Moderated milk replacer restriction of ewe lambs alters gut immunity parameters during the pre-weaning period and impairs liver function and animal performance during the replacement phase

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¹ *Abbreviations:* ADL, *ad libitum*; AST, aspartate aminotransferase; BHB, beta-hydroxybutyrate; Cq, quantification cycle; FRAP, ferric reducing ability of plasma; GALT, gut-associated lymphoid tissue; GGT, gamma glutamyl transpeptidase; HDL, high-density lipoprotein; IFN- γ , interferon gamma; IL, interleukin; iPP, ileal Peyer's patches; LBW, live body weight; LDL, low-density lipoprotein; MR, milk replacer; NEFA, non-esterified fatty acids; PEM, protein-energy malnutrition; PON, paraoxonase; RES, restricted; ROM, reactive oxygen metabolites; SED, standard error of the difference; sIgA, secretory immunoglobulin A; SOD, superoxide dismutase; TGF- β , transforming growth factor beta; TLRs, toll-like receptors; TMR, total mixed ration.

ABSTRACT

Undernutrition during the first stages of life may impair maturation of the immune system, harming the health status permanently. Thus, the aim of the present study was to define the impact of moderated milk replacer (MR) restriction during the artificial rearing period on the gut-associated lymphoid tissues of pre-weaned ewe lambs and long-term effects on biochemical profile and animal performance during the replacement period. Forty female Assaf lambs [average live body weight (LBW) 4.73 ± 0.116 kg] were penned individually. The first group of lambs (n=20) was fed MR *ad libitum* (ADL) whereas the second one (restricted, RES; n=20) only received approximately 62.5% of the MR intake measured in the ADL group. All the lambs were weighed twice a week until they were 35-days-old. Then, 8 lambs from each group (pre-weaned) were harvested and ileal samples collected. The rest of the lambs (12 ADL vs. 12 RES) were weaned progressively, and then offered a total mixed ration (TMR) *ad libitum* until the end of the experiment (7.5-months-old). The lower nutrient intake of RES pre-weaned lambs caused greater percentages for T lymphocytes (CD4⁺, CD8⁺, WC1⁺) and an increased secretory immunoglobulin A (sIgA) production in the ileum compared to the ADL group ($P<0.05$). These results, together with the significant differences in the expression of toll-like receptors and cytokines at this level (e.g., interferon gamma was increased in the RES group; $P<0.05$) suggest the existence of pro-inflammatory conditions in the ileum of pre-weaned lambs promoted by early feed restriction. Moreover, the biochemical profile revealed that liver function (e.g., albumin, gamma glutamyl transpeptidase) was impaired during several months once the feed restriction period was finished, whereas the average daily gain during the replacement phase was reduced for the early feed restricted lambs (182 vs. 202 g/day for RES and ADL groups, respectively; $P<0.05$). Therefore it can be concluded that moderated MR

restriction promoted both, pro-inflammatory conditions at ileal level in the pre-weaned lambs and an altered liver function which lasted several months after the end of feed restriction. Both circumstances might have impaired animal performance during the replacement phase of the ewe lambs.

Key words: feed restriction, metabolic programming, feed efficiency, inflammation, microbiome, GALT

1. Introduction

Nutrition is a critical determinant of immune response, so a deficient supply of nutrients may influence cell-mediated immunity, cytokine production, the complement system, phagocytic functions and secretory immunoglobulin A (sIgA) concentrations in humans according to the review reported by Chandra (1997). These effects can be worsened if feed restriction takes place during early life, because the lack of nutrients may impair not only animal growth, but also the adequate development of the gut-associated lymphoid tissue (GALT), thus having negative consequences on the health status along the whole life. Moreover, there is a complex interplay between nutrients, bacteria attached to the intestinal mucosa (epimural bacterial community) and the epithelial barrier of the GALT which plays a crucial role in the maturation of the immune system during the first months of life (Celi et al., 2017), so any factor modifying the colonization of the gut may also have long-term consequences on the health of the animals. However, even though there are several evidences in both humans and laboratory animal models (Chandra, 1997) and a recent experiment has described the effects on newborn calves (Schäff et al., 2016), to our knowledge there are no studies explaining the short and long-term effects of milk replacer (MR) restriction on GALT immunity and biochemical parameters of artificial reared ewe lambs. Extending

the knowledge about the mechanisms underlying nutritional programming by early feed restriction is crucial, since this circumstance –which may take place in the farm as a consequence of environmental, health or non-optimal feeding conditions– may affect the health status and animal performance of the animals along the whole life, thus decreasing the profitability of the dairy farm (e.g., reduced milk production or delay in the onset of puberty).

Therefore, the aim of the current study was to investigate the impact of moderated MR restriction on replacement dairy ewe lambs. Our working hypothesis was that early feed restriction during the artificial rearing period influences negatively GALT development, promoting gut inflammation and long-lasting changes on biochemical profile and animal performance of ewe lambs during the replacement phase.

2. Material and methods

2.1. Care and Use of Animals

All handling practices involving animals 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).

2.2. Animals and milk replacer during the artificial rearing period

A flock of newborn Assaf lambs was separated from their dams just after parturition. The dams were hand-milked and the colostrum offered to the lambs by bottle-feeding for 2 d. Lambs were bottle-fed for another 2 d with commercial MR (200 g dry matter/L) (Cordevit, Leches Maternizadas S.A., León, Spain). All newborn lambs were

treated with an intramuscular injectable selenium + vitamin E complex at the prescribed dose (Vitasel: 5 g α -tocopherol acetate, 110 mg sodium selenite, excipient, per 100 ml; Laboratorios Ovejero, León, Spain) to prevent nutritional muscular dystrophy (white muscle disease). Thereafter, 40 female lambs were selected from the flock to minimise the range of both, live body weight (LBW) and day of birth. Subsequently, all the lambs were stratified on the basis of LBW (on average 4.73 ± 0.116 kg), penned individually, and allocated randomly to one of two treatment groups (n=20 per treatment). All the lambs were fed MR three times daily (8:30, 14:00 and 20:30 hours) during the first week, and only twice daily (08:30 and 19:30 hours) during the rest of the suckling period. However, whilst the first group of lambs was fed MR *ad libitum* (ADL, approx. 2.5 times maintenance energy requirements to gain 250 g/day), the second one (restricted, RES) only received approximately 62.5% of MR intake measured in the ADL group (approx. 1.5 times maintenance energy requirements to gain 100-120 g/day) (NRC, 1985; Giráldez et al., 1999). A bucket with a teat was used to feed each lamb, being removed 20 minutes later when the lambs either had finished the offered MR or stopped eating because they were satisfied. All the lambs were weighed twice a week throughout the suckling period and the average daily weight gain (ADG, g/d) was estimated as the regression coefficient (slope) of LBW against time using the REG procedure of the SAS package (SAS Inst. Inc., Cary, NC). Ingredients and chemical composition of the MR administered are summarized in Table 1.

[INSERT TABLE 1 NEAR HERE, PLEASE]

2.3. Slaughter procedure and ileal parameters of pre-weaned lambs

When lambs were 35-days-old, eight lambs from each group (16 lambs in total) were stunned and harvested 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. Total fat depots (omental, mesenteric and perirenal) were separated and weighed. Additionally, and immediately after slaughter, 2 g of ileal contents were collected in two different tubes (10 ml each) containing 6 ml of distilled water and 0.25 mL of 20% sulfuric acid solution to acidify the medium and stop the fermentation. Fermentation end-products (volatile fatty acids) were determined according to Carro and Miller (1999). A 10 cm segment of ileum from each animal was collected proximal to the ileocecal valve immediately after slaughter. Ileal samples were rinsed three times with sterile phosphate-buffered saline solution (pH=7.0) to remove the digesta and divided into several portions preserved in different ways for analysis of epimural bacterial community (stored at -80°C 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), sIgA quantification (stored at -20°C), and gene expression of cytokines and toll-like receptors [stabilized with RNA_{later} (Invitrogen, Carlsbad, CA, USA) overnight at 4°C, and stored at -80°C]. Ileal epimural microbiota was assayed by DNA extraction and quantitative real-time PCR (qPCR); sIgA quantification in ileal mucosa was determined by ELISA; gene expression of cytokines and toll-like receptors was performed by RNA extraction of ileal samples and real-time reverse transcription PCR; all these procedures (assessed in duplicate for each parameter and lamb), together with flow cytometry (assayed in a single ileal sample per lamb), histological or immunohistochemical techniques (ten different fields were examined per parameter and lamb) performed on ileal samples were carried out according to Frutos et al. (2018a).

2.4. Animal performance and biochemical profile of replacement ewe lambs

The rest of the ADL ewe lambs ($n = 12$) were weaned progressively starting at a mean LBW of 13.5 kg (ADL), whereas the RES ewe lambs ($n = 12$) started weaning either with 11.5 kg or 70-days-old in order to ensure the viability of the lambs, but reducing at the same time the differences in age or weight at weaning. The progressive weaning lasted one week for each lamb and consisted in restricting MR intake and allowing free access to a starter feed for replacement lambs (Babymix, Inatega, León, Spain), barley straw and alfalfa hay. After weaning, the lambs were moved to a single feedlot for 42 days (1st post-weaning period), and fed *ad libitum* the same starter feed, barley straw and alfalfa hay supplied in separate feeders. After this period, the ewe lambs were fed *ad libitum* a total mixed ration (TMR) in feedlot until the end of the experimental period (7.5-months-old). All the ewe lambs were weighed once a week during the replacement period and the ADG during this phase estimated as previously mentioned.

Samples of the TMR offered were collected weekly and analysed for dry matter (ISO 6496:1999), ash (ISO 5984:2002), crude protein (ISO 5983:2009), aNDF (NDF was assayed with a heat stable amylase and expressed inclusive of residual ash; Ankom Technology Corp., Macedon, NY, USA) and ADF (ADF was expressed inclusive of residual ash; Ankom Technology Corp., Macedon, NY, USA). Ingredients and chemical composition of the TMR administered are summarized in Table 2.

[INSERT TABLE 2 NEAR HERE, PLEASE]

The 24 ewe lambs (12 animals per group) were blood sampled three times along the experiment early in the morning, at the end of the feed restriction period (1st month) or

once the replacement ewes were being fed exclusively the experimental TMR (5 and 7.5 months-old). Blood samples were collected by jugular venipuncture into tubes containing lithium-heparin, placed in iced water and centrifuged at $3520 \times g$ for 16 min at 5°C. Plasma samples were stored at -80°C until biochemical profile analysis.

Plasma samples were analysed for haptoglobin, ceruloplasmine, beta-hydroxybutyrate (BHB), paraoxonase (PON), reactive oxygen metabolites (ROM), myeloperoxidase, ferric reducing ability of plasma (FRAP), superoxide dismutase (SOD), Ca, Mg, Zn, retinol, tocopherol, aspartate aminotransferase (AST), gamma glutamyl transpeptidase (GGT), total bilirubin, creatinine, glucose, insulin, urea, protein, albumin, globulin, cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), non-esterified fatty acids (NEFA) and triglycerides as described by Frutos et al. (2018b). Globulin was calculated as the difference between total protein and albumin. Moreover, insulin, specific for sheep, was measured using ELISA assay (Mercodia Ovine Insulin ELISA, Uppsala, Sweden) in microplates (Synergy 2, BioTek, Winooski, VT, USA). Plasma vitamins A and E were measured by extraction with hexane and reverse-phase high-performance liquid chromatography using Spherisorb ODS-2, 3 μ m, in a 150×4.6 mm column (Alltech, Deerfield, IL, USA); an ultraviolet/visible detector set at 325 nm (for vitamin A) or 290 nm (for vitamin E); and 80:20 methanol:tetrahydrofurane as the mobile phase.

2.5. Statistical analysis

Average daily gain data, together with those of total fat depots, intestinal fermentation parameters, quantification of epimural bacterial community, flow cytometry, morphometric analysis, immunohistochemistry, sIgA quantification and gene expression of toll-like receptors (TLRs) and cytokines were analyzed by one-way

ANOVA using the GLM procedure of SAS (SAS Institute Inc., Cary, North Carolina) with the dietary treatment (*ad libitum* or restricted during the artificial rearing phase) as the only source of variation. In all cases, the individual lamb was considered as the experimental unit. Significance was declared at $P<0.05$ and a tendency at $P<0.1$.

Data corresponding to biochemical parameters measured at several growth stages (1st, 5th and 7.5th months of life of replacement ewe lambs) were analyzed as a repeated measures design using the MIXED procedure of SAS. Dietary treatment and growth stage were included in the model (the main effects of both factors and their interaction) as fixed effects. In all cases, the individual lamb was considered as the experimental unit and included in the model as a random effect. For each variable, the statistical model was fitted with different competing covariance structures, selected with the smallest value for Akaike's and Bayesian criteria. The Tukey-Kramer multiple-comparison test was used to compare means when the interaction between dietary treatment and growth stage was significant.

3. Results

3.1. Animal performance

Both ADG during the artificial rearing period (116 vs. 237 g/day for the RES and ADL groups, respectively; SED=20.0; $P<0.001$) and the weight at weaning (11.2 vs. 13.7 kg; SED=0.25; $P<0.001$) were lower in RES lambs due to MR restriction. Thereafter, a compensatory growth period for the RES ewe lambs (non-slaughtered) was observed after weaning (274 vs. 238 g/day for the RES and ADL lambs, respectively; SED=15.76; $P<0.05$), but the ADG was reduced for the early feed restricted lambs once all animals were being fed *ad libitum* the TMR during the

replacement phase (182 vs. 202 g/day for RES and ADL groups, respectively; SED=8.61; $P<0.05$). The LBW at the end of the experiment (7.5-months-old) was numerically lower for the RES ewe lambs, but no significant differences were detected (45.1 vs. 50.4 kg; SED=3.11; $P>0.05$).

3.2. Ileal epimural bacterial community and volatile fatty acids of pre-weaned lambs

No differences were observed for total bacteria or the relative abundance of *Bifidobacterium* spp. or *Prevotella* spp. in the ileal epimural bacterial community between experimental groups (ADL vs. RES lambs, Figure 1). Acetate measurements were lower in the RES group compared to the ADL lambs (0.71 vs. 1.42 mmol/L; $P<0.05$, SED=0.242). The rest of volatile fatty acids (e.g., propionate, butyrate...) were not detected.

[INSERT FIGURE 1 NEAR HERE, PLEASE]

3.3. Ileal immunity parameters of pre-weaned lambs

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

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

Greater percentages for T lymphocytes (CD4⁺, CD8⁺, WC1⁺) were detected in the ileum of the RES group when compared to the ADL lambs ($P<0.05$). Moreover, morphometric analysis revealed a reduced thickness of ileal submucosa layer in the RES

group ($P<0.05$) where the ileal Peyer's patches (iPP) are located (Figure 2). In addition, the infiltration of B-cells in the ileal lamina propria (Table 3, Figure 2) and the concentration of sIgA at ileal mucosa level were reduced ($P<0.05$) and increased ($P<0.05$), respectively in the RES lambs. On the contrary, the infiltration of T-cells in the ileal lamina propria (Table 3, Figure 2) was not affected significantly by early feed restriction, but numerically higher values could be observed for the RES lambs (204 vs. 176 cells per field 40×). Also, a trend towards significantly higher percentages ($P<0.1$) of negatively labelled regions in the epithelium region of the iPP domes was observed when M-cell marker was assessed in the RES group (Table 3, Figure 2). This was corroborated by the H-index for M-cell, which tended to be lower in the RES group ($P<0.1$).

Cytokines and TLRs gene expression at the ileal tissue (including iPP) is shown in Table 4. The mRNA expression of interferon gamma (IFN- γ) was greater in the RES group ($P<0.05$) whereas transforming growth factor β (TGF- β ; $P<0.05$) and interleukin 10 (IL-10, $P<0.05$) were down-expressed in these lambs (a smaller ΔC_q value equates to more abundant transcript). Also, interleukin 4 (IL-4, $P<0.1$) showed a trend towards significantly lower values in the RES lambs. In addition, gene expression of several TLRs was significantly down-regulated in the early feed restricted group (e.g., TLR-1, TLR-6, TLR-7, TLR-9; $P<0.05$).

[INSERT TABLE 4 NEAR HERE, PLEASE]

3.4. Biochemical profile of replacement ewe lambs

The effects of early feed restriction on biochemical profile during the artificial rearing (1st month) and replacement periods (months 5th and 7.5th of life) are presented

in Table 5. Several parameters related to energy (e.g., NEFA, BHB, cholesterol, LDL), protein metabolism (e.g., creatinine, urea, and total protein), minerals (e.g., Mg) or antioxidant and anti-inflammatory conditions (e.g., PON and FRAP) were either not modified or significantly reduced in the RES lambs only during the suckling period (e.g., PON and FRAP were reduced 22.5% and 19.3%, respectively during the feed restriction period; $P < 0.05$ for the interaction between treatment and day). However, albumin and GGT reductions persisted in RES ewe lambs several months after the end of the restriction period. No significant changes were observed in the rest of parameters of the biochemical profile (e.g., haptoglobin, ceruloplasmin, ROM, myeloperoxidase, SOD, Ca, Zn, retinol, tocopherol, AST, total bilirubin, glucose, insulin, globulin, HDL and triglycerides).

4. Discussion

In humans it has been observed a secondary immunodeficiency attributable to the poor nutritional status of undernourished children (Bourke et al., 2016). Accordingly, the reduction of submucosa thickness in the ileum (where iPP are located) of the RES lambs, together with the decreased staining intensity of M-cells, probably related to a reduced M-cell function (e.g., antigen uptake from the gastrointestinal tract; Mabbot et al., 2013; Prims et al., 2017) might have impacted negatively Peyer's patches maturation in the early feed restricted lambs (Rios et al., 2016).

On the other hand Manhart et al. (2000) mentioned that B lymphocytes of Peyer's patches may be more resistant to malnutrition than other immune cells. Accordingly, Rho et al. (2017) detected an increased frequency of long-lived plasma cells which can secrete large volumes of IgA in the small intestinal lamina propria of mice under severe protein-energy malnutrition (PEM) conditions. In addition, Beatty et al. (1983) also

reported an enhanced synthesis of sIgA in duodenal mucosal biopsies from children with acute kwashiorkor (PEM), whereas others have observed that the transport of IgA through the epithelium can be increased by inflammatory (IFN- γ) and stress conditions (Reyna-Garfias et al., 2010). All these results seem to be in agreement with the higher levels of ileal sIgA observed in the RES lambs. It must be stated, however, that this would not be in contradiction with the reduced counts of B-cells observed in these RES lambs detected by both, flow cytometry (where only numerically lower values of CD21⁺ were observed) and immunohistochemistry analysis, since not all the B-cells are differentiated into IgA-secreting plasma cells (Rios et al., 2016).

Furthermore, all the parameters measured by flow cytometry that were related to T lymphocytes (CD4⁺, CD8⁺, WC1⁺) at ileal level were increased in the RES group. Also numerically higher values of T cells could be observed by immunohistochemistry in early feed restricted lambs. Contrarily Fló et al. (1993 and 1994) described that B-cell and T-cell maturation were delayed in the GALT of 28 days undernourished rats. Nevertheless, in accordance with our results Campbell et al. (2003) and Amadi et al. (2009) found that T cells infiltrating the lamina propria rose and B cell numbers decreased in malnourished African children, whereas Márquez et al. (2000) also observed an increase in the number of T cells in the gut lamina propia and in the epithelium of rats fed a protein-free diet. Together all these data refute a generalized reduction in immune responsiveness caused by suboptimal nutrition, supporting instead a model of phenotypic plasticity in mucosal immunity that responds to nutrient availability, age, timing or severity of undernutrition (Bourke et al., 2016). However, the age of the lambs and the lack of nutrients during the early life make our study more comparable to those found in malnourished African children (Campbell et al., 2003; Amadi et al., 2009).

In any case, the ileal down-regulation of genes encoding anti-inflammatory cytokines (IL-4, IL-10 and TGF- β), together with the significant upper transcription of IFN- γ seemed to indicate a pro-inflammatory condition in the small intestine of RES lambs. These results are, again, in accordance with Campbell et al. (2003) and Amadi et al. (2009), who reported a Th1-dominated response in the small bowel of malnourished children, with a decrease of TGF- β expression and an up-regulated expression of IFN- γ . Regarding ruminants, a previous study (Frutos et al., 2018) describing the long-term effects of early feed restriction during the fattening phase of lambs (once re-feeding was restored) suggested a relationship between increments of *Prevotella* spp. (concomitant with associated decreases in *Bifidobacterium* spp.) and pro-inflammatory conditions at ileal level. However, although a similar trend could be observed in the present study, our ileal samples were collected early during the restriction phase, which probably prevented the observation of significant changes in the relative abundances of *Prevotella* spp. or *Bifidobacterium* spp. in the RES lambs. Moreover, in agreement with Sun et al. (2001), Gomes de Morais et al. (2017), Da Costa et al. (2017) and Frutos et al. (2018) a down-regulation of some TLRs [pattern-recognition receptors of pathogen-associated molecular patterns located in several cell types such as macrophages, T and B cells or non-immune cells] was observed in RES lambs, which has been suggested as a mechanism implemented to keep the homeostasis at intestinal level (Zhang and Ghosh, 2002; Malmuthuge et al., 2012; Villena et al., 2014). In any case, all these alterations in the gut could be, at least, partially responsible for the reduced animal performance of RES lambs.

The decreased energy and protein intake of RES lambs during the suckling period might have impaired liver functionality as suggested by the lower levels of albumin, GGT, FRAP, PON and cholesterol observed for the RES lambs during the first month

of life (Bionaz et al., 2007, Bertoni and Trevisi, 2013). Moreover, the lower levels of NEFA and BHB in the RES lambs during the suckling period, together with the lower total fat depots of these animals (292 vs. 111 g for ADL and RES lambs, respectively; SED= 4.81; $P<0.001$) suggest that adipose tissue was exhausted after a month of MR restriction. In addition, the lower levels of creatinine in the pre-weaned RES lambs seem to be indicating a reduced muscle mass in absolute terms (Agenäs et al., 2006). Most of these effects on the biochemical profile disappeared, at least partially (no significant effects, but numerically different values during the 5th and 7.5th months of life), once the refeeding conditions were restored, whereas the rest of biochemical parameters (e.g., haptoglobin, ceruloplasmine, ROM, myeloperoxidase, SOD, Ca, Zn, retinol, tocopherol, AST, total bilirubin, glucose, insulin, globulin, HDL and triglycerides) were not modified at any time. However, the lower values of albumin and GGT in the RES lambs are remarkable because they persisted several months after the feed restriction period was ended, suggesting negative residual effects on liver functionality which might have further impaired animal performance along the replacement phase. It must be considered that GGT enables the metabolism of antioxidants, inflammatory molecules, drug metabolites, glutathione and glutathionylated xenobiotics (Koenig and Seneff, 2015). Therefore, the lower levels of GGT observed in the RES ewe lambs along the whole experimental period might be associated to several indicators of intracellular stress and inflammatory response (e.g., phosphatidylserine compounds and prostaglandin E2-biotin) that were over accumulated in the plasma of these animals according to their metabolome profile published previously elsewhere (Santos et al., 2018). Moreover, other studies have shown that malnutrition is related to oxidative stress conditions as a consequence of chronic inflammation events (Ece et al., 2007). In the present study a transitory

imbalance in the antioxidant status during the suckling period could be observed in the pre-weaned RES lambs (e.g., lower FRAP and PON values), which also manifested pro-inflammatory conditions at ileal level. However, the lack of significant differences in the positive acute-phase proteins involved in inflammation (e.g., haptoglobin and ceruloplasmin) or in those parameters related to metabolic disorders such as insulin resistance (e.g., glucose and insulin) differ from those results previously observed in early feed restricted fattening lambs (Frutos et al., 2018b). This apparent contradiction might be explained by the different diets administered during the refeeding period in each experiment: whilst the replacement ewes were fed a TMR rich in fibre, the fattening lambs were fed an acidogenic diet (e.g., rich in cereals and carbohydrates) which together with the pro-inflammatory conditions promoted by early feed restriction might have compromised the health status under stressful diet conditions.

5. Conclusions

In conclusion, under the conditions of the present study our results suggest that the reduced animal performance observed in early feed restricted ewe lambs is associated with pro-inflammatory conditions in the gut and a persistent impaired liver functionality after the period of feed restriction.

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Table 1. Ingredients and chemical composition of the commercial lamb milk replacer

Ingredients, g/kg	
Skim milk powder	500
Milk whey powder	160
Vegetable oil (cocoa)	140
Animal fat (pork lard)	120
Whey protein powder	40
Hydrolysed wheat protein	15
Yeast/sodium bicarbonate /Dextrose	15
PreVIT mineral ^a	10
Chemical composition, g/kg dry matter	
Dry matter, g/kg	960
Crude protein	235
Ether extract	260
Ash	70
Metabolisable energy, kcal/kg DM	5089

^a Provided per kg milk replacer: vitamin A, 80,000 IU; vitamin D₃, 4250 IU; iron, 40 mg; cobalt, 0.2 mg; copper, 5 mg; manganese, 25 mg; zinc, 30 mg; selenium, 0.2 mg

Table 2. Ingredients and chemical composition of the total mixed ration administered to replacement lambs

Ingredients, g/kg	
Barley	360
Lucerne	160
Barley straw	157
Vetch	133
Molasses (sugar cane)	50
Corn	50
Sunflower	43
Soybean meal 44	30
Vitamins and minerals	17
Chemical composition, g/kg dry matter	
Dry matter, g/kg	892
Neutral detergent fibre (aNDF) ^a	355
Acid detergent fibre (ADF)	215
Crude protein	125
Ash	75
Metabolisable energy, kcal/kg DM	2303

^a NDF was assayed with a heat stable amylase and expressed inclusive of residual ash

Table 3. Lymphocyte counts by flow cytometry, morphometric analysis, immunohistochemistry, and secretory immunoglobulin A (sIgA) production in the ileum of pre-weaned lambs being fed *ad libitum* (ADL) or restricted (RES) during the artificial rearing period.

	ADL	RES	SED ^a	P-value
Lymphocytes, %^b				
CD4 ⁺	6.1	9.9	1.764	0.049
CD8 ⁺	8.1	13.7	2.587	0.047
WC1 ⁺ (gamma-delta T cells)	6.4	9.3	1.143	0.021
CD21 ⁺	18.8	12.6	3.763	0.120
CD45R ⁺	18.3	14.2	4.213	0.347
CD4 ⁺ /CD8 ⁺	0.77	0.81	0.118	0.724
Ileal thickness, μm				
Mucosa	980	958	61.0	0.717
Submucosa	1023	757	97.0	0.016
Muscular	178	162	21.4	0.487
Lymphocytes, number per field 40$\times$				
CD20 ⁺ (B cells)	3.52	2.34	0.368	0.006
CD3 ⁺ (T cells)	176	204	16.6	0.138
CK18⁺ (M-cells), %^c				
High positive	3.77	1.91	6.064	0.171
Medium positive	16.0	11.0	3.75	0.238
Low positive	51.9	45.2	4.65	0.221
Negative	28.4	41.9	6.30	0.065
H-index ^d	95.1	72.9	11.00	0.083
sIgA, pg/μg total protein	10.2	14.5	1.70	0.023

^a SED, standard error of the difference

^b Percentage of positive stained cells in sample populations of 10,000 individual cells

^c Percentage of positive labelled area in the epithelial cells of ileal Peyer's patches dome

^d "Histo" score (from 0 to 300), higher values indicating higher-intensity labelled area of epithelial cells according to Hirsch et al. (2003).

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

	ADL	RES	SED ^a	P-value
Cytokines, ΔCq^b				
IFN- γ^c	10.84	9.14	0.683	0.039
TGF- β^d	3.50	4.03	0.152	0.005
IL-4 ^e	11.89	12.88	0.506	0.081
IL-10 ^f	8.76	9.39	0.217	0.016
TLRs, ΔCq				
TLR ₁	8.00	8.70	0.291	0.036
TLR ₂	-	-	-	-
TLR ₃	10.15	10.07	0.210	0.710
TLR ₄	7.52	7.66	0.236	0.585
TLR ₅	16.74	16.28	0.742	0.558
TLR ₆	10.21	11.01	0.262	0.015
TLR ₇	8.12	8.88	0.183	0.003
TLR ₈	9.22	9.01	0.175	0.313
TLR ₉	6.73	7.43	0.289	0.037
TLR ₁₀	4.28	5.11	0.408	0.097

^a SED, standard error of the difference

^b Cq, quantification cycle. $\Delta Cq = Cq$ (cytokines or TLRs) – Cq (β -actin). Lower Cq represents higher mRNA abundance level

^c IFN- γ , interferon gamma

^d TGF- β , transforming growth factor beta

^e IL-4, interleukin 4

^f IL-10, interleukin 10

Table 5. Biochemical parameters of lambs fed *ad libitum* (ADL) or restricted (RES) during the artificial rearing period.

	<u>1st month</u>		<u>5th month</u>		<u>7.5th month</u>		SED ₁ ¹	SED ₂ ²	<u>P-value</u>		
	ADL	RES	ADL	RES	ADL	RES			T ³	D ⁴	T*D ⁵
Haptoglobin, g/L	0.510	0.556	0.272	0.402	0.171	0.387	0.1701	0.1772	0.450	0.341	0.893
Ceruloplasmine, µmol/L	3.86	4.24	2.70	2.97	2.53	2.76	0.445	0.308	0.514	<0.001	0.967
BHB ⁶ , mmol/L	0.574 ^c	0.147 ^a	0.518 ^c	0.523 ^c	0.279 ^b	0.282 ^b	0.0402	0.0425	0.002	<0.001	<0.001
PON ⁷ , U/L	173 ^b	134 ^a	159 ^b	149 ^{ab}	166 ^b	152 ^b	12.5	4.86	0.106	0.455	0.012
ROM ⁸ , mg/100 MI	20.5	20.8	15.3	16.3	14.3	15.3	1.74	1.24	0.672	<0.001	0.951
Myeloperoxidase, U/L	550	486	470	481	532	495	49.4	48.8	0.550	0.630	0.747
FRAP ⁹ , µmol/L	155 ^b	125 ^a	134 ^a	133 ^a	134 ^a	131 ^a	5.77	5.32	0.065	0.289	0.018
SOD ¹⁰ , U/L	1.03	0.962	1.25	1.22	1.34	1.46	0.125	0.086	0.952	<0.001	0.500
Ca, mmol/L	2.84	2.79	2.91	2.84	2.83	2.84	0.027	0.0327	0.151	0.160	0.483
Mg, mmol/L	1.031 ^c	0.852 ^a	0.931 ^b	0.906 ^b	0.907 ^b	0.911 ^b	0.0166	0.0161	<0.001	0.130	<0.001
Zn, mmol/L	22.6	20.3	17.2	15.8	18.3	17.3	1.13	1.15	0.172	<0.001	0.852
Retinol, ug/100 mL	22.0	22.5	53.3	45.2	58.0	49.1	3.92	3.21	0.174	<0.001	0.286
Tocopherol, ug/mL	1.16 ^b	1.42 ^b	0.782 ^a	0.700 ^a	0.681 ^a	0.620 ^a	0.115	0.071	0.732	<0.001	0.036
AST ¹¹ , U/L	90.7	85.0	139	127	141	127	6.40	5.86	0.116	<0.001	0.776
GGT ¹² , U/L	90.4	74.2	58.3	51.1	62.1	52.3	3.89	2.40	0.009	<0.001	0.170
Total bilirubin, µmol/L	2.65	2.37	0.77	0.86	0.62	0.56	0.314	0.381	0.796	<0.001	0.887
Creatinine, µmol/L	80.8 ^c	67.9 ^a	76.7 ^{bc}	73.3 ^b	78.9 ^{bc}	74.0 ^b	2.37	1.55	0.007	0.382	0.008
Glucose, mmol/L	4.64	5.40	5.63	5.24	5.00	5.13	0.196	0.236	0.411	0.166	0.064
Insulin, ng/L	96.2	105	297	199	265	270	48.03	59.73	0.563	0.032	0.313
Urea, mmol/L	7.29 ^{bc}	4.63 ^a	6.95 ^b	6.12 ^b	8.03 ^c	7.25 ^{bc}	0.459	0.411	0.005	<0.001	0.042
Protein, g/L	61.1	55.7	70.1	68.0	70.6	69.2	1.24	1.20	0.028	<0.001	0.211
Albumin, g/L	33.6 ^b	30.4 ^a	37.1 ^d	35.8 ^c	36.7 ^{cd}	36.0 ^c	0.39	0.36	<0.001	<0.001	0.005
Globulin, g/L	27.6	25.3	33.1	32.3	33.8	33.3	1.12	1.10	0.297	<0.001	0.713
Cholesterol, mmol/L	2.78 ^c	2.17 ^b	1.93 ^a	1.71 ^a	1.88 ^a	1.80 ^a	0.123	0.078	0.022	<0.001	0.005
LDL ¹³ , mmol/L	0.561 ^b	0.298 ^a	0.574 ^b	0.457 ^b	0.493 ^b	0.461 ^b	0.040	0.028	0.002	0.016	<0.001
HDL ¹⁴ , mmol/L	1.46	1.43	0.989	0.913	1.03	0.987	0.090	0.048	0.577	<0.001	0.891
NEFA ¹⁵ , g/L	1.13 ^c	0.486 ^b	0.117 ^a	0.123 ^a	0.136 ^a	0.182 ^a	0.056	0.069	0.002	<0.001	<0.001

Triglycerides, mmol/L	0.302	0.398	0.171	0.164	0.160	0.176	0.0256	0.0238	0.178	<0.001	0.085
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^{a, b, c, d} Values within a row with different superscripts differ significantly at P<0.05 for dietary treatment or growth stage

¹ SED₁, standard error of the difference to compare dietary treatments

² SED₂, standard error of the difference to compare days

³ T, dietary treatment

⁴ D, day

⁵ T*D, interaction between treatment and day

⁶ BHB, beta-hydroxybutyrate

⁷ PON, paraoxonase

⁸ ROM, reactive oxygen metabolites

⁹ FRAP, ferric reducing ability of plasma

¹⁰ SOD, superoxide dismutase

¹¹ AST, aspartate aminotransferase

¹² GGT, gamma glutamyl transpeptidase

¹³ LDL, low-density lipoprotein

¹⁴ HDL, high-density lipoprotein

¹⁵ NEFA, non-esterified fatty acids

Figure captions

Fig. 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 the $\log_2$ ratio of normalized abundances. No significant differences ($P>0.05$) calculated using Tukey's method (SAS) were observed.

Fig. 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).