Early feed restriction of lambs modifies ileal epimural microbiota and affects immunity parameters during the fattening period

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Abstract

Bacteria firmly attached to the gastrointestinal epithelium during the pre-weaning phase may show a significant impact on nutrient processing, immunity parameters, health and feed efficiency of lambs during post-weaning phases. Thus, the aim of this study was to describe the differences in the ileal epimural microbiota (e.g., total bacteria, *Prevotella* spp., *Bifidobacterium* spp. and *Lactobacillus* spp.) of fattening lambs promoted by early feed restriction during the suckling phase trying to elucidate some of the underlying mechanisms behind changes in feed efficiency during the fattening period. Twenty-four Merino lambs (average BW 4.81 ± 0.256 kg) were used, twelve of them (*ad libitum*, **ADL**) kept permanently in individual pens with their mothers, whereas the other 12 lambs were separated from their dams for 9 h each day to be exposed to milk restriction (**RES**). After weaning (BW = 15 kg) all the animals were penned individually, offered the same complete pelleted diet (35 g/kg BW per day) and slaughtered at a BW of 27 kg. During the fattening period, reduced gain:feed ratio (0.320 vs. 0.261, $P < 0.001$) was observed for the RES group. Moreover, increments of *Prevotella* spp. were detected in the ileal epimural microbiota of RES lambs ($P < 0.05$). There were also higher numbers of infiltrated lymphocytes (T and B cells) in the ileal lamina propria ($P < 0.05$), a higher M-cell labelling intensity in ileal Peyer´s patches domes ($P < 0.05$) and a trend towards a thickening of the submucosa layer when compared to the ADL group ($P = 0.057$). Some other immunological parameters, such as an increased immunoglobulin A **(IgA)** production (pg IgA/µg total protein) and increments in CD45+ cells were also observed in the ileum of RES group ($P < 0.05$), whereas transforming growth factor β and toll-like receptor gene expression was reduced ($P < 0.05$). In conclusion, early

feed restriction during the suckling phase promoted changes in ileal epimural microbiota and several immunity parameters that could be related to differences in feed efficiency traits during the fattening period of Merino lambs.

Keywords: feed efficiency, Peyer's patches, *Prevotella* spp., inflammation, metabolic programming

Implications

Differences in epimural microbiota and immunity parameters at ileal level together with reduced feed efficiency of fattening lambs are described as a consequence of early feed restriction during the suckling period. This information may allow implementing different strategies to increase feed efficiency of early feed restricted animals (udder problems, lack of lamb vitality, or low milk production), thus reducing the units of feedstuff consumed by the animal during the fattening period to produce a kilogram of meat and increasing the profitability for the farm.

Introduction

The World Organization for Animal Health has estimated that approximately 20% of animal production is lost due to unhealthy animals. This is due to the fact that immune defenses are energetically very expensive, so nutritional resources directed toward an immune response are removed from other functions such as growth (Rauw, 2012). Therefore, in order to increase animal performance, is fundamental to understand the different factors modulating the immune response (Animal Task Force, 2013).

One of these factors might be related to early feed restriction of animals (Greenwood and Cafe, 2007) during the postnatal phases, when the colonization of mucosal epithelium of the gastrointestinal tract by commensal bacteria takes place (Taschuk and Griebel, 2012). Interestingly, once established the epimural microbiota seems to be less influenced by the diet than microbiota associated to digesta contents (Petri *et al.*, 2013; Yáñez-Ruiz *et al.*, 2015), so any factor (e.g., early feed restriction) modifying the composition of the microbiota firmly attached to these cells may modulate not only the nutrient processing (Santos *et al.*, 2018a and 2018b) but also the gut-associated lymphoid tissue, thus exerting nutritional programming of immune health and hence long-term effects on lifetime feed efficiency.

However, even though several studies have described the composition of microbiota in the rumen, jejunum, colon and caecum contents of steers or lambs differing in feed efficiency (Myer *et al.*, 2015a, 2015b, 2015c and 2016; Santos *et al.*, 2018b), there are scarce studies focused on ileal epimural microbiota of ruminants (Mao *et al.*, 2015). This is surprising, since ileum is involved not only in nutrient absorption but also in the development of immune response (ileal Peyer´s patches, **iPP**) during early life. Moreover, ileum is a far smaller, physiologically and anatomically homogenous region of the small intestine (Verdonk *et al.*, 2001). Consequently, ileum was chosen as the target region of the small intestine in order to study if both ileal epimural microbiota and some parameters related to the mucosal associated lymphoid tissue are altered by early feed restriction, thus promoting long-term effects on feed efficiency traits of Merino lambs during the fattening period. Our working hypothesis was that early feed restriction modifies both ileal epimural microbiota and immunity parameters at this level, thus adversely impacting feed efficiency traits during the fattening period of Merino lambs.

Material and methods

Animals and diets

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 2015-04).

Twenty-four male Merino lambs, penned individually with their corresponding ewe during the suckling period, were used in this experiment. The lambs were stratified on the basis of live body weight at birth (average BW 4.81 ± 0.256 kg), treated with a subcutaneous injection of vitamin E and Se (1 mL per animal of Selevit Complex, Syva) to prevent white muscle disease, and then assigned randomly to one of two experimental treatments (n=12 per dietary treatment) during the suckling period. The first group of lambs (*ad libitum*, **ADL**) was kept permanently with the sheep whereas the other group (restricted, **RES**) was separated from the dams from 0900 h to 1800 h and milk restricted. Dams of the RES group were injected with oxytocin to remove alveolar milk and then milked at 1700 h before the reintroduction of lambs. Lambs were weighed twice a week throughout the experiment and vaccinated against enterotoxaemia and *pasteurella* (Heptavac P Plus, MSD Animal Health) at four weeks of age. When each lamb reached 13.5 kg of BW it was weaned progressively [free access to a complete pelleted diet and alfalfa, whereas it was allowed only two hours with the dam] until it weighed 15 kg. Then, all the animals were penned individually, had free access to fresh drinking water and were offered the same complete pelleted diet at the same level (35 g/kg BW per day) to avoid differences in DM intake during the fattening period as explained elsewhere (Santos *et al.*, 2018b).

The average daily gain was calculated by linear regression between the lamb body weight and the weighing day. All the lambs received the complete pelleted diet once a day at 0900 h, with the amount of feed offered being adjusted twice a week on the basis of the BW. Ingredients and chemical composition of the complete pelleted diet are summarized in Table 1.

[INSERT TABLE 1 NEAR HERE, PLEASE]

Sampling and storage

All the animals were slaughtered after a fattening period of at least 50 days when they reached the target BW of 27 kg. Feed was withdrawn 6 h before slaughtering; then lambs were weighed, stunned, slaughtered by exsanguination from the jugular vein, eviscerated and skinned according to the Council Regulation (EC) N° 1099/2009 on the protection of animals at the time of killing. A 10 cm segment of ileum was collected proximal to the ileocecal valve immediately after slaughter. Ileal samples were rinsed three times with sterile phosphate-buffered saline solution to remove the digesta and divided into several portions. Tissue samples were preserved in different ways for analysis of microbial composition (stored at -80 ºC during 48 hours, then freeze-dried), flow cytometry (sterile phosphate-buffered saline solution), histological or immunohistochemical examination (fixed by immersion in 10% buffered formalin for one week), immunoglobulin A (**IgA**) quantification (stored at -20 ºC), and gene expression (RNA_{later} Invitrogen, Lithuania; stored at -80 ºC).

DNA extraction and quantitative real-time PCR of bacteria attached to the ileal mucosa

The luminal part of the freeze-dried ileal mucosa (15 mg) was scraped with a scalpel and subsequent microbial DNA purification with the Ultra-Deep Microbiome Prep kit (Molzym, Bremen, Germany) was performed according to manufacturer´s instructions. The concentration of DNA was measured in each sample with a NanoDrop UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware), and then quantitative real-time PCR was used to quantify total bacteria attached to the ileal mucosa and the relative abundance of some genus very related to the health status of the host (e.g., *Prevotella* spp., *Lactobacillus* spp. and *Bifidobacterium* spp.) according to the procedures described by Andrés *et al.* (2016), Vargas *et al.* (2017) and Delroisse *et al.* (2008), respectively. Finally, quantitative real-time PCR data were normalized according to the previous concentration of DNA, and results were expressed as fold change (log_2 of "number of copies" per milligram of genomic DNA, RES/ADL) of overrepresented species in ileal epimural microbiota (positive value if RES/ADL > 1; negative value if ADL/RES > 1).

Mechanical disruption of ileal tissue and flow cytometry

In order to quantify leukocyte population in the ileum, a single color flow cytometry analysis was performed. Mucosal leukocytes were labelled with the leukocyte common antibody CD45 (Charavaryamath *et al.*, 2011). The mucosa of ileal samples was scraped off with a scalpel; the homogenates were mixed with 8 mL of cold phosphate-buffered saline solution (pH 7.4) and digested in a masticator paddle blender (IUL instruments, Barcelona, Spain). Contents were filtered, added carefully to 8 mL of Lymphoprep (Axis-Shield PocAS, Oslo, Norway) and centrifuged at 1000 × g and 21 ºC for 30 min. Layer of leukocytes was extracted, moved to a falcon tube with 20 mL of Hanks´ Balanced Salt Solution at 4 ºC and centrifuged at 270 × g and 4

ºC for 10 min. Then, the pellet was washed twice by centrifugation at 270 × g and 4 ºC for 10 min in RPMI 1640 medium with 10% fetal bovine serum (Lonza, Basilea, Switzerland) and 1% antibiotic antimycotic solution. The pellet was re-suspended in 5 mL cold of RPMI–fetal bovine serum. Cells were counted using Bio-Rad TC10 (Bio-Rad Laboratories, Hercules, California), and adjusted with RPMI–fetal bovine serum to a final concentration of 2 x 10^6 cells/mL. For antibody labelling, briefly, 50 µL of suspension were incubated for 60 min at 4 °C with a specific primary monoclonal antibody against CD45 antigen (Corpa *et al.*, 2001) diluted 1:5 in phosphate-buffered saline solution, centrifuged at 142 × g and 4 ºC for 3 min, washed twice by centrifugation at 270 × g and 4 ºC, then re-incubated for 30 min at 4 °C in the dark with 50 µL of fluorescein isothiocyanate conjugated rabbit antimouse immunoglobulin (Dako, Glostrup, Denmark) diluted 1:50. After two further washes, cells were kept at 4 °C until analysis on a FACScan flow cytometer (BD, San Jose, California) equipped with CellQuest software (BD, San Jose, California). Results were expressed as the percentage of positive stained cells in sample populations of 10 000 individual cells.

Histological and morphometric analysis

Formalin-fixed samples from ileum were trimmed and processed for paraffin embedding and histological examination (haematoxylin-eosin staining). Slides were examined with a Leica DM2000 LED microscope and digital pictures were taken at 4× magnification. Thickness of mucosa, submucosa and *tunica muscularis* was measured at 10 different sites in each picture using the image processing and analysis software ImageJ v1.6.0_14 (National Institutes of Health – NIH, USA).

Immunohistochemical analysis

Immunohistochemical analysis was performed according to the procedure described by Arranz-Solís *et al.* (2016). Briefly, cross sections were cut from the ileum wall samples and placed onto poly-L-Lysine coated slides for immunohistochemical labelling of T (CD3 antigen), B (CD20 antigen) and M-cells (cytokeratin 18 antigen). Endogenous peroxidase activity was blocked in deparaffinised sections by immersion in 3% hydrogen peroxide in methanol for 30 min in darkness at room temperature and rehydrated slides were rinsed The antigen retrieval was performed using heat-based methods (Table 2). After washing, sections were incubated with 100 µL of the primary antibodies diluted in phosphate-buffered saline solution overnight at 4 °C in a humidified chamber.

[INSERT TABLE 2 NEAR HERE, PLEASE]

After washing, sections were incubated for 40 min at room temperature with 100 µL of EnVision+/HRP solution (Dako, Glostrup, Denmark). After washing, antibody localization was determined using 100 µL of 3.3-diaminobenzidine (Sigma-Aldrich Corp., Saint Louis, Missouri) as chromogenic substrate for peroxidase. Sections were counterstained with haematoxylin for 30 s and mounted. Quantification of labelled cells was performed under a light microscope with a 40× objective. The number of T and B cells was counted in ten random fields within the lamina propria of the ileum.

Quantification of M-cells labelling intensity was performed measuring the % of positive labelled area in the total area of epithelial cells in iPP dome with the plugin IHC Profiler (Varghese *et al.*, 2014) of ImageJ. Domes were photographed under a 20× objective, and epithelial cells of domes were delimited as regions of interest. Positive labelling was automatically measured by the software.

Ileal mucosa antibody recovery and immunoglobulin A quantification

Immunoglobulin A was quantified according to the procedure described by Ahmed *et al.* (2015). Briefly, ileal samples were unfrozen overnight at 4 ºC; then the surface was scraped with a scalpel and 2 g were collected in a falcon tube with 6 mL of ice cold phosphate-buffered saline solution supplemented with protease inhibitors (Sigma-Aldrich Corp., Saint Louis, Missouri). Samples were vortexed, incubated overnight at 4 ºC and centrifuged at 3000 × g for 30 min at 4 ºC. Protein concentration of each supernatant was measured with the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, Massachusetts), and adjusted to 500 µg/mL using phosphate-buffered saline solution supplemented with protease inhibitors. The amount of IgA was measured in ileal mucosa using a Genorise ELISA IgA kit following manufacturer´s instructions (Genorise Scientific, Devon-Berwyn, Pennsylvania).

RNA extraction and real-time reverse transcription PCR

Total RNA was extracted from 100 mg of ileal samples (including iPP) according to the procedure described by Abecia *et al.* (2017). Briefly, samples were homogenized with 0.9 mm stainless steel bead and 1 mL of TRIzol Reagent (Invitrogen, Carlsbad, California), using a Bullet Blender homogenizer (Next Advance, Troy, New York). The homogenate was incubated for 5 min at room temperature before adding 200 µL chloroform mL^{-1} of TRIzol containing tissue homogenate. The aqueous phase was precipitated with 500 µL isopropanol mL^{-1} of TRIzol containing tissue homogenate, and applied to an RNeasy Mini-column (Qiagen, Hilden, Germany). RNA bound to the column was DNase treated using RNase-Free DNase Set (Qiagen, Hilden, Germany) and collected in 50 µL volume eluent. The RNA quantity was measured

using ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware) and RNA integrity number was evaluated using Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California).

Total RNA (1 µg) was reversed transcribed using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to manufacturer´s instructions. RNA was used as template for real-time reverse transcription PCR analysis to evaluate the expression of genes encoding 10 toll-like receptors (**TLRs**) in the ileal epithelium using the gene specific primer pairs described in Malmuthuge *et al.* (2012) relative to β-actin expression whereas for cytokines the amplification conditions and primers were those described by Martínez-Pérez *et al.* (2014). For each PCR reaction, 25 ng of cDNA were amplified with each primer set using the parameters described by Abecia *et al.* (2017) on the Bio-Rad iCycler (Bio-Rad Laboratories Ltd., Mississauga, Ontario). Amplification data were expressed as change in quantification cycle (Δ**Cq** value) and calculated as follows: ΔCq = Cq (cytokines or TLRs) – Cq (β-actin). A smaller ΔCq value equates to more abundant transcript.

Statistical Analysis

Growth performance data together with those of quantification of microbiota, flow cytometry, morphometric analysis, immunohistochemistry, IgA quantification and gene expression were analyzed by one-way ANOVA using the GLM procedure of SAS (SAS Institute Inc., Cary, North Carolina) with the suckling regime (*ad libitum* or restricted) as the only source of variation. In all cases, the individual lamb was considered as the experimental unit. Significance was declared at $P < 0.05$.

Results

Animal performance

Average daily gain during the suckling phase was reduced for RES lambs (267 vs. 191 g/day; $P < 0.001$) and the age at weaning (BW of 15 kg) increased (42 vs. 55 days; $P < 0.001$). Furthermore, significant differences in gain to feed ratio (average daily gain / DM intake) were observed during the fattening phase (0.320 vs. 0.261 for the ADL and RES groups, respectively; $P < 0.001$) despite the similar DM intake during this period of time. Thus, RES animals required more days (74 vs. 62 days; $P < 0.001$) to reach the intended BW at slaughter (27 kg).

Ileal epimural microbiota

No differences were observed for total bacteria, *Lactobacillus* spp. or *Bifidobacterium* spp. between experimental groups (ADL vs. RES lambs, Figure 1). However, a significant increase of the relative abundance of *Prevotella* spp. in the ileal epimural microbiota of early feed restricted lambs was detected (Figure 1; $\log_2FC = 2.09$).

[INSERT FIGURE 1 NEAR HERE, PLEASE]

Immunity parameters

The results regarding the counts of CD45+ cells (flow cytometry), thickness of ileal layers (histologic examination), M-cells labelling intensity, B or T infiltrating lymphocytes counts (immunohistochemistry), and IgA concentration in ileal mucosa (ELISA) are summarized in Table 3.

[INSERT TABLE 3 AND FIGURE 2 NEAR HERE, PLEASE]

Greater percentages for CD45+ were detected in the ileum of the RES group when compared to the ADL lambs (P = 0.049). Moreover, morphometric analysis revealed a trend towards a greater thickness of ileal submucosa layer in the RES group (P = 0.057) where the iPP are located (Figure 2). Likewise, a higher infiltration of B and T cells in the ileal lamina propria was observed in the RES lambs (P = 0.007 and P = 0.042, respectively; Table 3, Figure 2). Also, significantly higher percentages of the epithelium region in the iPP domes were labelled with high (P = 0.068) or medium intensity (P = 0.008) when M-cell marker was assessed in the RES group (Table 3, Figure 2), whereas those negatively labelled showed a trend towards being significantly higher (P = 0.067) in the ADL group. Similarly, secretory IgA at ileal mucosa level was greater for the early feed restricted lambs (P = 0.037).

Cytokines and TLRs gene expression at the ileal epithelium (including iPP) is shown in Table 4. No significant differences in mRNA expression of interferon gamma **(IFN-γ)**, interleukin 4 **(IL-4)** and interleukin 10 **(IL-10)** were observed. However, transforming growth factor β **(TGF- β)** was down-expressed in the RES group (P = 0.043). In addition, gene expression of several genes was significantly down-regulated in the early feed restricted group (e.g., TLR-3, P = 0.022; TLR-7, P = 0.012; and TLR-10, P = 0.016).

[INSERT TABLE 4 NEAR HERE, PLEASE]

Discussion

Early feed restriction may impact colonization patterns of gastrointestinal mucosa during early postnatal period and immunity parameters at this level, thus promoting

signalling events along the lifespan of the animal and adversely impacting feed efficiency during post-weaning phases (Greenwood and Cafe, 2007).

In the present study, the number of total bacteria attached to enterocytes was similar in both groups; nevertheless a higher presence of *Prevotella* spp. in the ileal epimural microbiota of the RES group was observed. *Prevotella* spp. strains are Gram-negative, anaerobic bacteria showing multiple virulence factors such as lipopolysaccharide, which facilitate the adhesion and invasion of host cells and chronic inflammatory conditions in the gut (Lukens *et al.*, 2014; Ley, 2016). It must be remarked that these studies are relative to humans, whereas there is scarce information about the role of *Prevotella* spp. in the intestine of ruminants (Mao *et al.*, 2015). However, it is tempting to speculate that the adhesion of *Prevotella* spp. to the ileal mucosa might have promoted pro-inflammatory conditions at this level and, consequently, lower feed efficiency traits for this group of animals as will be explained below. In any case, in accordance with our results, the relative abundance of *Prevotella* spp. has been shown to be increased in the ruminal liquid of inefficient (high residual feed intake) bulls (McCann *et al.*, 2014) and in the colon from steers showing low feed efficiency traits (Myer *et al.*, 2015c). Regrettably, the reasons behind different efficiency traits in these studies were not ascertained.

The outgrowth of *Prevotella* spp. has been described to occur with a concomitant reduction in the relative abundance of lactic acid bacteria such as *Lactobacillus* spp. in the intestinal microbiome, especially under autoinflammatory disease conditions (Lukens *et al.*, 2014). This is important, since *Lactobacillus acidophilus* and *Bifidobacterium* spp. are beneficial organisms due in part to their ability to attach to enterocytes, thus inhibiting the binding of enteric pathogens by competitive exclusion.

Lactic acid bacteria also generate signals for the production of cytokines modulating immune response, and decreasing inflammatory-intestinal damage (Villena *et al.*, 2014; Lim *et al.*, 2016). However, in the present study, although numerically lower values were observed for these bacterial groups in the RES lambs, the differences in the abundance of *Lactobacillus* spp. and *Bifidobacterium* spp. did not reach the significance level, probably due to the low levels of these bacterial groups detected in both groups of lambs.

In addition, a clear influence of early feed restriction in several parameters related to the immune response was observed according to the results of the present study. In this sense it was enticing to speculate, and warrants further investigation, that the differences in the colonization of gastrointestinal mucosa promoted by early feed restriction during the suckling period of these lambs might have triggered those changes found in the immunological parameters evaluated. In fact, it has been suggested that a different exposure to pathogenic bacteria might alter the ontology of M-cells, which are essentially epithelial cells of the gastrointestinal mucosa specialized in sampling and translocating luminal antigens to the underlying lymphoid tissue (Prims *et al.*, 2017). Therefore, a plausible hypothesis explaining these variations would be that the increased relative abundance of *Prevotella* spp. in the RES group might have contributed to enhance the expression of cytokeratin 18 antigen in the surface of M-cells, as suggested by the higher staining intensity of M-cells in the iPP domes of these animals. This is important, since the expression of cytokeratin 18 has been related to the M-cell function (e.g., antigen uptake; Gebert *et al*., 1994; Prims *et al.*, 2017). Thus, this circumstance might explain, at least partially, the higher levels of CD45+ (flow cytometry), the higher infiltrations of B and T cells in the lamina propria, and the trend towards a greater thickness of the ileal submucosa

layer (where iPP are located) in the RES lambs. In any case, the analysis of a more diverse immune cell population and *in vitro* functional studies are necessary to adequately evaluate this hypothesis.

Moreover, there were also significant differences between both groups of lambs as far as the amount of IgA in the ileal mucosa is concerned. IgA works across the gastrointestinal tract as an inhibitor of bacterial/viral adherence and penetration into the underlying epithelium, providing mucosal immune protection (Cerutti and Rescigno, 2008). Therefore, it might be feasible to have a higher concentration of ileal IgA in the RES group produced to neutralize microbial products such as lipopolysaccharide in intestinal epithelial cells.

Finally, no differences in the level of expression of several cytokines were observed excepting for TGF-β, which was reduced in the RES lambs. According to Letterio and Roberts (1998) the downregulation of this cytokine in the RES group might be indicative of the existence of a negative regulatory mechanism to reduce lymphocytes infiltration in the lamina propria and the inflammatory response. In agreement with this hypothesis, the down-regulation of some TLRs [pattern-recognition receptors of pathogen-associated molecular patterns located in several cell types such us macrophages, T and B cells or non-immune cells] observed in the ileal mucosa of RES lambs might have been implemented in an attempt to keep the homeostasis at intestinal level (Zhang and Ghosh, 2002; Malmuthuge *et al.*, 2012; Villena *et al.*, 2014). However, as stated beforehand, further functional studies are necessary to adequately evaluate this hypothesis.

Conclusions

Early feed restriction during the suckling period of Merino lambs modifies both bacterial microbiota attached to the ileum and immunity parameters at this level. These changes might be related to a decreased amount of nutrients towards productive processes (e.g., growth or fattening) during the fattening period. Actions to improve gut microbiome towards a healthier profile (e.g. prebiotics, probiotics, and nutritional management to decrease *Prevotella* spp. abundance) should be tested during the early life of lambs as a feasible way to minimize the negative effects on feed efficiency of those animals exposed to feed restriction during the suckling period.

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Declaration of interest

None

Ethics statement

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 2015-04).

Software and data repository resources

None

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Table 1 *Ingredients and chemical composition of the complete pelleted diet fed during the fattening period.*

Ingredients (g/kg)	
Barley	433
Corn	150
Soybean meal 44	237
Barley straw	150
Vitamin-mineral premix[1]	30
Chemical composition (g/kg DM)	
DM (g/kg)	900
NDF	227
ADF	121
CP	174
Fat	30
Ash	68
Metabolisable energy (kcal/kg DM)	2464

[1] 10% NaCl plus 20% premix [manufactured by DMS Nutritional Products, S.A. and containing (per kg) 2.25 g vitamin A, 0.02 g vitamin D_3, 10 g vitamin E, 0.25 g vitamin K_3, 0.50 g vitamin B_1, 0.50 g vitamin B_2, 2.50 g vitamin B_5, 0.50 g vitamin B_6, 5 g vitamin B_{12}, 5 g vitamin B_3, 50 g choline chloride, 17.50 g Fe, 15 g Zn, 2.5 g Cu, 20 g Mn, 0.05 g Co, 0.25 g I, 0.10 g Se, 259 g Ca and 50.5 g Mg]

Table 2 *Primary antibodies used in immunohistochemistry of ileal samples from fattening lambs.*

Target	Cell population	Type	Antigen retrieval	Dilution	Source
CD3[1]	T lymphocytes	Polyclonal antibody A-0452	Heat induced, pH 6.0, citrate buffer	1:300	Dako, Denmark
Cytokeratin 18	M-cells	Monoclonal antibody C-04	Heat induced, pH 6.0, citrate buffer	1:750	Abcam, United Kingdom
CD20	B lymphocytes	Polyclonal antibody RB-9013-P	None	1:200	ThermoFisher, Spain

[1] CD = cluster of differentiation.

Table 3 *Leukocyte counts measured by flow cytometry in the ileum, thickness of ileal layers, infiltrating lymphocyte counts in ileal lamina propria, intensity of staining of M-cells in ileal mucosa and ileal immunoglobulin A concentration of fattening lambs being fed ad libitum (ADL) or restricted (RES) during the suckling period.*

	ADL	RES	RSD[1]	*P*-value
Leukocytes (%)				
CD45 positive[2]	9.80	23.0	13.08	0.049
Thickness (µm)				
Mucosa	2279	2264	374.9	0.927
Submucosa	3376	3958	674.4	0.057
Muscular	481	480	86.8	0.979
Lymphocytes (number per field 40×)				
CD20 positive (B cells)	4.51	5.92	1.142	0.007
CD3 positive (T cells)	196	249	57.3	0.042
Intensity of cytokeratin 18 positive (M-cells)				
High positive	0.072	0.682	0.7148	0.081
Medium positive	2.00	6.78	3.434	0.008
Low positive	43.1	53.3	15.97	0.182
Negative	54.8	39.2	17.39	0.067
IgA[3] (pg IgA/µg total protein)	22.7	28.7	5.60	0.037

[1] RSD = residual standard deviation.

[2] CD = cluster of differentiation.

[3] IgA = immunoglobulin A.

Table 4 Cytokines and toll-like receptors (TLRs) mRNA expression in the ileal mucosa of fattening lambs being fed *ad libitum* (ADL) or restricted (RES) during the suckling period.

	ADL	RES	RSD[6]	*P*-value
Cytokines (ΔC_q[1])				
IFN-γ[2]	12.5	12.2	0.79	0.427
TGF-β[3]	5.60	5.87	0.264	0.049
IL-4[4]	13.6	13.8	0.84	0.589
IL-10[5]	10.3	10.2	0.42	0.785
TLR (ΔC_q)				
TLR_1	7.58	8.28	1.249	0.202
TLR_2	19.8	19.1	2.95	0.798
TLR_3	11.8	12.8	0.91	0.022
TLR_4	8.60	8.96	0.933	0.389
TLR_5	20.6	21.0	1.27	0.423
TLR_6	7.83	8.30	1.013	0.139
TLR_7	9.48	10.3	1.239	0.012
TLR_8	8.13	8.78	0.795	0.080
TLR_9	9.16	9.63	0.936	0.260
TLR_{10}	8.72	10.3	1.495	0.016

[1] Cq = quantification cycle. $\Delta C_q = C_{q(\text{cytokines or TLRs})} - C_{q(\beta\text{-actin})}$. Lower C_q represents higher RNA abundance level.

[2] IFN-γ = interferon gamma.

[3] TGF-β = transforming growth factor β.

[4] IL-4 = interleukin 4.

[5] IL-10 = interleukin 10.

[6] RSD = residual standard deviation.

Figure captions

Figure 1 Relative quantitation compared to *ad libitum* lambs (ADL) of 16S rRNA copy numbers of microbial groups attached to the ileal mucosa after early feed restriction (RES) during the suckling period. Fold-changes for specific amplicon groups were calculated as $\log_2$ ratio of normalized abundances. * Significant difference in copy number ($P < 0.05$) was calculated using Tukey's method (SAS).

Figure 2 Thickness of submucosa layer (arrows) in ileal segments of *ad libitum* (ADL) (A) and early feed restricted (RES) lambs (B). B cells labelled in the lamina propria of ADL (C) and RES lambs (D). T cells labelled in the lamina propria of ADL (E) and RES animals (F). Cytokeratin 18 labelling (M-cells) in Peyer´s patches domes of ADL (G) and RES lambs (H). Magnification was 4× for pictures A and B; 40× for pictures C, D, E and F; and 20× for pictures G and H.