the Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). DNA was diluted to 5 ng/μL for sequencing.

Amplicon library preparation and sequencing

The extracted DNA was used as template to prepare 16S rRNA gene fragments for sequencing using the 16S Metagenomic Sequencing Library Preparation methodology (Illumina, 2013). Rumen bacteria and archaea were sequenced using universal primers that target the V6-V8 region of the 16S rRNA gene (itag926F and itag1392wR, see Table 1).

The 25 μL PCR reaction comprised 12.5 ng of template DNA, 0.2 μM of each primer and 1x KAPA HiFi HotStart ReadyMix (Kapa Biosystems Inc., Wilmington, MA, USA). PCR amplification involved an initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s and a final extension step at 72°C for 5 min. The resulting PCR product was purified to remove any free primers or primer dimers using Agencourt AMPure XP beads according to the manufacturer's instructions (Beckman Coulter Australia Pty Ltd, Lane Cove, NSW, Australia).

Dual indices and Illumina sequencing adapters (Illumina, 2013) were added to the amplified product with an additional PCR step. The 50 μL PCR reaction consisted of 5 μL of template DNA, 5 μL of each index primer and 25 μL of 2x KAPA HiFi HotStart ReadyMix. Amplification was achieved through an initial denaturation step of 95°C for 3 min, followed by eight cycles, comprising denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s and a final elongation step of 72°C for 5 min. The resultant PCR product was purified using Agencourt AMPure XP beads according to the manufacturer's instructions. The final indexed PCR product was then sequenced using the MiSeq sequencing platform (Illumina, San Diego, CA, USA). Raw sequencing reads were submitted to the NCBI Sequence Read Archive under accessions SRR6316319-SRR6316413.

Bioinformatic analysis

Sequencing reads in the form of fastq files were first trimmed using Trimmomatic v0.32 (Bolger *et al.*, 2014). The trimming process removed primer sequences, reads with less than 250 bp and poor quality sequences that had an average quality score of less than 15 over four bases. The trimmed files were converted to fasta files and processed using the pick_open_reference_otus.py script in the QIIME pipeline (Caporaso *et al.*, 2010). Sequences were clustered and OTUs (Operational Taxonomic Units) were assigned using a 99% similarity

parameter to cluster OTUs at the species level (Bosshard *et al.*, 2003).Those sequences with less than 0.05% abundance were removed and remaining sequences were placed into an OTU table. Chimeric sequences introduced during the PCR process were detected and removed from the representative OTU sequences using Chimeraslayer (Haas *et al.*, 2011). For taxonomic classification, representative OTU sequences were aligned against sequences in the Greengenes database v2013/05 (DeSantis *et al.*, 2006) using BLAST (Altschul *et al.*, 1990). Variations in relative abundance caused by differences in 16S rRNA gene copy number were adjusted using CopyRighter v0.46 (Angly *et al.*, 2014). Differences in rumen microbial communities between the Control and H57 fed animals were identified using principle component analysis (PCA) on a Hellinger transformed OTU table (Rao, 1995). Using a Hellinger adjusted OTU table reduces skewing caused by highly abundant OTUs (Rao, 1995; Legendre and Gallagher, 2001). All analyses were performed using R v3.1.2 (R Core Team, 2013) with the vegan package (Dixon, 2003). Significant differences in OTU relative abundance were assessed using a Student's t test (Tarasińska, 2005).

Quantitative PCR

The quantification of H57 in rumen fluid samples was performed using a real-time PCR protocol developed by Yong *et al.* (2013). The protocol is specific to *B. amyloliquefaciens*, using primes and probe that target a specific region of the pgsB gene (Table 1). Real-time quantitative PCR (qPCR) amplification and detection was performed on a Rotor-Gene RG-6000 (Qiagen, Valencia, CA, USA). Reaction volumes of 25 µL were performed using the RealMasterMix Probe 1x (5 PRIME, Gaithersburg, MD, USA) with primer and probe concentrations of 0.9 µM and 0.05 µM, respectively. Reactions were performed in triplicate with 5 µL of template DNA. Amplification was performed with cycling parameters that

consisted of an initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing and extension at 60°C for 34 s (Yong *et al.*, 2013).

Aliquots of H57 at concentrations from 10^{10} to 10^{4} cells mL^{-1} were used to generate a standard curve. Using a Petroff-Hausser Counting Chamber (Hausser Scientific, Horsham, PA, USA), an overnight culture of H57 was enumerated, as described previously (Ouwerkerk *et al.*, 2002). The culture was then diluted to specified concentrations in rumen fluid to account for the presence of any potential inhibitory compounds (Ouwerkerk *et al.*, 2002). DNA was then extracted from each standard using the RBB + C method (Yu and Forster, 2005). A curve of H57 standards was generated through plotting the cycle threshold values against the log10 cell numbers determined microscopically. The number of H57 cells mL^{-1} for each sample was then calculated based on regression analysis of cycle thresholds with standard curve.

Results

16S rRNA gene amplicon profiling

From sheep rumen fluid samples, 38,402 ± 3,727 (mean ± standard error of the mean; SEM) reads $sample^{-1}$ were sequenced with 29,427 ± 2,874 remaining after quality filtering. The calf samples produced a mean total of 92,246 ± 3,377 reads with 47,227 ± 2,271 remaining after quality filtering.

A PCA analysis of the 16S rRNA gene amplicon sequencing data from the sheep showed clustering of all samples taken at the week eight time point, before the addition of H57 (Fig 1). The first principle component (PC1) represents 30.3% of variance while the second principle component (PC2) represents 9.9% of variance. At week 13 after the H57 sheep were fed H57 for five weeks, the animals of each treatment group formed distinctly different clusters (Fig 1), demonstrating the formation of a significantly different bacterial community between treatment groups (P = 0.001). The rumen fluid of Control sheep at week 13 did not show a

significant difference in bacterial community to the rumen fluid of sheep in both treatment groups at the week eight time point (P = 0.053). The trend towards significance between the week 13 sheep and week eight control sheep is likely due to slight differences that have developed over the five week time gap.

For a comparison of bacterial diversity between the communities, sequencing data was viewed at the phylum, family and genus level (Fig 2). Fig 2 A shows that at the phylum level there are substantial differences between treatment groups at week 13, with an increased abundance of Firmicutes in the H57-fed verses Control sheep (33 ± 3% vs 17 ± 4%; mean relative abundance ± SEM). The reduction in Firmicutes in the Control sheep was the result of an increase in Proteobacteria with relative abundances of 19 ± 4% and 3 ± 1% for the Control and H57-fed sheep, respectively (Fig 2 A). In both treatment groups, the bacterial community was dominated by Bacteroidetes (55 ± 5%, Control and 60 ± 3%, H57 fed sheep), primarily represented by the *Prevotellaceae* family with relative abundances of 52 ± 5% and 53 ± 3% for the Control and H57-fed sheep respectively (Fig 2 B). Within the Firmicutes, *Veillonellaceae* was the dominant family in Control sheep (10 ± 4%) while *Lachnospiraceae* (27 ± 3%) was the dominant family in H57-fed sheep (Fig 2 B). The dominant families within the respective treatment groups are in turn dominated by individual genera. In the *Prevotellaceae* family, *Prevotella* spp. were dominant in each treatment group, in fact *Prevotella* was the most dominant genus overall (51 ± 5%, Controls; 48 ± 3%, H57-fed; Fig 2 C). At the genus level, the greatest differences between treatment groups were larger *Roseburia* populations in the H57 fed sheep and a larger *Succinivibrionaceae* population in Control sheep.

Prevotella spp. dominated the bacterial community profiles of both the Control and H57-fed sheep, therefore the data was analysed for differences in the relative abundance of individual *Prevotella* OTUs. Two *Prevotella* spp. (OTUs 814711 and 647403), as well as a *Succinivibrionaceae* OTU (9357; Fig 3) dominated the rumen fluid of ewes not fed H57

(Control wk 8 and wk 13, H57 wk 8). By contrast, a different *Prevotella* OTU (216587) was dominant in the H57-fed sheep (H57 wk 13) as well as a *Roseburia* OTU (4463709; Fig 3**)**. To assess the species level classification of dominant OTUs, the sequences were aligned against the Greengenes database v2013_08 (DeSantis *et al.*, 2006) using BLAST (Altschul *et al.*, 1990). *Prevotella* (OTU 647403) showed all sequence alignments with 100% similarity over the partial 16S rRNA gene sequence belonging to *P. ruminicola*, suggesting that this species is the most likely representative of OTU 647403. A BLAST search of *Prevotella* OTU 216587 showed 100% identity with the 16S rRNA gene of both *Prevotella sp.* DJF_CP65 and *Homeothermaceae*, although all alignments with 98-99% identity also belong to *Prevotella sp.* DJF_CP65, suggesting that this is the species designation of *Prevotella* OTU 216587. The search results of *Roseburia* OTU 4463709 suggest that it is most likely a *Roseburia faecis* species with 100% identity over the partial 16S rRNA gene sequence.

Though less abundant, there were a number of other OTUs that were significantly different in abundance between the ewes of each treatment group (Fig 3). This included *Methanobrevibacter* sp. AbM4 (OTU 103975), which was more abundant in the Control sheep compared to the H57 fed sheep, with abundance of 8.1 ± 2.34% and 2.29 ± 0.62% (mean ± SEM) respectively.

No *Bacillus* OTUs were observed within the community profiles of H57 fed sheep, suggesting that H57 was either not present or at such low concentrations that it does not reach the detection threshold of this assay. An analysis of the complete OTU table before the removal of low abundant OTUs, revealed only three reads to classify as *Bacillus* (OTU 4385535), two from the H57-fed ewe 391 and one read sequenced from the H57-fed ewe 611 (S1 Table). A BLAST search of the reference sequence for OTU 4385535 against the 16S rRNA gene of the H57 genome (Schofield *et al.*, 2016), showed 100% identity over the 250 bp sequence, suggesting that this OTU is a true representation of H57 within the rumen samples.

For the calves there was no significant difference in bacterial populations between the Control animals and those fed H57 at either time point (Fig 4). However, there was a periodic change in bacterial populations between the samples at week four to those sampled at week 12. At week four the dominant OTUs were *Prevotella* (OTU 572743) and *Phascolarctobacterium* (OTU 22697), while at week 12 *Succinivibrionaceae* (OTU 805647) and *Prevotella* (OTU 216587) were the most dominant OTUs (Fig 4). No *Bacillus* OTUs were observed in the community profiles of calves fed H57 inoculated pellets for eight weeks (week 12). Like the sheep, an analysis of the complete OTU table before the removal of low abundant OTUs revealed three reads to classify as *Bacillus* (OTU 4385535), two from the H57-fed calf 1468 and one read sequenced from the H57-fed calf 468 (S1 Table). In the calves two other *Bacillus* OTUs were observed, though still low abundant with a total of four reads for OTU 4442381 and two reads for OTU 4364491 (S1 Table).

Quantitative PCR of *B. amyloliquefaciens* H57 in rumen fluid samples

H57 was quantified in the rumen fluid of both sheep and calves using a *B. amyloliquefaciens* spp. specific qPCR assay (Yong *et al.*, 2013). Due to intrinsic limitations of the assay it was only possible to accurately quantify populations greater than 1×10^4 cells mL^{-1}. Counts below this threshold are unable to be confidently differentiated from non-specific PCR products (Yong *et al.*, 2013; Mojà, 2014).

The populations of H57 present in sheep rumen fluid averaged at $2.65 \times 10^4 \pm 1.72 \times 10^3$ cells mL^{-1} (mean ± SEM) in H57 fed animals, whilst H57 was not detected (above the threshold) in any of the Control animals (Fig 5).

Detection of H57 by qPCR in rumen fluid from calves at week 12 of the feeding trial showed that the H57 fed calves possessed similar populations of H57 to the H57 fed sheep. The H57 numbers in the H57-fed calves averaged $3.59 \times 10^4 \pm 2.06 \times 10^3$ cells mL^{-1} with the

exception of calf 1468, which with a population above 2 x 10^5 cells mL^{-1}, was 10 fold higher than in all other treated calves and therefore regarded as a statistical outlier and removed (Fig 6). No H57 was detected above the detection threshold in the Control calves at week 12 (Fig 6).

To understand why H57 was not present in the 16S rRNA gene amplicon data, we calculated the sequencing coverage depth to determine if sequences of H57 were expected to be present given the concentration of H57 in the rumen. For the sheep we retained 29,427 ± 2,874 (mean ± SEM) reads $sample^{-1}$ after quality filtering. For one read of H57 to be sequenced it needed to be within 0.003% of the population (1/29,427 x 100). Total bacteria in the rumen have been quantified as between 4.0 x 10^9 to 8.8 x 10^{10} cells mL^{-1} for sheep and cattle (Hungate, 1966; Guo *et al.,* 2010), therefore for H57 to be sequenced it would need to be present in numbers greater than 1.39 x 10^5 cells mL^{-1} (0.003% of 4.0 x 10^9), at the most dilute estimate of ruminal bacterial density. In the calf samples we retained 47,227 ± 2,271 reads after quality filtering. Therefore H57 needs to be at least 0.002% of the bacterial population. With a total rumen microbiome population of 4.0 x 10^9 cells mL^{-1}, H57 needs to be present in numbers greater than 6.7 x 10^4 cells mL^{-1} for at least one read to be sequenced. The qPCR data shows that H57 is not at sufficient cell numbers to appear within the sequencing data.

Discussion

The goal of this study was to determine the effect, if any, the probiotic *B. amyloliquefaciens* H57, has on rumen bacterial community structure in sheep and calves.

The prokaryotic communities of all 24 pregnant ewes were relatively similar prior to the feeding of H57, with the dominant OTUs being *Prevotella sp.* (OTU 814711) and *Prevotella sp.* (OTU 647403, identified as a *P. ruminicola*). In the rumen *Prevotella* spp. generally contribute a significant portion of the total bacterial population with relative

abundances ranging from 42% to 60% (Stevenson and Weimer, 2007; Castro-Carrera *et al.*, 2014). There is considerable diversity within the *Prevotella* genus but in the rumen the most well known species are *P. ruminicola*, and *P. bryantii*. A comparative genome analysis showed *P. ruminicola* and *P. bryantii* have an abundance of enzymes associated with the digestion of hemicellulose and pectin components of the plant cell wall (Dodd *et al.*, 2010; Purushe *et al.*, 2010). These *Prevotella* spp. also demonstrate strong proteolytic activity in rumen fluid (Griswold *et al.*, 1999). The abundance of *Prevotella* spp. (in particular *P. ruminicola*) in our baseline rumen fluid samples demonstrate that before the addition of H57 the dominant rumen microbiome is consistent with previously known rumen bacterial communities, and these dominant organisms likely contribute to the digestion of proteins and non-structural components of the plant cell wall.

After feeding H57 for five weeks a significant increase in weight gain compared to the Control sheep had resulted (Le *et al.*, 2016b), along with a shift in the dominant bacterial populations. With a different *Prevotella* OTU (*Prevotella sp.* DJF_CP65 OTU 216587) as well as *Roseburia faecis* (OTU 4463709) being the dominant populations. Little is known about the potential role of *Prevotella sp.* DJF_CP65, as the only data available on this species is the unpublished data deposited in GenBank (Clark *et al.*, 2015) under the accession EU728727.1, stating that it was isolated from pig intestine in Denmark. It is possible that whatever influence H57 has had on the rumen, allowed this particular *Prevotella* species to proliferate, which may digest plant material more effectively for increased host weight gain.

The second most abundant OTU within the H57 fed sheep was *Roseburia faecis*, a strictly anaerobic gram-variable rod shaped bacterium that has been isolated from human faeces (Duncan *et al.*, 2006). Biochemical growth studies showed that *R. faecis* are capable of producing butyrate and formate as major end products and lactate as a minor product from the fermentation of glucose (Duncan *et al.*, 2006). VFAs such as butyrate are energy substrates

that are absorbed across the rumen epithelial wall to be utilised for cellular growth and development (Rémond *et al.,* 1995). It was thought that increased butyrate production could contribute to the increased weight gain observed in the H57 fed sheep. However, rumen fluid VFA analysis conducted by Le *et al.* 2016 (Le *et al.*, 2016b) only showed a slight increase in butyrate levels of H57 fed sheep (14.2% vs 17.2%; % of total molar VFAs), that was not significant (P = 0.09).

Although not as abundant, a *Methanobrevibacter* OTU was shown to be significantly more abundant in the Control sheep compared to the H57 fed sheep. *Methanobrevibacter* belong to a group of archaea called methanogens, which play a key ecological role in the rumen, removing hydrogen, so that fermentation can proceed efficiently (Saengkerdsub and Ricke, 2014). In doing so, methanogens produce methane gas that is eructated into the atmosphere, which is a considerable environmental problem for the agriculture industry. The reduction of methanogenic populations within the H57 fed sheep could lead to reduced methane emissions. Although this was an unexpected outcome of this study and methane measurements were not recorded to confirm this hypothesis.

Unlike the sheep, there were no significant differences found in the bacterial community structure of the Control and +H57 calves at week 12, after calves had been fed H57 for eight weeks. This was unexpected as Le *et al.* (2016a) observed a 20% greater live weight gain in the H57 fed calves compared with the Control calves. The H57 fed calves also suffered less diarrhoea compared to the Control calves suggesting that H57 was contributing to animal health (Le *et al.*, 2016a). It is possible that the same dominant populations between treatment groups are having a different influence on the rumen digestive process, or that the probiotic effect of H57 is not only restricted to affecting the rumen bacterial community, but may also interact with the host physiology.

At week 12 the dominant populations in both groups of calves were classified as belonging to the family *Succinivibrionaceae* (OTU 805647) and *Prevotella sp.* DJF_CP65 (OTU 216587). The latter is the same *Prevotella* OTU that was dominant in the pregnant ewes given pellets +H57 at week 13. Members of the *Succinivibrionaceae* family have a capacity to breakdown glucose and other carbohydrates producing succinate and acetate as major end products (Bryant *et al.*, 1958; O'Herrin and Kenealy, 1993; Stackebrandt and Hespell, 2006), which may be of benefit as the calves transition to the pelleted diet.

H57 was not detected in the 16S rRNA gene amplicon data of treated sheep and calves. Even when the threshold was dropped to include all sequenced OTUs, only 3 reads were identified as belonging to H57 in both the sheep and calves. It was determined by qPCR that the relative abundance of H57 within the rumen community was too low to appear in the amplicon sequencing data. The low relative abundance of H57 in the rumen makes it difficult to propose the mechanism for its positive impact on growth rate (Le *et al.*, 2016a; Le *et al.*, 2016b).

The genome of H57 encodes a number of genes that could impact rumen fermentation, including a number of antimicrobials, as well as genes involved with carbohydrate metabolism (Schofield *et al.*, 2016). The expression of these genes would be expected to be dependent on H57 spores germinating into vegetative cells. The H57 genome shows it may have the potential to grow under anaerobic conditions as it has the potential to reduce nitrate and therefore theoretically to respire anaerobically (Schofield *et al.*, 2016), but it is unclear if it does so, especially as the numbers found in the rumen were low. *Bacillus subtilis* spores can be carriers for carbohydrate degrading enzymes with the enzymes displayed and stabilised on the spore coat surface, fusing with spore coat proteins such as CotG and OxdD (Potot *et al.*, 2010). These enzymes retained their enzymatic activity and were stabilised by attachment to the spore coat. Sirec *et al.* (2012) demonstrated that the adsorption of β-galactosidase to the spore coat of *B.*

subtilis stabilised its enzymatic activity even when exposed to high temperatures (75°C and 80°C) for over an hour and low pH (pH 4). Perhaps antimicrobial lipopeptides and enzymes produced by the vegetative H57 cells during fermentation may absorb onto the H57 spores thus stabilising them against the high temperatures (c.80°C) of the feed pelleting process so that they remain active when fed to the animals. H57 spores may also benefit ruminants through the adsorption and stabilisation of fibre-degrading enzymes within the rumen allowing for greater degradation of plant materials. But further analyses would need to be performed to determine the mechanism or mechanisms of action that enable ruminants to benefit from H57 probiotically enhanced feed.

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Conflict of Interest

No conflict of interest declared.

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Table 1. PCR primers and probe

Primer	Sequence (5'-3')	Target gene	Annealing temperature	References
itag926F	AAACTYAAAKGAATTGRCGG	16S rRNA	50.0	(Engelbrektson *et al.*, 2010)
itag1392wR	ACGGGCGGTGWGTRC	16S rRNA	57.1	(Engelbrektson *et al.*, 2010)
pgsB726-f	TGGCGCCATGAGAATCCT	pgsB	58.7	(Yong *et al.*, 2013)
pgsB791-r	GCAAAGCCGTTTACGAAATGA	pgsB	58.9	(Yong *et al.*, 2013)
pgsB-probe	[a]FAM-CCGCTGCTCAGCACGAAGGAGC-TAMRA[b]	pgsB	69.3	(Yong *et al.*, 2013)

[a] FAM (6-carboxy-fluorescein).

[b] TAMRA (6-carboxy-tetramethylrhodamine).

Fig 1. Principal component analysis plot representing variations of OTU abundance in sheep. Individual ewes represented by symbol (△ = wk 8 Control sheep; ▲ = wk 8 H57 sheep; ◇ = wk 13 Control sheep; ◆ = wk 13 H57 fed sheep). Principal components 1 and 2 represent 30.3% and 9.9% of the variance, respectively.

Fig 2. Mean relative abundance of phyla, family and genera in sheep rumen fluid at week 13, identified by 16S rRNA gene sequencing. A) Phylum classification: ■ Actinobacteria, ⊞ Bacteroidetes, ◘ Euryarchaeota, ▨ Firmicutes, ▭ Nanohaloarchaeota, ▩ Proteobacteria,

Spirochaetes, Tenericutes. B) Family classification: *Atopobiaceae*, *Bifidobacteriaceae*, *Erysipelotrichaceae*, *Eubacteriaceae*, *Haloredivivales*, *Homeothermaceae*, *Lachnospiraceae*, *Methanobacteriaceae*, *Neisseriaceae*, *Prevotellaceae*, RF39, *Ruminococcaceae*, *Sphaerochaetaceae*, *Succinivibrionaceae*, *Veillonellaceae*. C) Genus classification: *Acidaminococcus*, *Butyrivibrio*, *Homeothermaceae*, *Lachnobacterium*, *Lachnospiraceae*, *Methanobrevibacter*, *Prevotella*, *Prevotellaceae*, RC9 YRC16, *Roseburia*, *Ruminobacter*, *Ruminococcaceae*, *Sporomusa*, *Succiniclasticum*, *Succinivibrionaceae*, Veillonellaceae hoa13_70F09. Genus legend only shows genera with greater than 1% relative abundance.

Fig 3. Heat map showing the relative abundance of OTUs present in sheep rumen fluid with a threshold of greater than 0.5% abundance. Each column represents an individual ewe at a certain time point, either at week 8 (prior to the addition of H57 to the diet) or week 13 (after five weeks of H57 in the diet). Each row represents a different OTU named to the lowest phylogenetic level possible, with OTU number in brackets. * = $P < 0.05$; ** = $P < 0.01$ indicates the significance of differences between treatment groups at the week 13 time point.

Fig 4. Heat map showing the relative abundance of OTUs present in calf rumen fluid. Each column represents an individual calf at a given time point. Each row represents a different OTU named to the lowest phylogenetic level possible, with OTU number in brackets. OTUs were selected at a threshold of greater than 0.6% relative abundance to remove low abundant OTUs.

Wk 4: prior to the addition of H57 to the diet; Wk 12: After the H57 treated calves were fed H57 for eight weeks. . * = P < 0.05; ** = P < 0.01 indicates the significance of differences between week 4 and 12 time points.

Fig 5. Enumeration of H57 populations in sheep rumen fluid by qPCR in Control and H57 fed sheep at week 13. Assays were run in triplicate and error bars show the standard error of the mean.

Fig 6. Enumeration of H57 populations in calf rumen fluid by qPCR in Control and H57 fed calves at week 12. Assays were run in triplicate and error bars show the standard error of the mean.

Supporting Information

S1 Table. Unfiltered read count of *Bacillus* OTUs compiled from sequencing of the 16S rRNA gene PCR amplicon in sheep and calf rumen fluid samples.