Grain grinding size of cereals in complete pelleted diets for growing lambs: effects on ruminal microbiota and fermentation

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Abstract

Lambs from intensive dairy sheep production systems are sold out either after weaning as milk-fed lambs or after a fattening characterized by high intakes of concentrate feeds (mainly cereals) which can lead to ruminal disorders. Therefore, it is essential to explore different feeding alternatives in order to reach a balance between economic or productive benefits and animal welfare. Twenty weaned male lambs (6 to 8 weeks age, 14.8 ± 0.16 kg body weight) were allocated randomly to one of two different groups (n=10 per group) to study the effect of feeding a complete pelleted diet (CPD) in which cereal grains were ground at two different sieve sizes (either 2-mm with 13.5% dry matter (DM) of the particles > 1.7 mm or 6-mm with 47.1% DM of particles > 1.7 mm) on DM intake, nutrient digestibility, ruminal microbiota and fermentation. Lambs were fed the corresponding CPD *ad libitum* and slaughtered when they reached 27 kg. DM intake was greater in the lambs fed 2-mm grains than in those fed 6-mm grains ($P<0.05$). Feeding 2-mm grains in comparison to 6-mm grains resulted in darker colour of ruminal mucosa (100 vs. 127, from a scale where 0 is black and 256 white; SED=9.8; $P=0.017$) and thicker stratum corneum (45.3 vs. 30.9 μm ; SED=3.67; $P=0.001$), suggesting more severe acidotic conditions in the rumen with the smaller cereal particle size. Analysis of microbial communities revealed that the presence of ruminal bacteria such as *Prevotella* and *Quinella*, were increased in the digesta of animals fed the 2-mm diet. These data were corroborated by the odd- and branched-chain fatty acid profile of ruminal contents, which would indicate a shift from cellulolytic to amylolytic bacteria in the rumen of lambs fed the 2-mm diet. In conclusion, feeding 6-mm in comparison to 2-mm ground cereal grains included in CPD formulated for growing lambs is an alternative strategy that may reduce ruminal acidosis through changes in the microbiota.

Key words: cereal processing, digestion, lamb, rumen health, ruminal fatty acids, ruminal microbiota

1. Introduction

In the Mediterranean area, dairy sheep are primarily reared to produce milk for high-quality cheese and also meat lambs (Milán et al., 2011). In dairy sheep farms, lambs are separated from the mothers soon after birth, and then fed with milk replacers. Most lambs are sold after 3-4 weeks as milk-fed lambs, a valuable product of dairy sheep production systems due to their organoleptic properties, and its demand is strongly tied to local traditions and consumer preference (De Rancourt et al., 2006). Due to seasonal variations in lamb production and demand, its price strongly fluctuates throughout the year (Milán et al., 2011; European Commission, 2017), with periods where market value does not cover production costs. An alternative strategy to break the seasonal variation and get a better return is to produce fattening lambs efficiently to expand the market and promote exports of lamb to other countries (Rodriguez et al., 2011). These lambs are fed high concentrate diets from weaning to slaughter weight. Complete pelleted diets (CPD) simplify feedlot feeding management of fattening lambs when compared to the traditional *ad libitum* supply of concentrates and cereal straw in separate feeding troughs (Manso et al., 1998). It has been shown that feed efficiency attained with CPD is similar to that with the traditional feeding system (Blanco et al., 2015). However, CPD developed so far for fattening lambs do not mitigate ruminal acidosis when compared to feeding concentrates and cereal straw in separate troughs (Blanco et al., 2014).

A possible approach to reduce the fermentation rate of starch in the rumen would be to modify the cereal particle size (grinding size) included within the pellet, as it is well known that lambs can use the whole grain cereal efficiently (Ørskov and Fraser, 1975). Feed intake, rumen ecosystem and fermentation parameters can be affected by cereal particle size (Pérez-Torres et al., 2011; Gimeno et al., 2015a; Kazemi-Bonchenari et al., 2017), but to the authors'

knowledge there are no studies integrating all this information in weaned lambs intensively fattened during short periods of time (less than 50 days) with CPD. Changing grinding size of grain may cause changes in the composition of ruminal microbiota, affecting the rate of fermentation and the formation of end-products, and then the composition of ruminal contents and digesta reaching the abomasum. Consequently, the effect of different grinding sizes of cereal must be properly evaluated from a holistic perspective to get the best balance between physiological, welfare and economic criteria. Therefore, the objectives of the present study were to investigate the effects of increasing the grinding size of cereal grain included in CPD on dry matter intake (DMI), nutrient digestibility, ruminal mucosa and fermentation, and composition of ruminal microbiota in growing lambs.

2. Materials and Methods

All handling practices followed the recommendations of the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes and the IGM-CSIC Animal Experimentation Committee (protocol number 2010-E80).

2.1. Animals and diets

Twenty intact male Merino lambs (age 47 ± 0.78 days, all lambs were between 6 and 8 weeks old) were used in this experiment. The lambs were allocated randomly to one of two different groups (n=10 per dietary treatment) balanced by body weight (14.8 ± 0.16 kg). Both groups were fed the same CPD (43% barley, 15% maize, 24% soybean, 15% barley straw, and 3% vitamin-mineral premix). Soybean meal and the premix were in a finely milled form, and the barley straw used in both diets had been ground using a 2-mm screen size (average particle size 550 μ m, with 97, 82 and 15% of the particles with a size < 1700, 1180 and 300

µm, respectively). The only difference between diets was the grinding size of the cereals included in the CPD before pelleting. The cereals included in the CPD of one group were ground using a sieve with a screen diameter of 2-mm (2-mm diet or grains) while for the other group the screen diameter was 6-mm (6-mm diet or grains). A 100-horsepower hammermill (VRE, Rosal, Spain) equipped with screens of either 2 or 6-mm openings was used to grind cereal grains (barley and maize). Particle size distribution of both CPD was measured by dry sieving analysis (ADSA, 1970; Waldo et al., 1971), and shown in Table 1. After mixing the ground cereals with the other ingredients, both diets were steam-conditioned at 70°C and then extruded through a 6 mm (hole diameter) × 44 mm (thickness) die using a 150-horsepower pellet mill (PVR, MABRIK, Spain). The die thickness to hole diameter ratio was 7.3. Physical characteristics of the pellets for both diets are shown in Table 1. Length and diameter of each pellet were measured using a digital caliper (0.01 mm resolution). It was confirmed that in all cases pellet diameter was 6 mm. Bulk density was determined by weighing the amount of pelleted feed required to fill a 155 (height) x 120 (diameter) glass cylinder. Pellet hardness was determined using a Kahl tester (Amandus Kahl GmbH & Co. KG, Reinbek, Germany). Durability index was determined with 100 g samples (in triplicate for each diet) using a Holmen tester equipped with 2.5-mm diameter hole mesh (NHP100, Holmen, UK).

Data on dry matter (ISO 6496:1999), ash (ISO 5984:2002), crude protein (ISO 5983:2009), neutral detergent fibre (NDF), acid detergent fibre (ADF) and total fat composition of both CPD are summarized in Table 1. NDF and ADF were determined by the automated fibre analysis methods for feeds and forages using an ANKOM 200 Fiber Analyzer (Ankom Technology Corp., Macedon, NY, USA), and total fat by the acid hydrolysis filter bag technique using the Ankom^{HCl} Hydrolysis System. Physically effective

fibre for each diet was calculated by multiplying the proportion of particles retained on the 1.18-mm sieve by the NDF content of the feed (Grant and Cotanch, 2005).

[INSERT TABLE 1 NEAR HERE, PLEASE]

Animals were housed in individual concrete-floor pens (1.45 m width $\times$ 1.40 m length $\times$ 1.30 m height) with sawdust bedding and equipped with individual feeding troughs and automatic drinkers. After seven days of adaptation, lambs received the experimental diets *ad libitum* at 09:00 h daily. The amount of feed offered to each lamb was adjusted daily according to the previous day intake to allow refusals of approximately 200 g/kg of feed offered. Samples of both the feed and refusals were collected daily for subsequent analyses after pooling all the subsamples refused by each animal after one week. The total duration of the experiment was 48 days including the adaptation period (7 days), until lambs reached a weight of 27 kg.

2.2. Digestibility

Four male Merino lambs per treatment (22.8 ± 2.45 kg) were used for a digestibility trial. The number of animals used was the minimum considered necessary according to both statistical and welfare criteria. After being fed with the experimental diets for 21 days, these lambs were confined to individual metabolic cages (0.65 m width $\times$ 1.10 m length $\times$ 0.90 m height) with individual feeding troughs and drinkers, and fitted with specific devices to collect faeces and urine separately. During the digestibility trial, DMI was adjusted to 35 g/kg body weight to avoid differences in the rate of passage due to possible variations in DMI of both CPD. After two days of adaptation to the pens, faeces were collected for five days. The faeces of each animal were collected daily, weighed, mixed thoroughly and sampled (10%). Aliquots from each lamb were pooled and stored at -30 °C until analysis of NDF, ADF and

starch. Starch was analyzed by spectrophotometry after enzymatic analysis on fresh feeds and refusals and on lyophilized fecal samples (Faisant et al., 1995).

2.3. Ruminal fermentation and histological examination of ruminal mucosa

When each animal reached a weight of ≥ 27 kg, food and water were withdrawn, and after 1 h the lamb was stunned and slaughtered by exsanguination from the jugular vein, eviscerated and skinned. Total rumen contents of each lamb were sampled and immediately frozen at -80°C for fatty acid profile determination (Morán et al., 2013) and DNA extraction. A fraction of rumen contents from each animal was collected and strained through two layers of cheese cloth in order to obtain ruminal liquid samples to determine pH and concentrations of volatile fatty acids (VFA), lactate and ammonia nitrogen ($\text{NH}_3\text{-N}$) according to Carro and Miller (1999). Then, two square (3×3 cm) samples of rumen wall from two locations (posterior part of dorsal sac and anterior part of ventral sac) were placed into histology cassettes, washed under tap water and fixed by immersion in 10% buffered formalin for one week in order to measure colour of the ruminal epithelium according to Benavides et al. (2013). Digital gross pictures (image size 6 mp) of the ruminal mucosa were taken and then converted to grey values to derive a mean grey value (in a scale from 0-black to 255-white) as indicator of the degree of keratinization of the ruminal epithelium. Tissue samples were trimmed and processed for histologic examination (hematoxylin-eosin staining). From each sample, two sections with five or more papillae were examined and digital pictures of three ruminal papillae case were taken. Histological measurement of the stratum corneum thickness was carried out on the digital pictures using ImageJ analysis software. Briefly, in each papilla, straight lines were drawn across the width of the stratum corneum in an area representative of the overall thickness of the papilla.

2.4. DNA extraction, quantitative real-time PCR (qPCR) and T-RFLP of ruminal microbiota

Frozen ruminal content samples were freeze-dried and thoroughly homogenized before DNA extraction by physical disruption (1 min) using a bead beater (Mini-bead Beater, BioSpec Products, Bartlesville, OK) and subsequent DNA purification with the QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA), with the modification of a greater temperature (95 °C) to improve cell lysis. Samples of DNA were used as templates for quantitative real-time PCR (qPCR) amplification and T-RFLP analysis according to the procedures described by Andrés et al. (2016). Additionally, qPCR for quantification of total bacteria and bacterial groups involved in fatty acid biohydrogenation (Andrés et al., 2016), *Megasphaera elsdenii* (Ouwerkerk et al., 2002), and *Selenomonas ruminantium* (Stevenson and Weimer, 2007) was performed according to the procedures described in the corresponding references.

2.5. Calculations and statistical analysis

The study was laid down according to a completely randomised design with 10 experimental units in each of 2 treatments (cereal grain grinding size). The individual lamb was considered as the experimental unit. Data on intake, digestibility, ruminal fermentation parameters, colour of ruminal mucosa, thickness of stratum corneum, qPCR, ruminal fatty acid profile and relative height of terminal restriction fragments (TRF) were analyzed by one-way analysis of variance using the GLM procedure of SAS (SAS Institute Inc., Cary, NC) with the experimental diet (cereal grinding size) as the only source of variation. Significance was declared at $P < 0.05$, and a tendency when $0.05 < P < 0.10$.

The T-RFLP data obtained for each rumen sample were analyzed by principal component analysis using SAS (SAS Inst. Inc., Cary, NC).

3. Results

3.1. Feed intake, digestibility and ruminal fermentation parameters

Data in DMI and nutrient digestibility are shown in Table 2. Significant differences in DMI ($P<0.05$) between the two groups studied were observed, with the lambs fed the diet with 2-mm ground cereals showing a greater daily DMI (7%) than the lambs fed the 6-mm diet. No significant differences were observed in digestibility, with similar average dry matter, NDF, ADF, and starch digestibility coefficients for 2-mm and 6-mm diets (Table 2).

Regarding ruminal fermentation parameters there were no significant effects on ruminal pH or total VFA (Table 2). However, a trend towards significantly increased molar proportions of propionate (Table 3, 41.9 vs. 37.2 mmol/100 mmol, $P=0.067$), and a decreased acetate/propionate ratio (Table 1, 1.12 vs. 1.38, $P=0.029$) were observed in the lambs fed 2-mm grains when compared to the lambs fed 6-mm grains. Moreover, no significant differences were observed between groups for rumen lactate and $\text{NH}_3\text{-N}$ concentrations.

[INSERT TABLE 2 NEAR HERE, PLEASE]

3.2. Characteristics of ruminal mucosa

In a grey scale ranging from 0 to 256, where 0 is black and 256 is white, the ruminal mucosa of the lambs fed 2-mm grains had a darker colour (100 vs. 127; $\text{SED}=9.8$; $P=0.017$) in comparison to the lambs fed 6-mm grains. Moreover, the histological examination of the rumen wall demonstrated increased numbers of keratinocytes in the stratum corneum of the epithelium in lambs fed 2-mm grains (Fig. 1). Additionally, a thicker stratum corneum in the

ruminal epithelium was observed in lambs fed 2-mm grains when compared to those fed 6-mm grains (45.3 vs. 30.9 μm ; SED=3.67; $P=0.001$; see Fig. 1).

[INSERT FIG. 1 NEAR HERE, PLEASE]

3.3. Ruminal microbiota

Total ruminal bacteria or some sub-acute ruminal acidosis (SARA) related bacteria, such as *Megasphaera elsdenii* or *Selenomonas ruminantium*, were not different between experimental groups (diets with 2-mm vs. 6-mm ground cereals, Table 3). Neither the presence of other SARA related bacteria, such as *Streptococcus bovis*, which was undetectable in this study (data not shown) was affected by the experimental diets. *Butyrivibrio* stearic acid producing bacteria (BSA; 2.64 vs. 3.24 number of copies $\times 10^5$ /ng total DNA) and *Butyrivibrio* vaccenic acid producing bacteria (BVA; 0.00204 vs. 0.00225 pg/ng total DNA), which are related to the biohydrogenation of fatty acids at ruminal level (Wallace et al., 2007) showed no significant differences due to cereal grinding size (Table 3).

[INSERT TABLE 3 NEAR HERE, PLEASE]

The principal component analysis with the matrix data of peak relative heights (indicative of relative abundance of the species) for each sample showed well-defined differences in ruminal bacterial communities and hence different clusters along axis of principal component 2 could be observed as a consequence of different grinding sizes (Fig. 2). Nevertheless, there were few cases classified ambiguously, which were probably on the border of both groups.

[INSERT FIG. 2 NEAR HERE, PLEASE]

The principal component analysis not only allowed proper discrimination of samples according to T-RFLP data (Fig. 2), but also the identification of the main variables

responsible for this classification (Fig. 3). In fact, Fig. 3 reflects the loading weights of the predictors (X-variables, relative heights of TRFs) contributing to explain the response variation along the first two principal components (PC1 and PC2). Thus, PC1 and PC2 accounted for 25% and 20% of the explained variance. Since the variables showing loadings weights closer to the centre can be considered irrelevant, just five predictors (relative heights of TRFs with 99.4, 101.9, 102.4, 182.5 and 402.7 bp) were the main responsible for the clusters detected in Fig 2.

[INSERT FIG. 3 NEAR HERE, PLEASE]

Based on this information, there was an increase of both the *Prevotella* and *Quinella* bacteria in the rumen of lambs fed the diet with 2-mm ground cereals since the relative heights of the TRFs associated with these species (101.9 bp for *Prevotella spp*: 4.12 vs. 2.82, SED=0.749, $P=0.099$; and 99.4 bp for *Quinella*: 6.14 vs. 3.48, SED=1.270, $P=0.050$) showed at least a trend towards significant increments in this group of animals when compared to lambs fed the diet with 6-mm ground cereals. In contrast, the relative height of the other two peaks (182.5 and 402.7 bp) likely related to non-identified clostridia in ruminal contents was not significantly affected by cereal grinding size.

3.4. Ruminal fatty acid profile

[INSERT TABLE 4 NEAR HERE, PLEASE]

Several odd-chain fatty acids (C13:0 and C15:0), *iso*-acids (e.g., *iso* C15:0 and *iso* C16:0) and *anteiso*-C15:0 were significantly increased in the rumen contents of lambs fed the diet with 2-mm ground cereals (Table 4). Moreover, C18:1 isomers, including C18:1 *trans*-10 and C18:1 *trans*-11, at least showed a trend towards significant differences between these two groups of animals (Table 4). Thus, a shift from the *trans*-11 to the *trans*-10 pathway of

biohydrogenation in the rumen of fattening lambs receiving the diet with 2-mm ground cereals was observed.

4. Discussion

A proper evaluation of the effects of increasing sieve grinding size of cereal grains included in CPD formulated for growing lambs on feed intake, nutrient digestibility and rumen status and conditions is necessary before any recommendation is given for lambs raised in intensive production systems.

Mean retention time of digesta in the rumen is affected by the particle size (Udén, 1988), so that the smallest particles leave the rumen faster (Lammers et al., 1996; Kononoff et al., 2003). It is possible that the average retention time in the digestive tract (in particular in the rumen) was shorter in the diet with 2-mm ground cereals than the diet with the 6-mm ground cereals, thus increasing the rate of passage and the DMI of animals fed the more extensively ground diet (Lammers et al., 1996). With fibrous diets (>280 g NDF/kg feed) with a slow rate of fibre fermentation, high passage rate (short retention time in the rumen) and high DMI are known to cause a decrease in fiber digestibility (Gherardi et al., 1992). However, in our study lambs were fed a high concentrate CDP and no significant reduction was observed in ADF or NDF digestibility due to different cereal grinding size. It must be considered that smaller grinding size reduces particle size, thus increasing the surface for microbial attack (Lammers et al., 1996) which might have counteracted the negative effects of the rate of passage on fibre digestibility. Increasing the diameter of pellets is an effective way to decrease the rate of fermentation and the risk of ruminal acidosis in beef cattle fed high concentrate diets (Castrillo et al., 2013). In our study the pellets of both diets had the same size, and the only difference between diets was the grinding size of the cereal grains contained in the pellets. A greater particle size of cereals may also have an effect on fermentation rate in the rumen.

Kazemi-Bonchenari et al. (2017) observed that feeding calves with barley more finely ground caused a decline in ruminal pH and reduced growth performance.

As cereal grains are more finely ground the rumen starch fermentation rate is increased, whereas this is slowed down with whole or coarsely ground grains (Offner et al., 2003). As a consequence, the amount of dietary starch escaping from rumen fermentation could have been greater in lambs fed CPD with grains ground at 6-mm. This could explain the trend towards significantly higher proportions of propionate in ruminal fluid samples, and the significantly lower acetate/propionate ratio observed with CPD containing grains ground at 2-mm when compared to those ground more coarsely (6-mm). This effect would be compatible with the lack of differences in starch digestibility, because it might have been digested in the small intestine or further fermented in the hindgut (Huntington et al., 2006; Larsen et al., 2009). A smaller percentage of starch fermentation in the rumen, and hence a higher percentage of starch reaching the small intestine might have promoted higher levels of glucose absorbed into the blood stream (Gathercole et al., 2011).

Regarding ruminal acidosis, similar ruminal pH values were observed with both experimental diets. However, only one sample of ruminal fluid was taken during the day, so it was not possible to verify if differences in rumen pH could become noticeable at other times after feeding, or to quantify the time (hours/d) than pH was below of 6.0. Nevertheless, colour and histological features of ruminal mucosa of lambs fed the diet with 2-mm ground cereals were indicative of a higher degree of parakeratosis characteristic of more severe ruminal acidotic conditions, suggesting that lambs fed this CPD would apparently remain more time with a low acidotic ruminal pH than lambs fed the the diet with coarser cereal grains (6-mm). The thickening of the stratum corneum in the ruminal epithelium observed in lambs fed the diet with 2-mm ground cereals was caused by the increased number of layers of

keratinocytes (hyperkeratosis). Besides, remnants of the cellular nucleus remained in several of these keratinocytes, a sign of parakeratotic hyperkeratosis or parakeratosis (Benavides et al., 2013; Uzal et al., 2016).

A more severe ruminal acidosis could also potentially led to changes in ruminal bacterial populations, mainly increments in both *Prevotella* and *Quinella* spp (Tolosa Álvarez, 2006; McCann et al., 2016). The greater abundance of *Prevotella* spp. in the rumen of lambs fed 2-mm ground cereals might be an adaptation mechanism of the ruminal microbial ecosystem to minimise the impact of the period of time with low ruminal pH, since some of these species (e.g., *Prevotella bryantii*, strain 25A) compete for starch to produce succinate (which is transformed into propionate), rather than lactate (Chiquette et al., 2012). The abundance of the *Prevotella* was increased in the rumen of cattle following an acidotic challenge (Petri et al., 2013). The darker colour of the ruminal mucosa and the thicker stratum corneum as observed in lambs fed the diet with 2-mm ground cereals would suggest faster rates of starch fermentation due to a smaller grinding size of cereals. *Prevotella* spp. are potentially an important source of lipopolysaccharide responsible for inflammation within the rumen (Al-Zahal et al., 2014), which would also explain the damage to the ruminal mucosa of the lambs fed the diet with 2-mm ground cereals regardless of fermentation characteristics of these species. Similarly, *Quinella*-type bacteria are more prevalent in the rumen of steers fed diets containing a high proportion of molasses (50 and 75% molasses) and may become dominant members of the bacterial community under these conditions (Tolosa Álvarez, 2006). *Quinella* fermentation products are mainly propionate and acetate (Krumholz et al., 1993), and it seems to be better adapted to acidotic conditions, thus incompatible with cellulolytic bacteria and their fermentation end products, acetate and hydrogen, the latter serving as substrate for methanogens. Again, these findings regarding the ruminal microbial population seem to be

supportive of a more severe acidotic condition in the lambs fed the diet with 2-mm when compared to diet with 6-mm ground cereals.

All of these changes in ruminal bacterial populations might be related to different fatty acid profiles observed in the rumen contents of the lambs being fed both experimental diets (Colman et al., 2013). In fact, it has been suggested that odd- and branched-chain fatty acids originating from rumen bacteria can be used as biomarkers for the detection of changes in ruminal bacterial populations related to SARA (Colman et al., 2013). Thus, in agreement with previous studies (Fievez et al., 2012; Colman et al., 2013; Li et al., 2014; Gimeno et al., 2015b), fatty acids more related to the presence of amylolytic bacteria (C13:0 and C15:0) were significantly increased in the rumen contents of the lambs fed the diet with 2-mm ground cereals, that is to say, those lambs supposed to have undergone faster rates of starch ruminal fermentation. Moreover, according to Allison et al. (1962), the increase of *Prevotella* spp. in the rumen of lambs fed the diet with 2-mm ground cereals is consistent with the significant increase in *iso*-acids observed in these lambs (e.g., *iso* C15:0 and *iso* C16:0). Likewise, the shift in the rumen towards a more acidotic environment, thus impairing cellulolytic bacteria, could explain the increased proportion of *anteiso*-C15:0 observed in the lambs fed the diet with 2-mm ground cereals, as suggested by Alves et al. (2013). However, those authors also observed an increase in *anteiso*-C17:0 in lambs fed the most acidotic diet; the different diets administered to the animals might have induced the proliferation of different bacterial communities, thus changing slightly the ruminal fatty acid profile when compared to our results. Therefore, all of the data regarding the ruminal long-chain fatty acid profile seem to corroborate the shift from cellulolytic to amylolytic bacteria in the rumen of lambs fed the diet with 2-mm ground cereals.

Acidotic conditions have been reported to cause a negative effect on the abundance of species involved in fatty acid biohydrogenation, such as *Butyrivibrio* spp. However, unexpectedly, the percentage of 18:0 in the ruminal fatty acid profile was higher with the 2-mm ground cereal diet when compared to the 6-mm ground cereal diet, so other bacterial species might have been involved in the ruminal biohydrogenation process. According to Colman et al. (2013), our results of the fatty acid composition in rumen contents demonstrated a shift from the *trans*-11 to the *trans*-10 pathway in the rumen of growing lambs receiving the diet with 2-mm ground cereals.

5. Conclusion

Lambs fed a high concentrate CPD formulated with cereal grains of different grinding size show changes in ruminal mucosa and microbiota and in the odd- and branched-chain fatty acid profile of ruminal digesta that might be indicators of a reduced degree of ruminal acidosis in lambs fed a diet formulated with coarser ground cereal grains (6-mm grinding size). Although no differences between groups in ruminal pH and nutrient digestibility were observed, the overall effects detected in the rumen would suggest that diets with coarser ground cereal grain (6-mm) may enhance ruminal health reducing the risk of digestive disorders in growing lambs.

Conflict of interest

The authors declare that there are not any conflict of interest.

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References

- ADSA, 1970. A Report: Committee on classification of particle size in feedstuffs. *J. Dairy Sci.* 53, 689.
- Allison, M.J., Bryant, M.P., Doetsch, R.N., 1962. Studies on the metabolic function of branched-chain volatile fatty acids, growth factors for ruminococci. 1. Incorporation of isovalerate into leucine. *J. Bacteriol.* 83, 523-532.
- Alves, S.P., Santos-Silva, J., Cabrita, A.R.J., Fonseca, A.J.M., Bessa, R.J.B., 2013. Detailed dimethylacetal and fatty acid composition of rumen content from lambs fed lucerne or concentrate supplemented with soybean oil. *PloS one* 8, e58386. doi:10.1371/journal.pone.0058386
- Al-Zahal, O., Dionissopoulos, L., Laarman, A.H., Walker, N., McBride, B.W., 2014. Active dry *Saccharomyces cerevisiae* can alleviate the effect of subacute ruminal acidosis in lactating dairy cows. *J. Dairy Sci.* 97, 7751-7763.
- Andrés, S., Bodas, R., Tejido, M.L., Giráldez, F.J., Valdés, C., López, S., 2016. Effects of the inclusion of flaxseed and quercetin in the diet of fattening lambs on ruminal microbiota, in vitro fermentation and biohydrogenation of fatty acids. *J. Agric. Sci.* 154, 542-552.
- Benavides, J., Martínez-Valladares, M., Tejido, M.L., Giráldez, F.J., Bodas, R., Prieto, N., Pérez, V., Andrés, S., 2013. Quercetin and flaxseed included in the diet of fattening lambs: Effects on immune response, stress during road transport and ruminal acidosis. *Livest. Sci.* 158, 84-90.
- Blanco, C., Bodas, R., Andrés, S., López, S., Giráldez, F.J., 2014. Concentrate plus ground barley straw pellets can replace conventional feeding systems for light fattening lambs. *Small Rumin. Res.* 116, 137-143.
- Blanco, C., Giráldez, F.J., Prieto, N., Benavides, J., Wattegedera, S., Morán, L., Andrés, S., Bodas, R., 2015. Total mixed ration pellets for light fattening lambs: effects on animal health. *Animal* 9, 258-266.

- Carro, M.D., Miller, E.L., 1999. Effect of supplementing a fibre basal diet with different nitrogen forms on ruminal fermentation and microbial growth in an in vitro semicontinuous culture system (RUSITEC). *Br. J. Nutr.* 82, 149-157.
- Castrillo, C., Mota, M., van Laar, H., Martin-Tereso, J., Gimeno, A., Fondevila, M., Guada, J.A., 2013. Effect of compound feed pelleting and die diameter on rumen fermentation in beef cattle fed high concentrate diets. *Anim. Feed Sci. Technol.* 180, 34–43.
- Chiquette, J., Allison, M.J., Rasmussen, M., 2012. Use of *Prevotella bryantii* 25A and a commercial probiotic during subacute acidosis challenge in midlactation dairy cows. *J. Dairy Sci.* 95, 5985-5995.
- Colman, E., Khafipour, E., Vlaeminck, B., De Baets, B., Plaizier, J.C., Fievez, V., 2013. Grain-based versus alfalfa-based subacute ruminal acidosis induction experiments: Similarities and differences between changes in milk fatty acids. *J. Dairy Sci.* 96, 4100-4111.
- De Rancourt, M., Fois, N., Lavín, M.P., Tchakérian, E., Vallerand, F., 2006. Mediterranean sheep and goat production: an uncertain future. *Small Rumin. Res.* 62, 167-179.
- European Commission, 2017. Public European Commission data on sheep and goat statistics. <https://circabc.europa.eu/faces/jsp/extension/wai/navigation/container.jsp> (accessed 20.01.2017).
- Faisant, N., Planchot, V., Kozłowski, F., Pacouret, M.P., Colonna, P., Champ, M., 1995. Resistant starch determination adapted to products containing high levels of resistant starch. *Sci. Alim.* 15, 83–89.
- Fievez, V., Colman, E., Castro-Montoya, J.M., Stefanov, I., Vlaeminck, B., 2012. Milk odd- and branched-chain fatty acids as biomarkers of rumen function—An update. *Anim. Feed Sci. Technol.* 172, 51-65.
- Gathercole, L.L., Morgan, S.A., Bujalska, I.J., Hauton, D., Stewart, P.M., Tomlinson, J.W., 2011. Regulation of lipogenesis by glucocorticoids and insulin in human adipose tissue. *PloS one* 6, e26223. doi:10.1371/journal.pone.0026223

- Gherardi, S.G., Kellaway, R.C., Black, J.L., 1992. Effect of forage particle length on rumen digesta load, packing density and voluntary feed intake by sheep. *Aust. J. Agric. Res.* 43, 1321-1336.
- Gimeno, A., Alami, A., Abecia, L., de Vega, A., Fondevila, M., Castrillo, C., 2015a. Effect of type (barley vs. maize) and processing (grinding vs. dry rolling) of cereal on ruminal fermentation and microbiota of beef calves during the early fattening period. *Anim. Feed Sci. Technol.* 199, 113–126.
- Gimeno, A., Alami, A., Toral, P.G., Frutos, P., Abecia, L., Fondevila, M., Castrillo, C., 2015b. Effect of grinding or pelleting high grain maize- or barley-based concentrates on rumen environment and microbiota of beef cattle. *Anim. Feed Sci. Technol.* 203, 67-78.
- Grant, R.J., Cotanch, K.W., 2005. Physically effective fiber for dairy cows: Current perspectives. In: *Proceedings of the 2005 Cornell Nutrition Conference*. Cornell University, Ithaca, NY, pp. 61-70.
- Huntington, G.B., Harmon, D.L., Richards, C.J., 2006. Sites, rates and limits of starch digestion and glucose metabolism in growing cattle. *J. Anim. Sci.* 84, E14-E24.
- Kazemi-Bonchenari, M., Salem, A.Z.M., López, S., 2017. Influence of barley grain particle size and treatment with citric acid on digestibility, ruminal fermentation and microbial protein synthesis in Holstein calves. *Animal* 11, 1295-1302.
- Kononoff, P.J., Heinrichs, A.J., Buckmaster, D.R., 2003. Modification on the pen state and total mixed ration particle separator and the effect of moisture content on its measurement. *J. Dairy Sci.* 86, 1858-1863.
- Krumholz, L.R., Bryant, M.P., Brulla, W.J., Vicini, J.L., Clark, J.H., Stahl, D.A., 1993. Proposal of *Quinella ovalis* gen. nov., sp. nov., based on phylogenetic analysis. *Int. J. Syst. Bacteriol.* 43, 293-296.
- Lammers, B.P., Buckmaster, D.R., Heinrichs, A.J., 1996. A simple method for the analysis of particle sizes of forage and total mixed rations. *J. Dairy Sci.* 79, 922-928.
- Larsen, M., Lund, P., Weisbjerg, M.R., Hvelplund, T., 2009. Digestion site of starch from cereals and legumes in lactating dairy cows. *Anim. Feed Sci. Technol.* 153, 236-248.

- Li, F., Yang, X.J., Cao, Y.C., Li, S.X., Yao, J.H., Li, Z.J., Sun, F.F., 2014. Effects of dietary effective fiber to rumen degradable starch ratios on the risk of sub-acute ruminal acidosis and rumen content fatty acids composition in dairy goat. *Anim. Feed Sci. Technol.* 189, 54–62.
- Manso, T., Mantecón, A.R., Giráldez, F.J., Lavín, P., Castro, T., 1998. Animal performance and chemical body composition of lambs fed diets with different protein supplements. *Small Rumin. Res.* 29, 185-191
- McCann, J.C., Luan, S., Cardoso, F.C., Derakhshani, H., Khafipour, E., Loor, J., 2016. Induction of subacute ruminal acidosis affects the ruminal microbiome and epithelium. *Front. Microbiol.* 7, 701. doi: 10.3389/fmicb.2016.00701.
- Milán, M.J., Caja, G., González-González, R., Fernández-Pérez, A.M., Such, X., 2011. Structure and performance of Awassi and Assaf dairy sheep farms in northwestern Spain. *J. Dairy Sci.* 94, 771–784.
- Morán, L., Giráldez, F.J., Panseri, S., Aldai, N., Jordán, M.J., Chiesa, L.M., Andrés, S., 2013. Effect of dietary carnosis acid on the fatty acid profile and flavour stability of meat from fattening lambs. *Food Chem.* 138, 2407–2414.
- Offner, A., Bach, A., Sauvant, D., 2003. Quantitative review of in situ starch degradation in the rumen. *Anim. Feed Sci. Technol.* 106, 81-93.
- Ørskov, E.R., Fraser, C., 1975. The effects of processing of barley-based supplements on rumen pH, rate of digestion and voluntary intake of dried grass in sheep. *Br. J. Nutr.* 34, 493-500.
- Ouwerkerk, D., Klieve, A.V., Forster, R.J., 2002. Enumeration of *Megasphaera elsdenii* in rumen contents by real-time Taq nuclease assay. *J. Appl. Microbiol.* 92, 753-758.
- Pérez-Torres, A., Sierra, I., de Vega, A. Keli, A., 2011. Effect of grinding size and sunflower oil addition on intake, digestibility, rumen function and microbial protein synthesis in sheep fed a dry total mixed ration. *Spanish J. Agric. Res.* 9, 1186-1196.
- Petri, R.M., Schwaiger, T., Penner, G.B., Beauchemin, K.A., Forster, R.J., McKinnon, J.J., McAllister, T.A., 2013. Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge. *PLoS One* 8, e83424. doi: 10.1371/journal.pone.0083424

- Rodríguez, A.B., Bodas, R., Landa, R., López-Campos, O., Mantecón, A.R., Giráldez, F.J., 2011. Animal performance, carcass traits and meat characteristics of Assaf and Merino×Assaf growing lambs. *Livest. Sci.* 138, 13-19.
- Stevenson, D.M., Weimer, P.J., 2007. Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl. Microbiol. Biotechnol.* 75, 165-174.
- Tolosa-Álvarez, M.T., 2006. Molecular ecology of rumen bacterial populations in steers fed molasses diets (PhD Thesis). The University of Queensland Brisbane, Australia.
- Udén, P., 1988. The effect of gridding and pelleting hay on digestibility, fermentation rate, digesta passage and rumen fecal particle size in cows. *Anim. Feed Sci. Technol.* 19, 145-147.
- Uzal, F.A., Plattner, B.L., Hostetter, J.M., 2016. Chapter 1 - Alimentary System, in: Grant Maxie, M. (Ed.), *Jubb, Kennedy & Palmer's Pathology of Domestic Animals: Volume 2 (Sixth Edition)*, Elsevier, St. Louis, Missouri, pp. 1-257.e2.
- Waldo, D.R., Smith, L.W., Cox, E.L., Weenland, B.T., Lucas, H.L., 1971. Logarithmic normal distribution for description of sieved forage materials. *J. Dairy Sci.* 54, 1465-1469.
- Wallace, R.J., McKain, N., Shingfield, K.J., Devillard, E., 2007. Isomers of conjugated linoleic acids are synthesized via different mechanisms in ruminal digesta and bacteria. *J. Lipid Res.* 48, 2247-2254.

Table 1. Particle size distribution (cumulative % w/w of feed retained in each sieve size) of diets before pelleting, physical characteristics of the pellets and chemical composition (g/kg dry matter unless otherwise stated) of the complete pelleted diets formulated with cereals ground at 2-mm and 6-mm

	2-mm	6-mm
Particle size distribution		
> 2360 μm	2.3	20.4
> 1700 μm	13.5	47.1
> 1180 μm	36.1	65.4
> 300 μm	82.0	91.8
> 105 μm	99.6	99.9
Mean particle size of mash diets before pelleting (μm)	640	1270
Pellet characteristics [mean (standard deviation)]		
Pellet length (mm) ¹	1.08 (0.106)	0.96 (0.112)
Bulk density (kg/m^3) ²	651 (6.55)	674 (5.94)
Kahl-hardness (kg) ¹	7.6 (1.06)	6.8 (1.61)
Pellet durability index (%) ²	96.7 (2.34)	95.1 (3.06)
Chemical composition		
Dry matter, g/kg feed	898	895
Crude protein	174	175
Neutral detergent fibre	224	218
Acid detergent fibre	112	109
Physically effective fibre	81	143
Starch	345	346
Total fat	27	28
Ash	76	72

¹ Measured in 15 pellets randomly selected from each diet (n=15)

² Measured in triplicate (n=3)

Table 2. Intake, digestibility and ruminal fermentation parameters of growing lambs fed the same complete pelleted diet formulated with cereals ground at 2-mm and 6-mm

	2-mm	6-mm	SED	<i>P</i> -value*
Dry matter intake, g/d	881	820	23.3	0.021
<u>Nutrient digestibility</u>				
Dry matter digestibility	0.710	0.734	0.0099	0.177
NDF digestibility	0.371	0.379	0.0298	0.876
ADF digestibility	0.322	0.350	0.0349	0.631
Starch digestibility	0.998	0.998	0.0004	1.000
<u>Ruminal fermentation parameters</u>				
Ruminal pH	5.71	5.75	0.254	0.890
Total VFA, mmol/L	123	122	18.4	0.953
VFA molar proportions, mmol/100 mmol total VFA				
Acetate	46.7	48.6	1.54	0.220
Propionate	41.9	37.2	2.33	0.067
Isobutyrate	0.825	0.792	0.2489	0.900
Butyrate	6.06	7.63	1.044	0.175
Isovalerate	1.09	1.31	0.381	0.591
Valerate	3.13	3.69	0.426	0.242
Caproate	0.272	0.260	0.1146	0.923
Acetate/propionate	1.12	1.38	0.109	0.029
NH ₃ -N, ug/mL	253	168	83.3	0.345
Lactate, ug/mL	53.3	62.5	11.71	0.470

* Within a row, *P*-values < 0.05 represent significant differences between experimental treatments 2-mm and 6-mm.

Table 3. Quantitative real-time PCR analysis of ruminal content samples (log of number of copies/ng total DNA unless otherwise stated) of growing lambs (n=10 per treatment) fed the same complete pelleted diet formulated with cereals ground at 2-mm and 6-mm.

	2-mm	6-mm	SED	P-value
Total bacteria	8.79	8.64	0.089	0.285
<i>Megasphaera elsdenii</i>	5.07	4.36	0.425	0.256
<i>Selenomonas ruminantium</i>	6.60	6.63	0.078	0.753
BSA ¹	5.37	5.38	0.107	0.928
BVA ² , pg/ng total DNA	0.00204	0.00225	0.00155	0.897

¹*Butyrivibrio* stearic acid producing bacteria

²*Butyrivibrio* vaccenic acid producing bacteria

Table 4. Effect of dietary treatment on fatty acid profile (% of total FAME¹) in the ruminal contents of growing lambs (n=10 per group) fed the same complete pelleted diet formulated with cereals ground at 2-mm and 6-mm

	2-mm	6-mm	SED	P-value
SFA²	33.0	31.5	1.80	0.415
13:0	0.318	0.199	0.0365	0.005
15:0	1.46	1.02	0.165	0.014
16:0	20.8	22.6	1.71	0.298
18:0	3.60	3.00	0.240	0.020
BCFA³	1.04	0.931	0.1552	0.484
<i>iso</i> -15:0	0.392	0.245	0.0470	0.006
<i>anteiso</i> -15:0	0.181	0.084	0.0213	0.006
<i>iso</i> -16:0	0.190	0.095	0.0310	0.007
<i>anteiso</i> -17:0	0.278	0.508	0.1326	0.100
MUFA⁴	22.2	23.3	0.627	0.092
Total 18:1 <i>cis</i> ⁵	17.7	18.8	0.360	0.006
Total 18:1 <i>trans</i> ⁶	2.39	2.66	0.386	0.492
t10-18:1	1.85	1.34	0.297	0.102
t11-18:1	0.107	0.849	0.3909	0.073
PUFA⁷	36.9	39.8	2.236	0.210
n-6 ⁸	33.3	36.7	2.031	0.112
18:3n-3	3.64	3.15	0.403	0.236
CLA⁹	0.109	0.099	0.0191	0.610
UNKNOWN	4.21	2.38	0.624	0.009

¹ FAME: fatty acid methyl esters.

² SFA: saturated fatty acids = sum of 6:0, 7:0, 8:0, 9:0, 10:0, 11:0, 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0.

³ BCFA: total branched chain fatty acids (sum of *iso* 15:0, *anteiso* 15:0, *iso* 16:0, *anteiso* 17:0).

⁴ MUFA: monounsaturated fatty acids= sum of *cis*- and *trans*-MUFA.

⁵ Total 18:1 *cis*: sum of *c9*-18:1, *c11*-18:1, *c12*-18:1.

⁶ Total 18:1 *trans*: sum of *4t*-18:1, *5t*-18:1, *6t/7t/8t*-18:1, *9t*-18:1, *10t*-18:1, *11t*-18:1, *12t*-18:1, *13t/14t*-18:1, *15t*-18:1, and *16t*-18:1.

⁷ PUFA: polyunsaturated fatty acids = sum of 18:2n-6, 20:2n-6, 20:4n-6, 18:3n-3.

⁸ n-6: sum of 18:2n-6, 20:2n-6, 20:4n-6.

⁹ CLA: conjugated linoleic acid = sum of *9c*, *11t*-18:2, *7t*, *9c*-18:2, *9t*, *11c*-18:2, *11t*, *13c*-18:2, *t/t*-CLA

* Within a row, *P*-values < 0.05 represent significant differences between experimental treatments 2-mm and 6-mm.

Figure captions

Figure 1. Histological detail of ruminal mucosa epithelium of growing lambs fed a complete pelleted diet containing cereal grain ground at 2-mm (A) or 6-mm (B). The thickness of the stratum corneum is showed in both pictures between the arrowheads. Note the excessive sloughing of keratin and the retention of nuclei in the squamous cells of group A (asterisk). Both pictures were taken at the same magnification. Bar=150µm. Hematoxylin-eosin staining.

Figure 2. Discriminant analysis of microbial diversity in ruminal contents performed on matrix data showing the relative height of peaks detected in terminal restriction fragment length polymorphism profiles with HhaI restriction enzyme.

Figure 3. Terminal restriction fragments loading weights in principal component analysis of the ruminal bacterial community.